The influence of humic and fulvic acids on the absorption of selected vitamins in the everted gut model

by

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This thesis is dedicated in memory of Ms. Adri Prinsloo, thank you for all your guidance and support over the years.

"There is no medicine like hope, no incentive so great and no tonic so powerful as the expectation of something better tomorrow"

Jai Shri Krishna

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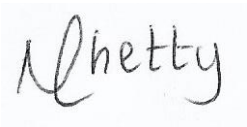
Declaration by candidate:

The experimental work contained in this dissertation was carried out by Nayna Chetty (author) from the Department of Pharmacology, Faculty of Health Sciences, University of Pretoria under the supervision of Prof A.D. Cromarty.

I declare that the work contained in this dissertation is my own work and has not been previously submitted for a degree at this, or any other tertiary institution.



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Abstract:

Nutritional deficiencies and illnesses are prevalent in society and there is a need to prevent them from occurring. The use of natural or complementary medicine when compared to the use of conventional western medication has increased in recent years. Complementary and natural medication preparations are however often unregulated and are not required to adhere to the strict safety and efficacy regulations enforced by the South African Health Products Regulatory Authority for prescription medication. These products are easily accessible to the public because they do not require a prescription and are often marketed as “safe” based on their natural ingredients. Many of these natural medications have unfounded claims of “benefits without side effects”. Humic acid and fulvic acid preparations have been reported to have antiviral, anti-inflammatory, antibacterial and profibrinolytic properties, however their efficacy may be overstated and therefore there is a need to investigate the claims regarding these natural products. The aims of this study were to evaluate the potential benefits of humic substances, and to determine the effects on the intestinal absorption of water-soluble vitamin when in the presence of humic substances. The method of choice for the evaluation of the effect of humic and fulvic acids on water soluble vitamin absorbance was an *ex vivo* method using the everted mouse gut model. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to quantitate vitamin absorption.

The absorption of different water-soluble B vitamins in the gastrointestinal tract (GIT) are affected by a number of factors including solubility, complexation ability, molecular size, the GIT region and the transporters available at the site of absorption. The influence of absorption of nutrients when taken in combination with humic substances was investigated and it was found that humic acid negatively affects absorption of most water-soluble vitamins while fulvic acid displays favourable absorption of select vitamins. There are potential benefits of fulvic acid in aiding nutrient absorption for treatment of nutritional deficiencies. Applications of humic acid as an additive in nutritional supplements is not recommended and should be avoided.

This project set out to evaluate the potential benefits of humic substance supplementation in combination with B-vitamins but the results indicate that humic acid

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provides no benefit and fulvic acid, despite increased absorption of select vitamins, should be used with caution to avoid selective vitamin uptake.

Keywords: vitamin B complex, humic acid, fulvic acid, humic substances, absorption, everted mouse gut model

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Glossary of Abbreviations:

ºC	degree Celsius
%	percentage
µg	micrograms
µL	microlitre
ABC	ATP-Binding Cassette
ATP	Adenosine triphosphate
$CaCl_2$	Calcium chloride
Caco-2	Colorectal adenocarcinoma 2
CD1	Cluster of differentiation 1
CHD	Carbohydrate derived
cm	centimetre
Da	Dalton
ESI	electrospray ionisation
FA	Fulvic Acid
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
g	grams
GIT	Gastrointestinal tract
GMP	Good Manufacturing Processes
HA	Humic Acid
HFBA	Heptafluorobutyric acid
HS	Humic Substances
Ig	Immunoglobulins
KCl	potassium chloride
KRB	Krebs Ringer Buffer
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg	milligram

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$MgCl_2$	magnesium chloride
min	minimum
mL	millilitre
mM	millimolar
mm	millimetre
MRM	Multiple reaction monitoring
MS	Mass spectrometry
msec	millisecond
NaCl	sodium chloride
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NaH_2PO_4	Sodium sulphate
$NaHCO_3$	Sodium bicarbonate
PAMPA	Parallel Artificial Membrane Permeability Assay
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
PLP	Pyridoxal-5-phosphate
ppm	parts per million
RDI	recommended daily intake
RFC	reduced folate carrier
RFVT1	riboflavin transporter 1
RFVT2	riboflavin transporter 2
RFVT3	riboflavin transporter 3
SLC	solute carrier
SPE	solid phase extraction
SVCT1	Sodium dependent Vitamin C transporter 1
SMVT	Sodium dependent multivitamin transporter
TC 199	Tissue culture media 199
THTR-1	Thiamine transporter 1
THTR-2	Thiamine transporter 2
TPP	Thiamine pyrophosphate

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ULOQ	Upper limit of quantification
UPBRC	University of Pretoria Biomedical Research Centre
Vitamin B_1	Thiamine
Vitamin B_2	Riboflavin
Vitamin B_3	Niacin
Vitamin B_5	Pantothenic acid
Vitamin B_6	Pyridoxine
Vitamin B_7	Biotin
Vitamin B_9	Folic acid
Vitamin B_{12}	Cobalamin
w/v	weight per volume

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Chapter 1: Literature review

1.1 Background

Nutritional deficiencies and illnesses are prevalent in society and there is a need to prevent them from occurring. The World Health Organization defines health as “a state of complete physical, mental and social wellbeing, and not merely the absence of disease or infirmity”. Illness has been described as being more of a psychological experience in which the patient experiences ill health, in contrast to disease which is a pathological process (Boyd, 2000). Illnesses can be divided into two broad categories based on their duration of presentation: namely, acute illnesses or chronic illnesses.

Murrow *et al.* (1996) highlighted the main differences and similarities between acute and chronic illnesses. The main differences distinguishing between acute and chronic illnesses are the duration as well as the severity of the condition. Chronic conditions require long term and more intensive treatment with more health care resources to maintain normal health. The treatment commonly involves intervention linked to multiple biological pathways or systems for example hypertension, diabetes and hypercholesterolemia. In contrast to chronic illnesses, acute illnesses are usually isolated to one bodily area, example pneumonia, croup, bronchitis or the common cold and are often easier to treat and have relatively rapid recovery times.

Both chronic and acute illnesses are normally treated with a form of medication which is administered for the purpose of controlling either the cause or the symptoms of the illness. Due to safety and efficacy concerns as well as the potential adverse effects of many of these therapeutic drug-based interventions, regulatory control has been imposed by the regulatory authorities, including the requirement of a prescription to access certain medication. This is especially true of medication with potential psychological or habit-forming effects. In recent years a growing trend has emerged where unregulated “natural” or herbal based medication not requiring a prescription is being self-administered (Eichhorn *et al.*, 2011).

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Natural medicine can be defined simply as “any system of medicine that complements and enhances the body’s natural capacity to heal by restoring balance without the use of synthetic drugs of chemicals”.

The South African Medicines and Related Substances Act, Act 101 of 1965 defines a complementary medicine as:

“any substance or mixture of substances that

a) Originates from plants, minerals or animals
b) Is used or intended to be used for, or manufactured or sold for use in assisting the innate healing power of a human being or animal to mitigate, modify, alleviate or prevent illness or the symptoms thereof or abnormal physical or mental state”

The use of natural or complementary medicine has increased worldwide in recent years when compared to the use of conventional “western” medication, partially due to these preparations often not being registered as medication and therefore not required to adhere to the strict regulations enforced by the regulatory authorities. In South Africa the South African Health Products Regulatory Agency, which replaced the South African Medicines Control Council in 2018 is the regulatory authority is responsible for assessing and registering medicinal products. Conventional medicinal preparations undergo strictly controlled drug development processes that includes testing to prove consistency and both safety and efficacy. These drugs must be manufactured using standardized Good Manufacturing Processes (GMP) and undergo quality control procedures. Conventional medicinal preparations are monitored for adverse effects through post-marketing surveillance after product release for patient use. Herbal and complementary medicinal preparations are generally not required to undergo this strict testing and registration process (Neergheen-Bhujun, 2013). These natural or complementary medicines are easily accessible to the public because they do not require a prescription and are often marketed as “safe” based on their natural origin and ingredients. Many of these natural medications have unfounded or anecdotal claims of health benefits or claims of no side effects (Zhang *et al.*, 2015). In a study conducted by *Zhang et. al (2015)* it was reported that despite their natural components, many herbal medications do have the potential to cause adverse effects and harm to the body. They

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suggested that herbal products be monitored for effective dosing, and that the course of treatment be monitored to avoid abuse. Toxicity studies of herbal products such as *Radix bupleuri chinensis* have been investigated, as prolonged use resulted in reported adverse effects (Yang *et al*., 2017). Despite their "natural" components, Aydin *et al.* (2016), reported side effects such as gastro-intestinal disturbances, allergic reactions, hepato- and renal toxicity effects, and cardiovascular complications. Sharwan *et al.,* (2015) noted that although natural products may be deemed safe, many herbs contain heavy metal such as lead, cadmium, mercury and arsenic, which may cause toxic effects. Their efficacy is often overstated. Izzo *&* Ernst *(2001)* concluded almost 20 years ago that herbal medicines are a popular option in healthcare, despite numerous safety issues, which are often under-researched. The levels of toxicity associated with herbal extracts are often difficult to predict because of the complex mixtures of compounds and large batch variability found in herbal medicines. It has been pointed out that it is difficult to determine which compounds within these extracts are pharmacologically active because of the large variability and undefined composition of herbal medicines (Izzo, 2001).

Some of the reasons patients report as to why they choose to use complementary and alternative medicines over conventional medicines is because of their dissatisfaction with conventional treatment, which may be related to access through prescription only, be expensive, are ineffective or are associated with unacceptable side effects. The desire for patients to be actively involved in making their own health care decisions and being more assertive about their participation in medical care are further reasons reported why patients chose to use complementary medicine (Hsiao *et al.*, 2006).

Haetzman *et al.* (2003) found that most patients preferentially used complementary medicine rather than conventional medicine because they claimed that conventional medicine failed to cure their problem. Complementary medicine has been used frequently in conjunction with conventional therapy, but this can pose the risk for interactions between herbal medicines and synthetic drugs and may have serious clinical consequences (Izzo and Ernst, 2001). The use of the natural or complementary medicine is often not reported to the prescribing doctor, which can then have a direct effect on the safety and efficacy of the conventional medication (Saad *et al.*, 2006).

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Changes that result in the pharmacological effects of a drug when used concomitantly with another drug, a herbal substance or food are known as drug interactions. This can occur as a result of pharmaceutical, pharmacodynamics or pharmacokinetic changes. Pharmacodynamic changes occur as a result of synergistic or antagonistic effects while pharmacokinetic changes occur when the drug absorption, distribution, biotransformation or excretion are affected. Variation in drug uptake can occur when a drug binds to another drug in the gut affecting its absorption. Gastric and intestinal motility also affect drug absorption. Biotransformation of drugs are affected by enzyme inducers or inhibitors and is sensitive to many drugs and exogenous compound exposure. (Renton, 1986).

1.2 The human alimentary canal

The major function of the digestive system is absorption of nutrients. The human alimentary canal or gastrointestinal tract (GIT) consists of the mouth, pharynx, oesophagus, stomach, small and large intestines, rectum and anus as illustrated in Figure 1. Studies have shown that the majority of nutrients and minerals are absorbed from the small intestine, in part due to the very high surface area of almost 200 m^2 but also the relatively long transit time of ingested substances. The intestinal absorption of very diverse substances is influenced by physicochemical properties of the substances being absorbed, the concentration of the substances in the GIT, breakdown of complex molecules by secreted enzymes or gut bacteria, complexation to form soluble substances and the mechanism by which trans-membrane transport takes place. The region of the intestine also has an influence on the absorption rate, due to the presence of different compound-selective cell membrane transporters (Vertzoni *et al.*, 2019).

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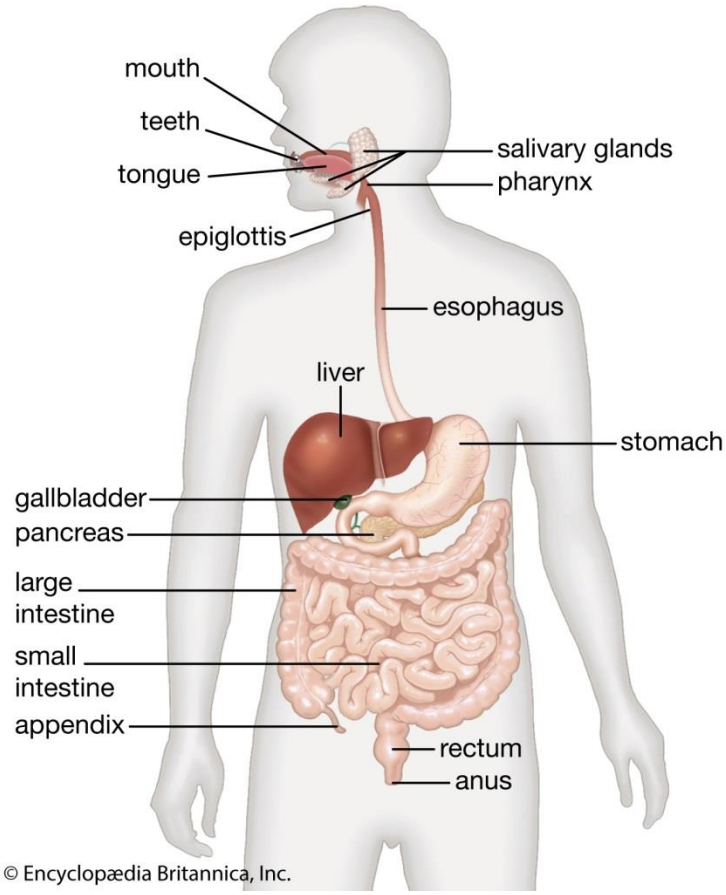

Figure 1: Gross anatomy of human alimentary canal, accessed 02 February 2022, https://www.britannica.com/science/gastrointestinal-tract#/media/1/15454/1087 (with permission)

The small intestine is the highly convoluted tubular organ extending from the stomach to the colon with an approximate diameter of 2.5 cm when not dilated and has an average length of 6 m in an adult. It is divided into three anatomically different divisions: the duodenum, jejunum and ileum. The duodenum is the first section attached to the stomach by the pyloric valve. It is approximately 25 cm long and is the region where both bile salts and pancreatic enzymes are introduced into the intestine to aid in pH control, digestion and solubilisation of ingested substances. The jejunum is the longest division of approximately 2.5 m long and is folded into coiled loops within the abdominal cavity where it is held in a flexible position by a membranous structure called the omentum. The final section of the small intestine is the ileum, which terminates into the colon. The abdominal cavity is filled with loosely attached coiled loops of the intestines.

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The luminal surface of the small intestine has millions of tiny finger-like projections, known as villi and illustrated in Figure 2. Each villus contains an arteriole, venule and lymph vessel (lacteal) which aid in the absorption process by carrying any absorbed substances to the draining portal vein system or to the lymphatic drainage system. Microvilli are tiny hair-like projections found on each of the villi that constitute the brush boarder of the intestine. The brush boarder cells produce enzymes that participate in the final process of digestion. The anatomical structure of the villi with their brush border increases the surface area available for absorption in the small intestine by approximately 160 times, making the small intestine the main site of absorption based on surface area. Absorption is facilitated by the presence of simple columnar epithelial cells called enterocytes. The lack of an extracellular matrix between these cells forming the epithelial layer permits close contact between neighbouring epithelial cells that are joined by intercellular junction complexes, which make up the tight junctions (Hooton *et al.,* 2015).

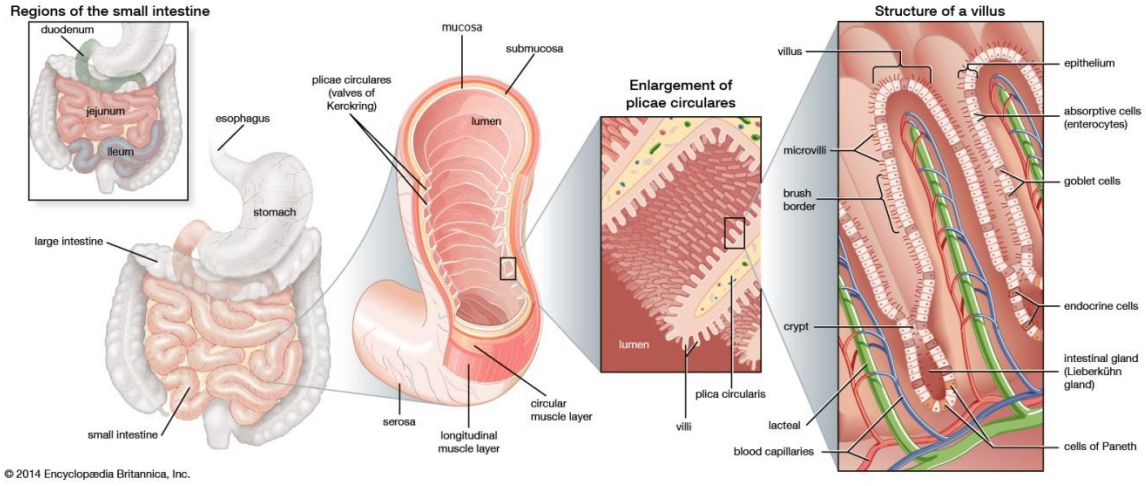

Figure 2: Illustration of villi and brush border cells, accessed 02 February 2022, https://www.britannica.com/science/brush-border#/media/1/82328/68637 (with permission)

Interspersed goblet cells secrete mucin which provides a protective layer to prevent digestion of the epithelial layer by the enzymes present in the lumen of the GIT.

Humans have submucosal folds which cannot be flattened by distention. They are fixed structures each containing a core of muscularis mucosae and mucosae (Wilson, 1967). The mucous coat contains permanent circular folds, *plicae circulares,* crypts of

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Lieberkühn, Brunner's glands, Payer's patches, and lymph nodes. The muscular layers of the small intestine are present in two distinct layers, an inner layer of circular fibres and an outer layer of longitudinal fibres. The fibroserous outer serosal layer of the small intestine, the visceral peritoneum, is continuous with the mesentery (Ba-Ssalamah *et al.*, 2013).

1.3 Absorption from the Gastrointestinal Tract

Absorption is the transport of digested material (amino acids, monosaccharides, vitamins, water, minerals or salts, fats) from the lumen through the different layers of the intestinal tract wall into the blood or lymphatic fluid.

There are different routes or mechanisms by which absorption can occur across the intestine, either through paracellular diffusion, which may be mediated by tight junctions, transcellular passive diffusion, carrier mediated transcellular transport or efflux (Kiela *et al.,* 2016).

In order for nutrients to be utilized efficiently they need to be absorbed from the GIT into the bloodstream. The small intestine is where most absorption occurs compared to the rest of the gastrointestinal tract because of the anatomical adaptations.

Absorption can occur using two different mechanisms, either passive diffusion or by active absorption. Villi and microvilli assist in absorption by increasing available surface area for both diffusion and active absorption. Both these absorption processes are dependent on a nutrient concentration gradient between the lumen and the blood vessels. The presence of microvilli with selective transmembrane transporters, promotes both passive and energy requiring active transport to transport compounds across the intestinal membranes. This process is known as carrier mediated transport (Barthe *et al.*, 1999). Most lipophilic compounds are absorbed by passive diffusion across intestinal epithelium and into the circulation. The rate of absorption is influenced by the extent of the concentration gradient of a particular compound and the surface area available for absorption at specific sites. Passive diffusion processes encompass lipid diffusion or aqueous diffusion, which are generally restricted to molecules with molecular weights of less than 200 Da. Small polar compounds like glucose, minerals

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and water-soluble vitamins use mainly the active absorption pathway. Transport of polar nutrients are regulated largely by selective carrier mediated transport mechanisms (Upadhyay, 2014).

If membrane permeable nutrient concentrations are higher in the lumen compared to the absorptive cells, the nutrients will diffuse from the area of higher to lower concentration. This is known as passive absorption. When nutrients are present in higher concentrations within the absorptive cells than the lumen, a carrier protein and energy is required to aid in transporting the nutrients from the lumen.

Solubility in aqueous solutions, molecular size, ionic status and hydrophobicity of compounds are factors that influence the transcellular transport rate or flux across the gastrointestinal barrier (Barthe *et al.*, 1999)).

Compounds that are rapidly and completely absorbed such as low molecular weight, lipophilic compounds move readily across cell membranes of the intestinal epithelium. Many compounds that are rapidly and completely absorbed are absorbed via the passive transcellular diffusion route. Hydrophilic compounds are slowly and incompletely absorbed by passive diffusion due to the lipophilic character of the middle zone of the cell membranes and the charge of the phospholipid head region that immobilises any ionic compounds at the membrane surface. These ionic compounds diffuse poorly into cells and therefore require active transport or water filled pores via the paracellular pathway (Stenberg *et al.*, 1999). Tight junctions between adjacent epithelial cells of the villi normally restrict paracellular transport of large molecules and are considered rate limiting barriers.

Diffusion of any solute can also occur down an electrochemical potential gradient of the solute. It is estimated that approximately 90% of drugs are absorbed from the gastro-intestinal tract via this pathway. The parameters indicated in Fick's first law of diffusion influence the rate of flux during passive diffusion. These are: diffusion coefficient, available membrane surface area, partition coefficient, volume of solute at the acceptor side, thickness of the membrane and concentration gradient across the membrane. The concentration gradient is the driving force for passive diffusion (Stilwell, 2016).

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Paracellular transport is a mechanism of intestinal absorption used as an alternative to other transport mechanisms, where substances are transported through opened tight junctions between epithelial cells. The extent of absorption is determined by the number of available tight junctions, which are the rate-limiting step. In the small intestine, the surface area presented by the paracellular route constitutes only a small fraction (0.01%) of the total membrane surface area (Schoultz and Keita, 2020).

Specific transporter systems exist in the intestinal mucosa for amongst others phosphate, glucose, amino acids, folic acid, benzoic acid, salicylic acid, choline, bile acids, fatty acids and other monosaccharides. In the small intestine, uptake and efflux transporters may both be expressed within the same cell membrane (Suzuki and Sugiyama, 2000).

Two forms of active transport play an important part in the uptake and efflux of different compounds and solutes. The process where membrane transport directly couples with Adenosine triphosphate (ATP) hydrolysis, is known as primary active transport.

Secondary active transporters utilize symporters or antiporters to transport specific compounds across the membrane in an orientation specific direction.

Pharmacologically important transporters may mediate drug absorption or efflux. An important efflux transporter is the P-Glycoprotein (P-gp) (Swed *et al.*, 2009).

Another transport mechanism is transcytosis in which large macromolecules are engulfed by the cell membrane and an endocytotic vesicle is formed (Heyman *et al.*, 1990). During infancy when immune substances from milk are absorbed, this process occurs.

Transcytosis is the route for the absorption of large molecules and high molecular mass compounds, mainly due to their size, however the disadvantage is that the membrane vesicles formed during transcytosis may also contain proteolytic enzymes that degrade these ingested compounds if they are protein based.

Examples of compounds absorbed by transcytosis are vitamin B_{12} and the intestinal absorption of immunoglobulins (Ig) from the colostrum by new-borns (Pierzynowska *et al.*, 2020)

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Receptor mediated endocytosis describes the process where macromolecules are taken up after binding to specific membrane receptors. A conformational change occurs in the membrane which triggers invagination. This causes the membrane to indent around the compound then pinch off to form a vesicle, which is transported across the cell to the basolateral membrane.

