School of Biosciences

Cobamide extraction procedures for analysis of tissue and microbiome samples

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Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

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1. Abstract

The structure of cobalamin has an upper β- axial ligand group and a lower α-axial ligand base. Different groups can attach to the upper ligand to form different forms of cobalamin. When the lower ligand base is changed, the molecule becomes unusable for humans. These are called the lower ligand analogues and are found most prominently in faecal samples. This study sets out to use a cobamide extraction procedure to identify the types of analogues present in faecal samples. It aims to investigate the effect of supplementation with B_{12} on the cobamides detected in racehorses' microbiome. Additionally, it aims to identify possible relations between treatment responses and cobamides detected in faecal samples of pernicious anaemia patients. Purification of cobamides is done with a His-tagged cobalamin binding protein and identification of them are done by HPLC-MS. The results show that supplementation does not have an effect on racehorses' cobamides detected and patients who take B_{12} sublingual spray or tablets have higher amounts of cyanocobalamin in their microbiome. Lastly, this procedure can successfully be adapted to study cobamides in tissue samples.

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2. Acknowledgements

I would like to first thank Professor Martin Warren who gave me this opportunity to immerse myself into a research lab working on a great project. Secondly, I would like to thank Dr. Evelyne Deery for teaching me everything that is to do with the lab and most importantly to work steady. I thank Dr. Andrew Lawrence for always being there for any questions and his help in using the HPLC-MS.

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3. List of abbreviations

HBAH: Hydrogenobyrinic acid hexamide

HPLC-MS: High performance liquid chromatography- Mass spectrometry

IF: Intrinsic Factor

IMAC: Immobilised metal ion affinity chromatography

PAS: Pernicious Anaemia Society

TFA: Trifluoroacetic acid

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5. Introduction

The basis of this project revolves around identifying the different types of cobamides found in tissue and faecal samples.

5.1 Cobamides

“Cobamide”, is an umbrella term used to describe molecules belonging to the corrinoid family with a cobalt containing centre along with an upper and lower ligand group attached (1). It is synthesised exclusively by microbial synthesis (2). The structure of the most studied cobamide, “cobalamin” was solved by Dorothy Hodgkin in 1955 by X-ray crystallography (3). This water soluble molecule consists of a tetrapyrrole corrin ring comprising of a cobalt ion bound to 4 nitrogen atoms. This molecule is able to have varying upper β-axial ligand groups and varying lower α-axial ligand bases (Figure 1 – lower ligand shown as 5, 6-Dimethylbenzimidazole) (4). Normally referred to as vitamin B_{12}, cobalamins varying in their upper ligand groups can be specifically termed cyanocobalamin (Figure 1), methylcobalamin, adenosylcobalamin, or hydroxocobalamin.

Biologically, vitamin B_{12} is usually found in its adenosylcobalamin or methylcobalamin form (5). Commercially, vitamin B_{12} is sold as cyanocobalamin or methylcobalamin (5). This is because cyanocobalamin and methylcobalamin is more stable than the rest. Cyanocobalamin (Figure 1) is the most stable of the four and is usually found in oral supplements (6, 7).

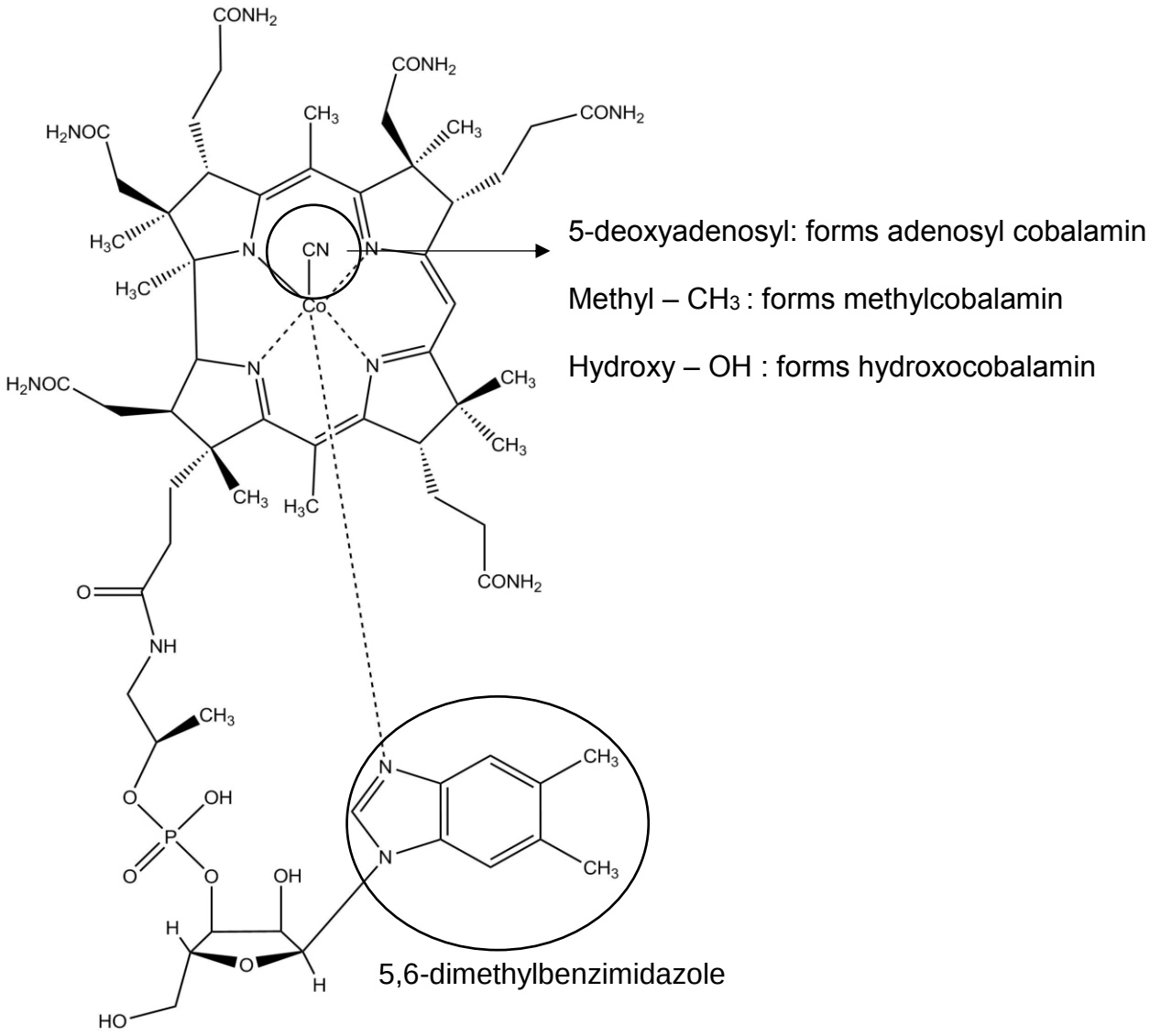


Figure 1: Structure of cyanocobalamin. This structure shows the corrin ring coordinating 5, 6-Dimethylbenzimidazole as its lower axial ligand base and CN in its upper axial ligand. The upper ligand group can have different groups as shown.

5.2 Importance of vitamin B_{12}

Ever since the discovery of vitamin B_{12} as the anti-pernicious factor by Minot and Murphy, much effort have been put into understanding its importance and function (8).

Although cobalamin deficiency is not as common in animals as it is in humans, a low level of cobalamin can still affect cell division, neuropathy, nervous system development, and mood (9–11). Thus, serving an important purpose towards their overall wellbeing. Deficiency in humans can be due to uptake disorders such as

pernicious anaemia (autoimmune disorder that attacks the intrinsic factor) or dietary reasons (12). These vitamin B_{12} deficient patients usually go through treatment regimens such as change in dietary habits, or consumption of supplements. Where pernicious anaemic patients are concern, intramuscular B_{12} injections are administered along with supplements if needed. In humans, vitamin B_{12} is a cofactor for two enzymes: methionine synthase and methyl malonyl coenzyme A mutase (13). Methionine synthase catalyses the methylation of homocysteine to methionine and mutations in this gene are associated with methylcobalamin deficiency. When vitamin B_{12} deficiency happens, the activity of this enzyme is decreased which decreases other connected folate enzymes resulting in defective DNA synthesis in cells and anaemia (14). Methyl malonyl coenzyme A mutase on the other hand catalyses the conversion of methylmalonyl-CoA to succinyl-CoA using adenosylcobalamin as a cofactor (15). This enzyme is found in high levels in crucial organs such as the kidney, heart, brain, and liver. Mutations in the gene encoding for this enzyme (*mut* gene) causes methylmalonic acidemia and is observed to also appear in children with inability to metabolise vitamin B_{12} .The deficiency or defect in this enzyme will lead to a build-up of the substrate L-methylmalonyl-CoA. This substrate will form methylmalonic acid which is toxic to the brain (16).

5.3 Cobalamin and the gut

Absorption of vitamin B_{12}

Since there are only two B_{12} dependent enzymes in humans, this means that vitamin B_{12} is only required in small quantifies per day. This is consumed through our diet as only microorganisms possess the ability to synthesize cobalamins (2). Therefore, the consumption and metabolism of cobalamins through our digestive system is crucial. Firstly, cobalamin from food sources will bind to haptocorrin. This forms the cobalamin-

haptocorrin complex (Figure 2). This complex prevents the breakdown of cobalamin due to the acidic environment in the stomach (17). As it travels towards the intestines, pancreatic proteases release the cobalamin from that complex by digesting haptocorrin. The released cobalamin then binds to intrinsic factor (IF) in the small intestine. This intrinsic factor-cobalamin complex is recognised by the receptors on the enterocytes at the terminal ileum. Once absorbed by the enterocytes, intrinsic factor is degraded and cobalamin is free to enter the blood stream. In the bloodstream, cobalamin binds to transcobalamin II. When needed, lysosomes from cells will degrade transcobalamin II and releases the cobalamin to be used (18). Vitamin B_{12} is mainly stored in hepatocytes where it usually take years to deplete this reservoir in a healthy human being (11).

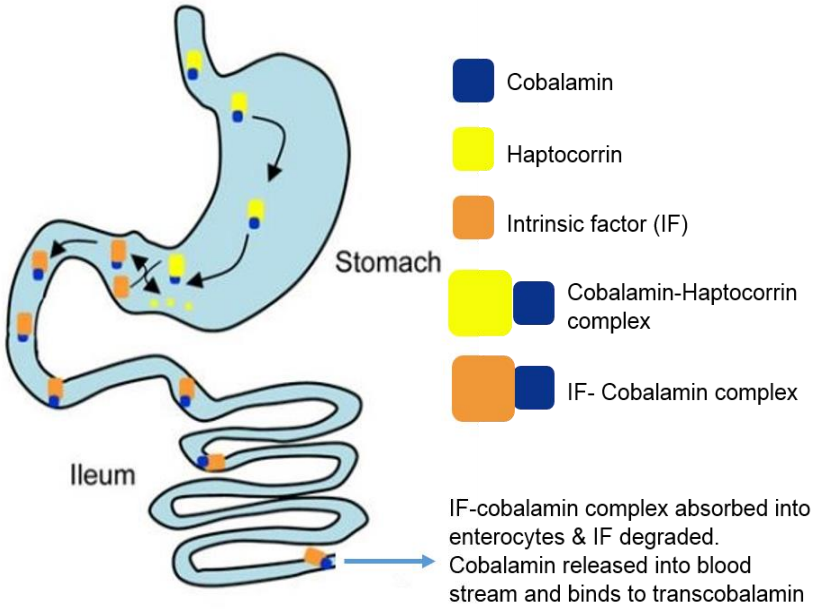


Figure 2: Absorption of cobalamin in the human digestive system. Cobalamin from food sources is first bounded to Haptocorrin which protects cobalamin from breaking down. Cobalamin then binds to IF at the small intestine for recognition by receptors at the enterocytes. Degradation of IF release cobalamin to the systemic circulation where it binds to transcobalamin. Figure adapted from study by Kozyraki and Cases, 2013 (18).

Cobalamins and the gut microbiome

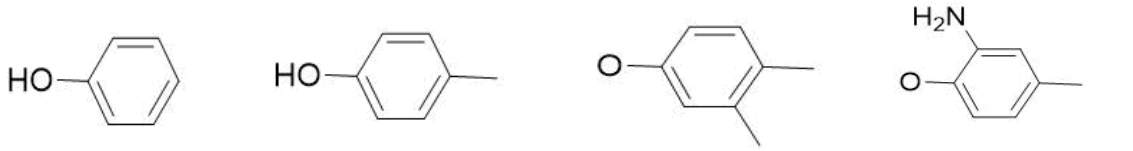
The gut is home to trillions of microbes dominated by Bacteroidetes and Firmicutes (19). The microbiome of the gut plays an important role in producing various metabolites used by the host which influences the host's health (20). The microbiota is not only important for vitamin production but they impact nutrient digestion and absorption, the immune system, normal development, and behaviour of the host (9, 20–23). In terms of vitamin B_{12} for humans, it is important to consume enough vitamin B_{12} in through their diet because the microbiome competes with the human host for cobalamins (24). This is unlike animals such as horses where their gut microbiome in the hindgut is important in producing and supplying enough vitamin B_{12} (25, 26).

Interestingly, even when enough vitamin B_{12} is consumed or supplemented, some vitamin B_{12} deficient people still lack cobalamin in their system. This could be due to a lack of intrinsic factor in combination with the competition of cobalamins from the gut bacteria (23, 27). With regards to this, establishing the relationship cobalamins and the gut microbiome. It has been shown that the composition of gut bacteria is affected by competition and exchange of cobalamins within the environment (23, 28). The bacteria requiring cobamides outweighs the biosynthesis in the human gut which indicates the need for many bacteria to take up cobamides from their environment and modify it for usage. This produces cobalamin analogues where the lower ligand base of the cobalamin structure is switched to other groups (Figure 1) (23, 29, 30).

Although there is a difference between the gut microbiome of humans and animals, the exchange of lower ligand bases done by the bacteria can still be observed (25, 31, 32).

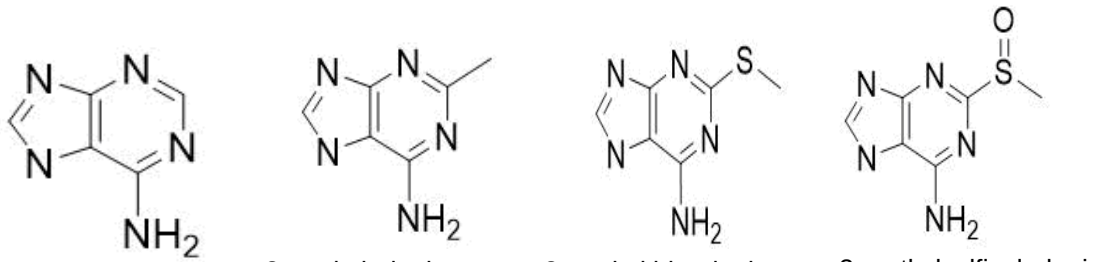
5.4 Lower ligand analogues

Lower ligand analogues are cobalamin molecules only differing in their lower ligand base (Figure 1). The lower base shown in Figure 1 (5,6-dimethylbenzimidazole) is the useful base that allows absorption into the human body to be used by cells. Once the base changes from 5,6-dimethylbenzimidazole, it is no longer useful to humans. The affinity of haptocorrin, intrinsic factor, and transcobalamin towards specific cobalamins help humans filter out unwanted cobalamins with other lower bases which would not be useful to humans (18). Although these are in place to filter non-useful analogues, some are still able to bypass this due to their similarity in structure (33–35). This would inhibit efficient uptake of useful cobalamin. There are a variety of cobalamin lower ligand analogues discovered to date which can be groups into three classes and cobinamide (23, 30, 36–38). Cobinamide is the incomplete corrinoid that is missing the lower ligand base. The three classes are the phenols (Figure 3), purines (Figure 4), and benzimidazoles (Figure 5).



Phenol p-cresole 3,4-dimethylphenol 2-amino-p-cresol

Figure 3: Structure of the lower ligand bases belonging to the phenols group



Adenine 2-methyladenine 2-methylthioadenine 2-methylsulfinyladenine

Figure 4: Structures of the lower ligand bases belonging to the purines group

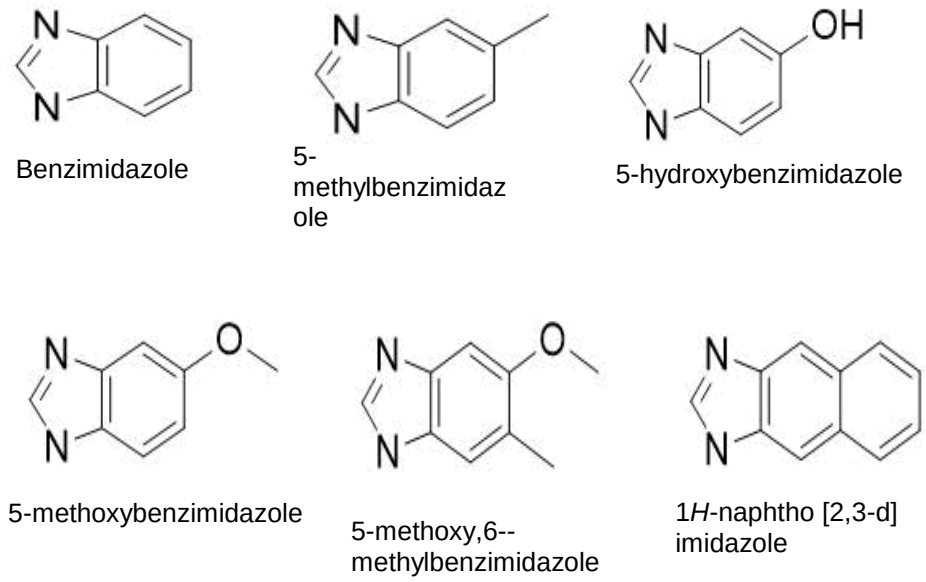


Figure 5: Structure of the lower ligand bases belonging to the benzimidazoles group

Haptocorrin is shown to bind to all cobalamin analogues with high affinity. Binding with haptocorrin delivers these analogues to hepatocytes where the retention of these useless cobamides by the liver could act as a shield to prevent the body from using them (34, 39). These analogues are then excreted in the urine and faeces over time (34, 39). Intrinsic factor binds to cobinamide, purine, and phenol analogues with low affinity while transcobalamin binds to cobinamide and phenol analogues with low affinity (33–35, 39). After entering into the bloodstream, these analogues can either go to the hepatocytes where it is stored, excreted, or transported around the circulatory system where cells can utilize them.

Not much is known about these lower ligand analogues except they are a product of microbial modification. The role of these analogues are not well established but it can be noted that the analogues could have implications in influencing the composition of the microbiome and subsequently the health of the host. This is further illustrated when the abundant presence of cobalamin analogues is observed in faecal samples (30,

40). Faeces contain a large amount of microbes and it has been shown that different cobalamin analogues can alter the population of microbes differently (23, 41, 42).

Humans and microbes both compete for cobalamins (23, 24, 27). Humans only need the 5,6-dimethylbenzimidazole analogue (cobalamin) while different bacteria are able to utilise different analogues for metabolism. This means that bacteria can take up the ‘useful’ cobamide and the product of their metabolism is molecule with a modified the lower ligand base. This competition with the host is amplified if the person has a bacterial overgrowth in the gut (27, 43). This results in even lower levels of useful cobalamin within the blood stream available for the host. Moreover, cobalamins produced by the gut bacteria located in the large intestine are unable to be absorbed as the site of absorption for humans is further upstream. This can be linked towards vitamin B_{12} deficiency where a lack of cobalamin uptake means increased competition for useful cobalamin since majority of the human gut microbiome is shown to require exogenous corrinoids (24).

The competition for cobalamin analogues can also be seen in certain animals. Mainly non-ruminants and animals who are not observed to practice coprophagy (44). The animals that practice coprophagy are capable of obtaining cobalamin produced by their gut microbes by consuming their faecal matter which contains the needed cobalamins. This allows them to move the cobalamins to the upper digestive tract where absorption takes place (32, 44). Ruminants are able to absorb cobalamins produced by the bacteria in the rumen where it is located before the absorption site of cobalamin (31). These can compensate for the competition for cobalamins at the site of absorption between the host and the gut microbes. Thus, deficiency is not as prevalent in animals as in the human population.

Moreover, the distribution of cobalamin analogues in animal tissues are different from one another which reinforces the relevance of these analogues in the wellbeing of an animal (38)(45). In this report, where analogues are mentioned, it refers to the lower ligand analogues.

5.5 Aim

The main aim of this project is to utilise a cobamide extraction procedure developed to identify cobamides in faecal samples. Secondly, the protocol is changed with an aim to adapt it for use on tissue and serum samples. It involves the use of the *E. coli* BtuF protein involved in cobalamin uptake to capture these cobamides (46).

5.5.1 Faecal sample trial extraction analysis

The protocol was first familiarised and tested using rabbit and hedgehog faecal samples as they were readily available. These two animals are different in their habits and thus, the cobamides in their gut should be different and should be shown if the protocol works well (17, 44).

5.5.2 Optimisation of extraction protocol with horse faecal samples

The protocol was then optimised using horse faecal samples as they were available in abundance. The aim here is to improve or maximise the types of cobamides being able to be detected by High performance liquid chromatography- mass spectrometry (HPLC-MS). It will investigate how different mass of faecal samples and changing the time period the faecal samples are incubated with the His-BtuF nickel resin will affect HPLC-MS detection.

5.5.3 The effect of supplementation on cobamide extraction profile of racehorse faecal samples

Once optimised, the protocol is used to investigate the effect of vitamin B_{12} supplementation on the composition and types of lower ligand analogues (cobamides) in racehorses' faecal samples. Because of cobalamin's association with metabolism, it can be investigated for its effect on stamina or performance of racehorses in this case (25, 26). It has been observed that supplementing horses with vitamin B_{12} is crucial in preventing anaemia and increasing appetite which could have ripple effects on its performance (26). This experiment will be analysed in parallel with a related study (supplementary S13) to see if there are any implications towards their performance. Racehorses live a high stress and strict diet life which can have a negative effect on its gut microbiome. This is important because the gut microbiome for horses is crucial in supplying them with enough vitamins including B_{12} which are needed for their wellbeing or performance (25, 26). Thus, if a shift in composition of cobamides is observed after supplementation, it indicates a shift in the microbiome of the horse which could identify potential markers of a racehorses' wellbeing and predicted performance.

5.5.4 Relationship between the cobamide composition in faecal samples and the treatment responses of pernicious anaemia patients

Lastly, this project aims to find if there is a relationship between the cobamide composition of pernicious anaemia patients and their treatment responses from three monthly Vitamin B_{12} intramuscular injections. Two groups of patients who are either happy or unhappy (needs more frequent treatments) with their injections are studied. This is studied because pernicious anaemia patients have varied responses towards their injections and the reason for this is not fully understood. One of it could be due to the difference in gut microbiome which can be reflected through the types of

cobamides present as different bacteria are able to make changes to the lower ligand base of the cobalamin molecule (23, 27, 30). Therefore, it will be of interest to look at their cobamide composition in disease states particularly when different treatment outcomes are concerned.

The data will be analysed along with a corresponding study (supplementary S15) whereby the types of bacteria present in the patients' faecal samples were identified by sequencing. This will reveal any correlation between the types of microbiome found, the cobamides that are present, and their treatment responses. By combining these, we hope to elucidate more clues into why there are different reactions to treatments.

5.5.5 Tissue and serum sample adaptation

The cobamide extraction protocol was altered for use on animal tissue and serum samples. This is done to see if the methodology is capable for tissue or serum cobamide analysis studies.

6. Materials and Methods

Overview:

1) Preparation of His-BtuF (His-tagged cobamide binding protein) nickel resin
2) Production and purification of HBAH molecule (spiked into samples for normalisation of data)
3) Extraction and purification of cobamides from faecal, tissue, or serum samples
4) HPLC-MS detection of cobamides found in samples
5) How HPLC-MS data was analysed

6.1 Preparation of His-BtuF IMAC nickel resin for the capture of cobamides

Expression and purification of E.coli His-BtuF in *E.coli* BL21 star (DE3) pLysS

Plasmid: pET14b-btuF^{his} gift from Dr. Evelyne Deery

Competent cell: *E.coli* BL21 star (DE3)pLysS from Promega (Catalog No: L1191)

Transformation Protocol:

The plasmid pET4b-btuF^{his} was transformed into BL21 star (DE3)pLysS for the production of His-BtuF.

50 µL of *E.coli* BL21 star (DE3)pLysS was mixed with 0.5 µL of pET14b-btuF. The mixture was left on ice for 15 minutes before a 42 ^{o}C heat shock for 1 minute. The mixture was then placed back on ice for 2 minutes before adding 200 µL of SOC medium (Table 1). The mixture was left in 37 ^{o}C water bath for an hour before being plated on LB-agar with 100 µg/mL of ampicillin (Melford, Catalog no: 40801-43025) and 34 µg/mL of chloramphenicol (Melford, Catalog no: 100M0061V). The plate is left to incubate at 37 ^{o}C overnight.

Table 1: Composition of SOC growth medium for per 100 mL:

Component	**Amount**
Tryptone	2 g
Yeast extract	0.5 g
NaCl	0.01 M
KCl	00025 M
Mg^{2+} *	0.02 M
Glucose	0.02 M

Tryptone, yeast extract, NaCl, and KCl were dissolved in distilled water then autoclaved. The other components were then added to make up to 100 mL. *Composition of Mg^{2+} = 2 g of $MgCl_2.6H_2O$ and 2.5 g of $MgSO_4,7H_2O$ to 10 mL of distilled water then filter sterilised

Inoculation protocol for starter culture:

In a sterile environment, a single colony of *E.coli* picked up from the LB-agar plate, it was inoculated into 10 mL of LB-medium containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. This is incubated at 37 ^{o}C with shaking overnight.

Inoculation of starter culture into 1 L flask for large scale growth:

In a sterile environment, 10 mL of starter culture was added to 1 L of LB-medium containing 100 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. This was left to shake at 37 ^{o}C until OD_{600} reaches 0.6 – 1. Then, 400 µM of IPTG (Melford, Catalog no: 40719-41032) was added to the flask and left to incubate at 18 ^{o}C shaking overnight.

Cell lysis:

1 L of growth culture was spun down at 2392 RCF for 20 minutes. The pellet was resuspended in 30 mL of binding buffer containing 20 mM of imidazole, 20 mM of pH 7.5 Hepes, and 500 mM of NaCl. The resuspended pellet was sonicated for 5 minutes with 30 seconds/30 seconds of pulse and 55% amplitude. The lysed cells were then centrifuged for 20 minutes at 26 000 RCF. The supernatant was kept on ice.

Purification of His-BtuF (recombinant protein) using IMAC and making His-BtuF resin.

In an empty column, 10 mL of Chelating Sepharose^{TM} resin (from GE healthcare, Catalog No: 10280810) was added and charged with 20 mL of 0.1 m $NiSO_4$. It was then washed with 60 mL of binding buffer containing 20 mM of imidazole, 20 mM of pH 7.5 hepes, and 500 mM of NaCl. The supernatant containing His-BtuF was loaded. The column was then washed with 50 mL of the same binding buffer. Then it was washed with 30 mL of wash buffer containing 60 mM of imidazole, 20 mM of pH 7.5 hepes, and 500 mM of NaCl. Next, the column was washed with 20 mL of final buffer containing 20 mM of pH 7.5 hepes and 100 mM of NaCl. The resin in the column was mixed evenly before being transferred into a beaker for storage at 4 ^{o}C. The his-tagged protein was not chelated from the nickel column and thus the His-BtuF resin can be used to capture cobamides.

Check the binding of His-BtuF to cyanocobalamin:

The His-BtuF resin was checked for its ability to bind to cobamides by loading 1 mL of 100 µM cyanocobalamin onto 800 µL of the His-BtuF resin on a mini-chromatography column (from Bio-Rad, Catalog no: 732-6207). The mini column was washed with final buffer until drops become clear. The resin should stay pink which indicate binding of

cyanocobalamin to the resin. Then, cyanocobalamin was eluted from the resin by denaturing the His-BtuF protein on the column. It was eluted with 8 M urea buffer solution containing 20 mM of pH 7.5 hepes and 100 mM of NaCl. The denaturation of His-BtuF releases cyanocobalamin (pink elution fractions) while still remaining bound to the column.

6.2 Production of Hydrogenobyrinic acid hexamide (HBAH) for the normalisation of HPLC-MS data

The molecule HBAH is used for normalisation of integrated peak areas of peaks detected in the HPLC-MS (High performance liquid chromatography-Mass spectrometry). The same concentration (7.5 µg/mL) of HBAH is spiked into each sample and the recovery of this molecule represented by its signal peak in the HPLC-MS is used to standardize the data values of cobamides. This serves as an internal standard and enables fairer comparison of integrated peak areas across different batches of samples going through the HPLC-MS.

Strain ED663: *E.coli* BGEC043 with (T7P)-AIG*JFMKLH-AlvBQ-E*- integrated in the fim operon and ΔlacZYAΩ(T7RNAP) ΔbtuF gift from Dr. Evelyne Deery

Inoculation into starter culture:

A single colony of *E.coli* strain ED663 was inoculated into 10 mL of LB-medium containing 0.2% glucose. This culture is incubated at 28 ^{o}C overnight with shaking.

Inoculation of starter culture into 1 L of '2YTNN' medium:

10 mL of starter culture was inoculated into 1 L of 2YTNN medium (Table 2). This was incubated at 28 ^{o}C shaking until OD_{600} is 1 – 1.5. Then, 400 µM of IPTG was added and the culture was left to shake at 28 ^{o}C overnight.

Table 2: Composition of 2YTNN medium per litre of distilled water

Component	Amount
Yeast extract	10 g
Tryptone	16 g
NaCl	5 g
Na_2HPO_4	10.99 g
NaH_2PO_4	2.71 g
NH_4Cl	1 g

Purification of HBAH using His-BtuF resin:

The 1 L culture was centrifuged at 2392 RCF for 20 minutes. The supernatant containing HBAH was kept on ice. His-BtuF resin from 3 L of culture was loaded onto an empty column. The supernatant was loaded onto the column containing His-BtuF resin. The column was then washed with 50 mL of binding buffer containing 20 mM of imidazole, 20 mM of pH 7.5 hepes, and 500 mM of NaCl. Then it was washed with 30 mL of wash buffer containing 60 mM of imidazole, 20 mM of pH 7.5 hepes, and 500 mM of NaCl. Next, the column was washed with 30 mL of final buffer containing 20 mM of pH 7.5 hepes and 100 mM of NaCl. HBAH was eluted with 8 M urea buffer solution containing 20 mM of pH 7.5 hepes and 100 mM of NaCl. The coloured fractions were collected and kept away from light (light sensitive) at 4 ^{o}C or –20 ^{o}C.

Concentrating the elution fractions using RP18 columns from Merck:

Purified HBAH fractions from IMAC was loaded into a RP18 column equilibrated with 5 column volumes of 0.1% of trifluoroacetic acid (TFA). The column was then washed with 3 column volumes of 0.1% TFA. Then, the column was washed with 5, 10, 20, 30, and 50% methanol to elute the concentrated HBAH. The coloured fractions (pink) was collected and kept in the dark at 4^{o}C or -20 ^{o}C. This sample was then sent for HPLC-MS to check its purity and confirm the correct molecule was produced.

Re-suspending HBAH in binding buffer to obtain a concentration of 7.5 µg/mL:

The eluted fractions from RP18 column were vacuum dried using a rota-evaporator. The methanol was first removed before switching the settings to remove water. Once the HBAH is dried, it was resuspended in 5 mL of 0.1 M pH 7.6 potassium phosphate buffer (KPi buffer). 0.1 M of pH 7.6 KPi buffer was made by adding 86.6 mL of 1 M K_2HPO_4 and 13.4 mL of 1 M KH_2PO_4 . The volume was then adjusted to 1 L using distilled water.

The resuspended HBAH solution was checked for its concentration by finding the peak on the UV-Vis scan (peak at around 330 nm) and calculating the concentration (Extinction coefficient = 50 000 M^{-1} cm^{-1}). The concentration of HBAH was then diluted down using KPi buffer to obtain a concentration of 7.5 µg/mL. This was stored at -20 ^oC and defrosted before use.

6.3 Extraction from faecal samples and conversion of cobamides into its cyano-form

Release of cobamides and conversion into the cyano-form of those cobamides (cyanocobalamin analogues):

Faecal samples were thawed overnight at 4 ^oC and weighed out into 50 mL falcon tubes. Each sample was then vortexed with 30 mL of 0.1 mg/mL KPi/KCN buffer (Table 3). Adding this buffer allows the conversion of all cobalamins into the cyano-form which is more stable.

Table 3: Composition of 0.1 mg/mL KPi/KCN buffer per 300 mL

Component	Amount
0.1 M pH 7.6 KPi buffer	300 mL
KCN (potassium cyanide)	0.03 g (30 mg)

The samples were then kept in boiling water bath with the lids off for 20 minutes with stirring every 5 minutes. This denatures cells and proteins which releases the cobamides into the solution. The cyanide in the buffer should make all the cobamides

in the solution to have the cyano-group attached to the upper ligand. Thus, all cobamides will only differ in their lower ligand base (lower ligand analogues).The lids were placed back on and samples were then allowed to cool to room temperature. These were then incubated in 4 ^{o}C overnight.

The samples containing KPi/KCN were then centrifuged at 4000 RPM for 45 minutes. The supernatant was transferred into new 50 mL falcon tubes. 100 µL of 7.5 µg/mL HBAH was added into the supernatant of each falcon tube. Then, 800 µL of His-BtuF resin was added into each falcon tube. The entire mixture is left at 4 ^{o}C for 18 hours with gentle shaking.

Checking the elution volume of His-BtuF resin on the mini chromatography columns:

800 µL of His-BtuF resin was pipetted into a mini chromatography column. The column was washed with 3 mL KPi buffer then 1 mL of 100 µM cyanocobalamin was loaded onto the column. The column was washed with KPi buffer until the drops turn clear. The resin should be observed to be pink. Wash the column in 200 µL of 8 M urea elution buffer solution containing 20 mM of pH 7.5 hepes and 100 mM of NaCl one at a time. The void volume (volume before pink is eluted out) and the elution volume (volume from first drop of pink to the last drop) were recorded.

Purifying cobamides:

The falcon tubes containing the His-BtuF resin, supernatant of stool sample, and HBAH is taken out of 4 ^{o}C and centrifuged for 60 seconds at 500 RPM. This is to collect the His-BtuF resin at the bottom. The supernatant in each falcon tube was carefully decanted (beware not to pour out the resin) into a KCN waste bottle. The pellet was then re-suspended with KPi buffer. A fresh mini chromatography column

was prepared for each sample and 800 µL of His-BtuF resin was pipetted into each column. A plastic pipette was used to transfer all the resuspended pellets (resuspended resin) from the falcon tubes to run through the mini columns containing His-BtuF resin. Then, two washes of KPi buffer were done on each mini column. The void volume of 8 M urea elution buffer solution containing 20 mM of pH 7.5 hepes and 100 mM of NaCl was loaded onto the column and flow through not collected. Then, the elution volume of the same urea elution buffer was loaded and flow through was collected in 2 mL eppendorf tubes. For each sample, 400 – 500 µL of the elution fraction was pipetted into HPLC-MS vials. The samples are now ready for HPLC-MS analysis.

Method testing with pet hedgehog and pet and wild Rabbit

The above procedure (Section 6.3) was repeated for hedgehog and rabbit faecal samples. 4 g of samples were used for pet hedgehog, pet rabbit, and wild rabbit samples.

Optimisation of protocol with horse samples to maximise the types of cobamides detected

i) Varying the mass of samples

The extraction procedure was the same as above (Section 6.3) except the masses of the samples were changed. The incubation time with the His-BtuF resin was kept constant at 18 hours. The masses investigated were: 2, 4, 6, 8, 10, and 12 grams.

ii) Incubating samples with His-BtuF resin for different time periods

The extraction procedure was the same as above (Section 6.3) except the incubation time with the His-BtuF resin was changed. The mass used for these samples were 6 g. The time periods investigated were: 1, 3, 6, 18, and 24 hours.

Racehorse faecal samples

The protocol was conducted same as above (Section 6.3). Racehorse faecal samples from three different months (June, August, and September) were sent to find if there are differences in the cobamides present between the control racehorses and supplemented racehorses that were injected with hydroxocobalamin in the month of June 2018.

i) JUNE (Month of supplementation)

The mass of all control and treatment group samples used for this month was 6 g.

ii) AUGUST (2 months after supplementation)

Table 4 and 5 shows the mass of samples used for the control and treatment group in the month of August. Abbreviations of the names are in brackets and will be used in place of the full name for this report. Treatment group horses will be differentiated with a (+) beside their abbreviated name. Example: COR (+).

Table 4: Mass of faecal samples used for control group

Control group	**Mass (g)**
Ashington (ASH)	5.01
Buxted Dream (BD)	5.00
Coolongolook (COOL)	5.09
Drill (DR)	5.58
Edelline (EDE)	4.00
Farewell to you (F2U)	5.08
God Given (GG)	5.35
La Rav (LR)	5.05
Loveheart (LH)	5.04
Recollect (REC)	5.00

Table 5: Mass of faecal samples used for treatment group

Treatment group (+)	Mass (g)
Alwaysandforever (A&4E)	5.20
Cortado (COR)	NO SAMPLE RECEIVED
Drap d'or (DDR)	5.34
Fairlight (FL)	5.10
Floria Tosca (FT)	4.00
Four white socks (4WS)	5.00
Plentiful (PL)	NO SAMPLE RECEIVED
Swansdown (SD)	5.11
Valyrian (VAL)	NO SAMPLE RECEIVED
Warsaw road (WR)	4.92

iii) SEPTEMBER (3 months after supplementation)

Table 6 and 7 shows the mass of samples used for the control and treatment group in the month of September.

Table 6: Mass of faecal samples used for control group

Control group	Mass (g)
Ashington (ASH)	5.04
Buxted Dream (BD)	5.26
Coolongolook (COOL)	5.01
Drill (DR)	4.83
Edelline (EDE)	5.25
Farewell to you (F2U)	NO SAMPLE RECEIVED
God Given (GG)	5.03
La Rav (LR)	5.56
Loveheart (LH)	5.33
Recollect (REC)	5.50

Table 7: Mass of faecal samples used for treatment group

Treatment group (+)	Mass (g)
Alwaysandforever (A&4E)	5.05
Cortado (COR)	NO SAMPLE RECEIVED
Drap d'or (DDR)	5.04
Fairlight (FL)	5.29
Floria Tosca (FT)	5.03
Four white socks (4WS)	5.02
Plentiful (PL)	NO SAMPLE RECEIVED
Swansdown (SD)	4.85
Valyrian (VAL)	NO SAMPLE RECEIVED
Warsaw road (WR)	5.07

Human faecal samples (Vitamin B_{12} deficient patients)

Table 8 shows the mass of the human faecal samples sent by the pernicious anaemia society (PAS) that were used for cobamide extraction.

Table 8: Mass of samples used for the human faecal samples

Patient sample code	Mass of sample
H2	6.21
H5	5.48
H7 *	1.55
H9	4.94
H10	4.24
H11	5.04
H12	2.05
H13	6.81
H15	3.96
U1	5.74
U2	3.02
U3	5.44
U4	4.34
U5	2.87
U6 *	0.11
U7	5.34
U9	7.87
U12 *	0.19

*Masses used for these samples are lower than 2 g and would need to be taken into account when analysing the results,

6.4 Extraction of cobamides from tissue samples

Method testing with lamb liver

i) Without methanol

Tissue samples were thawed overnight at 4 ^{o}C and transferred onto a clean pestle and mortar. Liquid nitrogen is poured onto the sample and the sample is ground. The freeze, thaw, and grind process is repeated 3 times to get a fine powder. 0.1 mg/mL of KPi/KCN buffer is poured into the pestle and mortar to mix with the fine powder. The mixture was poured into a 50 mL falcon tube. The sample was vortexed to mix it evenly. Caps off, the falcon tube was placed in boiling water bath for 10 minutes. The sample is left to cool and kept at 4 ^{o}C overnight.

Then, the sample was centrifuged at 4000 RPM for 10 minutes. The supernatant was collected into a new 50 mL falcon tube. 800 µL of His-BtuF resin and 100 µL of 7.5 µg/mL HBAH was added to the falcon tube. The mixture was left in 4 ^{o}C for 18 hours with gentle shaking. The cobamides was then purified and treated the same way as described previously.

ii) With methanol

The same method for without methanol was performed for the trial with methanol. The only difference was the addition of 10 mL of 50% v/v Methanol was added to the fine powder after grinding along with 0.1 mg/mL of KPi/KCN.

Mice Kidney and Liver

The same method described for method testing with lamb liver without methanol was used (Section 6.4 part (i)).

6.5 Extraction of cobamides from serum samples

The procedure was adapted from the faecal sample procedure and two other studies (47, 48).Table 9 shows the volume of serum used for the cobamide extraction procedure. The serum and twice the serum volume of 0.6 M pH 4 acetate buffer with 0.1 mg/mL of KCN was added into a 50 mL falcon tube. The samples were placed in boiling water bath for 10 minutes with stirring every 2 minutes. The samples were allowed to cool and left in 4 ^{o}C overnight. The samples were then centrifuged for 10 minutes at 4000 RPM. The supernatants were collected onto a new falcon tube and 1.5 times the serum volume of 0.3 N NaOH neutralizing buffer was added. Then, 1 serum volume worth of 0.1 M pH 7.6 KPi buffer was added. The mixture was vortexed then 800 µL of His-BtuF resin and 100 µL of 7.5 µg/mL HBAH was then added. The

mixture was left in 4^{o}C for 18 hours with gentle shaking. The cobamides was then purified and treated the same way as described previously.

Table 9: Volume of serum samples analysed

Sample	Volume (mL)
Racehorse DDR (+) JUN	2.5
Racehorse DDR (+) AUG	3.0
Racehorse DR JUN	3.5
Racehorse DR AUG	3.0
Mice 1	0.5

6.6 HPLC-MS detection of cyanocobalamin analogues found after IMAC

400-500 µL of sample were pipetted into HPLC-MS vials ready for the HPLC-MS (Agilent 1100 series HPLC coupled to a micrOTOF-Q (Bruker) mass spectrometer). The solvents used were 0.1% v/v of TFA as solvent A and 100% acetonitrile as solvent B. 50 µL of each sample was injected. Table 10 shows the 16 different lower ligand bases looked for and their corresponding masses.

Table 10: Lower ligand analogues and their masses in ascending order searched in the HPLC-MS

Lower ligand base	Mass ($[M+H]^{2+}$)
Cobinamide	508.25
Phenol	652.27
p-cresole	659.28
Benzimidazole	664.27
3,4-Dimethyl phenol	666.28
2-amino-p-cresol	666.78
5-methylbenzimidazole	671.28
5-hydroxybenzimidazole	672.27
Adenine	672.78
5,6-dimethylbenzimidazole	678.29
5-methoxybenzimidazole	679.28
2-methyladenine	679.78
5-methoxy,6-methylbenzimidazole	686.29
1*H*-naptho[2,3-d]	689.28
2-methylthioadenine	695.77
2-methylsulfinyladenine	703.77

6.7 Analysing HPLC-MS data

An example of the method for collecting and analysing HPLC-MS data is shown in Figures 6 and 7. For example: Collecting the results for racehorse BD's August sample. Peaks will appear where a signal is found in the corresponding mass. In Figure 6, the mass looked for is 672.78 (adenine). A clear peak is seen and the isotopic pattern for that detection further confirms the presence of this analogue. Thus the peak area of this analogue is integrated. An example where no peak is detected (just noise signals) can be seen in Figure 7. This means that this analogue is not present in the sample.

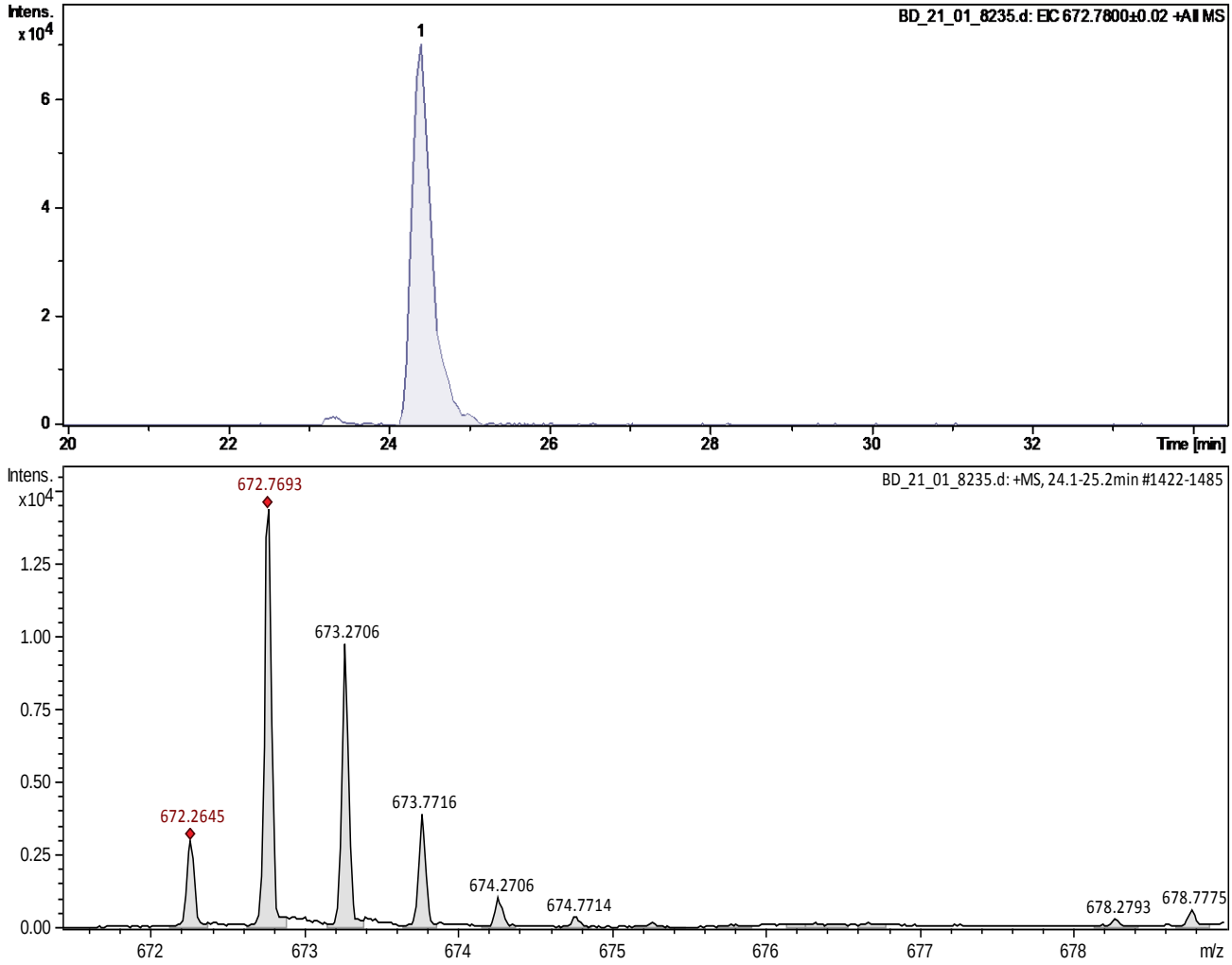


Figure 6: Detection of the cobamide with adenine as its lower base in the HPLC-MS

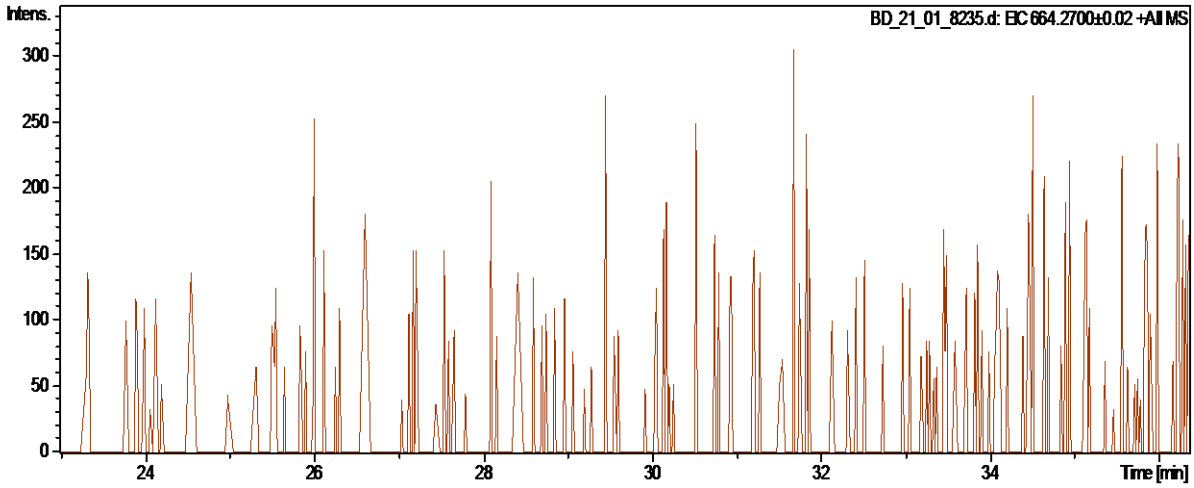


Figure 7: Example of an analogue not detected. No clear peak is seen.

