The Potential For A Novel Alcoholic Drink Prepared From The New Zealand Native Plant *Cordyline australis* (ti kōuka)

Minaxi Patel

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Cordyline australis

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to a substantial extent, has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed……

Date……

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I wish to express my sincere thanks to Assoc Prof Owen Young, my supervisor, for his supervision, encouragement, patience and endless support through the course of this study. I would like to thank Thoughts Group Limited and Berri Schroder for the Scholarship. I would also like to thank all the chemistry technicians in the Applied Science department at AUT University. Finally, I would like to thank my husband and two sons for their continuous moral support throughout this study.

Abstract

Some New Zealand indigenous plants may offer unique qualities that can be used to secure an exclusive niche in the alcoholic drinks market in the same way that Scotch whisky and tequila are strongly identified with the country of origin, Scotland and Mexico. Tequila is a spirit distilled from a fermented agave, dry adapted lily. Agave is in the family Agavaceae, a notable New Zealand member of which is the common cabbage tree or ti kōuka (*Cordyline australis*). Similarly, to the agave having a fermentable core, ti kōuka has carbohydrate (inulin) content in its young stems and roots that can be hydrolysed in acidic suspensions or by enzyme hydrolysis to yield fructose.

The main objective of this thesis was to systematically research the feasibility of the production of a tequila-like spirit from ti kōuka stem, profiling the chemical properties of the spirit with a view of future commercial production of an iconic New Zealand spirit.

The initial stage of the thesis focused on extracting inulin from the ti kōuka stem and hydrolysing (by both acid and enzyme) it to yield reducing sugar. The sugar concentration yielded was too low (~ 10 to 15%) to be fermented and distilled economically. Rather, the ti kōuka extract was evaporated to produce flavoured products by the Maillard reaction, a reaction between amino acids and sugars. The flavoured compounds were then infused with potable ethanol. In outline, the dried stem was hydrolysed with an inulinase at 60°C for 1 hour. The pH was adjusted to 10 with sodium hydroxide and evaporated at 60°C for 65 hours. The dried extract was reconstituted with water, centrifuged and the supernatant infused with portable ethanol to yield final different concentrations of 80, 67, 57 and 50%. The ethanol treatments simultaneously extracted flavour and colour to varying degrees.

Next, sugars and amino acids were analysed in the ti kōuka stems by liquid chromatography. The most abundant sugar present in the ti kōuka after inulinase hydrolysis was fructose and the dominant amino acids were arginine, leucine, lysine, and aspartic acid/aspargine and glutamic acid/glutamine. Amino acids and reducing sugar were also analysed at different stages of the spirit production. The reducing sugar

content decreased during each step of the process. The relative concentrations of arginine, leucine and lysine decreased while that of aspartic and glutamic acids increased during the whole process of making the spirit.

Model systems were then used to simulate the reactions taking place between the amino acids and reducing sugar present in the ti kōuka extract. The colour of the models became darker as a function of time, accumulating more brown pigment containing the flavoured compounds. Increasing the pH and concentration of the amino acids in the reaction mixture also increased the browning pigment formation.

Dichloromethane and n-pentane and diethyl ether solvent extraction of the spirits and analysis of volatiles by gas chromatography- mass spectrometry revealed that the chemical profiles of the spirits were different from those of the commercial spirits, gin, tequila and whisky.

Sensory evaluation was performed on four variations of the spirit, and demonstrated that the creations were consumer-acceptable.

The costs and other issues involved in producing and marketing such a spirit were identified, the major selling point being geographical exclusivity.

CHAPTER 1

Conventional and distinct alcoholic drinks

1.1 Introduction

Alcoholic drinks produced and sold in New Zealand are – with the possible exception of uniquely-flavoured vodkas produced by 42 Below Limited, Auckland – copies of Old World models. Thus, beer and whisky (from barley), wine (grapes), and gin (alcohol flavoured with plant extracts) all have their antecedents in Europe.
In the case of beer and white wine particularly, these drinks dominate domestic consumption, as is obvious by inspection of supermarkets and liquor stores. In the annual 2007 New Zealand wine growers report, it was reported that the number of grapes growers is increasing every year (http://www.nzwine.com). Domestic sales of New Zealand wine has increased from 30.9 million litres in June 1995 to 45.0 million liters in June 2005 to 51 million litres in June 2007, while the export volume has increased from 7.8 million litres to 51.4 million litres to 76 million litres, respectively. The current New Zealand wine projection forecast for domestic sales in 2010 is $0.5 billion with exports of $1 billion (Vieceli, 2005). These increasing domestic consumption figures cannot be attributed to tariff barriers against imports, because tariff barriers are low by world standards (Goff, 2007).

Thus, New Zealand's alcoholic drinks are generally good, even excellent copies and parallels of international models. However, in one instance, a wine is so distinct that it has become an international icon of its type, and has similarly conferred its region of origin with an international reputation that extends beyond the particular grape and wine (Apstein, 2005). The region is Marlborough, on the northeastern tip of New Zealand's South Island. The wine is made from the Loire Valley (France) grape variety S*auvignon blanc*, literally 'wild white'. Of the 76 million litres of wine exported (mentioned above) in the year ending June 2007, 56.6 million litres was *Sauvignon blanc*.

Wine producers in other regions of New Zealand are striving to achieve a distinctive style(s) to match the achievement of Marlborough sauvignon blanc. Their motive is clear. It is a fundamental tenant of successful marketing that your commercial offering must be distinct from others so as to avoid competition based on price alone (Baker,

2007). Thus, commodities such as oil, wheat, and sugar are traded internationally on price within defined and measurable quality grades. Whereas some very large wine producers can and will make profits selling a branded but mediocre wine in large volumes, smaller producers aim for distinctiveness to avoid price competition.

Looking beyond wine there are many good examples of distinctiveness in other alcoholic drinks. Scotch whisky and American Bourbon whisky are two examples, both of which rely on a geographical origin to secure their market position. For example, Laphroaig is a single malt whisky from Islay, Scotland. Promotional language like: "Love it or hate it, its powerful, structured flavours of peat smoke, medicinal seaweed, iodine, diesel oil and tar still…" (www.bakersandlarners.com), epitomises the image that distinctiveness can create. With modern chemical knowledge, it is likely that Laphroaig flavour and appearance could be recreated elsewhere, but the geographical distinctiveness is inextricably linked to the product and will always set it apart. Combined with iconic packaging, Laphroaig and similar drinks occupy the high priced end of the market.

Gin, on the other hand, is made all over the world; however, each gin maker has its own distinct recipe, which makes that maker's spirit unique. Gin consists of neutral spirits distilled or redistilled with juniper berries (*Juniperus* species, primarily *Juniperus communis*) and other botanical ingredients. The term neutral does not refer to acidity/alkalinity, rather to the fact that the spirit is flavourless beyond the basic taste of ethanol in water. The English word 'gin' is an abbreviation of the word 'geneva', a corruption of either the French word *genièvre* or the Dutch word *jenever*, both meaning juniper. Each gin brand has its own (and often secret) recipe, with juniper being the primary flavoring ingredient. To this, the producer might include lemon peel, orange peel, anise, orris root, angelica root, cardamom, coriander, licorice root, cinnamon, and cassia, and sometimes other various flavourings to give a distinctive flavour.

Practically all premium quality gin is distilled (Lea and Piggott, 2003). There are two methods for producing distilled gin, direct distillation and redistillation. Distilled gin is made by distilling neutral alcohol and water in physical contact with juniper berries and the other botanical ingredients. Even before heat is applied, many of the potential volatiles of the botanical congers become soluble in the ethanol/water mixture. As heat is applied through the steam coils in the bottom of the still, these volatiles co-distill with

ethanol. As the temperature rises (towards the boiling point of the individual solvent in the mixture) more volatiles will be extracted and co-distilled. The resulting vapour is condensed, and after the ethanol concentration (between 80 and 90% v/v) is adjusted to around 40% v/v, the spirit becomes gin as we know it.

Redistillation differs from distillation only in that the ethanolic vapour passes through a 'gin head' packed with the flavouring materials. Thus, extraction of volatiles nominally occurs in the vapour phase rather than by physical contact with the ethanol/water mixture. (The word nominally is used because at the point of extraction at the molecular level, ethanol as a liquid may be present (Lea and Piggott, 2003)).

Turning now to New Zealand, 42 Below Limited produces New Zealand's only locally produced premium vodka which has won many gold, silver and bronze awards at events like Sialkot International Airport Limited (le SIAL d'OR), Monde Selection, World Spirit Competition and International Wine and Spirit Competition, between 2002 and 2008 (www.42below.com). The company's original product is the "country's only super premium vodka 42 Below", a brand that is "unashamedly New Zealand", says Mr Steel, the Commercial Manager. "We have created the cleanest vodka because we have one of the purest water supplies in the world and we are the most awarded vodka in the world because of our pristine environment", says Geoff Ross, the founder of 42 Below.

From a food chemistry perspective, the validity of these claims is very doubtful, but they do illustrate the potential value of geographical distinctiveness, real or perceived.

Four varietal flavours have been introduced by 42 Below – feijoa, manuka honey, passionfruit and kiwifruit. 42 Below's products have been successfully marketed internationally, catching the attention of international liquor giants to the point that the company has been sold to the world's largest privately held spirits company, Bacardi Limited (Knowles, 2006)[1].

Tequila is another spirit with clear geographic distinctiveness. Tequila is distilled from a fermented core of the Mexican blue agave (*Agave tequilana* Weber *var azul*). It is often but mistakenly thought to be a cactus, but in fact is a lily (amaryllis) family that

[1] CEE – Food Industry.com Bacardi Limited acquires New Zealand Vodka producer 42 Below Limited (Accessed 28-09-2006)

adopts a cactus-like habitat suited to dry environments. The blue agave is in the botanical family Agavaceae, a notable New Zealand member of which is the common cabbage tree, *Cordyline australis*, or ti kōuka as was called by Māori.

The process of making tequila begins when the blue agave plant is 'ripe', usually eight to twelve years after planting: After five or six years of growth, when the plant normally starts flowering, the leaves are cut off. After eight years, the agave plant begins to dry as it matures. When the inside of the leaf bases close to the piña (also called the heart) turn green-yellow, it is an indication that the piña is ripe for harvesting. Harvesting takes place all year round because the plants mature at different times in the field. When ready for harvesting, the carbohydrate rich piñas are dug out of the ground (with its roots). The roots, stalk and leaves are cut off from the piña leaving the piña looking like a pineapple (Kretchmer, 1999). The piña, which weighs anywhere between 20 to over 100 kilogram, is cut into halves or quarters for the next step of the process i.e. the cooking process (Cedeño, 1995).

The traditional method for cooking piñas is in a brick or concrete oven at 100°C for about 24 to 36 hours while in modern methods, it is autoclaved for 7 hours at 121°C (Cedeño, 1995). During the cooking process, the carbohydrates (inulin) in the piñas are hydrolysed to fermentable sugars. (The nature of inulin is discussed later in this chapter.)

In ancient days (before 1950), the fibrous piñas were crushed with a stone wheel at a grinding mill. In the traditional method (after the 1950s), the cooked agave was passed through a cutter to be shredded and with a combination of milling and water extraction, sugar rich juice was extracted. Water was sprayed on the fibres, washing the converted sugars off the fibre. The extracted juice was then transported to the fermentation vessels (Kretchmer, 1999).

During the fermentation process, which takes place for about 30 to 48 hours, yeast (typically *Saccharomyces cerevisiae*) acts upon the extracted sugar juices of the piñas converting them into alcohol and carbon dioxide (Kretchmer, 1999). In the final stage, the fermented alcohol is double distilled in their traditional copper stills or modern stainless steel or in continuous distillation towers. At this point, the liquor is tequila (www.ianchadwick.com) with alcohol content between 35 and 55%.

Tequila has a flavour like no other drink. This feature combined with its geographic distinctiveness will, like *Laphroaig*, always set it apart. Resurrecting the practices the early Māoris used for obtaining sugar from the cabbage tree, and modifying the process of extracting the sugars and fermenting and distilling it, New Zealand could have its own distinctive ti kōuka flavoured spirit.

1.2 *Cordyline australis* used for producing illicit alcoholic drinks

The New Zealand cabbage tree, *Cordyline australis* or ti kōuka is a New Zealand native and is the world's largest lily (www.uneli.unitec.ac.nz). In pre-and early European times, Māori used its stems and rhizomes as a staple carbohydrate source. Mexicans used the agave piña as a carbohydrate in its own right and as a source of fermented liquor; Māori on the other hand never fermented ti kōuka (Best, 1976). Although there is no evidence that the Māori made any alcoholic beverages from *Cordyline australis*, early missionaries who settled in New Zealand prepared a 'beer' of sorts and there is a record of illicit spirit distillation (Simpson, 2000). In the 1840s, an Irishman Owen McShane living near Invercargill, made and sold an illicit spirit prepared from the carbohydrate-rich rhizome of ti kōuka. The rhizomes were chopped with a hatchet, and the slices placed in a vat and covered with water. The mixture was left to ferment, and the resulting liquor distilled to produce the spirit (Simpson, 2000). The fermentable carbohydrate in ti kōuka is not starch, rather a polymer, called inulin, of the sweet sugar fructose[2]. On heating in aqueous solution, inulin tends to hydrolyse to the monosaccharide, fructose. Complete hydrolysis by acid hydrolysis or with the aid of a specific enzyme can be rapidly achieved (as will be discussed in more detail in Chapter 2). Once the carbohydrate is extracted, it can be fermentable and subsequently distilled.

Thus, there is a historical precedence for ti kōuka spirit, has the historical evidence of a spirit, which if resurrected, may become a commercial reality protected by geographic, and possibly flavour distinctiveness.

1.3 Development of Ti Koka spirit

This thesis reports the development of a ti kōuka spirit, such a liquor that will be called 'Ti Koka' from the outset, although this name will not be used until the final chapter on

[2] On a weight basis fructose is about 15% sweeter than sucrose and 65% sweeter than glucose (Belitz & Grosch, 2004).

commercial possibilities. The name Ti Koka is clearly a corruption of the Māori name, ti kōuka. Under New Zealand trademark law, a specific or common name is not allowed to be used (www.med.govt.nz. Trade Mark Protection in New Zealand). The proposition is that Ti Koka is probably protectable, is easy to say (*tee coke a*), and when pronounced in this way, it equates with the formal pronunciation of ti kōuka.

This project began with the premise that Ti Koka production would follow the traditional path, the path by which most spirits are prepared (i.e. extraction, fermentation and distillation). However, early in this project it was realised that the costs involved in these steps may be too high. It was known that the yields of inulin per hectare from ti kōuka were markedly lower than those of competing carbohydrates like sucrose from sugar cane or sugar beet (Harris and Mann, 1994).

As the research evolved, it became clear that the best commercial strategy lay in using ti kōuka as a source of flavour and colour, while deriving the ethanol from other potable sources. This evolution in thinking will be further described in Chapter 2. However, ti kōuka remains central to the production creation, so this chapter describes the botany and ethnobotany of the plant and lays the groundwork for the study.

1.4.1 *Cordyline* botany

Cordyline australis is a native New Zealand monocotyledon plant of the order Asparagales, and the family Agavaceae (Simpson, 2000). *Cordyline* species occur mainly in arid tropical and sub-tropical climates throughout many parts of the world e.g. Malaysia, Australia, India and Polynesia (Salmon, 2001).

Five species of the genus *Cordyline* are endemic to New Zealand (Fankhauser & Brasch, 1988). Another member of the genus, *Cordyline terminalis*, later named *Cordyline fructicosa*, is widespread throughout the Pacific Islands. Roua, son of Tamatea-nukoroa, chief of the Nukutere colonizing canoe, introduced *Cordyline fructicosa* to New Zealand and became known as *ti pore* (Simpson, 2000). Of the genus *Cordyline*, *C. australis*, the ti kōuka of Māori – commonly called the cabbage tree by New Zealanders of European descent – has the widest range, occurring naturally throughout both main islands, especially in open places and forest margins. The species shows a preference for sites near swamps (Harris *et al*., 2001). The *Cordyline australis* species is distributed over 99% (Figure 1.1) of New Zealand (Simpson, 2000).

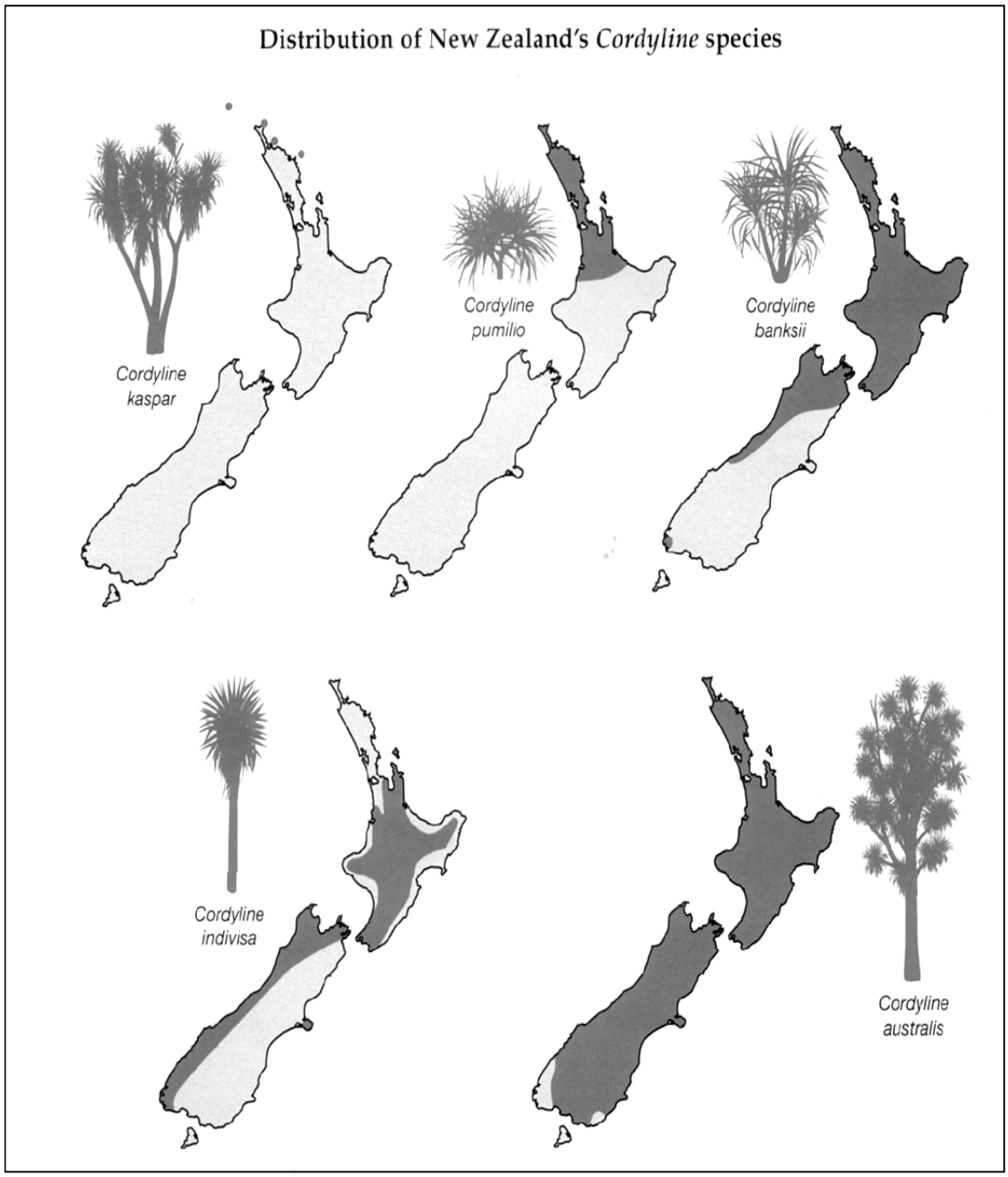


Figure 1.1 The distribution of New Zealand's *Cordyline* species (Simpson, 2000)

1.4.2 Ethnobotany of *Cordyline australis*

Māori from different parts of New Zealand have different names for the cabbage tree; and in earlier times, the plants were put to many uses. The main species of *Cordyline* are listed in Table 1.1 with their botanical, Māori and common names.

Table 1.1 Species of *Cordyline* in New Zealand

Systematic botanical names	Māori names	Common names
Cordyline fructicosa	ti pore	Short cabbage tree Pacific cabbage tree Polynesian cabbage tree
Cordyline kaspar	ti tawhitirahi, ti tawhitinui	Three Kings Island cabbage tree
Cordyline australis	ti awe, ti manu, ti kōuka, ti kauka, ti pua, ti puatea, ti rakau, ti tī, ti whanake, kiokio, kaore	Cabbage tree
Cordyline indivisa	ti toi, ti kapu, ti kupenga, ti matuka-tai	Mountain cabbage tree or broad-leaved cabbage tree
Cordyline banksii	ti ngahere, ti parae, ti torere, tituruki kiokio, hauora	Forest cabbage tree
Cordyline pumilio	ti koraha, ti papa, ti rauriki, mauku, kopuapua, korokio	Dwarf cabbage tree
Cordyline cultivars	ti para, ti tawhiti, ti towhiti, ti tahanui, ti mahonge, ti tohea	Cordyline 'Thomas Kirk'

Simpson, 2000

1.4.3 *Cordyline australis* used as food

The Māori were early New Zealand settlers of Polynesian descent. They introduced the three tropical crops, sweet potato or kumara, taro and ti, all of which are still eaten in New Zealand today (Cambie and Ferguson, 2003), particularly the former two. The name ti used here is to signify a genus *Cordyline* in its New Zealand context. Ti kōuka was not only a staple food for Māori but ti in general was also the source of food for other animals (e.g. keruru (wood pigeon), caterpillars of ti moths) which the Māoris collected and ate.

When the leaves of the stem are stripped off, what remains is the kōata, the cabbage-like stem that when cooked becomes kōuka, a nutritious but somewhat bitter vegetable. (Kōata is the botanical equivalent of the agave piña.) The fresh stem and rhizomes are rich in carbohydrate (inulin and reducing sugars) and fibre (refer to Table 1.2).

Table 1.2 Nutritional value of ti kōuka, percentage by wet weight[1]

	Water	Carbohydrate	Fat	Fibre	Protein	Energy (kJ 100 g^{-1})
Stem	68.3	14.9	1.5	13.6	0.4	71
Rhizome	64.0	23.6	1.4	10.3	0.4	103
Stem tips	81.5	8.8	3.2	4.5	1.4	68

[1]Adapted from Crowe, 1997

According to Fankhauser and Brasch (1985), the polysaccharide content (mainly glucofructan-type polysaccharide) in the rhizomes and stem was reported as 55 and 34.6% respectively in summer, and 43 and 17.5% respectively in winter. These percentages were based on the dry weight of the original rhizomes and stems. The fructan concentration in ti kōuka plant increases as it ages and reaches a maximum as the plant begins to flower in spring (Crowe, 1981). The dried rhizomes and stem of ti kōuka contains 3.3 and 2.8% unpolymerised, i.e. free fructose respectively in summer, and 5.8 and 3.5% respectively, in winter.

The polysaccharide of ti kōuka is inulin, a glucofructofuranan, or fructan. An identifying feature of inulin is its β-(2→1) bonds between adjacent fructose monomers (see Chaper 2). Inulin is not digested in the upper gastrointestinal tract, as starch is (Niness, 1999). Whereas the human digestive system can hydrolyse starch, principally by the action of α-amylase, it lacks the enzyme inulinase required to hydrolyse β-(2→1) bonds (Simpson, 2000). However, on moist heating, inulin can be hydrolysed to the sweet monosaccharide fructose, which is digestible.

James Hay observed that Māori carried out the practice of extracting sugar from ti kōuka stem by cooking it in a hangi for hundreds of years (Best, 1976). Hay described the hangi which the Māori used as deep holes (or pits), 2.4 m long, 1.2 m wide and 1.5 to 1.8 m deep in which a layer of stones that were heated by fire beforehand, were placed at the bottom of the hole. Layers of *Cordyline australis* were placed on top of the stones followed by water to generate steam. Once the steam was produced, the

holes were covered and left for many days. The sugar-rich fibre that was produced was used as a food source as well as using it to sweeten tea (Best, 1976)[3] .

1.4.4 Cordyline australis as medicine

Māori had a wide range of medicinal uses for the various parts of the cabbage tree and various species of *Cordyline*, and many remedies are still in use (Whistler, 1992). *Cordyline fructicosa* has been used for a variety of ailments of the mouth and stomach. *Cordyline fructicosa* contains a compound closely related to cinchophen (Figure 1.2) (which has analgesic properties) which was used to sooth the gastric system and for reducing inflammation (Brooker *et al*., 1981). However, cinchophen (a uricosuric drug used for the treatment of gout) can cause liver damage (Cutrin *et al*., 1991).

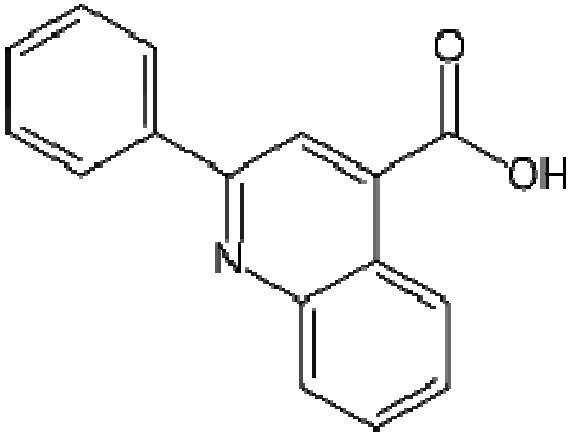


Figure 1.2 Structure of cinchophen (from Wikipedia, http://en.wikipedia.org/wiki/cinchophen)

The kōuka (see above) also contains saponins, which as the name suggests are soap-like insofar as they are surface active. Saponins consist of a polycyclic aglycones attached to one or more sugar chains (Figure 1.3). The aglycones part (also called sapogenin) is either a steroid or a triterpene. The foaming ability of saponins is caused by the combination of a hydrophobic (fat-soluble) sapogenin and a hydrophilic (water-soluble) sugar part. (Importantly for this thesis, saponins are bitter, which may be important in flavour of ti kōuka spirit.) According to Simpson (2000) kōuka as a food was regarded

[3] In Maori Agriculture Dominion Museum Bulletin No9 A.R. Shearer Government Printer Wellington, New Zealand.

as a ‘blood purifier’, relating particularly to cleansing fat from the blood. Certainly, saponins have anti-cancer (Rao and Sung, 1995) and anti-tumour properties.

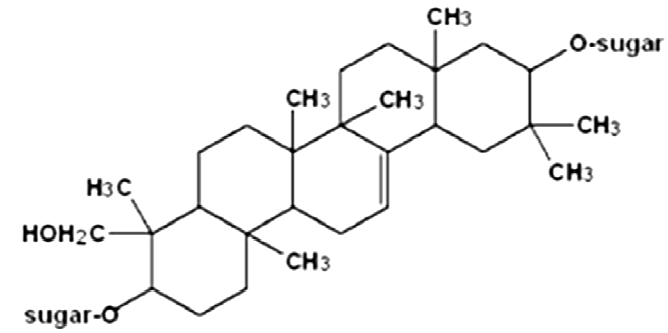


Figure 1.3 Structure of saponin (www.phtochemicals.info)

Saponins can stimulate the central nervous system and have anti-stress properties (Simpson, 2000), immune-stimulating, antifungal, and antiviral activities (Lacaille-Dubois and Wagner, 2000). Softened kōuka was applied as an ointment to cuts, cracks in the skin and sores (Simpson, 2000; Brooker *et al*., 1981). The seeds were used to treat skin disorders and heart ointments. Any therapeutically valid effect may be related to the fact that the seeds contain high concentrations (75 to 89%) of linoleic acid, a metabolically essential fatty acid (Morice, 1962). The young inner shoots and top of the stem were boiled and eaten by nursing mothers to stimulate milk flow (Simpson, 2000) and were also given to infants with colic problem (Brooker *et al*., 1981).

1.4.5 Cordyline australis as fibre

Ti kōuka leaves were useful as a structural material because the longitudinally-oriented fibres have a very high tensile strength, so were useful for making strings and ropes used for fishing lines and anchor ropes and fishing lines. Both surfaces of the leaf are covered in a wavy layer, which serves as a waterproofing agent. Ti kōuka leaves were also commonly used for weaving baskets for different purposes. For example, cylindrical baskets were used for retaining food when immersion-cooking was carried out in thermal pools, throwaway lunch baskets were made from fallen leaves, and large elongated baskets were made during each harvesting season of ti kōuka. The durability of ti kōuka was useful in making clothing items e.g. cloaks, sandals, waist mats, rain cloaks etc. (Simpson, 2000). There is a potential opportunity to use *Cordyline australis*

as a leaf crop for the production of novelty goods that relate to its history of traditional use, and to capitalise on New Zealand's tourist market.

1.5 Literature review of ti kōuka as a source of carbohydrate

In 1934, Lyon showed that the roots of *Cordyline terminalis* contained a water-soluble polysaccharide built of fructofuranosyl residues (as reported by Tanimoto, 1939). Boggs & Smith (1956) also isolated a glucofructofuranan polysaccharide from *Cordyline terminalis*. Analysis revealed it comprised one α-D-glucosyl residue linked to about 14 β-D-fructofuranosyl residues per molecule.

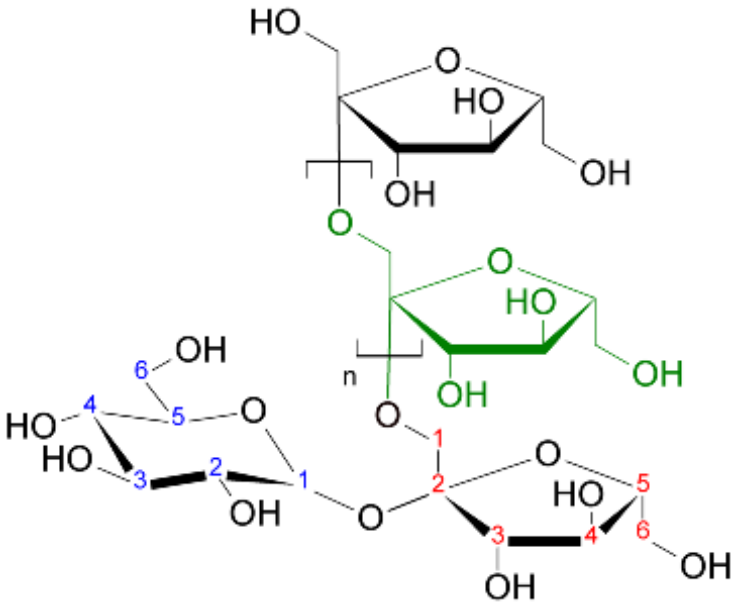


Figure 1.4 Structure of glucofructofuranan polysaccharide (inulin) (from Wikipedia, http://en.wikipedia.org/wiki/inulin)

The glucofructofuranan was subject to methylation (using Hakomori method) as a method of determining which hydroxyl groups were involved in the cross links (Hakomori, 1964) The analysis showed that it contained both 1→2-linked and 2→6-linked β-D-fructofuranosyl residues, with some branching. In 1988, Brasch *et al.* confirmed the main structural features of this fructose-rich polymer from the rhizome of *Cordyline australis* using a ^{13}C nuclear magnetic resonance method.

Later in 1994, Harris and Mann investigated the suitability of ti kōuka as a crop for fructose production. They found that ti kōuka is capable of producing at least 4 tonne (4,000 kg) of (fermentable) sugar – largely fructose – per hectare. Compared to this,

the yield of sugar from sugar beet grown in experimental trials in New Zealand was between 4.4 and 17 tonnes (i.e. 4400 to 17000 kg) per hectare (Harris and Mann, 1994). Although the estimated yield of fructose from ti kōuka is low, Harris and Mann made the point that no attempt has ever been made to selectively breed ti kōuka for yield. The authors suggested "that *Cordyline* syrup could provide the basis of novelty products such as pancake syrup, sweets, biscuits, soft drinks, beer, wine or liqueur. Such products would have a unique and distinctly indigenous character of interest to both tourists and residents of New Zealanders." For instance, it could be used as an alternative substitute for sugar sweeteners or used as a unique flavouring, e.g. in gin.

Harris and Mann also proposed that *Cordyline australis* would be a suitable feedstock for ethanol production for fuel, and that the fibrous bagasse left after extraction of fructose could be used as a mulch and soil conditioner, as cattle feed and poultry litter.

The traditional Māori practice (before 1851) for large-scale harvesting of *Cordyline australis* involved coppicing stems of trees on a four-year rotation. Coppicing may be a better option than growing it as an annual crop. For instance, it would reduce soil cultivation cost, lower tooling and labour cost and time spent on harvesting. It would also give the flexibility of harvesting time (Harris and Mann, 1994).

1.6 Sugar extraction of agave compared with ti kōuka

The objective of this thesis is to systematically research the production, and the chemical and organoleptic properties of the ti kōuka spirit, with a view to future commercial production of an iconic New Zealand spirit. In making this iconic New Zealand spirit, the procedure for extracting the sugars from the cabbage tree initially parallels the procedure for extraction of agave.

There were similarities in the process of extracting sugar as historically carried out by the Mexican people and early Māori. As a first step, the external leaves of agave, and the leaves and bark from ti kōuka, were removed. In both cases the piñas and kōata were steam heated in closed pits, known in New Zealand as hangi. For both species, the aim was to hydrolyse the storage carbohydrate to fermentable reducing sugars. As explained above the contemporary hydrolysis of agave piñas is now an industrial process in large steam ovens, sometimes under pressure (autoclave process), but the result is the same. Fructose is liberated as it is for kōata in a hangi.

1.7 Aims of this thesis

The main aim of this thesis was to systematically research the feasibility of production of a tequila-like spirit from ti kōuka stem. The major research emphasis was on the following:

- Extraction of reducing sugars (water, acid and enzyme hydrolysis) from different parts of the ti kōuka stem. Due to the low reducing sugar concentration in the ti kōuka extract, fermentation was not carried out; rather the ti kōuka extract was infused with portable ethanol. These procedures are discussed in Chapter 2.
- Sugar, protein and amino acid profiles of the ti kōuka stem (using HPLC) are discussed in Chapter 3.
- Amino acid profiles during different stages of the Ti Koka spirit production are discussed in Chapter 4.
- Analysis of sugar and amino acid model systems (to approximately simulate the Maillard reaction taking part in the ti kōuka stem is described in Chapter 5.
- Comparison of volatile compounds in the Ti Koka and commercial spirits (tequila, gin and whisky) by GC-MS analysis is discussed in Chapter 6.
- Sensory evaluation of Ti Koka is discussed Chapter 7.
- In Chapter 8, the production concepts on industrial scale and marketing issues for Ti Koka spirits including route to market is discussed.

CHAPTER 2

Development of ti kōuka spirit

Tequila is the Mexican spirit prepared from the *Agave tequila* Weber *var azul*, and as discussed in Chapter 1, this species is in the same family as *Cordyline australis* (ti kōuka). Thus at the outset it seemed sensible to prepare the ti kōuka spirit in a manner analogous to that of tequila. In the event, the preferred method of spirit production was by infusion into potable alcohol, where the plant served only to supply colour and flavour. However, the development was initially along the conventional path: extraction of potentially fermentable carbohydrate, hydrolysis, fermentation and distillation.

According to Fankhauser and Brasch (1985), the rhizome and the stem of ti kōuka are both storage organs for a carbohydrate, not starch rather fructan or inulin. This is dominantly a polymer of fructose rather than a polymer of glucose. The human digestive system cannot hydrolyse inulin as it can starch, so whether inulin is used as a food source or source of fermentable sugar, it has first to be hydrolysed to metabolisable or fermentable fructose.

Quantitative methods for the determination of inulin involve acidic or enzymatic hydrolysis of inulin followed by measurement of the liberated reducing sugars by techniques including the p-hydroxybenzoic acid hydrazide (PAHBAH) method and high pressure liquid chromatography (HPLC) with refractive index detection.

The main aim of the ti kōuka extraction and hydrolysis process was to obtain as much fructan (inulin) as possible from stem extraction and as much fructose from the hydrolysis process as possible. To achieve this, the following variables were assessed:

- Grounding of ti kōuka stem and extraction of inulin
- Moisture content of ti kōuka stem
- Choice of primary solvent
- Optimum conditions for extracting carbohydrate from ti kōuka stem
- Effect of stem position on the content of reducing sugars
- Comparison of acid-catalysed hydrolysis with pH-neutral hydrolysis
- Effect of the concentration of substrate (i.e. ti kōuka tissue) on acid hydrolysis
- Comparison of different enzymes on the hydrolysis of ti kōuka stem

- Effect of temperature and enzyme dilution
- Effect of incubation time on the hydrolysis of ti kōuka stem
- Comparison of acid hydrolysis with enzyme hydrolysis
- Effect of the surface area of ti kōuka tissue on the quantity of carbohydrate extracted
- Effect of moisture content of ti kōuka stem sample on the quantity of sugar extracted

2.1 Grounding of ti kōuka stem and extraction of inulin

Method

Initially, the *Cordyline australis* (ti kōuka) stem (approximately 1 m long) was collected mostly from residential areas from the suburb of Glenfield (with lot of exposure to sun), Auckland (Northern Island of New Zealand). The main soil type in Glenfield is the low fertility clay soil.Since the stems were randomly collected, the age of ti kōuka trees were unknown. The leaves and brown bark were removed from the stem as shown in Figure 2.1. The stem was transversely cut longitudinal and horizontal with an electric kitchen knife into about 1 cm cube pieces before further comminution with a domestic coffee grinder.

Figure 2.1 Upper ti kōuka stem with some of the leaves removed

Result and discussion

The fibrous nature of ti kōuka stem tissue (Figure 2.2) presented some challenges for processing with domestic food processing techniques, which employ low torque motors. However, trial and error showed that a domestic coffee grinder could successfully comminute wet stem pieces, which could be subsequently dried or used wet. Because

stem tissue could not be obtained easily (the AUT campus is located in the central city), the decision was made to work with dried tissue.

Figure 2.2 The fibrous stem tissue of ti kōuka

The comminuted stem tissue material was routinely dried by spreading it out in trays, and air dried, over several days, at room temperature yielding a fibrous mass (Figure 2.3).

Figure 2.3 Coarsely ground ti kōuka stem

The dried material was further processed by using a domestic kitchen sieve (0.5 mm mesh) to recover finely ground matter from the coarser matter. The latter was further ground, with a coffee grinder, and added to the fine matter.

2.2 Moisture content of ti kōuka stem

In the analysis of plant material, the collection of a representative sample is difficult due to variability of individual plants among a species or variety. In addition, the water content depends on the development stage of the *Cordyline australis* plant and the cultivation conditions (Romanik *et al*., 2007).

Cordyline australis stems were collected throughout the research period from different geographical locations in the Auckland region. The wet and dry weights of the ground matter were recorded and the estimated percent weight loss due to air-drying and moisture content were calculated (Table 2.1).

Table 2.1 Moisture content of the ti kōuka stem samples, due to air-drying

Date of stem collection	Moisture content of ground ti kōuka (%)
22-08-05	79.3
22-08-05	77.5
22-08-05	76.5
22-08-05	74.1
22-08-05	78.9
22-08-05	67.1
22-08-05	84.9
22-08-05	74.5
16-01-06	76.6
12-02-06	65.1
11-08-06	68.6
22-02-07	69.6
17-04-07	77.1
Mean ± SD	74.6 ± 5.6

SD=standard deviation

The average moisture content of the ti kōuka stems (including the stem and stem tip) collected was 74.6 ± 5.6%. This value is in close agreement with Crowe's (1997) finding (Table 1.2, Chapter 1). Crowe estimated that the fresh stem and stem tips had 68.3 and 81.5% water content respectively (average = 74.9%). Agave tissue also contains approximately 83.4% water (Peña-Alvarez *et al*., 2004).

2.3 Choice of primary solvent

The extraction of flavoured compounds from plant material is usually achieved by treating the raw material with a selected solvent in which the desired compounds are maximally soluble. The solvent that may be used is water, aqueous ethanol or aqueous methanol (Smith, 2003). Water was chosen as the solvent for extracting the ti kōuka tissue because carbohydrates in general are often soluble in water. The temperature and pH of extraction medium, extracting time, and solvent to sample ratio are important factors affecting the extraction yield of inulin (Lingyun *et al*., 2007).

Method

The ti kōuka stem sample extraction was initially carried out as described by Fankhauser and Brasch (1985). The prepared dry ground stem material (2 g) was extracted in magnetically-stirred water at ambient temperature, nominally 21°C, for 1 hour. The ratio of water: dried tissue varied from 1:5 to 1:25 depending on the coarseness of the matter being extracted (finer matter requiring more water). Extraction was followed by filtration, using Whatman No 4 paper and washing with deionised water. All extracts were made to a final 50 mL volumes.

Firstly, different parts of the stem were investigated to see if they had different contents of reducing sugars. The top 1 m of a ti kōuka stem was transversely cut into 10 cm segments. Where present, the bark of each segment was removed by an electric kitchen knife, ground and dried separately as above (Section 2.1.1). After progressively processing the first six segments from the top, it became apparent that the stem was becoming increasingly fibrous, to the point that the lower four segments was very difficult to cut and grind. Therefore, extraction of ti kōuka stem was limited to the top 60 cm of stem, and was cold water-extracted separately at room temperature for 1 hour as described above and analysed for carbohydrate by refractometry. A refractometer (ATAGO Model 311 range 0 – 32%, Japan) calibrated in percent sugar (w/v) (also known as Brix) was used. (Brix refractometer is an optical instrument that measures the sucrose concentration in a sucrose and water solution as a function of the index of refraction of the solution.)

Secondly, the effect of temperature on extraction of ti kōuka stem was also investigated. Extraction of the top 20 cm of the ti kōuka stem was compared at 20 and 60°C.

Results

The refractometer gave a Brix value of 1 to 2% (w/v) for segments 1 to 4 (i.e. tip to 40 cm) and 0.5% (w/v) for segments 5 and 6.

The refractometer values obtained at the two temperatures were unvarying. Inulin is known to be water soluble and as a soluble carbohydrate would contribute to refraction. However, since most samples contains substances other than sugar – such as salts, minerals and proteins – the Brix percentage represents the total concentration of all soluble solids in the sample i.e. this value cannot solely ascribe to inulin. Moreover, it seems surprising that the results were unaffected by temperature because inulin, like all carbohydrates are more soluble in warmer water. However, 20°C was useful as 60°C. Therefore, the following extractions were done at room temperature using the top 60 cm ti kōuka stem (unless otherwise stated).

2.4 Chemical determination of inulin and its hydrolysis products

Methods for analysing samples of plant and other materials for their constituent carbohydrates include various physical methods (e.g. hydrometry and refractometry), chemical and biochemical methods (both often employing spectrophotometry) and chromatographic techniques (e.g. high performance liquid chromatography and gas chromatography). Chemical techniques can be further subdivided into methods for total sugar determination and those methods for analysing individual sugars enzymatically. Enzymatic methods are suitable for quantifying individual sugars present in mixtures but specific enzyme must be available for each analyte (Blunden and Wilson, 1985). These methods range from simple and inexpensive to the more complex and expensive methods.

Both physical methods (hydrometry and refractometry) are cheap, quick and widely used methods, but have limited accuracy. Spectrophotometric methods use specific enzymes that react with both glucose and fructose to give accurate results for these sugars.

As described in Chapter 1, inulin is a polymer of mostly fructose, and as a polymer cannot be digested nor fermented, and is not a (copper) reducing carbohydrate, except for its terminal fructose residue. To be metabolised or fermented, the inulin has to be

hydrolysed. In this study, inulin has been extracted from ti kōuka, and hydrolysed to its composite reducing sugars, nominally fructose and glucose. The reducing sugars were monitored using the Blakeney and Mutton (1980) method, which is a simple manual colorimetric method for the determination of reducing sugar using the p-hydroxybenzoic acid hydrazine (PAHBAH) reaction (Refer to Appendix I for preparation of reagents).

In this assay, aldehydes or ketone group of the reducing sugar reacts with PAHBAH in alkaline solution. The resulting bis-benzoylhydrazones associate with Ca^{2+} ions (from calcium chloride) to form a soluble yellow complex that can be quantified by spectrophotometry. The advantage of using PAHBAH reaction is that the colour complexes with glucose and fructose – which are both present in inulin – have the same molar extinction coefficient. This means that whatever the composition of the carbohydrate source in ti kōuka, the absorbance data will give a reliable measure of fermentable sugar. Moreover, very few other reducing substances interfere with the PAHBAH reaction (Lever *et al*., 1973). Although glucose could be used to prepare calibration curves, it was more fitting to use fructose as the calibration standard, because that sugar is known to dominate ti kōuka carbohydrate (as will be discussed later in Chapter 3). Therefore, fructose standard solutions ranging from 0 to 5.0 g L^{-1} was used for calibration curve for all the reducing sugar analyses, unless mentioned otherwise.

The assay procedure for PAHBAH method was as follows. In two glass test tubes, 100 µL of the water extracted ti kōuka stem was pipetted. To one tube was added 400 µL of phosphoric acid (85% v/v) and to the other (the blank) 400 µL of deionised water. The tubes were incubated at room temperature for 30 minutes. To these mixtures was added 5 mL of PAHBAH solution. The tubes were incubated in a boiling water bath for 4 minutes then immediately cooled with running cold water. After addition of 10 mL of deionised water, the content of the tubes were mixed and absorbance of the samples were measured in a spectrophotometer (Pharmacia Biotech® Ultraspec 21000 model) at 415 nm using a 1 cm disposable plastic cuvette. (Samples were diluted with deionised water when required.) All determinations were done in triplicate.

2.5 Hydrolysis of inulin in ti kōuka to fermentable sugars

Inulin occurs as a carbohydrate reserve in many plants e.g. onion, chicory, Jerusalem artichoke and dahlia tubers. Inulin is a polymer comprising mainly of fructose units and typically has a terminal glucose unit. The fructose units are joined by a β(2→1) glycosidic bond and a glucose unit linked by an α(1→2) bond. Plant inulin generally contains approximately between 20 to 60 fructose units with the exception of inulin from artichoke globe, which has up to several hundred fructose units (Izydorczyk *et al*., 2005).

Hydrolysis literally means a chemical reaction with a water molecule. A reactant molecule is cleaved into two parts by the addition of a molecule of water in a chemical process. One fragment of the parent molecule gains a hydrogen atom (H) from the additional water molecule while the other group collects the remaining hydroxy group (OH). In polysaccharides, monosaccharide molecules are linked by glycosidic bonds. These can be cleaved by hydrolysis to yield monosaccharides. In the case of inulin, a hydrogen atom from the water is added to the oxygen on the glucose residue (Figure 2.4). The hydroxy group is added to the carbon on the fructose, thus yielding glucose and fructose.

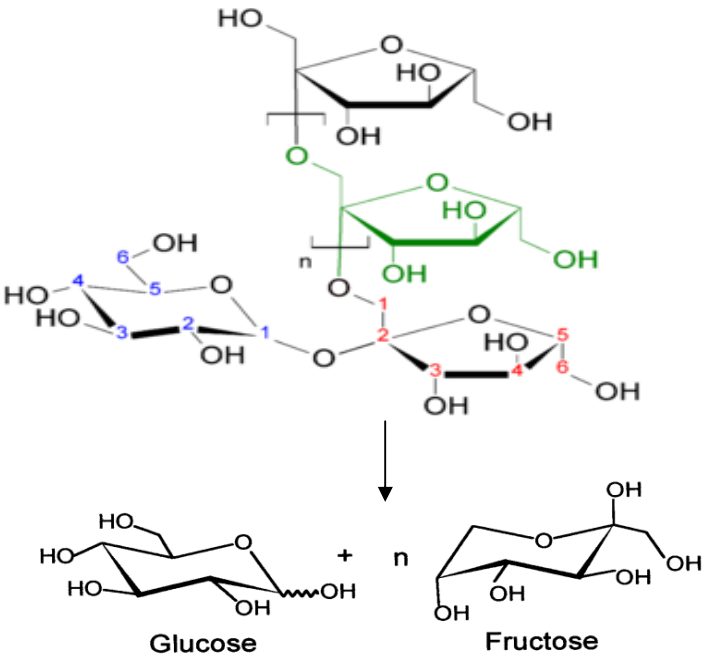


Figure 2.4 Schematic diagram of inulin hydrolysis (where n denotes the number of fructose residue in the initial inulin molecule) (www.wikipedia.com)

Chemical hydrolysis of inulin can be carried out by the treatment with dilute organic or mineral acids (Belgrov and Golubev, 2004), enzymes or by using acidic catalysts such as acid-cation exchange resins (Matsumoto and Yamazaki, 1986), zeolites (Abasaeed and Lee, 1995) or oxidised activated carbon (Heinen *et al*., 2001).

Several different methods for hydrolysing inulin from ti kōuka stem were tested i.e. water, acidic solution and enzyme assisted.

2.6 Comparison of acid hydrolysis with water hydrolysis

Abasaeed and Lee (1995) used sulphuric, hydrochloric, phosphoric, citric, oxalic and tartaric acids to catalyse the hydrolytic reaction of inulin to fructose. Fleming and Groot-Wassink (1979) compared various acids (hydrochloric, sulphuric, citric and phosphoric) and strong acidic catalyst such as acid-cation exchange resin for their effectiveness in inulin hydrolysis. They reported that no differences were noted among the acids or resin in their influence on inulin hydrolysis. For the present study, phosphoric acid hydrolysis was carried out using the method described by Fankhauser and Brasch (1985). This acid is certainly acceptable in the food industry (Food Standard Australia New Zealand) (www.nzfsa.govt.nz).

Method

Two grams of the dried ti kōuka stem was placed in two conical flasks. Fifty millilitres of deionised water was added to both the mixtures and were magnetically stirred for 1 hour at ambient temperature. Both mixtures were washed and filtered through Whatman No. 4 paper. One filtrate served as the control and the other was acidified to pH 3 with 45% (v/v) phosphoric acid (UN 1805 Univer Analytical Grade). Each mixture was boiled under reflux at atmospheric pressure for 30 minutes, cooled to room temperature, and the acidified filtrate was neutralised to pH 7.1 using 1 M $Ca(OH)_2$ (May and Baker Ltd. MP 7145). The precipitated calcium phosphate was removed by filtration using a Whatman No 4 paper. The filtrate was decolourised with activated charcoal (Merck 5272846) and re-filtered. The final filtrates were made up to 50 mL and analysed for reducing sugars using the PAHBAH method, making 1 in 5 and 1 in 10 dilutions in water before it was assayed. The diluted phosphoric acid hydrolysed samples were assayed at 100 and 400 µL, while the water hydrolysed samples were only assayed at 100 µL. Measurement of the same samples were repeated three times.

Results

Figure 2.5 shows that the phosphoric acid-hydrolysed samples gave higher absorbancies than samples hydrolysed with water alone.

It was observed that when the samples were diluted two fold, the absorbance decreased approximately proportionally for both the 100 µL and 400 µL substrate concentration (data not shown). The ti kōuka sample with 400 µL substrate concentration and 1 in 5 dilution gave the highest absorbance followed by 1 in 10 dilution.

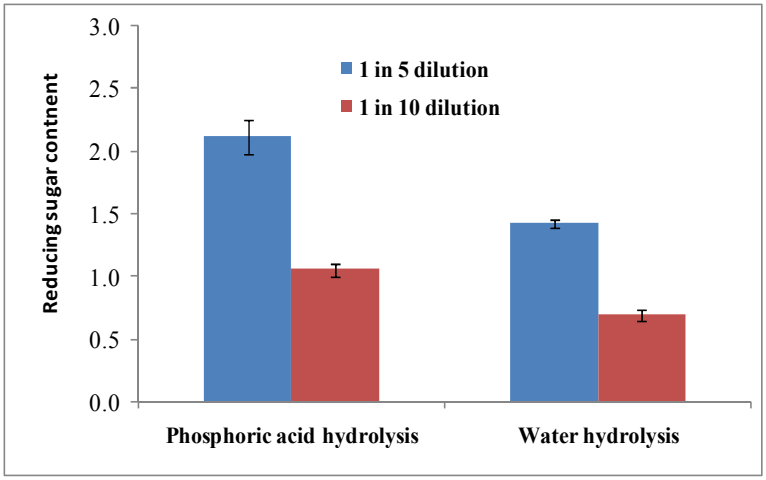


Figure 2.5 Comparison of water-hydrolysed and phosphoric acid-hydrolysed ti kōuka stem at 100 µL. The vertical bars are ± standard deviation

From this experiment, it was clear that phosphoric acid hydrolysis (with 400 µL substrate concentration) was more effective than hydrolysis by water alone.

2.6.1 Effect of different enzymes on the hydrolysis of ti kōuka stem

Acid hydrolysis is the traditional method of hydrolysis but enzyme catalysed hydrolysis exhibits some substantial advantages over acid. For example, there is no need for high temperature or pressure in enzyme-assisted hydrolysis (Yankov *et al*., 1986). It is well known that the hydrolysis pattern of polysaccharides differs with different enzymes. For instance, α-amylase hydrolyses starch in a random manner, while β-amylase removes successive identical units from one end of the chain (McClendon, 1975). The available literature supports the view that the dominant carbohydrate in ti kōuka is inulin, so an inulinase was an obvious choice. However, other enzymes were also trialled because a use of inulinase alone would necessarily assume that inulin was the sole carbohydrate.

Therefore, the aim was to identify an industrially available enzyme that would be effective in the commercial hydrolysis of the ti kōuka extract. The hydrolytic enzymes that were tested were invertase (BDH 39020) at pH 4.5, Fructozyme® (Fructozyme L - kindly donated by Novozyme, Australia Pty. Ltd.) at pH 4.6, α-amylase (AG 300L AMNO 4404 from Novozyme)at pH 4.3 and Enzidase® (Enzidase L 300 from Zymus International Ltd) at pH 4.3. (For a full description of these enzymes, refer to Appendix II.)

The disaccharide sucrose is catalysed by the hydrolysis of invertase to invert sugar i.e. mixture of the monosaccharides glucose and fructose at 1:1 ratio. Fructozyme is a commercial inulinase isolated from an *Aspergillus*, comprising a mixture of exo-inulinase and endo-inulinase, which is suitable for industrial hydrolysis of inulin in the production of fructose syrup. Endo-inulinase hydrolyses the internal β(2→1) fructose bonds, while exo-inulinase successively splits off the terminal fructose units. In the limiting case the final bond to be hydrolysed is the α(1→2) bond with glucose. That disaccharide is sucrose, so one activity of Fructozyme is invertase activity. α-Amylase is capable of attacking any internal α(1→4) bond in a random fashion along the starch chain. There are two kinds of starch polymers. Amylopectin, a branched molecule, has the same chemical composition as amylose but has α(1→4) and α(1→6) bonds, the latter resulting in branching. The result of α-amylase hydrolysis of amylopectin is a mixture of linear fragments (e.g. maltose), as for amylose, but also larger fragments that contain the α(1→6) bond, which cannot be broken by α-amylase (e.g. limit dextrins). Enzidase, a commercial form of amyloglucosidase, is used to produce glucose, starting from the non-reducing ends of starch chains and limit dextrin. During hydrolysis, the amyloglucosidase activity removes glucose units in a stepwise manner from the non-reducing end of the substrate molecule.

The main factors affecting enzyme hydrolysis are the temperature, pH of the medium, surface area of the substrate and the ratio of substrate to enzyme (Fennema, 1996). Determination of optimum conditions can be a major study in itself. The aim here was to 'broad brush' these variables and to obtain optimum conditions for extracting and hydrolysing reducing sugars in the ti kōuka stems.

Method

Dried ti kōuka stem tissue (5.113 g) was extracted with 50 mL of deionised water for 1 hour at 20°C and filtered through Whatman No.4 paper, washed and the filtered extract

was made up to 50 mL. Because enzymes catalyse most effectively when an excess of substrate is available (as was seen in the previous experiment), the substrate volume was increased from 100 µL to 400 µL for all the PAHBAH analyses from here forth. The enzyme volume (that replaced the phosphoric acid) was decreased from 400 µL to 100 µL, so total reaction volume remained the same. The substrates chosen for this part of study were fructose (Scientific Supplies SSC-R157-250), glucose (BDH AnalaR 101176K), inulin (Sigma I-5625), starch (Sigma S-9765) (each made up as 1 g L^{-1} dry w/v) and ti kōuka extract. Thus, each reference reaction mixture contained 400 µg of each substrate, while the 400 µL of ti kōuka extract was equivalent to 40,900 µg of the original dried stem.

The Blakeney and Mutton (1980) colorimetric method is based on the determination of reducing sugars (total fructose plus fructose in sucrose) before and after invertase digestion using PAHBAH. Therefore, sucrose (BDH AnalaR grade 102744B) ranging from 0 to 1 g L^{-1} was used as standard curve.

The substrates/enzymes experiments were conducted at 20, 60 and 100°C and at three different initial enzyme concentrations. These were the stock solutions, that diluted 1 in 2 and that diluted 1 in 4 (Table 2.2).

Table 2.2 Units of different enzyme added during enzyme-assisted hydrolysis in the 500 µL reaction mixtures

Units of enzyme added	Invertase	Fructozyme®	α-Amylase	Enzidase®
Working stock solution	27.2 EU	0.000211 INU	24 AGU	24 GAU
1 in 2 dilution	13.6 EU	0.000106 INU	12 AGU	12 GAU
1 in 4 dilution	6.8 EU	0.000053 INU	6 AGU	6 GAU

INU = International Units, GAU = Glucose Amyloglucosidase Units, EU = Enzyme Units, AGU = Amyloglucosidase Unit

Results and discussion

Pilot experiments with α-amylase and Enzidase yielded many absorbances that were >+3, which was the upper dynamic limit of the spectrophotometer. It was suspected that both enzymes were contaminated with high concentrations of reducing sugars, probably carried forward from production in the partial purification of these enzymes. This was confirmed when the enzyme stock solutions were diluted 1 in 20 and beyond,

where absorbances finally came on scale. In view of this contamination and the fact that both enzymes work only on starch, it was decided to stop experimentation with them. Only Fructozyme and invertase were tested in the proceeding experiments, but being aware that they too may be contaminated with reducing sugars.

