Isolation and structure determination of a heteroglycan from whiteworm lichen (*Thamnolia subuliformis*)

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Raunvísindadeild
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15 ECTS credit thesis submitted in partial fulfillment of a *Baccalaureus Scientiarum* degree in chemistry

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Isolation and chemical structure determination of a heteroglycan from whiteworm lichen (*Thamnolia subuliformis*)
Einangrun og byggingaákvörðun heteróglýkans úr ormagrösum (*Thamnolia subuliformis*)
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Útdráttur

Áður hefur verið sýnt fram á að fléttuheteróglýkanið thamnolan, úr fléttunni ormagrösum (*Thamnolia subuliformis*), hafi einstaka byggingu borið saman við aðrar fléttufjölsykrur. Það samanstendur af löngum galaktófúranósa keðjum og rhamnópýransósýlkjarna. Skyld fléttutegund hefur verið notuð frá alda öðli í alþýðulækningum og rannsóknir hafa sýnt fram á ónæmisstýrandi lífvirkni thamnolan. Einstök bygging thamnolan gæti átt stóran þátt í lífvirkni hennar og þrátt fyrir að byggingu hennar hafi verið lýst að stórum hluta hefur hún ekki verið að fullu staðfest með óyggjandi hætti.

Markmið þessa verkefnis var að seyða fram, einangra og hreinsa thamnolan úr ormagrösum og greina síðan byggingu þess. Seyðing og einangrun fór fram með heitum vatnsútdrætti, etanól útfellingu, díalýsu, frostþurrkun, jónaskiptaskiljun og gelsíun. Sýni af hreinsuðu útfellingunni Fraction III-a-1 (sem talið er að sé thamnolan) var greint með metanólýsu og gasgreiningu og metýleringsgreiningu á GC-MS til að greina einsykruhlutföll og tengjagerðir. Metanólýsan sýndi að Fraction III-a-1 innihélt einsykrurnar ara/rha/xyl/man/gal/glc í hlutföllum 3,2:25,3:7,0:3,5:50,0:11,0. Meðalmólþyngd Fraction III-a-1 var ákvörðuð með gelsíun á tveimur mismunandi súlum og reyndist hún 1420 kDa eða 1260 kDa. Fengin gildi úr mælingum voru síðan borin saman við þekkt gildi úr fyrri rannsóknum á thamnolan. Þær rannsóknir sýndu að thamnolan hafði meðalmólþyngdina 1450 kDa og einsykruinnihald í hlutföllunum rha/xyl/man/gal/glc 27:8:4,5:49:12,5. Niðurstöður þessarar seyðingar hljóta því að teljast í góðu samræmi við fyrri rannsóknir og því benda þær á það að tekist hafi að einangra fjölsykruna thamnolan í þessu verkefni. Þó þarf að gera hlutahýdrólýsu á sykrunni og 1D og 2D NMR mælingar til að ákvarða endanlega byggingu hennar.

Abstract

The lichen heteroglycan thamnolan, from the whiteworm lichen (*Thamnolia subuliformis*), has previously been shown to have a unique structure among other lichen polysaccharides. The heteroglycan is composed of long galactofuranosyl chains and a rhamnopyranosyl core. A related lichen species has been used historically in folk medicine and thamnolan's immunomodulating bioactivity has been demonstrated in other studies. Thamnolan's unique structure may play an important role in its bioactivity and although its structure has been described to a large extent, its structure has not been conclusively confirmed.

The objective of this research project was to extract, isolate and purify thamnolan from whiteworm lichen and then analyze its structure. Extraction and isolation was achieved using hot aqueous extraction of the lichen, ethanol fractionation, dialysis, lyophilization, ion exchange chromatography and gel filtration. A sample of the purified Fraction III-a-1 (thought to be thamnolan) was analyzed using methanolysis and gas chromatography (GC) and methylation and GC-MS analysis to discern its monosaccharide ratios and linkage types. Methanolysis revealed Fraction III-a-1 to contain monosaccharides in a ratio of 3.2:25.3:7.0:3.5:50.0:11.0. The mean molecular weight of Fraction III-a-1 was determined using size exclusion chromatography on two different columns and was determined to be 1420 kDa or 1260 kDa. The obtained values from these analyses were then compared to known values from previous studies of thamnolan. These studies showed thamnolan to have a mean molecular weight of 1450 kDa and a monosaccharide composition in a ratio of rha/xyl/man/gal/glc 27:8:4.5:49:12.5. The results of this extraction should therefore be considered consistent with previous studies and point to thamnolan‘s successful extraction. However, in order to confirm its detailed structure, partial hydrolysis and 1D and 2D NMR analysis is required.

Hereby I declare that this essay was written by me and has not been used by part or whole to a higher degree.

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Hlynur Árnason, kt. 041090-2279

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1 Introduction

1.1 Lichens

Lichens are the cohabitation of a fungus, called the mycobiont, and one or more species of photosynthesizing organisms, called the photobiont. The fungus provides shelter for the whole organism by weaving a protective shell made of fungal filaments around the photobiont and makes up most of the lichens volume. The outer layer of the fungus consists of specialized filaments which bind the lichen to the substrate as well as providing protection from external impact. Inside this protective shell, the fungus maintains a healthy level of moisture for the photobiont to live in and, in some cases, also processes minerals from the substrate. The role of the photobiont is to provide sustenance for the lichen by harvesting the sun's energy. The photobiont is usually either a green alga or a cyanobacterium, although a number of lichen species contain both. An important difference between these is that green algae synthesize polyhydric sugar alcohols while cyanobacteria synthesize glucose. Cyanobacteria can also fix nitrogen from the atmosphere (Óttarsdóttir 1999, Petersen 1997, Speer & Waggoner 1997, Lepp 2012).

Lichens have a varied morphology, some being almost microscopic and others spreading over vast areas of land. They are tough organisms, surviving in all but the most extreme climates and conditions. This makes them excellent pioneers in barren territories where they are often the first organism to settle. Upon death, the lichen residue converts to soil which makes habitation possible for less sturdy plants. This, however, is a slow process due to the fact that many lichens only grow a few millimeters each year.(Petersen 1997) An important part of the lichens considerable survivability is their ability to withstand drought. In arid environments, a lichen may dry up completely and become brittle, entering a kind of hibernation. When moisture is reintroduced, the lichen quickly absorbs water and resumes its usual functions (Haraldsdóttir 2001).

The reproduction of lichens can occur in two ways, sexually and asexually. In most lichens neither way is particularly preferred and both can occur simultaneously. The sexual reproduction produces small spores that are carried far by the wind while the asexual one produces larger spores that are carried shorter distances. The benefit of the latter is that the asexual spores usually end up in an environment closely resembling the one where the parent lichen grew and since the parent lichen was able to reproduce in this environment, the offspring is likely to do the same. The sexual spores are the pioneers, they may land in a completely different environment but they then have the added benefit of possibly combining their genes with spores from other lichens and thus create offspring with a broader genetic makeup that might survive under these new conditions (Lepp 2011, Lepp 2012).

1.2 Polymers

Polymers are macromolecules composed of many smaller units, called monomers. Polymers are classified on whether the monomers are identical or varied. Should the monomers be identical the polymer is called a homopolymer but if the monomers are varied it is a copolymer. Copolymers are then divided into random copolymers or block copolymers depending on whether the monomer sequence is random or if they arrange in regularly repeating blocks of monomers (Scott 1999).

Polymers play an increasingly big role in modern life. Whether they are man made or naturally obtained, the potential of their usability seems to be nowhere near exhausted as new ways to synthesize, manipulate and utilize polymers are constantly being discovered. Since the development of the first major industrial polymer in the early 19th century, the field of polymer chemistry has expanded rapidly and now synthetic polymers are used all over the world for example for packaging, coating and clothing (Davis 2004).

Although the chemistry of synthesized polymers is highly captivating, the focus of this thesis is on natural polymers, more specifically polysaccharides derived from lichens. Plants and algae produce polysaccharides through a process called photosynthesis in which the plant transforms water and carbon dioxide in combination with solar energy into chemical energy in the form of carbohydrates. A carbohydrate is, as the name suggests, a hydrate of carbon. They are fairly large molecules consisting in either aldehyde or ketone form. Carbohydrates are classified into mono-, oligo- and polysaccharides depending on the quantity of saccharides in the carbohydrate. A monosaccharide is the simplest form of carbohydrate, a single aldehyde or ketone consisting of a chain of 3-7 carbon atoms that are either linked in a straight chain or in the stable penta- or hexagonal form. The oligo- and polysaccharides consist of many monosaccharides bound together through glycosidic linkages, where oligosaccharides are made of 2-6 monosaccharide units and polysaccharides are any carbohydrates that contain more than 6 monosaccharide units. These units form long chains with some polysaccharides having branches at regular intervals (Bohlin & Samuelsson 2009). A significant difference between polysaccharides and other natural polymers such as proteins, nucleic acids, glycoproteins and glycolipids is that polysaccharides consist of regularly repeating units (Óttarsdóttir 1999). The most abundant carbohydrates structured this way are starch and cellulose, made from combining the glucose monosaccharide. However, numerous other carbohydrates are produced through this process and their structure ranges from simple to complex (Bohlin & Samuelsson 2009).

1.3 Chemistry of Lichens

Lichens abound with unique primary and secondary metabolites. The majority of the secondary metabolites are considered to be produced by the mycobiont using the carbohydrates provided by the photobiont. The mycobiont uses three major pathways, the acetyl-polymalonyl pathway, the mevalonic acid pathway

and the shikimic acid pathway, to produce varied substances from the limited variation of starting materials provided by the photobiont. The secondary metabolites serve many purposes, some act as antibiotic compounds to rid the lichen of any malicious microorganisms while others even act to limit the growth of other lichen and moss species in order to provide a competitive advantage for the lichen. Some aid photosynthesis by regulating optimal conditions for the photobiont and others protect the lichen from herbivorous invertebrates by adversely affecting the invertebrates growth (Lepp 2012).