This method of analysis is applied to every analogue in every sample to create a profile of integrated peak areas according to the analogues detected. This can then be normalised by dividing it against the integrated peak area of HBAH to make the 'normalised peak area'. The prevalence of each analogue as a percentage of the total cobamides detected within each sample are calculated from the normalised peak areas. The integrated peak areas of each detected analogue for each sample can be found in the supplementary material (S1-S12)

7. Results

Extraction of cobamides was performed on faecal samples from hedgehog, rabbit, horse, and humans. Firstly, the procedure was tested on hedgehog and rabbit faecal samples while the horse samples was used to optimise the procedure before using it for the racehorse faecal samples. Then, extraction was performed on the human faecal samples (pernicious anaemia patients) sent by PAS. Finally, this extraction procedure was adapted to try and find the types of cobamides present in mice tissue and horse or mice serum samples.

The analysis of results involved a comparison of the integrated peak area/HBAH/g of each lower ligand analogue within each sample (normalised peak area). This comparison allows an indication of the relative amounts found between samples since quantification of the amount of each analogue was not possible without standards. Additionally, it is possible to calculate the relative amount of each analogue with respect to the total level of cobamides within the sample (prevalence of a certain analogue as a percentage of the total cobamides detected within each sample).

Since vitamin B_{12} (cyanocobalamin) is commercially available, it is possible to more accurately determine the level of the analogue with the lower base 5,6-dimethylbenzimidazole through the use of standards. This allows further analysis of this analogue in terms of quantifying it in ng of cyanocobalamin per gram of sample.

Finally, the data were analysed using the three methods and results will be shown where significant observations are noted from either of the three comparisons (Normalised peak area, percentage prevalence, and cyanocobalamin quantification).

7.1 Cyanocobalamin standard curve

Table 11 below shows the amount of cyanocobalamin in ng injected into the HPLC-MS for detection and the integrated peak area for each amount. It is translated into a graph in Figure 8 to generate the line of best fit equation. This graph is used for quantifying the analogue with 5,6-dimethylbenzimidazole as lower ligand base when needed for further analysis. 0.014 ng or (2 nM) of cyanocobalamin was loaded but the HPLC-MS was not sensitive enough to be detect it.

Table 11: Integrated peak area values of different cyanocobalamin amounts injected into the HPLC-MS.

Amount of cyanocobalamin injected (ng)	Integrated peak area
0.068	841
0.680	16457
6.780	204461
67.770	2038392

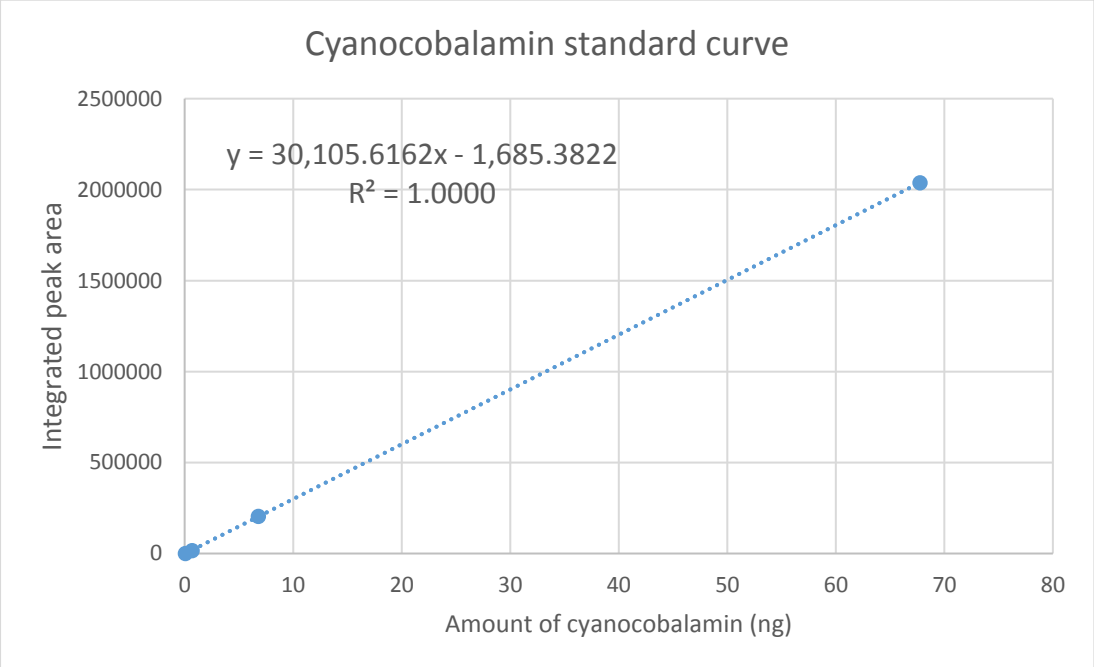


Figure 8: Cyanocobalamin standard curve. R^2 of 1 indicates a reliable trend line and thus, the equation of it can be used to quantify the amount of cyanocobalamin detected in samples.

7.2 HPLC-MS detection of purified HBAH

HPLC-MS run was performed for purified HBAH to check the purity of the molecule before using it for subsequent experiments (Figure 9). The HPLC-MS data indicated that this batch of HBAH is pure enough for use in subsequent experiments for normalising integrated peak areas of analogues detected. This enables HBAH to act as an internal standard allowing the peak areas to be compared fairly.

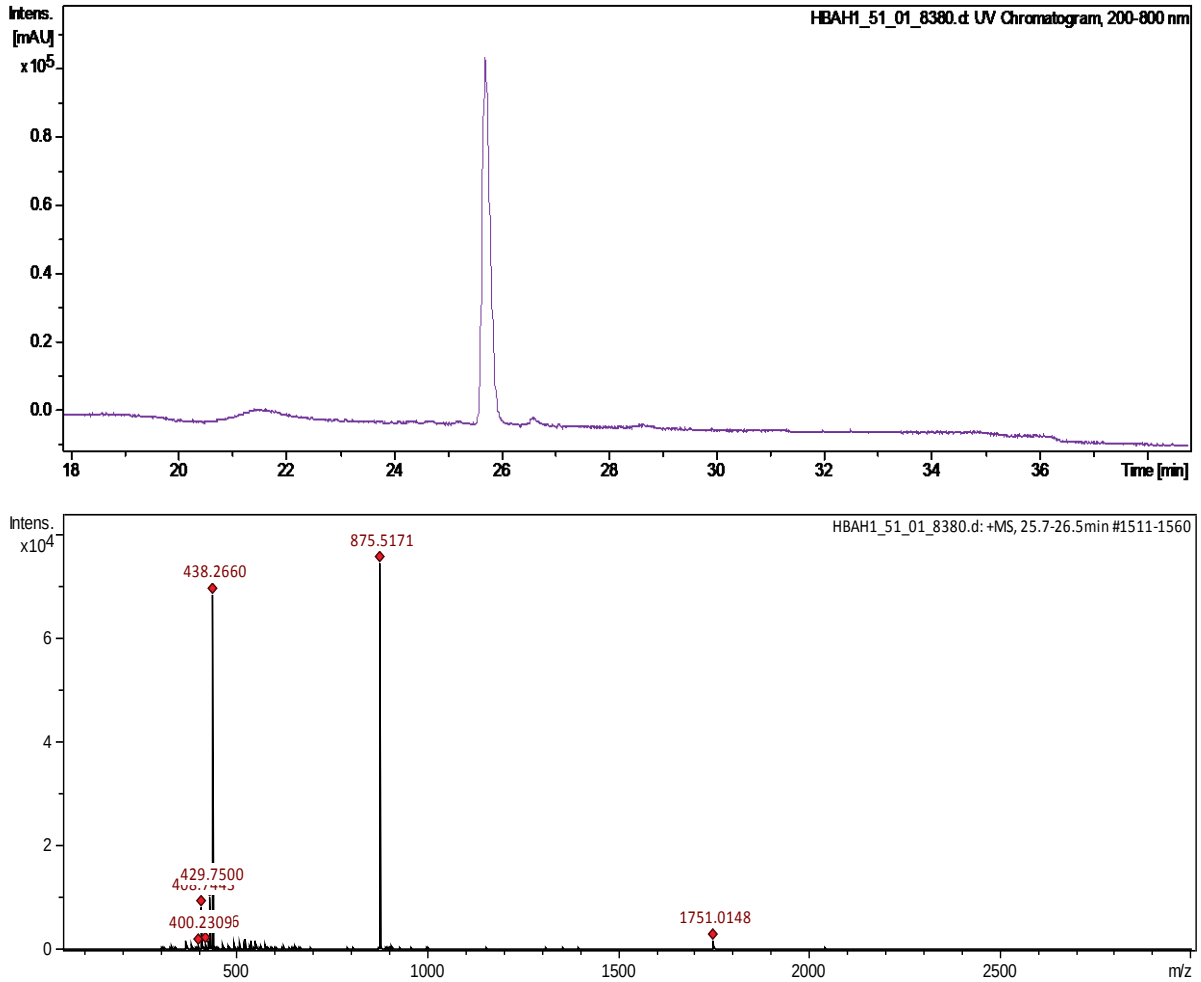


Figure 9: HPLC-MS of HBAH.
HPLC-MS profile of purified HBAH. There is just one large clear peak indicating a successful purification. Further analysis confirmed the peak is due to the presence of HBAH as the mass detected showed was the expected value at 875.5.

7.3 Trial extraction and optimisation using faecal samples from various animals

This methodology was tested with hedgehog and rabbit samples as a test run and to become familiar with the protocol before optimisation with the more abundant horse samples.

7.3.1 Pet Hedgehog and Wild and Pet Rabbit – Trial extraction

A trial extraction was performed to familiarise with the protocol using hedgehog and rabbit faecal samples.

Pet Hedgehog

Table 12 shows the types of cyanocobalamin analogues detected in the HPLC-MS and its corresponding integrated peak area per gram, normalised with HBAH. Table 13 shows the percentage each detected analogue represents compared to the total picked up in each sample. This indicates that in this hedgehog, analogues with 2-methyladenine and 2-methylthioadenine as its lower ligand base forms represent the majority of the cobamides found in its microbiome. The lowest level of analogue was cobinamide, the incomplete cobamide that is missing the lower loop. The amount of cyanocobalamin present per g is 3.44 ng (Table 14).

Table 12: Normalised integrated peak area per gram of detected lower ligand bases' peaks.

Lower ligand bases	Integrated peak area/HBAH/g
cobinamide	0.033
3,4-dimethyl phenol	0.047
2-amino-p-cresol	0.308
adenine	0.116
5,6-dimethylbenzimidazole	1.060
2-methyladenine	4.968
2-methylthioadenine	4.902

Table 13: Prevalence of each analogue as a percentage of the total cobamides detected in the sample

Lower ligand bases	Percentage total in each sample (%)
cobinamide	0.22
3,4-dimethyl phenol	0.42
2-amino-p-cresol	2.71
adenine	1.03
5,6-dimethylbenzimidazole	9.28
2-methyladenine	43.46
2-methylthioadenine	42.88
Total	100

Table 14: Quantification of 5,6-dimethylbenzimidazole

Sample	Amount of cyanocobalamin per g (ng)
Pet hedgehog	3.44

Wild and Pet Rabbit

The same analysis performed on the hedgehog samples were done on wild and pet rabbits to see if any significant differences could be observed perhaps reflecting a different living environment. Table 15 gives a profile of which analogues were detected. The difference observed between the wild and pet rabbit were the presence of cobamides with cobinamide as its lower ligand base in pet rabbit samples and the presence of cobamides with 5-methoxy,6-methylbenzimidazole as its lower ligand base in wild rabbit samples. The percentage total data in Table 16 shows that the most prevalent cobamide detected was with 2-methyladenine as its lower ligand base for both wild and pet rabbit samples. The proportions of other analogues were similar to each other except for 5,6-dimethylbenzimidazole whereby it is significantly higher at 19% in wild rabbit than pet rabbit at 8%. Lastly, comparing the amount of cyanocobalamin in each sample in Table 17, wild rabbit is observed to have higher amounts than pet rabbit.

Table 15: Normalised integrated peak area per gram of detected lower ligand bases' peaks in wild and pet rabbit faecal samples.

	Integrated peak area/HBAH/g	
Lower ligand bases	**Wild Rabbit**	**Pet Rabbit**
cobinamide	-	0.707
3,4-dimethyl phenol	0.039	0.085
2-amino-p-cresol	0.033	0.038
adenine	0.057	0.370
5,6-dimethylbenzimidazole	0.906	1.021
2-methyladenine	2.512	7.115
5-methoxy,6-methylbenzimidazole	0.077	-
2-methylthioadenine	1.142	3.344

Note: "– " denotes no detection in the HPLC-MS and thus, no peaks integrated.

Table 16: Prevalence of each analogue as a percentage of the total cobamides detected in the sample

	Percentage total in each sample (%)	
Lower ligand bases	**Wild Rabbit**	**Pet Rabbit**
cobinamide	-	5.58
3,4-dimethyl phenol	0.83	0.69
2-amino-p-cresol	0.68	0.29
adenine	1.20	2.91
5,6-dimethylbenzimidazole	19.01	8.04
2-methyladenine	52.70	56.12
5-methoxy,6-methylbenzimidazole	1.61	-
2-methylthioadenine	23.97	26.37
Total	100	

Table 17: Quantification of 5,6-dimethylbenzimidazole (cyanocobalamin)

Sample	**Amount of cyanocobalamin per g (ng)**
Wild rabbit	1.27
Pet rabbit	0.81

7.3.2 Horse – Optimisation for more variety of analogues detected

The method tested on hedgehog and rabbit samples showed that the extraction procedure can be utilized to obtain cobamide extraction profiles from animal faeces via HPLC-MS. This procedure was further optimised with a horse named ‘Ziggy’ in preparation for extraction of cobamides from racehorse samples. The two main factors investigated were the optimum time samples should be incubated with His-BtuF nickel resin and the optimum mass of the sample per falcon tube. The purpose of this is to maximize the types of cobamides detected when running the racehorses’ samples.

i) Varying the mass of faecal samples

Table 18 shows the different lower ligand bases detected when different masses of sample were used. This allows the determination of the minimum mass that gives a reliable result and therefore, results from samples with lower masses needs to be omitted or analysed with precaution. From three trials, it is better to use samples of more than 2 g for HPLC-MS analysis as the number of analogues detected was lowest with low level of material although the quantity of 5,6-dimethylbenzimidazole was not the lowest as shown in Table 19. If the provided sample was below 2 g, the concentration of cobinamides present was likely not high enough to yield a reliable result. This will need to be taken into account for observing patterns and deriving conclusions from samples in later experiments. From the trials conducted, it is observed that using 8 g of sample for every 50 mL falcon had the highest number of analogues detected.

Table 18: List of lower ligand bases detected when using different masses of faecal samples and their respective integrated peak area/HBAH/g values.

	Integrated peak area/HBAH/g					
Lower ligand base	**2 g**	**4 g**	**6 g**	**8 g**	**10 g**	**12 g**
cobinamide	1.625	2.401	2.094	0.563	0.710	0.319
p-cresole	-	-	-	0.038	-	0.023
3,4-dimethyl phenol	-	0.141	-	0.036	-	-
2-amino-p-cresol	-	0.309	0.251	0.086	0.176	0.057
5-hydroxybenzimidazole	0.995	1.488	1.681	0.236	0.421	0.172
Adenine	-	1.384	-	-	-	-
5,6-dimethylbenzimidazole	2.007	2.030	2.495	0.442	0.757	0.234
5-methoxybenzimidazole	-	0.630	-	-	-	-
2-methyladenine	13.437	16.146	19.913	3.002	5.470	1.837
5-methoxy,6-methylbenzimidazole	-	-	-	-	-	0.011
2-methylthioadenine	0.352	0.253	0.418	0.063	0.084	0.032

Table 19: Quantification of 5,6-dimethylbenzimidazole averaged from three trials.

Weight of sample used (g)	Amount of cyanocobalamin per g (ng)
2	0.75
4	0.68
6	0.63
8	0.74
10	0.53
12	0.43

ii) Incubating samples with His-BtuF nickel resin for different time periods.

Table 20 shows the different cobamide with different lower ligand bases detected in the HPLC-MS. The samples were incubated with His-BtuF nickel resin (cobalamin binding protein attached to nickel resin) for different time periods to check if it has an effect on the number of analogues detected. Five different time points were used. As seen in Table 20, both 1 hour and 3 hour incubations had one less analogue detected compared to the other time points. This indicates that 1 hour and 3 hours incubation

may be inconsistent. The base 2-methylthioadenine was not detected in the 24 hour time point when it was detected in all others. On the other hand, only the 24 hour time point had the base 5-methoxy,6-methylbenzimidazole present. Next, the amount of 5,6-dimethylbenzimidazole detected is compared in Table 21. The amount of cyanocobalamin in the 24 hour incubation is higher than the rest. This could indicate that 24 hour incubations are better for capturing more cyanocobalamin, however the number of cobamides detected may vary. Therefore to standardise and reduce inconsistencies, the incubation time with His-BtuF resin for racehorses' samples was set between from 6 to 18 hours as it is observed to have the same detection profiles (Table 20).

Table 20: List of lower ligand bases of cobamides detected when incubated with His-BtuF nickel resin for different time periods and their respective integrated peak area/HBAH/g values.

	Integrated peak area/HBAH/g at different time points				
Lower ligand base	**1 hour**	**3 hours**	**6 hours**	**18 hours**	**24 hours**
cobinamide	0.227	0.249	0.262	0.352	0.339
p-cresole	0.034	0.030	0.035	0.053	0.042
3,4-dimethyl phenol	0.006	-	0.009	0.011	0.011
2-amino-p-cresol	-	0.007	0.013	0.011	0.014
5-hydroxybenzimidazole	0.081	0.076	0.106	0.165	0.129
Adenine	0.130	0.129	0.142	0.210	0.169
5,6-dimethylbenzimidazole	0.057	0.066	0.101	0.139	0.265
2-methyladenine	0.797	0.733	1.101	1.689	1.399
5-methoxy,6-methylbenzimidazole	-	-	-	-	0.006
2-methylthioadenine	0.006	0.006	0.011	0.017	-

Table 21: Quantification of 5,6-dimethylbenzimidazole

Incubation time (hour(s))	**Amount of cyanocobalamin per g (ng)**
1	0.57
3	0.67
6	1.04
18	0.88
24	1.85

7.3.3 Summary of results from trial extraction and optimisation

The extraction procedure was tested on hedgehog and rabbit faecal samples and it is observed that the 5-methoxy,6-benzimidazole analogue is only present in wild rabbits compared to pet rabbit and hedgehogs. On the other hand, wild rabbits do not have the cobinamide analogue which is present in pet rabbit and hedgehog samples. The most prevalent analogue as a percentage of the total cobamides within each sample is 2-methyladenine for both groups. However, the percentages for 2-methyladenine and 2-methylthioadenine are within 1 % of each other and could between them represent the most prevalent analogues. This was not observed in rabbit samples where the second most prevalent analogue (also 2-methylthioadenine) is 30 % lower than the highest. Comparing the amount of the 5,6-dimethylbenzimidazole analogue, pet hedgehog was observed to have the highest amount of this analogue in its faeces at 3.44 ng per gram of sample. Next was the wild rabbit samples at 1.27 ng per gram and lastly pet rabbit samples were found to contain 0.81 ng per gram. This difference could be an indication of difference in gut microbiome or dietary habits.

Optimisation of the protocol using the horse samples showed that if 2 g samples were used for analysis, the number of cobamides detected will be lower than if 4 g of samples were used. Furthermore, incubating samples with His-BtuF nickel resin for different time periods reveal that 1 and 3 hour incubations showed inconsistencies although longer incubations had higher amounts of 5-dimethylbenzimidazole detected. Therefore, to standardise the protocol for racehorse samples, between 4 – 8 g of samples are used and they were incubated for 18 hours with the resin.

7.4 Racehorses' faecal sample data

The aim here is to identify any differences between treatment and control group racehorses in the level of analogues in their faecal samples. The treatment group horses are supplemented with 3mL of hydroxocobalamin by injections weekly for one month from June 2018. The control group received no injections. The two groups of thoroughbred racehorses studied are shown in Table 22. Their faecal samples were collected for 3 months, June (month of injection), August (2 months after injection), and September (3 months after injection).

Table 22: The names of the racehorses used in the study.

Control group	Treatment group (+)
Ashington (ASH)	Alwaysandforever (A&4E)
Buxted Dream (BD)	Cortado (COR) *
Coolongolook (COOL)	Drap d'or (DDR)
Drill (DR)	Fairlight (FL)
Edelline (EDE)	Floria Tosca (FT)
Farewell to you (F2U) *	Four white socks (4WS)
God Given (GG)	Plentiful (PL) *
La Rav (LR)	Swansdown (SD)
Loveheart (LH)	Valyrian (VAL) *
Recollect (REC)	Warsaw road (WR)

*Normalisation of peak area for VAL was not possible because the HBAH peak was undetected. Only June and August data available for F2U. Only June data available for COR (+), and PL (+). Thus, these have been omitted in the results.

Table 23 shows the lower ligand bases present in the racehorse samples Of the 16 different cobamide lower ligand masses looked for, no more than 10 was detected in each racehorse sample. The detected analogues will be discussed according to the groups these analogues belong in (phenols, benzimidazoles, purines, and cobinamide). Where applicable, bar charts are used to display any observable patterns. The three bars within each sample represents the values for June, August, and September (left to right). Where there are no bars, it indicates that the specific analogue was not detected.

Table 23: The lower ligand bases detected in the racehorse samples arranged according to the groups they belong in (phenols, benzimidazoles, purines, or cobinamide).

Phenols	Benzimidazoles	Purines	Cobinamide
p-cresole	5-hydroxybenzimidazole	adenine	cobinamide
3,4-Dimethyl phenol	5,6-dimethylbenzimidazole	2-methyladenine	
2-amino-p-cresol	1*H*-naphtho[2,3-d] imidazole	2-methylthioadenine	

i) Phenols

The three phenolic analogues present are p-creole, 3,4-dimethyl phenol, and 2-amino-p-cresol. No observable differences between the two groups were noted for the results of 3,4-dimethyl phenol and 2-amino-p-cresol.

Overall, the normalised peak area values of these three analogues in the month of June is much higher than in August or September shown in Figures 10-1 to 10-3. This observation is true for all samples except for COOL and SD (+) where the normalised peak area value of 2-amino-p-cresol in the month of August is higher (Figure 10-3).

A difference between the treatment and control group's normalised peak area values for p-cresole is observed. Figure 10-1 shows a gradual decrease in the normalised peak area levels of p-cresole from June to September in all treatment group samples which was not observed in the treatment group samples. However, when looking at the percentage prevalence of this analogue shown in Figure 10-4, no patterns were observed.

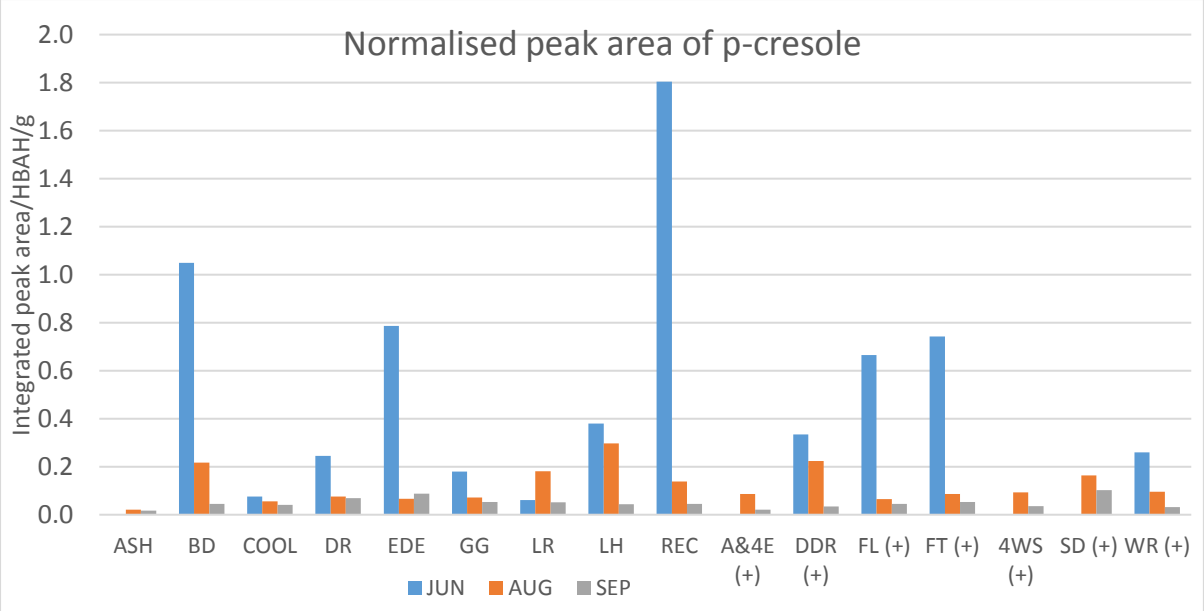


Figure 10-1: Integrated peak area/HBAH/g of p-cresole in June, August, and September in racehorse samples.

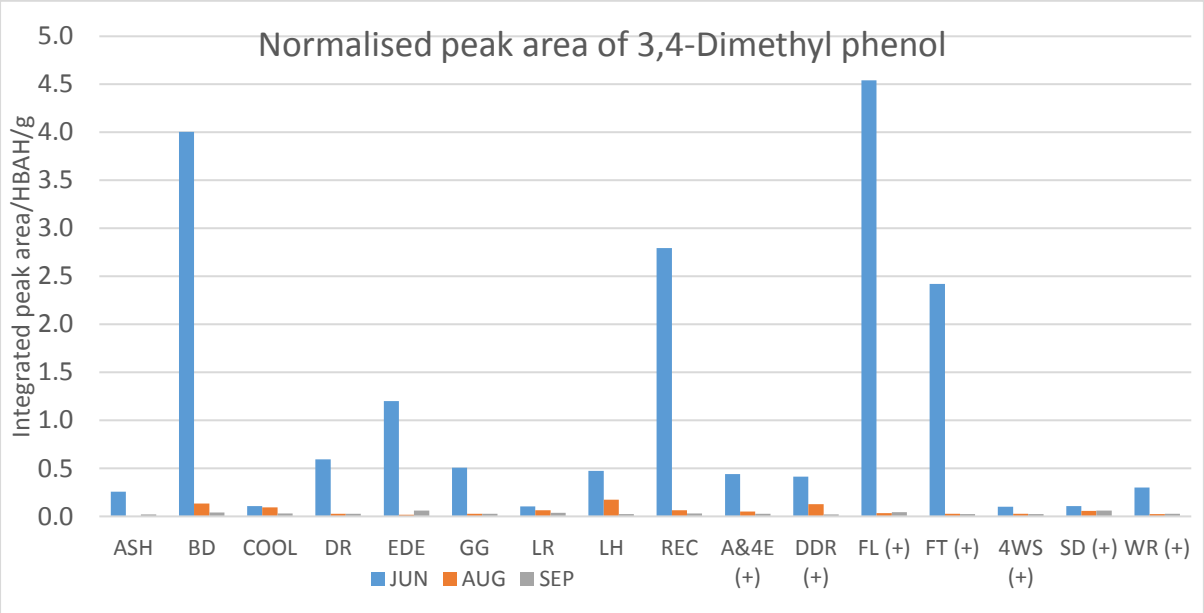


Figure 10-2: Integrated peak area/HBAH/g of 3,4-Dimethyl phenol in June, August, and September in racehorse samples.

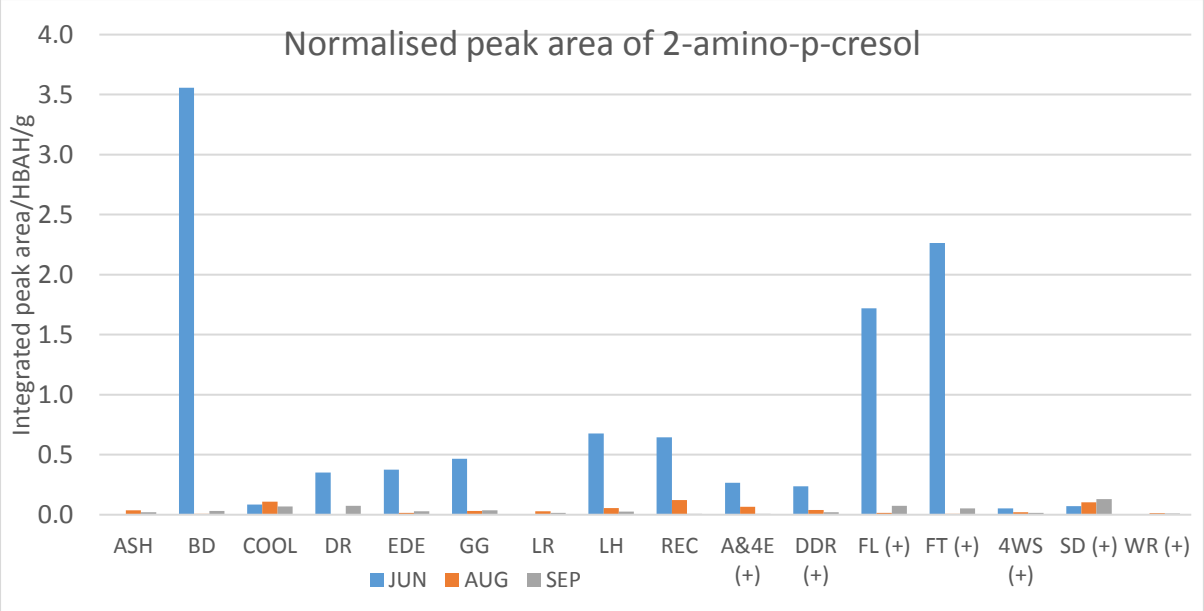


Figure 10-3: Integrated peak area/HBAH/g of 2-amino-p-cresol in June, August, and September in racehorse samples.

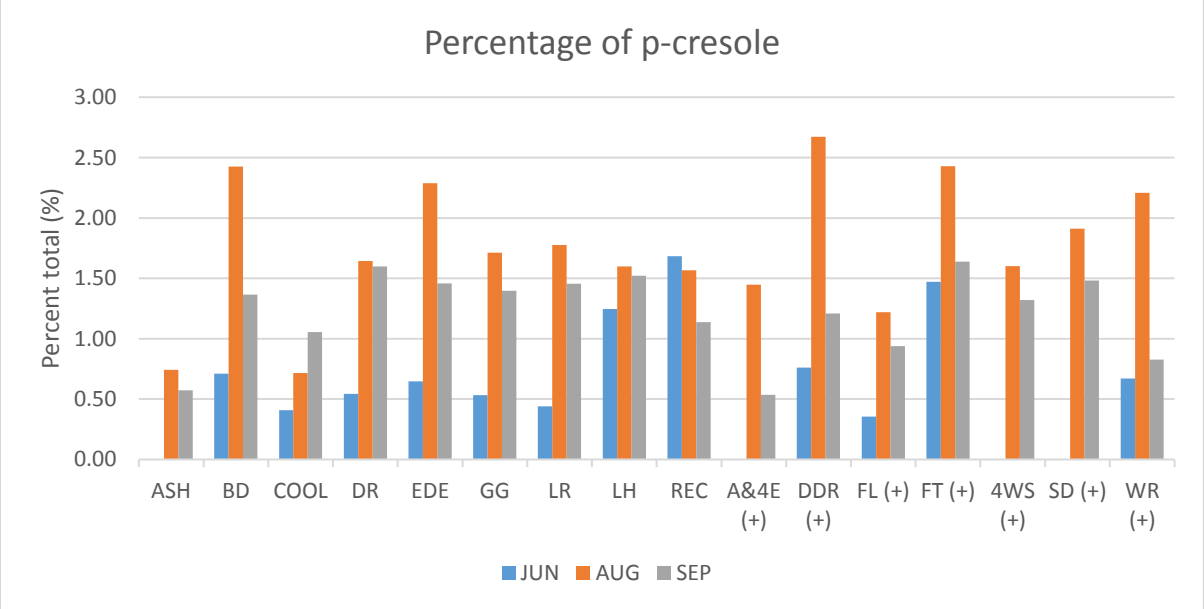


Figure 10-4: Prevalence of p-cresole as a percentage of the total cobamides detected within each sample.

ii) Benzimidazoles

No observable differences for the normalised peak area or percentage prevalence was noted between the two groups for this class of analogue. Quantification of 5,6-dimethylbenzimidazole show no observable differences between the control and treatment group.

However, a similarity is that the normalised peak area values for June is much higher than August and September for all samples shown in Figures 11-1 to 11-3. Moreover, for the 5-hydroxybenzimidazole analogue, both groups showed a gradual decrease in 5-hydroxybenzimidazole detection levels from June to September in Figure 11-1 except for DR, EDE, and DDR (+). Lastly, 1*H*-naphtho [2,3-d] imidazole only present in 5 of the 16 samples. This could indicate the low requirement of this analogue towards the horses' wellbeing.

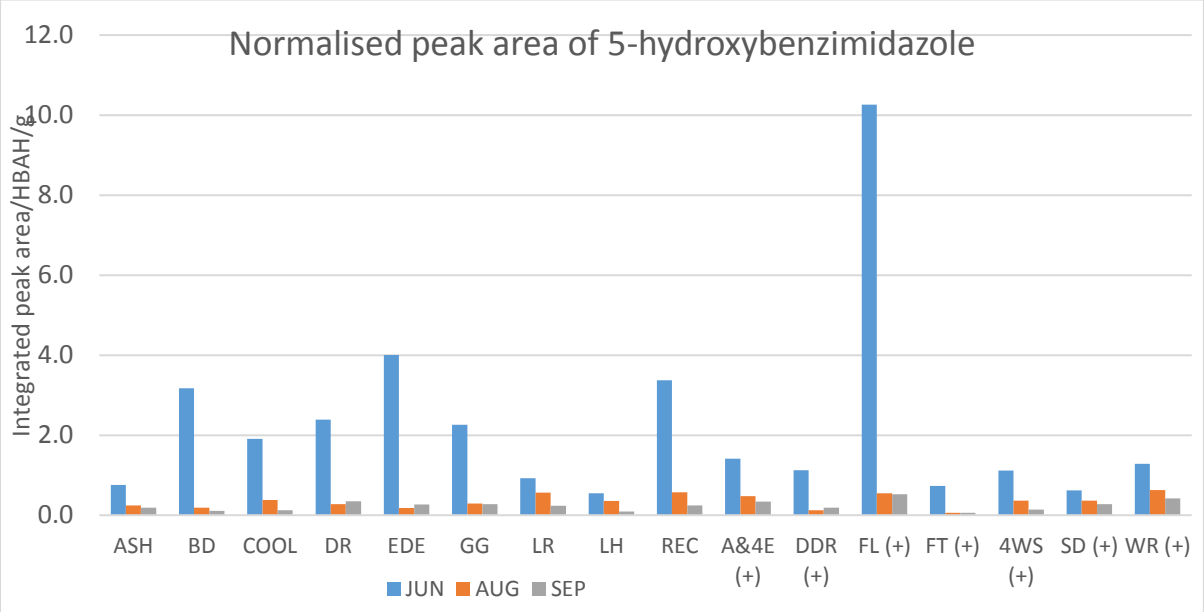


Figure 11-1: Integrated peak area/HBAH/g of 5-hydroxybenzimidazole in June, August, and September for racehorse samples

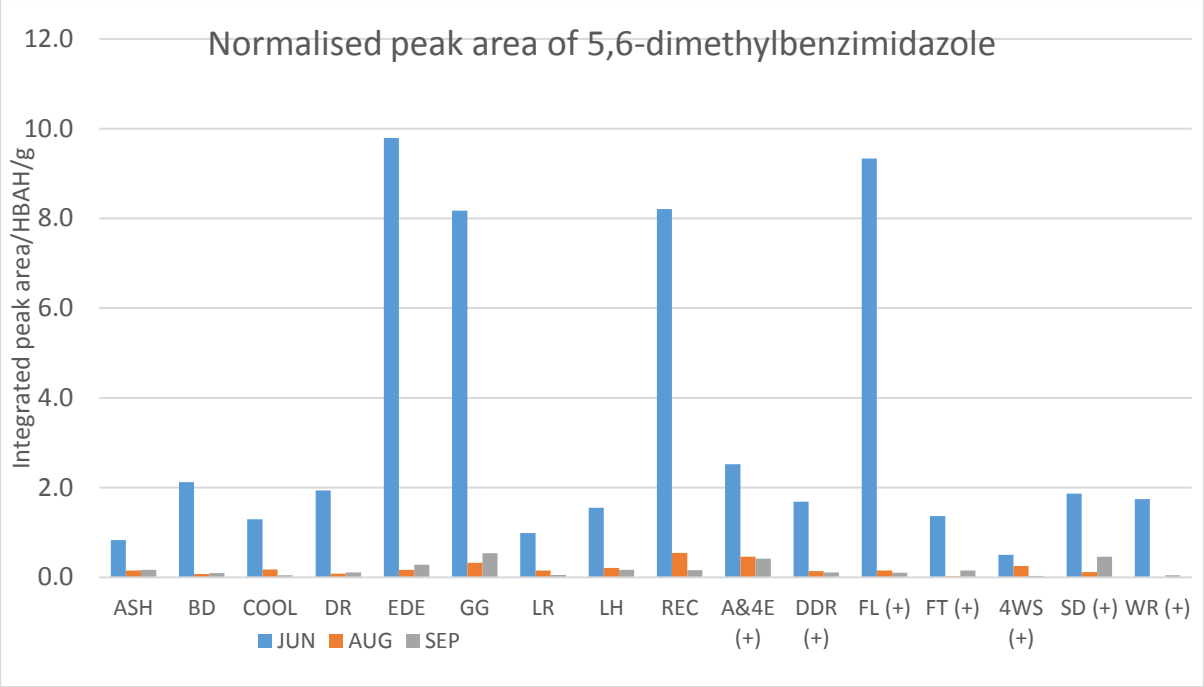


Figure 11-2: Integrated peak area/HBAH/g of 5,6-dimethylbenzimidazole in June, August, and September for racehorse samples

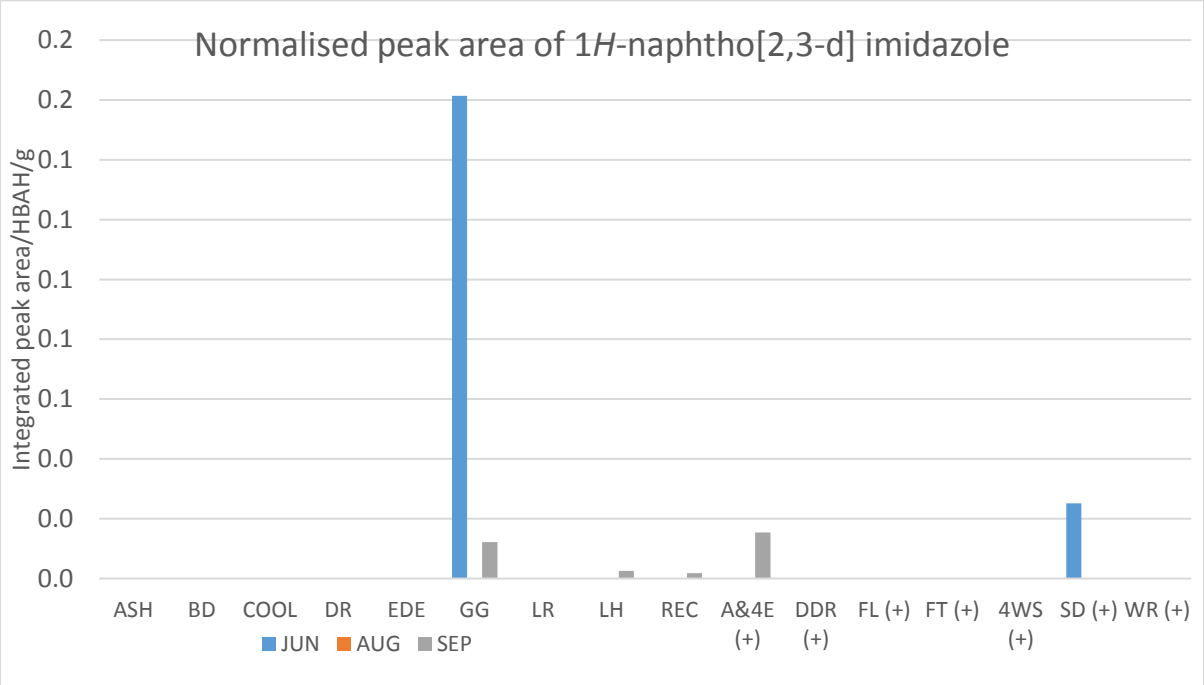


Figure 11-3: Integrated peak area/HBAH/g of 1*H*-naphtho [2,3-d] imidazole in June, August, and September for racehorse sample

iii) Purines

No significant differences were observed between the control and treatment groups for all the analogues detected within this class. All three analogues showed higher levels of normalised peak values in June than August and September as shown in Figures 12-1 to 12-3. Moreover, there is a gradual decrease in the normalised peak area values from June to September observed in adenine (except for EDE) and 2-methyladenine (except for EDE and WR (+) where September values are higher than August). Lastly, 2-methyladenine is the most prevalent analogue for all samples in terms of its percentage relative to the total cobamides detected within each sample as shown in Figure 12-4. No patterns were seen in the treatment group from June to September and showed no significant difference compared to the control groups.

