Development of Analytical Methods for the Stability Assessment of Parenteral Nutrition

A thesis submitted for the degree of

Master of Philosophy in Cardiff University

by

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May 2018

Cardiff School of Pharmacy and Pharmaceutical Science

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Acknowledgements

I would like to express my immense gratitude to Allan and Bec; this truly would not have been possible without them. Their help, guidance and faith in me has been endless; they have gone above and beyond in their support and I am extremely grateful to them for all they have done for me.

I would like to thank Fresenius Kabi for providing materials and support for this project. I would like to give a special mention to Lisa Cole, whom it has been a pleasure to work with.

To Emma, Sean and Helen, whose friendship and support have seen me through this every day.

My final thanks are to my family, for their continued support and encouragement; celebrating the milestones with me and providing reassurance during the challenging times. A special thank you to my mother, father and Owen for proof reading my thesis.

Summary

Parenteral nutrition (PN) provides intravenous nutritional support to patients with reduced gastrointestinal function. A PN bag comprises the basic building blocks of the food groups: lipids, glucose, amino acids, vitamins, electrolytes and trace elements. Recently there has been an increase in demand for extended storage periods for PN bags, to ease management of an increasing home care market. Prior to a PN formulation being deemed safe for a patient, a laboratory simulation is carried out on the proposed admixture under the requested storage and administration conditions. Currently only the physical stability is assessed; physical testing provides no information on the quantity of each component remaining in the bag after storage. Consequently, there is a need for assessing the chemical stability of PN to indicate the quantity of each component that remains in the PN bag.

A commonly used amino acid product, Aminoven® 25, contains 16 amino acids; this work aimed to develop a HPLC assay capable of quantifying the amino acids in an aqueous PN bag containing Aminoven® 25. Fluorescence detection was used as it is a highly selective method of detection, which was preferable due to the number of components in PN. To detect the amino acids, as they don't naturally fluoresce, derivatization was carried out using ortho-phthalaldehyde to form a fluorescing derivative. The developed assay resulted in validation of thirteen of the amino acids in Aminoven® 25. In addition, the method was shown to be unaffected by the

presence of aqueous PN components, so this method is suitable for quantifying thirteen amino acids in aqueous PN containing Aminoven® 25. This assay can be used for assessing the stability during stability testing and confirming the quantity of amino acids after compounding for quality control release.

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List of Abbreviations

3-MPA - 3-mercaptopropionic acid

ADAM - 1-aminoadamantane

Dns-Cl - dansyl chloride

Em – emission wavelength

Ex – excitation wavelength

FDA – Food and Drug Administration

FMOC - 9-fluorenylmethyl chloroformate

GI – Gastrointestinal

HPLC – high performance liquid chromatography

ICH - International Conference on Harmonisation

LOD – limit of detection

LOQ – limit of quantification

ML – multilayer

NICE – National Institute for Health and Care Excellence

OPA – ortho-phthalaldehyde

PN – parenteral nutrition

QC – quality control

RSD – relative standard deviation

S:N – signal to noise ratio

UV – ultraviolet

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1 Introduction

1.1 Parenteral Nutrition

Parenteral nutrition (PN) provides intravenous nutritional support to patients with reduced gastrointestinal (GI) function. The National Institute for Health and Care Excellence (NICE) recommends that PN should be considered if patients are malnourished or at risk of malnutrition, when combined with restricted oral and/or enteral access and a non-functioning or perforated GI tract (NICE 2006). PN may be used as complete nutritional intake, but also to supplement patients who are unable to fully meet their nutritional requirements orally or enterally. Where possible, it is preferable to feed via the oral or enteral route; a pathway showing the stages that result in administration of PN is shown in figure 1.1. Conditions that commonly cause impairment of the GI tract include short bowel syndrome and fistulas. PN formulations can be administered to neonates, paediatrics and adults on a short or long-term basis, in hospital and at home. Individual requirements are determined by numerous factors, for example: patient weight, activity level, underlying conditions and location of catheter. Patients who require PN on a long-term basis, usually at home, have an individualised regimen and are regularly monitored by a multidisciplinary team for signs of malnutrition. Short bowel syndrome is the most common indication in the UK for home PN, accounting for 59 % of all home PN patients in 2010 (Smith *et al.* 2011). In 2010 the British Artificial Nutrition Survey found that in the UK approximately 800 people were receiving PN at home. The modal age for home PN patients in the UK is 51-60 years of age (Smith *et al.* 2011).

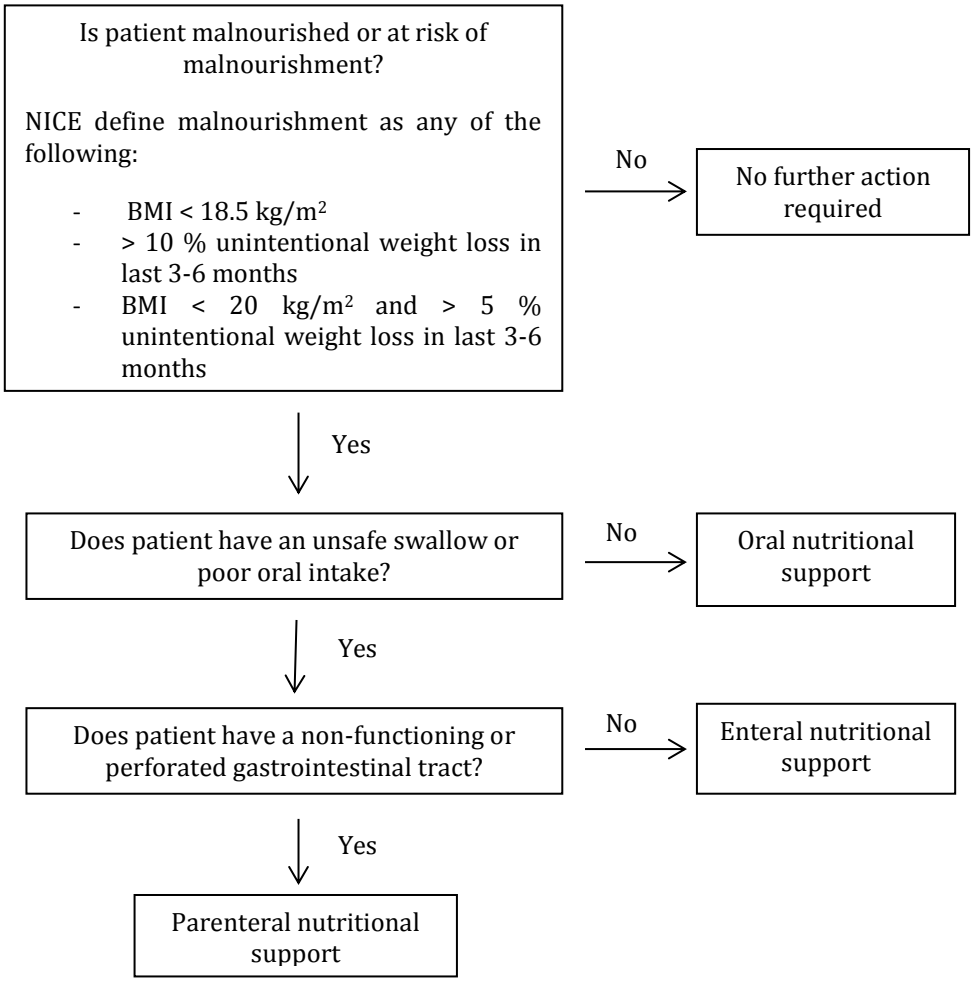


Figure 1.1 Overview of decision pathway leading to prescription of PN (Adapted from (Manuel & Maynard 2009)).

1.2 Components of Parenteral Nutrition

In order to meet the nutritional requirements of a patient receiving PN, the admixture comprises the basic building blocks of the necessary food groups. PN admixtures may include amino acids, lipids, glucose, water, electrolytes, vitamins, and trace elements; this complex formulation can result in upwards of 50 components being mixed together. Generally speaking there are two types of PN, aqueous and lipid; the main difference being the inclusion of lipid products in lipid PN.

1.2.1 Amino Acids

Humans cannot store amino acids, unlike fat and glucose, so they must be provided readily from nutrition. NICE (2006) recommends a nitrogen intake of 0.13-1.24 g/kg/day for adults receiving PN. ESPGHAN (2005a) recommends 1.5 to 4 g/kg/day for preterm infants and 1 to 2.5 g/kg/day for children. The amino acid requirements of a patient receiving PN is reduced compared with that of a patient obtaining nutrition orally or enterally; it is estimated that 30-50 % of ingested amino acids are not available to extra-intestinal tissues as they are utilised within the intestinal mucosa (Wu 1998). Duran (2005) found that parenteral feeding causes minimal intestinal atrophy in infants receiving PN; this is similar to findings by Raul et al. (1984), who studied parenterally fed rats.

1.2.2 Lipids

Lipid emulsions are included in PN as an energy source and to provide essential fatty acids, which are those that the body cannot synthesise; these are linoleic and α-linolenic acids. Currently there are a number of lipid emulsions available, where the oil is derived from plant or animal sources, to provide a variety of hydrocarbon chain lengths. Soybean oil is commonly found in PN lipid emulsions to provide the essential fatty acids linoleic acid and α-linolenic acid. Emulsions may also contain palm kernel oil, olive oil and fish oil. Lipid emulsions used in PN have an average globule size diameter of 0.25-0.5 μm, similar to the diameter of naturally occurring chylomicrons (Pertkiewicz and Cosslett 2000).

1.2.3 Glucose

Glucose is not essential due to human's endogenous ability for gluconeogenesis; however, it is used as a source of calories in PN. Excess glucose intake can cause lipogenesis, resulting in fat deposition (Wolfe *et al.* 1980). In addition, high levels of blood glucose have also been linked to an increase in infection related mortality in patients receiving PN (Berghe 2004). In the UK, intravenous glucose solutions are available in a range of concentrations, 5 % to 70 %; this allows glucose and volume needs of a patient to be met.

1.2.4 Electrolytes

Electrolytes included in PN are: salts of sodium, potassium, calcium, magnesium and phosphate. Requirements are met with additions of individual salt solutions; the monovalent cations in PN, sodium and potassium, are primarily sourced from chloride salts. Magnesium is added as magnesium sulphate. There are a variety of calcium and phosphate containing products available as these polyvalent ions particularly affect stability of PN; providing a range of products delivers the greatest likelihood of providing PN that is both clinically appropriate and physically stable.

1.2.5 Vitamins

Vitamins are categorised into fat-soluble and water-soluble vitamins. Fat soluble vitamins include A, D, E and K; water soluble vitamins include B vitamins, and vitamin C. Products containing fat-soluble and water-soluble vitamins, separately and together, are available.

1.2.6 Trace Elements

There are fifteen trace elements that are known to have physiological roles, however, only ten are generally considered to be essential to consume and included in PN: zinc, copper, selenium, chromium, iron, manganese, molybdenum, fluorine, cobalt (as a component of hydroxocobalamin) and iodine.

1.3 Physical Stability of Parenteral Nutrition

The large number of components in a PN formulation results in a high likelihood of interactions between components; so assessing the stability of an admixture is paramount when considering patient safety. As increasing numbers of patients manage their PN at home there is an increasing demand for extended storage times, resulting in reduced waste and less frequent deliveries, which in turn reduces costs. The primary physical stability issue when considering aqueous PN is the formation of precipitates; for lipid PN it is the integrity of the lipid emulsion. Precipitates in aqueous PN are commonly caused by reactions involving electrolytes, and less commonly trace elements. In 1994 the Food and Drug Administration (FDA) issued a safety alert due to two deaths and two patients in respiratory distress that occurred as a result of receiving PN. Autopsies revealed that the two deceased patients presented with microvascular pulmonary emboli containing calcium phosphate. Other reported deaths due to the infusion of calcium phosphate have been documented (Reedy 1999; Hill et al. 1996). Instability of a lipid emulsion

can ultimately cause the lipid emulsion to crack, resulting in free oil. The FDA and British Pharmaceutical Nutrition Group recommend the use of in-line filters during the administration of PN to effectively protect the patient from microbial contamination and stability problems related to PN. 0.2 μm and 1.2 μm filters are recommended for aqueous and lipid PN respectively.

1.3.1 Physical Stability Issues

Aqueous PN instability is likely to result in precipitate formation; the most researched and documented is calcium phosphate. As previously mentioned, there have been several fatalities reported due to the infusion of PN containing calcium phosphate precipitates. Calcium phosphate precipitation mainly occurs in admixtures with particularly high concentrations of calcium and phosphate; these include small volume regimens for adults with a low fluid requirement, infants and neonates (Ronchera-Oms *et al.* 1996). Calcium phosphate formation is affected by a complex interaction of numerous factors: pH, glucose concentration, amino acid composition and concentration, source of calcium and phosphate salts, time elapsed since admixing and temperature (Pertkiewicz and Cosslett 2000).

It is widely accepted that pH is the most important influence on the formation of calcium phosphate precipitates due to its effect on the phosphate ions. In PN, monobasic and dibasic phosphate ions can exist; monobasic ions are commonly seen at a lower pH (< 6.4), whereas dibasic

phosphate ions are formed at higher pH. Formation of dibasic calcium phosphate is not immediate and occurs during storage of the PN bags (Pertkiewicz and Cosslett 2000). Calcium phosphate formed with monobasic phosphate has a solubility of approximately 18 g/L, whereas when formed with dibasic phosphate it has a solubility of approximately 0.3 g/L (Parikh *et al.* 2005). The addition of amino acid solutions decreases the pH of PN and reduce the likelihood of calcium phosphate formation. Furthermore, amino acids interact with electrolytes to form soluble complexes, thereby improving stability by reducing the number of free calcium and phosphate ions (Manning and Washington 1992).

In addition to calcium phosphate, trace elements have also been linked to precipitation issues in PN; though little research has been carried out to identify their prevalence and mechanisms. Allwood et al. (1998) have shown that a reaction between copper and cysteine is more likely when there are no vitamins and air is present; suggesting that vitamins and the exclusion of air have a protective effect. The authors also demonstrated that a reaction between iron and phosphate is less likely with an increased amino acid concentration and the addition of vitamins. Manganese and zinc have been reported to react with phosphate to form precipitates (Allwood and Greenwood 1992). Finally, selenium has been shown to precipitate in PN. Selenium is added to the admixture as the soluble selenite ion; which is selenium in the +IV oxidation state. In PN, ascorbic acid reduces selenite to insoluble selenium; the effect is more pronounced in the presence of copper (Postaire *et al.* 1989).

In lipid PN the lipid emulsion is at risk of physical instability, and may present in three stages: creaming, coalescence and cracking. Creaming presents as a white layer at the top of a bag; coalescence is the generation of larger lipid droplets; cracking occurs when free oil is released from the emulsion. Creaming is safe if the cream layer disperses when agitated; coalescence and cracking are irreversible and the bag is unsafe to administer (Pertkiewicz & Cosslett 2000). High concentrations of amino acids and glucose improve lipid stability and reduce the tendency for creaming.

1.3.2 Extrinsic Factors Affecting Parenteral Nutrition Physical Stability

The physical instability in PN discussed above is influenced by extrinsic factors. These include light, temperature, bag material and method of filling.

Light and temperature can catalyse reactions and increase degradation rates (Hempoonsert *et al.* 2010; Allwood 2000; Keerati-u-rai and Corredig 2009); to overcome these issues bags are stored in pharmaceutical refrigerators after compounding and light impermeable covers can be used over the bags during administration. Refrigerating PN can extend the shelf life, which is especially important for allowing transportation and delivery time for home PN patients.

Bag material plays a role in PN stability; ethylene vinyl acetate bags are semi permeable to atmospheric gases, allowing gases to dissolve in PN (Allwood *et al.* 1996), which may react with the components of PN. This

problem can be addressed in two ways: the compounded bag can be overwrapped in a gas impermeable material or the bag can be manufactured from a gas impermeable material (Allwood *et al.* 1996). Multilayer (ML) bags are commonly used as they are impermeable to gases, so prevent gases entering the bag after compounding.

The method of filling affects the stability of an admixture as it determines the level of aeration during manufacture (Ball and Barnett 1996). The PN bag can be made using a compounder, or made manually. Compounders introduce more air which can react with components of PN and with ML bags becoming more common place, any dissolved air is retained in the bag. Mixing order can also have a detrimental effect on the stability of PN; for example, calcium and phosphate products should not be added consecutively as it is likely that calcium phosphate will form. Care must be taken to ensure lines are flushed between products that are known to react.

1.3.3 Stability Indicating Methods

Currently only the physical integrity of a PN formulation is assessed to determine whether it is safe for administration. Stability testing procedures for aqueous PN involves visual inspection and monitoring the change in turbidity, pH and colour of the solution; tests for lipid PN are visual inspection, particle size analysis, microscopy and pH. Once the formulation has been shown to be stable, it may be replicated by homecare companies or hospitals for administration to patients. PN compounded for

patient infusion is subject to more straightforward QC tests; before release for use the bags are weighed and visually inspected.

During stability testing there is no routine quantification of individual components prior to deeming an admixture safe for a patient. Recently there has been an emerging interest in the chemical stability of individual components in PN. There are two aspects to monitoring the chemical stability; firstly, to ensure that the patient is receiving the expected dose, and secondly to be aware that if there is significant degradation this will result in degradation products, some of which could be harmful. One addition to PN that has received little attention is the amino acids, they are important for the integrity of the PN formulation and for the patient so need researching.

1.4 Amino Acids

1.4.1 Amino Acid Biochemistry

Amino acids are characterised by a primary amino group, a carboxyl group and a hydrogen atom attached to the α-carbon atom; the general formula for an amino acid is shown in figure 1.2.

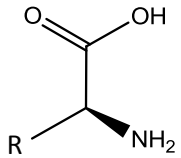
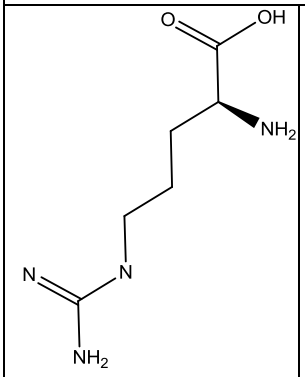
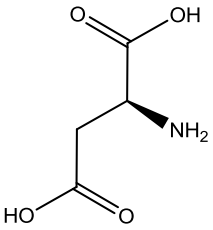
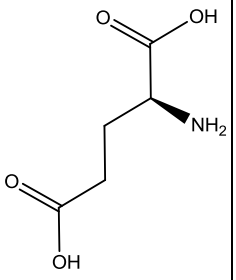
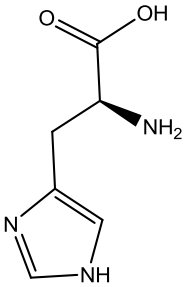
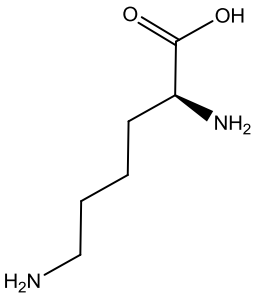
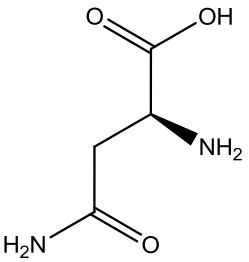
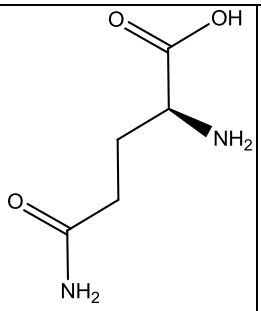
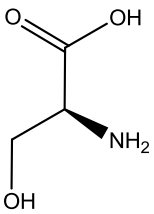
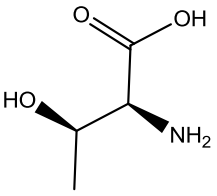


Figure 1.2 General formula of an amino acid

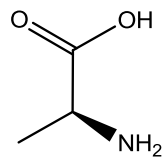
The R group of an amino acid is charged, polar or non-polar in nature and may be aliphatic, aromatic or heteroaromatic. The different structures of the amino acids are demonstrated in table 1.1. All amino acids are chiral, except for glycine as its R group is a single hydrogen atom. Chirality results in two enantiomers, for amino acids these are termed L and D; the L-configuration is naturally occurring.