The secondary metabolites provide important ways to identify lichen species through spot tests. These tests, although often crude and poor at determining lichen species conclusively, are a good way to get a general idea of the lichen species in-field. The secondary metabolites are also often the main reason for the utilization of lichens by humans, but lichens have been used in folk medicine for a long time to treat numerous ailments. They have also been used in the production of dyes, perfumes and as acid/base indicators (Egan 2014).

The most noteworthy primary metabolites of lichens are their polysaccharides. These constitute the bulk of the mycobiont and therefore of the whole lichen. Their study has seen a rising interest in recent years as many of them exhibit potential for use in medicine. Many lichen polysaccharides have been shown to have immunostimulatory and antitumor effects (Ólafsdóttir & Ingólfsdóttir 2001, Olafsdottir, Omarsdottir, Smestad Paulsen & Wagner 2003, Omarsdottir, Peterson, Paulsen, Togola, Duus & Olafsdottir 2006, Omarsdottir, Freysdottir & Olafsdottir 2007) and since the mechanism of these effects have not yet been established in detail, their study promises to be a worthwhile one.

The polysaccharides isolated from lichens have mainly been linear or lightly substituted α- or ß-glucans as well as branched galactomannans. A few complex heteroglycans have also been isolated and thamnolan fits into this category (Ólafsdóttir & Ingólfsdóttir 2001) Much of the lichen polysaccharides are produced by the mycobiont but around 60% of the saccharides produced by the photobiont is transported to the fungal walls where it is rearranged to produce the characteristic lichen polysaccharides (Óttarsdóttir 1999). Polysaccharides produced by algae are relatively simple for the most part and the fact that the fungus can transform the photosynthesized algae saccharides into various more complex polysaccharides signifies that the polysaccharides of fungi are structurally unique.

1.4 Whiteworm lichen

The whiteworm lichen (*Thamnolia subuliformis*) derives its name from its characteristic snow white offshoots that resemble worms. It grows throughout the world but is more common in the northern hemisphere. This lichen belongs to the Icmadophilaceae family (Thompson 1984). Although this lichen has not been commonly used in modern medicine, the chemical variant *Thamnolia vermicularis* var. *vermicularis* has been used in folk medicine in the Yunnan province of China where it is ingested in the form of a tea to treat psychic

disorders, high blood pressure and inflammatory conditions of the respiratory tract (Olafsdottir et al. 2003, Omarsdottir et al. 2007).

The lichen has been found to contain the secondary metabolites baeomycesic acid which is a low molecular weight depside and can be used to distinguish *Thamnolia subuliformis* from its close relative, *Thamnolia vermicularis* which contains squamatic acid (Kranner, Beckett & Varma 2002). *Thamnolia subuliformis* also contains a branched (1→3)-(1→6) ß-glucan in a great abundance. Three complex water soluble heteroglycans Ths-4, Ths-5 and thamnolan have also been isolated from this lichen species (Olafsdottir 2003, Omarsdottir 2006). All of these have shown immunomodulating activities *in vitro* (Omarsdottir et al. 2007, Olafsdottir, Omarsdottir, Smestad Paulsen, Jurcic & Wagner 1999).

The whiteworm lichen used for this project was collected by Elín Soffía Ólafsdóttir in August 2013 on grey moss beds in Þrengsli, Iceland.

Figure 1.1 – Whiteworm lichen (Thamnolia subuliformis) (www.floraislands.is)

1.5 Thamnolan

The heteroglycan thamnolan was first described in 1999 in the journal Phytomedicine. The article was called 'Rhamnopyranosylgalactofuranan, a new immunologically active polysaccharide from *Thamnolia subuliformis*' and covered the discovery of this unique and complex polysaccharide, that consisted mostly of rhamnopyranosyl and galactofuranosyl units. It was shown to have a monosaccharide composition of gal/rha/glc/xyl/man in a ratio of

40:31:13:10:6. Its mean molecular weight was found to be 1450 kDa and its *in vitro* immunostimulating activity was demonstrated (Olafsdottir et al. 1999).

However, in a subsequent study, its structure was elucidated further and corrected using more precise equipment and methods. The heteroglycan is composed of a core of rhamnopyranosyl units with two different galactofuranosyl chains, (1→3) Gal*f* and (1→5) Gal*f*. The rhamnan-rich core has been described as being composed of 2,3)-α-Rhap(1→ ; 2)-α-Rhap(1→ ; 3)-α-Rhap(1→ and 2,4-α-Rhap(1→ residues. The galactofuranosyl chains are likely attached to the core although the branching points could not be detected in the intact polymer. Methanolysis of thamnolan has shown its improved monosaccharide composition to be gal/rha/glc/xyl/man in ratios of 49:27:12.5:8:4.5 (Ómarsdóttir, S. 2006). A glycan structure such as this had not been previously described, making thamnolan quite unique amongst other lichen heteroglycans.

Since thamnolans structure has not been fully described, it was necessary to isolate more of the polysaccharide. The purified thamnolan was then subjected to methanolysis, gas chromatography, methylation GC-MS analysis and NMR spectroscopy in order to finalize the structure determination and get a clear idea of its monosaccharide ratios and linkage types. In order to do that, partial hydrolysis is used to cleave the large polysaccharides into smaller units. Unfortunately, there was not enough time to finish these analyses and so this thesis will only contain results for yield, mean molecular weight determination and monosaccharide analysis of the polysaccharide considered to be thamnolan. That polysaccharide will hence be referred to as Fraction III-a-1.

2 Materials, equipment & procedure

2.1 Materials

Deionized water	ELGA Purelab option, Hagi
Milli-Q water	MILLIPORE Milli-Q Academic
Ethanol	Spiritus fortis, Ethanolum 96%, Gamla apótekið
Methanol	Methanol, Sigma Aldrich ≥99,9%
Acetone	Acetone, Merck ≥99,9%
Petroleum ether	Petroleum ether, Sigma Aldrich ≥99,9%
Phenol	Phenol GR for analysis, Merck
Sulfuric acid	Sulfuric acid 95-97%, Merck
Gel for size exclusion chromatography	Sephacryl™ S-400 High resolution, GE Healthcare
Gel for ion exchange chromatography	DEAE Sepharose Fast Flow®, GE Healthcare
Lichen material	*Thamnolia subuliformis*, collected in Þrengsli (N63°58,274‘ W021°27,600‘) in August 2013
Sodium chloride	NaCl pro analysis, Merck
Sodium dihydrogen phosphate	NaH_2PO_4 pro analysis, Merck
Carbon dioxide	CO_2, Ísaga ehf.

2.2 Equipment

Grinder	CLATRONIC elektrische kaffeemühle KSW3307
Lyophilizer	SCANVAC CoolSafe™
Sonicator	Cole-Parmer 8892
Ion exchange column	Pharmacia Biotech, XK26/70
Size exclusion column	PL aquagel-OH 60 10µm
	PSS Suprema Linear XL
Pump	Pharmacia Biotech Pump P-1
Membrane tubing for dialysis	Spectra/Por® Dialysis Membrane, MWCO 6-8000Da
Hot plate	DEMKO 2392
Fraction collector	Pharmacia Fine Chemicals, Fraction Collector FRAC-100
Rotavapor	Büchi Rotavapor R-3
Refrigerator	Kelvinator Scientific R-22
RI detector	Hewlett Packard HP 1047A RI Detector
Data logger	Pharmacia Biotech REC 102
Scales	METTLER PM4800 DeltaRange®
	METTLER TOLEDO NewClassic MS
Spectrophotometer	Amersham Pharmacia Biotech Ultrospec® 2100 PRO
Magnetic stirrer	Heidolph MR3002
Centrifuges	HERMLE Z320
	Hettich Zentrifugen ROTINA 35R
HPLC	HP Series 1100 IsoPump G1310A

