Enhancement of “flavours” through enzymatic release of glutamate

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Abstract

The sensory characteristics of food, in particular the flavours of food, play a very crucial role in the food industry. It is often what consumers associate most from food, and it is therefore crucial for food manufacturers like The Tatua Co-operative Dairy Company to produce flavour ingredients that are unique and of the highest quality.

The Dairy Flavour Ingredients Business Unit at Tatua produces a range of dairy flavour ingredients including Butter, Cream and Milk, and Cheese Flavours. Tatua exports most of their flavour products worldwide to various food manufacturers, enhancing the flavour of the products that such manufacturers produce. Umami or savouriness is one of the five primary tastes. It is one of the most important flavour characteristic in dairy flavourings. In particular, umami is most needed in many of the different cheese flavourings produced by Tatua.

This project focuses on optimising the release of glutamate from casein. Liberated glutamate with a commercial name HCP337 is used as a flavour enhancer, increasing the umami of the dairy flavourings that Tatua makes. Alternatively, the addition of monosodium glutamate (MSG) can also increase the umami of the flavours that Tatua makes, however this is not possible due to a couple of reasons: MSG has a negative reputation in the consumers' mind, as many believe consumption of MSG can cause negative health effects; another reason is that addition of MSG would prevent Tatua from labelling their products as "natural". Thus, the glutamate used for Tatua's flavour ingredients is derived from the proteolysis of casein.

Currently, the problem is that very little free glutamate is present in the HCP337 product even after a very long proteolysis. Therefore, the purpose of this project is to comprehensively examine the biochemistry of glutamate release in order to find the optimum proteolysis condition and use this knowledge to optimise the current process, increasing glutamate release.

Three techniques were developed over the initial part of the study to quantify glutamate, glutamine, and pyroglutamic acid. A thorough set of samples from the HCP337 production process were then characterised. We found that indeed there was low glutamate content in the HCP337 product, and there was pyroglutamic acid in significant amounts. Stability tests of both glutamate and glutamine showed that

glutamate is stable and not affected by the processing conditions. On the other hand, glutamine is very unstable and converted into pyroglutamic acid spontaneously under the processing conditions.

With this information, various tests were performed with the focus of maximising glutamate yield from casein. These tests included: testing the proteolytic capacity of new and different enzymes at differing pH values with the hope of finding a better and more suitable enzyme at liberating glutamate from casein, the use of different acidifying agents in the HCP337 production process, and the screening of glutaminase activity within the new enzymes. The presence of glutaminase may further increase the glutamate yield, as glutaminase will convert the free glutamine released into glutamate instead of it spontaneously forming pyroglutamic acid.

In summary, this study demonstrated that the current HCP337 production process is not designed to optimally release glutamate from casein. Altering the pH and enzyme combinations during hydrolysis did not appear to be effective strategies to increase glutamate release from casein. More tests on more enzyme should be conducted in the future in order to find enzymes that are most effective at releasing glutamate under the appropriate production process.

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Chapter 1

Literature Review

Founded in 1914, the Tatua Co-operative Dairy Company is the oldest independent dairy company in New Zealand. One of the key areas that Tatua focuses on is flavour ingredients. The flavour ingredients are made from natural, high quality dairy ingredients (Tatua, n.d.-b). The purpose of developing flavour ingredients is to enhance the flavour and mouthfeel of food products, and reduce the cost in a wide range of food formulations (Tatua, n.d.-b). Today, Tatua exports more than 94% of its products to more than 60 countries worldwide each year (Tatua, n.d.-a).

Over the years, Tatua has developed a core range of dairy flavour ingredients including butter, Cream and Milk, and Cheese Flavours. One important subdivision of the flavour sector is the development of cheese flavour ingredients (Tatua, n.d.-b). Cheese flavour ingredients enhances processed cheese flavours, reduces formulation costs by replacing or reducing cheese usage, and reduces supply chain costs associated with sourcing, grading, and storing cheeses (Tatua, n.d.-a).

An important component of the natural cheese flavours that Tatua produces is an ingredient called HCP337. HCP337 is a component of a flavour made from the digestion of casein. The digestion of casein ultimately liberates free glutamate which is needed to enhance the umami of the cheese flavours.

In the food industry, glutamate is a popular flavour enhancer used to boost the umami/ savoury taste (i.e., the fifth basic taste) of food (Bellisle, 1999). Instead of liberating glutamate from casein, Tatua can also achieve the same objective by adding MSG (Mono Sodium Glutamate) to their flavourings. The problem is the addition of MSG to foods has bad reputations among consumers and many people prefer foods that are MSG-free and therefore regarded as “organic” and “natural”.

The purpose of this project is to comprehensively examine the biochemistry of glutamate release from casein (the HCP337 production process), finding the optimum proteolysis condition, and using this knowledge to optimise the current process and increase glutamate release.

According to Tatua, the present proteolysis treatment (involving the sequential breakdown of casein from a very large protein into smaller peptides and eventually

free amino acids) releases a small proportion of glutamate (approximately 20%) present in casein. Long proteolysis times are subsequently required to maximise the release of glutamate from casein hydrolysate. From the point of view of Tatua, the long proteolysis period is very uneconomical.

According to Tatua, the enzymes currently used are non-specific and thus other amino acids that make up casein are also liberated in the break-down process. In particular, the amino acid glutamine is also liberated from casein. Glutamine does not have umami flavour characteristic, but it can be easily converted to glutamate via glutaminase (Dura *et al.*, 2002). However, if glutamine is not immediately converted into glutamate, it can form into pyroglutamic acid spontaneously (Schilling *et al.*, 2004). It was speculated by Tatua that most of the glutamine released from the HCP337 production process is lost to pyroglutamic acid.

This literature review will identify knowledge gaps around this topic:

- To identify and explain the importance and functions of glutamate in foods and food flavours (Section 1.1).
- To review existing information on the known structure of casein (the raw ingredient used to release glutamate) (Section 1.2).
- To identify what and how pyroglutamic acid is formed. To identify all other relevant reactions surrounding glutamate, glutamine, and pyroglutamic acid (Section 1.3).
- To review existing information on known methodology for quantifying glutamate, glutamine, and pyroglutamic acid (Section 1.4).
- To review existing information on the stability of glutamate, glutamine, and pyroglutamic acid (Section 1.5).
- To review existing information on the enzyme glutaminase (Section 1.6).

1.1 Glutamate and Umami

Food ingredients containing lots of free amino acids has been used historically in order to enhance the sensory qualities of various foods (Bellisle, 1999). The first identification of umami was in the year 1908 by a Japanese scientist called Kikunae Ikeda (Ikeda, 1909). Ikeda managed to extract the amino acid glutamate from seaweed, and concluded that glutamate is the primary source of umami (Ikeda, 1909). After discovering glutamate as the source of umami, Ikeda patented the

production method of making MSG to a company called Ajinomoto (Ikeda, 1909). MSG is a food additive that enhances flavour and increases umami in food products (Bellisle, 1999). Today, MSG widely used in cooking and food production due to its flavour enhancement ability (Bellisle, 1999).

The mechanism through which taste stimuli for umami is perceived has been well studied (Bellisle, 1999). In particular, the biochemistry associated with the umami taste was first described in 1987 (Kurihara, 1987). It starts with the adsorption of a chemical stimulus with an umami characteristic (Glutamate) to the receptor membranes in the taste buds (Kurihara, 1987). This adsorption induces a receptor potential in receptor cells and causes a chemical transmitter to be released (Bellisle, 1999). The release of a chemical transmitter then triggers an impulse in the nerves that mediates gustatory sensitivity (these nerves may be the chorda tympani, and/or the glossopharyngeal) (Bellisle, 1999). Ultimately, this leads to successive brain relays conveying the message to the primary and secondary cortex where the message and impulses are processed and recognised (Bellisle, 1999).

Glutamate and glutamine are considered as non-essential amino acids (Bellisle, 1999). These amino acids can be synthesised endogenously from other amino acids and precursors in acceptable quantities (Abdallah *et al.*, 2013).

Endogenous glutamate (glutamate that are synthesised within the body) has been observed to participate in many functions from providing a principal fuel in the liver and intestinal mucosa and acting as the substrate for urea formation (Christensen, 1990).

Glutamate can also be synthesised in the brain endogenously (Bellisle, 1999). This endogenous brain glutamate is not related to any other glutamate found in the body (Roth *et al.*, 1995). In the brain, glutamate is a major excitatory neurotransmitter in the mammalian brain (Fonnum, 1984; Stanley *et al.*, 1993). Glutamate synthesised in the brain is also an important precursor of the neurotransmitter *g*-aminobutyric acid (GABA) (Fonnum, 1984).

Glutamate is present naturally in many foods consumed by humans. Within those foods, glutamate can either be in the free form or bound form (Giacometti, 1979). The levels of glutamate has been calculated in various foods, below are some examples:

Table 1: Glutamate in various foods, (Bellisle, 1999).

	Bound glutamate (mg/100g)	Free glutamate
Salmon	2216	36
Tomatoes	238	140
Bovine milk	819	2
Parmesan cheese	9847	1200
Beef	2846	33

The levels of glutamate in various proteins has also been calculated. Below are some examples:

Table 2: percentage (%) by weight of amino acid in food protein. * = essential amino acid. (Yildiz, 2010).

Amino acid	Egg white	Tuna	Beef	Chicken	Whey	Casein	Soy	Yeast
Alanine	6.6	6	6.1	5.5	5.2	2.9	4.2	8.3
Argnine	5.6	6	6.5	6	2.5	3.7	7.5	6.5
Aspartic acid	8.9	10.2	9.1	8.9	10.9	6.6	11.5	9.8
Cystine	2.5	1.1	1.3	1.3	2.2	0.3	1.3	1.4
Glutamic acid	13.5	14.9	15	15	16.8	**21.5**	19	13.5
Glycine	3.6	4.8	6.1	4.9	2.2	2.1	4.1	4.8
Histidine*	2.2	2.9	3.2	3.1	2	3	2.6	2.6
Isoleucine*	6	4.6	4.5	5.3	6	5.1	4.8	5
Leucine*	8.5	8.1	8	7.5	9.5	9	8.1	7.1
Lysine*	6.2	9.2	8.4	8.5	8.8	3.8	6.2	6.9
Methionine*	3.6	3	2.6	2.8	1.9	2.7	1.3	1.5
Phenylalanine*	6	3.9	3.9	4	2.3	5.1	5.2	4.7
Proline	3.8	3.5	4.8	4.1	6.6	10.7	5.1	4
Serine	7.3	4	3.9	3.4	5.4	5.6	5.2	5.1
Threonine*	4.4	4.4	4	4.2	6.9	4.3	3.8	5.8
Tryptophan*	1.4	1.1	0.7	1.2	2.2	1.3	1.3	1.6
Tyrosine	2.7	3.4	3.2	3.4	2.7	5.6	3.8	5
Valine*	7	5.2	5	5	6	6.6	5	6.2

The examples presented above demonstrates that glutamate is a very common in most pretentious foods. Furthermore, glutamate is the most abundant amino acid in the human diet (Christensen, 1990).

1.2 Casein

Casein is the primary protein found in bovine milk (Farrell *et al.*, 2004). Casein can be further divided into four main groups; α_{s1}-, α_{s2}-, β-, and κ-caseins. The α_{s1}-casein family constitutes about 40% of the whole casein fraction from bovine milk (Farrell, *et al.*, 2004). It consists of 199 amino acid residues and has a molecular weight of 23, 615 (Farrell, *et al.*, 2004). The α_{s2} –casein family constitutes about 10% of the whole casein fraction in bovine milk (Farrell, *et al.*, 2004). The predominant form of this protein in bovine milk contains a disulphide bond, consists of 207 amino acid residues, and has a molecular weight of 25, 226 (Farrell, *et al.*, 2004). The β-casein family constitutes of about 45% of the whole casein fraction from bovine milk (Farrell, *et al.*, 2004). This protein is very complex due to the action of milk protease plasmin (Farrell, *et al.*, 2004). The κ-casein family constitutes of about 5% of the whole casein fraction (Farrell, *et al.*, 2004).

The amino acid residues that make up casein (Table 3) revealed that glutamate and glutamine are found in abundance relative to other amino acids. Furthermore, casein is readily available at Tatua and thus makes it a great substrate for the making of flavour components high in glutamate content.

Table 3: Amino acid residues that make up caseins *(Farrell, et al., 2004)*.

Amino Acids		Casein Proteins	α_{s1}-casein	α_{s2}-casein	β-casein	κ-casein
Arginine	Arg		6	6	4	5
Histidine	His		5	3	5	3
Lysine	Lys		14	24	11	9
Aspartic acid	Asp		7	4	4	4
Glutamic acid	**Glu**		**25**	**24**	**19**	**12**
Serine	Ser		8	6	11	12
Post translational phosphorylation site	P-Ser		8	11	5	1
Threonine	Thr		5	15	9	15
Asparagine	Asn		8	14	5	8
Glutamine	**Gln**		**14**	**16**	**20**	**14**
Cysteine	Cys		0	2	0	2
Glycine	Gly		9	2	5	2
Proline	Pro		17	19	35	20
Alanine	Ala		9	8	5	14
Isoleucine	Ile		11	11	10	12
Leucine	Leu		17	13	22	8
Methionine	Met		5	4	6	2
Phenylalanine	Phe		8	6	9	4
Tryptophan	Trp		2	2	4	4
Tyrosine	Tyr		10	12	4	9
Valine	Val		11	14	19	11
Total amino acids			**199**	**207**	**209**	**169**

1.2.1 Cheese and free amino acids

In cheese making, flavour development occurs during the ripening process (Smit *et al.*, 2000). The ripening process is defined by the breakdown of caseins through proteolysis and peptidolysis (Smit, *et al.*, 2000). During proteolysis, caseins are broken down into many large peptides (soluble-nitrogen fraction) that are responsible for the bitterness flavour in cheese (Smit, *et al.*, 2000). These large peptides are then further broken down into smaller peptides and eventually free amino acids that contribute to the basic cheese flavour (Smit, *et al.*, 2000). These reactions are mediated by proteases and peptidases that may originate from starter cultures (Smit, *et al.*, 2000).

It has been observed that cheese that contains a high amount of free amino acids has the strongest cheese taste (Molina *et al.*, 1999). The main amino acids were glutamate, valine, leucine, glutamine, asparagine, aspartate, threonine, and isoleucine (Molina, *et al.*, 1999). The majority of free amino acids have sensory properties, but glutamate is the only ones that could give a umami taste (Schiffman & Engelhard, 1976).

This observation was supported by several other studies; for example, in 1995, a water-soluble extract of comté cheese containing high levels of glutamate and small amount of aspartic acid was described as umami (Salles *et al.*, 1995), and similarly just recently in 2013, Mayer and Fiechter found that glutamate make up most of the total free amino acid of seven commercially produced long ripening Italian extra hard parmesan (Fiechter *et al.*, 2013). These findings confirmed the influence of free glutamate towards the umami/ savoury flavour.

1.3 Pyroglutamic acid/ pyroglutamate

Pyroglutamic acid, also known as 5-oxo-L-proline pyrolidone 2-carboxylic acid, is an amino acid derivative where the amino group of glutamine or glutamate cyclises to form a lactam ring (cyclic amide) (Kumar & Bachhawat, 2012). Pyroglutamic acid has also been known based on its structure to be an internally cyclized glutamate (Abraham & Podell, 1981).

Pyroglutamic acid can be formed in a number of ways. In living cells, it can be derived from the degradation of glutathione (Kumar & Bachhawat, 2012). Pyroglutamic acid can also be formed from the degradation of proteins containing pyroglutamic acid at the N-terminus (Figure 1) (Kumar & Bachhawat, 2012). Cleavage of N-terminus pyroglutamic acid is done by the enzyme pyroglutamyl peptidase (Cummins & O'Connor, 1998). Many peptides and proteins have been found to have pyroglutamic acid at the N-terminus (Kumar & Bachhawat, 2012). N-terminus pyroglutamic acid is known to play a role in stability and activity of the protein (Abraham & Podell, 1981). In the cell, N-terminus pyroglutamic acid is made post-translationally by the enzyme glutaminyl cyclase (converting glutamate and/ or glutamine into pyroglutamic acid) (Schilling, *et al.*, 2004).

Glutamine cyclase, catalyses the production of pyroglutamic acid from bound glutamine and/or glutamate (Kumar & Bachhawat, 2012). In the absence of glutamine cyclase, pyroglutamic acid can still be formed from bound glutamine

and/or glutamate but at a much slower rate (Kumar & Bachhawat, 2012). This reaction is spontaneous and only occur in proteins that have glutamine or glutamate as the N-terminus residue (Schilling, *et al.*, 2004).

Alternatively, free glutamate can also be transformed into pyroglutamic acid non-spontaneously via several key enzymes (γ-glutamyl cysteine synthase, glutamine synthase, and glutamate-5-kinase) and energy (ATP) to form the γ-glutamyl phosphate intermediate (Orlowski & Meister, 1971; Seddon *et al.*, 1989; Krishnaswamy *et al.*, 1998; Kumar & Bachhawat, 2012). γ-glutamyl phosphate intermediate can then be spontaneously converted into pyroglutamic acid (Figure 1) (Cummins & O'Connor, 1998).

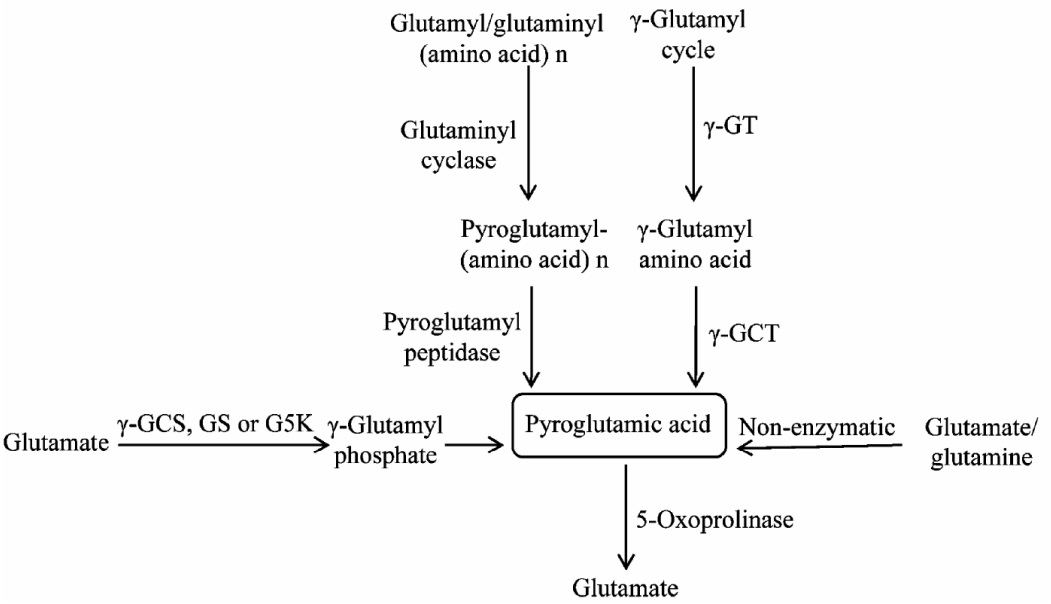


Figure 1: Different possible pathways for the generation of pyroglutamic acid (Kumar & Bachhawat, 2012).

Pyroglutamic acid is very stable and resistant to degradation by amino peptidases (Fietzek *et al.*, 1974; Kumar & Bachhawat, 2012). Despite its stability, there is one known enzyme called 5-oxoprolinase that can hydrolyse pyroglutamic acid into glutamate (Figure 1) (Kumar & Bachhawat, 2012). 5-oxoprolinase is the only known enzyme to act on pyroglutamic acid, and is an ATP-dependant enzyme containing an 'actin-like ATPase fold' (Kumar & Bachhawat, 2010, 2012). Boiling pyroglutamic acid under reflux (heating pyroglutamic acid solution with an attached condenser to prevent the it from escaping) for 60 minutes at pH 2.5 and below, or pH 11 and higher can also revert pyroglutamic acid back to glutamate (Airaudo *et al.*, 1987).

In terms of casein, previous studies have shown that κ-casein found in milk contains N-terminal pyroglutamic acid residues (Alais, 1984). Another milk protein of the ribonuclease superfamily called angiogenine 2 also contains N-terminal pyroglutamic acid residues (Strydom *et al.*, 1997).

1.4 Quantification of glutamate, glutamine, and pyroglutamic acid

In order to comprehensively examine the biochemistry of glutamate release from casein three techniques involving the quantification of the three main amino acids would have to be developed.