There are two compounds that are included when discussing amino acids that are not truly amino acids. Proline is classified as an imino acid, however is generally considered as an α-amino acid, whereby the amino group is involved in the closure of the pyrrolidine ring. Taurine is not an amino acid as it does not have a carboxyl group, but it is often referred to as one; as it does contain an amino group, more correctly it is an amino sulphonic acid.

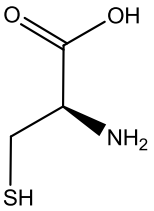
Table 1.1 Structures of amino acids

Charged side chains			
 Arginine	 Aspartic Acid	 Glutamic Acid	 Histidine
 Lysine			
Polar side chains			
 Asparagine	 Glutamine	 Serine	 Threonine

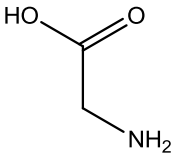
Non-polar side chains



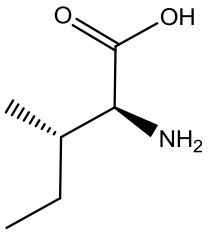
Alanine



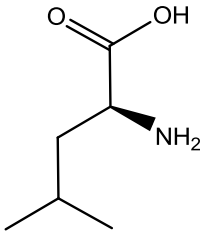
Cysteine



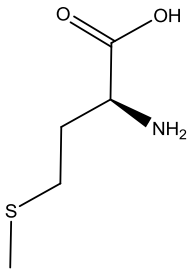
Glycine



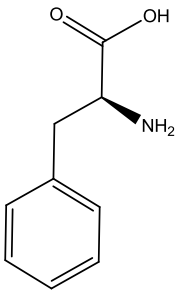
Isoleucine



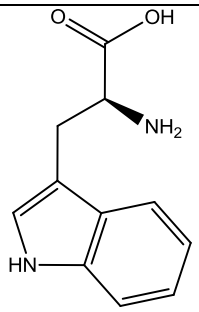
Leucine



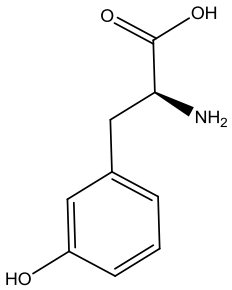
Methionine



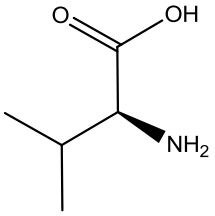
Phenylalanine



Tryptophan

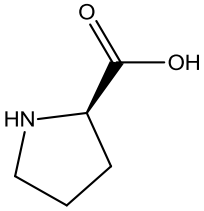


Tyrosine

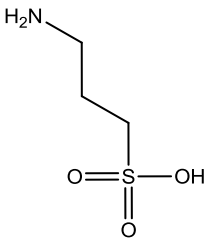


Valine

Other



Proline



Taurine

1.4.2 Amino Acids in Nutrition

Amino acids are the building blocks of proteins, and are involved in numerous roles within the body. Amino acids are categorised as essential, non-essential and conditionally essential. Essential amino acids are those which cannot be synthesised by the body; non-essential amino acids can be synthesised by the body, often from other amino acids; conditionally essential amino acids are those which are essential for some patients, often resulting from an underlying condition or due to age. Deficiency in an essential amino acid results in the body breaking down its own protein to obtain the amino acid. Humans can endogenously synthesise twelve of the twenty-one required amino acids, the remaining nine, the essential amino acids, must be provided by food intake. The essential amino acids are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The non-essential amino acids are alanine, asparagine, aspartic acid, glutamic acid, serine and taurine.

Several amino acids are considered conditionally essential: arginine, glycine, cysteine, tyrosine, glutamine and proline. Endogenous arginine and glycine synthesis does not compensate for inadequate intake or depletion, so can be required from nutrition (Castillo et al. 1994; Wang et al. 2013). New-borns require exogenous cysteine until they mature enough to synthesise the amino acid from methionine (Gaull *et al.* 1972), therefore cysteine is considered conditionally essential for this age group. Patients with liver disease also require exogenous cysteine. In addition, patients with liver disease require exogenous tyrosine; it may also be

essential for premature infants (Manning and Washington 1992). Glutamine is considered conditionally essential for patients whose bodies are in a stressed state and unable to meet the glutamine demands (Lacey and Wilmore 1990). Whilst proline is often categorised as a conditionally essential amino acid, no information detailing the instances when it becomes essential have been found.

1.4.3 Amino Acids in Parenteral Nutrition

Amino acids are generally thought of as improving the stability of PN, however little is documented about their susceptibility to reactions that may result in their degradation.

Amino acid solutions exert a protective influence on PN admixtures due to their buffering capacity (Pertkiewicz and Cosslett 2000); increased concentrations of amino acids improve the protective effect by enhancing the buffering capacity (Allwood and Kearney 1998). Amino acids solutions also decrease the pH of a PN admixture, which decreases the likelihood of calcium phosphate forming; the buffering capacity of amino acids helps maintain the lower pH.

Glutamine is unstable in solution and is not soluble at high concentrations; consequently, it is not included in standard amino acid solutions. In order to meet the needs of severely ill patients, for whom glutamine may have become conditionally essential, manufacturers have developed synthetic dipeptides of glutamine and alanine (Dunnett 2007). The synthetic dipeptides are stable in solution but rapidly hydrolyse in the plasma after

intravenous infusion, thereby allowing the inclusion of glutamine in parenteral nutrition (Ackher *et al.* 2000).

One study has shown that whilst riboflavin has a protective effect on glycine and leucine, it has a detrimental effect on methionine, proline, tryptophan and tyrosine (Bhatia *et al.* 1983).

A well-documented reaction involving amino acids is the Maillard reaction; the amino group of an amino acid reacts with the aldehyde group of a reducing sugar (glucose in PN). The reaction is enhanced with time, elevated temperatures and increased pH (Mirtallo *et al.* 1981). The Maillard reaction is characterised by a browning effect, this colour change occurs at later stages of the reaction and also indicates that insoluble compounds have formed (Laegeler *et al.* 1974). The initial stages of the reaction are normally colourless, so observing colour change is not an ideal method for monitoring the initial stages of the Maillard reaction. In the later stages of the Maillard reaction, when the solution has become dark brown, the reacted amino acid has been destroyed (Moughan 2003). It is possible for the early stages of the reaction to be reversed under laboratory conditions using a strong acid; however, this reversion cannot be achieved in the body. It has been shown that when Maillard products are given parenterally approximately 90 % of the products are excreted in the urine (Stegink *et al.* 1981). The presence of Maillard products in PN has also been linked to an increase in zinc excretion; most likely due to zinc chelating with the Maillard products (Freeman *et al.* 1975).

A study by Fry and Stegink (1982) investigated the formation of Maillard products in a formulation of heat sterilised glucose and amino acids, with and without electrolytes, for one, seven and thirty days at 4 ^{o}C and 25 ^{o}C. The greatest average loss was 7.43 % which was seen after thirty days at 25 ^{o}C. The authors found that serine, threonine, tryptophan and methionine were the most likely to form Maillard products.

Amino acids are also susceptible to photo-oxidation, which, similarly to the Maillard reaction, can be identified by a darkening in colour over time (Allwood *et al.* 1996). Amino acids most susceptible to oxidation are cysteine, histidine, methionine, tryptophan and tyrosine (Li *et al.* 1995). In the presence of oxygen, two cysteine molecules oxidise to form cystine. Methionine is oxidized by atmospheric oxygen forming methionine sulphoxide and methionine sulphone (Patel *et al.* 2011); is has not been established if these chemicals are commonly formed in PN or if they are a potential risk to patients. In Europe, in order to extend the shelf lives of the amino acid products manufacturers purge solutions with an inert gas, for example nitrogen, and use gas impermeable packaging (Allwood *et al.* 1996), but these benefits are lost once the packaging is opened and the solution is used in an admixture. Conversely, amino acids interacting with oxygen has a protective effect for ascorbic acid; ascorbic acid is susceptible to oxidation but amino acids compete for the oxygen (Kearney *et al.* 1998). However, the effects of oxygen should be limited due to the development of ML bags. Furthermore, if light protective bags are used this would further help protect the contents of PN bags from photo-oxidation.

Bouchoud and colleagues (2010) investigated amino acid stability in a formulation for neonates, composed of amino acids, glucose and electrolytes. They reported that there was no significant loss (>10 %) in amino acid concentration over a twelve-week storage period at 2-8 ^{o}C. The authors, however, failed to detail the individual amino acids that were quantified. The amino acid solution the authors used, Vaminolact®, contains proline and cysteine which are not detected with the method used in the study, this was not mentioned by the authors. Jeppsson & Tengborn (1987) found that lipid PN formulations with amino acids, glucose, electrolytes, vitamins and trace elements degraded by less than 10 % after four weeks refrigeration followed by one day at room temperature. Another study showed that after two months refrigeration of an amino acid, glucose, electrolyte and trace element formulation, none of the amino acids had degraded by more than 10 %. However after six months refrigeration, only tyrosine, lysine and histidine retained more than 90 % of their original concentration; greatest losses were seen with alanine, proline, methionine, glycine, arginine and threonine (Nordfjeld *et al.* 1983). Whilst six months refrigeration is perhaps an unrealistic length of time that a bag would be stored, increasingly times of longer than two months are being seen.

There are a few points to be considered when quantifying the amino acids in PN. Firstly, the implications of the degradation of amino acids; loss of amino acids means the patients will not receive the prescribed dose. Secondly, which amino acids should be routinely quantified as part of PN

stability testing; the literature on amino acid stability in PN is limited with no recent data to indicate their stability. Initially it would be ideal for all of the amino acids to be quantified, and then once the most reactive amino acids have been identified in a range of formulations, it may be possible to use some amino acids as markers for stability. As there is such limited data, and the majority of studies conducted on amino acid stability in PN omit many of the available PN components from the tested formulations, it is not currently possible to identify the most reactive amino acids to use as markers. Finally, another consideration for amino acid stability is thinking about what level of degradation would be clinically acceptable. Currently there are no guidelines to determine what would be an acceptable level of degradation to permit infusion of the admixture. Commonly in the pharmaceutical industry, a loss of more than 10 % is deemed unacceptable.

1.4.4 Amino Acid Stability Indicating Methods

It is clear that the stability of amino acids in PN needs to be monitored, the next section discusses the possible methods for this. High-performance liquid chromatography (HPLC) can separate, identify and quantify analytes, which makes it the ideal method when considering how to determine stability of an analyte in a complex matrix. HPLC can be utilised to separate the amino acids from the many other components in PN, to identify and quantify the amount of each amino acid in PN. Amino acid analysers are available; which are specialist, automated HPLC systems; unfortunately, they are hugely expensive. However, HPLC systems are

commonly found in analytical laboratories. A less specialised HPLC is a more practical method of analysis than an amino acid analyser as it is less costly, more likely to be found in Quality Control (QC) labs and has numerous alternative applications. If a suitable HPLC assay was available it is likely that quantification of amino acids would be carried out in a laboratory as part of determining chemical stability of a regimen and a QC department would have more interest in using the assay to confirm the quantity of amino acids added to the bag.

Most amino acids lack intrinsic properties for adequate detection with HPLC without derivatisation. There are two well established approaches to amino acid derivatisation; ninhydrin derivatization and ortho-phthalaldehyde (OPA) derivatization.

Amino acid analysers use the ninhydrin method; amino acid analysers have been used previously for analysis in PN (Ronchera-Oms *et al*. 1995; Nordfjeld *et al*. 1983; Jeppsson and Tengborn 1987). The ninhydrin method involves ion-exchange chromatography and ultraviolet (UV) detection; this method is used by amino acid analysers. Derivatization of amino acids with ninhydrin results in the R-group being removed during the reaction so the same product is formed from each amino acid, so derivatization must occur after separation as a post-column reaction. An advantage of the ninhydrin method is it reacts with primary and secondary amino acids; however, the reaction will only occur at temperatures greater than 100 ^{o}C and the assay requires a long run time

to separate the amino acids prior to derivatization. An additional drawback to the ninhydrin method is the need for additional equipment to derivatize and detect the amino acids after separation (Wahl and Holzgrabe, 2016).

The OPA method has been used for to detect amino acids in many biological samples: blood (Bartolomeo and Maisano 2006; Frank and Powers 2007); sweat (Delgado-Povedano *et al*. 2016); neurotransmitters (Perucho *et al*. 2015). OPA has also been used to detect amino acids in foodstuffs: tea (Hung *et al*. 2010); wine (Arrieta and Prats-Moya 2012); milk (Bennett *et al*. 2016). The OPA method uses pre-column derivatization and fluorescence detection; OPA does not itself fluoresce, so there are no issues of interference from excess reagent. Fluorescence detection is more sensitive and selective than UV detection. The selectivity is an important consideration when assaying PN, as there are numerous components; the sensitivity is less important as amino acids are present in abundance in PN. In addition, the OPA derivatization process is quick and occurs at room temperature.

It has been reported that proline does not react with OPA and therefore cannot be detected with this derivatization method (Chernobrovkin *et al.* 2004; Godel *et al.* 1984). It has also been reported that whilst cysteine reacts with OPA, the product is weakly fluorescent and unstable (García Alvarez-Coque *et al.* 1989; Bruckner *et al.* 1995; Chernobrovkin *et al.* 2004; Godel *et al.* 1984).

There are alternative derivatization methods for amino acids that are less widely used, for example 9-fluorenylmethyl chloroformate (FMOC) and dansyl chloride (Dns-Cl).

FMOC has been used to analyse alanine, arginine and glutamine in supplements (Baxter and Johns 2011). The reaction between FMOC and amino acids results in the formation of hydrolysis products that have similar properties to the derivatized amino acids, so interfere with detection; as does excess FMOC. It is possible to remove the hydrolysis products and excess FMOC with 1-aminoadamantane (ADAM); as ADAM is particularly hydrophobic this will elute slowly. It has been reported that using FMOC results in insoluble products (Heems *et al.* 1998) which would need to be removed from the sample before injection as they will cause damage to the column and reduce its lifespan. Dns-Cl has been used to analyse amino acids in food samples, but the sample preparation is over an hour and it is not possible to automate the derivatization process (Jia *et al*. 2011; Tuberoso *et al*. 2015).

There are also methods which can detect amino acids without derivatization. One method that has been used to detect underivatized amino acids in breast milk is HPLC electrospray ionization tandem mass spectrometry (ESI-MS/MS); the authors developed the method as a replacement for the ninhydrin method which resulted in a reduction in analysis time from two hours to two minutes (Sanchez *et al*. 2012). While analysis times are very short, it may be more useful as an identification

method than a quantitative method as repeatability was poor. Another detector that has been used without derivatization is a charged aerosol detector (CAD), however there is still sample preparation required to acidify some of the amino acids so they can be detected with CAD (Crafts *et al*. 2011; Socia and Foley 2016). Whilst there are methods that involve no derivatization, the detectors are not commonly found in laboratories so a method would not be widely available.

Due to the variety of advantages and disadvantages of the discussed analysis methods, it is becoming more common to use a combination approach; for example, using two derivatizing reagents, OPA and FMOC (Buha *et al.* 2011; Garcia-Rodenas *et al*. 2016); or two detection methods, such as UV and CAD (Wahl and Holzgrabe 2016).

1.4.5 Method Justification

To be viable for use in any stability laboratory or QC laboratory, the method requires minimal and quick sample preparation, no specialist equipment and a detection method that detects only the amino acids in PN. Considering the requirements of the method, a pre-column method was selected over a post-column method as this reduces the amount of HPLC equipment required. From the selection of pre-column derivatizing reagents, OPA was chosen as it has a short reaction time, that occurs at room temperature. OPA has been considered the method of choice for amino acid analysis due to its simplicity, sensitivity, selectivity and short sample preparation time (Hanczkó and Molnár-Perl, 2003). Furthermore,

OPA forms derivatives of amino acids that fluoresce; the ability to use fluorescence detection over UV is important as it is more selective so less likely to suffer interference from the numerous other components in a PN formulation. There are no existing assays for amino acids in PN that use pre-column OPA derivatization and fluorescence detection. However, as this method has been utilised widely in the food industry and for biological samples, work from this field was a good basis for beginning method development for PN analysis.

An application note (VWR 2012; appendix 1) that uses pre-column OPA derivatization with fluorescence detection is used initially as it was used for foodstuffs and provides detailed information about the derivatization procedure, so practically it is easier to replicate.

1.5 Summary

Amino acids are a vital part of PN, both for the patient and the physical stability of a PN bag. However, very little is known about their chemical stability in PN, which has potential implications for patient health. Therefore, there is a requirement to have a method that can identify and quantifying individual amino acids in the presence of other PN components, which can then be used to build up a stability profile for each amino acid.

1.6 Thesis Format

The next two sections have been presented to detail method development (section 2) and method validation (section 3) separately. The method development details the adaptations to optimise the separation of amino acids in PN. Following this, the suitability and limits of the assay are reported in section 3. Both topics are then discussed in section 4.

1.7 Aims

- Develop a stability indicating assay capable of quantifying amino acids in aqueous PN that is suitable for use in a stability laboratory or a QC laboratory

1.8 Objectives

- Minimise the number of steps in a derivatization process with OPA
- Separate the amino acids in PN with HPLC and detect the fluorescing OPA derivatives in a short runtime
- Validate the method with Aminoven® 25 and compare it with an aqueous PN formulation

2 Method Development

2.1 Initial Development Plan

Initially the assay was trialled exactly as stated in the application note (VWR 2012), using Aminoven® 25, to determine whether this assay was worth continuing development. Aminoven® 25 was chosen as the amino acid solution as it is widely used and there is no published stability data. Aminoven® 25 contains the amino acids listed in appendix 2.

2.1.1 Chemicals

HPLC grade acetonitrile and methanol (Fisher Scientific, Loughborough, UK). 3-Mercaptopropionic acid (≥98%), alanine (≥98%), ammonium acetate (≥98%), arginine (≥98%), glycine (≥98%), histidine (≥98%), hydrochloric acid (~37%), isoleucine (≥98%), leucine (≥98%), lysine (≥98%), methionine (≥98%), phthaldialdehyde, phenylalanine (≥98%), proline (≥98%), serine (≥98%), sodium tetraborate (99.99%), taurine (≥99%), threonine (≥98%), tryptophan(≥99%), tyrosine (≥99%) and valine (≥98%) (Sigma-Aldrich, Gillingham, UK).

2.1.2 Sample Preparation

Derivatization was carried out using the OPA reagent, borate buffer, hydrochloric acid and methanol, the reaction is shown in figure 2.1. A thiol is required as part of the OPA derivatization reagent, the two most commonly used are 2-mercaptoethanol and 3-mercaptopropionic acid (3-MPA), the latter was chosen due to forming more stable compounds (Molnár-Perl, 2011). The OPA reagent consisted of 10 mg OPA, 0.9 ml methanol, 100 µl borate buffer and 10 µl 3-MPA; the OPA reagent was

made fresh every week due to reduced fluorescence intensity if kept longer (Buha *et al.* 2011). The borate buffer was made by dissolving 2.515 g sodium tetraborate in 100 ml water and adjusting the pH to 9.5 with 1 N hydrochloric acid, then heating in boiling water for 5 minutes; this was used until it ran out.

