Determination of Toxicological and Nutritional Factors of *Crotalaria* species Used as Indigenous Vegetables

by

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ABSTRACT

Food frequency questionnaire and 24-hour recall methods were used to assess the general consumption of leafy vegetables and fruits within the Luo of northern Tanzania. The frequency questionnaire focused specifically on the consumption of Crotalaria brevidens, a leafy vegetable in a genus characterised by toxic pyrrolizidine alkaloids. Leafy vegetables were the most frequently consumed food group, contributing 23% of the total frequency scores. Crotalaria brevidens contributed 1.7% of the total frequency scores although some individuals consume significantly more. High consumption frequency of Crotalaria sp. is negatively associated with the consumption of fruits, fats and oils, vitamin A animal sources and consumption of animal products. Edible portions of Crotalaria sp. contain toxic pyrrolizidine alkaloids detected by thin layer chromatography. HPLC analysis showed that the plant contains high amounts of β-carotene. Quantification of the toxic alkaloids was not possible due to the low sensitivity of the methods used and low quantities present in the edible portions. Based on reported detection limits for the methods used a low and high estimated intake of toxic alkaloids was calculated and risk/benefit is assessed in relation to its nutrient content.

Résumé

Des questionnaires de fréquence de consommation et des rappels de 24-heures on été utilisés pour evaluer la consommation de légumes verts et de fruits chez les Luo du Nord de la Tanzanie. Le questionnaire de frequence de consommation s'est concentre sur Crotalaria brevidens, un legume vert dont le genre est caracterise par la presence d'alkaloides pyrrolizidiques toxiques. Les légumes verts furent les aliments les plus souvent consommes, contribuant 23% du total des points de fréquence. Crotalaria brevidens representait 1.7% du total des points de frequence mais certains individus en consomment beaucoup plus. La grande consommation de Crotalaria sp. est inversement liee a la vitamine A de source animale et de produits alimentaires animaux. Les parties comestibles de Crotalaria sp. contiennent des alkaloides pyrrolizidiques détectés par la chromatographie en couche mince. La haute teneur en β-carotène de cette plante fut révélée par son analyse par chromatographie en phase liquide à haute pression. Il n'a pas éte possible de quantifier l'alkaloide à cause de la faible sensibilite des methodes utilisées et des faibles quantités presentes dans la partie comestible de la plante. D'aprés les seuils de detection rapportés pour les méthodes utilisées, une ingestion elevee ou faible d'alkaloides toxiques a été evaluee. Le rapport des risques et des avantages a été calcule en fonction de la valeur nutritive de Crotalaria brevidens.

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CHAPTER 1
INTRODUCTION

The importance of green leafy vegetables as sources of nutrients in societies where consumption of animal based food products is low is well recognized (FAO 1988). Green leafy vegetables and certain fruits are important sources of pro-vitamin A carotenoids in addition to other nutrients (Simpson and Tsou 1985; Teply 1986). Vitamin A is essential for cellular differentiation, normal vision, growth and reproduction.

Although plant sources of pro-vitamin A are abundant worldwide, vitamin A deficiency is still a major nutritional and public health problem in a number of developing countries. Increased consumption of green leafy vegetables and their cultivation is one of the strategies promoted for the control of vitamin A deficiency in developing countries (WHO 1982).

Traditional cooking methods of green leafy vegetables especially bitter ones normally involve steam blanching, followed by water straining and further prolonged cooking and stewing. These cooking methods are aimed at removing the unacceptable bitter taste contained in the vegetables. A number of studies have documented high losses of the water soluble vitamins, minerals, thermal decomposition and isomerisation of the naturally occurring trans carotenoids to cis isomers which have lower vitamin A activity. There is growing concern with regard to nutrient losses as a result of these cooking methods.

The fact that these vegetables are intensely bitter, a taste which is unacceptable and normally is associated with the presence of toxic alkaloids, terpenoids or other anti-nutritional factors, has often been overlooked when evaluating the effect of traditional cooking methods. There

is little information on the chemical composition of these leafy vegetables and the components responsible for the bitter taste. Little attention has been given to the detoxification attained by traditional cooking methods without which a number of these leafy vegetables would be unacceptable and possibly pose a greater risk of toxicity.

The present study examines some of the above issues. The study aimed at establishing consumption patterns of wild plants used as leafy vegetables and fruits in general. The focus of this study was the consumption of Crotalaria brevidens Benth var intemedia (Kotschy) Polhill, an edible leafy vegetable containing toxic pyrrolizidine alkaloids. The importance of this vegetable as a dietary source for β-carotene was assessed on the basis of availability of other sources of this nutrient. A seven day food frequency questionnaire and a 24-hour recall were used for dietary data collection. A specific questionnaire looking at particular traditional cooking methods of Crotalaria leafy vegetable was administered to document these methods and relate them to nutrient loss and detoxification.

Phytochemical analysis of the edible parts of the plant was done in order to identify and quantify the amounts of pyrrolizidine alkaloids present in these edible portions. The amount thus found was assessed in relation to possible health risk posed to this population given their current consumption patterns established on the basis of the food frequency questionnaire. The β-carotene content of this vegetable was analyzed to assess the importance of this vegetable as a source of this nutrient.

LITERATURE REVIEW

Importance of Leafy Green Vegetables

Diets in most developing countries are composed of a main dish based mainly on carbohydrate staples like maize,

sorghum, rice or sweet potatoes. This is normally accompanied by a side dish or relish composed of leafy vegetables with or without legumes. Consumption of animal based foods may be low but most societies on the average have adequate nutritional status. Leafy vegetables form a substantial proportion of the diets of peasants in Nigeria and elsewhere in Africa (Ifon and Bassir 1979; Ogle and Grivetti, 1985 and Imungi et al.,1983). Ifon et al.(1979) observed that on the average Nigerians consume about 65 g of fresh vegetables daily. In the hunting and gathering society of Sandawe from Tanzania and other similar societies it has been shown that foodstuffs derived from plant substances are consumed more frequently than meat (Newman 1980; Grivetti 1978; 1979). In agricultural societies it has generally been assumed that the use of wild plant foods is supplementary or of emergency nature (Getahun 1974), implying that they are not eaten regularly or they are somehow peripheral to the diet. Fleuret (1979) has observed the contrary in a case study of the Shambaa people of Lushoto, Tanzania. Ogle and Grivetti (1985) have made similar observations in Swaziland.

Traditional societies have been able to maintain adequate nutritional status through a wide use of a variety of food staples together with wild leafy vegetables and fruits (Grivetti 1978; Ogle and Grivetti 1985). Although the bulk of these diets are composed of staples which contain low levels of nutrients other than carbohydrates, nutritional adequacy is maintained on the average due to their wide diversity in consumption of wild leafy vegetables and fruits. The majority of these staples are low in protein (Ndiokwere 1984) and in most cases with incomplete essential amino acid composition when compared to animal sources. The use of a wide variety of vegetables, even though low in the amount of protein tend to complement each other in amino acids, leads to an overall adequate protein intake (Ogle and Grivetti 1985).

Rural populations in most tropical countries are familiar with edible wild vegetables and fruits. It is not uncommon to find them planted in compound gardens or selectively cultivated in maize or cassava fields. For most of these plant species there is scanty information about their identity let alone nutrient value and chemical components. The nutrient data that are available indicate that these plants can be good sources of carotene, ascorbate, folic acid, riboflavin and the minerals iron, calcium and magnesium (West et al.,1988 and FAO,1968). They car also be important sources of trace minerals and protein (Ogle and Grivetti 1985, Shanley and Lewis 1969, Santos Oliveira and de Carvalho 1975; Faboya 1983, Ifon and Bassir 1979, Keshinro 1983; Imbamba 1973; Maclaren 1961 and Imungi et al.,1983).

Some of the indigenous food plants encountered in household gardens as weeds and wild plants are not only highly nutritious but they are also strategic reserves of essential nutrients that are available at certain critical periods of the year when other more common sources of these nutrients are scarce or completely unavailable (Okigbo 1977). Some trees or perennial leafy vegetables produce leaf flushes during the dry seasons when conventional annual vegetables are scarce or unavailable. It has also been shown that leaf flushes can be regulated or stimulated by pruning (Okigbo 1977).

The value of vegetables as dietary sources for minerals could be adversely affected by high contents of oxalate, phytate and cyanogenic glycosides. However it has been observed that traditional cooking methods rid vegetables of most of the soluble oxalate when the cooking water is discarded. This would otherwise complex with divalent metals in the diet rendering them unavailable for human absorption. At the same time prolonged cooking rids vegetables of cyanogenic glycosides (Ifon and Bassir 1979). However

prolonged cooking denature proteins and destroy heat sensitive nutrients such as β- carotene.

Traditional cooking methods have been of concern to nutritionists with regard to nutrient loss, especially of water soluble vitamins and minerals. Lyimo et al.(1991) looked at the effect of prolonged cooking time and traditional storage methods of vegetables. Prolonged cooking resulted in significant losses in protein, fat and vitamins. Fafunso and Bassir (1976) have studied the effect of cooking and wilting on vitamin C loss in 12 leafy vegetables in Nigeria. Cooking resulted in losses ranging from 25-38%, while wilting for up to 10 hours resulted in 38-66% loss of the vitamin. Studies by Keshinro et al.(1979) looked at three different cooking methods. Losses as high as 100% of vitamin C for the bitter vegetables that need debittering before cooking were recorded. Other studies by Ajayi et al.,1979 reported the effect of blanching with losses of vitamin C ranging from 62.2-93.1%. Sreeramulu et al.(1983) observed losses as high as 98.5% when vegetables were cooked with large amounts of water. These authors recommended the consumption of cooking water when no bitter compounds are present.

Gomez (1981) investigated carotene content of some green leafy vegetables in Kenya and the effects of processing on carotene retention. This study found that cooking results in a slight increase in carotene content as a result of increased tissue breakdown and accessibility of carotene to the extracting solvent. However Ogunlesi and Lee (1979) have found that this slight increase in β-carotene is due to the loss of soluble solids leached out into the cooking water. A similar slight increase in β-carotene after cooking of leafy vegetables has been observed by Imungi et al. (1983). However Park (1987), Shaheen et al.(1977) have observed no change in β-carotene content as a result of cooking. Rahman et al. (1990) evaluated β-carotene losses

using three different traditional cooking methods. Boiling followed by frying in oil in an open pot with stirring resulted in the highest loss of β-carotene (31 to 43 %). The other two methods investigated were simple boiling in a pot with lid and placing vegetables on top of partially cooked rice until cooking is done, then mashing into paste with condiments. These resulted into 11 to 14 % and 2.3 to 11 % β-carotene loss respectively.

Chen and Han (1990) have reported slightly higher losses of β-carotene using traditional cooking compared to steam and microwave methods but the differences were not statistically significant. Trans-cis isomerisation of carotenoids after cooking and heat processing has been reported in a number of studies (van der Pol et al.,1988; Nagra et al., 1988; Ogunlesi et al.,1979; Sweeney et al.,1971).

In general oxidation is the major cause of β-carotene destruction. Thermal processing of foods and all forms of dehydration of leafy vegetables leads to destruction and isomerization of β-carotene to isomers with lower vitamin A activity (Cottrell 1991; Park 1987; Chen and Han 1990).

Folacin loss after cooking vegetables has been reported by Imungi et al.(1983). For cowpea leaves a loss of up to 66% was observed. Only 20% free folacin and 12% total folacin were recovered in the cooking water. It has been observed that the loss of minerals and vitamins can be reduced by cooking vegetables in a minimum amount of water. If the cooking water is consumed, the proportion of nutrient recovered will increase.

Given the importance of leafy vegetables as nutrient sources to the diets of most developing countries Okigbo (1977) has expressed concern about the little attention they have received in horticulture and economic development efforts. Chweya (1985), have identified some indigenous leafy vegetables used in Kenya which could be developed as cultivated or semi-cultivated crops. The identification is

on the basis of their β-carotene, vitamin C, mineral and protein content. Crotalaria brevidens the plant of the present study is among the identified plants. Others are Amaranthus hybridus, Solanum nigrum, Gynandropsis gynandra and Erucastrum arabicum. These are common indigenous vegetables in most parts of Eastern Africa (Sreeramulu et al.,1983; Abe and Imbamba 1977). There is little information about their chemical composition other than the nutrient data. Ethnobotanical reports on some of these wild vegetables indicate that they are very bitter and most need special treatment to reduce their bitterness (Kokwaro 1976; Watt et al.1962). Bitterness in leafy vegetables is mainly associated with alkaloids, diterpenes and phenols.

Ethnobotany of Crotalaria Species

Young leafy shoots of Crotalaria brevidens Benth var.intermedia are used as vegetables by the Luo and neighbouring ethnic groups of both Kenya and Tanzania. A study on the food plants of the Luo of Siaya District, Kenya by Johns et al.(1991) reported this plant as cultivated and edible. In an earlier study by Tallantire and Goode (1975) on food plants of the West Nile and Madi districts of Uganda the same plant is reported as a food source. The leaves are picked, cut into small pieces, boiled in water and local salt added. These leaves are either cooked with or without beans. This study does not mention the taste of the vegetable. Kokwaro (1972, 1976) has also reported that the leaves of Crotalaria brevidens are edible but have a bitter taste. The same author reported that the leaves are used as a vegetable and they are a traditional remedy for general stomach pains and swellings.

Other species of the same genus are reported edible. These includes C. ochroleuca G. Don, which is cultivated in Uganda (Tallantire and Goode 1975). This plant is available throughout the year. C. mucronata is a food plant among the

Sukuma ethnic group (of Shinyanga district, Tanzania) who preserve this for use in dry periods as reported by Glegg (1945). Dried powdered leaves are mixed with Sesamum radiatum Schum and this powder is boiled for 15 minutes then served with other staples. The Sonjo of Tanzania eat the flowers of another Crotalaria species, probably C. polysperma (Johns personal communication). Martin (1971) reported that young leafy buds of C. mucronata Hochst (syn. C. striata) are consumed either raw or cooked as vegetables in Cambodia. Standley and Steyermarker (1946) reported that C. longirostrata Hook & Arn.is an important food plant in Guatemala. Large quantities are sold in Guatemalan vegetable markets (Booth personal communications), and young leafy shoots are cooked and consumed as a vegetable or as herbal teas. However eating of this species is reported to result in drowsiness which is suspected to be due to the presence of alkaloids. Roots of the same plant when mixed with maize are used as a bait to poison wild animals. Watt et al.(1962) have documented medicinal and other uses of Crotalaria species found in Southern and Eastern Africa. This includes the potential use of some species as fodder crops. The authors have also included the economic significance of certain toxic species responsible for the production of serious intoxication in domestic animals and birds. Toxic effects of Crotalaria species in this region have been documented as far back as 1904.

Sources of Pyrrolizidine Alkaloids

The genus Crotalaria is characterized by the presence of pyrrolizidine alkaloids. Seeds of Crotalaria brevidens contain toxic pyrrolizidine alkaloids, namely integerrimine and usaramine (Smith et al.,1981; WHO,1988). Pyrrolizidine alkaloids also occur in the following plant families: Boraginaceae (all genera), Compositae, (tribes Senecionae and Eupatoriae) and Leguminosae, genus Crotalaria. Species

belonging to these families are spread throughout the world. It is estimated that about 3% or over 6000 species of the world's flowering plants contain pyrrolizidine alkaloids (Smith and Culvenor,1981; Holzer et al.1987).

BIOLOGICAL ACTIVITY OF PYRROLIZIDINE ALKALOIDS

Plants containing pyrrolizidine alkaloids have been of medicinal interest since very early times and recently of renewed interest due to their varied and remarkable tumour inhibitory activity and toxic effects. Zalkow et al.(1979) reported that medicinal and other uses related to treatment of tumours and cancer dates far back as the 4 th. century B.C. and 10 th. century A.D. respectively. Tumour-inhibitory activity is widely distributed among the pyrrolizidine alkaloids and significant activity has been demonstrated with extracts of Heliotropium indicum (Boraginaceae). The active component has been identified as indicine N-oxide. Antitumour activities are also reported with monocrotaline, which is said to be more destructive to malignant cells than normal ones (Zalkow et al.,1979). Molyneux (1988) has isolated australine from Castanospermum australe which has high glucosidase inhibitory activity. The antitumour activity of these alkaloids is attributed to the strong antimitotic activity of their metabolites but this has not been conclusively established (WHO 1988).

Toxic Effects

Plants containing pyrrolizidine alkaloids, especially those belonging to the genera Senecio (Compositae), Crotalaria (Leguminosae) and Heliotropium (Boraginaceae) have attracted a great deal of attention due to their causative effects in heavy loss of livestock in many countries. The first report of pyrrolizidine poisoning to livestock appeared in the 18 th century (Zalkow et al.,1979). The wide distribution of the families Compositae,

Leguminosae and Boraginaceae has resulted in pyrrolizidine alkaloid poisoning being a global problem. Qualls et al.(1978) reported pyrrolizidine alkaloid poisoning be an increasing problem in Western USA. A 900% increase of cases was reported over ten years due to contamination of alfalfa hay.

Pyrrolizidine alkaloids have been found to be toxic for all animal species (Schoental et al., 1963; WHO 1988). However some species, notably the guinea-pig, sheep, goat and rabbit are somewhat resistant (Chesney and Allen, 1973; Swick et al.,1982). The pattern of toxicity varies between animal species, individual alkaloid and dosage. Pyrrolizidine alkaloids poisoning leads to hepatotoxicity producing liver necrosis and cirrhosis. A veno-occlusive disease characterized by the dominant occlusive lesion of the centrilobular veins of the liver in both man and animal is caused by these alkaloids. In small amounts they induce arterial hypertension (Lalich and Ehrhart,1962). The primary organ of toxicity is the liver which metabolizes these alkaloids. Effects on other organs like the lungs, kidney and the central nervous system are a result of spillover of these toxic metabolites (WHO 1988; Roth et al.,1981). A number of pyrrolizidine alkaloids are considered hepato-carcinogens as well.

Structure and Toxicity

Pyrrolizidine alkaloids constitute a class of more than 200 compounds and the general structure includes a methylpyrrolizidine nucleus with one or more hydroxyl groups esterified by organic acids (fig 1.1) (Vollmer et al.,1987). Alkaloids containing a 1:2 unsaturated pyrrolizidine ring together with ester substituent are hepatotoxins producing veno-occlusive disease, hepatomegaly and some have been found to produce liver cancer in experimental animals. Schoental (1957) found that among other things the allylic

ester function is essential for liver toxicity. Substitutions at the α- position of the acid and esterification of the C-7 hydroxy group both enhance the toxicity of the alkaloids (fig 1.1) (WHO 1988). Thus, according to Mattocks (1968), rosmarinine, retrocine, and other similar structures are not toxic (fig 1.2).

Invivo Activation of Pyrrolizidine Alkaloids to Toxic Metabolites

There are three major metabolic routes of unsaturated pyrrolizidine alkaloids in animals (fig 1.3) (WHO 1988). These alkaloids can undergo N-oxidation, induced by hepatic microsomal enzymes (path a) or they can undergo hydrolysis of their ester groups (path b). Dehydrogenation of the pyrrolizidine nucleus (path c) is induced by the hepatic mixed function oxidase enzyme system. This pathway has the requirement for cytochrome P450, oxygen and NADPH (WHO 1988).

Pathways (a) and (b) are believed to be detoxification mechanisms, whereas path (c) leads to toxic metabolites and appears to be the major activation mechanism. The dehydro alkaloids thus formed are highly reactive alklylating agents (Mattocks 1968, Huxtable 1980). These can attack nucleophilic centres of nucleic acid or protein such as sulfyhydril groups forming tissue bound pyrroles. Hydrolysis of these dehydro alkaloids leads to less reactive dehydro necine which are still very toxic. A more reactive metabolite (E)-4-hydroxy hex-2-enal and other metabolites are a result of the breakdown of these dehydro necines *in vivo* (Segall et al.,1985 and WHO 1988). Although other minor routes of metabolism are known, the process above accounts for the major known toxic effects of these alkaloids (WHO 1988).

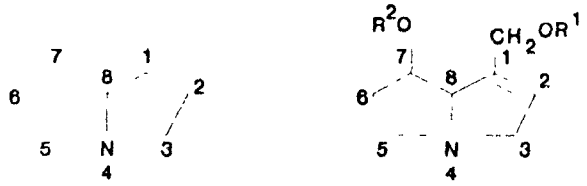


Fig 1 1Molecular Structure of Pyrrolizidine Alkaloids

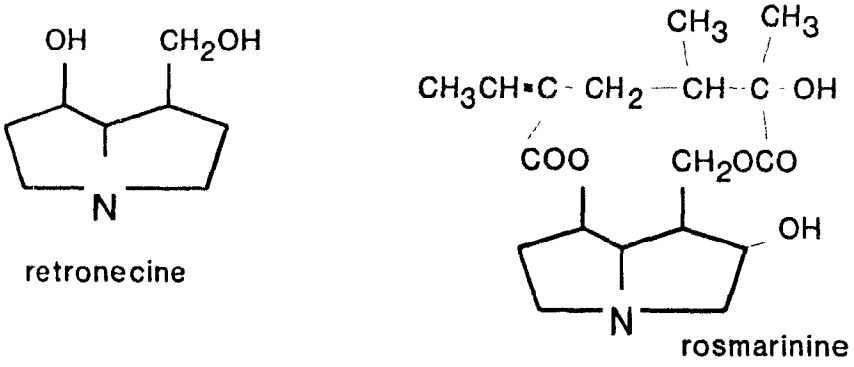
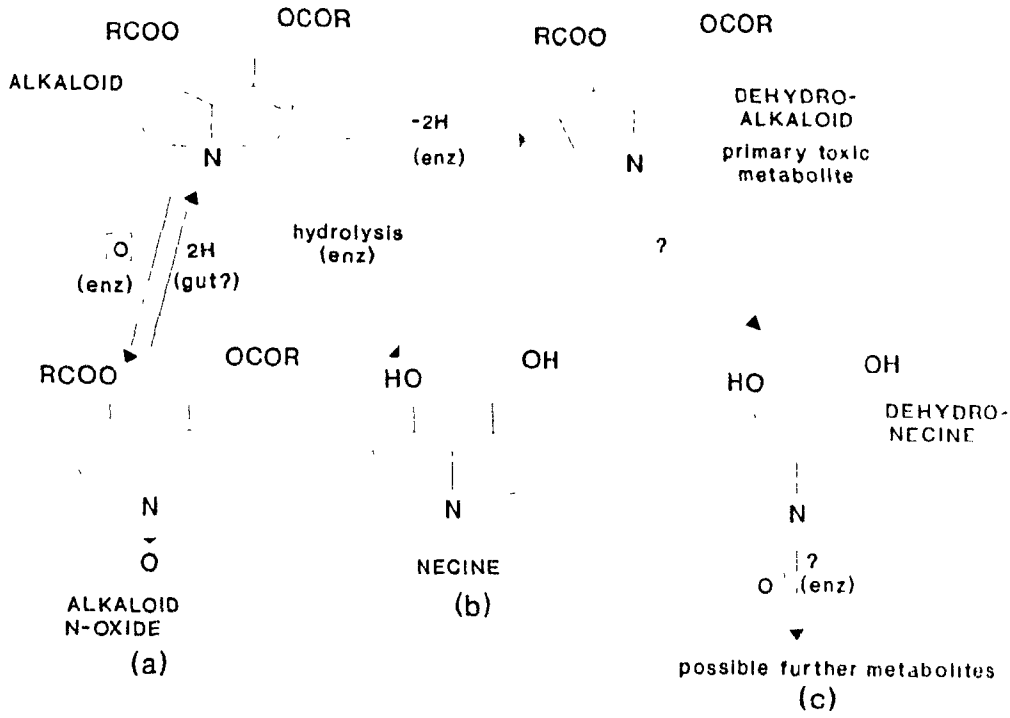


Fig.1.2 Non Toxic Pyrrolizidine Alkaloids

Fig 1 3 Major metabolic routes of unsaturated pyrrolizidine alkaloids



Pyrrolizidine Alkaloid Toxicity and Interaction with Nutrients

Based on the above proposed activation mechanisms researchers have tried to find out the protective effects of thiol compounds and other nutrients. Hayashi and Lalich (1968) investigated the protective effects of mercaptoethylamine and cysteine against monocrotaline intoxication in rats. Cysteine was found to be protective. The same protective effects was demonstrated by Buckmaster et al.,1976.

Further studies by Garret et al.(1984) found that a combination of dietary branched chain amino acids, butylated hydroxyanisole and cysteine increased survival times of rats fed tansy ragwort (Senecio jacobea). Addition of vitamin B_{12} and folic acid to the above treatments was found to improve the effectiveness of the therapeutic substances. The vitamins by themselves did not lead to increased survival time of the rats.

Pyrrolizidine alkaloids are metabolised to highly toxic, tissue damaging pyrrole metabolites by the mixed function oxidase enzyme system present in the liver. Several studies aimed at establishing the above proposed metabolic activation have been reported. Allen et al.(1972) studied the effect of pretreatment with phenobarbital (a Cytochrome P450 inducer) and chloramphenicol (an inhibitor of the enzyme system) on monocrotaline induced toxicity. Increased survival time was observed with chloramphenicol pretreatment as compared to phenobarbital and/or monocrotaline alone (table 1.1). Studies on similar lines, done by Petry and Sipes (1987), used phenobarbital and 2-dimethylamino-2,2-diphenyl valerate (SKF 525-a) as a cytochrome P450 inhibitor. Their results confirmed that monocrotaline is activated metabolically into reactive pyrroles by cytochrome P450 enzyme system.

In evaluating species susceptibility Allen and Chesney

Table 1.1
The Effect of Pretreatment on Monocrotaline-Injected Rats

Treatment	No. of Rats	50% mortality (days)	Location of major lesions	Body weight on day 14 (g)
Phenobarbital	15	-	-	179±9
Phenobarbital plus monocrotaline	30	14	lungs	118±20
Chloramphenicol	15	-	-	180±6
Chloramphenicol plus monocrotaline	30	-	lungs liver	175±10
Monocrotaline	30	25	lungs	163±16

Allen et al.,1972

Table 1.2
Rates of Administration of Pyrrolizidine Alkaloids Leading to Tumours in Rats

Alkaloid	Dosing Schedule	Approximate equivalent (mg/kg/day)	Rats developing tumours
Monocrotaline	a) 25 mg/kg,ip, 1/wk for 4 wk, for 38 wk	3.5 for 4 wk then 1.1 for 38 wk	10/50
	b) 5 mg/kg sc, once per 2 wk for 52 wk	0.36	43/60
Retrorsine	a) 30 mg/kg, ip, single dose	-	7/29
	b) 0.03 mg/ml in water, 3d/wk, to death	1.3	4/14
	c) 0.03-0.05 mg N-oxide/ml in water, 3d/wk for 20 months	1.3-2.0	10/22

Ref. Culvenor (1983)

(1972) showed that the *in vitro* microsomal ability to convert monocrotaline to its N-oxide was the same for both rats and guinea pigs. However the level of dehydrogenation activity in rats was considerably higher.

Tumours have been developed experimentally in rats by a number of researchers following administration of different levels of monocrotaline and retrorsine. Different dosing schedule composed mainly of an initial high dose followed by a maintenance dose for several weeks, resulted in tumour development in the test animals. However in an earlier study reported by Schoental and Bensted (1963), development of tumours was with a single high dose of retrorsine (30 mg/kg BW) (table 1.2).

