Half-year report: 'Insect-based ingredients in aquafeed'

Manon Eggink, 01.08.2020

February

76h

During February, I have worked on a time schedule for the three year project of the PhD and design of the different trials that will be performed during the PhD. A more detailed schedule of the PhD project can be found in Appendix I. One pre-trial and a larger scale trial will be performed at ENORM to investigate the influence of the rearing substrate on the nutritional composition of black soldier fly larvae. Furthermore, different inclusion levels of ENORMs black soldier fly meal will be tested in fish trials with rainbow trout and Nile tilapia. Lastly, the effects of the replacement of currently used protein sources with black soldier fly meal on fish health will be investigated.

Furthermore, a desk study has been performed on the use of black soldier fly larvae as fish feed ingredients. The most important findings were that black soldier fly meal can be used as replacement of fishmeal to a certain extent, however, it is likely that crystalline amino acids need to be added to sustain growth, although this needs to be confirmed with analysis of ENORM samples. Additionally, it will be difficult to include black soldier fly larvae meal high in fat to replace current protein sources, therefore appropriate defatting processes are required. Lastly, black soldier fly oil can only replace current lipid sources to a small extent, due to the high amount of saturated fatty acids, which can negatively affect fish health and performance.

March

155.4h

March was used to elaborate and discuss the time schedule for the three years with the supervisors Anne Johanne Tang Dalsgaard and Ivar Lund. Additionally, the PhD proposal has been handed in, and accepted by the PhD board.

The first work package of the PhD project is to determine the nutritional composition of black soldier fly larvae and to quantify the chitin. Chitin is found in the exoskeleton of insects, and provides structure and protection. It is a polymer consisting of N-acetylglucosamine subunits connected by β1,4-linkages, whereas chitosan is its deacetylated derivative consisting of N-glucosamine units with β1,4-linkages. Several methods have been previously described to determine or quantify chitin, including HPLC, NMR, and FT-IR. Due to the absence of most of these machines at the DTU location in Hirtshals, it was decided to start with a method based on weight before and after extraction as previously described by Liu et al. (2012). In this method, samples are treated with an acid (HCl) to remove catechols & minerals and a base (NaOH) to remove cuticle proteins. For more details, see the protocol used in Appendix 2.

There were some observed issues with this method: 1) large sample sizes were required (5g) which makes it difficult to use when investigating larvae samples, 2) large quantities of HCl and

NaOH are needed, and 3) the method is not very accurate because sample is lost during the filtration step and it can be that there are still other components in the sample left e.g. lipids that can lead to an overestimation of the chitin. It has therefore been decided to investigate the use of two different methods to quantify chitin: spectrophotometry and fluorescence. For the future, it would be interesting to investigate the use of HPLC for chitin quantification, although, Manon has currently no experience in HPLC and therefore needs to do a course in HPLC which was originally planned in June but because of corona, the course has been delayed to late October- beginning of November. The corona outbreak had also affected the possibilities to perform lab analysis in March.

April

140.6h

During April, different fractions of larvae samples send by ENORM have been analyzed for proximate and amino acid composition in our lab. The fractions that were sent: hele larver, fiber juice, larvejuice juicer, fibre baader, larvae juice baader, fibre baader, larvae juice baader. It was observed that large differences in proximate composition were found between these different fractions, mainly in DM, protein, and fat content. Also for the amino acid profile, differences in the fractions were observed (Appendix 3). It should be mentioned that total organic nitrogen was determined using the Kjeldahl method, with a conversion factor of 6,25 to approach the protein content. However, recent findings have showed that the use of 6,25 overestimates the protein content for black soldier fly larvae due to the presence of non-protein nitrogen (Janssen et al., 2017). Janssen et al. (2017) recommended the use of 4,67 as conversion factor, and calculations using this conversion factor have therefore been included.