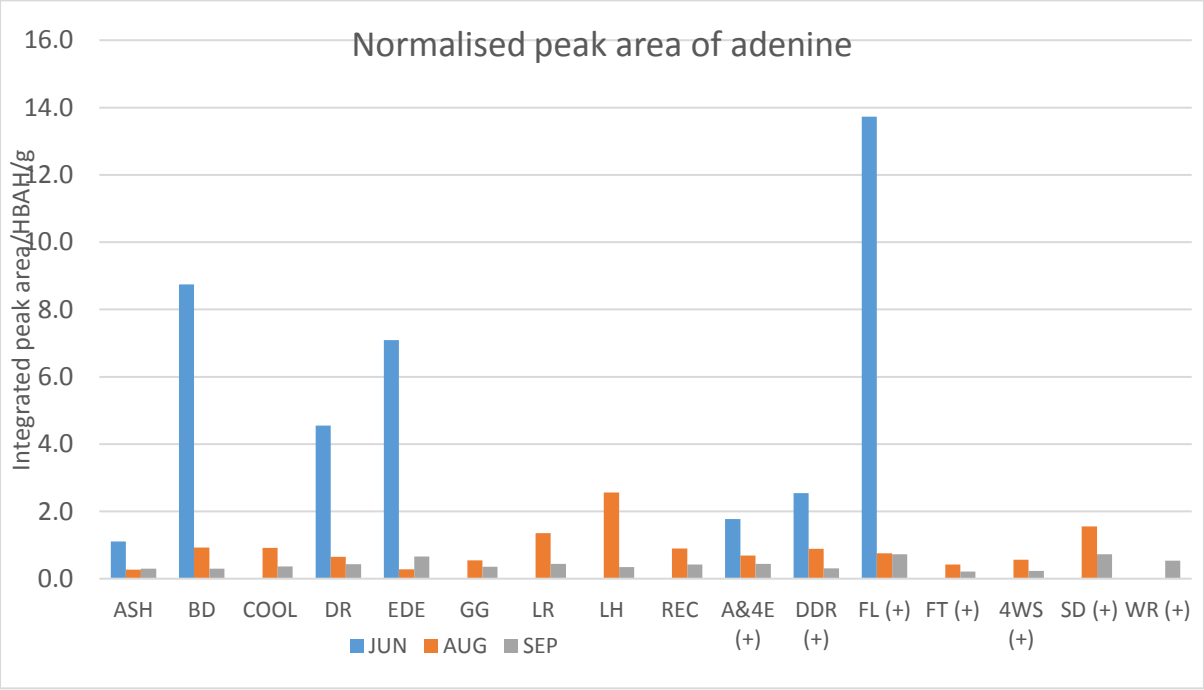


Figure 12-1: Integrated peak area/HBAH/g of adenine in June, August, and September for racehorse samples

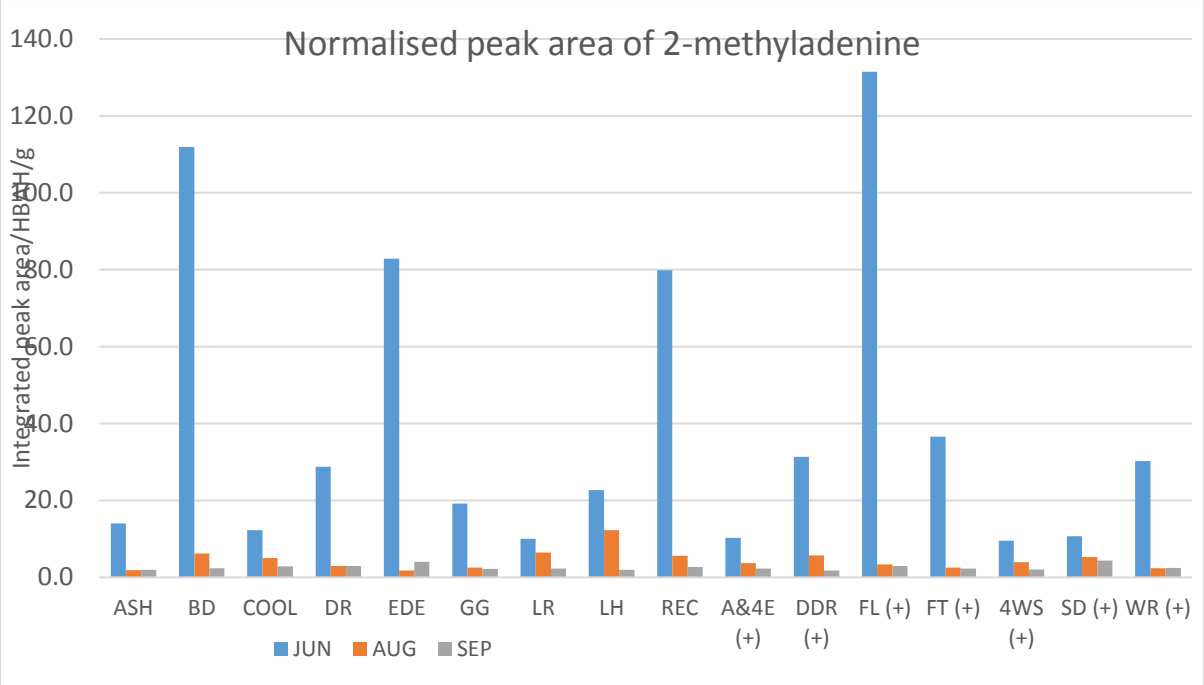


Figure 12-2: Integrated peak area/HBAH/g of 2-methyladenine in June, August, and September for racehorse samples

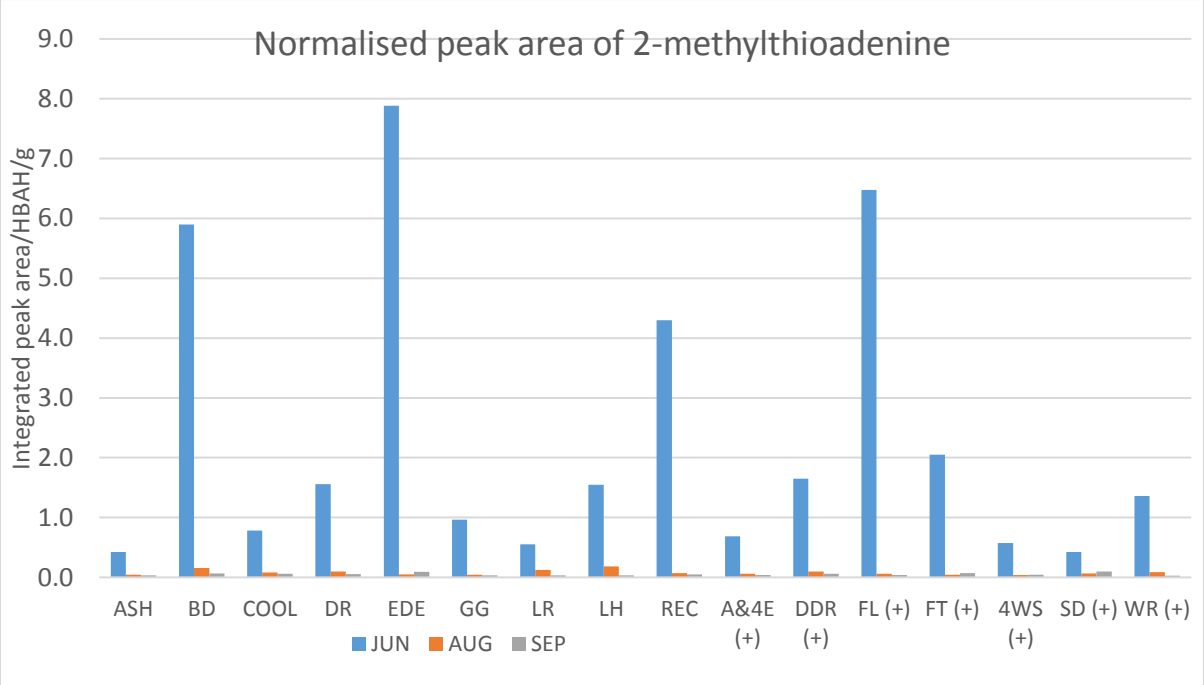


Figure 12-3: Integrated peak area/HBAH/g of 2-methylthioadenine in June, August, and September for racehorse samples

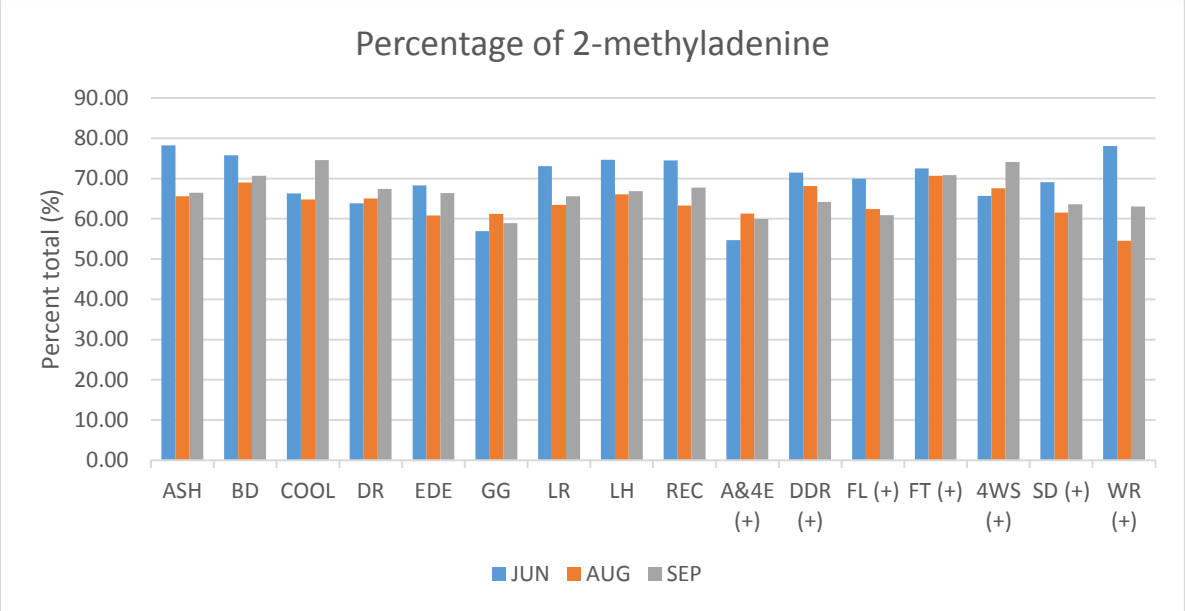


Figure 12-4: Prevalence of 2-methyladenine as a percentage of the total cobamides detected within each sample.

iv) Cobinamide

A difference is observed between the treatment and the control group. Shown in Figure 13-1, treatment group samples showed a gradual decrease in this analogue's normalised peak area values from June to September which was not observed in the control group. However, when comparing the percentage prevalence, there were no significant patterns observed (Figure 13-2).

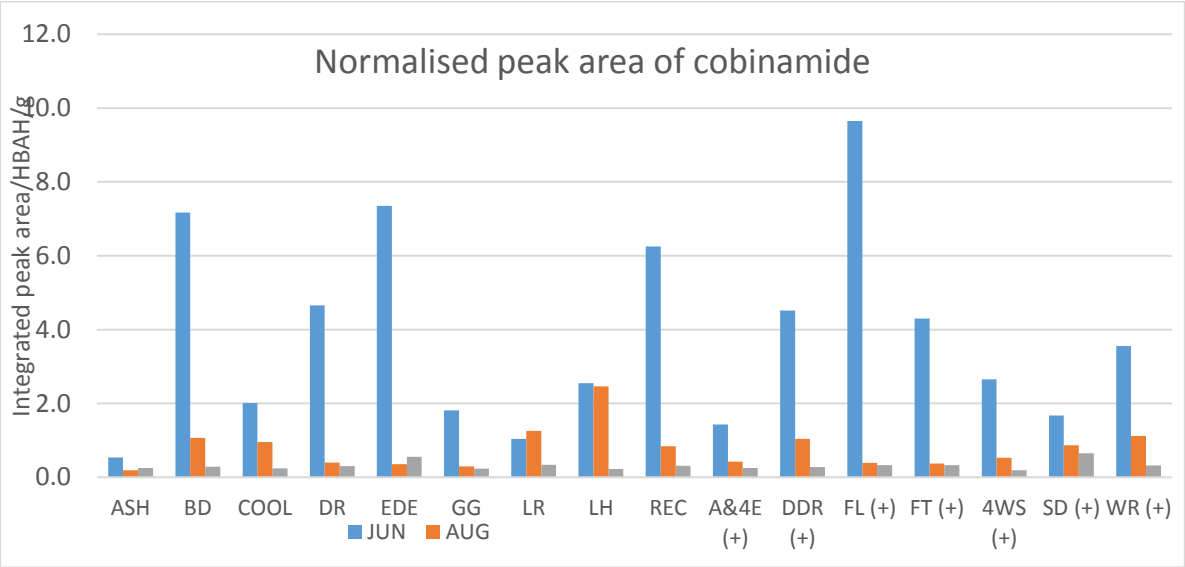


Figure 13-1: Integrated peak area/HBAH/g of cobinamide in June, August, and September for racehorse samples

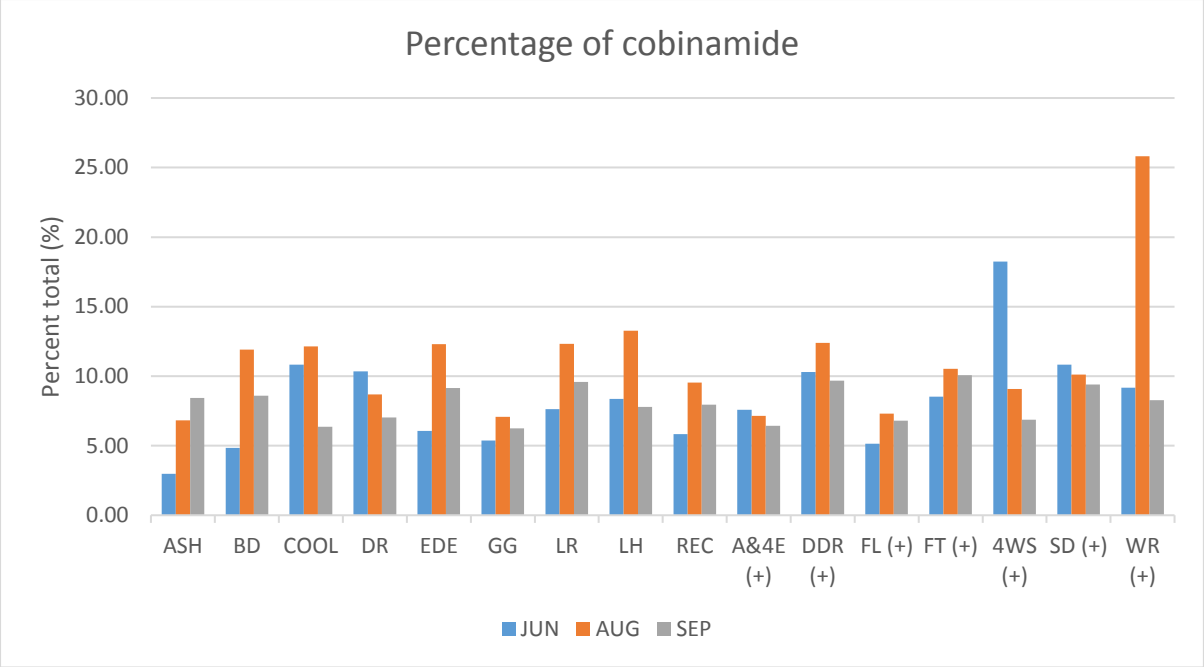


Figure 13-2: Prevalence of cobinamide as a percentage of the total cobamides detected within each sample.

7.4.1 Summary of racehorse faecal sample results

The similarities between the two groups (supplemented and control racehorses) of samples are; firstly, the cobamide with 2-methyladenine as its lower ligand base is the most prevalent analogue due to highest percentage prevalence (Figure 12-4). Secondly, most detected cobamides had higher 'normalised peak area' values in June than August or September except for cobamides with 2-amino-p-cresol, cobinamide, and p-Cresole as its lower loop ligand.

The last similarity noted is the gradual decrease of normalised peak area values of 2-methyladenine and adenine from June to September (except the sample: EDE and ASH, Figures 12-1 and 12-2).

For differences between the two groups, the normalised peak area value of cobamide and p-cresole showed a pattern of gradual decrease from June to August to September in the treatment group. This was not observed in the control group where fluctuations were observed between the months.

7.5 Vitamin B_{12} deficient human patient faecal samples

The aim here is to find a correlation between the treatment responses of pernicious anaemia patients and the composition of cobamides or lower ligand analogues found in their faecal samples. These patients are treated by three monthly hydroxocobalamin intramuscular injections. Faecal samples from 18 patients split into two groups of 9 were investigated.

Group H: patients who are happy with their three monthly injection

Group U: patients who are unhappy with their injection and require more frequent treatment to maintain wellbeing.

Table 24 shows the information about remarks made by the patients regarding their treatment responses and if additional supplements were taken. This will be taken into account when analysis the HPLC-MS data of their faecal samples.

Table 24: Patient's answers towards their reaction to their treatment and additional supplementations.

Patient code	Days since last injection from day of sample collection	Happy with 3-monthly injections? or Frequency of injection	Time when symptoms appear after injection	Additional Vitamin B_{12} supplements
H2	45	Yes	-	-
H5	No information provided			
H7	14	Injections every 2 months	-	-
H9	~365 (12 months)	Yes	-	-
H10	~120 (4 months)	Yes	-	-
H11	75	Yes	10–11 weeks	Tablets, Spray
H12	65	No	2 months	-
H13	31	Yes	-	-
H15	No information provided			
U1	0	No, monthly injections	-	-
U2	8	No	1.5 weeks	-
U3	1	No	2 weeks	Multi-Vitamin with Vitamin B_{12}, Spray
U4	3	No, monthly injections	2 weeks	Spray
U5	24	No, monthly injections	1 month	Occasional Tablets but not for the last 6 months
U6	No information provided			
U7	14 (2 weeks)	No	1 week	Methylcobalamin Tablets, Boost sublingual spray
U9	13	No, monthly injections	3.5 weeks	-
U12	14	No	2 months	

Table 25 shows the lower ligand analogues detected for the unhappy group (group U). On a whole, the Table indicates that each patient has a different profile although certain analogues are detected for every patient (they are cobamides with lower ligand base: Cobinamide, 2-amino-p-cresol, Adenine, 5,6-dimethylbenzimidazole, 2-methyladenine, and 2-methylthioadenine).

Table 25: The extraction profiles of patient samples belonging to group U.

	Integrated peak area/HBAH/g								
Lower ligand bases	U1	U2	U3	U4	U5	U6 *	U7	U9	U12 *
Cobinamide	0.117	0.127	1.987	0.044	0.096	-	0.105	0.074	-
p-cresole	0.028	0.015	0.224	-	0.028	-	0.019	0.018	-
3,4-Dimethyl phenol	0.021	0.036	0.257	0.020	0.008	0.025	-	0.003	-
2-amino-p-cresol	0.016	0.038	0.196	0.025	0.004	-	0.145	0.027	-
5-methylbenzimidazole	-	-	-	-	-	-	0.004	-	-
5-hydroxybenzimidazole	0.117	-	-	-	0.111	-	-	-	-
Adenine	0.351	0.259	3.458	0.118	0.121	0.867	0.167	0.077	0.237
5,6-dimethylbenzimidazole	0.046	0.048	16.947	0.075	0.089	0.281	0.630	0.132	-
2-methyladenine	0.544	4.341	3.166	1.720	0.651	3.479	1.437	1.031	1.636
5-methoxy,6-methylbenzimidazole	-	-	0.099	-	-	-	0.039	-	-
1*H*-naphtho[2,3-d] imidazole	-	-	0.263	-	0.003	-	0.013	0.005	-
2-methylthioadenine	0.235	0.865	4.396	1.293	0.103	0.675	0.188	0.120	0.212

* Note: The low variety of U6 and U12 detected could be due to low amounts of sample available. About 0.11 g and 0.19 g respectively

The same Table can be made for the happy group (group H) of patients (shown in Table 26). Comparisons between the extraction profiles of happy and unhappy patients can be made to identify any obvious or consistent differences and observations. For example, detection of the cobamide with 1*H*-naphtho [2,3-d] imidazole lower base is more prevalent in unhappy patients than happy patients. It is only detected once in happy group out of a total of 9 patients while it was detected 4 times out of 9 in the unhappy group. Moreover, the cobamide with 5-methoxy,6-methylbenzimidazole as the base was only detected in group U. From both Tables, the lower ligand base 2-methyladenine has the highest integrated peak area/HBAH/g value compared to rest of the lower ligand bases. This indicates that this base had the strongest signal being detected due to its high amounts.

Table 26: The extraction profiles of patient samples belonging to group H.

	Integrated peak area/HBAH/g								
Lower ligand bases	H2	H5	H7	H9	H10	H11	H12	H13	H15
Cobinamide	-	-	-	0.053	-	0.062	0.098	0.555	0.128
p-Cresole	-	0.018	-	0.010	-	0.014	0.025	0.135	0.110
Benzimidazole	-	-	-	-	-	-	-	0.006	-
3,4-Dimethyl phenol	-	-	-	0.008	-	0.021	0.055	0.028	0.023
2-amino-p-cresol	0.131	0.014	-	0.018	-	0.014	-	0.060	0.039
5-hydroxybenzimidazole	-	-	-	-	-	-	-	0.336	-
Adenine	0.208	0.047	0.362	0.050	-	0.307	0.312	0.395	0.202
5,6-dimethylbenzimidazole	0.008	-	-	-	-	0.357	0.082	0.046	0.012
2-methyladenine	2.345	0.935	1.107	0.839	0.139	2.379	4.537	2.492	1.170
1*H*-naphtho[2,3-d] imidazole	-	-	-	-	-	0.004	-	-	-
2-methylthioadenine	0.298	0.547	0.710	0.358	0.096	0.331	0.589	0.204	1.017

Again, quantification is not possible without standards but their prevalence of each cobamide can be looked at. This is seen in Table 27 and 28 where the percentage of each analogue for each patient sample can be compared. Across all samples, 2-methyladenine was seen to have the highest peak area in all samples except U3. The percentage for it within each sample is also the highest except for U3. For group U, the second most prevalent lower ligand base varies between, adenine, 5-6-dimethylbenzimidazole, and 2-methylthioadenine. However for group H, it is mainly 2-methylthioadenine except for H15 where it is adenine.

Table 27: Percentage of each detected analogue relative to the total cobamides detected within the same sample for group U samples.

	Percentage (%)								
Lower ligand bases	U1	U2	U3	U4	U5	U6*	U7	U9	U12*
Cobinamide	7.94	2.21	6.41	1.32	7.90	-	3.83	4.97	-
p-Cresole	1.93	0.26	0.72	-	2.29	-	0.68	1.20	-
3,4-Dimethyl phenol	1.42	0.62	0.83	0.60	0.64	0.46	-	0.18	-
2-amino-p-cresol	1.05	0.66	0.63	0.76	0.36	-	5.28	1.84	-
5-methylbenzimidazole	-	-	-	-	-	-	0.14	-	-
5-hydroxybenzimidazole	7.95	-	-	-	9.13	-	-	-	-
Adenine	23.80	4.52	11.16	3.58	9.95	16.27	6.08	5.16	11.38
5,6-dimethylbenzimidazole	3.09	0.84	54.68	2.27	7.31	5.28	22.92	8.91	-
2-methyladenine	36.89	75.79	10.21	52.23	53.75	65.31	52.32	69.35	78.45
5-methoxy,6-methylbenzimidazole	-	-	0.32	-	-	-	1.42	-	-
1*H*-naphtho[2,3-d] imidazole	-	-	0.85	-	0.21	-	0.49	0.33	-
2-methylthioadenine	15.93	15.09	14.18	39.25	8.46	12.68	6.84	8.05	10.17
Total	100								

* Note: The low variety of U6 and U12 detected could be due to low amount of sample (0.11 g and 0.19 g respectively).

Table 28: Percentage of each detected analogue relative to the total cobamides detected within the same sample for group H samples.

	Percentage (%)								
Lower ligand bases	H2	H5	H7	H9	H10	H11	H12	H13	H15
Cobinamide	-	-	-	3.93	-	1.77	1.72	13.03	4.72
p-Cresole	-	1.16	-	0.79	-	0.41	0.44	3.16	4.07
Benzimidazole	-	-	-	-	-	-	-	0.15	-
3,4-Dimethyl phenol	-	-	-	0.59	-	0.60	0.97	0.65	0.86
2-amino-p-cresol	4.38	0.89	-	1.34	-	0.39	-	1.41	1.43
5-hydroxybenzimidazole	-	-	-	-	-	-	-	7.89	-
Adenine	6.96	3.02	16.62	3.75	-	8.79	5.47	9.28	7.46
5,6-dimethylbenzimidazole	0.25	-	-	-	-	10.24	1.43	1.09	0.43
2-methyladenine	78.43	59.90	50.78	62.81	59.17	68.18	79.61	58.54	43.33
1*H*-naphtho[2,3-d] imidazole	-	-	-	-	-	-	0.12	-	-
2-methylthioadenine	10.17	9.98	35.03	32.60	26.79	40.83	9.49	10.34	4.80
Total	100								

Translating the data from Tables 25 and 26 into bar charts to compare each cobamide detected across all patient samples like in Figures 14-1 and 14-2. They show a clearer picture of any pattern observed. The same can be shown for all detected cobamides with varying lower ligand bases (lower ligand analogues).

i) Cobinamide

In this Figure, a more balanced chart is observed in comparison to Figure 14-1. The peak area in U3 is the highest in terms of signal strength however the percentage is at about 6% which indicates there must be another analogue with stronger signals. Overall, the percentage of cobinamide in group U and group H show no real pattern with H13 having the most in the sample.

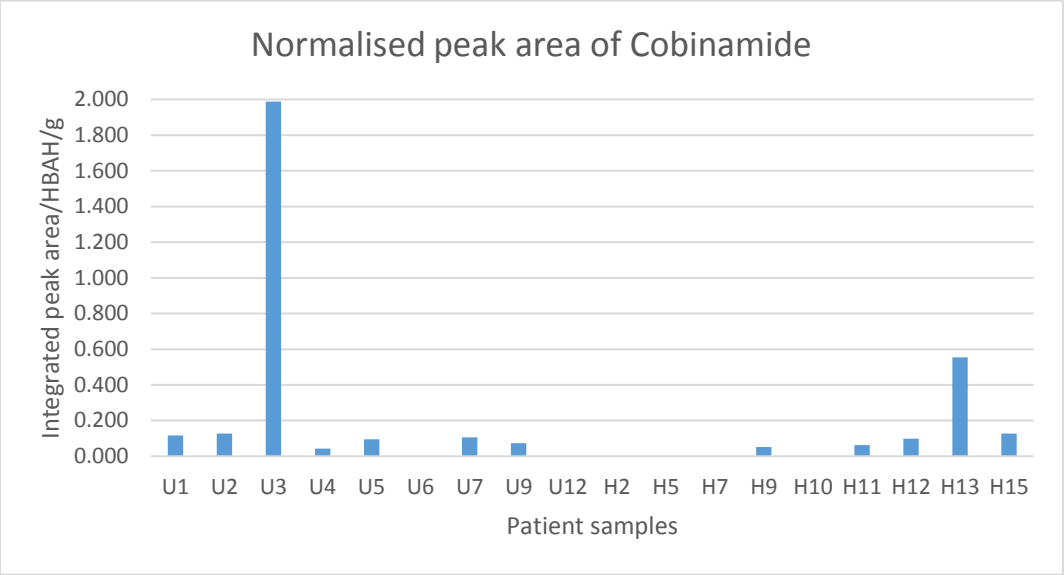


Figure 14-1: Integrated peak area/HBAH/g of cobinamide in each patient sample. No real pattern is observed. Sample U3 had the highest value while sample H13 was the highest amongst group H.

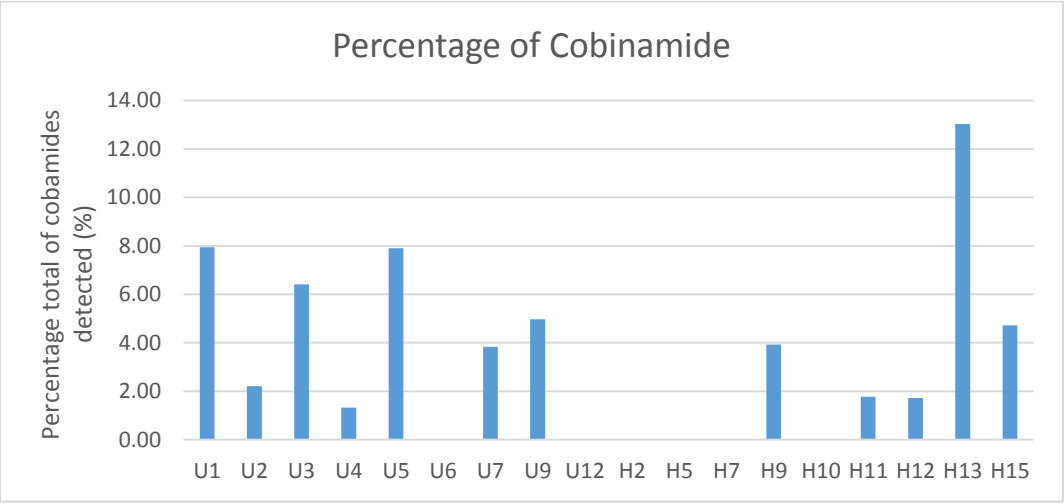


Figure 14-2: Prevalence of cobinamide as a percentage of the total cobamides detected within each sample.

ii) p-Cresole

The chart for p-cresole in Figure 15-1 shows that again, U3 has the highest peak area value while H13 had the highest value in group H. The percentage chart in Figure 15-2 shows that the percentage of this this cobamide is not as high as cobinamide. p-cresole had the highest prevalence in H15 at around 4%.

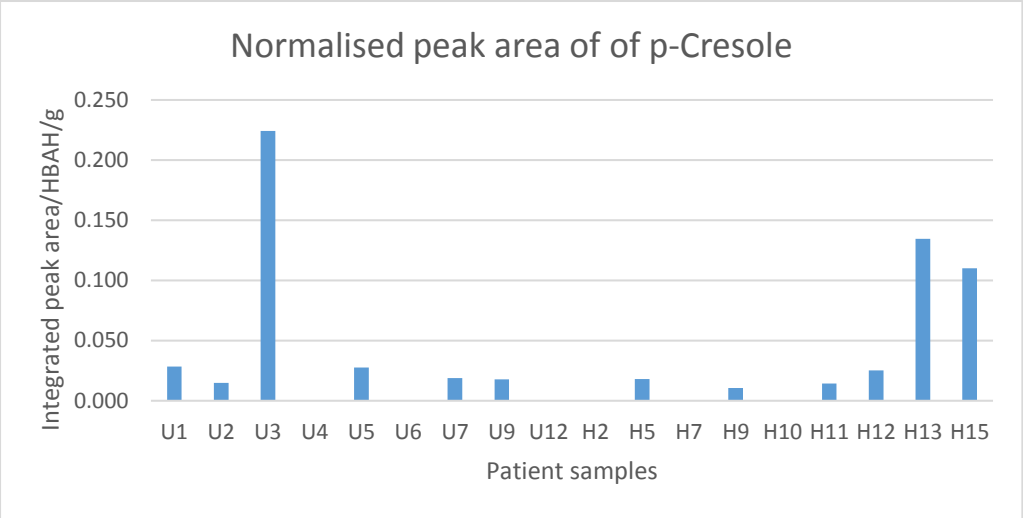


Figure 15-1: Integrated peak area/HBAH/g of p-cresole in each patient sample

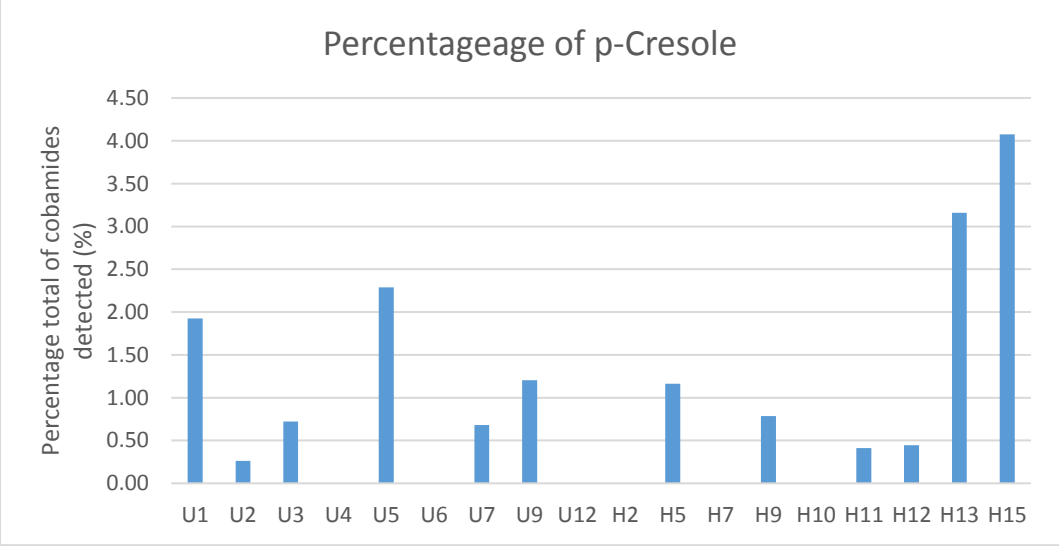


Figure 15-2: Prevalence of p-cresole as a percentage of the total cobamides detected within each sample .

iii) Benzimidazole

The cobamide with benzimidazole as its lower ligand base was only detected in H13 across all patient samples as seen in Figures 16-1 and 16-2. The peak area value is a lot lower than the values seen in cobinamide and p-cresole indicating how weak the signal for this cobamide was. Thus, as expected, the percentage for this cobamide is lower.

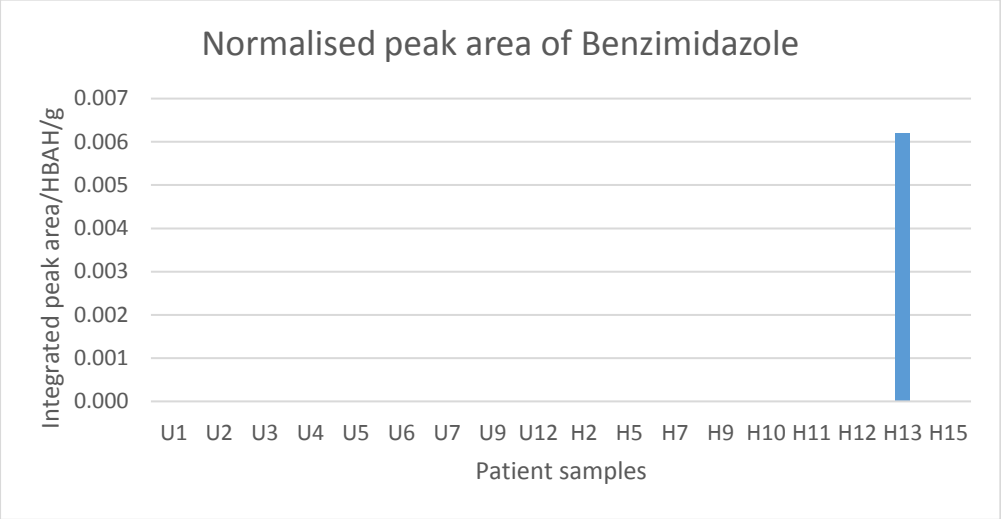


Figure 16-1: Integrated peak area/HBAH/g of benzimidazole in each patient sample

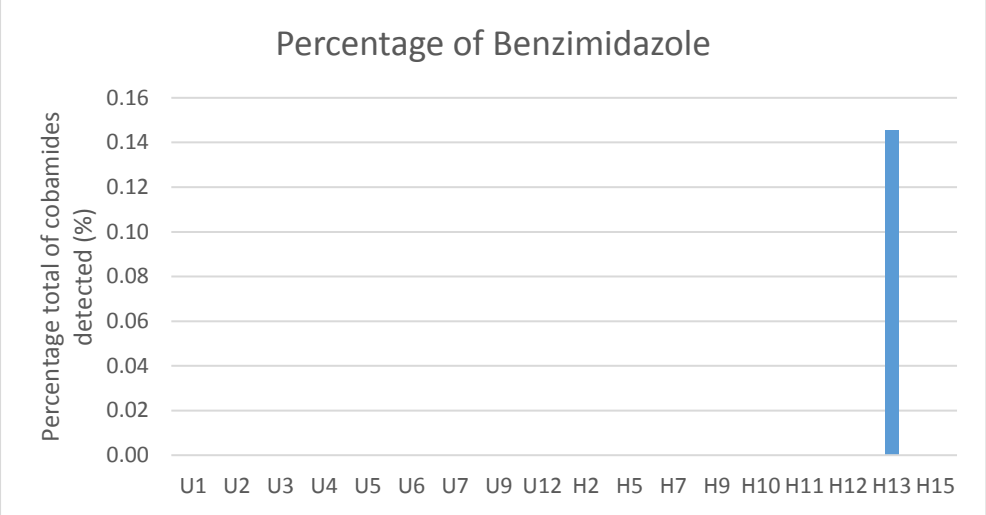


Figure 16-2: Prevalence of benzimidazole as a percentage of the total cobamides detected within each sample .

iv) 3,4-Dimethyl phenol

In Figures 17-1 and 17-2, U3 again has the highest value of 3,4-dimethyl phenol compared to the rest of the samples. In group H, H12 had the highest value. However, when looking at percentage of total cobamide, this analogue is most prevalent in U1 and in H12 for group H. Additionally, group H seems to have similar percentage with values from 0.60 to 1 % although more fluctuation is observed in group U.

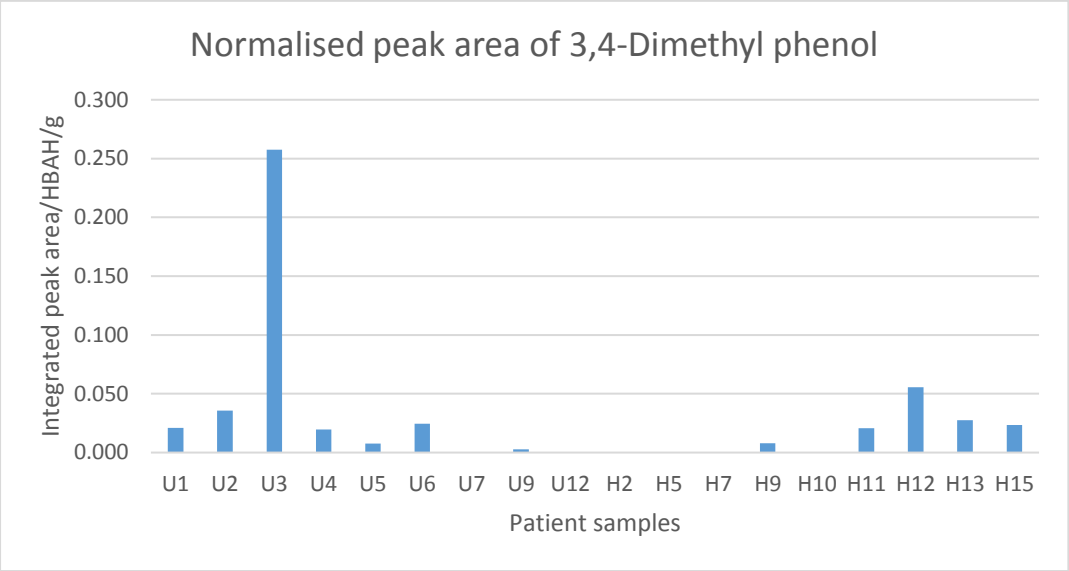


Figure 17-1: Integrated peak area/HBAH/g of 3,4-dimethyl phenol in each patient sample

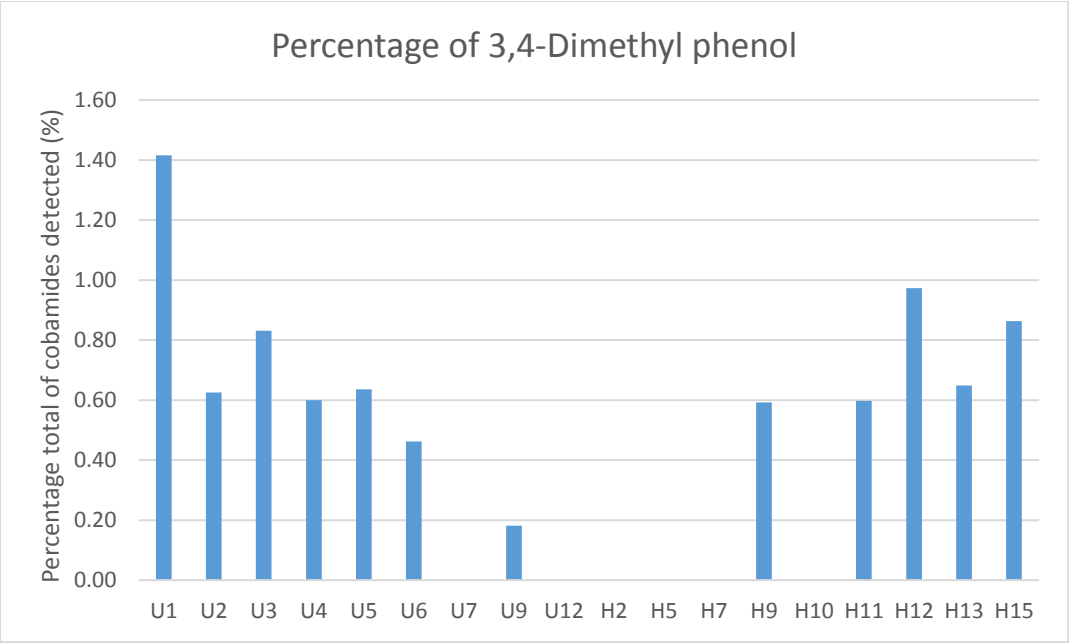


Figure 17-2: Prevalence of 3,4-dimethyl phenol as a percentage of the total cobamides detected within each sample

v) 2-amino-p-cresol

From Figure 18-1 and 18-2 no clear pattern can be observed between group U and H. A still consistent observation is that U3 also have the highest value for integrated peak area/HBAH/g across all samples. Fluctuations in percentage was observed and no clear pattern can be deduced.

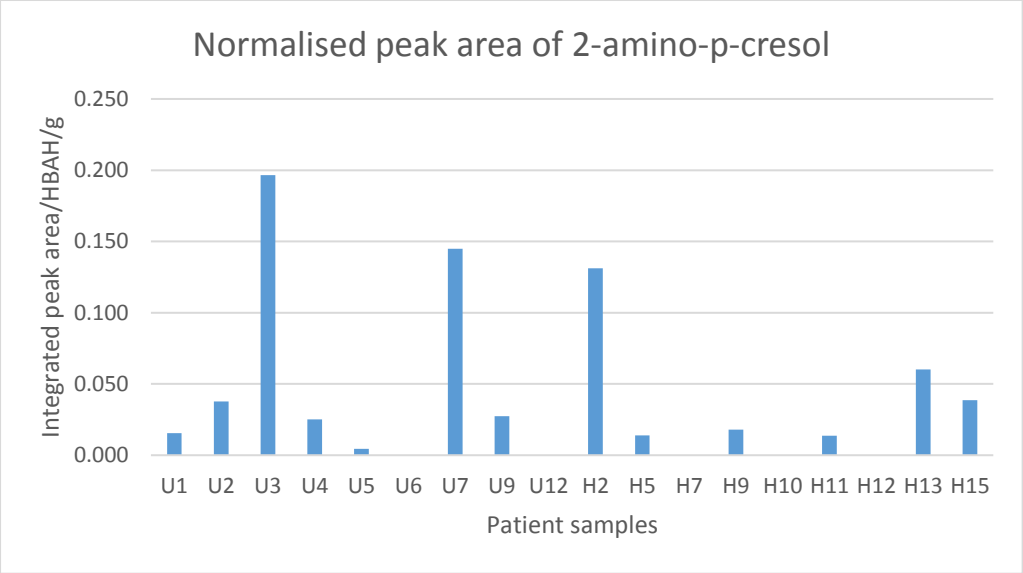


Figure 18-1: Integrated peak area/HBAH/g of 2-amino-p-cresol in each patient sample

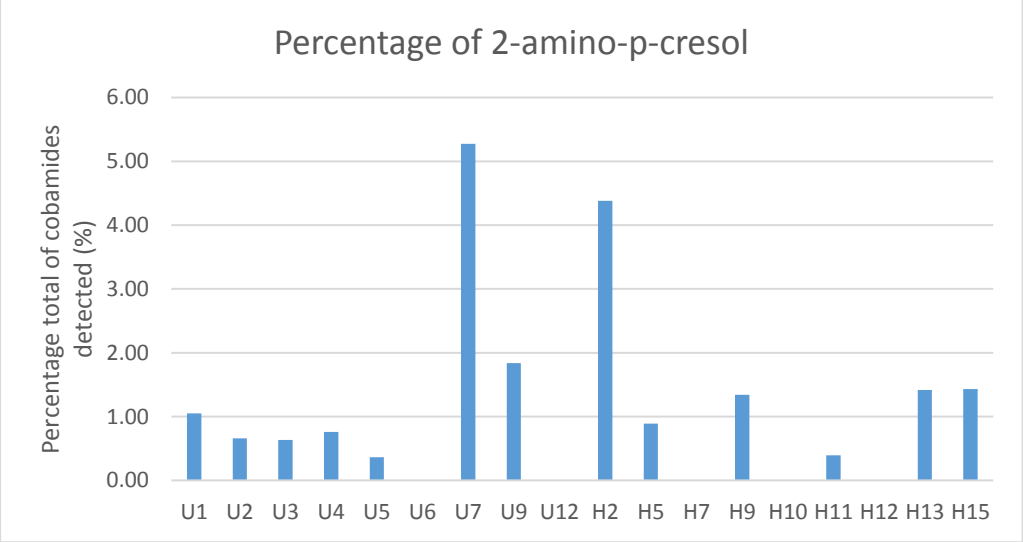


Figure 18-2: Prevalence of 2-amino-p-cresol as a percentage of the total cobamides detected within each sample .

vi) 5-methylbenzimidazole

This is another analogue where it is only detected in one sample in U7. Like benzimidazole mentioned above, the percentage and the peak area value seen in Figures 19-1 and 19-2 is very low compared to other analogues which could explain why it was only detected in one sample.

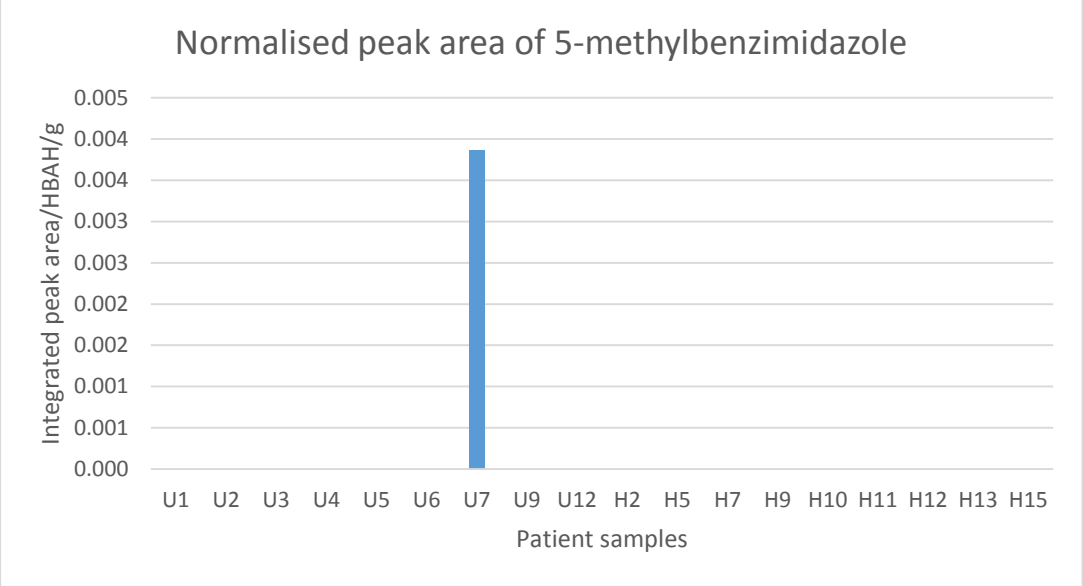


Figure 19-1: Integrated peak area/HBAH/g of 5-methylbenzimidazole in each patient sample

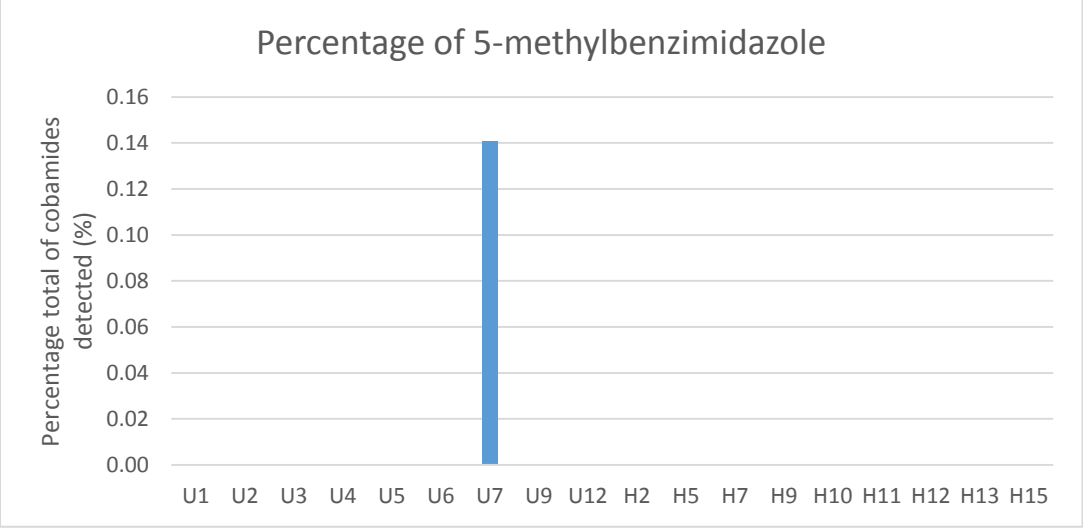


Figure 19-2: Prevalence 5-methylbenzimidazole as a percentage of total cobamides detected within each sample.

vii) 5-hydroxybenzimidazole

The frequency detected for this analogue as seen in Figures 20-1 and 20-2 is just 3, 2 of which are detected in group 2. The integrated peak area values are not as low as in those that were only detected once. Additionally, their percentage is around 8 to 9 %. This could indicate that weak signals are unlikely the cause of this low frequency of detection.

