Development of a novel alcoholic spirit from the New Zealand native plant *Cordyline australis*

Weiqing Huang

Supervisor: Associate Professor Owen Young

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***Cordyline australis*（ti kouka）in New Zealand**

Contents Page number

Cover i
Picture of *Cordyline australis* ii
Table of contents iii
List of figures vii
List of tables ix
List of appendices x
Attestation of authorship xi
Acknowledgements xii
Abstract xiii

CHAPTER 1 1

Introduction 1
1.1 Distilled alcohol drinks 1
1.2 Geographical distinctiveness in alcoholic drinks 4
1.3 New Zealand alcoholic drinks 5
1.4 Botany of *Cordyline australis* 7
1.5 Māori uses of *Cordyline australis* 9
1.6 *Cordyline australis* as a source of carbohydrate 12
1.7 Sugar extraction of *Cordyline australis* compared with agave 13
1.8 *Cordyline australis* use for producing illicit alcoholic drinks 14
1.9 The purpose of this research 15

CHAPTER 2 16

Pilot Development of Ti kouka Spirit 16
2.1 Introduction 16
2.2 Materials and methods 16
2.2.1 Yeast 16
2.2.2 Preparation of *Cordyline australis* stems 16
2.2.3 Fermentation treatments 17
2.2.4 Simultaneous distillation and extraction of volatiles from *Cordyline australis* *stem* 18

2.3 Results and discussion 19
2.4 Brief summary 21
CHAPTER 3 21
Kinetics Study on the Exponential Phase of the Ti kouka Fermentation Process 22
3.1 Introduction 22
3.1.1 Mode of batch fermentation 22
3.1.2 Components of fermentation 22
3.1.3 Production of alcohol 22
3.1.4 Production of other flavour compounds 24
3.2 Materials and methods 25
3.2.1 Chemicals 25
3.2.2 Preparation and characterization protocols of fermentation 26
3.2.3 Physicochemical analyses 27
3.2.4 Microbiological analyses 29
3.2.5 Data analysis 29
3.3 Results and discussion 30
3.3.1 Kinetics of colour during fermentation 30
3.3.2 Kinetics of yeast growth and biomass production 34
3.3.3 Kinetics of pH and CO_2 production 36
3.3.4 Kinetics of sugar content expressed as Brix 37
3.3.5 Kinetics of ethanol concentration and sugar concentration 38
3.4 Mathematical model 42
3.5 Brief summary 43
CHAPTER 4 45
Study of the Maillard Reaction in a Top Stem/Fructose Model System 45
4.1 Introduction 45
4.1.1 Modelling of the Maillard reaction 45
4.1.2 Classes of aroma compounds formed in the Maillard reaction 47
4.1.3 Detection of compound formation in the Maillard reaction 48

4.1.4 Experimental design 48
4.2 Materials and methods 48
4.2.1 Preparation and characterization protocols of reflux 48
4.2.2 Physicochemical analyses 49
4.2.3 Statistical analysis 50
4.3 Results and discussion 50
4.3.1 Colour development from incubations of ti kouka stem with frutcose 50
4.3.2 Description of colour and odour from incubations of ti kouka stem with fructose 54
4.3.3 Volatile compounds generated in Top stem/fructose model system 56
4.4 Brief summary 62

CHAPTER 5 63

Characterization of Major Volatile Profiles in Distillation Fractions by Gas Chromatography with Flame Ionization Detection (GC-FID) 63
5.1 Introduction 63
5.1.1 Distillation to achieve fractionation of flavour 63
5.1.2 Distillation in tequila production 63
5.2 Materials and methods 64
5.2.1 Chemicals 64
5.2.2 Fermentation protocol 64
5.2.3 Distillation protocol 64
5.2.4 Physicochemical analyses 65
5.2.5 Statistical analysis 66
5.3 Results and discussion 67
5.3.1 Colour and odour of distilled fractions 67
5.3.2 Major volatile compounds analysed by the GC-FID 71
5.3.3 Distribution of dominant volatiles in Fractions 1 to 4 of Top stem distillates 77
5.4 Brief Summary 78

CHAPTER 6 79

Determination of Terpenes and Ethyl Esters in Four Spirits by Solid Phase Microextraction and Gas Chromatography-Mass Spectrometry 79

6.1 Introduction 79
6.2 Materials and methods 80
6.2.1 Spirits 80
6.2.2 Optimization of the SPME procedure 80
6.2.3 Sample preparation 81
6.2.4 Extraction procedure 81
6.2.5 Statistical analysis 82
6.3 Results and discussion 82
6.4 Brief summary 87
CHAPTER7 88
Overall Conclusion 88
References 90

List of Figures

Figure 1.1 Distribution of ecotypes of *Cordyline australis* in New Zealand 8
Figure 1.2 Structure of cinchophen 11
Figure 1.3 Typical structure of a saponin, in this case based on a sterol structure 11
Figure 1.4 Structure of smilagenin 12
Figure 1.5 Structure of inulin 13
Figure 2.1 Cutting steps of *Cordyline australis* stem 17
Figure 2.2 Scheme of the micro-SDE apparatus 18
Figure 2.3 Changes in biomass of three fermentation treatments 19
Figure 2.4 Changes in accumulated CO_2 of three fermentation treatments, corrected for weight loss from biomass sampling 20
Figure 2.5 Chromatogram of raw *Cordyline australis* analyzed by SDE-GC-FID 21
Figure 3.1 Growth of a typical microbial culture in batch conditions 22
Figure 3.2 Basic routes by which yeasts form the major flavour groups during fermentation 25
Figure 3.3 The Hunter meter with a shroud covering the crystallizing dish that was placed centrally over the optical path 27
Figure 3.4 Development of the Lightness (L*) of six treatments during fermentation 30
Figure 3.5 Development of the redness (a*) of six treatments during fermentation 31
Figure 3.6 Development of the yellowness (b*) of six treatments during fermentation. 32
Figure 3.7 Yeast growth curves of six treatments during fermentation 34
Figure 3.8 Changes of biomass of treatments during fermentation 35
Figure 3.9 pH and CO_2 changes in two treatments during fermentation 36
Figure 3.10 Brix changes in two treatments during fermentation 37
Figure 3.11 Calibration curve used to quantify ethanol concentration 38
Figure 3.12 Calibration curve used to quantify sugar concentration 38
Figure 3.13 Changes of reducing sugar and ethanol concentrations in two treatments during fermentation 39
Figure 4.1 Phases of the Maillard reaction 46
Figure 4.2 Absorbance at three wavelengths of three pH treatments at 60°C and 100°C when ti koiuka stem was incubated with fructose solution 51
Figure 4.3 Interaction plot of absorbance with three factors 53
Figure 4.4 Chromatogram of compounds from the Top stem/fructose incubations at pH 4 and 60°C 56
Figure 4.5 Chromatogram of compounds from the Top stem/fructose incubations at pH 7 and 100°C 57

Figure 4.6 Chromatogram of compounds from the Top stem/fructose incubations at pH 9 and 100°C 57
Figure 5.1 Chromatograms of Top stem (sample) (Fraction 2), Control (Fraction 2) and four commercial spirits analysed by GC-FID 72
Figure 5.2 Box charts of concentration of 4 typical volatiles between 4 different fractions (n=5) 77
Figure 6.1 Influence of the sampling time on the extraction efficiency for terpens and ethyl ester, during dynamic SPME extraction using a 65mm PDMS/DVB fibre at 60 °C), expressed as peak area 82
Figure 6.2 Typical chromatograms of Jose Cuervo 100% agave tequila and ti kouka Top stem analysed by GC-MS 84
Figure 6.3 Bi-plots of principal components 1 and 2 of the volatile compounds of four treatments 86

List of Tables

Table 1.1 Percent composition of ti kouka and other foods 10

Table 3.1 Mean values of sugar and ethanol concentration in the Top stem treatment measured from Brix and chemical analysis methods of samples 40

Table 3.2 Comparison of kinetic parameters of Top stem and Control treatments 41

Table 4.1 Phases of the Maillard reaction 46

Table 4.2 Analysis of variance for absorbance as affected by pH, temperature and wavelength . 52

Table 4.3 Description of colour and aroma at different pH values and heating temperatures ... 55

Table 4.4 Maillard compounds found in Top stem/fructose model system under three conditions 59

Table 5.1 Colour measurement and ethanol concentration of 4 distillation fractions per treatment (ti kouka and control) 67

Table 5.2 Description of colour and odour of 4 distillation fractions per treatment (ti kouka and control) 68

Table 5.3 Concentration of major volatile compounds in mg L^{-1} present in 6 treatments by GC-FID, odour threshold and descriptors of each compound 73

Table 6.1 Relative abundance of ethyl esters and terpenes in four spirits 85

List of Appendices

Appendix I 94

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 nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university of other institution of higher learning, except where due acknowledgement and citing is made in the Acknowledgements and References.

Signature:

Date:

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Abstract

The main objective of this study was to systematically research the variations in the production process of a tequila-like spirit from *Cordyline australis* (ti kouka) stem. The potential future outcome is the commercial production of a tequila-like with a geographical distinctiveness arising from the fact that ti kouka is a New Zealand native plant in the same way the agave lily, from which tequila is prepared, is a native of Mexico. The two plants are botanically closely related. The production option in this research was a conventional fermentation of added fructose in the presence of comminuted stem, followed by distillation. First, batch fermentations with fructose at 150 g L^{-1} were done to evaluate the technological potential of *Cordyline australis* as fermentation juice. Next, reflux of *Cordyline australis* stem in fructose model system was used to study the resulting Maillard reaction products by gas chromatography-mass spectrometry. Finally, the chemical properties of the final spirit were be profiled by solid-phase microextraction-gas chromatography- mass spectrometry (SPME-GC-MS) and gas chromatography flame ionization detector (GC-FID) compared with four commercial spirit products to show similarities and differences. A distinctive spirit was produced with similarities to tequila.

CHAPTER 1

Introduction

1.1 Distilled alcoholic drinks

Alcoholic beverage is the product of a yeast-mediated anaerobic fermentation that converts sugars like glucose, fructose and sucrose to ethanol (alcohol) and carbon dioxide. The most common base ingredients of alcoholic drinks are fruits, cereals, molasses and vegetables (Julyan, 2008). The resulting products are called beers and wines. These fermented liquors can also be distilled to generate spirits. The word 'spirit' refers to an alcoholic drink which is low in sugar and containing at least 35% alcohol by volume (v/v). Some famous spirits are vodka, gin, whisky, rum, brandy and tequila.

Vodka was originated in Russia and Poland but is now produced worldwide. Initially, it is distilled in a continuous still from which a clear neutral spirit with about 96% abv is produced. The term neutral refers to the fact that the spirit lacks the flavour that would have been present if the alcoholic ferment were distilled to a lower alcoholic concentration. (Neutral spirit is often referred to as white spirit but this term is logically inaccurate. Rather it is clear and colourless.) To achieve the high concentration of alcohol free of congeneric materials, vodka is usually multiply distilled or filtered through charcoal filters to remove congeneric materials. Finally, the vodka is brought down to the required strength for sale, typically 35 and 45%[1], by the addition of distilled water. It is claimed that the best vodka is made from rye, molasses, a mixture of these, or from other grains (Julyan, 2008), although with multiple distillation and filtration it is likely that any alcoholic base can generate high quality neutral spirits. Beyond a defining ethanolic note, vodka is odourless and flavourless and thus is ideal for mixed drinks and cocktails. Vodka may also be flavoured using a variety of materials, the four most popular being lemon, orange, raspberry and vanilla.

Gin is fundamentally another neutral spirit made by distilling a ferment made from malted

[1] Throughout this thesis % alcohol/ethanol always means % (v/v)

barley, maize or rye. What separates gin from vodka is flavouring. Gin is flavoured with botanicals either by passing the vapour from the distillate through the flavourings, held in a metal basket or similar, or by macerating the botanicals in the spirit and distilling mixture. Sometimes the botanicals are added to the grain ferment that is distilled in such a ways as to retain the required flavour. The gin-defining botanical ingredient is juniper berry, the fruit of *Juniperus communis* in the family Cupressaceae. Other botanicals are lemon peel, orange peel, anise, orris root, angelica root, cardamom, coriander, licorice root, cinnamon, and cassia (Julyan, 2008). Each gin maker has its own distinct botanicals recipe, which makes that maker's spirit unique, but all include juniper berry. The berry is also widely used as a flavour ingredient in traditional southern European cuisine, which probably explains its parallel use in spirits.

Whiskies are also distilled alcoholic beverages, and are usually prepared from ferments of cereals. Fundamentally they have the same origins are vodka and gin. But the major difference is they are subsequently flavoured from contact with the interior of the oak barrels they are matured in whisky. The maturation process can take up to 15 years. It is important that the barrels used in maturation have been previously used to mature fortified wines like sherry and port. Thus the flavour of whisky derives from the components of toasted[2] oak and the residual wine. Generally, maturation should produce a significant improvement in flavour quality, which is based on the development of mellow or mature characteristics from the wood and a loss of the harsh or immature characteristics of the original distillate. Both the magnitude and the rate of change during maturation are dependent on the type of cask used. The legal minimum maturation time of whisky for most countries is three years. Maturation of whiskies can be monitored by sensory evaluation (Piggott & Conner., 2003) and chemical analysis.

There are many possible ways of producing whiskies, within the limitations set by the materials and processes available, and details vary depending on custom and regulation in

[2] Barrel interiors are heated to about 200°C during manufacture, and this generates pyrolytic products that are soluble in strong ethanol solutions.

producing countries. Corn (maize), rye, barley, and wheat are the major cereals used for whisky. These grains can provide high level of starch content for whisky production (Piggott & Conner., 2003). India is, curiously, a major whisky producer where the base carbohydrate source is molasses rather than grains, again confirming the fact that the ferment required to produce neutral spirit is not particularly important in the final flavour of vodka, gin and whisky.

Rum is made in many countries of the world and was made popular from product exported from the West Indies. Although sugar cane by-products are usually used, it has been made from sugar beet as well. Molasses and other residual leftovers from the manufacture of the sugar are used from both the sugar cane and sugar beet plants. The yield from 100 L of molasses is around 85 L of consumable rum.

Brandy is the distillation of the fermented juice of grapes without the addition of any other spirits, so it can be made wherever wine is produced (Julyan, 2008). Because of the nature of the fermented grape juice and the way brandies are produced, they contain far fewer volatile substances than other dark spirits. The making of brandy is usually a way to salvage defective wines or production surpluses. However, there are some good quality brandies made from wines specially grown for the purpose.

Tequila is a spirit distilled from fermented juice of the blue agave plant (*Agave tequilana*, Weber) so named from the town of Tequila, in the Jalisco state, Mexico. The unique blue agave is desert-adapted lily which is native to Mexico and is in the botanical family Asparagaceae.

The first step in tequila production begins with the harvesting of the agave plants. Generally, they are between seven and nine years of age before harvesting, the minimum maturity needed to attain a high inulin content in the agave's vegetative core, called the piña[3]. Inulin is a

[3] The word piña is pineapple in Spanish, so-called because agave cores trimmed of leaves resemble pineapples.

polymer of fructose, the fermentable sugar. At this point the piñas weight between 20 and 60 kg (Faria, 2003).

The next step is to cook piña halves in a ceramic oven at 100°C for about 24 to 36 hours, while in more modern factory, they are autoclaved for 7 hours at 120°C. The heating step generates a juice, known as agave juice, a fructose-rich syrup hydrolysed from the insulin. The agave juice is collected for batch fermentation (Faria, 2003), typically for 24 to 72 hours, either with specific yeast strains or strains endogenous to fermentation in a particular factory. In the latter case fermentation it can last 10 days. However, other parameters such as the required alcohol concentration are also taken into account before stopping the fermentation process (Faria, 2003).

Distillation systems used in the tequila-making process include pots still and rectification columns. After blending and dilution to commercial spirit strength it can be sold as conventional tequila, nominally white tequila, but is more accurately described as clear and colourless. White tequila can also be matured in oak barrels as is done for many other spirits, notably whiskey. Maturation in this way adds to cost, but generates more complex flavours in the tequila, which also develops an attractive golden hue. Depending on barrel type and maturation time tequilas will have very distinctive flavour characteristics, as is like the case with some other spirits.

The consumption and exportation of tequila has high relevance in Mexico both economically and culturally. Tequila is certainly one of the most recognized Mexican icons not only nationally but also internationally. In this respect tequila is a geographically-distinct drink as is discussed in more detail in the next section.

1.2 Geographical distinctiveness in alcoholic drinks

In Western countries, if someone was asked to identify the country most associated with champagne, the most common answer would undoubtedly be France. Similarly, whiskey

would be indentified with Scotland and tequila with Mexico. The distinctive attribute of these alcohol beverages has high commercial value that can be used to secure an exclusive niche in the competitive alcoholic drinks market.

For example, from the annual report of two largest global distilled spirits marketers, Diageo and Pernod Ricard, during 2008-2009, the top five spirits brands were entirely nationally explicit. The major spirits brands to be attributed in the top 10 in amount were Diageo's Smirnoff vodka (Russia) and Pernod Ricard's Absoult vodka (Swedish). There are many other examples of distinct regional brands of flavoured spirits. In India, there are many local brands based on neutral alcohol plus distinctive flavourings. These spirits are given the local designations of whiskey, brandy, and rum and are known as 'Indian-made foreign liquors'. Cachaca is regionally distinct rum distilled from sugar cane in Brazil (Chaipongrattana, 2008).

The Mexican government legally enforced the areas in which the blue agave can be grown as the source of authentic tequila. They include Jalisco state and some regions of other states all of which have similar reddish volcanic soil and climate (Chaipongrattana, 2008). With modern technology, it is likely that tequila flavour and appearance could be recreated elsewhere, but the geographical distinctiveness is inextricably linked to the product and will always set it apart.

1.3 New Zealand alcoholic drinks

Turning now to the New Zealand alcoholic drinks, they 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. This geographical area is Marlborough, on the northeastern tip of New Zealand's South Island. The wine is made from sauvignon blanc, a green-skinned grape variety originating from the Bordeaux region of France which was first introduced to New Zealand in the 1970s.

New Zealand other wine producers had been striving to achieve a distinctive style to match the achievement of Marlborough sauvignon blanc for many years. However, with the exception of Marlborough sauvignon blanc, New Zealand alcoholic drinks have no iconic status internationally except perhaps for the name 'New Zealand' itself. The proposal here is, in order to develop iconic drinks it is important to focus on plants strongly identified with New Zealand as a source of flavour.

In this respect, one New Zealand Company, 42 Below[4] Limited, had been successfully marketed internationally, and had won many awards in world spirit and wine competitions. Four various flavours have been marketed by 42 Below – feijoa, manuka honey, passionfruit and kiwifruit. The international success of 42 Below vodka may be due in part to the relative uniqueness and geographical distinctiveness of these four flavours. They illustrate the potential value of geographical distinctiveness, real or perceived.

Therefore, in AUT, several studies have been done to develop models of geographically-distinct wines and spirits with unique flavors derived from native species strongly identified with New Zealand. Patel (2010) is particularly relevant because the authorused extracts of *Cordyline australis* to produce a geographically-distinct New Zealand spirit. *Cordyline australis* is the common cabbage tree, or ti kouka as was called by Māori, and like the blue agave is a member of the Asparagaceae. The aim of her study was to produce a tequila-like spirit but with New Zealand distinctiveness and a New Zealand name. In the event she produced a spirit that was more like rum than tequila, and although pleasant enough to drink lacked flavours reminiscent of tequila. The present research aims to develop a spirit containing these flavours.

The next section describes the botany and natural world of this plant and lays the groundwork for the study.

[4] 42 Below now a trademark of the multinational company Bacardi Limited.

1.4 Botany of *Cordyline australis*

Cordyline australis is a widely branched monocotyledonous tree in the Asparagaceae family. With its unusual and distinctive shape, *Cordyline australis* is one of the features of the New Zealand landscape. They occur almost everywhere in New Zealand along the edges of forests and swamps or on riverbanks, or standing alone in fields or hillsides (Simpson, 2000).

Six species of the genus *Cordyline* occur in New Zealand. One is limited to Norfolk Island, located on the submarine Norfolk ridge about halfway between New Zealand and New Caledonia. Although Norfolk Island is politically part of Australia, it is geographically and ecologically closer to New Zealand (Simpson, 2000). Although the six species have the same chromosome number, each of has a specific habit.

Of the genus *Cordyline*, *C. australis*, the ti kouka 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. *C. australis* is also the largest ones of all tree lilies and can create dense forest themselves in swampland. Ten ecotypes (regional variants) of *Cordyline australis* is distributed over 99% of New Zealand (Figure 1.1).

There are north-south, east-west, and lowland-mountain trends in cabbage tree habit. Within this complex pattern there are several recognizable forms: wharanui in the west and south, with board flaccid leaves; tarariki in the north and east, with narrow, spiky leaves; tītī in the Far North, with either relatively short, broad leaves (west) or narrow, stiff leaves (east); and tī manu in the central North Island, with large straight leaves. Within these forms are local variations according to soil and climate, and there are usually broad transition zones (Simpson, 2000).

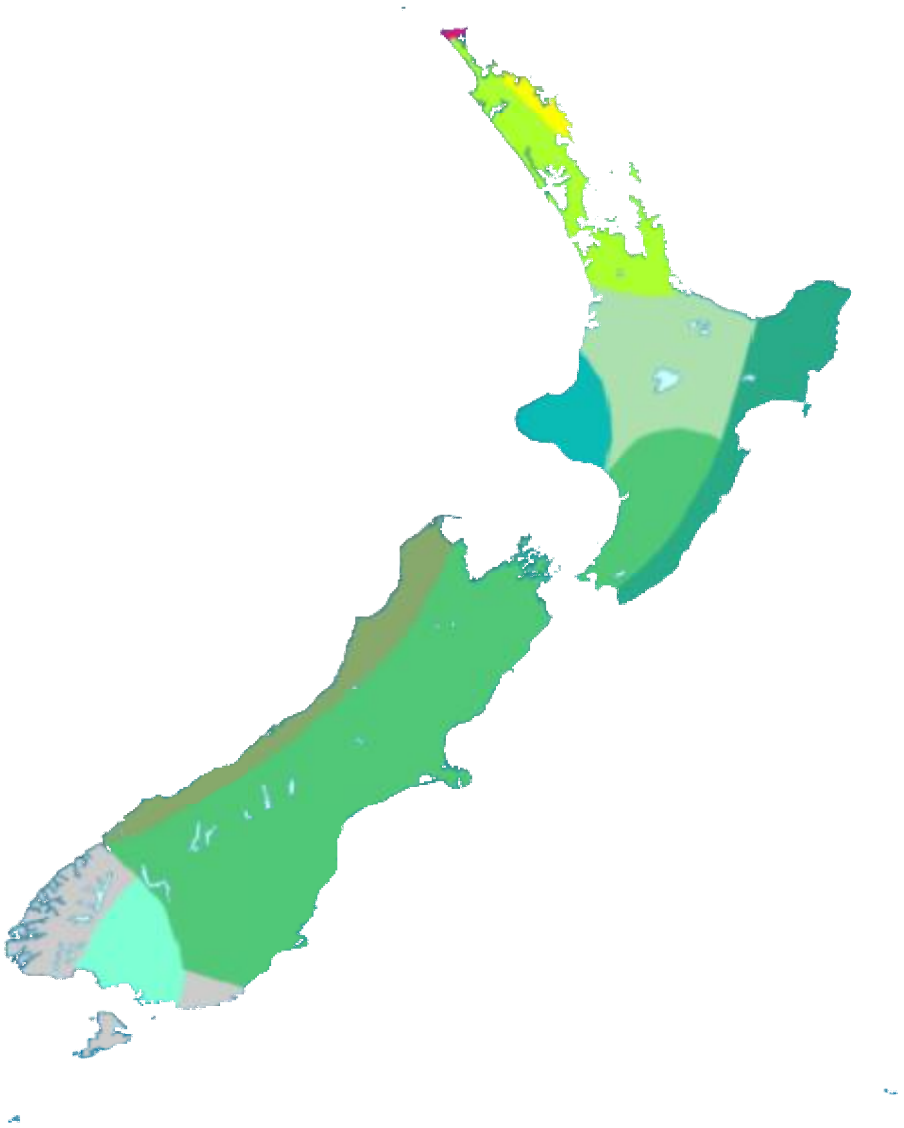


Figure 1.1 Distribution of ecotypes (regional variants) of *Cordyline australis* in New Zealand (http://en.wikipedia.org/wiki/File:Cordyline-australis-ecotype.png)

- Possible hybridisation with *C. pumilio*. Leaves floppy. North Cape
- Possible hybridisation with *C. obtecta*. Leaves straight, dark green, narrow. Eastern Northland
- Spindly trees with short broad leaves: Māori name Tītī. Northland and Auckland
- Robust trees with straight broad leaves, few branches: Māori name Tī manu. Central North Island
- Leaves narrow throughout, but lax in north, stiff in south: Māori name Tarariki. East Coast of the North Island
- Compact, densely branched trees. Taranaki
- Leaves long, relatively broad, flexible: Māori name Wharanui. Southern North Island (west of main divide), South Island (east of main divide)
- Robust trees with bluish leaves on the coast; tall trees with narrow, green leaves on river flats. South Island West Coast
- Robust trees with broad, lax leaves. Southland
- *C. australis* naturally absent. Catlins, Fiordland, Stewart Island

1.5 Māori uses of *Cordyline australis*

In traditional times, Māori had a rich knowledge of ti kouka, including spiritual, ecological and many practical aspects of its use.

When the leaves of the stem of were stripped off, what remains is the kōata, a tapering structure that resembles a elongated brassica stem, hence the European name. The kōata can be eaten raw or cooked as a vegetable, where they were called kōuka. The overall nutritional value of the kōata is similar to that of the agave piña, and includes carbohydrate and non-digestible fibre (Table 1.1). Since kōata is also low in protein and fat content, it is valued as an accompaniment to fatty food such as tuna, keruru (wood pigeon) and other birds, in modern times, pork, mutton, and beef.