Sample ID	Dry matter (DM)	Ash (%DM)	Protein (%DM)	Fat (%DM)
Hele larver 1.1 100%	30,8	9,8	37,0[a] 27,7[b]	38,0
Fiber juicer 1.1	58,7	10,3	67,8[a] 50,7[b]	7,2
Larvejuice juicer 1.1 91.3%	27,4	9,9	29,6[a] 22,1[b]	44,9
Fiber baader 1.1 20%	39,9	8,7	54,4[a] 40,6[b]	21,3
Larvejuice baader 1.1	27,6	9,9	32,2[a] 24,1[b]	42,4
Fiber baader 12%	46,0	10,1	59,8[a] 44,7[b]	8,7
Larvejuice baader 88%	29,8	9,3	31,2[a] 23,3[b]	50,0

[a] Conversion factor 6,25; [b] Conversion factor 4,67

May

133.2h

In May, a desk study was performed on the different probable rearing substrates for black soldier fly rearing. After the desk study, different substrates were selected for the insect trials: crushed mussels, crushed shrimp waste, and brewer's mash. These are by- and waste-products widely available in Denmark. For external comparison, one group will be fed chicken feed whilst for internal comparison, one group will be fed ENORM mix. These substrates will be tested first on small-scale, and later on larger scale when observed that the larvae can uses the substrate for growth.

Furthermore, ENORM has sent samples of the grax and protein fraction, which is samples before and after the drying process, respectively. Samples 1/3-3/3 are obtained from different containers but are replicates. It can be seen that the dry matter content is much higher in the protein fraction, which is expected as the protein fraction was dried. On dry matter basis, ash, protein, and fat content are a bit higher in the grax fraction compared to the protein fraction, indicating that the drying has a minor effect on the proximate composition. The amino acid profile of the protein and grax fraction were overall similar, showing that the drying process did not have large effects on the amino acid profile (Appendix 4).

Sample ID	Dry matter (DM)	Ash (%DM)	Protein (%DM)	Fat (%DM)
Protein 1/3	94,9	7,5	55,6[a] 41,6[b]	20,4
Protein 2/3	94,9	7,5	55,2[a] 41,3[b]	20,9
Protein 3/3	95,1	7,5	55,1[a] 41,2[b]	20,8
Grax 1/3	31,9	7,9	59,6[a] 44,5[b]	22,5
Grax 2/3	31,8	8,0	59,1[a] 44,2[b]	22,3
Grax 3/3	31,9	8,0	59,6[a] 44,5[b]	22,3

[a] Conversion factor 6,25; [b] Conversion factor 4,67

June

155.4h

In June, ENORM has sent us the samples that were mechanically separated on size (0-200 um, 200-400 um, and >400 um) assuming that the largest fraction (>400 um) has the highest quantity of chitin. Additionally, a proto-type of insect meal has been sent to us to investigate the nutritional composition. It was found that the mechanical separation seem to increase protein and fat content whilst decreasing ash content. However, the mechanical separation has to be performed several times to investigate whether this trend is also seen in different batches of insect samples.

Sample ID	Dry matter (DM)	Ash (%DM)	Protein (%DM)	Fat (%DM)
0-200 um	93,3	8,4	53,7[a] 40,1[b]	23,9
0-400 um	94,6	7,6	55,0[a] 41,1[b]	21,2
200-400 um	94,0	6,9	56,1[a] 41,9[b]	19,7
>400 um	94,4	6,5	57,6[a] 43,1[b]	17,2
Insect meal	99,8	14,6	58,7[a] 43,9[b]	13,6

[a] Conversion factor 6,25; [b] Conversion factor 4,67

July

170.2h

In July, most of the focus was put on finding a method for chitin quantification using either fluorescence methods or spectrophotometry. Two spectrophotometry methods were tested (Lehmann and White, 1975; Han and Heinonen, 2020) and one fluorescence method (Henriques et al., 2020). The spectrophotometry method is based on the reaction of hexosamines that deminate into 2,5-anhydrohexoses that react with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and $FeCl_3$ that yield into an intense blue colour. Whereas the fluorescence method is based on staining the chitin with calcofluor.

For the methods described by Lehmann and White (1975) and Henriques et al. (2020), large variations were observed between replicates as seen by the large standard deviation (Appendix 6 & 7). These variations are likely due to the difficulties with homogenization of these samples in distilled water in early steps of the quantification. However, the spectrophotometry method of Han and Heinonen (2020), which is also based on the reaction of MBTH and $FeCl_3$ similar to that of Lehmann and White (1975), and showed potential. Therefore, the protocol of Han and Heinonen (2020) is currently optimized to enhance repeatability (Appendix 5).