Fat soluble vitamins are non-polar and have poor aqueous solubility. Their absorption in the intestinal lumen occurs by hydrolysation and micelle formation (Thompson, 1971).

Receptor-mediated pinocytosis and non-specific fluid phase pinocytosis aid with nutrient uptake (Muir and Bowyer, 1984).

1.4 Vitamin transporters

Much of the B-vitamins absorbed are normally produced by the gut microbiome. *Bacteriodes*, *Bifidobacterium* and *Enterococcus,* common in the distal intestine produce vitamins such as thiamine, folate, biotin, riboflavin, pantothenic acid (Morowitz *et al.,* 2011).

Vitamin B_1 is absorbed by the intestinal epithelium only after conversion to free non-phosphorylated thiamine. Free thiamine is extensively absorbed in the colon by thiamine transporters such as Thiamine transporter 1 (THTR-1) and Thiamine transporter 2 (THTR-2). TPP produced in the colon cannot be converted to free thiamine, because the colon does not secrete alkaline phosphatase (Yoshii *et al.*, 2019).

Approximately 50% of thiamine is in an absorbable form in the colon (Said, 2011).

Vitamin B_2 absorption occurs via carrier-mediated transport by the brush border membrane of the villus cells (Sundaram, 2000).

Riboflavin absorption uses a sodium-independent carrier mediated mechanism. The main site of absorption is the apical brush border membrane. Bacterial riboflavin is produced in the colon in humans. Luminal riboflavin is also absorbed in the intestine (Said, 2011).

Niacin absorption occurs across the enterocyte brush border membrane. Human organic anion transporter-10 is the main transporter in intestinal niacin uptake at

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physiologic concentrations. At higher pharmacological doses, nicotinic acid is absorbed via the sodium-coupled monocarboxylate transporter (Gasperi *et al.*, 2019).

Pantothenic acid is absorbed by sodium-dependent multivitamin transporters on the brush border membranes of the small intestine, (Yoshii *et al.*, 2019).

Vitamin B_6 is absorbed in the jejunum of the small intestine via passive diffusion (Said, 2011).

Biotin is absorbed in the brush border by a sodium-gradient driven carrier mediated system. Sodium independent carrier mediated systems transport biotin across the basolateral membrane. Biotin uptake is highest in the duodenum, followed by the jejunum. The ileum shows minimal uptake in comparison to the duodenum and jejunum (Said, 2011).

The ascorbic acid or Sodium dependent vitamin C transporter 1 (SVCT1) is expressed in the small intestine epithelial cells. It preferentially transports L-ascorbic acid over other isomers. If large quantities of vitamin C are absorbed by the small intestine, it attracts water and thus causes diarrhoea as a side effect (Macdonald *et al.*, 2002).

The biotin transporter, Sodium-dependent multivitamin transporter (SMVT) is found in intestinal tissue and mediate the transport of biotin, pantothenate and lipoate (Chatterjee *et al.*, 1999).

Folate is required to ensure purine and pyrimidine nucleotide synthesis. A carrier-mediated pH dependent, folate uptake mechanism is responsible for folate absorption in the intestine. Among the solute carrier group (SLC) of transporters, the Reduced Folate Carrier (RFC, SLC 19A1) are primarily responsible for delivery of folate in mammalian cells and tissues (Hou and Matherly, 2014). Human proton-coupled high affinity folate transporters also assist in folate uptake. Higher levels of folate are found in the acidic duodenum and the proximal jejunum. The absorption gradient of folate is reduced from the jejunum to the colon.

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1.5 Bioavailability of nutrients:

Bioavailability of a drug is defined in terms of the percentage of the administered drug to reach the systemic circulation or central compartment. Cell membranes serve as barriers to absorption into the circulatory system and distribution from the circulation into the different tissues.

Bioavailability of nutrients is in a similar manner expressed as the fraction of an ingested nutrient that is available for use and storage in the body (Melse-Boonstra, 2020).

At a physiological level, the bioavailability of nutrients may have beneficial effects, however may cause toxicity due to excessive intake. Factors identified that may influence bioavailability include nutrient concentration, dietary factors, chemical form, nutrient interactions as well as nutrition and health of an individual (Hambidge, 2010).

Factors that influence bioavailability of nutrients include both external and internal factors. External factors refer to the environmental influences such as proteolytic enzymes, chelating agents and competitive inhibitors of nutrient metabolism. Intrinsic factors influencing absorption are the organic aspects of the nutrient and physiological conditions (Moran *et al.*, 2018).

Bioavailability of nutrients may be measured by using the following methods: intake excretion balance, serum concentration, cumulative urinary excretion, effect on target systems and *in vitro* tests such as dissolution and disintegration (Heaney, 2001).

1.6 Factors that influence absorption

Physiologically selective transmembrane transporters assist in the pharmacokinetic processes of absorption, distribution and excretion. Active transport of many molecules across cell membranes are dependent on molecule selective membrane transporters. Two classes of cell membrane transporters are the ATP-binding cassette (ABC) family and the solute carrier (SLC) family (Liang *et al.*, 2015).

Polar and ionic nutrients may have several different but selective transporters, each with a different affinity or transport rate for the molecule. The level of expression of these

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transporters depends on the abundance of the substrates. In conditions where the substrates are in abundance, the low affinity transporters are favoured, and in conditions where the substrates are scarce, high affinity transporters are expressed (Bosdriesz *et al.*, 2018).

The vitamin transporters generally have low capacities, which makes them poor targets to increasing oral vitamin bioavailability (Hsu *et al.*, 2019).

Secretions from the duodenum enterocytes which are triggered by the presence of chyme, are regulated by the hormone, enterocrinin, which causes release of intestinal mucus in response to stimulation by hydrochloric acid and presence of food in the gastrointestinal tract (Pelaseyad *et. al.,* 2014).

1.7 Methods to assess absorption:

1.7.1 Everted gut sac method:

The everted gut sac technique was first used by Wilson and Wiseman as a technique to assess the transportation of sugars and amino acids (Wilson and Wiseman, 1954). The rat everted sac is the preferred choice for the everted gut sac technique, although different animals such as frog, catfish, rabbit, sheep, chicken, goldfish, turtle, pigs, guinea pig and mice have been used (Alam *et al.*, 2012). After the gut is excised, it is everted to expose the luminal side to the surrounding fluids. This technique involves tying off an intact segment of intestinal tissue at both ends after everting it so that the luminal side faces outwards. The original model developed by Wilson and Wiseman (1954) used a simple salt medium for culture The use of this media resulted in tissue degradation within 2 hours. It has since been modified to improve tissue viability and to preserve the physiological functioning of cells. Houston introduced the use of a different medium, TC 199, to preserve the physiological functioning for up to 2 hours (Barthe *et al.*, 1998).

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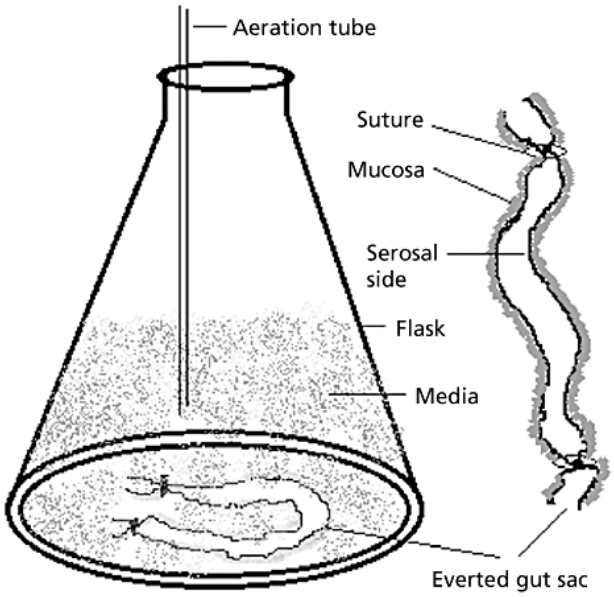

Figure 3: The everted gut sac method to assess intestinal absorption. (Alam *et al.,*2011) (with permission)

Some of the applications of this technique include investigating drug transport mechanisms through intestine and identifying the carriers responsible for the transportation of compounds. It is also used to confirm *in vivo* pharmacokinetic findings (Alam *et al.*, 2012). The limitations of this technique are that it is an *in vitro* technique and the correlation between the everted gut sac technique and an *in vivo* model is at times poor, probably due to lack of innervation and hormonal controls. However, this technique is relatively cost-effective, simple and reproducible.

1.7.2 Parallel artificial membrane assays

Parallel artificial membrane assays (PAMPA) are a technique that was used by Kansy *et al.* (2004) for absorption studies. The parallel membrane permeability assay consists of a 96 well plate, which uses a small amount of an organic solvent supported on a permeable supporting membrane that acts as a lipophilic artificial membrane. The organic solvent then interacts with the filter well plate and forms a thin lipophilic layer that mimics a cell membrane lipid bilayer. The final concentration of a compound in the recipient chamber is measured after a select incubation period starting when the compound is added to the donor chamber.

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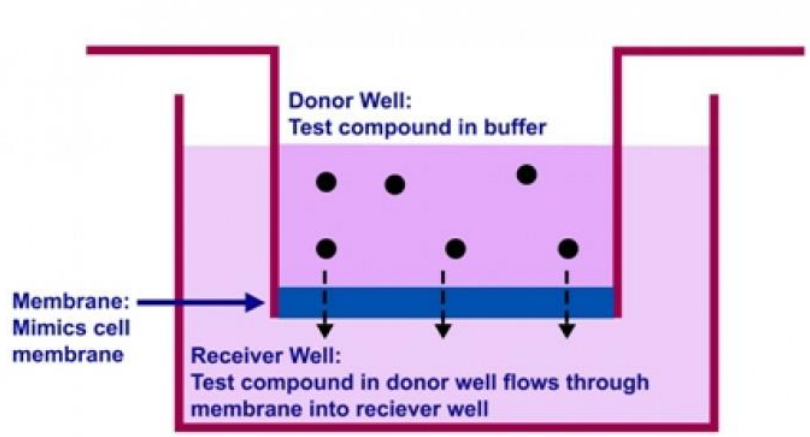

Figure 4: Diagram illustrating the setup for a PAMPA assay.

This technique was found to be useful for assessing intestinal absorption where passive diffusion only is monitored as there are no transporter molecules or paracellular mechanisms (Kansy *et al.*, 2004).

1.7.3 Caco-2 monolayers

Cell culture models are commonly used to model transport of compounds where all the potential transport mechanisms are measured together. The most commonly used cell line for these absorption assays is the colon cancer derived Caco-2 cell line that was developed by Fogh et al. Similar to the PAMPA assay the system consists of two chambers, a donor chamber and a receiver chamber. The test sample compounds are applied to the donor chamber and transport is assessed by withdrawing samples from either chamber at different times. The disadvantage of Caco-2 cell cultures are the time taken to form sealed confluent cell monolayers and that it lacks *in vivo* correlation for carrier mediated and paracellular-transported compounds (Meunier *et al.*, 1995).

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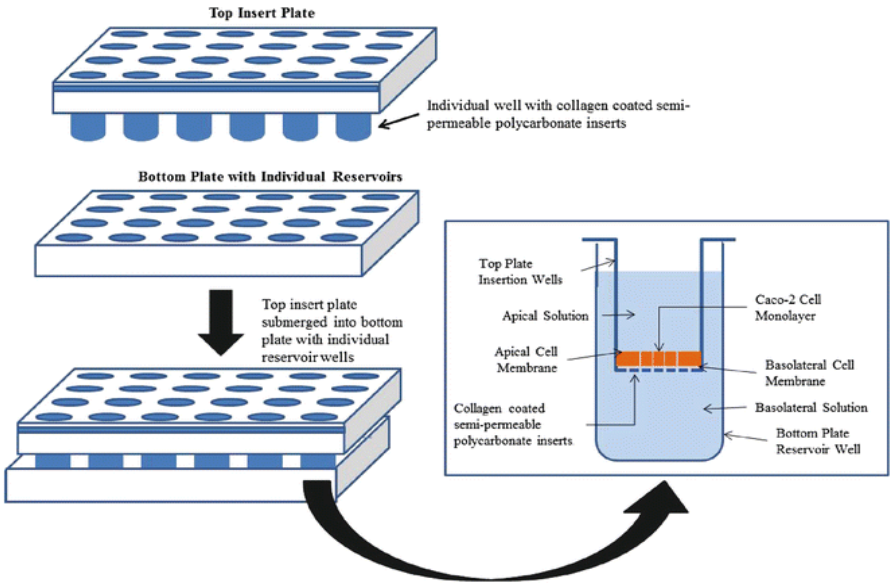

Figure 5: An illustration of Caco-2 transport assay to assess trans-GIT transport of substances

1.7.4 The Ussing chamber

The Ussing chamber was developed by Hans Ussing to investigate the effect of ionic compounds as they move across an intact physiological membrane (eg: skin, lungs or tissue). It can be used as a physiological system to measure the transport of ions, nutrients and drugs across epithelial tissue (Clarke, 2009).

The Ussing chamber was originally developed to understand the transport of sodium and chloride ions. The Ussing chamber model again uses two chambers, a donor chamber and receiver chamber, but is separated by a layer of isolated tissue to asssess the transport of compounds across the tissue. The use of this method assesses the permeability of substances by measuring the concentration of samples obtained from either chamber. While this technique may be advantageous because of its ability to mimic the *in vivo* conditions, it poses several disadvantages including, variation in tissue viability in samples, prolonged time, and large quantities of animal tissues and compounds required (Thomson *et al.*, 2019)

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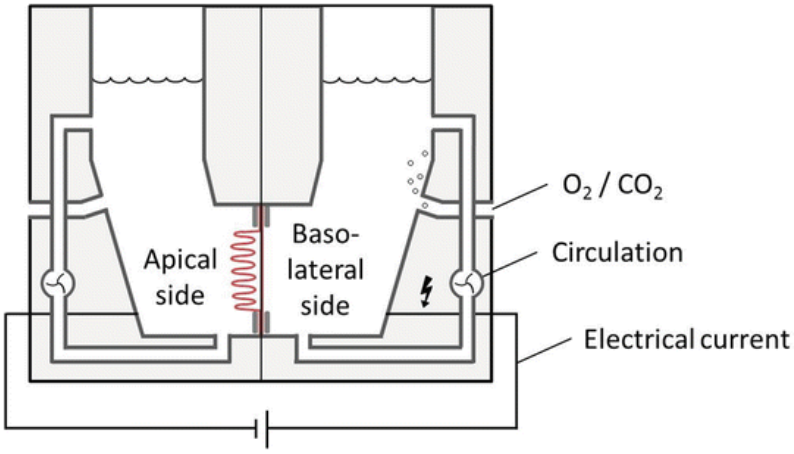

Figure 6: Ussing chamber used to measure intestinal permeability

1.8 Nutrient absorption in the gastrointestinal tract

The World Health Organization defines nutrition as “the science of food, the nutrients and the process by which an organism ingests, digests, absorbs, transports, utilizes and excretes food substances.” Nutrients are chemical substances in food that are essential parts of a diet. Nutrients serve as a source of building blocks, energy and they are important for growth and maintenance of homeostasis. They function to provide energy, assist in building the body and regulate chemical processes in the body. Nutrients are present in the food consumed, and certain diets with restricted or excessive nutrients of a particular class may pose risk factors for chronic diseases such as hypertension, diabetes and anaemia. Some nutrients taken in excess may be harmful. Because the body cannot manufacture certain nutrients that are required in specific minimal quantities, these must be obtained from the diet and referred to as essential nutrients (Hayes, 2008).

Vitamins and minerals have functions as key regulators and structural parts in the body. Vitamins and minerals are essential for good health, but are only needed in relatively small amounts in the body as they are usually catalysts or co-factors that are not altered during the biological processes in which they are involved, but excessive amounts may cause toxic side effects.

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Vitamins are carbon containing catalyst compounds required for the release of energy stored in carbohydrates, proteins and fats. Minerals have diverse roles in the body, from body structure, catalytic role in enzymes and form key components of parts of the blood.

Vitamins are needed in sufficient quantities to perform various catalytic functions in the body. Several vitamins can be synthesized by humans but most are obtained from the diet or supplements. The absence of sufficient vitamin quantities may impair particular functions resulting in symptoms of deficiency. When dietary vitamins are lacking, accumulated body stores may be depleted resulting in specific diseases associated with lack of a vitamin. A deficiency of Vitamin B_1 may be caused by chronic alcoholism, malnutrition, malabsorption syndrome, diarrhoea, diuretic use and hyperthyroidism. Dry beri-beri is associated with central nervous side effects and neurological defects. Wet beri-beri is associated with cardiovascular complications, resulting in fluid retention (Wiley and Gupta, 2021).

Riboflavin deficiency results from endocrine abnormalities, chronic diarrhoea, liver disorders, alcoholism and haemodialysis. Iron absorption can be impaired by riboflavin deficiency causing anaemia. Symptoms of riboflavin deficiency include fatigue, swollen throat, blurred vision, depression, dermatitis around the mouth and hair loss (Mahabadi *et al.*, 2021).

Chronic alcoholism, diarrhoea, inflammatory bowel disease decreases levels of niacin in the body and may lead to pellagra. Pellagra presents as dermatitis, dementia and diarrhoea (Savvidou, 2014).

Vitamin B_5 deficiencies present with fatigue, headache, malaise, personality changes, numbness, muscle cramps, paraesthesia, abdominal cramps, nausea and impaired muscle co-ordination (Sanvictores *et al.*, 2021).

Vitamin B_6 deficiency symptoms include seizures, rash, mental status changes, anaemia, cheilitis, glossitis and depression (Brown *et al*., 2020).

The main site of folate absorption is the jejunum, via both passive and active transport mechanisms. Alcoholism, pregnancy, celiac disease, dialysis and haemolytic anaemia

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may result in folate deficiency. Symptoms present with a painful, red tongue, anorexia, cognitive impairment, depression and dementia (Khan and Jialal, 2021).

Biotin or vitamin B7 deficiency can either be acquired or congenital. Patients taking medications such as isotretinoin or anticonvulsants, such as valproic acid, may be at risk for biotin deficiency. Prolonged use of antibiotics, and conditions that interfere with normal intestinal flora may cause deficiency states. Alcoholism, pregnancy and impaired intestinal absorption cause folate deficiency. Some of the symptoms of folate deficiency include alopecia, skin rashes, dermatitis, conjunctivitis, neurological symptoms, depression, lethargy, hypotonia and seizures (Patel and Sobczyńska-Malefora, 2017).

Vitamin B_{12} deficiency states result from auto-immune conditions such as pernicious anaemia, malabsorption syndrome and Crohn's disease. Medications such as oral contraceptives, hormone replacement therapy, metformin, proton pump inhibitors and histamine H_2 receptor antagonists can impair absorption of Vitamin B_{12} and result in deficiencies. Symptoms of deficiency include haematological, neurological (neuropathy) and neuropsychiatric, such as Alzheimer's, depression, mania, delirium and psychosis (Shipton and Thachil, 2015).

Vitamins, when taken in excess may cause toxic symptoms. Vitamins are divided into two main categories, the fat-soluble vitamins which include vitamin A, D, E and K and the water-soluble ascorbic acid and the B-Vitamin complex.

1.9 Vitamins

Nutrients including vitamins are required in sufficient quantities necessary for normal growth and functioning of the human body. Nutrients can be obtained from diet or through supplementation where the Recommended Daily Intake (RDI) system used to assess the daily intake amount of specific nutrients that is sufficient for bodily functions based on the requirements of 97-98% of healthy individuals.

Vitamins can be classified into two main classes, fat-soluble vitamins or water-soluble vitamins. The fat-soluble vitamins are: Vitamin A, Vitamin D, Vitamin E, and Vitamin K.

Fat soluble vitamins diffuse passively with the end products of fat digestion and are mainly stored in the liver and adipose tissue using special carrier proteins in the plasma

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assist with their distribution. Water soluble vitamin absorption is largely passive and are absorbed with water through diffusion mechanisms. Vitamin B_{12} absorption is affected by the presence of intrinsic factor and uses receptor mediated endocytosis as a transport mechanism for absorption.

The Vitamin B group of vitamins are all water-soluble therefore are more easily excreted by the kidney than the fat-soluble vitamins bound to transporter proteins. The different vitamin B molecules function directly or as part of as co-enzymes that activate enzymes. Three water soluble B-group vitamins, Vitamin $B_{6,}$ Vitamin B_9 and Vitamin B_{12} are involved in regulating homocysteine levels in blood (Fratoni and Brandi, 2015).

Vitamin $B_{2,}$ known as riboflavin, aids in skin health and is important for metabolism. Sources of riboflavin in the diet include milk, mushrooms, spinach, liver and grains. Deficiency symptoms include inflammation of the mouth and tongue and eye disorders.

Vitamin B_3, niacin is important for the nervous system, the digestive system and healthy skin. Dietary sources include mushrooms, bran, tuna, salmon, chicken, liver, peanuts and grains. Deficiency symptoms may result in pellagra, diarrhoea, dermatitis and dementia (Redzic *et al.,* 2020).

Vitamin B_6 is needed for protein metabolism and required for the formation of red blood cells. It also assists in the synthesis of haemoglobin and several neurotransmitters. Dietary sources include spinach, broccoli, bananas, salmon and sunflower seeds. Deficiency symptoms often result in headaches, anaemia, convulsions, nausea, vomiting and flaky skin (Brown *et al.,* 2020).

Vitamin B_9 is known as folic acid and is an essential vitamin as the human cannot synthesise this vitamin. Dietary sources of folate include green leafy vegetables, orange juice, sprouts and sunflower seeds. Folic acid functions as a co-enzyme involved in methyl group transfer required for synthesis of thymine a building block for DNA synthesis. Deficiencies of folic acid result in megaloblastic anaemia, diarrhoea, poor growth and mental disorders (Khan and Jialal, 2021).

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Vitamin B_{12} is essential for normal red blood cell production and maturation. Insufficient levels of intrinsic factor decreases Vitamin B_{12} absorption and may result in pernicious anaemia if insufficient vitamin B_{12} is present in the bone marrow.

Vitamin C, ascorbic acid, is required for the formation of collagen. It assists in hormone and neurotransmitter synthesis. Dietary sources include citrus fruits, strawberries, broccoli and greens. Deficient levels of Vitamin C result in a condition known as scurvy. Other symptoms of Vitamin C deficiencies may include poor wound healing, haemorrhages, bleeding gums and oedema (Gordon *et al.*, 2020).

1.9.1 Vitamin B_1: Thiamine

Background:

Thiamine (Vitamin B_1), is needed for enzyme function, nerve functioning and metabolism. Dietary sources include meat, sunflower seeds, grains, beans and peas. Deficiencies in Vitamin B_1 result in beri-beri, nerve tingling, poor co-ordination, oedema, heart remodelling and weakness.

When carbohydrates are ingested, they need to be broken down to form monosaccharides like glucose for cells to generate energy efficiently. The co-enzyme form of vitamin B_1 is involved in carbohydrate metabolism, which releases glucose via glycolysis, to provide energy to the cells (Lonsdale, 2006).

The bioavailability of thiamine is generally considered to be high, however because of its water solubility, most nutritional value is degraded by cooking because the enzyme thiaminase is inactivated by heat (Smithline *et al.*, 2012).