Māori also used stems and fleshy rhizomes of *C. australis* to produce kāuru, a carbohydrate-rich food used to sweeten other foods. Generally, Māori dug the rhizomes in spring or early summer just before the flowering of the plant, when *C. australis* were at their sweetest. The harvested stems or rhizomes were steamed for 24 hours or more in a hangi, an underground oven that in fact replicates the ovens used to cook agave piña. Layers of *C. australis* material were placed on top of the heated hangi stones followed by water to generate steam. The hangi was immediately covered with soil and leaves or mats to retain steam and heat. On the next day the soil was removed. The softened stems were beaten flat for easy storage, then carried to the platform for protection from rats and dogs, and covered for protection from rain.

There was a great deal of hard, physical work in preparing kāuru, but nutritional studies (Table 1.1) show that the energy derived from kōata is five times that expended in making it.

Table 1.1 Percent composition of ti kouka and other foods (Simpson, 2000)

	Water	Ash	Fat	Carbohydrate	Fibre	Protein	Cal/100g
Potato	79.8	0.9	0.1	17.1	0.5	2.1	76
Taro	72.5	1.2	0.2	24.2	0.9	1.9	104
Agave piña	72.3	0.7	1.2	21.3	8.7	0.4	95
Ti kōuka							
Rhizome	64.0	0.9	1.4	23.6	10.3	0.4	103
Stem	68.3	0.7	1.5	14.9	13.6	0.4	71
kōata	81.5	1.7	3.2	8.8	4.5	1.4	68

On the basis of a 3000-calorie daily energy requirement, 1 kg of cooked, dried kōata (bottom row) would have supplied sufficient energy and would have been "ideal as a carry-along food for activities such as hunting and travelling" (Simpson, 2000).

However, humans do not have the enzymes to digest the raw carbohydrate, inulin, stored in cabbage trees, and therefore cooking is required. Inulin is a glucofructofuranan, which upon cooking hydrolyses into monosaccharides, dominated by the very sweet sugar fructose. The characteristics of inulin are discussed in more detail in the next section.

Inulin is most concentrated in the underground rhizomes. However, in the spring the sap rises and the fructan content increases in the stem. It reaches its greatest concentration in the spring and summer, and not only declines in the winter but becomes less able to be converted to monosaccharides by cooking.

Ti kouka trees were not only food in themselves but are the source of food for other species that predated on the plant, presumably attracted by the inulin content. Māori had a wide range of medicinal uses for the various parts of *Cordyline australis* to treat injuries and illnesses, either boiled up into a drink or pounded into a paste. Many treatments are still in use. *Cordyline australis* contains an agent closely related to cinchophen (Figure 1.2) which is an analgesic drug with anti-inflammatory properties frequently used to treat gout.

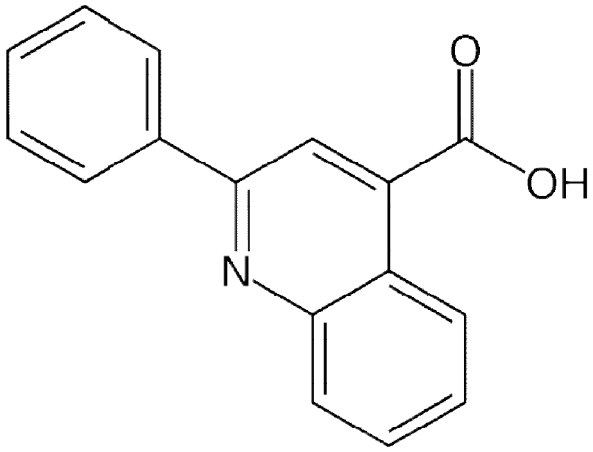


Figure 1.2 Structure of cinchophen (http://en.wikipedia.org/wiki/cinchophen)

Another important compound class found in *Cordyline australis* is the saponins a typical example of which is solanine from potato (Figure 1.3) (which unlike many saponins is toxic).

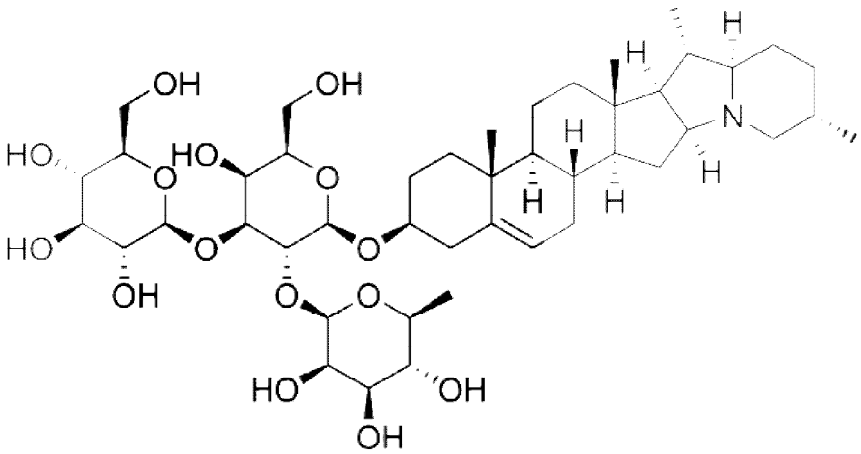


Figure 1.3 Typical structure of a saponin, in this case based on a sterol structure (http://en.wikipedia.org/wiki/File:Solanine_chemical_structure.png)

Saponins are surface active because they have fat soluble moiety – either a terpenoid or a sterol – linked to water soluble sugar molecules. They have been used for centuries to disperse fats and oils. All of them have a sugar (water-soluble) and aglycone (fat-soluble) component. When treated with a glycosidase or an acid, the sugar is lost, yielding the so-called aglycone or sapogenin. Sapinogens can be used for the synthesis of drugs such as cortisone and birth-control hormones. There are fourteen steroidal saponins in the fruits of *Cordyline australis*, including smilagenin (Figure 1.4), which has been used in cortisone

synthesis.

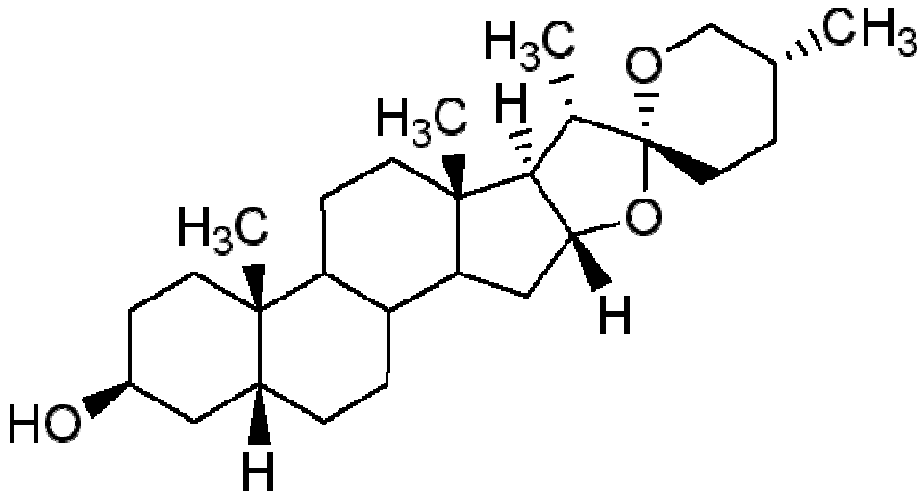


Figure 1.4 Structure of smilagenin (http://sigmaaldrich.com/catalog/ProductDetai)

Many health claims have been made for saponins of various kinds. At least one has been clinically proven. Plant sterols like stanol (as esters) in the diet can significantly reduce serum cholesterol concentrations and are sold over the counter in specialised margarine brands, such as *Take Control*, *Benecol*, and *Benecol Light*.

1.6 *Cordyline australis* as a source of carbohydrate

Boggs & Smith (1956) were the first to show that the rhizome of *Cordyline australis* contained a water-soluble glucofructofuranan polysaccharide. Further analysis showed it comprised a single α-D-glucosyl residue linked to about 14 β-D-fructofuranosyl residues per molecule.

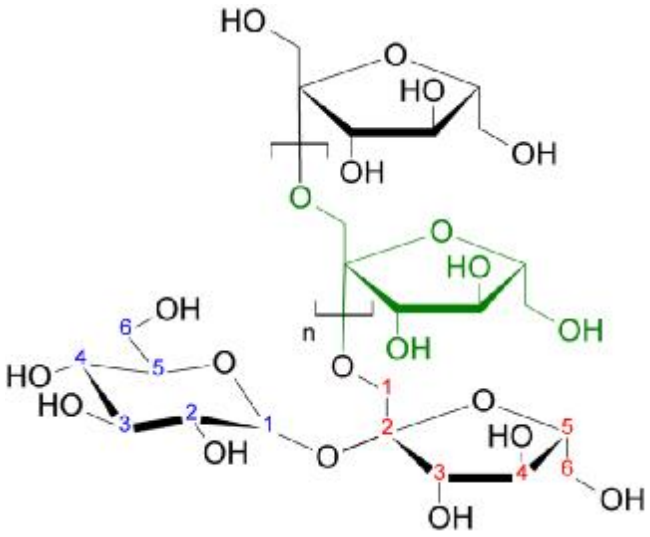


Figure 1.5 Structure of inulin (http://en.wikipedia.org/wiki/File:Inulin_strukturformel.png)

Brasch et al. (1988) further confirmed the polymer is a highly branched glucofructofuranan containing mainly (1→2)-linked β-D-fructofuranosyl residues, with branching at O-6 of 15% of the D-fructosyl residues (but not shown in Figure 1.4).

Harris & Mann (1994) investigated the suitability of ti kouka as a crop for fructose production. They found the maximum fructose yields were estimated to be 4 t ha^{-1} at densities of 80 000 plants ha^{-1}. However, compared with the sucrose from sugar beet grown in experimental trials in New Zealand (4.4 to 17 t ha^{-1}), the estimated yield of fructose from ti kouka was low. Harris and Mann made the point that there is scope to improve yield and harvest index by selection of variation present in wild populations. The research also showed that samples of the syrup and shortbread made using the ti kouka syrup shown to the public attracted considerable interest. In addition, Harris and Mann noted the species would be a suitable feedstock, for ethanol production, for fuel, and that the bagasse left after extraction of fructose could be used for fuel, as soil conditioner, for poultry litter, and as cattle feed.

1.7 Sugar extraction of *Cordyline australis* compared with agave

In history, Mexican people and Māori used similar methods to extract sugars from blue agave and *Cordyline australis*. Firstly, both of them cut off the leaves of blue agave and *Cordyline australis*. In both cases the piñas and kōata were steam heated in specific pit to hydrolyse the

stored carbohydrate to fermentable reducing sugars.

However, according to the research from a Patel (2010), the yield of fermentable sugar from ti kouka was only about 7 to 18% of fresh weight. This would require about 10 fold mass of ti kouka stem to produce one unit of sugar. While large-scale ti kouka cultivation and harvesting may be viable, the subsequent sugar extraction, fermentation and distillation would unlikely be commercially viable. The capital costs would be prohibitive, unless sugars were added to the fermentation process.

1.8 *Cordyline australis* used for producing illicit alcoholic drinks

Mexicans and Māori respectively used blue agave and ti kouka as staple carbohydrate source, but only Mexicans used their plant to make alcoholic drinks. Early Christian missionaries to New Zealand used ti kouka to brew a beer of sorts. Importantly, in the 1830s, an Irishman named Owen McShane undertook the illicit manufacture of spirit from ti kouka growing in coastal Southland near Invercargill. 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 was distilled to produce the spirit. McShane's spirit had several names. The most famous one was called 'Cabbage Tree Rum'. It was said to be a coarse and disagreeable brew but was relished by the whalers anchored at nearby Bluff. McShane was not the only one to experiment making with ti kouka spirit. In 1860, a resident of Waikouaiti near Dunedin made 'whiskey' from ti kouka and sold it to local Māori, on one occasion escaping the law by fleeing into the bush with the still on his back (Simpson, 2000).

Therefore, there is a historical precedence for spirit produce from ti kouka, and presents an opportunity to create a commercial spirit protected by geographical exclusivity, and possibly flavor distinctiveness. In a Ph.D. project, Patel (2010) produced a rum-like spirit from the stems of ti kouka. The method for this novel spirit avoided usual ways of producing spirits, fermentation followed by distillation, relying only on the plant as a source of flavour. She did this for the economic reasons discussed above. Her method was infusion of evaporated

extract of ti koukastems and potable ethanol. A distinctive colour and flavour were generated from the Maillard compounds resulting from the evaporation procedure, which concentrated the available fructose and reactive amino acids in ti kouka stem. The ethanol concentration of the final spirit was based on the composition of the potable ethanol infused. Although the spirit could probably be made economically by this method, there was a major disadvantage. The process lacked fermentation and distillation process that in the tequila production, yields flavour compounds that impart a distinctive tequila note. Patel's spirit was more like rum than tequila, and lacked a plant derived distinctiveness.

To overcome this disadvantage, one of Patel's examiners suggested producing a spirit from ti kouka by simply adding a fermentable sugar to a fermenting mixture of comminuted ti kouka stem. Fermentable sugars are cheap, and this would overcome the cost disadvantage inherent in ti kouka stem with its limited inulin content. The sugar of choice would be fructose because that is the main monomer of inulin. This concept is the basis of this thesis.

1.9 The purpose of this research

The purpose of this research is to systematically research the production methods of a tequila-like spirit from ti kouka stem. This involves simple batch fermentation with added fructose with and without comminuted ti kouka stem, refluxing comminuted stem and fructose mixtures before fermentation, and distillation. The outcomes of these experiments were studied by basic analytical methods and gas chromatography coupled to flame ionisation and mass spectrometry. The chemical attributes have also been compared with those of commercial spirits.

CHAPTER 2

Pilot Development of Ti kouka Spirit

2.1 Introduction

In Chapter 1 the major processes of mainstream spirits production including tequila was outlined. Further, the botanical links between *Cordyline* and the dry-adapted lily used for tequila production *Agave tequilana* (Weber) was examined. It was proposed that *Cordyline australis (*ti kouka in Maori) could be used to produce a spirit analogous to tequila, and it was noted there was an historical example of such a spirit. To make the spirit the ti kouka stem has to be fermented and as was discussed in Chapter 1, this required addition or additional fermentable sugar to a stem matter and water. The sugar chosen for use here was fructose, recognizing that inulin in *Agave tequilana* and ti kouka is a polymer of fructose. In this chapter three pilot fermentations are described, along with a trial to confirm or deny that there were potentially useful flavour volatiles in ti kouka stem. That work was done with a simultaneous distillation and extraction technique.

2.2 Materials and methods

2.2.1 Yeast

A commercial yeast wine S*accharomyces cerevisiae* MA33 was from the Vintner's Harvest Winemaking Ltd. (Auckland, New Zealand).

2.2.2 Preparation of *Cordyline australis* stems

Cordyline australis stems (approximately 50 cm long) were collected from the abundant roadside specimens growing around the town of Kumeu, West Auckland. The stems were randomly collected at random, so the ages of source ti kouka trees were unknown. The leaves and brown bark were removed from the stems with a box cutter and discarded. The stems were then transversely sliced into discs about 30 mm in diameter and 10 mm thick with an electric band saw. These discs were vertical diced into eight wedges, although quarters are shown in Figure 2.1.

Figure 2.1 Cutting steps of *Cordyline australis* stem

2.2.3 Fermentation treatments

There were three treatments, the Fresh stem, the Stem extract and the Control. In the Fresh stem treatment, 150 g fresh stem pieces was made by using the above procedure. This was mixed with 0.35 g of yeast wine, 1 g of yeast nutrients, 120 g of fructose and tap water to a final volume of 900 mL in a 1 L blue-capped Schott bottle as the designated as fermentation bottle.

The Stem extract treatment involved the following steps, which are analogous to the way tequila is prepared commercially. A pulp mass of 200 g of stem was prepared by dry-blending the diced pieces of stem in a domestic drink blender. This mass was placed in a beaker with 700 mL of water, which heated on a thermostatically-controlled hot plate at 80°C for 48 hr with magnetic stirring. This process softened the stem fibres and extracted the stem matter without caramelisation. The suspension was then vacuum filtered through (Whatman No.4 paper) to obtain a substantially clear preparation for further use. The suspension was made to 900 mL in a 1 L Schott bottles with yeast, nutrient and fructose as described above. The Control treatment, also to 900 mL, contained no stem or stem extract.

These three fermentation treatments were held in a conventional laboratory oven at 25°C for 84 hours. With this timing, experiments could be comfortably done in a working week. In the next 6 Biomass and accumulated mass of CO_2 were recorded every 12 h to monitor fermentation.

The mass of CO_2 released was determined by measurements of weight loss. Yeast biomass was determined by measuring dry weight of washed samples of the fermentation mixtures. Ten milliliter aliquots the fermentation mixtures were centrifuged at 3000 gravities at 20°C for 20 min, rinsed with the same volume of distilled water, and dried at 100°C until a constant cooled weight was obtained.

2.2.4 Simultaneous distillation and extraction of voltiles from *Cordyline australis* stem

Simultaneous distillation–solvent extraction (SDE) was carried out in an apparatus, as showed in Figure 2.2, which was the author's design.

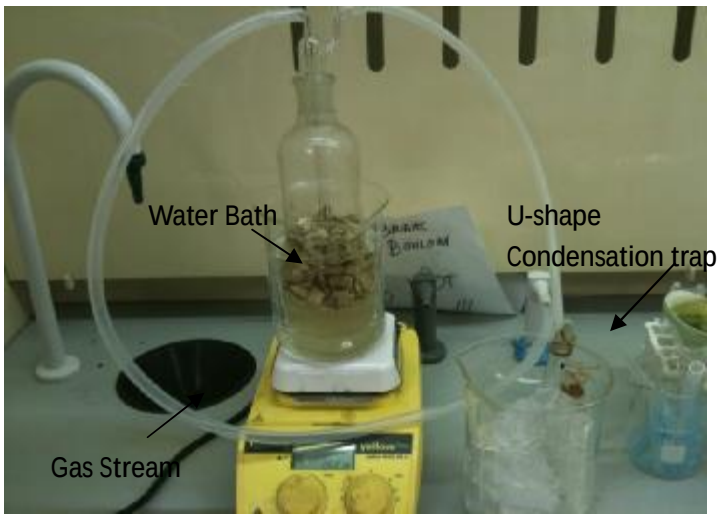


Figure 2.2 Scheme of the micro-SDE apparatus

For extraction, 100 g of fresh ti kouka stem pieces, 50 mL of distilled water and 50 mL of ethanol were placed in a 500-mL flask in turn placed in a water bath heated to 100°C on a thermostatically-controlled hot plate. Ten milliliters of dichloromethane was placed in a U-shape condensation tube immersed in ice. Volatiles were released into the headspace and trapped in the cold dichloromethane. Distillation was continued for 75 min. The dichloromethane extract was concentrated to 1.0 mL by heating in a water bath at 60°C. The concentrated samples were collected and stored in a Teflon-sealed glass vials until required for gas chromatographic (GC) analysis.

The concentrated dichloromethane extract was subjected to GC analysis, using a Shimadzu

GC17A gas chromatograph (Shimadzu, Japan) equipped with a split/splitless injector and a flame ionisation detector (FID). A capillary column coated with ZB-Wax, 25 m long with an internal diameter of 0.25 mm was used. The temperature of the injector and detector were set to 140°C and 250°C respectively. The oven temperature was held at 80°C, for 3 min, then programmed to rise from 80 to 220°C, at 20°C min^{-1}, and finally held at 220°C for 5 min.

2.3 Results and discussion

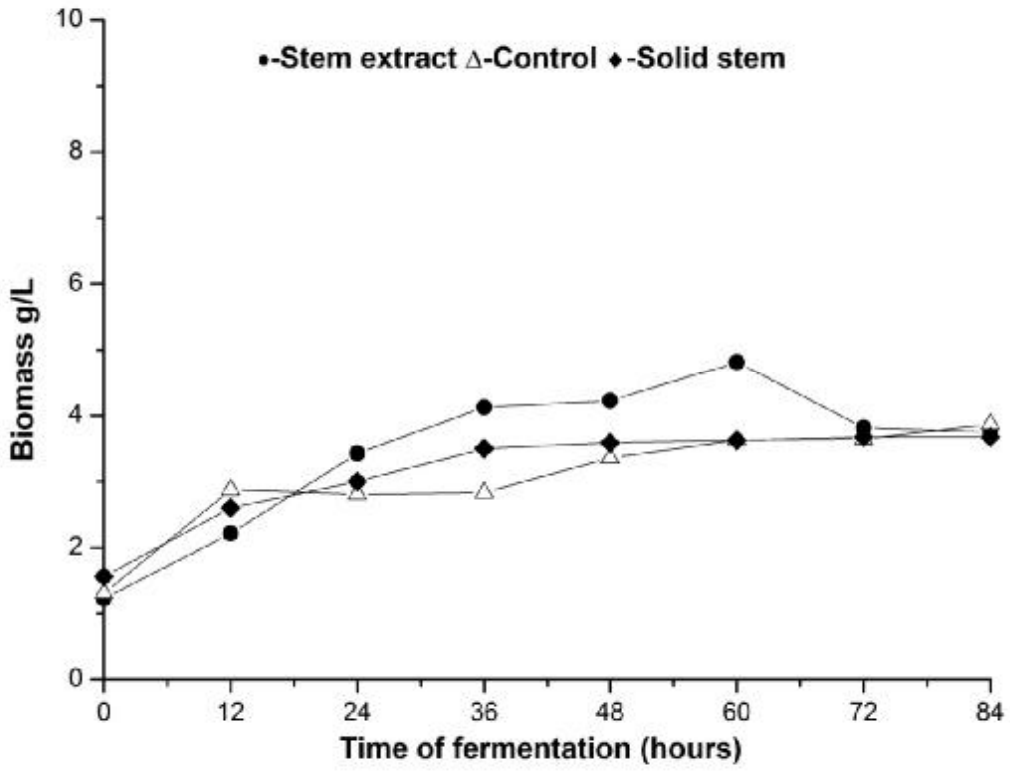


Figure 2.3 Changes in biomass of three fermentation treatments

Figure 2.4 shows the kinetics of yeast biomass for the three treatments, Fresh stem, Stem extract and Control, but with no replication that would allow statistical analysis. Numerically, the Stem extract produced the best result in terms of yeast growth, and is not surprising because the 40 h incubation would allow greatest extraction of nutrients to support subsequent fermentation. The apparent loss of mass for Stem extract at 48 h may be caused by yeast

autolysis.

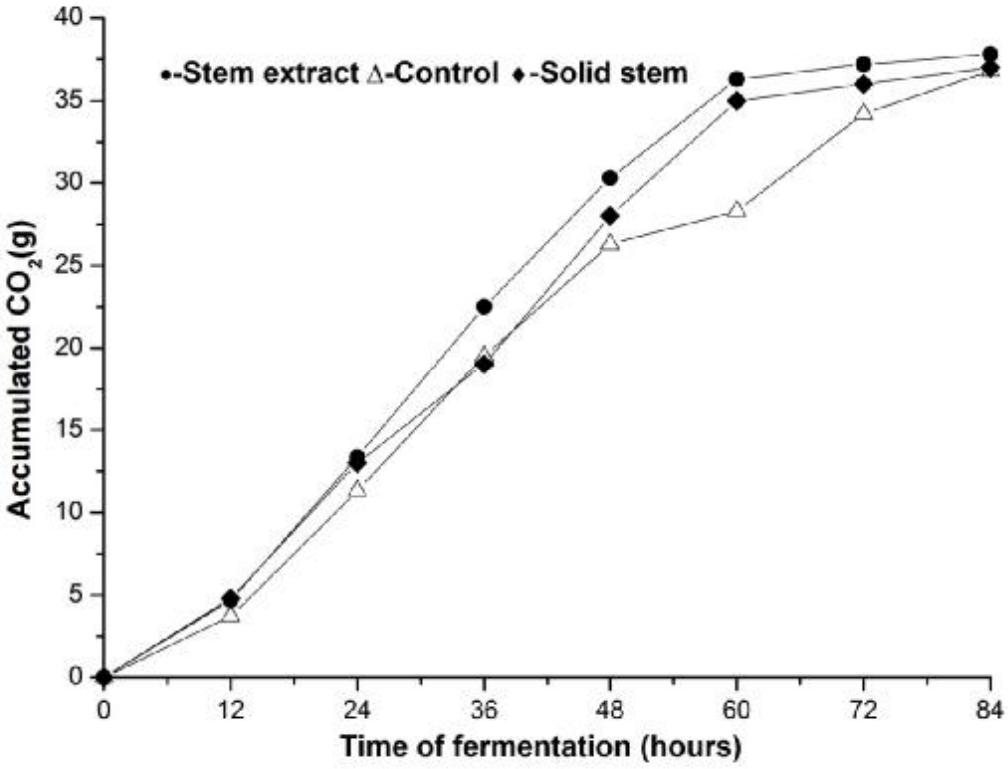


Figure 2.4 Changes in accumulated CO_2 of three fermentation treatments, corrected for weight loss from biomass sampling

Results shown in Figure 2.5 confirmed the outcome above. The Stem extract treatment produced numerical CO_2 up to 72 hours, but by 84 h the treatments were indistinguishable. On the basis of Figures 2.3 and 2.4, the Stem extract treatment, which is analogous to treatment used in commercial tequila production, was chosen to be the working model for all subsequent work, and for which treatments were duplicated so that the results could be statistically analysed.

