Development of the delivery system of an amino acids based protein supplement for the dietary management of Phenylketonuria (PKU)

By

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Abstract

Phenylketonuria (PKU) is an inherited metabolic disorder in which the patient cannot metabolise phenylalanine into tyrosine and other metabolites which leads to irreversible brain damage and neurological disorders if left untreated. The majority of the patients are treated using dietary therapy where the patient receives an amino acid based protein supplement. However these supplements exhibit poor organoleptic properties due to the bitter and sulphurous nature of certain amino acids, which can lead to poor patient compliance.

In order to reduce the negative organoleptic attributes masking technologies have been previously applied but have only had limited success. Therefore the use of alginate beads was investigated to encapsulate the amino acids. Although the use of alginate beads for encapsulation is well known and are used for pharmaceutical materials, the techniques had not been applied to amino acids. Therefore there was no prior knowledge of how to construct the beads, and the effect of encapsulation on the organoleptic properties and amino acid bioavailability. Experiments were undertaken to firstly develop and refine the method of manufacture utilising laser particle defraction techniques to determine bead size and nitrogen content quantification by Dumas to determine amino acid recovery. Initially the beads manufactured where too large, in excess of 2mm, however by reducing the aperture of the nozzle used to produce the droplet and milling the amino acids to a finer particle size it was possible to produce beads between 1 and 2mm, which was the optimal size for the beads to avoid gastric straining and aid the transit of the beads through the stomach.

A design of experiment methodology was then implemented to determine the relationship between the factors; calcium content, alginate content and the time in the gelling solution, and the amino acid recovery (output). The amino acid content remained constant at 10% (w/w) throughout the experiments. It was found that the alginate and time in the gelling solution had a significant effect on the amino acid recovery when the calcium chloride concentration was between 10 and 30% (w/w). The best recovery, of 78%, was produced with a bead containing 10% (w/w) amino acid and 3% (w/w) alginate using a 30% (w/w) calcium chloride gelling solution and a 1 minute residence time.

The beads were subjected to an *in vitro* digestion model and it was found that the amino acids encapsulated in the beads were still bioavailable as there was a reduction in amino acid content of the beads from 10g/100g of bead to 2.95 g/100g of bead after *in vitro* digestion.

To determine if the encapsulation had had an effect on the sensory attributes a triangle test with a maltodextrin containing placebo was carried out. In the test 12 expert panellists trained in tasting amino acid products tasted the beads and only 6 panellists identified the different sample. This was less than the 8 correct identifications needed for significance at 95% and therefore the beads could not be identified from the placebo. Therefore the method applied successfully produced an amino acid alginate bead that masked the flavour but did not compromise the bioavailability.

1. Literature Review

1.1 Introduction

1.1.1 Disorders of Amino Acid Metabolism

Protein is a major component of living cells and comprises 17% of the weight of an adult (Emery, 2015) . On consumption proteins are digested in the stomach and small intestine using enzymes that hydrolyse the proteins to poly peptides which are further digested into amino acids which enter the circulatory system. Once in the body the amino acids have three outcomes; they can be oxidised utilising transamination and excreted as urea, combined with other amino acids to produce proteins or be metabolised to produce non protein molecules (Emery, 2015).

However in some individuals with certain inherited metabolic disorders the enzymes or proteins required for the metabolism to non-protein molecules is defective which can lead to the formation of alternative or intermediate products that can have acute or toxic effects. Examples of such disorders are maple syrup urine disorder which affects the metabolism of branched chain amino acids, homocystinuria affecting methionine metabolism, tyrosinaemias affecting tyrosine metabolism and phenylketonuria affecting phenylalanine metabolism (Shaw, 2015).

1.1.2 Phenylketonuria

Phenylketonuria is present in around 1:10,000 live births in Europe (Blau, 2010) and is an is an autosomal recessive inborn error caused by certain mutated genes . As it is an autosomal recessive inherited condition the faulty genes are passed down from the parents to child, which results in either the child being a gene carrier or developing the condition. Phenylalanine is an essential amino acid and has two functions within the body; firstly the synthesis of tissue protein and secondly being hydroxylated to form tyrosine (Shaw, 2015).

In the normal metabolic pathway phenylalanine is metabolised in the liver utilising the phenylalanine hydroxylase (PAH) system which converts phenylalanine to tyrosine. The PAH system also requires the use of the tetrahydrobiopterin enzyme (BH_4) which during the hydrolysis reaction is converted to dihydrobiopterin (BH_2), but is regenerated by dihydrobiopterin reductase (qBH_2) (figure 1.0).

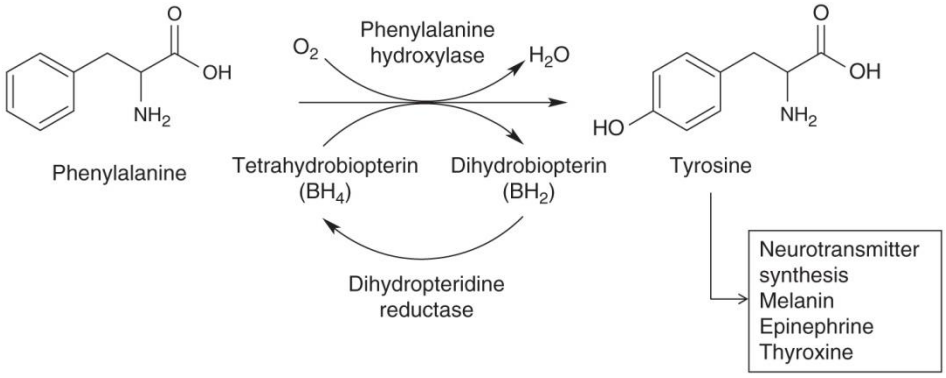


Figure 1.0 Hydroxylation of Phenylalanine (Shaw, 2015)

In a patient with phenylketonuria there is a reduction in activity or defect in either the PAH system or the BH_4 recycling system (Walter, 2012). This leads to the inhibition of hydroxylation of phenylalanine leading to an increase in the levels of phenylalanine in the blood from a normal level of around 2 to 10 mg/l to greater than 20mg/l (Acosta, 2010). This abnormal level of phenylalanine crosses the blood brain barrier and enters the brain acting like a neurotoxin, the mechanism of which is not fully understood (Imperlini, 2014).

The reduction in these systems also leads to a reduction in tyrosine which is not being produced from phenylalanine. If the phenylketonuria is caused by a defect in the BH_4 recycling system then the levels of BH_4 will be reduced. This leads to a reduction in the rate of activity of tyrosine hydroxylase and tryptophan hydroxylase as BH_4 is co-factor in these reactions. The effect of this is to reduce the synthesis of biogenic amines normally produced by those reactions such as dopamine, serotine and norepinephrine which are important for normal brain function (Walter, 2012). In addition high phenylalanine levels in the blood affects the transport of other large neutral amino acids to the brain, which further reduces the levels of tyrosine and serotonin (Walter, 2012).

These abnormal biochemical changes lead to brain dysfunctions although the actual mechanisms are not understood (Imperlini, 2014). These dysfunctions are presented by mental symptoms, with significant delays in developmental milestones, hyperactivity with autistic features and seizures. In addition there are also physical symptoms with eczema, a musty skin colour and light pigmentation being typical. If untreated the damage to the brain is irreversible with lifelong disability to the patient (Christ, 2010) with untreated patients not possessing the abilities for self-care or independent living (Acosta, 2010).

However the brain damage and poor outcomes can be avoided with early detection at birth and the use of measures to control the blood phenylalanine level. In the developed world this is achieved by new born screening via a heel prick blood test. In the UK blood is taken from the heel of a baby within 5 to 8 days of birth and tested for phenylalanine and tyrosine using tandem mass spectrometry (Shaw, 2015). If there is a level of 240µmol/l of phenylalanine or higher, and, or a phenylalanine to tyrosine ratio greater than 3 then child may be suffering from PKU, and further tests would be undertaken in order to diagnose the child (Cleary, 2010).

There are several different treatment options that can be applied to reduce and control the blood phenylalanine level and include gene therapy, enzyme replacement therapy, amino acid transport competition at the blood brain barrier and dietary management (figure 1.1).

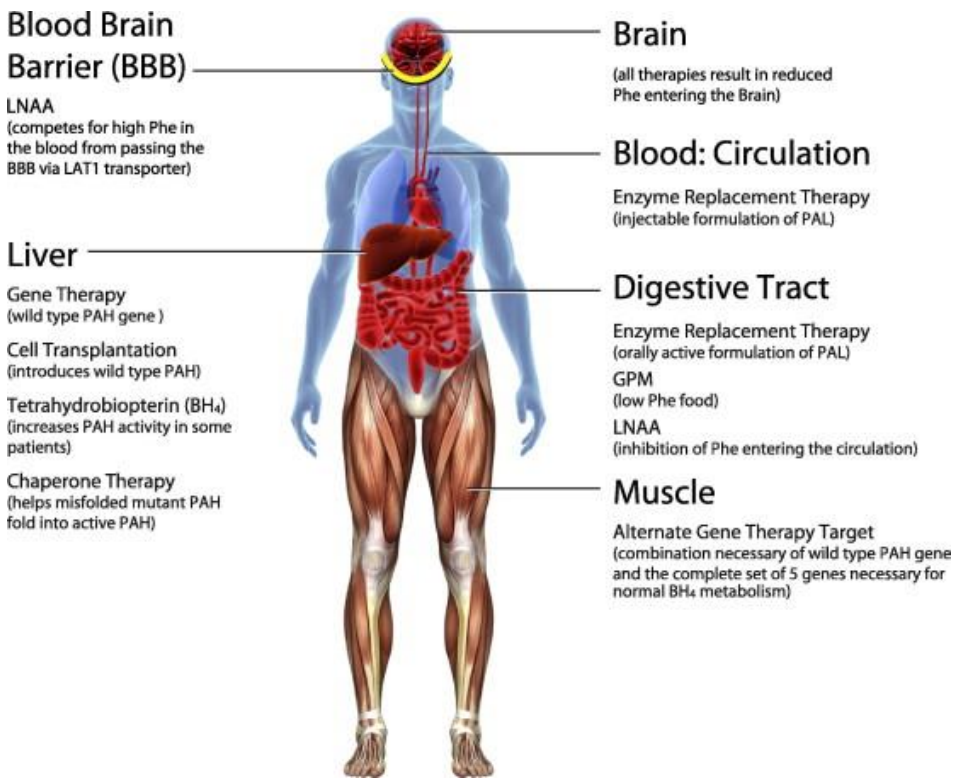


Figure 1.1 Different treatment options available for PKU at different body locations (Sprosen, 2010)

However some of these treatment options are not currently available to the patient, with gene and cell therapy being only at a pre-clinical stage and enzyme therapy currently only used in human trials. The current mainstay treatment throughout the world is by dietary management utilising a phenylalanine restricted diet (Sprosen, 2010).

In this diet the amount of natural protein from food sources is controlled and is mainly a very low protein diet containing mostly fruits and vegetables, which are low in. Produce that is high in protein thus high in phenylalanine, such as meat, fish and dairy products is controlled or excluded from the diet depending upon the severity of the PKU. As phenylalanine is an essential amino acid a small amount is required to be present in the diet so a small amount of protein is permitted. Foods naturally low in phenylalanine containing less than 20 – 25 mg phenylalanine per 100g, are freely permitted for consumption (Ahring, 2009) and are generally fruits, vegetables, certain cereals, fats and oils.

However it is not possible to thrive on a low protein diet alone, as protein is required to remain healthy and the diet avoids certain food groups it is deficient in certain micronutrients. Therefore the patients consume a protein replacement supplement daily, containing a source of L amino acids and micronutrients that mimics the amino acid composition of a normal diet containing meat, fish

and dairy products, but is free from phenylalanine (table 1.0). The amount of supplement consumed depends upon the protein requirement of the patient and is related to their age, weight and activity levels.

Table 1.0 Typical amino acid composition of a PKU protein supplement

Amino acid	g per 100g supplement
L-Alanine	0.93
L-Arginine	1.6
L-Aspartic Acid	1.4
L-Cystine	0.41
Glycine	1.5
L-Histidine	0.63
L-Isoleucine	0.99
L-Leucine	1.7
L-Lysine	1.3
L-Methionine	0.27
L-Phenylalanine	nil
L-Proline	1.6
L-Serine	0.87
L-Threonine	0.83
L-Tryptophan	0.33
L-Tyrosine	1.5
L-Valine	1.1
L-Carnitine	0.01
Taurine	0.08

(SHS 2010)

If this diet is followed the long term outcome of the patient is good with most patients health and wellbeing markers comparable to the healthy population (Shaw, 2015). As the patients get older the need for the supplementation reduces as the brain is less plastic, and the patients generally show less motivation to maintain the diet as the effects are less immediate. However it is important to remain on diet as elevated phenylalanine levels can lead to neurological effects such as an increase in irritability, poor concentration, headaches and a reduction in quality of life scores. The PKU patient is generally unaware of these symptoms and they are usually reported by family members, but if the patient returns on diet these symptoms alleviate and the quality of life score increases (Macleod, 2010).

1.1.3 Compliance

Compliance is an indicator of a patient's behaviour, a fully compliant patient would be consuming their protein supplement, following the diet and attending clinic as per the prescriber's recommendations (MacDonald, 2010). In PKU it is well known that significant non-compliance exists with only 38% of young people consuming all the required amount of protein supplement, and between 57% to 75% consuming less than prescribed, which can lead to a failure to thrive and an increase in serum phenylalanine levels due to patients being off a phenylalanine-restricted and low protein diet (Macdonald, 2000). Compliance can be influenced by many factors from cultural and environmental factors such as the stigma of PKU and the availability of health care, family support

and convenience of the protein supplement. Emotional and physiological factors can also effect compliance and it is well known that the unsatisfactory organoleptic properties of PKU supplements can be a barrier to the patients consuming as prescribed (Sarkissian, 1999) with both powdered and 'ready to drink' products being poor in terms of flavour and palatability (Gokmen-Ozel, 2009; Prince, 1997). These poor organoleptic properties are an expression of flavour of the individual amino acids which can exhibit bitter and or sulphurous flavours especially the essential amino acids (highlighted in table 1.1 below) which are required to be within the supplement.

Table 1.1 – Amino acids taste properties

Amino Acid	Taste - powder form	Texture - powder form	Taste - 5% water dilution
Alanine	Sweet	Fine/gritty	Sweet
Arginine	Bitter	Gritty/Dissolving	Bitter
Aspartic Acid	Sour	Gritty	Sour
Cystine	Neutral	Gritty crystals	Neutral
Glutamic Acid	Sour	Powdery	Sour
Glycine	Sweet	Chalky,gritty,melts in mouth	Sweet
Histidine	Bitter	Gritty	Bitter
Iso leucine	Bitter, Beefy	Flakes,crystalline	Bitter
Leucine	Bitter	Fine powder	Bitter
Lysine Glutamate	Salty, cabbage, a bit of sweet	Smooth, melts in mouth	Umami, cabbage
Lysine Hydrochloride	Salty, Bitter, Sour	bubbly, gritty	Bitter, sour, salty
Methionine	Beefy/cabbage, fishy	Fine	Beef/cabbage, burnt rubber
Proline	Sweet, bitter	Gritty, dissolves in mouth	Sweet, bitter
Serine	Sweet	Gritty, dissolves in mouth	Sweet, salty, umami
Threonine	Neutral, sweet	Gritty	Sweet, Neutral
Tryptophan	Bitter, sour, umami	Fine	Bitter, umami
Tyrosine	Neutral	Smooth, melts in mouth	Neutral. Umami
Valine	Bitter, sweet, salty	Flaky, crystalline	Bitter, sweet
Asparagine Monohydrate	Neutral	Gritty	Neutral, umami
Glutamine	Neutral, sweet	Gritty	Neutral, sweet,
Lysine Acetate	Sour, umami	Gritty	Sour, sweet, umami
Carnitine	Sweet, sour	Hot in the mouth and dissolves	Neutral. Bitter
Taurine	Neutral	Gritty	Neutral, Sour
Lysine Aspartate	Sour, umami	Gritty	Umami, sour
Citrulline	Sweet, Neutral	Hot in the mouth and dissolves	Sweet, slightly bitter
Magnesium Aspartate	Bitter, umami,		Bitter, umami, cabbage
Acetyl Methionine	Sour, bitter		Sour

(Anders 2012)

Therefore if a supplement could be produced that was convenient, with improved organoleptic characteristics from masking the negative attributes of amino acids, then this would aid compliance.

1.2 Masking technologies

Taste masking has been applied in both pharmaceutical and food industries for some years with differing techniques being applied to reduce negative flavour attributes. Simple flavour masking techniques have been used for many years for PKU supplements, such as the addition of sucrose and sweeteners to mask unpleasant bitter notes, however these techniques are not very successful and therefore new approaches are necessary (Sohi, 2005)

1.3 Double Emulsions

One approach is the use of double emulsions. Double emulsions have been used for encapsulation of active or sensitive components in the pharmaceutical and cosmetics industries for several years and now this technology is being applied to the food industry for encapsulation. (Dickinson, 2011)

In a double emulsion, an emulsion is formed which is then used as the dispersed phase for another emulsion thus producing an emulsion within an emulsion. They are manufactured using a 2 stage process, as seen in figure 1.2, in which an initial emulsion is formed from oil and water phases in combination with an oil soluble emulsifier and homogenisation. This water in oil emulsion is then combined with a water phase with a water soluble emulsifier to produce a water in oil in water (WOW) emulsion using homogenisation.

