Lancaster University

A Method for the Quantitative Recovery of Mononucleotides from Fermentation Waste by Precipitation

By

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ABSTRACT

Several thousand litres of liquid effluent waste are generated from the production of mycoprotein, at the Quorn Foods production facility in Billingham (UK) every day. All of this waste currently undergoes effluent treatment before disposal, despite the fact that potentially valuable mononucleotides are known to be present at significant concentrations in the waste. To harness these nucleotides, an extraction procedure was necessary. Polynucleotide extraction procedures are well reported, using methods such as precipitation, phenol-chloroform extraction or polyethylene glycol (PEG) extraction, but application of these methods for mononucleotide has not been reported, so significant adaptation of conventional protocols was necessary to ensure good recovery of mononucleotides, with specific consideration of the process operating industrially. Precipitation of nucleotides was identified as the most favourable option, after comparison of conventional laboratory-based extraction protocols. The effects of salt choice, salt molarity, solvent choice, solvent ratio, pH, incubation temperature, pre-treatment and product washing were all tested as a function of yield, purity, protein retention and salt contamination, using UV-Visible spectrophotometry, the bicinchoninic acid (BCA) assay and inductively-coupled plasma optical emission spectroscopy (ICP-OES). Results showed that 0.06 M magnesium acetate was effective at extracting mononucleotides at a yield of up to 50%, although higher yields may be possible following recommended further work. It is hoped that this method may be the basis for an industrial procedure for the large-scale extraction of nucleotides from waste, which can then be distributed to a number of active markets across the UK, Europe and beyond.

Keywords

Dietary Supplement; Extraction; Flavour enhancer; Industrial; Infant formula; Nucleotides; Phenol-chloroform; Polyethylene glycol; Precipitation; UV-Visible Spectrophotometry; Waste Utilisation.

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My thanks must also go to Rachel Hoyle, who has dedicated so much time and energy into the project. I have a huge amount of admiration for you Rachel, your single-handed efforts in founding and building Nucleotide Nutrition in the often harsh world of business are nothing short of inspiring. But more than that, you've shown me time and time again this year how much of a thoughtful and considerate friend you are, so thank you for everything, nothing went un-noticed.

Dr Peter Köppel, you are one of the best role models I've ever come across, both as a scientist and person. You have dedicated your life's work to the world of nucleotides, and still appear incredibly enthusiastic and ambitious about the future of your work. Thank you for sharing your encyclopaedic knowledge of nucleotides and showing me your beautiful country, you are a true gentleman in every sense of the word.

I hope that I may thank both Dr Muyiwa Akintoye, Dr Tim Finnigan and all at Quorn foods, collectively. Muyiwa and Tim, you are both clearly very successful professionals, and you were always at the back of my mind when I needed motivation to continue and make this project succeed. I thoroughly enjoyed sharing your company, thank you for your feedback and encouragement.

Of course, without the Centre for Global Eco-Innovation (CGE), this collaborative project would never have arisen or been funded through the European Regional Development Fund (ERDF), so I must say thank you to all involved in its conception and organisation. In particular, I would like to thank Dr Andy Pickard, Carolyn Hayes and Jacob Lawson, for your advice and efforts throughout the past year.

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ABBREVIATIONS

Abs- Absorption

ADP- Adenosine Diphosphate

AMP- Adenosine Monophosphate

APRTase- Adenine phosphoribosyltransferase

ATP- Adenosine Triphosphate

BCA- Bicinchoninic Acid

BOD- Biological Oxygen Demand

BSA- Bovine Serum Albumin

cAMP- cyclic Adenosine Monophosphate

cDNA- complimentary Deoxyribonucleic Acid

CDP- Cytidine Diphosphate

CEO- Chief Executive Officer

CGE- Centre for Global Eco-Innovation

cGMP- cyclic Guanosine Monophosphate

CMP- Cytidine Monophosphate

COD- Chemical Oxygen Demand

CTP- Cytidine Triphosphate

dADP- deoxy Adenosine Diphosphate

dATP- deoxy Adenosine Triphosphate

dCDP- deoxy Cytidine Diphosphate

dCMP- deoxy Cytidine Monophosphate

dCTP- deoxy Cytidine Triphosphate

dGDP- deoxy Guanosine Diphosphate

dGTP- deoxy Guanosine Triphosphate

DNA- Deoxyribonucleic Acid

dsDNA- double stranded Deoxyribonucleic Acid

dTDP- deoxy Thymidine Diphosphate

dTMP- deoxy Thymidine Monophosphate

dTTP- deoxy Thymidine Triphosphate

dUDP- deoxy Uridine Diphosphate

dUMP- deoxy Uridine Monophosphate

ERDF- European Regional Development Fund

ETF- Effluent Treatment Facility

EU- European Union

GDP- Guanosine Diphosphate

GMP- Guanosine Monophosphate

GTP- Guanosine Triphosphate

HGPRTase- Hypoxanthine-Guanine Phosphoribosyltransferase

HMW- High Molecular Weight

HPLC- High Performance Liquid Chromatography

ICI- Imperial Chemical Industries

ICP- Inductively Coupled Plasma

ICP-OES- Inductively Coupled Plasma Optical Emission Spectroscopy

IMP- Inosine Monophosphate

mRNA- messenger Ribonucleic Acid

MSG- Monosodium Glutamate

OPA- O-phthaldialdehyde

ORP- Oxidation-Reduction Potential

PARNUTs- (Foodstuffs for) PARticular NUTritional (uses)

PEG- Polyethylene Glycol

PPM- Parts Per Million

PRPP- Phosphoribosyl Pyrophosphate

RCF- Relative Centrifugal Force

RNA- Ribonucleic Acid

RNase- Ribonuclease

RPM- Revolutions Per Minutes

RSD- Relative Standard Deviation

SDS- Sodium Dodecyl Sulphate

ssDNA- single stranded Deoxyribonucleic Acid

SWR- Standard Working Reagent

tRNA- transfer Ribonucleic Acid

TVC- Total Viable Count

UDP- Uridine Diphosphate

UMP- Uridine Monophosphate

UV- Ultraviolet

UV-Vis- Ultraviolet-Visible

λ_{max}- Wavelength of maximum absorbance

1- INTRODUCTION

1.1- INTRODUCTION TO THE RESEARCH OPPORTUNITY

In 2012, Rachel Hoyle, founder and CEO of nucleotide supplement producer Nucleotide Nutrition LTD, begun collaborating with a large Dutch company called Vitablend, which purchased purified nucleotides for distribution into the infant formula market. One of Hoyle's long-standing contacts, Dr Peter Köppel, a biochemist and immunologist and CEO of Swiss nucleotide producer ProBio AG, had by this time conducted a number of clinical trials in collaboration with Hoyle- to specifically demonstrate the benefit of dietary nucleotide supplementation. These promising findings, combined with an increase in commercial activity in the human health supplement market, led to a tripartite collaboration between Nucleotide Nutrition, ProBio and Vitablend. During early discussions between the companies, it became evident that a mutual interest of the companies was to find an alternative, European source of nucleotides, as all the companies currently obtained their nucleotide products from Chinese suppliers, via notoriously unpredictable supply lines.

In 2013, Hoyle delivered a short presentation at a grants meeting in Birmingham, UK, to explain her company's work. A consultant of UK company Quorn who was in attendance, later approached Hoyle. After discussions, an exploratory meeting was set up between Hoyle and Tim Finnigan, director of research and development at Quorn, where it was discussed that Quorn produce a significant quantity of nucleotide-rich waste at their production facility in Billingham, UK. It was quickly realised that, if the nucleotides could be extracted from the waste industrially, this could create a new European nucleotide production hub to supply Nucleotide Nutrition, ProBio and Vitablend with nucleotides. With this in mind, samples of the waste were sent to Köppel for some preliminary analysis, the results of which were largely inconclusive, as the physical properties of the waste made analysis challenging.

Although Nucleotide Nutrition still classifies as a micro business, the company operates from the large Daresbury Innovation Centre in the North West of England, where several grants and

business opportunities are regularly advertised. One such opportunity was noticed by Hoyle, which presented the opportunity to receive funding for a collaborative project between a small North West business and a larger businesses, via the Centre for Global Eco-Innovation (CGE). The centre facilitates the collaboration between businesses and appoints a researcher to complete a research project to benefit all parties involved, funded by Lancaster University, the University of Liverpool, the European Regional Development Fund (ERDF) and Inventya, a third party company which provides international commercialisation consultancy to those involved in the project, to help increase the impact of the research and development work.

The application for the grant was accepted, which created a 1-year Master's by Research project, where a researcher would aim to devise and refine an extraction procedure to isolate nucleotides from the waste produced by Quorn. This project was advertised as an industry-led Master's by Research project to be completed at Lancaster University, and advertised to high attaining graduates in chemistry or biochemistry. After selecting amongst applicants, Jonathan Longden, a recent biochemistry graduate from Lancaster University, was accepted for the post. Longden would complete the project at Lancaster University's engineering department, under the direct supervision of Dr Vesna Najdanovic, a recently appointed Faculty Research Fellow in the department. Professor Peter Fielden and Professor Jamshed Anwar, both from the Department of Chemistry, were also appointed as additional supervisors for the project.

1.2- RESEARCH AIMS AND OBJECTIVES

The aim of this study is to devise an experimental procedure to extract and purify nucleotides from the neat waste emerging from the Quorn production facility. After evaluation of extraction methods that are currently employed for similar purposes, a suitable method can be identified and iteratively refined via experimentation and scrutiny of results.

During modification of the process, there are several considerations that must be fulfilled by the final process:

- **Yield and Purity**- The final process must extract nucleotides from the waste at a high yield. Initial work must be performed to determine the nucleotide concentration within the waste, and continuous analysis of yielded samples from various extraction conditions must be carried out using an appropriate method, to determine% yield. Additionally, the process must be effective in removing contaminants, to give a sufficiently pure product for sale or further processing.
- **Safety**- As the yielded nucleotides will ultimately be destined for food applications, it is imperative that the process uses food safe reagents to ensure the nucleotides remain safe for human and animal consumption. Careful and comprehensive analysis of the final nucleotide product should be used to ensure the product is free harmful levels of any potential impurities**.**
- **Scaling** - As an industrial process, the final process must be suitable for scaling to industrial levels, so must not be heavily reliant on tools or processes that can only be performed effectively at small-scales.
- **Throughput and simplicity**- As the Quorn production facility produces a huge amount of waste daily, the process must be able to cope with this output and process this to extract nucleotides from it. This relies on the process being quick and straightforward.
- **Cost**- As an industrial process which produces a product for sale, the process must not rely on costly reagents or processes which require significant energy input, to ensure the process is cost-effective.
- **Sustainability**- One of the wider objectives of this project falls in line with those of the project sponsor- the Centre for Global Eco-Innovation (CGE). These ambitions state that an environmental benefit must result from the endeavour, and so the process must divert the waste from its current effluent treatment process to production of a saleable product. Additionally, the process must integrate measures that limit the emission of greenhouse gases, use of natural resources and waste minimisation and re-use. To achieve this, the use of environmentally damaging or scarce resources, and significant use of any other

resources should be minimised, and recycling and reuse of these resources should be considered where appropriate.

2- LITERATURE REVIEW

2.1- NUCLEOTIDES: AN OVERVIEW

Nucleotides are biomolecules that fulfil a plethora of different structural, energetic, metabolic, catalytic and regulatory functions. Principally, nucleotides act as the subunits of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), which carry genetic information within cells and facilitate the transcription and translation of proteins. In addition to this, nucleotides also act as energy carrier molecules; such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP), as well as cell signalling mediators such as cyclic adenosine monophosphate (cAMP). Nucleotides are also incorporated into a variety of cofactors, which play a key role in enzymatic reactions (Rudolph, 1994).

Nucleotides are composed of β-D-ribofuranose, bound to a heterocyclic nitrogenous base (or *nucleobase*) via an *N*-glycosidic linkage, and one to three inorganic phosphate groups (see Figure 1). When devoid of any phosphate groups, the molecule is referred to as a nucleoside, and the number of bound phosphate groups can be specified by nomenclature, e.g. nucleoside mono-/di-/tri-phosphate. The pentose sugar is 2-deoxy-D-ribose in the case of *deoxyribonucleotides* (also referred to as *deoxynucleotides*), which are destined to be incorporated into DNA, or D-ribose in the case of *ribonucleotides*, which are incorporated into RNA.

Commonly, nucleotides have a phosphate group bound to the 5’ carbon of this pentose sugar, but 3’ nucleotides, where the phosphate is bound to the 3’ carbon of the sugar, are also present within cells, although these cannot be utilised in the formation of nucleic acid strands. Similarly, 2’ nucleotides are occasionally observed within cells. There are two basic types of nitrogenous bases that can be incorporated into nucleotides; purine bases and pyrimidine bases. The presence of a particular base determines the type of nucleotide, and also many of its key functions. For example,

purine nucleotides only bind, via hydrogen bonding, to a complementary pyrimidine nucleotide during the formation of nucleic acid double strands.

Purine bases within nucleotides share a common structure of a pyrimidine ring fused to an imidazole ring. The two purine bases adenine and guanine predominate in cells, which are constituents of the nucleotides adenosine and guanosine, respectively. A third purine base, hypoxanthine, is also present in cells, which is part of the nucleotide inosine. Inosine nucleotides are key intermediates in the synthesis of purine nucleotides, the purine nucleotide cycle also contributes some inosine nucleotides into the cellular nucleotide pool. However, inosine nucleotides are not incorporated into nucleic acid strands. The purine bases xanthine and uric acid also occur naturally in the body, but are associated with degradation of nucleotides, and so are not present in mature nucleotides. Fragments of polynucleotide strands, 2' and 3' monophosphates and modified bases can also be observed in the cytosol of cells, but these too are derived from degradation of nucleic acid strands (Devlin, 2011).

Pyrimidine bases, on the other hand, all derive from a simple pyrimidine ring. There are three types of pyrimidine bases; cytosine, thymine and uracil, that are incorporated into the nucleotides; cytidine, thymidine and uridine, respectively. Cytidine can be incorporated into RNA and DNA, when it contains the appropriate sugar. Thymidine can only be incorporated into DNA (in its deoxyribose form), while uridine can only be incorporated into RNA strands (in its ribose form).

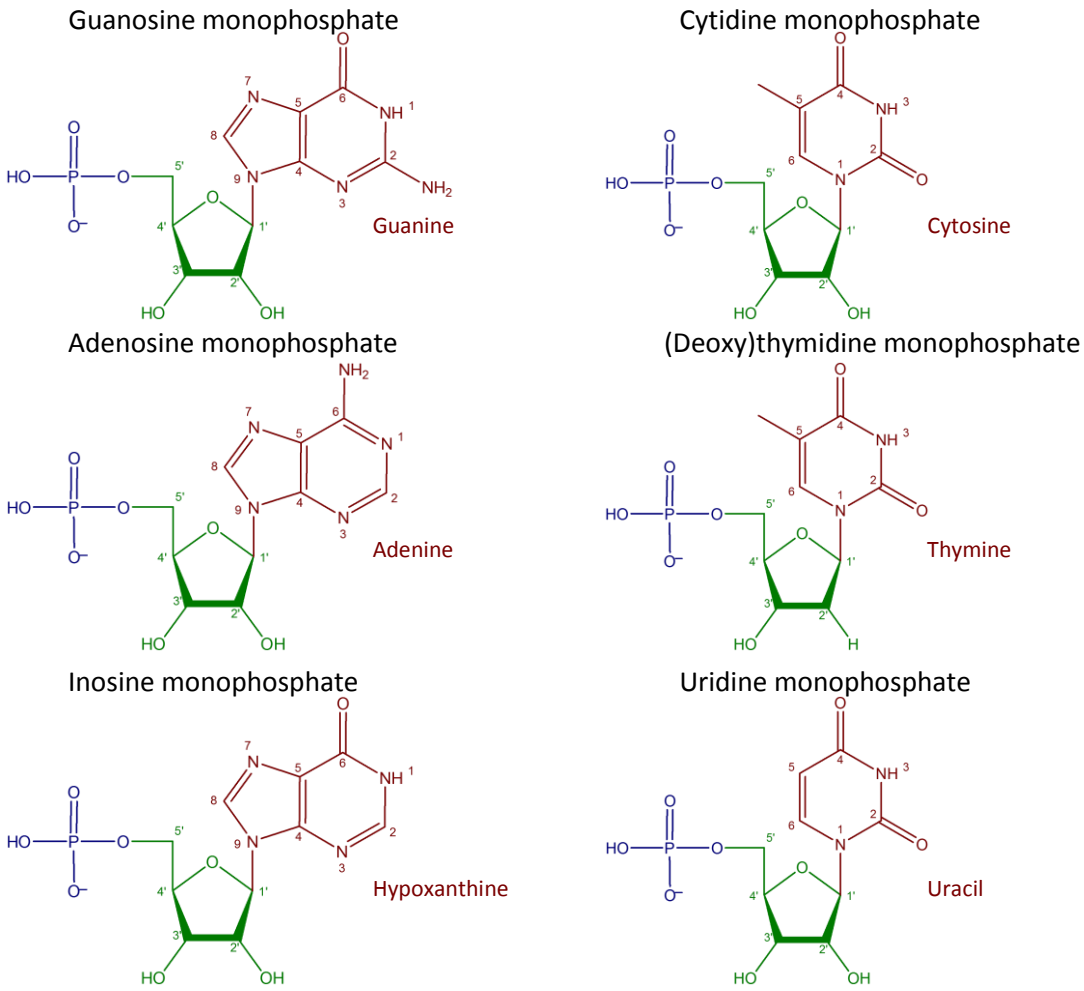


***Figure 1*- Structures of the six nucleotides that predominate in cells**. Purines are shown on the left and pyrimidines are on the right. Each has been colour coded to show the monophosphate group (blue), the pentose sugar (green) and the nitrogenous base, which is also labelled (red).

The purine nucleosides adenosine and guanosine can also form cyclic nucleotides when the 5'-monophosphate also binds to the 3'-carbon of the ribose sugar. These cyclic nucleotides- cAMP (cyclic adenosine 5'-monophosphate) and cGMP (cyclic guanosine 5'-monophosphate) act as vital second messengers in a number of cellular signalling transduction pathways (Duman and Nestler, 1999)

Humans are able to generate nucleotides via *de novo* synthesis, salvage pathways from nucleotide fragments left following cellular turnover, as well as interconversion of nucleotides. In *de novo* synthesis, each atom of a nucleotide base is derived from amino acids, either directly or indirectly, as shown in Figure 2. Both purine and pyrimidine nucleotides originate from 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) (shown in Figure 3), which is also adjoined to salvaged bases to

generate mature, usable nucleotides. PRPP is synthesised via the pentose pathway, from ribose-5-phosphate, or from phosphorylysis of nucleotides.

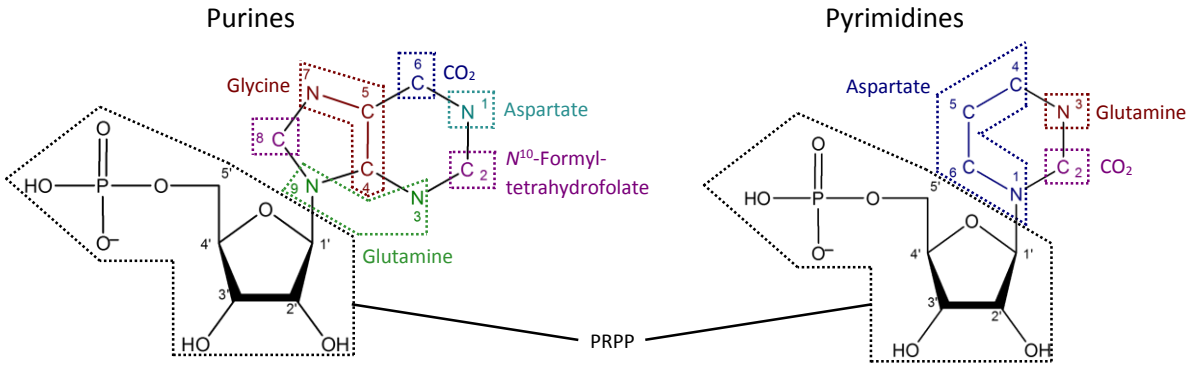


***Figure 2*- The biosynthetic origin of the atoms of purine and pyrimidine nucleotides, as generated by *de novo* synthesis.**
Adapted from a schematic from Rudolph, 1994 & Devlin, 2011.

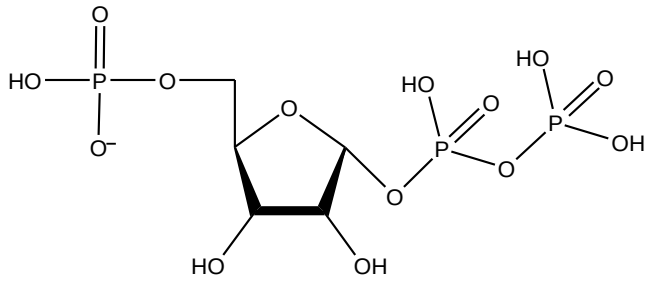


***Figure 3*- The structure of 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP).**

The amino acid glutamine has several other key roles in the synthesis of nucleotides. During some stages of purine and pyrimidine synthesis, glutamine is required for nitrogen donation to nucleotide precursors.

De novo purine nucleotides synthesis starts with PRPP and progresses via a 10-step process, which is reliant on various cytosolic enzymes. A mature purine ring is not created until the final product, inosine 5'-monophosphate (IMP), is formed. IMP can then subsequently used to produce adenosine monophosphate (AMP) and guanosine monophosphate (GMP). Six moles of ATP (or equivalent) are required to produce one mole of IMP, so the process comes at a significant energy cost (Devlin, 2011). An overview of this process is shown in Figure 4.

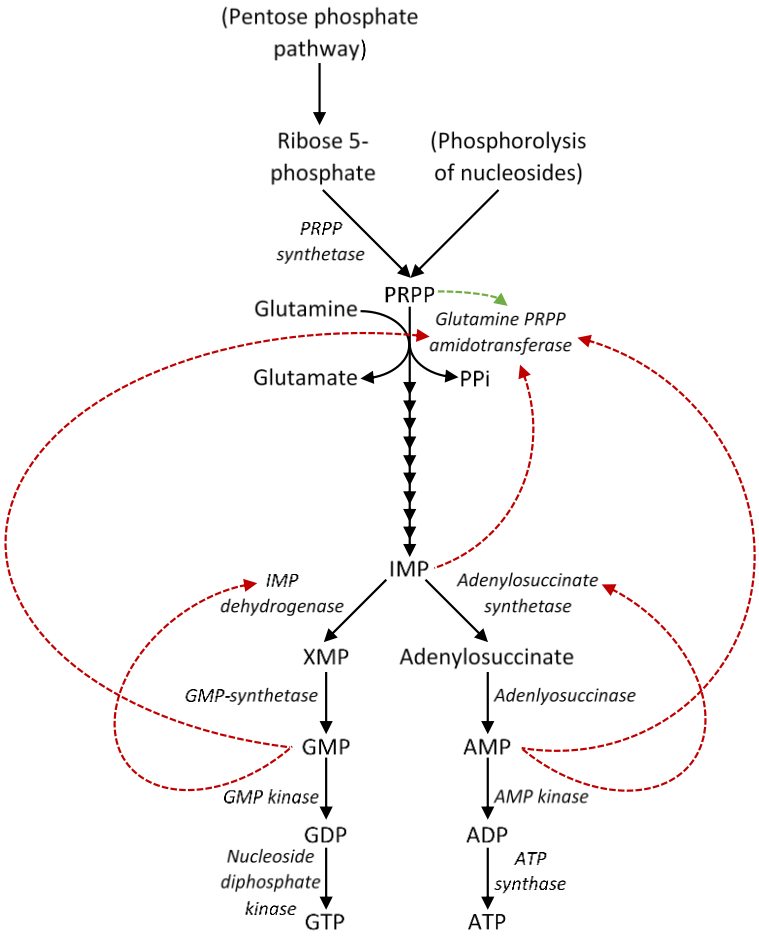


***Figure 4*- An overview of purine nucleotide synthesis.** Dashed lines show regulation- green lines show the action of positive effectors, red lines show the action of negative effectors (adapted from Devlin, 2011 and Salway, 1999).

Pyrimidine nucleotide synthesis progresses via a six step process, starting with glutamine, carbon dioxide and ATP. The enzymes that catalyse each step of the process are not all cytosolic, dihydroorotate dehydrogenase, which catalyses the oxidation of dihydroorotate to orotate, is mitochondrial. Again, an overview of pyrimidine synthesis is shown in Figure 5.

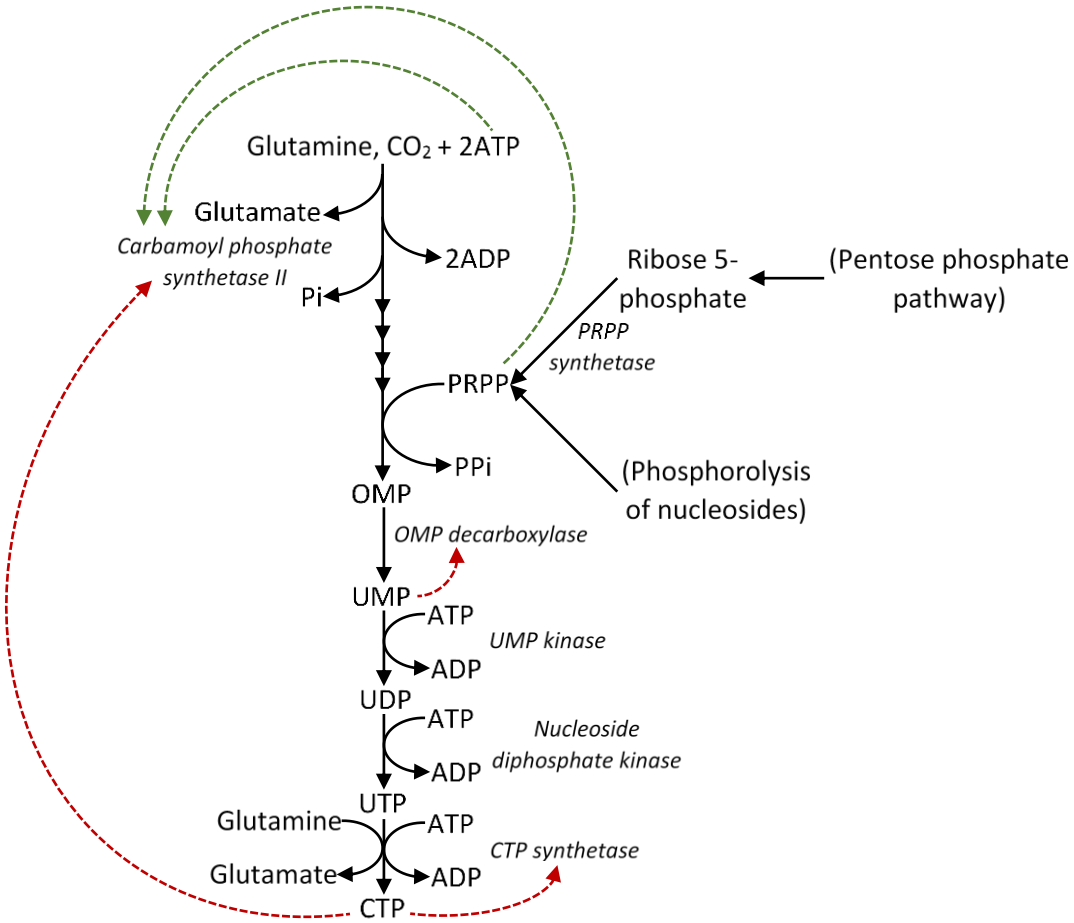


***Figure 5*- An overview of pyrimidine nucleotide synthesis.** Dashed lines show regulation- green lines show the action of positive effectors, red lines show the action of negative effectors (adapted from Devlin, 2011 and Salway, 1999).

Both purine and pyrimidine synthesis yield ribonucleotides. All deoxyribonucleotides are hence synthesised via the enzyme nucleoside 5'-diphosphate reductase (ribonucleotide reductase), which converts both purine and pyrimidine ribonucleotides to their deoxyribose equivalents. The substrate for ribonucleotide reducatase is a nucleotide diphosphate, so in the formation of deoxycytidine monophosphate (dCMP), CTP must first be hydrolysed to cytidine diphosphate (CDP) by a nucleotidase. This is shown in Figure 6.

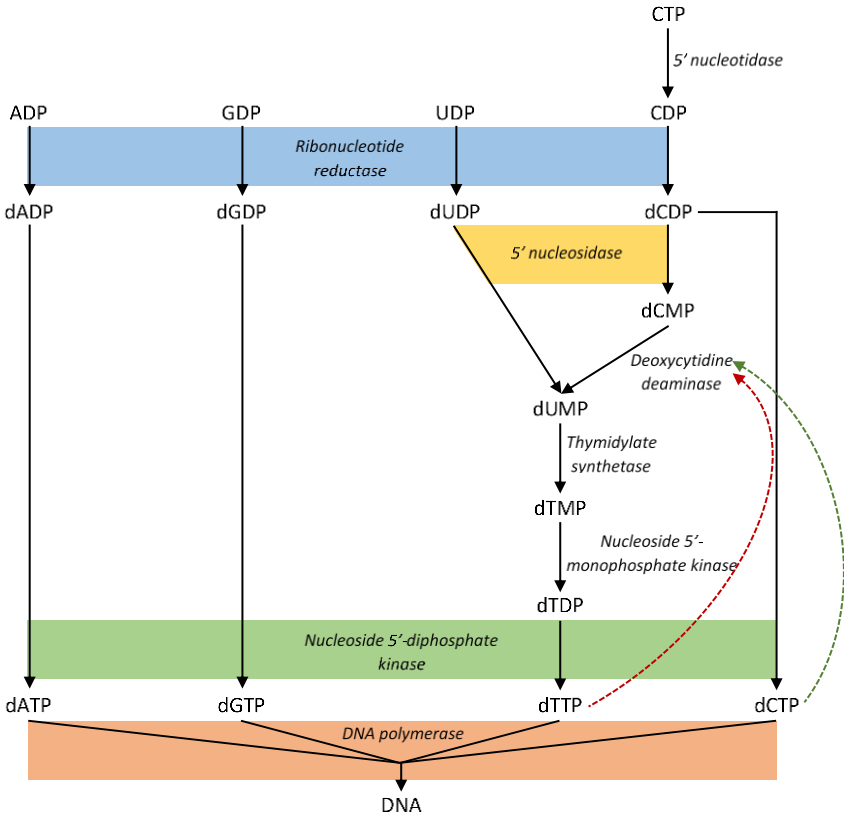


***Figure 6*- An overview of deoxynucleotide triphosphate synthesis.** Dashed lines show regulation- green lines show the action of positive effectors, red lines show the action of negative effectors (adapted from Devlin, 2011 and Salway, 1999).

Because of the high metabolic cost of *de novo* synthesis, conserved salvage pathways also exist which make use of free bases and preformed nucleosides, which can exist intracellularly as a product of degraded nucleotides, or from exogenous sources. PRPP is required as a ribose phosphate donor in the regeneration of nucleotides.

In the salvage of purine nucleobases, two phosphoribosyl transferases exist which have specificity for different bases. Hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) catalyses the reaction between PRPP and hypoxanthine or guanine, while adenine phosphoribosyl transferase (APRTase) catalyses the reaction between PRPP and adenine. From these reactions, a nucleoside monophosphate is produced, along with inorganic pyrophosphate.

Because PRPP is a common substrate in both salvage pathways and *de novo* synthesis, the relative affinity of each of the transferase enzymes determine the predominance of a specific pathway. APRTase has the highest affinity, followed by HGPRTase and PRPP amidotransferase. But because HGPRTase can catalyse salvage-based synthesis with two generally abundant nucleosides, this

enzyme is the most significant user of PRPP. Because PRPP is preferentially consumed by the enzymes that participate in salvage-based synthesis, this has the effect of downregulating *de* novo synthesis (Ramakrishnan et al, 2001). Because of the metabolic saving of salvage-based synthesis compared to *de* novo synthesis, enzymatic regulation also exists that allows crosstalk between the pathways. IMP and GMP are competitive inhibitors of HGPRTase, while AMP is a competitive inhibitor of APRTase. IMP, GMP and AMP produced from salvaged bases and nucleosides are also able to inhibit *de novo* purine nucleotide synthesis at the PRPP amidotransferase step, which reinforces the preferential use of preformed bases and nucleosides. This process is summarised in Figure 7.

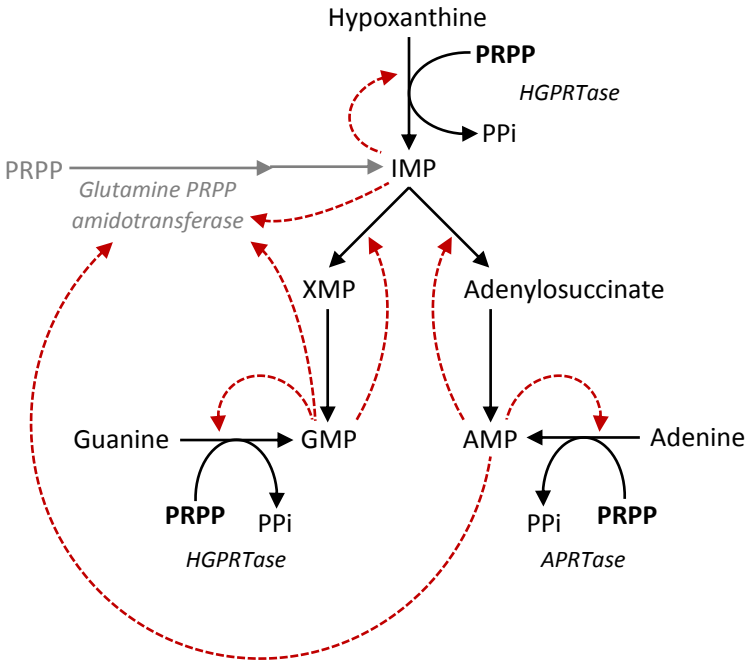


***Figure 7*- An overview of purine salvage**. The regulatory network that exists within the pathway is also shown by dashed lines- red lines show the action of negative effectors. The *de novo* purine synthetic pathway is summarised in grey to show pathway interaction.

RNA is known to be around 1000 times more concentrated within cells than DNA (Barness, 1994). Deoxyribonucleotide concentration is cell cycle stage dependent, but generally ranges around 2-60 $\mu molL^{-1}$. Cell-cycle dependent regulatory mechanisms exist that increase the levels of deoxyribonucleotides in anticipation of DNA replication, so considerable variation is observed in the levels of these nucleotides within cells (Devlin, 2011). Conversely, ribonucleotide

concentration remains somewhat more stable, with most of the ribonucleotide content within cells being contributed by ATP, which alone, ranges between 2-10 $mmolL^{-1}$. However, any decrease in ATP concentration during a cellular process results in a corresponding increase in ADP or AMP, so the contribution of ATP/ADP/AMP, and other nucleotide-derived energy carrier molecules for that matter, remains essentially constant in cells with a fixed energy state (Devlin, 2011). All other ribonucleotides range in concentration between 0.05-2$mmolL^{-1}$ (Rudolph, 1994).

Nucleoside triphosphates are generally more abundant than di- and mono- phosphates (Rudolph, 1994). This is primarily due to the fact that the high concentration ATP energy carrier is a nucleoside triphosphate, also DNA and RNA polymerases are only able to use nucleoside triphosphates when elongating a growing nucleic acid strand (Berg et al., 2002). According to the generally accepted semi-conservative model of DNA replication, the process involves the unwinding and partial cleavage of a complete DNA strand, to which complementary deoxyribonucleotides bind in the 5' to 3' direction, where the reaction is catalysed by the DNA polymerase complex. This results in 2 daughter double strands, connected via hydrogen bonds between complimentary bases. The finished strands then adopt an antiparallel double helical structure. RNA replication proceeds in a similar manner, whereby a DNA template unwinds and cleaves, and then complimentary ribonucleotides bind in a reaction catalysed by an RNA polymerase complex. The mature *messenger RNA* (mRNA) single strand then dissociates, rather than forming an RNA double strand, which are not usually present in eukaryotic cells. RNA strands can then assume a range of different structures to suit a particular downstream function, such as transfer RNA (tRNA) to carry amino acids to a ribosome for RNA synthesis or ribosomal RNA (rRNA), which along with proteins, make up the ribosome. The general structure of a DNA double strand segment is shown in Figure 8.

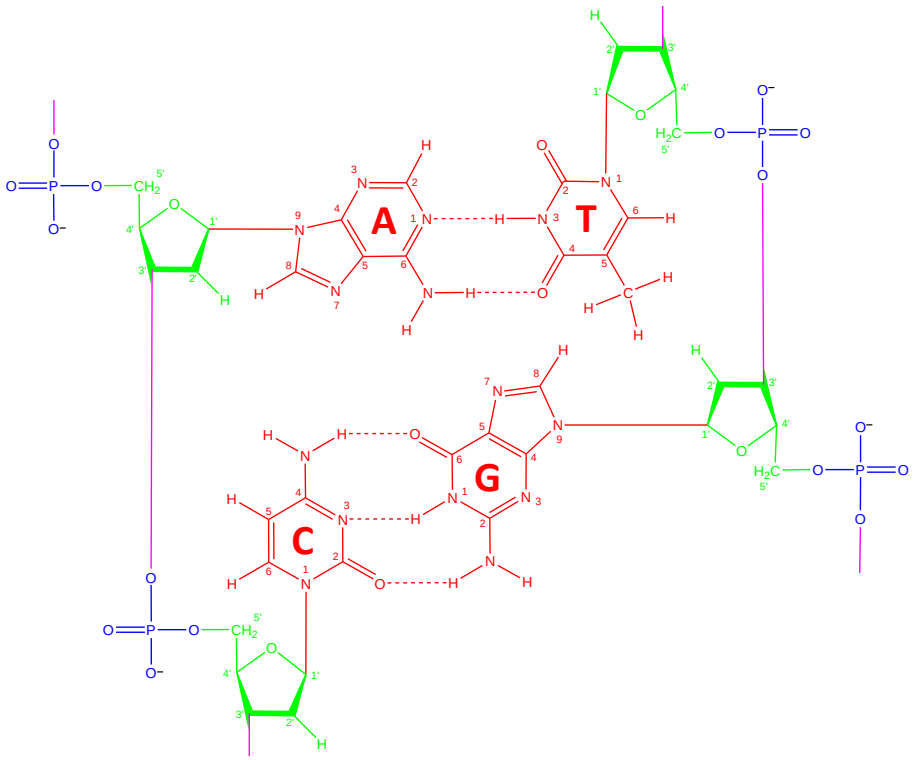


***Figure 8*- The structure of a short of a DNA double strand.** Two hydrogen bonds link adenine (A) and thymine (T) bases, while 3 hydrogen bonds like cytosine (C) and guanine (G) bases. The pink bonds show those formed during strand polymerisation, which are part of the phosphodiester backbone of the DNA strand.

2.1.2- Nucleotides as Dietary Supplements

All foods of an animal or vegetable origin contain *nucleoprotein*, or a protein linked to a nucleotide. The abundance of nucleoprotein in different foods is dependent on cell density (Hess and Greenberg, 2012). Absorption of dietary nucleotides occurs in the intestine, where the nucleoprotein is proteolytically cleaved, to yield nucleic acids. Nucleases and phosphodiesterases then cleave the nucleic acid strands into monomeric nucleotides, which can then be absorbed by enterocytes that line the intestinal wall. The site of cleavage at the phosphodiester bond is significant, as either 5' or 3' nucleotides can be generated. However, nucleosides are better absorbed by enterocytes, so alkaline phosphatases are able to remove the phosphate group from nucleotides in the lumen to give nucleosides. Unabsorbed nucleosides in the intestinal lumen are degraded by nucleosidases to give free bases, which can also be absorbed by the enterocytes.

Following absorption, only a small proportion of nucleosides, particularly adenine and uracil nucleosides, are directly incorporated into nucleotide pools within the body. This process of

incorporation into nucleotide pools is not significant in terms of the levels of absorbed nucleotides, the majority of which are actually degraded and their products excreted in urine.

In addition to their numerous intracellular functions, a wealth of research has begun to recognise the value of purified nucleotides, used as dietary supplements. In contrast to nucleotide absorption from food sources, which require extensive degradation throughout digestion, purified nucleotide supplements are highly bioavailable.

Nucleotides may be classified as semi-essential nutrients, as humans possess the capability to synthesise them *de novo*. However, under certain physiological stresses, nucleotide demand may be increased. During growth and development, recovery, immune system activation and gastrointestinal distress, the body's nucleotide demand is increased, as new cells are required, which require an abundance of nucleotides. In this case, *de novo* synthetic mechanisms are often too energetically costly to supply this increased requirement, and sufficient nucleotide fragments may not be available for salvage-based synthesis. Additionally some cells, such as those of the gastrointestinal tract, those derived from bone marrow, healthy gut floral bacteria and certain brain cells lack the capacity to produce their own nucleotide supply *de novo*, so preferentially utilise the salvage pathway. Exogenous nucleotides from dietary origin may become key in this case (Uauy, et al., 1996). Figure 9 shows the nucleotide contents of a variety of foodstuffs.