1.4.1 Quantification of glutamate

For the quantification of glutamate, several methods can be utilised:

1. <u>High Performance Liquid Chromatography (HPLC)</u>

   Glutamate has to first be derivatised (transformed into a product with a similar chemical structure) with OPA-ME (*o*-phthalaldehyde - 2-mercaptoethanol) solution in order for it to be detectable (Shih, 1985). Acetonitrile-0.04 M sodium acetate buffer at pH 5.4 was found to be the best buffer to use as the mobile phase (Shih, 1985). It was found that pH 5.4 was the best as it minimises interference between glutamate and aspartic acid derivatives (Shih, 1985). Lowering the pH of the buffers causes interference to occur (Figure 2) (Shih, 1985). Derivatised glutamate are then detected using a UV detector at 200nm (Shih, 1985).

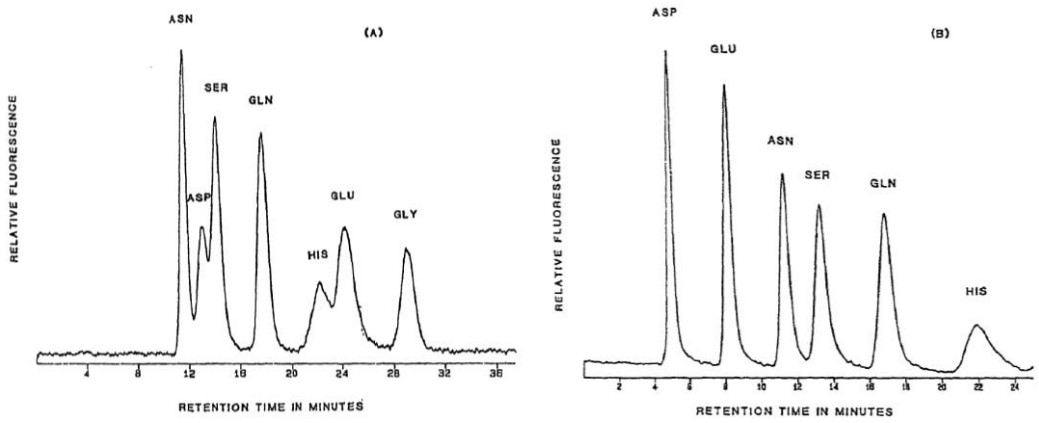


Figure 2: Elution profile of amino acid standards derivatised by OPA-ME. A) Shows the elution profile at pH 4.2. B) Shows the elution profile at pH 5.4 (Shih, 1985).

2. Enzymatic assay

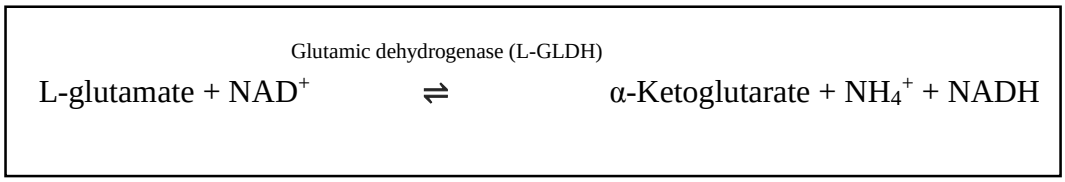


Figure 3: Dehydrogenation of glutamate and reduction of NAD^+.

The quantification of glutamate through enzymatic assay requires glutamic dehydrogenase (L-GLDH) (E.C 1.4.1.3) which is used to convert glutamate into α-Ketoglutarate (Figure 3) (Lund, 1986). NAD^+ is simultaneously reduced to NADH, and the level of NADH at the end of the reaction is used to determine the level of glutamate as the stoichiometry between glutamate and NADH is 1 to 1 (Lund, 1986). NADH is quantified by measuring the absorbance value at 340 nm (Lund, 1986).

A glutamate standard curve will have to be made by measuring known concentrations of glutamate in order to translate absorbance values into concentration values (Lund, 1986).

1.4.2 Quantification of glutamine

For the quantification of glutamine, several methods can be utilised:

1. High Performance Liquid Chromatography (HPLC)

The quantification of glutamine by HPLC is the same as the procedure described in Section 1.4.1 for the quantification of glutamate by HPLC.

2. Enzymatic assay

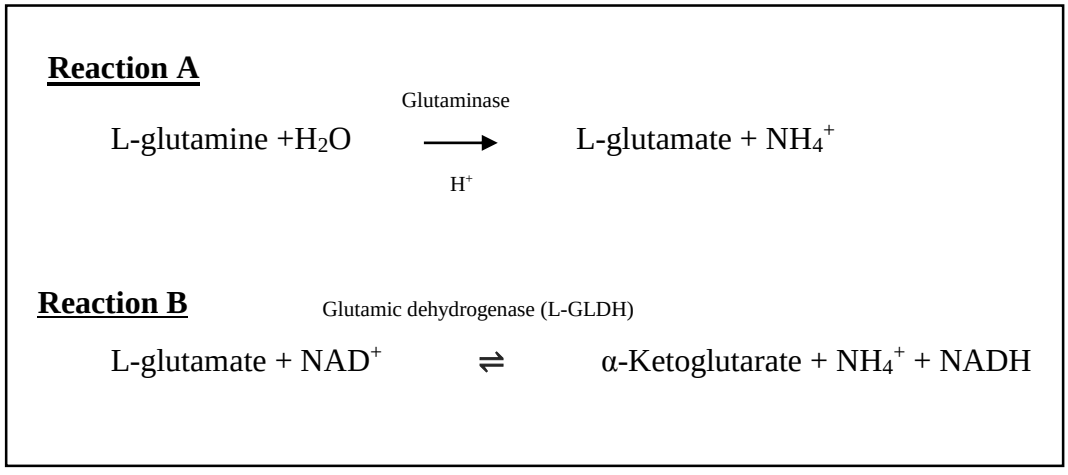


Figure 4: Quantification of glutamine.

Quantification of glutamine through enzymatic assay requires two steps (Figure 4). Endogenous glutamate (glutamate already present in a sample and is not glutamate formed by the conversion of glutamine to glutamate via glutaminase) concentration must first be determined using the procedures described in Section 1.4.1 (Glutamate assay). Once the concentration of endogenous glutamate has been determined, glutamine must be deamidated into glutamate by glutaminase, Figure 4 (i.e., Reaction A). Glutamate quantification is once again done and the values generated now will reflect the glutamate concentrations of endogenous origin and of glutamine origin (Lund, 1986).

Similarly, a glutamine standard curve will have to be made by measuring known concentrations of glutamine in order to translate absorbance values into concentration values (Lund, 1986).

1.4.3 Quantification of pyroglutamic acid

For the quantification of pyroglutamic acid, several methods can be utilised:

1. <u>High Performance Liquid Chromatography (HPLC)</u>

   Several HPLC methods has been described in the literature in order to quantify L-pyroglutamic acid.

   - Pyroglutamic acid from the casein hydrolysate can be quantified using reversed-phase high performance liquid chromatography with 0.1% phosphoric acid as the mobile phase. Pyroglutamic acid is detected using a UV detector at 200nm (Figure 5) (Shih, 1985).

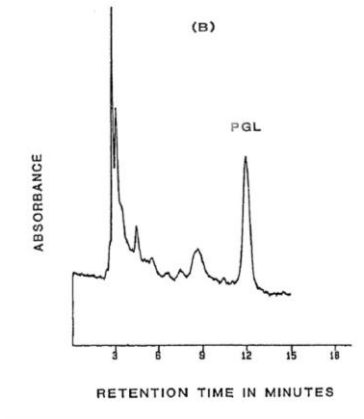


Figure 5: Elution profile of underivatised enzymatic casein hydrolysate from a commercial source. Mobile phase was 0.1% phosphoric acid (PGL= Pyroglutamic acid) (Shih, 1985).

- Gandini et al. described an easy and reliable method of determining pyroglutamic acid using HPLC. The method described was done using a 10µl loop, C_{18} precolumn, and an ODS Hypersil column (Gandini *et al.*, 1993). The method involves an isocratic elution at 1 ml/min with 7mM phosphate buffer, Na_2HPO_4 (pH 3.5) as the mobile phase (Gandini, *et al.*, 1993). The downside for this method is the interference of methionine peak with pyroglutamic acid peak (Gandini, *et al.*, 1993). The study showed that in order to minimise any interference from methionine peaks, samples should be treated with 35m/v H_2O_2 for one hour at room temperature (Gandini, *et al.*, 1993). This method allowed for a clear pyroglutamic acid peak to be formed with the retention time of 2.93 minutes (Gandini, *et al.*, 1993).

2. <u>Enzymatic assay:</u>
    - Simultaneous assay method for L-glutamate and L-pyroglutamic acid using 5-oxoprolinase (without ATP hydrolysing activity). This method was first described by Nishimura et al. in 2001 (Nishimura *et al.*, 2001).

      The method is composed of two steps. First, L-glutamate is measured (Reactions A and B, Figure 6). Reactions A and B are done simultaneously in a single reaction vessel. The conversion of L-glutamate via L-glutamate peroxidase produces H_2O_2, which is then used in Reaction B (Nishimura, *et al.*, 2001). H_2O_2 then reacts with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3, 5-dimethoxyaniline (DAOS), and 4-aminoantipyrine (4-AA) (Nishimura, *et al.*, 2001). Ultimately a blue pigment is then developed and its absorbance can be measured at 600 nm (Nishimura, *et al.*, 2001). This first set of reactions simply determines the amount of L-glutamate. Once this value has been determined, 5-oxoprolinase (without ATP hydrolysing activity) can then be added to the reaction mixture. 5-oxoprolinase converts L-pyroglutamic acid into L-glutamate (Nishimura, *et al.*, 2001). The resulting L-glutamate is measured again by the same process as described above. The difference in absorbance is used to calculate the amount of L-pyroglutamic acid originally present (Nishimura, *et al.*, 2001). Alternatively, L-pyroglutamic acid can also be quantified by converting it first into L-

glutamate via 5-oxoprolinase and then applying the glutamate assay to quantify glutamate (Figure 7).

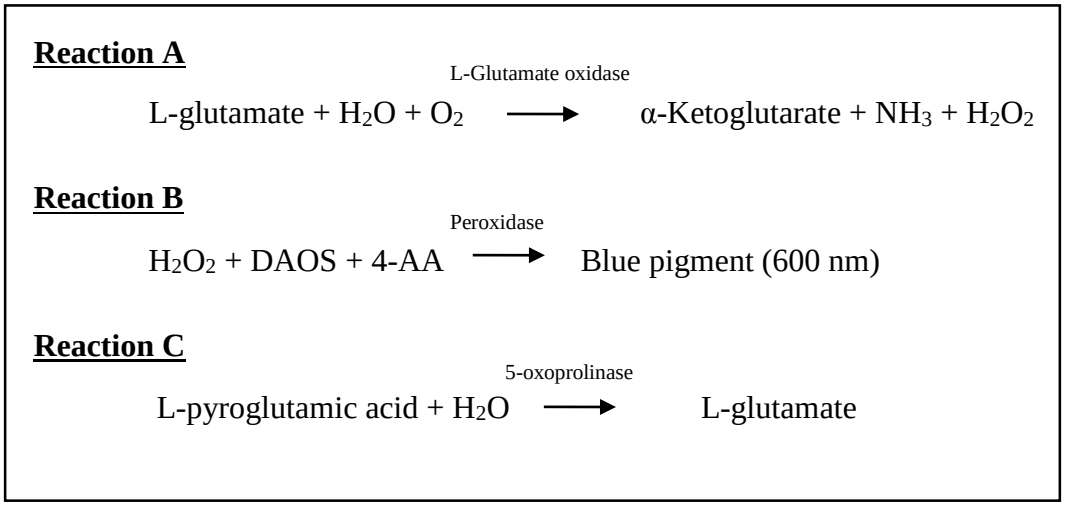


Figure 6: Simultaneous assay for L-pyroglutamate quantification.

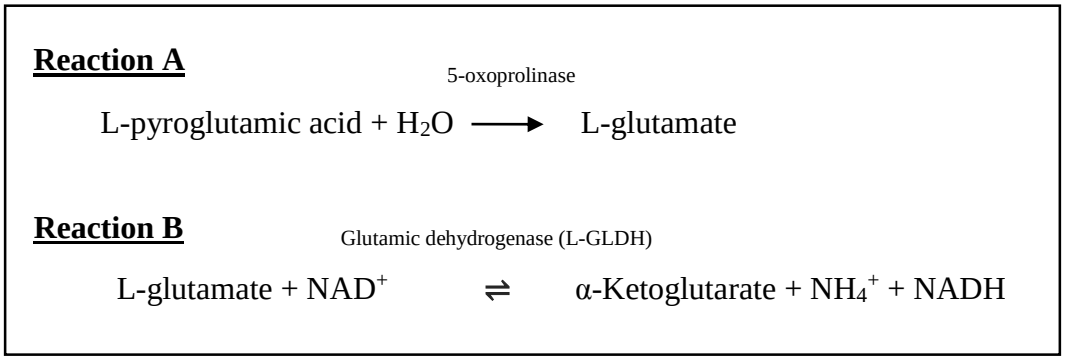


Figure 7: Alternative assay method for the quantification of L-pyroglutamic acid.

Despite the different pathways L-pyroglutamic acid can be determined, both methods relies on the use of 5-oxoprolinase (without ATP hydrolysing activity).

1.4.4 Methods Chosen

The ability to reliably quantify glutamate, glutamine, and pyroglutamate are the 'keys' to carrying out this study.

Even though the techniques on quantifying glutamate, glutamine, and pyroglutamic acid described in the previous sections are well established, it does not mean that all of the published techniques are reliable and usable in the context

of this study. After a lot of discussions, it was decided that the best and most realistic approach for quantifying glutamate and glutamine were through enzymatic assay.

Ideally, the quantification of pyroglutamic acid would be done using an enzymatic assay. This would keep the methods consistent and simple. However, 5-oxoprolinase (without ATP hydrolysing activity), which was discovered in 1999 in a cell extract of *Alcaligenes faecalis* (Nishimura *et al.*, 1999) is needed to convert pyroglutamic acid into glutamate. Today, it is near impossible to attain this particular enzyme. After a lot of searching and attempts to obtain this enzyme, it was found that this enzyme is very rare and very expensive when it is available. From this reason only, it was then decided that quantifying pyroglutamic acid through enzymatic assay was not a viable option. Quantification of pyroglutamic acid through HPLC was then chosen as the more realistic option.

1.5 Glutamate, glutamine, and pyroglutamic acid stability

The stability of glutamate, glutamine, and pyroglutamic acid is particularly important in this study as this will give a better understanding on how these amino acids behave in the HCP337 production process.

A study by Arii et al. looked at the stability of glutamine. Arii et al. used HPLC in order to quantify glutamine (Arii *et al.*, 1999). Citrate buffer was used to accommodate pH range from 1.21 to 6.41, and borate buffer was used to accommodate pH range from 7.39-11.01 (Arii, *et al.*, 1999).

This study found that degradation of glutamine in an aqueous solution follows a first-order reaction kinetics (Arii, *et al.*, 1999). This was determined by the linear relationship between the incubation time and the log of percentage of remaining glutamine (Figure 8) (Arii, *et al.*, 1999).

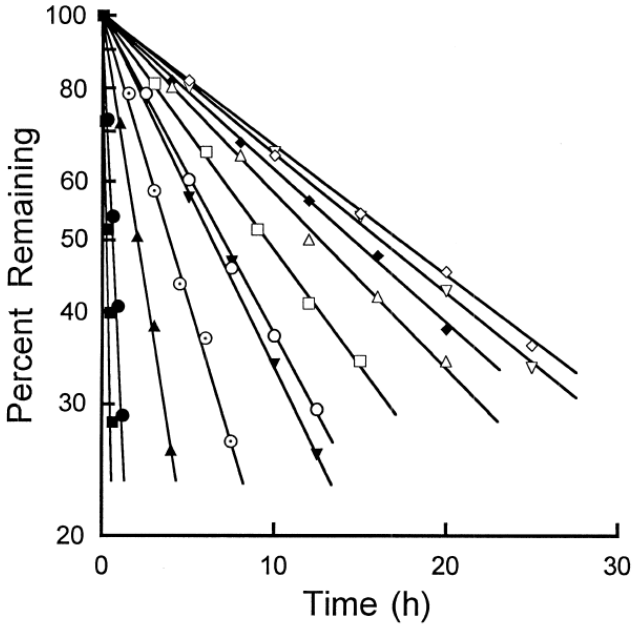


Figure 8: Apparent first-order plots for the degradation of Gln at 70˚C in buffer solution. pH values: (■) 1.21, (●) 1.93, (▲) 2.87, (▼) 3.93, (♦) 4.80, (▽) 5.61, (◊) 6.41, (△) 7.39, (□) 8.31, (○) 9.31, (○ dot) 11.01 (Arii, et al., 1999).

Figure 8 shows that the stability of glutamine decreases at the extreme ends of the pH scale. The rate of degradation for glutamine is a lot slower at near neutral pH values (pH 5.61 – 6.41). Degradation of glutamine was found to be due to the intramolecular cyclization of glutamine to pyroglutamic acid instead of the hydrolytic deamination of glutamine (Arii, *et al.*, 1999). This observation was made by analysing the reaction mixtures using HPLC and the results showed a close agreement between the loss of glutamine and the formation of pyroglutamic acid (Arii, *et al.*, 1999). Overall, this study found that glutamine is most stable at the pH range from 5 to 7.5 (Arii, *et al.*, 1999).

On the other hand, in 1985, a paper by Gayte-Sorbier et al, looked at the stability of glutamate. The aim of this study was to identify and study the different factors that can influence the conversion of glutamate under storage and processing, and also to identify the conversion product of glutamate (Gayte-Sorbier *et al.*, 1985). This study focused on four main factors: pH, oxygen exposure, temperature, and storage time in relation to the stability of glutamate (Gayte-Sorbier, *et al.*, 1985).

The study found that at room temperature, glutamate solutions were very stable at the extreme ends of the pH scale (pH 0 and 14) (Gayte-Sorbier, *et al.*, 1985). Glutamate was found to be least stable in the middle of the scale (between pH 5 and

6) (Gayte-Sorbier, *et al.*, 1985). Furthermore, glutamate was more stable in the absence of oxygen (Gayte-Sorbier, *et al.*, 1985).

This study also looked at the effects of boiling and autoclaving (135˚C) to glutamate under all pH values. When boiled, glutamate were very stable (over 60 minutes) in a very acidic, almost neutral and very alkaline pH (Gayte-Sorbier, *et al.*, 1985). This study also found two lability maxima (pH 11, and pH 2) when glutamate is boiled (Gayte-Sorbier, *et al.*, 1985). On the other hand, when autoclaved, glutamate showed greatest stability in the extreme ends of the pH scale, and poorest stability at pH 2 to 3.5 (Gayte-Sorbier, *et al.*, 1985). Although not as much as between pH 2 to 3.5, glutamate also showed a degree of instability between pH 8 to 13. As expected, the rate of conversion is much faster at the autoclaving temperature than that of the boiling temperature. In both cases glutamate converted to pyroglutamic acid only (Gayte-Sorbier, *et al.*, 1985).

Additionally, the stability of glutamine, glutamate, and pyroglutamic acid was also covered in another paper. Stability was determined by dissolving these amino acids in solutions of different pH, and at different temperatures (Shih, 1985). The levels of the amino acids were then analysed at regular intervals using the methods described above (Section 1.4) (Shih, 1985). The results were:

- Pyroglutamic acid: very stable and under extreme conditions (2M hydrochloric acid, at 100˚C for 2h), it can be converted into glutamate spontaneously (Shih, 1985).
- Glutamate: Stable at room temperatures, but has the tendency to form pyroglutamic acid at higher temperatures and extended storage time (Shih, 1985).
- Glutamine: Extremely unstable, and is readily deamidated at alkaline pH values, long storage time, and elevated temperature (Shih, 1985). Most of the reaction product was pyroglutamic acid, rather than glutamate (Shih, 1985).

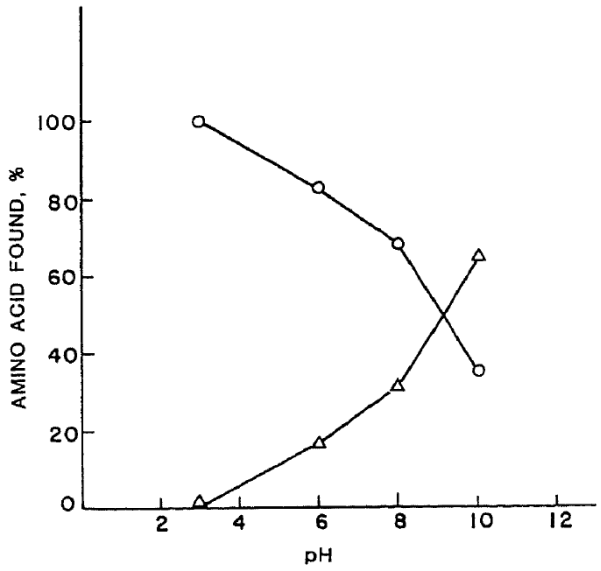


Figure 9: Effect of pH on glutamine (o) and pyroglutamic acid (Δ) at 37˚C for 24 h in 0.1M sodium phosphate buffer adjusted to the according pH by phosphoric acid or sodium hydroxide (Shih, 1985).

