The production of a potable alcoholic spirit from New Zealand dairy proteins, lactose and whey ethanol

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Attestation of Authorship

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

Signed……

Date……

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Abstract

With the alcoholic beverage market growing globally, there is always the potential for new and innovative products to gain market share. One of the ways in which alcoholic beverages can be marketed is the geographical exclusivity where a drink, or more generally a food, comes from one location. The aim of the research was to produce an alcoholic spirit derived solely from milk components on the basis that the dairy industry, although not unique to New Zealand, is nonetheless strongly identified with New Zealand. The original aim of the research was to use caseins, whey and lactose to generate flavours through the Maillard reaction that are suitable for flavouring whey ethanol. However, products derived from the reflux of dairy components were all opaque, difficult to work with, and regarded as unattractive for flavouring an alcoholic spirit. Hence amino acids instead of dairy proteins were reacted with lactose to produce suitable Maillard reaction products. Seventeen amino acids screened produced a wide range of colours from colourless to dark brown, and aromas ranging from non-existent to sweet and flowery. Four amino acids that were suitable for preparation of Maillard reaction products that could be used in spirits included alanine, leucine, phenylalanine and valine. A preliminary trial showed that Maillard reaction products when introduced into spirits increased in colour intensity with time. Spirits incorporated with Maillard reaction products of each amino acid were then stored for 0, 5, 10, 15 and 30 weeks. The spirits were assessed by 60 consumers for intensity of aroma with time and overall liking of aroma. Spirits prepared using flavours derived from the Maillard reaction of lactose and leucine, and phenylalanine were liked significantly more than other amino acids.

Chapter 1

Introduction

1.1 History of alcoholic beverages

In contemporary cultures, consumption of alcohol centers on socialising as alcohol increases sociability by increasing a person's self-confidence, and can act as a relaxant. People also consume alcohol because they like the flavour, for example the malt and bitterness of beer, and the fruitiness and complexity of wine (Durkan, 1997). Consumption of alcoholic beverages dates back to thousands of years. The remains of the world's oldest brewery have been discovered in Ancient Egypt, possibly dating back to 3400 BC. In this society, beer was considered a drink for the workers, while wine that was predominantly drunk by the elite was associated with power and wealth (Gately, 2008). Wine making may have been considered as a means of preserving grapes in Iran between 5000-5400 BC. There is some evidence that alcohol preparation began approximately 8000BC after the establishment of agriculture (Gately, 2008).

1.2 Market growth in sales of alcoholic beverages

Figure 1 shows the global regional prospects for alcoholic drinks from 2009-2014. New Zealand was predicted to have low growth, while countries such as India and China, which were predicted to have high growth, were known as being emerging markets.

Global Regional Prospects

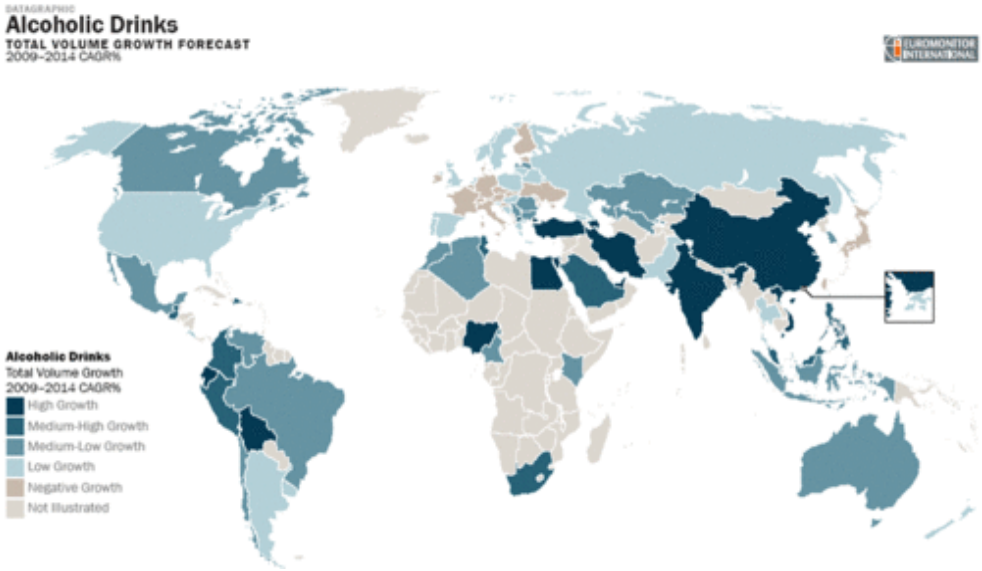


Figure 1. Global regional prospected growth in alcoholic drinks from 2009-2014. Retrieved from http://www.euromonitor.com/Copyright 2010 Euromonitor International from National statistics and trade resources

One approach used in marketing alcoholic drinks is through 'geographical exclusivity'. Good examples include Scotch whisky, which by definition comes only from Scotland. Similarly, tequila comes only from Mexico. Some alcoholic products from the emerging Indian and Chinese markets are even available in New Zealand. For example, Kingfisher beer, which comes from India and often sold at Indian restaurants, is marketed as possessing geographical exclusivity. In New Zealand, Marlborough sauvignon blanc and manuka honey have achieved a geographical status.

Globally the market for spirits is growing significantly (Figure 2) however total volume percent growth has been experiencing a steady decline from mid 2007.

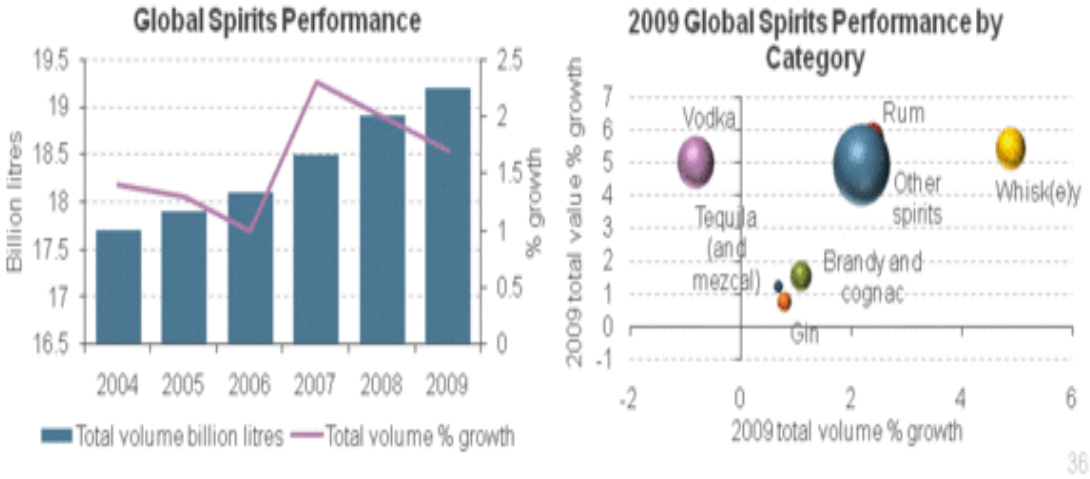


Figure 2. Global spirits performance from 2004 to 2009 and 2009 global spirits performance by category. Retrieved from http://www.euromonitor.com/ Copyright 2010 by Euromonitor International from national statistics and trade sources.

Performance by category (Figure 2) showed that whisky experienced the most growth of almost 5%, while vodka actually declined in growth. This indicated that a significant proportion of growth in the global spirits market could be due to the growth in volume of whisky due to it being an entry-level product for the emerging India and China markets.

There is also total volume growth in the 'other spirits' category. This category would include spirits such as liqueurs which are often flavoured with fruits, herbs, or spices to give them a distinctive and unique flavour. Growth in this category could indicate that consumers are becoming increasingly keen to try other non-traditional spirits. Many spirits fall under this category and this could be the other reason for the growth in this category. This indicates that there is a good potential for a uniquely flavoured spirit to be successful in the alcoholic spirit market.

1.3 Flavour development in alcoholic beverages

Time and money are important factors in the development of alcoholic drinks. To produce an alcoholic drink with strong flavor via traditional methods, generally requires time, and a major capital outlay. The traditional method of producing alcoholic spirits involves processes like fermentation, distillation, maturation, and in some cases blending. Grains, fruits and vegetables are the ingredients required to undergo

fermentation to produce alcohol. The alcohol produced depends on the ingredient used. For example, grains are used to produce beer and whisky; grapes are used to produce wine and cognac, while blue agave can be used to produce tequila. The ingredients described can also produce compounded flavoured spirits. For example, various fruit, grain and grapes can be used to produce flavoured liqueurs.

Distillation involves the extraction of alcohol from the fermented products and gives alcohol its distinctive flavour. Maturation is the process where the alcohol is stored in vats for a period of time. The material of the vat imparts flavour on the alcohol that influences the flavour of the final alcohol product. Much of the flavour developed during these processes can be attributed to the Maillard reaction.

1.3.1 The Maillard reaction

The Maillard reaction is a nonenzymic browning process and comprises a series of complex interactions that occur between amino acids and carbohydrates that ultimately generate flavour and colour compounds (Hodge 1953; O' Brien 1998; Nursten 2005). It occurs spontaneously in foods and drinks where carbohydrates and amino acids are both present, and is responsible for browning as well as the production of flavour and volatile compounds. There are three main stages to the Maillard reaction, as published by Hodge (1953) in the first major review on the Maillard Reaction.

The first stage occurs when carbohydrate-amine condensation produces N-substituted glycosamine, and rearrangement of Amadori products. The products of these reactions are colourless. During the second stage, sugar dehydration and/or fragmentation occur. These products can be colourless or yellow. The final stage involves aldol condensation, aldehyde-amine concentration, and the formation of heterocyclic nitrogen compounds. Products of the final stage are highly coloured melanoidins (Nursten, 2005; O'Brien, 1998).

1.3.2 Colour and flavour development during the Maillard reaction

Numerous studies on the Maillard reaction have helped us understand factors that influence the reaction, and more about its intermediates and products. The information gained has vast implications in food science and technology. In a review, Oliver et. al. (2006) proposed the creation of novel functional proteins via the Maillard reaction that can be used to develop functional and novel food ingredients by a feasible preparation method, and the application of optimal processing conditions.

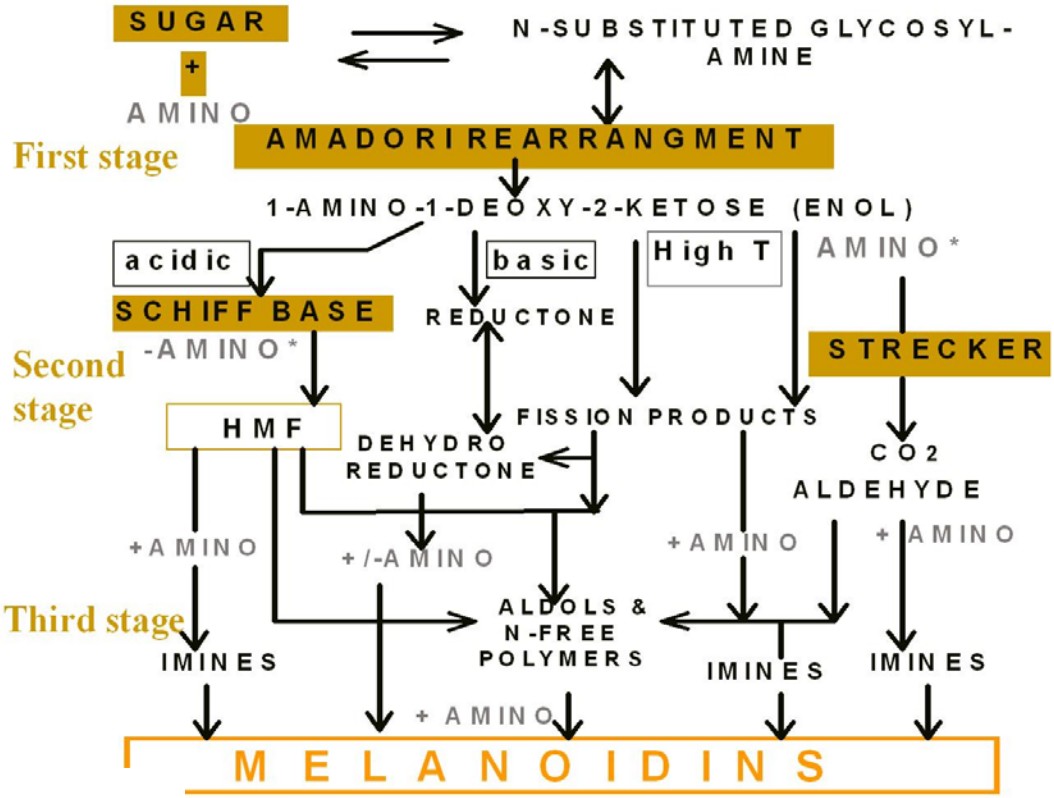


Figure 3. Scheme of the Maillard reaction . Retrieved from http://cdavies.wordpress.com/2007/02/06/the-hodge-scheme/

A number of studies have been carried out on the Maillard reaction to determine how the reactants influence the products formed. Mastrocola (2000) observed the effects of storage time on the Maillard reaction, and Moreno (2003) observed the effects of reactants, as well as the effect pressure had on the Maillard reaction and products. As the Maillard reaction occurs, the number of primary reactants decreases. Ketose sugars react better then aldose sugars (Brands & van Boekel, 2001). However, Yeboah et al. (1999) found that glucose (an aldose) had a faster initial utilisation of amino groups than fructose (a ketose). The proposed reason for this was the higher level of electrophilicity of the aldehyde group of ketose in comparison to the keto group of fructose. It was concluded that the carbonyl groups electrophilicity was important in determining the rate of the reaction. The amino acids which reacted best with carbohydrates to produce Maillard reaction products are lysine, tryptophan, histidine and arginine (Oliver, Melton, & Stanley, 2006). Sulphur-containing amino acids tend to generate ‘meaty’ flavours and aromas which may not be a desirable flavour in an alcoholic spirit and their Maillard reaction products are known to be quite significant in the generation of flavours in cooked meat (Rowe, 2005).

Many researchers have also studied conditions that affect the Maillard reaction. Oxygen acts as a catalyst for the Maillard reaction in the initial stages, with D-fructose having a higher sensitivity to the presence or absence of oxygen in all stages of the reaction (Yeboah, Alli, & Yaylayan, 1999). The formation and degradation of Amadori rearrangement products increases in the presence of a high pressure. This in turn leads to an increase in intermediate and advanced reaction products (Monero, Molina, Olando, & Lopez-Fandino, 2003). The high pressure acted as a catalyst, speeding up the formation and subsequent degradation of Amadori rearrangement products. Breakdown of peptides in solutions by buffer salts, catalyse the breakdown of peptides in solution, increasing the amino acids availability to react (Bell, 1997). It has been known for some time that the formation of high molecular weight coloured melanoidins via the Maillard reaction favour a low water activity environment (Belitz, Grosch, & Schieberle, 2009). However it is important that the reactants have analogous structures (Knerr, Lerche, Pischetsrieder, & Severin, 2001). Shen & Wu (2004) observed the Maillard reaction in ethanolic solution by measuring hydroxymethylfurfural (HMF) concentration. Heating equimolar mixtures of glucose and glycine in buffered pH 4.3 ethanolic solutions produced more HMF then heating glucose in ethanolic solutions alone. This suggests that reduced water activity alone is not the sole reason for acceleration of the Maillard reaction rate. The Maillard reaction favours high pH and supports the fact that Shen & Wu (2004) found an increased concentration of HMF in a pH 4.3 buffered ethanolic solution. The findings of the previously mentioned studies are of significant relevance to the proposed research as alcohol (ethanol) has a low water activity. Oliver et. al. (2006) found the optimum water activity for Maillard reaction to be between 0.5-0.8. Shen & Wu (2004) found that water activity above 0.81, or an ethanol concentration below 30% increased the rate of the Maillard reaction.

The Maillard reaction also produces compounds which have antioxidant activity which is why moderate alcohol consumption is considered to have health benefits. There is a correlation between the colour and antioxidant capacity (Mastrocola & Munari, 2000). Increased browning causes an increase in antioxidant activity. Goldberg and coworkers conducted research on a number of distilled spirits based 1999, and confirmed the presence of antioxidants in whisky, brandy and liqueurs. Phenol and furan concentrations were found to be parallel to antioxidant capacity.

The Maillard reaction is very important in the production of flavour in a number of types of alcohol including beer, wine and spirits. Furfuryl ethyl ether is responsible for a solvent-like stale flavour in beer (Vanderhaegen B. , Neven, Verstepen, Delvaux, Verachtert, & Derdelinckx, 2004). Vanderhaegen B. , Neven, Stefan, Verstrepen, Verachtert, & Derdelinckx, (2003) found the greatest increase in Maillard reaction products in beer after 6 months of storage at various temperatures. Beer samples stored at the highest temperature of 40°C showed the greatest increase in Maillard reaction products. The increase in Maillard reaction products correlated with the sensory panels' detection of increased solvent flavour, as well as increased sweet, and port flavours. The results from these studies confirm that an increase in Maillard reaction products in alcohol correlates to more intense flavour.

During the production of Tequila, the stems of the *Agave tequilana* plant are cooked for 32 hours at approximately 100 °C. The cooking process causes the hydrolysis of inulin present in the stem of the Agave tequilana. (Mancilla-Margalli & Lopez, 2002). Many Maillard compounds were identified in the exudates by Mancilla-Margalli & Lopez (2002) including furans, pyrans, aldehydes, and nitrogen and sulfur compounds. The most abundant Maillard compounds were methyl-2-furoate, 2,3-dihydroxy-3,5-dihydro-6-methyl-4(H)-pyran-4-one, and 5-(hydroxymethyl) furfural. Mancilla-Margalli & Lopez (2002) have demonstrated how the plant used for alcohol production can affect the development of flavour via the effects of the Maillard reaction in the final alcoholic spirit.

Phenols are commonly found in spirits that are aged during production and maturation in wood (Goldberg, Hoffman, Yang, & Soleas, 1999). The type of wood used can have a significant effect on the flavour of the spirit. Physical properties of the wood being used during maturation are important in flavour development. Tree age and growth rate- growth rate affects the grain of the wood. The resulting grain can either be, fine or course, and porosity can be affected as well. These factors can have an effect on how the spirit (or wine) undergoes oxidation which has an effect on the flavour of the spirit (Goldberg, Hoffman, Yang, & Soleas, 1999).

Wood preparation before it is used for maturation can also have a significant effect on flavour of the spirit. Singleton (1995) cited a study which showed that charring of wood resulted in a very low aromatic aldehyde content when compared to wood that had been toasted at 150°C. In that study, wood was toasted at 100°C, 150°C, and

200°C. The highest aromatic aldehyde content was that of the spirit which was allowed to mature in wood that had been toasted at 150°C.

Ethanol concentration of the spirit itself has an effect on the flavour of the product. Singleton (1995) reported results from a study published in 1959 that found that whiskey aged eight years in American oak was judged as being normally flavoured at 59% alcohol, less mature at 63% ethanol, and was judged as being weaker with a ‘spicy green oak’ taste at 77% alcohol. Details of the sensory panel and how this study was conducted could not be found because this study was conducted in 1959, therefore the reliability and validity of these results is questionable.

It is known that Maillard reactions can be produced in a relatively short time compared to traditional methods of producing flavour compounds. Furthermore the reactants required are of low cost, and are readily available in New Zealand. Research in the production of Maillard reaction products to produce food flavourings could have great potential in food technology as the reactants involved are already present in food, requiring no additional chemicals, and are therefore products that be considered safe for human consumption. Consumers today are more aware of the chemical processes involved in food processing. Hence the ability to generate natural flavour compounds for use in food would be beneficial to the consumers as well as food technologists.

1.4 The use of dairy components to create a potable spirit

Important factors affecting the Maillard reaction are the concentration of reactants, reactant type, pH, time, and temperature (O'Brien, 1998). Water activity also has an effect on the Maillard reaction (Belitz, Grosch, & Schieberle, 2009) and numerous studies have been conducted on how changes in reactants, pH, time, temperature and water activity affect the Maillard reaction for reasons other than the development of flavour and colour compounds and include the development of novel functional Maillard reaction products with nutritional purpose (Oliver, Melton, & Stanley, 2006). By adjusting the above factors, (pH, time, temperature and water activity), there can be some control over the colour and flavour products formed. The intention for the proposed research is to alter the concentration of reactants, pH of the conditions, temperature and time of the Maillard reaction in order to produce desirable flavour and colour compounds for use in an alcoholic spirit.

As mentioned previously, a marketing approach used by some manufacturers involved the claim of 'geographical exclusivity' of their products. Whilst dairy ingredients are produced worldwide, the dairy industry in New Zealand has an international status. This can provide opportunities to develop a potable spirit based solely on New Zealand dairy ingredients could contend for a geographical exclusivity status. The New Zealand dairy industry is a major low cost producer of potable alcohol at two sites in New Zealand (Edgecumbe and Reporoa). Lactose in whey is converted to ethanol by yeast capable of hydrolysing lactose to galactose and glucose. Both sugars are then fermented to ethanol and carbon dioxide. The first ingredient that can form the basis of the new product is whey alcohol and the second is the dairy carbohydrate, lactose. Lactose is a reducing sugar by virtue of the free anomeric carbon atom of the glucose moiety. The lactose disaccharide consists of glucose and galactose linked via a $\beta(1\rightarrow4)$ (Coultate, 2002) glycosidic linkage and is quite stable as shown in Figure 4 below.

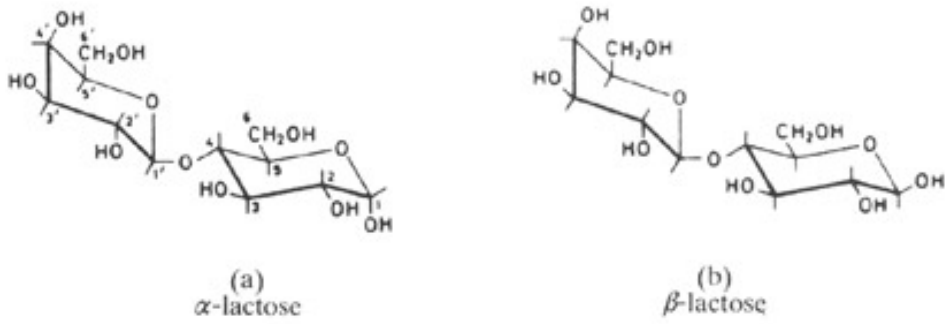


Figure 4. Fischer projections of the Alpha (a) and beta (b) structures of lactose. Retrieved from Dairy chemistry and biochemistry, Fox & McSweeney, 1998 P 24.

Lactose undergoes hydrolysis to glucose and galactose in the presence of the enzyme lactase, which is secreted by the mucosal cells in the intestine to aid the digestion of lactose in humans (Coultate, 2002). Galactose and glucose are both capable in participating in Maillard reactions.

When heat is applied to carbohydrates, caramelisation occurs, which is a dehydration reaction involving the development of characteristic sweet volatile chemicals and brown coloured products (Belitz, Grosch, & Schieberle, 2009). Under strongly acidic conditions, lactose is degraded to strongly acidic products and monosaccharides. However under alkaline conditions and slightly raised temperatures, lactose undergoes the Lobry de Bruyn-Alberda van Ekenstein rearrangement of aldoses to ketoses (Fox & McSweeney, 1998). A number of aroma compounds are also formed via the cleavage and fragmentation of 1,2-and 2,3-enediols. Examples of these aroma compounds are saccharinic acid, lactic acid, 2,4 dihydrohy butyric acid, ethyl alcohol, aromatic compounds, benzene, malitol, catechol and benzaldehyde (Cui, 2005).

Caramelisation is also favoured at low water activity, as does the Maillard reaction (Rowe, 2005). Hence the application of heat and low water activity should ultimately favour the formation of volatile aromatic products.

Heating of lactose causes hydrolysis of the disaccharide to galactose and glucose, which will then form anhydrides that dehydrate to form 5-hydroxymethylfurfural (HMF). The 5-HMF then gives rise to furans, aldehydes and ketones that also participate in Strecker degradation to form aliphatic and aromatic hydrocarbons as shown in Figures 5 and 6.(Fox & McSweeney, 1998).

The presence of various components in whole milk act as catalyts, causing lactose to undergo isomerisation to lactulose. The glucose moiety then undergoes further

isomerisation to mannose, and then epilactose (Walstra, Wouters, & Geurts, 2006). It is difficult to predict how lactose will react in the Maillard reaction because of the catalyst effect that various milk components may have.

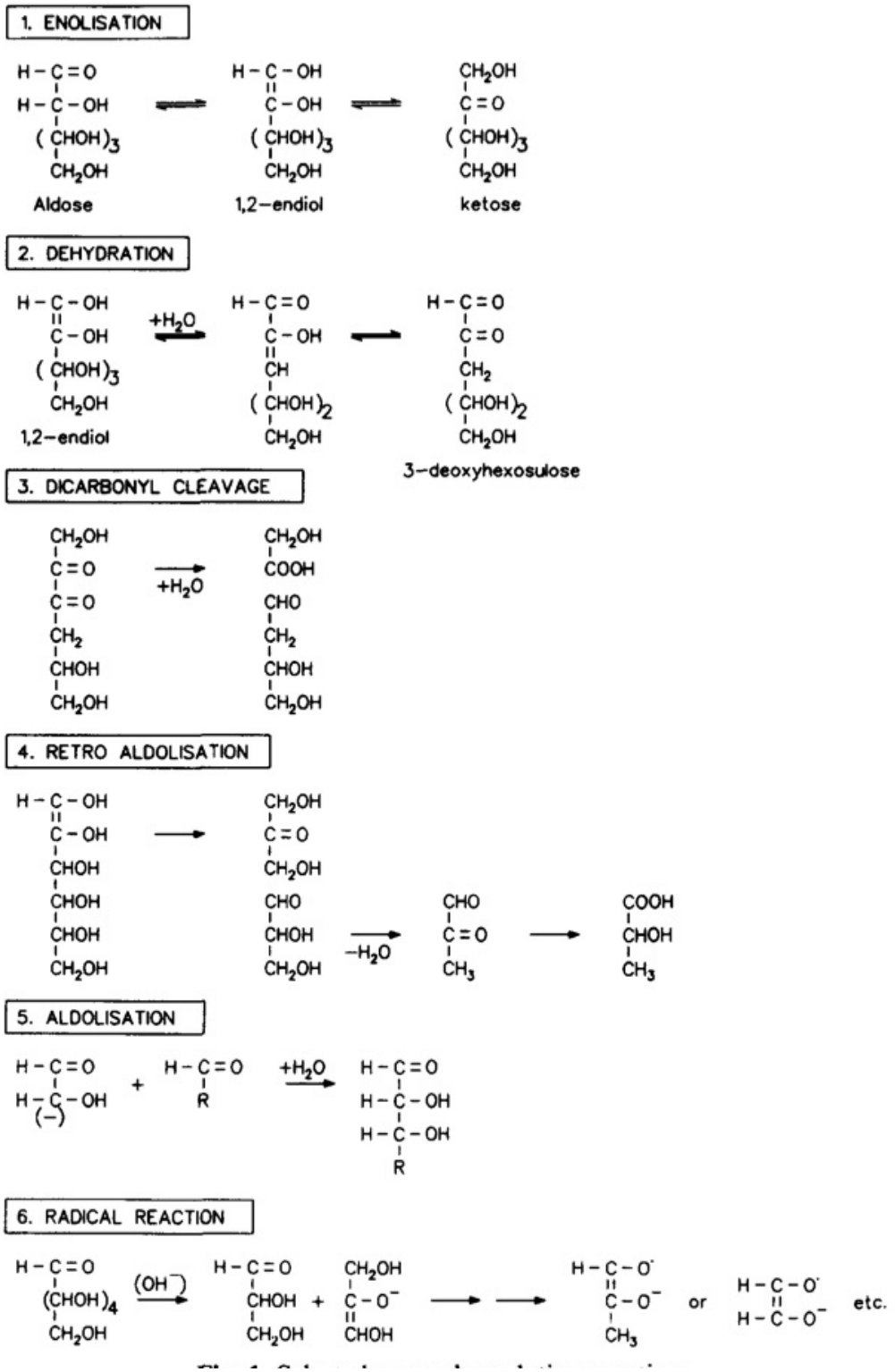


Figure 5. Selected degradation reactions. Retrieved from "Caramelisation in food and beverages," by Lothar W. Kroh, 1994, *Food chemistry*, 51, p373-379.

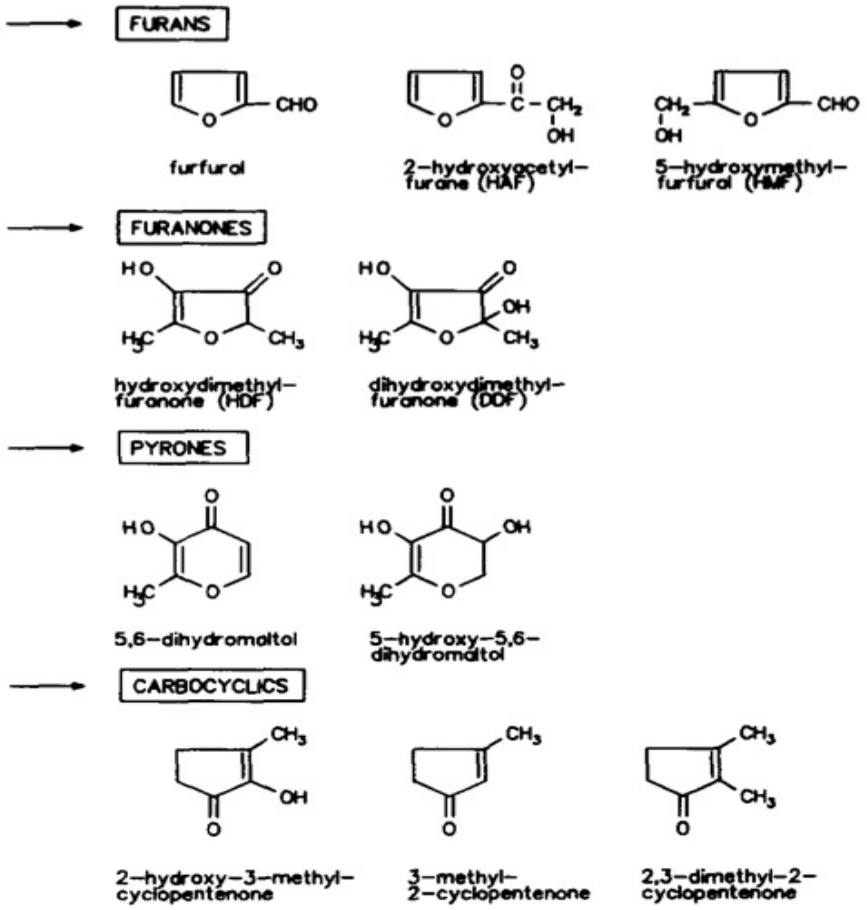


Figure 6. Aromatics produced via caramelisation. Retrieved from "Caramelisation in food and beverages," by Lothar W. Kroh, 1994, *Food chemistry*, 51, p373-379.

The main elements of milk are fat globules, lipoprotein particles, casein micelles, and globular proteins including whey, lactose, and various minerals, salts, vitamins and of course water. Casein includes α_{s1}-casein, α_{s2} casein, κ-casein, β-casein, γ_1-casein, γ_2-casein and γ_3 –casein. The whey proteins include β lactoglobulin, α-lactalbumin, serum albumin and immunoglobulin (Belitz, Grosch, & Schieberle, 2009; Fox & McSweeney, 1998).

It can be hypothesized that the addition of dairy proteins could promote the formation of Maillard reaction products. The other reactant class required is amino acids, initially ploymerised in dairy proteins like casein, whey protein isolate and other dairy fractions. If hydrolysed, these proteins yield amino acids, all of which can

participate in Maillard reaction. Table 1 shows the protein concentrations of each amino acid per 100 grams of protein in whey and casein.

Table 1. Amino acid composition (g AA/100 g protein) of total protein, casein and whey of bovine milk.

Amino acid	Total protein	Casein	Whey
Alanine	3.7	3.1	5.5
Arginine	3.6	4.1	3.3
Aspartic Acid	8.2	7.0	11.0
Cystine	0.8	0.3	3.0
Glutamic Acid	22.8	23.4	15.5
Glycine	2.2	2.1	3.5
Histidine	2.8	3.0	2.4
Isoleucine	6.2	5.7	7.0
Leucine	10.4	10.5	11.8
Lysine	8.3	8.2	9.6
Methionine	2.9	3.0	2.4
Phenylalanine	5.3	5.1	4.2
Proline	10.2	12.0	4.4
Serine	5.8	5.5	5.5
Threonine	4.8	4.4	8.5
Tryptophan	1.5	1.5	2.1
Tyrosine	5.4	6.1	4.2
Valine	6.8	7.0	7.5

The amino acid composition of casein and whey are both quite different. Casein contains a lot more glutamic acid and proline than whey. Table 2 shows that casein makes up approximately 80% of the total milk proteins. Hence the amino acid composition of casein would ultimately have a greater influence on the Maillard reaction products then whey would. α_s-casein and β-casein concentrations are particularly high.

Skim milk powder contains casein and whey, as well as lactose. Heating of skim milk powder dispersed in de-ionised water is expected to yield some Maillard reaction products because all of the reactants required for the Maillard reaction to occur are present. Whey protein or casein alone when refluxed with lactose would generate some Maillard reaction products as the amount of amino acids available to react with lactose would be more concentrated. Some caramelisation is expected to occur because lactose will be present in the reaction (Coultate, 2002). The Maillard reaction products produced by refluxing either whey or casein with lactose will differ because each protein has a different amino acid composition.