2.3 Procedure

A scheme of the isolation procedure for Fraction III-a-1 is given in Figure 2.1

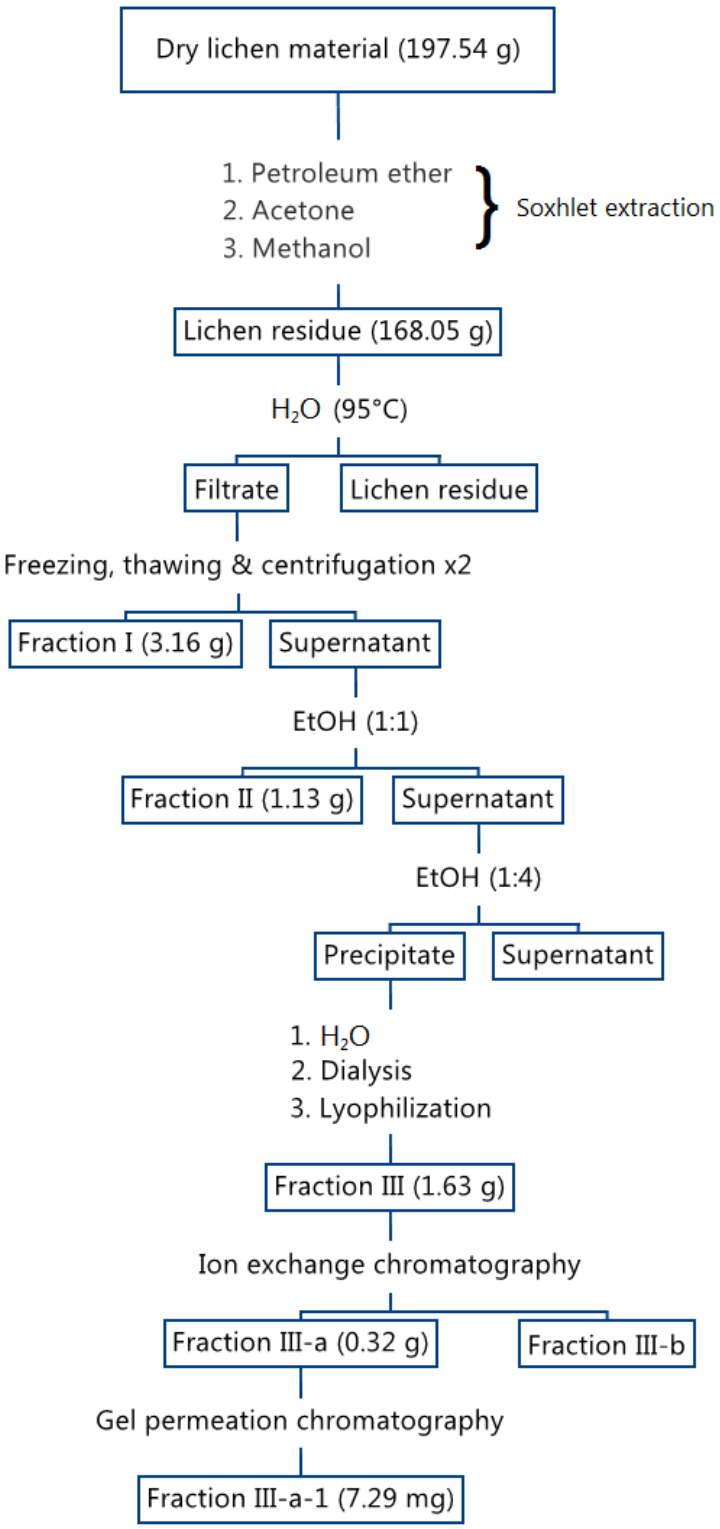

Figure 2.1 – Scheme for the isolation of Fraction III-a-1

2.3.1 Extraction of polysaccharides

The whiteworm lichen was initially extracted with three organic solvents in a soxhlet apparatus for 12 hours for each solvent. This was done in order to dispose the dried lichen material of secondary metabolites and the extracts can be used for further studies. The solvents used were petroleum ether, acetone and methanol, respectively. The large and polar polysaccharides were then extracted from the lichen material using hot, deionized water. The dried and powdered lichen material (168.05 g) was mixed with 2 l of 85 °C hot water and subsequently heated up to 95 °C. The resulting porridge was occasionally stirred with a metal spoon for 2 hours at constant temperature to avoid the mixture burning. Deionized water was regularly added to compensate for water lost by evaporation. The lichen material was filtered hot with a cloth gauze and the resulting extract was a light brown liquid with the consistency of maple syrup.

2.3.2 Isolation of Fraction I

The water extract was frozen overnight and thawed again. From this treatment there resulted precipitation that was separated from the water extract by use of a centrifuge spinning at 3000 RPM for 10 minutes. The water extract was frozen and thawed again and the resulting precipitate was treated in the same way. The precipitate was combined and then dried and ground. This precipitate was called Fraction I and had a weight of 3.16 g. It was not utilized further. The remaining water extract now had a volume of 1 l.

2.3.3 Isolation of Fraction II

The remaining water extract was treated with 96% ethanol in a ratio of 1:1 in order to precipitate Fraction II. The ethanol was added dropwise and the mixture was stirred overnight at 4 °C. The resulting precipitate was separated from the water extract by use of a centrifuge spinning at 3000 RPM for 15 minutes. The remaining water and ethanol mixture now had a volume of 2 l. The precipitate was dissolved in ca. 200 ml of deionized water and put in dialysis. The dialyzed mixture was then lyophilized and the resulting precipitate was called Fraction II. The lyophilized fraction weighed 1.3 g.

2.3.4 Isolation of Fraction III

Further precipitation from the water extract was acquired by treating with even more ethanol, now in a ratio of 1:4. In order to salvage ethanol the mixture was reduced to 1,1 l on a rotavapor. Then 4.4 l of 96% ethanol were added dropwise and the mixture stirred overnight at 4°C. The resulting precipitate was separated from the mixture by use of a centrifuge spinning at

3000 RPM for 10 minutes. The precipitate was dissolved in ca. 200 ml of deionized water and put in dialysis. The dialyzed mixture was then lyophilized and the resulting precipitate was called Fraction III. The lyophilized fraction had a weight of 1.63 g.

2.3.5 Isolation of neutral polysaccharides

Neutral polysaccharides were separated from negatively charged ones with anion exchange chromatography. The column used was a Pharmacia Biotech XK 16/70 column. The column was packed following instructions for the column using a packing adaptor overnight with degassed Milli-Q water at a flow rate of ca. 100 ml/h.

The stationary phase used was DEAE Sepharose® Fast Flow gel which is a weak anion exchanger and therefore suitable for the task at hand. The gel consists of 6% cross linked agarose, giving the ion exchangers high chemical and physical stability.

The mobile phases used were water and solutions of sodium chloride at incrementally stronger concentrations (0.25 M, 0.50 M, 0.75 M and 1.00 M NaCl). This ensured the separation of neutral and charged polysaccharides. All water used, including the water that went into making NaCl solutions, was degassed Milli-Q water. The NaCl solutions were passed through a membrane filter before being used on the column.

Samples loaded onto the column were no more than 350 mg of Fraction III, dissolved in 50 ml of degassed Milli-Q water with heating to 70 °C to increase solubility. Thus, the column was loaded and run 5 times in total. A volume of 500 ml was passed through the column for each mobile phase at a flow rate of ca. 140 ml/h and samples were collected in a fraction collector, with ca. 11 ml of sample in each test tube. The column was flushed with 1.0 M NaCl and then water in between runs to ensure that no polysaccharides remained bound to the column before starting the next run.

From every third test tube, 100 µl of sample were removed and subsequently subjected to the phenol-sulfuric acid test (DuBois, Gilles, Hamilton, Rebers & Smith 1956). Those samples were then measured on the spectrophotometer to determine which test tubes contained polysaccharides. Spectroscopic measurements from the phenol-sulfuric acid test can be seen in Appendix C. The test revealed that the polysaccharides in the sample split into two groups. Neutral polysaccharides, which were flushed out with the pure water mobile phase, and slightly charged ones which were collected with the 0.25 M NaCl mobile phase. The polysaccharides were collected and the neutral ones called Fraction III-a while the charged ones were called Fraction III-b. Fraction III-a was dialyzed and lyophilized. The lyophilized fraction had a weight of 0.32 g.

2.3.6 Isolation of Fraction III-a-1

Fraction III-a-1 was isolated from other neutral polysaccharides using preparative high pressure gel permeation chromatography. At this point there remained 207.07 mg of Fraction III-a after a long stint of unsuccessful attempts at determining the mean molecular weights of individual polysaccharides in the sample (see 4.1). A sample of 10 mg/ml was prepared with the remainder of Fraction III-a and degassed Milli-Q water, hence the sample had a volume of 20.7 ml. The sample was heated to 70 °C to facilitate solution of the polysaccharides and centrifuged to remove all insoluble particles.

The polysaccharides were manually separated using preparative gel filtration and an RI detector to recognize individual polysaccharides. The column used was a pre-packed PL Aquagel-OH 60 10 μm and the mobile phase was degassed Milli-Q water. Batches loaded onto the column had a volume of 1.0 ml with a constant flow rate of 1.5 ml/min. Each run lasted for approximately 120 minutes, with the retention time of Fraction III-a-1 typically being between 70-80 minutes. A resulting graph can be seen in Appendix B.

2.3.7 Mean molecular weight determination

Two different columns were used to determine the mean molecular weight of Fraction III-a-1. Dextran and glucose standards of known molecular weights were used. The two methods will be described separately. Since the mean molecular weight of a molecule is correlated with its retention time on a column, the mean molecular weight of Fraction III-a-1 can be determined based on its retention time on the same column used for polysaccharide standards of a known molecular weight. A linear relationship should exist between the logarithm of the standards‘ mean molecular weight and their retention time. From this there can be obtained a best fit line and its equation used to determine the mean molecular weight of a sample of unknown weight. In order to determine the mean molecular weight of Fraction III-a-1 a standard curve was drawn using dextran standards of known molecular weight. According to previous studies the mean molecular weight of Fraction III-a-1 should be around 1450 kDa (Ólafsdóttir et al. 1999) and so standards were chosen in order for it to fall within that range.

Method A

Table 2.1 – Dextran standards used for mean molecular weight determination in method A and their molecular weights

Dextran standard	MW [kDa]
T10	10
40	40
70	70
500	500
T2000	2000

0.10 g of each standard was dissolved in 10 ml of degassed Milli-Q water to obtain standard solutions in the concentration of 10 mg/ml. A 1 mg sample of purified Fraction III-a-1 was dissolved in 100 µl of degassed Milli-Q water to get a solution of the same concentration as the standards. Using a pre-packed PL Aquagel-OH 60 10 µm column, the retention time for each standard solution and the sample solution were measured using an RI-detector. Each injection had a volume of 0.1 ml and the mobile phase was degassed Milli-Q water. The pump had a flow rate of 1.0 ml/min. Graphs of each run can be seen in Appendix A.

Method B

Table 2.2 - Standards used for mean molecular weight determination in method B and their molecular weights

Standard	MW [Da]
T2000	2,000,000
T500	500,000
T70	70,000
T40	40,000
T10	10,000
Glucose	180

This method used another column and mobile phase and additively used a glucose standard. It was performed by Oddný Þóra Logadóttir and is based on the same principle as method A. The column used for this method was a pre-packed PSS Suprema Linear XL column with a separation range of 5,000-3,000,000 Da. The mobile phase used was 1.0 mM NaCl solution.

2.3.8 Methanolysis

Methanolysis was performed by Prof. Berit S. Paulsen at the University of Oslo in order to determine the monosaccharide composition of Fraction III-a-1. Trimethylsilyated (TMS) derivatives of the methyl glycosides obtained by methanolysis of 1 mg of Fraction III-a-1 with mannitol as an internal standard were subjected to gas chromatography. The method used is comparable to the one described by Reinhold (1972) and its original descirption can be found in Kale, Freysdottir, Paulsen, Friðjónsson, Hreggviðsson & Omarsdottir (2013).

After being kept at 80 °C for 20 minutes, the reagents were removed by flushing nitrogen. Then 0.1 ml pyridine and 0.1 ml acetic anhydride was added, the mixture shaken and the reagent removed by nitrogen. 0.5 M HCl in methanol was subsequently added and the mixture was kept at 65 °C for 1 hour. Following drying by nitrogen, the TMS reagent was added and the sample subjected to GC. The following column temperature program was used: Injection temperature was 140 °C and increased to 170 °C with 1 °C/min increments. The increments were then increased to 20 °C/min until the column reached its final temperature of 250 °C.