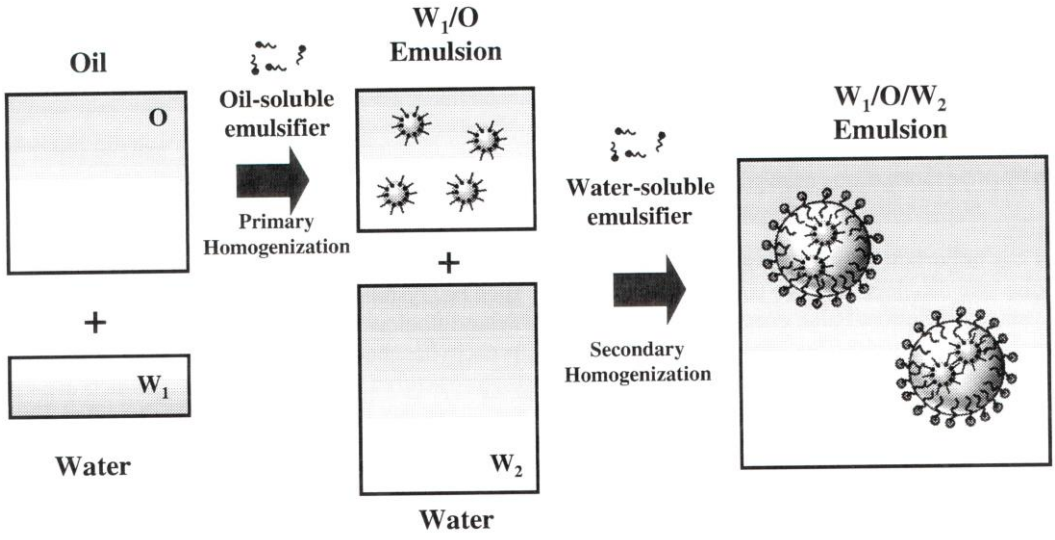


Figure 1.2 Double emulsion manufacturing process (McClements, 2012)

The component that is to be encapsulated is usually dispersed in water phase of the first emulsion, and as such is protected from interacting with the oral surfaces of the mouth (Dickinson, 2011) and thus any negative attributes associated with the component are masked.

However double emulsions can prove difficult and expensive to manufacture when compared to a simple emulsion, as two emulsions have to be produced involving stabilising multiple interfaces with differing emulsifiers, compared to a simple emulsion with one interface and one emulsifier (Dickinson, 2011). In addition double emulsions are also prone to instability and are easily destabilised by environmental and mechanical factors such as freezing, dehydration, thermal processing, pumping and mixing (McClements, 2012).

For an amino acid blend to be used in a PKU product the challenges are greater. Firstly, a large proportion of the pharmaceutical and cosmetic double emulsions use synthetic surfactants and polymers that are not permitted for use in a food product (Frash-Melnik, 2010). Double emulsions have been produced with food grade emulsifiers but the choices are limited with polyglycerol polyricinoleates (PGPR) being generally used for the initial water in oil phase and functional proteins used for the oil in water phase.

The use of PGPR in a PKU product would be problematic as the provision under the 1995 Miscellaneous Food Additives Regulations (1995:17) limits its use to low and very low fat spreads and dressings, and chocolate and chocolate based confectionary Therefore its use in a food for special medical purposes would not be permitted.

There have, however, been double emulsions produced without PGPR by utilising small fat crystals to stabilise the initial water phase via the Pickering mechanism. In this process the oil blend used contains a saturated monoglyceride and tripalmitin which produces small crystals in the blend. These small crystals then form a protective shell around the water droplets and therefore stabilise the initial water in oil emulsion which is then used to produce a double emulsion (Frash-Melnik, 2010).

But if this technique were applied there would still remain issues as the emulsifiers used for the final emulsion are functional proteins (Dickinson, 2011) which are not compatible with a low protein PKU diet and as such replacements would need to be found.

If the issue with the emulsifier were solved further problems would need to be overcome, as the materials that are generally encapsulated are solubilised in the first water phase. The amino acid blend used contains both water soluble and insoluble amino acids and therefore electrolytes, sugars or polysaccharides would have to be added to the final water phase in order to maintain an osmotic balance as any osmotic pressure can lead to a shrinkage or swelling of the inner emulsion droplets emulsion droplets which can lead to rupture (Dickinson, 2011). Also the insoluble amino acid crystals can physically rupture the droplets (Frash-Melnik, 2010). Once ruptured the negative tasting amino acids would be present in the final water phase and no longer be encapsulated and would be available to interact with the oral surfaces of the mouth .

1.4 Viscosity

Another approach for masking negative flavour attributes is to increase the viscosity of the product using a rheological modifier. This increase in viscosity acts to reduce the rate of diffusion of the negative attributes from the bulk into the saliva and onto the taste buds and therefore reduces their perception (Sohi, 2004).

This approached has already been applied to a PKU product where pectin was added to produce a selection of gels of increasing viscosity containing amino acids. These gels were then tested using a trained sensory analysis panel which produced favourable results with a reduction of negative taste attributes with increasing pectin addition, with a 20mm reduction on a 100mm intensity scale each 2% increase in pectin (Anders, 2011), figure 1.3.

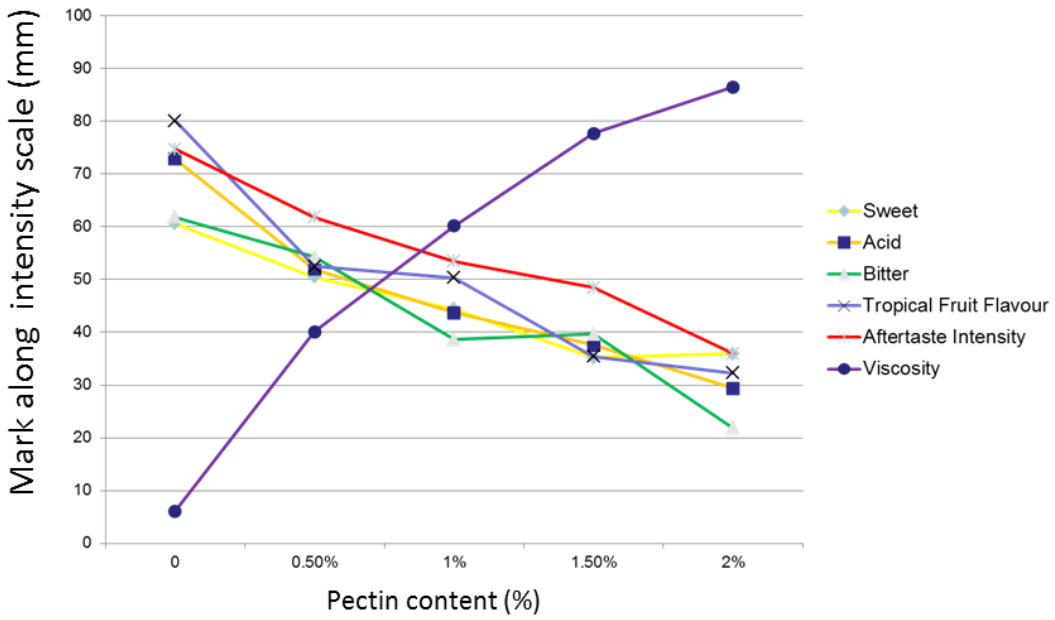


Figure 1.3 Flavour Attribute intensities with increasing viscosity (Anders, 2011).

This was then transferred to produce a commercial product, thickened by pectin, and the opinions of 60 consumers were sought, and although some participants liked the concept with 28% of participants perceiving an improved flavour over a liquid supplement, 53% did not perceive any flavour improvement (Rason, 2011) . In addition some participants did not like the gelatinous texture and there was concern about a reduction in convenience as the product required a spoon to eat. Therefore the technique was concluded to be unsuccessful.

1.5 Beads

Another technology to consider is the encapsulation of the negative flavour components in a hydrogel bead. This technology has successfully masked unpleasant bitter tastes, such as polyphenolic compounds in the pharmaceutical industry (Stojanovic, 2012) and has been used to mask the bitter taste of polypeptides and protein hydrolysates (Mohan, 2015) which are similar to amino acids. There is a wide range of materials that may be encapsulated in addition to water soluble materials, with hydrophilic and solid materials such as particles, yeast and bacterial cells being encapsulated into the gel matrix (Dordevic, 2015). This ability is important as the amino acid

blends required contain both water soluble and insoluble amino acids so the ability to encapsulate solid particles is desirable.

Once produced the beads would be consumed within a suitable carrier or food matrix. Alginate beads containing colours and flavours have been successfully added to beverages to provide a novel drinking experience. However the integrity of the bead and shelf life of the product can be influenced by the matrix and the composition of the bead (Genialab,2005).

In hydrogel manufacture the material to be encapsulated is mixed with the gelling material and extruded through an orifice to form a gel droplet. Once formed the droplet is immediately solidified to form a capsule by a physical (cooling, heating) or chemical process, thus encapsulating the material. (Dordevic, 2015). It also possible to produce encapsulates by firstly producing an 'empty' hydrogel which is then placed in a solution of the material, and the material allowed to diffuse into the beads. However this technique is limited to water soluble materials.

Several gelling materials may be used to construct hydrogels, however some pharmaceutical materials are not permitted for food use. In food applications hydrogels are generally produced from polysaccharides and proteins as they exhibit good technical properties (gelation) and are legally permitted in several applications (Dordevic, 2015). As the product is intended for PKU patients the use of proteins would not be permitted so a hydrogel would therefore have to be constructed using polysaccharides.

It is possible to produce gels using xanthan and guar gums as the mixture of gums has a synergistic effect giving enhanced gelling properties (Draget, 2010) . However they gel on the addition of water which would make them unsuitable for producing a hydrogel as it would be difficult to form a bead.

Pectin can also produce gels and comes in two main types high and low methylated ester pectins. High methylated ester pectins will only form gels in the presence of high levels of sugar, around 65% and a suitably low pH, around 3.4 to 3.2, and the gel is formed by cooling the solution down (Draget, 2010). Low methylated ester pectin relies less on the pH and sugar content and more on the interaction between the pectin and calcium ions to form gels, but there is a minimum pH (3.0 to 5.0) and soluble solids level (10-80%) required for gelatinisation (Draget, 2010). Therefore it would be possible to produce hydrogels using pectin, utilising the physical gelatinisation method of high methylated pectin or a chemical gelatinisation method of a low methylated method. However both require relativity high soluble solids content and a low pH which would not be suitable in combination with an amino acid based supplement due to the higher calorie product it would produce.

Another polysaccharide that can be considered for bead manufacture is carrageenan. There are three main types of carrageenan, Iota, Kappa and Lambda with the difference depending on the position and amount of ester sulphate groups on the galactose units which has an influence on the type of gels produced. Lambda, which contains the highest amount of ester sulphate groups, does not form a gel whereas Kappa which contains the least amount of ester sulphate groups, once dissolved in water at 70^{o}C or greater, will rapidly form strong, rigid gels once cooled in the presence of potassium as a gelling agent (Draget, 2010) . Therefore by utilising physical and chemical gelatinisation techniques it would be possible to produce a hydrogel using kappa carrageenan.

The main polysaccharide used in hydrogel manufacture is alginate which has been used extensively in both food and pharmaceutical applications (Bellich, 2011). Alginate rapidly forms firm gels in a calcium solution, which can be easily utilised for producing hydrogels. Alginate hydrogels have been shown to be both physically and chemically stable (Li, 2011) and have been previously used for peptide encapsulation (Mohan, 2015), therefore it should be possible to produce amino acid containing hydrogels.

1.6 Alginate Beads

Alginate is an extract of the marine brown algae and consists of linear, 1-4 linked β-D-Mannuronic acid (M unit) and α-L-Guluronic acid (G unit). The G and M units join together in one of three different blocks, GG…, MM… and MG… as seen in figure 1.4, and it is the proportion, distribution and length of these blocks which gives alginate gels their properties.

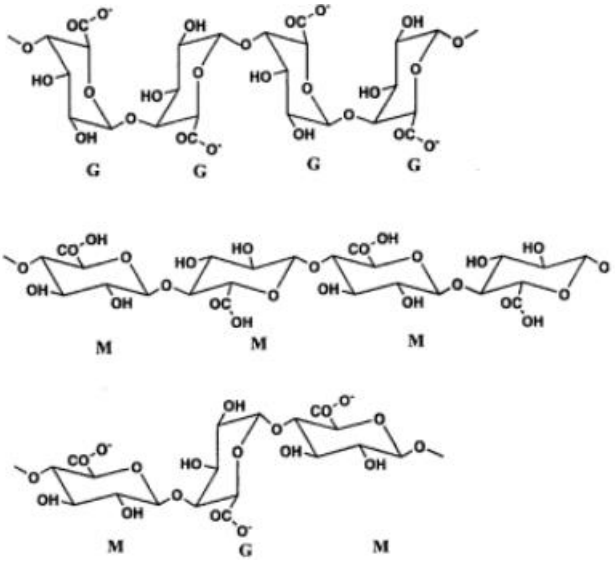


Figure 1.4 Alginate Structure (Somasundaran, 2006)

In alginate, gelling occurs when a cation (usually calcium) falls into the void in the alginate chain produced by 2 G units (G Block). These G blocks can further join with other G blocks which gives arise to a 3 dimensional network, this is often referred to as the 'egg box model' as shown in figure 1.5. This reaction requires no pre-heating and rate of gelling and gel strength can be determined by the concentration of calcium, temperature and amount of, and length of G units, with the firmest gels being generally formed with the highest concentration and longest G blocks in the highest calcium concentration. The gels produced by this mechanism can be produced rapidly without any heat and are thermally irreversible.

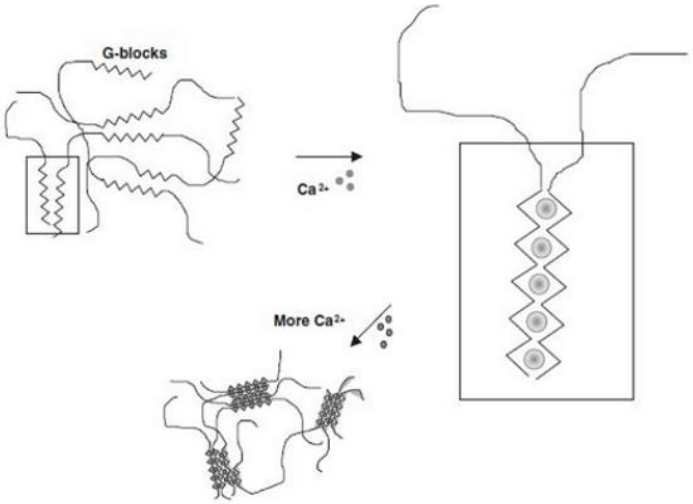


Figure 1.5 Alginate 'Egg Box' gelling model (Draget, 2010)

To produce hydrogels using alginates, the solution of the encapsulate material and alginate need to be exposed to the gelling solution which is usually a calcium solution. This can be achieved by two differing techniques; internal setting and diffusion setting.

In internal setting calcium is released within the product by controlling the pH, the solubility of the calcium used and using a sequestering agent in order to slowly set the alginate. An example of this is to use a calcium salt in combination with alginate and slowly reduce the pH, thus making the calcium soluble and able to gel the alginate (Imeson 2011). As the gelation rate utilising this method is very slow it would not lend itself to hydrogel bead formation.

In diffusion gelling the solution of alginate and encapsulate are extruded into a droplet which is introduced into the calcium solution. Once in contact with the calcium solution the calcium will quickly diffuse into the alginate. At the calcium / alginate interface gelling will occur and once the interface is saturated with calcium ions, the interface will then move as the calcium further diffuses in to the alginate. This causes further gelling, until the alginate is completely saturated with calcium and is gelled (Imeson, 2011). As this process is a diffusion process it requires a small droplet size, but it does offer rapid gelatinisation

1.7 Droplet Formation

The hydrogel beads produced are formed by initially forming a droplet which then combines with the gelling material to form the beads. There are several different ways that droplets can be formed, from simply dripping through to more complex methods such as electrostatic extrusion, coaxial air flow, vibrating jets and spinning discs depending on the amount of droplets required and the physical properties of the material (Dordevic, 2015)

The physical property of the droplets is determined by the Plateau – Rayleigh instabilities and is related to surface tension of the liquid. These instabilities explain why the falling stream of liquid from the aperture break up into the smaller packets which eventually become droplets (figure 1.6)

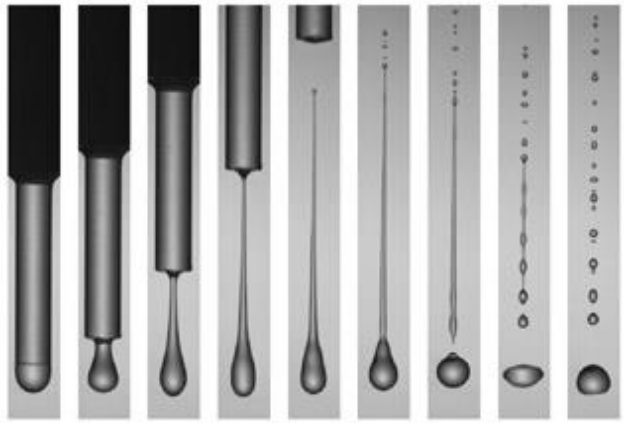


Figure 1.6 Plateau Raleigh Instability (Vincent, 2014)

In this model the rate at which the fluid column forms into droplets is determined by perturbations in the fluid column. These perturbations are small areas of instability within the column and at a certain point the instabilities will become large enough for the column to decay into droplets. (Dressen, 2013)

The time it takes for these perturbations to grow and decay in the column is affected by several factors. The width of the column has a direct effect on the time taken to decay, as a thin column cannot withstand the growth of the instabilities as long as a large column. The width of the column is determined by aperture of the nozzle, but also by the viscosity of the fluid.