Table 2. Approximate proportions of major proteins found in milk protein (%). Adapted from Food-the chemistry of its components (sourced from T. P. Coultate, 2002, p142)

Component	
Whey (20% of total protein)	
Lactalbumin	3.3-5.0
Lactoglobulin	6.6-13.3
Immunoglobulins	2.3-2.7
Other	3.0-7.0
Casein (80% of total protein)	
α_s-casein	31.0-45.0
κ-casein	3.0-5.0
β-casein	24.0-34.0
γ-casein	1.0-1.5

At temperatures above 100°C, the pH of whole milk will decrease to approximately 5.8, which is the point of coagulation (Fox & McSweeney, 1998). Factors contributing to this decrease include the formation of organic acids, precipitation of calcium phosphate, loss of CO_2 and release of H^+ as well as the formation of saccharinic acids (Fox & McSweeney, 1998). Coagulation must be avoided so that the amino acids are available to partake in the Maillard reaction. The rate at which the Maillard reaction occurs will increase when the temperature of the condition is elevated. Heating of milk influences the heat stability of milk via formation of di-sulfide bonds (Gerrard, 2002) and the formation of a β-1g/κ-casein disulphide linked complex (Swaisgood & Jang, 1990). Whey proteins contain sulphydryl and/or disulphide residues, which become exposed upon heating, and even more so at pH above 7.5 (Fox & McSweeney, 1998). These are more likely to participate in this reaction and ultimately produce more Maillard reaction products. Table 1 shows that the typical whey proteins contained a slightly higher concentration of the sulfur containing amino acid, methionine, compared to the casein proteins. Literature has shown that the presence of sulfur containing amino acids contributes to meaty flavours which would be unattractive in an alcoholic spirit and therefore the concentration of methionine and cysteine would need to be kept to a minimum.

1.5 Aim and scope of project

The aim of the proposed research was to produce an alcoholic spirit derived solely from milk components, specifically the proteins (caseins, whey), lactose, and indirectly ethanol. New Zealand dairy products are recognized internationally as being of high quality. Using dairy components to create an alcoholic spirit will mean that the product can be marketed on geographical exclusivity. There are currently no products on the market that are flavoured using dairy components therefore; this alcoholic spirit will be truly unique and can be marketed as having all natural flavours.

Because this spirit will be marketed on geographical exclusivity, the target market should primarily be the overseas market.

The proposed method for producing flavour was inexpensive and quick in comparison with the traditional methods such as fermentation, distillation, and maturation in wooden barrels and blending. Using resources only from New Zealand meant that the final product will be a unique 'made in New Zealand' product. The flavours generated using the Maillard reaction were 'natural flavours'.

Proteins and lactose were used to generate flavour-active Maillard reaction products that were blended with dairy-derived ethanol to produce the final spirit at an ethanol concentration of around 50 % (v/v), which is the strength of many commercial spirits. A number of changes attributed to the Maillard reaction were monitored including browning, and development of colour, odour (aroma) and flavour. These changes were measured using instrumental and sensory methods. Flavour generated was attractive however the final alcoholic spirit produced was not aesthetically pleasing because it remained opaque after centrifugation and filtration.

Amino acids in powder form were used as sources of protein rather than dairy powders so that the final alcoholic spirit was clear. Changes in colour and odour attributed to the Maillard reaction were monitored over time.

Finally a consumer sensory trial was carried out to assess the spirit samples containing the Maillard reaction product orthonasally.

Chapter 2

Materials and Basic Methods

2.1 Dairy ingredients and amino acids

A number of dairy powders were used. These were Anchor skim milk powder, milk protein concentrate, whey protein concentrate, and sodium caseinate. Milk protein concentrate, whey protein concentrate and sodium caseinate (as a source of casein) were all provided by Westland Milk Products (Hokitika). Each of the dairy powders had different compositions (Table 3) and was stored at room temperature in a dark cupboard before use.

Table 3. Composition of proteinaceous and lactose dairy powder(s) used

	Content per 100 g of powder (g)				
Powder	Protein	Carbohydrate	Fat	Moisture	Ash
Skim milk powder	37.58	50.31	0.61	3.90	7.60
Milk protein concentrate 85	76.26	17.91	1.30	4.53	Not available
Whey protein concentrate 80	82.25	6.21	4.50	4.54	2.50
Sodium caseinate	92.22	None claimed	0.90	4.40	3.60
Lactose monohydrate	0.00	99.00	0.00	0.00	

Amino acids were obtained from a variety of sources by way of AUT's chemical store (Table 4). Purity dates were not always available.

2.2 Miscellaneous chemicals and equipment

Chemicals used included hydrochloric acid (1 M) (HCl) and sodium hydroxide (1M) (NaOH). Ethanol 99% was used to create the alcoholic spirits however this was not food grade and was therefore not potable. A Meterlab PHM201 Portable pH meter was used to measure pH after calibration with pH 4 and pH 7 standards.

Table 4. Amino acids used, their purity and supplier information

Name	Claimed purity (% w/w)	Supplier
Phenylalanine		Sigma Chemical Company
L Lysine monohydrate		AppliChem
β-Alanine		BDH Chemicals
L Leucine	Not less than 99	BDH Chemicals
Glycine	99	BDH Chemicals
DL-Valine	98.5	AppliChem
L-Methionine		BDH Chemicals
L-Serine		Sigma Chemical Compounds
L-Proline		BDH Chemicals
L-Tyrosine	Not less than 98.5	BDH Chemicals

Figure 7. The standard reflux configuration with a 100 mL round bottom flask.

Refluxing involved use of a Horst GmBH mantle as a source of heat, a round bottom flask (100 mL or 500 mL), and a water-cooled condenser. The flask and condenser were linked by a 19/26 and 24/29 ground glass joint. A variety of Whatman filter papers were used including glass microfiber filter papers and cellulose filter papers and will be described in individual experiments. Diatomaceous earth (Celite, 545) was sometimes used as a filter aid, and a centrifuge (Heraeus Megafuge 1.0R and DuPont Sorvall Instruments RC5C) were used to isolate soluble or less dense Maillard reaction products.

An ultraviolet/visible range spectrophotometer (Spectro Ultraspec 2100 pro) was used to measure absorbances that were monitored and recorded using BioDC Version 2.0 software (Biochrom Ltd). Wavescans were carried out in the range from 200-800nm. Although it was expected that the Maillard reaction products would mostly be brown, it was important that the absorbance be measured because the results from the absorbance readings give basic information about the extent of the reaction for comparison within and between treatments. Another reason for conducting wavescans is that some of the Maillard reaction products may absorb UV light in the portion of the spectrum which is not visible to the human eye. Because many of the Maillard reaction products were quite intense in colour the Maillard reaction products were diluted 1:10 in either de-ionised water or 99% ethanol depending on whether or not the Maillard reaction product had been blended in ethanol to produce a spirit. Quartz cuvettes were used because wavelength scans were being conducted in the ultra violet spectrum range.

For most work treatments were stored in a refrigerator at 4°C however, for an odour and colour stability trial treatments were stored at 30°C in the dark. The reason for storing the treatments at an elevated temperature was so that the effect a higher than average temperature will have on the Maillard reaction products and also to see the effect long term storage will have on the Maillard reaction product spirits.

2.3 An outline of the experimental sequence

What follows is a summary of the research path, the details of which will be presented in subsequent chapters, along with details of data analysis.

As a starting point skim milk powder (Table 3) was used as a source of amino groups for the development of Maillard reaction products. Skim milk powder was used rather than whole milk powder because whole milk powder typically contains 30 g of fat per 100 g (Anchor Milk Products, 2010). Fat oxidation products, if produced, would complicate the flavour where an initial simplicity was sought. However, lactose – the milk carbohydrate and at the same time a reducing sugar – was also added to the mixture to increase the concentration of carbohydrate available for the Maillard reaction. In a typical experiment, 20 g of skim milk powder was dispersed in 30 mL of deionised water and the pH was either unaltered or made acidic or basic with 1 M HCl or NaOH. The mixture was refluxed for 90 minutes. These experiments were followed by similar experiments where milk protein concentrate (Table 3) was used instead of skim milk powder on the rationale that the concentrate would provide more amino groups to contribute to the Maillard reaction. The outcome of the skim milk trials

showed that useful colours and odours could be generated. However, the milk protein concentrate was curiously ineffective in generating useful colours and odours. Therefore further experiments with milk protein concentrate were not conducted. Subsequently, the experiments were extended to casein (as sodium caseinate) and whey protein concentrate in acid, neutral and alkali conditions. Whey protein powder and casein both have much higher concentrations of protein than skim milk powder, and each of the proteins differ in amino acid composition. It was hypothesised that by deferring the amino acids available in the reaction, the resulting Maillard reaction products would also be different. Lactose was also added to the reaction mixture because these fractions have low or negligible concentrations of lactose (Table 3). As judged by the researcher and associates, the most attractive outcomes in terms of both aroma and colour were those produced from skim milk powder under alkali conditions. However, the mixture was opaque and unsuited to production of a clear spirit, the stated aim of this product development research.

Several methods were used to create a clear coloured and flavoured isolate, while realising that the potentially useful Maillard reaction products may themselves be insoluble in water and perhaps in alcohol. The first method trialed was filtration with various types of filter paper as well as with diatomaceous earth (Celite) which is a filtering aid. These methods were unsuccessful, therefore the Maillard reaction products were then diluted to 50% ethanol solutions and the processes of filtration were trialed again but were again unsuccessful. The next step was to try centrifugation at 3000 rpm for fifteen minutes. Products were separated into three layers after centrifugation. The dark brown coloured Maillard reaction products became trapped in a gelatinous later within a layer in the test tube and could not be used to colour or flavour ethanol.

Zyactinase is a kiwifruit extract which is known to hydrolyse proteins. Zyactinase was therefore added to skim milk powder and boiled prior to refluxing with lactose. The results of this experiment were also deemed unsuccessful because the Maillard reaction products remained cloudy after filtering with filter paper and celite.

With the failure to produce a clear coloured and flavoured isolate, it was decided to reduce the mass of skim milk powder added to the reflux reaction to see if enough Maillard reaction products could still be produced for use in an alcoholic spirit without being cloudy. Decreasing the mass of skim milk powder caused a decrease in opaqueness and intensity of colour and sweet odour. A clear solution could not be produced without compromising colour and odour at this stage. These products were

then diluted with 99% ethanol to create spirits with a final ethanol concentration of 50%. The purpose of this was so that the effect ethanol has on these products could be observed.

Repeated trials failed to produce the desired result; therefore it was decided to further simplify the experimental approach by using amino acids in place of dairy proteins.

The amino acid experiments were carried out using the same reflux method where pH was altered to 9 by addition of NaOH before starting the reaction. A number of amino acids were trialed initially (Table 4) to see which would produce the most desirable alcoholic spirit product. Wavescans were conducted with all products, aroma was assessed and colour was observed visually by the researcher. Four amino acids were found to have produced promising results. These were then experimented with even further. Length of reflux time and shelf life, as well as the addition of low concentrations of amino acids to see if any increase in colour intensity could be made were observed.

A general observation was that aroma and visible colour were changing over time. The researcher conducted all odour assessments on her own judgment. The researcher had no formal training in this area. A hedonic sensory trial was then conducted for aroma only to help determine if this was indeed true. Details of the sensory methods are presented in the relevant Chapter 5.

2.4 Data analysis

Data handling was all performed in Microsoft Excel (Microsoft Excel 2007). In particular the wavescan data were manipulated in Excel to that absorbances were corrected for dilution. Plotting was performed in Excel.

Statistical analysis of the results of the sensory trial, were conducted with Minitab 16 and ExelStat. Details of the analysis of variance are described in Chapter 5.

Chapter 3

Results and discussion of experiments with dairy proteins

3.1 Introduction

As stated in previous chapters, the main objective was to produce Maillard reaction products to flavour whey ethanol, creating a final potable alcoholic spirit. Firstly, skim milk powder was used as a source of amino acids, with lactose added. This meant that the final product would be completely derived from dairy ingredients

Following the skim milk powder experiments, whey protein and sodium caseinate powders were experimented with. All of the experiments were conducted in acid, neutral and alkaline conditions to observe the effect of pH. Of all of the experiments, the most suitable combination for flavouring whey ethanol was that of skim milk powder and lactose in alkaline conditions.

The next step was to work on filtering the Maillard reaction products to produce a coloured but transparent and aromatic product. A number of methods were tried including various filtration methods and centrifugation, as well as alteration of the mass of reactants in the products.

3.2 Preliminary trials with milk proteins

3.2.1 Development of Maillard reaction with skim milk powder under different pH conditions

The objective was to find how different pH values would affect the Maillard reaction. Skim milk powder was refluxed for 90 minutes under acid, neutral and alkali conditions with quantities as described in Table 5, generating liquids with a consistency approximating that of condensed milk.

Table 5. Development of Maillard reaction products in skim milk suspensions under different pH conditions

Component	pH condition: Acid added (pH = 4.02)	Neutral	Alkali added (pH = 6.62)
Water (mL)	30	32	30
Skim milk powder (g)	20	20	20
NaOH, 1 M (mL)	0	0	2
HCL, 1 M (mL)	2	0	0
Lactose (g)	10	10	10
Total mass (g)	62	62	62
pH	6.87	7.02	7.59
	Results		
Consistency	Thick paste	Very thick paste	Thick paste
Colour	Pale yellow/ brown patches	Not consistent, dark yellow/ light and dark brown	Dark yellow/ dark brown patches
Odour	Milky	Burnt milk	Sweet milky
Photograph	*this phtograph was lost due to file corruption issues		

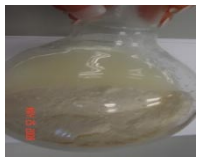
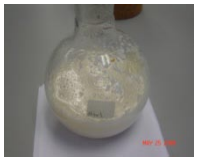
In the three cases, the consistency was paste like after refluxing, but at the nominal neutrality the paste was thicker. The reason for this is not known. It is possible that the high concentration of proteins is causing some protein and/or Maillard cross-linking to occur. The colour of the NaOH added treatment was the darkest and because of the agreeable odour this treatment appeared to be the most promising. Following this experiment, the work was extended to other dairy proteins, but doubling volumes and using a 500 mL round-bottom flask for a uniform boiling temperature.

The incidental observation was made that the skim milk suspension did not precipitate on acid addition. This was surprising because at low pH levels casein usually precipitates to form curd. This principle is the basis on which much of cheese making is based.

3.2.2 Development of Maillard reaction products with whey protein concentrate under different pH conditions

Table 6 shows the results obtained with whey protein concentrate (WPC). A greater mass of lactose was added in the whey protein concentrate and the subsequent sodium caseinate experiments than it was in the skim milk powder experiments because skim milk powder already contains large amounts of lactose (50.3 g per 100 g) whereas whey protein concentrate has only 6.21 g per 100 g of protein and sodium caseinate has no reported lactose content as shown in Chapter 2 Table 3. Because whey protein concentrate contains almost no lactose, the mass of lactose added to the reaction was increased to 20 g. All of the treatments had a rather paste like consistency, more viscous then the skim milk powder treatments before refluxing.

Table 6. Development of Maillard reaction products in whey protein suspensions under different pH conditions

	pH condition		
Component (g)	Acid added	Neutral	Alkali added
Water (mL)	60	64	60
WPC (g)	40	40	40
NaOH, 1 M (mL)	0	0	4
HCL, 1 M (mL)	4	0	0
Lactose (g)	20	20	20
Total mass (g)	124	124	124
pH	6.60	7.03	7.77
	Results		
Consistency	Very thick, sponge-like, upper liquid phase also present. Boiled more vigorously than neutral and alkali	More viscous than water with some sponge-like parts	Sponge-like which burnt onto flask
Colour	No colour change	No colour change	No colour change
Odour	Milky odour, similar to neutral sample odour	Slight burnt milk odour	Burnt odour
Photographs			*this phtograph was lost due to file corruption issues

The results from the experiments with whey protein concentrate were not successful in terms of colour and odour. A brown or caramel colour was not obtained. The odours produced were not pleasant and not suitable for flavouring ethanol. It appears that the Maillard reaction in its usual context did not take place. For example, results for skim milk powder under alkaline conditions were suitable for flavouring ethanol to produce an alcoholic spirit (Table 5), but a similar reaction did not occur here.

In the acid and alkali added reactions, the consistency of the product after refluxing was sponge-like. Whey has a proportion of lysine at least as high as in caseins (Table 1, Chapter 1) and on the face of it there seems to be no reason why conventional browning reactions did not occur in the presence of the added lactose. The Maillard reaction favours reaction lysine and histidine (Coultate, 2002). The answer to this may lie in the formation of a spongy matter, and is discussed in the reasons for differences section 3.3 below.

3.2.3 Development of Maillard reaction products with sodium caseinate protein concentrate under different pH conditions

The experiment was then repeated using sodium caseinate as a source of amino acids. Sodium caseinate was very difficult to disperse in water and this was expected because casein is known to be relatively hydrophobic (Fox & McSweeney, 1998). It was finally found that the best way to disperse sodium caseinate in water was to progressively add the small amounts and to hot water. Because of this problem the proportion of sodium caseinate added to the incubation mixture was decreased to 10 grams.

The results for sodium caseinate were unsuccessful in terms of colour and odour. The development of a slightly darker colour in the alkali treatment was not intense enough to be considered a good result. Odours produced in the acid and alkali treatments were unpleasant, and unsuitable for flavouring alcohol, while in the neutral treatment odour did not change after refluxing at all.

Table 7. Development of Maillard reaction products in sodium caseinate protein suspensions under different pH conditions

Component (g)	Acid added	Neutral	Alkali added
Water (mL)	120	124	120
Sodium caseinate (g)	10	10	10
NaOH, 1 M (mL)	0	0	4
HCL, 1 M (mL)	4	0	0
Lactose (g)	20	20	20
Total mass (g)	134	134	134
pH	5.96	7.47	10.83
		Results	
Consistency	Not much change more viscous than water	No change but more viscous than water	Liquid consistency
Colour	No change, milky, cloudy, off-white colour	Slight colour change, more yellow	Slight colour change, darker yellow then neutral sample
Odour	Slight burnt milk smell	No change in odour	Very faint burnt notes detected.
Other observations	Upon adding HCl proteins coagulated as expected for acid precipitation of casein. Exact pH was difficult to record as the reading kept decreasing.		
Photographs	*this phtograph was lost due to file corruption issues		

3.3 Reasons for differences

The most attractive outcomes were generated from skim milk powder (Table 5) and the question must be asked as to why it behaved so differently from the two other powders. Whey protein concentrate contains mainly β-lactoglobulin and lactalbumin.

Skim milk powder contains casein and whey, as well as lactose. It was expected that perhaps whey protein alone, or casein alone when refluxed with lactose would generate some Maillard reaction products as the amount of amino acids available to react with lactose would be more concentrated. Some caramelisation would also have been expected to occur because lactose had been added to the reaction (Coultate, 2002), however there was no evidence of this occurring at all.

Heating milk and denaturing should influence the heat stability of milk via formation of di-sulfide bonds (Gerrard, 2002) and the formation of a β-1g/κ-casein disulphide linked complex (Swaisgood & Jang, 1990). Whey proteins contain sulphydryl and/or disulphide residues which become exposed upon heating, more so at pH above 7.5 (Fox & McSweeney, 1998) and are therefore likely to be more available to take part in this reaction. This could be a reason for why the results of the experiments using whey protein concentrate and casein alone seemed to have undesirable consistencies. Whey protein concentrate does not contain any κ-casein and sodium caseinate does not contain any β-lactoglobulin whereas skim milk powder which contains both β-lactoglobulin and κ-casein and was still liquid in consistency after refluxing. Heating of skim milk powder may have allowed other amino acids to partake in the Maillard reaction while formation of the disulfide bonds between denatured β-lactoglobulin and κ-casein maintained heat stability.

Table 1 (Chapter 1) shows that the typical whey proteins contain higher concentrations of sulfur compared to the casein proteins. Table 2 (chapter 2) shows the proportions of the major proteins found in whey. The results show that after refluxing a sponge-like product was forming. It is possible the formation of disulfide bonds may have influenced the formation of a final product with the sponge-like consistency. The concentration of lysine is relatively high in both whey and casein, and it is known that the Maillard reaction favours lysine which indicates that lysine may have been involved in some other reactions during heating rather than the Maillard reaction. Another possibility is that some other type of cross-linking reaction may be occurring, possibly a phenomenon favoured by lysine with its ε-amino group. What it is reacting with is not

clear. Certainly the mixture contains no transglutaminase which could catalyse the ε(γ-glutamyl) lysine link between glutamine and lysine. Transglutaminase is often used in food processing to form ε(γ-glutamyl) lysine crosss-links between glutamine and lysine because of its ability to improve nutritional and textural properties of foods.

Upon heating, the sulphydryl and/or disulphide residues in the whey proteins (lactalbumin, lactaglobumin and immunoglobulin's) become exposed and a number of reactions can occur.

The other possible issue with the whey protein concentrate treatment may be the low lactose content because lactose protects the protein from denaturing during spray drying perhaps via lactose's capacity to replace exposed hydrophobic pockets (Jost, 1993). This means that some of the proteins in the whey protein concentrate would already have been denatured prior to refluxing and in theory this should mean that amino acids are even more readily available to partake in the Maillard reaction. This does not explain why the Maillard reaction did not appear to have occurred.

Casein also failed to produce any useful Maillard reaction products. While the consistency remained liquid after refluxing, there was no generation of colour or odour after refluxing. It would have been expected that the acid added treatment would have produced some changes in consistency because it is known that in cheese and yoghurt making the formation of curd via the precipitation of casein occurs. Although the mass of sodium caseinate added to these treatments was lower than in the skim milk powder and whey protein concentrate experiments, some Maillard reaction products were expected to have been produced. The unpleasant odour produced is most likely due to the presence of sulfur (Table 1, Chapter 1).

Caseins are relatively hydrophobic, and contain few disulphide bonds and phosphorylated which gives caseins an increased ability to bind calcium and form aggregates and precipitates, and also affects their heat stability. Caseins are not generally susceptible to denaturation via exposure to high temperatures. Sodium caseinate can be heated at 140°C for up to an hour without and visible physiochemical changes (Fox & McSweeney, 1998). Casein may have not formed Maillard reaction products because of these factors.

3.4 Further trials with SMP

Skim milk powder gave the best results so a further trial was conducted with this source of protein. The literature generally shows that the Maillard reaction is favoured by alkaline conditions (Coultate, 2002). In Table 5, alkalinisation was attempted with NaOH but the quantity added was insufficient to raise the pH above 7, presumably due to the buffering capacity of skim milk powder. To explore the effect of true alkalinisation the pH of the reaction was raised to 9 as some literature has found that this is an ideal pH for the reaction to occur (Patel, 2010). Importantly the pH was measured before and after reflux and pilot experiments conducted earlier showed that if the starting pH was 9 it would drop to pH 7 after reflux.

The new trial involved double volumes and the pH was successfully raised to 9.10. The purpose of this experiment was firstly to observe the changes in pH before and after refluxing, and to produce a greater volume of Maillard reaction products so that various filtration methods could be trialed. Table 8 shows the mass of each of the components used as well as pH before and after refluxing. This experiment confirmed that the pH drops to 6.62 on reflux. The resulting mixture was dark brown and potentially useful as a source of and flavour colour because of its colour and sweet caramel like odour (Figure 8).

Table 8. Mass of components added for the development of an increased volume of Maillard reaction products in skim milk powder buffered pH 9

Component	
Water (mL)	240
Skim milk powder (g)	20
NaOH, 1 M (mL)	5
Lactose (g)	20
Total mass (g)	285
pH before reflux	9.10
pH after reflux	6.62

Figure 8. Appearance of a SMP /lactose mixture after reflux at an initial pH of 9.10 and final of 6.62.

At this point, the question must also be asked: why did the pH decrease? The presence of salts (calcium phosphate, citrate, and bicarbonate), and acidic and basic amino acid side chains on milk proteins increase the buffering capacity of milk proteins. The Maillard reaction has the affinity to follow different pathways depending on the pH of the condition. At pHs above 7, some of the products of the Maillard reaction may be acidic which causes pH to decrease after refluxing. Products of caramelisation are also acidic. Some of these products include saccharinic acid, lactic acid, 2,4 dihydrohy butyric acid, ethyl alcohol and other various acidic aroma compounds.

The chemical concept of pH is based on aqueous solutions and non-spirituous alcoholic drinks are generally acidic, as exemplified by wines and beers. In spirits however, which are only 60% water the concept is probably not as useful. But intuitively it seems more sensible to have a spirit that is neutral or acidic rather than alkaline. Moreover, diluents of spirits, water or compound soft drinks, are either neutral (water) or acidic. Thus the decrease in pH from 9 to 7 would appear to be a beneficial rather than a problem.

Overall, the use of skim milk powder in alkali conditions was found to produce the most favourable Maillard reaction products with added lactose. At this point the mixture was cloudy (Figure 8) and required clarification if a clear spirit much like a whisky or rum were to be produced. Thus, the next step was to clarify the mixture.

3.4.1 Clarification of cloudy reflux mixtures of SMP and lactose

The Maillard reaction products produced from refluxing skim milk powder had useful odour and colour, however, the products were cloudy. The aim was to finally produce a clear but coloured and aromatic alcoholic spirit like product. The two main methods that were considered were filtration and centrifugation. Filtration was the preferred method to trial first as this method is commonly used in for example the wine industry, and a series of continuous decanter centrifuges to completely clarify a cloudy mixture is likely to be prohibitively expensive.

To begin the Maillard reaction product was filtered using Whatman 541 filter paper, which although not fully quantitative is fast draining. The paper was mounted in a Buchner system evacuated with a water pump. The product remained opaque after filtration (Figure 9) and remained so after the filtered mixture was mixed 50:50 with 99% ethanol.

Figure 9. Maillard reaction products after filtering.

This work was repeated with Whatman grades 1, 42, and 542 papers and glass microfiber filter paper. Diatomaceous earth was used as a filter aid. Diatomaceous earth (celite) is a fine powdery substance which is often used as a filter aid in chemistry because it has the ability to prevent small particles from passing through or clogging filter paper during filtration. In this particular experiment the objective of using celite was to prevent the particles causing cloudiness in the Maillard reaction products from passing through the filter paper thereby allowing the Maillard reaction products to be clear. Celite was mixed with the products after refluxing, and the solution was then allowed to pass through the Buchner flask with the use of vacuum. None of the

experiments involving Celite produced a clear Maillard reaction product containing solution and the results of this experiment were deemed unsuccessful.

Because none of the filtration methods used were successful, the SMP and lactose reaction products were then centrifuged at 3000 gravities for 15 minutes. This resulted in the formation of a three layered product. A small white pellet formed at the bottom of the tube. A dark brown gelatinous layer formed on top of this, and a light yellow liquid portion. The dark brown gelatinous layer contained all of the coloured Maillard reaction products while the colour was lost from the other two components (pellet and liquid portion). No odour was detected. Because the coloured Maillard reaction products were trapped in a gelatinous layer they could not be used to colour and flavour ethanol. The results of centrifugation were not deemed successful.

3.4.2 The use of Zyactinase to hydrolyse dairy proteins

Zyactinase is an enzyme derived from kiwifruit and is known to hydrolyse proteins. Zyactinase is often used in health supplements that are designed to aid and improve digestion in humans. The expected outcome for this experiment was that by boiling milk powder in the presence of Zyactinase (in powder form) the amino acids from the proteins would be hydolysed resulting in a clear amino acid containing solution The other possible outcome was that the solution produced after boiling would be able to be filtered to produce a clear amino acid containing solution. This solution would then be used as the source of amino acids for the production of Maillard reaction products for flavouring ethanol. Twenty grams of skim milk powder was therefore boiled in the presence of Zyactinase at 90 °C for 90 minutes to hydrolyse the proteins. The aim was to produce a clear liquid containing the amino acids necessary which would still have contained all the desired Maillard reaction products. A control experiment containing SMP without Zyactinase was also conducted. The hydrolysed product will then be used as the source of amino acids and refluxed in alkali conditions to produce a clear Maillard reaction product.

The hydrolysed skim milk powder was then refluxed with lactose at pH 9 for 180 minutes.

Table 9. Mass of components added for hydrolysis of proteins in skim milk powder using Zyactinase

Component	Hydrolysed skim milk powder	Control
Water (mL)	100	102
Skim Milk Powder (g)	20	20
Zyactinase (g)	2	0
Total mass	102	102
Observation	Product contained white 'clumps' after boiling which settled after some time. The clear portion of the solution was very faint yellow.	No colour change, white and opaque in colour.

Table 10. Development of Maillard reaction products using skim milk powder hydrolysed with Zyactinase as a source of amino acids.

Component	
Water (mL)	110
Hydrolysed Skim Milk Powder (mL)	10
Lactose (g)	10
NaOH 1 M (mL)	0.8
Total Mass (g)	128
pH before reflux	8.94
pH after reflux	6.8
Observations	
Colour/consistency	Clear, yellow/dark yellow. White 'clumps' of proteins still present. Cloudy appearance
Odour	Milky with very very faint sweet notes.

The results from the experiment with zyactinase were not ideal. The Maillard reaction product was cloudy and had white 'clumps' which were most likely protein. The odour of the product was not desirable either.

The next step was to alter the mass of skim milk powder in the reaction to see if lowering the protein content in the reaction could still produce a Maillard reaction product that would still have good results for colour and for odour.

3.4.3 Altered mass of SMP in reaction- 10g, 1g, 0.1g + made 50% EtOH solutions.

The mass of skim milk powder may have been too high in previous experiments, causing the Maillard reaction products to remail opaque after reflux, centrefugation and filtration. The mass was then lowered to see if decreasing the skim milk powder content would decrease or eliminate the cloudiness without compromising the colour and odour of the Maillard reaction product.

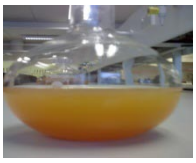
Ethanol was then added to the products to produce 50% ethanol solutions. The results for this experiment are on Table 11. The general observation was that as skim milk powder concentration decreased, the darkness of the product decreased. This indicates that the Maillard reaction products decreased. As skim milk powder concentration decreased, colour went from opaque dark brown to clear yellow, and odour went from noticeably sweet to very faint sweet odour. Although having very low concentrations of skim milk powder in the reaction produced a clear product, it seemed that the colour and odour were compromised. The control which contained lactose only resulted in an ideal and attractive colour; however the odour had been compromised. Lactose is unstable under milk alkaline conditions in milk at mild temperatures whereby lactose undergoes re-arrangement of aldoses to ketoses.

Opaqueness in milk is generally caused by fat globules and casein micelles, however, these would not be the cause of opaqueness in the experiments carried out because fat content in all of the dairy powders was very low, and casein was present in small amounts in the skim milk experiments, and was not present at all in the whey protein concentrate suspension.

The possible cause of opaqueness could have been protein cross linking due to the high mass of skim milk powder in the reaction. It is also possible that some of the dairy powders did not break down and disperse in the water completely.

The biggest obstacle in this experiment still remains to be the production of a dark brown colour, and pleasantly flavoured Maillard reaction product that is clear.

Table 11. Development of Maillard reaction products at pH 9 using various mass of skim milk powder as a source of amino acids.

Component				
Water (mL)	120	120	120	120
Skim Milk Powder (g)	10	1	0.1	0
NaOH 1 M (mL)	2.5	0.2	0.7	2
Total Mass (g)	132.5	121.2	120.8	122
pH after reflux	7.63	6.08	5.61	5.05
Observations				
Colour	Dark brown, opaque, more viscous than water	Dark brown, same colour as 10g product, slightly opaque	Clear, yellow	Dark yellow/orange clear
Odour	Slightly sweet, some burnt milky notes	Very faint burnt milk	Very faint sweet	Faint caramel
Photographs				
Observations of 50% EtOH solutions				
Colour	Slightly lighter than no EtOH sample	Slightly lighter than no EtOH sample	Slightly lighter than no EtOH sample	Slightly lighter than no EtOH sample
Odour	Condensed/caramel milk	Slightly sweet/caramel	Slightly sweet	Sweet but not milky
Other observations	Upon adding EtOH a white precipitate formed on top which dissolved after shaking- final solution remained murky	Opaque		
Photos				

Chapter 4

Amino Acid Experiments

4.1 Introduction

Results presented in Chapter 3 showed that without elaborate filtration or centrifugation methods, the use of dairy proteins to generate Maillard reaction products with lactose was not productive. Attention was switched to free amino acids and lactose because intuitively it seemed likely that initial clear amino acid solutions would yield clear Maillard reaction outcomes, and would therefore be more suitable visually for flavouring a potable alcoholic spirit. The approach was to screen many amino acids with a view to narrowing the range to a few amino acids with desirable flavour and colour properties. Lactose was maintained as the carbohydrate as the original aim was to produce a flavour for spirit using dairy components.

In the event, four potentially useful amino acids were found to produce likable products to flavour ethanol. These were alanine, leucine, phenylalanine and valine. These amino acids were then refluxed for a longer time to help decide the optimum time for reflux. At many points of this work, wavelength scans were conducted to quantify colour changes.

4.2 Development of Maillard reaction products in amino acid lactose mixtures.

4.2.1 Method

The amino acids chosen are listed in Table 12. The use of amino acids containing sulphur was avoided as it was known from previous experiments that sulphur-containing amino acids yielded unpleasant odours. Compounds containing sulphur are known to contribute to ‘cooked meat’ flavours (Coultate, 2002) and this would have been unpleasant in an alcoholic spirit.

A 7 M solution of lactose was prepared by dissolving lactose in its powder form in de-ionised water. Solutions or solution/suspensions of amino acids were also prepared

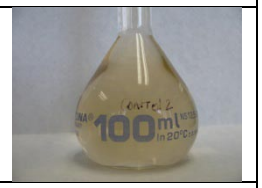
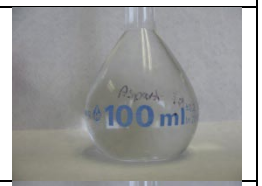
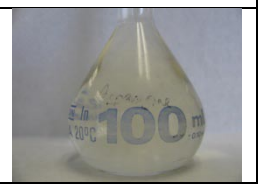
in the same way, also to 7 M. Equal volumes of 65 mL of lactose and amino acids were combined, resulting in final concentrations of 3.5 M. The pH was then adjusted to 9 using 1M NaOH and was measured using a Meter Lab PHM201 pH meter (Radiometer, Copenhagen). After the pH had been adjusted, enough deionised water was added to the flasks so that the final volume of the reactant was 140 mL, each reactant being nominally 3.25M if in solution.

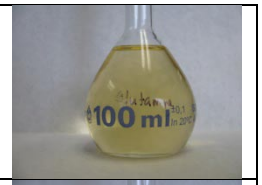
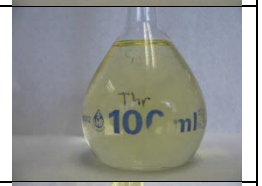
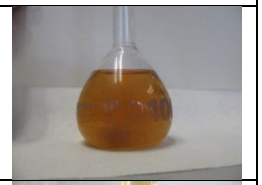
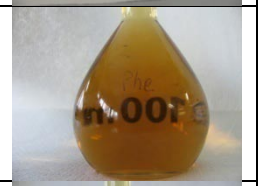
After 90 minutes of reflux with antibumping chips, the mixtures were assessed by the researcher for odour and visual colour. Whereas this assessment method was clearly subjective (an assessor replicate of 1), it was the only practical way that treatments could be rapidly screened. Some treatments had residual solid matter – quite likely undissolved amino acids – so all mixtures were filtered through Whatman No. 1 paper on a Buchner water vacuum funnel. All of the solutions were clear after filtration.