3 Results & Discussion

3.1 Yield of Isolated Fractions

3.1.1 Results

The procedure used to obtain the values in table 3.1 can be seen in sections 2.3.1-2.3.6.

Table 3.1 – Yield of fractions throughout the isolation process described in sections 2.3.1-2.3.6

	Weight [g]	Weight compared to weight of dry lichen material
Dry lichen material	197.54	100%
Lichen residue	168.05	86%
Fraction I	3.16	1.6%
Fraction II	1.13	0.57%
Fraction III	1.63	0.83%
Fraction III-a	0.32	0.16%
Fraction III-a-1	**0.00729**	**0.0037%**

3.1.2 Discussion

Thamnolan has been extracted from the whiteworm lichen a few times before (Olafsdottir et al. 1999, Ómarsdóttir, S. 2006). It is therefore apt to compare the measured yield of Fraction III-a-1 to those obtained by previous extractions of thamnolan.

Those values can be seen in table 4.1: #1 is the yield of this extraction, #2 is the yield from the article 'Rhamnopyranosylgalactofuranan, a new immunologically active polysaccharide from *Thamnolia subuliformis*' (Olafsdottir et al. 1999) and #3 is the yield from 'The lichen heteroglycan thamnolan – revised structure' (Ómarsdóttir, S 2006).

Table 3.2 – Yield of thamnolan and Fraction III-a from prior extractions compared to the obtained yields of Fraction III-a and Fraction III-a-1 where #1 is this extraction, #2 is from Olafsdottir et al. (1999) and #3 is from Ómarsdóttir, S. (2006)

	#1	**#2**	**#3**
Dry lichen material [g]	197.54	459	108
Fraction III-a [mg]	320	251	N/A
Thamnolan [mg]	N/A	102	14.1
% Yield of Fraction III-a	0.162%	0.055%	N/A
% Yield of thamnolan	N/A	0.022%	0.013%
Fraction III-a-1 [mg]	7.29		
% Yield of Fraction III-a-1	0.0037%		

Compared to the other extractions, the yield of Fraction III-a-1 from this extraction is poor, being only 0.0037% as compared to 0.022% and 0.013% from the previous extractions of thamnolan (Table 3.2). A plausible explanation of this difference in yield is the difference in ambient conditions where the collected lichens grew, such as climate, date of collection and quality of soil. The lichens used in #2 and #3 were collected in Mosfellsheiði, Úlfarsfell, Brúarskarð and Borgarfjörður while the lichen used for this extraction was collected in Þrengsli. Another reason which cannot be ignored is the authors own clumsiness, since a small amount of Fraction III-a was lost when its' solution was spilled.

Some interesting comparisons can, however, be made. The juxtaposition of #1 and #2 shows that even though Fraction III-a showed a poorer yield in #2, the yield of thamnolan was proportionally higher. The proportion of Fraction III-a-1 compared to Fraction III-a in #1 is considerably lower, indicating that the whiteworm lichen used in this extraction contained a proportionally higher amount of other neutral polysaccharides than thamnolan. These are probably Ths-4 and Ths-5 (Omarsdottir et al. 2006) although they were not subjected to further analysis.

3.2 Mean Molecular Weight Determination

3.2.1 Results

Method A

The measured retention times of the standards mentioned in 2.3.7 (Method A) were used to draw a standard curve. A best fit line was drawn with the obtained values (table 3.3) and its equation used to determine the mean molecular weight of Fraction III-a-1 (table 3.4). The standard curve and best fit line for method A can be seen in figure 3.1.

Table 3.3 – Measured retention times of dextran standards used in method A, their molecular weight and logarithm used to draw Figure 3.1

Dextran standard	RT [min]	MW [kDa]	log(MW)
T2000	108.0	2000	3.30
500	139.0	500	2.70
70	143.0	70	1.85
40	150.5	40	1.60
T10	171.5	10	1.00

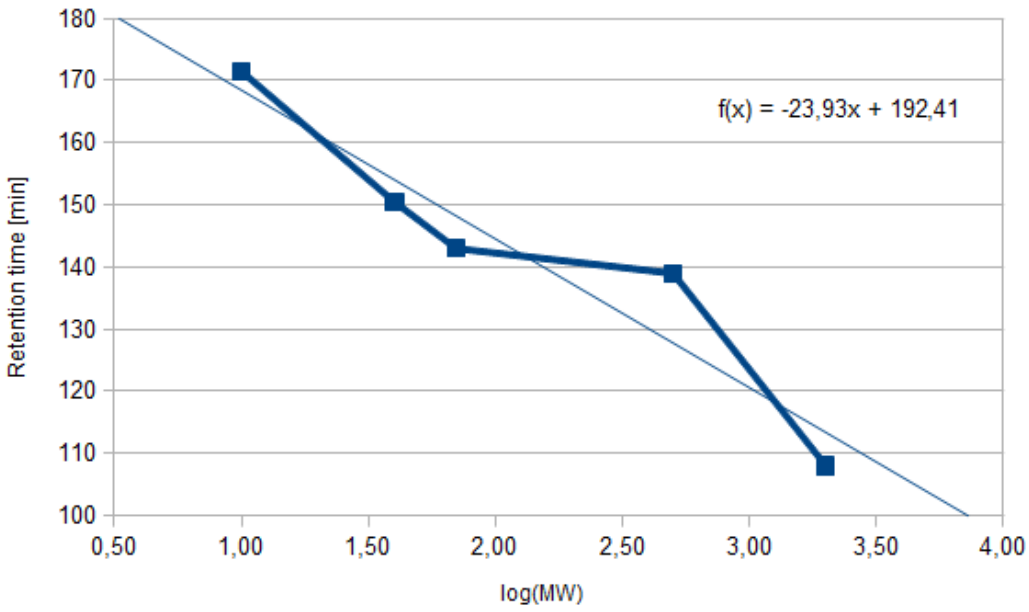

Figure 3.1 – Standard curve, best fit line and its equation drawn using the measured retention times for standards used in method A (values in table 3.3)

Using the equation for the best fit line in figure 3.1, y = -23.93x + 192.41 R = 0.90 and substituting the sample's measured retention time of 117 min for x gives the log of the mean molecular weight of Fraction III-a-1. Calculating 10 to that power gives the mean molecular weight of Fraction III-a-1, 1420 kDa. These results can be seen in table 3.4.

Table 3.4 – Retention time and mean molecular weight of Fraction III-a-1 using method A obtained using the equation for best fit line in figure 3.1

RT [min]	log(MW)	MW [kDa]
117.0	3.15	1420

Method B

The measured retention times of the standards mentioned in 2.3.7 (Method B) were used to draw a standard curve. A best fit line was drawn with the obtained values (table 3.5) and its' equation used to determine the mean molecular weight of Fraction III-a-1 (table 3.6). The standard curve and best fit line for method B can be seen in figure 3.2.

Table 3.5 - *Measured retention times of standards used in method B, their molecular weight and logarithm used to draw Figure 3.2*

Standard	RT [min]	MW [Da]	log(MW)
T2000	35	2,000,000	6.30
T500	37	500,000	5.70
T70	40	70,000	4.85
T40	41	40,000	4.60
T10	43	10,000	4.00
Glucose	50	180	2.26

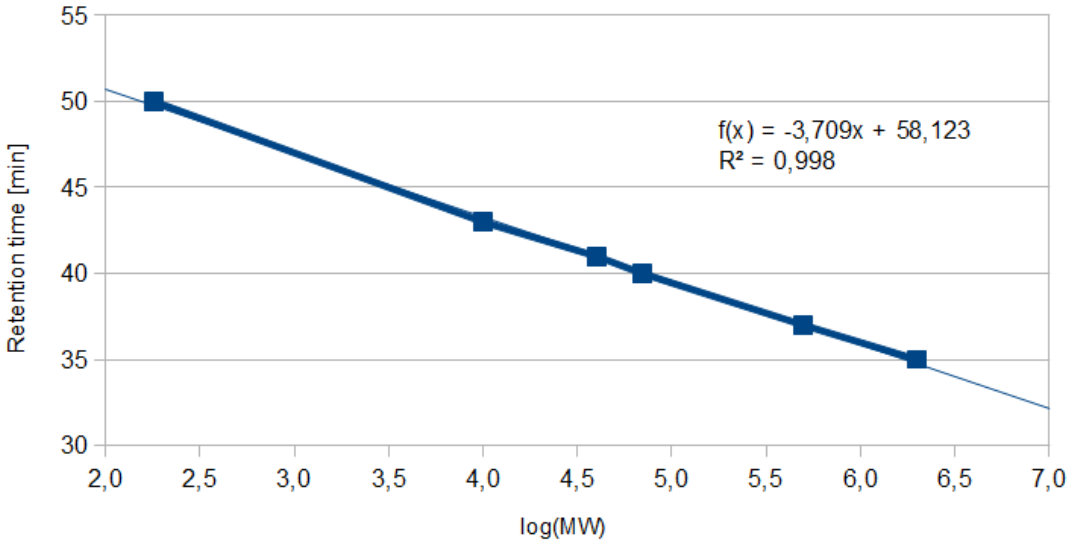

Figure 3.2 – Standard curve, best fit line and its equation drawn using the measured retention times for standards used in method B (values in table 3.5)

Using the equation for the best fit line in figure 3.2, y = -3.709 + 58.123 R = 0.998 and substituting the samples measured retention time of 35.5 min for x gives the log of the mean molecular weight of Fraction III-a-1. Calculating 10 to that power gives the mean molecular weight of Fraction III-a-1, 1260 kDa. These results can be seen in table 3.6.

Table 3.6 – Retention time and mean molecular weight of Fraction III-a-1(Method B)

RT [min]	log(MW)	MW [kDa]
35.5	6.01	1260

3.2.2 Discussion

Method A

The result of the mean molecular weight determination of Fraction III-a-1 gives the value of 1420 kDa using method A. It is consistent with the value from a previous study which estimates thamnolan to have a mean molecular weight of 1450 kDa (Olafsdottir et al. 1999).