A typical condition for enzyme hydrolysis is at 37˚C for 24 h (Shih, 1985). Figure 9 shows the effect of pH on glutamine when exposed to the normal conditions for enzyme hydrolysis. Very clearly, as pH rises, glutamine loss increases, and the formation of pyroglutamic acid increases (Shih, 1985). This observation is contradictory to the ones made in the previous study (Figure 8) where glutamine was found to be most stable at just under neutral pH and most unstable in low pH. The differing results from the two papers may be due to the temperatures that the test were carried out at. The test in Figure 8 was done at 70˚C, whereas, the test in Figure 9 was done at 37˚C.

1.6 Glutaminase

Glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme that deamidate L-glutamine in the presence of water and produces L-glutamate and ammonia (McCauley *et al.*, 1999). Depending on the origin of the enzyme, it may have different characteristics. For example, L-glutaminase from *Streptomyces avermitilis* is active at pH 3-11, exhibits optimum activity at pH 7-8, and has an optimal temperature of 30˚ C (Abdallah, *et al.*, 2013). L-glutaminase from *Debaryomyces* spp. is active at the pH range from 5.5-10 with an optimum pH of

8.5, and has an optimal temperature of 40˚ C (Dura, *et al.*, 2002). L-glutaminase from C. albidus that has an optimum activity at temperatures of 70˚ C (Dura, *et al.*, 2002).

There is a very close analogue of EC 3.5.1.2 called protein glutaminase (PG) (EC 3.5.1.44) that deamidates glutamine residues in protein, to form glutamate residues (Hashizume *et al.*, 2011). In contrast to glutaminase, protein glutaminase cannot deamidate free glutamine (Hashizume, *et al.*, 2011; KEGG, n.d.).

The use of glutaminase in the HCP337 production process can further increase the glutamate yield from the HCP337 production process. Unfortunately, at the time of this study, the use of pure glutaminase in the production of flavours at Tatua was not permitted by the Ministry for Primary Industries.

Chapter 2

Methodology

This chapter is dedicated for the refinement, optimisation, and validation of the methodology that were chosen in Section 1.4.4.

2.1 Glutamate assay

As a starting point, the method described in the Sigma Product information for Glutamine/ Glutamate determination kit (GLN1) which was derived from Lund, 1986 (Section 1.4.1) was used as a general outline in the development and validation of an optimised glutamate assay for this study.

Briefly, the quantification of glutamate was done through the reduction of NAD^+ to NADH, and the amino group of glutamate oxidised to α-Ketoglutarate. The whole reaction is mediated by the enzyme glutamic dehydrogenase (L-GLDH) (EC 1.4.1.2) (Figure 10).

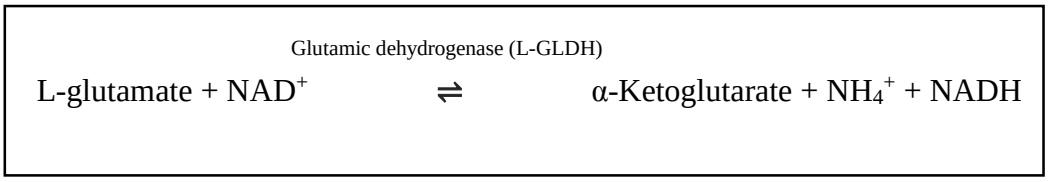


Figure 10: Dehydrogenation of glutamate and reduction of NAD^+.

The quantification of glutamate was done by measuring the change in absorbance value of the mixture by putting it in a spectrophotometer at a wavelength of 340nm at room temperature. The spectrophotometer used for this set up was a Biochrom Libra S60 Double Beam Spectrophotometer.

2.1.1 Buffers

The buffer suggested in the sigma Product information for Glutamine/ Glutamate determination kit was Tris (0.1M)-EDTA (0.002M) buffer (TE buffer) adjusted to pH 9. Before testing TE buffer, phosphate buffer was first trialled at pH 7, 7.5, and 8. This was done as phosphate buffer was an acceptable buffer for food processing.

NAD^+ (C-NAD-25G, from Megazyme, Wicklow, Ireland) was tested at 10mM. ADP (216J0000 from Calzyme Laboratories San Luis Obispo, CA, USA) which is an activator of the enzyme glutamic dehydrogenase was tested at 1mM (Prough *et al.*, 1973). Glutamic dehydrogenase (GLDH, 080A0010 from Calzyme Laboratories, San Luis Obispo, CA, USA) was reconstituted to 5mg/mL with phosphate buffer at pH 7, and glutamate (the substrate, purchased from Sigma-Aldrich with the product code G1251) was tested at 1mM. A summary of the components for the tests are shown below:

Table 4: Components for glutamate assays. Phosphate buffer tests.

Phosphate buffer test 1
1mM Glutamate
10mM NAD^+
1mM ADP
5mg/mL GLDH
Phosphate buffer (pH 7, 7.5, and 8)
Reaction volume: 1.025mL

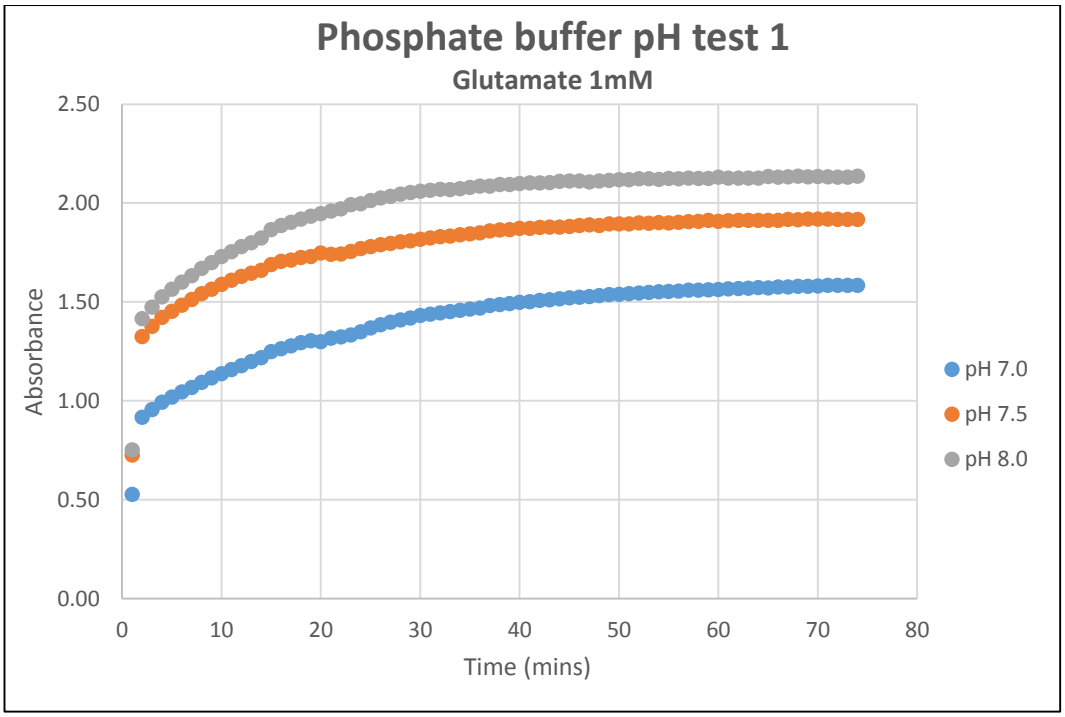


Figure 11: Phosphate buffer pH test 1 results. Absorbance vs. time.

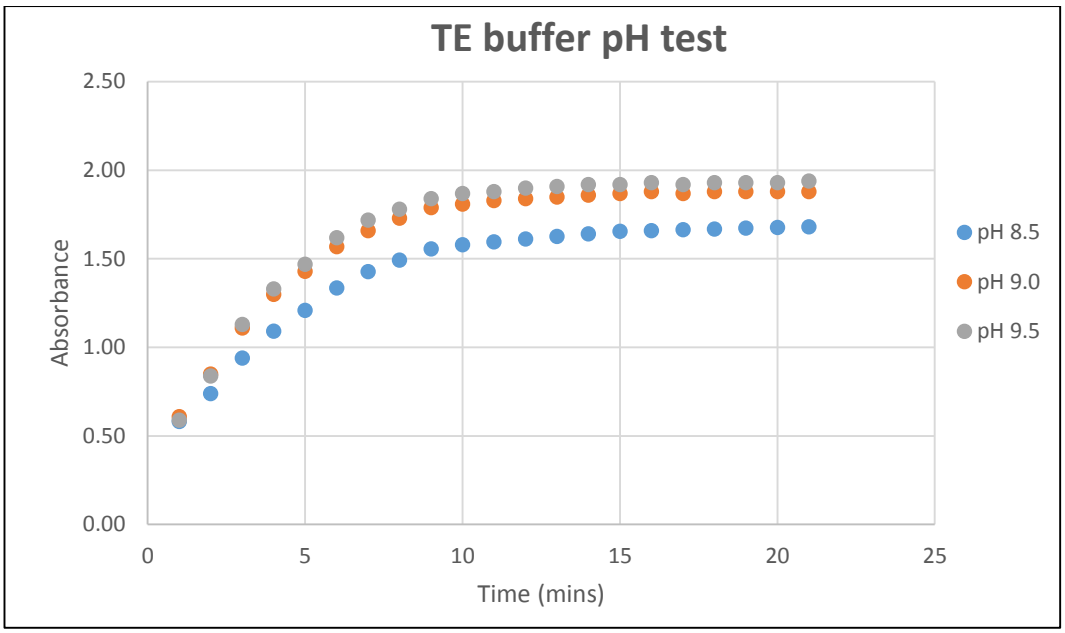


Figure 12: TE buffer pH test results, absorbance vs. time, 0.4mM glutamate.

The conclusion of these tests revealed that the higher the pH of phosphate buffer, the faster the reaction reached equilibrium (evident by the plateau). On top of this the results showed a lot of inconsistencies evident by the differing values of final absorbance even though all of the tests used a consistent concentration of glutamate of 1mM (Figure 11). It is speculated that the inconsistencies in the final absorbance values may be due to the glutamic dehydrogenase operating at unfavourable pH.

For the TE buffer, three pH values (pH 8.5, 9, and 9.5) were made and tested. Initially, a concentration of 1mM of glutamate was used for the test. 1mM NADH (derived from glutamate, 1 to 1 stoichiometry) however was found to be too high for the detection limit of the spectrophotometer. The graph obtained from this test (data not shown) showed minimal increase in absorbance, as the starting absorbance values were already too high. For this reason, a much lower concentration of glutamate of 0.4mM was then found to be much suitable for the detection limits of the spectrophotometer. Other reagents needed to complete the reaction were kept constant to the previous test.

The results for the TE buffer pH test concur with the previous phosphate buffer pH tests. The higher the pH of the buffer, the faster the reaction reaches equilibrium evident by the plateauing of the slope (Figure 12). In this case, TE buffer at pH 8.5 did not seem to allow for all of the glutamate to be converted into α-Ketoglutarate.

This is evident by the lower final absorbance value compared to the other two pH values (Figure 12). Nevertheless, this test signifies the importance of buffer pH and its ability to hinder a complete reaction if the pH is sub-optimal for the enzyme mediating the reaction. Compared to the phosphate buffer, TE buffer reached equilibrium faster. Reactions in phosphate buffer reached equilibrium (evident by the plateauing of the slope) around 40 to 60 minutes depending on the pH of the buffer (Figure 11). In contrast, reactions in TE buffer reached equilibrium around 10 to 15 minutes depending on the pH of the buffer (Figure 12). From these results it was then concluded that TE buffer at pH 9.5 will be used for the glutamate assay.

Theoretically, the concentration of NADH can be calculated using the formula:

$$\Delta A = \varepsilon C l$$

Where ΔA is the change in absorbance, ε is the extinction coefficient of NADH at 340nm, C is concentration of NADH, and l is the path length. The extinction coefficient of NADH is 6220 $M^{-1}cm^{-1}$, and the path length is 1cm. Using 0.4mM as the concentration, the change in absorbance should be about 2.48. Since the reagents for the glutamate assay already have a baseline absorbance value of about 0.6, the final absorbance value should be around 3.08. However, this theoretical value does not correspond to the experimental results generated. For example, in (Figure 12) 0.4mM of glutamate resulted in a plateauing just under the absorbance value of 2. This disagreement is due to the reversible nature of the reaction (Figure 10). For this reason, the quantification of glutamate will have to be done using a standard curve.

2.1.2 NAD^+ Optimization

The next step was to find if the glutamate in the reaction presented in Figure 13 is fully converted. Incomplete glutamate conversion could occur if not enough NAD^+ is used in the assay. It is important to remember that the reaction in Figure 13 is reversible, and if the driving force for the forward direction is weak, then the reverse reaction is favoured transforming α-Ketoglutarate back into L-glutamate.

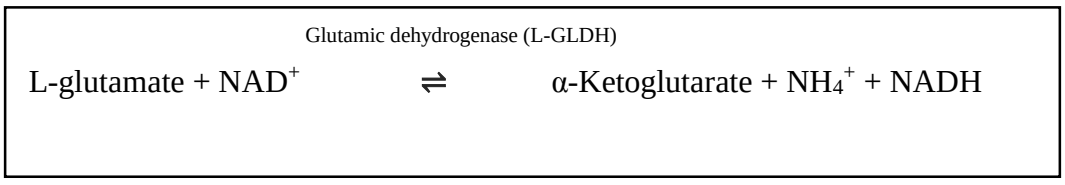


Figure 13: Dehydrogenation of glutamate and reduction of NAD^+.

The reverse reaction in all cases should be prevented as it will diminish the accuracy of the results. Thus, in order to reduce the undesired reverse reaction, NAD^+ should be at a high enough concentration so that the forward reaction is always favoured.

To test this, three NAD^+ concentrations (12, 14, and 15mM) were tested along with 0.3mM of glutamate as the substrate, 1mM of ADP, 5mg/ml GLDH, and TE buffer, pH 9.5. The results are presented in Figure 14.

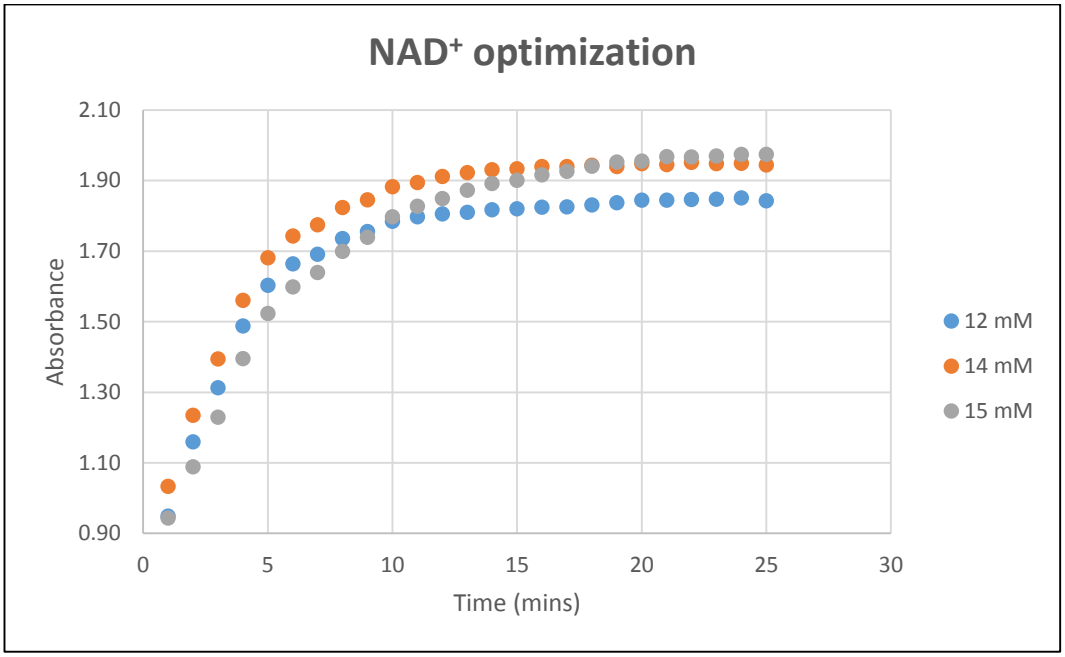


Figure 14: NAD^+ optimization results. Absorbance vs. time.

By increasing the NAD^+ concentration, it was clear that the forward reaction is more favourable, evident from the final absorbance values of the reactions (Figure 14). In this case 12mM of NAD^+ was still not enough to allow full conversion of the glutamate into α-ketoglutarate evident by the lower final absorbance value compared to the other two NAD^+ concentrations (Figure 14). On the other hand, both 14 and 15mM showed a very similar final absorbance values, suggesting that these concentrations of NAD^+ were sufficient enough to drive the forward reaction only. At these concentrations equilibrium were reached in about 15 minutes (Figure 14).

2.1.3 ADP Optimization

As stated before, ADP is needed for this reaction as it is an activator for glutamic dehydrogenase. To investigate the importance of ADP, an identical assay to the one performed for the NAD^+ test was done using no ADP and the results showed no change in absorbance value. ADP is thus a crucial component of the glutamate assay. How much ADP is required for this reaction to operate optimally? The previous tests used 1mM of ADP as a starting point, however this may be too much and thus wasteful.

To test this, two ADP concentrations below 1mM were tested (0.5mM and 0.7mM) along with 0.3mM of glutamate, 15mM NAD^+, 5mg/ml GLDH, and TE buffer, pH 9.5. The results are presented in Figure 15.

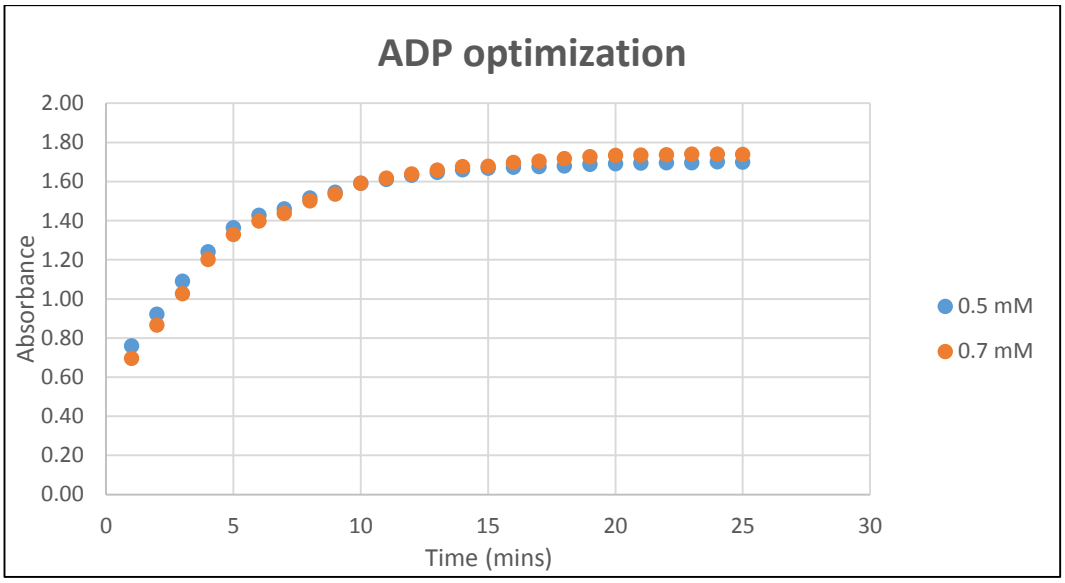


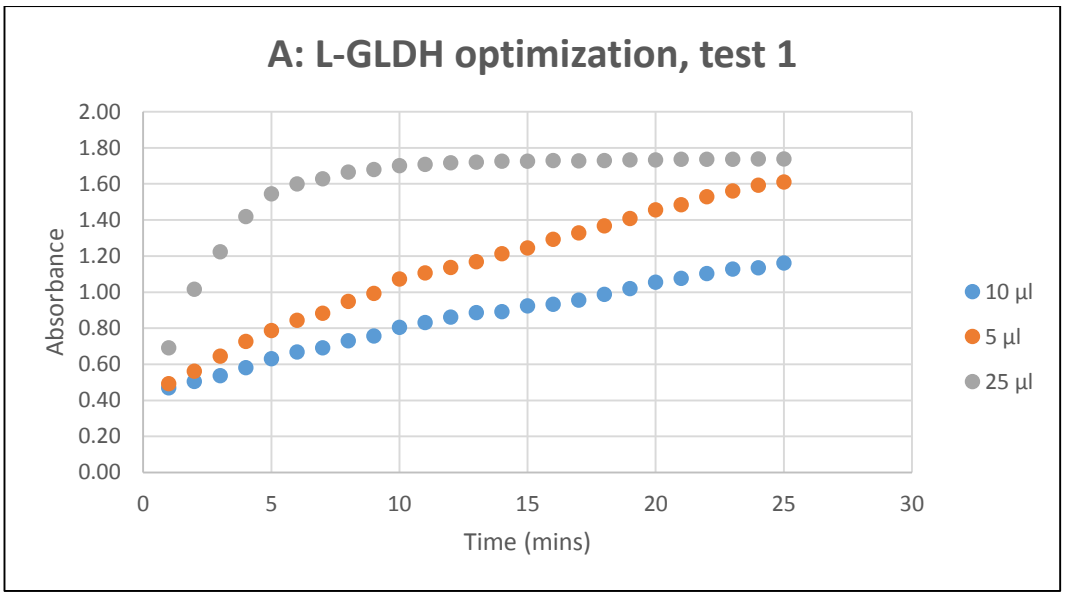
Figure 15: ADP optimization result. Absorbance vs. time.