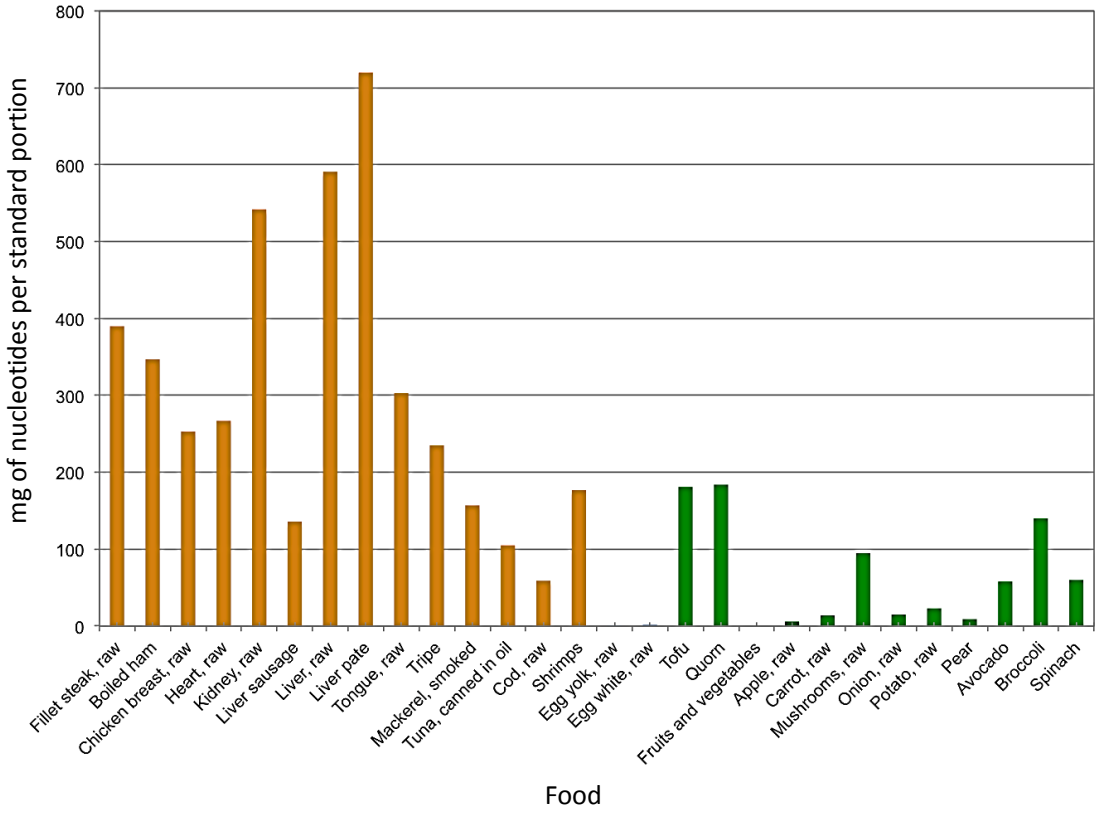


***Figure 9*- Evaluation of total nucleotide content of a range of meat (orange) and vegetarian (green) protein sources according to typical single portions.** Taken from Verkerk and Köppel, 2011.

As expected, meats have a higher nucleotide content overall, as they have a higher cell density. Organ meats and offal, which were often staples of ancestral diets, are shown to have the highest nucleotide content, yet these foods have gradually faded out of the modern western diet (Verkerk and Köppel, 2011). However, as shown in Figure 10, all foods were found to be primarily rich in purine nucleotides.

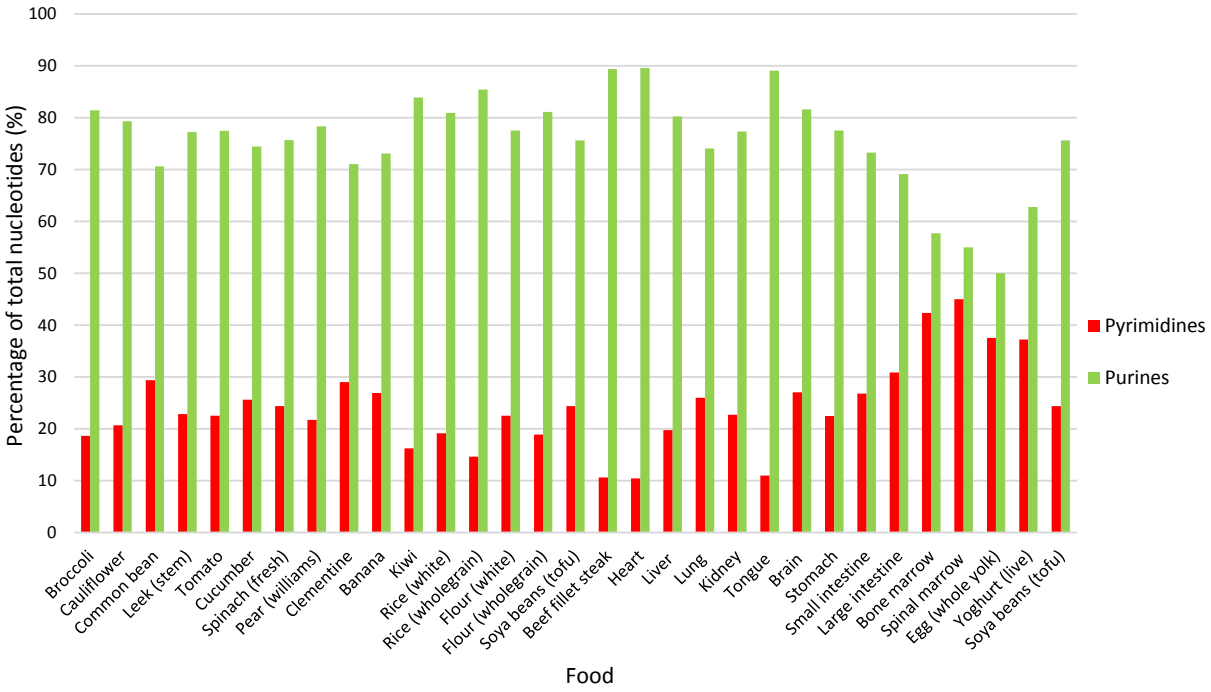


***Figure 10*- Evaluation of purine and pyrimidine contents of various foods.** Produced from supplementary unpublished data, courtesy of Dr Peter Köppel, 2009.

For normal cell functioning, the body must maintain equimolar concentrations of purine and pyrimidine nucleotides, as both this is necessary for nucleic acid strand formation, one nucleotide cannot be substituted for another. More balanced nucleotide profiles are found in foods where new cells are produced, in foods such as egg yolk and bone marrow, although the overall nucleotide content in these foods per portion is very low. Even when nucleotide-rich foods are consumed, a healthy gut is required for the effective absorption of nucleotides from food.

Increased nucleotide demand is often brought about due to a need for additional cells. Lymphoid cells of the immune system proliferate rapidly during immune activation, so require a significant amount of nucleotides to divide fast enough to tackle an invading foreign microbe, which generally proliferate much faster than human immune cells. In this case, dietary nucleotide supplementation has been shown to be beneficial in this case (Gil, 2002), by supporting a broad range of immune responses (Carver, 1994). A number of *in vivo* studies in mice have shown dietary nucleotide supplementation results in; increased lymph node proliferation (Yamauchi et al., 2002), higher natural killer cell and macrophage activity (Carver et al., 1990), increased resistance to microbial

challenge (Kulkarni et al., 1986) and stimulation of bone marrow cell proliferation and peripheral blood neutrophil populations following infection (Matsumoto et al., 1995).

Cells that line the gut have the highest rate of turnover in the body, so require a significant proportion of the body's free nucleotides to regenerate. When the gut is in need of repair, following damage or infection for example, this nucleotide demand grows significantly. Again, several studies have shown nucleotide supplements to be beneficial in maintaining and repairing the gut lining, as shown in Figure 11. Observations from a number trials have shown that dietary nucleotide supplementation results in improved intestinal healing following chronic diarrhoea or gastrointestinal distress, and improved intestinal ultrastructure (Carver, 1999)(Bueno et al., 1994). Uauy, 1998, also explains that bifidobacteria, which are normal bacterial residents in the gut flora, use nucleotides as growth factors in the intestinal lumen, and so proliferate more effectively in the presence of sufficient bioavailable nucleotides.

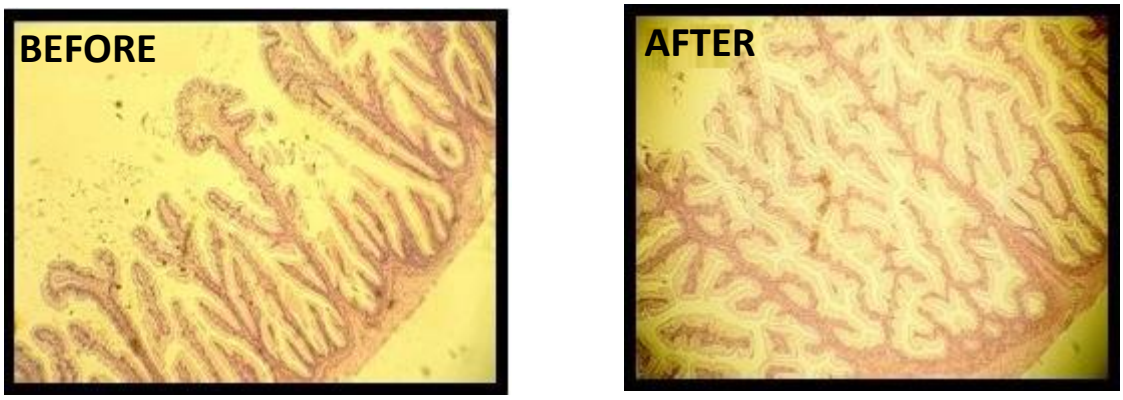


***Figure 11*- Histological slides of intestinal villi, before and after 3 weeks of nucleotide supplementation.** Seventy-fold magnification, haematoxylin and eosin stained samples, Atlantic salmon model used (Burrels et al., 2001).

Following injury or strenuous exercise, nucleotide demand increases, so dietary nucleotide supplements are thought to be beneficial for elite athletes in a number of ways. Studies have shown that nucleotides have a protective effect against stress (Riera et al., 2013) and lower stress thresholds and reduce cortisol build-up (McNaughton et al., 2006). Additionally, as nucleotides are vital intermediates in protein synthesis (Lopez-Navarro, 1996), nucleotide supplementation has been shown to preferentially divert nutrients toward muscle growth, as opposed to fat storage.

In addition, several studies have demonstrated the positive effects of nucleotides on metabolism (Kabal and Ramey, 1965), appropriate hormone signalling through correct supply of nucleotide-derived second messengers (Denninger and Marletta, 1999)(McNaughton et al., 2006), tissue repair and wound healing (Rudolph et al., 1990), oxidative damage on DNA (Salobir et al., 2005), lipid synthesis (Gella et al., 2011)(Sanchez-Pozo et al., 1995), cognitive ability (Sato et al., 1995)(Chen et al., 2000), liver repair (Uauy, 2011) and cellular detoxification (Frankic et al., 2006).

Based on these findings, a range of commercial nucleotide dietary supplements have become available for humans, livestock and domestic pets.

2.1.3- Adding Nucleotides to Infant Formula

Human breast milk is known to maintain fairly constant levels of different soluble ribonucleotides, totalling around 210 $\mu molL^{-1}$, or around 71.8 mgL^{-1} (Hess & Greenberg, 2012). This being said, the levels of certain nucleotides are known to change somewhat during the course of lactation, possibly to accommodate for the changing needs of an infant. It is possible that infants may not have sufficient surplus energy to synthesise nucleotides *de novo*, and may have a higher nucleotide requirement than adults, due to their faster rate of growth (Stein and Mateo, 2005). In an effort to replicate the natural composition of breast milk, an increasing number of milk formulas are now supplemented with nucleotides to support babies and infants during this period of rapid growth (Yu, 1998). However, human breast milk contains more nucleotides than formulae, and more than cow's milk for that matter (Barness, 1994). Despite the somewhat minor contribution of nucleotides to human breast milk and nucleotide-enriched formula, infants fed on natural breast milk or nucleotide-enriched formula are seen to benefit from improved; immune function, response to immunisation (Carver et al., 1991), gastrointestinal health and mesenteric blood flow, lipid metabolism and growth (Hess & Greenberg, 2012).

The supplementation of infant formula with nucleotides is strictly regulated. The European Union states an upper limit of 5 mg of total nucleotides per 100 kcal of formula, as well as limiting the quantity of each individual nucleoside monophosphate added to formula, as follows; guanosine

monophosphate (GMP) 0.5 mg per 100 kcal, adenosine monophosphate (AMP) 1.5 mg per 100 kcal, inosine monophosphate (IMP) 1.0 mg per 100 kcal, cytidine monophosphate (CMP) 2.5 mg 100 kcal and uridine monophosphate (UMP) 1.75 mg 100 kcal. The United States simply sets an upper limit for the nucleotide content of formula to 16 mg 100 kcal.

2.1.4- Nucleotides as Flavour Enhancers

Some nucleotides, despite having little inherent flavour, are known to contribute to the umami (or savoury) taste of foods, even at low concentrations. The purine nucleotide disodium 5'-guanylate (GMP) is a very active flavour enhancer, alongside disodium 5'-inosate (IMP), which is somewhat less active. To produce IMP in sufficient amounts industrially, adenosine monophosphate (AMP) is commonly enzymatically converted to IMP with AMP deaminase (Chae, 2001).

Nucleotides only have flavour enhancing properties in their 5'-monophosphate salt form, 2' or 3' monophosphates, nucleosides or nucleobases have little or no flavouring properties (De Palma Revillion, et al., 2003)(Yamaguchi and Ninomiya, 1998). 5'-adenylic acid, a derivative of adenosine, is a weak flavour enhancer, which is often found naturally in foods, but not generally used as a commercial food additive (Yamaguchi and Ninomiya, 1998).

Monosodium L-glutamate is very commonly used flavour enhancer, but MSG is around 100 times less active in comparison to GMP and IMP (Vieira et al, 2013). MSG and IMP and GMP have been shown to work synergistically in savoury foods, with an overall cost reduction and no negative effect on the organoleptic properties of the product. The flavour enhancing properties imparted by 100 g of MSG can be replicated with the use of only 17 g of MSG, when used in combination with just 0.9 g of a 50:50 blend of GMP and IMP (Lölinger, 2000).

2.1.5- Industrial Nucleotide Production and Economic Value

The industrial production of nucleotides is normally achieved by fermentation of yeasts, which are known to give a high ribonucleotide yield, with little deoxynucleotide content (Perlman, 2012). Often, culture strains which have been genetically modified to increase their ribonucleic acid

production, or produce a higher proportion of the economically significant purine nucleotides, are used (Arora et al., 1991). Strains are then fermented at large scales, often in countries such as Brazil or China, where inexpensive carbon sources are easily accessible, using culture conditions that are optimised for nucleic acid production.

Once sufficient polynucleotides have been produced, cells can be disrupted to release intact strands, which can hence be hydrolysed to give 5'-mononucleotides in solution, following simple chemical or enzymatic phosphorolysis. Although laboratory methods for nucleic acid extraction are well-documented, accounts of industrial scale methods are poorly reported, perhaps as specific method conditions are employed by different manufacturers.

Following hydrolysis, nucleotides are often purified with high performance liquid chromatography (HPLC) methods, which give highly pure mononucleotides (Gill et al., 2012). Such methods are well-established, so following extraction from waste, nucleotides may be directed to these refining facilities, before distribution to target markets. Table 1 shows the potential markets for nucleotide-based products, which may be explored.

***Table 1*- Market information for nucleotides**. Values are given in US dollars. (Compilation of unpublished market data, 2014)

Product	Legislation	Purity	Unit value
Animal Market			
Protein carrier – partial replacement for a thermolysed yeast carrier for animal health products	EU feed additive regulations	Basic (RNA/polynucleotides approx. 30% purity)	$1/kg
RNA	EU feed additive regulations	Basic (60% RNA purity)	$20/kg
Fishmeal replacer add-on –with similar amino acid composition	EU feed additive regulations	Basic extract (RNA/polynucleotides approx. 30% purity)	$1/kg
Human Market			
Pyrimidine nucleotides	EU food supplement regulations	Food grade (purity >60% single nucleotides)	$200/kg
Purine nucleotides – for health and flavour enhancer products	EU food supplement regulations	Food grade (purity >60% single nucleotides)	$75/kg
Infant Formula Market			
Pyrimidine nucleotides	PARNUTs regulations	Pharmaceutical grade (>95% single nucleotide)	$250/kg
Purine nucleotides	PARNUTs regulations	Pharmaceutical grade (>95% single nucleotide)	$125/kg

2.2- Mycoprotein (Quorn^{TM})

In 1967, as part of a major effort to identify an alternative protein source to supply the growing animal and human population, British company Rank Hovis McDougall discovered mycoprotein, derived from the filamentous fungus *Fusarium venenatum*. However, the expected protein shortages never emerged as they were expected to, so efforts were diverted into developing mycoprotein into a commercially viable product, as it showed promise as an economically viable alternative protein source, both physically and nutritionally, to cater for a growing vegetarian market. In 1984, in a joint venture with Imperial Chemical Industries (ICI), large-scale production of mycoprotein began. In the same year, mycoprotein was approved for sale as food by the Ministry of Agriculture, Fisheries and Food in the United Kingdom (Weibe, 2002), then approved for sale in Europe in subsequent years. In 1985, mycoprotein was launched commercially under the brand name *Quorn*, and the now extensive range of meat analogues products all derive from the single-cell protein yielded from aerobic fermentation of the *F. venenatum*.

Approximately 3,000 different fungi were investigated during early explorations, as well as other microbial sources such as bacteria and algae. *F. venenatum* strain A3/5 (ATCC PTA-2684) was eventually selected, as it suited the application ideally. Aside from having a high protein content, the hyphae of the fungus also develop into branched structures, approximately one branch per 300 μm, which creates a fibrillar structure which resembles meat. The hyphae themselves are similar in length (400-700 μm) and width (3-5 μm) to muscle fibres, so the texture of meat can be convincingly reproduced in the finished product (Rodger, 2001; Hosseini et al., 2009). Mycoprotein itself is inherently bland, but this allows for a great deal of versatility when it comes to flavouring.

Careful and comprehensive evaluation of mycoprotein with regard to its safety continued for 12 years after its initial discovery. Several years after the commercial launch of mycoprotein across Europe, the Food and Drug Administration approved mycoprotein for food use in the USA, in 2002, following yet more research (Miller & Dwyer, 2001). Studies have consistently shown that the A3/5 strain of *F. venenatum* does not produce mycotoxins, and the production process has been

adapted to make mycotoxin production unfavourable. Despite this, regular testing of the mycoprotein takes place at 6-hour intervals at the Quorn production facility, to ensure that the product is indeed free of any toxins or exogenous contaminants (Weibe, 2002).

2.2.1- Quorn Production

All Quorn products are manufactured at their facility in Billingham, UK. The production process occurs in 50 metre tall (Thrane, 2002), 155,000 litre (Milmo, 2005), sterile airlift fermenters in which the fungus is cultured aerobically on a medium of food-grade glucose syrup, with other added essential minerals and micronutrients, such as biotin and salts, under anexic conditions (Rodger, 2001). Glucose is delivered to the facility as 75% glucose syrup, while salts (such as zinc sulphate, iron sulphate, copper sulphate and manganese sulphate) are delivered as powders, which are used to produce the appropriate solutions before addition into the fermenters. Phosphoric and sulphuric acids are also used to dissolve certain salts (such as zinc sulphate), while also acting as buffering components, alongside ammonia.

Prior to fermentation, the entire fermenter system is sterilised with steam at 140°C, for at least four hours. 0.2 micron filters are used to sterilise all gases that enter the fermenter, while all media, water, nutrients and micronutrients are all sterilised also. This assures the fermentation proceeds anexically. In the past, water was demineralised on site to ensure the process was completely standardised, but this demineralising equipment is now disused and normal sterile water is used in the process. Similarly, 100% demineralised glucose is now un-favoured as it has been seen to result in minor colour changes in the final product.

Various ports throughout the fermenter facilitate the inflow of nutrients. The fermentation vessel contains no moving parts- continuous looping of the culture broth as its density changes provides agitation. This method of agitation is preferable as opposed to harsher mechanical methods, which can inflict damage to the fungi cell walls, as well as offering improved transfer of oxygen and nutrients, efficient removal of carbon dioxide and reduced generation of heat (Thrane, 2002).

Initially, sterile media is introduced into the fermenter followed by the *F. venenatum* production strain. The strain is pre-prepared before being added to the fermenter, via a patented process, beginning with a freeze-dried sample of *F. venenatum* A3/5 (Rodger, 2001). For this, a pure *F. venenatum* sample is inoculated into a small Erlenmeyer shake flask for initial growth. Several cultures are produced, then the ideal culture is selected following histological examination and other quality control measures, such determination of the culture's dry cell weight, to be transferred into a fermenter in a sterile five litre vessel.

After an initial four days of batch growth in a main fermenter, compressed air and ammonia are introduced into the main vessel via a sparge bar, which provide oxygen and nitrogen sources, respectively, although supplementary oxygen, from a reservoir of liquid oxygen on site, can be supplied if dissolved oxygen levels fall in the fermenter (Rodger, 2001). Addition of nitrogen and oxygen causes the culture to rise up through the 'riser' tube. At the top of the riser, the carbon dioxide produced by the fermentation process is removed, which results in an increase in density and subsequent falling of the culture broth down the 'downcomer tube.' The evolution of carbon dioxide is used to determine the flow rate, as it directly indicates the biomass concentration (Weibe, 2002). The broth then passes through a heat exchanger, which ensures the temperature of the vessel remains at a stable 28–30°C. The broth then returns to the main vessel and the process repeats itself. This process becomes continuous four days after initial inoculation, whereby 26 m^3 per hour of media and nutrients are fed into the fermenter, while the same amount of mycoprotein broth is removed. The environment within the fermenter is automatically controlled from a control centre, including mycoprotein broth harvesting and pH control, which is controlled to a consistent pH 6. All nutrients are also kept in excess, in doing so, preventing any metabolic change in the organism, as mutations could potentially induce mycotoxin production during fermentation.

The process operates continuously for around 31 days at a time, during which the typical biomass yield from this process is around 300-350 kg of biomass per hour from the facilities two fermenters (Weibe, 2002), which are referred to as *Q2* and *Q3*. During continuous operation, the mycoprotein

maintains a density of 25 g per litre (dry weight) and a glucose density of 6 g per litre. Fermentation is staggered between these two fermenters. During continuous operation, each fermenter is capable of producing 45 tonnes of mycoprotein per day, but at certain times, the operation of the two fermenters overlap, meaning the production facility's total output can reach 90 tonnes per day.

The Quorn range is the most popular meat alternative brand worldwide, and its popularity is increasing. As all Quorn for the entire global market is produced at a single facility, to supply this growing demand, a third fermenter, designated as *Q4*, started production in June 2015. This new fermenter is approximately 10% larger than the two existing fermenters, so production could potentially increase. Initially, however, because subsequent stages of the production line are not equipped to cope with the output of three fermenters running simultaneously, only two will run at a time.

A single fermentation is commercially referred to as a *campaign*. After around 31 days, an alternative morphological variant of *F. venenatum* begins to accumulate during fermentation. The normal *A* variant is desirable, as its characteristic final morphology resembles meat structure. However, it has been found that an alternative *C* variant begins to propagate after 31 days of fermentation, which has a highly branched, dense morphology that is incapable of forming a meat-like structure in a finished product. The C variant is faster growing than the A variant, so quickly begins to predominate after initial appearance, so when the C variant percentage exceeds a given threshold, the campaign is ended. The cue for the switch between the A and C variants is currently unexplained, but this switch generally never occurs before 31 days of fermentation. Work is currently taking place to identify the prompt for this switch, and if it is preventable, this could mean the process could run continuously for an indefinite period.

After being harvested, the mycoprotein has an RNA content of around 10%. Without reduction, the finished product would contain a high proportion of purine ribonucleotides, which when ingested, are converted within the body to uric acid. Accumulation of uric acid in the bloodstream can lead to gout, so the mycoprotein is transferred into a separate vessel and heated using heat

that originates from the facility's centrifuges, which is transferred to the RNA reduction vessels via a heat exchanger. This induces autolysis and activation of the endogenous RNase enzymes within the cells, which degrade RNA strands into monomeric ribonucleotides, which freely diffuse out of the cells into the supernatant. This reduces the RNA content of the mycoprotein to below the necessary 2%, by weight. In the past, the contents of the RNA reduction vessels were heat-shocked with steam for around 20 minutes at 64-65°C, but this harsher method resulted in an overall net loss of 35-38% of the yielded biomass. The newer, gentler method results in the same RNA reduction, with a yield increase of around 5%, compared to the original method. However, the process still results in a loss of proteins and other cellular components. Crucially, the heat treatment also renders the yielded mycoprotein insoluble.

The contents of the RNA reduction vessel are then heated to 80°C, to both kill the organism and maintain sterility. The contents of the vessel are then transferred to centrifuges, three large 5,000 RPM centrifuges serve each fermenter continuously, which centrifuge and filter the biomass from 1.5% (w/v) to 25-30% (w/v) (Weibe, 2002; Thrane, 2002). The dewatered solids are then cooled in vacuum chillers for around 1 hour to below 7°C. The production process is summarised in Figure 12.

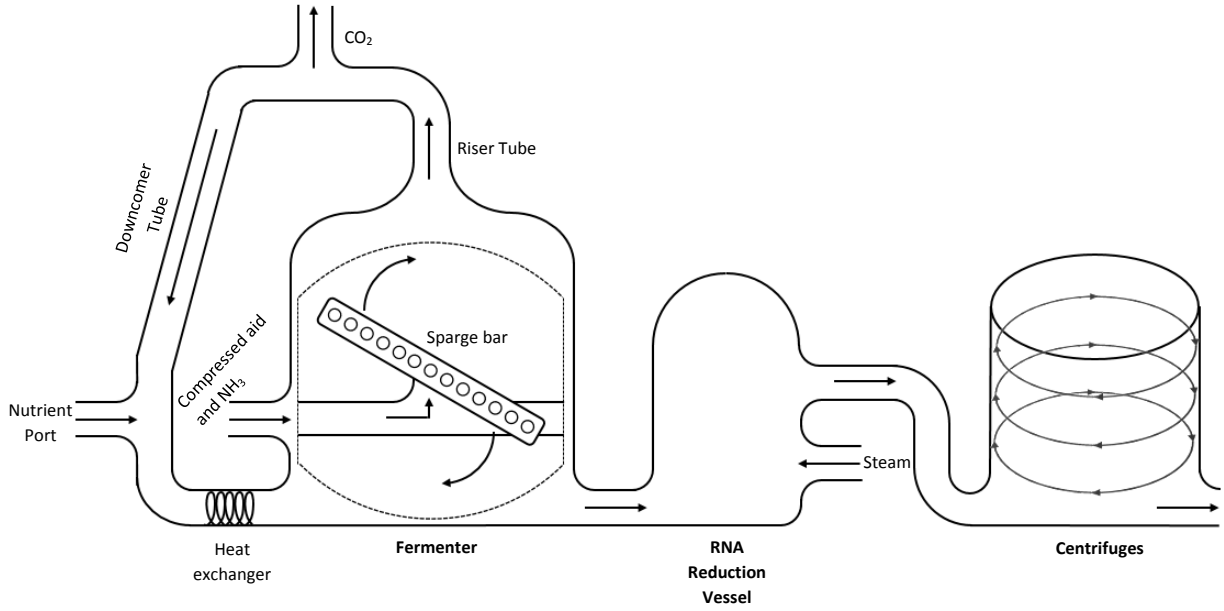


***Figure 12*- An overview of the Quorn production process, from fermentation to centrifugation.**

After being discharged from the chillers, the mycoprotein product resembles bread dough, and is commercially referred to as *paste*. This paste is then delivered to one of two facilities, either in Stokesley, North Yorkshire, or Methwold, Norfolk. These facilities process this paste into final products, such as meat alternatives for sliced chicken, or beef burgers, for example. The timescale within which the paste must be processed is directly affected by the temperature at which that batch was discharged from the chillers, and the batch weight, which may necessitate different storage requirements for the paste, if the discharge weight is over a given limit. For international supply, the products are either shipped as finished products, or in large batches of so-called *bulk intermediates*, such as beef-burger alternatives, which are then processed further by third parties.

Despite having the fibrillar structure of meat, cross-linking structures are absent in the mycoprotein paste, so at the processing facilities, free-range egg albumin is added to align bind the fibril structures, along with any colourants and flavourings required for the particular product. The company's reliance on egg albumin is significant. As well as not completely suiting the company's image as a completely vegetarian product producer, any disruption to the availability of egg albumin, such as an avian flu outbreak, could severely disrupt production and distribution. Projects have been carried out in the past to reduce this reliance on egg albumin, which could potentially result in a product to serve the vegan market. Varying the proportions of binder and mycoprotein and additional vegetable fat allows the finished texture of the product to be controlled, to accurately replicate the texture of different meats. Finally, the products are gently heated to gel the binder, then shaped and frozen. The freezing process is crucial for accurately reproducing a meat texture, as the ice crystals that form radically transform the product's texture to give the desired fibrillar structure.

A significant amount of quality control occurs throughout the production process, including microbiology analysis by agar plating. At regular daily intervals, samples are taken from various stages of fermentation are plated on nutrient and malt extract agar. At this point, the aforementioned C variant is easily distinguishable as a dense colony, whereas the normal *A* variant generally produces a diffuse colony. The mycoprotein paste is also regularly tested with a colour

analyser, which provides three values to indicate the consistency of the final product colour between batches that are discharged from the chillers. Glucose assays are also carried out on site, while some tests, including a total viable count (TVC) for some samples, and protein and total amino acid analyses (via the Kjeldahl method (Kjeldahl, 1888)) are outsourced. In addition to this, a radioactive source is used to determine the amount of culture at the top of the fermenter. If this level is too low, this indicates that circulation is not occurring in the fermenter, which will eventually result in the death of the culture.

Following centrifugation, the harvested material from mycoprotein production is divided into solid biomass and a supernatant. The supernatant is considered as waste for the process, which is optimised for biomass production. Currently, as only two fermenters are operational at the facility, around 500 million litres of this waste is produced annually, although this is set to increase significantly when the facility's third fermenter comes on stream. The waste itself has a chemical oxygen demand (COD) of around 12,000 mgL^{-1}, and a biochemical oxygen demand (BOD) of around 900 L^{-1}. If this waste was directly discharged into local rivers, it would deplete the dissolved oxygen in the water, to the detriment of the indigenous aquatic life. To comply with the license requirements for disposal of this waste, the waste currently undergoes a lengthy treatment process including deep shaft fermentation and polishing, to reduce the COD, followed by discharge into local rivers. The waste must also comply with maximum allowable limits for suspended solids, ammonia, phosphorus and mercury, amongst other potential pollutants.

When the waste, or *centrate*, is discharged from the centrifuges, it is diverted to the effluent treatment facility (ETF). After an anti-foaming agent is added, the centrate is piped to a large, deep shaft effluent treatment tank. Here, the centrate is aerobically digested to reduce its COD, with the aid of a non-specific microbial population. After digestion, the waste is transferred to a large *clarifier* tank, which slowly stirs the vessel to sediment solids and collect waste liquids free of suspended solids and pollutants, which are discharged directly into the nearby River Tees, along with the effluent treatment waste from other neighbouring facilities. The solids are subsequently centrifuged. Some of the solids are used to periodically inoculate the effluent treatment plant,

while most is diverted to agriculture, for use as a soil improver. In rough terms, the use of one tonne of glucose translates to the yield of one tonne of mycoprotein, but also the production of one tonne of solid waste. Potentially, the waste may be treated by anaerobic digestion in the future, which could generate biofuels to generate electricity for the production facility, and for resale to the national grid.

However, the waste is known to contain a number of commercially valuable nutrients, so in the past, this waste has been processed to harness these. The intention of this scheme was to generate a saleable product, while diverting some waste away from the ETF, which has obvious benefits in terms of environmental impact.

Some independent analysis has been carried out through Quorn to identify, characterise and quantify the components present in the centrate over a five week period. The results of these analyses have been summarised, where relevant, in Table 2.

***Table 2*- A summary of the centrate's composition.** Only relevant results have been included (unpublished Technical Report, 2014)

Component	Amount shown to be present in centrate	Basis of method used
Monoucleotides	0.6-1.1 gL^{-1}	HPLC with spectrophotometric detection at 254 nm (modified from Oruna-Concha et al., 2007)
Sugars	2.5-7.7 gL^{-1}	Gas chromatography following derivatization
Free amino acids	0.9-1.3 gL^{-1}	Modified spectrophotometric O-phthaldialdehyde (OPA) method, read at 340 nm (Church et al., 1985, Nielsen et al., 2001), followed by derivatization with gas chromatography-mass spectrometry.

2.2.2- Mycoscent Production

Overall, the centrate, is known to contain cell debris, nucleotides, sugars, alcohols, protein, amino acids and other volatiles. One of the most commercially valuable components within the centrate is nucleotides. Quorn's independent tests showed that only monomeric and short chain polynucleotides were present in the centrate. The waste contains around 1.46% total dry mass, of which 6-15% is nucleotides. During a five week testing period, the proportion of the nucleotides in

this waste remained fairly stable, where the ribonucleotide monophosphates showed the general pattern of abundance as shown in Figure 13.

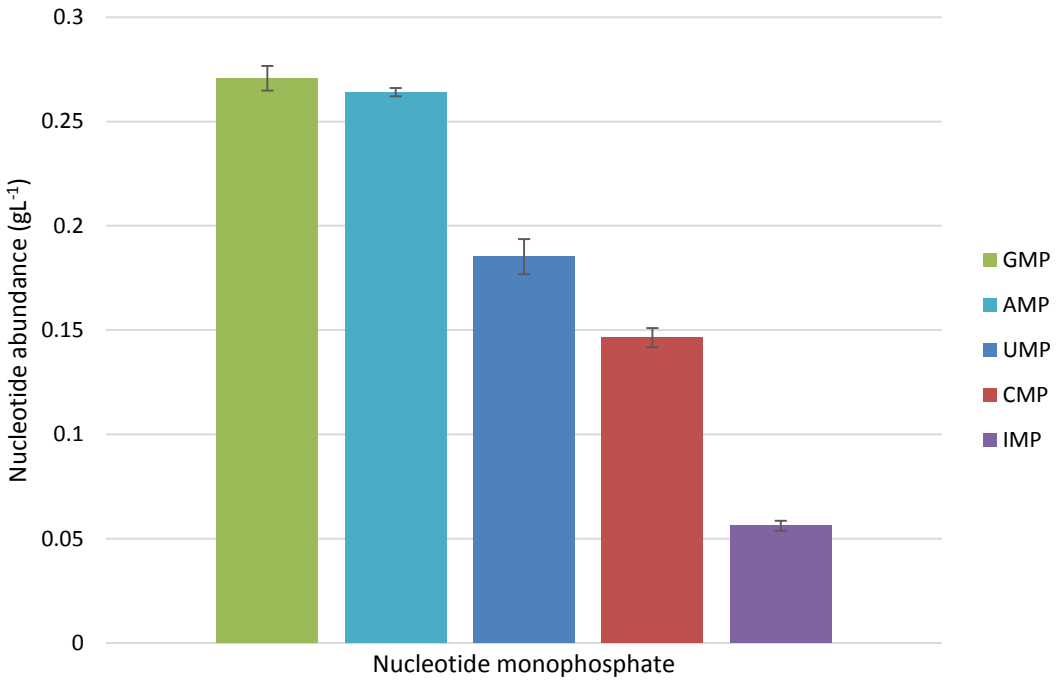


***Figure 13*- A graph to show the average abundance of mononucleotide monophosphates present in the centrate, as tested over a 5 week period.** Averages of results obtained from a five week test period (Unpublished report, 2014).

The Quorn manufacturing facility was extended to include a new range of industrial equipment to process the waste and harness the nucleotides, which would then be sold and marketed as *Mycoscent* (or *Quessent*), a flavour enhancer.

In practice, the centrate was processed by the method explained in Figure 14, to produce the powdered Mycoscent product. Essentially, the large solids are removed from the centrate by a bulk filtration step, followed by nano-filtration to selectively retain nucleotides, then a two-step process to dewater the product.

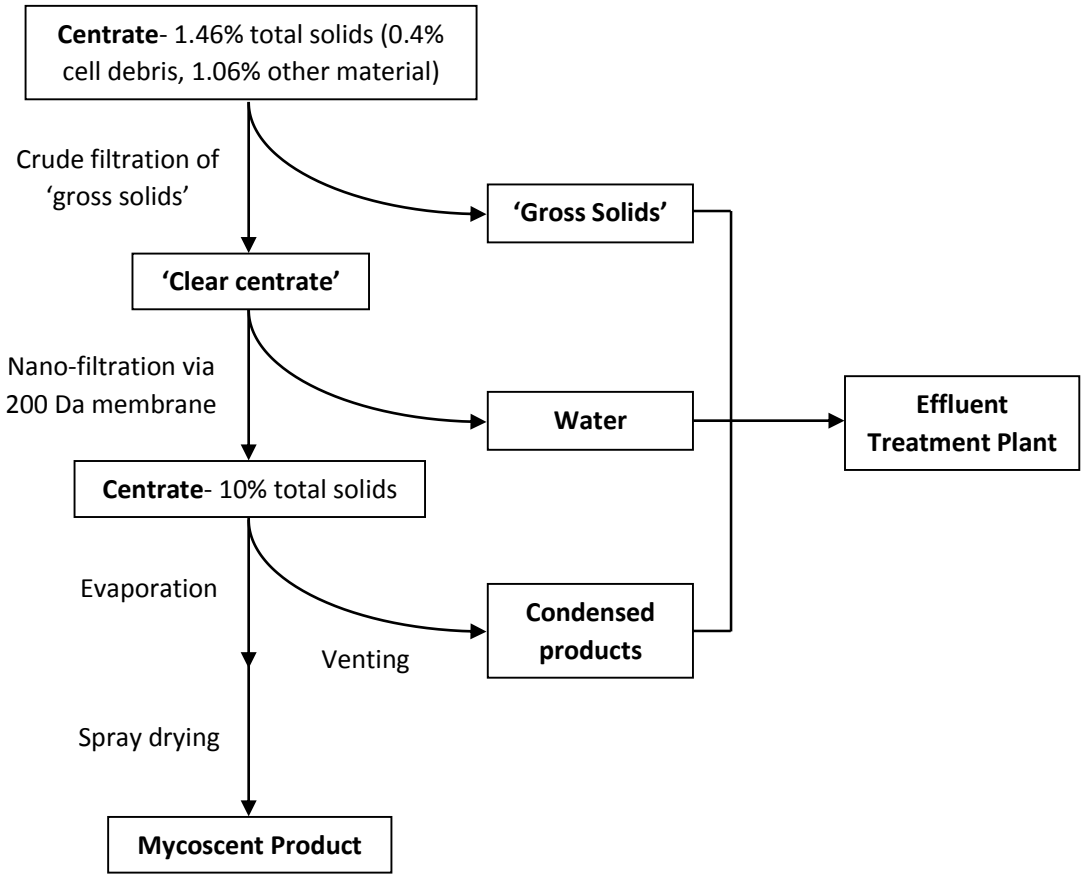


***Figure 14*- A flowchart summarising the method used to process Quorn centrate to yield the *Mycoscent* product.**

However, when the method was applied at an industrial scale, it was found that the 200 Da nano-filter quickly became blocked, which almost immediately arrested the process. It has been speculated that the blockages were due to the remaining cell membranes, which quickly obstructed the entire inner surface of the filter. Since 2010, the Mycoscent has not been produced, but there are several potential avenues for re-establishing the production process and so resurrecting the currently unused array of industrial equipment, which represents a significant investment from Quorn.

The most likely option for re-establishing the process is to introduce an additional centrifugation step to pellet the cell membranes. This step would be included in the process directly before the nano-filtration step, where the supernatant would then undergo nano-filtration and progress through the process as it is currently. This will obviously require a substantial initial outlay to modify the current process, but would also mean that the finished Mycoscent product is devoid of

any cell debris, which may impart some contribution in terms of the product's quality, but also its quantity.

However, there is also a second option to explore. Potentially, an enzymatic degradation step could be introduced prior to nano-filtration, to break down the cell membrane debris into fragments that will pass through the nano-filter. Again, this option would require significant costs to modify the process, with additional ongoing costs to supply fresh active enzyme to the process. However, cellular components, such as cell wall proteins, are likely to be retained in the finished product, which may afford some benefits to the quality of the finished product, but will almost certainly increase the overall quantity of useable product also.

Following the modification and optimisation of the process, Quorn had ambitions to incorporate an anaerobic digestion stage into the process, to harness power from the residual glucose in the untreated centrate, although this may not be an economically viable venture.

2.3- Experimental avenues for exploration

2.3.1- Extraction- Precipitation

Precipitation of nucleotides presents the best prospects for an industrial process in terms of the *Research Aims and Objectives* defined in section 1.2. The process essentially relies on adding a small quantity of inorganic salt, then selectively precipitating nucleotides out with a benign organic solvent, which can potentially be recycled throughout the process.

The method is commonly used in laboratories to concentrate intact, biologically active nucleic acids for molecular biology applications (Zeugin and Hartley, 1985). A summary of this method is shown in Figure 15.

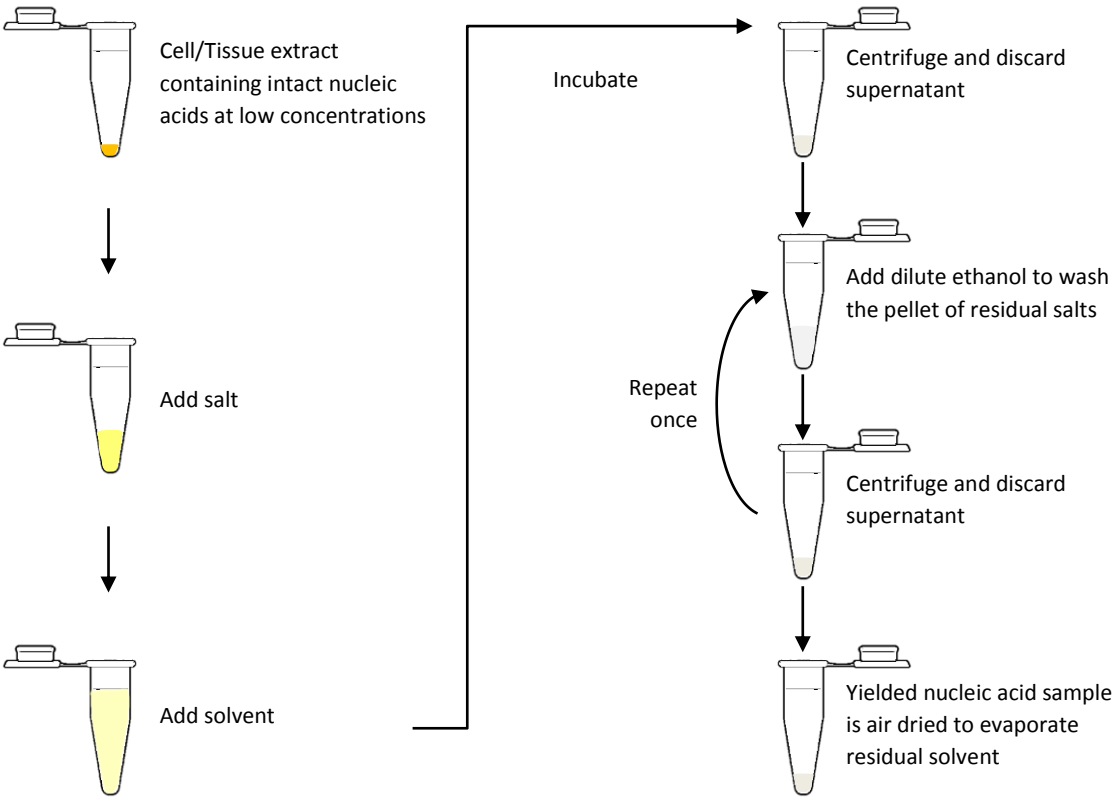


***Figure 15*- A summary of precipitation of nucleic acids by salting out.** Compiled from Zeugin and Hartley (1985), Sambrook and Russell (2000), Cathala et al. (1983), Crouse and Amorese (1987), Mulhardt (2010), Nothwang and Hildebrandt (2013) and Ausubel et al., (1999).