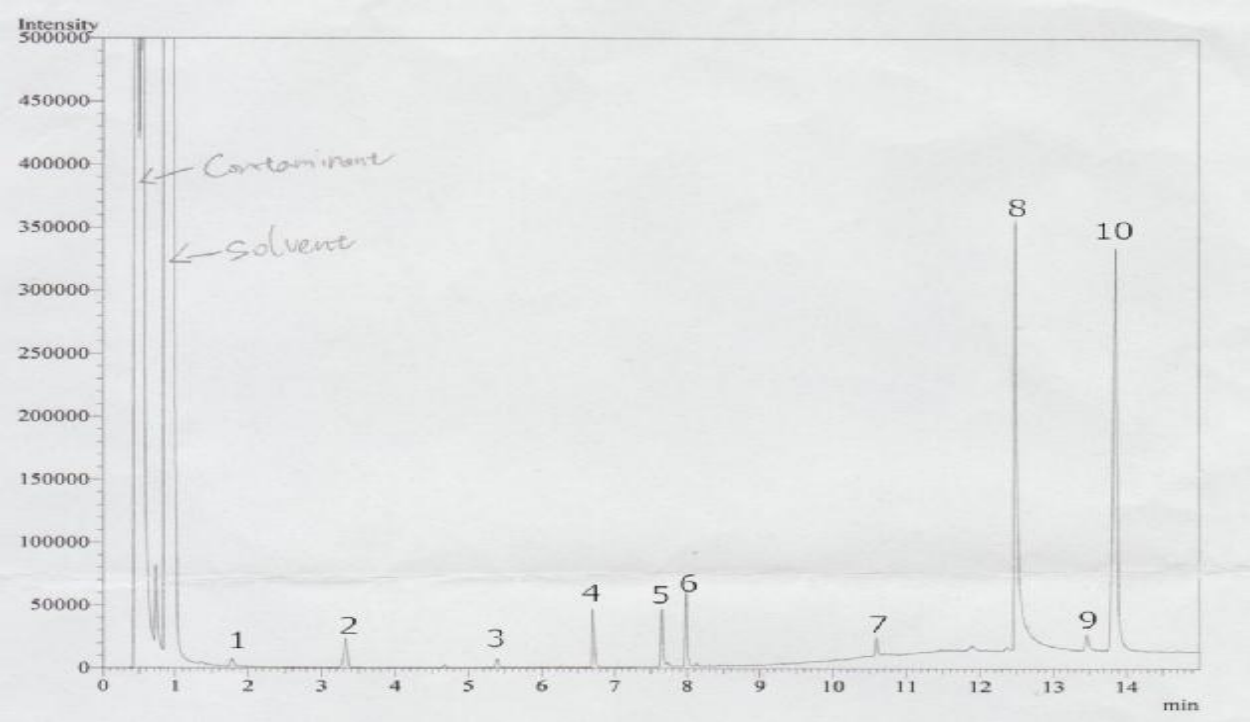


Figure 2.5 Chromatogram of raw *Cordyline australis* analyzed by SDE-GC-FID

After extraction by SDE, there were 10 reasonable peaks appearing totally using GC-FID, which indicated *Cordyline australis* contained some specific volatile compounds. These volatiles cannot be confirmed at this stage due to the lack of standards. However, the volatiles from the *Cordyline australis* probably can contribute to some distinctive and unique flavour for the final products.

2.4 Brief summary

The pilot experiments above showed fermentation proceeded better in the juice (stem extract) of *Cordyline australis.* Some of the volatile peaks were indentified roughly in the extraction of *Cordyline australis* by GC-FID. The next chapter reports the physical, microbiological and chemical properties of fermentation samples from the stem extract of *Cordyline australis*.

CHAPTER 3

Kinetics Study on Exponential Phase of the Ti kouka Fermentation Process

3.1 Introduction

3.1.1 Mode of batch fermentation

Fermentations may be carried out as batch, continuous and fed-batch processes. Generally, alcoholic beverage is produced in a batch process contacting a fermentable substrate with yeast cells encapsulated within a porous, semi-permeable material. Batch culture is a closed culture system which contains an initial, limited amount of nutrient. The inoculated culture will pass through a number of phases, as illustrated in Figure 3.1.

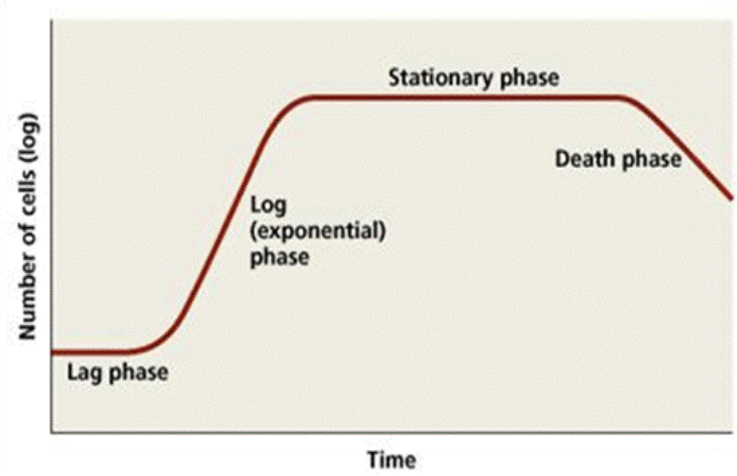


Figure 3.1 Growth of a typical microbial culture in batch conditions (Stanbury, 1995)

After an initial lag phase the number of cells increases exponentially resulting in a straight line when the ordinate is on a logarithmic scale. The growth in number of cells eventually plateaus where no further growth occurs. This is followed by progressive cell death. The study will mainly focus on the exponential phase of growth.

3.1.2 Components of fermentation

Fermentation requires three basic ingredients: water, yeast and sugar. As simple as this seems, however, the endless variations in which these ingredients can combine changing the final product in many ways. Other ingredients may also be added to achieve different products and results (Schmid, 2008).

Yeast is a one-celled, chlorophyll-free plant that lives on simple sugar. The characteristics of the yeast will affect the final product, and in order to produce a consistent product, care must be taken with the type of yeast used (Rande & Luciani, 2001). There are endless varieties of yeast, and different fermented beverages require different strains of yeast. Any strain of yeast used for fermentation must be alive. Heating at temperature at 60°C is lethal to yeast, but yeast can be frozen or freeze-dried and revived for later use by thawing or reconstituting. In this part of research, yeast type is studied as a variable.

In addition to requiring a carbon source and nitrogen source yeast also require for a range of metals such as iron zinc, and copper and other inorganic nutrients such as chloride, sulfur, and phosphate. It also has a requirement for vitamins, such as biotin, inostiol and nicotinic acid. In this experiment, specific nutrients will be involved to supply these requirements of yeast (Berry & Slaughter, 2003). The two product classes of interest in fermentation – and subsequent distillation are alcohol and other flavour compounds.

3.1.3 Production of alcohol

The fermentation reaction can be presented by the simple equation:

$$\mathbf{Yeast + C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2 + Energy}$$

The amount of alcohol from fermentation depends on the amount of sugar available for the yeast to consume. If the yeast is mixed with a liquid containing a high concentration of sugar, however, the yeast will continue to metabolize the sugar and produce alcohol to a final concentration of about 15 % (v/v). The yeast stops functioning at this point because the alcohol creates a toxic environment for the yeast (Schmid, 2008).

When the molecular weights are applied to the equation above, it can be shown that a given mass of sugar will produce about one-half its mass of alcohol, which means a standard bottle of spirit 750 mL (37.5% alcohol) requires 562 g of sugar. Calculations by Patel (2010) showed that the known concentrations of fermentable sugar in ti kouka stem and rhizome, would require about 10 kg of this matter to yield one 750 mL bottle of spirit. This would be commercially unrealistic, Therefore, extra sugar , specifically fructose, was added to the

fermentation mixture. Any fermentable sugar like glucose or fructose would work, but fructose was chosen because the fermentable sugar from inulin is fructose, and that may contribute to a characteristic ti kouka flavour in the same way that fructose may contribute to a characteristic tequila flavour. Moreover, the assay to measure sugar was specific for reducing sugar. Had sucrose been used, this would require an initial acid hydrolysis before sugar determination to monitor fermentation.

3.1.4 Production of other flavour compounds

When yeast ferments sugars, ethanol is not the only product. It is possible to demonstrate that several hundred other compounds are generated as well by using gas-liquid chromatography. Many of them contribute to the flavour of the final product. These compounds can be divided to several categories based on their metabolic origins within the cell (Figure 3.2). The most abundant of these are the higher alcohols whose production parallels that of ethanol. However, many compounds present in smaller amounts also play an important role in flavour development. Fatty acids such as acetic, caproic, and caprylic contribute to the flavour directly or through being involved in the formation of esters. Carbonyl compounds such as acetaldehyde and diacetyl are present in even lower concentrations but have very low flavour thresholds so they can play a key role in the flavour of the product. The yeast may also be involved in the production or modification of sulphur compounds, which can be of critical importance in the development of flavours and off-flavours in alcoholic beverages (Berry & Slaughter, 2003).

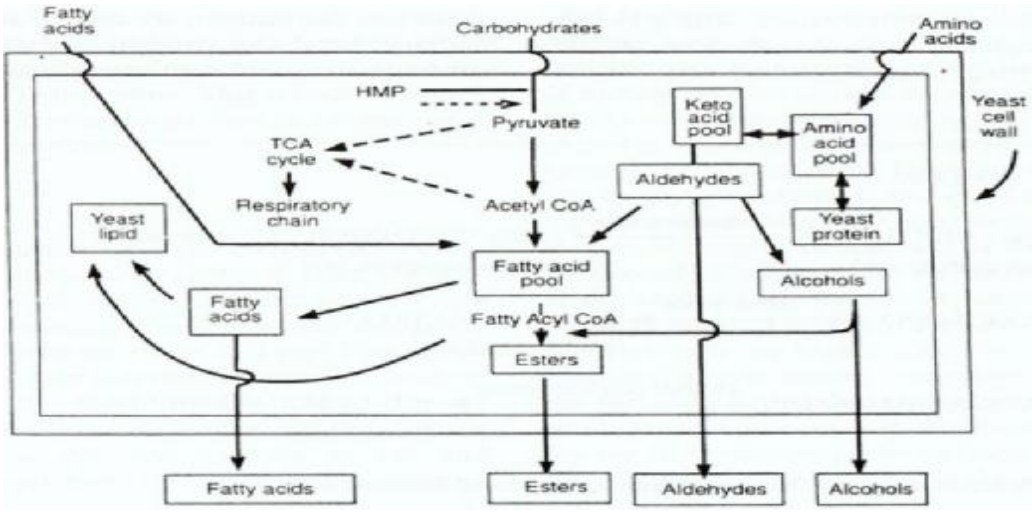


Figure 3.2 Basic routes by which yeasts form the major flavour groups during fermentation (Berry & Slaughter, 2003)

In this study, batch fermentations were performed with six treatments at 150g L^{-1} sugar concentration in order to compare and evaluate the technological potential of *Cordyline australis* as fermentation juice.

3.2 Materials and methods

3.2.1 Chemicals

For the nicotinamide adenine dinucleotide (NAD) enzymatic method for ethanol determination, NAD and alcohol dehydrogenase (ADH) were obtained from Sigma, U.S.A, types 43410 and A7011 (300 units mg^{-1} protein), respectively. The reference analytical ethanol 96 % (v/v) was obtained from BDH, U.K. Tris-(hydroxymethyl)aminomethane (Tris) and L-lysine HCl were analytical grades obtained from Applichem, Ottoweg, Germany.

For the Parahydroxybenzoic hydrazide (PAHBAH) method to determine reducing sugars, trisodium citrate (10242) and calcium chloride (BDH 27586) were purchased from BDH. Parahydroxybenzoic hydrazide (PAHBAH) was sourced from Sigma (St. Louis, MO, USA). Fructose was obtained from Scientific Supplies Ltd.

To determine the growth of yeast, acidified potato dextrose agar (PDA), peptone water, and methylene blue Pirth solution were purchased from Fort Richard, Difco, Auckland, New Zealand.

3.2.2 Preparation and characterization protocols of fermentation

Two strains of yeast were used. These were S*accharomyces cerevisiae* MA33, a commercial yeast wine, and a commercial still spirits classic turbo yeast, both from the Vintner's Harvest Winemaking Ltd. (Auckland, New Zealand). The latter is claimed to quickly generate high ethanol concentrations. They are identified as Yeast wine and Yeast turbo.

The standard production procedure of must[5] was described in Chapter 2, such that 15% of the sugar came from heated *Cordyline australis* macerate in tap water and the rest from fructose. To this was added ammonium sulfate (0.5 g L^{-1}) to support yeast growth. This mixture was held simmering at 100°C for 10 min on a hot plate to kill vegetative microbes, then cooled to ambient. Fermentation treatments followed this sequence: Yeast cells were grown for 12 h at 30°C with shaking (250 rpm) in 500-mL Erlenmeyer flasks with 150 mL of must. Yeast population and viability were determined using Methylene Blue staining. Methylene Blue Pirth solution (0.1 g methylene blue in 1 L of 2% sodium citrate) was added volume for volume of must on a microscope slide. Under a conventional transmission light microscope (400 x objective), yeast viability was determined, where viable yeast cells were colourless and non-viable cells stained blue. Incubations were considered to be effective where colourless cells greatly exceeded numbers of blue-stained cells.

The 150-mL aliquots of must in the Erlenmeyer flasks that were successfully fermenting after 12 h were poured into the 1 L blue-capped laboratory bottles and made to 900 mL with the respective must. The bottles were lightly capped and fermentations were continued at 30°C. Since there were two types of yeast (Yeast wine and Yeast turbo) and three different treatments (Top stem, Whole stem and Control). Thus there were 2 x 3 = 6 fermentation treatments,

[5] The term 'must' normally refers to a grape extract prior to fermentation, applied here to any fermentable mixture

each performed in duplicate.

3.2.3 Physicochemical analyses

Colour of fermenting musts were determined with a Hunter colorimeter (Model 45/0 Hunterlab ColorFlex, Reston, Virginia, U.S.A.) in L*, a*, b* space (CIE, 1976), where L*, a* and b* indicate lightness, redness/greenness, and yellowness/blueness, respectively. A daylight (D65/10°) illuminant/observer combination was selected to record L*, a* and b* values. From each duplicate ferment within each treatment, 10 mL of fermenting liquid was sequentially placed in placed a glass crystallising dish (10 cm diameter) that sat beneath a black shroud (Figure 3.3). Three readings were taken of each. The mean blank value (due to the empty dish) was subtracted from those 2 x 3 reading. Corrected values were then averaged within duplicate. Finally the duplicate values were averaged and the standard deviation calculated.

Figure 3.3 The Hunter meter with a shroud covering the crystallising dish that was placed centrally over the optical path

The pH of the sample was determined using a portable pH meter (Meterlab, U.K.). The mass of CO_2 released was determined by measurements of fermentation weight loss at 12 h intervals during fermentation.

Total soluble solids (Brix), was measured throughout the fermentation process using a Reichert® Refractometer, 0 to 30% Brix with automatic temperature compensation to 20°C.

The reducing sugar concentration was measured according to Blakeney & Mutton (1980), based on the colour reaction with p-hydroxybenzoic hydrazide (PAHBAH) in alkaline solution. The assay procedure was started by making duplicate 1:100 dilutions of samples from the duplicate fermentations in each treatment. One milliliter samples of the diluted ferments were mixed with 400 µL of phosphoric acid (85% v/v) and 400 µL of deionised water (blank) respectively. To this was added 5 mL of PAHBAH solution and incubated in a boiling water bath for 4 minutes to develop colour. The mixture was cooled immediately under running cold water, centrifuged lightly, and the absorbance of the clear supernatantwas measured in triplicate for each incubation test tube in a spectrophotometer at 420 nm (Ultraspec 2100 pro, U.K.), using 1-cm plastic cuvettes. The sugar content was calculated from a standard curve of fructose and expressed as gram fructose L^{-1} of ferment. Presented data are means of duplicate ferments and their standard deviations

The ethanol concentration was determined according to Cornell & Veech (1983) where the acetaldehyde product is trapped in a stable hydrazone that serves to drive the oxidation reaction to completion. The extent of reaction is monitored by an ultraviolet colour change due to NAD/NADH. Duplicates aliquots (0.24 mL) of the 1:10000 dilutions of duplicate ferments within treatment were mixed with 1 mL Tris/lysine/NAD[6], 1.45 mL of water and 0.1 mL enzyme or heated enzyme solution (blank) to make the final volume of 2.79 mL. The enzyme solution was a freshly prepared solution containing 600 units mL^{-1}. Absorbance was measured at 340 nm using an Ultraspec spectrophotometer. After subtracting the absorbance of the heated enzyme blank, the ethanol concentration was calculated from a standard curve. As for other assays, determinations within duplicate ferments were averaged, and then the mean and standard deviation were calculated for each treatment.

3.2.4 Microbiological analyses

[6] The term ‘NAD’ refers to Nicotinamide Adenine Dinucleotide

Yeast biomass was determined by measuring dry weight. The cellular dry weight was obtained by collecting the cells from 10 mL of the culture medium by centrifugation (3000 gravities, at 20°C, for 20 min), rinsed with the same volume of distilled water, and desiccated at 100℃ until a constant cooled weight was obtained.

The growth rate of yeast was determined by the aerobic plate count technique (APC) using acidified PDA according to the method of the Association of Official Analytical Chemists (AOAC, 2000). A 1 mL sample of ferment was aseptically transferred to 9 mL of peptone water to make 1 in 10, then 1 in 100 dilutions and so on. Aliquots of the dilution series (1 mL) were plated in duplicate on PDA Petri dishes and incubated at 37°C for two days. The enumeration of yeast growth is reported as log colony-forming units per mL of sample (cfu mL^{-1}), corrected for decimal dilution.

3.2.5 Data analysis

Data were first marshaled by routines within Origin 8.0 (OriginLab Corporation, Northampton, Massachusetts, USA), where means were also calculated, and graphs prepared. More detailed analysis of variance was performed with Minitab 15 (Minitab Inc., State College, Pennsylvania). Comparisons between individual means were done with the Tukey test in that routine.

Alcohol production efficiency is a measure of fermentation efficiency and was calculated from the ratio of the experimental ethanol concentration produced at the end of fermentation and the theoretical ethanol concentration from the biochemical conversion of sugar. Specific biomass growth rate, sugar consumption rate and ethanol formation rate fitted to a mathematical model using Curve Expert 1.4. The maximum specific rates were obtained from the maximum slopes of each respective response factor.

3.3 Results and discussion

3.3.1 Kinetics of colour during fermentation

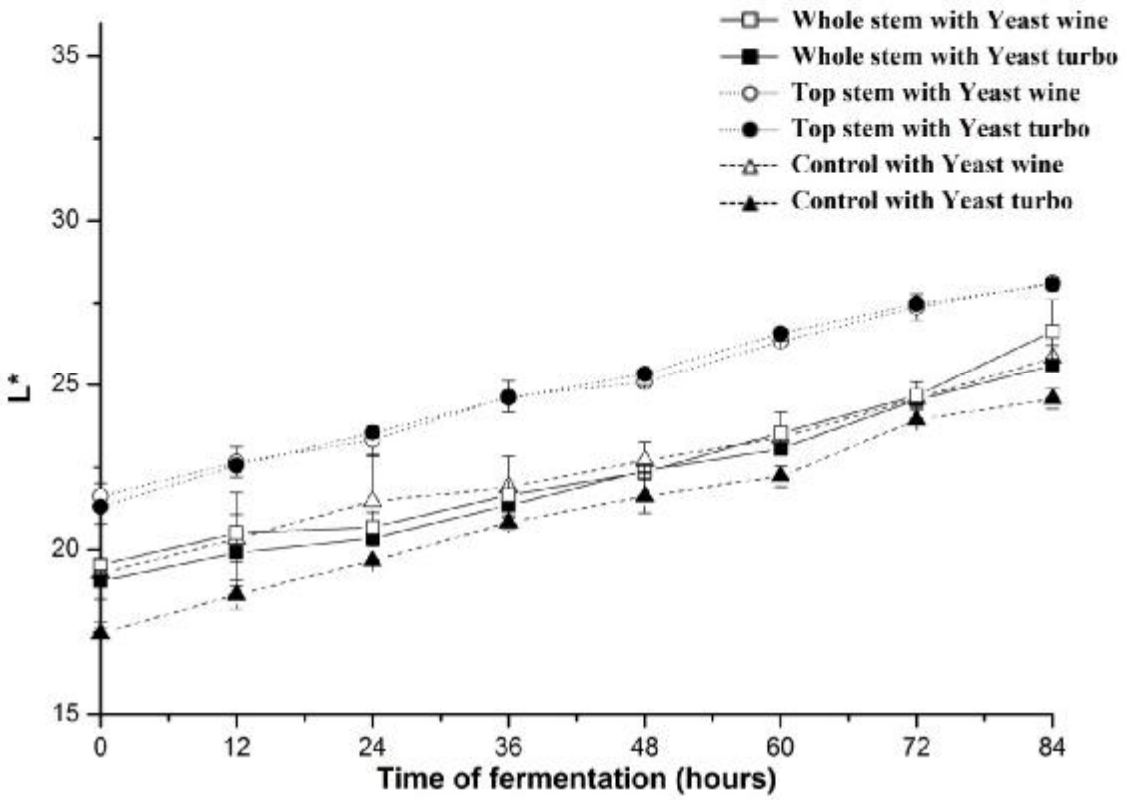


Figure 3.4 Development of the reflectance (L*) of six treatments during fermentation. Bars indicate standard deviation from duplicate fermentations

The L* values are shown in Figure 3.4. Overall, it is obvious that the top stem had the highest L* value (light reflectance), followed by the whole stem and control treatments. This result appears to be consistent with the visual observations. The cloudiest treatments were the Top stem and Whole stem, and it might be expected that these should reflect more light.

All treatments showed increased light reflectance with time of fermentation ($p < 0.001$). This phenomenon is presumably results from the fact of more alcohol being produced due to fermentation. It is well known that ethanol has higher reflective index than carbohydrate. On the other hand, the significant increasing L* value also demonstrate that the fermentation process develops well. In the Top stem and Whole stem treatments there was no significantly

difference due to yeast, but there clearly was in the Control, again for unknown reason.

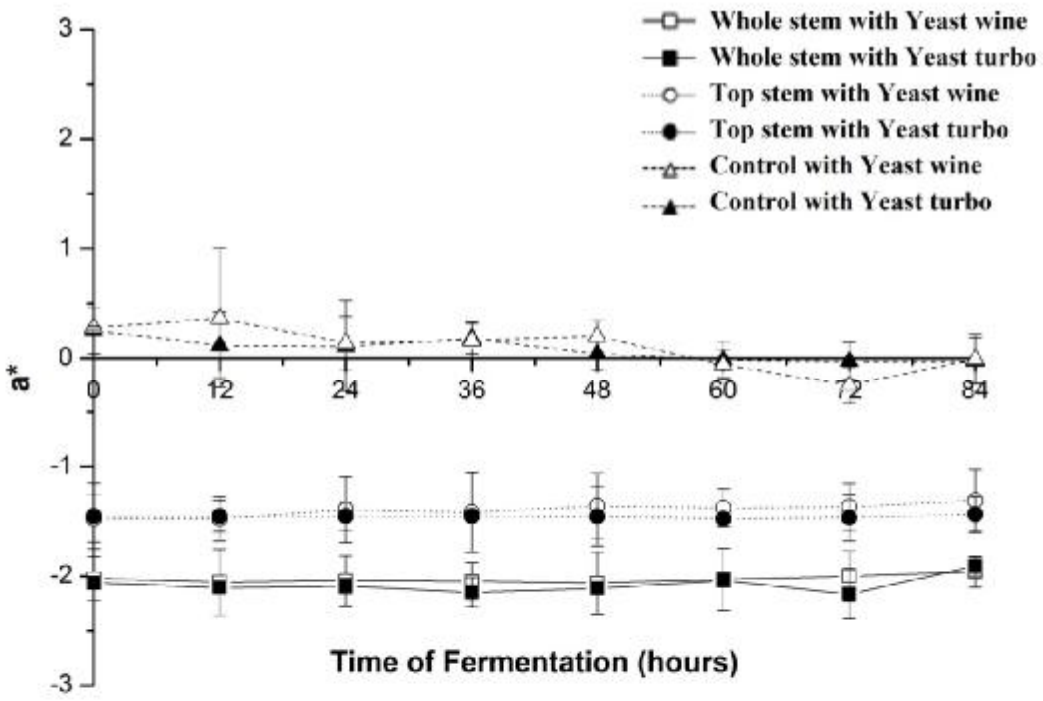


Figure 3.5 Development of the redness (a*) of six treatments during fermentation. Bars indicate standard deviation from two determinations

The kinetics of a* values are shown in Figure 3.5. The a* values were the highest for the control, but so close to zero that the colour was neither red nor green. By contrast, the Top stem and Whole stem treatments were slightly greener, particularly for Whole stem. The greenness presumably derives from the stem material and may be derived from traces of cholorphyll. The differences between the three treatments were significant ($p < 0.001$), but did not change with time of fermentation nor yeast type.