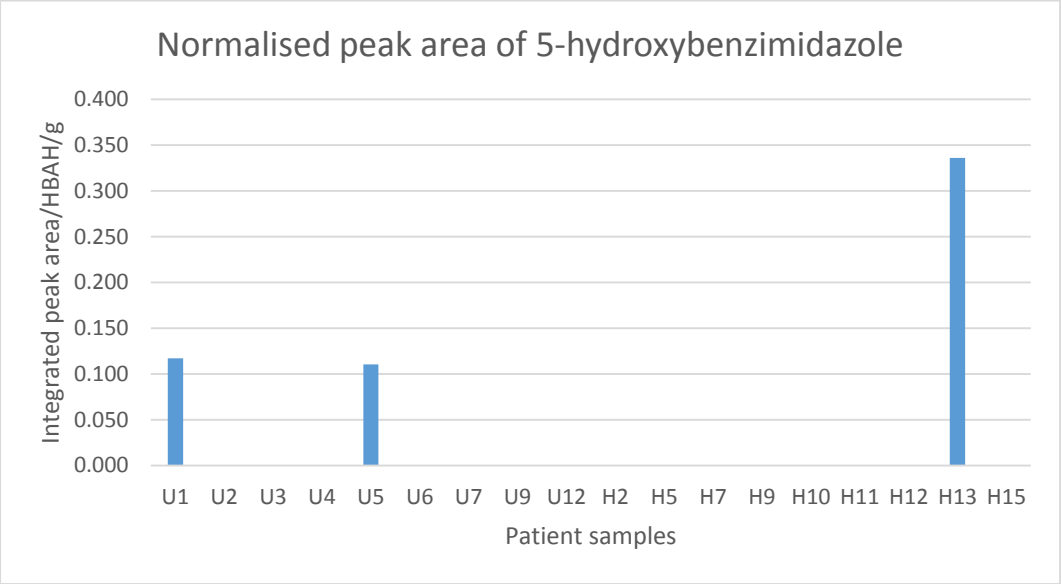


Figure 20-1: Integrated peak area/HBAH/g of 5-hydroxybenzimidazole in each patient sample

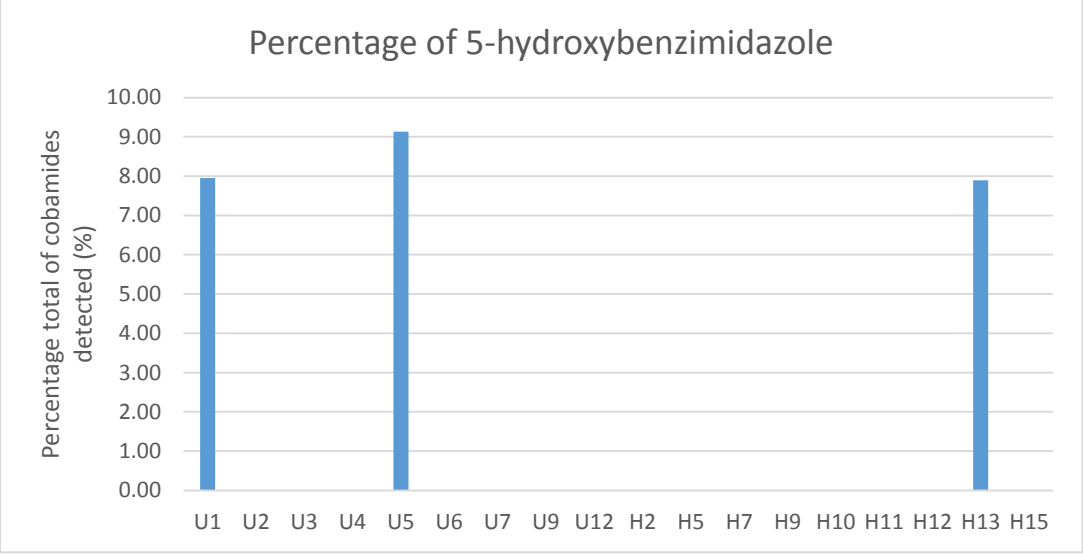


Figure 20-2: Prevalence of 5-hydroxybenzimidazole as a percentage of the total cobamides detected within each sample .

viii) Adenine

In Figures 21-1 and 21-2, U3 again has the highest integrated peak area/HBAH/g value but as a percentage, U1 is the highest. This analogue is detected in all patients except for H10. The percentage or integrated peak area/HBAH/g values of adenine fluctuates and do not display any obvious pattern.

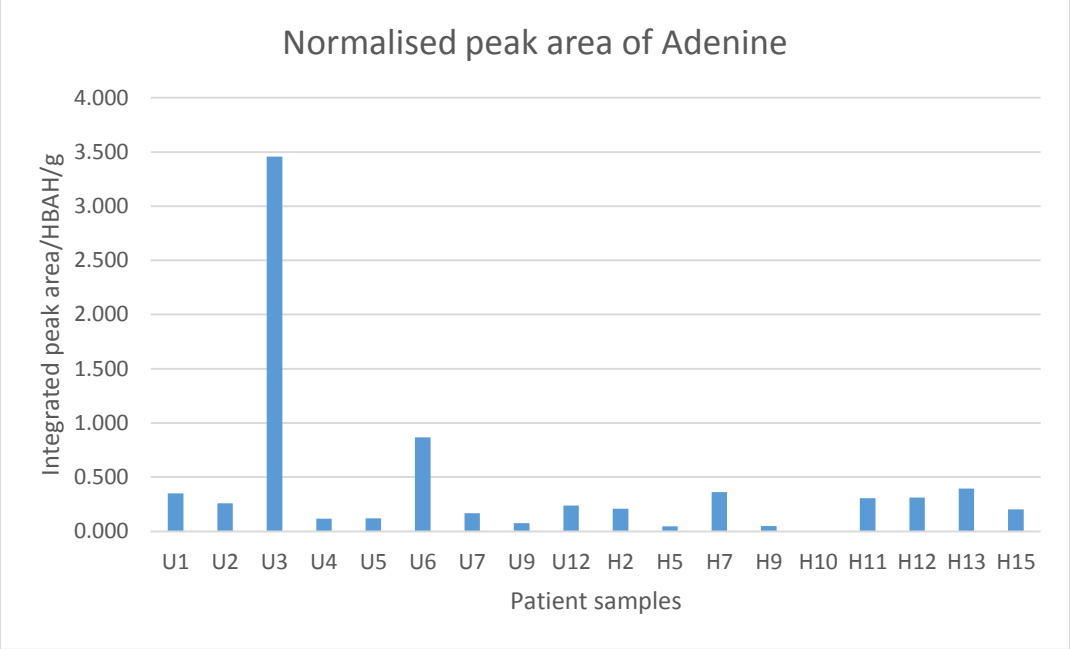


Figure 21-1: Integrated peak area/HBAH/g of adenine in each patient sample

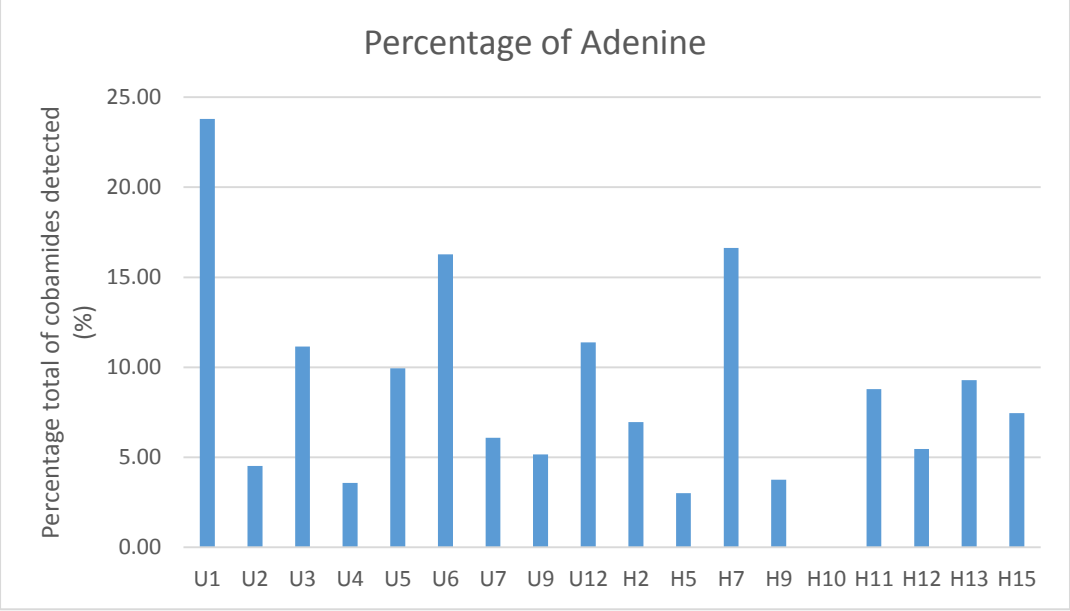


Figure 21-2: Prevalence of adenine as a percentage of the total cobamides detected within each sample.

ix) 5,6-dimethylbenzimidazole (cyanocobalamin)

This lower ligand is the usual base that is ‘useful’ for humans. In Figure 22-1, U3 again has the highest value. Due to the vast difference, U3 is removed in order to see the values of other samples clearer in Figure 22-2. It is observed that this analogue is more frequently detected in group U than group H. It is detected in 8 of 9 samples in group U and only 5 of 9 samples in group H. Furthermore, when looking at the percentage, in Figure 22-3 and 22-4, the group U percentage is higher than the group H percentage except for samples H11 and U2.

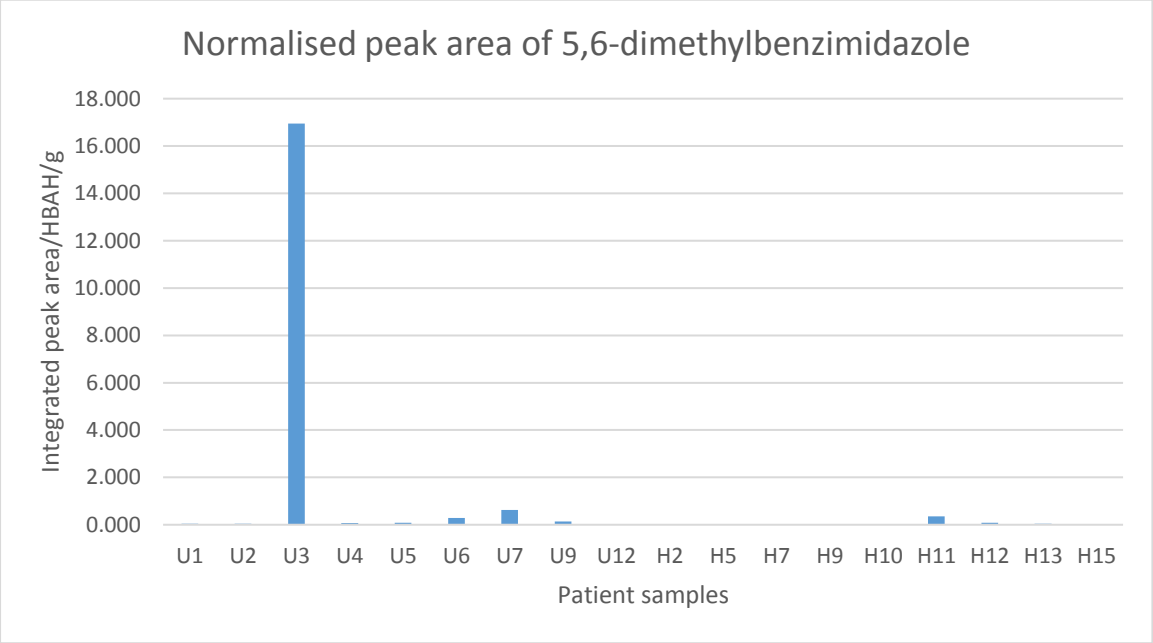


Figure 22-1: Integrated peak area/HBAH/g of 5,6-dimethylbenzimidazole in each patient sample

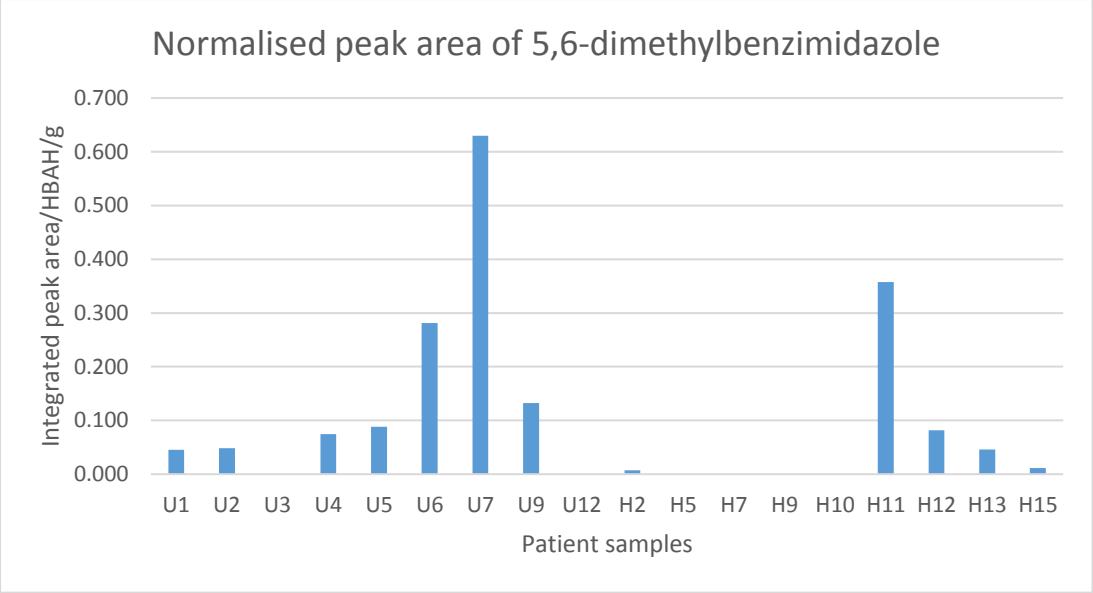


Figure 22-2: Integrated peak area/HBAH/g of 5,6-dimethylbenzimidazole in each patient sample. This is a chart without U3 to see the bars of other samples clearer due to the vast difference in value between U3 and the rest.

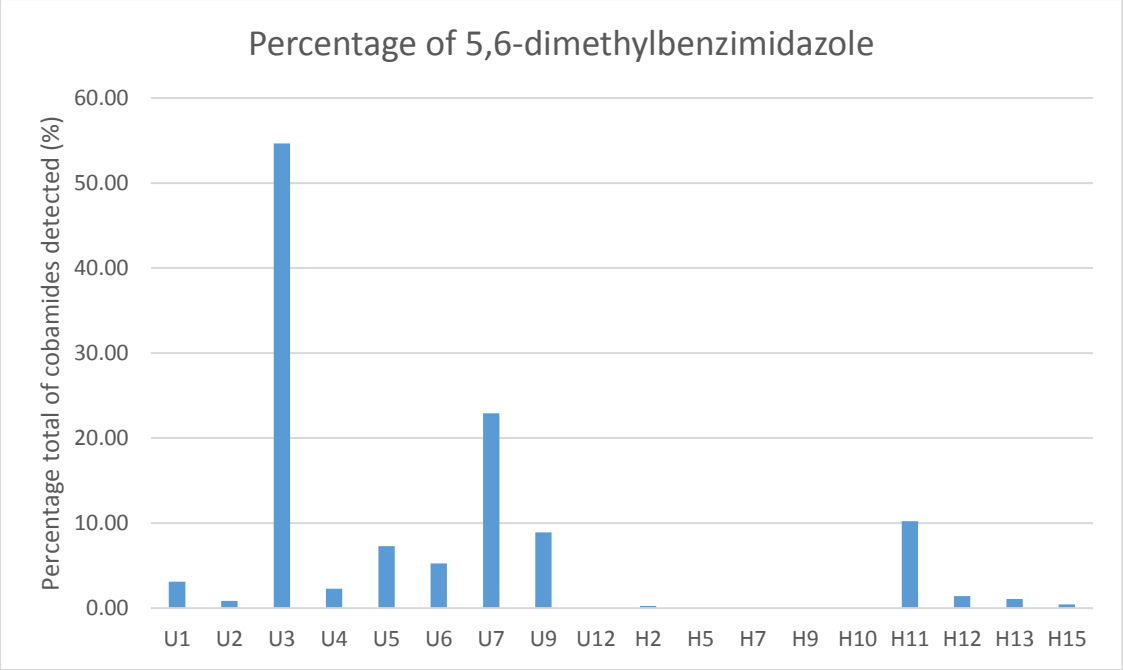


Figure 22-3: Prevalence of 5,6-dimethylbenzimidazole as a percentage of the total cobamides detected within each sample.

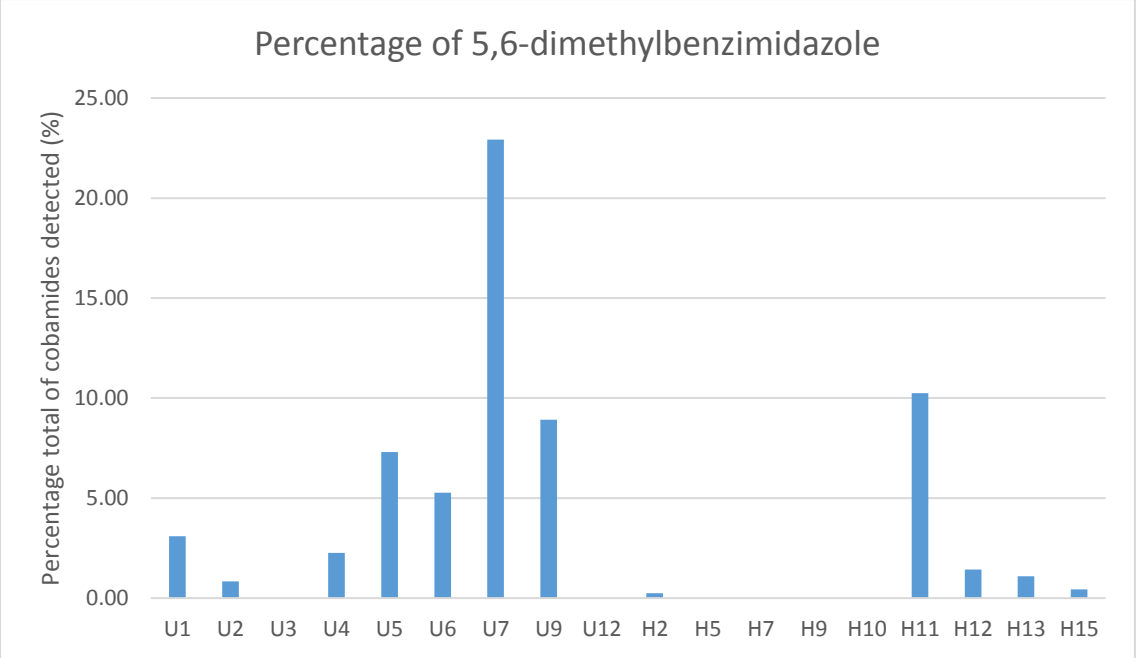


Figure 22-4: Prevalence of 5,6-dimethylbenzimidazole as a percentage of the total cobamides detected within each sample. This is a chart without U3 to see the bars of other samples clearer due to the vast difference in value between U3 and the rest.

Quantification of the amount of cyanocobalamin detected per gram in each sample

Tables 29-1 and 29-2 shows the amount of cyanocobalamin quantified for group U and H respectively. This is graphically presented in Figures 22-5 and 22-6. These Figures are similar to that of the integrated peak area/HBAH/g figures (Figure 22-1, 22-2). Nonetheless, the pattern observed remains similar.

Table 29-1: Amount of cyanocobalamin in ng in each gram of group U sample

Sample	U1	U2	U3	U4	U5	U6	U7	U9	U12
Amount per g (ng)	0.443	0.335	31.679	0.409	1.399	4.160	7.314	3.167	0

Table 29-2: Amount of cyanocobalamin in ng in each gram of group H sample

Sample	H2	H5	H7	H9	H10	H11	H12	H13	H15
Amount per g (ng)	0.066	0	0	0	0	3.518	0.514	0.149	0.156

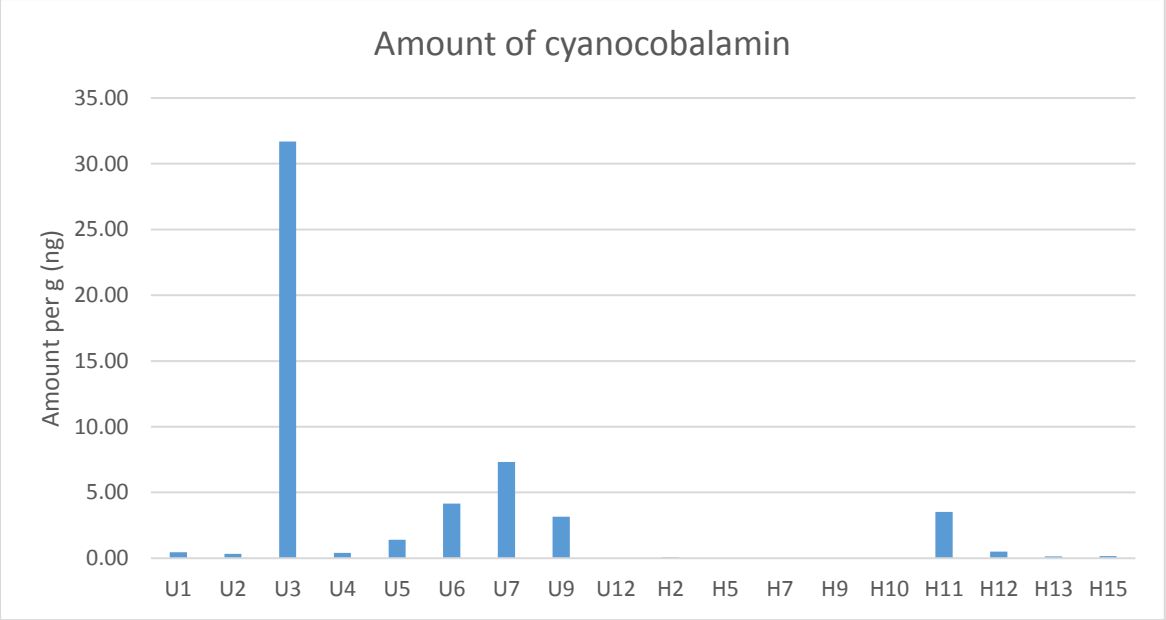


Figure 22-5: Amount of cyanocobalamin quantified for each patient sample

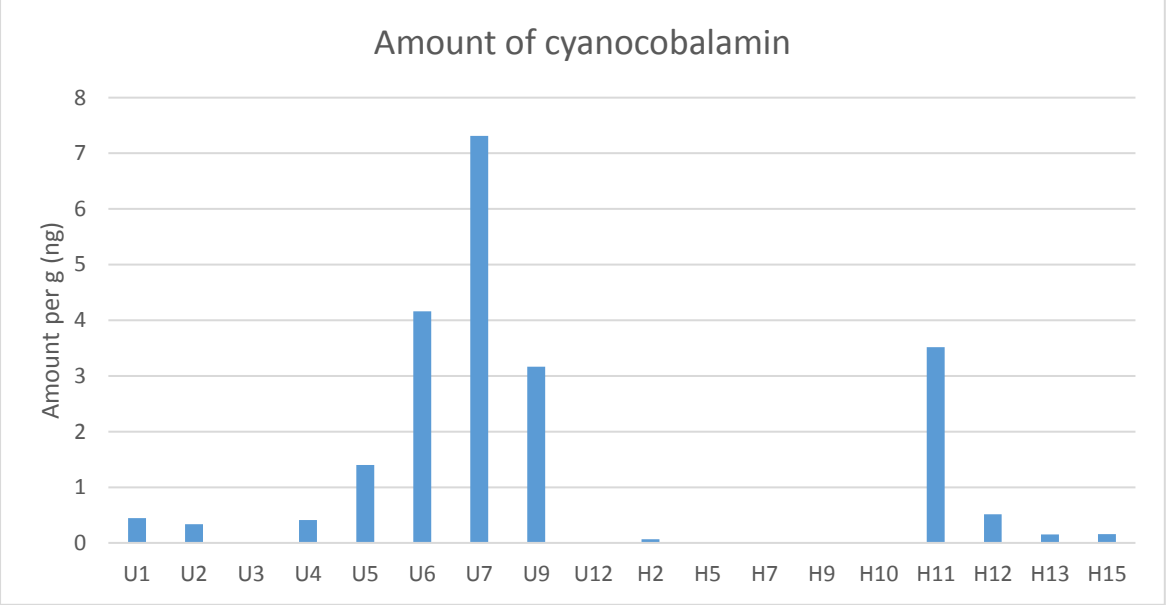


Figure 22-6: Amount of cyanocobalamin quantified for each patient sample. This is a chart without U3 to see the amount present in other samples due to the vast difference in value between U3 and the rest.

x) 2-methyladenine

2-methyladenine is detected in all samples and its integrated peak area/HBAH/g value is the highest compared to other analogues except for U3 seen in Figure 23-1. Looking at both Figures 23-1 and 23-2, there does not seem to be any observable differences between group U and group H but the similarity is that this analogue is the most prevalent with the highest percentage.

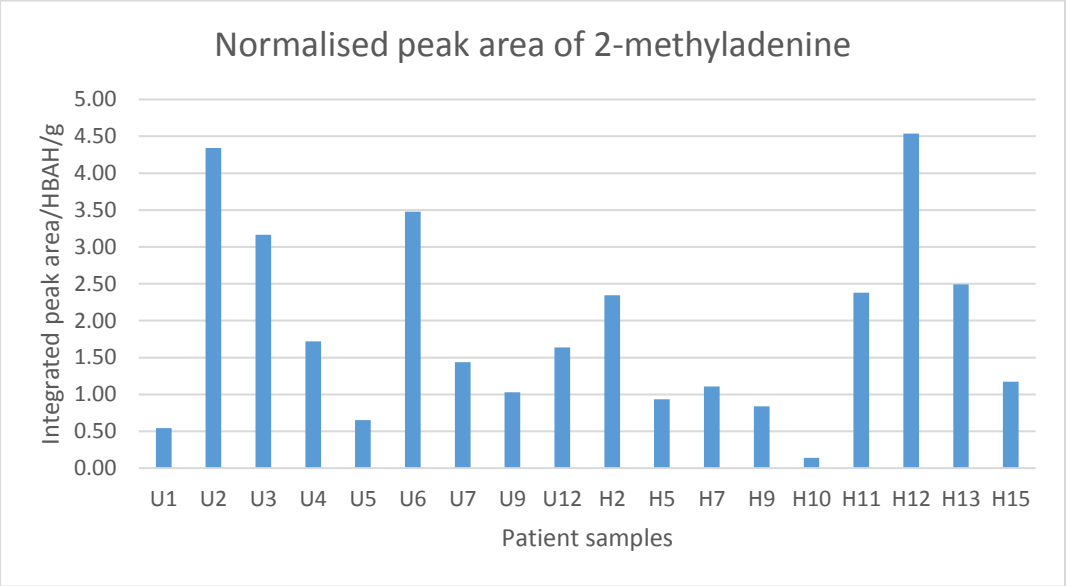


Figure 23-1: Integrated peak area/HBAH/g of 2-methyladenine in each patient sample

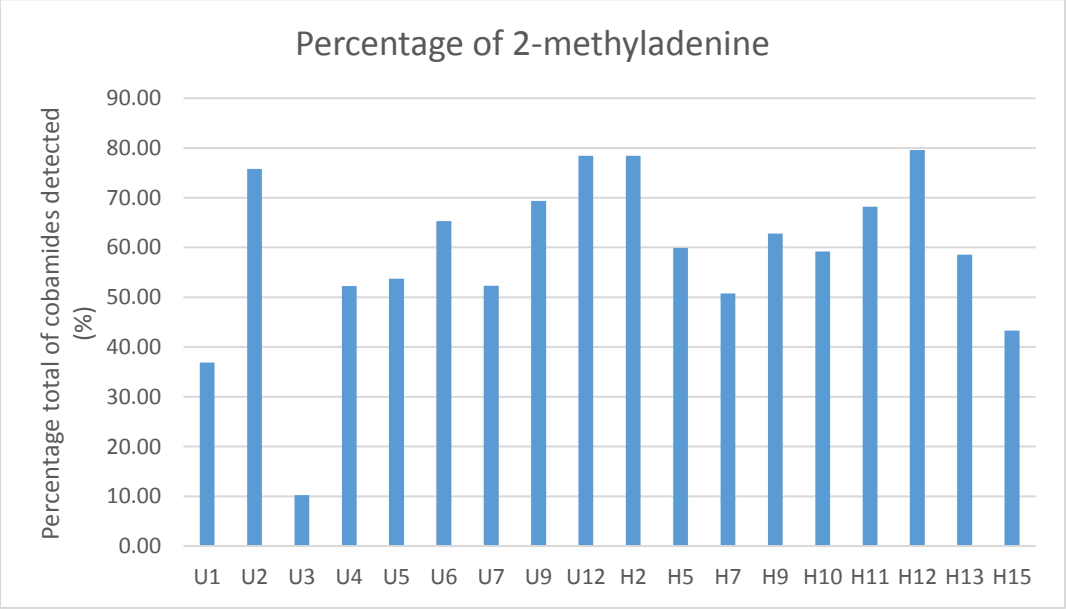


Figure 23-2: Prevalence of 2-methyladenine as a percentage of the total cobamides detected within each sample

xi) 5-methoxy,6-methylbenzimidazole

This analogue is only detected in group U patients. The integrated peak area/HBAH/g values in Figure 24-1 is not as low as in 5-methylbenzimidazole or benzimidazole however it is lower when compared to 5-hydroxybenzimidazole. Thus, the weak signal could be a reason why this compound is not more often detected. This is supported by looking at the percentage (Figure 24-2). This analogue represents only 1.4 % for U7 and 0.3% for U3 which implies there are very low amounts of this analogue in the sample to begin with.

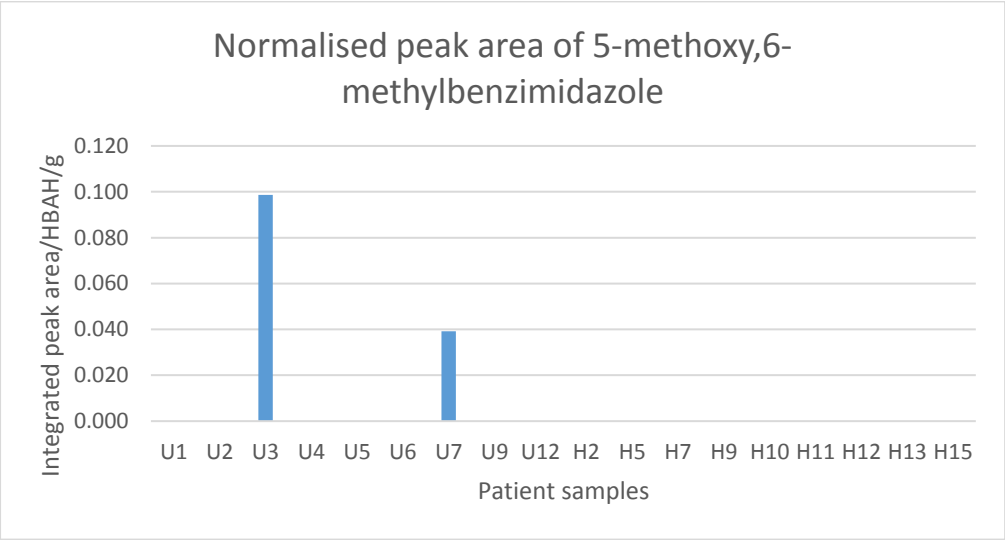


Figure 24-1: Integrated peak area/HBAH/g of 5-methoxy, 6-methylbenzimidazole in each patient sample

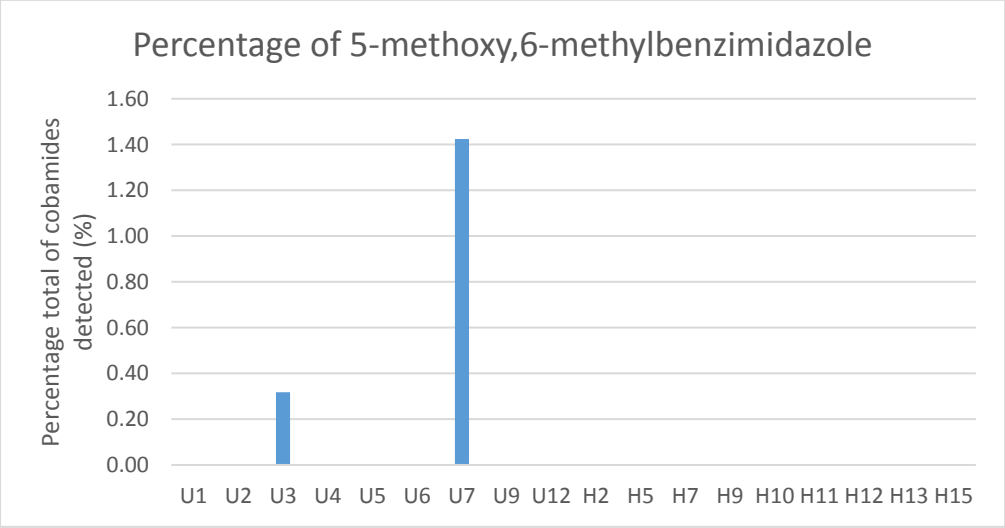


Figure 24-2: Prevalence of 5-methoxy,6-methylbenzimidazole as a percentage of the total cobamides detected within each sample

xii) 1*H*-naphtho[2,3-d] imidazole

This analogue is detected more frequently in group U than in group H. As seen in Figure 25-1, it is seen in 4 samples in group U while it only appears once in group H. U3 again has the highest value for the integrated peak area/HBAH/g for this analogue. Additionally, U3 has the highest percentage compared to other samples seen in Figure 25-2. The percentage value in group U is higher than the group H samples.

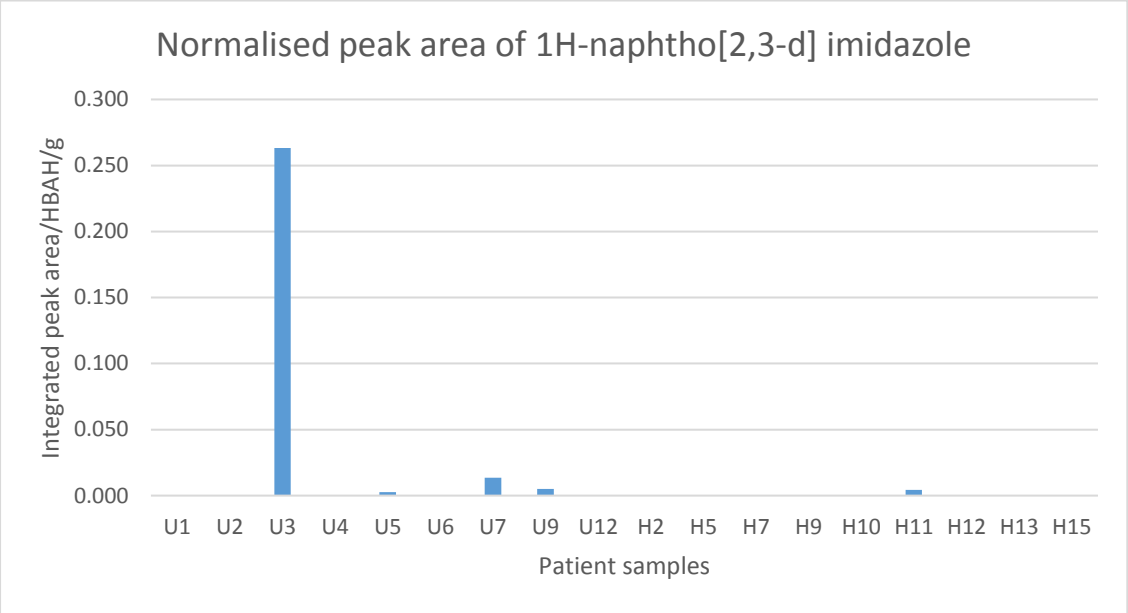


Figure 25-1: Integrated peak area/HBAH/g of 1*H*-naphtho[2,3-d] imidazole in each patient sample

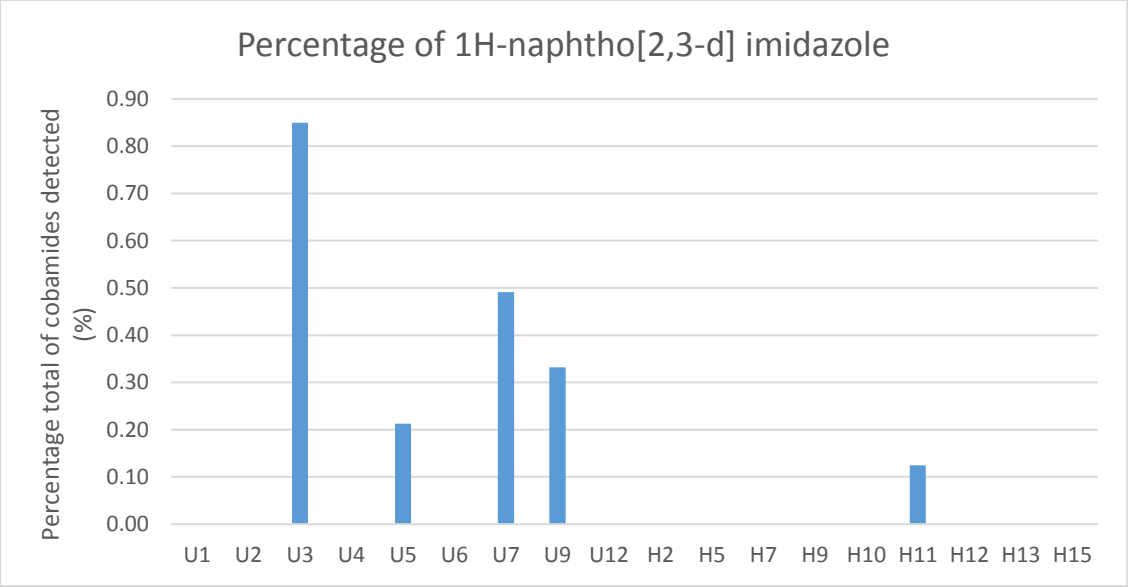


Figure 25-2: Prevalence of 1*H*-naphtho[2,3-d] imidazole as a percentage of the total cobamides detected within each sample

xiii) 2-methylthioadenine

This analogue is detected in every sample as seen in Figure 26-1. Again, U3 has the highest integrated peak area/HBAH/g value compared to the rest of the samples. Considering the percentage values in Figure 26-2, most of group H samples had higher percentages than group U samples except for U4.

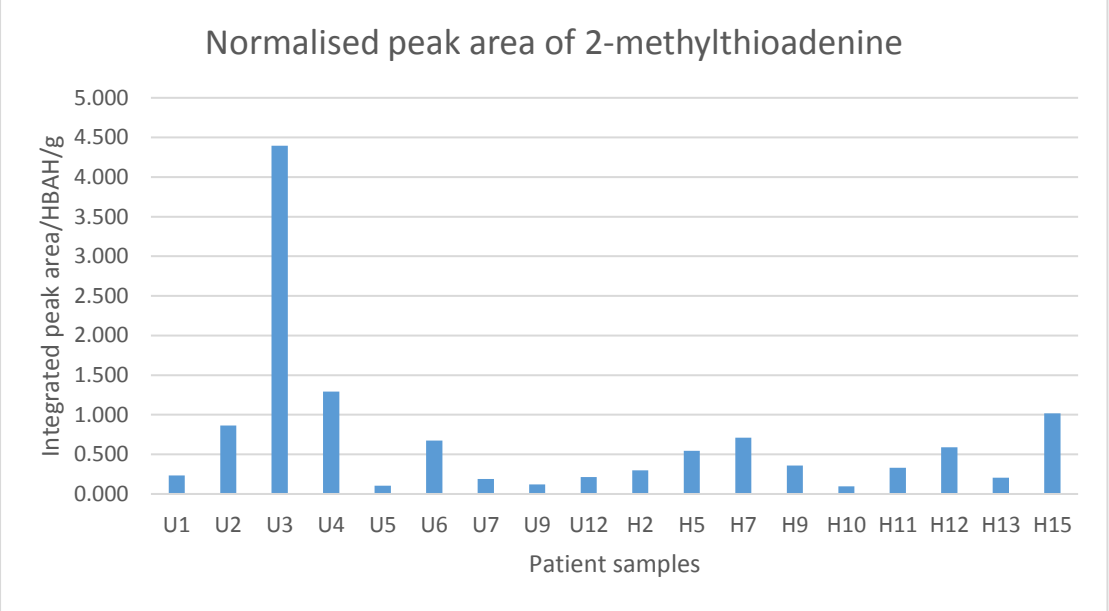


Figure 26-1: Integrated peak area/HBAH/g of 2-methylthioadenine in each patient sample

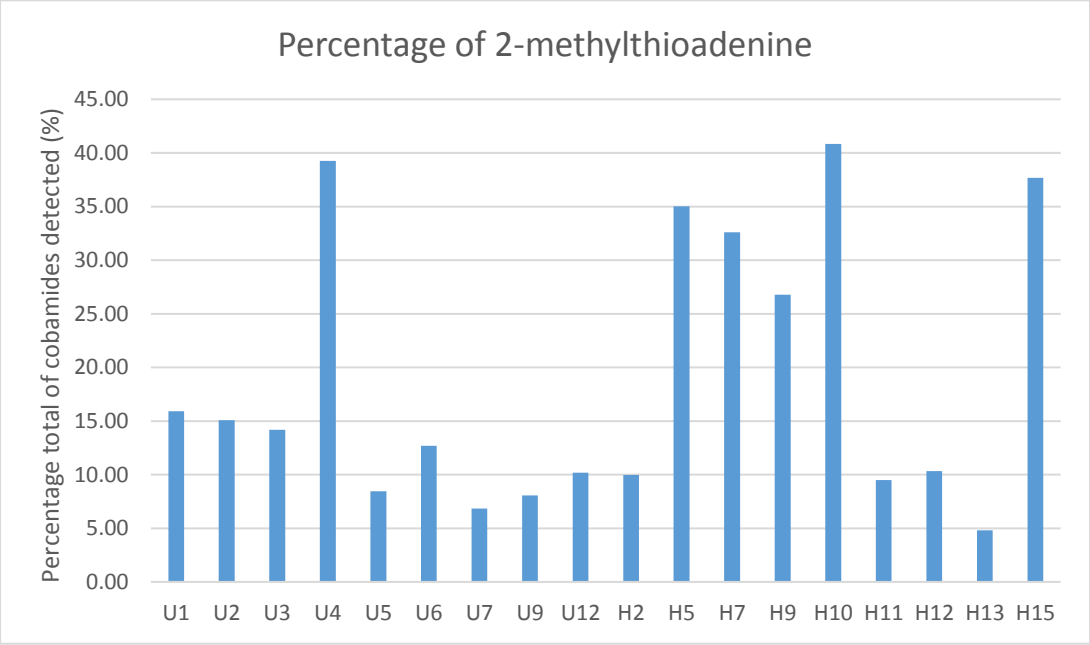


Figure 26-2: Prevalence of 2-methylthioadenine as a percentage of the total cobamides detected within each sample

7.5.1 Summary of human faecal sample results

In terms of percentage across all samples, the cobamide with 2-methyladenine as its lower ligand base has the highest prevalence except for patient U3's sample. The second most prevalent cobamide for group U (unhappy) and H (happy) is different. In group U samples, it can be cobamides with either adenine (4 samples), 5-6-dimethylbenzimidazole (2 samples), or 2-methylthioadenine (3 samples) as lower loop bases. For group H samples, it is mainly 2-methylthioadenine (7 samples).

One recurring observation is that sample U3 has the largest normalised peak area values for several cobamides compared to rest of the samples (Table 25, highest for 7 of the 13 detected analogues – cobinamide, p-cresole, 3,4-dimethyl phenol, 2-amino-p-cresol, adenine, 5,6-dimethylbenzimidazole, and 2-methylthioadenine). Despite this, the percentage prevalence of these analogues was not the highest. Another interesting observation about sample U3 is that the cobamide with 2-methyladenine as its base is not the most prevalent analogue contrary to rest of the patient samples (Figure 23-2). On top of this, the percentage value for U3 is much lower than the rest at 10% while the rest was above 30% (Figure 23-2).

Another observation is the low frequency of detection for analogues belonging to the benzimidazoles. Six in total were detected: benzimidazole, 5-methylbenzimidazole, 5-hydroxybenzimidazole, 5,6-dimethylbenzimidazole, 5-methoxy,6-methylbenzimidazole, and 1*H*-naphtho[2,3-d] imidazole. Of these, only 5-6-dimethylbenzimidazole was detected in more than 3 samples. The benzimidazole analogue was detected only in sample H13 which was barely present at 0.14% (Figure 16-2). 5-methylbenzimidazole was only detected in U7 also at a percentage of 0.14% (Figure 19-2). 5-hydroxybenzimidazole was detected in 3 samples (U1, U5, and H13) but at a much higher percentage level of 8-9% (Figure 20-2). 5-methoxy,6-

methylbenzimidazole was detected in 2 samples in group U (U3, and U7) representing 0.3 and 1.4 % of total cobamides present in these samples respectively (Figure 24-2). 1*H*-naphtho[2,3-d] imidazole was only present in 5 samples all at less than 0.90 % prevalence (Figure 25-2). Therefore, not only is this group of analogues occur remarkably infrequently, it is also present at very low levels except for 5-hydroxybenzimidazole.

The 'useful' base for humans, 5-6-dimethylbenzimidazole (cyanocobalamin) was detected more frequently in group U than group H. It also had higher prevalence values detected in group U than group H samples (excluding H11 and U2 due to low sample weight). The quantification of this analogue revealed no new patterns however when analysed with the patients' profiles in Table 24, it can be seen that taking additional supplements affects the amount of this cobamide in their faeces. All the patients taking additional supplements had higher amounts of cyanocobalamin in their faecal samples (U3, U4, U5, U7, and H11 – Figures 22-5 and 22-6). Patients taking both a tablet containing Vitamin B_{12} and a boost sublingual spray is observed to have higher amounts of cyanocobalamin in their faeces. U3 had the highest amount and the patient take both multi-vitamin (with vitamin B_{12}) and sublingual spray. Likewise, U7 and H11 takes methylcobalamin tablets and sublingual spray and has a higher amount of cyanocobalamin detected than patients who took only tablet or spray. The patient U4 who only took sublingual spray had no higher amounts of cyanocobalamin detected than some patients who took no additional supplements (lower than U1, U9, and H12). Finally, U5 only takes Vitamin B_{12} tablets and is shown to have higher amounts of cyanocobalamin detected compared to U4 and those who did not take any supplements except U9.

Other observations include the higher frequency of detection of analogue with base 1*H*-naphtho [2, 3-d] imidazole in group U than H samples (4 times in group U and 1 time in group H).

Lastly, the percentage of the 2-methylthioadenine found in group H is generally higher than that found in group U. As observed in this experiment, 5 of the 9 samples from group H had more than 25 % of total cobamides as 2-methylthioadenine while only 1 of 9 sample from group U was above this level.

7.6 Animal tissue samples

Adapting the faecal sample extraction protocol for extraction of cobamides in tissue samples. Three mice liver and kidney samples were sent to determine the types of lower ligand analogues present in these tissues. Initial testing of method was done with lamb liver before extraction of mice samples.