Once a sample has been gained that is free of particulate matter, a certain molarity of inorganic salt is added to the sample. This is commonly done by adding a particular volume of a highly concentrated stock salt concentration, such as 3.0 M sodium acetate, to adjust the final sample volume to the desired molarity.

Following this, a certain volume of solvent is added to the sample, to precipitate the nucleic acids in the sample. Two to three volumes of cold ethanol is most commonly used for the precipitation (Sambrook and Russel, 2000), but cold isopropanol is generally seen to be more effective at precipitating nucleic acids, hence a smaller amount is required to precipitate the same amount of nucleic acids, compared to ethanol (Nothwang and Hildebrandt, 2013).

Following solvent addition, the sample is normally incubated for a few minutes to several hours. Early usage of nucleic acid precipitations suggested incubation at very cold temperatures, but studies have since shown that incubation temperature does not have a significant benefit in terms of nucleic acid recovery (Crouse and Amorese, 1985)(Zeugin and Hartley, 1985).

During incubation, the nucleotides form insoluble precipitates, which are subsequently pelleted during high speed ultracentrifugation. The supernatant is then removed, leaving a pellet of nucleic acids.

To remove traces of co-precipitated salts, dilute ethanol is added to the pellets. This 'washing' procedure is carried out with 70-95% ethanol, where the volume selected is dependent on the pellet size, but it is generally considered that it is necessary to cover the pellet to achieve effective salt removal. After washing, the pellet is centrifuged again and the supernatant is removed. This washing procedure is repeated once more to remove remaining traces of salts, leaving a nucleic acid pellet, ready for further analysis. For this, the pellet is normally re-dissolved in water or an appropriate buffer.

In precipitation of intact nucleic acid strands, it is imperative to handle the samples delicately at each stage, particularly mixing. To avoid shearing of nucleic acid strands, initial mixing or mixing during washing is normally done by gently inverting the tube a number of times, gentle vortexing or flicking of the centrifuge tube. Similarly, most stages, including centrifugation, should be carried out at chilled temperatures to limit the activity of endogenous nucleases in the sample (Nothwang and Hildebrandt, 2013). Exogenous nucleases, particularly the more ubiquitous RNases, can also lead to spontaneous ribonucleic acid strand breakage, so use of RNase-free reagents and equipment and proper laboratory technique is necessary to maintain intact strands.

The theory behind this process is fairly complex. Many of the bonds within nucleotides exhibit a permanent dipole due to a difference in electronegativity in the atoms. Because of the presence of these bonds, nucleotides, and therefore nucleic acids, are referred to as *polar* molecules. Non-polar solvents are not able to dissolve polar substances, whereas polar solvents are, therefore nucleotides and nucleic acids are soluble in water, which is also polar due to the differences in electronegativity between the oxygen and hydrogen atoms present in the molecule.

At 25°C, each liquid water molecule interacts with neighbouring water molecules via 2.357 hydrogen bonds per water molecule (Zielkiewicz, J., 2005). During solvation, the electronegative (δ-) oxygen atoms of polar water molecules associate with the exposed electropositive (δ+) atoms in the nucleic acids. The water molecules assemble themselves in a so-called *solvation shell*, which is often referred to as a *hydration shell*, when specifically applied to water as a solvent. When water molecules directly associate, via dipole-dipole interactions, with the atoms within a nucleic acid, they form *primary* hydration shells, and disrupt the hydrogen bond network between neighbouring water molecules. The orientation of the water molecules in the hydration shell creates a net charge on the outer shell face, allowing additional water molecules to associate in a *secondary* hydration shell. An overview of the conformation of hydration shells arranged around a short DNA double strand is shown in Figure 16.

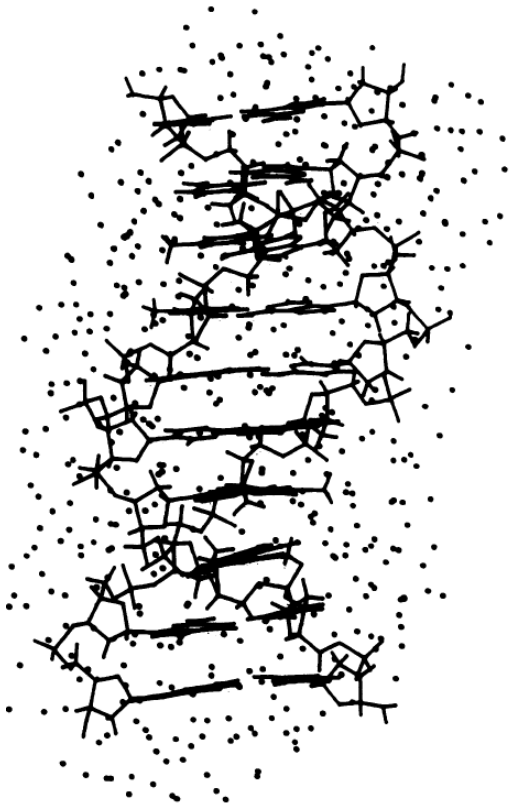


***Figure 16*- A DNA double helix surrounded by primary and secondary hydration shells.** Dots indicate electronegative oxygen atoms, oriented towards a polarised charge concentration. From Chuprina et al. (1991).

Precipitation of nucleic acids relies on depletion of hydration shells, which causes the nucleic acids to form solid precipitates. Following the addition of an inorganic salt, the salt dissociates in solution into its constituent ions. In nucleic acid precipitation, salts that give rise to monovalent cations are

commonly used, but the choice of salt is often a combination of several considerations, as per Table 3.

***Table 3*- An overview of the attributes of commonly used precipitating salts.** Compiled from Mulhardt (2010), Sambrook and Russell (2000), Cathala et al. (1983).

Salt	Considerations
Ammonium Acetate (NH_4CH_3COO)	To be used when the nucleotides (particularly dNTPs) or oligonucleotides below 30bp in length are undesired. These fragments are not precipitated with ammonium acetate. If the target DNA will be phosphorylated in subsequent experimentation, ammonium acetate should not be used, as bacteriophage T4 polynucleotide kinase is inhibited by ammonium ions.
Lithium Chloride (LiCl)	Highly soluble in ethanol-rich matrices. Not to be used when target RNA is to be reverse transcribed, as chloride ions commonly inhibit cell-free protein synthesis and inhibit RNA-dependent DNA polymerases. Loss of oligonucleotides below 300bp is observed.
Potassium Acetate (KCH_3COO)	Frequently used in precipitation of plasmid DNA with alkali lysis. Potassium forms a solid precipitate with SDS, so SDS can be easily removed from a sample with potassium.
Sodium Acetate ($NaCH_3COO$)	Most frequently used salt in nucleic acid precipitations. One tenth volume (with respect to the sample) of 3.0 M sodium acetate stock solution (pH 5.2) is normally added to achieve a final concentration of 0.3M.
Sodium Chloride (NaCl)	Ideal where the sample contains sodium dodecyl sulphate (SDS), as SDS remains soluble in 70% ethanol when sodium chloride is present. As with lithium chloride, sodium chloride should be avoided when target RNAs are to be used in cell free translation or is to be reverse transcribed.

Dissociated monovalent cations are able to interact with the negatively charged phosphodiester backbone of a nucleic acid strand, while, the dissociated monovalent anions are able to associate with water molecules. Additionally, complex anions are able to sequester water molecules, which has the overall effect of reducing the number of free water molecules available for solvation in the matrix, effectively augmenting the depletion of solvation shells around the nucleic acids.

In salting-out, a high salt concentration can be created to decrease the solubility of a proteins (Arakawa and Timasheff, 1984) in solution. This is achieved because the high concentrations of salt results in the water molecules occupying themselves in solvating the ions. This makes freely available water molecules scarce, so the protein becomes less soluble at higher salt concentrations, as the salt floods the solution, as shown in Figure 17.

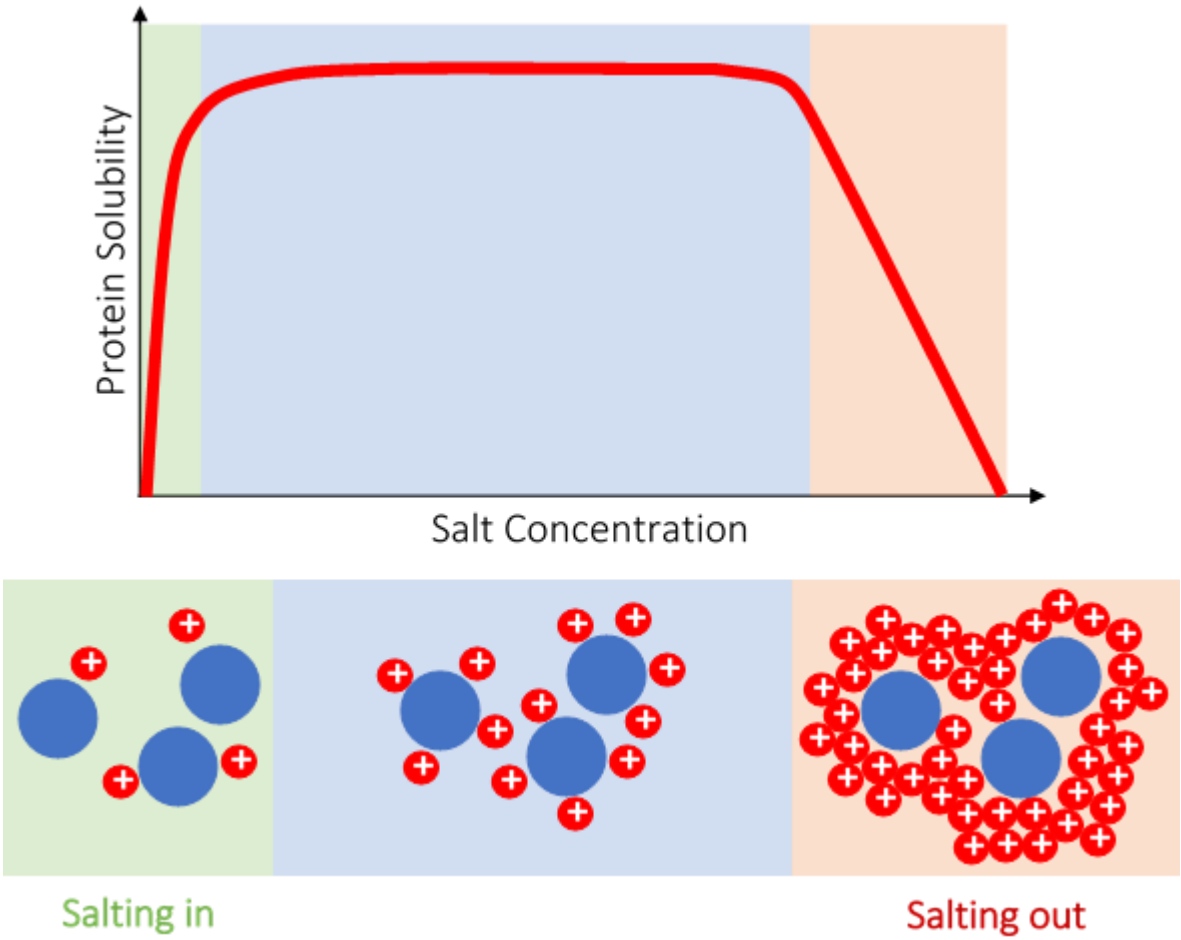


***Figure 17*- A schematic to demonstrate the mechanism of salting out.**

Precipitation of nucleic acids functions by a similar mechanism, but relies on the action of a solvent to achieve extraction. In nucleic acid precipitation, lower salt concentrations are used. The dissolved monovalent cations associate with the negatively charged phosphodiester backbone of the nucleic acid strand. The association of the cations and the nucleic acids is strongly dependent on the *dielectric constant* of the solvating environment.

The dielectric constant of a material refers to its relative permittivity, in relation to the dielectric constant of a vacuum. By definition, the dielectric constant of a vacuum is 1. A solvent's permittivity directly accounts for that solvents ability to insulate the charges of ions within it, from each-other. For example, in high permittivity solvents, such as water, the negatively charged nucleic acids are well-insulated from the positively charged anions, due to the resistance of the solvent. A solvent's permittivity also accounts for the solvent's polarity (Smith, 2000), which also influences the precipitation of a solute in a solvent-aqueous matrix of changing composition.

When in a 100% water solution, the dielectric constant of the solvent is very high, so the electrostatic interactions between the dissolved cations and the nucleic acids is relatively low, meaning the nucleic acids remain solvated. However, when a solvent with a low dielectric constant is added to the solution, the overall dielectric constant of the matrix is decreased, enhancing the interactions between the cations and nucleic acids in the solvent, due to depletion of solvation shells around the nucleic acid. These interactions are often referred to as Coulomb forces, as Coulomb's Law can be used to predict the forces between charged solutes in a solvent with a given dielectric constant (Baigrie, 2006). When the Coulomb interaction between the charged nucleic acids and the cations is sufficiently increased, stable ionic bonds are formed between the ions, resulting in the neutralisation of the net charge across the nucleic acids, and hence a reduction in their solubility, resulting in the nucleic acids forming solid precipitates in solution.

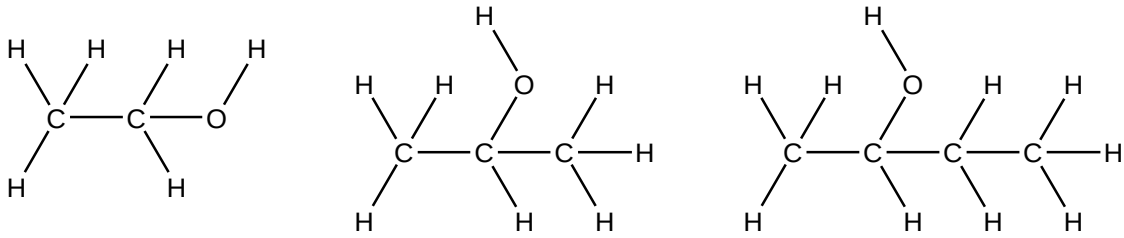
To sufficiently reduce the dielectric constant of the sample's solvent matrix, a solvent with a low dielectric constant is added. The chosen solvent must be also miscible in water, so that the solvent may influence the matrix's dielectric constant. The solvent must also be volatile, so that traces of the solvent can be evaporated from the final nucleic acid pellet.

Ethanol remains the most commonly used solvent for routine nucleic acid precipitations. It has a low dielectric constant of 25.08 at 298.15 K (Patil, 2001). Ethanol also has a high vapour pressure of 7.916kPa at 298.15 K (Nasirzadeh et al., 2004), which relates to the solvent's volatility.

Isopropanol is also regularly used for nucleic acid precipitations due its exceptionally low dielectric constant of 19.255 at 298.15 K (Patel, 2000). This is of particular advantage when working with larger sample volumes, as less isopropanol can be used to sufficiently reduce the dielectric constant of the matrix to achieve nucleic acid precipitation. However, as isopropanol has a longer carbon chain length, the vapour pressure is lower than that of ethanol, 2.880kPa at 298.15 K (Patel, 2000), indicating that the solvent is less volatile, which eventually necessitates longer evaporation times to remove all traces of isopropanol from the final nucleotide pellet. Due to the longer chain length, the influence of the polar hydroxyl (-OH) group on isopropanol has less effect on the overall

polarity of the molecule, compared to ethanol. Because isopropanol is less polar, more salt may co-precipitate with nucleic acids, as it fails to dissolve into the isopropanol-rich matrix.

Several chemical characteristics correlate with the carbon chain length in these solvents (see Figure 18). It could be assumed that butanol, which has an extra carbon than isopropanol, may be a suitable solvent in ethanol precipitation, due to its low dielectric constant. However, this is not the case, as butanol is much less miscible in water. It would be expected that a high level of co-precipitation of salts would occur if butanol were used as a solvent, due to its low polarity. The solvent may be very difficult to remove from the final nucleic acid pellet as its volatility is so low. Despite this, nucleic acids may be concentrated in a sample using *sec*-butanol. This multi-step method differs greatly from conventional precipitation, and relies on phase splitting to gradually concentrate the nucleic acids in a sample (Ausubel, 1999)(Mulhardt, 2010).



Ethanol	**Isopropanol**	***sec*-Butanol**
Short chain length	Intermediate chain length	Long chain length
High dielectric constant	Intermediate dielectric constant	Low dielectric constant
Very volatile	Somewhat volatile	Poorly volatile
Very polar	Somewhat polar	Weakly polar

***Figure 18*- A diagram to illustrate the structures of ethanol, isopropanol and *sec*-butanol, and the trends that correlate with their chain lengths.**

When a precipitation is performed, low incubation temperatures were traditionally used for several reasons, including decreasing the solubility of target nucleic acids, preventing nucleic acid strand shearing and limiting the activity of nucleases. However, at lower temperatures, the dielectric constant of any material decreases, so this may prove counter-productive in precipitating nucleic acids effectively. Additionally, at very low temperatures, the viscosity of the solvent-aqueous matrix increases, meaning the migration and aggregation of target nucleic acids is hindered, particularly with lower molecular weight fragments (Zeugin and Hartley, 1985). Furthermore, although the solubility of target nucleic acids decreases at lower temperatures, the solubility of other solutes in the matrix, particularly salts, also decreases, leading to increased co-

precipitation of salts and other endogenous contaminants present in the sample. Cold solvent is commonly added to the sample after salt addition, presumably to accelerate the precipitation, but potentially to prevent absorption of moisture into the hygroscopic solvent.

When working with exceptionally low nucleic acid concentrations in a sample, it may be necessary to add a carrier substance, otherwise known as a co-precipitant. Carriers are used to flocculate the nucleic acids into well-developed complex, which form a large, visible pellet following centrifugation. In the absence of a carrier, nucleotides of 2 µg or less in mass form small pellets (Hengen, P., 1996) which are easily perturbed, but the addition of a carrier aids in removal of supernatants without disturbing the pellet and potentially reducing overall yield. Several carriers have been shown to quantitatively increase recovery of nucleic acids from dilute solutions. However, the effect of carriers has not previously been tested in their effect on precipitating nucleotides, where they are present at high concentrations in the sample. Carriers can be purchased easily from commercial suppliers, but are generally rather expensive. However, carriers are added to give a very low final concentration, so there may be an argument for including carriers in an industrial process, if they result in a significantly increased yield. Like salts, carriers are chosen based on a number of considerations, as shown in Table 4.

***Table 4*- An overview of the attributes of common carriers in nucleic acid precipitations.** Compiled from Palukaitis (2013), Hengen (1996), Zyskind and Bernstein (1989), Baugh (2001), Michelson and Orkin (1982), Wang et al. (2002), Tracy (1981), Gaillard and Strauss (1990), Aruffo and Seed (1987), Strauss and Varshavsky (1984).

Carrier	Final Concentration	Considerations
Glycogen	50-150 μgmL^{-1}	Inert purified polysaccharide. Does not inhibit restriction endonucleases ≤30 $mgmL^{-1}$, T4 DNA ligases ≤7 $mgmL^{-1}$ nor nucleic acid hybridisation reactions. Nucleotide fragments as small as 8bp can be recovered. Commercial samples have been shown to be contaminated with trace amounts of nucleic acids. May interfere with DNA-protein interactions. May also inhibit transcription of large DNA templates, in a concentration-dependent manner.
Linear Polyacrylamide	10-20 μgmL^{-1}	Inert polymer, which can be prepared directly from monomeric acrylamide. Does not inhibit DNA:Protein interactions, cloning, electrophoresis or enzyme reactions, including polynucleotide kinase reactions or ligation by T4 DNA ligase. However, fragments below 20bp are not precipitated.
Spermine	10-20 μgmL^{-1} (5-10 mmol)	Not commonly used as a carrier in conventional nucleic acid precipitations. Can be added to dilute cDNA solutions prior to precipitation. Does not precipitate nucleotide fragments below 60bp and is difficult to remove from product. The same concentration range of salmon sperm DNA can be used as an inexpensive source of nucleic acid to increase the sample's nucleic acid concentration, to aid in forming a visible pellet.
Yeast transfer RNA (tRNA)	10-20 μgmL^{-1}	Biologically active carrier. Free 3'-OH terminus of tRNA competitively inhibits polynucleotide kinase and terminal transferase. Also inhibits tailing. When an oligo(dT)-T7 primer is used, cDNA synthesis is and *in vitro* transcription by T7 RNA polymerase are not inhibited. In the absence of a primer, template-independent low molecular weight products are generated. Nucleobases can contribute to an increase in absorbance at 260 nm, as yeast tRNA is co-precipitated into the final product.

Precipitation of nucleic acids is often carried out with very small sample volumes, perhaps no more than a few microliters, which explains why salt solutions are often added to achieve a desired final salt concentration. Achieving a specific salt molarity in such a small starting volume, using powdered salts, is unrealistic. The ultimate aim of nucleic acid precipitation is to purify or concentrate intact nucleic acid strands at an acceptable yield. Therefore, protocols often incorporate complex preparation procedures, followed by a meticulously controlled and lengthy protocol. This juxtaposes the objectives of industrial mononucleotide extraction. For this, yield will be prioritised, followed by purity. Preservation of strand structure is not required however, so the effect of nucleases need not be a concern, as nucleotides are pre-monomerised during Quorn's RNA reduction procedure. Additionally, mixing, washing and solubilisation steps may be carried out more thoroughly, as delicate handling will not be necessary in nucleotide precipitation. The process must also be simplified to allow high throughput and decrease energy expenditure, to

ensure cost-effectiveness. Thorough re-evaluation of each protocol parameter will be necessary to determine which conditions are optimal for the recovery of mononucleotides.

2.3.2- Extraction- Phenol-Chloroform Extraction

Phenol chloroform extraction is a widely used liquid-liquid extraction method to remove protein from samples of nucleic acids, which relies on separating a sample into two immiscible phases, which distribute nucleic acids and proteins differently. This procedure was originally pioneered for RNA isolation by Chomczynski and Sacchi in 1987, and has since become commonplace in laboratories.

Despite the popularity of the technique, prospects for the process operating at larger scales are bleak, not least because the solvents phenol and chloroform are hazardous and toxic. But additionally, preparation of chemicals requires time and meticulousness, and the method must often be repeated several times to improve deproteinisation, then normally proceeded by conventional nucleic acid precipitation to concentrate the target nucleic acids. Nonetheless, a brief summary of this method is described in Figure 19, along with a discussion of the theory.

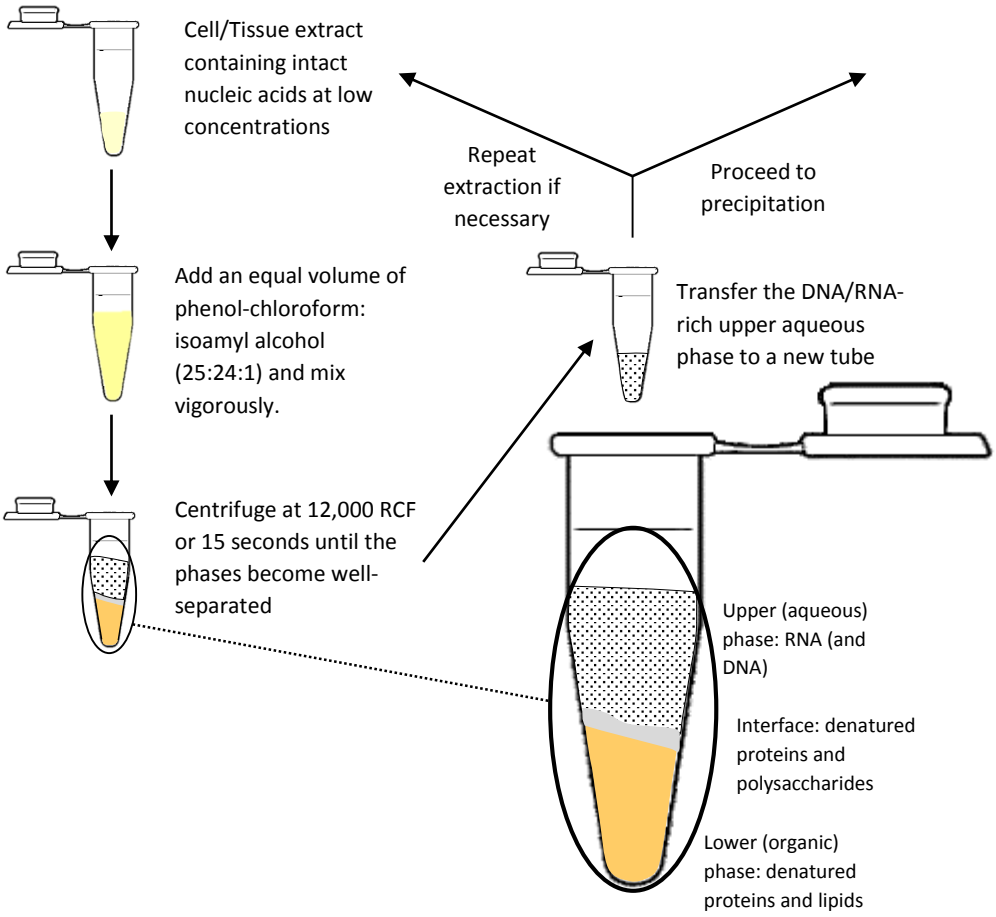


***Figure 19*- An overview of phenol-chloroform extraction for the purification and concentration of nucleic acids.** (Sambrook and Russell, 2000)(Chomczynski and Sacchi, 1987)(Chomczynski and Sacchi, 2006)(Ausubel et al., 1999).

A pre-prepared mix of phenol:chloroform:isoamyl alcohol (25:24:1) is added in equal volumes to a nucleic acid sample, before being vigorously mixed to produce an emulsion. A short centrifugation step then induces phase separation, where the denser phenol and chloroform solvents, alongside dissolved lipids, form the lower organic phase. The upper aqueous phase localises the nucleic acids. An interphase also forms, which is composed of denatured proteins and other biological polymers.

The aqueous phase is then transferred to a fresh tube, before the extraction is repeated again, until no visible interphase layer is present following centrifugation (Sambrook and Russell, 2000). Precipitation often proceeds phenol-chloroform extraction, to further recover and concentrate the nucleic acids from the aqueous solution.

In principal, phenol or chloroform may be added to a sample in isolation and phase separation may be achieved, as both solvents are non-polar, and so are capable of denaturing cytosolic proteins.

However, when phenol and chloroform are used in combination, proteins are efficiently denatured, the partitioning of mRNAs with long poly(A) tracts into the organic phase is reduced, and formation of insoluble nucleoprotein complexes is reduced at the interphase (Perry et al., 1972). The different densities between the two solvents allows them to form a dense mixture that efficiently separate from distinct phases, allowing localisation of target nucleic acids into the upper aqueous phase. Isoamyl alcohol is commonly added to reduce foaming and aids in the deactivation of nucleases. Guanidinium thiocyanate, like guanidinium chloride, can be added as a chaotropic salt, which further denatures proteins. This is done particularly to inactivate RNases, so RNA strands are undamaged.

The basis of this process relies on the different physical characteristics of biological molecules, particularly their polarity and hence, solubility in different solvents. In cells, DNA arranges in double strands, with the negatively charged phosphodiester backbones arranged on the strand exterior, meaning the strands are polar. Similarly, RNA strands are present in cells as single strands, with a single negatively charged phosphodiester side, and a face of exposed polar nitrogenous bases. Proteins, conversely, arrange as highly folded structures. Proteins are essentially polymers of amino acids, some of which contain highly polar (or *hydrophilic*) side chains, while others contain non-polar (or *hydrophobic*) side chains. *Cytosolic* proteins (i.e. proteins that locate and function in a cell's cytosol) normally arrange with the hydrophilic residues on their exterior, which permits solubility and is energetically favourable.

The conformation of proteins is drastically changed upon addition of a non-polar solvent such as phenol-chloroform. The change in environment results in the hydrophobic residues relocating from the protein interior to the protein exterior, changing the protein structure via a process called *denaturation*. Some proteins may localise into the organic phase if they are sufficiently soluble in the non-polar phenol-chloroform, whereas most form an insoluble flocculent at the interface of the aqueous and organic phases.

pH is a very important factor in phenol-chloroform extraction (see Figure 20), as it effectively determines which nucleic acids are localised in the aqueous phase. At acidic pHs, protonation of the phosphodiester backbone occurs in both DNA and RNA, effectively neutralising the charge here. In DNA, the neutralisation of the exposed backbones on the strand result in total neutralisation of the strand, making it insoluble in in the aqueous phase, so it migrates to the organic phase. In RNA, positive charges are still maintained in the exposed nitrogenous bases, meaning hydrogen bonds can still associate, and so solvation can still occur.

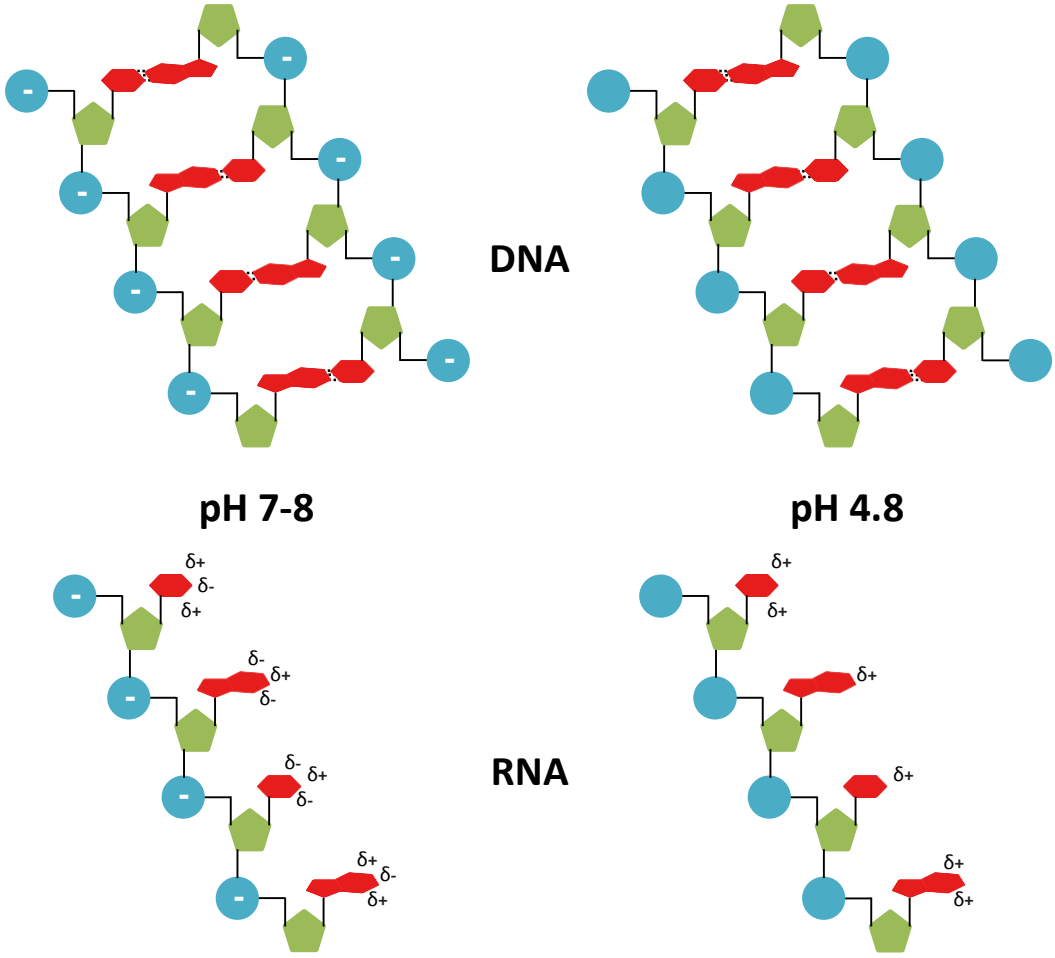


***Figure 20*- A figure to demonstrate the effect of pH on DNA and RNA solubility during phenol-chloroform extraction.** At neutral or slightly alkaline pH (left) both DNA (top) and RNA (bottom) maintain their negatively charged phosphodiester backbone. At an acidic pH, (right) DNA is neutralised as the phosphodiester backbone is protonated, whereas RNA (bottom), retains its solubility in the aqueous phase as positively charged nitrogenous bases are still able to form hydrogen bonds with water molecules.

The real value of this technique lies in its ability to isolate RNA from DNA, when performed at acidic pHs. Alternative methods, such as silica column based methods can be employed to achieve the same outcome, with benefits in terms of health and safety, convenience, and potentially

throughput when working with multiple samples (Shafer et al., 1997)(Salvo-Chirnside et al., 2011). However, phenol-chloroform extraction is commonly employed as the process does not necessitate the use of specialist and costly equipment and reagents. The convenience of the phenol-chloroform extraction procedure can be improved with the use of pre-prepared commercially available extraction reagents, such as TRIzol (Ambion- Life Technologies), TRI reagent (Sigma-Aldrich), Trisure (Bioline), and STAT-60 (Tel-Test). These pre-prepared reagents eliminate the need to equilibrate and mix the phenol-chloroform solvent, which can be laborious, although unsurprisingly, the higher cost of these reagents reflect savings in terms of time.

2.3.3- Extraction- Polyethylene Glycol Extraction

Polymers of ethylene glycol, otherwise known as polyethylene glycol (PEG) can be used as precipitants to extract nucleic acids. The procedure is similar to conventional solvent precipitation of nucleic acids, which relies on adding salt to a nucleic acid sample. However, instead of a solvent, polyethylene glycol is added to the sample to promote nucleic acid precipitation.

There are some considerable advantages to this procedure over conventional solvent precipitation, chiefly that PEGs have a low toxicity (Froehlich et al., 2011), which are already used as food additives in the EU (Food Standards Agency, 2014), and are widely used for drug applications in humans (Di Palma et al., 2002)(Parveen and Sahoo, 2012). Also, if solid PEG and salts were added to the aqueous nucleic acid sample to achieve the desired final concentration of each (rather than pre-prepared stock solutions), a much higher volume of sample could be processed per extraction for a given vessel size, in comparison to solvent precipitation.

On face value, this process certainly seems amenable to upscaling, but there is a fundamental flaw in the process, as it has been repeatedly reported that PEG is not effective in precipitating short oligonucleotides below 150 bp (Paithankar and Prasad, 1991)(Lis and Schleif, 1975), although Schmitz and Riesner, 2006, more recently reported recovery of 120 nt fragments. Unless overcome, this makes the process ineffective for recovering monomerised nucleotides. Paithankar and Prasad, 1991, determined that both PEG and ethanol extraction gave comparable recoveries

of digested DNA when optimised, but ethanol precipitation was able to efficiently recover fragments as small as 26 bp, whereas PEG was not able to recover small nucleic acid fragments, meaning the process would not be effective in recovering the very short oligonucleotides or monomeric nucleotides present in the centrate.

Again, an overview of the process is described here in Figure 21:

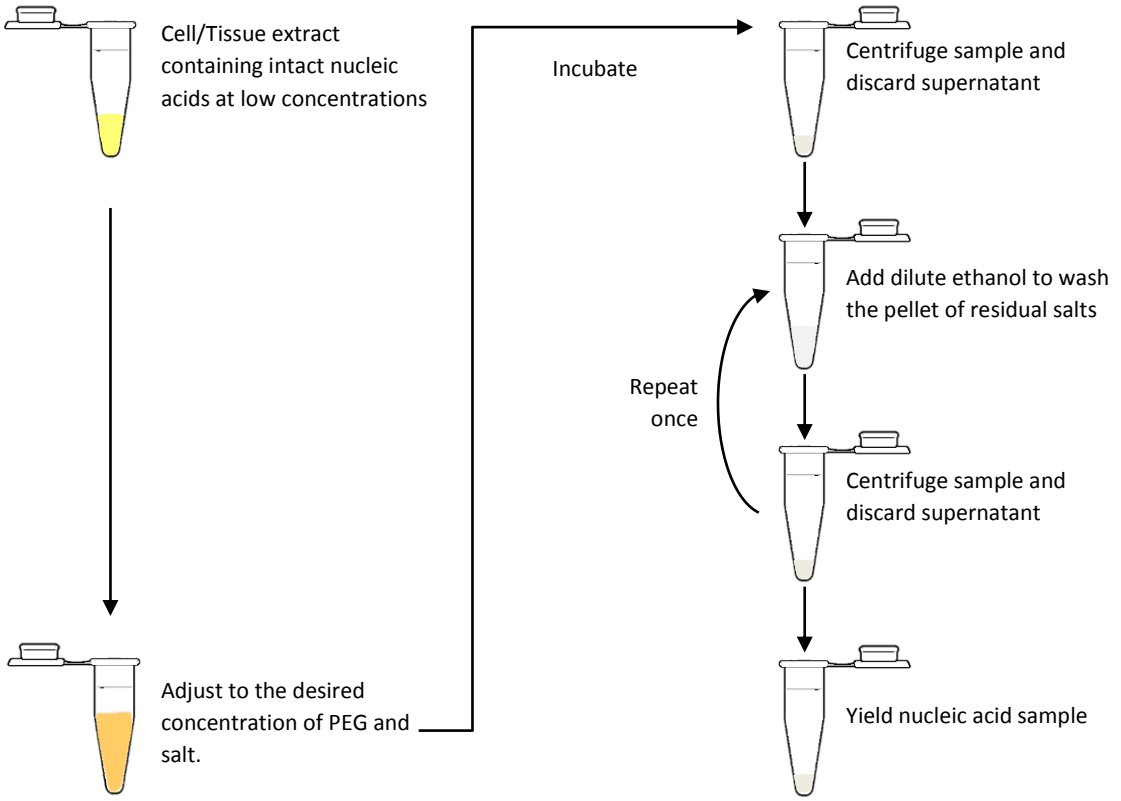


***Figure 21*- An overview of polyethylene glycol (PEG) extraction for the recovery of nucleic acids.** (Mulhardt, 2010)(Lis and Schleif, 1975)(Paithankar and Prasad, 1991)

Polyethylene glycol is a synthetic water-soluble polymer which has a repeating ethylene oxide unit, in the structure as shown below in Figure 22.

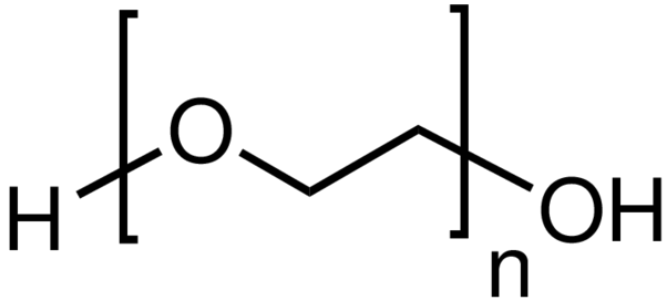


***Figure 22*- The chemical structure of polyethylene glycol (PEG).** *n* designates the number of repeated ether [-O-CH_2-CH_2-] units.

PEG compounds are available commercially in a range of molecular weights, although PEG compounds with an average molecular weight of 6000 and 8000 $gmol^{-1}$ are commonly reported for nucleic acid precipitation (Paithankar and Prasad, 1991)(Lis and Schleif, 1975). The theoretical basis for this process is somewhat more complex than traditional solvent precipitation. PEG is able to reduce the dielectric constant of an aqueous environment (Naimushin et al., 2000), so when sufficient cations are present and associated with the phosphate groups in the nucleic acids, their Coulomb attraction is increased as the dielectric constant of the matrix decreases, resulting in precipitation of the nucleic acids, as solvation shells are depleted, reducing the solubility of the nucleic acids. However, Froehlich et al., 2011, demonstrated specific stable hydrophobic and hydrophilic interactions formed between DNA and PEG, even at low concentrations. Changes in nucleic acid strand secondary structure may also be induced (Naimushin et al, 2000),

Theoretical basis notwithstanding, there are some obvious trends that have consistently demonstrated during PEG-based extractions of DNA. Perhaps the strongest influencing factor on nucleic acid yield is the influence of PEG concentration. At low PEG concentrations, only nucleic acids of high molecular weight (HMW) are recovered. A minimum of 5% PEG is required to precipitate large (46.5Kbp) nucleic acids, whereas a 15% PEG solution was effective in precipitating HMW nucleic acids, as well as oligonucleotide fragments as low as 150 bp (Lis and Schleif, 1957).

Herein lies the true value of PEG-based extraction. A certain concentration can be used to selectively a nucleic acid strand of a specified size, while conveniently removing unwanted low molecular weight nucleic acid fragments (Paithankar and Prasad, 1991). This principle is often applied in the presence of a solid phase to act as reversible carriers, such as negatively charged carboxyl-coated paramagnetic beads, which bind the nucleic acids until they have been washed and eluted (Hawkins et al., 1994)(He et al., 2013).

Although some reports portray PEG-based nucleic acid extraction as selective, as effective deproteinisation is shown, this is normally as a result of sample pre-treatment, such as phenol-chloroform extraction, which is used prior to PEG extraction (Peng et al., 2014). Comparable protocols can be used to isolate proteins using PEG (Ingham, 1984), again, where higher PEG

concentrations result in the recovery of smaller proteins (Sim et al., 2012), so protein contamination in a simplified process is a possibility. Additionally, it is likely that a PEG-based extraction would come at a greater overall cost when compared to a conventional solvent extraction. With this in mind, and considering conventional solvent extraction has been reported in greater depth, it seems that precipitation is an overall more suitable option as a potential industrial process.