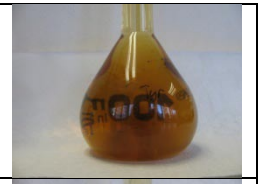
Prior to wavelength profile scanning the filtrates were diluted 1:10 solutions with deionised water. This was done because some of the colours were intense to the point that absorbances at some wavelengths were predicted to have exceed the allowable zero to three absorbances. After setting a baseline with deionised water, the diluted filtrates were wavelength-scanned from 200 to 800 nm in 3 mL quartz curvettes using a 'Spectro' Ultraspec. 2100 pro (Armasham Biosciences, U.K.). The absorbances were captured by computer using BioDC version 2.0 software (Biochrom Ltd), the output of which is in the form of an Excel spreadsheet. Wavelength scans are routinely presented corrected for dilution.

Fifty percent ethanol solutions of each of the undiluted filtrates were made up by mixing 25 mL of filtrate with 25mL of 99% ethanol. Odour and colour were re-assessed by the researcher. This was done because the compounds in the original filtrate were likely to exhibit a different volatility in ethanolic solution, depending on the relative vapour pressures of each Maillard compound in water compared with 50% ethanol. For rescanning, the ethanolic solutions were diluted 1:10 with a 50% ethanol: water mixture using a 1:10 dilution.

4.2.2 Results and discussion

Table 12. Results of refluxing lactose with different amino acids for 90 minutes.						
Amino acid	pH after reflux	Colour	Odour	Odour of 50% EtOH solution	Other observations	Photos
Control (lactose only)	5.2	Very faint yellow	Slightly sweet	EtOH, very faint sweet		
Acidic polar amino acids						
Aspartic acid	4.09	No change	Rubbery	None other than EtOH		
Glutamic acid	7.50	No change	No odour	None other than EtOH	Difficult to disperse in water, required 11 mL of NaOH and pH still did not increase to 9	
Neutral polar						
Asparagine	8.65	Very pale yellow/almost colourless	Milky	Solvent like, milky smell lost		

Glutamine	7.68	Pale peach/brown	Acetone like	None other than EtOH		
Threonine	8. 66	Very pale yellow	Solvent like	Solvent like, felt tip pen		
Serine	8.64	Bright/dark yellow	Faint sweet/burnt notes	None other than EtOH	Required 5mls of NaOH to raise pH to 9	
Neutral non-polar amino acids						
Alanine	8.2	Light golden brown	Toffee like, tangy/yoghurt like	Faint sweet		
Phenylalanine	8.55	Dark brown	Flowery/potpourri - quite strong	Solvent like/faint floral		
Leucine	8.45	Medium/dark yellow	Slightly sweet/ musty/damp	Sweet/solvent/ tequila like		
Glycine	8.40	Orange red/brown	Faint sweet/damp	None other than EtOH		

Tyrosine	8.80	Dark yellow/murky	Faint sweet/wet	Sweet/potato like		
Proline	5.26	Bright yellow	Damp/musty/wet	Sweet/savory/biscuity/potato		
Valine	8.29	Yellow	Chlorine like, mild sweet	Slightly sweet/tequila like		
Basic polar						
Lysine	8.34	Black/brown	Superwine biscuit/pastry	Superwine biscuit/pastry	No NaOH was added, starting pH was 9.38	No photograph available but noted to be as dark as arginine treatment
Arginine	11.0	Reddish/black/brown	Slightly sweet	Slightly sweet/solvent like	No NaOH was added, starting pH was 10.89	
Histidine	8.56	Pale yellow	Slightly sweet	None other than EtOH		

Ornithine	8.29	Dark orange/brown	Sour cream and chives potato chip like	Slightly sweet/biscuit	Required 6.5 mL of 1 M NaOH to raise pH to 9	

Prior to refluxing, the pH of the treatments were all raised to 9 by the addition of 1 M NaOH. The volume of 1 M NaOH required was variable, ranging from 0.5 mL to 3 mL for most amino acids. However the serine and ornithine treatments required 5 and 6.5 mL respectively. The hydroxy group on serine is titratable with a pKa of 9.21 (Dawson and others 1986), presumably accounting for the excess NaOH required. Glutamic acid required a greatest volume to raise the pH to 9, at least 11 mL, and this can be traced to the two carboxy groups that need to be fully titrated to achieve pH 9 (Figure 9). Similar arguments would apply to all the amino acids used, also depending on the exact ionic form present in the reagent bottle.

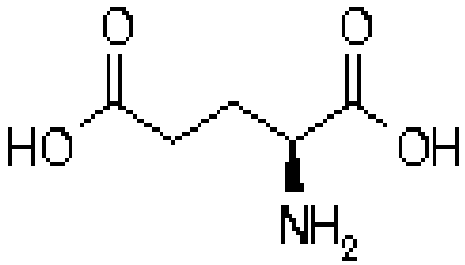


Figure 10. Structure of glutamic acid

Table 12 shows that the majority of the treatments' pHs decreased after reflux by between 0.5 and 1.5 units. Carabasa-Giribet & Irbarz-Ribas (2000) had similar findings when asparagine, glutamine and aspartic acid were refluxed with glucose, however, the reason for this was not known. It was suggested that because the Maillard reaction is so complex there may be a number of products contributing to the decrease in pH.

Some treatments were, however, exceptional. The lactose treatment with no amino acid added fell to pH 5.2. There are several probable of possible reasons for this. First, the presence of the amino acids in the other treatments may exhibit a degree of buffering capacity resisting the decrease in pH. Because no amino acid was present in the lactose treatment to help resist the change in pH, the lactose treatment had the potential to change the most if some source of acidity were generated. According to Belitz and others (2004), monosaccharide's produce saccharinic acids and other acidic products in strongly alkali conditions (Figure 11).

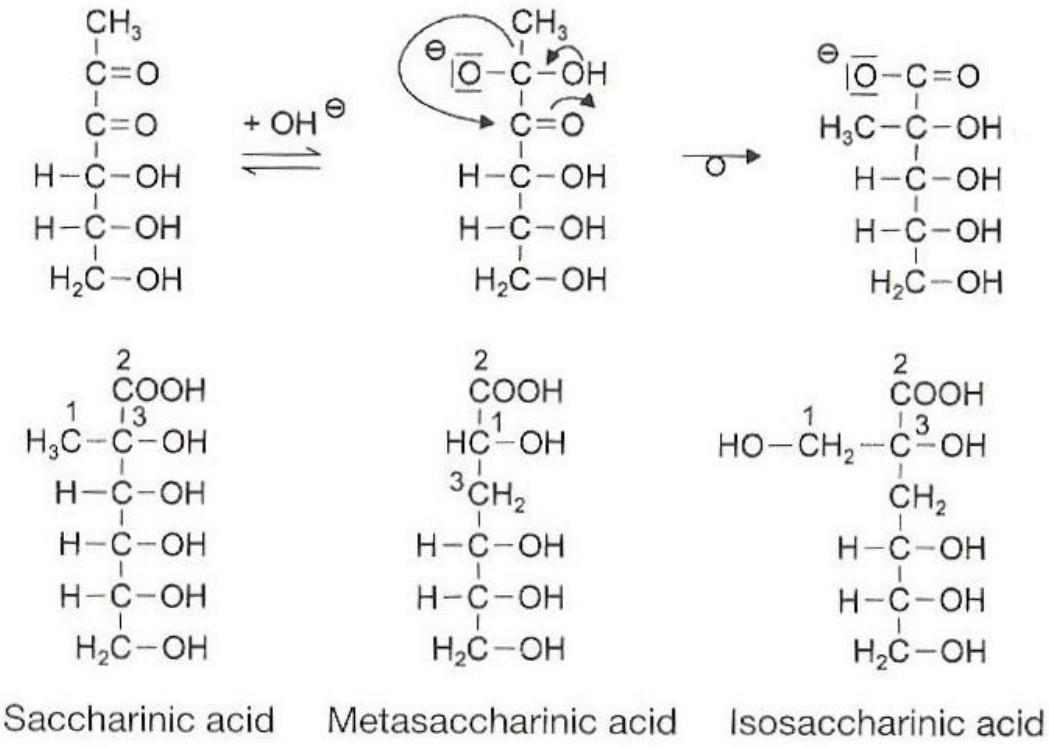


Figure 11. Structure of saccarinic, metasaccarinic, and isosaccharinic acids

Although lactose is a disaccharide, it is possible that lactose may be undergoing a similar process and forming saccharinic acids (Belitz, Grosh, & Schieberle, 2004). For example it could also be possible that lactose is hydrolysed to glucose and galactose and is producing saccharinic acids in this way. As mentioned in Chapter 1, lactose undergoes a series of changes when heated. Some of these changes other then the production of saccharinic acids include mutarotation, isomerisations and the formation of a number of volatile compounds including furfural, hydroxymethylfurfural, CO_2 and CO. Lactose is relatively unstable in mild conditions at moderate temperatures, however as heat has been applied it is hypothesised that any reaction that may occur in mild conditions would occur at an even faster rate with heat because heat acts as a catalyst.

When amino acids were included the pH usually fell, but not by nearly as much as in the control experiment. There are several reasons for this, one being the buffering capacity of amino acids as noted above. Second, the production of the saccharinic acids could still occur in parallel to the Maillard reaction, so lowering the pH. According to Nursten (2005), the two causes for the decrease in pH as a result of the Maillard reaction are the formation of acids via the process of sugar degradation resulting in fission products in the third stage of the Maillard reaction (Figure 3), and the conversion of amino groups into less basic heterocyclic compounds in the final stage (Figure 3).

Upon closer examination, it is difficult to see how sugar fragmentation into fission products would produce some acidic compounds that are responsible for lowering the pH. Fissions products include acetol, butanedione, and 2-oxopropanal, dehydroascorbic acid, only one of which would be significantly acidic, dehydroascorbic acid.

However, dehydroascorbic is a weak acid compared with ascorbic acid and carboxylic acids like saccharinic acids and acetic. Aqueous solutions of ascorbic acid attain pH 3, whereas solutions of dehydroascorbic acid are neutral (Dawson and others 1986). Thus the exact cause of pH reduction for most amino acid treatments is unclear.

The pH of the aspartic acid treatment decreased from 9 to a 4.09, a 100,000 fold increase in acidity. Although aspartic acid is relatively insoluble in water (2.09 g L^{-1} at 0°C and 68.9 g L^{-1} at 100°C (Belitz, Grosh, & Schieberle, 2004) the act of titration allowed a concentration of 3.25 M to be achieved. Thus, the massive drop in pH cannot be attributed to solubilisation of aspartic acid on reflux yielding free protons from the two carboxy groups (Figure 12). It is also possible that aspartic acid may not have taken part in the Maillard reaction, and rather than pH decreasing due to the presence of the Maillard reaction products, the caramelisation of lactose and production of saccharinic acids may have decreased the pH. However, the exact reason for massive drop is unknown. The proline treatment declined to pH 5.2 and again there is no obvious reason for the fall. Again, saccharinic acids may be responsible if proline is unreactive in the Maillard reaction.

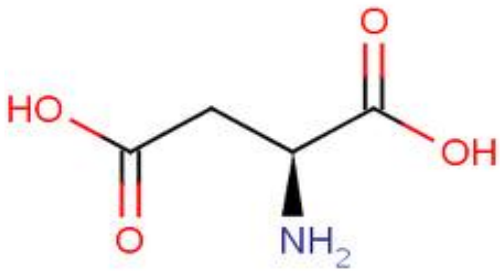


Figure 12. Structure of aspartic acid

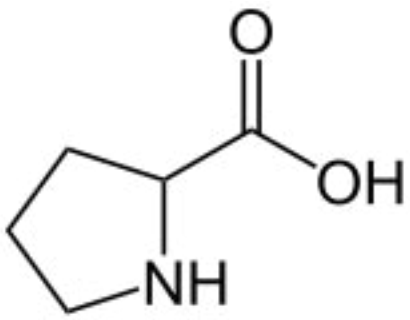


Figure 13. Structure of proline

The four basic amino acids used were arginine, lysine, histidine and ornithine. Lysine and arginine did not require the addition of NaOH to raise the pH because their

native starting pH values were 9.38 and 10.89 respectively. Ornithine by contrast needed 6.5 mL, indicating that the ionic form was ornithine hydrochloride. The pH of the arginine treatment was effectively unchanged after reflux, but the pH of the other three treatments decreased similarly to most amino acids, to between pH 8 and 9.

Turning now to colour and odour, the colours produced after reflux ranged from colourless to very dark brown and – in the opinion of the researcher – a range of pleasant and unpleasant odours were produced (Table 12). The development of colours and odour are both good indicators of the extent of the Maillard reaction. The dark compounds produced as a result of the Maillard reaction are complex mixtures of low and high molecular weight compounds. The darkest treatments after reflux were lysine and arginine. The two acidic amino acid treatments (aspartic acid and glutamic acid) were colourless after reflux, which indicates that the Maillard reaction may not have proceeded to any extent at all. Moreover, they were remarkably odourless. These two amino acid treatments suffered falls in pH (especially aspartic acid) that might be attributed to the formation of saccharinic acids (Figure 11) as discussed above. Aspartic acid and Glutamic acid may infact inhibit caramelisation. Consider that lactose alone generated more colour. All other treatments generated some colour and odour.

The high reactivity of lysine is well known on account of its two available amino groups. Arginine also has two available amino groups as well as a primary imine group. Judged by pH change, colour and odour, arginine was slightly less reactive than lysine implying that the primary imine was unreactive. Lysine and arginine showed the development of highly intense brown colours, almost to the point of black, while ornithine developed dark orange/brown colours. In aqueous solution, the ornithine treatment exhibited a ‘sour cream and chives potato chips’ odour, but in 50% ethanol, it exhibited sweet biscuit-like odour, similar to that of the lysine treatment. Figures 14 and 15 show the structure of lysine and ornithine respectively. The structures of the two amino acids are similar, presumably accounting for the similarity in a biscuit odour. The biscuit-like odour was not detected in any other treatments.

Certainly the secondary imine of histidine did not appear to be reactive, because the histidine treatment was only pale yellow.

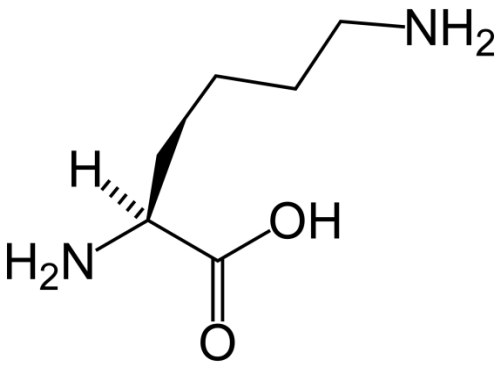


Figure 14. Structure of lysine

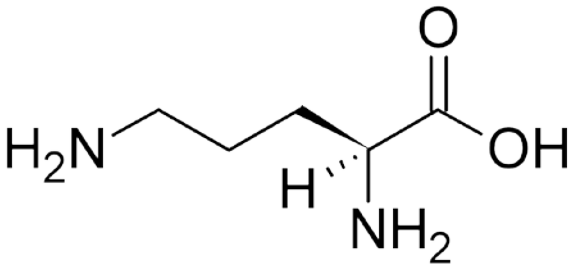


Figure 15. Structure of ornithine

Of the neutral polar amino acid group, serine developed the most intense bright yellow colour. Asparagine, glutamine and threonine all developed relatively pale yellow colours. None of the odours produced from the polar neutral amino acid group were judged to be particularly intense or even pleasant. The pale colours and weak odours indicate that these amino acids did not react extensively in the Maillard reaction. The odours that were produced by this group were mostly undetectable after the addition of ethanol, suggesting that the odour-active compound(s) were more soluble in ethanol/water than in water, and were not released to the headspace above 50% ethanol. Therefore the final ethanolic products from this group were eliminated as possibilities for further research as alcohol flavourants. Equally the colours developed from the neutral polar amino acid group were not intense enough to be useful.

The neutral non-polar amino acid group developed more intense colours and more intense and suitable sweet odours for flavouring ethanol than the neutral polar amino acid group. The colours from the neutral non-polar group had a range of tones from yellow to brown, and in the case of glycine, an orange red/brown. The phenylalanine and glycine treatments developed the darkest colours of this group. In respect of primary and secondary amine and imines, proline is a secondary amine (Figure 13) and is clearly reactive (Table 12). The pH also declined markedly. In this group the most likeable odour was produced by phenylalanine, which was intense and floral. Although most of the odours produced after reflux were acceptable, the addition of ethanol to the products caused the odours to change, presumably for the reason outlined in the previous paragraph. However, alanine, phenylalanine, leucine and valine showed the most suitable results for odour even after the addition of ethanol. The colours developed from this group were also quite suitable, therefore these amino acids were chosen for further work, as is described in later sections. The odour produced from phenylalanine was judged to be one of the best because of its uniqueness. The Maillard reaction products of phenylalanine had a pleasant flowery aroma, matched by no spirits currently

on the New Zealand liquor market. It is noted that phenylalanine as a dry amino acid powder had a detectable floral odour.

Considering now all the amino acids in Table 12, absorbance profiles of the reflux products diluted 1:10 are shown in tabular form in Appendix 1. Although data were collected between 200 and 800 nm, all the interesting information was contained in the range 200 to about 450 nm. Therefore, the data presented in graphic form below are truncated at 450 nm. The main focus was on the reflux mixtures diluted in ethanol to 50%, because the outcomes of this research are directed at alcoholic drinks. These data (Figures 15, 16, 17, 20, and 21) are similarly truncated because the results above 450 nm approached zero; none of the solutions appeared blue and this was consistent with minimal absorbance at longer wavelengths. Figures 15, 16, 17, 20, and 21 are the wavelength scans for the reflux mixtures diluted to 50% ethanol, and then further diluted 1:10 with 50% ethanol to bring many but not all absorbances into the dynamic range of the spectrophotometer. Note that all ordinate axes are corrected for dilution and are scaled identically, 0 to 29.

Absorbances were also measured prior to reflux and showed that most of the lactose and amino acid treatments had very low absorbances and some were almost 0.

Lactose alone generated ultraviolet absorbances peaking at around 225 nm at a dilution-corrected absorbance of 9 (Figure 16). The least reactive amino acids were glutamic and aspartic acid, both of which are acidic polar amino acids (Figure 17). The highest absorbances appeared to occur at 200 nm or below, below being outside the spectrophotometers' scan range. Aspartic and glutamic acids showed peak absorbances at approximately 215 nm. The neutral polar (Figure 18) and neutral non-polar amino acid (Figure 21) groups absorbed more extensively at longer ultraviolet wavelengths, suggesting the formation of more complex molecules than for glutamic and aspartic acids. Although scans for many amino acids were off scale, it is clear that basic polar amino acids displayed the highest readings overall (Figure 22). This result is presumably reflected in the results in Table 12 where the lysine and arginine treatments produced very dark colours in comparison to the treatments containing other amino acids.

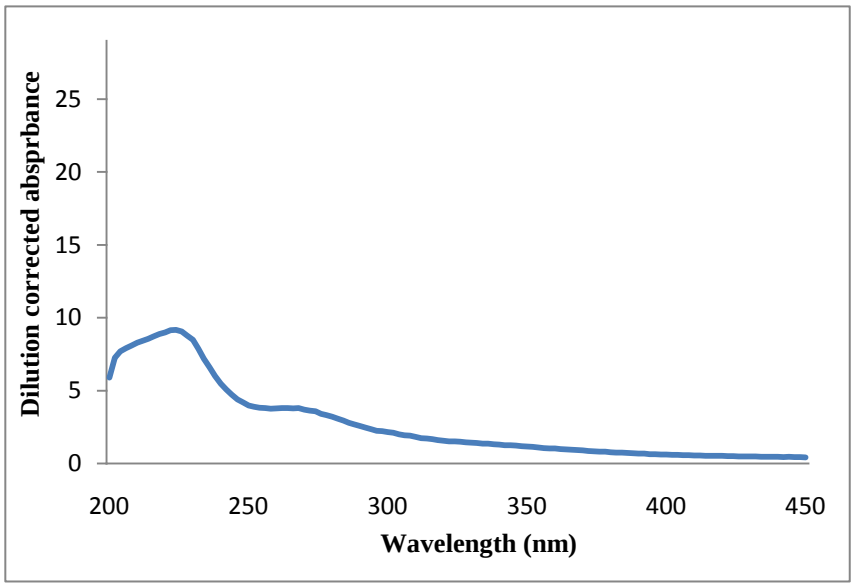


Figure 16. Wavelength scanning results for lactose products after adjusting ethanol concentration to 50%

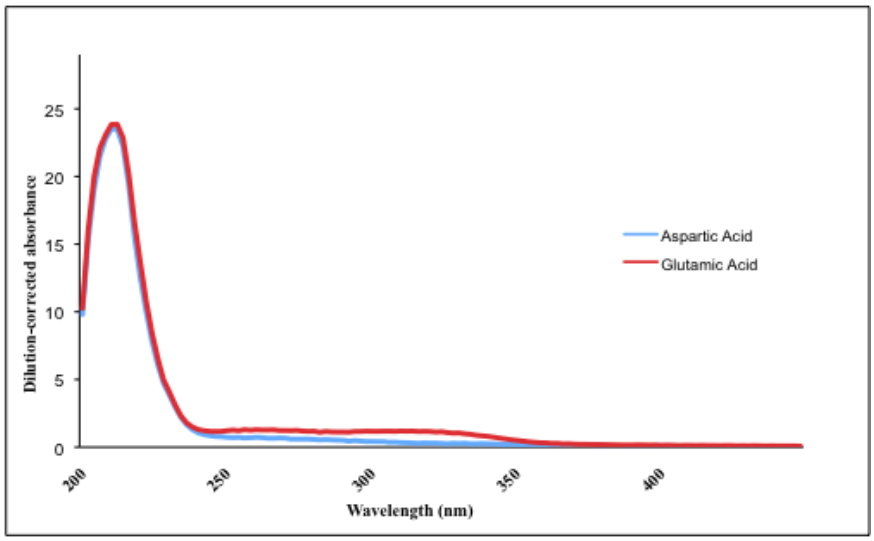


Figure 17. Wavelength scanning results for acidic polar amino acid products after adjusting ethanol concentration to 50%

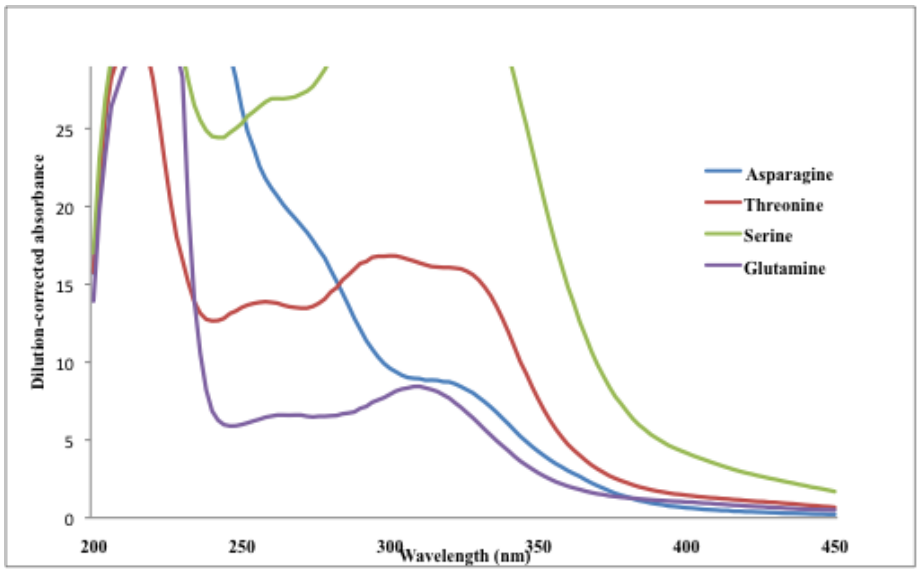


Figure 18. Wavelength scanning results for neutral polar amino acid products after adjusting ethanol concentration to 50%.

Figure 18 shows the results for wavelength scans of neutral polar amino acids. Absorbances were much higher than for the acidic amino acids. The result also show that asparagine and glutamine behaved rather similarly because their peak absorbances are likely to be at approximately the same wavelengths, but clearly off scale. Serine and threonine also showed similar results to each other, their peak absorbances being at longer wavelengths compared to the asparagine and glutamine treatments. Of serine and threonine, the former was the most reactive, presumably reflecting the greater colour development reported in Table 12. Serine is a primary alcohol and theonine is a secondary alcohol, and they may be the reason for the difference in reactivity.

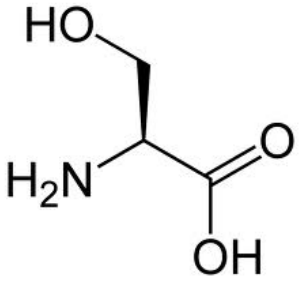


Figure 19. Structure of serine

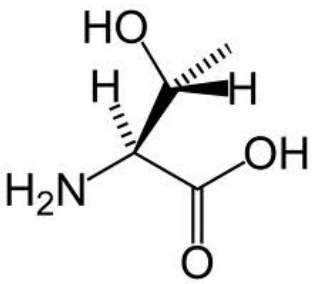


Figure 20. Structure of threonine

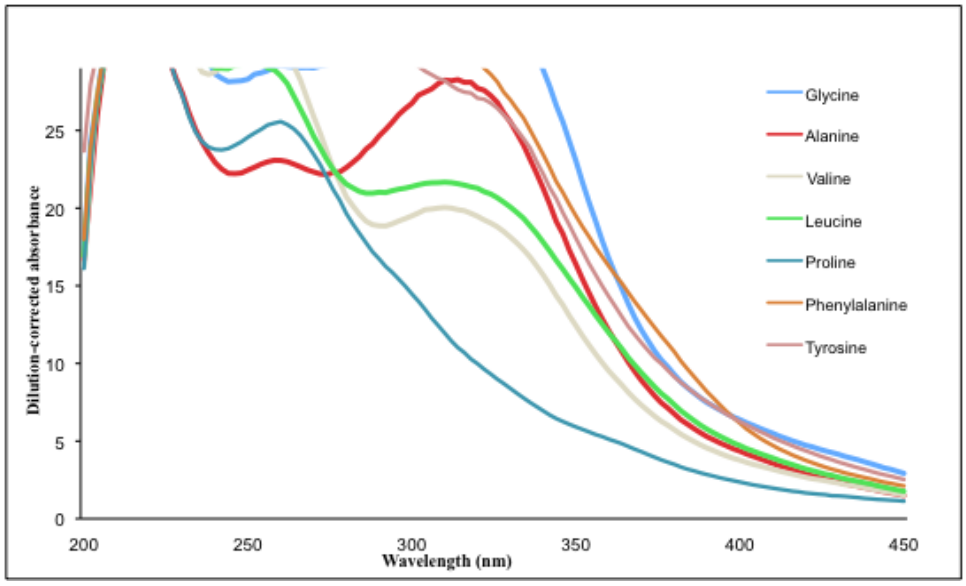


Figure 21. Wavescanning results for neutral non polar amino acid products after adjusting ethanol concentration to 50%

In the neutral non-polar amino acid group (Figure 21), absorbances were greater for the other amino acid groups. Although peaks were not identified, a comparison of Figure 17 and 18 shows that more of the ultraviolet spectrum was occupied by absorbing compounds in Figure 21.

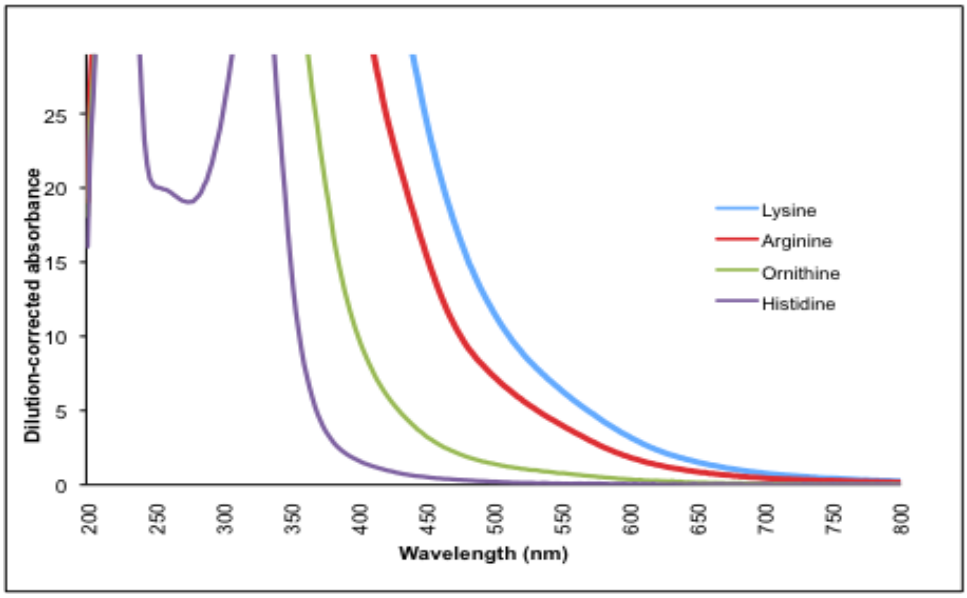


Figure 22. Wavelength scanning results for basic polar amino acid products after refluxing in a 50% ethanol mixture.

Basic amino acids appeared to be highly reactive, with absorbances spreading into the visible range to a great extent. Therefore in Figure 22, wavelength axis is scaled to 800 nm. If this adjustment is taken into account, lysine and arginine can be seen to absorb massively in the ultraviolet range. The overall pattern of absorbance appeared similar within this family of amino acids. As judged by absorbance, the sequence of

increasing reactivity from these data is: histidine, ornithine, arginine, and lysine. Although no photograph was available for the lysine treatment, the sequence of increasing reactivity appears to match that in Table 12.

The general observation from these results was that groups of amino acids react similarly. Based on the results for colour and odour after reflux, and results for wavelength scans, acidic amino acids appeared to be the least reactive. Neutral polar amino acids were slightly more reactive then the acidic amino acid groups, neutral non-polar amino acids were more reactive then both of these groups and produced odours and colours that were judged to be suitable for flavouring ethanol to produce a spirit. Most of the amino acids in the basic amino acid group were the most reactive of all however the odours produced were biscuity and while results for colour were relatively good, the odours produced were not attractive.

The results for the wavelength scans loosely correlate to the results for odour and colour in Table 12. The more reactive amino acids generally display higher peaks for absorbance and those amino acid treatments with more intense colours show that they have some level of absorbance at longer wavelengths. The wavelength scanning results were not considered to be factors that affected the choice of amino acid for flavouring ethanol to create a spirit but were of academic interest.

4.3 Determination of optimum length of reaction time for creating suitable Maillard reaction products.

The results of this experiment showed that there were four amino acids that had the potential to produce Maillard reaction products which produced likable ethanolic spirits. These amino acids were alanine, leucine, valine and phenylalanine and all happen to be neutral and nonpolar.

4.3.1 Method

The volume and mass of the reactants were doubled to produce a greater volume of the final product so that a number of assessments could be made. The ratio of the lactose to amino acids was still 1:1. The reactants were allowed to reflux for 180 minutes in total. The length of time was limited to 180 minutes because this was judged as being a long enough reflux time to show the effect of time on the reaction and refluxing for longer than this did not appear to be necessary. Twenty millilitre aliquots were taken by a pipette after 60, 120 and 180 minutes without stopping the reflux to

demonstrate the effect of time. These aliquots were cooled immediately on ice to halt the reaction. Aroma, and observed colour were assessed by the researcher, pH was measured using a Meter Lab PHM201 pH meter (Radiometer, Copenhagan). After setting a baseline with deionised water, the diluted filtrates (1:10) were wavelength-scanned from 200 to 800 nm in 3 mL quartz curvettes using a 'Spectro' Ultraspec. 2100 pro (Armasham Biosciences, U.K.). The absorbances were captured by computer using BioDC version 2.0 software (Biochrom Ltd), the output of which is in the form of an Excel spreadsheet. Fifty percent ethanol solutions of the aliquots were prepared after 180 minutes and visual colour, odour and wavelength scans were repeated as above. Fifty percent ethanol solutions of the samples taken at 60 and 120 minutes were not prepared because of resource constraints at the time. Because 50% ethanol spirits were prepared in the first amino acid experiment after refluxing for 90 minutes the 60 and 120 minute results were not deemed as being crucially important by the researcher. Graphs of the absorbances from each of the time points were plotted using Microsoft Excel

4.3.2 Results and discussion

All treatments were clear and colourless before reflux. Phenylalanine was difficult to dissolve in de-ionised water but dissolved with vigorous stirring. Once the phenylalanine treatment was slightly warmed to approximately 30 °C all of the amino acid powder had dissolved.

Table 13. Results for determination of optimum length of reaction time for creating suitable Maillard reaction products.