PA Poisoning and Public Health

Culvenor (1983) reviewed all cases of pyrrolizidine alkaloid poisoning in humans in the world. A total of 12 different alkaloids have been involved (table 1.3) and there has been no recorded development of tumours so far. He suggested follow-up studies for the survivors in some of the epidemics since the population was exposed to levels which are comparable to doses leading to tumours in rats.

Pyrrolizidine alkaloid poisoning is a public health problem in many areas in the world. This is partly due to the wide distribution of pyrrolizidine alkaloid containing plants. Several Crotalaria, Senecio and Heliotropium species are weeds which flourish well after prolonged periods of drought. This conditions leads to increased chances of grain contamination in the subsequent harvests and increases chances of human exposure, and hence higher risk of toxicity.

Pyrrolizidine alkaloids toxicity sometimes has prolonged latent period before symptoms are seen. This kind of toxic effect is difficult to relate to pyrrolizidine alkaloid consumption due to the long latent period with

Table 1.3
Estimated Rates of Consumption of Pyrrolizidine alkaloids by Humans

Alkaloid	Intake Rate (mg/kg.d)	Duration	Effect
Heliotrine	4-10	20-50 days	Liver necrosis, veno-occlusion
Heliotrine and Other PA	0.05	2 year	Liver necrosis, veno-occlusion
Echimidine and related PA	0.01-0.1	Indefinite	Nil
Indicine N-oxide	3-50 (iv)	5 days	Myelosuppression
Crotananine, cronaburmine	up to 1	(Several months?)	Liver necrosis, veno-occlusion
Retrorsine, riddeline	a) about 1.4-3 b) 0.7-1.5	< 2wk 2 wk	Liver necrosis Liver fibrosis, cirrhosis

Ref. Culvenor (1983)

no apparent ill-effects. Therefore it is considered that pyrrolizidine alkaloid poisoning might be a bigger problem than currently recognized (WHO, 1988). Pyrrolizidine alkaloids also act synergistically with aflatoxin and hepatitis B virus (WHO 1988) thus making it even more difficult to differentiate these effects.

There are two sources of human exposure to these alkaloids: 1) accidental contamination of food and, 2) deliberate use of pyrrolizidine containing plants in herbal preparations or as leafy vegetables. Recent epidemics reported in India and Afghanistan were due to accidental contamination of food grains by seeds of Heliotropium and Crotalaria species (Mohabbat et al.,1976; Tandon et al.,1976). The Afghanistan case involved more than 1600 people, and in the Indian case 67 people were involved. Most died due to veno-occlusive disease.

Chronic contamination of food grains is reported in South Africa and the offending plants are Crotalaria and Senecio species. Senecio poisoning by this route is considered to be responsible for the high incidence of primary liver carcinoma in this area. It has also been suggested that, in view of the widely observed hepatotoxicity of pyrrolizidine alkaloids in experimental animal studies, consumption of plants containing these alkaloids might be of etiological significance in human liver disease, especially in developing countries where they are consumed as food or herbal medicines (WHO 1988). Huxtable (1980) reported two cases of pyrrolizidine poisoning by herbal teas among infants in the USA. In assessing the problem further he observed that mortality due to liver cirrhosis in Arizona is consistently much higher than the national average. He attributed this to the cultural custom of the Mexican and American Indians for drinking herbal teas prepared from plants containing these alkaloids. It is also a concern to know that large

populations in some countries might be exposed to low levels of these alkaloids in commonly available foods such as milk, honey (Australia) and comfrey (Sympytum officinale) used in salad in many countries (Panter and James, 1990; Dickison et al. 1976 and Culvenor et al.,1981).

SUMMARY

The importance of leafy vegetables in the diets of most tropical populations is now well recognized and has recently received reasonable attention by researchers. A number of wild leafy vegetables have been identified and to some extent their nutrient composition has been determined.

Traditional cooking methods and the rationale for such practices have been documented as well. Some traditional cooking methods have been simulated in the laboratory set up to study their effects on the nutrient composition of these vegetables. The concern raised by some nutritionists about high nutrient loss following traditional cooking methods should be considered with caution. This is based on the fact that there is no information available about these vegetables other than their nutrient composition. This then calls for urgent need of detail phytochemical analysis of these wild vegetables.

Ethnobotanical surveys and other similar studies indicate that Crotalaria species are important food plants in a number of populations in the tropics and these are bitter vegetables. The consumption of Crotalaria species is of concern given that the genus is characterized by the presence of pyrrolizidine alkaloids. Given the fact that unsaturated pyrrolizidine alkaloids are hepatotoxins it has also been suggested that the consumption of these plants should be considered an etiological factor to liver cirrhosis. With the foregoing in mind the following is the hypothesis of the present study:

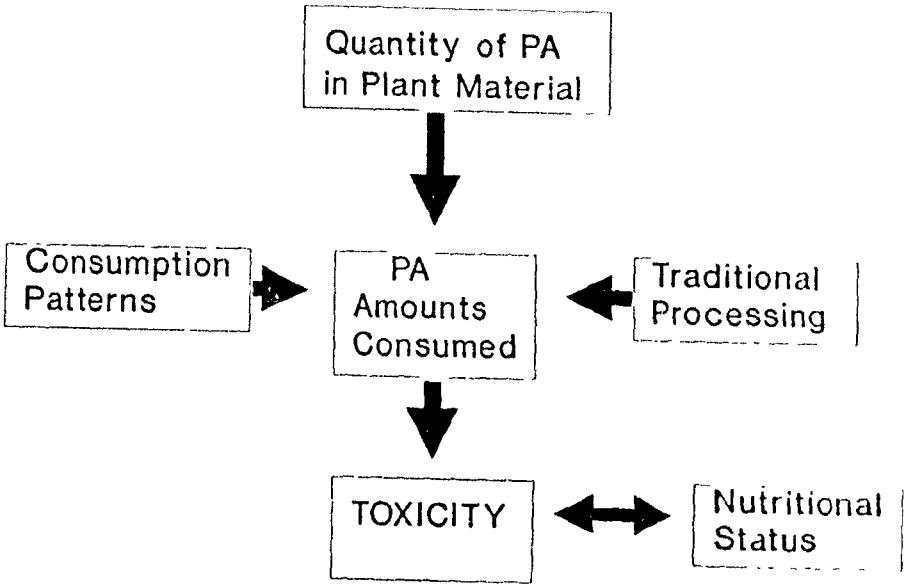
Crotalaria species, important leafy vegetables, contain alkaloids which are detrimental to humans in the pattern in which they are consumed.

Conceptual Framework of the Study

It is assumed that the amount of pyrrolizidine alkaloids consumed by an individual using Crotalaria species as vegetable is directly related to the original quantity of these alkaloids in the plant material. This amount can be modified by two human functions: (1) the traditional processing the vegetables undergo before consumption and (2) the total consumption pattern of these individuals. Given such modifying factors the amount consumed can lead to either acute or chronic toxicity.

The manifestations of toxic effects will have a direct effect on the nutritional status of the individual and population as a whole. At the same time nutritional status can ameliorate or attenuate, to some extent, the toxic effects given that sulphur-containing amino acids and other nutrients involved in the synthesis of the cytochrome P450 enzyme system can modulate the toxicity of these alkaloids. Therefore nutritional status directly affects toxicity and the reverse is also true (fig 1.4).

Figure 1.4 Conceptual Framework of the Study



CHAPTER 2

Study design

The study was divided into two parts: Part one was a descriptive field study done in Mara region, Tanzania, where consumption patterns and traditional processing techniques were defined. Dietary information was collected using a 24-h dietary recall and a seven day food frequency questionnaire. Information about traditional processing was collected separately. Part two of the study was a laboratory based chemical analysis and data analysis. The chemical analysis aimed at quantifying and identifying the particular alkaloids present in the edible portion of the plant in order to correlate this to any potential health risk posed to this population due to their consumption of this leafy vegetable. B-carotene content of this vegetable was also analyzed. A summary of this design is given below.

STUDY DESIGN

STAGE 1: DESCRIPTIVE METHODS
- i. Preparation methods
- ii. Consumption patterns/frequency
- iii Quantification/portion size
- iv. Sample collection

STAGE 2: CHEMICAL ANALYSIS & DATA PROCESSING
- i Alkaloids
- ii. B-carotene
- iii Data preparation & Analysis
- iv. Interpretation of results

Research Goals

The study had the goal of establishing consumption patterns of wild leafy vegetables and fruits in general with the specific aim of determining consumption patterns of Crotalaria brevidens. It also sought to assess the importance of this vegetable as a dietary source of β-carotene taking into account the existence of other dietary sources of this nutrient. The study also sought to compare consumption patterns of Crotalaria brevidens in urban and rural population thus indirectly looking at social economic differences and wild leafy vegetable consumption. Methods used to attain all the above goals were a seven day food frequency questionnaire and 24-hour recall administered at the same time.

A specific questionnaire was used to determine particular traditional cooking methods, treatments and preparations for Crotalaria brevidens which might be used to detoxify the leafy vegetable.

Phytochemical analysis of the edible portions of Crotalaria sp. was carried out to identify and quantify the amounts of pyrrolizidine alkaloids present in these portions. Amount of pyrrolizidine alkaloids thus found was evaluated to see if they pose any health risk to the population given their current consumption levels. Assessment of the risk to toxicity was based on extrapolation from usual portion size established on the basis of the food frequency questionnaire and the amounts found on the basis of phytochemical analysis.

Study Area

The study area was in Tarime district, Mara region, Northern Tanzania at Latitude 1.15^{0}S and Longitude 34.15^{0}E (appendix 1) (Mmbando 1987). The district has borders with Kenya in the north, Musoma in the south, Serengeti in the east and Lake Victoria in the west. Rainfall in this

district is highly variable. A major part of the district falls within the low rainfall range of 800-1000 mm per annum, while some areas receive between the 1000-1200 mm yearly and a small portion is within the 1200-1400 mm per annum range.

The soil is a mixed loam and sand and a small portion has clay soil. Vegetation is basically Savannah grassland with scattered short trees and thickets. Main activities in Tarime district involve fishing in Lake Victoria, cattle herding and cultivation. Main food crops are sorghum, cassava and to a limited extent maize. In the high rainfall area coffee and bananas are cultivated as cash crops.

Based on the 1978 National census, the population was 723,827 and given an annual increase rate of 3% it was projected to be 996,404 people in 1990. The disease pattern of Mara region as compared to the whole country record for 1989 appears in appendix 2.

Study Population

Women involved with food preparation in a household were the subjects of the present study. This was because they were the ones conversant with food plants used as vegetables and their preparation methods. The respondents were mainly of the Luo ethnic group from Nchage within the Tarime town area, Girango, Luimbo and Nyanja divisions in Tarime district.

It was not feasible to divide the study population into two groups, ie. urban and rural, in order to compare their consumption patterns as originally planned. This was due to the small number of Luo ethnic group in the urban centre. This centre contained more than 10 different ethnic groups and the Luo were the least represented (District cultural office unpublished reports). Given that food and culture are closely related we decided to restrict our choice of subjects to the Luo ethnic group. Therefore the dietary data

was collected on one single group.

The study population lived within 1200 and 1400 meters above sea level. This excluded the low rainfall area where Crotalaria was not available.

Subject Selection

Due to shortage of time, insufficient funds, inadequate transport facilities and the poor road system in the area, it was not possible to conduct a systematic random sample selection as initially planned. Instead subjects were recruited through the church and women groups affiliated to the church or through the women's organization under the umbrella of the ruling political party.

A date for an introductory meeting was fixed with these representatives and they undertook to inform people to assemble for the meeting. People who turned out for these meetings (which included both men and women) were informed of the purpose of the study and the women were requested to participate. After explaining the purpose of the study and the necessary formalities of introduction and inspection of research permit by their representative the rest of the people were dismissed and only those who were to be interviewed remained. People coming long distances from the interview site were interviewed first on the day of the introductory meetings.

A contact person was identified and this person had the responsibility of informing the other people when we arrived at the site for the subsequent interviews. In two locations (Tatwe and Shirati) a house to house interview was conducted for a period of one week each. In both locations we had to move with a representative woman from the area who introduced us to the subjects. These representatives were identified during the initial meetings and they were mostly in the leadership position either in the church or other organization.

Interview days were fixed such that they did not coincide with market days in the particular location to minimize the disruption of the survey on the subjects daily activities. Market days are held every alternate day and these days varied from one location to the other. This led to having all the days of the week well represented in the 24-hour recalls except Saturday since we were unable to do interview on Sundays. However the daily food intake in this area is thought to be practically the same, there is no weekend effect expected on any of their meal patterns. Since the subjects are not restricted to a single market location and the market locations are fairly close together their food intakes are not affected by market day either.

The initial aim of the study was to interview 120 women. Only 74 subjects were interviewed in the present study due to low attendance and communication problems encountered in the study. The distribution patterns of the interviewed subjects in terms of divisional location and altitude, appears in table 2.1.

Sample collection

Samples for alkaloid analysis were collected each day from each of the locations covered in the dietary survey. Only aerial parts of the plant were collected, leaves were plucked from the stems in the evening and air-dried under the shade. These daily collection samples were pooled into one lot analysis sample. Other samples collected in Kenya in 1988 and 1991 were bought from the vegetable markets.

Vegetable samples, for β-carotene analysis were

DIVISIONAL DISTRIBUTION OF STUDY SUBJECTS AND RESPECTIVE ALTITUDE

Altitude (m)	Division	Ward	Village	No: Subjects
1380	Nchage	Nyamasangura	Biafra	3
to				
1610		Turwa	Nyamwanga	5
1200	Luimbo	Rabur	Makongoro	7
to				
1300		Nyaturogo	Omugajuu	9
1300	Girango	Mika	Mika	1
to				
1400		Koria	Utegi	4
		Nyassoro	Nyassoro	1
		Goribe	Tatwe	16
		Mirare	Riagoro	4
		Mirare	Ingri	4
1200	Nyanja	Mukoma	Shirati	16
to		Kirogo	Sokolaboro	3
1300		Suba	Nyanjabakenya	1

collected at the end of the interview period, steam blanched and stored in a freezer prior transport to the laboratory for analysis. A weighed portion of the un-blanched sample was used for moisture determination.

Plant identification

Plant identification in the field was done by Dr. T. Johns of McGill University, Canada, and Mr. B. Mhoro a staff member of the Traditional Medicine Research Unit, Dar es Salaam University Tanzania. Herbarium staff of the department of Botany in Dar es Salaam University also helped with the identification.

GENERAL APPARATUS AND CHEMICALS FOR ANALYSIS

High Performance Liquid Chromatography.

The modular HPLC (LC-6A) apparatus consisted of glass bottle solvent reservoir, equipped with a low pressure mobile phase filter with a pore size of 2 micron, and a single small plunger pump (Shimadzu, Japan). The HPLC had a system controller module (SCL-6A) with a built-in CRT display which controlled the solvent delivery unit, automatic sample injector, column and oven, detectors and channel selection valves.

An automatic injector Model SIL 6A (Shimadzu) and a variable wavelength UV detector model SPD - 6AV were used. Chromatograms were processed by a Shimadzu C-R4A chromatopac recording integrator.

Gas Liquid Chromatography

A Varian model 3700 gas chromatography was used. This was fitted with a Shimadzu C-R6A Chromatopac integrator and recorder. Peak detection was by flame ionization detector. Carrier gas was Helium at a flow rate of 20 ml/min and combustion gas was a mixture of air and hydrogen at a flow rate of 25 ml/min.

UV Spectrophotometer

A Beckman (DU-40) spectrophotometer utilizing a single beam

technology was used. Readings were in absorbance. Fixed wavelength mode was used for quantitation while the scanning mode was used to record spectral characteristics of the compounds. Calibration of the instrument in both operating modes was done using a solvent blank.

Nuclear Magnetic Resonance Spectroscopy

NMR was done on Varian XL 300. Spectra width was 4000 Hz and pulse width was 11.0 μ sec. Acquisition time was 3.752 sec., and offset of 700 Hz. Pulse delay was 10.0 sec with 64 transients and NNN decouple mode. Samples were run in $CDCL_3$ solvent.

Thin Layer Chromatography

Plates were Silica gel 60 F_{254} pre-coated (20x20 cm), layer thickness 0.25 mm (E.Merck, Darmstadt). Sample spotting was done using calibrated (10 μl) pipettes. Detecting reagents for alkaloids were Dragendorf reagent (Munier modification), Potassium iodoplatinate, Mayer's, and UV lamp at wavelength 254 and 356 nm. The 1:2 unsaturated toxic pyrrolizidine alkaloids were detected with reagents prepared according to Mattocks (1967a,1967b). Authentic reference samples of monocrotaline, retrorsine and retrorsine N-oxide were always co- chromatographed with the samples.

Solvents and Chemicals

Solvents for extraction and preparation of reagents were obtained mainly from BDH Company (ACS reagent grade). For HPLC work OmniSolv grade was used. Potassium phosphate was obtained from Aldrich Chemical Co. and their Gold label grade was used throughout the alkaloid separation and preparative work. Monocrotaline, retrorsine and retrorsine N-oxide were also obtained from Aldrich. Senecionine is a donation from Dr.R.Molyneux (USDA, Western Regional Laboratory, Albany, California). β-and α-carotene were obtained from Sigma chemicals.

CHAPTER 3
DESCRIPTIVE FIELD STUDY

Review of Dietary Survey methods

Methods used in dietary surveys to characterize consumption patterns and usual intake of certain food items which might contain toxins or contaminants considered a health risk are not well defined. A combination of nutritional assessment methods containing variables assessing cultural practices, food habits and food beliefs or a focused questionnaire are considered adequate (Gibson 1990).

There are two approaches used to assess food consumption of individuals or populations. Methods quantifying amounts and types of daily consumption are based on individual recalls or records of food consumed. These methods measure quantity of individual food items consumed over a short time period. The quantities measured are used to calculate nutrient intake for the individual respondents using food composition tables (Stuff 1983). The second approach to assess food consumption measures usual or habitual intake. These methods collect retrospective information on patterns of food use over longer periods. They can also be used to assess consumption patterns of specific food groups.

The methods frequently used in the quantitative estimation are the 24-hour recall (24-h), the weighed food records, or slight modifications of these two. The 24-h recall is a development from the reported two day detailed quantitative diet history of aircraft workers by Wiehl (1942). Quantitative intake information in the 24-h recall is usually collected using common household measures. The volumes are then converted to weights before calculating individual's nutrient intake. In a weighed food record the subject or researcher weighs all foods and beverages consumed by the subject during a specified time. The weighed

food record has a high respondent burden and food consumption patterns might be distorted to simplify the measuring process or to impress the interviewer. This second limitation also applies to the 24-h dietary recall method. The success of the 24-h dietary recall depends on the subject's memory, the accurate estimation of portion size, the degree of motivation and persistence of the interviewer. However this is easily administered and has low respondent burden which leads to high compliance (Gibson 1990, Beaton et al.,1979). Weighed dietary food records and 24-h recalls provide quantitative information on intake but normally they do not measure habitual intake; this can be obtained by increasing the number of dietary records or recalls (Block, 1982; Gibson, 1990). It is generally accepted that a single one day record or recall is a poor estimation of an individual habitual intake (Beaton et al.,1983 and Todd et al.,1983, Hankin 1987). However a single 24-h recall is most appropriate for assessing average intake of foods and nutrients for large groups (Beaton et al.,1979), since errors associated with individuals due to over or under estimation tend to average out.

A dietary history or a food frequency questionnaire are the methods generally used to obtain information on habitual food intake. These methods provide qualitative information on dietary patterns; however quantitative intake of individuals food items using these methods are often inaccurately reported (Bingham 1987).

For the dietary history method subjects are required to keep a diary of foods and beverages for prolonged periods of time. This is normally validated by a 24-h recall of actual food intakes administered at certain intervals during the record period. This method is expensive, has a high respondent burden and it is not suitable for developing countries where communication facilities and literacy levels are low.

The other method frequently used is the food frequency questionnaire whose origin is also attributed to Wiehl (1942). Similar questionnaires have subsequently been developed either for completion by the subjects themselves or for administration by a trained interviewer (Block 1982). An extensive food frequency questionnaire can be used to estimate total food intake and food diversity of a population or group. The objective of a food frequency questionnaire is to assess the frequency with which certain food items or food groups are consumed during a specified period (either weekly, monthly or yearly). These questionnaires may be purely qualitative (Acheson and Doll, 1964; Stefanik and Trulson, 1962) or they may collect quantitative information of intake as well as frequency (Epstein et al.,1970; Hankin et al.,1975; Jain et al.,1982; Abramson et al.,1963).

Other modifications of the food frequency questionnaire have led to the development of a focused food frequency questionnaire. This questionnaire uses a specific combination of certain foods as a predictor for intakes of certain nutrients or anti-nutrients provided that the dietary components are concentrated in a relatively small number of foods or specific food groups. Such focused questionnaires can also be used as a predictor of the consumption of toxins known to be present in specific food items (Gibson 1990).

Results of a food frequency questionnaire generally represent usual or habitual intakes over an extended period of time. A food frequency questionnaire is often used in epidemiological studies investigating possible associations between dietary habits and disease patterns (Gibson 1990).

The validity of the food frequency questionnaire was tested by Abramson et al.(1963) in a single interview which included questions assessing frequency of consumption and amounts of food items consumed. Correlation coefficients

between quantity and number of times per week an item was consumed were between 0.5 and 0.96 for various food items. Morgan et al. (1978) have also found a food frequency interview to provide reliable results. Other studies have yielded variable results with correlation coefficients between dietary methods ranging from essentially zero (Stuff et al.,1983) to very high 0.69-0.94) (Bolagh et al.,1968).

Reproducibility and validity of the semi-quantitative food frequency questionnaire was tested by Willett et al.(1985) using four one-week diet records. Intra-class correlation coefficients for nutrient intakes estimated by the one-week records ranged from 0.41 to 0.79 and these were similar to those computed from the frequency questionnaire (range 0.49 to 0.71). This indicates that a simple self administered questionnaire can provide useful information about nutrient intakes over a one year period. Although food the frequency questionnaire is not a very precise survey instrument its simplicity, low cost and the avoidance of the need to convert the data to nutrient intake outweighs its limitations.

Other studies, which have addressed reliability and validity of existing dietary assessment methods have been reviewed by Block (1982) and Bingham (1987). It is generally accepted that food frequency questionnaire has a role in-case finding studies and possibly in nutritional surveillance programs. It can identify persons who are likely to need detailed dietary investigation and treatment. In epidemiology, where quantitative precision of food intake is not required, the food frequency questionnaire is a valid research method. This method locates individuals on the distribution in broad categories of low, medium and high intake. These categories provide rough estimation and correlation of dietary intake with nutritional status or disease prevalence but do not permit precise estimation and association of dietary intakes with the parameters

investigated. However it is also accepted that there is no ideal dietary method for use, although there are preferred methods for particular purposes (Beaton et al.1983; Muller et al.,1984; Bingham, 1987; Gibson, 1990).

METHODS

Description of the 24-h dietary recall procedures

Complete information was obtained about all food and beverage intake during the preceding 24 hours (appendix 3). The information obtained included a description of each item consumed, the methods of preparation, and the quantities of each food item consumed. Portion size for the 24-h recall was estimated by the subjects using measuring cups, bowls and other household measures obtained from their local markets. This included different sizes of gourds marked to correspond cup volume measures.

A standard set of questions were used to elicit a complete list of food items consumed. For example after all foods for a particular meal were described by the subjects, the list was read to the subject. The subjects were then asked, "Is this every thing you ate?". If foods were mentioned which are consumed in a combination with other foods the subject was asked, "With what did you take this?". If tea, coffee or porridge was taken then the subject was asked if it was taken with milk, sugar or fruits such as lemons as it is commonly done in this area. Leading questions were not used; i.e. the subjects were asked "what was the first food you ate yesterday after waking up" instead of "what did you eat for breakfast". The same type of approach was used for other meal and snack times.

Description of the 7-day food frequency questionnaire.

The food frequency questionnaire was designed to obtain information about habitual weekly food consumption patterns during the months of June and July (appendix 4). This is

towards the end of the rain season and most vegetation including Crotalaria brevidens and other leafy vegetables are in full bloom and abundant supply. This time of the year was expected to correspond to a period of increased leafy vegetable consumption.

Usual average portion size was also obtained. The questionnaire focused specifically on the consumption of different species of vegetables and fruits. This was planned such that consumption frequencies of Crotalaria sp. could be used to predict intakes of the non-nutrient toxic components (pyrrolizidine alkaloids) occurring in the plant.

Food categories defined for the food frequency questionnaire were as follows: cereals, leafy vegetables, vegetables, animal products, legumes & seeds, fruits and juices and fats & oils. Under each category a list of respective foods followed. This acted as a memory prompt to the subjects and to the interviewer. The food list was extensive covering all available foods in the area, especially vegetables and fruits. This was done in order to provide information on diversity and sources of β-carotene in general. List of foods commonly available in the area was compiled from food list appearing in the Tanzania and East Africa food tables (Marealle 1974; West et al., 1988) and other sources. A list of wild leafy vegetables and fruits was supplied by the district cultural officer in Tarime town and other people interviewed during the initial week when the questionnaires were pre-tested. This list of wild fruits and leafy vegetables was repeated to the subjects after the conventional list was completed.

Description of the vegetable preparation and sample collection data sheets.

A special questionnaire to find out specific preparation methods of Crotalaria sp. was used (appendix 5). This was designed to determine attitudes towards the use of the study

vegetable and bitterness in foods as a whole. Details of preparation methods were sought, cooking time and any condiments added to the vegetable were recorded. The questionnaire also included a question about tradition storage and preservation methods used to store vegetables.

A sample collection form was prepared. This was intended to collect information on general condition of plant, maturity stage (i.e. flowering, fruiting etc.). This form also contained a section on sample preparation for transportation. This kind of sheet was not used since it was not possible to collect many plant samples. A copy of this questionnaire together with the informed consent form appear in the appendix 6 and 7 respectively.

Data Entry and Analysis

The 24-hour recall data was grouped into main meal times as follows: 1) Breakfast - any reported intake before eleven o'clock. 2) Lunch - any reported intake between eleven o'clock and three o'clock. 3) Dinner - any reported intake after three o'clock and midnight. Number of individuals who recalled intakes within these broad categories was added and percent main meal recalled is based on total number of subjects who recalled the main meals and total subjects interviewed (74). To get type of food group total all the recalled main foods were recorded and number of individuals recalling each food recorded according to meal time was added. Percent calculation is based on total main foods per meal time and the particular main food. Overall percent calculation is based on total number of main foods recalled by the study group and the respective main food group total.

Dietary consumption frequencies of each recorded food item were converted into weekly consumption frequency scores by multiplying the number of meals the item was consumed in a day with the consumption frequency in a week. Frequency

consumption scores for each individual were entered on a spread sheet using Lotus 123 computer software program. Total frequency scores for different food groups and a total of all the food items were computed for all the individuals interviewed and for the whole group using the same program. Total consumption frequency scores were computed on the basis of recorded frequencies irrespective of whether the food item is a main staple food, eaten alone or is a minor component of a side dish to a main meal.