From the results above, using both 0.5 and 0.7mM of ADP has no indistinguishable differences on the rates and the final absorbance value (Figure 15). Therefore the assays were done using the lower ADP concentration of 0.5mM, reducing waste of reagents.

2.1.4 Glutamic dehydrogenase (L-GLDH) optimization

The last component to optimise for the glutamate assay was the enzyme glutamic dehydrogenase. All previous tests have used this enzyme at a concentration of 5mg/mL at 20µl.

To start, three volumes (5, 10, and 25µl) of glutamic dehydrogenase at 5mg/mL were tested with 0.3mM glutamate, 15mM NAD^+, 0.5mM ADP, and TE buffer, pH 9.5. The results are presented in Figure 16A.



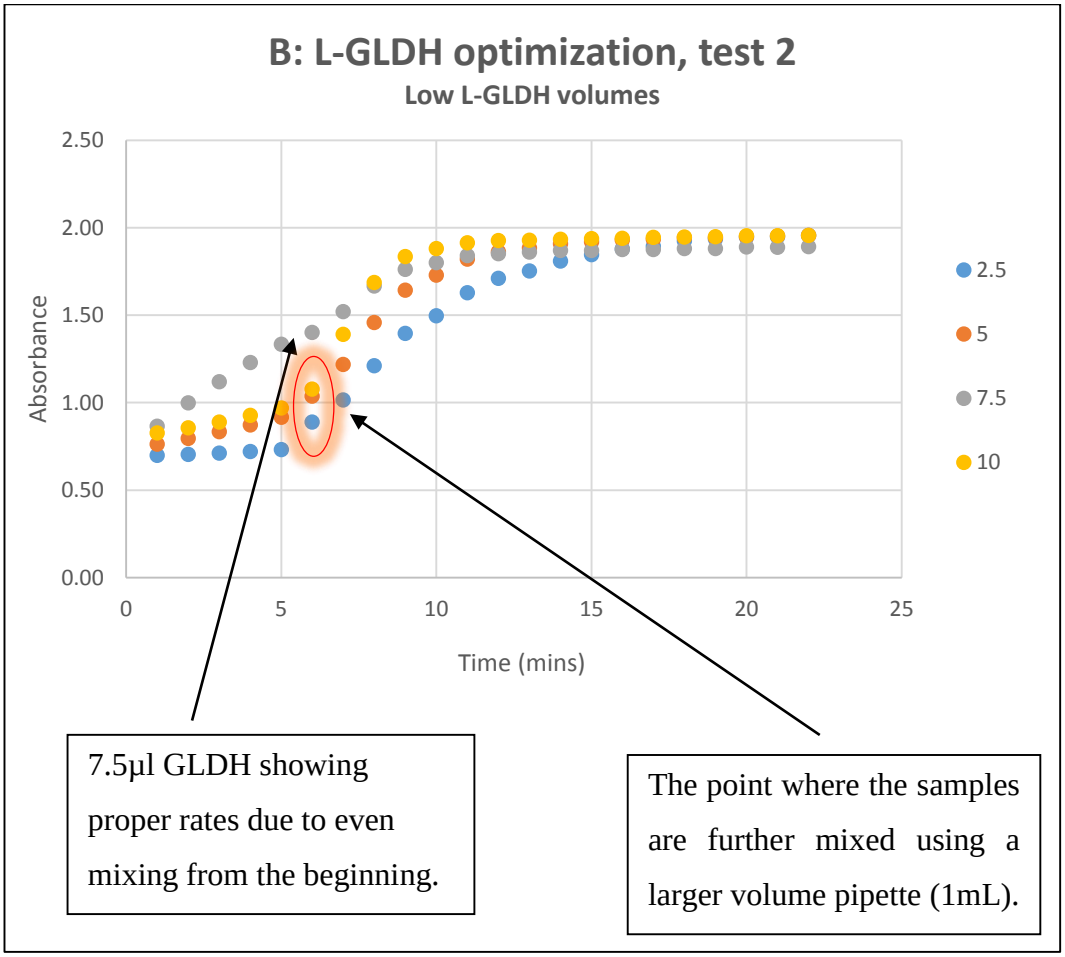


Figure 16: A) Glutamic dehydrogenase (L-GLDH) optimization, test 1 results. Absorbance vs. time. B) Debunking the irregular trends observed from the previous Glutamic dehydrogenase (L-GLDH) optimization tests.

At first glance, Figure 16A looks unusual as the rate for the reaction with only 5µl of L-GLDH is faster than the reaction with 10µl of L-GLDH. However it was then found that the culprit behind this phenomena is improper mixing of reagents before the reaction started (Figure 16B).

To conclude, the rate of reaction is proportional to the volume of L-GLDH as expected. 25µl of L-GLDH at 5mg/mL gave the fastest rate of reaction and was thus chosen as the final amount and concentration for the glutamate assay.

2.1.5 Optimised glutamate assay

The final set of conditions are selected based on the results of the tests presented above.

Table 5: Final set of conditions for glutamate assay. The values described below are representative for one assay.

Sample containing Glutamate	100µl (Glutamate concentration ≤ 0.4mM)
NAD^+	150µl (final concentration of **15mM**)
ADP	5µl (final concentration of **0.5mM**)
L-GLDH	25µl at **5mg/mL**
Buffer	745µl TE buffer, pH 9.5
Reaction volume	**1.025mL**
Detection wavelength	340nm

2.1.6 Glutamate assay standard curve

Using the conditions set out for the glutamate assay, a standard curve was generated in order to determine samples with unknown concentration of glutamate. Four glutamate concentrations ranging from 0.1mM to 0.4mM were made and measured multiple times to get an average reading. The result were as follows:

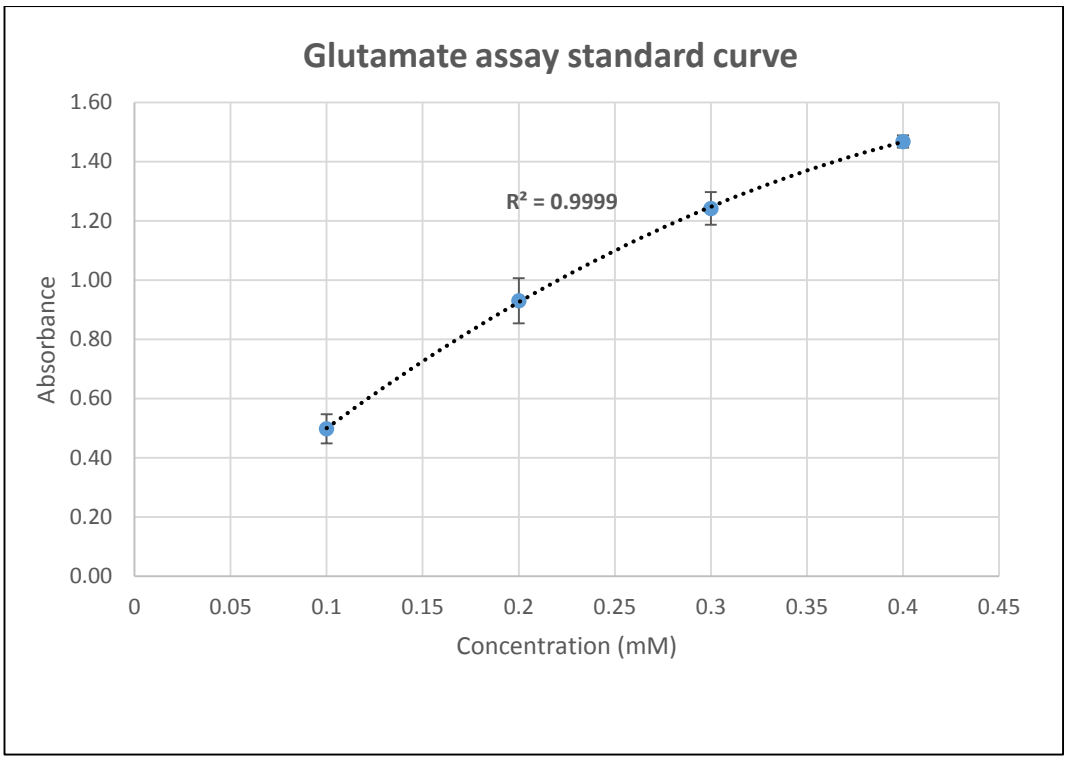


Figure 17: Glutamate assay standard curve along with equation.

The formula used to calculate the concentration of glutamate from absorbance is:

$$y = -5.1562x^2 + 5.7994x - 0.0287$$

2.2 Glutamine assay

The glutamine assay is a coupled reaction with the glutamate assay. The first step of the glutamine assay is the conversion of glutamine to glutamate via glutaminase (EC 3.5.1.2) (Figure 18).

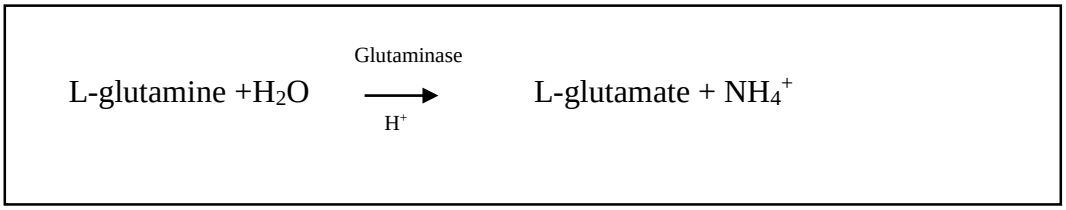


Figure 18: conversion of glutamine into glutamate via the enzyme glutaminase.

Glutamate is then quantified through the already established glutamate assay. If the samples tested contains both glutamate and glutamine, to properly quantify glutamine, the concentrations of glutamate derived from glutamine and endogenous

glutamate should be determined separately, and the concentration of glutamine calculated from their difference.

Once again, the method from Sigma (GLN1) was used as a baseline to start the development of the glutamine assay.

The Sigma approach to the glutamine assay requires 0.2mL of 0.5M of acetate buffer at pH 5, 0.45mL of deionised water, 0.25mL of glutamine containing sample, and 0.1mL of glutaminase diluted first in 10% of the 0.5M acetate buffer. All of these equates to a reaction volume of 1mL and 1 unit of glutaminase is used. The reaction was then carried out at 37˚C for 1 hour. The Sigma protocol for glutamine assay was replicated and tested. The glutaminase (E-GLUTEC from Megazyme located in Ireland) was supplied as a suspension in 2.5M of lithium sulphate at 1250 U/mL at 25˚C or 3029 U/mL at 37˚C. To mimic the Sigma protocol, 0.4µL (1.212 U) of the Megazyme glutaminase was used per assay. 0.4µL of glutaminase was mixed with 5mM sodium acetate buffer at pH 4.9 (used only for mixing with glutaminase before each assay) to make 50mL before used for the assay. Once all of the glutamine has been converted into glutamate, an aliquot of 100µL from the glutamine assay reaction chamber will then be used for the glutamate assay.

The next step was to find the concentration limit of glutamine for each assay. Several calculations were made in order to find the ideal concentration, and it was found that starting with 3mM of glutamine will equate to 0.3mM glutamate and 0.3mM of NADH as 100µL aliquot is transferred from the glutamine assay into the glutamate assay. 0.3mM of NADH is within the detection limit of the spectrophotometer.

Future work on samples with an unknown concentration of glutamine will have to be diluted down if it contains more than 6mM of glutamine in order to stay within the detection limits of the spectrophotometer. This may have to be done through trial and error.

The next step was to find out the required time needed for the conversion of glutamine to glutamate (incubation time). To test this, 100µL of 6mM glutamine, 50µL of diluted glutaminase (1.212 U), 200µL 0.5M acetate buffer, and 650µL deionised water were made and used for the glutamine assay.

The glutamine samples were then incubated and 16 samples were taken at 16 different time points. The results are presented in Figure 19.

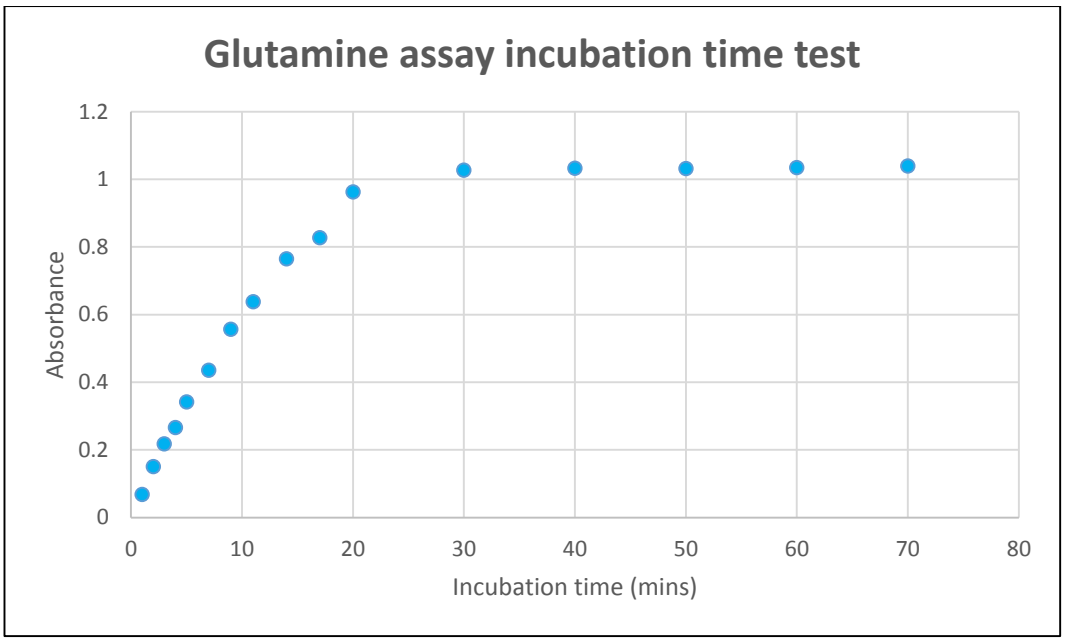


Figure 19: Glutamine incubation time test results.

From the graph above, an appropriate incubation period can be determined for this assay. The one hour incubation period suggested by Sigma-Aldrich is excessive and a shorter period of about 40 minutes should suffice.

To minimise work and accelerate the whole process, an alternative procedure for this assay was tested. This procedure combines both the glutamine and glutamate assay together in a single reaction chamber. To test this, both reagents needed for the glutamine assay and the glutamate assay were put into one reaction chamber. These included NAD^+, ADP, glutamine, glutaminase, and buffers (TE buffer at pH 9.5 and Acetate buffer 0.5M, pH 5) at concentrations and volumes specified earlier. The mixture were then incubated at 37˚C for 40 minutes. Straight after the incubation period, GLDH are then added to the mixture and NADH are quantified using the spectrophotometer.

Unfortunately, there were no change in the absorbance value after GLDH was added to all of the samples tested. This demonstrates that the two assay cannot be done simultaneously. It is speculated that the presence of both buffers may be causing inhibition for glutaminase to operate.

2.2.1 Optimised glutamine assay

The final set of conditions were selected based on the results of the tests and calculations presented above.

Table 6: Final set of conditions for glutamine assay. The values described below are representative for one assay. *Not to be confused with 5mM sodium acetate buffer at pH 4.9 used to mix the glutaminase before each assay.

Sample containing glutamine	**100µL at (3mM)**
Acetate buffer*	200µL at 0.5M, pH 5
Deionised water	650µL
Glutaminase (0.4µL diluted to 50µL with 5mM of sodium acetate buffer at pH 4.9)	50µL at 1.212U
Incubation at 37˚C for 40 minutes.	
50µL is then used for glutamate assay.	

2.2.2 Glutamine assay standard curve

Using the conditions set out for the glutamine assay, a standard curve was generated in order to determine samples with unknown concentration of glutamine. Four glutamine concentrations ranging from 0.1mM to 0.4mM were made and measured multiple times to get an average reading. The results are presented in Figure 20.

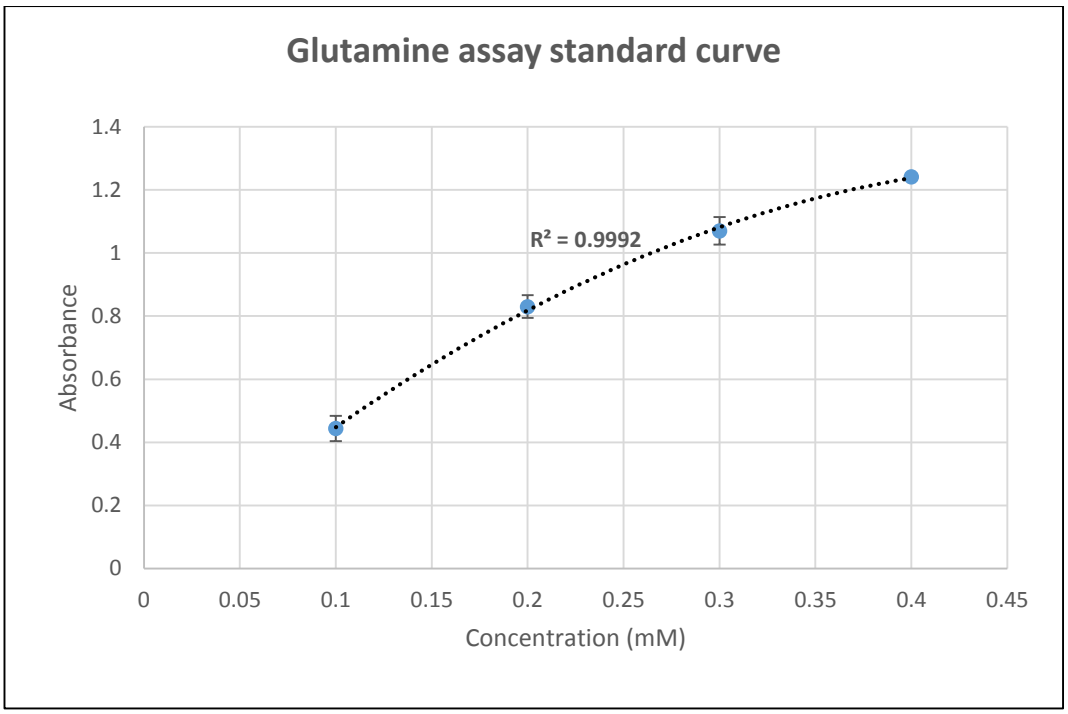


Figure 20: Glutamine assay standard curve along with equation.

The formula used to calculate the concentration of glutamine from absorbance is:

$$y = -5.35x^2 + 5.309x - 0.0295$$

2.3 Glutamate and glutamine stability under glutamine assay condition

Out of the two methods presented above, only the glutamine assay requires a substantial incubation period. The incubation time was necessary in order to convert the glutamine into glutamate, before it is quantified using the glutamate assay. The incubation is at 37˚C for 40 minutes. A test was then conducted to ensure the glutamate and glutamine does not degrade during the incubation period. To test this, glutamine (6mM) and glutamate (0.4mM) were subjected to a 80 minute incubation at 37˚C along with all of the reagents required for the glutamine assay (acetate buffer, 0.5M at pH 5) with the exception of glutaminase. The results showed that even when subjected to twice the incubation period, both glutamine and glutamate did not show any sign of degradation (data not shown).