	Time (mins)	pH	Colour	Odour	pH of EtOH spirit	Odour of 50% EtOH spirit
Control	60	5.19	Very faint/light peach/brown colour	Very slight sweet odour		
	120	4.44	No obvious colour change from T60	Slightly more intense		
	180	4.89	Slightly lighter in colour then previous samples	Very faint sweet aroma, less than previous sample	8.27	None other then EtOH
Alanine	60	8.09	Yellow/orange	Slight/faint sweet aroma		
	120	6.84	Dark orange	Very very faint sweet aroma		
	180	5.59	Dark red/maroon colour	Very very faint sweet aroma	6.20	None other then EtOH
Leucine	60	8.28	Dark yellow	Solvent smell, slightly sweet		
	120	8.00	Darker yellow than T60 leucine sample	Faint sweet aroma, ammonia/solvent notes detected		
	180	7.39	Dark orange, darker than T120	Savoury/pastry/cracker aroma	7.24	Slightly sweet, solvent like
Phenylalanine	60	8.62	Faint/light yellow colour	Flowery/ potpourri		
	120	8.35	Darker/brighter/more intense yellow colour	Stronger flowery aroma, some sweetness detected		
	180	8.41	Intense dark orange	Very intense flowery/potpourri aroma, slightly sweet	8.32	Flowery notes, slightly sweet
Valine	60	8.37	Dark yellow	Ammonia smell detected		
	120	7.94	Dark yellow	Faint sweet smell, ammonia smell gone		
	180	7.43	Dark orange, darker than T120	Pastry aroma	7.18	Slightly sweet

pH of most of the treatments decreased from a starting pH of 9 to approximately 7 or 8 after refluxing for 180 minutes. The pH of the control decreased from pH 9 to 5.19 after 60 minutes, and decreased further to 4.44, however the pH then increased to 4.89 after 180 minutes. If the reflux was allowed to continue perhaps the pH would have increased even further. The decrease in pH is most likely to be the result of the development of saccharinic acids. The increase in pH could be caused by the production of basic coloured compounds or the breakdown of saccharinic acid (Belitz, Grosch, & Schieberle, 2009) and other acidic products. Interestingly, the pH of the T180 control sample increased significantly from 4.89 to 8.27 after addition of ethanol to create a 50% ethanol solution. This could have been due to an instrument error. Alanine showed the greatest decrease in pH of all the amino acids after 180 minutes, which is likely to be due to the development of basic Maillard reaction products. The pH of the T180 sample increased from 5.59 to 6.20 after the addition of ethanol to create the 50% mixture. The leucine and valine treatments showed similar results for changes in pH, the pH of both of these treatments decreased over time to final pH's of 7.39 and 7.43 respectively. Both leucine and valine showed decreases in pH after the addition of ethanol. The pH of the phenylalanine decreased slightly from a starting pH of 9 to 8.41 after refluxing for 180 minutes and decreased further when ethanol was added and a 50% ethanolic alcoholic spirit was created.

The pH of the alanine, leucine and phenylalanine treatments all decreased after the addition of ethanol. Ethanol is relatively neutral therefore this result was quite unexpected.

All treatments were clear and colourless before the start of reflux. Intensity colour of all of the treatments apart from the control increased with reflux time. This was expected because an increased reaction time is almost always associated with an increased yield of products.

The colour of the lactose treatment increased from the start of reflux to 60 minutes, then showed no change after 120 minutes of reflux, and then decreased in colour intensity after 180 minutes. Lactose could have reacted differently because it is undergoing caramelisation rather than the Maillard reaction. It is possible that the yellow and brown coloured compounds may have broken down and taken part in other reactions sometime between 60 and 120 minutes.

Results for aroma over reflux time demonstrate the complexity of the production of volatile compounds as a result of the Maillard reaction. The treatments had no detectable aroma before the start of refluxing.

The odour of the control increased in intensity of sweetness from 0 to 120 minutes and then decreased again at 180 minutes. The decrease in odour could be due to the breakdown of the sweet smelling volatile compounds produced as a result of caramelisation. The results for colour showed similar results. It is possible that the products of caramelisation are taking part in further reactions after 120 minutes.

Alanine developed a faint sweet aroma, which did not increase much over time, and when ethanol was added to the T180 sample the researcher could not detect the volatile Maillard reaction products. Leucine developed some sweet and solvent like notes, after 180 minutes of refluxing the Maillard reaction products from the leucine treatment developed a savoury and cracker like odour which was quite similar to the odours produced by some of the basic amino acid treatments in the preliminary experiment. The ethanolic spirit created using the T180 sample was still rather sweet and solvent like and not unlike some of the alcoholic spirits currently on the market. Phenylalanine produced very desirable results, the intensity of the characteristic flowery aroma increased with reflux time and the development of a slightly sweet odour also occurred which were still detectable after the addition of ethanol. The results for odour of valine treatment was interesting, ammonia like notes were detected at 60 minutes of reflux time, which then disappeared after 120 minutes, and a pastry like odour was detected after 180 minutes of reflux. The addition of ethanol to the T180 sample of the valine treatment resulted in a slightly sweet smelling spirit. The results for odour of the ethanolic spirits of the leucine, phenylalanine and valine treatments were deemed ideal because the odours produced as a result of the Maillard reaction were still detectable after the addition of ethanol.

The development of sweet odours may be due to caramelisation while the development of flowery or cracker and pastry like odours may be occurring as a result of the Maillard reaction. The reason for some of the treatments having both sweet and biscuit like odours could be the fact that both caramelisation and the Maillard reaction are occurring simultaneously. The sweet notes detected in the alanine, leucine and valine treatments also decreased with reflux time, a similar observation was made with the control as well. This could reinforce the fact that some of the caramelisation products undergo very complex reactions over time.

The results of this experiment showed that the intensity of the aroma and colour increased with time allowed for refluxing. It was decided that the optimum length of time for the Maillard reaction to produce likable products for flavouring ethanol was 180 minutes. After 180 minutes the results gave stronger odours and the intensity of the colours also increased.

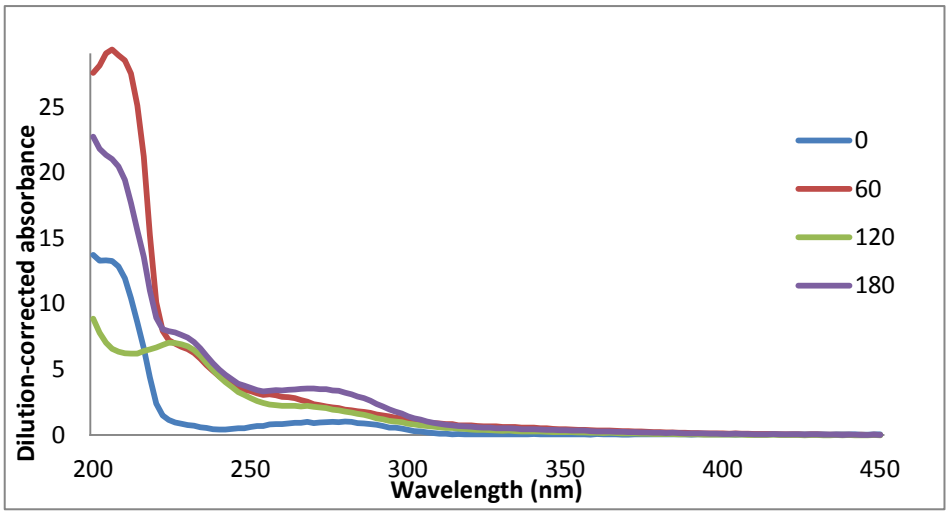


Figure 23. Wavescanning results for control over 180 minutes reflux

Figure 23 shows the wavescanning results for the control taken before reflux (0 minutes), and then after 60, 120, and 180 minutes of refluxing. Each time point showed a peak at approximately 210 nm. At zero time was displayed a peak at approximately 200 nm with an absorbance of approximately 14. After 60 minutes of refluxing the peak increased by approximately 15 unites to 29. Absorbance decreased again after 120 minutes. Absorbance then increased again after 180 minutes. A number of compounds appear to be produced after 60 minutes of reflux however it is likely that they may break down at some point between 120 and 180 minutes of reflux and possibly take part in other reactions.

The wavescanning results of the alanine treatment (Figure 24) over time show a general increase in absorbance with reflux time. All time points show the highest peaks at below 200nm which is not visible to the human eye. The 60 and 120-minute samples display second peaks at a wavelength of approximately 320 nm with a slight decrease at 180 minutes. Visible colour also increased in intensity with reflux time therefore the results for wavescanning and visible colour correlate and re-enforce the importance that reaction time has on Maillard reaction product rate.

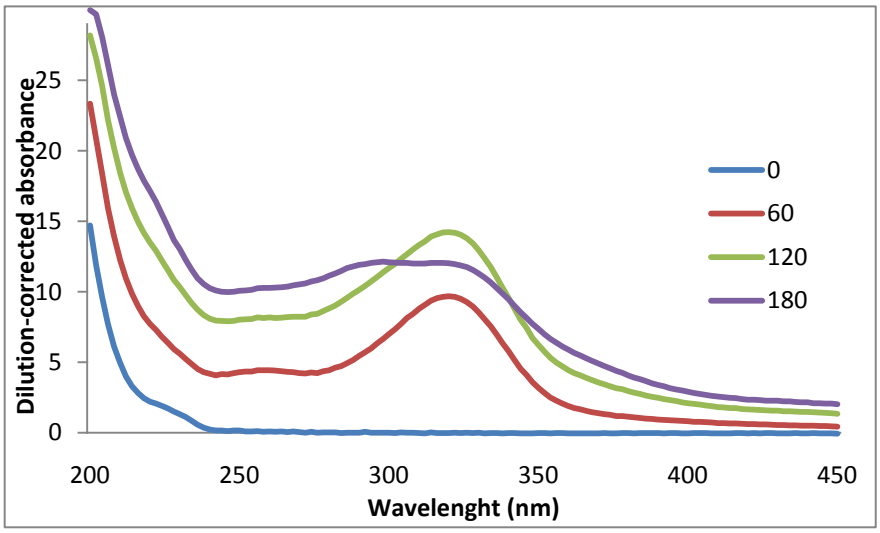


Figure 24. Wavescanning results for alanine over 180 minutes reflux

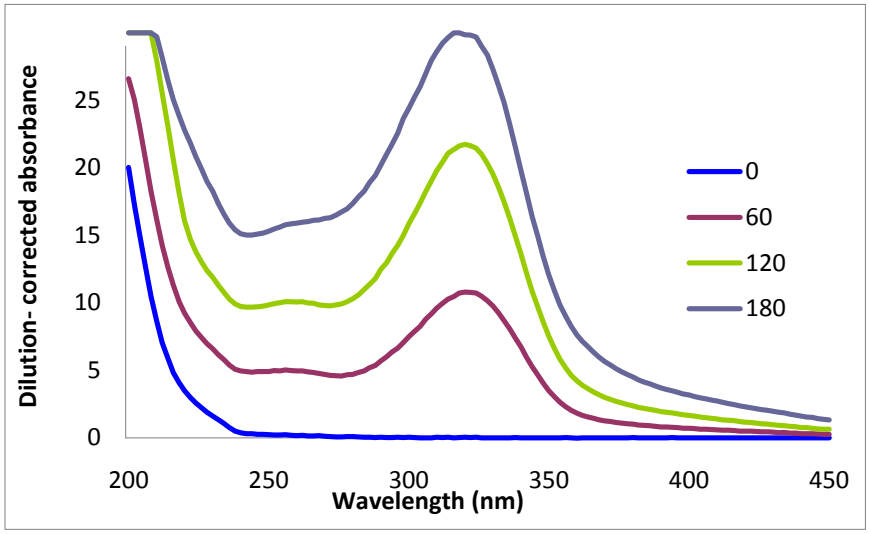


Figure 25. Wavescanning results for leucine over 180 minutes reflux

The wavescanning results for absorbance of leucine (Figure 25) over time show an increase absorbance with reflux time. All time points show peaks at 200nm or less, and second peaks at approximately 325 nm. These results were expected.

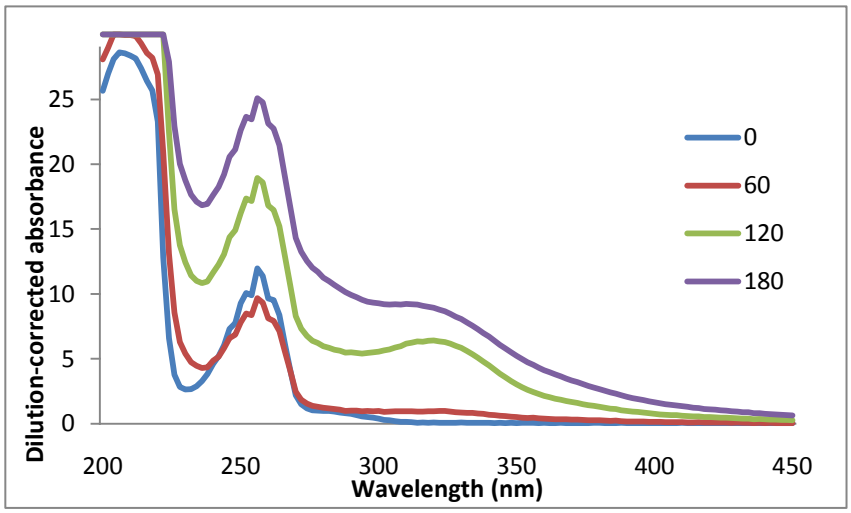


Figure 26. Wavescanning results for phenylalanine over 180 minutes reflux

The absorbances from each of the time points (Figure 26) (including zero time) display peaks at approximately 210 nm and then again at approximately 260 nm. As reflux time increases the absorbance peaks increase.

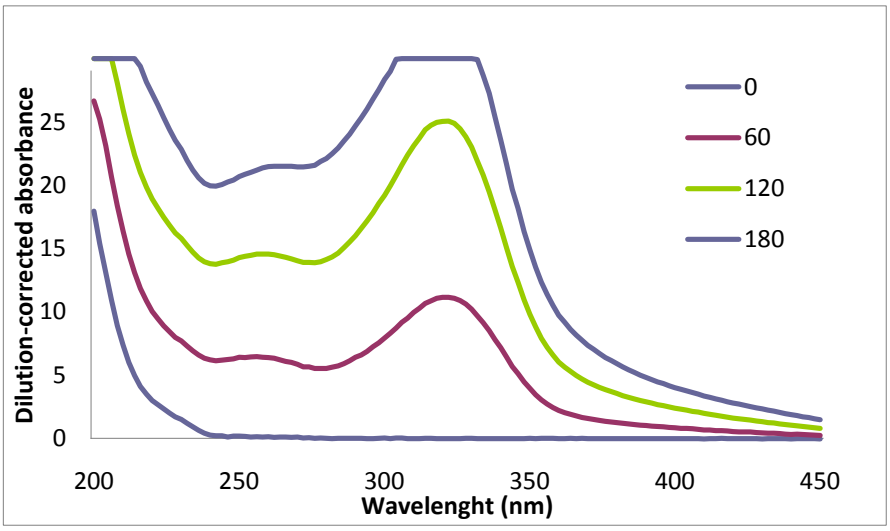


Figure 27. Wavescanning results for valine over 180 minutes reflux

As with the leucine and phenylalanine treatments, as reflux time increased the absorbances also increased (Figure 27). The valine treatment samples from all of the time points showed high peaks at less than 200 nm and then peaked again at 325 nm.

All of the treatments showed a general decrease in pH with reflux time, and an increase in intensity of colour and odour. Some of the sweet odours smelt in some of the treatments were lost after between 120 and 180 minutes of reflux and this observation was also seen in the results for the control containing lactose alone.

The control containing lactose alone exhibited quite unique behaviour because as reflux time increased the intensity of colour and odour actually decreased. A possible reason for this could be the breakdown of a number of the caramelisation products from this reaction.

Odours changed significantly after the addition of ethanol. The faint sweet odours of the control and alanine treatments were lost after the addition of ethanol however because these odours were faint after 180 minutes of reflux this result was not unexpected. The biscuit like odours of the leucine and valine were lost after the addition of ethanol and the final spirits created were rather pleasant and slightly sweet and deemed acceptable for an alcoholic spirit by the researcher because they were similar to products that are already on the market. Phenylalanine was deemed to have the most unique odour of all of the treatments even after addition of ethanol. The 50% ethanolic spirit created using phenylalanine had quite a strong flowery aroma with some sweetness, which was rather pleasant and was also very unique. There are currently no spirits on the market that exhibit this type of odour.

It was then decided that the products resulting from the Maillard reaction with phenylalanine were the most likable in terms of both colour and aroma when used to create a 50% ethanolic solution. For this reason further work was carried out using phenylalanine.

4.4 Refluxing phenylalanine in the presence of various concentrations of ethanol to create Maillard reaction products.

Phenylalanine was found to be one of the most likeable amino acids to use for flavouring ethanol to create an alcoholic spirit. The odour produced by phenylalanine was flowery and rather unique compared to spirits that are currently on the market. Knerr, Lerche, Pischetsrieder, & Severin (2001) conducted an experiment using D-glucose as a source of cabohydrates and various amino acids and found that Maillard browning was favoured at low water activity. The next experiment involved refluxing phenylalanine in ethanol to observe what the outcome would be. The break down of lactose will produce galactose and glucose therefore the results of the study conducted by Knerr, Lerche, Pischetsrieder, & Severin (2001) indicate that refluxing lactose and the amino acid in the presence of ethanol may produce a high concentration of Maillard reation products. If this experiment produced an ideal result this would mean that a final Maillard reaction flavoured spirit would be produced in one less step then the

previously trialled methods which in turn means that the process would involve the use of less resources and time and could be more economical.

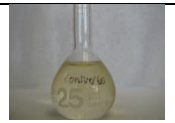
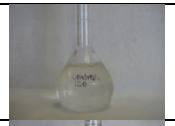
The outcome of this experiment would be quite interesting because low water activity and an increase in pH typically increases the rate of the Maillard reaction. However, the presence of ethanol will cause the temperature of the reaction conditions to decrease. It is difficult to predict whether the effect of low water activity and elevated pH will be more influential on the rate of reaction then the lowered temperature caused by the presence of ethanol. The other issue with this experiment is that the intermediate stage of the Maillard reaction involves the loss of water, therefore at high water activities the reactants will be dilute which slows the rate of reaction down, while on the other hand a low water activity the concentration of the reactants decreases however the reactants lose their mobility (Nursten, 2005). The optimum water activity would be one where there is enough water to allow the reactants to be mobile without causing the reactants to be too dilute.

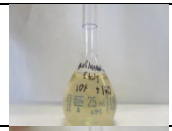
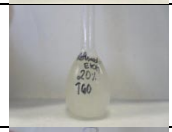
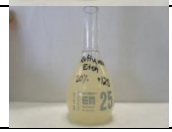
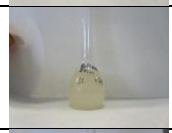
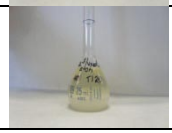
4.4.1 Method

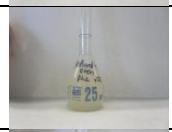
The concentrations of phenylalanine and lactose were the same as they were in the previous experiments, each of the treatments contained 3.25M of phenylalanine and lactose. Solutions with the final concentrations of 10%, 20%, 30%, 40% and 50% ethanol in de-ionised water were made up. Treatments were prepared as they were in previous experiments by addition of phenylalanine and lactose to 130mL of solutions. The pH was adjusted to 9 and the treatments were then made up to 140mL by the addition of the rest of ethanol/deionised water solutions. Two control experiments were also conducted at the same time. Control 1 contained 3.24M of lactose dissolved in a 50% ethanol: water solution. The purpose of this control was to observe the extent of the reaction in the absence of phenylalanine because the effect of water activity would be demonstrated if the results from this control were to be compared to the 50% ethanol treatment. The second control contained lactose only, no ethanol or phenylalanine was added to this treatment

4.4.2 Results and discussion

Table 14. Results for refluxing phenylalanine in the presence of various concentrations of ethanol to create Maillard reaction products.

	Time Point (minutes)	pH	Aroma	Colour	Photo
Control Lactose only in 50% EtOH (no Phe)	60	6.72	Strong EtOH aroma, no other aroma	Very very pale yellow	
	120	7.84	Strong EtOH aroma	Still very very pale yellow	
	180	6.27	Strong EtOH aroma	No significant colour change from T60 sample	
Control Lactose only in water (no EtOH or Phe)	60	5.05	Faint sweet aroma	Very very slight yellow tinge, almost clear/colourless	
	120	4.76	Faint sweet aroma, a little stronger then T60 but not much	Not much colour change from T60 sample	
	180	4.37	Burnt, slightly sweet aroma	Very pale yellow, slightly darker then T60 and T120 samples	
10% EtOH	60	8.58	Pleasant flowery aroma, cannot detect EtOH aroma	Very pale yellow, colourless with a tinge of yellow.	

	120	8.57	Flowery aroma, no EtOH aroma detected	Pale yellow, slightly darker then the T60 sample	
	180	8.19	Flowery aroma, quite strong and pleasant, EtOH aroma not detected	Medium yellow, slightly bright	
20% EtOH	60	8.84	Faint flowery aroma, very slight hint of EtOH aroma	Clear/colourless with a yellow tinge	
	120	8.74	Flowery aroma, very slight EtOH detected	Pale yellow, darker then T60 sample (slightly more pale then T120 10% sample)	
	180	8.53	Flowery aroma and EtOH aroma are both obvious	Medium yellow, lighter then 10% sample	
30% EtOH	60	8.81	Faint flowery aroma, EtOH aroma stronger than the 20% sample	Colourless/very very slight yellow colour	
	120	8.71	Faint flowery aroma, EtOH aroma is still strong, overpowers flowery aroma	Very pale yellow, lighter then 10% and 20% samples	
	180	8.50	Strong EtOH aroma, very very faint flowery aroma	Yellow, not dark	
40% EtOH	60	8.60	Flowery aroma, faint EtOH aroma	Very slight yellow tinge, slightly darker then control 2 T60	
	120	8.55	EtOH aroma quite strong, overpowers the faint flowery aroma	Slightly more yellow then T60 sample, transparent pale yellow	

	180	8.27	Strong EtOH aroma, completely overpowers any flowery aroma	Medium yellow, bright, darker then T60 and T120	
	60	8.67	Strong EtOH aroma , very faint flowery aroma	No significant colour change, very very pale yellow	
	120	8.67	Strong EtOH aroma, flowery aroma also detected	Slightly darker yellow colour compared to t60	
	180	8.32	Flowery aroma, EtOH aroma was no longer very strong	Slightly brighter yellow colour compared to T60 and T120- straw yellow	

The results from this experiment showed that at higher ethanol concentrations, the water activity was low enough to significantly decrease the rate of the Maillard reaction significantly. All samples other then control 1 containing 50% ethanol/water and lactose only began to exhibit cloudiness after they were allowed to stand for 20 minutes. This could be because phenylalanine has low solubility in ethanol.

.The pH of control 1 containing lactose and ethanol decreased from a starting pH of 9, to 6.72 after reflux- a decrease of 2.28 units. pH then increased after 120 minutes by 1.12 units and then decreased again after 180 minutes. It is difficult to predict whether the pH would have decreased or increased if the reflux was allowed to continue. Control 2, which contained lactose alone decreased in pH by 4 units and then steadily decreased as the reflux proceeded. The presence of ethanol in control 1 had an inhibiting effect on the caramelisation reaction because the pH of this reaction did not decrease as much as the pH for the control 2 containing no lactose did.

There was no significant decrease in pH of the treatments containing 10 %, 20%, 30%, 40% or 50% ethanol after reflux. The pH's of all of the reactions decreased by only 1 unit even after 180 minutes of reflux from 9 to 8. These results suggest that the presence of ethanol in the reaction has an inhibitory effect on the rate of the Maillard reaction.

Control 1 produced ethanol like odours after reflux; there was no evidence of the characteristic sweet notes of caramelisation while control 2 produced some sweet odours. The treatment containing 10% ethanol showed an increase in pleasant flowery odour with reflux time while the odour of the ethanol remained undetected. The treatment containing 20% ethanol showed an increase in both the flowery and ethanol odours with reflux time. The 30% and 40% ethanol treatments behaved similarly in terms of odour. For both of these treatments the strength of ethanol odour overpowered the strength of the flowery odour. The 50% ethanol treatment showed an increase in ethanol odour after 60 and 120 minutes of reflux however after 180 minutes of reflux the ethanol odour disappeared and the intensity of the flowery aroma appeared to increase. It is difficult to tell whether the decrease in the strength of the odour of ethanol increased or if indeed the flowery odour did increase in intensity.

The results for the colour of control 1, which contained 50% ethanol/water and lactose, were similar to the results for the control 2, which contained lactose alone

however the treatments containing lactose alone produced slightly darker yellow colours.

As the concentration of ethanol in the reaction increased, the intensity of the yellow colour decreased. This indicates that the ethanol may have lowered the temperature of the reaction and inhibiting the Maillard reaction from occurring.

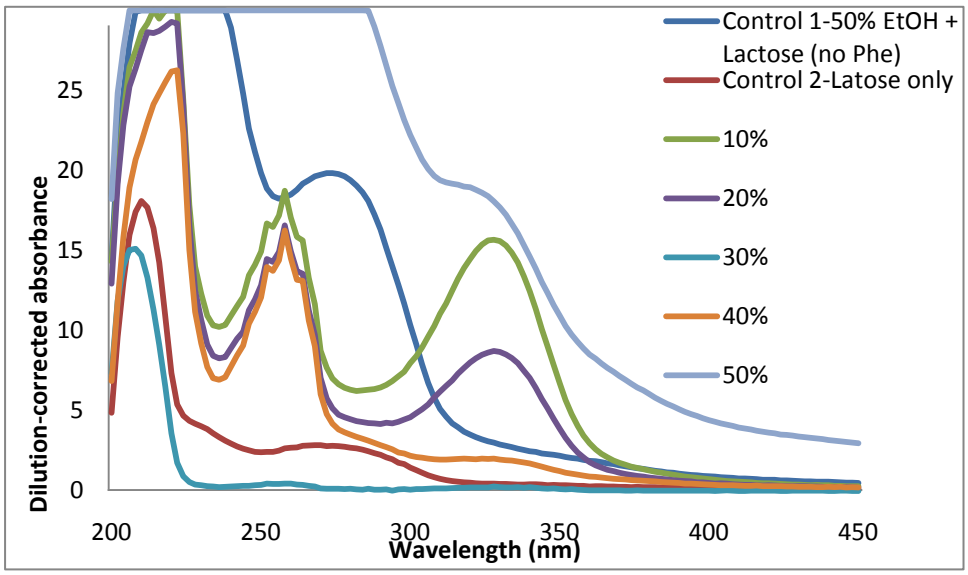


Figure 28. Wavescanning results for the treatments after refluxing in ethanol for180 minutes.

Figure 28 shows that control 2 which contained lactose alone, and the treatment containing 30% ethanol resulted in quite similar results. Because one of the observations made was that the products became cloudy after being allowed to stand and cool after reflux these wavescanning results are likely to be inaccurate. This data is included in Appendix 2. The particles in the solutions causing cloudiness would have interfered with the wavescans.

The results from refluxing in the presence of ethanol were unsuccessful because the products became cloudy and unattractive on standing. At high concentrations of ethanol the temperature of the reaction was lowered so significantly that the rate of the reaction decreased and not enough Maillard reaction products were produced to create the desirable colour and odour.

4.5 Refluxing valine, leucine and phenylalanine in the presence of 0.2M of lysine to observe the effects lysine has on colour

The results from the preliminary experiment showed that lysine produced the most intense result in terms of colour; however the odour of this treatment was not ideal. Arginine also produced a suitable intense colour; however the odour was not ideal for

flavouring ethanol. The next step was to observe the effect that the addition of small amounts of lysine would have when added to treatments which also contained a 1:1 ratio of the amino acid and lactose. Treatments were prepared in the same way as previous experiments however 0.2M of lysine was also added to each treatment.

4.5.1 Method

A 7 M solution of lactose was prepared by dissolving lactose in its powder form in de-ionised water. Solutions or solution/suspensions of amino acids were also prepared in the same way, also to 7 M. Equal volumes of 130 mL of lactose and amino acids were combined, resulting in final concentrations of 3.5 M. The pH was then adjusted to 9 using 1M NaOH and was measured using a Meter Lab PHM201 pH meter (Radiometer, Copenhagen). After the pH had been adjusted, enough deionised water was added to the flasks so that the final volume of the reactant was 280 mL, each reactant being nominally 3.25 M if in solution. 0.2M of lysine was also added to the reaction.

After 60, 120 and 180 minutes of reflux with antibumping chips, the mixtures were assessed by the researcher for odour and visual colour. All mixtures were filtered through Whatman No. 1 paper on a Buchner water vacuum funnel.

Prior to wavelength profile scanning the filtrates were diluted 1:10 solutions with deionised water. This was done because some of the colours were intense to the point that absorbance at some wavelengths were predicted to have exceeded the allowable 0 to 3 absorbances. After setting a baseline with deionised water, the diluted filtrates were wavelength-scanned from 200 to 800 nm in 3 mL quartz curvettes using a 'Spectro' Ultraspec. 2100 pro (Amersham Biosciences, U.K.). The absorbance were captured by computer using BioDC version 2.0 software (Biochrom Ltd), the output of which is in the form of an Excel spreadsheet. Wavelength scans are routinely presented corrected for dilution.

Fifty percent ethanol solutions of each of the undiluted filtrates were made up by mixing 25 mL of filtrate with 25mL of 99% ethanol. Odour and colour were re-assessed by the researcher. This was done because the compounds in the original filtrate were likely to exhibit a different volatility in ethanolic solution, depending on the relative vapour pressures of each Maillard compound in water compared with 50% ethanol. For rescanning the ethanolic solutions were diluted 1:10 with a 50% ethanol: water mixture using a 1:10 dilution of the mixture to zero the spectrophotometer.

The amino acids treatments involved in this experiment were leucine, phenylalanine and valine. A control experiment involving refluxing lactose alone was also conducted.

4.5.2 Results and discussion

The control and leucine experiments were conducted on one day and the phenylalanine and valine experiments on the next. Because the results were not deemed ideal for these three amino acids the researcher decided not to carry out the experiment with the alanine treatment. Table 15 shows the results for this experiment.

Although results for colour were good, biscuit like odours were still detected in each of the samples. The control reacted as expected however an experiment containing lactose and 0.2M lysine should have been conducted. The ratio of lactose to amino acid was 1:1, and 0.2M of lysine was also added to the reaction which means that lactose had a greater likelihood of reacting with any of the two amino acids included in the treatment. Because of this, the development of more intense odours and colours as a result of the Maillard reaction were expected. Unfortunately due to technical errors the photographs of the results were lost and could not be included in the table of results (Table 15).

The results for pH changes with reflux time in the amino acid treatments containing lysine were not significantly different from the results for pH observed in the determination of optimum length of reaction time for creating suitable Maillard reaction products which was conducted previously. The addition of ethanol caused the pH of the treatments to increase which was quite unexpected because in the previous experiments, adding ethanol to the treatments post-reflux actually caused a decrease in pH. The reason for the slight decrease is not known.

The results for colour of all of the treatments were quite similar. All three treatments were colourless before reflux and developed yellow tones after 60 minutes of reflux. After 120 minutes the development of brown and red tones were apparent, and after 180 minutes dark brown/red/maroon colours developed. The red tones were similar to those produced by the lysine treatment when the preliminary experiment was conducted.

Table 15. Results for refluxing valine, leucine and phenylalanine in the presence of 0.2M of lysine to observe the effects lysine has on colour				
	Time (mins)	pH	Colour	Aroma
Control	60	5.19	Very faint/light peach/brown colour	Very slight sweet odour
	120	4.44	No obvious colour change from t60	Slightly more intense
	180	4.89	Slightly lighter in colour than previous samples	Very faint sweet aroma, less than previous sample
	50% EtOH	8.62	Very faint with yellow tint	None other than EtOH
Valine	60	8.51	Dark yellow	Biscuit/pastry aroma
	120	7.88	Medium brown	Biscuit/pastry aroma
	180	7.28	Dark brown/maroon/reddish	Savoury/cracker aroma, slightly sweet
	50% EtOH	7.56	Dark brown but lighter than t180	Solventy, slightly sweet
Leucine	60	8.61	Dark yellow	Damp/ammonia notes
	120	8.09	Medium brown with reddish tones	Sweet aroma, slight biscuity aroma
	180	7.26	Dark brown/maroon/reddish	Cracker/savoury aroma
	50% EtOH	7.52	Same colour as valine EtOH solution	Solventy/sweet aroma, slight savoury aroma
Phenylalanine	60	8.50	Yellow	Slight flowery aroma with biscuit/pastry notes
	120	8.11	Medium brown	Flowery with savoury biscuity aroma
	180	7.70	Dark brown/maroon/reddish	Faint biscuit aroma, slightly sweet, still a bit flowery

All of the treatments developed biscuit and cracker like odours after 180 minutes of reflux. This observation was also made with the lysine treatment in the preliminary experiments. Leucine developed some sweet notes after 120 minutes however the sweet notes became overpowered by the biscuit like odours after 180 minutes. Phenylalanine developed a characteristic floral odour, faint biscuit notes were detected after 120 minutes of reflux. Unfortunately, after 180 minutes of reflux the flowery odour was over powered by the biscuit/cracker like odour. The biscuit/cracker like odour was detected after only 60 minutes of reflux of the valine treatment indicating that the amino acid lysine was a lot more reactive in the Maillard reaction then valine.

In the leucine and phenylalanine treatments, the biscuit/cracker odours are detected after 120 minutes of reflux. It is possible that at some time point between 60 and 120 minutes of reflux the leucine and phenylalanine have reacted completely and therefore lysine begins to react with the lactose at this time point, allowing the development of the odours that are characteristic of the lysine treatment.

A slight biscuit/savoury like odour still remained after the addition of ethanol to the leucine and phenylalanine treatments. A slight sweet note was detected in the valine treatment after addition of ethanol however the result was still rather solvent like and unattractive.

The results for colour in this experiment were ideal. The dark brown/maroon and reddish tones were attractive and characteristic of many of the alcoholic spirits currently on the market. Unfortunately the biscuit and cracker like odours produced were unattractive, therefore this experiment was deemed to be not ideal by the researcher. Lysine favours the Maillard reaction therefore lysine was the preferred amino acid for taking part in the reaction. The extent of reaction of the other amino acids present would have been relatively low compared to that of lysine. Wavescanning results showed an increase in absorbance with reflux time which was expected (Appendix 3). The next step was to then observe what the effects of lowering the concentration of lysine in the reaction

4.6 Refluxing phenylalanine in the presence of various concentrations of lysine for colour

As the results for phenylalanine were deemed to be quite ideal in terms of flavour, the idea of adding an additional lysine to increase the intensity of the colour after refluxing was trialed. While phenylalanine produced quite pleasant results for odour

after refluxing, an increase in the intensity of colour would produce an alcoholic spirit with a more intense colour and may be perceived by the consumer as having a more intense flavour. Lysine produced the darkest and most intense colour therefore lysine was chosen as the additional amino acid. The previous experiment involved refluxing leucine, phenylalanine and valine in the presence of 0.2M of lysine. Although colours produced were ideal, odours were rather biscuit like and unattractive therefore the concentration of lysine in the reaction was thought to have been too high. The next step was to experiment with lower concentrations of lysine in the reaction to see whether the desirable dark brown/maroon colours would still develop while eliminating the biscuit like odour. The treatments for this experiment involved lactose, phenylalanine and addition of the following concentrations of lysine- 0.2M, 0.1M, 0.05M and 0.025M.