The consumption frequencies obtained for Crotalaria sp. were ranked in an ascending order using Lotus computer program. Several regression analyses were used to determine associations and the type of relationship between the consumption of this suspected toxic leafy vegetable and other foods. The assumption was that if the relationship was completely random then the slope of the regression line would be zero. The following are the relationships which were evaluated in relation to Crotalaria sp. consumption scores keeping Crotalaria sp. consumption scores as the independent variable throughout:

1) Cultivated Leafy Vegetables. 2) Wild Leafy Vegetables (excluding Crotalaria scores). 3) Green Leafy Vegetables (excluding those of Crotalaria). 4) Vegetables High in β-Carotene. 5) Total Intake of Vegetables. 6) Vitamin A Animal Sources. 7) Total Animal Product Intake. 8) Total Consumption of Seeds and Legumes. 9) Consumption of Fruits High In β-Carotene Content. 10) Consumption of Fruits in General. 11) Consumption of Fats and Oils which are Vitamin A Sources. 12) Total Consumption of Oil. 13) Total Consumption of Vitamin A Animal Sources. 14) Total Consumption of Vitamin A Plant Sources (excluding Crotalaria scores). 15) Total Animal and Plant Product Vitamin A Sources (excluding Crotalaria scores).

RESULTS

FOOD CONSUMPTION PATTERNS

(a) Consumption Patterns Observed From Food Frequency Questionnaire

The staple food of the Luo in Tarime district is a stiff porridge known throughout Tanzania as 'ugali'. Starchy cereals likes sorghum and maize or tubers like cassava are used to make the flour for this porridge. This is served with a side dish composed of either stewed fish, meat and/or leafy vegetables. The consumption of cereals from this group averaged was 19% of all the foods consumed (table 3.1).

Leafy vegetables accounted for almost 23% of the total frequency scores fo foods consumed during the months of June-July. This is the highest contributor overall when considering the seven major categories of food used in this study (table 3.1). The other categories contributed less percent wise to the total frequency score.

A total of 31 different leafy vegetables were documented and specimens of the less common species were collected for botanical identification (appendix 8). Six of these leafy vegetables have not been identified. The list is composed of all the cultivated and wild leafy vegetables collected in the area. A total of 23 of these are collected in the wild whereas the rest are cultivated. Although 31 different leafy vegetables were identified as edible plants, only 24 species appeared on the 7-day food frequency data and only 13 were wild. This means that 7 species out of the available wild leafy vegetables were not eaten on a regular basis. As explained by the subjects the reminder were considered to be an emergency alternative when other sources are not available.

Table 3.1
Percentage Contribution of Different Food Groups to the Total Frequency Scores

Source	Percent	Source	Percent
CEREALS	19.35	SEED AND LEGUME	6.31
LEAFY VEGETABLES	22.69	FRUITS	11.10
cultivated	13.25	High β-carot. fruits	5.75
wild leafy	7.70	Other fruits	5.35
Crotalaria sp.	1.74	OIL	8.56
Total wild leafy	9.44	High β-carotene source	2.14
VEGETABLE TOTAL	19.86	Other	6.42
High β-carot. veg.	13.50	ANIMAL PRODUCT	12.14
Other veg.	6.35	High vitamin A sources	5.42
		Other animal products	5.72
	TOTAL FREQUENCY	100%	

Fruit Consumption Pattern

Fruits in most African societies are eaten as snacks on a casual basis whether cultivated or gathered from the wild (Fleuret, 1986). This makes collection of consumption frequency data rather imprecise since the subjects are less accurate in recall of food items eaten outside of meal times and outside the home.

Another problem is that within this culture, fruits are considered items for children to eat while at play or when waiting for the next meal to be prepared.

While fruits are culturally identified as a child's food this does not mean that adults do not eat them. However it is hard to elicit information of such consumption among adults.

When asking for such information the interviewer had to ask as though they just tasted fruits rather than implying

that they ate this food item.

Appendix 9 lists 25 different fruits recorded from the food frequency interviews. Botanical specimens were collected for all the wild fruits for identification, seven of which have not been identified. Of the reminder 11 fruits are cultivated and 14 are gathered in the wild. The data collected indicates that, on the average, fruits contributed about 11% of all foods consumed. The ones categorized as high in β-carotene contributed slightly less than 6%. Classification by β-carotene content has been drawn mainly from the cultivated ones, since nutrient data on the wild species was not available. Nutrient data are mainly available for mangoes, papaya and oranges. Therefore a contribution of 6% represents an underestimation. It should be noted that the months of June and July are off season for mangoes. The high season for mangoes is in December. A small amount of mangoes was still available in the market possibly obtained from other places. To obtain a better indication of fruit and leafy vegetable consumption patterns a larger study covering different seasons is needed.

(b) Consumption Patterns obtained From 24-Hour Recall

Most of the women interviewed consumed two main meals on the average and the majority (86.5%) had breakfast as well. Forty out of the seventy four (54.0%) women interviewed had breakfast composed of porridge together with sweet potatoes, whereas others had roasted maize alone or some leftover food from the previous night. Only 10 people out of 74 or 13.5% did not have some form of breakfast. Two people (2.7%) did not have lunch and only one person (1.4%) out of the whole group did not take dinner.

The main food item eaten for both dinner and lunch was ugali. The proportions of the two flours ranged from a 1:1 mixture up to 1:3 sorghum/cassava mix, depending on how much of each the individual had at home. For other respondents

this ugali was made from only sorghum. This is because for the particular year of study (1990) the local cassava crop was diseased which led to a number of plants drying out and resulted in a very low harvest. As a result most people were using lower proportions of cassava flour with sorghum than usual.

Ugali was eaten 115 time out of the 145 main meals recorded (79.31%) (table 3.2). Other foods eaten for lunch and dinner in descending order of frequency of use were boiled maize with beans or groundnuts, sweetpotatoes, squash, rice, plantains, and porridge alone.

Ugali is normally served with a side dish. This side dish can either be leafy vegetables or animal products. In this particular study ugali was taken with animal products 107 times out of the total 115 ugali meals (93.0%) and in only 15.7% of the meals (18/115) it was eaten with leafy vegetables. Leafy vegetables were the only side dish taken with ugali in 7% of the recorded main meals (8/115) while in 10 out of the recorded ugali meals (8.7%) these leafy vegetables were part of meat or fish dish (table 3.2).

Therefore leafy vegetable intake as a percent of recorded main meals was 12.4% (18/145) (table 3.2). All the other foods taken as main meals were not accompanied with leafy vegetables. This percent is about half the value obtained in the 7-day food frequency above. However, during the study year (1990), the rainy season was much shorter than usual and drought conditions were prevalent throughout the district. The interviews were done during the dry part of the season when leafy vegetables were not easily available. The seven day food frequency questionnaire collected information on usual intake for the months of June and July while the 24-h recall was a reflection of the prevailing situation.

Table 3.2

Main Food Consumption Pattern Based on 24-h Recall. Percent Ugali and Accompanying Specific Foods Are Given (n=145).

Food Type	Percent
Ugali	79.31
Others	20.69
Ugali Accompanying Food	
Animal Product	93.04
Leafy Vegetable Total	15.65
Leafy vegetable alone	6.97
Leafy vegetable & meat	8.70
Leafy Vegetable as a Percent of total meals	12.41
Animal products as a percent of total meals	76.55
Animal Product source	
fish	63.55
Dagaa	61.99

Animal products as a percentage of total main meal intake was 76.6% (111/145). Fish contributed 64% (71/111) of the total animal product intake. 'Dagaa' (Restrineobola argentatus) (a very small dried fish) was the major type of fish consumed (62% of total fish or 44/71).

Details of the reported meal patterns are presented in table 3.3. This data is divided by meal type ie. Lunch or Dinner, and food type, ie. Ugali or Others. The table also gives details on sources of animal product, ie. Fish or Meat, and the fish source is broken down to the major contributor, ie. Dagaa or Others. The other main type of fish eaten was the Nile perch (Lates niloticus) and some species of Tilapia obtained from Lake Victoria.

Preparation and cleaning of fish before cooking involves removal and discarding of all internal organs including liver. This is possibly because these organs spoil easily. Therefore fish was not considered a source of vitamin A since liver was not eaten. The small fish (dagaa) is dried and eaten as a whole. No information is available about its vitamin A content.

A quantitative estimation of food intake using the 24-h recall was not performed due to difficulties to obtain accurate and reliable estimates of the quantities of food consumed by the individuals interviewed. Food in this area is usually eaten from a common dish shared among several individuals, which makes it difficult for the subjects to estimate their individual intake. Attempts were made to collect information on individual portions but it proved erratic and unreliable. Quantitative estimates of intake were obtained using gourds and bowls turned upside down to mimic the shape of ugali. Subjects tried to estimate the amount eaten in a group but even that was unreliable as well. Most of the times they could not recall how many people shared the food.

Table 3.3

Main Meal Patterns Based on 24-h Recall. The Percentage of Meals of Each Class That Contain Specific Component Are Listed.

LUNCH (n=72)		DINNER (n=73)	
Food Type	**Percent**	**Food type**	**Percent**
Ugali	76.39	Ugali	82.19
Other	23.61	Other	17.81
Ugali Accompanying Foods			
Animal Product	90.91	Animal Product	81.67
Leafy Veg.	9.09	Leafy Vegetables	8.33
Others	-	Others (beans)	10.0
Sources Of Animal Product Accompanying Ugali			
Fish	62.00	Fish	66.67
Meat	38.00	Meat	33.33
Contributors Of Fish Sources			
Dagaa	74.19	Dagaa	57.50
Others	25.81	Others	42.50

Crotalaria Consumption Patterns

The regression analyses described in the methods section were performed not as a model fitting tool, but as a tool for detecting linear trends. The linear fits were extremely poor: R^2 was very close to zero ($R^2 < 0.02$ in general) (table 3.4). Only for wild leafy vegetables (R^2=0.120) and for green leafy vegetable (R^2=0.038) were these values slightly higher than the observed trend. Nonetheless the following general trends were observed: 1) A negative slope was observed in relation to Crotalaria consumption with the following consumptions frequencies: i) fruits high in β-carotene. ii) total fruit consumption iii) total animal product consumption iv) total oil consumption v) fats and oil which are vitamin A sources vi) high vitamin A animal sources and vii) total vitamin A animal sources (table 3.4).

Table 3.4

Regression Analysis of Ranked *Crotalaria* Frequency Scores Relative to Consumption Frequencies of Other Food Groups.

Food Group	Slope value	R squared
Total leafy veg.	0.844	0.0383
cultivated	0.039	0.0002
wild	0.805	0.120
Vegetables	0.441	0.0164
Seeds & Legumes	0.010	0.00001
Vitamin A Plant source	0.766	0.0154
Fruits total	-0.511	0.0181
High carotene	-0.415	0.0226
Animal product total	-0.309	0.0172
Vitamin A source	-0.362	0.0085
Fats & oils total	-0.494	0.0324
carotene source	-0.207	0.0182

This suggests that individuals with high frequency of Crotalaria consumption tend to consume less of all the sources showing negative slope. The most negative slopes were -0.415 and -0.511 corresponding to the high β-carotene fruit group and total fruit consumption, respectively. High frequency scores for Crotalaria sp. had negative association with animal product consumption frequency scores as well. Although there is a negative slope when total leafy vegetable frequency consumption scores are compared to total animal product consumption (-0.04582), the absolute magnitude is much lower than the values obtained for Crotalaria consumption in relation to animal product consumption (-0.30878). This means that there is a specific relationship due to Crotalaria sp. alone other than the general one observed with leafy vegetables as a whole.

2) A positive slope was observed in relation to Crotalaria consumption with the following frequencies: i) cultivated leafy vegetables ii) wild leafy vegetables iii) total leafy vegetables iv) high β-carotene vegetables v) total vegetable consumption vi) total seeds and legumes consumption and vii) total vitamin A plant sources (table 3.4).

Again this suggests that individuals with high consumption frequency of Crotalaria tend to consume more of these other sources. The highest slope value obtained were 0.766 and 0.805 for total vitamin A plant sources and wild leafy vegetables respectively.

This outcome is not unexpected given that a number of leafy vegetables are eaten in combination with other leafy vegetables which tends to increase the overall leafy vegetable scores.

Student's t-test

To test whether the observed trends were significant for any of the above food groups a Student's t-Test

statistical analysis was done (Draper and Smith 1981). The analysis showed that there was a significant relationship between Crotalaria consumption frequency and the following:1) Wild leafy vegetable 2) Total green leafy vegetable. This is in agreement with the trends observed with the regression analysis.

The relationship between Crotalaria consumption frequency and the following:i) fruits high in β-carotene and ii) total oil consumption frequency was negative and almost significant at the $p < 0.1$ level. This relationship might be more distinct with a larger sample size. A study looking more specifically at vegetable cooking methods and the use of oil and milk may explain the negative relationship observed with oil consumption frequency. For all other relationships tested, there were no relationships which were significant at the $p < 0.1$ level (table 3.5). This means that either there is no real relationship between the consumption of these food groups and Crotalaria sp. or there is a relationship and insufficient evidence. This relationship might be clarified with a larger sample size.

Attitudes Towards Bitterness

A total of 21 people out of 38 who volunteered taste information about this vegetable said that it was bitter. Seventeen people said it was not bitter. When asked why they eat this vegetable despite its bitter taste, the majority of them replied that it is food and this is good for the stomach and general health as a whole. Some people said they like the bitter taste and when cooked with milk it is not too bitter. One person observed that this vegetable is not eaten very frequently but most people consider it as food and medicine as well. Other people said that the cultivated type is much more bitter than the wild one. They observed that the wild one is more or less like okra. This information is confusing when one considers food selection

Table 3.5

Significance Based on t-Test Statistics of Slope Values of Fitted Regression Line Between Crotalaria sp. Consumption Frequency Scores and Those of Other Food Groups.

Food Group	t-value Cal.	Tabulated P(value)	Comments
Cultivated green lv.	0.111	0.91	NS $p < 0.05$
Wild green lv.	3.138	0.002	S $p < 0.05$
Total green lv.	1.695	0.094	S $p < 0.10$
High β-carotene veg	1.053	0.296	NS $p < 0.1$
Total veg.	1.096	0.276	NS $p < 0.1$
Vitamin A animal	-0.786	0.434	NS $p < 0.1$
Animal product	-1.122	0.266	NS $p < 0.1$
Seed and legumes	0.023	0.982	NS $p < 0.1$
High β-carotene fruit	-1.289	0.202	NS $p < 0.1$
Total fruit	-1.153	0.252	NS $p < 0.1$
β-carotene oil	-1.156	0.252	NS $p < 0.1$
Total oil	-1.553	0.124	NS $p < 0.1$
Vit.A animal source	-1.227	0.224	NS $p < 0.1$
Vit.A plant source	1.062	0.292	NS $p < 0.1$
Total vit.A source	0.494	0.622	NS $p < 0.1$

and domestication of wild plants for cultivation. There was no way to find out if these people had eaten the cultivated variety. Most of the Crotalaria consumed was collected from the wild and we did not see much Crotalaria species under cultivation apart from three individuals.

One of these was in Ingri, the other one was in Shirati and third one was in Tatwe area. All the three women had their origins in Kenya where it is reported to be cultivated on a more regular basis (Johns et al.,1991).

Cooking Methods of Crotalaria sp.

On the whole two different cooking methods were recorded with slight variations documented between individuals. Initial vegetable preparation is basically the same for the two methods. This involves leaf and flower collection, cleaning and sample reduction by cutting before the actual cooking.

(a) Leaves prepared as above are added into boiling water containing locally prepared magadi soda (bala). The prepared leaves are then cooked for up to 3 hours. The cooking water is discarded, with more water added and then discarded again. This process can be repeated up to three times depending on individuals. Milk is then added, followed by simmering for some time. The vegetable is removed from the fire and kept aside. After 12 hours more milk is added with subsequent simmering. This is repeated every 12 hours for up to three days or even 5 days before the vegetable is eaten. A slight modification of this method involves stewing the vegetables in the last day before serving.

This is the main cooking method recorded. Out of 37 people who gave information on cooking method, 20 used this method. Four people reported that the vegetable can be boiled with water containing the local salt for 30 minutes then milk can be added, followed by simmering for a while followed by immediate serving.

(b) In the second method of preparation this vegetable is cooked together with other vegetables. The vegetables with which it is frequently mixed with are pumpkin leaves, Gynandropsis gynandra, Solanum nigrum or certain species of wild Amaranthus. This mixture is cooked for about one hour in water containing local salt, then stewed with onions and tomatoes fried in cooking oil. After stewing, milk is added and the vegetables are further cooked at simmering heat for

some time before serving. Ghee or butter can be added to the vegetable during eating. This method does not involve the prolonged cooking observed in the previously described method, and the vegetables are eaten on the same day although the same dish can be served for more than one meal or one day.

A slight variation to this cooking method involves discarding the cooking water before the stewing stage. When these vegetables are cooked together with the slimy type of vegetables such as Corchorus sp.(Apoth) or Sesamum sp.(Anyiim) milk is not added to the vegetables but they are stewed as usual. A total of 13 people used this type of cooking method out of the 37 who responded.

Leafy Vegetables and Preferences

After conducting the 24-h recall, subjects were asked which vegetable they preferred most and which one they did not like. Table 3.6 presents a compilation of their preference. Gynandropsis gynandra or 'mgagani' is the leafy vegetable prefered most followed by Corchorus sp. and Amaranthus sp. For Amaranthus sp. this includes the cultivated species and the wild ones since there is no distinction most of the time when these vegetables are collected for cooking. Crotalaria sp. is on the low side of preference, only 5 times prefered and 3 times as disliked leafy vegetable. The most avoided leafy vegetable was Vigna unguiculate or cowpea leaves. Most people complained that when they eat this leafy vegetable they have stomach problems with a lot of rumbling probably due to high fibre content or other factors (West et al.,1988).

Gynandropsis gynandra is commonly refereed to as 'mgagani' but there are names like 'deck' and 'alotdeck'. As Johns and Kokwaro (1991) observed that the meaning of these names underlines the importance of this leafy vegetable to the Luo ethnic group as a whole. Deck is synonymous with

Table 3.6

Number of Women Avoiding and Preferring Different Leafy Vegetables in Tarime District.

Botanical Name	Vernacular Name	Preference Scores	
		Favoured	Avoided
Gynandropsis gynandra	Mgagani/Deck	28	4
Corchorus olitorius	Apoth/Mlenda	21	1
Amaranthus hybridus	Mchicha	17	2
Vigna unguiculate	Boo/majani kunde	9	29
Brassica oleracea var. *capitate*	Kabichi	7	1
Brassica sp.	Sukumawiki/Kandhira	7	0
Crotalaria brevidens var. *intermedia*	Mitoo	5	3
Curcubita sp.	Susa	3	3
Spinacia oleracea	Spinach	2	0
Crotalaria sp.	Nyassamo	1	0
Hibiscus esculentus	Bamia	1	0
Oxygonum sinuatum	Awayo	1	1
Manihot esculata	Kisamvu	1	5
Bassela rhubra	Ndemra/Kirerema	1	6
Solanum nigrum	Osuga/Mnavu	0	1
Justicia matamensis	Piupiu	0	2

vegetable while alotdeck means vegetable food. This in a way explains the high number of preferences obtained for this leafy vegetable in the present study. Only 4 people disliked this vegetable.

Conclusion

Over half (69%) of the respondents reported having consumed Crotalaria brevidens leafy vegetable at least once a month within the period (June-July) of the food frequency recall. This finding to some extent is in agreement with our original hypothesis that Crotalaria brevidens is an edible food plant in the test population. High consumption of Crotalaria brevidens is associated with low consumption of fruits, vitamin A animal sources and total intake of animal products (table 3.4), even though these trends were not statistically significant at the $p < 0.1$ level (table 3.5). We think that given a large sample these trends might be shown to be significant. A similar negative trend was observed with frequency scores of leafy vegetables and animal product scores as a whole. The above observed trends are in agreement with findings reported by Grivetti (1979; 1978); Newman (1980) and Fleuret (1979) in similar study groups in Tanzania and else where in Africa.

The results obtained on the basis of the food frequency questionnaire underlines the important position leafy vegetables, and to some extent Crotalaria brevidens, occupy in the provision of essential nutrients that complement the high carbohydrate staples consumed as the sole food item by the subjects of the present study.

Although the 24-hour recall was intended to validate the food frequency questionnaire it was not possible to carry out the validation. This was due, in part, to the encountered drought conditions prevailing at the time of the survey (1990). This led to scarcity of leafy vegetables and forced the population to rely more on animal products and

mainly dried fish. Given that the food frequency questionnaire is an accepted survey instrument in epidemiology and nutritional surveillance (Gibson 1990; Block 1982; Abramson 1963) we feel confident in assuming that the data is, on the average representative of the consumption patterns of Crotalaria brevidens and that of leafy vegetables in general during the months of June-July. The consumption patterns of fruits and other food groups investigated in the present study was also obtained. We conclude that the respondents relied more on leafy vegetables and fruits as sources of vitamin A as opposed to animal sources during the months of June and July. This is in agreement with what is generally accepted by most vitamin A deficiency prevention programs and organizations (WHO 1982; McLaren 1986). Fruits might be found to be consumed more frequently if the study is repeated at another time of the year especially during the high fruit season reported to be December in this particular area. To get a year round picture of leafy vegetable consumption a longer study covering different seasons is needed.

CHAPTER 4
BETA CAROTENE ANALYSIS

Review of analysis methods

The importance of vitamin A for growth, reproduction, cellular differentiation and vision is well recognized (Goodman 1980; Simpson 1981). In food preformed vitamin A is always present as retinyl esters. The major sources of preformed vitamin A include liver, fish oil and dairy based food products. Precursors of vitamin A occur in plants as carotenoids (Simpson 1981; 1983) and to a limited extent in fat storage site in animal tissues (Simpson et al.,1986).

Vitamin A activity varies within the pro-vitamin A carotenoids depending on their structure. All-trans-β-carotene has the highest vitamin A activity. β-carotene is a common, but not always the predominant, carotenoid in most orange coloured fruits and dark green leafy vegetables. Other pro-vitamin carotenoids are α-carotene, γ-carotene and β-cryptoxanthin, whose activity is around 50% of that of β-carotene. Other minor carotenoids have lower activity. Dietary sources of pre-formed vitamin A and carotenoids vary depending on the population. Recent statistics have indicated that carotene from vegetables contribute 68% of dietary vitamin A on a worldwide basis and 82% in developing countries (Simpson 1983).

Carotenoids are lipid soluble, and readily dissolve in benzene, petroleum ether, chloroform and carbon disulphide but dissolve only sightly in ethanol (Simpson et al.1985). This class of compounds undergoes isomerization and oxidation in the presence of oxygen, heat and light. A prolonged cooking time for leafy vegetables results in an extensive isomerization of β-carotene to less active pro vitamin A isomers (Chen and Han 1990; Nagra et al.,1988; Ogunlesi et al.,1979). Carotenoids are altered or partially destroyed by acids, by bases and by certain enzymes. Light

is especially destructive in the presence of oxygen and some metals. The degradative steps results in the formation of cis-trans isomers, epoxides and chain cleavage, leading to destruction of vitamin A activity. To avoid these changes, manipulations such as sample collection, extraction and storage must be carried out in subdued light and in the absence of drastic reagents. Samples should be stored in the dark at low temperature and preferably in an inert atmosphere (Simpson et al.,1985)

Carotenoids exhibit characteristic absorption spectra around 450 nm. The major absorption maxima of some common carotenoids in different solvent system have been used for their identification and quantification (Simpson et al.,1985; Braumann et al.,1981). Compound such as magnesium oxide, alumina, microcel C and silica gel that are used in chromatographic procedures may shift the absorption spectra of carotenoids and xanthophyll. Silica gel shifts the spectrum of β-carotene considerably. Alumina and diatomaceous earth are considered less chemically reactive chromatographic stationary phases and are used for the separation of this compound (Simpson et al.,1985; de Leenheer et al.,1988).

Separation and quantification of carotenoids is based on physicochemical methods. All the procedures use some form of extraction, usually but not always followed by a saponification step and physical methods of separation of the pigments. Quantification is based on their light absorption properties.

Thin layer chromatography (TLC), colorimetry or fluorometry have been used to a limited extent for carotenoid analysis. These methods are time consuming and show poor sensitivity due to interference of other components present in plants (Braumann et al.,1981). Gas liquid chromatography (GC) has had limited application particularly due to the thermolabile nature of the

isoprenoids and the high operational temperatures needed for this method.

Conventional methods involve extraction with acetone/hexane mixture, followed by separation. The lipid fraction is then separated on a packed activated magnesia-diatomaceous earth using hexane-acetone mixture. The eluted pigment is then quantified spectrophotometrically at 436 nm (AOAC, 1984). The method gives reliable estimates for tissues that contain β-carotene as the only carotenoid, but tends to over-estimate if complex carotenoid mixtures are present as found in fruits and in some leafy vegetables. Certain modifications have been suggested that utilize the correct extinction coefficient. A saponification step is necessary for some of the fruits or oil samples which contain esterified carotenoids.

Modern carotenoid assay techniques are based on liquid chromatography (LC). A standard analytical scheme consists of sample reduction and extraction of plant tissue with a mixture of acetone/hexane as in the conventional methods. This is then followed by lipid extraction. Saponification does not appear to be necessary but is some times performed especially with fruit samples where xanthophyll occurs in an esterified form. Speek et al.,(1986) included the saponification step for all the vegetables analyzed in order to utilize a single method to all the food samples they analyzed. The organic layer was recovered, evaporated to dryness under nitrogen and then the residue was dissolved in a suitable solvent prior to injection on an LC column.

Adsorption chromatography on silica based column is unattractive for quantitative work with carotenoids. Un-derivatized silica is suspected to cause on-column degradation and these packings show little or no retention for β-carotene. These packing also failed to separate positional double bond isomers like α- and β- carotene. The major application for underivatized silica based column lies

in its ability to resolve geometric isomers (cis / trans). Alumina packing are considered less reactive compared with silica and they are capable of separating positional isomers (α- and β-carotene). Alumina also has a higher affinity for β-carotene. However this system requires very stringent precautions and control of both the water content of the mobile phase and temperature in order to obtain reproducible results.

The High Performance Liquid Chromatography (HPLC) separation of carotenoids on micro particulate supports is a rapid, reproducible, and sensitive method for carotenoid analysis. The limit of detection for β-carotene has been reported to be 38.5 pmoles (Simpson et al.,1985). Pigments are identified by retention times and quantification by peak height and area. Within the 20-2,000 pmole range, there is a linear relationship between the amount of pigment injected and peak area, with a linear correlation coefficient of 0.991-0.998 (Simpson et al.,1985).

HPLC results are considered more precise than those obtained by the AOAC method of analysis. However HPLC is not without problems. With an isocratic solvent system on a normal-phase column it is difficult to develop a complete carotenoid profile. Gradient elution has been used successfully but the major drawback with the approach is the long re-equilibration time required with initial solvent system (Simpson et al.,1985; Lambert et al.,1985).