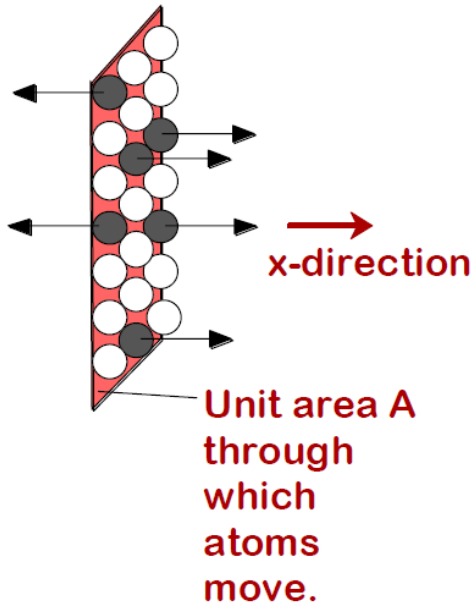
As the liquid falls from the nozzle the diameter of the column decreases as it is stretched. However in a more viscous product the perturbations are dampened by the viscosity and therefore the column is able to stretch further gaining a smaller diameter before they destabilise the column into droplets. This can be seen in the difference between a water column which breaks in around 10cm and a honey column which can go over 10m before breaking up (Ball, 2013). Therefore viscous fluids can break up into longer columns and produce larger droplets.

1.8 Diffusion

In hydrogels diffusion is an important physical parameter to consider. Diffusion not only controls the rate of gelatinisation in carrageenan and alginate hydrogels, but also that ability of the hydrogel to retain encapsulated material as there is the capacity for water soluble materials to diffuse out of the hydrogel into the gelling solution (Dordevic, 2015).

Diffusion is the net movement of molecules from an area of high concentration, i.e. The gelling solution to an area of low concentration i.e. the droplet and can be described by Flicks laws. In flicks laws the rate of diffusion is referred to as the diffusion flux (J) and is used to determine how fast the diffusion occurs. The flux can be defined in a few different ways but is generally described as the amount of mass diffusing per unit area per time eg. $kg/m^{-2}s$ (Callister, 2015) as seen in equation 1.0

$$J = \frac{1}{A}\frac{dM}{dt} \Rightarrow \left[\frac{kg}{m^2 s}\right] \text{ or } \left[\frac{atoms}{m^2 s}\right]$$



Equation 1.0 Diffusion Flux (Callister, 2015)

In Flicks first law, under steady state conditions the diffusion flux is proportional to the concentration gradients and can be expressed as the equation below

$$J = -D\frac{\emptyset}{x}$$

Where J= Diffusion Flux mol/m^2s^{-1}

D = Diffusion co-efficient or diffusivity m^2/s

x = Length diffused along (m)

Ø= Concentration (mol/m^3)

In order to determine the diffusion flux the diffusion co-efficient is required; fortunately diffusion coefficients are available in literature and are shown in tables 1.2 and 1.3.

Table 1.2 Diffusion coefficients of amino acids (Young, 2005)

Amino acid	D (m^2/s)
L -Alginate	8.58
Glycine	10.0
Serine	9.16
Arginine	7.01
Isoleucine	7.61

Table 1.3 Diffusion coefficients of minerals (Archer, 1948)

Mineral	D ($m^{-2}s$)
Potassium Chloride	1.83
Calcium Chloride	1.165

1.9 Digestion models

When new food forms or delivery systems, such as the hydrogels, are developed work is required in order to test their effectiveness, as no matter how good the encapsulate system is at masking the amino acid flavours they still need to be bioavailable in order for the patients to thrive.

By using *in vivo* feeding methods it is possible to obtain very accurate results by feeding the hydrogels to humans and or animals and then monitoring and measuring parameters over time (Sun, 2011). However although highly accurate, *in vivo* methods are very time consuming and costly and as such would not be practical for the hydrogel study and therefore an *in vitro* model is required.

In vitro models are widely used with the food and pharmaceutical industries to model the digestibility and release of components under simulated gastrointestinal conditions. Due to the physiochemical and physiological complexity of digestion there are several models available, with differing digestion times, ionic composition, temperatures, pH, mechanical stress and enzymes used depending on which parts of digestion is being modelled e.g. mouth, stomach, duodenum, and what outcome is desired (Sun, 2011).

One of the most important components in an *in vitro* model is the enzymes used, with specific enzymes used to give maximum digestibility and rate of hydrolysis. The enzymes can be added as a biological mixture containing several enzymes, however it is often advantageous to use single enzymes as this aids consistency and greater predictability is achieved especially if investigating the digestibility of a single nutrient (Coles, 2005). There are three main enzymes used in digestion models, lipases which are generally present in the stomach and pancreas which breakdown lipids into fatty acids, proteases which are mainly present in the stomach and small intestine which breakdown proteins and polypeptides into amino acids, and amylases which are present in the mouth and stomach and are involved in the breakdown of starches into oligosaccharides and monosaccharides (Sun, 2011). In the digestion of amino acid containing hydrogels the use of enzymes is limited if required at all as the components of the hydrogels are not affected by digestive enzymes. The hydrogels contain no lipids so there is no requirement for lipase, the amino acids present are already elemental so can undergo no further hydrolysis by a protease and the alginate is an indigestible polysaccharide and as such will not be broken down by amylase (Brownlee, 2005).

The pH is important factor when considering *in vitro* systems as the pH will change depending upon which step or steps of the digestion process is being modelled. In hydrogels, however the pH is more critical as the release of encapsulated materials can be determined by the size and shape of the hydrogel (Bellich, 2011) which is influenced by the pH. At lower pH values shrinkage occurs due to a decrease in the repulsive charge due to protonation of any free carboxyl groups on the alginate molecules, also the calcium ions can dissociate from the chains at a low pH which allows the alginate chains to come closer together (Zeeb, 2015). This shrinkage leads to a reduction of the dimensions of pores within the hydrogel matrix and can affect the release of particles and diffusion from the hydrogel (Zeeb, 2015).

In terms of time and temperature most digestion occurs at 37^{o}C with a time that mimics the typical digestion time in humans. However this time is dependent on the particle size of the 'food' particles, with larger particles moving through the stomach slower than small particles as the size of the particles have to be small enough to pass through the pylorus valve separating the stomach and the small intestine (Sun, 2011).

Therefore it is possible to produce an *in vitro* digestion model to determine the bioavailability of the amino acids within the alginate hydrogel.

1.10 Design of Experiment methodology

In the design of experiment methodology a systematic approach is used to determine the relationship between inputs and outputs so that cause and effect relationships can be (Minitab, 2015).

In this model the inputs into the experiment are broken down into factors and levels. A factor is a major independent variable, and a level is a subdivision of the factor, for example calcium and alginate in the recipe which could have been added at different levels. The factors were the calcium and alginate and the different amounts added where the levels If you have two factors each with two levels it can be expressed as 2x2 or 2^2 factorial design; where the 2 is the level, and the superscript 2 is the factor i.e. #levels$^{\#factors}$. If we use this example in the calculation it can show how many experimental conditions are required, ie 2^2 = 4 experimental conditions, 2^5 = 5 factors with 2 levels = 32 experimental conditions. (Trochim, 2006).

As the levels for each factor can be continuous rather than discrete it can be possible to produce several levels and therefore a large amount of experiments which would not be feasible.

To reduce the number of experiments down to a more manageable level a two level Plackett Burman factorial design method can be used (steele, 2012). Using this method it is possible to vastly reduce the number of experiments, but still learn a great deal from the data as it can be possible to learn as much from a two level design as a four level design using this method. This is especially true when conducting sequential experiments, where the learned outcomes can be fed into future experiments (steele, 2012).

However the success of a design of experiment model is dependent on the amount of factors and levels used, and it is often useful to carryout exploratory experiments in order to help define the experimental space (Barry, 2011). In addition centre points can be added to increase the model accuracy as it can determine if the data is linear or curvature points.

1.11 Regression analysis

Using regression analysis it is possible to understand the relationships between the various factors (predictors) and the outcome (response) in the form of a mathematical model. There are different types of regression, depending upon whether the predictors and responses are continuous or categorical. When a continuous predictor and response are used both linear and non-linear regression models can be used.

A linear regression model is based around two components and is widely used. As seen in equations 1.1 and 1.2 below, a deterministic component which is determined by changes in the predictor variables, and a stochastic component which is the randomness and unpredictability and explains the difference between the expected value and the observed value.

Equation 1.1

Response = (constant + predictor) + error

Equation 1.2

Response = deterministic + stochastic

(Frost, 2012)

For the regression model to function correctly this randomness and unpredictability are critical, as in a perfect regression model the deterministic components would explain the relationship so well that the difference between the observed and expected values would be solely down to inherent randomness. This can be determined by plotting the residuals, or observed errors, as if they have a constant spread throughout the range and are not systematically high or low and generally appear random, it can be assumed that the observed error is based on stochastic errors only (Frost, 2012).

As in the design of experiment method it is also useful to undertake preliminary experiments in order to know which variables to include in the model. To help with the model accuracy only three predictors should be used (Frost, 2013) and a normal probability plot of the residual completed, as the residuals should be normally distributed to gain the best model (Frost, 2014).

Non-linear regression models can also be used, and although useful can have several drawbacks as they are difficult to set up and often the effect of each predictor has on a response is not often clear. Also it is not possible to calculate P values for predictors and it is not always possible to calculate confidence intervals (Frost, 2011).

In both linear and non-linear regression it should also be noted that they are models, and correlation does not necessarily mean causation and it is advisable to understand the fundamental causal relationship (Frost, 2013).

2.0 Methodology

The experiments were conducted at Nutricia Liverpool, who develop and manufacture protein supplements for metabolic disorders .

2.1 Materials

Sodium Alginate – Protanal GP5450 supplied by Danisco, Marlborough, UK.

Calcium Lactate – Analytical grade supplied by Fisher Scientific, St. Helens, UK.

Calcium Chloride – Analytical grade supplied by Fisher Scientific, St. Helens, UK

Maltodextrin – DE21 supplied by Cargill, Manchester, UK

Sodium chloride – Analytical grade supplied by Fisher Scientific, St. Helens, UK

Potassium chloride – Analytical grade supplied by Fisher Scientific, St. Helens, UK

Sodium hydrogen carbonate – Analytical grade supplied by Fisher Scientific , St. Helens, UK

Xanthan gum – 200 Mesh supplied by Danisco, Marlborough, UK.

Cherry flavour 96323-33 – Supplied by Givaudan, Milton Keynes UK

Sucrose – Granulated supplied by Tate and Lyle, London UK

1M hydrochloric acid – Analytical grade supplied by Fisher Scientific, St. Helens, UK

Anamix Junior amino acid blend – Supplied by Nutricia Liverpool (composition as shown in table 2)

Table 2 – Amino acid blend composition

Amino Acid	g/100g
ALANINE	13.65
GLYCINE	13.1
GLUTAMINE	8.657
TYROSINE	7.6
THREONINE	7.2
LEUCINE	6.504
PROLINE	6.5
CYSTINE	5.45
ARGININE	5
VALINE	4.8
ISO-LEUCINE	3.854
LYSINE ACETATE	7.45
SERINE	3.435
METHIONINE	2.1
HISTIDINE	2
Mg ASPARTATE DIHYD.	1.5
TRYPTOPHAN	1.2

2.2 Methods

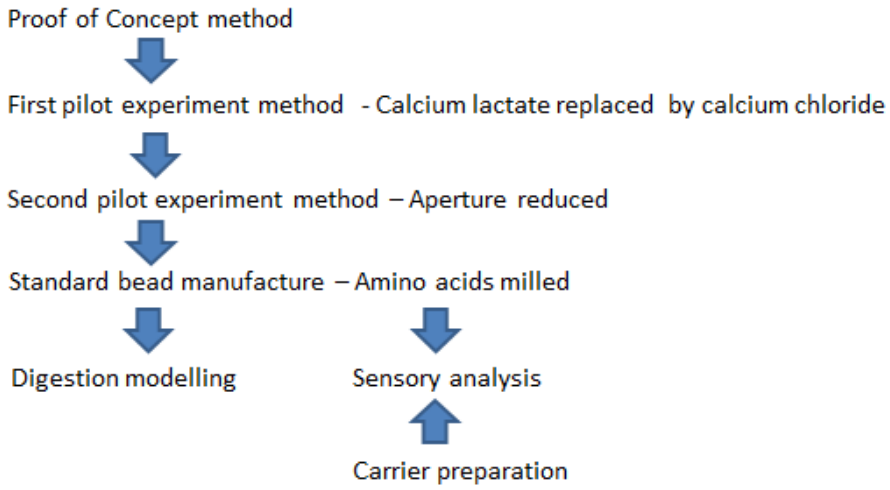


Figure 2.0 Experimental overview

2.2.1 Proof of concept bead manufacture method

The following method was developed to test the concept of producing an amino acid containing lginate bead.

1. 10g of amino acid mixture was dry blended with 1g alginate for 5 mins.

2. 89g of distilled water was weighed into a beaker and the dry blended amino acids and alginate blend added whilst mixing with a Silverson L5T high-sheer mixer at 5000rpm, until a homogenous mix was produced.

3 The viscosity of feed was measured using a Anton Paar NCR 200 rheometer at 25^{o}C using a 50mm cone and plate measuring system and a sheer rate of 100s^{-1}, (as per method outlined in appendix 2).

4. 297ml of distilled water was measured and 3g of calcium lactate added in a 500ml beaker and stirred until dissolved.

5. Alginate / amino acid feed was dropped using a 2ml pipette with a 3 mm aperture from a height of 50cm into the an agitated calcium lactate solution.

6. The beads remained in the solution overnight.

7. The beads were then removed using a screen and washed with distilled water for 2 minutes, dried on blue roll and stored in a 100ml Sterilin jar.

2.2.2 First pilot experiments bead manufacture method

This method is based upon the proof of concept method but the amounts of amino acids, alginate and calcium where varied and the mass of the beads were measured.

1. The amino acid mixture was dry blended with alginate using the amounts shown in table 2.1

2. Distilled water was weighed into a beaker and the dry blend added whilst being mixed using a Silverson L5T high sheer mixer at 5000rpm, until mix was homogenous. (Amount of water dependant on experiment as shown in table 2.1) .

3 The viscosity of feed was measured using a Anton Paar NCR 200 rheometer at 25^{o}C using a 50mm cone and plate measuring system and a sheer rate of 100s^{-1}, (as per method outlined in appendix 2)

4. 300ml of calcium chloride solution was prepared in a 500ml beaker using the amounts shown in table 2.1.

5. Alginate / amino acid feed was dropped using a 2ml pipette with a 3 mm aperture from a height of 50cm into the an agitated calcium chloride solution.

6. The beads remained in the solution overnight.

7. The beads were then removed using a screen and washed with distilled water for 2 minutes, dried on blue roll and stored in a 100ml Sterilin jar.

8. The beads were randomly selected into batches of 10, weighed. This was repeated twice more and the mean was calculated.

2.2.3 Second pilot experiments bead manufacture method

This method was based upon the first pilot method and the amounts of amino acids, alginate and calcium were varied but a smaller aperture was used to form the droplets and the bead volume was measured.

1. The amino acid mixture was dry blended with alginate using the amounts shown in table 2.2

2. Distilled water was weighed into a beaker and the dry blend added whilst being mixed using a Silverson L5T high sheer mixer at 5000rpm, until mix was homogenous. (Amount of water dependant on experiment as shown in table 2.2) .

3 The viscosity of feed was measured using a Anton Paar NCR 200 rheometer at 25^{o}C using a 50mm cone and plate measuring system and a sheer rate of 100s^{-1}, (as per method outlined in appendix 2).

4. 300ml of calcium chloride solution was prepared in a 500ml beaker using the amounts shown in table 2.2.

5. Alginate / amino acid feed was dropped using a 2ml pipette with a 0.4 mm aperture from a height of 50cm into the an agitated calcium chloride solution.