The results should nevertheless be taken with a grain of salt. Taking the standard deviation of residual errors when compared to the fitted linear relationship for the measured standards and then applying it to the sample shows that the molecular weight should be in the range of 760-2643 kDa. Raising the measured log-value of the measured weight to the power of 10 inevitably amplifies the errors in the result greatly.

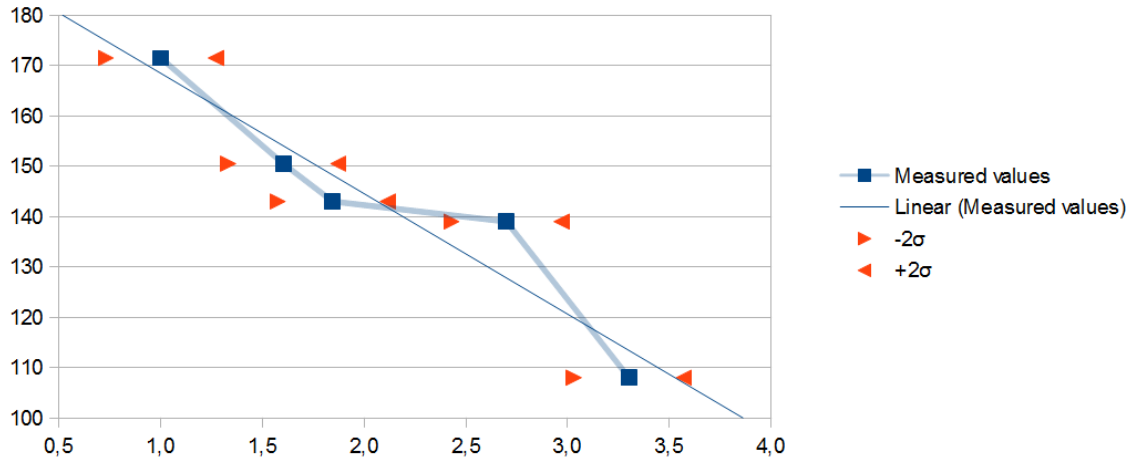

Figure 3.3 – Standard curve and its best fit line drawn using the measured retention times for standards used in method A and ±2σ residual errors, values obtained from tables 3.3 and 3.7

Table 3.7 – Values used to calculate standard deviation of mean molecular weight in method A

log(MW) of standards (xs)	log(MW) for standards from curve (xc)	abs(xs - xc)	Standard deviation
3.30	3.53	0.23	0.14
2.70	2.23	0.47	
1.85	2.06	0.21	
1.60	1.75	0.15	
1.00	0.87	0.13	

Since the method is highly sensitive to even the slightest deviations, the only reliable way of using it would be if an all but perfectly straight standard curve was obtained through measurements. This would require the standards to be of impeccable quality and as can be seen from the graphs for the standard runs (Appendix A), the peaks are broad which might indicate that they have deteriorated significantly. This is most obvious from the peak for the T2000 dextran standard, which is comparatively small and shows lingering residues.

A reason for this is likely the fact that the standards were well past their expiry date so a certain level of deterioration is to be expected. Having a few standard values above the measured log-value of the weight would have improved the accuracy of the weight determination significantly. In this case, only the least reliable standard provides a weight reference above the measured sample log-value. Another reason might be that the column used was intended for preparative work rather than analytical. The reason this column was used is that another column more fit for the mean molecular weight determination was suspected to be contaminated and therefore not

used. That column was, however, later used when all suspicion of contamination had been eradicated and is the one used in method B. More sophisticated machines should also be used for a reliable determination, for example a viscosity meter, but due to a string of malfunctions these machines were not used.

To further demonstrate the amplification of the errors of the method used, one can give the measured sample value hypothetical confidence limits and view them in relation to the measured standard deviation. A visual demonstration of ±5% hypothetical confidence limits can be seen in figure 3.4.

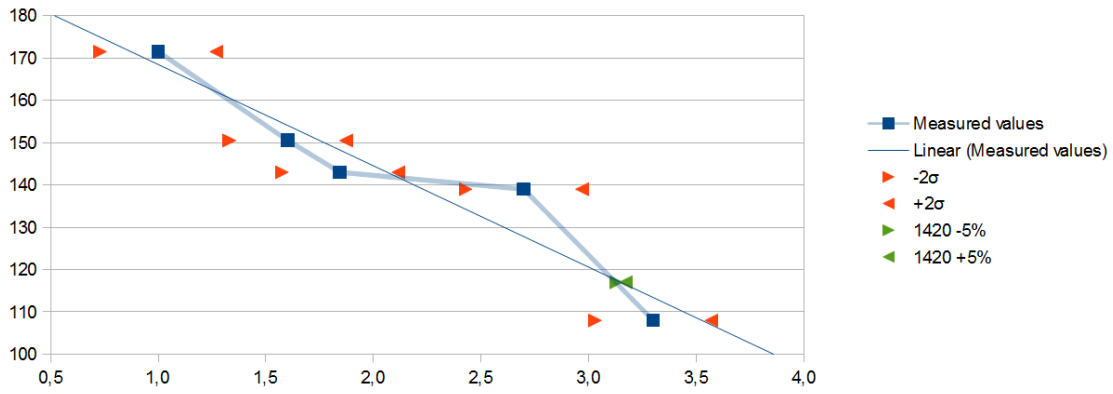

Figure 3.4 – Standard curve and its best fit line drawn using the measured retention times for standards used in method A, ±2σ residual errors and ±5% hypothetical confidence limits, values obtained from tables 3.3 and 3.7(hypothetical confidence limits drawn from table 3.8)

The hypothetical ±5% confidence limits show how any reasonable confidence limit would be hard to achieve and would require precise equipment and execution.

Table 3.8 – Measured sample value in method A with hypothetical ±5% confidence limits, used in figure 3.4

	Lower limit (95%)	Sample value	Upper limit (95%)
log(MW)	3.13	3.15	3.17
MW [kDa]	1345	1420	1487

In conclusion, the measured value for the sample in method A is a consistent one, albeit with an inconsistent deviation.

Method B

Luckily, proper equipment was available to perform a reasonable mean molecular weight determination in method B. As seen in figure 3.2, the retention times and the logarithm of the standards' mean molecular weight show a much better linear relationship in this method and might even fall within the aforementioned ±5% hypothetical confidence limits. Although the result for method B is not as consistent with the previous study (Olafsdottir et al. 1999), this method should be considered the more precise one. This mean molecular weight determination was done on a pre-packed PSS Suprema Linear XL column which is much better suited for analytical work than the one used in method A. This method also showed a much better peak for the T2000 standard. The reason for that might be the fact that the T2000 standard solution was put in a sonicator in method A which might have broken down linkages in the large dextran molecules.

A mean molecular weight of 1260 kDa can still be considered fairly consistent with the previous study. This lighter result might for instance indicate that side chains in Fraction III-a-1 are shorter than in the previous extraction although the heteroglycans core structure probably remains the same. Graphs for runs in method B can be seen in Appendix A.

3.3 Methanolysis

3.3.1 Results and discussion

Methanolysis revealed the monosaccharide composition of Fraction III-a-1. It can be seen in table 3.9 along with comparisons to other extractions of thamnolan for comparison. Similarly to 3.1.2, #1 is the monosaccharide composition from this extraction and #2 and #3 are from Olafsdottir et al. (1999) and Ómarsdóttir, S. (2006) respectively.

Table 3.9 – Monosaccharide ratios of Fraction III-a-1 (#1) and thamnolan from Olafsdottir et al. (1999) (#2) and Ómarsdóttir, S. (2006) (#3)

	Ratios		
Monosaccharide	**#1**	**#2**	**#3**
Galactose	50	40	49
Rhamnose	25.3	31	27
Glucose	11	13	12.5
Xylose	7	10	8
Mannose	3.5	6	4.5
Arabinose	3.2		

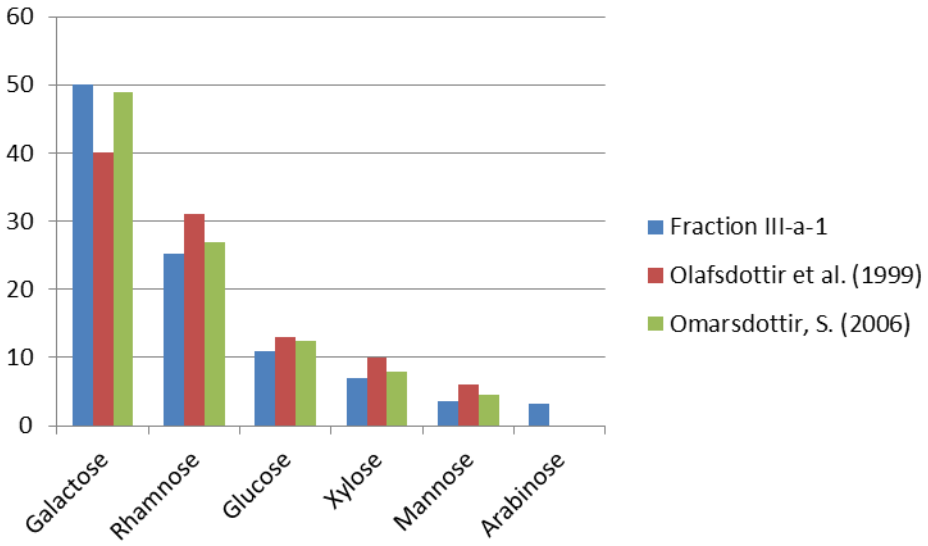

Figure 3.5 – Visual representation of monosaccharide ratios for Fraction III-a-1 and thamnolan from previous studies, values obtained from table 3.9

The monosaccharide composition of Fraction III-a-1 was consistent with previous studies of thamnolan as can be seen from table 3.9 and figure 3.4. The most important comparison is to Ómarsdóttir, S. (2006) as it was an improvement on the one from Olafsdottir et al. (1999) and clear consistency can be seen between the monosaccharide composition of Fraction III-a-1 and thamnolan from Ómarsdóttir, S. (2006). Of these monosaccharides, the high quantity of galactose and rhamnose is important, to support the idea that the heteroglycan is composed of a rhamnopyranosyl core with galactofuranose sidechains. Interestingly, Fraction III-a-1 contained a marginal amount of arabinose which was not detected in the two previous studies. Whether this might be because of more a precise method being used this time or the fact that the whiteworm lichen used for this extraction might contain polysaccharides of slightly different composition is unclear and trivial for the end result.