Consider invertase first. Table 2.3 shows the absorbances of the five substrates and the standard sucrose hydrolysed for 30 minutes at the three invertase dilutions at 20°C (the quoted optimum operating temperature of invertase). As the concentration of invertase decreased, the absorbances decreased, but as the concentration of sucrose increased, the absorbances increased due to hydrolysis to glucose and fructose. Fructose and glucose had similar absorbance values to absorbance due to 400 µg sucrose at all three enzyme dilutions. This stands to reason because invertase hydrolyses sucrose to fructose and glucose in a 1:1 ratio.

Table 2.3 Mean absorbances at 415 nm of different substrates at different invertase concentrations at 20°C for 30 minutes (n=3)

Substrate	Absorbances of stock invertase solution at 20°C	Absorbances of 1 in 2 dilution of stock invertase solution at 20°C	Absorbances of 1 in 4 dilution of stock invertase solution at 20°C	Absorbances with no invertase 20°C
0 µg Sucrose (water)	1.184 ± 0.04	0.632 ± 0.04	0.273 ± 0.08	0.010 ± 0.01
80 µg Sucrose	1.422 ± 0.03	0.783 ± 0.02	0.463 ± 0.02	0.014 ± 0.01
160 µg Sucrose	1.587 ± 0.04	0.885 ± 0.04	0.587 ± 0.06	0.014 ± 0.01
240 µg Sucrose	1.692 ± 0.18	1.149 ± 0.22	0.715 ± 0.07	0.015 ± 0.02
320 µg Sucrose	1.802 ± 0.20	1.295 ± 0.07	0.938 ± 0.16	0.012 ± 0.02
400 µg Sucrose	2.233 ± 0.34	1.454 ± 0.01	1.101 ± 0.05	0.016 ± 0.01
400 µg Fructose	1.853 ± 0.07	1.397 ± 0.07	1.070 ± 0.06	0.729 ± 0.01
400 µg Glucose	1.911 ± 0.06	1.253 ± 0.21	1.032 ± 0.03	0.694 ± 0 02
400 µg Starch	1.063 ± 0.15	0.593 ± 0.06	0.394 ± 0.03	0.010 ± 0.01
400 µg Inulin	1.213 ± 0.10	0.623 ± 0.05	0.253 ± 0.06	0.033 ± 0.01
Ti kōuka extract	1.794 ± 0.29	1.292 ± 0.01	0.919 ± 0.01	0.486 ± 0.05

Starch and inulin gave absorbances similar to absorbances at 0 µg sucrose concentration (water control) at all three dilutions, clearly indicating that invertase was inactive against these two substrates. In the absence of invertase, the ti kōuka treatment (last column of Table 2.3) had a mean absorbance of 0.486 at 415 nm, compared with water control (first row) of a negligible 0.010. This absorbance was due to reducing sugar

equivalent to about 273 µg originally from 40,900 µg of dried stem. That translates to about 0.7% reducing sugar that is immediately available before any enzymatic hydrolysis. When enzyme was included in the reactions mixtures (columns 2 to 4), all absorbances increased, obviously due to contaminating reducing sugars in the invertase. (At 0 µg sucrose concentration (the water control), the absorbances recorded for the three enzyme dilutions were 1.184, 0.632 and 0.273 respectively.) What is now of most interest, is what increase in absorbance due to invertase can be shown for ti kōuka. This can be extracted from Table 2.3, but is better seen in Figures 2.6 to 2.8 where the absorbance values are due only to invertase hydrolysis.

These bar charts were constructed from hydrolyses done at 20, 60 and 100°C (raw data presented in Appendix III). The values were obtained by subtracting the absorbance values with no invertase from the absorbance values with invertase, and then subtracting the absorbance value of the water control, 0 µg sucrose (e.g. 1.184 in Table 2.3) from that result. The values in these figures are therefore invertase-dependent absorbances.

In these depictions, fructose and glucose should and do show values close to zero because, the invertase has no effect on them. By contrast, sucrose should and did yield increasing values, due to reducing sugar generation, as sucrose concentration increased.

Inspection of these bar charts shows that absorbances for fructose, glucose, starch and inulin vary considerably with no distinctive pattern between the three temperatures. Equally, the sucrose series shows much variation. These variations might seem surprising given that the values are means of triplicates. However, because the background absorbances due to contaminating reducing sugar in the invertase were high, the absolute differences in absorbances (the values of interest) were difficult to see. In plain language, shades of grey had to be measured against a grey background. High errors are to be expected.

Invertase activity against sucrose ostensibly occurred at 100°C as is obvious from Figures 2.6 to 2.8. The quoted maximum temperature for invertase activity is 55°C, implying that enzyme stability, and thus activity, decreases because of denaturation at higher temperatures. Assuming that the enzyme is ultimately 100 % inactive at 100°C,

hydrolysis must have already started taking place as soon as the enzyme was added to the reaction mixture at ambient temperature before the contents reached 100°C.

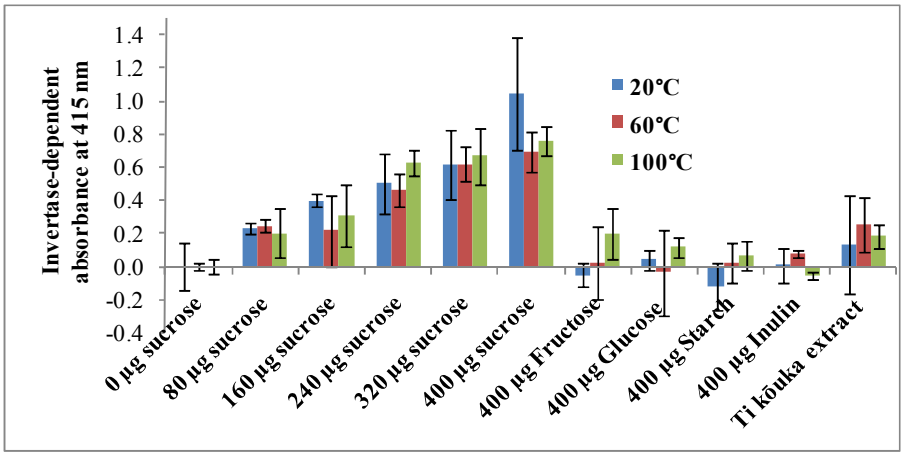


Figure 2.6 Substrate hydrolysis with stock invertase solution

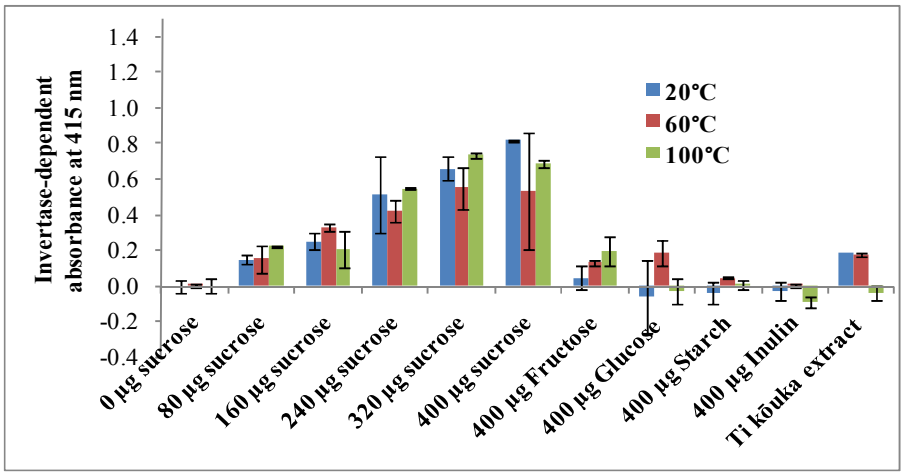


Figure 2.7 Substrate hydrolysis with 1 in 2 dilution of stock invertase solution

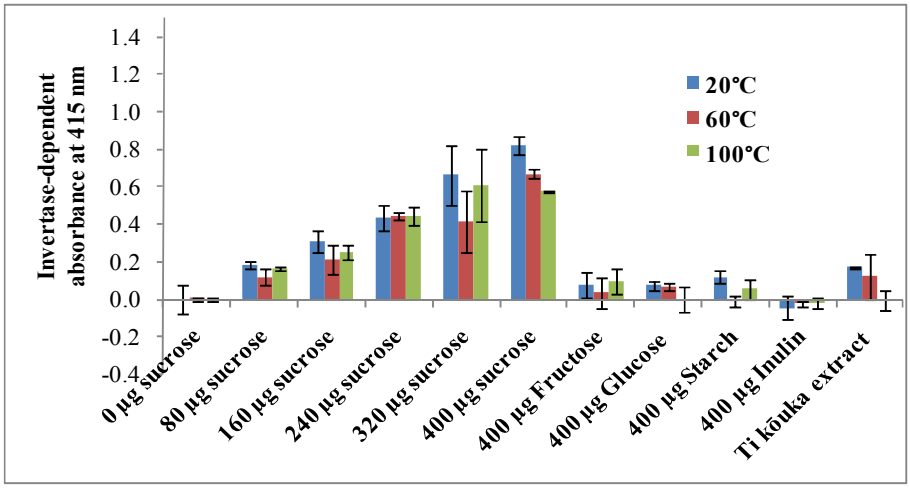


Figure 2.8 Substrate hydrolysis with 1 in 4 dilution of stock invertase solution

Starch and inulin showed no significant reaction with invertase, showing only the expected experimental error due to contaminating reducing sugars (Figures 2.6 to 2.8). This means that the invertase was not contaminated with starch and inulin hydrolysing activities. By contrast, ti kōuka showed some reactivity. Although the values were numerically similar to the errors associated with fructose and glucose, the results at 20 and 60°C were consistently positive. What is the substance in the ti kōuka extract that might be reacting with invertase? The obvious answer is sucrose, which is the limit carbohydrate arising from a (putative exo-inulinase) endogenous to ti kōuka stem. This model is supported by the fact that free reducing sugars are already present in ti kōuka stem extract (Table 2.3), meaning that some limit sucrose could be present. The data also indicate that the inulin used was completely sucrose-free.

The above discussion of these results has focused on the relative reactivities of the substrates at different temperatures. What was the effect of enzyme dilution? Inspection of Figures 2.6 to 2.8 shows that enzyme dilution had little effect in the 30 minute incubations. This suggests that all hydrolyses had gone to completion.

Turning now to Fructozyme, Table 2.4 shows the absorbances of the five substrates and the standard sucrose hydrolysed at the three Fructozyme dilutions at 60°C. (The quoted optimum operating temperature of Fructozyme is in the range 57 to 63°C.) As with invertase, as the concentration of Fructozyme decreased (columns 2 to 4), the absorbance values decreased, but as the concentration of sucrose increased, the absorbances increased at all three dilutions. It was apparent from the results that Fructozyme had invertase activity as was discussed above.

As with invertase, the ti kōuka treatment (last column of Table 2.4) gave a mean absorbance value greater than zero (0.247) in the absence of Fructozyme compared to water control (0.017) confirming that reducing sugar was present in the ti kōuka extract. When Fructozyme was included in the reaction mixtures (columns 2 to 4), all absorbances increased, obviously due to contaminating reducing sugars in the Fructozyme. (At 0 μg sucrose concentration (the water control), the absorbances recorded for the three Fructozyme dilutions were 0.898, 0.563 and 0.183 respectively.)

Table 2.4 Mean absorbances at 415nm of different substrates at different Fructozyme concentrations at 60°C for 30 minutes (n=3)

Treatment	Absorbances of stock Fructozyme solution at 60°C	Absorbances of 1 in 2 dilution of stock Fructozyme solution at 60°C	Absorbances of 1 in 4 dilution of stock Fructozyme solution at 60°C	Absorbances with no Fructozyme at 60°C
0 µg Sucrose(water)	0.898 ± 0.11	0.563 ± 0.01	0.183 ± 0.02	0.017 ± 0.01
80 µg Sucrose	1.194 ± 0.01	0.705 ± 0.04	0.322 ± 0.01	0.018 ± 0.01
160 µg Sucrose	1.234 ± 0.12	0.893 ± 0.01	0.400 ± 0.07	0.021 ± 0.01
240 µg Sucrose	1.504 ± 0.05	0.907 ± 0.18	0.570 ± 0.02	0.021 ± 0.01
320 µg Sucrose	1.675 ± 0.01	1.138 ± 0.01	0.700 ± 0.01	0.034 ± 0.01
400 µg Sucrose	1.540 ± 0.24	1.231 ± 0.04	0.779 ± 0.02	0.024 ± 0.01
400 µg Fructose	1.536 ± 0.17	1.175 ± 0.19	0.893 ± 0.01	0.650 ± 0.07
400 µg Glucose	1.649 ± 0.61	1.210 ± 0.04	0.780 ± 0.11	0.643 ± 0.02
400 µg Starch	1.244 ± 0.23	0.792 ± 0.08	0.407 ± 0.04	0.022 ± 0.01
400 µg Inulin	1.678 ± 0.11	1.096 ± 0.04	0.763 ± 0.08	0.040 ± 0.01
Ti kōuka extract	1.669 ± 0.05	1.226 ± 0.04	0.713 ± 0.01	0.247 ± 0.01

Figures 2.9, 2.10 and 2.11 show Fructozyme-dependent absorbance values, created in the manner of the invertase bar charts (raw data presented in Appendix IV). As with invertase absorbances due to sucrose, fructose and glucose were 'noisy' from high background absorbances due to contaminating reducing sugars. Starch showed an inconsistent pattern for the three temperatures. However, there was some evidence of starch-hydrolysing activity at 60°C, perhaps due to contaminating activity.

Fructozyme clearly hydrolysed inulin, as it was purported to do, with evidence of higher absorbances at 60 and 100°C. All concentrations of the enzyme were similarly effective. Fructozyme clearly hydrolysed ti kōuka extract with evidence of higher absorbances at 20 and 60°C. The storage polysaccharide of ti kōuka is claimed to be inulin, therefore one would expect both inulin and ti kōuka to be hydrolysed with similar efficiency. At 100°C, the absorbance values decreased by approximately half (compared to 20°C) probably due to the enzyme denaturation.

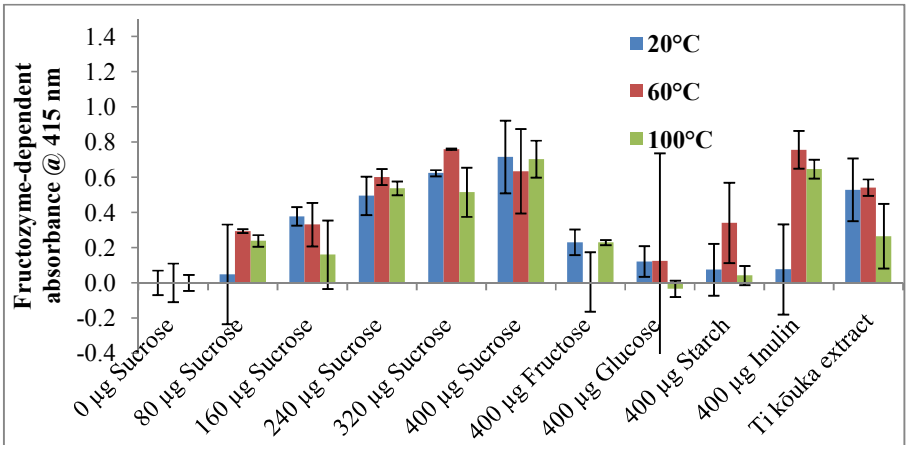


Figure 2.9 Substrate hydrolysis with stock Fructozyme solution

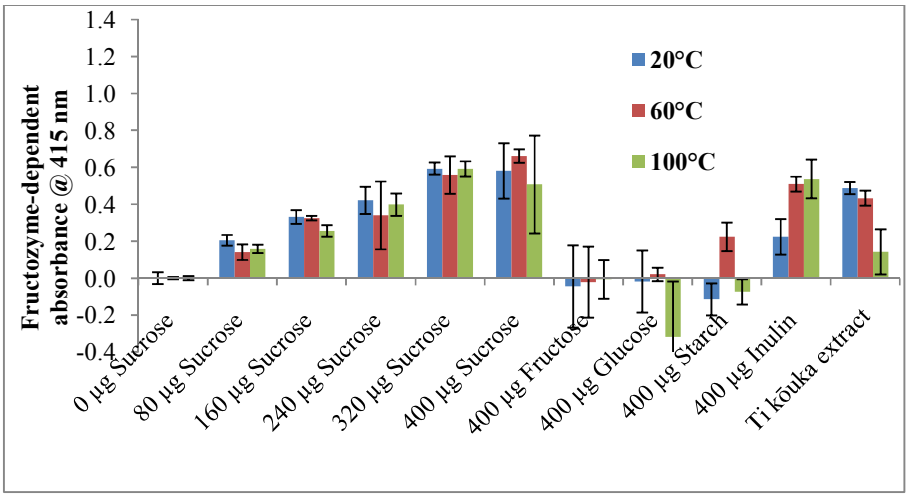


Figure 2.10 Substrate hydrolysis with 1 in 2 dilution of stock Fructozyme solution

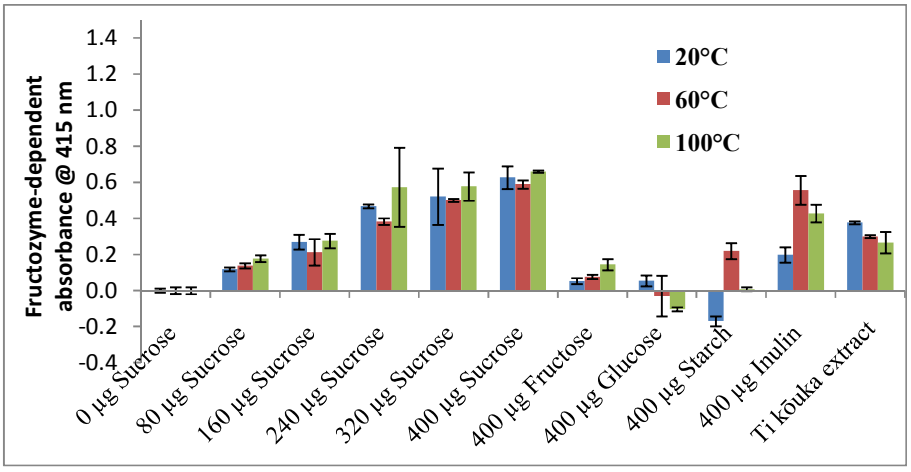


Figure 2.11 Substrate hydrolysis with 1 in 4 dilution of stock Fructozyme solution

Compared to invertase, Fructozyme was more effective at hydrolysing inulin and ti kōuka at all temperatures and dilutions. Therefore, Fructozyme was chosen as being the most appropriate enzyme for hydrolysing the ti kōuka extract.

It was calculated above that the concentration of free reducing sugars in the ti kōuka extract in the absence of exogenous enzyme was about 0.7% of dry weight in the invertase experiment. For the Fructozyme experiment, the result was about 0.4%. Fructozyme, which also has invertase activity, elicited a further hydrolysis yielding a maximum absorbance of 0.54 at 415 nm (60°C at the highest enzyme concentration). Assuming 100% hydrolysis for inulin and ti kōuka extract occurred in the 30 minutes, the mass of inulin in the 400 µL of ti kōuka extract was approximately 240 µg. This translates to about 0.6% of dry weight. This value added to the mean value of free reducing sugars (0.55%) yields a total of say 1.1%. This was an unexpectedly low figure, compared with the values obtained in the later experiments. This suggests that much of the inulin was retained on the filter paper even though the procedure of washing and re-filtering was repeated. A different approach for extracting the reducing sugar had to be explored (discussed in Section 2.8.1).

Fructozyme was effective at hydrolysing ti kōuka at both 20 and 60°C. Industrial inulin hydrolysis is usually carried out at 60°C in order to prevent microbial contamination (Gill *et al*., 2004). The commercial Fructozyme L used in this project was produced from *Aspergillus niger*. Gill *et al*. (2004) purified an exo-inulinase from *Aspergillus fumigatus* whose optimum temperature for enzyme hydrolysis was 60°C. This was in accordance with previous reports from other *Aspergillus* species, namely *Aspergillus ficuum*, *Aspergillus versiclor* (Kochhar *et al*., 1998) and *Aspergillus awamori* (Arand *et al*., 2002). Therefore, for all subsequent experiments, Fructozyme hydrolysis was conducted at 60°C (unless stated otherwise) and fructose used for the standard curve.

The next step was to explore the effects of time and temperature on the hydrolysis of ti kōuka extract by Fructozyme.

2.7 Effect of incubation time on the hydrolysis of ti kōuka stem

Chemical reactions, whether catalysed by enzyme or not, are all affected by temperature and time. The effects of time and temperature are explored here.

Method

In multiple tubes, 400 µL of ti kōuka extract (the same extract that was used in Sections 2.6 and 2.6.1) was added to all the tubes and placed in a waterbath at 60°C and at ambient temperature (~ 20°C). Fructozyme (100 µL yielding 0.000106 INU) was added to the tubes at 30 second intervals. At the end of a total 300 seconds incubation period, all the tubes were removed simultaneously, and 5 mL of PAHBAH solution was rapidly added to all the tubes using an automated dispenser, starting with the zero time tube and progressing to longer times. After the standard PAHBAH procedure, samples were read in a spectrophotometer at 415 nm. Deionised water was used as the blank. As a control, ti kōuka extract (400 µL) was similarly incubated with 100 µL of deionised water at the two temperatures for up to 300 seconds, and PAHBAH procedure followed.

Results

As reported in the previous section, the Fructozyme preparation was contaminated with reducing sugar, so the absorbance data have been adjusted to show the reducing sugars present before enzyme addition and the response due to enzyme activity but not the contaminating reducing sugars. In the presence of Fructozyme there was immediate rapid increase in reducing sugar concentration that plateaued in less than 60 seconds, where the plateau value was higher at 60°C (2.50) than at 20°C (2.25) (Figure 2.12).

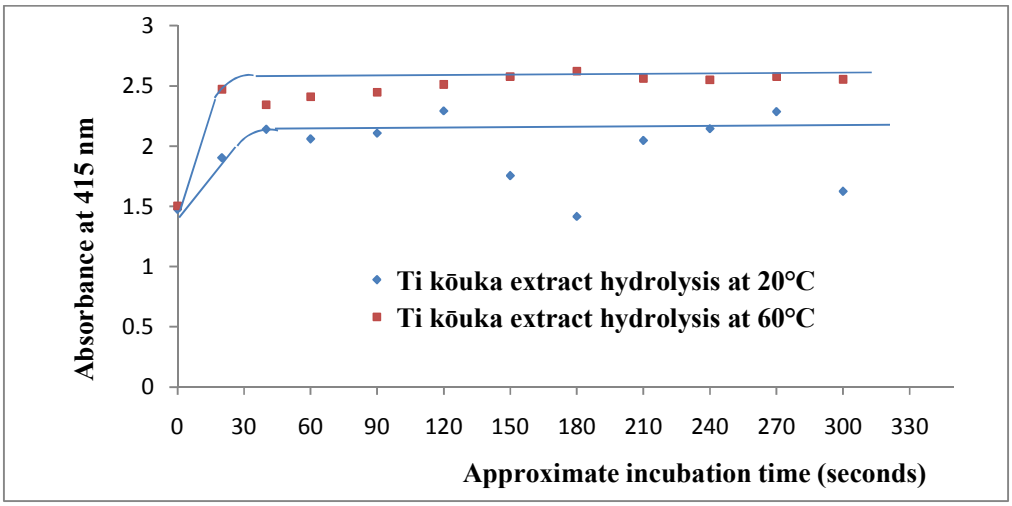


Figure 2.12 Kinetics of ti kōuka extract hydrolysis due to Fructozyme at different temperatures

Of these plateau values, absorbance at time zero (1.54 and 1.51 respectively) were due to reducing sugars present in the ti kouka extract as used. Thus, the increases in reducing sugars due to Fructozyme were about 66% at 60°C and 50% at 20°C. These increases occurred in less than 60 seconds and confirm the assumption made in the previous section that there was 100% hydrolysis within the 30 minute incubation period. What is not easily explained is why the incubation at 20°C does not progressively increase to the higher final concentration of reducing sugars seen with 60°C. This question was not pursued.

2.8 Efficacy of enzyme catalysed hydrolysis of ti kōuka stem at 60°C and the effect of moisture content on the quantity of sugar extracted

So far, dried ti kōuka stem had been extracted with distilled water at 20°C for 1 hour and the final extract was used for hydrolysis experiments (Sections 2.6, 2.6.1 and 2.7). From the results of these experiments, it was decided that from all the enzymes tested, Fructozyme was the most effective enzyme to hydrolyse the ti kōuka extract at 60°C. However, values of reducing sugars yielded after hydrolysis were low. In this experiment, the efficacy of directly hydrolysing ti kōuka stem tissue with Fructozyme at 60°C (without the water extraction) was explored using fresh and dried ti kōuka stem.

Method

After ti kōuka stem tissue was ground, 50 g of fresh and 50 g dried ti kōuka stem sample were extracted with 150 mL and 250 mL of deionised water (respectively), along with an equal volume of Fructozyme (1.5 mL yielding approximately 10.55 INU). Three treatments each were prepared for both stem tissue types. One comprised ti kōuka plus water plus Fructozyme, a second comprised ti kōuka and water (blank) but no Fructozyme, and the third comprised no ti kōuka plus water plus Fructozyme (control). All six treatments were placed in a water bath starting at ambient temperature (~ 20°C) with heating to 60°C in about 45 minutes. The treatments were held at 60°C for 1 hour after which they were filtered, washed twice with water, and adjusted to known approximate equal volumes. The filtrates were analysed for reducing sugar using the PAHBAH method.

Results and discussion

After adjusting for contaminating reducing sugar in the Fructozyme preparations, the results of the reducing sugar yields are summarised in Table 2.5.

Table 2.5 Recovery of reducing sugars from dried and fresh ti kōuka stem in the presence of Fructozyme

	Extractable reducing sugar (% w/w of air-dried weight)	Extractable reducing sugar (% w/w of fresh weight)	Extractable reducing sugar (% w/w of fresh weight expressed as air-dried weight)
Ti kōuka extract + deionised water (blank)	4.07 ± 0.21^{b}	1.71 ± 0.16^{a}	6.84 ± 0.16^{a}
Ti kōuka extract + Fructozyme	7.30 ± 0.34^{b}	2.38 ± 0.14^{a}	9.52 ± 0.14^{a}

Data are means of three replicates ± standard deviation
[a,b] Means bearing different letters in column groups are significantly different at $P < 0.05$

In the dry stem preparation, water alone recovered 4.07 ± 0.21% reducing sugar per gram dry weight. Addition of enzyme increased to 7.30 ± 0.34%, a 44% increase, roughly similar to that observed in the previous section. The equivalent data for the fresh stem tissue were 1.71± 0.16 and 2.38 ± 0.14 respectively on a wet weight basis. Moisture contents were not recorded in this experiment but Table 2.1 shows that the mean weight loss on air-drying ti kōuka stem tissue is approximately 75%. Applying a correction factor of 4 to the data in Table 2.5, shows that the calculated value for the extractable reducing sugar in the wet weight sample would be 9.52% (ti kōuka and Fructozyme) and 6.84% (for the ti kōuka and deionised water). This result shows that using fresh ti kōuka stem tissue gave better yield of reducing sugar than dried stem, but not markedly so. However, for convenience, it was decided to continue with dry stem tissue (unless stated otherwise).

2.9 Effect of the ti kōuka particle size on reducing sugar recovery by phosphoric acid and Fructozyme hydrolysis

In an enzyme-catalysed reaction when the activity of enzyme is not limiting, the rates of reaction obviously depend on the available concentration of the substrate, in this case inulin. If that is bound in some way to inert matter like cellulose particles, then

reducing the particle size may affect the reaction rates. This was tested here for both acid and enzyme catalysed hydrolysis of ti kōuka stem.

Method

Fresh ti kōuka stem samples were ground in a domestic coffee grinder, and dried at ambient temperature. With a domestic kitchen sieve (0.5 mm mesh), quantities of coarse material were separated from fine material (as described in Section 2.1).
In two separate experiments, these samples were extracted in three different combinations:

- 100% finely ground ti kōuka stem
- 50% finely ground and 50 % coarsely ground ti kōuka stem
- 100% coarsely ground ti kōuka stem

Nominally insoluble stem tissue was not included in the incubation trials because hydrolysis of insoluble stem tissue with phosphoric acid might yield reducing sugars from cellulose rather than inulin. Thus, samples of these three preparations were extracted with deionised water at ambient temperature for 1 hour, followed by filtration and water washing through Whatman No 4 paper. Final volumes were recorded. Aliquots of these volumes were subjected to phosphoric acid and Fructozyme hydrolysis, followed by reducing sugar determination using the PAHBAH method. The hydrolysed samples were analysed at four concentrations (i.e. undiluted, diluted 1 in 5, 1 in 10 and 1 in 20), each measured three times. In the case of phosphoric acid hydrolysis, the target pH was 3 (as described in Section 2.6). After boiling these acidified mixtures under reflux conditions for one hour, the mixtures were neutralized to pH 7 ± 0.3 with 1M $Ca(OH)_2$. Fructozyme hydrolysis was conducted at 60°C as discussed above (Section 2.8).

Results and discussion

As might be expected, the coarsely ground sample was easier and quicker to filter than the finely ground sample or the 50:50 mixture. After acid hydrolysis, the finely ground treatment was very dark compared to the coarsely ground treatment, presumably because finely ground matter escaped being trapped on the filter. The finer the stem preparation the more phosphoric acid was required to acidify the samples before hydrolysis (i.e. 150 µL for fine, 100 µL for 50:50 and 75 µL for coarsely ground sample) in the 50 mL extraction volumes. The pH of the extracts remained consistent (between 2.75 to 3.08) during the phosphoric acid hydrolysis. The quantities of acid

required to acidify the sample was reflected in the volumes of calcium hydroxide required to neutralize them (i.e. 2000 μL of 1 M $Ca(OH)_2$ was required for the finely ground sample, 1250 μL for the 50:50 mixture and 950 μL for the coarsely ground sample) in the 50 mL reflux volume. The results of reducing sugar extraction are summarised in Table 2.6.

Table 2.6 Effect of the ti kōuka particle size on sugar extraction by phosphoric acid and Fructozyme hydrolyses

Hydrolysis treatment	Finely ground sample	50% fine + 50% coarse mixture	Coarsely ground sample
Acid hydrolysis - extractable reducing sugar (% w/w) air-dried weight	7.70 ± 0.19^{a}	16.16 ± 0.11^{c}	10.08 ± 1.39^{b}
Enzyme hydrolysis - extractable reducing sugar (% w/w) air-dried weight	9.73 ± 0.37^{a}	17.95 ± 0.29^{c}	12.38 ± 1.46^{b}

The Fructozyme-hydrolysed treatment of the ti kōuka sample was marginally more effective than acid hydrolysis under these conditions ($P < 0.001$). Yields were higher by about 8 percentage points. Recoveries from the coarsely ground sample were higher than from the finely ground ($P < 0.001$), but curiously less than the 50:50 mixture ($P>0.05$). The possible reason is that the extraction solution of the finely ground sample was more viscous therefore making it difficult to filter and possibly leaving behind small amount of extractable reducing sugar on the filter paper (as was observed previously in Section 2.6.1). The coarsely ground sample was more fibrous therefore making it easier to filter and recovering most of the extractable reducing sugars. The enzyme hydrolysis activity was most effective with the 50:50 mixture, followed by the coarsely ground and finely ground samples for unknown reason, possibly due to surface area.

When the total experiment was repeated (data not shown), very similar results as above were obtained. It was apparent from the two experiments that Fructozyme hydrolysis was more effective than phosphoric acid hydrolysis. The 50:50 ground mixture gave the most reducing sugar therefore this combination of ground sample was used from here forth.

Conclusion

From all the above experiments, the following conclusions were made:
Acid hydrolysis was more effective than water hydrolysis, and enzyme hydrolysis in turn was more effective than acid hydrolysis. Using the ti kōuka extract (substrate) at 400 µL in the PAHBAH method was more effective than 100 µL sample. From the enzyme experiments, it was observed that Fructozyme (using working stock solution) was the most suited enzyme to hydrolyze the ti kōuka extract at 60°C. In the presence of Fructozyme there appeared to be an immediate rapid increase in reducing sugars that plateaued in less than 60 seconds, where the absorbance plateau value at 415 nm was higher at 60°C (2.50) than at 20°C (2.25). The surface area of the raw material of the ti kōuka did have an effect on the amount of sugar extracted. The best combination being a mixture of 50% finely ground sample and 50% of the coarsely ground sample.

In all the proceeding experiments from here forth, the wet ti kōuka sample was ground, dried at ambient temperature for 2 to 3days, and further processed (mixture of 50% finely ground and 50% coarsely ground) and stored until required. An aqueous solution of the dried material was incubated with Fructozyme in a waterbath, starting at ambient temperature and bringing the temperature up to 60°C, and held at 60°C for 1hour. After one-hour incubation, the samples were cooled at room temperature, filtered with Whatman No 4 paper and washed with deionised water. The sample is now ready for the next step in the process of making the spirit i.e. fermentation and distillation.

2.10 Fermentation and distillation as production options

Fermentation in its strictest sense is the energy-yielding anaerobic metabolic breakdown of a nutrient molecule, such as glucose, without net oxidation. Ethanol has been made since ancient times by the fermentation of sugars derived from grapes (for wine), barley, corn, potatoes (for beer and whisky), molasses (for rum) etc. All beverage ethanol and more than half of industrial ethanol is still made by this process, in which simple sugars are the raw material (Stone and Nixon, 2000). Yeast changes the simple sugar into ethanol and carbon dioxide. The fermentation reaction can be presented by the simple equation:

$$\underset{\text{Sugar (180 g mol}^{-1}\text{)}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Ethyl alcohol (92 g mol}^{-1}\text{)}}{2CH_3CH_2OH} + \underset{\text{Carbon dioxide (88 g mol}^{-1}\text{)}}{CO_2}$$

In practice however, an ethanolic fermentation is rather more complex. Different cultures of yeast produce varying amounts of other substances e.g. glycerol, various organic acids, and higher alcohols collectively called fusel oils (Kardos and Mulcock, 1977, Stone and Nixon, 2000). If higher concentrations of alcohol accumulate in the fermentation tank, the alcohol tends to variously denature fermentative enzymes and reduce the viability of the yeast cells. The concentration of alcohol tolerance by yeast varies from about 5 to 21% (v/v). For example, brewers' yeasts cannot withstand much beyond 5 or 6% alcohol while wine yeasts are more tolerant up to about 15%. Specially selected cultures of yeast can withstand alcohol concentrations up to 21% alcohol. High percentage alcohol beverages (e.g. spirits) are produced by distilling the fermented alcohol (Stone and Nixon, 2000).

When the molecular weights are applied to the equation above, it can be approximated that a given weight of sugar will produce about one-half its weight of alcohol (Stone and Nixon, 2000). Therefore, to obtain 315 mL of alcohol (100%) – enough for a standard 750 mL bottle of spirit (42%) – requires 630 g of sugar. Thus to obtain large volumes of alcohol on a commercial scale for making the ti kōuka spirit, would require large quantities of sugar extracted from ti kōuka. Because the yield of fermentable sugar from hydrolysis was only about 7to 18% of wet/air dried weight (from Sections 2.8 and 2.9 above), this would require about 10 fold mass of ti kōuka stem. While large-scale ti kōuka cultivation and harvesting may be viable, the subsequent sugar extraction, fermentation and distillation would unlikely be commercially viable. The capital costs would be prohibitive. (The argument for this is elaborated in Chapter 8.) An alternative cheaper method of making ti kōuka spirit had to be found.

New Zealand is the world's cheapest producer of potable alcohol (from fermentation of whey) (www.nzic.org.nz), so the next option would be to distill the ti kōuka extract with the commercial potable alcohol. The thesis is that flavour components in ti kōuka extract would co-distil with ethanol.

Work to this point with ti kōuka extracts showed that not only was the sugar concentration probably too low to be fermented economically, but also that the extracts

lacked a distinctive aroma. Much of what people describe as flavour is in fact aroma (Belitz *et al*., 2004). Because all alcoholic drinks, with the obvious exception of conventional vodka, have a distinctive flavour, it seemed clear that a flavour would have to be generated in some way that did not involve the addition of exogenous flavour compounds. The reason for this is that the plant itself must be the point of difference, by way of geographical uniqueness and distinctive flavour.

At this point, it was reasoned that one way of generating flavour would be through heating, knowing that the ti kōuka extract contained reducing sugars and probably contained protein, the two key reactants in the Maillard reaction.

2.11 The Maillard reaction

The Maillard reaction has been named after the French chemist Louis Maillard who first described it (Maillard, 1912) but it was only in 1953 that the first coherent scheme was put forward by one John Hodge (Hodge, 1953).

In the initial step of the Maillard reaction, a reducing sugar condenses with a compound possessing a free amino group to give a condensation product. In food, the sugar is typically glucose, maltose, fructose or lactose, and the amino compound is typically an amino acid. The Maillard reaction subsequently comprises complex reactions (in parallel and series) that produce a large number of Maillard reaction products (Figure 2.13) such as odour-active cyclic sugar derivatives, ultraviolet absorbing intermediates and dark brown polymeric compounds called melanoidins (Wijewickreme *et al*., 1997).

Melanoidins formed through the Maillard reaction have been reported to have functional chemical characteristics such as antioxidative activities (Osada and Shibamoto, 2006; Alaiz *et al*., 1996), antimutagenic activities, and nitrite scavenging ability (Cheigh *et al*., 1990).

A key feature of the Maillard reaction is the loss of water at several points in the chain of events, and for this reason, the reaction is promoted under lower water activity conditions. It is also accelerated by heating (Ajandouz and Puigserver, 1999), as is common for chemical reactions.

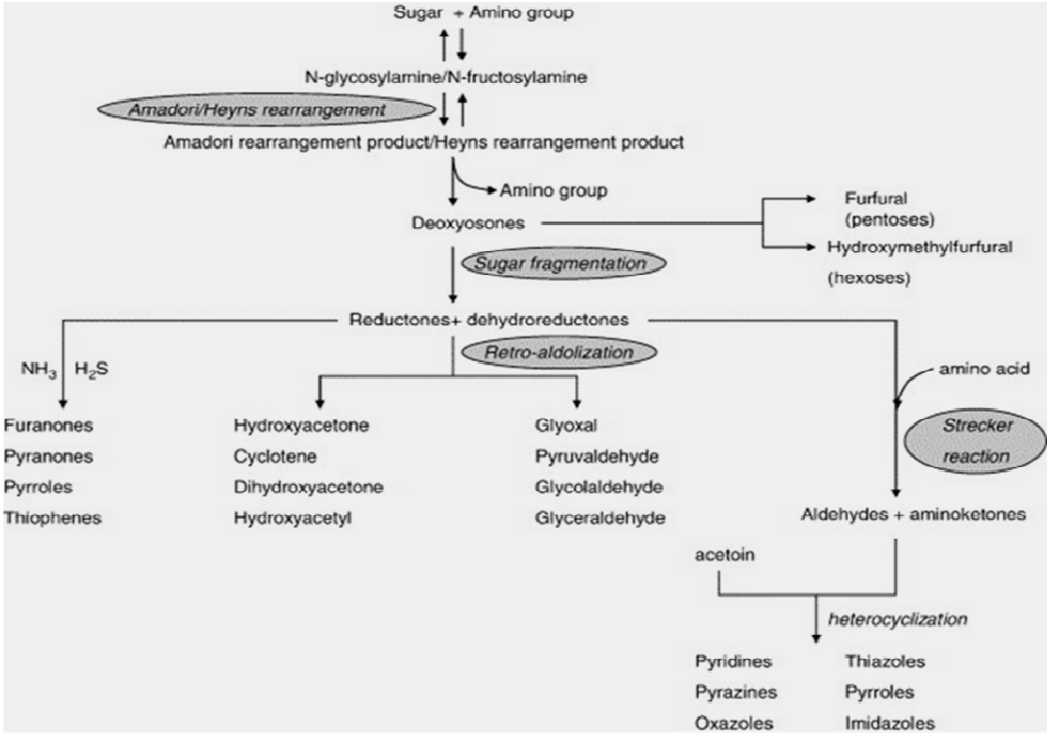


Figure 2.13 General overview of the Maillard reaction showing flavour compounds as end products (from Ho, 1996)

2.12 Generation of a distinct flavour profile in ti kōuka extract by the Maillard reaction

Knowing that heat and lower water activities promote the Maillard reaction, it was decided to heat the ti kōuka extracts in a conventional oven (i.e. evaporate) after enzyme-assisted hydrolysis and filtration process.

Method

A large sample of dried ground ti kōuka (68.8 g) was hydrolysed in water with 1.5 mL of stock Fructozyme (10.55 INU) in a volume of 600 mL. After one hour, the mixture was filtered (Whatman No. 4), washed and made to a final volume of 415 mL. (The dried stem tissue imbibed a substantial volume.) Four aliquots (50 mL each) were placed in 135 mm Schott-Duran glass Petri dish (Figure 2.15), which were incubated uncovered in a conventional oven at 60°C for 90 hours. Under these conditions, the water evaporated although the rate of loss was not monitored. These conditions were chosen to avoid compromising the speed of drying over the extent of the Maillard reaction. For example, if the samples were evaporated at 100°C, the samples would evaporate in short time yielding only the later stages of the Maillard reaction

compounds. On the other hand, at 60°C a mixture of early and intermediate stage of the Maillard reaction products could also be yielded.

After 90 hours, all four samples were reconstituted with deionised water to its original 50 mL, and pooled together. One part (10 mL) of the reconstituted pooled sample was used for PAHBAH analysis, another part of the sample was used for distillation (Section 2.13) and the remaining sample was stored at 4°C for further analysis (Section 2.14).

Results and discussion

As expected, the 90 hour incubation resulted in a complete loss of water (Figures 2.14 and 2.15). When the PAHBAH analysis was carried out on the original hydrolysed extract and the reconstituted extract after heating, the reducing sugar concentrations due to ti kōuka were calculated at 9.94 ± 0.57% and 19.79 ± 1.01% of the original dry weight, respectively. The value 9.94 was comparable to the values found in Section 2.9 (Table 2.6), but below that which might have been expected, up to 18%. What was surprising was the apparent increase to 19.79% after the 90 hours incubation. The results in previous sections suggested that Fructozyme acted very quickly supposedly hydrolysing all the inulin. The data here suggests the situation may be more complicated. One possibility is as follows. In the PAHBAH reaction, the hydrazine reacts with aldehyde and ketone groups of the sugars. Other aldehydes and ketones arising from the Maillard reaction, e.g. reductones and Stecker aldehydes (Figure 2.13), might also be reactive, giving the illusion of an increase in the reducing sugar concentration. Significantly, the reconstituted extract showed clear signs that the Maillard reaction had occurred: The brown residue had a sweet caramel aroma reminiscent of the mash process in beer brewing (Belitz *et al*., 2004).

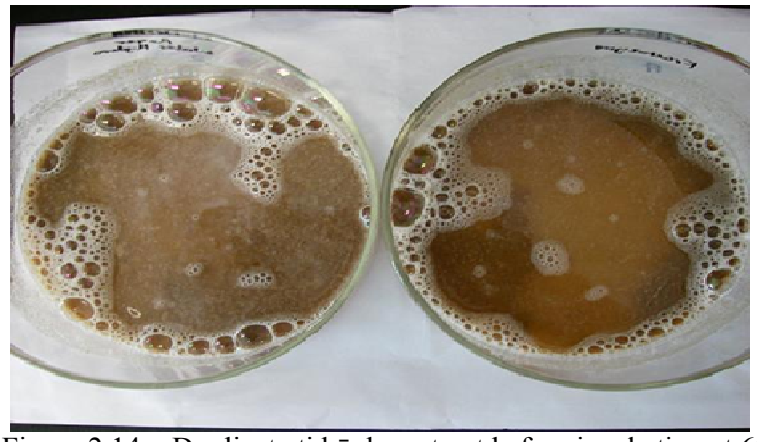


Figure 2.14 Duplicate ti kōuka extract before incubation at 60°C for 90 hours

Figure 2.15 A ti kōuka extract after incubation at 60°C for 90 hours

In the beer brewing process, several inter-related chemical and physical changes take place. The hydrolytic actions of amylases and proteases convert the carbohydrates and proteins into fermentable sugars and amino acids. During the final stage of malting of barley in the kiln, moisture is removed by drying, and colour is developed. Important flavoured compounds are formed during the kilning. These compounds are the Maillard products (Fix, 1999). It is assumed here that similar chemical and physical changes during the final stages of malting are also taking place in the Petri dish, hence the caramel aroma of the Maillard products. This can be related to the evidence that early missionaries, who settled in New Zealand, called their fermented drink a beer. The connection to beer may be through the distinctive aroma encountered. The dried residue was the obvious starting point to recover flavour and colour from the ti kōuka extract for the production of the spirit. As noted earlier, the concentration of reducing sugar was low, therefore making the fermentation path very costly. It was then decided to add potable ethanol to the extract and to attempt distillation.

2.13 Distillation of ti kōuka extracts with ethanol

The aim was to reconstitute the heated ti kōuka extract with water and then to attempt distillation in the presence of different concentrations of potable alcohol.

Method

The reconstituted ti kōuka extract from Section 2.12 was used for this work. Potable ethanol (99.9%) was obtained from Scientific Supplies, New Zealand (HPLC Reagent

Grade, Scharlau ET 0015-2500). The ethanol was mixed with 30 mL of reconstituted extract and water to achieve a range of final concentrations in a final volume of 150 mL (Table 2.7). The five treatments were distilled over heating mantle and 10 mL fractions were progressively collected until the stillhead temperature in Treatments 1 to 4 reached almost 100°C (boiling point of water) at which point nearly all the ethanol must have been recovered. The stillhead temperature in Treatment 5, with no alcohol involved, was held at 100°C.

The refractive index was read for all the fractions collected for all the treatments using a Prisma Abbe Bench refractometer (Ceti, Belgium) and the temperature was recorded. Visual colour was also recorded for all the fractions (Table 2.8).

Results and discussion

The range of the refractive indices of all the treatments are tabulated in Table 2.7 along with the number of fractions collected for each treatment.

As the ethanol concentration decreased from Treatments 1 to 5, the number of fractions collected decreased because it took less distillation to approach 100°C (Table 2.7).

Table 2.7 Distillation of evaporated and reconstituted ti kōuka extract with different concentrations of potable ethanol

Treatment	Volume of reconstituted ti kōuka extract added (mL)	Volume of ethanol added (mL)	Volume of deionised water added (mL)	Final concentration of ethanol before distillation (%)	Number of fractions	Refractive index (at 25°C) of the distilled fractions from first to last
1	30	120	0	80	12	1.363 to 1.361
2	30	90	30	60	10	1.363 to 1.349
3	30	60	60	40	7	1.363 to 1.339
4	30	30	90	20	5	1.361 to 1.340
5	30	0	120	0	5	1.336 to 1.334

The refractive indices of ethanol and water are 1.3614 and 1.3333 respectively at 20°C (The Merck Index, 2001). A correction factor of – 0.00045 per °C can be applied to compensate for a different temperature, in this case 25°C. The respective values were calculated to be 1.359 and 1.331 at 25°C. If all the distillate fractions were mixtures of

ethanol and water alone, all the values would be expected to lie between these two values. The values are displaced to higher refractive indices implying that there was more dissolved matter in the fraction than just ethanol and water. However, as expected the refractive indices decreased as the ethanol concentration decreased from 80% to 0%.

All 12 fractions of Treatment 1 (80%) were colourless (Table 2.8), as were Fraction 1 to 8 of Treatment 2 (60%). However, Fractions 9 and 10 were clear but yellow, setting a trend with decreasing ethanol concentration such that coloured and often turbid fractions were recovered earlier. Thus the fractions collected from Treatment 5 (0% ethanol) were all turbid and coloured.

Table 2.8 Description of fractions collected after distillation of ti kōuka extract with different concentrations of ethanol

Fraction number	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
1	Colourless	Colourless	Colourless	Colourless	Turbid, yellow /brown
2	Colourless	Colourless	Colourless	Clear, v. p. yellow	Turbid, yellow brown
3	Colourless	Colourless	Colourless	Clear, p. yellow	V. p. yellow/ brown
4	Colourless	Colourless	Colourless	Turbid, yellow	Pale yellow
5	Colourless	Colourless	Colourless	Turbid, yellow brown	Pale yellow
6	Colourless	Colourless	Pale, clear yellow		
7	Colourless	Colourless	Turbid, light yellow		
8	Colourless	Colourless			
9	Colourless	Yellow			
10	Colourless	Dark yellow			
11	Colourless				
12	Colourless				

v = very; p = pale

Ethanol has a boiling point of 78.5°C at atmospheric pressure (The Merck Index, 2001). As the treatment mixtures reached this temperature, they started to boil and the early fractions were dominated by ethanol. Clearly, the coloured compounds did not co-distil with ethanol (e.g. Treatment 1), indicating that they were preferentially water-soluble. As the proportion of water increased, the tendency for coloured compounds to co-distil increased, so coloured compounds distilled over in progressively earlier fractions.

The higher alcohol treatments (Treatments 1 and 2) yielded a clear, colourless liquor with an aroma reminiscent of tequila, and as noted above only the two water-rich treatments (Treatments 4 and 5) were markedly brown. More intense aromas occurred

in the later fractions of later treatments. Thus, the water-soluble flavoured and coloured matter generated by the Maillard reaction was poorly recovered by ethanolic distillation.

After standing the fractions from these five treatments for several weeks, those that were originally clear became slightly turbid. The precipitate was physically reminiscent of protein, but is unlikely to be the cause because proteins are not volatile in distillations. Filtration through Whatman GF/B glass microfiber paper solved the precipitation problem. The chemical origin of the precipitate was not pursued because the primary goal was to prepare an attractive spirit.

The treatments in Table 2.8 that carried most flavour and colour were undoubtedly those with higher water content. The decision was made at this point to infuse the brown aqueous extract of ti kōuka with potable alcohol to simultaneously extract flavour, aroma and colour rather than distilling the brown aqueous ti kōuka extract with ethanol.

2.14 Infusion of ti kōuka flavour with ethanol

As the work with ti kōuka extract progressed, it was apparent that a spontaneous browning reaction was occurring as extracts were hydrolysed and evaporated at 60°C over two days. The aromas of the dried and reconstituted extracts were beer-like as described earlier. Whisky-like aroma notes were also evident. These aromas are almost certainly derived from the Maillard reaction. In the previous section, it was established that distillation of ethanolic mixtures could not simultaneously recover flavour and colour compounds. Therefore, as a starting point to recover these, the reconstituted brown extract of ti kōuka was infused with different concentrations of potable alcohol.

Method

The remaining evaporated and reconstituted extract from Section 2.12, which had been stored at 4°C, was centrifuged at 3700 gravities for 15 minutes in a Heraeus Labofuge 400e centrifuge. The supernatant was collected and infused with potable ethanol and deionised water in three different combinations (Table 2.9) for 15 minutes. The three treatment solutions were filtered using GF/B glass microfiber paper. The odour, refractive indices, and colours were then assessed or measured.

Results and discussion

The results obtained are summarised in Table 2.9. As was previously observed, as the concentration of ethanol decreased, the refractive index decreased. However, the values were higher than expected for pure water/ethanol mixtures confirming the presence of other dissolved matter. The high alcohol (80%) treatment (Treatment 1) yielded a clear, pale yellow liquid, while the zero alcohol treatment (Treatment 3) yielded a turbid, brown liquid. The infusions had different smells. The high alcohol (80%) treatment has a faint caramel smell. At 40% ethanol this was lost. However, at 0% ethanol there was a fruity, sweet smell.

Thus, colour and flavour are probably more likely to be due to hydrophilic compounds (water loving) generated by the Maillard reaction (Imafidon and Spanier, 1994), between amino acids and simple sugars. Both are present in the ti kōuka stem, and when heated and slowly dried at 60°C, they react to form flavoured compounds.

Table 2.9 Infusion of reconstituted ti kōuka extract with different concentrations of ethanol, refractive index, colour and smell

	Treatment 1	Treatment 2	Treatment 3
Volume of reconstituted ti kōuka extract (mL)	5	5	5
Volume of ethanol added (mL)	20	10	0
Volume of water added (mL)	0	10	20
Final ethanol concentration (%)	80	40	0
Refractive Index (at 23°C)	1.366	1.356	1.336
Colour of ti kōuka extract after filtration	Clear, pale yellow	Yellow	Turbid, dark brown
Odour of ti kōuka extract after filtration	Faint caramel alcohol	Alcohol	Fruity, sweet

Thus, distinctive colour and flavour outcomes could be generated by infusions of ethanol in different concentrations, indicating that this line of development was promising. The decision was made to follow this path but to also precede it with systematic manipulation of the Maillard reaction that is responsible for flavour and colour. The following section describes colour changes that occur in the Maillard reaction and how they could be systemically monitored in the present project.

2.15 Colour development and monitoring techniques

The early stages of the Maillard reaction result in colourless compounds that subsequently polymerise to produce the brown colours that are distinctive of the reaction (Bailey *et al*., 1996). The change in colour is usually measured by absorbance or reflectance over a range of wavelengths.

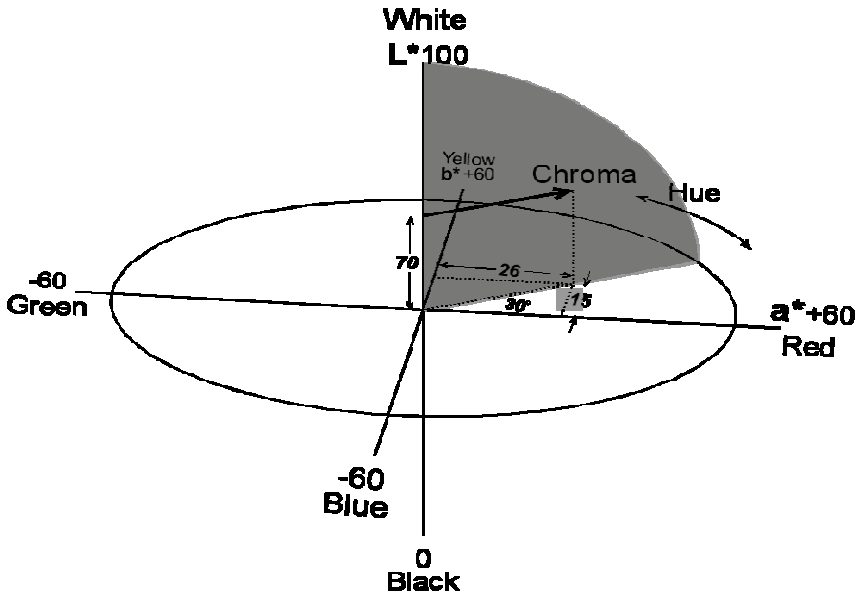
The compounds formed early in the Maillard reaction absorb in the ultraviolet region of the spectrum, e.g. 5-hydroxymethyl furfuraldehyde (Bailey *et al*., 1996) but do not absorb in the visible region of the spectrum. As the Maillard reaction progresses, the spectrum changes and visible colour develop. This is typified by very strong absorption in the ultraviolet region, the longer wavelength tail end of which progressively protrudes into the visible spectrum and causes the yellow, orange and brown colours to appear. Therefore, as a rule, the intensity of non-enzymatic browning resulting from the Maillard reaction is based on absorbance measurement at 294 nm for the early Maillard reaction products, at 320 nm for soluble pre-melanoidins, and at 420 nm for the advanced Maillard reaction products (Wijerickreme *et al*., 1997).

The development of colour is an important feature of the Maillard reaction, but the human colour memory is quite poor, and the verbal communication is not precise either (Coultate, 2002). The human eye uses the cone and rod cells of the retina to perceive the colour of an object. The cone cells respond best to three wavelengths, i.e. 445, 535 and 570 nm. Our perception of the colour is based on the ratio between the three responses, combined with the response of the rod cells. The rods are more sensitive than the cones and have their peak sensitivity at 505nm. Therefore, it should be possible to describe any colour, as perceived by the human eye in terms of the final ratios. However, scientific instruments such as reflectance colorimeters used in colour definition have a more even distribution of wavelengths across the visible spectrum, corresponding to the three primary colours red, green, and blue of the trichromatic system (Coultate, 2002).

Based on the principles of the trichromatic system, the light for each colour, formed by the combinations of the three primary colours (red, green and blue) is assigned numerical values. Ideally, the numbers involved should be easily interpretable in terms of visual attributes such as hue, colour intensity (also known as chroma or saturation) and lightness. The widely accepted and used Commission Internationale de l'Eclairage

(CIE) system (1976) is based on the concept of colour space (Figure 2.16), where colour can be fundamentally defined by the three values, lightness (L*), redness/greenness (a*) and yellowness/blueness (b*) (Coultate, 2002).

The a* and b* axes are at right angles, and the circumference of the circle they define basically represents the colours of the rainbow. These colours are the hue. Hue is the term used for general classification of colour – the region of the visible spectrum (380 nm to 750 nm) – in which the greatest reflectance of light occurs. In Figure 2.16, the rotation of the thick arrow can be defined by the ratio of a*/b* or b* /a*. These simple expressions are however not very useful because, for example, pure yellow defined by b* /a* would have the numerical value of infinity. The angle superimposed on the circle by the thick arrow gives a better idea of hue and for this reason hue is defined as hue angle i.e. arctan (b*/a*), usually expressed in radians. Thus, the scale ranges from 0 to 6.28 (= 2π).



In L*a*b* colour space, the tip of the thick arrow is defined by its lightness (70 on a scale of 0 to 100), its redness (+26 on a scale of -60 to +60) and yellowness (+15). The hue is arctangent 15/26 (=30°) and the chroma, or intensity, is the length of thick line, √(152 + 262) i.e. 30 (Young and West, 2001)

Figure 2.16 L*a*b* colour space

Chroma expresses the measurement of hue intensity or saturation. Saturation describes the vividness/dullness of a perceived colour. A highly saturated hue has a bright,

intense colour, while a less saturated hue appears gentler. It can be measured independently of hue. Chroma is calculated as $\sqrt{[(a^*)^2 + (b^*)^2]}$.

The vertical coordinate L* is lightness from 0 (total light absorbance and therefore completely black) through grey (50) to 100 (complete light reflectance i.e. white).