4.6.1 Method

A 7 M solution of lactose was prepared by dissolving lactose in its powder form in de-ionised water. Solutions or solution/suspensions of amino acids were also prepared in the same way, also to 7 M. Equal volumes of 130 mL of lactose and amino acids were combined, resulting in final concentrations of 3.5 M. Lysine was then added to each of the dispersions so that each of the solutions had a final lysine concentration of 0.2M, 0.1M, 0.05M and 0.025M. The pH was then adjusted to 9 using 1M NaOH and was measured using a Meter Lab PHM201 pH meter (Radiometer, Copenhagen). After the pH had been adjusted, enough deionised water was added to the flasks so that the final volume of the reactant was 280 mL, each reactant being nominally 3.25 M if in solution.

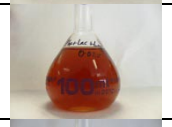
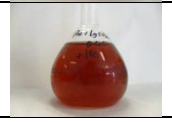
After 60, 120 and 180 minutes of reflux with antibumping chips, the mixtures were assessed by the researcher for odour and visual colour. All mixtures were filtered through Whatman No. 1 paper on a Buchner water vacuum funnel.

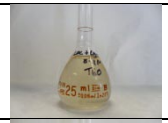
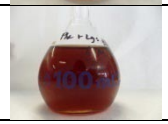
Prior to wavelength profile scanning the filtrates were diluted 1:10 solutions with deionised water. This was done because some of the colours were intense to the point that absorbances at some wavelengths were predicted to have exceeded the allowable 0 to 3 absorances. After setting a baseline with deionised water, the diluted filtrates were wavelength-scanned from 200 to 800 nm in 3 mL quartz curvettes using a ‘Spectro’ Ultraspec. 2100 pro (Amersham Biosciences, U.K.). The absorbances were captured by computer using BioDC version 2.0 software (Biochrom Ltd), the output of which is in the form of an Excel spreadsheet. Wavelength scans are routinely presented corrected for dilution.

Fifty percent ethanol solutions of each of the undiluted filtrates were made up by mixing 25 mL of filtrate with 25mL of 99% ethanol. Odour and colour were re-assessed by the researcher. This was done because the compounds in the original filtrate were likely to exhibit a different volatility in ethanolic solution, depending on the relative vapour pressures of each Maillard compound in water compared with 50% ethanol. For rescanning the ethanolic solutions were diluted 1:10 with a 50% ethanol: water mixture using a 1:10 dilution of the mixture to zero the 4.6.2 Results and discussion.

While the results for colour from these experiments were ideal the results for odour were unpleasant. The presence of lysine in the treatment caused the development of rather biscuit like odours, which were deemed to be unpleasant for flavouring ethanol by the researcher, therefore no further work with lysine was carried out. Wavescanning results (Appendix 6) showed an increase in absorbance over reflux time however there were no significant differences between the treatments containing differing concentrations of lysine. This indicates that lysine is highly reactive with lactose and Maillard reaction products created by lysine are extremely intense in colour.

Table 16. Results for refluxing phenylalanine in the presence of various concentrations of lysine for colour

	Time	**pH**	**Colour**	**Aroma**	**Photo**
Control (Phe alone)	60	8.62	Faint/light yellow colour	Flowery potpourri aroma	
	120	8.35	Darker/brighter/more intense yellow colour	Stronger flowery aroma, some sweetness detected	
	180	8.41	Intense dark orange	Very intense flowery/potpourri aroma, slightly sweet	
0.025M	60	8.66	Pale yellow	Sweet/biscuit/pastry/very faint flowery notes	
	120	8.38	Bright yellow	Slightly flowery	
	180	8.12	Dark orange/brown	Biscuit/pastry/sweet with faint flowery notes	
0.05M Lysine	60	8.62	Pale yellow	Sweet/slightly biscuity	
	120	8.41	Bright yellow	Biscuit/pastry/sweet. Very faint flowery notes	
	180	8.03	Brown/orange/dark	Sweet, slightly flowery, cracker like	

0.1M Lysine	60	8.74	Pale yellow	Biscuit like, stronger then 0.025M and 0.05M.	
	120	8.48	Dark yellow	Sweet/biscuit	
	180	8.03	Dark brown	Slightly sweet, faint flowery aroma	
0.2M Lysine	60	8.50	Yellow	Slight flowery aroma with biscuit/pastry notes	* Photograph lost due to file corruption issues
	120	8.11	Medium brown	Flowery with savoury biscuity aroma	Photograph lost due to file corruption issues
	180	7.70	Dark brown/maroon/reddish	Faint biscuit aroma, slightly sweet, still a bit flowery	Photograph lost due to file corruption issues

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4.7 Assessment of visible colour, wavelength and odour of chosen amino acids over 30 weeks.

Time is an important factor in the Maillard reaction. Previous experiments had shown that the Maillard reaction continues to produce Maillard reaction products as time progresses. Further experiments to observe the changes in Maillard reaction products and the effect of more long term storage were conducted. The researcher had been storing the samples from the earlier experiment involving the control, alanine, leucine, phenylalanine and valine in storage to observe any potential changes in product quality over a long length of time. Over time the changes in colour and aroma had been noted.

4.7.1 Method

Samples were then prepared represent 0,5,10 and 15 weeks of storage. The method for preparing these samples was identical to that described in the section 4.3 which was titled “Determination of optimum length of reaction time for creating suitable Maillard reaction products”. The purpose of preparing these samples was to confirm the odour and colour change observations made by the researcher by carrying out a sensory trial.

4.7.2 Results and discussion

Changes in odour and colour were observed over thirty weeks. Samples were assessed almost every week, the results for which are in Appendix 5.

The control developed a sweet odour at approximately 5 weeks, which over time changed, and the development of pungent odours began to occur. The pungent odour disappeared after 30 weeks. The odour of the alanine treatment did not change much however at 30 weeks salty notes appeared to develop. Leucine developed pleasant tequila like odour. Phenylalanine was quite pleasant and unique; the flowery odour became even stronger after 30 weeks. The valine treatment had biscuit like odours which eventually disappeared and allowed rather pleasant chocolate and rum like odours to develop. The disappearance of the biscuit like odours of valine indicates that perhaps if the samples from the experiments involving amino acids plus small amounts of lysine for colour would have been allowed to develop for a long period of the unattractive biscuit/cracker like odours would have disappeared allowing the development of more attractive odours.

Table 17. Results for changes in aroma and colour of ethanolic Maillard reaction products over thirty weeks					
	Amino acid				
	Changes in aroma				
Week	Control	Alanine	Leucine	Phenylalanine	Valine
Week 0	No aroma other then EtOH	Sweet aroma at first, then burnt notes come through	Solvent aroma, slightly sweet/biscuity	Flowery, slightly sweet, pleasant	Solvent aroma biscuity, savoury
Week 5	Faint sweet	Slightly sweet, slight EtOH aroma	Sweet, chocolate like, EtOH very very faint	Faint flowery, very slight EtOH aroma	Slight EtOH, very faint eucalyptus aroma
Week 10	Burnt caramel aroma, slightly pungent	EtOH/solvent aroma, rather spirit like	Solvent aroma, slight burnt caramel smell. Burnt smell is not particularly pleasant or pungent	Slight flowery aroma, very very faint sweet aroma	Solvent with a faint burnt toffee aroma. Slightly sweet
Week 15	Pungent, unpleasant aroma	EtOH aroma obvious, characteristic spirit	Solvent like, slightly sweet aroma-pleasant	Very faint sweet EtOH aroma, flowery smell is no longer evident-maybe lost due to transfer of contents into another flask?	Sweet aroma, pleasant and rum-like
Week 20	Burnt sugar, pungent aroma, very faint EtOH aroma	Solvent aroma, faint sweet aroma	Pleasant, sweet, very very faint EtOH aroma, slightly solvent like	Faint flowery aroma present this time, very very faint EtOH	Very sweet and pleasant, no EtOH aroma
Week 25	Sharp, pungent, unpleasant	Sweet, faint EtOH	Sweet, smooth, faint EtOH	Faint flowery odour, faint EtOH odour	Smooth, sweet, chocolate like
Week 30	Strong EtOH, very faint sweet notes	Salty notes, solvent like, slightly sweet	Typical spirit like aroma (tequila)	Strong flowery aroma, faint EtOH	Sweet caramel/rum like, pleasant

	Amino acid				
	Changes in colour				
Week	Control	Alanine	Leucine	Phenylalanine	Valine
Week 0	Very pale yellow	Dark brown	Dark brown	Dark brown	Dark brown
Week 5	Very pale yellow	Medium reddish brown	Dark brown, reddish	Medium reddish brown	Dark brown, reddish
Week 10	Almost clear/colourless	Dark yellow/orange-brown	Dark yellow	Dark yellow/orange colour	Orange/brown
Week 15	Very very pale, almost colourless with a yellow/brown tinge	Dark yellow	Dark yellow/orange. Slightly darker then leucine	Medium brown	Medium brown
Week 20	Clear, almost colourless with a tinge of brown	Yellow/orange-quite dark	Dark yellow	Dark yellow/orange/ brown tinge	Medium brown
Week 25	Clear, almost colourless with a yellow tinge	Dark yellow/orange	Medium yellow	Dark yellow	Brown/orangey
Week 30	Almost colourless, slight yellow tinge	Orange/yellow	Golden yellow	Dark yellow/gold	Light brown

Colour changes were also observed by the researcher. The control containing lactose alone lost colour over time. The reason for this is not known. All other treatments started off brown in colour, then developed red colours after 5 weeks, then gradually lightened in colour. This is unusual because coloured Maillard reaction products usually develop and darken over time. Lactose undergoes a number of reactions under heat and under alkali conditions. These reactions include Lobry de Bruyn Alberda van Ekenstein transformation and subsequent degradation, (Morales & JimeÂnez-PeÂrez, 2001), as well as the production of saccharinic acid, and various other acid compounds. Some of these products may be the brown coloured compounds that are seen and may be undergoing further reactions such as breakdown, degredation, or cleavage overtime, causing changes in colour. Hofmann (1999) found that after refluxing L-alanine and glucose for 180 minutes, the amount of the pale yellow coloured carbohydrate degradation compound glyoxal decreased, while other carbohydrate degradation prducts decreased.

The changes in the odours over time are mostly due to the Maillard reaction.

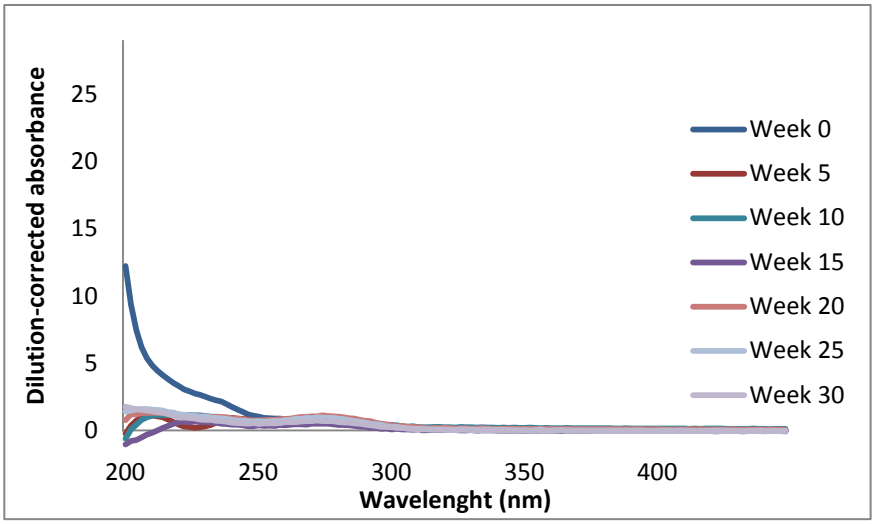

Figure 29. Wavescanning results for control over a period of thirty weeks.

The wavescanning results for control (Figure 29) have a slight correlation with the observations for colour from table 17, a more intense colour is observed at week 0 while the peak for week 0 seen on the wavescanning results for control (figure 29) is highest. The week 0 peak is the highest, while the rest of the weeks have consistently low absorbance. These results indicate that there was a low level of activity occurring during storage.

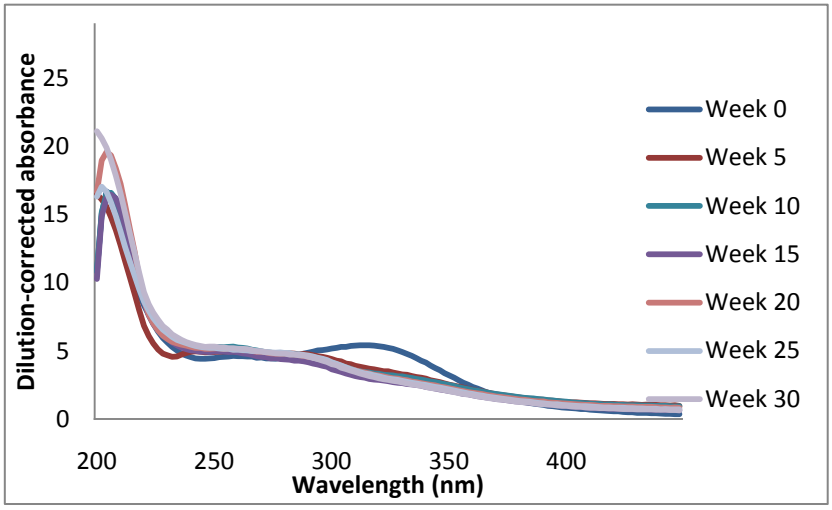

Figure 30. Wavescanning results for alanine over a period of thirty weeks.

Alanine shows no significant changes in absorbance over time (Figure 30).

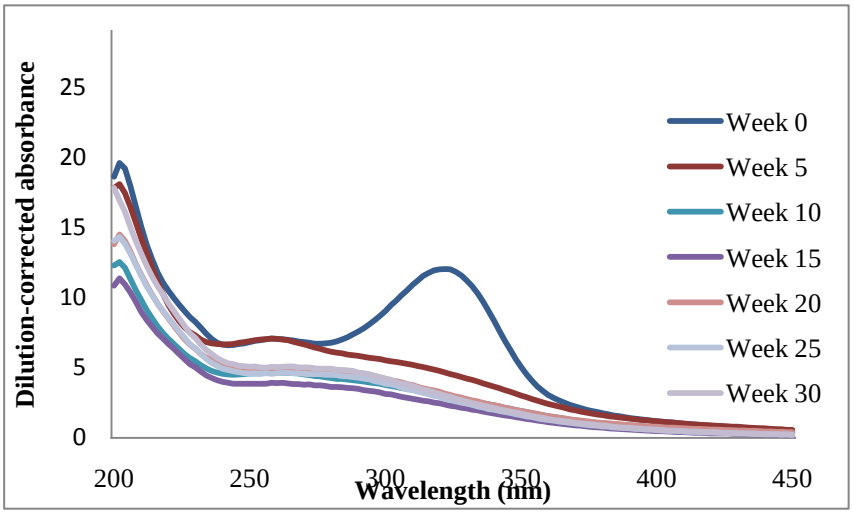

Figure 31. Wavescanning results for leucine over a period of thirty weeks.

The week 0 leucine treatment (Figure 31) showed a peak at approximately 325 nm where an absorbance of approximately 12 was observed. This peak then disappeared.

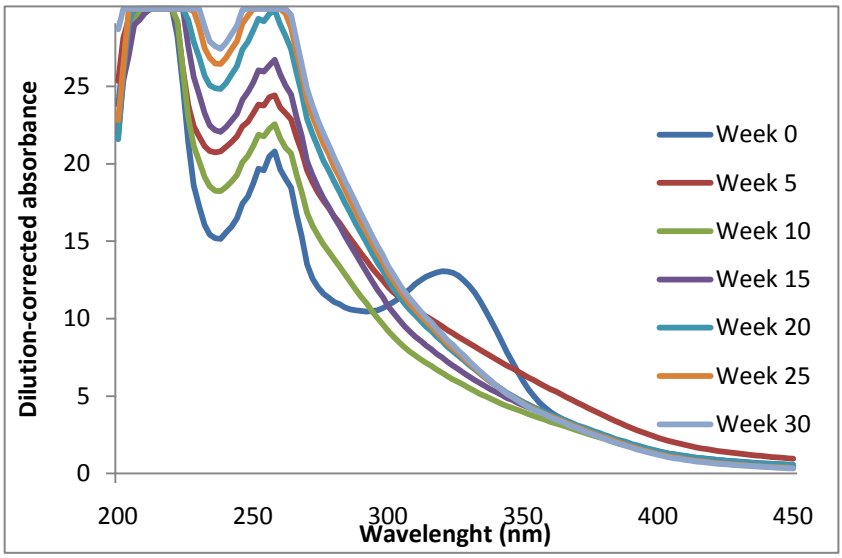

Figure 32. Wavescanning results for phenylalanine over a period of thirty weeks.

Absorbance results for the phenylalanine treatment (Figure 32) consistently increased at every time period. This indicates that the Maillard reaction continues to occur over time. The small peak seen at 325 nm at week 0 dissappeared after 5 weeks.

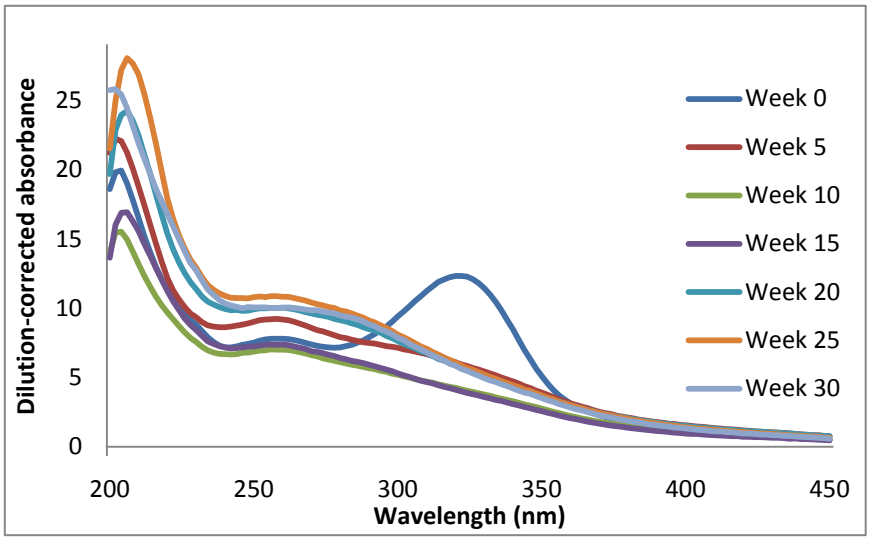

Figure 33. Wavescanning results for valine over a period of thirty weeks.

Generally absorbance for valine (Figure 33) increased over time, the peak seen at 325 nm at week 0 disappeared after 5 weeks. A similar peak was seen in the phenylalanine sample.

The colour and flavour compounds produced by the Maillard reaction in each of the treatments involving the different amino acids are the results of a number of complex chemical reactions. Stability of aroma chemicals is dependent on a number of factors

including the stability of the flavour compound itself, flavour changes during processing and storage, and reactions of flavour components such as degradation, rearrangement and/or oxidation (Rowe, 2005). There is also the possibility of evaporation of aroma compounds and in perhaps in the case of phenylalanine, crystallisation of non-soluble components. Ethanol can act as a preservative of flavour compounds however ethanol can react with aldehydes and ketones to form acetals and ketals (Rowe, 2005).

Chapter 5

Sensory Trial

5.1 Introduction

As discussed in Chapter 1, the aroma of a food contributes immensely to its perceived flavour, and much flavour information can be gained from smelling alone. In sensory science, eating and drinking as assessments methods are inherently more difficult than all other methods except perhaps assessment by touch. Vision, hearing, and smelling methods are non-contact. Assessment by orthonasal smell was chosen as the drinks were alcoholic spirits and may restrict the consumer base, for reasons including religion, age, and unfamiliarity with spirits.

5.2 Methods

5.2.1 Sample preparation

Spirits were flavoured using Maillard reaction products derived from lactose and either alanine, leucine, phenylalanine or valine. The control contained only lactose. Samples were prepared at 0, 5, 10, 15, and 30 weeks prior to the sensory trial. Thus, the total number of samples to be assessed was 25. These samples were prepared in the same way as described in Chapter 5 for the experiment in section 4.3 titled Determination of optimum length of reaction time for creating suitable Maillard reaction products.

After the samples were refluxed to create Maillard reaction products, the samples were made up to 50% ethanol solutions and stored at 32°C in a dark room for at specified storage times in 250 mL volumetric flasks with a small airspace in each flask. Colour and odour were observed on a weekly basis, and wavelength scans were conducted weekly in the same manner as described in Chapter 4 under the Development of Maillard reaction products in amino acid lactose mixtures section.

Each treatment was allocated a random three digit code, selected from telephone directory numbers, and presented in an opaque black wine glass (Figure 34) to mask colour differences. The phenomenon of associating depth of colour with intensity of

odour (or flavour) is referred to as logical error (Kemp, Hollowood, & Hort, 2009) and presenting samples in black glasses eliminated the likelihood of this occurring.

Figure 34. Black wine glass labeled with a randomised 3 digit number for treatment. The colour of sample was masked

5.2.2 Recruitment of consumers

Consumers were invited to take part in the trial via an email that was sent to staff and students at AUT University. Consumers evaluated the samples at any time between 9am -4pm on Thursday the 18th of March. Sixty consumers were recruited. All consumers were known to have a normal sense of smell.

5.2.3 Sensory trial room set up and execution of the trial

The sensory trial was conducted in a well-ventilated laboratory. Samples were assessed for liking of odour and intensity. Samples from five different treatments were assessed for intensity at each of five time points. Five bays were set up for assessment of intensity. Each bay contained samples from the alanine, leucine, phenylalanine, valine and control treatments at 0, 5, 10, 15 and 30 weeks. A sixth bay was set up for the evaluation of liking. This bay contained the week-15 spirit samples for alanine, leucine, phenylalanine, valine and control. Week 15 samples were chosen because they were incubated long enough to exhibit the putatively intense aromas that developed with time. Letter A, L, P, V and 1 identified the six bays. A glass of water was presented in each bay for consumers to sniff so as to minimise nasal fatigue. Because consumers were assessing the odour of the samples, this trial was regarded as having minimum risk.

Figure 35. Set up of room for sensory trial. Each bay contains five samples plus a glass of water

On arrival, those who were interested were given a short information sheet (Appendix 6) to read that explained the purpose of the trial. Consent was implied by participation. An immediate reward of a chocolate was offered for completion of the trial and mobile telephone numbers were elicited for a $50 draw. It was made clear that the only reason a consumer would be contacted for was to advise success in the draw.

Two ballots were prepared, one to assess liking (Appendix 7) on a 9-point scale from like extremely (9) to dislike extremely (1) and one to assess intensity Appendix 8) on a five point scale ranging from extremely intense (5) to no aroma (1). Consumers were also asked to identify their gender, and age group in a range to explore possible gender and age group effects.

The ballots were presented in a completely randomised design with respect to order of bay and order of treatment within bay to avoid any possible order effect (MacFie and others 1989). A randomised design meant that all consumers had to follow a prescribed order in which bay they were to evaluate the samples.

5.3 Results & Discussion

5.3.1 Consumer liking results

Table 18. Demographic data of consumers who participated in sensory trial	
Total number	60
Gender (% of total consumers)	
Male	65
Female	32
Not declared	3
Age group (% of total consumers)	
18-30	40
31-45	22
46+	36
Not declared	2

Table 18 shows the demographics of the consumers who had participated in the sensory trial. The consumers fell in either the 18-30 years, 31-45 years or 46+ years age group category.

Prior to conducting statistical analysis, the results from the ballots were entered into Excel. Statistical analysis was conducted using Minitab version 16 (Minitab Inc.) and XLSTAT (version 2010.5.02 Addisoft 1995-2000).

The results for liking of treatment were analysed using Analysis of variance (ANOVA) using XLSTAT to help determine if there were any statistically significant differences in liking between any of the treatments. The results of ANOVA showed that there was a statistically significant difference between mean liking scores for the spirit flavoured using Maillard reaction products derived from different amino acids. (P=0.021). Least square difference was conducted to determine which sprit(s) flavoured with Maillard reaction products derived from amino acids was 'liked' significantly more.

Table 19 showed that the leucine, and phenylalanine treatments were liked significantly more than the control and valine treatments. Alanine was not significantly different from either leucine and phenylalanine, or valine and control. ANOVA was conducted and showed that there were no significant differences in liking scores between different genders or age groups.

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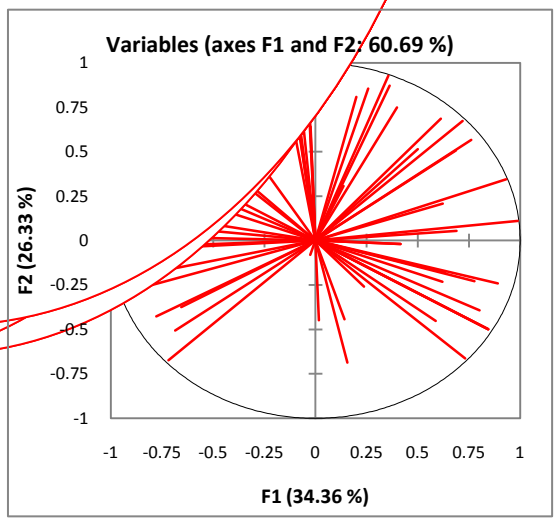
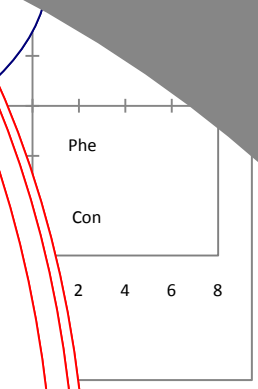


Figure 36 Loading plot (PC1 vs. PC2)



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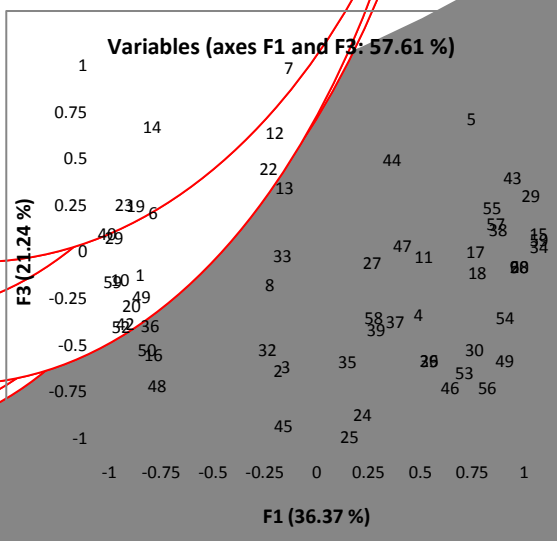

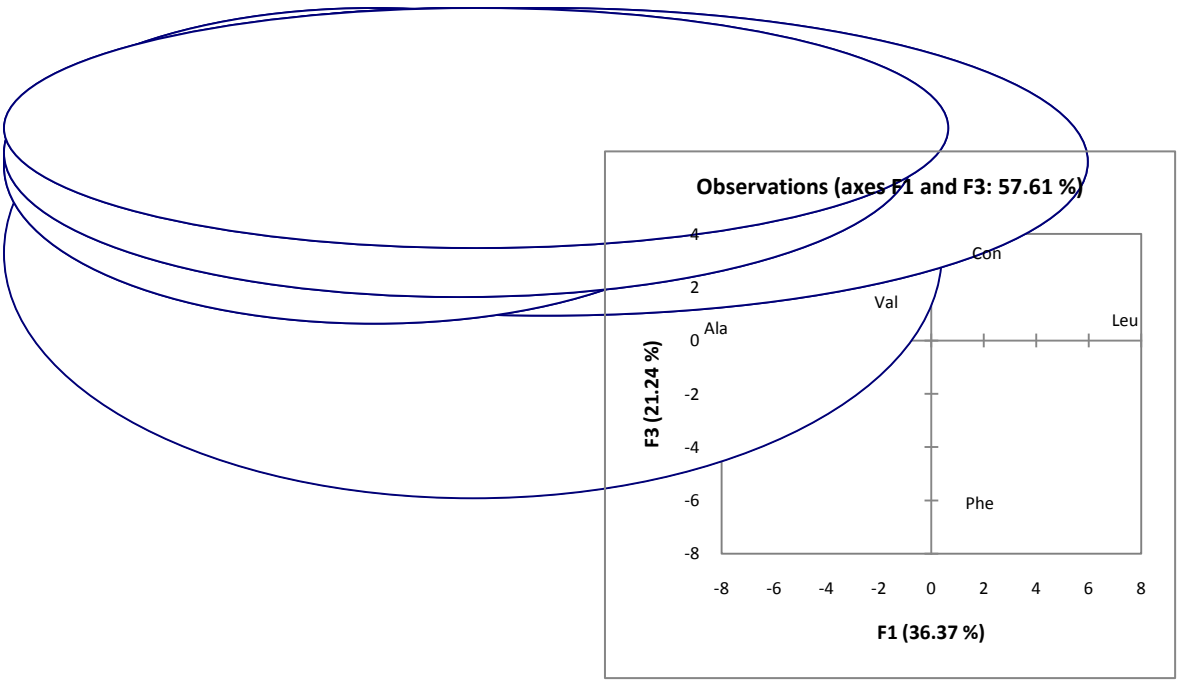

Figure 39. Principal component analysis (PC1 vs. PC3) of liking of spirit flavoured with Maillard reaction products derived from different amino acids.

A further 21.24% variance was explained along factor 3 (Figures 37 and 39).

Table 20. Strecker aldehydes produced from alanine, leucine, phenylalanine and valine and their aroma description and odour threshold.

Amino acid precursor	Strecker aldehyde produced	Aroma description	Odour threshold value (μg/l of water)
Alanine	Ethanal	Sharp, penetrating, fruity	10
Leucine	3-Methylbutanal	Malty	0.2
Phenylalanine	2-Phenylethanal	Flowery, honey like	1
Valine	Methylpropanal	Malty	4

Table 20 describes the Strecker aldehydes produced from each of the amino acids. Strecker aldehydes are one of the most important compounds that contribute to flavor. The spirits flavoured with Maillard reaction products derived from leucine and phenylalanine were significantly most liked, and were described by the researcher as having tequila like and flowery aromas respectively (Chapter 4 Table 17).

3-Methylbutanal is one of the most potent odorants in all tequilas (Piggot & Lea, 2003) and has a low odour threshold meaning that consumers may have been able to detect this aroma and would therefore have scored the leucine spirit high for liking because the aroma was familiar. Compounds known to have floral aromas include Phenylethyl acetate and 2-Phenylethyl alcohol and are also found in some tequilas

(Piggot & Lea, 2003). It is highly likely that these compounds will be present in the spirit flavoured with Maillard reaction products derived from phenylalanine. Phenylethyl acetate is also known to have fruit and wood notes. The Strecker aldehyde derived from phenylalanine is 2-Phenylethanal and has a relatively low odour threshold value meaning that consumers would have been able to detect this compound. Most of the Maillard reaction products derived from the reflux of phenylalanine with lactose are reported to have floral and honey like aromas (Burdock, 2005). Consumers may have rated this product highly for liking because the floral aroma was obvious and pleasant.

Sprits flavoured using products derived from lactose, and the Maillard reaction products of alanine were the least like of all.

5-hydrohymethyl furfural is a product of caramelisation and furural is known to have a characteristic penetrating odour. Fufural is also known to be naturally occurring in eucalyptus and could possibly be responsible for some of the eucalyptus like aromas noted in some of the samples over time (Appendix 5). Because lactose was used as the source of carbohydrate for all of the samples it is not surprising that this aroma developed in most of the samples.

Table 21 shows the means for results of perceived intensity of different amino acid treatments at different storage time. One-way analysis of variance results showed that storage time had a significant effect on perception of intensity for the phenylalanine treatment, and that the responses for control were significantly different for the age groups. To examine mean differences, least square difference (LSD) was carried out.

5.3.2 Consumers perceived intensity results

Table 21. Mean results for intensity over time as affected by gender and age

Treatment	Week				
	0	5	10	15	30
Control	3.7 ± 0.67	3.8 ± 0.64	3.8 ± 0.56	3.8 ± 0.71	3.8 ± 0.69
Alanine	3.8 ± 0.61	3.9 ± 0.63	3.8 ± 0.47	3.8 ± 0.66	3.8 ± 0.74
Leucine	3.7 ± 0.56	3.6 ± 0.61	3.7 ± 0.47	3.8 ± 0.66	3.9 ± 0.67
Phenylalanine	3.7 ± 0.60	3.9 ± 0.74	3.7 ± 0.54	3.0 ± 1.00	3.8 ± 0.65
Valine	3.1 ± 1.07	3.1 ± 0.93	3.1 ± 0.88	3.2 ± 1.01	3.1 ± 1.09
		Age group (years)			
		18-30	31-45	46+	Not declared
Control		3.87 ± 0.68	3.72 ± 0.63	3.64 ± 0.60	3.67 ± 0.58
Alanine		3.84 ± 0.60	3.85 ± 0.59	3.70 ± 0.66	4.00 ± 1.00
Leucine		3.78 ± 0.61	3.82 ± 0.63	3.66 ± 0.67	3.40 ± 0.55
Phenylalanine		3.69 ± 0.80	3.66 ± 0.70	3.43 ± 0.79	3.20 ± 0.45
Valine		3.12 ± 1.06	3.22 ± 0.96	3.18 ± 0.93	3.60 ± 0.55
		Gender			
		Male	Female	Not declared	
Control		3.77 ± 0.63	3.79 ± 0.69	3.67 ± 0.58	
Alanine		3.83 ± 0.63	3.76 ± 0.59	4.00 ± 1.00	
Leucine		3.78 ± 0.64	3.70 ± 0.62	3.40 ± 0.55	
Phenylalanine		3.61 ± 0.78	3.64 ± 0.79	3.20 ± 0.45	
Valine		3.16 ± 1.06	3.13 ± 0.87	3.60 ± 0.55	
		One way ANOVA			
			P-value		
Effect of:		Storage time	Age	Gender	
Control		0.884	0.025	0.669	
Alanine		0.920	0.204	0.317	
Leucine		0.068	0.193	0.210	
Phenylalanine		0.000	0.076	0.957	
Valine		0.975	0.848	0.703	

A 5-point scale (1=No aroma, 5= extremely intense) was used to assess intensity. *P*=0.05

Table 22. Mean intensity scores for spirits flavoured with Maillard reaction products derived from phenylalanine

Storage time (weeks)	Mean Intensity
5	3.860^{A}
30	3.789^{A}
10	3.696^{A}
0	3.690^{A}
15	3.017^{B}

Means with no common superscript differ ($P < 0.05$)

The results for mean intensity of phenylalanine over time (Table 22) showed that there was a decrease in average perception of aroma intensity between week 10 and 15, and then an increase again at week 30. There is a possibility that the Maillard reaction products may have undergone further reactions at 15 weeks, making volatile products unavailable for detection, and then producing more volatile products at approximately 30 weeks. The period between the week 15 and week 30 samples was significantly long; therefore it is difficult to determine what was happening in between this time period.