2.3.4- Quantification- Ultraviolet-Visible Spectrophotometry

Ultraviolet-Visible (UV-Vis) Spectrophotometry can be used to quickly assess the nucleic acid concentration in a sample, as nucleic acids intrinsically absorb ultraviolet light in the region of 260 nm. This property arises because of the aromatic structure of the nucleobases within nucleic acids. Within the nucleobases, p-orbitals that arrange themselves perpendicularly to the plane of the ring, are able to delocalise into a π (pi) system. The electrons in the π system can transition to an excited π* (pi-star) state when they absorb high-energy UV light, which is then emitted when the electrons in the π system returns to its ground state (Schmid, 2001).

The procedure for quantifying nucleic acid concentration with UV-Visible spectrophotometry is very simple and convenient, in so lending itself well for use as a quality control measure at industrial scales. The technique is also sensitive, non-destructive and requires a small sampling volume (usually 2mL in macro cuvettes, but as little as 10µL when micro cuvettes can be accurately analysed) (Schmid, 2001).

Essentially, the sample is dissolved in a transparent solvent and placed in a quartz cuvette before being placed into a spectrophotometer, where a light source (usually a deuterium lamp) emits an ultraviolet light beam through the sample. A detector detects light exiting the sample, either at a pre-defined wavelength of interest, or over a range of wavelengths in more sophisticated systems, presented as an absorption spectrum. As nucleobases in nucleotides vary in structure, their exact absorption maxima vary slightly (see Table 5). All have a λ (lambda) maxima around 260 nm, so in polynucleotides, a broad peak is present at 260 nm which is used to determine the nucleotide

concentration in the sample from a calibration curve following analysis of known nucleic acid standard solutions of known concentrations.

***Table 5*- A summary of the λ_{max} values for each of the different common nucleotides and their derivatives.** Adapted from Sambrook and Russell, 2000.

Letter Code	Nucleoside/Derivative and λ_{max} (nm)					
A	Adenine	Adenosine	Adenosine 5'-monophosphate	Adenosine 5'-diphosphate	Adenosine 5'-triphosphate	2'-Deoxyadenosine 5'-triphosphate
	260.5	260.0	259.0	259.0	259.0	259.0
C	Cytosine	Cytidine	Cytidine 5'-monophosphate	Cytidine 5'-diphosphate	Cytidine 5'-triphosphate	2'-Deoxycytidine 5'-triphosphate
	267.0	271.0	271.0	271.0	271.0	272.0
G	Guanine	Guanosine	Guanosine 5'-monophosphate	Guanosine 5'-diphosphate	Guanosine 5'-triphosphate	2'-Deoxyguanosine 5'-triphosphate
	276.0	273.0	252.0	253.0	253.0	253.0
T	Thymine	2'-Deoxythymidine	2'-Deoxthymidine 5'-monophosphate		2'-Deoxythymidine 5'-triphosphate	
	264.5	267.0	267.0		267.0	
U	Uracil	Uridine	Uridine 5'-monophosphate		Uridine 5'-triphosphate	
	259.0	262.0	260.0		260.0	
H	Hypoxanthine			Inosine		
	249.5			248.5		
X	Xanthine					
	267.0					

This method exploits the principle of the Beer-Lambert Law (Perkampus, 1992), which states that the absorbance of light by a material is directly proportional to its path length. When this principle is applied specifically to spectrophotometry, an equation can be used to express this relationship:

$$A = \varepsilon c l$$

Where A is the absorbance of the material, ε is the molar extinction coefficient, c is the concentration of the material and l is the light path length. This can be rearranged to give:

$$c = A / \varepsilon l$$

When this is specifically applied to determination of nucleotide concentration, an absorbance value of 1.0 can be used to represent the direct relationship between given nucleotide concentration and that absorbance, where path length is kept constant (usually 10 mm in standard quartz cuvettes). An absorbance of 1.0 at 260 nm, with a 10 mm light path relates to a dsDNA concentration of 50 μgmL^{-1}, a ssDNA concentration of 33 μgmL^{-1} and an RNA concentration of 40

μgmL^{-1} (Barbas et al., 2007). These values are well-reported in literature and can be used to approximate a nucleic acid concentration from a given OD260 value. However, these values are subject to variation which arises due to a number of factors. For instance, if any nucleic acids preparations become contaminated with phenol, a peak at 270 nm forms due to the intrinsic absorptive properties of the aromatic ring structure of phenol at this wavelength, with the overall effect of overestimating the nucleic acid concentration (Lee et al., 2014). Physiological factors such as pH and temperature also have an influence here. As pH decreases, the increased proton concentration results in the protonation of the nucleobase, and thus alterations in its absorptive properties. Significant temperature shifts and degradation of a polynucleotide chain can result in changes in the extinction coefficient for a nucleic acid solution, due to a phenomenon called hyperchromicity. In the case of native double-stranded DNA, as illustrated in Figure 23, the strands are arranged so that the nucleobases are in the interior of the strand, stabilised by non-covalent forces between nucleobases called *stacking interactions*. Essentially, a given concentration of polynucleotides will give a lower absorption at 260 nm than the same concentration of mononucleotides, as densely organised bases in nucleotide chains aren't able to interact with UV light as effectively as they would when dispersed as monomers in solution.

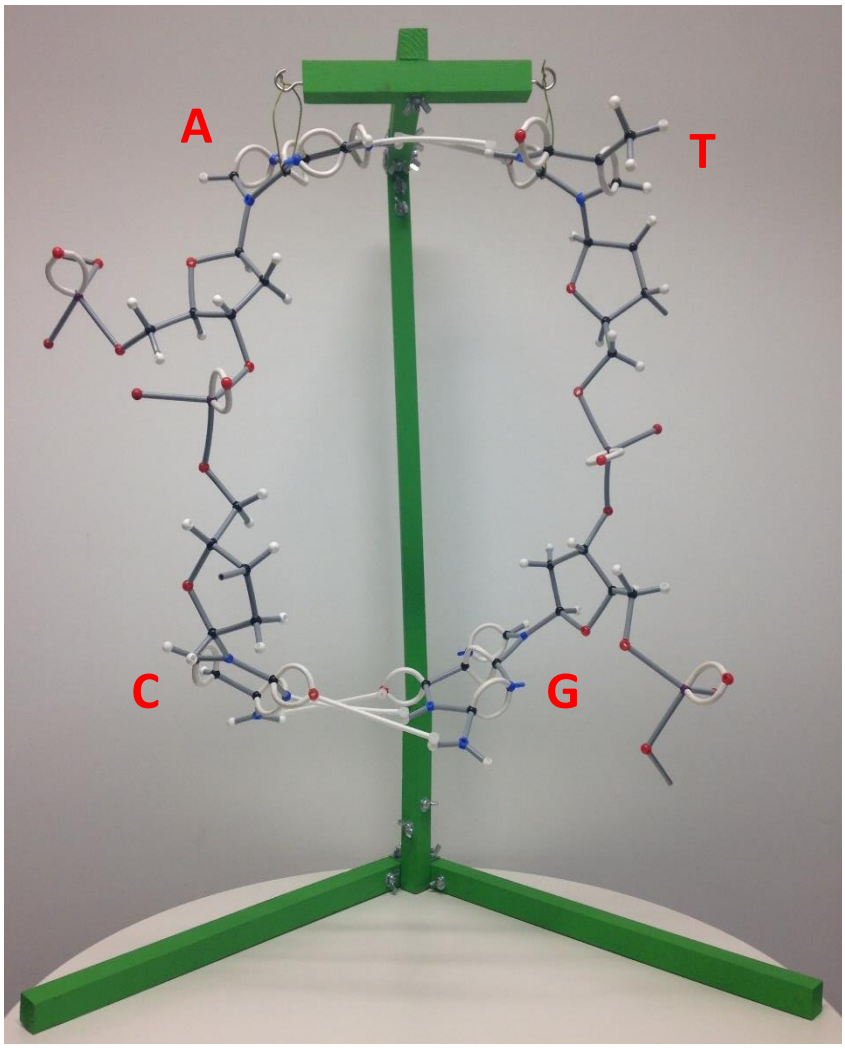


***Figure 23*- An atomic model of a short DNA oligonucleotide.** The nucleotides (A- 2'-Deoxyadenosine, T- 2'-(Deoxy)thymidine, C- 2'-Deoxycytidine, G- 2'-Deoxyguanosine) orient in a specific way, where the deoxyribose sugars face the exterior of the antiparallel strands. The nucleobases face the interior of the strand and are stabilised on a plane by stacking interactions with neighbouring bases (bonds from stacking interactions not shown). (Designed and constructed by the author for the GRØN DYST [Green Challenge] 2015, Denmark)

UV-Visible spectrophotometry can also be used to indicate the protein contamination within the nucleotide sample, which will be of critical importance in determining how much protein has been co-precipitated and retained into the final product. Of the 26 amino acids that make up proteins, there are only two amino acids that have aromatic side chains which strongly absorb near-UV light- tryptophan and tyrosine. To a lesser extent, phenylalanine residues and the disulphide bonds that form between cysteine residues also have the ability of absorb near-UV light, as shown in Figure 24 (Aitken & Learmonth, 2001).

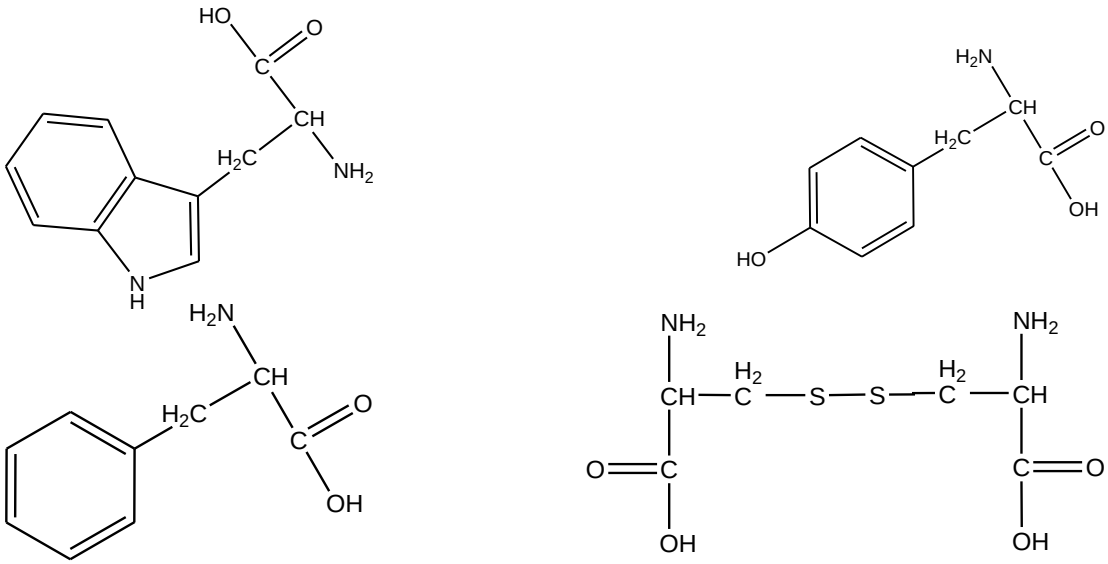


***Figure 24*- The amino acids with intrinsic absorbance at 280 nm.** Tryptophan (top left), Tyrosine (top right), Phenylalanine (bottom left) and two residues of cysteine bonded via a disulphide bond to give a cystine/dicysteine residue (bottom right).

In a native folded protein, exposed external residues that absorb UV light will do so differently than those that are situated on the interior of the protein, although this difference is normally less than 5%. The λ maxima of proteins is generally considered to be 280 nm, which is a general average of the UV-absorbing residues shown above. An accurate determination of protein concentration can be calculated from the absorbance of a protein sample, if the protein primary sequence is known (Schmid, 2001). However, when the protein sequence is unknown, it is very difficult to determine the protein concentration as the protein may be rich or poor is tyrosine or tryptophan residues, leading to obvious inaccuracies in the calculated protein concentration.

Despite this, in extracted nucleic acid samples, the ratio of absorbance at 260 nm to the absorbance at 280 nm (or 260:280) can provide insights into the protein contamination in the sample. Proteins absorb UV light much more weakly than nucleic acids, so nucleic acid contamination of protein samples cannot be determined with this method. However, reference values for 260:280 absorbance ratios are well reported and can be related to the percentage of protein in a nucleotide sample, as shown in Table 6.

***Table 6*- Theoretical 260:280 ratios and their relation to protein and nucleotide content within a sample.** From Glasel, 1995.

% Protein	% Nucleotide	260:280
100	0	0.57
95	5	1.06
90	10	1.32
85	15	1.48
80	20	1.59
75	25	1.67
70	30	1.73
65	35	1.78
60	40	1.81
55	45	1.84
50	50	1.87
45	55	1.89
40	60	1.91
35	65	1.93
30	70	1.94
25	75	1.95
20	80	1.97
15	85	1.98
10	90	1.98
5	95	1.99
0	100	2.00

The percentage of nucleotide in the sample can be directly calculated from the 260:280 value using:

(11.16 [260:280] – 6.32)/(2.16 – [260:280])

3- METHODS

3.1- Materials

The reagents and materials presented in Table 7 were purchased and used during research.

***Table 7*- A table to show all reagents purchased for experimentation.** Stated purity and the chosen suppliers have been listed.

Reagent	Stated Purity	Supplier
Ammonium Acetate	≥96%	Scientific Laboratory Supplies
Sodium Acetate Anhydrous	≥98%	Scientific Laboratory Supplies
Sodium Chloride	≥99.5%	Sigma-Aldrich
Magnesium Chloride Hexahydrate	99-102%	Sigma-Aldrich
Magnesium Acetate Tetrahydrate	97.5%	Acros Organics
Tripotassium Phosphate	≥98%	Sigma-Aldrich
Potassium Carbonate	99-100.5%	Sigma Aldrich
Ethanol Industrial	99%	Scientific Laboratory Supplies
Isopropanol	*No purity stated*	Barretine Industrial
Ribonucleic Acid from Torula Yeast	*No purity stated*	Sigma-Aldrich
Guanosine 5'-monophosphate disodium salt hydrate	≥99%	Sigma-Aldrich
Glycogen, from Oyster	*No purity stated*	Acros Organics
Yeast tRNA (10 $mgml^{-1}$ solution)	*No purity stated*	Invitrogen
Sodium Bicinchoninate Anhydrous	*No purity stated*	Alfa Aesar
Sodium L-(+)- Tartrate Dihydrate	99-101%	Alfa Aesar
Copper Sulphate Pentahydrate	≥99%	Acros Organics
Sodium Carbonate	98%	Alfa Aesar
Sodium Hydrogen Carbonate	99%	Alfa Aesar
Sodium Hydroxide Pellets	98%	Sigma-Aldrich
Bovine Serum Albumin, New Zealand Origin, Standard Grade, Chromatographically Purified	*No purity stated*	Alfa Aesar
Hydrochloric Acid	37% solution in water	Acros Organics
Acetic Acid	≥99.7%	Sigma-Aldrich
Nitric Acid	70% solution in water	Sigma-Aldrich

Samples of centrate and mycoscent were supplied by Quorn. Due to the presence of some insoluble particulate matter in the centrate, before sampling, aliquots were either vacuum filtered with a 25 µm cellulose filter (Whatman, GE Healthcare Life Sciences, Buckinghamshire, UK), or pre-centrifuged in a Sigma 1-14 microfuge, with polypropylene fixed angle rotor (Sigma, Germany) at 16,163 RCF for 30 minutes, to pellet insoluble matter. Following precipitation of nucleotides, any insoluble matter in the original sample is pelleted following centrifugation, so methods of removing insoluble matter were compared. Following pre-treatment, samples of centrate were

stored as 200 mL aliquots at -20°C until required, at which point, a sample was thawed in a 20°C water bath and vortexed before sampling. Storage at sub-zero temperatures was necessary to prevent rancidification and microbial growth, due to the glucose content in the centrate.

Initially, access to samples of the centrate was delayed, so the protocol was tested twice with a substitute, made *in situ* from samples of *Asperguillus oryzae*, kindly provided by Dr Peter Köppel. For this 'simulated centrate,' frozen inactivated *A. oryzae* culture was thawed from -80°C to 20°C in a water bath, and weighed. The sample was then placed in a large beaker and placed on a heated stirring plate with a magnetic stirrer. The sample was then heated to approximately 65°C for 20 minutes, with continuous stirring, to replicate the RNA reduction procedure used during Quorn's production process. After heating, the fungus was allowed to cool to room temperature before being weighed again. The mass loss was used to accurately reconstitute the fungus with ultrapure water, before the vessel was stirred for a further 10 minutes. Following this, the contents were vacuum filtered using a 25 µm cellulose filter (Grade 114 Qualitative filter paper, wet strengthened with chemically stable resin, Whatman, GE Healthcare Life Sciences, Buckinghamshire, UK), after which the solid matter was discarded and the supernatant was collected and stored as 10 mL aliquots at -20°C in a laboratory freezer. Using this method, around 48 mL of lysate was collected from 67.5 g of thawed culture. When required, the aliquots were thawed in a 20°C water bath and vortexed before sampling for extraction. Some information was collected during initial extractions with the *Aspergillus* supernatant, but no major changes were made until extraction begun with the centrate shortly after.

For the Bicinchoninic Acid Assay (BCA), stock solutions was prepared for all assays during the experimental period. The solution was prepared as per the protocol described for a standard assay by Walker, 2010. *Reagent A* was prepared by adding 0.25g of sodium bicinchominate, 5.0g of sodium carbonate, 0.4g of sodium tartrate dihydrate, 1.0g of powdered sodium hydroxide (crushed with a mortar and pestle) and 2.375g of sodium hydrogen carbonate, into a 250 mL volumetric balloon. After addition of the salts, ultrapure water was added to a final volume of 250 mL in the volumetric balloon. After all salts were fully dissolved, the solution was placed on a stirring plate

with a stirrer bar, and a HI-2211 pH/ORP meter (Hanna Instruments, Bedfordshire, UK) was used to measure pH, with a general plastic pH electrode (Eutech Instruments, Landsmeer, The Netherlands). With this setup, finely ground sodium hydroxide powder was added in small increments to achieve a pH of exactly 11.25. Prior to any pH measurement during the experimental period, the pH meter was calibrated using the manufacturer's recommended 2-point calibration protocol, where pH 7 and pH 4 colour coded buffer solutions (Scientific Laboratory Supplies) were used to calibrate the meter. *Reagent B* was prepared by adding 0.4g of copper sulphate pentahydrate to a 10 mL volumetric balloon, then adding ultrapure water to a final volume of 10 mL. Both stock solutions were kept at room temperature until required, at which point the necessary amount of standard working reagent (SWR) was prepared by combining reagent A and reagent B at a ratio of 50:1. Samples of SWR were used immediately and were not stored for later use.

For all experimentation, ultrapure (Type I) water was used where required, as dispensed from a Direct-Q 3 UV Water Purification System (Millipore, Molsheim, France).

3.2- General Overview of Experimental Design

After some initial modifications of conventional solvent precipitation protocol, different variables were altered to determine their effect on nucleotide yield, as determined by UV-Visible Spectrophotometry. All trials to investigate the role of any variable were carried out in duplicate or triplicate. As per the general protocol for solvent precipitation of nucleic acids, a certain molarity of salt was achieved in the filtered of centrifuge centrate (Figure 25) before a solvent was added. After incubation, the sample was centrifuged to yield a pellet (henceforth referred to as *Pellet 1*- P1) of precipitated nucleotides, after aspiration of the supernatant (henceforth referred to as *Supernatant 1*- S1). A centrifugation speed of 16,163 RCF, the maximum speed for the centrifuge used, was used for all centrifugation steps, as low molecular weight mononucleotides require a high force to migrate and pellet efficiently. The pellet was hence washed in dilute ethanol, followed by centrifugation to yield *Pellet 2*- P2 and *Supernatant 2*- S2. This washing was repeated once more

to yield *Pellet 3*- P3 and *Supernatant 3*- S3 before the residual solvents were evaporated in a fume hood. For analysis, the pellets were fully dissolved in 2 mL of ultrapure water, diluted appropriately, and analysed for yield, protein content and salt contamination. Many supernatants resulting from the three centrifugation steps were also retained for analysis of nucleotide content and for salt concentration.

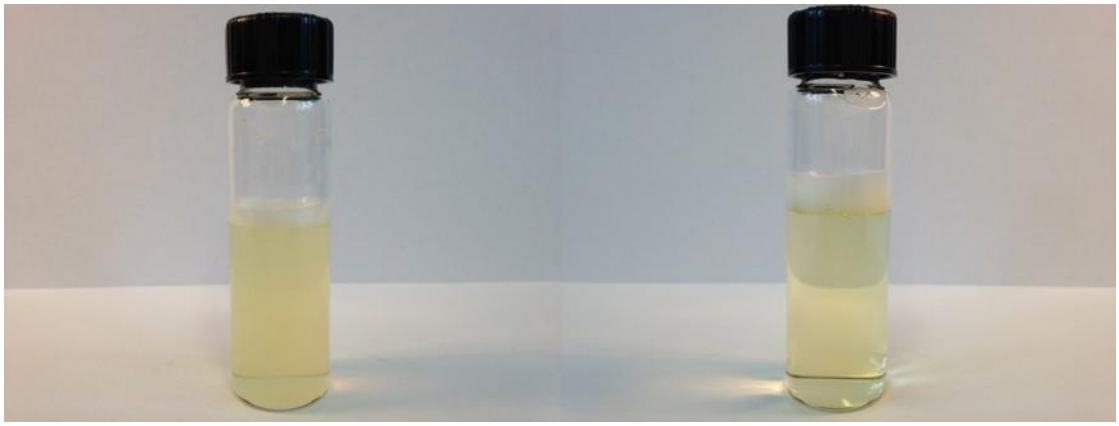


***Figure 25*- Photographs of centrate samples in clear glass vials.** (Left) following vacuum filtration, (right) following centrifugation at 16,163 RCF for 30 minutes.

3.3- Assessment of Yield

To analyse the yielded products, ultraviolet-visible (UV-Vis) spectrophotometry was used. Although several papers quote that an absorbance of 1.0 indicates an RNA concentration of 40.0 μgmL^{-1}, when a cuvette with a 10 mm light path is used, it was necessary to generate new calibration curves for this experimentation, as any inaccuracies would be exaggerated when accounting for high dilution factors. Commercially purchased RNA was used to generate several calibration curves. Polymeric RNA is very difficult to solubilise in water at room temperature, it quickly coagulates to form a congealed mass.

This is in stark contrast to the highly soluble nature of RNA. During experimentation, a completely saturated RNA solution was prepared by adding 3.0 g of RNA to 14 mL of water in a sealed vial. After 4 weeks of continuous stirring on a magnetic stirring plate with a small stirring bar at room temperature, a saturated amber solution was produced, with a sediment of undissolved RNA after stirring was ceased. This was diluted by a factor of nearly 9000 before UV-Vis interrogation, to

allow the solution to fall within the boundaries of the calibration curve, which after correction, showed the saturated solution had an RNA concentration of 194.3 $mgmL^{-1}$.

To prepare stock solutions for standard solutions for calibration curves, 100 mg of RNA was weighed into a volumetric flask and fully solubilised in a final volume of 100 mL, to give a 1 $mgmL^{-1}$ stock solution (S1). Solubilisation was aided with the use of an ultrasonic bath (FB11005-Fisherbrand, Fisher Scientific, UK). Volumetric flasks were submersed in the ultrasonic bath and sonicated at 100% power until the solution was clear. This has the added advantage of sporadically shearing the RNA strands to give shorter chain polynucleotides, so stacking interactions are lessened and the solution has a more similar nucleotide composition to the centrate. Additional vortexing was used where necessary. As sonication generates some heat, the stock solution was cooled to 20°C in a water bath before it was used to prepare a secondary stock solution (S2). Initially, curves were produced by analysing ten freshly prepared solutions, with a concentration range of 10 $ugmL^{-1}$ to 100 gmL^{-1}, by first preparing S2 by diluting the 1 $mgmL^{-1}$ S1 by a factor of ten with water into a fresh 100 mL volumetric flask. The S2 solution was hence used to simply prepare ten 4 mL dilutions by diluting the appropriate ratios of S1 and ultrapure water. Stock solutions were freshly prepared before the production of standard solution series, as RNA solutions were seen to form precipitates or sediments when stored, regardless of concentration, even with refrigeration. These precipitates were seen to be difficult to resolubilise.

A far-UV quartz 3 mL macrocuvette with a 10 mm light path (Scientific Laboratory Supplies, Yorkshire, UK) was used for spectrophotometric interrogation of all samples. Before analysis, the cuvette was cleaned with 100% ethanol, as per the manufacturer's recommendations. Standard solutions were briefly vortexed before being used to fill the cuvette to a minimum volume of 2 mL, before placement into the Evolution 220 UV-Visible Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), which generated results via the coupled Thermo INSIGHT software. Initially, the scan function was used to analyse the absorption of UV light in the samples across a broad range of wavelengths from 500 nm to 200 nm, so any significant abnormal spectral features could be identified. A peak detector was also used to detect peaks based on height, with a sensitivity

setting of 100%, in the range 290 nm and 250 nm, to identify peaks relating to nucleotides (260 nm) and proteins (280 nm). All other parameters remained in their default settings (baseline correction at 100%T baseline, a bandwidth of 1 nm, integration time of 0.05 seconds, interval of 1.00 nm and a scan speed of 1200 nmminute^{-1}). A blank of ultrapure water was used and between each sampling, the cuvette was evacuated completely with the aid of compressed air, then rinsed with ultrapure water and evacuated once more with compressed air. Absorption values for each concentration were taken at the λ_{max} for each spectra and used to construct calibration curves against the exact concentrations of the solutions. Curves were produced in Microsoft Excel, where an origin at zero was set. Curves were consistent despite absorbance values ranging between around 0.25 and 3.0 A.U. An equivalent calibration curve was produced with a pure sample of GMP, which solubilised much more readily than native RNA. Perhaps because of this, calibration curves were near perfectly linear over a concentration range of 10-100 µgmL^{-1}, although the λ_{max} and regression equations were understandably different to RNA standards.

After repeat attempts with a producing an RNA calibration using concentrations between 10 µgmL^{-1} and 100 µgmL^{-1}, some inter-trial variability was evident, as well as some deviation from the trendline in some data points. Inter-trial variability may have occurred due to differences in extraneous conditions, such as temperature and humidity, but significant differences were more likely to arise as a result of inconsistencies in weighing, diluting and solubilising during preparation of stock solutions. However, in an attempt to limit this variation, modifications were made. S1 was diluted to produce an S2 solution with a concentration of 37-40 µgmL^{-1}, which was predicted to give an absorbance of 1.0, based on previously data. This diluted as before with water to give 10 standard solutions with concentrations ranging between 3.37-4.00 µgmL^{-1} and 33.70-40.00 µgmL^{-1}. Additionally, the UV-Vis spectrophotometer was operated in the fixed mode for analysis, which analysed samples at one or more pre-defined wavelengths, where default settings of 0.40 second integration time and 1 nm bandwidth were used. Wavelengths of 260 nm and 280 nm were inputted, and the 260 nm absorbance values were used in the formation of the calibration curves, as opposed to the individual λ_{max} values for each spectrum. Analysis of these solutions in this way

gave a very consistent trendlines, with absorbance values ranging between 0.1 and 1.0 A.U, the regression equation of which was used to determine the nucleotide concentrations of the yielded nucleotide solutions produced from extractions.

It was necessary to determine the nucleotide concentration in the centrate to allow expression of the yield as a percentage. Information provided by Quorn following some independent testing of the centrate stated that the nucleotide concentration in the centrate was seen to vary between 0.6-1.1 gL^{-1} (see *Quorn Production*). However, due to the differences in methods used, it was necessary to directly analyse the centrate using UV-Vis spectrophotometry to ensure the readings were consistent. A dilution factor of 160 was used to dilute the centrate, by diluting 25 µL of centrate to a final volume of 4 mL with ultrapure water. Two batches of centrate were received during the experimental period, which were both analysed separately. Filtered and centrifuged centrate samples were also analysed separately to determine the effect of different pre-treatments on nucleotide concentration. Similarly, the *Aspergillus* lysate, which was used in lieu of the centrate in early trials, was also analysed for nucleotide concentration in this way.

Following extractions, a washed nucleotide pellet is produced. For yield analysis, this pellet was fully solubilised in 2 mL of ultrapure water. The centrifuge tube was filled completely to increase the chances of the pellet solubilising completely, and physical perturbation of the pellet was avoided and solubilisation was enhanced with the use of an ultrasonic bath, which was used to sonicate the samples for 90 minutes. After solubilisation, the solutions were diluted appropriately before quantification with UV-Vis. As per the procedure for analysis of RNA standard solutions, the samples were vortexed before being placed in a clean quartz macrocuvette, then analysed in fixed mode to give absorbance values at 260 nm and 280 nm. Absorbance values were multiplied by the value as specified in the regression equation of the calibration curve to give a nucleotide concentration, then accounted for dilutions to allow determination of % yield from 1 mL of centrate.

Similarly, supernatants following centrifugation could be interrogated by UV-Vis spectrophotometry to give an indication of:

- The amount of nucleotides that were not precipitated during the initial precipitation- supernatant 1.
- The amount of nucleotides lost as a consequence of pellet washing steps- supernatants 2 and 3.

However, supernatant 1 is a solution of nucleotides in 1:2.5 water:ethanol or water:isopropanol (in routine extractions) and 75% or 95% ethanol in supernatants 2 and 3 following the pellet washing steps. It was therefore necessary to construct calibration curves of RNA in solutions of different proportions of solvents, as it was unknown if this would have an influence on the regression equations. This was found to be exceptionally difficult, and the production and analysis of the standard solutions necessitated extreme meticulousness to ensure the resulting curves would be of acceptable quality. This was chiefly due to the inorganic solvents having such significant differences in density compared to water. The following protocol were deemed absolutely necessary in generating a good quality calibration, after several failed attempts. Firstly, four stock solutions in different solvent-aqueous mixtures were carefully prepared as below in Figure 26.

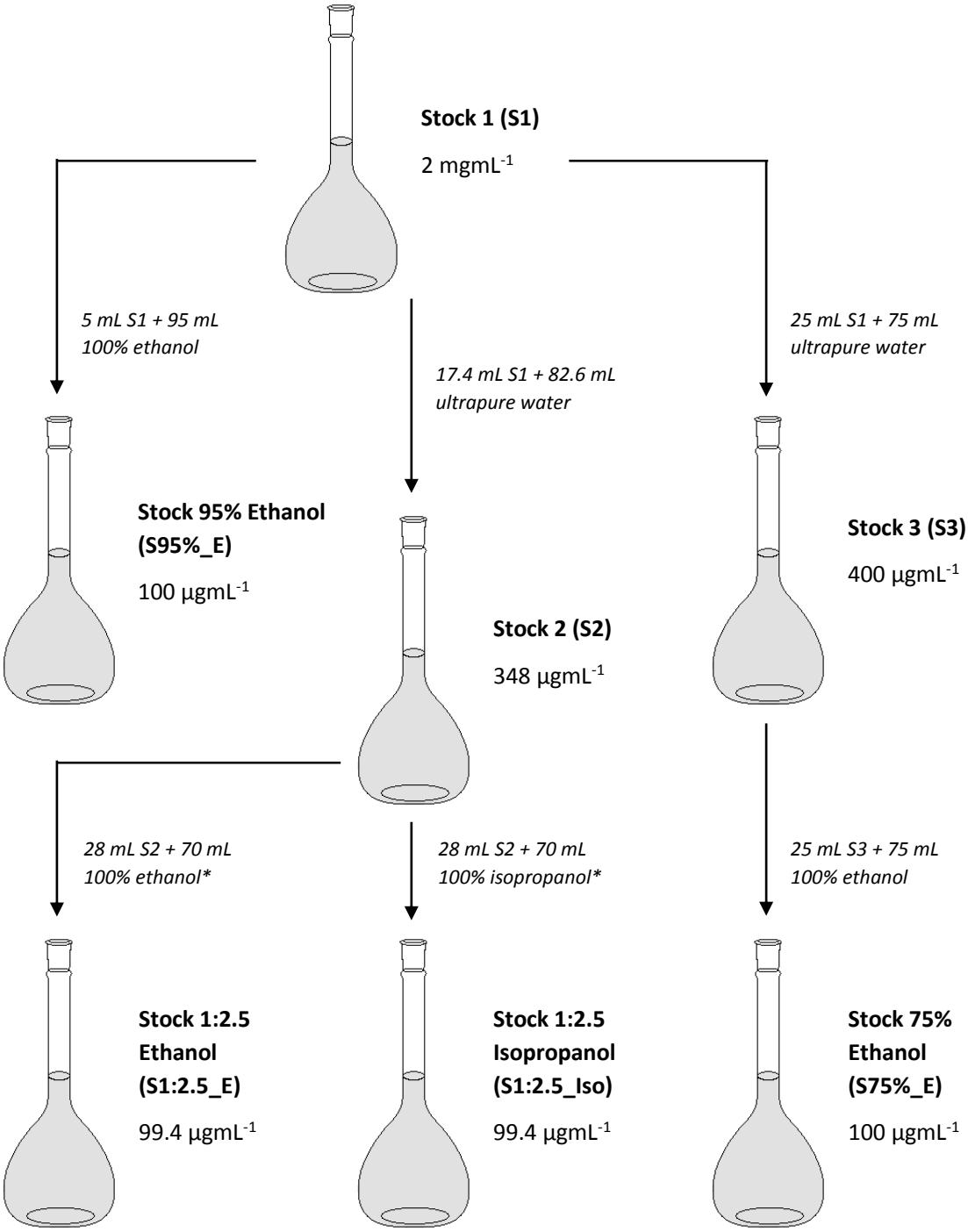


***Figure 26*- Preparation of stock solutions of RNA in different aqueous-solvent matrices, for analysis via UV-Vis spectrophotometry.** A mother solution was prepared by weighing 200 mg of RNA into a 100 mL volumetric flask, which was then used to prepare daughter solutions in fresh 100 mL volumetric flasks as above. Stock solutions were added with a burette *S1:2.5_E and S1:2.5_Iso relied on adding **both** components with a burette.

Aqueous-solvent stock solutions were sonicated for 30 minutes to ensure thorough mixing of the solvents. Diluents were also pre-prepared by combining the appropriate amounts of solvent and ultrapure water into 200 mL aliquots, using a burette. These aliquots were stored alongside the stock solutions in a 20°C water bath before being used to make ten standard solutions ranging

from 10 μgmL^{-1} to 100 μgmL^{-1}, from each stock solution, by pipetting appropriate amounts of stock solutions and diluents into a final volume of 4 mL. A broad concentration range was chosen as opposed to the narrow range used in the standard calibration. The same diluents used in preparation of the stock solutions were later used as blanks during spectrophotometry, which was again run in fixed mode, and also to rinse the cuvette between analyses of stock solutions. When these measures were taken, consistent curves were produced, which could be used to quantify the RNA content in the supernatants, which were diluted using the pre-prepared aqueous-solvent diluents. The resulting absorbance values could be used to determine the nucleotide loss per 1 mL of centrate, as a percentage.

3.4- Salt and Solvent Testing

To consider the *Research Aims and Objectives*, salt addition was done during experimentation by adding solid powdered salts, as opposed to stock solutions. This was advantageous because this allowed more centrate to be added to a vessel, leaving sufficient free space for solvent addition. This also eliminates the need to purchase or prepare salt stock solutions. For initial experimentation, salts were added to the centrate to achieve a concentration based on those that had been stated in literature. Subsequent extractions analysed yields to compare different salts on a like-for-like basis, where molarities were kept the same between salts. During further testing, some salts were also directly analysed to determine the effect of salt molarity on nucleotide yield, with salts which had previously shown to give good nucleotide recovery. This was achieved by achieving a range of molarities of a certain salt in the centrate, in separate aliquots. The precipitation was then performed and the yields were compared following spectrophotometric interrogation of the final nucleotide solutions.

Ammonium acetate, sodium acetate, sodium chloride, magnesium chloride and magnesium acetate were all tested for their effectiveness. In general, addition of salt was achieved adding a certain mass of salt to a small (5-30 mL) beaker, which was then filled with a certain volume of centrate to achieve the desired molarity. Samples were then stirred until the salt had fully

dissolved, after which, the sample was pipetted, using a micropipette, to 2 mL round-bottom polypropylene DNA Lo-Bind centrifuge tubes (Eppendorf, Hamburg, Germany), ready for solvent addition.

Both ethanol and isopropanol were both investigated during the experimental period for their effectiveness in recovering nucleotides. Initially, ratios of 1:2 for centrate/salt:ethanol and 1:1 for centrate/salt:isopropanol were used during extractions, which generally matches the ratios used in literature. However, this was quickly changed to a ratio of 1:2.5 for both solvents, so the nucleotide recoveries for both solvent could be compared on a like-for-like basis. The use of a high solvent ratio was necessary to ensure minor increases in yield as a result of changes to other variables were obvious, and not masked due to the limitations of the solvent ratio. Solvents were stored at -20°C in a laboratory freezer to accelerate nucleotide precipitation upon addition to the centrate. Cold solvents were added directly to centrifuge tubes containing centrate and salt, using a micropipette. During later experimentation, solvent ratios were directly tested by changing the ethanol and isopropanol ratios in otherwise identical trials. To achieve this, it was necessary to ensure all conditions gave a final volume of 2 mL, as nucleotides may migrate more efficiently to the bottom of a tube during centrifugation, if a smaller total volume was used. Solvent ratios were tested as per Table 8.

***Table 8*- Ratios of solvent (ethanol or isopropanol) to centrate/salt tested to determine the effect of solvent ratios on final nucleotide yield.**

Volume of centrate/salt added	Volume of solvent added	Ratio achieved
875 µL	875 µL	1:1
500 µL	1250 µL	1:2.5
350 µL	1400 µL	1:4
290 µL	1460 µL	1:5.03
250 µL	1500 µL	1:6
220 µL	1530 µL	1:6.99

During incubation, extraction samples were also chilled in the removable centrifuge rotor at -20°C freezer for 30 minutes. This step may not be absolutely necessary, but aided in the fixing of the pellet to the bottom of the tube during centrifugation.

3.5- pH Testing

pH manipulation was carried out to determine the effect of pH during precipitation. This was originally considered to be a key factor, as it was hypothesised that an acidic environment would result in protonation of the exposed charged region of the nucleotides, which would result in a loss of a site for cations to interact. Adjusting the pH beyond *biologically relevant* limits may also result in some damage to the target nucleotides, and extreme pH levels are undesirable at industrial scales, in terms of hazards and effects on equipment. It was initially decided that a buffer system would be the most appropriate here, and a sodium acetate/acetic acid buffer system was initially used for several reasons:

- Sodium acetate had been previously been shown to be effective in precipitating nucleotides, so may enhance precipitation in certain conditions
- Both sodium acetate and acetic acid are used as food additives.
- Both components would be used at fairly low concentrations to achieve a desired pH, so would pose few industrial hazards.
- Crucially, both components are soluble in ethanol and isopropanol, so will not precipitate upon solvent addition.

One disadvantage of this buffering system is the limited buffering range, from around 3.7-5.6. Nevertheless, buffering was achieved by fully solubilising a near-saturated solution of sodium acetate to a final molarity of 2.7M, while a highly concentrated solution of acetic acid was prepared with a final molarity of 11.2M. Highly concentrated solutions were used so that the volume of samples were not significantly influenced by buffer additions, although this could be accounted for afterwards when calculating yields. These solutions were added to 5 mL aliquots of centrate in varying proportions to one-another, to achieve a range of pH levels, prior to addition of salts. Ratios were determined simply based on Michaelis-Menten kinetics, Hendersen-Hasselbach approximations and some initial testing (Berg et al., 2002). As per these equations, the pH range

that was targeted using this buffering system is as shown in Table 9, where target pH levels were verified by measuring the pH with a meter.

***Table 9*- Exploratory pH testing to determine the buffering capabilities of the sodium acetate/acetic acid buffering system, in the centrate.**

Target pH	Volume of 2.7 M sodium acetate solution added (µL)	Volume of 11.2 M acetic acid solution added (µL)	Moles of sodium acetate in 5 mL aliquot (µmol)	Moles of acetic acid in 5 mL aliquot (µmol)	Theoretical pH in centrate (native pH measured at 6.26)	pH achieved
3.7	55.7	82.4	149	919	3.97	3.97
4	87.4	78.1	234	871	4.19	4.14
4.5	175.6	53.6	469	598	4.66	4.99
5	275.6	32.0	737	357	5.07	5.60
5.5	341.7	9.7	913	108	5.67	5.74

From this data, three 10 mL aliquots of centrate were successfully buffered to pH 4.13, pH 5.15 and pH 5.78, and extractions were carried out with a variety of salts at concentrations of 0.5M, where a control trial with unbuffered centrate (pH measured at 6.43), was also performed.

An alternative approach was used later, whereby 10 mL aliquots of centrate were prepared, placed on a magnetic stirring plate with a stirring bar and the pH monitored with a pH probe and meter. A pipette was used to gradually add 0.5 µL additions of concentrated hydrochloric acid to decrease the pH of the centrate, or small grains of powdered sodium hydroxide (prepared from sodium hydroxide pellets in a pestle and mortar) were gradually added to increase the pH of the centrate. Using this method, aliquots of the centrate were adjusted to pH 7.54, pH 7.00, pH 6.01 and pH 5.47. This method is advantageous when compared to buffering in terms of simplicity, but additionally, the effect of precipitation enhancement or reduction as a result of additional ions is minimal, as the required concentrations of hydrochloric acid or sodium hydroxide are incredibly low. Also, the centrate may be adjusted to any desired pH, as the method is not limited by the buffering capacity of a specific system.