Absorption of Thiamine:

Humans cannot synthesize thiamine and therefore must consume the vitamin from dietary sources. Specialized organic cation/H^+ antiporter transmembrane transporter systems with substrate specificity transport thiamine across the intestine and into the liver. Thiamine pyrophosphate (TPP) is a co-factor involved in many biochemical reactions. It is produced by the enzyme thiamine diphosphokinase. Its main role is in

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carbohydrate and amino acid metabolism. Alkaline phosphatase is the primary phosphatase responsible for converting TPP to free thiamine in the intestinal lumen (Smithline *et al.*, 2012).

The absorption of thiamine is dependent on a number of factors including: temperature, metabolic energy, and pH, and is a saturable process. The biological half-life of thiamine in the body is 9 to 18 days.

Functions in the body of thiamine:

TPP is the most abundant phosphorylated form of thiamine found in cells. It plays an important role in nerve signal transmission. TPP is a co-enzyme for three mitochondrial enzymes which play a role in the oxidative decarboxylation of α-keto acids (Mayr *et al.*, 2011).

Deficiency and high doses:

Deficiency most commonly occurs because of poor diet, however other factors such as chronic alcoholism or conditions where fluid loss is significant or malabsorption can cause deficiency symptoms.

Deficiency results in beri-beri. Three types of beri-beri present depending on severity, dry (wasting) beri-beri, wet (oedematous) beri-beri and fulminating (cardiac) beri-beri. Symptoms of beri-beri present with weakness, loss of appetite, irritability, nerve tingling throughout the body, poor arm and leg co-ordination, and deep muscle pain in the calves. Beri-beri results because of a deficiency in TPP, resulting in insufficient glucose supply to tissues and cells (Wiley and Gupta, 2021).

Deficiency of thiamine also affects diet resulting in anorexia and gastro-intestinal disturbances. TPP is responsible for nerve function, when deficient may present symptoms of neuropathy and cardiovascular irregularities. Wernicke-Korsakoff syndrome results from a deficiency of thiamine resulting from chronic alcohol intake.

Thiamine in high doses is non-toxic, because of the solubility characteristics, any excess amounts are rapidly excreted in urine.

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Properties: Thiamine

IUPAC ID: 2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethanol
Formula: $C_{12}H_{17}N_4OS$
Molar mass: 265,35 g/mol
Bioavailability: 3.7% to 5.3%
Log P: -3.1

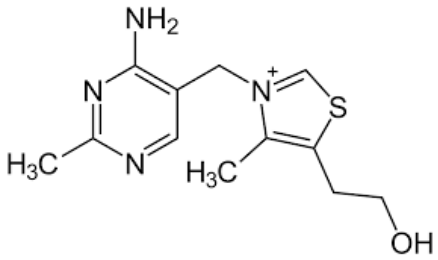

Figure 7: The chemical structure of thiamine (Vitamin B_1)

1.9.2 Vitamin B_2: Riboflavin

Background:

Vitamin B_2 exists in the active form of flavins, riboflavin, riboflavin-5'-phosphate (flavin mononucleotide, FMN) and riboflavin-5'-adenosyl-diphosphate (flavin adenine dinucleotide, FAD) (Liu *et al.,* 2020). Riboflavin based co-enzymes are used in many energy-yielding metabolic pathways.

Deficiency symptoms present with inflammation of the mouth and tongue. Other symptoms may include dermatitis, cheilosis, eye disorders and sensitivity to the sun.

Dietary sources include yeast, liver, kidney, wheat bran, eggs, meat, milk and cheese.

The bioavailability of vitamin B_2 is higher from meat and dairy products in comparison to plant-based products

Absorption of Vitamin B_2:

Riboflavin is not synthesized in the body and thus adequate intake from dietary sources or supplements is necessary for adequate absorption. The upper region of the small

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intestine, which contain brush border cells, hydrolyse flavin co-enzymes to riboflavin for absorption (Ross *et al.,* 1992). Bile salts aid in the absorption of riboflavin from the intestinal lumen.

Functions in the body:

The main functions include oxidoreductive processes and playing a role in intermediary metabolism.

Deficiencies and high doses:

Deficiencies of riboflavin are difficult to identify because symptoms are linked to deficiencies of vitamins B_1, B_3 and B_6.

Ophthalmic symptoms of superficial vascularization of the cornea and intense photophobia have been reported with severe riboflavin deficiencies (Mahabadi *et al.*, 2021).

Properties: Riboflavin

IUPAC ID: 7,8-Dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4-dione
Formula: $C_{17}H_{20}N_4O_6$
Molar mass: 376,36 g/mol
Bioavailability: 95%
pKa: 10.2 (Sheraz *et al.,* 2014)
pKa = 10.2; pKb = 1.7

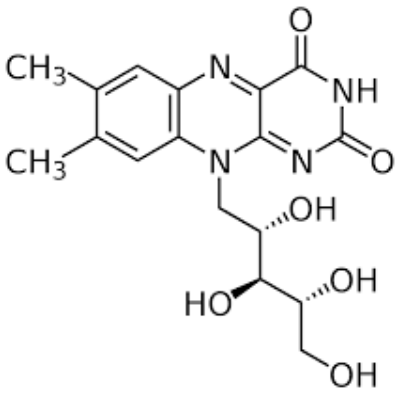

Figure 8: The chemical structure of Riboflavin (Vitamin B_2)

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1.9.3 Vitamin B_3: Niacin, nicotinic acid and nicotinamide

Background:

Nicotinamide forms the active portion of the co-enzymes Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP).

Niacin is synthesized from the amino acid L-Tryptophan. Niacin is essential for fat synthesis and in cellular metabolic pathways.

The bioavailability is increased in an alkaline pH, and this releases free nicotinic acid.

Absorption of Vitamin B_3:

NAD and NADP are the predominant forms found in dietary products. Nicotinamide is converted using these co-enzymes.

Functions in the body:

Vitamin B_3 is involved in oxidation-reduction reactions

Deficiency and high doses:

Deficiency results in pellagra, which is characterised by dermatitis, inflammation of the mucous membranes, diarrhoea and psychiatric disturbances.

Toxic or excessive doses cause peripheral vasodilation and flushing of the skin. When administered in high doses, it competes with uric acid and may cause gout symptoms. Sever liver damage, including jaundice have been reported with use of doses of 750 mg per day (Habibe *et al.,* 2021).

Properties:
Names: Niacin, nicotinic acid and nicotinamide
IUPAC ID: pyridine-3-carboxylic acid
Formula: $C_6H_6N_2O$
Molar mass: 123,11 g/mol
pKa: 2.0, 4.85
Log P: 0.219

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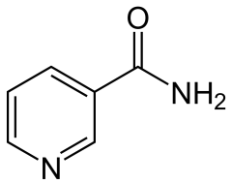

Figure 9: The chemical structure of nicotinamide

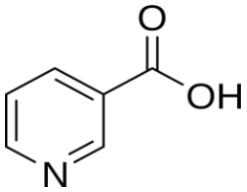

Figure 10: The chemical structure of Niacin also known as Nicotinic acid

1.9.4 Vitamin B5: Pantothenic acid.

Background:

Pantothenic acid is a building block of co-enzyme A, which participates in energy yielding metabolic pathways for especially lipids. Dietary sources include mushrooms, peanuts and eggs (Sanvictores *et al.,* 2021).

Lean meat, milk, potatoes and green leafy vegetables contain lower quantities of vitamin B_5, while sugars, fats and other refined foods lack vitamin B_5.

The bioavailability ranges from 40% to 61% with an average of 50%

Absorption of Vitamin B_5:

Pantothenic acid can be converted to pantetheine, which is a precursor for co-enzyme A, prior to absorption. The jejunum is the main site of absorption of pantothenic acid. The predominant form, pantothenate anion is mostly present within the alkaline medium of the intestinal chyme (Sanvictores *et al.,* 2021).

Functions in the body:

Vitamin B_5 is involved in the biochemical functions of co-enzymes A which is an acyl carrier protein in cellular metabolism.

Properties:

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Names: Pantothenic acid

IUPAC ID: 3-[(2,4-dihydroxy-3,3-dimethylbutanoyl)amino]propanoic acid

Formula: $C_9H_{17}NO_5$

Molar mass: 219,23 g/mol

pKb: 9.698 Log P: -1.4

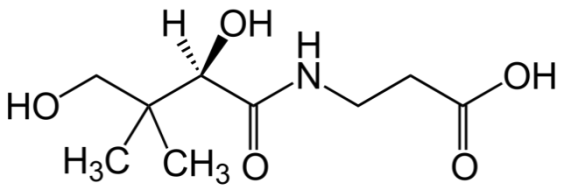

Figure 11: The chemical structure of pantothenic acid (Vitamin B_5)

1.9.5 Vitamin B6: Pyridoxine

Background:

Vitamin B_6 consists of a family of three related and chemically interchangeable compounds; pyridoxine, pyridoxamine and pyridoxal. These compounds can be changed enzymatically in the body to the active co-enzyme form.

The main functions of vitamin B_6 are carbohydrate, protein and fat metabolism. Amino acids are necessary building blocks for proteins and vitamin B_6 helps metabolise amino acids. Vitamin B_6 is also essential for nerve signal transmission through the requirement of this vitamin for the synthesis of neurotransmitters.

Dietary sources:

Vitamin B_6 is abundant in all natural unprocessed foods. Yeast, wheat and bran are dense sources. Other sources include whole grain cereals, nuts, pulses, lean meat, fish, kidney, potatoes and many other vegetables.

The bioavailability of Vitamin B_6 from plant sources is less compared to animal sources. Vitamin B_6 is thermolabile, so cooking foods for long periods may result in less of the vitamin being available for absorption.

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Absorption of Vitamin B_6:

The major site of absorption is the jejunum, mostly by passive diffusion but the absorption is dependent on the pH. At lower pH levels the vitamin-protein complexes are more easily dissociated compared to higher pH levels.

The active form of Vitamin B_6, Pyridoxal-5-Phosphate (PLP) is first transported to the liver, which is the primary site of Vitamin B_6 metabolism then distributed to the tissues.

Functions in the body:

Vitamin B_6 plays an essential role in many biochemical functions, such as amino acid metabolism. It also assists in the biosynthesis of biogenic amines, and nucleic acids. Vitamin B_6 also plays a role in glycogenolysis, the breakdown of glycogen to glucose which aids the cell in energy processes. Vitamin B_6 assists in lipid metabolism, regulation of steroid hormones and immune functions.

Deficiency and high doses:

The lack of Vitamin B_6 results in symptoms such as depression, vomiting, skin disorders, impaired immunity and nerve problems.

Gastrointestinal disturbances occur.

Alcoholics are susceptible to deficiencies because acetaldehyde competes with Vitamin B_6 enzymes for displacement.

Toxic doses produce symptoms of neuropathy; however, this is noticed with long term use of doses up to 50 mg. Symptoms of toxicity are walking difficulties and numbness of the limbs.

Properties:

Names: Pyridoxine, Pyridoxamine,

IUPAC ID: 4,5-Bis(hydroxymethyl)-2-methylpyridin-3-ol

Formula: $C_8H_{11}NO_3$

Molar mass: 169,18 g/mol

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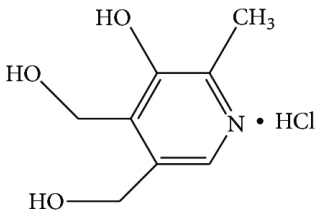

Figure 12: The structure of pyridoxine

1.10 Humic substances

Background:

Humic substances (HS) are complex organic compounds of unknown structure found in natural organic matter such as soil, coal, peat or surface water (Pena-Mendez *et al*., 2004). They arise from plant and animal material through a process known as humification.

Humic substances can be characterised into three main components based on their solubility characteristics. Humic acids (HA) and fulvic acids (FA) are soluble in alkaline and acidic solutions respectively, and the third fraction, humin, is insoluble at any pH in aqueous solutions. Humic acid can be separated from fulvic acid in aqueous solutions by precipitation with an acid. Humic substances can further be differentiated by their characteristic colour. Fulvic acid is a mixture of light-yellow to a dark yellow-brown colour. Humic acid is dark-brown to black in colour, and humin is mostly black in colour (Kumada, 1965).

The exact structure of humic substances are unknown, however there are basic elemental structures present, such as carbon, oxygen, nitrogen and sulphur (Pena mendez et al., 2007). The presence of specific functional groups may influence the ability to dissolve in acidic solutions and can thus further differentiate between humic substances (Eshwar *et al.,* 2017). Fulvic acids contain more acidic functional groups than humic acids where there is an increase in the degree of polymerisation with an increase in molecular weight. The structure of humic acid allows for the formation of

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molecular aggregates and molecules of highly complex structures and possibly supramolecular structures with higher molecular weights.

As a result of humification, various structures such as amino acids, lignins, pectins, or complex hydrolysis-resistant carbohydrates are retained through intermolecular forces (Pena-Mendez *et al*., 2004). These intermolecular forces, including donor- acceptor, ionic, hydrophilic or hydrophobic interactions, allow humic substances to bind to each other and to other molecules or compounds to form strongly associated complexes.

The ability of humic substances, particularly humic and fulvic acids, to bind to and form complexes or chelate with molecules, such as vitamins and minerals, influences the solubility characteristics of both the humic substance as well as the bound molecules. The change in solubility may affect the concentrations of these compounds that are absorbed and thus may affect the safety and efficacy.

Humic substances applications fall into four main categories; agriculture, industry, environment and biomedicine (Pena-Mendez *et al*., 2004). Medicinal applications include peat therapy where humic substances are used to treat musculo-skeletal, gynaecological, and skin conditions among others (Schepetkin *et al.*, 2002)

Common medical uses of humic substances are anti-viral, anti-inflammatory, pro-inflammatory, anti-oxidant and wound healing properties.

Table 1: Commercially available fulvic acid containing preparations:

Brand name	Manufacturer
Humic monolaurin complex®	Nutricology
Fulfixer – fulvic acid®	Nature's Alchemist
Supreme fulvic and humic complex®	Supreme Fulvic
Fulvic force®	Wellness Warehouse
Amorganic fulvic acid detox nutrients	Amorganic
Quality Health fulvic acid capsules	Quality Health

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The focus of this study was to determine what the effect of a carbohydrate derived fulvic acid and a reference synthetic humic acid have on the absorption of the water-soluble class of B vitamins using the everted gut model on living *ex vivo* GIT tissue.

The purpose of this study was to evaluate if there were any positive or negative changes in vitamin B absorption rates when these vitamins were combined with either humic acid or fulvic acid.

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1.11 Research Statement:

Humic and fulvic acid preparations are sold as supplements to promote health and claimed to improve nutrient absorption, but questions as to whether it has a measurable interaction with essential vitamins have been raised. There are contradictions and unanswered questions around whether they inhibit or promote nutrient uptake. This project aimed to address the question whether the absorption of water-soluble B vitamins is influenced by the presence of the two most commonly used supplement related humic substances. There are various types of commercially available preparations containing either humic or fulvic acids. The aim was to test whether the claims of a synthetic humic and synthetic fulvic acid are valid, in terms of improving water soluble vitamin absorption efficacy.

1.12 Aim:

To assess selected uptake of water-soluble B-vitamins in the presence of physiologically relevant concentrations of humic and fulvic acid.

1.13 Objectives:

a) To use ion-pairing Liquid Chromatography/Mass Spectrometry-Mass Spectrometry (LC-MS/MS) methods to simultaneously quantitate selected B-vitamins
b) To perform the everted mouse gut absorption assay of control vitamin solution as well as the same vitamins in the presence of synthetic humic acid and fulvic acid preparations.
c) To assess the influence of humic substances on the absorption of selected water-soluble B-vitamins.

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Chapter 2: Materials and methods:

2.1 Animal work:

2.1.1 Animal ethics:

All animal work was carried out in accordance with the SANS 10386:2008 (The care and use of animals for scientific purposes) and the University of Pretoria Bioresearch Centre (UPBRC) guidelines for performing animal research. Ethical clearance was obtained from the University of Pretoria Animal Ethics Committee (Ethics Approval number H011-16).

2.1.2 Animals used

A total of 45 CD1 female mice were used to conduct the study. Mice were healthy approximately 6 - 8 weeks of age and weighing between 18 and 20 grams.

2.1.3 Animal housing and care

Care of the mice was undertaken by the UPBRC, where they were housed in groups of 6 with controlled environmental conditions of 12-hour light dark cycles, 23°C and 50 – 60% relative humidity.

They were appropriately monitored for any change in behaviour. The mice had *ad libitum* access to clean water and standard mouse chow pellets.

The mice were acclimatised to the laboratory conditions at least one week prior to the commencement of the experiment. Sterile additional nesting materials and enrichment items were provided.

2.1.4 Euthanasia

Mice were euthanized by a qualified veterinary technician using an overdose of isoflurane. Isoflurane was used as it is a more “humane” technique for euthanizing mice and unconsciousness is induced rapidly while the isoflurane is reputed to not have any effect of the GIT or non-neural tissues.

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2.1.5 Dissection and removal of the tissue

Immediately following euthanasia, the limbs of the mouse were pinned to a dissection board exposing the ventral regions. The abdominal area was disinfected using 70% ethanol. An incision was made through the abdominal wall along the midline moving in an upward direction from the lower abdomen to the xiphoid process. The skin and muscle layers were folded aside, exposing the gut. The tissue was dissected out of the abdominal cavity from the end of the colon, preceding the rectum back to the stomach. The tissue was dissected below the pyloric valve of the stomach. The removed GIT was quickly rinsed with Krebb's Ringers buffer solution (KRB) and sectioned either side of the caecum then any food debris removed from the intestine sections by using the assisted peristalsis technique. The segments were kept viable by frequent submerging in warm (37ºC) KRB solution. After clearing the bulk of the food material and chyme, a 2 mL syringe fitted with an 18G Teflon catheter tube was used to flush warm saline solution through the GIT to ensure no food particles remained in the gut.

2.1.6 Mouse carcass disposal

The carcasses of dissected mice and organs used after experimentation were disposed of by UPBRC staff in accordance with the Standard Operating Procedure. As no infectious or toxic compounds were used, standard burial procedures were used.

2.2 Materials

2.2.1 Test compounds:

The test compounds were a synthetic carbohydrate derived fulvic acid and a synthetic humic acid made through the oxidation of ligneous coal respectively.

The fulvic acid solution with product name AP 5000Da was used. The sample had Batch code: 470C 06/02/17 and a reported concentration of 8% in water.

A dry synthetic humic acid powder labelled as Oxihumate that was manufactured by Enerkom (Pretoria, RSA) with a batch number N_8 was donated for use in this study.

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The water-soluble vitamins were obtained from a generic Vitamin B complex tablet (Vitaforce Nutri-B, Dischem, SA) containing the following concentrations of the individual vitamins per unit.

Vitamin B_1	Thaimine monohydrate	10.1mg
Vitamin B_2	Riboflavin	12.5mg
Vitamin B_3	Nicotinamide	12.5mg
Vitamin B_5	Calcium d-Pantothenate	17.2mg
Vitamin B_6	Pyridoxine hydrochloride	10.3mg
Vitamin B_7	Biotin	15.0µg
Vitamin B_9	Folate	200 µg
Vitamin B_{12}	Cyanocobalamine	12.5 µg

One tablet was crushed and ground to a fine powder then quantitatively transferred to a 50 mL volumetric flask with approximately 40 mL deionised water and dissolved by placing the flask in an ultrasonic bath for 15 minutes with regular vortex mixing to ensure the vitamins dissolved completely. The flask was filled to the mark and well mixed before transferring to a centrifuge tube and centrifuged at 3500 *g* for 15 minutes to clarify the solution and remove the insoluble excipients. The fresh supernatant was used for diluting into the three treatment preparations.

Preparation of solutions:

Unless specifically mentioned, all chemicals used were of analytical grade or better and purchased from either Merck (Darmstadt, Germany) or Sigma Aldrich (St Louis, USA). Solvents for mass spectrometry (MS) were of MS grade Romil products purchased from MicroSep (Johannesburg, RSA). Water was produced in-house using an Elga Genetics system producing >18 MΩ water with TOC of less than 2 parts per million (ppm).

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KRB solution: pH 7.4 (dissolved in 1 L de-ionised water)

115 mM NaCl
5.9 mM KCl
1.2 mM $MgCl_2$
1.2 mM NaH_2PO_4
1.2 mM Na_2SO_4
2.5 mM $CaCl_2$
25 mM $NaHCO_3$
10 mM Glucose

The control treatment solution consisted of KRB buffer spiked with the clarified vitamin B complex solution at a ratio of 10 mL vitamin B solution to 490 mL KRB.

Preparation of humic acid solution:
A 0.51% w/v solution of was prepared by dissolving 2.55 g of the humic acid powder in 500 mL KRB solution with ultrasonic bath (Bransonic® M, Branson 120 W) treatment for 15 minutes. The humic acid incubation solution was prepared by spiking 490 mL of the 0.51% humic acid solution with 10 mL of the clarified vitamin B complex stock solution and mixing by inversion till homogenously mixed.

Preparation of fulvic acid solution:
An 8% v/v Fulvimed AP 5000Da solution with Batch code: 470C was used for this study. A volume of 6.375 mL of the 8% Carbohydrate derived (CHD) FA fulvic acid solution was diluted with 93.625 mL of KRB solution to prepare a final concentration of 0.51% fulvic acid solution which was further used for the vitamin absorption studies. The fulvic acid incubation solution was prepared by spiking 490 mL of the 0.51% fulvic acid solution with 10 mL of the clarified vitamin B complex solution to give a final 0.5% fulvic acid solution.

For each of the fulvic and humic acid solutions tested, as well as the control KRB solution a set of 10 mice were assayed, split into groups of 5 mice per day. The small intestine consists of three distinct regions, namely the duodenum, jejunum, and ileum which show different absorption rates for the different vitamin B compounds. Each

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mouse GIT provided one duodenum, four jejunum and one ileum segment. This provided ten data points for the duodenum and ileum each and a total of forty jejunum segments per treatment to ensure statistical power.

Following dissection of the GIT and clearing of the entire small intestine of any remaining food and chyme, the gut was everted then cut into approximately 6 cm segments that included sections representing the duodenum, jejunum and ileum for the incubation as described below. This was to evaluate whether absorption is differentially influenced in different GIT regions as the absorption of the vitamin B is known to take place differentially along the GIT.

2.3 Methods:

2.3.1 Everted mouse gut method:

The everted mouse gut model uses relatively short segments of the small intestine of the mouse to evaluate the absorption of specific compounds after incubation for set incubation times. Dissection and removal of the small intestine from the mouse specimen took place immediately after euthanasia. After clearing any food by simulated peristaltic movement and rinsing out with saline solution (at room temperature) to remove any food particles that were present that could possibly affect the results of this experiment the whole GIT was everted in a single procedure. To achieve this the entire GIT was gently pushed over an 18G Teflon catheter through which a silk thread had been inserted, ensuring that the tissue was well wetted with KRB at all times. When the entire GIT was over the Teflon catheter the end of the GIT was tied off with the silk thread and the GIT gently pushed off the catheter and over the tied off end to evert the GIT. The everted GIT was rinsed in KRB then sequentially cut into the required segments followed by tying off one end of each segment with a 20 cm long thread at about 2 mm from one end. Each segment was filled with 400 µL of Krebs Ringers buffer solution without any vitamin B and again tied off at the open end with a silk suture. The longer thread was weighted with a paper clip to ensure that the filled segment would be submerged throughout the incubation period.