Reversed-phase systems are capable of separating positional double bond isomers of carotenoids, such as α- and β-carotene. The non-polar bonded phase pose low risk of on column carotenoid degradation. Unfortunately reversed-phase columns do not lend themselves very well to separation of geometric isomer. Very often the trans peak is only partially resolved from a composite cis peak (Lambert et al.,1985). Isocratic non-aqueous solvent systems are the most popular mobile phases and have been used successfully

for the separation of several carotenoids using different types of reversed-phase LC columns (Nelis and Leenheer 1983; Simpson et al.,1983; Broich et al.,1983; Bushway et al.,1982; 1986). Polar mobile phases containing little or no water are necessary to elute carotenoids from reversed-phase columns. Different solvent systems have been used. Acetonitrile: dichloromethane or Tetrahydrofuran (THF): methanol mixtures or mixtures of more than two solvents have been used. Introduction of methanol in a mixture of acetonitrile:dichloromethane improves separation (Lambert et al.,1985). Speek et al.(1986) used an aqueous solvent system composed of methanol:acetonitrile:chloroform:water (200:250:90:11) on a home packed ODS-Hypersil 3μm column. Simpson (1983) separated α- and β-carotene and other carotenoids using an isocratic THF/Methanol (10:90) solvent system on a RCM-C18 column. There is no one single isocratic solvent system that separates all pigments in a single step. However by careful selection of column and solvent it is possible to resolve major pro-vitamin A active carotenoids in a single isocratic system.

There is no suitable internal standard for β-carotene HPLC analysis. The proposed dimethyl-β-carotene is not commercially available, it is very unstable and can not be stored without deterioration not even at -70°C. Other derivatives of β-carotene have been proposed as internal standards but they are either too polar and hence elute from the reverse-phase columns well before β-carotene, or they show substantial bathochromic shift in their absorption maxima (λ max 502 nm compared to λ max 450 nm for β-carotene). This necessitates addition of excess amount of internal standard to equal the response of β-carotene at 450 nm (de Leenheer et al., 1988). Sudan 1 (1-(phenylazo)2-napthalenol) has been used as an internal standard by Quackenbush and Smallidge (1986), but this also elutes well before the β-carotene peak.

METHODS OF ANALYSIS AND RESULTS

(a) Calibration Curve

A sample of β-carotene (5 mg, type iv, no: C0126) obtained from Sigma Chemical Company was dissolved in hexane and the solution was diluted to 50 ml in a volumetric flask. From this stock solution several dilutions were prepared with hexane and their absorbance was read on the spectrophotometer. Absorbance readings for the calibration curve concentrations was between 0.0 and 1.0, and these were 3, 2, 1, and 0.5 μg/ml.

HPLC separation was performed using a Supelcosil LC-18 ODS C_{18} 5μm reversed phase column (Supelco), a stationary phase which had been used successfully for separating carotenoids in human serum by Broich et al.(1983). The eluting isocratic solvent system consisted of 10% tetrahydrofuran in methanol at a flow rate of 1.2 ml/min and run time of 30 minutes. Conditions for separation have been tested with standards and found to be reproducible and they are based on unpublished reports by Tsou and Simpson (Simpson, 1983). The injection volume, 2μl, was applied to the head of the column using an auto sample injector. Two replicates injections of each concentration was performed and the β-carotene peak areas were used to establish a calibration curve after calculating the exact concentrations using the following formula: Conc. = (A x path length x 10)/$E^{1\%}_{cm}$ (Simpson et al.1985). The curve thus obtained was used to determine the amount of β-carotene in the Crotalaria sp. samples analyzed.

(b) Crotalaria sp. analysis

Two batches of samples were collected in the field towards the end of July 1990. These samples were blanched in boiling water, transferred into zip-lock plastic freezer bags and frozen before being shipped by airfreight to McGill University in early August 1990. Upon arrival they were

stored in a freezer at -80^{0}C to await analysis in June 1991. Extraction method of carotenoids from plant samples is a modification of that reported by Zakaria et al. (1979) and Simpson et al. (1985) omitting the saponification step.

For HPLC analysis 3 replicate 10 g sub-samples were separately blended with acetone (75-80 ml) in a Waring commercial blender then filtered with a suction pump. This was repeated several times until completely extracted. The combined acetone extracts were extracted with hexane (40 ml) (3 times) and washed with water several times to remove residual traces acetone. The hexane extract was then dried with Na_2SO_4, filtered and the solvent was removed under vacuum using a rotary evaporator. The residue thus obtained was redissolved in hexane, transferred into a 25 ml volumetric flask and the volume was made up to the mark with hexane. The absorbance of this solution was determined at 452 nm. When this was within the expected range then HPLC separation was done, otherwise appropriate dilutions were made with the same solvent. The same conditions used for the calibration curve were used for this separation.

Three dilutions of the initial extract were made resulting into four different concentrations for analysis. Solutions were with dilution factor 1, 2, 10 and 100. Absorbance of each concentration was measured and only the one with 100 dilution factor was within the expected range on the spectrophotometer. On scanning it was found that there was a lot of interfering components whose absorption maxima was less than 452 nm. Because of this interferences the 100 fold dilution runs were not included in the calculation of the amount of β-carotene present in this plant material.

Duplicate HPLC separations were performed for each of the three remaining concentrations. Separation conditions were as in (a) above. On looking at the peak areas it was decided to disregard the first two high concentrations since

these areas were not within the ones obtained for the β-carotene calibration curve. Fig. 4.1 shows the HPLC separation of this plant sample together with the β-carotene one. The sample shows a small peak for α-carotene (Rt 12.0). The retention time for standard β-carotene (Rt 13.2) and plant sample (Rt.13.1) are similar to the reported in the literature for similar chromatographic conditions (Simpson 1983).

Peak areas for the duplicate runs were averaged and used to calculate the amount of β-carotene in the leafy vegetable. The calculation is based on the calibration curve equation obtained with standard β-carotene. The results are presented on table 4.1 together with reported value by Gomez (1981).

The one reported value for the β-carotene level in Crotalaria brevidens is higher than our findings. The method used to determine this reported value is based on an open column separation followed by spectrophotometric quantification (Gomez 1981). This method tends to over estimate the true value of carotenoids for some foods especially those containing both α- and β-carotene. This should not have been a problem in the present study since for leafy green vegetables the active carotenoids are almost exclusively β-carotene (Simpson et al.,1985).

There could be a number of explanations to this difference: i) the reported literature value was determined from the cultivated variety of Crotalaria brevidens and our analysis is based on the wild variety. ii) other factors like maturity stage, geographic and environmental difference might affect the amount of β-carotene present in the parts analyzed. iii) the fact that the sample was stored for several months before analysis might be a reason to explain the low value obtained. However the precautions taken during the storage and transportation were considered to be adequate to minimize loss.

Fig. 4.1 HPLC Separation of (a) carotenoid fraction from Crotalaria sp. leafy vegetable and (b) β-carotene standard sample using tetrahydrofuran-methanol 1:9 (v/v), a 5 μm SupelcoSel C18 reversed phase column and detection at 452 nm.

Absorbance 452 nm
Time (min)
a
b
2.094
3.640
5 085
7.732
10 452
13 108
16 385
18 477
23 417
2 092
7 830
13 216

Table 4.1

β-Carotene Content of Edible Portions of Crotalaria brevidens

Source	Moisture	Carotene (μg/100g)		
	%	Range	Average	Mean
Batch 1	79.93	5624 - 5810	5717*	4907± 922
Batch 2	79.93	3817 - 4497	4096	"
Reported	84.8	6250 - 7750	-	7000 ± 842

*Batch 1 is based on two duplicates and batch 2 is based on 3.

Since the column used was of non polar phase and this type of phase is inadequate for the separation of geometric isomers (Lambert et al.,1985), we were unable to determine whether the sample had undergone trans / cis stereoisomerization. This is not suspected either since the samples were stored in an inert atmosphere at low temperature. Carotenoids are prone to this type of isomerisation only if exposed to high temperatures. Therefore factors (i) and (ii) are considered to provide more plausible explanations for the difference between the values we obtained and those reported by Gomez (1981).

Conclusion

The present analysis established that the edible portions of Crotalaria brevidens contain a high amount of β-carotene (>4900 μg / 100 g edible portion). These findings emphasize the importance of this leafy vegetable as a dietary source of provitamin A, protein (Imungi et al.,1983) and possibly other nutrient to the present study group. The amount of β-carotene in this leafy vegetable is comparable to values reported for other frequently consumed leafy vegetables in this region (Gomez 1981; McLaren 1961; West et

al., 1988; Speek et al.,1986). The reported species which are also frequently consumed by the respondents in the present study are Amaranthus sp., Gynandropsis gynandra, Corchorus sp., Sesamum sp., Vigna unguiculata, Solanum nigram, Ipomea batatas, Manihot ultilisima and Brassica sp. β-carotene content for these leafy vegetables is higher than 5000 μg / 100 g fresh edible portion (Gomez 1981; McLaren 1961). These vegetables could be recommended as substitutes for Crotalaria brevidens if its consumption is considered a high risk factor to the health of this population. Acceptance of the recommendation will of course depend on individual taste, flavour preference. Other factors like other nutrients in the leafy vegetable have to be taken into consideration before recommendation.

CHAPTER 5
ANALYSIS OF PYRROLIZIDINE ALKALOIDS

Review of analysis methods

In view of the adverse toxic effects of pyrrolizidine alkaloids, investigations of plants containing these alkaloids have been of prime importance since the early nineteenth century. Methods for separation and identification have followed the same gradual developmental trend of chemistry as a whole. Originally pure organic chemistry, involving degradative methods and forming derivatives were the only tools available and used (Warren,1955, 1966). Over the past 40 years, non-degradative chromatography and spectroscopic techniques have been introduced. These techniques have had a profound effect on pyrrolizidine alkaloid research.

In the 1950s and 1960s thin layer chromatography (TLC) in combination with colour reagents was used predominantly. Pyrrolizidine alkaloids do not offer much advantage in terms of their structure to the application of these techniques. Most TLC detecting reagents are neither specific nor very sensitive for this specific class of compounds. The use of iodine vapour is of course non specific in general for all compounds, but such reagents like Dragendorf (Sharma et al.,1965) and iodoplatinate (Mattocks,1964) do not offer much advantage either in terms of specificity as compared to other classes of alkaloids (Mattocks,1967, 1968).

The advent of the ultraviolet (UV) spectroscopy in the 1950s did not have a profound effect on pyrrolizidine alkaloid analysis due to the absence of a strong absorbing chromophore within in their molecular structure. Nonetheless efforts to utilise UV spectroscopy for their analysis resulted in development of rather novel techniques for detection. Based on Polonovsky reaction applied to 3-pyrrole ring, a colour reaction was developed by Dann (1960).

Mattocks (1967a) applied this reaction for TLC detection. This involves an on-plate oxidation of the eluted alkaloids to their N-oxides by spraying with hydrogen peroxide followed by subsequent heating. The TLC plate is further sprayed with acetic anhydride which on heating dehydrogenates the N-oxides bases to form pyrroles. The pyrroles thus formed can couple with 4-dimethylaminobenzadehyde (Ehrlich reagent) to give coloured complex which can be quantified visually or spectrophotometrically (Mattocks, 1967a; 1967b). This method is rather long and cumbersome but a modification by Molyneux et al.,(1980) using ortho-chloranil, thus skipping the need to convert these alkaloids to their N-oxides before coupling with Ehrlich reagent is reported to result in some false positive results. The method is specific for basic moieties with 3-pyrroline ring. False positive with 2-hydroxyl groups e.g. rosmarinine (See fig.1.2) has been explained as probably due to dehydration. The hydrogen atom at position 8 and the two hydrogens at position 3 are crucial for a positive reaction (Fig.1.1).

Another spectrophotometric determination of retronecine esters has been described by Habib et al.(1978). This method involves coupling of purified acid extracts with bromocresol purple, extraction of the coupled compounds with chloroform, release of the coupled dye by re-extraction with sodium hydroxide followed by spectrophotometric quantification of the coupled dye. This method like the previous ones, involves too many steps thus compromising on the accuracy of the quantification obtained. The presence of other compounds other than pyrrolizidine alkaloids might lead to higher values. Therefore so far the only dependable method is still the one developed by Mattocks (1967a).

HPLC Separation and Isolation

Separation and isolation of pyrrolizidine alkaloids in

the 1960s and early 1970s was predominantly based on preparative TLC and simple gravity dependant column chromatography. The first use of High Performance Liquid Chromatography (HPLC) was reported by Quall et al.(1978). The separation used a 10μ Bondapack CN column (30 cm x 4 mm) utilizing a gradient elution technique. Solvents used were chloroform, tetrahydrofuran (THF) and 30% 0.01 M ammonium carbonate (pH 7.8). A 13% to 26% THF gradient for 30 min and a flow rate of 1.8 ml/min. was used. These investigators also examined the use of isocratic combinations and suggested 16% THF at pH 7.8 to be optimal. Columns other than 10 μ Bondapack CN were also examined but were found to give poor separation. Detection was by UV at 235 nm. Fractions were collected and their mass spectral data were compared with reported values for known compounds.

This method involved the use of high pH mobile phases which shortens column life and leads to fast wearing of seals in the whole pumping system. The use of reversed phase chromatography (C_8, C_{18}) at pH 7.9 eluting with methanol - tetrahydrofuran as eluting solvents reported by Segall et al. (1978; 1979) did not achieve better separation and this involved the use of high buffer pH eluting solvent system which shortens column life. Significant peak tailing resulted in reduced resolution. The use of gradient elution as a whole has special problems in general with regards to base-line drifts and high noise level.

Dimena et al.(1980) has used a semi-preparative 10 μ C_{18} reversed phase column to isolate monomers, diesters and macrocyclic diesters of pyrrolizidine alkaloids from Senecio jacoboea and Amsinckia intermedia. Solvent system was isocratic methanol- 0.01 M KH_2PO_4 (17.5:82.5) and pH 4.79. Fractions were collected, methanol evaporated, pH adjusted to 9 and extracted with chloroform. The recovered samples were analyzed with GC-MS.

Pieters and Vlietinck (1986) reported a quantitative

HPLC method for the analysis of Senecio vulgaris alkaloids. Narceine-hydrochloride was used as an internal standard and a reversed phase 10 micron C_{18} Bondapack (30 cm x 3.9 mm) column was used. The mobile phase was a mixture of methanol-0.01 M KH_2PO_4 at pH 6.3. Detection was by UV at 219 nm. Good separations for the alkaloids were obtained but it was not possible to distinguish between the Z and E geometric isomers.

Gas Chromatography

Initial pioneering work on the application of gas chromatography (GC) technique to pyrrolizidine alkaloids was done by Chalmers et al.(1965). This was done using a laboratory constructed apparatus from glass components with a 6 ft column coated with 4% SE-30 siloxane polymer on Gas Chrom P packing material. The interior surface of the column, inlet and outlet were all silanized by methyldichlorosilane. Introduction of sample to the column needed special capillary arrangement which was withdrawn in the analysis process. A short (2 cm) silanized Gas Chrom pre-column at the inlet was used to collect non-volatile constituents or carbonaceous pyrolysis products. This had to be replaced at least once every week. Inlet temperature was 250^0C and column temperature at 140^0C using an argon ionization detector. A total of 58 alkaloids were analyzed and their relative retention times using both TLC and gas chromatography were analyzed with respect to C_7-OH orientation (A or B). The relative retention times were further correlated with molecular structure.

From the above treatments it was obvious that pyrrolizidine alkaloids were rather unstable at the high temperatures needed to vaporise them for gas chromatography. Subsequent work by Culvenor et al.(1981) using GC-MS for the identification of pyrrolizidine alkaloids in honey was achieved by derivatization. Butylborate & trimethylsilylate

derivatives were used for identification. Peaks were compared to those produced by known amounts of authentic samples. GC-MS analyses were performed using (1 m x 2 mm ID) glass-lined stainless steel column packed with 1% SE 30(U/P) on a Chromosorb W (HP) mesh size 80-100. Injection temperature 210^{0}C and oven temperature was programmed from 150-220^{0}C at increments of 6^0 C/min. The GC-MS interface temperature was 220^{0}C and carrier gas was helium at a flow rate 15 ml/min. Luthy et al.(1981) used capillary GC-MS analysis for pyrrolizidine alkaloids contained in hay and silage having both been used for cattle feed. A 20 metre SE 54 capillary column with helium as carrier gas were used in this study. Similar temperature programming with injection temperature at 259 were utilised. Quantification of alkaloids was done by peak integration, a total of 9 compounds were thus identified. Electron-Impact Mass Spectrometry (EI-MS) for all the nine peaks were reported. In this study, capillary columns improved the separation as compared to previous studies.

Further capillary GC and capillary GC-MS of pyrrolizidine alkaloids has been reported by Bicchi et al.1985. The analysis was done using a 20 mm x 0.32 mm (id) Soda-lime and Duran-50 glass capillary columns pre-treated by high-temperature silylation, coated with 0.1 μ OV-1. For the capillary GC carrier gas was hydrogen at a flow rate of 3 ml/min. Quantitation was carried out with respect to a suitable C_{24} hydrocarbon in hexane solution as an internal standard added to the alkaloid solution. The GC-MS carrier gas was helium and data was compared with reported values for pure compounds. The reported analytical conditions could not achieve a good separation of the compounds senecivernine and senecionine.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) is a very important

spectroscopic method for chemical structure elucidation. This is due to the fact that the method gives information on both the type and number of hydrogen in a molecular structure. This technique has been applied extensively to pyrrolizidine alkaloid structure elucidation (Zalkow et al., 1979), and it has also been used for quantification of toxic alkaloids in mixtures of plant extracts (Molyneux et al.,1979; Johnson et al.,1985 and Vollmer et al.,1987).

Although the NMR spectra of pyrrolizidine alkaloids is rather complex, with most of the signals due to the pyrrolizidine nucleus being obscured by signals derived from the esterifying necic acids, Molyneux et al.(1979) has observed that the vinylic H-2 proton of the basic system can easily be identified as a low field broadened singlet or multiplet. This signal appears at δ 6.2 in the macrocyclic diesters and around δ 5.8 in the acyclic di- and mono-esters when the spectra is run in $CDCL_3$ solution.

Integration of this single vinyl proton is used for quantitation of total alkaloids in a sample. Concentration of the alkaloids in a sample can be determined by relating the integration of the vinyl proton to that of an internal standard. By addition of a known amount of *p*-dinitrobenzene (DNB) as an internal standard the total unsaturated pyrrolizidine alkaloids in an NMR sample can be calculated by comparing the integrated area of the vinyl proton to that of the four protons in the internal standard (DNB). The following formula has been used for the calculation:

$$\text{Wt. of PA} = \frac{\text{Mol. wt.of PA}}{\text{Mol.wt.of DNB}} \times \frac{A(H\text{-}2)}{0.25A\ (DNB)} \times \text{Wt.of DNB}$$

The molecular formula weight of pyrrolizidine alkaloid is based on an average value where the composition of the mixture is known or on a representative major alkaloid when the composition of the mixture is not known (Molyneux et al, 1979; Vollmer et al.,1987).

It has also been observed that the vinyl H-2 proton signal is rarely obscured by other signals. It should be noted that for alkaloids with vinyl protons other than the one from the basic ring system, these will resonate within the same area. Structures like senecionine/integerrimine, retrorsine/usaramine have a vinyl H-20 proton in addition to the H-2 vinyl proton. Other structures like symphytine also contain vinyl protons. Therefore quantitative NMR procedure using both continuous wave and Fourier transform technique are both limited by the complexity of the NMR spectra.

Carbon-13 NMR spectroscopy has been used extensively in pyrrolizidine alkaloid structure elucidation (Roeder 1990) and a combination of ^{1}H and ^{13}C-NMR Fourier transform technique for PA quantitation has been reported by Pieters et al.(1985, 1987, 1988 and 1989a). A combination of these two techniques have led to the detection of the occurrence of several pyrrolizidine alkaloids together with their corresponding E geometric isomers in a number of Senecio species. Structural information was also inferred from the same spectra data (Pieters et al.,1986; 1989b).

The performance of quantitative ^{1}H and ^{13}C-NMR spectroscopy has been compared with capillary gas chromatography (Pieters et al.,1989a) and HPLC (Pieters et al.,1986). NMR is considered superior to all these methods in terms of precision and the fact that it is non destructive, but overall it's sensitivity is low compared to the other methods (WHO,1988; Pieters and Vlietinck, 1986).

METHODS OF ANALYSIS AND RESULTS

Thin layer chromatography (TLC)

Several TLC solvent systems were evaluated. Single, binary and tertiary eluting solvent systems were used. Chloroform or ethylacetate alone showed no movement of the components, whereas methanol resulted in most of the components moving to the solvent front with a lot of tailing being observed.

Table 5.1 gives some of the solvent mixtures used for evaluation and the respective R_f values obtained with the standards (namely monocrotaline, retrorsine and retrorsine N-oxide or isatidine).

Other solvent mixtures which were tried were toluene/ ethylacetate / diethylamine (70:20:10); chloroform/methanol/ammonium hydroxide in mixtures of the following proportions (75:24:1); (70:29:1) and (50:49:1). For all these mixtures there was very little separation or movement. On the whole there was a lot of tailing with most of the material remaining at the baseline.

A mixture of chloroform/methanol/ammonium hydroxide/water (70:26:2:2) gave the best separation with

Table 5.1

TLC Retention For Authentic Pyrrolizidine Alkaloids

Solvent System	Rf values	Comments
CH_2Cl_2/MeOH/NH_4OH (85:14:1)	0.8 Retrorsine 0.8 Monocrotaline	Too polar
$CHCl_3$/MeOH (90:10)	0.2 Monocrotaline 0.3 Retrorsine 0.05 Isatidine	Tailing
$CHCl_3$/MeOH (80:20)	0.29 Monocrotaline 0.27 Retrorsine 0.10 Isatidine	Tailing
$CHCl_3$/MeOH/NH_4 (80:19:1)	0.25 Monocrotaline 0.24 Retrorsine 0.05 Isatidine	
$CHCL_3$/$(C_2H_5)_2NH$ (90:10)	No movement	remained at baseline

minimum tailing. R_f values for the standards were 0.75 (Monocrotaline), 0.73 (Retrorsine) and 0.40 (Retrorsine N-oxide or isatidine). Consistent results were observed for

plates sprayed with any of the following reagents: Potassium iodoplatinate, Dragendorf or Mattocks test.

Gas liquid chromatography

A DB5 Megabore (15m x 0.53 mm id) column was used. Carrier gas was helium at a flow rate of 20ml/min and combustion gas was a mixture of air and hydrogen. Injection temperature was 240^{0}C and a flame ionization detector was used at 260^{0}C. Run time was 20 minutes.

Only solutions of the reference standards were evaluated. These were made up in methanol at an approximate concentration of 3mg/ml and 2 micro litres of these solutions were injected on the GC column. These standards were evaluated alone or in mixtures of two or three to establish separation conditions for analysis of isolated alkaloids (table 5.2).

Several temperature programmes were carried out as shown. Samples containing mixtures of the three compounds could be separated as only two components. On column reduction of retrorsine N-oxide to the free base was suspected leading to only one peak appearing for both retrorsine and its N-oxide. Causes for this could be the operating high temperature but it was not possible to operate at lower temperatures than 180^{0}C since no components could be eluted even after 30 minutes of run time.

EXTRACTION SEPARATION AND ISOLATION

Extraction

Both samples preserved in methanol and air dried samples were extracted following these specific procedures:

(a) Samples which were collected and stored in methanol during field collection were macerated in a blender with the same solvent, filtered, and the process repeated 3-5 times until a clear extract was obtained.

Table 5.2

GC Retention Times For Authentic Pyrrolizidine Alkaloids

Programme	Retention Time	Source
1) Initial 185^{0}C (5 min)	8.2	Mixture*
8^0/min to 210^0 (8 min)	12.8	
2) Initial 185^0 (5 min)	7.0	Mixture
8^0/min to 230^0 (7 min)	9.5	
3) Initial 180^0 (5 min)	8.5, 8.8	Mixture
10^0/min to 230^0 (5 min)	11.1, 11.3	
4) Initial 180^0 (7 min)	9.7	"
5^0/min to 220^0 (7 min)	13.7	
5) Initial 180^0 (5 min)	11.0	"
2^0/min to 210 (5 min)	18.2	
6) Initial 150^0 (3 min)	11.5,	Retrorsine
8^0/min to 230^0 (7 min)	14.0,	Monocrotaline
	11.5, 14.1	Mixture

*Mixture contained retrorsine, monocrotaline and isatidine.

The combined extracts obtained were concentrated to dryness on a rotary evaporator under vacuum at 40^{0}C. This yielded a crude extract which was weighed and analyzed further as explained in the appropriate sections below.

(b) Dried plant material was powdered and extracted with methanol using a soxhlet extractor. The solvent was changed after every 12 hrs. On the average, the extraction was completed within 48 hrs. The combined extracts thus obtained were concentrated under the same conditions as in part (a) above and analyzed accordingly.

The following plant samples were extracted: (i) green house cultivated plant material; (ii) Samples collected from Kenya in 1988 and 1991; (iii) Samples collected from Tanzania in 1990. The Tanzanian samples are wild plants and

this included leaves, flowers and one cooked vegetable collected during the field part of the study.

Yield: Dried plant material after exhaustive methanol extraction yielded between 21 and 25% of crude extract of which 4 to 6 percent separated as lipid soluble. The rest was taken up as aqueous acid soluble. The flower sample yielded a higher percent of crude extract (29.24%) (table 5.3).

Table 5.3
Crude Extraction Yields

Plant Source	MeoH soluble fraction (%)
1988 Collection (Kenya)	21.24
Greenhouse Cultivated	25.24
1990 Collection (Tanzania)	
(a) Leaves	24.91
(b) Flowers	29.24
1991 Collection (Kenya)	26.11

Partition and separation

The fractions obtained from above extractions were partitioned in two different ways.

(a) Crude dried methanolic extract was triturated sequentially in different solvents in order of ascending polarity. The order was as follows: hexane, diethylether, ethylacetate, chloroform, methanol and ethanol. Solubilization was performed using a sonicator bath. Samples were shaken for 5 to 10 minutes, solvent decanted into a separating flask and the process was repeated 5 to 6 times until no more colour was extracted with the respective

solvent. Any undissolved material was removed before concentration.

A small portion of each fraction was set aside for thin layer chromatography to find out if any alkaloids were extracted by any of the solvents. Neither the hexane nor diethylether fractions contained any alkaloids on the basis of thin layer chromatography and colour reagents. Ethylacetate, chloroform and methanol fractions had components showing positive reaction for alkaloids with both iodoplatinate and Mattocks reagents (fig 5.1). The spot at Rf 0.5-0.52 seemed to be the major components whereas the ones at Rf 0.95, 0.40 and 0.28 were minor on the basis of colour intensity. Dragendorf reagent did not result in a conclusively positive reaction, a brownish colour was observed instead of the expected orange colour. For fractions with positive alkaloid spots different separation and isolation procedures were attempted including preparative thin layer chromatography and elution on an open column.