For this project, a Hunter colourimeter (ColourFlex, Hunter Associates, Virginia, USA) was used for measuring reflected colour (Figure 2.17).

Figure 2.17 Hunter Colorimeter (ColourFlex, Hunter Associates, Virginia, USA)

A Duran cylindrical glass dish (Schott, Germany) measuring 10 cm in diameter was placed in the illuminant path of the instrument and was covered with a cylindrical black shroud (Figure 2.17). Daylight D65/10° illuminant/observer combination was selected to measure daylight colour that measures the colour terms of L*, a*, b*. The calibrations standards used were a white tile, whose reflectance properties have been determined using a reference instrument (L* = 93.94, a* = –0.94, b* = 0.94), and a black tile, with colour values unstated. Five readings were taken for each sample.

The change of colour and the caramel smell obtained after evaporating the extracts in a conventional oven at 60°C gave an indication that Maillard reaction was occurring during the evaporation process in the oven. The rate of the Maillard reaction and the nature of the products are governed by its immediate environmental conditions e.g. temperature, pH, water activity, and the concentration and/or molar ratios of sugar to amino acids can affect the rate and extent of the Maillard reaction (Renn and Sathe,

1997). To achieve the best flavour, aroma and colour for the ti kōuka spirit, optimal conditions of the Maillard reaction and the best ratio of alcohol to ti kōuka extract for infusion had to be established.

In the preceding sections (Sections 2.16 to 2.18), dried ti kōuka sample was extracted with Fructozyme, and subsequently evaporated to varying degrees of temperature, different parts of ti kōuka stem and different pHs and time of incubation to establish the most suitable combination for flavour generation.

2.16 Effect of temperature on Maillard reaction in ti kōuka extracts

The Maillard reaction is a complex reaction, which is initiated by reacting a reducing sugar with amino compound followed by a cascade of consecutive and parallel reactions (Figure 2.13). It is influenced by many factors such as temperature, pH, time of heating, water activity, type and concentration of buffer, reactant source and sugar involved (Wijewickreme *et al*., 1999). Changing any of these factors will alter the reaction rate, reaction pathways and reaction end-products.

Each step within the Maillard reaction network may therefore have different temperature dependence and, as a result, temperature may strongly determine which reaction route prevails (Martins and van Boekel, 2005). In the Maillard reaction, the active form of the sugar is the open chain, the concentration of which increases with temperature (van Boekel, 2001). Not only does temperature generally increase reaction rates, but the effect of temperature is synergetic because more of the reactive species becomes available. For both these reasons, increasing the reaction temperature would lead to an increase of the reactivity between the sugar and the amino group (Martins *et al*., 2001). The aroma profile also depends on the temperature, time of heating and in turn the reaction route (Ames, 1998). Therefore, for any given temperature-time combination, a unique aroma would be produced, which is not likely to be produced at any other combination of heating and time. In this section, the effect of temperature on the evaporation of ti kōuka extracts was explored at three temperatures.

Method

After carrying out the by-now standard procedure of grounding, drying, hydrolysis with Fructozyme, and filtering the ti kōuka extract, the extract was placed into three 135 mm Schott Duran glass Petri dishes. These were heated at ambient (23), 60 and 100°C for

65 hours. After drying, the extracts were reconstituted to the original volume with deionised water. Ten milliliters of all three samples were retained for PAHBAH analysis. The remaining sample was centrifuged at 3700 gravities for 15 minutes. Aliquots of the supernatant of each of the three samples were infused with potable ethanol and deionised water in five combinations (Table 2.10).

All 15 treatments were then filtered using GF/B glass filter paper. Colour measurements, wavescans, and refractive indices were measured.

Table 2.10 Infusion of reconstituted ti kōuka extract with different proportions of ethanol

Treatment	Supernatant volume of ti kōuka extract (mL)	Volume of pure ethanol added (mL)	Volume of deionised water added (mL)	Final ethanol concentration (%)
1	10	20	0	66.7
2	10	15	5	50.0
3	10	10	10	33.3
4	10	5	15	16.7
5	10	0	20	0.0

Results and discussion

The sample held at ambient temperature evaporated only 10% of its total volume. It smelt woody. The sample treatment at 60°C evaporated completely leaving a dark brown layer with a sweet caramel smell, while the sample evaporated at 100°C was very dark brown with a strong caramel smell.

The brown colour and the caramel aroma are indicators of Maillard reaction product(s). Archetypal caramel-smelling compounds like 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, trademark Furaneol) and 5-hydroxymethyl-2- furaldehyde are formed by thermal degradation of sugars e.g. fructose in the presence of amines or amino acids (Mills and Hodge, 1976), which were both present in the ti kōuka extract. Blank and Fay (1996) proposed that Strecker aldehydes (intermediate stage of Maillard reaction) may play a role in generating odor-active furanones. This suggests that the caramel-like smell produced at 60°C evaporation must have been the product(s) of the intermediate stage of the Maillard reaction and presumably later stages of the Maillard reaction at

100°C evaporation. For the precise identification of these compounds, GC-MS analysis needs to be was carried out.

The percentages of original reducing sugar of the dried ground ti kōuka stem were 13.5 ± 0.6%, 12.6 ± 0.1% and 2.4 ± 0.2%, after evaporation at ambient, 60 and 100°C, respectively. Interestingly, there was little difference between the percentage values between ambient and 60°C, although only 10% of the extract was evaporated at ambient temperature. However, it was clear from the caramel smell that the Maillard reaction took place at 60°C, possibly yielding intermediate stage products e.g. reductones and Stecker aldehydes. Therefore, in effect, the concentration of reducing sugar should decrease. However, as was explained in Section 2.12, the hydrazine (in the PAHBAH reaction) may have also reacted with the ketone and aldehyde groups of the Maillard reaction products, giving the illusion of a high reducing sugar concentration. The percentage of reducing sugars was lowest at 100°C, probably because most of the available reducing sugars present in the extract was used up, reaching the final stages of the Maillard reaction and yielding melanoidins (heterocyclic aromatic compounds) which do not react with the PAHBAH reaction.

The refractive indices and calculated colour measurements are tabulated in Table 2.11 (the original a* and b* values are listed in Appendix V). As the alcohol concentration decreased from Treatment 1 to Treatment 5, the refractive indices decreased slightly, although the values were consistent for all the treatments for all the corresponding three temperatures. The refractive indices of ethanol and water are 1.3614 and 1.3333 respectively at 20°C (The Merck Index, 2001). Applying the correction factor of –0.00045 per °C to compensate for difference in temperature (in this case 23°C), the respective values were calculated to be 1.3600 and 1.3319. Again, (as was previously observed in Section 2.14), the refractive indices values were higher than pure ethanol and water. There was an average increment of 0.004 and 0.005 for 0 and 67% ethanol respectively. This implies that dissolved matter was present in the treatments.

Table 2.11 Description of colour measurements of ti kōuka supernatants (after evaporation at different temperatures, reconstitution, infusion with different proportions of ethanol and centrifugation)

Temperature of evaporation	Treatment	Final ethanol concentration (%)	Refractive Index at 23°C	L*	Saturation $\sqrt{[(a^*)^2 + (b^*)^2]}$	Hue angle $\arctan[(b^*)/(a^*)]$
Ambient	1	66.7	1.365	18.85 ± 0.13	12.67 ± 0.29	1.20 ± 0.01
	2	50.0	1.361	9.17 ± 0.02	8.61 ± 0.06	1.18 ± 0.03
	3	33.3	1.353	2.52 ± 0.10	2.84 ± 0.23	1.01 ± 0.11
	4	16.7	1.344	0.74 ± 0.39	2.21 ± 1.05	0.70 ± 0.07
	5	0.0	1.336	0.59 ± 0.06	1.29 ± 0.06	0.77 ± 0.11
60°C	1	66.7	1.365	16.70 ± 0.17	15.03 ± 0.30	1.19 ± 0.01
	2	50.0	1.356	5.89 ± 0.06	6.12 ± 0.11	1.12 ± 0.04
	3	33.3	1.353	2.37 ± 0.07	2.91 ± 0.26	1.08 ± 0.08
	4	16.7	1.344	2.24 ± 0.06	1.30 ± 0.14	0.83 ± 0.19
	5	0.0	1.336	1.03 ± 0.13	1.66 ± 0.05	0.87 ± 0.16
100°C	1	66.7	1.365	12.68 ± 0.48	16.27 ± 0.25	1.10 ± 0.01
	2	50.0	1.360	0.96 ± 0.05	0.98 ± 0.04	1.33 ± 0.10
	3	33.3	1.354	1.15 ± 0.06	0.56 ± 0.24	-0.72 ± 1.21
	4	16.7	1.345	1.10 ± 0.06	0.38 ± 0.10	-0.79 ± 0.39
	5	0.0	1.336	0.59 ± 0.05	0.42 ± 0.15	0.70 ± 1.18

Values are the means ± 1 standard deviation (n = 5)

The most obvious trend to the eye was that low alcohol treatments were much darker brown and it was of interest to see how appearance related to objective colour values. The L* values (lightness) decreased drastically as the volume of ethanol decreased from Treatment 1 to 5 for all the three temperatures (96.8, 93.8 and 95.4% for ambient, 60 and 100°C respectively). The corresponding L* values of each treatment (1 to 5) decreased as the temperature increased from ambient to 60 to 100°C (18.9 ± 0.13 to 16.7 ± 0.17 to 12.7± 0.48 respectively, for Treatment 1). The lowest values, in Treatments 5, were close to zero meaning that very little light was reflected from those treatments, i.e. the colour were darker to the eye.

At the same time – and independent of L* – the saturation or colour intensity decreased as ethanol concentration decreased for all the three temperatures. However, the saturation values for Treatment 1 increased as the temperature increased (from 12.67 ± 0.29 to 15.03 ± 0.03 to 16.27 ± 0.25) suggesting that at lower temperature the colour of Treatment 1 was more intense.

The hue angle for the corresponding temperatures of Treatments 1 to 4 decreased with increase in temperature. Hue angle values for Treatment 4 and 5 at ambient and 60°C were similar but the values at 100°C decreased significantly indicating a shift in colour i.e. becoming redder. Looking at it, it suggests that at 20°C, the hue was yellow and as temperature of evaporation increased, the hue became redder. Thus, the appearance was most dependent on light absorbance rather than colour as such. One possible explanation of the hue being yellow at lower temperature is that in the Maillard reaction water is generated. This water may mix with the rest of the matrix causing dilution of the soluble reactants and hence lower the browning rate. Additional water may dilute the concentration of the reactants to the point where the rate of reaction diminishes. If the water generated does not readily diffuse into the total matrix it could be considered that a growing aqueous layer could be generated around the reactants. This could encourage solubility and further the colour and water generation (Mustapha *et al*., 1998). On the other hand, lower water activity and higher surface temperature (in this case, 100°C) favour the production of colour and flavour (Reineccius, 2006).

2.17 Infusion of different parts of enzyme-treated ti kōuka stem with ethanol

It was apparent from Section 2.3 that when the ti kōuka stem was progressively processed from the top of stem downwards, the stem was becoming increasingly fibrous, to the point that the lower segments of a 1 m stem was very difficult to cut and grind. Therefore, extraction of ti kōuka stem was limited to the top 60 cm of the stem. There was a very small decrease in the refractometry Brix value from the top of the stem going down (Section 2.3), however this method has a limited accuracy. It was later observed (discussed in Chapter 3) that the concentration of some of the individual amino acids also decreased progressively down the stem.

The absolute concentration and molar ratios of sugars to amino acids affects the rate and extent of the Maillard reaction (Renn and Sathe, 1997). The next set of experiments was to evaporate different parts of the ti kōuka stem at different temperatures to explore this effect and to see if it could be exploited in spirit development.

Method

Three fresh ti kōuka stems (60 cm long from the tip) were collected and cut into 20 cm lengths. The corresponding 20 cm lengths were pooled and ground, and designated top, second and third sections respectively.

The three ground samples (100 g each) were extracted (without drying) with deionised water and Fructozyme at 60°C as described in Section 2.8. The extracts were cooled, and filtered using Whatman No 4 filter paper, with multiple washes of deionised water, and each was made to 300 mL. The filtrates of the three samples were each distributed into three 135 mm Schott Duran glass Petri dishes (giving a total of nine) to be evaporated at ambient (~23), 60 and 100°C respectively, in conventional ovens for approximately 18 hours. All the nine samples were reconstituted to their original volume with deionised water. Reducing sugar concentration was measured by the PAHBAH method (Table 2.12). The reconstituted samples were then centrifuged at 3700 gravities for 15 minutes and colour measurements were made on the supernatant using CIELAB colour space (Table 2.13). The supernatant were then infused with different combinations of potable ethanol and deionised water (Table 2.10), and then filtered using GF/B glass microfiber filter paper. Wavescans were performed between 200 and 800 nm wavelength using a single quartz cuvette with 1cm optical path in a

Pharmacia Biotech® Ultraspec 2100pro spectrophotometer. Known dilutions were made with deionised water before performing the wavescans to ensure the absorbancies were between 0 and 3. Presented data (Table 2.14) are corrected for these dilutions. The researcher also assessed smells and recorded colour.

Results and discussion

As was previously observed (Section 2.16) and expected here, only 10% of the volume of the three stems evaporated at ambient temperature was lost. Treatments at 60 and 100°C had completely evaporated leaving a dry dark brown layer in the Petri dishes.

Moisture contents were not recorded in this experiment but Table 2.1 shows that the mean weight loss on air-drying the ti kōuka sample was about 75% (w/w). Applying this value to the results obtained from PAHBAH analysis, the approximate predicted air-dried weights of reducing sugars for the wet weight samples are summarised in Table 2.12.

Table 2.12 PAHBAH analysis of different sections of fresh ti kōuka sample at different incubation temperature

Incubation temperature (°C)	Extractable reducing sugar (% w/wet weight expressed as air dried weight) Top	Extractable reducing sugar (% w/ wet weight expressed as air dried weight) Second	Extractable reducing sugar (% w/ wet weight expressed as air dried weight) Third
20	7.80 ± 0.16^{a}	8.04 ± 0.16^{a}	8.32 ± 0.08^{a}
60	8.32 ± 0.11^{b}	9.52 ± 0.12^{b}	9.04 ± 0.13^{b}
100	4.52 ± 0.15^{c}	5.20 ± 0.15^{c}	4.56 ± 0.18^{c}

Values are mean ± standard deviations of three replicates
[a,b,c] Means bearing different leeters incolumn groups are significantly different at $P<0.05$

There was little difference between the three sections ($P=0.073$), but treatments evaporated at different temperatures had significant effect on the reducing sugar ($P<0.001$). Samples evaporated at 60°C had the highest PAHBAH values and those at 100°C had the least ($P<0.001$). (As noted in Section 2.12, some Maillard reaction products may react with PAHBAH, creating a falsely high measure of reducing sugar content.) It is proposed that the progression of the Maillard reaction at 100°C was responsible for the apparent major loss in reducing sugar.

Colour measurements showed that as the temperature increased from 20 to 100°C, the L* (lightness) values markedly decreased for all samples (Table 2.13). The third section was the darkest and top section was the lightest, but only by a small margin.

Table 2.13 Hunter colour measurement of different parts of ti kōuka stem extracts evaporated at different temperatures

Stem section	Temperature (°C)	Averages of L*	Saturation $\sqrt{[(a^*)^2 + (b^*)^2]}$	Hue angle $\arctan[(b^*)/(a^*)]$
Top	20	14.40 ± 0.02	7.04 ± 0.18	1.53 ± 0.90
	60	6.12 ± 0.03	7.79 ± 0.17	1.14 ± 0.94
	100	1.39 ± 0.10	1.74 ± 0.24	1.09 ± 0.50
Second	20	13.60 ± 0.04	7.36 ± 0.11	1.50 ± 1.23
	60	3.63 ± 0.06	2.58 ± 0.33	1.05 ± 0.64
	100	1.36 ± 0.05	1.36 ± 0.25	1.08 ± 0.72
Third	20	13.40 ± 0.07	9.52 ± 0.20	1.42 ± 0.69
	60	3.65 ± 0.03	2.63 ± 0.17	1.05 ± 0.77
	100	1.73 ± 0.05	1.92 ± 0.21	1.16 ± 0.45

Values are mean ± 1 standard deviation of five replicates

For each three samples, saturation decreased with increasing evaporation temperature i.e. at higher temperature the colour had lower intensities (except for top section of the stem). For this sample, the saturation increased slightly at 60°C and then decreased at 100°C as for the other samples.

The hue angle for the corresponding temperatures of the three samples decreased slightly with increase in temperature. The data suggest that at 20°C, the hue was yellow and as the temperature of evaporation increased, the hue became redder for all the three stem sections. As is discussed later, these hue changes suggest a progression of the Maillard reaction.

After ethanol infusion, the final ethanol concentrations were again 66.7, 50.0, 33.3, 16.7, and 0%. The three treatments without ethanol (Treatments 5) developed a turbid precipitate the nature of which was unknown and not pursued. It was removed by filtration that was applied to all 45 treatments (3 samples x 3 evaporation temperature x 5 ethanol concentrations).

Similar absorbance patterns were observed in the 45 wavescans measured after ethanol infusion. Figure 2.18 shows a visual example of a typical wavescans measured in this experiment at 60°C for the top section of the stem at five ethanol concentrations.

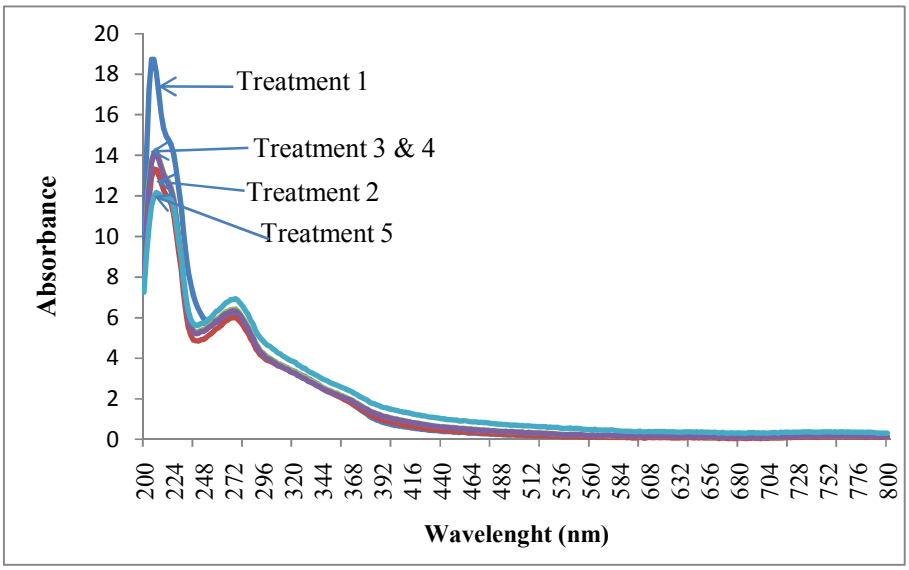


Figure 2.18 Dilution-corrected wavescan of the top section of stem evaporated at 60°C and infused to five final ethanol concentrations

At first sight it appears from Figure 2.18 that very little colour should be visible to the eye (i.e. above 400 nm), but it must be remembered that the absorbance scale is greatly expanded to cope with the high absorbance in the ultraviolet region. The compounds formed early in the Maillard reaction absorb in the ultraviolet region of the spectrum but do not absorb in the visible (MacDougall and Granov, 1998). As the Maillard reaction progresses, the spectrum changes and visible colour develop. This is typified by continuing strong absorption in the ultraviolet region, but with the longer wavelengths of the absorption curve progressively protruding into the blue region of the visible spectrum. This causes the yellow, orange and brown colours to appear progressively.

For comparison of the scans, Table 2.14 presents the absorbance values of the two main peak ranges of the 45 wavescans.

In all the infusion treatments, the absorbance of the first peak range which is highlighted in grey in Table 2.14 (about 206 to 210 nm) was always higher than that of the second peak (about 266 to 284 nm), the latter peak range being characteristic of low molecular weight Maillard reaction products (Kim and Lee, 2008; Bailey *et al*., 1996).

Table 2.14 Absorbance of wavescan peaks of different sections of ti kōuka stem evaporated at different temperatures and infused with different ethanol concentrations

Stem section/ treatment	Absorbance for samples evaporated at 20°C			Absorbance for samples evaporated at 60°C			Absorbance for samples evaporated at 100°C		
	206 to 210 nm	266 to 284 nm	420 nm	206 to 210 nm	266 to 284 nm	420 nm	206 to 210 nm	266 to 284 nm	420 nm
Top									
1	14.98	4.10	0.12	18.73	6.21	0.53	20.67	14.07	1.20
2	10.79	4.07	0.18	13.32	6.00	0.60	22.93	15.54	2.39
3	11.18	4.10	0.22	14.25	6.42	0.70	23.04	15.34	2.43
4	11.03	4.04	0.23	14.09	6.34	0.79	21.99	14.45	2.55
5	10.29	5.81	0.12	12.17	6.93	1.21	22.35	14.26	2.57
Second									
1	15.50	10.21	0.02	12.14	5.13	0.40	27.66	8.42	2.41
2	10.89	4.19	0.01	14.69	5.73	0.62	28.08	18.69	2.70
3	10.70	3.63	0.03	15.46	7.10	1.37	29.41	18.54	3.01
4	10.82	3.62	0.01	16.32	8.22	1.87	29.70	19.01	3.35
5	8.68	3.66	0.15	12.95	5.79	0.94	29.89	19.09	3.38
Third									
1	15.83	4.85	0.32	15.20	5.31	0.38	27.80	17.97	2.34
2	16.42	5.13	0.35	17.10	6.01	0.58	>30.00	20.97	2.94
3	13.76	4.47	0.32	15.91	5.66	0.61	29.51	18.81	2.66
4	14.85	4.74	0.38	16.58	5.95	0.71	29.35	19.57	2.75
5	13.99	5.87	0.85	19.90	9.13	1.98	28.85	18.48	2.85

The absorbances fluctuated (i.e. decreased and then increased or vice versa) for Treatments 1 to 5 at all three temperatures and stem sections. However, for all three sections of the stem, as the temperature of evaporation increased the absorbancies increased as well for each treatment. The increase was greatest at 100°C suggesting that at lower temperatures the colour was lighter and became darker as the temperature increased. This result compliments the result of the calculated values of the CIELAB colour space (Table 2.13).

Appearance and odour results are shown in Table 2.15. As the temperature of evaporation was increased, the colour changed from pale yellow to brown for all three sections. Treatments 5 (with no ethanol) were turbid at 20 and 60°C for the top and second section, but were brown at 100°C. The eye could only perceive the brown colour and not the different degree of redness in the alcohol treatments, as was calculated in CIELAB colour space in Table 2.13.

Table 2.15 Dominant colour and odour profile after infusion of different concentration of ethanol with different parts of ti kōuka stem at different evaporation temperature

Stem section	Treatment 1		Treatment 2		Treatment 3		Treatment 4		Treatment 5	
	Colour	Odour	Colour	Odour	Colour	Odour	Colour	Odour	Colour	Odour
Top										
20°C	Light yellow	Alcohol	Light yellow	Woody	Light yellow	Alcohol	Light yellow	Woody	Murky, pale yellow	Woody
60°C	Light yellow	Alcohol	Light yellow	Alcohol	Light yellow	Alcohol	Light yellow	Woody	Murky, pale yellow	Off
100°C	Brown	Alcohol	Brown	Woody	Brown	Caramel	Brown	Caramel	Brown	Woody
Second										
20°C	Light yellow	Ester	Light yellow	Alcohol	Light yellow	Alcohol	Light yellow	Woody	Light yellow, turbid	Woody
60°C	Pale brown	Alcohol	Light brown	Alcohol	Light brown	Alcohol	Light brown	Alcohol	Light brown, turbid	Off
100°C	Brown	Alcohol	Brown	Alcohol	Brown	Alcohol	Brown	Woody	Brown	Woody
Third										
20°C	Light yellow	Alcohol	Light yellow	Alcohol	Light yellow	Alcohol	Light yellow	Woody	Light yellow	Woody
60°C	Light brown	Alcohol	Light brown	Alcohol	Light brown	Woody	Light brown	Woody	Light brown	Off
100°C	Brown	Alcohol	Brown	Caramel	Brown	Rum	Brown	Rum	Brown	Caramel

Most of the 45 treatments had either woody or alcohol odour after ethanol infusion. The odour of caramel or rum (highlighted in grey in Table 2.15) was only achieved at 100°C for the top (Treatments 3 and 4) and third section (Treatments 2 to 5) of the stem. Odour of alcohol was dominant for most of the Treatments 1 and 2 at all temperatures. On the other hand, Treatments 5 (with no ethanol) had a woody or 'off' odour which suggests that water alone extracted unfavorable flavour notes, as assessed by odour. Although not commonly realized, odour is a major component of flavour. Retronasal detection of volatiles by the olfactory lobe in the nasal cavity strongly influences human perception of flavour during mastication and swallowing (Reineccius, 2006).

The above experiments showed that consumer-acceptable spirits could be made by manipulating temperature and ethanol infusion ratios. The next section describes how other variables could be exploited.

2.18 Effect of pH and evaporation time on the enzyme-treated ti kōuka extract

Just as for temperature, the reactivity of the sugar and amino group is also influenced by pH (Hidalgo *et al*., 1999). The open chain form of the sugar and the unprotonated form of the amino group, which are the reactive forms, are favoured at higher pH (Martins *et al*., 2001). The lower the pH, the more protonated amino groups become (Martins *et al*., 2001) and therefore less reactive with sugar. The initial step of the Maillard reaction is thus highly influenced by the initial pH of the reaction. However, since the Maillard reaction consists of several reaction steps, which are variously acid or base catalysed, pH can have a considerable effect on which reaction route prevails and which products are therefore formed (Martins and van Boekel, 2005). Generally, the reaction rate increases as pH increases (Apriyantono and Ames, 1993). The next set of experiments was carried out at different pH values and times of evaporation to see if they could be exploited in spirit development.

Method

Dry ti kōuka stem was grounded and extracted with Fructozyme and deionised water. The filtrate (1600 mL) was divided into four parts of 400 mL each. The pH of three of the parts was adjusted to pH 3.0, pH 7.0 and pH 10.0 with 1 M citric acid or 1 M sodium hydroxide as required using a portable pH meter (Meter Lab pH M201). The

pH of the fourth solution (4.92) was left unaltered. Each of the four-pH treatments were further divided into four parts, giving a total of 16 solutions (100 mL each). Four, representing each pH, were evaporated for 0, 24, 48 and 65 hours at 60°C. They were subsequently reconstituted to the original volume of 100 mL with deionised water. The pH of all 16 solutions was recorded before evaporation and after reconstitution (Table 2.17). The solutions were then centrifuged at 3700 gravities for 15 minutes. Colour in CIELAB space was recorded before and after centrifugation of the 16 treatments (Table 2.18). Different volumes of the supernatant of all 16 solutions were added to fixed volume of ethanol i.e. 20 mL (Table 2.16) giving a total of 64 samples.

The ethanol-treated mixtures were further centrifuged at 3700 gravities for 15 minutes. The supernatants were vacuum-filtered through Whatman GF/B glass micro-fibre filter paper. The colours of the 64 mixtures were observed. The mixtures were subsequently stored for 18 months to see if any changes occurred.

Table 2.16 Different ratio of ti kōuka extract infused with ethanol

Alcohol treatment	Volume of ti kōuka added (mL)	Volume of ethanol added (mL)	Final concentration of ethanol (% v/v)
1	5	20	80.0
2	10	20	66.7
3	15	20	57.1
4	20	20	50.0

Results and discussion

All treatments heated for zero hours remained in liquid state and they all had a woody smell (Table 2.17). Treatments heated at 24 hours were variously still liquid or dried, but all were brown. They all had some degree of caramel odour. All treatments heated for 48 and 65 hours evaporated completely leaving a dark brown layer in each treatment. They all had caramel odour except for the two treatments around pH 3 which had an odour of rum even though no ethanol had been added at that point. There were differences in the caramel smells from the different treatments that could be ascribed to the final balance of flavour compounds from the Maillard reaction (Reineccius, 2006).

Table 2.17 Changes in pH before and after evaporation and state of ti kōuka extract at different pH and time of evaporation

Time of evaporation (hours)	pH before evaporation	pH after evaporation and reconstitution	State of ti kōuka extract after evaporation	Odour after evaporation
0	3.00	3.00	All liquid	Woody
0	4.92	4.92	All liquid	Woody
0	7.00	7.00	All liquid	Woody
0	10.00	10.00	All liquid	Woody
24	3.00	3.27	Partially dried	Caramel
24	4.92	4.79	All liquid	Caramel
24	7.00	5.90	Dry, brown layer	Faint caramel
24	10.00	6.86	All liquid	Faint caramel
48	3.00	3.29	Dry, dark brown layer	Rum
48	4.92	4.80	Dry, dark brown layer	Sweet caramel
48	7.00	6.00	Dry, dark brown layer	Sweet caramel
48	10.00	6.60	Dry, dark brown layer	Faint caramel
65	3.00	3.41	Dry, dark brown layer	Rum
65	4.92	4.81	Dry, dark brown layer	Sweet caramel
65	7.00	5.76	Dry, dark brown layer	Sweet caramel
65	10.00	6.62	Dry, dark brown layer	Sweet caramel

The immediate chemical environment of the Maillard reaction governs the rate and the nature of the coloured products. One such factor is the water activity (Labuza, 1980). Water availability can influence the rate of numerous Maillard pathways thereby influencing the rate of overall flavour formation and possibly flavour character (Reineccius, 2006). This is expected because some chemical reactions yield water as a product (e.g. sugar dehydration), others require water as a substrate (e.g. hydrolysis), while yet others are promoted by water (e.g. aldol condensation). In the present study, at ambient temperature hardly any evaporation was taking place. Thus, water diluted the concentration of the reactants in the ti kōuka extract, and reduced the rate of reaction. Hence, there was no browning. At 60°C, as the time of incubation was increased and evaporation took place, the reactants became more concentrated and so promoted the Maillard reaction. There would be clearly a limit to this. Molecular mobility (Fennema, 1996) finally decreases, as the matrix becomes dry, and in turn slows the reaction because the reactants cannot diffuse and react. Another factor is

temperature itself. Reactions go faster as temperature increases. Thus, a range of influences will be operating in the evaporation step, including possible changes in pH. There was no change in the pH of the ti kōuka extract at pHs 3.0 and 4.92 at any incubation time (Table 2.17). However, the pH 7.0 treatments decreased by about 1 pH unit when ti kōuka extract was heated for any period. A profound change in pH was observed with ti kōuka extract at pH 10.0. The pH changed from 10.0 to between 6 and 7.

Clearly, at lower pH and incubation time, fewer unprotonated (i.e. reactive form) amino compounds would be present in the mixtures. Hence, no browning was observed. Van Boekel (2001) had proposed that the percentage of unprotonated amino group (at room temperature) will typically be less than 1% at pH <7 and it decreases with lower pH values.

The Maillard reaction has a strong influence on pH (Apriyantono and Ames, 1993). In unbuffered systems, as these were, pH falls during the reaction due to the disappearance of basic amino groups at the early stages of the reaction (Hill *et al*., 1996). Amino acids are involved in more than one distinctive stage of the Maillard reaction, e.g. during the initial degradation of sugar and the polymerization of intermediates. Because they have different compositions and structures, they may react in different ways, resulting in a large variety of compounds, which can influence the pH changes during the whole reaction (Carabasa-Giribet and Ibarz-Ribas, 2000).

It has been known for many years that formic and acetic acids are formed by degradation of glucose and fructose during the Maillard reaction (van Boekel and Brand, 1998). Recent data indicates that acetic acid is formed in high concentrations from Amadori compounds (Brands and van Boekel, 2001). The importance of these organic acids is that they cause a substantial reduction in pH, and as a result, the Maillard reaction slows (van Boekel, 2001). The subsequent degradation of the Amadori products is dependent on the pH of the system (refer to Figure 5.3, Chapter 5). At pH 7 or below, it undergoes mainly 1,2-enolisation with the formation of hydroxymethylfurfural when hexose sugars are involved in the reaction. This compound is a typical components of caramel flavour (Kroh, 1994), hence the caramel smell noted in many of these experiments. At pH >7 the degradation of the Amadori compound is thought to involve mainly via 2,3 enolisation, where reductones such as 4-

hydroxy-5-methyl-2,3-dihydrofuran-3-one and other products including acetol, pyruvaldehyde and diacetyl are formed (Martins *et al*., 2001).

Overall inspection of colour analysis (Table 2.18) shows that variances (standard deviation/mean) within each of the 16 treatments were low to the point that trends could be discussed without the need for a formal statistical analysis.

Before centrifugation, the differences in L* values (lightness) between the 16 treatments were minor for all evaporation times except 65 hours, where values were clearly lower. Within initial pHs i.e. 3.00, 4.92 etc., L* values were lower at initial pH 10.0, particularly at 65 hours, where the means fell from 14.6 at pH 3 to 4.3 at pH 10.0. L* values after centrifugation were lower in all treatments, presumably because reflective particulate matter was lost. The percentage loss of reflectance due to centrifugation had no clear relationship to pH or incubation time (data not shown).

Within each incubation group (0, 24 hours etc.) saturation decreased with increasing pH, both before and after centrifugation. Stated another way, more alkaline treatments had lower colour intensities. Centrifugation caused a consistent loss in saturation, presumably because coloured particulate matter was no longer present.

There was not much difference in the hue angle at the different pHs and evaporation times before and after centrifugation.

MacDougall and Granov (1998) showed that the compounds produced in the Maillard reaction under alkaline conditions using different combinations of sugar and amino acids exhibited distinct characteristics in both the ultraviolet and visible spectral regions with different peak absorbance values during their development. However, in the previous section (Section 2.17) it was observed that the hue angle decreased going down the ti kōuka stem. Differences in hue are caused by both the concentration of the pigments formed and wavelength distribution of the spectrum (MacDougall and Granov, 1998).

Table 2.18 Colour analysis of ti kōuka extracted at different pH and evaporation times before and after centrifugation

pH/heating treatment	Evaporation time (hours)	Initial pH	Averages of L*		Saturation $\sqrt{[(a^*)^2 + (b^*)^2]}$		Hue angle arctan[(b*)/(a*)]	
			Before	After	Before	After	Before	After
1	0	3.00	18.2 ± 0.98	9.1 ± 0.09	11.6 ± 0.93	11.2 ± 0.13	1.1 ± 0.79	1.0 ± 1.09
2	0	4.92	18.0 ± 1.11	4.1 ± 0.13	10.0 ± 0.56	5.1 ± 0.36	1.2 ± 0.86	1.0 ± 0.85
3	0	7.00	14.4 ± 0.56	2.9 ± 0.03	4.7 ± 0.26	3.0 ± 0.19	1.1 ± 0.23	1.1 ± 0.55
4	0	10.00	13.7 ± 0.70	2.5 ± 0.09	4.5 ± 0.32	1.5 ± 0.27	1.2 ± 0.97	1.3 ± 0.93
5	24	3.00	18.1 ± 0.40	8.0 ± 0.09	12.8 ± 0.66	10.3 ± 0.37	1.1 ± 1.16	1.0 ± 0.41
6	24	4.92	19.0 ± 1.60	4.4 ± 0.08	11.0 ± 0.54	5.2 ± 0.32	1.2 ± 1.46	1.0 ± 0.88
7	24	7.00	18.5 ± 0.61	1.6 ± 0.06	4.5 ± 0.17	1.7 ± 0.22	1.2 ± 0.37	0.9 ± 1.28
8	24	10.00	11.3 ± 0.97	2.2 ± 0.03	3.2 ± 0.30	0.9 ± 0.23	1.2 ± 1.17	0.8 ± 0.96
9	48	3.00	16.8 ± 0.34	1.8 ± 0.09	13.3 ± 0.23	2.1 ± 0.29	1.1 ± 1.03	0.9 ± 0.55
10	48	4.92	16.4 ± 0.64	1.4 ± 0.11	11.4 ± 0.52	1.5 ± 0.30	1.1 ± 0.53	0.8 ± 0.81
11	48	7.00	15.7 ± 1.05	1.3 ± 0.08	5.0 ± 0.22	0.8 ± 0.26	1.2 ± 0.83	0.8 ± 0.87
12	48	10.00	15.1 ± 0.62	2.5 ± 0.06	1.7 ± 0.27	0.8 ± 0.27	1.0 ± 1.33	0.9 ± 0.72
13	65	3.00	14.6 ± 1.54	1.4 ± 0.07	13.9 ± 0.40	1.9 ± 0.26	1.1 ± 0.65	1.2 ± 0.89
14	65	4.92	12.5 ± 1.30	1.0 ± 0.08	11.0 ± 0.45	1.5 ± 0.29	1.2 ± 1.17	1.3 ± 0.69
15	65	7.00	6.6 ± 0.88	1.2 ± 0.03	4.8 ± 0.20	1.4 ± 0.29	1.1 ± 0.73	1.3 ± 0.23
16	65	10.00	4.3 ± 0.78	2.0 ± 0.07	3.0 ± 0.33	1.3 ± 0.31	1.2 ± 1.11	1.4 ± 0.81

Values are the mean ± standard deviation (n = 5)

Although the wavescans for this set of experiments were not measured, it was evident from Table 2.14 that evaporating the enzyme-treated different sections of ti kōuka extract at different temperatures yielded different peak absorbance values.

For all the changes noted for centrifugation in Table 2.18, parallel changes in colour were observed in the centrifugation pellets. Generally speaking, the pellets became darker as pH increased. However, CIELAB colour space data was not recorded.

Another observation was made. As initial pH increased, the pellets become softer and looser. This may be due to proteins in the precipitate, such that at final pHs between ~3 and ~ 6.7 (Table 2.17), the water-holding capacity of the proteins could change markedly, thus altering texture (Nursten, 2005). It is well known that water holding capacity reduces on either side of the isoelectric point of a protein (Belitz *et al*., 2004). In the present case, this would imply that the isolectric point of the proteins from ti kōuka stem would be low. As will be shown in Chapter 3, the ti kōuka stem proteins are rich in the acidic amino acids, aspartic and glutamic, and/or in their amide forms aspargine or glutamine respectively.

The 16 pH/heat treatments (Table 2.17) were subsequently mixed in ethanol in the ratios shown in Table 2.16. As the volume of ti kōuka extract increased from alcohol Treatments 1 to 4 (Table 2.16) the colour changed from light yellow to brown, as expected from previous infusions. Alcohol Treatments 1 and 2 (80.0 and 66.7%) had a tequila-like odour, whereas alcohol Treatments 3 and 4 (57.1 and 50.0%) had rum-like odours. Within the alcohol Treatments, there were other subtle odour differences due to pH and heating treatments, not described here.

Overall, the colour and flavour can be controlled by the choice of ti kōuka and the ethanol volume in the mixture before final dilution to the commercial spirit strength.

On 18 months storage at ambient temperature in the dark, all the 64 treatments showed some degree of precipitation except for four. These were alcohol Treatments 1 evaporated for 48 and 65 hours at pH 7.0 and 10.0 respectively. As the ratio of ti kōuka increased from alcohol Treatments 1 to 4, more precipitation was observed for all the pHs and evaporation time (data not shown). The nature of these precipitates is unknown, as noted earlier. These precipitates were quite different from the turbid suspensions observed in prior distillation work, reported in Section 2.13. The pH

greatly affects the mechanism of the Maillard reaction itself and it may cause polymeric materials, particularly proteins, to become less soluble (Nursten, 2005). According to Refsgaard *et al*. (1996), formation of precipitates in alcoholic beverages has been shown to be related to the antioxidative properties of the flavonoids, as phenol oxidation initiates co-precipitation of the oxidized flavonoids with proteins and sugars.

When ti kōuka extract was distilled with ethanol/water mixtures (Section 2.13), treatments with more water content exhibited murkiness in the later distillation fractions. Similar observations were also made when ti kōuka extract was infused with ethanol/water mixtures (Section 2.14). None of the 64 treatments with ethanol showed any murkiness indicating that ethanol alone was a better option for infusion as opposed to different combinations of ethanol and water. It is possible that water extracts water-soluble protein from ti kōuka that later flocculates in the presence of alcohol. Formation of precipitates in alcoholic beverages has been shown by Refsgaard *et al*. (1996) to be related to the antioxidative properties of the flavonoids, as phenol oxidation initiates co-precipitation of the oxidized flavonoids with proteins and sugars.

The most obvious choice of conditions for making the ti kōuka spirit would therefore be the conditions of the above four alcohol treatments. From the experimental evidence, the best pH chosen for evaporating the ti kōuka extract was 10.0, and 65 hours evaporation gave the best results. It was also decided that ethanol alone would be the best option for infusing the ti kōuka extract. The process in developing the ti kōuka spirit is summarised in Figure 2.19.

To summarise, ti kōuka stems were ground, dried and stored until needed. An aqueous suspension of the dried ground stem was incubated with Fructozyme enzyme for 1 hour at 60°C, filtered, then simultaneously dried and caramelised for 65 hours at 60°C, at an optimum initial pH of 10.0. After drying the extract, it was reconstituted to its original volume with water. The preparations were then centrifuged at 3700 gravities for 15 minutes. The supernatant was infused with potable ethanol to give 80, 67, 57 and 50% v/v final ethanol concentrations - for 15 minutes. The alcohol treated ti kōuka extract was further vacuum filtered through GF/B glass micro-fibre paper. The aqueous yellow/brown extract of ti kōuka was then diluted with water to yield a final alcohol concentration i.e. 43% v/v – a typical strength of a premium spirit.

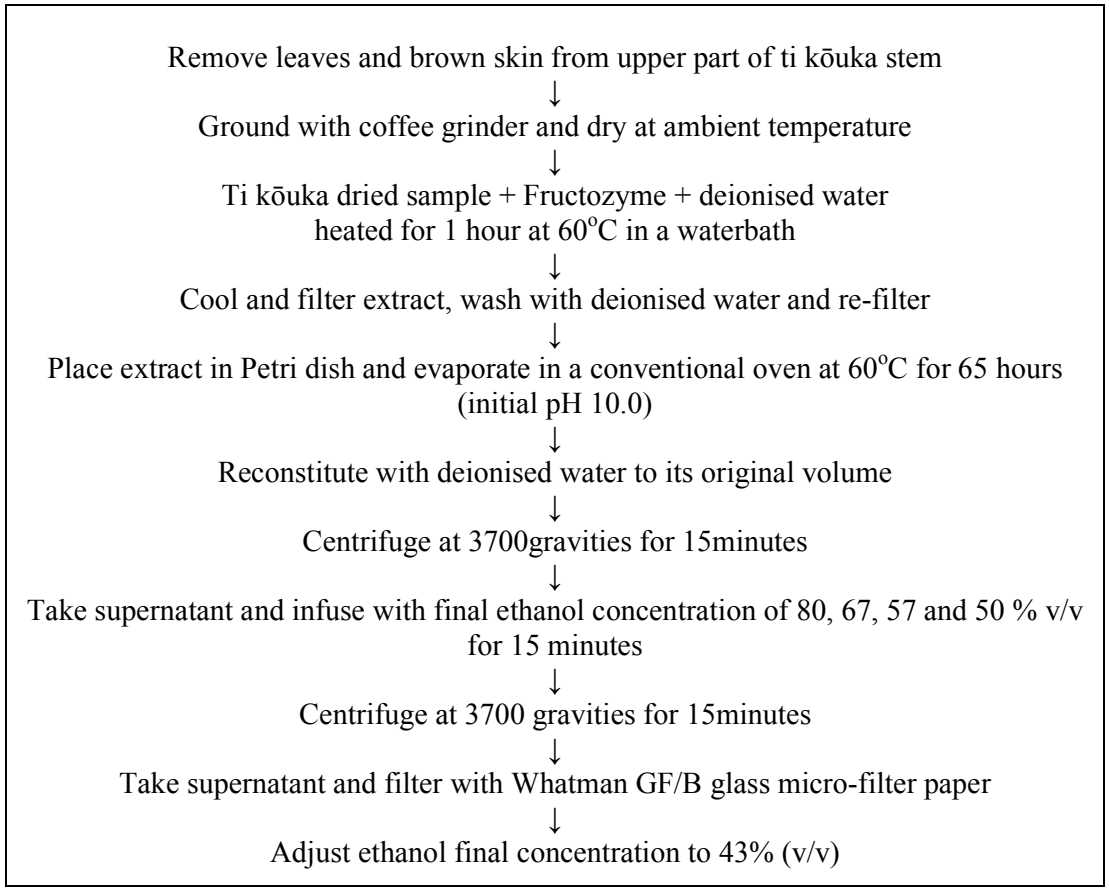


Figure 2.19 Diagrammatic scheme of ti kōuka spirit development

Having created the ti kōuka spirit, the aim of the next step was to analyse the sugars and amino acids in the ti kōuka stem extracts, using high performance liquid chromatography. This work is discussed in Chapter 3.

CHAPTER 3

Sugar, protein and amino acid analyses of the ti kōuka stem

In the previous chapter (Chapter 2), ti kōuka spirit was prepared by extracting the ti kōuka stem with Fructozyme and water followed by filtration, evaporation (promoting the Maillard reaction), reconstitution, centrifugation, and infusion with ethanol. The formation of yellow and brown colours, common indicators of intermediate and advanced Maillard products, were monitored by wavescans between 200 and 800 nm, as well as measurement in CIELAB colour space. For any reducing sugar/amino acid combination at defined pH and temperature, the ultraviolet and visible spectrum will have a distinctive absorption profile (MacDougall and Granov, 1998), as was seen from the results in the previous chapter. Obviously, reducing sugar(s) is reacting with amino acid(s) to produce Maillard reaction products that define the drink flavour.

3.1 Sugar analysis of the ti kōuka extract

The formation of Maillard reaction products is greatly affected by the source of reactants (i.e. carbonyls and amines) and the reactant conditions (Baisier and Labuza, 1992; Cammerer and Kroh, 1995; Wijewickreme *et al*., 1997b). It has been known for a long time that there is a big difference in the browning capacity of sugars (van Boekel, 2001). This can be attributed to the relative concentration of the open chain form of the various sugars. Sugars with a higher concentration of open chain form – which has the free and reactive carbonyl group – brown faster. In general, the rate of the Maillard reaction is as follows: pentose sugars (xylose or arabinose) > hexose sugars (glucose and fructose) > disaccharides (lactose or maltose) > trisaccharides > corn syrup solids > starch (Reineccius, 2006).

As discussed in Chapter 1, ti kōuka rhizome and stem are both storage organs for inulin, a polymer of fructose. Upon hydrolysis with Fructozyme, inulin is hydrolysed mainly to fructose (Section 2.6), which presumably is taking part in the Maillard reaction. Quantitative analysis of purported fructose in the Fructozyme-hydrolysed ti kōuka stem had been monitored by the PAHBAH method throughout the development stage of the spirit. Confirmation of the presence of any other sugar(s), for example glucose and/or sucrose that might be present in the hydrolysed ti kōuka stem was carried out here using high performance liquid chromatography (HPLC). For most of the HPLC analyses of

carbohydrates, the columns are packed with either cation-exchange resins (eluted with pure water or diluted mineral acid) or amino-modified silica gel (water and acetonitrile).

Method

After carrying out the procedure of grounding, drying, hydrolysis with Fructozyme, and filtering the ti kōuka stem, the extract was diluted 10 times in preparation for injection. Standards of 1% (w/v) sucrose (BDH AnalaR grade 102744B), fructose (Scientific Supplies SSC-R157-250), glucose (Sigma G-5000) and mannitol (BDH 443907) were prepared in Millipore water.

Separation was carried out using an ICI LC1100 pump system equipped with a Phenomonex Luna 100A amino column, 5 μm particle size, 4.6 mm (internal diameter) × 250 mm, mounted in a Shimadzu (Tokyo) HPLC system with an AJ4310 amino guard column kept in a thermostated jacket at 40°C, and a Shimadzu refractive index detector-(10A RI). The solvent was acetonitrile (Unichom HPLC Grade 2315-2.5L from Ajax Finechem) and MilliQ water (85:15 v/v) and the flow rate was 1.5 mL min^{-1}. Sample injection was via a Rheodyne 7725 valve with a 20 μL sample loop.

An identical aliquot (500 μL) of each standard, ti kōuka extract and the solvent were injected separately into the column system for analysis. Peaks were identified by reference to the retention times of the four standard sugars and solvent injected separately.

Results

Only two peaks were identified in the ti kōuka extract (chromatogram not shown), one being the solvent peak (acetonitrile and MilliQ water) and the other peak that had a retention time closest to fructose (Table 3.1). Retention times can be variable for any given analyte depending on a number of experiment variables not all of which can be controlled (Bidlingmeyer, 1992). Importantly, retention times can be matrix dependent; the ti kōuka extract matrix was much more complex than the sugar standards. The results indicate that the only significantly important sugar in the hydrolysed ti kōuka extract was fructose.

Table 3.1 Retention times of solvent, standard sugar solutions and ti kōuka extract

Standard/Sample	Retention time (minutes)
Solvent	1.32
1% (v/v) Fructose	3.68
1% (v/v) Glucose	5.01
1% (v/v) Sucrose	9.12
1% (v/v) Mannitol	14.53
Ti kōuka extract	3.43

3.2 Protein and amino acid analysis

The Maillard reaction is also dependent upon the amino acids present and their availability to take part in the Maillard reaction. Proteins are highly defined but complex polymers made up of 20 different amino acids. In the polymeric form, only the N-terminal amino group and five other amino acids (lysine, histidine, arginine, asparagine, and glutamine) can take part in the Maillard reaction, but proteolysis – which could occur during the drink preparation procedure – would obviously liberate more amino groups capable of reaction. At the most basic level, however, the protein content of any protein-containing matter should give some guide to the potential Maillard reactivity. Therefore, the next initial step was to determine the protein content in the ti kōuka stem.

3.2.1 Protein determination by Kjeldahl method

Various methods are available for measuring the protein content in biological samples. The most widely used method for the determination of protein is the Kjeldahl method, which is composed of three steps – digestion, distillation and titration. The method is based on two facts. First, protein is the only macronutrient in food containing significant quantities of nitrogen. Second, upon digestion with concentrated sulphuric acid and catalysts, all nitrogen in the sample is quantitatively converted to ammonia as the non-volatile ammonium ion (NH_4^+). After digestion, the mixture is made strongly alkaline generating the volatile NH_3 that is (in one common method) collected into an accurately known volume and concentration of dilute sulphuric acid containing the indicator methyl red. Critically the moles of acid must be greater than the moles of NH_3. An alkali titration is then used to determine the moles (as mass) of NH_3 collected.

A factor of around 6.25 is used to estimate the mass of protein from which the NH_3 was derived.

Method

For the materials and method for the Kjeldahl analysis refer to Appendix VI. Bovine serum albumin (Sigma A-9647) was used as a protein control. Six ti kōuka samples (five of which were previously used in Chapter 2) and the sixth ti kōuka stem, collected in April (2007), were used. This last sample had come from stem that had recently fruited (Figure 3.1).

Figure 3.1 Ti kōuka stem when fruited

Samples of approximately 0.5 g of dried ti kōuka and BSA were weighed accurately into Kjeldahl digestion tubes. Next, 10 mL of concentrated sulphuric acid (nitrogen free) and 3 g catalyst was digested at 400°C in a fume hood until the entire sample in the tubes were digested completely and the solution turned bright green. The tubes were cooled to room temperature, and 20 mL of deionised water was added to the digested sample, followed by 10 mL of 35% sodium hydroxide. The steam distillate was trapped in the collecting Erlenmeyer flask containing 10 mL of 0.05 M sulphuric acid and 3 drops of methyl red indicator. Approximately 150 mL of distillate was collected. The excess sulphuric acid was titrated with 0.05 M NaOH to the colourless endpoint.

Results

An estimate of the protein concentration of a food is obtained by multiplying its Kjeldahl nitrogen content by multiplying the nitrogen-to-protein conversion factor. The value of 6.25 is applicable for most plant and animal proteins, on the assumption that on

average their proteins contain approximately 16% nitrogen. However, nitrogen-to-protein conversion factors vary from one food to another. For example, the factor for rice is 5.95 while that of milk is 6.38. At this stage, the conversion value of 6.25 was used to calculate the protein concentration in the ti kōuka stem.

The results are tabulated in Table 3.2. The percent protein varied in the different months, being lowest in January for these particular samples. It was apparent from the results that the concentration of protein as a fraction of dry weight decreased with maturation, essentially confirming that the shoot tips are the most useful part of the stem generating Maillard reaction products.

Table 3.2 Protein content in selected ti kōuka stem and in a control protein BSA by the Kjeldahl method

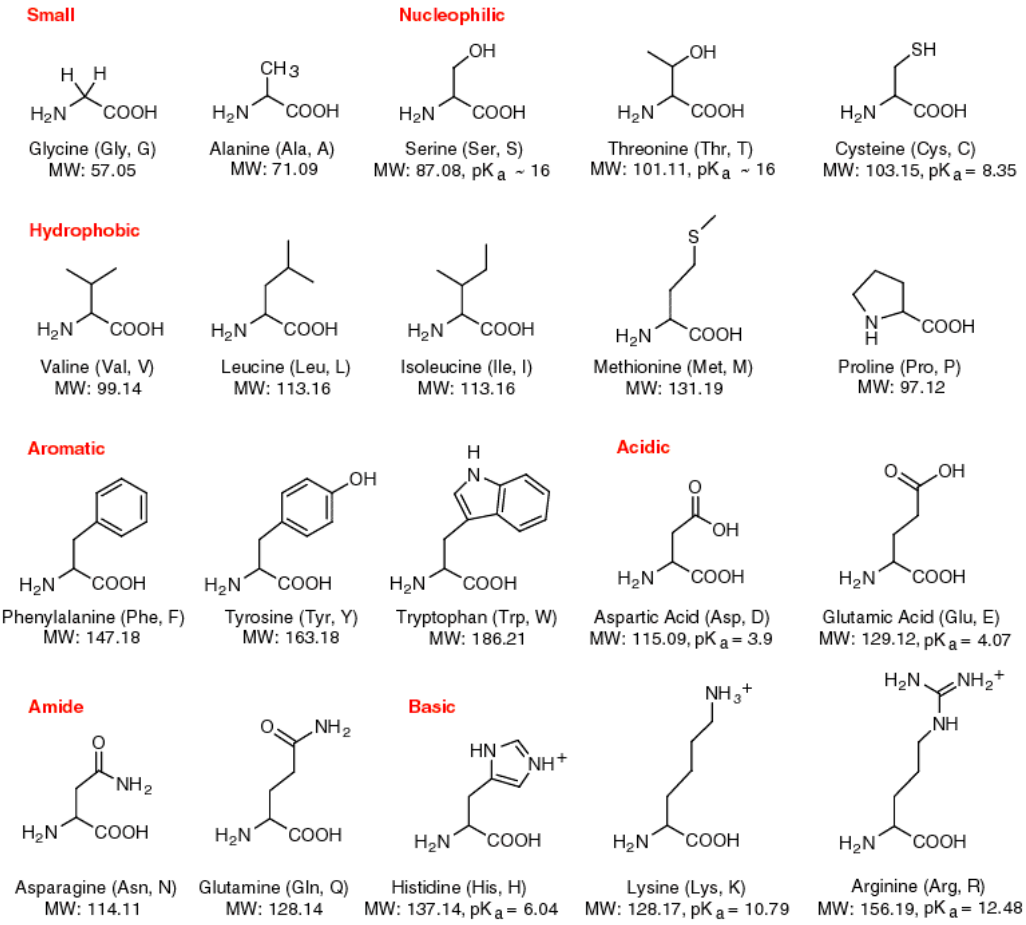
Section of ti kōuka stem/Month of collection	Nitrogen content (% dry weight)	Protein content (% dry weight)
Top section (tip to 20 cm) collected in January	0.48	3.0
Top section (tip to 20 cm) collected in February	2.64	16.5
Second section (20 to 40 cm) collected in February	1.89	11.8
Third section (40 to 60 cm) collected in February	1.10	6.9
Top section (tip to 20 cm) collected in April	2.20	13.8
Top section (tip to 20 cm) collected in August	1.70	10.6
Bovine serum albumin (BSA)	13.40	83.8

The protein content of the BSA was calculated to be 83.8% assuming the 6.25 conversion factor. The claimed content of the BSA was 97%, and assuming all that is protein the conversion factor should have been (6.25 x 97)/83.8 = 7.23. Similarly, the conversion factor for ti kōuka may not be 6.25. By knowing the percent of amino acid content in a protein or protein mixture and the nitrogen content of each amino acid (which is known exactly), it is possible to calculate the correct nitrogen-to-protein conversion factor. Therefore, to obtain accurate conversion factor for the ti kōuka stems and BSA, the amino acid profiles had to be obtained from HPLC analysis.

The subject of the following sections is the determination of the amino acid contents in different parts of the ti kōuka stem and stems collected at different times of the year.

3.3 Amino acid analysis of the ti kōuka stems

In nature there are 20 amino acids recognized by the genetic code (Fennema, 1996), and these dominate amino acid profiles in all proteins. Proteins are made by linking amino acids in peptide bonds, a special case of the more general amide bond. The peptide bond is formed in a condensation reaction between the amino group (–NH2) of one amino acid and the carboxyl group (–COOH) of any other amino acid. Proteins, which can include hundreds of peptide bonds, are thus amino acid polymers linked in a systematic way defined by the genetic code. As a result, they have different molecular structures, nutritional attributes and physiochemical properties. Figure 3.2 shows the structure of the common amino acids.



Each amino acid is accompanied by its three- and one-letter code, residue molecular weight (actual weight minus one equivalent of water i.e. 18 g mol^{-1}) and side chain pK_a, where appropriate (www.neb.com)

Figure 3.2 The structure of common amino acids

The general formula for the so-called α-amino acids is $H_2NCHRCOOH$ where the R group in the formula stands for the functional portion of the amino acid. They are also known as side chains. Thus, α-amino acids vary only in the composition of their R groups (with the exception of proline).

Analysis of amino acids involves four steps, namely hydrolysis, derivatisation, separation of derivatised amino acids, and data interpretation and calculation.

Prior to analysis of the amino acid in proteins, the proteins have to be completely hydrolysed to yield free amino acids. Acids, alkalis or enzymes may be used for this; however, acid hydrolysis is the most common procedure. Acid hydrolysis can be performed by heating the sample to be analysed in the presence of high concentrations of acids using thermal or microwave radiation energy (Weiss *et al*., 1998).