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Thirty millilitre glass tubes containing one of the three treatments spiked with the vitamin B mixture were placed in a water bath at 37±1°C at least 20 min prior to dissecting the mice to ensure that the incubation temperature was reached and gassed slowly with oxygen. The KRB filled, everted GIT segments were then incubated for 90 minutes at 37°C in these different treatments after which the everted segments were removed from the vitamin solutions and briefly rinsed with PBS solution by dipping the segments in a PBS solution several times. The segments were then doubled over into appropriately labelled 1.5 mL Eppendorf tubes and carefully cut to collect the contents for analysis by LC-MS/MS. In general, a volume of about 100 – 200 µL could be recovered from each GIT segment after incubation.

2.3.2 Sample analysis

All post-incubation samples collected from the everted GIT segment were recovered and analysed simultaneously for all the B vitamins using a validated LC-MS/MS developed and optimised for the analysis of these different vitamins. Due to the low concentrations used and the dilution effect of the samples during the sample clean-up procedure, three of the vitamins (biotin, folic acid and cyanocobalamin) were present at concentrations below the Limit of quantitation (LOQ) and could not be quantitated although they could be detected.

2.3.2.1 Sample preparation:

Sample for tuning and method development

Tuning of the mass spectrometer, to achieve highest sensitivity for the five different B vitamins to be quantitated in the samples, was done to ensure that the LC-MS/MS system conditions for each analyte and that the maximum signal for each of the vitamin B analyte precursor and fragment or product ion transitions were optimised under the source and fragmentation settings on the equipment available.

Initially one vitamin B complex tablet which weighed approximately 250 mg and containing the following amounts of active ingredients of water-soluble B-Vitamins was dissolved in a total volume of 50 mL 5% methanol in deionised water extraction solution:

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Vitamin B_1	Thiamine monohydrate	10.1 mg
Vitamin B_2	Riboflavin	12.5 mg
Vitamin B_3	Nicotinamide	12.5 mg
Vitamin B_5	Calcium d-Pantothenate	17.2 mg
Vitamin B_6	Pyridoxine hydrochloride	10.3 mg
Vitamin B_7	Biotin	15 µg
Vitamin B_9	Folate	200 µg
Vitamin B_{12}	Cyanocobalamin	12.5 µg

One tablet was ground to a fine powder and dissolved in a 50 mL volumetric flask in extraction solution using an ultrasonic bath (Bransonic M, 120 W, Branson, USA) operated at ambient temperature for 15 minutes with regular interspersed vortex mixing.

A sample of the extract was centrifuged at 16000 x *g* for 10 minutes to clarify and to remove insoluble excipients. For the tuning of the analytes a 50 µL aliquot of this supernatant was diluted 1000-fold, using a volumetric flask and a 50:50 mixture of mobile phases A and B that each had 0.1% formic acid and 0.025% heptafluorobutyric acid (HFBA), and thoroughly mixed by inversion.

Using a Harvard syringe pump, the diluted sample was directly infused into a Sciex 4000QTrap triple quadrupole system at a flow rate of 10 µL/min. Manual tuning was done to optimize the source and mass spectrometer fragmentation parameters for each of the vitamins including ionisation voltage, source gas flow rates, source temperature, fragmentation voltage and collision cell gas flow. Suitable MRM mass transitions were selected for both the quantitation and qualification fragment ions with dwell times of 100 msec.

2.3.2.2 GIT sample analysis:

Sample preparation of test compounds:

The sample volumes obtained from the gut segments were insufficient for solid phase extraction and preconcentration during sample clean up, therefore Solid phase extraction (SPE) removal of interfering matrix components from the sample was not

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possible and a simple acetonitrile protein crash followed by a centrifugation step was included to remove possible protein or mucin components.

After the incubation period, the outside of the GIT segments were washed by dipping the segment several times into a tube with clean saline, lightly dabbed with absorbent paper to remove any saline clinging to the GIT segment and the content of each segment collected into Eppendorf tubes by cutting off one end of the GIT segment while hanging inside the tube. The tubes were centrifuged to remove any particulates and tissue before collecting exactly 50 µL from each sample and diluting this with 100 µL of acetonitrile containing 0.025% heptafluorobutyric acid (HFBA). After standing on ice for 30 minutes, the solution was clarified by centrifugation at 16 000 *g* after which the supernatant was transferred to tapered autosampler vial inserts and used directly for quantitative analysis. Concentrations of each of the vitamin B analytes per segment were compared to a calibration curve prepared from vitamin B standards that were run using the same sample preparation and analytical LC-MS/MS conditions as used for the GIT segment samples.

Analyte concentrations from each GIT segment sample for the two humic substances were compared to the equivalent KRB based segment absorption to determine the effect of the humic substance treatment on the vitamin absorption per GIT segment zone and per treatment.

Concentrations of each analyte in each of the samples were determined by comparison to the calibration curves prepared using vitamin B standards under the same analytical conditions and run before and after each batch of unknown samples that were interspersed at intervals of ten unknown samples with a QC standard. Peak areas were integrated by the Analyst 1.5.2 software and peak area integration confirmed manually to ensure proper peak identity and integration limits of each analyte. Concentrations were collated in MS Excel and exported to Prism GraphPad 6 software for graphing and statistical analysis.

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2.3.3 Sample vitamin concentrations analysis using LC-MS/MS:

A previously developed High Performance Liquid Chromatography separation using a volatile ion pairing reagent and a solvent gradient protocol was adapted to the system dead volume and the column dimensions used in this study to separate the vitamin B analytes in the samples.

A Sciex 4000QTrap triple-quadrupole mass spectrometer, with a Turbo-V ESI source was operated in positive ionisation mode for all the analytes. Analyst 1.5.2 software was used for system control and data analysis, including the peak area integration and to establish analyte calibration curves for each vitamin. Individual LC-MS/MS mass transitions were optimised for each of the different vitamin B analytes during method optimisation. The individual analytes could be very selectively identified by the mass spectrometer with high sensitivity and although the very low concentrations of folic acid, biotin and vitamin B12 could be clearly detected, these three analytes were below the lower limit of quantitation of the method.

Sciex 4000QTrap optimised source conditions used:

Mode:	Positive ESI mode
Curtain gas:	23 nominal units
Ion spray voltage:	+5500 V
Source temperature:	On
Ion source gas 1:	35 nominal units
Ion source gas 2:	36 nominal units
Drying gas temperature:	500°C

Dwell time for all Multiple reaction monitoring (MRM) transitions: 100 milliseconds

De-clustering potential and collision energy were optimised per analyte and summarised in the Table below

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The following scan types were done:

An initial untargeted Q_1 scan, followed by enhanced resolution scans of identified vitamin B analytes, MS^2 (product ion) scan, enhanced product ion scan and optimized MRM Scan.

Table 2: Optimised values of the de-clustering potential and collision energies of the different analytes for LC-MS/MS analysis of vitamin B sample:

Vitamin	**Q_1**	**Q_3**	**De-clustering potential**	**Collision energy**	**Cell exit potential**
Vitamin B_1	265.8	122.2	26	23	12
Vitamin B_2	377.5	243.1	100	33	14
Vitamin B_3	123.2	80.0	50	45	12
Vitamin B_5	220.4	90.1	58	18	10
Vitamin B_6	170.3	134.1	50	45	12

2.3.4 LC consumables:

Column used	Agilent Eclipse XDB 150 x 4.6 mm (5-micron particle size)
Column temperature	35^{o}C
Mobile phase A	0.1% FA and 0.025% HFBA (Heptafluorobutyric acid) in deionized water.
Mobile phase B	75:25 methanol:acetonitrile solution with 0.1% FA and 0.025% HFBA.

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Injection volume	6 microliters
Needle wash	75:25 methanol:water

2.3.5 LC Gradient conditions

A Sciex 4000 QTrap triple-quadrupole mass spectrometer was used for analysis of the water-soluble B vitamins. Analyst 1.5.2 software controlled the chromatographic system and analysed the data including integration and comparison to standard curves.

Vitamins were separated on an Agilent Eclipse XDB 150 x 4.6 mm (5-micron particle size) column, with a 0.1% FA and 0.025% Heptafluorobutyric acid (HFBA) in deionised water (Solvent A) and 75:25 methanol:acetonitrile solution with 0.1% FA and 0.025% HFBA (Solvent B). Flow rate was set at 1.0 mL/minute running the following linear gradient profile (A:B) starting at 83:17 from 0 to 0.25 minutes changing to 50:50 at 5 minutes. Ratio changed to 2:98 at 5.2 minutes for column clean-up and held at a constant ratio of 2:98 from 5.2 to 7.2 minutes. At 7.5 minutes the ratio was returned to 83:17 and the column re-equilibrated till 9.5 minutes. Total run time was 9.5 minutes. Injection volume was 6 microliters. Column temperature was kept constant at 35ºC. Quantitation of vitamins were done using peak areas of the optimised MRM fragments for each analyte with peak confirmation using the confirmatory fragment.

2.3.5.1 Validation techniques:

In order to correctly assess whether the method for analysing the analytes using LC-MS/MS matched its purpose, method validation was performed. The objective of validation was to show the suitability of the technique for its intended purpose.

The International Conference on Harmonisation (ICH) (Borman and Elder, 2017) established basic guidelines that describe the common analytical procedures used for validation. These include selectivity, accuracy, precision, interference, linearity, limit tests for the quantitative range used in test samples, and identification tests.

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Selectivity:

The test sample contained a mixture of compounds. The process of selectivity in analytical validation refers to the ability to identify a particular analyte without interference from other components. Specificity was supported by individual retention times, by identifying the precursor masses of compounds and determining the transition masses during the fragmentation process of LC-MS/MS detection. An analysis of a series of blank samples of everted GIT content after incubation with KRB where no B-vitamins were added, showed no compounds at the retention times of the analytes with any of the masses matching precursor ions of the vitamins being analysed.

Accuracy, precision and recovery:

Accuracy is validated by doing replicate analyses using known concentrations of analytes and separately prepared quality control standards. The accuracy of an analytical method describes the closeness of the mean of test results obtained by the method to the true concentration of the analyte (referenced against the QC standards).

Precision:

The degree of variation of the results from repeated analysis of the same homogenous sample, done under the same conditions at intervals is used to measure the precision. The three levels in precision are: repeatability, intermediate precision and reproducibility (ICH, 2005). Repeatability conditions are used to assess the closeness of the results obtained from the samples using the same procedure, method and equipment. Acceptable repeatability is considered a variation of less than 15% in results obtained when repeatedly testing the same sample.

Intermediate precision, also known as “within-lab” reproducibility, differs from repeatability in the time aspect. It is the results obtained in a single laboratory over a longer period of time where analyst or reagents may be from different batches.

Reproducibility is the concept of measuring precision of results obtained from different laboratories.

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Recovery:

The recovery of a method is an expression of the mean percent difference in peak areas measured when spiking the original blank sample matrix with a known concentration of analyte then extracting the analytes compared to the same concentration being spiked into the blank samples after performing all the extraction steps.

Other criteria for validation include detection limits, quantitation limits, linearity, range and robustness.

2.3.6 Calibration (standard curves) and Limit Of Quantitation

Changes in the concentrations of the analytes are measured against the response of the instrument using calibration curves. A linear relationship was established between concentration and the measured response.

The Limit of quantitation (LOQ) is the lowest concentration in the standard curve where the signal to noise exceeds 10 or where the determined concentration variability is less than 20% for repeat analysis of the same concentration of analyte. It must be a measured value and may not be inferred. The ULOQ was higher than the highest concentration used in the calibration which was above the range of the sample concentrations used in this study (20 µg/mL). This provided adequate range for determining the concentration of each of the vitamins in the samples as well as to prove the linearity of the assay for each of the analytes where acceptable correlation coefficients were determined.

Quantitative analysis was performed using the largest fragment peak area for each of the analytes and the identity of each was then confirmed using the retention time and the qualifier fragment of each analyte under optimal fragmentation conditions.

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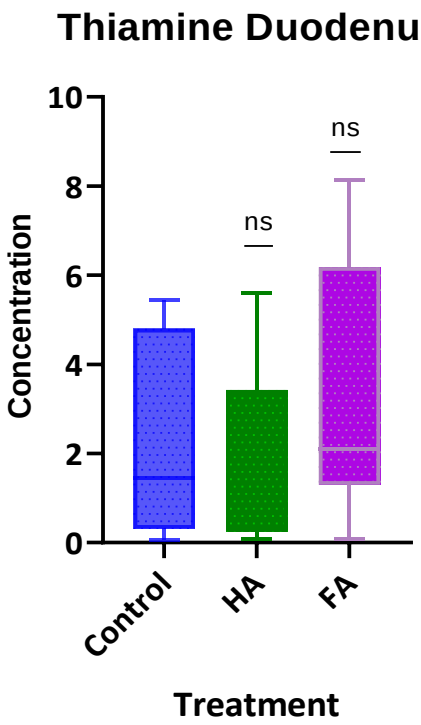
Chapter 3: Results:

3.1 Thiamine:

3.1.1 Duodenum



Graph 1: Comparative absorption of Vitamin B_1 (Thiamine) in mouse duodenum in control, humic acid and fulvic acid conditions. N = 10

Three treatment conditions were examined to compare the effect on the absorption of the different vitamin B analytes.

Condition 1: HA at 0.5% together with the vitamin mixture
Condition 2: FA at 0.5% together with the vitamin mixture
The control was the vitamin mixture in KRB.

Samples obtained from individual segments of the gut were analysed to observe vitamin absorption when used in combination with one of the humic substances. Results

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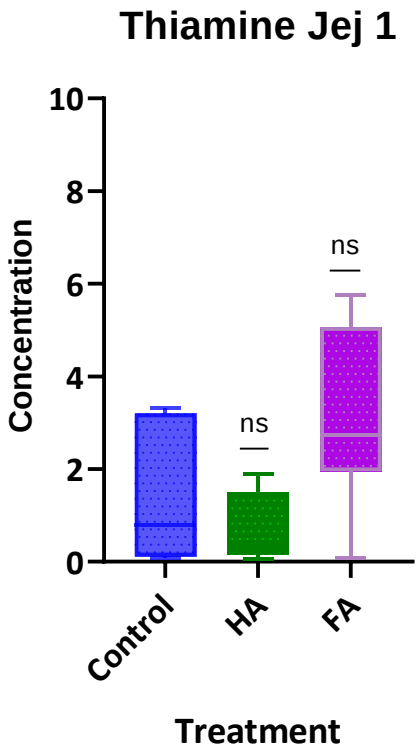
indicated that there is no significant difference between the control and either treatment conditions in the duodenal segment for thiamine absorption.

3.1.2 Jejunum segment 1:

The jejunum of this mouse was longer in length and could be divided into four individual segments for analysis. Jejunum segment 1 is the segment which is starts at the end of the duodenum.



Graph 2: Comparative absorption of Vitamin B_1 (Thiamine) in mouse jejunum segment 1 in control, humic acid and fulvic acid conditions. N = 10

Thiamine in the 1st jejunum segment when used in combination with both humic and fulvic acid versus the control solution (Vitamin B) showed no significant differences although a clear trend of increased absorption in the fulvic acid treated group was observed.

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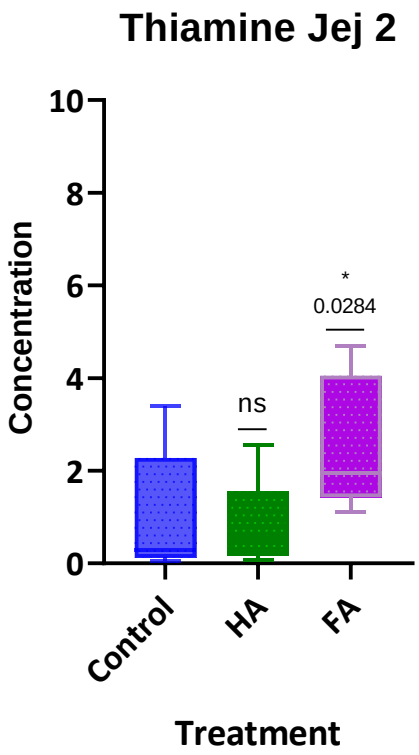
3.1.3 Jejunum segment 2:



Graph 3: Comparative absorption of Vitamin B_1 (Thiamine) in mouse jejunum 2 segment in control, humic acid and fulvic acid conditions. N = 10 *P=0.0284

The presence of humic acid in the 2nd jejunum segment does not affect the absorption of thiamine. There is no significant difference in the absorption.

The presence of fulvic acid in the 2nd jejunum segment increases the absorption of thiamine with a significance P-value of 0.0284.

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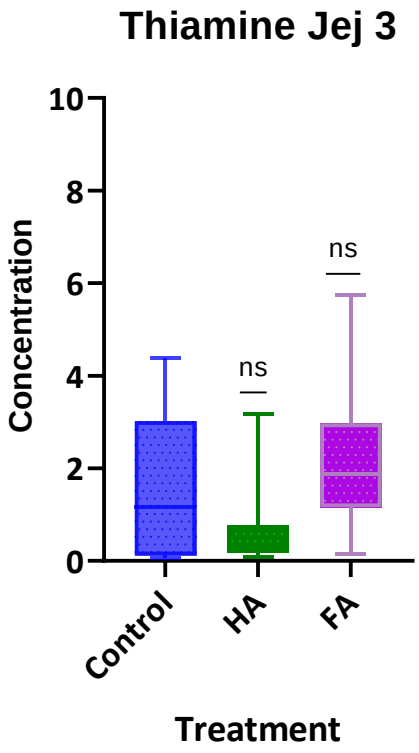
3.1.4 Jejunum segment 3:



Graph 4: Comparative absorption of Vitamin B_1 (Thiamine) in jejunum 3 of mouse in control, humic acid and fulvic acid conditions. N = 10

Results indicated that there is no significant difference between the control and treatment conditions in the third jejunum segment for thiamine absorption although there is a trend of reduced absorption in the humic acid treatment group.

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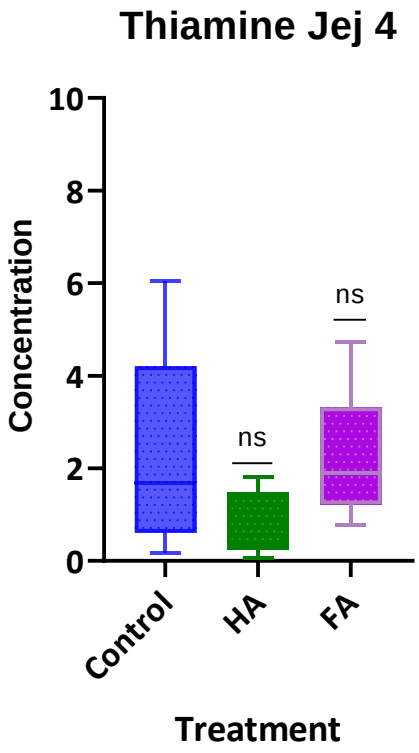
3.1.5 Jejunum segment 4:



Graph 5: Comparative absorption of Vitamin B_1 (Thiamine) in jejunum 4 of mouse in control, humic acid and fulvic acid conditions. N = 10

Results indicated that there is no significant difference between the control and treatment conditions in the fourth jejunum segment for thiamine.

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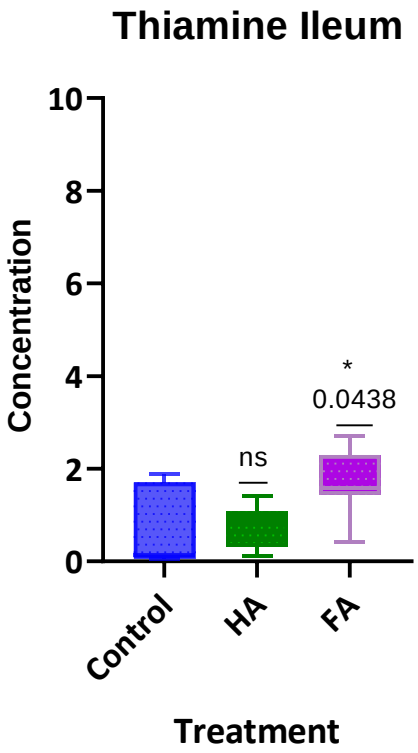
3.1.6 Ileum:



Graph 6: Comparative absorption of Vitamin B_1 (Thiamine) in ileum of mouse in control, humic acid and fulvic acid conditions. N = 10 * P=0.0438

The presence of humic acid does not affect the absorption of thiamine significantly in the ileum. The presence of fulvic acid does increase absorption of thiamine significantly in the ileum (P-value of 0.0438).

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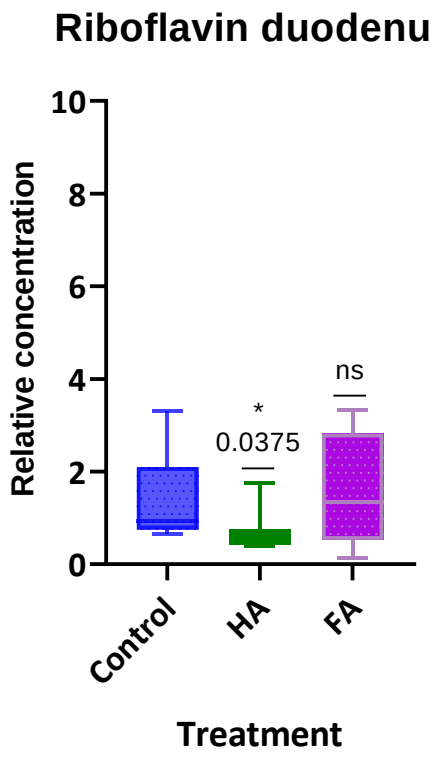
3.2 Riboflavin:

3.2.1 Duodenum



Graph 7: Comparative absorption of Vitamin B_2 (Riboflavin) in duodenum of mouse in control, humic acid and fulvic acid conditions. N = 10 * P=0.0375

Results indicated that there is no significant difference between the control and fulvic acid condition in the duodenal segment for riboflavin absorption although there is a significantly lower absorption in the humic acid treatment group.

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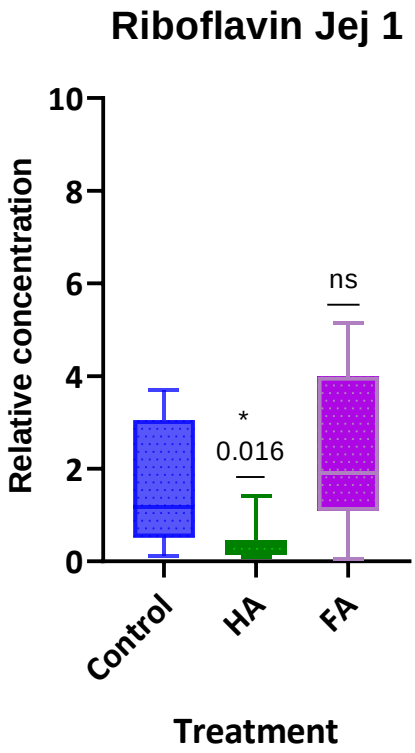
3.2.2 Jejunum segment 1:



Graph 8: Comparative absorption of Vitamin B_2 (Riboflavin) in jejunum 1 segment of mouse in control, humic acid and fulvic acid conditions. N = 10 *P=0.0160

The presence of humic acid in the jejunum negatively affects the absorption of riboflavin

Humic acid showed a significant change in absorption with a P-value of 0.016 when compared to the control. Fulvic acid did not affect the absorption significantly of riboflavin in the 1st jejunum segment when compared to the control.