ANOVA was also carried out on the intensity results for control to determine which age groups were significantly different in their intensity results from each other.

Table 23. Mean intensity scores for spirit flavoured with Maillard reaction products derived from the control by age group

Age group	Mean intensity score
18-30	3.872^{A}
31-45	3.719^{AB}
46+	3.638^{B}

Means with no common superscript differ. ($P < 0.05$)

Table 23 showed that there were statistically significant differences between responses for intensity of the control by age group. The lower age group (18-30) rated intensity significantly higher than the older two age groups (31-45 and 46+).

The following bar charts showed the percentage of responses of perceived intensity of aroma for each sample treatment for each treatment over time. In a commercial situation, participants taking part in such a trial would have been trained. Because consumers in this trial were not trained and a majority of the consumers did not

have much experience with participating in consumer trials previously, the data obtained from this trial will not be completely reliable.

A large proportion of the consumers rated the samples as either moderate or intense; the 'extremely intense' response was not used much. Consumers may have based their response based on the intensity of ethanol aroma rather than Maillard reaction product. If the products had lower ethanol concentrations, the aroma of the Maillard reaction products might have been stronger and would have given more valuable information about the strength of the Maillard reaction products. As both Maillard reaction products and ethanol are volatile, the aroma from the ethanol might have interfered with Maillard reaction product aroma

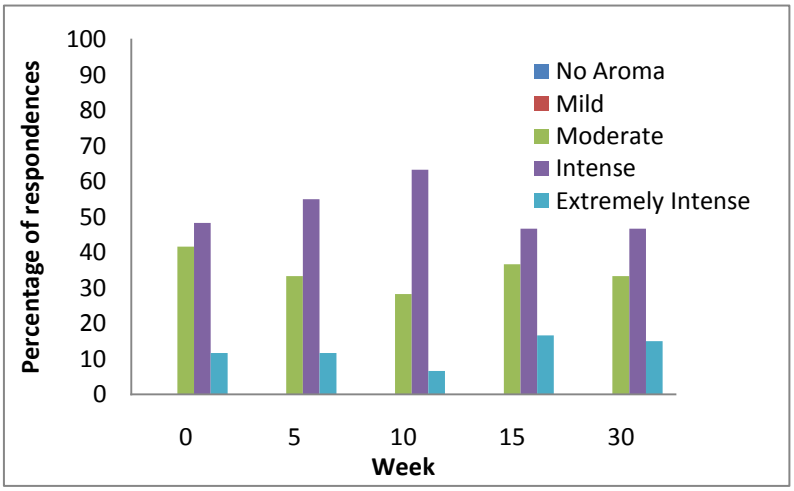

Figure 40. Percentage of responses for intensity by week of spirit flavoured using products derived from the control.

Figure 40 show that at week 10 a relatively high percentage (approximately 60%) of respondents perceived the sample to be 'Intense'

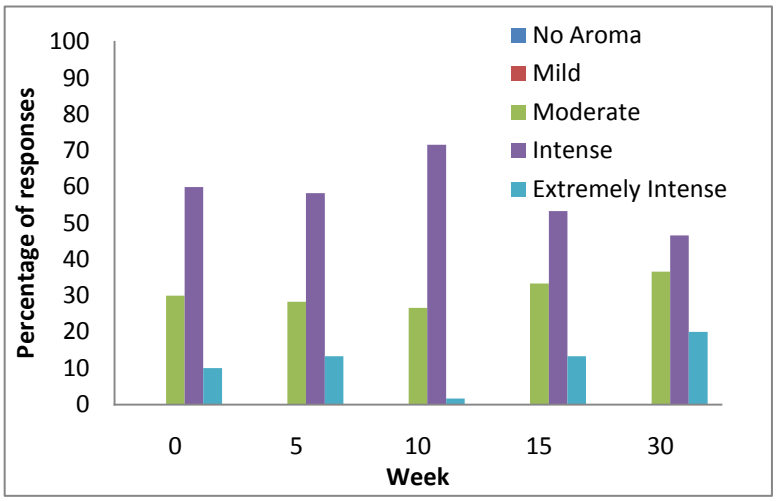

Figure 41. Percentage of responses for intensity by week of the spirits flavoured using Maillard reaction products derived from alanine.

The responses for intensity of the control and the alanine treatment appeared to be quite similar. A high percentage of respondents found the aroma of the week 10 samples to be the most ‘intense’.

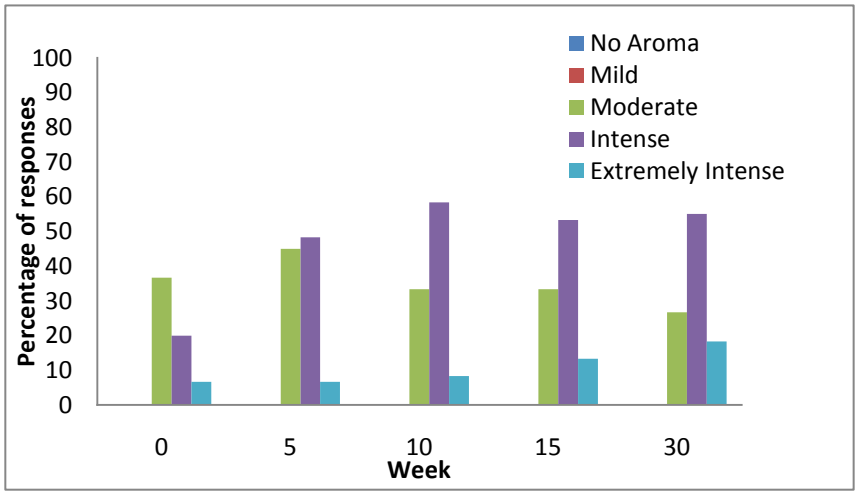

Figure 42. Percentage of responses for intensity by week of the spirits flavoured using Maillard reaction products derived from leucine.

Most consumers rated the week 10, alanine and leucine samples as being the most intense (Figure 42).

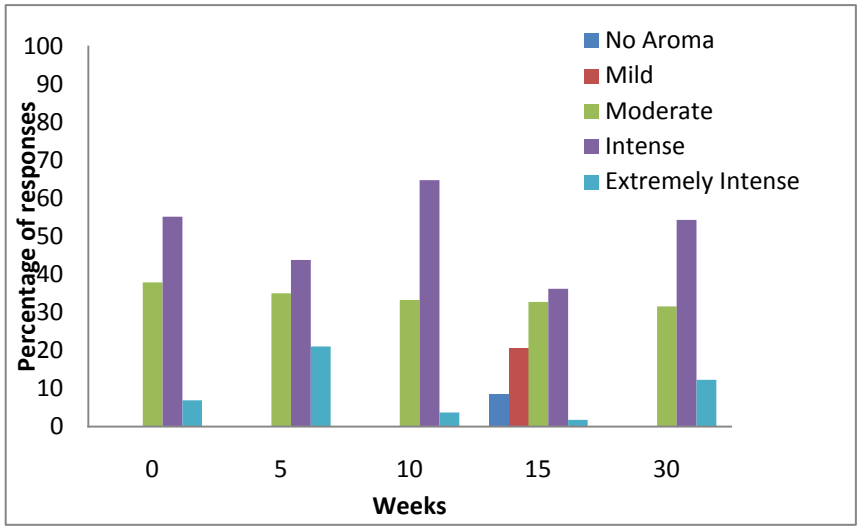

Figure 43. Percentage of responses for intensity by week of the spirits flavoured using Maillard reaction products derived from phenylalanine.

Table 21 showed that phenylalanine showed more obvious changes in perceived intensity of aroma over time. Figure 41 showed that with the phenylalanine treatment, aroma decreased at 15 weeks with approximately 8% of consumers perceiving ‘no aroma’ response for this sample. The mean intensity for phenylalanine at week 15 was 3.0, which corresponded to ‘moderate’. The week 10 sample was rated ‘intense’ by

approximately 65% of consumers which is a relatively high percentage. Unlike the control, alanine and leucine treatments, many of the consumers reported the phenylalanine samples as having ‘moderate’ intensity.

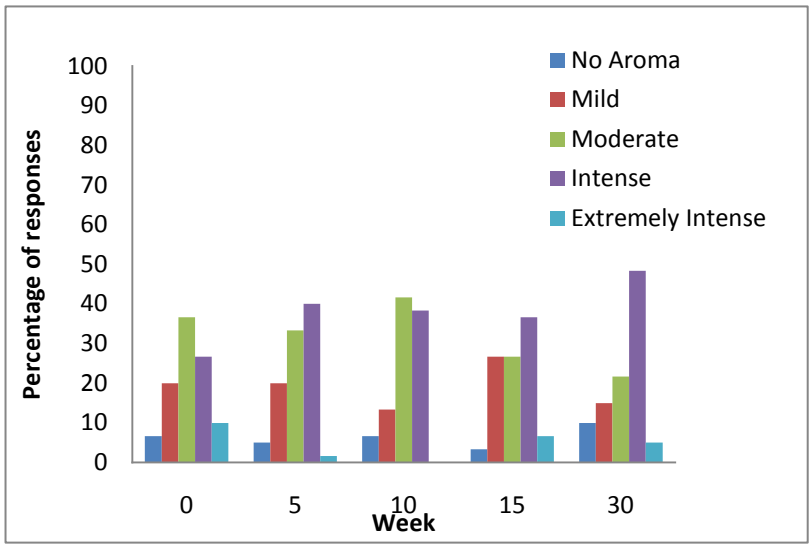

Figure 44. Percentage of responses for intensity by week of the spirits flavoured using Maillard reaction products derived from valine.

As both the Maillard reaction products and ethanol are volatile, some consumers may have had difficulty distinguishing between the two aromas. This may have affected the individual’s responses and could have resulted in them rating all samples in terms of intensity of ethanol, rather than the Maillard reaction products. If the Maillard reaction products alone were to be assessed, the results would have then been different; however the objective of this study was to assess the sensory properties of the final ethanol product containing the Maillard reaction products.

Chapter 6

Conclusion

Refluxing of lactose and various different types of dairy powders dispersed in distilled in water should have theoretically yielded Maillard reaction products that are able to be used to flavour ethanol, thereby creating a potable spirit via the exclusive use of dairy components. A number of unknown factors caused these Maillard reaction products to be opaque and unattractive

A number of methods were experimented with to remove the opaqueness from the Maillard reaction products created by refluxing skim milk powder and lactose, however none were successful. Some of the opaqueness was removed via centrifugation; however the pleasant sweet flavours were lost. It may be possible that the flavour compounds were bound to the compounds that were causing opaqueness. A number of other methods were also trialed but were unsuccessful. The possible cause of opaqueness could have been protein cross linking due to the high mass of skim milk powder in the reaction however decreasing skim milk powder concentration decreased Maillard reaction product yield.

Suitable results for flavour and colour were achieved via the use of amino acid powders and lactose as Maillard reaction reactants. Four amino acids were found to produce suitable results for flavouring ethanol when refluxed with lactose in a 1:1 ratio at an elevated pH of 9. These four amino acids were alanine, leucine, phenylalanine and valine. pH decreased after reflux due to the production of saccharinic acids, H+, lactic acid, and other acidic compounds. A control experiment containing lactose alone was used as a source of carbohydrate because it is a dairy component. Time and high temperature increase the rate of the Maillard reaction and this effect was indeed noted when reflux time was increased from 90 to 180 minutes, and when the prepared Maillard reaction spirits were stored for a period of 30 weeks at 32° C. Flavour assessed as aroma and colour increased in intensity over time. Some of the spirits flavoured with Maillard reaction products derived from the various amino acids were more attractive than others.

One of the advantages of using this method to produce an alcoholic spirit was that a shorter period of time was required in comparison to traditional methods of producing alcohol which involve fermentation, distillation, maturation and in some cases blending- these processes can take months. If alcoholic spirits were to be produced by reflux on a commercial scale less time and resources would be required, resulting in more efficient overall production which will be economical.

A sensory trial involving assessment of aroma only showed that out of all four of the amino acids used to create flavours, and the control, the sprits flavoured with Maillard reaction products derived from leucine and phenylalanine were significantly liked the most. The researcher noted that the leucine derived spirit had tequila like aroma and literature showed that the Strecker aldehyde produced from leucine is one of the most potent known flavor compounds found in tequila. Consumers may have liked this spirit because the aroma was familiar to them. Consumers also significantly liked the spirit flavoured with Maillard reaction products derived from phenylalanine which had a unique and pleasant floral aroma. The spirit flavoured with Maillard reaction products derived from phenylalanine was the only spirit that had a significant result for change in aroma over time.

New Zealand is well known for its dairy industry. Dairy products potentially contain all of the components needed to create an alcoholic spirit. Lactose is already used to produce potable ethanol in New Zealand. The Maillard reaction is a non-enzymic browning process which requires amino acids and carbohydrates for it to occur, both of which are present in milk.

There is always potential on the market for new and innovative products alcoholic beverages. While certain countries such as China and India are developing markets (GMID), New Zealand is already relatively well known for producing high quality alcoholic beverages and has the potential to create a product that can be marketed on its geographical exclusivity and innovative production method.

6.1 Further research

The idea of creating a spirit using flavours derived from the Maillard reaction products of dairy components is still feasible. Further research into the pathways involved in the Maillard reaction in dairy components needs to be carried out to determine which of the components are causing opaqueness and when these reactions are occurring. A method whereby these components can either be eliminated from the

reaction, or inhibited from reacting can then be developed so that clear Maillard reaction products can be produced. The successful development of these flavours would not only have industry applications in the alcoholic beverages category, but in other food and beverage categories as well.

The extent of the Maillard reaction could have been determined by measuring lactose concentration before and after reflux. By gaining more information about the extent of the Maillard reaction, optimum conditions for the Maillard reaction to occur could be determined and used to create Maillard reaction products for use as flavourants more efficiently. Colour could have been measured using a more reliable method such as by the measurement of L*a*b* values, rather than visually. This would have increased reliability and validity of the results for colour and would have given more accurate information of the determination of the extent of the Maillard reaction.

The sensory trial showed that the use of Maillard reaction products derived from the reflux of leucine or phenylalanine and lactose creates a potable spirit that is liked by consumers. The researcher had observed that the aroma of leucine was rather tequila like. 3-Methylbutanal is a Strecker aldehyde derived from leucine and is an important odourant in tequila this explains the researchers observation. Gas chromatography-mass spectrometry analysis and refractive index measurements would have provided a valuable amount of information here. A comparison of the Maillard reaction products with samples of tequila would have shown if the products are indeed similar at all. Tequila is a popular spirit and if these flavours can be created in a laboratory in a relatively short period of time involving the use of few resources then there is the potential for a patent of this method. The successful production of tequila like flavours could have applications in the alcoholic spirits market, and the RTD market. The same potential would be noted for development of the floral flavour created via the Maillard reaction of phenylalanine. The phenylalanine derived floral flavours could also be used in food and beverage categories other then alcoholic spirits.

A trained panelist on flavour would have been provided more reliable

The consumer trial for the assessment of intensity had shown that only phenylalanine showed a change in intensity of aroma over time. This result was most likely inaccurate and may even have been due to an experimental error. The information gained from the sensory trial would have been more valuable if consumers had been asked to rank samples in order of intensity rather than rate sample intensity.

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Appendix 1 Wavescanning results for Development of Maillard reaction products in amino acid lactose mixtures.

Wavelengtl	Glutam	Aspara	Threo	Proline	Alanin	Pheny	Valine	Leucir	Serine	Glycin	Tyros	Aspa	Ornitl	Arginir	Lysin	contro
200.0	18.6	12.9	10.0	10.6	10.9	15.1	12.1	11.6	11.8	12.2	15.3	24.7	23.7	30.0	30.0	5.9
202.0	23.7	18.8	15.2	16.4	15.1	21.3	17.8	16.4	16.9	16.6	21.1	30.0	30.0	30.0	30.0	7.3
204.0	27.8	23.0	18.0	19.7	16.7	25.3	21.2	18.3	19.3	17.7	25.0	30.0	30.0	30.0	30.0	7.7
206.0	30.0	25.7	18.1	21.0	16.5	27.9	22.7	18.5	19.5	17.4	28.0	30.0	30.0	30.0	30.0	7.9
208.0	30.0	26.6	16.7	21.3	15.3	30.0	22.2	17.4	18.3	16.3	29.4	30.0	30.0	30.0	30.0	8.1
210.0	30.0	27.6	14.9	21.0	14.1	30.0	21.1	15.9	16.4	15.2	30.0	30.0	30.0	30.0	30.0	8.3
212.0	28.5	28.4	12.8	20.3	12.6	30.0	19.2	14.3	14.9	14.0	30.0	30.0	30.0	30.0	30.0	8.4
214.0	26.1	28.8	11.0	19.4	11.3	30.0	17.1	12.8	13.3	13.1	30.0	30.0	30.0	30.0	30.0	8.6
216.0	23.7	28.7	9.4	18.1	10.2	30.0	15.0	11.4	11.8	12.1	30.0	28.8	30.0	30.0	30.0	8.7
218.0	20.7	28.8	7.9	16.5	9.2	30.0	13.0	10.3	10.6	11.3	30.0	27.9	30.0	30.0	30.0	8.9
220.0	18.0	28.3	6.9	14.6	8.4	30.0	11.1	9.4	9.5	10.6	30.0	26.8	30.0	30.0	30.0	9.0
222.0	16.2	26.9	5.9	12.6	7.7	30.0	9.8	8.7	8.7	10.1	30.0	26.2	30.0	30.0	30.0	9.2
224.0	15.2	24.5	5.1	10.4	7.0	30.0	8.9	8.1	8.0	9.6	30.0	25.5	30.0	30.0	30.0	9.2
226.0	14.6	21.6	4.4	8.5	6.4	28.1	8.4	7.6	7.3	9.1	30.0	25.0	30.0	30.0	30.0	9.1
228.0	14.2	18.7	3.8	6.8	6.0	23.0	7.8	7.2	6.7	8.6	30.0	24.6	30.0	30.0	30.0	8.8
230.0	14.0	16.6	3.5	5.8	5.6	20.4	7.5	7.0	6.4	8.3	30.0	24.4	30.0	30.0	30.0	8.5
232.0	13.8	14.4	3.2	4.9	5.3	18.4	7.1	6.7	6.0	7.8	30.0	24.1	30.0	30.0	30.0	7.9
234.0	13.6	12.4	2.9	4.3	5.0	17.2	6.7	6.4	5.7	7.3	30.0	23.8	30.0	30.0	30.0	7.2
236.0	13.5	11.1	2.8	4.0	4.8	16.8	6.5	6.3	5.5	6.9	24.5	23.6	30.0	30.0	30.0	6.6
238.0	13.5	9.9	2.7	3.8	4.7	16.6	6.4	6.2	5.3	6.7	16.9	23.4	30.0	30.0	30.0	6.0
240.0	13.5	8.8	2.7	3.7	4.5	17.1	6.4	6.2	5.3	6.5	12.6	23.2	30.0	30.0	30.0	5.5
242.0	13.3	8.0	2.7	3.7	4.5	17.5	6.5	6.1	5.2	6.4	11.1	23.1	30.0	30.0	30.0	5.1
244.0	13.4	7.4	2.7	3.7	4.4	18.0	6.6	6.2	5.2	6.4	10.6	23.0	30.0	30.0	30.0	4.7
246.0	13.3	6.8	2.7	3.7	4.4	19.1	6.7	6.2	5.3	6.4	10.6	22.9	30.0	30.0	30.0	4.4
248.0	13.4	6.4	2.8	3.7	4.5	19.8	6.8	6.2	5.3	6.4	10.8	22.9	30.0	30.0	30.0	4.2
250.0	13.4	5.9	2.8	3.8	4.5	20.5	6.9	6.2	5.3	6.4	11.2	22.8	30.0	30.0	30.0	4.0
252.0	13.4	5.6	2.9	3.9	4.5	22.1	7.0	6.3	5.4	6.4	11.8	22.8	30.0	30.0	30.0	3.9
254.0	13.4	5.4	2.9	3.9	4.6	21.9	7.1	6.3	5.5	6.4	12.3	22.7	30.0	30.0	30.0	3.8
256.0	13.4	5.1	2.9	3.9	4.6	22.4	7.0	6.2	5.5	6.4	13.3	22.6	30.0	30.0	30.0	3.8
258.0	13.4	4.9	2.9	3.9	4.6	23.6	6.9	6.2	5.5	6.4	14.3	22.5	30.0	30.0	30.0	3.8
260.0	13.3	4.8	2.9	4.0	4.6	22.0	6.8	6.0	5.5	6.4	15.4	22.5	30.0	30.0	30.0	3.8
262.0	13.3	4.6	2.8	3.9	4.6	20.7	6.6	5.9	5.5	6.4	16.6	22.4	30.0	30.0	30.0	3.8
264.0	13.3	4.5	2.8	3.8	4.6	20.2	6.4	5.7	5.5	6.3	18.1	22.4	30.0	30.0	30.0	3.8
266.0	13.2	4.4	2.8	3.8	4.6	18.0	6.1	5.6	5.5	6.3	19.9	22.3	30.0	30.0	30.0	3.8
268.0	13.1	4.4	2.8	3.7	4.5	16.3	5.9	5.4	5.6	6.3	21.6	22.2	30.0	30.0	30.0	3.8
270.0	13.0	4.2	2.8	3.6	4.5	13.7	5.5	5.2	5.6	6.2	23.2	22.1	30.0	30.0	30.0	3.7
272.0	13.0	4.1	2.8	3.5	4.5	12.1	5.3	5.0	5.6	6.3	24.7	22.1	30.0	30.0	30.0	3.6
274.0	12.9	4.1	2.8	3.4	4.5	11.4	5.0	4.9	5.7	6.3	25.9	22.1	30.0	30.0	30.0	3.6
276.0	12.9	3.9	2.9	3.2	4.4	10.8	4.8	4.7	5.8	6.2	27.0	22.0	30.0	30.0	30.0	3.4
278.0	12.8	3.8	2.9	3.0	4.5	10.5	4.6	4.6	5.9	6.3	27.1	21.9	30.0	30.0	30.0	3.3
280.0	12.8	3.6	3.0	3.0	4.6	10.2	4.4	4.5	6.1	6.3	25.9	21.9	30.0	30.0	30.0	3.2
282.0	12.8	3.5	3.1	2.9	4.6	9.9	4.3	4.5	6.2	6.4	24.9	21.9	30.0	30.0	30.0	3.1
284.0	12.7	3.3	3.2	2.8	4.7	9.6	4.2	4.5	6.4	6.5	23.6	21.8	30.0	30.0	30.0	2.9
286.0	12.7	3.2	3.2	2.7	4.7	9.4	4.1	4.4	6.5	6.5	20.5	21.7	30.0	30.0	30.0	2.8
288.0	12.7	3.0	3.3	2.6	4.8	9.2	4.1	4.4	6.7	6.6	16.0	21.7	30.0	30.0	30.0	2.7
290.0	12.7	2.8	3.3	2.5	4.9	9.0	4.0	4.4	6.8	6.6	12.2	21.6	30.0	30.0	30.0	2.6
292.0	12.6	2.7	3.4	2.5	4.9	8.8	4.0	4.4	6.9	6.7	9.6	21.6	30.0	30.0	30.0	2.5
294.0	12.6	2.5	3.4	2.5	5.0	8.6	4.0	4.4	7.0	6.7	8.4	21.5	30.0	30.0	30.0	2.3
296.0	12.5	2.4	3.4	2.4	5.1	8.4	4.0	4.4	7.1	6.7	7.8	21.5	30.0	30.0	30.0	2.3
298.0	12.5	2.3	3.3	2.4	5.1	8.2	4.0	4.4	7.1	6.7	7.5	21.5	30.0	30.0	30.0	2.2
300.0	12.5	2.2	3.3	2.4	5.2	8.1	4.1	4.4	7.1	6.8	7.4	21.4	30.0	30.0	30.0	2.2
302.0	12.5	2.1	3.2	2.3	5.2	7.9	4.1	4.4	7.1	6.8	7.3	21.4	30.0	30.0	30.0	2.1
304.0	12.4	2.1	3.2	2.2	5.3	7.8	4.1	4.5	7.1	6.9	7.2	21.4	30.0	30.0	30.0	2.0
306.0	12.4	2.0	3.2	2.2	5.3	7.7	4.1	4.4	7.1	6.9	7.1	21.3	30.0	30.0	30.0	1.9
308.0	12.4	2.0	3.2	2.2	5.4	7.5	4.1	4.5	7.2	6.9	7.0	21.3	30.0	30.0	30.0	1.9
310.0	12.4	2.0	3.2	2.1	5.4	7.5	4.1	4.4	7.2	7.0	6.9	21.3	30.0	30.0	30.0	1.8
312.0	12.4	2.0	3.1	2.0	5.4	7.4	4.1	4.5	7.2	7.0	6.9	21.2	30.0	30.0	30.0	1.7
314.0	12.3	2.0	3.1	2.0	5.4	7.3	4.1	4.4	7.2	7.1	6.8	21.2	30.0	30.0	30.0	1.7
316.0	12.3	1.9	3.1	1.9	5.4	7.2	4.1	4.4	7.3	7.1	6.8	21.2	30.0	30.0	30.0	1.7
318.0	12.3	1.9	3.1	1.9	5.4	7.1	4.1	4.4	7.4	7.2	6.7	21.1	29.6	30.0	30.0	1.6
320.0	12.2	1.9	3.1	1.8	5.4	7.0	4.0	4.3	7.4	7.2	6.6	21.1	29.6	30.0	30.0	1.6
322.0	12.2	1.8	3.1	1.8	5.3	6.9	4.0	4.3	7.5	7.2	6.5	21.0	29.6	30.0	30.0	1.5
324.0	12.2	1.8	3.1	1.7	5.2	6.8	4.0	4.3	7.5	7.2	6.5	21.0	29.3	30.0	30.0	1.5
326.0	12.2	1.8	3.1	1.7	5.2	6.6	4.0	4.2	7.5	7.2	6.4	21.0	29.4	30.0	30.0	1.5
328.0	12.1	1.7	3.0	1.6	5.1	6.5	3.9	4.2	7.4	7.2	6.3	20.9	29.2	30.0	30.0	1.5
330.0	12.1	1.7	3.0	1.6	4.9	6.4	3.8	4.1	7.3	7.1	6.2	20.9	29.1	30.0	30.0	1.4
332.0	12.0	1.6	2.9	1.5	4.8	6.2	3.8	4.0	7.2	7.0	6.1	20.9	28.9	30.0	30.0	1.4
334.0	12.0	1.5	2.8	1.5	4.6	6.0	3.7	4.0	7.0	6.9	6.0	20.8	28.6	30.0	30.0	1.4
336.0	12.0	1.5	2.7	1.4	4.5	5.9	3.6	3.9	6.8	6.7	5.8	20.8	28.4	30.0	30.0	1.4
338.0	11.9	1.4	2.6	1.4	4.3	5.7	3.5	3.8	6.5	6.5	5.7	20.8	28.2	30.0	30.0	1.3
340.0	11.9	1.3	2.4	1.3	4.1	5.5	3.4	3.7	6.2	6.3	5.5	20.8	27.9	30.0	30.0	1.3

Appendix 2 Wavescanning results for determination of optimum length of reaction time for creating suitable Maillard reaction products for flavouring ethanol.