7.6.1 Method testing with lamb liver

Extraction procedure tested with lamb liver to determine the difference between presence and absence of 50 % methanol in the extraction process. Table 30 below shows the types of analogues detected and its normalised peak area values with and without methanol. It is observed that both procedures have the same variety of cobamides detected. However, the normalised peak area values are all higher in the extraction procedure without methanol. Additionally, the amount of 5,6-dimethylbenzimidazole was quantified. The sample with methanol in the extraction process had 43 ng of cyanocobalamin per gram while the sample without methanol had 66 ng per gram. This could indicate that the presence of methanol in the extraction solvent reduced the amount of cobamides released or captured by the resin. Thus, the mice kidney and liver samples were extracted without methanol as the extraction solvent.

Table 30: The lower ligand bases detected in liver samples from extraction procedure with and without 50 % v/v methanol.

	Integrated peak area/HBAH/g (normalised peak area)	
Lower ligand base	**With 50 % v/v methanol**	**Without methanol**
5-hydroxybenzimidazole	0.058	0.158
5,6-dimethylbenzimidazole	1.809	4.189
1*H*-naphtho[2,3-d] imidazole	0.065	0.101

7.6.2 Mice Liver

Table 31-1 below shows the different analogues detected within the sample and their respective normalised peak area values. It is observed that the type of analogues detected are the same in all 3 samples and the normalised peak area values are similar. The prevalence of each analogue in each liver sample also shows similar values as the percentages are just within 2 % of each other (Table 31-2). Lastly, 5,6-dimethylbenzimidazole is the most prevalent analogue in each sample.

Table 31-1: The lower ligand bases detected in 3 mice liver samples and their normalised peak area values

	Integrated peak area/HBAH/g		
Lower ligand base	**Mice 1**	**Mice 2**	**Mice 3**
Adenine	0.019	0.007	0.011
5,6-dimethylbenzimidazole	1.060	1.018	1.339
2-methyladenine	0.066	0.044	0.067
1*H*-naphtho[2,3-d] imidazole	0.034	0.037	0.047
2-methylthioadenine	0.025	0.018	0.027

Table 31-2: The lower ligand bases detected in 3 mice liver samples and the prevalence of each analogue as a percentage of the total cobamides detected within each sample

	Percentage (%)		
Lower ligand base	**Mice 1**	**Mice 2**	**Mice 3**
Adenine	1.56	0.65	0.74
5,6-dimethylbenzimidazole	88.02	90.56	89.84
2-methyladenine	5.49	3.90	4.50
1*H*-naphtho[2,3-d] imidazole	2.83	3.33	3.14
2-methylthioadenine	2.10	1.56	1.78
Total	100		

7.6.3 Mice Kidney

All kidney samples are observed to have the same type of cobamides detected as seen in Table 32-1. Their normalised peak area values are not as similar as they are in the liver samples. This is reiterated in Table 32-2, where bigger percentage differences between the analogues are seen in the kidney samples than the liver samples.

Table 32-1: The lower ligand bases detected in 3 mice kidney samples and their normalised peak area values

	Integrated peak area/HBAH/g		
Lower ligand base	**Mice 1**	**Mice 2**	**Mice 3**
Adenine	0.013	0.014	0.008
5,6-dimethylbenzimidazole	0.413	2.317	0.593
2-methyladenine	0.030	0.067	0.042
1*H*-naphtho[2,3-d] imidazole	0.017	0.124	0.036
2-methylthioadenine	0.006	0.012	0.017

Table 32-2: The lower ligand bases detected in 3 mice kidney samples and the prevalence of each analogue as a percentage of the total cobamides detected within each sample

	Percentage (%)		
Lower ligand base	**Mice 1**	**Mice 2**	**Mice 3**
Adenine	2.70	0.53	1.19
5,6-dimethylbenzimidazole	86.14	91.45	85.28
2-methyladenine	6.36	2.63	5.98
1*H*-naphtho[2,3-d] imidazole	3.54	4.90	5.17
2-methylthioadenine	1.26	0.49	2.38
Total	100		

7.6.4 Quantifying cyanocobalamin

Further analysis is done for the mice liver and kidney samples by quantifying the amount of cyanocobalamin present in each sample shown in Figure 27. This figure shows that the amount of cyanocobalamin present in liver are far higher than in kidney. Moreover, liver samples show similar amounts of the 5,6-dimethylbenzimidazole analogue to each other compared to the kidney samples. This value however, is just

indicative since it does not account for their sample weight. Thus, the difference in levels could differ.

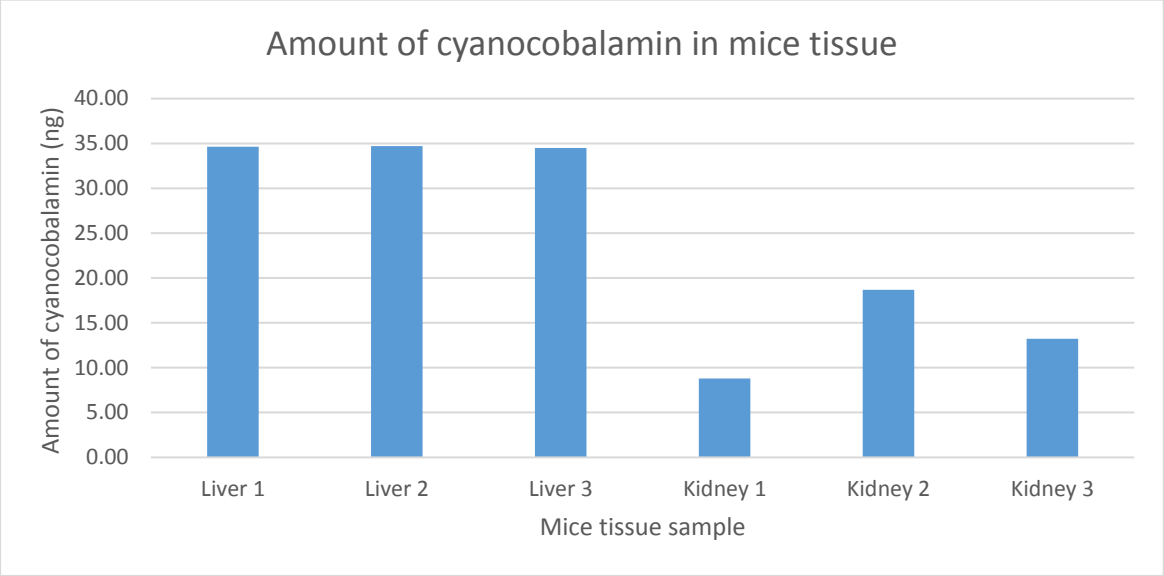


Figure 27: Comparison between the amounts of cyanocobalamin detected in all mice tissue samples

7.6.5 Summary of results from animal tissue testing

The adapted procedure for cobamide extraction enabled the detection of various lower ligand bases present in the tissue samples. Presence of methanol decreased the normalised peak area values and does not need to be part of the extraction solvent. Similarities are observed between the liver and kidney samples. The types of cobamides detected and the most prevalent analogue (5,6-dimethylbenzimidazole) for both mice liver and kidney tissue are identical. However, the prevalence of each cobamide is different. Bigger percentage differences between the analogues are seen in the kidney samples than the liver samples. The quantification of 5,6-dimethylbenzimidazole showed that liver tissues have a much higher and more consistent amount of cyanocobalamin than kidney samples. These could have indication into the usage of cobalamin in these tissues for mice.

7.7 Animal Serum samples

Horse and mice serum samples were sent to determine the type of analogues that are present in the sample. The extraction procedure adapted for the serum did not detect any cobamides in the horse samples. No signals for any cobamide masses searched for was detected. One of the three mice serum samples had detected levels of only the 5,6-dimethylbenzimidazole analogue. Shown in Figure 28, the intensity of the signal is low, the presence of a peak at the mass for this analogue indicates that the sample might contain traces of this analogue. The amount quantified using the standard curve for this peak is 0.061 ng/mL or 61 pg/mL. More sample volume or a more sensitive method may be needed to capture the cobamides in serum samples to increase the detection of other possible analogues and improve the signal intensity.

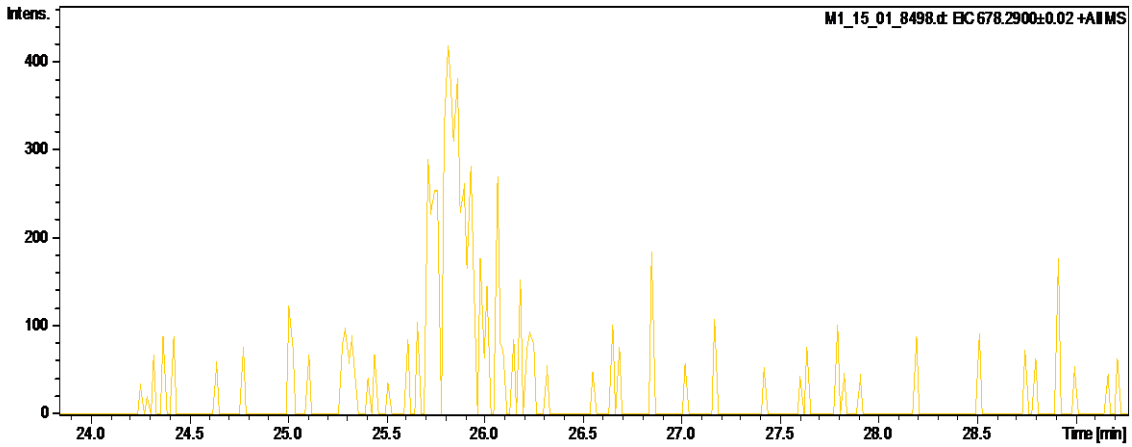


Figure 28: HPLC-MS of mice serum sample. A peak is seen at the associated mass of 5,6-dimethylbenzimidazole (678.29).

8. Discussion

This project tested the cobamide extraction procedure and used it on different specimens. It is tested primarily on faecal samples with a view towards the microbiome. Faecal samples from various animals (hedgehog, rabbit, and horses) were obtained for testing and optimisation of the extraction protocol. Subsequently, it was used to investigate the effect of supplementation on the cobamide extraction profiles of racehorses' microbiome. Next, the procedure it was used to analyse human faecal samples with an aim to find any relationship between the treatment responses of Vitamin B_{12} deficient patients and the types of cobamides detected. Lastly, this procedure was adapted for use on tissue and serum samples.

8.1 Cobamide trial extraction procedure for faecal samples

Although a larger sample size from different rabbits and hedgehogs would be required to confirm observed differences between them, the method testing showed that this procedure worked well for faecal samples and was able to capture a variety of cobamides for detection. The percentage difference between 2-methyladenine and 2-methylthioadenine in hedgehogs is way lesser than that in rabbits. This could be explained by their difference in gut microbiome or different dietary habits. For instance, hedgehogs belong to the erinaceidae family and are not well documented to practice coprophagy like rabbits (44). This practice could explain why hedgehogs have more than twice the amount of cyanocobalamin in their faecal samples (Tables 14 and 17). Coprophagy allows rabbits to consume cobalamin produced by their gut bacteria that are present in cecotropes. These results in less cobalamin being present in the faecal pellets (49). Moreover, the frequency of coprophagy is affected by diet and thus, cobamide rich pellets will less likely be consumed (32).The difference between the pet and wild rabbit could be due to their different dietary habits and again, would require

a larger sample size to confirm that the differences are true (Table 17). This could be an area for future studies, whereby the effect of different dietary habits on the cobamide levels present in faeces is investigated. This could open doors into finding ways in controlling the gut microbiome in those organisms.

Due to availability of samples, horse faecal samples were used to optimise the extraction protocol after method testing. Two factors, mass of sample and incubation time with His-BtuF nickel resin was explored. It showed that faecal samples less than 2 g will yield inconsistent results and less number of cobamides will be detected by the HPLC-MS (Table 18). Moreover, His-BtuF resin is stable when incubated with the supernatant of faecal samples and needed to be incubated more than 3 hours to allow more binding to occur between the cobamides and the resin (Table 20). This allows more consistent and reliable results to be obtained.

8.2 Racehorse faecal samples

Supplementation of racehorses with hydroxocobalamin does not seem to have a significant effect on the types of cobamides present in the racehorses' faecal samples. No significant difference or pattern was noted between the supplemented and control group racehorses when comparing the normalised peak area and percentage prevalence values. There are only two observed differences noted between the supplemented and control group horses.

Firstly, cobinamide, the incomplete cobamide that is missing the lower loop is seen to have a pattern of gradual decrease in the normalised peak area values from June to September in the supplemented group only (Figure 13-1). Secondly, this pattern was observed for the p-cresole analogue as well (Figure 10-1). This could be an indication towards the effect of supplementation on the physiological change that is occurring.

The pattern can be due to the alternation in intestinal flora due to the exposure of extra Vitamin B_{12} .P-cresole is only known to be synthesized by *Veillonellaceae*, and thus, a gradual shift in the levels of this analogue could reflect the abundance of this taxon in the racehorses' gut (29, 50). Despite this observation, the percentage prevalence of this analogue did not show the progressive decrease from June to September as observed when comparing the normalised peak area values (Figure 10-4). For the treatment group, the normalised peak area values decreased from June to August (Figure 10-1), but the percentage prevalence increased from June to August (Figure 10-4). This indicates that the amount of p-cresole synthesized within the microbiome did not decrease relative to the total amount of all cobamides in its gut. The percentage spiked in the month of August from June could be related to the shift in the microbiome composition.

This experiment was done in parallel with another study by Professor Hunter (supplementary- S13). In this study, the effect of supplementation on the serum levels of vitamin B_{12} and performance is investigated. The injections increased the level of vitamin B_{12} in the blood but no changes to performance was noted by the trainer. This increase is temporary (noted in June to July 2018) and the higher levels disappeared in the subsequent months (August and September 2018).

Combining the two, no obvious relationship could be noted. Further investigations would be needed to delve deeper into studying why there is no significant difference or how differences can be caused by other factors. It could be argued that this is due to the sufficient supply of cobalamins from the hind gut of the horses through microbial synthesis to sustain themselves. Thus, most of the injected cobalamin is possibly excreted via the urine (51).

8.3 Human faecal samples

This area of the project set out to investigate the potential link between the gut microbiome and the types of cobamides present in the faecal samples of patients with pernicious anaemia. Two groups of patients were investigated for differences in the cobamides present in their faeces. Patients in group H are happy with their current vitamin B_{12} treatment (only needing their hydroxocobalamin injections every 3 months or more) while patients in group U are unhappy with their treatment (symptoms of pernicious anaemia appear before their next 3 monthly injection is due). With this, the final objective is to find potential areas of focus to improve the outcomes of patients' response to Vitamin B_{12} deficiency treatment.

The results of this experiment showed that 2-methyladenine is the most prevalent analogue in all the human faecal samples. The second most abundant analogue is different between the group U (unhappy) and group H (happy) patients. The second most abundant analogue in the happy group is 2-methylthioadenine (Figure 23-2). The unhappy group patients are split between adenine, 5,6-dimethylbenzimidazole, and 2-methylthioadenine. There is a low frequency of detection for cobamides with lower base belonging to the benzimidazoles observed except for 5,6-dimethylbenzimidazole. 5,6-dimethylbenzimidazole (lower loop of cyanocobalamin) is detected more frequently in group U than H (Figure 22-1). Lastly, the analogue 1*H*-naphtho [2,3-d] imidazole is detected more frequently in unhappy patients' samples than the group happy patients' samples.

Comparing these observations to a previous study (supplementary-S14), 2-methyladenine remains the most prevalent analogue detected and the second most abundant is 2-methylthioadenine for majority of the samples. The supplementary study also showed that analogues with the lower base in the benzimidazoles class had lower

frequencies of detection. However, the differences between the supplementary study and this study is that 5,6-dimethylbenzimidazole and 1*H*-naphtho [2,3-d] imidazole was not more frequently detected in group U patient samples in the supplementary study.

To sum up the observations between this project and supplementary study (S14), 2-methyladenine is the most abundant analogue in human faecal samples. The second most abundant is most likely 2-methylthioadenine. The analogues belonging to the benzimidazoles group are not prevalent and not frequently detected. No significant observable differences or patterns were noted between the group H and U samples. Further studies can be done with larger sample sizes to test to what extent are these observations true. Because of sample size, statistical analysis was not performed. Therefore, larger sample sizes will be needed to have statistically reliable observations and predictions.

Taking the patient profile in Tablet 24 into consideration, differences can be seen when patients take additional supplements such as multi-vitamin tablets containing B_{12}. Additional B_{12} supplements taken on top of their 3-monthly injections increased the amounts of 5,6-dimethylbenzimidazole (cyanocobalamin) in faecal samples (U3, U4, U5, U7, and H11). Patients who took both tablets and spray (U3, U7, and H11) had higher amounts of cyanocobalamin than those who took just spray (U4) or tablet (U5) in their samples. Additionally, the patient who took tablets (U5) had higher cyanocobalamin than the patient who only took the spray (U4). This could indicate the efficiency of absorbing vitamin B_{12} in these patients. Having more cyanocobalamin in their system could mean either the patients are not absorbing it into the system and thus being excreted into the faeces. It could also indicate an excess in cyanocobalamin within the body (10).

Studies have been done to compare the effectiveness of these treatments as a replacement therapy for one another but a combination of all remains to be fully investigated (52–54). In these studies, oral supplements and sublingual vitamin B_{12} spray was deemed to be as effective as intramuscular injections in treating patients with cobalamin deficiency in obtaining short term responses (52–54). Future investigations can be conducted to study the difference in absorption between the patients. Patients could be put under strict cobalamin free diets and the amount of cobalamins with 5,6-dimethylbenzimidazole as base consumed through treatments can be recorded. The amount excreted can be monitored through urine and faeces to give an indication of how much of the vitamin is retained. The difference in retention can be associated to the gut bacteria within the patients.

A supplementary study by Cultech (supplementary-S15) was done to find out if there are any differences between the gut microbiome between the two patients groups and the control group. One point of interest could be the higher abundance of *Lactobacillus* in their microbiome in unhappy group patients compared to the happy and control group patients. *Lactobacillus* is shown to compete for available cobalamins and produce only pseudo-cobalamin (cobamide with adenine as its lower ligand base) which is not useful for humans (55).

Other significant observations from this study include lesser abundance of *Bifidobacterium* in patients (group U and H) compared to non-patients. It has been found in other studies that *Bifidobacterium* can produce several B-vitamins including vitamin B_{12} (56). Therefore, it could be suggested that increasing this genus of bacteria might improve the symptoms of these patients. Lastly, *Klebsiella* and an unidentified genus were found to be higher in abundance for patients than non-patients. *Klebsiella* is known to also compete for cobalamins with the host (27). This could be a potential

reason why some patients are unhappy with their treatments. Having bacterial overgrowth in the small intestine have been shown to contribute to the level of vitamin B_{12} absorption although it could be reversed with antibiotic therapy (43, 57).

Thus, to improve absorption or treatment outcomes by reducing the competition via alteration of gut microbes could be a feasible strategy. Multiple studies have also shown the connection between vitamin B_{12} and the gut by proving its association with inflammatory bowel disease (23, 27, 43, 58). Moreover, because different bacteria also require specific cobamides, the gut microbiome can be shifted by changing the levels of specific cobamides (23)(28). This could go beyond implications in treating cobalamin deficiencies. It can be potentially serve as a foundation towards making cobalamin treatments targeting intestinal flora and gut diseases in the future

8.4 Animal tissue and serum samples

The adapted procedure was able to identify the types of cobamides present in tissue samples. However, this procedure was not sensitive enough for serum samples. Only one analogue (5,6-dimethylbenzimidazole) was detected in the mice sample and none was detected in the racehorse serum samples (Figure 28). Although the mean vitamin B_{12} level in horse serum varies, the average can be considered around 6300 pg/mL (59). The lowest detected cyanocobalamin level shown in the standard curve is 1360 pg/mL. Therefore, there should be a signal if cyanocobalamin is present in normal amounts (samples sent were 1.5 mL to 2 mL each). This could be due to the loss of these molecules during the extraction process or the concentration of these analogues are too low for the HPLC-MS to pick any signal up. Therefore, more samples should be pooled together for a large volume, or a more sensitive assay or extraction procedure needs to be done for the serum analysis.

For the tissue samples, the composition of 5,6-dimethylbenzimidazole as a percentage of total cobamides in the sample for both liver and kidney tissue were above 85 % (Tables 31-2 and 32-2). This could be an indication to the importance of these organs in cobalamin storage (45). The quantification of cyanocobalamin in the respective tissues show that liver had higher amounts than kidney (Figure 27). This amount however did not account for the sample weight. Studies have shown that kidney is an important organ for vitamin B_{12} homeostasis and have higher amounts of vitamin B_{12} accumulation in kidneys than liver (38, 45, 60). It would be interesting to create standards for other cobalamin analogues and compare their amounts to see if the amount of other analogues present in liver tissues are higher than that in kidney tissues. Having these comparisons can open up studies that investigate the effect of any treatment on the changes in accumulation of different cobamides in these crucial organs.

8.5 Conclusion

This project showed that the extraction protocol for cobamides was able to reveal a complex variety of cobamides in faecal and tissue samples at different compositions. The protocol was shown to be effective and reproducible for faecal samples with masses more than 2 g. This procedure was successfully adapted for usage on tissue samples but a different or more sensitive method will be needed to identify the cobamides present in serum samples.

Testing on racehorse faecal samples revealed that supplementing the racehorses with hydroxocobalamin injections for one month does not significantly alter their cobamide composition compared to the non-supplemented horses. Furthermore, supplementation does not affect their observed performance or have a lasting effect on serum B_{12} levels. Future work such as prolonging the supplementation period or

feeding them Vitamin B_{12} fortified foods instead of supplementation by injection can be done to affirm these conclusions

Although major correlations were not noted between the patient faecal samples' data and their treatment responses, the differences in the analogues' composition observed (Section 7.5) indicates why vitamin B_{12} deficient patients within the same 'group' can develop varied responses to treatments. Despite the lack of significant differences between the two groups of patients, the cobamide extraction method could potentially be used to trace the amount of cyanocobalamin in faeces after consumption through various routes (example: tablets or sprays). Likewise, this procedure could prove useful in establishing the types and quantities of cobamides in foods and to track its movement after consumption. For example, to explore how consuming probiotics can affect the cobamide composition or wellbeing of pernicious anaemia patients (61). Furthermore, we can define the patient samples into more groups such as age or dietary habits to investigate their influence on cobamides present. Additionally, we can extend the quantification comparisons to beyond cyanocobalamin alone by making standards for other cobalamin analogues. Such investigations could help elucidate the possible roles these cobalamin analogues play in pernicious anaemia and find potential therapeutic targets for treating the disease.

Lastly, if larger sample quantities can be collected, statistical analysis should be performed to confirm observable patterns.

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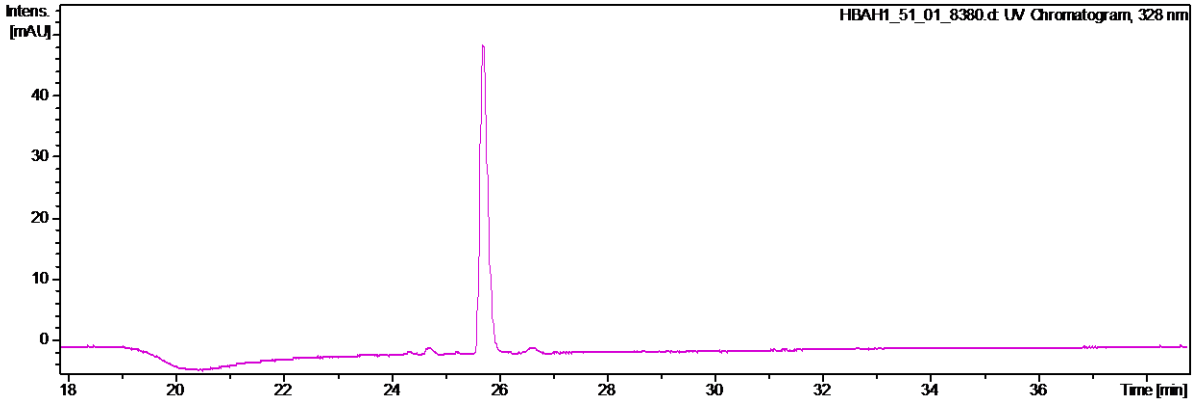
10. Supplementary data

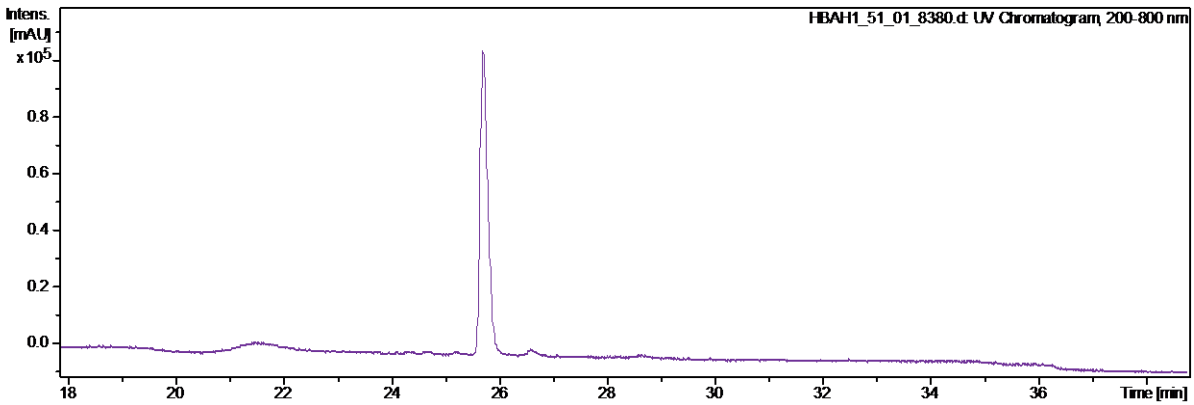
S1 Cyanocobalamin standards

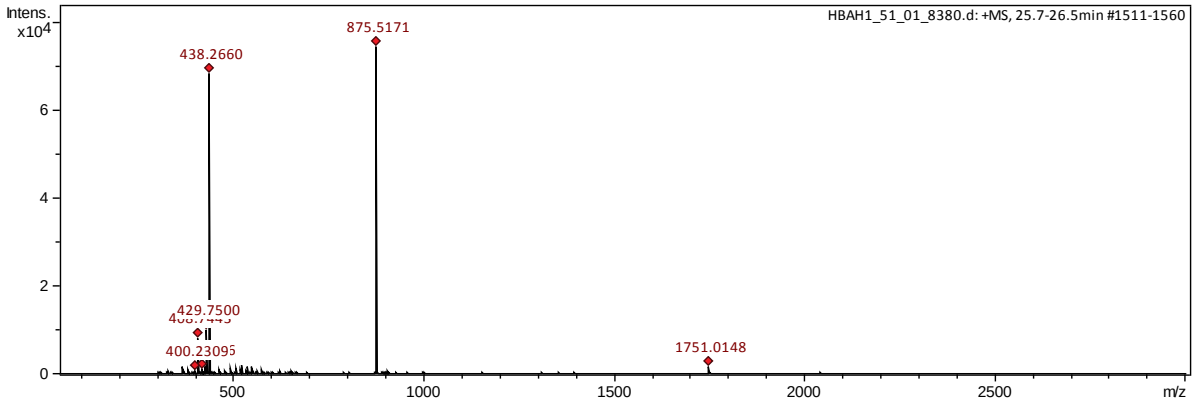
Integrated peak area (Area) of the signal of cyanocobalamin (CN-Cbl) from HPLC-MS with increasing concentrations of CN-Cbl loaded.

[CN-Cbl] (µM)	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
10	1	25.8	19542186	Manual	1091676	4491.1	EIC 678.29	678.3083	
1	1	25.8	2038392	Manual	124872	762.3	EIC 678.29	678.3029	
0.1	1	25.9	204461	Manual	13692	101.1	EIC 678.29	678.2975	
0.01	1	25.5	16457	Manual	1268	10.3	EIC 678.29	541.2606	
0.001	1	25.7	841.16	Manual	232	1.9	EIC 678.29	542.2563	

S2 HBAH - Chromatogram of the HBAH molecule signal on the HPLC-MS







S3 Pet Hedgehog - Integrated peak area (Area) of the detected chromatograms of cobamides in Pet hedgehog faecal samples from the HPLC-MS

#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	22.1	7486.4	Manual	476	2.7	EIC 508.2500±0.02 +All MS	409.178	
8	23.6	5180.6	Manual	468	2.7	EIC 508.2500±0.02 +All MS	678.2907	
5	22.9	18147.2	Manual	1348	8.4	EIC 666.2800±0.02 +All MS	679.7883	
2	22.2	119700.4	Manual	9444	58.6	EIC 666.7800±0.02 +All MS	791.8315	
3	22.7	45098.1	Manual	3004	26.1	EIC 672.7800±0.02 +All MS	679.7879	
7	23.6	412376.8	Manual	26980	160.4	EIC 678.2900±0.02 +All MS	678.2906	
4	22.9	1933631	Manual	133732	961.2	EIC 679.7800±0.02 +All MS	679.7879	
6	23.4	1907640	Manual	137088	782.6	EIC 695.7700±0.02 +All MS	695.7745	
9	24.9	97295.6	Manual	8092	45.2	EIC 875.5000±0.02 +All MS	875.5056	

S4 Pet Rabbit - Integrated peak area (Area) of the detected chromatograms of cobamides in Pet rabbit faecal samples from the HPLC-MS

#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	22.1	37314.8	Manual	3204	23.7	EIC 508.2500±0.02 +All MS	409.1789	
8	23.6	29378	Manual	1968	14.7	EIC 508.2500±0.02 +All MS	541.2575	
5	22.9	7987.3	Manual	560	4.8	EIC 666.2800±0.02 +All MS	679.7894	
2	22.2	3544.6	Manual	780	6.7	EIC 666.7800±0.02 +All MS	791.8316	
3	22.8	34899.1	Manual	2664	24.8	EIC 672.7800±0.02 +All MS	679.7891	
7	23.6	96269.7	Manual	6144	42.8	EIC 678.2900±0.02 +All MS	305.0865	
4	22.9	670856.4	Manual	47656	323.4	EIC 679.7800±0.02 +All MS	679.789	
6	23.4	315255	Manual	22156	182.9	EIC 695.7700±0.02 +All MS	695.7737	
9	24.9	23570.8	Manual	1940	11.6	EIC 875.5000±0.02 +All MS	875.5112	

S5 Wild Rabbit - Integrated peak area (Area) of the detected chromatograms of cobamides in wild rabbit faecal samples from the HPLC-MS

#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
4	22.8	6556.1	Manual	496	4.3	EIC 666.2800±0.02 +All MS	679.7836	
1	22.2	5477.1	Manual	620	6.5	EIC 666.7800±0.02 +All MS	791.8269	
2	22.7	9504.5	Manual	796	8.7	EIC 672.7800±0.02 +All MS	679.7833	
6	23.5	151075.3	Manual	11328	73.5	EIC 678.2900±0.02 +All MS	541.2542	
3	22.8	418800.4	Manual	32824	228	EIC 679.7800±0.02 +All MS	679.7833	
7	24.5	12808.7	Manual	1124	7.4	EIC 686.2900±0.02 +All MS	511.244	
5	23.3	190450.4	Manual	14072	124.3	EIC 695.7700±0.02 +All MS	695.7685	
8	24.9	41681.6	Manual	3380	18	EIC 875.5000±0.02 +All MS	875.503	

S6 Ziggy horse 1- Integrated peak area (Area) of the detected chromatograms of cobamides in horse (named Ziggy) faecal samples of varying weights (2, 4, 6, 8, 10, and 12 g) from the HPLC-MS

2g	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
	9	26.6	4997	Manual	552	5.1	EIC 508.25	499.2461	
	8	25	4381.3	Manual	448	4.2	EIC 508.25	465.2549	
	6	23.5	12950.1	Manual	1168	10.9	EIC 508.25	541.2554	
	1	21.9	12918.6	Manual	1176	11	EIC 508.25	409.1782	
	3	22.8	21583.6	Manual	1372	13.9	EIC 672.27	679.7849	
	5	23.5	43531.8	Manual	2936	21.8	EIC 678.29	541.2563	
	2	22.7	291389.5	Manual	18076	147.5	EIC 679.78	679.7847	
	4	23.4	7641.1	Manual	740	7	EIC 695.77	695.768	
	7	24.8	10842.5	Manual	1048	9.4	EIC 875.50	875.501	

4g	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
	1	22	94787.8	Manual	3920	29	EIC 508.25	409.1783	
	6	23	5560.7	Manual	516	5.4	EIC 666.28	679.7859	
	5	22.9	12196.1	Manual	504	4.7	EIC 666.78	679.7869	
	4	22.8	58751.7	Manual	4072	40.9	EIC 672.27	679.7854	
	2	22.8	54628.7	Manual	3844	37.1	EIC 672.78	679.7857	
	9	23.6	80151.5	Manual	5552	48	EIC 678.29	678.2881	
	8	23.5	24854.9	Manual	1584	12.3	EIC 679.28	678.2884	
	3	22.8	637396.9	Manual	48624	364.4	EIC 679.78	679.7856	
	7	23.3	9998.9	Manual	928	8.6	EIC 695.77	497.2287	
	10	24.9	9869.2	Manual	972	7.2	EIC 875.50	451.2235	

6g	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
	10	26.9	11922.3	Manual	788	5.8	EIC 508.25	435.2295	
	9	25.1	14264.9	Manual	1104	7.9	EIC 508.25	361.2129	
	7	23.6	17573.2	Manual	1388	9.8	EIC 508.25	541.2544	
	1	22.1	50865.7	Manual	3808	26.6	EIC 508.25	409.1778	
	2	22.2	11336.8	Manual	1224	12.3	EIC 666.78	791.8306	
	3	22.8	75952.9	Manual	5204	47.2	EIC 672.27	679.786	
	6	23.6	112710	Manual	7644	56.8	EIC 678.29	678.2897	
	4	22.8	899503.5	Manual	56788	417.2	EIC 679.78	679.7859	
	5	23.4	18889.6	Manual	1548	16.8	EIC 695.77	393.1823	
	8	24.9	7528.5	Manual	776	6.4	EIC 875.50	451.2295	

8g	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
	1	22.1	91946	Manual	7648	50.3	EIC 508.25	409.1803	
	8	23.7	72188	Manual	5360	35.3	EIC 508.25	678.2959	
	10	25.2	29245	Manual	1928	12.8	EIC 508.25	771.3303	
	12	27	29837	Manual	2184	14.5	EIC 508.25	499.2465	
	11	26.9	14934	Manual	1320	6	EIC 659.28	499.246	
	3	22.9	14284	Manual	1104	7.9	EIC 666.28	679.7915	
	2	22.3	34171	Manual	2748	22.3	EIC 666.78	791.8367	
	5	22.9	93733	Manual	6576	46.7	EIC 672.27	679.7919	
	7	23.7	175469	Manual	12360	80.6	EIC 678.29	678.2956	
	4	22.9	1191147	Manual	95744	771.6	EIC 679.78	679.792	
	6	23.5	25123	Manual	2188	19.2	EIC 695.77	695.773	
	9	25	49595	Manual	4440	24.2	EIC 875.50	875.5112	

10g	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
	10	26.8	15120	Manual	1284	11.5	EIC 508.25	419.2526	
	9	25.1	24417	Manual	2040	18.6	EIC 508.25	361.2106	
	6	23.6	28612	Manual	2456	22.3	EIC 508.25	678.2876	
	1	22.1	78977	Manual	5932	52.5	EIC 508.25	409.1765	
	2	22.2	36518	Manual	2404	21	EIC 666.78	791.8256	
	4	22.9	87258	Manual	5304	45	EIC 672.27	679.7831	
	7	23.7	156860	Manual	10172	71.2	EIC 678.29	678.2875	
	3	22.9	1133841	Manual	73560	533	EIC 679.78	679.7833	
	5	23.4	17429	Manual	1652	15.4	EIC 695.77	695.7646	
	8	25	20730	Manual	1656	12.9	EIC 875.50	361.2101	

12g	#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
	1	22.1	91049.9	Manual	6260	35.3	EIC 508.25	409.1815	
	7	23.7	64126.4	Manual	4772	26.8	EIC 508.25	541.2616	
	10	25.2	24429.6	Manual	2212	11.7	EIC 508.25	673.3377	
	11	26.9	30816.3	Manual	2168	12	EIC 508.25	499.2455	
	12	26.9	14954	Manual	1356	6.7	EIC 659.28	499.2457	
	2	22.3	37352.3	Manual	2972	27.7	EIC 666.78	791.8369	
	3	22.9	113313.3	Manual	8308	49.7	EIC 672.27	679.7917	
	6	23.7	154556.2	Manual	10148	65.4	EIC 678.29	541.2614	
	4	22.9	1211596	Manual	83468	700.4	EIC 679.78	679.7918	
	8	24.6	7174	Manual	744	5.8	EIC 686.29	629.3153	
	5	23.4	21428.9	Manual	2068	18.6	EIC 695.77	695.773	
	9	25	54958.6	Manual	4684	27.4	EIC 875.50	875.514	

S7 Ziggy Horse 2 - Integrated peak area (Area) of the detected chromatograms of cobamides in horse (named Ziggy) faecal samples of varying incubation times with the His-BtuF resin (1, 3, 6, 18, and 24 hours)from the HPLC-MS

1H	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	1H
	12	26.7	90702	Manual	5968	32	EIC 508.2500±0.02 +All MS	499.2489	
	10	25	136361	Manual	7952	43.6	EIC 508.2500±0.02 +All MS	301.1759	
	8	23.5	103371	Manual	7748	41.6	EIC 508.2500±0.02 +All MS	1015.496	
	1	21.9	217971	Manual	16160	87.7	EIC 508.2500±0.02 +All MS	508.253	
	13	26.7	34036	Manual	2624	9	EIC 659.2800±0.02 +All MS	499.2485	
	11	25	47417	Manual	3160	10.9	EIC 659.2800±0.02 +All MS	301.1764	
	5	22.8	13779	Manual	1096	7.7	EIC 666.2800±0.02 +All MS	679.7937	
	3	22.7	196185	Manual	13836	95.2	EIC 672.2700±0.02 +All MS	679.7932	
	2	22.7	312857	Manual	21396	184.1	EIC 672.7800±0.02 +All MS	679.793	
	7	23.5	136764	Manual	8768	61.2	EIC 678.2900±0.02 +All MS	1015.496	
	4	22.8	1920960	Manual	133812	986.9	EIC 679.7800±0.02 +All MS	679.7932	
	6	23.3	13734	Manual	1328	9	EIC 695.7700±0.02 +All MS	695.7751	
	9	24.8	301449	Manual	20676	109.6	EIC 875.5000±0.02 +All MS	875.5164	

3H	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
	1	21.9	198215	Manual	14488	56.2	EIC 508.2500±0.02 +All MS	508.2512	
	8	23.5	159277	Manual	12564	48.2	EIC 508.2500±0.02 +All MS	1015.495	
	10	24.9	126104	Manual	8372	32.3	EIC 508.2500±0.02 +All MS	301.1734	
	12	26.7	115927	Manual	7524	29.3	EIC 508.2500±0.02 +All MS	499.2462	
	11	25	33644	Manual	2276	6.4	EIC 659.2800±0.02 +All MS	301.1733	
	13	26.7	39097	Manual	2848	8	EIC 659.2800±0.02 +All MS	499.2461	
	2	22.1	16221	Manual	1604	12.4	EIC 666.7800±0.02 +All MS	791.8363	
	4	22.7	183886	Manual	13548	96.5	EIC 672.2700±0.02 +All MS	679.7903	
	3	22.7	310425	Manual	22072	172.4	EIC 672.7800±0.02 +All MS	679.7904	
	7	23.5	160100	Manual	9888	67.1	EIC 678.2900±0.02 +All MS	1015.495	
	5	22.7	1767201	Manual	128804	1039.8	EIC 679.7800±0.02 +All MS	679.7904	
	6	23.2	13822	Manual	1188	10.7	EIC 695.7700±0.02 +All MS	305.0868	
	9	24.8	301552	Manual	23680	129.2	EIC 875.5000±0.02 +All MS	875.5149	

6H	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
	1	21.8	217256	Manual	16348	64.5	EIC 508.2500±0.02 +All MS	508.2478	
	9	23.3	189223	Manual	14648	57.4	EIC 508.2500±0.02 +All MS	1015.488	
	12	24.8	121892	Manual	8748	33.9	EIC 508.2500±0.02 +All MS	301.1713	
	13	26.6	116210	Manual	8436	32.7	EIC 508.2500±0.02 +All MS	499.2421	
	11	24.8	38682	Manual	2616	6.8	EIC 659.2800±0.02 +All MS	301.1712	
	14	26.6	46724	Manual	3236	8.3	EIC 659.2800±0.02 +All MS	499.2419	
	4	22.6	22230	Manual	1412	10.6	EIC 666.2800±0.02 +All MS	679.7859	
	2	21.9	32854	Manual	2584	19.1	EIC 666.7800±0.02 +All MS	791.8309	
	6	22.6	261356	Manual	17816	109.2	EIC 672.2700±0.02 +All MS	679.7856	
	3	22.6	349144	Manual	23372	209.8	EIC 672.7800±0.02 +All MS	679.7856	
	8	23.3	249235	Manual	15944	101.8	EIC 678.2900±0.02 +All MS	1015.488	
	5	22.6	2707120	Manual	195428	1419	EIC 679.7800±0.02 +All MS	679.7856	
	7	23.1	26815	Manual	2028	17.6	EIC 695.7700±0.02 +All MS	305.0873	
	10	24.6	307476	Manual	22612	170.9	EIC 875.5000±0.02 +All MS	875.5091	

18H	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
	1	21.9	191748	Manual	15204	55.1	EIC 508.2500±0.02 +All MS	508.2443	
	9	23.4	132636	Manual	10724	38.3	EIC 508.2500±0.02 +All MS	1015.482	
	11	24.9	104739	Manual	7688	26.4	EIC 508.2500±0.02 +All MS	301.1689	
	13	26.6	103475	Manual	7648	27.9	EIC 508.2500±0.02 +All MS	499.2393	
	12	24.9	39598	Manual	2308	6	EIC 659.2800±0.02 +All MS	301.169	
	14	26.6	40377	Manual	3024	7.5	EIC 659.2800±0.02 +All MS	499.2395	
	6	22.7	16355	Manual	1280	10	EIC 666.2800±0.02 +All MS	679.7826	
	2	22.1	16761	Manual	1024	8.3	EIC 666.7800±0.02 +All MS	791.8257	
	5	22.7	249525	Manual	16600	126.4	EIC 672.2700±0.02 +All MS	679.7825	
	3	22.7	317731	Manual	20996	193.9	EIC 672.7800±0.02 +All MS	679.7825	
	8	23.4	209727	Manual	12980	75.8	EIC 678.2900±0.02 +All MS	1015.482	
	4	22.7	2556487	Manual	177652	1173.8	EIC 679.7800±0.02 +All MS	679.7824	
	7	23.2	25384	Manual	1776	15.9	EIC 695.7700±0.02 +All MS	305.0849	
	10	24.8	189158	Manual	15252	94	EIC 875.5000±0.02 +All MS	301.1684	

24H	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
	1	21.7	242156.5	Manual	16792	66.6	EIC 508.2500±0.02 +All MS	508.2537	
	8	23.3	120075.4	Manual	10140	39.3	EIC 508.2500±0.02 +All MS	678.3026	
	12	24.9	112676.8	Manual	7184	27.7	EIC 508.2500±0.02 +All MS	301.1758	
	13	26.6	92914.9	Manual	6728	26.7	EIC 508.2500±0.02 +All MS	499.2485	
	11	24.8	37630.7	Manual	2000	6.1	EIC 659.2800±0.02 +All MS	301.1759	
	14	26.6	33328.8	Manual	2708	8.3	EIC 659.2800±0.02 +All MS	499.2483	
	6	22.6	18116.7	Manual	1060	8	EIC 666.2800±0.02 +All MS	679.7945	
	2	22.1	24084.6	Manual	1640	13.5	EIC 666.7800±0.02 +All MS	791.838	
	5	22.6	215557.4	Manual	15880	105	EIC 672.2700±0.02 +All MS	679.7944	
	3	22.6	282460.5	Manual	19540	166	EIC 672.7800±0.02 +All MS	679.7942	
	7	23.3	6863489	Manual	443868	2128.7	EIC 678.2900±0.02 +All MS	678.3025	
	4	22.6	2343428	Manual	165216	1299.3	EIC 679.7800±0.02 +All MS	679.7935	
	9	24.3	9688.3	Manual	988	7.1	EIC 686.2900±0.02 +All MS	629.316	
	10	24.7	209327.1	Manual	15840	124.4	EIC 875.5000±0.02 +All MS	875.5171	

S8 Racehorse - Integrated peak area (Area) of the detected chromatograms of cobamides in racehorse faecal samples collected in June, August, and September 2018

JUNE – Month of supplementation

4WS								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	23	238811.2	Manual	16288	63.5	EIC 508.2500±0.02 +All MS	508.2476	
7	24.6	86677.2	Manual	4948	19.4	EIC 508.2500±0.02 +All MS	1015.484	
10	26.1	24555.3	Manual	1704	6.2	EIC 508.2500±0.02 +All MS	499.2417	
11	28	13320.4	Manual	1244	4.5	EIC 508.2500±0.02 +All MS	499.2432	
5	23.9	13715	Manual	788	5	EIC 666.2800±0.02 +All MS	679.7859	
2	23.4	7194.1	Manual	592	4.8	EIC 666.7800±0.02 +All MS	791.8326	
4	23.9	153090.4	Manual	9236	61.1	EIC 672.2700±0.02 +All MS	679.7858	
8	24.7	68739.5	Manual	3684	26.6	EIC 678.2900±0.02 +All MS	1015.483	
3	23.9	1307852	Manual	76296	531.9	EIC 679.7800±0.02 +All MS	679.7859	
6	24.4	78540	Manual	5260	49	EIC 695.7700±0.02 +All MS	695.7699	
9	26	34192.6	Manual	2356	15.2	EIC 875.5000±0.02 +All MS	301.1719	