2.4 Quantification of pyroglutamic acid

The HPLC set up was as follows:

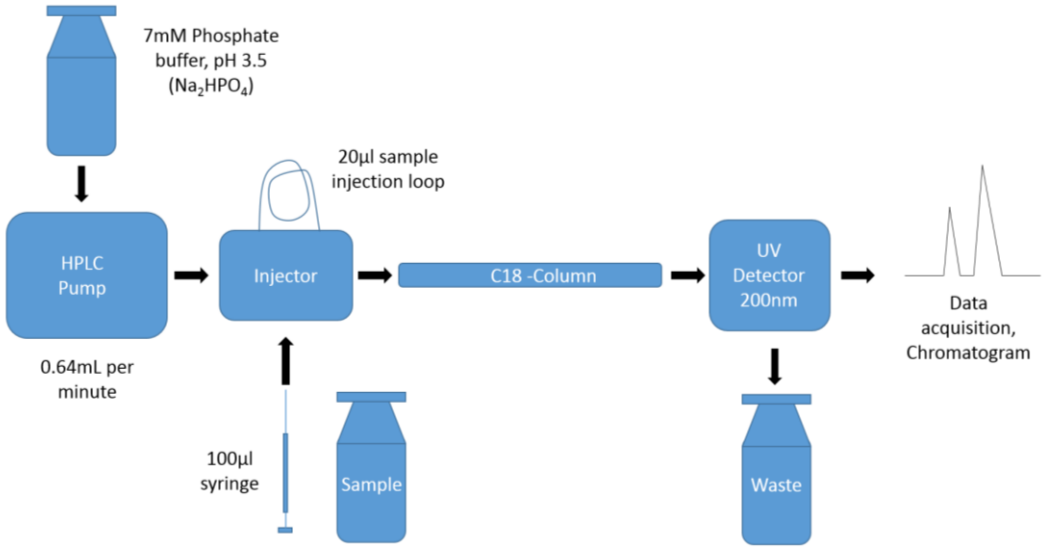


Figure 21: HPLC set up.

A Waters 515 HPLC pump running isocratically was used for this assay. The flow rate was chosen based on the capacity of the column (Brownlee™ Columns, Spheri-5 RP-18, 5µm, 220 X 4.6 mm), which has a maximum pressure limit of 2000 psi. 0.64mL per minute was found to be the fastest flow rate while keeping the pressure below 2000psi. A Hamilton® HPLC syringe 51mm in length with 22s ga (blunt tip) needle was used for sample injection. 100µL was chosen as the sample volume in order to sufficiently fill the 20µL injection loop. A Waters 2996 photodiode array detector set to 200nm was used to detect pyroglutamic acid (Shih, 1985).

The system functioned well and gave clear results. In this case, the retention time for pyroglutamic acid at these conditions was about 5.9-6.0 minutes (Figure 22).

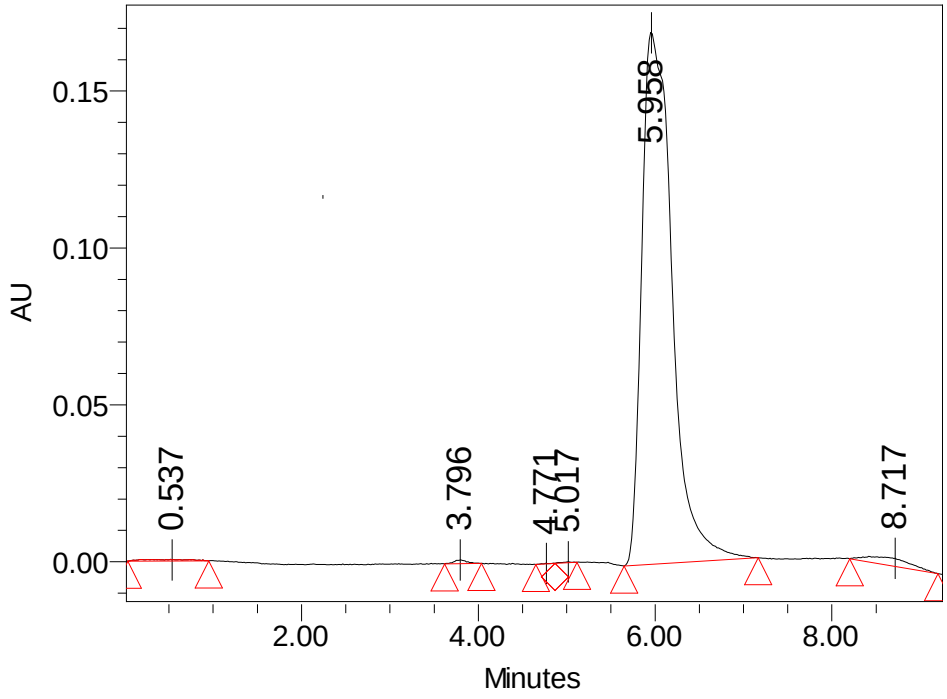


Figure 22: Chromatogram for pyroglutamic acid only.

2.4.1 Methionine interference

Previous studies have reported that methionine is also detected at 200nm (Gandini, *et al.*, 1993), but the retention time for methionine was unknown for this set up. Trials were carried out to examine potential interference from methionine with the pyroglutamic acid detection.

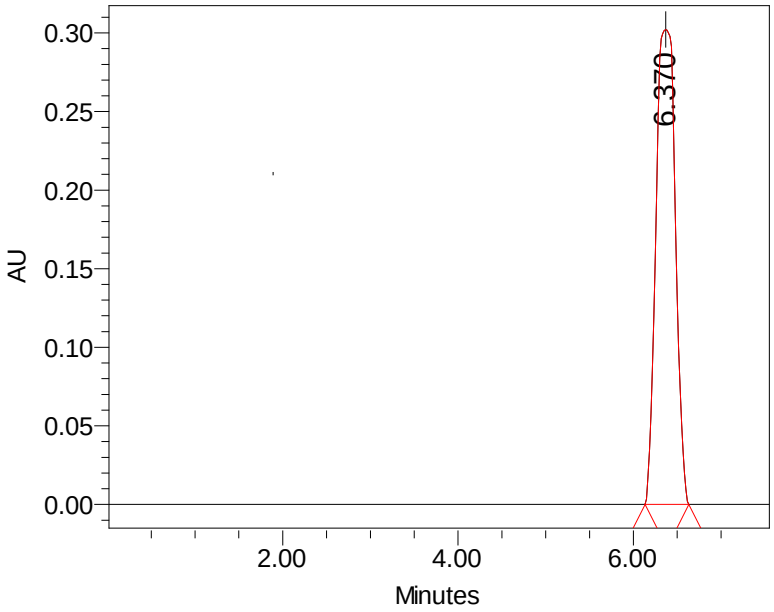


Figure 23: Chromatogram for methionine at 0.5mM dissolved in the mobile phase.

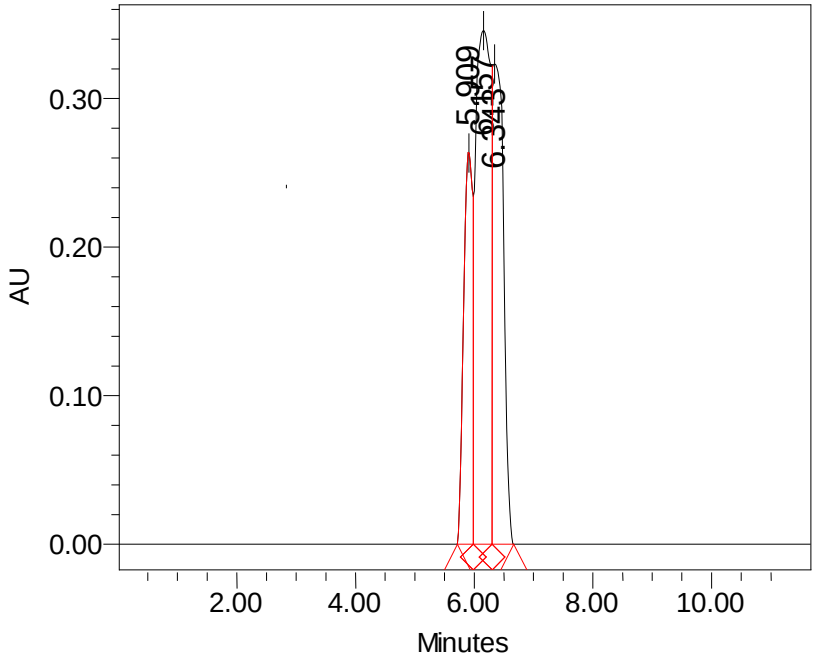
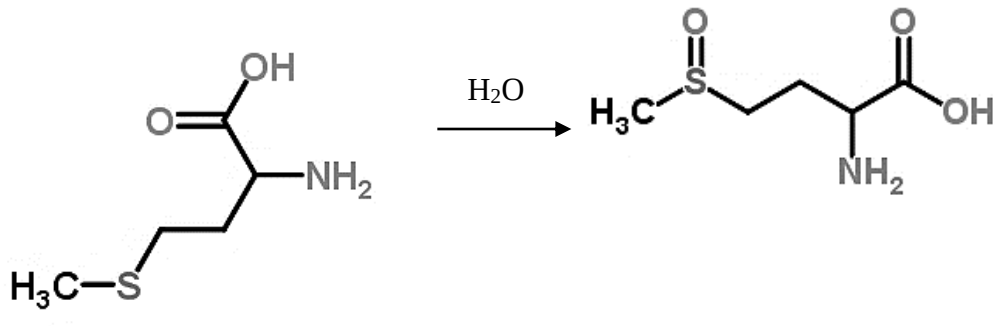


Figure 24: Chromatogram for pyroglutamic acid and methionine combined at 0.5mM dissolved in the mobile phase.

Unfortunately, the retention time for methionine under this set up is around 6.4 minutes (Figure 23). Combining both methionine and pyroglutamic acid together showed the peaks overlapping each other (Figure 24), and thus a way of eliminating methionine while preserving pyroglutamic acid is needed. Previously, treatment of

samples containing both pyroglutamic acid and methionine with 35% m/v H_2O_2 for one hour at room temperature was found to be sufficient in eliminating the presence of methionine but preserving the concentration and integrity of pyroglutamic acid (Gandini, *et al.*, 1993). The addition of H_2O_2 (an example of a reactive oxygen species) oxidises the SH- group of methionine into methionine sulfoxide (Figure 25) (Luo & Levine, 2009) (Rapoport *et al.*, 1984).



Methionine Methionine Sulfoxide

Figure 25: Conversion of methionine into methionine sulfoxide in the presence of H_2O_2.

Tests were carried out to confirm that H_2O_2 treatment eliminates methionine from detection while preserving pyroglutamic acid. Condition for the tests are presented in Table 7.

Table 7: Methionine elimination test outline (PGA=Pyroglutamic acid and MET=Methionine).

Conditions	**1**	**2**	**3**	**4**	**5**	**6**
Components	PGA	Met	PGA	Met	PGA + Met	Buffer only
H_2O_2 treatment (1hr)	No	No	Yes	Yes	Yes	Yes

Conditions 1 and 2 were the controls of this test. The chromatograms from these conditions were as expected, with a single peak for both pyroglutamic acid and methionine at approximately the same retention time (data not shown). Conditions

3 and 4 were the samples that were treated with 30% m/v H_2O_2 for one hour. The chromatogram from condition 3 gave a clear peak (with similar peak height to condition 1) at around 6 minutes, and the chromatogram from condition 4 showed no peak at the expected retention time for methionine (data not shown). The chromatogram from condition 5 also gave a clear single peak at around 6 minutes and no other peaks were visible (identical to Condition 3), indicating the absence of methionine (data not shown). It is also important to mention that for every samples treated with H_2O_2, there is always a peak at 4 minutes retention time. The peak at 4 minutes was found to be a signature peak for H_2O_2. The chromatogram from condition 6 (data not shown) showed a single peak at 4 minutes retention time.

The conclusion of this test reveals that 30% m/v H_2O_2 treatment for one hour was sufficient in eliminating methionine detection.

2.4.2 Pyroglutamic acid HPLC standard curve

Using the set up described above, a standard curve was then generated in order to determine samples with unknown concentration of pyroglutamic acid. The first trial in making a standard curve for pyroglutamic acid used a range of concentrations from 0.1mM to 5mM. After examining the peaks from these concentrations, it was found that pyroglutamic acid peaks for concentrations above 0.8mM were shouldering (Figure 26).

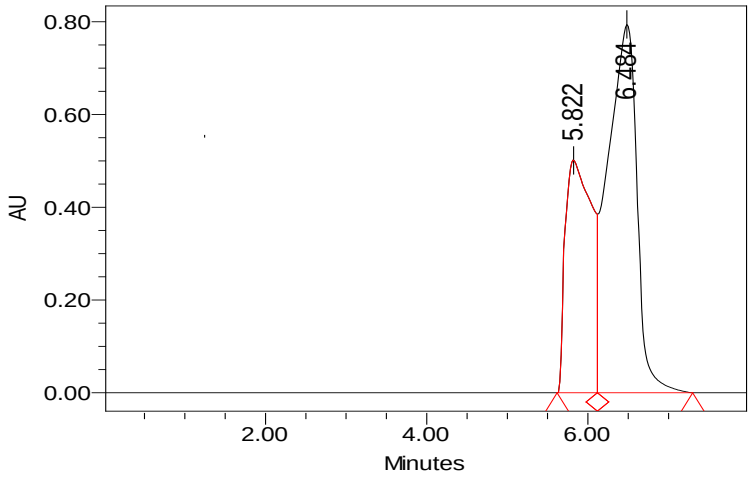


Figure 26: Peak shouldering example. Chromatogram of pyroglutamic acid only at a concentration of 2mM.

This shouldering will effectively alter the accuracy of the results as the primary results are based on the height of the peaks. Another set of lower concentrations were then made in an attempt for another standard curve. This time, the standard curve was made with concentrations limited to 0.8mM. No shouldering occurred and the results are presented in Figure 27.

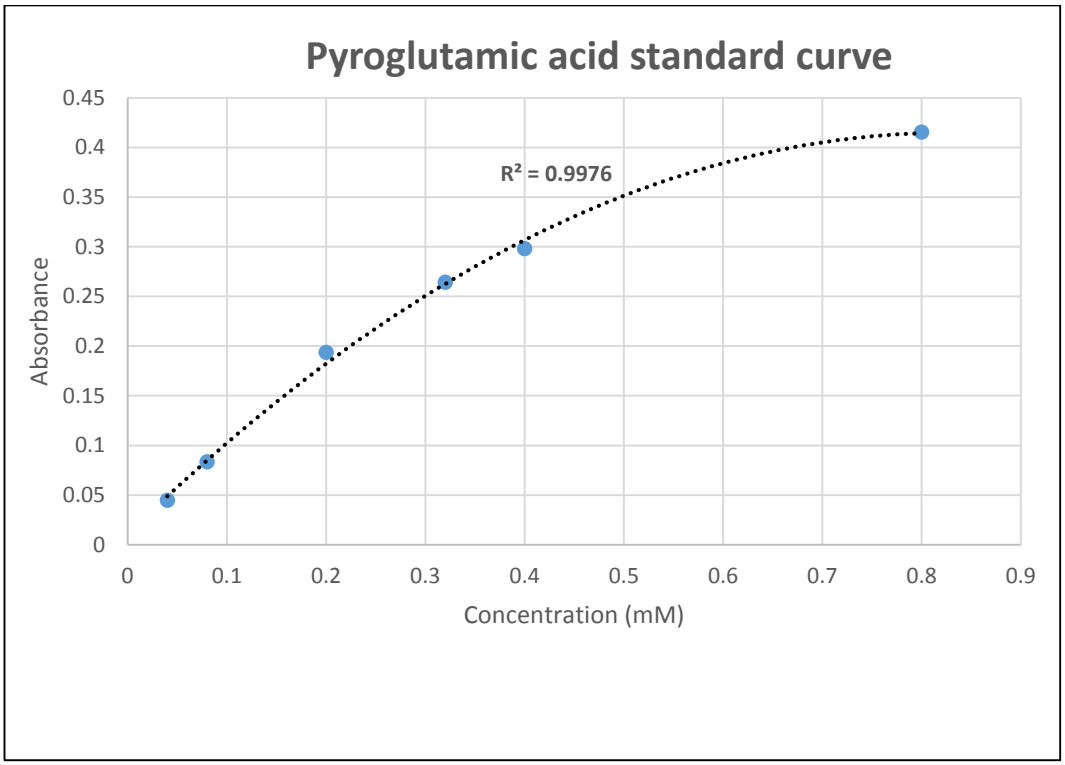


Figure 27: Pyroglutamic acid standard curve along with equation.

The formula used to calculate the concentration of glutamine from absorbance is:

$$\mathbf{y = -0.5892x^2 + 0.9761x + 0.0106}$$

Chapter 3

HCP337

3.1 HCP337 production process

Using the methods described in Chapter 2, a commercial flavour component called HCP337 from The Tatua Co-operative Dairy Company was characterised to understand the biochemistry of glutamate release during its production.

HCP337 is a flavour component that is made by digesting casein hydrolysate by several different proteases and peptidases. The main goal of the HCP337 production process is to digest casein hydrolysate protein and liberate as much free glutamate as possible. The free glutamate in the final product can then be used as a flavour ingredient and substitute for MSG in the production of flavours at Tatua. The HCP337 product is most important in the production of cheese flavour ingredients as umami/savouriness is crucial for many of Tatua's cheese flavour products.

Currently, the HCP337 production process takes around 83 hours (Table 8). The whole process consists of two major hydrolysis stages using three different proteases and peptidases. pH adjustments and heat treatments are performed after each hydrolysis step in order to deactivate the enzymes and as a control for microbial growth. Once the two hydrolysis steps are done, the HCP337 slurry is put through a centrifuge and separated by density. The centrifugation process separates HCP337 into two forms: "clarification overflow" (supernatant) and "clarification discharge" (solid pellet residue). The clarification discharge is discarded, while the clarification overflow goes into the final processing steps and becomes the HCP337 powder/product. During the final steps, the clarification overflow is evaporated under vacuum and concentrated. This process leads to the last major step, the spray drying of the thick slurry and transforming it into a fine powder.

The challenge for Tatua is that not much of the glutamate theoretically available from casein is actually present in the final product. On average, about 20-25g/kg of glutamate is present in the final HCP337 powder (recognising that inter-batch variations exist), whereas in theory a complete hydrolysis of casein releasing all of its glutamate would yield 134.5g/kg of glutamate (Yildiz, 2009).

Table 8: Descriptive profile of the HCP337 production process outlining the time, pH and temperature changes.

Description	Time (h)	pH	Temp (°C)	Samples taken for testing
Half of the casein added	0	1.7	55	After tipping of casein was completed
Enzyme 1 added (Start of first hydrolysis)	6.2	2.4	55	• 8 hours into the first hydrolysis • 16 hours into the first hydrolysis • 24 hours into the first hydrolysis, before the pH adjustment
Tipping start second half casein	6.33	2.8	54	
Tipping finished	7.2	3.8	54	
Enzyme 2 add (total hydrolysis 24h from 1st enzyme)	7.33	3.8	53	
Heat treatment for 93 seconds	31.2	5.2	125	After the first heat treatment
Enzyme 3 add (start of second hydrolysis) (total hydrolysis 34h)	35.3	5.12	53	• 12 hours into the second hydrolysis • 24 hours into the second hydrolysis • 34 hours into the second hydrolysis, before the pH adjustment
Heat treatment for 77 seconds	80.4	6.7	125	A sample was taken after the second heat treatment.
Clarifier	80.5	6.7	55	Two samples: clarification solids and overflow
Evaporation	82.7	6.7	65	
Spray dry	83	6.7	95	HCP337 powder

Table 9: Average concentrations of glutamate and glutamine from the HCP337 production process samples (December 2015 batch), and the corresponding standard deviation. Important to note that the glutamine values represent glutamine and endogenous glutamate.

	Glutamate		**Glutamine + Glutamate**	
Samples	**Mean Concentration $\overline{x}$ mM**	**Standard deviation s**	**Mean Concentration $\overline{x}$ mM**	**Standard deviation s**
Casein	-	-	-	-
Tipping complete	0.000	0.000	0.000	0.000
1st hydrolysis 8hr	1.117	0.008	5.602	0.262
1st hydrolysis 16hr	3.885	0.107	9.420	0.271
1st hydrolysis finish, before pH adjustment	5.686	0.472	12.212	0.780
After 1st heat treatment	6.419	1.136	10.694	0.928
2nd hydrolysis 12hr	15.896	0.228	19.347	0.374
2nd hydrolysis 24hr	18.737	0.408	26.917	0.988
2nd hydrolysis finishes before pH adjustment	20.509	1.097	25.349	1.039
After 2nd deactivation	18.069	0.721	23.940	0.862
Clarifier solids	12.453	0.954	19.437	0.600
Overflow	17.169	0.089	22.104	0.888
HCP337 Powder	18.768	0.501	23.229	0.427

Table 10: Quantitative results of the HCP337 production process from December 2015 batch. Concentration in g/kg (according to the total solid % of each sample). Glu=Glutamate, Gln= Glutamine, and PGA= Pyroglutamic acid.