(b) Crude dried methanol extract was taken up with 2M HCL and defatted with hexane followed by diethylether. The aqueous extract thus obtained was basified followed by extraction of the liberated free bases with chloroform to result in a free alkaloid fraction. The remaining aqueous basic extract was acidified to pH 2. Zinc dust was added to reduce the N-oxide bases. This was done at room temperature for 12 hours with frequent stirring. After reduction of the N-oxides the mixture was basified to pH 9-10 and the reduced alkaloids were extracted with chloroform as above to give the reduced alkaloid fraction (fig 5.2). The resulting free or reduced alkaloid extracts was dried with anhydrous sodium sulphate, filtered and the solvent evaporated under vacuum. The resulting residues were extracted again with 2M HCL and any colouring or non basic material was partitioned into dichloromethane before releasing the alkaloids with ammonium

Fig.5.1 (a) Thin layer chromatography of different crude *Crotalaria* sp. plant extracts and authentic alkaloid samples.
1) Hexane extract, 2) Diethylether extract, 3) Ethylacetate extract, 4) Chloroform extract, 5) Retrorsine N-oxide, 6)Retrorsine, 7) Monocrotaline, 8) Methanol extract, 9) Ethanol extract.
(b) Thin layer chromatography of alkaloid positive fractions after preparative TLC separation.
1 & 2 Fractions from the ethylacetate soluble fractions
3 & 4 Fractions from the chloroform soluble fractions.
S start, R R_f and F solvent front. Eluting solvent is as explained in the text. Detection was by Mattocks test.

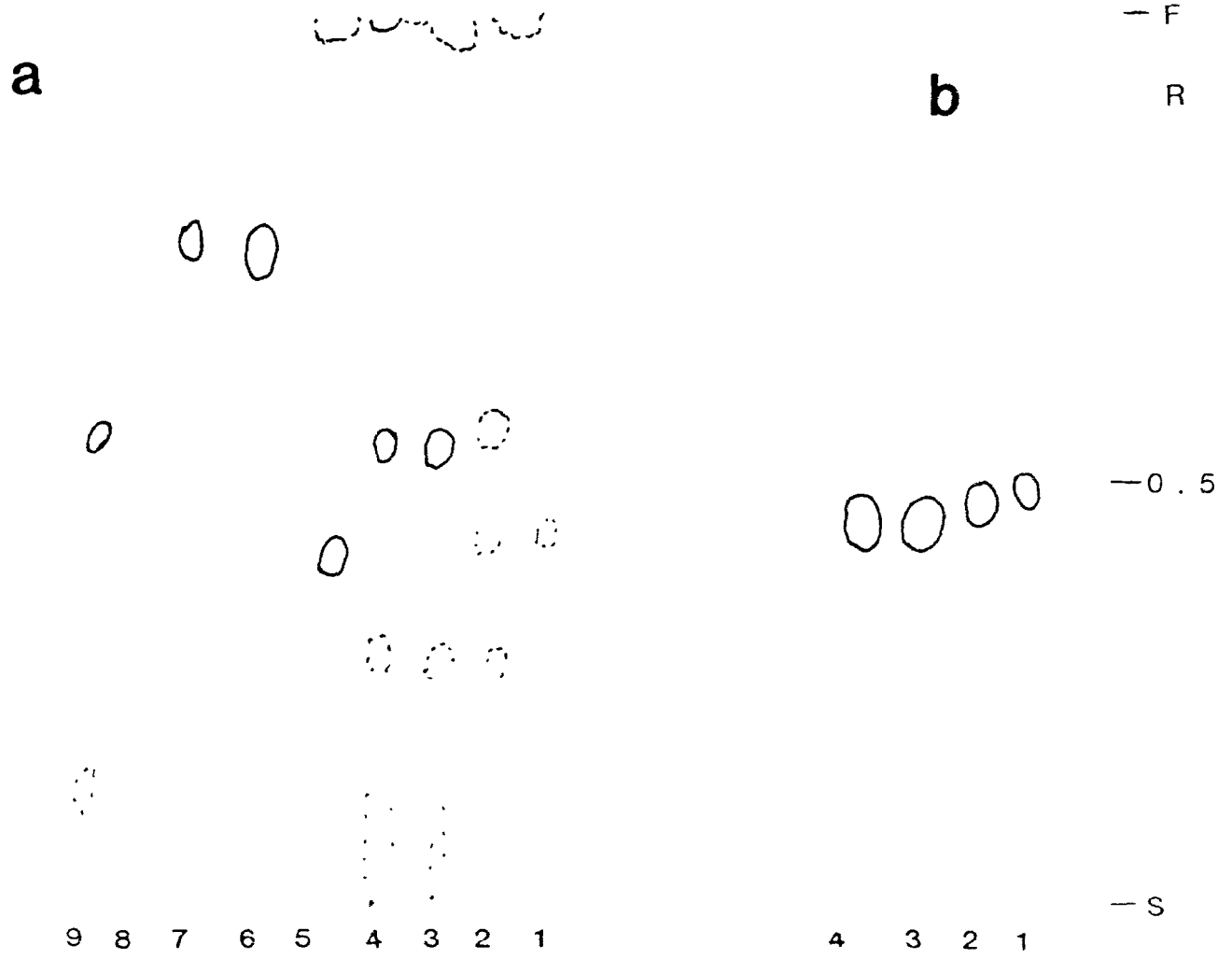
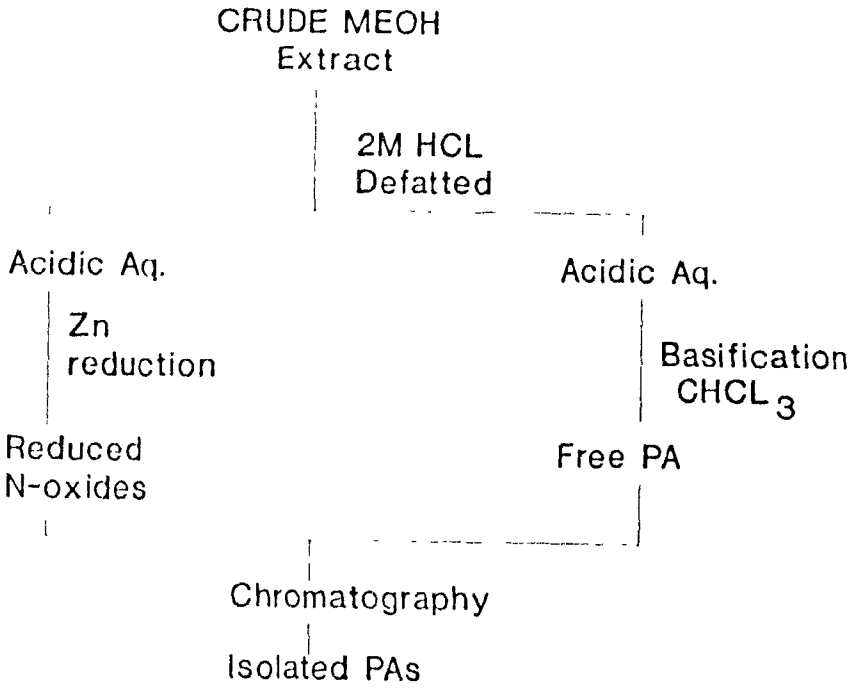

a
9 8 7 6 5 4 3 2 1
b
F
R
0.5
S
4 3 2 1

Figure 5.2 Extraction, Separation and Isolation Scheme.



hydroxide at the same pH as stated above. Chloroform was used to recover the basic components, the organic phase was dried with anhydrous Na_2SO_4 filtered and the solvent evaporated under vacuum.

The residues were weighed and analyzed further as explained in the respective sections. The crude basic fraction yields for the different plant samples extracted are given in table 5.4.

The thin layer chromatographic behaviour of the different basic fractions was compared with the behaviour of authentic alkaloid standards (appendix 10). The cooked basic fraction did not show any alkaloid positive spots. This means that either the cooking methods leads to detoxification or else the amount of sample used was too small for the alkaloids to be detected given their low quantity in the uncooked sample (amount of cooked sample was 100 g; this was a mixture of two leafy vegetables cooked in milk and drained). The other samples showed alkaloid positive spots.

Table 5.4

Crude Basic Material Yields

Plant Source	Yields in Percent	
	Free PA	Reduced PA
1988 Collection (Kenya)	0.08	0.09*
Greenhouse Cultivated	0.01-0.014	0.016-0.02
1990 Collection (Tanzania)		
(a) Leaves	0.0053	0.0101
(b) Flowers	0.026	0.013
1991 Collection (Kenya)	0.0125	0.002

*The 1988 sample was not double extracted with hydrochloric acid.

Open column chromatography separation

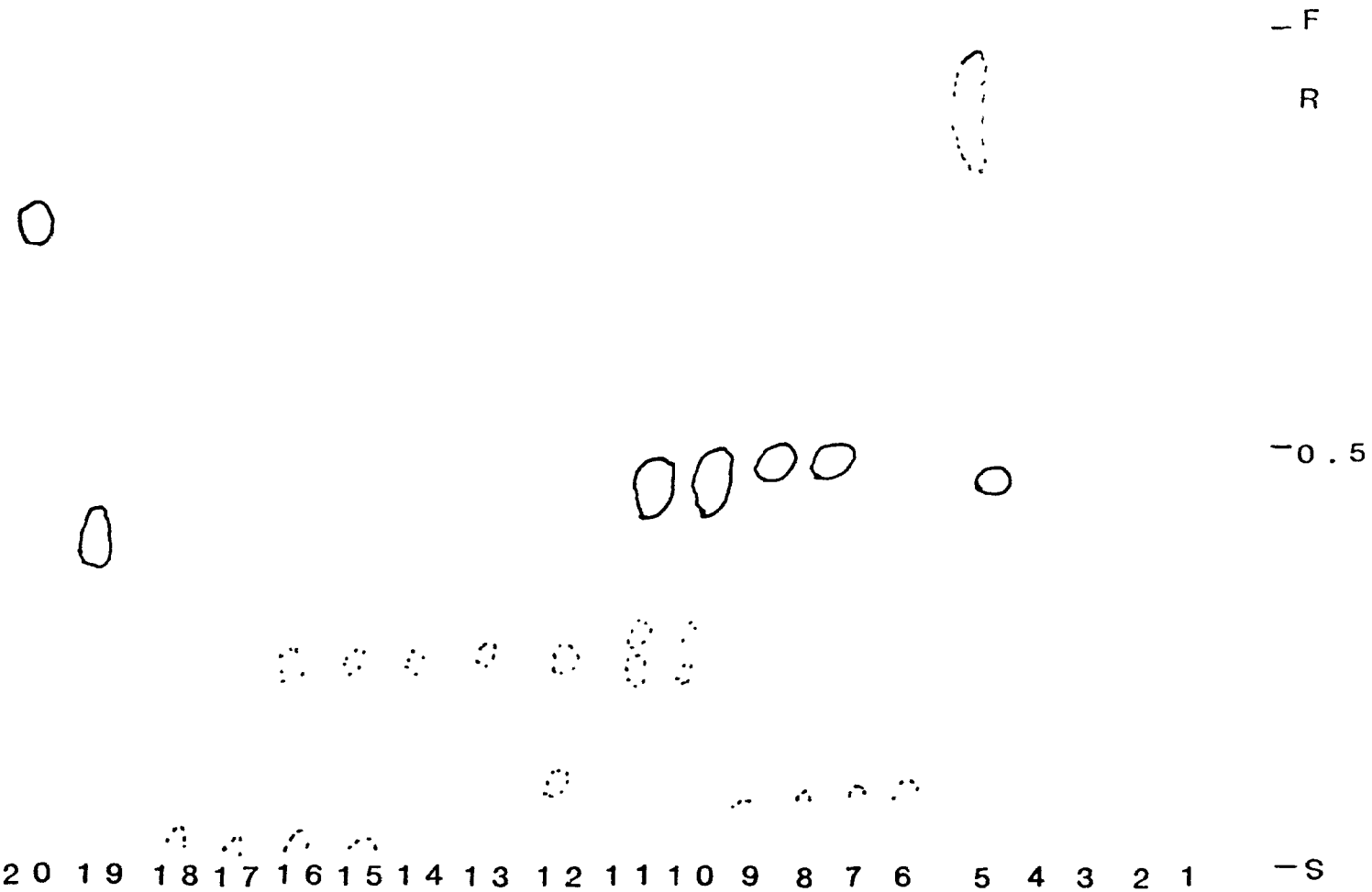
The ethyl acetate and chloroform extracts from the separation method (a) above contained spots positive for pyrrolizidine alkaloids. These were each eluted on a silica gel 60(Merck) (100 g) column (column diameter 2.5 cm height 30 cm). The column was packed in chloroform and eluted with the same, followed by 5:1 ($CHCl_3$/MeOH). Elution was continued with mixture of chloroform containing increased proportions of methanol, down to pure methanol in variations of a factor of one for each fraction collected. Fractions of 50 ml were collected and all the methanol washing were collected as one fraction. In total 21 fractions were collected for the ethylacetate soluble fraction and 40 for the chloroform soluble one. Fractions 8-10 of the ethylacetate extract contained iodoplatinate positive components for pyrrolizidine alkaloids (fig 5.3), whereas for the chloroform extract fractions 25-30 contained components with similar positive reaction. These components were combined and preparative thin layer chromatography was carried out,using a total of 16 (20x20) precoated plates.

Separation on these plates was monitored by UV light since with any of the alkaloid detecting reagents one cannot reverse the reaction to recover the alkaloids. Components of the same R_f were scraped as single bands from the plates. The scrapings from each plate were combined and exhaustively extracted with methanol.

Analytical thin layer chromatography for each of the fractions was carried out using the following solvent system: $CHCL_3$/MEOH/NH_4OH/H_2O (70:26:2:2). Detection was with iodoplatinate and Mattocks reagents. Positive spots were detected with both reagents (R_f 0.5 and 0.52), which were not comparable with the standards Retrorsine and Monocrotaline (R_f 0.73 and 0.75 respectively) (fig 5.1).

The amount of alkaloid positive material obtained was only enough for thin layer chromatography and detection so

Fig 5.3 Thin layer chromatogram of fractions eluted from the open column chromatography separation.
1 - 18 Fractions eluted from column in increasing order of elution, 19) Retrorsine N-oxide and 20) Monocrotaline. S start, R R_f and F solvent front. Eluting solvent is as explained in the text. Detection was by Mattocks test.


F
R
0.5
S
20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

no further work could be done on this portion. There was insufficient material left even for co-chromatography with reference standards, retrorsine N-oxide, monocrotaline and retrorsine. The component is considered to be different from these standards since co-chromatography of the crude fractions with the reference standards had earlier shown no corresponding components in terms of their R_f values (see fig. 5.7 page 91).

HPLC SEPARATION AND ISOLATION

Semi-preparative Separation

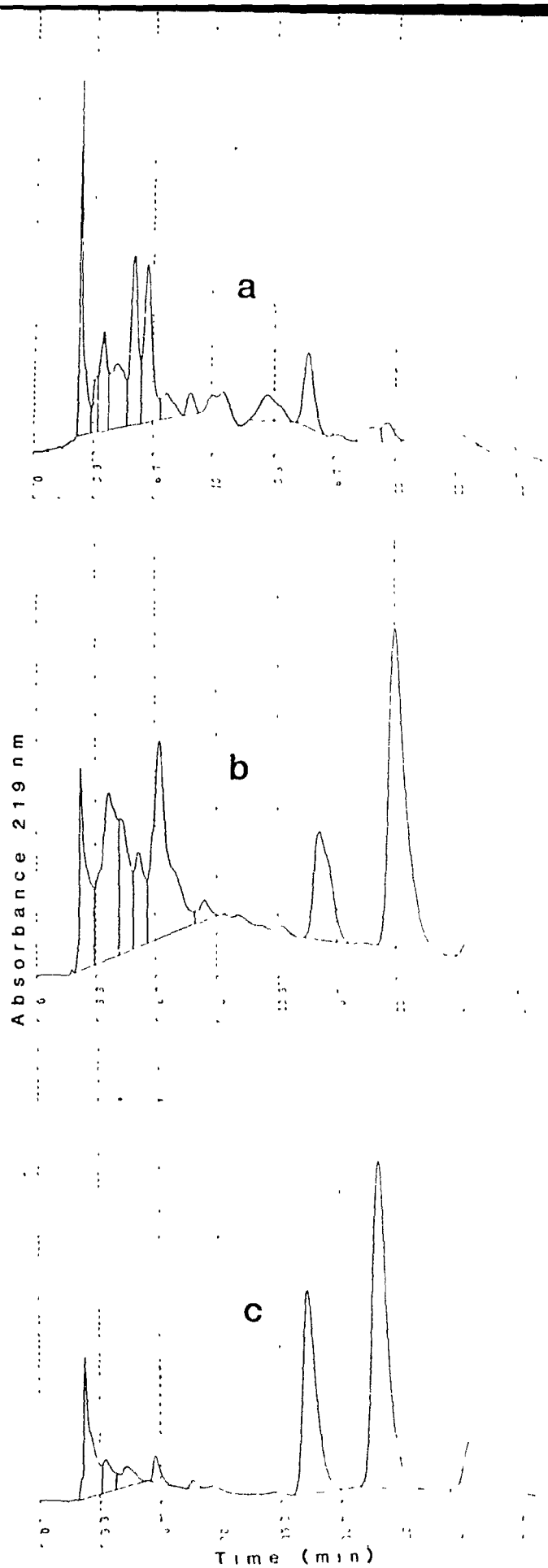
The mobile phase was a degassed Methanol-0.01 M KH_2PO_4, mixture. Different proportions and pH of this mixture were evaluated to establish optimum conditions. The pH was varied between 3.2 and 6.5; pH 6.3 was found to give reasonable separation of the reference standards. Solvent proportions varied from 90:10 to 20:80 MeOH/KH_2PO_4 mixtures. The 30:70 proportion gave the best separation. Samples for injection were dissolved in methanol and detection was by a variable UV detector (SPD-6AV) at 219 nm. The detection wavelength was established after scanning the standards; retrorsine, retrorsine N-oxide and monocrotaline solutions (conc. 0.3 mg/ml) on a UV/VIS spectrometer and establishing the λ_{max}.

The column used was a reversed phase 10 micron C_{18} Bond clone (Phenomenex, USA) (30 cm x 3.9 mm). Run time was 30 min and injection volume was 2 μl.

Under these conditions monocrotaline and retrorsine N-oxide eluted in close proximity to each other (Rt 7.2 and 6.9 respectively) but retrorsine was well separated (Rt 10.4) although its elution profile showed some tailing. Senecionine eluted rather erratically with appreciable tailing (Rt 18.0- 22.3).

The reduced pyrrolizidine alkaloid plant sample was used for all HPLC analysis work. The separation showed a total of at least 14 different peaks (fig. 5.6a). Semi-

Fig. 5.4 HPLC Separation of Crotalaria brevidens (a) crude basic extract chromatogram, (b) chromatogram of fraction number 4 and (c) chromatogram of fraction number 14. Eluting solvent isocratic methanol / 0.01 M KH_2PO_4 30:70 and column 10 μm C_{18} Bond clone 30 cm x 3.9 mm. Run time 30 minutes, injection volume 4 μl. Detection UV 219 nm.


a
b
c
Absorbance 219 nm
Time (min)

preparative separation was done using the same conditions. The sample (4ul) was injected several times (120 runs in total) and fractions were collected as their UV absorbance peaks were observed on the C-R4A Chromatopac monitor. The different fractions were combined accordingly. These were then evaporated on the rotary evaporator at 40^0C under vacuum until all the methanol was distilled out. The remaining aqueous solution was basified with 4% KOH solution to pH 9-10. These were then individually extracted with chloroform (20 ml, 5 times). The combined chloroform extracts were dried with anhydrous sodium sulphate, evaporated into dryness in the usual way using a rotary evaporator.

A thin layer chromatography was done on all the 14 fractions collected using Chloroform/Methanol/NH_4OH/H_2O (70:26:2:2) mixture as eluting solvent system. Detection, as usual used Mattocks reactions. Only fraction 4 and 14 contained toxic alkaloid positive spots with Rf values close to the standards retrorsine and monocrotaline (fig.5.5 & 5.6). All the other fractions showed no positive reaction.

The fractions were re-run on the HPLC under the same conditions as previously. All the fractions contained a lot of contaminants and fraction 14 showed an addition peak at 23.948 whose retention time corresponded to reported literature value for senecionine (Pieters and Vlietinck 1986) (fig 5.4c). The peak for fraction 4 was overlapped by some impurities at 6.817 (fig. 5.4b). A sample of senecionine was obtained from Prof R. Molyneux but as explained above the separation of this sample on our HPLC system was erratic so it was not possible to establish conclusively the identity nor similarity of these compounds by this method. The quantity separated was very small and because the TLC detection method used was destructive no further analysis could be done on this sample.

Fig 5.5 Thin layer ch.omatography of semi-preparative HPLC fractions and authentic reference alkaloid standards.
(1 - 7) Fractions from HPLC separation in ascending order of elution, 8) Crude reduced basic plant extract, 9) Monocrotaline, 10) Retrorsine, 11) Retrorsine N-oxide, S start, R R_f and F solvent front. Eluting solvent is as explained in the text. Detection was by Mattocks test.

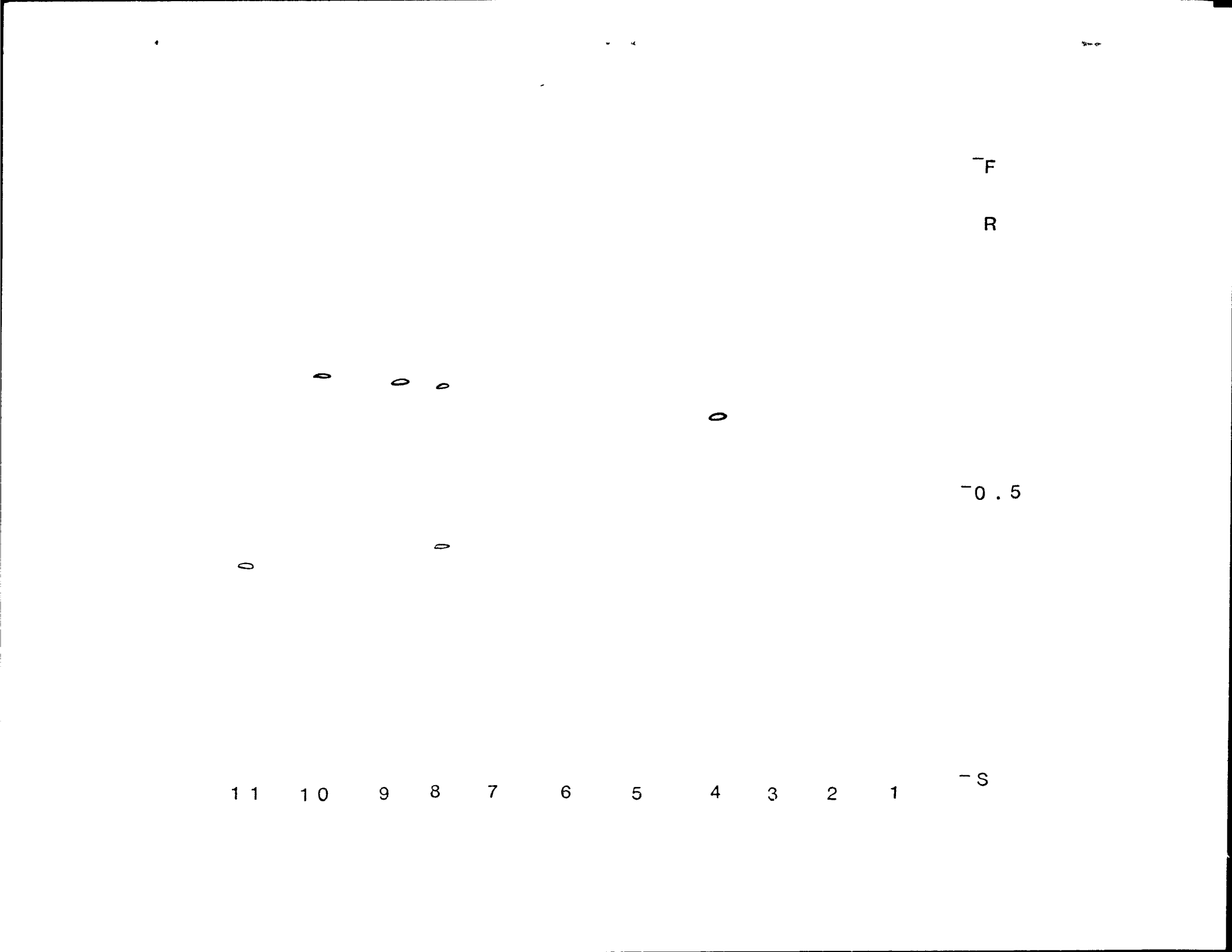
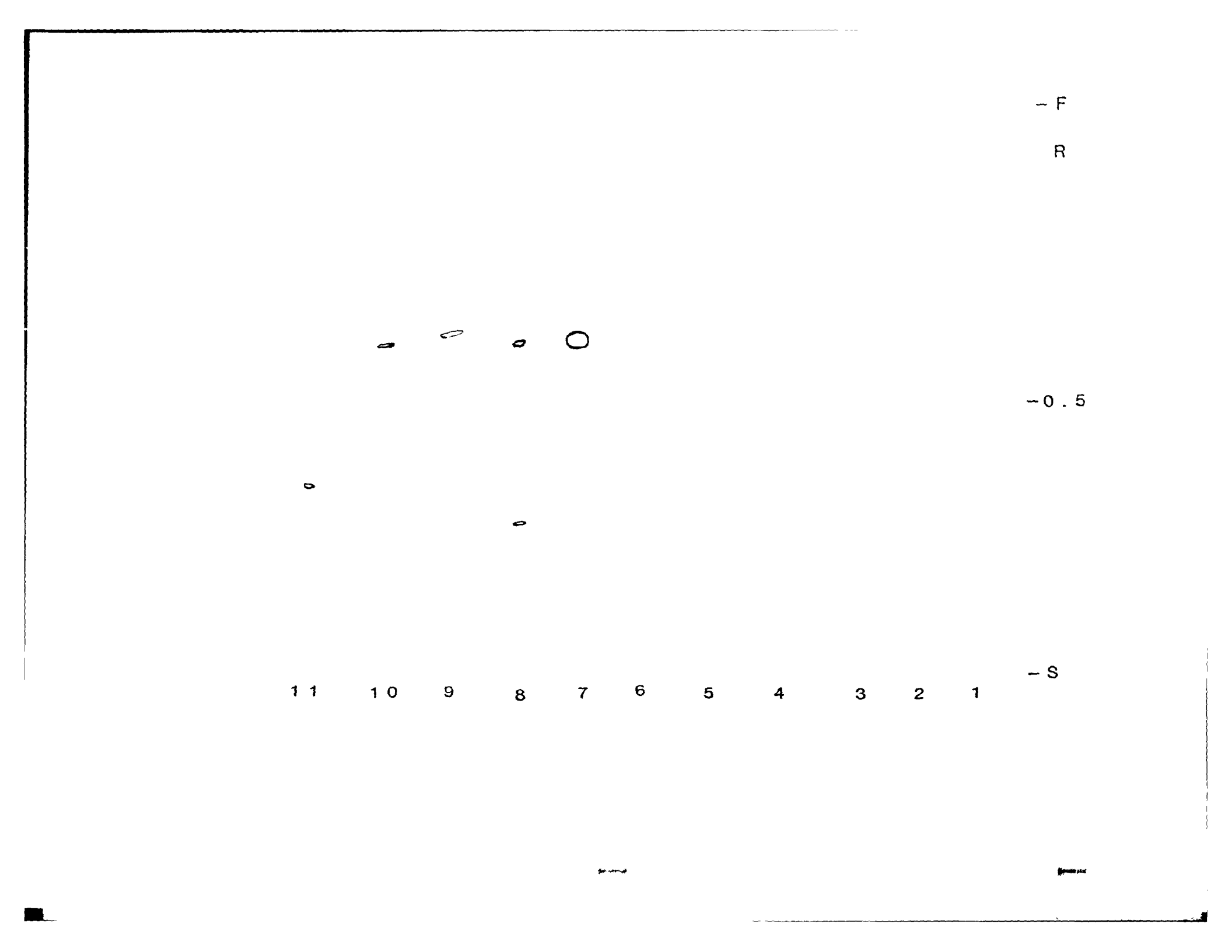

F
R
0.5
S
11
10
9
8
7
6
5
4
3
2
1

Fig 5.6 Thin layer chromatography of fractions from semi-preparative HPLC separation and authentic reference alkaloid standards.
(1 - 7) Fractions number 8 to 14 in ascending order, 8) crude reduced basic plant extract, 9) Monocrotaline, 10) retrorsine, 11) retrorsine N-oxide, S start, R R_f and F solvent front. Eluting solvent is as explained in the text. Detection was by Mattocks test.