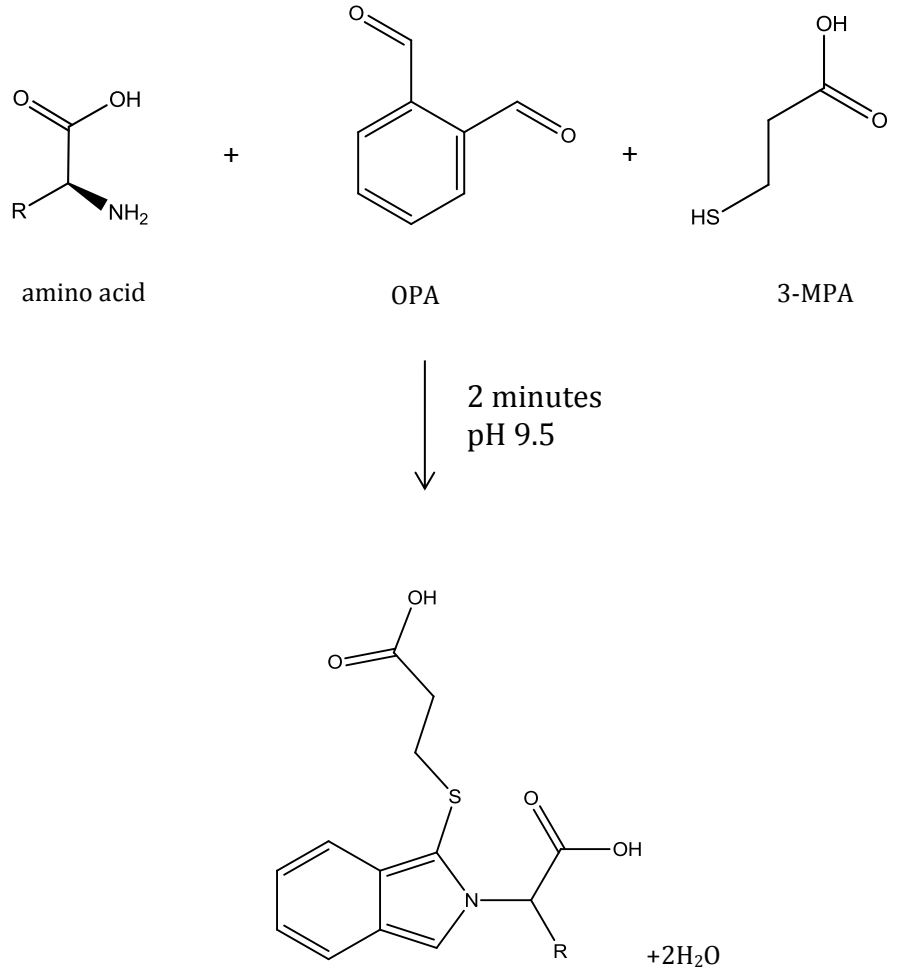


Figure 2.1 Schematic of the reaction between amino acids, OPA and 3-MPA during derivatization.

To prepare a sample for HPLC analysis, 10 µl of Aminoven® 25 (Fresenius Kabi, Runcorn, UK) was mixed with 0.5 ml 50 % methanol, 200 µl borate buffer and 100 µl OPA reagent, mixed and then left for 200 seconds. Next,

100 µl of 0.75 N hydrochloric acid was added and mixed, and then finally 0.5 ml 50 % methanol was added. This resulted in a sample too concentrated for the detector and overloaded it, causing the run to be terminated. The derivatized sample was diluted in 50 % methanol to a suitable concentration for the fluorescence detector.

2.1.3 HPLC Assay

The HPLC conditions were as follows: the run time was 65 minutes, the excitation wavelength (Ex) was 330 nm and the emission wavelength (Em) was 440 nm for the duration of the run. The injection volume was 10 µl. Two mobile phases were used in a gradient run (table 2.1): 50 mM ammonium acetate (pH 7) as A and methanol (HPLC grade) as B; with a flow rate of 1 ml/min. The ammonium acetate was prepared fresh every day. A Merck LiChroCart Purospher STAR RP-18e (5 µm) 250-4.5 mm column was used with a column oven at 40 ^{o}C. HPLC analysis was carried out on ThermoFinnigan SCM100, P2000 and F3000 and data were interpreted using Chromquest 4.2 Chromatography data system.

Table 2.1 Mobile phase gradient elution

Time (min)	%B
0	25
5	25
25	36
49	75
52	75
52.1	25
65	25

The first chromatogram of Aminoven® 25 analysed with the conditions detailed above, is shown in figure 2.2A. Before continuing with improving

separation and determining the identity of each peak, a sample of an aqueous PN formulation containing Aminoven® 25 was injected to see if there was any interference from other PN products and whether this assay looked worth pursuing (the formulation used is detailed in appendix 3). The chromatogram was largely unchanged by the presence of the numerous other components in PN, see figure 2.2B.

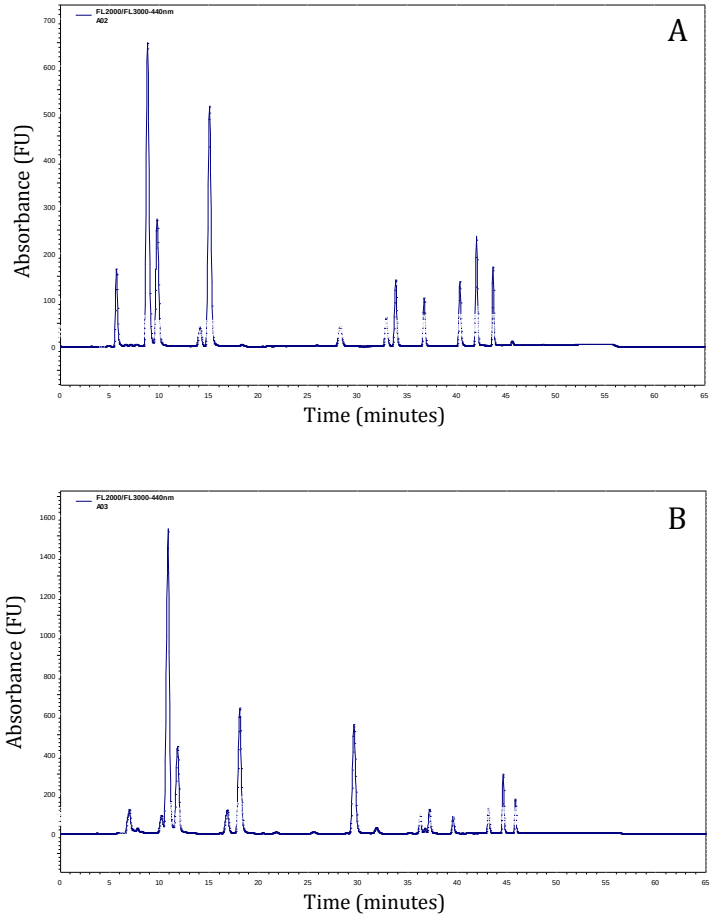


Figure 2.2 Initial HLPC analysis:
A – Aminoven® 25 derivatised with OPA
B – Aqueous PN formulation containing Aminoven® 25 derivatised with OPA

2.2 Peak Identification

Using standards for each amino acid in Aminoven® 25, work began to identify which amino acid/s corresponded to each peak. Solutions were prepared daily using a standard dissolved in water then derivatized; chromatograms for each amino acid are shown in figures 2.3 to 2.17. Aminoven® 25 was then spiked with each amino acid at twice the concentration found in Aminoven® 25.

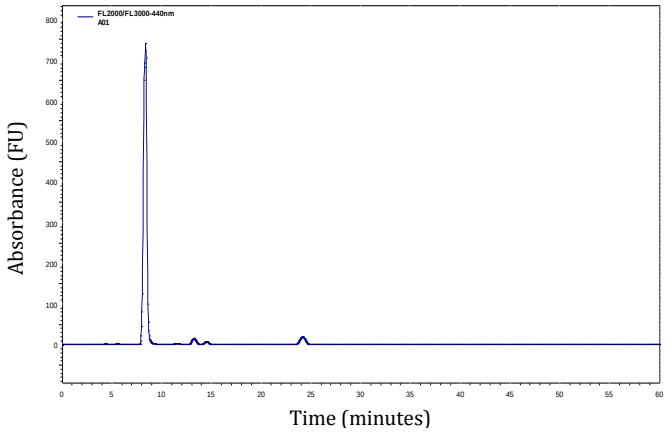


Figure 2.3 Chromatogram showing OPA derivative of serine standard

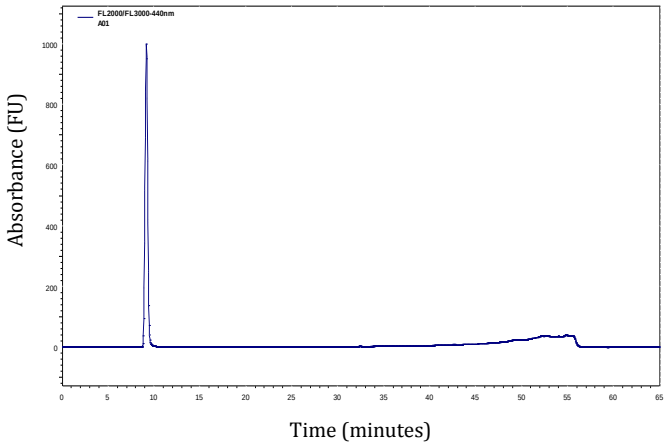


Figure 2.4 Chromatogram showing OPA derivative of threonine standard

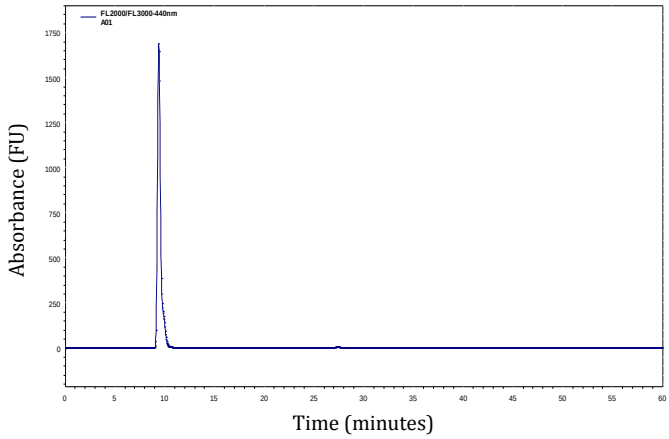


Figure 2.5 Chromatogram showing OPA derivative of glycine standard

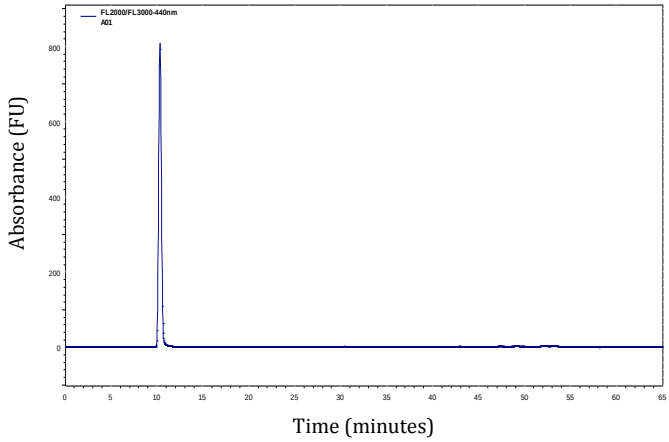


Figure 2.6 Chromatogram showing OPA derivative of arginine standard

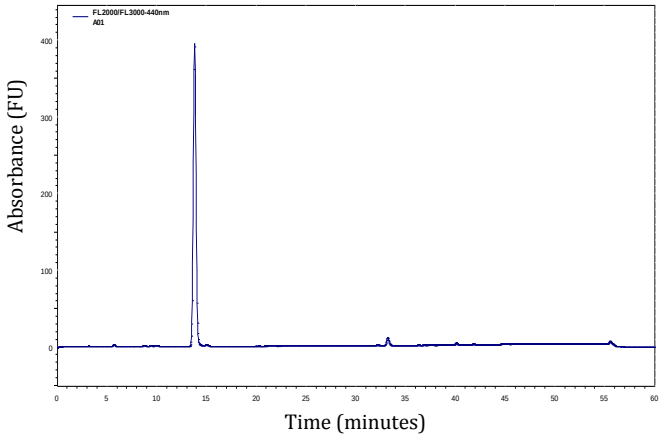


Figure 2.7 Chromatogram showing OPA derivative of taurine standard

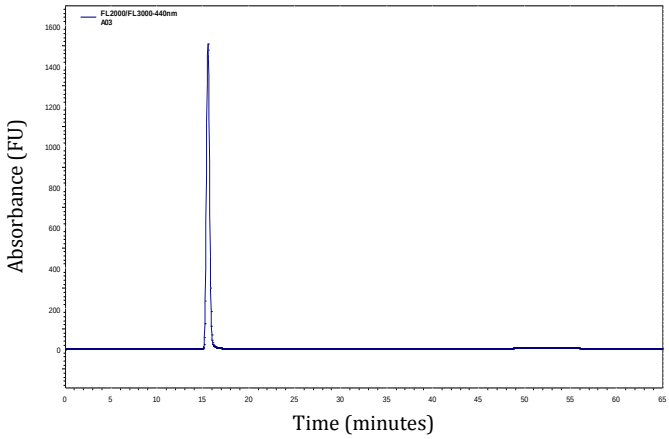


Figure 2.8 Chromatogram showing OPA derivative of alanine standard

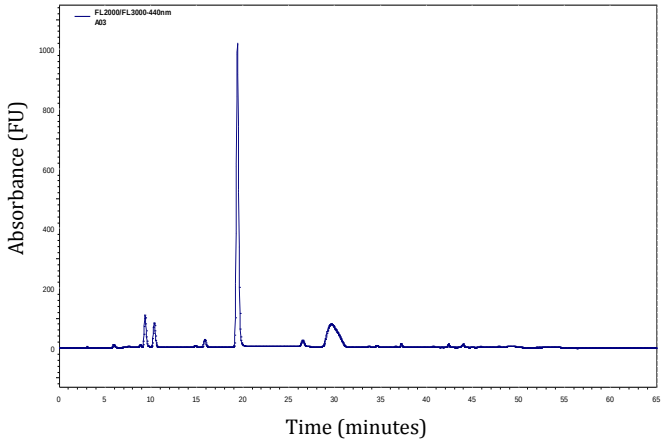


Figure 2.9 Chromatogram showing OPA derivative of tyrosine standard

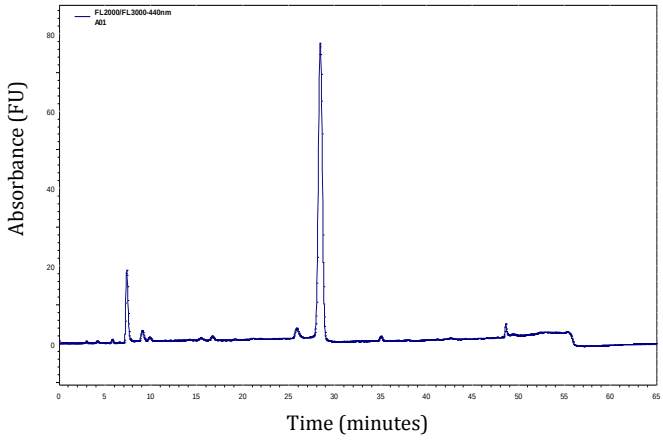


Figure 2.10 Chromatogram showing OPA derivative of histidine standard

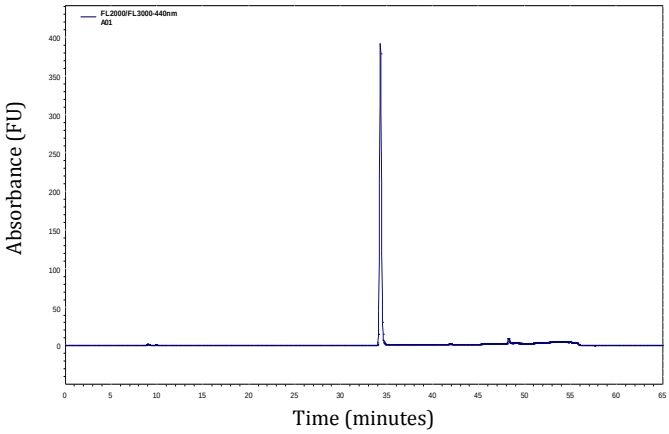


Figure 2.11 Chromatogram showing OPA derivative of methionine standard

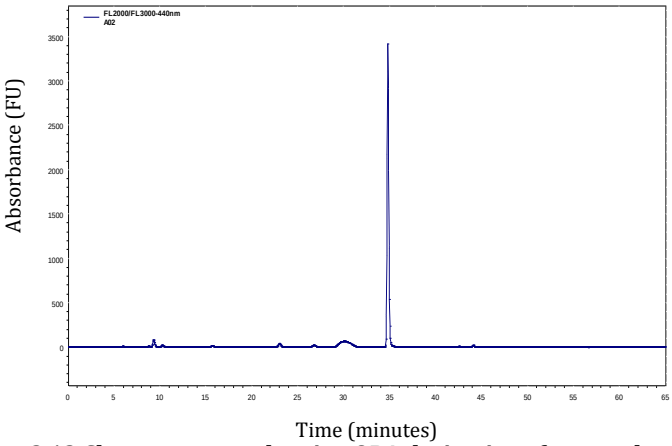


Figure 2.12 Chromatogram showing OPA derivative of tryptophan standard

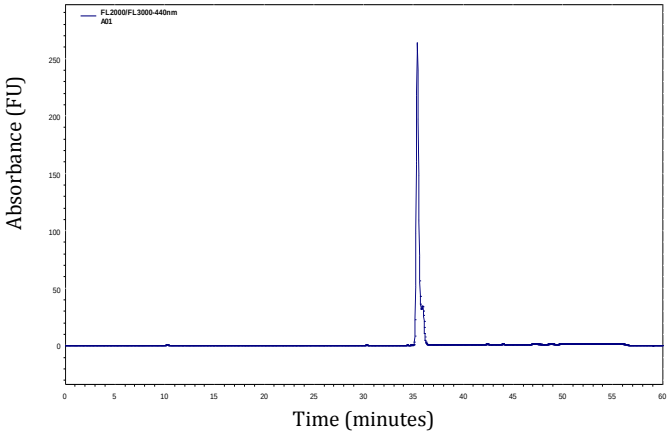


Figure 2.13 Chromatogram showing OPA derivative of valine standard

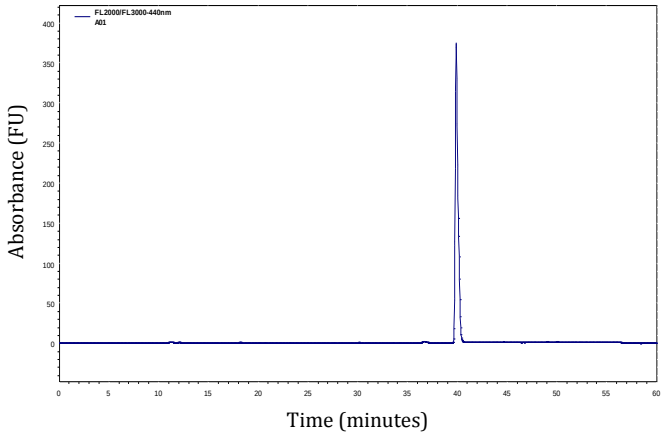


Figure 2.14 Chromatogram showing OPA derivative of phenylalanine standard

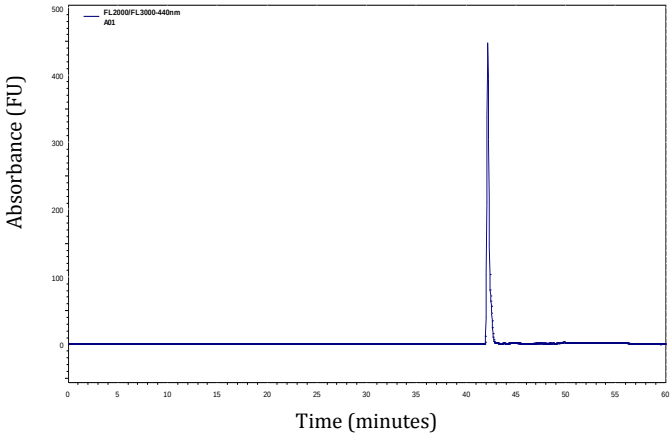


Figure 2.15 Chromatogram showing OPA derivative of isoleucine standard

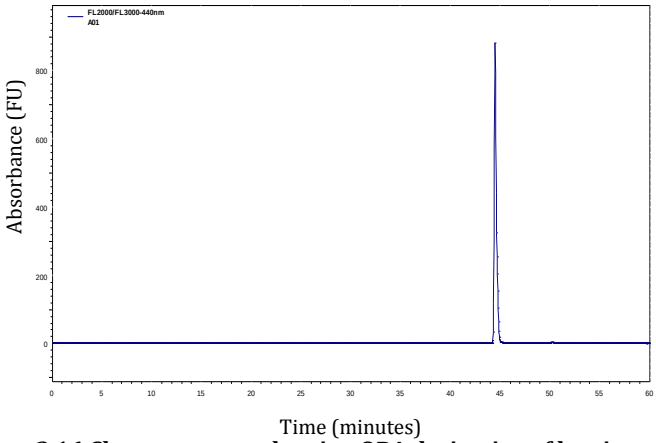


Figure 2.16 Chromatogram showing OPA derivative of leucine standard

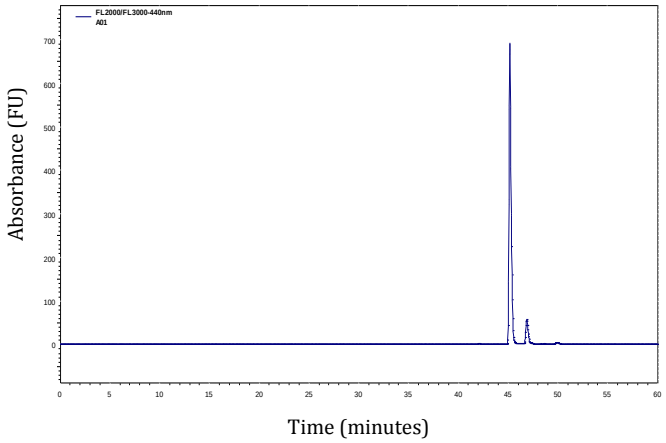


Figure 2.17 Chromatogram showing OPA derivative of lysine standard

Threonine and glycine were confirmed as co-eluting and efforts were made to separate them by adjusting the gradient. Changing the ratio of aqueous and organic phase affects the adsorption to the column; by reducing the rate that the concentration of methanol increases, glycine and threonine interact with the stationary phase for longer and are more likely to separate. This was unsuccessful despite reducing the rate that methanol increased, as well as trying isocratic elution. Reducing the temperature of the column may encourage the peaks to elute separately, but this was not attempted due to concerns that decreasing the temperature would increase the run time. A chromatogram of Aminoven® 25 with the identified peaks labelled is shown in figure 2.18.