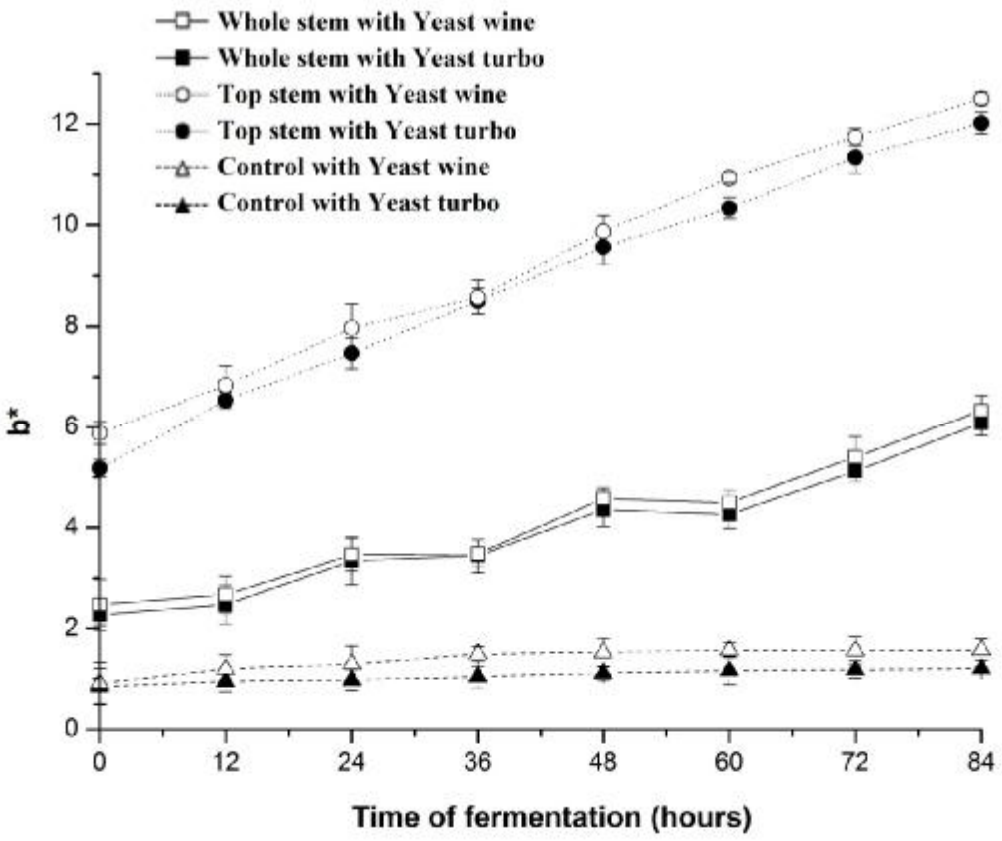


Figure 3.6 Development of the yellowness (b*) of six treatments during fermentation. Bars indicate standard deviation from two determinations

The kinetics of b* values of six treatments are depicted in Figure 3.6. At 0 hours, the b* value of the top stem treatment was about 6, which means maillard reaction might occur during the cooking process of *Cordyline australis* leading to the yellowness of the treatment.

During fermentation, the b* value of the two stem treatments increased markedly and significantly ($p < 0.001$), while the Control treatment showed little increase, but was nonetheless significant ($p < 0.05$). The increase in yellowness was most marked in the Top stem treatment. Yeast type had no clear effect.

The increase in yellowness in the stem treatments may be due to a continuation of the Maillard reaction generating compounds such as furfural, 5-(hydroxymethyl)furfural, and pre-melanoidins. As discussed in Section 1.6, stem material contains reducing sugars – notably the highly reactive sugar fructose (Waleckx et al., 2008) – and amino groups

associated with proteins, the two reactants required for the Maillard in foods. The increase in yellowness was most marked in the Top stem treatment (Figure 3.6), and is consistent with the fact that the tips of growing plants are richer in unpolymerised metabolites including amino acids and sugars. Indeed in the case of *Cordyline australis*, Crowe (1997) showed that on a wet weight basis the concentration of protein in the stem tip was about three times higher than in lower stem. The change in pH with fermentation (Section 3.33) may also be important here, because the directions of the Maillard reactions are affected by pH. The reaction is also favoured by low water activity, which was necessarily lowered when sucrose was added to the fermentation mixture (Section 3.2.2). Also, the water activity of an increasingly alcoholic mixture necessarily decreases. In addition to the Maillard reaction, caramelisation of sugars under fermentation condition might lead to an increase in yellowness. However, this seems unlikely because the yellowness of the control hardly changed. According to Mancilla-Margalli & Lopez (2002) the Maillard reaction that occurs at least during the juice production phase of tequila manufacture, has an important effect on the flavour of the tequila. Thus, it seems likely that the change in yellowness observed during fermentation (Figure 3.6) will be responsible for some flavour effects in the final distilled product.

3.3.2 Kinetics of yeast growth and biomass production

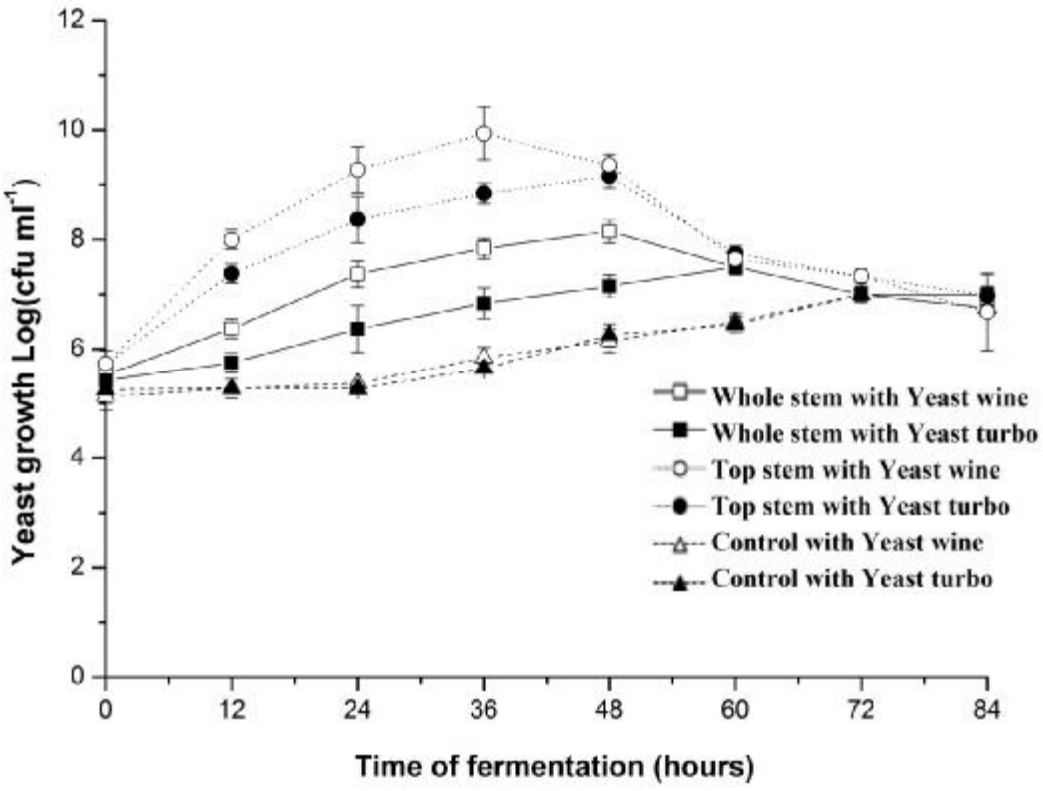


Figure 3.7 Yeast growth curves of six treatments during fermentation. Bars indicate standard deviation from two determinations

The kinetics of yeast growth is shown in Figure 3.7. The initial microbial load for each yeast was about 5.0 x 10^5cfu mL^{-1}. Yeast growth was marked for the Top stems treatments, less so for the Whole stem treatment, and was minimal for the Control. The stem treatments ultimately showed a decline in active yeast cells. The final concentration was 7.0 x 10^5cfu mL^{-1} for all treatments. In both stem treatments it was clear that the yeast wine was the more effective in terms of growth.

The most important result was that the Top stem stimulated yeast growth the most, suggesting that more nutrients were available to the yeasts from the Top stem than the Whole stem. This result is indirectly consistent with the greater yellowing shown in Section 3.3.1; the same nutrients that were reactive in the Maillard reaction could also support yeast growth.

Because the Yeast wine and the Top stem together showed the best results in terms of yeast growth and pigment development, further work with Turbo yeast and Whole stem was discontinued. The terms Yeast wine and Top stem are retained.

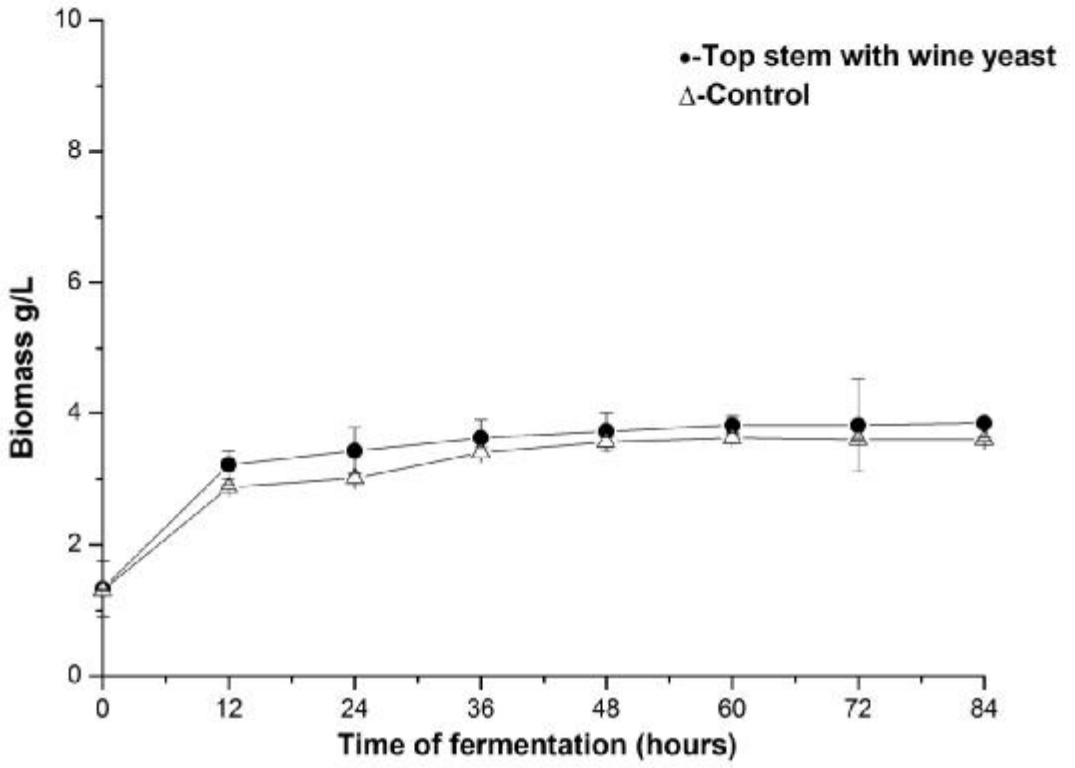


Figure 3.8 Changes of biomass of treatments during fermentation. Bars indicate standard deviation from two determinations

Biomass production was similar between the Top stem and Control treatment with maximum specific growth rates of 0.032 and 0.046 h^{-1}, respectively, but there was no overall statistically significant difference between two treatments ($p=0.59$).

3.3.3 Kinetics of pH and CO_2 production

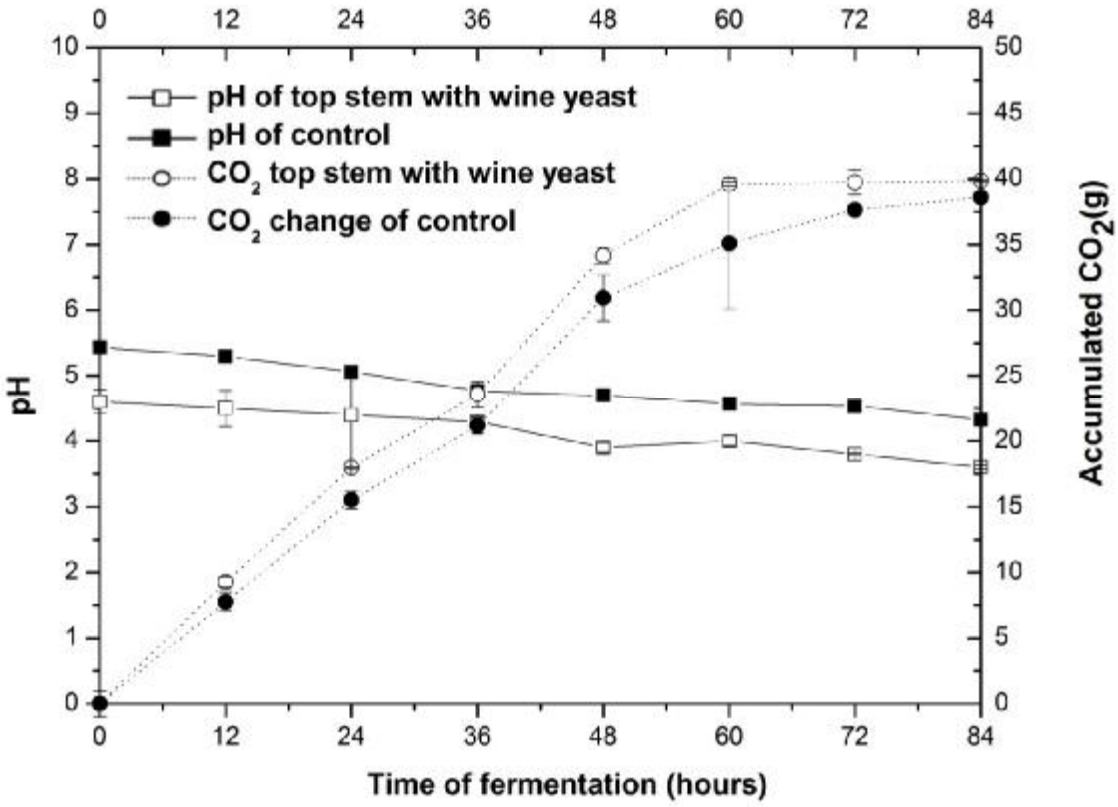


Figure 3.9 pH and CO_2 changes in two treatments during fermentation. Bars indicate standard deviation from two determinations

Changes in pH of the two treatments during fermentation are shown in Figure 3.9. Generally, the pH of both treatments decreased significantly during fermentation ($p < 0.001$ within each treatment). At all times the pH was lower in the Top stem treatment, probably because it was initially lower, possibly from organic acids present in the plant. The lowering of pH is associated with the well-described production of organic acids during fermentation.

The kinetic of accumulated CO_2 is also shown in Figure 3.9. CO_2 production increased with increasing fermentation time, but there was no statistically significant overall difference between two treatments ($p = 0.774$), but at many sample times the Top stem treatment was generating more CO_2 (statistics not shown), a result consistent with the data in Figure 3.7.

3.3.4 Kinetics of sugar content expressed as Brix

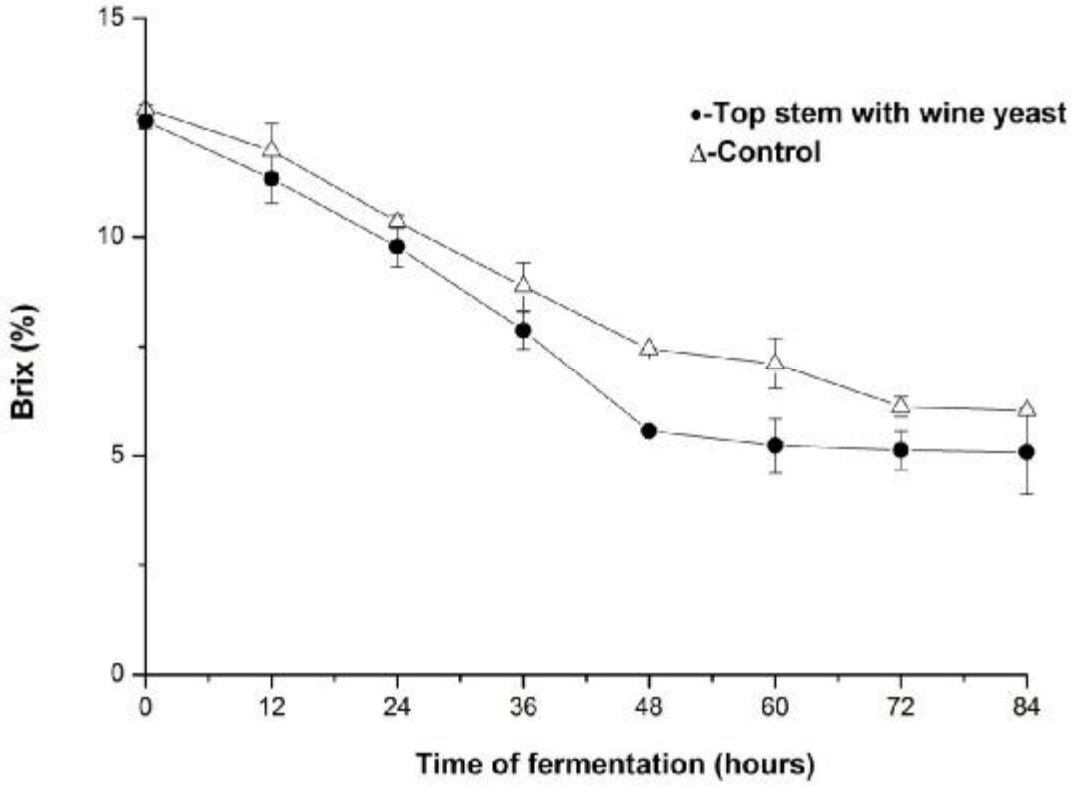


Figure 3.10 Brix changes in two treatments during fermentation. Bars indicate standard deviation from two determinations

Figure 3.10 shows the change of Brix of the two treatments during fermentation. Brix declined as expected for both treatments of both treatments decreased significantly during fermentation, again with significant differences between the treatments at many time points (statistics not shown).

3.3.5 Kinetics of ethanol concentration and sugar concentration

Figures 3.11 and 3.12 show the calibration curves used to determine changes in reducing sugar and ethanol concentration shown in Figure 3.13.

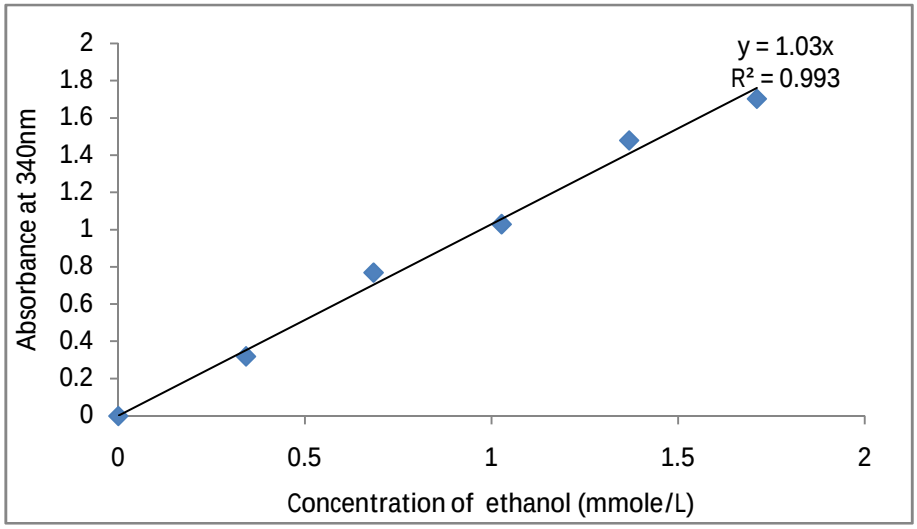


Figure 3.11 Calibration curve used to quantify ethanol concentration. The absorbance at 340 nm is described by the equation: Absorbance=1.03x, where x is the ethanol concentration. The R^2 value is 0.993. After solving for x, the ethanol concentration is quantified.

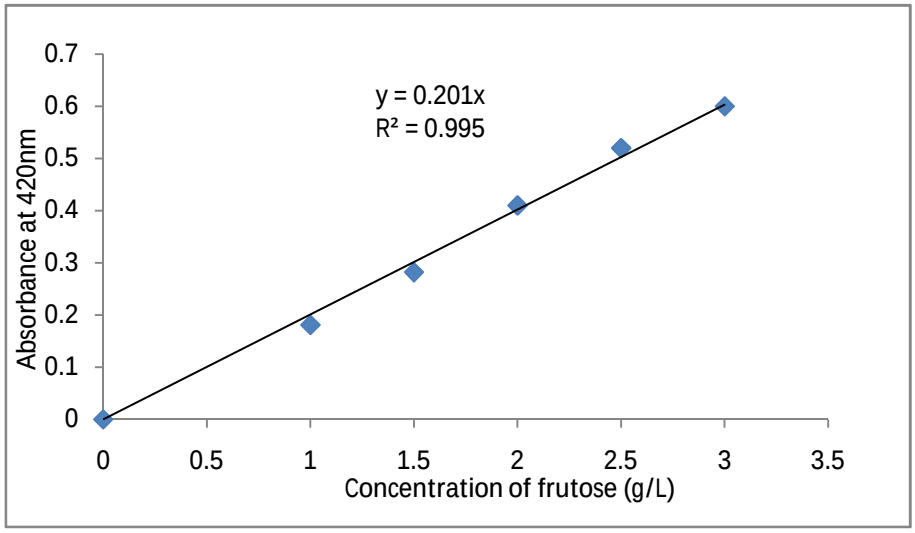


Figure 3.12 Calibration curve used to quantify sugar concentration. The absorbance at 340 nm is described by the equation: Absorbance=0.201x, where x is the sugar concentration. The R^2 value is 0.995. After solving for x, the sugar concentration is quantified.

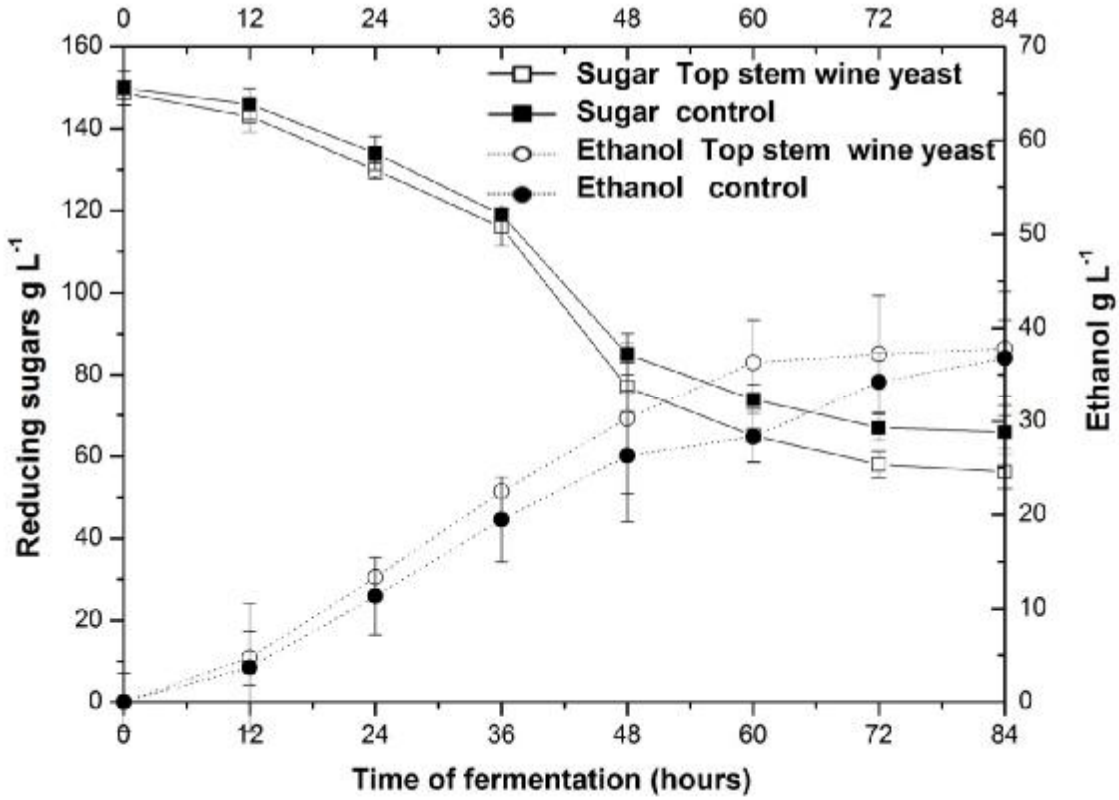


Figure 3.13 Changes of reducing sugar and ethanol concentrations in two treatments during fermentation. Bars indicate standard deviation from two determinations

Figure 3.13 shows the change of reducing sugar and ethanol concentrations during fermentation. The change of reducing sugar of both treatments decreased significantly during fermentation ($P < 0.001$ within each treatment), but there were no statistically significant difference between two treatments ($p = 0.76$), but with some indication of an interaction. Conversely, the production of ethanol in both treatments increased significantly during fermentation as expected, but no significant differences between two treatments ($p = 0.67$). Again there was some evidence of an interaction.

Table 3.1 shows the Brix value (Column 1), and the calculated sugar concentration from Brix in g L^{-1} compared with the results of the PABAH determination (Columns 2 and 3).

The relationship between Brix and sugar content of sample treatment can be calculated as followed: For example, at 0 hours, the mass of must = density x volume = 1180 g L^{-1} x 0.9 L=1062 g and Brix = 12.5%, which gave a sugar content of 132.75 g. Therefore sugar concentration = 132.75/0.9 =147.5 g L^{-1}. This compares with the results of the chemical method, 150 g L^{-1}. The results are the same within experimental error ($p < 0.001$, but standard deviation data not shown). The results of the two methods were comparable at all times (Table 3). Therefore, increasing ethanol did not affect sugar values calculated from Brix. It follows that the theoretical ethanol values calculated from Brix (1 mole of fructose yields 2 mole of ethanol) in Column 4 should be valid. Actual ethanol values determined by the enzymatic method are shown in Column 5.

Table 3.1 Mean values of sugar and ethanol concentration in the Top stem treatment measured from Brix and chemical analysis methods of samples

Time of fermentation (hours)	Parameter				
	Column 1	Column 2	Column 3	Column 4	Column 5
	Brix (%)	Sugar conc. from Brix (g L^{-1})	Sugar conc. from chemical method (g L^{-1})	Ethanol conc. calculated from Brix sugar value (g L^{-1})	Ethanol conc. from chemical method (g L^{-1})
0	12.5	147.5	150.0	0	0
12	12.2	143.9	142.3	3.1	3.8
24	10.8	127.4	128.5	11.6	11.3
36	8.8	103.8	115.2	23.6	25.0
48	5.8	68.4	69.1	41.7	34.6
60	5.2	60.9	60.1	45.5	39.4
72	5.1	60.2	59.9	45.9	39.4
84	5.0	59.0	59.2	46.5	39.4
Pearson correlation (r^2)		0.995		0.990	
p value of the correlation		< 0.001		< 0.001	

The maximum production efficiency of ethanol in the Top stem treatment was calculated as follows: the mean theoretical ethanol concentration was 27.23 g L^{-1} (mean of column 4), whereas the mean experimental value was 24.11 g L^{-1}. The alcohol efficiency was therefore 24.11/27.23=0.88 (Table 3.2), which indicated there were still some reason(s) leading to inhibition.