Additionally, ENORM has performed another time of mechanical separation with a different batch. Samples 101-104 (0-200 um) and 121-124 (200-400 um) were separated once, whereas the largest fraction (>400 um) was again separated to sample 201 (0-200 um), 221 (200-400 um), and 241-244 (>400 um). It can be seen that the fat content seems to decrease with mechanical separation, as was seen previously in June. The difference in ash and protein content between the different fractions as was seen in June, was less pronounced this time.

Sample ID	Dry matter (DM)	Ash (%DM)	Protein (%DM)	Fat (%DM)
101-104 (0-200 um)	96,5	13,6	54,9[a] 41,0[b]	21,5
121-124 (200-400 um)	96,4	13,5	55,7[a] 41,6[b]	20,2
201 (0-200 um)	96,3	13,6	54,9[a] 41,0[b]	19,2
221 (200-400 um)	96,7	11,9	56,2[a] 42,0[b]	16,3
241-244 (>400 um)	98,1	10,6	56,1[a] 41,9[b]	13,3

[a] Conversion factor 6,25; [b] Conversion factor 4,67

Han, X., & Heinonen, M. (2020). Development of ultra-high performance liquid chromatographic and fluorescent method for the analysis of insect chitin. *Food Chemistry*, 127577.

Henriques, B. S., Garcia, E. S., Azambuja, P., & Genta, F. A. (2020). Determination of Chitin Content in Insects: An Alternate Method Based on Calcofluor Staining. *Frontiers in Physiology*, *11*, 117.

Janssen, R. H., Vincken, J. P., van den Broek, L. A., Fogliano, V., & Lakemond, C. M. (2017). Nitrogen-to-protein conversion factors for three edible insects: Tenebrio molitor, Alphitobius diaperinus, and Hermetia illucens. *Journal of Agricultural and Food Chemistry*, *65*(11), 2275-2278.

Lehmann, P. F., & White, L. O. (1975). Chitin assay used to demonstrate renal localization and cortisone-enhanced growth of Aspergillus fumigatus mycelium in mice. *Infection and immunity*, *12*(5), 987-992.

Purkayastha, D., & Sarkar, S. (2020). Physicochemical Structure Analysis of Chitin Extracted from Pupa Exuviae and Dead Imago of Wild Black Soldier Fly (Hermetia illucens). *Journal of Polymers and the Environment*, *28*(2), 445-457.

Waśko, A., Bulak, P., Polak-Berecka, M., Nowak, K., Polakowski, C., & Bieganowski, A. (2016). The first report of the physicochemical structure of chitin isolated from Hermetia illucens. *International Journal of Biological Macromolecules*, *92*, 316-320.

Technical University of Denmark

Appendix I

Study and project plan - Time schedule

Please list your planned activities below. A Gantt diagram will be generated automatically.
You must enter the start date of your PhD project for the Gantt diagram to work.