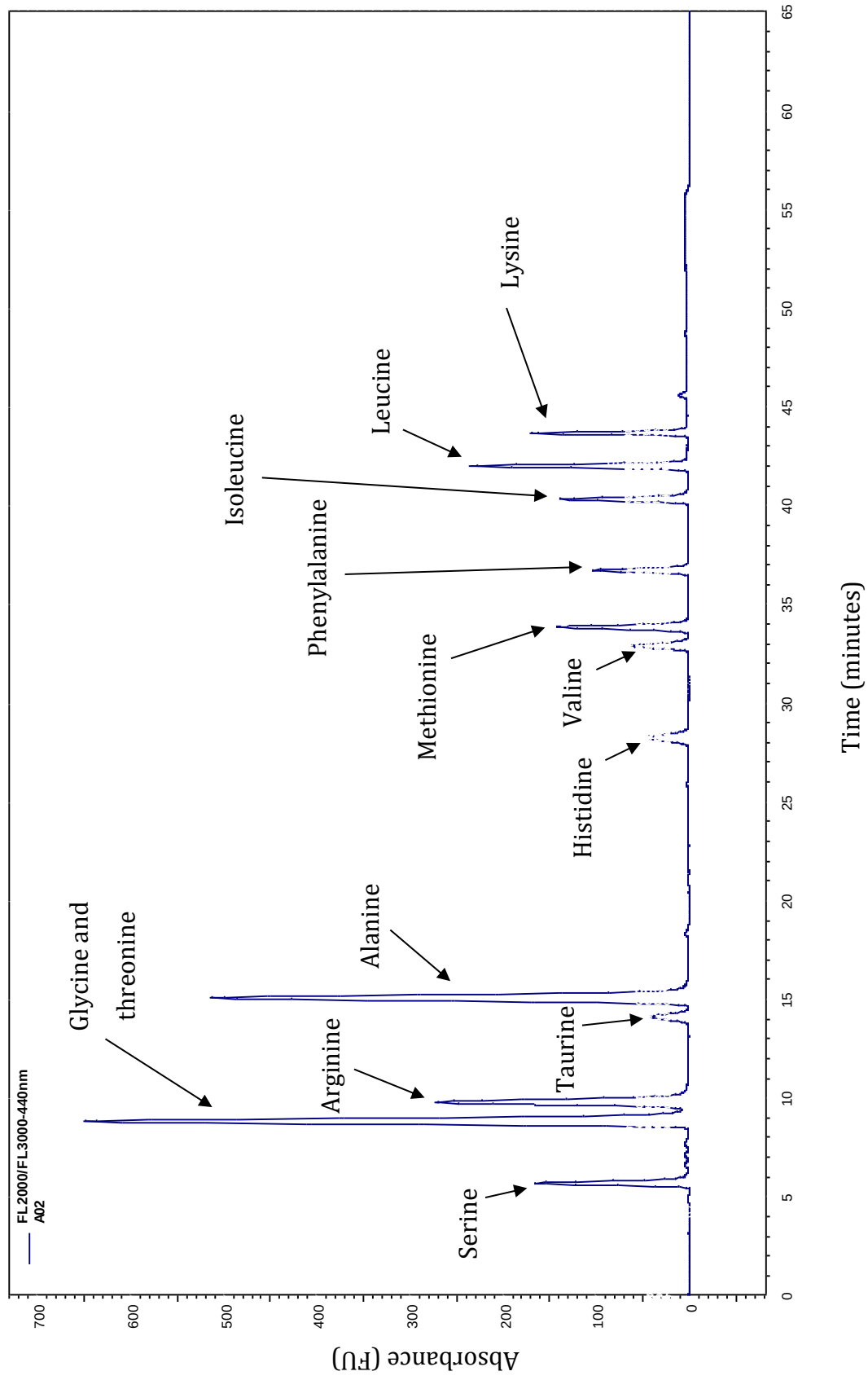


Figure 2.18 OPA derivative of Aminoven® 25 chromatogram with labels to show the identity of each peak

2.3 Reducing Run-Time

The initial work was presented at an NHS QA Symposium, feedback suggested that there was interest in an assay for confirming the quantity of amino acids added during compounding, but the run time was too long to be feasible in that environment. Currently PN bag checks before release involve weighing and visually inspecting the bags. Neither of these measures indicates if the correct volume of an individual PN product has been added. A shorter run time may make this assay accessible to QC labs to confirm the correct amount of amino acids has been added.

Firstly, to try and decrease the run time, the flow rate was increased from 1 ml/min to 2 ml/min, but this had little effect so the column oven temperature was increased to 50 ^{o}C, but again this had little effect. As these simple changes were not successful, the next step was to adjust the mobile phase. Speeding up the rate of the increase in methanol, for more solvent strength, reduced the run time but also worsened the separation and resulted in co-elution of many peaks. The reduction in run time required was not possible with these condition changes. The organic solvent portion of the mobile phase was changed to include acetonitrile, as it is a stronger solvent so the amino acid derivatives should elute more quickly which will reduce the runtime. Initially a mix of methanol: acetonitrile (75 %: 25 %) was tried, and then the proportion of acetonitrile was increased by 25 % until mobile phase B was 100 % acetonitrile. The inclusion of acetonitrile decreased the run time and

maintained reasonable separation. With 100 % acetonitrile, the threonine and glycine peak slightly split (figure 2.19), this was the first time it had been possible to separate them.

Figure 2.19 First time threonine and glycine OPA derivatives peak split

Initially the gradient conditions were kept the same as previous (table 2.1); as expected this significantly reduced retention times and caused co-elution of peaks because acetonitrile is a stronger solvent than methanol. Subsequently the gradient was adjusted by reducing the speed that the concentration of acetonitrile increased, however even when the run became isocratic with 25 % acetonitrile there was still co-elution. So, the starting concentration of acetonitrile was reduced in 5 % intervals; satisfactory separation was seen when the starting concentration of acetonitrile was 5 %. This resulted in glycine and threonine successfully separating from each other. The order of elution was then determined, as seen in figure 2.20. A gradient delay can help achieve better separation; a gradient delay for the first two and a half minutes gave the best

separation, shorter gradient delay times were tried but this resulted in worse separation.

With the starting mobile phase conditions optimised adjusting the rest of the mobile phase amounts was done by adding each subsequent amino acid then tweaking and extending the assay for each additional amino acid. Where amino acids were co-eluting the rate of increase of acetonitrile concentration was slowed; if an amino acid was eluting a long time after the previous one, the rate the acetonitrile concentration increased sped up. Work began on serine, glycine, threonine and arginine; these were well resolved from each other, as shown in figure 2.20. Taurine was then added to the sample and separated, as shown in figure 2.21.

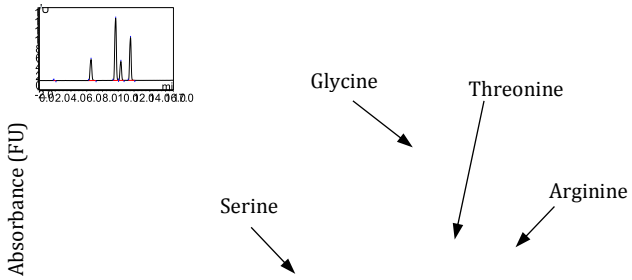


Figure 2.20 Chromatogram showing OPA derivatives of serine, glycine, threonine, arginine separated

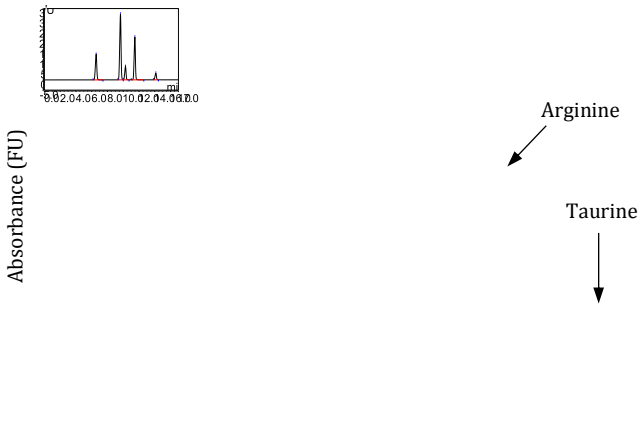


Figure 2.21 Chromatogram showing OPA derivatives of taurine separated from arginine

Unexpectedly when alanine was added to the sample, it now had a shorter elution time than taurine, as shown in figure 2.22, but they were still well resolved from each other.

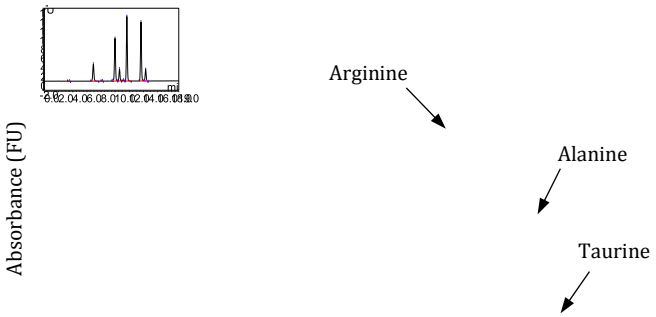


Figure 2.22 Chromatogram showing OPA derivatives of alanine separated from arginine and taurine

When histidine was added, no additional single resolved peak was detected, small peaks were detected at 9, 16.5 and 20.5 minutes, as seen in figure 2.23.

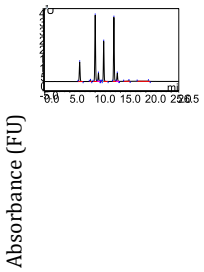


Figure 2.23 Chromatogram showing OPA derivatives of histidine added to serine, glycine, threonine, arginine, alanine and taurine

Histidine was derivatized individually; this confirmed that no single peak was detected, as shown in figure 2.24.

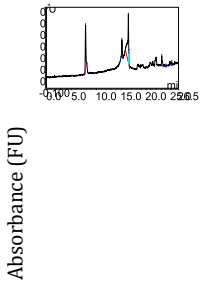


Figure 2.24 Chromatogram showing OPA derivative of histidine standard

Valine and methionine were next to be added to the sample; it was confirmed that valine is the first to elute (figure 2.25).

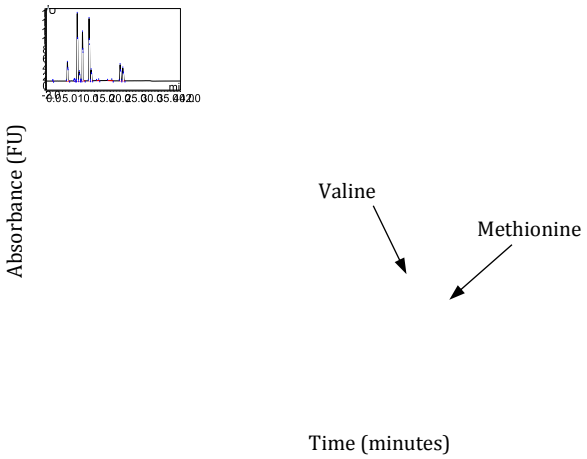


Figure 2.25 Chromatogram showing OPA derivatives of valine and methionine separated

Norvaline and phenylalanine were added to the sample. Norvaline was included in the assay development as it may be required at a later date as an internal standard during stability testing, so it seemed sensible to ensure it was separated from the Aminoven® 25 peaks at this stage. Norvaline eluted shortly after the methionine peak, as shown in figure 2.26, the identity was confirmed by doubling the amount of norvaline in the sample, the peak area doubled.

N.B. The split peaks in figure 2.25 and 2.26 are due to an old guard column.

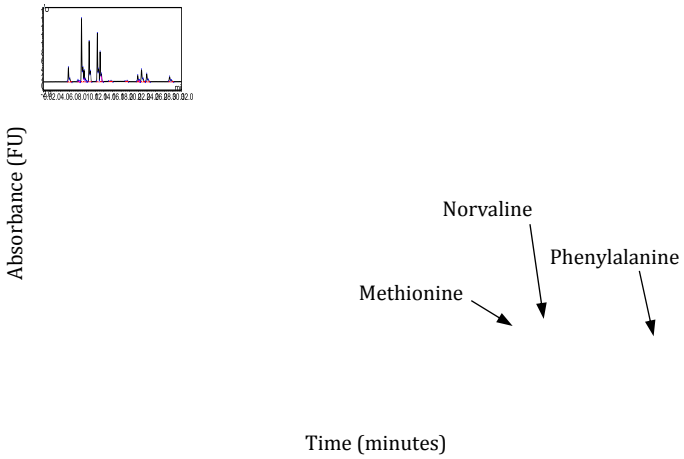


Figure 2.26 Chromatogram showing OPA derivatives of norvaline and phenylalanine separated from methionine

Isoleucine, leucine and lysine were added to the sample; they didn't separate as well as the separations with methanol as mobile phase B. As shown in figure 2.27 there were co-eluting peaks at 28 minutes and a well resolved peak at 30.5 minutes. The identity of the peaks was confirmed by analysing each amino acid individually. As can be seen in figure 2.28 isoleucine and leucine are well separated, with isoleucine eluting first.

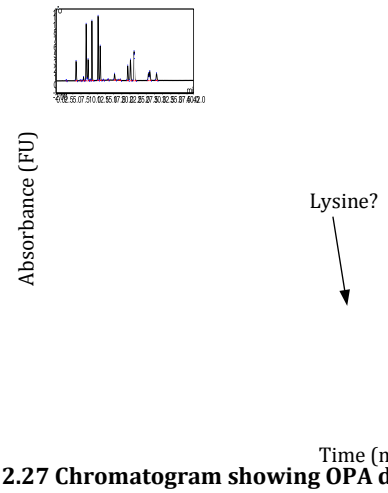


Figure 2.27 Chromatogram showing OPA derivatives of isoleucine, leucine and lysine

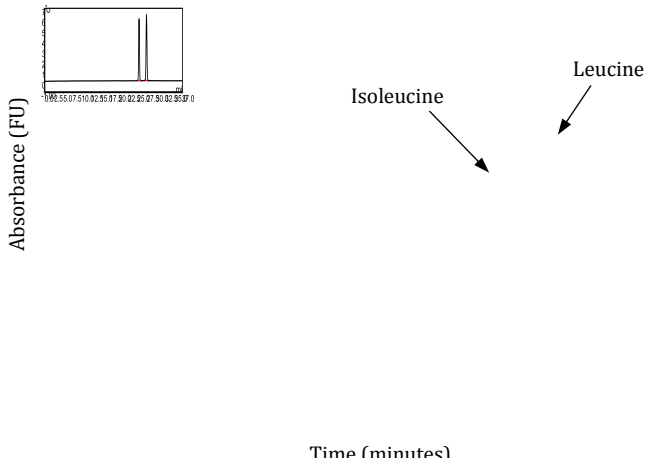


Figure 2.28 Chromatogram showing OPA derivatives of isoleucine and leucine

When phenylalanine was added it confirmed that it co-elutes with isoleucine (figure 2.29). To determine which was isoleucine and

phenylalanine, the amount of phenylalanine in the sample was doubled and the area of the second peak doubled, shown in table 2.2; this is the limiting pair.

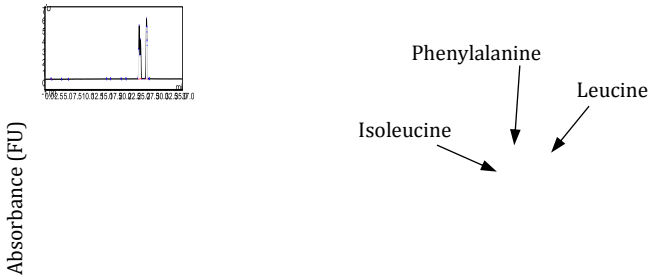


Figure 2.29 Chromatogram showing OPA derivatives of isoleucine, phenylalanine and leucine

Table 2.2 Peak areas confirming the identity of isoleucine and phenylalanine peaks

Isoleucine:Phenylalanine	Isoleucine peak area	Phenylalanine peak area
1:1	3.502	2.854
1:2	3.509	5.784

When isoleucine, leucine and lysine were added a peak was detected at 18 minutes (figure 2.27) which possibly is lysine, however when lysine was derivatized individually there is not a single peak but several small peaks (figure 2.30).

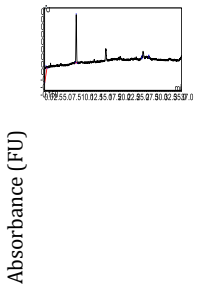


Figure 2.30 Chromatogram showing OPA derivative of lysine standard

Tyrosine and tryptophan were confirmed as being detected, as shown in figure 2.31 and 2.32 respectively.

N.B. The split peaks in figure 2.31 and 2.32 are due to an old guard column.

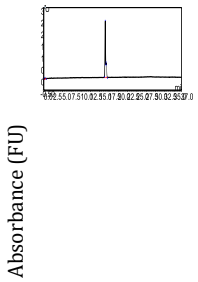


Figure 2.31 Chromatogram showing OPA derivatives of tyrosine standard

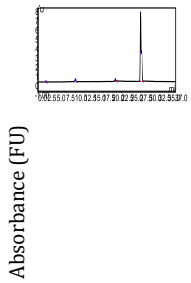


Figure 2.32 Chromatogram showing OPA derivative of tryptophan standard

To demonstrate the selectivity of the assay a sample of water was derivatized which resulted in no peaks; this confirms that the OPA alone is not fluorescent. A sample of underivatized Aminoven® 25 was also injected, unexpectedly there was a small peak at 7.5 minutes, see figure 2.33. Aminoven® 25 contains glacial acetic acid and malic acid; malic acid has been reported as being detected by a fluorescence detector.

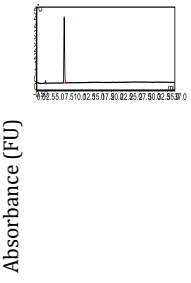


Figure 2.33 Chromatogram of underivatized Aminoven® 25

Once the process of separating each amino acid was complete, the time for the elution of the final peak had been reduced from the original time of 46 minutes with methanol, to 30 minutes with acetonitrile. A labelled

chromatogram of the identified amino acids in Aminoven® 25 when acetonitrile is used is shown in figure 2.34.