		Control 1			Control 2			10% EtOH			20% EtOH			30% EtOH			40% EtOH			50% EtOH	
Waveleng	60	120	180	60	120	180	60	120	180	60	120	180	60	120	180	60	120	180	60	120	180
200	1.51	1.46	1.44	0.53	0.56	0.48	1.11	0.99	1.43	1.13	1.22	1.29	1.05	1.36	0.73	0.45	0.71	0.68	1.47	1.52	1.82
202	2.04	2.16	2.11	1.01	1.07	0.96	1.65	1.54	1.96	1.67	1.78	1.91	1.59	1.93	1.19	0.95	1.25	1.18	2.23	2.1	2.48
204	2.49	2.55	2.48	1.39	1.44	1.3	2.03	1.93	2.36	2.08	2.16	2.28	1.97	2.35	1.4	1.31	1.65	1.58	2.58	2.64	2.76
206	2.83	2.74	2.79	1.67	1.72	1.6	2.32	2.16	2.64	2.32	2.42	2.52	2.2	2.62	1.5	1.61	1.92	1.89	2.82	2.86	> +3
208	2.86	2.92	2.99	1.82	1.84	1.74	2.42	2.28	2.75	2.41	2.55	2.64	2.34	2.79	1.51	1.81	2.11	2.07	3	> +3	> +3
210	3	> +3	> +3	1.93	2.01	1.81	2.51	2.36	2.86	2.51	2.62	2.75	2.4	2.81	1.47	1.89	2.3	2.18	> +3	> +3	> +3
212	> +3	> +3	> +3	2.03	2.01	1.77	2.56	2.39	2.92	2.58	2.67	2.86	2.46	2.89	1.33	2	2.4	2.3	> +3	> +3	> +3
214	> +3	> +3	> +3	1.98	1.95	1.64	2.6	2.42	> +3	2.62	2.74	2.86	2.51	2.96	1.13	2.08	2.51	2.41	> +3	> +3	> +3
216	> +3	> +3	> +3	1.8	1.77	1.42	2.59	2.4	2.95	2.62	2.76	2.87	2.48	2.98	0.91	2.11	2.56	2.48	> +3	> +3	> +3
218	> +3	> +3	> +3	1.41	1.42	1.08	2.61	2.4	> +3	2.63	2.75	2.9	2.5	> +3	0.65	2.11	2.63	2.55	> +3	> +3	> +3
220	> +3	> +3	> +3	0.91	1.03	0.73	2.62	2.31	> +3	2.65	2.79	2.93	2.48	> +3	0.35	1.82	2.73	2.62	> +3	> +3	> +3
222	> +3	> +3	> +3	0.66	0.82	0.54	2.55	1.6	> +3	2.6	2.78	2.92	2.31	> +3	0.17	1.11	2.75	2.63	> +3	> +3	> +3
224	> +3	> +3	> +3	0.57	0.75	0.47	1.92	0.97	2.44	2.07	2.34	2.41	1.52	> +3	0.09	0.64	2.69	2.23	> +3	> +3	> +3
226	> +3	> +3	> +3	0.54	0.71	0.43	1.16	0.58	1.78	1.25	1.54	1.66	0.89	2.43	0.05	0.38	2.2	1.5	> +3	> +3	> +3
228	> +3	> +3	> +3	0.53	0.69	0.42	0.75	0.4	1.4	0.83	1.09	1.25	0.58	1.79	0.04	0.26	1.59	1.11	> +3	> +3	> +3
230	> +3	> +3	> +3	0.52	0.66	0.4	0.59	0.33	1.23	0.64	0.88	1.07	0.44	1.46	0.03	0.21	1.27	0.92	> +3	> +3	> +3
232	2.87	> +3	> +3	0.51	0.63	0.38	0.47	0.27	1.09	0.51	0.73	0.91	0.34	1.22	0.03	0.17	1.02	0.77	> +3	> +3	> +3
234	2.74	> +3	> +3	0.49	0.59	0.35	0.42	0.25	1.03	0.44	0.65	0.84	0.29	1.12	0.02	0.15	0.9	0.7	> +3	> +3	> +3
236	2.58	> +3	> +3	0.46	0.55	0.33	0.42	0.25	1.02	0.44	0.64	0.82	0.29	1.11	0.02	0.14	0.89	0.69	> +3	> +3	> +3
238	2.42	> +3	> +3	0.42	0.51	0.31	0.44	0.25	1.03	0.46	0.66	0.83	0.3	1.14	0.02	0.14	0.92	0.71	> +3	> +3	> +3
240	2.25	2.92	2.89	0.37	0.47	0.29	0.5	0.27	1.09	0.51	0.72	0.89	0.35	1.24	0.02	0.16	1.03	0.77	> +3	> +3	> +3
242	2.1	2.76	2.69	0.33	0.44	0.27	0.57	0.3	1.15	0.58	0.79	0.95	0.39	1.37	0.02	0.17	1.15	0.84	> +3	> +3	> +3
244	1.97	2.6	2.48	0.3	0.41	0.26	0.62	0.32	1.21	0.64	0.85	0.99	0.42	1.48	0.03	0.19	1.25	0.9	> +3	> +3	> +3
246	1.82	2.45	2.26	0.28	0.38	0.25	0.75	0.38	1.34	0.77	1	1.12	0.52	1.72	0.03	0.22	1.48	1.04	> +3	> +3	> +3
248	1.73	2.34	2.12	0.27	0.36	0.24	0.81	0.4	1.41	0.83	1.06	1.19	0.56	1.85	0.03	0.24	1.6	1.11	> +3	> +3	> +3
250	1.66	2.28	1.98	0.26	0.35	0.24	0.9	0.43	1.49	0.91	1.15	1.28	0.62	2.01	0.03	0.26	1.74	1.21	> +3	> +3	> +3
252	1.62	2.25	1.89	0.26	0.35	0.24	1.06	0.5	1.67	1.08	1.34	1.45	0.74	2.32	0.04	0.3	2.04	1.39	> +3	> +3	> +3
254	1.6	2.2	1.84	0.25	0.34	0.24	1.04	0.49	1.65	1.06	1.31	1.43	0.73	2.28	0.04	0.29	2	1.37	> +3	> +3	> +3
256	1.61	2.26	1.82	0.25	0.35	0.25	1.1	0.53	1.72	1.12	1.38	1.49	0.77	2.39	0.04	0.31	2.11	1.44	> +3	> +3	> +3
258	1.64	2.31	1.83	0.26	0.37	0.26	1.27	0.58	1.87	1.29	1.56	1.66	0.9	2.68	0.04	0.35	2.38	1.62	> +3	> +3	> +3
260	1.67	2.25	1.85	0.25	0.37	0.26	1.1	0.52	1.7	1.13	1.39	1.49	0.78	2.39	0.04	0.31	2.11	1.45	> +3	> +3	> +3
262	1.69	2.26	1.88	0.25	0.38	0.27	0.98	0.47	1.58	1	1.25	1.37	0.69	2.17	0.03	0.28	1.91	1.31	> +3	> +3	> +3
264	1.74	2.29	1.92	0.26	0.4	0.28	0.98	0.47	1.56	1	1.23	1.35	0.69	2.12	0.03	0.28	1.87	1.31	> +3	> +3	> +3
266	1.78	2.22	1.94	0.25	0.4	0.28	0.75	0.38	1.33	0.76	0.98	1.12	0.52	1.71	0.03	0.23	1.48	1.06	> +3	> +3	> +3
268	1.8	2.17	1.96	0.24	0.41	0.28	0.6	0.31	1.16	0.6	0.81	0.97	0.42	1.37	0.02	0.19	1.19	0.9	> +3	> +3	> +3
270	1.82	2.09	1.97	0.23	0.41	0.28	0.33	0.2	0.87	0.33	0.51	0.7	0.23	0.87	0.01	0.12	0.72	0.6	2.5	> +3	> +3
272	1.83	2.07	1.98	0.23	0.41	0.27	0.22	0.16	0.77	0.23	0.38	0.57	0.15	0.67	0.01	0.09	0.53	0.48	1.97	2.74	> +3
274	1.83	2.06	1.98	0.23	0.41	0.28	0.17	0.13	0.7	0.17	0.32	0.51	0.11	0.56	0.01	0.08	0.43	0.42	1.62	2.39	> +3
276	1.82	2.05	1.98	0.22	0.41	0.27	0.15	0.12	0.66	0.15	0.29	0.47	0.09	0.51	0.01	0.07	0.38	0.38	1.46	2.2	> +3
278	1.81	2.04	1.96	0.22	0.41	0.27	0.14	0.11	0.64	0.14	0.27	0.46	0.08	0.48	0.01	0.06	0.36	0.36	1.41	2.12	> +3
280	1.79	2.02	1.94	0.22	0.41	0.26	0.13	0.11	0.63	0.13	0.26	0.44	0.08	0.47	0.01	0.06	0.35	0.35	1.37	2.05	> +3
282	1.76	1.99	1.9	0.22	0.41	0.26	0.13	0.11	0.62	0.13	0.26	0.43	0.08	0.46	0	0.06	0.34	0.33	1.34	2	> +3
284	1.72	1.95	1.86	0.22	0.4	0.25	0.13	0.11	0.62	0.13	0.25	0.43	0.08	0.45	0	0.06	0.33	0.32	1.31	1.93	> +3
286	1.66	1.89	1.81	0.21	0.39	0.24	0.12	0.1	0.63	0.12	0.24	0.42	0.07	0.44	0.01	0.06	0.32	0.31	1.26	1.86	3
288	1.59	1.81	1.72	0.2	0.37	0.23	0.11	0.1	0.63	0.11	0.23	0.42	0.07	0.42	0	0.06	0.31	0.29	1.2	1.77	2.9
290	1.5	1.71	1.63	0.2	0.36	0.22	0.11	0.09	0.64	0.11	0.22	0.41	0.06	0.41	0	0.05	0.29	0.28	1.12	1.67	2.78
292	1.4	1.6	1.52	0.19	0.33	0.21	0.1	0.09	0.66	0.1	0.21	0.42	0.06	0.4	0	0.05	0.28	0.27	1.04	1.57	2.66
294	1.3	1.48	1.4	0.18	0.31	0.19	0.1	0.09	0.68	0.09	0.2	0.42	0.05	0.39	-0	0.05	0.27	0.26	0.96	1.46	2.53
296	1.2	1.36	1.28	0.16	0.28	0.17	0.09	0.09	0.71	0.09	0.2	0.43	0.05	0.39	0	0.04	0.25	0.23	0.88	1.37	2.42
298	1.09	1.24	1.17	0.15	0.26	0.16	0.09	0.09	0.74	0.08	0.19	0.44	0.04	0.39	0.01	0.05	0.25	0.23	0.81	1.28	2.32
300	0.97	1.1	1.03	0.13	0.23	0.14	0.08	0.09	0.79	0.07	0.18	0.46	0.04	0.39	0	0.04	0.23	0.21	0.73	1.18	2.22
302	0.86	0.97	0.91	0.12	0.2	0.12	0.07	0.09	0.84	0.07	0.18	0.48	0.03	0.39	0.01	0.04	0.22	0.21	0.66	1.1	2.14
304	0.75	0.84	0.79	0.11	0.17	0.11	0.07	0.09	0.89	0.06	0.18	0.51	0.03	0.4	0.01	0.04	0.22	0.2	0.58	1.02	2.07
306	0.65	0.72	0.67	0.09	0.14	0.09	0.06	0.09	0.96	0.05	0.18	0.54	0.03	0.4	0.01	0.04	0.21	0.2	0.52	0.95	2.01
308	0.57	0.63	0.58	0.08	0.12	0.08	0.06	0.1	1.03	0.05	0.18	0.58	0.02	0.42	0.01	0.03	0.21	0.19	0.46	0.89	1.96
310	0.51	0.56	0.51	0.07	0.1	0.07	0.06	0.1	1.1	0.05	0.18	0.62	0.02	0.43	0.01	0.03	0.21	0.19	0.42	0.85	1.94
312	0.46	0.51	0.46	0.07	0.09	0.06	0.05	0.11	1.17	0.05	0.19	0.65	0.02	0.45	0.01	0.03	0.21	0.19	0.4	0.82	1.92
314	0.42	0.47	0.42	0.06	0.08	0.06	0.06	0.11	1.25	0.04	0.19	0.69	0.02	0.47	0.01	0.03	0.22	0.19	0.38	0.79	1.92
316	0.4	0.45	0.39	0.06	0.07	0.05	0.06	0.12	1.32	0.05	0.2	0.73	0.02	0.49	0.01	0.03	0.22	0.2	0.37	0.78	1.91
318	0.37	0.42	0.37	0.05	0.07	0.05	0.05	0.12	1.39	0.04	0.2	0.76	0.02	0.51	0.02	0.03	0.22	0.2	0.35	0.76	1.9
320	0.36	0.4	0.35	0.05	0.06	0.05	0.06	0.12	1.44	0.04	0.21	0.8	0.02	0.53	0.02	0.03	0.23	0.2	0.34	0.75	1.89
322	0.34	0.39	0.33	0.05	0.06	0.04	0.05	0.13	1.49	0.04	0.21	0.82	0.02	0.54	0.02	0.03	0.23	0.19	0.33	0.73	1.88
324	0.32	0.38	0.32	0.05	0.06	0.04	0.06	0.13	1.53	0.04	0.21	0.85	0.02	0.55	0.02	0.03	0.23	0.2	0.32	0.72	1.86
326	0.31	0.37	0.31	0.04	0.05	0.04	0.06	0.13	1.56	0.04	0.21	0.86	0.02	0.56	0.02	0.03	0.23	0.19	0.32	0.71	1.84
328	0.3	0.36	0.3	0.04	0.05	0.04	0.06	0.13	1.57	0.04	0.21	0.87	0.02	0.56	0.02	0.03	0.23	0.2	0.31	0.69	1.81
330	0.29	0.35	0.29	0.04	0.05	0.04	0.05	0.13	1.56	0.04	0.21	0.86	0.02	0.55	0.02	0.03	0.23	0.19	0.31	0.67	1.77
332	0.28	0.34	0.28	0.03	0.05	0.04	0.05	0.13	1.53	0.04	0.21	0.85	0.01	0.54	0.02	0.03	0.23	0.19	0.3	0.66	1.72
334	0.27	0.33	0.27	0.03	0.05	0.04	0.05	0.12	1.49	0.04	0.2	0.83	0.01	0.52	0.02	0.03	0.22	0.19	0.29	0.64	1.67
336	0.26	0.32	0.26	0.03	0.05	0.04	0.05	0.12	1.42	0.03	0.19	0.79	0.01	0.5	0.02	0.03	0.21	0.18	0.28	0.61	1.61
338	0.25	0.3	0.25	0.03	0.05	0.04	0.04	0.11	1.34	0.03	0.18	0.75	0.01	0.47	0.01	0.03	0.2	0.17	0.27	0.59	1.53
340	0.24	0.29	0.24	0.03	0.05	0.04	0.04	0.11	1.24	0.03	0.17	0.7	0.01	0.45	0.02	0.03	0.2	0.17	0.26	0.57	1.46
342	0.24	0.28	0.24	0.03	0.05	0.04	0.04	0.1	1.13	0.03	0.16	0.64	0.01	0.41	0.01	0.03	0.18	0.16	0.25	0.54	1.38
344	0.23	0.27	0.23	0.03	0.04	0.03	0.04	0.09	1.01	0.03	0.14	0.57	0.01	0.37	0.01	0.02	0.17	0.15	0.24	0.51	1.3
346	0.22	0.27	0.22	0.03	0.04	0.03	0.03	0.08	0.9	0.02	0.13	0.51	0.01	0.33	0.01	0.02	0.15	0.14	0.23	0.49	1.23
348	0.22	0.26	0.22	0.03	0.04	0.03	0.03	0.07	0.78	0.03	0.12	0.45	0.01	0.29	0.01	0.02	0.14	0.13	0.23	0.47	1.16
350	0.21	0.25	0.21	0.03	0.04	0.03	0.03	0.06	0.67	0.02	0.11	0.38	0.01	0.26	0.01	0.02	0.13	0.12	0.22	0.45	1.09
352	0.21	0.24	0.21	0.03	0.04	0.03	0.02	0.05	0.56	0.02	0.09	0.33	0.01	0.22	0.01	0.02	0.12	0.11	0.21	0.42	1.03
354	0.2	0.23	0.2	0.02	0.04	0.03	0.02	0.04	0.48	0.02	0.08	0.28	0.01	0.19	0.01	0.02	0.11	0.1	0.2	0.41	0.97
356	0.2	0.23	0.2	0.02	0.04	0.03	0.02	0.04	0.4	0.02	0.08	0.24	0.01	0.17	0	0.02	0.1	0.1	0.2	0.39	0.93
358	0.19	0.22	0.19	0.02	0.03	0.03	0.02	0.03	0.34	0.02	0.07	0.21	0	0.15	0	0.02	0.09	0.09	0.19	0.38	0.89
360	0.18	0.21	0.18	0.02	0.03	0.02	0.01	0.03	0.29	0.02	0.06	0.17	0.01	0.13	-0	0.02	0.08	0.08	0.19	0.36	0.85
362	0.18	0.21	0.18	0.02	0.03	0.03	0.01	0.02	0.25	0.02	0.06	0.16	0	0.12	-0	0.02	0.08	0.08	0.18	0.36	0.82
364	0.17	0.2	0.17	0.02	0.03	0.02	0.01	0.02	0.23	0.01	0.05	0.14	0	0.11	-0	0.02	0.08	0.08	0.18	0.35	0.79
366	0.17	0.2	0.17	0.02	0.03	0.02	0.01	0.02	0.21	0.01	0.05	0.13	0	0.1	-0	0.02	0.08	0.08	0.17	0.34	0.76
368	0.16	0.19	0.16	0.02	0.03	0.02	0.01	0.02	0.19	0.01	0.05	0.12	0	0.1	-0	0.02	0.07	0.07	0.17	0.33	0.74
370	0.16	0.18	0.16	0.02	0.03	0.02	0.01	0.02	0.17	0.01	0.04	0.11	0	0.09	-0	0.02	0.07	0.07	0.17	0.32	0.71
372	0.15	0.17	0.15	0.02	0.03	0.02	0.01	0.02	0.16	0.01	0.04	0.1	0	0.09	-0	0.02	0.07	0.07	0.16	0.31	0.69
374	0.15	0.17	0.14	0.02	0.02	0.02	0.01	0.02	0.15	0.01	0.04	0.09	0	0.08	-0	0.02	0.07	0.06	0.16	0.3	0.67
376	0.14	0.16	0.14	0.02	0.03	0.02	0.01	0.01	0.14	0.01	0.04	0.09	0	0.08	-0	0.02	0.06	0.06	0.15	0.29	0.64
378	0.13	0.15	0.13	0.02	0.02	0.02	0.01	0.01	0.13	0.01	0.04	0.09	0	0.08	-0	0.02	0.06	0.06	0.15	0.28	0.62
380	0.13	0.15	0.13	0.02	0.02	0.02	0.01	0.01	0.12	0.01	0.03	0.08	0	0.07	-0	0.02	0.06	0.06	0.15	0.27	0.6
382	0.12	0.14	0.12	0.02	0.02	0.02	0.01	0.01	0.12	0.01	0.03	0.08	0	0.07	-0	0.02	0.06	0.05	0.14	0.26	0.58
384	0.12	0.13	0.12	0.02	0.02	0.02	0.01	0.01	0.11	0.01	0.03	0.07	0	0.07	-0	0.02	0.06	0.05	0.14	0.25	0.56
386	0.11	0.13	0.11	0.02	0.02	0.02	0.01	0.01	0.1	0.01	0.03	0.07	0	0.06	-0	0.02	0.06	0.05	0.14	0.24	0.54
388	0.11	0.13	0.11	0.02	0.02	0.02	0	0.01	0.1	0.01	0.03	0.06	0	0.06	-0	0.02	0.05	0.05	0.13	0.24	0.52
390	0.1	0.12	0.1	0.02	0.02	0.02	0	0.01	0.09	0.01	0.03	0.06	0	0.06	-0	0.02	0.05	0.05	0.13	0.23	0.51
392	0.1	0.12	0.1	0.01	0.02	0.01	0	0.01	0.08	0.01	0.02	0.06	-0	0.06	-0	0.02	0.05	0.04	0.13	0.22	0.49
394	0.1	0.11	0.1	0.01	0.02	0.02	0	0.01	0.08	0.01	0.02	0.05	0	0.05	-0	0.02	0.05	0.04	0.12	0.21	0.48
396	0.09	0.11	0.09	0.01	0.02	0.01	0	0.01	0.08	0.01	0.02	0.05	-0	0.05	-0	0.02	0.05	0.04	0.12	0.21	0.46
398	0.09	0.1	0.09	0.01	0.02	0.01	0	0.01	0.07	0.01	0.02	0.05	0	0.05	-0	0.02	0.05	0.04	0.12	0.2	0.45
400	0.09	0.1	0.09	0.01	0.01	0.01	0	0.01	0.07	0.01	0.02	0.04	0	0.05	-0	0.02	0.05	0.03	0.12	0.2	0.44
402	0.08	0.1	0.08	0.01	0.01	0.01	0	0	0.07	0.01	0.02	0.04	0	0.05	-0	0.02	0.04	0.03	0.12	0.19	0.43
404	0.08	0.09	0.08	0.01	0.01	0.01	0	0	0.06	0.01	0.02	0.04	-0	0.04	-0	0.02	0.04	0.03	0.12	0.19	0.42
406	0.08	0.09	0.08	0.01	0.01	0.01	0	0	0.06	0.01	0.02	0.04	-0	0.04	-0	0.02	0.04	0.03	0.12	0.18	0.41
408	0.08	0.09	0.08	0.01	0.01	0.01	0	0	0.05	0.01	0.02	0.04	-0	0.04	-0	0.02	0.04	0.03	0.12	0.18	0.4
410	0.07	0.08	0.07	0.01	0.01	0.01	0	0	0.05	0.01	0.02	0.04	-0	0.04	-0	0.01	0.04	0.03	0.12	0.17	0.39
412	0.07	0.08	0.07	0.01	0.01	0.01	0	0	0.05	0	0.01	0.03	-0	0.04	-0	0.02	0.04	0.03	0.12	0.17	0.39
414	0.07	0.08	0.07	0.01	0.01	0.01	0	0	0.05	0	0.01	0.03	-0	0.04	-0	0.02	0.04	0.03	0.12	0.17	0.38
416	0.07	0.08	0.07	0.01	0.01	0.01	-0	0	0.05	0	0.01	0.03	-0	0.04	-0	0.02	0.04	0.03	0.12	0.16	0.37
418	0.07	0.08	0.07	0.01	0.01	0.01	0	0	0.04	0	0.01	0.03	-0	0.04	-0	0.02	0.04	0.03	0.11	0.16	0.37
420	0.06	0.07	0.06	0.01	0.01	0.01	0	0	0.04	0	0.01	0.03	-0	0.04	-0	0.02	0.04	0.02	0.11	0.15	0.36
422	0.06	0.07	0.06	0.01	0.01	0.01	0	0	0.04	0	0.01	0.03	-0	0.03	-0	0.02	0.04	0.02	0.11	0.15	0.35
424	0.06	0.07	0.06	0.01	0.01	0.01	-0	0	0.04	0	0.01	0.03	-0	0.03	-0	0.02	0.04	0.02	0.11	0.15	0.35
426	0.06	0.07	0.06	0.01	0.01	0.01	0	0	0.04	0	0.01	0.03	-0	0.03	-0	0.02	0.04	0.02	0.11	0.15	0.35
428	0.06	0.07	0.06	0.01	0.01	0.01	-0	-0	0.04	0	0.01	0.03	-0	0.03	-0	0.02	0.04	0.02	0.11	0.14	0.34
430	0.06	0.07	0.06	0.01	0.01	0.01	0	0	0.04	0	0.01	0.02	-0	0.03	-0	0.01	0.04	0.02	0.11	0.14	0.34
432	0.05	0.06	0.06	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.04	0.02	0.11	0.14	0.33
434	0.05	0.06	0.05	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.04	0.02	0.11	0.13	0.33
436	0.05	0.06	0.05	0.01	0.01	0.01	-0	0	0.03	0	0.01	0.02	-0	0.03	-0	0.01	0.04	0.02	0.11	0.13	0.32
438	0.05	0.06	0.05	0.01	0.01	0.01	0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.03	0.02	0.11	0.13	0.32
440	0.05	0.06	0.05	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.04	0.02	0.11	0.13	0.31
442	0.05	0.06	0.05	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.04	0.02	0.1	0.12	0.31
444	0.05	0.05	0.05	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.03	0.02	0.1	0.12	0.31
446	0.05	0.05	0.05	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.01	0.03	0.02	0.1	0.12	0.3
448	0.04	0.05	0.05	0.01	0.01	0.01	-0	-0	0.03	0	0.01	0.02	-0	0.03	-0	0.02	0.03	0.02	0.1	0.12	0.3
450	0.04	0.05	0.05	0.01	0.01	0.01	-0	-0	0.02	0	0.01	0.02	-0	0.03	-0	0.01	0.03	0.02	0.1	0.12	0.29

Appendix 3. Wavescanning results for Refluxing valine, leucine and phenylalanine in the presence of 0.2M of lysine to observe the effects lysine has on colour

Wavelength	Phenylalanine				Leucine				Valine			
	60	120	180	50% EtO	60	120	180	50% EtO	60	120	180	50% EtO
200	29.83	30	30	0.83	27.99	30	30	0.26	27.51	30	30	0.13
202	30	30	30	0.74	29.85	30	30	0.31	28.53	30	30	0.36
204	30	30	30	0.85	30	30	30	0.36	27.8	30	30	0.16
206	30	30	30	0.86	30	30	30	0.27	26.25	30	30	0.11
208	30	30	30	0.85	30	30	30	0.23	23.63	30	30	0.09
210	30	30	30	0.75	30	30	30	0.16	20.89	30	30	0.03
212	30	30	30	0.7	29.67	30	30	0.15	18.64	30	30	0.03
214	30	30	30	0.59	27.4	30	30	0.14	16.53	30	30	0.02
216	30	30	30	0.48	23.95	30	30	0.11	14.65	30	30	-0.02
218	30	30	30	0.36	19.38	27.55	30	0.11	13.08	30	30	-0.03
220	30	30	30	0.25	15.04	24.69	30	0.1	11.73	30	30	-0.02
222	30	30	30	0.16	12.36	22.86	30	0.09	10.71	26.47	30	-0.03
224	25.28	30	30	0.11	10.74	21.31	30	0.09	9.81	23.83	30	-0.04
226	18.66	30	30	0.12	9.59	20.05	30	0.11	9.04	22.08	29.38	-0.03
228	14.61	29.19	30	0.11	8.78	19.01	28.86	0.11	8.44	20.87	28.38	-0.02
230	12.93	27.45	30	0.1	8.3	18.31	27.97	0.12	8.05	20.16	27.48	-0.02
232	11.74	25.98	30	0.06	7.83	17.56	26.78	0.1	7.67	19.43	26.48	-0.01
234	11.38	25.38	30	0.07	7.52	17.02	25.76	0.1	7.41	18.96	25.74	-0.02
236	11.43	25.16	30	0.02	7.3	16.65	25.24	0.08	7.22	18.68	25.33	-0.03
238	11.8	25.43	30	0.05	7.16	16.41	24.76	0.1	7.12	18.58	25	-0.01
240	12.7	26.26	30	0.04	7.15	16.35	24.45	0.08	7.14	18.65	24.91	-0.03
242	13.45	26.87	30	0.05	7.13	16.31	24.36	0.07	7.13	18.72	24.87	0
244	14.37	27.87	30	0.05	7.15	16.33	24.29	0.11	7.14	18.89	24.87	0.01
246	16.03	29.35	30	0.02	7.3	16.43	24.36	0.07	7.22	19.16	24.98	-0.05
248	16.73	30	30	0.06	7.35	16.53	24.49	0.07	7.3	19.35	25.17	-0.03
250	18.19	30	30	0.05	7.43	16.66	24.6	0.05	7.35	19.62	25.29	-0.04
252	19.64	30	30	0.04	7.58	16.85	24.82	0.08	7.42	19.87	25.54	-0.02
254	19.36	30	30	0.04	7.66	16.9	24.92	0.07	7.46	19.93	25.61	-0.06
256	21.44	30	30	0.03	7.73	16.97	25.08	0.05	7.49	20.23	25.76	-0.04
258	21.34	30	30	0.03	7.72	17.05	25.24	0.03	7.48	20.16	25.81	-0.05
260	19.11	30	30	0.03	7.64	16.97	25.27	0.03	7.41	19.85	25.82	-0.04
262	18.66	30	30	0.06	7.54	16.89	25.24	0.03	7.35	19.72	25.69	-0.03
264	17.21	30	30	0.06	7.48	16.78	25.26	0.06	7.27	19.41	25.58	-0.01
266	14.42	27.52	30	0.07	7.28	16.66	25.31	0.03	7.1	18.98	25.48	-0.01
268	11.45	24.58	30	0.01	7.12	16.55	25.3	0.03	6.96	18.56	25.36	-0.04
270	8.78	22.16	30	-0.01	6.99	16.49	25.38	0.01	6.82	18.21	25.31	-0.05
272	7.67	20.96	30	0.02	6.88	16.49	25.49	0.01	6.7	18.03	25.26	-0.06
274	7.05	20.22	30	0.03	6.83	16.58	25.69	0.01	6.65	18	25.22	-0.03
276	6.74	19.79	30	0.03	6.85	16.83	25.8	0.02	6.61	18.06	25.38	0
278	6.5	19.53	30	0	6.84	17.1	26.11	0.01	6.62	18.18	25.5	-0.04
280	6.35	19.49	30	0.06	6.99	17.56	26.46	0.05	6.68	18.64	25.8	-0.02
282	6.25	19.48	30	-0.01	7.14	18.06	26.86	0.03	6.72	19.01	26.16	-0.05
284	6.17	19.67	30	0	7.41	18.74	27.22	0.02	6.92	19.81	26.54	-0.05
286	6.16	19.89	30	-0.01	7.75	19.64	27.74	0	7.25	20.52	27.08	-0.06
288	6.1	20.17	30	-0.01	8.03	20.49	28.2	0.02	7.51	21.31	27.63	-0.05
290	6.12	20.61	30	0.01	8.38	21.46	28.75	0.03	7.74	22.35	28.18	-0.04
292	6.1	21.14	30	0	8.8	22.66	29.38	0.01	8.11	23.3	28.72	-0.04
294	6.15	21.65	30	-0.01	9.25	23.86	29.69	0.02	8.47	24.62	29.37	-0.04
296	6.2	22.28	30	-0.01	9.79	25.17	30	-0.01	9	26.07	30	-0.04
298	6.22	22.93	30	-0.01	10.3	26.56	30	0.02	9.41	27.39	30	-0.09
300	6.33	23.81	30	0.01	11	28.03	30	-0.01	9.92	28.8	30	-0.06
302	6.41	24.71	30	-0.02	11.55	29.25	30	-0.01	10.5	30	30	-0.06
304	6.57	25.57	30	0.01	12.2	30	30	0	11.09	30	30	-0.06
306	6.63	26.43	30	0.01	12.81	30	30	0.01	11.58	30	30	-0.06
308	6.8	27.42	30	0	13.5	30	30	0.01	12.21	30	30	-0.06
310	6.98	28.26	30	-0.02	14.16	30	30	-0.02	12.76	30	30	-0.08
312	7.04	28.89	30	-0.03	14.66	30	30	-0.03	13.18	30	30	-0.09
314	7.16	29.65	30	-0.02	15.19	30	30	-0.01	13.62	30	30	-0.06
316	7.21	29.94	30	-0.02	15.56	30	30	-0.02	13.98	30	30	-0.07
318	7.34	30	30	0.01	15.93	30	30	0.02	14.29	30	30	-0.04
320	7.34	30	30	0	16.07	30	30	0	14.37	30	30	-0.05
322	7.25	30	30	0	16.05	30	30	-0.02	14.32	30	30	-0.04
324	7.18	30	30	0.03	15.98	30	30	0.01	14.24	30	30	-0.04
326	7.01	29.66	30	0	15.64	30	30	0	13.91	30	30	-0.04
328	6.88	28.96	30	-0.02	15.22	30	30	-0.03	13.52	30	30	-0.05
330	6.66	27.96	30	0.01	14.61	30	30	0.01	12.98	30	30	-0.04
332	6.36	26.82	30	0.02	13.86	30	30	0	12.3	30	30	-0.05
334	6.07	25.49	30	0	13.03	30	29.63	-0.01	11.56	30	30	-0.04
336	5.71	23.92	30	0	12.06	29.56	28.33	0.01	10.69	29.7	28.94	-0.04
338	5.35	22.34	30	0	11	27.65	26.7	-0.01	9.78	27.46	27.4	-0.05
340	4.97	20.43	30	-0.01	10.01	24.85	24.94	-0.01	8.88	24.91	25.45	-0.06
342	4.57	18.6	30	-0.01	8.92	22.35	23.27	-0.01	7.93	22.33	23.63	-0.05
344	4.17	16.8	29.51	0	7.82	19.99	21.42	0	7	20.04	21.66	-0.03
346	3.83	15.34	28.27	-0.02	6.95	17.9	20.05	-0.01	6.23	17.92	20.08	-0.05
348	3.47	13.63	26.43	-0.02	6	15.59	18.3	-0.01	5.42	15.79	18.33	-0.04
350	3.15	12.22	24.78	-0.02	5.22	13.69	16.93	-0.02	4.76	13.91	16.85	-0.05
352	2.87	10.97	23.25	-0.02	4.55	11.97	15.63	0	4.15	12.26	15.46	-0.06
354	2.63	9.9	21.66	-0.02	3.97	10.53	14.38	0	3.66	10.85	14.21	-0.04
356	2.43	9	20.37	-0.02	3.51	9.39	13.45	-0.01	3.26	9.7	13.14	-0.05
358	2.27	8.26	19.24	-0.02	3.11	8.42	12.54	0	2.91	8.74	12.21	-0.04
360	2.08	7.59	18.17	-0.03	2.78	7.6	11.77	-0.01	2.61	7.94	11.39	-0.05
362	2	7.15	17.33	-0.03	2.56	7.03	11.16	-0.01	2.44	7.39	10.82	-0.04
364	1.89	6.7	16.46	-0.03	2.36	6.51	10.55	-0.01	2.26	6.84	10.19	-0.05
366	1.79	6.29	15.61	-0.03	2.18	6.06	10.01	-0.01	2.1	6.38	9.64	-0.04
368	1.7	5.94	14.88	-0.03	2.03	5.67	9.5	-0.02	1.96	5.98	9.15	-0.05
370	1.63	5.63	14.18	-0.03	1.92	5.34	9.03	0	1.84	5.63	8.69	-0.04
372	1.55	5.36	13.59	-0.03	1.81	5.06	8.66	0	1.74	5.33	8.27	-0.04
374	1.48	5.1	12.99	-0.04	1.7	4.79	8.25	-0.02	1.64	5.04	7.88	-0.06
376	1.42	4.85	12.42	-0.03	1.62	4.55	7.88	0	1.55	4.79	7.54	-0.04
378	1.35	4.63	11.95	-0.03	1.54	4.36	7.6	-0.01	1.49	4.59	7.25	-0.04
380	1.29	4.44	11.41	-0.03	1.47	4.16	7.27	-0.01	1.42	4.38	6.94	-0.05
382	1.22	4.22	10.89	-0.03	1.39	3.95	6.95	0	1.34	4.16	6.62	-0.04
384	1.18	4.03	10.43	-0.03	1.34	3.8	6.68	-0.01	1.29	3.97	6.34	-0.05
386	1.12	3.84	9.98	-0.03	1.28	3.62	6.41	0	1.23	3.8	6.07	-0.04
388	1.07	3.66	9.56	-0.04	1.22	3.49	6.17	-0.02	1.16	3.63	5.83	-0.05
390	1.03	3.51	9.14	-0.04	1.18	3.34	5.91	-0.02	1.14	3.47	5.59	-0.05
392	0.98	3.35	8.76	-0.02	1.12	3.2	5.69	-0.01	1.08	3.33	5.37	-0.05
394	0.93	3.21	8.4	-0.03	1.08	3.07	5.48	0	1.03	3.18	5.15	-0.03
396	0.89	3.07	8.07	-0.03	1.03	2.95	5.27	-0.01	0.98	3.05	4.95	-0.04
398	0.85	2.92	7.74	-0.04	0.99	2.82	5.06	-0.02	0.94	2.91	4.76	-0.05
400	0.82	2.8	7.43	-0.03	0.96	2.72	4.88	-0.01	0.91	2.81	4.58	-0.05
402	0.77	2.68	7.11	-0.04	0.91	2.59	4.69	-0.01	0.86	2.67	4.39	-0.06
404	0.74	2.57	6.85	-0.03	0.87	2.5	4.53	-0.02	0.83	2.57	4.22	-0.05
406	0.71	2.45	6.58	-0.03	0.82	2.38	4.36	-0.01	0.78	2.44	4.04	-0.05
408	0.68	2.36	6.32	-0.02	0.8	2.3	4.2	-0.01	0.77	2.35	3.9	-0.03
410	0.65	2.24	6.1	-0.04	0.77	2.2	4.02	-0.02	0.72	2.25	3.74	-0.05
412	0.63	2.18	5.85	-0.04	0.74	2.11	3.86	-0.02	0.7	2.16	3.61	-0.06
414	0.62	2.08	5.65	-0.04	0.71	2.03	3.73	-0.02	0.67	2.07	3.46	-0.05
416	0.58	1.99	5.45	-0.03	0.67	1.94	3.59	-0.01	0.64	1.98	3.32	-0.05
418	0.57	1.94	5.28	-0.06	0.65	1.87	3.48	-0.03	0.62	1.92	3.22	-0.06
420	0.54	1.86	5.1	-0.03	0.63	1.8	3.36	-0.02	0.6	1.83	3.1	-0.05
422	0.52	1.8	4.93	-0.04	0.61	1.74	3.25	-0.01	0.56	1.77	2.99	-0.05
424	0.48	1.72	4.79	-0.04	0.58	1.67	3.15	-0.01	0.53	1.69	2.89	-0.05
426	0.51	1.69	4.67	-0.04	0.57	1.63	3.08	-0.01	0.54	1.66	2.81	-0.05
428	0.47	1.63	4.52	-0.05	0.56	1.59	2.97	-0.03	0.52	1.61	2.73	-0.06
430	0.46	1.56	4.4	-0.03	0.53	1.52	2.9	-0.01	0.49	1.56	2.63	-0.04
432	0.45	1.54	4.26	-0.05	0.51	1.48	2.8	-0.01	0.48	1.51	2.57	-0.06
434	0.43	1.5	4.15	-0.04	0.51	1.45	2.73	-0.02	0.47	1.48	2.51	-0.05
436	0.42	1.44	4.04	-0.04	0.48	1.39	2.64	-0.02	0.45	1.42	2.41	-0.05
438	0.39	1.39	3.92	-0.04	0.46	1.33	2.56	-0.02	0.42	1.36	2.34	-0.05
440	0.39	1.37	3.81	-0.04	0.45	1.3	2.49	-0.01	0.41	1.32	2.28	-0.04
442	0.37	1.31	3.72	-0.04	0.44	1.26	2.41	0	0.41	1.28	2.2	-0.04
444	0.37	1.3	3.62	-0.05	0.43	1.23	2.35	-0.02	0.41	1.26	2.15	-0.06
446	0.35	1.23	3.52	-0.03	0.41	1.18	2.28	-0.01	0.37	1.19	2.09	-0.04
448	0.34	1.2	3.43	-0.04	0.39	1.14	2.21	-0.02	0.37	1.16	2.02	-0.04
450	0.33	1.17	3.34	-0.04	0.37	1.12	2.15	-0.03	0.36	1.13	1.97	-0.05

Appendix 4. Wavescanning results for refluxing phenylalanine in the presence of various concentrations of lysine for colour