Time	Sample	Total Solids (%)	Glu (g/kg)	Gln (g/kg)	PGA (g/kg)
	Acid Casein	13.4	0	0	
7:15	Tipping complete	13.4	0	0	
13:20	1st hydrolysis 8hr	13.4	1.2	4.9	
21:20	1st hydrolysis 16hr	13.4	4.3	6.1	
5:20	1st hydrolysis finish, before pH adjustment	13.4	6.2	7.1	
11:05	After 1st heat treatment	13.4	7.0	4.7	
0:00	2nd hydrolysis 12hr	13.4	17.5	3.8	
11:20	2nd hydrolysis 24hr	13.4	20.6	9.0	
21:20	2nd hydrolysis finishes before pH adjustment	13.4	22.5	5.3	
2:20	After 2nd deactivation	13.4	19.8	6.4	
2:30	Clarifier solids	17.0	10.8	6.1	
2:30	Overflow	14.4	17.5	5.1	24.9
	HCP337 Powder	13.1	21.1	5.0	32.4

3.2 HCP337 (December 2015)

Quantitative analysis was done for samples taken from the HCP337 production process. Glutamate and glutamine assays were done in triplicates, while pyroglutamic acid quantifications were only done in duplicates. Even though the retention time for pyroglutamic acid is around 6 minutes, the total time for the system to expel the sample was around 40 minutes (before another sample can be injected and tested). The results are presented in Tables 9 and 10:

Unlike glutamate and glutamine, pyroglutamic acid was only quantified in the final two samples of the HCP337 production process. The final two samples contained high enough pyroglutamic acid for a peak to be visible at 6 minutes retention time. Other samples prior to this step did not show a clear peak as it was hidden beneath the noise due to the complexity of the samples tested.

At the beginning of the process, casein was found to have no free glutamate or glutamine. The first hydrolysis yielded 6.2g/kg of glutamate and 7.1g/kg of glutamine over 24 hours. The first heat treatment was done at 125˚C with no apparent negative effect on glutamate concentration. In fact, the concentration of glutamate seemed to increase from 6.2 to 7.0g/kg after the first heat treatment. The increase of glutamate may be due to the residual activity of the enzyme that managed to survive the heat treatment. The same observation however cannot be said for glutamine. The first heat treatment appeared to have decreased the concentration of glutamine from 7.1 to 4.7g/kg. This drop suggest that glutamine is unstable under the heat treatment conditions, which is consistent with previous studies done on glutamine stability (Section 1.5).

After the first heat treatment, the second hydrolysis starts with the addition of the third protease. The second hydrolysis is different to the first one in several aspects: only one protease is involved in this hydrolysis, the total hydrolysis time is 34 hours, and the pH for the second hydrolysis was kept constant at 5.1.

12 hours into the second hydrolysis, the concentration of glutamate increased very rapidly. At this point, the samples gave an average reading of 17.5g/kg of glutamate. In contrast, the concentration of glutamine has dropped even more from the previous sample point. In theory, the concentration of glutamine should have increased as the protease break down the casein peptides even more. However, this is not the case. This may be due to the rate of glutamine degradation being faster

than the rate of glutamine release by the second hydrolysis. At the 24 hour mark into the second hydrolysis, glutamate concentration had increased even more, however the rate of increase is significantly lower compared to the first 12 hours. The decelerating rate of glutamate release is evident for the rest of the hydrolysis period, where the concentration of glutamate had only increased by a small amount at the 34 hour mark compared to the 24 hour mark. On the other hand, there was a substantial increase of glutamine from the 12 hour mark to the 24 hour mark but this was then followed by a significant drop in glutamine concentration from the 24 hour mark to the 34 hour mark. Unlike the first deactivation process, the second deactivation process affected the concentration of glutamate negatively, evident by the drop in glutamate concentration from 22.5g/kg to 19.8g/kg after the second deactivation process.

As mentioned above, at this point the slurry is separated in a centrifuge into clarifier solids and overflow. The results showed that the clarifier solids, which comprise most of the undissolved particulates, contain a large amount of glutamate and a smaller amount of glutamine. This suggests that the precipitate in the clarifier solids sample contains a lot of trapped glutamate that is discarded.

The overflow sample in this case has a glutamate concentration of 17.5g/kg, glutamine content of 5.1g/kg, and remarkably a very high concentration of pyroglutamic acid of 24.9g/kg.

At the end, the HCP337 powder contains 21.1g/kg of glutamate, 5.0g/kg of glutamine, and 32.4g/kg of pyroglutamic acid. To verify the accuracy of the data presented here, an independent quantification of glutamate by AgResearch was carried out. The method of quantification done by AgResearch employed a free amino acid analysis which is based on precipitation of peptides with 2% sulphosalicylic acid followed by ion exchange chromatography with a lithium buffer system. AgResearch found that the HCP337 powder from the same batch used for this study has a glutamate concentration of 23.2g/kg. This value is very close to the 21.1 g/kg of glutamate presented from this study.

3.3 HCP337 (March 2016)

The results presented so far on the HCP337 production process were done using samples from a single batch. To gain further understanding on this process and the inter-batch variations, replications from another batch was done. However, due to

the infrequent nature of HCP337 production, only two batches were analysed during the course of this study.

The second batch of HCP337 was produced in March, 2016. Unfortunately, when the decision to analyse the samples was made, the production for HCP337 was already in process, and thus samples were only obtained from the clarification stage onwards. The results are presented in Tables 11 and 12.

Table 11: Average concentrations of glutamate and glutamine from the HCP337 production process samples (March 2016 batch), and the corresponding standard deviation. Important to note that the glutamine values represent glutamine and endogenous glutamate.

		Glutamate		Glutamine	
Sample	**Sampling Period**	**Mean Concentration $\overline{x}$ mM**	**Standard deviation s**	**Mean Concentration $\overline{x}$ mM**	**Standard deviation s**
Clarification Feed Flow	Start	22.756	0.100	2.467	0.799
	Middle	24.605	0.507	34.170	0.165
	End	23.910	0.660	33.045	0.325
Clarification Overflow	Start	15.509	0.196	27.787	0.945
	Middle	16.936	0.286	33.480	0.821
	End	17.257	0.110	34.243	0.218
Clarification Discharge	Start	17.892	0.814	27.467	0.108
	Middle	19.418	0.241	28.528	0.487
	End	18.628	0.223	26.940	1.141
Evaporator Sample	Start	57.261	0.670	82.102	0.423
	Middle	57.542	0.629	87.250	0.807
	End	59.555	0.336	87.978	0.812
Concentration tank Sample	Start	49.699	0.386	70.408	0.603
	Middle	58.538	0.125	86.815	0.725
	End	58.610	0.072	91.817	0.748
HCP337c Filtered		48.475	0.628	86.815	0.523
HCP337 powder		24.634	0.163	28.105	0.174

Table 12: Quantitative results of the HCP337 production process from March 2016 batch. Concentration in g/kg (according to the total solid % of each sample). Glu= Glutamate, Gln= Glutamine, and PGA= Pyroglutamic acid.

Sample	Sampling Period	Total Solids (%)	Glu (g/kg)	Gln (g/kg)	PGA (g/kg)
Clarification Feed Flow	Start				
	Middle				
	End				
Clarification Overflow	Start	14.4	15.8	12.6	17.4
	Middle	14.4	17.3	16.9	
	End	14.4	17.6	17.4	
Clarification Discharge	Start	17.0	15.5	8.3	
	Middle	17.0	16.8	7.9	
	End	17.0	16.1	7.2	
Evaporator Sample	Start				
	Middle				
	End				
Concentration tank Sample	Start				
	Middle				
	End				
HCP337c Filtered					
HCP337 powder		13.1	27.6	3.9	36.8

The concentrations of glutamate from the overflow samples of the two batches were similar (Table 13). However the concentration of glutamine in the new overflow sample was a lot higher compared to the previous batch. On top of these, the pyroglutamic acid concentration in the new overflow samples were lower relative to the previous batch. At this stage, it was assumed that when glutamine is lost, it is converted to pyroglutamic acid. If this assumption is true, then the low concentration of glutamine in overflow sample from the old batch would be due to its spontaneous conversion into pyroglutamic acid.

Similarly, the clarification discharge samples from the new batch (the same as clarified solids samples from the previous batch) also contained a high concentration of glutamate and a lower concentration of glutamine relative to glutamate. This once again shows that the discarded clarification discharge products contain a lot of glutamate and to a lesser extent glutamine.

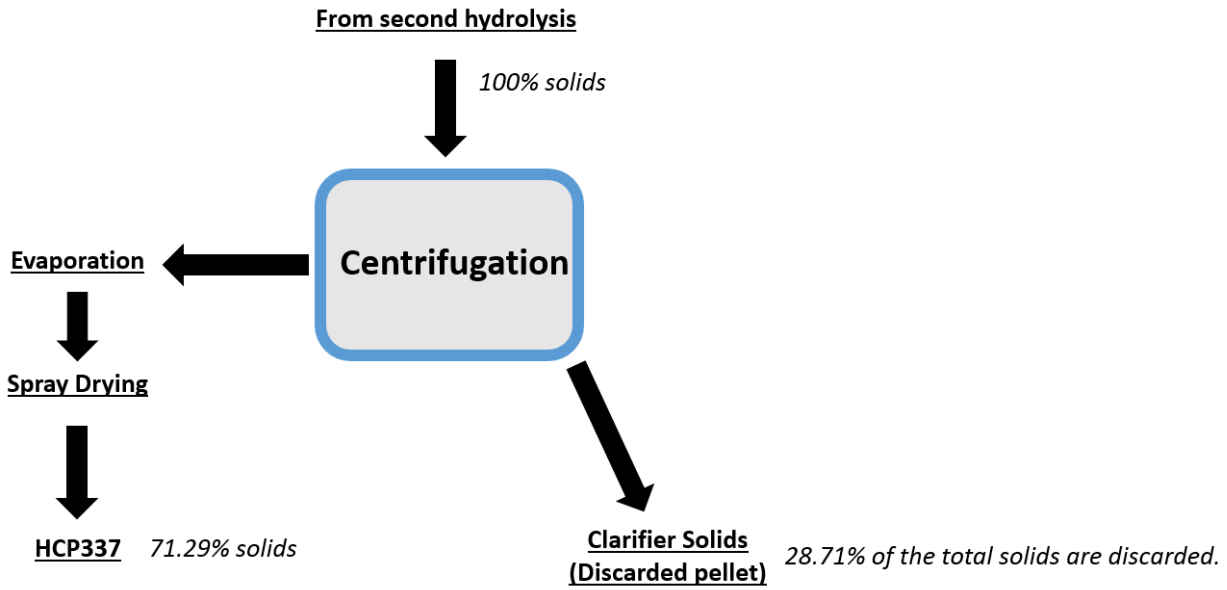
Lastly, the HCP337 powder from the new batch contains a slightly higher concentration of glutamate, slightly lower concentration of glutamine, and slightly higher concentration of pyroglutamic acid when compared to the old batch (Table 13). Unlike the old batch, data for the new batch suggest that more than half of the pyroglutamic acid present in the final powder is formed during the drying process at the end. The high temperature of 200˚C in the spray drying process is the likely culprit for the formation of pyroglutamic acid (Gayte-Sorbier, *et al.*, 1985; Arii, *et al.*, 1999).

Table 13: Glutamate, glutamine, and pyroglutamic acid concentration comparison between the two HCP337 batches. *values depicted are the average value of the three sampling periods.

Sample	Glu (Old batch) (g/kg)	Glu (New batch) (g/kg)	Gln (Old batch) (g/kg)	Gln (New batch) (g/kg)	PGA (Old batch) (g/kg)	PGA (New batch) (g/kg)
Clarifier solids	10.8	16.1*	6.1	7.8*		
Overflow	17.5	16.9*	5.1	15.6*	24.9	17.4
HCP337 Powder	21.1	27.6	5.0	3.9	32.4	36.8

3.4 Clarification solids

The tests done on the HCP337 samples reveal that clarified solids discarded from the HCP337 production process have a relatively high glutamate content. To better understand how much glutamate is wasted from discarding the clarifier solids, a mass balance analysis of the HCP337 production process was carried out. The information presented below represents the last few steps within the HCP337 production process according to Pinlei Lu, technologist in the flavours sector of Tatua.



Batch	December 2015	March 2016
Glutamate concentration (g/kg)	21.1	27.6
Glutamate weight (kg)	93.7	122.6

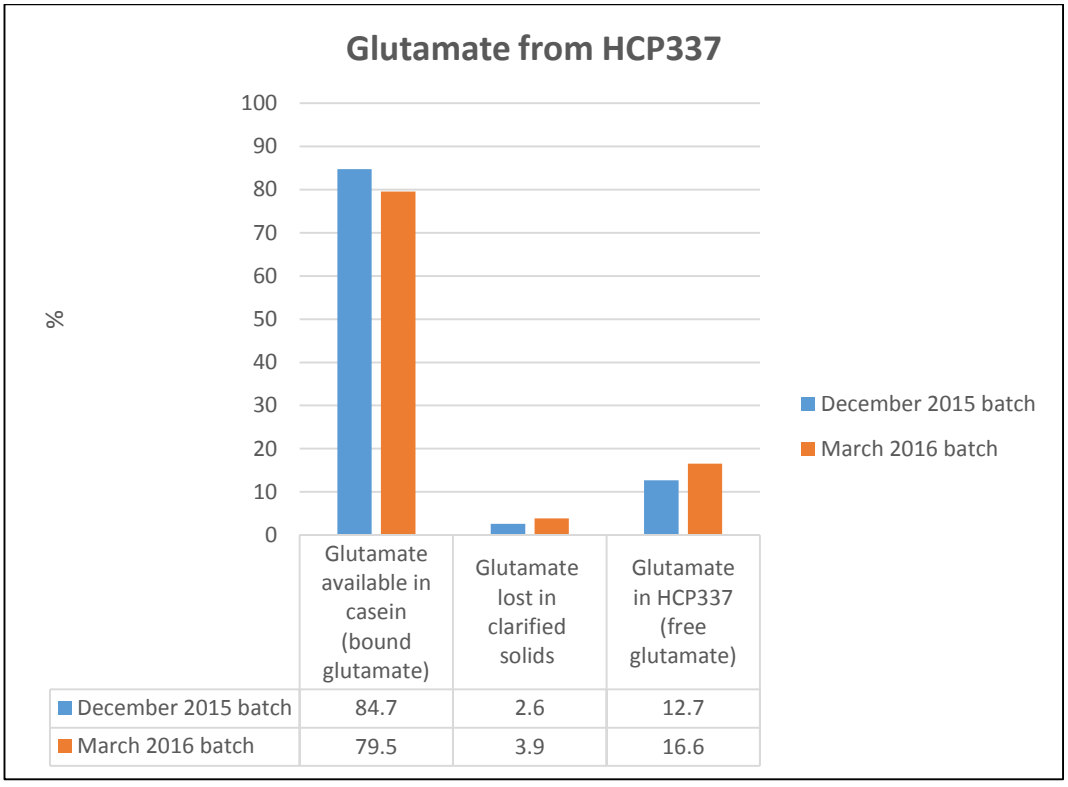
Batch	December 2015	March 2016
Glutamate concentration (g/kg)	10.8	16.1
Glutamate weight (kg)	19.3	28.8

Figure 28: Final steps of the HCP337 production process mass balance.

From the calculations above, it is clear that the amount of glutamate wasted from discarding the clarifier solids is significant.

3.4.1 The big picture

The purpose of this section is to analyse and gain perspective on the glutamate present in the HCP337 product, the glutamate loss in the clarification process, and the potential glutamate still available in casein. The HCP337 production process for both the December 2015 and March 2016 batch started with *x* kg of lactic casein as the substrate. Theoretically, casein contains 134.5g/kg of glutamate (Yildiz, 2010). Thus, in theory, the potential glutamate that is present in the casein used to make HCP337 is *y* kg (this is 100% of the potential glutamate present in casein). Below is a graph representing the percentage of glutamate utilised in the HCP337 production process.



	Glutamate available in casein (bound glutamate)	Glutamate lost in clarified solids	Glutamate in HCP337 (free glutamate)
December 2015 batch	84.7	2.6	12.7
March 2016 batch	79.5	3.9	16.6

Figure 29: Perspective view (from both batches) on the glutamate present in the HCP337 products (free glutamate), the glutamate loss in the clarification process, and the potential glutamate still available in casein (bound glutamate).

It is fair to say that relative to the glutamate present in the final HCP337 powder, the amount of glutamate lost from discarding the clarified solids is significant. Furthermore, the amount of bound glutamate still present in the casein polypeptides is enormous relative to the amount of free glutamate in the HCP337 product. Presumably most of the undigested casein polypeptides are discarded in the clarified solids. These findings are true for the two batches tested in this study.

3.5 Glutamate and glutamine stability test

The results confirmed sub-optimal glutamate released by the HCP337 production process. The results also confirmed the presence of pyroglutamic acid in the final HCP337 product in significant amounts. The next step was to find the origin of pyroglutamic acid. Both glutamate and glutamine were tested by subjecting them to the two hydrolysis conditions. The buffer chosen for this test was acetate buffer at 0.2M. Both glutamate and glutamine were dissolved with the acetate buffer (at differing pH) at a concentration of 0.3mM. The dissolved glutamate and glutamine were then transferred into 1.5mL Micro tube, and were sealed with Parafilm in order to prevent any moisture loss due to evaporation of water during the test. The individual Micro tube were then put into an Eppendorf Thermomixer which was set to 53˚C and constantly mix at 800 RPM. For 'Condition 1' samples were taken at 0, 3, 6, and 24 hour time intervals. For 'Condition 2', samples were taken at 6, 24, and 34 hour intervals. The results of the tests are presented in Table 14.

Table 14: Raw results of glutamine concentrations from all of the sample points in condition 1 (conditions of the first hydrolysis) and 2 (conditions of the second hydrolysis).

Condition 1				**Condition 2**		
Hours	**Concentration (mM)**	**%**		**Hours**	**Concentration (mM)**	**%**
0	0.313	100		**0**	0.328	100
3	0.302	96.4		**9**	0.310	94.5
9	0.261	83.3		**24**	0.259	78.9
24	0.177	56.5		**34**	0.229	69.8

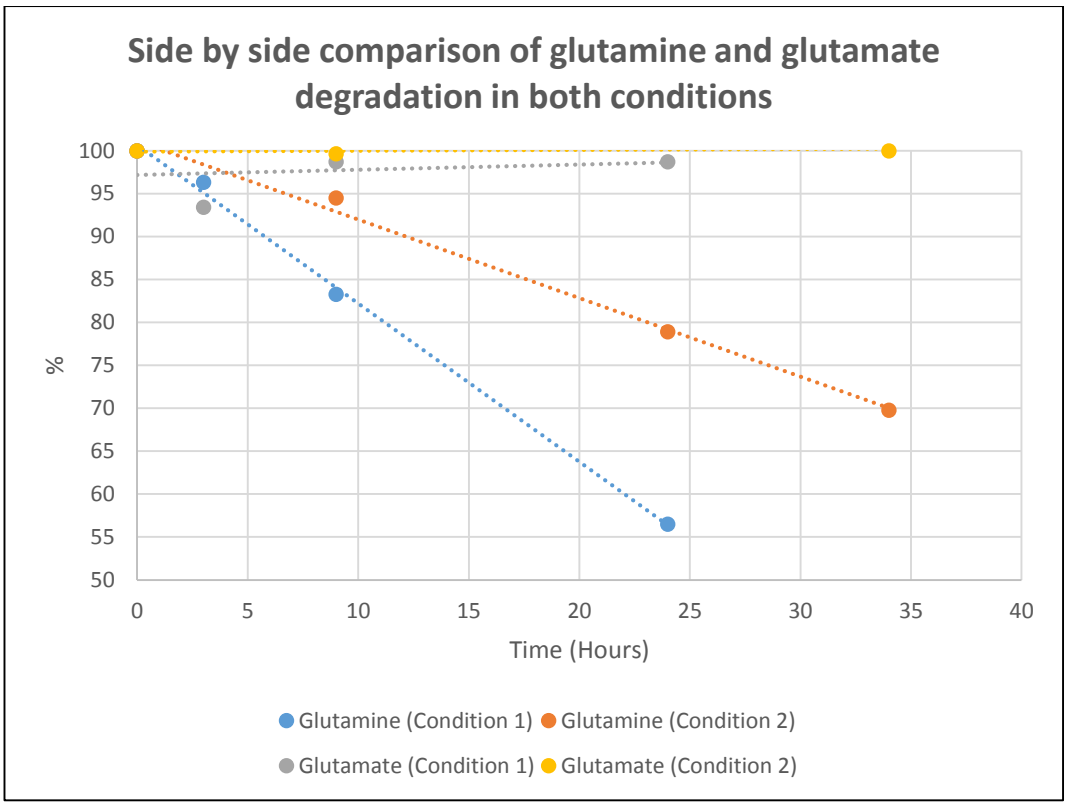


Figure 30: Side by side comparison of glutamine and glutamate degradation from condition 1 and condition 2.