Figure 2.34 Aminoven® 25 derivatised with OPA

2.4 Secondary Derivatization

In Aminoven® 25, proline is the only amino acid that cannot react with OPA, as it does not have an amino group. In order to analyse proline it is possible to perform a two stage derivatisation; firstly with OPA, and then add FMOC to derivatize proline (Buha *et al.* 2011). Using two reagents simultaneously to derivatize all the amino acids in a sample will result in all the amino acids being identified in one injection. FMOC is insoluble with methanol, so acetonitrile replaced any methanol in the derivatization process. FMOC is known to form a precipitate when added to the reaction, so the amount required to react 1:1 with proline was used; there was no precipitate visible to the naked eye.

2.4.1 Sample Preparation

The FMOC reagent was made by dissolving 4 µg in 100 µl of acetonitrile; the FMOC reagent was usable for seven days when refrigerated (Buha *et al.* 2011). Samples were derivatized with the same method as detailed in section 2.1.2, however after the HCl was added 5 µl of FMOC reagent was added before dilution for injection.

2.4.2 HPLC Conditions

The FMOC derivative requires a different wavelength to the OPA derivatives, so this was added to the method (table 2.3).

Table 2.3 Gradient and wavelength conditions for OPA and FMOC derivatives

Time (min)	Acetonitrile (%)	Wavelength (nm)
0	5	Ex 330 Em 440
2.5	5	
12	13	
25	20	
32	20	Ex 266 Em 310
48	90	
48.1	5	
50	5	

2.4.3 Initial work

Preliminary work showed that the addition of FMOC to the derivatization process allowed proline to be detected, see figure 2.35. However, when the two-stage derivatization was carried out with Aminoven® 25 the proline and FMOC peaks were less well defined, as shown in figure 2.36. FMOC does fluoresce unreacted, but is more hydrophobic than the OPA derivatives so does not interfere with separation of OPA derivatives, as seen in figure 2.36. Work on secondary derivatization was not pursued any further, development of the assay continued with solely OPA derivatization.

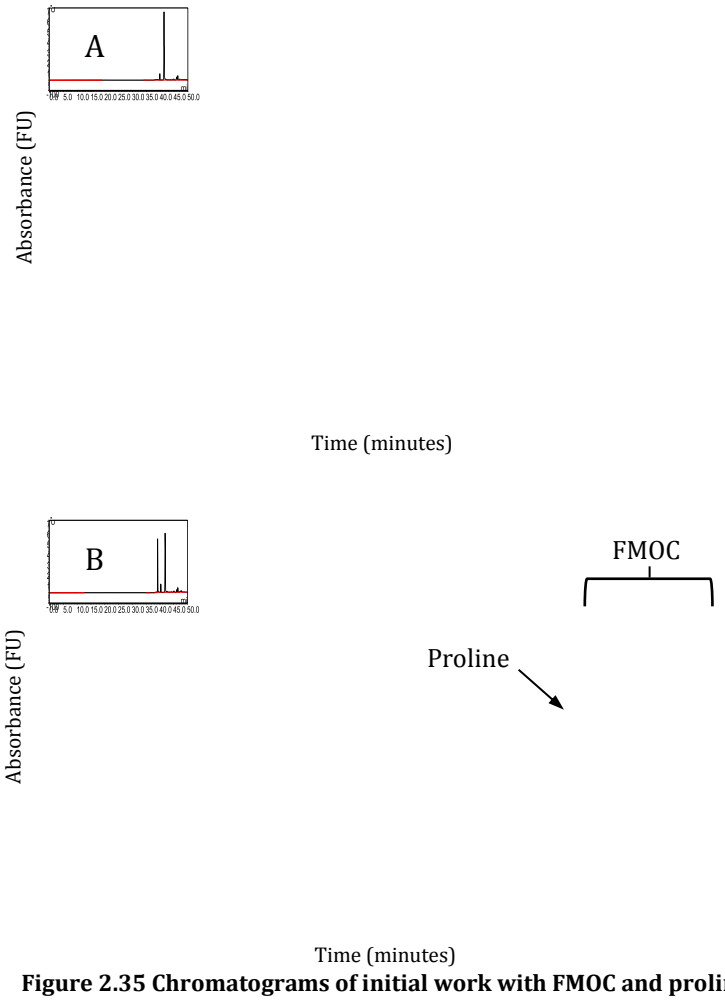


Figure 2.35 Chromatograms of initial work with FMOC and proline
A – FMOC
B – Proline derivatized with FMOC

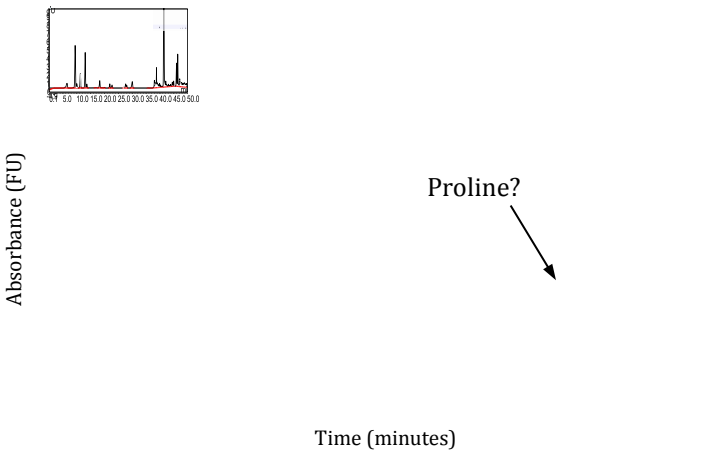


Figure 2.36 Chromatogram of Aminoven® 25 derivatized with OPA and FMOC

2.5 Repeatability

Peak areas were decreasing with each subsequent intra-vial injection; this lack of consistency must be resolved in order for the assay to be viable as a stability indicating assay. The most likely reason is the lack of stability of the amino acid derivatives (García Alvarez-Coque *et al.* 1989; Fürst *et al.* 1990); glycine and lysine derivatives are particularly susceptible to degradation (Bruckner *et al.* 1995). This is worsened by the presence of any excess OPA and 3-MPA which form isoindoles that can attack the amino acid OPA derivatives. Amino acids, OPA and 3-MPA react in a 1:1:1 ratio, so in theory they should be present in this ratio to minimise the formation of isoindoles; however, in the application of stability assessment, the quantity of amino acids is unknown after day 0.

To overcome the poor stability of the derivatives, derivatisation needs to be carried out prior to each injection. Ideally, the derivatization procedure would be undertaken by an autosampler with on-board derivatization capabilities, as this ensures continuity throughout the preparation of amino acids for analysis (Bartolomeo and Maisano 2006). A suitable autosampler was purchased for this application. To use this function a program has to be written detailing the derivatization procedure. When writing the program on Chromeleon 7 to instruct the autosampler of the sequence for derivatization the same volumes as manual derivatisation were used, unfortunately this overloaded the detector. Additional wash steps were added to prevent contamination by the needle between

reagent vials and smaller volumes were programmed but this still overloaded the detector. Volumes of reagents were reduced again and again until the detector was no longer overloaded, however there was a large amount of disruption to the baseline that interfered with several peaks, particularly at shorter elution times, between 6 and 14 minutes, as can be seen in figure 2.37.

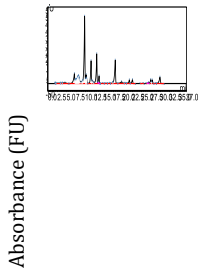


Figure 2.37 Chromatogram of Aminoven® 25 with on-board OPA derivatization

Fresh reagents were used, but no improvement was seen. It is probable that the cause is not the reagents or the sample, but the program written for the autosampler; however, after extensive investigation and discussion with the manufacturer no resolution was found. Therefore, validation of this assay was conducted with manual derivatization prior to each injection.

2.6 Final Method

As a summary of the work completed, a comparison of the method from the application note and the finished method that has been developed for

this project is shown in table 2.4, table 2.5 and figure 2.38. The validation process is then required to determine the limits of the assay.

Table 2.4 Comparison of methods, between application note and the final method

	Method from application note	Final method
OPA reagent	100 mg OPA 9 ml acetonitrile 1 ml borate buffer 100 µl 3-MPA	16 mg OPA 0.9 ml acetonitrile 0.1 ml borate buffer 10 µl 3-MPA
Run time (minutes)	65	35
Column oven (°C)	40	50
Mobile phase A	50 mM ammonium acetate (pH 7)	50 mM ammonium acetate (pH 7)
Mobile phase B	Methanol	Acetonitrile
Flow rate (ml/min)	1	2
Ex - Em (nm)	330 - 440	330 - 440
Injection volume (µl)	10	10
Column	Merck LiChroCart Purospher STAR RP-18e, 5 µm, 250-4.6 mm	Merck LiChroCart Purospher STAR RP-18e, 5 µm, 250-4.6 mm
HPLC equipment	**Pump:** Thermo Finnigan SCM100 **Autosampler:** Thermo Finnigan P2000 **FL detector:** Thermo Finnigan FL3000 **Software:** Chromquest 4.2 Chromatography	**Pump:** Dionex UltiMate 3000 RS **Autosampler:** Dionex UltiMate 3000 RS **Column Compartment:** Dionex UltiMate 3000 RS **FL detector:** Thermo Finnigan FL3000 **Software:** Chromeleon 7

Table 2.5 Change in gradient conditions from the application note (left table) to the final assay (right table)

Time (min)	Methanol (%)
0	25
5	25
25	36
49	75
52	75
52.1	25
65	25

Time (min)	Acetonitrile (%)
0	5
2.5	5
12	13
25	20
32	20
32.1	5
35	5

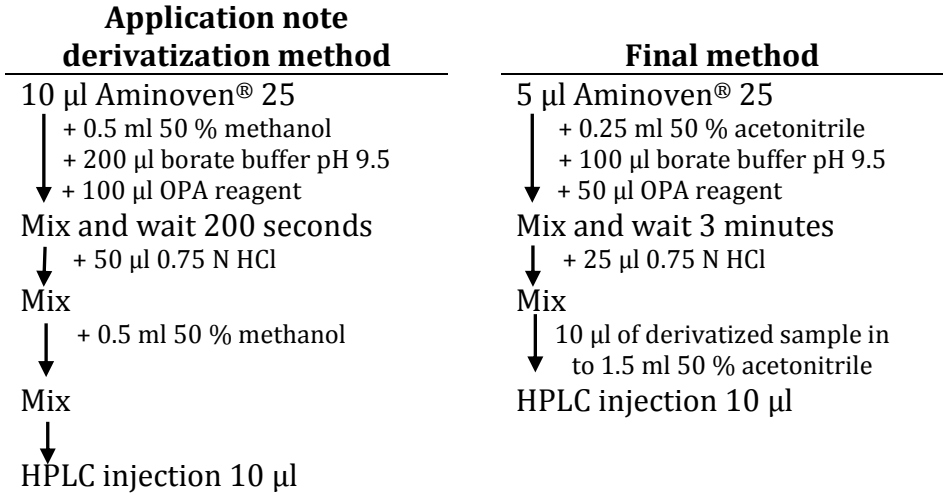


Figure 2.38 Comparison of derivatization method from the application note (left) and the final method (right)

3 Method Validation

3.1 Validating a HPLC Assay

Method validation is a crucial part of developing a HPLC assay, as it demonstrates the suitability and limits of the assay. In this case, the International Conference on Harmonisation (ICH) document, Validation of Analytical Procedures Q2(R1), was used as a guide to validation processes. The ICH was set up to co-ordinate regulatory authorities for pharmaceutical product registration from Europe, Japan and the United States so that the same protocols are followed to eliminate duplicate testing in each area. As this assay is not for use for product registration, the ICH guidelines have only been used as a guide and the relevant parts followed. These are range, linearity, precision, accuracy, detection and quantitation limit; in addition, resolution and selectivity has been measured and a degradation study conducted.

3.1.1 Range

The range of a HPLC assay is defined as the upper and lower amounts of an analyte that show suitable accuracy, precision and linearity. The range for each amino acid used for assay validation is detailed in table 3.1. The ratio of the amino acids is fixed as Aminoven® 25 is a pre-prepared product.

Table 3.1 Upper and lower amounts of each amino acid for validation

	Lower (ng/ml)	Upper (ng/ml)
Serine	247	739
Glycine	476	1425
Threonine	221	662
Arginine	514	1540
Alanine	643	1925
Taurine	51	154
Tyrosine	10	31
lValine	142	424
Methionine	98	293
Tryptophan	41	123
Isoleucine	142	424
Phenylalanine	134	400
Leucine	229	685

3.1.2 Linearity

Linearity is a measure of how accurately the detector responds to changes in concentration of the analyte. This is essential in a stability assay, as if the analyte degrades the reduction in peak area should be directly proportional to the loss of the amino acid. The ICH (2005) recommend that linearity should be determined using at least five different amounts of an analyte; the peak areas are then used to generate the calibration graph with a standard deviation error bar. Linearity is expressed as a value: R^2, this is measure of how well the data points fit to a fitted regression line. To generate the calibration graph, the amount of amino acid derivatized was plotted against absorbance. Usually a R^2 value greater than 0.999 is acceptable, though due to the number of analytes and the derivatisation steps, a value of 0.99 may be acceptable (Snyder et al. 1997). Figures 3.1 to 3.13 show the calibration plots for each amino acid and the corresponding R^2 value.

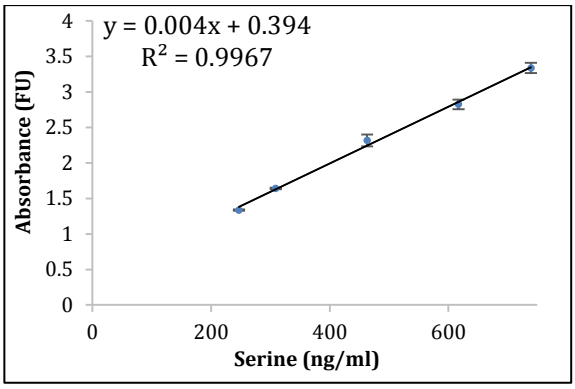


Figure 3.1 Calibration plot of serine n=3

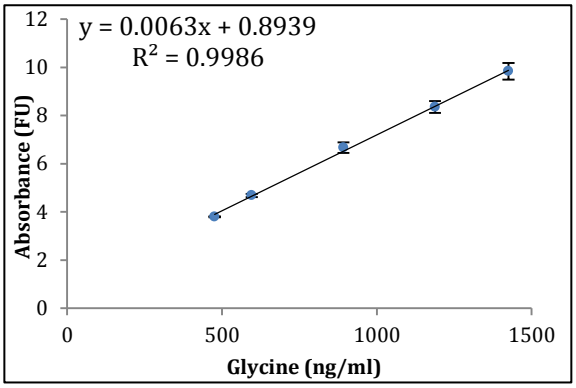


Figure 3.2 Calibration plot of glycine n=3

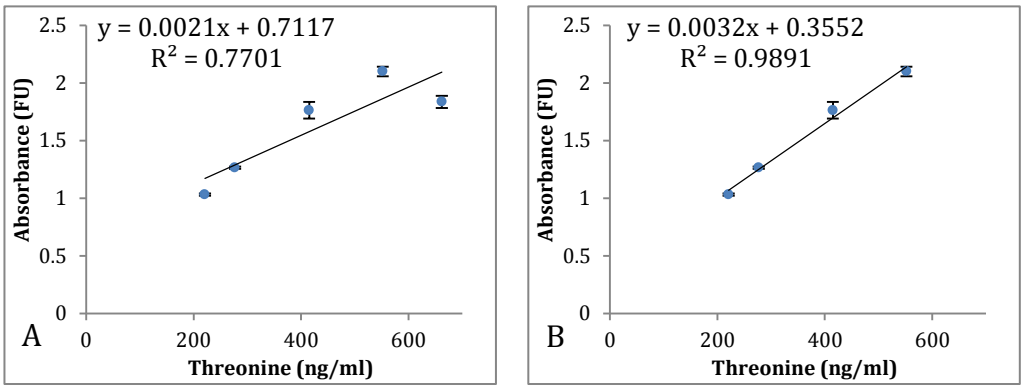


Figure 3.3 Calibration plots of threonine n=3.
A – Calibration plot with five concentrations from 221 ng/ml to 662 ng/ml
B – Calibration plot with four concentrations from 221 ng/ml to 552 ng/ml

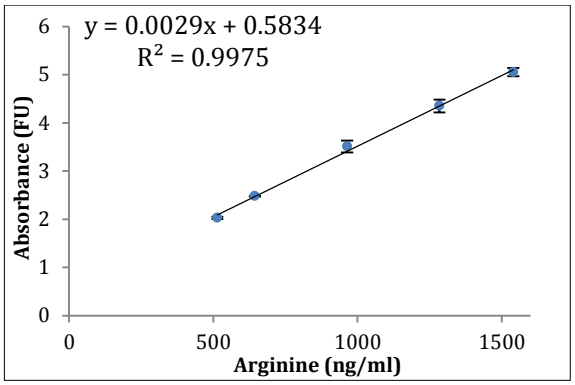


Figure 3.4 Calibration plot of arginine n=3

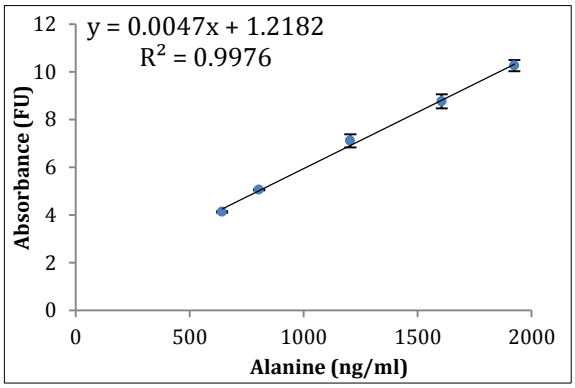


Figure 3.5 Calibration plot of alanine n=3

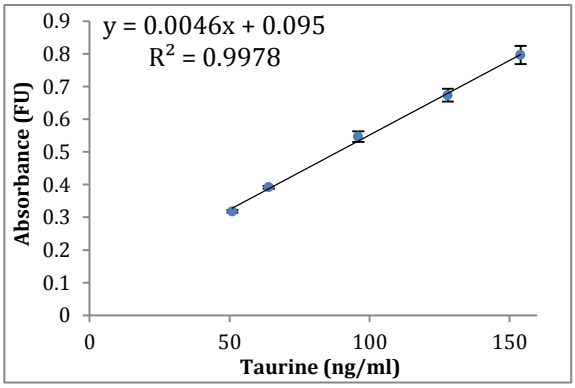


Figure 3.6 Calibration plot of taurine n=3

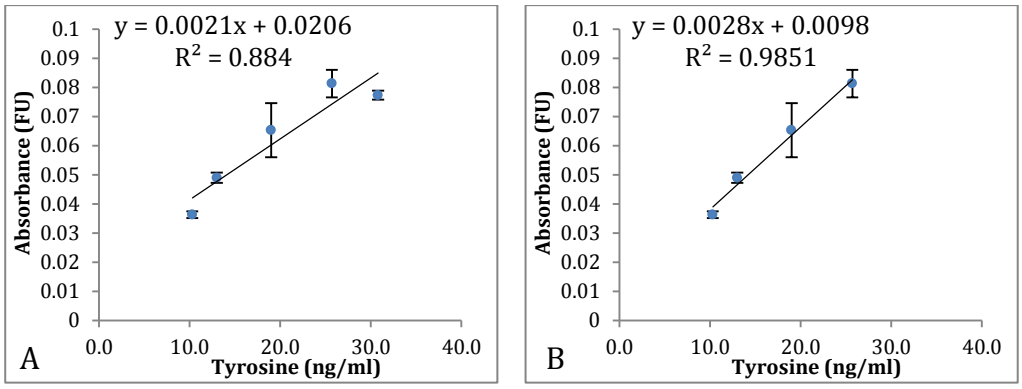


Figure 3.7 Calibration plots of tyrosine n=3
A – Calibration plot with five concentrations from 10 ng/ml to 31 ng/ml
B – Calibration plot with four concentrations from 10 ng/ml to 26 ng/ml