3.4 Further analyses

A 2 mg sample of Fraction III-a-1 was sent to Prof. Berit S. Paulsen at the University of Oslo for methanolysis and gas chromatography and methylation on GC-MS to discern its monosaccharide ratios and linkage types. In order to confirm its detailed structure, partial hydrolysis and 1D and 2D NMR analysis will be performed if the prior analyses are successful. Results from these analyses, aside from the methanolysis, will not be published in this thesis since they are still being performed as of the writing of this text. This is unfortunate but unavoidable due to time constraints.

4 Conclusion

Based on the fact that the results of all performed analyses on Fraction III-a-1 have been consistent with prior extractions and studies of thamnolan, the author feels secure in concluding that the heteroglycan Fraction III-a-1, is in fact pure thamnolan. The results of all further analyses, mentioned in 3.4, will hopefully be sufficient to finally elucidate the heteroglycan‘s structure conclusively. Ideally they will show beyond any reasonable doubt where the galactofuranose sidechains connect to the rhamnopyranosyl core.

The fact that the two different methods used for the mean molecular weight determination showed similar values gives the result considerable reliability. Method B should be considered more reliable since the equipment used for that measurement was better suited for analytical work and although that result showed a slight deviation from previously obtained values, it can be explained by the fact that whiteworm lichen collected in different locations, season and climate might have slightly differing polysaccharide compositions.

The difference in thamnolan‘s yield between this extraction and previous studies is significant but the fact that this extraction had a higher yield of neutral polysaccharides in total might also point to whiteworm lichen possibly producing polysaccharides in varying ratios based on environmental conditions.

Finally, the fact that the thamnolan from this extraction is slightly lighter than the one previously studied but has the same overall monosaccharide ratios might suggest that the basic composition is the same but with a smaller core and/or shorter sidechains. This should confirm the fact that *Thamnolia subuliformis* produces this complex heteroglycan as it has now been found in dry lichen material of the lichen species in two different locations.

All in all this extraction should be considered a success and a legitimate, albeit minute, addition to humanities unending quest of exploring and understanding their environment.

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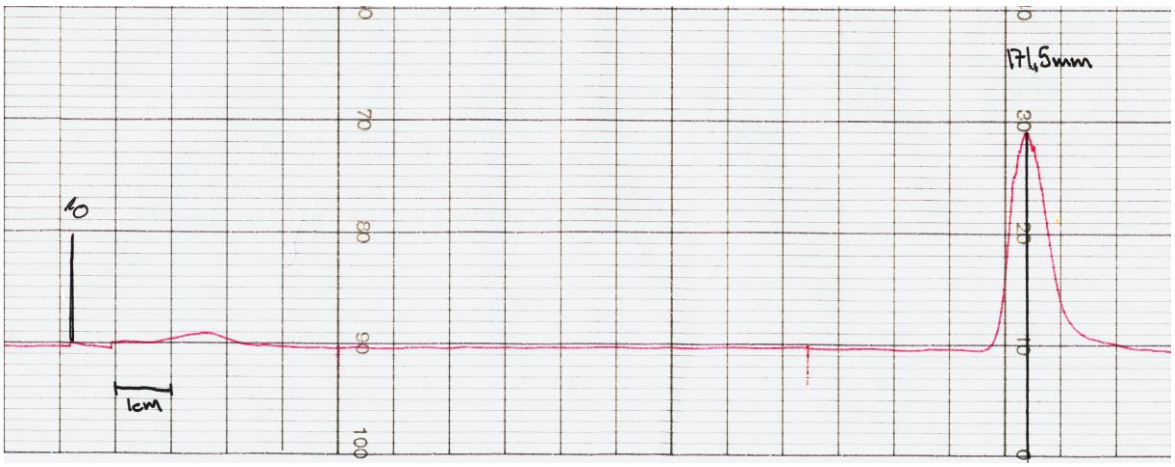
Appendices

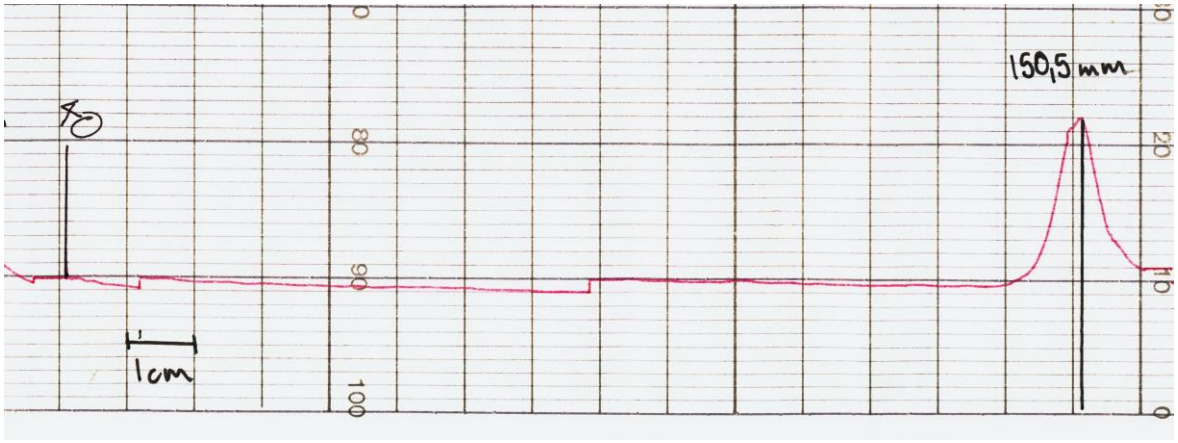
Appendix A

Graphs of runs for mean molecular weight determination in methods A & B. The width of a column in each of the graphs is 1 cm. The data collector operated at 1 mm/min and therefore one column represents 10 minutes of runtime.

