Developing a New Agritech Product to Increase and Enhance the Uptake of Vitamin D and Other Vitamins and Minerals in Mushrooms

By

Michael B. Williams, BSc

In collaboration with

NutriGain Ltd. & Drinkwater Mushrooms Ltd.

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Supervisors:
Prof. Martin McAinsh & Prof. Kirk Semple

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Abstract

This study aimed to test and refine a new method of mushroom vitamin and mineral enrichment with the use of novel ‘during-growth’ and ‘water-on’ liquid supplements. Through experimental trials, different formulations and dosages, as produced by NutriGain Ltd, were compared along with untreated and UV treated mushrooms, all in commercial conditions, through collaboration with Drinkwater Mushrooms Ltd. This aimed to assess the product in its suitability for providing nutritionally relevant content of Vitamin D, as well as the B-vitamins; B1, B2, B6 and B12, and Selenium. Vitamin D is the most important target of enrichment due to the possibility in replacing the current energy-intensive and high associated carbon emitting method of UV enrichment, which comes with many issues such as limited application (only brown mushrooms) and slowed packaging for application after growth. This has great potential benefits and opportunities for novel food products and public health by increasing levels of vitamins to combat deficiencies which are becoming more common in the UK and globally, especially vitamin D deficiencies.

The experiment involved commercial growth trials using white button mushrooms conducted at Drinkwater Mushrooms Ltd., with applications of product produced by NutriGain and analysis of vitamins and mineral content at Lancaster Environment Centre using HPLC and ICP-OES methods. A dosage rate in vitamin D of around 125-150 mg/m^2 in a calcium-based formulation of the liquid supplement was identified to yield higher vitamin D contents than other formulations tested, such as potassium-based, and was successful in matching UV-produced levels of vitamin D, without discolouration of white mushrooms. Enrichment with the novel MycroNutrient product gave a significant improvement compared to untreated samples, at levels of around 30 µg/g DW, whilst also providing nutritionally relevant levels of B-vitamins. All at a predicted annual carbon emission reduction of 99% in the process alone through the replacement of the UV method for this new treatment being applied to all mushrooms, both white and brown.

Declaration and Signatures

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own and has been approved for submission by both supervisors and business partners.

Candidate Signature: 

Lead Supervisor Signature: 

Second Supervisor Signature:

First Business Partner Signature:

Second Business Partner Signature: 

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1. Introduction

1.1 Cultivation

Commercial cultivation of mushrooms started in France around 1707 and is now the largest horticultural 'cash-crop', grown inside in controlled conditions (Dhar, 2017). Throughout history the white button variety have been seen as more attractive and popular among consumers. They also provide opportunities in sustainable agriculture, food security, socio-economic growth, and nutrition in many lower income countries (Chang and Wasser, 2017), which often have lower inputs available for agricultural practices, such as capacity for addition of other supplements or application of technology needed for processes like UV-treatment. Mushrooms show reliable and stable yield and income (Higgins et al., 2017), as well as increasing importance for innovation in the sustainable food and agricultural sectors (Zhang et al., 2014).

The market for mushrooms is large, $45 billion in 2005 (encompassing all sections) (Chang, 2006) and 3.2 million tons produced in 2003, 37% of which was produced in Europe (Bernaś et al., 2006). The sector has grown rapidly in recent years, with production globally estimated between 10-20% increase per year (Yoo et al., 2016) and is expected to increase much further. A major source of this market expansion is their importance in supplementing vegan and vegetarian diets which are also becoming far more popular, with an expected 16 million meat-free people in the UK by 2023 and currently 14% of adults, made up mostly of younger (Gen-Z) people (Johnson, 2021). Diet statistics can be found in **Figure 1.1**, to highlight the expanding market in catering for meat-free diets.

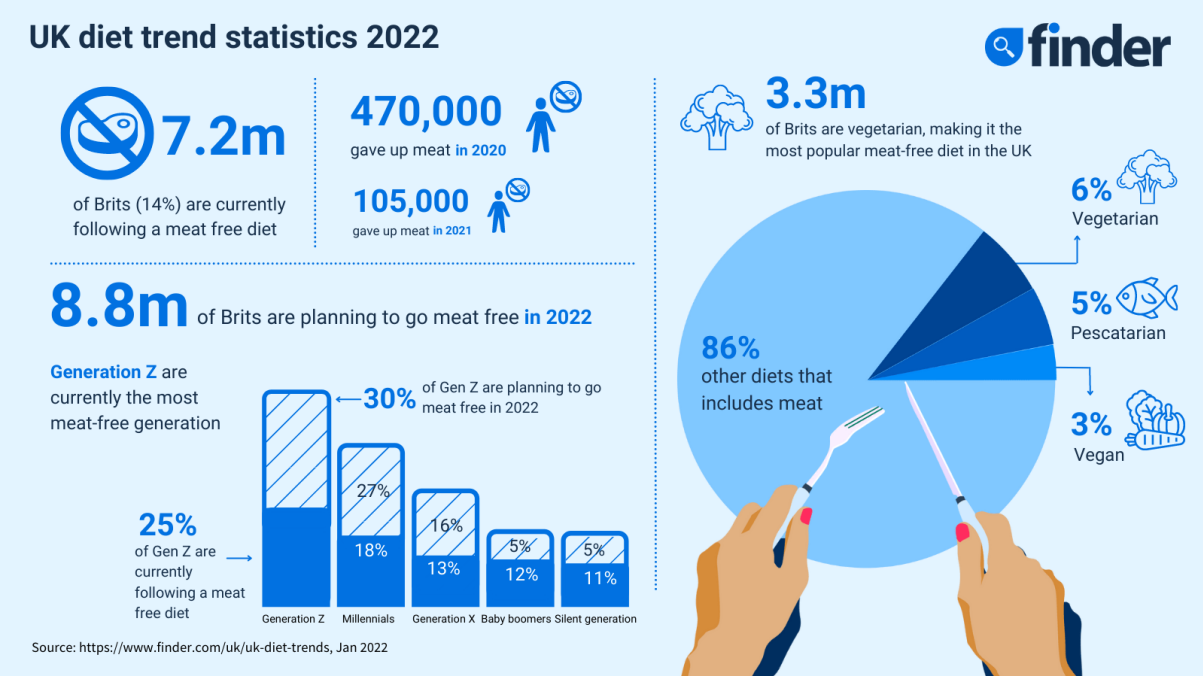

Figure 1.1 Diet statistics and predictions/ trends of meat-free or meat-reduced diets in the UK detailing generational differences and types of diets (from Johnson, 2022).

There is increasing evidence and research to indicate great potential for mushrooms as functional foods, supplementation for deficiencies in diets, and role as a nutraceutical. Many recommendations and requirements have been made for the necessary improvement to mushrooms cultivation, such as advances in growth and spawning technology, alternatives to peat use in casing materials, promotion of compost waste uses and strain improvements for variety protection (Sonnenberg et al., 2011) . In the case of the latter, on a global scale, mushrooms have limited genetic variability which can cause issues in disease resistance, therefore research to improve genetic understanding and subsequently selection and breeding of current cultivated strains is needed (Chakravarty, 2011). To this end, one of the industrial partners for this research, Drinkwater Mushrooms Ltd., is considered a leading player in the expanding market of mushroom cultivation and consumption (Yoo et al., 2005, Chang, 2006, Sumit, 2022 and Verified Market Reports, 2022), making developments in the sector of great interest to them.

The contribution of mushroom cultivation to agricultural sustainability and food security is acknowledged through their recognition as being considered a relatively ‘green’ industry and environmentally friendly crop as a source of protein (Chakravarty, 2011). Life-Cycle Assessments (LCA) such as that carried out by

Leiva et al., 2015 estimated greenhouse gas emissions at 4.42 kg CO_2eq (Carbon Dioxide equivalent) per kg of product, though figures vary with Robinson et al., 2018 reporting roughly 2.54 kg CO_2eq per kg and Dorr et al., 2021 reporting 3.1 kg CO_2eq per kg. There is also potential to reduce this figure further through green initiatives as recommended by Robinson et al., 2018, such as the use of on-site renewable power, employed by producers such as Drinkwater Mushrooms Ltd with solar panels to reduce external energy usage.

The most widely cultivated and commercially grown edible mushroom globally and within the UK is *Agaricus bisporus* (*A. bisporus*) (Sumit, 2022), making up 80% of production in Europe (Biswas et al., 2011) and 30% globally (Kertesz and Thai, 2018). Coming in both white and brown varieties and going by many names, such as button, chestnut, portobello and cremini, the scientific classification is *Agaricus bisporus* of the Basidimycota fungi. As with most commercially grown mushrooms, they are epigeous (aboveground fruiting) (Miles and Chang, 1997) saprotrophs which rely on the breakdown of non-living organic matter (usually a form of lignocellulosic waste) often provided by agricultural waste to make the compost or substrate and spawn layers (Sebaaly et al., 2021). A casing layer is then applied over the this which stimulates fruitification and ‘pin-head’ formation (production of mushrooms) via a comparative reduction in nutrients and influence of microbes to promote primordium initiation (Murmu, et al., 2020) (**Figure 1.2**). This usually occurs three times in the cropping cycle, resulting in three flushes (harvests), where yield is still commercially viable. Casing is very important in mushroom cultivation, often considered the major factor in yield and quality of mushrooms (Sonnenberg et al., 2022).

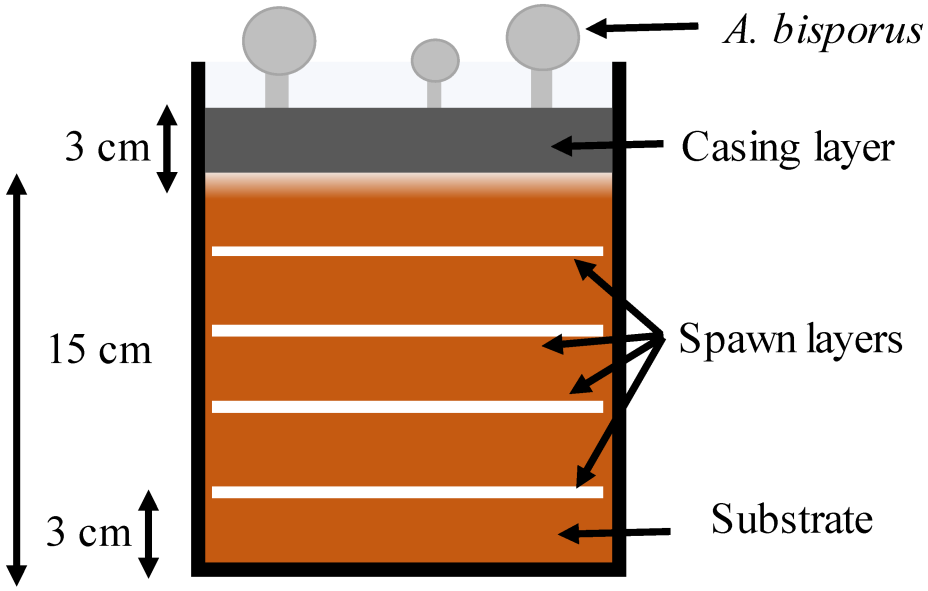

Figure 1.2 Typical structure of growth mediums for mushroom cultivation displaying substrate, spawn layers and casing layers designed to provide room for growth, nutrition, and induction of fruitification (from Kumar et al., 2022).

A. bisporus is a fungus that evolved as a degrader in a humic-rich leaf-litter environment, demonstrated by its expression of many enzymes targeted towards cellulose, hemi-cellulose (aided by many bacteria species) (Kertesz and Thai, 2018), lignin and other more complex 'humic' substrates (Morin et al., 2012). These fungi display cytoplasmic continuity and complexity with hyphal fusion (anastomoses) which can be very important for distribution of resources throughout the organism, which makes up the entire crop (Simonin et al., 2012).

1.2 Problems and Challenges

Mushrooms are an increasingly important food product due to their current status and further potential as a highly nutritious source of both protein, vitamins and minerals, including vitamin D with enrichment. Vitamin D is produced endogenously in animals in response to ultraviolet (UV) light triggering its synthesis, but it can also be obtained from the diet. Rates of vitamin and mineral deficiencies are rising, especially in the UK with vitamin D deficiency the most prevalent due to lack of sunlight exposure, only emphasized by the COVID-19 pandemic and lockdown (Tiwari et al., 2021). Vitamin deficiencies in the population also increase with age with approximately 1 in 10 of the of people over 75 and 1 in 20 of those aged 65-74

in the UK deficient in vitamin B12, increasing to 11% among vegans (Fleming, 2017). Vitamin B12 unlike vitamin D, comes solely from diet and is not available from plant sources. Similarly, the intake of selenium (Se), which is also diet derived, has fallen well below recommended daily intakes (RDI), with vegetarians, vegans and the elderly the most at risk of Se deficiency (Jackson et al., 2003). Governments and health bodies often promote and encourage fortification and enrichment of food to benefit public health (de Lourdes Samaniego-Vaesken, et al., 2012). Mushrooms are recognised as a good source of Se whilst enrichment methods are used to increase the levels of vitamin D most commonly.

Mushrooms are versatile and often used as a major protein source in meat-reduced diets especially (You et al., 2022). They are currently enriched with vitamin D using UV irradiation to convert ergosterol in the mushrooms to vitamin D2, a bioavailable form of vitamin D for humans. A high content can be achieved that is practical for supplementation in diets with regular mushroom consumption such that serum content is improved and deficiency prevented (Tiwari et al., 2021). However, this is an energy intensive process significantly adding to production costs as well as mushroom producers CO_2 emissions. Also, only brown capped mushrooms can be treated due to discolouration of white capped mushrooms. The treatment is also often uneven as mushrooms are UV treated in punnets, with the top layer of mushrooms having greater exposure to UV light. Therefore, new approaches to the enrichment of mushrooms are required to increase the uniformity of vitamin D content and to expand the vitamins and minerals that can be fortified in mushrooms.

1.2.1 Nutrition

Mushrooms have a range of important nutritional qualities, they are a low-calorie source of protein, vital vitamins and minerals. They have also been shown to have many medicinal properties such as anti-tumour, anti-diabetic, anti-microbial, anti-inflammatory and antioxidant (Glamočlija et al., 2015, Atila et al., 2017) leading to their classification and use as a nutraceutical, mainly through the presence of ergosterol (provitamin D2) (Rangsinth et al., 2023).

A. bisporus contains roughly 20-30% protein (by dry weight). Button mushrooms are also low in fat and high in fibre which make them a common meat-alternative for vegans and vegetarians, as well as providing a range of promissory bioactive compounds and natural sources of vitamin Bs like thiamine (B1) and riboflavin (B2) (Furlani and Godoy, 2008), and cobalamin (B12) which is often rare in meat-free/reduced diets and can be enriched in mushrooms, offering a practical way to supplement them and aid in reducing meat consumption for both health and sustainability (Koyyalamudi et al., 2009a, Goyal et al., 2020 and Pérez-Montes et al., 2021). Another interesting mineral that is commonly insufficient or deficient in populations (1 billion people deficient globally) is Se, an important co-factor in the human body, contained in mushrooms (Prange et al., 2019) and has potential to be enriched via supplementation of the growth substrate. Se is highly affected by climate-soil interactions and it is predicted that this will result in greater losses from crops in the future and thereby increasing the prevalence for Se deficiency due to insufficient dietary intake (Jones et al., 2017). Mushrooms are also considered to be an excellent source of potassium (K) and phosphorous (P), and contain mainly polyunsaturated fats and linoleic acid which can make them a good choice for people with chronic conditions such as diabetes and heart disease etc. (Goyal et al., 2020).

Mushrooms grown in sunlight or through treatment with UV-irradiation will contain vitamin D2 (Urbain and Jakobsen, 2015). In addition to D2 mushrooms sometimes also contain much lower contents of vitamins D3 and D4 which are the common dietary source of vitamin D found in animal products (Cardwell et al., 2018). Vitamin D is incredibly important for human health in both general function and in specific diseases. It plays an important role in calcium (Ca) uptake and muscle function (Cardwell et al., 2018), which is essential for the elderly who are most at risk of vitamin D deficiencies. Other diseases are lowered in risk by sufficient vitamin D intake such as diabetes, Crohn's, rickets, respiratory issues and neurodegenerative disorders (Tiwari et al., 2021), including lower incidence rates of dementia among those with greater vitamin D exposure or uptake (Ghahremani et al., 2023), again important for the elderly. Vitamin D has associated deficiencies in many countries and not just the UK. In developed and developing countries alike vitamin D is very environment dependent and indicative of the diversity of food consumption, so more alternatives to the traditional fish-oil based products are required (Wahlqvist, 2013).

This highlights the importance of developing new 'low-tech' methods of nutritional enrichment, especially in a dependable, widely cultivated and sustainable crop, to improve vitamin dietary intake globally.

Fungi and mushrooms are often considered a vegetable in dietary advice but are nutritionally distinct by the presence of vitamin B12 which is normally considered a meat-available vitamin due to its common sourcing in meat or meat products. In mushrooms B12 is produced by concomitant bacteria (see **Section 1.3.1**) which has the added benefit of supporting health through microbial interactions, contributing to adaptive immunity through immune conditioning (Feeney et al., 2014a). Other distinct factors of fungi bridge the gap between plant and animal products such as the presence of chitin, found in cell walls and a source of dietary fibre (usually found in crustaceans and shellfish) and ergothioneine which is an important bioactive antioxidant with a unique and specialised transport system, SLC22A4 in humans, proving its importance (Borodina et al., 2020). Ergothioneine has also been shown to have great potential to aid in reducing the risk of many age-related and inflammatory diseases (Beelman et al., 2019 and Borodina et al., 2020). Ergothioneine is also considered an important nutraceutical which is not produced by plant or animals and instead by fungi and some other microbes, making mushrooms the most easily available form (Borodina et al., 2020), with white button mushrooms containing 1.4 mg per serving of 80g (fresh) (Dubost et al., 2007).

Mushrooms are also considered a highly sustainable food choice by 2015 dietary guidelines and a novel culinary solution to meat consumption and other areas of future sustainable diet discussion (Feeney et al., 2014b). Mushrooms also show good content of Se, Zn, vitamins B1, B6, B7 and C (Muszyńska et al., 2017). Fungal protein is also pointed to for being easily digestible and the texture allows for potential replacement in meat products, aiding in reducing meat consumption and production (Pérez-Montes et al., 2021 and Feeney et al., 2014a) by combining with beef in making burgers and mince for example. This also reduces the fat content and aids nutrition but can cause change in texture and flavour (Patinho et al., 2019). It is also known that cooking methods influence the nutrition of mushrooms and decay of vitamins. This research concluded that microwaving and grilling were best for retention of vitamins and minerals in the mushrooms (Roncero-Ramos et al., 2016).

Other studies found that lower cooking temperatures, shorter cooking times and the addition of lemon juice (to alter pH) when boiling led to increased retention of vitamin D specifically (Ložnjak and Jakobsen, 2018).

There is considerable interest in increasing/enriching nutrition in mushrooms especially in the context of meat-free/reduced diets and the implications this has for vitamin B12 uptake (Nakos et al., 2017). Mushrooms also provide a palatable taste or umami which is measured in Equivalent Umami Content (EUC), which has increased their popularity and use in development of new food products (Wasser, 2005). The many economic and health benefits they therefore provide (see **Figure 1.3**) mean that there are many advantages to enhancing and enriching the content of vitamins and minerals in mushrooms through both technological and agronomic approaches (Poiroux-Gonord et al., 2010), highlighting the importance for future research in this area.

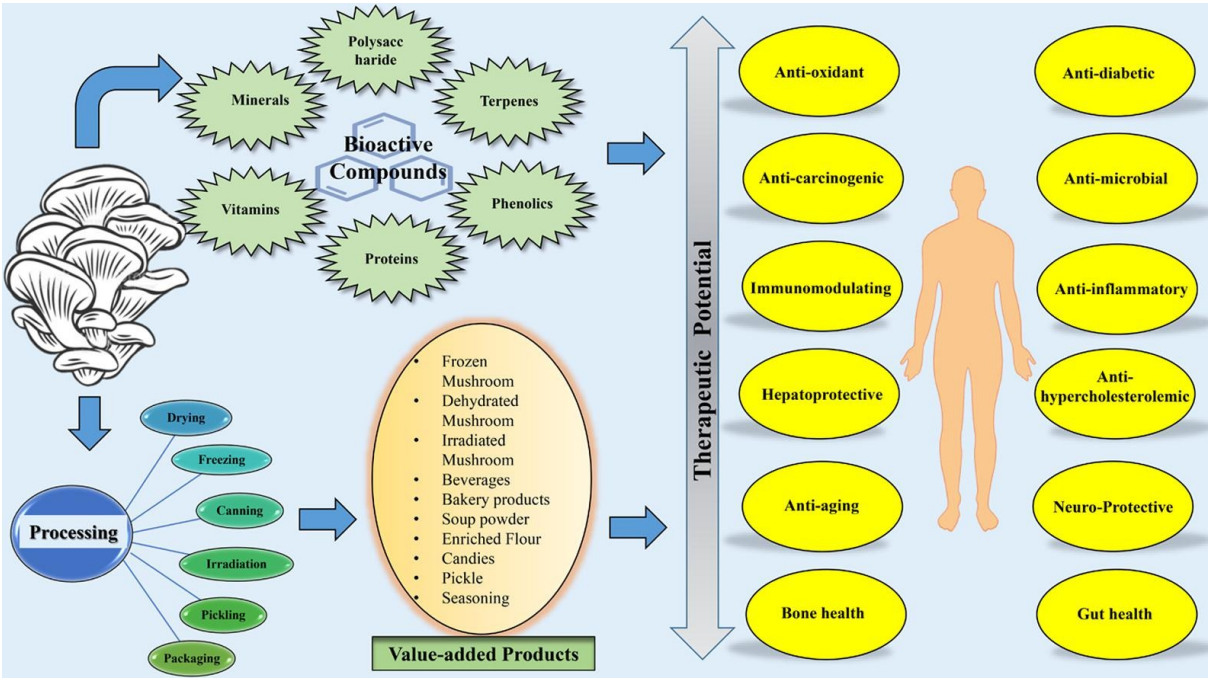

Figure 1.3 Elements and compounds contained in mushrooms and their potential and characteristics for therapeutics and health benefits as well as possible processing and food products available (from Yadav and Negi, 2021).

1.2.2 Current method of enrichment

White and brown mushrooms have similar macronutrient compositions, however only brown mushrooms are currently enriched with UV-irradiation to increase vitamin D content as it causes discolouration in white mushrooms, affecting saleability (Reis et

al., 2012). UV-irradiation works by exposing the mushrooms to UV-B or UV-C (Taofiq et al., 2017) wavelength light to catalyse ergosterol (provitamin D2) through photochemical and thermal reactions into vitamin D2 (ergocalciferol) which is called ergosterol enrichment (Simon et al., 2013, Urbain et al., 2016 and PubChem, 2022). This is shown for D2, D3 and D4 in **Figure 1.4**

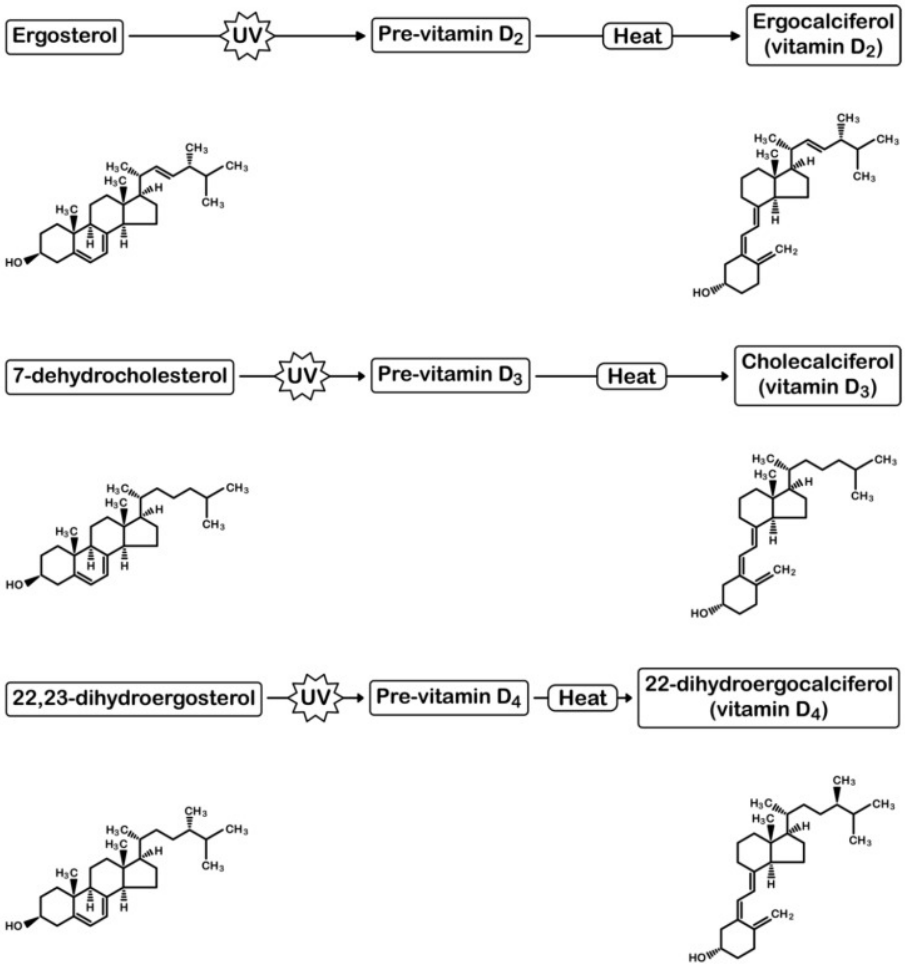

Figure 1.4 Conversion of different provitamins into vitamin D2, 3 and 4 by UV-irradiation (from Cardwell et al., 2018).

Ergosterol is an important membrane protein analogous to cholesterol and regulates fungi membrane fluidity (Walker and White, 2017). Mushrooms grown in/exposed to sunlight such as wild mushrooms will also contain vitamin D because of this same process, however generally mushrooms are cultivated in the dark in controlled climate conditions, but UV treatment can achieve equivalent vitamin D levels to wild mushrooms (Simon et al., 2013). *A. bisporus* has the lowest rate of conversion of ergosterol to vitamin D2 of cultivated species, the highest rates seen in oyster and

shiitake varieties (Jasinghe et al., 2007), although *A. bisporus* shows the highest levels of the precursor ergosterol. The orientation of the mushrooms also makes a difference to conversion of ergosterol, with gills exposed rather than caps, conversion is 4x higher with optimum conditions at treatment of 78% moisture content and 35°C (Jasinghe and Perera, 2005).

UV-irradiation is commonly practiced in developed countries where higher inputs are more easily achievable and is considered practical, safe, efficient, and reliable (Simon et al., 2011 and Simon et al., 2013) for benefitting consumer health by enrichment with vitamin D (Koyyalamudi et al., 2009b). Most achieve high doses with short timings and high intensity. Vitamin D produced in this manner usually has a postharvest stability of roughly 1-4 days and has a decay rate contestant of 0.025 h^{-1} (Roberts et al., 2008). **Figure 1.6** shows the typical UV-irradiation equipment for packaging lines, such as those used at Drinkwater Mushrooms Ltd.

Figure 1.5 Example conveyer UV-irradiation system for installation on packaging lines (from JenAct, 2022).

The main issue with UV-irradiation as a common practice of enriching mushrooms with vitamin D is the associated costs, including energy, replacing UV lamps and carbon emissions, as well as issues with slowing packaging processes to allow UV-doses. UV can also not be used for white capped mushrooms as they become discoloured as previously mentioned, whilst also being a larger market segment than brown capped mushrooms (Sumit, 2022 and Verified Market Reports, 2022), a hindrance to the positive effects for public health. The UV lamps use an estimated 874 kWh of electricity per week (see **Appendix D**) and must be replaced between 4-5 times a year at a cost of £1000, this equates to an estimated £9090 and 14,043 kg of CO_2 equivalent emissions per year. The lamps also lose intensity over time so mushrooms irradiated towards the end of the life of the bulb will have lower vitamin D contents (Drinkwater Mushrooms Ltd, personal communications, 2022).

Packing is also reduced by UV treatment, from 110 packs per minute to 80, slowing production (Drinkwater Mushrooms Ltd, personal communications 2022). Therefore, a new 'during-growth' applied product to provide vitamin and mineral enrichment would greatly benefit both producers, consumers and the environment. Vitamin D distribution throughout the punnet of mushrooms will also likely be more even than when irradiated. There are other practices with similar results such as irradiating with gamma-radiation which also has the benefit of increasing shelf-life by killing microbes. Additionally it was found to increase the measure of umami flavour of the mushrooms as well (Tsai, Mau and Huang, 2014).

1.2.3 Quality preservation and shelf-life

Quality preservation is a major issue in mushrooms as they decay quickly and have a relatively short shelf-life. The causes are commonly humidity (Ares et al., 2007) and temperature due to mushrooms having no barrier against water loss combined with continued high respiration and biochemical processes. White caps often turn brown and only have a shelf-life of roughly 1-3 days at normal ambient temperatures (Singh et al., 2010) and 3-5 days at 2°C, making them highly perishable with characteristic aspects such as veil breaking, sliminess and browning etc. (Surabhi and Devina, 2014), with the main cause of browning identified as tyrosinase activity (Nerya et al., 2006). Many treatments seek to improve preservation during storage

such as modified atmosphere packaging, tyrosinase inhibitor additions, ozone treatments and humectants (moisture retainers) for example (Singh et al., 2010), some of which could possibly be included in supplements. Other methods use anti-browning solutions and electron-beam irradiation for killing of microbes and prevention of spoilage which results in longer shelf-lives and increased satisfaction for customers (Yurttas et al., 2013). Post-harvest storage has many different processing options to try and improve shelf-life and slow spoilage. These can include simple refrigeration which is common for large producers, modified condition packaging (atmosphere and humidity) and washing with reducing agents such as sodium sulphite to inhibit polyphenoloxidases and subsequently reduce browning and microbial load (Wakchaure, 2011).

There are many methods tested and employed to try and increase quality preservation as well as reduce the decay of nutrients within the mushrooms or improve their stability to increase/prolong shelf-life. The nutrient profile is heavily affected by pre-treatment and storage time. Research shows that vitamin B1 was the most affected, and decayed most quickly, D2 was stable (Bernaś and Jaworska, 2016, Cardwell et al., 2018 and Nölle et al., 2017), but only for 24 hours before reducing by 50% over 7 days, even in cold storage, due to photodegradation (Salemi et al., 2021). Hybrid methods are recommended, implementing both thermal/physical and chemical solutions as well as novel, non-thermal methods such as ultrasound treatments. UV has been shown to increase shelf-life somewhat by reducing microbial load (Zhang et al., 2018). interestingly, other studies found that vitamin D2 produced by UV-irradiation has also been shown to be stable for 24 hours and after cooking (Salemi et al., 2021), but for 10 days at 4°C, with vitamin D2 levels increasing for the first 6 days (Slawinska et al., 2017), though this disparity in results is not explained. Ergosterol increases over 14 days of cold storage whilst vitamin D in the caps decreases, but increases in the stems (Guan et al., 2016). Therefore, it is important to determine the impact of novel enrichment methods on shelf-life, deterioration, discolouring and loss of firmness of treated mushrooms which are all predicted to be improved due to a predicted improvement in dry weight/matter and nutrition resulting from the use of novel supplement treatments (Sassine, 2021). Potential improvements in packaging efficiency/speed must also be considered

allowing shorter harvest-to-sale periods and increasing overall shelf-life for the consumer.

Different storage methods are also more applicable depending on the type of product. For raw mushrooms sold directly to the consumer, refrigeration and packaging are most common, but other mushroom products, drying and dehydration processes may be used, with greater nutrient retention as a result (Tian et al., 2016 and Sławińska et al., 2016). Some drying processes can also be used whilst converting ergosterol to vitamin D2 through use of thermal drying in the presence of UV (Jiang et al., 2020), which could be suitable for some enriched products, or the production of vitamin D2 for other purposes. Freeze drying and microwave vacuum drying are usually considered the most effective and energy efficient forms (Pei et al., 2013).

Drying methods are also very important when considering testing of samples. Fresh samples can be tested but commercial labelling and accuracy of quantification is often presented as a concentration in dry weight, which requires dehydration of the samples for accuracy. The most popular choices are freeze or oven-drying, however these can present issues such as nutrient decay resulting from the process itself, or timing of the processes. Freeze-drying has been seen to show no change in ergosterol or vitamin D2 (Yadav and Negi, 2021) but other literature suggests a reduction in vitamin B3 as a result of freezing and a recommendation to not store frozen B-vitamin samples more than 6 months (Bernaś and Jaworska, 2016). This is not a vitamin of interest in this experiment and for sample testing flash-freezing in liquid nitrogen and storage at -80°C is used, rather than conventional cryogenic freezing at -30°C. Consequently, the relative merits of freeze-drying compared to both oven-drying and the testing of fresh samples has been considered in selecting methods for sample preparation and storage.

1.3 Enrichment Targets

Many different vitamins are of keen interest for enrichment or fortification in food. Often those where deficiency rates are increasing, especially in the elderly or where some are difficult to acquire, depending on different dietary restrictions, such as

vegan and vegetarian diets which struggle with getting mainly meat or dairy-derived vitamins and minerals like B12, Se and zinc (not used in this study) (Zeuschner et al., 2012).

The following section will describe the various benefits around some vitamins and minerals of interest and their current status in mushrooms in terms of content or enrichment.

1.3.1 Vitamin D

Vitamin D is a very important fat-soluble vitamin and the main subject for enrichment in this study. Low intake is common in the UK due to lack of sunlight exposure and insufficient dietary intake. Deficiency in the UK has a rate of 19% of men and 15% women, ages 19-64 (Bates et al., 2016). Mushroom derived vitamin D2 supplementation, from UV treatment, has been shown to be effective at maintaining and supporting intake in active individuals, as well as in improving vitamin D status in low intake individuals (Pinto et al., 2020). A study by Stepien et al., 2013 showed that supplementation with enriched mushrooms could increase D2 intake by 128%. In regard to human health, D2 is sometimes seen as less effective than D3 for supplementation. When comparing human blood serum, D3 was found to raise levels of 25-hydroxyvitamin D (measure of vitamin D status in blood) by a greater amount (Houghton and Vieth, 2006). However, D2 does not show effects of hypercalcaemic properties that is sometimes seen with D3 supplementation (Tiwari et al., 2021).

1.3.2 Vitamin B12

B12 is a water-soluble B-complex vitamin, it is not directly produced by the fungi and instead thought to be provided by interaction with microbes in the environment (mainly concomitant bacteria) in which the mushrooms are grown (Koyyalamudi et al., 2009a). Cobalamin is a very important vitamin for human health and is involved in many essential activities and functions such as regulation of the nervous and immune systems (Calderón-Ospina and Nava-Mesa, 2019 and Gay and Meydani, 2001). Vegans, vegetarians, and the elderly are considered high risk for deficiency, as B12 is not produced by any plants and only accumulates in animal and fungi

tissues, with *A. bisporus* having a good uptake efficiency (Watanabe and Bito, 2017). B12 can also be used as a key biomarker for nutrition and public health and is often deficient in strict vegetarians (Allen et al., 2018 and Zeuschner et al., 2012). Fortification in foods and supplementation is encouraged to aid these deficient subgroups due to its functional benefit and increasing risk of deficiency with age (Fleming, 2017 and Allen, 2008), as well as many other benefits such as correlation with reduced risk of chronic diseases and combinations with folic acid to reduce birth defects for pregnant women (Ryan-Harshman and Aldoori, 2008, Dror and Allen, 2012 and Zeuschner et al., 2012).

1.3.3 Vitamin B1

Thiamine is another water-soluble vitamin essential for human health. It has far less risk of deficiency in developed countries than B12 but B1 deficiency can still be common in nutritionally compromised populations and can even be supplemented in diets to benefit recovery from sepsis showing further nutraceutical potential of mushrooms enriched with this vitamin (Moskowitz and Donnino, 2020). B1 is available in plant-based foods so does not pose the same risk of deficiency in meat-reduced diets but is still very important for neurological and nervous system function (Calderón-Ospina and Nava-Mesa, 2019). Addition or enrichment in plants has also been seen to increase crop yields (Fitzpatrick and Chapman, 2020) which may be applicable to supplementation in mushrooms as well. B1 is required in concentrations of 10^{-6} (g per g of mushrooms) for growth as well as biotin (B7) at concentrations of 10^{-10}. These B-complex vitamins are usually produced by microbes in the compost or substrate (Calvo, 2010).

1.3.4 Vitamin B2

Riboflavin is another vitamin with which enrichments have been widely tested through additions to substrate and shown to have many benefits to yield and disease resistance (Guhr et al., 2017). Riboflavin is a water-soluble antioxidant important to human health and function, usually of low risk of deficiency except in more vulnerable groups such as the elderly and vegetarians, though not to such extremes as B12 due to wide plant-source availability (Powers, 2003). B2 can also be important for the crop itself, involved in stress-priming and allowing for better

respiration during stress conditions such as drought, though as cultivation conditions are controlled with mushrooms, this is likely not an issue or of particular benefit of addition. Mushroom nutrition is not enhanced particularly by these additions as it accumulates mainly in the hyphae rather than fruiting bodies but may still be beneficial (Guhr et al., 2017), the exact pathway and translocation systems require more research to fully understand. In general, B2 and B3 (niacin) are the most abundant vitamins in mushrooms (Bernaś and Jaworska, 2016).