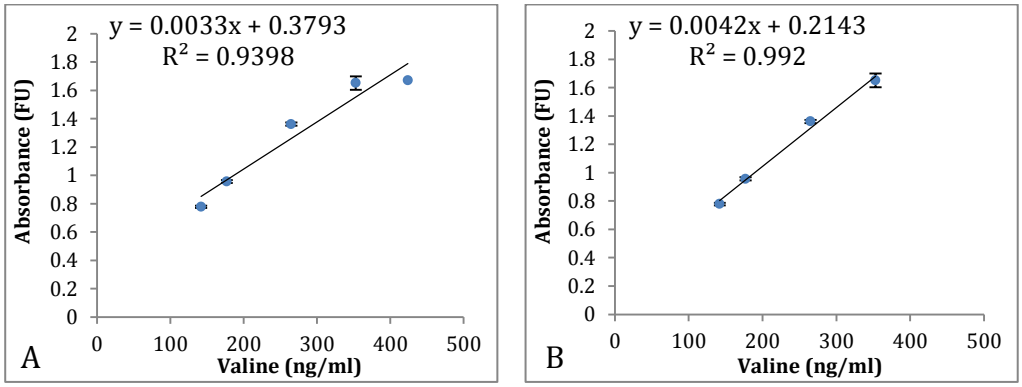


Figure 3.8 Calibration plots of valine n=3
A – Calibration plot with five concentrations from 142 ng/ml to 424 ng/ml
B – Calibration plot with four concentrations from 142 ng/ml to 353 ng/ml

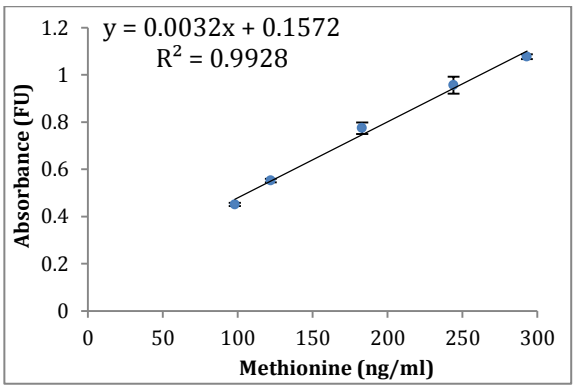


Figure 3.9 Calibration plot for methionine n=3

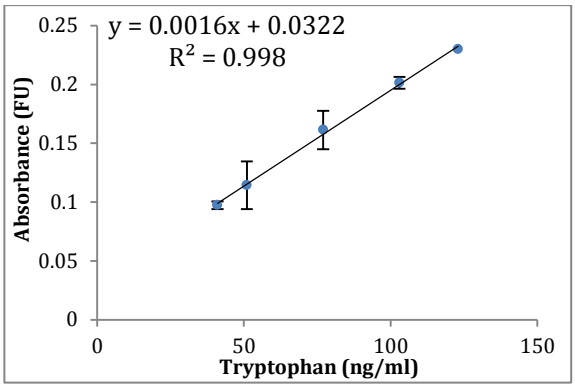


Figure 3.10 Calibration plot for tryptophan n=3

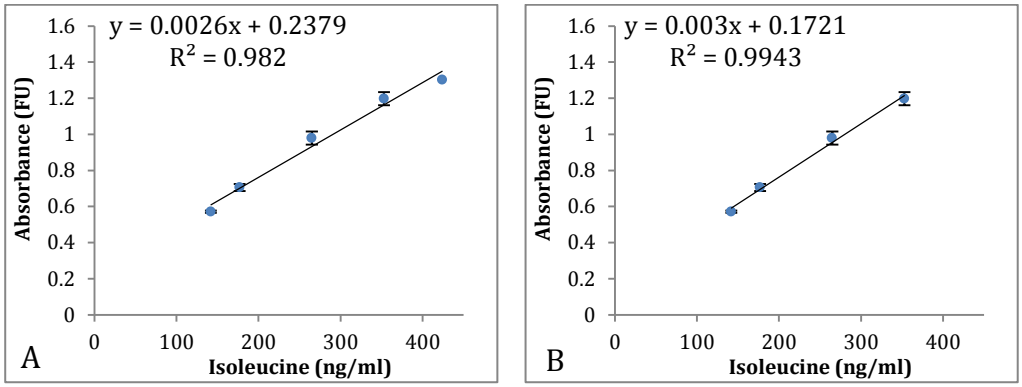


Figure 3.11 Calibration plots for isoleucine n=3
A – Calibration plot with five concentrations from 142 ng/ml to 424 ng/ml
B – Calibration plot with four concentrations from 142 ng/ml to 353 ng/ml

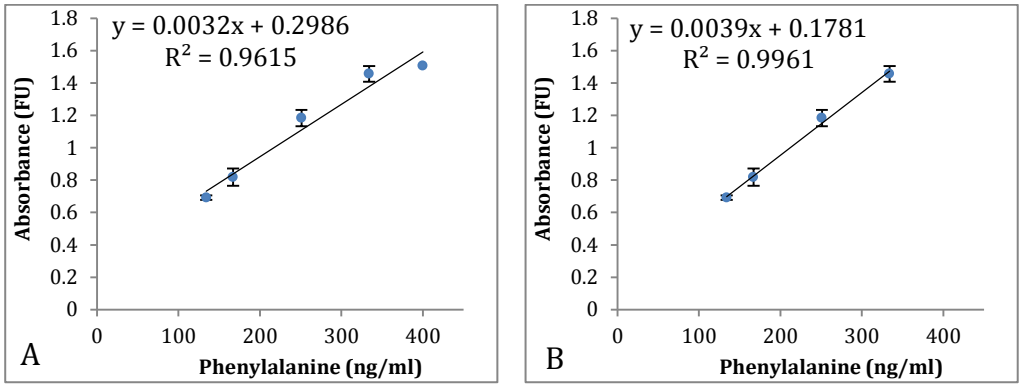


Figure 3.12 Calibration plots for phenylalanine n=3
A – Calibration plot with five concentrations from 134 ng/ml to 400 ng/ml
B – Calibration plot with four concentrations from 134 ng/ml to 334 ng/ml

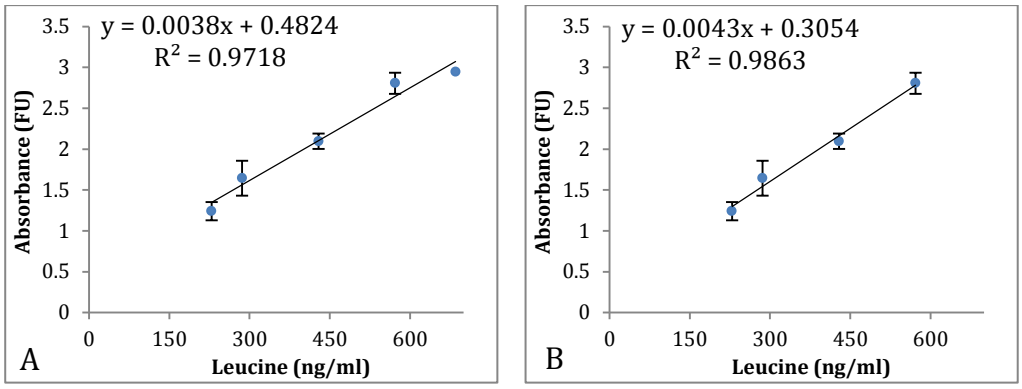


Figure 3.13 Calibration plots for leucine n=3
A – Calibration plot with five concentrations from 229 ng/ml to 685 ng/ml
B – Calibration plot with four concentrations from 229 ng/ml to 572 ng/ml

Serine, glycine, arginine, alanine, taurine, methionine and tryptophan demonstrated linearity greater than 0.99. Threonine, tyrosine, valine, isoleucine, phenylalanine and leucine had poor linearity. The y-intercept for all the calibration graphs does not pass through zero as would be expected.

When reviewing the data of those with poor linearity it was apparent that the peak area for the largest amount of those amino acids derivatized was very similar to that of the next highest amount. This could be due to the amount of derivatizing reagent used in the reaction, which was calculated on the total moles of amino acids in the uppermost amount of the range, so for every mole of amino acids there was a mole of OPA and 3-MPA present. Threonine, tyrosine, valine, isoleucine, phenylalanine and leucine showed poor linearity; it seems likely that the results do not reflect the true amount of these amino acids due to the total amount not reacting with OPA. Therefore, the data for the largest amount was removed from the calibration plots of threonine, tyrosine, valine, isoleucine, phenylalanine

and leucine; the R^2 value improves considerably, this can be seen in figures 3.3B, 3.7B, 3.8B, 3.11B, 3.12B and 3.13B. The altered calibration plot results in R^2 greater than 0.99 for valine, phenylalanine and isoleucine; table 3.2 details the R^2 and RSD values for the amino acids.

Table 3.2 Linearity of amino acids in Aminoven® 25

	Linearity (R^2) n=5	RSD (%)	Linearity (R^2) n=4	RSD (%)
Serine	0.9967	2.93		
Glycine	0.9986	2.74		
Threonine	0.7701	11.52	0.9891	3.62
Arginine	0.9975	2.76		
Alanine	0.9976	2.98		
Taurine	0.9978	2.89		
Tyrosine	0.8840	11.67	0.9851	8.42
Valine	0.9398	7.09	0.9920	3.03
Methionine	0.9928	3.62		
Tryptophan	0.9980	8.02		
Isoleucine	0.9820	4.90	0.9943	3.42
Phenylalanine	0.9615	6.43	0.9961	4.11
Leucine	0.9718	8.69	0.9863	8.67

3.1.3 Accuracy

Accuracy is defined as the agreement between the true value and the measured value. Values between 90 and 110 % are acceptable (Snyder *et al*. 1997).

Table 3.3 Percentage of recovery

	Recovery (% ± SD)
Serine	99.68 ± 3.13
Glycine	99.92 ± 2.17
Threonine	100.74 ± 4.50
Arginine	100.83 ± 2.74
Alanine	100.37 ± 2.78
Taurine	99.07 ± 2.74
Tyrosine	100.87 ± 6.82
Valine	98.59 ± 3.67
Methionine	99.72 ± 4.53
Tryptophan	102.15 ± 2.02
Isoleucine	98.16 ± 3.46
Phenylalanine	99.27 ± 2.26
Leucine	100.67 ± 6.18

All amino acids showed acceptable accuracy; as shown in table 3.3, values ranged from 98.16 % to 102.15 %.

3.1.4 Precision

Precision is a measure of how reproducible results are; this is measured in various ways and expressed as a relative standard deviation (RSD). The ICH guidelines define precision as; repeatability, intermediate precision and reproducibility (ICH 2005). Repeatability is the result of repeated injections over a short period of time, in this case three injections of the same sample were carried out immediately one after another (results shown in table 3.4). Intermediate precision is the measure of reproducibility within a laboratory, in this case samples were analysed on two different days and the results compared (results shown in table 3.4).

Reproducibility is the precision between laboratories; this has not been carried out as part of the validation process as it is not required according to the ICH guidelines (2005); however, would be required should another laboratory require this method. Suggested limits for precision vary, but the lower the RSD value the better. Less than 2 % is considered ideal (Paithankar 2013); however, this is a guideline for drug products so may be unnecessarily restrictive for this application.

Table 3.4 Repeatability and intermediate precision RSD values for each amino acid

	Repeatability RSD % (n=3)	Intermediate precision RSD % (n=5)
Serine	2.41	2.81
Glycine	2.89	3.40
Threonine	2.02	3.48
Arginine	3.07	3.54
Alanine	3.34	3.60
Taurine	2.91	2.98
Tyrosine	5.81	21.98
Valine	2.89	3.47
Methionine	3.76	4.23
Tryptophan	2.50	4.16
Isoleucine	3.34	4.04
Phenylalanine	3.03	3.23
Leucine	4.61	4.89

Repeatability of the amino acids ranged from 2.02 % to 5.81 % (table 3.4), although apart from tyrosine and leucine all were less than 4 %. The results for intermediate precision are similar to the repeatability, apart from tyrosine, which increases to 21.98 %. One of the injections on the second day of testing for the intermediate precision resulted in lower peak areas compared to the other injections; this data has been identified as an

anomaly and removed from the intermediate precision data. The peak areas for all six injections are detailed in appendix 4.

3.1.5 Resolution and Selectivity

Resolution is a measure of baseline separation of two peaks which is necessary for accurate integration. Determining resolution is not included in the ICH guidelines, but has been measured during the validation process due to the number of analytes. Resolution was calculated by Chromeleon using the following equation, which is based on the European Pharmacopeia method:

$$R_S = \frac{1.18(T_2 - T_1)}{(W50_1 + W50_2)}$$

Where:

T_1 / T_2 are the retention times of the two peaks

$W50_1$ / $W50_2$ are the time of the two adjacent peaks at 50 % of the peak height

The resolution value for a peak indicates the resolution for that peak from the subsequent peak. The higher the R_S value, the further apart the peaks are, consequently increasing resolution increases the run time, so the two must be balanced. A resolution greater than 1.5 indicates baseline resolution; however for samples containing more than ten analytes, as in this case, a value of $R_s > 1$ may be deemed acceptable (Snyder *et al.* 1997).

Selectivity is a measure of the quality of the peak separation by determining how will analytes are chemically distinguished from one another. Selectivity values greater than 1 indicates good separation; a

value of 1 results from co-eluting peaks. Selectivity was calculated by Chromeleon using the following equation:

$$\alpha = \frac{T_2 - T_0}{T_1 - T_0}$$

Where:

T_1/T_2 are the retention times of the two peaks

T_0 is the dead time (time between injection and the solvent front)

Table 3.5 Resolution and selectivity of amino acid peaks (n=3)

	R_S (RSD %)	α (SD)
Serine - Glycine	6.55 (2.62)	1.56 (0.00)
Glycine - Threonine	1.96 (0.59)	1.07 (0.00)
Threonine – Arginine	4.02 (0.50)	1.13 (0.00)
Arginine – Alanine	5.87 (0.95)	1.17 (0.00)
Alanine – Taurine	2.16 (0.80)	1.06 (0.00)
Taurine – Tyrosine	8.82 (1.61)	1.24 (0.00)
Tyrosine – Valine	5.51 (0.46)	1.37 (0.00)
Valine - Methionine	2.17 (1.07)	1.04 (0.00)
Methionine – Tryptophan	10.05 (1.85)	1.21 (0.00)
Tryptophan - Isoleucine	1.39 (2.31)	1.02 (0.00)
Isoleucine - Phenylalanine	0.94 (2.22)	1.02 (0.00)
Phenylalanine - Leucine	4.07 (0.74)	1.08 (0.00)

Most peaks were well resolved, with a R_S value greater than 1. As reported in table 3.5 isoleucine and phenylalanine did not resolve to baseline, as

shown by the R_S value of 0.93, so cannot be integrated separately. Selectivity values were all greater than 1, indicating good peak separation.

3.1.6 Detection Limit and Quantitation Limit

The limit of detection (LOD) is defined as the smallest amount of an analyte that can be distinguished from the baseline. The limit of quantification (LOQ) defines the smallest amount of an analyte that can be quantified with acceptable precision and accuracy under the stated assay conditions. The LOD and LOQ were calculated from the linearity plot by using the standard deviation of the response and the slope of the linearity plot.

LOD and LOQ are calculated by:

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

Where: σ = standard deviation of the response

S = slope of the calibration curve

LOD and LOQ values for the amino acids detected are shown in table 3.6.

Table 3.6 Limit of detection and limit of quantification values

	LOD (ng/ml)	LOQ (ng/ml)
Serine	56	169
Glycine	70	212
Threonine	95	287
Arginine	100	304
Alanine	123	373
Taurine	9	28
Tyrosine	5	16
Valine	52	157
Methionine	33	99
Tryptophan	7	22
Isoleucine	44	133
Phenylalanine	34	104
Leucine	110	335

3.1.7 Degradation

A stability indicating assay must be able to detect the analytes without interference from degradation products. In order to test this, a forced degradation study is conducted. Ideally during the degradation study the analyte/s should be degraded by approximately 10 % (Snyder et al. 1997). Degrading the product by this much will ascertain if any of the degradation products are detected during the assay, and if so whether they are co-eluting with the analyte/s for quantification. For this degradation study an uncapped bottle of Aminoven® 25 was exposed to air and sunlight, as amino acids are susceptible to degradation by these conditions (Allwood *et al*. 1996; Li *et al.* 1995; Patel *et al*; 2011). Peak areas reduced by 11 % or more (table 3.7) and there was no interference by the degradation products, see figure 3.14.

Table 3.7 Percentage of peak area reduction after forced degradation

	Degradation (%)
Serine	23
Glycine	11
Threonine	26
Arginine	25
Alanine	21
Taurine	12
Tyrosine	34
Valine	38
Methionine	29
Tryptophan	38
Isoleucine	35
Phenylalanine	32
Leucine	11

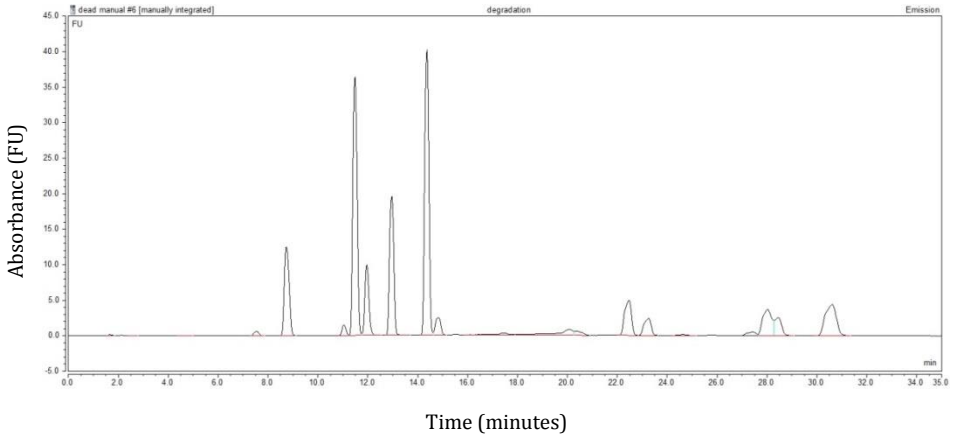


Figure 3.14 Chromatogram of degraded Aminoven® 25

3.2 Aqueous Parenteral Nutrition Containing Aminoven® 25

The assay validation process was conducted with Aminoven® 25; however, the future application of this assay is to quantify amino acids in PN. In order to test the suitability of this assay for analysing PN samples a mock paediatric aqueous PN sample was made and derivatized; the formulation is detailed in table 3.8. Repeatability of aqueous PN was assessed and the peak areas were compared to Aminoven® 25 (table 3.9).

Table 3.8 Aqueous PN formulation used for comparison to Aminoven® 25

Component	Volume (ml)
Aminoven® 25 (Fresenius Kabi, Runcorn, UK)	30
Peditrace® (Fresenius Kabi, Runcorn, UK)	2
Glucose 70% (Baxter, Newbury, UK)	5
Sodium glycerophosphate 21.6% (Fresenius Kabi, Runcorn, UK)	5
Water for injections (Fresenius Kabi, Runcorn, UK)	44
Sodium chloride 30% (Torbay, Paignton, UK)	2
Potassium chloride 15% (Torbay, Paignton, UK)	4
Potassium acetate 49% (Torbay, Paignton, UK)	2
Magnesium sulphate 50% (Torbay, Paignton, UK)	1
Calcium chloride 1 mmol/ml (Martindale, Buckinghamshire, UK)	4
Solivito® N (in WfI) (Fresenius Kabi, Runcorn, UK)	1

Table 3.9 Repeatability of aqueous PN formulation (n=3) and aqueous PN formulation compared to Aminoven® 25 (n=6)

	Repeatability of aqueous PN RSD (%)	Aminoven® 25 compared to aqueous PN RSD (%)
Serine	1.09	1.68
Glycine	5.07	7.27
Threonine	8.57	8.32
Arginine	3.38	2.94
Alanine	2.90	2.83
Taurine	4.30	3.53
Tyrosine	0.83	9.60
Valine	6.21	7.13
Methionine	1.40	2.79
Tryptophan	5.18	3.78
Isoleucine	2.11	6.39
Phenylalanine	2.21	2.69
Leucine	2.84	3.58

Repeatability of the amino acids when derivatized as part of an aqueous PN formulation ranged from 0.83 % to 8.57 %. Precision results for the comparison of Aminoven® 25 with an aqueous formulation containing Aminoven® 25 were encouraging, RSD values ranged from 1.68 % to 9.60 %.