Results parallel to those shown in Table 3.1 were also calculated for the Control, but key outcomes are summarized in Table 3.2 which compares the Top stem and Control fermentations.

Table 3.2 Comparison of kinetic parameters of Top stem and Control treatments

Kinetic parameter	Treatment	
	Top stem	Control
Max. specific growth rate (h^{-1})	0.046	0.032
Max. specific sugar consumption rate (g L^{-1} h^{-1})	1.30	1.19
Max. specific ethanol production rate (g L^{-1} h^{-1})	0.87	0.73
Alcohol efficiency (%)	88	83

Low alcohol efficiency can result from many factors that affect the yeast's performance. These include high sugar concentration (osmotic stress), micronutrients limitation, high ethanol concentration, low pH, oxygen low levels, poor mixing, extreme temperature and yeast-toxic substances (Thatipamala et al., 1992). Many of these could not apply here. The most likely cause in the present experiments is micronutrients limitation, because the Control treatment (with no added stem matter) had the lower efficiency, 0.83. Yeast nutrient was added to both treatments but may be lacking in some compound(s) important for efficiency.

Table 3.1 also shows that the correlation between physical method and chemical for sugar and ethanol determination was excellent good (r^2 = 0.99). As for the PAHBAH method for sugar determination, the advantage of using this method is that the color complexes have the same molar extinction coefficient for glucose and fructose, and both of these were present in the

fermentation musts. In addition, the PAHBAH complexes are stable, and there are few interfering factors (Lever M, 1973). In respect of the NAD enzymatic method for ethanol determination, this method has been applied widely in food chemistry because of its speed, simplicity, and accuracy.

3.4 Mathematical model

From a chemistry perspective, we approximate the whole fermentation process as consecutive unimolecular reactions: S → intermediate → E, where S stands for the reducing sugar, intermediate (I) for pyruvate or acetaldehyde and E for the ethanol. We set the rate constant for S → I as K_a, and I → E as K_b. K_a and K_b are always positive. Both reactions are first-order, thus the intermediate is formed from S at a rate of $K_a[S]$ and transforms to E at a rate of $K_b[I]$.

In terms of the first-order rate law

$S = S_0 e^{-Kat}$ ……Equation 3.4.5

Where S_0 is the original sugar concentration, so, the net rate of formation of intermediate is $d[I]/dt = K_a[S] - K_b[I]$, When S is substituted from Equation 3.4.5, we obtain after rearrangement

$d[I]/dt + K_b[I] = K_a S_0 e^{-Kat}$. This differential equation has a standard form (dy/dx+yf (x)=g (x), here f(x) is a constant K_b and after setting $[I]_0 = 0$, the integrated solution is

$[I] = \frac{Ka}{Kb-Ka}(e^{-kat} - e^{-kbt})S_0$ ……Equation 3.4.6

At all times $[S] + [I] + [E] = [S]_0$, so it follows that

$[E] = (1+\frac{K_a e^{-K_b t} - K_b e^{-K_a t}}{K_b - K_a})S_0$ ……Equation 3.4.7

The outcome is that Equations 3.4.5 and 3.4.7 predict how the concentrations of sugar, intermediate and ethanol change. The [S] keeps going down until zero while [E] rises from zero towards $[S]_0$. The two equations are plotted in

Equation all has terms of $[S]_0$, $K_a t$ and $K_b t$, we can sketch two theoretical trend line for sugar

and ethanol respectively which has an X axis of K_at and Y axis of $[M]/[M]_0$, without knowing the values of $[R]_0$, K_a and K_b.

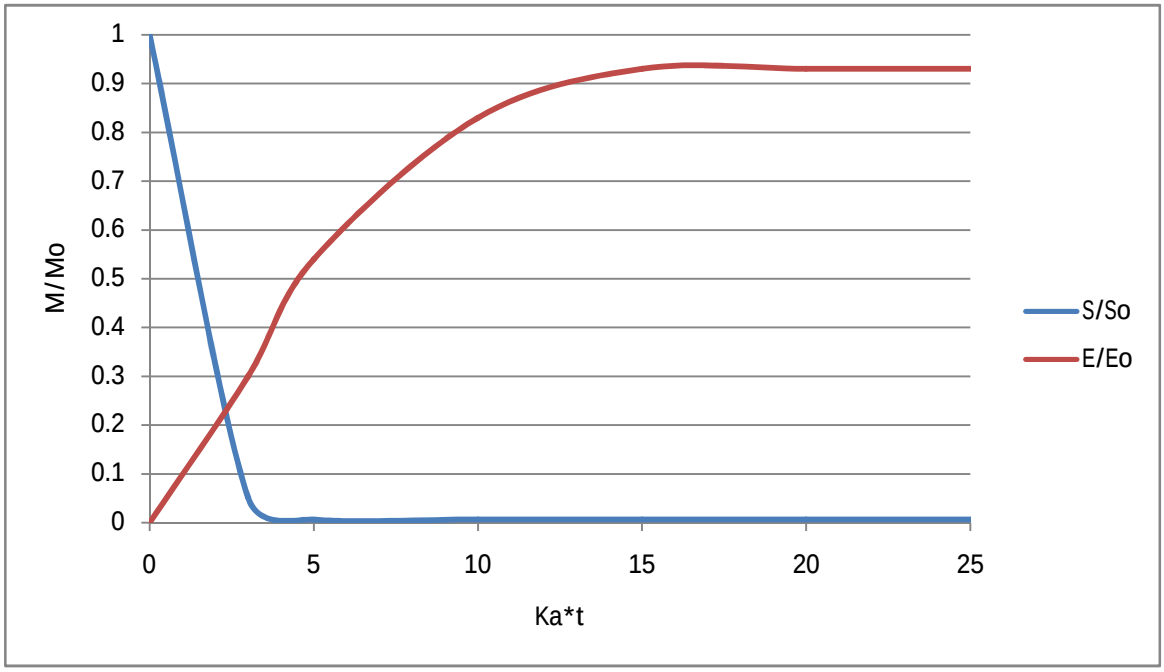


Figure 3.14 The concentrations of S and E from equation 3.4.5 and 3.4.7 with $K_a = 5K_b$

The model showed a satisfactory prediction on ethanol behavior; however, as for the sugar content a good prediction could not be obtained. As mentioned above, there were many factors which might affect the microbial performance during fermentation process, which might result in lower reaction rate of sugar decomposition. This also conformed to the result of low alcohol efficiency and lower maximum specific sugar consumption rate of two treatments (Table 3.2).

3.5 Brief Summary

Research results of this chapter showed: in the microbiology aspect, yeast growth pattern and biomass production of all six treatments had similar mode of development. However, top stem treated with wine yeast was characterized as the treatment, which exhibited the best fermentation. Therefore, all the subsequent analyses in the next stage were concentrated on this treatments and its responding control treatment. It turned out there was a potential opportunity to use *Cordyline australis* as a leaf crop for the production of novelty alcoholic

goods although some negative factors still existed, which led to the ethanol inhibition in the fermentation products. In addition, colour measurement of the fermentation products indicated it was possible that Maillard reaction took place in the fermentation process. Hence, in the next chapter, Maillard reaction would be studied in details to see its role on the formation of the flavour volatiles from the *Cordyline australis*-fructose system.

CHAPTER 4

Study of the Maillard Reaction in a Top stem/Fructose Model System

4.1 Introduction

As described in Chapter 1, the procedure for the production of the spirit followed this sequence: Stem comminution and maceration at 80°C for two days; coarse filtration; adjust of the sugar concentration to 150 g L^{-1}; fermentation; coarse filtration; distillation. In the tequila industry by contrast the agave *piñas* are coarsely comminuted, and moist heated at 60 to 80°C for two to three days to hydrolyse the inulin before fermentation. At the same time the Maillard reaction and caramelisation will be active because the agave contains fructose and amino acids. If in the future the path to spirit production from ti kouka top stem were to parallel that for tequila production, the comminuted top stem might be heated with added fructose, to generate Maillard reaction products, before conventional fermentation and distillation. Thus as a way of keeping production options open this alternative pathway is explored here, with a focus on Maillard reaction products that can be generated before fermentation. This can be done at several pH values and at different temperatures

4.1.1 Modeling of Maillard reaction

The Maillard reaction results in the development of colour when most foods are heated. Although the Maillard reaction has been known since 1912, its complexity still leaves some uncertainty as to the exact mechanism of the formation of several of its typical products.

A simplified scheme of the reactions involved was proposed by Hodge (1953) (Figure 4.1) where the letters A to G symbolize reaction types shown in Table 4.1.

Table 4.1 Phases of the Maillard reaction		
Phase	Color of products	Reaction type
Initial phase	Colorless	A Sugar-amine condensation
	Without absorption in the ultraviolet (280nm)	B: Amodori rearrangement
Intermediate phase	Colorless or yellow Strong absorption in the ultraviolet	C: Sugar dehydration D: Sugar fragmentation
Final phase	Highly colored	F: Aldol condensation G: Aldehyde-amine condensation and formation of heterocyclic nitrogen compounds H: Free radical

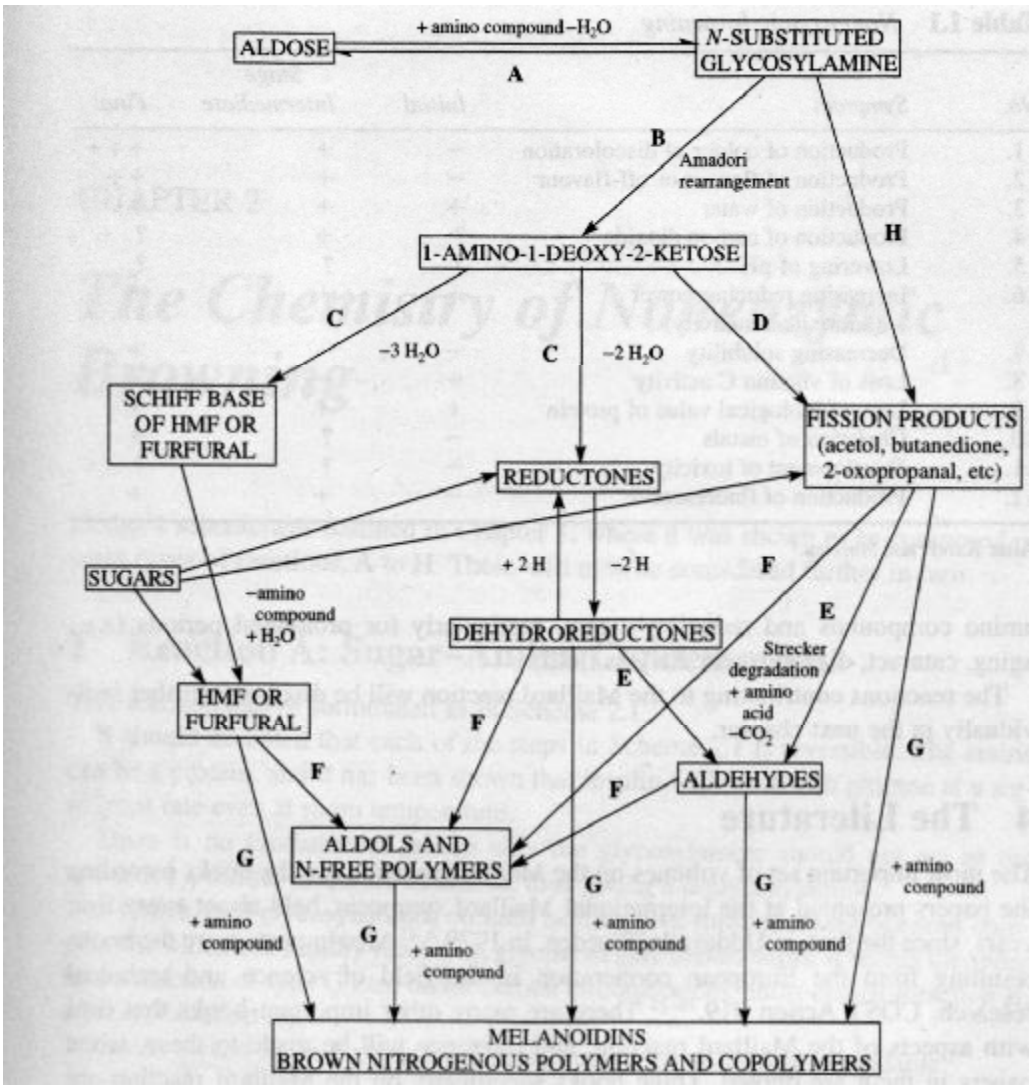


Figure 4.1 Phases of the Maillard reaction. Based on Hodge (1953)

The final products of Maillard reaction are called melanoidins which is to distinguish them from the melanins produced by enzymatic browning. It has been proposed that melanoidins have functional chemical characteristics such as antioxidative activities (Osada & Shibamoto, 2006), antimutagenic activities (Alaiz et al., 1996), and nitrite scavenging ability (Cheigh et al., 1990). However, in practice, it is difficult to classify the dark brown products formed in foods because they are very complex mixtures and are chemically intractable.

In addition, heating promotes the Maillard reaction, as is common for chemical reactions. Another key feacture of the Maillard reaction is the loss of water at the initial stage in the chain of reaction; therefore, the reaction is accelerated by lower water activity.

4.1.2 Classes of aroma compounds formed in the Maillard reaction

The complex mixture of aroma volatiles derived from the Maillard reaction has been divided into three classes by Nursten, 2005.

Class 1: Sugar dehydration/fragmentation products such as furans, pyrones, and carbonyl compounds.

Class 2: Amino acid degradation products such as aldehydes, sulphur compounds, and nitrogen compounds.

Class 3: Volatiles produced by further interactions such as pyridines, thiophenes, and furanthiols.

Intermediates from the breakdown of Amadori compounds (1-amino-1-deoxy-2-ketoses) lead to the Class 1 compounds in the early stages of the reaction. These compounds are similar to those found in the caramelisation of sugars. and play important role in the aspect of food flavor.

Class 2 includes simple aldehydes, sulphides and amino compounds that result from the Strecker degradation occurring between amino acids and dicarbonyl compounds.

Products from Classes 1 and 2 are capable of further reaction which involves the interaction of furfurals, furanones and dicarbonyls with other reactive compounds such as amines, amino acids, hydrogen sulphide, thiols, ammonia, acetaldehyde and other aldehydes. These reactions results in many important kinds of flavour compounds in Class 3.

4.1.3 Detection of compound formation in the Maillard reaction

Formation of coloured compounds is a primary characteristic of the Maillard reaction. The colour produced can be readily measured by reading the absorbance in the visible region of the spectrum, typical wavelengths used being 360 and 420 nm. Another, but more complex method of analysis, is to determine the compounds generated. Liquid and gas chromatography are the most common methods employed (Finot, 1990). Both colorimetric and chromatographic methods have been used here.

4.1.4 Experimental design

As discussed above, temperature, pH, heating time and water activity are four important factors in the Maillard reaction. In this study to model the generation of compounds in a reflux mixture of Top stem and fructose, temperature (60 and 100°C) and pH (4, 7 and 9) were the chosen variables.

4.2 Materials and methods

4.2.1 Preparation and characterization protocols of reflux

Top stem was transversely sliced into discs about 30 mm in diameter and 2 mm thick with band saw. Samples of 50 g were heated for 3 hours under reflux conditions with 200 mL of fructose solution at 150 g $L^{-1.}$ The resulting suspension was then vacuum-flitered through Whatman No 4 paper in preparation for analysis. Since there are three variables of pH factor and two variables of temperature, there were six reflux treatments in total.

4.2.2 Physicochemical analyses

Absorbances were measured against a water blank at three wavelengths (210, 320, and 420 nm) by using a single quartz cuvette with 1 cm optical path in a Pharmacia Biotech® Ultraspec 2100pro spectrophotometer. Dilutions were made with deionised water before determining absorbances to ensure the value were between 0 and 3. Presented data are corrected for these dilutions. All determinations were done in triplicate.

Determination of major aroma volatiles in was done by gas chromatography-mass spectrometry (GC-MS), using a Trace GC-Ultra gas chromatography (DSQ Thermoelectron Corp.) equipped with a Shimadzu QP5000 Mass Spectrometer. Separations were performed using a 25 m x 0.25 mm internal diameter capillary column, coated with a 0.25 μm film of ZB-Wax stationary phase (Phenomenex Ltd Auckland NZ). Helium was used as the carrier gas.

An aliquot of 5 mL sample was added with 100 μL of 1-octanol (1000mg/L) as internal standard. About 1μL sample was injected.

The chromatographic conditions were 80°C for 3 minutes, increasing at 8°C min^{-1} to 120°C for 3 minutes, 5°C min^{-1} to 250°C, and maintaining at this temperature for 20 min, making 57 min in all. The injector and detector temperatures were 200°C and 250°C, respectively. The MS ionisation potential was 70 ev, the transfer line temperature was 200°C, and the scan mode was 40 to 700 m/z. The compounds were tentatively identified by comparing their mass spectra with those obtained in the National Institute of Standards and Technology (NIST) library of the MS database.

Colour and odour of each treatment were informally assessed by two researchers (Chinese and French) and Dr. Owen Young.

4.2.3 Statistical analysis

See section 3.2.6

4.3 Results and discussion

4.3.1 Color development from incubations of ti kouka stem with frucose

The incubations were very effective in generating brown pigments to the point that some mixtures were almost black, and most required extensive dilution before the absorbances could be read. It was clear that caramelisation/Maillard reactions were occurring, and this was confirmed by absorbances in the ultraviolet range (Figure 4.2).

Figure 4.2 shows the absorbance at three wavelengths of three pH treatments at two temperatures. The absorbance at 210 nm was always higher than that at 320 nm, the latter wavelength being characteristic of low molecular weight Maillard reaction products (Bailey et al., 1996), presumably formed from the reaction of added fructose, and fructose derived from inulin in the stem, with amino acids known to be present in the stem (Patel, 2010). All the absorbances at 420 nm were significantly lower than for the other two treatments ($p < 0.001$), which means that most of the colour changes were occurring in the ultraviolet range. The reaction was clearly favoured by the higher temperature.

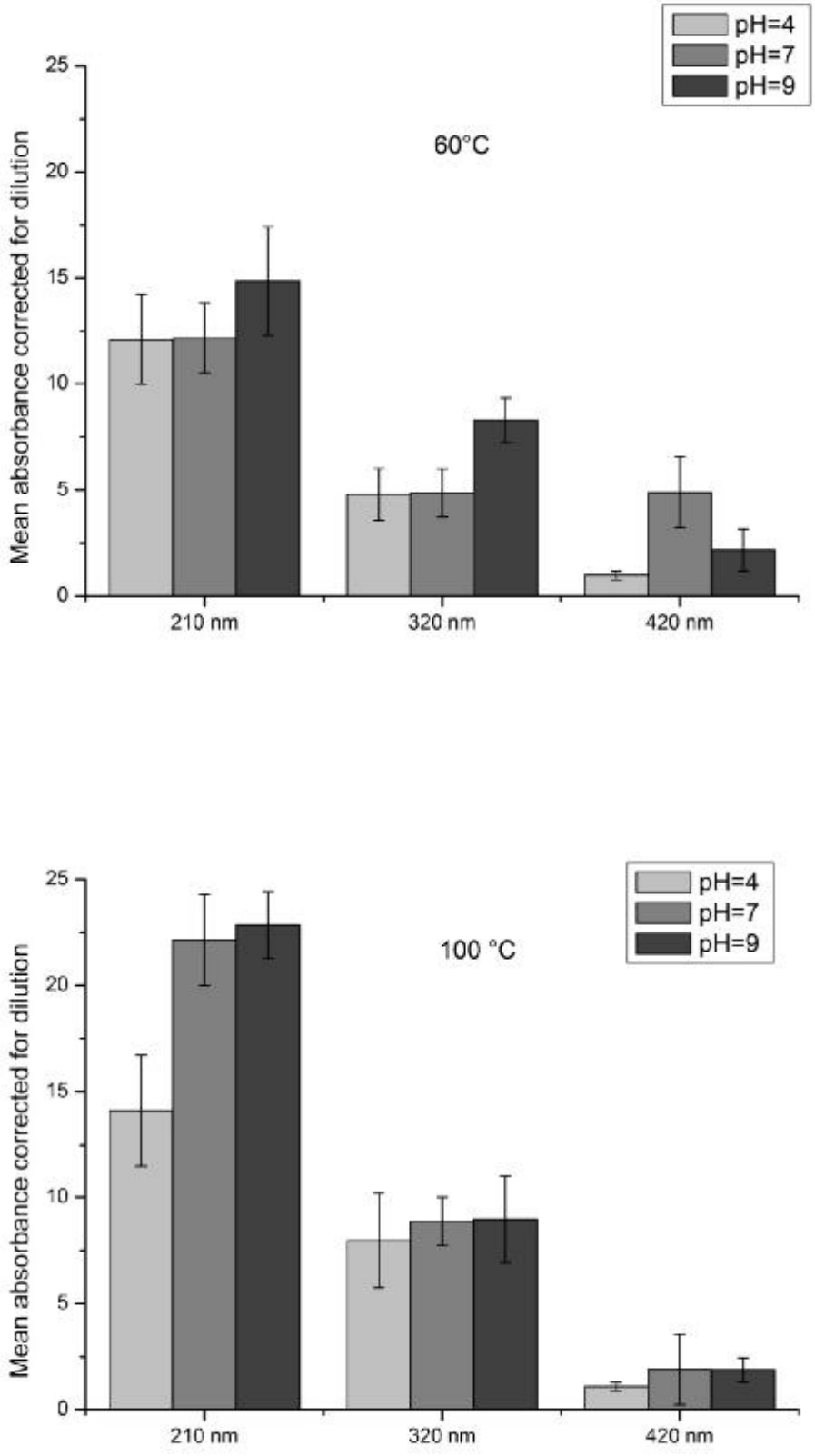


Figure 4.2 Absorbance at three wavelengths of three pH treatments at 60°C and 100°C when ti kouka stem was incubated with fructose solution. Bars are standard deviations from three replicate absorbances

pH exerts a crucial effect on the mechanism and kinetics of Maillard reaction. Generally, reaction rates increase with increasing pH above 7 up to a maximum at 9 to 10. Incubation at pH 9 was most effective (Table 4.2), but the differences were not numerically great.

One explanation for this could be the relatively long reaction times (3 hours) used in this experiment such that the reactions may have gone to completion. In hindsight, the colour changes could have been monitored over the three hour period. Another uncertainty is as follows: not all absorbance may be due to caramelisation/Maillard reactions; during incubation for 3 hours ultraviolet-absorbing compounds would have leeched from the comminuted ti touka. Again in hindsight, control incubations lacking added fructose and control incubations lacking stem would have given better insights as to the chemical detail, but would have added markedly to the work required. Of more practical interest was the odour generated by these treatments.

Table 4.2 Analysis of variance for absorbance as affected by pH, temperature and wavelength

General Linear Model: Mean of Abso versus pH, Heating Temp, Wavelength

Factor	Type	Levels	Values
pH	fixed	3	4, 7, 9
Heating Temperature	fixed	2	60, 100
Wavelength	fixed	3	210, 320, 420

Analysis of Variance for Mean of Absorbance, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	2	24.445	24.445	12.223	52.39	0.001
Heating Temperature	1	37.700	37.700	37.700	161.60	0.000
Wavelength	2	731.538	731.538	365.769	1567.82	0.000
pH*Heating Temperature	2	0.052	0.052	0.026	0.11	0.897
pH*Wavelength	4	2.237	2.237	0.559	2.40	0.209
Heating Temperature*Wavelength	2	53.276	53.276	26.638	114.18	0.000
Error	4	0.933	0.933	0.233		
Total	17	850.181				

S = 0.483009 R-Sq = 99.89% R-Sq(adj) = 99.53%

In this part of the experiment, we had three factors, pH, heating temperature, and wavelength. From Figure 4.3, we can find wavelength is a leading factor. Response of absorbance was

much depenent on this factor. When three-factor interactions of ANOVA was done, it showed denominator of F-test was zero. Therefore, **pH*heating temperature*wavelength** was exclude from the interaction model. Final factorial design in ANOVA was based on these three factors except the item of pH*heating temperature*wavelength to see the interaction between them.

Two of the two factor interactions were not significant (p=0.897&0.209), but the **Heating temperature*wavelength** interaction was significant (p=0.000). The interaction plot shows that although absorbance at 210 nm have higher value, this difference is much greater for higher heating temperature.

Since pH does not interact with the other two factors, the main effect of pH is focused on. This is significant (p<0.001). From the interaction plot, it is clear that for all combinations of the other variables, higher pH have higher absorbance than lower pH.