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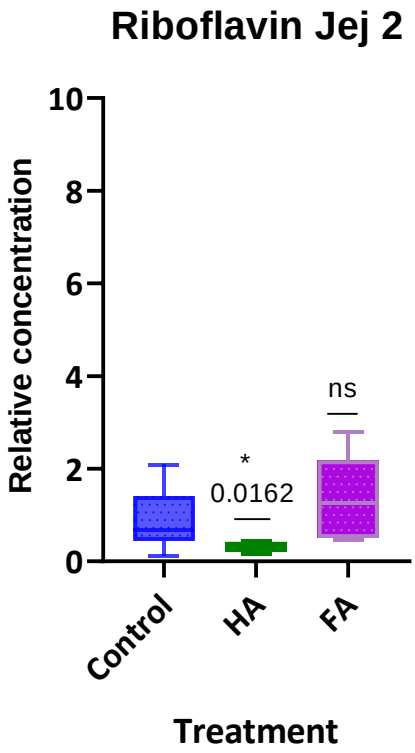
3.2.3 Jejunum segment 2:



Graph 9: Comparative absorption of Vitamin B_2 (Riboflavin) in jejunum 2 segment of mouse in control, humic acid and fulvic acid conditions. N = 10 *P=0.0162

The presence of humic acid in the 2nd jejunum segment does negatively affect the absorption of riboflavin, with a P-value of 0.0162 when compared to the control solution. The presence of fulvic acid does not significantly affect the absorption of riboflavin in the 2nd jejunum segment although a trend of increased absorption was evident.

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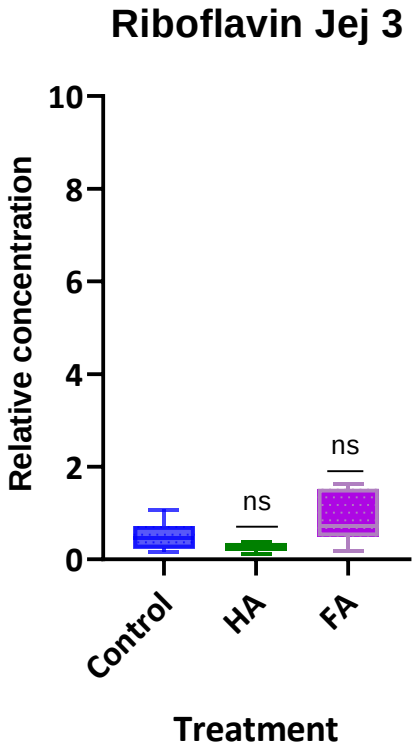
3.2.4 Jejunum segment 3:



Graph 10: Comparative absorption of Vitamin B_2 (Riboflavin) in jejunum 3 segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the third segment of the jejunum did not significantly affect the absorption of riboflavin despite a negative trend for humic acid and a slight increased absorption trend for the fulvic acid being observed.

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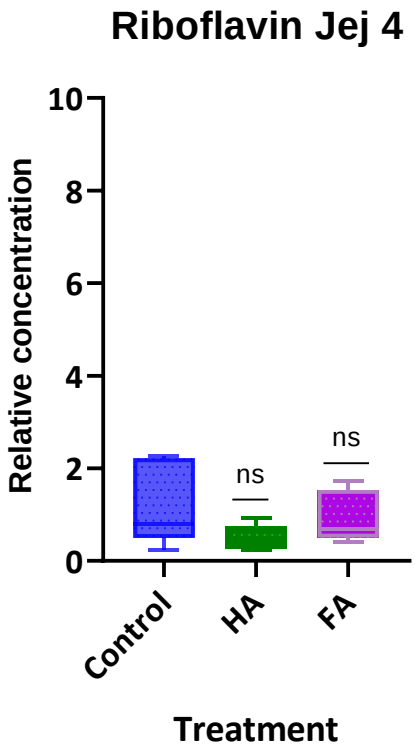
3.2.5 Jejunum segment 4:



Graph 11: Comparative absorption of Vitamin B_2 (Riboflavin) in jejunum 4 segment of mouse in control, humic acid and fulvic acid conditions. N = 10

Results indicated that there is no significant difference between the control and either treatment conditions in the fourth jejunum segment for riboflavin absorption. As seen with the previous segments there was a slight decrease in absorption for the humic acid treatment group.

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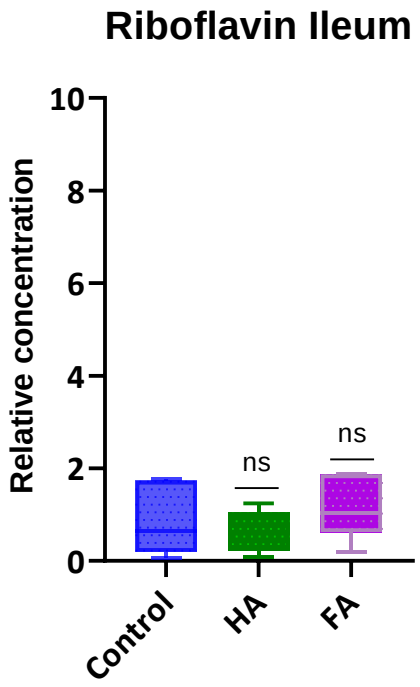
3.2.6 Ileum:



Graph 12: Comparative absorption of Vitamin B_2 (Riboflavin) in ileum segment of mouse in control (N=8)*, humic acid (N=10) and fulvic acid (N=9)** conditions

*Two missing segments due to degradation of tissue sample
**Missing segment due to tear in tissue sample

Results indicated that there is no significant difference between the control and treatment conditions in the ileum segment for riboflavin absorption.

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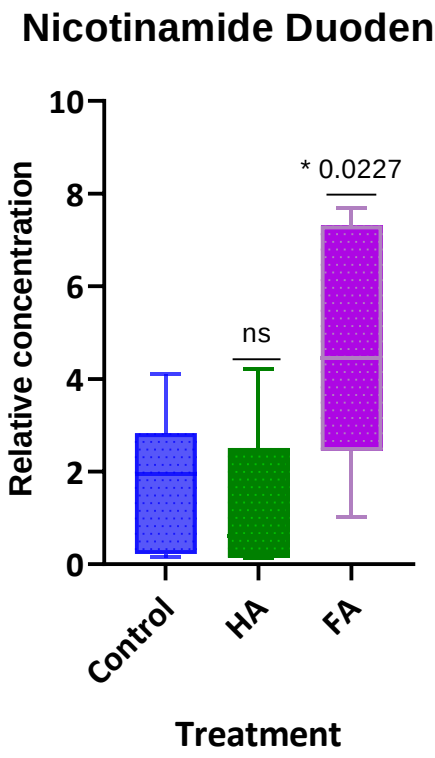
3.3 Nicotinamide:

3.3.1 Duodenum:



Graph 13: Comparative absorption of Vitamin B_3 (Nicotinamide) in duodenum segment of mouse in control, humic acid and fulvic acid conditions. humic acid (N=10) and fulvic acid (N=9) *P=0.0227

The presence of humic acid in the duodenum does not affect the absorption of nicotinamide significantly. The presence of fulvic acid does affect the absorption of nicotinamide significantly in the duodenum, with a P-value of 0.0227.

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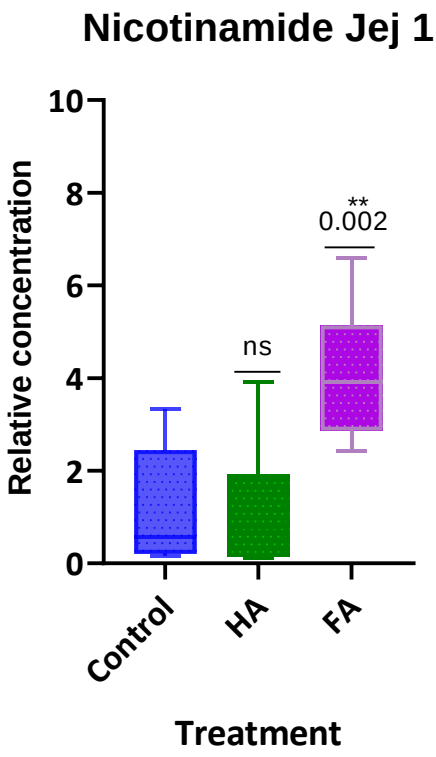
3.3.2 Jejunum 1:



Graph 14: Comparative absorption of Vitamin B_3 (Nicotinamide) in jejunum segment 1 of mouse in control, humic acid and fulvic acid conditions. N = 10 *** P=0.002

The presence of humic acid in the 1st jejunum segment does not significantly affect the absorption of nicotinamide when compared to the control solution. The presence of fulvic acid in the 1st jejunum segment significantly increases the absorption of nicotinamide when compared to the control solution. (P-value=0.002)

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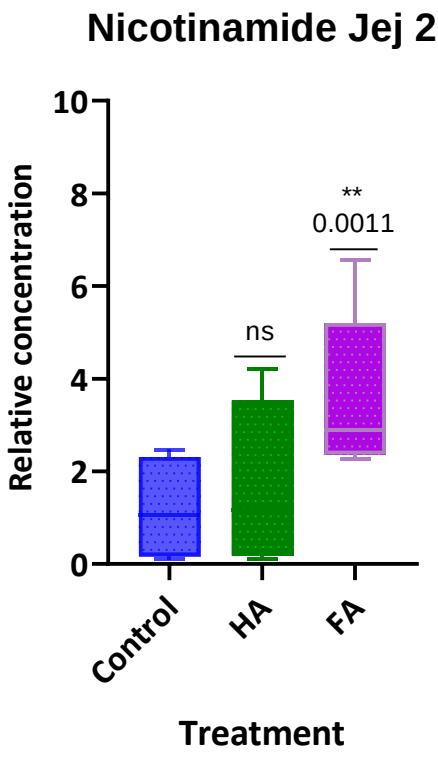
3.3.3 Jejunum 2:



Graph 15: Comparative absorption of Vitamin B_3 (Nicotinamide) in jejunum segment 2 of mouse in control, humic acid and fulvic acid conditions. N = 10 **P=0.0011

The presence of humic acid in the 2nd jejunum segment does not significantly affect the absorption of nicotinamide when compared to the control solution. The presence of fulvic acid in the 2nd jejunum segment significantly increases the absorption of nicotinamide when compared to the control solution. (P-value=0.0011)

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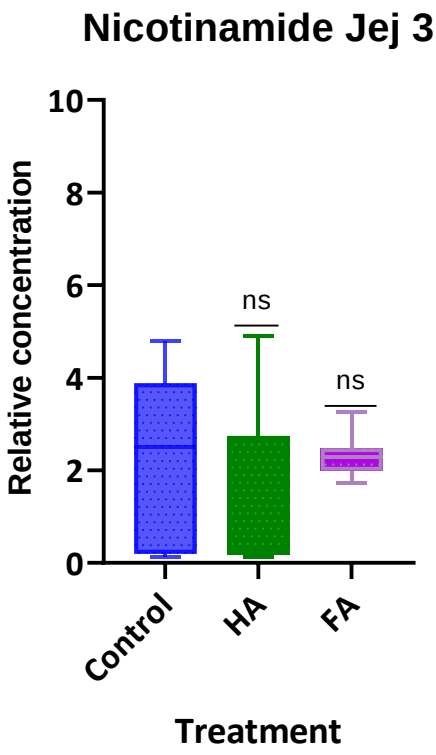
3.3.4 Jejunum 3:



Graph 16: Comparative absorption of Vitamin B_3 (Nicotinamide) in jejunum segment 3 of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the third jejunum segment does not affect the absorption of nicotinamide. There is no significant difference amongst the mean absorption from all three treatments. This is in contrast to other GIT segments where fulvic acid has consistently increased the absorption of nicotinamide.

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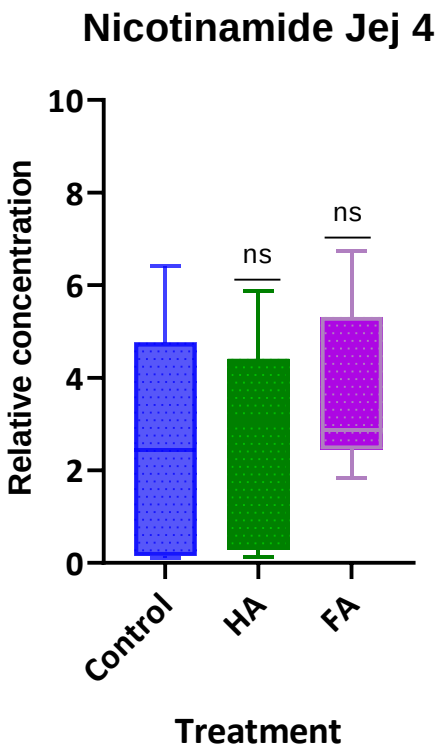
3.3.5 Jejunum 4:



Graph 17: Comparative absorption of Vitamin B_3 (Nicotinamide) in jejunum segment 4 of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the fourth jejunum segment does not significantly affect the absorption of nicotinamide although a slight increased absorption trend is observed for fulvic acid. There is no significant difference amongst the mean absorption from all three treatments.

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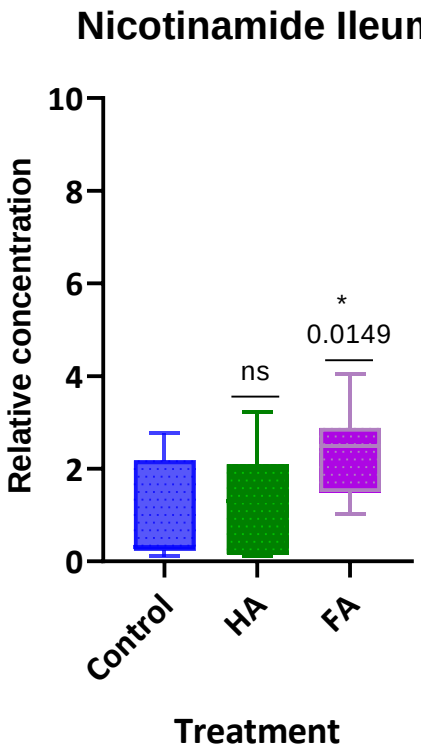
3.3.6 Ileum:



Graph 18: Comparative absorption of Vitamin B_3 (Nicotinamide) in the ileum of mouse in control, humic acid and fulvic acid conditions. N = 10 *P=0.0149

The presence of humic acid in the ileum segment does not show any affect the absorption of nicotinamide when compared to the control solution. The presence of fulvic acid in the ileum segment does significantly increase the absorption of nicotinamide, with a P-value of 0.0149.

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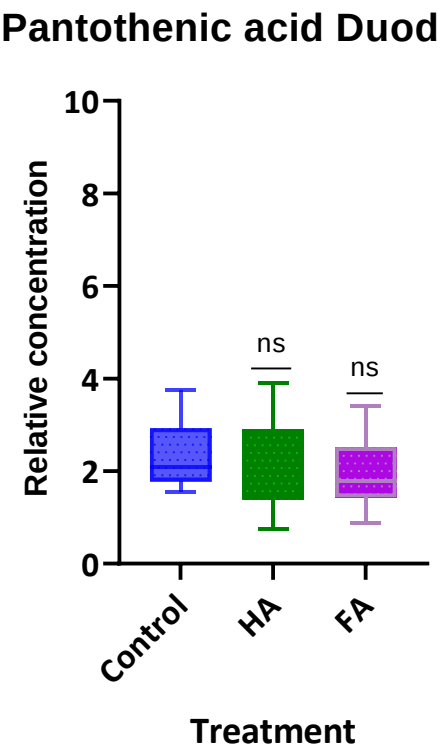
3.4 Pantothenic acid:

3.4.1 Duodenum:



Graph 19: Comparative absorption of Vitamin B_5 (Pantothenic acid) in the duodenum of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the duodenal segment does not affect the absorption of pantothenic acid. There is no significant difference amongst the mean absorption from all three treatments.

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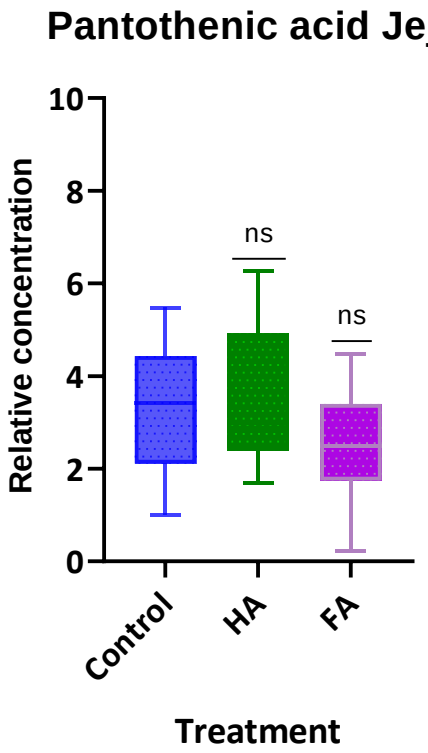
3.4.2 Jejunum segment 1:



Graph 20: Comparative absorption of Vitamin B_5 (Pantothenic acid) in the 1st jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the 1st jejunum segment does not affect the absorption of pantothenic acid. There is no significant difference amongst the mean absorption from all three treatments.

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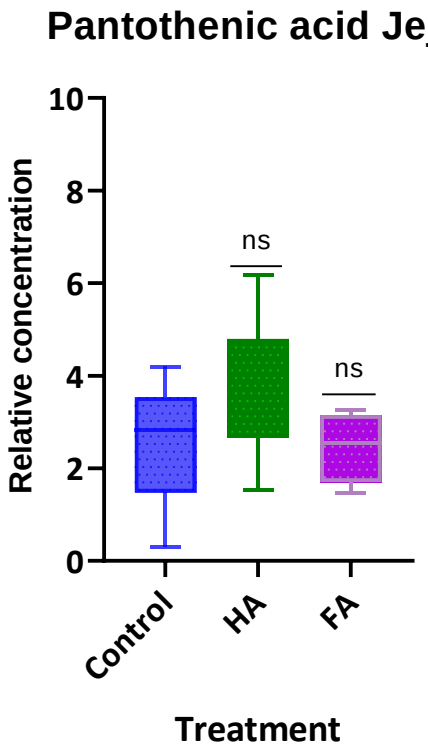
3.4.3 Jejunum segment 2:



Graph 21: Comparative absorption of Vitamin B_5 (Pantothenic acid) in the 2nd jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the 2nd jejunum segment does not significantly affect the absorption of pantothenic acid although the humic acid treatment did show a trend of increased absorption. There is no significant difference amongst the mean absorption from all three treatments.

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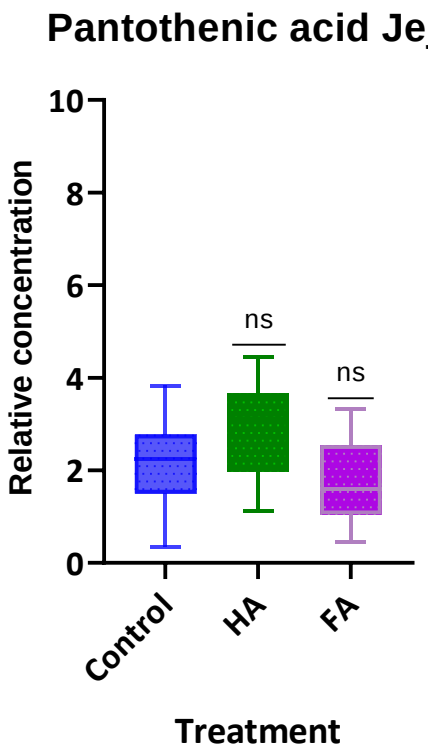
3.4.4 Jejunum segment 3:



Graph 22: Comparative absorption of Vitamin B_5 (Pantothenic acid) in the 3rd jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid and fulvic acid in the 3rd jejunum segment does not affect the absorption of pantothenic acid although the humic acid treatment did show a trend of increased absorption. There is no significant difference amongst the mean absorption from all three treatments.

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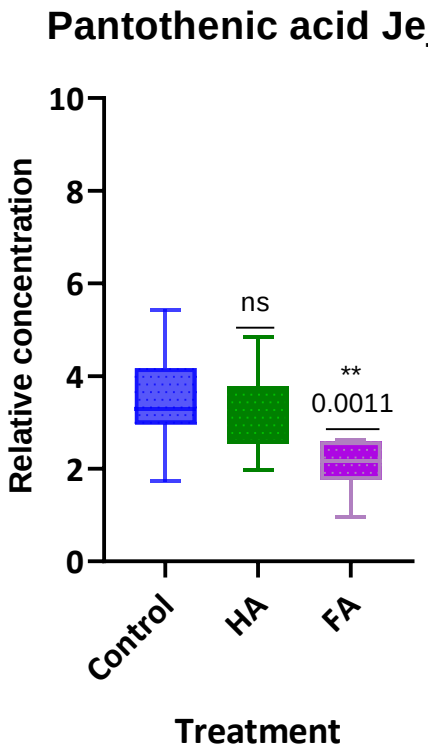
3.4.5 Jejunum segment 4:



Graph 23: Comparative absorption of Vitamin B_5 (Pantothenic acid) in the 4th jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10 **P=0.0011

The presence of humic acid in the 4th jejunum segment shows no significant change in absorption of pantothenic acid in comparison to the control. The presence of fulvic acid in the 4th jejunum segment shows a significant decrease in absorption of pantothenic acid in comparison to the control with a P-value of 0.0011.

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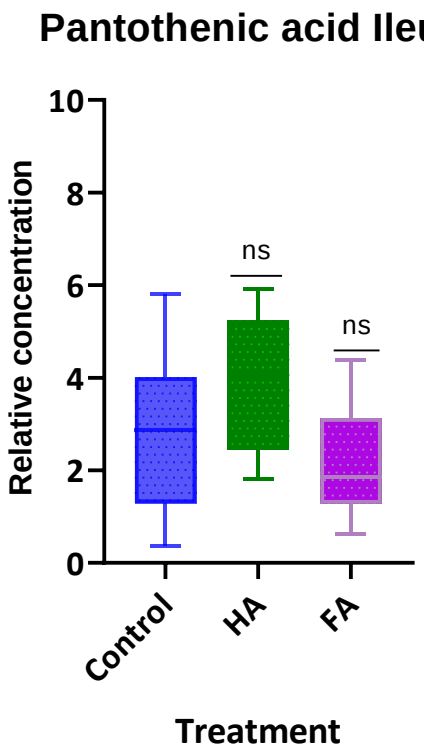
3.4.6 Ileum:



Graph 24: Comparative absorption of Vitamin B_5 (Pantothenic acid) in the ileum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of either humic or fulvic acid in the ileum segment did not significantly affect the absorption of pantothenic acid although there was again a slight trend of increased absorption for the humic acid treatment group.