The conditions used to hydrolyse proteins with hydrochloric acid (HCl) are vital in determining amino acid compositions because they necessarily represent a compromise aimed at yielding the best estimate of amino acid composition. Variations in ease of peptide bond cleavage and differences in amino acid stabilities prohibits a single method of hydrolysis that will quantitatively hydrolyse every peptide bond and at the same time cause no destruction of any amino acid (White and Hart, 1992).

Different hydrolysis methods can have varying effects on individual amino acids. In acid hydrolysis for example, aspargine and glutamine are converted to aspartic (Asp) and glutamic (Glu) acids respectively, so the proportions of the amides and acids cannot be known and are therefore defined as Asx and Glx. Tryptophan is unstable under acid condition (Belitz *et al*., 2004) and is destroyed by acid hydrolysis (Krishnamurti *et al*., 1984); therefore, tryptophan peaks were not detected. Methionine and cysteine are partially oxidised during acid hydrolysis, so complicating the resulting profile. However, hydrolysis with HCl is widely practiced, because it generally yields over 95 % recoveries for the following amino acids: aspartic and glutamic acids (amide plus acid), glycine, alanine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine (White and Hart, 1992). Amino acids such as isoleucine, leucine and valine are released slowly during hydrolysis because peptide bonds involving these residues are more resistant to hydrolysis.

Two acid hydrolysis methods were attempted in this study; however, both methods had some drawbacks. The performic acid hydrolysis oxidizes the sulphur containing amino acid cystine and methionine to yield their more stable forms i.e. cysteic acid and methionine sulfone respectively, however destroying tyrosine. The hydrochloric acid hydrolysis method partially oxidises methionine. However, by performing hydrolysis under an atmosphere of nitrogen gas can reduce the degree of oxidative destruction. In both methods, tryptophan is completely destroyed by acid hydrolysis. Phenylalanine, histidine and arginine are labile amino acids. Aspartic acid, alanine and leucine on the other hand are both stable to acid hydrolysis and are easily hydrolysable.

3.3.1 Performic acid oxidation with acid hydrolysis (Association of Analytical Communities 16th Edition, 1995 Official Method 994.12, Alternative I)

In this method cysteine (and its cross-linked form cystine) and methionine are oxidised by performic acid to cysteine sulfonic acid and methionine sulphone, respectively (Jingquan *et al*., 2005). Sodium metabisulphite is then added to decompose performic acid. Amino acids are subsequently liberated from protein by heat-assisted hydrolysis in 6 M HCl. The pH of the hydrolysate is adjusted to ~2 to 3 and the individual amino acids are separated by high performance liquid chromatography (HPLC).

Method

For the materials and preparation of the reagents, refer to Appendix VII. A dried ti kōuka sample (0.05 g) was accurately weighed into a 10 mL test tube. The sample tube was placed in an ice bath (0°C) over a magnetic stirrer. After addition of 5 mL of performic acid reagent to the sample, the tube was capped and gently stirred. After 16 hours, the sample was removed from the ice bath and 1.5 mL of 1M sodium metabisulphite (Univar A487-500g) solution was added to the tube to decompose performic acid. The solution was further stirred for 15 minutes to liberate sulphur dioxide gas. To the approximate 6.5 mL of sample mixture was added 3 mL of 6 M HCl-phenol solution. The sample was reflux digested at 110°C for 24 hours after which the tube were cooled and the pH was adjusted to between 2.0 and 3.0. The sample was filtered with a 0.4 μm syringe filter. This method proved to be very time consuming, therefore a faster method had to be found. The acid hydrolysis method with flushing nitrogen gas in the sample mixture (i.e. giving some oxidation protection) (AOAC Official Method 994.12 – Alternative III) was followed.

3.3.2 Acid hydrolysis method (AOAC Official Method 994.12 Alternative III)

In this method, amino acids are liberated from protein by hydrolysis in 6M HCl-phenol solution.

Method

A dry 0.08 g sample of ground ti kōuka stem was weighed in an acid-washed and oven dried 15 mL boiling tube. Three milliliters of deionised water and 3 mL of 12 M HCl containing 0.02% phenol was added, yielding a final acid concentration of 6 M. The tube was flushed with nitrogen (to minimise oxidation) and sealed immediately before digestion at 110°C for 18 to 24 hours in a fume hood. After cooling to room temperature, the pH of the mixture was adjusted to pH 7.0 ± 0.20 with 6 M sodium hydroxide solution, and filtered through a 0.45μm filter. The filtrate was distributed into 3 mL aliquots that were stored at –20°C until required. A control hydrolysis was carried out with 0.052 g of bovine serum albumin (Sigma A-9647).

3.3.3 Derivatization of amino acids

Most amino acids exhibit neither ultraviolet light absorption nor fluorescence, therefore they cannot be easily detected by liquid chromatographic profiles unless they have been derivatised to a detectable form. For detection at trace levels, fluorescence derivatisation is commonly used. Many HPLC methods involving pre- or post-column derivatisation techniques have been described for amino acid quantitation (Molnar-Perl, 2003). Although the use of a number of different types of fluorescent labeling reagents, such as o-phthalaldehyde (OPA), 4-chloro-7-nitro-2,1,3-benzoxadiazole, 9-fluorenylmethyl chloroformate, phenylisothiocyanate and other less common reagents has been described, each of these methods has advantages and disadvantages.

OPA is probably the most commonly derivatisation reagent used in reverse-phase HPLC for the determination of free amino acids (Molnar-Perl, 2001; Dorresteijn, *et al*., 1996). OPA, which reacts with the liberated primary amino groups, may be applied as either a pre-column or post-column derivatising agent (Molnar-Perl, 2001). The technique has been reported to be 500 times more sensitive than the tradition ninhydrin colorimetric method (Fisher *et al*., 2001), and that technique can only be applied post-column. The reaction between OPA and primary amino acids takes place in the presence of reducing agent such as 2-mercaptoethanol to give a complex which can be

measured by fluorescence. OPA, however, does not react with secondary amines (e.g. proline) under these reductive conditions. Therefore, proline peaks were not detected.

3.3.4 Separation of derivatized amino acids by High Performance Liquid Chromatography (HPLC)

The analysis of amino acids has traditionally been performed using commercial instrumentation based on the technology developed by Moore and Stein (1951), in which amino acids with free amino groups are separated by cation exchange chromatography, reacted with ninhydrin by a post-column derivatisation procedure, and detected by absorbance in the visible region at one or two wavelengths. This method is reliable, with usually excellent resolution. However analysis times are long, sensitivity is limited, peak broadening can occur, and the post column derivatisation procedure is difficult to apply consistently (White and Hart, 1992). A more powerful technique applies a pre-column derivatisation procedure followed by the separation of the amino acid derivatives by reverse-phase chromatography (RP-HPLC).

For this study the OPA pre-column derivatisation technique was chosen, because it has several advantages, such as a relatively simple derivatisation procedure and high sensitivity (picomolar range) when compared with other techniques like 9-fluorenylmethyl chloroformate (Yunwei *et al*., 2006) and pheylisothiocyanate-derivatisation (Paramás *et al*., 2006). Paramás *et al*. (2006) method for the column resolution of derivatised amino acids was followed here.

3.3.5 Equipment for amino acid analysis

Amino acids were resolved and quantified with a Shimadzu HPLC system, which consisted of a SIL-10A auto injector, with sample cooling system, a FCV-10A four way liquid mixer system, a DGU-2A degassing system using helium, a LC-10AD pump and a RF-10A fluorescence detector. Fluorometric detection was carried out using excitation and emission wavelengths of 340 nm and 426 nm respectively. The data collecting system consisted of CBM 10A (Communications Bus Module) unit with data processing function coupled with Shimadzu Class LC-10 analyser.

3.3.6 Separation column, mobile phase and elution conditions

Separation was carried out at ambient temperature in a Waters Nova-Pak® (Ireland) reverse phase C18 column, 4 μm particle size, 3.9 mm (internal diameter) x 150 mm.

A wide variety of solvent systems are used in HPLC analyses, usually containing water, ethanol, methanol, acetonitrile, and less frequently tetrahydofuran as the organic component of the mobile phase (White and Hart, 1992). In this study, the solvents used were ethanol (99.7% HPLC Reagent Grade, Scharlau ET 0015-2500), methanol (Chromasolv 34860) and tetrahydrofuran (BDH 1.08114.2500). Separation of the derivatised amino acids was carried out in a gradient mode comprising Eluent A, sodium phosphate buffer (10 mM, pH 7.3): methanol: tetrahydrofuran (80:19:10 v/v), and Eluent B comprising sodium phosphate buffer: methanol (20:80 v/v). All solvents were degassed before use. The elution gradient applied is shown in Table 3.3.

Table 3.3 Elution gradient for the separation of derivatized amino acids

Step	Time (min)	Flow rate (mL min^{-1})	Eluent A (% v/v)	Eluent B (% v/v)
1	0.01	0.5	100	0
2	3.00	0.5	100	0
3	3.01	1.0	100	0
4	5.00	1.5	100	0
5	14.50	1.5	85	15
6	24.50	1.5	70	30
7	29.50	1.5	60	40
8	45.00	1.5	30	70
9	50.00	1.5	20	80
10	50.01	0.5	100	0
11	70.01	STOP		

It took 50 minutes for a complete run and an additional 20 minutes period for the system to return to initial condition, 100% Eluent A.

3.3.7 Preparation of OPA derivative

OPA was prepared in a 25 mL volumetric flask by dissolving 500 mg of reagent OPA (Acros Product 13108) in 22.5 mL of 99.7% v/v ethanol (99.7% HPLC reagent grade, Scharlau ET 0015-2500) made up to 25 mL with 0.4 M borate buffer, pH 10.0. (Borate buffer was prepared by dissolving 24.73 g boric acid (Scharlau AC-0578) in 1 L of Millipore water and adjusting the pH to 10.0 with potassium hydroxide pellets.) After mixing well, 400 µL of 2-mercaptoethanol (Fluka 63700) was added and mixed well. The mixture was stored in the dark at 4°C.

3.3.8 Internal standard preparation

The internal standard was 3,5-dibromotyrosine (Aldrich S448933). It was chosen because it does not occur in proteins and because it can be resolved chromatographically from other amino acids. A stock solution was prepared by dissolving 118 mg of 3,5-dibromotyrosine in 1322 µL of Millipore water. A working solution was prepared by diluting the stock 100-fold (with a final concentration of 0.01 mM in test sample).

3.3.9 Amino acid standards preparation

Amino acids were purchased from Sigma, BDH and Merck (Appendix VIII). Standards of each amino acid (0.01 M) were prepared in Millipore water and filtered using 0.4 µm syringe filter. The 20 individual amino acid standards were analysed by HPLC three times, to get an average retention time for each. After obtaining the retention time, the reference concentrations were adjusted in an attempt to yield similar peak areas for all amino acids (Table 3.4).

Table 3.4 Adjusted weights of amino acids in standard solution mixture

Amino acid	Empirical formula	Adjusted weight (g) of amino acids in 100 mL MilliQ water (standard)	Molarities (mM) of amino acids in standard solution
Aspartic acid (Asp)	$C_4H_7NO_4$	0.119	8.33
Glutamic acid (Glu)	$C_5H_9NO_4$	0.055	3.71
Aspargine (Asn)	$C_4H_8N_2O_3$	0.202	15.31
Serine (Ser)	$C_3H_7NO_3$	0.079	7.52
Glutamine (Gln)	$C_5H_{10}N_2O_3$	0.022	1.49
Histidine (His)	$C_6H_9N_3O_2$	0.292	13.93
Glycine (Gly)	$C_2H_5NO_2$	0.047	6.30
Threonine (Thr)	$C_4H_9NO_3$	0.156	13.12
Alanine (Ala)	$C_3H_7NO_2$	0.014	1.58
Taurine (Tau)	$C_2H_7NO_3S$	0.038	3.03
Arginine (Arg)	$C_6H_{14}N_4O_2$	0.076	4.38
γ-Amino butyric acid (GABA)	$C_4H_9NO_2$	0.330	32.01
Tyrosine (Tyr)	$C_9H_{11}NO_3$	0.025	1.38
Methionine (Met)	$C_5H_{11}NO_2S$	0.116	7.75
Valine (Val)	$C_5H_{11}NO_2$	0.185	15.80
Phenylalanine (Phe)	$C_9H_{11}NO_2$	0.071	4.30
Isoleucine (Ileu)	$C_6H_{13}NO_2$	0.176	13.41
Leucine (Leu)	$C_6H_{13}NO_2$	0.369	28.12
Ornithine (Orn)	$C_5H_{12}N_2O_2$	0.186	11.04
Lysine (Lys)	$C_6H_{14}N_2O_2$	0.072	3.96

One standard amino acid mixture was prepared using the adjusted weights of the 20 individual amino acids in a final volume of 100 mL. The subsequently filtered standard was derivatised and analysed at different dilutions as described below.

3.3.10 OPA derivatisation of standard amino acid solutions, ti kōuka and BSA acid hydrolates

Method

Kutlan and Molnar-Perl (2001) found that the stability and reproducibility of reaction can be improved by performing the reactions with refrigerated stock solution and maintaining that temperature before injecting the sample (preferably at 4°C), and by injecting the analysis of derivatives at the derivatisation pH.

Fisher *et al*. (2001) carried out a study to determine the time of development of the fluorescence and the time at which maximum fluorescence occurs with OPA reagents. They found that the maximum fluorescence is reached within 1 to 2 minutes after mixing the sample with the reagent, and that the fluorescence is stable for up to about 30 minutes. To minimise fluctuations in fluorescence response due to the differential stability of the various fluorescence OPA amino acid derivatives, the time between derivatisation and injecting the sample was kept constant. Samples were injected 5 minutes after derivatisation.

The above refinements were incorporated into the method used: 500 µL of standard amino acid solution was added to a 2 mL screw thread vial, to which 50 µL of working solution internal standard and 100 µL of derivatised OPA mixture was added. The solution was shaken and allowed to stand for 5 minutes in the autoinjector's cooling system. Finally, 5 µL of the derivatised sample solution was directly autoinjected into the column system for analysis. Hydrolysed and neutralised ti kōuka extracts (diluted as required) and the BSA control was prepared for injection by replacing the volume of standard amino acid solution with the relevant solution.

3.3.11 HPLC chromatogram analysis — elution order and regression equations

The elution order of amino acids is determined by complex interactions between specific ions in the eluent and the OPA derivatives (Krishnamurti *et al*., 1984). For each amino acid, the peak area relative to the area of the internal standard was used to calculate the amino acid concentrations. Since a known amount of internal standard

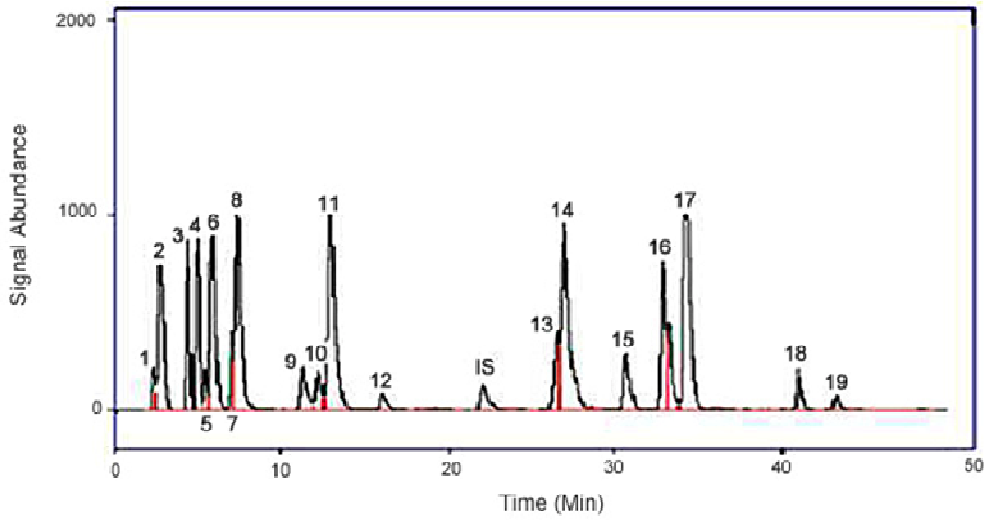
was added to each derivatised sample, the amount of each amino acid present in the sample can be calculated.

Method

Peaks were identified by reference to the retention times of the 20 standard amino acids injected separately. For the quantitation of the amino acids, peak areas (relative to the area of internal standard) were measured and regression equations developed from the fluorescence response to concentration curve of each derivatised individual standard amino acid.

Results

An elution profile of the amino acids in the standard amino acid mixture is shown in Figure 3.3. In general, the elution order and relative retention times obtained in the standard amino acid solution in this study were comparable to those reported by Paramás *et al*. (2006), who had used similar chromatographic equipment and pre-column OPA derivatisation. However, the notable difference from the results of Paramás *et al*. (2006) was that the elution of alanine co-eluted with taurine in the samples and control under the conditions of this study.



1=Aspartic acid, 2=Glutamic acid, 3= Aspargine, 4=Serine, 5= Glutamine, 6= Histidine, 7=Glycine, 8=Threonine, 9=Alanine + Taurine, 10=Arginine, 11= γ-amino butyric acid (GABA), 12=Tyrosine, IS= Internal Standard, 13=Methionine, 14=Valine, 15=Phenylalanine, 16=Isoleucine, 17=Leucine, 18=Ornithine, 19=Lysine

Figure 3.3 HPLC chromatogram of standard amino acids derivatised with OPA in the presence of mercaptoethanol. The vertical axis is signal abundance in arbitrary units.

The regression equation and coefficient determinations of the standard amino acid are tabulated in Table 3.5. The regression equations are in the form y = mx + b, where y is the peak area, m is the slope, x is the amino acid concentration and b is the y intercept, always zero by definition. Most of the amino acids were found to yield good linear responses with the square correlation coefficient (r^2) always greater than 0.850. These regression equations were used to quantify the amino acid profile in ti kōuka samples and a control protein.

Table 3.5 Retention times, regression equations and correlation coefficients derived from the standard amino acid mixture (n=15)

Amino acid	Retention time (minutes)	Regression equation	Coefficient of determination (r^2)
Aspartic acid (Asp)	3.0 ± 0.2	y = 0.013x	0.985
Glutamic acid (Glu)	3.4 ± 0.2	y = 0.022x	0.971
Aspargine (Asn)	5.5 ± 0.3	y = 0.033x	0.969
Serine (Ser)	6.6 ± 0.3	y = 0.029x	0.985
Glutamine (Gln)	7.9 ± 0.4	y = 0.003x	0.914
Histidine (His)	10.3 ± 0.5	y = 0.206x	0.931
Glycine (Gly)	11.0 ± 0.5	y = 0.014x	0.990
Threonine (Thr)	12.8 ± 0.6	y = 0.079x	0.974
Arginine (Arg)	14.8 ± 0.6	y = 0.170x	0.856
Alanine (Ala)	15.9 ± 0.6	y = 0.018x	0.980
Taurine (Tau)	17.0 ± 0.7	y = 0.009x	0.957
γ-amino butyric acid (GABA)	18.1 ± 1.1	y = 0.081x	0.928
Tyrosine (Tyr)	20.7 ± 0.7	y = 0.026x	0.998
Internal standard (IS)	29.6 ± 0.7		
Methionine (Met)	31.1 ± 0.8	y = 0.010x	0.966
Valine (Val)	33.5 ± 0.6	y = 0.092x	0.989
Phenylalanine (Phe)	36.0 ± 0.8	y = 0.008x	0.924
Isoleucine (Ileu)	37.5 ± 1.0	y = 0.008x	0.896
Leucine (Leu)	42.6 ± 1.1	y = 0.943x	0.948
Ornithine (Orn)	46.7 ± 0.6	y = 0.109x	0.862
Lysine (Lys)	49.9 ± 0.7	y = 0.256x	0.919

Before analysis of ti kōuka samples for amino acid content it was prudent to test the quantitation protocol on the control protein, bovine serum albumin (BSA). As noted earlier the method chosen for this subsequent work with ti kōuka was the AOAC Official Method 994.12 Alternative III (Section 3.1.2).

3. 3.12 Analysis of the reference protein, BSA

When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified BSA (Sigma A-9647 Fraction V) was used as a protein standard.

Method

The BSA was acid hydrolysed, derivitized with OPA and analysed using HPLC (as described in sections 3.3.2 to 3.3.10).

Results

The eluted amino acids obtained for BSA in this study was in close agreement with the National Institute of Standards and Technology (www.abrf.org). The calculated percentage contents of amino acids from hydrolysed BSA control are presented in Table 3.6.

Table 3.6 Percent amino acid values for the reference protein, BSA

Amino acid	BSA (% of total amino acids)	BSA literature values* (% of total amino acids)
Aspartic acid + asparagine (Asx)	8.90	nd
Aspartic acid	nd	10.54
Glutamic acid + glutamine (Glx)	13.01	nd
Glutamic acid	nd	15.17
Serine	5.27	4.47
Histidine	2.80	3.00
Glycine	2.80	2.87
Threonine	5.77	5.98
Alanine + taurine	7.74	nd
Alanine	nd	8.58
Arginine	4.28	4.26
γ-amino butyric acid (GABA)	0.00	0.00
Tyrosine	3.46	3.23
Methionine	0.82	0.74
Valine	6.26	6.51
Phenylalanine	4.94	5.00
Isoleucine	2.47	2.49
Leucine	10.71	11.13
Ornithine	0.00	1.00
Lysine	9.88	11.00
Proline	4.61	5.04

*Values reported by Cohen and Michaud, 1993
nd = not determined

The results obtained were in good agreement with previously reported results by Cohen and Michaud (1993). The percentage values from literature for aspartic acid (10.54), glutamic acid (15.17) and alanine (8.58) were higher than the maximum possible values determined in this study (8.90, 13.01 and 7.74 respectively). One possible reason being that in this study, the amino acids were derivatized with OPA, while Cohen and Michaud used 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as their derivatising agent. The other possible reason is that the separation procedure used by Cohen and Michaud was different. Their column was maintained at 37°C, the mobile phases were different, and gradient conditions were different.

Nonetheless, the results presented above showed that the analysis method was robust, and was ready to apply to ti kōuka sample. The amino acid profile of BSA achieved in this section can be used to calculate the nitrogen-to-protein conversion factor (Section 3.4).

3.4 Analysis of amino acids concentrations at different times of the year

Both amino acids and reducing sugars take part in the Maillard reaction. Different amino acids have different browning effect on the reducing sugars. Therefore, the next step was to find if there were any seasonal variations in the amino acid profile in ti kōuka stem which could play a part in the production of distinctive flavours in ti kōuka spirit. This immediately raised the issue of biological variability, in that variations might be due to genotype and to developmental and environmental factors. Harris *et al*. (1998) confirmed the anecdotal perception that *Cordyline australis* trees in different parts of New Zealand differ in their phenotype. Harris *et al*. (2003) also demonstrated that phenotypic differences between populations under differ environmental conditions are indicative of population genotype interactions with environmental factors. The only way to overcome this issue would be through a systematic sampling procedure of many samples from the same plants. At this stage of development, it was decided simply to see what sort of variability might be present in samples collected at different times of the year. However, three sections of a single plant were also analysed which will eliminate variation due to genotype and environment. In all cases, the aim was to look for big differences that might be significant.

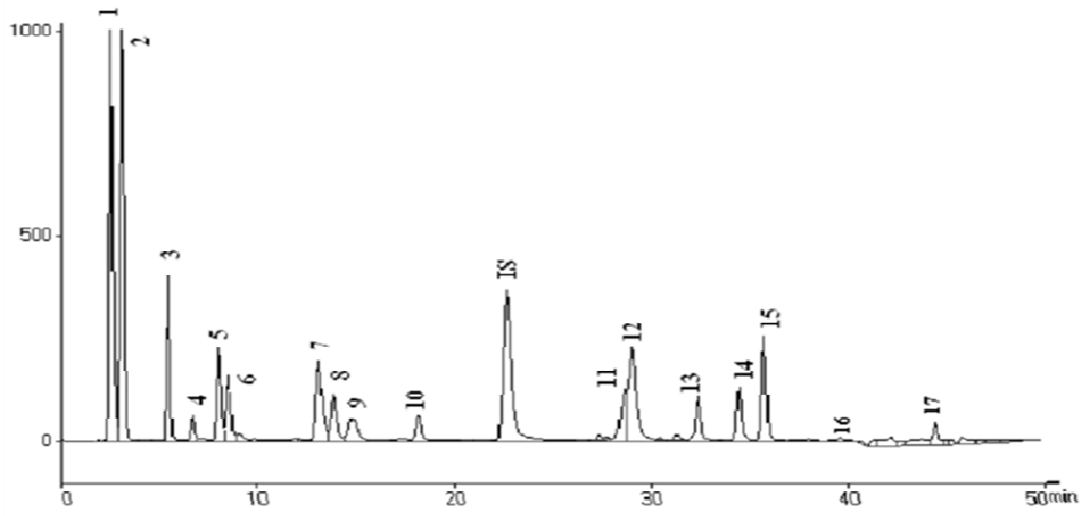
Method

Six ti kōuka samples (previously analysed in the Kjeldahl method) were used. The dried ground ti kōuka stems were acid hydrolysed, derivatised with OPA and analysed for amino acid using HPLC (as described in Sections 3.3.2 to 3.3.10).

Results

An example of an elution profile of the amino acids in the top section of a ti kōuka stem (collected in February) is shown in Figure 3.4.

The calculated percentage contents of amino acids from hydrolysed ti kōuka stem collected at different times of the year are shown in Table 3.8. The percentage of each amino acid contents in the sample was multiplied by the percentage of nitrogen content of each amino acid (from textbook) and then calculated as the inverse of the multiple (Table 3.9) to give the nitrogen-to-protein conversion factor.



1=Aspartic acid + Aspargine (Asx), 2=Glutamic acid + Glutamine (Glx), 3=Serine, 4= histidine, 5=Glycine, 6=Threonine, 7=Arginine, 8=Alanine + Taurine, 9=GABA (γ-amino butyric acid), 10=Tyrosine, IS= Internal Standard, 11=Methionine, 12=Valine, 13=Phenylalanine, 14=Isoleucine, 15=Leucine, 16=Ornithine, 17=Lysine (The vertical axis is signal abundance in arbitrary units.)

Figure 3.4 HPLC chromatogram of top section of ti kōuka stem derivatized with OPA in the presence of mercaptoethanol.

The nitrogen-to-protein conversion factor for BSA was 7.663 by HPLC (Table 3.8) compared to 7.46 by Kjeldahl method (Section 3.2.1) showing that the method for calculating the nitrogen factor by HPLC method is feasible and reliable. The difference between the two values may be due to the fact that the Kjeldahl procedure is able to

digest the protein fully and release nitrogen whereas 6 M HCl hydrolysis does not completely digest the protein (Dintzis *et al*., 1988). The nitrogen-to-protein conversion factors of the ti kōuka stem collected at different times of the year ranged from 6.42 to 6.74 (highlighted grey in Table 3.8). The three different sections of the stem had similar values with a slight increase with maturation (Table 3.8).

Subject to the issue of variability being due to causes other than season, the relative concentrations of Asx, serine, glycine, alanine + taurine, tyrosine, phenylalanine and isoleucine were higher in the ti kōuka stem collected in January compared to the other months (Table 3.7). In contrast, lysine was markedly lower. However, the data from within one plant sampled in February 2007 (Table 3.7) revealed concentration differences due only to maturity. Within one plant sampled in February 2007, the relative concentrations of valine and lysine were highest in the top section of the ti kōuka stem, while those for Asx, Glx, serine, threonine, methionine and leucine were highest in the third and most mature section. Overall, the relative concentrations of Asx, serine, threonine, alanine + taurine and leucine clearly increased with maturity. On the other hand, the relative concentrations of GABA and lysine decreased with maturity (Figure 3.5).

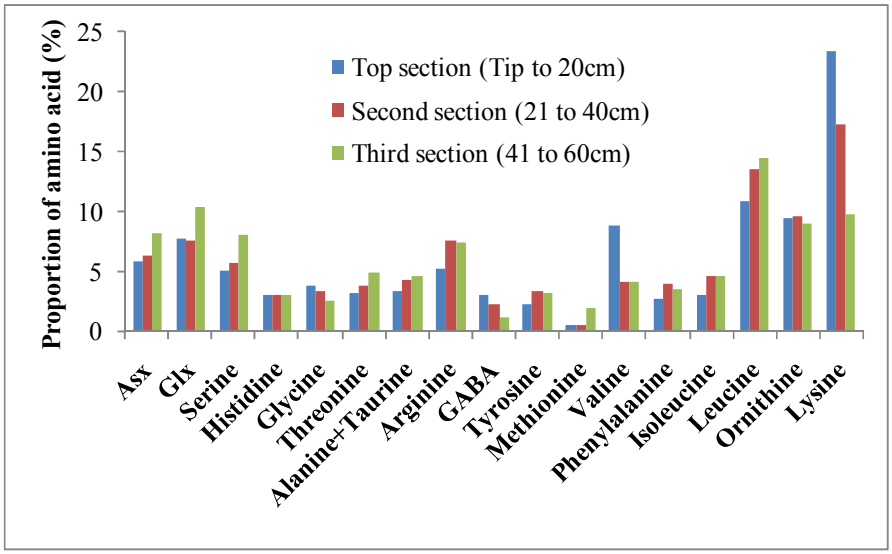


Figure 3.5 Comparison of amino acids in different parts of the stem

Table 3.7 Total percentage of amino acids in ti kōuka samples collected at different times of the year

Amino Acid	January total % of amino acid	February Top section (tip to 20cm) total % of amino acid	February Second section (20 to 40cm) total % of amino acid	February Third section (40 to 60cm) total % of amino acid	April total % of amino acid	August total % of amino acid
Aspartic acid + Aspargine (Asx)	8.86	5.76	6.30	8.17	7.63	4.35
Glutamic acid + Glutamine (Glx)	8.37	7.61	7.52	10.36	7.54	5.51
Serine (Ser)	13.03	4.98	5.70	8.05	6. 98	9.27
Histidine (His)	7.46	2.97	3.00	2.93	5.06	11.29
Glycine (Gly)	5.26	3.68	3.26	2.48	4.48	1.26
Threonine (Thr)	2.97	3.09	3.78	4.91	5.27	0.49
Alanine + Taurine	4.56	3.23	4.27	4.59	4.41	2.74
Arginine (Arg)	13.41	5.11	7.47	7.33	16.31	11.15
γ-Amino butyric acid (GABA)	4.40	2.92	2.23	1.08	3.02	6.55
Tyrosine (Tyr)	5.02	2.24	3.36	3.14	2.99	2.74
Methionine (Met)	0.70	0.42	0.43	1.88	1.65	1.07
Valine (Val)	6.51	8.74	4.07	4.13	4.10	4.38
Phenylalanine (Phe)	5.73	2.68	3.92	3.49	3.11	3.50
Isoleucine (Ileu)	4.55	3.00	4.50	4.46	3.50	3.33
Leucine (Leu)	4.99	10.81	13.45	14.38	13.92	14.79
Ornithine (Orn)	1.67	9.44	9.57	8.89	1.37	1.46
Lysine (Lys)	2.51	23.32	17.19	9.74	10.18	16.12

Table 3.8 Percentage of nitrogen content and conversion factor of ti kōuka samples collected at different times of the year

Amino Acid	% N in Standard amino acid by weight*	Total amount of nitrogen in ti kōuka sample collected in January	Total amount of nitrogen in top section of ti kōuka sample collected in February	Total amount of nitrogen in second section of ti kōuka sample collected in February	Total amount of nitrogen in third section of ti kōuka sample collected in February	Total amount of nitrogen in ti kōuka stem collected in April	Total amount of nitrogen in ti kōuka sample collected in August	Total amount of nitrogen in BSA
Aspartic acid + Aspargine (Asx)	10.52	0.0093	0.0060	0.0066	0.0086	0.0060	0.0046	0.0094
Glutamic acid + Glutamine (Glx)	9.58	0.0080	0.0073	0.0072	0.0099	0.0072	0.0053	0.0125
Serine (Ser)	16.09	0.0210	0.0080	0.0092	0.0130	0.0123	0.0149	0.0081
Histidine (His)	27.07	0.0202	0.0077	0.0081	0.0079	0.0155	0.0306	0.0076
Glycine (Gly)	18.65	0.0098	0.0068	0.0061	0.0046	0.0075	0.0024	0.0052
Threonine (Thr)	11.72	0.0035	0.0037	0.0044	0.0058	0.0066	0.0006	0.0068
Arginine (Arg)	32.15	0.0211	0.0081	0.0117	0.0115	0.0258	0.0175	0.0138
Alanine + Taurine	15.71	0.0146	0.0103	0.0137	0.0148	0.0153	0.0088	0.0139
γ-amino butyric acid (GABA)	13.59	0.0034	0.0025	0.0017	0.0008	0.0039	0.0051	0.0000
Tyrosine (Tyr)	7.73	0.0068	0.0031	0.0046	0.0043	0.0030	0.0037	0.0027
Methionine (Met)	9.38	0.0007	0.0004	0.0004	0.0018	0.0009	0.0010	0.0008
Valine (Val)	11.95	0.0078	0.0104	0.0049	0.0049	0.0060	0.0052	0.0092
Phenylalanine (Phe)	8.48	0.0049	0.0023	0.0033	0.0030	0.0030	0.0030	0.0042
Isoleucine (Ileu)	10.67	0.0049	0.0033	0.0048	0.0048	0.0042	0.0035	0.0026
Leucine (Leu)	10.67	0.0053	0.0113	0.0144	0.0153	0.0157	0.0158	0.0131
Ornithine (Orn)	21.18	0.0035	0.0200	0.0203	0.0188	0.0014	0.0031	0.0000
Lysine (Lys)	19.15	0.0048	0.0446	0.0329	0.0187	0.0168	0.0309	0.0206
Total Sum		0.1496	0.1556	0.1543	0.1484	0.1483	0.1559	0.1312
Nitrogen conversion factor		6.686	6.421	6.481	6.739	6.609	6.415	7.663

*Values calculated from textbook (Belitz *et al*., 2004)

The fruited ti kōuka stem (collected in April) had higher relative concentrations of threonine and arginine compared to all the other (non-fruited) stems (Table 3.8). Of all the stems, the sample analysed in August had very much the lowest relative concentration of threonine.

The two acid hydrolysis methods were also compared for the nitrogen content of the liberated amino acids in the ti kōuka stem. Using the amino acid profiles obtained from the HPLC analyses, the percentage of each amino acid in the sample was multiplied by the known percentage of nitrogen content of each amino acid (calculated from Table 3.8). The nitrogen-to-protein conversion factor was calculated as the inverse of the total mass.

Table 3.9 Comparison of total nitrogen in protein from three sections of ti kōuka stem (collected in February) by two hydrolysis methods

	Performic acid hydrolysis		Hydrochloric acid hydrolysis	
	Total mg of nitrogen in 1 mg of protein	Nitrogen conversion factor	Total mg of nitrogen in 1 mg of protein	Nitrogen conversion factor
Nitrogen in top section	0.1465	6.824	0.1557	6.421
Nitrogen in second	0.1480	6.755	0.1542	6.481
Nitrogen in third section	0.1493	6.694	0.1483	6.739

The two hydrolysis methods gave some variability (Table 3.9) which could be due to the fact that during acid hydrolysis tyrosine is preserved while it is destroyed in the performic acid hydrolysis. The conversion factors calculated for the ti kōuka stem were all markedly greater than 6.25.

Having established that individual amino acids varied at different times of the year, the next chapter (Chapter 4) will be focused on analysing the amino acids at different stages of the ti kōuka spirit production.

CHAPTER 4

Amino acid Analyses During Ti Kōuka Spirit Production

4.1 Introduction

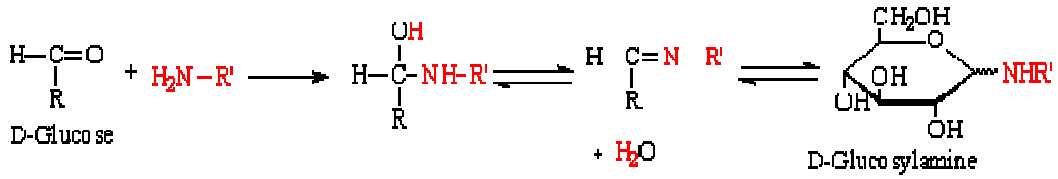
In the previous chapter (Chapter 3), the amino acids present in the different parts of the ti kōuka stem, and in stem collected at different times of the year were determined by the process of acid hydrolysis followed by derivatization with OPA and separation by liquid chromatography. It was also established that the dominant sugar in the hydrolysed ti kōuka extract was fructose. Reactions occurring in amino acids-reducing sugar systems produce a broad spectrum of coloured and flavoured compounds due to the Maillard reaction (Maillard, 1912), and it was expected that these reactions would continue and evolve through the several steps in the spirit production. This chapter outlines the main features of the Maillard reaction, then describes the fate of amino acids and fructose at the various stages of ti kōuka spirit production in representative stem samples.

4.2 The Maillard reaction

Although studied since 1912, the Maillard reactions are so complex that many reactions and pathways are still obscure. Nonetheless, the Maillard reaction is often considered as occurring in three main steps (Hodge, 1953).

The first step is the condensation of unprotonated amino group with the aldehyde group of a reducing sugar (e.g. glucose) in its open chain form, thus losing a molecule of water to form a Schiff's base, specifically an aldosylamine also known as an Amadori compound. Glucosylamine is the archetypical aldosylamine (Belitz & Grosch, 2004), where the double erows represents a mixture of the α and β anomers (Equation 1).

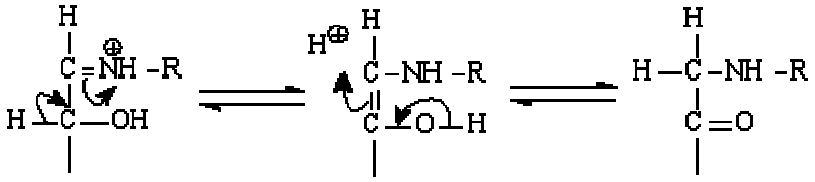
Equation 1:



R =Carbonyl moiety of the sugar
R' = N-terminal of amino acid

The Amadori compounds subsequently isomerise, by the Amadori rearrangement, into 1-amino-1- deoxyketoses (Equation 2).

Equation 2

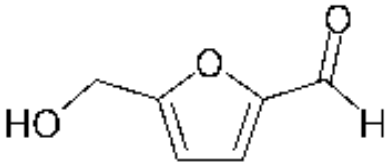


1-amino-1-deoxyketose

If ketohexoses (e.g. fructose) react with an amine or an amino acid, an analogous ketosylamine is the first product formed. This subsequently isomerises to a 2-amino-2-deoxyaldose.

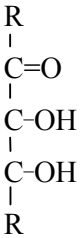
1-Amino-deoxyketoses and 2-amino-2-deoxyaldoses subsequently and readily react according to one or all of the following pathways.

One pathway comprises deamination and further dehydration yielding caramel products, typified by the classic caramel compound 5-hydroxymethyl-2-furaldehyde (Nursten, 2005; Blank and Fay, 1999). This pathway is favoured by acidic conditions.



5-Hydroxymethyl-2-furaldehyde (HMF)

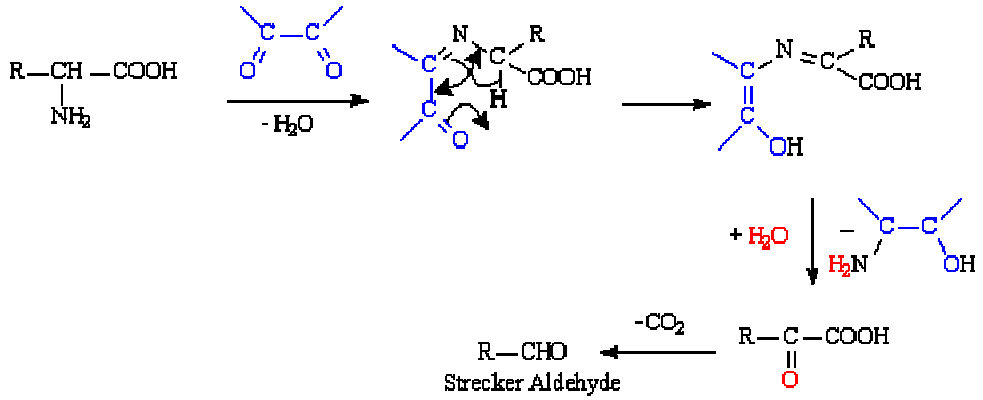
Above pH 7 there are two further pathways. Deamination and dehydration leads to the formation of reductones and their oxidised form dehydroreductones (Figure 5.3). Reductones are characterised by a carbonyl group adjacent to an endiol, and are powerful antioxidants.



The second alkaline-favoured pathway is the production of short chain hydrolytic fission products such as diacetyl, acetol, pyruvaldehyde etc. (Figure 5.3). These can then undergo the Strecker degradation (Martins *et al*., 2001) with amino acids to form

Strecker aldehydes (Equation3) that bear the side chain of the original amino acid. Thus, the basic 20 amino acids potentially yield 20 Strecker aldehydes that have a range of odours.

Equation 3



(www.chm.bris.ac.uk)

In the third and very messy stage of the Maillard reaction, many of the products formed above can react further with themselves, and with unreacted sugars and amino acids to ultimately form the brown pigments and flavour active compounds collectively called melanoidins. These are brown nitrogenous polymers, and are common in heated foods. The outcome will depend on which amino acids and sugars are available, and what the pH, temperature, and concentrations are.

Amino acids that produce the most intense Maillard browning are lysine, glycine, tryptophan and tyrosine. Moderately reactive amino acids include alanine, valine, leucine, isoleucine, phenylalanine, proline, methionine, aspargine and glutamine. The two basic amino acids (histidine and arginine), the two acidic amino acids (aspartic and glutamic acid), and the thiol-containing amino acid (cysteine) are the least reactive (Lamberts *et al*., 2008).

In the next set of experiments, the concentrations of fructose and of individual amino acids present at the different stages of the ti kōuka spirit production have been monitored. The steps are hydrolytic extraction, evaporation and reconstitution, centrifugation, and infusion with ethanol. The aim was to find which amino acids were taking part in the Maillard reaction to create the flavoured ti kōuka spirit. Table 4.1 gives a summary of the ti kōuka stem sections used in the proceeding experiments including their hydrolytic extraction conditions and nitrogen content.

Table 4.1 Ti kōuka stem sections and their nitrogen contents to be analysed in the following experiments

Collection date	Section of ti kōuka stem analysed	Described in section	Extraction/hydrolysis conditions	Nitrogen in dried ground ti kōuka stem analysed by HPLC (mg g^{-1})
February 2007	Top[1]	4.3	Fructozyme	1.945
February 2007	Second	4.3	Fructozyme	1.929
February 2007	Third	4.3	Fructozyme	1.855
April 2007	Top	4.4	Fructozyme and water	1.854
August 2006	Top	4.5	Fructozyme	1.949

[1] Top is tip to 20 cm, second is 20 to 40 cm, and third is 40 to 60 cm

4.3 Amino acid analysis at different stages of the ti kōuka spirit process using three stem sections

Method

Samples (10 g) of each of the three ground and dried sections (tip to 20 cm, 20 to 40 cm and 40 to 60 cm) of the ti kōuka stem collected in February 2007 were incubated with Fructozyme at 60°C as described in Section 2.4. The extracts were cooled and filtered using Whatman No. 4 filter paper, with multiple washes of deionised water. Each extract was made up to its original starting volume, the pH was adjusted to 10 with 1 M sodium hydroxide, and 3-mL samples were retained for amino acid analysis. The three filtrates were then evaporated at 60°C for 65 hours, and the residues reconstituted to their original volume with deionised water. Samples of each were retained for amino acid analysis. The remaining volumes of the three treatments were centrifuged at 3700 gravities for 15 minutes, and samples retained for amino acid analysis.

All nine samples (3 hydrolysed with Fructozyme, 3 evaporated and reconstituted, and 3 centrifuged) were placed in acid-washed screw cap test tubes and hydrolysed with 6 M HCl (Section 3.1.2) and analysed for amino acid composition by HPLC (Sections 3.2 and 3.3).

Results

As was previously observed (Section 3.4), the relative concentrations of several amino acids in dried ground stem sections changed with stem maturity (Figure 4.1). The most striking absolute difference was for leucine, the amino acid that dominated the three maturity profiles, and was up from 29 to 56% in the bottom, most mature section

(Appendix IX). In contrast, the absolute proportion of arginine fell from 18, to 13, to 8% with increasing maturity. With the exception of lysine, which showed no pattern with maturity, all other amino acids declined in proportion, presumably due to the large increase in the proportion of leucine.

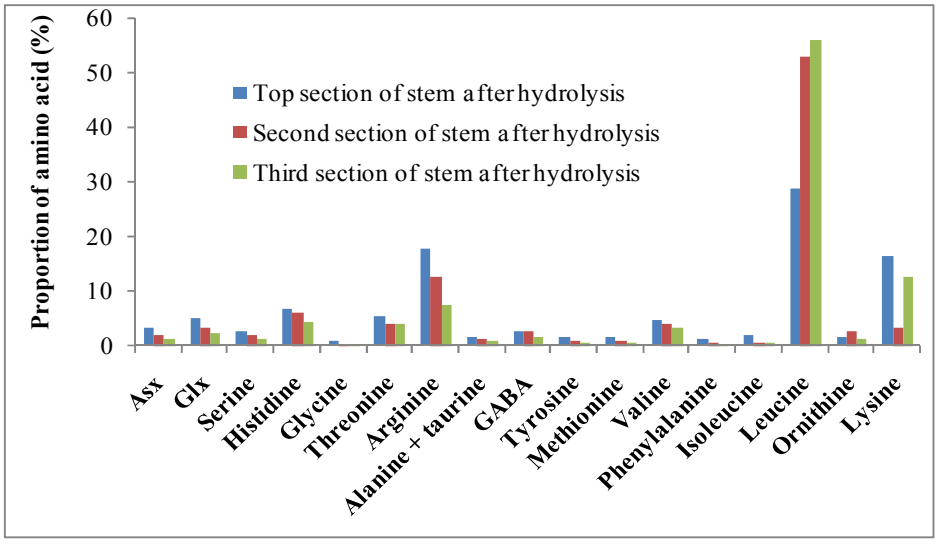


Figure 4.1 Amino acid proportions in the three dried ground ti kōuka stem sections

Turning now to changes within stem sections at different stages, within the top section, the relative proportions of leucine and lysine (and less obviously Asx, Glx, glycine and GABA) decreased during evaporation and reconstitution (Figure 4.2), indicating that these amino acids dominated the Maillard reaction, or less likely, were lost in some other way.

The proportion of leucine dropped from 29 to 7% during evaporation and reconstitution. Concomitant increases in proportion were seen for many other amino acids suggesting they were relatively unreactive. After the final centrifugation step the relative proportions of leucine and lysine increased while the relative proportions of many others decreased, indicating they were preferentially retained in the pellet, perhaps still polymerized in proteins. All these results suggest that leucine and lysine were physically and chemically the most available for reaction and were also the most reactive.

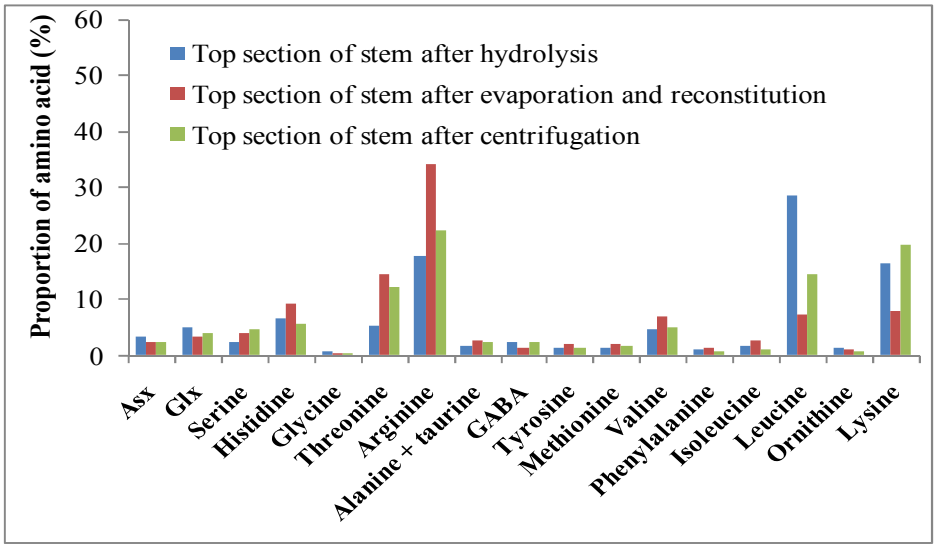


Figure 4.2 Amino acid proportions in the top section of ti kōuka stem at different stages of ti kōuka spirit process

In the second section of the stem (Figure 4.3), the pattern of proportions at the different stages had similarities and differences to that in the top section. Thus, the proportion of leucine decreased the most from 53 to 17% during evaporation and reconstitution. Again, this clearly indicates that leucine was taking part in the Maillard reaction.

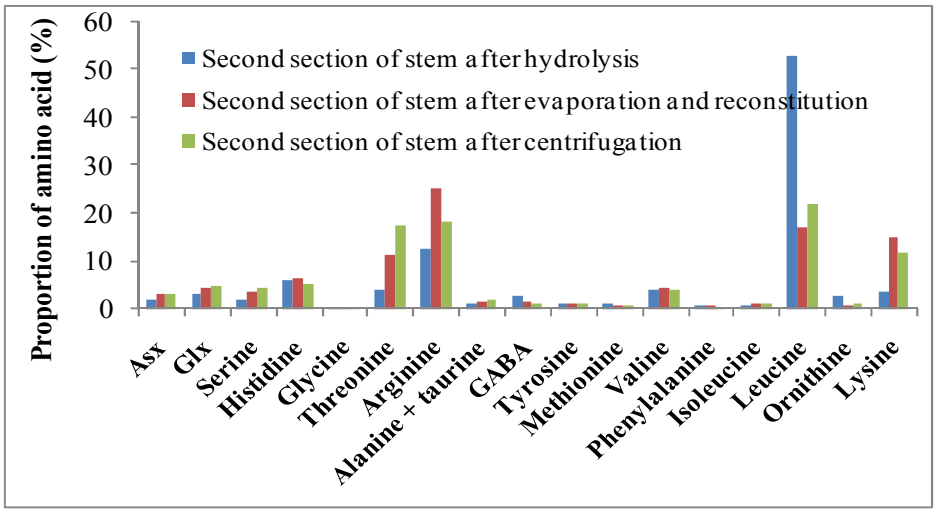


Figure 4.3 Relative amino acid concentrations in the middle section of the ti kōuka stem at different stages of ti kōuka spirit process

As a result of leucine's loss the proportion of most other amino acids increased. Lysine was included in this group. Its increase in the middle section thus contrasted with its decrease in the first section, suggesting it was more available or reactive in the top section of the stem. As in the top section, increases in the proportion of threonine and

arginine dominated the profile after evaporation and reconstitution. After the final centrifugation step there was a further readjustment of proportions as some amino acids were lost to the pellet.

In the third section (Figure 4.4), the absolute proportion of leucine similarly decreased markedly during the evaporation process from 56 to 25%. Changes in the proportion of lysine were less marked than in the two other stem sections. As before, arginine was the amino acid that showed the greatest absolute increase (from 8 to 23%) presumably due the loss of leucine.

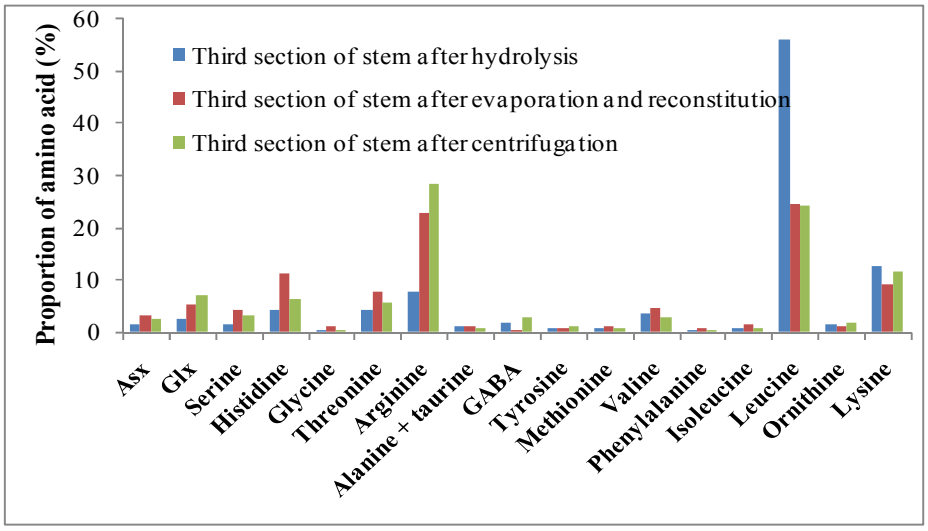


Figure 4.4 Relative amino acid concentrations in the bottom section of the ti kōuka stem at different stages of ti kōuka spirit process

Discussion

In seeking to build a chemical model of these changes in proportions of amino acids, it is important to identify the principal drivers of the Maillard reaction particularly during the evaporation step. To take part in the Maillard reaction, the amino groups associated with amino acids not only have to be available, i.e. not polymerised in peptide bonds, but also have to be unprotonated. Because the pool of free amino acids in plant tissue is low compared with the pool of protein (Macnicol, 1983) this implies that hydrolysis of proteins, probably enzyme-mediated, is occurring during the evaporation reconstitution step. Plant enzymes are usually unstable/inactive at the evaporation temperature of 60°C (www.loomisenzymes.com), but it is important to realise that while the oven temperature was 60°C, the temperature of the extract would be less than 60°C due to evaporative cooling until such time as the readily evaporable water was lost.

Endogenous ti kōuka proteases could thus be active, generating the available amino groups. However, to be active they would have to survive the preliminary grounding and drying step. These actions have unknown effects on the stability of proteases.

Whatever the role of proteases, evaporation would steadily increase the concentration of available fructose and amino acids, further favouring the Maillard reaction. The temperature, although less than 60°C, would certainly be higher than ambient and this too would accelerate reaction.

Throughout the evaporative process, pH is likely playing a key role. Unprotonated amine groups are required for the initial reaction between reducing sugar and amino acids, and this requirement is clearly favoured by the initial pH 10 conditions. The pKa values of the α-amino groups of free amino acids are, with the exception of proline, all below pH 10 (Belitz *et al*., 2004). Thus under the starting conditions of the evaporative procedure virtually all the amino acids would be potentially reactive (if free). Even the ε-amino group of lysine is close to its pKa of 10.28. On the other hand, a pH of 10 would be unlikely to favour proteolysis by enzyme activity (www.loomisenzymes.com).

But on balance, the Maillard reaction is clearly occurring and it remains to account for the marked changes that occur in the proportions of different amino acids (Figures 4.2, 4.3, 4.4). The marked fall in the proportion of leucine can be most simply explained by the fact that leucine is the most abundant of the amino acids recovered after acid hydrolysis (Figure 4.1), and by the law of mass action is the most likely to react. At the same time, the increases in proportions of many other amino acids have to increase to compensate for this large loss. This does not preclude reaction by these amino acids. It is simply that leucine dominates the Maillard reaction in this system.

Turning now to lysine, which is the most reactive of all amino acids in the Maillard reaction (Belitz *et al*., 2004), Figure 4.1 shows that the proportion of lysine is lowest in the second stem section at the point of hydrolytic extraction. Again, by the law of mass action, it means that less lysine would be available for reaction. This implies that the second section – but not the top and third sections – should show an increase in proportion of lysine on evaporation and reconstitution: less to react and therefore more affected by the loss in leucine. Inspection of the graphs shows this to be the case.

The next step was to compare the effects on amino acid profiles of unassisted hydrolytic extraction (deionised water) with Fructozyme-assisted hydrolytic extraction.

4.4 The effects of Fructozyme-assisted hydrolytic extraction of ti kōuka stem of the amino acid profiles and colour at different stages of the spirit process

In Chapter 2, it was found that when dried ground ti kōuka stem was hydrolytically extracted with Fructozyme, more reducing sugar was obtained than when it was extracted with an equivalent volume of deionised water (Table 2.3). Maillard reactions are influenced by factors such as types and concentrations of sugars and amino acids (Baisier and Labuza, 1992), temperature, pH, time etc. Therefore, it is possible that the different sugar concentrations arising from the hydrolysis treatments will affect and influence the relative proportions of individual amino acid taking part in the Maillard reaction, hence producing different Maillard reaction products and flavours. This possibility was tested here.

Method

A sample (8 mg) of the fruited dried ground ti kōuka stem collected in April 2007 (Figure 3.1, Section 3.2.1) was set aside for amino acid analysis. Another two lots of the dried ground stem (10 g each) was hydrolysed with and without Fructozyme, followed by evaporation and reconstitution with deionised water and centrifugation (as in Section 4.3). Samples at each step (i.e. hydrolysis, evaporation and reconstitution, and centrifugation) were retained for amino acid content analysis.

All seven samples (1 dried ground sample + 3 from water + 3 from Fructozyme) were placed in acid-washed screw cap test tubes, hydrolysed with 6 M HCl (Section 3.1.2), and analysed in triplicate for amino acid composition by HPLC (Sections 3.3.2 to 3.3.10).

The final supernatants of the two treatments (water, Fructozyme) were infused with 99.7% ethanol to yield a final concentration of 80% ethanol. The ethanolic infusions were vacuum filtered through Whatman GF/B glass paper.

Colour measurements (described in Section 2.15) were recorded for all eight liquid samples (4 from deionised water + 4 from Fructozyme).

What is immediately striking in these profiles is that the proportion of leucine is lower than found in other stems (Figures 4.1 to 4.4), indicating biological, seasonal or other variation as discussed in Chapter 3. (In this respect note that Figures 4.1 to 4.5 are all scaled the same way to permit easy comparison.) Importantly, the nitrogen content of this fruited stem was lower than in the other ti kōuka stems samples analysed (Table 4.1). Thus not only was the total protein content lower in fruited stem, but the amino acid profile was also affected.