3.6- Protein Quantification

As proteins could theoretically be co-precipitated by the mechanism used in this instance, it was necessary to quantify the amount of protein present in the final nucleotide products. An indication

of the protein contamination could be taken from the 260:280 ratios when the nucleotide solutions, so results for absorbance at both wavelengths were recorded. However, some inconsistencies were observed, such as low 260:280 ratios from analysis of pure nucleotide samples and ratios above a value of two. The bicinchoninic acid assay was chosen for its convenience and broad sensitivity range, and was performed as per Walker, 2001. Reagents A and B were prepared from commercially bought reagents (see *Materials*) and combined at a ratio of 50:1 when necessary to give a standard working reagent (SWR). Standard protein solutions were prepared alongside experimental samples, by preparing five solutions of bovine serum albumin (BSA), ranging from 200 μgmL^{-1} to 1000 μgmL^{-1}. Yielded nucleotide products were dissolved in 2 mL of ultrapure water to give a solution, which were added to 3.5 mL disposable plastic macrocuvettes with a volume of 100 µL, alongside 100 µL samples of BSA protein standards and a blank of ultrapure water. To all samples, 2 mL of SWR was added and all samples were incubated in a 60°C water bath for 30 minutes. After cooling to room temperature, all samples were analysed by UV-Vis spectrophotometry in fixed mode to determine the absorbance of samples at 562 nm. Based on the calibration curve generated from the absorbance values from protein standards, the protein concentration in the yielded nucleotide products were determined. The protein concentration was similarly determined in the centrate, although the presence of residual reducing salts (chiefly glucose) may have resulted in an overestimation of protein content. Data provided from Quorn's independent analysis could be used for the initial protein content in the centrate.

3.7- Pellet Washing

Following centrifugation, supernatants were carefully aspirated away with a micropipette. Following the first two centrifugation steps, the resulting pellets were washed in dilute ethanol to remove residual salts. Early trials with the *Aspergillus* lysate showed high volumes of 70% ethanol led to significant RNA losses, so just 500 µL of aqueous-ethanol was used for all subsequent washes. Both 75% and 95% ethanol were tested for their effectiveness in removing salts, while nucleotide yields and losses were also compared for the two solvents. In general, following the

first centrifugation, 500 µL of 75% or 95% ethanol was pipetted into the centrifuge tube to submerge the pellet, which was then sonicated in an ultrasonic bath for 20 minutes. Following resuspension of the pellet, the samples and centrifuge rotor were chilled for 30 minutes at -20°C to aid in fixing of the pellet to the base of the centrifuge tube during the subsequent centrifugation. After centrifugation, the supernatant was aspirated away once more and the pellet was washed again in fresh 75% or 95% ethanol. After resuspension via sonication for 20 minutes in an ultrasonic bath, the samples and centrifuge rotor were chilled once more at -20°C for 30 minutes prior to the final centrifugation. After the final centrifugation, the supernatant was aspirated away to leave the yielded nucleotide pellet, which was left in a fume hood for 15 minutes to evaporate residual ethanol. Although all supernatants were removed, many were retained for later analysis.

3.8- Incubation Temperature Testing

Throughout the experimental period, samples were incubated at -20°C for 12 hours, to ensure that insufficient incubation was unable to conceal the potential benefits of other variable changes. Once favourable conditions had been identified, the effect of incubation temperature was quantified by comparing trials that were prepared using a protocol that included the conditions which had been shown to be favourable. After preparing samples by adding the appropriate amount of salt to aliquots of centrate and adding in 500 µL aliquots to centrifuge tubes, 1,250 µL of freshly dispensed, room temperature ethanol or isopropanol was added to conditions. The use of cold ethanol was chosen against in this instance, as this may have enhanced or constrained the precipitation in samples incubated at higher temperatures. Samples were then incubated at either; 20°C in a water bath, 4°C in a laboratory refrigerator, -20°C in a laboratory freezer or -70°C in an ultra-low temperature freezer, for 12 hours. After incubation, all samples were collected, then processed and analysed simultaneously.

3.9- Carrier Testing

Carrier substances glycogen and yeast transfer RNA (tRNA) were both tested to determine whether they are effective in enhancing the recovery of nucleotides from the centrate. Yeast tRNA has a disadvantage of having an intrinsic absorbance at 260 nm, so during quantification, some increases in yield may be due to the absorbance of the added tRNA. However, any increase in absorbance is likely to be minimal as the starting concentrations of tRNA are so low. Glycogen has no intrinsic absorption. Glycogen has the added benefit of being overall cheaper, when directly compared to yeast tRNA, based on the costs of our chosen suppliers. Spermine and linear polyacrylamide were not purchased or tested as they have been previously shown to be ineffective in recovering smaller nucleotide fragments. For tRNA testing, 15 µL of 10 $mgmL^{-1}$ yeast tRNA solution was added directly to a 10mL aliquot of centrate and salt, to give a final concentration of 15 μgmL^{-1}. The appropriate concentration of salt was then added to this centrate to 500 µL of this centrate was then added to centrifuge tubes. For glycogen testing, a solution of glycogen was prepared in a volumetric flask, by dissolving 21.53 mg of glycogen into 25 mL of ultrapure water, giving a glycogen solution with a final concentration of 861.2 μgmL^{-1}. Centrate and salt were combined appropriately and added in 471 µL aliquots to centrifuge tubes, 29 µL of the fully solubilised glycogen solution was then directly added to the samples to give a final concentration of 49.9 μgmL^{-1} of glycogen in the samples. This standardises the volume of the aqueous component in the sample to 500 µL, so all conditions and controls can be directly compared. Despite different volumes of centrate being used in these conditions, any difference was likely to be negligible and direct comparison is straightforward, as yields were expressed as percentages, so differences in centrate sampling could be accounted for later. The influence of carriers was tested alongside incubation testing (see *Incubation testing*), where samples with the addition of carriers were compared to controls, where conditions were incubated at a range of temperatures.

3.10- Assessment of Salt Contamination

Inductively-coupled plasma optical emission spectroscopy (ICP-OES) was used to determine the concentration of magnesium ions in the centrate, supernatant 1 samples, supernatant 2 samples, supernatant 3 samples and final products from a range of different extraction conditions where magnesium acetate was used. As the magnesium cations associate with the nucleotides, it is necessary to quantify these, rather than the acetate anions, to give an indication of the salt concentrations at each stage. The samples were sent to a third party for analysis with an iCAP 6300 ICP Spectrometer (Thermo Fisher Scientific, Massachusetts, USA), with source settings of: radiofrequency (RF) power of 1150W and an auxillary gas of nitrogen, at a flow rate of 0.5 $Lmin^{-1}$. Unfortunately, the instrument used for analysis was unable to analyse samples in a matrix that contains solvent, and a minimum sampling volume of 5 mL was required. As supernatants from extractions are primarily solvated in ethanol or isopropanol, it was necessary to completely evaporate all solvents from these samples, and reconstitute them with the appropriate volumes of ultrapure water, before analysis. Additionally, as only 500 µL of supernatants 2 and 3 are produced per extraction, twelve identical extractions were ran for each condition (eight conditions were tested) to produce enough sample for analysis. For supernatants, these were combined into large 15 mL glass vials and evaporated completely at 55°C before being reconstituted with ultrapure water in the same vial. All samples were sonicated for 30 minutes to ensure complete solubilisation of residues. Similarly, yielded products were solubilised in 2 mL of ultrapure water per extraction, with the aid of an ultrasonic bath, which was used to solubilise the pellets for 90 minutes. These samples were combined and sent for ICP-OES analysis. Following preparation of aqueous solutions, approximated PPM values for Mg^{2+} were used to determine a dilution factor for each sample, before each was diluted appropriately. All samples were then acidified to 0.1 M nitric acid, a condition used for magnesium standard solutions during analysis. Each sample was analysed in triplicate and an average value was recorded, where the relative standard deviation (RSD) was <5%. A summary of the samples is shown in Table 10.

***Table 10*- A summary of each of the 30 samples prepared for ICP-OES analysis.**

Sample condition	Sample Code	Quantity Provided	Dilution provided	Acidified to (HNO_3) (M)
Filtered centrate	FSP	10 mL	Neat	0.10046M
Centrifuged centrate	CSP	10 mL	Neat	0.10046M
Filtered supernatant 1 Ethanol	FS1E	10 mL	1:5	0.10046M
Filtered supernatant 1 Isopropanol	FS1I	10 mL	1:5	0.10046M
Centrifuged supernatant 1 Ethanol	CS1E	10 mL	1:5	0.10046M
Centrifuged supernatant 1 Isopropanol	CS1I	10 mL	1:5	0.10046M
Filtered supernatant 2 Ethanol 75%	FS2E75	6 mL	1:10	0.09889M
Filtered supernatant 2 Ethanol 95%	FS2E95	6 mL	1:10	0.09889M
Filtered supernatant 2 Isopropanol 75%	FS2I75	6 mL	1:10	0.09889M
Filtered supernatant 2 Isopropanol 95%	FS2I95	6 mL	1:10	0.09889M
Centrifuged supernatant 2 Ethanol 75%	CS2E75	6 mL	1:10	0.09889M
Centrifuged supernatant 2 Ethanol 95%	CS2E95	6 mL	1:10	0.09889M
Centrifuged supernatant 2 Isopropanol 75%	CS2I75	6 mL	1:10	0.09889M
Centrifuged supernatant 2 Isopropanol 95%	CS2I95	6 mL	1:10	0.09889M
Filtered supernatant 3 Ethanol 75%	FS3E75	6 mL	1:10	0.09889M
Filtered supernatant 3 Ethanol 95%	FS3E95	6 mL	1:10	0.09889M
Filtered supernatant 3 Isopropanol 75%	FS3I75	6 mL	1:10	0.09889M
Filtered supernatant 3 Isopropanol 95%	FS3I95	6 mL	1:10	0.09889M
Centrifuged supernatant 3 Ethanol 75%	CS3E75	6 mL	1:10	0.09889M
Centrifuged supernatant 3 Ethanol 95%	CS3E95	6 mL	1:10	0.09889M
Centrifuged supernatant 3 Isopropanol 75%	CS3I75	6 mL	1:10	0.09889M
Centrifuged supernatant 3 Isopropanol 95%	CS3I95	6 mL	1:10	0.09889M
Filtered Final Product Ethanol 75%	FFPE75	10 mL	Neat	0.10046M
Filtered Final Product Ethanol 95%	FFPE95	10 mL	Neat	0.10046M
Filtered Final Product Isopropanol 75%	FFPI75	10 mL	Neat	0.10046M
Filtered Final Product Isopropanol 95%	FFPI95	10 mL	Neat	0.10046M
Centrifuged Final Product Ethanol 75%	CFPE75	10 mL	Neat	0.10046M
Centrifuged Final Product Ethanol 95%	CFPE95	10 mL	Neat	0.10046M
Centrifuged Final Product Isopropanol 75%	CFPI75	10 mL	Neat	0.10046M
Centrifuged Final Product Isopropanol 95%	CFPI95	10 mL	Neat	0.10046M

Results were generated as PPM values for Mg^{2+}, which were corrected for any dilution factor used. This value could be converted to a molarity of Mg^{2+} ions, and therefore microgram mass of Mg^{2+} ions in each sample, as present at each stage of the extraction. These values could therefore be used to track the Mg^{2+} concentration throughout each stage of the extraction, from the inherent Mg^{2+} presence in the centrate, to the amount of Mg^{2+} ions that do not participate in precipitation (i.e. supernatant 1), to the Mg^{2+} that is effectively removed during pellet washing (i.e. supernatant 2 and 3), to the residual Mg^{2+} present in the final product.

3.12- Assessment of Sample Composition

Although the protocol for UV-Vis spectrophotometric nucleotide yield determination can be reliably used to quantitate the nucleotide concentration in a given sample, its power to determine the sample's composition is limited. To determine this, twenty-four samples were processed simultaneously and the final nucleotide pellets were fully solubilised in 1 mL of water with the aid of sonication, for 90 minutes. The resulting nucleotide solutions were poured into an evaporating dish and evaporated in a laboratory oven at 55°C and left until a dry powdered residue was collected, where 33.2 mg of powder was collected in total. The yielded powder was taken to Chemorforma Laboratories, Augst, Switzerland, along with finely ground samples of mycoscent powder and commercially purchased samples of RNA and GMP, as previously used in experimentation. The samples were analysed via a sophisticated HPLC-based method, the specifics of which have not been disclosed. The resulting data gave valuable information about the composition of the nucleotides in samples.

4- RESULTS AND DISCUSSION

4.1- RNA Calibration Curves

To permit quantification, calibration curves were produced by analysis of standard solutions of RNA in water. Several calibrations were performed (ref. Appendices 10-25), but a single curve was used in the determination of yield following all extractions. Some variation between curves was evident, but this was likely to be due to variations in weighing of RNA or pipetting for example, rather than physical conditions such as temperature or humidity. Analysing a new series of RNA standards alongside yielded solutions may be advised, but as RNA is known to solubilise slowly and RNA has been seen to precipitate out of solution after storage, this is often time-consuming and impractical. The calibration curve used in yield determination is shown in Figure 27.

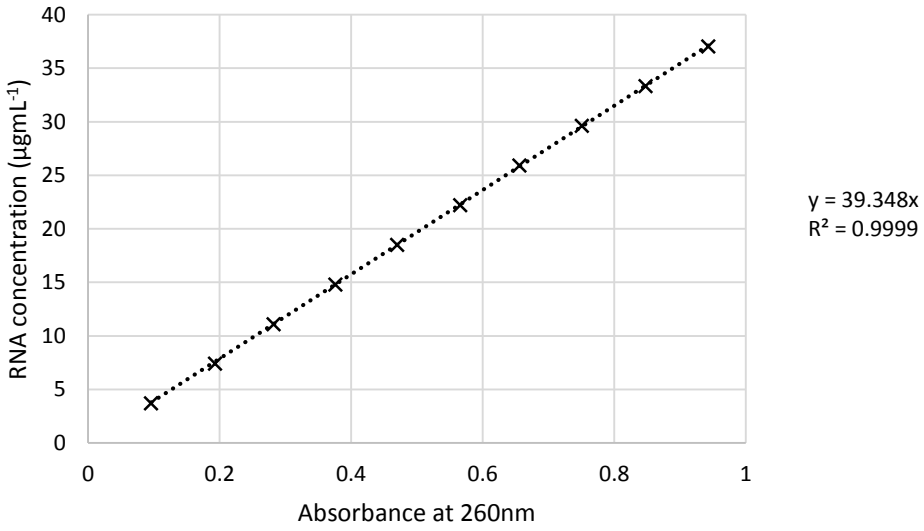


***Figure 27*- A calibration curve produced from RNA solutions in water.** RNA solutions were diluted to a range of 3.7-37.0 µgmL^{-1}, then analysed in fixed mode at 260 nm via UV-Vis spectrophotometry. Results from calibration 7 from 15/06/2015, ref. Appendix 22.

Using this calibration curve, samples of *Aspergillus* lysate and centrate were analysed for their nucleotide concentrations, to allow expression of yield as a percentage. The results from this analysis are shown in Table 11.

***Table 11*- Nucleotide concentrations of samples used for nucleotide extraction**.

Sample	Absorbance at 260 nm	Dilution Factor	Nucleotide Concentration (μgmL^{-1})	Date range used
Aspergillus lysate	0.249	160	1567.62	12/02/2015
Centrate Batch 1	0.536		3374.48	03/03/2015-13/05/2015
Centrate Batch 2 (Filtered)	0.488		3072.29	09/06/2015-31/07/2015
Centrate Batch 2 (Centrifuged)	0.438		2757.51	15/06/2015-22/07/2015

The nucleotide concentration of the filtered centrate was used in the determination of the % yield of extractions where centrifuged centrate had been used. Calibration curves were also produced from RNA solutions in different proportions of aqueous-solvent, to determine whether the solvent has any impact on the absorbance of solutions. This was done to permit accurate analysis of supernatants, which contain nucleotides in different aqueous solvents. Calibration curves for 1:2.5 ethanol, 1:2.5 isopropanol, 75% ethanol and 95% ethanol are -all shown in Figure 28.

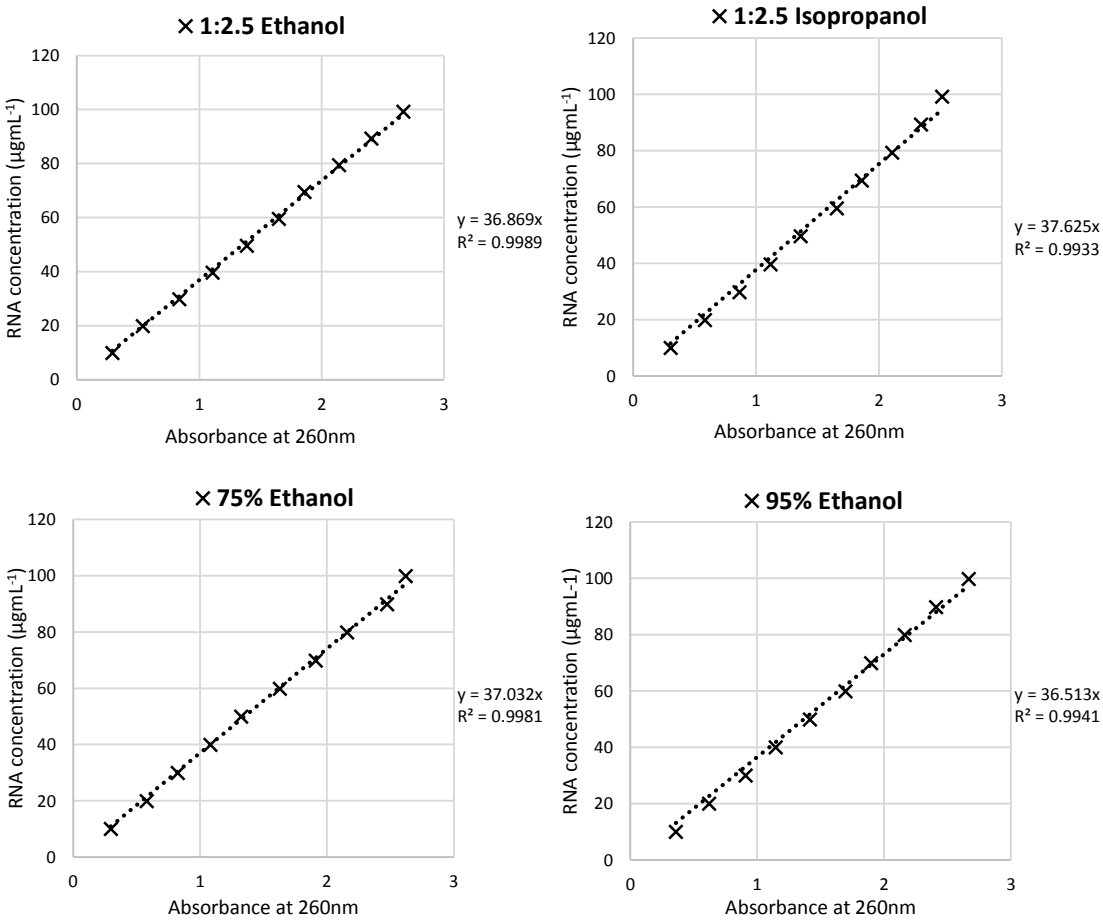


***Figure 28*- Calibration curves for RNA in different aqueous-solvents.** Results from calibrations 8-11 from 31/07/2015, ref. Appendices 25-28**.**

4.2- The Effect of Salt and Solvent Choice

A preliminary extraction with a lysate from *Aspergillus oryzae* gave low yields, when comparing salts that are commonly used in nucleic acid precipitation, in conjunction with solvent ratios of 1:2.5 ethanol or 1:1 isopropanol. Overall yields fell in the region of 3-8%. This was thought to be primarily due to the use of a 1.5 mL of 70% ethanol during the two washing steps, which resulted in overall nucleotide losses of 6-25% as a result of pellet washing (data not shown ref. *Appendix 1*). A similar trial was later conducted with filtered centrate, which was found to have a nucleotide concentration of 3374.48 μgmL^{-1}. Yields during this trial were slightly improved, falling in the range of 3-16%, but this is thought to be due to the use of 0.5 mL of 75% ethanol during washing, which resulted in a slight decrease in overall nucleotide losses to 10-22%, as a result of pellet washing. However, addition of 0.01 M magnesium chloride to conditions was found to result in a significant yield increase, as much as 15% in some cases, with some slight decreases in nucleotide losses as a result of washing, in the order of 0-5% (data not shown ref. *Appendix 2*). A trial was then conducted to compare commonly used salts, at a fixed molarity of 0.5M, used in conjunction with 1:2.5 ethanol or 1:2.5 isopropanol. Magnesium chloride was trialled as a standalone salt, and Tripotassium phosphate and potassium carbonate were also investigated as non-conventional salts.

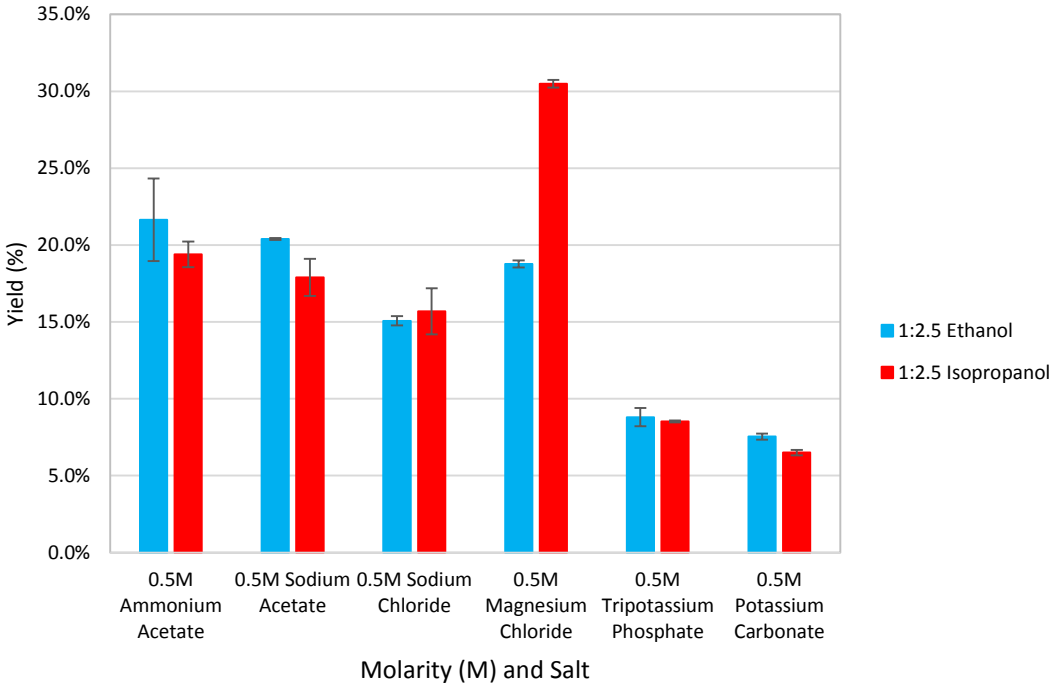


***Figure 29*- The effect of salt choice on yield during precipitation of nucleotides.** Filtered centrate was adjusted to the indicated salt molarity with powdered salts, followed by a solvent at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extraction from 11/03/2015, ref. Appendix 3.

As shown in Figure 29, conventional salts ammonium acetate, sodium acetate and sodium chloride were shown to give respectable yields of 15-22%, when used at a molarity 0.5M. Non-conventional salts were shown to give poor yields of 7-9%. Both tripotassium phosphate and potassium carbonate are insoluble in ethanol and isopropanol, so precipitated upon solvent addition, and therefore failed to aid in the precipitation of nucleotides. Magnesium chloride showed the best yield when used in conjunction with isopropanol, giving an average yield of 31%. Additionally, this condition gave the highest 260:280 ratio, an average of 1.56, which relates to a nucleotide purity of 18.5%. In general, ethanol was seen to result in higher yields than isopropanol, except in the case of the two chloride salts. When magnesium chloride was used at a lower molarity of 0.2 M the average yield achieved was 33%, when used in conjunction with 1:2.5 isopropanol, where the nucleotide purity was 35%, as indicated by the 260:280 ratio of 1.78 (data not shown ref. *Appendix 4*). To quantify the effect of salt concentration, extractions were conducted with varying molarities of magnesium chloride. Magnesium acetate was also tested for its effectiveness as it was thought

the acetate anion may improve precipitation. Sodium acetate had also been previously shown to increase the pH of the centrate, which was due to the buffering capabilities of the acetate anion, which may be replicated with the use of magnesium acetate. The pH of the centrate was measured following salt addition to identify any relationship between centrate pH and yield. The results of this experimentation are shown in Figure 30. Overall, magnesium acetate was shown to be 10% more effective in precipitating nucleotides, on average. An average yield of 49% was achieved with 0.05 M magnesium acetate, while the average purity of the product was shown to be 40%, as indicated by the 260:280 ratio of 1.81.

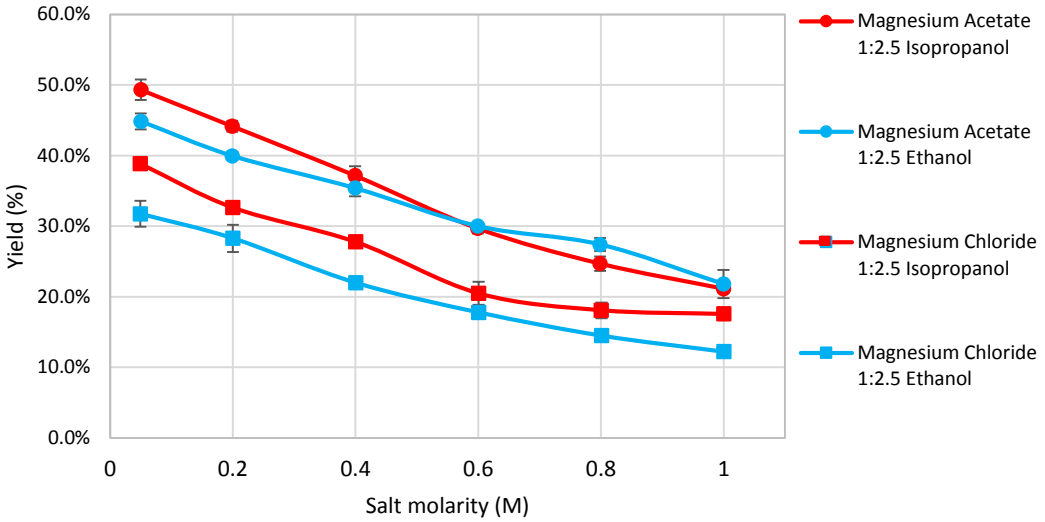


***Figure 30*- The effect of salt molarity on yield during precipitation of nucleotides.** Filtered centrate was adjusted to the indicated salt molarity with powdered salts, before a solvent was then added at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extractions 07/05/2015, ref. Appendix 7 and 13/05/2015, ref. Appendix 9.

Here, isopropanol was seen to be generally more effective in increasing nucleotide yield, compared to ethanol. When the magnesium chloride concentration was increased in the centrate the pH measured in the centrate was shown to decrease, as shown in Figure 31. This trend was reversed in the case of magnesium acetate as expected, although pH shifts weren't as significant.

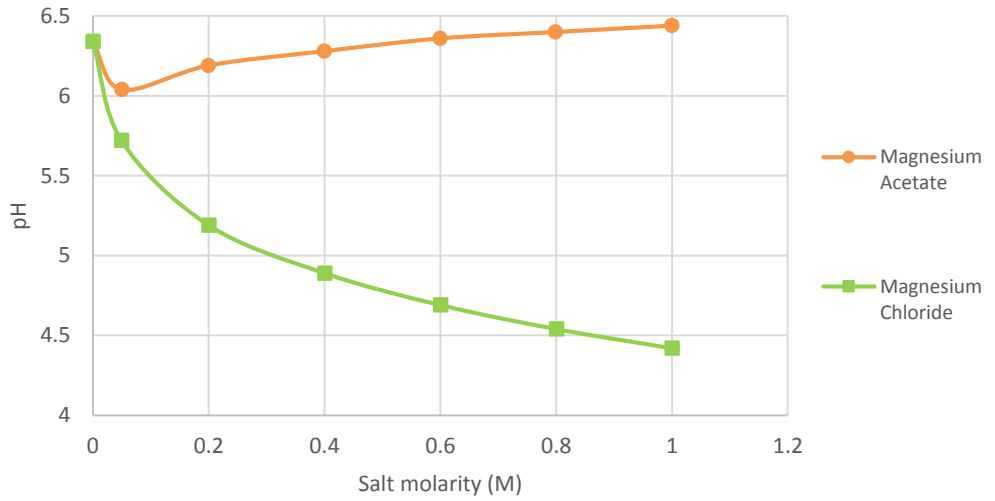


***Figure 31*- The pH of centrate samples, following addition of various molarities of salt.** After salts had been added to filtered centrate to a given molarity, the pH of the sample was taken. Samples were hence used in extractions 07/05/2015, ref. Appendix 7 and 13/05/2015, ref. Appendix 9.

From 09/06/2015 a new batch of centrate was used for extractions, which was found to have a lower nucleotide concentration, at 3072.29.00 μgmL^{-1}, but this was accounted for in yield determination. To pinpoint the optimum molarity of magnesium acetate, two trials were carried out to test a narrow range of molarities, the results of which are presented in Figure 32.

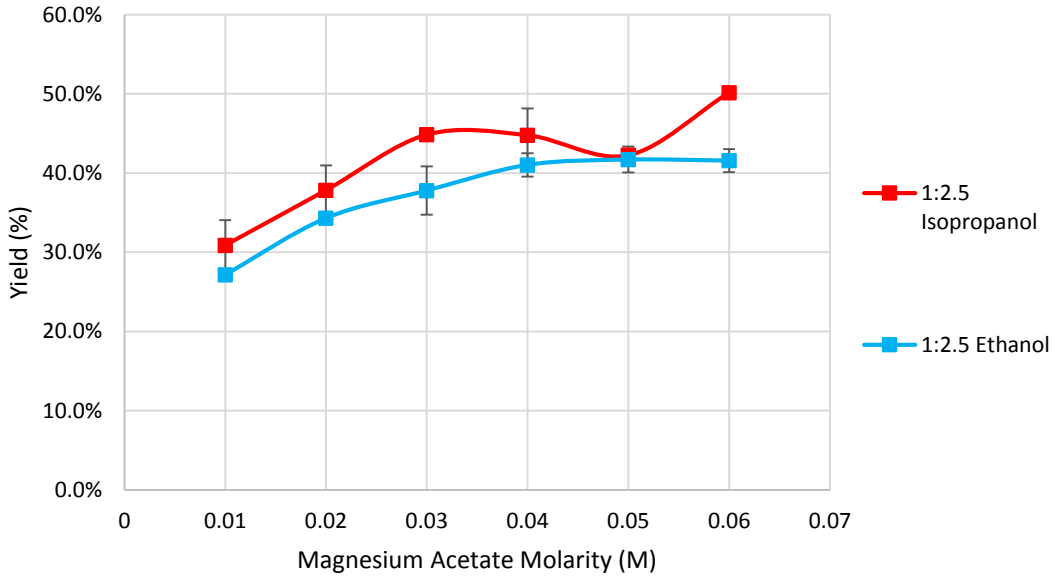


***Figure 32*- The effect of magnesium acetate molarity on yield during precipitation of nucleotides.** Filtered centrate was adjusted to the indicated salt molarity with powdered salts, followed by a solvent at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extractions 09/06/2015, ref. Appendix 10.

The results of these extractions showed some inconsistent trends. However, at a magnesium acetate concentration of 0.06 M, the highest average yield of the experimental period was achieved, at 50%, with a product purity of 32%, as indicated by the 260:280 ratio at 1.75. 0.06 M magnesium acetate was hence used during all subsequent extractions. Isopropanol was again seen to result in higher yields than ethanol, but experimentation with ethanol continued throughout experimentation.

Ammonium acetate, sodium acetate and sodium chloride are all commonly used in routine precipitations of nucleic acids. These salts all give rise to monovalent cations, which are able to associate with the exposed phosphodiester backbone of nucleic strands to aid in depletion of solvation shells, and hence precipitation, upon addition of solvent. However, in monomeric nucleotide monophosphates, the presence of an exposed oxygen atom in the exposed phosphate confers a greater negative charge to the molecule. Consequently, a divalent cation may attract a mononucleotide more effectively, resulting in a greater Coulomb force of attraction between the ion pairs, following solvent addition. In this instance, the magnesium salts consistently resulted in higher yields of nucleotides when directly compared to commonly used monovalent salts.

The Mg^{2+} ion is placed higher in the Hofmeister series (Hofmeister, 1888) monovalent cations, which classifies ions based on their ability to salt out proteins. Although the mechanism of protein salting out is distinct from nucleic acid precipitation, the classification of ions set out in the Hofmeister series may give an indication of ions which may co-precipitate proteins at higher levels in nucleic acid precipitation. Despite this, as well as giving high yields, magnesium salts also produce nucleotide solutions that give higher 260:280 ratios than products from extractions with monovalent salts, indicating purities of up to 40%, compared to 7-18% in the case of monovalent salts.

Magnesium acetate was found to increase nucleotide yield by an average of 10%, when compared to magnesium chloride. Similarly, sodium acetate was found to result in an average 4% yield increase, compared to sodium chloride. This is likely to be due to the effect of the acetate ion in

solution, as it sequesters free water molecules more effectively than the small chloride ion, as it solvated. In effect, this augments the depletion of solvation shells around the nucleotides, therefore increasing the yield of nucleotides. As one mole of magnesium acetate dissociates to give two moles of acetate ions, this may explain why the yield increase is greater in the case of magnesium salts, compared to sodium salts, which dissociate to give only one acetate ion.

In later trials with magnesium salts, isopropanol was shown to give an average of 4.3% higher yields when directly compared to ethanol. This is simply due to the higher dielectric constant of isopropanol as a solvent, which results in enhanced precipitation of nucleotides. This trend wasn't obvious in early trials, probably due to low yields masking these solvent trends.

One consistent trend which is not easily explained is that of the decreasing yields at higher magnesium salt concentrations. It is true that the pH is directly affected by salt concentration, but pH trends were not shown to correlate with yield trends and was hence not thought to have significantly influenced yield. In any case, the direct implication of these findings is that a much lower amount of magnesium acetate is necessary to achieve good yields, which bodes well for the process operating cost-effectively at industrial scales.

4.3- The Effect of pH

The effect of pH on yield was initially quantified by buffering the centrate with sodium acetate and acetic acid. After some initial testing, the centrate was buffered to pH 4.13, 5.15 and 5.78, and each buffered batch was used for extraction, alongside an un-buffered control batch. During this testing, ammonium acetate, sodium acetate, sodium chloride and magnesium chloride were all used, to determine whether pH was the primary reason why monovalent salts weren't capable of achieving yields in the range of magnesium chloride. Two trials were carried out and the results in which the centrate was buffered to three pH levels with highly-saturated sodium acetate and acetic acid, prior to salt addition, as per Table 12.

***Table 12*- Molarities of sodium acetate and acetic acid in the centrate to achieve the observed pH levels.**

pH achieved	Final molarity of sodium acetate in centrate	Final molarity of acetic acid in centrate
4.13	234 µmol	871 µmol
5.15	586 µmol	464 µmol
5.78	913 µmol	108 µmol

The results of these extractions are presented in Figure 33.

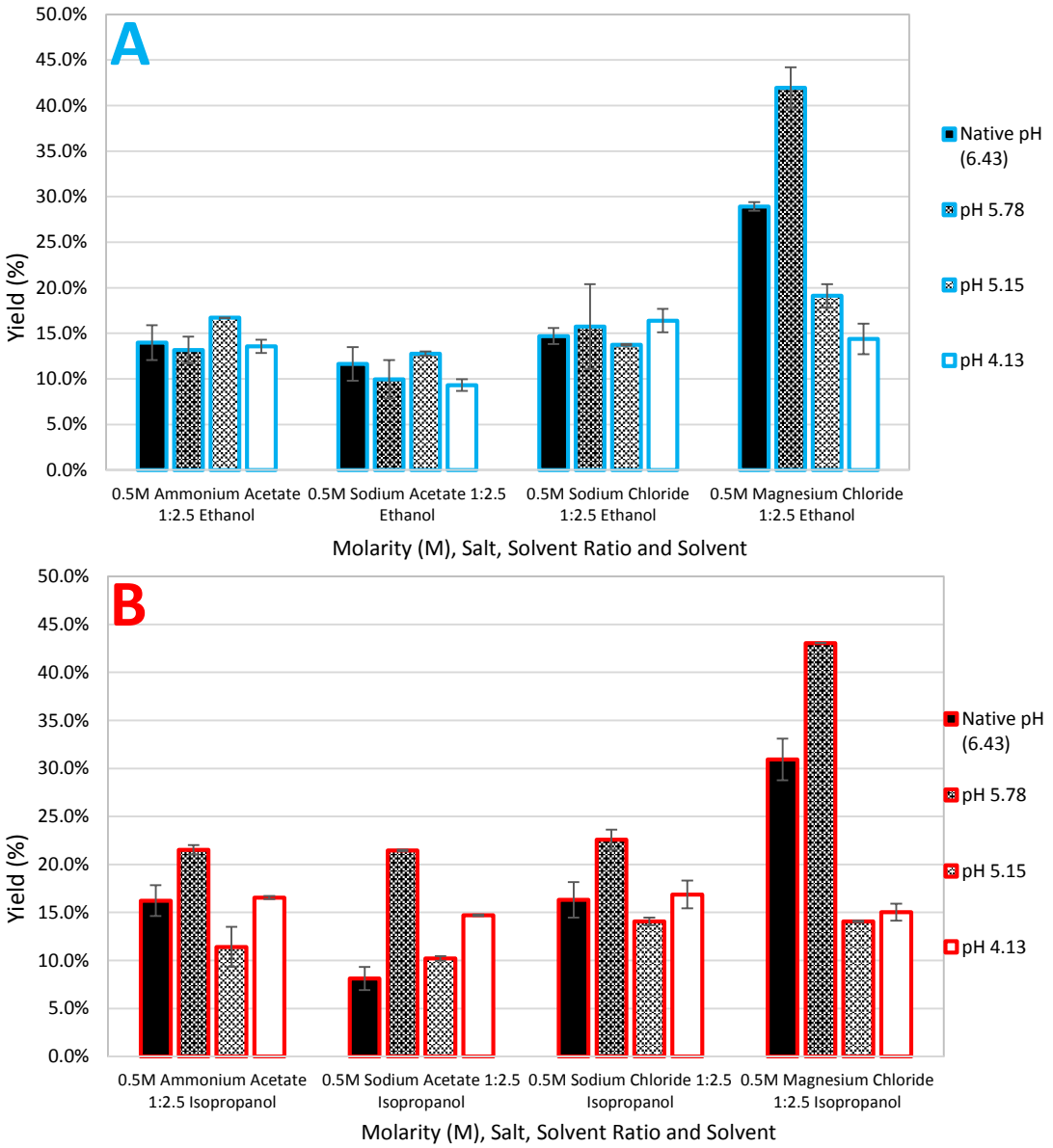


***Figure 33*- The effect of pH on yield during precipitation of nucleotides.** (A) 1:2.5 Ethanol and (B) 1:2.5 Isopropanol. Filtered centrate was adjusted to the indicated pH with highly-saturated solutions of sodium acetate and acetic acid. Powdered salts were then added to the indicated molarity, followed by a solvent at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extractions 30/04/2015, ref. Appendix 5.

Results from pH trials were somewhat inconsistent and few definite conclusions could be drawn from the resulting data. When isopropanol was used, in all cases, yield was greater at pH 5.78 than at the native pH, however, this trend was not always observed when ethanol was used as a solvent. An identical extraction was performed later, which showed similarly inconsistent trends (data not shown, ref. Appendix 6). Inconsistencies may simply be down to a practical error during extraction, however, as sodium acetate is an effective precipitating salt, having differing, albeit low, molarities present in conditions may disguise the direct effect of pH changes on nucleotide yield. Similarly, as the acetate ion can sequester free water molecules in solution, different molarities of acetic acid, which associates to give acetate ions, may lead to inconsistent trends in yield in buffered centrate samples. The molarities of both buffering components are relatively low, but to investigate the effect of pH on yield more directly, the pH was manipulated with powdered sodium hydroxide or concentrated hydrochloric acid. This method would not sustain a specific pH level, but pH differences in samples would remain consistently separate throughout the extraction. pH levels of 5.47 and 6.01 were achieved with the addition of hydrochloric acid, while pH levels of 7 and 7.54 were achieved by adding powdered sodium hydroxide. Again, batches of un-buffered centrate, measured at pH 6.32, were also used alongside buffered batches. These trials were carried out with 0.06 M magnesium acetate, all other salts were excluded. Results from these trials are shown in Figure 34.

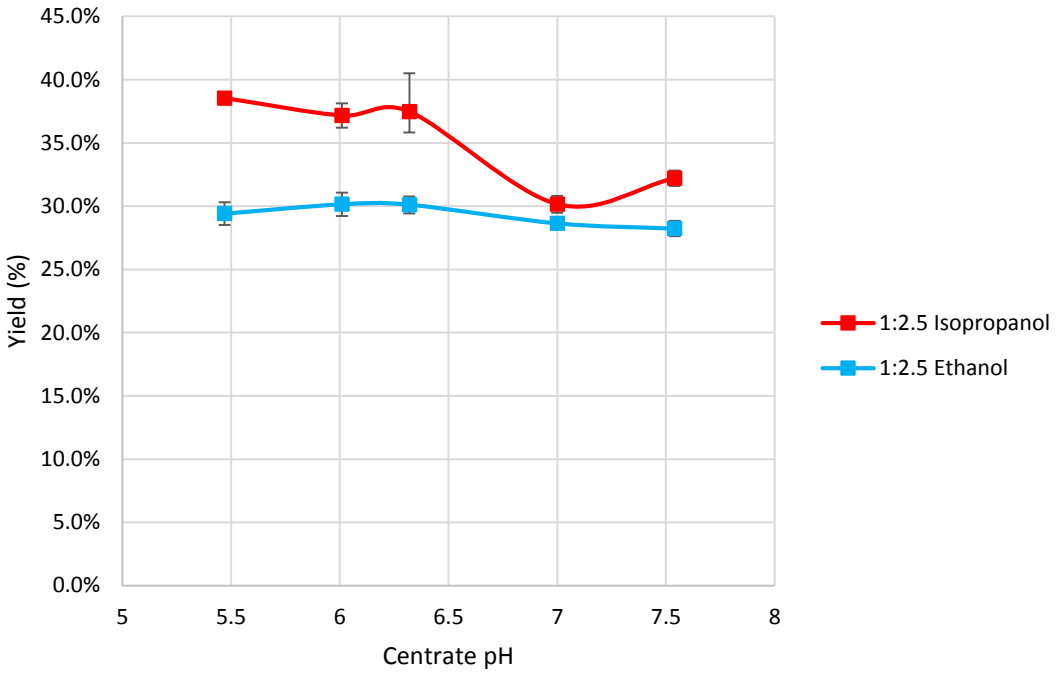


***Figure 34*- The effect of pH on yield during precipitation of nucleotides (2).** Filtered centrate was adjusted to the indicated pH with small amounts of powdered sodium hydroxide or concentrated hydrochloric acid. Powdered magnesium acetate was then added to a molarity of 0.06M, followed by a solvent at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extractions 02/07/2015 (alkalised centrate), ref. Appendix 12 and 07/07/2015 (acidified centrate) ref. Appendix 13.