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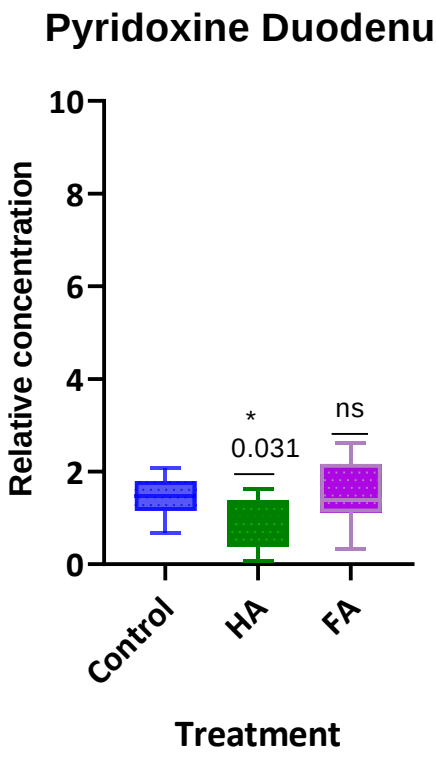
3.5 Pyridoxine:

3.5.1 Duodenum:



Graph 25: Comparative absorption of Vitamin B_6 (Pyridoxine) in the duodenum segment of mouse in control, humic acid and fulvic acid conditions. N = 10 *P=0.031

The presence of humic acid in the duodenum segment negatively affected the absorption of pyridoxine. There is a significant difference between the means of the humic acid treatment group and the control (P= 0.031).

The presence of fulvic acid in the duodenum segment does not affect the absorption of pyridoxine. There is no significant difference in absorption compared to the control.

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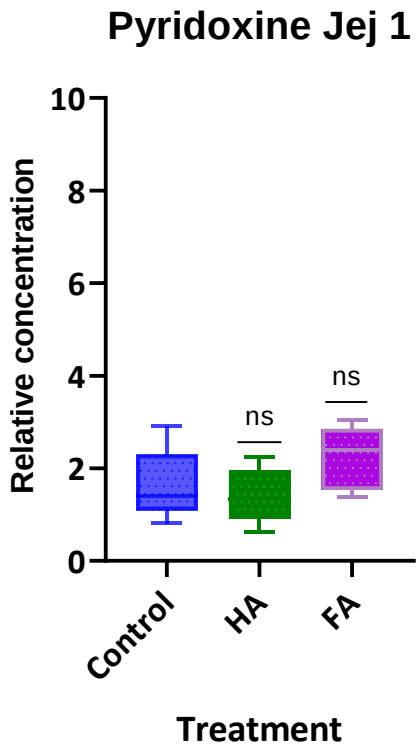
3.5.2 Jejunum 1:



Graph 26: Comparative absorption of Vitamin B_6 (Pyridoxine) in the 1st jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid nor fulvic acid in the 1st jejunum segment affected the absorption of pyridoxine.

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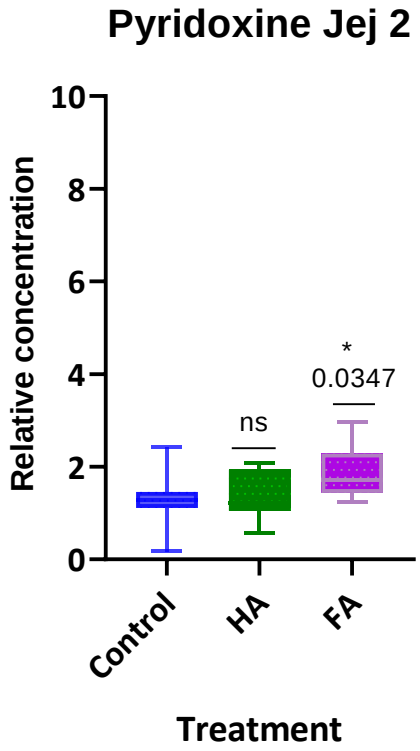
3.5.3 Jejunum 2:



Graph 27: Comparative absorption of Vitamin B_6 (Pyridoxine) in the 2nd jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10 *P=0.0347

The presence of humic acid in the 2nd jejunum segment does not affect the absorption of pyridoxine. There is no significant difference between the means of the humic acid treatment group and the control.

There is a small but significant increase in absorption in the fulvic acid treatment group compared to the control, with a P-value of 0.0347.

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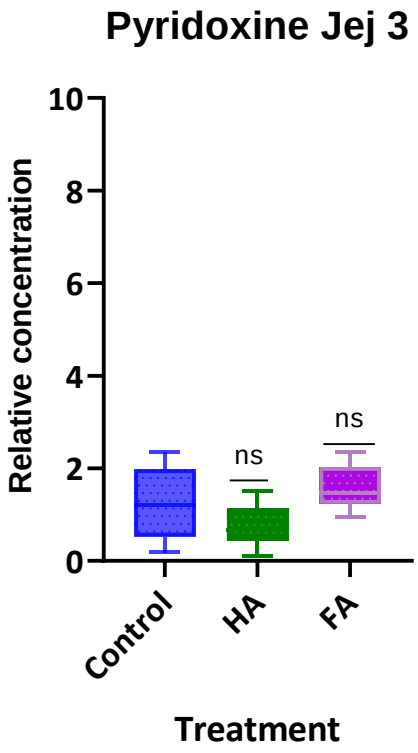
3.5.4 Jejunum 3:



Graph 28: Comparative absorption of Vitamin B_6 (Pyridoxine) in the 3rd jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid nor fulvic acid in the 3rd jejunum segment affected the absorption of pyridoxine.

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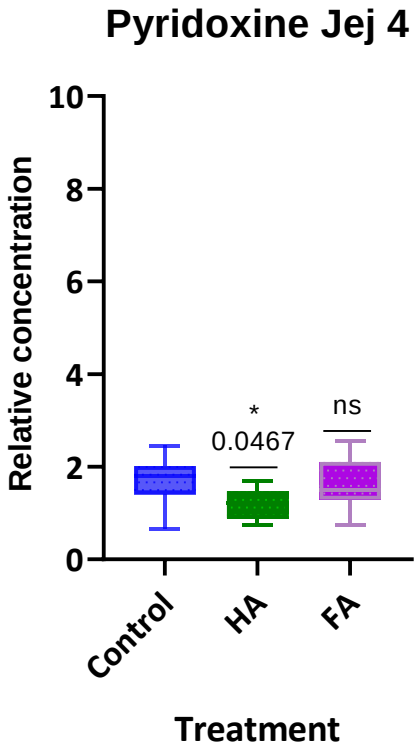
3.5.5 Jejunum 4:



Graph 29: Comparative absorption of Vitamin B_6 (Pyridoxine) in the 4th jejunum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of humic acid in the 4th jejunum segment does significantly decrease the absorption of pyridoxine with a P-value of 0.0467.

The presence of fulvic acid in the 4th jejunum segment does not affect the absorption of pyridoxine. There is no significant difference between the mean of the control group.

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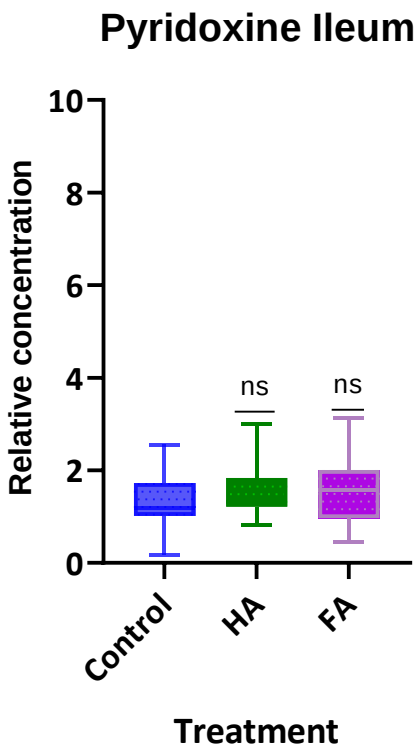
3.5.6 Ileum:



Graph 30: Comparative absorption of Vitamin B_6 (Pyridoxine) in the ileum segment of mouse in control, humic acid and fulvic acid conditions. N = 10

The presence of neither humic acid nor fulvic acid in the ileum segment affect the absorption of pyridoxine. There is no significant difference amongst the means of either treatment group and the untreated control group.

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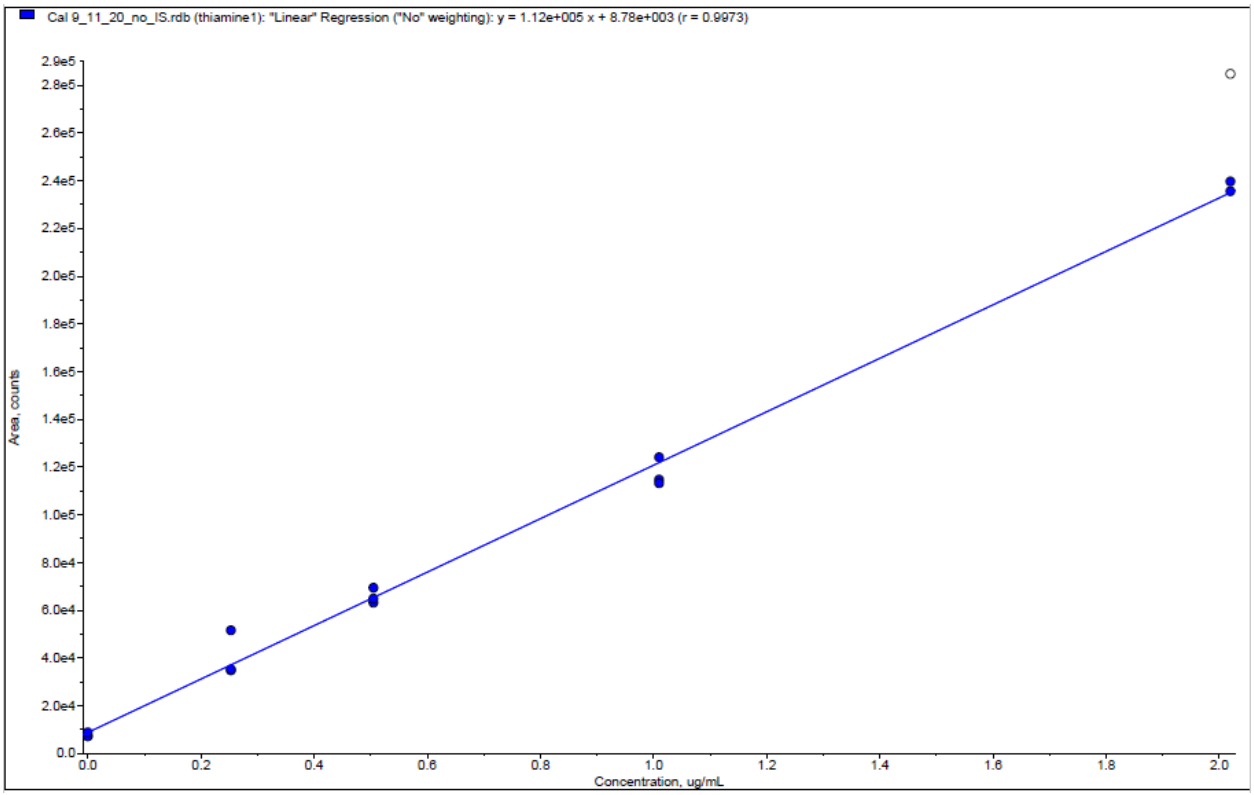
3.6 Standard curves:

The standard curves were derived from analysing the standards of the different B vitamins following the same sample preparation method as used for the GIT samples and as made up for different days over a two-week period. This is equivalent to making use of matrix matched calibration curves and takes the full extraction, drying and reconstitution of the standards into account for the technical variability of the methodology.

3.6.1 Thiamine:



Graph 31: Standard curve of Vitamin B_1 (Thiamine)

Regression equation: $y= 1.12x10^5x+8730$ (r=0.9973)

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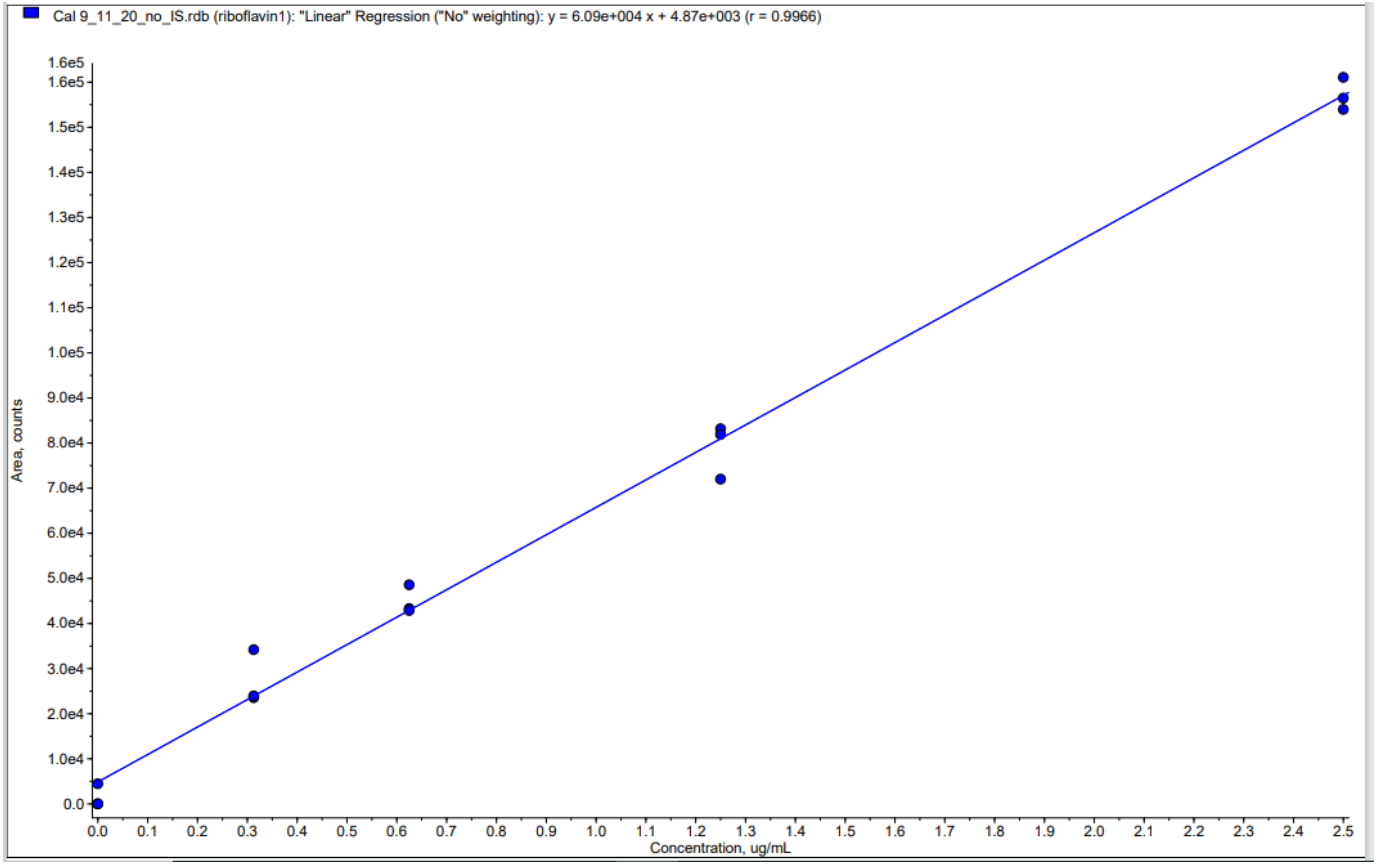
3.6.2 Riboflavin:



Graph 32: Standard curve of Vitamin B_2 (Riboflavin)

Regression equation: $y=6.09x10^4X+4870$ ($r=0.9966$)

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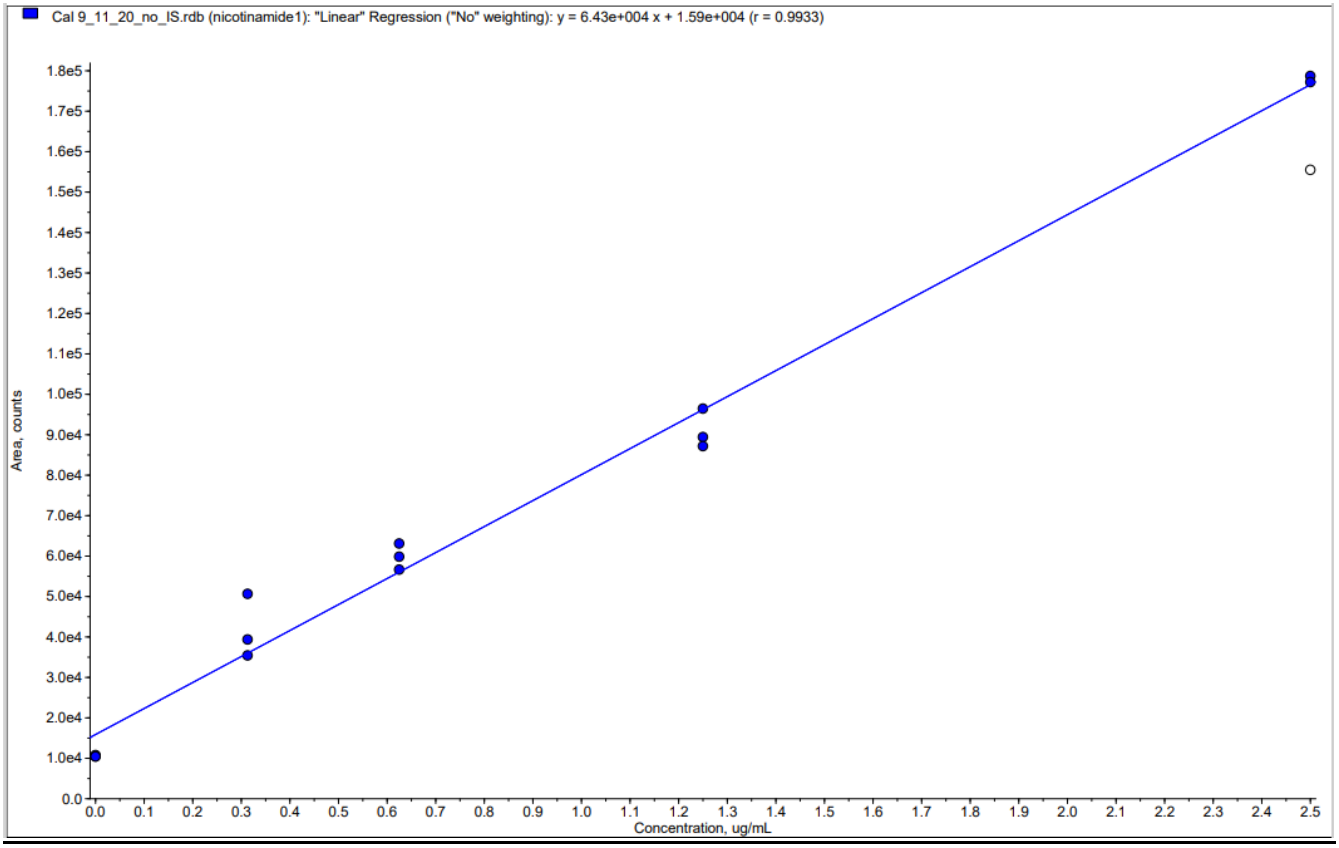
3.6.3 Nicotinamide:



Graph 33: Standard curve of Vitamin B_3 (Nicotinamide)

Regression equation: $y=6.43x10^4X+1590$ ($r=0.9933$)

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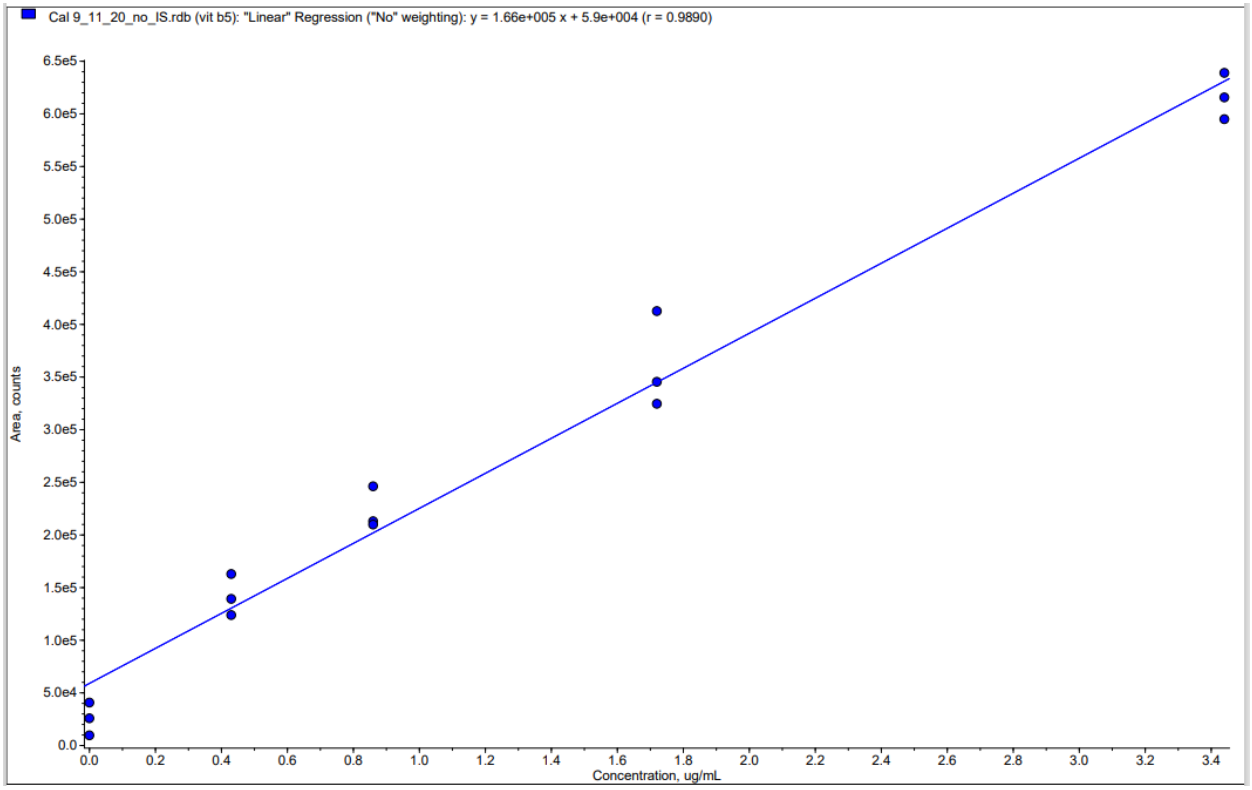
3.6.4 Pantothenate:



Graph 34: Standard curve of Vitamin B_5 (Pantothenate)

Regression equation: $y= 1.66x10^5X+59000$ ($r=0.9890$)