After hydrolytic extraction of dried ground stem with water or Fructozyme, the overall amino acid profile (Appendix X) was similar to that obtained with direct acid hydrolysis of dried ground stem (Figure 4.5). This is perhaps not surprising given that the Maillard reaction would be only beginning in the evaporation process at 60°C, but even subtle differences here may lead to substantial differences in flavour after the progression through to the final product.

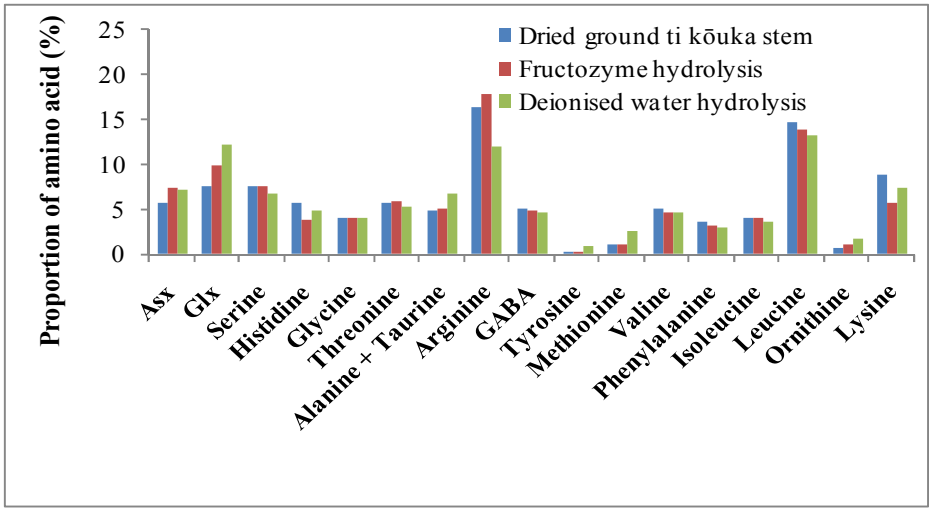


Figure 4.5 Relative amino acid proportions of dried ground stem (collected in April) and after water and Fructozyme-assisted hydrolytic extraction

In Figures 4.6 and 4.7, the results have been extended through to the point of centrifugation and rescaled to emphasize which amino acids proportionately changed the most and the least during the spirit production. These figures showed that some flattening of the profiles did occur but was minor. Moreover, there was little difference between the Fructozyme and the deionised water treatments.

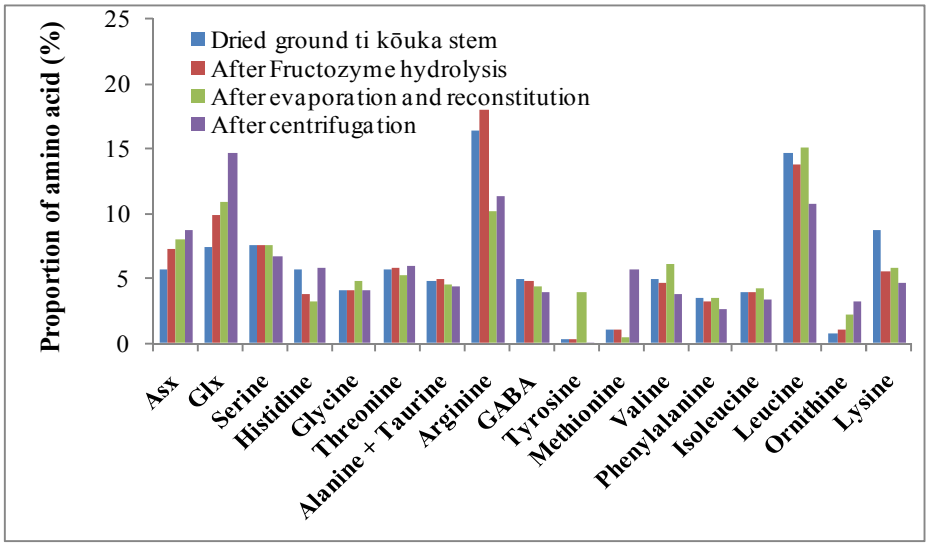


Figure 4.6 Relative amino acid proportions in dried ground stem (collected in April), and after Fructozyme-assisted hydrolytic extraction, evaporation and reconstitution, and centrifugation

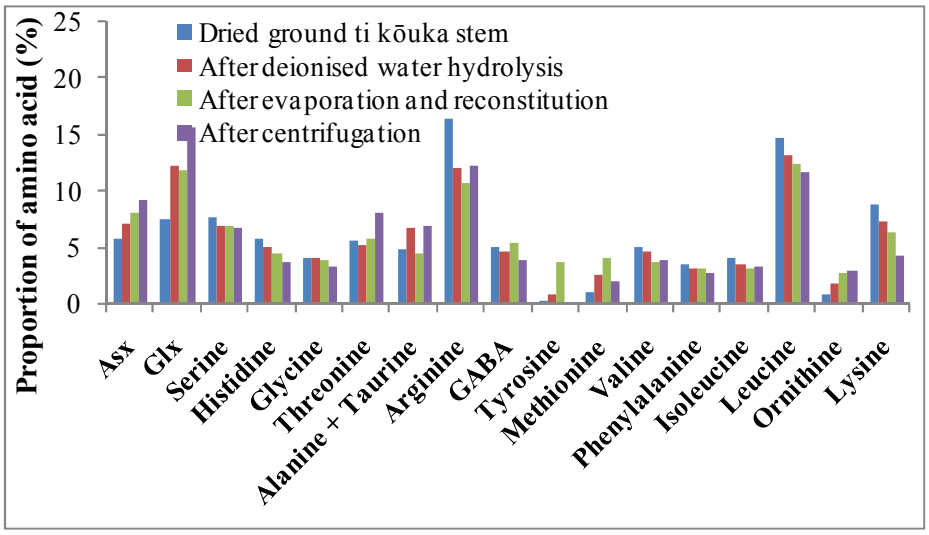


Figure 4.7 Relative amino acid proportions in dried ground stem (collected in April), and after water-assisted hydrolytic extraction, evaporation and reconstitution, and centrifugation

Thus, the greater availability of fructose in the Fructozyme treatment compared with the water treatment did not markedly affect the amino acid profile as the Maillard reaction progressed. For water and Fructozyme hydrolytic extractions, probably histidine, GABA, valine, phenylalanine, leucine and lysine were the most reactive amino acids as shown by their declines in proportion - and therefore being lost during the hydrolytic extraction process (Figure 4.8).

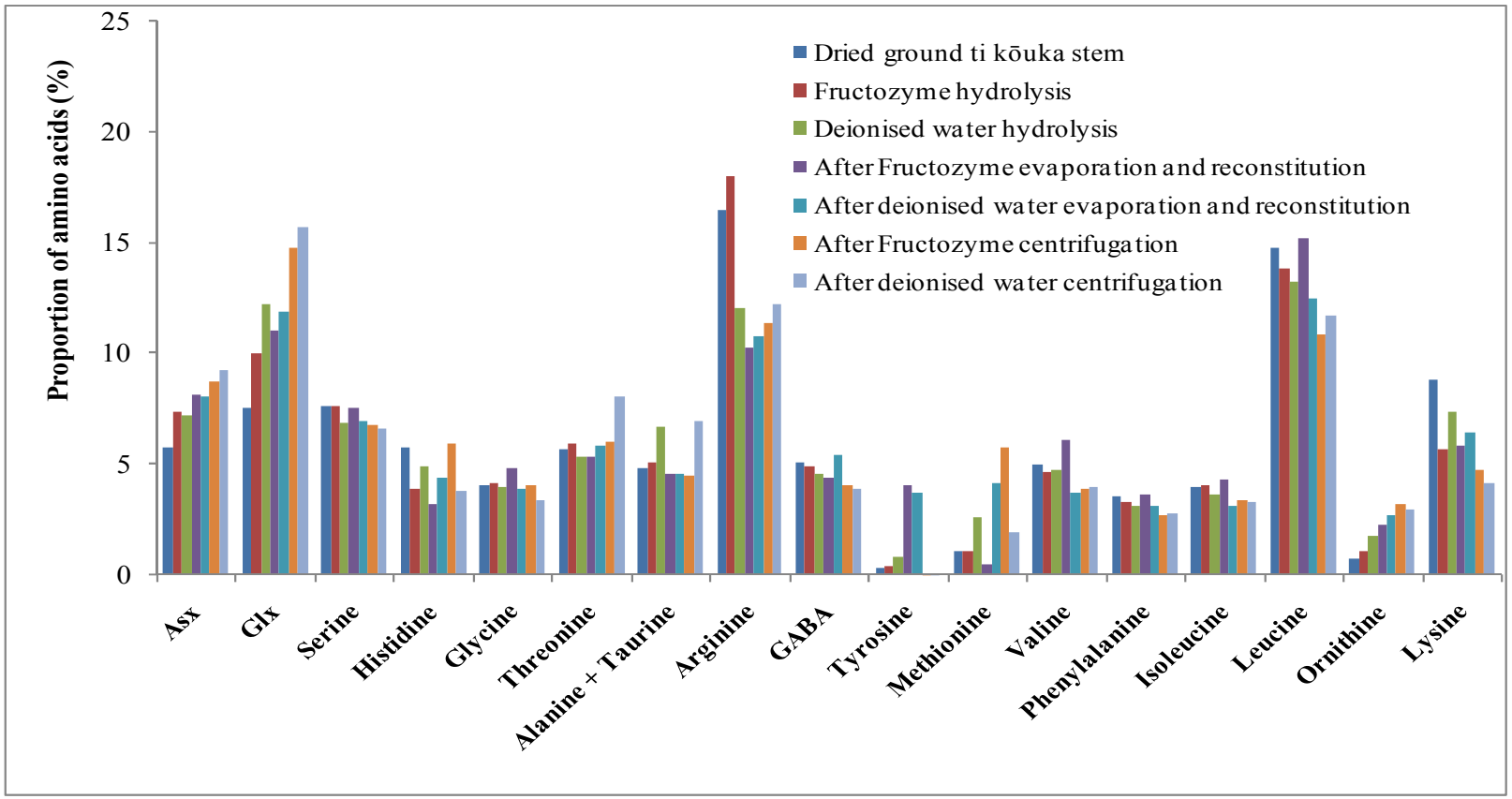


Figure 4.8 Proportion of amino acid (%) in dried ti kōuka stem (collected in April, 2007) and after Fructozyme and deionised water-assisted hydrolytic extraction, evaporation and reconstitution and centrifugation

Asx, Glx, alanine and taurine, methionine and ornithine presumably were reacted the least because their proportions increased. Other amino acids reacted in an intermediate or otherwise complicated manner.

These reactions of amino acids and fructose were reflected in the colour of the reconstituted liquids obtained at each step. Inspection of Table 4.2 shows that at each step in the process there were few Hunter colour differences due to Fructozyme versus water hydrolysis. At the point of centrifugation, there was a difference in L* values between the two treatments, but there are no obvious reasons from the perspective of amino acid losses.

Table 4.2 Colour measurements of fruited ti kōuka stem (collected in April 2007) at different stages of the spirit production with acid hydrolysis

Stages in the production of ti kōuka spirit	L*	Saturation $\sqrt{[(a^*)^2 + (b^*)^2]}$	Hue angle arctan [(b*)/(a*)]
After Fructozyme hydrolytic extraction	42.39 ± 1.61	20.13 ± 0.25	1.33 ± 0.00
After deionised water hydrolytic extraction	43.30 ± 1.07	21.45 ± 0.56	1.34 ± 0.03
After evaporation and reconstitution (Fructozyme)	35.07 ± 1.89	21.85 ± 0.07	1.24 ± 0.02
After evaporation and reconstitution (water)	35.67 ± 1.69	19.96 ± 0.41	1.25 ± 0.00
After centrifugation (Fructozyme)	7.20 ± 0.21	7.27 ± 0.58	1.28 ± 0.10
After centrifugation (water)	12.99 ± 0.09	8.27 ± 0.73	1.47 ± 0.00
After ethanol infusion (80% v/v) (Fructozyme)	2.94 ± 0.50	0.32 ± 0.12	-0.53 ± 1.08
After ethanol infusion (80% v/v) (water)	2.89 ± 0.85	0.28 ± 0.17	-0.11 ± 0.71

Now considering the data pooled across the water and Fructozyme treatments, at each step of the process from hydrolytic extraction to ethanol infusion, the L* (lightness) values decreased, i.e. the liquids were becoming less reflective and therefore appeared darker. The effect was marked, reflecting an average of 43, 35, and finally 10 % of light at the points of hydrolysis, evaporation and reconstitution, and centrifugation. Some of this change may reflect transmission of light as the liquids became progressively clearer. This effect continued after ethanol infusion where L* values dropped to 2.9 in these substantially clear liquids.

The saturation values decreased on centrifugation and after ethanol infusion, suggesting that the colour before centrifugation and ethanol infusion was more intense. The higher

molecular weight coloured Maillard compounds formed during evaporation are presumably less soluble in water and ethanol, therefore precipitated out in the pellet during centrifugation and ethanol infusion. The hue angle decreased markedly upon ethanol infusion indicating a shift in colour from browner to redder.

4.5 A further amino acid and fructose analysis of Fructozyme-assisted hydrolytic extraction of ti kōuka stem to the point of infusion with ethanol

The differences between the amino acid profiles in dried stem matter collected in February and in April 2007 prompted a repeat analysis of kōuka stem, this time collected in August 2006. At the same time, it was decided to simultaneously analyse for fructose, and to extend the analysis through to the point of ethanol infusion.

Method

The method followed those detailed in Sections 4.3 and 4.4. In outline, dried ground ti kōuka stem collected in August – which had also been used in previous experiments in Chapter 3 – was hydrolysed with Fructozyme at 60°C, cooled, filtered, washed and made up to its original starting volume. The pH was adjusted to 10 with 1 M sodium hydroxide and evaporated at 60°C for 65 hours. The dried residue was reconstituted to the original volume with deionised water. After centrifugation, the supernatant was infused with 99.7% v/v ethanol to yield final ethanol concentrations of 80, 66.7, 57 and 50% v/v. These were centrifuged, and filtered with GF/B glass filter paper.
Samples of each of the ground stem, and eight mixtures or solutions hydrolysed with 6 M HCl (Section 3.1.2) were analysed for amino acid composition by HPLC (as in Sections 3.2 and 3.3), reducing sugars (by PAHBAH method), and colour measurements (by CIELAB, as described in Section 2.15).

Results and discussion

In descending order of relative proportion, the most abundant amino acids present in the dried ground stem were lysine (29%), arginine (17%) and leucine (12%) (Figure 4.9), each of which was variously dominant in the two preceding trials. After hydrolytic extraction for 1 hour at 60°C, the relative concentration of leucine increased (12 to 38%) because all other amino acids decreased in relative concentration. On evaporation and reconstitution, the relative proportion of all amino acid remained similar as the hydrolytic value or decreased slightly with the exception of histidine, arginine and

leucine, which increased from 4.81 to 7.44%, 14 to 17% and 38 to 41% respectively. These results imply that leucine, which was dominant in the Maillard reaction in the two previous analyses (Sections 4.3, 4.4), was less reactive in this particular sample.

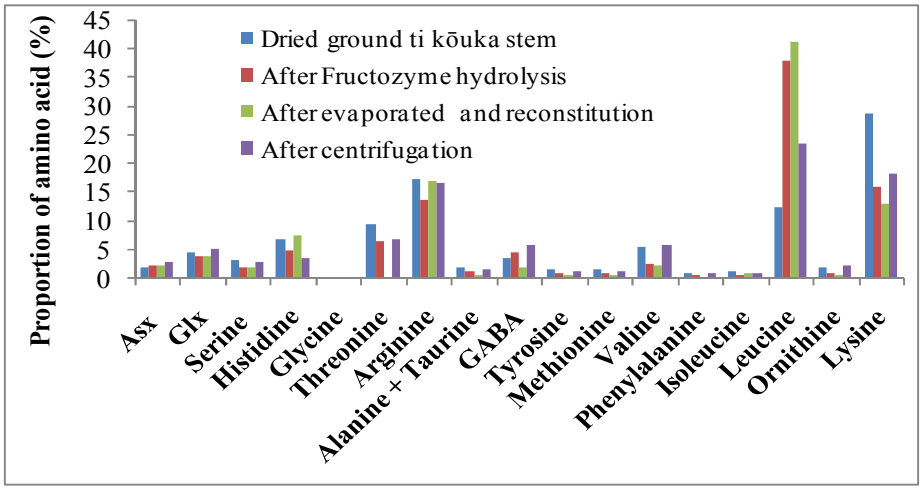


Figure 4.9 Relative amino acid proportions in dried ground stem (collected in August 2006) and after Fructozyme-assisted hydrolytic extraction, evaporation and reconstitution, and centrifugation

Similarly, arginine was not particularly reactive. The reason for these differences in reactivity between the analyses described in Section 4.3, 4.4 and this Section is not known. However, one possible reason is that reactivity will depend on the liberation of the α-amino group due to protein hydrolysis caused by an endogenous enzyme remaining active in dried ground stem, or non-enzymatic hydrolysis at the starting pH of 10. If endogenous enzyme activity were to vary from sample to sample then the profile of amino acids available for reaction could vary. In support of this argument is the fact that in common with other stem samples collected in February and April, the proportion of lysine decreased at each step. The reactivity of lysine does not depend on protein hydrolysis because its ε-amino group is always available for reaction particularly under alkaline conditions where the amino group would be unprotonated (Belitz *et al*., 2004). In this respect Ajandouz *et al*. (2001) observed a complete loss of lysine when heated with fructose (pH 9.0 to 12.0) while no significant loss was observed when the amino acid was heated in the absence of fructose, during the early stages of browning.

Infusion with the different concentrations of ethanol markedly affected the relative concentration of the amino acids (Table 4.3, Figure 4.10). What differences there were

– for example, the relative concentration of lysine increased as ethanol concentration decreased – probably reflect the different solubility of amino acids in ethanol/water mixtures.

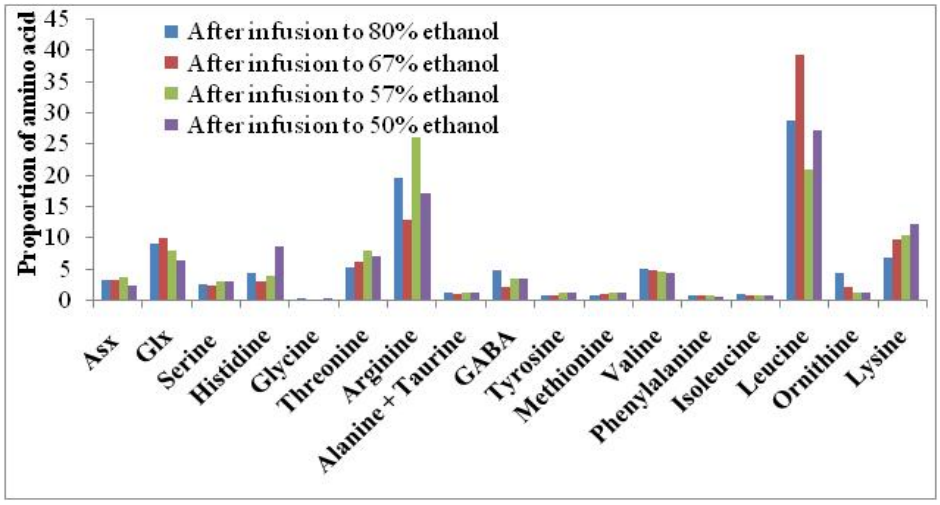


Figure 4.10 Relative amino acid proportions in dried ground stem (collected in August 2006), after ethanol infusion

In contrast to the results obtained with stem samples collected in April (Table 4.2), the L* (lightness) values increased after evaporation and reconstitution (Table 4.4). However, as before, the reflectance of light decreased markedly after centrifugation as particulate matter was removed in the pellet. It did not change on alcohol infusion. These results were roughly paralleled by changes in saturation. With particulate matter removed, the colour intensity was reduced. Within the ethanol series saturation increased as the concentration decreased to 50%, implying that more water soluble coloured matter was being infused in the spirit. Hue angle also changed, with lower ethanol infusions being browner and higher ethanol infusions being redder.

Table 4.3 Amino acid profile (Percentage of total mg dry weight of amino acid) of ti kōuka sample collected in August at different stages of process

Amino Acid	Dried ground stem	After Fructozyme hydrolysis	After evaporation and reconstitution	After centrifugation	After infusion to final 80% ethanol	After infusion final to 67% ethanol	After infusion to final 57% ethanol	After infusion to final 50% ethanol
Asx	1.83	2.29	2.39	2.98	3.30	3.20	3.80	2.52
Glx	4.28	3.97	3.94	5.08	9.09	9.88	8.07	6.56
Serine	3.18	2.07	2.07	3.07	2.71	2.33	3.22	3.03
Histidine	6.63	4.81	7.44	3.51	4.53	2.98	3.90	8.65
Glycine	0.07	0.29	0.48	0.42	0.37	0.16	0.219	0.54
Threonine	9.32	6.41	3. 90	6.73	5.36	6.20	8.01	7.10
Arginine	17.19	13.63	17.00	16.54	19.56	12.88	26.05	17.20
Alanine + Taurine	1.56	1.29	0.79	1.52	1.34	0.97	1.35	1.31
GABA	3.49	4.65	2.05	5.78	4.80	2.21	3.59	3.52
Tyrosine	1.38	1.06	0.75	1.46	0.80	0.76	1.41	1.31
Methionine	1.27	0.94	0.81	1.43	0.99	0.96	1.38	1.37
Valine	5.43	2.66	2.20	5.88	5.07	4.79	4.60	4.49
Phenylalanine	0.74	0.57	0.48	0.82	0.91	0.76	0.78	0.72
Isoleucine	1.19	0.77	1.07	0.98	1.22	0.83	0.92	1.00
Leucine	12.22	37.76	40.97	23.40	28.65	39.20	20.91	27.18
Ornithine	1.60	1.01	0.74	2.30	4.46	2.20	1.25	1.27
Lysine	28.53	15.83	12.92	18.10	6.86	9.69	10.55	12.26

Table 4.4 Reducing sugar concentration and colour measurements of dried ti kōuka stem sample (collected in August) at different processing stages

Stages in the production of ti kōuka spirit	Reducing sugar (mg g^{-1} dry weight)	L*	Saturation $\sqrt{[(a^*)^2 + (b^*)^2]}$	Hue angle arctan $[(b^*)/(a^*)]$
Dried ground ti kōuka stem	–	–	–	–
After Fructozyme-assisted hydrolytic extraction	163.6 ± 1.48	34.99 ± 1.60	22.74 ± 1.00	1.40 ± 0.01
After evaporation and reconstitution	146.5 ± 1.14	42.63 ± 2.23	15.37 ± 1.84	1.24 ± 0.02
After centrifugation	156.8 ± 1.70	3.71 ± 0.04	13.39 ± 0.26	1.34 ± 0.03
Ethanol infusion (80% v/v)	12.4 ± 0.02	4.04 ± 0.24	0.43 ± 0.18	0.50 ± 0.37
Ethanol infusion (66.7% v/v)	27.6 ± 0.09	3.43 ± 0.08	0.99 ± 0.25	-0.93 ± 0.18
Ethanol infusion (57.1% v/v)	45.7 ± 0.35	3.46 ± 0.10	1.48 ± 0.32	-1.22 ± 0.06
Ethanol infusion (50% v/v)	62.7 ± 0.61	3.48 ± 0.07	1.58 ± 0.17	-1.27 ± 0.11

To summarise, from the results of all three experiments (Sections 4.3 to 4.5), it is clear that differences in the physical and probably chemical properties of the ti kōuka spirit as prepared by the methods described are not very much affected by the use of Fructozyme, or simply using hydrochloric acid or water for hydrolysing the ti kōuka stem. Rather larger differences probably arise from differences in amino acid profiles in the stem as affected by physiological maturity (position in stem), and almost certainly other factors like time of year (pre/post fruiting) and factors not examined like genotype and environment. As is seen from Figure 1.1 (Chapter 1), *Cordyline australis* is distributed in 99% of New Zealand, covering both the North and South island. With time and financial restrains, ti kōuka stems from other areas in New Zealand were not analysed. Neither were stems collected in each month of the year analysed. There is no documented data available, which could be sourced for the amino acid profiles.

As was observed from the results of the experiments in this chapter and previous chapters, the rate of the Maillard reaction and the nature of the products are governed by its immediate chemical environment, including water activity, pH, heating time, chemical composition and temperature. The effect of these variables in the Maillard reaction can be investigated by using model systems in which sugars and amino acids react under simplified conditions. The next chapter (Chapter 5) will be focused in creating model systems with ti kōuka extract to approximately simulate the ti kōuka spirit production.

CHAPTER 5

Reaction of sugars and amino acid taking part in the Maillard reaction in model systems

5.1 Introduction

In the previous chapter (Chapter 4), the fate of amino acids and fructose was monitored at the different stages of ti kōuka spirit production using ti kōuka stems collected at different times of the year, and also using different sections of the same stem. It was established that the principal amino acids available for the Maillard reaction were Asx, Glx, arginine, leucine and lysine.

The rate of the Maillard reaction and the type of products and flavours formed depends on factors such as types and concentrations of sugars and amino acids (Baisier and Labuza, 1992), temperature, pH, time, and water activity etc. (Martins and van Boekel, 2005). At early and intermediate stages of the Maillard reaction, ultraviolet light-absorbing but otherwise colourless compounds are formed (Wijewickreme and Kitts, 1997a), whereas dark-brown polymeric compounds, collectively called melanoidins, are formed at later stages (Lamberts *et al*., 2008).

The mechanisms and the profile of products of the Maillard reaction in foods can be very complicated. Therefore a common method to investigate non-enzymatic browning reaction is the use of model systems in which known concentrations of sugars and amino acids react under controlled conditions of temperature, time and pH etc. (Carabasa-Giribet and Ibarz-Ribas, 2000).

The objective of the following experiments was to monitor the colour formation of the Maillard reaction products using different combinations of sugar and/or amino acids, identified as reactive in the ti kōuka stem (Chapter 4), at different concentrations, pHs, temperature and reaction times.

5.2 Effect of different combinations and ratios of sugar and amino acids on the Maillard reaction

Materials and Methods

Amino acids were purchased from Sigma, Merck and BDH, and fructose was purchased from Scientific Supplies, New Zealand (Appendix VIII). Twenty-one model systems containing fructose and amino acids with different combinations and ratios were

weighed (as shown in Table 5.1), and made up in 100 mL of deionised water and placed in 135 mm-diameter Schott Duran glass Petri dishes.

Table 5.1 Concentrations and ratios of fructose and amino acids used in the model systems. All values are mg 100 mL^{-1} final concentration

Ratio of fructose to amino acid	Fructose	Arginine	Lysine	Leucine	Aspartic acid	Glutamic acid
	500	0	0	0	0	0
	0	500	0	0	0	0
	0	0	500	0	0	0
	0	0	0	500	0	0
	0	0	0	0	500	0
	0	0	0	0	0	500
2 : 1	500	250	0	0	0	0
1 : 1	500	500	0	0	0	0
1 : 2	250	500	0	0	0	0
2 : 1	500	0	250	0	0	0
1 : 1	500	0	500	0	0	0
1 : 2	250	0	500	0	0	0
2 : 1	500	0	0	250	0	0
1 : 1	500	0	0	500	0	0
1 : 2	250	0	0	500	0	0
2 : 1	500	0	0	0	250	0
1 : 1	500	0	0	0	500	0
1 : 2	250	0	0	0	500	0
2 : 1	500	0	0	0	0	250
1 : 1	500	0	0	0	0	500
1 : 2	250	0	0	0	0	500

In the same manner of preparing the ti kōuka drink, the Petri dishes were placed in a conventional oven at 60°C. After 65 hours, the model samples were cooled, reconstituted with deionised water to their original volume (100 mL).

Among the most commonly used indicators of the Maillard reaction are spectrophotometric measurements at 294 nm for the early Maillard reaction products (pyrazines and other heterocyclics), at 320 nm for soluble melanoidins, and at 420 nm for the advanced brown Maillard reaction products which includes the presence of furans, pyrroles, pyridines and pyrazines components (Carabasa-Gibibet and Ibarz-Ribas, 2000). Wavescans were therefore performed between 200 and 800 nm wavelengths (as in Section 2.17). All work was done with a single quartz cuvette

capable of transmitting light in the ultraviolet range of interest. Known dilutions were made with deionised water before performing the wavescans to ensure the absorbances were between 0 and 3. Presented data in tables and figures are corrected for dilutions.

It must be pointed out that advanced brown Maillard reaction products do not demonstrate a peak at 420 nm (Figure 5.1). Rather this wavelength is useful indicator of brownness. By contrast, the wavescan in the ultraviolet range demonstrated clear peaks (Figure 5.1). The peaks observed in the wavescan are real peaks and not background electronic noise, because the scans were reproducible.

Appearance and odour were also assessed.

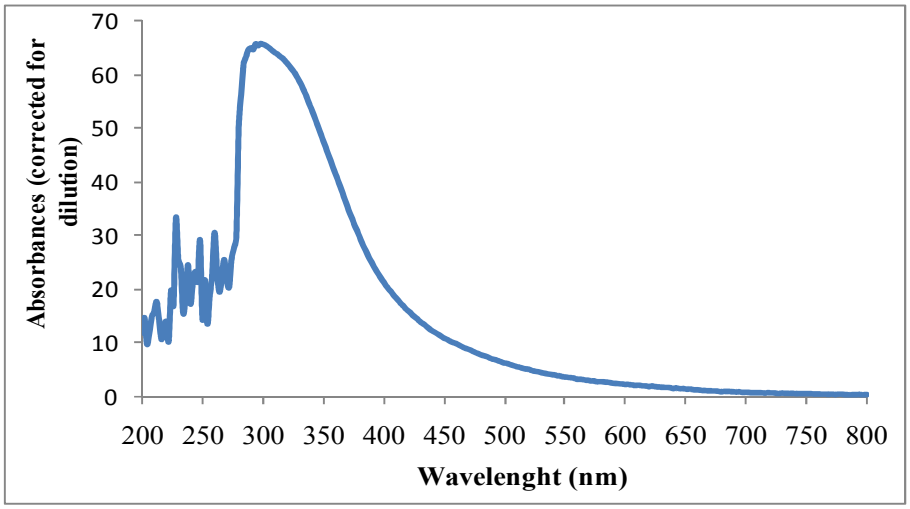


Figure 5.1 Wavescan of fructose + lysine (1 to 2 ratio)

Results and Discussion

All combinations of fructose with arginine, glutamic and aspartic acids and lysine were soluble prior to evaporation and after reconstitution. Leucine, on the other hand, was largely insoluble (at 1:1 and 1:2 sugar to amino acid ratio) in water before evaporation. (The solubility of leucine is 243 mg 100 mL^{-1} at 25°C.) On evaporation, a white layer with crystal-like specks was observed at these two ratios. After reconstitution with water, some leucine still remained insoluble.

Results of the appearance and odour are summarised in Table 5.2. All amino acids were colourless and odourless when evaporated in the absence of fructose. Likewise, fructose evaporated on its own was colourless and odourless. Some caramelisation

might be expected at 60°C over a prolonged period, but such was not the case. Evidently, the temperature was not high enough to achieve caramel flavours and browning. However, lack of obvious caramelisation does not preclude generation of colourless, low-molecular weight reductones (Rhee and Kim, 1975) as precursors to true caramelisation, which can be detected by absorbance in the ultraviolet range (Ajandouz *et al*., 2001).

Table 5.2 Appearance and odour profile of different sugar/amino acid combinations and ratios after evaporation (at 60°C for 65 hours) and before reconstitution with deionised water

Model system/(Ratio)	Appearance after evaporation and before reconstitution	Odour after evaporation and before reconstitution
Fructose	Colourless	Odourless
Arginine	Colourless	Odourless
Lysine	Very light yellow layer	Faint woody
Leucine	White	Odourless
Aspartic acid	White layer	Odourless
Glutamic acid	White layer	Odourless
Fructose + Arginine (2 to 1)	Light brown	Faint caramel
Fructose + Arginine (1 to 1)	Light brown	Faint caramel
Fructose + Arginine (1 to 2)	Dark brown	Caramel smell
Fructose + Lysine (2 to 1)	Very dark brown	Burnt caramel
Fructose + Lysine (1 to 1)	Very dark brown	Burnt caramel
Fructose + Lysine (1 to 2)	Very dark brown	Burnt caramel
Fructose + Leucine (2 to 1)	White	Sweet sugar
Fructose + Leucine (1 to 1)	White	Sweet sugar
Fructose + Leucine (1 to 2)	White	Sweet sugar
Fructose + Aspartic acid (2 to 1)	Yellow/brown layer	Very faint sugar smell
Fructose + Aspartic acid (1 to 1)	Very light brown layer	Very faint sugar smell
Fructose + Aspartic acid (1 to 2)	White layer	Odourless
Fructose + Glutamic acid (2 to 1)	White layer	Odourless
Fructose + Glutamic acid (1 to 1)	White layer	Odourless
Fructose + Glutamic acid (1 to 2)	White layer	Odourless

When sugars are subjected to heat in the absence of water or are heated in concentrated solution, a series of reactions occurs that finally leads to caramel formation. The typical caramel flavour is the result of a number of sugar fragmentation and dehydration

products, including diacetyl, acetic acid, formic acid, and more complex degradation products like 4-hydroxy-2,5-dimethyl-3(H)-furanone. Caramels contain high and low molecular weight coloured compounds, as well as a variety of volatile components (de Man, 1999).

Aspartic and glutamic acids in combination with fructose did not yield any distinctive colour or odours at any of the three ratios tested (Table 5.2), having the lowest absorbance values of any fructose amino acid combination, barely visible in Figure 5.2. Lamberts *et al*. (2008) also documented that these two acidic acids belong to the group of the least Maillard-reactive amino acids.

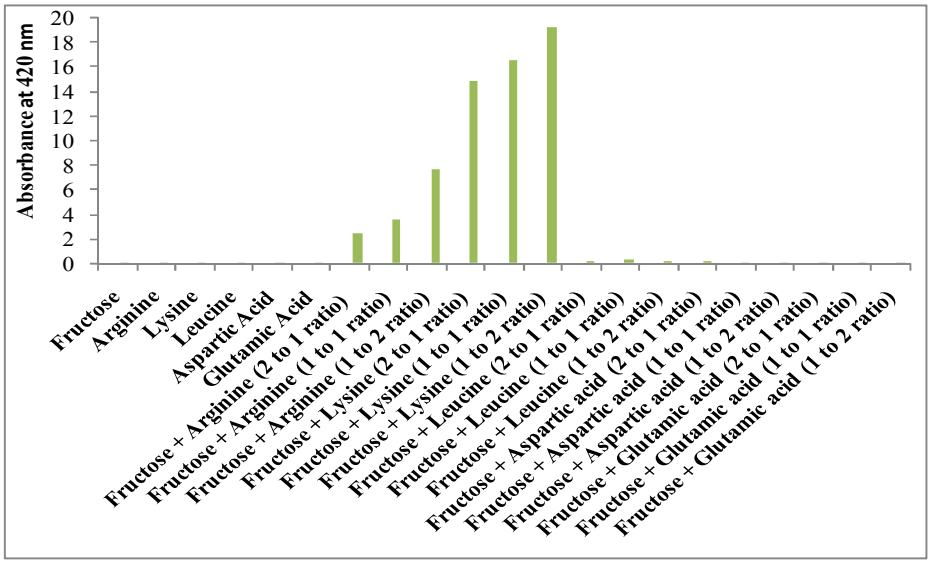


Figure 5.2 Comparison of absorbances (at 420 nm) of fructose and amino acids in different combinations and concentration ratios

Leucine in combination with fructose did not yield any distinct colour at all three ratios, but displayed a sweet sugar smell. Lysine was the most reactive amino acid in combination with fructose yielding a very dark brown colour and a strong, distinctive caramel, burnt odour. Lysine is often reported as being the most reactive amino acid in the Maillard reaction (O'Brien and Morrissey, 1997). Fructose + arginine combinations were less brown, with fainter caramel odours. Visual appearances of the brown colour (Table 5.2) showed similar high absorbances at 420 nm (Figure 5.2).

Evaporating fructose + arginine and fructose + lysine combinations clearly produced caramel compounds at 60°C. These are likely to include compounds such as the archetypal Maillard reaction compound 5-hydroxymetyl-2-furaldehyde (HMF). This

compound is yellow/brown (The Merck Index, 2001) and has a pleasant caramel smell. The dark brown colour, observed for fructose + lysine combination is probably due to caramel compounds and melanoidins (soluble nitrogen-containing polymers), the latter being generated in the later stages of the Maillard reaction (Wijewickreme *et al*., 1997). To identify these compounds, GC-MS analysis needs to be done. This will be discussed in Chapter 6.

Turning now to absorbances in the ultraviolet light (Table 5.3), individual amino acids and fructose incubated at 60°C for 65 hours showed no significant absorbances in the ultraviolet light range. (Data have been rounded to whole numbers for clarity.) In the case of fructose, this means that no reductones were being formed (see earlier), because reductones (e.g. ascorbic acid) absorb in the ultraviolet range. For example, ascorbic acid absorbs at 243 nm.

No significant absorbance was detected for fructose + aspartic acid nor fructose + glutamic acid combinations at all ratios. Fructose + leucine did show some absorbances but these were insignificant compared to results with arginine and lysine (Table 5.3). The most striking effect was observed for fructose + arginine and fructose + lysine combination displaying ultraviolet absorbance patterns peaking between 241 and 260 nm and 281 and 320 nm. These absorbance ranges have been associated with early-stage and low molecular weight Maillard reaction products (Kim and Lee, 2008, Bailey *et al*., 1996). The low molecular weight substances in turn are able of cross-link with free amino groups of lysine or arginine to produce the high-molecular-weight coloured melanoidins (i.e. the later stages of the Maillard reaction), as was observed particularly with fructose-lysine combinations (Table 5.2). In most cases, the higher amino acid combination ratio (i.e. 1 part sugar to 2 parts amino acid) gave the highest absorbances.

For the melanoidins compounds to be formed, free amino moieties have to be available in the reaction mixture in the later stages of the Maillard reaction to produce the darker brown compounds.

Table 5.3 Dilution-corrected absorbances at approximate wavescan peaks of different fructose + amino acids combinations after evaporation and reconstitution

Model system	Ratio of fructose to amino acid	200 – 210 nm	211 – 220 nm	221 – 230 nm	231 – 240 nm	241 – 250 nm	251 – 260 nm	261 – 270 nm	271 – 280 nm	281 – 320 nm
Fructose										
Arginine										
Lysine										
Leucine										
Aspartic acid										
Glutamic acid										
Fructose + Arginine	2 to 1	3	4	5	5	6	5	6		16
Fructose + Arginine	1 to 1	3	4	6	6	6	6	6		18
Fructose + Arginine	1 to 2	6	7		12	16	12	0		39
Fructose + Lysine	2 to 1	10	8	21		15	20	17	18	50
Fructose + Lysine	1 to 1	9	10	17		21	17	19		
Fructose + Lysine	1 to 2		18	33	24	29	30	26		66
Fructose + Leucine	2 to 1									1
Fructose + Leucine	1 to 1						1			2
Fructose + Leucine	1 to 2									1
Fructose + Aspartic acid	2 to 1				1					2
Fructose + Aspartic acid	1 to 1									1
Fructose + Aspartic acid	1 to 2									
Fructose + Glutamic acid	2 to 1									
Fructose + Glutamic acid	1 to 1									
Fructose + Glutamic acid	1 to 2									

Blanks mean no peak was observed

Fructose-lysine also had peaks between 221 and 240 nm suggesting that early Maillard reaction products were also formed. In a given Maillard reaction mixture, a heterogeneous mixture of melanoidins may be present, each of which has a different maximum absorbance. The colour that was observed was the result of a highly concentrated mixture of ultraviolet active compounds (Davies and Labuza, year not available for referencing).

MacDougall and Granov (1998) also reported that the compounds produced in the Maillard reaction using different combinations of sugar and amino acids exhibited distinct characteristics in both the ultraviolet and visible spectral regions with different peak absorbances during their colour development.

The results show that the type and ratio of amino acid plays an important role in the contribution of the Maillard reaction. This factor will play an important part in the development of the ti kōuka spirit. It was evident from Section 3.4 that there were seasonal variations as well as genotype and environmental differences in the amino acid profile of the ti kōuka stem collected from different geographical location and at different times of the year. For a consistent stream of ti kōuka spirit production, sugar and amino acid content in the ti kōuka stem will have to be consistent. *Cordyline australis* will have to be grown from the same seed-raised progeny under the same environmental conditions and stems collected at the same time of the year. This will be discussed in more detail in Chapter 8.

5.3 The effect of sugar/amino acid models on Maillard reaction under different pH and reaction times at 80°C

In the previous section, the pH of the amino acid mixture was uncontrolled, governed as it was by the 'natural'pH determined by the particular amino acid as supplied. The pH of the Maillard reaction is an important parameter in the characteristics of ultraviolet light-absorbing intermediates (Ames *et al*., 1997) and the rate and final outcomes of the Maillard reaction. In Chapter 2 (Section 2.18) it was shown that adjusting the pH of ti kōuka stem incubations affected the characteristics of the spirit finally produced.

In this present section the reactivities of fructose (and glucose) in combination with arginine, lysine and leucine (the dominating amino acids of the ti kōuka stem) are

compared at different pH values and times. The intention was not to reproduce ti kōuka spirit formation with sugars and selected amino acids, but rather to explore the Maillard reaction outcomes where the initial pHs covered the range pH 3, 5, 7 and 9, but was also buffered around those values. In the ti kōuka experiments, no buffers were used and the pH dropped from 10 to about 7 (Table 2.17), indicating that hydrogen ions were produced during the Maillard reaction. Buffers minimize this effect and permit a more controlled study of the reaction (Jousse *et al*., 2002; Ames *et al*., 1993). Equally however, the anion of the buffer could interact with the reactants in some way enhancing the Maillard reaction (de Kok and Rosing, 1994) or it may exert a specific effect as a catalyst on the reaction (Rizzi, 2004).

The method chosen to explore the effects of controlled pH was that of Kwak and Lim (2004) who employed a higher temperature (80°C) than used in ti kōuka incubations (60°C) with no evaporative step.

Materials and methods

Amino acids, fructose, glucose, disodium hydrogen phosphate and citric acid were purchased from Sigma, BDH and Scientific Supplies, NZ (Appendix VIII). Disodium hydrogen phosphate (28.4 g) and citric acid (21.0 g) solutions were made up in 1 L deionised water (i.e. 0.2 M and 0.1 M, with pH 9.5 and 2.1 respectively). These two solutions were mixed in different proportion to yield citrate-phosphate buffered solutions with final pHs of 3.0, 5.0, 7.0 and 9.0. Six combination of fructose (0.01 M) and glucose (0.01 M) with arginine (0.02 M), lysine (0.02 M) and leucine (0.02 M) were made up in 100 mL of the above four pH buffers giving a total of 24 stock solutions(as shown in Table 5.4). Individual solutions of fructose, glucose, arginine, lysine and leucine were also prepared in pH 9.0 buffer, giving a final total of 29 stock solutions.

From the 100 mL of each of the total 29 stock solutions, 15 mL of each was sampled into three sets of screw-sealed glass tubes (87 tubes in total). The three sets of tubes were heated at 80°C for 60, 180 and 300 minutes. After heating, each set of tubes was removed from the waterbath, cooled immediately under running tap water and the development of yellow/brown colour was measured without delay at 420 nm against water as blank. The pH was also measured.

Results and Discussion

The results of the pH changes are tabulated in Table 5.4.

Table 5.4 Changes in pH of fructose and glucose heated at 80°C with different amino acid combination for different incubation times

Sugar/Amino acid made up in 100 mL pH buffered solution	pH before heating	pH after 60 minutes heating at 80°C	pH after 180 minutes heating at 80°C	pH after 300 minutes heating at 80°C	Odour after 300 minutes heating at 80°C
Fructose + Arginine	3.0	5.9	6.0	5.8	Odourless
Fructose + Lysine	3.0	6.2	6.1	6.1	Faint popcorn
Fructose + Leucine	3.0	3.2	3.2	3.2	1-Butanol
Glucose + Arginine	3.0	5.9	5.8	5.8	Faint popcorn
Glucose + Lysine	3.0	6.2	6.1	6.0	Faint popcorn
Glucose + Leucine	3.0	3.3	3.2	3.2	1-Butanol
Fructose + Arginine	5.0	8.7	8.5	8.3	Faint popcorn
Fructose + Lysine	5.0	8.9	8.6	8.3	Strong popcorn
Fructose + Leucine	5.0	5.0	5.0	5.0	1-Butanol
Glucose + Arginine	5.0	8.5	8.0	7.6	Faint caramel
Glucose + Lysine	5.0	8.6	7.8	7.3	Strong popcorn
Glucose + Leucine	5.0	5.0	5.0	5.0	1-Butanol
Fructose + Arginine	7.0	9.7	9.4	9.2	Popcorn
Fructose + Lysine	7.0	9.7	9.3	9.0	Strong popcorn
Fructose + Leucine	7.0	6.9	6.9	6.8	1-Butanol
Glucose + Arginine	7.0	9.6	9.4	9.1	Popcorn
Glucose + Lysine	7.0	9.5	9.0	8.6	Strong popcorn
Glucose + Leucine	7.0	6.9	6.8	6.8	Strong 1-Butanol
Fructose + Arginine	9.0	10.1	9.8	9.6	Strong popcorn
Fructose + Lysine	9.0	10.0	9.6	9.4	Burnt popcorn
Fructose + Leucine	9.0	8.2	8.0	7.8	Strong 1-Butanol
Glucose + Arginine	9.0	10.7	9.8	9.6	Strong popcorn
Glucose + Lysine	9.0	9.8	9.4	9.1	Burnt popcorn
Glucose + Leucine	9.0	8.1	7.7	7.5	Strong 1-Butanol
Fructose	9.0	8.4	8.1	7.9	Faint caramel
Glucose	9.0	8.6	8.3	8.1	Sweet sugar
Arginine	9.0	10.5	10.5	10.4	Odourless
Lysine	9.0	10.3	10.3	10.3	Odourless
Leucine	9.0	8.5	8.5	8.6	Odourless

At pHs 3, 5, and 7, the fructose + leucine and glucose + leucine combinations showed no significant pH change at all three heating times. At pH 9, both combinations showed a decrease of about 1 to 1.5 pH units. By contrast, for the other four sugar + amino acid

combinations, the pH increased by about 3, 2 to 4, 2 to 3, and 1 pH units at pHs 3, 5, 7, and 9 respectively. In short, pH values increased with arginine and lysine at all pHs, and decreased with leucine but only at pH 9. The least pH increase (1 pH unit) was at pH 9. A drop in pH could be an advantage for the spirit production as most alcoholic spirits are either neutral or slightly acidic.

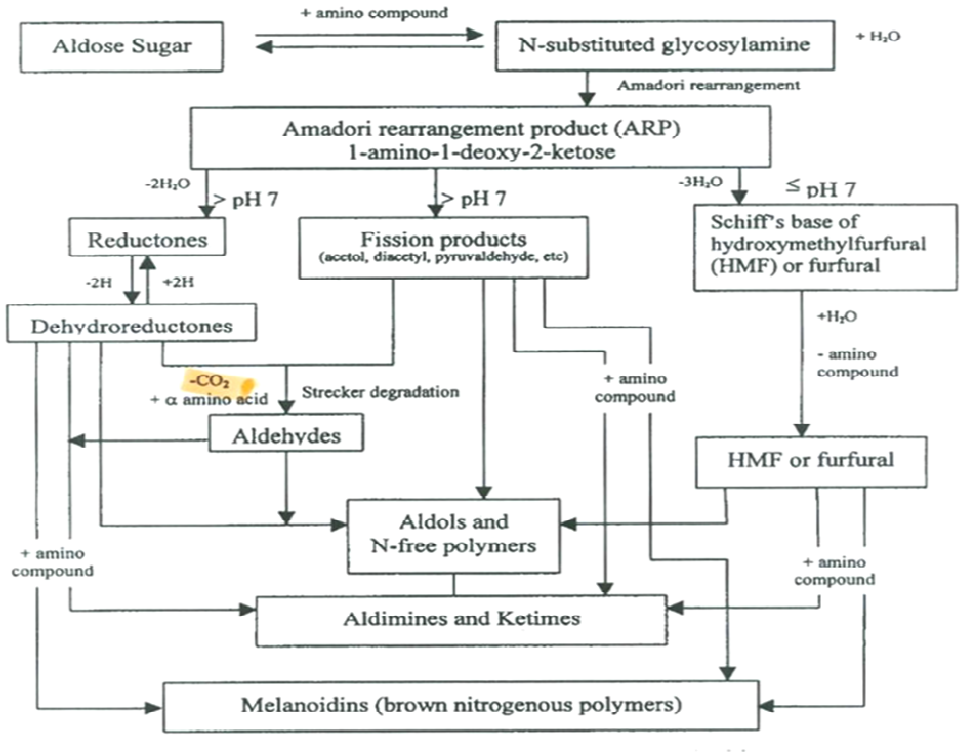


Figure 5.3 Maillard reaction scheme adapted from Hodge (1953)

A possible reason for the increase in pH of lysine and arginine (basic amino acids) may be due to Strecker degradation. In acidic conditions, sugar degradation takes place via 1,2-enolization with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (when hexoses are involved). In alkaline conditions (at pH >7), highly reactive dicarbonyl e.g. reductones (left hand side of the pathway in Figure 5.3) and a variety of fission products, including acetol, pyruvaldehyde, and diacetyl (middle pathway in Figure 5.3) are favoured. Dicarbonyl compounds initiate the Strecker reaction. In the presence of the α-dicarbonyl compound, α-amino acid are deaminated and decarboxylated (highlighted in Figure 5.3), leading to the generation of Strecker aldehydes and alpha-aminoketones and production of carbon dioxide which in turn may elevate the pH. An observation was made when the test tubes were open to read the

absorbances. A "pop" sound was heard. This suggests that carbon dioxide must have been produced during the heating process. However, a wooden splint test for carbon dioxide was not done, not realising at the time that it could be an important factor.

The pH dependence of the initial step of the Maillard reaction is related to the amount of unprotonated amine present (Martins *et al*., 2001). At pH 3 the amino groups of the amino acids are largely protonated and therefore the concentration of unprotonated amino acid available for participation in the Maillard reaction is small. At the pKa of the amine group, half the amine is present as the protonated $-NH_3^+$ state preventing electron transfer to the carboxyl group of the open-chain form of the sugar. Thus, the rate of the Maillard reaction is lower at a pH lower than the pKa of the reactive amino group. The pKa values of the α-amino group of arginine, lysine, and leucine, are 9.04, 8.95, and 9.74, respectively (Belitz *et al*., 2004). These values are in close proximity to the highest buffered model system used i.e. pH 9. Moreover, the pKa of the ε-amino group of lysine is 10.54. At pH 9, one might expect the rate of reaction to be faster than the other pHs used in the model system, which is exactly what was observed here.

As a general rule, the Maillard reaction from ketoses (e.g. fructose) proceeds through imine intermediates that favour the formation of Heyns products, while the aldose sugars (e.g. glucose) readily react to form Amadori products (Hodges, 1953). The rate of browning of Heyns products is documented to be slower than that of Amadori products (Pilkova *et al*., 1990). However, the differences observed in Figure 5.4 – which compares browning at 420 nm – were minor but nonetheless favoured glucose. One reason why fructose may remain almost reactive as glucose (Figure 5.4) may lie in the proportions of the open-chain forms of glucose and fructose. Naranjo *et al*. (1998) pointed out that fructose had the highest proportion of reactive open chain form compared to glucose, lactose and maltose.

The buffer type and reactants concentration also have a significant impact on the Maillard reaction. On the face of it, the reduced browning could be attributed to pH alone as pH governs protonation of amino groups. Bell (1997) proposed that the buffer's anions might affect the reaction. He found that citrate was neither an inhibitor nor a promoter of the Maillard reaction. However, he found that phosphate enhanced the rate of amino acid loss and brown pigment formation in solutions at pH 7 even at

25°C. Bell concluded that phosphate acts as an acid/base catalyst to speed up the glycosylation of amino acids.

Absorbances at 420 nm (corrected for any required dilution) are shown in pH categories in Figure 5.4. The raw data are presented in Appendix XI.

It is clear that the models became darker with time. (Plots of absorbances as a function of time as continuous data showed that the increase in absorbances were roughly linear.) Fructose and glucose yielded a browner colour with lysine than with arginine at all pHs and incubation times. Bates *et al*. (1994) also found that in the pH range between 3.4 and 7.7, the browning of glucose + lysine system increased with increase in pH, and this was confirmed here. However, Gögüs *et al*. (1998) found that fructose was more colour yielding than glucose in acid media.

Figure 5.4 shows that when fructose and glucose were heated in the absence of amino acids at an initial pH 9, brown pigments were generated. This phenomenon is likely to have contributed to the absorbances observed for the other sugar/amino acid combinations at pH 9. For instance, comparison of the absorbance values of fructose and fructose + leucine at pH 9, shows the absorbance of the latter was only slightly higher. However, the other combinations did exhibit convincingly higher values when amino acids were present. Leucine was not very reactive with fructose. Overall, pH 9 was the most effective pH in the generation of Maillard browning products. Therefore, further experiments were conducted at this pH.

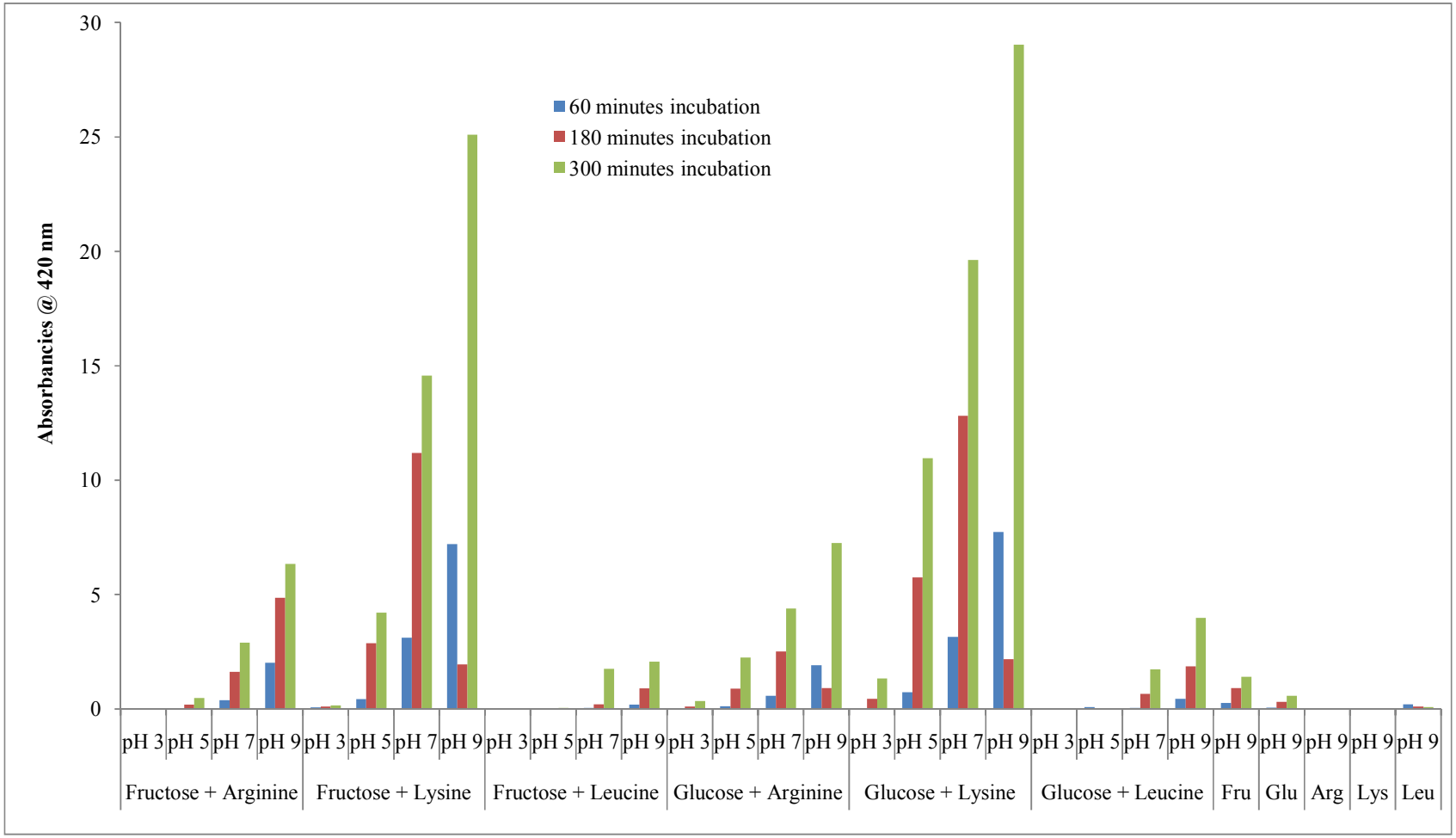


Figure 5.4 Absorbances of the 29 sugar/amino acid model system (at 420 nm) at different pH and incubation times

5.4 The effect of sugar/amino acid models on evaporation at 60°C, followed by subsequent infusion with ethanol

The previous section followed the Kwak and Lim (2004) method to explore the Maillard reaction with amino acids and sugars of interest under buffered conditions, with no evaporation step i.e. the whole process was carried out at 80°C in a waterbath, where the reactants remain in a liquid phase throughout the incubation period. There was clear evidence that buffering failed, possibly due to decarboxylation by the Strecker reaction leading to increased alkalinity. Nonetheless, it was clear that an initial pH of 9 was the most effective in generating Maillard reaction products. This result was consistent with the ti kōuka results in Chapter 2 (Section 2.18) where the best conditions for evaporating the ti kōuka extracts were at 60°C for 65 hours at pH 9.0 to 10.0.

Using the same amino acids and sugars as in Section 5.3, this section explores the approximate in vitro simulation of the ti kōuka spirit production. Here, the reactants are in liquid phase to start with but eventually evaporate into a dry layer.