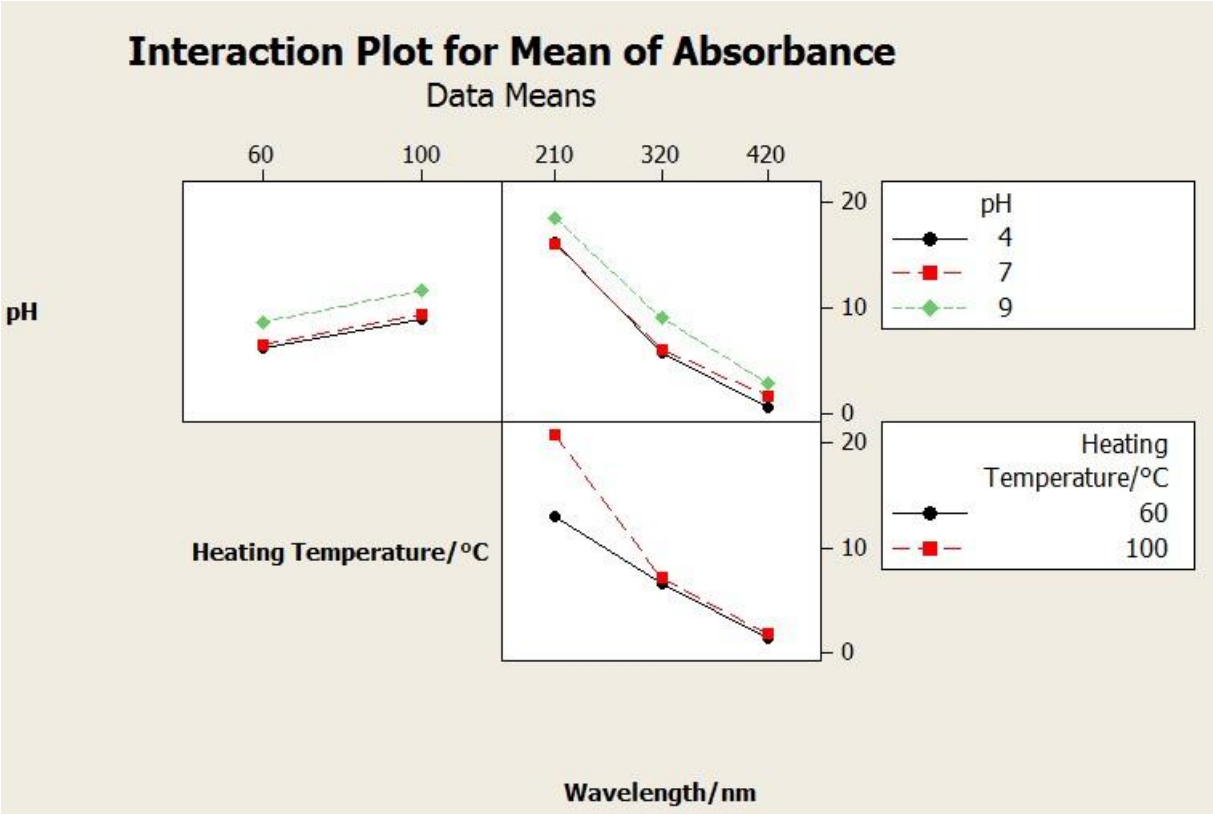


Figure 4.3 Interaction plot of absorbance with three factors

4.3.2 Description of colour and odour from incubations of ti kouka stem with fructose

Table 4.2 shows the results of an informal sensory assessment by the researcher and female colleague aged about 22 years. There were clear differences between the pH treatments, with caramel notes evident at pH 9, but not elsewhere. pH 7 would clearly be the most useful in generating a tequila-like note. Importantly the condition pH 7, and 100°C most closely resembles the tequila process where the agave cores are incubated in ovens for extended periods. However, in that situation fructose is not added, and any Maillard reaction relies on endogenous fructose hydrolysed from agave's inulin. But from this result it is not possible to identify the chemical cause of this unique flavour.

Table 4.3 Description of color and aroma at different pH values and heating temperatures

Temperature (°C)	pH = 4		pH = 7		pH = 9	
	Colour	Odour	Colour	Odour	Colour	Odour
60	Pale yellow	Woody	Yellow	Smoky	Yellow	Sugar, caramel
100	Pale yellow	Vinegar	Yellow	Tequila	Brown	Caramel, chocolate

4.3.3 Volatile compounds generated in Top stem/fructose model system

Table 4.3 shows the colour and odour outcomes of six combinations of temperature and pH. These six treatments were further examined by GC-MS. This was done by direct injection of the solutions to which the internal standard, 1-octanol has been routinely added.

Of the six treatments chromatograms are shown only for three conditions, pH 4 at 60°C, pH 7 at 100°C and pH 9 at 100°C. Together these show the main peaks observed.

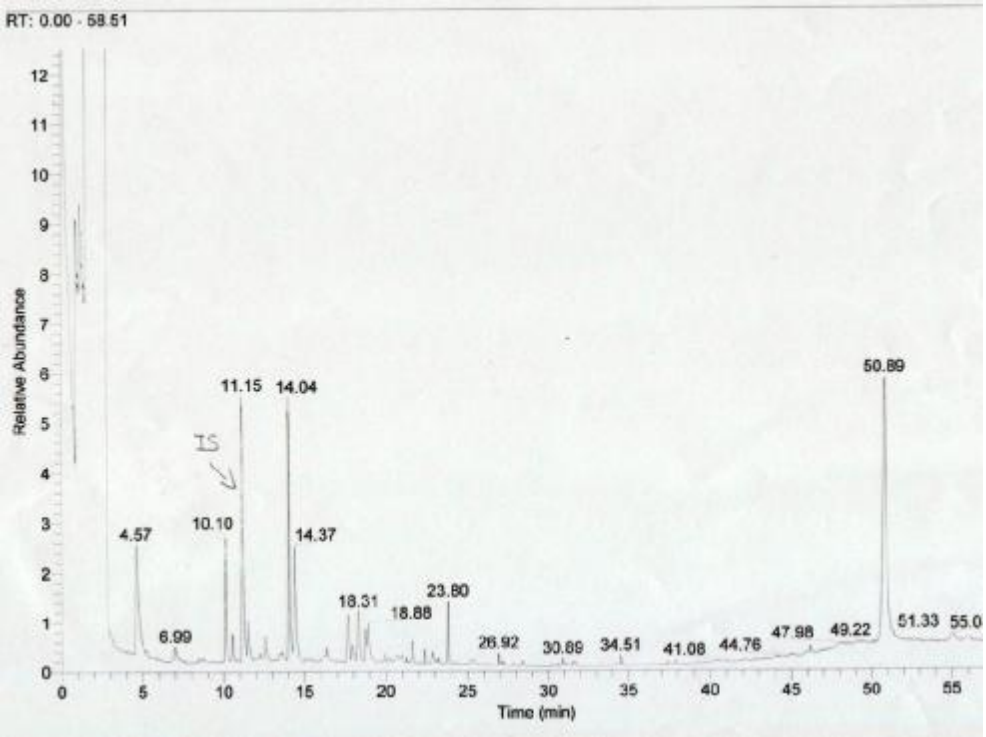


Figure 4.4 Chromatogram of compounds from the Top stem/fructose incubations at pH 4 and 60°C. The internal standard is indicated, IS

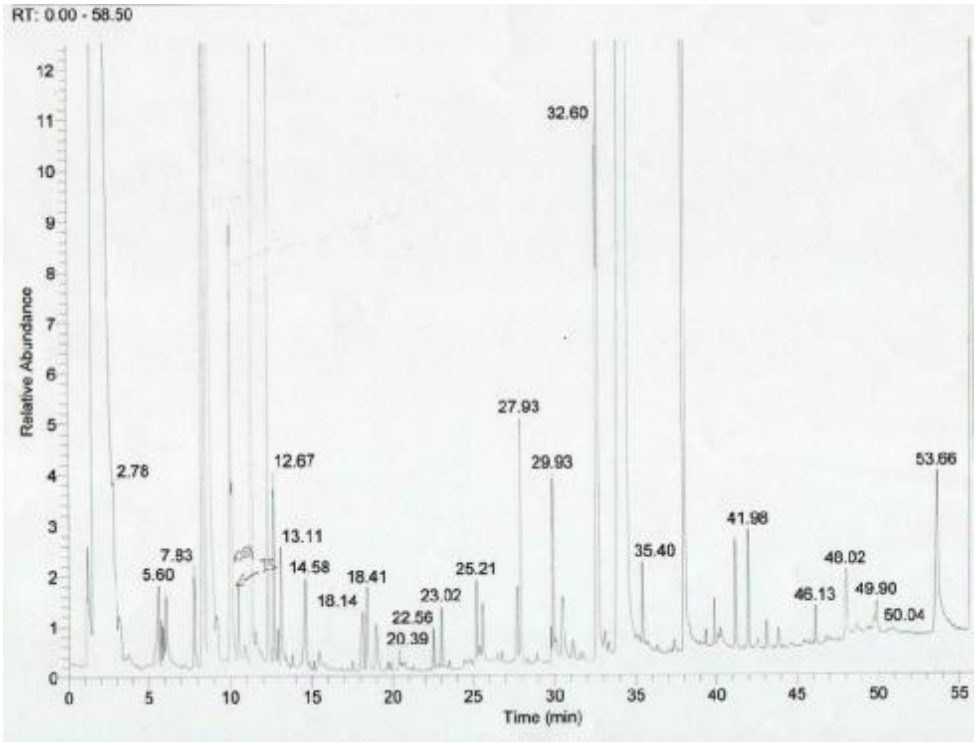


Figure 4.5 Chromatogram of compounds from the Top stem/fructose incubations at pH 7and 100°C. The internal standard is indicated, IS

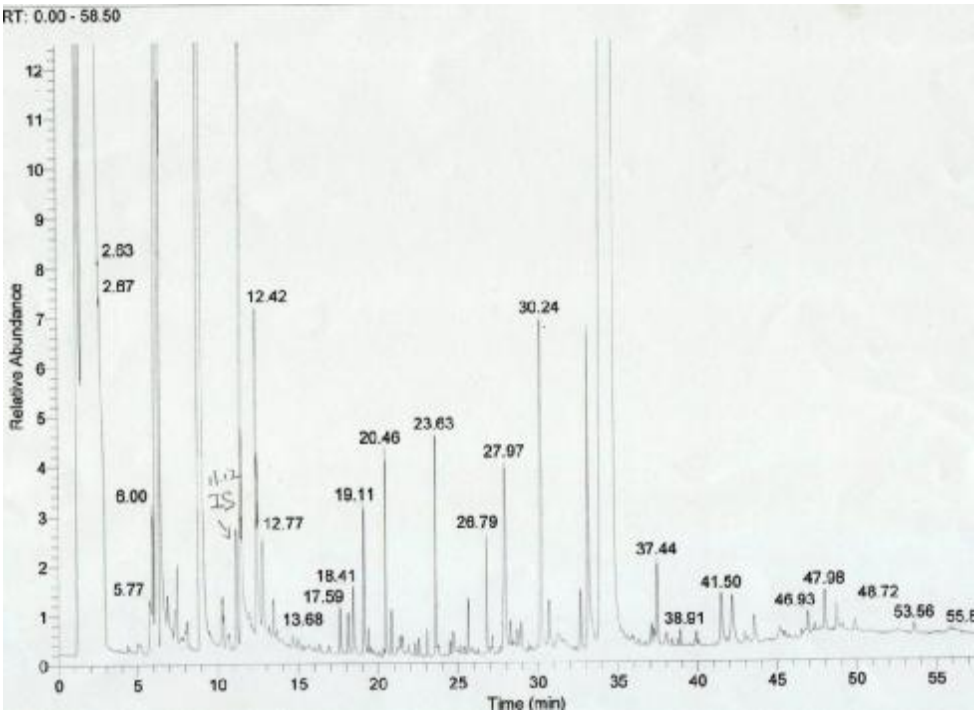


Figure 4.6 Chromatogram of compounds from the Top stem/fructose incubations at pH 9 and 100°C. The internal standard is indicated, IS

Inspection of the peaks relative to the internal standard (IS) show that the pH 7 and 9 treatments at 100°C generate many more compounds that pH 4 at 60°C.

A quantitative analysis of the individual compounds was not done. For comparison, relative abundance of the internal standard was used to normalise the relative abundance of compounds, many of which were tentatively identified from the mass spectral library.

Chromatograms obtained from at three different conditions (4/60, 7/100, and 9/100) showed very complex profiles with 75, 93, and 131 components were respectively found. Table 4.3 lists 50 principal volatile compounds that were first: present in high abundance; second, generated a mass spectrum that had a high probability of being correct; and third, was plausibly in the mixture. Acids and alcohols were the most abundant compounds, followed by aldehydes, terpenes, furans and pyrans. Some were found in all treatments.

It has been proposed that there are significant differences in the Maillard reaction products due to the sugar involved (Hollnagel & Kroh, 2000). Generally, simple heterocyclic compounds are produced when monosaccharides involved and the presence of these compounds such as simple furans and pyrans is evidence that Maillard reaction took place during the reflux of ti kouka stem with fructose. By contrast, disaccharides and oligo- and polysaccharides generate more complex products. These were either overlooked in the profile, were not present by virtue of low volatility or were not generated at all. The most likely conclusion is that they were not present because the cooking conditions would favour the total hydrolysis of endogenous inulin toward fructose generation.

Table 4.4 Maillard compounds found in Top stem/fructose model system under three conditions

Ret. time (min)	Likely compound	Concn. relative to internal std.			Odour description	Ret. time (min)	Likely compound	Concn. relative to internal std.			Odour description
		4/60	7/100	9/100				4/60	7/100	9/100	
2.78	Unknown(s)	NC	NC	NC		29.93	Butyrolactone		0.4	8.4	
3.18	*3-Methyl-1-butanol	0.01	2.0	0.35	Banana	31.10	*2-Furanmethanol	0.04	1.2	1.4	
5.60	*2-Methyl-1-propanol	0.03	0.4	4.4	Polish	31.55	Squalene		1.4	0.8	
7.83	1-Butanol			2.4	Alcohol	32.72	Pentanoic acid		3.8	30.4	
8.59	3-Methyl-2-hexanol		2.2	31		33.08	3-Methylbutanoic acid			2.2	
9.51	1-Hexanol		0.2		Coconut	34.51	*Phenylethyl alcohol	0.03	0.4	18.1	Floral
10.04	*Cyclotene	0.37	0.8	21.8		35.40	Hexanoic acid		2.4	0.2	Goaty
10.52	3-Methylcyclopentanone	0.09		3.2		36.72	4-Ethyl-2-methylpyrrole		0.2		
10.89	**1-Octanol**	**1**	**1**	**1**	**IS**	37.35	4-Methoxybenaldehyde	0.01		0.8	
11.36	*Pyridine	0.19	21.8	35.8		38.05	Octanoic acid			9.3	Fatty acid
12.33	*Linalool	0.04	0.2	23.3	Aniseed	39.86	Nananoic acid		2.0	1.6	
12.67	2-Nonanone	0.13		5.8		40.22	*Decanoic acid	0.01	4.2	2.6	
13.11	2-Butylfuran		18.5	6.2		41.18	Maltol		0.2	3.8	
13.78	*3-Hydroxy-2-butanone	0.07	2	0.6		41.98	Dodecanoic	0.01		4.0	
14.58	*Phytol	0.62	17.8	6.4		42.59	Benzeneacetic		1.2	1.0	
15.43	*2-Pyrrolidinone	0.01	0.20	1.8		43.10	Tetradecanoic			1.0	
18.41	Furfural	0.3		8.6	Paper	43.44	2,4,5-Trimethylthiazole			0.2	
18.96	*1-(2-Furanyl)ethanone	0.36	5.6	3.0		43.86	2-Cyclopropylthiophene	0.04		1.0	
19.70	3-Furaldehyde		0.4	0.6		45.20	Dibenzofuran	0			
20.39	Acetic acid	0.06	12.2	1.2	Vinegar	46.13	*1-Hydroxylinalool	0.05	2.2	1.2	
22.56	*2,3-Butanediol	0.07	0.8	1.4		46.80	Vanillin	0.01		1.0	Creamy
23.02	Benzaldehyde		1.8	2.6	Nutty	48.02	*Hexadecanoic	0.09	1.2	5.0	

Ret. time (min)	Likely compound	Concn. relative to internal std.			Odour description	Ret. time (min)	Likely compound	Concn. relative to internal std.			Odour description
		4/60	7/100	9/100				4/60	7/100	9/100	
25.21	*Propanoic acid	0.06	0.6	3.8		49.90	4-Hydroxybenzaldehyde		1	3.4	
25.57	2-Methylpropanoicacid			2.6		50.82	Syringaldehyde		0.8	1.4	
27.93	Butanoic acid		0.2	12.4		53.66	Octadecanoic			16.4	
NC, Not calculated; IS, Internal standard; Blank values mean not detected; *Compounds found in all treatments											

Most organic acids observed in the profiles probably came from cell membranes that were chemically degraded during the cooking treatment, although acetic acid (20.39 min) is also generated by the Strecker degradation of alanine (Hofmann et al., 2000). Alcohols such as 2-methyl-1-propanol (5.60), 3-methyl-2-hexanol (8.59), and phenylethyl alcohol (34.51) have been considered to be fermentation products, which are responsible for the unique notes of most alcoholic beverages, but their presence in the profiles suggests that the Maillard reaction is another pathway for their formation. It is well known that aldehydes such as 3-furaldehyde (19.70) and benzaldehyde (23.02) are formed during the resting or aging of alcoholic beverages through contact with the wood oaks; however, these compounds are also found in the profiles, and have been reported in the cooked exudates of agave *piñas* (Mancilla-Margalli et al., 2002). These authors also showed that their concentrations increase during aging of tequila in oak (Mancilla-Margalli et al., 2002). Terpenes such as linalool, phytol might come from the plant itself.

The volatile profile coming from the Maillard reaction plays an important role in flavouring development and sensory characteristic to final products in food system. Although acids were the most abundant compounds during the reflux process modeled here for ti kouka stem, they do not show a constant concentration during the whole production process of tequila. In tequila production industry, most of them like hexanoic and tetradecanoic acids are lost during production because their concentrations are notably lower than in exudates. In contrast, many of the alcohols such as 3-methyl-1-butanol and phenylethyl alcohol persist in the final product, influencing the overall flavor. Aldehydes such as benzaldehyde and phenylacetaldehyde have a green, floral, and flower notes with a low threshold. Terpenes are known for their sweet, fruity, and flowery odor. Many of these compounds have received much attention as biologically active constituents in foods for their antioxidative properties (Fuster et al., 2000) and may have a similar role in spirits. Finally, furans and pyrans usually add sweet notes.

4.4 Brief summary

During the thermal process (reflux) of ti kouka Top stem with fructose, many volatiles, mainly Maillard compounds, were produced, many of which may have a significant impact on the overall flavor of final spirit. Differences in chromatographic profiles showed that of the six treatments it was pH 9 at 100°C that generated the most Maillard reaction products in the Top stem/fructose system. However, the most tequila-like flavour was observed at pH 7 and 100°C, as decided by two observers. Many Maillard compounds were identified in the chromatographic profiles including acids, alcohols, furans, pyrans, aldehydes, and terpenes. Many of them have been reported as a powerful odorants, responsible for unique flavours. However, in the tequila production process, many volatiles generated during cooking stage (equivalent to the reflux performed here) are transformed to other compounds during distillation. Therefore, in the next chapter, major volatile components from the distillation process will be studied quantitatively by gas chromatography-Flame ionization detector (GC-FID).

CHAPTER 5

Characterization of Major Volatile Profiles in Distillation Fractions by Gas Chromatography with Flame Ionization Detection (GC-FID)

5.1 Introduction

5.1.1 Distillation to achieve fractionation of flavour

Distillation of fermented juices with an alcoholic strength between 5 and 15% v/v is the main technological step in the production of distilled spirits by which ethanol and flavour compounds are separated and transferred into the distillate; ethanol is initially distilled as a ethanol:water azeotrope at 78.15 °C, together with other volatile compounds (Christoph & Bauer-Christoph, 2007). After the ethanol concentration in the distillation vessel falls below a critical value the distillation temperature rises as a greater proportion of water distils. The temperature steadily approaches 100°C.

The most basic type of device for the production of heavily flavoured distillates is the batch distillation with a pot still, which is an enclosed copper pot that narrows into an overhead-vapor pipe at the top to collect alcohol vapour. The pipe bends downwards off the top of the pot to a water-cooled condenser which causes the alcohol vapor to condense back into liquid. It is possible to separate different fractions in this type of distillation. The first fraction called the ‘head cut’ contains volatiles such as acetaldehyde meanwhile the last faction called ‘tail cut’ (below 40% v/v) is composed of high-boiling compounds like long-chain fatty acids; since both fractions contain aroma compounds considered undesirable, these substances can be separated off from the ‘heart cut’ which is rich in aroma compounds important for sensory quality (Christoph & Bauer-Christoph, 2007).

5.1.2 Distillation in tequila production

There are two steps in the batch distillation of tequila. First, fermented juice is distilled to remove solid particles, yeast, proteins and mineral salts. The alcohol content attained is 25 to 30%. In the second step, the liquid from the first stage is redistilled. The main objective is

to increase the alcohol content up to 55%, but also provides the opportunity to separate head, heart and tail cuts. Final products are diluted with water to achieve a final alcohol concentration of typically 38% (Faria et al., 2003; Cedefio, 1995).

5.2 Materials and methods

5.2.1 Chemicals

Identification standards for gas chromatography with flame ionization detection (GC-FID) were sourced from the AUT chemistry laboratory. These were acetaldehyde, ethyl acetate, methanol, 2-butanol, 1-propanol, 1-butanol, 2-methylpropanol, 2-methyl-1-butanol, acetic acid and 2-phenylethanol with claimed purity of 99%. 1-Octanol was chosen as the internal standard.

The reference commercial spirits were tequila (Pepe Lopez, Premium Silver), gin (Gordon's London Dry) and whisky (Johnnie Walker Red Label), and were purchased from Liquor Park (11 Beach Rd., Auckland). Tequila (Jose Cuervo 100% Agave Gold) was sourced from Dr. Owen Young.

5.2.2 Fermentation protocol

Fermentation of duplicate Top stem and Control treatments was carried out as described in Chapter 3. After fermentation was complete the fermented liquor, brown in the Top stem treatment and colourless in the Control, was decanted to minimize yeast content in the distillation process.

5.2.3 Distillation protocol

Distillation of 250 mL-fermented broth was carried out in a 1 L round-bottom flask. Before distillation, however, each 250-mL sample was heated under reflux conditions for 30 min to encourage further generation of flavour compounds. Treatments were then distilled over heating mantles and 10 mL fractions were progressively collected until the stillhead

temperature reached almost 100°C (boiling point of water) at which point nearly all the ethanol would have been recovered. Four fractions were collected from each duplicate within treatment.

5.2.4 Physicochemical analyses

Colour was measured using a Hunter meter (Meterlab, U.K.). The concept and basic principle of this colour monitoring technology was briefly discussed in Section 3.2.3. L* is lightness from 0 (total light absorbance and therefore completely black) through grey (50) to 100 (complete light reflectance i.e. white). The other values returned by the Hunter meter are a* and b*, redness-greenness and yellowness-blueness, respectively. However, the simple expressions of a* and b* are not very useful for categorical responses because they have a numerical value to infinity. Therefore, in this part of the project, two new paramters 'hue' and 'chroma' were used to qauntify colour development on different distillation Fractions.

Hue is defined as arctangent (b*/a*) and represents rotation about the a* and b* axes. It is usually expressed in radians. The scale ranges from 0 to 6.28 (= 2π). As the name suggests, hue refers to the gradation of colour within the visible spectrum of light, 380 nm to 750 nm. Chroma is calculated as $\sqrt{(a*^2 + b*^2)}$, and is the intensity of a hue. For example, a highly saturated hue has a vivid, intense colour while a less saturated hue appears 'washed out'.

The distilled fractions were evenly spread, in a Petri dish (100 mm diameter). This was then placed in the illuminant path (sample port), covered with an opaque metallic black shroud. A daylight D65/10° illuminant/observer combination was selected to measure reflected daylight colour as L*, a*, and b*. Within a treatment, the colour of each duplicate was measured three times. The means of the triplicates were used to calculate the final reported means and standard deviations.

Refractive index was measured for each fraction per treatment throughout the distillation process using Prisma Abbe Bench refractometer (Ceti, Belgium) and the ethanol content (v/v) was calculated from one specific refractometry concentration table (Appendix I).

Descriptive analysis of distillation fractions of colour and odour was carried out as depicted in section 4.2.3.

Major volatile constituents of the distill fractions were analyzed directly, without any previous treatment, on a Shimadzu GC17A gas chromatograph (Shimadzu, Japan) equipped with a Split/Splitless injector and a flame ionization detector (FID). A capillary column coated with ZB-Wax (25 m x 0.25 mm) was used. The temperature of the injector and detector were set to 140°C & 250 °C respectively. The oven temperature was held at 80 °C, for 3 min, then programmed to rise from 80 °C to 220 °C, at 20°C /min, and finally held at 5 min at 220°C. Nitrogen at 80 psig was used as carrier gas, and the split vent was set to 20 ml/min. Before injection of 1 µl sample, 1 µl 100 mg/l 1-octanol was added to the sample (internal standard). Quantification of volatiles, as 1-octanol equivalents, was performed by comparing retention indices with those of pure standard compounds. Before injection to GC, each treatment was adjusted to around 38%.

Standards of known concentration were mixed with the same concentration of IS as in the spirits and were injected to GC-FID. The ratio was calculated by peak area of standard/peak area of IS. In the next analysis stage:
Concentration of sample = Ratio x Concentration of IS x (Peak area of sample/peak area of IS)
Each compound was identified by a comparison of the retention times of the individual reference.

5.2.5 Statistical analysis

See section 3.2.6

5.3 Results and discussion

5.3.1 Colour and odour of distilled fractions

Table 5.1 Colour measurement and ethanol concentration of 4 distillation fractions per treatment (ti kouka and control)

Treatment	Fraction	Refractive Index at 20°C	Ethanol concn.[a] (v/v %)	Hue angle[a] arctangent (b*/a*)	Chroma[a] $\sqrt{(a*^2 + b*^2)}$	L*[a]
Top stem	1	1.3650	90	1.30 ± 0.24	14.11 ± 0.41	13.23 ± 0.12
	2	1.3590	42	1.02 ± 0.19	8.24 ± 0.12	18.46 ± 0.03
	3	1.3535	30	0.98 ± 0.02	7.91 ± 0.45	19.37 ± 0.02
	4	1.3440	16	0.91 ± 0.02	6.97 ± 0.57	19.93 ± 0.04
Control	1	1.3646	92	1.31 ± 0.07	14.23 ± 0.29	12.45 ± 0.03
	2	1.3575	35	1.08 ± 0.13	13.65 ± 0.31	17.65 ± 0.04
	3	1.3524	28	1.01 ± 0.02	6.68 ± 0.30	18.54 ± 0.06
	4	1.3425	14	0.81 ± 0.03	5.01 ± 0.21	18.15 ± 0.02

Values are the means ± 1 standard deviation (n = 2 x 3). [a] Differences within fraction between treatment were not significant.