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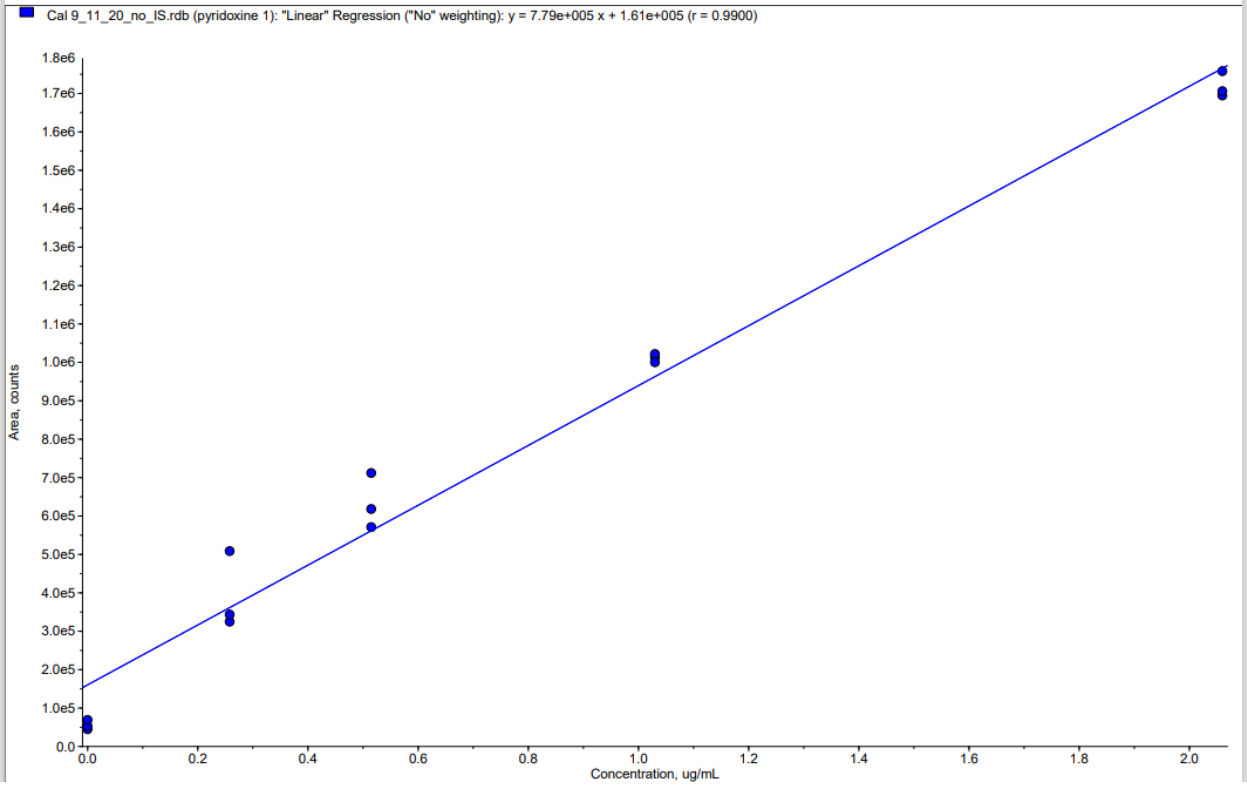
3.6.5 Pyridoxine:



Graph 35: Standard curve of Vitamin B_6 (Pyridoxine)

Regression equation: $y= 7.79x10^5X+161000$ (r=0.9900)

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Chapter 4. Discussion of results:

Humic substances are available commercially as food supplements that claim to have several health benefits including that of improved uptake of various beneficial nutrients. These large water-soluble compounds are regarded as supramolecular structures, are generally of natural origin and are responsive and sensitive to changes in pH and presence of different inorganic cations as demonstrated by their change in solubility in the presence of certain mineral salts. Both strongly acidic or alkaline conditions influence the micellar structure of dissolved humic substances where the hydrophobic arrangement of these molecules' change under acidic conditions due to the acid penetrating the interior of the micellar aggregates and changing the ionic form of internally located molecular moieties leading to molecular rearrangements. Negative charges present under alkaline conditions interfere with the conformation of humic substances, causing larger aggregates to separate into smaller micelles (Piccolo, 2001) altering their ability to bind other molecules present in the solution. The hydrated size, polarity, net ionic status and hydrophobicity of a molecule or molecular complex influence the molecules' ability to be absorbed across the intestinal mucosa via passive diffusion. Low molecular weight, lipophilic molecules are more easily absorbed. According to Fick's law the rate of absorption is proportional to the molecules concentration and the surface area of the GIT through which it is absorbed. Previous studies of the effect of humic substances on the absorption of several vitamins and minerals indicated that there may be a beneficial effect on the GIT absorption of several of the more polar and water-soluble vitamins of the B vitamins (Willis, 2015).

The different water-soluble B vitamins have relatively smaller molecular sizes, different polarities and can easily associate with the larger humic substances, which may influence the GIT absorption of the B vitamins if administered in combination with humic or fulvic acids, either favourably or by reducing its absorption.

Vitamin B_1:

Vitamin B_1 is a highly water-soluble essential vitamin that functions as a co-enzyme in metabolic pathways. Thiamine absorption is dependent on a number of factors

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including: activity and number of specific transmembrane transporters available, metabolic energy, temperature and pH.

The GIT uptake of thiamine is Na^+-independent and is driven by an outward proton gradient. Thiamine uptake is competitively inhibited by cation transporter inhibitors.

Thiamine transport is reported to be absorbed fastest in the proximal and distal regions of the small intestine by a selective transmembrane carrier-mediated process, or an ATP- independent uptake occurring at the intestinal brush border through membrane vesicles (Dudeja *et al.*, 2001).

Absorption of thiamine is favoured in the duodenum and proximal jejunum. While diffusion dominates at higher concentrations (Rindi *et al.*, 2000) this absorption is concentration dependent, with the process being saturable at higher concentrations where this saturation of the vitamin absorption appears to be pH dependent. The active transport based absorption of thiamine requires ATP and the sodium, potassium and Mg-ATPase trans-membrane transporters in the intestinal villi to promote absorption at low thiamine concentrations (Szymański, 1988). Active transport of this vitamin is mediated by thiamine transporters-1 (hTHTR-1) and -2 (hTHTR-2) (Pacei *et al.*, 2020) as well as other carrier proteins; such as the solute-carrier anion transporters, alkaline phosphatase transport systems and human extraneuronal monoamine transporters (Manzetti *et al.*, 2014).

Thiamine uptake is competitively inhibited in the duodenum by the thiamine analogue, pyrithiamine, (Laforenza *et al.*, 1997).

Using the ANOVA statistical test, the results indicated that there was no significant difference found in the absorption of thiamine when used in combination with either humic acid or fulvic acid in the duodenum segment.

The jejunal segment 1, immediately following the duodenum, does not show significant changes in thiamine absorption in the presence of either humic or fulvic acid when compared to the control. The jejunum is exposed to higher flow and higher concentrations of nutrients compared to the ileum (Spiller *et al.*, 1987). The binding of different molecules within humic and fulvic acids are environment dependant, causing

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them to change macro-structure and interact with other molecules more or less readily. The complexing ability of both humic and fulvic acids allows them to associate strongly with thiamine and affect its absorption along the jejunum segment.

The 2nd jejunal segment showed increased absorption of thiamine in the presence of fulvic acid (P=0.0284). The presence of humic acid showed no significant change in the absorption patterns of thiamine.

Neither of the 3rd and 4th jejunum segments showed any significant differences in absorption in combination with either humic acid or fulvic compared to the control.

The ileum segment, after analysis with Kruskal-Wallis test, did show a significant increase in absorption in the presence of fulvic acid compared to the control solution (P=0.0438). Humic acid did not significantly affect the absorption of thiamine in the ileum. Kerckring's folds enlarge the surface and are more separated in the ileum (Keuchel *et al.*, 2013). The larger surface area could account for increased absorption of the vitamin.

Vitamin B_2:

Vitamin B_2 functions in the form of the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) co-enzymes involved in various oxido-reductive reactions required for cellular function and cell growth.

Specific enzymes located in the brush border cells hydrolyse flavin co-enzymes to free riboflavin. Riboflavin absorption from the GIT is regulated by sodium-independent solute-specific carrier mediated mechanisms (Said, 2011). The most important intestinal riboflavin transporters are RFVT1, RFVT2 and RFVT3 (Kiela and Ghishan, 2016). The pH of the intestine appears to affect the absorption rate of the vitamin and when present in higher concentrations, the vitamin appears use passive diffusion as the transport mechanism.

The presence of fulvic acid did not significantly affect the uptake of riboflavin by the duodenum, although the trend did indicate reduced absorption when combined with humic acid (P=0.0375). The transporter RFVT3 is more active than RFVT1 and RFVT2,

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but is most active at acidic pH's. This could account for reduced absorption of riboflavin in the duodenum.

The 1st jejunal segment showed significant changes in riboflavin absorption patterns for humic acid, with a P-value of 0.0160, but did not show any significant changes in absorption with fulvic acid.

The 2nd jejunal segment showed significant changes in absorption patterns for humic acid. Humic acid decreases the absorption of riboflavin. The P-value calculated for the with Kruskal-Wallis test was 0.162. Fulvic acid does not affect the absorption of riboflavin in the 2nd jejunal segment.

The 3rd jejunal segment did not show any significant changes in absorption patterns for both humic and fulvic acids.

Humic acid is less soluble in the Krebs Ringer buffer compared to fulvic acid. A characteristic black precipitate was seen on the GIT tissue as well as in the bottom of the tubes after the incubation period and this was washed off from the everted sacks before harvesting the sack contents. This suggests that the absorption of the vitamin is reduced due to possible binding with the humic acid that is forming this observed precipitate. It has also been seen in previous experiments that the presence of calcium appears to promote humic acid flocculation and precipitation (Yu *et al.,* 2003).

The 4th jejunal segment did not show any significant changes in absorption patterns with both humic and fulvic acid, in comparison to the control.

The ileum did not show any significant changes in absorption patterns with either humic or fulvic acid, in comparison to the control. The lack of significant changes in both the terminal jejunum and ileum may be explained by the presence of fewer transmembrane transporters in this region of the GIT (Drozdzik *et al.,* 2014). The pH of the jejunum to the ileum varies between 7 and 9, and the higher pH could influence the absorption rate of riboflavin, as the transporters are reported to function better under acidic conditions.

Vitamin B_3:

Vitamin B_3 has functions in the digestive, skin and nervous systems. Vitamin B_3 is very water soluble and it appears that the favoured mechanism of transport would either be

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active transport or through the paracellular route. It appears that a concentration gradient that is formed across the brush boarder membrane facilitates the absorption of the vitamin in its active form.

Humic acid does not affect the absorption of nicotinamide in the duodenum, while fulvic acid increased the absorption of nicotinamide The presence of fulvic acid significantly affected the uptake of nicotinamide in the duodenum. The Kruskal-Wallis test indicated a P-value of 0.0227. At lower concentrations, nicotinamide is absorbed by carrier-mediated facilitated diffusion, and at higher concentrations, passive diffusion is reported to occur but because of the ionic status of the compound this is unlikely and paracellular transport would appear to be a logical alternative route (Sadoogh-Abasian *et al.,* 1980).

Segments of both jejunum 1 and jejunum 2 showed that the uptake of nicotinamide in the presence of fulvic acid were significantly affected. ANOVA analysis reported a P-value of 0.0006 for the jejunum 1 and 0.0012 for jejunum 2 respectively. The absorption of nicotinamide in the 1st and 2nd jejunum segments were unaffected by the presence of humic acid.

Both jejunum 3 and jejunum 4 segments showed that the presence of humic or fulvic acid did not significantly affected the absorbance of nicotinamide.

The nicotinamide absorption in the ileum showed significant changes in the presence of fulvic acid. The Kruskal-Wallis test reported a P-value of 0.0149. Humic acid does not affect the absorption of nicotinamide in the ileum compared to the control. The larger surface area in the ileum probably favours absorption over other sections in the gastro-intestinal tract.

Vitamin B_5 (pantothenic acid):

The synthesis and metabolism of proteins, carbohydrates and fats are dependent on Vitamin B_5. It is also important in the synthesis of co-enzyme A, essential for fatty acid synthesis and catabolism for energy production.

The pH of the intestine affects absorption of this vitamin. The presence of chyme in the intestine, presents a more alkaline environment and this causes the pantothenate ionic form of the vitamin to predominate. Enzymes such as 3- ketoacyl synthase present in

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the intestinal mucosa catalyse reactions to release the active form of the vitamin in the lumen for absorption. Absorption of the vitamin takes place mostly in the jejunum. The rate of absorption is not affected by the region of the intestine. Potential difference across the brush boarder cells and the Na^+ gradient using carrier mediated transport is reported to be the mechanism of absorption used by Vitamin B_5.

The presence of both humic or fulvic acid did not significantly affect the uptake of pantothenic acid in the duodenum. A sodium-dependent multivitamin transporter is responsible for the uptake of pantothenic acid in the small intestine. This transmembrane transporter is also involved in biotin and lipoate absorption (Said, 2011).

Jejunum segments 1, 2 and 3 showed that the uptake of pantothenic acid in the presence of humic or fulvic acid were not significantly affected.

Pantothenic acid absorption in the 4^{th} jejunum segment was not affected in the presence of humic acid. There was a significant change in absorption with fulvic acid in the presence of pantothenic acid, with a P-value of 0.0011.

The terminal end of the jejunum, the 4^{th} segment, did however show a significant change in absorption of pantothenic acid when used in combination with humic or fulvic acid. Both humic acid and fulvic acid reduced the absorption of pantothenic acid in the terminal jejunum segment. Analysis with the Kruskal-Wallis test showed a P-value of 0.0023.

The ileum showed no significant change in absorption of pantothenic acid when used in combination with humic and fulvic acid.

There was a general trend that the absorption of pantothenic acid was slightly increased in all GIT segments in combination with humic acid while fulvic acid combinations appeared to decrease the absorbance slightly for all the segments, although both these changes in absorbance were insignificant. Fulvic acid decreased the absorption of pantothenic acid in the ileum to the greatest extent. A sodium-dependent carrier mediated uptake mechanism is responsible for the absorption of pantothenic acid. Pantothenic acid occurs mostly in the form of Coenzyme A and must be hydrolysed first

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before being absorbed. Humic acid, with its larger molecular weight than fulvic acid, may bind to the hydrolysed form more easily and promote absorption in this way.

Vitamin B_6:

Amino acid, glucose and lipid metabolism require the Vitamin B_6 co-enzyme for essential physiological functioning. Vitamin B_6 and protein complexes tend to be dissociated more easily in conditions of low pH, such as those found in gastric acid. Alkaline phosphatase hydrolyses the vitamers of Vitamin B_6 in the intestinal lumen before absorption takes place. Simple diffusion across the brush boarder appears to be the transport mechanism favouring absorption in the long jejunum. The increase in water absorption causes a higher concentration of the active form of the vitamin, which allows absorption to increase from the intestinal lumen.

The presence of humic acid in the duodenum segment does affect the absorption of pyridoxine. There is a significant difference amongst the means (P= 0.0309).

The presence of fulvic acid in the duodenum segment does not affect the absorption of pyridoxine. There is no significant difference in absorption.

The presence of both humic or fulvic acid significantly affected the uptake of pyridoxine from the duodenum. Analysis with the Kruskal-Wallis test gave a P-value of 0.0439. Fulvic acid showed favourable absorption of pyridoxine. Humic acid decreased absorption of pyridoxine.

The presence of both humic or fulvic acid did not significantly affect the uptake of pyridoxine in the first jejunum segment.

The presence of both humic acid did not significantly affect the uptake of pyridoxine in the second jejunum segment. Fulvic acid shows favourable absorption of pyridoxine in the 2nd jejunal segment, with a P-value of 0.0347.

The presence of both humic or fulvic acid did not significantly affect the uptake of pyridoxine in the third jejunum segment.

The presence of fulvic acid did not affect the absorption of pyridoxine in the 4th jejunum segment. Humic acid showed a significant decrease in absorption of pyridoxine (P=0.0467) in the 4th jejunal segment.

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The presence of humic or fulvic acid did not significantly affect the uptake of pyridoxine in the ileum segments.

There is a trend observed in the different segments of the gastro-intestinal tract where water-soluble B-vitamins used in combination with humic acid tend to reduce absorption of the vitamins. In comparison to controls, fulvic acid has shown an increase in absorption of the vitamin, specifically in the jejunum segments, when used in combination.

Upon collection of samples, a black precipitate was found to be attached to the segments of tissue. Humic acid displays a black colour. Due to the solubility characteristics of humic acid, it may explain the reduction in absorption. It may be that due to the complexing ability of humic acid, it forms an insoluble precipitate after vitamin complexation thereby effectively removing it from the solution and reducing the free unbound concentration that would influence its absorption.

Fulvic acid is more soluble in comparison to humic acid. This makes it easier to be absorbed with the vitamins, causing a possible increase in absorption. The molecular weight of fulvic acids are also smaller compared to humic acid (MacFarlane, 1978). This would explain easier interactions with the vitamins, causing favourable absorption.

Humic acid preparations should not be taken together with vitamin B supplements as they decrease the absorption of vitamin B. There is no beneficial effect. Impaired absorption over time may result in deficiency symptoms such as dry skin, weakness and general fatigue, depression, insomnia, skin rashes, weak immune system, low energy and mood.

Fulvic acid may be beneficial when used in combination with vitamin B supplements, however this should be monitored as increased absorption may also result in toxicity symptoms.

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Chapter 5. Concluding remarks

Illnesses are prevalent in society and many people prefer treatment using complementary medicine. Humic acid and fulvic acid preparations are a popular choice as they are marketed as “natural products”, which are unregulated and untested. Supplements of humic acid or fulvic acid have become a popular option for patients to improve general health. These preparations may sometimes be used in combination with other multivitamins to boost immunity and maintain general health. However, the risk of taking these two concomitantly has never been assessed. It is not known whether these preparations taken together with multivitamins would have either a beneficial or detrimental effect. Changes in absorption patterns of different vitamins contained in the multivitamin may be affected when taken in combination with humic substances.

Due to the popularity and claims of the benefits of humic acid and fulvic acid preparations, patients are more likely to opt for taking these compared to conventional medicine. These preparations are easily available and do not need a prescription.

The structure of humic substances has still not been unequivocally established but are regarded as supramolecular structures that can change their conformation according to pH or the presence of mineral ions. Their ability to form complexes with other compounds makes their structure subject to constant change. The associations between humic substances are easily altered resulting in conformational changes. This may explain why when combined with vitamins, the absorption of these vitamins can be either increased or decreased.

There has been research done regarding the medicinal properties of humic substances. Their claimed applications in treating medical conditions has resulted in marketing of these preparations as an alternative to conventional medicine.

Nutrients are needed for a variety of functions, including maintaining bodily functions and for growth and development. Vitamins are carbon containing catalytic compounds that enable critical energy transferring chemical reactions used for different processes to occur in the body.

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Vitamins are needed in sufficient quantities as prescribed by guidelines based on average needs. If the diet is deficient in certain vitamins, supplements may be needed to be taken. Vitamin deficiencies occur when there is a reduced amount available in the diet, resulting in unwanted conditions that may be detrimental to health.

The purpose of this study was to assess the effects that humic acid and fulvic acid have on the absorption of selected water-soluble B vitamins using the well documented and validated mouse everted gut model.

The vitamins assessed in this study were selected water-soluble vitamins B, which could easily form complexes with both humic and fulvic acids.

Thiamine's bioavailabilty is generally around 3.7% to 5.3%. The molecular mass of thiamine is 265.35 g/mol. Thiamine is an essential nutrient and cannot be manufactured in the body, and therefore must be taken, in sufficient quantities, either through diet or supplements.

The structure of thiamine consists of an aminopyrimidine and thiazolium ring, both of which are linked together by a methylene bridge. Thiamine monohydrate was the salt form used in the tablet for the study. This salt form is more stable compared to thiamine hydrochloride. Thiamine monohydrate is non-hygroscopic, and is less likely to absorb water. Thiamine monohydrate reacts with water to release nitrate. The binding reactions between the thiamine and humic or fulvic acids can have an influence on the absorption capacity of the vitamin.

Sulphites break the methylene bridge, destroying the link between the pyrimidine and thiazolium structure. Humic substances that originate from plant matter are often reported to contain caffeic acid, that is a known antagonist of thiamine. It interacts with thiamine by oxidizing the thiazole ring and hinders absorption of the vitamin.

Phosphatase and pyrophosphatases release thiamine in the upper small intestine. Transport mechanisms used for absorption are both carrier mediated and passive diffusion. Carrier mediated transport occurs at low concentrations, but is a saturable process. The Na^+ dependent ATPase transport system controls the release of the vitamin on the serosal side of the intestine. When higher concentrations of thiamine are

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present, passive diffusion occurs as a mode of transport, as an alternative to the carrier-mediated transport. Active transport of thiamine alone is greater in both the jejunum and ileum. Phosphorylated forms of thiamine are less likely than thiamine monophosphate and free thiamine to cross the cell membrane.

Humic acid preparations seem to negatively affect the absorption of thiamine, and would not be recommended for use in combination with multivitamins. Fulvic acid shows increased absorption in the jejunum and ileum segments, however their benefits regarding concomitant use with multivitamins would be subject to more studies, as it has been proven that the absorption of thiamine itself is concentrated in the jejunum and ileum segments.

Riboflavin's molecular mass is 376.369 g/mol. Riboflavin is absorbed in the proximal small intestine. The main site of absorption is the jejunum. The absorption of riboflavin is dependent on the dephosphorylation of riboflavin-5-phosphate in the GI lumen. Riboflavin absorption occurs through carrier-mediated transport. At higher concentrations, passive diffusion processes occur in the cell. It appears that the maximum absorbable dose of riboflavin is approximately 27 mg, after this dose, minimal further amounts are absorbed or it is excreted. After tissue saturation occurs, the rate of excretion becomes proportional to the level of intake. The biological half-life of riboflavin in 66-84 minutes.

There are benefits to taking a riboflavin/fulvic acid preparation to treat riboflavin associated deficiencies, as the absorption of riboflavin is favoured when used in combination with fulvic acid. Humic acid preparations should not be used together with vitamin supplements containing riboflavin, as the absorption of the vitamin is reduced when in the presence of humic acid.

Nicotinamide's molar mass is 122,12 g/mol and is a highly water-soluble vitamin. The structure consists of a pyridine ring and a primary amide group, allowing the structure to undergo electrophilic substitution reactions. Humic substances are highly chemically reactive compounds that undergo conformational changes in the presence of other substances. The presence of fulvic acids is beneficial in the absorption of nicotinamide and can be used concomitantly in supplements to aid deficiencies such as pellagra.

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However, dosages need to be monitored as excessive nicotinamide absorption may lead to liver problems. Humic acid supplements taken together with nicotinamide would decrease the absorption, and are not recommended.

Vitamin B_5 has a molar mass of 219.237 g/mol. Vitamin B_5 obtained in foods is bound to acyl carrier proteins and needs to be converted to free pantothenic acid to be absorbed. Absorption is mostly by active transport, which is a saturable process. Following excessive levels, after saturation has occurred, some passive transport may aid in vitamin absorption. Fulvic acid preparations should not be taken with Vitamin B_5 supplements as there is a decrease in absorption. Humic acid shows a general decrease in absorption when taken together with vitamin B_5.

Pyridoxine's molar mass is 169.18 g/mol. The main site of absorption of pyridoxine is the jejunum. Passive diffusion is the transport mechanism used for absorption following dephosphorylation of this vitamin. Fulvic acid preparations may be used to increase the levels of absorption of pyridoxine. Fulvic acid aids in the absorption of pyridoxine, and may be used to treat deficiencies such as microcytic anaemia, dermatitis, cheilosis, glossitis, weakened immune function, depression and confusion. It is important to monitor the levels of pyridoxine absorbed when taken with fulvic acid, as excessive dosing may result in toxicity symptoms such as sensory neuropathy and ataxia. Humic acid preparations were shown to decrease the absorption of pyridoxine. (Hemminger *et al.,* 2021).