1.3.5 Vitamin B6

Pyridoxine/pyridoxal/pyridoxamine is again a water-soluble vitamin used in many essential coenzymes. Deficiency in B6 is often because of renal failure and alcohol abuse and not often dietary lack, but it can be very important to maintain during pregnancy (Abosamak and Gupta, 2020) and to maintain maternal health and healthy foetal development (Dror and Allen, 2012). Deficiency often affects neurological function, especially age-associated dementia and is thought to contribute to mental decline so supplementation to the diets of the elderly could be especially promising (Malouf and Grimley Evans, 2003).

1.3.6 Minerals

Global mushroom cultivation shows great diversity in macronutrients but similar quantities of micronutrients, with the most variation in Ca, K, Mg, Na and S. Mushrooms are also far more efficient than plant and animal tissues at accumulating minerals and assimilate them well in the edible fruiting bodies (Nagulwar et al., 2020) so biofortification is a practical proposal (Siwulski et al., 2020). Many enrichments of mineral have been tested, mainly involving Se. Issues arise with co-occurrence with toxic metals which are also taken up and accumulated, and many papers seem to display erroneous data when determining the compound concentrations (Falandysz and Borovička, 2012). Se is of particular importance and highlighted as an aim for enrichment in this study as deficiency or low intake is common in the UK. Se deficiency is linked to higher risks in many pathologies and cancers due to its involvement in proper immune function (Jackson et al., 2003).

1.4 Factors Affecting Enrichment

1.4.1 Vitamin and mineral uptake

The uptake and translocation of added vitamins and minerals to the fruiting bodies is extremely important in order for nutrition to be supplied in the edible mushroom, whilst unwanted minerals are excluded. Efficient uptake may be achieved through the addition of vitamins alongside fungal-required vitamins as is the basis of this study. Extreme bioaccumulation can occur in mushrooms especially among some metal ions such as K, Na, Zn, Cu and Mn, with differences in concentrations between caps and stipes (Georgescu et al., 2015). It has been shown that specific carrier molecules may be responsible for this and could possibly be employed for enrichment and bioaccumulation of certain minerals in the fruiting bodies (Vetter, 2019). Supplementation experiments are usually done by modification of the substrate/growing medium and can be a practical way to increase concentrations in the mushrooms (Rzymski et al., 2016). It is noted that timing and application of supplements are essential and *A. bisporus* is receptive to improving nutrition in this way. However, the main drawback to this method of supplementation is uneven and decreasing results between flushes, which makes it hard to employ and make claims in a commercial environment (Rzymski et al., 2016). Multiple-application 'spray-on' supplements may be able to improve this through continued addition of nutrients throughout growth and between flushes. There is also lots of interest in bioinoculants to improve the microbiome of the substrate, which could improve nutrition of vitamins, like B12, which are provided for by microbes (Carrasco et al., 2018).

Se has been used for many enrichment tests with different application methods. Maseko et al. (2013) showed that irrigation of sodium selenite solution onto the compost increased mushroom content from 13.8-60.1 µg to 14.1-137 µg Se per g (dry weight) with Selenocysteine (SeCys) accumulating most in the fruiting bodies of *A. bisporus*. *A. bisporus* is considered a very good accumulator of Se and converter of inorganic to organic and available Se which can be used to produce Se-rich food products (Witkowska, 2014).

K accumulates more in caps than stipes of *A. bisporus* which has the highest natural K values of cultivated mushrooms tested and a 100:1 ratio with sodium (Na). Zinc

(Zn), Copper (Cu) and Manganese (Mn) also accumulate in deceasing concentrations respectively, these are all done through specific binding organic molecules which can be utilized to achieve extreme bioaccumulation in some cases (Vetter, 2019). Cu, Zn and Nickel (Ni) has high transfer to fruiting bodies, whereas Ca is not very bioavailable in comparison and has a much lower concentration in mushrooms even when relatively high in the substrate. This is likely due to a more inefficient uptake translocation pathway (Lee et al., 2009). There is evidence that there is no dependence between bioaccumulation of metal ions and growth and that iron (Fe) accumulates to a greater extent than Zn (Umeo et al., 2020). Mushrooms also show bio-exclusion from fruiting bodies of some metal ions leading to reduced content in the fruiting bodies, these include Arsenic (As), Beryllium (Be), Ca, Cadmium (Cd), Cobalt (Co), Chromium (Cr), Iron (Fe), Mn, Ni and Silicon (Si) (Rasalanavho et al., 2020).

Uptake and translocation are important subjects in supplementing and enriching nutrients in mushroom cultivation. Hyphal tips (apical hyphae) are the primary site of uptake and nutrients are transported around the mycelium by tip-directed pressure-driven mass flow for translocation of water and solutes (Herman and Bleichrodt, 2021). They are also the site of mineral sensing and selection before control of fruiting body nutrient selection which occurs at the gill interface (Gramss and Voigt, 2013). Tip selection is achieved through a combination of biomechanical and biochemical action and adhesive forces to access, target and uptake minerals from the substrate (Li et al., 2022). Redistribution of nutrients throughout the mycelium is essential for foraging, directing growth and forming mushrooms/fruitification (Bleichrodt and Wösten, 2022). The nutrients are absorbed by the vegetative hyphae, which must then by translocated to the fruiting bodies facilitated by mycelial cords and directional growth aids, which translocate to zones of growth (Herman et al., 2020). These may prove to be useful acting as chaperones in novel supplement/enrichment approaches. Ca chelator addition has been shown to improve utilization of nutrients absorbed, which could also be beneficial as it can reduce Ca accumulation, which has been shown to block hyphal tips and limit yields in later flushes (Beyer et al., 1998).

1.4.2 Mushroom formation

The ability of supplements to improve yield and provide better control of fruitification timing have been explored. Many studies have been conducted into the causes of fruitification, mainly impacted by the conditions of the casing layer such as microbes, decrease in nitrogen availability and moisture conservation to allow transport of dissolved nutrients (Murmu et al.,2020), combined with nutritional exhaustion of the substrate (McGee, 2017 and Kües and Liu, 2000). Flushing is also induced by depletion of specific nutrients by outgrowing primordia as the process is demand-driven rather than supply-driven (Straatsma et al., 2013). No phototropic response is required for fruiting and microbes are widely considered to induce fruitification through removal of a mycelial-generated inhibitory factor (Baars et al., 2020). This points to possibilities of bioinoculants for use in greater controlling fruitification timing beyond changes to conditions currently used like lowering temperature (down 5°C to 16-18°C) and changing CO_2 concentration, humidity and salinity. These affect the formation and maturation of hyphal knots leading to fruitification (Kües and Liu, 2000). An example of condition changes throughout the different phases of the cultivation process can be seen in **Figure 1.7**.

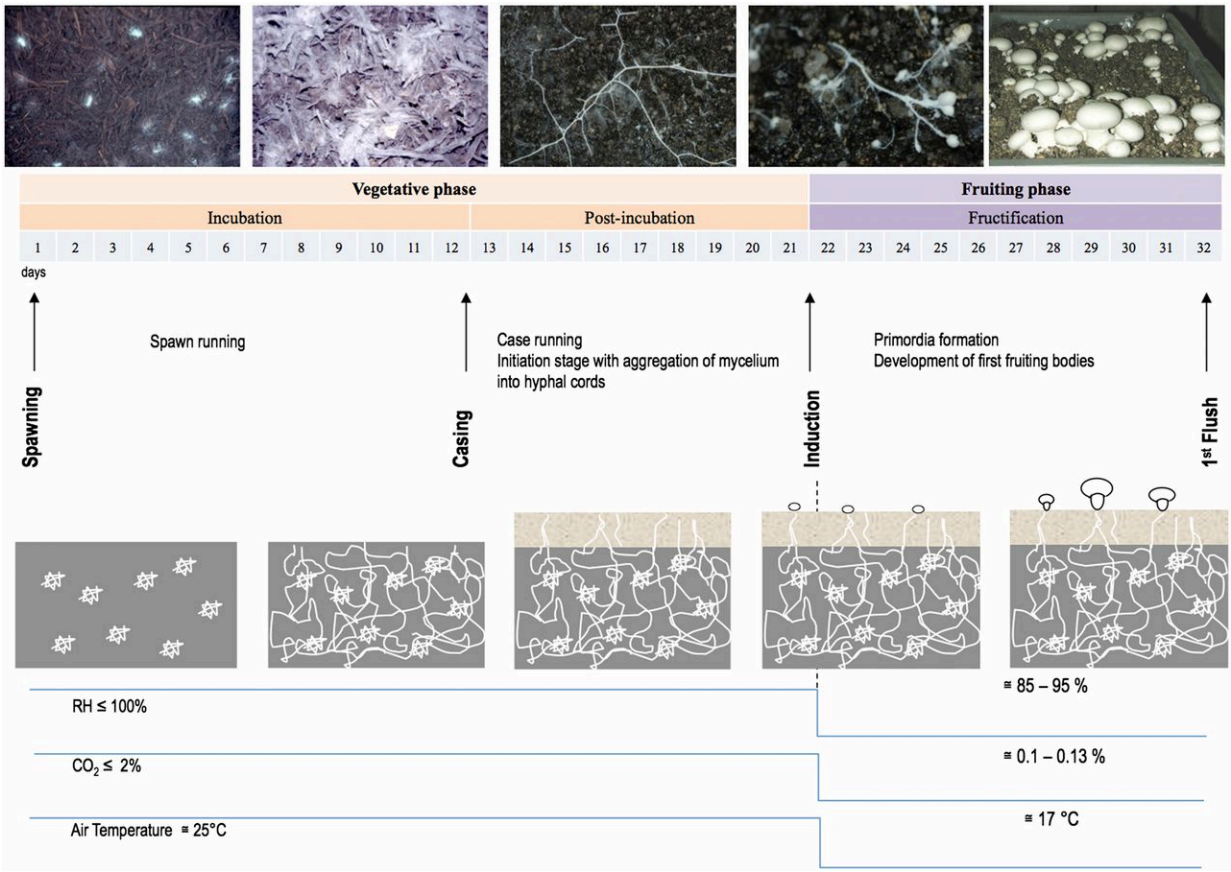

Figure 1.6 Timing and phases of mushroom cultivation up to the first flush with stages of development with condition optimums (from Morin et al., 2012).

Biogas digester liquid (BDL) used as a nutrient supplement has been shown to increase yields, biological efficiency (by 66%) and uptake efficiency of nitrogen (N), carbon (C) and phosphorous (P), and to reduced cultivation times (Malayil et al., 2016). Ca chloride and lactate irrigation together as a liquid spray has been shown to increase the dry matter of mushrooms and yield, whilst Ca lactate alone resulted in a decrease in yield (Kałużewicz et al., 2015). This raises the possibility that further additions to an irrigated supplement could result in greater increases in yield and control of fruitification, assuming nutritional additions do not interfere with flush induction by limiting nutritional exhaustion, which would likely be dependent on application timing, and should therefore be applied at the beginning of a new cycle/after a flush is harvested. Alternatively, the inclusion of delayed release supplements applied at different times so as to not interfere with specific nutritional availability conditions required for spawning and mushroom formation could provide the required yield increases and control of fruitification (Carrasco et al., 2018).

Enriched culture mediums have shown greater bioaccumulation of nutrients and vitamins in the fruiting bodies of later crops compared to those spawned on non-enriched mediums in *A. bisporus,* showing that optimization of liquid culture can also increase uptake of nutrients in future spawn (Krakowska et al., 2016). Application timings are very important for the uptake of nutrients due to food source cycling and timings of respiration and activity bursts which synchronise with hyphal fusion and growth, also having effects on associated gene expression (Vos et al., 2020). This allows for greater efficiency of substrate degradation by timing the application of certain nutrients to coincide with this cycling. Vegetative compatibility if multiple strains are used can greatly affect rates of anastomosis and proteomics which in turn affects the cycling, uptake, and translocation of nutrients (via cytoplasmic exchange) and subsequently fruitification timing (O'Connor et al., 2020).

In general, higher nutrition supplied, and therefore higher nutrient availability leads to greater uptake, higher yields, and better nutritional quality of mushrooms (Sardar et al., 2017). As such, much of the research conducted has been looking at different substrates and their nutritional availability and the effect this has on the mushrooms produced. This is exemplified in the results of Sardar et al. (2017) which showed that cotton waste substrate had the highest biological efficiency which was reflected in the highest antioxidant activity and accumulation of phenols in the mushroom crops produced. There is also evidence of the effects of supplements applied to the casing layer with corn and soybean resulting in increased levels of vitamins, protein and antioxidants in fruiting bodies (Adibian and Mami, 2015).

1.5 Project rationale and potential

1.5.1 Product rationale

NutriGain Ltd has previously developed a basic MycroNutrient product formulation (without vitamins D, B12 and B6 or Se) which has been shown to result in both yield and dry weight increases in white-cap mushrooms (Noble and Dobrovin-Pennington, 2018). The aim of this study is to investigate the viability of a new agri-tech product from NutriGain Ltd. to enhance the vitamin D, B complex and mineral content of commercially grown mushrooms, both brown and white capped, in collaboration with Drinkwater Mushrooms Ltd.

Enrichment of the mushrooms focuses on vitamin D, B complex and minerals, especially Se. This would be done in a more easily applicable way which is more beneficial both economically and environmentally, as well as providing benefits to energy usage, packing speeds, compost fill rates and nutrition distribution within punnets. This would subsequently improve carbon costs and would provide opportunities in increasing sustainability of the industry.

The main priority is vitamin D as this is the currently enriched value product already on the market and which the new supplement will seek to replace the enrichment process of UV irradiation of the mushrooms. UV produces D2 in mushrooms, but in these experiments, D3 is applied due to its benefits for human health and easier supply access. Further work could be done in the future to test with D2 which may be favoured for uptake and translocation to fruiting bodies as it is known to be a common vitamin present in mushrooms grown in sunlight and undergoing UV-treatment, unlike D3 which is not usually present in mushrooms. Applied vitamin D2 or D3 in the product would likely be a synthesized form. For D2 this is most often done through the treatment of fungal material like hyphae and mycelium and un-harvested fruiting bodies from mushroom cultivation which then undergo UV treatment by the same principle as enrichment of mushrooms (Moyad, 2009). However, this is on a much larger scale and can be done more efficiently as it does not impact food production packaging lines and does not require whole mushrooms, therefore it is not so energy intensive. Vitamin D3 is synthesized in a similar manner using cholesterol which is most commonly sourced from lanolin in sheep wool (National Institute of Health, 2023). These systems could show the opportunity for circular and sustainable methods for the use of mushroom production waste to feed into the more widely applicable enrichment of growth and nutrition in the consumer product, whilst also reducing overall emissions and energy use especially for the grower.

Other benefits may be found with yield improvements, changes to mushroom formation and fruitification timings, as well as improvements to flush and punnet heterogeneity of nutrient distribution etc. Yield and dry weight increases have already been seen in the basic form of NutriGain's Mycronutrient product formulation (without vitamin D etc.). It is also found that nutrients in the top third of the substrate

are used for the first flush but the bottom two-thirds are used for subsequent flushes (Sonnenberg et al., 2022). This would suggest that any novel supplement products applied in this manner would give the greatest benefit to first flushes due to application on the casing. Though this is likely only to be an issue with a single supplementation and further applications, as used with MycroNutrient, would benefit the second and third flush to give a more uniform result.

There is also likely a commercial benefit to expand the market for exports of the new novel version of MycroNutrient (with vitamin D etc.) having the potential to aid in sustainable development, nutrition, and food security in developing countries (Tiwari et al., 2021) through the improvement and easier access to mushroom enrichment. Consumers may also benefit from a reduced premium on nutritionally enriched mushrooms which is a growing market, especially among meat-reduced diets increasing into the future.

1.5.2 Commercial importance

There are also many advantages and possibilities posed by a new ‘water-on’ liquid, ‘in-growth’ mushrooms supplement for the enrichment of vitamins and minerals in commercially produced mushrooms, aside from delivery of commonly deficient vitamins or beneficial minerals. This is true especially in expanding markets of mushroom cultivation in both high and low input (cost and materials) farms, especially as the product is non-toxic and safe to handle and could be applied without specialist spray equipment (NutriGain Ltd, personal communications, 2023). Validation of this supplement would support sales and enable higher value products, importantly including enriched white capped mushrooms, which is the largest section of the market and currently not enriched, as well as reducing operating costs and emissions. It is expected that a more even distribution of vitamins and minerals will be achieved than seen with vitamin D distribution towards the surface from UV treatment. NutriGain exports over 90% of products overseas (NutriGain Ltd. personal communications, 2022), this study could allow them to access new markets in countries like the USA, Canada, much of the EU, Australia, New Zealand and Japan which currently use UV treatments in mushroom production (Tiwari et al., 2021). Another potential market is likely to be India where it could have great effects on

food security and economic growth, already seen in this industry with *A. bisporus* being the most cultivated mushroom in the region (Mehta et al., 2011 and Cağlarirmak, 2011). The product would be particularly suitable for single supplement use in low input conditions due to its potential in yield improvements and 'water-on' application for ease of use.

The eventual goal is that these treated mushrooms would be practical in providing a relevant proportion of vitamin and mineral RDAs at a cost-effective price to retailers and consumers, and at an improvement to current energy intensive UV methods. This is an important priority for the producer NutriGain Ltd. and growers such as Drinkwater Mushrooms Ltd, since large supermarket chains and other retailers are already committed to selling mushrooms high in vitamin D (NutriGain Ltd. and Drinkwater Ltd. personal communications, 2022).

1.5.3 Sustainability and opportunity

Mushroom cultivation is considered a good industry for sustainability already, through the use of lignocellulosic waste as the main substrate, mainly from agricultural practices, as well as an important biotechnological industry with mushroom cultivation referred to as the 'non-green revolution' (Fan et al., 2008). There are also many possibilities for using waste from mushroom cultivation, the spent mushroom substrate (SMS) can be used for fertilisers, compost, animal feed production, bioremediation agents, biofuel, biomaterial production and as an enzyme source etc. (Grimm and Wösten, 2018, Mohd Hanafi et al., 2018, Chiu et al., 2000 and Elisashvili, 2005). SMS is also an aspect of mushroom cultivation which can contribute to offsetting carbon emissions for the grower, however this is relatively small in comparison to overall emissions (Robinson et al., 2018). These possibilities do however improve recycling and contribute to sustainable and circular agricultural practices, helping optimise mushroom production and reduce emissions (Dorr et al., 2021). The quality of the SMS would also be improved by nutritional supplements leading to more possibilities of growers benefitting from these developments with resale of the SMS to other industries.

The largest issue facing the mushroom cultivation industry is the unsustainable practice and difficulty in replacing peat use in the casing layer, currently irreplaceable due to its great water retention and induction of fruitification. Very few replacements have shown promise, with severely decreased yields but research and testing is being carried out for the future, due to the importance of peat's replacement to align with government initiatives. The downsides of using peat alternatives can sometimes be compensated using drip irrigation to maintain water content, and supplement addition to maintain biological conditions (Sassine, 2021). Therefore a new 'water-on' or irrigated product, such as those made by NutriGain Ltd. could help in these ways and provide a new avenue for replacement of peat use in the mushroom cultivation industry. This is dependent on the timing of application, so as to not interrupt the nutritional exhaustion in casing helping to induce the reproductive stage and subsequent fruitification, whilst still allowing transport of dissolved nutrients to the fruiting bodies, which are also added by the product (Sassine, 2021). Another possibility which is undergoing research is the use of treated SMS as an alternative casing layer, which would be very sustainable but less suitable and likely to not achieve necessary yields (Sassine, 2021).

There is also interest in using mushrooms for addition to animal feed as a high protein source (Bederska-Łojewska et al., 2017). This would help to reduce the high carbon emissions from the import of soy products for this purpose and could also be improved by NutriGain's novel product by increasing their nutritional content, contributing to agricultural sustainability in a different way and showing areas for opportunity.

1.6 Aims and Objectives

There are many areas of mushroom cultivation which are a target for biotechnical advances and many possibilities for improving sustainability and opportunities that may arise as a result. This novel MycroNutrient product aims to contribute to all of these factors by enrichment with vitamins for greater nutritional value, improving shelf-life and reducing energy usage and emissions. This gives potential opportunities and possibilities of new enriched products, greater market expansions

and more applicable enrichment to lower input growers which could have sustainable social and economic impacts.

The main objectives for the project relate to testing the dosage rates and efficiency of uptake of nutrients to the mushrooms by this type of novel liquid supplement. Also, this must be compared to the current method of UV irradiation to judge the effectiveness and commercial viability in replacing that enrichment method.

The novel MycroNutrient product utilises a ‘water-on’ application with desired minerals and vitamins which will be applied to the casing layer at certain timings for efficient uptake. It aims to improve cultivated mushroom vitamin and mineral contents before harvest, and without need for extra processing. Therefore, a more efficient and sustainable solution than current methods. This could also benefit public health by making mushrooms a more accessible and healthier source to aid in meeting RDAs and reducing deficiency rates of key vitamin and mineral requirements in the UK and further afield.

1.6.1 Objectives

- Analyse dose response of the product on mushroom vitamin D content and identify an optimum commercial dose for delivery of vitamin D
- Analyse the effect of flush on content of vitamin D
- Identify most effective formulation of product for vitamin D uptake
- Identify most effective formulation of product for B-vitamin uptake
- Analyse the effect of application regime and flush on content of vitamin D
- Compare product effectiveness in comparison to UV and no treatment
- Analyse viability of novel mushroom enrichment in commercial conditions, in terms of carbon emission cost, success of enrichment and ease of application.

2. Materials & Methods

2.1 Growth Conditions

White and brown cap mushrooms (*A. bisporus*) were grown under commercial conditions at Drinkwater Mushrooms Ltd, Hampson Farm, Hampson Lane, Lancaster, Lancashire, LA2 0JB. The substrate used is Phase 3 Compost from Tunnel Tech North Ltd. (UK), this is pre-spawned with strain A15 from Sylvan Ltd. (US). The casing used is peat, from MacGregors Peat, Bogbain Farm, Inverness.

2.1.1 Growing site management

All experimental trials underwent normal management conditions used at Drinkwater Mushrooms Ltd. in climate-controlled grow houses. Mushrooms are grown in approximately 30-day cycles, during which the climatic conditions within the growth house were changed for the different phases of growth and induction of flushes. Growth substrate was treated with water treated with hypo-chloride or SporGon disinfectant (Scully Growth Supplies, Ireland) for sterilization (Drinkwater Mushrooms Ltd, personal communications, 2023). See **Appendix A** for full timetable of standard cultivation regime and conditions.

Total watering for white mushrooms during growth is 40 L/m^2 with each m^2 producing an average of 35 kg of mushrooms over all 3 flushes harvested (Drinkwater Mushrooms Ltd, personal communications, 2023).

2.1.2 Product Treatment application

A total of 180 mL of the novel MycroNutrient product per m^2 was applied in two doses, consisting of:

1st application of 60 mL/m^2 at day 4, just after ruffling of the casing.

2nd application of 120 mL/m^2 after first flush (between days 17-19) just after picking and watering is finished.

All applications were diluted into 1 L of water per m^2, having no significant effect on the normal watering schedule. The diluted mixture was agitated before application to

promote even distribution. Application was performed by hand with simple equipment (e.g. watering can).

2.2 Product Treatments

Experimental trials were performed using different formulation 'carriers', consisting of the standard Ca (water-soluble) base in Trial 1 (T1) and new K (insoluble) base in Trial 2 (T2), using Calcium and Potassium Citrate respectively, in which the vitamins and minerals were applied. In Trial 3 (T3), a '5x' dosage of vitamin D was tested using the K formulation. Each experimental trial also consisted of 2-3 flushes (F1-3). A control was also included in T1 and T2 of a basic MycroNutrient product including the Ca or K base but without the addition of further vitamin D, but still containing B-vitamins and Se. The standard MycroNutrient product contains: Water, Calcium or Potassium Lignosulphinate (Binder), Hydrated Lime, Citric Acid, Propionic Acid, Acetic Acid. The exact quantities and formulation are commercially confidential.

2.2.1 Vitamin D

The dosage concentrations of vitamin D tested are given in **Table 2.1**. These were chosen based on the predicted levels of vitamin D in mushrooms as a result of each treatment based on the assumptions:

1) 5% uptake to mushrooms
2) Dry weight (DW) makes up roughly 15% of fresh weight (FW) (after harvest)
3) All applied nutrients will be evenly distributed between all 3 flushes

Treatments were as follows:

Table 2.1 Dosage rates of vitamin D applied in each section and their predicted contents achievement.

Treatment	Dosage rate (per m^2)	Predicted contents (per 100g DW)
'Low'	31.5 mg	10 µg (RDA)
'Medium'	180 mg	55 µg
'High'	315 mg	100 µg
'5 x (High)'	1575 mg	500 µg
Control (MN)	0 mg	0 µg / trace

The range of predicted concentrations were chosen based on 'nutritionally relevant' contents of vitamin D (Cardwell et al., 2018). 10 µg is the Nutrient Reference Value (NRV), formerly Recommended Dietary Allowance (RDA) per day (NHS, 2020).
The control treatment consisted of an application of the original MycroNutrient product containing no extra additions of vitamins and minerals but still containing the Ca or K base to check that the treatments are the primary factor in a possible increase in vitamin and mineral contents of the mushrooms. Vitamin D was added to concentrated product before dilution as 2.5% in lanolin oil.

2.2.2 Vitamin B and Minerals

The concentrations of B-vitamins tested are given in **Table 2.2**. B-vitamins were added to concentrated product as a premade solution containing 4.78 L water, 220g $MgSO_4$, 220g $ZnSO_4$, 14g B1, 14g B6, 14g B12. Se is added as sodium selenite (Na_2SeO_3) to the concentrated product prior to dilution as well and this addition is recorded as the concentration of Se only.
Subsequent dosage rates are shown below:

Table 2.2 Final dosages of applied B-vitamins and minerals per m^2. Se (selenium)

Vitamin/Mineral	Dosage rate (per m^2)
B1	4.67 g
B6	4.67 g
B12	4.67 g
Se	0.13 g

2.3 Experimental Design

Experiments were carried out in one 'section' of growth bed shelving units within a standard commercial growth shed at Drinkwater Mushrooms Ltd premises. This section measures 1.5 m x 1.32 m (roughly 2 m^2), this was split into 4 sections for 3 treatments and a control (**Figure 2.1**). The areas were separated by metal partitions through the substrate and casing to prevent leakage or sharing of nutrients between the treatments, as such each treatment has an area of 0.5 m^2.

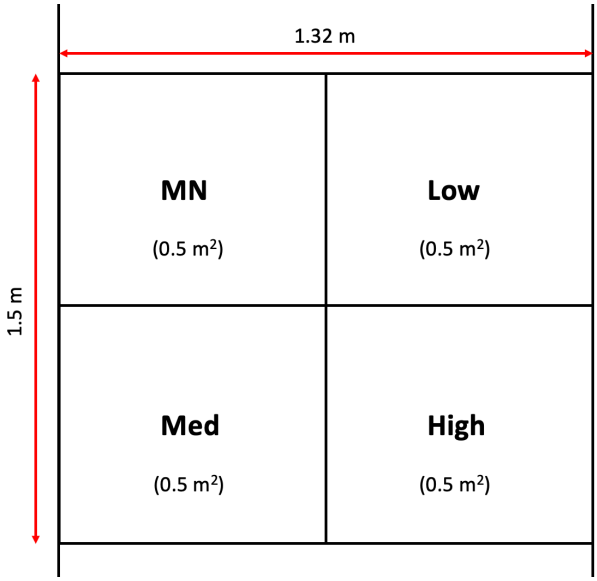

Figure 2.1 Diagram of the experimental layout in the shelves of growth sheds. The labels refer to treatments in first 2 trials (Ca and K formulations), a 3rd trial was also carried out in which a '5 x' vitamin D dosage treatment was tested using the K formulation. MN refers to the base MycroNutrient product without vitamin D addition, Low, Med and High denote the different dosages of vitamin D, as detailed in Table 2.2.

2.3.1 UV treatment

The current method of vitamin D enrichment used by Drinkwater Mushrooms Ltd. is UV treatment which is applied after harvest on packaging lines using a high energy UV lamp irradiating mushrooms with UV-C wavelength light. The UV bulb used was a standard UV-C lamp, 400 V 3-phase supply, 10 A per phase (20.8 kW).

2.4 Sample Preparation

Mushrooms were collected locally from Drinkwater Mushrooms Ltd. in light-proof containers to avoid exposure to sunlight/UV and refrigerated. Samples were cut into smaller pieces and then either flash frozen in liquid nitrogen and freeze-dried or oven-dried at 60°C for 24 hours (or until a constant weight) for storage. Samples were then ground into a powder using a ball mill ready for extractions. Triplicate samples from each vitamin D treatment were taken for vitamin D, B-vitamins and minerals extraction and analysis.

2.4.1 Vitamin Extraction

Vitamins were extracted from prepared samples via different methods, vitamin D extracted separately from a simultaneous extraction of all B-vitamins. Both were

performed in subdued light conditions and out of direct sunlight to ensure little reaction with UV to the light-sensitive vitamins being analysed.

Vitamin D:

Vitamin D was extracted according to Malik et al., (2022) and Cardoso et al., (2021). 1.25g powdered sample was mixed with 5 mL DMSO and vortexed thoroughly. The sample was then ultrasound oscillated in a sonicator water bath for 30 mins and 45°C. 5 mL 50% (v/v) methanol and 10 mL hexane was added before vortexing and oscillated again in the same manner. The sample was then centrifuged at 8000 rpm for 5 mins and the hexane supernatant layer collected. Extraction on the remaining sample was then carried out once more with 10 mL hexane, vortexing, oscillating and centrifuging again and the hexane layer extracted. The hexane extracts were combined and underwent nitrogen blow-down till dryness and then re-dissolved in 1 mL methanol or propan-1-ol (change in method to allow greater solubility of D3) and kept in a freezer until ready for analysis.

B vitamins:

All B vitamins are extracted simultaneously using a modified method by (Albawarshi et al., 2022).

0.1g powdered sample was mixed with 10 mL each of 0.1% (w/v) sodium ascorbate and 0.1% (w/v) EDTA and then vortexed for 1 min. The sample was then heated in a shaking water bath for 15 mins at 50°C then centrifuged for 10 mins at 6000 rpm. The supernatant was collected and precipitate re-extracted with 5 mL of each 0.1% sodium ascorbate and 0.1% (w/v) EDTA and the extraction repeated (vortex, heating and centrifuging again). Supernatant was collected again and combined before storage in a freezer until analysis.

2.4.2 Vitamin analysis

Vitamin analysis was carried out using reverse-phase HPLC with a Photo Diode Array (PDA) detector and C18 columns. HPLC analysis was performed using a Shimadzu Nexera X2 UHPLC instrument consisting of a LC-30AD liquid chromatography pump, a SIL-30AC autosampler, a CTO-20AC column oven, and a

SPD-M30A diode array detector. Both vitamin extracts (Bs and D) were syringe filtered (0.2 µm) before injection of a 20 µL sample by autosampler.

Quantification was achieved using an external calibration curve from 0.005 mg/mL to 0.5 mg/mL, with a limit of quantification (LOQ) at 0.0001 mg/mL. Concentrations given were then calculated for the sample size and extract to be represented in a mass per 100 g dry weight as is the standard for nutritional information.

Data was gathered using associated Shimadzu analytical software to identify peaks, produce graphs, calibration curves and analyse peak areas for quantification calculations.

Vitamin D:

An Agilent Poroshell 120 EC C18 column with dimensions 3.0 x 100 mm, 4 µm film thickness and associated guard column (Poroshell 120 EC) with dimensions 2.1 x 5 mm, 2.7 µm film thickness was used for vitamin D analysis.

Vitamin D3 was detected and quantified at an optimum wavelength of 265 nm using the following conditions: column temperature 35°C, autosampler tray temperature 10°C, flow rate 1.0 mL/min with isocratic mobile phase of HPLC grade methanol for 20 mins per sample. Normal retention time for identification of peak was 1.4 minutes in vitamin D3 standards.

For Vitamin D2 analysis of UV samples, Chromeleon software was used on separate equipment. This equipment was a Dionex ICS-3000 HPLC system consisting of a Dionex ICS-3000 pump, Dionex ICS-3000 DC (degasser) and UltiMate 3000 Variable Wavelength Detector with Dionex AS-1 autosampler. The column used was the Zorbax Eclipse Plus, C18 4.6 x 100 mm, 3.5 µm and Agilent Poroshell 120 EC C18 with dimensions 3.0 x 100 mm, 4 µm film thickness with the same conditions as mentioned previously with the exception of the use instead of an isocratic mobile phase of 70:30 Acetonitrile:Methanol.

B vitamins:

A Zorbax Eclipse Plus, C18 4.6 x 100 mm, 3.5 µm and Agilent Poroshell 120 EC C18 with dimensions 3.0 x 100 mm, 4 µm film thickness and associated guard column (Poroshell 120 EC) with dimensions 2.1 x 5 mm, 2.7 µm film thickness were used for B-vitamin analysis. The PDA detector was set to a wavelength range of 200-400 nm with optimum wavelengths for quantification of each B-vitamin selected for calibration at 250 (B1), 267 (B2), 290 (B6) and 361 nm (B12) using the following conditions: column temperature 35°C, autosampler tray temperature 14°C, flow rate 0.7 mL/min and gradient elution of 0.03% (v/v) trifluoroacetic acid (TFA) and acetonitrile (ACN). The gradient used varied throughout the elution in the profile:

0-2 mins: 100% TFA → 83% TFA and 17% ACN

2-10.5 mins: 83% TFA and 17% ACN

10.5-11.5 mins: 83% TFA and 17% ACN → 100% TFA

11.5-20 mins: 100% TFA

Retention times from test standards were used for identification of peaks and compounds at roughly 2.73 (B1), 6.10 (B2), 4.05 (B6) and 5.77 (B12) minutes with the Zorbax column and 1.20 (B1), 4.45 (B2), 2.10 (B6) and 4.17 (B12) minutes with the Poroshell column.

2.4.3 Mineral extraction

Mineral extraction was performed via microwave-assisted acid digestion as follows: 0.25g powdered sample was mixed with 5 mL nitric acid (HNO_3) and left for 15-20 mins before microwave treatment at 200°C for 30 mins (15 min 'ramp-up') then left to cool for 1 h. Samples were then diluted to 20% (v/v) nitric acid (4 mL Milli-Q de-ionised (DI) water and 1 mL digested solution) for storage and further diluted to a 2% (v/v) nitric acid matrix (9 mL DI-water and 1 mL of the 20% [v/v] solution), syringe filtered (0.45 µm) and refrigerated, ready for ICP-OES analysis.

2.4.4 Mineral analysis

Mineral analysis was carried out using ICP-OES (Inductively Coupled Plasma - Optical Emission Spectroscopy) with a Thermo iCAP Duo view ICP 6300 with wavelength range 166-847 nm. This allows simultaneous detection and measurement of Se, Ca and K. These elements were chosen to identify efficiency

and success of enrichment as well as to check any uptake of carriers and undesired minerals. These are elements involved in and making up the product formulation or as ‘carriers’ for the application of the novel MycroNutrient products, particularly K and Ca.

All samples were put through a 0.45 µm filter before injection of the sample. Detection analysis lines are picked dependent on samples and calibration of a standard within the runs, with further calibration and checks between sets of 10 samples. The analysis lines picked for each element were as follows: Ca 3933, K 7664 and Se 1960-2.

2.5 Validation of Methods

Vitamin standards were run at different concentrations to determine retention time and then create calibration curves for the quantification of HPLC results within the Chromeleon software used for D2 or Shimadzu software used for D3 and all B-vitamins.

Calibration was achieved through standards of the concentrations: 0.005 mg/mL, 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, and 0.5 mg/mL, if still linear in relationship, some examples of these can be found in **Figures 2.2-2.4**. A lone standard at the concentration of 1 mg/mL was used firstly to identify retention time before any mixed samples were employed for calibration and identification in the case of the B-vitamins. Some examples of standards and calibration curves are displayed in **Figures 2.5 and 2.6.** The full set of initial chromatograms are shown in **Appendix B**.