ASH								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
2	23.1	24219.3	Manual	1820	7.9	EIC 508.2500±0.02 +All MS	409.1809	
9	24.6	9804.6	Manual	776	3.3	EIC 508.2500±0.02 +All MS	678.794	
12	27.8	13161.9	Manual	948	4.1	EIC 508.2500±0.02 +All MS	499.2474	
1	21.6	5117.4	Manual	456	2.9	EIC 666.2800±0.02 +All MS	666.277	
3	23.4	2690.9	Manual	276	1.7	EIC 666.2800±0.02 +All MS	791.8342	
6	23.9	14776.1	Manual	1124	7.3	EIC 666.2800±0.02 +All MS	679.7895	
7	23.9	66149	Manual	4260	40.4	EIC 672.2700±0.02 +All MS	679.7893	
4	23.9	97226.8	Manual	5632	59	EIC 672.7800±0.02 +All MS	679.7891	
10	24.7	73052.7	Manual	4856	28.3	EIC 678.2900±0.02 +All MS	678.2937	
5	23.9	1236984	Manual	80044	571.6	EIC 679.7800±0.02 +All MS	679.7891	
8	24.5	37471.5	Manual	2440	22.7	EIC 695.7700±0.02 +All MS	695.7737	
11	26	21988.3	Manual	1808	17.4	EIC 875.5000±0.02 +All MS	301.1732	
BD								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	22.9	31869.9	Manual	2108	8.1	EIC 508.2500±0.02 +All MS	784.8317	
12	25.7	74196.9	Manual	3704	14.3	EIC 508.2500±0.02 +All MS	301.1732	
14	27.5	41014.8	Manual	2624	10.1	EIC 508.2500±0.02 +All MS	499.2451	
13	25.8	21530.4	Manual	1384	3.9	EIC 659.2800±0.02 +All MS	771.3302	
1	21.3	20380	Manual	1324	7.1	EIC 666.2800±0.02 +All MS	666.2835	
2	22.4	36131.5	Manual	2408	12.9	EIC 666.2800±0.02 +All MS	666.2838	
8	23.6	25674.5	Manual	1756	9.4	EIC 666.2800±0.02 +All MS	679.7936	
4	23	72987.7	Manual	2900	24.3	EIC 666.7800±0.02 +All MS	791.8396	
6	23.6	65062.8	Manual	4068	26.9	EIC 672.2700±0.02 +All MS	679.7937	
5	23.6	179364.2	Manual	11268	80.4	EIC 672.7800±0.02 +All MS	679.7936	
10	24.4	43614.1	Manual	3508	18.7	EIC 678.2900±0.02 +All MS	678.2948	
7	23.6	2296691	Manual	143884	1001.9	EIC 679.7800±0.02 +All MS	679.7935	
9	24.1	121049.9	Manual	7608	58	EIC 695.7700±0.02 +All MS	695.7763	
11	25.7	5129.3	Manual	884	6.1	EIC 875.5000±0.02 +All MS	301.1713	

A&4E								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	23.8	47125	Manual	2880	11.1	EIC 508.2500±0.02 +All MS	314.0925	
9	25.3	16441	Manual	1120	4.3	EIC 508.2500±0.02 +All MS	678.2925	
12	26.7	38309	Manual	2440	9.4	EIC 508.2500±0.02 +All MS	301.1721	
13	28.4	18223	Manual	1296	5	EIC 508.2500±0.02 +All MS	499.2435	
1	22.3	18549	Manual	1160	8.1	EIC 666.2800±0.02 +All MS	666.2776	
2	23.5	18519	Manual	1432	10	EIC 666.2800±0.02 +All MS	436.6981	
4	24	22288	Manual	1544	11.8	EIC 666.7800±0.02 +All MS	791.8347	
7	24.6	118712	Manual	8188	60.5	EIC 672.2700±0.02 +All MS	679.7871	
5	24.5	149252	Manual	8988	80.7	EIC 672.7800±0.02 +All MS	679.7868	
10	25.4	212371	Manual	11044	60.2	EIC 678.2900±0.02 +All MS	678.2925	
6	24.6	866886	Manual	57332	479.9	EIC 679.7800±0.02 +All MS	679.7868	
8	25.1	57905	Manual	3944	30.5	EIC 695.7700±0.02 +All MS	316.1077	
11	26.7	21047	Manual	1220	10.2	EIC 875.5000±0.02 +All MS	301.1718	

COOL								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	23.3	151836	Manual	9444	41.6	EIC 508.2500±0.02 +All MS	508.2499	
8	24.9	52997.4	Manual	3460	15.3	EIC 508.2500±0.02 +All MS	678.2936	
10	26.4	28037.5	Manual	1680	7.4	EIC 508.2500±0.02 +All MS	301.1735	
11	26.4	8758.4	Manual	832	2.1	EIC 659.2800±0.02 +All MS	301.1716	
5	24.2	12247.7	Manual	800	5	EIC 666.2800±0.02 +All MS	679.789	
2	23.5	9639.7	Manual	852	7.4	EIC 666.7800±0.02 +All MS	791.8327	
4	24.2	221089.2	Manual	13728	88.5	EIC 672.2700±0.02 +All MS	679.789	
7	24.9	149505.7	Manual	8120	49.3	EIC 678.2900±0.02 +All MS	678.2933	
3	24.2	1425616	Manual	96148	766.3	EIC 679.7800±0.02 +All MS	679.789	
6	24.7	90643.1	Manual	5164	44.8	EIC 695.7700±0.02 +All MS	695.7715	
9	26.3	28932.5	Manual	1988	14.7	EIC 875.5000±0.02 +All MS	301.1739	
COR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	23.1	141205	Manual	8780	34.6	EIC 508.2500±0.02 +All MS	508.2553	
7	24.6	52320.7	Manual	3364	13.2	EIC 508.2500±0.02 +All MS	1015.497	
10	26.1	87178.1	Manual	4696	18.4	EIC 508.2500±0.02 +All MS	605.2558	
12	27.8	43245.9	Manual	2464	9.7	EIC 508.2500±0.02 +All MS	499.2494	
11	26.2	21783.4	Manual	1780	4.7	EIC 659.2800±0.02 +All MS	499.2494	
13	27.9	18972.3	Manual	1308	3.4	EIC 659.2800±0.02 +All MS	499.2492	
5	24	8163.9	Manual	1020	7.3	EIC 666.2800±0.02 +All MS	679.7977	
4	23.9	79723	Manual	5288	40.3	EIC 672.2700±0.02 +All MS	679.7961	
2	23.8	211511.2	Manual	12200	98.5	EIC 672.7800±0.02 +All MS	679.796	
8	24.7	23016.9	Manual	1680	10.5	EIC 678.2900±0.02 +All MS	1015.494	
3	23.9	1867344	Manual	121332	920.6	EIC 679.7800±0.02 +All MS	679.7959	
6	24.4	151332.5	Manual	10328	86.4	EIC 695.7700±0.02 +All MS	695.7785	
9	26	26034.4	Manual	2192	17.8	EIC 875.5000±0.02 +All MS	301.1758	

#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	24.1	172589	Manual	10592	42.6	EIC 508.2500±0.02 +All MS	508.2466	
10	25.7	53815	Manual	3320	12.8	EIC 508.2500±0.02 +All MS	1015.482	
12	27.2	73656	Manual	4944	19.2	EIC 508.2500±0.02 +All MS	301.17	
14	28.8	31230	Manual	2724	9.7	EIC 508.2500±0.02 +All MS	499.2402	
13	27.2	24464	Manual	1568	4.7	EIC 659.2800±0.02 +All MS	605.2445	
2	23.8	12940	Manual	1220	7.7	EIC 666.2800±0.02 +All MS	666.2758	
6	24.9	17438	Manual	1236	7.8	EIC 666.2800±0.02 +All MS	679.7856	
1	23.7	17354	Manual	976	8.5	EIC 666.7800±0.02 +All MS	508.2459	
5	24.9	82481	Manual	4724	31.2	EIC 672.2700±0.02 +All MS	679.7852	
4	24.9	186175	Manual	11016	85.3	EIC 672.7800±0.02 +All MS	679.7853	
9	25.7	123770	Manual	6216	34	EIC 678.2900±0.02 +All MS	678.2891	
7	25	2298476	Manual	141840	989.6	EIC 679.7800±0.02 +All MS	679.7852	
8	25.5	120714	Manual	7844	57.2	EIC 695.7700±0.02 +All MS	695.7676	
11	27.1	18320	Manual	1264	9.3	EIC 875.5000±0.02 +All MS	301.1695	
DR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
2	23	111455	Manual	6880	27.9	EIC 508.2500±0.02 +All MS	508.2527	
9	24.5	36297	Manual	2492	10.1	EIC 508.2500±0.02 +All MS	1015.492	
12	25.9	40532	Manual	2388	9.4	EIC 508.2500±0.02 +All MS	301.1743	
14	27.7	19292	Manual	1608	6.2	EIC 508.2500±0.02 +All MS	499.2468	
13	26	10915	Manual	716	2.3	EIC 659.2800±0.02 +All MS	301.1766	
1	21.5	12774	Manual	1148	7.6	EIC 666.2800±0.02 +All MS	666.2806	
7	23.8	13675	Manual	876	5.8	EIC 666.2800±0.02 +All MS	679.7908	
3	23.2	15674	Manual	1420	13.2	EIC 666.7800±0.02 +All MS	791.8364	
6	23.8	106357	Manual	6696	52.6	EIC 672.2700±0.02 +All MS	679.7908	
4	23.7	202941	Manual	11112	107.5	EIC 672.7800±0.02 +All MS	679.7906	
10	24.5	86245	Manual	4992	32.1	EIC 678.2900±0.02 +All MS	678.2969	
5	23.8	1282349	Manual	81304	728.5	EIC 679.7800±0.02 +All MS	679.7908	
8	24.3	69511	Manual	4688	34.7	EIC 695.7700±0.02 +All MS	695.7745	
11	25.8	11148	Manual	792	7.5	EIC 875.5000±0.02 +All MS	301.1749	

EDE								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
2	23.5	48769.9	Manual	3768	16.1	EIC 508.2500±0.02 +All MS	508.2517	
9	25.1	15618.2	Manual	1728	7.4	EIC 508.2500±0.02 +All MS	678.2971	
11	26.5	37278.8	Manual	2148	9.2	EIC 508.2500±0.02 +All MS	301.1754	
14	28.3	19253.6	Manual	1384	5.9	EIC 508.2500±0.02 +All MS	499.2472	
13	26.6	12940.7	Manual	848	2.5	EIC 659.2800±0.02 +All MS	605.2536	
1	22	4298.7	Manual	556	3.9	EIC 666.2800±0.02 +All MS	666.7811	
7	24.4	15468.4	Manual	912	6.4	EIC 666.2800±0.02 +All MS	679.7925	
3	23.7	6184.1	Manual	612	5.3	EIC 666.7800±0.02 +All MS	791.8355	
5	24.3	65805.7	Manual	4124	32.4	EIC 672.2700±0.02 +All MS	679.7925	
4	24.3	116667.8	Manual	7344	67.6	EIC 672.7800±0.02 +All MS	679.7922	
10	25.2	161069.9	Manual	9316	56.5	EIC 678.2900±0.02 +All MS	678.297	
6	24.4	1363601	Manual	80556	594.4	EIC 679.7800±0.02 +All MS	679.7922	
8	24.9	129677.3	Manual	8912	79.5	EIC 695.7700±0.02 +All MS	695.7763	
12	26.5	4112.2	Manual	704	6.3	EIC 875.5000±0.02 +All MS	301.1697	
F2U								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	23.4	80223	Manual	5900	23.9	EIC 508.2500±0.02 +All MS	508.2552	
7	25	27470	Manual	1768	7.2	EIC 508.2500±0.02 +All MS	1015.493	
10	26.5	62446	Manual	3060	12.4	EIC 508.2500±0.02 +All MS	301.1755	
12	28.3	28517	Manual	1856	7.2	EIC 508.2500±0.02 +All MS	499.2496	
11	26.5	17147	Manual	976	2.8	EIC 659.2800±0.02 +All MS	301.1754	
5	24.3	16970	Manual	964	7.1	EIC 666.2800±0.02 +All MS	679.7936	
4	24.2	141126	Manual	9656	73.7	EIC 672.2700±0.02 +All MS	679.794	
2	24.2	189689	Manual	10716	107.5	EIC 672.7800±0.02 +All MS	679.7939	
8	25.1	77471	Manual	4288	26.2	EIC 678.2900±0.02 +All MS	585.3749	
3	24.2	1234660	Manual	80408	610.3	EIC 679.7800±0.02 +All MS	679.7939	
6	24.8	85449	Manual	5676	59.4	EIC 695.7700±0.02 +All MS	695.7769	
9	26.4	34246	Manual	2748	23.2	EIC 875.5000±0.02 +All MS	301.1757	

FL								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	23.4	90935	Manual	6544	27.8	EIC 508.2500±0.02 +All MS	508.2546	
10	25	32582.8	Manual	1928	8.3	EIC 508.2500±0.02 +All MS	678.2996	
14	26.5	51405.1	Manual	3324	14	EIC 508.2500±0.02 +All MS	301.1759	
12	26.4	12067.4	Manual	740	2.4	EIC 659.2800±0.02 +All MS	301.1734	
1	21.9	34344.6	Manual	2332	14.3	EIC 666.2800±0.02 +All MS	666.2846	
2	23	21277.5	Manual	1456	8.9	EIC 666.2800±0.02 +All MS	666.2873	
8	24.3	26713.2	Manual	1600	9.8	EIC 666.2800±0.02 +All MS	679.7962	
4	23.7	31182.4	Manual	2140	16.8	EIC 666.7800±0.02 +All MS	791.8407	
7	24.3	185939.2	Manual	11944	111.2	EIC 672.2700±0.02 +All MS	679.7963	
5	24.3	248916	Manual	13928	119.2	EIC 672.7800±0.02 +All MS	679.7961	
11	25.1	169237.8	Manual	9812	64.8	EIC 678.2900±0.02 +All MS	678.2997	
6	24.3	2383093	Manual	158096	1238	EIC 679.7800±0.02 +All MS	679.7961	
9	24.9	117349.9	Manual	6988	53.5	EIC 695.7700±0.02 +All MS	695.7783	
13	26.4	4531.5	Manual	684	6.4	EIC 875.5000±0.02 +All MS	301.1836	
WR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	23	97266	Manual	6352	23.1	EIC 508.2500±0.02 +All MS	508.2537	
6	24.6	34737	Manual	2376	8.6	EIC 508.2500±0.02 +All MS	1015.497	
9	26	54090	Manual	2996	10.9	EIC 508.2500±0.02 +All MS	301.1743	
11	27.9	23309	Manual	1416	4.9	EIC 508.2500±0.02 +All MS	499.2486	
10	26.2	15285	Manual	1060	3.1	EIC 659.2800±0.02 +All MS	499.2485	
4	23.9	17724	Manual	1344	8.2	EIC 666.2800±0.02 +All MS	679.7949	
3	23.9	75485	Manual	5752	42.5	EIC 672.2700±0.02 +All MS	679.7949	
7	24.7	102791	Manual	5828	36.7	EIC 678.2900±0.02 +All MS	678.299	
2	23.9	1782163	Manual	120164	913	EIC 679.7800±0.02 +All MS	679.7948	
5	24.4	80053	Manual	4864	43.7	EIC 695.7700±0.02 +All MS	316.1096	
8	26	14716	Manual	1128	10.9	EIC 875.5000±0.02 +All MS	301.1749	
FT								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
2	23.7	83193	Manual	6228	24.3	EIC 508.2500±0.02 +All MS	508.2471	
7	25.2	27831	Manual	1968	7.6	EIC 508.2500±0.02 +All MS	1015.483	
10	26.8	72207	Manual	3632	13.7	EIC 508.2500±0.02 +All MS	771.3225	
12	28.5	32170	Manual	2000	7.6	EIC 508.2500±0.02 +All MS	499.2415	
11	26.8	24573	Manual	1236	3.1	EIC 659.2800±0.02 +All MS	771.321	
13	28.5	12612	Manual	892	2.3	EIC 659.2800±0.02 +All MS	499.2403	
1	23.3	121240	Manual	3188	16.7	EIC 666.2800±0.02 +All MS	679.7927	
3	24	113286	Manual	3776	28.8	EIC 666.7800±0.02 +All MS	791.8282	
5	24.6	36576	Manual	2048	16.6	EIC 672.2700±0.02 +All MS	679.7861	
8	25.3	68362	Manual	3888	22.2	EIC 678.2900±0.02 +All MS	678.2888	
4	24.6	1832482	Manual	116036	710.8	EIC 679.7800±0.02 +All MS	679.7858	
6	25.1	102742	Manual	6208	48.8	EIC 695.7700±0.02 +All MS	695.767	
9	26.6	12517	Manual	1112	8.1	EIC 875.5000±0.02 +All MS	301.1695	

GG								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
4	23.2	35862.7	Manual	2840	11.3	EIC 508.2500±0.02 +All MS	508.2488	
12	24.8	11914.7	Manual	844	3.4	EIC 508.2500±0.02 +All MS	678.2931	
13	26.1	29079.8	Manual	2004	8	EIC 508.2500±0.02 +All MS	301.1701	
16	27.9	12895.1	Manual	1116	4.5	EIC 508.2500±0.02 +All MS	499.2424	
15	26.2	8913.9	Manual	724	1.9	EIC 659.2800±0.02 +All MS	301.1737	
1	21.8	5254.1	Manual	584	4	EIC 666.2800±0.02 +All MS	666.2771	
2	22.4	2839.6	Manual	264	1.8	EIC 666.2800±0.02 +All MS	365.1535	
3	22.8	7282.4	Manual	696	4.7	EIC 666.2800±0.02 +All MS	666.2833	
7	24	9881.4	Manual	796	5.4	EIC 666.2800±0.02 +All MS	679.7873	
5	23.4	23077.9	Manual	1512	12.7	EIC 666.7800±0.02 +All MS	791.8319	
8	24.1	112281.8	Manual	6992	53.3	EIC 672.2700±0.02 +All MS	679.7871	
11	24.8	405441.5	Manual	21584	115.7	EIC 678.2900±0.02 +All MS	678.2924	
6	24	952815.3	Manual	57572	425	EIC 679.7800±0.02 +All MS	679.7872	
10	24.8	8010.2	Manual	716	5	EIC 689.2800±0.02 +All MS	678.291	
9	24.6	47971.3	Manual	2968	27.6	EIC 695.7700±0.02 +All MS	695.7735	
14	26.1	12407.5	Manual	1036	7.9	EIC 875.5000±0.02 +All MS	301.1711	
LH								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
2	23.2	68392	Manual	4288	20	EIC 508.2500±0.02 +All MS	508.2544	
7	24.8	26400	Manual	2052	9.6	EIC 508.2500±0.02 +All MS	678.3029	
10	26.1	55954	Manual	2688	12.5	EIC 508.2500±0.02 +All MS	301.1753	
12	27.8	28717	Manual	1524	7.1	EIC 508.2500±0.02 +All MS	499.2494	
11	26.2	16201	Manual	876	2.3	EIC 659.2800±0.02 +All MS	771.3337	
13	27.8	10558	Manual	1000	2.6	EIC 659.2800±0.02 +All MS	499.248	
1	22.8	33414	Manual	1704	10.2	EIC 666.2800±0.02 +All MS	666.2854	
3	23.4	47809	Manual	2800	21.4	EIC 666.7800±0.02 +All MS	791.8418	
5	24	38785	Manual	2432	18	EIC 672.2700±0.02 +All MS	679.7961	
8	24.8	109230	Manual	6488	34.4	EIC 678.2900±0.02 +All MS	678.3017	
4	24	1601906	Manual	100908	746	EIC 679.7800±0.02 +All MS	679.7958	
6	24.5	109150	Manual	7408	58.2	EIC 695.7700±0.02 +All MS	695.7797	
9	26	17631	Manual	1852	13.7	EIC 875.5000±0.02 +All MS	301.1762	
LR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	22.9	41479.7	Manual	2760	11.2	EIC 508.2500±0.02 +All MS	485.3546	
6	24.4	15619.9	Manual	1112	4.5	EIC 508.2500±0.02 +All MS	678.2942	
9	26	32377.1	Manual	1928	7.8	EIC 508.2500±0.02 +All MS	301.1717	
11	27.8	16911.1	Manual	1080	4.4	EIC 508.2500±0.02 +All MS	499.2455	
10	27.6	6150.1	Manual	592	1.5	EIC 659.2800±0.02 +All MS	499.245	
4	23.8	10607.5	Manual	828	5.5	EIC 666.2800±0.02 +All MS	679.7907	
3	23.8	94197.1	Manual	6108	38.4	EIC 672.2700±0.02 +All MS	679.7907	
7	24.6	101089.6	Manual	6060	33.9	EIC 678.2900±0.02 +All MS	585.3715	
2	23.8	1018954	Manual	69984	499.6	EIC 679.7800±0.02 +All MS	679.7907	
5	24.3	56425.7	Manual	3524	28.5	EIC 695.7700±0.02 +All MS	695.7753	
8	25.8	25484.9	Manual	1736	13.2	EIC 875.5000±0.02 +All MS	301.1714	

PL								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	23.7	54265	Manual	3388	14.8	EIC 508.2500±0.02 +All MS	508.2552	
9	25.4	17952.6	Manual	1020	4.4	EIC 508.2500±0.02 +All MS	678.2994	
12	26.7	21345.9	Manual	1332	5.8	EIC 508.2500±0.02 +All MS	301.1768	
14	28.4	13059.8	Manual	1048	4.6	EIC 508.2500±0.02 +All MS	499.2515	
13	26.8	6410.7	Manual	524	1.8	EIC 659.2800±0.02 +All MS	301.1788	
1	22.1	8521.6	Manual	732	4.6	EIC 666.2800±0.02 +All MS	666.2743	
2	23.3	6649.8	Manual	828	5.2	EIC 666.2800±0.02 +All MS	666.2766	
4	23.9	20774.8	Manual	1812	15.2	EIC 666.7800±0.02 +All MS	791.8384	
5	24.5	90971.1	Manual	5724	41.1	EIC 672.2700±0.02 +All MS	679.7936	
8	25.4	212020.1	Manual	11088	70	EIC 678.2900±0.02 +All MS	678.2989	
6	24.5	754689.9	Manual	47668	353.4	EIC 679.7800±0.02 +All MS	679.7935	
10	25.4	2356.9	Manual	380	2.8	EIC 689.2800±0.02 +All MS	678.2995	
7	25	49185.3	Manual	3160	27.5	EIC 695.7700±0.02 +All MS	695.7792	
11	26.6	4133.5	Manual	468	3.4	EIC 875.5000±0.02 +All MS	301.1768	
REC								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
8	24.4	29718	Manual	1836	7	EIC 508.2500±0.02 +All MS	678.2988	
11	25.9	63708.8	Manual	3080	11.7	EIC 508.2500±0.02 +All MS	499.2465	
13	27.6	33156.8	Manual	2432	9.3	EIC 508.2500±0.02 +All MS	499.2493	
12	26	24029.4	Manual	1480	3.9	EIC 659.2800±0.02 +All MS	301.1739	
14	27.6	12520.5	Manual	880	2.3	EIC 659.2800±0.02 +All MS	499.2501	
1	21.5	20948.9	Manual	1584	10.2	EIC 666.2800±0.02 +All MS	666.2863	
2	22.6	15871.3	Manual	1264	8.1	EIC 666.2800±0.02 +All MS	436.6992	
6	23.8	19806.1	Manual	1332	8.6	EIC 666.2800±0.02 +All MS	679.7933	
3	23.2	13050.8	Manual	864	6.4	EIC 666.7800±0.02 +All MS	791.8387	
5	23.8	68274.7	Manual	4320	30.1	EIC 672.2700±0.02 +All MS	679.7934	
9	24.6	166309.1	Manual	9740	56.4	EIC 678.2900±0.02 +All MS	678.2983	
4	23.8	1618317	Manual	104640	751	EIC 679.7800±0.02 +All MS	679.7933	
7	24.3	87136.2	Manual	5940	45.4	EIC 695.7700±0.02 +All MS	695.7775	
10	25.8	5065.3	Manual	904	7.3	EIC 875.5000±0.02 +All MS	301.1793	
SD								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
12	28.4	27302.6	Manual	1412	6.2	EIC 508.2500±0.02 +All MS	499.247	
11	26.7	43349	Manual	2456	10.8	EIC 508.2500±0.02 +All MS	301.1717	
7	25.2	27928.8	Manual	1968	8.6	EIC 508.2500±0.02 +All MS	678.2936	
2	23.6	90790.2	Manual	5688	25.1	EIC 508.2500±0.02 +All MS	508.2503	
1	23.3	11963.7	Manual	816	5.4	EIC 666.2800±0.02 +All MS	437.1946	
3	23.9	8011.5	Manual	668	5.9	EIC 666.7800±0.02 +All MS	791.8257	
5	24.5	69851.6	Manual	4076	26.2	EIC 672.2700±0.02 +All MS	679.7885	
8	25.3	211050.9	Manual	10984	65.9	EIC 678.2900±0.02 +All MS	678.2936	
4	24.4	1208026	Manual	82516	624.2	EIC 679.7800±0.02 +All MS	679.7885	
9	25.3	2835.2	Manual	256	2	EIC 689.2800±0.02 +All MS	678.2923	
6	25	48165.9	Manual	2856	24.7	EIC 695.7700±0.02 +All MS	695.772	
10	26.5	28238.4	Manual	2000	14.4	EIC 875.5000±0.02 +All MS	301.173	

VAL								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	24.3	54525	Manual	3356	12.7	EIC 508.2500±0.02 +All MS	791.8283	
7	25.7	20851	Manual	1376	5.2	EIC 508.2500±0.02 +All MS	678.2884	
9	27.1	88447	Manual	4764	18	EIC 508.2500±0.02 +All MS	301.1698	
12	28.9	40863	Manual	2208	8.3	EIC 508.2500±0.02 +All MS	499.24	
10	27.1	23661	Manual	1396	3.7	EIC 659.2800±0.02 +All MS	301.1705	
11	28.7	13621	Manual	904	2.4	EIC 659.2800±0.02 +All MS	499.2411	
1	22.8	56704	Manual	2408	11.1	EIC 666.2800±0.02 +All MS	666.2773	
2	23.9	49991	Manual	2724	12.6	EIC 666.2800±0.02 +All MS	436.6925	
4	25	345300	Manual	19184	113.5	EIC 672.2700±0.02 +All MS	679.785	
8	25.9	160717	Manual	8624	50.4	EIC 678.2900±0.02 +All MS	678.2883	
5	25.1	2687457	Manual	162340	1132.7	EIC 679.7800±0.02 +All MS	679.7851	
6	25.6	158308	Manual	11524	80.6	EIC 695.7700±0.02 +All MS	695.7692	

AUGUST

WR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	22.6	700386	Manual	43620	181.8	EIC 508.2500±0.02 +All MS	508.2457	
7	24.1	443918	Manual	26252	109.3	EIC 508.2500±0.02 +All MS	1015.483	
10	25.7	192876	Manual	10696	44.1	EIC 508.2500±0.02 +All MS	499.2397	
11	27.4	154218	Manual	8576	35.3	EIC 508.2500±0.02 +All MS	499.2409	
9	25.6	59276	Manual	3580	9.4	EIC 659.2800±0.02 +All MS	499.2397	
12	27.4	68345	Manual	4328	11.3	EIC 659.2800±0.02 +All MS	499.2408	
5	23.4	34122	Manual	2200	13.5	EIC 666.2800±0.02 +All MS	679.7837	
2	22.8	16802	Manual	1276	10.3	EIC 666.7800±0.02 +All MS	791.8247	
4	23.4	835487	Manual	50124	393.7	EIC 672.2700±0.02 +All MS	679.7836	
3	23.4	3151911	Manual	190032	1443.3	EIC 679.7800±0.02 +All MS	679.7836	
6	24	118775	Manual	7640	53.3	EIC 695.7700±0.02 +All MS	1015.482	
8	25.5	271925	Manual	16832	124.3	EIC 875.5000±0.02 +All MS	875.5017	
4WS								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	23	397189	Manual	25684	105.3	EIC 508.2500±0.02 +All MS	508.2445	
8	24.7	118595	Manual	5376	22.2	EIC 508.2500±0.02 +All MS	1015.48	
11	26.2	55566	Manual	2644	7.3	EIC 659.2800±0.02 +All MS	499.2389	
12	28	35456	Manual	2148	6	EIC 659.2800±0.02 +All MS	553.2843	
6	23.9	27638	Manual	1984	12.8	EIC 666.2800±0.02 +All MS	679.784	
2	23.2	21182	Manual	1208	9.8	EIC 666.7800±0.02 +All MS	791.8236	
5	23.9	351569	Manual	20464	139	EIC 672.2700±0.02 +All MS	679.7834	
3	23.9	550415	Manual	30444	238.4	EIC 672.7800±0.02 +All MS	679.7833	
9	24.8	249488	Manual	13280	74.2	EIC 678.2900±0.02 +All MS	678.2864	
4	23.9	3838775	Manual	231404	1616.5	EIC 679.7800±0.02 +All MS	679.7834	
7	24.5	37298	Manual	2564	19.8	EIC 695.7700±0.02 +All MS	347.1939	
10	26.1	195245	Manual	11364	69.7	EIC 875.5000±0.02 +All MS	499.2396	
A&4E								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	26.7	117939	Manual	7108	33.1	EIC 508.2500±0.02 +All MS	508.2518	
7	28.3	27671	Manual	1752	7.8	EIC 508.2500±0.02 +All MS	678.2961	
11	29.6	150372	Manual	7932	36.4	EIC 508.2500±0.02 +All MS	499.2466	
12	31	77487	Manual	4168	19.4	EIC 508.2500±0.02 +All MS	499.2477	
10	29.5	44732	Manual	2376	8.9	EIC 659.2800±0.02 +All MS	499.2468	
13	31	30953	Manual	2140	8	EIC 659.2800±0.02 +All MS	499.2474	
4	27.5	45653	Manual	3032	18.1	EIC 666.2800±0.02 +All MS	679.7919	
2	26.9	58669	Manual	1644	14.5	EIC 666.7800±0.02 +All MS	679.7922	
3	27.5	598243	Manual	34332	253.8	EIC 672.7800±0.02 +All MS	679.7915	
8	28.4	399801	Manual	22844	129.7	EIC 678.2900±0.02 +All MS	678.2959	
5	27.6	3208070	Manual	195456	1421.4	EIC 679.7800±0.02 +All MS	679.7918	
6	28.1	51791	Manual	2744	23	EIC 695.7700±0.02 +All MS	958.4334	
9	29.4	168226	Manual	11372	86.6	EIC 875.5000±0.02 +All MS	875.5121	
6	27.7	419508	Manual	22156	146.6	EIC 672.2700±0.02 +All MS	679.7916	
ASH								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	25.3	155273	Manual	8636	41.7	EIC 508.2500±0.02 +All MS	508.2519	
7	26.8	51872	Manual	2648	12.8	EIC 508.2500±0.02 +All MS	678.2977	
9	28.2	84965	Manual	5336	25.9	EIC 508.2500±0.02 +All MS	875.514	
12	29.9	39426	Manual	2664	12.9	EIC 508.2500±0.02 +All MS	499.2472	
11	28.3	22871	Manual	1300	4.8	EIC 659.2800±0.02 +All MS	875.5156	
13	29.9	13239	Manual	888	3.3	EIC 659.2800±0.02 +All MS	499.2467	
2	25.5	64382	Manual	2072	17.4	EIC 666.7800±0.02 +All MS	679.7928	
5	26.1	423731	Manual	25112	161.1	EIC 672.2700±0.02 +All MS	679.7926	
3	26.1	470583	Manual	28744	288.8	EIC 672.7800±0.02 +All MS	679.7925	
8	27	269547	Manual	14324	103	EIC 678.2900±0.02 +All MS	678.2964	
4	26.1	3191297	Manual	199940	1570.1	EIC 679.7800±0.02 +All MS	679.7926	
6	26.7	77519	Manual	5396	50.2	EIC 695.7700±0.02 +All MS	695.7748	
10	28.2	346186	Manual	20752	153.4	EIC 875.5000±0.02 +All MS	875.5143	

BD								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
3	23.6	449287	Manual	23684	93.9	EIC 508.2500±0.02 +All MS	508.2415	
10	25.1	139808	Manual	8484	33.3	EIC 508.2500±0.02 +All MS	1015.476	
14	26.7	564694	Manual	27668	108.9	EIC 508.2500±0.02 +All MS	499.2386	
15	28.4	261165	Manual	14300	55.5	EIC 508.2500±0.02 +All MS	499.2378	
13	26.6	181442	Manual	8736	21.3	EIC 659.2800±0.02 +All MS	499.2388	
16	28.4	106970	Manual	6108	14.9	EIC 659.2800±0.02 +All MS	499.2378	
1	22.1	10603	Manual	776	4.8	EIC 666.2800±0.02 +All MS	666.2791	
2	23.2	25615	Manual	1136	7	EIC 666.2800±0.02 +All MS	436.69	
8	24.5	72041	Manual	4424	27.1	EIC 666.2800±0.02 +All MS	679.7822	
4	23.9	11424	Manual	676	5.5	EIC 666.7800±0.02 +All MS	791.8182	
7	24.5	251467	Manual	13176	77.9	EIC 672.2700±0.02 +All MS	679.782	
5	24.4	1229071	Manual	69800	487.4	EIC 672.7800±0.02 +All MS	679.7819	
11	25.2	95035	Manual	4592	30.4	EIC 678.2900±0.02 +All MS	1015.476	
6	24.4	8207801	Manual	461568	2547.5	EIC 679.7800±0.02 +All MS	679.7821	
9	25	209537	Manual	14260	108.6	EIC 695.7700±0.02 +All MS	695.7622	
12	26.5	266213	Manual	15880	109.4	EIC 875.5000±0.02 +All MS	499.2384	
COOL								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
15	29.6	97144	Manual	5820	22.9	EIC 508.2500±0.02 +All MS	499.2418	
13	28	193674	Manual	9796	38.9	EIC 508.2500±0.02 +All MS	499.2425	
10	26.6	158617	Manual	8972	35.8	EIC 508.2500±0.02 +All MS	1015.486	
3	25	507319	Manual	31684	126.4	EIC 508.2500±0.02 +All MS	508.2477	
14	28	56472	Manual	2984	9.6	EIC 659.2800±0.02 +All MS	499.2424	
8	25.9	59695	Manual	3540	23.4	EIC 666.2800±0.02 +All MS	679.7876	
2	24.7	18098	Manual	1096	7.3	EIC 666.2800±0.02 +All MS	436.6924	
1	23.5	15417	Manual	1552	10.3	EIC 666.2800±0.02 +All MS	666.2772	
4	25.3	108136	Manual	3328	27.9	EIC 666.7800±0.02 +All MS	679.788	
7	25.9	385101	Manual	20344	121.8	EIC 672.2700±0.02 +All MS	679.7875	
5	25.8	922522	Manual	55656	499.6	EIC 672.7800±0.02 +All MS	679.7876	
11	26.6	172816	Manual	9044	51.7	EIC 678.2900±0.02 +All MS	1015.486	
6	25.9	5103137	Manual	319964	2476.5	EIC 679.7800±0.02 +All MS	679.7876	
9	26.4	81547	Manual	4580	37.1	EIC 695.7700±0.02 +All MS	1015.487	
12	27.9	197688	Manual	12340	91.2	EIC 875.5000±0.02 +All MS	499.2426	
DDR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
17	29.5	162799	Manual	9144	37.2	EIC 508.2500±0.02 +All MS	499.2434	
14	27.8	368447	Manual	18560	75.9	EIC 508.2500±0.02 +All MS	499.2431	
11	26.4	68052	Manual	4256	17.5	EIC 508.2500±0.02 +All MS	1015.49	
3	24.9	226576	Manual	12712	52.5	EIC 508.2500±0.02 +All MS	508.2496	
16	29.4	56695	Manual	3200	10.1	EIC 659.2800±0.02 +All MS	499.2435	
15	27.9	103812	Manual	5276	16.6	EIC 659.2800±0.02 +All MS	499.2432	
6	25.6	17470	Manual	1208	3.8	EIC 659.2800±0.02 +All MS	679.7905	
9	25.8	59855	Manual	3804	23.3	EIC 666.2800±0.02 +All MS	679.7903	
2	24.5	22052	Manual	1212	7.4	EIC 666.2800±0.02 +All MS	436.6959	
1	23.5	19085	Manual	1336	8.2	EIC 666.2800±0.02 +All MS	666.2786	
4	25.2	32045	Manual	2352	17	EIC 666.7800±0.02 +All MS	791.8313	
8	25.8	95632	Manual	5648	36.4	EIC 672.2700±0.02 +All MS	679.79	
5	25.6	706204	Manual	44140	426.7	EIC 672.7800±0.02 +All MS	679.79	
12	26.5	108197	Manual	6848	44.1	EIC 678.2900±0.02 +All MS	1015.489	
7	25.8	4536757	Manual	274312	2087.8	EIC 679.7800±0.02 +All MS	679.7899	
10	26.3	77567	Manual	4200	34.1	EIC 695.7700±0.02 +All MS	695.7691	
13	27.8	148803	Manual	9820	60.2	EIC 875.5000±0.02 +All MS	499.2429	
DR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
11	27.3	217694	Manual	13280	53.7	EIC 508.2500±0.02 +All MS	499.241	
9	25.5	436464	Manual	24864	102.5	EIC 508.2500±0.02 +All MS	605.2465	
6	24	133600	Manual	9104	37	EIC 508.2500±0.02 +All MS	958.4277	
1	22.4	364908	Manual	21744	89.6	EIC 508.2500±0.02 +All MS	508.246	
12	27.3	90734	Manual	5224	14.1	EIC 659.2800±0.02 +All MS	499.2407	
10	25.6	127635	Manual	6572	17.8	EIC 659.2800±0.02 +All MS	605.2466	
4	23.3	76653	Manual	3836	23	EIC 666.2800±0.02 +All MS	679.7864	
2	23.2	1878406	Manual	112264	723.6	EIC 672.7800±0.02 +All MS	679.7866	
7	24.1	242328	Manual	13248	79.3	EIC 678.2900±0.02 +All MS	958.4254	
3	23.2	8632524	Manual	555056	3027.9	EIC 679.7800±0.02 +All MS	679.7866	
5	23.8	278816	Manual	17000	115	EIC 695.7700±0.02 +All MS	958.4276	
8	25.4	517333	Manual	32384	193.8	EIC 875.5000±0.02 +All MS	605.2463	
4	23.2	800551	Manual	50140	278.4	EIC 672.2700±0.02 +All MS	679.7866	

FT								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
13	27	135220	Manual	7640	30.5	EIC 508.2500±0.02 +All MS	499.2418	
10	25.1	119418.2	Manual	6096	24.3	EIC 508.2500±0.02 +All MS	605.2466	
7	23.6	156319.5	Manual	9516	37.6	EIC 508.2500±0.02 +All MS	1015.483	
12	26.9	54202.7	Manual	3140	9.2	EIC 659.2800±0.02 +All MS	499.2417	
11	25.1	40716	Manual	2016	5.9	EIC 659.2800±0.02 +All MS	605.2468	
4	22.8	30822.7	Manual	1916	12.4	EIC 666.2800±0.02 +All MS	679.7853	
1	22.3	7401.6	Manual	772	5.9	EIC 666.7800±0.02 +All MS	791.8246	
3	22.8	62614.1	Manual	3628	23.4	EIC 672.2700±0.02 +All MS	679.7852	
2	22.8	465753.5	Manual	27796	258.8	EIC 672.7800±0.02 +All MS	679.7852	
8	23.7	25056.4	Manual	1344	8.3	EIC 678.2900±0.02 +All MS	1015.483	
5	22.9	2760478	Manual	178360	1118.5	EIC 679.7800±0.02 +All MS	679.7852	
6	23.4	48712.3	Manual	2956	24.3	EIC 695.7700±0.02 +All MS	1015.48	
9	25	276286.9	Manual	17364	111.9	EIC 875.5000±0.02 +All MS	875.5051	
GG								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
13	28.7	68733	Manual	4792	21.1	EIC 508.2500±0.02 +All MS	499.2416	
11	27.1	130156	Manual	6084	26.8	EIC 508.2500±0.02 +All MS	499.2422	
9	25.6	24241	Manual	1740	7.7	EIC 508.2500±0.02 +All MS	678.2899	
1	23.8	59523	Manual	4116	17.7	EIC 508.2500±0.02 +All MS	508.2456	
14	28.7	25292	Manual	1656	4.8	EIC 659.2800±0.02 +All MS	499.2413	
12	27.1	43149	Manual	2336	6.7	EIC 659.2800±0.02 +All MS	499.2419	
6	24.7	25384	Manual	1676	11.4	EIC 666.2800±0.02 +All MS	679.786	
2	24.1	28734	Manual	1776	13.5	EIC 666.7800±0.02 +All MS	791.8282	
5	24.7	275713	Manual	14716	89.1	EIC 672.2700±0.02 +All MS	679.7855	
3	24.7	519252	Manual	28396	254.9	EIC 672.7800±0.02 +All MS	679.7856	
8	25.6	306343	Manual	16156	101.4	EIC 678.2900±0.02 +All MS	678.2889	
4	24.7	2446730	Manual	142136	1019.6	EIC 679.7800±0.02 +All MS	679.7857	
7	25.3	43507	Manual	2964	21.6	EIC 695.7700±0.02 +All MS	695.7688	
10	26.9	178117	Manual	9964	75.9	EIC 875.5000±0.02 +All MS	875.5036	
LH								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
2	24.8	280914.1	Manual	17100	62.1	EIC 508.2500±0.02 +All MS	508.2493	
9	26.3	84084.8	Manual	4972	17.5	EIC 508.2500±0.02 +All MS	1015.49	
12	27.8	192164	Manual	9916	35.6	EIC 508.2500±0.02 +All MS	499.2446	
14	29.4	83952.1	Manual	5528	19.5	EIC 508.2500±0.02 +All MS	499.246	
13	27.8	46109.7	Manual	2444	7.9	EIC 659.2800±0.02 +All MS	499.245	
15	29.4	31171.6	Manual	1824	5.9	EIC 659.2800±0.02 +All MS	499.2458	
1	24.5	8012.2	Manual	748	5.1	EIC 666.2800±0.02 +All MS	437.1978	
7	25.7	37322.3	Manual	2360	16	EIC 666.2800±0.02 +All MS	679.791	
3	25.1	14092.1	Manual	1056	9.2	EIC 666.7800±0.02 +All MS	791.8334	
6	25.7	93367.5	Manual	5588	34	EIC 672.2700±0.02 +All MS	679.7908	
4	25.6	665286.7	Manual	41440	316.6	EIC 672.7800±0.02 +All MS	679.7909	
10	26.5	54859.7	Manual	3304	23.1	EIC 678.2900±0.02 +All MS	678.293	
5	25.7	3192941	Manual	196200	1409.4	EIC 679.7800±0.02 +All MS	679.7908	
8	26.2	47501	Manual	3372	31.5	EIC 695.7700±0.02 +All MS	695.7716	
11	27.7	51597.5	Manual	3108	23.3	EIC 875.5000±0.02 +All MS	499.2444	

EDE								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
13	27.5	83444	Manual	4304	18.3	EIC 508.2500±0.02 +All MS	499.2398	
11	25.6	119511	Manual	5728	24.4	EIC 508.2500±0.02 +All MS	499.24	
8	24	57901	Manual	3396	14.5	EIC 508.2500±0.02 +All MS	678.288	
1	22.4	100905	Manual	6032	25.7	EIC 508.2500±0.02 +All MS	508.2458	
14	27.6	26298	Manual	1784	6	EIC 659.2800±0.02 +All MS	499.2398	
12	25.7	40911	Manual	1920	6.4	EIC 659.2800±0.02 +All MS	499.2392	
6	23.3	16764	Manual	892	8.1	EIC 666.2800±0.02 +All MS	679.7834	
2	22.7	15008	Manual	1020	8.8	EIC 666.7800±0.02 +All MS	791.8244	
4	23.3	185830	Manual	12276	96.5	EIC 672.2700±0.02 +All MS	679.7835	
3	23.3	279503	Manual	14680	141.9	EIC 672.7800±0.02 +All MS	679.7834	
9	24.1	173016	Manual	10008	64.6	EIC 678.2900±0.02 +All MS	678.2876	
5	23.3	1787563	Manual	111268	696.9	EIC 679.7800±0.02 +All MS	679.7835	
7	23.8	52385	Manual	4152	38.6	EIC 695.7700±0.02 +All MS	695.766	
10	25.4	254779	Manual	13652	104	EIC 875.5000±0.02 +All MS	875.5019	
F2U								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
12	28.2	74620	Manual	3760	14.1	EIC 508.2500±0.02 +All MS	499.2376	
10	26.5	91869	Manual	4544	17	EIC 508.2500±0.02 +All MS	875.5003	
1	23.5	13525	Manual	1128	4	EIC 508.2500±0.02 +All MS	784.8148	
13	28.2	28305	Manual	1944	5.5	EIC 659.2800±0.02 +All MS	499.239	
11	26.5	33681	Manual	1684	4.8	EIC 659.2800±0.02 +All MS	875.5004	
6	24.4	36502	Manual	2264	14.6	EIC 666.2800±0.02 +All MS	679.7818	
2	23.8	24566	Manual	1524	10.9	EIC 666.7800±0.02 +All MS	791.8227	
5	24.4	237044	Manual	13004	78.6	EIC 672.2700±0.02 +All MS	679.7812	
3	24.3	537520	Manual	28436	245.9	EIC 672.7800±0.02 +All MS	679.7812	
8	25.2	40807	Manual	2636	15.1	EIC 678.2900±0.02 +All MS	541.2478	
4	24.4	3531383	Manual	210700	1428.4	EIC 679.7800±0.02 +All MS	679.7813	
7	24.9	57658	Manual	3728	32.3	EIC 695.7700±0.02 +All MS	695.7632	
9	26.4	456476	Manual	28068	197.2	EIC 875.5000±0.02 +All MS	875.5011	
FL								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	23.5	161818	Manual	8444	35.1	EIC 508.2500±0.02 +All MS	508.2475	
6	25.1	65717	Manual	3884	16.1	EIC 508.2500±0.02 +All MS	958.4254	
10	26.7	150390	Manual	6768	28.1	EIC 508.2500±0.02 +All MS	499.2417	
11	28.3	76958	Manual	4608	19.1	EIC 508.2500±0.02 +All MS	499.2416	
9	26.5	47374	Manual	2376	7.2	EIC 659.2800±0.02 +All MS	499.2416	
12	28.4	28720	Manual	2236	6.8	EIC 659.2800±0.02 +All MS	499.2416	
4	24.4	39443	Manual	2336	15.3	EIC 666.2800±0.02 +All MS	679.7875	
2	23.8	18356	Manual	1332	10.5	EIC 666.7800±0.02 +All MS	791.8284	
3	24.3	875639	Manual	49748	416.4	EIC 672.7800±0.02 +All MS	679.7874	
7	25.2	174352	Manual	9504	68.3	EIC 678.2900±0.02 +All MS	678.2896	
5	24.9	71690	Manual	4180	37.5	EIC 695.7700±0.02 +All MS	958.4264	
8	26.5	227426	Manual	15456	97.1	EIC 875.5000±0.02 +All MS	875.5061	
5	24.4	3890481	Manual	232200	1666.1	EIC 679.7800±0.02 +All MS	679.7875	
6	24.4	632887	Manual	34456	163.4	EIC 672.2700±0.02 +All MS	679.7874	