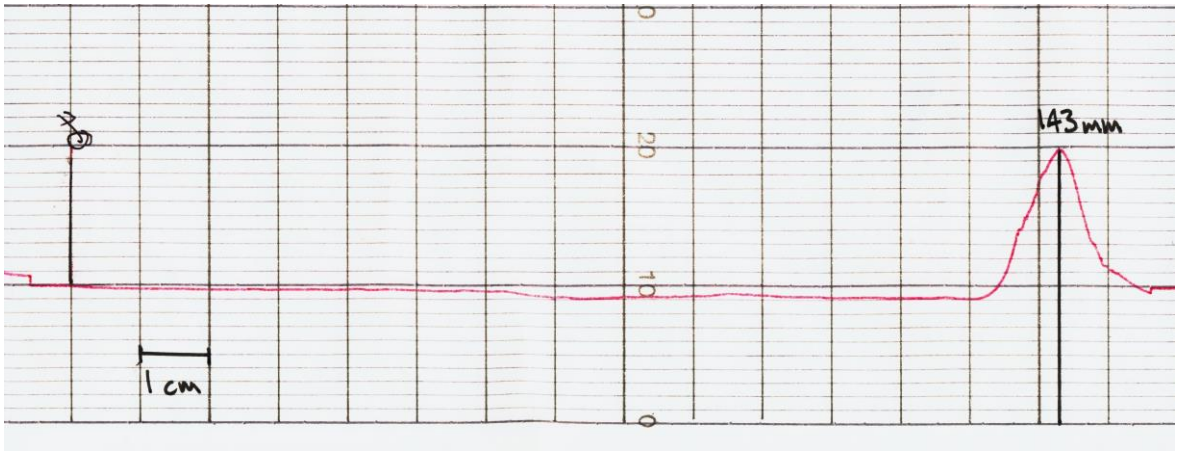
Method A



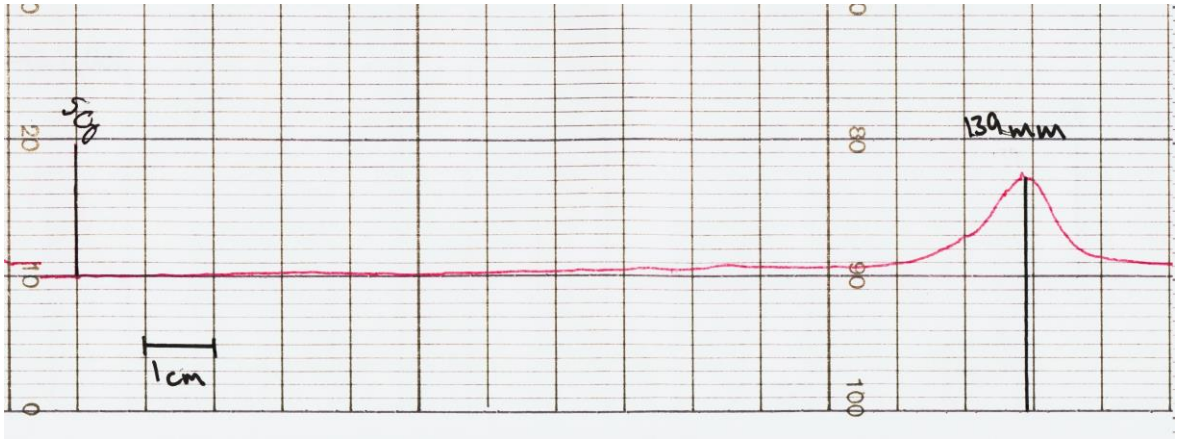
Graph 6.1 – Run of dextran T10 standard



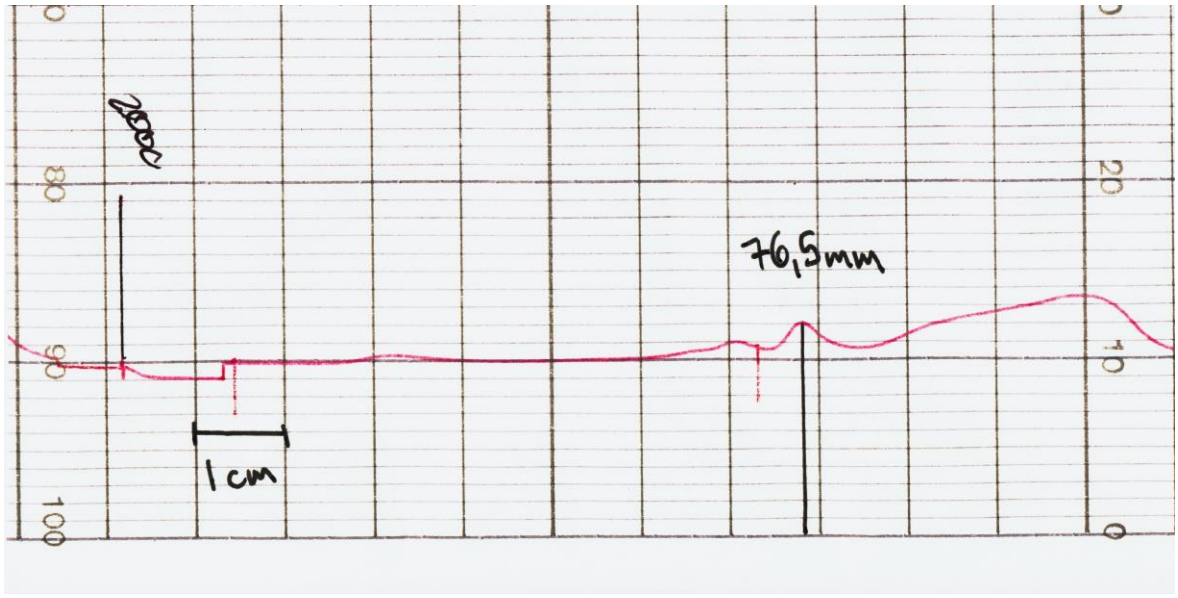
Graph 6.2 – Run of dextran 40 standard



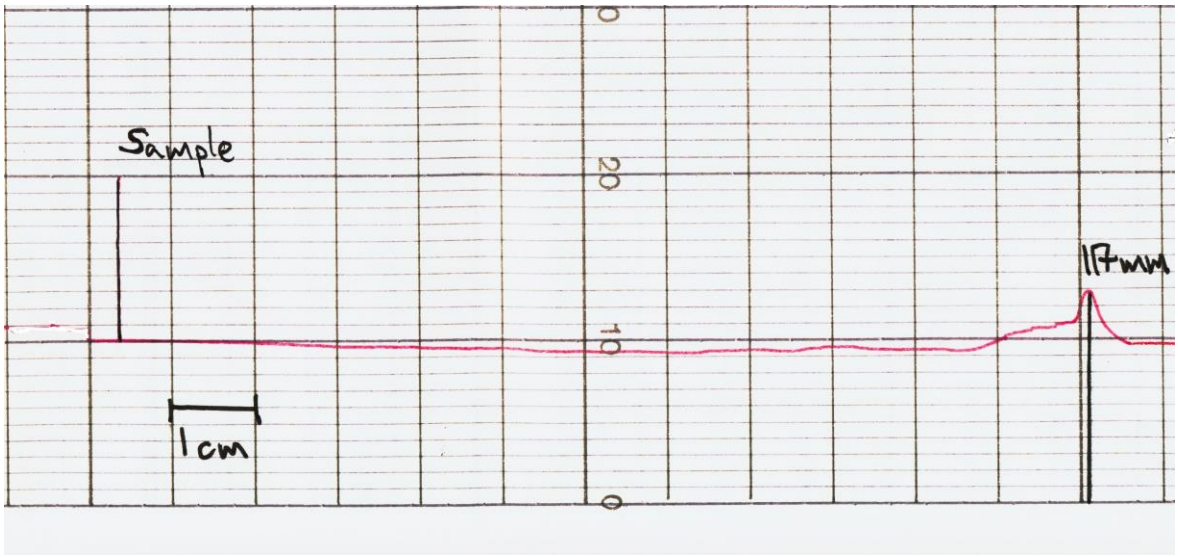
Graph 6.3 – Run of dextran 70 standard



Graph 6.4 – Run of dextran 500 standard