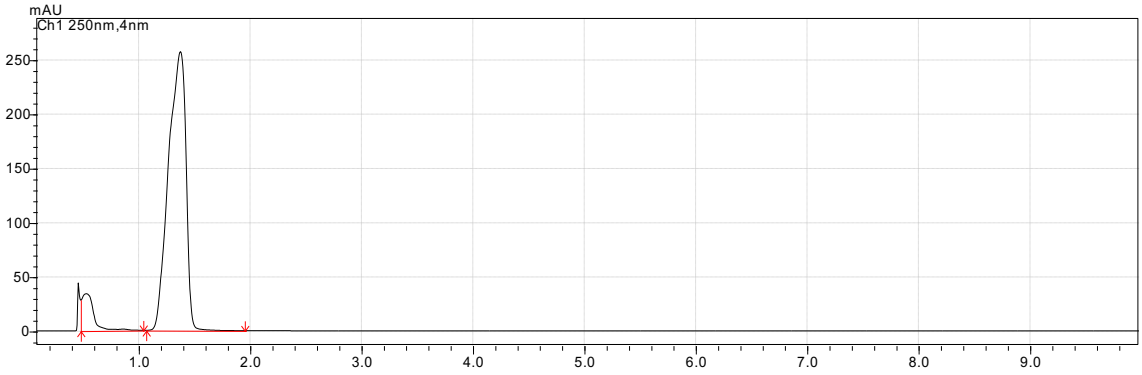

Figure 2.2 Chromatogram of Vitamin D3 at 0.1 mg/mL Detection at 250 nm

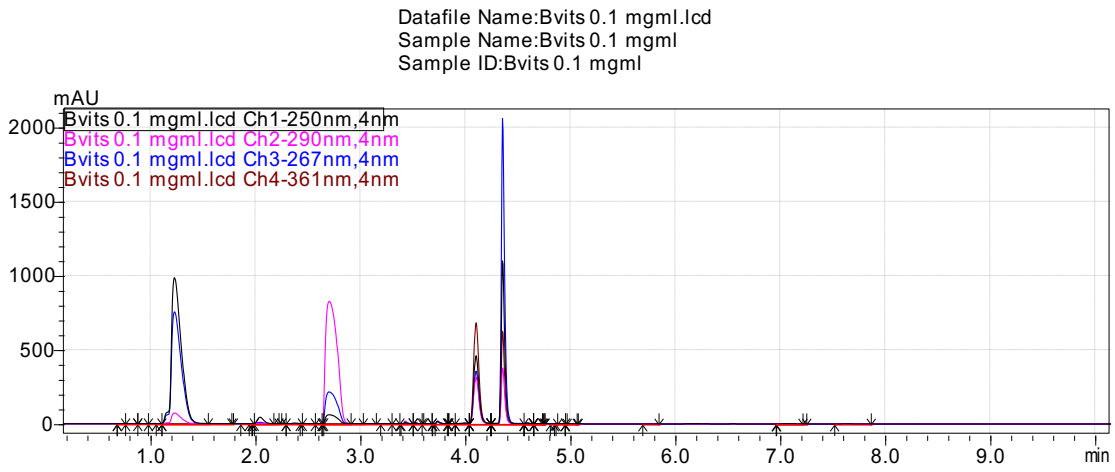

Figure 2.3 Chromatogram of mixed B-vitamins sample at 0.1 mg/mL showing detection at all wavelengths on Poroshell column (see Section 2.4.2)

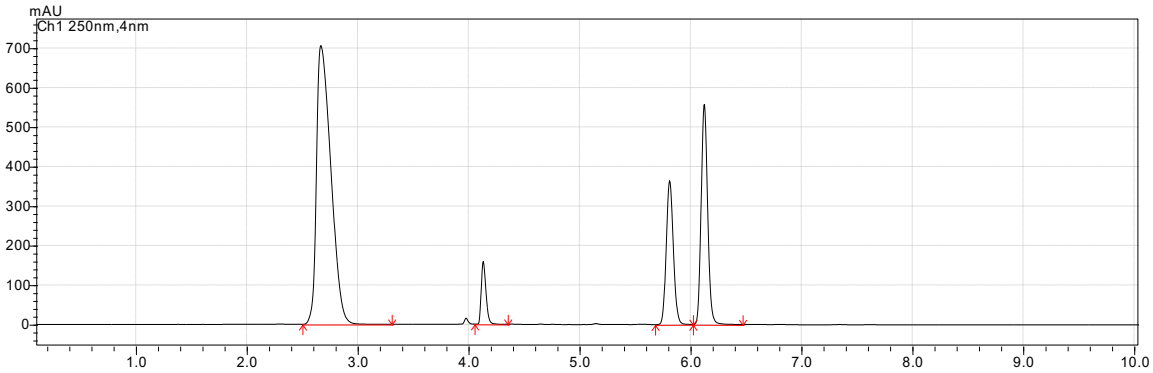

Figure 2.4 Chromatogram of mixed B-vitamins sample at 0.1 mg/mL at one wavelength (250 nm) on Zorbax column (see Section 2.4.2)

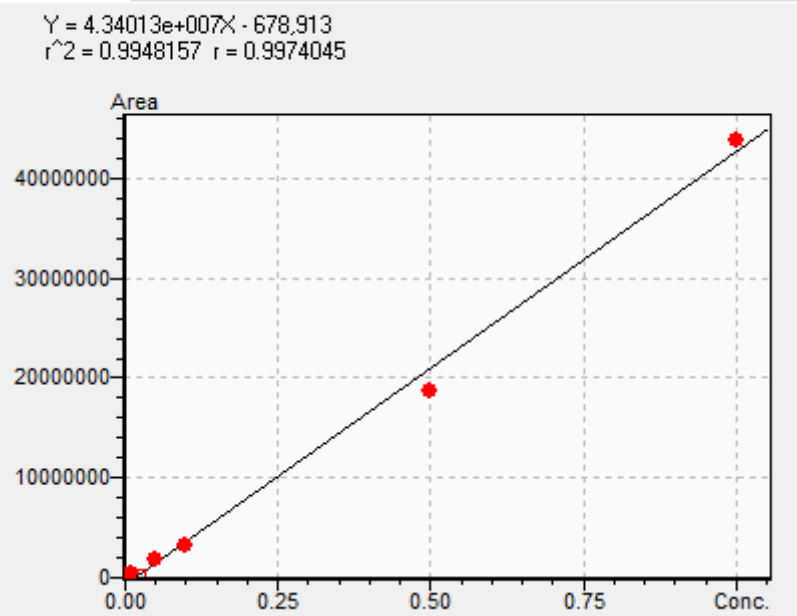

Figure 2.5 Vitamin D Run 1 D3 standard calibration curve.

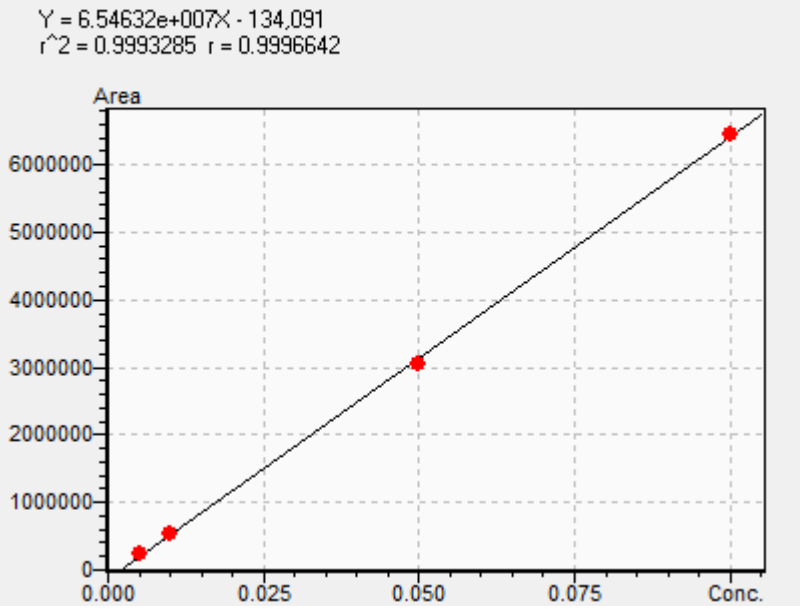

Figure 2.6 B-Vitamins Run 2 B1 standard calibration curve.

2.6 Statistics

Statistics were used to compare data between dosage, flush and formulation where possible. These comparisons between dosages were performed within flushes and flushes compared within trials to ensure robust comparison. Overall averages are

taken for formulations to make comparisons between different groups and to give a most representative value that might be expected from any flush. Statistical tests were also used to compare different dosages and formulations to UV treated samples and untreated controls.

Statistical tests used were One-Way ANOVA in order to identify significance within groups then Bonferroni corrected significance thresholds of 0.05 and 0.01 applied to post-hoc two-sample t-tests (assuming equal variance) to identify differences between means of specific dosage treatments, flushes and formulations. Statistics are reported using thresholds of 0.05 and 0.01.

3. Results

3.1 Vitamin D

The effect of the novel MycroNutrient product on the levels of the fat-soluble vitamin, vitamin D in mushrooms, which is important for Ca uptake, bone health and muscle function in humans (see **Section 1.2.1**), was analysed in three independent experimental trials. The effects of the dose of the product (control (MN), low, medium, high and 5 x high (see **Table 2.1**) and formulation (Ca or K) on the vitamin D content in the different flushes of mushrooms was assessed.

3.1.1 Trial 1

The Ca formulation for the vitamin and mineral carrier within the novel MycroNutrient product was used in T1. Four doses of vitamin D were included and the vitamin D content of mushrooms from F1 and F2 analysed. It was not possible to harvest F3 in this trial due to the growhouse at Drinkwater Mushrooms Ltd used for the trial being steamed after F2 had been harvested.

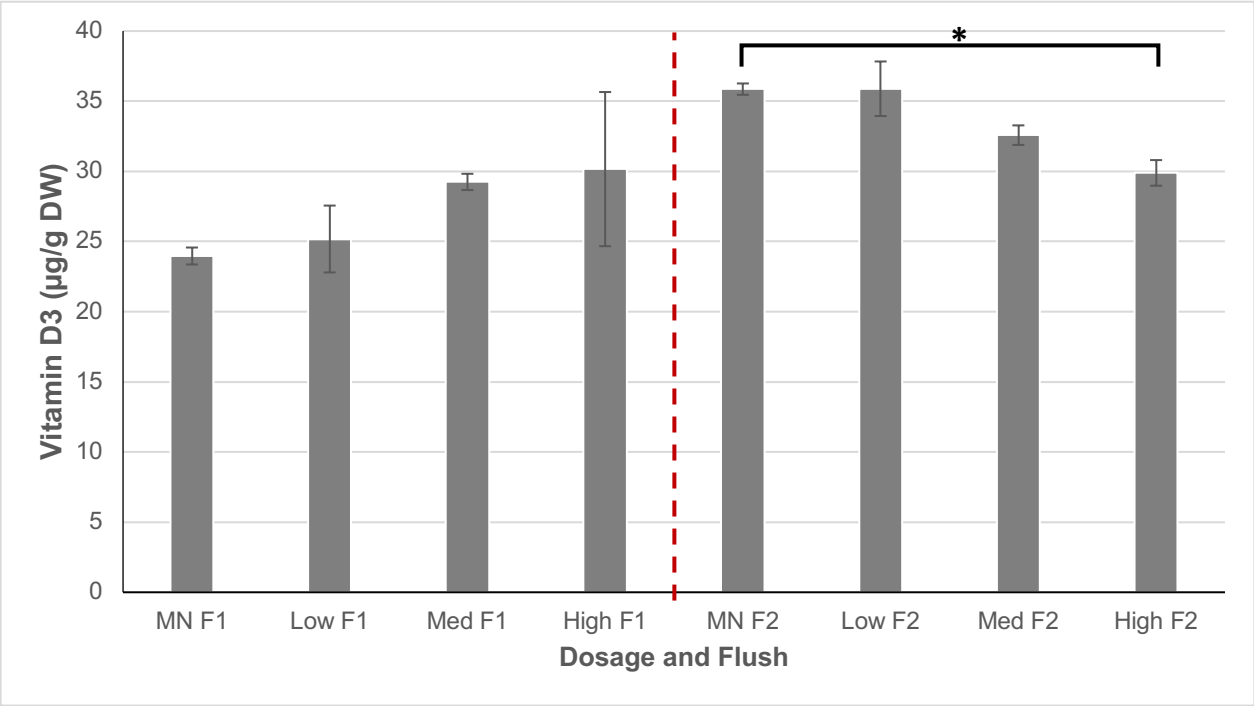

Figure 3.1 The effect of dose of the novel MycroNutrient product formulated with the Ca carrier on the vitamin D content of mushrooms harvested from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3 in all but Med F1 and High F2 where n = 2). The '*' and bracket represents significance (P-value < 0.05) between the dosages MN and High in F2. ANOVA P-values were 0.06 for F1 and 0.036 for F2.

Figure 3.1 shows that in F1, the vitamin D content of mushrooms increased with increasing dose of vitamin D in the novel MycroNutrient product. In contrast, in F2, the opposite was observed with the vitamin D content being negatively correlated with dose. In addition, the vitamin D content of mushrooms in F2 appears to be slightly higher than in F1. However, these trends were not statistically significant; only the difference between the MN (control) and High dosage in F2 was statistically significant.

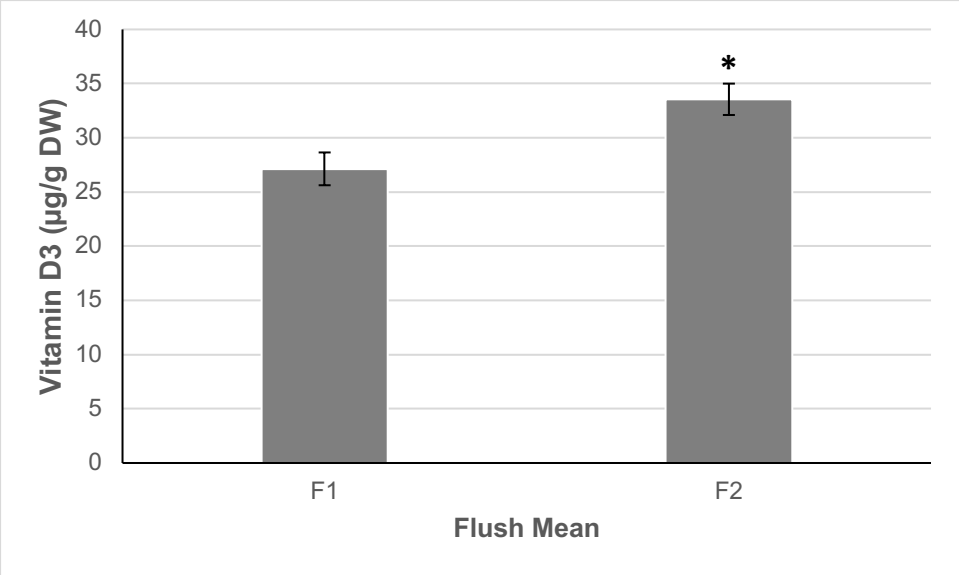

Figure 3.2 The effect of flush (F1-2) on the vitamin D content of mushrooms harvested from the Ca novel MycroNutrient formulation in Trial 1 (T1). Values are means of all dosages within the flush +/- SEM (n = 4). '*' denotes significance (P-value < 0.05).

When the total vitamin D content of mushrooms in T1F1 and T1F2 were calculated, there was a significantly higher vitamin D content overall in T1F2 compared to T1F1 (**Figure 3.2**). This suggests that whilst vitamin D uptake has occurred there appears to be a delay in the uptake of the vitamin although unfortunately, no third flush was taken from this trial and therefore it was not possible to obtain a full picture of the effects of flushes on vitamin D uptake.

Taken together, the results of T1 suggest that vitamin D uptake has occurred in mushrooms although the lack of statistically significant differences between most dosages in both flushes suggests that all sections of the trial have received roughly the same dosage.

3.1.2 Trial 2

The K formulation for the vitamin and mineral carrier within the novel MycroNutrient product was used in T2. This trial again included four doses of vitamin D and the vitamin D content of mushrooms from all three flushes analysed. As **Figure 3.3**

shows, the vitamin D content of mushrooms from F1 were below the detection threshold of the analysis used across all dosages.

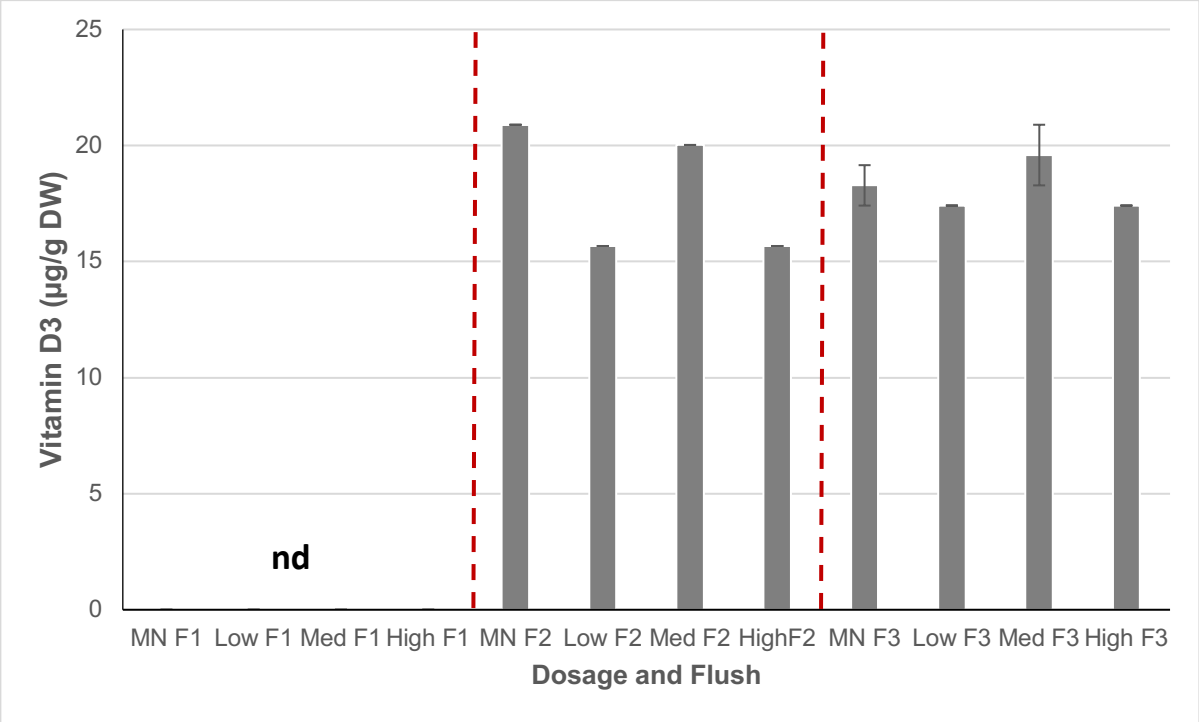

Figure 3.3 The effect of dose of the novel MycroNutrient product formulated with the K carrier on the vitamin D content of mushrooms harvested from flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 1 for all F2 values, Low F3 and High F3 and 2 for MN F3 and Med F3). 'nd' stands for non-detectable, as none of the samples from F1 were detectable in vitamin D when tested. There was no significant difference (P-value > 0.05) between any of the dosage rates within the individual flushes.

Whilst the levels of vitamin D in F2 and F3 were above the detection threshold they were nevertheless lower than for the corresponding dosages in T1 (approx. 15-20 ug/g DW compared with approx. 25-35 ug/g DW). In addition, there were no clear trends in the effect of dose on vitamin D content in either flush (T2F2 and T2F3); there were no statistically significant differences between any of the dosages within flushes and no statistically significant difference between the flushes either (**Figure 3.4**).

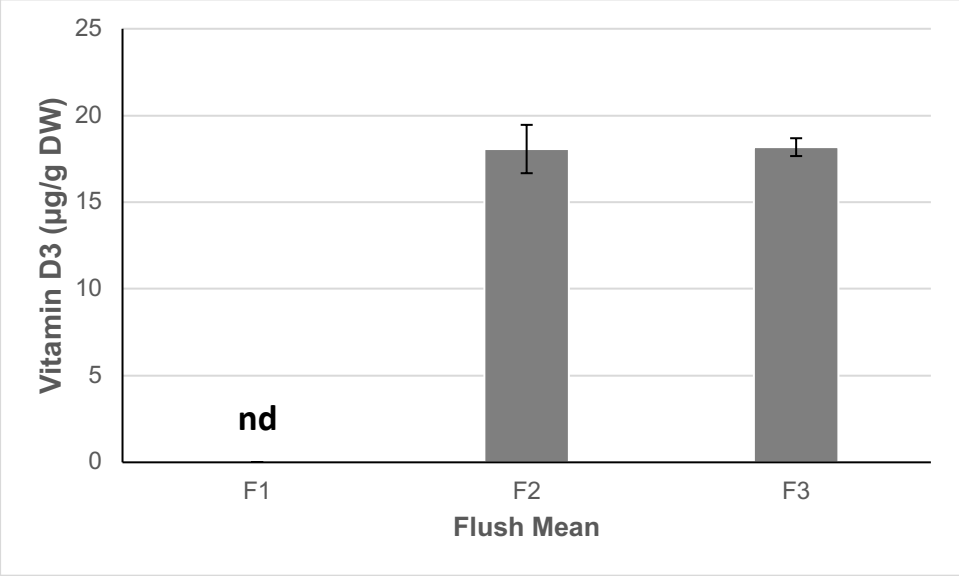

Figure 3.4 The effect of flush (F1-3) on the vitamin D content of mushrooms harvested from the K novel MycroNutrient formulation in Trial 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 4). 'nd' stands for non-detectable, as none of the samples from Flush 1 (F1) were detectable in vitamin D when tested. There was no significant difference (P-value > 0.05) between Flush 2 (F2) and Flush 3 (F3).

When the mean vitamin D content of mushrooms in these flushes were calculated, with the exception of F1 being non-detectable, there was no statistically significant difference in the overall vitamin D content between F2 and F3 (**Figure 3.4**); the low errors calculated are indicative of relatively consistent uptake in all cases. Taken together, despite the non-detectable levels in F1, the results from T2F1 and T2F3 suggest a more consistent effect of the novel MycroNutrient product on vitamin D levels with the K formulation (T2) than with the Ca formulation (T1) shown in **Figure 3.3**, which had a statistically significant difference between the 2 flushes available for analysis, but that the levels of vitamin D achieved are higher with the Ca formulation.

3.1.3 Trial 3

The K formulation for the vitamin and mineral carrier within the novel MycroNutrient product was again used in T3 although this time with only the '5x High' dosage rate of vitamin D. **Figure 3.5** shows the effect of the '5xK' across the three flushes.

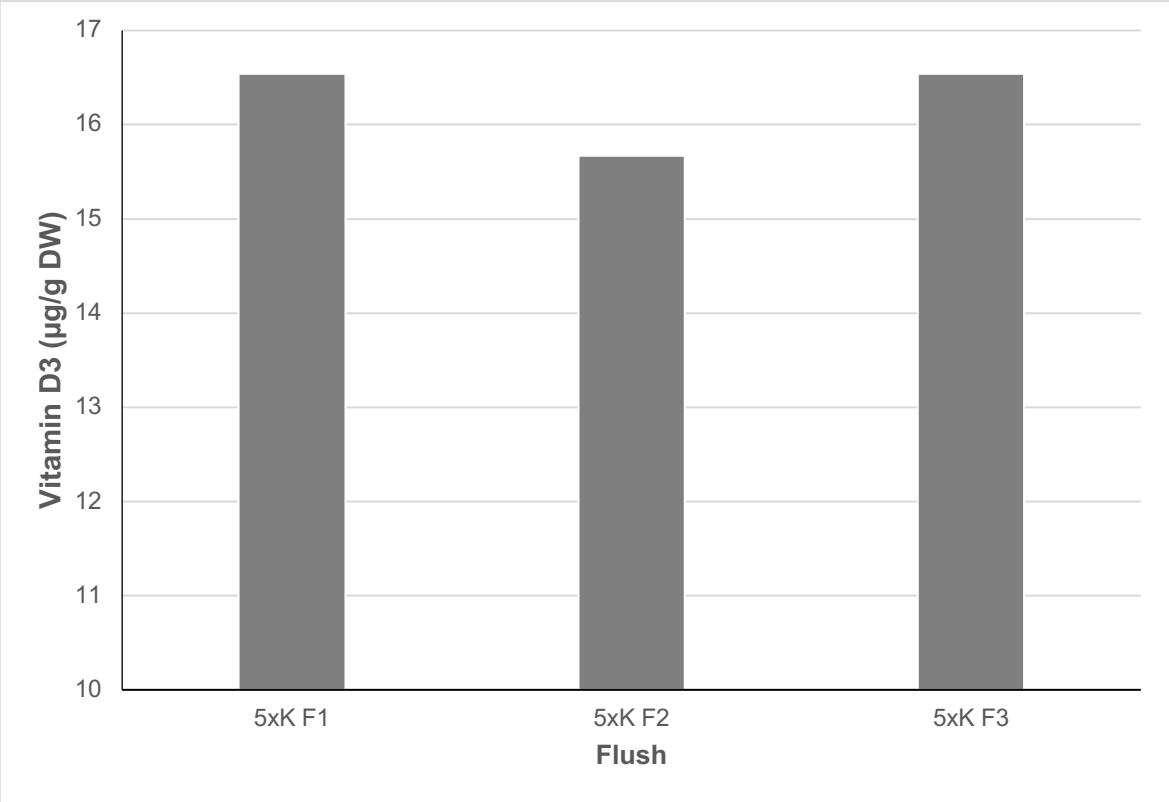

Figure 3.5 The effect of flush (F1-3) on the vitamin D content of mushrooms harvested from the 5xHigh (5xK) novel MycroNutrient formulation in Trial 3 (T3). No error bars are available due to representing single values (n = 1) as others were non-detectable. Also, no significance could be calculated from the single values.

No statistical significance could be tested from these single values, others being non-detectable. The variation between flushes is relatively small, at only 0.8 µg, giving a sense of consistency although this cannot be fully confirmed.

3.1.4 Formulation and Treatment Comparison

When considering which of the Ca or K formulations of the novel MycroNutrient product could be most commercially applicable for the replacement of UV treatment used by Drinkwater Mushrooms Ltd. for the enrichment of mushrooms with vitamin D, it is important to establish a baseline of vitamin D content achieved using their standard UV treatment. However, whereas the novel MycroNutrient product treatments contains vitamin D3 and therefore the D3 content of treated mushrooms is analysed, UV treatment stimulates the production of the naturally occurring form (in mushrooms), vitamin D2, (see **Section 1.2.2**) which was therefore analysed for in UV+ and UV- treatments.

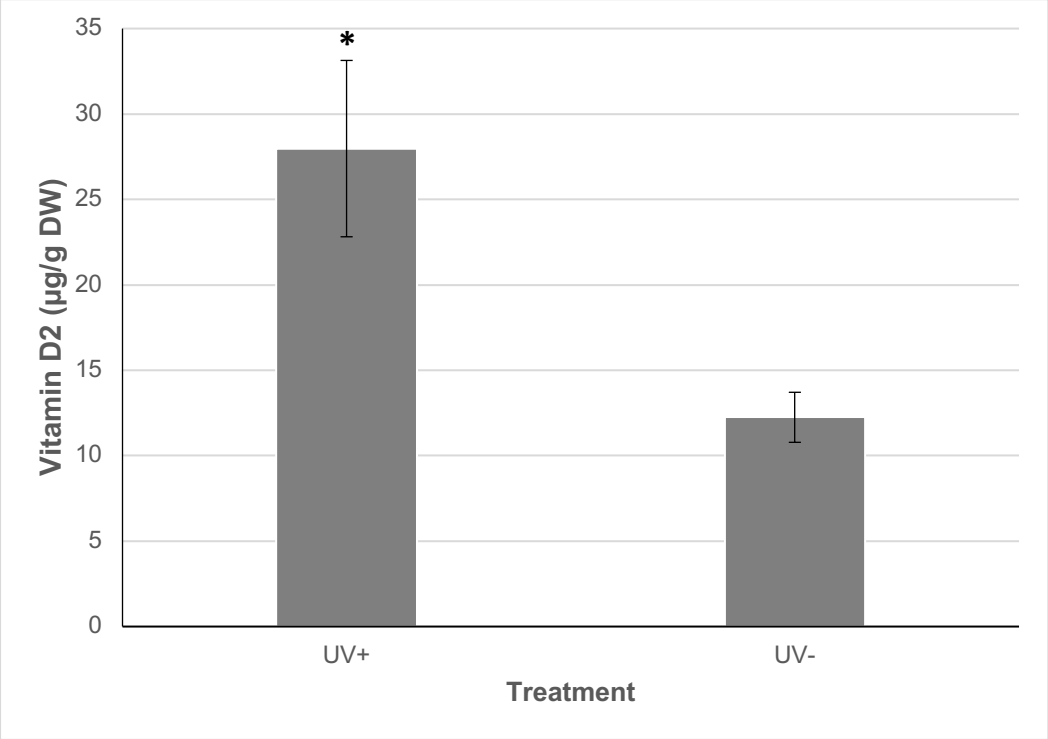

Figure 3.6 The effect of UV treatment on vitamin D2 content of mushrooms. Values are means +/- SEM (n = 3). '*' denotes significance of P < 0.05, UV+ shows a significantly higher (P-value < 0.05) content of vitamin D2 than that of UV- which acts as the control for these experiments.

Figure 3.6 shows that UV treatment does, as expected, result in a significantly higher vitamin D content in the mushrooms than untreated (UV-) controls, causing an increase of approximately 2.3x. However, unexpectedly, the untreated UV- mushrooms nevertheless do contain some vitamin D content, approx. 12.5 µg/g DW, as opposed to trace or non-detectable levels.

Figure 3.7 shows the total vitamin D content of mushrooms from all flushes and dosages in T1 (Ca formulation), T2 (K formulation) and T3 (5xK formulation) compared against the content of UV-treated (UV+) and untreated (UV-) mushrooms.

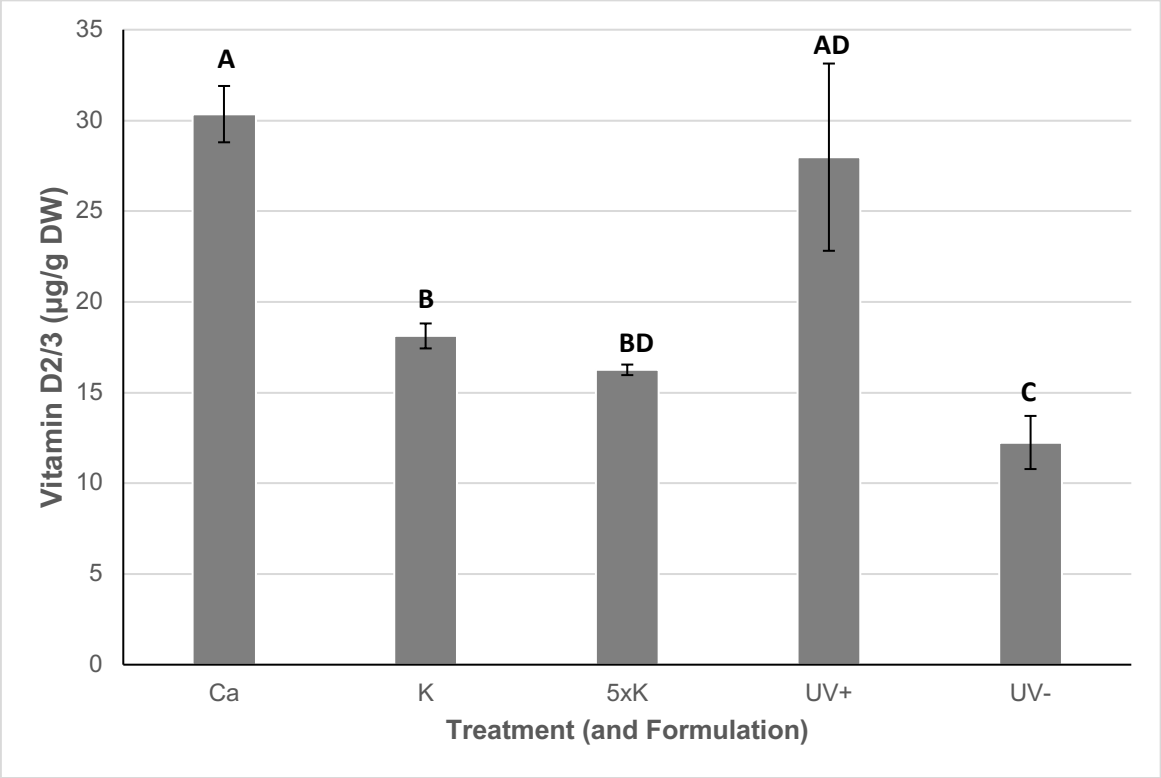

Figure 3.7 The effect of formulation (novel MycroNutrient) and treatment on vitamin D content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 8 for Ca and K and n = 3 for UV+, UV- and 5xK). Letters are used to represent significance (P-value < 0.05), shared letters indicate non-significance.

Interestingly, UV+ results in a markedly higher vitamin D content than both the K formulations which result in similar levels of vitamin D; importantly there is no significant difference between the standard K treatment and 5xK treatment. However, despite the notable difference between the UV+ and 5xK treatment there is no statistical significance (P-value = 0.097) . This is likely to be as a result of the small number of samples in which vitamin D levels were above the detection threshold. The Ca formulation is the only product treatment which induces similar levels of vitamin D content to UV treatment. There was no statistically significant difference between Ca and UV+ whilst Ca was statistically significantly different from UV-, with a roughly 2.5x increase in vitamin D over the latter. However, it is noteworthy that the standard error bars for all of the product treatments are smaller

than that of UV+ suggesting that the novel MycroNutrient product results in a more consistent and homogenous delivery of vitamin D than is achievable through UV treatment of mushrooms.

3.2 B-Vitamins

The effect of the novel MycroNutrient product on the levels of the B-vitamins B1, B2, B6 and B12 were analysed at the same dosage across all experiments, alongside the dosages of vitamin D in the same trials. Dosage of vitamin D, flush and formulation are all assessed for each B-vitamin individually to understand any possible effects on their uptake and delivery. These B-complex vitamins are all water-soluble, important for human health and encouraged in food enrichment, see **Section 1.2.1**.

3.2.1 Vitamin B1

Dosage:

Any effect of vitamin D dosage on the content of vitamin B1 was first determined. This was done in both Ca and K formulations of the product, Ca in T1 (**Figure 3.8**) and K in T2 (**Figure 3.9**).

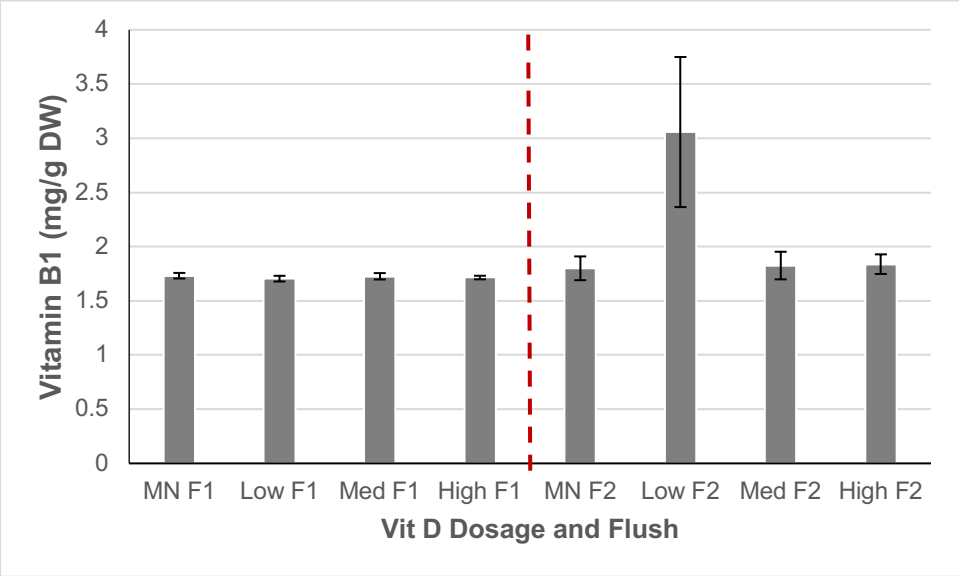

Figure 3.8 The effect of vitamin D dose on the content of vitamin B1 in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). Vitamin D dosage had no significant effect on the content of B1 in T1 (P-value > 0.05) within either flush. ANOVA P-values were 0.87 (F1) and 0.097 (F2).

Despite the notable increase in the Low dosage of T1F2 shown in **Figure 3.8** there was no statistical significance within either flush. There is very little variance within the means with the exception of the same Low dosage possibly pointing to an anomaly here. It can be concluded from this data that vitamin D dosage in the Ca formulation has no effect on vitamin B1 uptake.