6. The beads remained in the solution overnight.

7. The beads were then removed using a screen and washed with distilled water for 2 minutes, dried on blue roll and stored in a 100ml Sterilin jar.

8. The beads were then measured using the liquid dispersion cell of the Mastersizer analyser filled with distilled water (as described in appendix 3). 10g of beads were added to the cell and a dispersant refractive index of 1.342 (Prakash, 2003) and a suspension refractive index of 1.33 (Hale, 1973) used analysis. The volume weighted mean (equation 2.0) was calculated automatically by the Mastersizer software.

Equation 2.0

$$Volume\ weighted\ mean = \frac{\sum d^4}{\sum d^3}$$

Where d = diameters of the spheres under measurement

(Malvern, 2010)

2.2.4 Third pilot and standard bead manufacture method

This method was based upon the second pilot method and the amounts of amino acids, alginate and calcium were varied and in addition to the smaller aperture the amino acid blend was milled to a smaller particle size. This method was the standard method used in all subsequent experiments.

1. Annamix Jnr amino acid mixture was milled using a Retsch ZM100 bench top mill (18,000 rpm, 0.5mm ring). To avoid excessive heating during milling only 500g of amino acid was milled at one time.

2. The particle size pre and post milling was measured using the powder cell on the Mastersizer analyser (as described in appendix 3)

3. Amino acid mixture was dry blended with alginate, using the amounts shown in tables 2.4, 2.5 and 2,6.

4. Distilled water was weighted into a beaker and the dry blend added whilst mixing using a Silverson L5T high sheer mixer at 5000rpm, until mix was homogenous. Amount of water dependant on experiment as shown in tables 2.4, 2.5 and 2.6.

5. 300ml of calcium chloride solution was prepared in a 500ml beaker using the amounts shown in tables 2.4, 2.5 and 2.6.

6. Alginate / amino acid feed was dropped using a 2ml pipette with a 0.4 mm aperture from a height of 50cm into the an agitated calcium chloride solution.

7. The beads remained in the solution for a fixed amount of time, dependant on the experiment.

8. The beads were then removed using a screen and washed with distilled water for 2 minutes, dried on blue roll and stored in a 100ml Sterilin jar.

9 The beads were then measured using the liquid dispersion cell of the Mastersizer analyser filled with distilled water (as described in appendix 3). 10g of beads were added to the cell with a dispersant refractive index of 1.342 (Prakash, 2003) and a suspension refractive index of 1.33 (Hale, 1973) used for analysis. The volume weighted mean (equation 2.0) was calculated automatically by the Mastersizer software.

2.2.5 Digestion model

In order to determine if the amino acid contained in the beads was available for digestion it was necessary to model the digestion. Although utilising an *in vivo* method would have provided the most accurate results these methods are time consuming and expensive (Sun, 2011), therefore an *in vitro* model was utilised.

There were several *in vitro* models available and in order to decide which model was the most suitable it was necessary to examine the composition of the beads and from this determine which digestive enzymes would be the most suitable. The beads are composed of amino acids, calcium, chloride and alginate. Alginate is an indigestible polysaccharide and is a source of dietary fibre and as such it is not broken down by amylase (Brownlee, 2005).Amino acids are the basis of protein and cannot be further broken down by protease and there is no lipid contained in the beads. Therefore there is no requirement for lipase so the use of digestive enzymes within the *in vitro* model was not required.

The pH and ionic concentrations are an important part of a model, as the pH and ionic composition within the digestive tract can vary from acidic conditions within the stomach to more neutral

conditions in the intestines (Sun, 2011). Therefore the digestion was modelled in two stages, with a more acidic 'stomach' phase containing a simulated gastric solution, and an intestinal phase at a higher pH with a simulated intestinal solution.

The time and temperature of digestion were also considered with digestion modelled at 37^{o}C to simulate body temperature (Rayment, 2009). The digestion time can vary depending upon the age, condition, sex, type of food and time of the day (Sun, 2011). For the *in vitro* model a stomach digestion time of one and half hours and an intestinal digestion time of 2 hours was used (Rayment, 2009).

The method below was used to perform an *in vitro* digestion model.

1. The beads were manufactured by the standard method as described in 2.2.4, with 10% (w/w) amino acid and 3% (w/w) alginate feed being dropped into a 30% (w/w) calcium chloride solution and left for 1 minute. A sample of the beads was tested for nitrogen content (as per Dumas method described in Appendix 1).

2. A simulated gastric solution containing 2.86g NaCl, 0.865g KCl and 0.4g $CaCl_2$ per litre (Rayment, 2009) and a simulated intestinal solution containing 6.5g NaCl, 0.835g KCl, 0.22g $CaCl_2$ and 1.386g $NaHCO_3$ per litre (Rayment, 2009) were prepared.

3. 200ml of simulated gastric solution was heated to, and held at 37^{o}C using a Stuart SD162 temperature regulating hot plate. Agitation was provided by a magnetic stirrer.

4. 10g of amino acid alginate bead was added, after 10 minutes 1M hydrochloric acid was slowly added until pH 2.0 was reached. The solution was agitated for 90 minutes.

5. After 90 minutes the beads were removed from the solution and placed into 200ml of simulated intestinal solution at 37^{o}C with a magnetic stirrer providing agitation. After 120 minutes in the solution, the beads were removed and rinsed in distilled water, dried and then analysed for nitrogen content using the Dumas method.

6. The nitrogen content of the beads before and after digestion was measured in order to determine the amount of amino acids remaining within the beads.

2.2.6 Carrier preparation

This method was used to prepare the carrier for the placebo and active beads in the sensory testing.

1. 0.1g Xanthan gum and 120g sucrose were dry blended together.

2. 79.7g distilled water was weighed into a 300ml beaker and the xanthan / sucrose blend added whilst agitating using a Silverson L5T high sheer mixer at 5000rpm, until mix was homogenous

3. The cherry flavour was then added and the beaker zeroed on the scale.

4. The beaker was then heated on the Stuart SD162 temperature regulating hot plate until all the sucrose had dissolved. Agitation was provided by a magnetic stirrer.

5. The beaker was returned to scale and water added until the scaled returned to zero, to replace any condensate loss.

6. The carrier was stored overnight to cool and for all entrapped air to be released.

2.2.7 Nutricia Methods

Nitrogen determination Dumas method	Appendix 1
Viscosity measurement	Appendix 2
Particle size measurement using laser particle diffraction	Appendix 3
Inductively coupled plasma spectrophotometry	Appendix 4
Chloride analysis	Appendix 5
A_w analysis	Appendix 6

2.3 Proof of concept experiment

In order to make the alginate beads the diffusion setting method was used. In this method, the alginate solution was dropped using a pipette into a bath of gelling solution of Ca^{2+}, allowing the calcium to diffuse into the droplet causing rapid gelatinisation to produce a bead. Several methods exist for producing droplets, but as the viscosity of the material to form the droplets is relatively low and there were no requirements for a large amount of beads to be produced, the simple dropping method proved the most viable as it was simple to prepare and required no specialist equipment.

To produce the alginate beads a 0.6 to 3% sodium alginate solution and a 0.05 to 1.5 M calcium chloride gelling solution can be used (Dordevic, 2015). In the initial experiment a solution of 1% sodium alginate (w/w) and 10% (w/w) Anamix Junior amino acid mixture was used to manufacture the beads as per the method described in 2.1.1.

Several amino acid mixtures were available for the treatment of PKU but for the experiments the Anamix Junior amino acid mixture, as shown in Table 2, was chosen as it contained higher levels of amino acids with positive flavour attributes compared to other PKU amino acid blends. The level of alginate and amino acids were kept to a minimum as it was important to maintain a relatively low feed viscosity in order to form droplets.

Calcium lactate was initially chosen over calcium chloride for the gelling solution, as calcium chloride can have a salty taste due to the chloride ions present. The beads remained in the solution with light agitation overnight and were harvested from the solution using a fine screen and examined visually for bead integrity. On examining the beads, it was noted that although a roughly-spherical bead was produced they were slightly flaccid and not fully gelled.

2.4 First pilot experiments

In these experiments, the beads were manufactured using the initial bead method but the levels of amino acids and calcium varied with a constant level of alginate. In addition, the calcium source was changed from calcium lactate to calcium chloride as calcium chloride contains a higher amount of calcium and is more soluble; 36% by mass calcium, 102g/100ml solubility, compared to 18% by mass calcium, 7.9g/100ml solubility. The amounts of amino acid blend used were 10, 20 and 30% (w/w) which were dispersed into a 1% (w/w) alginate solution and the viscosity measured as before. This mixture was then dropped into 1, 2 and 3% calcium chloride solution. The experiment is outlined in table 2.1 and the beads manufactured as per the method described in 2.1.2

Table 2.1 First pilot experimental outline

Calcium Chloride solution (% w/w)	Amino Acid blend (g/100g feed solution)	Alginate (g/100g feed solution)
1	10	1
2	10	1
3	10	1
1	20	1
2	20	1
3	20	1
1	10	1
2	20	1
3	30	1

Initially it was intended for the beads to be characterised by laser particle diffraction in order to determine the size, however the measuring cell has a maximum diameter of 2mm and the majority of the beads produced where visibly larger than this. Therefore the beads were characterised by mass, with the mass of 10 randomly selected beads measured. This was repeated three times and an average taken.

2.5 Second pilot experiments

In these experiments the beads were manufactured as in experiment 2.1, but in addition to varying the calcium and amino acids the alginate content was varied with 1, 2 and 3% (w/w) levels being used. The experiments are outlined in table 2.2 and the beads were manufactured as per the method described in 2.1.3.

Table 2.2 Second pilot experimental outline

Calcium chloride solution (% w/w)	Amino acid blend (g/100g feed solution)	Alginate (g/100g feed solution)
1	10	1
2	10	1
3	10	1
1	20	1
2	20	1
3	20	1
1	30	1
2	30	1
3	30	1
1	10	2
2	10	2
3	10	2
1	20	2
2	20	2
3	20	2
1	30	2
2	30	2
3	30	2
1	10	3
2	10	3
3	10	3
1	20	3
2	20	3
3	20	3
1	30	3
2	30	3
3	30	3

As the beads produced in experiment 2.1 where too large, being greater than 2mm in diameter, the aperture of the pipette was reduced from 3mm to 0.4mm. The size of the bead is determined by the size of the droplet which can be influenced by several factors, one of which is diameter of the aperture as this affects the diameter of the initial liquid column that will form the droplet (Dressen, 2013).

As before the amino acids were dispersed into the alginate solution, however it was not possible to produce high amino acid and alginate combinations as the higher viscosity alginate solutions meant it was not possible to homogenously disperse all the amino acids and a lumpy dispersion was produced. Also, some amino acid/alginate dispersions, which even though homogenous, produced high viscosities which produced elongated strings instead of beads.

Laser particle diffraction was then used to characterise the bead size using a Mastersizer produced by Malvern Technologies using the liquid dispersion cell with distilled water as the carrier and the method outlined in appendix 3.

In order to determine the amino acid recovery the nitrogen content of beads was used as a proxy measurement. The Dumas method was used in preference to the Kjeldhal method as it is a rapid method and requires no harmful or toxic reagents to be used (Francini, 2003) and was carried out using the Nutricia method as described in Appendix 1. The recovery was then found by calculating the theoretical total amount of nitrogen from the amino acids present as shown in table 2.3 and then comparing it to the nitrogen found by analysis.

Table 2.3 – Total Nitrogen in bead calculation

Amino Acid in blend	**%**	**Nitrogen content ***	**Nitrogen / 100g**
ALANINE	13.65	15.72	2.146
GLYCINE	13.10	18.65	2.443
GLUTAMINE	8.65	19.17	1.660
TYROSINE	7.60	7.72	0.587
THREONINE	7.20	11.76	0.847
LEUCINE	6.50	10.68	0.695
PROLINE	6.50	12.17	0.791
CYSTINE	5.45	11.66	0.635
ARGININE	5.00	32.16	1.608
VALINE	4.80	11.96	0.574
ISO-LEUCINE	3.85	10.68	0.412
LYSINE ACETATE	7.45	13.58	1.012
SERINE	3.43	13.33	0.458
METHIONINE	2.10	9.39	0.197
HISTIDINE	2.00	27.09	0.542
Mg ASPARTATE DIHYD.	1.50	8.63	0.129
TRYPTOPHAN	1.20	13.72	0.165
		Total Nitrogen / 100g amino acid (g)	14.899

* (FAO, 2002)

10% Amino acid bead = $\frac{14.899}{100} \times 10 = 1.489$g Nitrogen

20% Amino acid bead = $\frac{14.899}{100} \times 20 = 2.979$g Nitrogen

30% Amino acid bead = $\frac{14.899}{100} \times 30 = 4.469$g Nitrogen

Nitrogen content of 10% amino acid bead (by Dumas) = 0.37g

$\therefore \frac{0.37}{1.4899} \times 100 = 24.83\%$ recovery

2.6 Third pilot experiment

In these experiments beads were produced in the same manner as in 2.4 with a variance in the calcium, alginate and amino acid content. However, combinations that produced an alginate solutions that were too thick to form, or produced strings instead of beads, were omitted. The experiments are outlined in table 2.4 below and the beads manufactured as per the method described in 2.1.4. This method of bead manufacture became the standard way in which all the beads were manufactured for subsequent experiments.

Table 2.4 Third pilot experimental outline

Calcium Chloride solution (% w/w)	Amino Acid blend (g/100g feed solution)	Alginate (g/100g feed solution)
1	10	1
2	10	1
3	10	1
1	20	1
2	20	1
3	20	1
1	30	1
2	30	1
3	30	1
1	10	2
2	10	2
3	10	2

It was noted in experiment 2.1 that large particles of amino acid mix were present in the feed solution which lead to blockages in the 0.4 mm aperture pipette when forming the droplets and also may have led to poor homogeneity and possibly the poor recovery.

In order to remove these large crystals, the amino acid blend was milled using a Retsch ZM100 bench top centrifugal mill and the particle size measured for comparison using the Mastersizer produced by Malvern Technologies using the powder dispersion cell.

The beads produced were then characterised for size by laser particle diffraction and nitrogen content by the Dumas method.

2.7 First design of experiment

After completing the pilot experiments to establish the methodology and the experimental parameters it was decided to investigate how these different parameters affect the bead and therefore a design of experiment methodology was applied.

The experiments conducted are outlined in table 2.5 below , and the beads were manufactured as per the method described in 2.1.4.

Table 2.5 First design of experiment outline

Alginate (g/100g in Feed)	Calcium chloride solution (% w/w)	Time (mins)
1.0	3	20.0
1.0	1	5.0
2.0	3	20.0
1.5	2	12.5
2.0	3	5.0
2.0	1	5.0
2.0	3	5.0
1.0	3	20.0
2.0	1	20.0
1.0	1	20.0
1.0	3	5.0
2.0	1	20.0
1.0	1	5.0

Once the beads were produced they were analysed for their nitrogen content to determine the amino acid recovery which was fed back into the model as an output and used to produce a main effects plot and a standard effects chart.

From this data it appeared that all the factors had an effect on the recovery, but the recovery levels were still sub-optimal as they were less than 80 percent of the added level. Therefore, it was decided to work out the relative rates of diffusion for a selection of amino acids to compare to the calcium using Flick's first law of diffusion (equation 1.0) , as there needed to be adequate time for the calcium to diffuse and set the alginate without the amino acids diffusing out of the bead.

∴ Flicks first law

$$J = -D\frac{\Delta \emptyset}{\Delta x}$$

Where: $\Delta\emptyset$ = Concentration gradient (mol/m^3)

Δx = Lengh (m) i.e. Radius of a bead (determined from the volume-weighted mean by laser diffraction using $v = \frac{4}{3}\pi r^3$)

D = Diffusion Co-efficient / diffusivity (m^2/s)

J = Diffusion flow mol/m^{2}s^{-1}

∴ for Alanine in a 10% (w/w) amino acid solution, where D= 8.589 m^2/s (Young 2005)

$$J = -8.589\frac{0.15}{5.126 \times 10^{-7}}$$

$$J = -2.51 \times 10^{6} \text{ mol/m}^2\text{s}^{-1}$$

∴ for Glycine in 10% (w/w) amino acid solution, where D= 10 m^2/s (Young 2005)

$$J = -10\frac{0.174}{5.126 \times 10^{-7}}$$

$$J = -3.390 \times 10^{6} \text{ mol/m}^2\text{s}^{-1}$$

∴ for a 1% (w/w) Calcium chloride solution , where D= 1.165 m^2/s (Archer, 1948)

$$J = -1.165\frac{0.088}{5.126 \times 10^{-7}}$$

$$J = -2.045 \times 10^{9} \text{ mol/m}^2\text{s}^{-1}$$

∴ for a 10% Calcium solution , where D= 1.165 m^2/s (Archer, 1948)

$$J = -1.165\frac{0.88}{5.126 \times 10^{-7}}$$

$$J = -5.910 \times 10^{6} \text{ mol/m}^2\text{s}^{-1}$$

2.8 Second design of experiment

In these experiments a Plackett Burman factorial design was conducted with 3 factors of 2 levels with centre points as in the first experiment. However, in this second experiment the values for the levels were adjusted based on the outcomes from the first experiment. The experimental outline is shown in table 2.6 below and the beads where manufactured as per the method described in 2.1.4

Table 2.6 Second design of experiment outline

Calcium chloride solution (% w/w)	Alginate (g/100g feed solution)	Time (mins)
10	3	4.0
10	3	1.0
30	1	1.0
20	2	2.5
10	1	1.0
30	1	4.0
10	1	4.0
30	1	4.0
30	3	1.0
30	3	4.0
10	1	1.0
10	3	4.0
30	3	1.0

The beads were analysed for their nitrogen content to determine their amino acid recovery which was fed back into the model as an output. This data was then used to produce a main effects plot and a standard effect chart as in experiment 1.