Results from these trials were more conclusive, it appeared deviations from the native centrate pH resulted in minor decreased in yield. No increase in purity, as indicated by the 260:280 ratio of products, was observed at non-native pH levels. It was originally suspected that a basic pH may preserve the single-bonded oxygen atoms at the phosphate groups of the nucleotides, which would otherwise be protonated in an acidic environment. The presence of negatively charged oxygen atoms would therefore provide a free site for cations to associate, with the overall effect of increasing yield. However, this seems to not be the case. This has positive implications for the process operating industrially, however, as good yields can be achieved without the need for buffering reagents, which would inevitably result in an overall greater cost of the process.

4.4- The Effect of Solvent Ratio

A single trial was conducted to examine the exact relationship between solvent ratio and yield, where 0.05 M magnesium chloride was used in all conditions. Both ethanol and isopropanol were tested. The results of this trial are presented in Figure 35.

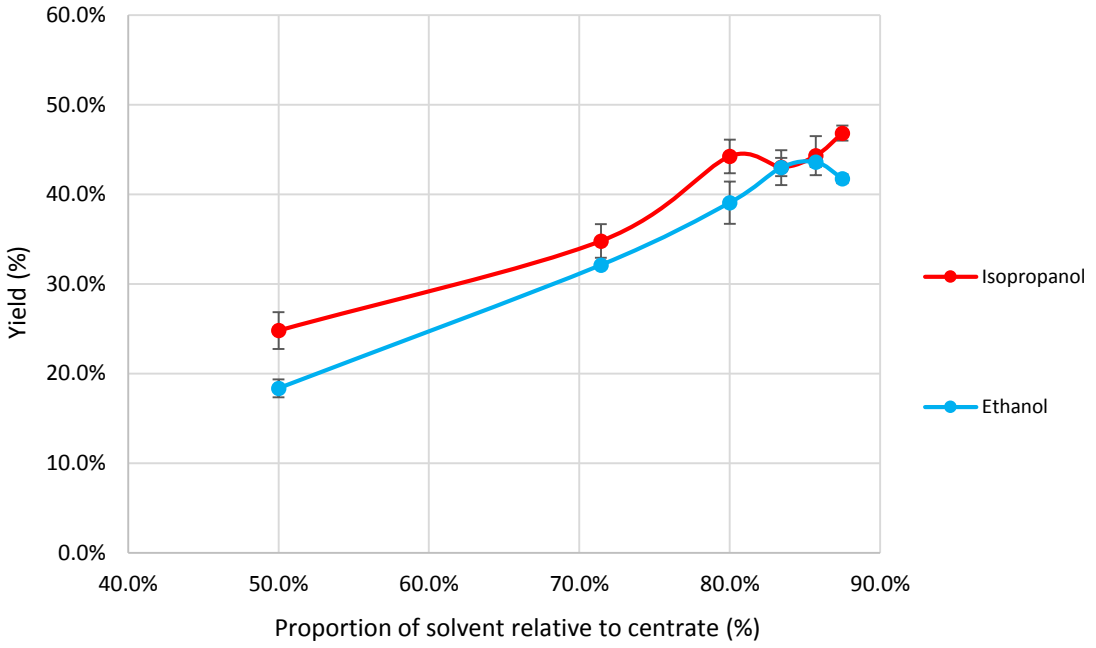


***Figure 35*- The effect of solvent ratio on yield during precipitation of nucleotides.** Powdered magnesium chloride was added to filtered centrate a molarity of 0.05M, followed by a solvent at the ratio indicated. All samples had a final volume of 1.75 mL prior to incubation. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extraction 13/05/2015, ref. Appendix 8.

Results showed that, generally, yield was higher when the ratio of solvent was higher. Again, isopropanol was shown to be a more effective solvent in terms of yield. Some deviations from trends were observed at higher solvent ratios, but this is simply thought to be a result of the salt choice, which may have limited further increases in yield. Solvent ratio was also shown to directly impact product purity, as shown in Figure 36.

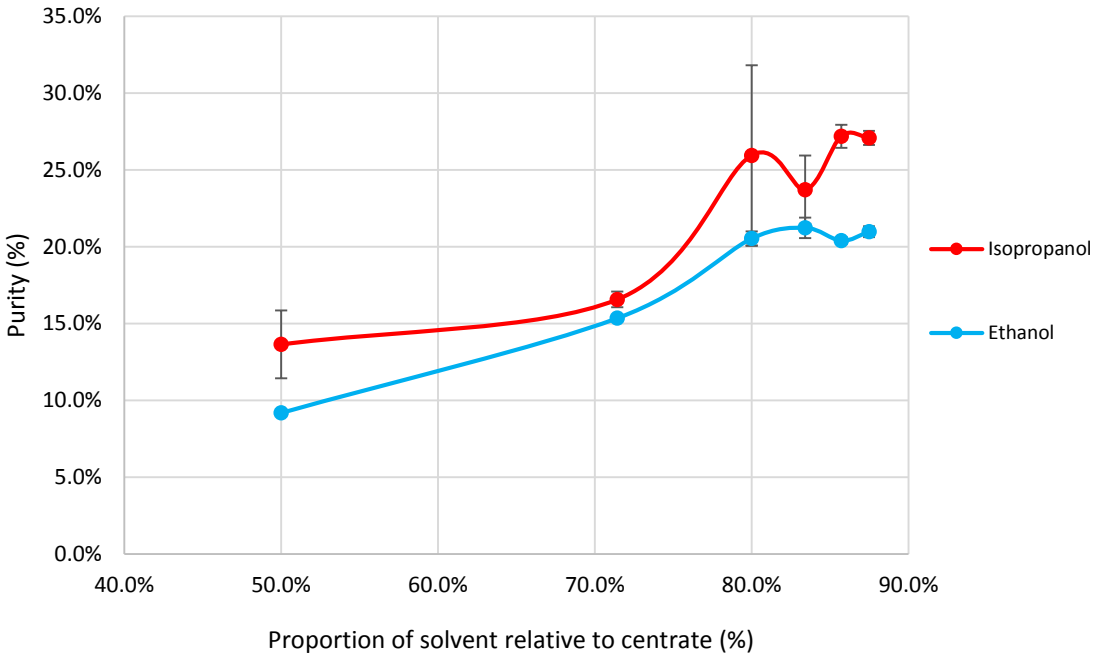


***Figure 36*- The effect of solvent ratio on product purity during precipitation of nucleotides.** Products from extraction 13/05/2015 were analysed via UV-Vis spectrophotometry and their absorbance at 260 nm and 280 nm were recorded. The ratio of absorbance values (260:280) was used to determine the nucleotide purity, as per Glasel, 1995. Results of extraction 13/05/2015, ref. Appendix 8.

It is evident that, as well as increasing nucleotide yield, a higher solvent ratio also generally results in a greater product purity. Trends here are consistent with theory stated in literature. The dielectric constants were approximated for each solvent-proportion used in testing, from values stated in Akerlof, 1932. These values were plotted against yield for each condition, as presented in Figure 37.

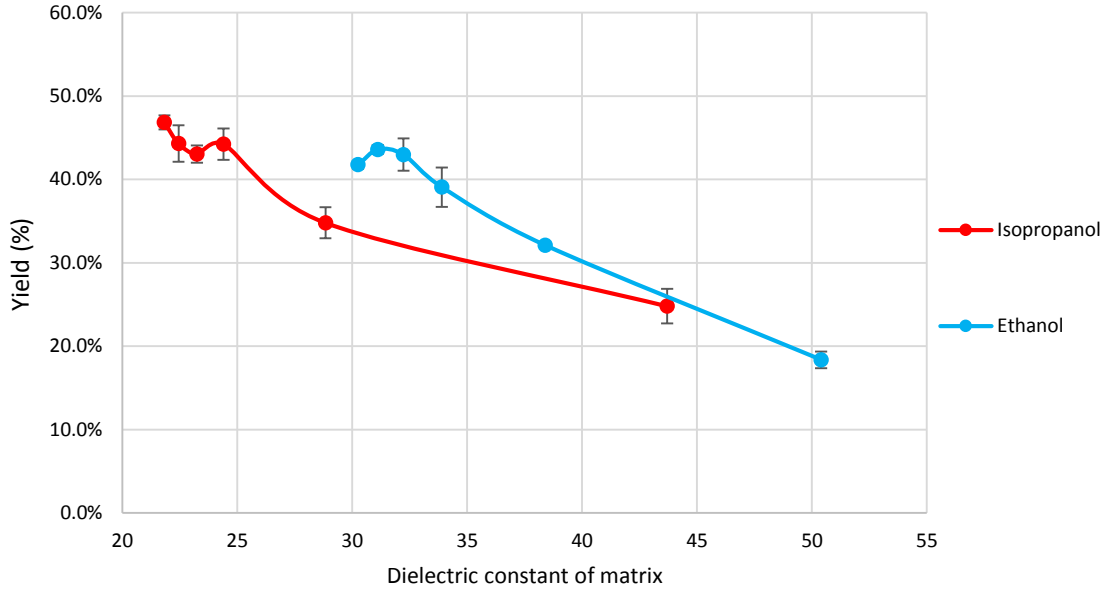
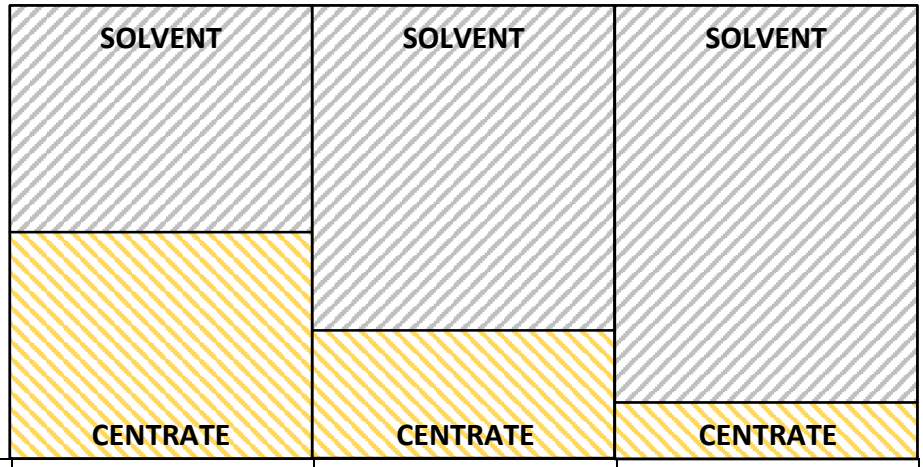


***Figure 37*- The effect of dielectric constant of an aqueous-solvent mixture on yield during precipitation of nucleotides.** Dielectric constants of solvent ratio conditions were calculated from Akerlof, 1932 and plotted against yield data from extraction 13/05/2015, ref. Appendix 8.

As expected, at higher lower dielectric constants, yield is increased. It may be expected that overlap would occur between solvents, where the same dielectric constant is achieved. This is not the case, particularly at lower dielectric constant values. However, to achieve a given dielectric constant, more ethanol is required than isopropanol. For this reason, a sample with a higher proportion of solvent will inevitably be less dense, allowing nucleotides to pellet more effectively during centrifugation, which may partially explain why yield appears higher at a given dielectric constant with ethanol, than isopropanol.

Although results with regard to yield and purity both indicate a higher solvent ratio would be preferable, there are other key considerations which may strongly influence the solvent ratio used. Above all, a higher solvent ratio results in a much lower throughput of the process, and obviously result in a greater solvent consumption. Although the yield per volume of centrate used is greater at higher proportions of solvent, the yield for a given vessel size is often lower at higher proportions of solvent, as shown in Figure 38, where a hypothetical vessel size of 1 litre has been used for demonstration.

Extraction vessel size of 1 litre



Solvent Ratio	1:1	1:2.5	1:6.99
Centrate used	500 mL	286 mL	125 mL
Solvent used	500 mL	714 mL	875 mL
Indicated % yield- Ethanol	18%	32%	42%
Indicated % yield- Isopropanol	25%	35%	47%
Indicated mg nucleotides yielded- Ethanol	310 mg	310 mg	176 mg
Indicated mg nucleotides yielded- Isopropanol	419 mg	336 mg	198 mg

***Figure 38*- A figure to demonstrate the trends in nucleotide production at different proportions of solvent.** Values determined from extraction 13/05/2015, ref. Appendix 8.

Although it wasn't tested, it is likely that a higher solvent ratio will also result in a greater co-precipitation of salts, as they become less soluble in more concentrated solvent solutions. Higher solvent ratios have been shown to result in less co-extraction of proteins, as indicated by trends in the 260:280 ratio. But increased salt contamination may necessitate more extensive pellet washing, which would not only influence yield, but would also lengthen the overall process and/or lead to a great overall cost. With this in mind, a choice must be made which provides an appropriate compromise of these factors, when considering the process industrially.

4.5- Comparison of Centrate Pre-treatment Methods

Throughout early experimentation, all centrate samples were vacuum filtered via a 25 µm cellulose filter before extraction. The aim of this was to remove insoluble matter, such as cell debris, that would otherwise pellet with target nucleotides upon centrifugation. However, relatively poor purities of yielded nucleotide solutions were thought to be as a result of co-precipitation of insoluble proteins and nucleoproteins, which evade filtration. Batches of centrate were centrifuged in 2 mL aliquots at 16,163 RCF and stored at -20°C until required. Analysis of the centrifuged centrate showed a nucleotide concentration of 2757.51 μgmL^{-1}, around 10% lower than the equivalent filtered centrate. The results of a trial, which was conducted with 0.06 M magnesium acetate to directly determine the difference in yield between the two batches, are presented in Figure 39.

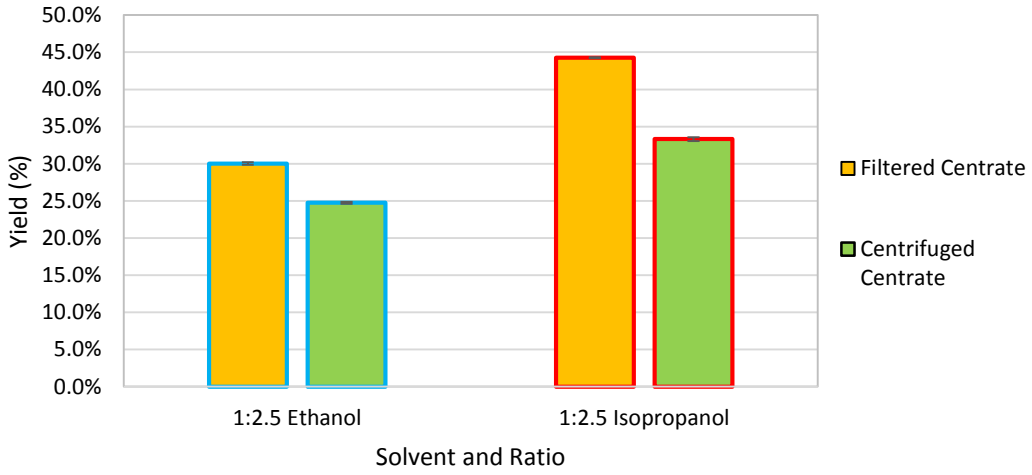


***Figure 39*- The effect of centrate pre-treatment on yield during precipitation of nucleotides.** Powdered magnesium acetate was added to filtered or centrifuged centrate a molarity of 0.06M, followed by a solvent at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extraction 22/07/2015, ref. Appendix 14.

Yields were understandably lower with the use of centrifuged centrate, compared to the equivalent filtered centrate. This difference was lower with the use of ethanol, but more significant at 11% when isopropanol was used. Nevertheless, the aim of centrifugation is to remove

contaminating substances which may ultimately impact the purity of the yielded nucleotide product. The purities of products are shown, as determined by the 260:280 ratio, in Figure 40.

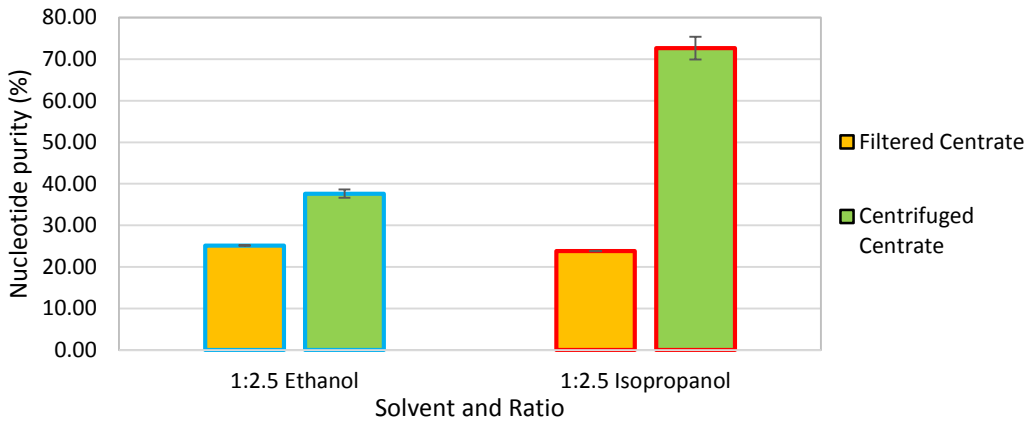


***Figure 40*- The effect of centrate pre-treatment on product purity during precipitation of nucleotides.** Products from extraction 22/07/2015 were analysed via UV-Vis spectrophotometry and their absorbance at 260 nm and 280 nm were recorded. The ratio of absorbance values (260:280) was used to determine the nucleotide purity, as per Glasel, 1995. Results of extraction 22/07/2015, ref. Appendix 14.

Perhaps unsurprisingly, after centrifugation, final product purity is significantly higher than after crude filtration. The significance of this difference is remarkable, particularly with isopropanol, which results in a product purity of 73% with centrifuged centrate, nearly 50% higher than with use of filtered centrate. The results indicated that in the filtered centrate, many of the nucleotides may associate with other biomolecules, which can be pelleted with centrifugation. After centrifugation, overall yield is decreased as complexes of nucleotide with other biomolecules aren't extracted, but absence of these contaminating substances in the final product therefore results in a higher product purity. To validate the purities indicated by the 260:280 ratios, the bicinchoninic acid (BCA) assay was carried out to test the amount of protein present in yielded protein products, the results of which are summarised in Figure 41.

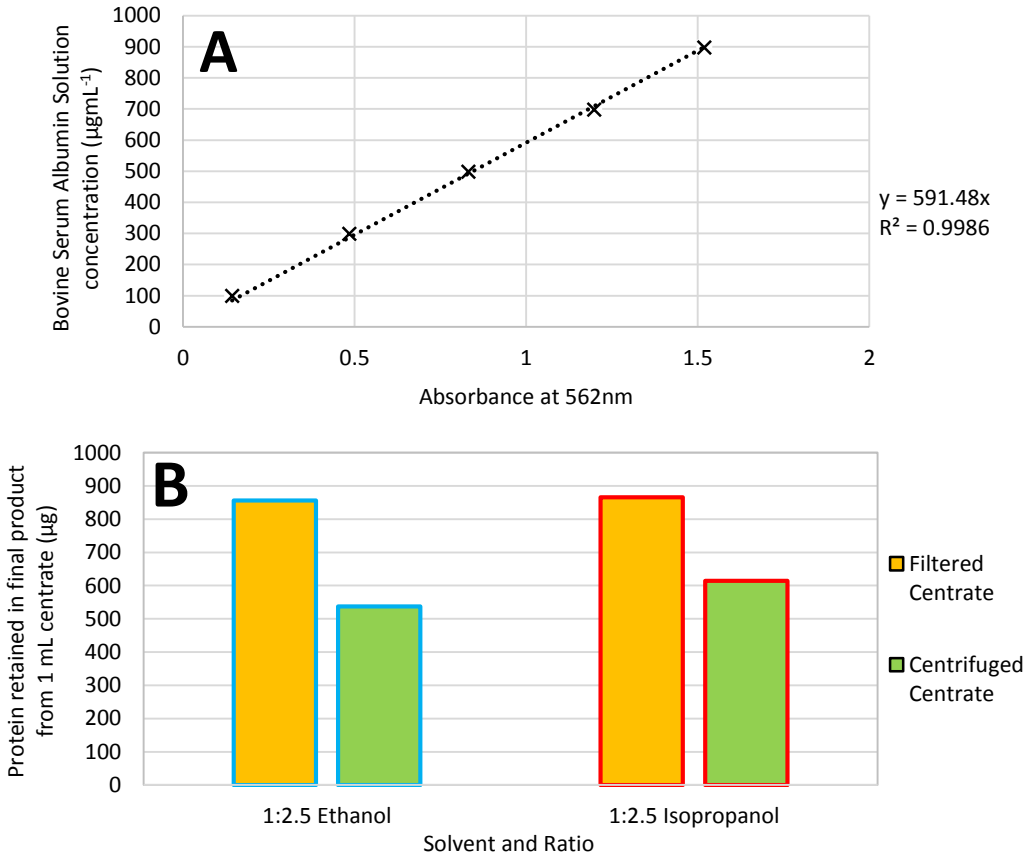


***Figure 41*- The effect of centrate pre-treatment on protein retention during precipitation of nucleotides.** Products from extraction 22/07/2015 tested with the BCA assay. Standard solutions of bovine serum albumin were prepared and ran alongside yielded nucleotides to generate a calibration curve (A), which was hence used to determine the protein concentration in yielded nucleotides (B). Results of BCA extraction 08/07/2015, ref. Appendix 27.

Results from BCA assays showed very similar trends to those demonstrated by the 260:280 ratios. Nucleotide yields and protein retention data can be compared here to determine an *expected* 260:280 ratio, which were found to match astonishingly closely to the actual 260:280 ratios observed during UV-Vis analysis (ref. Appendix 27). What seems apparent however, is that solvent choice does not have a significant influence on the retention of proteins, as similar levels were retained with the use of ethanol or isopropanol. With this in mind, it seems isopropanol is a better solvent choice, as it appears that use of ethanol a lower yield, but the same level of protein retention.

4.6- Comparison of Ethanol Concentration in Pellet Washing

In initial trials with the *A. oryzae* lysate, pellets were washed twice by dispersing them in 1.5 mL of 70% ethanol, which was shown to result in significant nucleotide losses, as nucleotides solubilised in the water component of the washing solution so were removed following centrifugation. Hence, for most early extractions, pellets were washed in 0.5 mL of 75% ethanol, but 95% ethanol was used in some trials, and the resulting effect on yield has been shown in Figure 42.

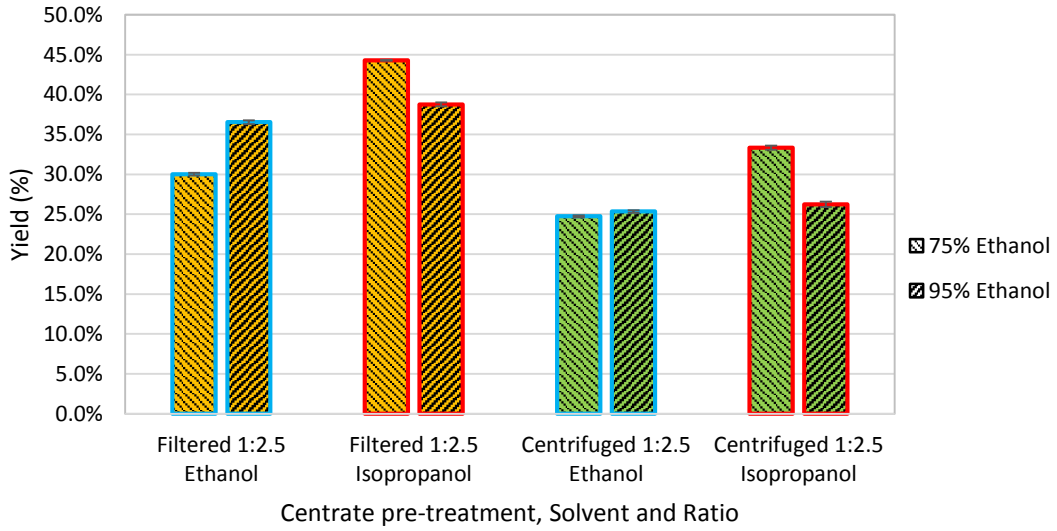


***Figure 42*- The effect of ethanol concentration during pellet washing on yield during precipitation of nucleotides.** Powdered magnesium acetate was added to filtered or centrifuged centrate a molarity of 0.06M, followed by a solvent at the ratio indicated. After 12 hours of incubation at -20°C, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% or 95% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% or 95% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extraction 22/07/2015, ref. Appendix 14.

A higher solvent proportion should, in theory, lead to a higher yield, as fewer nucleotides can dissolve in the water component of the aqueous solvent. This was not always observed in this case, as a 95% ethanol wash either resulted in a negligible increase in yield, or a decrease in yield. Of course, the aim of pellet washing is to remove residual salts from pelleted nucleotides, so results from ICP-OES analysis of centrate, supernatants and products are summarised in Figure 43.

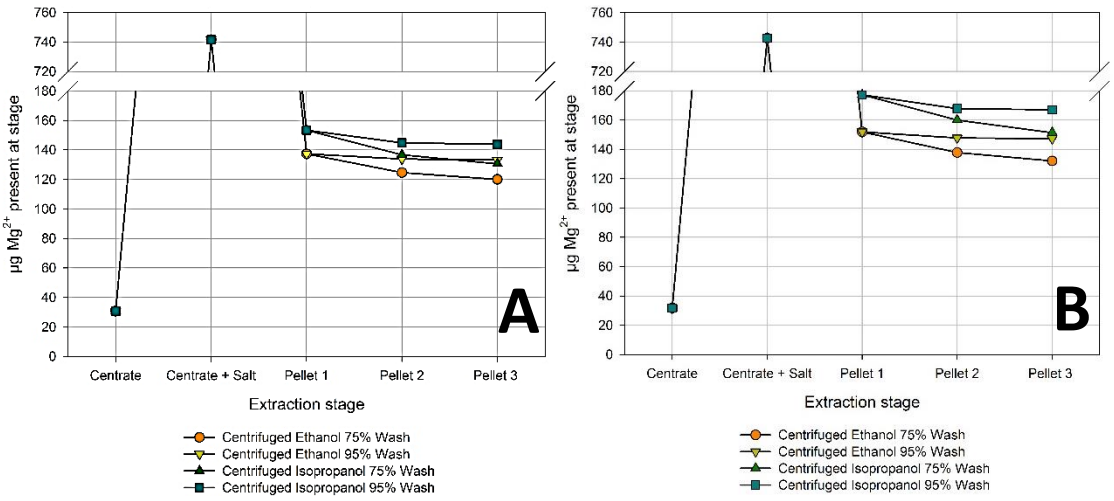


***Figure 43*- Results calculated from ICP-OES data from analysis of extraction solutions.** Centrifuged (A) and filtered (B) centrate samples were analysed directly by ICP-OES. Solvents from supernatants from extraction 22/07/2015 were evaporated and reconstitued with ultrapure water, before analysis. PPM values were used to determine the microgram amount of magnesium present at each stage of the extraction, taking into account the amount of magnesium acetate added prior to incubation. Results of ICP-OES testing 30/07/2015, ref. Appendix 29.

Both the centrifuged and filtered centrate were found to have 62 µg of Mg^{2+} present per mL, with the centrifuged having marginally less. Following addition of magnesium acetate, around 740 µg of Mg^{2+} is present in each 0.5 mL sample of centrate. Interestingly, around 70-80% of salt is removed following the initial precipitation and centrifugation into supernatant 1, leaving 20-30% of the overall Mg^{2+} content present in pellet 1. Isopropanol was seen to co-precipitate around 10-15% more Mg^{2+}, compared to ethanol.

Of the remaining Mg^{2+} in pellet 1, around 9-11% of Mg^{2+} is removed following an intial wash with 75% ethanol, but only around 2-6% is removed following washing with 95% ethanol. Similarly, of the Mg^{2+} present in pellet 2, around 3-5% is removed following a second wash with 75% ethanol, but less than 1% is removed following a second wash with 95% ethanol. During washing, a higher proportion of Mg^{2+} is removed from conditions where isopropanol was used, but this likely due to the fact that more Mg^{2+} is present in pellets from isopropanol conditions following precipitation and centrifugation. Overall, samples from centrifuged centrate were seen to have a lower amount of Mg^{2+} present, possibly because there are fewer additional biomolecules present which may associate with the magnesium cations during the initial precipitaion.

It is important to account for the yield when considering co-precipitation of salts, so the amount of Mg^{2+} per µg of yielded nucleotides was calculated, the results of which are shown in Figure 44.

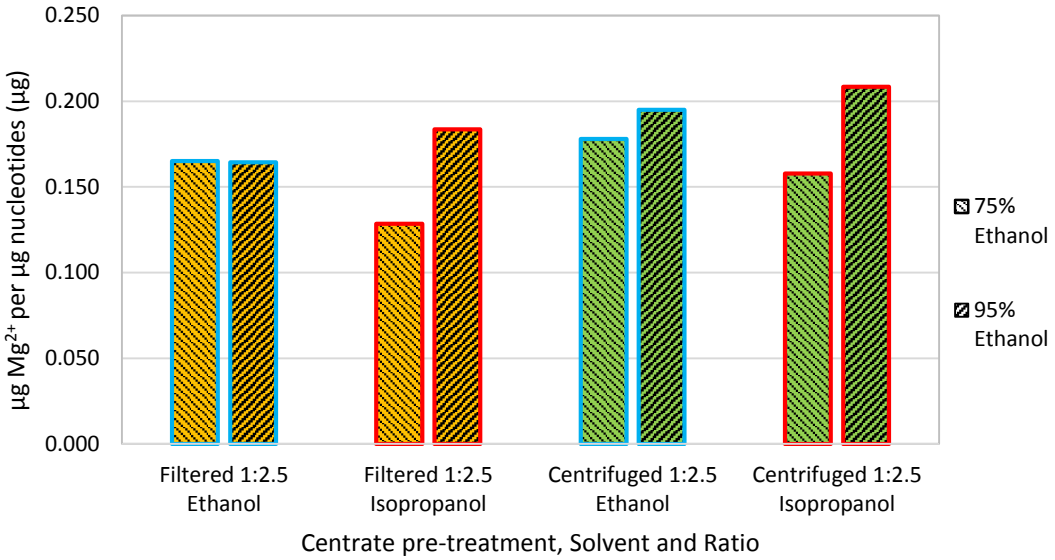


***Figure 44*- The effect of centrate pre-treatment, solvent choice and washing solution on contamination of nucleotides with Mg^{2+}.** Yield data from 22/07/2015 was combined with ICP-OES data from 30/07/2015 to determine the amount of Mg^{2+} present, per µg of yielded nucleotides. Results of extraction 22/07/2015, ref. Appendix 14 and ICP-OES testing 30/07/2015, ref. Appendix 29.

Generally, 95% ethanol washing results in a greater contamination of Mg^{2+}, compared to 75% ethanol washing. This is thought to be due to the higher solubility of Mg^{2+} in water than ethanol, so a greater aqueous component of the employed washing solution results in more effective Mg^{2+} removal. Additionally, based on physical observations, it appears a greater aqueous component of the washing solution results in better disruption of the pellet during washing, so the interior of the pellet is effectively rinsed of Mg^{2+}.

Overall, these results are promising, as a lower ethanol proportion in salt washing steps is overall less costly. Additionally, as so little Mg^{2+} is retained into the yielded nucleotides, it seems a single washing step will be adequate to remove enough Mg^{2+} during extraction. This is a significant improvement on conventional laboratory-based protocols for nucleic acid precipitations, as it simplifies and abridges the process, resulting in a greater throughput and a lower overall process cost.

4.7- The Effect of Incubation Temperature and Addition of Carriers

Carrier substances yeast tRNA and glycogen were both tested for their influence on nucleotide yield in extraction conditions at different temperatures for 12 hours, alongside control trials with no carriers present. The results of this trial are shown in Figure 45.

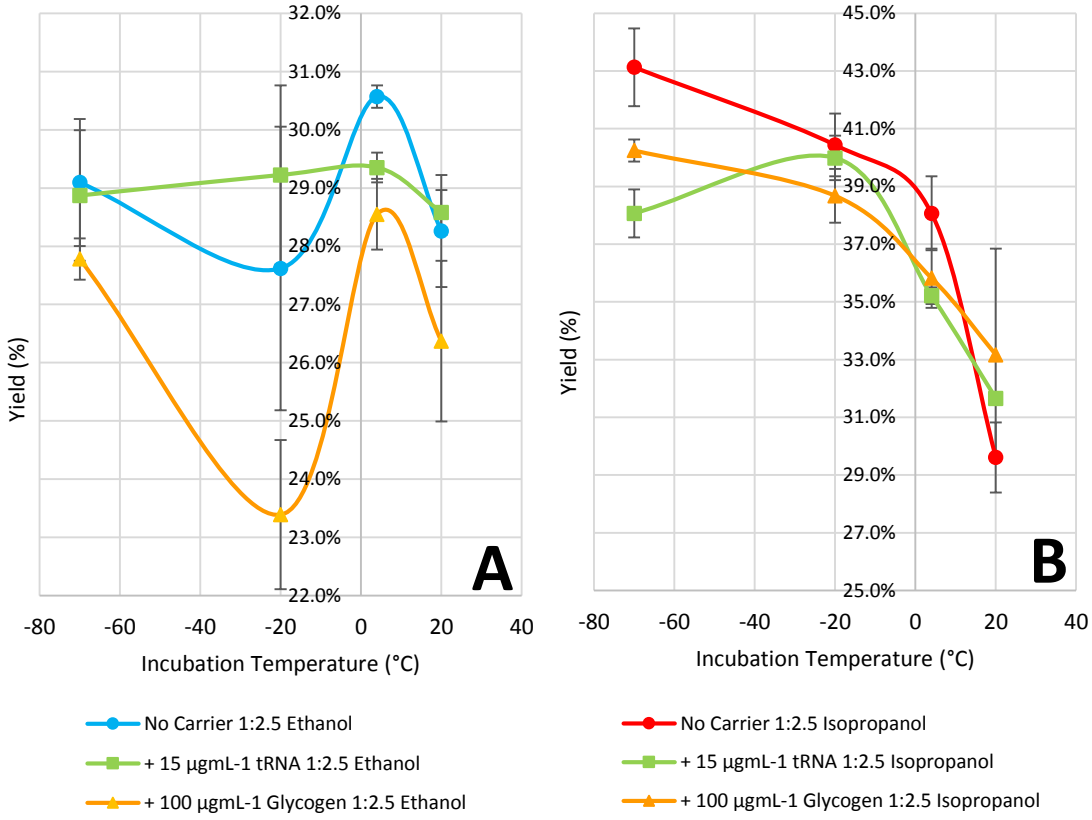


***Figure 45*- The effect of incubation temperature and carrier addition on yield during precipitation of nucleotides.** Powdered magnesium acetate was added to filtered centrate a molarity of 0.06M, followed by carrier solutions to achieve the amount indicated, at a final volume of 0.5 mL. Ethanol (A) or Isopropanol (B) was then added at the ratio indicated. After 12 hours of incubation at the temperature indicated, the samples were centrifuged at 16,163 RCF for 30 minutes, before pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75% ethanol. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 2 mL of ultrapure water, diluted appropriately and analysed via UV-Vis spectrophotometry. Results of extraction 31/07/2015, ref. Appendix 15.

From this data, it seems that carrier substances as used here, are generally ineffective at increasing nucleotide yield. Trends of incubation temperature are inconsistent between solvents, where the trends observed with isopropanol and no carrier, which have a narrow margin of error, seem to correspond best with theoretical understanding. Although some literature states that low incubation temperatures aren't necessary for good recovery in nucleic acid precipitation (Crouse

and Amorese, 1985)(Zeugin and Hartley, 1985), it appears this is not the case in terms of mononucleotide precipitation. At lower temperatures, all solutes will become less soluble in the solution, including mononucleotides, hence why the best yields were observed at a lower incubation temperatures. It may be the case that additional salts will co-precipitate at lower incubation temperatures, but when centrifugation occurs at ambient temperatures, these salts are likely to re-dissolve into the supernatant. Other complex biomolecules may not re-solubilise into the solvent so easily, despite a raise in temperature following incubation, so it is important to identify any trends in product purity following incubation at different temperatures. The results of the product purities, as indicated by the 260:280 ratio, are shown in Figure 46.

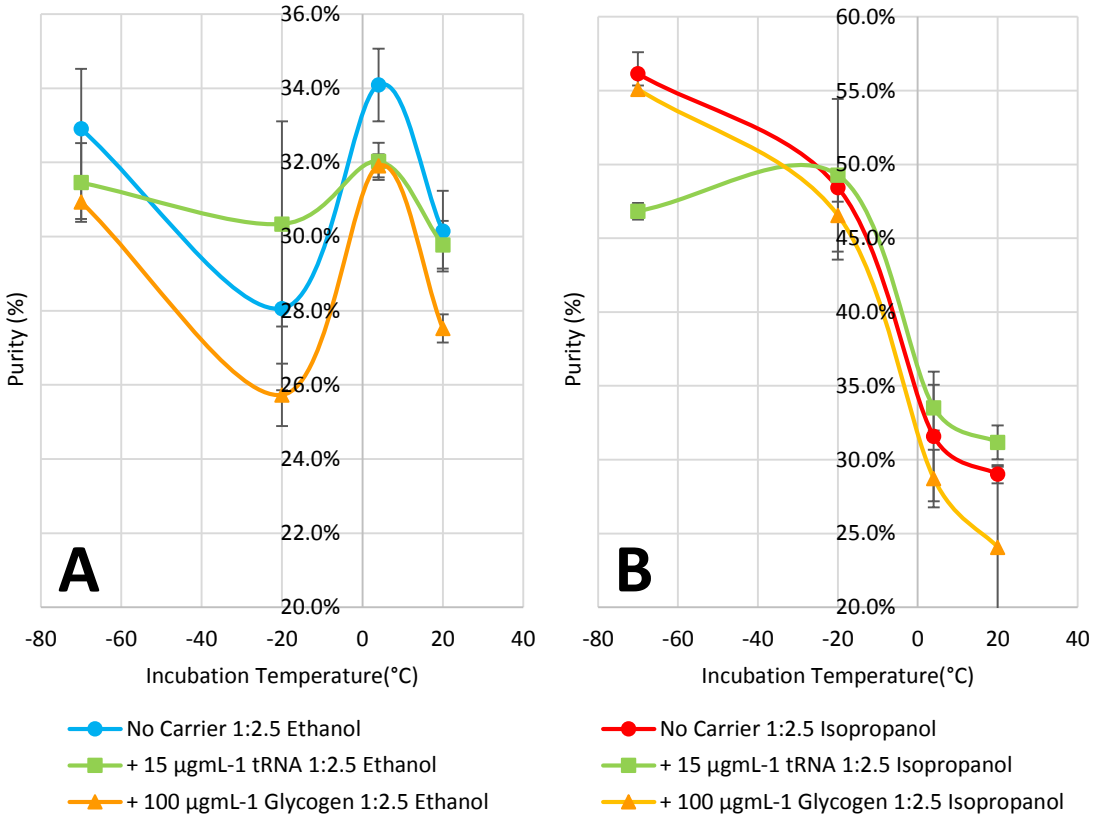


***Figure 46*- The effect of incubation temperature and carrier addition on product purity during precipitation of nucleotides.** Products from extraction with ethanol (A) and isopropanol (B) from 31/07/2015 were analysed via UV-Vis spectrophotometry and their absorbance at 260 nm and 280 nm were recorded. The ratio of absorbance values (260:280) was used to determine the nucleotide purity, as per Glasel, 1995. Results of extraction 31/07/2015, ref. Appendix 15.

Again, some inconsistent trends were evident, but carrier substances were generally ineffective in significantly increasing product purity. At temperatures between -20°C and 20°C, the addition of yeast tRNA did marginally increase product purity, where isopropanol was used. Although this

trend isn't consistent, this is likely due to the retention of yeast tRNA into the product may have resulted in an increase in absorbance at 260 nm, and hence a higher estimation of yield. After incubation at -70°C, samples became frozen solid. In a solid state, no precipitation events can occur, yet high yields were observed in these conditions. Incubation at these temperatures is costly and impractical at large scales, particularly when working with large volumes of sample. However, if the sample can be briefly brought to around -70°C, this may be sufficient to achieve a high yield.

4.8- Composition Analysis

For analysis of composition, yielded nucleotides were taken in powder form to Chemoforma laboratories, Augst, Switzerland, to be analysed via a sophisticated HPLC method. Twenty-four millitres of filtered centrate was extracted to give 33 mg of powdered nucleotides, which was analysed via HPLC. Nucleotide concentrations were calculated as grams per litre of centrate, shown in Figure 47.