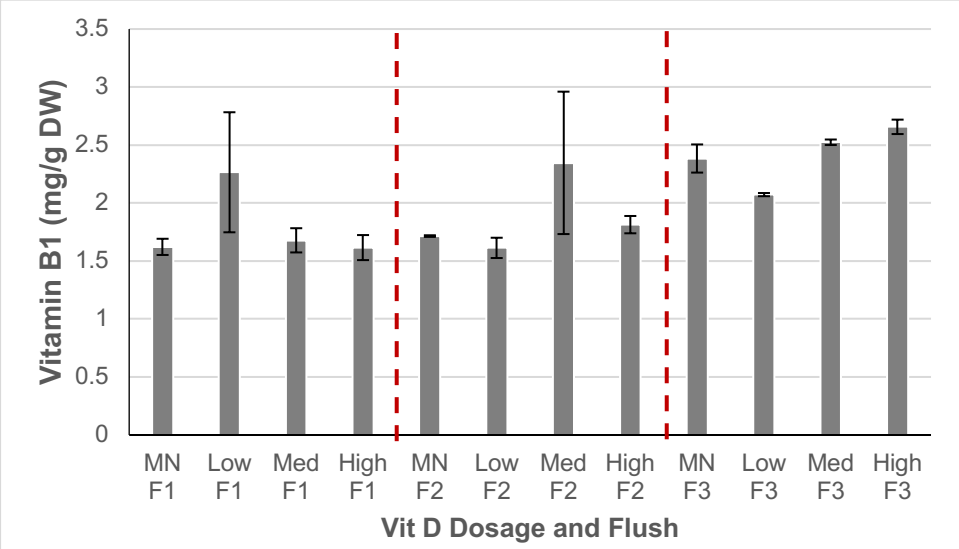

Figure 3.9 The effect of vitamin D dose on the content of vitamin B1 in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 3). Vitamin D dosage had no significant effect on the content of B1 in T2 (P-value > 0.05) within all flushes. ANOVA P-values were 0.32 (F1), 0.41 (F2) and 0.21 (F3).

Dosage shows an irregular pattern in vitamin B1 content with large variation in values for both Low dosage in T2F1 and Medium (Med) dosage in T2F2, no trend is clearly visible, and no statistical significance was recorded within any of the flushes. Thus, it can be determined that in the K formulation there is also no effect of vitamin D dosage on vitamin B1 uptake.

Flush:

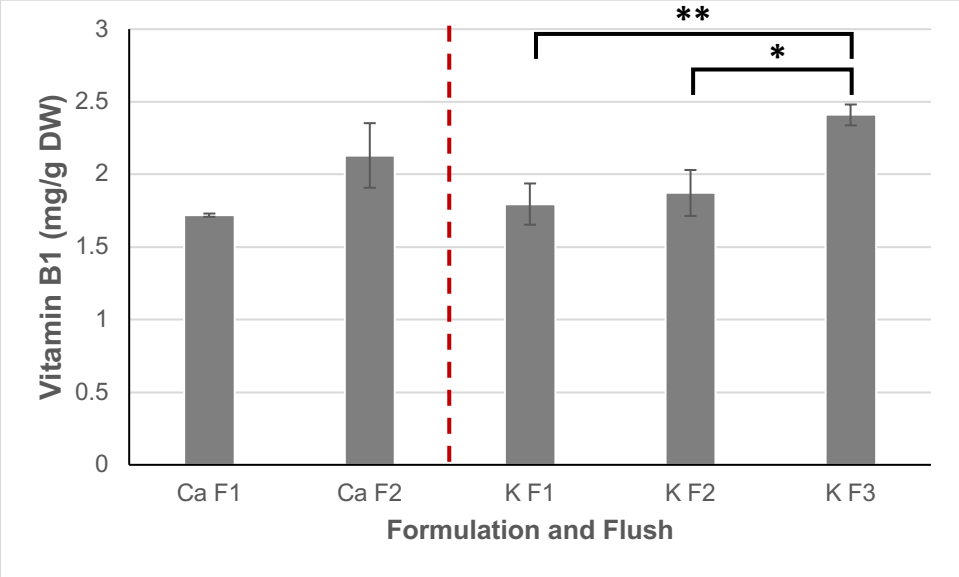

Figure 3.10 The effect of flush (F1-3) on the vitamin B1 content of mushrooms harvested from the Ca and K product formulations in Trials 1 and 2 (T1 and T2). Values are means of all dosages within the flush +/- SEM (n = 12). No significance (P-value > 0.05) was found between flushes of T1. T2F3 had significantly higher B1 content than both T2F1 (P-value < 0.01) and T2F2 (P-value < 0.05). T2F1 and T2F2 were not significantly different (P-value > 0.05). ANOVA P-values were 0.08 (Ca) and 0.004 (K).

Whilst trends in **Figure 3.10** would appear to show an increase with each flush in both trials, only in T2 was this pattern statistically significant. Without the third flush for T1 it is again not possible to obtain a full picture of a possible pattern in this trial as well.

Formulation:

To identify the most suitable carrier for B1, the formulations were compared to each other as well as to controls in the form of UV treated and untreated mushrooms, as seen in **Figure 3.11**. The B-vitamins should improve upon these baselines and can

be applied alongside vitamin D, in the replacement of UV treatment for mushroom enrichment.

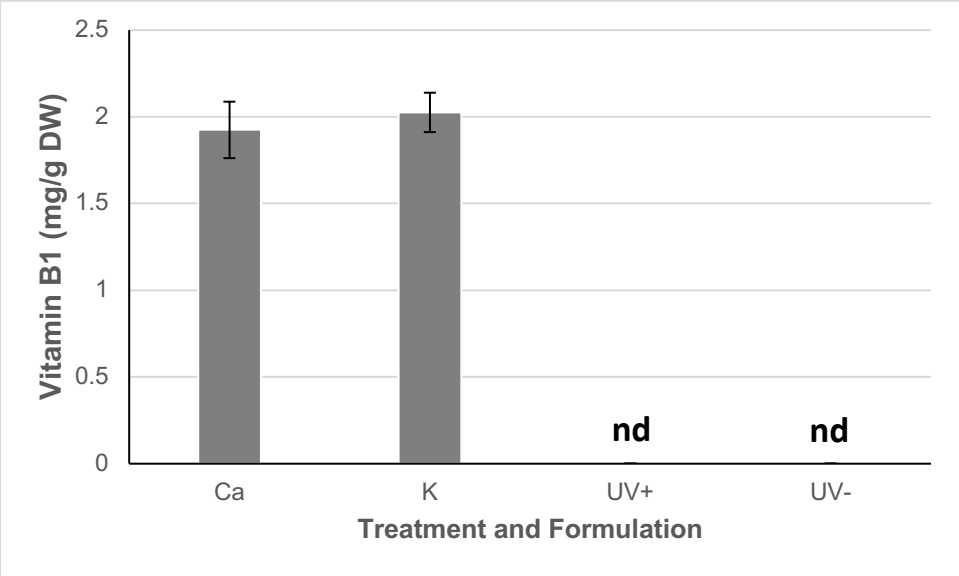

Figure 3.11 The effect of novel MycroNutrient formulation and treatment on vitamin B1 content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca) and 36 (K)). Both UV+ and UV- were non-detectable (nd) and likely trace contents. No significance (P-value > 0.05) was found between the Ca and K formulations.

The different formulations show little, and no significant, difference in vitamin B1 content. Both demonstrate relatively consistent and similar variance. UV+ and UV- were both non-detectable for B1 and therefore no statistical analysis could be performed for these treatments. However, this data indicates that either formulation would be appropriate in delivery of vitamin B1 alongside vitamin D in the novel MycroNutrient product.

3.2.2 Vitamin B2

Dosage:

Any effect of vitamin D dosage on the content of vitamin B2 is first determined. This is done in both formulations of the product, Ca in T1 (**Figure 3.12**) and K in T2 (**Figure 3.13**).

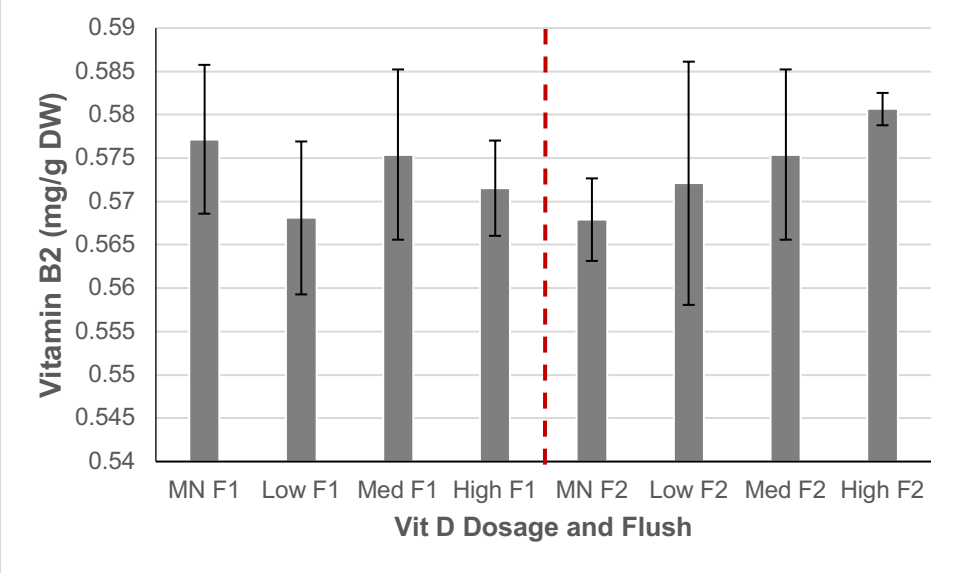

Figure 3.12 The effect of vitamin D dose on the content of vitamin B2 in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). Vitamin D dosage had no significant effect on the content of B2 in T1 (P-value > 0.05) within either flush. ANOVA P-values were 0.87 (F1) and 0.78 (F2).

Figure 3.12 shows no effect of vitamin D dosage on the content of vitamin B2 in the Ca formulation. There are no obvious trends in the data within the flushes, mainly due to the variation shown by the error bars and the very small range between all values.

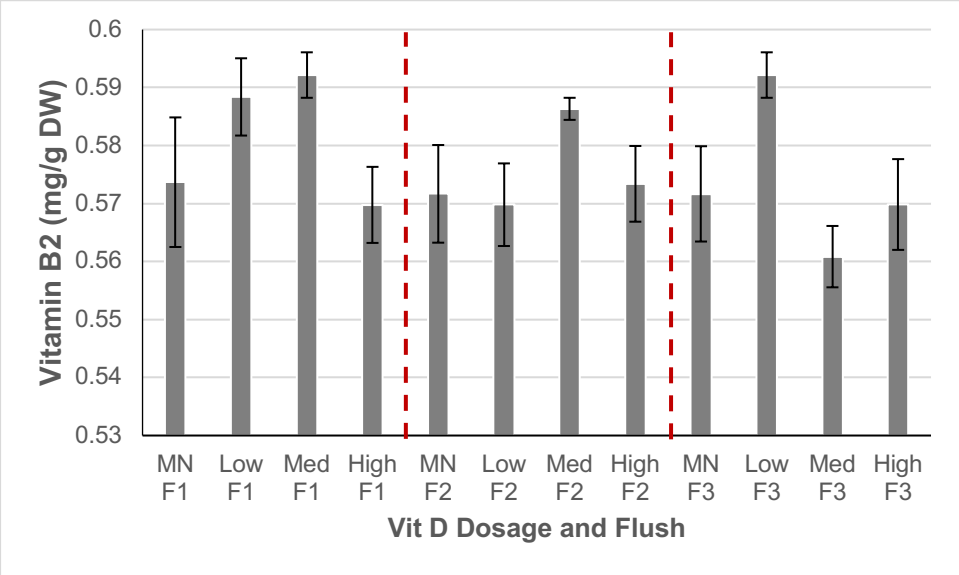

Figure 3.13 The effect of vitamin D dose on the content of vitamin B2 in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 3). Vitamin D dosage had no significant effect on the content of B2 in T2 (P-value > 0.05) within all flushes. ANOVA P-values were 0.18 (F1), 0.32 (F2) and 0.05 (F3).

Figure 3.13 similarly shows no effect of vitamin D dosage on the content of vitamin B2 in the K formulation. There are again no obvious trends in the data within the flushes.

Flush:

Flushes of formulations in T1 and T2 are compared in **Figure 3.14** to determine a change in uptake or any effect application regime might have on vitamin B2 content.

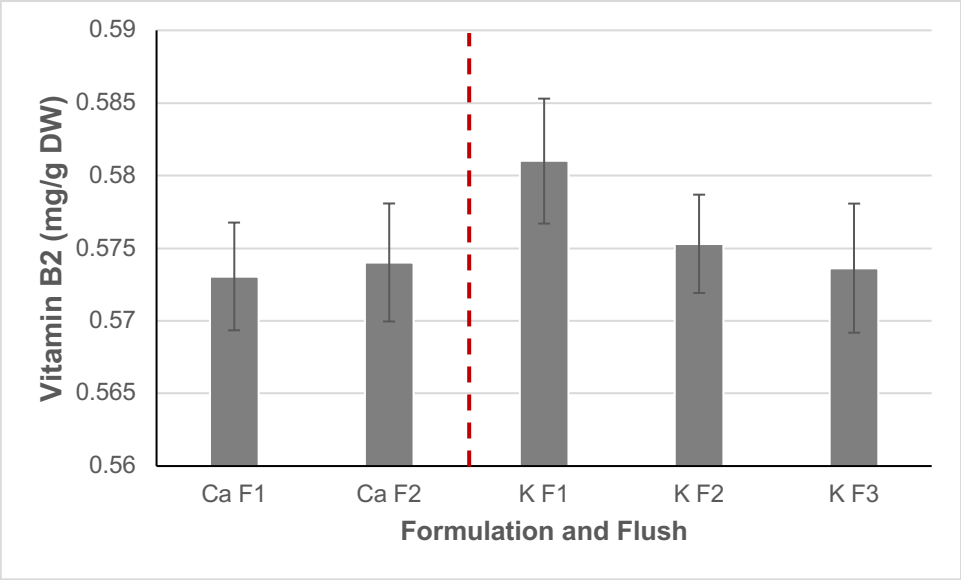

Figure 3.14 The effect of flush (F1-3) on the vitamin B2 content of mushrooms harvested from the Ca and K novel MycroNutrient formulations in Trials 1 (T1) and 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 12). There was no significance (P-value > 0.05) between flushes of T1 (Ca formulation) or T2 (K formulation). ANOVA P-values were 0.86 (Ca) and 0.42 (K).

There appears to be a small decrease in each subsequent flush observed in T2, whilst a small increase is observed in between T1F1 and T1F2, however neither to a statistically significant extent. The variation in values is also small despite the appearance of the error, showing ranges of roughly 0.007 mg (7 µg).

Formulation:

To identify the most suitable carrier for B2, the formulations were compared to each other as well as to controls in the form of UV treated and untreated mushrooms, as seen in **Figure 3.15**. The B-vitamins should improve upon these baselines and can be applied alongside vitamin D, in the possible replacement of UV treatment for mushroom enrichment.

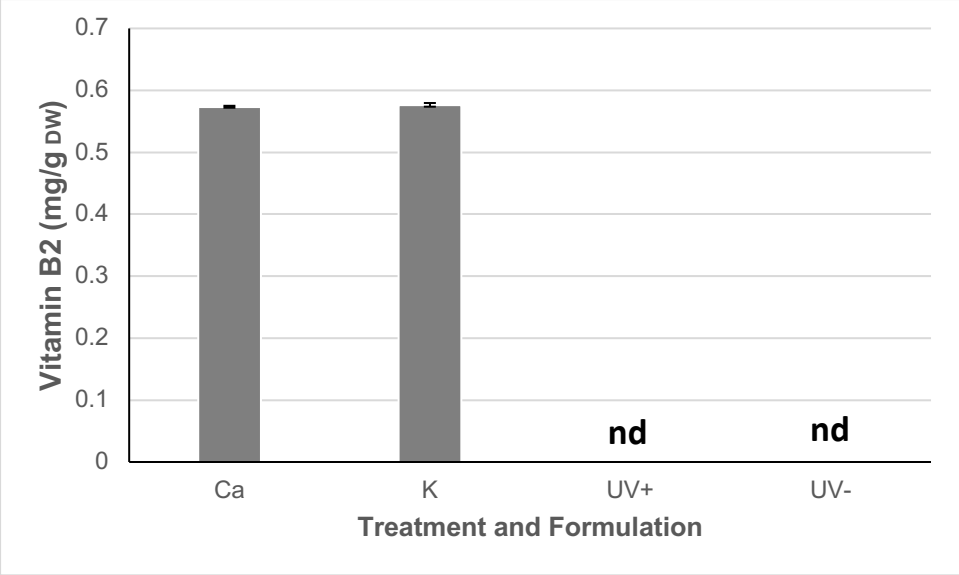

Figure 3.15 The effect of novel MycroNutrient formulation and treatment on vitamin B2 content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca) and 36 (K)). Both UV+ and UV- were non-detectable (nd) and likely trace contents. No significance (P-value > 0.05) was found between the Ca and K formulations with very little variability in either (low standard error).

The different formulations show little, and no statistically significant, difference in vitamin B2 content. Both demonstrate relatively consistent and similar variance. UV+ and UV- were both non-detectable for B2 and therefore no statistical analysis could be performed for these treatments. However, this data indicates that either formulation would be appropriate in delivery of vitamin B2.

3.2.3 Vitamin B6

Dosage:

Any effect of vitamin D dosage on the uptake of vitamin B6 is first determined. This is done in both formulations of the product, Ca in trial 1 (**Figure 3.16**) and K in trial 2 (**Figure 3.17**).

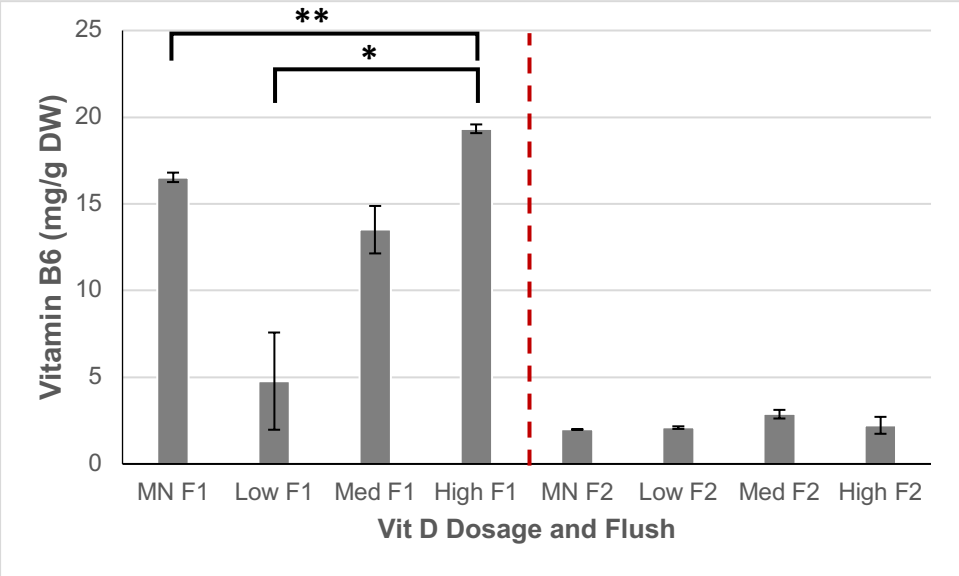

Figure 3.16 The effect of vitamin D dose on the content of vitamin B6 in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). In F1 the High dosage of vitamin D has a significantly higher content of vitamin B6 than the MN dosage (P-value < 0.01) and the Low dosage (P-value < 0.05) but no significance from Medium (Med). ANOVA P-values were 0.0009 (F1) and 0.19 (F2).

In T1F1 there is statistical significance between a few dosages however they are not in any expected pattern with the dosages of vitamin D. F2 has no statistical significance between any vitamin D dosages and shows a notable decrease compared to the F1, as explored in **Figure 3.18**.

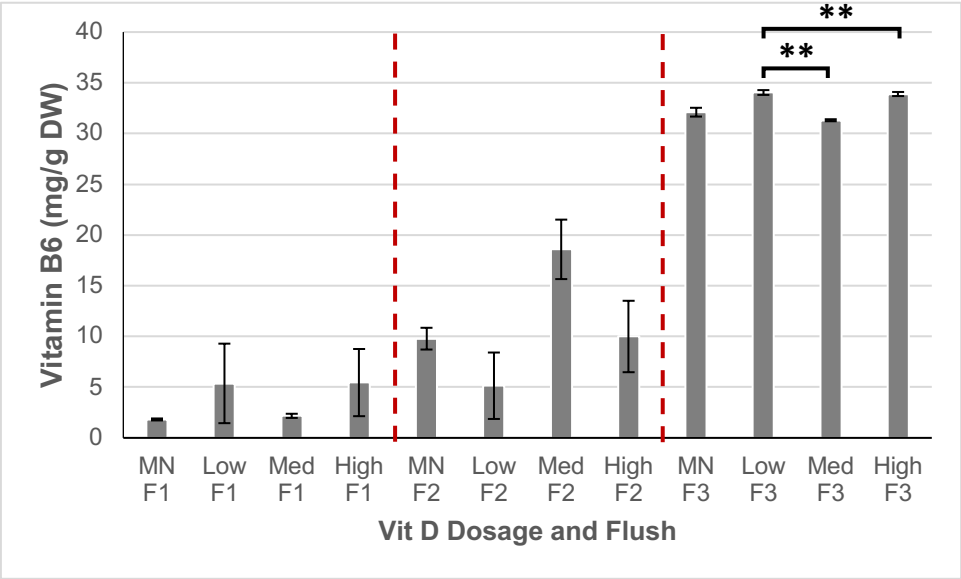

Figure 3.17 The effect of vitamin D dose on the content of vitamin B6 in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 3). In the third flush the Low dosage of vitamin D has a significantly higher (P-value < 0.01) content of vitamin B6 than both the Med and High dosages. No other flushes showed any significance (P-value > 0.05). ANOVA P-values were 0.59 (F1), 0.057 (F2) and 0.0002 (F3).

Only T2F3 showed any statistical significance, all others had no statistical significance within the flushes and do not follow any obvious trend of vitamin D dose response. Overall, vitamin B6 increases in each subsequent flush, in opposition to the pattern observed in T1. Overall flush effects are shown in **Figure 3.18**.

Flush:

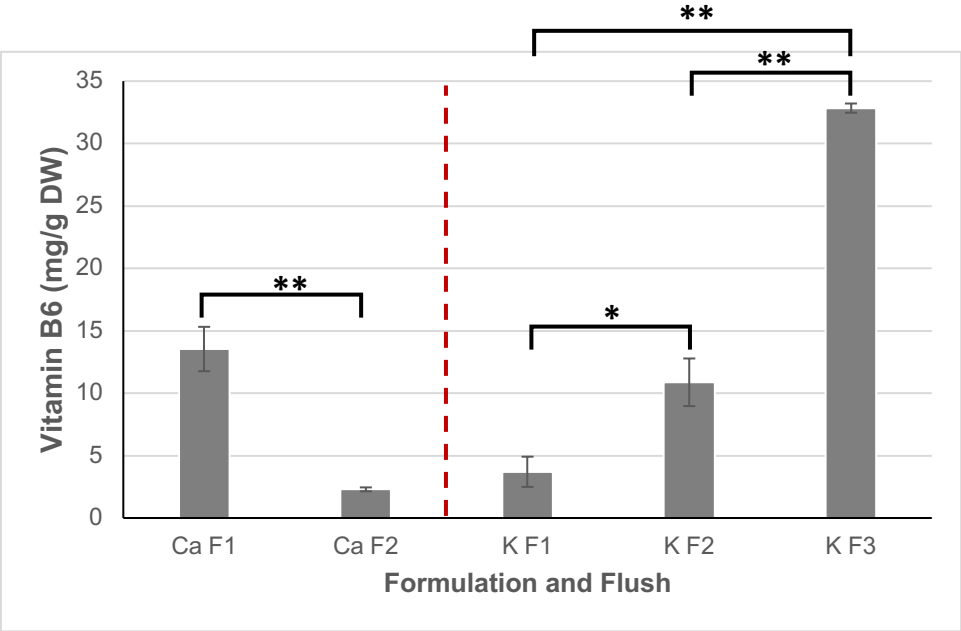

Figure 3.18 The effect of flush (F1-2) on the vitamin B6 content of mushrooms harvested from the Ca and K novel MycroNutrient formulations in Trials 1 (T1) and 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 12). T1 shows a large significance (P-value < 0.01) between the higher F1 and lower F2. T2 shows a significant increase with flush, F2 higher than F1 (P-value < 0.05) and F3 higher than both F1 (P-value < 0.01) and F2 (P-value < 0.01). ANOVA P-values were $2.4x10^{-6}$ (Ca) and $1.8x10^{-16}$).

The patterns of the flushes have been shown to be statically significant in the expected pattern, decreasing with flush in T1 and increasing with flush in T2, opposite patterns between the different formulations. Overall, the K formulation would appear to have higher contents of B6, this is directly compared in **Figure 3.19**.

Formulation:

To identify the most suitable carrier for B6, the formulations were compared to each other as well as to controls in the form of UV treated and untreated mushrooms, as seen in **Figure 3.19**. The B-vitamins should improve upon these baselines and can be applied alongside vitamin D, in the potential replacement of UV treatment for mushroom enrichment.

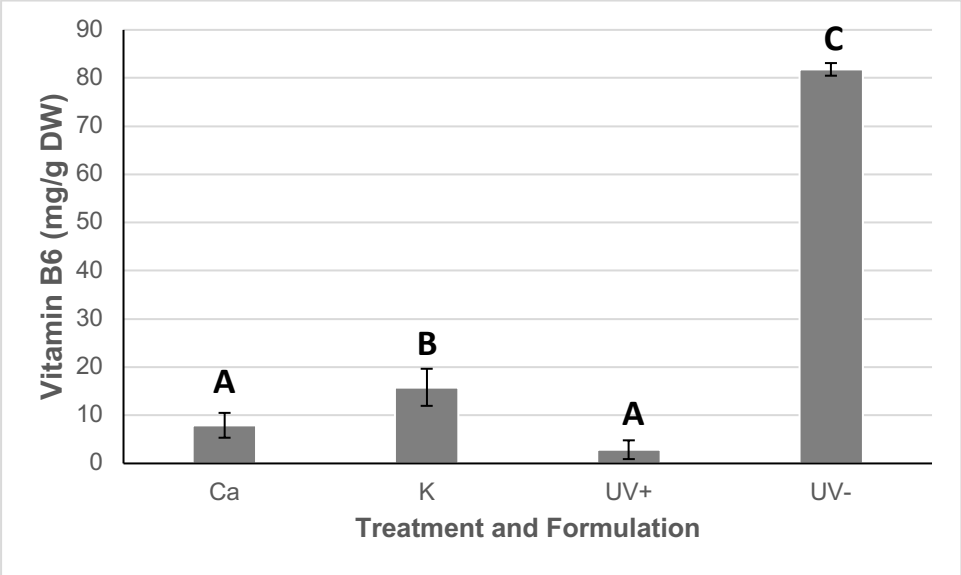

Figure 3.19 The effect of novel MycroNutrient formulation and treatment on vitamin B2 content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca), 36 (K), 2 (UV+) and 3 (UV-)). Letters are used to represent significance (P-value < 0.05), shared letters indicate non-significance.

Figure 3.19 shows that the K formulation results in significantly higher vitamin B6 content than the Ca formulation, but neither are significantly higher than the UV+ treatment. The untreated control (UV-) has far higher B6 content than all treatments and is significant in this regard.

3.2.4 Vitamin B12

Dosage:

Any effect of vitamin D dosage on the uptake of vitamin B12 was first determined. This was done in both formulations of the product, Ca in T1 (**Figure 3.20**) and K in T2 (**Figure 3.21**).

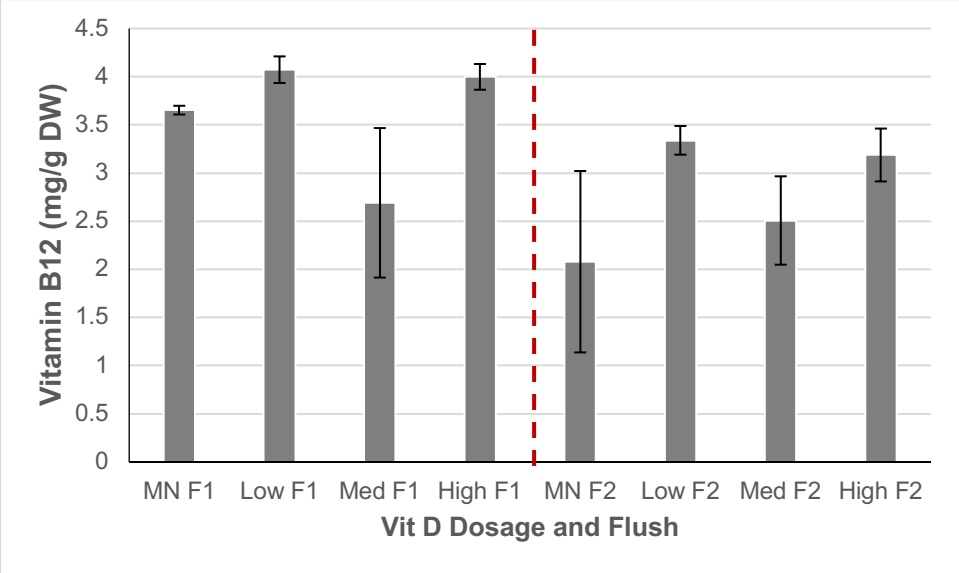

Figure 3.20 The effect of vitamin D dose on the content of vitamin B12 in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). Vitamin D dosage had no significant effect on the content of B12 in T1(P-value > 0.05) within either flush. ANOVA P-values were 0.13 (F1) and 0.44 (F2).

No effect of vitamin D dosage has proven statistically significant in the Ca formulation, there are no obvious trends in the data, within or between flushes in the Ca formulation. Differences in flush is examined in **Figure 3.22**.

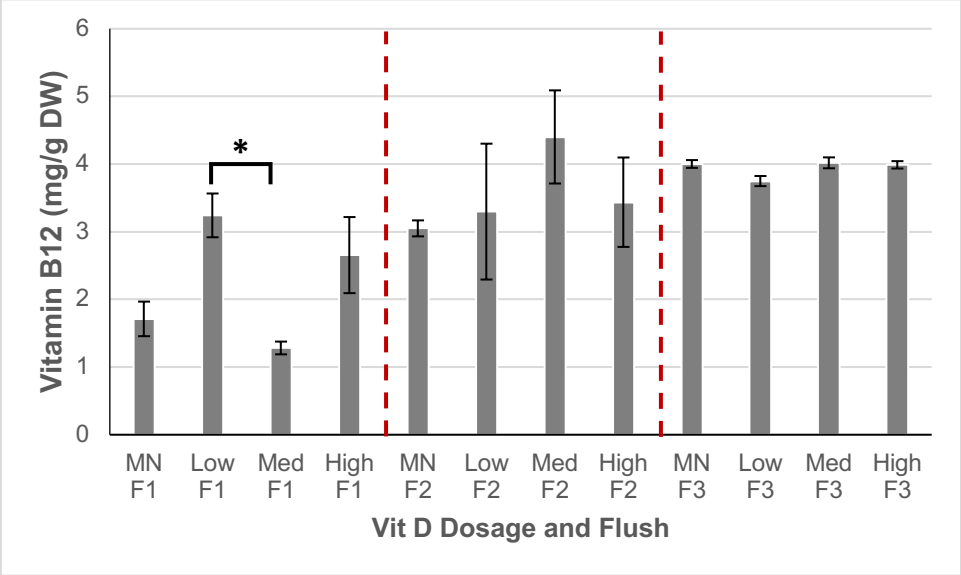

Figure 3.21 The effect of vitamin D dose on the content of vitamin B12 in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 1 (T1). Values are means +/- SEM (n = 3). In F1 the Low dosage of vitamin D has a significantly higher content of vitamin B12 than the Med dosage (P-value < 0.05). ANOVA P-values were 0.016 (F1), 0.54 (F2) and 0.06 (F3).

In **Figure 3.21** there is statistical significance between two dosages of T2F1, however there is no obvious pattern with the dosages of vitamin D. T2F2 and T2F3 both have no significance between any vitamin D dosages. Any significance between flushes is explored in **Figure 3.22**.

Flush:

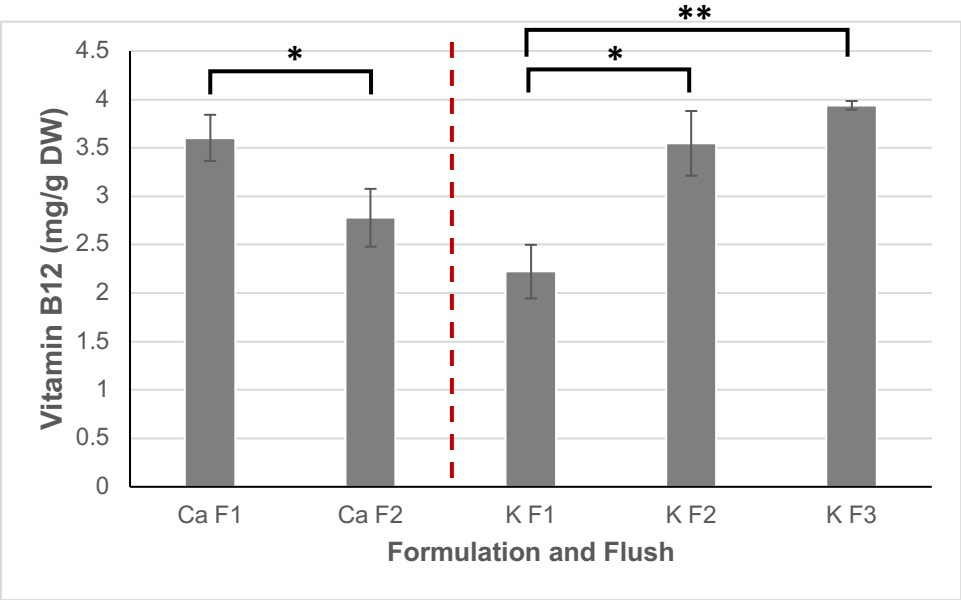

Figure 3.22 The effect of flush (F1-3) on the vitamin B12 content of mushrooms harvested from the Ca and K novel MycroNutrient formulations in Trials 1 (T1) and 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 12). T1 shows a significant (P-value < 0.05) reduction between F1 and F2. T2 shows an increase in B12 content with flush, F2 (P-value < 0.05) and F3 (P-value < 0.01) significantly higher than F1, but no significance between F2 and F3. ANOVA P-values were 0.033 (Ca) and $7.8x10^{-5}$ (K).

The patterns of the flushes have been shown to be statistically significant, suggesting that whilst in the Ca formulation, vitamin B12 content decreases with subsequent flushes, but with the K formulation it increases.

Formulation:

To identify the most suitable carrier for B12, the formulations were compared to each other and to UV+ and UV- controls, as seen in **Figure 3.23**. The B-vitamins should improve upon these baselines and can be applied alongside vitamin D, in the potential replacement of UV treatment for mushroom enrichment.

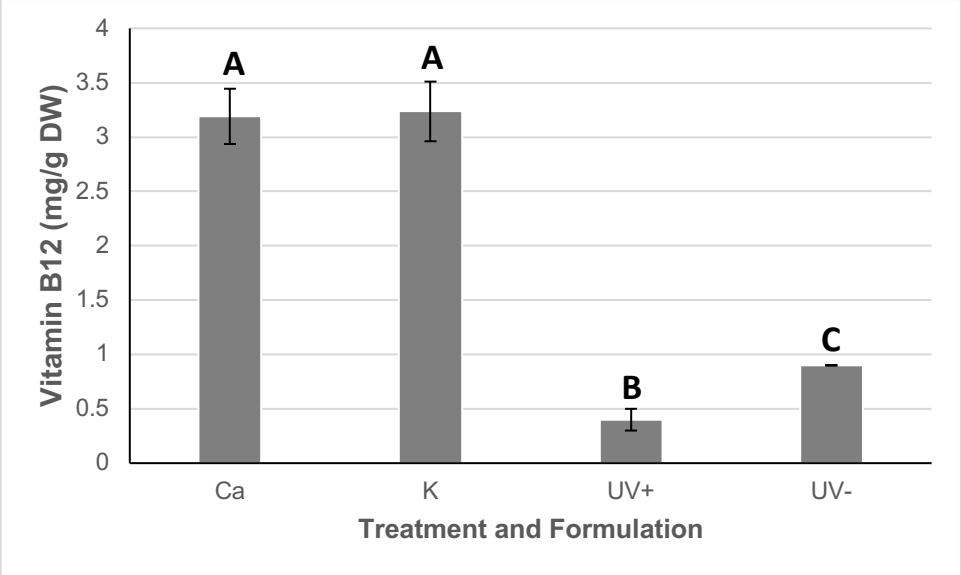

Figure 3.23 The effect of novel MycroNutrient formulation and treatment on vitamin B12 content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca), 36 (K) and 3 (UV= and UV-)). Letters are used to represent significance (P-value < 0.05), shared letters indicate non-significance.

In **Figure 3.23**, both formulations show an equally significant improvement in vitamin B12 content when compared to UV+ and UV- treatments and are not significant from each other. UV- is also significant from the UV+ treatment, suggesting that UV treatment could reduce B12 content, whilst the product treatments may add upon the 'normal' content.