- F
R
- 0 . 5
- S
11 10 9 8 7 6 5 4 3 2 1

Preparative HPLC Separation

The above semi-preparative conditions were scaled up to a preparative scale using a 10 μm 30 cm x 22.5 mm Bond clone reversed phase C_{18} HPLC column and same eluting solvent system. Equal mixture (v/v) retrorsine, senecionine, monocrotaline and retrorsine N-oxide solution in methanol (0.3 mg/ml) was prepared. Different volumes of this mixture were injected on the HPLC column to find out an appropriate volume for sample injection. 40 μl up to 200 μl were tried. 120 μl gave reasonable results. Different flow rate were tried starting with 4 ml/min to 7 ml/min. For all these flow rates it was not possible to separate the four reference compound into single peaks. The base line was drifting most of the times especially at the low flow rate. At 7 ml/min the components eluted with the solvent front. Flow rates 4 and 5 ml/min were too slow and components took more than three hours to elute. At flow rate 6ml/min two distinct peaks eluting very close together were obtained irrespective of whether the mixture contained three or four components. Retention times for these peaks were 38.3 and 40.2 minutes or 39.3 and 41.3 minutes. Solutions for each individual standard were also injected and the following are the retention times obtained: Retrorsine N-oxide 22.2 min (drifting), Monocrotaline 37.7 min and Retrorsine 42.4 min. Elution of senecionine was rather erratic, highly peak drifting and tailing was observed. For some of the runs the peak started at 16 to 64 minutes which was not detectable by the detector.

The basic plant material was eluted at a flow rate of 6 ml/min and run time was 140 minutes. Several injections(20 in total) of 120 μl samples were eluted on this column and a total of 30 fractions per run were collected each time. Fraction collection was on the basis of absorbance at 219 nm monitored by the C-R4A-Chromatopac monitor. The collected fractions were mixed accordingly and separation from the

buffer was done in the same way as in the semi-preparative method.

The released basic material thus obtained was dried in a vacuum desiccator containing sodium hydroxide pellets for 24 hours after which the vials were flushed with nitrogen covered and sent for proton NMR analysis. A total of 10 fractions out of the 30 collected were analyzed by ^{1}H-NMR and subsequently all 30 fractions were subjected to TLC. The spectroscopic analysis was performed in an effort to detect alkaloids on the basis of the C-2 vinylic proton resonance which had been used to characterize toxic pyrrolizidine alkaloids.

None of all the ten proton NMR spectra obtained covering different sections of the chromatogram displayed a resonance peak in the vicinity of δ 6.2 ppm (the characteristic region for these compounds). Each spectra contained a set of two multiplets between δ 7.5-8 ppm and δ 4.2-4.4 ppm. These resonance peaks could not be assigned to any pyrrolizidine alkaloid proton resonance peaks. The two multiplets around δ 7.5-8 ppm appear in the aromatic region or highly deshielded protons in other systems like pyrroles. Three oxo-pyrroline compounds have been isolated from Senecio species. These nitrogen containing compounds are not basic but they are highly polar and difficult to separate from the toxic pyrrolizidine alkaloids (WHO 1988). Since these structures contain a pyrrole ring system they give false positive reaction with the Mattocks test as well.

A pyrrole selective TLC detection reaction based on Mattocks reaction was carried out for a portion of the above fractions collected from HPLC separation. Some of the fractions showed trailing brown coloured components with Ehrlich reagent but different R_f from the one observed for pyrrolizidine alkaloid positive spots. The same brown colour was observed with the unseparated reduced alkaloid sample when a similar test was done. This could well be due to a

pyrrole compound or any other compound which forms coloured complexes with Ehrlich reagent. The presence of pyrroles in our plant samples cannot be ruled out on the basis of the results obtained so far and it cannot be established whether these pyrroles are of the 3-oxo type isolated from Senecio species either.

Thin layer chromatography was done on all the 30 fractions obtained from the preparative column chromatography. Very faint alkaloid positive components were detected in fractions 10-12 on the basis of colour reactions using Mattocks test. The retention times for these peaks was between 36 to 42 minutes, which on the basis of the values obtained for the reference standards (37.684 monocrotaline and 41.269 retrorsine) could be either of these two. The thin layer chromatography R_f values also corresponded to those obtained for senecionine and monocrotaline or retrorsine.

The initial material used for preparative HPLC separation contained more than two alkaloid positive components. It seems the process of separation and extraction resulted in diluting the compounds into different fractions whose detection was no longer possible under the operating conditions of this study.

Thin Layer Chromatography of Crude Basic Material

Extensive TLC was done with the crude basic plant material in comparison with the reference compounds. The R_f values for two of the alkaloid positive components were similar to those of retrorsine or monocrotaline and senecionine respectively. Two spots appeared slightly higher and lower than retrorsine N-oxide but their colour was not conclusive enough. This had a touch of brown on the mauve colour instead of the expected distinct purple or mauve. Co-chromatography of the crude basic material with the different reference compounds was done. On the basis of this

method it was possible to show that this plant sample did not contain detectable amount of monocrotaline but contained two components with similar R_f values to senecionine and retrorsine (fig 5.7). Co-chromatography with these two compounds always resulted in one alkaloid positive spot at the respective R_f value while with monocrotaline two very closely moving spots could be detected.

QUANTFICATION OF PYRROLIZIDINE ALKALOIDS

Proton NMR method

A reduced pyrrolizidine alkaloid fraction obtained from an accurately weighed quantity of plant material extracted following method (b) above was sent to Prof. Vlietinck and Pieters in Antwerp university Belgium for proton NMR. This was supposed to be quantified in chloroform using para nitrobenzene as an internal standard but preliminary scanning in chloroform without the internal standard showed no resonance peak at δ 6.2 ppm even after accumulation of 250 scans. δ 6.2 ppm is the resonance position for the C-2 vinyl proton of the pyrrolizidine ring system. Quantification by this method is based on integrating the resonance peak of the C-2 proton compared with that of the 4 equivalent proton of the standard.

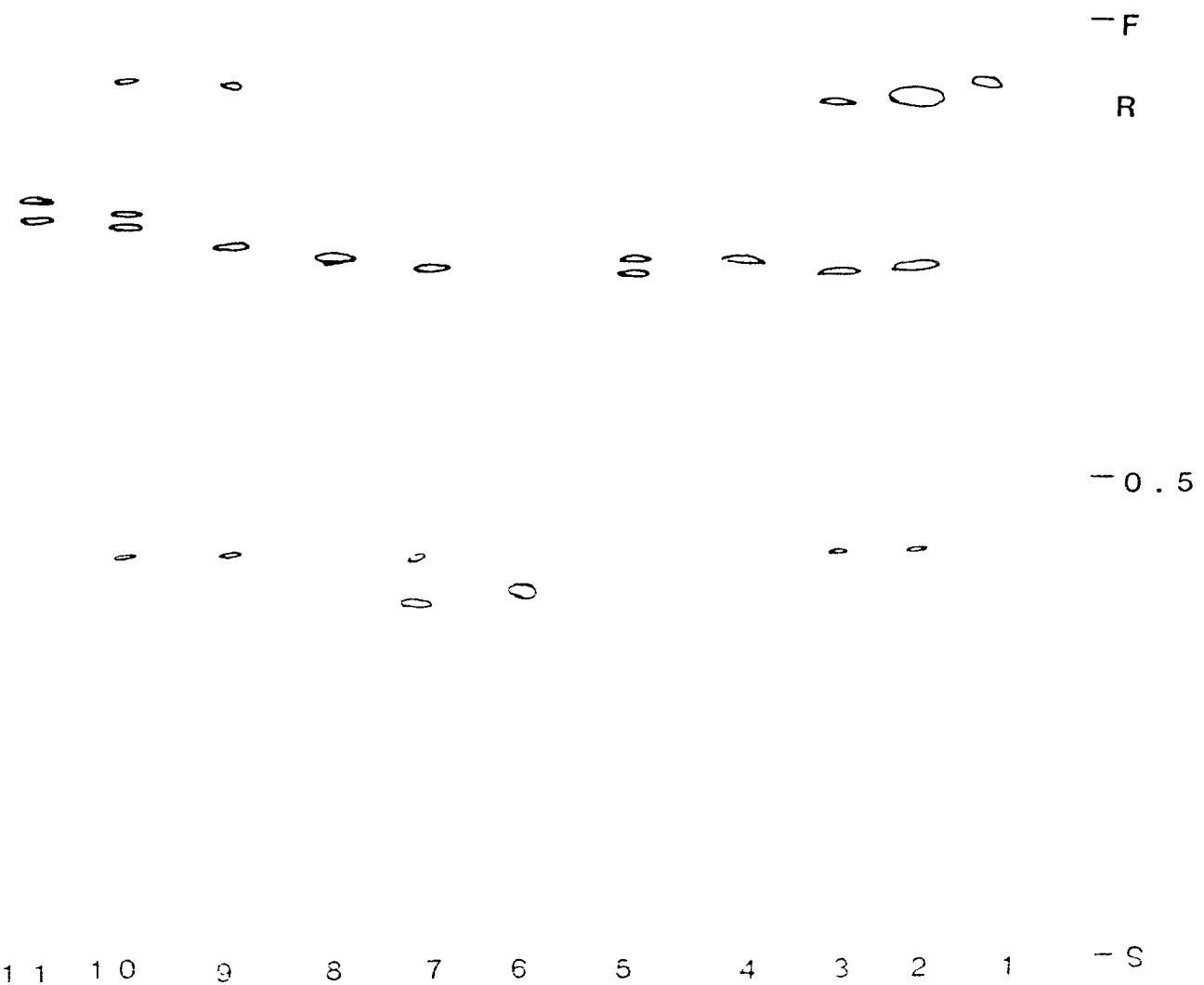
The amount of alkaloid present in the fraction separating as basic material was very small (8.6 mg from 40 g of plant material). The NMR spectra did not show any distinguishable resonance at δ 6.2 ppm; the expected range for the C-2 proton. It was not therefore possible to quantify the alkaloids on the basis of NMR.

Spectrophotometric Method

(a) Calibration Curve.

Monocrotaline (17.2 mg) was dissolved in absolute methanol and diluted to 50 ml in a volumetric flask.

Fig. 5.7 Thin layer co-chromatography of crude reduced extract and authentic alkaloid standards.
1) Senecionine, 2) Senecionine + crude reduced basic plant extract, 3) Crude reduced basic plant extract, 4) Monocrotaline, 5) Monocrotaline + crude reduced basic plant extract, 6) Retrorsine N-oxide, 7) Retrorsine N-oxide + crude reduced basic plant extract, 8) Retrorsine, 9) Retrorsine + crude reduced basic plant extract, 10) Monocrotaline + crude reduced plant extract, 11) Retrorsine + Monocrotaline, S start, R R_f and F solvent front. Eluting solvent is as explained in the text. Detection was by Mattocks test.


F
R
0.5
11 10 9 8 7 6 5 4 3 2 1
S

From this stock solution appropriate dilutions were made resulting in concentrations between 5 and 30 µg/ml (Mattocks,1967b). Five different concentrations were used for the calibration curve (3.44, 4.3, 8.6, 17.2 and 34.4 ug/ml). One millilitre of each concentration was used. To each sample 0.5 ml of dilute hydrogen peroxide was added and the sample was heated on a boiling water bath for 30 minutes (Mattocks, 1967b). To avoid water condensing in the tubes a low stream of air was blown on the tubes frequently. The tubes were then cooled to room temperature then 1 ml of diglyme was added followed by 0.1 ml of acetic anhydride. The tubes were again heated in the boiling water bath for 2 minutes. These tubes were then cooled to room temperature and the water bath was cooled to around 65^{0}C. After cooling 1 ml of Ehrlich reagent was added to the sample followed by subsequent heating at 60-65^{0}C for 5 to 6 minutes. The solution was cooled and diluted with 4.0 ml of absolute methanol, then the absorbance at 565 nm was measured in a 1-cm cuvette against a blank prepared in the same way but with the omission of the standard sample (monocrotaline). The spectrum between 450 and 565 nm was also recorded.

The absorbance values and the corresponding concentrations were used to plot a calibration curve and to compute a linear regression using the Lotus computer software program.

(b) Pyrrolizidine Quantification

Accurately weighed plant material (39 g representing a collection from Tanzania and two other 10 g samples , collected from Kenya) was macerated in a blender with methanol (200 ml for the first lot and 80 ml for the other lots), filtered and the process was repeated several times until the material was exhaustively extracted and clear in colour. The combined extracts were concentrated under vacuum using a rotary evaporator. The dried extract was taken up with 2N hydrochloric acid, defatted with hexane and

diethylether. Zinc dust was then added and the reaction mixture was left standing for 12 hours with frequent stirring. After reduction the solution was filtered, then basified to pH 9-10 with ammonium hydroxide. This solution was then extracted 5 times with chloroform (15 ml). The combined chloroform extracts were dried as above and the residue was redissolved in 2N HCL and the extraction process repeated again as above. The residue obtained after this process was dissolved into 2 ml of absolute methanol, then divided into two parts. One part was used for background absorption reading to which only the complexing reagent (Ehrlich reagent) was added. To the other tube the oxidizing and dehydrogenating reagents were added together with complexing reagent following the same procedure as in (a). After complexing with Ehrlich reagent absorbance of the samples was measured at 565 nm in absolute methanol against a blank prepared in the same way but without the plant sample.

The absorbance of the background sample was subtracted from the absorbance of the test sample to obtain the actual absorbance of alkaloid material. The collection from Tanzania and one of the test sample from the Kenya collection gave negative absorbance values when the background absorbance was subtracted. Only one sample gave a very low positive absorbance (0.009);and on fitting this to the calibration curve this concentration (0.7μg) was below the detection limit by this method (i.e. 5 μg, Mattocks 1967b). The absorbance due to the background in this method is also suspected to be due to pyrroles or 3-oxo derivatives as explained previously.

HPLC Method

High performance liquid chromatography has been used for quantitation of pyrrolizidine alkaloids using narceine hydrochloride as an internal standard (Pieters and Vlietinck

Table 5.5
Spectrometric Quantification of Pyrrolizidine Alkaloids in Crude Plant Samples

Source (Wt in gm)	Absorbance Total	Background	Actual	Conc µg
Tanzania coll. (39)	0.133	0.274	-0.147	-
Kenya coll. (10)	0.068	0.059	0.009	0.7021
Kenya coll. (10)	0.102	0.122	-0.02	-

1986). However the same method could not be used in the present study because the internal standard is no longer available from chemical suppliers. The manufacturers have discontinued synthesis of narceine hydrochloride due to its high toxicity.

Conclusion

All the edible potions of Crotalaria brevidens analyzed in the present study contain detectable amounts of pyrrolizidine alkaloids (detection range 0.25 to 1 µg) (Mattocks 1967b). In our case this detectable amount was contained in approximately 4.65 g of dried leafy plant material. These alkaloids could not be quantified in the present study. Their identity has been tentatively established as possibly retrorsine and senecionine or isomers of these two alkaloids. A large scale extraction might be able to accumulate enough material for quantitation and complete identification.

These findings support the original hypothesis that Crotalaria brevidens contain toxic pyrrolizidine alkaloids. Whether the amount present in the edible portion of the leafy vegetable pose any risk to toxicity depends on the quantity consumed and the effect of traditional processing on these alkaloids. The present study could not evaluate

traditional processing as it affects the toxic alkaloids due to difficulties in detecting the alkaloids in the unprocessed material. Because these alkaloids were only marginally detected without processing it is unlikely they can easily be detected in cooked samples unless one performs a large scale extraction. Therefore we can only speculate on how much toxic alkaloid the subjects are exposed to assuming no appreciable detoxification is attained during traditional processing.

CHAPTER 6

DISCUSSION AND CONCLUSION

Several factors beyond the control of the present study have affected the results reported. Drought, transport and other technical factors affected in a number of ways the outcomes reported. Usual consumption patterns of leafy vegetables, fruits and Crotalaria brevidens are based only on data collected using the food frequency questionnaire since the 24-hour recall data were more a reflection of the then prevailing unusual drought conditions.

Poor roads led to sampling difficulties and this resulted into a smaller number of subjects being reached and interviewed than planned. The subjects had to be treated as a single group, therefore one of the original goals of looking at social economic differences and leafy vegetable consumption could not be assessed. Low sensitivity of the analytical techniques used and the small amount of toxic pyrrolizidine alkaloids in the edible portions of Crotalaria brevidens made it impossible to conclusively prove our original hypothesis, even though toxic alkaloids were detected. Other unforeseen problems associated with food serving and social cultural practices limited our efforts to quantify portion size and risk/benefit assessment. This has therefore been expressed in broad terms of upper and lower limit of exposure.

Not withstanding the above general limitations, the following are the specific observations and the inferred conclusions pertaining to the present study.

The seven day food frequency data show that leafy vegetables as a group are the most frequently consumed (22.69%) food items in this population during the months of June-July. This implies that leafy vegetables are also a very important source of vitamin A given that the frequency of consumption of the preformed vitamin A sources was only 5.5% of the total frequency scores.

The 24-hour recall which is specific to the actual time of interview found that animal products especially fish and the staple food 'ugali' were the most frequently consumed food items in this population at the time of the interview. As explained this was due to the drought conditions at the time of the interview. Therefore the food frequency findings are more a reflection of the usual food patterns for this group during the months of June-July. These findings are in agreement with other reported findings in similar societies (Fleuret 1979; Ogle et al., 1985).

The study group consumed a variety of leafy vegetables including Crotalaria brevidens. The seven day food frequency for Crotalaria brevidens shows that this leafy vegetable although not the major one consumed, occupies an important role as a food plant to this population. This leafy vegetable is not equally important to the whole group interviewed since 23 out 74 (33.78%) people do not consume it. On the other hand high consumption frequency scores for Crotalaria brevidens is associated with low frequency scores of fruits in general and vitamin A animal sources. The same overall relationship applies to the frequency of consumption of animal products. Although a similar trend was observed between high leafy vegetables frequency scores and those of the above food groups in general, the magnitude of the slope was bigger for Crotalaria sp. consumption. The β-carotene content of this leafy vegetable is high (>4900 μg/100 g edible portion). This underlines the important role as a source of vitamin A and possibly of protein and other nutrients this leafy vegetable plays in the diets of some of the people interviewed. The high content of β-carotene found in the present study is in agreement with other nutrient data reported by Gomez 1981 and Imungi et al.,1983.

High frequency consumption of Crotalaria sp. was associated with low consumption of fats and oils as a whole which might compromise the availability and absorption of

lipid soluble vitamins. This is partly due to the cooking and preparation methods of this leafy vegetable which involves addition of milk. People who cook with milk do not use cooking fat frequently since they used milk as a substitute of cooking fat. When whole milk is used there may be enough butter to help absorption but there is no information about the way the milk is processed within the present study group.

Phytochemical analysis showed the presence of at least two 1:2 unsaturated pyrrolizidine alkaloids. This was conclusively shown on the basis of thin layer chromatography and colour detecting reagents. Co-chromatography with reference standard compounds showed that the leafy vegetable possibly contains senecionine and retrorsine or their isomers. Integerrimine and usaramine both isomers of retrorsine have been isolated from the seeds of Crotalaria brevidens (WHO 1988; Segall et al.,1985; Smith et al.,1981). The structure of both alkaloids contain the 1:2 unsaturated pyrrolizidine nucleus and a macrocyclic diester ring system which are toxic to humans and animals (Culvenor 1983; WHO 1988).

Proton NMR of the crude basic plant material could not detect the presence of toxic pyrrolizidine alkaloids due to the low sensitivity of the technique. The resonance of the 1:2 vinylic proton was not distinguishable from the general high noise level of the crude plant sample. Separation of these alkaloids using both open column chromatography and reversed phase high performance liquid chromatography resulted in partial separation of the alkaloids. The separated alkaloid material could not be analyzed further due to the small quantity obtained and the low amount of alkaloid present in the plant material in general.

The detection of alkaloids in only a few of the fractions obtained using both open column chromatography and HPLC separation shows that separation was achieved. If

larger quantities had been available these alkaloids could have been isolated and identified as well.

Quantification of these alkaloids using both proton NMR and spectrophotometric method was not conclusive due to the low amounts. The NMR technique was not even able to detect the presence of toxic pyrrolizidine alkaloids. Spectrophotometric analysis of the same sample indicated that these alkaloids were present at levels below the detection limit for this method thus making quantification impossible.

The reported detection limits on the basis of thin layer chromatography are much lower (0.1 μg retrorsine N-oxide and 0.25 μg retrorsine) (Mattocks 1967a and Molyneux et al.1980) than spectrophotometric quantification detection limits (5 μg) (Mattocks 1967b). Therefore it was possible to conclusively detect these toxic alkaloids on the basis of TLC but it was not possible to conclusively quantify the amount of alkaloid present in this edible portions of the plant. This means that the actual amount extracted in the leafy material is higher than the TLC detection limit but lower than the spectrophotometric detection limit. If one takes both limits as the lower and upper limit we can estimate the probable amount of toxic alkaloid a person will be exposed to upon consumption of one 250 ml cup of cooked drained leafy vegetable (approximate weight 190 g) (average portion size obtained from the food frequency questionnaire).

Assuming that the amount of toxic alkaloid detected was 0.25 μg, and this was contained in 40 μl (the amount spotted on TLC plate) of a crude basic solution (concentration 2.6 mg/ml), then the amount of crude material spotted was 104 μg and this contained at least 0.25 μg of toxic alkaloid. Based on 1991 Kenyan collection (table 5.4) 116.23 g of dried plant material yielded 2.6 mg of crude basic material. Therefore 104 μg of crude extract was extracted from 4.65 g

of plant material. Consumption of 100 g of dried plant material corresponds to an intake of 5.38 μg of toxic alkaloid.

The moisture content of this leafy vegetable was 79.93%. Therefore consumption of 100 g dried plant material is equivalent to 498.26 of fresh leafy material. If we assume that cooked and drained leafy material weighs the same as the fresh plant to get a rough estimate, then for a person who eats a 250 ml cup full of leafy material weighing 190 grams consumes 2.10 μg of toxic alkaloid.

Following a similar argument using the spectrophotometric detection limit as a basis for calculation this amount corresponds to 38.13 μg of toxic alkaloids per 190 leafy vegetable meal. That is assuming 5 μq of toxic alkaloids were contained in 1 ml of crude basic extract used for spectrophometric quantification (Kenyan collection 1991, table 5.5). Since the extract from 10 g plant material was dissolved in 2 ml absolute methanol and only 1 ml of this was used for quantification then the 10 g material contained twice the amount assumed detected. Total amount contained in the plant will be a bit higher than this since we do not have 100% extraction of these alkaloids.

Assuming the average weight of an adult woman to be 56 Kg then the lowest intake is 3.7×10^{-5} mg/Kg Bw and the highest is 6.8×10^{-4} mg/Kg Bw. Assuming that this leafy vegetable is eaten only once a day the amount remains the same but if this is eaten twice a day then this amount will double. The amount estimated for a single meal intake is lower (about 1/1000 to 1/100 th fraction) than the estimated amounts of intake in the epidemics of acute toxicity that occurred in Afghanistan and India and other incidence of human toxicity (table 1.3) (Culvenor, 1983). The estimated intake in the present study is even lower than reported estimated intakes of alkaloids from the consumption of comfrey salad (0.01-0.1) (table 1.3, echimidine and related

alkaloids). Comfrey consumption has not been associated with toxic effect so far, even though one can not rule out long term chronic effects as cautioned by most of the studies. It should also be noted that comfrey alkaloids are acyclic diester type of alkaloids which are not as toxic as the macrocyclic diesters unsaturated pyrrolizidine alkaloids, detected in the plant of the present study.

People who eat Crotalaria brevidens believe that it has medicinal properties and it is good for general abdominal problems. Similar attitudes are held for most of the wild and bitter leafy vegetables as a whole (Johns et al.1991). Given the very low amounts of toxic alkaloids detected in this leafy vegetable and the purported medicinal properties one might argue that under normal circumstances and occasional consumption the nutritional and medicinal benefits from eating this leafy vegetable outweighs the detrimental effects. When consumption is increased due to environmental problems such as scarcity of other food items, the detrimental effects might outweigh the benefits especially for the nutritionally compromised individuals like children.

This leafy vegetable undergoes a very elaborate cooking which might result in detoxification. There are no reported studies which have evaluated the effects of these cooking methods on the toxic alkaloids or on nutrients as a whole. It was not possible to do so in the present study due to time shortage and difficulties encountered with the phytochemical analysis and detection. This vegetable has high β-carotene content and assuming that detoxification of some sort is attained by the cooking methods, and given that very low amounts of alkaloids were detected even without detoxification, then the benefits definitely outweighs the detrimental effects of the alkaloids present in Crotalaria sp.

These alkaloids are known hepatotoxins and carcinogens.

We have detected them in edible portions of C. brevidens and thus must accept the original hypothesis of this study that the leafy vegetable contain toxic alkaloids that are detrimental to humans in the pattern in which they are consumed. Thus there is a possibility of toxicity especially to young children who are more susceptible to the toxicity of these alkaloids (WHO 1988). Other people with low nutritional status might also be at risk since nutritional status plays a role in the bioactivation and metabolism of these alkaloids (Garret et al.,1984; Buckmaster et al., 1976; Hayashi et al.,1968). It should also be remembered that the toxic effects of pyrrolizidine alkaloids have a long latent period and their effects are more like that of mycotoxin. The high incidence of primary liver carcinoma among Africans has been suspected to be due in part to these type of alkaloids and other alkylating agents from mycotoxin (Schoental et al.,1963; WHO 1988). There were no data available on the incidence of liver diseases in Tarime (appendix 2), therefore it was not possible to find out if they have much higher incidence than places where this leafy vegetable is not eaten.