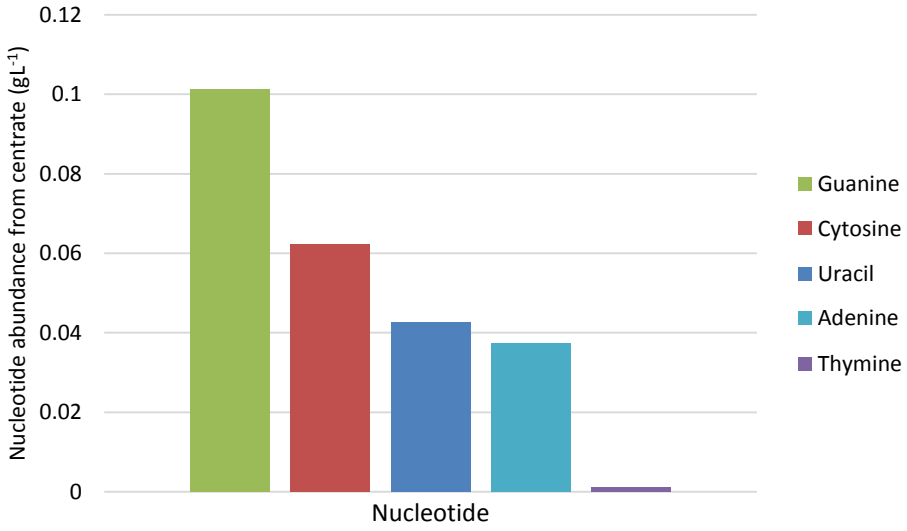


***Figure 47*- Results ofHPLC analysis of yielded nucleotide powder, extracted from centrate.** Powdered magnesium acetate was added to 1 mL filtered centrate to a molarity of 0.06M, followed a 1:1 ratio of isopropanol. After 12 hours of incubation at -70°C, the samples were centrifuged at 16,163 RCF for 30 minutes. Following this, pellets were re-suspended in 0.5 mL of 75% ethanol, with the aid of sonication, before being chilled for 30 minutes at -20°C. The samples were re-centrifuged at 16,163 RCF for 15 minutes and the resulting pellets were washed once more in 75%. After aspiration of supernatants, residual solvents were evaporated before pellets were dissolved in 1 mL of ultrapure water, then 24 identical product solutions were completely evaporated at 55°C, to give 33 mg powder which was analysed by HPLC. Results of analysis 25/08/2015, ref. Appendix 28.

Similarly, mycoscent samples were also taken for analysis. Again, nucleotide concentrations were calculated as grams per litre of centrate, shown in Figure 48.

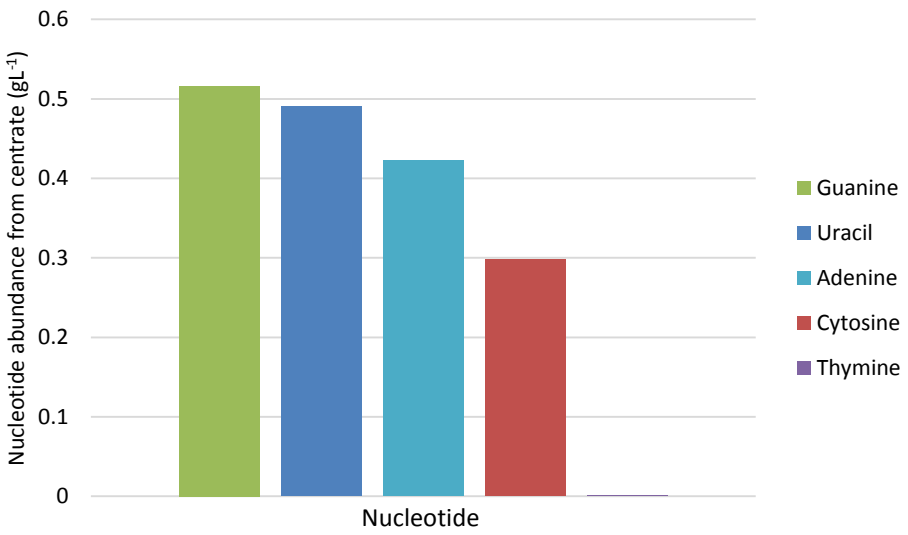


***Figure 48*- Results of HPLC analysis of mycoscent powder.** Mycoscent powder, as supplied by Quorn, was directly analysed via HPLC. Results of analysis 25/08/2015, ref. Appendix 28.

The composition of the yielded nucleotide powder was similar to that of the centrate, with retention of all five nucleotides. Table 12 shows the abundance of nucleotides in mg per gram of powder, for both the mycoscent powder.

***Table 12*- A summary of the nucleotide abundances in both the yielded nucleotide product and mycoscent.**

Nucleotide	Mycosent powder	Yielded nucleotide product
Uracil	33.6	31.0
Cytosine	20.4	45.2
Guanine	35.3	73.7
Thymine	0.1	0.9
Adenine	29.0	27.1

5- CONCLUSIONS

5.1- Conclusions and Suggestions for Further Work

A complete protocol will be presented here which includes favourable variable choices, which offer a compromise between yield, purity and salt retention, with considerations for practicality, simplicity and cost in mind. The protocol will be explained as a laboratory-scale process, where points of care are emphasised. Generic volumes are described here, but scaling is certainly possible. Considerations for industrial up-scaling will be discussed later. This protocol doesn't include quality control measures such as protein assays, analysis of supernatants, magnesium quantification by ICP-OES or purity/composition analyses via HPLC. An overview of the process is shown in Figure 49.

PRE-TREATMENT

1 Centrifuge the centrate at 16,163 RCF for 30 minutes in 2 mL aliquots. Aspirate the supernatant with a micropipette and discard pelleted material. ◄ CARE –Ensure the pelleted material is not physically perturbed, as this could result in contamination of the centrate with insoluble matter, which will inevitably pellet during centrifugation.

2 Pre-treated centrate should be stored in 200 mL aliquots at -20°C until required.

PRECIPITATION

3 When required, fully thaw an aliquot of pre-treated centrate in a 20°C water bath. When thawed, briefly vortex before proceeding. ◄ CARE – Ensure centrate is fully thawed and homogenous before proceeding to extraction.

4 Accurately weigh 129 mg of magnesium acetate tetrahydrate into clean beaker.

5 Add 10 mL of thawed, pre-treated centrate with a volumetric pipette, Mohr pipette or large volume micropipette with several additions, to achieve a molarity of 0.06 M of magnesium acetate in the centrate. ◄ CARE – Aim to rinse the edges of the beaker with the centrate to dissolve as much of the added salt as possible.

6 Add a magnetic stirrer bar to the centrate/salt and place on a magnetic stirring platform at a medium speed, until the salts have fully dissolved.

7 Add 400 µL of centrate to 24 fresh 2 mL Eppendorf centrifuge tubes with a micropipette.

8 Add 1,600 µL of -20°C isopropanol to each tube, and vortex briefly until the mixture has a homogenous appearance.

9 Arrange each tube in a 28-hole microcentrifuge rotor and incubate all samples in the rotor for 12 hours at -70°C in an ultra-low temperature freezer.

10 Centrifuge the samples at 16,163 RCF for 30 minutes immediately following their removal from the laboratory freezer.

11 After centrifugation, carefully aspirate the supernatant with a micropipette. ◄ CARE – Do not physically perturb the nucleotide pellet, as this may result in loss of nucleotides and hence lower yield. Using the stated quantities, pellets are easily visible, so measures such as marking the face of the tube oriented away from the centrifuge centre are not deemed necessary. This supernatant can be retained in a fresh vessel for quantification of un-precipitated nucleotides.

PELLET WASHING

12 Add 500 µL of 75% ethanol to each tube and add sonicate in an ultrasonic bath at 100% power, at room temperature, for 20 minutes. ◄ CARE – Additional vortexing may be required in some samples to aid in dispersion of the pellet.

13 Incubate all samples in the centrifuge rotor for 30 minutes at -20°C.

14 Centrifuge the samples at 16,163 RCF for 15 minutes immediately following their removal from the laboratory freezer.

15 After centrifugation, carefully aspirate the supernatant with a micropipette. ◄ CARE – Do not physically perturb the nucleotide pellet, as this may result in loss of nucleotides and hence lower yield.

PELLET SOLUBILISATION

16 Place the samples in open centrifuge tubes in a fume hood, to evaporate residual solvents from the product, for 15-20 minutes.

17 Add 2 mL of ultrapure water to each tube and sonicate in an ultrasonic bath at 100% power, at room temperature, for 90 minutes. ◄ CARE – Additional vortexing may be required in some samples to aid in dispersion of the pellet. Prolonged sonication can generate heat, it is recommended that the temperature be monitored and the bath water changed if it exceeds 60°C.

YIELD DETERMINATION

18 Prepare a 1:12.5 dilution of the nucleotide product solution by pipetting 160 µL of solution into a fresh Eppendorf tube, with 1,840 µL of ultrapure water.

19 Prepare a UV-Vis spectrophotometer by analysing a blank of ultrapure water, in a cleaned quartz macrocuvette.

20 Place the diluted nucleotide solution in the cuvette and analyse in fixed mode at wavelengths of 260 nm and 280 nm. ◄ CARE – Between sampling, evacuate the cuvette with compressed air, rinse with ultrapure water and evacuate once again with compressed air.

21 The absorbance value at 260 nm can be directly converted to a nucleotide yield in µg per mL of centrate by multiplying the value 1967.4. ◄ CARE – This multiplication accounts for dilution factors and converts absorbance to nucleotide concentration, as per the calibration curve. However, this is only strictly valid in the limits for the calibration, from absorbance values from 0.1-1.0.

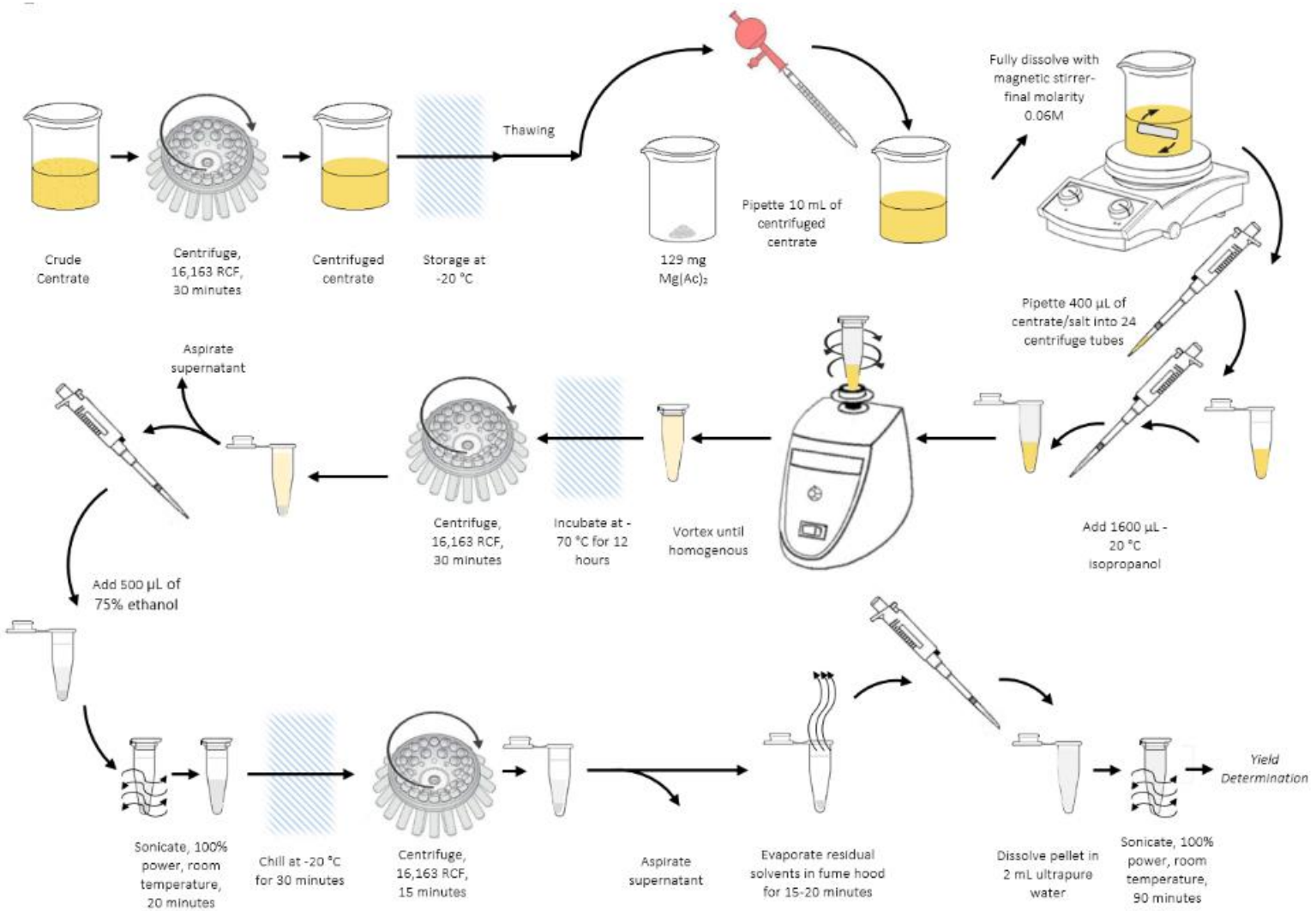


***Fig 49*- An overview of a complete protocol for the precipitation of mononucleotides.** Procedures for yield determination are not shown.

Centrifugation is recommended for the centrate as a pre-treatment method, because this was shown to result in a significant increase in product purity and a minor decrease in salt retention. An overall decrease in yield is observed compared to filtration, but as problems were previously experienced with filtration techniques at the Quorn production facility during mycoscent production, centrifugation seems a more practical choice for centrate pre-treatment.

Storage requirements are included as part of the protocol, but at larger scales, there is no reason why centrate cannot be immediately processed following its exit from the centrifuges. At this stage, the centrate is hot, at around 78°C, so some cooling may be required before processing can begin.

Magnesium acetate tetrahydrate, used at a final of 0.06 M in the centrate, is recommended following several high-yielding trials with the salt, equating to around 13 gL^{-1} of salt in the centrate. Some inconsistency with pH-controlled results calls for repeats of these trials. In the case of alkalised trials, it may be preferable to use magnesium hydroxide as opposed to sodium hydroxide to achieve a desired pH, as the molarity of magnesium can be accounted for in each condition, without the addition of sodium cations, which may influence yield in some way.

Isopropanol is recommended for use as a solvent during precipitation. In later trials, isopropanol was seen to be consistently more effective at achieving a high yield, as well as a higher product purity. Ethanol did result in a slightly lower retention of salts, but this difference is fairly insignificant. Overall, process economics may have dictated that ethanol may be a better choice, particularly as ethanol must be used during washing, so could be purchased in greater bulk to fulfil the solvent needs for both precipitation and washing stages. However, as such significant differences in yield were consistently observed between the two solvents, and ethanol purchase includes a tax in the UK, isopropanol appears to be a better option overall. Solvent consumption

for the process will inevitably be significant, so as a foresight, solvent recycling measures should be considered which may be integrated into the industrial scale process.

A solvent ratio of 1:4 (80% solvent) is recommended, as it presents a good compromise of high yield and purity, along with reasonable solvent use and throughput and, expectantly, a reasonably manageable level of salt co-precipitation. Despite trends matching those stated in literature, solvent ratio testing should be repeated with 0.06 M magnesium acetate, as the single trial was conducted with 0.05 M magnesium chloride. Some inconsistent results at high solvent ratios was thought to have arisen due to the salt choice limiting further increases in yield.

As a laboratory procedure, it is fairly straightforward to incubate a small number of low-volume samples at -70°C, but this is obviously more difficult at larger scales. Results showed that high yields and product purities were generally achieved at lower incubation temperatures, but no trials were carried out to determine the effect of incubation time on yield or purity. Samples incubated at -70°C froze after 12 hours of incubation, so less than 12 hours is likely to be adequate to achieve the same yield.

A centrifugation speed of 16,163 RCF was used throughout experimentation, for centrate pre-treatment and following initial precipitation and washing. A centrifugation time of 30 minutes is used for centrate pre-treatment and pelleting following precipitation, and 15 minutes following pellet washing. These speeds and times were chosen in search for optimal yields, but such speeds are unrealistic in large, industrial centrifuges, so it is necessary to quantify the effect of centrifugation speed and time on product yield in further work, and carry out trials where practical industrial centrifugation speeds are used. Chilled centrifugation is often called for in laboratory-scale methods but was not tested during experimentation, primarily because chilling during large-scale centrifugation is a very costly operation. The benefits of chilled centrifugation include limiting the effect of nucleases, which therefore preserves nucleic acid strand structure, and improved fixing the pellet to the base of the centrifugation tube, resulting in easier removal of the supernatant. The former benefit does not apply in mononucleotide precipitation, but to improve fixing of the pellet, samples were chilled for 20 minutes at -20°C prior to centrifugation steps.

Nucleotides may be gained at higher yield if the initial centrifugation took place at lower temperatures, as nucleotides would remain very less soluble throughout centrifugation. Likewise though, salts and potentially other biomolecules would also remain less soluble during centrifugation, so high salt retention may occur in this case. Larger volumes will also require additional centrifugation, so investigation of the relationship between sampling volume and centrifugation should be quantified to demonstrate the significance of this.

During washing, a pellet wash of 0.5 mL of 75% ethanol is recommended. The same volume of a 95% ethanol was not shown to result in any yield increase and also resulted in higher retention of salts. It is possible that a higher volume of 95% ethanol may result in effective pellet washing, but this is likely to result in a higher overall cost. Depending on what is deemed to be an acceptable level of Mg^{2+} retention in the final product, a lower volume of 75% ethanol may be called for, which is likely to result in a yield increase. During pellet washing, sonication is recommended to disrupt the pellet and improve salt removal from the pellet and its interior. Sonication may not be possible for very large vessel sizes, but some form of agitation of the vessel contents, which avoids physical perturbation, is necessary to achieve the efficient salt washing observed during experimentation. Physical perturbation of nucleotide pellets is likely to result in a sub-optimal yield. The results of ICP-OES study were very logical and consistent, but preparation of samples was very laborious, so the analysis of samples via ion-exchange chromatography is recommended for future work, as samples can be directly analysed in solvent matrices, with a sampling volume of a few microliters. At industrial scales, a magnesium ion-selective electrode would supply adequately accurate data for quality control measures.

Final products can be analysed by UV-Visible spectrophotometry following dissolving in water, for yield and purity. This may be done as a quality control measure at larger scales, but nucleotides are yielded as powders, which can be diverted to HPLC-based nucleotide purification systems for analysis, but also further refining, to then be distributed to a number of European markets.

As the Quorn production is non-specific, it is also necessary to determine the exact nucleotide make-up in the centrate, in terms of the concentration of deoxyribo- and ribo-nucleotides, and the

presence of residual intact polynucleotides. High molecular weight polynucleotide chains are in one respect desirable, as they are likely to be extracted more effectively during precipitation. Polynucleotides can then be easily and cheaply hydrolysed, either chemically or enzymatically, prior to HPLC refinement. Products can similarly be analysed for their nucleotide composition and chain length, it is likely that some nucleotide chain shearing will occur due to the lack of nuclease-free reagents and equipment, and indelicate treatment of samples throughout extraction. Characterisation of the polynucleotide strand in samples can be achieved with urea gel electrophoresis, where a conjugated fluorophore can be used for detection of fragments.

Other further work may include investigation of the relationship between yield and starting nucleotide concentration of the centrate. If the yield is found to be higher at higher initial nucleotide concentrations, there may be an argument for pre-concentrating the centrate prior to extraction to achieve these yields. Effectively, more centrate can then be processed in a given vessel size, increasing the throughput of the process. Testing in this case is very simple, as solutions of centrate of various concentrations can be easily produced by dissolving mycoscent powder in water.

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Appendix 1- Table summarising results from extraction 1, completed 12/02/2015. 500 µL of *Aspergillus oryzae* lysate was used, where the product was diluted by a factor of 5 before analysis. 1.5 mL 70% ethanol wash was used. Supernatant 2 was diluted by a factor of 2, while supernatant 3 was undiluted, before analysis. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Nucleotide yield from 1mL culture (µg)	Average yield (µg)	Average % yield	S2 Abs	λ_{max}	Nucleotide lost from 1ml of culture (µg)	Average % loss	S3 Abs	λ_{max}	Nucleotide lost from 1ml of culture (µg)	Average % loss
0.5M Ammonium Acetate	1:2.5 Ethanol	0.270	254.578	106.24	95.81	6.1%	0.842	257.791	186.97	11.9%	0.367	260.963	40.77	2.6%
		0.242	255.650	95.38										
		0.218	255.736	85.82										
	1:1 Isopropanol	0.149	253.692	58.43	62.29	4.0%	0.468	257.982	103.87	6.6%	0.160	267.155	17.78	1.1%
		0.168	254.881	65.99										
		0.159	253.338	62.45										
2.5M Ammonium Acetate	1:2.5 Ethanol	0.158	255.555	62.33	61.26	3.9%	0.508	257.929	112.76	7.2%	0.445	257.680	49.44	3.1%
		0.158	253.909	62.09										
		0.151	254.249	59.38										
	1:1 Isopropanol	0.106	253.330	41.79	39.78	2.5%	0.372	257.409	82.66	5.3%	0.141	261.002	15.66	1.0%
		0.096	254.607	37.89										
		0.101	253.421	39.66										
0.3M Sodium Acetate	1:2.5 Ethanol	0.329	255.032	129.61	122.61	7.8%	1.328	258.244	294.96	18.8%	0.833	257.735	92.54	5.9%
		0.278	255.054	109.19										
		0.328	255.684	129.02										
	1:1 Isopropanol	0.187	253.783	73.66	69.13	4.4%	0.799	258.710	177.53	11.3%	0.575	257.319	63.88	4.1%
		0.143	253.138	56.43										
		0.197	253.810	77.32										
0.2M Sodium Chloride	1:2.5 Ethanol	0.295	255.429	116.04	116.51	7.4%	1.330	258.626	295.52	18.8%	0.798	257.514	88.65	5.6%
		0.297	255.658	116.98										
		0.296	254.662	116.51										
	1:1 Isopropanol	0.187	253.755	73.54	69.53	4.4%	0.535	257.484	118.76	7.6%	0.154	257.107	17.11	1.1%
		0.162	253.175	63.63										
		0.182	253.332	71.42										

Appendix 2- Table summarising results from extraction 2, completed 03/03/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 15 before analysis. 0.5 mL 75% ethanol wash was used. Supernatants from washes were diluted by a factor of 6 before analysis. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield	Supernatant 2 Absorbance	λ_{max}	Nucleotide lost from 1ml of centrate (µg)	Average % loss	Supernatant 3 Absorbance	λ_{max}	Nucleotide lost from 1 mL of centrate (µg)	Average % loss
0.5M Ammonium Acetate	1:2.5 Ethanol	0.333	252.985	0.228	1.46	14.32	392.89	301.44	8.9%	0.710	257.789	157.66	4.7%	0.782	256.076	173.65	5.1%
		0.354	253.031	0.240	1.47	14.67	417.48										
	1:1 Isopropanol	0.390	253.692	0.297	1.31	9.84	93.96	419.69	12.4%	1.519	259.912	337.55	10.0%	1.042	256.349	231.61	6.9%
		0.378	253.585	0.293	1.29	9.28	91.05										
0.5M Ammonium Acetate + 0.01M Magnesium Chloride	1:2.5 Ethanol	0.637	253.590	0.407	1.57	18.85	752.52	807.96	23.9%	0.573	258.914	127.26	3.8%	1.030	257.746	228.89	6.8%
		0.731	256.344	0.458	1.60	20.47	863.40										
	1:1 Isopropanol	0.816	256.975	0.657	1.24	8.19	393.16	395.91	11.7%	0.794	259.250	176.49	5.2%	1.029	258.491	228.55	6.8%
		0.827	256.298	0.666	1.24	8.21	398.67										
0.3M Sodium Acetate	1:2.5 Ethanol	0.482	253.779	0.328	1.47	14.65	569.09	522.45	15.5%	1.670	259.836	371.01	11.0%	1.298	255.862	288.44	8.5%
		0.403	253.896	0.271	1.49	15.29	475.81										
	1:1 Isopropanol	0.488	254.317	0.306	1.59	20.26	117.53	112.49	3.3%	1.983	259.867	440.66	13.1%	1.336	257.049	296.95	8.8%
		0.446	254.314	0.312	1.43	13.20	107.44										
0.3M Sodium Acetate + 0.01M Magnesium Chloride	1:2.5 Ethanol	0.838	257.179	0.529	1.59	19.79	989.70	1015.49	30.1%	1.445	260.072	321.11	9.5%	1.260	259.648	279.93	8.3%
		0.882	256.684	0.545	1.62	21.65	1041.29										
	1:1 Isopropanol	0.791	256.010	0.623	1.27	8.80	381.35	395.22	11.7%	1.901	260.592	422.28	12.5%	1.289	258.318	286.51	8.5%
		0.849	255.195	0.625	1.36	10.99	409.10										
0.2M Sodium Chloride	1:2.5 Ethanol	0.319	253.312	0.223	1.43	13.20	376.74	389.16	11.5%	1.726	259.531	383.61	11.4%	0.818	255.55	181.71	5.4%
		0.340	253.904	0.235	1.44	13.71	401.57										
	1:1 Isopropanol	0.372	253.560	0.288	1.29	9.33	89.63	90.71	2.7%	1.865	259.901	414.46	12.3%	1.142	255.919	253.85	7.5%
		0.381	253.400	0.296	1.29	9.19	91.79										
0.2M Sodium Chloride + 0.01M Magnesium Chloride	1:2.5 Ethanol	0.455	254.027	0.314	1.45	13.95	537.52	541.13	16.0%	1.161	259.237	258.04	7.6%	0.758	256.684	168.32	5.0%
		0.461	254.613	0.314	1.47	14.67	544.75										
	1:1 Isopropanol	0.533	255.006	0.613	0.87	2.62	256.72	253.57	7.5%	1.847	260.023	410.37	12.2%	0.897	257.194	199.40	5.9%
		0.519	255.282	0.620	0.84	2.29	250.42										

Appendix 3- Table summarising results from extraction 3, completed 11/03/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 15 before analysis. 0.5 mL 75% ethanol wash was used. Supernatant 2 was diluted by a factor of 5, while supernatant 3 was diluted by a factor of 3 before analysis. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Abs 279.96nm	260:280	% Nucleotide	Nucleotide yield from 1mL centrate (µg)	Average yield (µg)	Average % yield	Supernatant 2 Absorbance	λ_{max}	Nucleotide lost from 1 mL of centrate (µg)	Average % loss	Supernatant 3 Absorbance	λ_{max}	Nucleotide lost from 1 mL of centrate (µg)	Average % loss
0.5M Ammonium Acetate	1:2.5 Ethanol	0.695	260.982	0.590	1.18	6.96	820.98	730.35	21.6%	1.073	257.640	198.61	5.9%	0.721	256.191	80.08	2.4%
		0.542	260.959	0.430	1.26	8.61	639.72										
	1:2.5 Isopropanol	0.579	260.794	0.440	1.32	9.91	683.11	654.90	19.4%	1.429	258.589	264.62	7.8%	0.957	256.269	106.28	3.1%
		0.531	253.449	0.384	1.38	11.73	626.70										
0.5M Sodium Acetate	1:2.5 Ethanol	0.582	260.811	0.455	1.28	9.04	686.48	688.65	20.4%	1.742	259.190	322.58	9.6%	1.589	256.819	176.49	5.2%
		0.585	260.762	0.438	1.34	10.41	690.82										
	1:2.5 Isopropanol	0.477	254.324	0.310	1.54	17.63	563.55	604.29	17.9%	1.802	259.863	333.70	9.9%	2.239	258.237	248.74	7.4%
		0.546	253.978	0.381	1.44	13.39	645.02										
0.5M Sodium Chloride	1:2.5 Ethanol	0.422	260.909	0.339	1.25	8.33	498.47	508.59	15.1%	1.466	257.294	271.43	8.0%	1.001	260.673	111.16	3.3%
		0.439	260.928	0.352	1.25	8.32	518.72										
	1:2.5 Isopropanol	0.406	260.762	0.323	1.26	8.49	479.19	529.56	15.7%	1.681	259.577	311.24	9.2%	1.487	256.417	165.15	4.9%
		0.491	253.432	0.369	1.33	10.33	579.94										
0.5M Magnesium Chloride	1:2.5 Ethanol	0.530	260.965	0.392	1.35	10.84	626.22	633.69	18.8%	0.415	257.676	76.79	2.3%	0.381	257.749	42.31	1.3%
		0.543	260.886	0.389	1.40	12.15	641.16										
	1:2.5 Isopropanol	0.865	257.064	0.544	1.59	19.98	1021.04	1029.24	30.5%	0.540	257.804	99.93	3.0%	0.456	257.721	50.70	1.5%
		0.879	257.809	0.576	1.53	16.90	1037.43										
0.5M Tripotassium Phosphate	1:2.5 Ethanol	0.235	261.051	0.203	1.16	6.61	277.20	297.20	8.8%	0.450	261.068	83.25	2.5%	0.420	261.203	46.62	1.4%
		0.269	261.139	0.238	1.13	6.12	317.21										
	1:2.5 Isopropanol	0.242	261.141	0.212	1.14	6.32	285.87	287.80	8.5%	NO DATA	NO DATA	#VALUE!	#VALUE!	0.621	261.186	68.96	2.0%
		0.245	261.101	0.223	1.10	5.66	289.73										
0.5M Potassium Carbonate	1:2.5 Ethanol	0.221	261.028	0.191	1.16	6.59	261.29	254.54	7.5%	0.437	261.072	80.87	2.4%	0.543155	261.196	60.34	1.8%
		0.210	261.013	0.176	1.20	7.28	247.79										
	1:2.5 Isopropanol	0.191	261.134	0.181	1.05	4.92	225.13	219.35	6.5%	0.479	261.013	88.70	2.6%	0.323647	261.105	35.96	1.1%
		0.181	261.181	0.173	1.05	4.82	213.56										

Appendix 4- Table summarising results from extraction 4, completed 27/03/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 16 before analysis. 0.5 mL 75% ethanol wash was used. Supernatant 1 was diluted by a factor of 80, supernatant 2 was diluted by a factor of 10, and supernatant 3 was diluted by a factor of 5, before analysis. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield	Supernatant 1 Absorbance	λ_{max}	Nucleotide un-precipitated from 1ml of centrate (µg)	Average % loss	Supernatant 2 Absorbance	λ_{max}	Nucleotide lost from 1ml of centrate (µg)	Average % loss	Supernatant 3 Absorbance	λ_{max}	Nucleotide lost from 1 mL of centrate (µg)	Average % loss
0.5M Ammonium Acetate	1:2.5 Ethanol	0.367	254.198	0.246	1.49	15.40	461.59	459.98	13.6%	0.338	257.737	2994.81	88.7%	0.625	257.241	231.58	6.9%	0.396	256.785	73.35	2.2%
		0.375	254.722	0.250	1.50	15.87	472.44														
		0.354	254.259	0.235	1.51	16.15	445.92														
	1:2.5 Isopropanol	0.332	253.940	0.219	1.52	16.60	418.20	478.86	14.2%	0.314	257.701	2832.39	83.9%	0.924	257.700	342.36	10.1%	0.547	256.673	101.33	3.0%
		0.414	254.323	0.277	1.50	15.71	521.85														
		0.394	254.135	0.254	1.55	18.06	496.54														
0.5M Sodium Acetate	1:2.5 Ethanol	0.420	254.245	0.278	1.51	16.17	529.08	524.26	15.5%	0.321	257.443	2836.73	84.0%	1.464	259.700	542.17	16.1%	1.308	256.913	242.16	7.2%
		0.394	254.037	0.252	1.56	18.59	496.54														
		0.435	254.372	0.281	1.55	17.94	547.16														
	1:2.5 Isopropanol	0.458	254.690	0.295	1.55	17.97	576.08	583.31	17.3%	0.291	257.621	2626.23	77.8%	1.275	259.983	472.04	14.0%	1.747	257.792	323.45	9.6%
		0.477	254.284	0.305	1.56	18.54	600.19														
		0.456	254.423	0.293	1.56	18.26	573.67														
0.2M Sodium Chloride	1:2.5 Ethanol	0.309	251.006	0.210	1.47	14.80	389.28	418.60	12.4%	0.264	257.759	2339.41	69.3%	1.074	257.386	397.75	11.8%	0.516	256.427	95.47	2.8%
		0.351	251.251	0.237	1.48	14.99	442.31														
		0.337	251.271	0.222	1.52	16.55	424.23														
	1:2.5 Isopropanol	0.359	251.233	0.243	1.48	14.87	451.95	537.11	15.9%	0.224	257.585	2021.66	59.9%	1.559	260.008	577.15	17.1%	0.873	256.512	161.63	4.8%
		0.332	253.815	0.227	1.46	14.27	418.20														
		0.589	257.277	0.365	1.61	21.25	741.19														
0.2M Magnesium Acetate	1:2.5 Ethanol	0.575	257.062	0.357	1.61	21.17	724.32	641.56	19.0%	0.265	257.592	2348.37	69.6%	0.342	257.428	126.66	3.8%	0.326	257.093	60.30	1.8%
		0.554	257.126	0.313	1.77	34.22	697.81														
		0.399	253.811	0.251	1.59	20.08	502.57														
	1:2.5 Isopropanol	0.817	257.237	0.561	1.46	14.18	1029.23	1115.20	33.0%	0.179	257.126	1619.16	48.0%	0.520	257.801	192.63	5.7%	0.509	259.450	94.33	2.8%
		0.925	257.546	0.516	1.79	37.20	1164.22														
		0.915	257.264	0.521	1.76	33.01	1152.16														

Appendix 5- Table summarising results from extraction 5, completed 30/04/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in scan mode during UV-Vis.

Condition	pH	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.5M Ammonium Acetate	Native (6.43)	1:2.5 Ethanol	0.273	254.419	0.198	1.38	11.50	536.91	471.83	14.0%
			0.207	259.889	0.143	1.44	13.62	406.75		
		1:2.5 Isopropanol	0.306	260.294	0.229	1.34	10.46	601.99	547.76	16.2%
			0.251	255.020	0.187	1.34	10.48	493.52		
	4.13	1:2.5 Ethanol	0.245	258.596	0.193	1.27	8.86	482.68	458.27	13.6%
			0.221	258.495	0.171	1.29	9.29	433.87		
		1:2.5 Isopropanol	0.287	255.779	0.232	1.24	8.13	564.03	558.60	16.5%
			0.281	257.688	0.234	1.20	7.37	553.18		
	5.15	1:2.5 Ethanol	0.287	255.276	0.226	1.27	8.79	564.03	564.03	16.7%
			0.287	260.074	0.223	1.28	9.14	564.03		
		1:2.5 Isopropanol	0.232	259.229	0.171	1.35	10.93	455.56	385.06	11.4%
			0.160	257.169	0.116	1.38	11.67	314.55		
	5.78	1:2.5 Ethanol	0.201	254.985	0.149	1.35	10.85	395.90	444.71	13.2%
			0.251	255.625	0.193	1.30	9.52	493.52		
		1:2.5 Isopropanol	0.361	256.298	0.292	1.24	8.09	710.46	726.73	21.5%
			0.378	260.128	0.303	1.25	8.29	743.00		
0.5M Sodium Acetate	Native (6.43)	1:2.5 Ethanol	0.232	255.163	0.185	1.25	8.47	455.56	393.19	11.6%
			0.168	259.136	0.132	1.27	8.84	330.82		
		1:2.5 Isopropanol	0.119	259.881	0.085	1.39	11.85	233.20	273.88	8.1%
			0.160	254.659	0.119	1.35	10.77	314.55		
	4.13	1:2.5 Ethanol	0.149	254.608	0.135	1.10	5.65	292.86	314.55	9.3%
			0.171	260.756	0.135	1.27	8.72	336.25		
		1:2.5 Isopropanol	0.251	255.686	0.207	1.21	7.63	493.52	496.23	14.7%
			0.254	258.984	0.210	1.21	7.57	498.95		
	5.15	1:2.5 Ethanol	0.223	255.384	0.185	1.21	7.54	439.29	431.15	12.8%
			0.215	257.171	0.179	1.20	7.37	423.02		
		1:2.5 Isopropanol	0.179	256.234	0.141	1.27	8.93	352.52	344.38	10.2%
			0.171	257.272	0.132	1.29	9.32	336.25		
	5.78	1:2.5 Ethanol	0.207	257.300	0.163	1.27	8.85	406.75	336.25	10.0%
			0.135	256.465	0.105	1.29	9.27	265.74		
		1:2.5 Isopropanol	0.369	258.841	0.303	1.22	7.72	726.73	724.01	21.4%
			0.367	255.277	0.300	1.22	7.76	721.30		
0.5M Sodium Chloride	Native (6.43)	1:2.5 Ethanol	0.267	254.932	0.207	1.29	9.36	526.06	496.23	14.7%
			0.237	254.565	0.182	1.30	9.59	466.41		
		1:2.5 Isopropanol	0.311	255.235	0.245	1.27	8.82	612.84	550.47	16.3%
			0.248	260.706	0.196	1.27	8.77	488.10		
	4.13	1:2.5 Ethanol	0.259	258.131	0.218	1.19	7.17	509.79	553.18	16.4%
			0.303	255.661	0.254	1.20	7.28	596.57		
		1:2.5 Isopropanol	0.314	259.244	0.270	1.16	6.68	618.26	569.45	16.9%
			0.265	254.983	0.218	1.22	7.66	520.64		
	5.15	1:2.5 Ethanol	0.234	256.225	0.193	1.21	7.65	460.98	463.69	13.7%
			0.237	259.586	0.193	1.23	7.93	466.41		
		1:2.5 Isopropanol	0.234	256.340	0.187	1.25	8.38	460.98	474.54	14.1%
			0.248	259.375	0.204	1.22	7.68	488.10		
	5.78	1:2.5 Ethanol	0.190	254.201	0.146	1.30	9.57	374.21	531.49	15.7%
			0.350	257.501	0.287	1.22	7.78	688.76		
		1:2.5 Isopropanol	0.405	260.701	0.317	1.28	9.01	797.23	761.98	22.6%
			0.369	256.744	0.300	1.23	7.95	726.73		
0.5M Magnesium Chloride	Native (6.43)	1:2.5 Ethanol	0.488	257.160	0.328	1.49	15.28	959.93	976.20	28.9%
			0.504	259.468	0.328	1.54	17.43	992.47		
		1:2.5 Isopropanol	0.493	255.539	0.336	1.47	14.51	970.78	1043.99	30.9%
			0.568	256.695	0.391	1.45	13.92	1117.21		
	4.13	1:2.5 Ethanol	0.276	259.843	0.232	1.19	7.18	542.33	485.39	14.4%
			0.218	256.781	0.179	1.22	7.67	428.44		
		1:2.5 Isopropanol	0.273	255.337	0.232	1.18	6.96	536.91	507.08	15.0%
			0.243	258.278	0.207	1.17	6.87	477.25		
	5.15	1:2.5 Ethanol	0.350	260.631	0.248	1.41	12.59	688.76	645.38	19.1%
			0.306	255.583	0.212	1.44	13.60	601.99		
		1:2.5 Isopropanol	0.240	260.046	0.176	1.36	11.05	471.83	474.54	14.1%
			0.243	258.996	0.176	1.38	11.50	477.25		
	5.78	1:2.5 Ethanol	0.681	254.684	0.488	1.40	12.10	1339.56	1415.49	41.9%
			0.758	259.850	0.527	1.44	13.54	1491.42		
		1:2.5 Isopropanol	0.739	257.005	0.527	1.40	12.34	1453.45	1453.45	43.1%
			0.739	259.834	0.551	1.34	10.53	1453.45		

Appendix 6- Table summarising results from extraction 6, completed 05/05/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 15 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in scan mode during UV-Vis.