3.3 Minerals

The minerals tested in this experiment utilising ICP-OES (see **Section 2.4.4**) were Se, Ca, and K. Se was directly supplemented, and the other minerals are tested to understand their uptake as a possible 'side-effect' of application with Ca and K-based formulations of the novel MycroNutrient product. All these minerals are essential for human health and particularly Se is a target for fortification in food (see **Section 1.3.6**).

3.3.1 Selenium (Se)

Dosage:

Any effect of vitamin D dosage on the uptake of Se was determined. This was done in both formulations of the product, Ca in trial 1 (**Figure 3.24**) and K in trial 2 (**Figure 3.25**).

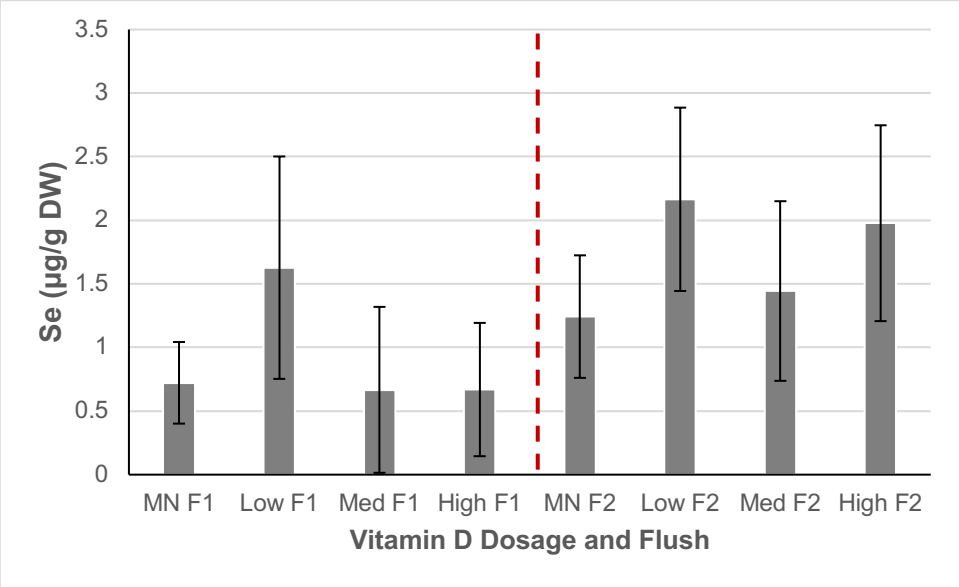

Figure 3.24 The effect of vitamin D dose on the content of Se in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). There is no significance between any of the dosages within flushes. ANOVA P-values were 0.83 (F1) and 0.86 (F2).

No trend is visible as a result of vitamin D dosages as well as no statistically significant differences between dosages within flushes, suggesting there is no effect of vitamin D dosage on the uptake of Se in T1 (**Figure 3.24**).

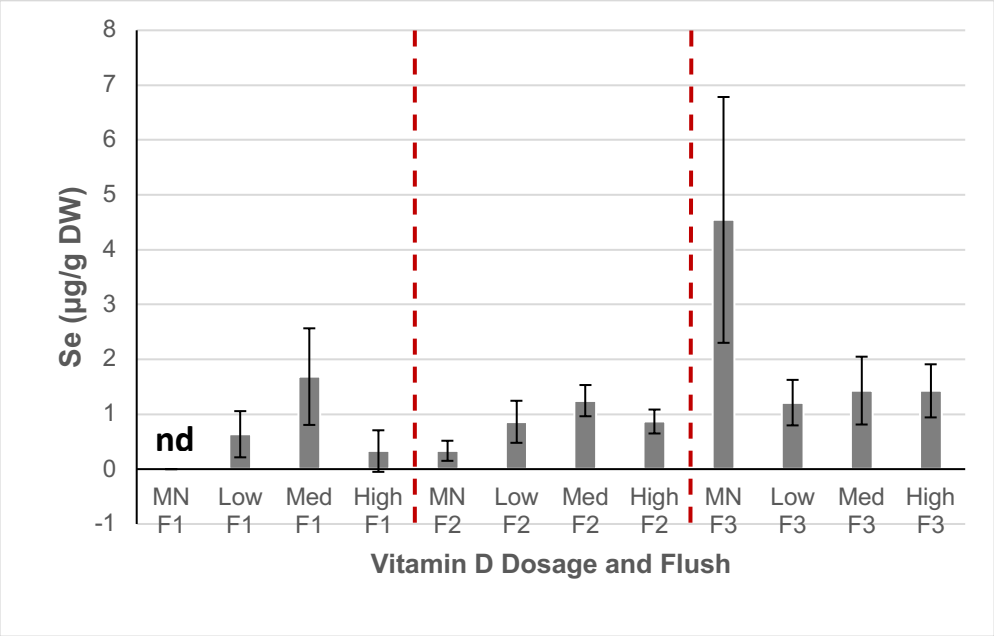

Figure 3.25 The effect of vitamin D dose on the content of Se in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 3). MN dosage of F1 was non-detectable (nd). There is no significance between any of the dosages within flushes. ANOVA P-values were 0.096 (F1), 0.11 (F2) and 0.054 (F3).

No trend is visible in **Figure 3.25** also as a result of vitamin D dosages as well as no statistically significant differences between dosages within flushes despite the increase in the MN dose of T2F3, though with large variation between repeats. The first flush MN dosage was also non-detectable and likely too low to be recorded appropriately at the concentration of sample. However, this data does suggest that there is no effect of vitamin D dosage on the uptake of Se in T2.

Flush:

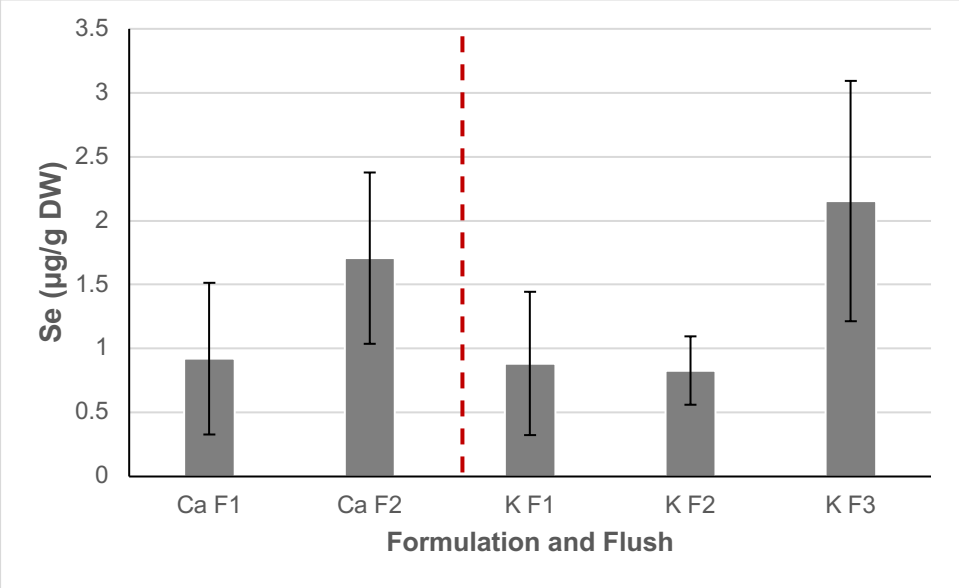

Figure 3.26 The effect of flush (F1-3) on the Se content of mushrooms harvested from the Ca and K novel MycroNutrient formulations in Trials 1 (T1) and 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 12 (Ca and K F2-F3) and 9 (K F1)). There is no significance between the flushes within the trials. ANOVA P-values were 0.14 (Ca) and 0.052 (K).

In T1 (**Figure 3.26**) Se content appears to increase with flush however not significantly and without T1F3 this pattern cannot be confirmed. In T2 a large increase can be seen in T2F3 but a decrease in F2 when compared to the F1, all these are again non-significant and so flush can be considered to not have a statistically significant effect on the content of Se in mushrooms treated with either product formulation.

Formulation:

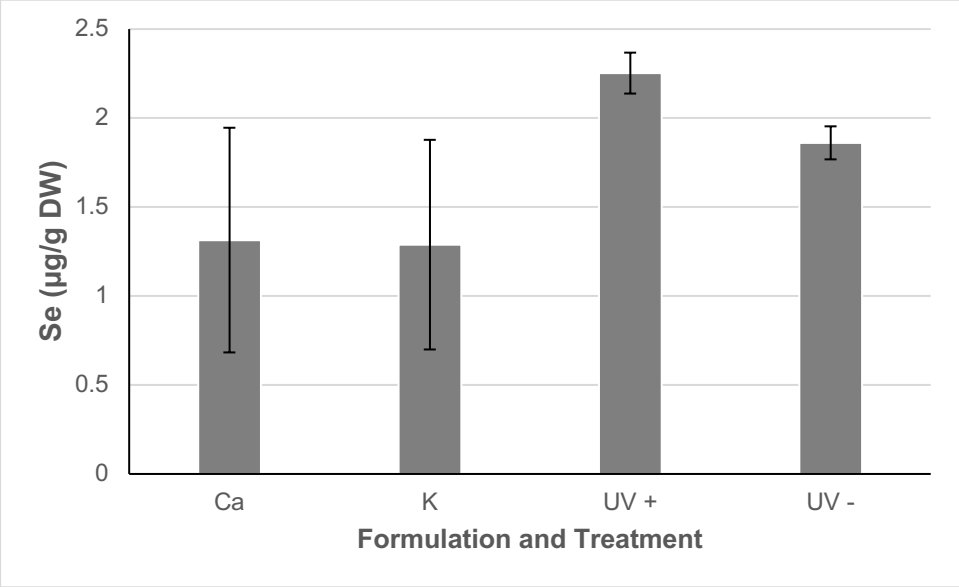

Figure 3.27 The effect of novel MycroNutrient formulation and treatment on Se content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca), 33 (K), 2 (UV+) and 3 (UV-)). There is no significance between any of the formulations or treatments.

Se content shows a small increase with UV treatment compared to untreated mushrooms, whilst both novel MycroNutrient treatments show a similar decrease in comparison to both UV+ and UV-. None of these changes are statistically significant however so it is suggested by the data in **Figure 3.27** that Se enrichment by novel MycroNutrient is ineffective.

3.3.2 Calcium (Ca)

Dosage:

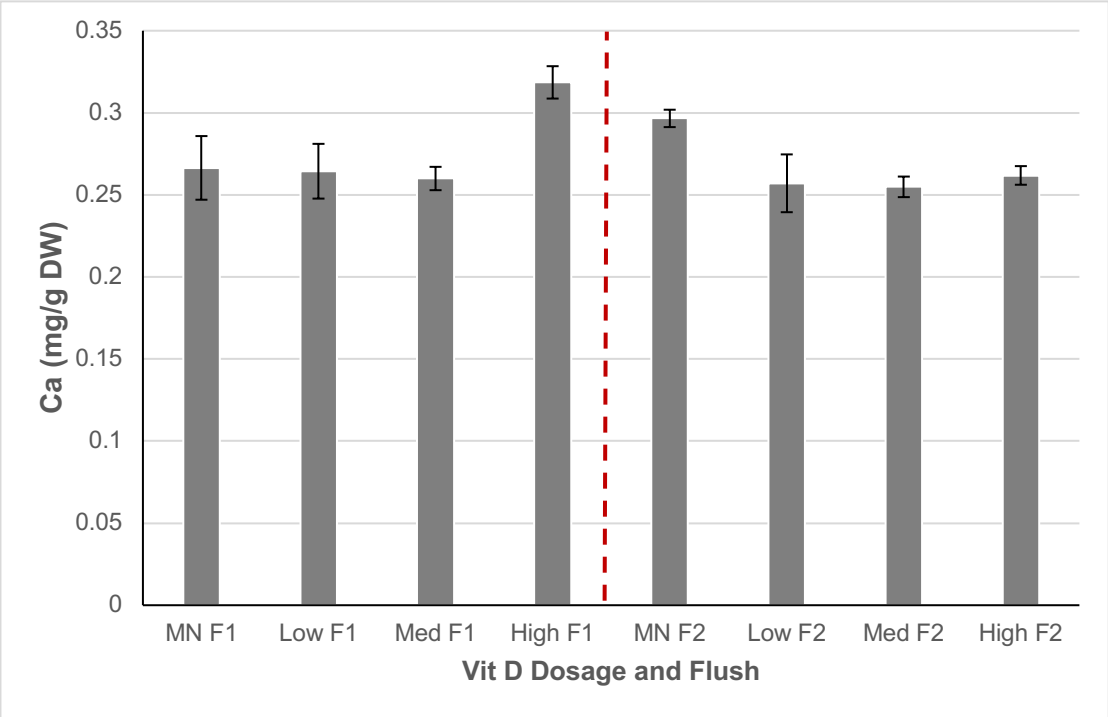

Figure 3.28 The effect of vitamin D dose on the content of Ca in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). There is no significance between any of the dosages within flushes. ANOVA P-values were 0.058 (F1) and 0.061 (F2).

Ca contents across all dosages and between flushes appear very similar with no discernible pattern or statistical significance within the flushes of T1 (**Figure 3.28**).

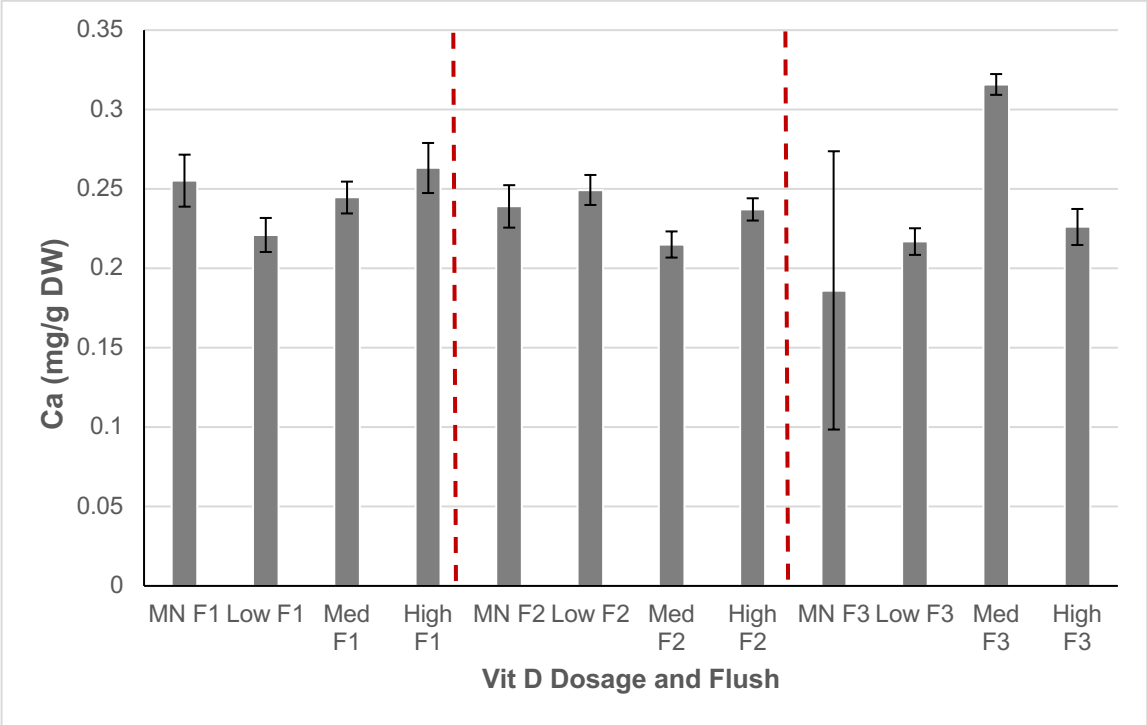

Figure 3.29 The effect of vitamin D dose on the content of Ca in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 3). There is no significance between any of the dosages within flushes. ANOVA P-values were 0.22 (F1), 0.16 (F2) and 0.27 (F3).

Similarly in **Figure 3.29**, there is very little variation or difference between any of the contents within the flushes of T2 with no statistical significance measured between them as well. The only outlier is MN in T2F3 which shows a larger variation than the others but is still very similar.

Flush:

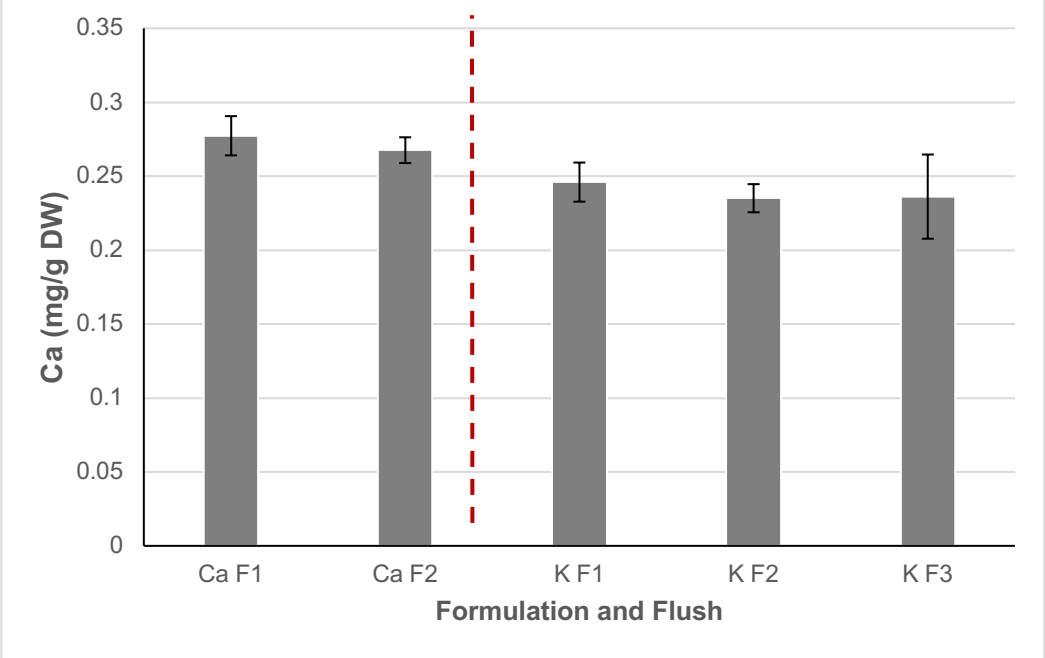

Figure 3.30 The effect of flush (F1-3) on the Ca content of mushrooms harvested from the Ca and K novel MycroNutrient formulations in Trials 1 (T1) and 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 12). There is no significance between the flushes within the trials. ANOVA P-values were 0.41 (Ca) and 0.84 (K).

In **Figure 3.30** flush shows very little effect on Ca content with no apparent pattern between the flushes and no statistically significant differences between the flushes within either formulation.

Formulation:

Ca content shows a notable and significant increase in both product formulations in comparison to the UV treated and untreated mushrooms, the Ca formulation is also significantly higher than the K formulation. This data in **Figure 3.31** would suggest that both novel MycroNutrient formulations have a statistically significant impact in increasing Ca content of mushrooms.

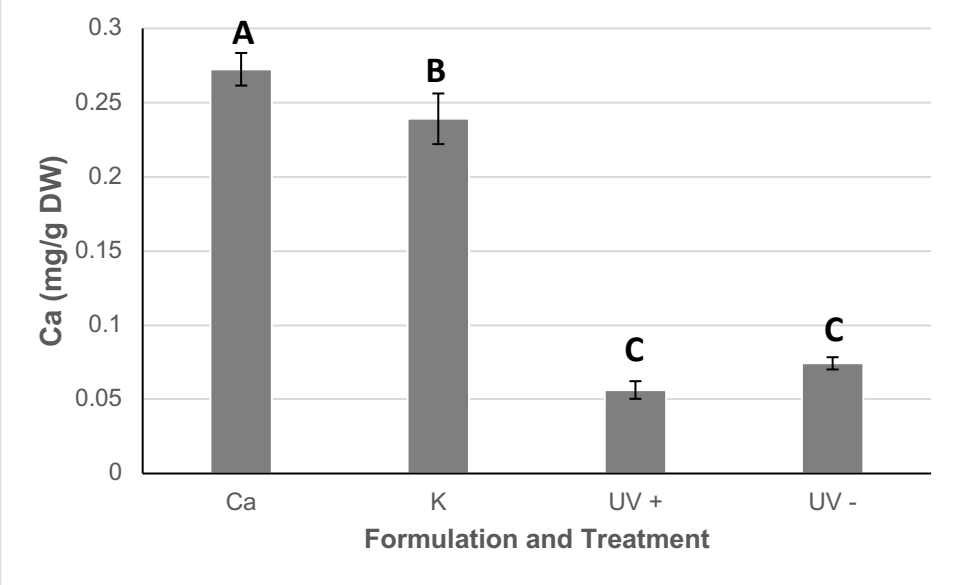

Figure 3.31 The effect of novel MycroNutrient formulation and treatment on Ca content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca), 36 (K) and 3 (UV+ and UV-)). Letters are used to represent significance (P-value < 0.05), each letter corresponding to significance from another group: 'A' denotes significance from K, 'B' denotes significance from UV+ and 'C' denoted significance from UV-.

3.3.3 Potassium (K)

Dosage:

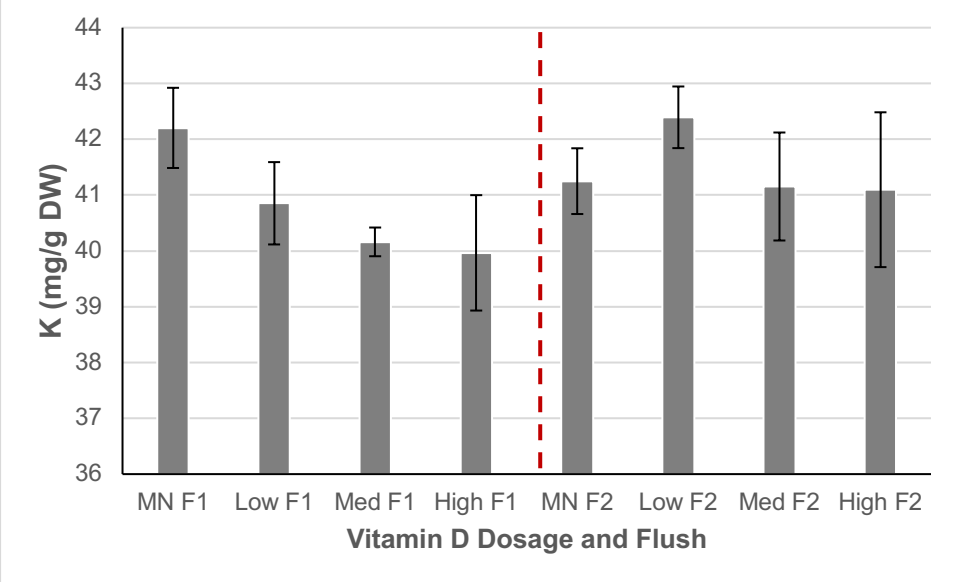

Figure 3.32 The effect of vitamin D dose on the content of K in the Ca formulation of the novel MycroNutrient product from the flushes (F1-2) of Trial 1 (T1). Values are means +/- SEM (n = 3). There is no significance between any of the dosages within flushes. ANOVA P-values were 0.21 (F1) and 0.73 (F2).

Similar to the other minerals, K shows little change in content and no statistical significance between dosages within the different flushes of trial 1 (**Figure 3.32**). Although, in the first flush there is a pattern of decrease with higher dosages, this is still over a relatively small difference and is non-significant.

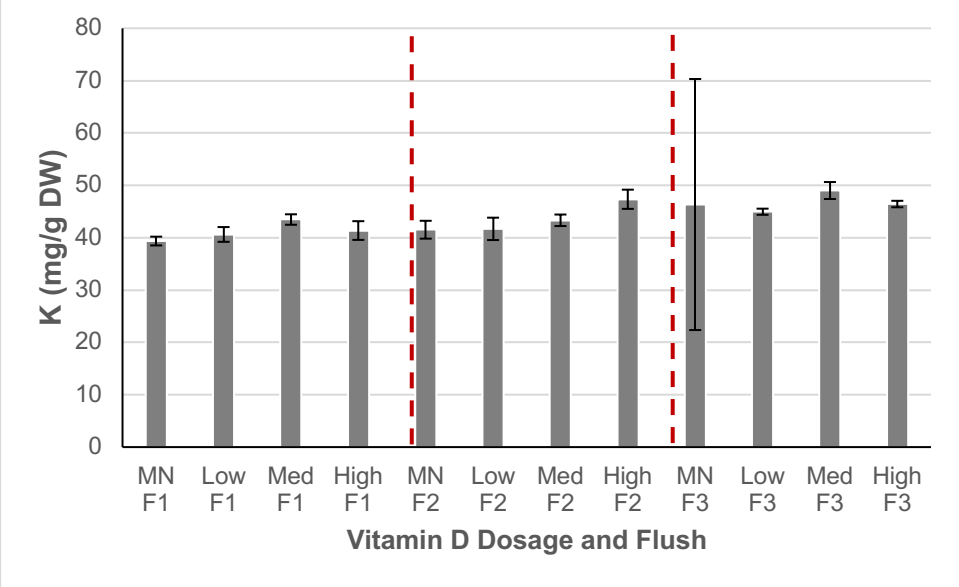

Figure 3.33 The effect of vitamin D dose on the content of K in the K formulation of the novel MycroNutrient product from the flushes (F1-3) of Trial 2 (T2). Values are means +/- SEM (n = 3). There is no significance between any of the dosages within flushes. ANOVA P-values were 0.24 (F1), 0.14 (F2) and 0.99 (F3).

T2 (**Figure 3.33**) also shows very little difference in K content between all dosages and between flushes. The only outlier is MN in T2F3 with a much larger variation. Again, no differences are significant and vitamin D dosage appears to have no effect on the content of K in either T1 or T2.

Flush:

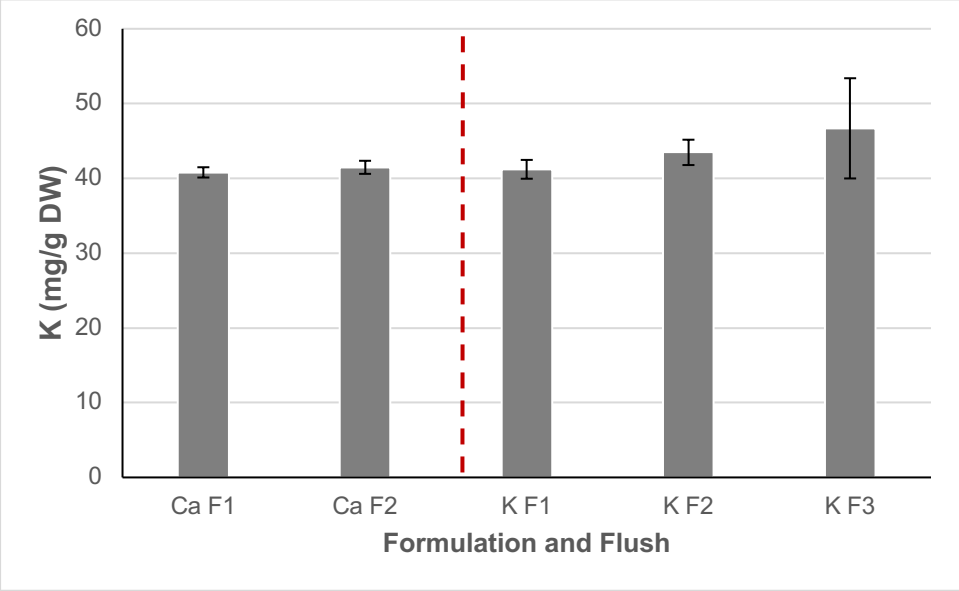

Figure 3.34 The effect of flush (F1-3) on the K content of mushrooms harvested from the Ca and K novel MycroNutrient formulations in Trials 1 (T1) and 2 (T2). Values are means of all dosages within the flush +/- SEM (n = 12). There is no significance between the flushes within the trials. ANOVA P-values were 0.26 (Ca) and 0.45 (K).

Figure 3.34 shows content of K between flushes is very similar, showing no pattern or statistical significance between them in either T1 or T2.

Formulation:

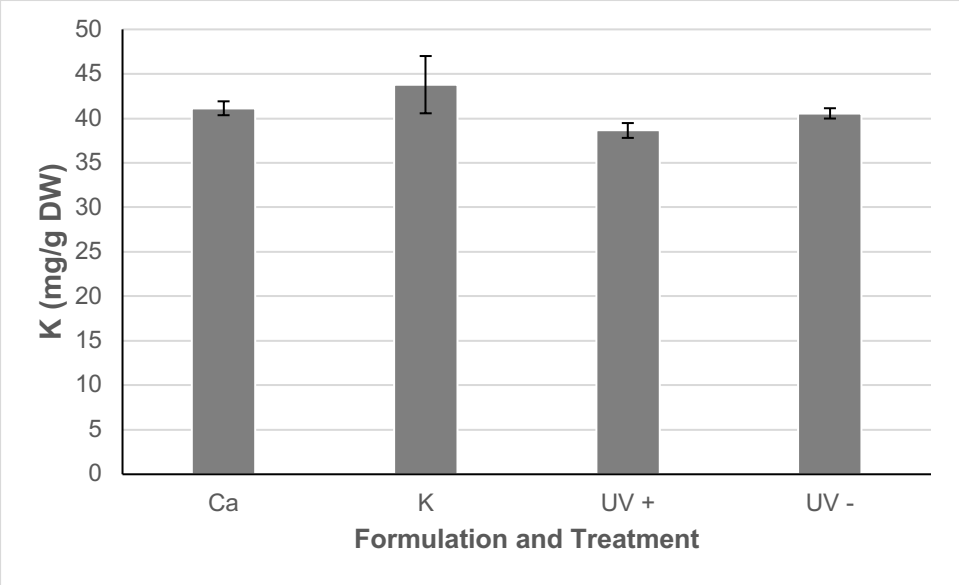

Figure 3.35 The effect of novel MycroNutrient formulation and treatment on K content of mushrooms. Values are a mean of all results from the respective trials and treatments +/- SEM (n = 24 (Ca), 36 (K) and 3 (UV+ and UV-)). There is no significance between any of the formulations or treatments.

Figure 3.35 shows little and no significant differences between any of the treatments for overall K content with both novel MycroNutrient formulations, UV treated and untreated mushrooms. Therefore, K content is likely to be unchanged and unaffected by any of the treatments.

3.4 Yield

As mushrooms were harvested, yield was also recorded by weighing punnets of mushrooms collected before taking samples. An average yield from untreated mushrooms was also provided by Drinkwater Mushrooms Ltd. for comparison. The product trials are shown in **Figure 3.36** and the K treatment in T2 (having all 3 flushes reported) is compared to the 'standard' average in **Figure 3.37**.

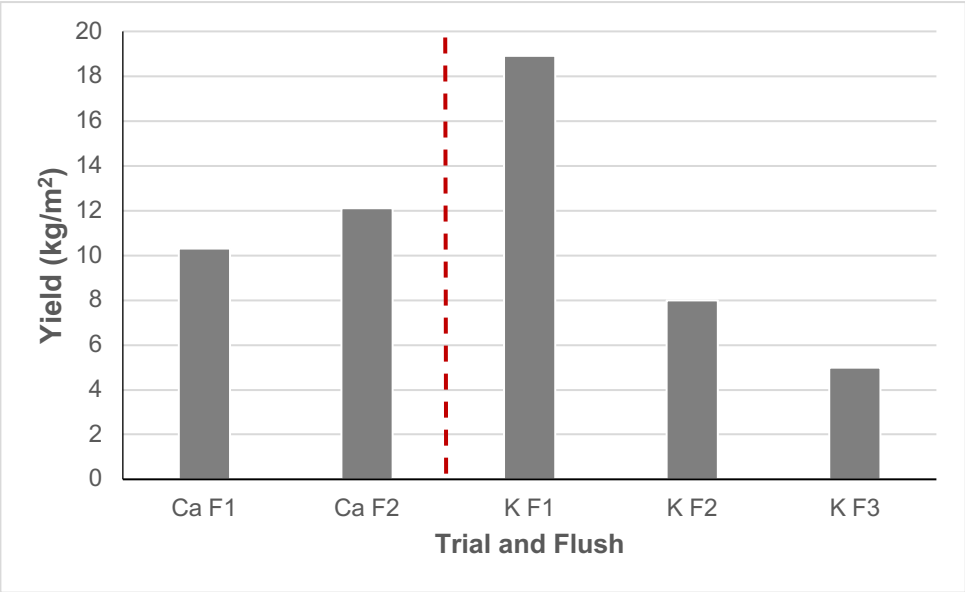

Figure 3.36 The effect of novel MycroNutrient product on yield of crop by flush (F1-3), in the Ca formulation of Trial 1 (T1) and K formulation of Trial 2 (T2).

Yield unexpectedly increases in T1F2 compared to T1F1. T2 follows more of the expected pattern with yield of each subsequent flush decreasing by roughly half each time.

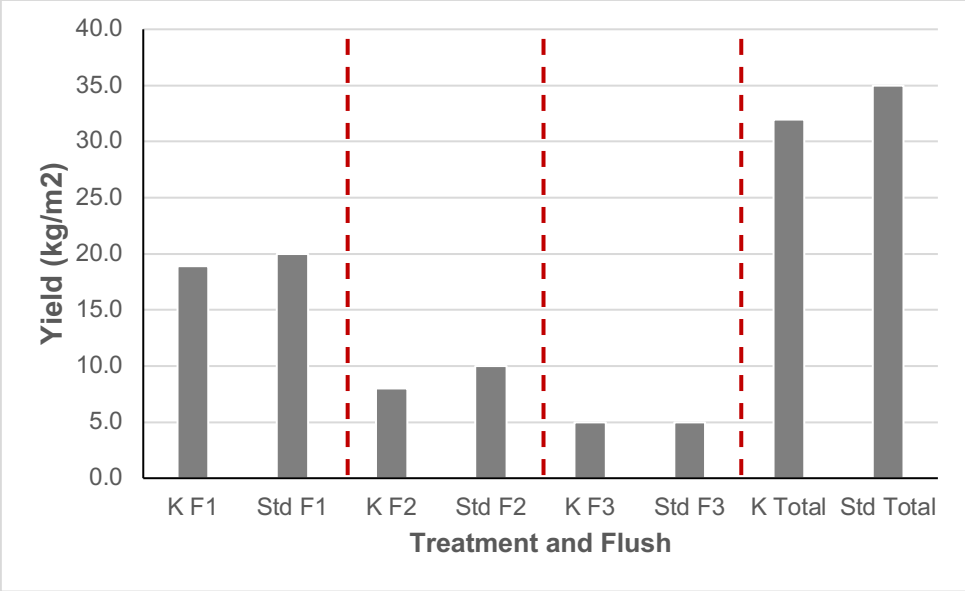

Figure 3.37 'Side-by-side' comparison of yield in the K formulation novel MycroNutrient of Trial 2 (T2) and standard (Std) flushes and totals.

Figure 3.37 shows a direct comparison between the yields of T2 and the average reported 'standard' yield. They both follow a similar pattern of decrease in yield with flush as well as similar totals.

3.5 Carbon Reductions

Alongside the main experiments a separate carbon report was conducted to compare the novel MycroNutrient treatment to UV treatment in terms of associated carbon emissions, mainly as a result of water or energy use respectively. These baselines and predictions were calculated using data collected from NutriGain Ltd. and Drinkwater Mushrooms Ltd.

The following is an excerpt from the annual report provided to both business partners as part of the Eco-I NW scheme:

'Values given are those associated with only the carbon emissions from enrichment processes (UV and novel MycroNutrient treatment), covering here only application to brown capped mushrooms as UV treatment is not applied to white capped mushrooms.

Total application to all mushrooms:

29.12 kg (browns) + 74.41 kg (whites) = 103.53 kg CO_2 eq (per year)

UV comparison:

14,043 kg (UV, only browns) – 103.53 kg (novel product, all mushrooms)

= 13,939.47 kg / 13.94 t CO_2 eq savings (per year – all mushroom production)

99.3% reduction in carbon emissions associated with enrichment process

When comparing application of the novel MycroNutrient treatment to all mushrooms there is still a marked reduction in associated emissions:

Comparison of Processes (browns):
Carbon emissions associated with UV (baseline) = **14,043 kg CO_2 eq** (per year)
Carbon emissions associated with product treatment = **29.12 kg CO_2 eq** (per year)
UV – Product = **14,013.88 kg CO_2 eq** per year savings

Carbon equivalent emissions from the novel MycroNutrient treatment in this scope (comparable to running of UV lamps) is generated from associated emissions from water-use in diluting the product for application onto mushrooms during growth and has no alterations to other normal watering or energy use for the crop.

Overall emissions for crop production were also calculated and reported within the **Carbon Report** with reduction figures shown as a percentage of overall production emissions, reported at a 6.99% reduction.

Data was acquired directly from energy reports undertaken for Drinkwater Mushrooms Ltd (the grower and second business partner for the project), they also provided the information of growing conditions and normal energy and water use and running of UV equipment. NutriGain (business partner) provided information on the application regime and dilution needed for the product for associated water-use figures.'