Clearly, glutamate is very stable when subjected to both hydrolysis conditions (Figure 30). No degradation was observed under either condition.

The results here agree with a previous report of glutamate's stability under various conditions (Gayte-Sorbier, *et al.*, 1985).

3.5.1 Glutamine stability

In contrast, glutamine was found to be much less stable (Figure 30). In the first hydrolysis condition glutamine dropped to 57% of its original concentration in 24 hours (from 0.313mM to 0.177mM). In the second hydrolysis condition, where the pH was slightly higher, glutamine was found to be more stable as it only degraded to 79% of its original concentration after 24 hours (from 0.328mM to 0.259mM). After 34 hours, the glutamine dropped even further to 70% of its original concentration (to 0.229mM). The rate of glutamine degradation from both conditions showed linear decrease over time. The degradation of glutamine observed here followed a first-order reaction kinetics. The first-order plots from

both conditions showed an R^2 of over 0.99 (data not shown). The rate of glutamine degradation is much faster at pH 4 compared to pH 5.1.

The results for glutamine degradation were also supported by a previous study done in 1999 by Arii et al which found that at 70˚C and pH 3.93, the rate of glutamine degradation is much faster than that at pH 4.80 (Arii, *et al.*, 1999). It takes about 8 hours before the glutamine concentration reach half of its original value at pH 3.93 (Arii, *et al.*, 1999). On the other hand it takes about 17 hours before the glutamine concentration reach half of its original value at pH 4.80 (Arii, *et al.*, 1999). These results were in line with the results from this stability test. Extrapolating the data from Figure 30 suggests that in condition 1, it takes 27.5 hours before the glutamine reach half of its original value. On the other hand it takes about 55.9 hours before the glutamine concentration reach half of its original value in condition 2. The longer times required to reach half of the glutamine's original value is due to the lower temperature used for this test.

3.6 Origin of pyroglutamic acid

The question at this stage was: Does spontaneous conversion to pyroglutamic acid explain all of the glutamine losses?

To answer this question, HPLC tests were performed for the end samples (24 hours and 34 hours) of the stability tests in order to see if there is any pyroglutamic acid present. First, glutamine samples at time zero were tested to find the retention time for glutamine under HPLC conditions used (detection at 200nm).

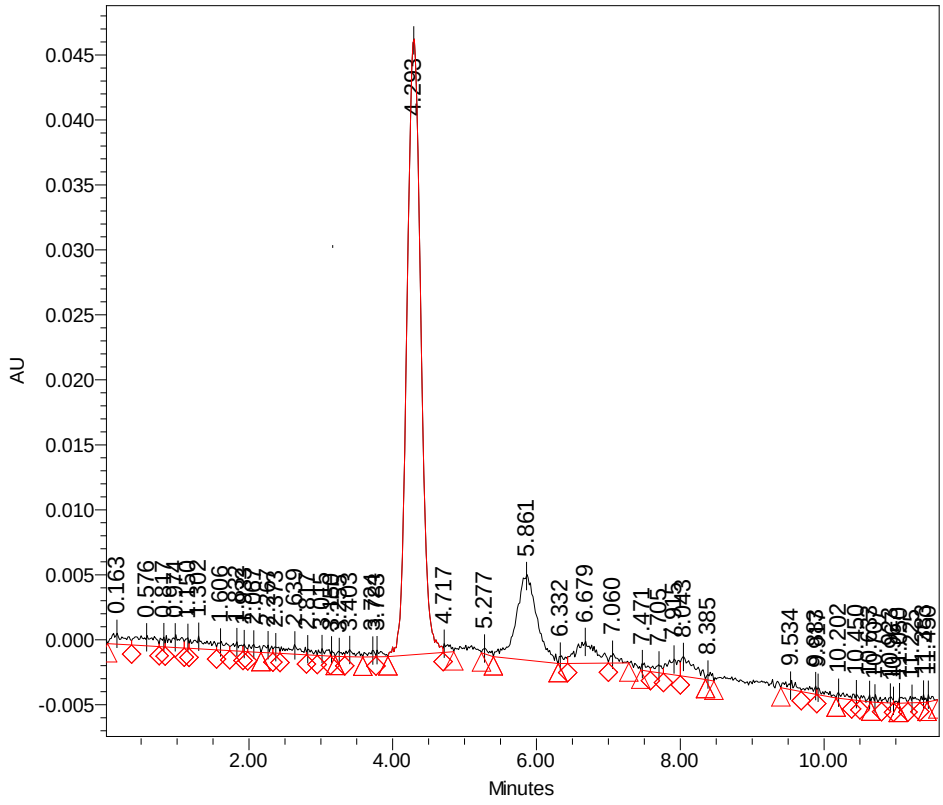


Figure 31: Chromatogram of the untreated glutamine sample, 0.64ml/min.

In Figure 31, there is a clear peak at 4.29 minutes which corresponds to glutamine, and analysis of additional samples showed a similar profile. The chromatogram however also showed a very small peak at 5.86 minutes which corresponds to pyroglutamic acid. In a perfect world, these samples of untreated glutamine should have no pyroglutamic acid peak at all, however all of the samples in this test were subjected to freezing before it was tested. The freezing and thawing effect may be responsible for the minute formation of pyroglutamic acid from glutamine, or it could be due to contamination in the glutamine substrate.

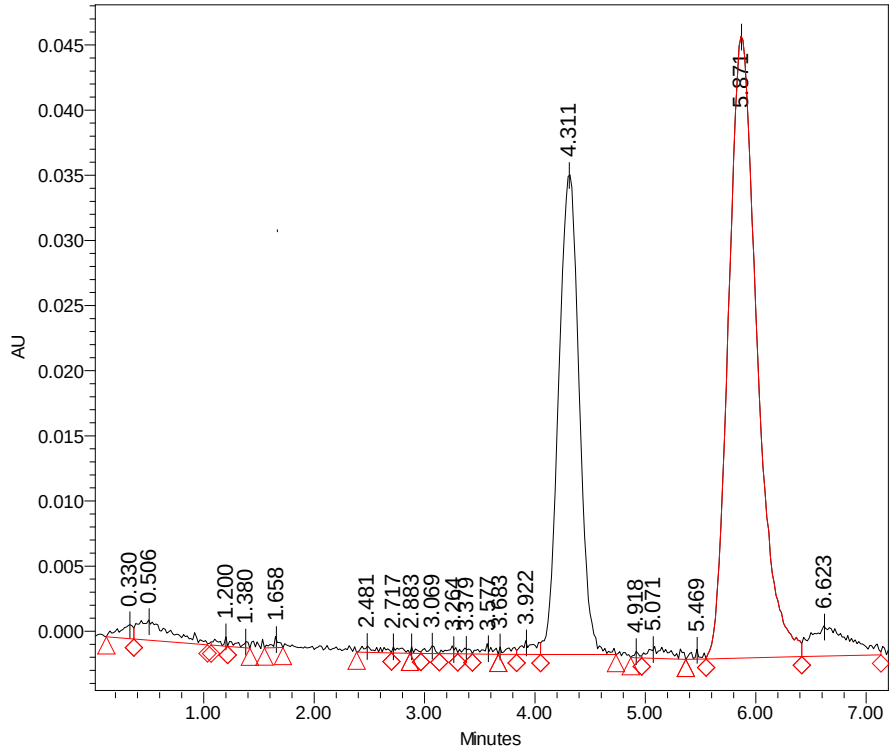


Figure 32: Chromatogram of the end product of 'condition 2', at 200nm, 0.64ml/min.

Figure 32 portrays a chromatogram of the end product of the glutamine sample at pH 5.1 (condition 2). The chromatogram showed two distinct peaks at 4.31 minutes that corresponds to glutamine at 200nm and one at 5.87 minutes. The pyroglutamic acid peak appeared uninterrupted by the glutamine peak. The results from both conditions were then collected and presented as below.

Table 15: Pyroglutamic acid results converted from peak height to concentration.

Samples	**Run 1**	**Run 2**	**Average peak height**	**Absorbance**	**Concentration (mM)**
Condition 1 end product	58859	59930	59394.5	0.059395	**0.13598**
Condition 2 end product	47830	49374	48602	0.048602	**0.09978**

Since the stoichiometry of glutamine to pyroglutamic acid conversion is 1 to 1, it is then possible to calculate the concentration of pyroglutamic acid formed from

the degradation of glutamine solely by subtracting the initial glutamine concentration by the final glutamine concentration at the end of the tests.

Under condition 1, the decrease in glutamine concentration is 0.136mM after 24 hours. This observation consistent with the detected pyroglutamic acid concentration of 0.136mM. In condition 2, the difference in glutamine concentration is 0.099mM after 34 hours. This conjecture was proven to be correct when the end sample of condition 1 was tested in the HPLC and revealing a pyroglutamic acid concentration of 0.100mM.

These results confirms that all of the glutamine loss from the hydrolysis conditions were spontaneously converted to pyroglutamic acid.

Chapter 4

Maximising glutamate release

4.1 Introduction

Quantitative analyses of glutamate, glutamine, and pyroglutamic acid of the HCP337 production process revealed that indeed there was sub-optimal glutamate levels in the end product relative to the theoretical glutamate concentration found in casein. Knowing that glutamate is stable under the conditions within the HCP337 production process, the next step was to find ways in increasing glutamate release from casein.

On the other hand, speculation of the presence of pyroglutamic acid was proven. Pyroglutamic acid was present in substantial amount in the end products, and further testing showed that glutamine is responsible for the formation of pyroglutamic acid during the production of HCP337 due to its instability. Knowing this, a logical step was to find a way to convert the glutamine released to glutamate before it converts into pyroglutamic acid spontaneously.

The names of the enzyme tested in this chapter (including enzymes used in the HCP337 production process) are withheld as they are proprietary information.

4.2 Effects of phosphoric acid and hydrochloric acid on enzyme activity

The current HCP337 production process uses phosphoric acid in the initial step of the process to decrease the pH. Does the use of phosphoric acid have any negative effects towards the enzyme used for proteolysis? To answer this, two of the three enzymes currently used in the HCP337 production process (Enzyme A and Enzyme B) were tested at pH 3-7 and 53˚C for 4 hours. The substrate used for this test is the HCP337 powder, which evidently still contains a lot of bound glutamate (see Chapter 3). No buffer was used in this test as the HCP337 powder has buffering capacity of its own. Acidification were done using phosphoric acid and hydrochloric acid for comparison. For phosphoric acid, samples were first acidified to pH 3.8, then titrated back to the desired pH using sodium hydroxide or more phosphoric acid in the case of pH 3. This is done to ensure that every samples have

phosphoric acid present despite the different pH. In contrast, samples that were acidified with hydrochloric acid did not undergo the initial acidification to pH 3.8; rather, hydrochloric acid was used to adjust the samples straight to the desired pH. The substrate concentration for this test was 10% of the total volume, and the enzyme concentrations was 0.1%. The results are presented in Figures 33 and 34.

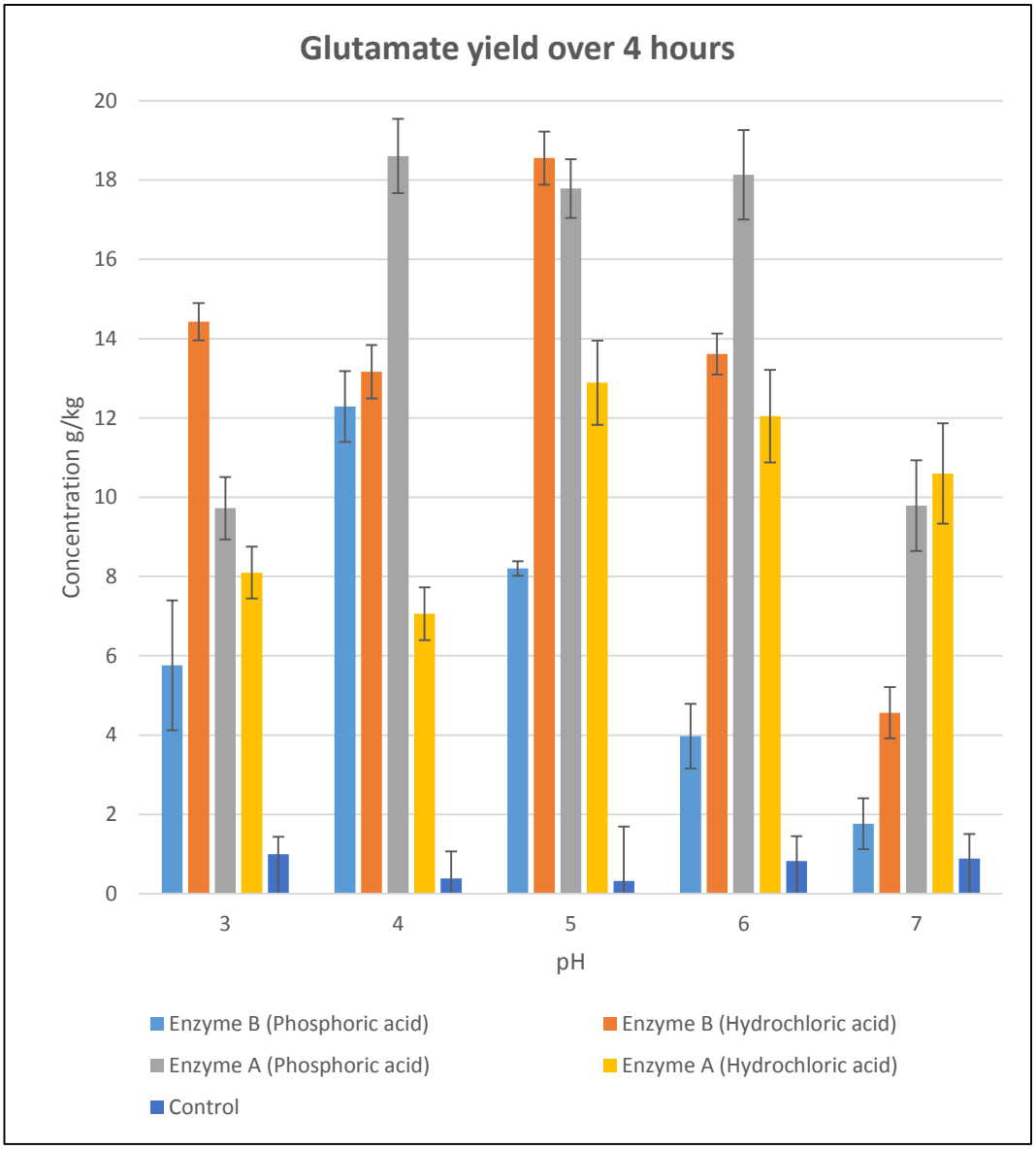


Figure 33: glutamate yield (loss) for both enzymes under the two different conditions.

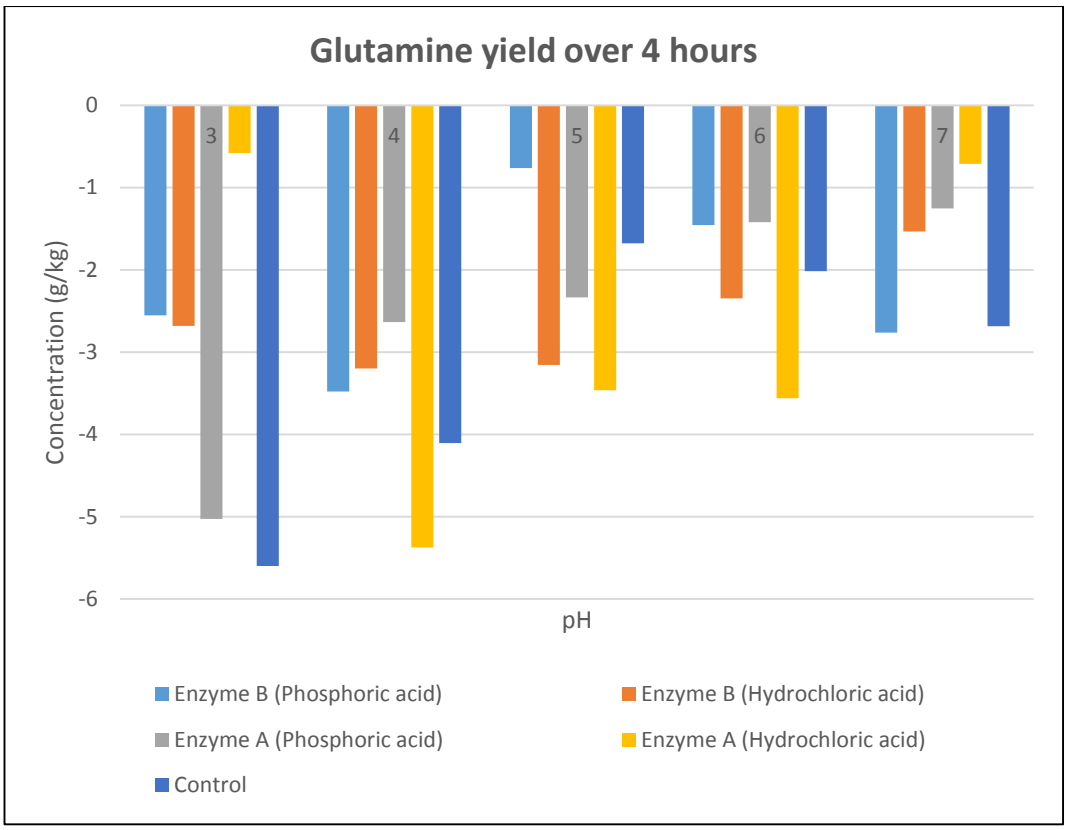


Figure 34: Glutamine loss for both enzymes under the two different conditions.

Table 16: Difference in proteolytic activity of both enzymes in or without the presence of phosphoric acid in terms of P-value.

Enzyme A	
pH	**p-value**
3	**0.002**
4	0.375
5	**0.001**
6	**0.003**
7	**0.002**
Enzyme B	
pH	**p-value**
3	0.098
4	**0.012**
5	**0.031**
6	**0.001**
7	0.582

4.2.1 Methodology

To support the results obtained in this test, paired t-tests were performed using Microsoft Excel 2013, and the P-values (Table 16) were compared. The P-values were interpreted in terms of a significance level (α) of 0.05.

The data presented in Figure 33 is the mean difference in glutamate yield after four hours of digesting HCP337 powder. To obtain the difference standard deviation (σ_f) between time zero and time four hours, this formula was used:

$$\sigma_f = \sqrt{\sigma_A^2 + \sigma_B^2 - 2cov_{AB}}$$

Where σ_A^2 is the square of the standard deviation at time 0, σ_B^2 is the square of the standard deviation at time 4, and cov is the covariance from time 0 and time 4.

4.2.2 Discussion

The null hypothesis is that phosphoric acid does not alter the ability of the enzymes to release glutamate from casein, and thus no difference in enzyme activity is seen. Overall, the experimental data reject the null hypothesis.

Enzyme B appears more efficient at releasing glutamate in the absence of phosphoric acid. The activity level at all pH values are better and in some cases significantly better in the absence of phosphoric acid (Figure 33). Furthermore, the pH optima of Enzyme B shifts from pH 4 in the presence of phosphoric acid, to pH 5 in the absence of phosphoric acid (Figure 33). On the other hand, Enzyme A activity was better in the presence of phosphoric acid, in most pH, except for pH 7 (Figure 33).

The control for this test used water instead of an enzyme and the results showed that there was very little spontaneous release of glutamate due to the processing conditions in the absence of any enzyme (Figure 33).

Lastly, glutamine in all of the samples for this test were also assayed and the results clearly shows that no further glutamine were gained. In fact, the amount of glutamine decreases from time zero to time four hours for every samples tested. The decrease in glutamine observed is most likely due to the instability of glutamine under the test condition.