Method

Stock solutions (with only pH 9.0) of fructose and glucose, and the amino acids arginine, leucine and lysine were prepared as in Section 5.3 (Materials and methods), and 50 mL of these stock solutions were evaporated at 60°C for 65 hours. The combinations were as described in Section 5.3. Appearance and odour were assessed before reconstituting the samples with deionised water to their original volume, 50 mL. Wavescans between 200 and 800 nm wavelength (Table 5.6) and pH were measured after reconstitution with water (Table 5.5). The reconstituted samples were then centrifuged for 15 minutes at 3700 gravities, and the supernatants infused with different combinations of potable ethanol to yield the usual final ethanol concentrations of 80, 67, 57 and 50% (v/v). Odour (Table 5.5) and wavescans (Table 5.7) were recorded on the infused samples.

The ethanol-treated mixtures were further centrifuged for 15 minutes at 3700 gravities. The supernatant were vacuum-filtered through Whatman GF/B glass micro-fibre filter paper.

Results and Discussion

All combinations of fructose and glucose with arginine and lysine were soluble prior to evaporation and after reconstitution. As was observed in Section 5.2, leucine was largely insoluble in water. When the leucine dispersion was mixed with fructose or glucose in deionised water, evaporated and reconstituted, what was almost certainly leucine remained insoluble. This observation alone suggested that leucine was unreactive. However, the reconstituted leucine-containing mixtures had odours that were not evident prior to reaction, suggesting some chemical reaction must have occurred.

After the 65 hours of evaporation, and reconstitution, the initial buffered pH value of 9 had decreased on average, by 0.3 to 0.9 pH units except for the fructose + leucine and glucose + leucine combinations in which case the pH decreased, on average, by 1.5 to 1.9 pH units respectively (Table 5.5). This may explain the observations made previously with ti kōuka stem extract (Section 4.3). In that work, the relative proportions of the dominating amino acids leucine and lysine decreased during evaporation and reconstitution, which indicated that these two amino acids dominated the Maillard reaction in the stem extracts. The decrease in pH may be due to the formation of organic acids (van Boekel and Brand, 1998), or due to the inability of amino moieties to act as bases when the amino compounds are reacting during the Maillard reaction (Mancilla-Margalli and Lopez, 2002). It could also be due to the disappearance of basic amino groups at the early stages of the reaction (Hill *et al.*, 1996).

These pH changes were in marked contrast to those experienced in the previous section where the mixture was heated at 80°C, but not evaporated. In that experiment, the pH values increased by about 1 pH unit where lysine and arginine were used, and decreased by about 1 pH unit where leucine was used. The mechanism of these differences between the two experiments is not known, but is probably related to water activity. A possible explanation is that in the initial stage of evaporation (and presumably Maillard reaction), enhancement of the reaction occurs thereby producing early Maillard reaction products. As the water evaporates, it concentrates the reactants so initially increasing reaction rates. However, the high reactant concentration ultimately leads to loss of mobility (Fennema, 1996). Several phases of the Maillard reaction effectively involve

dehydration of sugars. It follows that dehydration pathways will be favoured in the evaporation models explored in this section, but much less so in the aqueous model explored in the previous section (Section 5.3). The different pathways are likely to have different pH outcomes.

The combinations fructose + leucine and glucose + leucine produced light brown and dark brown residues respectively. All other combinations were dark-brown to black after evaporation. The fructose + leucine residue was the least odorous, a faint 2-butanol note, the origin of which is not obvious from inspection of known Maillard reaction schemes. Likewise, the origin of the almond note from glucose + leucine is unknown, realising that the characteristic almond smell is caused by benzaldehyde. Lysine gave a clear popcorn-like odour with both sugars, while arginine yielded caramel notes, both of which have been well described in model Maillard reaction systems (Wong *et al*., 2008).

When reconstituted fructose + leucine and glucose + leucine combinations were infused with ethanol to achieve final concentrations of 50, 57, 67 and 80%, all the purported leucine dissolved, as might be expected with ethanol as a less hydrophobic solvent (Belitz *et al*., 2004). The four fructose + leucine combinations exhibited an odour of alcohol (80, 67% ethanol) or 2-butanol (57, 50%), while the four glucose + leucine combinations exhibited 2-butanol rather than ester/almond before infusion. The 2-butanol note was confirmed by several colleagues comparing the odours with that of authentic 2-butanol. (However, this does not prove identity because other isomers and similar alcohols may have a closely similar odour.) The four combinations of fructose + arginine, fructose + lysine, glucose + arginine and glucose + lysine all gave burnt sugar or caramel odour, typical of later stages of Maillard reaction products.

As the concentration of ethanol decreased, the odour of the infusions became more pronounced. There maybe two reasons for this. Firstly, aromas that are very soluble in ethanol will be released to the gas phase less readily, and secondly ethanol will be released to gas phase more readily, potentially swamping other odours. Another possible reason could be that different ethanol concentrations correspond to different polarities of the solvent and different solubility for each solute.

Table 5.5 pH changes after evaporation and reconstitution and pH and odour after ethanol infusion

Model	pH before heating	pH after evaporation and reconstitution	Appearance after evaporation and before reconstitution	Odour after evaporation and before reconstitution	Alcohol concentration after infusion (%)	Appearn after ethanol infusion	Odour after ethanol infusion
Fructose + Arginine	9.0	8.5	Dark-brown to black	Faint caramel	80		Sweet candy
	9.0	8.5			67		Faint caramel
	9.0	8.5			57		Burnt caramel/alcohol
	9.0	8.5			50		alcohol
Fructose + Lysine	9.0	8.7	Dark-brown to black	Faint popcorn	80		Faint burnt sugar
	9.0	8.7			67		Faint caramel
	9.0	8.7			57		Caramel
	9.0	8.7			50		Caramel
Fructose + Leucine	9.0	7.5	Light brown layer	Faint 2-butanol	80		Alcohol
	9.0	7.5			67		Alcohol
	9.0	7.5			57		2-Butanol
	9.0	7.5			50		2-Butanol
Glucose + Arginine	9.0	8.4	Dark-brown to black	Very faint popcorn	80		Alcohol
	9.0	8.4			67		Sweet sugar/alcohol
	9.0	8.4			57		Faint caramel
	9.0	8.4			50		Faint caramel
Glucose + Lysine	9.0	8.1	Black layer	Popcorn	80		Faint burnt sugar
	9.0	8.1			67		Faint burnt sugar
	9.0	8.1			57		Faint burnt sugar/alcohol
	9.0	8.1			50		Caramel
Glucose + Leucine	9.0	7.1	Brown layer	Ester/almond	80		2-Butanol
	9.0	7.1			67		2-Butanol
	9.0	7.1			57		2-Butanol
	9.0	7.1				50	2-Butanol

Turning now to the wavescan peaks absorbances (Table 5.6), glucose combinations, on average, had higher absorbances than fructose combinations. Data have been rounded to whole numbers for clarity. Absorbances of the fructose + arginine, glucose + arginine and glucose + lysine combinations had high absorbances in most wavelength ranges, with the highest peaks occurring between 281 and 320 nm (Table 5.6). By contrast, fructose + lysine showed the highest absorbance between 231 and 240 nm. Maillard reaction products absorbing between 281 and 320 nm are so-called intermediate reaction products (see Section 5.2). This raises the question as to whether the absence of a peak for fructose + lysine in the range 281 to 320 nm means the overall reaction has not progressed much or has gone more toward completion. The colour of the residue, dark-brown to black suggests the latter is true, as is seen from the high absorbances at 420 nm (Table 5.6). Fructose + lysine and glucose + lysine combinations had the highest absorbances at 420 nm after evaporation and reconstitution, again proving the obvious that lysine is the most reactive amino acid. Fructose + leucine and glucose + leucine had the lowest absorbances. Leucine obviously was not involved in the final stages of the Maillard reaction.

Table 5.6 Absorbances of wavescan peaks (and 420 nm wavelength) of different model combinations evaporated at 60°C for 65 hours and reconstituted with deionised water

	200 – 210 nm	211 – 220 nm	221 – 230 nm	231 – 240 nm	241 – 250 nm	251 – 260 nm	261 -270 nm	271 -280 nm	281 – 320 nm	420 nm
Fructose + Arginine	37	41	68	61	74	80	72		192	43
Fructose + Lysine		58		137	87	78	86			69
Fructose + Leucine		3	6	5	8	7	7		23	4
Glucose + Arginine		41	72	59	66	76	85	70	173	50
Glucose + Lysine	49		88	84	129	91	77		256	71
Glucose + Leucine	19	21	31		35	43	31	37	76	16

Blank means no peak was observed

Table 5.7 Absorbances of wavescan (200 – 320 nm) and at 420 nm of sugar/amino acids combination after ethanol infusion

Sugar/Amino Acid	Ethanol concentration after infusion (%)	200 - 210 nm	211- 220 nm	221 – 230 nm	231 - 240 nm	241 – 250 nm	251 – 260 nm	261 – 270 nm	271 – 280 nm	281 – 320 nm	420 nm
Fructose + Arginine	80	2	2	5	3	5	4	5		13	2
	67	8	9	10	11	13	12	11	12	34	6
	57		16	29	27	26	28	29		60	11
	50		19	29	26	27	26	27		84	11
Fructose + Lysine	80	3	2	3	3	3	3	3		10	2
	67	4	6	7	8	9	8			24	5
	57	10		17	12	21	20		20	52	11
	50	17	19	26	36	21	29	25		68	15
Fructose + Leucine	80	1	1	2	2	2	2	2	2	5	1
	67		2	2	2	2	2	2		8	1
	57	1	2	3	2	3	3	3	3	12	2
	50	3	3	2	3	3				12	2
Glucose + Arginine	80	2	3	4	4	5	4		4	12	2
	67	4	5	7	7	10	7	7	8	29	5
	57		12	16		15	16	14	14	56	10
	50	13	25	26	30	27	20	26		82	17
Glucose + Lysine	80		19	28	23	29	26	29	24	83	1
	67	6	4	7	6	8	7			24	4
	57	8	12	12		13	14	12		55	12
	50	6	10	10	10	15	13	14		43	19
Glucose + Leucine	80	2	3	4	5	5	4	5	5	14	3
	67	4	4	7	5	8	7	7		24	5
	57	8	9	11	12	15	13	11	13	34	7
	50		11	12	11	12	12		12	40	8

What was immediately striking when ethanol was infused was that the absorbances of the wavescan increased drastically, with 50% ethanol infusion samples having the highest absorbances of the four-ethanol concentrations (Table 5.7). This suggests that the coloured Maillard reaction products formed during evaporation were more soluble in water than in ethanol. Fructose + arginine and glucose + arginine combination had the highest absorbance values followed by fructose + lysine and glucose + lysine, and glucose + leucine and fructose + leucine having the lowest absorbances. It makes sense that the ethanol infusion of leucine combinations had the lowest absorbances since the reactions did not go to completion. Therefore no brown coloured compounds were formed.

Compared to lysine, arginine had lower absorbances at 420 nm (with both fructose and glucose combinations) yet after ethanol infusion there was not much difference in the absorbances of the corresponding ethanol concentration of the four sugar- amino acid combinations. A possible explanation is that the Maillard reaction products of lysine (with fructose and glucose combination) were not as soluble in ethanol as the products from arginine.

5.5 Effect of different combinations of more than one amino acid with fructose on the Maillard reaction

In the previous experiments, only one amino acid with fructose or glucose was used in the model system to investigate the non-enzymatic reaction. It was clear from the HPLC analysis of different parts of the ti kōuka stem (Chapter 4) that more than one amino acid was available to take part in the Maillard reaction. Therefore, in this next set of experiments, different concentrations and combinations of fructose and more than one amino acid was used in the model systems. The amino acids were arginine, aspartic and glutamic acids, lysine and leucine, the major amino acids in ti kōuka stem.

Materials and Methods

Phosphate buffer (0.2 M, pH 9.0) was prepared as before (Section 5.3), and used to prepare 0.01, 0.05, 0.1 and 0.5M solutions of fructose, arginine, aspartic acid, glutamic acid, lysine and leucine. These stock solutions were used (with corresponding sugar/amino acid concentrations) to make model combinations (A to O) as shown in Table 5.8.

Table 5.8 Model systems containing combination of fructose and amino acid/s with total volume of 150 mL. There were four such sets corresponding to the 0.01, 0.05, 0.1 and 0.5 M concentrations. In all cases except Models M, N and O, the molar ratio of fructose to each amino acid was 5:1

Model	Volume of buffer (mL)	Fructose (mL)	Arginine (mL)	Aspartic acid (mL)	Glutamic acid (mL)	Lysine (mL)	Leucine (mL)
A	75	75	0	0	0	0	0
B	60	75	15	0	0	0	0
C	60	75	0	15	0	0	0
D	60	75	0	0	15	0	0
E	60	75	0	0	0	15	0
F	60	75	0	0	0	0	15
G	45	75	15	0	0	15	0
H	45	75	15	0	0	0	15
I	45	75	0	0	0	15	15
J	45	75	15	15	15	0	0
K	45	75	15	0	0	15	15
L	30	75	15	15	0	15	15
M	15	75	15	0	15	15	15
N	75	0	15	15	15	15	15
O	150	0	0	0	0	0	0

Fifteen milliliters of each model stock solution (at each corresponding sugar/amino acid concentrations) were sampled into 8 sets of screw-sealed glass tubes. The tubes were sealed as in Section 5.3 and placed in a waterbath at 80°C and each set (A to O) were heated for 0, 30, 60, 90, 120, 180, 240 or 300 minutes. At each time point, the tubes were immediately cooled under ice-cold water. As soon as was practicable after each final time point, brownness was measured in 1 cm cuvettes in a spectrophotometer at 420 nm using water as blank. Wavescans between 200 and 800 nm wavelength were also measured using a single quartz cuvette. Known dilutions were made with deionised water before performing the wavescans to ensure the absorbances were between 0 and 3. Absorbance data presented are corrected for dilutions, and are sometimes rounded to whole numbers for clarity. Appearance and odour were also recorded (Table 5.9). Model A containing only fructose was used to determine the contribution of caramelisation to non-enzymatic browning. Model N acted as a control containing all the amino acids with no fructose and Model O acted as a blank with no fructose or amino acid.

Results and Discussion

All stock solutions of fructose and amino acids (except leucine) were soluble in phosphate buffer at all concentrations. Leucine was soluble at 0.01 and 0.05 M, sparingly soluble at 0.1 M, and largely insoluble at 0.5 M even when heated. However, when the dispersed leucine stock solution/suspension was added to fructose and other amino acids to make up the Models A to O, the stock leucine solution became soluble in all the Models A to O at 0.01, 0.05 and 0.1 M. However, some leucine in Models M and L at 0.5 M remained insoluble.

The results of absorbance, appearance and odour for all four concentrations followed similar trend. All model combinations at all concentrations were colourless at time zero. As the time of incubation proceeded, colour developed, except in Models N and O, which consisted only of amino acids and buffer respectively. At 0.01 and 0.05 M concentration, some models yielded only very light yellow colours at 300 minutes while at 0.1 M concentration, light brown colours were produced (data not shown). Clearly, the concentration of the reactants at lower concentrations was not sufficient to allow maximum interaction between the reactants within the heating period, thus prohibiting the intermediate and final stages of the Maillard reaction. On the contrary, the 0.5 M concentration set yielded a range of colours from colourless to dark brown. Therefore, further discussion is focused mainly on the results obtained with the 0.5 M concentration set of 15 tubes.

Absorbances at 0 and 300 minutes are shown in Table 5.9 to focus first on the initial and final outcome of the reactions. Absorbances of the Models at 420 nm are presented in descending order (Table 5.9). There was an obvious trend in the final appearances (Table 5.9 and Figure 5.5) which could be divided into five colour categories – namely, dark brown, brown, yellow, light yellow and colourless. The increasing colour intensity indicated the progression from early, to intermediate, to final stages of the reaction.

Table 5.9 Absorbances, odour and appearance of fructose with different combinations of amino acids incubated at 80°C

Model System	Sugar/Amino acid combinations	Absorbance at 420 nm		Odour at 300 minutes incubation	Appearance at 300 minutes incubation
		0 minute incubation	300 minutes incubation		
G	Fructose + Arginine + Lysine	0.01	13.70	Popcorn	Dark brown
E	Fructose + Lysine	0.01	13.10	Popcorn	Dark brown
I	Fructose + Lysine + Leucine	0.04	12.00	Popcorn	Dark brown
K	Fructose + Arginine + Lysine + Leucine	0.01	9.73	Popcorn	Dark brown
B	Fructose + Arginine	0.00	4.72	Faint caramel	Brown
M	Fructose + Arginine + Glutamic acid + Lysine + Leucine	0.01	4.55	Aldehyde	Brown
L	Fructose + Arginine + Aspartic acid + Lysine + Leucine	0.01	4.43	Aldehyde	Brown
H	Fructose + Arginine + Leucine	0.04	3.90	Aldehyde	Light brown
F	Fructose + Leucine	0.00	2.07	Aldehyde	Yellow
A	Fructose	0.01	1.75	Faint caramel	Yellow
C	Fructose + Aspartic acid	0.03	0.46	Faint caramel	Light yellow
D	Fructose + Glutamic acid	0.03	0.45	Faint caramel	Light yellow
J	Fructose + Arginine + Aspartic acid + Glutamic acid	0.05	0.34	Very faint aldehyde	Light yellow
N	Arginine + Aspartic acid + Glutamic acid + Lysine + Leucine	0.00	0.04	Odourless	Colourless
O	Buffer	0.01	0.01	Odourless	Colourless

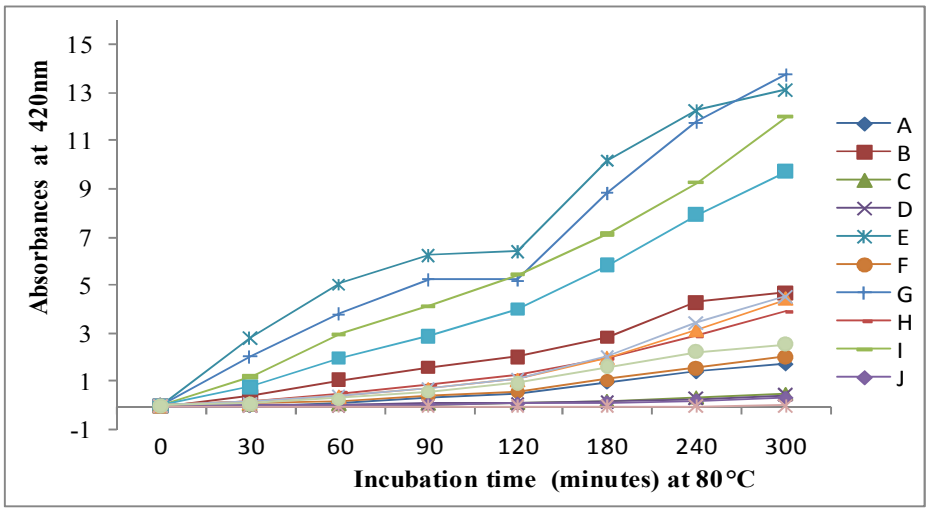


Figure 5.5 Kinetics of colour development for different fructose and amino acids combinations at 80°C

In Models where fructose was present with single amino acids, the overall browning was either faster (lysine (E), arginine (B) and leucine (F)) or slower (aspartic (C) and glutamic (D) acids) than for fructose alone (Table 5.9). Lysine and arginine yielded brown to dark brown colour suggesting formation of polymeric melanoidins compounds. Possible chemical identities that are contained in the brown products include furans, pyrroles, and pyridine components as well as pyrazines.

Models G, E, I and K (lysine being common amino acid in all these models) yielded the highest absorbances. The high reactivity of lysine was also observed previously in Section 5.3, Figure 5.4 and is in agreement with results of Ajandouz and Puigserver (1999), and Kwak and Lim (2004).

As noted earlier, fructose + leucine (F) gave a lower absorbance than equivalent Models of fructose + lysine (E) and fructose + arginine (B). Moreover, fructose + leucine gave a similar absorbance at 420 nm to fructose alone (fructose + leucine = 2.07 cf. fructose = 1.75). In a prior experiment (Section 5.3) at pH 9, where the molar proportion of fructose to amino acid was 1:2, the absorbances of fructose + lysine and fructose + arginine were roughly double compared to those in the present 5:1 experiment. However, the absorbance due to fructose + leucine was the same. Thus, leucine is relatively unreactive in any ratio with fructose.

Aspartic and glutamic acids in combination with fructose gave lower absorbances than fructose alone. These two acidic amino acids are known to be the least reactive in producing Maillard browning, as was also observed by Lamberts *et al*. (2008). Moreover, adding aspartic or glutamic acid to Models with arginine, lysine or leucine (i.e. Models J, L, M and N) also gave lower absorbances. These two acidic amino acids were clearly hindering the reaction to proceed to the latter stages of the Maillard reaction.

Fructose alone yielded yellow colour at 300 minutes incubation. Some reaction (caramelisation) similar to non-enzymatic browning was taking place. When fructose is heated, it causes dehydration (loss of water) of the sugar molecule. As with the Maillard reaction, odorous compounds are formed, liberating water and carbon dioxide. Intermediate products such as 3-deoxyosones and furans were probably formed (hence the yellow colour and caramel odour). Carboxylic acids and their salts, phosphates, and metallic ions (Nursten, 2005) accelerate caramelisation. Even though phosphate salts (present in the phosphate buffer that was used to make up the Models) would have facilitated the caramelisation reaction, a brown colour did not develop with fructose due to the reaction temperature not being high enough, normally requiring temperatures above 100°C.

As expected, heating amino acids and buffer alone (N and O) gave no brown colour. Wong *et al*. (2008) also observed in their study that none of the amino acids, with the exception of tryptophan, gave rise to browning in the absence of glucose. Inspection of the kinetics of absorbances at 420 nm shows that all combinations except two followed an essentially linear increase with time (Figure 5.5). The two exceptions were Models E (fructose + lysine) and G (fructose + lysine + arginine).

Consider the simpler Model E first. As noted earlier, lysine has two reactive amino groups with pKa values of 8.9 (α-amino) and 10.28 (ε-amino). Around pH 9, the more reactive group would probably be the α-amino group assuming the unprotonated amino group is the reactive species in the initial step of the Maillard reaction. It is proposed that this amino group reacts first and tends to saturate with respect to 420 nm absorbance at around 100 minutes. It is further proposed that the ε-amino group reacts more slowly (being partly protonated) and that after a lag phase lasting perhaps 80 minutes – where a pool of secondary reaction products accumulates – a second

browning phase occurs that adds to the browning derived from the α-amino group. However, the addition of arginine to the mix (Model G) complicates the proposition, because fructose + arginine alone is reactive (Table 5.9; Figure 5.5), yet it slows the proposed reaction with lysine except at the very end, 300 minutes, where the final absorbances were very similar. Thus, the effect of the additional arginine was not cumulative except perhaps at the very end. The inhibitive phenomenon was also seen in other Models, where addition of a further amino acid to a Model generally reduced browning. The most notable example of this is Model J. Adding more amino acids to fructose will create competition for available fructose. However, the molar concentration of fructose in all models (except Model N where it was absent) was always greater than the concentration of the amino acid(s). There appears to be no obvious kinetic scheme to explain the inhibitive phenomenon.

Turning now to wavescan data (Table 5.10), all Models (except N and O) had their highest peak in the 281 – 320 nm wavelength range, followed by 261 – 280 nm and 241 – 260 nm. Fructose + leucine in the ratio 5:1 (Table 5.8) yielded lower absorbance between 281 – 320 nm wavelength than equivalent Models of fructose + lysine and fructose + arginine. Again, Models G, E, I and K were on top of the table followed by B, M, L and H. All these eight models would have been involved in the sugar condensation, dehydration and fragmentation to produce early and intermediate Maillard reaction products.

The pattern of colour development may be further explored by more detailed analysis of the ratio of absorbances at wavelengths 294 nm (representative of intermediate compounds) and 420 nm (related to the content of brown polymers). The absorbance ratio A_{294}/A_{420} was calculated to monitor the transformation of UV-absorbing compounds into brown polymers (Benjakul *et al*., 2005). Figure 5.6 represents the highest peak absorbances between 281 to 320 nm, at 420 nm and the ration of these two absorbances for Models A to M. Models N and O were not included since no peak absorbances (Table 5.10) was observed at those wavelength.

Table 5.10 Dilution-corrected absorbances at approximate wavescan peaks of different fructose (0.5 M) and amino acid (0.5 M) combinations incubated at 80°C for 300 minutes

Model System	Fructose/ Amino acid combinations	200 – 210 nm	211 – 220 nm	221 – 230 nm	231 – 240 nm	241 – 250 nm	251 – 260 nm	261 - 270 nm	271 - 280 nm	281 – 320 nm	420 nm	Ratio of 294/420 nm
G	Fructose + Arginine + Lysine	11	13	22	17	25	22		24	94	14	6.7
E	Fructose + Lysine	9	12	19		23	24			89	13	6.8
	Fructose + Lysine + Leucine	16	11	29	22	32	33	33		91	12	7.6
K	Fructose + Arginine + Lysine + Leucine	7		23	13	16	14	18	15	67	10	6.7
B	Fructose + Arginine	7		8	7	11	9		12	45	5	9.0
M	Fructose + Arginine + Glutamic acid + Lysine + Leucine		6	8		10	10	10	10	57	5	11.4
L	Fructose + Arginine + Aspartic acid + Lysine + Leucine	7	5	9	9	13		11	10	48	4	12.0
H	Fructose + Arginine + Leucine	6	5	11	10	10	8	8	9	41	4	10.3
F	Fructose + Leucine	3	3	6	6	4	5		6	24	2	12.0
A	Fructose	1	1	3	2	2	3	3		17	2	8.5
C	Fructose + Aspartic acid	1		2		2	1	2		6		12.0
D	Fructose + Glutamic acid	1		1	1	2	2	2	2	7		14.0
J	Fructose + Arginine + Aspartic acid + Glutamic acid	1		2		2	2	2	1	7		
N	Arginine + Aspartic acid + Glutamic acid + Lysine + Leucine											

Blanks mean no peak was observed

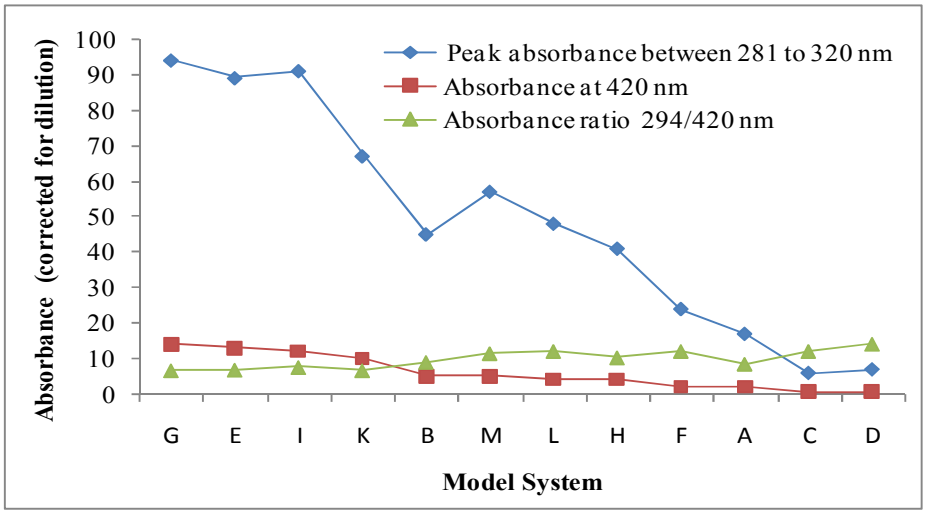


Figure 5.6 Absorbances of Models at 294 and 420 nm and their ratios

The absorbance ratio of the Models varied with the type of amino acid(s) present (Figure 5.6). The highest ratio values were from Models M, L, F, C and D, although visually that is not obvious in Figure 5.6 because the absorbance scale was expanded to cope with the high absorbance at 294 nm. The ratio values were lower from Models G, E, I and K, where lysine was common to all Models. In other words, a large proportion of the intermediate products produced in Models G, E, I and K acted as precursors, and in the presence of amino acid(s) underwent polymerisation to produce brown melanoidins compounds. Meanwhile, in Models M, L, F, C and D some intermediate products may still be in the process of being generated by the reactants. Lysine must be the key amino acid that was taking part in polymerising the intermediate Maillard reaction products. Models M, L, F, C and D contained aspartic acid, glutamic acid or leucine. As was observed earlier, the two acidic acids slowed the Maillard reaction; therefore, not enough intermediate Maillard products were produced to polymerise into brown polymers. The final appearances of colour (Table 5.9) somewhat relates to the calculated absorbance ratio (degree of polymerisation), where the brown to dark brown colour corresponds to lower ratio values than with fructose alone, while the light yellow to yellow corresponding to higher ratio values than for fructose alone (Table 5.10).

The Maillard reaction participates not only in the formation of colour but also in aroma generation i.e. during cooking and thermal processing in the food industry e.g. sweet and bakery aromas that are associated with roasted cocoa and nuts, chocolate, malt, popcorn and bread (Cerny, 2008). The volatiles produced from the Maillard reaction

are generally only present at trace concentrations e.g. nitrogen-containing aroma compounds are in a magnitude range of 0.001 to 10 mg kg^{-1} of food (Cerny, 2008). The differences in odour between the 15 combinations at all four concentrations (0.01 to 0.5 M) were similar and as might be predicted, the lowest and the highest concentrations (0.01 and 0.5 M) of reactants yielded the least and greatest intensities. The odour data for the 0.5 M set are shown in Table 5.9.

Fructose produced a caramel smell (Table 5.9). Models G, E, I and K (lysine being the common amino acid) gave a faint to strong popcorn smell, while Models M, L, H, and F (the common amino acid was leucine) and J gave faint to strong aldehyde odour. Clearly, lysine was responsible for the popcorn-like odour while leucine was responsible for the aldehyde note. Arginine, aspartic and glutamic acid in combination with fructose all yielded a faint caramel odour that may be traced to fructose alone.

The most likely cause of the aldeyhde odour is the Strecker pathway (Schönberg and Mouchacher, 1952). Aldehydes produced from amino acids are often major contributors to the aroma produced during nonenzymatic browning e.g. 3-methylbutanal from L-leucine.

When compounds containing primary amino acids (e.g. lysine) are reacted with fructose or glucose, the most important classes of compounds formed are pyrroles (BeMiller and Huber, 2008). Hence, the 2-acetyl-1-pyrroline (a pyrrole) is responsible for the typical popcorn-like aroma (Belitz *et al*., 2008) yielded by lysine.

The intensity of odours produced in this section was definitely higher than in the previous section (Section 5.4) where the Models were dried in the manner of ti kōuka spirit production before reconstitution and odour assessment. There are several possible reasons for this (as discussed previously). Differences in temperature and water activity may be playing a major part. Firstly, although the temperature of the oven is set at 60°C, the actual temperature of the ti kōuka extract would be less due to evaporation – cooling process that is going on (as was discussed in Section 4.3). Secondly, there is a loss of reactants' mobility as was discussed in Section 2.17 and Section 5.4. Thirdly, there may be loss of odour during evaporation. Some odorous compounds produced during the Maillard reaction would have been released from the Petri dishes in the oven at 60°C, therefore decreasing the intensity of the final odorous compounds. On the

other hand, in an enclosed environment, as these test tubes were at 80°C, any odorous compounds produced during the Maillard reaction would be retained within the test tubes. Finally, the overall temperature in the Model test tube would have retained close to 80°C, and it has been well documented that temperature plays a major role in the aroma profile of the Maillard reaction.

Turning now to ti kōuka stem, the percentages of total amino acid in ti kōuka stems collected at different times of the year (Table 3.9) showed that the most dominant amino acids were arginine, lysine and leucine. Analysis of the amino acids proportions during the different stages of the ti kōuka spirit production (Section 4.3) showed that leucine decreased and arginine increased during evaporation at 60°C in all three sections (tip to 20, 20 to 40 and 40 to 60 cm respectively) of the ti kōuka stem. The proportion of lysine increased in the second section (20 to 40 cm), but decreased in the top (tip to 20 cm) and third section (40 to 60 cm). Clearly, the three amino acids were the driving force of the Maillard reaction in the ti kōuka stem extract, which was also apparent in the Models. When ti kōuka stem extracts were evaporated at 60°C, they evaporated completely leaving a brown layer with a sweet caramel smell, while at100°C the extract layers were dark brown with strong caramel smell (Section 2.16). The caramel-like odour produced must be the product/s of the intermediate stages of the Maillard reaction. The difference between the ti kōuka stem and the model system is that the odours produced by the ti kōuka extract were not as intense as the models (as discussed above). Concentrations of fructose and amino acids were much less in ti kōuka extract compared to the Model system. Moreover, the pool of free amino acids in plant tissue is low, therefore the availability of unprotonated amino group may not have been high in the ti kōuka stem compared to the model system. However, calculating the ratios of fructose (Table 2.12) to arginine, lysine and leucine (Table 3.9) in the ti kōuka stem gave an approximate 1 to 2 ratio, same as the ratios used in Section 5.3. Clearly, there was evidence that Maillard reaction was taking place in the ti kōuka extract. The results obtained in previous chapters with the ti kōuka extract has a similar pattern to the results obtained using the simulation of the Model system.

In summary, the Maillard reaction can be used as a basis for the production of specific flavouring products by carefully selecting the sugars and amino acids, and controlling the reaction conditions. The type and ratio of amino acid, the concentration of the

reactants, pH and time of reaction plays an important role in controlling the type of odorous compounds and colour that are produced during the Maillard reaction. By increasing the pH, the intensity of browning increases, and prolonging the reaction time and concentration of reactants intensified the colour and odorous compounds. The approximate simulation of ti kōuka spirit production (Section 5.4) yielded desirable odorous and coloured compounds. This raises the question as to whether ti kōuka extract is essential to produce an alcoholic spirit.

In the next chapter (Chapter 6) analysis of volatile compounds in the ti kōuka spirit will be done. This will be compared with commercial spirits.

CHAPTER 6

Comparison of volatile compounds in the ti kōuka and commercial spirits

6.1 Introduction

In the previous chapter (Chapter 5), model system were used to simulate the reactions taking place between the amino acids and reducing sugar present in the ti kōuka extract. The model systems were carried out at different sugar to amino acid ratios and concentrations, pHs and reaction times.

As with the ethanol infusion with ti kōuka extract (Section 2.14), the sugar/amino acid models (Sections 5.2 to 5.5) also generated colour (ranging from colourless to brown) and aroma (including caramel, popcorn-like and aldehyde - all contributors to aroma produced during non-enzymatic browning). In both cases, colour and aroma were probably likely due to the hydrophilic compounds generated by the Maillard reaction, between simple sugars and amino acids.

The objective of this thesis was to systematically research the feasibility of producing a tequila-like spirit from ti kōuka stem and to compare the chemical properties of ti kōuka spirit (Ti Koka) with commercial spirits (i.e. tequila, gin and whisky). This chapter outlines the chemical properties of the ti kōuka spirit and compares it with tequila, gin and whisky, particularly tequila, because of the botanical similarity. Distilled spirits range from virtually pure aqueous alcohol, e.g. vodka, to aromatic products, such as tequila, whisky, gin, cognac, rum, and brandy. The flavourful distilled beverages are highly complex mixtures containing hundreds of individual compounds in an ethanol–water matrix that are variously generated or added at different stages of the spirit production.

Tequila is a product of complex process with many factors affecting the final composition of the spirit including cultivation region, harvesting, sugar extraction, hydrolysis, yeast, fermentation conditions, distillation, and aging in oak barrels (Peña-Alvarez *et al*., 2006). As a result of this process, different compounds e.g. alcohols (e.g. methanol, 1-propanol, 2-proponal, 1-butanol, 2-methyl propanol), fatty acids, esters, aldehydes (e.g. acetaldehyde), terpenes, phenols, lactones, sulphur compounds etc. has been reported as part of the aroma and flavour of the tequila spirit (Benn and Peppard, 1996).

Whiskies refer to a broad category of alcoholic beverages that are distilled from fermented grain mash and aged in oak barrels. Different grains are commonly used for different varieties, including barley, malted barley, rye, malted rye, wheat and maize (corn). In India, whisky is fermented from molasses, underscoring the fact that unaged whisky is clear, colourless and relatively flavourless. The flavours of the whiskies are derived largely from infused flavours in the barrel wood. Whiskies contain many volatile compounds, which belong to a great variety of chemical families such as ethyl esters, higher alcohols, fatty acids, higher alcohol acetates, carbonyl compounds (e.g. aldehydes and ketones), sulphur compounds, furanic compounds, lactones and volatile phenolics (Fitzgerald *et al*., 2000). As with tequila, many factors affect the final flavour of whisky (Caldeira *et al*., 2007).

Gin is a clear, colourless spirit that is by definition flavoured with at least juniper berries (*Juniperus communis*) and usually other plant material, the so-called botanicals (a varied assortment of herbs and spices). The adjunct botanicals can include aniseed, angelica root, cinnamon, orange peel, coriander seed, liquorice root, nutmeg and cassia bark. All gin makers have their own secret combination of botanicals, the number of which can range from as few as 4 to as many as 15. The combination used maintains a consistency of product flavour that can support brand identification. The spirit base of gin is primarily grain (usually wheat or rye), and as with whisky before aging, the spirit is clear, colourless and relatively flavourless. In a typical gin process, the botanicals and clear spirit are co-distilled to generate the final product. Gin is not barrel aged. As in all spirit beverages, several key volatiles and semi-volatiles strongly contribute to gin flavour perception e.g. the main monoterpenes in gin are α-pinene, β-myrcene and limonene followed by γ-terpinene, p-cymene, sabinene and β-pinene, which reflect the monoterpenic composition of juniper berry extract (Vichi *et al*., 2005).

As noted above for whisky and often for tequila, it is a common practice to store the spirits in oak barrels (Mosedale and Puech, 1998). In the case of tequila, this adds complexity to an already distinctive spirit, and in the case of whisky, largely defines the spirit flavour. During barrel construction, heat treatment is required to temporarily plasticise the staves. The lignin fraction of wood partially decomposes to form phenolic aldehydes (vanillin, springaldehyde, coniferaldehyde, sinapaldehyde) (Panossian *et al*., 2001). To a lesser extent, furanic compounds such as 2-furaldehyde, 5-methyl

furfuraldehyde and 5-hydroxymethyl furfuraldehyde are formed as the degradation products of hemicelluloses. These compounds slowly diffuse into the spirit, giving characteristic (whisky) or extra (tequila) flavour and colour to the spirit (Panossian *et al*., 2001).

It must be emphasized here that in the production of ti kōuka spirit, it was found that not only was the sugar concentration in the ti kōuka too low to be fermented economically but the ti kōuka extracts lacked a distinctive aroma (Sections 2.8 and 2.9). Distilling ti kōuka extract with different combinations of ethanol and deionised water (Section 2.10) showed that the coloured compounds did not co-distil with ethanol indicating that they were preferentially water soluble, which on storage, developed cloudiness (Section 2.13). Therefore, the brown aqueous ti kōuka extracts (after extraction, evaporation and reconstitution) were infused with potable alcohol to simultaneously extract colour, aroma and flavour. In other words, fermentation and distillation were not carried out in the ti kōuka spirit production. Due to time restraint, the ti kōuka spirits were not aged in barrel either. In choosing these processing options, lot of the flavoured volatiles such as acetals, acids, alcohols, aldehydes, esters, furans, ketones, phenols, pyrazines, sulphur compounds and terpenes, which are products of fermentation, distillation and aging, would most likely not be detected (or be present in negligible amounts) in the ti kōuka spirit samples.

It was known from the onset, as mentioned above, that lot of the volatiles would not be present in the ti kōuka spirit since the only process that was common between the ti kōuka and the commercial spirits was the Maillard reaction. Therefore, standard samples were not used in the GC-MS analysis, rather identifications of the most likely peak compounds was based on two comprehensive GC-MS libraries. The overall aim of the chemicals analysis was to look at the bigger picture - to compare gross differences of the profile between two extraction methods, and the ti kōuka and commercial spirits.

6.2 Materials and methods

6.2.1 Sample preparation

The ti kōuka spirit samples were prepared as summarised in Figure 2.19 (Chapter 2). In outline, dried ground ti kōuka stem was hydrolysed with Fructozyme at 60°C, cooled

and filtered, washed and made up to its original starting volume. The pH was adjusted to 10 with 1M sodium hydroxide and evaporated at 60°C for 65 hours. The dried residue was reconstituted to its original volume with deionised water. After centrifugation, the supernatant was infused with 99.7% v/v ethanol to yield final ethanol concentrations of 80, 67, 57 and 50% v/v (Treatments 1, 2, 3, and 4 respectively). These were further centrifuged, and the supernatant filtered with GF/B glass filter paper. The four ti kōuka spirits concentrations were further diluted to yield a final concentration of 20% v/v with deionised water, a concentration specified in the solvent extraction procedure (Fretz *et al*., 2005).

The alcohol content of the tequila (Pepe Lopez, Premium Silver), gin (Gordon's London Dry, Diageo) and whisky (Johnnie Walker Red Label) were also diluted to 20% v/v. The Pepe Lopez brand is a colourless tequila, and therefore unaged in oak as was also true for the Gordon's gin. The commercial spirits were purchased from Liquor King (New Zealand).

The most widely used extraction processes have traditionally been based either on different liquid extraction methods or on vapour-phase extraction methods. Extractions of the flavoured compounds in the ti kōuka spirit were carried out using two solvent extraction methods, the dichloromethane and n-pentane methods. Each is described in turn.

6.2.2 Dichloromethane extraction

Materials and method

Dichloromethane was purchased from Burdick and Jackson Company (HPLC, GC and spectrometry grade 10071685). 2-Octanol (Acros 12941-1000) was purchased from Scientific Supplies (New Zealand) and sodium sulphate (Riedel-de Haen 231-820-9) was purchased from BioLab (New Zealand).

The dichloromethane extraction method of Fretz *et al*. (2005) was followed with some modification. In an Erlenmeyer flask, 350 mL of the adjusted 20% (v/v) ti kōuka or commercial spirits was added to 50 mL of dichloromethane. As an internal standard, 100 μL of 2-octanol (70 mg 100 mL^{-1} in 50% ethanol) was also added. After passing nitrogen gas through the conical flask and sealing with Parafilm, the content of the flask

was stirred for 30 minutes on a magnetic stirrer. The aqueous and the organic phase were separated in a pear shaped separating funnel. The organic phase was collected in another conical flask. To the aqueous phase, a further 50 mL of dichloromethane was added and the whole extraction process was repeated once more. The two organic phases were mixed together and dried over anhydrous sodium sulphate (Na_2SO_4). The final organic phase was placed in a round bottomed flask equipped with a Vigreux column (20 to 25 cm long). Nitrogen gas was passed through it and the mixture was slowly concentrated to approximately 0.5 mL by heating in a waterbath at 50°C. The concentrated samples were collected and stored in a Teflon-sealed glass vials until required for GC-MS analysis. Ten microlitres of the concentrated samples were injected in the gas chromatograph for analysis.

6.2.3 n-Pentane and diethyl ether extraction

Materials and method

Finechem Ajax (Greenmount, Auckland, New Zealand) was the supplier of diethyl ether (1725 GL) and n-pentane (632 GL). Sodium hydrogen carbonate (BDH 10398 4T AnalaR) was purchased from BioLab (New Zealand). In an Erlenmeyer flask, 50 mL of 20% ti kōuka or commercial spirits was added to 50 mL of n-pentane and 50 mL of diethyl ether. As an internal standard, 100 µL of 2-octanol (70 mg 100 mL^{-1} 50% ethanol) was also added. After passing nitrogen gas through the mixture, it was stirred on a magnetic stirrer for 30 minutes. The organic and aqueous phase was separated in a separating funnel. The organic phase was washed three times with 30 mL of saturated sodium bicarbonate solution (108 g L^{-1}).

The organic layer was dried over anhydrous sodium sulphate powder, and a stream of nitrogen gas was passed through the final mixture before refluxing (as in the dichloromethane method). The concentrated samples were collected and stored in a Teflon-sealed glass vials until required for GC-MS analysis. Ten microlitres of the concentrated samples were injected in the gas chromatograph for analysis. The extractions were only done once since there was no time left to repeat it.

In subsequent text, the n-pentane and diethyl ether extraction method will be referred to as the n-pentane extraction method.

6.3 Gas chromatography-mass spectrometry (GC-MS)

The concentrated dichloromethane and n-pentane extracts and commercial spirit samples were subjected to GC-MS analysis, using a Trace GC-Ultra gas chromatography (DSQ Thermoelectron Corporation), equipped with Factor Four^{TM} Capillary Column VF- 5ms, (30 m length, 0.25 mm internal diameter, and 0.25 μm film). Samples (10 μL) were injected using split mode with a split ratio of 10. The helium carrier gas flow was set at a constant flow of 1.5 mL min^{-1}. The injector temperature was 200°C. The interface temperature between the column and detector was 250°C. The mass range was 50 to 650 m z^{-1}. Mass detection was by the χ-Calibar method. The National Institute of Standards and Technology (NIST) and Main EI-MS library were used as reference to tentatively identify the resolved peaks (as discussed earlier).

Dichloromethane and n-pentane have different polarity indices, 3.1 and 0.0 respectively (www.Phenomenex.com) which means the two extracted chemical profiles would be somewhat different. For example, Xu *et al*. (2005) found that n-pentane was particularly useful for extracting low-boiling-point volatiles from plant material. Different chemical profiles normally require different temperature programs, which are typically not isothermal. Indeed, isothermal programs may cause the later-eluting components to suffer from peak broadening. Therefore, different temperature programs were used for the two solvent extraction methods.

For the dichloromethane method, the temperature was held for 5 minutes at 60°C, then increased to 70°C at the rate of 2°C min^{-1}, held for 5 minutes, then increased to 80°C at the rate of 5°C min^{-1}, then held for another 5 minutes. The final temperature was increased to 280°C at the rate of 5°C min^{-1}, completing the whole cycle in 62 minutes. For the n-pentane method, the temperature was held for 5 minutes at 40°C, then increased to 50°C at the rate of 2°C min^{-1}, held for 5 minutes, then increased to 80°C at the rate of 5°C min^{-1}, held for another 5 minutes. The temperature was then increased to 250°C at the rate of 10°C min^{-1} and held for 5 minutes. The final temperature was increased to 280°C, at the rate of 5°C min^{-1} and held for 5 minutes, completing the whole cycle in 59 minutes.

6.4.1 Results and discussion of dichloromethane and n-pentane extraction of ti kōuka

As mentioned above, the quantitative analysis of the individual compounds was not done. For comparison, peak area of the internal standard was used as 100% to normalize the peak areas of compounds tentatively identified.

Typical chromatographs of the ti kōuka spirit (initial ethanol infusion concentrations of 67%, i.e. Treatment 2) extracted by the two solvent methods are presented in Figures 6.1 and 6.2, and the relative ratio (to the internal standard peak area) of dominant peak areas of the different ethanolic ti kōuka extracted spirits are shown in Table 6.1 and Table 6.2. In all dichloromethane-extracted samples, the retention time for the internal standard was between 5.11 to 5.23 minutes compared to 10.80 to 10.82 minutes for n-pentane. This difference was due to the lower starting temperature program used for the latter solvent.

Figures 6.1 and 6.2 compare typical chromatograms for the 67% (v/v) treatment as an example, where the data have been scaled to show an approximately equal peak size for the internal standard. It is clear that more total abundance was recovered by the dichloromethane extraction than the n-pentane extraction. The volume of spirit extracted with dichloromethane was 7-fold greater than for the pentane (as dictated by the published methods used), while the volume of the internal standard added was the same. Assuming for the moment an equal extraction ability, one would expect a 7-fold greater total abundance of non-solvent detected mass (relative to the internal standard) in the dichloromethane treatments.

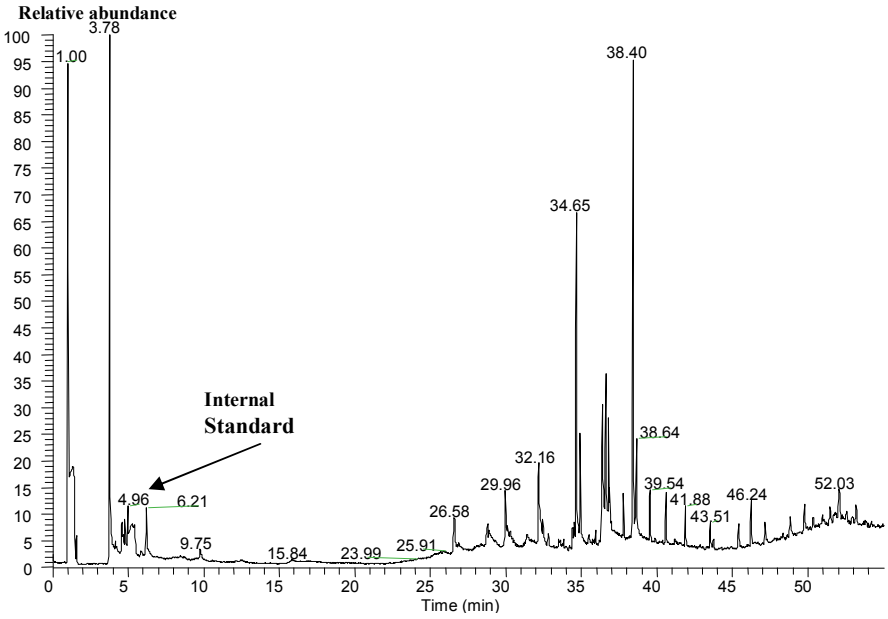


Figure 6.1 A gas chromatogram showing the dichloromethane extraction of the ti kōuka spirit (67% ethanol, Treatment 2).

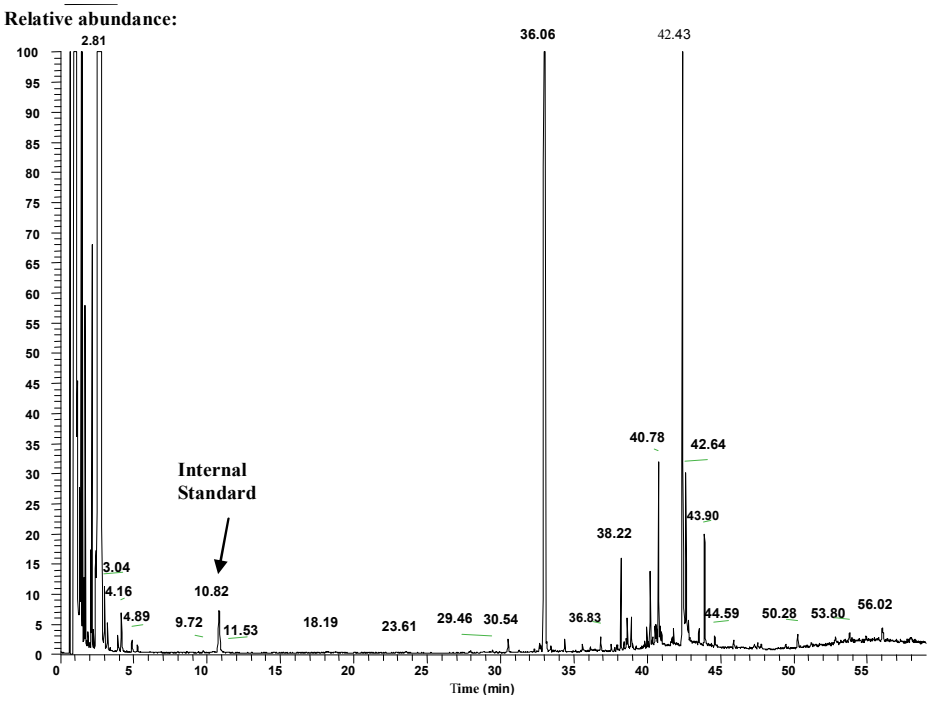


Figure 6.2 A gas chromatogram showing the n-pentane extraction of the ti kōuka spirit (67% ethanol, Treatment 2). Relative abundance scale magnified x 50.

Turning now to Tables 6.1 and 6.2, where areas have been normalized to the internal standard, it is evident that more non-solvent mass was present in the dichloromethane extracts, while at the same time realizing that many of the resolved compounds are different between the two solvents methods. The mean abundance for the two methods were 4640 ± 3090 (dichloromethane) and 1460 ± 190 (n-pentane). If the volume extracted by n-pentane had been 7-fold larger to match the dichloromethane extractions, the equivalent n-pentane abundance would be 7 x 1460 = 10220, assuming equal extraction capability. Thus, the n-pentane and diethyl ether extraction method may not be inferior, but certainly reveals a different profile from dichloromethane extraction.

Esters represented the largest number of volatiles in all four ti kōuka spirit samples extracted by the dichloromethane method, followed by acids, fatty acids and phytosterols (Table 6.1). (The scale of relative abundance was adjusted in these figures for better comparison of peaks with lower abundance values.) Hexadecanoic acid, hexadecanoic acid ethyl ester, octadecenamide and β-sitosterol were the common compounds tentatively identified in all four spirits. Octadecanoic acid, 4-hydroxy-2-methoxycinnamaldehyde, hexadecanamide, heptacosane, dioctyldiphenylamine and diosgenin were identified in three of the four treatments.

Esters are products of yeast metabolism or are formed subsequently during the aging process by esterification of fatty acids in the presence of ethanol at high concentration (Benn and Peppard, 1996). As was seen in Table 1.2, ti kōuka stem and stem tip have 1.5% and 3.2% (respectively) total fat (fresh weight) content. Even though the ti kōuka spirits were not fermented or aged, the brown extract was infused with different ethanol (80, 67, 57 and 50% v/v) concentration and the fat content in the ti kōuka stem (presumably including fatty acids) may have reacted with the alcohol to produce esters during the extraction process.

The results of ti kōuka (Table 2.14) and model system (Table 5.5) pH experiments showed that the pHs decreased after the heating process. At first sight, it may appear that these acids could be responsible for this decrease. However, the pKa values of these acids are all above 8.0 (Kanicky and Shah, 2002); in short they are very weak acids.

Table 6.1 Relative dominant peaks area of dichloromethane extraction compounds of different ti kōuka spirits infused with different ethanolic concentrations. Data are normalized to the internal standard (2-octanol) peak area which was set at 100. Grey areas are solvent or solvent impurities.

Likely compound	Retention time (min)	Molecular weight	Initial ethanol infusion concentration (%)			
			80	67	57	50
Dichloromethane	1.00	84	706	339	6417	12386
2-Octanol	**5.17**	**130**	**100**	**100**	**100**	**100**
2-Ethyl hexanol	6.14	130			11	
Propenyl alcohol	6.21	58		45		
Nonaldehyde	9.56	142	8			
2-Octen-1-ol	9.75	128		22		
Octadecanoic acid	15.84	282		10		
Ethyl octanoate	16.12	172			5	
Decanoic acid	26.58	172	6	65		
Dodecanoic acid, ethyl ester	26.86	200			7	
Butylated hydroxytoluene	28. 72	220		65	18	
Dodecanoic acid	29.96	198		81		4
4-Hydroxy-2-methoxycinnamadehyde	32.16	178	131	117		95
Tetradecanoic acid	32.46	228			92	
Hexadecanoic acid	34.65	256	178	174	822	331
Ethyl hexadecanoate	34.91	284	31	78	35	276
Octadecanoic acid	36.27	282	159	203		152
Ethyl octadecanoate	36.53	308			446	382
Hexadecanamide	36.78	255	358		745	515
Octadecane	37.80	254			141	
9-Octadecenamide	38.40	281	1391	272	4047	3068
Octadecanamide	38.64	283				625
Heptacosane	43.51	366	58	29	343	
Octacosane	44.50	394			679	
Dioctyldiphenylamine	46.24	393	102	39		22
Nonacosane	47.19	408			144	
Cholesterol	48.84	386			199	70
Stigmasterol	50.93	412			128	91
Diosgenin	51.43	414	75	16		33
β-Sitosterol	52.03	414	308	77	373	300
Heptatriacotanol	52.55	536	177	29		
Butanoic acid, octahydro-5-5a-dihydroxy	53.03	418	97		28	
Total abundance excluding peaks obviously due to solvent and solvent impurities			3079	1257	8245	5964

Table 6.2 Relative dominant peaks area of n-pentane extraction compounds of different ti kōuka spirits infused with different ethanolic concentrations. Data are normalized to the internal standard (2-octanol) peak area which was set to 100. Grey areas are solvent or solvent impurities.

Most likely compound	Retention time (min)	Molecular weight	Initial ethanol infusion concentration (%)/ (Treatment number)			
			80	67	57	50
3-Methyl pentane	1.08	86	23465	40539		12067
Ethyl pentane	1.49	114	511			
Octane	2.81	114	94561	85834	85748	90690
Xylene	4.16	106	49	43	42	39
Ethyl benzene	4.89	106		15		16
Methyl ethyl benzene	9.72	120	5	3		1
2-Octanol	**10.82**	**130**	**100**	**100**	**100**	**100**
Dihydro-4,4-dimethyl 2(3H)furanone	18.70	114			11	
3,5-Dimethyl octane	23.61	142		7		
Dimethyl ethyl benzoic acid	30.54	178	16	19	17	19
Butylated hydroxytoluene	33.06	220	6199	6498	5312	6379
Hexanoic acid, ethyl octyl ester	34.37	256	21	13	9	
Hexadecane	36.83	226			20	27
Hexadecanoic acid	38.63	256			41	89
Hexadecanoic acid, ethyl ester	38.22	284	74	54	86	124
Hexadecanamide	40.78	255		240	229	
Octadecenamide	42.43	281	767	1069	902	713
Octadecanamide	42.64	283	201			
Benzenecarboxylic acid, ethyl hexyl ester	43.90	278	167	109	93	90
Heptacosane	44.59	380	24	8	11	
Dioctyldiphenylamine	50.28	393	56	26	13	18
Heptatriacotanol	53.80	536	154	17		9
Stigmasterol	54.94	412			21	16
β-Sitosterol	56.02	414	57	38	60	67
Total abundance excluding peaks obviously due to solvent and solvent impurities			1537	1600	1513	1172

Phytosterols are widely distributed in the plant kingdom, e.g. stigmasterol, β-sitosterol and diosgenin belong to this group. Cholesterol also contain in plants (Behram and Gopalan, 2005). β-Sitosterol was identified in the dichloromethane extracts of the four spirits (80, 67, 57 and 50%). Cholesterol and stigmasterol on the other hand were only identified in the two lower ethanol treatments with the 57% treatment having the greater abundance ratio (Table 6.1). Comparison of the abundance of the phytosterols in the

four treatments revealed no consistent pattern with ratio of ti kōuka extract to ethanol during infusion. Recall that the 50% treatment had the highest ratio of ti kōuka extract to ethanol, and 80% had the lowest, and that phytosterols tend to be soluble in alcohols (including ethanol) but insoluble in water.