The full carbon report and figures can be found in **Appendix D**. Which further details the calculations and origins of the values shown here, resulting in the reduction of over 99% carbon emissions in the scope of application of the novel MycroNutrient treatment.

4. Discussion

This study sought to test and refine the efficacy of NutriGain's new MycroNutrient product for increasing vitamin and mineral content in commercially cultivated mushrooms. In particular, the study focused on the potential of 'water on' liquid supplements for increasing the vitamin D content of all mushrooms (white and brown capped) as a potential replacement for the current energy intensive UV treatment method of enrichment used as the industry standard, alongside the added addition of various B-vitamins and Selenium (Se). The objective of the study was to find optimum dosages and formulations of the novel MycroNutrient product in terms of the levels of vitamins and minerals applied to the mushroom crop in order to replicate or exceed the levels achieved by UV treatment to better inform the use of 'water-on' liquid supplements within the commercial production environment. Therefore, the effects of formulation and application regime on the vitamin and mineral content as well as the yield of mushrooms and uniformity and consistency across flushes was studied and comparisons to UV baselines and untreated controls made. The results obtained provide insights into the commercial viability of the novel MycroNutrient product and its possible use as a more sustainable alternative to UV treatment for mushroom enrichment for vitamin D alongside other important dietary vitamins and minerals. Additionally, the study highlights the potential reductions in carbon emissions and energy savings achievable using NutriGain's new MycroNutrient product compared to UV enrichment of mushrooms.

4.1 NutriGain's novel MycroNutrient 'water-on' liquid supplement product can enrich vitamin D content to levels equivalent to UV treatment

The focus of this study was mainly on Vitamin D, being the most important vitamin tested as it relates directly to the replacement of UV treatment in enrichment of mushrooms commercially (Taofiq et al., 2017). The application of Ca formulation MycroNutrient vitamin D3 was successful in matching vitamin D2 contents of UV treated mushrooms (at roughly 30µg/g DW) and thus in this regard can be considered a success.

4.1.1 The different dosages of vitamin D in the novel MycroNutrient product had little effect on the vitamin D content of mushrooms

Little difference in mushroom vitamin D content was observed within the flushes of different experimental trials using different dosages of vitamin D in the novel MycroNutrient product. With the exception of T1F1, there is little evidence to support dose-dependent effects, and few differences were significant. Importantly, most applications of the novel MycroNutrient product containing no additional vitamin D3 (MN) still resulted in uniform levels of vitamin D3, suggesting there was no limitation on uptake of vitamin D3 or its translocation from the mycelium into mushroom fruiting bodies (Herman et al., 2020).

Given there appears to be no limitation on the uptake or translocation of vitamin D3 by mushrooms, the lack of a dose-dependent effect of vitamin D in the novel MycroNutrient product suggests that there was no difference in the dosage of vitamin D received by mushrooms. One explanation for this might be that the partitioning used to divide the growing bed into the four experimental regions was not effective in separating and partitioning the dosages within the growing bed. Instead, all sections may have received the same 'combined' dose, whether through leakage of the product between sections or sharing of nutrients by connections of the mycelial network. The adaptable and efficient exchange of nutrients through a mycelial network connection to support the flow of resources evenly to areas lacking in particular nutrients in a demand-driven manner is well documented, particularly in 'young' fungi (Simonin et al., 2012; Straatsma et al., 2013; Fricker et al., 2017). Importantly, the present study used newly spawned substrate for commercial cultivation, in which the young fungi would be well placed to exchange nutrients (Fricker et al., 2017). If this is the case, an equivalent theoretical dosage of 131.6 mg/m^2 was applied across all the sections of each experimental trial based on the combined dosages present in the four different treatments (MN, Low, Med and High). This is supported by the relatively small differences between different dosages within flushes, only a few of which were significant. Therefore, this suggests that the cause of any differences in results is likely due to flush or formulation-dependent effects rather than the dosage, which looks likely to be shared across all treatments.

Nutrient uptake by mushrooms can be driven by supply and/or demand (Fricker et al., 2017). If, as hypothesised, all treatment sections receive the same dosage of vitamin D, this may also explain why each flush does not necessarily experience the same level of uniformity. If the sharing of nutrients is demand-driven rather than supply-driven (Fricker et al., 2017), vitamins are not 'saved' and shared between flushes and instead taken up and translocated to the fruiting bodies even if in larger dosages for later flushes, likely as a result of the larger second application and a possible limitation that is unclear from this testing. Also, the higher dosage used in the '5xK' treatment of T3 does not achieve higher vitamin D contents, suggesting they could be reaching a demand maximum, although this is only seen in the K formulation and could be different for Ca which achieves higher contents at lower dosages. As a result, the levels of vitamin D achievable through the uptake of applied nutrients appears much lower than the levels achievable through the conversion of precursor compounds with UV treatment (Taofiq et al., 2017). Concentrations as high as 100 µg/g DW have been reported in response to UV treatment (Taofiq et al., 2017), although the reported allowed maximum using UV treatment in a commercial context is 10 mg/100g and ≤10 µg/100g is considered repeatable (O'Leary, 2017). However, the effects of UV treatment will vary with the age of the UV bulb and exposure time; the levels reported by O'Leary (2017) will likely only be achieved with the use of a new high intensity UV bulb and long exposure time, not suitable or applicable to commercial conditions. There is considerable variation in the reported levels of vitamin D in mushrooms and how they are affected by UV treatment (Taofiq et al., 2017), and in the way vitamin D content is quantified. Values are reported per unit dry weight or fresh weight, per gram or per 100g, with many reports not specifying the specific units used which complicates comparisons with the 'industry standard' effects of UV treatment (Taofiq et al., 2017). Additionally, there is considerable variation in methodologies with the use of different wavelengths of light or a range, as well as differences of intensity, duration of treatment and distance to the bulb, to orientation of mushrooms treated which can all affect vitamin D content achieved (Jasinghe and Perera, 2005). This contributes to the frequently inconsistent and varied effectiveness of UV treatments for mushroom enrichment.

Despite the issues highlighted above, in all cases treatment with the new MycroNutrient product achieved nutritionally relevant levels of vitamin D with an overall average (mean of all trials) at 22.97 µg/g DW, which is comparable to µg/100g fresh weight (FW) on the basis of 1% DW to FW when freeze-dried (United States Department of Agriculture, 1992)), which is more than double the NRV (Nutrient Reference Value) set at 10 µg per day in the UK (British Nutrition Foundation, 2021) and 5 µg per day globally (Cambridge Commodities, 2019). Using the British standard, this would equate to an approximate portion size of 43.5 g of fresh treated mushrooms for suitable daily vitamin D intake, when the suggested serving size is 80 g or approximately 14 button mushrooms (Shubrook, 2023). Whilst the dosages used in this study were based on the calculation of DW as being 15% of FW from preliminary studies of oven-dried mushrooms rather than freeze-dried mushrooms used subsequently (see **Section 2.2.1**), the use of the 1% figure from the United States Department of Agriculture (1992) might have resulted in the use of higher dosages being calculated and applied. However, as seen with the results of experimentation with the '5xK' dosage, it would seem that higher dosages had little effect on improving final vitamin D content, and would likely be no more effective, less cost effective and less commercially viable.

4.1.2 All flushes of novel MycroNutrient-treated mushrooms have nutritionally relevant contents of vitamin D

Vitamin D contents of all flushes were very similar despite the significant difference between the T1F1 and T1F2, the difference being only approximately 6.37 µg. In both cases, the vitamin D content remained at nutritionally relevant levels of above 10 µg (NRV) (British Nutrition Foundation, 2021). However, whether mushrooms can be considered an enriched food product (de Lourdes Samaniego-Vaesken et al., 2012) is a complex matter and likely needs further consideration since mushrooms would contain no vitamin D without some exposure to sunlight or UV (McCance and Widowson, 2021) and therefore any increase would constitute a new dietary source. For this reason, it is important that nutritional relevance and comparisons of vitamin D content are made to the NRVs and a UV baseline rather than simply enrichment effect or success alone.

Consistent effects of the novel MycroNutrient-treatment across flush is important as enrichment is necessary for all mushrooms sold, no matter which flush they are harvested from (Sonnenberg et al., 2022). This is especially the case when comparing with UV treatment, which is applied to post-harvest allowing for consistency of enrichment, at least across flushes (JenAct, 2022). In the present study, only T2 and T3 were taken to third flush due to Drinkwater's commercial consideration and even then, some measurements in T2 and all in T3 were from single values. Therefore, experiments would likely need additional repeats to fully confirm the consistency of vitamin D content between flushes, achieved by the novel MycroNutrient product, though the present data provide a good indication of consistent treatment effects. The lack of significance between the flush data does however lend itself to being used to calculate means for the entire data set among trials for comparisons of formulations as a whole, helping to identify the most optimal product formulation for the achievement of the highest average vitamin D content across all flushes.

4.1.3 The calcium formulation of the novel MycroNutrient treatment provided the highest vitamin D content and matched UV treatment

The Ca formulation and UV treatments both resulted in a roughly 2.5x increase in vitamin D content compared to untreated controls. The difference in contents of the UV and Ca formulation treatments were non-significant; thus, the Ca formulation was successful in matching content achieved with UV treatment. The Ca formulation also resulted in a roughly 1.7x increase compared to the combined mean of the K formulations (both standard and 5x. Both formulations achieve a higher vitamin D content than the NRV (British Nutrition Foundation, 2021), and therefore nutritionally relevant levels of vitamin D content, making them both applicable for the purpose of vitamin D enrichment of mushrooms (Pinto et al., 2020), with the Ca formulation performing best. Additionally, the effects of the novel MycroNutrient treatment are more consistent than UV-treatment, based on the variability in the data indicated by the standard error bars, which is as expected, due to irregularity in the application method as described in **Section 1.2**.

As previously mentioned, there was no significant difference in the level of vitamin D enrichment between the standard K and 5xK treatment both of which were lower than the levels achieved with the Ca formulation or through UV treatment (**Figure 3.7**). This could be due to the K formulation, in which vitamin D is soluble (Thermo Fisher Scientific, 2017), possibly lowering the vitamin D uptake by the crop. Alternatively, there could be degradation of the vitamin D during storage of the novel MycroNutrient product used in the 5xK treatment, although the impact of this would be expected to be much larger due to the degradation rates of vitamin D in unsuitable storage conditions (Sharifi and Jahangiri, 2017) which is carried in lanolin oil when added to the novel MycroNutrient product (see **Section 2.2.1**). However, a similar pattern was not observed in the B-vitamins making storage issues unlikely. It may therefore be that the Ca formulation, in which vitamin D is insoluble, allowed a more stable and better delivery, possibly due to creating an oil-in-water nanoemulsion which has been shown to be very stable for vitamin D and other fat-soluble vitamins for food fortification supplements (Öztürk, 2017).

Current uptake (percentage of the applied vitamin contained within the tested samples) is about 8% for the Ca formulation when using the overall formulation mean. This is relatively good as this is contained only within the harvested fruiting bodies and some is likely contained within the rest of the mycelium, similar to that of D2 in UV-treatment (Huang et al., 2015). Though this could allow opportunity for greater value on the mushroom waste (see **Section 4.6**). Comparatively the K formulation has an uptake between 4-5%. This data is calculated on the assumption of the 'leakage' or sharing dosage rate discussed earlier.

Interestingly, the untreated controls (UV-) also showed a high content of vitamin D2, above the NRV, despite care being taken to prevent light exposure during handling and extraction (see **Section 2.4**). It is possible however, that incident exposure to sunlight may have taken place before collection of samples for analysis from Drinkwater's, from the time of harvest to transport to the packhouse. This is consistent with reports in the literature where untreated controls are frequently shown to contain vitamin D2 (Guan et al., 2016). In some cases of deliberate and lengthy exposure to sunlight, vitamin D levels have been recorded to match that of UV treatment over a much longer time period (Simon et al., 2011). However, how

this might have affected shelf-life and other parameters for consumption has not been studied, although exposing mushrooms to sunlight before cooking is often recommended as a household practice (Simon et al., 2011; Urbain and Jakobsen, 2015), but this is likely to be an inconsistent and unreliable method of vitamin D enrichment. It is not possible to determine whether any accidental UV exposure may have affected experimental treatments, possibly resulting in higher overall levels of vitamin D in the form of D2 which was not measured in the novel MycroNutrient product treated mushrooms. This could provide opportunity of even greater vitamin D enrichment achieved with a combination of liquid product and UV or sunlight treatment.

The novel MycroNutrient product contains vitamin D3 whereas UV-treated and sunlight exposed mushrooms produce vitamin D2. Vitamin D3 is much more common in our diets coming from foods such as fish products as well as being the form of the vitamin we produce from sunlight exposure in a similar way to the mushrooms, there is also evidence that D3 is retained better after cooking, and greater retention is observed with lower cooking temperatures (Ložnjak and Jakobsen, 2018), which could be an unforeseen benefit to this change in enrichment strategy, actually delivering a larger proportion of the vitamin D content to the consumer, in a more realistic scenario than the reported for raw mushrooms. There are also suggestions that supplementation with D3 is more effective and potent than D2 for the raising of serum status and generally more beneficial in humans (Heaney et al., 2011; Balachandar et al., 2021), but considered clinically equivalent in high doses, well above NRV levels (Bouillon et al., 2016), possibly due to the mitochondrial CYP (cytochrome P450 mixed-function oxidases), CYP27A1, which cannot hydroxylate vitamin D2 unlike D3, highly present for this purpose in the liver but also elsewhere in the body (Bikle, 2014). Thus, D3 is normally recommended for supplementation for the purpose of reaching NRVs and prevention of deficiency, especially during winter months when vitamin D deficiency is most prevalent, especially in the UK and northern Europe (Vieth, 2020; Tripkovic et al., 2012; Logan et al., 2013). Some literature such as Houghton and Vieth (2006) also points to lesser stability resulting in shorter effective shelf-life of D2 in supplements or fortified products, recommending that only D3 is used for this purpose in regard to human health. Therefore, the argument could be made that despite similar levels of vitamin

D content between the liquid supplement and UV treatments, the MycroNutrient-treated mushrooms could be more effective in supplementing diets for greater health benefit and reduction of vitamin D deficiency rates, as well as making up dietary intake without the need for supplements whilst being more accessible to vegans and vegetarians especially as many dietary sources are animal-derived products, whilst also providing approaches with mixed protein sources and meat alternatives to reduce meat consumption, and contribute to more sustainable food security (Das et al., 2021). Nevertheless, it is possible that inclusion of vitamin D2, rather than D3, in the novel MycroNutrient product could result in greater contents in the mushrooms, as vitamin D2 is the normal 'mushroom-available' vitamin (Cardwell et al., 2018).

In addition to the enrichment of vitamin D achievable using the novel MycroNutrient product, another benefit as a result of not treating mushrooms with UV could be maintaining higher contents of ergosterol which is usually reduced in the conversion to ergocalciferol (vitamin D2) by UV irradiation (Hu et al., 2020), although this is dependent on the wavelength of UV used as no significant decrease in ergosterol content has been observed with UV-C (Guan et al., 2016). This may have been shown by a second large peak that was observed after the elution of D3 (See **Figure 4.2**) although this would require further analysis to be confirmed as ergosterol or alternatively as a possible isomer of vitamin D that may have been produced by the mushrooms.

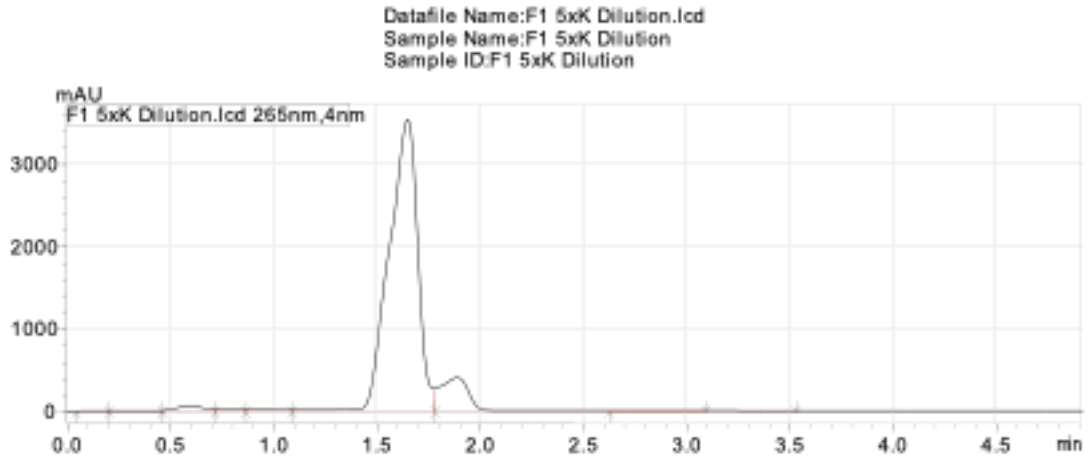

Figure 4.1 Unknown observed peak at a 10x sample dilution for better resolution of the peak

Ergosterol is largely considered a highly beneficial natural bioactive component of mushrooms with many related health benefits, particularly as an antioxidant

(Rangsinth et al., 2023). This putative increase in ergosterol in novel MycroNutrient product treated mushrooms, compared to UV-treated mushrooms, could indicate that they would have greater health benefits than those enriched with UV, as well as the additonal nutrition with the provision of B-vitamins possible alongside (see **Section 4.2**).

The present data show the potential of the novel MycroNutrient product to increase the vitamin D content in button mushrooms, to levels which match that achievable using UV but without the need for energy intensive UV treatment during packaging. It should also be noted that treatment with the novel MycroNutrient product caused no browning or discolouration of the white mushrooms used in this experiment, suggesting applicability to both white and brown mushrooms, increasing output of enriched products and to a greater proportion of the market (Sumit, 2022; Verified Market Reports, 2022). Specifically, the Ca formulation of the new MycroNutrient product would be recommended as it had the greatest effect on Vitamin D content. However, to apply this commercially further testing for consistency and validation by official channels, such as the Food Standards Agency (GOV.UK, 2017), would be required. These products may allow for a more sustainable, widespread, and effective solution for accessible vitamin D in a healthy meat alternative than UV irradiation. For both deficient groups and the general population in sufficient amounts to affect more vulnerable elderly and other deficiency-prone groups even during winter months with this non-sunlight reliant and year-round produced crop (Tiwari et al., 2021).

4.2 The novel MycroNutrient product was successful in providing nutritionally relevant levels of B-vitamins in treated mushrooms

B-complex vitamins B1, B2, B6 and B12 were added alongside vitamin D in the novel MycroNutrient product. These are important for human health highlighting the benefit of enrichment in food products to increase dietary intake with B6 and B12 being particularly important for vegetarians and vegans (Zeuschner et al., 2012) due to mainly animal-based derivation (see **Section 1.3**). The B-vitamins, unlike vitamin D, are not increased by UV treatment and, therefore, this allows for the possibility of

greater health benefits and vitamin provision, whilst also reducing carbon emissions associated with enrichment treatments (see **Section 3.5**).

4.2.1 Vitamin D dosage has no discernible effect on B-vitamin content

No consistent effect was observed of the level of vitamin D in the novel MycroNutrient product on the B-vitamin content of mushrooms. No significant differences were observed in vitamins B1 and B2 as a result of dosage within any flush with either the K or Ca formulation. In contrast, significant differences in the content of vitamins B6 and B12 were observed although these were not dose-dependent. It is likely therefore that these represent natural variation as seen in the literature (Bernaś and Jaworska, 2016), as despite significance, the difference is relatively low (Furlani and Godoy, 2008). Additionally, assuming a shared dosage across all treatments (see **Section 4.1.1**), these data suggest that vitamin D did not have an effect on B-vitamin content in the fruiting bodies in this study, although this would need further confirmation.

4.2.2 Flush has an effect on uptake of different B-vitamins

In contrast to vitamin D, differences in B-vitamin content were observed between flushes. The K formulation showed increases in B1, B6 and B12 to significant extents whereas B2 decreased non-significantly. In contrast, the Ca formulation showed small non-significant increases with flush in B1 and B2 but significant decreases in B6 and B12. All the B-vitamins are water-soluble (Thermo Fisher Scientific, 2017) and were applied as part of the novel MycroNutrient product at the same time so that differences in solubility and uptake of the different B-vitamins are unlikely to explain these patterns. However, despite these changes between flushes, all values remain nutritionally relevant and exceed or reach at least half (B2) the NRVs set for them, which are (as a mean of male and female UK recommendations): 0.9 mg for B1, 1.2 mg for B2, 1.3 mg for B6 and 1.5 µg for B12 (British Nutrition Foundation, 2021). The most notable being B12 in which the contents recorded far exceed the NRV, especially as B12 is not naturally produced by the mushrooms and is usually provided by concomitant bacteria in the substrate (Watanabe and Bito, 2017). Overall, despite flush differences, the vitamin-B content within flushes is reasonably

consistent and therefore any mushrooms from any flush can still provide a relevant dietary vitamin content in a serving.

4.2.3 B-complex vitamins B1, B2 and B12 were successfully enriched when compared to UV and untreated mushrooms to nutritionally relevant levels

Although UV treatment cannot provide B-vitamins to the mushrooms it is still used as a comparison to understand any effects UV treatment may have upon the often UV or light-sensitive B-complex vitamins, especially when present together (Monajjemzadeh et al., 2014). A comparison to untreated samples is also required to understand how the product application has differed and to assess the success of enrichment.

The present study showed non-detectable or trace amounts of vitamins B1 and B2 in the UV and untreated samples, whereas the novel MycroNutrient product formulations (Ca and K) achieved similar (non-significant) contents, with good consistency across replicates. This points to a definite improvement and success of enrichment compared to UV and untreated mushrooms. Mushrooms have also been shown to incur large decreases in B1 content when exposed to UV light and sunlight, though this does not appear to affect the standard contents of B6 or other B-vitamins normally contained within non-enriched mushrooms (Simon et al., 2011). The novel MycroNutrient product treatment would be able to either rectify or counteract this process and therefore improve B1 content and bioavailability in mushrooms reaching consumers.

Treatment with the novel MycroNutrient product achieved slightly higher contents of B6 than that of UV treatment, with the K formulation recording significantly higher levels than the Ca formulation; this was the only B-vitamin to have a significant difference between the two formulations. The UV- control content of B6 showed a large, likely anomalous and unexplained result, reaching 81 mg/g DW, far beyond any expectation or standard content (McCance and Widowson, 2021). UV exposure could be a cause of reduced content in the UV+, though with the anomalous control it is hard to determine a pattern for B6 and confirm its successful enrichment over untreated mushrooms and non-significant increase over UV treated mushrooms. B12

follows a similar pattern with reduction after UV treatment, whilst novel MycroNutrient product treatment adds significantly to the content of the vitamin compared to control. This would suggest that UV treatment causes degradation of B12 which the product treatment would not, allowing higher contents to be reached. However, all results are particularly high when looking at the normal scale of B12 content in button mushrooms which is considered trace (McCance and Widowson, 2021).

The levels of B-vitamins achieved in novel MycroNutrient product treated mushrooms are all greater than the standard contents of button mushrooms set out by the CoFID (Composition of Foods Integrated Dataset) by McCance and Widowson (2021), as shown in **Table 4.1** for comparison to the present study's data.

Table 4.1 Comparison of B-vitamin contents, standard (McCance and Widowson, 2021), UV- controls and product treatments. Values are per 100g FW and per g DW which are roughly comparable for freeze-dried mushrooms (United States Department of Agriculture, 1992).

Vitamin	Standard	UV- (control)	Ca	K
B1	0.13 mg	Trace	1.9 mg	2.0 mg
B2	0.27 mg	Trace	0.57 mg	0.57 mg
B6	0.1 mg	**81.8 mg**	7.9 mg	15.8 mg
B12	Trace	0.9 mg	3.19 mg	3.2 mg

The most notable increases were in B1, B6 and especially B12 (by an order of magnitude). B6 and B12 are considered the most important vitamins to enhance due to their importance to human health and most commonly deficient in diets and thus the most common targets of many enrichment and fortification schemes (Titcomb and Tanumihardjo, 2019). B12 content can vary widely between different farms as the mushrooms themselves do not produce the vitamin and rather it is synthesised by bacteria in the substrate and compost then taken up by the fungi. Therefore, variation is often caused by differences in bacterial flora and source of the substrates etc. (Watanabe and Bito, 2017; Koyyalamudi et al., 2009a), which might also contribute to the B12 enrichment in this study in addition to that resulting from the novel MycroNutrient product treatment. High B12 contents are very beneficial and unlikely to cause issues with larger intakes, having no 'tolerable upper intake level'

(Kubala, 2023). It is also very important to have vegan and vegetarian-accessible sources due to its predominantly animal-derived nature (Titcomb and Tanumihardjo, 2019). Consequently, the enrichment observed here could have a great impact for this market in improving vitamin intake and help prevent deficiencies for those with reduced or no meat/animal product diets (Zeuschner et al., 2012).

Taken together these results show that the formulation (K or Ca) of the new MycroNutrient product had little difference in their effect on levels of the B-complex vitamins in mushrooms, except for B6 with higher content in the K formulation, which also showed the least enrichment of vitamins. Whilst this suggests that either formulation would be suitable for the delivery of B-vitamins, the better performance of the Ca formulation with respect to vitamin D enrichment, makes it more commercially viable for enrichment of button mushrooms (white and brown capped) in both vitamin D and B-vitamins.

4.3 Target mineral contents were not successfully enriched through novel MycroNutrient product treatment

There was little effect of the novel MycroNutrient product on the mineral content (Se, Ca and K) of mushrooms throughout the growth cycle. The Se content was not significantly different from untreated and UV treated controls, between 1.25-2.25 µg/g (DW), all of which were markedly lower than the reported standard of 17 µg/100g (FW) (McCance and Widowson, 2021). These values are roughly comparable due to the 1% conversion of dry and fresh weights after freeze-drying (United States Department of Agriculture, 1992). This could be due to a shortage of Se in the growth substrate; this is typically the greatest determinant of mushroom Se content, addition to the substrate being an effective method of Se enrichment in mushrooms (de Oliveira and Naozuka, 2019). Therefore, this study suggests that the liquid ‘water-on’ application of the novel MycroNutrient product to the casing is not effective in enrichment of Se, which does not appear to be reaching the mycelium in the substrate for uptake. However, the reported standard concentrations in the literature are disputed (Falandysz 2013) suggesting the standard nutritional value is a lot lower than reported in most studies. The Se content of mushrooms is also variable and much lower in *A. bisporus*, which although the most commonly

cultivated edible white mushroom, is not directly specified as the species in standard reports of the CoFID (McCance and Widowson, 2021). Nevertheless, values of treated mushrooms in these experiments are still non-significant, and no increase is observed from untreated or UV-treated mushrooms and thus enrichment through this method appears to be ineffective.

K content followed a similar trend as Se and was not affected by the novel MycroNutrient product or UV treatment, even in the K formulation, suggesting that the product has no effect on uptake of K and the form of the carrier is not bioavailable to the mushrooms. K was not a target for enrichment and due to already relatively high contents of potassium in mushrooms (Nasiri et al., 2013) is not a concern for addition. In contrast, Ca does appear to be increased as a result of the novel MycroNutrient product treatment in both the Ca and K formulation. This suggests that the Ca carrier is not the source of this increase but rather the novel MycroNutrient product has an effect on Ca uptake or content within the mushrooms. Ca has previously been suggested as a target for enrichment (Tang et al., 2023), such that this increase offers commercial benefits. However, the values for both Ca and K are very low when compared to standards set out in the CoFID (see **Table 4.2**) although this could again be as a result of deficiency in substrate as discussed previously.

Table 4.2 Comparison of mineral contents, standard (McCance and Widowson, 2021), UV- controls and product treatments. Values are per 100g FW and per g DW which are roughly comparable for freeze-dried mushrooms (United States Department of Agriculture, 1992).

Mineral	Standard	UV- (control)	Ca	K
Se	17 µg	1.86 µg	1.31 µg	1.29 µg
Ca	3 mg	0.07 mg	0.27 mg	0.23 mg
K	378 mg	40.6 mg	41.1 mg	43.8 mg

These values are also not large enough to be considered nutritionally relevant when compared to the NRVs of 68 µg, 700 mg and 3500 mg for Se, Ca and K respectively (British Nutrition Foundation, 2021), both as the standard and treated mushrooms.

Overall, the study shows that enrichment of minerals using' water on' liquid supplements such as the novel MycroNutrient product used here is unlikely to represent a viable option for the mineral enrichment of mushrooms. Other methods, such as those demonstrated in other mushroom species using Se enrichment of the substrate before spawning (Falandysz, 2008) use selenium selenite (da Silva et al., 2012), the same as was added in this product, are likely to prove a more successful alternative.

4.4 Yield was likely unaffected by novel MycroNutrient treatment

In the present study, yields obtained using the Ca formulation of the novel MycroNutrient product only reached 22.4 kg in 2 flushes (standard is 30 kg – Drinkwater Ltd. personal communications, 2023) with the K formulation having only a small decrease from the average standard yield. This result contrasts with the results of previous studies using the previous MycroNutrient product without vitamin D which have reported increased yields (Noble and Dobrovin-Pennington, 2018). However, T1, using the Ca formulation, only had 2 flushes and increased in yield in T1F2 to levels akin to those usually reported despite a very poor yield in the first flush. This could be due to a delay in filling and subsequently early picking on the first flush, or due to edge effects affecting growth which normally yield lower quantities (Sabeh et al., 2006) due to its end position within the room and end of the lowest shelf. Additionally, interruption of the substrate and casing when partitioning the sections for the experiments, shifting yield towards the second flush could have affected yields (Sonnenberg et al., 2022). Furthermore, the partition of the substrate to allow the different treatments occurred after ruffling and at that time the mycelium was already partly established in the substrate, even if not growing into the casing layer yet, which could cause delays in growth and reduce the overall yield in comparison to other areas (Sonnenberg et al., 2022). However, these considerations are unlikely to be an issue when the novel MycroNutrient product is applied in a larger-scale or commercial setting, as no partitions would be present.

4.5 Application associated Carbon reductions were over 99% for the novel MycroNutrient Product compared to UV treatment

There is a marked reduction in predicted carbon emissions when comparing UV enrichment to the application of the novel MycroNutrient product. This is not surprising as the UV treatment is a highly energy intensive process, the energy requirement being the main source of carbon equivalent emissions. UV treatment also slows production, likely leading to further unforeseen carbon costs in refrigeration and storage as well as reduced production capacity, that were not accounted for in the scope of this carbon assessment. The novel MycroNutrient product treatment allows enrichment to take place during growth and so will not cause these associated emissions. These carbon emissions are far lower due to the only business and application associated emissions coming from water-use for the dilution of the novel MycroNutrient product. Application simplicity could also reduce carbon as specialist equipment is not required and can utilise existing structures in place in developed mushrooms farms for the best efficiency. The method is still applicable to all farms with simple application methods, such as using a watering can, as used for this experiment. The carbon reduction of roughly 14 tonnes CO_2eq per year would also be beneficial in terms of cost to the producer, especially considering energy prices, with the change of treatment allowing a decrease of just under 7% in carbon emissions across the whole business but over 99% in terms of just the application, even when applying to the entirety of mushrooms produced, rather than just browns which make up roughly 20-25% of production. The replacement of UV treatments with the novel MycroNutrient product is therefore likely to greatly increase operational performance as well as allowing far greater sustainability and future energy and carbon security (Gallego-Álvarez et al., 2015) for producers adopting the product over UV, whilst also creating more valuable products in terms of enrichment with more vitamins and not impacting food security whilst reducing emissions and reducing contributions to climate change (Frank et al., 2017). This combined with other strategies for reducing carbon emissions such as renewable energy sources and such, which are already employed by Drinkwater Mushrooms Ltd. can have great benefits for sustainability and further environmentally friendly improvements (Leiva et al., 2017).

4.6 Further Work

Whilst the present study has provided tantalising glimpses into the efficacy of the novel MycroNutrient product, further work will enable optimisation of the product's formulation and application allowing better understanding of interactions between vitamins and their uptake and translocation.

Whilst all samples were stored in either propan-1-ol or methanol to prevent the degradation of vitamins (Temova Rakuša et al., 2021), extraction of each of the B-vitamins individually from separate samples combined with the use of an immunoaffinity or preparative column, as is common with analysis of vitamin B12 alone (Koyyalamudi et al., 2009a) would allow for greater purity and less interference when analysing samples on the HPLC, by separating the specific vitamins rather than analysing the whole sample of extract. Additionally, mass spectrometry (MS) could be employed to confirm the identify of each vitamin and the quantity present in samples (Porter and Lodge, 2021). Calculation of the extraction efficiency using samples 'spiked' with pure forms of the compounds could allow further optimisation of sample extractions and analyses. Whilst the majority of studies of mushroom mineral content utilise ICP-OES variations, or AAS for studying Se in mushrooms, other methods are also available for use on *A. bisporus* specifically (Falandysz 2013), however these were not available to the present study.

In order to determine the wider potential of the novel MycroNutrient product in widespread application to the mushroom industry, future studies should include both white and brown mushrooms combined with an assessment of different application regimes. An assessment of the uniformity of effects between flushes and across production systems as a whole would also add weight to the commercial applicability of the product. It may also prove insightful to test other changes within the make-up and dosages of the product and compare them to those of the present study. Changes to dosages should be made in separate trials and later tested on larger scale commercial conditions, when possible, for businesses to undertake. Nevertheless, the present study shows that the novel MycroNutrient product and its continued development shows great promise in the expanding area of enrichment and biotechnical innovation in mushroom cultivation.

Specific areas where future studies could be targeted include the assessment of the impacts of including vitamin D at a higher purity grade and/or concentration to limit volume of the concentrated product as well as aiding in stability and storage shelf-life (Sharifi and Jahangiri, 2017). In the present study, the novel MycroNutrient product was formulated using vitamin D3, which is the human produced form accepted to be a more effective supplement to human diets than vitamin D2 (Heaney et al., 2011; Balachandar et al., 2021), due to supply limitations with the availability of vitamin D2 which is the form of the vitamin produced naturally by mushrooms exposed to UV light (Simon et al., 2011). Additional studies could be done to compare D2 and D3 to see if D2 may result in greater contents as currently policies do not make the distinction between enrichment and supplementation between the different forms of the vitamin (Bouillon et al., 2016), and greater content could counteract the lesser efficacy. Vitamin D2 is often synthesised from larger scale and more efficient exposure of mushroom waste, such as unused stems after harvesting, to UV (Cardoso et al., 2021). Therefore if it were found that inclusion of vitamin D2 increased further the efficacy of the novel MycroNutrient product, whilst this does mean UV would still be used in the production it could be far more efficient and move carbon costs away from the grower whilst also contributing a circular and far more sustainable economic practice.

Vitamin D4 has also been found to be present in many mushrooms exposed to UV or sunlight (Urbain et al., 2016), as well as coeluting alongside vitamin D3 in many HPLC procedures of analysis (Phillips et al., 2012). This raises the possibility that a combined treatment of the novel MycroNutrient product as well as UV or sunlight could allow for an even greater boost in overall ‘vitamin D’ content of the mushrooms, which could be confirmed with analysis mentioned above to separate the different vitamin D types within the samples and accurately quantify them (Phillips et al., 2012). This way a greater maximum content could be reached utilising different types of vitamin D. There is also opportunity to better understand how exposure to sunlight as a more common practice could be beneficial (Urbain and Jakobsen, 2015), combined with treatment application, with potential for greater benefits (Simon et al., 2011).

In terms of product stability and vitamin retention in normal consumer conditions, vitamin D2 decreases in post-harvest cold storage in the caps but actually increases in the stipes (Guan et al., 2016). D2 is stable for 24 hours at room temperature, though this is likely not relevant for a consumer of commercially produced mushrooms, and after 7 days overall mushroom D2 content had halved, even with cold storage (Salemi et al., 2021). It is unknown whether D3 would show a similar pattern and further work could be carried out to study the effects of storage and shelf-life and see if this pattern may change with the application of vitamin D3 instead of the production of D2, though in other studies of fortified foods little difference was observed between D2 and D3 (Tabibian et al., 2017). Studies on vitamin stability and shelf-life would be necessary, as nutritive labelling of food products must be based on the least stable and fastest deteriorating component (Berry Ottaway, 2010). It would also be important to test this in the concentrated product as well to confirm optimal storage conditions before dilution and application during use.

For mineral enrichment, it would be interesting to assess other methods such as direct application to the substrate as discussed previously in **Section 4.3** (Falandysz, 2008; da Silva et al., 2012). Combined treatments alongside separate novel MycroNutrient and substrate additions would give a better understanding of interactions and differences between the methods for uptake efficiency and possibilities in this area. Combined treatments like those suggested here have the greatest opportunity in wide-range enrichment of both vitamins and minerals, for greater impacts on public health and deficiencies (Wimalawansa, 2013).

For future development of enrichment products, research also needs to consider the uptake and translocation of vitamins and minerals by mushrooms, possibly allowing for more specific, targeted and efficient supplements. To date, little attention has been paid to vitamin and mineral processes in fungi therefore more research in this area could lead to biotechnological advancements for this industry to target health benefitting vitamins, minerals and other compounds more specifically to the fruiting bodies for harvest. Genetic research could also be performed for these purposes and have great potential due to the adaptability and plasticity of fungi and mycelial networks (Fricker et al., 2017).