To demonstrate that the peaks detected when analysing a PN sample represent only amino acids, another PN sample was made similar to that detailed in table 3.8, except Aminoven® 25 was replaced with water so if there were any remaining PN components reacting with OPA, they would be detected; no peaks were detected when this sample was derivatized and analysed, therefore none of the PN components in the formulation detailed it table 3.8 interfere with the assay.

3.3 Summary

Method validation has tested the suitability and limits of the method, by measuring the range, linearity, accuracy, precision, resolution, detection limit, quantitation limit, degradation and compatibility with aqueous PN. Serine, glycine, arginine, alanine, taurine, valine, methionine and tryptophan met the criteria set for the method validation. Tyrosine did not show adequate linearity or intermediate precision, therefore is not suitable for quantification in a stability study. Threonine and leucine did not show adequate linearity, although their R^2 values were significantly improved by removing the upper amount from the range. Phenylalanine and isoleucine showed linearity, repeatability, intermediate precision and were above the quantification limit, however the two peaks did not resolve well enough from each other.

4 Discussion

4.1 General Discussion

There is an increasing demand for longer shelf lives for PN bags, but the longer the bags are stored the greater the likelihood of reactions occurring within the bag. Therefore, there is a definite need for more comprehensive stability testing of PN to maintain patient safety and patient health. The MHRA have requested that data to indicate the chemical stability of PN components should be incorporated in to stability testing. It is possible that the concentration of components patients are receiving is less than the original dose added to the PN bag, so data on degradation of components will be vital for clinicians. Amino acids are a crucial component of PN, both for patient nutrition and the physical integrity of the PN formulation but there is limited data, particularly new data, on their stability in PN formulations. Previous methods have required lengthy sample preparation and analysis time, making them unpractical for generating the volume of chemical stability data required for clinical practice. In addition, previous methods have required expensive equipment, making it less accessible to stability and QC laboratories.

This thesis shows the development and validation of a simple method to analyse amino acids in PN by their fluorescing OPA derivatives; resulting in a short sample preparation and analysis time, without the need for additional equipment. Aminoven® 25, which has not previously been the subject of analysis was used during this work. The method has been validated for thirteen of the sixteen amino acids in Aminoven® 25, in 35

minutes. Furthermore, the method has been shown to be applicable to aqueous PN formulations; this is the first time that a PN formulation containing amino acids, glucose, electrolytes, trace elements and vitamins has been analysed.

Amino acids in Aminoven® 25 that were not successfully detected were proline, lysine and histidine. It is well known that proline cannot react with OPA due to the absence of an amino group, so it was expected that it would not be possible to detect proline with this method. Lysine may have sometimes been detected, but this was not confirmed. It has previously been noted that lysine has a poor fluorescent yield, therefore poor quantification (Godel *et al.* 1984) but it is not clear if this was the reason for the issues detailed in this thesis. Histidine was not detected with the final assay, so consequently could not be quantified during the method validation. The gain of a significantly reduced run time using acetonitrile resulted in the loss of two amino acids (histidine and lysine) being detected.

Histidine and lysine were detected when methanol was part of the mobile phase; they were also both shown in the application note. This suggests that the change of mobile phase to acetonitrile may be the cause. This could be due to the change from a protic solvent, methanol, to an aprotic solvent, acetonitrile, and the way the derivatized histidine and lysine (which are both basic) interact with the solvents. Although it is worth noting that arginine is also basic and was detected when methanol and

acetonitrile were used. It has been reported that changes in HPLC conditions can affect histidine derivatives (Csampai *et al.* 2004). If a quaternary pump was available it would have been possible to introduce methanol to the mobile phase after glycine and threonine had separated; as histidine and lysine were both detected when methanol was the organic phase this may have resulted in their detection.

Linearity values were greater than 0.985; linearity values are not stated in the VWR (2012) application note, or in previous papers that have analysed amino acids in PN with the ninhydrin method (Ronchera-Oms *et al*. 1995; Nordfjeld *et al*. 1983; Jeppsson and Tengborn 1987). However, analysis of foodstuffs and biological samples with OPA and fluorescence detection show a range of linearity values from 0.98 to 0.999 (Perucho *et al*. 2015; Hung *et al*. 2010; Buha *et al.* 2011; Frank and Powers 2007; Bartolomeo and Maisano 2006; Delgado-Povedano *et al*. 2016); suggesting that this method has acceptable linearity.

Linearity was initially poor ($R^2 < 0.99$) for threonine, tyrosine, valine, isoleucine, phenylalanine and leucine, although when the greatest concentration was removed from those calibration graphs valine, phenylalanine and isoleucine show increased linearity. The key reason identified as the cause of poor linearity at the upper range was the molar ratio between amino acids, OPA and 3-MPA; they were present in equal moles based for the uppermost range of the amino acids, and therefore in excess for lower concentrations of Aminoven® 25. It is plausible that not

all of the amino acids reacted when there is a not an excess of reagents; it has been reported that the derivatizing reagents should be present in a large excess (Lindroth and Mopper 1979).

Precision results were larger than the 2 % often given as an acceptable limit; repeatability in the application note was 4 %. Other studies using OPA to analyse amino acids in foodstuffs and biological samples report repeatability values of 12 % (Herbert *et al.* 2000; Frank and Powers 2007) and 19.8 % (Pripis-Nicolau *et al.* 2001); precision of Aminoven® 25 and aqueous PN samples were considerably below these values, indicating that the repeatability values in this work are acceptable. Tyrosine showed the poorest precision results; it had the highest repeatability and intermediate precision results, 5.81 % and 21.98 % respectively. Tyrosine has previously been reported as having a larger precision RSD than other amino acids, though not with values as high as 21.98 % (Arrieta and Prats-Moya 2012).

The method showed good separation in terms of resolution and selectivity, apart from isoleucine and phenylalanine which were co-eluting. The application note (VWR 2012) does not report any resolution values, however Buha *et al.* (2011) reported a $R_S < 1$ for isoleucine and phenylalanine.

The method does not always comply with regulatory method validation standards; however, these are intended for pharmaceutical product

registration. This method has validation results comparable to those reported when assaying amino acids in foodstuffs and biological samples.

4.2 Limitations

There are two main limitations with this method. Firstly, the samples had to be manually derivatized due to unresolvable issues with the on-board derivatization program for the autosampler. It has been widely reported that there are issues with repeatability when samples are manually derivatized; this may have affected the validation results. In addition, manually derivatizing is not ideal for conducting stability studies as this will limit the number of samples that can be run as it is time consuming for a member of staff.

The second limitation is the assumption that the conversion rate when derivatizing the amino acids was 100 %. OPA derivatization of amino acids is a widely used method and publications do not routinely include an assessment of the conversion rate. However, one study has reported that the conversion rate of alanine, arginine, glycine, histidine, isoleucine, leucine, serine, threonine, tryptophan, tyrosine and valine is 96.6 % ± 6 % (Aminot and Kérouel, 2006). The authors found that reaction time and pH were the most important determinants of the reaction; the time and pH for the reaction in the method detailed in this thesis were optimal. Although the paper does not analyse the conversion rate of all the amino acids in Aminoven 25®, it is an indicator that the rate of conversion is high for this method.

4.3 Future work

The next stage for this work is to resolve the issue with the program for the on-board derivatization sequence. If assessment of amino acid stability is going to be required on a regular basis the automated derivatization will be essential, as it significantly reduces labour time and will allow more samples to be analysed. In addition to the more practical benefits of the on-board derivatization, it will also improve the repeatability of the assay. Once the on-board capabilities are resolved there are some further steps to improve the assay.

Firstly, as there was no obvious cause for the y-intercept of the calibration curve to be greater than zero, the linearity should be retested. Finally, development of the secondary derivatization process with FMOC needs completing; this will allow the detection of proline.

4.4 Conclusion

The MHRA has requested stability data for amino acids in PN as currently clinicians have no information on the degradation rates of amino acids in PN which ultimately may lead to poor patient outcomes. Previous methods used for analysis of amino acids in PN have long sample preparation and runtimes so are not feasible in a stability or QC laboratory where sample throughput is high. This work has resulted in a simple and quick method that is validated and appropriate for use in stability and QC laboratories. In the future, the method can be used as part of routine stability studies or

as a safety check to confirm the correct quantity has been added before it is administered to a patient.

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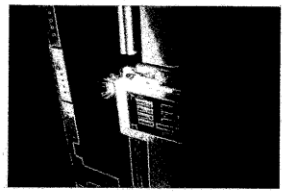
6 Appendices

VWR

Application Note
C-10225

March 2012

Separation of amino acids in feedstuff by manual precolumn derivatision with OPA using the Chromaster system with fluorescence detector



Abstract:

Amino acids are molecules containing an amine group, a carboxylic acid group and a side chain that varies between different amino acids. These molecules are particularly important in biochemistry, where this term refers to alpha-amino acids with the general formula H2NCHRCOOH, where R is an organic substituent. In an alpha amino acid, the amino and carboxylate groups are attached to the same carbon atom, which is called the α–carbon. The various alpha amino acids differ in which side chain (R group) is attached to their alpha carbon.

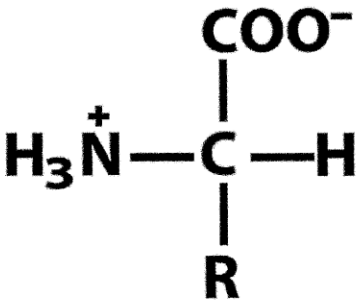
These side chains can vary in size from just a hydrogen atom in glycine, to a methyl group in alanine, through to a large heterocyclic group in tryptophan. Amino acids are critical to life, and have many functions in metabolism. One particularly important function is as the building blocks of proteins, which are linear chains of amino acids. Every protein is chemically defined by this primary structure, its unique sequence of amino acid residues, which in turn define the three-dimensional structure of the protein. Just as the letters of the alphabet can be combined to form an almost endless variety of words, amino acids can be linked together in varying sequences to form a vast variety of proteins. Amino acids are also important in many other biological molecules, such as forming parts of coenzymes, as in S-adenosylmethionine, or as precursors for the biosynthesis of molecules such as heme. Due to this central role in biochemistry, amino acids are very important in nutrition. Amino acids are commonly used in food technology and industry. For example, monosodium glutamate is a common flavor enhancer that gives foods the taste called umami. They are also used in industry.

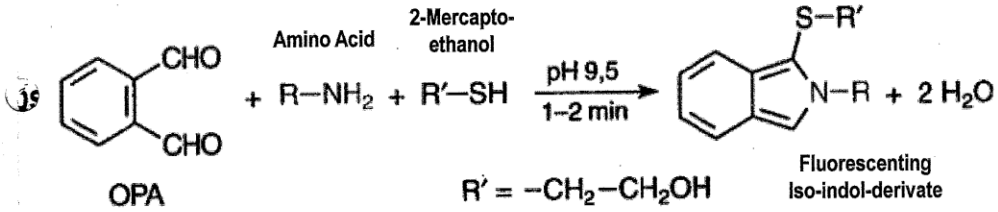
4U™ Chrom for You

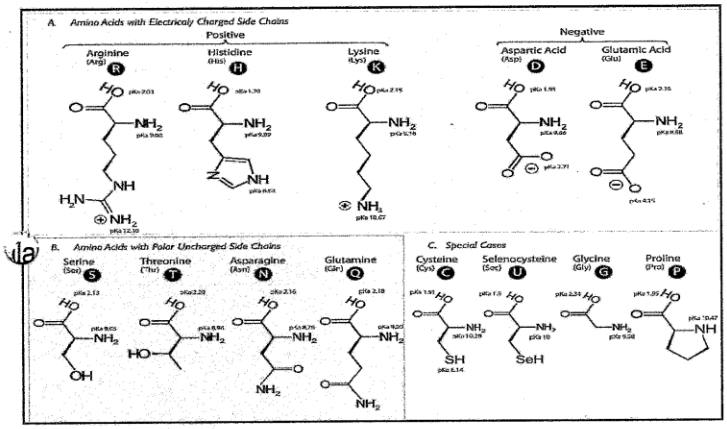
Segment: Industrial – F&B

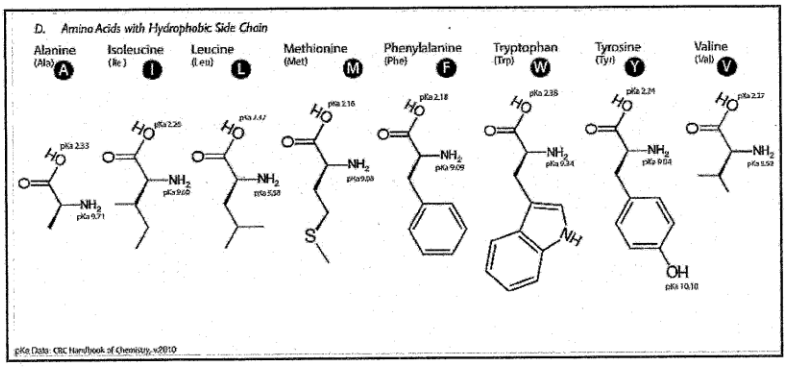
All amino acids have an amino group and a carboxylic group connected through a single carbon (called alpha-carbon shown in blue).

The 20 amino acids differ from one another in the chemical structure of the side chain connected to the alpha carbon (called side-chain shown as a red R)









Chromatographic conditions and ordering numbers

Pump:	CM 5110 incl. Standard Static Mixer (700 µl), Gradient Mode: HFM, art. HITA892.0104
Low pressure gradient unit:	art. HITA 892-0152
Degasser:	6-Channel degassing unit, art. HITA892.0156
Autosampler:	CM 5210 (Cut-Method, Lead: 5 µl, Rear: 30 µl; Syringe: 175 µl), art. HITA892.0204
Oven:	CM 5310, art. HITA892.0304
Detector:	CM 5440, art. HITA892.0464
Wavelength:	EX 330 nm / EM 440 nmEM BW: Standard, PMT-Voltage: Medium
Organizer:	CM Organizer, art. HITA892.0804
IF Control Board:	IFCB, art. HITA892-0900
Elects:	A: 50 mM Ammonium acetate pH 7, B: Methanol
Gradient:	0 min 25 % B 5 min 25 % B 25 min 96 % B 49 min 75 % B 52 min 75 % B 52.1 min 25 % B 65 min 25 % B
Flow rate / Run Time:	1.0 ml/min / 30 min
Pressure:	180 bar
Oven Temperature:	40°C
Column:	Merck LiChroCart® Purospher® STAR RP-18e, (5 µm) 250-4.6 mm
Injection Volume:	10 µl

Tools:

Pipettes to pipette 50, 100, 200 and 500 µl solutions
VWR analog dry block heater, VWR art. no. 460-3249
Single block heater for 12 mm culture tubes, VWR art. no. 460-3279
12 mm culture tubes with screw-cap (50 pieces), VWR art.no. 391-4022
Whatman filters Spartan 30 – 0.45 µm, 500 pieces, VWR art.no. 514-1230
Vortex shaker analog, VWR art.no. 444-2790
Column Merck LiChroCart® Purospher® STAR RP-18e, (5 µm) 250-4.6 mm, VWR art.no.1.50359.0001
Manu CART NT cartridge holder, VWR art.no. 1.51486.0001
Guard columns

Chemicals:

Ortho-Phthaldialdehyd , VWR art.no. 8.21027.0010
Sodium tetraborate Na2B4O7, water free, Suprapur, VWR art.no. 1.06309.0025
2-Mercaptoethanol, VWR art.no. 8.18125.0005
Hydrochloric acid, 32 %, VWR art.no. 1.00391.1000
Sodium hydroxide pellets, VWR art.no. 1.06462.1000
Methanol, gradient grade, VWR art.no. 1.06007.1000
Sigma amino acid standard containing 22 amino acids, Sigma art.no. A 2908
Sigma amino acid standard containing 18 amino acids, Sigma art.no. AA-S-18
Ammonium acetate

haracterisation of the OPA method

application range:	50 pmol/ml - 5 mmol/ml per amino acid in the sample (100 fmol - 10 µmol per amino acid injected with 3-mercaptopropionic acid higher sample concentrations can be derivatized)
limit of detection:	50 pmol/ml per amino acid in the sample (100 fmol per amino acid injected)
linear range:	5 nmol/ml - 5 µmol/ml per amino acid in the sample (10 pmol-10 nmol per amino acid injected)
reproducibility: peak areas:	σ% = 2-4% for standards , retention times: σ = 0.01-0.2 min for standards
advantages:	• fast reaction • the OPA reagent itself is non-fluorescent and therefore, excess reagent must not be removed: only one derivatization step is necessary • the amino acids are relatively easy to be separated in a gradient elution • tryptophane can be detected by the OPA method
disadvantages:	• secondary amino acids as proline or hydroxyproline are not derivatized by OPA • the OPA reagent is not stable and should be prepared fresh daily • cysteine and cystine are derivatized by OPA, but the fluorescence quantum yield is very low. Therefore, fluorescence detection is not sensitive enough for these amino acids. However, UV detection at 230 nm is possible.

Buffers and Solutions

1. **Borate buffer:**

   Sodium tetraborate pH 9.5

   2,515 g sodium tetraborate ($Na_2B_4O_7$ water free) is dissolved in 100 ml water, pH is adjusted with 1 n HCl, the solution is boiled 5 min. The solution should be stored at room temperature (crystallization occurs at lower temperatures).