RECOMMENDATIONS

1.Improved analytical methods are required for detection and determination of very low levels of pyrrolizidine alkaloids.
2.There is a need to establish a network for researchers in the field of pyrrolizidine alkaloids to facilitate exchange or provision of standard reference alkaloid samples for analytical work. Several analytical techniques applied for separation, characterization and quantification of these alkaloids are based on comparison with authentic samples few of which are available commercially.
3.More ethnobotanical and taxonomic studies are required to provide information on the use of other Crotalaria sp. for medicinal and dietary purpose.

4.There is a need to carry out detailed phytochemical analysis of all edible and medicinal Crotalaria species to find out if they contain high levels of pyrrolizidine alkaloids and discourage their use.

5.Further studies should evaluate the documented traditional cooking methods to find out if they attain any tangible detoxification. The interaction of milk, the local salt 'bala' and prolonged heating can be evaluated with authentic alkaloid samples at levels comparable to those found in the edible portions. It is known that high temperatures and acids leads to hydrolysis and decomposition of the some of the pyrrolizidine alkaloids.

6.Contracted epidemiological surveys should be carried out in Tarime and in areas with similar consumption to determine if there is a higher incidence of primary liver cancer compared to populations with similar diet habits and living in similar environment but who do not consume Crotalaria species or other pyrrolizidine containing plants as leafy vegetables and herbal remedies. This will help to determine whether the consumption of Crotalaria species, although shown to contain very low levels of toxic alkaloids, have a cumulative or chronic toxic effects to this population.

7.There is a need to inform the populations consuming Crotalaria species whether for food or medicinal purpose of the possible hazards of such consumption.

8. Improved methods of dietary evaluation in this food system are needed.

9. Seasonal variations in intakes of green leafy vegetable and fruits needs to be studied to give the picture of year-round average intake of individuals.

REFERENCES

Abe L.O. and Imbamba S.K.,1977. Levels of Vitamin A and C in Some Kenyan Vegetables. E. Afr. Agric. For. J. 42; 316-321.

Abramson J.H, Slome M.B, Kosovsky C., 1963. Food Frequency Interview as an Epidemiological Tool. Am. J. Pub. Health 53(7); 1093-1101.

Acheson E.D. and Doll, 1964. Dietary Factors In Carcinoma Of The Stomach: A Study Of 100 Cases And 200 Controls. Gut 5; 126-131.

Ajayi S.O., Oderinde S.F. and Osibanjo O., 1980. Vitamin C losses in Cooked Fresh Leafy Vegetables. Food Chemistry 5;243-247.

Allen J.R., Chesney C.F. and Frazee W.J., 1972. Modification of Pyrrolizidine Alkaloid Intoxication Resulting from Altered Hepatic Microsomal Enzymes. Toxicology and Applied Pharmacology 23; 470-479.

AOAC 1984. Official Methods Of Analysis. 14th Ed. Vitamins And Other Nutrients. Food And Administration. Arlington, VA. pg 830-837.

Beaton G.H.,Milner J.,Corey P.,McGuire V.,Cousins M. Stewart E.,de Ramos M.,Hewitt D.,Grambsch P.V.,Kassim N.and Little J.A., 1979. Sources of Variance In 24-Hour Dietary Recall Data: Implications For Nutrition Study Design and Interpretation. Am. J. Clin. Nutr. 32; 2456-2559.

Beaton G.H., Milner J., McGuire V., Feather T.E. and Little J.A.,1983. Sources of Variance In 24-H Dietary Recall Data: Implications For Nutrition Study Design And Interpretation. Carbohydrate Sources, Vitamins And Minerals. Am. J. Clin. Nutr. 37; 986-995.

Bicchi C.,D'amato A. and Cappelletti E., 1985. Determination Of Pyrrolizidine Alkaloids In Senecio inaequidens D.C. By Capillary Gas Chromatography. Journal of Chromatography 349; 23-29.

Bingham S.A. 1987. The Dietary Assessment of individuals; Methods, Accuracy,New Techniques and Recommendations. Nutritional Abstracts and Reviews (Series A) 57: 705-742.

Block G. 1982. A Review of Validations of Dietary

Assessment Methods. Am.J. Epidemiol. 115(4); 492-505.

Bolagh M., Medalie JH., Smith H. et al., 1968. The Development Of Dietary Questionnaire For Ischemic Heart Disease Survey. Isr. J. Med. Sci. 4; 195-203.

Braumann T. and Grimme L.H. 1981 Reversed-Phase High-Performance Chromatography of Chlorophylls and Carotenoids. Biochica et Biophysica Acta 637: 8-17.

Buckmaster G.W., Cheeke P.R. and Shull L.R., 1976. Pyrrolizidine Alkaloid Poisoning in Rats: Protective Effects of Dietary Cysteine. Journal of Animal Science 43; 464-473.

Bull G.F.M.(ed), 1988. Fat Soluble Vitamin Assays in Food Analysis. A Comprehensive Review. Elsiview Science Publication Ltd.NY pp 190-199.

Bushway R.J., 1986. Determination of a- and B- Carotene in Some Raw Fruits and Vegetables by High-Performance Liquid Chromatography. J. Agric. Food Chem. 34; 409-412

Bureau J.L. and Bushway R.J., 1986. HPLC Determination of Carotenoids in Fruits and Vegetables in the United States. J. Food Sci. 51(1); 128-130.

Chalmers A.H., Culvenor C.C.J. and Smith L.W., 1965. Characterization Of Pyrrolizidine Alkaloids By Gas, Thin Layer and Paper Chromatography. J. Chrom. 20; 270-277.

Chandler LA., and Schwartz SJ., 1988. Isomerization And Losses Of Trans-β-Carotene In Sweet Potatoes As Affected By Processing Treatments. J. Agric. Food Chem. 36; 129-133.

Chen B.H. and Han L.H., 1990. Effects Of Different Cooking Methods On The Yield Of Carotenoids In Water Convolvulus (Ipomea aquatica). J. Food Protection 53(12); 1076-1078.

Chesney C.F. and Allen J.R., 1973. Resistance of the guinea pig to Pyrrolizidine Alkaloid Intoxication. Toxicology and Applied Pharmacology 26; 385-392.

Chweya J.A., 1985. Identification And Nutritional Importance Of Indigenous Green Leaf Vegetables In Kenya. Acta Hortculturae 153; 99-108.

Cottrell RC., 1991. Introduction: Nutritional Aspects Of

Palm Oil. Am. J. Clin. Nutr. 53; 989s-1009s.

Culvenor C.C.J.,Edgar J.A. and Smith L.W., 1981. Pyrrolizidine Alkaloids In Honey From Echium planagineum L. J. Agric. Food Chem. 29; 958-960.

Culvenor C.C.J., 1983. Estimated Intakes of Pyrrolizidine Alkaloids by Humans. A Comparison with Doses Causing Tumours in Rats. Journal of Toxicology and Environmental Health 11; 625-635.

Dann A.T. 1960. Nature, 186; 1975.

De Leenheer A.P., Nelis H.J., Lambert W.E. and Bauwens R.M., 1988. Review Chromatography of Fat Soluble Vitamins in Clinical Chemistry. J. Chrom. 429; 3-58.

Dickison J.O., Cooke M.P., Kings R.R. and Mohamed P.S., 1976.Milk Transfer Of Pyrrolizidine Alkaloids In Cattle. J.A.V.M. 169; 1192-1196.

Dimenna G.P., Krick T.P. and Segall H.J., 1980. Rapid High-Performance Liquid Chromatography Isolation Of Monoesters, Diesters and Macrocyclic Diester Pyrrolizidine Alkaloids From Senecio jacobaea and Amsinckia intermedia. J. Chrom. 192; 474-478.

Draper N.R. and Smith H., 1981. Applied Regression Analysis. Second Edition. John Wiley & Sons, New York, Toronto. pg. 1 - 55.

Faboya O.O.P., 1983. The Mineral Content of Some Green Leafy Vegetables Commonly Found in The Western Part of Nigeria Food Chemistry 12;213-216.

Fafunso M. and Bassir O., 1976. Effect of Cooking on Vitamin C Content of Fresh Leaves And Wilted Leaves. J. Agric. Food Chem. 24(2); 354-355.

---------; 1979. Nutritional Effects Of Food Processing Of Some Nigerian Leafy Vegetables. Journal of Plant Foods 3; 187-190.

FAO , 1988. Traditional Food Plants. Food and Nutrition Paper 42. Food And Agriculture Organization Of The United Nations, Rome.

Fleuret,Anne, (1979). The role of Wild Plants in the Native Diets . A Case Study From Lushoto, Tanzania. Ecology of Food and Nutrition 8; 87-93.

Garret B.J. and Cheeke P.R., 1984. Evaluation of Amino

acids, B Vitamins and Butylated hydroxyanisole as Protective Agents Against Pyrrolizidine Alkaloid Toxicity in Rats. Journal of Animal Science 58(1); 138-144.

Getahun A., 1974. The Role of Wild Plants in the Native Diets of Ethiopia. Agro-Ecosystems 1; 45-56.

Gibson R.S., 1990. Principles of Nutritional Assessment New York Oxford University Press pg. 37-152.

Glegg C.G., 1945. Native Food Stuffs in Tanganyika. The Preparation and Use of Local Foodstuffs in the Shinyanga District of Sukuma land, Tanganyika Territory. Tropical Agriculture 22(2); 32-38.

Goodman Dewitt S., 1980. Vitamin A Metabolism Federation Proceedings 39 (10) ; 2716-2722.

Gomez M.I., 1981. Carotene Content of Some green Leafy Vegetables of Kenya and Effects of Dehydration and Storage on Carotene Retention. Journal of Plant Foods 3; 231-244.

Grivetti L.E., 1978. Nutritional Success in a Semi-arid Land. Examination of the Tswana Agro-Pastoralists of the Eastern Kalahari, Botswana. Am.J. Clin. Nutr. 31; 1204-1220.

Grivetti L.E., 1979. Kalahari Agro-Pastoral-Hunter-Gatherers. The Tswana Example. Ecology of Food and Nutrition 7; 235-256.

Habib Abdel Azim M. and El-Sebakhy Nadia A., 1980. Microestimation Of Retronecine Ester Alkaloids Of Crotalaria madurensis R.Wight. Egypt J. Pharm. Sci. 19(1-4); 71-76 CA:30464h.

Hankin LH., Rhoads GG. and Glober GA., 1975. A Dietary Method For An Epidemiology Study Of Gastrointestinal Cancer. Am.J. Clin. Nutr. 28; 1055-1060.

Hankin J.H., 1987. Dietary Methods For Estimating Vitamin A And Carotene Intakes In Epidemiologic Studies Of Cancer. Journal of Canadian Dietetic Association 48(4); 219-224.

Harborne J.B.,1988. Introduction to Ecological Biochemistry. 3rd Ed. Academic Press, London, pp 110-114.

Hayashi L. and Lalich J.J., 1968. Protective Effect of Mercaptoethyamine and Cysteine Against Monocrotaline

Intoxication in Rats. Toxicology and Applied Pharmacology 12; 36-43.

Holzer G., Zalkow L.H. and Asibal L.H., 1987. Capillary Supercritical Fluid Chromatography of Pyrrolizidine Alkaloids. Journal of Chromatography 400; 317-322.

Huxtable R.j., 1980. Herbal Teas and Toxins: Novel Aspects of Pyrrolizidine Poisoning in the United States. Perspectives in Biology and Medicine 24(1); 1-14.

Ifon E.T. and Bassir O., 1979. The Nutritive Value of Some Nigerian Leafy Green Vegetables - Part 1: Vitamin and Mineral Contents. Food Chemistry 4(4); 263-267.

Imbamba S.K., 1973. Leafy Protein Content Of Some Kenya Vegetables. E.A. Agric. and Forestry J. 38(3); 246-251.

Imungi J.K. and Potter N.N., 1983. Nutrient Contents of Raw and Cooked Cowpea leaves. J.Food Sci. 48; 1252-1254.

Jain MG., Howe GR, Johnson KG, Miller AB., 1980. Evaluation of Dietary History Questionnaire for Epidemiologic field Study. Am. J. Epidemiol. 111; 212-9.

Jain MG., Harrison L., Howe GR., Miller AB., 1982. Evaluation Of A Self Adiminstered Dietary Questionnaire For Use In A Cohort Study. Am. J. Clin. Nutr. 36; 931-935.

Johns T. and Kokwaro J.O., 1991. Food Plants of the Luo of Siaya District, Kenya. Economic Botany 45(1); 103-113.

Johnson A.E., Molyneux R.J. and Merril G.B., 1985. Chemistry of Toxic Range Plants. Variation in Pyrrolizidine Alkaloid Content of Senecio, Amsinckia, and Crotalaria species. J. Agric. Food Chem. 33; 50-55.

Keshinro O.O. and Ketiku A.O., 1979. Effect Of Traditional Cooking Methods On The Ascorbic Acid Content Of Some Nigerian Leafy And Fruit Vegetables. Food Chem. 4(4); 303-310.

Keshinro O.O., 1983. The Free and Total Folate Activity in Some Commonly Available Tropical Foodstuffs. Food Chemistry 11; 87-93.

Kokwaro J.O., 1972. Luo - English Botanical Dictionary. East African Publishing House, Nairobi, Dar er Salaam, Kampala. pg 39, 41.

_____, 1976. Medicinal Plants of East Africa. East African Literature Bureau, Kampala, Nairobi, Dar er Salaam. pg 132.

Lalich J.J. and Ehrhart L.A., 1962. Monocrotaline Induced Pulmonary Arteritis In Rats. Journal of Atherosclerosis Research 2; 482-492.

Lambert W.E., Nelis H.J., de Ruyther M.G.M. and de Leenheer A.P., 1985. Vitamin A: Retinol, Carotenoids and Related Compounds In: de Leenheer A.P., Lambert W.E., de Ruyter M.G.M. (Eds). Modern Chromatographic Analysis of Vitamins. Chromatographic Science Series Vol. 30 Marcel Dekker Inc. NY. pg 46-55.

Lyimo M.H., Nyagwegwe S.and Mnkeni A.P., 1991. Investigations On The Effect Of Traditional Food Processing, Preservation And Storage Methods On Vegetable Nutrients: A Case Study In Tanzania. Plant Food For Human Nutrition 41; 53-57.

Marealle A.L.D., 1974. Tanzania Food Tables. Muhtasari Wa Vyakula Tanzania. East Africa Literature Bureau. Dar es Salaam, Nairobi, Kampala.

Martin M.A., 1971. Introduction A'Ethnobotanique du Cambodge. Centre National de la Recherche Scientifique. pg. 154.

Mattocks A.R., 1967a. Detection of Pyrrolizidine Alkaloids on Thin-layer Chromatograms. Journal of Chromatography 195; 412-415.

----------, 1967b. Spectrophotometric Determination of Unsaturated Pyrrolizidine Alkaloids. Analytical Chemistry 39(4); 443-447.

----------, 1968. Toxicity of Pyrrolizidine Alkaloids. Nature 217; 723-728.

Mclaren D.S., 1961. Sources of Carotene and Vitamin A in Lake Province, Tanganyika. Acta Trop. 18(1);78-81.

-------; 1986. Global Occurence Of Vitamin A Deficiency. In Bauernfeid C.J. (editor) Vitamin A Deficiency And Its Control Nutrition: Basic And Applied Science A Series Of Monographs. Academic Press pg.1-14.

Mmbando JS., 1987. Atlasi kwa Shule za Msingi Tanzania.

Wizara ya Elimu Tanzania. Macmillan Publisher Ltd. pg. 9-17.

Mohabbat O., Younos M.S., Merzad A.A., Srivastava R.N., Sediq G.G. and Aram G.N., 1976. An Outbreak of Veno-occlusive Disease in North-West Afghanistan. The Lancet Aug 7; 269-271.

Molyneux R.J., Johnson A.E., Roitman J.N. and Benson M.E., 1979. Chemistry of Toxic Range Plants. Determination of Pyrrolizidine Alkaloid Content and Composition in Senecio species by Nuclear Magnetic Resonance Spectroscopy. J. Agric. Food Chem. 27(3); 494-499.

Molyneux R.J. and Roitman J.N., 1980. Specific Detection of Pyrrolizidine Alkaloids on Thin-layer Chromatograms. Journal of Chromatography 195; 412-415.

Molyneux R.J., Benson M., Wong R.Y., Tropea J.E. and Elbein A.D., 1988. Australine, A Novel Pyrrolizidine Alkaloid Glucosidase Inhibitor From Castanospermum australe. J. Nat. Prod. 51(6); 1193-1206.

Morgan R.W., Jain M., Miller A.B., Choi N.W.,Mathews V. Munan L., Burch JD., Feather J. Howe Gr. and Kelly A., 1978. A Comparison of Dietary Methods in Epidemiologic Studies. Am.J. Epidemoil. 107; 488-498.

Muller B.J., Krantzler N.J., Grivetti L.E. Schutz H.G., Meiselman H.L., 1984. Validity of Food Frequency Questionnaire For Determination of Individual Food Intake. Am. J. Clin. Nutr. 39: 136-42.

Nagra S.A. and Khan S., 1988. Vitamin A (β-Carotene) Losses In Pakistan Cooking. J. Sci.Food Agric. 46; 249-251.

Nelis H.J.C.F. and De Leenheer A.P., 1983. Isocratic Nonaqueous Reversed-Phase Liquid Chromatography Of Carotenoids. Anal. Chem. 55; 270-275.

Newman J.L., 1980 Dimensions of The Sandawe Diet in Food Ecology and Culture. Readings in The Anthropology Of Dietary Practice Ed. by John R.K. Robson Gordon and Breach Science Publishers pg. 27-33

Ndiokwere Ch.L., 1984. Analysis of Various Nigerian Foodstuffs For Crude Protein and Mineral Contents By Neutron Activation. Food Chemistry 14; 93-102.

Ogle B.M. and Grivetti L.E., 1985. Legacy of The Chameleon: Edible Wild Plants In The Kingdom Of Swaziland, Southern Africa. A Cultural, Ecological, Nutritional Study. Part 4 Nutritional Analysis and Conclusions. Ecology of Food and Nutrition 17; 41-64.

Ogunlesi AT. and Lee CY., 1979. Effect Of Thermal Processing On The StereoisomarisationOf Major Carotenoids And Vitamin A Value Of Carrots. Food Chem. 4; 311-318.

Okigbo B.N., 1977. Neglected Plants of Horticulture and Nutritional Importance in Traditional Farming Systems of Tropical Africa. Acta Hortculturae 53; 131-151.

Panter K.E. and James L.F., 1990. Natural Plant Toxins In Milk: A Review. J. Anim. Sci. 68; 892-904.

Park Y.W., 1987. Effect of Freezing, Thawing, Drying, and Coking on Carotene Retention in Carrots, Broccoli and Spinach. J. Food Sci. 52(4); 1022-1025.

Pepping F.. Vencken C.M.J. and West C.F., 1988. Retinol and Carotene and Carotene Content of Foods Consumed in East Africa Determined By High Performance Liquid Chromatography. J. Sci.Food Agric. 45; 359-371.

Petry T.W. and Sipes I.G., 1987. Modulation of Monocrotaline-induced Hepatic Genotoxicity in Rats. Carcinogenesis 8(3); 415-419.

Pieters L.A.C. and Vlietinck A.J., 1985. Quantitative ^{1}H Fourier Transform Nuclear Magnetic Resonance Spectroscopy Analysis of Mixtures of Pyrrolizidine Alkaloids From Senecio vulgaris. Fresenius Z Anal Chem. 321; 355-358.

Pieters L.A., Dommise R.A., Corthout J., Totte' J. and Vlietinck A.J., 1986. Identification and quantitative Determination of Pyrrolizidine Alkaloids From Senecio sp. By ^{1}H, ^{13}C-NMR and HPLC. Bio-Organic Heterocycles 1986 - Synthesis, Mechanism and Bioactivity. Proceedings of the 4th. FECHEM Conference on Heterocycles in Bio-Organic Chemistry, Houthalen, Belgium, 25-28 May 1986 pg 259-263.

Pieters L.A. and Vlietinck A.J., 1986. Comparison of High-Performance Liquid Chromatography With ^{1}H Nuclear Magnetic Resonance Spectrometry For Quantitative Analysis of Pyrrolizidine Alkaloids From Senecio vulgaris. Journal of Liquid Chromatography 9(4); 745-755.

Pieters L.A.C. and Vlietinck A.J., 1987. Quantitative Analysis of Pyrrolizidine Alkaloid Mixtures From Senecio vulgaris By Carbon-13 Nuclear Magnetic Resonance Spectroscopy. Magnetic Resonance in Chemistry 25; 8-10.

----------, 1988. Spartioidine and Usaramine, Two Pyrrolizidine Alkaloids From Senecio vulgaris. Planta Medica Journal of Medicinal Plant Research 1988 (2); 95-100.

Pieters L.A., Hartmann T., Jamssens J. and Vlietinck A.J., 1989a, Comparison of Capillary Gas Chromatography with ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectroscopy For The Quantitation of Pyrrolizidine Alkaloids From Senecio vernalis. Journal of Chromatography 462; 387-391.

Pieters L.A., Van Zoelen A.M., Vrieling K. and Vlietinck A.J., 1989b. Determination of The Pyrrolizidine Alkaloids From Senecio jacobae By ^{1}H and ^{13}C- NMR Spectroscopy. Magnetic Resonance in Chemistry 27; 754-759.

Pilbeam D.J., Lyon-Joyce A.J. and Bell E.A., 1983. Occurrence of Pyrrolizidine Alkaloid Monocrotaline in Crotalaria Seeds. Journal of Natural Products 46(5); 601-605.

Pol F., Purnomo SU. and Rosmalen HA., 1988. Trans-cis Isomerisation Of Carotenes And Its Effect On Vitamin A Potency Of Some Common Indonesian Foods. Nutritional Reports International 37(4); 785-793.

Quackenbush F.W. and Smallidge R.L., 1986. Non-aqueous Reverse Phase Liquid Chromatographic System For Separation And Quantitation Of Provitamin A. J. Assoc. Off. Anal. Chem. 69(5); 767-772.

Qualls C.W. and Segall H.J., 1978. Rapid Isolation And Identification Of Pyrrolizidine Alkaloids (Senecio vulgaris) By Use Of High-Performance Liquid Chromatography. J. Chrom. 150; 202-206.

Rahman M.M., Wahed M.A., and Akbar Ali M., 1990. β-Carotene Losses During Different Methods of Cooking Green Leafy Vegetables in Bangladesh. Journal of Food Composition And Analysis 3; 47-53.

Raynal J., Troupin G. and Sita P., 1985. Medicine Traditionnelle et Pharmacopee. Contribution aux Etudes Floristiques au Rwanda. pg. 154.

Roeder E., 1990. Review Article Number 50. Carbon-13 NMR Spectroscopy Of Pyrrolizidine Alkaloids. Phytochemistry 29(1); 11-29.

Roth R.A., Dotzlaf L.A., Baranyi B.,Kuo C.H. and Hook J.B., 1981. Effect Of Monocrotaline Ingested On Liver, Kidney and Lungs Of Rats. Toxicology And Applied Pharmacology 60; 193-203.

Santos Oliveira J. and Fidelgo de Carvalho M., 1975. Nutritional Value of Some Edible Leaves Used in Mozambique. Eco. Bot. 29; 255-263.

Schoental R., 1957. Hepatotoxic Action of Pyrrolizidine (Senecio) Alkaloids in Relation to Their Structure. Nature February 16, 179; 361-368.

Schoental R. and Bensted J.P.M., 1963. Effects of Whole Body Irradiation and of Partial Hepatectomy on the liver lesions Induced in Rats by a Single dose of Retrorsine, a Pyrrolizidine (Senecio) Alkaloid. Br. J. Cancer 17; 242-251.

Segall H.J., Wilson D.W. Dallas J.L.and Haddon W.F., 1985. Trans-4-Hydroxy-2-Hexanal: A Reactive Metabolite From Macrocyclic Pyrrolizidine Alkaloid Senecionine. Science, N.Y. 229; 472-475.

Shanley B.M.G. and Lewis O.A.M., 1969. The Protein Nutritional Value of Wild Plants Used as Dietary Supplements in Natal. Plant Food Hum. Nutr.1; 253-258.

Sharma R.K., Khajuria G.S. and Atal C.K., 1965. Thin Layer Chromatography Of Pyrrolizidine Alkaloids. J. Chrom. 19; 433-434.

Shaheen A.M., Morsi K.S., Bahgat M.A. and Rofael N., 1977. Effect Of Processing On β-Carotene Content Of Carrots. J. Drug Res. Egypt 9(1-2); 19-26.

Simpson K.L. and Chichester C.O., 1981. Metabolism and Nutritional Significance of Carotenoids. Ann. Rev. Nutr. 1; 351-374.

Simpson K.L., 1983. Relative Value of Carotenoids as Precursors of Vitamin A. Proc. Nutr. Soc. 42; 7-17

Simpson K.L., Tsou S.C.S. and Chichester C.O., 1985. Carotenes in: Augustin J., Klein B.P., Becker D., Venugopal P.B. (editors) For The Assoc. Of Vitamin Chemists. Methods Of Vitamin Assay 4th. Ed. John Wiley & Sons N.Y. pg. 185-209.

Simpson K.L., Tsou S.C.S., 1986. Vitamin A and Provitamin A composition of Foods. In: Bauerfeind J.C., Ed. Vitamin A Deficiency and Its Control. Academic Press, Orlando. pg.461-478.

Smith L.W. and Culvenor C.C.J., 1981. Plant Sources Of Hepatotoxic Pyrrolizidine Alkaloids. J. Nat. Prod. 44(1); 129-149.

Speek A.J., Temaliwa C.R. and Schrijver J., 1986. Determination of β-Carotene Content and Vitamin A Activity of Vegetables by High-Performance Liquid Chromatography. Food Chemistry 19; 65-74.

Sreeramulu N., Ndossi G.D. and Mtotomwema K., 1983. Effects of Cooking on Nutritive Value of Common Food Plants in Tanzania: Part 1 - Vitamin C in Some of the Wild Green Leafy Vegetables. Food Chemistry 10; 205-210.

Standley P.C. and Steyermark J.A., 1946. Flora of Guatemala. Fieldiana: Botany 24(v); 193-201.

Stefanik P.A, Trulson M.F., 1962. Determination Of The Frequency Intakes Of Foods In Large Group Studies. Am. J. Clin. Nutr. 11; 335-343.

Stuff J.E., Garza C., Smith H., Nichols B.l. and Montandon C.M., 1983. A Comparison Of Dietary Methods In Nutritional Studies. Am. J. Clin. Nutr. 37; 300-306.

Sweeney J.P. and Marsh A.C., 1971. Effect Of Processing On Provitamin A In Vegetables. Journal Of American Dietetic Association 59; 238-243.

Swick R.A., Cheeke P.R. and Buhler D.R., 1982. Effects of Dietary Senecio Alkaloids and Monocrotaline on Guinea pigs. Journal of Animal Science 55(6); 1411-1416.

Tallantire A.C. and Goode P.M., 1975. A Preliminary Study Of The Food Plants Of The West Nile And Maid Districts Of Uganda. The Utilization Of Leaves And Fruits Of Local And Mainly Indigenous Plants In Supplementing The Staple Foods. E.A. Agric. For. J. 40(3); 233-255.

Tandon B.N., Tandon H.D., Tandon R.K. and Narndranathan M., 1976. An Epidermic of Veno- occlusive Disease of the liver in Central India. The Lancet Aug 7; 271.