Table 5.2 Description of colour and odour of 4 distillation fractions per treatment (ti kouka and control)

Treatment	Fraction	Colour description	Odour description
Top stem	1	Clear, colourless	Alcohol/ fruity
	2	Clear, colourless	Alcohol/tequila
	3	Clear, pale yellow	Woody
	4	Turbid light yellow	Caramel
Control	1	Clear, colourless	Alcohol/sharp
	2	Clear, colourless	Alcohol/winey
	3	Clear, colourless	Smoky
	4	Clear, pale yellow	Sweet chocolate

Table 5.1 summarizes the ethanol concentration and colour development of the four distillation Fractions per treatment. There were no significant difference between Top stem and Control treatments for all colour attributes ($p>0.05$). However, for both Top stem and Control treatments, there were significant differences between all colour attributes from Fractions 1 to 4 ($p = 0.005$).

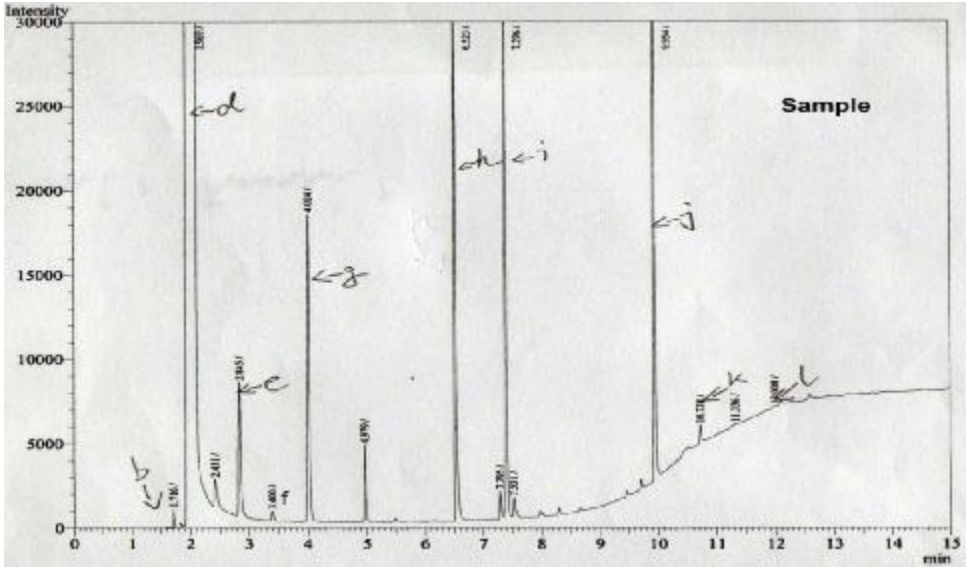
The L* value (lightness) increased gradually from fraction 1 to fraction 4, and was slightly higher in fraction 4 of Top stem than of Control. This may be due to the turbidity noted in Top stem but not Control. Table 5.2 showed colour changed from clear to pale yellow. These results suggested there were different volatile compounds among different fractions, which probably led to the distinguished flavour of each fraction.

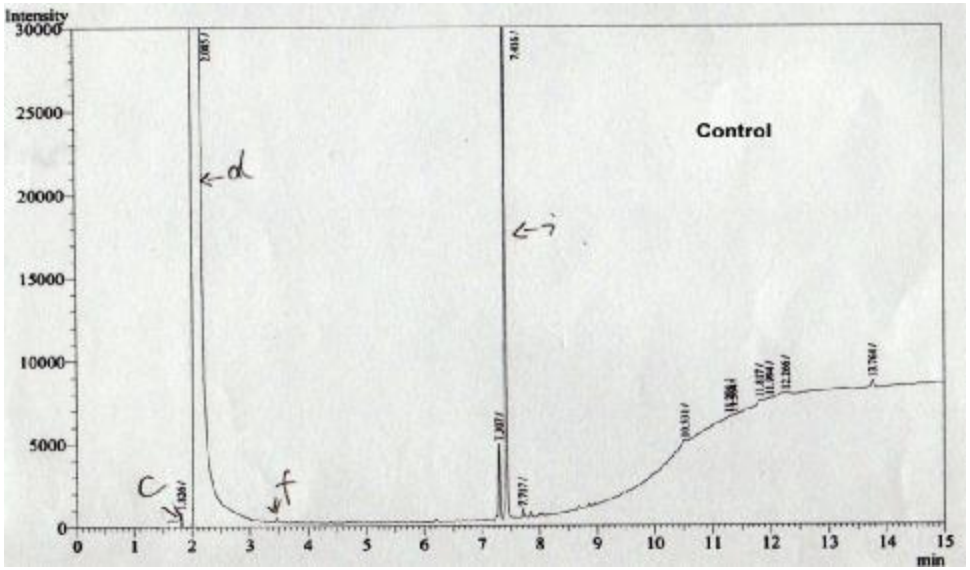
The hue angle and chroma decreased gradually from fraction 1 to fraction 4 which indicated a shift in colour. For the top stem, a* increased while b* decreased from Fraction 1 to Fraction 4. This result combined with Table 5.2 suggested the there are more Maillard reaction products being produced in the later stage of distillation. Because Fraction 1 was collected at 78.2°C which was a relatively low temperature for the formation of Maillard reaction products, low water activity resulting from the low temperature led to the dilution of the soluble reactants and hence lowered the browning rate.

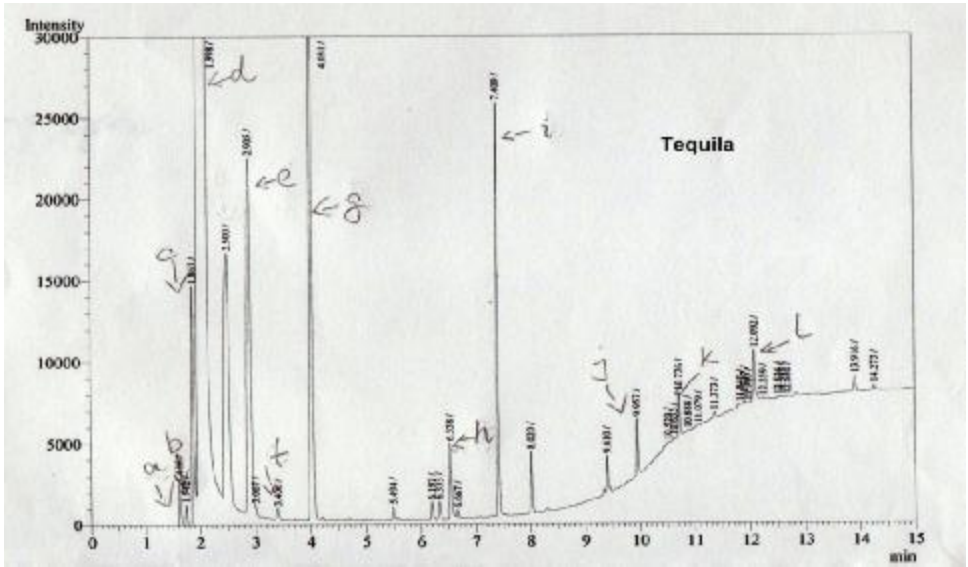
Fraction 4 of Top stem was slightly turbid. Whatever this was caused by was almost certainly derived from the stem in some way, and was insoluble in the 16 % ethanol. The compound(s) may be present in other fractions but not manifest as turbidity if the compound(s) were soluble in stronger ethanolic solutions.

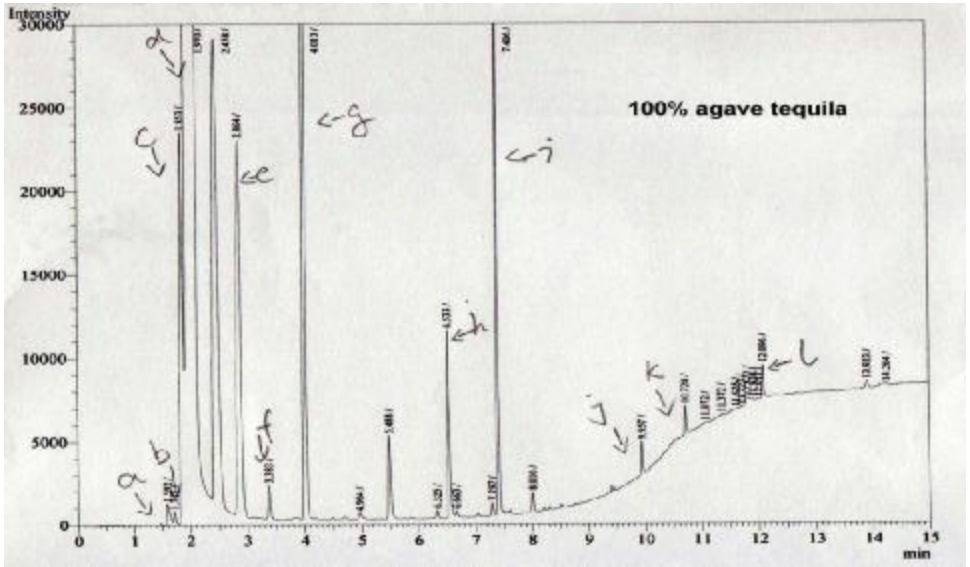
The sensory results of colour and odour as observed by the researcher and colleague are summarized in Table 5.2. For both Top stem and Control treatments, Fraction 2 and 3 usually had a pleasant aroma and appearance. Fraction 2 of the Top stem treatment had an aroma similar to that of tequila. Therefore, both Fractions 2 were first examined in detail by gas chromatography with flame ionisation detection (GC-FID) and compared with four

commercial spirits. This work, summarized in Table 5.3, is derived from a single example of a Fraction 2 spirit, examined by duplicate injections onto the ZB-Wax column. That work is described next.









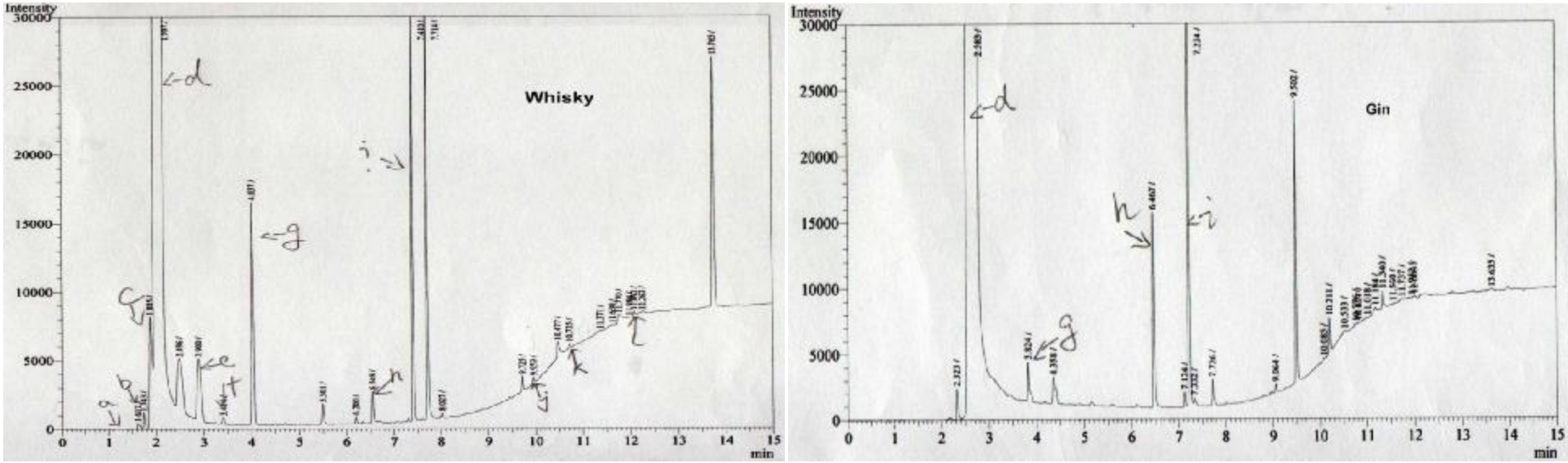


Figure 5.1 Chromatograms of Top stem (sample) (Fraction 2), Control (Fraction 2), and four commercial spirits analysed by GC-FID. a = Acetaldehyde b = Ethyl acetate, c = Methanol, d = Ethanol, e = 2-Butanol, f = 1-Propanol, g = 2-Methylpropanol, h = 3-Methyl-1-butanol, i = 1-Octanol (internal standard), j = Acetic acid, k = Furfural, l = 2-Phenylethanol

Table 5.3 Concentration of major volatile compounds in mg L^{-1} present in 6 treatments by GC-FID, odour threshold and descriptors of each compound

Retention time (min)	Compound	Spirit						Odour threshold* (mg L^{-1})	Odour description
		Top stem (Sample)	Control	Pepe Lopez silver	Jose Cuervo 100%	Gordon's Gin	Johnny W. Red Label		
1.67	Acetaldehyde	ND	ND	25 ± 1	15 ± 1	ND	9 ± 1	25[b]	Nutty
1.83	Ethyl acetate	18 ± 1	ND	23 ± 2	22 ± 1	ND	15 ± 4	12.3[b]	Fruity
1.96	Methanol	ND	13 ± 1	75 ± 6	85 ± 4	ND	55 ± 4	10	
2.02	Ethanol	29874	29871	29856	30189	30723	30117	5	Alcohol
2.93	2-Butanol	38 ± 2	7 ± 1	88 ± 3	80 ± 9	ND	55 ± 18	160[a]	Fuel
3.86	1-Propanol	8 ± 11	ND	12 ± 11	18 ± 3	ND	9 ± 1	750[a]	
4.01	2-Methyl-1-propanol	83 ± 11	ND	266 ± 15	394 ± 13	22±1	89 ± 19	65[c]	Banana
6.53	3-Methyl-1-butanol	295 ± 12	ND	35 ± 1	40 ± 6	58±3	21 ± 11	7[c]	Sweet
9.95	Acetic acid	93 ± 3	ND	25 ± 2	12 ± 1	ND	6 ± 1	300[c]	Vinegar
10.77	Furfural	1.2 ± 0.02	ND	1.1 ± 0.3	1.2 ± 0.1	ND	0.7 ± 1	0.5	Almond
12.03	2-Phenylethanol	2.8 ± 0.3	ND	1.6 ± 0	1.5 ± 0.1	ND	1.5 ± 2	14[c]	Rose

Values are means of duplicate injections of a single example of spirit ± standard deviations. ND = not detected; - no significant odour; [a] Odour hreshold in wine; [a]Meilgaard (1975); [b]Salo (1970); [c]Escudero et al. (2004).

Alcoholic fermentation generates series of products besides ethanol, including carbonyl compounds, alcohols, esters, acids and acetals, each of them influencing the quality of the finished product. The composition and concentrations of these compounds can vary widely. Some compounds appear in high concentrations (hundreds of mg L), while othersoccurs much lower concentrations, even as low as ng L^{-1} (Dragone & Oliveira 2009).

In the present study, 10 compounds (6 higher alcohols, 1 ester, 2 aldehydes, and 1 acid) were identified as major components in six spirits, with concentration values higher than 0.5 mg L^{-1} The GC-FID chromatograms of these major volatile compounds are shown in Figure 5.2 and the compounds and their respective concentrations are summarised in Table 5.3.

There were clear differences between the Control and any of the other treatments, and this is most obvious by inspection of Figure 5.1, where the vertical and horizontal axes are identically scaled for ease of comparison. The Control profile was relatively simple, as was the gin profile, possibly due to the fact the Gordon's gin is produced in New Zealand from a pure form of whey ethanol. There was a clear difference was between Top stem and Control, where the influence of the stem/incubation/reflux has resulted in a very much more complex profile than fermentation of fructose alone.

Many differences in the concentration of major compounds in each spirit were observed. The ester was ethyl acetate, which has a fruity aroma, and its presence is usually desirable in alcoholic beverages (Szambelan & Nowak 2005). However, its flavour becomes vinegary at concentrations above 150 mg L^{-1}, adding spoilage notes to a drink (Apostolopoulou et al., 2005). In the present study, the Top stem treatment and the two tequilas (Pepe Lopez, Jose Cuervo) had the highest concentration of ethyl acetate up to 23 mg L^{-1}, while the Red Label whisky had a concentration of 15 mg L^{-1}. Therefore, the ethyl acetate concentration in ti kouka spirit was at a level suitable to confer a desirable flavour. Generally, increased ethyl acetate concentrations are indicative of long term storage of the raw material and a probable acetic bacterial spoilage (Mingorance-Cazorla et al., 2003).

Acetaldehyde was also identified among the major volatile compounds in three spirits, Pepe Lopez, Jose Cuervo and Red Label with concentrations of 25 mg L^{-1}, 15 mg L^{-1} and 9 mg L^{-1}, respectively. Acetaldehyde is found in alcoholic beverages as a product of yeast fermentation, as an intermediate in the formation of higher alcohols (fusel oil), and as a result of alcohol oxidation at various stages of drink production. Acetaldehyde has been reported in tequila production where fermentation is increased by the addition of nitrogen nutrient, which leads to the rapid production of pyruvate due to the rapid glycolysis of fructose to pyruvate, which then converted to acetaldehyde by decarboxylation (Pronk et al., 1996). This could explain its relatively high concentration in Pepe Lopez. Acetaldehyde was not detected in the Top stem treatment. This result is positive because elevated acetaldehyde concentrations give a pungent irritating odour to a spirit, and can be a health hazard (Geroyiannaki et al., 2007). On the other hand, at low concentrations, the sensory descriptors for acetaldehyde range from 'nutty' to being reminiscent of overripe bruised apples.

Top stem also presented highest concentration of acetic acid. The high concentrations of acetic acid in sample treatment could be due to a major contamination of the fermentative musts with acetic acid bacteria or a poor distillation technique, and may cause an off-flavour at certain levels. However, the concentration of acetic acid in sample treatment (95 mg L^{-1}) was still in the acceptable range because of the high threshold value of acetic acid (300 mg L^{-1}) in wine, although it is realized that the Top stem spirit has a much higher ethanol concentration than does wine.

Besides acetic acid, 2-butanol and 1-propanol could also be the indicator of bacterial spoilage, because its presence could be a result of possible microbial spoilage during storage under unfavourable conditions of the raw material before distillation (Apostolopoulou et al., 2005). The concentrations of 2-butanol and 1-propanol in the Top stem treatment were similar to those in commercial tequila and whisky suggesting that fermentation and storage were properly controlled.

In alcoholic distillates of plant matter, methanol is a constituent formed from pectin by pectolytic enzymes, which hydrolyse the methoxy groups on carbon 6 of pectin galactose residues during fermentation (Soufleros et al., 2004). Ingestion or inhalation of methanol can cause blindness or death (Geroyiannaki et al., 2007), so its concentration in spirits is limited by law. The European

Union for example limits its concentration to 10,000 mg L^{-1} methanol in pure ethanol equivalents. None of the spirits approached this limit, and methanol was not determined in Top stem at all.

The concentration of methanol usually correlated with the fruit material (pulp) and the kind of raw material used for fermentation (Bauer-Christoph et al., 2003) Therefore, it should be a compound suitable for authentication of ti kouka. Methanol was not found in the sample treatment, which was benefic because of the highly toxic effect of this compound.

The higher branched alcohols such as 3-methyl-1-butanol and 2-methyl-1-propanol are typical components of spirits that are produced by catabolic conversion of the branched chain amino acids or by the anabolic formation of these amino acids from sugars (Arrizon & Gschaedler 2007). These two compounds constitute greater fraction of higher alcohols in most distilled beverages, and are considered predictors of sensory character in the distilled product (Silva et al., 1996). They have a fruity aroma that is desirable in spirits. In Top stem, the combined highest concentration of these two was a useful 380 mg L^{-1},

The 2-phenylethanol concentration in Top stem was 2.8 mg L^{-1}, which introduces a pleasant aroma to distillates. It is described as ‘rose-like’, and has a positive influence on the aroma of beverages when present at low concentration (Falqué et al., 2001). Similarly, furfural, a Maillard reaction product, has a positive influence when present at low concentrations, being reminiscent of almond. The concentration in Top stem was similar to that in the tequilas.

The preceding work has compared one distillate fraction of Top stem and Control (Fractions 2 for both) with several spirits including tequila. Fraction 2 was chosen because the Top stem fraction 2 had a tequila-like odour (Table 5.2). Fractions 1, 3 and 4 remain unexplored. The next section describes the concentration of higher alcohols, acetic acid, ethyl acetate and acetaldehyde in Fractions 1 to 4.

5.3.3 Distribution of dominant volatiles in fractions 1 to 4 of Top stem distillates

Figure 5.2 shows the differences between the fractions, and data are box plots of dominant volatiles

found in Table 5.3, for five pooled replicate distillations, where a single injection was made for each fraction of each replicate. There were significant difference for the concentration of higher alcohols, ethyl acetate, and acetaldehyde between four fractions ($p<0.001$) but not for the acetic acid ($p = 0.61$).

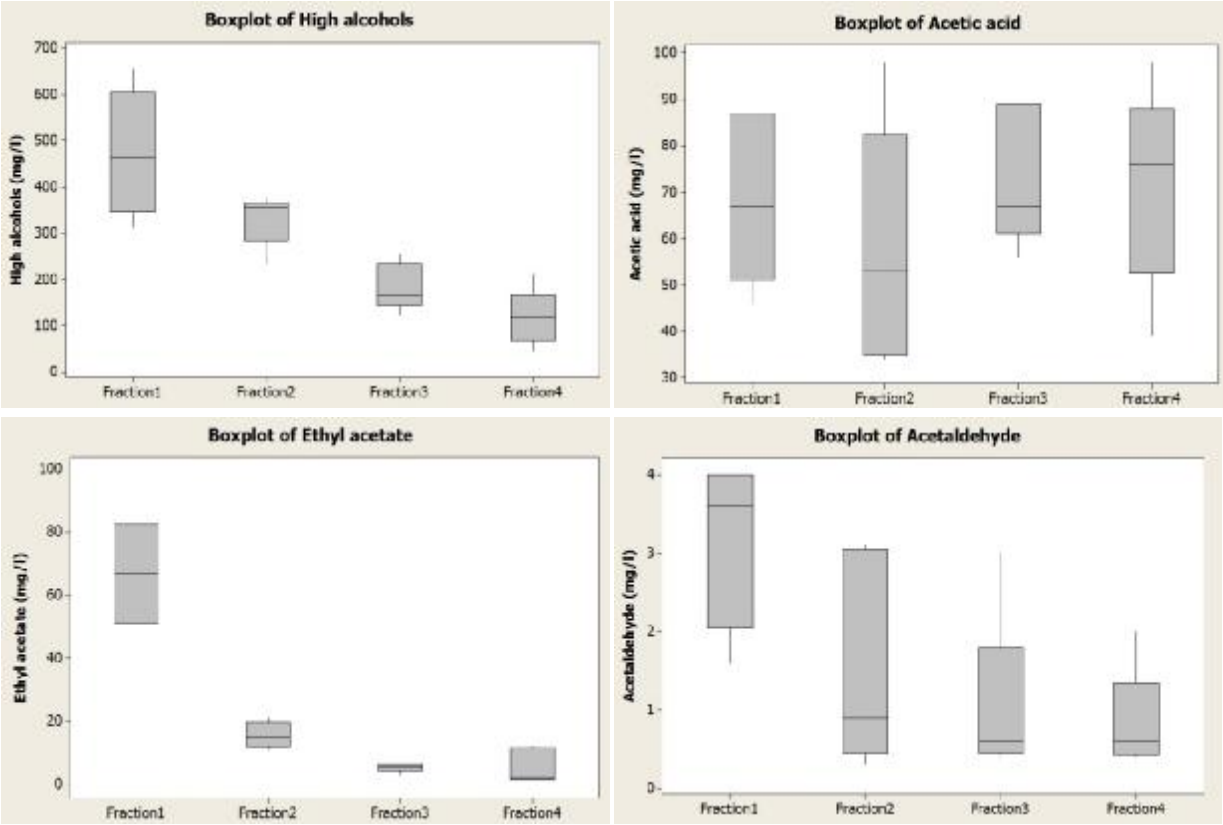


Figure 5.2 Box charts of concentration of 4 typical volatiles between 4 different Fractions (n=5)

The higher alcohols should be concentrated in certain fractions because their solubility is markedly different in high and low concentrations of ethanol in water (Walas, 1988). Thus, they may appear as head or tail products throughout the distillation process. Figure 5.2shows that in the Top stem treatment, they clearly behave as head products. The behavior of ethyl acetate and acetaldehyde were similar to that of the high alcohols as a group. They exhibited high concentrations at the beginning of distillation and then decreased towards the end of the distillation. In addition, acetic acid distilled at approximately the same rate during distillation.

Translating this data into meaning in terms of odour/flavour suggests that the identified concentrations of the four components in Fraction 2 are a necessary condition – but perhaps not

sufficient – for the generation of tequila flavour from ti kouka (Table 5.2).

5.4 Brief summary

The differences of colour and odour between different ti kouka distillation fractions were studied. The results showed that Fraction 2 had the most-tequila characteristic. Most of the major compounds found in ti kouka distillate were similar to those reported for tequila. Another important outcome showed the concentration of some major volatiles was varied in the different distillation fractions. In the next chapter, two important kinds of minor volatiles including terpenes and ethyl esters of ti kouka (Fraction 2) and other four commercial spirits would be examined by solid phase microextraction and gas chromatography-mass (SPME-GC-MS) to see the differences and similarities.