Turning now to the n-pentane extraction samples, these samples had very high 3-methyl pentane (retention time =1.08 minutes) and octane (retention time = 2.81 minutes) peak area ratios to the internal standard. Butylated hydroxytoluene (BHT, retention time = 33.06 minutes) was identified in high concentration as well (Table 6.2). BHT is often used as an antioxidant food additive and is used to stabilize solvents. All these compounds including xylene were identified when n-pentane alone (as blank) was injected in the GC-MS, and were thus impurities in the solvent. These are highlighted in grey in Table 6.2. Compounds that were derived from the spirits and common to all four treatments were dimethylethyl benzoic acid, benenecarboxylic acid ethyl ester, hexadecanoic acid ethyl ester, octadecenamide, dioctyldiphenylamine and β-sitosterol. Hexadecanoic acid ethyl octyl ester, heptacosane and heptatriacotanol were identified in three of the four treatments. As for dichloromethane extraction, stigmasterol was only identified in the 57 and 50% ethanol treatments subjected to n-pentane extraction.

Compounds identified in both solvent extractions were hexadecanoic acid ethyl ester, octadecenamide and β-sitosterol. Neither higher alcohols nor aldehydes were identified in the four ti kōuka spirits by the two extraction methods. Since the ti kōuka extract did not undergo fermentation, higher alcohols were not produced.

There was no major difference in the total non-solvent relative mass abundance between all the four treatments (Table 6.2) with n-pentane extraction compared to the vast differences in the dichloromethane extraction (Table 6.1) with Treatments 3 and 4 having the highest values followed by 1 and 2. Even though the number of compounds eluted by the four dichloromethane-extracted spirits were similar, the concentration of the compounds differed.

Notably, Maillard reaction products were not identified in any of the ti kōuka spirit (as was also true for whisky (Section 6.4.4) as they have been by Kaushal (2007) with wine infused with toasted oak chips (equivalent to barrel aging) and extracted by the same n-pentane/diethyl ether extraction method. There has been ample evidence of caramel

smell and brown colour (from previous chapters) that Maillard reaction was definitely taking place when ti kouka extract was heated. Possible reason for this will be addressed in detail later in this chapter (Section 6.5).

6.4.2 Results and discussion of dichloromethane and n-pentane extraction of tequila

Figures 6.3 and 6.4 show examples of chromatograms of tequila extracted by the dichloromethane and n-pentane methods. The ratios of dominant peak areas to the internal standard peak area of tequila are shown in Table 6.3 and Table 6.4 respectively. Again, more non-solvent compounds were resolved and tentatively identified by the dichloromethane extraction (23) than by the n-pentane method (18), but the same argument for ti kōuka spirit about the 7-fold difference in extraction volumes applies to tequila and the other commercial spirits.

Esters (ethyl octanoate, ethyl decanoate, ethyl dodecanoate, ethyl hexanoate), alcohols (phenyl ethyl alcohol, pentanol), acids (octanoic acid, decanoic acid), furan (5-methyl furfural, 5-ethenyltetrahydro-α-α,5-trimethyl-2-furanmethanol), terpenes (linalool, β-terpineol) and others (hexadecanamide, octadecenamide), were tentatively identified as the most likely compounds (Table 6.3) by both solvent methods. Exceptions were ethyl hexanoate, octanoic acid, decanoic acid, 5-ethenyltetrahydro-α-α,5-trimethyl-2-furanmethanol and β-terpineol which were extracted and resolved only by the dichloromethane method. Again, n-pentane impurity solvent peaks were identified as in the ti kōuka spirits. As for ti kōuka, solvent and impurities are greyed in these tables.

In terms of the number of components identified, esters represented the largest group. Benn and Peppard (1996) also identified the esters as being the largest group of volatiles in their study of tequila including the ones tentatively identified in this study. 5-Methyl furfural and 5-ethenyltetrahydro-α-α,5-trimethyl-2-furanmethanol are both Maillard reaction product and the latter is also found in the extrudes of *Agave tequilana* (Mancilla-Margalli and Lopez, 2002). Benn and Peppard (1996) also identified octanoic and decanoic acids as two of their three most abundant acids. Fermentation seems the most likely origin.

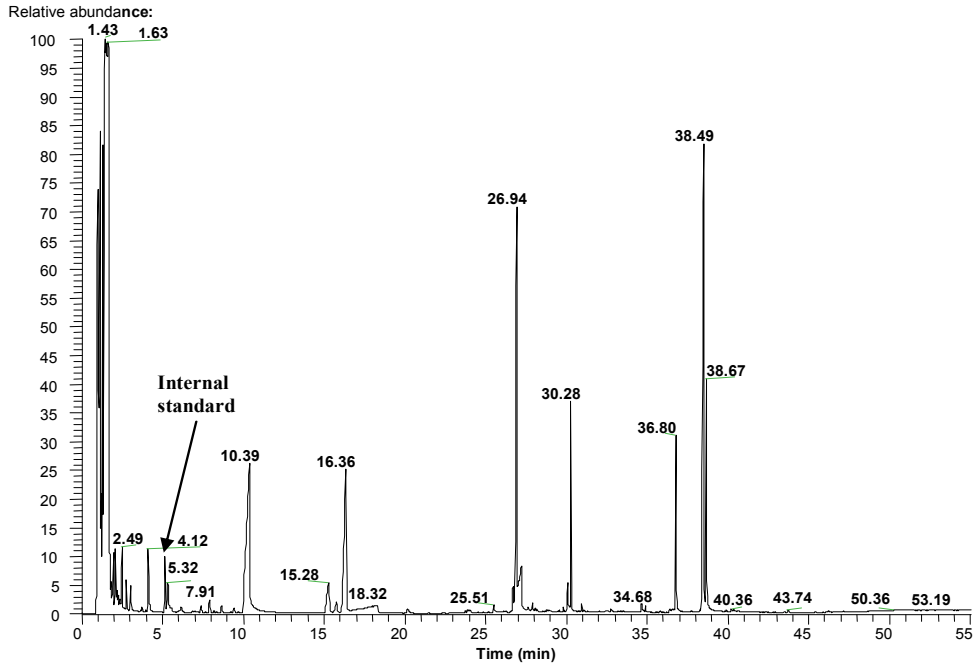


Figure 6.3 Gas chromatograph of tequila with dichloromethane extraction

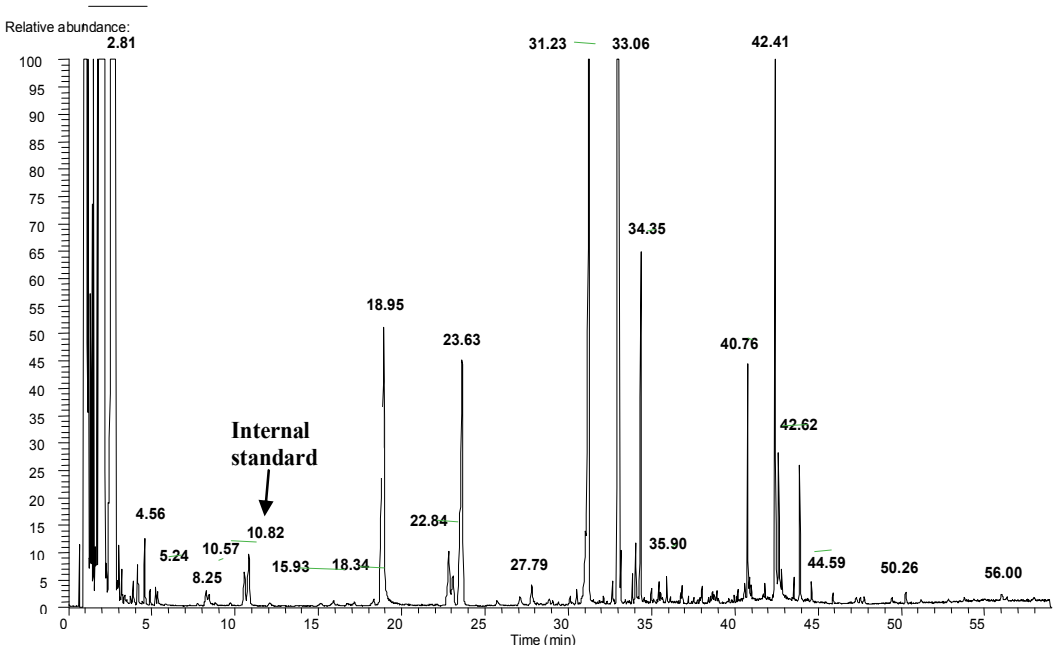


Figure 6.4 Gas chromatogram of tequila by n-pentane extraction (magnified x50)

Peña-Alvarez *et al*. (2004) identified 32 terpenes in tequila using *Agave tequilana*. Terpenes such as linalool and geraneoil probably come from the agave plant. Monoterpenes and sesquiterpenes have particularly desirable notes of flavour and pleasant odour that probably contribute to the flavour and aroma of tequila (Peña-Alvarez *et al*., 2004). Common terpenes identified in the present study were linalool (by both methods) and β-terpineol (by dichloromethane method) although the concentrations were very low.

Phenyl ethyl alcohol and 1-pentanol were tentatively identified as the most abundant alcohols in this study of tequila. These two were also identified by Benn and Peppard (1996) who considered them to be fermentation products. The yeast strain used during fermentation is the most important factor influencing the amount of higher alcohols produced. For example, in the case of tequila, higher amounts of isoamyl alcohol (3-methyl-1-butanol) and 2-butanol are produced from native strains of yeast isolated from natural fermentation of cooked agave juice as opposed to the yeast strain employed in bakeries (Lachenmier *et al*., 2006). Thus, yeast exerts a prominent role on the final organoleptic quality of the tequila (Arrizon *et al*., 2005). Mancilla-Margalli and Lopez (2002) also found phenyl ethyl alcohol present in pre-fermentation agave extrudes which suggested that fermentation is not the only pathway for their formation. It is also a product of the Maillard reaction (Mancilla-Margalli and Lopez, 2002).

Since the tequila used in this study was not aged in oak barrels, phenolic compounds such as vanillin, syringaldehyde, phenol, and phenol derivatives were not identified. These compounds are extracted from the contact of ethanol and the inner barrel wall. Besides the hydrolysis of inulin to generate fermentable sugars, production of volatile Maillard compounds is common to both tequila and ti kōuka spirit production. Maillard compounds formed during ti kōuka extract evaporation and agave pinãs cooking are dependent on temperature and time, as was described in Chapter 2. The ti kōuka extract was evaporated at 60°C for 65 hours while the agave piña is cooked at 80 to 95°C for 50 to 72 hours. The differences in the flavour characteristics between these spirits would therefore be evident. Therefore, the flavour can be changed, directed, or controlled during heating step.

Table 6.3 Relative areas of dominant peaks in dichloromethane extraction of different commercial spirits. Data are normalized to the internal standard (2-octanol) peak area which was set to 100. Grey areas are solvent or solvent impurities

Likely compound	Retention time (min)	Molecular weight	Pepe Lopez Tequila	London Dry Gin	Johnnie Walker Red Label Whisky
Dichloromethane	0.92	84		4761	15460
Cyclohexene	1.15	82		1264	16
1-Pentanol	1.43	88	5413		7024
3-Methylbutyl acetate	2.33	130			266
β-pinene	3.24	136		173	
Valeraldehyde, diethyl acetal	3.75	160	10		
5-Methylfurfural	4.12	110	115		
β-Phellandrene	4.14	136		106	
Hexanal	4.65	100	4		
2-Octanol	**5.16**	130	**100**	**100**	**100**
Ethyl hexanoate	5.36	144	179		
Cymene	5.85	134		21	
Limonene	5.99	136		33	
β-Terpineol	7.50	136	2	50	
2-Hydroxy-4-methyl pentanoic acid, ethyl ester	7.40	160	15		
5-Ethenyltetrahydro-α-α,5-trimethyl-2-furanmethanol	8.66	170	15		
Linalool	9.51	154	15	2748	
Phenyl ethyl alcohol	10.61	122	973		188
Camphor	11.65	152		226	
Terpinen-4-ol	14.18	154		232	
Butanedioic acid, diethyl ester	15.28	174	142		
Ethyl octanoate	16.14	172	621		714
Octanoic acid	17.50	144	73		78
Geranoil	20.13	154		86	
Phenyl ethyl acetate	20.15	164	21		84
Ethyl decanoate	26.91	200	920		2229
Decanoic acid	27.24	172	291		
α-Caryophyllene	27.83	204		13	
Dodecanoic acid	30.01	200			281
Ethyl dodecanoate	30.27	228	294		501
α-Cadinol	31.09	222		30	
Ethyl tetradecanoate	32.76	256			43
Hexadecanoic acid	34.66	254		35	223
Ethyl hexadecanoate	34.68	282	25		
Octadecanoic acid	34.68	282		17	
Hexadecanamide	36.77	255	217	254	445
Octadecenemide	38.41	281	1393	978	1532
Benzenedicarboxylic acid, dioctyl ester	39.98	390		9	14
Octadecanamide	40.36	283	20	150	251
Heptatricotanol	43.72	536		52	21
Squalene	43.74	410		44	
Dioctyldiphenylamine	46.23	393		18	
Eicosadienoic acid, methyl ester	46.23	322	7		34
3-Octadecyloxy -9-octadecenoate	53.19	592			65
Total abundance excluding peaks due to solvent and solvent impurities			10765	5275	13993

Table 6.4 Relative areas of dominant peaks in n-pentane extraction of different commercial spirits. Data are normalized to the internal standard (2-octanol) peak area that was set to 100. Grey areas are solvent or solvent impurities.

Likely compound	Retention time (min)	Molecular weight	Pepe Lopez Tequila	London Dry Gin	Johnnie Walker Red Label Whisky
Pentane	0.94	72	2997	4432	4152
3-Methylpentane	1.14	86	8258	9706	1942
1-Pentanol	2.10	88	27045		18550
Octane	3.02	114	47733	86691	64368
Xylene	4.22	106	27	50	26
3-Methylbutyl acetate	4.56	130	41		179
5-Methyl furfural	8.25	110	30		
α-Pinene	6.56	136		87	
Camphene	7.27	136		6	
β-phellandrene	8.60	136		42	
Trimethyl benzene	9.70	120			9
β-pinene	9.80	136		45	
Limonene	12.14	136		21	
3-Methylpentanoic acid, ethyl ester	10.57	144	41		
Ethyl hexanoate	10.58	144			54
2-Octanol	**10.89**	**130**	**100**	**100**	**100**
3-Carene	14.75	136		25	
Propionaldehyde, diethyl acetal	16.87	132			8
β-Terpineol	22.21	136		161	
Linalool	18.70	154	9	2359	
Phenyl ethyl alcohol	19.05	122	517		623
Camphor	20.39	152		148	
Butanedioic acid, ethyl ester	22.84	174	79		
Ethyl octanoate	23.69	172	383		440
2-Phenylacetic acid, ethyl ester	27.12	164			72
Geraniol	27.20	154		69	
Ethyl decanoate	31.27	200	819		1943
Germacrene	32.62	204		27	
Butylated hydroxytoluene (BHT)	33.13	220	3761	6187	5629
Elemene	33.84	204		34	
Ethyl dodecanoate	34.37	228	160		918
Tridecan-1-ol	35.43	200			103
Octadecane	36.85	254		23	
Hexadecanol	37.77	242			75
Hexadecanamide	40.83	255	107	435	455
9-Octadecenamide	42.50	281	459	3542	2806
Octadecanamide	42.62	283	89		
Tetradecanamide	42.68	227			481
Dodecanamide	42.70	199		494	
Diisooctyl phthalate	43.92	390	62	125	108
Heptacosane	44.64	380	12	46	40
Squalene	47.83	410		65	
Dioctyldiphenylamine	50.28	393	10		12
Heptatriacotanol	53.76	536	9		
β-Sitosterol	56.06	414	11		9
Total abundance excluding peaks due to solvent and solvent impurities			29883	7754	26885

6.4.3 Results and discussion of dichloromethane and n-pentane extraction of gin

Figures 6.5 and 6.6 show examples of chromatograms of London Dry Gin extracted by the dichloromethane and n-pentane methods. The ratios of dominant peak areas to the internal standard peak area of London Dry Gin are shown in Table 6.3 and Table 6.4 respectively.

As in all spirit beverages, the presence of unique volatiles and semi-volatiles strongly dominate to gin flavour perception. London Dry Gin is distilled from ethanol in the presence of juniper berries (*Juniperus communis*) and usually other plant material, the so-called botanicals. These botanicals, including the juniper berries, are rich in essential oils, which impart the characteristic flavour of most gins. Monoterpenes present in juniper berries and the degradation products derived from juniper berries, contribute significantly to the flavour and aroma of gin (Greer *et al*., 2008). The main terpenes detected in gin samples by Vichi *et al*. (2005) using solid phase micro extraction (liquid-liquid extractions followed by GC-MS analysis) were linalool, α-pinene, β-myrcene and limonene followed by γ-terpinene, p-cymene, sabinene, and β-pinene to mention a few. These compounds reflect the monoterpenic composition of juniper berry extract. Greer *et al*. (2008) revealed a substantial amount of linalool present upon distilling coriander alone (their observation was also supported by Kerrola and Kallio, 1993), while in the presence of juniper, α-pinene and β-mycrene were the most dominant.

In this present study of gin extracted by dichloromethane, the largest peak areas of the terpenes relative to the internal standard were linalool (2748) followed by terpinen-4-ol (232), camphor (226), β-pinene (176), β-phellandrene (106), geranoil (86), β-terpineol (50), squalene (44), limonene (33), α-cadinol (30), cymene (21) and α-caryophyllene (13). Linalool (2359) also had the largest relative peak area for the n-pentane extraction followed by β-terpineol (161), camphor (148), α-pinene (87), geraniol (69), squalene (65), β-pinene (45), β-phellandrene (42), elemene (34), germacrene (27), carene (25), limonene (21) and camphene (6). The majority of the common peak areas of the terpenes were higher in the dichloromethane extraction as opposed to the n-pentane extraction. However, both methods resolved similar number of terpenes. All these compounds were also identified by Vichi *et al*. (2005).

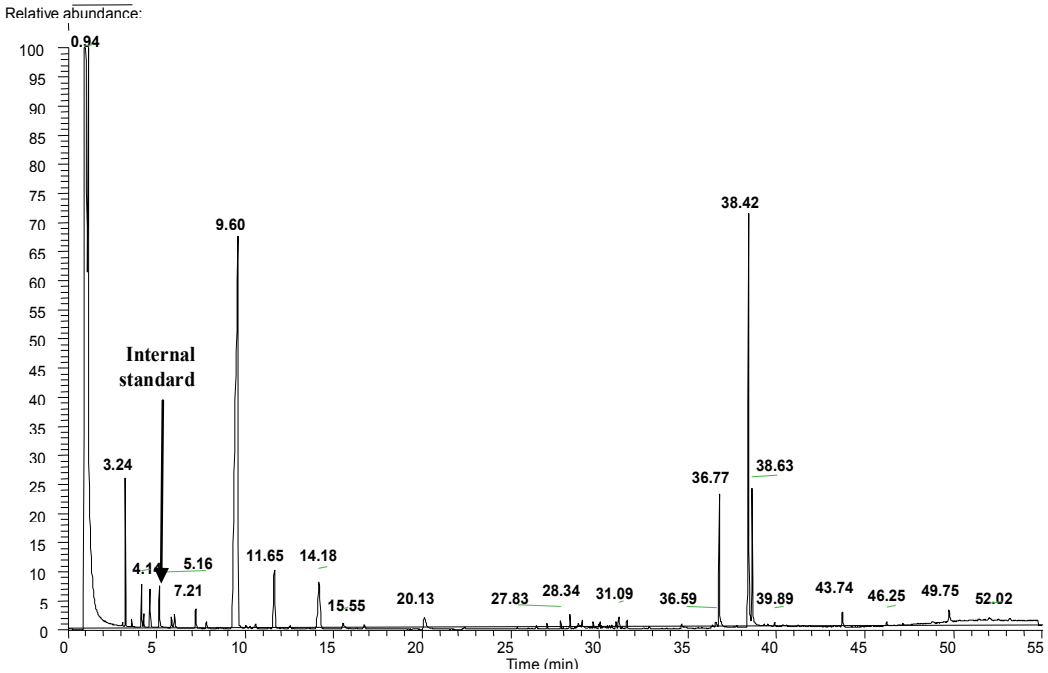


Figure 6.5 Gas chromatogram showing the dichloromethane extraction of London Dry Gin (magnified x2)

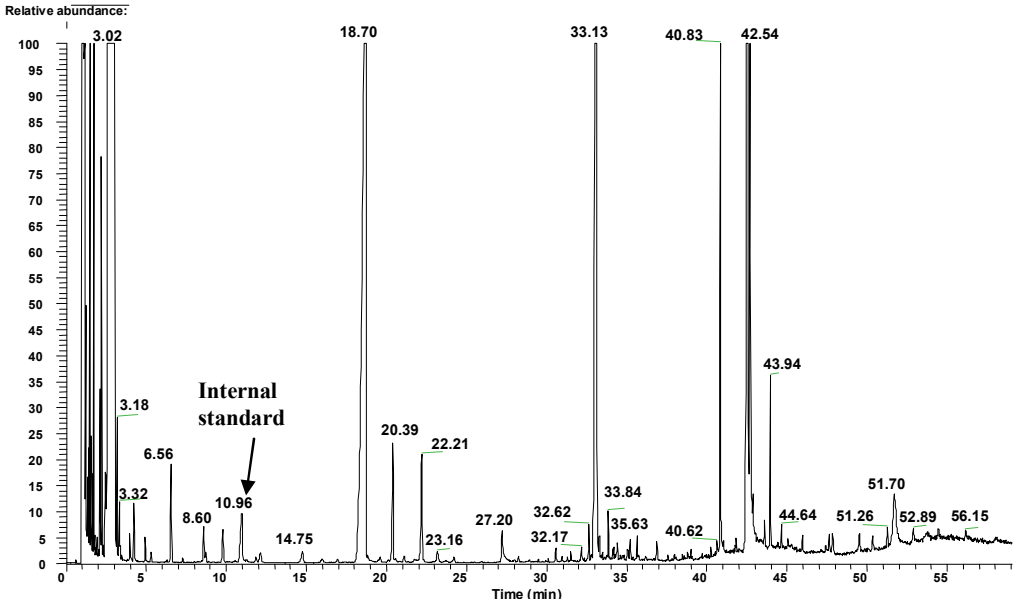


Figure 6.6 Gas chromatogram of London Dry Gin extracted by n-pentane extraction (magnified x37)

Common esters resolved by both extraction methods were ethyl hexanoate, ethyl octanoate, ethyl decanoate, and ethyl dodecanoate. In addition, butanedioic acid ethyl ester, 2-hydroxy-4-methyl pentanoic acid ethyl ester, tetradecanoic acid ethyl ester, ethyl hexadecanoate, and eicosadienoic acid methyl ester were also extracted and resolved by the dichloromethane method while 3-methylpentanoic acid ethyl ester, butanedioic acid ethyl ester and 2-phenylacetic acid ethyl ester were extracted and resolved by the n-pentane extraction method. Other compounds that were derived from the spirit and common to both extraction methods were hexadecanamide and octadecenamide.

In preparing the ti kōuka spirits, the ti kōuka extract was not distilled, hence any botanicals present in the ti kōuka stem would not have co-distilled with ethanol. Rather the fructans and amino acids reacted to form Maillard reaction products. The only resolved compound common to ti kōuka spirits and gin was octadecenamide.

6.4.4 Results and discussion of dichloromethane and n-pentane extraction of whisky

Examples of chromatograms of the Johnnie Walker Red Label Whisky using the dichloromethane and n-pentane extraction methods are shown in Figures 6.7 and Figure 6.8. The ratios of dominant peak areas to the internal standard peak area are shown in Tables 6.3 and Table 6.4 respectively.

Compounds tentatively identified included esters (ethyl decanoate, ethyl dodecanoate, and ethyl octanoate), alcohols (pentanol, phenyl ethyl alcohol), acetate (3-methylbutyl acetate) and others (hexadecanamide, octadecenamide) as the most likely compounds (Table 6.3) by both solvent methods. Exceptions were tetradecanoic acid ethyl ester, eicosadienoic acid methyl ester, octanoic acid, dodecanoic acid, hexadecanoic acid, phenyl ethyl acetate, octadecanamide and heptatricotanol, which were extracted and resolved only by the dichloromethane method. Hexadecanol, ethyl hexanoate, phenyl acetic acid ethyl ester, tetradecanamide, heptacosane and β-sitosterol were resolved only by the n-pentane method.

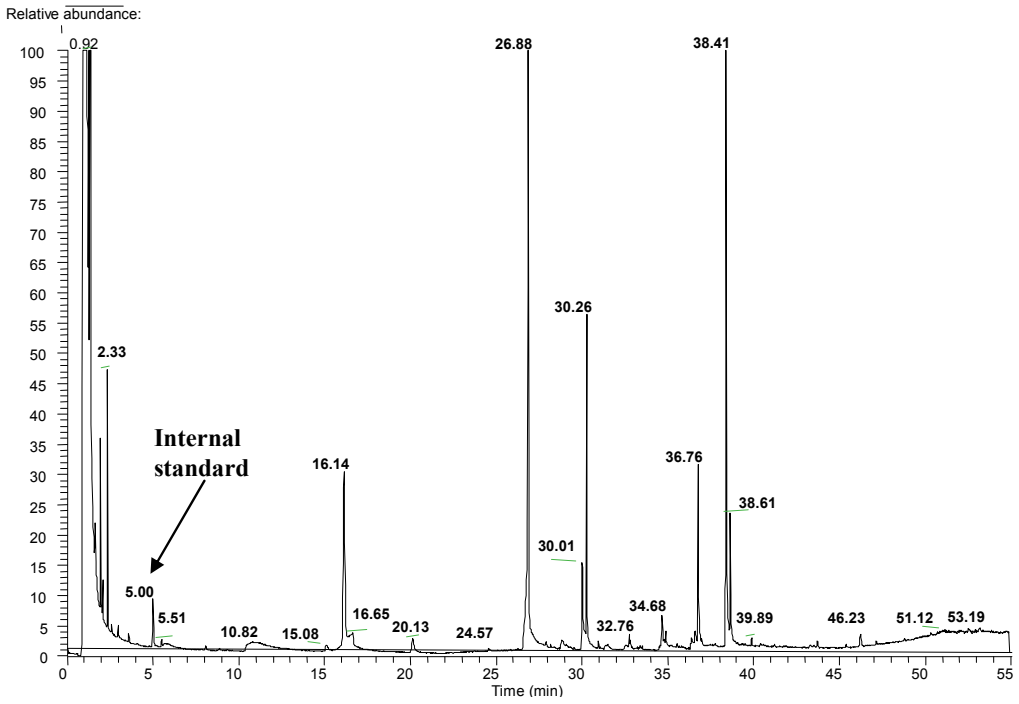


Figure 6.7 Gas chromatogram of Red Label Johnnie Walker Whisky by dichloromethane) extraction (magnified x7)

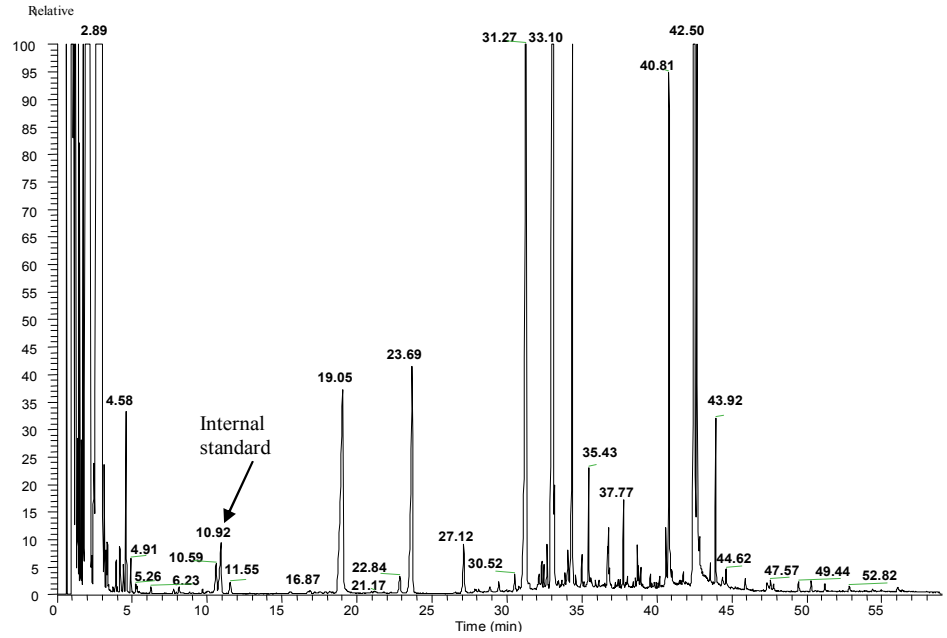


Figure 6.8 Gas chromatogram of Red Label Johnnie Walker Whisky by n-pentane extraction (magnified x25)

Ethyl esters were amongst the largest group of volatiles identified. Camara *et al*. (2006) found that ethyl esters contributed 60% of total volatiles in Black Label whisky. Ethyl esters are important among the aroma components of whiskies. Pentanol was markedly the most abundant higher alcohol in Red Label whisky in the present study followed by phenyl ethyl alcohol. The most abundant fatty acids and derivatives identified were octadecenamide followed by hexadecanamide. The higher alcohols and fatty acids constitutes important group of aroma compounds that give whisky the 'fruity' and 'fatty/cheese' sensory property (Caldeira *et al*., 2007, Camara *et al*., 2006).

As mentioned earlier, aging of the whisky in oak barrel forms phenolic aldehydes and to a lesser extent, furanic compounds such as 2-furaldehyde, 5-methyl furfuraldehyde and 5-hydroxymethyl furfuraldehyde. Possible reason being that during extraction, more than one step was involved before analyses. During solvent extraction and possibly solvent evaporation, some compounds may have been lost or degraded. Other authors, for example, Caldeira *et al*. (2007) used liquid-liquid microextraction method having the advantage of extracting the compounds in one step. Using this method they identified furfural, 5-methyl-2-furfural, benzeneacetaldehyde, springaldehyde, whisky lactones, 5-hydroxymethyl furfural and vanillin.

Again, the only common compounds resolved and identified in the four ti kōuka spirits and whisky were hexadecanamide and octadecenamide.

6.5 Evidence for Maillard reaction products in ti kōuka spirits

There is evidence from the results of experiments in previous chapters to suggest that the Maillard reaction did occur. Analysis of extractable reducing sugars from the top 20 centimeters of the ti kōuka stem (assisted by Fructozyme) showed the presence of reducing sugars, potentially available for the Maillard reaction (Chapter 2). Analysis of the sugar and amino acid profiles using HPLC showed the presence of fructose and most of the common amino acids in the ti kōuka stem (Chapter 3). The change of colour and the caramel smell obtained after evaporating the extracts in an oven at 60°C clearly indicated that the Maillard reaction was occurring (Chapter 2, Table 2.12). The typical caramel flavour is the result of a number of sugar fragmentation and dehydration products of the Maillard reaction (de Man, 1999). The reconstituted ti kōuka extract infused with different concentrations of potable alcohol also had a caramel smell (Table 2.14).

In Chapter 4, samples from each step of the ti kōuka spirit process (i.e. extraction, hydrolysis, evaporation, infusion with alcohol and centrifugation) were also analysed for reducing sugar and amino acid concentrations. The reducing sugar concentration decreased during each step of the process. The relative concentrations of arginine, leucine and lysine decreased during the whole process of making the spirit while that of aspartic and glutamic acids increased, suggesting that the former amino acids were particularly active in the Maillard reaction.

In the model systems, amino acid did not produce any coloured products when heated individually (Chapter 5). However, combination of fructose and amino acid(s) did produce coloured Maillard reaction production. The results obtained using the model system (approximately simulating the ti kōuka spirit production) had a similar pattern to the results obtained with the ti kōuka extract. The colour of the models became darker as a function of time, the longer incubation period of heating reducing sugar and amino acid/s accumulating more brown pigment formation. Increasing the pH and concentration of the amino acids in the reaction mixture also increased the brown pigment formation.

From the above body of evidence there is absolutely no doubt that the Maillard reaction was taking place. However, the Maillard reaction compounds were definitely not identified in the ti kōuka spirit chromatograms in the present chapter. From fundamental chemical principles, it is extremely unlikely that either extractive solvent could result in the loss or modification of Maillard reaction compounds. The likely explanation lies in the relative solubility of the Maillard compounds in water compared with organic solvents. Recall that in Section 2.13 (Chapter 2) evaporated and reconstituted ti kōuka extract infused with ethanol and water to yield different final concentrations of ethanol (Table 2.4). These were distilled in an attempt to produce an acceptable spirit. However, the flavoured and coloured matter generated by the Maillard reaction was poorly recovered in the distilled fractions, especially the treatment with highest water content. Although the classic Maillard reaction products, furfural and furfural alcohol are very soluble in alcohol and less soluble in water (The Merck Index, 2001), the coloured polymeric compounds from the intermediate and latter stages of the Maillard reaction may be relatively more soluble in water. They certainly were not co-distilling with ethanol. Looking now at the relative solubility of

these compounds in dichloromethane and n-pentane/diethyl ether, the same argument may apply: many compounds of interest were simply not extracted.

The two extraction methods also showed some variations in the compounds that were extracted and resolved in all the four ti kōuka and commercial spirits. Owing to the wide variety of the chemical species of the spirits, only with the combination of different extraction techniques it is possible for a complete analysis of the volatiles. More than one extraction method followed by GC-MS analysis could be a useful analytical strategy when low levels of odorants have to be detected in complex mixtures. In addition, there is a very likely chance that some of the peaks in this study may have co-eluted therefore a full spectrum of compounds may not have been identified.

Clearly, the chemical profiles of the ti kōuka spirits were different from those of the commercial spirits. Although agave and ti kōuka may belong to the same family, the path chosen to produce the ti kōuka spirits was different. The characteristic of the flavour and aroma of the ti kōuka spirits produced is therefore unique in its own right. In the next chapter (Chapter 7), sensory evaluation of the ti kōuka spirits will discussed, looking at the liking of the appearance, odour and taste of the four spirits.

CHAPTER 7

Sensory evaluation of ti kōuka spirit

7.1 Introduction

Chapter 2 outlined the steps leading the production of an infused spirit made from ti kōuka extract where the endogenous fructose and amino acids were reacted to create a complex mixture that included Maillard reaction products. It was found that a range of spirit types could be produced by adjusting the final concentration of ethanol used to infuse. Thus, lower final concentrations of ethanol in the infusion resulted in darker and seemingly more flavoured spirits. This was attributed to more water soluble Maillard reaction products being extracted. In contrast, higher final concentrations led to a spirit more reminiscent of a light whisky. These observations were only casual however, and it was necessary to formally evaluate these spirits with human subjects. At the outset, it was important that the spirits as presented had to have the same concentration of ethanol. Thus in all the work described here, the ethanol concentrations, initially 80, 67, 57 and 50% v/v were all diluted to 43% ethanol, often the concentration of a so-called premium spirit.

The definition of sensory evaluation prepared by the Sensory Evaluation Division of the Institute of Food Technologists (Chicago) is as follows. Sensory evaluation is a scientific discipline used to evoke, measure, analyse and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing (Stone and Sidel, 2004). In this trial the senses chosen were sight (appearance), smell (odour), and taste. These are obviously the senses of interest for ti kōuka spirit. However, as described in Chapter 2 odour and taste and their interaction are far from simple (Stone and Sidel, 2004). Thus, odour can be orthonasal (smelt through the nostrils) and retronasal (sensed from the back of the throat) as drinks are swallowed (Meilgaard *et al*., 1999). Unless elaborate precautions are taken, taste is inextricably linked with odour – together called flavour – because mastication of food generates volatiles that are retronasally sensed during swallowing (Reineccius, 2006). Drinks are not masticated but the same retronasal phenomenon occurs. However, untrained panellists, such as were used here, are unfamiliar with these complexities, so the questions asked used the simple terms appearance, odour, and taste.

Generally, sensory testing methods are in three categories: hedonic, comparative and descriptive (Meilgaard *et al.*, 1999). Hedonic[4] tests measure liking and disliking reactions as well as overall acceptability tests like preference tests and ranking tests. Hedonic tests are therefore always used within the scope of consumer tests and serve to characterise consumer behaviour. Comparative and descriptive sensory tests are referred to as 'expert tests' because they may be carried out only by trained persons and can give very detailed information about individual product parameters.

Hedonic tests were chosen here because with a completely novel spirit it was essential in the first instance to find out if people liked or disliked it, and how this might be affected by some characteristics of the people involved. Two characteristics that are commonly sought are gender and age. Asking consumers to pick a gender box is non-intrusive and besides, gender is obvious. Asking a person's age can be intrusive, but this can be solved by asking age in broad categories, as was done here. Thus the experimental design used here is based on liking of appearance, odour (orthonasal), and taste (really flavour) of spirits (4 treatments) at 43% ethanol, as affected by age category (3 levels) and gender (2 levels).

Materials and methods

Ti kōuka spirit at four extraction concentrations was prepared as described in Figure 2.19, Chapter 2) and diluted to a final concentration of 43% v/v with Natural Spring Water (Pam's Brand).

Participants were invited to take part in the sensory trial by advertising through the School of Applied Sciences. Ethical approval was obtained from the Auckland University of Technology Ethics Committee. Adults who were 18 years or older and who drank alcohol were recruited as participants, all on a strictly voluntary basis. When they arrived at the test site, a dedicated food laboratory, they were warmly greeted and provided with an information sheet (Appendix XII), and a consent form (Appendix XIII). The written information was first explained verbally, and after reading the written information sheet and being given a chance to ask any questions, they were asked if they were willing to participate. In all, 45 panellists were recruited,

[4] The term 'hedonic' is derived from the Greek *hedone*, having to do with pleasure

27 male and 18 female. Anonymity was kept by assigning each participant with a code number, which was used during the study.

Each panellist was presented with the four ti kōuka spirit treatments presented in wine glasses containing 15 mL samples of spirit. At 43% ethanol, each sample was equivalent to approximately half of a standard alcoholic drink if consumed in total. The glasses were arranged in a row (on four corresponding circles) on a clean white plastic tray and were marked with a random 3-digit code below the circles on a white sheet of paper as shown in Appendix XIV. The order of presentation was such that each of the four treatments followed each of the others with equal frequency (Wakeling and MacFie, 1995). Each panellist had to answer three ballots (Appendices XVI, XVII and XVIII) on each of which gender was to be selected along with age category, 18 to 30 years, 31 to 60 and 61 and over. Panellists scored for liking of appearance, odour, and taste on a nine-point scale by ticking the boxes. Panellists were advised to spit out the samples after tasting each of them and wash/drink water between each sample. If any swallowed, and some may have, there would be a retronasal contribution to the hedonic response. Plain crackers were also provided for panellist to remove any flavour/odour/residues from the mouth, if required to do so. After completing the assessment, panellists were rewarded with a chocolate.

The descriptive information recorded on the three ballots was converted into numerical values as shown in Table 7.1.

Table 7.1 Descriptors and numerical value of liking of the ti kōuka spirits

Liking descriptors	Numerical value
Like extremely	9
Like a lot	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike a lot	2
Dislike extremely	1

Data were analysed for variance using Minitab® Release 14 (SMBIOS Version 2.4). Results are reported as mean ± standard deviation with the associated probability values

for treatment, age and gender. Turkey's multiple range tests was applied where it could be.

Results

The statistical analysis is presented in Table 7.2, summarising the raw data in Appendix XV. Statistical interactions between alcohol treatments and age groups, and between alcohol treatments and gender were non-significant (P >0.05) for appearance, odour and taste. Therefore, the data could also be pooled across alcohol treatments and analysed for the effects of age group and gender separately.

Table 7.2 Mean scores, standard deviations, and analysis of variance for the liking of four ti kōuka spirits by 45 panellists. Data are analysed for effects of treatment, age, and gender

Treatment		Appearance	Odour	Taste
Concentration of ethanol at infusion (% v/v), before dilution to 43 % (v/v)	50	6.58 ± 1.8[a]	4.73 ± 2.3	4.69 ± 2.4
	57	6.38 ± 1.7[a]	5.24 ± 2.2	4.62 ± 2.3
	67	6.04 ± 1.2[ab]	5.31 ± 2.2	4.91 ± 2.1
	80	5.24 ± 1.9[b]	5.27 ± 1.9	4.58 ± 2.0
Age in years	18 – 30 (10 panellists)	5.88 ± 2.0[a]	4.58 ± 2.4[a]	3.88 ± 2.2[a]
	31 – 60 (30 panellists)	6.00 ± 1.6[a]	5.14 ± 2.1[ab]	4.73 ± 2.1[b]
	61 and over (5 panellists)	7.00 ± 1.3[b]	6.25 ± 1.7[b]	6.15 ± 1.8[c]
Gender	Male (27 panellists)	6.29 ± 1.6	5.43 ± 1.9	5.28 ± 2.0
	Female (18 panellists)	5.78 ± 1.9	4.71 ± 2.4	3.83 ± 2.2
Effect of treatment		P < 0.001	NS[1]	NS
Effect of age		P = 0.053	P = 0.039	P = 0.004
Effect of gender		NS	NS	P < 0.001

[a,b,c] Means bearing different letters in column groups are significantly different at P < 0.05

[1] NS = Not significant.

Bar graphs of the results were also prepared to visually identify the trends (which are not obvious to the eye from Table 7.2) of the liking of appearance, odour and taste at the different alcohol infusion concentrations (Figure 7.1) by three age groups (Figures 7.2, 7.3 and 7.4) and the two gender (Figures 7.5, 7.6 and 7.7). The effect of alcohol treatment had a significant effect (P < 0.001) on the liking of appearance of the ti kōuka

spirits (Table 7.2) as illustrated in Figure 7.1. The appearance of the darker coloured ti kōuka spirit derived from the lowest alcohol infusion concentration was preferred the most by all panellists as a group. Inspection of Figure 7.1 shows there is a steady decrease in liking of appearance as infusion concentration increased.

The infusion concentration had no significant effect (P = 0.523 and P = 0.869 respectively) on odour and taste.

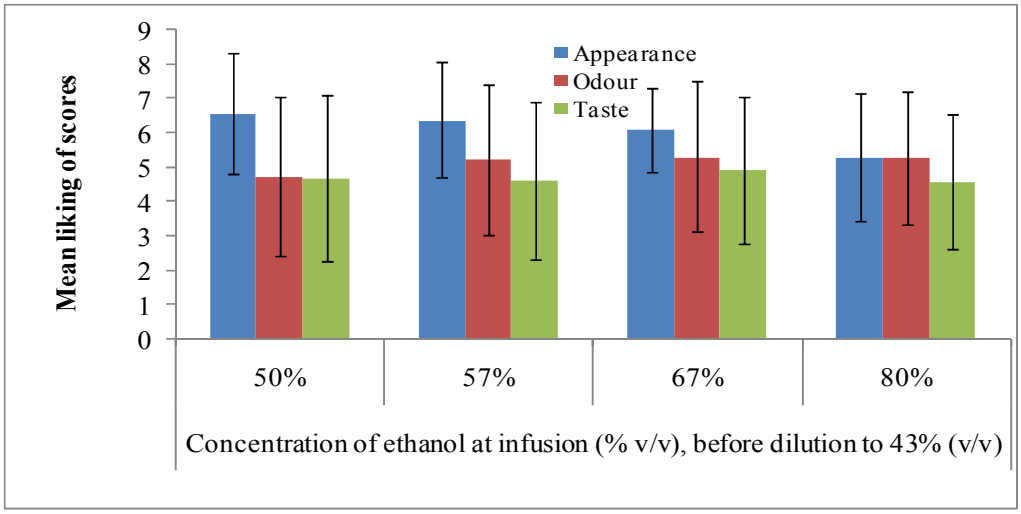


Figure 7.1 Liking of appearance, odour, and taste of the four ti kōuka spirits by all panellists as a group. Error bars represents the standard deviation.

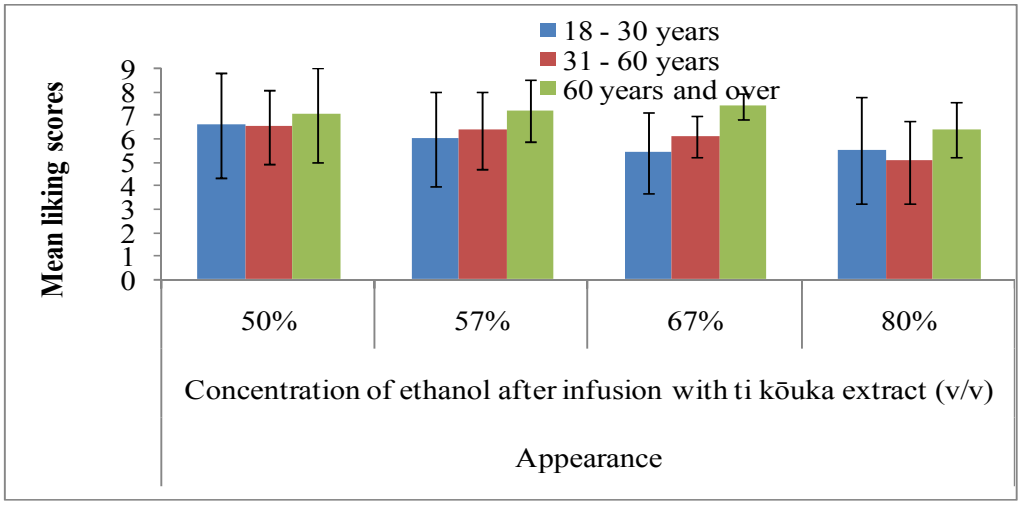


Figure 7.2 Liking of appearance of the four ti kōuka spirits by different age groups. Error bars represents the standard deviation.

Panellist age had a significant effect on the liking of appearance (P = 0.053), odour (P = 0.039) and taste (P = 0.004) of the ti kōuka spirits as a pooled group (Figure 7.2).

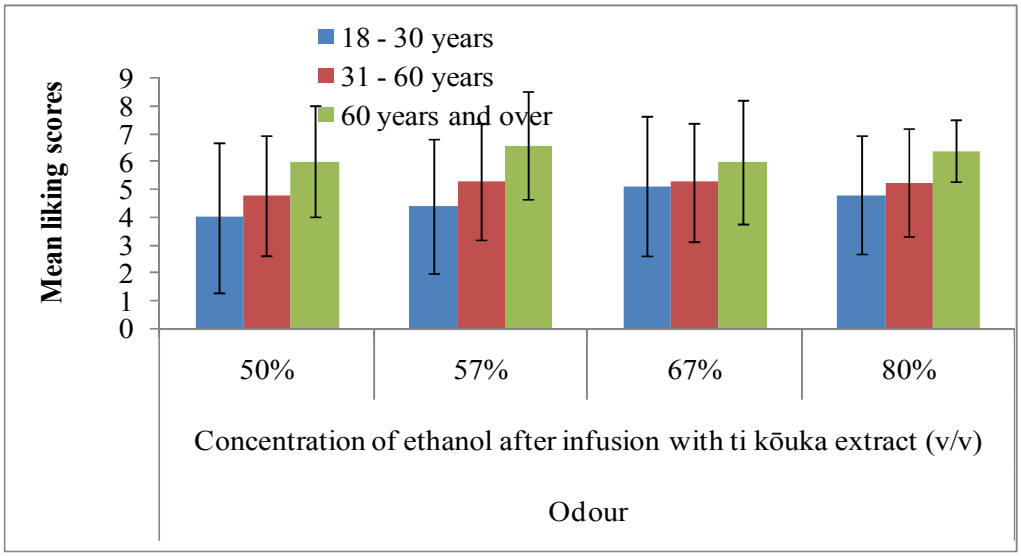


Figure 7.3 Liking of odour of the four ti kōuka spirits by different age groups. Error bars represents the standard deviation.

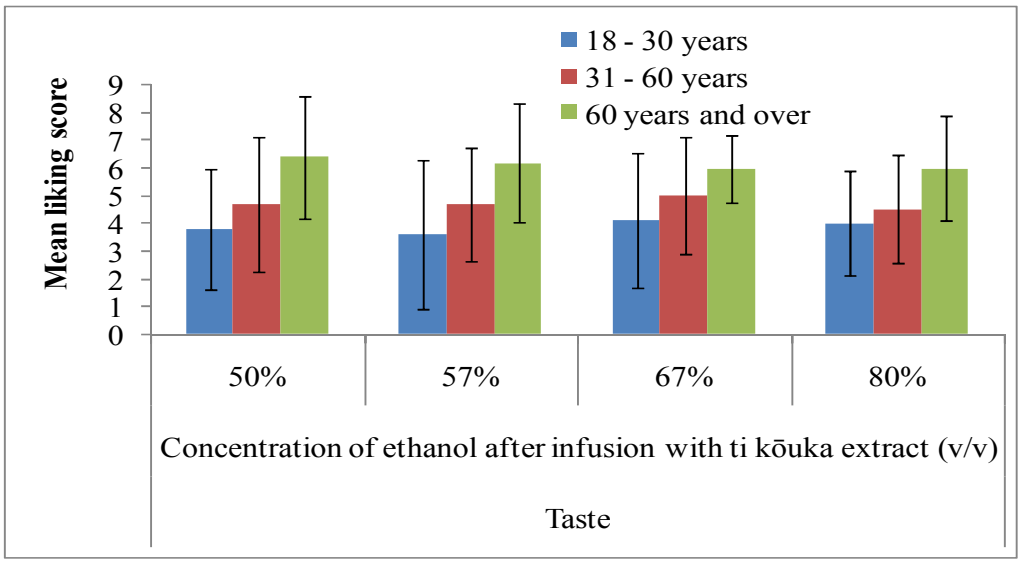


Figure 7.4 Liking of taste of the four ti kōuka spirits by different age groups. Error bars represents the standard deviation.

The older group (61 years and over) preferred the appearance, odour and taste more than the other two groups. With only two exceptions (appearance 50% and appearance 80 %), liking for these attributes decreased with age. Taste showed the biggest differences as is clear from the statistical analysis in Table 7.2 (P = 0.004).

Close inspection of the plots in Figure 7.2 to 7.4 suggests there are subtle statistical interactions between alcohol infusion concentration and age for appearance, odour and taste. However, in this limited sensory study these age-alcohol infusion interactions were considered unimportant compared with the main effects of age.

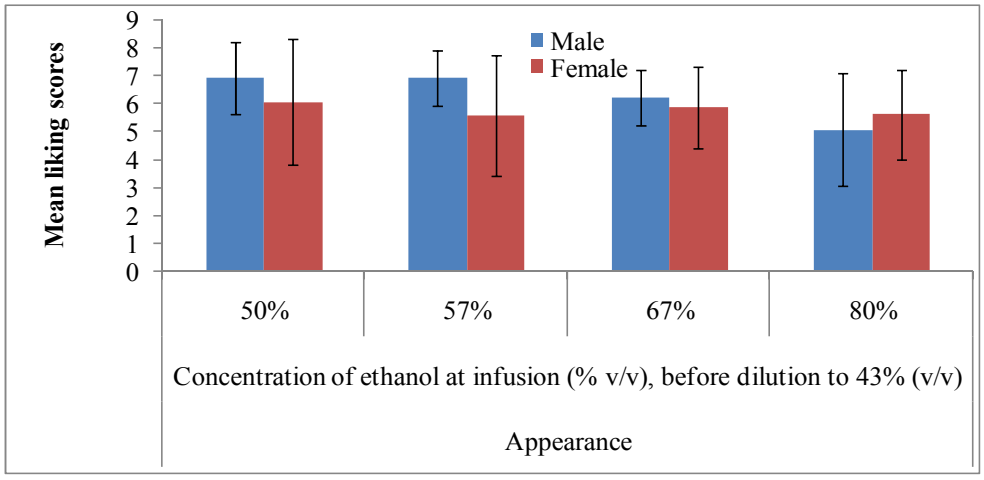


Figure 7.5 Liking of appearance of the four ti kōuka spirits by genders. Error bars represents the standard deviation.

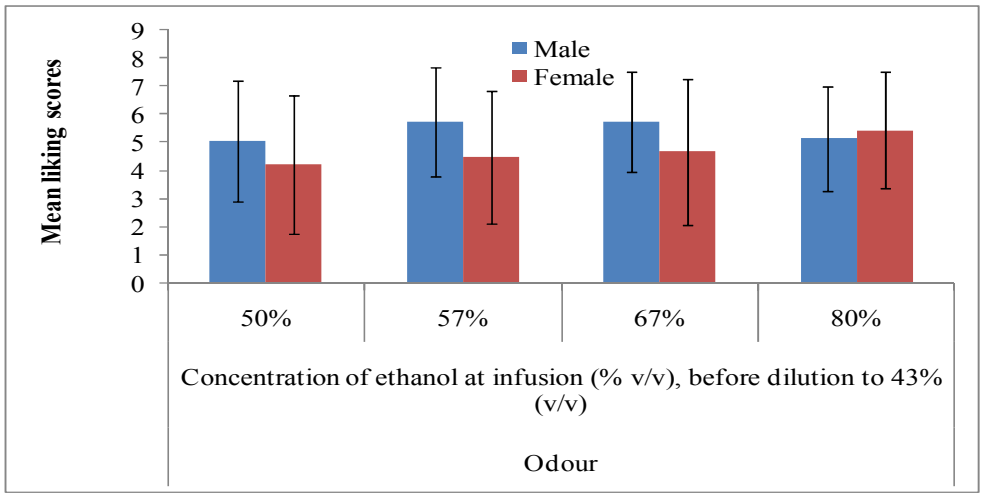


Figure 7.6 Liking of odour of the four ti kōuka spirits by genders. Error bars represents the standard deviation.

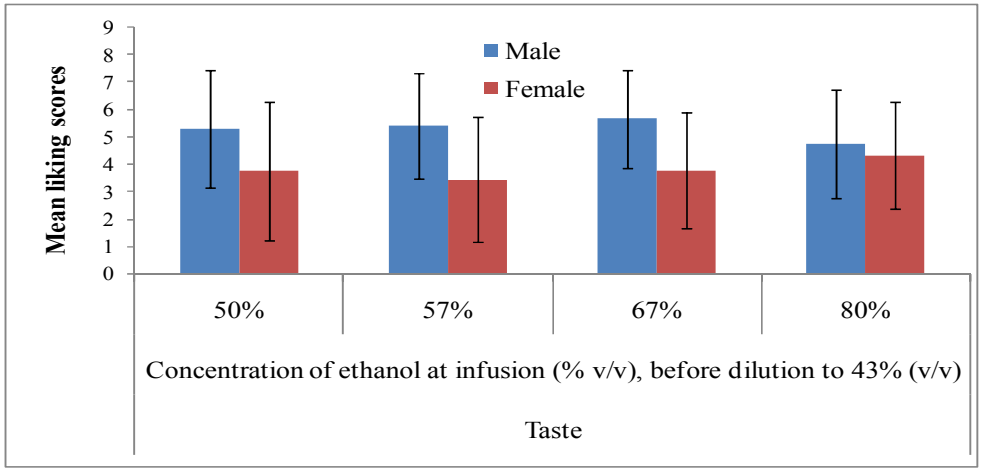


Figure 7.7 Liking of taste of the four ti kōuka spirits by genders. Error bars represents the standard deviation.

Gender had no significant effect on the liking of appearance (P = 0.095) or odour (P = 0.077) of the ti kōuka spirits (Figures 7.5 to 7.7). However, taste was liked much more (P < 0.001) by males, but it is clear by inspection (and by the Turkey test in Table 7.2) that the lighter high ethanol infusion was more equally liked. This interaction suggests that the caramel flavours extracted by the more aqueous infusions (67, 57, 50 %) were more appreciated by males. Also of interest was the fact that for 10 of the 12 individual bar plot comparisons in Figure 7.3, males showed a numerical higher preference. Males clearly liked ti kōuka more.

Discussion

Human olfactory sensitivity, and especially odour perception, can be affected by a number of factors such as age, gender, smoking habits, disease and injury (Doty, 2001). It is generally agreed that taste, odour, sound, and sight sensitivities decrease with age (Carpenter *et al*., 2000). However, when a new product to be tested is aimed at the entire population, participants of all ages are essential. In the case of the ti kōuka spirits, it was the elderly group which scored the highest for likeness of appearance, odour and taste. Younger people are more accustomed to drinking beer and pre-mix drinks which have a lower alcohol content, while the older people, are more familiar and adapted to stronger alcoholic drinks, e.g. port, rum, whisky, sherry etc. especially the male population.

The two ti kōuka spirits with the originally higher alcohol content were reminiscent of tequila (colour and odour) while the originally lower alcohol spirits were reminiscent of rum. The four ti kouka spirits tested here are in the highest alcohol category available (40 to 50%), and are more generally liked by the more matured population. The older age group had the highest scores for the three attributes. In fact, two of the panellists from the older age group did comment that they would have preferred the spirits even more if the spirits were matured. Maturation of the spirits does add more flavour to the ti kōuka spirits (as was discussed in Section 6.1, Chapter 6).

One of the new and increasingly popular beverages particularly among adolescent drinkers are ‘ready to drink’ (RTD) beverages. An RTD is a mixture of a spirit or wine and a non-alcoholic drink, served in a pre-mixed format. The most popular of these RTD products are mixtures of non-alcoholic beverages such as milk and soft drinks

with alcoholic beverages, typically spirits. In New Zealand, 18 to 24 years old are the most frequent drinkers of RTDs and hardly any people over 45 years (Ministry of Health, 2004).