Teply L.J., 1986. Control Of Vitamin A Deficiency By The

Agricultural Crop And HouseHold Food Approach. In Bauernfeind J.C. (editor) Vitamin A Deficiency And Its Control. Academic Press, Inc. London, NY, Toky, Toronto. pg. 319-324.

Todd K.S., Hudes M., Calloway D.H., 1983. Food Intake Measurements: Problems and Approaches. Am. J. Clin. Nutr. 37; 139-147.

Vollmer J.J., Steiner N.C., Larson G.Y., Muirhead K.M. and Molyneux R.J., 1987. Pyrrolizidine Alkaloids: Testing For Toxic Constituents Of Comfrey. Journal Of Chemical Education 64(12); 1027-1030.

Warren F,W., 1955. The Pyrrolizidine Alkaloids. Fortschr. Chem. Org. Naturst. 12; 198-269.

----------; 1966.The Pyrrolizidine Alkaloids ii. Fortschr. Chem. Org. Naturst. 24; 329-406.

Watt J.M. and Breyer-Brandwijk M.G., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2 nd Ed. E & S Livingstone Ltd. Edinburgh and London pg. 577-590.

West C.E., Pepping F. and Temaliwa C.R., 1988. The Composition of Foods Commonly Eaten in East Africa. Wageningen Agriculture University. Wageningen.

WHO, 1988. Pyrrolizidine Alkaloids Environmental Heath Criteria 80.

WHO, 1982. Technical Report Series No. 672. Control Of Vitamin A Deficiency And Xerophthalmia. Report Of A Joint WHO/UNICEF/USAID/Helen Keller International/IVACG Meeting pg. 30-65.

Wiehl D.G., 1942. Diets Of A Group Of Aircraft Workers In Southern California. Millbank Memorial Fund Quarterly, 20; 329-366.

Willett WC., Sampson L., Stampfer MJ., Rosner CB., Witschi J., Hennekens CH. and Speizer FE., 1985. Reproducibility And Validity Of A Semiquantitative Food Frequency Questionnaire. Am. J. Epidemiol. 122(1); 51-65.

Zakaria M., Simpson K., Brown P.,and Krstulovic A., 1979. Use Of Reversed-Phase High-Performance Liquid Chromatograghic Analysis For The Determination Of Provitamin A Carotenes In Tomatoes. J. Chrom. 176; 109-117.

Zalkow L.H., Boneth S., Gelbaum L., Gordon M.M., Path B.B., Shani A. and van Derveer D., 1979. Pyrrolizidine Alkaloid From Middle Eastern Plants. J. Nat. Prod. 42(6); 603-614.

Appendix 1 Map of Tanzania Showing The Districts of Mara Region

Shaded area is Tarime district the area of the present study.

Appendix 2

1989 Total Disease Summary For Tanzania Regions Compared With Mara Region Disease Summary From Monitoring Stations.

Disease Name	Tanzania Cases	Tanzania Percent	Mara Cases	Mara Percent
Diarrhoeal	466399	8.02	5225	8.40
Malaria	1783748	30.67	21130	33.97
Measles	32410	.56	42	.07
Acute Poliomylitis	120	.00	1	.00
Whooping cough	1768	.03	1	.00
Neonatal tetanus	105	.00	3	.00
Adult Tetanus	3596	.06	-	-
Intestinal worms	173129	2.98	1840	2.96
Skin Disease	199068	3.42	2186	3.51
Nutritional Disorders	47813	.83	506	.81
Anaemia	88230	1.52	1805	2.90
Normal pregnancy & Minor Complic.	69474	1.19	1585	2.55
Compl. pregnancy & Child birth & puerperium	19531	.34	235	.38
Gonorrhea	106327	1.83	1041	1.67
Upper Resp. Infec.	627728	10.79	6902	11.10
Pneumonia	241680	4.16	1237	1.99
Accidents (incl. burns,fractures)	130563	2.24	419	.67
Schistosomiasis	37482	.64	158	.25
Eye Disease	292652	5.03	1745	2.81
Ear disease	96060	1.65	494	.79
Mental disorders	17722	.30	114	.18
All others (diagn.)	733581	12.61	9336	15.01
Symptoms & ill-difined cond.	646735	11.12	6197	9.96
TOTAL NEW CASES	5815971	100.00	62204	100.00

Appendix 3
MCGILL University - School of Dietetics & Human Nutrition
Edible Greens Study

24 - Hour Recall
KUMBUKUMBU YA SAA 24

CODE/WARD----------Cell leader----------
CODE/Tarafa SHINA KIONGOZI

Household name----------House number----------Jina
la Familia Namba ya nyumba
Interviewee name----------Wt----Interviewer---------
Msailiwa/jina Uzito Msahili/jina

Date recalled----------
Tarehe ya kumbukumbu
Day of the week----------Time----------
Siku ya juma Wakati

Time Wakati	Food Type/part Aina ya chakula/sehemu	How prepared Matayarisho	Amount Kiasi

Note Do not forget lard, milk, sugar and vegetables that Kumbuka: Usisahau mafuta, maziwa, sukari, mbegu za mafuta are normally added as a condiment to stews. Also fruits na mboga ambazo zinaongezwa kwenye chuzi ili kuongeza that are eaten outside normal meals as snacks.
ladha. Pia matunda ambayo huliwa nje ya mlo wa kawaida.

Appendix 4

MCGILL University - School of Dietetics and Human Nutrition

7-DAY FOOD RECORD
KUMBUKUMBU YA MILO YA SIKU SABA

CODE/WARD----------Cell leader----------
CODE/TARAFA SHINA KIONGOZI

Family name----------House Number----------Jina la familia Namba ya nyumba

Interviewee----------Wt---Interviewer----------
Msahiliwa/jina Uzito Msahili/jina

Age/Umri----------

Date----------
Tarehe Day Month Year
Siku Mwezi Mwaka

All of the following questions refer to food eaten during the June-July only.
Maswali yote yanahusu milo iliyoliwa katika kipindi cha miezi ya Juni na Julai tu.

How frequently have the following foods been eaten by you within one week?
Ni mara ngapi vyakula vifuatavyo vililiwa katika familia yako katika muda wa juma moja?

FOOD CODE	FOOD	FREQUENCY Days/Week	AVERAGE NO: Meals/Days	AVERAGE PORTION SIZE
	CEREALS			
	NAFAKA/ UNGA			
	Maize Mahindi	_____	_____	
	Finger millet Ulezi	_____	_____	
	Sorghum Mtama	_____	_____	
	Rice Mchele	_____	_____	
	Cassava Mhogo	_____	_____	
	Others (specify) Nyinginezo(taja)	_____	_____	

Appendix 4 cont.

FOOD CODE	FOOD	FREQUENCY Days/week	AVERAGE NO: Meals/Day	AVERAGE PORTION SIZE
	LEAFY VEGETABLES Mboga za majani			
	Amaranthus Mchicha			
	Crotalaria brevidens Mitoo			
	Cowpea leaves Majani ya kunde			
	Pumpkin leaves Majani ya maboga			
	Cassava leaves Kisamvu			
	Cabbage Kabichi			
	Okra leaves Majani ya bamia			
	Black night shade			
	Jews malo Mlenda			
	Others (specify) Nyinginezo(taja)			

Appendix 4 cont.

FOOD CODE	FOOD	FREQUENCY Days/week	AVERAGE NO: Meals/day	AVERAGE Potion size
	VEGETABLES			
	Sweet potatoes			
	Viazi vitamu			
	Cassava			
	Mhogo			
	Banana			
	Ndizi			
	Squash			
	Maboga			
	Tomato			
	Nyanya			
	Pepper			
	Pilipili			
	Okra			
	Bamia			
	Onion			
	Vitunguu			
	Others (specify)			
	Vinginevyo(taja)			
	ANIMAL PRODUCTS			
	Vitokanavyo na wanyama			
	Meat			
	Nyama			
	Fish			
	Samaki			
	Liver (specify)			
	Maini (taja)			
	Eggs			
	Mayai			
	Milk			
	Maziwa			

Appendix 4 cont.

FOOD CODE	FOOD	FREQUENCY Days/week	AVERAGE NO: Meals/day	AVERAGE Portion size
	LEGUMES & SEEDS			
	KUNDE & MBEGU NYINGINE			
	Beans			
	Maharagwe			
	Cowpeas			
	Kunde			
	Pigeon-peas			
	Mbaazi			
	Groundnut			
	Karanga			
	Others (specify)			
	Nyinginezo(taja)			
	FRUITS & JUICES			
	MATUNDA NA MAJI YAKE			
	Papaya			
	Papai			
	Pineapple			
	Mananasi			
	Sweet orange			
	Machungwa			
	Other citrus (specify)			
	Aina nyingine			
	Mango			
	Maembe			
	Avocado			
	Maembe mafuta			
	Guava			
	Mapera			
	Tamarind			
	Ukwaju			
	Hoslundia opposita			
	Carrisa edulis			
	Others (specify)			
	Mengineyo(taja)			

Appendix 4 cont.

FOOD CODE	FOOD	FREQUENCY Day/week	AVERAGE NO: Meals/day	AVERAGE Portion size
	FATS, OILS & OIL SEEDS			
	MAFUTA NA MBEGU ZA MAFUTA			
	Vegetable oil			
	Mafuta			
	Butter			
	Siagi			
	Ghee			
	Samli			
	Animal fat (lard)			
	Mafuta ya nyama			
	Margarine			
	Majarine			
	Oil seeds (specify)			
	Mbegu za mafuta (taja)			

Are there any vegetables that are by custom designated or
Kuna mboga zozote ambazo kijadi zinatakiwa ziliwe na specially
favoured by certain members of the household? marika
mbalimbali katika familia yako? Kuna mboga zozote
Are there certain vegetables that are by custom
ambazo kijadi hazitakiwi kuliwa na marika mbalimbali.
undesirable or avoided by certain members of your
katika familia yako?
household?

Member RIKA	Custom desigd. Favoured Kijadi zapend. Zapendwa		Custom undesir. Avoided Kijadi zachuk. Zachukiwa
Young woman			
Pregnant			
Nursing			
Older woman			

Appendix 5

PYRROLIZIDINE ALKALOIDS AND NUTRITIONAL STUDY
UCHUNGUZI WA LISHE NA ATHARI ZA VYAKULA AINA ZA MBOGA ZA MAJANI

Mara Region Vegetable Preparation Data Sheet
Kumbukumbu Kuhusu Matayarisho na Mapishi Mkoa wa Mara

Code/ Ward_____ Cell Leader_____
Code/Tarafa Shina/Kiongozi
Family name_____ House Number_____
Jina la familia Nyumba/nambari
Interviewee_____ Interviewer_____
Msahiliwa Msahili
Date_____
Tarehe Day/siku Month/Mwezi Year/Mwaka

All the questions refer to preparation of the vegetable
Mswali yote yanahusu matayarisho na mapishi ya mboga aina Crotalaria brevidens.
Mitoo.

Do you consider this vegetable bitter?_____
Je, unaihisi mboga hii kuwa chungu?
If yes why do you eat this vegetable?_____
Kama ndio mbona unaila?
How do you prepare the leaves before cooking?_____
Unateyarishaje majani kabla ya mapishi?
Do you pound them before cooking?_____
Je, unayatwanga au kukatakata kabla ya kupika?
Do you steep them in water?_____
Je, unayaloweka kabla ya kupika?
How long are they steeped?_____
Kwa muda gani unayaloweka?
Do you add any thing to the cooking water?_____
Unaongeza chochote kwenye maji ya kupikia?
How long do you boil them in water?_____
Unayachemsha kwa muda gani kwenye maji?
Do you discard the boiling water?_____
Je, unayamwaga maji ya kuchemshia ?
How many times do you do this before the bitterness is removed?
Unafanya hivyo mara ngapi kabla ya mboga kuwa tayari kwa Mlo?
_____
_____

Appendix 5 cont.

Do you test them to see if they are no longer bitter? _____
Je, unonja kuona kama uchugu umeisha?
After this process do you do further cooking? _____
Baada ya matayarisho haya je unaedeleaje na mapishi?
What else do you add to the vegetable before it is served? _____
Ni kitu gani kingine ambacho unaongeza kwenye mboga hii kabla ya kuila?
Do you ever preserve this vegetable? _____
Je kuna namna zozote ambazo unatumia kuitunza mboga hii kwa matumizi ya baadaye?

Appendix 6

PYRROLIZIDINE ALKALOIDS AND NUTRITIONAL STUDY
UCHUNGUZI WA LISHE NA ADHARI ZA VYAKULA AINA YA MBOGA ZA MAJANI

Mara Region Vegetable Sample Collection Sheet
Karatasi ya kukusanyia mboga za majani katika Mkoa wa Mara

Date_____ Person Collecting Sample_____
Tarehe Jina la mkusanyaji sampuli
Day__ Month__ Year__ Person Packaging Sample_____
Siku Mwezi Mwaka Jina la mfungashaji

Name of Species_____ Vernacular Name_____
Jina la mmea Jina la jadi

Approximate Weight_____ Harvest Location_____
Uzito Mahali pa kukusanya
Harvest Date_____
Tarehe ya kukusanya

General Condition of the Plant_____
Hali ya mmea kwa ujumla
Presence of flowers_____
Mmea una maua
Presence of Fruits_____
Mmea una matunda
Presence of Seeds_____
Mmea una mbegu
Part Sampled_____
Sehemu iliyokusanywa
Time Dried_____
Muda wa kukausha Date/tarehe Time of the Day/wakati Time of Collection_____
Wakati wa kukusanya
Preparation of Sample if any; describe in detail.
Namna sampuli ilivyotayarishwa kwa urefu hasa kama Especially packing and Preserving with methanol for
imetindikizwa na kitu chochote kabla ya kufungashwa. cooked samples.

_____
_____
_____
_____
_____

Appendix 6 cont.

Label on packaged plant sample must include:
Kitambulisho kwenye sampuli kiwe kama ifuatavyo:

Name of plant sample
Jina la mmea
Part collected
Sehemu iliyokusanywa
State (cooked or raw)
Hali yake(imepikwa/sio)
Person who collected
Mkusanyaji
Collection location
Mahali pa kukusanya
Code number of the package
Nambari ya Kifurushi

Cooked Samples must be shipped preserved in methanol or
Sampuli zilizopikwa zinatakiwa zitindikizwe na methanoli
frozen to the laboratory for analysis.
kwa ajili ya uchunguzi wa maabara.

Appendix 7
MARA REGION DIETARY SURVEY
MKOA WA MARA SURVEY YA MILO

INFORMED CONSENT FORM
KARATASI YA MAELEZO NA MAKUBALIANO

THE PURPOSE OF OUR STUDY IS TO FIND OUT THE KINDS AND
UCHUNGUZI HUU UNATAKA KUFAHAMU AINA NA KIASI CHA
AMOUNTS OF FOODS EATEN BY THE LUO OF MARA REGION. WE WILL
VINAVYOLIWA MKOANI MARA. HII ITATUSAIDIA KUONA NI KIASI
THEN BE ABLE TO KNOW HOW MUCH GOOD NUTRITION AND HOW MUCH
CHAKULA VYA JADI VINAONGEZA LISHE NA KAMA GANI KUNA
PYRROLIZIDINE ALKALOIDS THE PEOPLE OF LUO ETHNIC GROUP AMBAZO
WAJALUO WANAWEZA KUZIPATA. ATHARI ZOZOTE TUTAFANYA ARE EXPOSED
TO. WE WILL DO A DIETARY SURVEY FOR TWO UCHUNGUZI WA MILO HII
KWA MIEZI MIWILI WAKATI WAMIEZI YA MONTHS DURING THE MONTHS OF
JUNE AND JULY 1990. AT THE
SITA NA SABA 1990. BAADA YA UCHUNGUZI TUNATEGEMEA
END OF THE STUDY THE LEADERS OF THIS PROJECT
KUWAELIMISHA WATU KAMA AINA FULANI ZA MBOGA
HOPE TO BE ABLE TO ADVICE THE LUO PEOPLE IF THERE IS ANY
ZINAATHARI ZOZOTE NA NAMNA YA KUEPUKANA NAZO.
RISK IN EATING SOME OF THE VEGETABLES AND THE PRECAUTIONS TO
BE TAKEN WHEN EATING THESE VEGETABLES.
IF YOU WANT TO HELP,THIS INTERVIEW WILL TAKE ABOUT 3/4 OF KAMA
UNATAKA KUSHIRIKIANA NASI KATIKA HUU UCHUNGUZI
AN HOUR OF YOUR TIME TO ANSWER ALL THE QUESTIONS ABOUT MASWALI
YATACHUKUA MUDA WA DAKIKA 45 YA MUDA WAKO.
THE FOODS YOU EAT. ALL INFORMATION WILL BE CONFIDENTIAL MAJIBU
YOTE YATAKUWA SIRI NA HAYATAAMBATANISHWA POPOTE NA AND NEVER
PUBLICLY ATTACHED TO YOUR NAME. NUMBER CODES WILL BE USED FOR
ALL THE FORMS.
JINA LAKO. TUTA"UMIA NAMBARI KATIKA HUU UCHUNGUZI.
THIS STUDY IS BEING DONE BY MCGILL UNIVERSITY IN
HUU UCHUNGUZI UNAFANYWA NA MCGILL UNIVERSITY
COOPERATION WITH THE TRADITIONAL MEDICINE RESEARCH UNIT
IKISHIRIKIANA NA MUHIMBILI MEDICAL CENTRE .
KITENGO CHA UTAFITI WA DAWA ZA ASILI - MUHIMBILI HOSPITALI.
THIS IS SPONSORED BY CIDA (CANADA) MY SPONSORS FOR THE
SHIRIKA LA CANADA KWA JINA CIDA AMBAO WANALIPIA MASOMO
GRADUATE STUDIES. YOUR HELP OR YOUR REFUSAL WILL NOT YANGU
NDIO WANALIPIA HUU UCHUNGUZI.KUKATAA AU KUKUBALI INFLUENCE
YOUR STANDING WITH ANY OF THESE ORGANIZATIONS KWALO
HUKUTAATHIRI KWAVYOVYOTE UHUSIANO WAKO NA MASHIRIKA IN ANY
WAY. AT ANY TIME YOU CAN REFUSE TO ANSWER ANY OR YALIYOTAJWA.
KWA WAKATI WOWOTE UNAWEZA UKAKATAA KUJIBU ALL QUESTIONS AND
ASK US TO LEAVE.
MASWALI YEYOTE NAUKATUELEZA TUONDOKE.
FEBRONIA C. UISO WHO IS HERE OFFICIALLY FOR THE STUDY,
FEBRONIA C. UISO AMBAYE YUKO HAPA RASMI KWA SHUGHULI YA
WILL ANSWER ANY QUESTIONS YOU MIGHT HAVE ABOUT NUTRITION

Appendix 7 cont.

UCHUNGUZI ATAJIBU MASWALI YEYOTE KUHUSU LISHE NA ATHARI AND THE ALKALOIDS SUSPECTED TO BE PRESENT IN VEGETABLES OF ZINAZOHISIWA KUTOKANA NA AINA ZA MBOGA ZINAZOFANYIWA INTEREST.
UCHUNGUZI.

DO WE HAVE YOUR PERMISSION TO BEGIN?
JE, UNATURUHUSU KUANZA KUULIZA MASWALI?

NAME
JINA
HOUSE NUMBER
NAMBARI YA NYUMBA

Research Supervisor: Dr. Timothy Johns
Msimamizi wa uchunguzi:
Professor of Human Nutrition
Macdonald College of McGill University
21,111 Lakeshore Road,
Ste. Anne de Bellevue,
Quebec H9X 1C0.

Appendix 8

GREEN LEAFY VEGETABLES CONSUMED IN TARIME DISTRICT

BOTANICAL NAME	LUO NAME	COMMON NAME English/Swahili
Amaranthus hybridus		Mchicha
Amaranthus spp.	Odoodo, Omboga Alkra	Mchicha pori
Crotalaria brevidens var.intermedia	Mitoo, Mtoo	
Crotalaria spp.	Nyassamo modongo Nyassomo matindo Nyabundege	
Vigna ungiculata	Boo	Cowpea leaves/ Majani ya kunde
Cucurbita spp.	Susa	Pumkin leaves/ Majani ya maboga
Manihot esculata		Cassava leaves/ Kisamvu
Brassica oleracea var.capitata	Kabichi	Cabbage/ Kabichi
Brassica spp.	Kandhira	Sukumawiki
		Spinach
Hibiscus esculentus	Bania	Okra / Bamia
Ipomea batatus	Marande Marando	Sweet potatoe leaves Matembele
Gynandropsis gynandra	Mgagani Deck	
Asystasia schimperi	Atipa	
Corchorus olitorius	Apoth	Mlenda
Bassela rhubra	Ndemra Kirerema	
Sesamum anguistifolia	Anyiim	
Solanum nigram	Osuga	Mnavu
Oxygonum sinuatum	Awayo	

Appendix 8 cont.

BOTANICAL NAME	LUO NAME	COMMON NAME English/Swahili
Justicia matammensis	Piupiu	
Vigna sp.	Dindi	
Commelina subulata	Angayo	
Portulaca spp.	Egetandu	
Cucumis spp.	Limbe	
Mormodica spp.	Inungu	
Launea cornuta	Nyabusungu	Mchunga
unidentifiedspecies	Nyadeckdan	
	Nyawendagwata	
	Nyayado	
	Amondii	
	Okuuro	
	Duumu	

Appendix 9

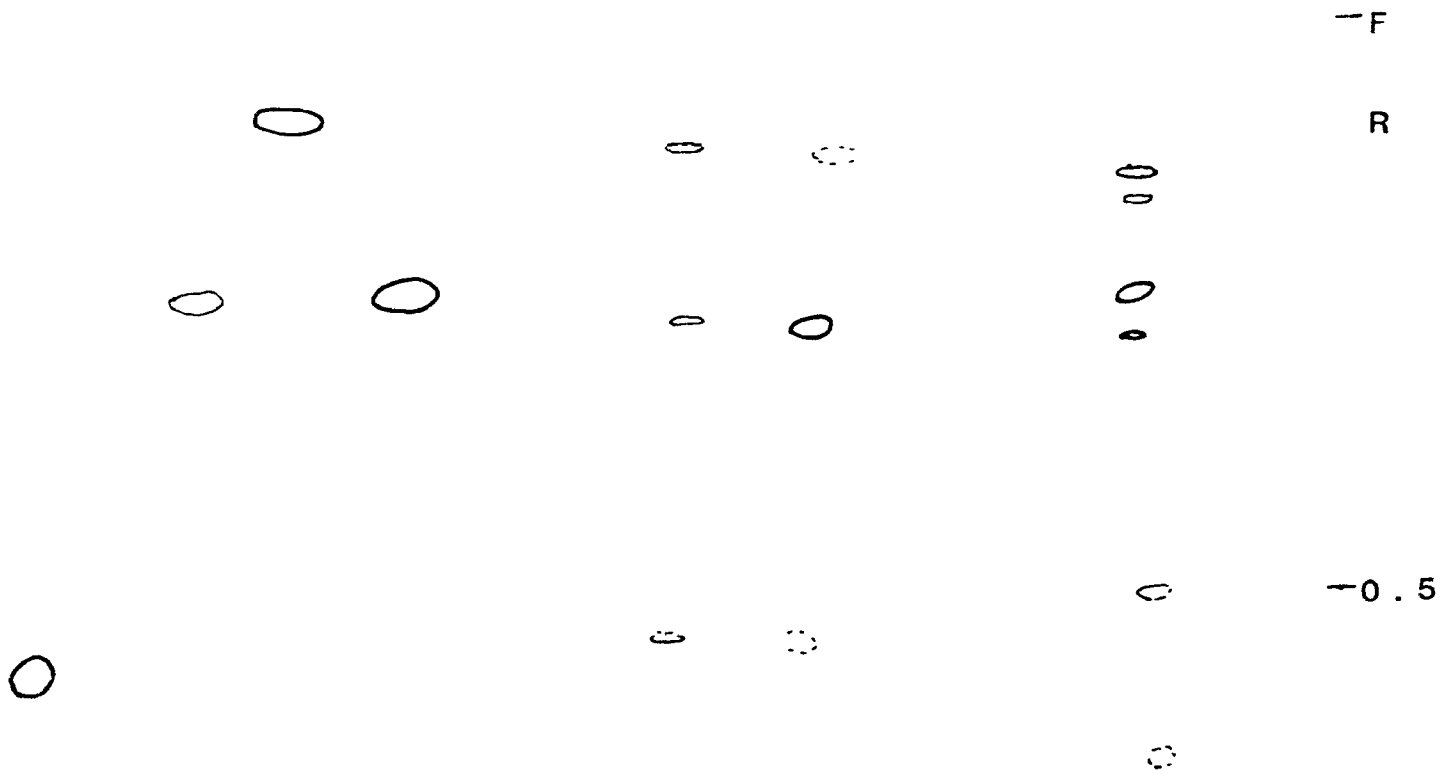
LIST OF FRUITS CONSUMED IN TARIME DISTRICT

BOTANICAL NAME	LUO NAME	COMMON NAME English/Swahili
Carica papaya	Poipoi	Papaya / Papai
Ananas comosa	Mananas	Pineaple / Nanasi
Citrus sinensis	Machunga	Oranges / Machungwa
C. limom	Malmao	Lemons / Malimao
C. reticulata	Sandhra	Tangerine / Machenza
C. paradisi or grandis	Balungi	Grape fruits / Madanzi
Mangifera indica	Maembe	Mangoes / Maembe
Persea americana		Avocadopea
Psidium guavaja		Guava / Mapera
Musa spp.	Rabolo	Bananas / Ndizi
Carrisa edulis	Ochuoga	
Toddalia zanzibarensis	Sangla	
Ximenia caffra	Olemo	
Afromomum senegalensis	Akuuna	
Tamarindus indica		Ukwaju
Grewia spp.	Powo Poo	
Annona senegalensis	Nyabolo obolo	Wild soursop
Passiflora edulis	Matunda	Passion fruits
unidentified species	Masungi	
	Mathaboro Ongara	
	Ambuuna	

Appendix 9 cont.

BOTANICAL NAME	LUO NAME	COMMON NAME English/Swahili
	Omuso	
	Ong'oora	
	Abang	
		Black berries

Appendix 10 Thin layer chromatogram of the different basic plant extracts with the authentic reference standards.
1) Free alkaloid flowers extract, 2) Reduced alkaloid flowers, extract, 3) Free alkaloid fraction from green house grown material, 4) Reduced alkaloid fraction from green house grown material, 5) Free alkaloid fraction from wild Crotalaria plant extract, 6) Reduced alkaloid fraction from wild Crotalaria plant extract, 7) Free alkaloid fraction from cultivated (Kenyan) plant extract, 8) Reduced alkaloid fraction from cultivated (Kenyan) plant extract, 9) Free alkaloid cooked (wild) fraction, 10) Reduced alkaloid cooked (wild) fraction, 11) Monocrotaline, 12) Senecionine, 13) Retrorsine, 14) Retrorsine N-oxide. S start, R R_f and F solvent front. Eluting solvent is as explained in the text. Detection was by Mattocks test.


F
R
0.5
14 13 12 11 10 9 8 7 6 5 4 3 2 1
S