The data was then subjected to a regression analysis. There are several different type of regression analysis available, but as the both the predictors and responses used were continuous and non-categorical then a regression model was used (Frost, 2012). A normal plot was then produced and it was determined that linear regression would be used.

The residuals were then plotted. As the residuals had a constant spread throughout the range and were not systematically high or low and generally appeared random, and therefore it was assumed that the observed error was based on randomness and unpredictability only (Frost, 2012).

The regression modelling was then carried out and the multiple linear regression equation was calculated and for each predictor the coefficient, standard error of the coefficient, T value, P value, R squared and adjusted R squared values were calculated.

In order to test for multicollinearity, where predictors correlate to each other, a variance inflation factor was also calculated (Heckman, 2015).

2.9 Food Safety

Before permitting the beads to be consumed by colleagues it was necessary to determine if the composition was safe. The composition of the beads was known in terms of alginate and amino acid but the calcium and chloride content from the diffusion was unknown. Therefore the calcium content was analysed using inductively coupled plasma spectrophotometry and chloride using a 926 Korning chloride analyser as per the Nutricia methods described in appendix 5 and 6. The levels were then normalised for dosage (10% w/w beads in a sucrose solution) and compared to the daily reference intakes outlined by the UK Department of Health.

2.11 Carrier

For the beads to be consumed a liquid carrier was needed. The liquid carrier needed to be of sufficient viscosity to suspend the beads to aid consumption and homogeneity. Generally the palatability of a carrier can be increased by making the carrier sweet (Rolls, 1982) with the use of fruit flavours and a syrupy texture (Pharmacy, 2015) .Therefore a thickened sucrose solution with a fruit flavour was used.

In order to avoid moisture movement from the beads to the sucrose solution the a_w needed to be considered and matched to the a_w of the beads. This is because the rate of moisture migration is proportional to the difference in a_w, with water migrating from an area of high a_w to an area of low a_w until an equilibrium is reached (Skilcast, 2011) . The a_w of the beads was measured using a Sprint TH-500 a_w analyser as per the Nutricia method described in appendix 7 and found to be 0.955, therefore a 60% (w/w) sucrose solution with an a_w of 0.95 was produced and the final composition can be seen in table 2.7 below and manufactured as per the method described in 2.1.6.

Table 2.7 Composition of thickened sucrose carrier

	% w/w	Supplier
Xanthan gum 200 mesh	0.05	Danisco, Marlborough, UK
Cherry flavour 96323-33	0.10	Givaudan , Milton Keynes, UK
Sucrose	60.00	Tate and Lyle, London, UK
Water	39.85	Distilled

2.12 Sensory methods

To determine the effectiveness of the flavour masking a difference test was undertaken with the amino acid beads tested alongside a blank placebo bead. There are generally three types of difference testing available, a paired comparison test, a duo-trio test and a triangle test (Stone, 2012) . The duo-trio test is generally used for products that have a relatively intense taste and odours, and the pair comparison test is recommended when there is limited product availability or the product characteristics require a limited exposure due to strong flavours or where excessive consumption would not be recommended (Stone, 2012). As the amino acid beads did not have an intense taste , strong flavour and can be readily consumed a triangle test was carried out.

To run the test a placebo was produced substituting the amino acid blend for a maltodextrin with the feed compositions of the placebo and actives shown below in Table 2.8

Table 2.8 bead feed composition.

Placebo Composition

	% w/w	Suppier
Maltodextrin, DE21	10	Cargil, Manchester, UK
Alginate protanal GP5450	3	Danisco, Marlborough, UK
Distilled water	87	

Active composition

	% w/w	Suppier
Milled amino acid	10	Nutricia UK
Alginate protanal GP5450	3	Danisco UK
Distilled water	87	

Beads were then manufactured as per the method described in 2.1.4, with the maltodextrin replacing the amino acid blend in the placebo.

The beads were then dispersed into the carrier solution (composition as per table 2.7) to produce a 10g bead / 90g carrier dispersion. It was evident that there was a visual difference between the placebo and active beads, even though the beads were of similar size (figure 2.2).

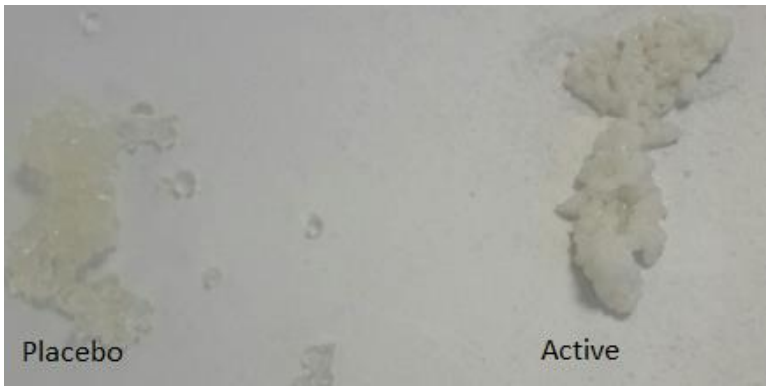


Figure 2.1 Photograph of placebo and active beads

The samples were presented in lidded cups to be consumed with a straw (figure 2.2) to prevent the panellists from seeing the dispersions. Each cup contained 100g of the bead dispersion.

Figure 2.2 Photograph of lidded sample cups.

The cups were then coded, with active cups coded 471 and 895, and placebo cups coded 279 and 794 Three cups were presented to the panellists (figure 2.4), with two cups containing the active and one the placebo, or two cups containing the placebo and one the active, and the panellists asked to decide which of the three samples was different. The panellists were also provided with a consent form and product information sheet.

Figure 2.3 Photograph of triangle test cups

At the end of the test, a tally of correct answers, where the different sample was identified, and incorrect answers was made to determine if the number of correct answers was significant. The amount of correct answers required in order to determine that the sample was different depended on the confidence interval required. The standard that Nutricia works to is a 95% confidence interval, and as such the amount of correct answers per total number of panellists required is shown in table 2.9.

Table 2.9 Minimum number of correct answers to establish significance

Number of panellists	Number required for significance @95%
5	4
6	5
7	5
8	6
9	6
10	7
11	7
12	8
13	8
14	9
15	9
16	9

(O'Mahoney 1985)

The panel contained 12 participants so for a significant difference 8 or more panellists were required to correctly identify the different sample.

When completing the triangle test it was possible to either use a selection of employees or use the Nutricia trained panel. For the trial the Nutricia trained panel was used as the panel is more accurate and can produce reproducible reliable data when compared to a panel consisting of normal employees (Lerma, 2013). This is because in order to become a member of the trained panel testing is given to assess the potential panellists' natural ability which is then enhanced by training over several months and exposure to amino acid based products.

3.0 Experimental Results

3.1 Proof of concept experiment

Figure 3.0 Photograph of beads produced in initial experiment.

3.2 First pilot experiments

Table 3.0 First pilot experiments

Calcium Chloride solution (% w/w)	Amino Acid blend (g/100g feed solution)	Alginate (g/100g feed solution)	10 x bead weight (g)	10 x bead weight (g)	10 x bead weight (g)	Mean x10 bead weight (g)	Standard deviation (g)	Feed Viscosity (Pa.S)
1	10	1	0.1980	0.1940	0.2149	0.202	0.108	0.144
2	10	1	0.2270	0.2238	0.2529	0.234	0.015	0.144
3	10	1	0.2720	0.2810	0.2614	0.271	0.009	0.144
1	20	1	0.2870	0.2593	0.2225	0.256	0.325	0.229
2	20	1	0.2720	0.2566	0.2728	0.267	0.009	0.229
3	20	1	0.2750	0.2741	0.2538	0.267	0.012	0.229
1	30	1	0.2960	0.3278	0.3277	0.317	0.120	0.460
2	30	1	0.3000	0.3443	0.2858	0.310	0.030	0.460
3	30	1	0.3390	0.3464	0.6457	0.444	0.175	0.460

3.3 Second pilot experiments

Table 3.1 Second pilot Experiments

Calcium Chloride solution (% w/w)	Amino Acid blend (g/100g feed solution)	Alginate (g/100g feed solution)	Feed Viscosity (Pa.S)	Nitrogen (g/100g bead)	% Amino acid Recovery (w/w)	Volume weighted mean (micron)
1	10	1	0.146	0.21	14.10	1245
2	10	1	0.146	0.21	14.10	1165
3	10	1	0.146	0.19	12.76	1166
1	20	1	0.230	0.20	6.71	894
2	20	1	0.230	0.19	6.38	1062
3	20	1	0.230	0.21	7.05	1039
1	30	1	0.469	0.21	4.70	1137
2	30	1	0.469	0.20	4.47	1055
3	30	1	0.469	0.19	4.39	1036
1	10	2	0.901	0.20	13.43	952
2	10	2	0.901	0.21	14.10	1137
3	10	2	0.901	0.21	14.71	852
1	20	2	1.452	-	-	Poor beads
2	20	2	1.452	-	-	Poor beads
3	20	2	1.452	-	-	Poor beads
1	30	2	1.705	-	-	Poor beads
2	30	2	1.705	-	-	Poor beads
3	30	2	1.705	-	-	Poor beads
1	10	3	2.363	-	-	Too thick
2	10	3	2.363	-	-	Too thick
3	10	3	2.363	-	-	Too thick
1	20	3	4.443	-	-	Too thick
2	20	3	4.443	-	-	Too thick
3	20	3	4.443	-	-	Too thick
1	30	3	Lumpy	-	-	Too thick
2	30	3	Lumpy	-	-	Too thick
3	30	3	Lumpy	-	-	Too thick

Figure 3.1 Photograph of elongated strings / Poor beads

3.4 Third pilot experiment

Amino Acid Base powder (Volume weighted mean)

Before Retsch milling = 0.145mm

After Retch milling = 0.077mm

Table 3.2 Third pilot Experiment

Calcium Chloride solution (% w/w)	Amino Acid blend (g/100g feed solution)	Alginate (g/100g feed solution)	Nitrogen (g/100g bead)	Amino acid recovery (% w/w)	Volume weighted mean (micron)
1	10	1	0.37	24.84	1281
2	10	1	0.32	21.48	1248
3	10	1	0.21	14.10	1100
1	20	1	0.53	26.23	1272
2	20	1	0.45	22.27	1328
3	20	1	0.75	37.12	1326
1	30	1	0.81	26.47	1330
2	30	1	0.96	31.37	1349
3	30	1	0.67	21.89	1375
1	10	2	0.56	37.63	1305
2	10	2	0.47	31.68	1308
3	10	2	0.47	31.68	1313

3.5 1st design of experiment

Table 3.3 Factors and outputs used in the factorial design

FACTORS			OUTPUT
Alginate (g/100g)	Calcium (g/100g)	Time (mins)	Amino acid recovery (%)
1.0	3	20.0	42.70
1.0	1	5.0	47.20
2.0	3	20.0	29.60
1.5	2	12.5	42.10
2.0	3	5.0	38.10
2.0	1	5.0	38.80
2.0	3	5.0	34.20
1.0	3	20.0	42.10
2.0	1	20.0	34.80
1.0	1	20.0	46.00
1.0	3	5.0	40.13
2.0	1	20.0	39.47
1.0	1	5.0	51.90

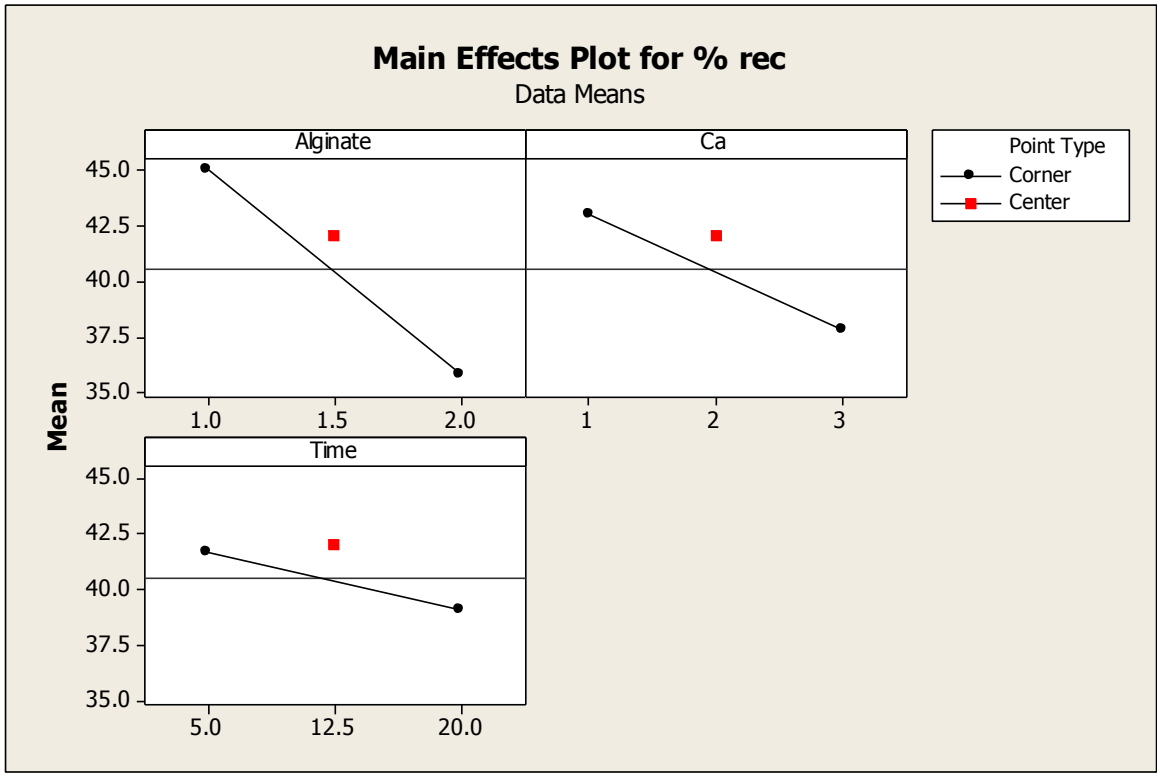


Figure 3.2 Main effects plot for factors in experiment 1, Y axis is % amino acid recovery, X axis are alginate content (g/100g) , calcium chloride concentration (g/100g) and time (mins).