Name of PhD student: *Manon Eggink*

Start date of PhD: *01-02-2020*

Gantt diagram updated on: *07-08-2020*

Activity	Start date (dd-mm-yyyy)	End date (dd-mm-yyyy)	Half year period 1	2	3	4	5	6	7	8
Study plan and literature research	01-02-2020	31-03-2020								
Course - Transdisciplinary approaches to sustainable marine aquaculture (GU)	10-02-2020	14-02-2020								
Course - Laboratory animal science (SDU)	24-02-2020	28-02-2020								
Course - Hands-on liquid chromatography-mass spectrometry (AU)	26-10-2020	06-11-2020								
Course - Insects as food and feed, from producing to consuming (WUR)	22-06-2021	26-06-2021								
Course - Sustainability, evaluation and communication (DTU)	01-08-2020	31-08-2020								
Course - Teaching and learning (DTU)	25-01-2021	31-01-2021								
Course - Aquatic science and living resources (DTU)	01-04-2020	01-04-2022								
WP1 - Experimental work on chitin quantification and proximate analysis in black soldier fly	01-04-2020	31-08-2020								
WP2 - Experimental work on use of black soldier fly meal in rainbow trout and Nile tilapia	01-11-2020	30-04-2021								
WP2 - Writing paper 1 + 2	01-05-2021	30-06-2021								
WP2 - Finalize and submit paper 1 + 2	01-07-2021	01-08-2021								
WP3 - Experimental work on use of different rearing substrates for black soldier fly	01-09-2020	01-12-2020								
WP3 - Writing paper 3	01-12-2020	31-01-2021								
WP3 - Finalize and submit paper 3	01-02-2021	30-04-2021								
WP4 - Experimental work on postprandial uptake of free and protein-bound amino acids in rainbow trout and	01-08-2021	30-06-2022								
WP4 - Writing paper 4	01-07-2022	31-08-2022								
WP4 - Finalize and submit paper 4	01-09-2022	01-11-2022								
Thesis writing	01-08-2022	31-01-2023								
Conference - International conference on aquaculture, environment, and waste management - paper 1 + 2 -	19-08-2021	20-08-2021								
Conference - International conference on fish health and nutrition - paper 3 - Berlin	20-05-2022	21-05-2022								
Conference - International conference on fish health in aquaculture - paper 4 - Rome	11-11-2022	12-11-2022								
External research stay	01-02-2022	01-05-2022								
Department work - Teaching fish physiology in aquaculture	01-03-2021	31-05-2021								
Department work - Teaching fish nutrition and bioenergetics	01-03-2021	31-05-2021								
Department work - Assistance with activities related to other projects	01-12-2020	31-01-2021								
Department work - Assistance with activities related to other projects	01-06-2021	31-07-2021								
Department work - Assistance with activities related to other projects	01-07-2022	01-08-2022								

Appendix 2

Chitin quantification based on Liu et al. (2012)

1. Dry samples for 2 days at 50 degrees Celsius
2. Micronize samples if needed
3. Store samples at 4 degrees until analysis
4. Weigh 5g sample in an Erlenmeyer
5. Treat the sample with 250 mL 1 M HCl at 100 degrees for 30 minutes
6. Filter the sample
7. Wash the sample with distilled water until neutrality is reached
8. Treat the sample with 250 mL 1 M NaOH at 80 degrees for 24 hours
9. Filter the sample
10. Wash the sample with distilled water until neutrality is reached
11. Dry the sample at 50 degrees in the oven until constant weight is achieved

Appendix 3

Relative amino acid content, % of total amino acids	Hele larver 1.1 100%	Fiber juicer 1.1	Larvejuice juicer 1.1 91.3%	Fiber baader 1.1 20%	Larvejuice baader 1.1	Fiber baader 12%	Larvejuice baader 88%
Hydroxyproline (Hypro)	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]
Histidine (His)	3,5	2,9	3,8	3,2	3,5	3,0	3,5
Taurine (Tau)	0,3	0,2	0,3	0,0	0,3	0,0	0,3
Serine (Ser)	4,9	5,7	4,3	5,7	4,4	6,2	4,6
Arginine (Arg)	5,6	4,6	6,2	4,6	6,3	4,1	6,0
Glycine (Gly)	5,7	7,3	4,7	7,3	4,6	8,5	4,9
Aspartate (+ asparagine) (Asp + Asn) (3)	10,7	7,9	12,5	8,2	12,6	6,9	11,3
Glutamate (+ glutamine) (Glu + Gln) (3)	13,7	8,9	15,9	10,7	15,5	9,9	16,2
Threonine (Thr)	4,6	4,3	4,8	4,2	4,8	3,9	4,6
Alanine (Ala)	7,1	10,3	5,2	10,1	5,2	11,1	5,9
Cysteine (Cys)	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Proline (Pro)	7,0	9,2	5,4	9,2	5,2	10,4	6,2
Cystine (Csn)	0,2	0,1	0,3	0,0	0,3	0,1	0,4
Lysine (Lys)	6,7	5,1	8,3	4,9	8,0	3,8	7,4
Tyrosine (Tyr)	6,2	7,9	5,1	7,6	5,5	8,2	5,6
Methionine (Met)	1,5	0,8	1,8	0,7	2,0	0,5	1,9
Valine (Val)	6,2	8,5	5,0	8,2	5,0	8,5	5,4
Isoleucine (Ile)	4,7	4,7	4,8	4,5	4,8	4,4	4,6
Leucine (Leu)	7,4	8,2	7,1	7,8	7,3	8,0	7,0
Phenylalanine (Phe)	4,1	3,4	4,6	3,1	4,7	2,5	4,3
Tryptophan (Trp)(4)	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]
Sum of amino acids	**100**	**100**	**100**	**100**	**100**	**100**	**100**