LR								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
14	29.9	106414	Manual	7036	24.4	EIC 508.2500±0.02 +All MS	499.2473	
12	28.2	287553	Manual	15032	55.5	EIC 508.2500±0.02 +All MS	499.2476	
9	26.9	172658	Manual	10628	39.5	EIC 508.2500±0.02 +All MS	1015.496	
1	25.2	553212	Manual	32380	120.9	EIC 508.2500±0.02 +All MS	508.2515	
15	29.9	43238	Manual	2492	8.6	EIC 659.2800±0.02 +All MS	499.2473	
13	28.3	89260	Manual	4356	15	EIC 659.2800±0.02 +All MS	499.2475	
3	26	28911	Manual	1408	4.9	EIC 659.2800±0.02 +All MS	679.7938	
7	26.1	56405	Manual	3320	19.4	EIC 666.2800±0.02 +All MS	679.7941	
2	25.5	24955	Manual	1556	12.2	EIC 666.7800±0.02 +All MS	791.8351	
6	26.1	502304	Manual	29608	212.6	EIC 672.2700±0.02 +All MS	679.7939	
4	26.1	1209600	Manual	68928	577	EIC 672.7800±0.02 +All MS	679.7938	
10	27	134969	Manual	7836	51.8	EIC 678.2900±0.02 +All MS	1015.495	
5	26.1	5761933	Manual	361508	2750.5	EIC 679.7800±0.02 +All MS	679.7939	
8	26.7	112920	Manual	6820	56.1	EIC 695.7700±0.02 +All MS	1015.494	
11	28.1	176940	Manual	12068	67.4	EIC 875.5000±0.02 +All MS	499.2478	
SD								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
1	23.4	203207	Manual	11004	43.9	EIC 508.2500±0.02 +All MS	508.2438	
8	25	70943	Manual	3564	14.2	EIC 508.2500±0.02 +All MS	1015.48	
11	26.5	243594	Manual	12832	51.2	EIC 508.2500±0.02 +All MS	499.2387	
14	28.3	124948	Manual	6456	25.8	EIC 508.2500±0.02 +All MS	499.24	
12	26.6	75656	Manual	3688	9.2	EIC 659.2800±0.02 +All MS	499.2389	
13	28.2	45767	Manual	2616	6.5	EIC 659.2800±0.02 +All MS	499.2398	
6	24.3	41946	Manual	2096	13	EIC 666.2800±0.02 +All MS	679.7843	
2	23.6	75727	Manual	2452	19.9	EIC 666.7800±0.02 +All MS	679.7844	
5	24.3	268398	Manual	13320	76.1	EIC 672.2700±0.02 +All MS	679.784	
3	24.3	1155657	Manual	63120	416.4	EIC 672.7800±0.02 +All MS	679.7839	
9	25.1	87594	Manual	4420	24.7	EIC 678.2900±0.02 +All MS	678.2879	
4	24.3	3908250	Manual	229296	1513.6	EIC 679.7800±0.02 +All MS	679.784	
7	24.9	49879	Manual	2824	20.3	EIC 695.7700±0.02 +All MS	679.7801	
10	26.4	145212	Manual	8356	70	EIC 875.5000±0.02 +All MS	499.2386	
REC								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]
12	30.3	73275	Manual	3940	17.3	EIC 508.2500±0.02 +All MS	499.2459	
10	28.8	143632	Manual	7248	31.9	EIC 508.2500±0.02 +All MS	936.4197	
7	27.4	43645	Manual	2408	10.6	EIC 508.2500±0.02 +All MS	678.2906	
1	26	131729	Manual	7940	35	EIC 508.2500±0.02 +All MS	508.2506	
13	30.4	25674	Manual	1584	6.4	EIC 659.2800±0.02 +All MS	499.245	
11	28.8	38679	Manual	2108	8.5	EIC 659.2800±0.02 +All MS	605.2493	
6	26.8	28993	Manual	1868	13.8	EIC 666.2800±0.02 +All MS	679.7904	
2	26.2	56562	Manual	2020	16.9	EIC 666.7800±0.02 +All MS	679.7907	
5	26.8	266257	Manual	17048	129.7	EIC 672.2700±0.02 +All MS	679.7901	
3	26.8	415357	Manual	24400	197.9	EIC 672.7800±0.02 +All MS	679.7899	
8	27.6	253909	Manual	15932	108.1	EIC 678.2900±0.02 +All MS	678.2911	
4	26.8	2599294	Manual	162432	1101.8	EIC 679.7800±0.02 +All MS	679.7899	
9	28.7	93065	Manual	6352	45.4	EIC 875.5000±0.02 +All MS	605.2492	

SEPTEMBER

FL SEP									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	15	29.4	58664	Manual	3736	14.2	EIC 508.2500±0.02 +All MS	499.2444	
	13	27.8	124866	Manual	6528	24.8	EIC 508.2500±0.02 +All MS	499.2441	
	10	26.4	63634	Manual	3948	15	EIC 508.2500±0.02 +All MS	958.4301	
	3	24.8	217647	Manual	12424	47.1	EIC 508.2500±0.02 +All MS	508.2501	
	14	27.8	34228	Manual	1652	4.3	EIC 659.2800±0.02 +All MS	499.2442	
	8	25.7	30041	Manual	2056	5.4	EIC 659.2800±0.02 +All MS	679.7891	
	2	24.4	22715	Manual	892	4.9	EIC 666.2800±0.02 +All MS	436.6975	
	1	23.4	40095	Manual	2644	14.4	EIC 666.2800±0.02 +All MS	666.2796	
	4	25	104315	Manual	3068	24.1	EIC 666.7800±0.02 +All MS	679.7891	
	7	25.7	743267	Manual	47440	331.2	EIC 672.2700±0.02 +All MS	679.7889	
	5	25.7	1031076	Manual	60532	524.7	EIC 672.7800±0.02 +All MS	679.7889	
	11	26.5	147892	Manual	8076	52	EIC 678.2900±0.02 +All MS	678.2911	
	6	25.7	4166985	Manual	273464	1964.3	EIC 679.7800±0.02 +All MS	679.7889	
	9	26.2	54075	Manual	3248	28.2	EIC 695.7700±0.02 +All MS	958.4313	
	12	27.7	268399	Manual	17484	129.1	EIC 875.5000±0.02 +All MS	875.5086	
4WS									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	13	30.3	107185	Manual	7236	30.3	EIC 508.2500±0.02 +All MS	499.2465	
	11	28.7	96024	Manual	4888	20.5	EIC 508.2500±0.02 +All MS	499.2436	
	8	27.3	41166	Manual	2504	10.5	EIC 508.2500±0.02 +All MS	1015.489	
	1	25.7	59821	Manual	3736	15.7	EIC 508.2500±0.02 +All MS	409.1783	
	14	30.3	34517	Manual	2028	5.5	EIC 659.2800±0.02 +All MS	499.2465	
	12	28.7	23979	Manual	1476	4	EIC 659.2800±0.02 +All MS	499.2442	
	5	26.6	38585	Manual	2776	18.8	EIC 666.2800±0.02 +All MS	679.7915	
	2	26	24495	Manual	1628	15.4	EIC 666.7800±0.02 +All MS	791.8324	
	4	26.6	223337	Manual	13000	75.1	EIC 672.2700±0.02 +All MS	679.7911	
	3	26.6	381875	Manual	21652	187.7	EIC 672.7800±0.02 +All MS	679.7911	
	9	27.4	47823	Manual	3072	19.1	EIC 678.2900±0.02 +All MS	678.2951	
	6	26.6	3283910	Manual	204888	1428.8	EIC 679.7800±0.02 +All MS	679.7913	
	7	27.1	68968	Manual	4484	36.4	EIC 695.7700±0.02 +All MS	695.7729	
	10	28.6	326093	Manual	18672	138	EIC 875.5000±0.02 +All MS	875.5102	
A&4E									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	14	30	35583.3	Manual	1832	7.3	EIC 508.2500±0.02 +All MS	499.2445	
	12	28.2	72837.1	Manual	3436	13.7	EIC 508.2500±0.02 +All MS	499.2447	
	8	26.9	18223.7	Manual	1020	4.1	EIC 508.2500±0.02 +All MS	678.2954	
	1	25.3	57018.7	Manual	3980	15.9	EIC 508.2500±0.02 +All MS	508.2497	
	13	28.3	15333.7	Manual	1060	3.8	EIC 659.2800±0.02 +All MS	499.2447	
	6	26.2	21221.8	Manual	1220	8.5	EIC 666.2800±0.02 +All MS	679.7894	
	2	25.5	5679.6	Manual	472	4	EIC 666.7800±0.02 +All MS	791.8335	
	5	26.2	248867	Manual	15408	115.6	EIC 672.2700±0.02 +All MS	679.7895	
	3	26.2	322470.3	Manual	18276	156.8	EIC 672.7800±0.02 +All MS	679.7892	
	10	27	310235.2	Manual	18800	118	EIC 678.2900±0.02 +All MS	678.2936	
	4	26.2	1712662	Manual	109092	942.6	EIC 679.7800±0.02 +All MS	679.7894	
	9	26.9	11411	Manual	632	5.5	EIC 689.2800±0.02 +All MS	678.2932	
	7	26.8	27022.2	Manual	1808	16.2	EIC 695.7700±0.02 +All MS	958.4295	
	11	28.2	146498	Manual	9780	76.8	EIC 875.5000±0.02 +All MS	875.5094	

ASH									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
	13	29.6	46030	Manual	2868	10.9	EIC 508.2500±0.02 +All MS	499.2445	
	11	28	47681	Manual	2356	9	EIC 508.2500±0.02 +All MS	875.5091	
	8	26.6	40836	Manual	2312	8.8	EIC 508.2500±0.02 +All MS	678.2936	
	1	25.1	66725	Manual	4108	15.6	EIC 508.2500±0.02 +All MS	409.1795	
	12	29.6	13689	Manual	1052	2.5	EIC 659.2800±0.02 +All MS	499.2452	
	6	25.9	17079	Manual	944	7.2	EIC 666.2800±0.02 +All MS	679.7893	
	2	25.3	16999	Manual	1100	9.2	EIC 666.7800±0.02 +All MS	792.332	
	5	25.9	150785	Manual	9188	70	EIC 672.2700±0.02 +All MS	679.7892	
	3	25.9	238425	Manual	14368	120.4	EIC 672.7800±0.02 +All MS	679.789	
	9	26.7	134835	Manual	8436	55.8	EIC 678.2900±0.02 +All MS	678.2929	
	4	25.9	1586065	Manual	98060	724.1	EIC 679.7800±0.02 +All MS	679.7891	
	7	26.5	26657	Manual	1636	14.7	EIC 695.7700±0.02 +All MS	695.7706	
	10	27.9	158529	Manual	10184	89.8	EIC 875.5000±0.02 +All MS	875.5092	
BD									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
	14	28.9	97941	Manual	6132	23.3	EIC 508.2500±0.02 +All MS	499.2353	
	13	27.3	189271	Manual	10624	40.5	EIC 508.2500±0.02 +All MS	499.235	
	9	25.8	56091	Manual	3848	14.7	EIC 508.2500±0.02 +All MS	1015.47	
	2	24.3	171872	Manual	11180	42.6	EIC 508.2500±0.02 +All MS	508.2398	
	15	28.9	27212	Manual	2096	5.8	EIC 659.2800±0.02 +All MS	499.2351	
	12	27.2	54565	Manual	3924	10.8	EIC 659.2800±0.02 +All MS	499.2348	
	7	25.2	51195	Manual	3492	19.5	EIC 666.2800±0.02 +All MS	679.7781	
	1	22.8	24866	Manual	2320	12.9	EIC 666.2800±0.02 +All MS	666.2685	
	3	24.6	58105	Manual	3344	28	EIC 666.7800±0.02 +All MS	791.8182	
	6	25.2	188102	Manual	10868	70	EIC 672.2700±0.02 +All MS	679.7779	
	4	25.1	540004	Manual	32596	256	EIC 672.7800±0.02 +All MS	679.7778	
	10	25.9	173293	Manual	10332	63.3	EIC 678.2900±0.02 +All MS	678.2799	
	5	25.2	4232072	Manual	274892	2032.3	EIC 679.7800±0.02 +All MS	679.7779	
	8	25.7	121644	Manual	7592	59.6	EIC 695.7700±0.02 +All MS	695.7592	
	11	27.1	342742	Manual	26480	178.6	EIC 875.5000±0.02 +All MS	875.4963	
COOL									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
	13	29.5	144466	Manual	8140	32.4	EIC 508.2500±0.02 +All MS	499.2381	
	11	27.8	253767	Manual	12932	51.6	EIC 508.2500±0.02 +All MS	499.2367	
	8	26.3	75955	Manual	4252	17	EIC 508.2500±0.02 +All MS	1015.474	
	1	24.8	176981	Manual	9688	38.7	EIC 508.2500±0.02 +All MS	508.2409	
	14	29.5	47323	Manual	2700	7.3	EIC 659.2800±0.02 +All MS	499.2383	
	12	27.8	60571	Manual	3316	9	EIC 659.2800±0.02 +All MS	499.2368	
	6	25.8	79443	Manual	4736	25.8	EIC 666.2800±0.02 +All MS	679.7808	
	2	25.1	180184	Manual	6724	51.2	EIC 666.7800±0.02 +All MS	679.7809	
	5	25.7	335315	Manual	17904	109.8	EIC 672.2700±0.02 +All MS	679.7806	
	3	25.6	964737	Manual	57272	478.8	EIC 672.7800±0.02 +All MS	679.7807	
	9	26.5	124261	Manual	6936	42.5	EIC 678.2900±0.02 +All MS	1015.473	
	4	25.7	7625599	Manual	469112	3190.1	EIC 679.7800±0.02 +All MS	679.7806	
	7	26.2	161225	Manual	9976	83.6	EIC 695.7700±0.02 +All MS	695.7621	
	10	27.7	528105	Manual	35040	231.5	EIC 875.5000±0.02 +All MS	875.4988	
DDR	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
	11	30.1	59502	Manual	2836	11.1	EIC 508.2500±0.02 +All MS	499.2425	
	9	28.4	135452	Manual	5844	23	EIC 508.2500±0.02 +All MS	499.2419	
	6	27	53449	Manual	3480	13.3	EIC 508.2500±0.02 +All MS	1015.483	
	1	25.3	173136	Manual	9392	36.9	EIC 508.2500±0.02 +All MS	508.2469	
	12	30.1	18271	Manual	1176	3.7	EIC 659.2800±0.02 +All MS	499.2422	
	10	28.4	34407	Manual	1844	5.9	EIC 659.2800±0.02 +All MS	499.2427	
	4	26.2	31310	Manual	1748	11	EIC 666.2800±0.02 +All MS	679.7875	
	2	25.6	33560	Manual	2048	17.2	EIC 666.7800±0.02 +All MS	791.8302	
	3	26.1	473805	Manual	25940	228.8	EIC 672.7800±0.02 +All MS	679.7872	
	7	27	170771	Manual	9904	69.1	EIC 678.2900±0.02 +All MS	678.2898	
	5	26.8	94452	Manual	5604	48.6	EIC 695.7700±0.02 +All MS	958.4257	
	8	28.3	306639	Manual	20584	172.5	EIC 875.5000±0.02 +All MS	875.5044	
	5	26.2	2796459	Manual	178352	1439.4	EIC 679.7800±0.02 +All MS	679.7874	
	6	26.2	285706	Manual	16692	107.6	EIC 672.2700±0.02 +All MS	679.7874	

DR									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	13	29.2	111792	Manual	6828	26	EIC 508.2500±0.02 +All MS	499.2358	
	10	27.5	169576	Manual	8768	33.9	EIC 508.2500±0.02 +All MS	499.2343	
	8	26.2	46130	Manual	3328	12.8	EIC 508.2500±0.02 +All MS	1015.473	
	1	24.6	82049	Manual	5608	21.7	EIC 508.2500±0.02 +All MS	1182.504	
	14	29.3	42244	Manual	2680	7.2	EIC 659.2800±0.02 +All MS	499.2358	
	12	27.6	50988	Manual	3016	8.2	EIC 659.2800±0.02 +All MS	499.2345	
	3	25.4	38594	Manual	2212	10.9	EIC 666.2800±0.02 +All MS	679.7784	
	2	24.9	99249	Manual	4212	35.3	EIC 666.7800±0.02 +All MS	679.7785	
	6	25.5	470007	Manual	27464	135.4	EIC 672.2700±0.02 +All MS	679.7781	
	4	25.5	572963	Manual	30712	233.9	EIC 672.7800±0.02 +All MS	679.778	
	9	26.3	146785	Manual	8528	51	EIC 678.2900±0.02 +All MS	678.2808	
	5	25.5	3933329	Manual	235104	1784.4	EIC 679.7800±0.02 +All MS	679.7781	
	7	26	70262	Manual	4096	34.3	EIC 695.7700±0.02 +All MS	695.7574	
	11	27.5	277507	Manual	17352	128.3	EIC 875.5000±0.02 +All MS	875.4955	
EDE									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	2	25.2	168424	Manual	9712	35.9	EIC 508.2500±0.02 +All MS	508.2451	
	9	26.7	55541	Manual	3788	14	EIC 508.2500±0.02 +All MS	1015.477	
	12	28.1	193835	Manual	9512	35.1	EIC 508.2500±0.02 +All MS	499.2395	
	14	29.7	98145	Manual	5700	21.1	EIC 508.2500±0.02 +All MS	499.2389	
	13	28.1	51410	Manual	2480	7.8	EIC 659.2800±0.02 +All MS	499.2394	
	15	29.7	30938	Manual	1840	5.8	EIC 659.2800±0.02 +All MS	499.2389	
	1	23.8	11443	Manual	788	5.3	EIC 666.2800±0.02 +All MS	666.27	
	7	26.1	44606	Manual	3100	20.5	EIC 666.2800±0.02 +All MS	679.784	
	3	25.5	26359	Manual	1488	13.9	EIC 666.7800±0.02 +All MS	791.8241	
	5	26	250909	Manual	16220	118.9	EIC 672.2700±0.02 +All MS	679.7838	
	4	26	614267	Manual	33256	278.3	EIC 672.7800±0.02 +All MS	679.7836	
	10	26.8	265966	Manual	13176	92.1	EIC 678.2900±0.02 +All MS	678.2852	
	6	26.1	3747311	Manual	229716	1673.1	EIC 679.7800±0.02 +All MS	679.7838	
	8	26.6	85607	Manual	6120	53	EIC 695.7700±0.02 +All MS	695.7662	
	11	28.1	178056	Manual	10760	74.1	EIC 875.5000±0.02 +All MS	499.2403	
WR									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	1	25.3	227803	Manual	12200	49.5	EIC 508.2500±0.02 +All MS	508.2477	
	8	26.8	63345	Manual	3444	14	EIC 508.2500±0.02 +All MS	958.4303	
	11	28.3	91283	Manual	4924	20	EIC 508.2500±0.02 +All MS	875.5091	
	13	29.9	41484	Manual	2808	10.9	EIC 508.2500±0.02 +All MS	499.2436	
	12	28.3	22952	Manual	1236	3.5	EIC 659.2800±0.02 +All MS	875.5087	
	14	29.9	19410	Manual	1140	3.2	EIC 659.2800±0.02 +All MS	499.2445	
	6	26.1	34828	Manual	2308	16.1	EIC 666.2800±0.02 +All MS	679.7897	
	2	25.6	12617	Manual	1084	9.4	EIC 666.7800±0.02 +All MS	791.8298	
	5	26.1	554661	Manual	32352	242.6	EIC 672.2700±0.02 +All MS	679.7896	
	3	26.1	718348	Manual	39744	344.3	EIC 672.7800±0.02 +All MS	679.7895	
	9	27	66042	Manual	4008	26.9	EIC 678.2900±0.02 +All MS	1015.487	
	4	26.1	3225671	Manual	199640	1433.8	EIC 679.7800±0.02 +All MS	679.7896	
	7	26.7	38501	Manual	1924	17.3	EIC 695.7700±0.02 +All MS	958.4303	
	10	28.1	262494	Manual	16584	143.7	EIC 875.5000±0.02 +All MS	875.5091	
FT									
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
	1	25.6	123740	Manual	7332	29.3	EIC 508.2500±0.02 +All MS	508.2495	
	7	27.1	77501	Manual	5084	20.3	EIC 508.2500±0.02 +All MS	1015.487	
	10	28.5	207585	Manual	9312	37.2	EIC 508.2500±0.02 +All MS	499.2444	
	12	30.3	164171	Manual	9032	36	EIC 508.2500±0.02 +All MS	499.2448	
	11	28.5	49266	Manual	2004	6.1	EIC 659.2800±0.02 +All MS	771.326	
	13	30.3	44015	Manual	2276	6.9	EIC 659.2800±0.02 +All MS	499.2447	
	4	26.4	43123	Manual	2588	15.1	EIC 666.2800±0.02 +All MS	679.7897	
	2	25.8	91149	Manual	3856	26.2	EIC 666.7800±0.02 +All MS	679.7916	
	3	26.4	361875	Manual	19104	145.5	EIC 672.7800±0.02 +All MS	679.7895	
	8	27.2	271064	Manual	15368	101.7	EIC 678.2900±0.02 +All MS	678.2916	
	5	26.5	4032892	Manual	238236	1812.6	EIC 679.7800±0.02 +All MS	679.7896	
	6	27	124937	Manual	7172	58.6	EIC 695.7700±0.02 +All MS	695.7713	
	9	28.5	346360	Manual	20724	130.2	EIC 875.5000±0.02 +All MS	875.5088	
	6	26.5	101151	Manual	5632	42.3	EIC 672.2700±0.02 +All MS	679.7895	

GG								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	1	25.5	28496.8	Manual	2040	8.5	EIC 508.2500±0.02 +All MS	409.1793
	10	28.4	79579.1	Manual	3808	16	EIC 508.2500±0.02 +All MS	499.245
	12	29.9	34026.4	Manual	1916	8	EIC 508.2500±0.02 +All MS	499.2454
	11	28.4	21044.1	Manual	1060	4	EIC 659.2800±0.02 +All MS	499.2435
	13	29.9	10784.4	Manual	736	2.8	EIC 659.2800±0.02 +All MS	499.2451
	6	26.3	15778.2	Manual	1160	8.6	EIC 666.2800±0.02 +All MS	679.7885
	2	25.7	21982.7	Manual	1768	14.3	EIC 666.7800±0.02 +All MS	791.8339
	5	26.3	165509.7	Manual	10728	79.3	EIC 672.2700±0.02 +All MS	679.7883
	3	26.3	209791.3	Manual	12860	115.4	EIC 672.7800±0.02 +All MS	679.7883
	4	26.3	1341102	Manual	86644	603	EIC 679.7800±0.02 +All MS	679.788
	8	27	7307.9	Manual	532	3.5	EIC 689.2800±0.02 +All MS	678.2935
	7	26.8	19840	Manual	1876	20.1	EIC 695.7700±0.02 +All MS	695.7696
	9	28.2	119212.9	Manual	7256	45	EIC 875.5000±0.02 +All MS	875.5088
	9	27.1	322738.4	Manual	21312	148.6	EIC 678.2900±0.02 +All MS	678.294
LH								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	13	29.3	94816.6	Manual	4888	19.8	EIC 508.2500±0.02 +All MS	499.2361
	12	27.7	112264.2	Manual	5188	21	EIC 508.2500±0.02 +All MS	875.4957
	7	26.3	39032.1	Manual	2772	11.2	EIC 508.2500±0.02 +All MS	678.2844
	1	24.7	63619.3	Manual	4420	17.9	EIC 508.2500±0.02 +All MS	508.2417
	14	29.3	30749.9	Manual	2352	7.1	EIC 659.2800±0.02 +All MS	499.2365
	11	27.6	29774.7	Manual	2008	6.1	EIC 659.2800±0.02 +All MS	499.2354
	4	25.5	31804.6	Manual	1908	12	EIC 666.2800±0.02 +All MS	679.7789
	2	25	34272	Manual	2584	23.2	EIC 666.7800±0.02 +All MS	791.8192
	3	25.5	468835.7	Manual	27796	211.7	EIC 672.7800±0.02 +All MS	679.7787
	9	26.4	227736.4	Manual	13272	83.3	EIC 678.2900±0.02 +All MS	678.2824
	5	25.6	2659938	Manual	165308	1332.4	EIC 679.7800±0.02 +All MS	679.7788
	8	26.3	3493.6	Manual	324	1.7	EIC 689.2800±0.02 +All MS	678.2842
	6	26.1	49722.2	Manual	3236	27.1	EIC 695.7700±0.02 +All MS	695.762
	10	27.6	257724.9	Manual	15208	131.8	EIC 875.5000±0.02 +All MS	875.4959
	6	25.6	131410.6	Manual	7976	46.6	EIC 672.2700±0.02 +All MS	679.7788
LR								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	14	30.4	114294	Manual	6476	23.9	EIC 508.2500±0.02 +All MS	499.2401
	12	28.9	218426	Manual	12148	44.3	EIC 508.2500±0.02 +All MS	499.24
	9	27.5	71968	Manual	4364	15.6	EIC 508.2500±0.02 +All MS	1015.482
	2	25.9	225773	Manual	13480	49.8	EIC 508.2500±0.02 +All MS	508.2456
	15	30.4	36560	Manual	2600	7.2	EIC 659.2800±0.02 +All MS	499.2402
	13	28.9	59246	Manual	2672	7.4	EIC 659.2800±0.02 +All MS	499.2397
	7	26.8	51120	Manual	3100	18.6	EIC 666.2800±0.02 +All MS	679.7861
	1	24.6	16421	Manual	1316	7.9	EIC 666.2800±0.02 +All MS	666.2809
	3	26.2	27626	Manual	1692	14.7	EIC 666.7800±0.02 +All MS	791.8264
	6	26.8	444234	Manual	24528	134.2	EIC 672.2700±0.02 +All MS	679.7858
	4	26.7	825483	Manual	48952	437.8	EIC 672.7800±0.02 +All MS	679.7858
	10	27.6	106068	Manual	5860	37.8	EIC 678.2900±0.02 +All MS	1015.481
	5	26.8	4316203	Manual	263532	2135.3	EIC 679.7800±0.02 +All MS	679.7859
	8	27.3	64722	Manual	3436	30.8	EIC 695.7700±0.02 +All MS	1015.484
	11	28.7	334915	Manual	20044	132.6	EIC 875.5000±0.02 +All MS	875.503
REC								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	2	24.7	228343.7	Manual	12192	46.2	EIC 508.2500±0.02 +All MS	508.2409
	9	26.2	96465.5	Manual	5952	22.7	EIC 508.2500±0.02 +All MS	958.4171
	13	27.6	277972.9	Manual	15404	58.6	EIC 508.2500±0.02 +All MS	499.2359
	15	29.2	132989.8	Manual	8204	30.5	EIC 508.2500±0.02 +All MS	499.2375
	14	27.7	60756.7	Manual	3720	9.8	EIC 659.2800±0.02 +All MS	499.2363
	16	29.3	44282.3	Manual	2444	6.4	EIC 659.2800±0.02 +All MS	499.2373
	1	23.1	16278.5	Manual	1260	8.3	EIC 666.2800±0.02 +All MS	666.2733
	7	25.5	58278	Manual	3496	23.1	EIC 666.2800±0.02 +All MS	679.7798
	3	24.9	16350.7	Manual	1692	12.5	EIC 666.7800±0.02 +All MS	791.8162
	6	25.5	567118.8	Manual	34508	204	EIC 672.2700±0.02 +All MS	679.7797
	4	25.5	991488.2	Manual	56284	468.3	EIC 672.7800±0.02 +All MS	679.7795
	10	26.3	370586.6	Manual	19528	129	EIC 678.2900±0.02 +All MS	678.2818
	5	25.5	6263254	Manual	402444	2519.8	EIC 679.7800±0.02 +All MS	679.7798
	11	26.4	4267.3	Manual	476	2.7	EIC 689.2800±0.02 +All MS	678.2844
	8	26	116119.3	Manual	7512	60.9	EIC 695.7700±0.02 +All MS	958.4163
	12	27.5	425995.6	Manual	27216	184.9	EIC 875.5000±0.02 +All MS	875.498

SD								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	2	24.8	153817	Manual	8024	34.2	EIC 508.2500±0.02 +All MS	508.2464
	9	26.4	40574	Manual	2456	10.5	EIC 508.2500±0.02 +All MS	678.2903
	12	27.8	198454	Manual	10056	42.8	EIC 508.2500±0.02 +All MS	499.2414
	15	29.5	89406	Manual	5356	22.8	EIC 508.2500±0.02 +All MS	499.2419
	13	27.8	44751	Manual	2600	6.9	EIC 659.2800±0.02 +All MS	499.2417
	14	29.4	31222	Manual	2000	5.3	EIC 659.2800±0.02 +All MS	499.2419
	1	23.4	11916	Manual	860	5.9	EIC 666.2800±0.02 +All MS	666.2788
	7	25.7	33883	Manual	2152	14.6	EIC 666.2800±0.02 +All MS	679.7868
	3	25.2	96549	Manual	4268	33.5	EIC 666.7800±0.02 +All MS	679.7873
	6	25.7	207313	Manual	12272	81.2	EIC 672.2700±0.02 +All MS	679.7868
	4	25.7	540857	Manual	30764	276.2	EIC 672.7800±0.02 +All MS	679.7867
	10	26.5	342418	Manual	18764	116	EIC 678.2900±0.02 +All MS	678.2899
	5	25.7	3261467	Manual	211432	1558.7	EIC 679.7800±0.02 +All MS	679.7868
	8	26.2	73287	Manual	5480	43	EIC 695.7700±0.02 +All MS	695.7702
	11	27.7	154074	Manual	10176	78.7	EIC 875.5000±0.02 +All MS	499.2418

S9 Animal tissue

LAMB LIVER - Integrated peak area (Area) of the detected chromatograms of cobamides in lamb liver samples treated with and without 50% Methanol (MetOH) from the HPLC-MS

With 50 % MetOH								
#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
1	24.1	33155	Manual	3156	21.4	EIC 672.27	672.2773	
2	24.9	1034606	Manual	63580	312.2	EIC 678.29	678.3009	
3	24.9	37129	Manual	2140	18.6	EIC 689.28	678.3009	
4	26.2	571787	Manual	42500	281.1	EIC 875.50	875.5171	
Without 50 % MetOH								
#	RT [min]	Area	Int. Type	I	S/N	Chromato	Max. m/z	FWHM [m
1	24.3	60347	Manual	4372	34.3	EIC 672.27	672.28	
3	25	1602762	Manual	95980	473.1	EIC 678.29	678.3021	
2	24.9	38547	Manual	2204	14.4	EIC 689.28	678.3023	
4	26.3	382605	Manual	28936	242.5	EIC 875.50	875.5182	

MICE KIDNEY - Integrated peak area (Area) of the detected chromatograms of cobamides in mice kidney samples from the HPLC-MS

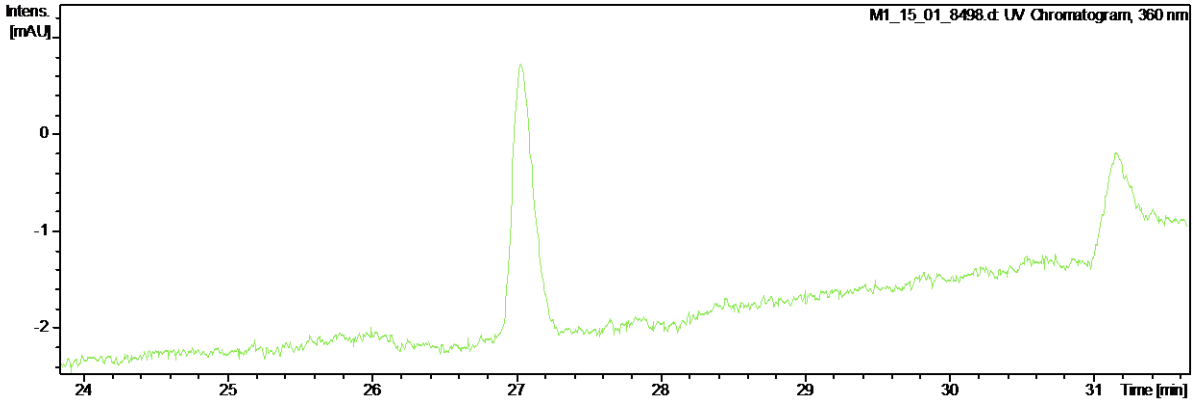
Mice kidney 1								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	1	24.1	8258.9	Manual	980	7.5	EIC 672.7800±0.02 +All MS	679.7867
	3	24.9	263171.3	Manual	19780	137.4	EIC 678.2900±0.02 +All MS	678.2963
	2	24.1	19426.8	Manual	1792	13.2	EIC 679.7800±0.02 +All MS	679.783
	4	25	10825.4	Manual	664	5.1	EIC 689.2800±0.02 +All MS	678.2969
	5	26.2	637862.5	Manual	52084	310.7	EIC 875.5000±0.02 +All MS	875.5169
		24.7	3844.2				**EIC 695.7700±0.02 +All MS**	
Mice Kidney 2								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	1	24.2	3267.7	Manual	572	4.5	EIC 672.7800±0.02 +All MS	679.7859
	4	25	560695	Manual	33092	203.8	EIC 678.2900±0.02 +All MS	678.2998
	3	24.9	30029.4	Manual	1884	14.4	EIC 689.2800±0.02 +All MS	678.2996
	2	24.7	2978.7	Manual	608	5.7	EIC 695.7700±0.02 +All MS	481.2585
	6	26.3	138028.9	Manual	21124	152.2	EIC 875.5000±0.02 +All MS	875.5073
	5	26.2	103923.9	Manual	22272	160	EIC 875.5000±0.02 +All MS	875.5155
		24.3	16114.7				**EIC 679.7800±0.02 +All MS**	
Mice kidney 3								
	#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z
	1	23.8	5548.2	Manual	528	4.5	EIC 672.7800±0.02 +All MS	679.7881
	4	24.7	396759.6	Manual	26828	156.5	EIC 678.2900±0.02 +All MS	678.297
	2	24	27833.4	Manual	2448	17.6	EIC 679.7800±0.02 +All MS	679.7878
	5	24.7	24078.5	Manual	1792	11.1	EIC 689.2800±0.02 +All MS	678.2974
	3	24.4	11082.7	Manual	964	7.8	EIC 695.7700±0.02 +All MS	695.7706
	6	25.9	669030.7	Manual	50120	323	EIC 875.5000±0.02 +All MS	875.5186

MICE LIVER - Integrated peak area (Area) of the detected chromatograms of cobamides in mice liver samples from the HPLC-MS

Mice 1			
RT [min]	Area	Chromatogram	Base
24.3	18416	EIC 672.7800±0.02 +All MS	Adenine (Pseudo)
25.1	1040967	EIC 678.2900±0.02 +All MS	5,6-dimethylbenzimidazole (B12)
24.2	64917	EIC 679.7800±0.02 +All MS	2-methyladenine
25	33449	EIC 689.2800±0.02 +All MS	1*H*-naphtho[2,3-d] imidazole
24.8	24843	EIC 695.7700±0.02 +All MS	2-methylthioadenine
26.3	982229	EIC 875.5000±0.02 +All MS	HBAH
Mice 2			
RT [min]	Area	Chromatogram	Base
24.2	7472.5	EIC 672.7800±0.02 +All MS	Adenine (Pseudo)
24.9	1042803	EIC 678.2900±0.02 +All MS	5,6-dimethylbenzimidazole (B12)
24.2	44923.4	EIC 679.7800±0.02 +All MS	2-methyladenine
25	38370.2	EIC 689.2800±0.02 +All MS	1*H*-naphtho[2,3-d] imidazole
24.7	17977.9	EIC 695.7700±0.02 +All MS	2-methylthioadenine
26.2	1024427	EIC 875.5000±0.02 +All MS	HBAH
Mice 3			
RT [min]	Area	Chromatogram	Base
24.3	8538.7	EIC 672.7800±0.02 +All MS	Adenine (Pseudo)
25.1	1037186	EIC 678.2900±0.02 +All MS	5,6-dimethylbenzimidazole (B12)
24.4	51963.3	EIC 679.7800±0.02 +All MS	2-methyladenine
25.2	36225.6	EIC 689.2800±0.02 +All MS	1*H*-naphtho[2,3-d] imidazole
24.9	20587.5	EIC 695.7700±0.02 +All MS	2-methylthioadenine
26.4	774342.2	EIC 875.5000±0.02 +All MS	HBAH

S10 Animal serum

MICE SERUM - Integrated peak area (Area) of the detected chromatograms of cobamides in mice serum samples from the HPLC-MS. Only CN-Cbl is detected and at very low levels.The UV chromatogram below reinforces the presence of cyanocobalamin (peak at ~27 mins).



#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [min]	
2	27	266753.2	Manual	54664	175.6	EIC 875.5000±0.02 +All MS	875.5132		
3	27.1	344488.8	Manual	51312	165.3	EIC 875.5000±0.02 +All MS	875.513		
1	25.8	2905.9	Manual	416	2.2	EIC 678.2900±0.02 +All MS	525.2878		

S11 Patient group H (happy) - Integrated peak area (Area) of the detected chromatograms of cobamides in human faecal samples belonging to the happy group from the HPLC-MS. Happy group means that these pernicious anaemia patients are satisfied with their three monthly Vitamin B_{12} injections.

H2								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	26.2	184588	Manual	10424	77.1	EIC 666.7800±0.02 +All MS	791.8381	
2	26.7	293147	Manual	20280	154.5	EIC 672.7800±0.02 +All MS	679.7962	
5	27.6	10575	Manual	816	6.6	EIC 678.2900±0.02 +All MS	347.2042	
3	26.8	3302931	Manual	218012	1336	EIC 679.7800±0.02 +All MS	679.7963	
4	27.3	420147	Manual	29512	190.6	EIC 695.7700±0.02 +All MS	695.7778	
6	28.8	226789	Manual	17184	123	EIC 875.5000±0.02 +All MS	875.5137	
H5								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
7	30.4	27984	Manual	2808	15.7	EIC 659.2800±0.02 +All MS	499.2532	
6	28.8	18863	Manual	1496	8.4	EIC 659.2800±0.02 +All MS	499.2527	
1	26.1	35849	Manual	2728	25.4	EIC 666.7800±0.02 +All MS	791.8395	
2	26.6	121772	Manual	9000	70.4	EIC 672.7800±0.02 +All MS	679.7977	
3	26.7	2416840	Manual	158308	812.1	EIC 679.7800±0.02 +All MS	679.7975	
4	27.2	1413403	Manual	93472	520.3	EIC 695.7700±0.02 +All MS	695.7818	
5	28.6	471940	Manual	34236	234.7	EIC 875.5000±0.02 +All MS	875.5163	
H7								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	26.6	145075	Manual	10408	81.8	EIC 672.7800±0.02 +All MS	679.7926	
2	26.7	443229	Manual	32476	205.8	EIC 679.7800±0.02 +All MS	679.7925	
3	27.2	284523	Manual	19392	135.2	EIC 695.7700±0.02 +All MS	695.7789	
4	28.7	258366	Manual	19212	130.5	EIC 875.5000±0.02 +All MS	875.516	
H10								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	26.7	60507	Manual	5060	37.4	EIC 679.7800±0.02 +All MS	679.7913	
2	27.2	41761	Manual	3356	22.2	EIC 695.7700±0.02 +All MS	695.7781	
3	28.6	102889	Manual	8284	59.6	EIC 875.5000±0.02 +All MS	875.5151	
H15								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	25.4	9865.4	Manual	860	5.1	EIC 508.2500±0.02 +All MS	784.8334	
8	27	11959.2	Manual	1072	6.5	EIC 508.2500±0.02 +All MS	608.1431	
12	28.4	57047.5	Manual	4200	25.1	EIC 508.2500±0.02 +All MS	771.3358	
14	30	80057.6	Manual	6000	35.9	EIC 508.2500±0.02 +All MS	499.2548	
11	28.3	14154.8	Manual	960	5.6	EIC 659.2800±0.02 +All MS	875.5223	
13	30	26441.2	Manual	2188	12.8	EIC 659.2800±0.02 +All MS	499.2549	
5	26.3	29053.3	Manual	2076	16.3	EIC 666.2800±0.02 +All MS	679.8	
2	25.7	48222.4	Manual	3632	33.7	EIC 666.7800±0.02 +All MS	791.8426	
3	26.2	251162.2	Manual	17480	162.7	EIC 672.7800±0.02 +All MS	679.7996	
9	27.1	14549.7	Manual	1068	7.1	EIC 678.2900±0.02 +All MS	541.2642	
4	26.2	694929.3	Manual	152756	1163.3	EIC 679.7800±0.02 +All MS	679.7915	
6	26.4	763209.9	Manual	103220	786.2	EIC 679.7800±0.02 +All MS	679.776	
7	26.8	1267680	Manual	93020	646.3	EIC 695.7700±0.02 +All MS	695.7837	
10	28.2	314684.8	Manual	25348	159.3	EIC 875.5000±0.02 +All MS	875.5189	

H9								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
7	28.4	37201	Manual	2276	12.7	EIC 508.2500±0.02 +All MS	875.5131	
9	30	55885	Manual	3876	21.7	EIC 508.2500±0.02 +All MS	499.2494	
8	30	18602	Manual	1608	7.6	EIC 659.2800±0.02 +All MS	499.2498	
4	26.3	14009	Manual	1132	7.5	EIC 666.2800±0.02 +All MS	679.7937	
1	25.7	31712	Manual	2628	20.6	EIC 666.7800±0.02 +All MS	791.8372	
2	26.2	88872	Manual	5844	45.9	EIC 672.7800±0.02 +All MS	679.7923	
3	26.3	1487142	Manual	108416	1009.3	EIC 679.7800±0.02 +All MS	679.7928	
5	26.8	634244	Manual	43648	365.4	EIC 695.7700±0.02 +All MS	695.7773	
6	28.3	358806	Manual	25816	175.4	EIC 875.5000±0.02 +All MS	875.5132	
H11								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
1	25.4	17264.6	Manual	1168	8	EIC 508.2500±0.02 +All MS	679.7923	
8	27	18898.9	Manual	1580	10.7	EIC 508.2500±0.02 +All MS	678.3017	
12	28.3	26515.3	Manual	1900	12.9	EIC 508.2500±0.02 +All MS	438.2691	
13	30	29459.4	Manual	2712	18.4	EIC 508.2500±0.02 +All MS	499.2528	
4	26.2	10581.2	Manual	740	3.6	EIC 659.2800±0.02 +All MS	679.7978	
14	30	10805	Manual	844	4.2	EIC 659.2800±0.02 +All MS	499.2529	
6	26.2	31025.7	Manual	2252	14.9	EIC 666.2800±0.02 +All MS	679.7978	
2	25.6	20460.5	Manual	1476	10.6	EIC 666.7800±0.02 +All MS	791.838	
3	26.2	456567.2	Manual	29584	218.1	EIC 672.7800±0.02 +All MS	679.7974	
9	27	532040.9	Manual	36828	220	EIC 678.2900±0.02 +All MS	678.3015	
5	26.2	3541595	Manual	244220	1533.2	EIC 679.7800±0.02 +All MS	679.7975	
10	27	6477.9	Manual	844	5.9	EIC 689.2800±0.02 +All MS	678.3035	
7	26.7	493004.2	Manual	34040	244.1	EIC 695.7700±0.02 +All MS	695.7808	
11	28.3	295422.4	Manual	27096	194.6	EIC 875.5000±0.02 +All MS	438.2689	
H12								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
7	28.3	16062.3	Manual	1304	9.1	EIC 508.2500±0.02 +All MS	673.3458	
10	30	20029.3	Manual	1688	11.8	EIC 508.2500±0.02 +All MS	499.2528	
9	30	9303.9	Manual	800	4.4	EIC 659.2800±0.02 +All MS	499.2505	
3	26.2	20371.8	Manual	1564	13.6	EIC 666.2800±0.02 +All MS	679.7985	
1	26.1	114549.6	Manual	7420	62.2	EIC 672.7800±0.02 +All MS	679.7981	
5	27.1	30008.5	Manual	2060	16.7	EIC 678.2900±0.02 +All MS	541.2677	
2	26.2	1666856	Manual	142612	872.1	EIC 679.7800±0.02 +All MS	679.7974	
4	26.7	216498.8	Manual	15188	119.3	EIC 695.7700±0.02 +All MS	695.7802	
6	28.1	85754.1	Manual	18484	99.1	EIC 875.5000±0.02 +All MS	361.2258	
8	28.3	93452.5	Manual	14364	76.8	EIC 875.5000±0.02 +All MS	499.2562	
H13								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [mi
10	28.4	155309.4	Manual	9692	64.4	EIC 508.2500±0.02 +All MS	499.2548	
12	30	192056.1	Manual	12792	84.6	EIC 508.2500±0.02 +All MS	499.2549	
9	28.3	30234.8	Manual	1828	10.9	EIC 659.2800±0.02 +All MS	875.5205	
13	30.1	54007.8	Manual	3288	19.7	EIC 659.2800±0.02 +All MS	499.2548	
14	30.9	3883.4	Manual	444	3.9	EIC 664.2700±0.02 +All MS	499.2541	
5	26.4	17297.9	Manual	1076	8.9	EIC 666.2800±0.02 +All MS	679.7976	
1	25.7	37717.7	Manual	2920	20.4	EIC 666.7800±0.02 +All MS	791.8415	
4	26.3	210421.2	Manual	15028	105.1	EIC 672.2700±0.02 +All MS	679.7979	
2	26.2	247384.3	Manual	15988	143.7	EIC 672.7800±0.02 +All MS	679.7977	
7	27	28952.9	Manual	1808	12.5	EIC 678.2900±0.02 +All MS	541.2664	
3	26.3	1560574	Manual	109288	654.1	EIC 679.7800±0.02 +All MS	679.7976	
6	26.8	127982	Manual	9004	62.9	EIC 695.7700±0.02 +All MS	695.7812	
8	28.2	62721.2	Manual	15608	99.2	EIC 875.5000±0.02 +All MS	876.5127	
11	28.5	29237.3	Manual	4692	29.9	EIC 875.5000±0.02 +All MS	771.3349	

S12 Patient group U (unhappy) - Integrated peak area (Area) of the detected chromatograms of cobamides in human faecal samples belonging to the unhappy group from the HPLC-MS. Unhappy group means that these pernicious anaemia patients are dissatisfied with their three monthly Vitamin B_{12} injections. They report symptoms of pernicious anaemia before their next injection is due and require more frequent treatment.