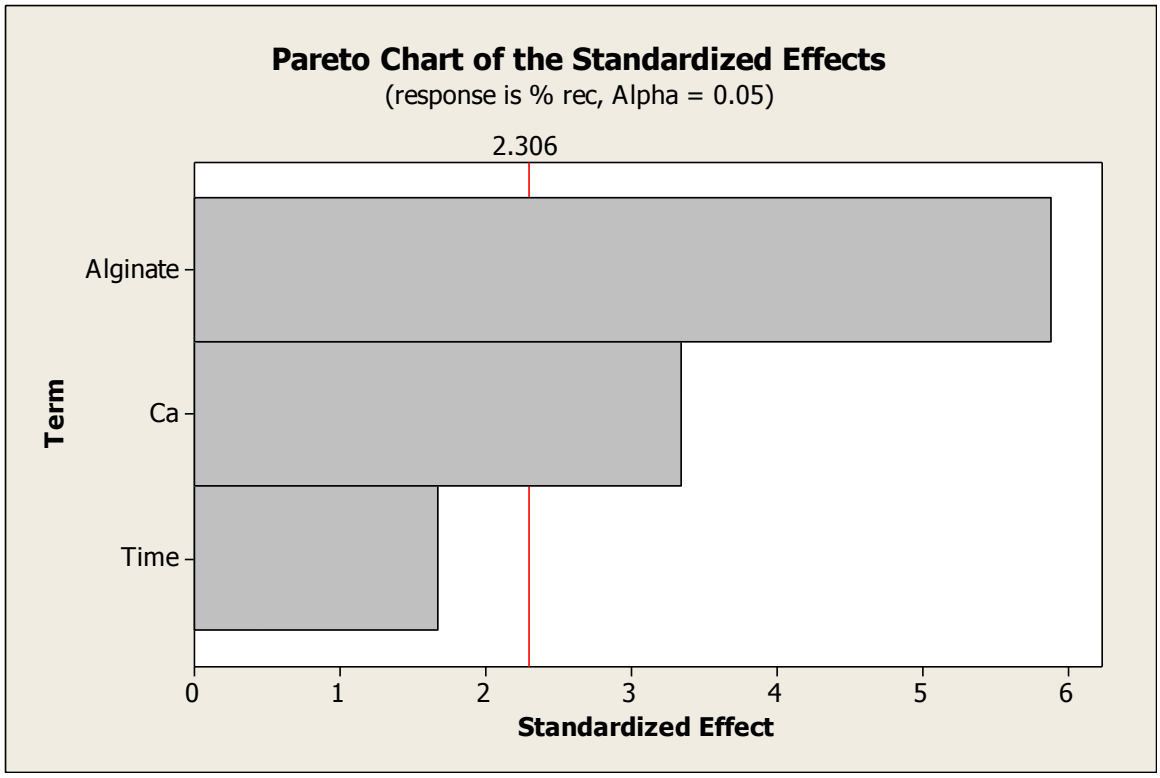


Figure 3.3 Pareto chart for % recovery of amino acids showing the standardised effects for experiment 2

3.6 2nd design of experiment

Table 3.4 Factors and outputs used in the factorial design

FACTORS			OUTPUT
Calcium (g/100g)	Alginate (g/100g)	Time (mins)	Amino acid recovery (%)
10	3	4.0	66.40
10	3	1.0	71.70
30	1	1.0	63.15
20	2	2.5	49.34
10	1	1.0	60.52
30	1	4.0	41.40
10	1	4.0	30.92
30	1	4.0	45.39
30	3	1.0	83.50
30	3	4.0	63.15
10	1	1.0	52.60
10	3	4.0	53.28
30	3	1.0	78.28

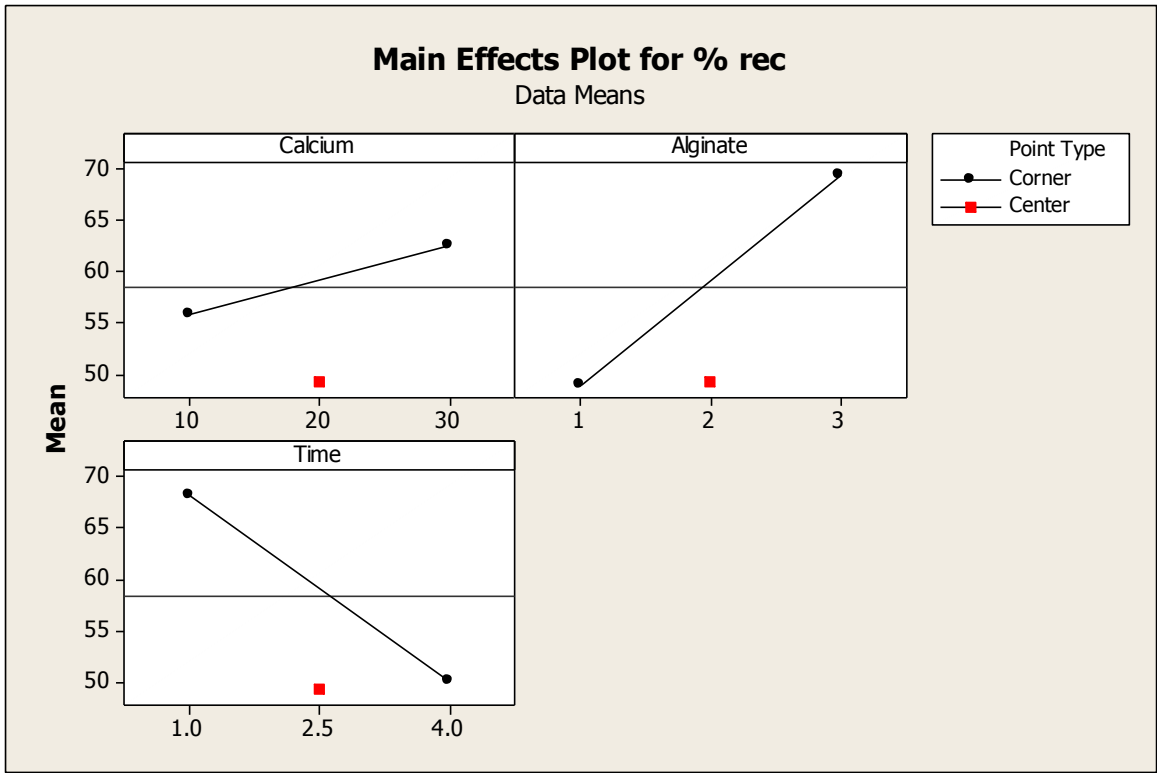


Figure 3.4 Main effects plot for factors for second experiment, were Y axis is % amino acid recovery, X axis are alginate content (g/100g) , calcium chloride concentration (g/100g) and time (mins).

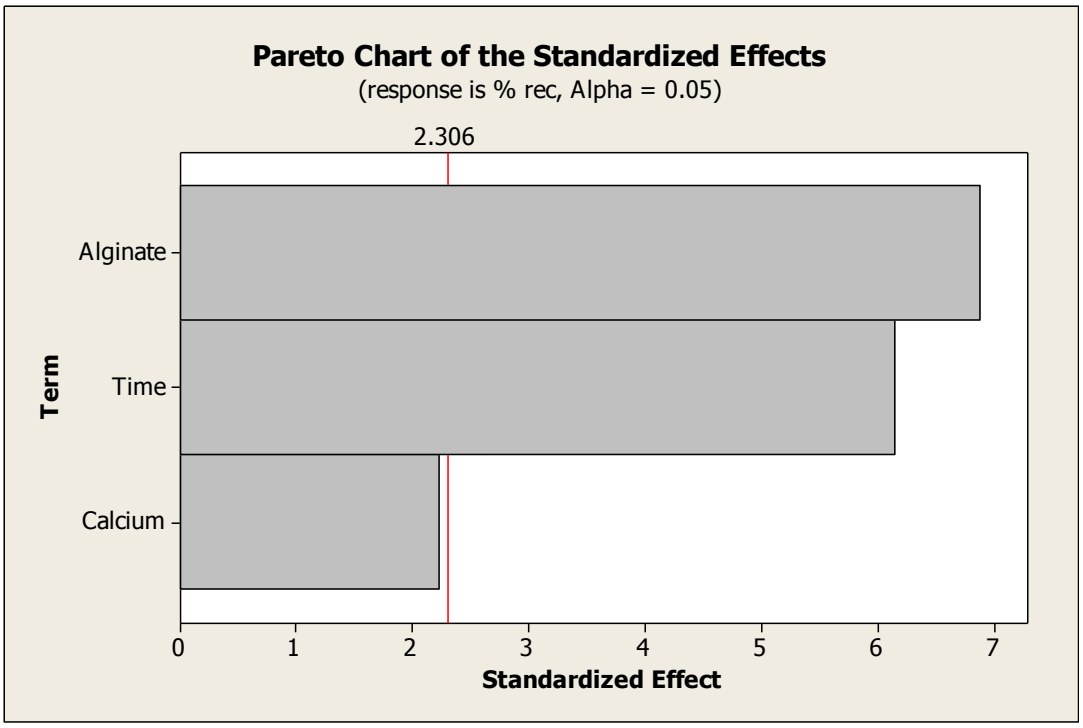


Figure 3.5 Pareto chart for % recovery of amino acids showing the standardised effects for 2nd experiment

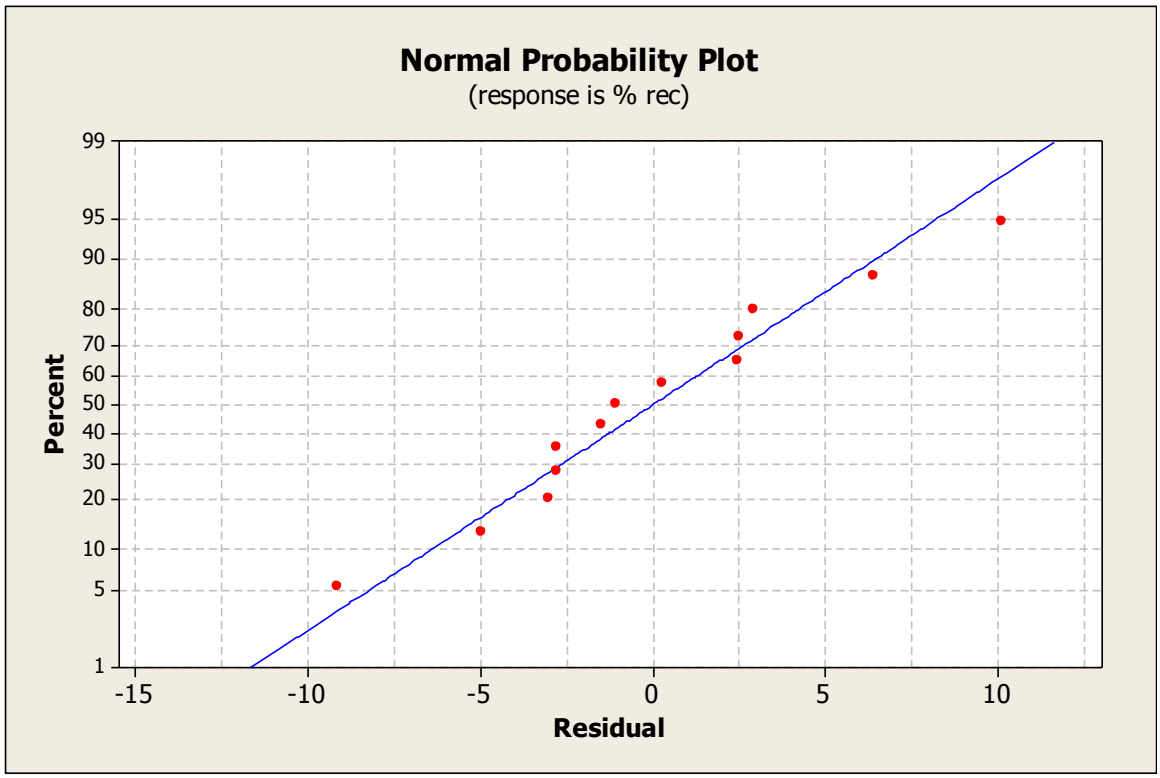


Figure 3.6 Normal probability plot for % amino acid recovery for the second experiment residuals

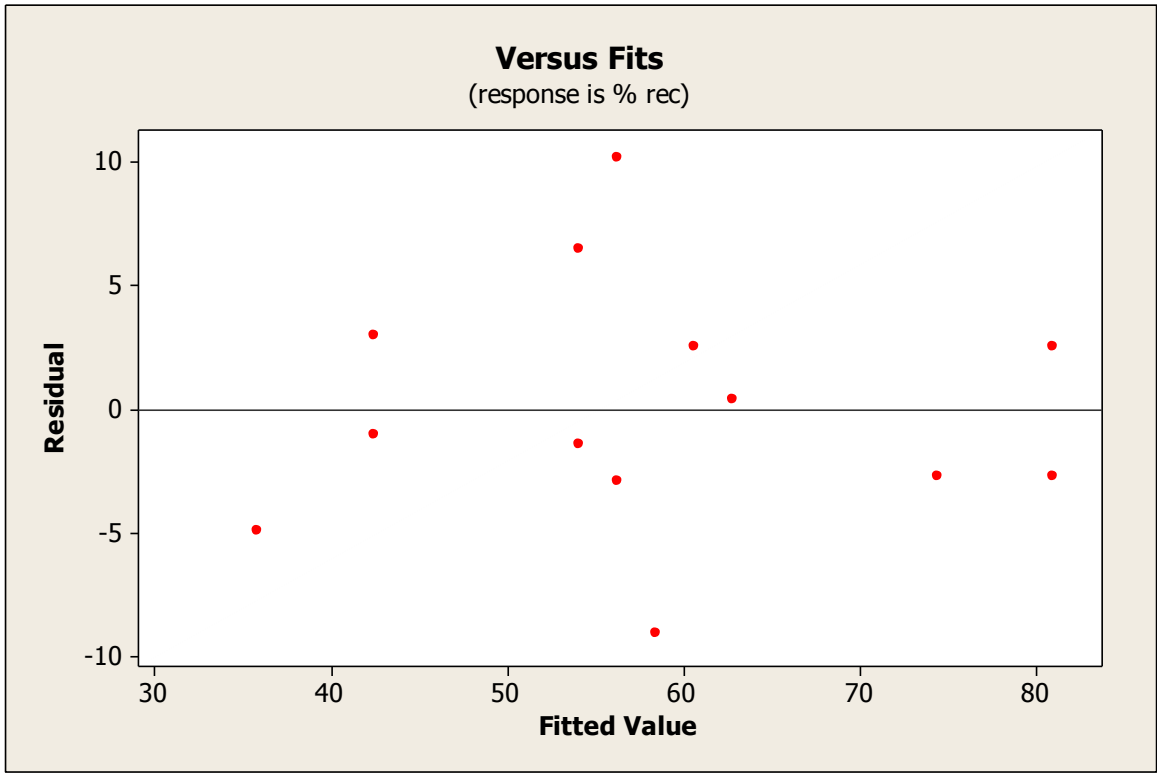


Figure 3.7 Residual Plots for % amino acid recovery for the second experiments.

Table 3.5 Regression Analysis

The regression equation is
% rec = 46.6 + 0.329 Calcium + 10.2 Alginate - 6.07 Time

Predictor	Coef	SE Coef	T	P	VIF
Constant	46.638	5.705	8.17	0.000	
Calcium	0.3288	0.1668	1.97	0.080	1.000
Alginate	10.194	1.668	6.11	0.000	1.000
Time	-6.067	1.112	-5.46	0.000	1.000

S = 5.77756 R-Sq = 88.8% R-Sq(adj) = 85.0%

3.7 Digestion Modelling

Table 3.6 Amino Acid recovery before and after *in vitro* digestion model

	Nitrogen (g/100g bead)	% recovery (w/w)
Bead prior digestion	1.17	78.57
Bead post digestion	0.12	8.06

Equation 3.1 Amino acid digestion

Amino acid digested = $(\text{nitrogen prior digestion} - \text{nitrogen post digestion}) \times \text{conversion factor}$

Conversation factor = 6.75 (Pritchard, 1995)

Amino acid digested = $(1.17 - 0.12) \times 6.75 = 7.05$g amino acid digested per 10g amino acid containing bead.

3.8 food safety

Table 3.7 Bead calcium and chloride content compared to Department of Health dietary reference values

	mg/100g bead	mg/10g bead dose	DRV *(mg)	Diff (mg)
Calcium	2346	234.6	700	-465.4
Chloride	1140	114.0	1,600	-1486

*Dietary reference value age 19-50 (Acheson, 2013)

3.9 Sensory

Table 3.8 Results from triangle test comparing active amino acid containing beads against maltodextrin containing placebo.

Participant	Sample codes	Taste order	Sample chosen	Correct	Comments
1	794,279,895	Placebo, placebo active	Active	Yes	odd sample less sweet
2	471,279,794	Active, placebo, placebo	Active	Yes	sweeter stronger flavour
3	895,794,471	Active, placebo, active	Placebo	Yes	Slight difference in taste and aftertaste.
4	471,895,279	Active, active, placebo	Active	No	Slightly stronger taste.
5	471,279,895	Active, placebo, active	Active	No	stronger taste
6	794,895,279	Placebo, active, placebo	Placebo	No	794 is less concentrated and more mildly flavoured
7	895,471,794	Active, active, placebo	Active	No	Appeared to be less sweeter than the other two and less lumpy
8	279,895,471	Placebo, active, active	Active	No	It tastes more medicinal, like cherry flavoured amoxycillin
9	279,794,471	Placebo, placebo, active	Active	Yes	a bit tangy
10	794,471,895	Placebo, active, active	Placebo	Yes	slightly moe sweet/almond
11	895,794,279	Active, placebo, placebo	Placebo	No	
12	279,471,794	Placebo, active, placebo	Active	Yes	appears more concentrated

Number of panellists correctly identifying different sample = 6

Number of panellists incorrectly identifying different sample = 6

Number of correct required for significance @ 95% confidence = 8

4.0 Discussion

The experiments can be characterised into four stages. In the first stage the experiments were concerned with the development of the bead and optimising the method of manufacture. The second stage experiments explored which factors affected the bead in terms of the retention of encapsulated amino acids. In the third stage the digestibility of the bead was investigated and finally in the fourth stage the sensory properties of the beads were examined.

4.2 Bead manufacture development

4.2.1 Proof of concept experiment

The initial experiment was carried out in order to test the concept of encapsulating an amino acid blend within an alginate hydrogel. Although the method of manufacture was crude, beads were produced and the amino acids were successfully encapsulated in the bead (figure 3.0). However the beads were large and soft to the touch. Initially the softness of the beads was attributed to the low calcium content and low solubility of calcium lactate, producing reduced calcium content in the gelling solution. However 3% calcium lactate would produce 0.54g calcium per 100ml, which is below the maximum solubility of calcium lactate (7.9g/100ml) and above the amount of calcium that would be available using a calcium chloride at the recommended level of 0.05M (0.0209g / 100ml) (Dordevic, 2015) . Therefore sufficient calcium was available to firmly gel the alginate, however the lactate can act as a chelating agent removing the calcium from the junction zones thus reducing the amount of cross linking and leading to a reduction in gel strength and soft beads (Rehm, 2009).Hence it was decided to use the calcium chloride instead.