[1]ND= not detected

Appendix 4

Relative amino acid content, % of total amino acids	**Protein 1/3**	**Protein 2/3**	**Protein 3/3**	**Grax 1/3**	**Grax 2/3**	**Grax 3/3**
Hydroxyproline (Hypro) (2)	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]
Histidine (His)	3,0	3,0	3,0	3,0	3,0	3,0
Taurine (Tau)	0,0	0,2	0,0	0,0	0,2	0,0
Serine (Ser)	5,0	5,0	5,1	5,0	5,1	5,2
Arginine (Arg)	4,7	4,7	4,7	4,5	4,6	4,3
Glycine (Gly)	6,4	6,4	6,3	6,4	6,5	6,6
Aspartate (+ asparagine) (Asp + Asn) (3)	9,2	9,2	9,3	9,6	9,2	9,0
Glutamate (+ glutamine) (Glu + Gln) (3)	11,2	11,2	11,3	11,5	11,1	11,2
Threonine (Thr)	4,6	4,6	4,6	4,6	4,6	4,5
Alanine (Ala)	8,8	8,7	8,7	8,7	8,8	9,1
Cysteine (Cys)	0,0	0,0	0,0	0,0	0,0	0,0
Proline (Pro)	7,5	7,5	7,4	7,4	7,5	7,7
Cystine (Csn)	0,2	0,2	0,2	0,2	0,1	0,1
Lysine (Lys)	5,7	5,7	5,7	6,1	5,9	5,8
Tyrosine (Tyr)	7,4	7,4	7,4	7,3	7,5	7,5
Methionine (Met)	1,6	1,5	1,6	1,3	1,3	1,3
Valine (Val)	6,9	6,9	6,9	6,7	6,8	6,9
Isoleucine (Ile)	5,1	5,1	5,1	5,1	5,0	5,0
Leucine (Leu)	8,3	8,3	8,2	8,3	8,3	8,3
Phenylalanine (Phe)	4,4	4,5	4,5	4,5	4,5	4,3
Tryptophan (Trp)(4)	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]	ND[1]
Sum of amino acids	**100**	**100**	**100**	**100**	**100**	**100**

[1]ND= not detected

Appendix 5

Sample preparation

1. Freeze samples at -20 degrees
2. Freeze dry to remove moisture until constant weight
3. Grind into a powder using micronisation and seal in plastic bags
4. Keep in desiccator under -20 degrees to keep the original composition

Preparation

Make a 0.5M NaOH solution

Cool the centrifuge to 5 degrees

Removal of protein

1. Add 0.5g sample to 10 mL 0.5M NaOH solution in a centrifuge tube

To remove proteins

1. Agitate with a stirrer for 2 hours
2. Centrifuge at 12000 rpm, 5 degrees, 15 minutes
3. Discard the supernatant
4. Wash the pellet with distilled water
5. Centrifuge at 12000 rpm, 5 degrees, 15 minutes
6. Add again 10 mL 0.5M NaOH solution to the sample
7. Agitate with a stirrer for 2 hours
8. Centrifuge at 12000 rpm, 5 degrees, 15 minutes
9. Wash pellet with distilled water
10. Move the pellet in buckets able to use for freeze drying
11. Freeze dry the samples until constant weight is achieved (~2-5 days)
12. Store in a desiccator at room temperature for later use

Demineralization

1. Hydrolyze 10 mg freeze dried deproteinized insect sample in 3 mL w/v 6 M HCl for 24h in a heating block at 100 degrees
2. Take 3 mL hydrolysate and add ±1.4 mL 12 M NaOH to adjust the pH to 6.0-6.5 by adding NaOH solution
3. Move the mixture into a centrifuge tube and add up to 10 mL with distilled water
4. Take 1 mL of the sample into a new centrifuge tube and for the reagent blank 1 mL distilled water