5.1 Constructive criticism of the study

The everted mouse gut model was used for this study. Although there are correlations between the mouse gut and human intestinal tissue, *ex vivo* models do not fully mimic living systems as there is little to no enzymatic action and the effect of the microbiome is removed. Tissue viability for extended experimental times is a problem that was identified during a previous study especially if the tissue is cooled to 4°C after dissection but before everting. Tissue viability had an influence on the time that could be taken from eversion of the gut segments until the end of the incubation time and this needed to be kept to less than 2 hours while retaining the segments in warm and well wetted conditions. Krebs Ringer buffer was used to provide the required minerals and nutrients

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to maintain tissue viability while maintaining a constant physiologically acceptable pH. One drawback of the study was that constant oxygenation of the samples during the incubation period was not performed due to several logistical factors.

The technique used during the eversion of the gut was altered to speed up the process and provided less tissue trauma than methods used previously where a glass rod was used. Instead of using the glass rod, an 18-gauge Teflon catheter was used through which a silk thread was passed. After the entire small intestine was pushed over the catheter the end was tied off with the silk thread and the gut everted as a whole intestine in one relatively quick action. The eversion was easy if the tissue was completely wetted with KRB and avoided the need to evert each segment separately. Once everted, the different segments were then cut to length, tied off on one end then filled with KBR and tied off as filled sacks within minutes of being dissected from the mice. This prevented the tissue tearing often seen when using the hard and thicker glass rod, allowed quick preparation of the everted GIT sacks, led to differences in starting to finishing the everted sack preparation to less than 20 minutes which led to more reproducible replicate results for GIT segments and comparable results from mice used on different days.

The combination of the adapted tissue preparation technique, the direct sample collection into tubes for further processing, the simplified GIT content work-up using the acetonitrile protein precipitation and the use of the ion-pairing vitamin B assay for the LC-MS/MS resulted in more reproducible results and allowed the complete animal study to be completed in a limited time.

Reliable and reproducible outcomes were achieved with a clear difference between the absorption of especially nicotinamide in the presence of fulvic acid at the low levels tested which would closely relate to the concentrations that would be expected in the GIT after administration of supplementary vitamin B complex tablets.

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References:

Agarwal, S. P., et al. (2008). "Complexation of furosemide with fulvic acid extracted from shilajit: a novel approach." Drug Development and Industrial Pharmacy **34**(5): 506-511.

Alam, M. A., et al. (2012). "Everted gut sac model as a tool in pharmaceutical research: limitations and applications." Journal of Pharmacy and Pharmacology **64**(3): 326-336.

Arancon, N. Q., et al. (2006). "Effects of humic acids from vermicomposts on plant growth." European Journal of Soil Biology **42**: S65-S69.

Awortwe, C., Fasinu PS, Rosenkranz B. (2014). "Application of Caco-2 cell line in herb-drug interaction studies: current approaches and challenges". Journal of Pharmacy & Pharmaceutical Sciences **17**(1): 1-19.

Aydin, A., et al. (2016). "A Guidance Manual for the Toxicity Assessment of Traditional Herbal Medicines." Natural Product Communications **11**: 1763-1773.

Ba-Ssalamah, A., et al. (2013). Mesentery, Omentum, Peritoneum: Embryology, Normal Anatomy and Anatomic Variants. Abdominal Imaging. B. Hamm and P. R. Ros. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 1563-1576.

Barthe, L., et al. (1999). "Gastrointestinal absorption of drugs: methods and studies." Fundamental & Clinical Pharmacology **13**(2): 154-168.

Bermejo, M., et al. (2004). "PAMPA—a drug absorption in vitro model: 7. Comparing rat in situ, Caco-2, and PAMPA permeability of fluoroquinolones." European Journal of Pharmaceutical Sciences **21**(4): 429-441.

Bosdriesz, E., et al. (2018). "Low affinity uniporter carrier proteins can increase net substrate uptake rate by reducing efflux." Scientific Reports **8**(1): 5576.

Boyd, K. M. (2000). "Disease, illness, sickness, health, healing and wholeness: exploring some elusive concepts." Medical Humanities **26**(1): 9.

Brown, M. J., et al. (2020). Vitamin B6 Deficiency, StatPearls Publishing, Treasure Island (FL).

Chatterjee, N. S., et al. (1999). "Molecular mechanism of the intestinal biotin transport process." American Journal of Physiology-Cell Physiology **277**(4): C605-C613.

© University of Pretoria

Clarke, L. L. (2009). "A guide to Ussing chamber studies of mouse intestine." American Journal of Physiology-Gastrointestinal and Liver Physiology **296**(6): G1151-1166.

Corti, G., et al. (2006). "Development and evaluation of an in vitro method for prediction of human drug absorption II. Demonstration of the method suitability." European Journal of Pharmaceutical Sciences **27**(4): 354-362.

Dinu, M. I. (2013). "Metals complexation with humic acids in surface water of different natural–climatic zones." E3S Web of Conferences **1**: 32011.

Drozdzik, M., et al. (2014). "Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine." Mol Pharm **11**(10): 3547-3555.

Dudeja, P. K., et al. (2001). "Mechanism of thiamine uptake by human jejunal brush-border membrane vesicles." American Journal of Physiology-Cell Physiology **281**(3): C786-C792.

Eichhorn, T., et al. (2011). "Self-medication with nutritional supplements and herbal over-thecounter products." Natural Products and Bioprospecting **1**(2): 62-70.

Eshwar, M. C., et al. (2017). "Characterization of Humic Substances by Functional Groups and Spectroscopic Methods." International Journal of Current Microbiology and Applied Sciences **6**: 1768-1774.

Eyheraguibel, B., et al. (2008). "Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize." Bioresource Technology **99**(10): 4206-4212.

Fratoni, V. and M. L. Brandi (2015). "B vitamins, homocysteine and bone health." Nutrients **7**(4): 2176-2192.

Gasperi, V., et al. (2019). "Niacin in the Central Nervous System: An Update of Biological Aspects and Clinical Applications." International Journal of Molecular Sciences **20**(4).

Genser, D. (2008). "Food and drug interaction: consequences for the nutrition/health status." Annals of Nutrition and Metabolism **52 Suppl 1**: 29-32.

Gordon, B. L., et al. (2020). "Vitamin C Deficiency: An Under-Recognized Condition in Crohn's Disease." ACG Case Reports Journal **7**(7): e00424-e00424.

Habibe, M. N. and J. Z. Kellar (2021). Niacin Toxicity. StatPearls. Treasure Island (FL), StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.

© University of Pretoria

Haetzman, M., et al. (2003). "Chronic pain and the use of conventional and alternative therapy." Family Practice **20**(2): 147-154.

Hambidge, K. M. (2010). "Micronutrient bioavailability: Dietary Reference Intakes and a future perspective." American Journal of Clinical Nutrition **91**(5): 1430s-1432s.

Hamilton, K. L. and A. G. Butt (2013). "Glucose transport into everted sacs of the small intestine of mice." Advances in Physiology Education **37**(4): 415-426.

Hayes, D. P. (2008). "Adverse effects of nutritional inadequacy and excess: a hormetic model." The American Journal of Clinical Nutrition **88**(2): 578S-581S.

Heaney, R. P. (2001). "Factors influencing the measurement of bioavailability, taking calcium as a model." The Journal of Nutrition **131**(4 Suppl): 1344s-1348s.

Hemminger, A. and B. K. Wills (2021). Vitamin B6 Toxicity. StatPearls. Treasure Island (FL), StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.

Heyman, M., et al. (1990). "Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2." Journal of Cellular Physiology **143**(2): 391-395.

Hooton, D., et al. (2015). "The Secretion and Action of Brush Border Enzymes in the Mammalian Small Intestine." Rev Physiol Biochem Pharmacol **168**: 59-118.

Hou, Z. and L. H. Matherly (2014). "Biology of the major facilitative folate transporters SLC19A1 and SLC46A1." Current Topics in Membranes **73**: 175-204.

Houston, J. B., et al. (1972). "The absorption of aliphatic carbamates by everted gut sacs of rat." Biochemical Journal **128**(4): 141P-142P.

Hsiao, A. F., et al. (2006). "Variations in provider conceptions of integrative medicine." Soc Sci Med **62**(12): 2973-2987.

Hsu, C.-Y., et al. (2019). "Use of Lipid Nanocarriers to Improve Oral Delivery of Vitamins." Nutrients **11**(1): 68.

Izzo, A. A. and E. Ernst (2001). "Interactions between herbal medicines and prescribed drugs: a systematic review." Drugs **61**(15): 2163-2175.

Jones, K. and W. Huang (2003). "Evaluation of toxicity of the pesticides, chlorpyrifos and arsenic, in the presence of compost humic substances in aqueous systems." Journal of Hazardous Materials **103**: 93-105.

Kansy, M., et al. (2004). "Advances in screening for membrane permeability: high-resolution PAMPA for medicinal chemists." Drug Discovery Today. Technologies **1 4**: 349-355.

© University of Pretoria

Keuchel, M., et al. (2013). "Normal Small Bowel." Video Journal and Encyclopedia of GI Endoscopy **1**(1): 261-263.

Khan, K. M. and I. Jialal (2021). Folic Acid Deficiency. StatPearls. Treasure Island (FL), StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.

Kiela, P. R. and F. K. Ghishan (2016). "Physiology of Intestinal Absorption and Secretion." Best Practice & Research: Clinical Gastroenterology **30**(2): 145-159.

Kumada, K. (1965). "Studies on the colour of humic acids." Soil Science and Plant Nutrition **11**(4): 11-16.

Laforenza, U., et al. (1997). "Thiamine uptake in human intestinal biopsy specimens, including observations from a patient with acute thiamine deficiency." American Journal of Clinical Nutrition **66**(2): 320-326.

Liang, Y., et al. (2015). "The physiological role of drug transporters." Protein & Cell **6**(5): 334-350.

Liu, S., et al. (2020). "Production of riboflavin and related cofactors by biotechnological processes." Microbial Cell Factories **19**(1): 31.

Lonsdale, D. (2006). "A review of the biochemistry, metabolism and clinical benefits of thiamin(e) and its derivatives." Evidence Based Complementary and Alternative Medicine **3**(1): 49-59.

MacDonald, L., et al. (2002). "Decreased expression of the vitamin C transporter SVCT1 by ascorbic acid in a human intestinal epithelial cell line." British Journal of Nutrition **87**(2): 97-100.

MacFarlane, R. B. (1978). "Molecular weight distribution of humic and fulvic acids of sediments from a north Florida estuary." Geochimica et Cosmochimica Acta **42**(10): 1579-1582.

Mahabadi, N., et al. (2021). Riboflavin Deficiency. StatPearls. Treasure Island (FL), StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.

Manzetti, S., et al. (2014). "Thiamin function, metabolism, uptake, and transport." Biochemistry **53**(5): 821-835.

Mayr, J. A., et al. (2011). "Thiamine pyrophosphokinase deficiency in encephalopathic children with defects in the pyruvate oxidation pathway." American Journal of Human Genetics **89**(6): 806-812.

© University of Pretoria

Melse-Boonstra, A. (2020). "Bioavailability of Micronutrients From Nutrient-Dense Whole Foods: Zooming in on Dairy, Vegetables, and Fruits." Frontiers in Nutrition **7**: 101-101.

Meunier, V., et al. (1995). "The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications." Cell Biology and Toxicology **11**(3-4): 187-194.

Milman, N. (2012). "Intestinal absorption of folic acid - new physiologic & molecular aspects." The Indian Journal of Medical Research **136**(5): 725-728.

Mirza, M. A., et al. (2011). "Comparative evaluation of humic substances in oral drug delivery." Results in Pharmaceutical Sciences **1**(1): 16-26.

Moran, N. E., et al. (2018). "Intrinsic and Extrinsic Factors Impacting Absorption, Metabolism, and Health Effects of Dietary Carotenoids." Advances in Nutrition (Bethesda, Md.) **9**(4): 465-492.

Morowitz, M. J., et al. (2011). "Contributions of intestinal bacteria to nutrition and metabolism in the critically ill." The Surgical Clinics of North America **91**(4): 771-viii.

Muir, E. M. and D. E. Bowyer (1984). "Inhibition of pinocytosis and induction of release of lysosomal contents by lysosomal overload of arterial smooth muscle cells in vitro." Atherosclerosis **50**(1): 85-92.

Murrow, E. J. and F. M. Oglesby (1996). "Acute and chronic illness: similarities, differences and challenges." Orthopedic Nursing **15**(5): 47-51.

Neergheen-Bhujun, V. S. (2013). "Underestimating the Toxicological Challenges Associated with the Use of Herbal Medicinal Products in Developing Countries." BioMed Research International **2013**: 804086.

Pacei, F., et al. (2020). "The Relevance of Thiamine Evaluation in a Practical Setting." Nutrients **12**(9).

Pandey, A., et al. (2000). "Stability Constants of Metal–Humic Acid Complexes and Its Role in Environmental Detoxification." Ecotoxicology and Environmental Safety **47**: 195-200.

Patel, K. R. and A. Sobczyńska-Malefora (2017). "The adverse effects of an excessive folic acid intake." European Journal of Clinical Nutrition **71**(2): 159-163.

Pelaseyed, T., et al. (2014). "The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system." Immunological Reviews **260**(1): 8-20.

© University of Pretoria

Peña-Méndez, E., et al. (2004). "Humic substances-compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine." Journal of Applied Biomedicine **3**.

Peña-Méndez, E. M., et al. (2007). "Characterization of humic substances of different origin by means of mass spectrometry and neural networks." Chemosphere **68**(11): 2047-2053.

Piccolo, A. (2001). "The supramolecular structure of humic substances." Soil Science **v. 166**(no. 11): pp. 810-832-2001 v.2166 no.2011.

Pierzynowska, K., et al. (2020). "Maternal Immunoglobulins in Infants—Are They More Than Just a Form of Passive Immunity?" Frontiers in Immunology **11**(855).

Redzic, S. and V. Gupta (2020). Niacin Deficiency, StatPearls Publishing, Treasure Island (FL).

Renton, K. W. (1986). "Factors affecting drug biotransformation." Clinical Biochemistry **19**(2): 72-75.

Rindi, G. and U. Laforenza (2000). "Thiamine intestinal transport and related issues: recent aspects." Proceedings of the Society for Experimental Biology and Medicine **224**(4): 246-255.

Ross, N. S. and T. P. Hansen (1992). "Riboflavin deficiency is associated with selective preservation of critical flavoenzyme-dependent metabolic pathways." Biofactors **3**(3): 185-190.

Rubas, W., et al. (1996). "Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue." Journal of Pharmaceutical Sciences **85**(2): 165-169.

Saad, B., et al. (2006). "Safety of traditional arab herbal medicine." Evidence Based Complementary and Alternative Medicine **3**(4): 433-439.

Sadoogh-Abasian, F. and D. F. Evered (1980). "Absorption of nicotinic acid and nicotinamide from rat small intestine in vitro." Biochimica et Biophysica Acta **598**(2): 385-391.

Said, H. M. (2011). "Intestinal absorption of water-soluble vitamins in health and disease." The Biochemical Journal **437**(3): 357-372.

Sanvictores, T. and S. Chauhan (2021). Vitamin B5 (Pantothenic Acid). StatPearls. Treasure Island (FL), StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.

© University of Pretoria

Savvidou, S. (2014). "Pellagra: a non-eradicated old disease." Clinics and Practice **4**(1): 637-637.

Schepetkin, I., et al. (2002). "Medical drugs from humus matter: Focus on mumie." Drug Development Research **57**: 140-159.

Schoultz, I. and Å. V. Keita (2020). "The Intestinal Barrier and Current Techniques for the Assessment of Gut Permeability." Cells **9**(8): 1909.

Sharwan, G., et al. (2015). "Toxicity profile of traditional herbal medicine." Journal of Ayurvedic and Herbal Medicine **1**: 81-90.

Sheraz, M. A., et al. (2014). "Photo, thermal and chemical degradation of riboflavin." Beilstein Journal of Organic Chemistry **10**: 1999-2012.

Shipton, M. J. and J. Thachil (2015). "Vitamin B12 deficiency - A 21st century perspective " Clinical Medicine (London, England) **15**(2): 145-150.

Shugarts, S. and L. Z. Benet (2009). "The role of transporters in the pharmacokinetics of orally administered drugs." Pharmaceutical Research **26**(9): 2039-2054.

Smithline, H. A., et al. (2012) Pharmacokinetics of high-dose oral thiamine hydrochloride in healthy subjects. BMC Clinical Ppharmacology **12**, 4

Spiller, R. C., et al. (1987). "Jejunal water and electrolyte absorption from two proprietary enteral feeds in man: importance of sodium content." Gut **28**(6): 681-687.

Steffansen, B., et al. (2004). "Intestinal solute carriers: an overview of trends and strategies for improving oral drug absorption." European Journal of Pharmaceutical Sciences **21**(1): 3-16.

Stenberg, P., et al. (1999). "Prediction of Membrane Permeability to Peptides from Calculated Dynamic Molecular Surface Properties." Pharmaceutical Research **16**(2): 205-212.

Stevenson, F.J. (1982). Humus Chemistry: Genesis, Composition, Reactions (1st ed). New York: John Wiley and Sons, Ltd.

Stillwell, W. (2016). "Membrane Transport." An Introduction to Biological Membranes: 423-451.

Sundaram, U. (2000). "Regulation of intestinal vitamin B2 absorption Focus on “Riboflavin uptake by human-derived colonic epithelial NCM460 cells”." American Journal of Physiology-Cell Physiology **278**(2): C268-C269.

© University of Pretoria

Suzuki, H. and Y. Sugiyama (2000). "Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine." European Journal of Pharmaceutical Sciences **12**(1): 3-12.

Swed, A., et al. (2009). "The role of P-glycoprotein in intestinal transport versus the BBB transport of tetraphenylphosphonium." Molecular Pharmacology **6**(6): 1883-1890.

Szymański, A. (1988). "New aspects in the study of the mechanism of intestinal absorption of thiamine in rats." Acta Physiology Poland **39**(5-6): 450-459.

Terashima, M., et al. (2004). "Influence of pH on the surface activity of humic acid: Micelle-like aggregate formation and interfacial adsorption." Colloids and Surfaces A-physicochemical and Engineering Aspects - Colloid Surface A **247**: 77-83.

Thompson, G. R. (1971). "Absorption of fat-soluble vitamins and sterols." Journal of Clinical Pathology. Supplement (Royal College of Pathologists). **5**: 85-89.

Thomson, A., et al. (2019). "The Ussing chamber system for measuring intestinal permeability in health and disease." BMC Gastroenterology **19**(1): 98.

Upadhyay, R. K. (2014). "Transendothelial Transport and Its Role in Therapeutics." International scholarly research notices **2014**: 309404-309404.

Vertzoni, M., et al. (2019). "Impact of regional differences along the gastrointestinal tract of healthy adults on oral drug absorption: An UNGAP review." European Journal of Pharmaceutical Sciences **134**: 153-175.

Wiley, K. D. and M. Gupta (2021). Vitamin B1 Thiamine Deficiency. StatPearls. Treasure Island (FL), StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.

Willis, K. (2015). An investigation of the effects of fulvic and humic acids on the absorption of selected drugs, vitamins and minerals using the everted mouse gut model. MSc dissertation. Available on: https://repository.up.ac.za/handle/2263/46044

Wilson, J. P. (1967). "Surface area of the small intestine in man." Gut **8**(6): 618-621.

Wilson, T. H. and G. Wiseman (1954). "The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface." The Journal of Physiology **123**(1): 116-125.

Yang, F., et al. (2017). *Radix Bupleuri*: A Review of Traditional Uses, Botany, Phytochemistry, Pharmacology, and Toxicology." BioMed Research International **2017**: 7597596.

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Yoshii, K., et al. (2019). "Metabolism of Dietary and Microbial Vitamin B Family in the Regulation of Host Immunity." Frontiers in Nutrition **6**: 48.

Yu, J., et al. (2003). "Characteristics of coagulation-flocculation of humic acid with effective performance of polymeric flocculant and inorganic coagulant." Water Science and Technology **47**(1): 89-95.

Zhang, J., et al. (2015). "The Safety of Herbal Medicine: From Prejudice to Evidence." Evidence-Based Complementary and Alternative Medicine **2015**: 316706.

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Appendix 1: Ethics approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**The influence of humic and fulvic acids on the absorption of vitamins and minerals in the everted mouse gut model**
PROJECT NUMBER	**H011-16 (Amend 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**N Chetty**

STUDENT NUMBER (where applicable)	**U_2514 6298**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Mice**	
NUMBER OF ANIMALS	**6**	
Approval period to use animals for research/testing purposes		**May 2017- May 2018**
SUPERVISOR	**Prof. D Cromarty**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 29 May 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

23 June 2021

Acknowledgement Certificate
Research Completed

Dear Miss N Chetty

Ethics Reference No.: 86/2020
Title: The influence of humic and fulvic acids on the absorption of selected vitamins in the everted gut model

The **Research Completed Report** as supported by documents received between 2021-05-11 and 2021-06-17 for your research, was acknowledged by the Faculty of Health Sciences Research Ethics Committee on 2021-06-17 as resolved by its quorate meeting.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

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Appendix 2: Letters from Animal Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

12 February 2020

TO WHOM IT MAY CONCERN

This note serves to confirm that Dr Richard Mavunganidze will be the attending veterinarian for the project; The influence of humic and fulvic acids on the absorption of selected vitamins in the everted mouse gut model, which will be conducted at the OVARU (Onderstepoort Veterinary Animal Research Unit).

Kind Regards

Richard Mavunganidze
Attending Veterinarian
012 529 8173

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

11.2.2020

TO WHOM IT MAY CONCERN

This letter confirms that Ilse Janse van Rensburg and M Chovheya will be the technologist/vet nurse performing technical procedures on project: The influence of humic and fulvic acids on the absorption of selected vitamins in the everted mouse gut model.

I Janse van Rensburg
Control Veterinary Technologist
012 529 8101

M Chovheya
Veterinary Nurse
012 529 8582

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

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YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

11.2.2020

TO WHOM IT MAY CONCERN

This letter confirms that project: The influence of humic and fulvic acids on the absorption of selected vitamins in the everted mouse gut model, will be conducted at the OVARU (Onderstepoort Veterinary Animal Research Unit).

I Janse van Rensburg
Control Veterinary Technologist
012 529 8101

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

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Faculty of Veterinary Science
Animal Ethics Committee

9 September 2021

Study completed

Miss N Chetty
Department of Pharmacology
Faculty of Health Science
University of Pretoria

Dear Miss N Chetty,

RE: Acknowledgement Study Completed for AEC

Protocol No	86/2020
Title	The influence of humic and fulvic acids on the absorption of selected vitamins in the everted gut model
Investigator	Miss N Chetty
Supervisor	Prof AD Cromarty
Sponsor	

We hereby acknowledge receipt of the documents received on 2021-06-23 and note the content thereof.

Please note that the AEC may ask further questions and/or seek additional information

Yours sincerely

H. Lutermann

Dr Heike Lutermann

DEPUTY CHAIRMAN: UP-Animal Ethics Committee

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