U1								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
9	28.4	89505	Manual	5696	34.1	EIC 508.2500±0.02 +All MS	736.9517	
12	30.1	102721	Manual	7340	43.9	EIC 508.2500±0.02 +All MS	1287.298	
10	28.4	18043	Manual	1500	8	EIC 659.2800±0.02 +All MS	1001.236	
11	30	28596	Manual	2396	12.8	EIC 659.2800±0.02 +All MS	1287.295	
4	26.3	34270	Manual	2384	15.2	EIC 666.2800±0.02 +All MS	679.8008	
1	25.8	25475	Manual	2544	16	EIC 666.7800±0.02 +All MS	791.8412	
3	26.3	192561	Manual	13588	106.5	EIC 672.2700±0.02 +All MS	679.8008	
2	26.2	576136	Manual	42096	319.2	EIC 672.7800±0.02 +All MS	679.8006	
7	27.1	74886	Manual	4712	32	EIC 678.2900±0.02 +All MS	678.3002	
5	26.4	893040	Manual	121576	898.8	EIC 679.7800±0.02 +All MS	679.7761	
6	26.8	385543	Manual	29036	197.3	EIC 695.7700±0.02 +All MS	695.7812	
8	28.2	286009	Manual	26772	156.1	EIC 875.5000±0.02 +All MS	875.5194	
U3								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	25.4	200892.8	Manual	14152	66	EIC 508.2500±0.02 +All MS	508.2594	
10	26.9	213149	Manual	15312	71.2	EIC 508.2500±0.02 +All MS	678.3087	
14	28.3	82535.4	Manual	5296	24.7	EIC 508.2500±0.02 +All MS	875.5227	
17	29.9	111565.7	Manual	7448	34.7	EIC 508.2500±0.02 +All MS	499.2532	
5	26.1	20349.4	Manual	1376	5.9	EIC 659.2800±0.02 +All MS	679.8027	
15	28.3	18908.8	Manual	1184	5	EIC 659.2800±0.02 +All MS	875.5225	
18	29.9	29343.3	Manual	2316	9.9	EIC 659.2800±0.02 +All MS	499.2533	
6	26.1	78789.6	Manual	5884	34.4	EIC 666.2800±0.02 +All MS	679.803	
2	25.6	60125.5	Manual	4444	33.9	EIC 666.7800±0.02 +All MS	791.8448	
4	26.1	1058404	Manual	70680	507.6	EIC 672.7800±0.02 +All MS	679.8026	
11	26.9	5186526	Manual	315912	1784.7	EIC 678.2900±0.02 +All MS	678.3087	
3	26	341483	Manual	69672	436.3	EIC 679.7800±0.02 +All MS	673.2929	
7	26.4	627419.9	Manual	80196	504	EIC 679.7800±0.02 +All MS	497.2398	
8	26.5	30186.3	Manual	1040	7.1	EIC 686.2900±0.02 +All MS	695.7855	
12	27	80593.5	Manual	4448	28.7	EIC 689.2800±0.02 +All MS	678.3086	
9	26.7	1345359	Manual	93788	589.4	EIC 695.7700±0.02 +All MS	695.7847	
13	28	6071.8	Manual	2144	14.9	EIC 875.5000±0.02 +All MS	361.2158	
16	28.3	50185.9	Manual	8596	59.9	EIC 875.5000±0.02 +All MS	771.3391	

U4								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
8	28.2	11802	Manual	1052	6.4	EIC 508.2500±0.02 +All MS	499.2521	
9	29.8	18395	Manual	1268	7.7	EIC 508.2500±0.02 +All MS	499.2516	
4	26.1	13695	Manual	960	7.8	EIC 666.2800±0.02 +All MS	679.7965	
1	25.5	17362	Manual	1356	12.4	EIC 666.7800±0.02 +All MS	791.841	
2	26	81658	Manual	6136	53.2	EIC 672.7800±0.02 +All MS	679.7964	
6	26.9	51714	Manual	3700	26.6	EIC 678.2900±0.02 +All MS	678.2992	
3	26.1	1192402	Manual	89880	564.5	EIC 679.7800±0.02 +All MS	679.7965	
5	26.6	896035	Manual	62520	455.5	EIC 695.7700±0.02 +All MS	695.7815	
7	28.1	159706	Manual	11956	77.2	EIC 875.5000±0.02 +All MS	875.5164	
U5								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
10	28.3	52626.4	Manual	3492	18.7	EIC 508.2500±0.02 +All MS	875.5167	
12	29.9	75396.4	Manual	5148	27.2	EIC 508.2500±0.02 +All MS	499.2514	
11	28.3	15315.1	Manual	1000	4.3	EIC 659.2800±0.02 +All MS	499.2511	
13	29.9	21779.7	Manual	1788	7.7	EIC 659.2800±0.02 +All MS	499.2519	
5	26.2	10308	Manual	700	5.5	EIC 666.2800±0.02 +All MS	679.7945	
1	25.5	5842.1	Manual	672	6.3	EIC 666.7800±0.02 +All MS	792.3403	
4	26.2	147955.8	Manual	11340	79.1	EIC 672.2700±0.02 +All MS	679.7943	
2	26.1	161229.4	Manual	9820	74.1	EIC 672.7800±0.02 +All MS	679.7943	
7	27	118384.9	Manual	6800	46.3	EIC 678.2900±0.02 +All MS	678.3006	
3	26.2	870822.4	Manual	63356	428.9	EIC 679.7800±0.02 +All MS	679.7944	
8	27	3447	Manual	440	4	EIC 689.2800±0.02 +All MS	541.266	
6	26.7	137093.9	Manual	8820	72.7	EIC 695.7700±0.02 +All MS	695.7795	
9	28.2	465909	Manual	36312	251.8	EIC 875.5000±0.02 +All MS	875.5172	
U6								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
3	26.1	1057.6	Manual	252	2.2	EIC 666.2800±0.02 +All MS	680.2962	
1	26	37284.7	Manual	2464	23.8	EIC 672.7800±0.02 +All MS	679.7931	
5	26.8	12089.3	Manual	848	7	EIC 678.2900±0.02 +All MS	541.2627	
2	26.1	149625.4	Manual	11312	91.7	EIC 679.7800±0.02 +All MS	679.793	
4	26.6	29050.5	Manual	2428	22.2	EIC 695.7700±0.02 +All MS	695.7789	
6	28	93290.8	Manual	26664	171.9	EIC 875.5000±0.02 +All MS	875.4888	
7	28.2	297730.4	Manual	43088	277.7	EIC 875.5000±0.02 +All MS	875.5098	

U7								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
2	25.3	44557.3	Manual	3568	17.6	EIC 508.2500±0.02 +All MS	1182.528	
7	26.8	29479.5	Manual	2104	10.4	EIC 508.2500±0.02 +All MS	678.3012	
13	28.2	57554.6	Manual	3904	19.2	EIC 508.2500±0.02 +All MS	771.3343	
14	29.8	64499.2	Manual	4500	22.2	EIC 508.2500±0.02 +All MS	499.2503	
12	28.1	19283.4	Manual	932	4.1	EIC 659.2800±0.02 +All MS	771.3352	
15	29.9	15619.1	Manual	1120	4.9	EIC 659.2800±0.02 +All MS	499.2501	
3	25.5	270333.9	Manual	11508	85.1	EIC 666.7800±0.02 +All MS	792.3379	
1	25.1	7194.5	Manual	504	3.8	EIC 671.2800±0.02 +All MS	1182.533	
4	26	311592.1	Manual	21008	170.3	EIC 672.7800±0.02 +All MS	679.7968	
8	26.9	1174159	Manual	76336	479.1	EIC 678.2900±0.02 +All MS	678.301	
5	26.1	2680448	Manual	188388	1126.9	EIC 679.7800±0.02 +All MS	679.7969	
10	27.7	72984.3	Manual	2584	17.3	EIC 686.2900±0.02 +All MS	790.846	
9	27	25152.1	Manual	1220	9.6	EIC 689.2800±0.02 +All MS	678.3009	
6	26.5	350214.8	Manual	24332	185.6	EIC 695.7700±0.02 +All MS	695.7795	
11	28.1	349255.5	Manual	24272	179.4	EIC 875.5000±0.02 +All MS	790.8387	

U2								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
8	30.3	8930.3	Manual	992	5.1	EIC 659.2800±0.02 +All MS	499.2529	
3	26.6	21402.1	Manual	1728	11.4	EIC 666.2800±0.02 +All MS	679.7993	
1	26	22526	Manual	1648	11.8	EIC 666.7800±0.02 +All MS	791.8416	
2	26.6	154918	Manual	10260	84.8	EIC 672.7800±0.02 +All MS	679.7987	
5	27.4	28779.2	Manual	1840	13.6	EIC 678.2900±0.02 +All MS	541.2642	
4	27.1	516821.9	Manual	38572	275.3	EIC 695.7700±0.02 +All MS	695.7816	
7	28.8	162203.3	Manual	19056	116.8	EIC 875.5000±0.02 +All MS	771.3372	
6	28.5	35747.7	Manual	11588	70.3	EIC 875.5000±0.02 +All MS	755.352	8
4	26.7	2595355	Manual	177200	839.4	EIC 679.7800±0.02 +All MS	679.7986	11
U9								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
10	28.3	174220	Manual	11752	57.9	EIC 508.2500±0.02 +All MS	499.2502	
11	29.9	243270	Manual	15480	76.3	EIC 508.2500±0.02 +All MS	499.2512	
9	28.2	42180	Manual	2564	9.1	EIC 659.2800±0.02 +All MS	771.334	
12	29.9	59015	Manual	3948	14	EIC 659.2800±0.02 +All MS	499.2508	
1	24	15252	Manual	1448	8.4	EIC 666.2800±0.02 +All MS	666.2819	
2	25.5	154329	Manual	6324	43	EIC 666.7800±0.02 +All MS	679.7986	
3	26.1	433594	Manual	31080	250.8	EIC 672.7800±0.02 +All MS	679.7972	
6	26.9	748776	Manual	45732	286.7	EIC 678.2900±0.02 +All MS	678.2983	
4	26.1	5826950	Manual	441884	2642.5	EIC 679.7800±0.02 +All MS	679.7972	
7	27	27912	Manual	2024	13	EIC 689.2800±0.02 +All MS	678.2986	
5	26.6	676669	Manual	46512	288	EIC 695.7700±0.02 +All MS	695.7789	
8	28.2	718063	Manual	52664	307.9	EIC 875.5000±0.02 +All MS	875.5166	

U12								
#	RT [min]	Area	Int. Type	I	S/N	Chromatogram	Max. m/z	FWHM [m
1	26.3	17265	Manual	1396	12.5	EIC 672.7800±0.02 +All MS	679.7932	
2	26.4	119016	Manual	9180	67.9	EIC 679.7800±0.02 +All MS	679.7931	
3	27	15427	Manual	1360	10.4	EIC 695.7700±0.02 +All MS	549.4776	
5	28.5	248617	Manual	37760	243.4	EIC 875.5000±0.02 +All MS	875.4876	
4	28.4	134221	Manual	34020	219.3	EIC 875.5000±0.02 +All MS	875.5097	

S13 Racehorses' sera and performance study – Overall result provided by Professor Hunter from a study investigating the effect of supplementation on the serum levels of vitamin B_{12} and on its observed performance by its trainer.

STUDY OF B_{12} STATUS OF THOROUBRED RACEHORSES DURING THE FLAT SEASON June – September 2018

This study was performed because the trainer was dissatisfied with the condition and performance of his horses in the early summer. Full veterinary assessment revealed no reason for this and I suggested the possibility of B_{12} deficiency as we see in so many human patients with chronic fatigue. B_{12} determinations were performed by the laboratories of Rossdales veterinary practice in Newmarket.

- Blood was collected from 20 horses in June, July August and September. During June-July, 10 horses received a supplementary B12 injection, (3mls hydroxycobalamin weekly for 4 weeks) which was stopped after the July blood sample. The trainer assessed his horses performance after supplementation in July, naming those he found to have improved, to see if this was related to B12 status.
- The concentration of B12 in the 20 horses ranged from 3085 pg/ml to 7177 pg/ml, all within the normal range suggested by Addenbrooke's (although normal ranges in man are still disputed and Martin may have some comments to make!) The yearlings at the stud ranged from 2439 to 5273 pg/ml and, rather to my surprise their levels were significantly lower than those of the horses in training (4935 +/- 905 n=20 v 3527+/- 734,n=10 t= 4.2583, p=0.0002).
- Before supplementation there was no difference between the control group and the supplemented (4955+/- 937 n=10 v 4897+/- 988 n=10, t= 0.132 p=0.89). After supplementation (July) this changed significantly (4711+/- 895 n=10 v 6160+/- 749 n=10, t=3.9237 p =0.001) Clearly the injections worked, and this was confirmed by the rise in the supplemented group alone (4955+/- 937 before n=10 v 6160 +/-749 after t= 3.1745, p=0.0052).
- The changes in performance noted by The Trainer however, did not correspond to changes in B12 concentration. There was no difference between his 'better -8 horses' and 'no better -12 horses ' at the start (5267+/- 632 v 4714+/- 1014, t= 1.3698 p= 0.1876) nor even after supplementation! (July results (5267+/- 632 v 5771 +/- 792, t=1.4059, p=0.1816).
- The effects of the supplementation rapidly disappeared (July level, n=10, 6079+/- 805 v August level n=8, 5118 +/- 1078 t= 6.0255 p = 0.0005). There was no evidence however, of progressive B12 depletion during the season (June level in unsupplemented horses,(n= 10) 4915+/-923 v September level n= 9, 4948+/-1015, t=0.1871 p=0.8557).
- 10 control samples were obtained from another yard. There are no faecal samples corresponding to these sera. The mean was 4377 +/- 704.53. (N=10) The mean of 16 values from the original trainers's string (September values as closest to time of control samples) 4950 +/- 1001.29
- By Student's t-test T= 1.5774 P= 0.128 NS.

In conclusion, therefore, this study suggests that these racehorses have normal vitamin B12 levels, which remain satisfactory throughout the season, that supplementary injections increase the blood concentrations very temporarily, and there is no correlation between B_{12} status and performance!

S14 Previous human faecal samples HPLC-MS analysis results – Below are figures representing the cobamide composition in pernicious anaemia patients' faecal samples. The patient samples are grouped in 4 groups.

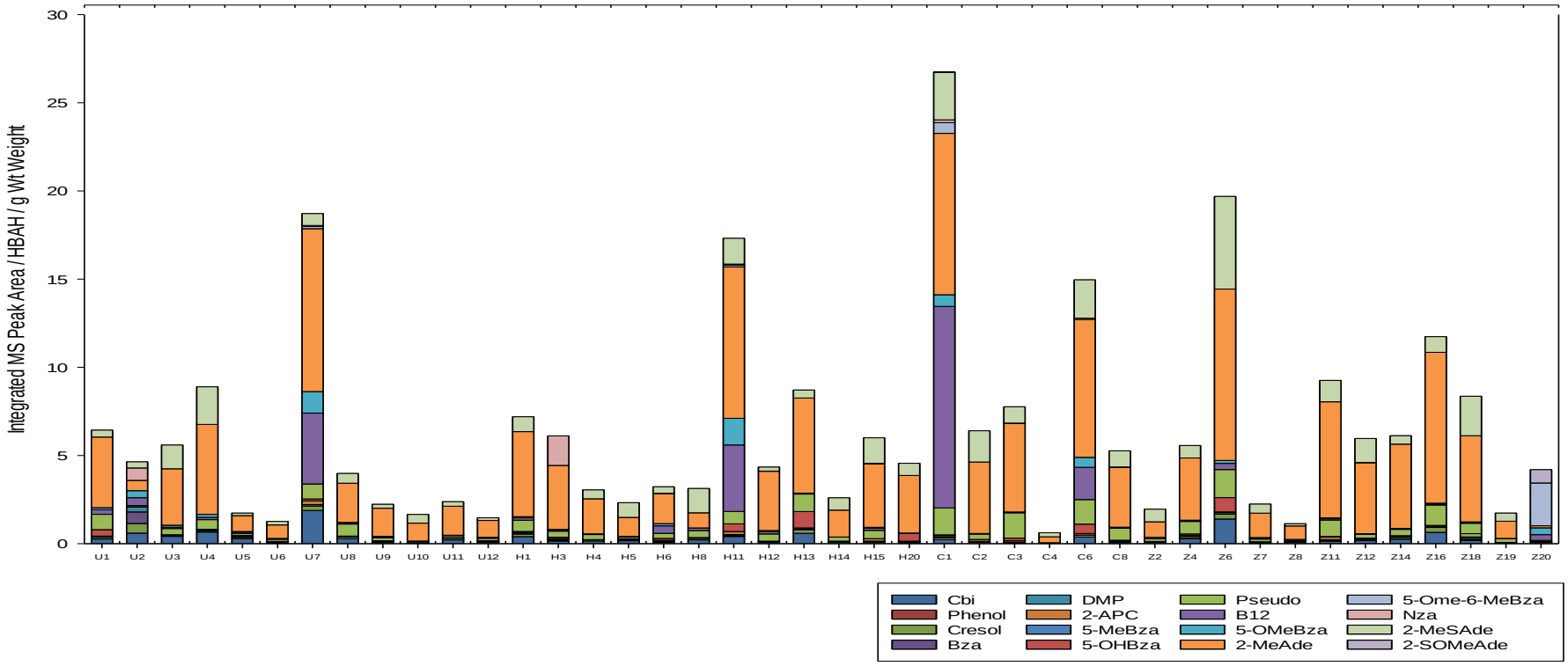
Group H: Happy group patients who are satisfied with their three monthly B_{12} injection

Group U: Unhappy group patients whose B_{12} deficient symptoms surface before with their next injection is due

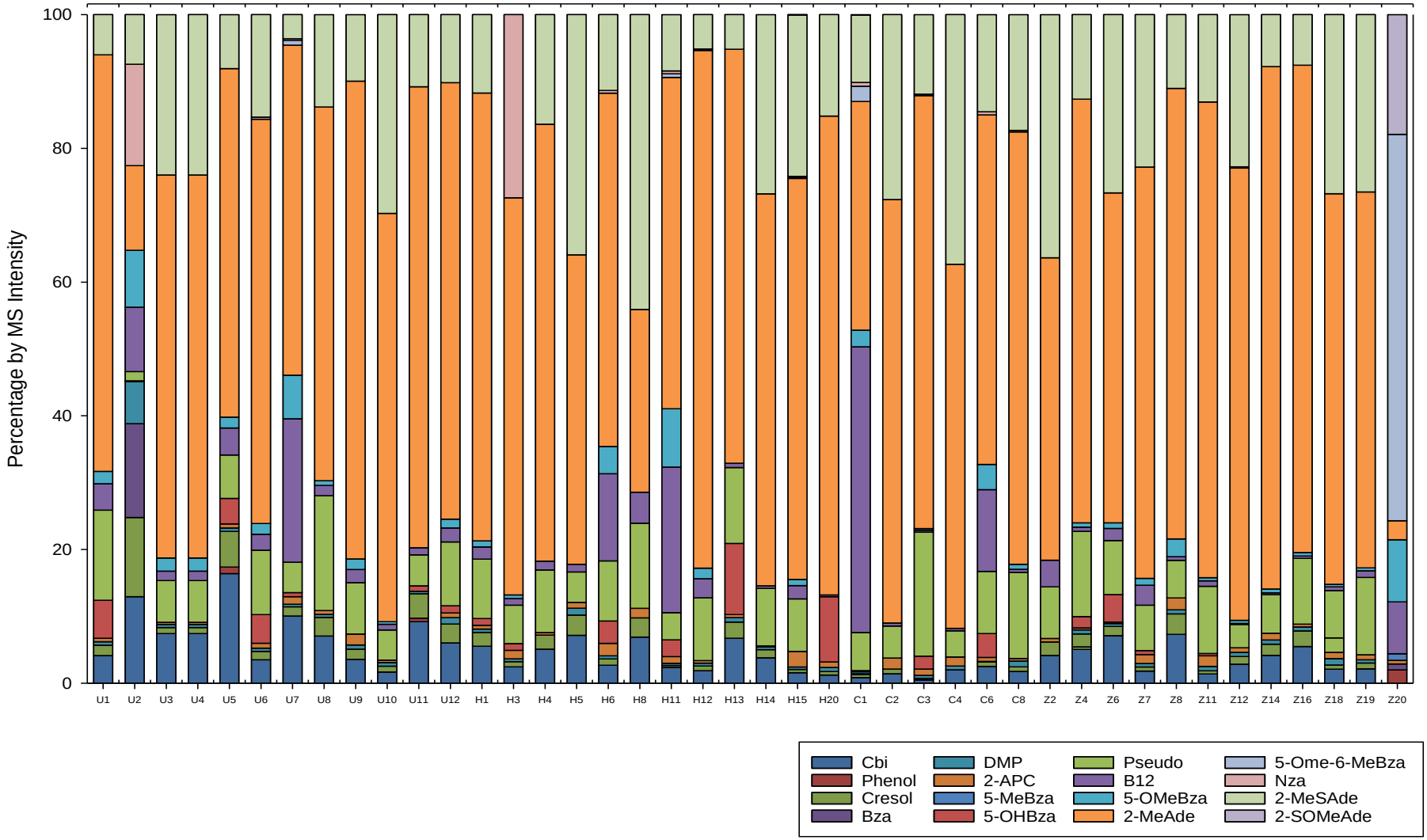
Group C: Control group from the general population who report no signs of deficiency

Group Z: Control group subjects who were serum tested to be non-deficient

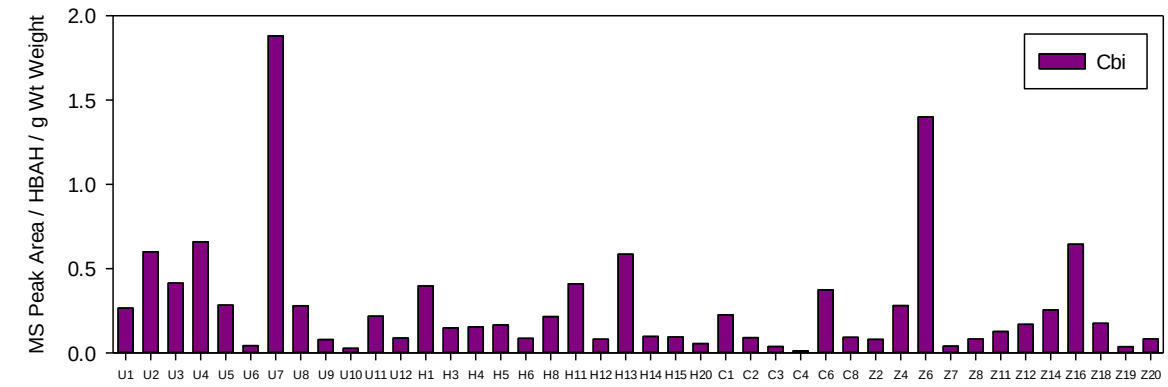
Normalised peak area of all cobamides present in each patients' faecal samples

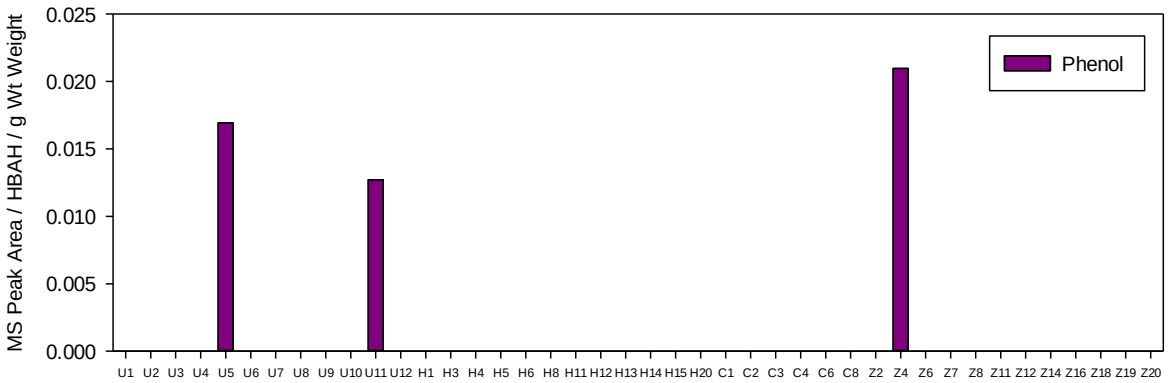


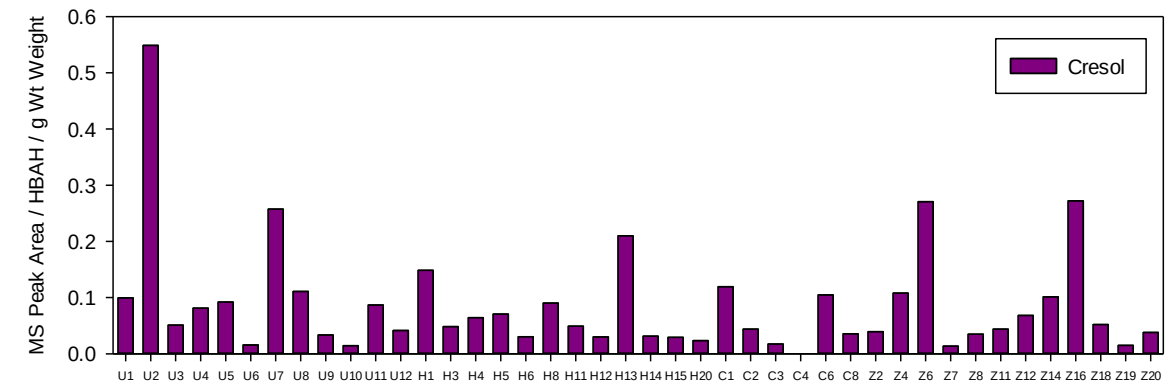
Prevalence of each analogue as a percentage of the total cobamides detected within each sample

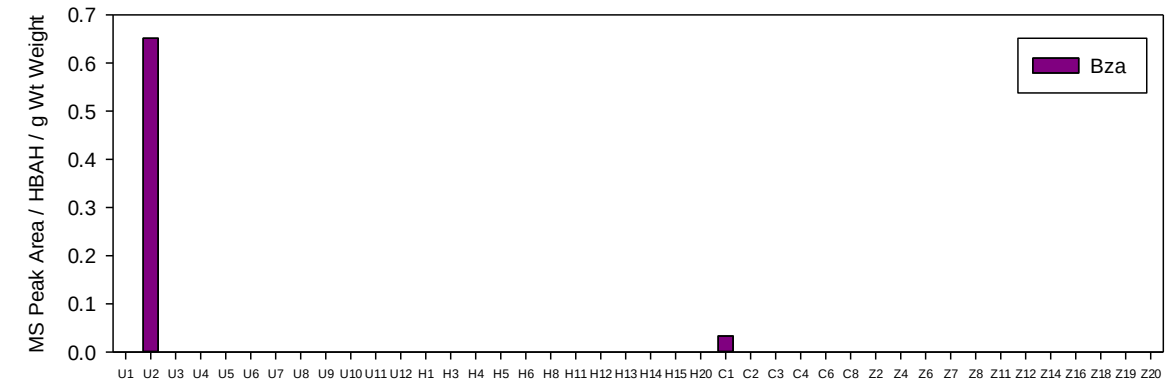


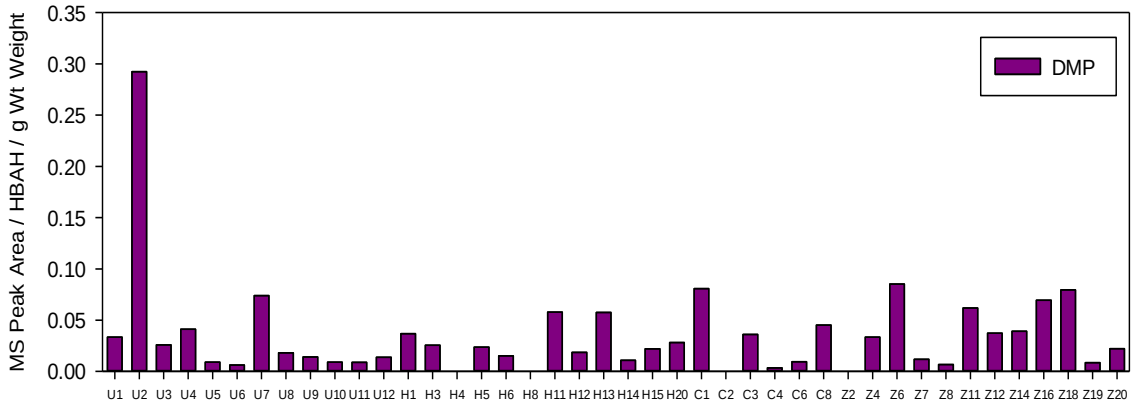
Levels of individual CN-Cbl analogues by normalised peak area

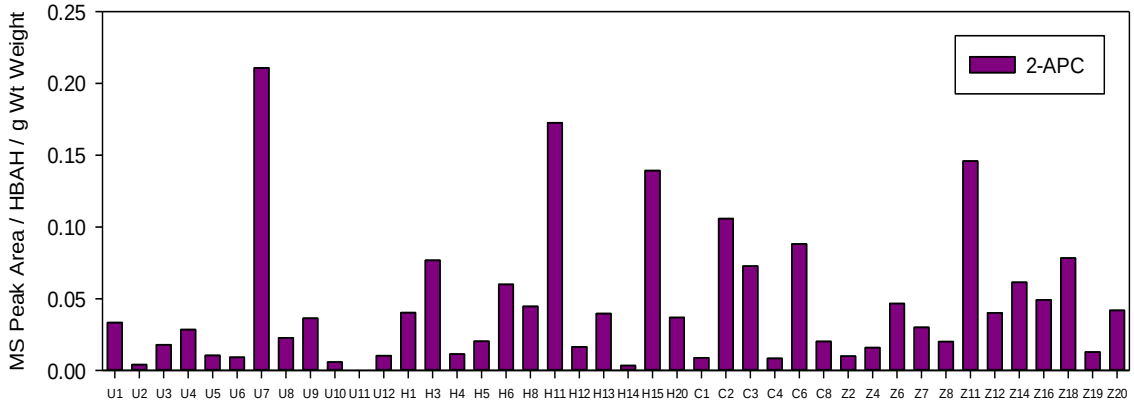


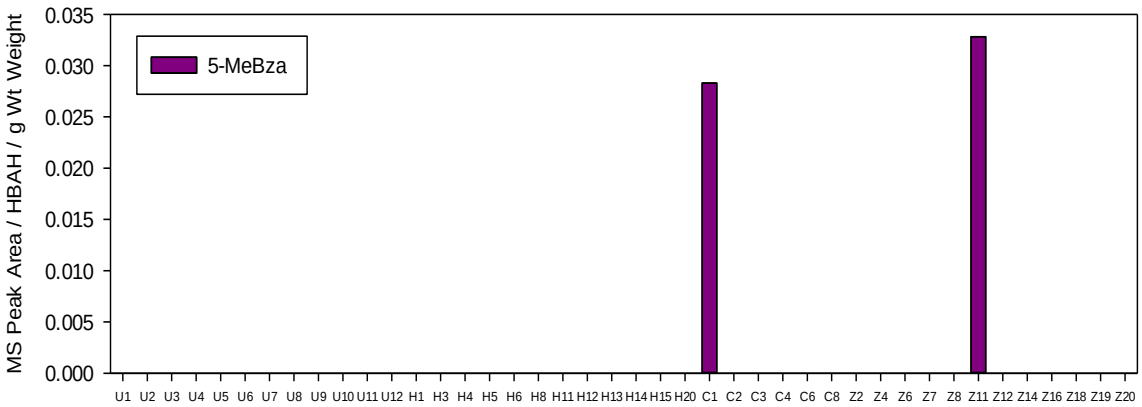







MS Peak Area / HBAH / g Wt Weight
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
DMP
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
0.25
0.20
0.15
0.10
0.05
0.00
2-APC
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
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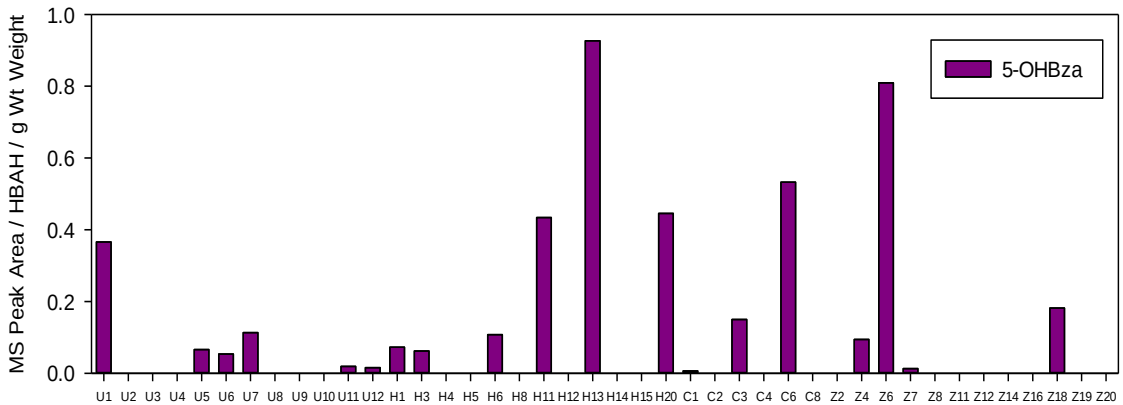
0.030
0.025
0.020
0.015
0.010
0.005
0.000
5-MeBza
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
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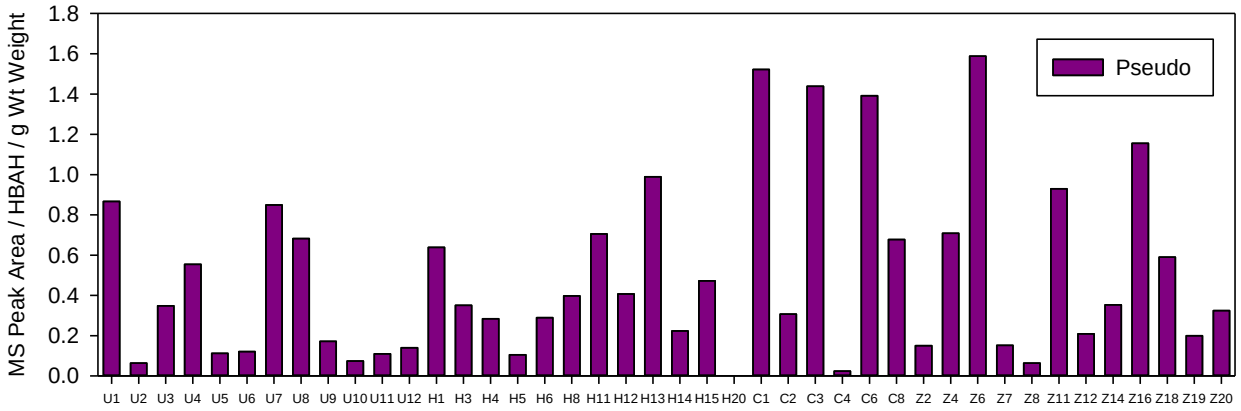
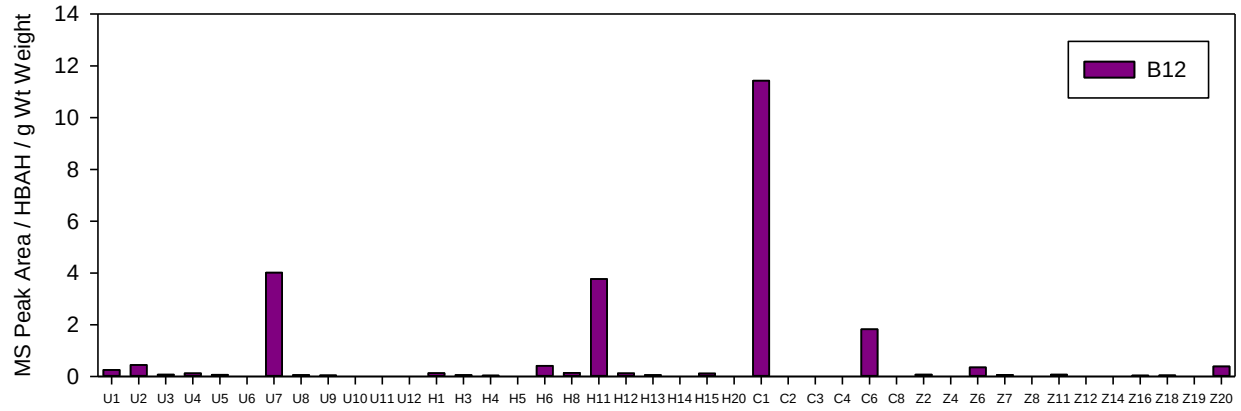
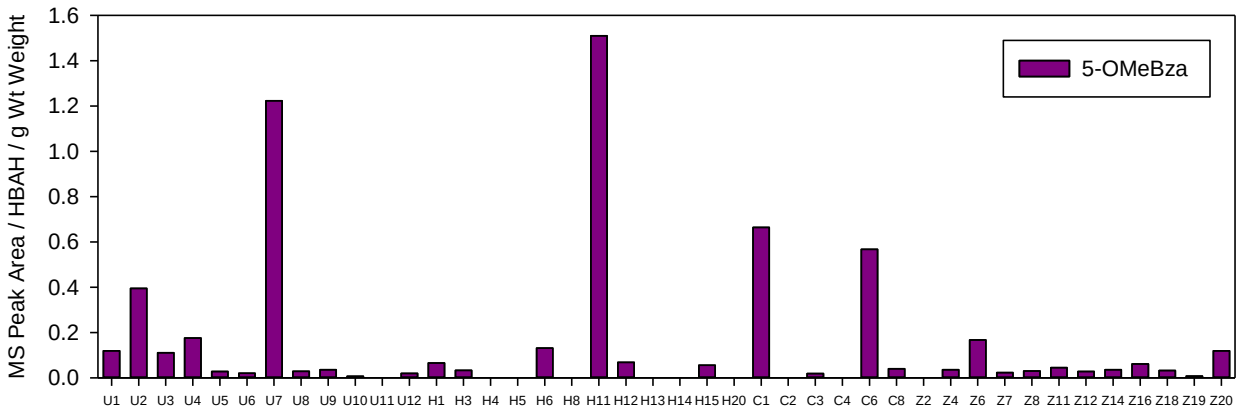
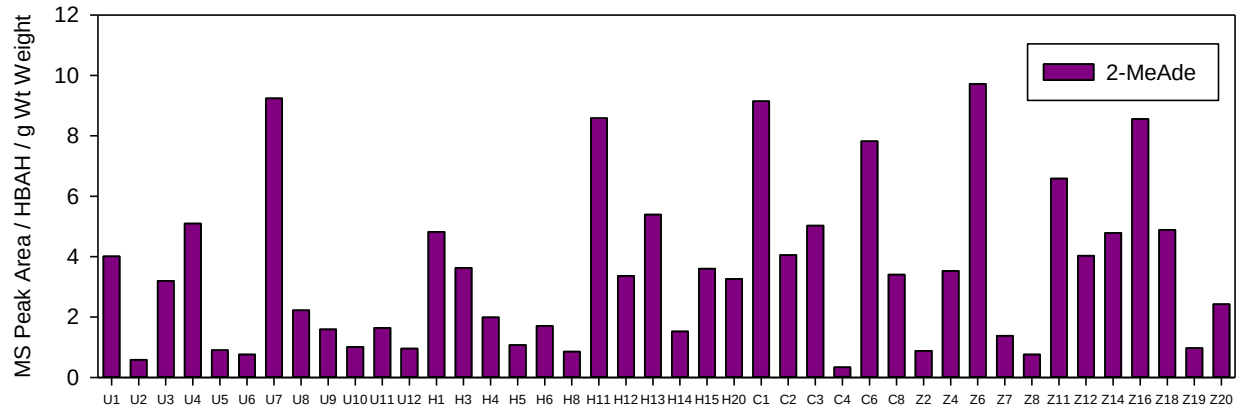
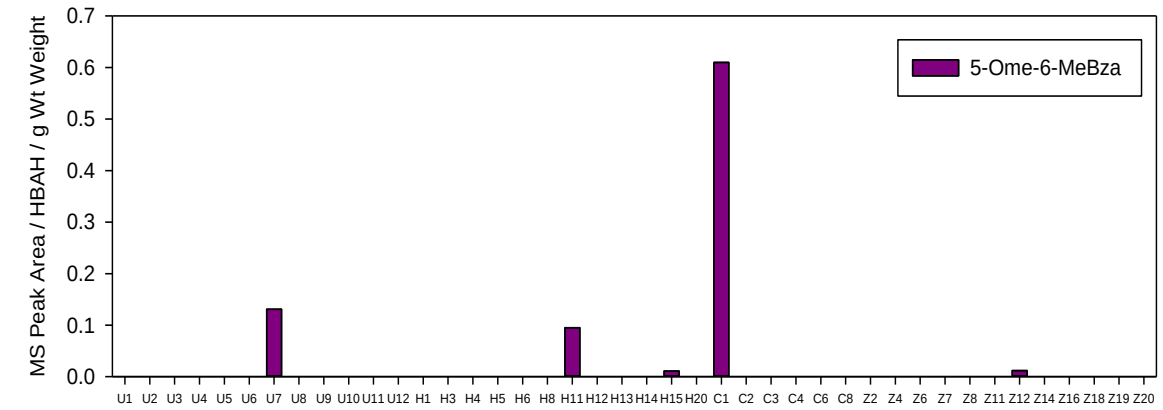
0.8
0.6
0.4
0.2
0.0
5-OHBza
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20

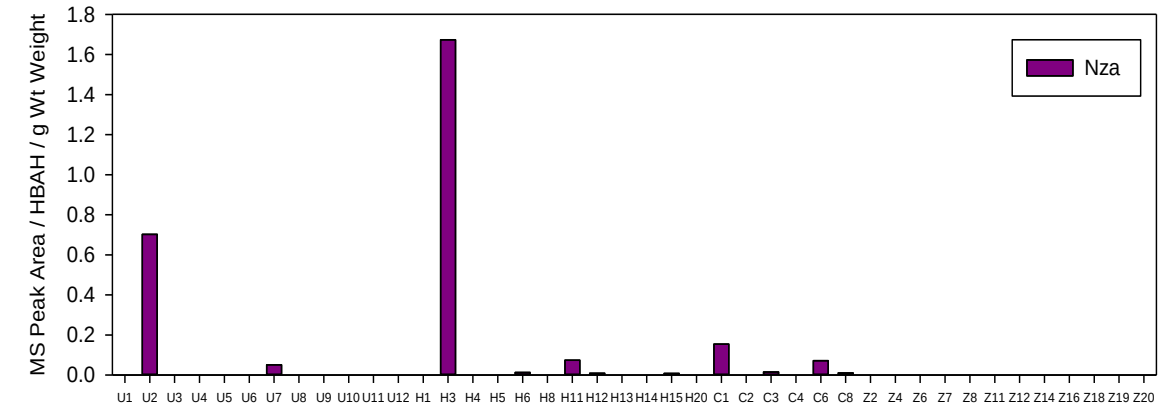

MS Peak Area / HBAH / g Wt Weight
Pseudo
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
B12
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
5-OMeBza
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
2-MeAde
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5-Ome-6-MeBza
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
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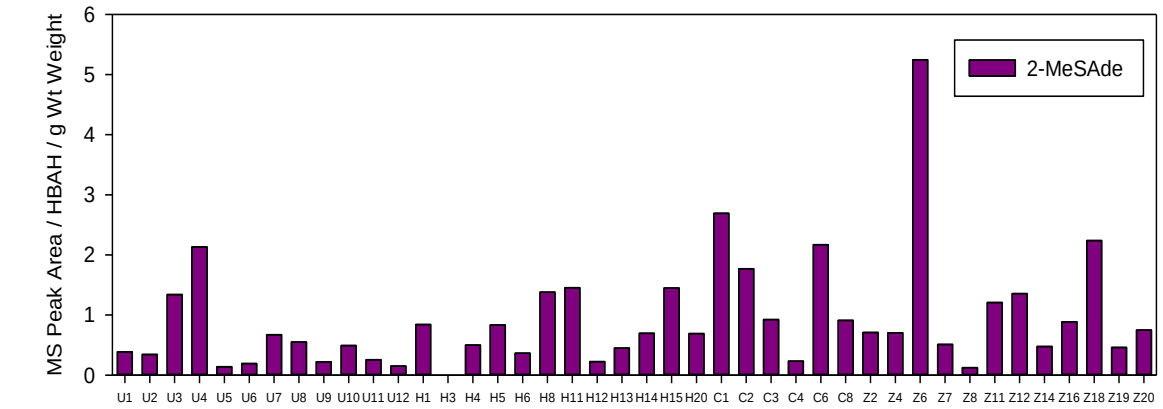
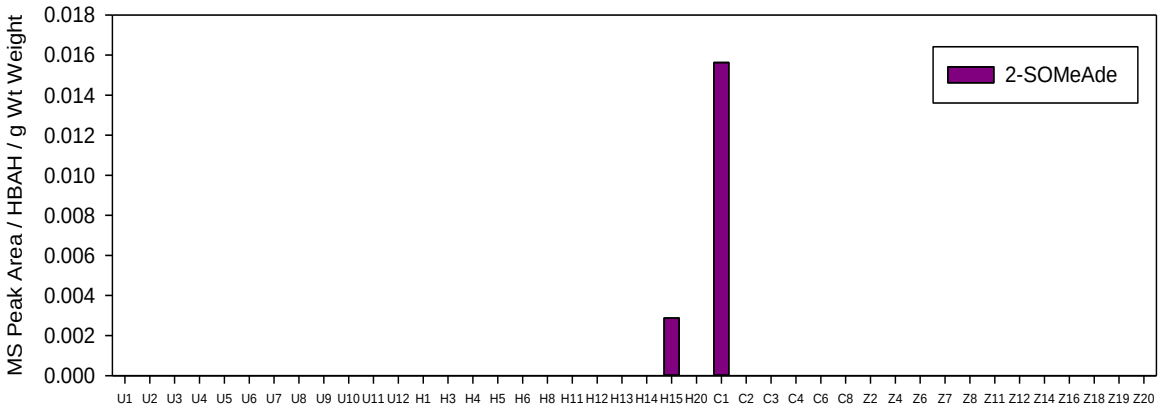
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Nza
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20


MS Peak Area / HBAH / g Wt Weight
6
5
4
3
2
1
0
2-MeSAde
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20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0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
2-SOMeAde
U1 U2 U3 U4 U5 U6 U7 U8 U9 U10 U11 U12 H1 H3 H4 H5 H6 H8 H11 H12 H13 H14 H15 H20 C1 C2 C3 C4 C6 C8 Z2 Z4 Z6 Z7 Z8 Z11 Z12 Z14 Z16 Z18 Z19 Z20

S15 – CULTECH study

Comparative Analysis of the Microbiota of a Population of Pernicious Anaemia Sufferers

This supplementary study was done by CULTECH to find potential biological markers associated with Pernicious Anaemia that could uncover more ways to improve the outcomes of patients receiving Vitamin B_{12} deficiency treatments. This study tested faecal samples to identify microbiological representation in the gut microbiota. They were processed using 16s rRNA gene sequencing. Three groups of patients were studied.

Group H: Happy patients who are satisfied with their 3-monthly supplementation

Group U: Unhappy patients who are dissatisfied with their 3-monthly supplementation. Patients whose Vitamin B_{12} deficient symptoms surface before their next 3-monthly injection is due.

Group C: Control, non-pernicious anaemia patients

In the following page is the summary of findings provided by CULTECH. Main findings include:

- Significantly lower levels of *Bifidobacterium* in patients than non-patients.
- Levels of *Lactobacillus* was found to be enriched in the Unhappy group patients compared to control.
- Levels of *Klebsiella* was found to be higher in patients than non-patients.
- An unidentified genus of bacteria was found to be in higher levels in patients than non-patients.

Comparative Analysis of the Microbiota of a Population of Pernicious Anaemia Sufferers

June 2018

CULTECH
BIOSPECIALITY PRODUCTS

COMPARATIVE ANALYSIS OF THE MICROBIOTA OF A POPULATION OF PERNICIOUS ANAEMIA SUFFERERS

Summary of Key Findings

A total of 36 faecal samples were provided to Cultech originating from 3 cohorts, Happy, Unhappy and a Control group. DNA was extracted and Next generation sequencing was completed. Quality control of DNA and sequencing data resulted in group sizes of 12,11 and 11 for the Happy, Unhappy and a Control groups respectively (i.e. 34 of the 36 samples provided were suitable for analysis).

Key findings from this study have shown no significant differences in the alpha or beta diversity. However significant differences were seen at the taxonomic level. Specifically, significant differences in the Firmicutes:Bacteroidetes and Actinobacteria:Bacteroidetes were observed. The relationship between these ratios has been linked to disease conditions.

A reduction in B*ifidobacteria* was seen in both the Happy and Unhappy groups in comparison to the Control group. Higher levels of the genus *Lactobacillus* were seen in the Unhappy group.

In the *Enterobacteriaceae*, significantly higher levels of Klebsiella and an "unknown" bacterium were seen in the Happy and Unhappy groups when compared to the control.

Therefore, overall this study has indicated significant differences in the microbiota composition of the three groups investigated. More in-depth analysis relating some of these outcomes to anthropomorphic data might prove useful but ideally this would be done with a larger population to gain a more meaningful understanding of these findings.