4.2.2 First pilot experiment

In these experiments the amount of amino acid blend and the calcium chloride content were varied and the concentration of alginate remained constant as shown in table 3.0 . The beads produced were firmer to the touch than those produced in the initial experiment. This was due to the gelling solution being changed from a calcium lactate to a calcium chloride solution. By changing the calcium source not only was there an increase in the calcium available but there was no chelation affect from the lactate (Rehm, 2009).

The feed viscosities for each amino acid level were the same. This was due to the amino acid / alginate feed being produced in bulk which was dropped into the calcium gelling solution. As the feed viscosity was measured prior to being introduced into the calcium gelling solution, the level of calcium would have no effect on the feed viscosity.

When the amount of amino acid was increased there was an increase in the feed viscosity due to an increase in the alginate concentration as the water was displaced by the amino acid. As the viscosity increased there was an increase in the bead mass this is shown in figure 4.0. Although the regression coefficient R^2 for this relationship was low (Hughes, 2010).

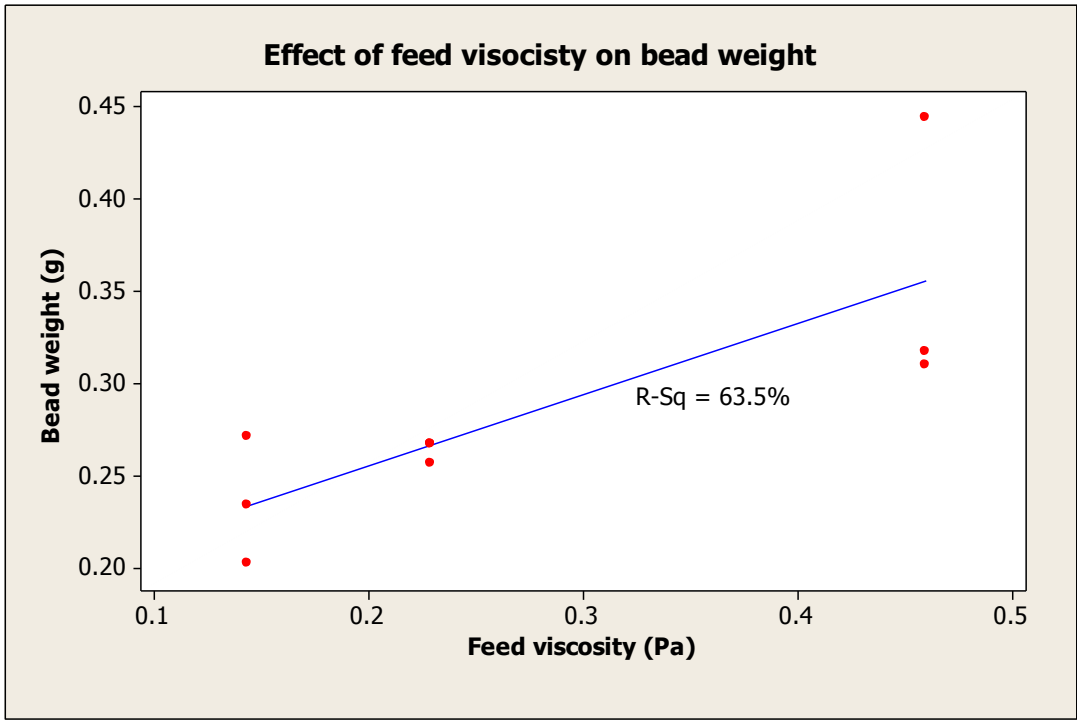


Figure 4.0 Effect of feed viscosity on bead weight

Therefore outlier analysis was undertaken using an r11 Dixon test that showed that the 0.444g mass was an outlier (figure 4.1).

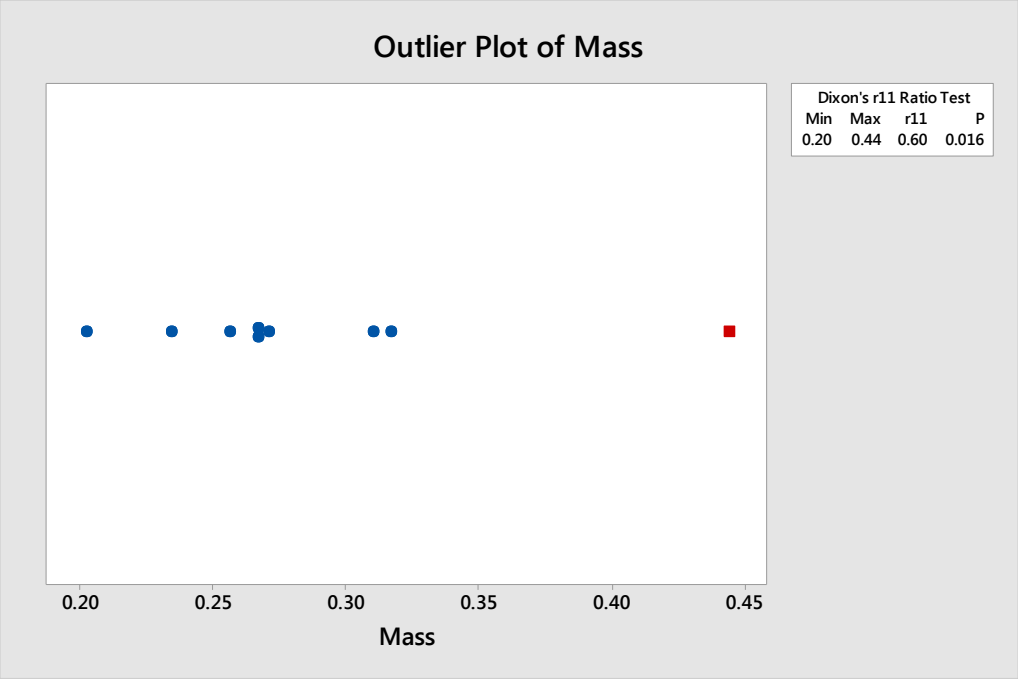


Figure 4.1 Dixon r11 Outlier plot

The Dixons outlier analysis was used as the sample size was low and one value was suspected as being an outlier (Watfish, 2006). The outlier value was then removed and the regression co-efficient

calculated to produce a R^2 of 73.7, which although is larger is still quite low. This may be due to the small number of data points recorded.

The increase in bead mass can be directly related to the mass of the drop that formed therefore the increase in viscosity was producing heavier droplets. This may be due to a damping effect, as the liquid leaves the aperture it forms a column inside this column natural instability waves form which eventually build up and lead to the disintegration of the column which due to surface tension forms the droplet. As the viscosity was increased the internal instability waves were dampened and the column grew larger before disintegrating and therefore a larger droplet was formed (Ball, 2013). The difference in bead weight could also be attributed to the difference in density, as the higher viscosity feed has an increased amino acid component thus a higher density, and would have a larger weight for the same size bead. Therefore measuring the weight and inferring a bead size was incorrect and measuring the size of the bead would have been preferred, however this was not possible due to the bead size being larger than the 2mm aperture of the Mastersizer measurement cell.

In addition to the beads being too large for size characterisation, the beads being larger than 2mm was not ideal for digestion as the particle size will affect the residence in the stomach. For liquids containing particles between 1mm and 2 mm in diameter the liquid and particles pass through the stomach relatively quickly through the pyloric opening. However if the particle size is larger than 2mm then they are too large to pass through the pyloric opening and will be ground down by contractions of the stomach with any larger particles remaining having to wait until the stomach completely empties (Kong, 2008). This leads to a longer residence time within the stomach and can cause bloating and nausea (Lunenfield, 2007).

In the 10 and 20% amino acid beads the calcium content of the gelling solution had no effect on the mass of the beads. In the 30% amino acid beads there was an increase in bead mass at 3% calcium; however this value was shown to be an outlier.

4.2.3 Second pilot experiment

In this experiment the scope was increased to include variations in the alginate content in addition to the amino acids and the calcium chloride as shown in table 3.1. As the droplets were too large in the previous experiment, a smaller aperture was used to produce smaller droplets. A smaller aperture produces smaller droplets due to its effect on the size of the initial fluid column produced. As previously discussed inside the fluid column instability waves are formed, however as the column was now narrower it was less robust to the instabilities and therefore disintegrated earlier to produce smaller droplets (Ball, 2013). The smaller aperture produced beads with diameters less than 2mm thus it was possible to measure the beads size and distribution and determine the volume weighted mean diameter, D[4,3] . The volume weighted mean was calculated as it showed which particle sizes made up the majority of the sample, Figure 4.2 (Malvern, 2012).

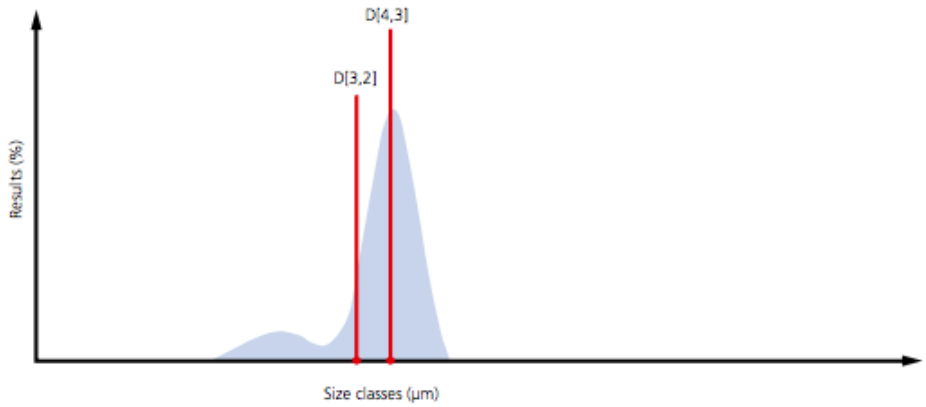


Fig 4.2 Example of particle size distribution (Malvern, 2012)

When the amount of alginate increased, again there was an increase in viscosity however this did not lead to an increase in bead size, as shown in figure 4.3 with a correlation coefficient of R^2 value of 22%. This would indicate that the weight increase seen in the first pilot experiment can be attributed to changes in bead density rather than changes in size.

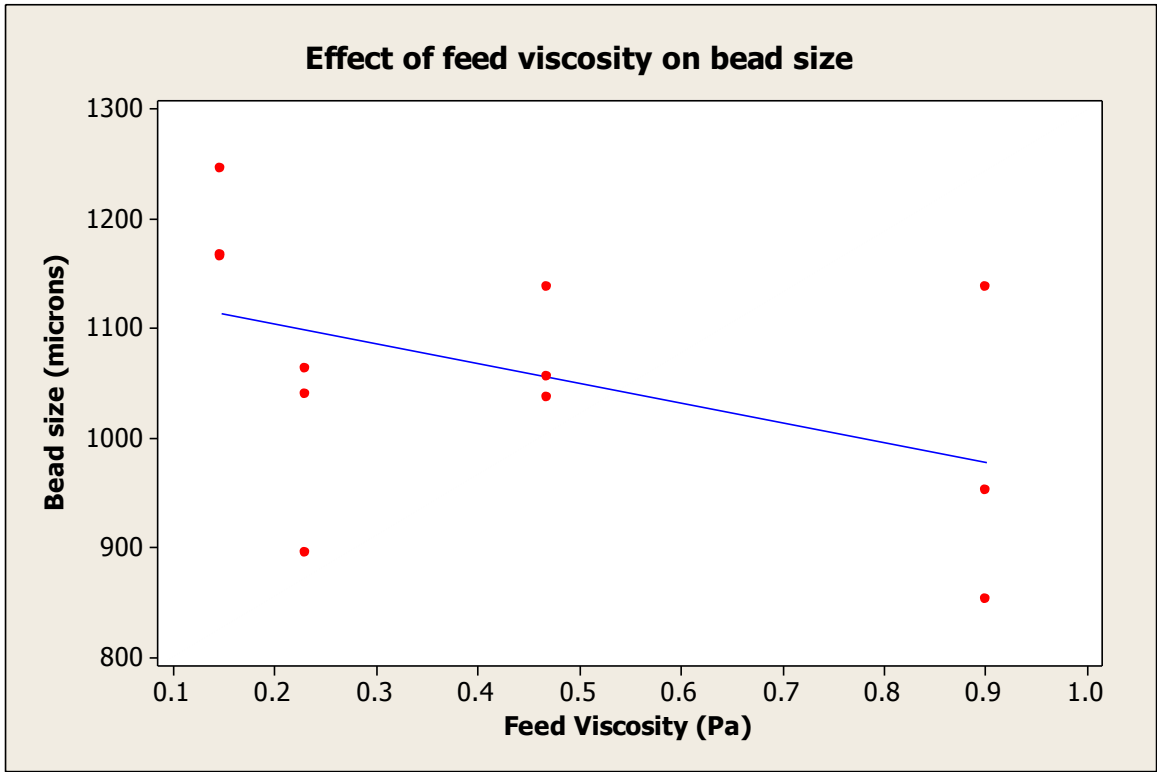


Figure 4.3 Effect of feed viscosity on bead size (volume weighted mean)

Also the feed viscosity measured may not be a true reflection of the viscosity at the time of droplet formation, as the feed will encounter a shear field when passing through the aperture resulting in a reduction in viscosity due to the shear thinning nature of the alginate thickened feed (FMC, 2003).

Using the smaller aperture produced beads within the required range of 1 to 2mm with a mean bead size of 1.295mm and a standard error of the mean of +/- 0.021mm . However at some viscosities it was not possible to produce beads as long strings were produced instead. In these cases the viscosities of the fluids were damping the internal waves to such an extent that a large column was produced before it broke. Once the column had broken the time taken to fall into the gelling solution was insufficient for the surface tension to pull the viscous column into a droplet (figure 3.1)

To determine if the beads were suitable nutritionally the nitrogen content was analysed and the percentage recovery calculated with a target recovery of greater than 80% (DFS, 2014). A lower recovery would render the beads unsuitable, as the patient would be at risk of dietary protein and calorie deficit that would impact on their health. The recovery for the beads produced was between 4% and 15% and as such is nutritionally poor.

The method of bead manufacture used in the pilot experiment was prone to issues with the aperture blocking which impeded bead production. Blockages can occur when there is a particle size to tube diameter ratio of around 0.3 – 0.4 (Sharp 2005). The amino acid blend used had a volume weighted mean diameter of 0.145mm and the aperture had a diameter of 0.4mm.Therefore a tube to particle diameter ratio of 0.36 was produced which is within this range and therefore the blocking of the tube was inevitable. However prior to a blockage forming the large particles can form an arch in the tube (Sharp 2005) thus straining the insoluble amino acids out of the feed stock in the aperture and therefore producing a droplet containing a reduced amino acid content and there may be inconsistencies in the initial amino acid content from bead to bead.

4.2.4 Third pilot experiment

In this experiment there were variations in the alginate content, amino acid and the calcium chloride contents as shown in table 3.2, however the combinations that produced strings were omitted.

To reduce the blockages and improve the homogeneity the amino acid blend was milled to produce a smaller particle size. This reduced the volume weighted mean size from 0.145mm to 0.077mm and produced a particle to tube ratio of 0.19 that is below the 0.3 ratio responsible for blockages. When manufacturing the beads no blockages were encountered so the milling had a positive effect. The milling also produced larger and more consistent bead sizes compared to pilot experiment 2 as seen in table 4.0

Table 4.0 Bead size comparison

	Mean volume weighted mean (µm)	Standard Deviation (µm)
Pilot Experiment 2	1061	117.0
Pilot Experiment 3	1294	70.9

Although the beads are larger than those produced in pilot experiment 2 they were still within the 1 to 2mm size range required. Therefore the modified method of bead manufacture with the amino acids being milled and a 0.4mm aperture pipette was established as the standard method of manufacture for all subsequent experiments.

Although the amino acid particle size reduction and the removal of straining prior to droplet formation produced a positive impact on the amino acid recovery with an increase when compared to pilot experiment 2, the recovery levels, between 14 and 38%, were still poor and sub optimal.

To understand the poor recovery the structure of the beads needs to be considered. The beads are not a solid impermeable mass entrapping the soluble and insoluble amino acids but are a porous gelled network as seen in the photographs and SEM micrographs (figure 4.4)

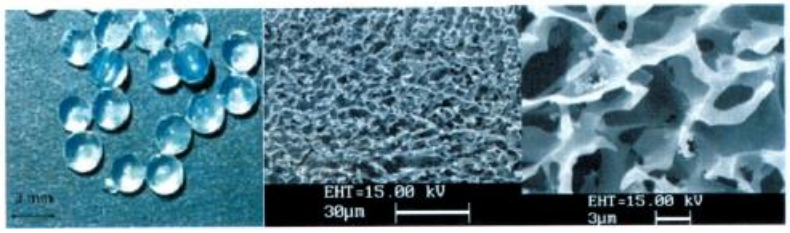
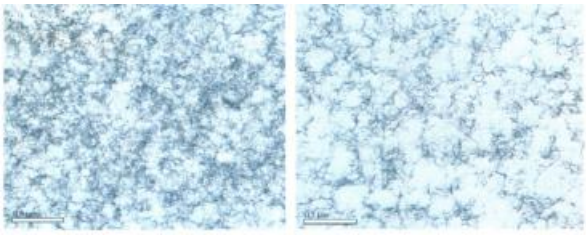


Fig 4.4 Photographs and SEM micrographs of an Alginate bead (Bellich, 2011)

As a gelled network the diffusion of the amino acids contained is reduced when compared to a normal solution, however it is still possible for the amino acid to diffuse out of the network and into the gelling solution.