Using hydrochloric acid instead of phosphoric acid may be the better option in terms of increasing glutamate yield for Enzyme B. However it was then pointed out that hydrochloric acid may have a negative impact over time on the apparatus used

to make the flavours at Tatua, which are mostly made out of stainless steel. In general, acids used on stainless steel should always have both reducing and oxidising properties in order to maintain the 'passive' corrosion resistant surface layer (British Stainless Steel Association, n.d.). Hydrochloric acid unfortunately lacks the ability to oxidise thus, prolong contact of stainless steel with hydrochloric acid can eventually cause pitting corrosion (British Stainless Steel Association, n.d.). Therefore, is the gain in glutamate yield by using hydrochloric acid worth the consequences of stainless steel pitting corrosion over time?

4.3 New enzyme peptidase activity test

Using the same set up and methodology from Section 4.2, proteolytic capacity of four new enzymes were tested. These enzymes are labelled as Enzyme C through Enzyme F. In this case, only phosphoric acid is used for acidification. The results were as follows:

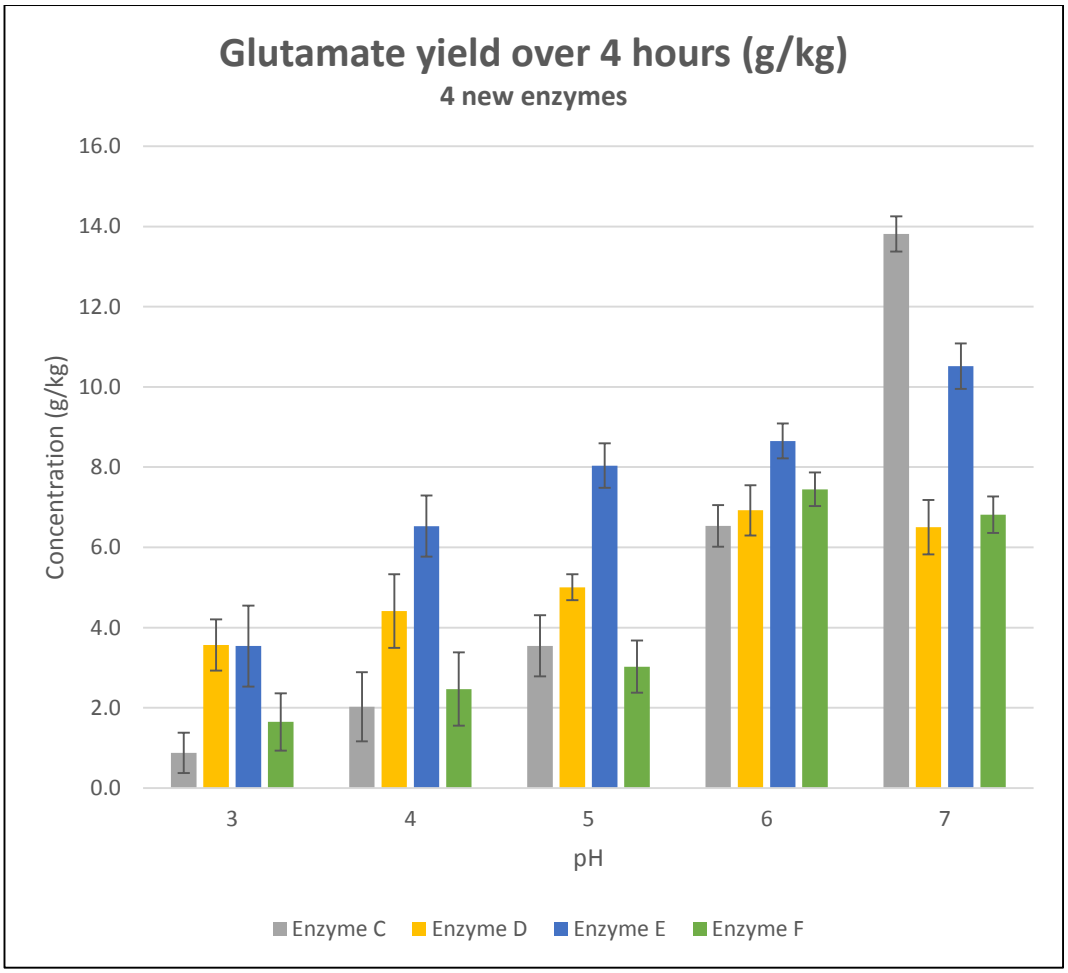


Figure 35: Proteolytic activity comparison between the new enzymes. Glutamate yield are measured in g/kg

4.3.1 Discussion

In general, all of the new enzymes tested show a tendency to be most efficient at releasing glutamate at neutral pH (Figure 35). This was expected as most of these enzymes are glutaminase-containing enzyme preparation that are most active at near neutral pH (Dura, *et al.*, 2002). Thus the efficacy of these enzymes at neutral pH is most likely due to the presence of glutaminase.

Enzyme C was seen to be the best enzyme in terms of glutamate yield after 4 hours at pH 7 (Figure 35). The second best enzyme was found to be Enzyme E, once again at pH 7 (Figure 35). Both Enzyme D and Enzyme F were very close together in terms of glutamate yield in the higher pH values. However, Enzyme D and Enzyme F was inferior when compared to the glutamate yield of Enzyme C and Enzyme E in the higher pH values (Figure 35). One major difference between these new enzymes and the current enzymes used in the making of HCP337 product is that these new enzymes have the tendency to be most active (operate optimally) in the neutral pH, as oppose to the more acidic pH of the enzymes currently used.

The standard deviations (error bars) for the mean concentration of glutamate from the 4 hour hydrolysis (Figure 35) were calculated using the formula stated in Section 4.2.1.

Glutamine was also assayed for each samples of this test, however the assays revealed very minimal glutamine presence, and the data were not included.

Since the conditions of this test is the same as the conditions done to test the two current enzymes (Section 4.2), the results from this test are thus comparable. Combining all of the results gives a better understanding and perspective on the efficacy of the new enzymes compared to the current enzymes.

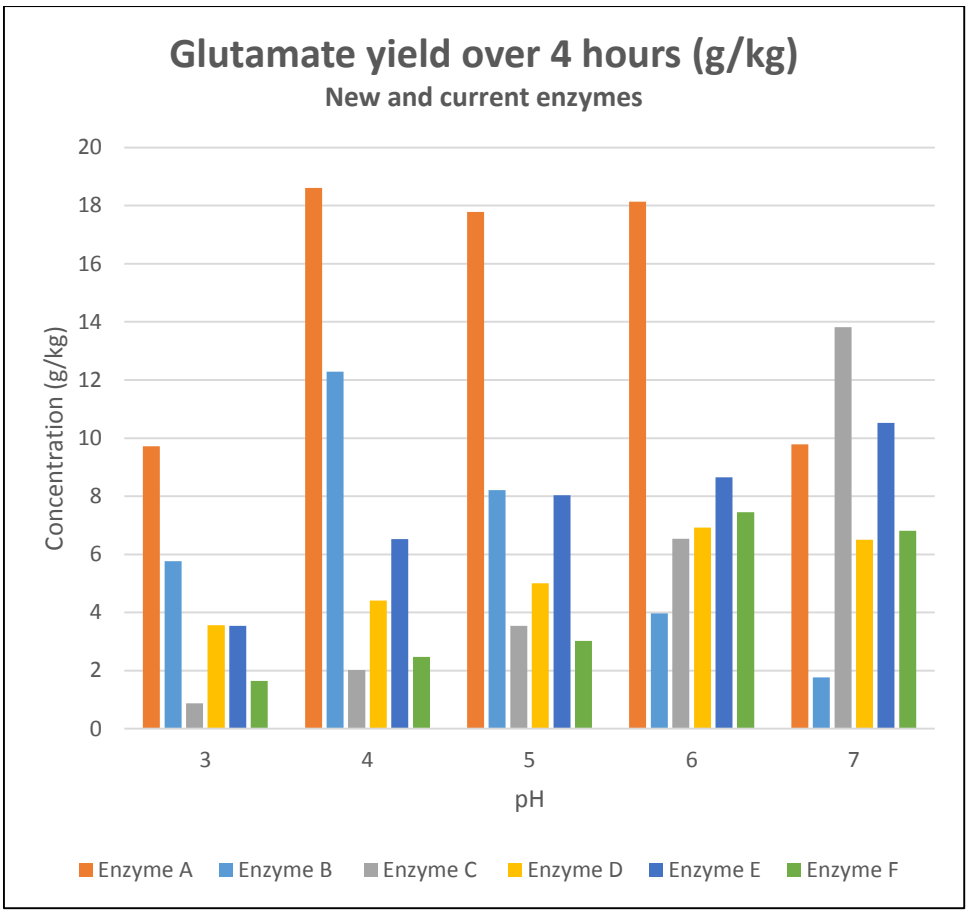


Figure 36: Proteolytic comparison between all of the enzymes (currently used [Enzyme A and B in phosphoric acid] and new), without error bars. Glutamate yield are measured in g/kg.

From the two tests, it is clear that Enzyme A was the most active at releasing glutamate over the 4 hour test period (Figure 36). Enzyme C was another enzyme with significant glutamate yield at pH 7 (Figure 36). Enzyme B has a similar maximum glutamate yield with Enzyme E (Figure 36), the only difference is the pH optima.

Lastly, the proportion of glutamate yield by the 4 hour tests (specifically by Enzyme A) was found to be a lot when compared to the average glutamate yield from the entire HCP337 production process which in total takes 58 hours of digestion time. What is the reason behind this anomaly? One speculation that can be made is that perhaps the enzyme are being denatured over the long period of HCP337 processing. Looking at the enzyme manufacture's guideline revealed that 80% of Enzyme A inactivation occurs at 50˚C for an unspecified time interval. On top of this, it is also important to note that the tests presented in this chapter used

HCP337 powder as the substrate, whereas the 58 hours of total digestion time from the actual HCP337 production process started from casein.

4.4 Glutaminase screening

At the time of this experiment, the use of pure glutaminase in the production of flavours at Tatua was not permitted by the Ministry for Primary Industries. The use of glutaminase can be of great value as it would help convert the unstable glutamine into glutamate before it is converted into pyroglutamic acid spontaneously. Even though pure glutaminase was not allowed, there are several enzyme preparations that are permitted and contain glutaminase as contamination or as a deliberate component of the enzyme preparation. Four enzymes (known to have glutaminase side activity) were then chosen and tested for their glutaminase activity along with the two current enzymes.

New enzymes (not currently used in the HCP337 production process) that may contain glutaminase activity:

- Enzyme C
- Enzyme D
- Enzyme E
- Enzyme F

Current enzymes:

- Enzyme A
- Enzyme B

Positive control

- Known glutaminase

Negative control

- Water

The enzymes were tested at five different pH values ranging from pH 3 to 7. The substrate for this test was glutamine and the buffer used were sodium citrate buffer for pH 3 to 6, and phosphate buffer for pH 6 to 7 (Table 17). Finding a single buffer that could span the pH range for this test would be perfect, unfortunately there was no buffer that could span from pH 3 to 7. The solution was to use two buffers and overlap at pH 6 to see if there is any differences between the buffer systems.

Table 17: Outline of the buffers used for each pH values.

pH	3	4	5	6	7
Buffer	Sodium Citrate	Sodium Citrate	Sodium Citrate	Sodium Citrate **&** Phosphate	Phosphate

The test was done at 53˚C for 3 hours. Samples were collected at three time intervals (30 minutes, 90 minutes and 180 minutes) in case glutamine is fully converted into glutamate before 180 minutes. Glutamine concentration was 6mM, and the enzyme concentration was 0.05% in a reaction volume of 2mL. The samples were then assayed for glutamate and the results were as follows:

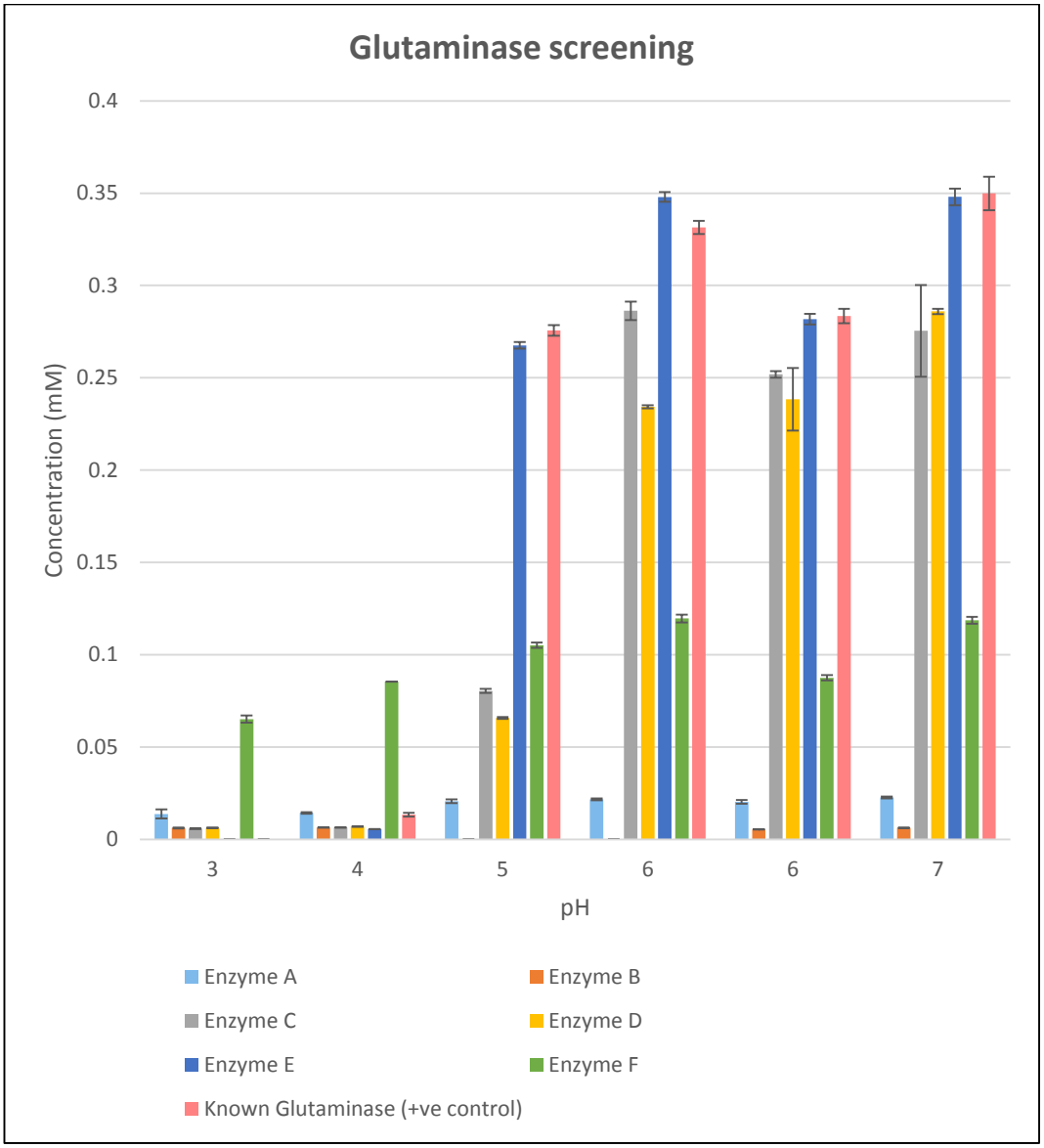


Figure 37: Glutaminase screening comparison with a positive control.

4.4.1 Discussion

Firstly, the negative control of this test showed that there were no spontaneous conversion of glutamine into glutamate in the absence of an enzyme (Figure 37), and the positive control was found to be active at pH 5 and above (Figure 37).

Enzyme A and Enzyme B both have very little or no glutaminase activity (Figure 37).

In contrast, the four new enzymes have relatively high glutaminase activity with the exception of Enzyme F. The activity of glutaminase from Enzyme C, Enzyme D and Enzyme E were very similar in terms of the amount of glutamine converted into glutamate (Figure 37). The glutaminase activity in these enzyme preparation showed a pH optima of near neutral to neutral (Figure 37). The activity of glutaminase in Enzyme F was still relatively high (Figure 37) when compared to the two current enzymes, however relatively low when compared to the other three new enzymes. Although not very defined, glutaminase in Enzyme F also show a tendency to be most active at neutral pH.

In terms of the different buffers used, the null hypothesis was that the different buffers will not cause different proteolytic activity. To test the effects of different buffers on the enzyme proteolytic activity, paired t-tests were done (Section 4.2.1) and the P-values are presented below.

Table 18: Difference in proteolytic activity of all enzymes between the two different buffers used at pH 6 in terms of P-value.

Enzyme	P-value
Enzyme A	0.175
Enzyme B	**0.002**
Enzyme C	**0.0116**
Enzyme D	0.766
Enzyme E	**0.002**
Enzyme F	**0.003**
Known glutaminase (+ve control)	**0.006**

Most of the enzymes (Enzyme C, Enzyme E, Enzyme F, Enzyme B, and the positive control) was less efficient at converting glutamine to glutamate in the presence of phosphate. This is evident from a minute decrease in glutamate concentration in phosphate buffer compared to sodium citrate buffer at pH 6 for these enzymes (P-values <0.05, Table 18). These observations were further supported by the P-values which were below 0.05. In contrast, the enzymes

(Enzyme D and Enzyme A) had very little difference in terms of proteolytic activity between the two buffer systems. This claim was supported by P-values which were above 0.05. As an overall, the experimental data reject the null hypothesis.

Chapter 5

Conclusion

The main objective of this research was to comprehensively examine the biochemistry of glutamate release from casein, finding the optimum proteolysis condition and using this knowledge to optimise the current process, increasing glutamate release.

In order to understand the biochemistry of glutamate release from casein, three techniques were first developed for this research. The quantification of glutamate, glutamine, and pyroglutamic acid were developed and were used extensively throughout this project to view and understand why so little glutamate is released from casein even with the extensive digestion time of the HCP337 production process.

Applying these techniques and testing the samples from the HCP337 production process revealed that there was very little glutamate yield in the HCP337 product relative to the theoretical concentration of glutamate in casein even after 83 hours of production time and two digestion processes. On top of this, the amount of glutamate wasted as the clarified solids was high relative to the amount of glutamate present in the HCP337 product. Additionally, the concentration of pyroglutamic acid in the HCP337 product was found to be very high. Stability tests on both glutamate and glutamine under production conditions revealed that glutamate is stable and did not show any sign of reduction in the processing conditions. In contrast, glutamine showed a linear decline over time when exposed to the production conditions. Glutamine was found to be more unstable when exposed to lower pH. Furthermore, testing the end products of the stability test proved that all of the glutamine lost was converted into pyroglutamic acid.

The next step in the project was to find a way to increase glutamate release and decrease pyroglutamic acid formation. Initially, it was speculated that the use of phosphoric acid in the production of HCP337 was causing a negative impact on the enzyme's ability to liberate glutamate. A test was done to compare the effects of two acidifying agents (phosphoric acid and hydrochloric acid) on the enzymes currently used in the HCP337 production process. The results showed that there

was a slight increase in glutamate yield for one of the two enzymes only. Moreover, the use of hydrochloric acid can have negative consequences over time to the stainless steel equipment.

Due to the prohibition of pure glutaminase use in the production of flavours at Tatua during the time of this research, a search into finding new enzyme preparations containing glutaminase activity was conducted. The presence of glutaminase can aid in the increase of glutamate yield in the HCP337 production process.

After testing several new enzyme preparations with possible glutaminase activity, one enzyme was found with a promising outcome (Enzyme C). However, this enzyme was still inferior in terms of its ability to yield glutamate when compared to one of the current enzyme used in the HCP337 production process (Enzyme A). Furthermore, it was found that all of the enzyme preparations with glutaminase activity showed a neutral pH optima. This was expected as the enzyme glutaminase is known to have a pH optima of near neutral.

These results causes a slight dilemma as the enzymes currently utilised in the HCP337 production process has lower pH optima. This means, even if glutaminase is allowed to be used, it cannot be combined with the current process as it will not be very active at the lower pH. In the likely event of glutaminase being approved to be used, more enzymes with near neutral pH optima (like the ones tested in Section 4.3) should be screened. Proteases and peptidases most active in neutral pH can be combined with glutaminase, thus further increasing the glutamate yield.

In summary, this project demonstrated that the current HCP337 production process does not optimally release glutamate from casein. Changing pH during hydrolysis and using alternative enzymes did not appear to effective strategies for increasing glutamate release, but the combination of test performed during this project is far from exhaustive. The use of glutaminase during the production process to convert glutamine into glutamate before it spontaneously converts into pyroglutamic acid is potentially an effective strategy for increasing glutamate yield, but the current production process will need to be modified to accommodate glutaminase that perform only at neutral pH.

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