2. **OPA reagent:**

   100 mg ortho-phthaldialdehyde
   9 ml methanol
   1 ml borate buffer
   100 ul 2-mercaptoethanol or 3-mercaptopropionic acid

3. **Hydrochloric acid:**

   0.75 n HCl

   7,367 ml HCl 32% ad 100 ml with water

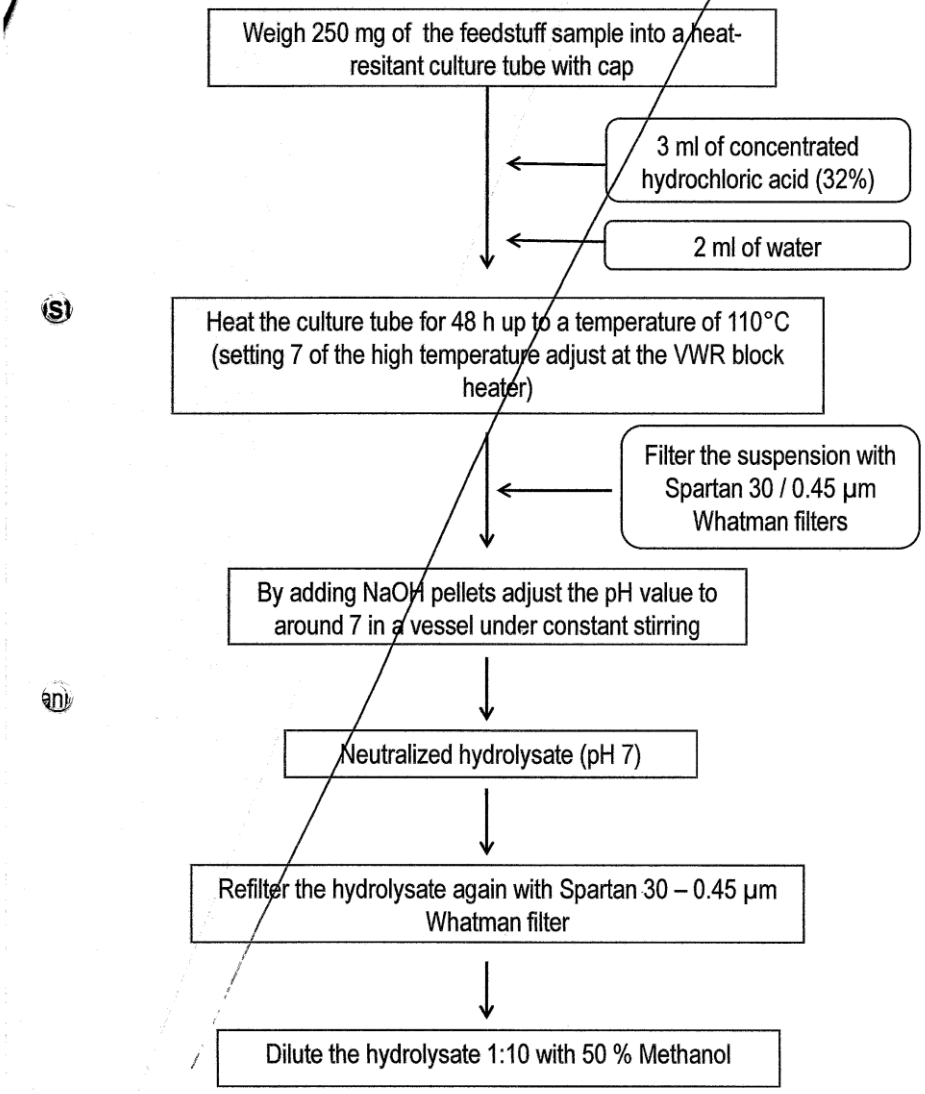
   - careful making.
   0.7367 ml up to 10 ml

4. **Dilution solution:**

   water/methanol (50/50, v/v)

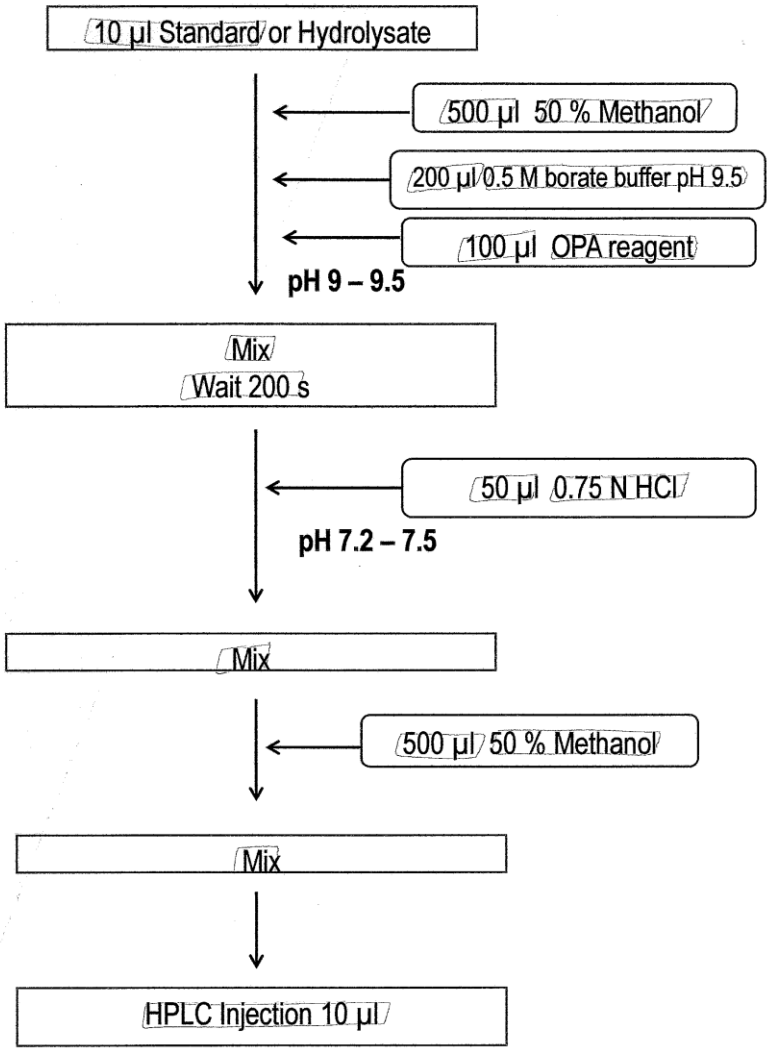
4

Hydrolysis of Proteins in Feedstuff



5

OPA Derivatisation Procedure



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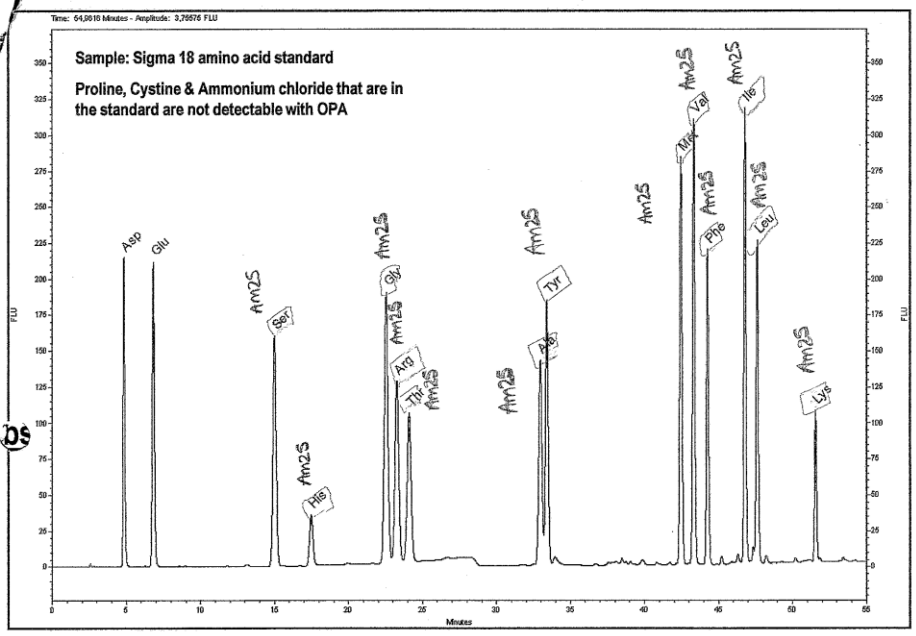
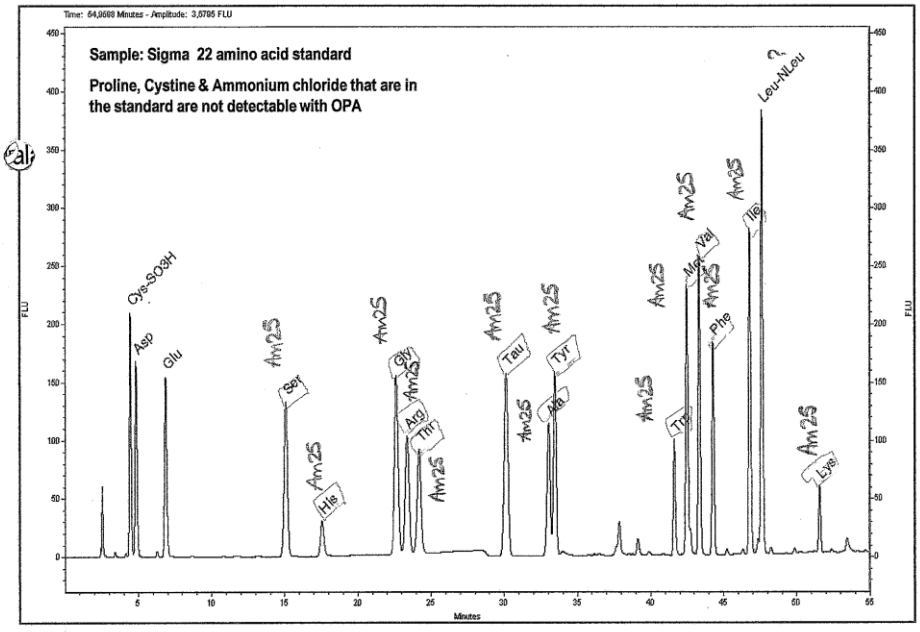
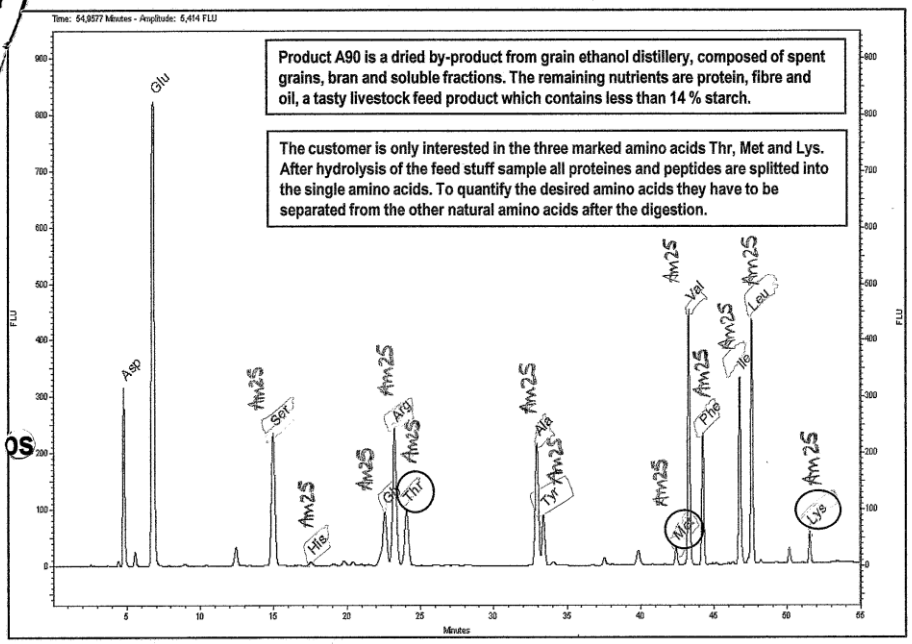
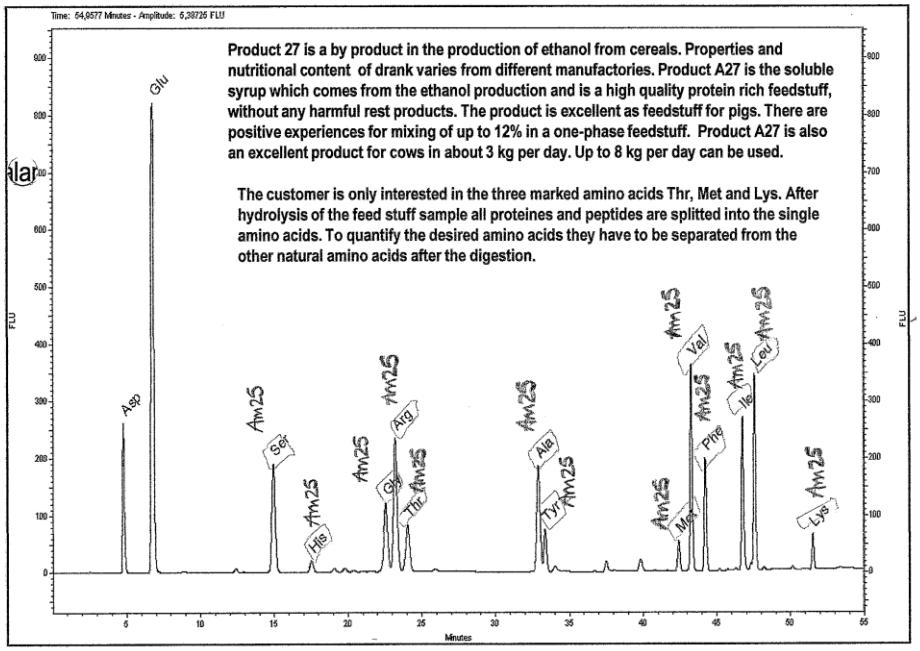

Sample: Sigma 18 amino acid standard
Proline, Cystine & Ammonium chloride that are in the standard are not detectable with OPA
Asp
Glu
Ser
His
Gly
Arg
Thr
Tyr
Met
Val
Ile
Phe
Leu
Lys
Minutes
FLU

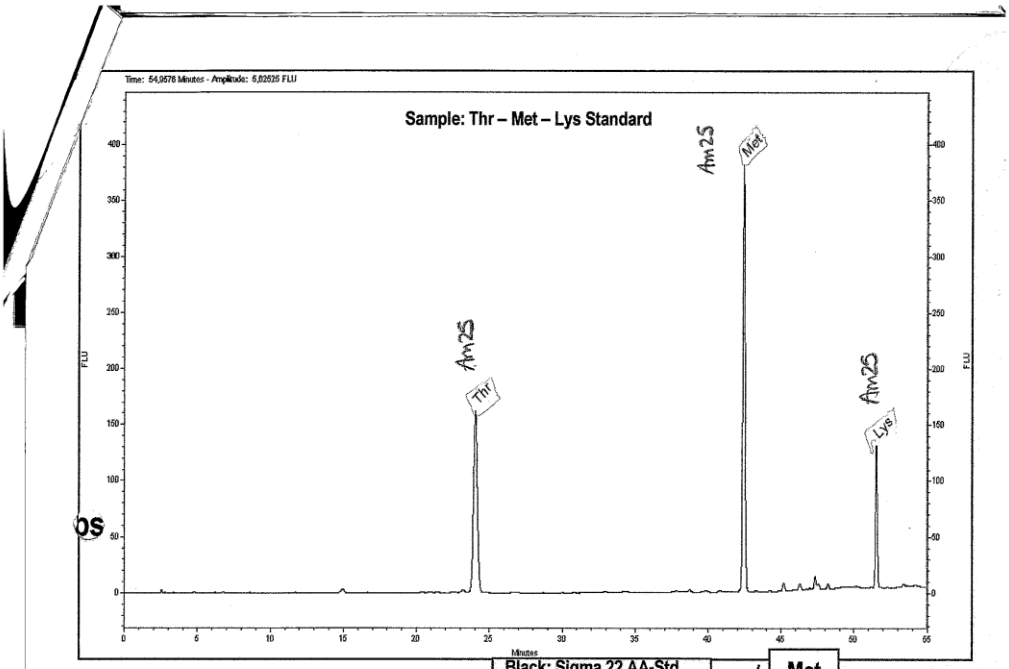

Sample: Sigma 22 amino acid standard
Proline, Cystine & Ammonium chloride that are in the standard are not detectable with OPA
Cys-SO3H
Asp
Glu
Ser
His
Gly
Arg
Thr
Tau
Tyr
Met
Val
Ile
Phe
Leu-NLeu
Lys
Minutes
FLU

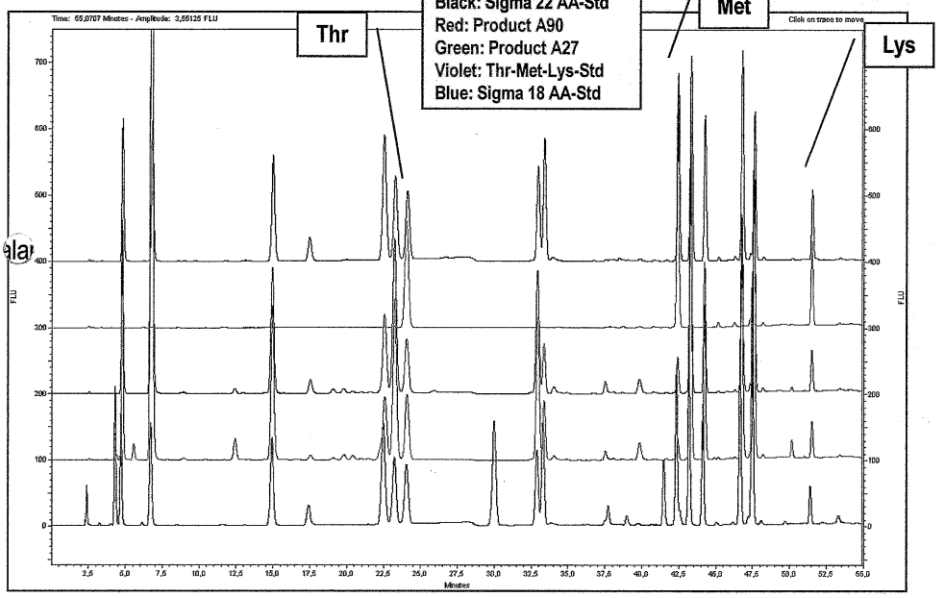
7


Time: 54,9577 Minutes - Amplitude: 5,414 FLU
Product A90 is a dried by-product from grain ethanol distillery, composed of spent grains, bran and soluble fractions. The remaining nutrients are protein, fibre and oil, a tasty livestock feed product which contains less than 14 % starch.
The customer is only interested in the three marked amino acids Thr, Met and Lys. After hydrolysis of the feed stuff sample all proteines and peptides are splitted into the single amino acids. To quantify the desired amino acids they have to be separated from the other natural amino acids after the digestion.
Asp
Glu
Ser
His
Gly
Arg
Thr
Ala
Tyr
Met
Val
Phe
Ile
Leu
Lys
Am25
FLU
Minutes


Time: 54,9577 Minutes - Amplitude: 5,39735 FLU
Product 27 is a by product in the production of ethanol from cereals. Properties and nutritional content of drank varies from different manufactories. Product A27 is the soluble syrup which comes from the ethanol production and is a high quality protein rich feedstuff, without any harmful rest products. The product is excellent as feedstuff for pigs. There are positive experiences for mixing of up to 12% in a one-phase feedstuff. Product A27 is also an excellent product for cows in about 3 kg per day. Up to 8 kg per day can be used.
The customer is only interested in the three marked amino acids Thr, Met and Lys. After hydrolysis of the feed stuff sample all proteines and peptides are splitted into the single amino acids. To quantify the desired amino acids they have to be separated from the other natural amino acids after the digestion.
Asp
Glu
Ser
His
Gly
Arg
Thr
Ala
Tyr
Met
Val
Phe
Ile
Leu
Lys
Am25
FLU
Minutes

8


Sample: Thr – Met – Lys Standard
Thr
Met
Lys
Minutes
FLU


Thr
Met
Lys
Black: Sigma 22 AA-Std
Red: Product A90
Green: Product A27
Violet: Thr-Met-Lys-Std
Blue: Sigma 18 AA-Std
Minutes
FLU

9

Appendix 2 Contents of 500 ml bottle of Aminoven® 25

	Amount (g)
Alanine	12.5
Arginine	10
Glycine	9.25
Histidine	3.65
Isoleucine	2.6
Leucine	4.45
Lysine	5.55
Methionine	1.9
Phenylalanine	2.75
Proline	8.5
Serine	4.8
Taurine	1
Threonine	4.3
Tryptophan	0.8
Tyrosine	0.2
Valine	2.75

Appendix 3 Aqueous formulation used for initial HPLC work

Constituents	Volume (ml)
Aminoven® 25	350.19
Glucose 70%	428.57
Additrace® (Fresenius Kabi, Runcorn, UK)	20
Solivito® N (in Water for Injections)	20
Sodium Chloride 30%	14.61
Potassium Chloride 15%	38.53
Potassium Acetate 49%	19.52
Calcium Chloride 1 mmol/ml	40
Magnesium Sulphate 50%	9.86
Glycophos® (Fresenius Kabi, Runcorn, UK)	50
Sodium Selenite 0.2 µmol/ml	2
Water for Injections	6.71

Appendix 4 Peak areas from intermediate precision. The shaded column shows the anomalous injection

	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Peak area 6	Peak area 6 compared to mean of peak areas 1-5 (%)
Serine	2.895	2.759	2.823	2.696	2.854	2.437	86.87
Glycine	8.619	8.148	8.293	7.843	8.201	7.083	86.16
Threonine	2.1	2.057	2.142	2.002	2.19	1.821	86.79
Arginine	4.496	4.235	4.318	4.079	4.331	3.694	86.07
Alanine	9.085	8.509	8.704	8.234	8.706	7.44	86.04
Taurine	0.694	0.655	0.671	0.644	0.681	0.587	87.74
Tyrosine	0.076	0.083	0.085	0.094	0.128	0.124	133.05
Valine	1.703	1.609	1.642	1.55	1.652	1.471	90.18
Methionine	0.996	0.926	0.947	0.886	0.945	0.816	86.81
Tryptophan	0.202	0.196	0.206	0.189	0.187	0.18	91.84
Isoleucine	1.508	1.412	1.447	1.351	1.454	1.229	85.68
Phenylalanine	1.238	1.167	1.189	1.14	1.214	1.035	87.00
Leucine	2.781	2.69	2.945	2.579	2.776	2.379	86.38