Condition	pH	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average% yield
0.5M Ammonium Acetate	Native (6.43)	1:2.5 Ethanol	0.292	254.13	0.137	2.13	497.00	344.20	366.70	10.9%
			0.330	255.07	0.213	1.54	17.74	389.20		
		1:2.5 Isopropanol	0.322	254.87	0.202	1.59	20.28	380.20	386.95	11.5%
			0.334	255.61	0.210	1.59	20.09	393.70		
	4.13	1:2.5 Ethanol	0.221	254.32	0.170	1.30	9.60	260.96	250.84	7.4%
			0.204	254.36	0.147	1.39	11.93	240.72		
		1:2.5 Isopropanol	0.248	254.17	0.173	1.43	13.16	292.46	286.84	8.5%
			0.238	254.36	0.173	1.37	11.46	281.21		
	5.15	1:2.5 Ethanol	0.170	254.00	0.122	1.39	11.96	200.22	214.85	6.4%
			0.194	256.80	0.137	1.42	12.77	229.47		
		1:2.5 Isopropanol	0.173	253.91	0.120	1.44	13.70	204.72	200.22	5.9%
			0.166	254.57	0.118	1.40	12.34	195.72		
	5.78	1:2.5 Ethanol	0.248	254.90	0.168	1.48	14.89	292.46	282.34	8.4%
			0.231	254.72	0.154	1.49	15.54	272.21		
		1:2.5 Isopropanol	0.196	254.93	0.132	1.49	15.50	231.72	247.47	7.3%
			0.223	254.17	0.149	1.50	15.79	263.21		
0.5M Sodium Acetate	Native (6.43)	1:2.5 Ethanol	0.372	251.35	0.242	1.54	17.32	438.69	444.31	13.2%
			0.381	254.56	0.253	1.50	15.94	449.94		
		1:2.5 Isopropanol	0.339	254.32	0.213	1.59	20.00	400.45	382.45	11.3%
			0.309	254.24	0.198	1.56	18.37	364.45		
	4.13	1:2.5 Ethanol	0.208	256.84	0.152	1.36	11.14	245.22	233.97	6.9%
			0.189	254.91	0.132	1.43	13.36	222.72		
		1:2.5 Isopropanol	0.177	256.87	0.130	1.37	11.29	209.22	202.47	6.0%
			0.166	259.06	0.124	1.34	10.49	195.72		
	5.15	1:2.5 Ethanol	0.196	256.84	0.137	1.43	13.22	231.72	226.09	6.7%
			0.187	254.00	0.130	1.44	13.58	220.47		
		1:2.5 Isopropanol	0.168	255.09	0.116	1.44	13.63	197.97	212.60	6.3%
			0.192	254.09	0.128	1.51	16.10	227.22		
	5.78	1:2.5 Ethanol	0.208	256.71	0.145	1.43	13.35	245.22	236.22	7.0%
			0.192	256.90	0.133	1.44	13.64	227.22		
		1:2.5 Isopropanol	0.215	254.39	0.149	1.45	13.84	254.22	238.47	7.1%
			0.189	254.99	0.137	1.38	11.50	222.72		
0.5M Sodium Chloride	Native (6.43)	1:2.5 Ethanol	0.299	256.67	0.196	1.52	16.82	353.20	380.20	11.3%
			0.345	255.63	0.223	1.55	17.85	407.19		
		1:2.5 Isopropanol	0.313	254.74	0.202	1.55	17.86	368.95	391.45	11.6%
			0.351	254.74	0.233	1.51	16.13	413.94		
	4.13	1:2.5 Ethanol	0.164	260.37	0.137	1.19	7.26	193.47	206.97	6.1%
			0.187	259.04	0.151	1.24	8.18	220.47		
		1:2.5 Isopropanol	0.088	259.25	0.076	1.15	6.45	103.49	161.98	4.8%
			0.187	259.08	0.160	1.17	6.74	220.47		
	5.15	1:2.5 Ethanol	0.183	253.95	0.135	1.35	10.85	215.97	204.72	6.1%
			0.164	259.17	0.122	1.34	10.63	193.47		
		1:2.5 Isopropanol	0.162	254.185	0.112	1.44	13.57	191.22	182.23	5.4%
			0.147	258.94	0.103	1.43	13.07	173.23		
	5.78	1:2.5 Ethanol	0.271	254.74	0.191	1.42	12.87	319.46	318.33	9.4%
			0.269	255.21	0.179	1.50	15.79	317.21		
		1:2.5 Isopropanol	0.271	254.65	0.181	1.49	15.57	319.46	308.21	9.1%
			0.252	253.83	0.175	1.43	13.36	296.96		
0.5M Magnesium Chloride	Native (6.43)	1:2.5 Ethanol	0.507	257.07	0.320	1.58	19.68	598.42	637.79	18.9%
			0.574	257.10	0.349	1.64	23.36	677.16		
		1:2.5 Isopropanol	0.884	257.13	0.509	1.74	30.97	1043.86	1017.99	30.2%
			0.840	257.19	0.492	1.71	28.30	992.11		
	4.13	1:2.5 Ethanol	0.229	259.19	0.172	1.33	10.35	269.96	316.08	9.4%
			0.307	259.35	0.219	1.40	12.24	362.20		
		1:2.5 Isopropanol	0.503	258.87	0.221	2.28	-164.67	593.92	574.80	17.0%
			0.471	257.14	0.299	1.57	19.15	555.67		
	5.15	1:2.5 Ethanol	0.486	256.33	0.305	1.59	20.25	573.67	561.30	16.6%
			0.465	257.14	0.288	1.62	21.53	548.92		
		1:2.5 Isopropanol	0.713	257.19	0.408	1.75	31.97	841.38	854.88	25.3%
			0.736	257.22	0.421	1.75	31.86	868.38		
	5.78	1:2.5 Ethanol	0.690	256.88	0.398	1.73	30.40	814.39	807.64	23.9%
			0.678	256.97	0.387	1.75	32.61	800.89		
		1:2.5 Isopropanol	0.583	256.92	0.345	1.69	26.73	688.41	732.27	21.7%
			0.658	257.01	0.385	1.71	28.18	776.14		

Appendix 7- Table summarising results from extraction 7, completed 07/05/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.05M Magnesium Chloride	1:2.5 Ethanol	0.513	256.792	0.332	1.55	17.77	1009.28	1071.25	31.7%
		0.576	256.909	0.368	1.57	18.74	1133.22		
	1:2.5 Isopropanol	0.666	257.070	0.401	1.66	24.47	1310.29	1310.29	38.8%
		0.666	256.829	0.406	1.64	23.07	1310.29		
0.2M Magnesium Chloride	1:2.5 Ethanol	0.452	257.004	0.296	1.53	16.94	889.26	954.19	28.3%
		0.518	257.297	0.340	1.52	16.78	1019.11		
	1:2.5 Isopropanol	0.562	257.181	0.339	1.66	24.26	1105.68	1100.76	32.6%
		0.557	257.045	0.338	1.65	23.57	1095.84		
0.4M Magnesium Chloride	1:2.5 Ethanol	0.369	257.606	0.251	1.47	14.62	725.97	741.71	22.0%
		0.385	257.635	0.260	1.48	15.02	757.45		
	1:2.5 Isopropanol	0.483	256.774	0.308	1.57	18.89	950.25	936.48	27.7%
		0.469	257.127	0.297	1.58	19.46	922.71		
0.6M Magnesium Chloride	1:2.5 Ethanol	0.290	257.430	0.206	1.41	12.48	570.55	600.06	17.8%
		0.320	256.995	0.227	1.41	12.54	629.57		
	1:2.5 Isopropanol	0.379	257.699	0.247	1.53	17.27	745.64	690.56	20.5%
		0.323	256.934	0.226	1.43	13.18	635.47		
0.8M Magnesium Chloride	1:2.5 Ethanol	0.258	257.278	0.191	1.35	10.82	507.59	488.90	14.5%
		0.239	256.167	0.171	1.40	12.17	470.21		
	1:2.5 Isopropanol	0.329	256.998	0.216	1.52	16.77	647.27	609.89	18.1%
		0.291	256.407	0.194	1.50	15.79	572.51		
1.0M Magnesium Chloride	1:2.5 Ethanol	0.199	255.138	0.149	1.34	10.41	391.51	412.17	12.2%
		0.220	255.877	0.163	1.35	10.79	432.83		
	1:2.5 Isopropanol	0.316	257.748	0.219	1.44	13.64	621.70	592.19	17.5%
		0.286	257.721	0.192	1.49	15.37	562.68		

Appendix 8- Table summarising results from extraction 8, completed 13/05/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.05M Magnesium Chloride	1:1 Ethanol	0.522	254.364	0.404	1.29	9.28	586.29	619.73	18.4%
		0.581	254.191	0.453	1.28	9.09	653.18		
	1:1 Isopropanol	0.683	256.807	0.497	1.37	11.45	767.29	837.13	24.8%
		0.807	256.109	0.537	1.50	15.85	906.97		
	1:2.5 Ethanol	0.554	256.779	0.371	1.49	15.52	1089.94	1084.04	32.1%
		0.548	256.915	0.369	1.49	15.19	1078.14		
	1:2.5 Isopropanol	0.629	256.412	0.411	1.53	17.09	1237.49	1174.54	34.8%
		0.565	255.973	0.375	1.51	16.06	1111.58		
	1:4 Ethanol	0.441	257.156	0.274	1.61	21.01	1239.46	1319.14	39.1%
		0.498	257.089	0.313	1.59	20.08	1398.82		
	1:4 Isopropanol	0.554	257.014	0.317	1.75	31.81	1556.21	1493.26	44.2%
		0.509	257.088	0.320	1.59	20.09	1430.30		
	1:5.03 Ethanol	0.408	257.576	0.252	1.62	21.91	1385.05	1450.96	43.0%
		0.447	256.859	0.280	1.60	20.58	1516.87		
	1:5.03 Isopropanol	0.418	257.554	0.249	1.68	25.94	1418.50	1452.92	43.0%
		0.438	257.052	0.271	1.62	21.50	1487.35		
	1:6 Ethanol	0.370	257.240	0.232	1.59	20.19	1453.91	1471.62	43.6%
		0.379	256.979	0.237	1.60	20.62	1489.32		
	1:6 Isopropanol	0.362	257.015	0.212	1.71	27.95	1422.43	1496.21	44.3%
		0.399	257.530	0.237	1.69	26.45	1569.99		
	1:6.99 Ethanol	0.312	256.893	0.193	1.61	21.34	1392.92	1409.64	41.8%
		0.319	257.148	0.199	1.60	20.63	1426.37		
	1:6.99 Isopropanol	0.347	257.344	0.205	1.69	26.64	1552.28	1580.81	46.8%
		0.360	257.594	0.212	1.70	27.56	1609.33		

Appendix 9- Table summarising results from extraction 9, completed 13/05/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in scan mode during UV-Vis.

Condition	Solvent	Absorbance	λ_{max}	Abs 279.57nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.05M Magnesium Acetate	1:2.5 Ethanol	0.750	257.348	0.424	1.77	34.31	1475.55	1513.91	44.8%
		0.789	257.275	0.441	1.79	36.79	1552.28		
	1:2.5 Isopropanol	0.821	257.169	0.459	1.79	36.74	1615.24	1664.42	49.3%
		0.871	257.390	0.476	1.83	42.71	1713.61		
0.2M Magnesium Acetate	1:2.5 Ethanol	0.676	279.570	0.389	1.74	30.96	1329.96	1348.65	40.0%
		0.695	257.275	0.391	1.78	35.34	1367.34		
	1:2.5 Isopropanol	0.771	257.906	0.430	1.79	37.31	1516.87	1490.31	44.1%
		0.744	257.321	0.416	1.79	36.71	1463.75		
0.4M Magnesium Acetate	1:2.5 Ethanol	0.587	257.198	0.351	1.67	25.31	1154.86	1194.21	35.4%
		0.627	257.176	0.377	1.66	24.64	1233.56		
	1:2.5 Isopropanol	0.615	257.888	0.354	1.74	30.91	1209.95	1254.22	37.2%
		0.660	257.253	0.386	1.71	28.35	1298.48		
0.6M Magnesium Acetate	1:2.5 Ethanol	0.515	257.045	0.314	1.64	23.05	1013.21	1012.23	30.0%
		0.514	257.258	0.310	1.66	24.27	1011.24		
	1:2.5 Isopropanol	0.497	257.398	0.296	1.68	25.82	977.80	1001.41	29.7%
		0.521	257.193	0.306	1.70	27.73	1025.02		
0.8M Magnesium Acetate	1:2.5 Ethanol	0.454	257.288	0.276	1.64	23.37	893.20	924.68	27.4%
		0.486	257.234	0.288	1.69	26.48	956.16		
	1:2.5 Isopropanol	0.406	257.054	0.242	1.68	25.72	798.76	833.19	24.7%
		0.441	257.204	0.265	1.66	24.71	867.62		
1.0M Magnesium Acetate	1:2.5 Ethanol	0.340	256.813	0.211	1.61	21.26	668.92	735.81	21.8%
		0.408	256.886	0.246	1.66	24.31	802.70		
	1:2.5 Isopropanol	0.354	257.021	0.222	1.59	20.30	696.46	713.18	21.1%
		0.371	257.120	0.228	1.63	22.22	729.91		

Appendix 10- Table summarising results from extraction 10, completed 09/06/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in fixed mode during UV-Vis.

Condition	Solvent	Absorbance at 260nm	Absorbance at 280nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.01M Magnesium Acetate	1:2.5 Ethanol	0.415	0.283	1.47	14.48	816.47	833.19	27.1%
		0.432	0.295	1.46	14.41	849.92		
	1:2.5 Isopropanol	0.432	0.281	1.54	17.41	849.92	947.30	30.9%
		0.531	0.353	1.50	15.96	1044.69		
0.02M Magnesium Acetate	1:2.5 Ethanol	0.529	0.340	1.56	18.28	1040.75	1052.56	34.3%
		0.541	0.350	1.55	17.79	1064.36		
	1:2.5 Isopropanol	0.541	0.332	1.63	22.37	1064.36	1160.77	37.8%
		0.639	0.398	1.61	20.92	1257.17		
0.03M Magnesium Acetate	1:2.5 Ethanol	0.542	0.336	1.61	21.36	1066.33	1159.78	37.8%
		0.637	0.392	1.63	22.08	1253.23		
	1:2.5 Isopropanol	0.689	0.414	1.66	24.72	1355.54	1376.20	44.8%
		0.710	0.427	1.66	24.61	1396.85		
0.04M Magnesium Acetate	1:2.5 Ethanol	0.617	0.382	1.62	21.49	1213.89	1259.14	41.0%
		0.663	0.408	1.63	22.08	1304.39		
	1:2.5 Isopropanol	0.646	0.372	1.74	30.84	1270.94	1374.23	44.8%
		0.751	0.443	1.70	27.11	1477.52		
0.05M Magnesium Acetate	1:2.5 Ethanol	0.625	0.384	1.63	22.25	1229.63	1279.79	41.7%
		0.676	0.409	1.65	23.91	1329.96		
	1:2.5 Isopropanol	0.648	0.360	1.80	38.24	1274.88	1296.52	42.2%
		0.670	0.391	1.71	28.68	1318.16		
0.06M Magnesium Acetate	1:2.5 Ethanol	0.626	0.375	1.67	25.09	1231.59	1275.86	41.6%
		0.671	0.400	1.68	25.70	1320.13		
	1:2.5 Isopropanol	0.781	0.446	1.75	32.34	1536.54	1539.49	50.1%
		0.784	0.449	1.75	31.81	1542.44		

Appendix 11- Table summarising results from extraction 11, completed 15/06/2015. 500 µL of filtered or centrifuged centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in fixed mode during UV-Vis.

Condition	Solvent	Centrate	Absorbance at 260nm	Absorbance at 280nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.05M Magnesium Acetate	1:2.5 Ethanol	Filtered	0.392	0.233	1.68	26.08	771.22	830.24	27.0%
			0.452	0.266	1.70	27.44	889.26		
		Centrifuged	0.411	0.221	1.86	48.07	808.60	795.81	25.9%
			0.398	0.213	1.87	49.86	783.03		
	1:2.5 Isopropanol	Filtered	0.532	0.294	1.81	39.59	1046.66	1016.16	33.1%
			0.501	0.274	1.83	42.49	985.67		
		Centrifuged	0.516	0.264	1.95	75.41	1015.18	994.52	32.4%
			0.495	0.257	1.93	64.87	973.86		
0.06M Magnesium Acetate	1:2.5 Ethanol	Filtered	0.397	0.236	1.68	26.06	781.06	840.08	27.4%
			0.457	0.268	1.71	27.95	899.10		
		Centrifuged	0.380	0.206	1.84	45.24	747.61	738.76	24.1%
			0.371	0.199	1.86	48.99	729.91		
	1:2.5 Isopropanol	Filtered	0.482	0.259	1.86	48.32	948.29	1032.89	33.6%
			0.568	0.310	1.83	43.11	1117.48		
		Centrifuged	0.497	0.253	1.96	79.78	977.80	1025.02	33.4%
			0.545	0.271	2.01	108.26	1072.23		
0.07M Magnesium Acetate	1:2.5 Ethanol	Filtered	0.400	0.236	1.69	27.08	786.96	818.44	26.7%
			0.432	0.257	1.68	25.97	849.92		
		Centrifuged	0.346	0.193	1.79	37.27	680.72	732.86	23.9%
			0.399	0.214	1.86	49.03	784.99		
	1:2.5 Isopropanol	Filtered	0.487	0.266	1.83	42.87	958.12	967.96	31.5%
			0.497	0.284	1.75	32.22	977.80		
		Centrifuged	0.500	0.259	1.93	66.34	983.70	982.72	32.0%
			0.499	0.258	1.93	67.58	981.73		
0.08M Magnesium Acetate	1:2.5 Ethanol	Filtered	0.384	0.226	1.70	27.43	755.48	760.40	24.8%
			0.389	0.226	1.72	29.38	765.32		
		Centrifuged	0.331	0.181	1.83	42.53	651.21	719.08	23.4%
			0.400	0.217	1.84	45.00	786.96		
	1:2.5 Isopropanol	Filtered	0.506	0.257	1.97	81.90	995.50	1001.41	32.6%
			0.512	0.274	1.87	49.88	1007.31		
		Centrifuged	0.484	0.249	1.94	71.10	952.22	965.99	31.5%
			0.498	0.255	1.95	74.74	979.77		
0.09M Magnesium Acetate	1:2.5 Ethanol	Filtered	0.358	0.205	1.75	31.84	704.33	797.78	26.0%
			0.453	0.266	1.70	27.76	891.23		
		Centrifuged	0.373	0.200	1.87	49.13	733.84	787.94	25.7%
			0.428	0.228	1.88	51.73	842.05		
	1:2.5 Isopropanol	Filtered	0.509	0.282	1.80	38.94	1001.41	1040.75	33.9%
			0.549	0.307	1.79	36.69	1080.10		
		Centrifuged	0.476	0.244	1.95	73.87	936.48	963.04	31.4%
			0.503	0.255	1.97	83.72	989.60		
0.1M Magnesium Acetate	1:2.5 Ethanol	Filtered	0.377	0.217	1.74	30.92	741.71	796.80	26.0%
			0.433	0.252	1.72	29.10	851.88		
		Centrifuged	0.372	0.203	1.83	43.15	731.87	731.87	23.8%
			0.372	0.202	1.84	44.70	731.87		
	1:2.5 Isopropanol	Filtered	0.480	0.272	1.76	33.83	944.35	975.83	31.8%
			0.512	0.278	1.84	44.72	1007.31		
		Centrifuged	0.473	0.243	1.95	72.15	930.58	931.56	30.3%
			0.474	0.245	1.93	67.78	932.55		

Appendix 12- Table summarising results from extraction 12, completed 02/07/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% or 95% ethanol wash was used. Products were analysed in fixed mode during UV-Vis.

Condition	pH	Solvent	Ethanol Wash	Absorbance at 260nm	Absorbance at 280nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.06M Magnesium Acetate	Native pH	1:2.5 Ethanol	75%	0.466	0.270	1.73	29.81	916.81	909.92	29.6%
				0.459	0.269	1.71	28.04	903.04		
			95%	0.414	0.235	1.76	33.49	814.50	889.26	29.0%
				0.490	0.283	1.73	30.34	964.03		
		1:2.5 Isopropanol	75%	0.563	0.310	1.82	40.56	1107.65	1103.71	36.0%
				0.559	0.311	1.80	37.89	1099.78		
			95%	0.588	0.325	1.81	39.54	1156.83	1180.44	38.5%
				0.612	0.335	1.83	42.23	1204.05		
	pH 7	1:2.5 Ethanol	75%	0.449	0.265	1.69	27.03	883.36	879.43	28.6%
				0.445	0.272	1.64	22.78	875.49		
			95%	0.549	0.313	1.75	32.65	1080.10	1050.59	34.2%
				0.519	0.293	1.77	34.60	1021.08		
		1:2.5 Isopropanol	75%	0.460	0.264	1.74	31.43	905.00	925.66	30.2%
				0.481	0.281	1.71	28.52	946.32		
			95%	0.594	0.317	1.87	50.99	1168.64	1165.68	38.0%
				0.591	0.325	1.82	40.91	1162.73		
	pH 7.54	1:2.5 Ethanol	75%	0.431	0.256	1.68	26.17	847.95	866.64	28.2%
				0.450	0.265	1.70	27.35	885.33		
			95%	0.456	0.266	1.71	28.74	897.13	915.82	29.8%
				0.475	0.278	1.71	28.24	934.52		
		1:2.5 Isopropanol	75%	0.512	0.289	1.77	34.64	1007.31	988.62	32.2%
				0.493	0.282	1.75	32.03	969.93		
			95%	0.565	0.310	1.82	41.55	1111.58	1146.01	37.3%
				0.600	0.328	1.83	42.62	1180.44		

Appendix 13- Table summarising results from extraction 13, completed 07/07/2015. 500 µL of filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% or ethanol wash was used. Products were analysed in fixed mode during UV-Vis.

Condition	pH	Solvent	Centrate	Absorbance at 260nm	Absorbance at 280nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average% yield
0.06M Magnesium Acetate	Native pH	1:2.5 Ethanol	Filtered	0.475	0.267	1.78	35.52	934.52	939.43	30.6%
				0.480	0.271	1.77	34.59	944.35		
			Centrifuged	0.474	0.254	1.87	49.36	932.55	910.91	29.7%
				0.452	0.241	1.88	51.36	889.26		
		1:2.5 Isopropanol	Filtered	0.585	0.307	1.91	58.73	1150.93	1197.16	39.0%
				0.632	0.334	1.89	55.26	1243.40		
			Centrifuged	0.508	0.260	1.95	75.11	999.44	1014.19	33.0%
				0.523	0.268	1.95	74.14	1028.95		
	pH 6.01	1:2.5 Ethanol	Filtered	0.456	0.262	1.74	31.23	897.13	925.66	30.2%
				0.485	0.273	1.78	35.22	954.19		
			Centrifuged	0.419	0.227	1.85	45.45	824.34	837.13	27.3%
				0.432	0.235	1.84	44.13	849.92		
		1:2.5 Isopropanol	Filtered	0.565	0.303	1.86	49.07	1111.58	1141.09	37.2%
				0.595	0.313	1.90	57.50	1170.60		
			Centrifuged	0.517	0.265	1.95	73.92	1017.15	1045.67	34.1%
				0.546	0.277	1.97	83.00	1074.20		
	pH 5.47	1:2.5 Ethanol	Filtered	0.445	0.254	1.75	32.43	875.49	903.04	29.4%
				0.473	0.269	1.76	33.12	930.58		
			Centrifuged	0.400	0.215	1.86	48.22	786.96	799.75	26.1%
				0.413	0.224	1.84	45.08	812.54		
		1:2.5 Isopropanol	Filtered	0.595	0.321	1.85	46.88	1170.60	1183.39	38.5%
				0.608	0.330	1.84	44.84	1196.18		
			Centrifuged	0.520	0.265	1.96	78.79	1023.05	1054.53	34.3%
				0.552	0.282	1.96	76.65	1086.00		

Appendix 14- Table summarising results from extraction 14, completed 22/07/2015. 500 µL of filtered or centrifuged centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% or 95% ethanol wash was used. Products were analysed in fixed mode during UV-Vis.

Condition	Solvent	Centrate	Ethanol Wash	Absorbance at 260nm	Absorbance at 280nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.06M Magnesium Acetate	1:2.5 Ethanol	Filtered	75%	0.466	0.279	1.67	25.16	916.81	921.73	30.0%
				0.471	0.282	1.67	25.15	926.65		
		Centrifuged	95%	0.393	0.215	1.83	42.40	773.19	778.11	25.3%
				0.398	0.215	1.85	46.43	783.03		
	1:2.5 Isopropanol	Filtered	75%	0.690	0.418	1.65	23.76	1357.51	1359.47	44.3%
				0.692	0.419	1.65	23.82	1361.44		
		Centrifuged	95%	0.404	0.176	2.30	-142.46	794.83	805.65	26.2%
				0.415	0.179	2.32	-123.42	816.47		
	1:2.5 Ethanol	Filtered	95%	0.566	0.317	1.79	36.33	1113.55	1121.42	36.5%
				0.574	0.320	1.79	37.40	1129.29		
		Centrifuged	75%	0.388	0.217	1.79	36.65	763.35	759.42	24.7%
				0.384	0.213	1.80	38.63	755.48		
	1:2.5 Isopropanol	Filtered	95%	0.601	0.257	2.34	-110.79	1182.41	1190.28	38.8%
				0.609	0.261	2.33	-113.77	1198.15		
		Centrifuged	75%	0.524	0.270	1.94	69.96	1030.92	1023.05	33.3%
				0.516	0.264	1.95	75.41	1015.18		

Appendix 15- Table summarising results from extraction 15, completed 31/07/2015. Filtered centrate was used, where the product was diluted by a factor of 25 before analysis. 0.5 mL 75% ethanol wash was used. Products were analysed in fixed mode during UV-Vis.

Condition	Solvent	Carrier Added	Incubation Temperature	Abs 260nm	Abs 280nm	260:280	% Nucleotide	Nucleotide yield from 1 mL centrate (µg)	Average yield (µg)	Average % yield
0.06M Magnesium Acetate	1:2.5 Ethanol	None	-70°C	0.437	0.251	1.74	31.29	859.75	893.20	29.1%
				0.471	0.266	1.77	34.52	926.65		
	1:2.5 Isopropanol			0.694	0.365	1.90	57.61	1365.38	1324.06	43.1%
				0.652	0.345	1.89	54.68	1282.74		
	1:2.5 Ethanol		-20°C	0.393	0.234	1.68	25.85	773.19	847.95	27.6%
				0.469	0.271	1.73	30.26	922.71		
	1:2.5 Isopropanol			0.648	0.349	1.86	47.49	1274.88	1241.43	40.4%
				0.614	0.329	1.87	49.39	1207.98		
	1:2.5 Ethanol		4°C	0.474	0.267	1.78	35.07	932.55	938.45	30.6%
				0.480	0.273	1.76	33.11	944.35		
	1:2.5 Isopropanol			0.574	0.322	1.78	35.97	1129.29	1168.64	38.1%
				0.614	0.362	1.70	27.18	1207.98		
	1:2.5 Ethanol		20°C	0.426	0.248	1.72	29.06	838.11	867.62	28.3%
				0.456	0.262	1.74	31.23	897.13		
	1:2.5 Isopropanol			0.443	0.259	1.71	28.40	871.56	908.94	29.6%
				0.481	0.279	1.72	29.63	946.32		
	1:2.5 Ethanol	+ 50 µg Glycogen	-70°C	0.414	0.237	1.74	31.40	863.69	852.87	27.8%
				0.403	0.233	1.73	30.47	842.05		
	1:2.5 Isopropanol			0.597	0.316	1.89	55.34	1247.33	1235.53	40.2%
				0.586	0.310	1.89	54.85	1223.72		
	1:2.5 Ethanol		-20°C	0.325	0.195	1.67	24.89	678.75	718.10	23.4%
				0.363	0.215	1.69	26.57	757.45		
	1:2.5 Isopropanol			0.555	0.302	1.83	43.55	1158.80	1187.33	38.7%
				0.582	0.312	1.87	49.56	1215.85		
	1:2.5 Ethanol		4°C	0.411	0.236	1.74	31.59	857.79	876.48	28.5%
				0.429	0.245	1.75	32.22	895.17		
	1:2.5 Isopropanol			0.542	0.320	1.69	26.78	1131.26	1099.78	35.8%
				0.512	0.295	1.73	30.67	1068.30		
	1:2.5 Ethanol		20°C	0.367	0.217	1.70	27.14	767.29	809.59	26.4%
				0.408	0.239	1.70	27.91	851.88		
	1:2.5 Isopropanol			0.542	0.347	1.56	18.61	1131.26	1018.13	33.2%
				0.433	0.252	1.72	29.53	905.00		
	1:2.5 Ethanol	+ 7.5 µg tRNA	-70°C	0.433	0.250	1.73	30.40	851.88	886.31	28.9%
				0.468	0.267	1.75	32.52	920.74		
	1:2.5 Isopropanol			0.581	0.314	1.85	46.27	1143.06	1168.64	38.1%
				0.607	0.327	1.86	47.40	1194.21		
	1:2.5 Ethanol		-20°C	0.432	0.254	1.70	27.57	849.92	897.13	29.2%
				0.480	0.273	1.76	33.11	944.35		
	1:2.5 Isopropanol			0.612	0.324	1.89	54.44	1204.05	1227.66	40.0%
				0.636	0.346	1.84	44.10	1251.27		
	1:2.5 Ethanol		4°C	0.454	0.259	1.75	32.53	893.20	901.07	29.4%
				0.462	0.265	1.74	31.53	908.94		
	1:2.5 Isopropanol			0.545	0.307	1.78	35.07	1072.23	1081.09	35.2%
				0.554	0.317	1.75	31.97	1089.94		
	1:2.5 Ethanol		20°C	0.440	0.254	1.73	30.42	865.66	877.46	28.6%
				0.452	0.263	1.72	29.14	889.26		
	1:2.5 Isopropanol			0.492	0.281	1.75	32.31	967.96	971.90	31.7%
				0.496	0.287	1.73	30.03	975.83		

Appendix 16- Table summarising results from calibration 1, completed 02/12/2014. RNA solutions of different concentrations in water were analysed in scan mode.

RNA concentration μgmL^{-1}	Absorbance	λ_{max}	Abs 280.49	260:280	% Nucleotide
10.126	0.240	257.071	0.121	1.98	89.59
20.252	0.602	257.454	0.294	2.05	147.10
30.378	0.800	257.145	0.393	2.04	131.84
40.504	0.954	257.929	0.451	2.12	386.72
50.630	1.156	257.368	0.550	2.10	294.53
60.756	1.539	257.782	0.778	1.98	86.64
70.882	1.820	257.872	0.947	1.92	63.53
81.008	2.089	258.100	1.122	1.86	48.49
91.134	2.351	258.201	1.294	1.82	40.67
101.260	2.576	259.013	1.413	1.82	41.63
y= 39.309 x			***R^2= 0.9938***		

Appendix 17- Table summarising results from calibration 2, completed 02/12/2014. GMP solutions of different concentrations in water were analysed in scan mode.

GMP concentration μgmL^{-1}	Absorbance	λ_{max}	Abs 280.49	260:280	% Nucleotide
10.045	0.334	252.152	0.203	1.65	23.40
20.090	0.636	253.022	0.373	1.71	27.94
30.134	0.910	252.878	0.516	1.76	33.70
40.179	1.217	252.579	0.698	1.74	31.55
50.224	1.514	252.701	0.854	1.77	34.78
60.269	1.805	252.300	1.015	1.78	35.44
70.314	2.116	252.723	1.194	1.77	34.70
80.358	2.385	252.949	1.345	1.77	34.83
90.403	2.669	252.418	1.508	1.77	34.43
100.448	2.975	252.044	1.693	1.76	33.00
y= 33.545 x			***R^2= 0.9993***		

Appendix 18- Table summarising results from calibration 3, completed 02/12/2014. RNA solutions of different concentrations in water were analysed in scan mode.

RNA concentration μgmL^{-1}	Absorbance	λ_{max}	Abs 280.49	260:280	% Nucleotide
5.050	0.135	258.091	0.069	1.96	76.25
10.101	0.267	257.422	0.130	2.05	156.39
20.202	0.522	257.380	0.238	2.19	-545.63
30.303	0.774	257.208	0.348	2.22	-288.46
40.404	1.020	257.625	0.483	2.11	357.85
50.505	1.277	257.766	0.619	2.06	172.21
60.606	1.517	257.601	0.742	2.04	142.79
70.707	1.759	257.946	0.903	1.95	72.72
80.808	2.004	257.964	1.032	1.94	70.37
90.909	2.249	258.412	1.200	1.87	51.06
101.010	2.492	258.843	1.368	1.82	41.40
111.111	2.718	259.558	1.507	1.80	38.74
121.212	2.962	257.154	1.663	1.78	35.78
131.312	3.202	258.164	1.803	1.78	35.15
141.413	3.365	259.410	1.943	1.73	30.38
151.514	3.541	260.240	2.103	1.68	26.19
y= 41.178 x			***R^2= 0.998***		

Appendix 19- Table summarising results from calibration 4, completed 09/02/2015. RNA solutions of different concentrations in water were analysed in scan mode.

RNA concentration μgmL^{-1}	Absorbance	λ_{max}	Abs 280.49	260:280	% Nucleotide
9.943	0.340	257.842	0.199	1.71	28.24
19.886	0.623	257.838	0.358	1.74	31.21
29.828	0.840	257.951	0.476	1.76	33.83
39.771	1.139	257.970	0.650	1.75	32.47
49.714	1.430	258.108	0.834	1.71	28.77
59.657	1.692	258.050	0.973	1.74	31.08
69.600	1.955	258.071	1.053	1.86	47.46
79.542	2.222	258.285	1.289	1.72	29.62
89.485	2.477	259.042	1.446	1.71	28.63
99.428	2.732	259.634	1.606	1.70	27.60
y= 35.746 x			***R^2= 0.9978***		

Appendix 20- Table summarising results from calibration 5, completed 20/02/2015. RNA solutions of different concentrations in 75% ethanol were analysed in scan mode.

RNA concentration μgmL^{-1}	Absorbance	λ_{max}	Abs 280.49	260:280	% Nucleotide
9.750	0.520	257.704	0.336	1.55	17.88
19.500	0.918	257.791	0.516	1.78	35.53
29.250	1.323	257.934	0.738	1.79	37.26
39.000	1.699	257.927	0.920	1.85	45.62
48.750	2.099	258.063	1.239	1.69	27.02
58.500	2.446	258.788	1.479	1.65	23.98
68.250	2.768	259.797	1.808	1.53	17.11
78.000	3.070	260.743	2.151	1.43	13.11
87.750	3.284	259.611	2.464	1.33	10.34
97.500	3.646	260.302	2.715	1.34	10.61
y= 25.223 x			***R^2= 0.9819***		

Appendix 21- Table summarising results from calibration 6, completed 27/03/2015. RNA solutions of different concentrations in water were analysed in scan mode.

RNA concentration μgmL^{-1}	Absorbance	λ_{max}	Abs 280.49	260:280	% Nucleotide
3.737	0.100	257.620	0.050	2.00	100.00
7.474	0.196	257.953	0.101	1.94	69.90
11.211	0.291	257.772	0.153	1.90	57.77
14.948	0.391	257.947	0.208	1.88	52.32
18.685	0.491	258.041	0.264	1.86	48.10
22.422	0.591	258.036	0.321	1.84	44.62
26.159	0.690	257.968	0.377	1.83	42.77
29.896	0.782	257.986	0.429	1.82	41.59
33.633	0.886	258.082	0.488	1.82	40.48
37.370	0.914	258.044	0.506	1.81	39.13
y= 38.711 x			***R^2= 0.9955***		

Appendix 22- Table summarising results from calibration 7, completed 15/06/2015. RNA solutions of different concentrations in water were analysed in fixed mode.

RNA concentration μgmL^{-1}	Abs 260nm	Abs 280nm	260:280	% Nucleotide
3.704	0.096	0.049	1.96	77.41
7.408	0.193	0.105	1.84	44.09
11.112	0.282	0.151	1.87	49.66
14.816	0.376	0.204	1.84	44.97
18.520	0.470	0.252	1.38	11.53
22.224	0.566	0.312	1.81	40.26
25.927	0.656	0.358	1.83	43.13
29.631	0.751	0.414	1.81	40.24
33.335	0.848	0.469	1.81	39.38
37.039	0.943	0.528	1.79	36.64
y= 39.348 x			***R^2= 0.999***	

Appendix 23- Table summarising results from calibration 8, completed 31/07/2015. RNA solutions of different concentrations in 1:2.5 ethanol were analysed in fixed mode.

RNA concentration μgmL^{-1}	Abs 260nm	Abs 280nm	260:280	% Nucleotide
9.923	0.289	0.159	1.82	40.79
19.846	0.539	0.312	1.73	29.97
29.769	0.835	0.505	1.65	23.95
39.692	1.109	0.683	1.62	22.00
49.615	1.387	0.857	1.62	21.68
59.538	1.649	1.024	1.61	21.20
69.461	1.858	1.145	1.62	21.94
79.384	2.141	1.343	1.59	20.27
89.307	2.405	1.498	1.61	20.91
99.230	2.668	1.667	1.60	20.63
y= 36.868 x			***R^2= 0.9989***	

Appendix 24- Table summarising results from calibration 9, completed 31/07/2015. RNA solutions of different concentrations in 1:2.5 isopropanol were analysed in fixed mode.

RNA concentration μgmL^{-1}	Abs 260nm	Abs 280nm	260:280	% Nucleotide
9.923	0.305	0.170	1.79	37.45
19.846	0.585	0.297	1.97	82.30
29.769	0.866	0.462	1.87	51.13
39.692	1.117	0.671	1.66	24.75
49.615	1.363	0.840	1.62	21.94
59.538	1.657	1.052	1.58	19.25
69.461	1.861	1.168	1.59	20.23
79.384	2.106	1.364	1.54	17.71
89.307	2.342	1.497	1.56	18.70
99.230	2.514	1.624	1.55	17.90
y= 37.625			***R^2= 0.9933***	

Appendix 25- Table summarising results from calibration 10, completed 31/07/2015. RNA solutions of different concentrations in 75% ethanol were analysed in fixed mode.

RNA concentration μgmL^{-1}	Abs 260nm	Abs 280nm	260:280	% Nucleotide
9.980	0.295	0.172	15.23	-12.52
19.960	0.577	0.347	0.85	2.42
29.940	0.822	0.499	1.16	6.56
39.920	1.080	0.659	1.25	8.33
49.900	1.322	0.802	1.35	10.71
59.880	1.628	1.001	1.32	10.03
69.860	1.911	1.172	1.39	11.91
79.840	2.157	1.330	1.44	13.43
89.820	2.474	1.508	1.43	13.22
99.800	2.620	1.621	1.53	16.90
y= 36.821 x			***R^2= 0.9981***	

Appendix 26- Table summarising results from calibration 11, completed 31/07/2015. RNA solutions of different concentrations in 95% ethanol were analysed in fixed mode.

RNA concentration μgmL^{-1}	Abs 260nm	Abs 260nm	260:280	% Nucleotide
9.980	0.358	0.237	11.25	-13.12
19.960	0.619	0.398	0.90	2.95
29.940	0.909	0.594	1.04	4.75
39.920	1.146	0.743	1.22	7.83
49.900	1.416	0.912	1.26	8.53
59.880	1.695	1.087	1.30	9.59
69.860	1.898	1.223	1.39	11.82
79.840	2.163	1.396	1.36	11.06
89.820	2.409	1.554	1.39	12.00
99.800	2.667	1.733	1.39	11.94
y= 36.513 x			***R^2= 0.9941***	

Appendix 27- Table summarising results from the bicinchoninic acid (BCA) assay calibration and testing of extracted products, completed 08/07/2015. The resulting calibration was used to determine the protein concentration in extracted nucleotide samples, diluted by a factor of 2, from 07/07/2015.

BCA Calibration									
Bovine Serum Albumin Standard Solution Concentration (μgmL^{-1})					Absorbance at 562nm				
99.8					0.143				
299.4					0.485				
499.0					0.832				
698.6					1.199				
898.2					1.520				
y= 591.48 x					***R^2= 0.9986***				
Product Analysis									
Condition	**Solvent**	**Centrate**	**Nucleotide yield from 1 mL centrate (µg)**	**Nucleotide % yield**	**Absorbance at 562nm following BCA assay**	**Protein content in final product from 1 mL extraction (µg)**	**Actual Nucleotide content%**	**Expected 260:280 value**	**Actual 260:280 value**
0.06M Magnesium Acetate	1:2.5 Ethanol	Filtered	944.35	30.8%	0.362	856.26	52.45%	1.88	1.77
		Centrifuged	889.26	29.0%	0.227	537.10	62.34%	1.92	1.88
	1:2.5 Isopropanol	Filtered	1243.4	40.5%	0.366	865.75	58.95%	1.91	1.89
		Centrifuged	1028.95	33.5%	0.260	614.31	62.62%	1.92	1.95

Appendix 28- Table summarising results from HPLC analysis of yielded products, mycoscent samples and commercially purchased RNA, completed 25/08/2015. Nucleotide abundances and purities are determined on a dry weight basis.

Product			Mycoscent		
Nucleotide monophosphate	mg of nucleotide per g powder	% of powder	Nucleotide monophosphate	mg of nucleotide per g powder	% of powder
Cytidine	45.224	4.52%	Cytidine	20.420	2.04%
Uridine	30.979	3.10%	Uridine	33.612	3.36%
Guanidine	73.672	7.37%	Guanidine	35.382	3.54%
Thymidine	0.855	0.09%	Thymidine	0.098	0.01%
Adenosine	27.105	2.71%	Adenosine	28.956	2.90%

Appendix 29- Table summarising results from ICP-OES testing, completed 30/07/2015. Products and supernatants from conditions (extracted 22/07/2015) were diluted as stated and their Mg^{2+} concentrations measured via ICP-OES. Nucleotide yields were also used in calculations. A molarity of 58.49mmol of Mg^{2+} was achieved in the centrifuged centrate and 58.50mmol of Mg^{2+} was achieved in the filtered centrate, when adjusted for the stated 97.5% purity of the magnesium acetate used.

Sample	Solvent	Ethanol Wash	Mg^{2+} PPM	Dilution Factor	Mg^{2+} PPM (Dilution corrected)	Mg^{2+} molarity (mmol)	Mg^{2+} per volume in extraction (µg)	Volume	Nucleotide yield per 1 mL of centrate (µg)	µg Mg^{2+} per µg nucleotides
Centrifuged Centrate	-	-	61.57	Neat	61.57	2.53	61.57	1 mL	3222.8	0.019
Centrifuged Centrate Supernatant 1	Ethanol	-	69.05	5	345.23	14.20	1208.305	3.5 mL	-	-
	Isopropanol	-	67.21	5	336.05	13.83	1176.175	3.5 mL	-	-
Centrifuged Supernatant 2	Ethanol	75%	2.53	10	25.28	1.04	25.28	1 mL	-	-
		95%	0.69	10	6.875	0.28	6.875	1 mL	-	-
	Isopropanol	75%	3.35	10	33.53	1.38	33.53	1 mL	-	-
		95%	1.72	10	17.17	0.71	17.17	1 mL	-	-
Centrifuged Supernatant 3	Ethanol	75%	0.93	10	9.317	0.38	9.317	1 mL	-	-
		95%	0.15	10	1.463	0.06	1.463	1 mL	-	-
	Isopropanol	75%	1.21	10	12.09	0.50	12.09	1 mL	-	-
		95%	0.20	10	1.968	0.08	1.968	1 mL	-	-
Centrifuged Final Product Ethanol	Ethanol	75%	33.79	Neat	33.79	1.39	135.16	4 mL	759.42	0.178
		95%	37.94	Neat	37.94	1.56	151.76	4 mL	778.11	0.195
	Isopropanol	75%	40.36	Neat	40.36	1.66	161.44	4 mL	1023.05	0.158
		95%	41.97	Neat	41.97	1.73	167.88	4 mL	805.65	0.208
Filtered Centrate	-	-	61.57	Neat	63.5	2.61	63.5	1 mL	3222.8	0.020
Filtered Centrate Supernatant 1	Ethanol	-	67.51	5	337.55	13.89	1181.425	3.5 mL	-	-
	Isopropanol	-	64.62	5	323.1	13.29	1130.85	3.5 mL	-	-
Filtered Supernatant 2	Ethanol	75%	2.81	10	28.146	1.16	28.146	1 mL	-	-
		95%	0.81	10	8.106	0.33	8.106	1 mL	-	-
	Isopropanol	75%	3.43	10	34.32	1.41	34.32	1 mL	-	-
		95%	1.89	10	18.859	0.78	18.859	1 mL	-	-
Filtered Supernatant 3	Ethanol	75%	1.15	10	11.47	0.47	11.47	1 mL	-	-
		95%	0.11	10	1.132	0.05	1.132	1 mL	-	-
	Isopropanol	75%	1.73	10	17.32	0.71	17.32	1 mL	-	-
		95%	0.16	10	1.617	0.07	1.617	1 mL	-	-
Filtered Final Product Ethanol	Ethanol	75%	38.05	Neat	38.05	1.57	152.2	4 mL	921.73	0.165
		95%	46.09	Neat	46.09	1.90	184.36	4 mL	1121.42	0.164
	Isopropanol	75%	43.69	Neat	43.69	1.80	174.76	4 mL	1359.47	0.129
		95%	54.61	Neat	54.61	2.25	218.44	4 mL	1190.28	0.184