CHAPTER 6

Determination of Terpenes and Ethyl Esters in Four Spirits by Solid Phase Microextraction and Gas Chromatography-Mass Spectrometry

6.1 Introduction

During the process of spirit production, the raw material (e.g. agave or ti kouka stem) undergoes many chemical reactions which generate a very complex distillate. The primary aroma compounds come from the raw material and the secondary aroma compounds come from cooking, fermentation, and distillation process (Medina & Diaz, 2004). Thus many factors, such as maturity of the raw material, cooking conditions, fermentation conditions, distillation conditions, affect the final quality of spirit as determined by aroma and flavour on consumption. Aroma and flavour are inextricably linked and in the report that follows the term flavour is taken to include aroma.

Many different compound classes are responsible for the flavour of tequila, such as fatty acids, esters, aldehydes, terpenes, and phenols (Capella & Labastida, 2006). Many of these are volatile and contribute markedly to flavour. Rosa,2006 reported more than 175 compounds in a dichloromethane extract of tequila. Twenty five were terpenes. Although terpenes are generally defined as the minor compounds in spirits due to their low concentration, their presence is considered very important in regard to flavour because they can harmonically synergize with other minor compounds to produce the outcome (Rosa, 2006). Ethyl esters are among the most numerous of volatiles detected in the spirits such as tequila, whiskey, cognac, and rum (Vallejo B, 2004). It also has been reported ethyl esters showed significantly qualitative and quantitative differences among the different type of spirits (Vallejo B, 2004). Ethyl esters are often characterized as possessing ‘fruity’ and ‘adhesive’ flavour note.

Several different analytical methods have been developed to determine volatile compounds in spirits, all ultimately using gas chromatography. Present headspace methods to prepare chromatography material for include simultanaeous distillation and extraction (Likens and Nickerson), solvent extraction, and dynamic purge and trap methods. These methods are generally

labour intensitive, can require exhaustive concentration steps, have memory effect problems (in solvent extraction), and can require dedicated gas chromatographs equipped with headspace sampling devices. A different approach was described by Steffen and Pawliszyn (2000), which called solid-phase microextraction (SPME). This analytical technique can be applied to the detection of volatile compounds in spirit. The method is solvent free and sample handling is minimized (Kataoka et al., 2000). In SPME, analytes establish equilibrium among the sample matrix, the headspace above the sample, and a stationary phase coated on a fused silica fiber; they are then thermally desorbed from the fiber to a capillary column. The main parameters that affect the SPME process including extraction time profile, desorption time, and salt addition were optimized before the final analysis.

The objective of this chapter is to use SPME with GC-MS to characterize the main terpenes and ether ester profile in four spirits, including ti kouka, to find chemical relationships between them. Differences and similarities in chemical relationships are likely to translate to differences and similarities in flavour, although that was not determined here.

6.2 Materials and methods

6.2.1 Spirits

Ti kouka from Top stem (Fraction 2) was produced as described in Chapter 2. The reference commercial spirits were tequila (Pepe Lopez, Premium Silver), whisky (Johnnie Walker Red Label) which were purchased from Liquor Park (11 Beach Rd., Auckland). Jose Cuervo 100% Agave Gold tequila, a tequila that had been aged in oak barrels was sourced from Dr. Owen Young. 1-Octanol was chosen as the internal standard.

6.2.2 Optimization of SPME procedure

A solid-phase assembly holder and 65 mm polydimethylsiloxane/divinylbenzene (PDMS/DVB) were from sourced Phenomonex Ltd. (Auckland, N.Z.). This coating is the most popular reported in the literature for headspace analysis of foods. Successful execution of the SPME equilibrium technique requires optimization of the extraction parameters to obtain high recoveries of volatiles.

Both the temperature and time of SPME exposure to the headspace affect extraction. Higher temperatures and longer exposure times generally give better results. Operating conditions were optimized for SPME extractions at an arbitrary 60°C at 10, 20, 30, 40 and 50 min exposure. Other factors such as sampling temperature and sampling volume were kept constant at 60 °C and 20 mL.

6.2.3 Sample preparation

All the samples were diluted to 15% (v/v) alcohol with distilled water and NaCl was added to a final concentration of 30% to improve the extraction efficiency of the apolar volatiles anticipated to be of interest. For apolar volatiles an increase in the polarity of the sample matrix, shifts to equilibrium to the headspace above the sample, and in turn to the stationary phase coated on the fibre. For extraction, 20 mL of sample was spiked with 0.5 mL of 400 ppm octanol, so that the final concentration of internal standard in the sample was 10 ppm. The spiked sample was in a 50 mL glass vial fitted with a Teflon septum with a magnetic stirring bar. There was approximately 30 mL of headspace.

6.2.4 Extraction procedure

The extraction procedure was as follows. The SPME fibres were first conditioned for 5 min at 240°C. The prepared vial was vigorously stirred at a constant rate and at 60°C. The needle of the SPME device was pierced through the septum of the vial, and the plunger is depressed to expose the fiber to the headspace of the solution. After exposure for a given time, the fibre was withdrawn into the needle, and the assembly was transferred to the injection port of the GC. An equilibration study was first performed (10, 20, 30, 40 and 50 min) to determine the most suitable extraction time for spirit volatiles.

The sample extracts were analyzed by GC-MS using a Trace GC-Ultra gas chromatography (DSQ Thermoelectron Corp.) equipped with a Shimadzu QP5000 mass spectrometer. Separations were performed using a 25 m x 0.25 mm internal diameter capillary column, coated with a 0.25 μm film of ZB-Wax stationary phase (Phenomenex Ltd. Auckland, N.Z.). Splitless injections were used, aiming to detect as much of the desorbed matter as possible. The initial oven temperature was set to 80°C for 2 min, and then increased in two steps: 80 to 120°C, at 5 °C min^{-1}; 120 to 240°C at 8°C

min^{-1}, and maintaining at this temperature for 5 min, making 30 min in all. The MS ionization potential was 70 ev, the injector temperature was 240°C, the transfer line temperature was 200°C, and the scan mode was 40 to 700 m/z. The compounds were tentatively identified by comparing their mass spectra with those obtained in the National Institute of Standards and Technology (NIST) library of the MS database.

6.2.5 Statistical analysis

Principal component analysis was performed by XLSTAT (Addinsoft Ltd, U.K.).

6.3 Results and discussion

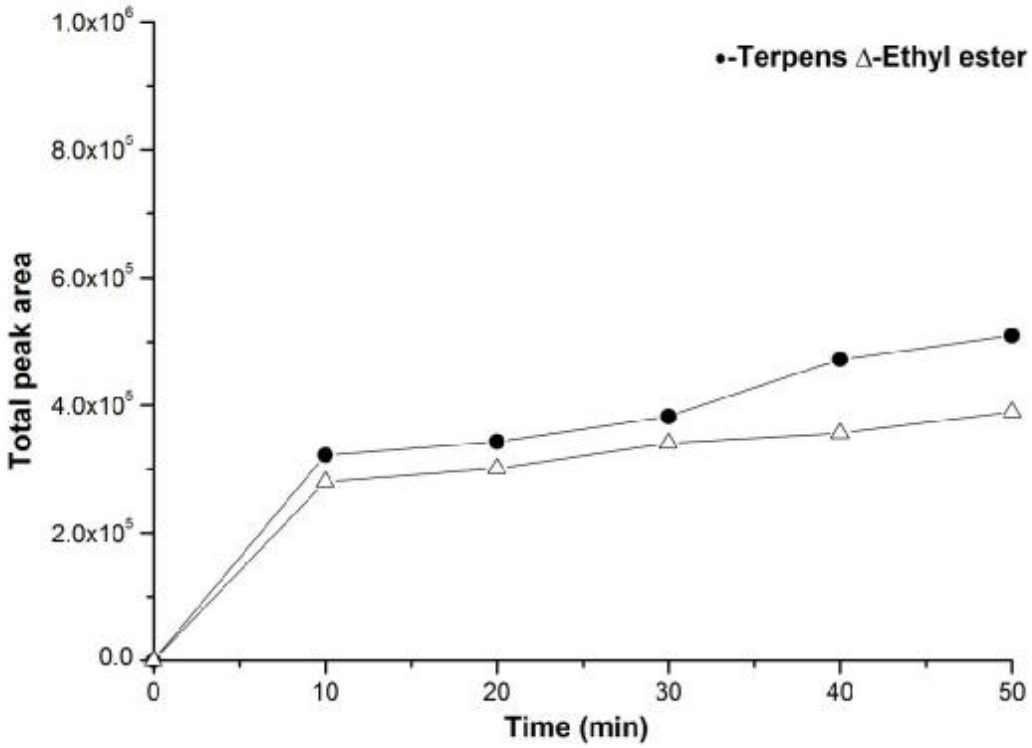
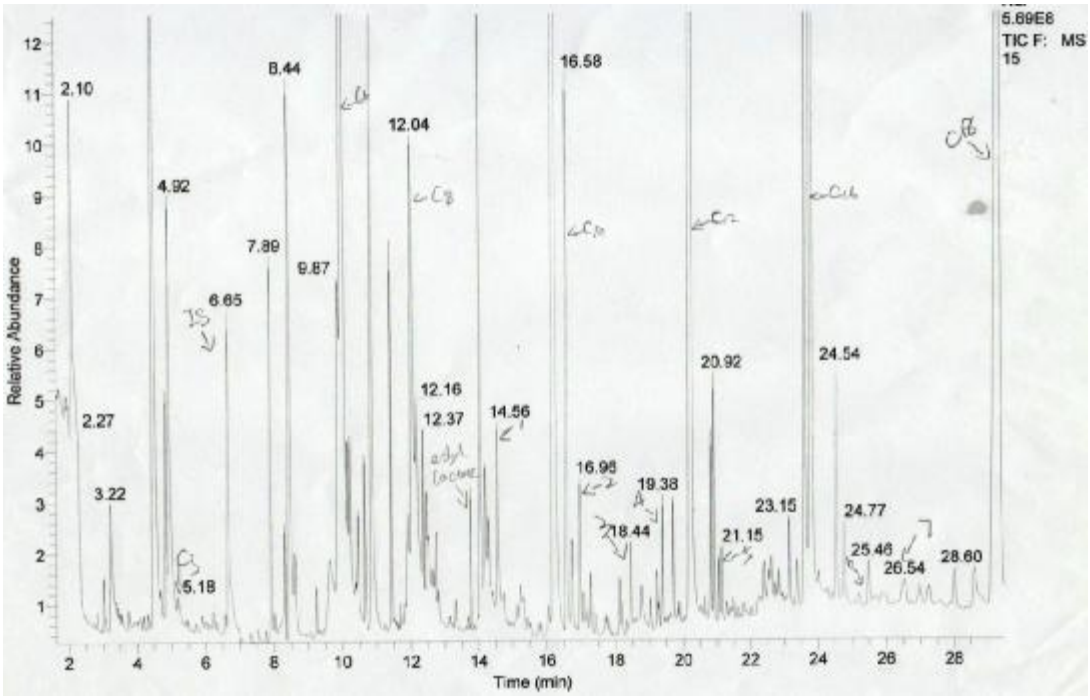


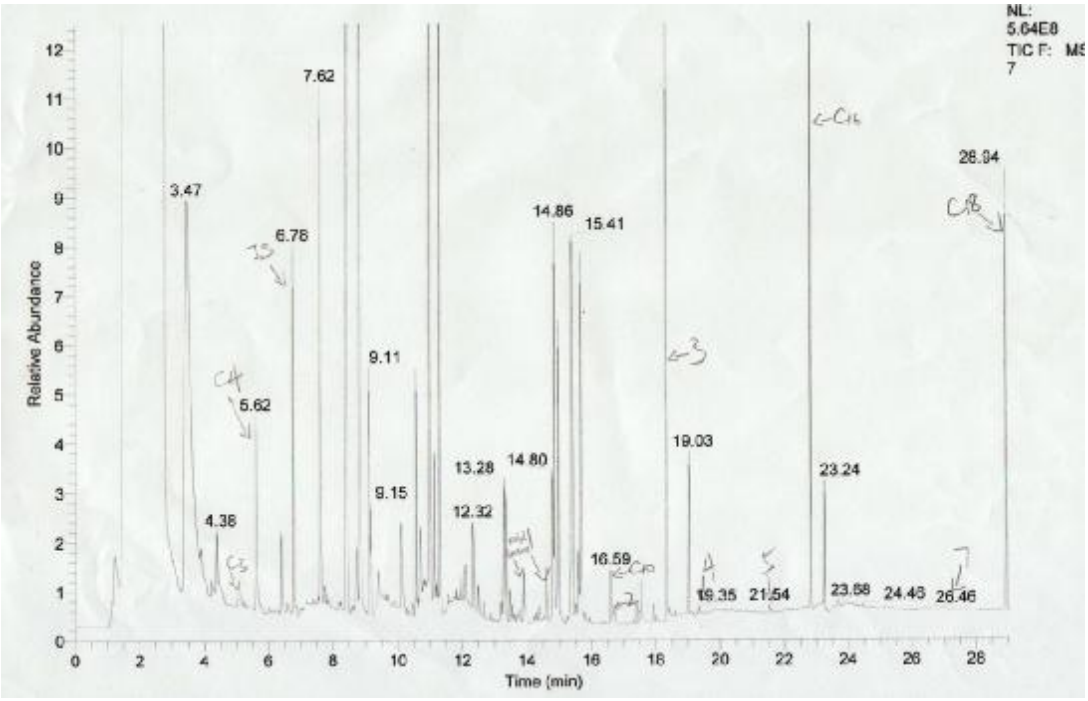
Figure 6.1 Influence of the sampling time on the extraction efficiency for terpens and ethyl ester, during dynamic SPME extraction using a 65mm PDMS/DVB fibre at 60 °C), expressed as peak area

Exploratory chromatography revealed that the profile was dominated by terpenes and ethyl esters. The total peak areas of these two classes were pooled to determine a suitable SPME exposure

period, a compromise between high peak areas obtained and a short exposure time. A graph of the total peak area against the SPME sampling period, for PDMS/DVB fibre desorption after different extraction times (0 to 50 min), for the pooled terpenes and ethyl esters is displayed in Figure 6.1. It was found that an initial rapid partitioning onto the fibre was followed by a slower uptake that did not appear to completely reach equilibrium between the fibre and the vapour phase in 50 min. An exposure time of 45 min was chosen for the subsequent experiments. Two representative chromatograms are shown in Figure 6.2 for Jose Cuervo 100% agave tequila and ti kouka from Top stem.



Jose Cuervo 100% agave tequila



Ti kouka top stem

Figure 6.2 Typical chromatograms of Jose Cuervo 100% agave tequila and ti kouka Top stem analysed by GC-MS. 1: Linalool, 2: Citronellol, 3: 2-Terpineol, 4: cis-Nerolidol, 5: (E)-Geraniol, 6: trans-Farnesol, 7: Squalene, IS: Internal Standard. C_3: Ethyl propionate C_4: Ethyl butyrate C_6: Ethyl hexanoate C_8: Ethyl octanoate C_{10}: Ethyl decanoate C_{12}: Ethyl dodecanoate C_{16}: Ethyl hexadecanoate C_{18}: Ethyl octadecanoate

Four spirits were analysed to determine qualitative compositions of ethyl esters and terpenes. Nineteen specific compounds were extracted from these four treatments using the SPME method, including 8 terpenes and 11 ethyl esters as tentatively identified by the mass spectral library (Table 6.1). For comparison, the abundance of the internal standard was used to normalise the relative abundance of compounds tentatively identified.

Several of the compounds detected appeared in all spirits. These volatile compounds were linalool, 2-terpineol, ethyl propionate, ethyl decanoate, ethyl hexadecanoate, ethyl octadecanoate, and ethyl lactate. Consistent occurrence of these compounds in all treatments suggested its importance in contributing to the fruity note of the spirits. It is well known that the mild floral and fruity aroma of spirit is due to the predominance of esters and aliphatic alcohol over other compounds. Johnny Walker Red Label whisky was notable for its relative lack of terpenes, and this is presumably a

useful distinction between whisky and tequila-like spirits.

Table 6.1 Relative abundance of ethyl esters and terpenes in four spirits

Retention time (min)	Compounds tentatively identified	Spirits			
		Johnny W. Red Label	Pepe Lopez silver	Jose Cuervo 100%	Ti kouka (Top stem)
6.70	Internal standard	1	1	1	1
14.56	Linalool	0.58	0.75	0.85	0.27
16.96	2-Terpineol	0.21	0.21	0.44	0.15
18.44	Citronellol	ND	0.38	0.73	1.52
19.38	cis-Nerolidol	ND	0.56	0.69	0.05
21.15	(E)-Geraniol	ND	ND	0.42	0.05
25.46	trans-Farnesol	ND	ND	0.39	ND
26.54	Squalene	ND	ND	0.13	0.02
23.78	Stigmasterol	ND	ND	ND	0.18
5.18	Ethyl propionate	0.23	0.11	0.21	0.23
5.62	Ethyl butyrate	0.16	0.09	ND	0.53
10.07	Ethyl hexanoate	2.34	ND	3.32	ND
12.04	Ethyl octanoate	3.11	0.98	1.76	ND
16.58	Ethyl decanoate	2.45	1.56	1.89	0.17
18.56	Ethyl undecanoate	1.43	1.21	ND	ND
20.53	Ethyl dodecanoate	4.67	2.89	4.57	ND
22.19	Ethyl tetradecanoate	5.13	1.24	ND	ND
24.05	Ethyl hexadecanoate	5.56	ND	6.32	7.56
29.08	Ethyl octadecanoate	4.56	2.02	8.14	1.23
13.87	Ethyl lactate	7.23	2.4	0.53	0.26

ND = not detected

In contrast, concentrations of ethyl esters of fatty acids were higher in the whisky (Table 6.1).

It has been reported that esters are be the result of yeast metabolism or are formed subsequently during the aging process by esterification of fatty acids in the presence of ethanol at high concentrations (Vallejo B, 2004), and occur in a wide range of spirits (Kaminski E, 1998). Ethyl hexadecanoate and octadecanoate were the most abundant ethyl esters of all tequila types (Vallejo B, 2004), but the former was not found in Pepe Lopez tequila.

Another fact was observed in Table 6.1 is that Red Label had highest concentration of ethyl lactate, which relate to the bacterial contamination of the malt by strains of *Lactobacillus spp*. and are responsible for a concurrent lactic fermentation. A possible source of these bacteria is associated to contaminated raw materials or the distillation apparatus itself (Rosa et al., 2006).

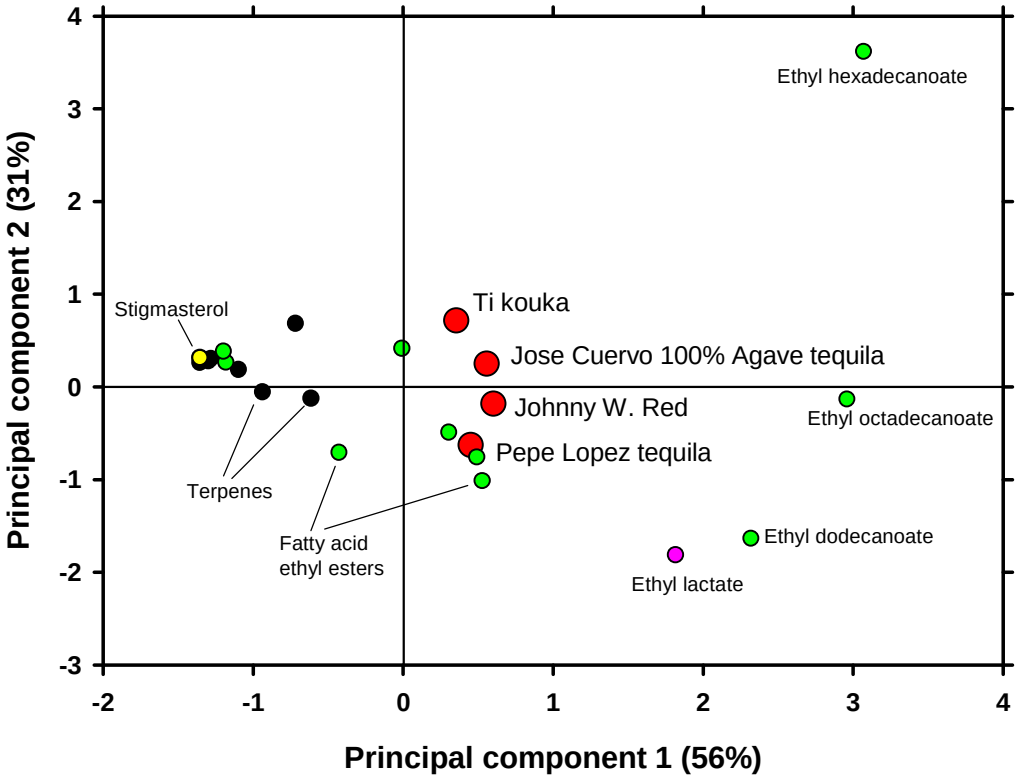


Figure 6.3 Bi-plots of principal components 1 and 2 of the volatile compounds of four treatments

A correlative principal components analysis was applied to the data in Table 6.1 as a way of visualizing the data. Ethyl hexadecanoate, ethyl octadecanoate, and ethyl dodecanoate were strongly loaded on principal component 1 (56% of information), but were variably positive and negative in principal component 2 (31%). Thus, ethyl hexadecanoate, also known as ethyl palmitate, was characterizing for ti kouka and Jose Cuervo 100% *Agave* tequila. Ethyl dodecanoate, also known as ethyl laurate, was more characterizing of Pepe Lopez tequila. The terpenes as a group were negative on principal component 1, but this does not mean that they were inversely related to the fatty acid ethyl esters in terms of abundance. Thus Table 6.1 shows that

the spirit with the greatest abundance of all volatiles was Jose Cuervo. The terpenes tended to be positive on principal component 2 and thus were slightly more characterizing of ti kouka and Jose Cuervo than of the other two spirits. Stigmasterol occurred only in ti kouka and was positive on principal component 2 as expected. Ethyl lactate was most dominant in Johnny Walker Red Label whiskey and in Pepe Lopez tequila and was located in the lower right quadrant.

6.4 Brief summary

Solid-phase microextraction sampling followed by GC–MS analysis provides a clean and selective way to characterize the terpenes and ethyl esters in spirits. Ethy esters predominated in aged-samples analysed. Although ti kouka contain less ethyl ester, it has similar terpenes with other two tequila samples.

CHAPTER 7

Overall Conclusion

The initial stage of the study focused on the development of ti kouka spirit. Its production process involves five major stages: stem cutting, cooking to convert polysaccharides (insulin) to a mixture of fructose (mainly) and fructose, filtration, fermentation with added fructose, and finally distillation.

Next, batch fermentations were performed with six treatments at an added fructose concentration of 150 g L^{-1} to evaluate the technological potential of *Cordyline australis* stem as a fermentable substrate, but realizing that source of carbohydrate was mainly added fructose. The six treatments were combinations of stem position, yeast type and controls. In terms of yeast growth and biomass production all six treatments had similar outcomes. However, the treatment with immature stem matter (called Top stem) and yeast wine was marginally better than the others and was chosen for further work.

A model system was developed to simulate a particular processing step in the production of tequila. This is heating of agave stem, with its endogenous fructose, which generates flavour-active Maillard reaction products some of which will be recovered in distillation. The simulation involved reflux of Top stem with endogenous and added fructose for three hours at different temperatures. It was also done at three pH values, something not done in tequila production. Many volatiles, mainly Maillard compounds, were produced, many of which may have a significant impact on the overall flavor of final spirit. Differences in chromatographic profiles by GC-MS showed that of the six treatments it was pH 9 at 100°C that generated the most Maillard reaction products in the Top stem/fructose system.

Finally, ti kouka spirit and four other spirits including Pepe Lopez silver tequila, Jose Cuervo 100% agave tequila, Gordon's Gin and Johnny Walker Red Label whiskey were analysed by gas chromatography with flame ionization detector, and by headspace solid-phase microextraction- gas

chromatography-mass spectrometry. Ten major volatile compounds (> 0.5 mg L^{-1}) were quantified. Most of the major compounds found in ti kouka distillate were similar to those reported for tequila. In addition, two important kinds of minor volatiles including terpenes and ethyl esters were examined by the principal component analysis technique to differentiate the four spirit treatments according to their unique flavour compounds. The first principal components explained 82% of the total variance.

The results from the analysis of GC-FID and SPME-GC-MS analysis point to the commercial opportunities for ti kouka because it has comparable properties with the commercial spirits, especially, tequila. Further research should focus on the sensory quality of ti kouka spirit so that the risk in launching products in the market can be minimised.

Finally, the volumes of spirit produced here are minute, and unsuitable for commercial assessment. Mr Andrew Bonner who is the owner of New Zealand wine brand *Toi Toi* and has extensive liquor industry contacts is interested in the industrial production of ti kouka. He has access to land where ti kouka thrives, and with AUT Enterprises Limited has provides funds to produce 10 L of ti kouka spirit at 40% (v/v) to be assessed by commercial liquor interests. This work begins in October 2011 aimed at producing a New Zealand unique spirit.

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Appendix I

Refractive indices of ethanol solutions at 20 °C

concentration %v/v	Refractive indices	concentration % v/v	Refractive indices
0	1.333	52	1.3621
2	1.3342	54	1.3626
4	1.3354	56	1.363
6	1.3367	58	1.3634
8	1.3381	60	1.3638
10	1.3395	62	1.3641
12	1.341	64	1.3644
14	1.3425	66	1.3647
16	1.344	68	1.365
18	1.3455	70	1.3652
20	1.3469	72	1.3654
22	1.3484	74	1.3655
24	1.3498	76	1.3657
26	1.3511	78	1.3657
28	1.3524	80	1.3658
30	1.3535	82	1.3657
32	1.3546	84	1.3656
34	1.3557	86	1.3655
36	1.3566	88	1.3653
38	1.3575	90	1.365
40	1.3583	92	1.3646
42	1.359	94	1.3642
44	1.3598	96	1.3636
46	1.3604	98	1.363
48	1.361	100	1.3614
50	1.3616		