		Control			0.025M Lys			0.05M Lys			0.1M Lys			0.2M Lys	
Wavelen	T60	T120	T180	T60	T120	T180	T60	T120	T180	T60	T120	T180	T60	T120	T180
200	28.09	30	30	27.18	30	30	26.95	29.27	30	26.99	30	30	29.83	30	30
202	29	30	30	28.6	30	30	28.85	30	30	28.32	30	30	30	30	30
204	30	30	30	30	30	30	30	30	30	29.89	30	30	30	30	30
206	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
208	29.96	30	30	30	30	30	30	30	30	30	30	30	30	30	30
210	29.96	30	30	30	30	30	30	30	30	30	30	30	30	30	30
212	29.89	30	30	30	30	30	30	30	30	30	30	30	30	30	30
214	29.31	30	30	30	30	30	30	30	30	30	30	30	30	30	30
216	28.56	30	30	29.55	30	30	29.61	30	30	29.36	30	30	30	30	30
218	28.21	30	30	29.41	30	30	29.36	30	30	28.9	30	30	30	30	30
220	26.92	30	30	28.96	30	30	28.89	30	30	28.37	30	30	30	30	30
222	20.91	30	30	26.29	30	30	26.51	30	30	26.23	30	30	30	30	30
224	13.08	22.44	27.9	18.13	24.32	30	18.7	23.95	30	18.31	27.7	30	25.28	30	30
226	8.57	16.52	22.94	11.51	18.06	28.85	11.89	18.06	28.17	11.43	21.57	30	18.66	30	30
228	6.29	13.74	20.03	8.51	15.08	25.88	8.54	14.84	25.44	8.01	18.06	30	14.61	29.19	30
230	5.4	12.43	18.76	7.27	13.7	24.41	7.29	13.53	24	6.67	16.53	28.79	12.93	27.45	30
232	4.73	11.43	17.66	6.34	12.72	23.12	6.35	12.52	22.76	5.72	15.28	27.29	11.74	25.98	30
234	4.46	11.01	17.1	6.11	12.37	22.5	6.11	12.17	22.16	5.48	14.88	26.53	11.38	25.38	30
236	4.3	10.83	16.85	6.22	12.45	22.39	6.24	12.24	22.08	5.59	14.91	26.38	11.43	25.16	30
238	4.33	10.97	16.93	6.61	12.81	22.64	6.6	12.59	22.24	5.98	15.28	26.49	11.8	25.43	30
240	4.85	11.65	17.64	7.39	13.68	23.42	7.5	13.4	23.06	6.7	16.15	27.27	12.7	26.26	30
242	5.15	12.27	18.24	7.96	14.25	24.12	8.15	14.12	23.71	7.34	16.91	27.99	13.45	26.87	30
244	5.81	13.05	19.2	8.83	15.31	25.04	9.06	15.13	24.66	8.17	17.86	28.91	14.37	27.87	30
246	6.6	14.38	20.58	10.21	16.67	26.69	10.52	16.58	26.24	9.6	19.51	30	16.03	29.35	30
248	6.86	14.91	21.13	10.79	17.23	27.29	11.08	17.18	26.78	10.06	20.08	30	16.73	30	30
250	7.8	16.23	22.63	12.18	18.69	28.79	12.68	18.77	28.37	11.51	21.77	30	18.19	30	30
252	8.49	17.35	23.66	13.32	19.89	29.85	13.78	19.82	29.43	12.65	22.95	30	19.64	30	30
254	8.36	17.16	23.46	13.14	19.71	29.75	13.58	19.67	29.3	12.47	22.79	30	19.36	30	30
256	9.67	18.95	25.09	14.96	21.43	30	15.46	21.56	30	14.25	24.72	30	21.44	30	30
258	9.33	18.6	24.77	14.76	21.27	30	15.36	21.15	30	14.12	24.3	30	21.34	30	30
260	8.11	16.82	23.14	13.01	19.5	29.69	13.46	19.36	29.21	12.36	22.39	30	19.11	30	30
262	7.91	16.47	22.74	12.61	19.06	29.24	13.07	18.94	28.73	11.99	21.89	30	18.66	30	30
264	7.12	15.21	21.46	11.47	17.64	27.83	11.77	17.47	27.37	10.83	20.36	30	17.21	30	30
266	5.58	12.93	19.07	9.05	15.12	25.34	9.27	15.04	24.96	8.44	17.52	28.71	14.42	27.52	30
268	4.08	10.63	16.68	6.84	12.85	22.87	6.89	12.59	22.56	6.25	14.79	26.35	11.45	24.58	30
270	2.46	8.3	14.32	4.47	10.24	20.38	4.44	10.08	20.18	3.95	12.12	23.8	8.78	22.16	30
272	1.85	7.32	13.25	3.57	9.23	19.25	3.47	9.02	19.04	3.05	10.96	22.63	7.67	20.96	30
274	1.55	6.75	12.55	3.08	8.55	18.44	2.95	8.34	18.26	2.59	10.24	21.9	7.05	20.22	30
276	1.37	6.39	12.02	2.83	8.12	17.93	2.7	7.93	17.77	2.37	9.8	21.5	6.74	19.79	30
278	1.31	6.22	11.69	2.68	7.8	17.56	2.57	7.61	17.43	2.24	9.51	21.26	6.5	19.53	30
280	1.22	5.98	11.26	2.59	7.55	17.29	2.49	7.38	17.13	2.18	9.28	21.07	6.35	19.49	30
282	1.17	5.84	10.99	2.52	7.31	17.07	2.4	7.15	16.95	2.11	9.1	21.02	6.25	19.48	30
284	1.13	5.71	10.68	2.46	7.14	16.96	2.35	6.95	16.78	2.04	8.94	20.96	6.17	19.67	30
286	1.08	5.62	10.41	2.34	6.92	16.88	2.25	6.76	16.73	1.98	8.8	21.04	6.16	19.89	30
288	0.99	5.48	10.15	2.28	6.75	16.85	2.16	6.63	16.73	1.88	8.7	21.22	6.1	20.17	30
290	1.01	5.51	9.93	2.2	6.61	16.89	2.07	6.48	16.7	1.79	8.59	21.39	6.12	20.61	30
292	1	5.45	9.76	2.1	6.42	16.97	1.97	6.31	16.8	1.72	8.46	21.59	6.1	21.14	30
294	0.95	5.4	9.54	2.01	6.3	17.17	1.9	6.18	16.99	1.64	8.38	21.99	6.15	21.65	30
296	0.97	5.46	9.4	1.92	6.17	17.41	1.8	6.07	17.15	1.53	8.3	22.26	6.2	22.28	30
298	0.95	5.49	9.33	1.84	6.05	17.68	1.71	5.97	17.43	1.45	8.2	22.67	6.22	22.93	30
300	0.97	5.56	9.27	1.76	6	18.11	1.64	5.88	17.82	1.39	8.15	23.24	6.33	23.81	30
302	0.91	5.64	9.21	1.71	5.92	18.63	1.56	5.82	18.23	1.34	8.11	23.8	6.41	24.71	30
304	0.92	5.72	9.17	1.65	5.9	19.08	1.51	5.79	18.72	1.27	8.05	24.36	6.57	25.57	30
306	0.96	5.87	9.2	1.61	5.85	19.67	1.45	5.75	19.22	1.23	8.04	24.97	6.63	26.43	30
308	0.96	5.98	9.19	1.56	5.8	20.24	1.38	5.69	19.75	1.18	7.98	25.55	6.8	27.42	30
310	0.96	6.17	9.23	1.51	5.77	20.79	1.37	5.68	20.23	1.14	7.94	26.17	6.98	28.26	30
312	0.96	6.24	9.21	1.52	5.75	21.31	1.34	5.65	20.65	1.12	7.94	26.74	7.04	28.89	30
314	0.94	6.34	9.18	1.47	5.7	21.75	1.31	5.59	21.06	1.08	7.86	27.09	7.16	29.65	30
316	0.93	6.32	9.08	1.48	5.67	21.93	1.31	5.58	21.34	1.1	7.85	27.39	7.21	29.94	30
318	0.96	6.39	9.01	1.46	5.64	22.19	1.28	5.51	21.49	1.06	7.76	27.53	7.34	30	30
320	0.95	6.41	8.94	1.44	5.55	22.17	1.28	5.44	21.52	1.06	7.7	27.6	7.34	30	30
322	0.98	6.34	8.78	1.4	5.47	22.13	1.26	5.35	21.42	1.03	7.6	27.55	7.25	30	30
324	0.97	6.29	8.66	1.4	5.38	21.9	1.23	5.29	21.23	1.05	7.49	27.35	7.18	30	30
326	0.92	6.16	8.47	1.37	5.28	21.52	1.21	5.18	20.88	1	7.37	26.82	7.01	29.66	30
328	0.87	5.99	8.25	1.35	5.18	20.97	1.2	5.08	20.35	0.99	7.23	26.29	6.88	28.96	30
330	0.86	5.84	8.06	1.3	5.02	20.27	1.14	4.93	19.7	0.95	7.03	25.47	6.66	27.96	30
332	0.84	5.61	7.8	1.26	4.86	19.4	1.11	4.77	18.85	0.93	6.83	24.47	6.36	26.82	30
334	0.83	5.36	7.56	1.23	4.7	18.52	1.07	4.62	18	0.91	6.63	23.41	6.07	25.49	30
336	0.78	5.07	7.27	1.15	4.51	17.46	1.02	4.43	17.03	0.86	6.37	22.1	5.71	23.92	30
338	0.77	4.79	6.99	1.08	4.3	16.3	0.95	4.21	15.94	0.8	6.08	20.75	5.35	22.34	30
340	0.69	4.49	6.7	1.04	4.13	15.21	0.93	4.05	14.89	0.78	5.81	19.32	4.97	20.43	30
342	0.66	4.16	6.39	0.97	3.9	13.97	0.85	3.81	13.71	0.72	5.5	17.89	4.57	18.6	30
344	0.61	3.84	6.06	0.93	3.7	12.78	0.82	3.61	12.52	0.68	5.21	16.28	4.17	16.8	29.51
346	0.58	3.57	5.8	0.87	3.5	11.76	0.78	3.42	11.59	0.64	4.96	15.07	3.83	15.34	28.27
348	0.54	3.28	5.49	0.8	3.3	10.69	0.71	3.22	10.53	0.59	4.67	13.72	3.47	13.63	26.43
350	0.5	3.04	5.24	0.75	3.12	9.76	0.68	3.04	9.65	0.55	4.42	12.57	3.15	12.22	24.78
352	0.47	2.81	4.98	0.7	2.94	8.92	0.63	2.87	8.85	0.51	4.17	11.5	2.87	10.97	23.25
354	0.44	2.61	4.74	0.66	2.78	8.16	0.59	2.72	8.14	0.49	3.95	10.58	2.63	9.9	21.66
356	0.45	2.43	4.54	0.61	2.65	7.54	0.57	2.57	7.53	0.45	3.74	9.79	2.43	9	20.37
358	0.41	2.28	4.31	0.57	2.51	6.99	0.53	2.44	7.01	0.42	3.56	9.08	2.27	8.26	19.24
360	0.38	2.14	4.11	0.54	2.37	6.53	0.48	2.31	6.54	0.38	3.37	8.48	2.08	7.59	18.17
362	0.37	2.04	3.96	0.52	2.28	6.15	0.47	2.22	6.2	0.38	3.22	8.02	2	7.15	17.33
364	0.35	1.93	3.8	0.49	2.18	5.83	0.44	2.12	5.86	0.36	3.1	7.59	1.89	6.7	16.46
366	0.35	1.85	3.65	0.48	2.09	5.51	0.42	2.03	5.54	0.35	2.96	7.18	1.79	6.29	15.61
368	0.33	1.75	3.48	0.45	1.99	5.21	0.4	1.94	5.25	0.32	2.82	6.8	1.7	5.94	14.88
370	0.31	1.67	3.33	0.43	1.92	4.96	0.38	1.86	4.98	0.32	2.71	6.48	1.63	5.63	14.18
372	0.31	1.6	3.2	0.41	1.84	4.73	0.37	1.79	4.76	0.3	2.61	6.18	1.55	5.36	13.59
374	0.28	1.5	3.05	0.37	1.74	4.48	0.33	1.69	4.51	0.28	2.48	5.88	1.48	5.1	12.99
376	0.26	1.42	2.9	0.37	1.66	4.27	0.33	1.62	4.29	0.26	2.38	5.6	1.42	4.85	12.42
378	0.27	1.38	2.79	0.34	1.59	4.09	0.31	1.54	4.12	0.24	2.28	5.38	1.35	4.63	11.95
380	0.26	1.3	2.66	0.31	1.51	3.87	0.28	1.47	3.9	0.23	2.17	5.13	1.29	4.44	11.41
382	0.23	1.23	2.53	0.29	1.43	3.66	0.26	1.4	3.7	0.21	2.07	4.87	1.22	4.22	10.89
384	0.21	1.16	2.41	0.27	1.37	3.49	0.25	1.33	3.52	0.2	1.98	4.65	1.18	4.03	10.43
386	0.23	1.12	2.31	0.26	1.31	3.32	0.23	1.27	3.35	0.18	1.9	4.45	1.12	3.84	9.98
388	0.2	1.04	2.19	0.24	1.24	3.16	0.21	1.2	3.17	0.17	1.81	4.25	1.07	3.66	9.56
390	0.17	0.98	2.06	0.22	1.17	2.99	0.2	1.12	3.02	0.16	1.73	4.05	1.03	3.51	9.14
392	0.17	0.93	1.98	0.21	1.1	2.85	0.19	1.08	2.88	0.14	1.66	3.88	0.98	3.35	8.76
394	0.17	0.9	1.9	0.19	1.05	2.71	0.16	1.02	2.76	0.12	1.57	3.72	0.93	3.21	8.4
396	0.16	0.86	1.81	0.18	1	2.6	0.16	0.98	2.64	0.12	1.51	3.56	0.89	3.07	8.07
398	0.15	0.8	1.73	0.17	0.97	2.48	0.15	0.93	2.5	0.11	1.45	3.4	0.85	2.92	7.74
400	0.14	0.76	1.65	0.14	0.9	2.36	0.13	0.88	2.39	0.09	1.37	3.26	0.82	2.8	7.43
402	0.13	0.72	1.58	0.13	0.86	2.26	0.12	0.84	2.28	0.09	1.32	3.1	0.77	2.68	7.11
404	0.12	0.69	1.51	0.13	0.82	2.16	0.12	0.8	2.19	0.08	1.26	2.99	0.74	2.57	6.85
406	0.12	0.67	1.45	0.12	0.79	2.07	0.11	0.76	2.1	0.07	1.2	2.87	0.71	2.45	6.58
408	0.11	0.65	1.41	0.11	0.75	2	0.1	0.72	2.02	0.07	1.15	2.76	0.68	2.36	6.32
410	0.11	0.62	1.35	0.1	0.72	1.91	0.09	0.69	1.95	0.06	1.11	2.65	0.65	2.24	6.1
412	0.1	0.58	1.29	0.1	0.68	1.84	0.08	0.66	1.85	0.05	1.06	2.55	0.63	2.18	5.85
414	0.1	0.57	1.24	0.09	0.65	1.78	0.08	0.63	1.79	0.05	1.01	2.46	0.62	2.08	5.65
416	0.11	0.55	1.21	0.08	0.63	1.7	0.08	0.59	1.72	0.04	0.97	2.35	0.58	1.99	5.45
418	0.08	0.52	1.14	0.08	0.6	1.65	0.06	0.58	1.66	0.03	0.94	2.26	0.57	1.94	5.28
420	0.09	0.5	1.1	0.07	0.58	1.58	0.06	0.54	1.59	0.03	0.9	2.18	0.54	1.86	5.1
422	0.1	0.49	1.08	0.08	0.55	1.53	0.05	0.53	1.54	0.04	0.86	2.13	0.52	1.8	4.93
424	0.09	0.47	1.03	0.07	0.54	1.48	0.05	0.52	1.49	0.04	0.85	2.04	0.48	1.72	4.79
426	0.08	0.45	1	0.05	0.51	1.43	0.04	0.49	1.44	0.02	0.79	1.99	0.51	1.69	4.67
428	0.07	0.44	0.96	0.06	0.5	1.39	0.06	0.47	1.41	0.03	0.79	1.93	0.47	1.63	4.52
430	0.09	0.42	0.93	0.05	0.47	1.33	0.03	0.44	1.33	0.02	0.74	1.86	0.46	1.56	4.4
432	0.06	0.39	0.89	0.05	0.46	1.29	0.05	0.43	1.31	0.01	0.72	1.81	0.45	1.54	4.26
434	0.07	0.38	0.87	0.04	0.43	1.24	0.02	0.4	1.27	0	0.7	1.76	0.43	1.5	4.15
436	0.05	0.35	0.82	0.04	0.42	1.2	0.03	0.4	1.23	0.01	0.67	1.7	0.42	1.44	4.04
438	0.06	0.36	0.8	0.04	0.39	1.17	0.03	0.38	1.19	0.01	0.65	1.65	0.39	1.39	3.92
440	0.05	0.33	0.75	0.03	0.38	1.12	0.02	0.36	1.14	-0.02	0.62	1.6	0.39	1.37	3.81
442	0.06	0.32	0.74	0.02	0.36	1.08	0.02	0.35	1.1	0	0.59	1.54	0.37	1.31	3.72
444	0.04	0.32	0.71	0.03	0.36	1.06	0.02	0.33	1.09	0.01	0.59	1.52	0.37	1.3	3.62
446	0.05	0.28	0.68	0.02	0.33	1.03	0.02	0.32	1.02	0	0.56	1.46	0.35	1.23	3.52
448	0.04	0.28	0.65	0.03	0.34	1.01	0.02	0.32	1	0.01	0.55	1.42	0.34	1.2	3.43
450	0.05	0.27	0.63	-0.01	0.31	0.94	0	0.28	0.98	-0.02	0.52	1.38	0.33	1.17	3.34

Appendix 5. Assessment of visible colour, and odour of chosen amino acids over 30 weeks.

	Treatment	**Aroma**	**Colour**
Week 0	Control (Lactose only)	No aroma other then EtOH	Very pale yellow
	Phenylalanine	Flowery, slightly sweet, pleasant	Dark brown
	Leucine	Solvent aroma, slightly sweet/biscuity	Dark brown
	Alanine	Sweet aroma at first, then burnt notes come through	Dark brown
	Valine	Solvent aroma biscuity, savoury	Dark brown
Week 1	Control (Lactose only)	Solvent like, very faint sweet aroma	Pale yellow
	Phenylalanine	No flowery aroma, no odour of anything	Dark brown
	Leucine	Sweet, pastry like	Reddish brown
	Alanine	Very faint solvent like aroma	Brown, dark
	Valine	Solvent like, no sweetness detected	Dark brown
Week 2	Control (Lactose only)	Very faint sweet aroma	Very pale yellow
	Phenylalanine	Very faint flowery aroma, no EtOH detected	Medium brown, reddish
	Leucine	Pleasant sweet caramel, very very faint EtOH	Medium brown
	Alanine	Very very faint sweet	Medium brown, reddish
	Valine	Strong EtOH, faint sweet notes, eucalyptus like	Medium brown
Week 3	Control (Lactose only)	Very very faint sweet	Very pale yellow
	Phenylalanine	Faint flowery aroma, faint sweetness	Medium brown, reddish tinge
	Leucine	Slight EtOH, solvent like	Medium brown, reddish tinge
	Alanine	Faint sweet	Medium brown, reddish tinge
	Valine	Faint sweet, eucalyptus like, faint EtOH	Medium brown, reddish tinge
Week 4	Control (Lactose only)	Very faint sweet aroma	Very very pale brown/yellow
	Phenylalanine	Faint flowery and sweet	Reddish brown
	Leucine	Sweet, caramel like	Dark brown/reddish
	Alanine	Faint sweet and faint solvent like	Brown-reddish
	Valine	Faint sweet, solvent like	Dark reddish brown

Week 5	Control (Lactose only)	Faint sweet	Very pale yellow
	Phenylalanine	Faint flowery, very slight EtOH aroma	Medium reddish brown
	Leucine	Sweet, chocolate like, EtOH very very faint	Dark brown, reddish
	Alanine	Slightly sweet, slight EtOH aroma	Medium reddish brown
	Valine	Slight EtOH, very faint eucalyptus aroma	Dark brown, reddish
Week 6	Control (Lactose only) pH 5.25	Sharp aroma, EtOH aroma is strong, no sweet smell	Pale yellow
	Phenylalanine pH 7.65	Flowery notes, burnt caramel smell, very slight EtOH aroma- not strong	Dark orange/brown
	Leucine pH 6.92	Strong/pungent solvent aroma, slight EtOH smell	Dark orange
	Alanine pH 5.60	Burnt caramel smell, solvent smell	Brown/orange
	Valine pH 6.18	Solvent aroma, not particularly pleasant	Dark reddish/orange -brown
Week 9	Control (Lactose only)	EtOH aroma is obvious, burnt caramel notes	Pale brown colour
	Phenylalanine	Slight/faint flowery aroma, slightly sweet	Slightly darker then leucine, golden yellow
	Leucine	EtOH and solvent aroma, like a felt tip pen	Golden yellow/orange
	Alanine	Slightly sweet, very faint sweet aroma, no obvious EtOH aroma	Golden yellow/orange colour, darker then phe and leu samples
	Valine	Sweet aroma, pleasant caramel smell, no EtOH aroma	Brown/orange, darker then all of the others
Week 10	Control (Lactose only)	Burnt caramel aroma, slightly pungent	Almost clear/colourless
	Phenylalanine	Slight flowery aroma, very very faint sweet aroma	Dark yellow/orange colour
	Leucine	Solvent aroma, slight burnt caramel smell. Burnt smell is not particularly pleasant or pungent	Dark yellow
	Alanine	EtOH/solvent aroma, rather spirit like	Dark yellow/orange-brown
	Valine	Solvent with a faint burnt toffee aroma. Slightly sweet	Orange/brown
Week 12	Control (Lactose only)	Strange pungent aroma	Very pale, almost clear and colourless

	Phenylalanine	Very sweet, faint flowery aroma, Slightly pungent with very faint EtOH aroma	Dark yellow
	Leucine	Burnt caramel aroma, EtOH smell is faint	Medium yellow
	Alanine	Faint sweet smell, faint EtOH aroma	Dark yellow, about the same as phenylalanine sample
	Valine	Slightly pungent with a faint EtOH aroma	Dark orange/brown
Week 13	Control (Lactose only)	Pungent/burnt unpleasant smell, no EtOH aroma detected	Very very faint light brown, almost colourless
	Phenylalanine	Faint flowery aroma, faint EtOH aroma	Yellow, slightly dark brown-ish
	Leucine	Solvent aroma, strong, almost pungent. EtOH aroma not obvious	Medium to dark yellow
	Alanine	Pleasant, quite typical aroma for a spirit	Dark yellow-orange/brown
	Valine	Faint sweet aroma, no obvious EtOH aroma	Medium brown
Week 14	Control (Lactose only)	Pungent/burnt aroma, unpleasant	Very pale, almost colourless, very very faint yellow/brown tinge
	Phenylalanine	Faint flowery and sweet aroma	Dark yellow, almost the same as leucine
	Leucine	EtOH aroma, strong and solvent like	Dark yellow, quite bright
	Alanine	Strong, almost rum-like aroma	Dark yellow/brown
	Valine	Sweet/rum like aroma, EtOH aroma	Brown, darkish orange
Week 15	Control (Lactose only)	Pungent, unpleasant aroma	Very very pale, almost colourless with a yellow/brown tinge
	Phenylalanine	Very faint sweet EtOH aroma, flowery smell is no longer evident- maybe lost due to transfer of contents into another flask?	Dark yellow/orange. Slightly darker then leucine
	Leucine	Solvent like, slightly sweet	Dark yellow
	Alanine	EtOH aroma obvious, characteristic spirit aroma-pleasant	Medium brown
	Valine	Sweet aroma, pleasant and rum-like	Medium brown
Week 16	Control (Lactose only)	Pungent/burnt/urine like-unpleasant	Almost colourless (clear). Very slight

			tinge of yellow/brown.
	Phenylalanine	Ammonia like aroma, very faint flowery aroma	Dark yellow
	Leucine	Strong EtOH aroma, slight sweet aroma, solvent like	Dark yellow
	Alanine	Strong EtOH aroma, faint sweet notes	Dark yellow/ orangey
	Valine	Sweet, rum like notes, quite pleasant	Medium brown
Week 17	Control (Lactose only)	Pungent/burnt sugar aroma	Almost colourless with a yellow/brown tinge
	Phenylalanine	Faint EtOH aroma, faint. Flowery smell is not evident	Dark yellow/brownish
	Leucine	EtOH/solvent aroma, faint sweet aroma	Dark yellow
	Alanine	EtOH aroma, faint burnt toffee smell, similar to control but less pungent	Dark yellow/brown
	Valine	Sweet and very pleasant, rum like	Dark/reddish brown
Week 18	Control (Lactose only)	Pungent, burnt sugar, urine like smell	Clear, almost colourless, yellow/brown tinge
	Phenylalanine	No obvious flowery aroma, slight hint of ammonia type aroma	Yellow, bright, similar to week 18 leucine sample
	Leucine	EtOH aroma, slightly sweet aroma	Yellow, bright
	Alanine	Strong EtOH aroma, faint sweet aroma	Yellow/brown colour
	Valine	Sweet, caramel aroma, pleasant	Medium-light brown
Week 20	Control (Lactose only)	Burnt sugar, pungent aroma, very faint EtOH aroma	Clear, almost colourless with a tinge of brown
	Phenylalanine	Faint flowery aroma present this time, very very faint EtOH	Dark yellow/orange/ brown tinge
	Leucine	Pleasant, sweet, very very faint EtOH aroma, slightly solvent like	Dark yellow
	Alanine	Solvent aroma, faint sweet aroma	Yellow/orange- quite dark
	Valine	Very sweet and pleasant, no EtOH aroma	Medium brown
Week 21	Control (Lactose only)	Pungent, slight burnt sweet notes	Clear, almost colourless with a yellow tinge
	Phenylalanine	Faint flowery/ potpourri	Dark yellow

		aroma, slight ammonia like smell, with burnt sweet notes (like control)	
	Leucine	Sweet notes, faint solvent like aroma	Yellow, like Phenylalanine sample.
	Alanine	Strong EtOH, faint caramel	Dark yellow/orange
	Valine	Sweet caramel, pleasant, faint EtOH	Medium brown
Week 22	Control (Lactose only)	Pungent burnt sugar, EtOH not detected	Almost colourless
	Phenylalanine	Faint flowery and ammonia odours, no EtOH detected	Medium/dark yellow
	Leucine	Sweet, pleasant, faint EtOH	Medium yellow
	Alanine	Solvent like, slightly sweet	Yellow/orange
	Valine	Sweet, caramel, chocolate like	Medium brown
Week 23	Control (Lactose only)	Sharp, pungent, unpleasant	Almost colourless- same as week 22
	Phenylalanine	Faint flowery odour, faint EtOH odour	Medium yellow
	Leucine	Sweet, smooth, faint EtOH	Medium yellow
	Alanine	Sweet, faint EtOH	Medium/dark yellow
	Valine	Smooth, sweet, chocolate like	Dark yellow/brown
Week 24	Control (Lactose only)	No change from week 23,	Pungent, burnt sugar
	Phenylalanine	Very very faint flowery odour, faint EtOH odour	Dark/bright yellow
	Leucine	Sweet, solvent	Medium yellow
	Alanine	Very faint sweet	Dark yellow/orange
	Valine	Sweet, caramel, chocolate like, faint EtOH	Dark yellow/brown
Week 25	Control (Lactose only)	Pungent burnt sugar, EtOH obvious	Same colour as week 24
	Phenylalanine	Faint flowery odour, faint EtOH, eucalyptus like	Dark yellow
	Leucine	Pungent like the control sample faint EtOH	Medium yellow
	Alanine	Sweet, chocolate like, sharper odour then valine	Dark yellow/orange

	Valine	Sweet, chocolate and rum like	Brown/orangey
Week 27	Control (Lactose only)	Pungent, burnt sugar	Very pale, almost colourless- solid particles settled on bottom of flask
	Phenylalanine	Faint flowery odour	Bright yellow/orange
	Leucine	Sweet odour, faint solvent odour	Medium/bright yellow
	Alanine	Solvent, sweet, smooth, chocolate like	Dark yellow
	Valine	Sweet chocolate like, pleasant	Dark brown
Week 29	Control (Lactose only)	Pungent, burnt sugar, unpleasant	Almost colourless, slight yellow tinge
	Phenylalanine	Flowery aroma, faint sweet notes	Dark yellow/gold
	Leucine	Sweet, pleasant, slight EtOH odour	Golden yellow
	Alanine	Solvent like, faint sweet notes	Orange/yellow
	Valine	Sweet, chocolate like, pleasant	Light brown
Week 30	Control (Lactose only)	Strong EtOH, very faint sweet notes	Almost colourless, slight yellow tinge
	Phenylalanine	Strong flowery aroma, faint EtOH	Dark yellow/gold
	Leucine	Typical spirit like aroma (tequila)	Golden yellow
	Alanine	Salty notes, solvent like, slightly sweet	Orange/yellow
	Valine	Sweet caramel/rum like, pleasant	Light brown

Appendix 6. Information sheet given to consumers prior to commencement of the sensory trial.

Assessment of Aroma of Spirits Produced From Whey Alcohol

I invite you to take part in my Masters project as an aroma assessor. You are not asked to drink the spirit, only sniff it. If there is anything that is not clear, or if you would like more information please feel free to ask. You can then decide to take part or not.

The purpose of the project is to develop an alcoholic spirit using only New Zealand dairy proteins, lactose and whey alcohol. The aim is to manipulate a reaction between lactose and amino acids to produce flavours and colours suitable for flavouring whey alcohol.

There are 30 samples in all, and each sample has been prepared in a different way. You will probably like some samples more than others, and I want to know which.

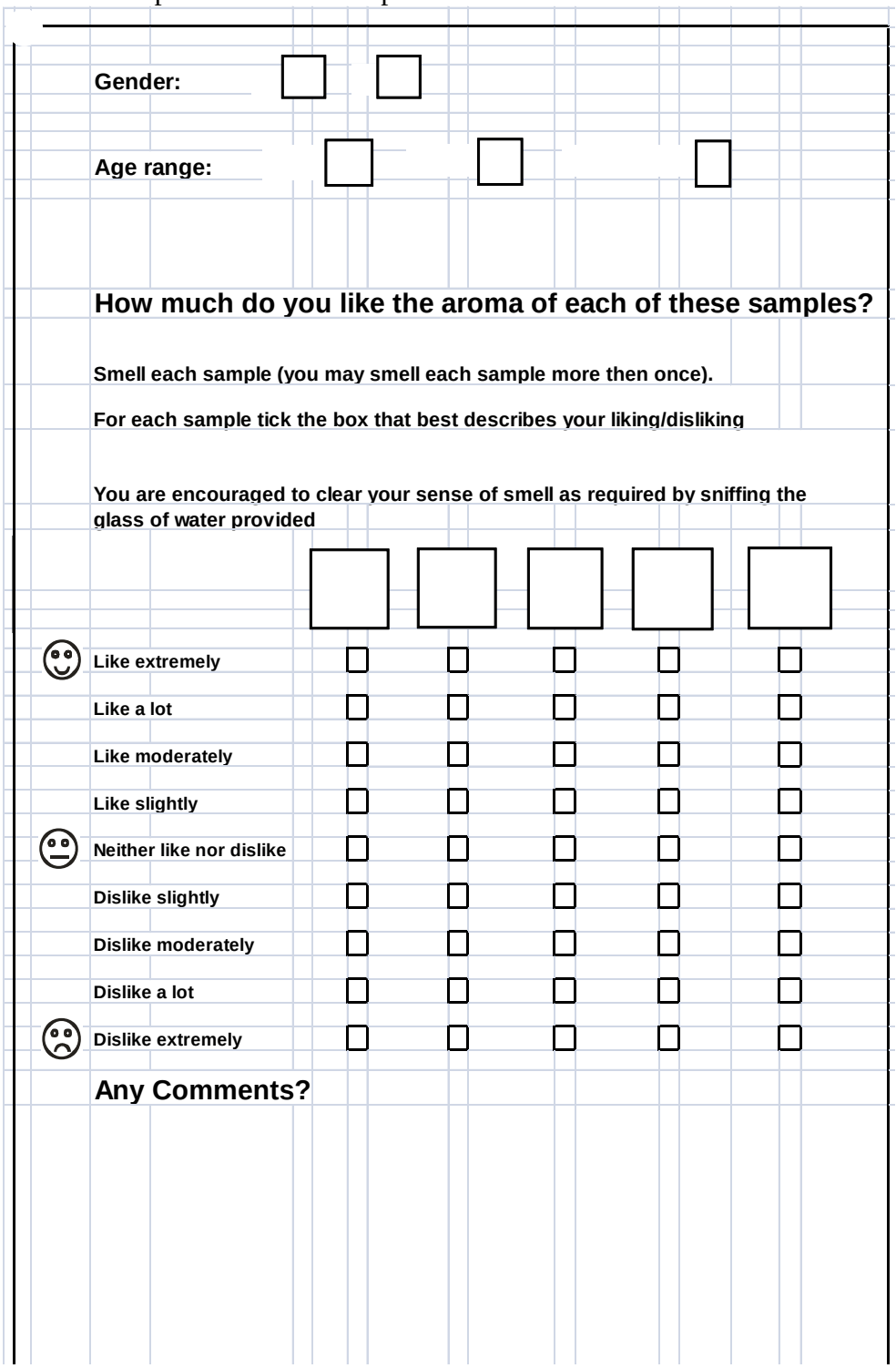
Your assessment will take about 10 minutes and participation in this research is entirely optional. Your name is not recorded. You will receive a small reward for taking part (**a chocolate bar**) and you can elect to enter a draw for **a $50 cash prize**. If you want to take part in the draw, you must supply a mobile phone number so we can text you. Winning is the only reason you would be contacted, and once a winner is drawn all contact information will be destroyed.

Taking part will involve you assessing the aroma of each sample and filling in a form to indicate how you feel about the aroma of each sample. Please feel free to leave a comment if you have one.

There are six 'bays' and each bay will contain a different set of samples. You are encouraged to smell of the glass of water provided to clear your sense of smell as required. I will be present at all times to assist you with this should you need any help.

Nisha Patel
AUT Masters student

Appendix 7. Sensory trial ballot for assessment of liking of aroma of Maillard reaction product flavoured spirits.



Gender: ☐ ☐

Age range: ☐ ☐ ☐

How much do you like the aroma of each of these samples?

Smell each sample (you may smell each sample more then once).

For each sample tick the box that best describes your liking/disliking

You are encouraged to clear your sense of smell as required by sniffing the glass of water provided

		☐	☐	☐	☐	☐
☺	Like extremely	☐	☐	☐	☐	☐
	Like a lot	☐	☐	☐	☐	☐
	Like moderately	☐	☐	☐	☐	☐
	Like slightly	☐	☐	☐	☐	☐
😐	Neither like nor dislike	☐	☐	☐	☐	☐
	Dislike slightly	☐	☐	☐	☐	☐
	Dislike moderately	☐	☐	☐	☐	☐
	Dislike a lot	☐	☐	☐	☐	☐
☹	Dislike extremely	☐	☐	☐	☐	☐

Any Comments?

Appendix 8. Sensory trial ballot for rating of intensity of aroma