In hindsight, an most important question that was not included in the questionnaire was to ask the panellist what their favourite and most often consumed drink was. Had it been asked, the pattern of popularity of certain drinks amongst certain age group, and maybe gender, would have been clarified.

Even though the results showed that the elderly group liked the ti kōuka spirits more than the other two groups, the number of participants taking part in this survey was not big enough to generalise the statement for the whole population of New Zealand, or even the University for that matter. However, assumption could be made here that consumer-acceptable spirits (at least for the older population) have been created.

CHAPTER 8

Commercial production and marketing of Ti Koka

8.1 Introduction

At the onset of the project, the objective was to extract the sugars from ti kōuka stem, to ferment them, and to distil the resulting mixture to make an iconic flavoured spirit which would be geographically distinct to New Zealand, and finally to propose a route to market.

In Best (1976), one James Hay described how the Māori used deep holes to steam-cook the ti kōuka stem, so hydrolysing the otherwise indigestible inulin. The Māori name for this cooking method is a hangi, and has parallels in several Pacific cultures (www.pigonaspit.com). In this respect, their practice was just another example of the universality of heating food before consumption in all human societies (Wrangham, 2009). Wrangham, an anthropologist, noted that in the case of starch and protein, cooking breaks down the starch molecules into more digestible fragments, while the protein is denatured so that the amino acid chain is unfolded and digestive enzymes can attack the protein more readily. The food also becomes softer and easier to chew. Overall, cooking increases absorption in the small intestine from about 50 to 90% (Wrangham, 2009).

Although there is no evidence that Māori made any alcoholic beverages from *Cordyline australis* (Best, 1976), early missionaries who settled in New Zealand prepared a 'beer' of sorts. In the mid 1880s, fermentation was taken a step further with the illicit production of 'cabbage tree rum'. An Irishman, Owen McShane living near Invercargill (South Island, New Zealand), made and sold an illicit spirit prepared from the carbohydrate-rich rhizomes of ti kōuka (Simpson, 2000). The rhizomes were chopped with a hatchet, and the slices placed in a vat and covered with water. The mixture was left to ferment, and the resulting liquor distilled to produce the spirit.

Harris and Mann (1994) investigated the technical feasibility of producing high-fructose syrups with fructose content acceptable to the food processing industry from *Cordyline australis*. They evaluated that fructose yield of 4,000 kg hectare^{-1} at densities of 80,000 plants hectare^{-1} were possible. This figure includes the average yield from both the rhizomes and the shoots of *Cordyline australis*. This 4,000 kg hectare^{-1}-fructose yield

can be compared to a 2,600 to 8,200 kg hectare^{-1}-sugar yield from chicory in New Zealand (Douglas and Poll, 1986).

It was calculated in Section 2.10 that to obtain a standard 750 mL (42%) bottle of ti kōuka spirit, 630 g of sugar would be required to generate the required 315 mL of 100% alcohol. Therefore, in effect, 4,000 kg yield of fructose could produce 6350 standard bottles of the spirit.

In the present study, it was found that the yield of fermentable sugar from the hydrolysis of ti kōuka stem was approximately 10 to 15% (w/w) dry weight. On a wet weight basis, this amounts to about 3% sugar. Using the data of Harris and Mann (1994), it is calculated that each bottle of spirit (750 mL) would require about 20 kg of wet stem/rhizome. Intuitively it was realized that this would be commercially unrealistic. The capital equipment costs would be very high for fermentation and distillation. In contrast, it takes 5.25 kg of piña to produce 750 mL of 100% agave tequila. The averge sugar content of fresh agave is between 23 to 27% which means approximately 1.2 to 1.4 kg of sugar is required to make a 750 mL of 100% agave tequila (www.ianchadwick.com).

However, a fermentation step would guarantee to produce flavours traced to yeast activity (esters, higher alcohols etc.) that would add to any volatile and therefore distillable flavor that might have its origins in the original ti kōuka stem. Another way was sought to generate flavor that could be infused into potable alcohol, so obviating the expensive sequence of extraction, fermentation and distillation. Potable alcohol is cheap in New Zealand (before taxes), so it seemed pointless to obtain that ethanol from fermentation of a low sugar source.

It was thus decided that one way of generating flavour would be through heating the ti kōuka extract to generate Maillard reaction products. The extract was simultaneously dried, reconstituted to its original volume, centrifuged, infused with different concentrations of ethanol, centrifuges and diluted to yield same final alcohol concentration of a typical strength of a premium spirit (i.e. 43%). The treatments prepared from higher ethanol infusions (80%) yielded a clear, colourless liquor with a flavour reminiscent of tequila, while lower ethanol infusions were clear but yellow to

orange/dark brown with a flavour reminiscent of rum. Results from sensory evaluation (Chapter 7) revealed that consumer-acceptable spirits were created.

If ti kōuka spirit produced from ti kōuka (*Cordyline australis*) stem is chosen for commercialisation, it must be continually available without significant legal and cultural obstacles. In addition, the commercial preparation of the spirits must be kept within well-defined cost limitations e.g. raw material, labour, plant overheads, labels, packaging, advertising and marketing (Fuller, 2005).

In this final chapter (Chapter 8), the main legal and cultural issues, production concepts from laboratory scale to industrial scale, marketing issues for Ti Koka spirits including route to market, promotion and price will be identified and discussed.

8.2 Legal and cultural issues

There is a claim before the Waitangi Tribunal (WAI 262) by members of six iwi (tribes) that claims all property associated with the indigenous flora and fauna of New Zealand. The claim is focused on ensuring appropriate recognition, protection and provision for Māori rights in relation to indigenous flora and fauna, Māori's special relationship with that flora and fauna, and all the knowledge and intellectual property rights that go with the relationship (Tuffery, 2008).

The Māori used ti kōuka as a food source, but never used it to make alcoholic drinks. European settlers however made illicit beer of sorts. Thus, this work is an extension of European activity, not indigenous culture. A research project of this nature is likely to generate intellectual property, and its relationship to the claim may not be simple. For this reason, the involvement of Māori in this research was fostered from the onset. Three Māori groups were approached to invite them to participate in this work. These groups were Ngai Tahu, Tuwharetoa and Tohu Wines. An agreement may have prevented any commercialisation of research by parties other than Māori. Each of the three groups were asked if they would like to contribute funds to support the work in return for a share of the intellectual property, with a view to commercialization. They declined for their individual commercial reasons.

Cultural and legal issues remain unclear. The unidentifiable risk could be either favourable if the Māori do not put their claim forward, or it could be unfavourable if

they decide to put a claim in which case there will be legal costs involved. For this reason, an unknown cost is ascribed to these aspects.

8.3 Land for a ti kōuka plantation and its cultivation

At present, the value of bare land in the Auckland region is approximately NZ $150,000 heactare^{-1} (www.realestate.co.nz). One alternative option would be to lease land, which would still be prohibitively expensive at around $600 heactare^{-1} month $^{-1}$ (www.realestate.co.nz). Another option would be to lease cheap marginal land realizing that ti kōuka is tolerant of a wide range of habitats including swampy land. The bare land prices for some of the other parts of North and South Island in New Zealand (www.waikatotimes.co.nz) are for example around $60,000 hectare^{-1} in the Matamata (Waikato area, North Island), $125,000 hectare^{-1} in Ngawha (North Island), $76,000 hectare^{-1} in Omanaia (North Island), $110,000 hectare^{-1} in Waimate (South Island), and NZ $66,000 hectare^{-1} in Te Anau (South Island). Some of these have volcanic land (with fertile soil) and have water supply nearby, both features could be beneficial for the *Cordyline australis* cultivation.

The most widely used and reliable method of establishing native plants is transplanting of glasshouse- or nursery-grown plants (Porteous, 1993). This method ensures a controlled environment in which seeds can germinate and have higher seedling survival compared to the direct sowing seeds in the field. However, the transplantation method may prove more expensive than the direct sowing method, each 30 to 60 cm plant costing around NZ $3 (www.oratianatives.co.nz, accessed 15-09-09). The direct method is cheaper and it also has the advantage of sowing seedlings in larger areas and the development of well-structured root systems (Douglas *et al*., 2007). For higher seedling survival, guaranteed viable seeds have to be used which may cost around NZ $1 g^{-1} (www.milliganseeds.co.nz, accessed 15-09-09).

The soil used for cultivating *C. australis* needs to contain all the right minerals as well as moisture. Therefore, cost of fertilisers and weed control agents also has to be considered. The cost of current universal weed killer containing 360 g L^{-1} glyphosate is approximately NZ $195 20 L^{-1} (www.coolstore.co.nz, accessed 14-09-09).

All trees are susceptible to disease and pests. In the case of ti kōuka, the most notable disease, called Sudden Decline, was first observed in the northern part of the North

Island in 1987 (Beever *et al*., 1996; Simpson, 1993; Rees-George *et al*., 1990). This outbreak developed into a New Zealand-wide epidemic. The etiology of the disease starts with the leaves turning yellow, followed by leaf fall until only dead branches remain. Rees-George and his co-workers found no apparent relationship between the topography of a location and incidence of Sudden Decline. Therefore, preventative measure, like spraying pesticides, would have to be taken into account in order to manage pests and diseases in ti kōuka.

The ti kōuka moth can also damage the leaves and thus the plant's photosynthetic capacity. Biological insecticides such as *Bacillus thuringiensis* can control these moths.

8.4 Harvesting

The traditional Māori practice (before 1851) for large-scale harvesting of ti kōuka involved coppicing of a perennial stem crop. Harris and Mann (1994) compared the practice of stem and rhizome harvesting – which requires a progressive replanting programme – with the traditional Māori practice of coppicing. In their work, a medium sized tree (after being cut down to a stump) was left to grow new shoots that was also analysed for fructose content after three years. The fructose content of the third order branches initially cut down gave the highest fructose reading of 28% on a dry weight basis. The most interesting results came from the three-year-old re-growth stems from the same tree. Harris and Mann found that the fructose content was actually higher than the upper *Cordyline australis* branches initially cut. From these data, Harris and Mann suggested that 3 to 4 year coppicing rotation could be used and could prove beneficial. For example, it would reduce the cost of soil cultivation, lower tooling and labour cost and the flexibility of harvesting time. This would be an advantage for the ti kōuka spirit production in that there would always be mature stem that could be harvested every year. At present, the minimal labour cost in New Zealand is \$12.50 $hour^{-1}$.

8.5 Processing and milling

The ti kōuka stems would have to be cut and the leaves removed in some way. Manually- or pneumatically-powered pruning shears would be the obvious choice for harvesting the ti kōuka stem. This operation might best be carried out after leaves had been trimmed, possibly by machete. Any of these operations will incur a labour cost. Since the structure of ti kōuka is similar to sugar cane, milling equipment used in the

sugar industry might be adapted to comminute ti kōuka stem. This equipment would require capital expenditure.

In Chapter 2 (Section 2.8), hydrolyses of ti kōuka stem tissue with Fructozyme at 60°C was explored using fresh and dried ti kōuka stem. The results showed that using fresh ti kōuka stem tissue gave markedly better yield of reducing sugar than dried stem. However, for convenience and availability of ti kōuka stem tissue, it was decided to use dry stem tissue throughout this project. By drying the stem tissue, it would be available whenever required but drying would incur a cost. At this point, the cost of drying the ti kōuka tissue has to be weighed against the availability of fresh ti kōuka stem.

Recalling Chapter 4, the results showed that the concentration of reducing sugar in the ti kōuka stem was greatest during summer months (i.e. in the fruited stage). The amino acid profiles were also different in different parts of the ti kōuka stems (Figure 3.5, Section 3.4) and in stems collected at different times of the year (Table 3.10, Section 3.4). Different combinations and ratios of sugar and amino acids also yielded different volatile compounds. On the face of it, for production of a consistent ti kōuka spirit, the seedlings must be raised from the same progeny under the same environmental conditions and the stems collected at the same time of the year. However, in large scale production systems of wine and whisky for example, variations are averaged out by blending. Blending would not be an option in the early stages of production.

Assuming that the stem tissue is not dried, the wet shredded ti kōuka stem can be hydrolysed with Fructozyme at 60°C in any sort of tank. Once hydrolysed, it could be feed through rollers, like sugarcane, to squeeze out the ti kōuka extract. The latter process can be repeated once more and the particulates can be removed by continuous filtration (as with the sugarcane). The extract can then be dried at 60°C, initially at pH 10 to produce the Maillard residue required for ethanol infusion. Centrifugation equipment is also likely to be required, again incurring a capital cost. The supernatant can then be infused with ethanol to different final ethanol concentrations as required, centrifuged and the supernatant filtered. The final filtrate can then be diluted with potable water to a final alcoholic concentration, typically between 40 and 43% v/v ethanol.

In summary, the cost of ti kōuka spirit production will include costs for rental or otherwise, of building, shredders, revolving knives, Fructozyme, filters, centrifugals, tanks, electricity, water supply, and ethanol.

The Fructozyme used in this project was kindly donated by Novozyme. However, the price sourced from Sigma would have costed NZ \$325 250 mL^{-1} (www.sigmaaldrich.com). The price markups of Sigma are understandably very high because of the hugely fragmented nature of sales. Therefore, a much wholesale supply would have to be identified.

New Zealand is one of the world's cheapest producers of ethanol (from fermentation of whey) (www.nzic.org.nz). For laboratory purposes, ethanol (99.7%) was bought for \$10 1 L^{-1} (www.ajaxfinechem.co.nz). However, the approximate wholesale price was telephone quoted by Anchor Ethanol of Auckland at \$2 L^{-1}.

8.6 Co-products

The shredded leaves from the ti kōuka stem could be used as a source of fibre for a number of low value applications, having the inherent advantage of biodegradability. The leaves could be used for weaving fabrics for different purposes (as discussed in Section 1.4.5, Chapter 1) as novelty products for tourists. The fibrous by-product left after comminuting and hydrolysing the ti kōuka stem could be sold to other farmers as livestock feed and as a mulch and soil conditioner. Fankhauser and Brasch (1985) noted that cabbage tree would be a suitable feedstock for ethanol production for fuel. These co-products could generate some income. As was mentioned in Chapter 1, Māori had a wide range of medicinal uses for the various parts of the cabbage tree and various species of *Cordyline*, and many remedies are still in use (Whistler, 1992). The traditional medicinal uses of ti suggest that co-products of medicinal value should also be investigated on a commercial scale. This could also generate money.

8.7 Flavour, stability and safety

The quality of most foods and beverages decreases with time. Exceptions include distilled spirits that develop desirable flavours during storage in wooden barrels, some wines that increase in flavour complexity in barrels and bottles, and many cheese varieties where aging leads to desirable flavour and texture (Robert, 2000). In the case of ti kōuka, the spirits were neither distilled nor aged in barrels. Recalling Section 2.18

(Chapter 2), precipitation and turbidity was observed in the spirits stored for 18 months. This problem was overcome by filtering the spirits through the Whatman GF/B glass micro-fibre filter paper. Therefore, an additional filtration process (which is already dealt with in Section 8.5) has to be considered to avoid precipitation and turbidity of the Ti Koka spirit.

From the analysis of the volatile compounds in the four ti kōuka spirits, esters represented the largest number of volatiles followed by acids, fatty acids and phytosterols. These compounds are commonly found in other spirits like whisky, gin and tequila. However, more work is still required (as is explained later in the chapter) to safely say that the ti kōuka spirits can be consumed without concern beyond the obvious effects of alcohol.

8.8 Packaging

Flavour and packaging interact as a result of three factors: migration of packaging or food components, permeation of the package by gas, water and organic vapours and exposure to light. Amongst the oldest packaging materials, glass remains popular in spite of its fragility and weight disadvantage. Using reusable containers (like glass) for packing also means that it can be recycled. Special designer bottles could be created to set it apart from the current available products. Plastic bottles are a cheaper option, but spirits packed in plastic are most definitely not considered premium. Therefore, this option would not be favoured. For sealing the bottles, metal cap is regarded as the best means of excluding oxygen. However, the choice of type of closure may well depend upon the buyer's specification, particularly when the buyer is a major supermarket group (Grainger and Tatterstall, 2005).

8.9 Labeling

Some standards that would apply for ti kōuka beverage are prescribed under the New Zealand Food Act 1981 and are also prescribed by Standards 2.7.1. (labeling of alcoholic beverages) and 2.7.5 (which defines spirits) (www.foodstandards.govt.nz). There may be some confusion as to where to place Ti Koka. Since a spirit is defined as a portable alcoholic distillate (e.g. whisky, brandy, rum, gin, vodka and tequila) that contains at least 37% alcohol by volume, produced by distillation of fermented liquor derived from food sources so as to have the taste, aroma and other characteristics

generally attributed to that particular spirit. In this regard, Ti Koka was not produced by distillation of fermented liquor, but it could not be regarded as non-alcoholic beverage either because it contains 43% v/v ethanol.

8.10 Advertising and marketing

In New Zealand, there are many routes for promoting products e.g. there are more than 200 online internet options, 40 television channels, 300 radio stations, 700 magazines, mailers, cinema, newspapers and outdoor (e.g. billboard, distributing pamphlets) options (www.media.co.nz, accessed 21-09-09). The possible main routes of advertising and marketing Ti Koka will be discussed below.

The internet opens up new opportunities for business to market and sell its products and interact with customers. Therefore, a well-designed website can be a powerful sales and marketing tool. There are many companies in New Zealand that focuses on web site design and web development e.g. Addent Interactive, Chameleon Marketing and Communications Ltd, Chubby Graphics Ltd, (www.idealog.co.nz, accessed 21-09-09) to mention a few, which could be used for setting up the website. Once a website is set up, a legal binding agreement could be set up for online users to accept a set of terms and conditions. One of these conditions should be that users under 18 years old are not allowed to access the website, and another condition is that Visa/MasterCard can be the only means of payment that can be accepted for purchasing the spirit. With the continuing evolution of internet technology, many business owners are now profiling their business on Facebook, providing yet another avenue to market.

Ti Koka spirit could also be marketed through television, although the Principles and Guidelines set out in the Codes for Advertising Liquor has to be obeyed for advertising on television. One such rule is that liquor advertising should not be shown on television between 6.00 am and 8.30 pm (www.asa.co.nz/code_liquor, accessed 21-09-09). One other option is to take the Ti Koka concept on a live current affairs program like Campbell Live, which is featured live on TV 3 during weekdays.

One other route of marketing is through bars. Management of bars could be approached with a proposal by which both parties' could benefits from it. Assuming an initially limited quantity of 300 bottles of the ti kōuka spirit were made, then 5 bottles could be used for free sample tasting for customers and 10 bottles for sale with a

condition of giving certain percentage profit from the sales of the spirit to the establishment. In this respect, 20 different bars could be approached. A similar strategy could also be applied to individually owned wine and liquor shops.

8.11 Future work

At first sight, it may not seem beneficial to increase the fructose of the ti kōuka plant. This is because the fructose is not a source of fermentable sugar. However, it is the main driver of the Maillard reactions and its closely related phenomenon of caramelisation. The fructose yield could be improved by selecting variation in the wild population. One possibility is changing the genetic structure of the wild population i.e. hybridisation. This would provide further variation for selection of plants with higher yields. Moore (1975) provided field evidence that *Cordyline* species could be crossed to form fertile hybrids. *Cordyline* species is well adapted to many New Zealand environments and once established, it is easy to grow. However, when choosing the proper variant of *C. australis*, it is especially important to take into account the plant's native area it is from (due to natural variations in *C. australis* over New Zealand), in relation to where it will cultivate and grow best in New Zealand. This will ensure that the trees will be able to withstand local environmental conditions.

When the volatile compounds were analysed by GC-MS, it was apparent from the two extraction methods that there was some variation in the compounds resolved. Therefore, a combination of different extraction techniques could be carried out for a complete analysis of the volatiles.

To improve odour and taste, the spirits are usually aged in oak wood barrels (Mosedale and Puech, 1998). During heat treatment of the inner part of barrels (toasting), lignin contained in wood decomposes with formation of phenolic aldehydes (vanillin, springaldehyde, coniferaldehyde, sinapaldehyde). To a lesser extent, furanic compounds such as 2-furaldehyde, 5-methyl furfuraldehyde and 5-hydroxymethyl furfuraldehyde are formed as the degradation products of hemicellulose. During aging process, aldehydes are extracted from wood to alcoholic beverage thus contributing in its final flavour (Panossian *et al*., 2001). Due to time and financial constrains, the ti kōuka spirit was not aged in barrels, but this remains an area for experimentation. Parallel research work at AUT University is exploring the use of toasted woods other than oak to flavour wine and spirits. The wood chosen for this work are generally New

Zealand natives (e.g. manuka), and are often totally unsuited as a barrel wood. The modern and greatly cheaper alternative is to toast chips of oak (or any other suitable wood), and to add these to the wine or spirit as required. The wood chip approach would be the direction of choice, using a New Zealand native like manuka to reinforce the concept of geographical exclusivity.

CHAPTER 9

Conclusion

The previous chapter identified the factors important in the potential commercialisation of the ti kōuka spirit in its four existing forms. All alcoholic drinks gain at least some of their appeal from the fact they all contain alcohol, a drug widely enjoyed and abused, particularly in Western societies. There is a vast array of drinks offered, and in introducing a new spirit to the New Zealand market in the first instance, there has to be a point of difference to set it apart from incumbent competitors, given that the spirit has an acceptable flavour to at least an identifiable group within the population over the age of 18 years.

The main aim of this thesis was to produce a tequila-like spirit from ti kōuka stem. The main point of difference for the spirit had to be the geographical exclusivity. Ti kōuka is a native plant of New Zealand and it can grow in a very wide range of habitats throughout New Zealand.

The Māori people had a wide range of uses for the different parts of ti kōuka trees, for example, it was used as a food source, for medicine purposes, for recreation and for fibre. The Māori never made any alcoholic drinks from ti kōuka. However, it was not until the early 1840, when the Europeans settlers who arrived in New Zealand used ti kōuka to ferment beer. Early missionaries in the Bay of Islands (northern part of the New Zealand) have also been reported to have made beer from cabbage trees by collecting the stems, boiling them and allowing the liquid to ferment. The bitterness of using cabbage tree gave the beer an excellent flavour (Simpson, 2000). Clearly, from pre-historic evidence, and with modern technology and knowhow, it is possible to make an alcoholic drink from the ti kōuka.

In this thesis, ti kōuka spirits were systematically produced by collecting the ti kōuka stems, hydrolysing it with an enzyme. Chemical analysis of the extract showed that fructose was the most abundant sugar present and the dominating amino acids were arginine, leucine, lysine Asx and Glx. The extract was then evaporated (to promote Maillard reaction - (reaction between reducing sugar and amino acids) in a conventional oven at 60°C for 65 hours and the dry layer was reconstituted with water. The reconstituted extract showed clear signs that the Maillard reaction had occurred, the

brown residue had a sweet caramel aroma reminiscent of the mash process in beer brewing. The evaporated extract was centrifuged and the supernatant was then infused with ethanol to yield different final ethanol concentrations. The ethanol mixtures were further centrifuged and filtered. Distinctive colour and flavour were generated by the infusions of ethanol in different concentrations, thus creating novel spirits. The chemical profiles of the ti kōuka spirits were different from those of the commercial spirits, thus showing that the characteristics of the aroma and flavour were unique in its own right. From the sensory evaluation of the spirits, it was revealed that the elderly group (61 years and over) liked the ti kōuka spirits more than the other two groups (aged between 18 to 60 years).

As was discussed in Chapter 8, a commercial scale production of the ti kōuka spirits is possible. By leasing land, *Cordyline australis* could be grown as a crop that could be utilised for commercial purposes. By controlling the pests, using fertilisers and using the traditional Māori practice of coppicing as a perennial stem crop, the yield of cabbage tree could be improved. By adopting milling equipment used in the sugar industry, the ti kōuka stem could be comminuted in similar manner. By manipulating and controlling the temperature, pH, reaction times and reactants, desirable aroma and flavour could be yielded to produce the final spirit. However, to proceed with this project, a large capital would be required to get this project off the ground.

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Appendix I Preparation of reagents for para-hydroxybenzoic acid hydrazine (PAHBAH)

Materials

- The alkaline reagent was prepared by dissolving 14.7 g tri-sodium citrate (BDH AnalaR 10242) and 1.47 g calcium chloride (BDH 27586) in 300 mL distilled water in a 1 L volumetric flask. In a beaker, 20 g sodium hydroxide (Scharlan ACS SO 0425) was dissolve in 200 mL distilled water. The sodium hydroxide solution was added to the volumetric flask, mixed and diluted to the 1 L mark.

- The colour reagent was prepared by completely dissolving 5 g PAHBAH (Sigma H-9882) in 1L of alkaline reagent. The colour reagent is unstable therefore; fresh reagent was made for each experiment.

- Maleic acid buffer (for making up Invertase solution) was prepared by dissolving 5.8 g of maleic acid (Sigma M-5757) and 2.1 g sodium hydroxide in 1L of distilled water and the pH adjusting to 5.5.

- Glucose (Sigma G-500) was prepared in distilled water (0.5g L^{-1} and 1.0 g L^{-1})

- Sucrose (BDH AnalaR 102744B) standard was prepared in distilled water in the range 0 to 1g L^{-1}.

- Fructose (Scientific Supplies, SSC-R157-250) standard was prepared in distilled water in the range of 0 to 5.0 g L^{-1}.

Appendix II Enzyme profile

- Fructozyme (Novozymes North America, Inc) is a mixture of exo-inulinase and endo-indulinase with temperature and pH profiles that are suitable for industrial hydrolysis of inulin in the production of fructose syrup. Fructozyme hydrolyses starch to reducing sugars. The recommendation (by the Novozyme Company) for the process parameters for hydrolysis with Fructozyme are:
  Temperature: Activity is optimal in the range of 57^{o}C to 63^{o}C
  pH: The optimum pH range for hydrolysis is broad, from 4 to 5
  Dosage: Dosage of 1.5 – 2.0 INU/g DS are recommended.

- Invertase (or sucrase), also known as β-fructofuranosidase (EC 3.2.1.26), catalyzes the hydrolysis of the non-reducing terminalof β-fructofuranoside residues i.e. the enzyme invertase catalyzes the hydrolysis of the disaccharide sucrose to invert sugar. Invert sugar is a mixture of glucose and fructose, which are both monosaccharide. When sucrose is hydrolyzed it forms a 1:1 mixture of glucose and fructose.

  Sucrose + H_2O = Glucose + Fructose

  A wide range of microorganisms produces invertase. Commercially, invertase is biosynthesized chiefly by yeast stains of *Saccromyces cerevisiae* or *Saccromyces carlsbergensis*. Invertase was purchase from Biolab (Auckland, New Zealand) BDH 39020 having a 340 EU mL^{-1} activity. Invertase exhibits relatively high activity over a broad range of pH (3.5-5.5), with optimum pH near 4.5. The maximum activity of the enzyme is at about 55^{o}C.

- Amylase (AG 300L, Novozyme AMNO 4404) is produced by submerged fermentation of a micro organism. AG 300L is a fungal glucoamylase, produced from a selected strain of *Aspergillus Niger*. The enzyme protein is separated and purified from the production organism. ά -Amylase acts as a catalyst for the step-by-step hydrolysis of 1,4-ά-D-glucosidic linkages in starch glucose being separated at the non-reducing end of the molecule. The main parameters to influence the process are temperature (between 70^{o}C and 80^{o}C), pH of the medium, concentration of substrate and of enzyme. The operating temperature for thermostable ά-amylase is most commonly 90-95^{o}C. The pH values are usually

between 6 and 8. Substrate concentrations of 30-40% dry weight content are important.

- Enzidase L 300(Amyloglucosidase) (Zymus International Limited) is a fungal glucoamylase derieved from strains of Aspergillus species. Typical application of this enzyme includes wine production, fruit juice production, baking, brewing and waste treatment. Enzidase is used to produce glucose, starting from the non-reducing ends of starch chains and dextrins. During hydrolysis, the amyloglucosidase activity removes glucose units in a stepwise manner from the non-reducing end of the substrate molecule. Enzidase has an activity of 300 GAU mL^{-1}. The pH range of activity is approximately from 3-5, with an optimum performance at pH 4.2 – 4.4. The activity of the enzyme is effective in the temperature range of 40 to 65^{o}C with an optimum performance at 60^{o}C. The exact optimum pH and temperature will depend on process variables including temperature, time, substrate nature and concentration. For the optimization of the enzyme dosage, enzyme rate of 0.75 L 1000 kg^{-1} starch (DS) is recommended.

Appendix III Mean invertase absorbances at 415 nm of different substrates at different enzyme concentrations and temperatures

Substrate	Incubation temperature	Absorbance of stock invertase solution at 415 nm	Absorbance of 1 in 2 dilution of stock invertase solution at 415 nm	Absorbance of 1 in 4 dilution of stock invertase solution at 415 nm
0 µg Sucrose	20	0.000 ± 0.04	0.000 ± 0.04	0.000 ± 0.08
	60	0.000 ± 0.02	0.000 ± 0.01	0.000 ± 0.01
	100	0.000 ± 0.05	0.000 ± 0.04	0.000 ± 0.01
80 µg Sucrose	20	0.234 ± 0.03	0.147 ± 0.02	0.186 ± 0.02
	60	0.247 ± 0.04	0.150 ± 0.07	0.119 ± 0.04
	100	0.204 ± 0.15	0.222 ± 0.01	0.166 ± 0.01
160 µg Sucrose	20	0.399 ± 0.04	0.250 ± 0.04	0.311 ± 0.06
	60	0.219 ± 0.21	0.327 ± 0.02	0.215 ± 0.08
	100	0.307 ± 0.19	0.204 ± 0.10	0.250 ± 0.04
240 µg Sucrose	20	0.503 ± 0.18	0.512 ± 0.22	0.437 ± 0.07
	60	0.465 ± 0.10	0.412 ± 0.06	0.446 ± 0.02
	100	0.628 ± 0.08	0.545 ± 0.01	0.443 ± 0.05
320 µg Sucrose	20	0.616 ± 0.20	0.661 ± 0.07	0.663 ± 0.16
	60	0.621 ± 0.11	0.550 ± 0.12	0.416 ± 0.16
	100	0.671 ± 0.17	0.734 ± 0.01	0.608 ± 0.19
400 µg Sucrose	20	1.043 ± 0.34	0.816 ± 0.01	0.822 ± 0.05
	60	0.691 ± 0.12	0.533 ± 0.33	0.671 ± 0.03
	100	0.762 ± 0.09	0.689 ± 0.02	0.578 ± 0.01
400 µg Fructose	20	-0.050 ± 0.07	0.046 ± 0.07	0.078 ± 0.06
	60	0.024 ± 0.22	0.129 ± 0.01	0.035 ± 0.09
	100	0.197 ± 0.15	0.191 ± 0.08	0.096 ± 0.07
400 µg Glucose	20	0.043 ± 0.06	-0.063 ± 0.21	0.075 ± 0.03
	60	-0.034 ± 0.26	0.189 ± 0.07	0.069 ± 0.02
	100	0.119 ± 0.06	-0.030 ± 0.07	-0.003 ± 0.07
400 µg Starch	20	-0.121 ± 0.15	-0.039 ± 0.06	0.121 ± 0.03
	60	0.022 ± 0.12	0.047 ± 0.01	-0.009 ± 0.03
	100	0.071 ± 0.09	0.004 ± 0.03	0.056 ± 0.05
400 µg Inulin	20	0.006 ± 0.10	-0.032 ± 0.05	-0.043 ± 0.06
	60	0.078 ± 0.02	0.002 ± 0.01	-0.022 ± 0.01
	100	-0.053 ± 0.03	-0.090 ± 0.03	-0.016 ± 0.03
Ti kōuka extract	20	0.134 ± 0.29	0.184 ± 0.00	0.170 ± 0.01
	60	0.252 ± 0.16	0.174 ± 0.01	0.122 ± 0.12
	100	0.185 ± 0.07	-0.043 ± 0.04	-0.002 ± 0.05

Values are the mean ± standard deviation of three replicates

Appendix IV **Mean Fructozyme absorbances at 415 nm of different substrates at different enzyme concentrations and temperatures**

Substrate	Incubation temperature	Absorbance of stock Fructozyme solution at 415 nm	Absorbance of 1 in 2 dilution of stock Fructozyme solution at 415 nm	Absorbance of 1 in 4 dilution of stock Fructozyme solution at 415 nm
0 µg Sucrose	20	0.000 ± 0.07	0.000 ± 0.03	0.000 ± 0.01
	60	0.000 ± 0.11	0.000 ± 0.01	0.000 ± 0.02
	100	0.000 ± 0.05	0.000 ± 0.01	0.000 ± 0.02
80 µg Sucrose	20	0.048 ± 0.28	0.205 ± 0.03	0.118 ± 0.01
	60	0.294 ± 0.01	0.141 ± 0.04	0.137 ± 0.01
	100	0.239 ± 0.03	0.159 ± 0.02	0.178 ± 0.02
160 µg Sucrose	20	0.378 ± 0.05	0.331 ± 0.04	0.269 ± 0.04
	60	0.331 ± 0.12	0.325 ± 0.01	0.213 ± 0.07
	100	0.160 ± 0.19	0.256 ± 0.03	0.276 ± 0.04
240 µg Sucrose	20	0.495 ± 0.11	0.422 ± 0.07	0.467 ± 0.01
	60	0.601 ± 0.05	0.340 ± 0.18	0.383 ± 0.02
	100	0.538 ± 0.04	0.398 ± 0.06	0.573 ± 0.22
320 µg Sucrose	20	0.624 ± 0.02	0.593 ± 0.03	0.521 ± 0.16
	60	0.760 ± 0.01	0.558 ± 0.01	0.500 ± 0.01
	100	0.515 ± 0.14	0.592 ± 0.04	0.577 ± 0.08
400 µg Sucrose	20	0.716 ± 0.21	0.580 ± 0.15	0.627 ± 0.06
	60	0.634 ± 0.24	0.661 ± 0.04	0.589 ± 0.02
	100	0.703 ± 0.11	0.507 ± 0.27	0.660 ± 0.01
400 µg Fructose	20	0.231 ± 0.07	-0.045 ± 0.22	0.053 ± 0.02
	60	0.005 ± 0.17	-0.022 ± 0.19	0.076 ± 0.01
	100	0.228 ± 0.01	-0.007 ± 0.11	0.145 ± 0.03
400 µg Glucose	20	0.121 ± 0.09	-0.018 ± 0.17	0.054 ± 0.03
	60	0.125 ± 0.61	0.020 ± 0.04	-0.030 ± 0.11
	100	-0.034 ± 0.05	-0.318 ± 0.30	-0.103 ± 0.01
400 µg Starch	20	0.074 ± 0.15	-0.115 ± 0.09	-0.170 ± 0.03
	60	0.341 ± 0.23	0.224 ± 0.08	0.219 ± 0.04
	100	0.042 ± 0.05	-0.074 ± 0.07	0.008 ± 0.01
400 µg Inulin	20	0.076 ± 0.26	0.224 ± 0.10	0.198 ± 0.04
	60	0.756 ± 0.11	0.509 ± 0.04	0.557 ± 0.08
	100	0.646 ± 0.05	0.537 ± 0.11	0.428 ± 0.05
Ti kōuka extract	20	0.529 ± 0.18	0.488 ± 0.03	0.377 ± 0.01
	60	0.541 ± 0.05	0.433 ± 0.04	0.300 ± 0.01
	100	0.265 ± 0.18	0.142 ± 0.12	0.266 ± 0.06

Values are the mean ± standard deviation of three replicates

Appendix V **Description of reconstituted ti kōuka extract (evaporated at different temperatures) infused with different proportions of ethanol**

Temperature	Final ethanol concentration (%)	L*	a*	b*
Ambient	66.7	18.9 ± 0.13	4.56 ± 0.11	11.8 ± 0.32
	50.0	9.17 ± 0.02	3.25 ± 0.20	7.97 ± 0.09
	33.3	2.52 ± 0.12	1.50 ± 0.23	2.40 ± 0.31
	16.7	0.74 ± 0.39	1.71 ± 0.86	1.39 ± 0.62
	0.0	0.59 ± 0.06	0.92 ± 0.10	0.80 ± 0.27
60°C	66.7	16.69 ± 0.17	5.59 ± 0.11	14.0 ± 0.33
	50.0	5.89 ± 0.06	2.64 ± 0.19	5.51 ± 0.17
	33.3	2.37 ± 0.07	1.35 ± 0.14	2.57 ± 0.32
	16.7	2.24 ± 0.56	0.87 ± 0.20	0.93 ± 0.20
	0.0	1.03 ± 0.13	0.89 ± 0.51	1.23 ± 0.25
100°C	66.7	12.7 ± 0.48	7.35 ± 0.12	14.5 ± 0.25
	50.0	0.96 ± 0.05	0.23 ± 0.10	0.94 ± 0.05
	33.3	1.15 ± 0.06	-0.16 ± 0.15	0.53 ± 0.22
	16.7	1.10 ± 0.06	-0.25 ± 0.13	0.25 ± 0.12
	0.0	0.59 ± 0.05	-0.12 ± 0.11	0.40 ± 0.14

Values are the means ± standard deviation (n = 5)

Appendix VI Kjeldahl Method

Materials

Concentrated sulphuric acid (BDH 30325 6G GPR)
Potassium sulphate (BDH AnalaR 10220.4B)
Copper sulphate (BDH AnalaR 103733C)
Anti bumping granules (BDH 330093Y)
Concentrated hydrochloric acid (Univar UN1789)
Sodium hydroxide (BDH AnalaR 10252.5P)
Methyl red (BDH CI 13020)
Methyl blue (George T. Gurr 17900)

The Kjeldahl method has three steps:

Digestion Step

During the digestion step, proteins and other forms of nitrogen are broken down and converted to ammonium ion.

Method

Samples (0.5 g each) were place in digestion tubes with 10 mL concentrated sulphuric acid, 3.0 g catalyst (9 parts potassium sulphate (K_2SO_4) and 1 part copper sulphate ($CuSO_4.5H_2O$)) and a few bumping granules. Heat was applied (approximately 370^oC-400^oC) until the entire sample was digested and the mixture gradually became clear. After cooling to room temperature, 20 mL of distilled water was added.

Organic N by-product	+ H_2SO_4	⟶ $(NH_4)_2SO_4$	+ H_2O	+ CO_2	+ Other sample matrix
Sample	Conc. sulphuric acid	Ammonium sulphate	Hydrogen converted to water	Carbon converted to carbon dioxide	

Distillation Step (using Velp Scientifica UDK 126A Distillation Unit)

Distillation involves the separation of ammonia-nitrogen from the digestate by raising the pH with sodium hydroxide to give a strong alkaline (pH >11) solution. This changes the ammonium (NH_4^+) ion to ammonia (NH_3)
The ammonium (NH_4+) ion ⟶ ammonia (NH_3)

$(NH_4)_2SO_4$	+ 2NaOH	⟶ $2NH_3$	+ Na_2SO_4	+ $2H_2O$
Ammonium sulphate	Sodium hydroxide	Ammonia gas	Sodium sulphate	Water

In the steam distillation unit, 10mL of 35% sodium hydroxide was dispensed automatically into the digesting tube. In the receiving Erlenmeyer flask, 10 mL of

0.05M sulphuric acid and three drops of Kjeldahl indicator was added. The indicator was prepared by dissolving 0.6 g of methyl red in 50 mL of 95% ethyl alcohol and then added to methyl blue solution (0.1 g methyl blue dissolved in 50 mL distilled water). Approximately 150mls of distillate was collected in the receiving Erlenmeyer flask. Receiving solution was kept below 45^{o}C during distillation to prevent loss of ammonia.

The Titration Step

The titration process indicates the ammonia present in the distillate with a colour change and allows for calculation of unknown concentrations. Ammonia is captured by the standardized sulphuric acid solution in the receiving flask. The excess acid in the receiving solution keeps the pH low, and the indicator does not change.

$2NH_3$	+	H_2SO_4	⟶	$(NH_4)_2SO_4$	+	H_2SO_4
Ammonia sulphate		Standard sulphuric acid		Ammonium sulphate		Excess sulphuric acid

(No colour change)

The excess acid solution is titrated with standardized alkaline base solution such as sodium hydroxide. A colour change is produced at the end point of the titration.

$(NH_4)2SO_4$	+	H_2SO_4	+	2NaOH	⟶	$(Na)_2SO_4$	+	$(NH_4)_2SO_4$
Ammonia sulphate		Measured excess acid		Measured excess sodium hydroxide		Sodium sulphate		Ammonium sulphate

(Colour change)

The following formula was used to calculate the percentage nitrogen:

% Protein = [0.007[*1] x (Vb – Vs) x F x 6.25[*2] x 20 x 100]/S
Where : Vs = mL of 0.05N NaOH used for titrating the sample
Vb = mL of 0.05N NaOH used for titrating the blank
F = Correction factor for the 0.05N NaOH standard solution (i.e. normality of NaOH/standard 0.05N NaOH)
S = Weight of sample (g)
[*1] = Each mLof 0.05N NaOH is equivalent to 0.0007 g of Nitrogen
[*2] = Nitrogen Factor (since protein is assumed to be 16% nitrogen, the factor 6.25 (i.e. 100/16) is used to convert total nitrogen to total protein, although the values for the factor depend on the kind of protein

Appendix VII Performic Acid Oxidation with Acid Hydrolysis – AOAC Official Method 99.12

Materials and Reagents

- Tri sodium citrate (BDH AnalaR 102242.4L)
- Thioglycol (Fluka 63700)
- Concentrated hydrochloric acid (Univar UN 1789)
- Sodium hydroxide (BDH AnalaR 10252.5P)
- Phenol crystal (Scharlau FE 0480)
- Formic acid BDH AnalaR 10115.6G)

Preparation of Reagents

- Sodium citrate buffer, pH 2.0 : Dissolve 19.60 g tri-sodium citrate dehydrate in 1 in about 800 mL distilled water. While stirring, add 10 mL 98% thioglycol solution and 15 mL concentrated HCl. Transfer solution quantitatively into 1L volumetric flask and dilute to mark with distilled water. Adjust pH to 2.2 with concentrated HCl or 2M NaOH.
- 6N HCl-Phenol solution: Weigh 1g phenol crystal into 1L beaker. Dissolve crystals in 500mL distilled water. While stirring, slowly add 500mL concentrated HCl.
- Performic acid reagent: Prepare in hood. Weigh 25 g phenol crystals in 25 mL test tube; then add 0.5 mL 30% H_2O_2, using a micropipette, and 4.5 mL of 88% formic acid solution. Cover test tube with stopper, and let mixture stand for 30 minutes at room temperature. After 30minutes, place the tube in an ice bath (0^oC) and cool the performic acid mixture for 15minutes. Prepare the reagent just before use.

Appendix VIII Brand and catalogue numbers of chemicals

Amino Acid	Brand/Catalogue Number
γ-Amino butyric acid (GABA)	Sigma A-2129
Arginine	Sigma A-5006
Cystine	Sigma C-8755
Glutamic acid	Sigma G-1251
Glutamine	Sigma G-3126
Histidine	Sigma H-8125
Lysine	Sigma L-5626
Ornithine	Sigma O-2375
Serine	Sigma S-4500
Threonine	Sigma T-8625
Tryptophan	Sigma T-0254
Glucose	Sigma G-5000
Glycine	BDH 28458
Hydroxyproline	BDH 37120
Iso-leucine	BDH 37124
Leucine	BDH 37121
Methionine	BDH 37132
Taurine	BDH 37149
Tyrosine	BDH 37156
Valine	BDH 37160
Disodium hydrogen phosphate	BDH 10249
Alanine	Merck 302
Aspargine	Merck 1565
Aspartic acid	Merck 123
Phenylalanine	Merck 7257
Fructose	SSC-R157-250
Citric acid	Scharlau ACO 725

Appendix IX **Amino acid profile (percent of total recovered amino acids) of ti kōuka stems sections (collected in February) at different stages of the ti kōuka process**

Amino acids	Top section (tip to 20 cm)			Second section (20 to 40 cm)			Third section (40 to 60 cm)		
	Dried ground stem	After evaporation and reconstitution	After centrifugation	Dried ground stem	After evaporation and reconstitution	After centrifugation	Dried ground stem	After evaporation and reconstitution	After centrifugation
Aspartic acid + Aspargine (Asx)	3.16	2.14	2.26	1.91	3.07	3.39	1.46	3.11	2.47
Glutamic acid + Glutamine (Glx)	4.77	3.15	3.76	3.29	4.56	4.95	2.28	5.42	7.05
Serine	2.26	3.72	4.44	1.95	3.63	4.28	1.47	4.06	3.18
Histidine	6.50	9.02	5.65	5.96	6.44	5.17	4.25	11.10	6.20
Glycine	0.61	0.26	0.30	0.29	0.49	0.51	0.29	1.08	0.37
Threonine	5.09	14.26	12.27	4.10	11.44	17.35	4.14	7.66	5.52
Arginine	17.70	34.13	22.32	12.65	25.26	18.07	7.50	22.75	28.59
Alanine + Taurine	1.49	2.54	2.39	1.24	1.72	1.84	0.85	1.08	0.58
GABA	2.32	1.09	2.19	2.55	1.45	1.33	1.74	0.44	2.69
Tyrosine	1.32	1.85	1.26	0.97	1.23	1.34	0.72	0.78	1.16
Methionine	1.37	1.87	1.59	0.96	1.00	1.00	0.68	1.12	0.68
Valine	4.59	6.81	4.96	4.10	4.66	4.12	3.46	4.69	2.84
Phenylalanine	0.93	1.31	0.77	0.60	0.91	0.54	0.43	0.67	0.34
Isoleucine	1.73	2.40	1.09	0.80	1.35	1.22	0.70	1.26	0.74
Leucine	28.67	6.99	14.32	52.63	16.93	21.89	56.00	24.57	24.21
Ornithine	1.24	0.76	0.67	2.55	0.92	1.08	1.30	1.11	1.83
Lysine	16.26	7.69	19.76	3.45	14.95	11.94	12.74	9.11	11.55

Appendix X **Amino acid profile (percent of total mass of amino acid) of fruited ti kōuka sample (collected in April) at different stages of process**

Amino Acid	Dried ground stem	After Fructozyme hydrolysis	After deionised water hydrolysis	After Fructozyme evaporation and reconstitution	After deionised water evaporation and reconstitution	After Fructozyme centrifugation	After deionised water centrifugation
Asx	5.67	7.34	7.11	8.05	7.97	8.72	9.18
Glx	7.46	9.95	12.19	10.96	11.83	14.69	15.68
Serine	7.61	7.58	6.81	7.49	6.85	6.70	6.60
Histidine	5.73	3.85	4.88	3.16	4.30	5.89	3.72
Glycine	4.01	4.06	3.92	4.75	3.85	4.04	3.31
Threonine	5.60	5.88	5.23	5.24	5.75	5.94	7.97
Alanine + Taurine	4.76	5.00	6.64	4.54	4.47	4.42	6.90
Arginine	16.43	17.96	12.02	10.18	10.75	11.29	12.17
GABA	4.99	4.81	4.54	4.32	5.33	4.00	3.81
Tyrosine	0.22	0.31	0.78	3.97	3.67	0.03	0.02
Methionine	0.98	1.06	2.54	0.45	4.05	5.73	1.92
Valine	4.95	4.63	4.66	6.04	3.69	3.83	3.92
Phenylalanine	3.47	3.20	3.03	3.53	3.03	2.67	2.77
Isoleucine	3.95	3.99	3.53	4.21	3.07	3.31	3.24
Leucine	14.72	13.80	13.16	15.12	12.42	10.83	11.68
Ornithine	0.68	1.00	1.66	2.23	2.62	3.17	2.95
Lysine	8.79	5.57	7.29	5.76	6.36	4.74	4.14

Appendix XI **Absorbances (at 420 nm) of sugar/amino acid model samples after heating at 80°C at different incubation times**

Model No.	pH	Model system	Absorbance of model samples incubated for 60 minutes	Absorbance of model samples incubated for 180 minutes	Absorbance of model samples incubated for 300 minutes
1	3	Fructose + Arginine	0.006	0.016	0.015
2	3	Fructose + Lysine	0.072	0.108	0.162
3	3	Fructose + Leucine	0.005	0.020	0.004
4	3	Glucose + Arginine	0.009	0.106	0.359
5	3	Glucose + Lysine	0.032	0.441	1.338
6	3	Glucose + Leucine	0.007	0.023	0.005
7	5	Fructose + Arginine	0.038	0.194	0.479
8	5	Fructose + Lysine	0.431	2.874	4.212
9	5	Fructose + Leucine	0.009	0.033	0.052
10	5	Glucose + Arginine	0.113	0.895	2.254
11	5	Glucose + Lysine	0.742	5.748	10.965
12	5	Glucose + Leucine	0.083	0.017	0.010
13	7	Fructose + Arginine	0.385	1.623	2.898
14	7	Fructose + Lysine	3.118	11.185	14.567
15	7	Fructose + Leucine	0.050	0.205	1.752
16	7	Glucose + Arginine	0.586	2.518	4.387
17	7	Glucose + Lysine	3.156	12.813	19.623
18	7	Glucose + Leucine	0.051	0.659	1.739
19	9	Fructose + Arginine	2.028	4.866	6.340
20	9	Fructose + Lysine	7.212	1.950	25.093
21	9	Fructose + Leucine	0.197	0.904	2.070
22	9	Glucose + Arginine	1.916	0.919	7.250
23	9	Glucose + Lysine	7.737	2.171	29.033
24	9	Glucose + Leucine	0.440	1.867	3.979
25	9	Fructose	0.264	0.915	1.408
26	9	Glucose	0.066	0.305	0.585
27	9	Arginine	0.036	0.010	0.009
28	9	Lysine	0.034	0.009	0.018
29	9	Leucine	0.211	0.104	0.081

Appendix XII Information Sheet

THE POTENTIAL FOR NEW ZEALAND INDIGENOUS PLANTS IN THE FORMATION OF NOVEL ALCOHOLIC DRINKS

I would like you to take part in a sensory trial as a part of my PhD degree. I want to know the preference of the appearance, odour, and taste of the alcoholic spirits I have created. These spirits have been made from a New Zealand native plant. The panellist will have to score the four alcoholic spirits for liking.

To take part in the trial you have to be 18 years or older. Your participation in this trial is entirely voluntary and you may withdraw at any time. Firstly, you will be asked if you would like to take part in this trial and if you do, you will be presented with four spirit samples. The four samples have to be scored by the preference of the appearance of the samples, the odour and finally the taste. Each sample contains 43% alcohol (v/v) which is equivalent to approximately 0.5 of a standard alcoholic drink; therefore, you are advised to spit out the samples after tasting each of them. You must wash with/drink water between each sample.

What do I do if I have concerns about this research?
At the completion of the work in May, 2008 you are invited to discuss the results with Minaxi (Meena) Patel (ext 8185) or Owen Young (ext 8150).

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor, Owen Young, owen.young@aut.ac.nz, 921 9999 ext 8150.

Concerns regarding the conduct of the research should be notified to the Executive Secretary, AUTEC, Madeline Banda, madeline.banda@aut.ac.nz , 921 9999 ext 8044.
Auckland University of Technology Ethics Committee approval for application given on 10/03/2008

Appendix XIII Consent Form

Project title: ***The Potential for New Zealand indigenous plants in the creation of novel alcoholic drinks***

Project Supervisor: ***Owen Young***

Researcher: ***Minaxi Patel***

- I have read and understood the information provided about this research project in the Information Sheet
- I have had an opportunity to ask questions and to have them answered
- I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way
- I have no known allergy to alcoholic spirits or flavours in spirits
- I agree to take part in this research by tasting the alcoholic spirits
- I am at least 18 years old

Participant's signature ……

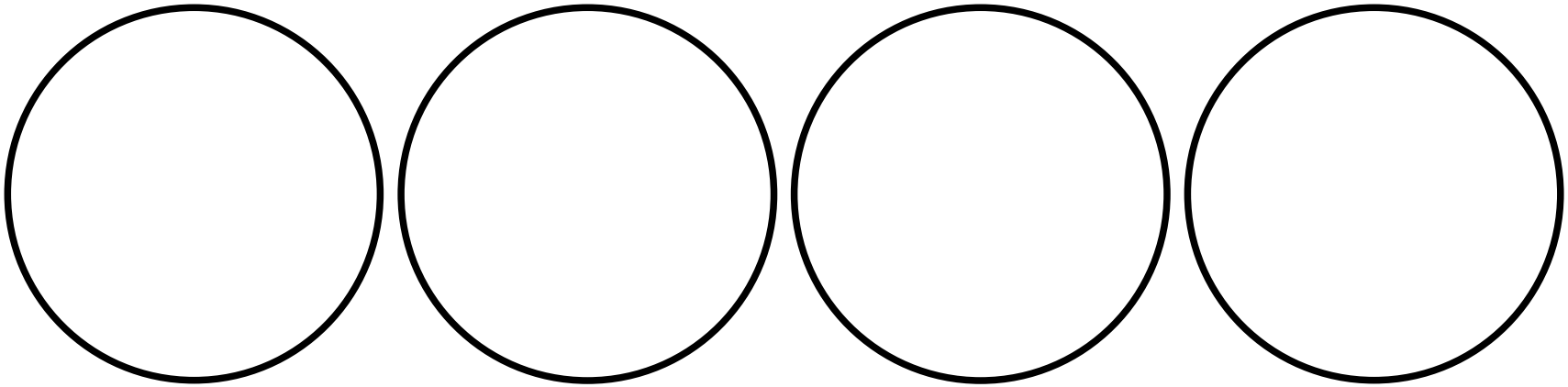
Participant's name …….

Date: …….

Approved by the Auckland University of Technology Ethics Committee on 10/03/2008

Appendix XIV Presentation of ti kōuka spirit for sensory evaluation

Panellist 01



Order of scoring ti kōuka spirits

Appendix XV Mean panellist liking scores and standard deviation grouped by gender and age groups

	Concentration of ethanol after infusion with ti kōuka extract (% v/v)	Total (N = 45)	Female (N = 18)	Male (N = 27)	Age Group A (18 – 30) N = 10	Age Group B (31 – 60) N = 30	Age Group C (61 and over) N = 5
Appearance	50	6.58 ± 1.8	6.93 ± 1.3	6.06 ± 2.2	6.60 ± 4.6	6.50 ± 1.6	7.00 ± 2.0
	57	6.38 ± 1.7	6.93 ± 1.0	5.56 ± 2.2	6.00 ± 2.0	6.37 ± 1.6	7.30 ± 1.3
	67	6.04 ± 1.2	6.22 ± 1.0	5.78 ± 1.5	5.40 ± 1.7	6.03 ± 1.0	7.40 ± 0.6
	80	5.24 ± 1.9	5.07 ± 2.0	5.61 ± 1.6	5.50 ± 2.3	5.03 ± 1.8	6.40 ± 1.1
Odour	50	4.73 ± 2.3	5.07 ± 2.2	4.22 ± 2.5	4.00 ± 2.7	4.77 ± 2.2	6.00 ± 2.0
	57	5.24 ± 2.2	5.74 ± 2.0	4.50 ± 2.4	4.40 ± 2.4	5.30 ± 2.1	6.60 ± 2.0
	67	5.31 ± 2.2	5.74 ± 1.8	4.67 ± 2.6	5.10 ± 2.5	5.27 ± 2.1	6.00 ± 2.2
	80	5.27 ± 1.9	5.15 ± 1.9	5.44 ± 2.1	4.80 ± 2.2	5.23 ± 1.9	6.40 ± 1.1
Taste	50	4.69 ± 2.4	5.30 ± 2.2	3.78 ± 2.5	3.80 ± 2.2	4.70 ± 2.4	6.40 ± 2.2
	57	4.62 ± 2.3	5.41 ± 1.9	3.44 ± 2.3	3.60 ± 2.7	4.70 ± 2.0	6.20 ± 2.2
	67	4.91 ± 2.1	5.67 ± 1.8	3.78 ± 2.1	4.10 ± 2.4	5.00 ± 2.1	6.00 ± 1.2
	80	4.58 ± 2.0	4.74 ± 2.0	4.33 ± 2.0	4.00 ± 1.9	4.53 ± 1.9	6.00 ± 1.9

Values are the mean ± standard deviation

Appendix XVI Panellist ballot form for liking of Appearance

Panelist number

Gender: M ☐ **F** ☐

Age range: **18-30** ☐ **31-60** ☐ **61 and over** ☐

How much do you like each of these spirits?
Colour preference from left to right
For each spirit tick the box that best describes your liking

	647	912	860	155
Like extremely	☐	☐	☐	☐
Like a lot	☐	☐	☐	☐
Like moderately	☐	☐	☐	☐
Like slightly	☐	☐	☐	☐
Neither like nor dislike	☐	☐	☐	☐
Dislike slightly	☐	☐	☐	☐
Dislike moderately	☐	☐	☐	☐
Dislike a lot	☐	☐	☐	☐
Dislike extremely	☐	☐	☐	☐

Any comments?

Appendix XVII Panellist ballot form for liking of Odour

Panelist number

Gender: M ☐ **F** ☐

Age range: **18-30** ☐ **31-60** ☐ **61 and over** ☐

How much do you like each of these spirits?
Colour preference from left to right
For each spirit tick the box that best describes your liking

	647	912	860	155
Like extremely	☐	☐	☐	☐
Like a lot	☐	☐	☐	☐
Like moderately	☐	☐	☐	☐
Like slightly	☐	☐	☐	☐
Neither like nor dislike	☐	☐	☐	☐
Dislike slightly	☐	☐	☐	☐
Dislike moderately	☐	☐	☐	☐
Dislike a lot	☐	☐	☐	☐
Dislike extremely	☐	☐	☐	☐

Any comments?

Appendix XVIII Panellist ballot form for liking of Taste

Panelist number

Gender: M ☐ **F** ☐

Age range: **18-30** ☐ **31-60** ☐ **61 and over** ☐

How much do you like each of these spirits?
Colour preference from left to right
For each spirit tick the box that best describes your liking

	647	912	860	155
Like extremely	☐	☐	☐	☐
Like a lot	☐	☐	☐	☐
Like moderately	☐	☐	☐	☐
Like slightly	☐	☐	☐	☐
Neither like nor dislike	☐	☐	☐	☐
Dislike slightly	☐	☐	☐	☐
Dislike moderately	☐	☐	☐	☐
Dislike a lot	☐	☐	☐	☐
Dislike extremely	☐	☐	☐	☐

Any comments?