As suggested previously, further work could also be done on the waste of mushroom cultivation, usually the spent mushroom substrate (SMS), as there are implications that this product could result in higher quality, biological and nutritional value after use of the product. There is also potential for greater sustainability by reusing waste from this industry for further uses in waste valorisation and SMS interests such as fertilisers, animal feed and fortification for other food sources (Chiu et al., 2000, Mohd Hanafi et al. 2018; Kumar et al., 2022a). These uses can also include the extraction of ergosterol from waste but also using this for the production of vitamin D2 for many other uses but also possibly for use as the additive to the novel MycroNutrient product if going down a D2 route as mentioned previously (Papoutsis et al., 2020).

Other added benefits which could be explored as a result of the addition of the novel MycroNutrient product (Noble and Dobrovin-Pennington, 2018) include increases in dry matter and also benefits in improving shelf life. Testing would be required to see whether the side-effect of increased shelf-life via UV irradiation and subsequent killing of microbes may decrease shelf-life if this new liquid supplement is used instead, although this change is relatively small, as mushroom shelf-life is short and greater determinants such as high respiration rates and high moisture contents (Guo et al., 2023), with dry matter being a greater affector. As such, future work could also include comparisons of shelf-life between UV and novel MycroNutrient product treated mushrooms.

4.7 Conclusions

This study shows that the Ca formulation of the novel MycroNutrient product with a vitamin D dosage of around 125-150 mg/m^2 is an appropriate replacement of UV treatment for enrichment of mushrooms with vitamin D; this results in consistent nutritionally relevant vitamin D levels across flushes, whilst also providing added B-vitamins alongside. The vitamin content recorded provide a suitable level of daily intake, as seen with comparisons to NRVs, with suitable serving sizes in both vitamin D and B-vitamins at the dosages seen in these experiments and without causing discolouration to white varieties of mushrooms. In contrast, the target of Se enrichment does not appear achievable through this treatment although it is possible

that this could be achieved through combinations of substrate enrichment methods. Further work would be necessary to test this possibility. However, the novel MycroNutrient product used in the study shows great promise for use in the future to replace high energy UV treatment and to markedly reduce carbon emissions associated with mushroom vitamin D enrichment for commercial sale, whilst also applying to a greater quantity of mushrooms. This provides opportunities for lowering costs but also opening other avenues for waste management and sustainability to the mushroom cultivation industry. This new enrichment method has the potential to also improve the range of vitamins available to create new fortified and novel mushroom products, aiding in creating more accessible options for preventing deficiencies and improving public health, especially among deficient and deficiency vulnerable groups.

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Appendices

Appendix A

Growth Management and Condition Control

(Any volumes refer to use over the entire shed)

DAY 0

1. Filling of substrate and compost layer into growth beds (substrate already spawned)
2. Air temperature at 15°C with 80-90% fan (prevent compost temperature rising)

DAY 1

1. Watering – 1600 litres total application
2. Roughed surface
3. Wash out and leave
4. Air temperature 15°C and 80-90% fan.

DAY 2

1. Application of 400 – 800 litres hypo-chloride solution added if needed.
2. Bed temperature up to 26°C on average with 15°C air temp, fan speed maintained

DAY 3

1. Application of 900 litres of hypo-chloride solution if needed.
2. Compost temperature starts to decrease, increase in average compost and air temperatures

DAY 4

1. Application of 1 – 3 x 2000 litres of hypo-chloride solution
2. Compost temperatures decrease again, air temperature increased to 22-23°C and compost temperature increased to 26.5-27°C
3. Fan speed slowed to 35-40%
4. Humidity increases
5. Ruffling of casing and **first application**

DAY 5

1. Application of 2-4 x 2000 litres hypo-chloride solution and ready-to-use disinfectant (SporGon – disinfectant and fungicide to kill remaining spores)
2. Temperature and fan speed maintained

DAY 6

1. Watering and disinfectant or just water (same as before)
2. Fans speed maintained (35-40%)

DAY 7

1. Airing if ready – mycelium evenly peeking through peat
2. CO_2 of 1400-1500 ppm from 6000+ over 2 – 3 days.
3. Air temperature 21-22°C at start of airing decreased to 18°C at pinning - roughly 2-3°C difference between compost and air temperature
4. When pins appear drop air temperature from 18-17°C for better spread of pins for picking over the next 5 days

DAY 8

1. Drop air temp by 1°C
2. Wet whites and floors x 2.

DAY 9

1. Wet whites and floors x 2 and rewash shed floor.
2. Depending as to how strong mycelium are moving drop air temp by 1°C

DAYS 10 TO 13

1. Wet floor x 2
2. Air temp now 19°C
3. Compost temperature 22-23°C
4. Fan at 38-40%
5. CO_2 maintained at 1400-1500 ppm
6. Fresh air 45-55%
7. Humidity at 90%
8. Maintain conditions until day 12-13 when the first pins appear then – compost temperature will be roughly 21-22°C
9. If not enough pins, decrease air temperature to 17°C
10. Maintain air and fans throughout until pea size pins are crooked - increase humidity 1-2% and decrease fan speed 3%

DAYS 14 TO 16

1. Once pins have set wait 2-3 days to increase in size.
2. Start to increase air temperature 0.5°C every 12 hours to 18.5°C
3. As mushrooms get bigger CO_2 begins to rise above 1500 ppm so more fresh air (5-10%)
4. Decrease humidity by 2%
5. Decrease air temperature back to 17.5-18°C – achieves better freshness and quality.

DAYS 17 – 19

1. Roughly 48 hours for picking before clear 1-2 watering (as before) and floor washed.
2. Air temperature increased back up to 18.5°C to gently dry overnight
3. Other controls: CO_2 should be at 1400ppm (39-42%) and fresh air at 55-90%.
4. **Second application** just after picking and watering of first flush

DAYS 20 – 23

1. Clearing and watering
2. Air temperature to 22°C for 12 – 24 hours, fan at 50%
3. Fresh air at 50-65%, humidity to 80-86%
4. Application of 3 x 2000litre hypo-chloride
5. Application of 1:1000 L (hypo-chloride:water)
6. Application of disinfectant (SporGon) and wash-off
7. Air temperature decreased to 19°C
8. CO_2 at 1300-1500 ppm
9. Application of 4 x 1000 litre hypo-chloride solution

DAYS 24 – 26

1. If after drying from watering off, not enough pins coming through, decrease air temperature to 17-18°C for 48 hours very slowly
2. Slow fan speed and introduce more fresh air over the next 1-3 days

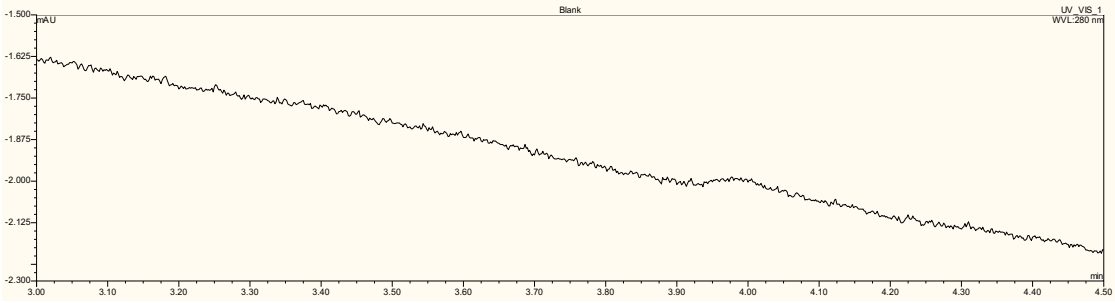
DAYS 27 – 30

1. Mushrooms growing larger, fan speed and fresh air slightly increased
2. CO_2 kept at 1200-1350 ppm and temperature kept at 17°C
3. When clear - air temperature increased to 20°C for 24 hours
4. Application of 2 x 2000 L hypo-chloride solution
5. Wash next day – air temp increased to 18.5°C
6. Application of 1 x 2000 L hypo-chloride solution
7. On 1st clear day – fan at 45-55%, fresh air 50-70% and humidity at 78-84% whilst drying off
8. When pinned – 900-1000 ppm CO_2, 38% fan speed, 48% fresh air and 82% humidity
9. 3rd clearing day – water and wick then add electrical box and apply steam pipe
10. All harvested mushrooms transported to pack house
11. Application of formaldehyde and 'cook out' compost temperature of 65°C for 10 hours
12. Once shed emptied – all washed and sterilized with formaldehyde then gas bottles applied 12-24 hours before next filling. Gas bottles taken out and air temperature decreased to 14-15°C

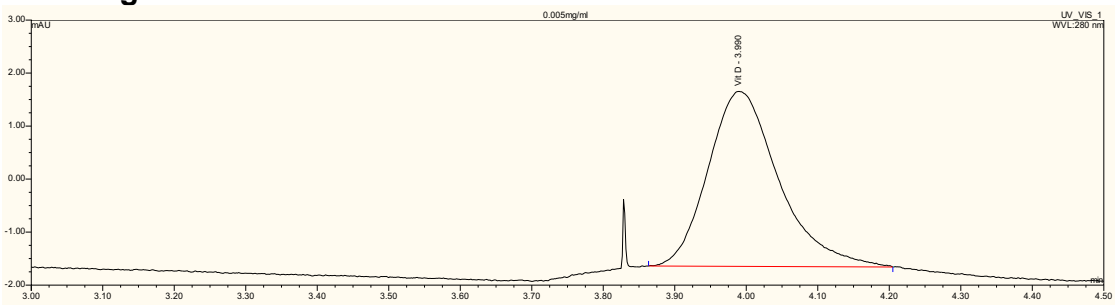
Appendix B

Vitamin D2 (Zorbax and Chromeleon) calibration chromatograms

Blank



0.005 mg/mL



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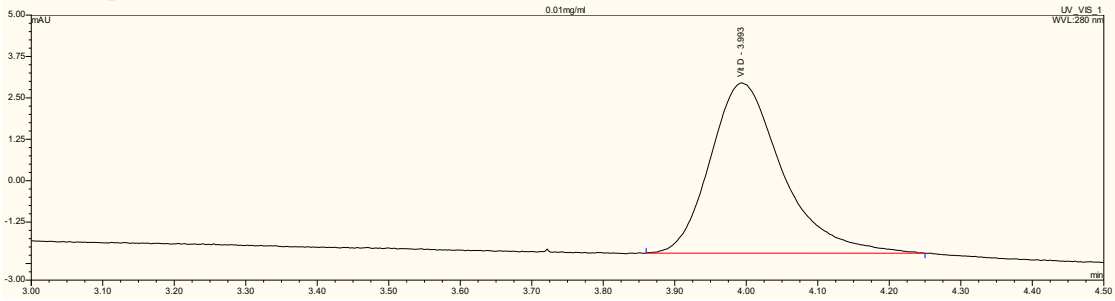
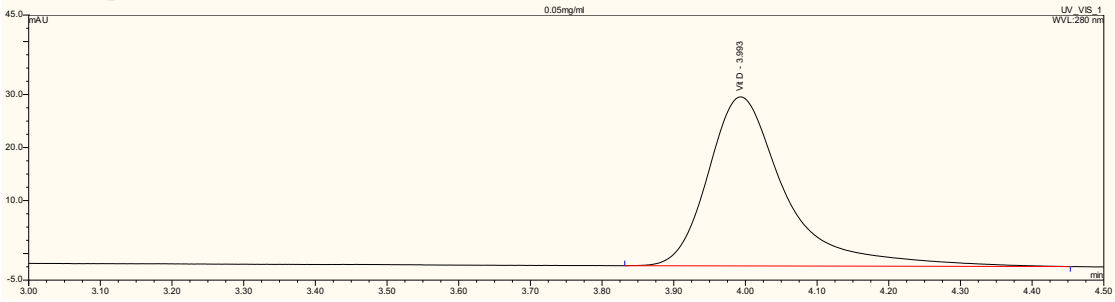


0.05 mg/mL



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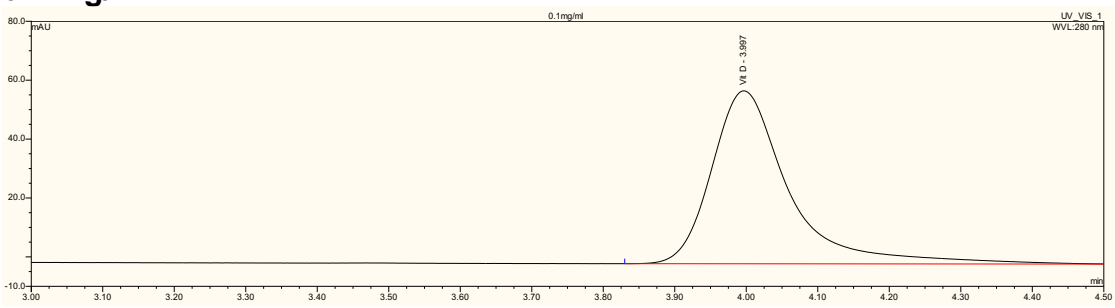
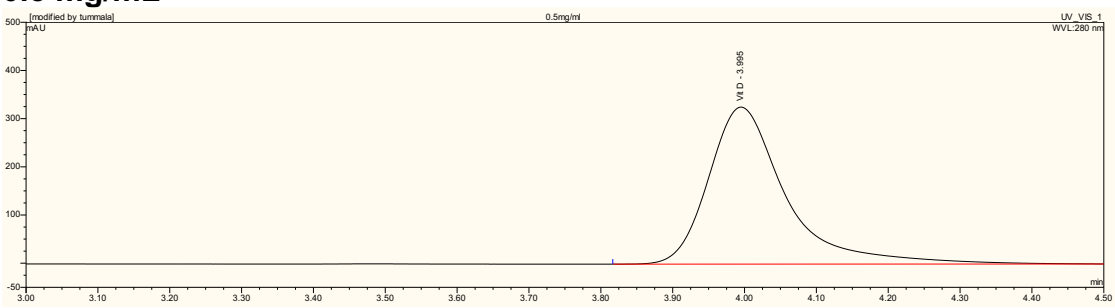
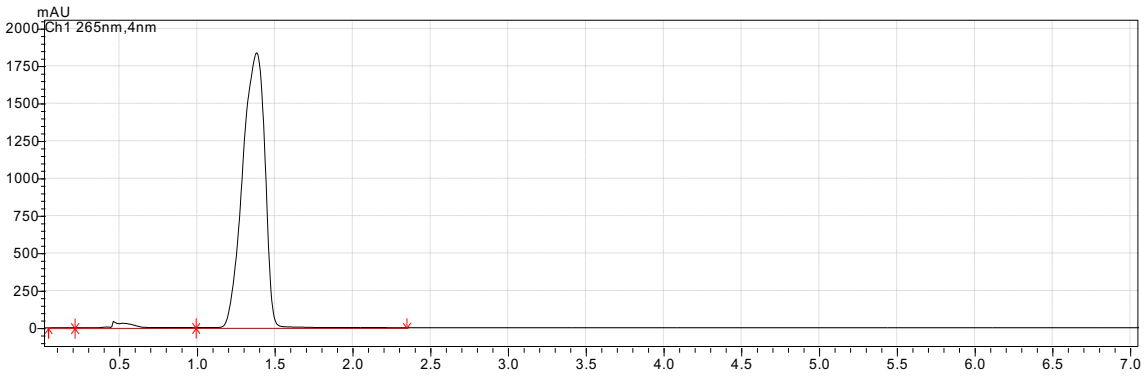


0.5 mg/mL

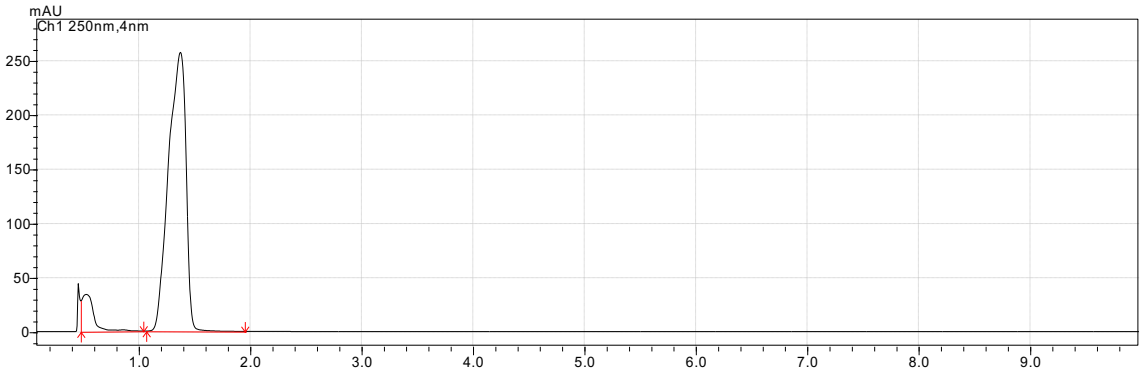


Vitamin D3 (Poroshell and Shimadzu) calibration chromatograms

0.005 mg/mL



0.01 mg/mL



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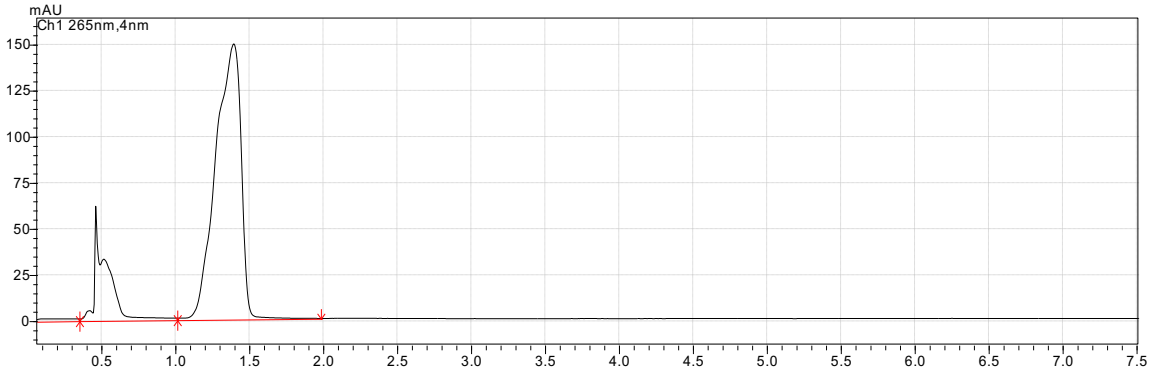
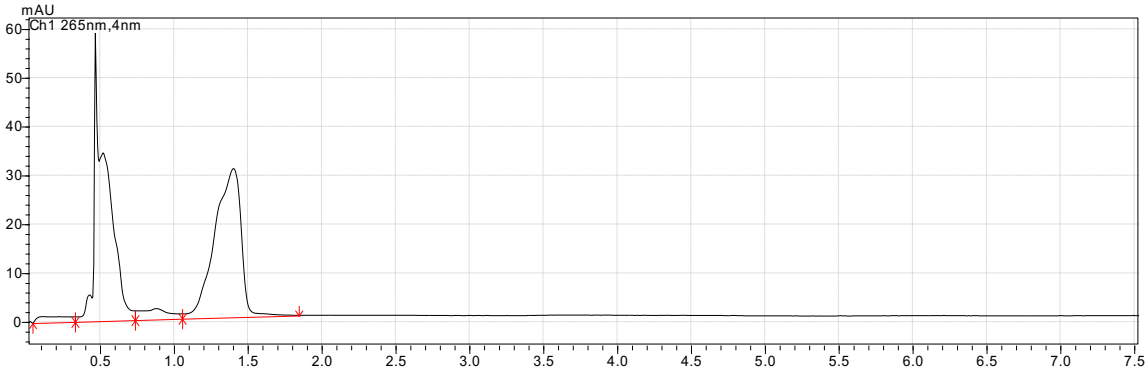
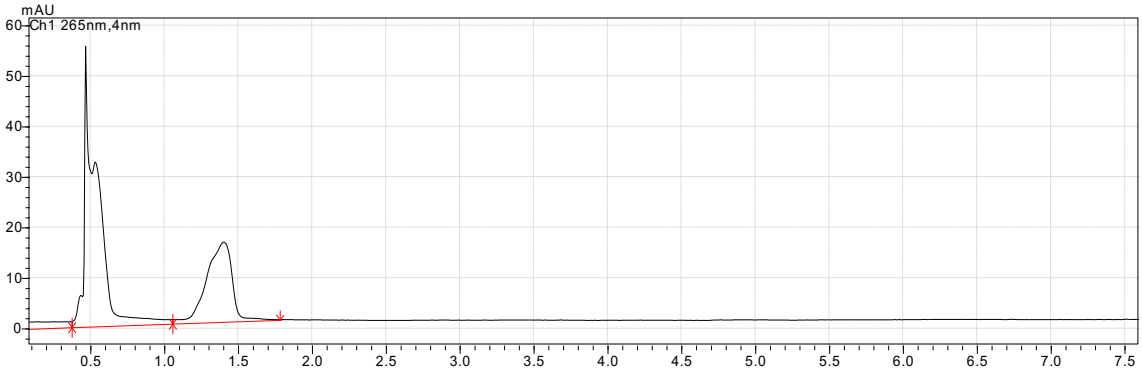


0.1 mg/mL

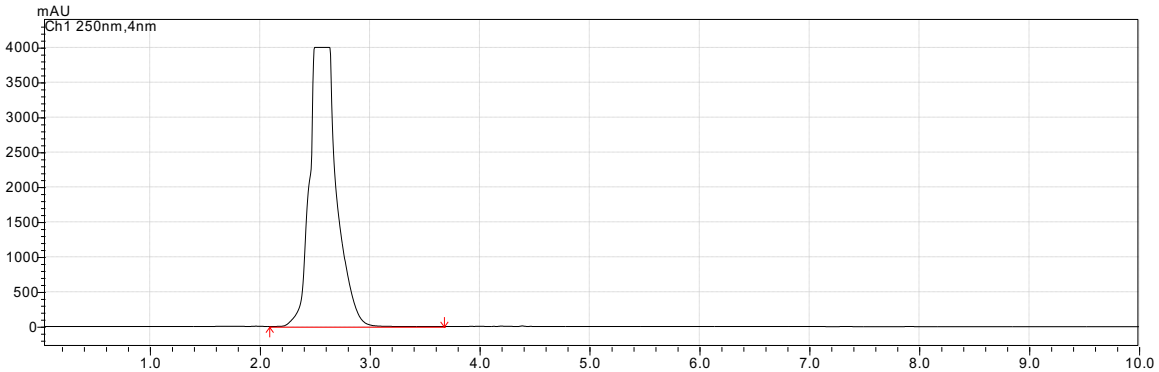


0.5 mg/mL

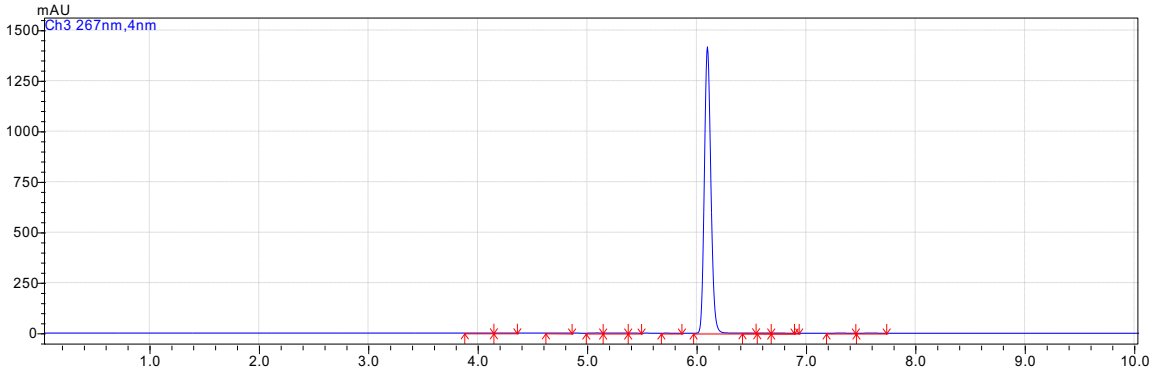


B Vitamin identifiers (Zorbax)

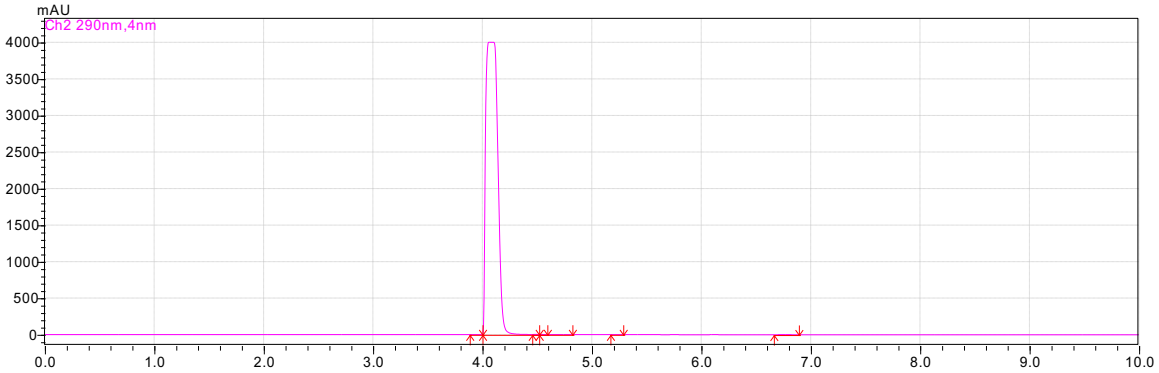
B1



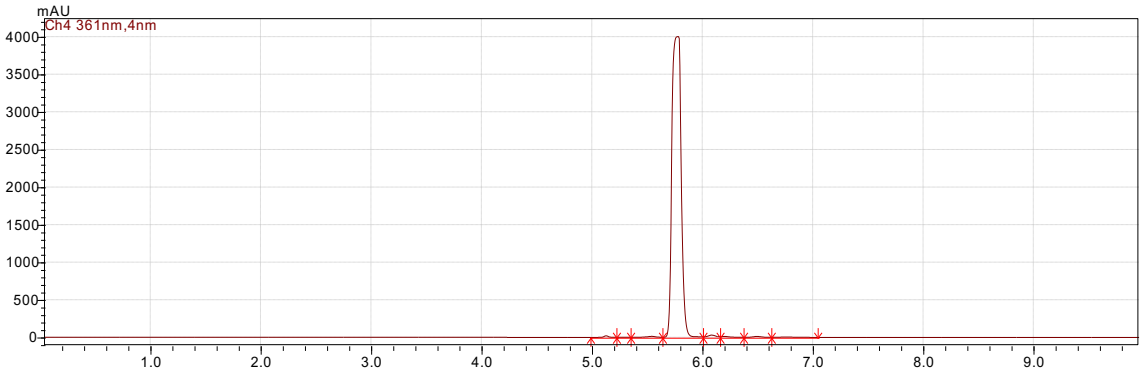
B2



B6

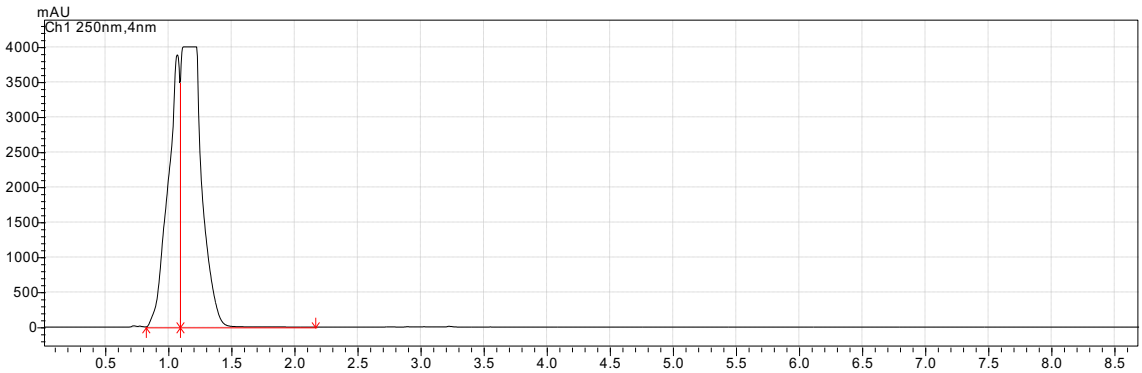


B12

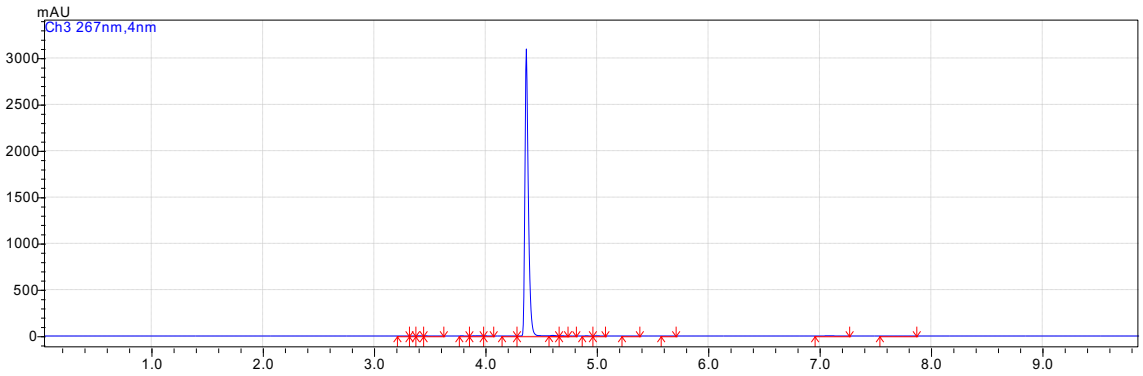


B Vitamin identifiers (Poroshell)

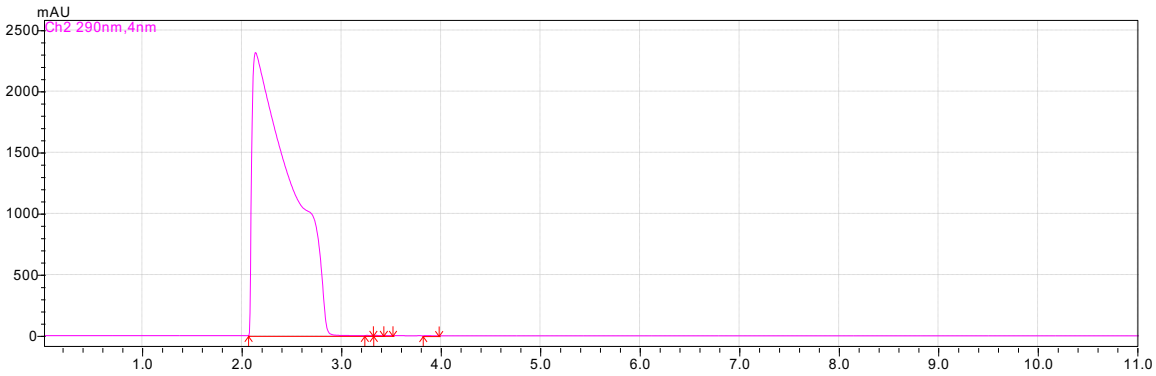
B1



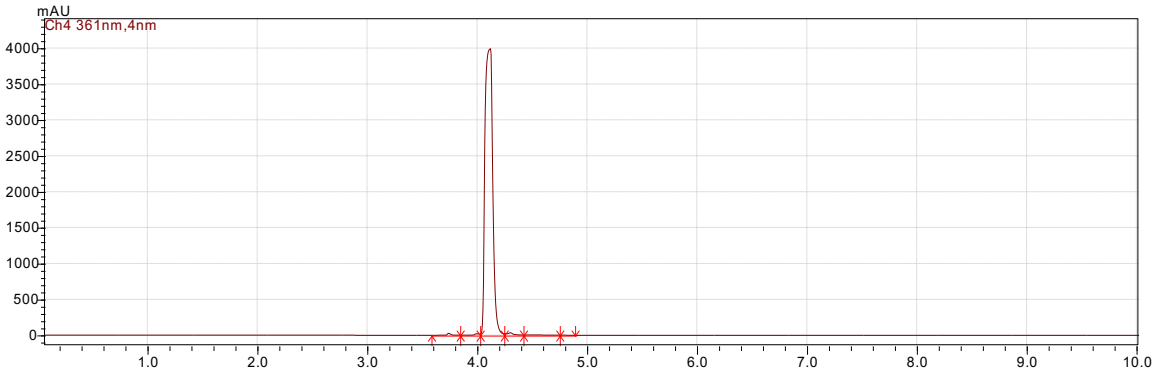
B2



B6



B12



B vitamin mixed samples (Poroshell)

0.005 mg/mL

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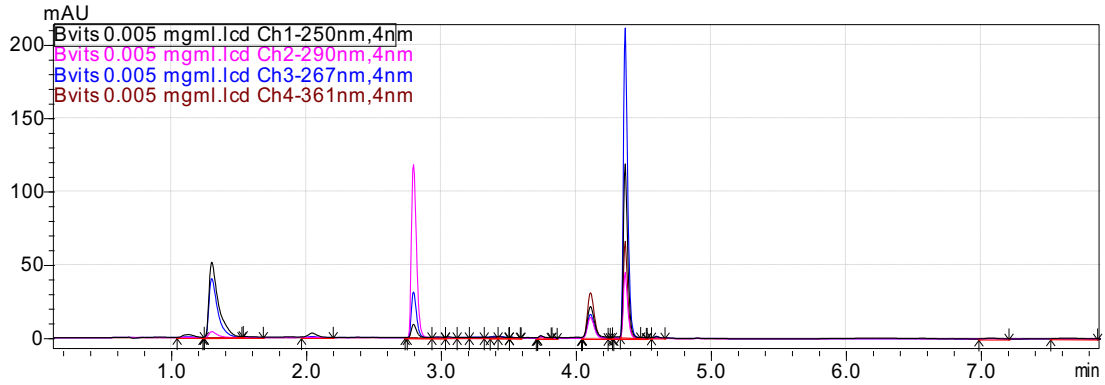
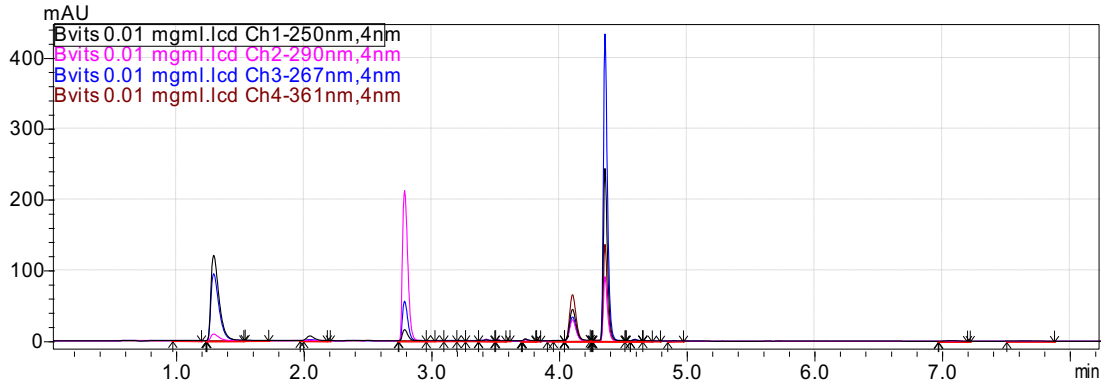
Sample Name:Bvits 0.005 mgml
Sample ID:Bvits 0.005 mgml



0.01 mg/mL

Datafile Name:Bvits 0.01 mgml.lcd
Sample Name:Bvits 0.01 mgml
Sample ID:Bvits 0.01 mgml