Therefore whilst the beads were in the gelling solution overnight, the calcium was diffusing into the beads but at the same time the amino acids were diffusing out of the beads. Therefore the time in gelling solution used in the pilot experiment was too long and a shorter time was required in order for the calcium to gel the solution before the amino acids diffused out of the matrix.

The percentage recovery of amino acids in the beads produced with 2% alginate was higher when compared to 1% alginate. This can be attributed to the increase in alginate producing a denser network (figure 4.5) which reduces the beads permeability and restricts the diffusion (Zeeb, 2015). Therefore the increase in alginate concentration had a positive effect on the amino acid recovery.



High Alginate conc Low Alginate conc

Figure 4.5 Effect of Alginate on network density (Rayment, 2009)

4.3 First design of experiment

In these experiments a Plackett Burman factorial methodology was utilised in order to discover the relationships between the factors and their effect on the recovery without completing a large number of experiments. Three of the four factors available were chosen, alginate, calcium chloride concentration and the residence time in the gelling solution; with the amino acid recovery being an output. The alginate and the residence time were chosen as from the pilot experiment it was hypothesised that these would affect the recovery. The amino acid content was omitted as a factor as it was known from the pilot experiments that it was not physically possible to produce beads with a combination of higher levels of amino acid and alginate due to the increased viscosity of the feed solution. The levels of 1% and 3% calcium chloride solution and 1% and 2% alginate where chosen as these were used successfully in the pilot experiments. The time in the gelling solution was reduced from overnight to between 5 and 20 minutes as it was indicated from the pilot experiments that overnight was too long. In addition to the two levels, centre points of 1.5% alginate, 2% calcium and 12.5 minutes where included to increase the accuracy of the model and to check for linearity.

Once the beads were manufactured the nitrogen was measured by Dumas method and the percentage recovery of amino acid calculated and inputted into the model (table 3.3).

From the model a main effect chart and a Pareto chat were produced (figures 3.2 and 3.0) to determine which factors had an effect on the output. The alginate had the greatest effect as it had the largest magnitude on the Pareto chart and had the steepest gradient on the main effects plot. This finding was similar to the findings of the third pilot experiment and can be attributed to the reduction in the beads porosity due to the increase in the alginate concentration.

The level of calcium also showed a significant effect on the output as its magnitude was greater than the reference line on the Pareto chart and the line is off the horizontal on the standard effects chart. The time in the gelling solution did not have a significant effect on the output as its magnitude is not greater than the reference line on the Pareto chart and the line is almost horizontal on the main effects plot.

Both of these effects can be explained by diffusion. Diffusion can be described by Flicks first law where the rate of diffusion, or diffusion flow, over a given length is related to the concentration gradient and diffusion coefficient. As can be seen in table 4.1 the rates of diffusion of alanine and glycine, which make up over a quarter of the amino acids in the blend, were greater than that of the calcium in the gelling solution.

Table 4.1 Calculated diffusion flows for amino acids and calcium

Material	Diffusion flow mol/m^2s^{-1}
Alanine (1.36% sol)	-2.51×10^6
Glycine (1.31% sol)	-3.39×10^6
Calcium (1% Calcium chloride sol)	-2.04×10^5
Calcium (3% Calcium chloride sol)	-5.90×10^5

Therefore it was hypothesised that whilst the beads were in the gelling solution the calcium was diffusing into the beads. At the higher levels of calcium there was a greater diffusion gradient and

therefore a higher diffusion flow, and the rate at which the calcium diffused through the bead to the core was increased (Blandino, 1999). Therefore the alginate network which negatively affects the beads porosity will form faster and inhibit the amino acid loss (Bellich, 2011).

However as the calcium is diffusing into the beads, the amino acids are diffusing out of the beads at a greater rate as indicated by the higher diffusion flow rates. Therefore there may be no significant effect shown by the residence time in the gelling solution, as the majority of the amino acids have already diffused out of the beads by the five minute time point, thus the twenty minute time point will show no further effect.

The percentage recovery from the experiments, between 30 and 52%, although better than the pilot studies, were still sub optimal and therefore further enhancements were needed to the bead production method.

4.4 Second design of experiment

In these experiments the findings from the previous experiments were used to update the Plackett Burman factorial design. The three factors and outputs used were the same ie. Alginate, calcium chloride, residence time in the gelling solution and recovery however the levels were changed (table 3.4). The levels of alginate were increased from 1 to 2%; to 1 to 3% as from the previous experiment it was shown that the level of alginate had a significant effect. Although the residence time was shown not to be significant it was hypothesised that this was due to the residence times of 5 to 20 minutes being too long with the majority of the amino acids already diffusing out of the beads within 5 minutes. Therefore the levels for the residence time were reduced to 1 and 4 minutes. As the residence time had been reduced it was necessary to increase the rate of diffusion of the calcium so that the calcium can diffuse to the core and fully gel the bead without the amino acids diffusing out. The levels of 10 to 30% were chosen so that the rate of calcium diffusion was higher than that for the amino acids (table 4.2).

Table 4.2 Calculated diffusion flows for amino acids and calcium

Material	Diffusion flow mol/m^2s^{-1}
Alanine (1.36% sol)	-2.51×10^6
Glycine (1.31% sol)	-3.39×10^6
Calcium (10% Calcium chloride sol)	-2.04×10^6
Calcium (30% Calcium chloride sol)	-5.9×10^6

As in the previous experiments in addition to the two levels (calcium 10 and 30%, alginate 1 and 3% and residence time of 1 and 4 minutes), centre points of 2% alginate, 15% calcium and 2.5 minutes were included to increase the accuracy of the model and to check for linearity. Once the beads were manufactured the nitrogen was measured by the Dumas method and the percentage recovery calculated and inputted into the model.

From the output a Pareto chart and main effects plot were produced (figures 3.5 and 3.5) and it was seen that levels of alginate and residence time had an effect on the output as both were above the

line of significance on the Pareto chart and were not horizontal on the main effects plot. The level of alginate had the greatest effect overall with the steepest gradient in the main effects plot and the largest magnitude on the Pareto chart. The level of calcium was not significant as its level was below the significant line on the Pareto chart and was almost horizontal on the main effects plot.

The effect of the alginate was similar to the effect in the first experiment with larger amount of alginate producing denser networks which reduced the beads permeability and further restricted the diffusion of the amino acids.

The effect of the residence time was different to what was observed in the first experiment in which the residence time was not significant. Therefore the significance of the reduced residence time in this experiment shows that the amino acids rapidly diffused out of the beads. Hence the hypothesis that the residence times used in the first experiment were too long was correct.

The calcium level which was a significant factor in the first experiment was not significant in this experiment. The lack of significance can be explained, as although the increase in the level of calcium lead to an increase in the rate of diffusion (table 4.2) there was only a finite number of binding sites available on the alginate for the calcium bind to and gel (Blandino, 1999). Once all the sites were occupied and the matrix fully formed, any further increase in calcium did not affect the matrix and did not change the porosity. Thus the effects of the higher level of calcium were not seen as the matrix had fully formed and gelled within a minute. On visual examination of the beads it was noted than after only 1 minute in the gelling solution there was no visual difference between the beads formed with different levels of calcium, and all the beads appeared gelled and firm

Therefore the reduction in residence time with an increase in calcium had a positive effect on amino acid recovery allowing for the calcium to diffuse at a sufficient rate to gel the beads before the amino acids had time to diffuse out of the bead.

To model the relationship between the 3 factors (predictors) and the output, a linear regression model was used. However before this could be completed it was first necessary to determine if the residuals were normally distributed as if the residuals are non-normally distributed the output of the regression analysis cannot be trusted (Frost, 2014). Therefore a normal plot of the residuals was produced, (figure 3.6), and as the plots were close to the normal distribution line with no outliners the residual were normally distributed.

The residuals were also plotted in order to determine if the output was derived from stochastic or deterministic errors. This was important as for the model to work correctly the randomness and unpredictability of stochastic errors was vital (Frost, 2012). On examining the residual plot (figure 3.7) the residuals had a constant spread throughout the range with no values systematically high or low , therefore the errors were stochastic and no deterministic errors were affecting the residuals (Frost, 2012). However an R^2 value of 88.8% and an adjusted R^2 value of 85% were achieved (table 3.5) for the model. In order to achieve higher R^2 values and a more accurate model the stochastic errors needed to be reduced. This could be achieved by increasing the amount of replicates within the design of experiment and should be incorporated into any further work.

To increase the model accuracy other regression models were investigated. When continuous predictors and outputs are used only linear and nonlinear models can be used. However nonlinear

regression analysis is difficult to achieve due to the large amount of functions that are available and therefore only linear regression was used (Frost, 2011).

Another factor that was considered was multicollinearity, where predictors are correlated with other predictors. This leads to an increase in the standard errors of the regression coefficients of the predictors and can overinflate them making some variables statistically insignificant when they should be significant. To check for multicollinearity the variance inflation factor (VIF) was calculated, if the VIF was greater than 1 then there would be evidence of multicollinearity (Martz, 2013). The VIF was equal to 1 for all predictors in the model and therefore there was no multicollinearity present.

The regression equation was determined with regression coefficients, associated P values and their standard errors (table 4.3)

Table 4.3 Regression analysis outputs

Predictor	**Coef**	**SE Coef**	**P**
Constant	46.638	5.705	0.000
Calcium	0.3288	0.1668	0.080
Alginate	10.194	1.668	0.000
Time	-6.067	1.112	0.000

The regression equation is

Percentage recovery of Amino acids = 46.6 + 0.329 Calcium + 10.2 Alginate - 6.07 Time

From the regression equation it can be seen that the alginate has the largest effect on the output with a 10.2% increase in the output per unit of alginate increase. The residence time has the next largest effect on the output, for every unit of time decrease there is a 6.07% increase in the recovery (it was a decrease as residence time was negatively correlated). Calcium has the lowest effect on the output with only a 0.329% increase in recovery per unit increase in calcium. The P values for the alginate and residence time are below 0.05 which shows that changes to these predictors values resulted in significant changes to the output. However the P value for the calcium is >0.05 with a value of 0.08, which can indicate that changes to this predictor did not have a significant effect on the output. This was expected as the Pareto chart and main effects plot did not show significance for calcium level therefore the regression equation was recalculated excluding calcium (table 4.4)

Table 4.4 Regression analysis outputs (calcium omitted)

Predictor	**Coef**	**SE Coef**	**P**
Constant	53.213	5.254	0.000
Alginate	10.194	1.893	0.000
Time	-6.067	1.262	0.001

The regression equation is

Percentage recovery of Amino acids = 53.2 + 10.2 Alginate - 6.07 Time

As can be seen the regression coefficients for the predictors have not changed, but there has been an increase in the regression constant. The P values for all the predictors are still <0.05 and are therefore still significant. Therefore from the regression equation it was seen that for the highest recovery the sample needs the highest alginate concentration and needs to be in the gelling solution for the shortest time period. The samples produced with 1 minute residence time and 3% alginate produced the best recovery of 78% and 84 % which was acceptable.

4.5 Digestion modelling

For the digestion modelling an *in vitro* model was used. Due to the elemental composition of the beads and the insoluble fibre nature of the alginate no digestive enzymes were used. However simulated gastric and intestinal solutions were used with a simulated gastric digestion period of 90 minutes and a simulated intestinal digestion period of 120 minutes carried out at 37^{o}C to model the conditions of *in vivo* digestion (Rayment, 2009). The nitrogen content of the beads was measured pre and post digestion to determine if the beads released the amino acids.

After the digestion it was seen that the amino acid content of the beads had reduced from an initial amino acid content of 7.9g / 100g beads to 0.85g / 100g of beads, however the beads themselves were visually unaffected by the digestion.

This can be attributed to the porous nature of the beads allowing the amino acids to diffuse out of the beads into the digestive fluids. It is known that due to alginate having a pKa of around 3.5, under acidic gastric conditions there is a change in the electrical characteristics, thus causing the alginate to lose its negative charge (Li, 2011). This results in the calcium ions dissociating from the gel junction zones allowing the alginate chains to come closer to each other causing the beads to contract and become less porous thus hampering diffusion. However in the more neutral intestinal conditions the beads will swell and become more porous thus allowing diffusion to occur at a higher rate (Rayment, 2009). From the second and third pilot experiments it was known that the amino acids will diffuse out of the beads quickly thus the 210 minute digestion time was adequate for 89% of the amino acids to diffuse out. The beads were not physically affected by the digestion which can be attributed to the alginate matrix being resistant to digestion. This has been seen in *in vivo* studies where the beads were not broken down in digestion and visible on an NMR in the small intestine (Hoad, 2009).

To determine the amount of amino acids digested the nitrogen content of the beads pre and post digestion was measured by Dumas, however amino acids contain differing amounts of nitrogen (FAO, 2002). Therefore measuring the nitrogen would not show if the amino acid composition of the bead was similar to the desired amino acid profile. A reduced nitrogen content, post digestion, could indicate a difference in the retention of particular amino acids within the beads, or that all the amino acids diffused out of the beads equally but not completely. It was possible to measure amino acid composition by ion exchange chromatography, however this technique used a solvent extraction step that was not validated for the beads so could not be used. Therefore although the physical beads were not digested and amino acids diffused out and thus were bioavailable, the composition of the amino acids is unknown.

4.6 Food safety and sensory testing

Before conducting the sensory testing it was necessary to check that the beads were safe for consumption, therefore the calcium and chloride content was measured by inductively coupled plasma spectrophotometry and a 926 Corning chloride analyser and compared to the dietary reference intake for an adult population. Although the calcium and chloride content of the beads were considerable, the amount of beads consumed in the sensory study was small therefore the amount of calcium and chloride consumed was below the reference nutritional intake and therefore was deemed safe.

The beads were then exposed to a triangle test against a placebo product. The placebo product contained maltodextrin in place of the amino acids in the beads manufacture, utilising the same carrier. The output from the triangle test was that of the 12 participants, 6 correctly identified the different sample and 6 incorrectly identified the different sample. The amount of participants required to correctly identify the sample for significance at a 95% confident level was 8, therefore as only 6 participants correctly identified the sample it can be concluded that the amino acid bead is not significantly different to the placebo bead, and therefore the encapsulation has successfully masked the amino acids.

5.0 Conclusion and recommendations

From the experiments it can be concluded that to produce beads the alginate content, calcium chloride content and the residence time in the gelling solution are critical for both the physical manufacture of the bead and the retention of the amino acids within the bead. The most successful beads in the study were the beads produced using a feed stock comprising 10% amino acids and 3% alginate dropped into a 10% calcium chloride solution with a residence time of 1 minute.

Due to the effect on viscosity of the feed stock resulting in poor beads it was not possible to investigate the effect of higher amino acid contents. This could, however be achieved if different techniques were utilised for bead manufacture which would accommodate higher viscosities such as spinning discs, instead of the dripping method used.

Although physical bead characterisation was conducted using laser particle diffraction techniques this was limited to the size of the beads. It may be useful to use scanning electron microscopy and transmission electron microscopy in order to examine the surface topography and internal structure of the beads (Burey, 2008).

Through the *in vitro* digestion model it was shown that although the amino acids were successfully encapsulated in the beads they were liberated during digestion and were bioavailable. Ideally an *in vivo* study would be conducted to validate the model, however *in vivo* studies are expensive and time consuming to undertake. The *in vitro* study utilised the nitrogen content to determine the level of amino acids liberated, it may be beneficial for further investigations to use different analytical techniques to determine the amino acid composition post digestion rather than relying on nitrogen content alone.

In the sensory study utilising a triangle test it was demonstrated that there was no significant difference between the amino acid containing beads and a placebo product. Although the triangle test used trained panellists that produce more accurate and reliable results, the number of participants was relatively low due to the manufacturing method. If it were possible to automate the manufacturing method to easily produce larger quantities of beads then a wider study with more participants could be conducted.

Although a bead was successfully produced and consumed with a carrier, no shelf life study was completed. It would be necessary to complete a shelf life study of the bead and carrier to determine any changes to the bead integrity and organoleptic properties over time.

Therefore the encapsulation of amino acids in an alginate bead can be used successfully as a delivery system for amino acid based protein supplementation for the dietary management of phenylketonuria (PKU).

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7. Appendices

Appendix 1 Nitrogen determination Dumas method

Appendix 2 – Viscosity measurement

Appendix 3 – Particle size measurement using laser particle diffraction

Appendix 4 - Inductively coupled plasma spectrophotometry

Appendix 5 – Chloride analysis

Appendix 6 - A_w analysis