Measurement of glucosamine

1. Add 1 mL 5% $NaNO_2$ and 1 mL of 5% $KHSO_4$ to each 1 mL sample solution and to a reagent blank
2. Leave solution standing for 15 minutes at room temperature with occasional shaking

Preparation

Make 0.5% w/v MBTH solution in distilled water

1. Add 1 mL of 12.5% $NH_4SO_3NH_2$ (slowly, as this gets foamy) and shake constantly for 5 minutes
2. Add 1 mL of 0.5% fresh MBTH and let it stand for 60 minutes at room temperature
3. Add 1 mL of 0.5% fresh $FeCl_3$ and let it stand for minimally 30 minutes
4. Measure the absorbance at 650 nm against the reagent blank

MBTH and $FeCl_3$ need to be made every three days and stored in the fridge.

Standard curve

A standard curve was constructed to determine the glucosamine content in the samples as a measurement for chitin.

GlcN-HCl standard solution:
1. Standard solution I
   30 mg/10 mL GlcN-HCl in distilled water
2. Standard solution II
   1 mL of standard solution I with 9 mL distilled water (1:10 dilution)

Standard solution II was diluted according to the scheme below to obtain a final volume of 10 mL per standard solution, all concentrations were made in duplicate:

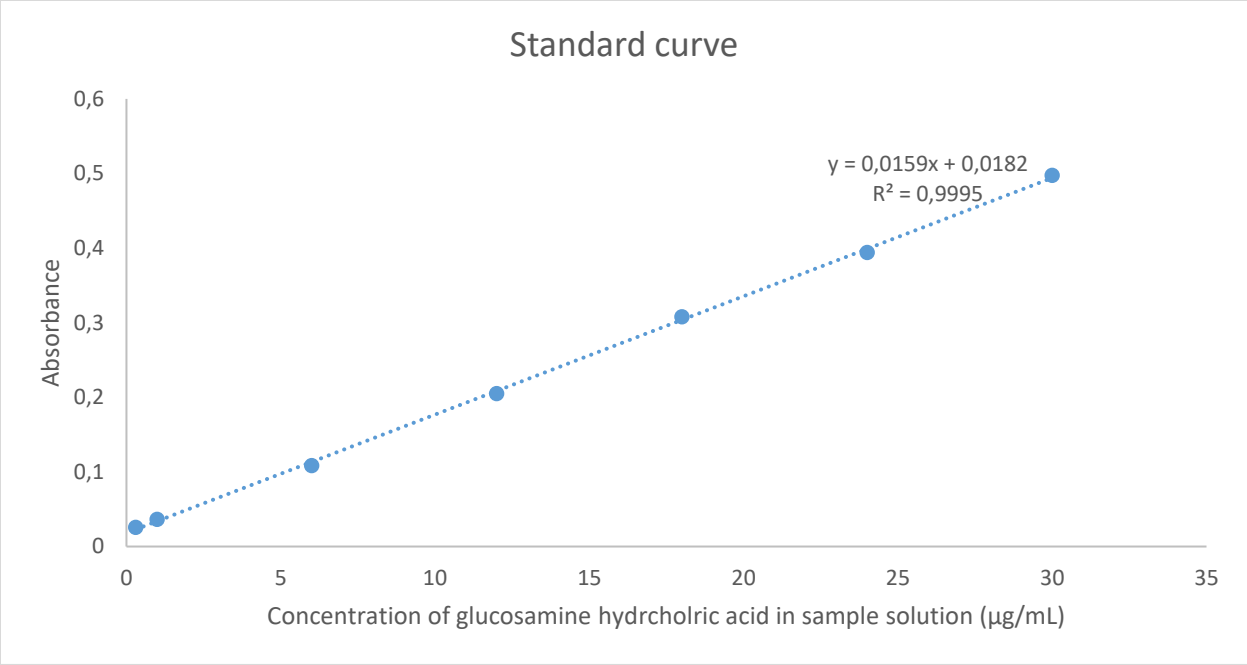
Standard solution concentration GlcN-HCl (µg/mL)	Pipetted volume standard solution II (µL)	Pipetted volume distilled water (µL)
0.3	10	9990
0.99	33	9977
6	200	9800
12	400	9600
18	600	9400
24	800	9200
30	1000	9000