0.05 mg/mL

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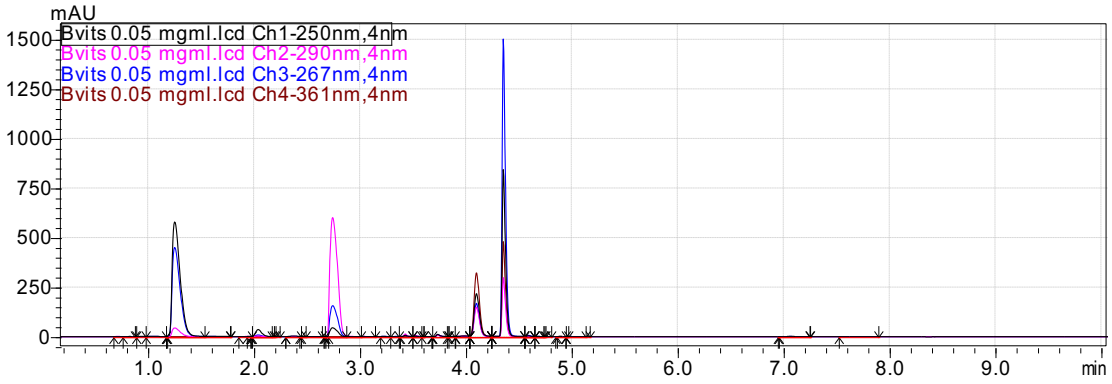
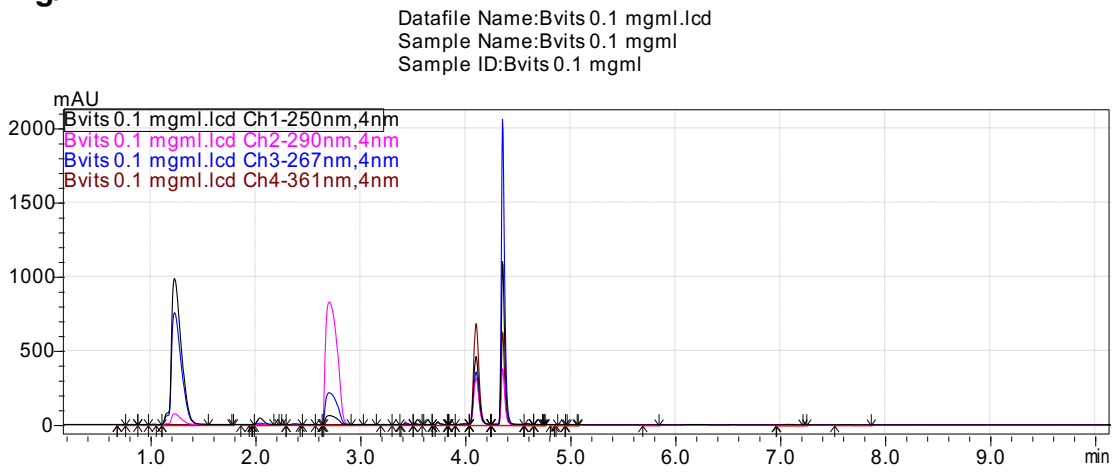
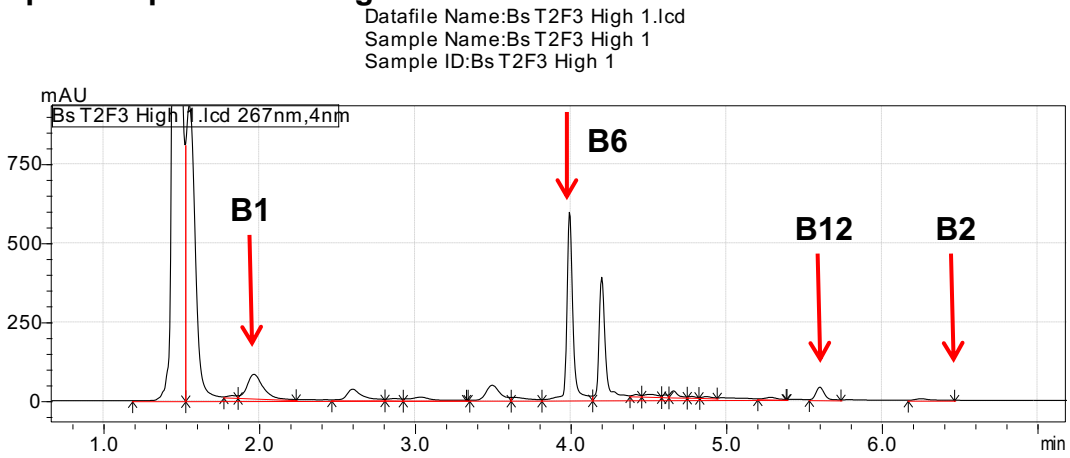
Sample Name:Bvits 0.05 mgml
Sample ID:Bvits 0.05 mgml



0.1 mg/mL



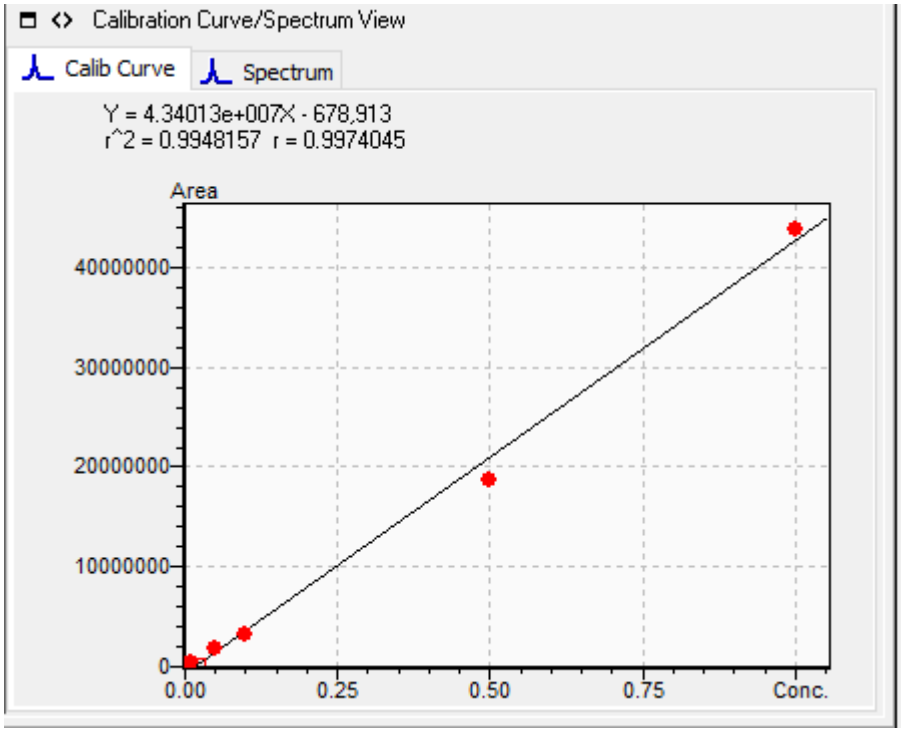
Example Sample Chromatogram



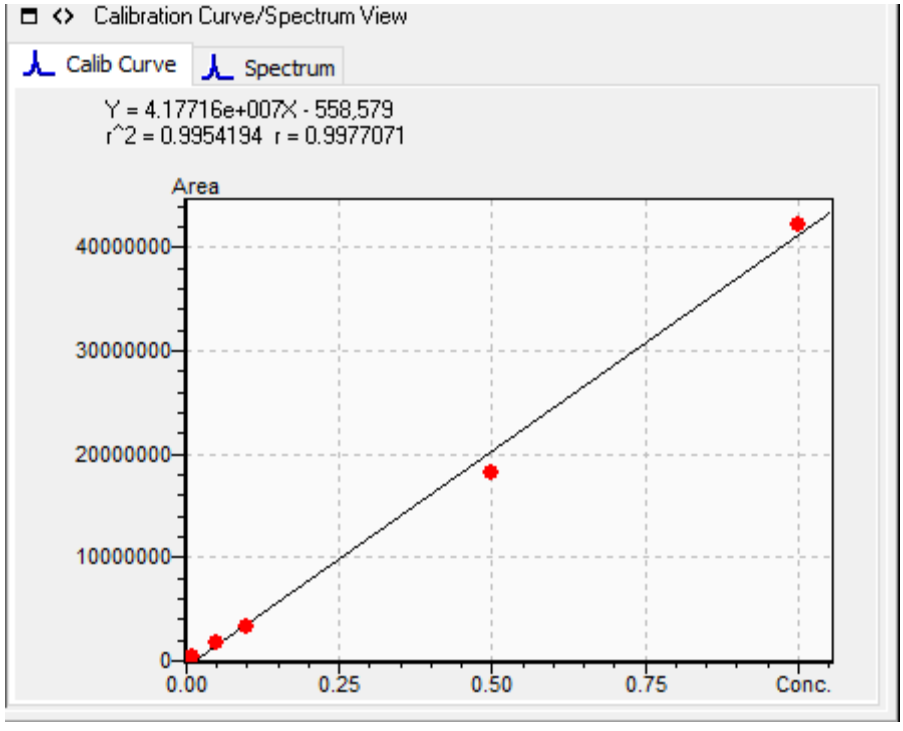
Appendix C

Vitamin D Calibration

Run 1



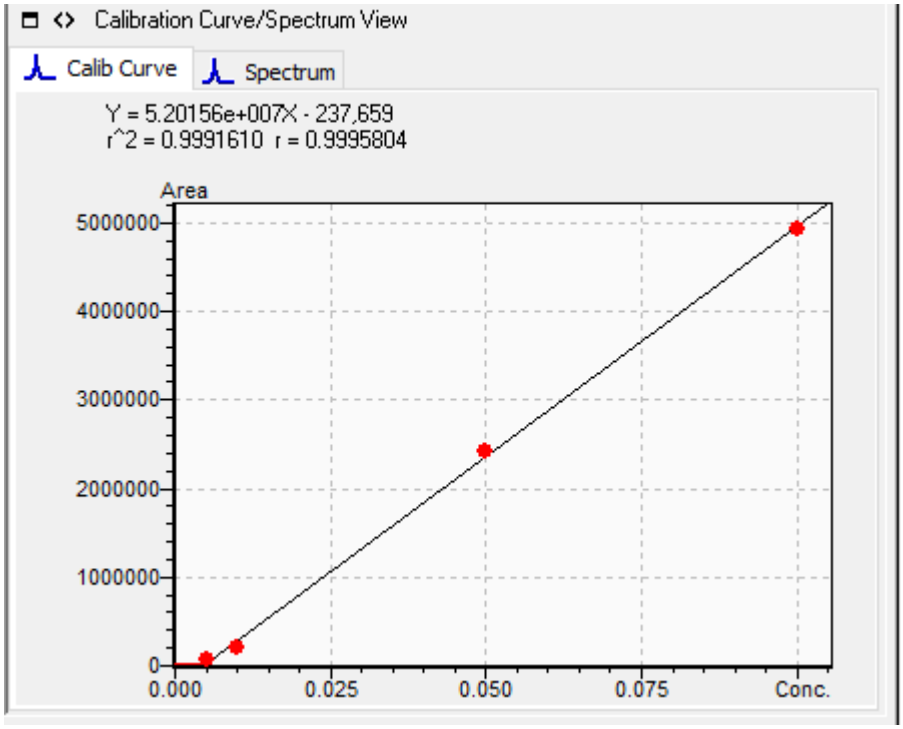
Run 2



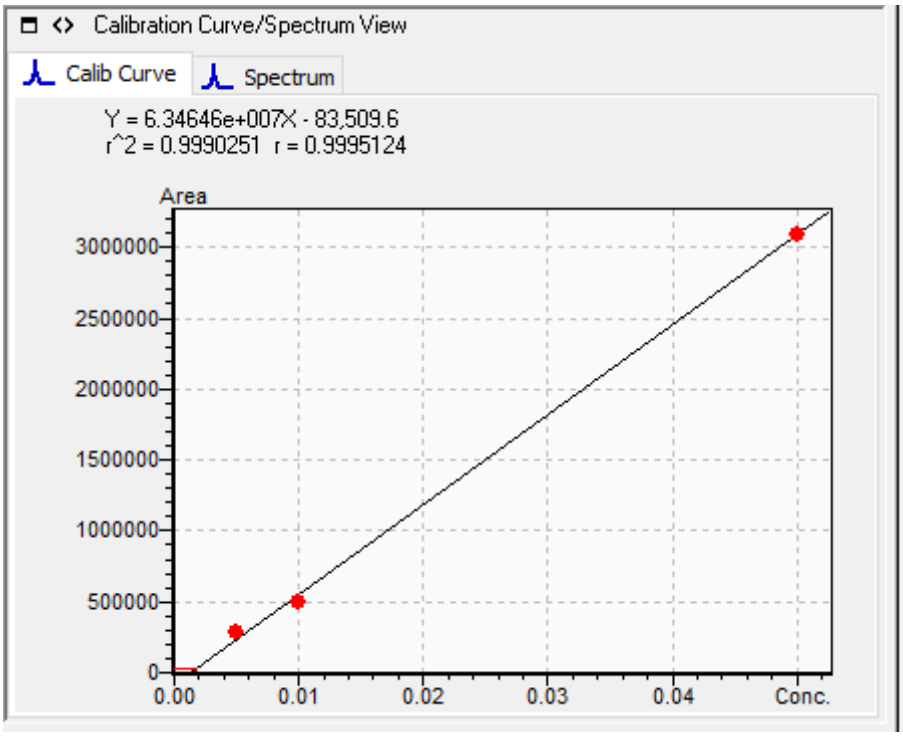
B Vitamin Calibration

Run 1

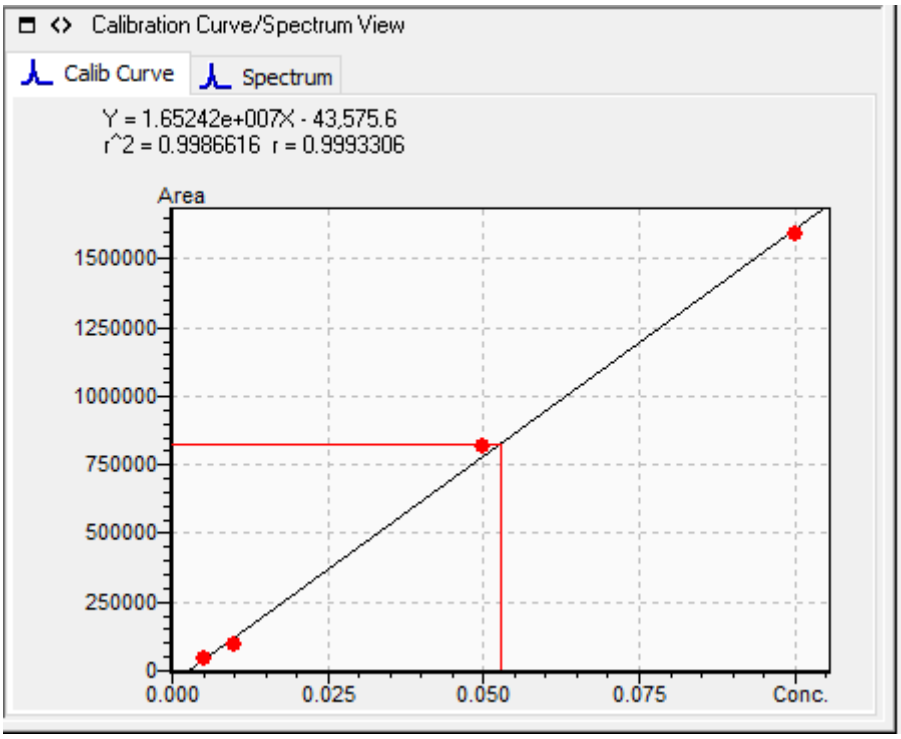
B1



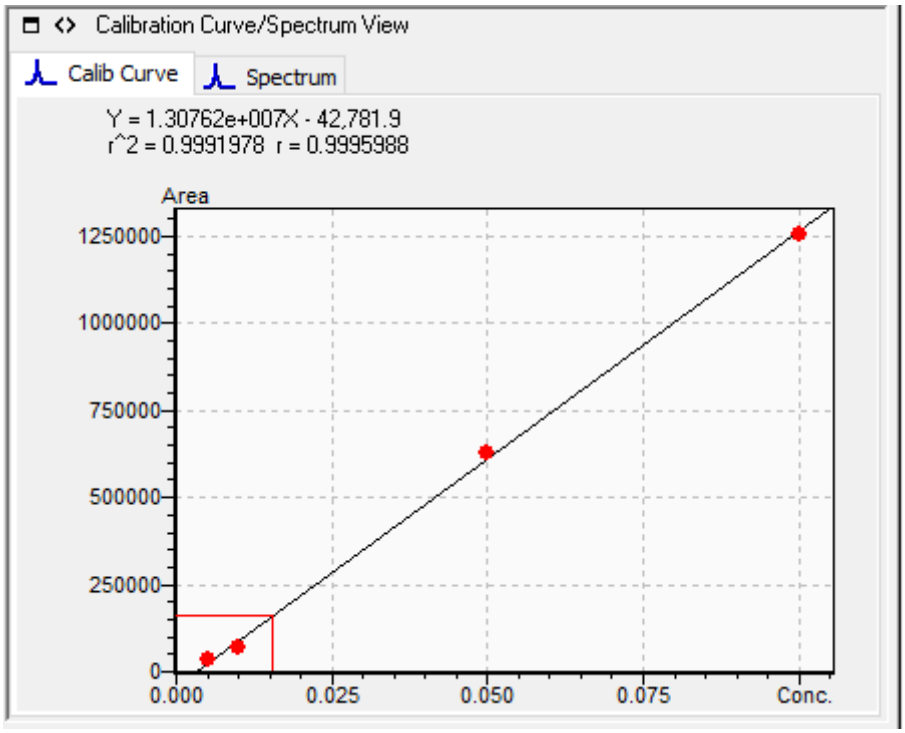
B2



B6

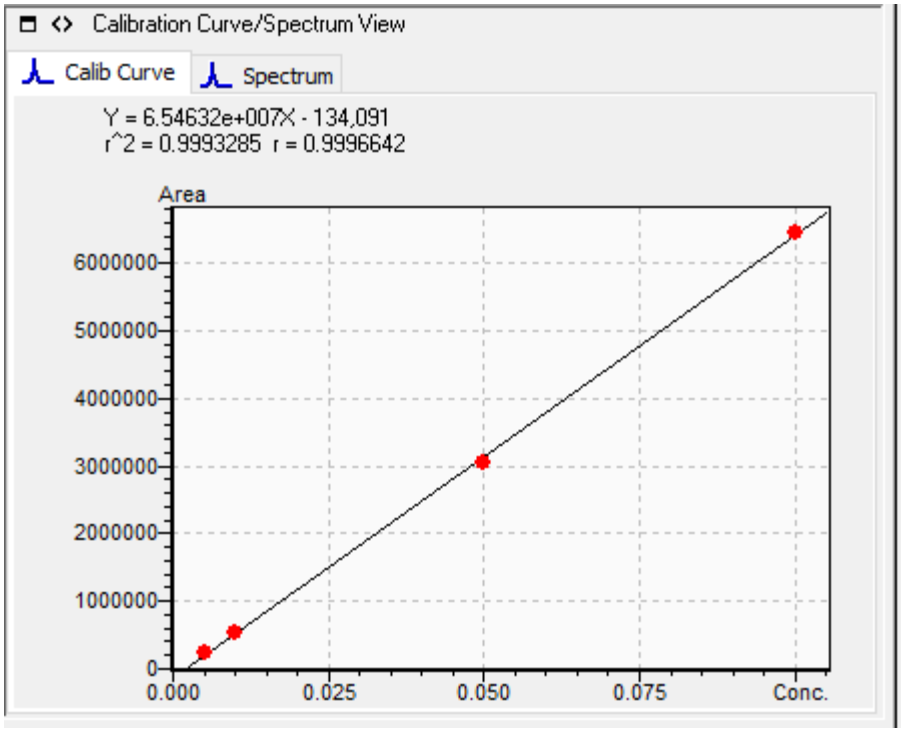

Calibration Curve/Spectrum View
Calib Curve
Spectrum
Y = 1.65242e+007X - 43,575.6
r^2 = 0.9986616 r = 0.9993306
Area
1500000
1250000
1000000
750000
500000
250000
0
0.000
0.025
0.050
0.075
Conc.

B12

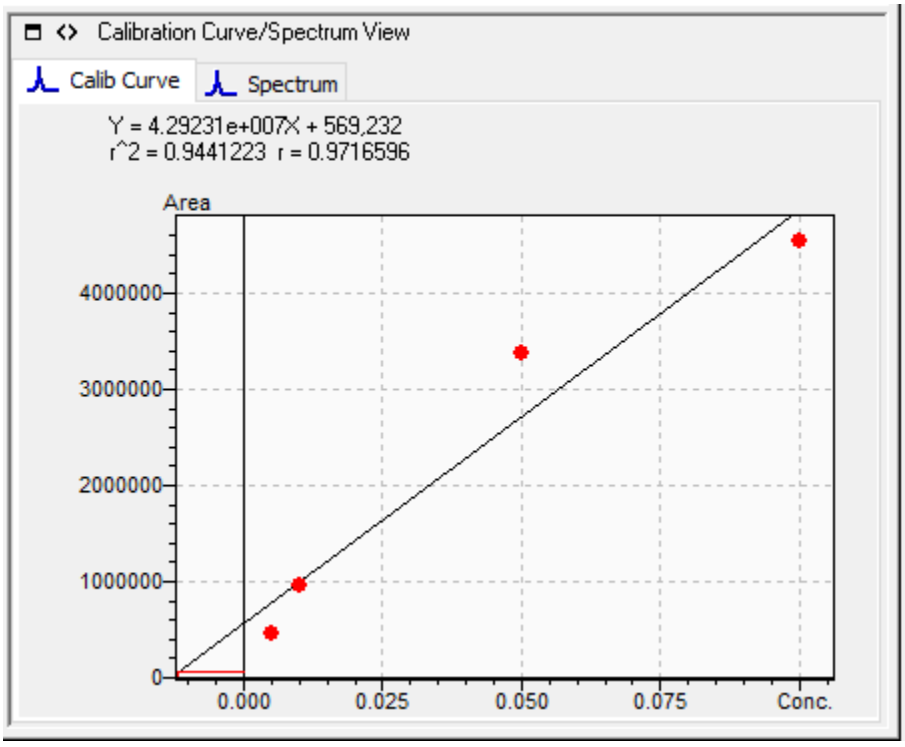

Calibration Curve/Spectrum View
Calib Curve
Spectrum
Y = 1.30762e+007X - 42,781.9
r^2 = 0.9991978 r = 0.9995988
Area
1250000
1000000
750000
500000
250000
0
0.000
0.025
0.050
0.075
Conc.

Run 2

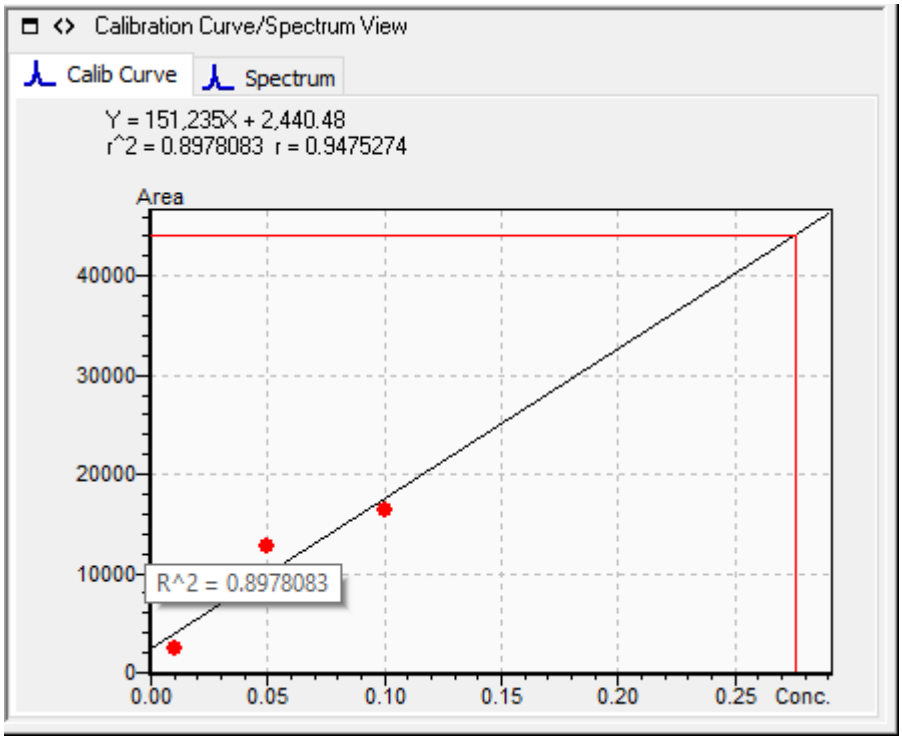
B1


Calibration Curve/Spectrum View
Calib Curve
Spectrum
Y = 6.54632e+007X - 134,091
r^2 = 0.9993285 r = 0.9996642
Area
6000000
5000000
4000000
3000000
2000000
1000000
0
0.000
0.025
0.050
0.075
Conc.

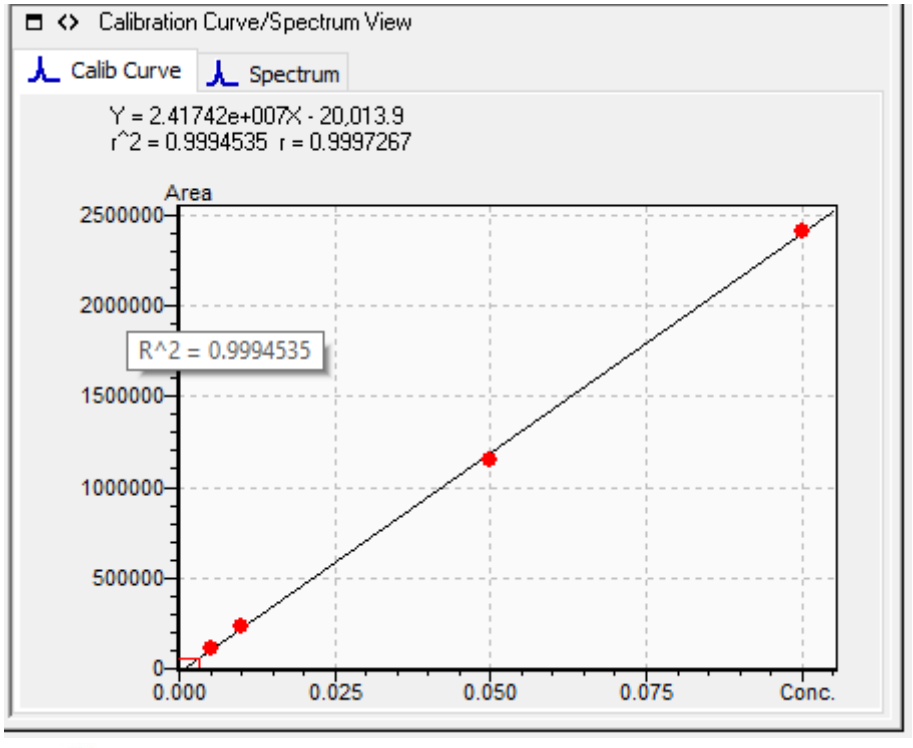
B2


Calibration Curve/Spectrum View
Calib Curve
Spectrum
Y = 4.29231e+007X + 569,232
r^2 = 0.9441223 r = 0.9716596
Area
4000000
3000000
2000000
1000000
0
0.000
0.025
0.050
0.075
Conc.

B6


Calibration Curve/Spectrum View
Calib Curve
Spectrum
Y = 151.235X + 2,440.48
r^2 = 0.8978083 r = 0.9475274
Area
40000
30000
20000
10000
0
R^2 = 0.8978083
0.00
0.05
0.10
0.15
0.20
0.25
Conc.

B12


Calibration Curve/Spectrum View
Calib Curve
Spectrum
Y = 2.41742e+007X - 20,013.9
r^2 = 0.9994535 r = 0.9997267
Area
2500000
2000000
1500000
1000000
500000
0
R^2 = 0.9994535
0.000
0.025
0.050
0.075
Conc.

Appendix D

Full Eco-I NW Carbon Report

CENTRE FOR GLOBAL ECO-INNOVATION

CO_2e Calculator

Name: Michael Williams
Project: Developing a new agritech product to increase and enhance the uptake of vitamin D and other vitamins and minerals in mushrooms
Industry Partner: NutriGain
Academic Supervisors: Martin McAinsh and Kirk Semple
Description of project: New product to enrich mushrooms with vitamin D and other vitamins and minerals seeking to replace current methods of enrichment such as UV-irradiation which is highly energy intensive and limits production as well as having limited application to mushroom varieties
Summary of GHG emission reduction: GHG emissions will be reduced by taking away the need for a very energy intensive process of enrichment and instead adding enrichment through an added supplement during growth of the crop. Reductions will be calculated in comparison to the emissions associated with application of the new product to the equivalent mushroom production. Calculations are for the following case study: **Drinkwater Mushrooms Ltd.** - The baseline is concerned with the emissions generated by the production and most importantly processing of enriched mushrooms which this project aims to replace with a carbon reduced alternative - Figures are calculated to a period of 1 year to calculate the annual emissions and reductions - This company are supporting the project and a client of NutriGain who are invested and interested in the use of the new product should it be a suitable replacement of these current methods.

GHG emissions before support	Current GHG emissions	Total GHG reduction	Percentage of reduction
200.592 t CO_2 eq (per year)	186.578 t CO_2 eq (per year)	14.014 t CO_2 eq (per year)	6.99% (for overall production)

Section one – Baseline of CO_2e emissions relating to original process, service or product

Scope one – Direct emissions from company owned and controlled operations

n/a

Scope two – Indirect emissions purchased by company

CO_2e from **purchased energy for own use (electricity, steam, heating and cooling)**

a) Energy used to power UV light and machinery installed on packaging line for vitamin D enrichment. UV lamps use a 3 phase, 10 A per phase electricity supply, standardly at 400 V. UV-irradiation takes place for 6 hours per day, 7 days per week.

1. Energy consumption = (400 V * 10 A * 3 phases) / 1000 = 20.8 kW
2. Per week = 20.8 kW * 6 hours * 7 days = 874 kWh
3. Per year = 874 kWh * 52 weeks = 45,448 kWh
4. Conversion factor = 0.309 kg CO_2 eq per kWh
5. Emissions = 45,448 kWh * 0.309 (cf) = **14,043 kg / 14.043 t CO_2 eq (per year)**

b) Energy use of Drinkwater mushrooms premises

1. Energy use by all Drinkwater mushrooms premises reported at 2,603,222.3 kWh
2. 2,603,222.30 kWh – 45,448 kWh (UV) = 2,557,774.3 kWh per year
3. 2,557,774.3 kWh * 0.25 (proportion browns production) = 639,443.6 kWh

4. Carbon equivalent emissions reported at 758.32 t CO_2 eq per year
5. 758.32 t – 14.043 t (UV) = 744.277 t CO_2 eq per year
6. 744.277 t * 0.25 (proportion browns production) = 186.1 t CO_2 eq per year

Reported values as provided by Energy & Environmental Review by Chamber Low Carbon for Drinkwater Mushrooms Ltd based on 2021

Scope three – Other indirect emissions from the supply chain owned and/or purchased by suppliers and consumers

Upstream e.g. suppliers

CO_2e embodied in **purchased goods and services**

Water applied during growth of mushrooms

1. For brown mushrooms 28 L/m^2 applied
2. 30 kg/m^2 produced
3. 28 L / 30 kg = 0.93 L per kg
4. 0.93 L / 1000 = $9.3*10^{-4}$ m^3 water per kg
5. Conversion factor = 0.344 kg CO_2 eq per m^3 water
6. $9.3*10^{-4}$ m^3 * 0.344 = **$319*10^{-6}$ kg CO_2 eq (per kg brown mushrooms)**
7. $319*10^{-6}$ kg CO_2 eq * 27,000 kg (per week) * 52 (weeks) = **449.2 kg CO_2 eq (per year)**

Downstream e.g. consumers (sold products)

n/a

Biogenic emissions – Other emissions related to flora, fauna, land and water

n/a

Total baseline emissions figure

Total emissions of brown mushrooms with UV enrichment **= 200.592 t CO_2 eq (per year)**

Or **200,592 kg CO_2 eq (per year)**

Section two – Reduction of CO_2e emissions relating to new process, service or product

Scope one – Direct emissions from company owned and controlled operations
n/a
Scope two – Indirect emissions purchased by company
CO_2e from **purchased energy for own use (electricity, steam, heating and cooling)** **Energy use of Drinkwater mushrooms premises** 1. Energy use by all Drinkwater mushrooms premises reported at 2,603,222.3 kWh 2. 2,603,222.30 kWh – 45,448 kWh (UV) = 2,557,774.3 kWh per year 3. 2,557,774.3 kWh * 0.25 (proportion browns production) = 639,443.6 kWh 4. Carbon equivalent emissions reported at 758.32 t CO_2 eq per year 5. 758.32 t – 14.043 t (UV) = 744.277 t CO_2 eq per year 6. 744.277 t * 0.25 (proportion browns production) = **186.1 t CO_2 eq per year**
Scope three – Other indirect emissions from the supply chain owned and/or purchased by suppliers and consumers
Upstream e.g. suppliers CO_2e embodied in **purchased goods and services** Water applied during growth of mushrooms: 1. For brown mushrooms 28 L/m^2 applied 2. 30 kg/m^2 produced 3. 28 L / 30 kg = 0.93 L per kg 4. 0.93 L / 1000 = $9.3*10^{-4}$ m^3 water per kg 5. Conversion factor = 0.344 kg CO_2 eq per m^3 water 6. $9.3*10^{-4}$ m^3 * 0.344 = $319*10^{-6}$ kg CO_2 eq (per kg brown mushrooms) 7. $319*10^{-6}$ kg CO_2 eq * 27,000 kg (per week) * 52 (weeks) = **449.2 kg CO_2 eq (per year)** (Same as normal watering schedule for growth) **Additional carbon associated with water use for dilution and application of the novel product (on browns):**

1. 2 applications of 60 mL/m^2 and 120 mL/m^2 made up to 1 L each time = 1.82 L water total for all applications
2. 27,000 kg produced per week with an average of 30 kg produced per m^2 (Drinkwater)
3. 27,000 kg / 30kg/m^2 = 900 m^2 estimate for production (per week)
4. 1.82 L * 900 m^2 = 1638 L (per week)
5. 1638 L / 1000 = 1.638 m^3 water used (per week)
6. 1.638 m^3 * 52 (weeks) * 0.344 (cf) = **29.12 kg CO_2 eq (per year)**

Alternatively:

1. 1.82 L / 35 kg = 0.052 L per kg mushrooms
2. 0.052 L / 1000 = 5.2*10^{-5} m^3 per kg mushrooms
3. 5.2*10^{-5} m^3 * 0.344 (cf) = **1.79*10^{-5} kg CO_2 eq (per kg mushrooms)**

(or 17.9 mg CO_2 eq)

Downstream e.g. consumers (sold products)

n/a

Biogenic emissions – Other emissions related to flora, fauna, land and water

n/a

Total reduction emissions figure (savings)

Total emissions for use of novel product (on browns) **= 186.578 t CO_2 eq per year**

Or **186,578 kg CO_2 eq per year**

Reduction (only browns):

1. UV total = 200.592 t CO_2 eq per year
2. Product total = 186.578 t CO_2 eq per year
3. UV – Product **= 14.014 t CO_2 eq savings per year**

Comparison of Processes (browns):

1. Carbon emissions associated with UV = 14,043 kg CO_2 eq (per year)
2. Carbon emissions associated with product treatment = 29.12 kg CO_2 eq (per year)
3. UV – Product = **14,013.88 kg CO_2 eq per year savings**

Further information and comparison (wider scope)

The novel product can also be applied to white mushrooms and has the opportunity for further carbon reductions on a wider scale whilst being applied to the full proportion of production. Below shows further calculations of carbon emissions associated with normal production and application of the product to white mushrooms and comparisons to UV with new carbon reductions.

Water use of whites: (extra)

Water applied during growth of mushrooms – would remain the same regardless of product application)

1. For white mushrooms 40 L/m^2 applied
2. 35 kg/m^2 produced
3. 40 L / 35 kg = 1.14 L per kg
4. 1.14 L / 1000 = $1.14*10^{-3}$ m^3 water per kg
5. Conversion factor = 0.344 kg CO_2 eq per m^3 water
6. $1.14*10^{-3}$ m^3 * 0.344 = **$393.14*10^{-6}$ kg CO_2 eq (per kg white mushrooms)**
7. $393.14*10^{-6}$ kg CO_2 eq * 80,000 kg (per week) * 52 (weeks) = **1635.5 kg CO_2 eq (per year)**

Carbon associated with water use for dilution and application of the novel product (on whites):

1. 2 applications of 60 mL/m^2 and 120 mL/m^2 made up to 1 L each time = 1.82 L water total for all applications
2. 80,000 kg produced per week with an average of 35 kg produced per m^2 (Drinkwater)
3. 80,000 kg / 35kg/m^2 = 2285 m^2 estimate for production (per week)
4. 1.82 L * 2285 m^2 = 4160 L (per week)
5. 4160 L / 1000 = 4.16 m^3 water used (per week)
6. 4.16 m^3 * 52 (weeks) * 0.344 (cf) = **74.41 kg CO_2 eq (per year)**

Total application to all mushrooms:

29.12 kg (browns) + 74.41 kg (whites) **= 103.53 kg CO_2 eq (per year)**

UV comparison:

14,043 kg (UV, only browns) – 103.53 kg (novel product, all mushrooms)

= 13,939.47 kg / 13.94 t CO_2 eq savings (per year – all mushroom production)

99.3% reduction in carbon emissions associated with enrichment process

References

Data provided by:
UV energy use, production, and water use information - **Drinkwater Mushrooms Ltd (2023)**

General energy use information - **Chamber Low Carbon, Shirley Newman, SHE Associates Ltd – Energy & Environmental Review, prepared for: Drinkwater Mushrooms Ltd (2022)**

Application rates and dilutions for novel product - **NutriGain Ltd (2023)**