Measurement

1. Add 1 mL 5% $NaNO_2$ and 1 mL of 5% $KHSO_4$ to each 1 mL standard solution and to a reagent blank (1 mL distilled water)
2. Leave solution standing for 15 minutes at room temperature with occasional shaking
3. Add 1 mL of 12.5% $NH_4SO_3NH_2$ (slowly, as this gets foamy) and shake constantly for 5 minutes
4. Add 1 mL of 0.5% fresh MBTH and let it stand for 60 minutes at room temperature
5. Add 1 mL of 0.5% fresh $FeCl_3$ and let it stand for minimally 30 minutes
6. Measure the absorbance at 650 nm against the reagent blank

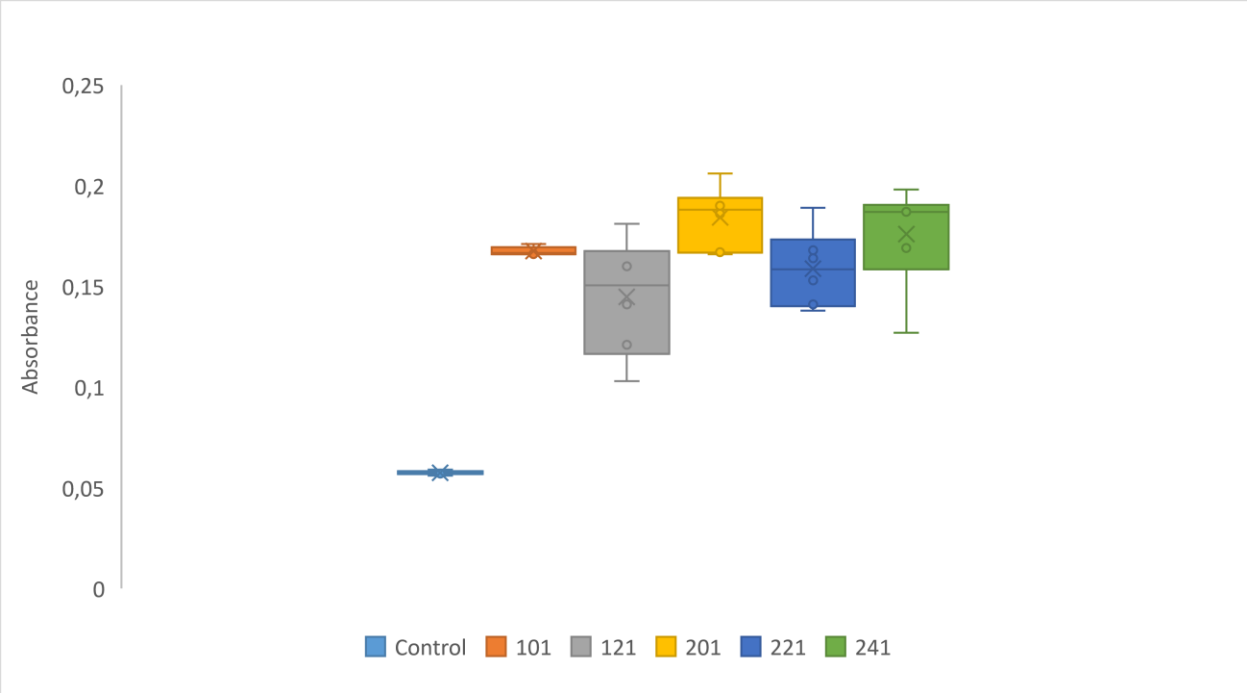
Standard curve calculations

Y-axis: standard absorbance minus the absorbance of the reagent blank (background deduction)
X-axis: concentration of GlcN-HCl (µg/mL)


Standard curve
y = 0,0159x + 0,0182
R² = 0,9995
Absorbance
0,6
0,5
0,4
0,3
0,2
0,1
0
0
5
10
15
20
25
30
35
Concentration of glucosamine hydrcholric acid in sample solution (µg/mL)

Appendix 6

Absorbance measured using spectrophotometry for internal control and samples 101-241 using the Lehmann and White (1975) method.



Appendix 7

Standard curve determined using fluorescence with chitin samples 1-50 mg initial weight