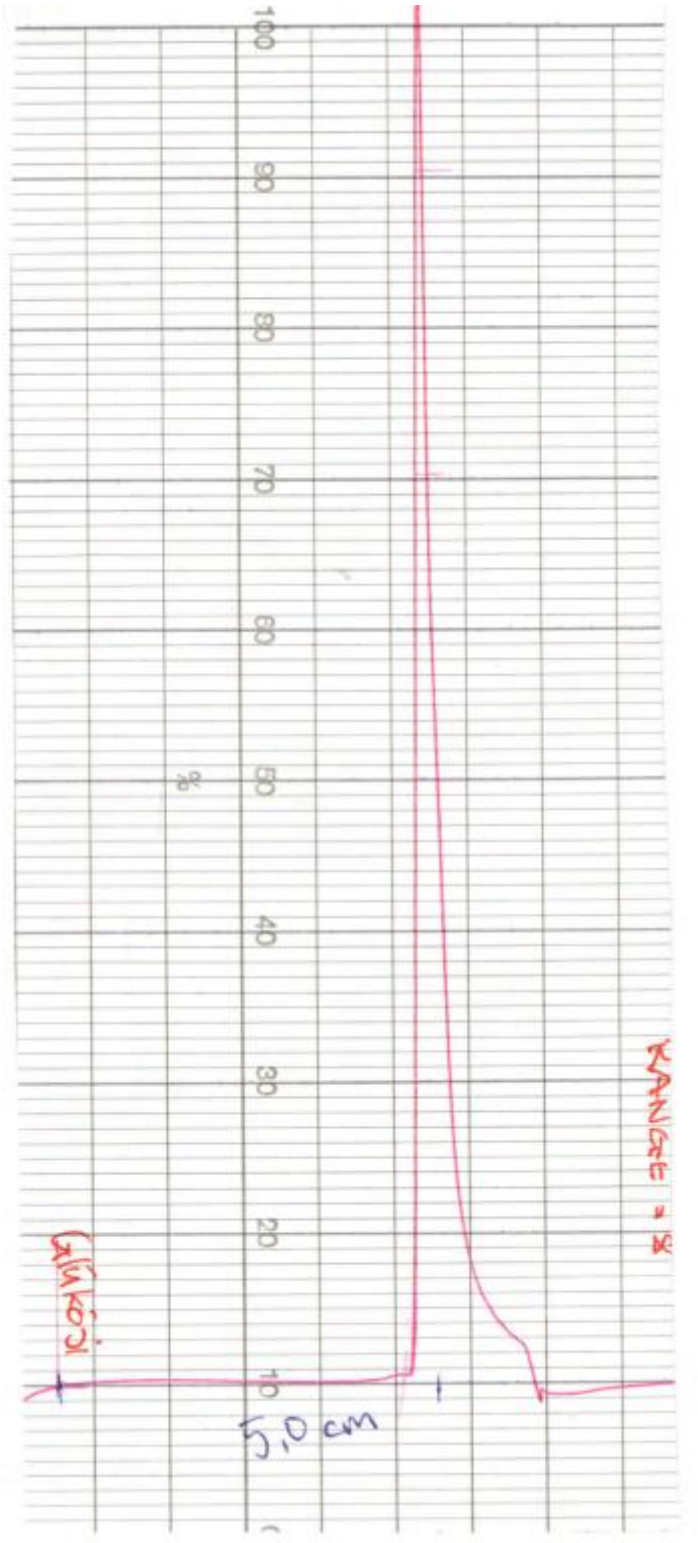


Graph 6.5 – Run of dextran T2000 standard

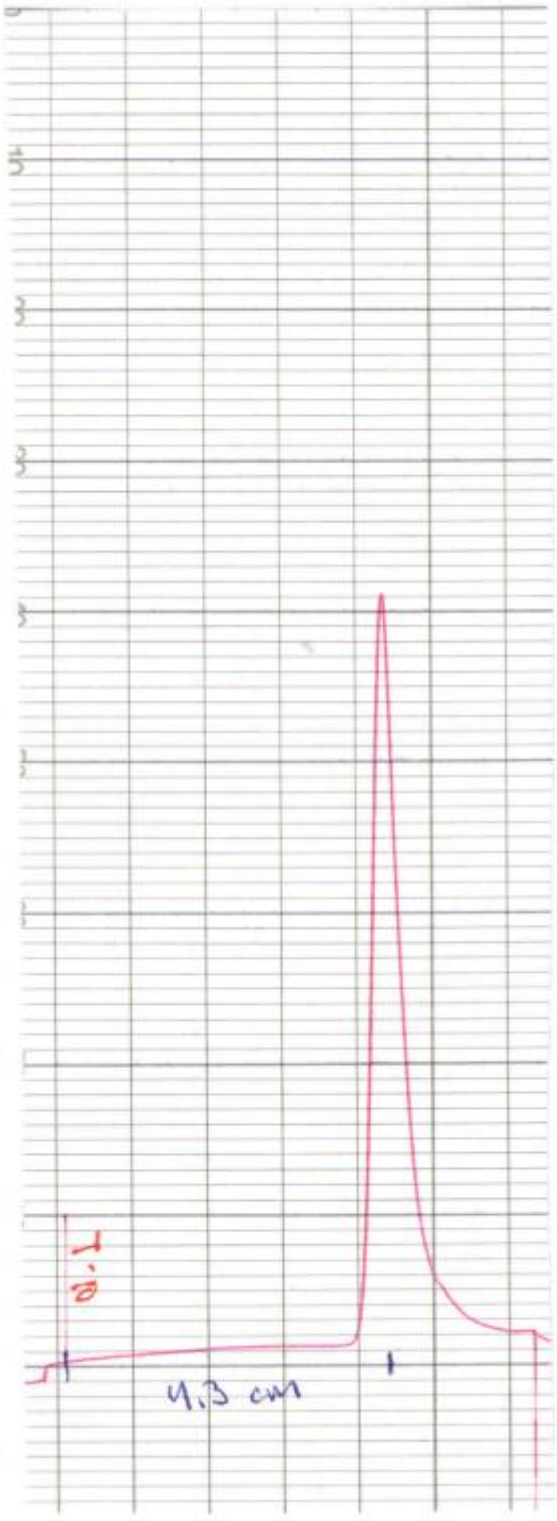


Graph 6.6 – Run of Fraction III-a-1

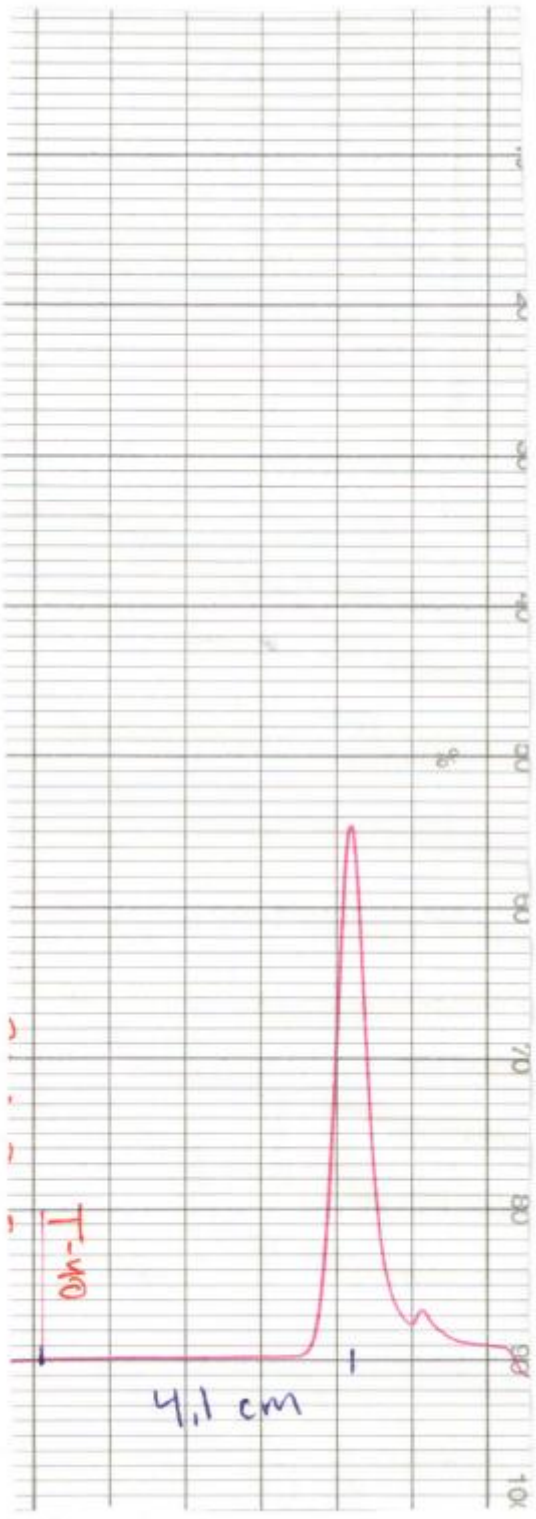
Method B



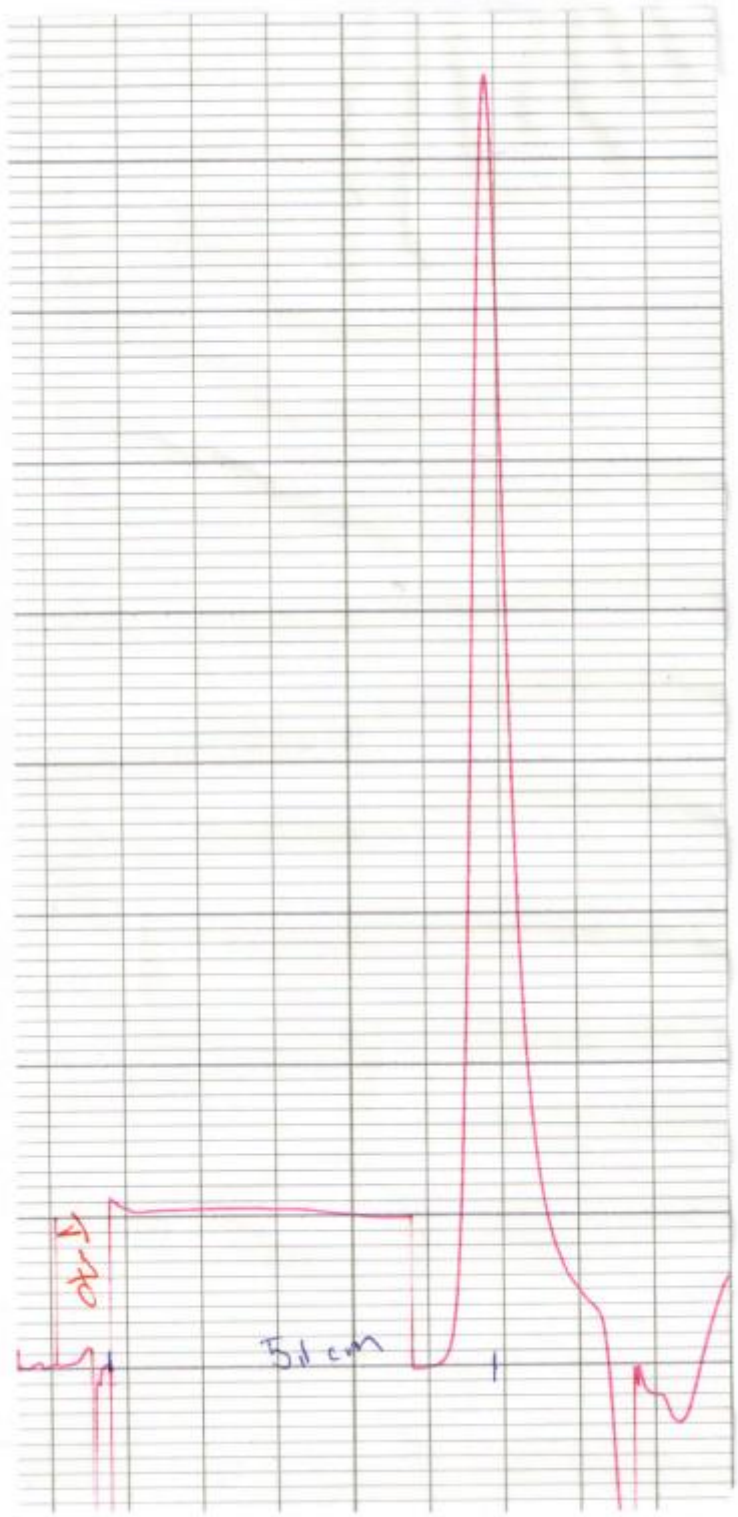
Graph 6.7 – Run of glucose standard



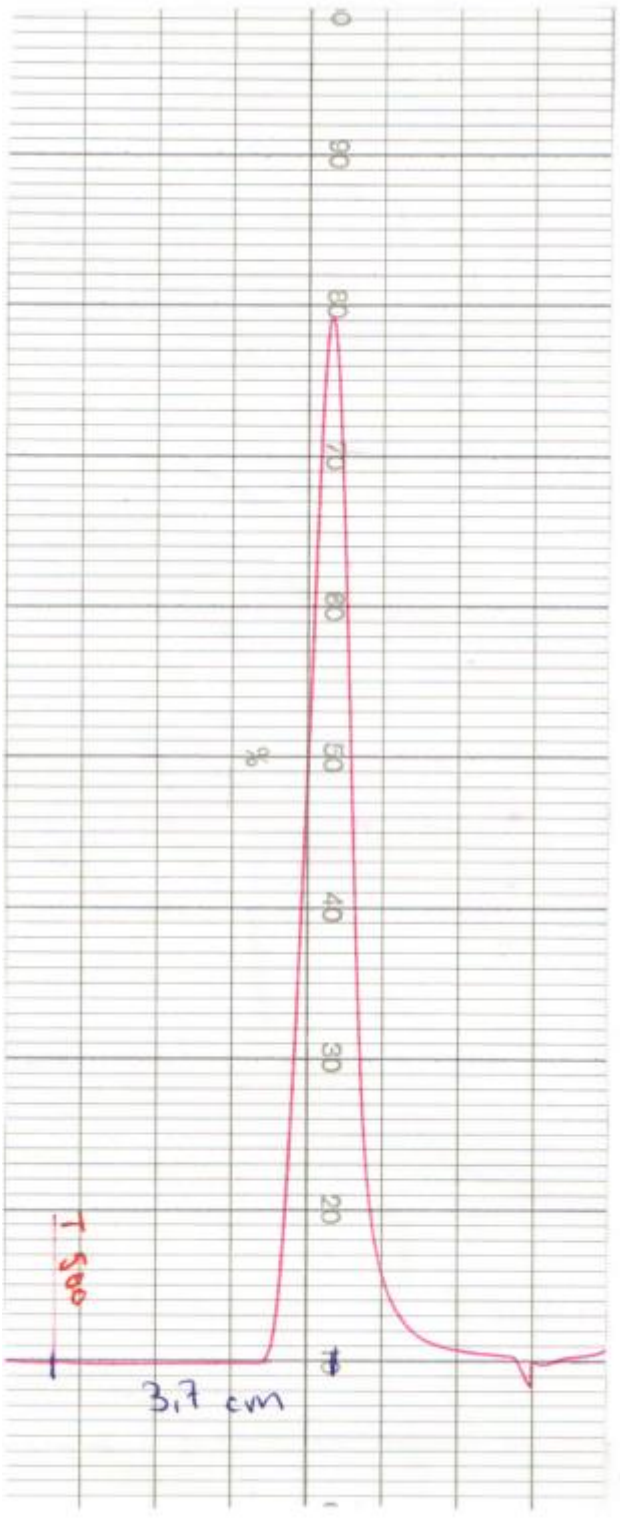
Graph 6.8 – Run of dextran T10 standard



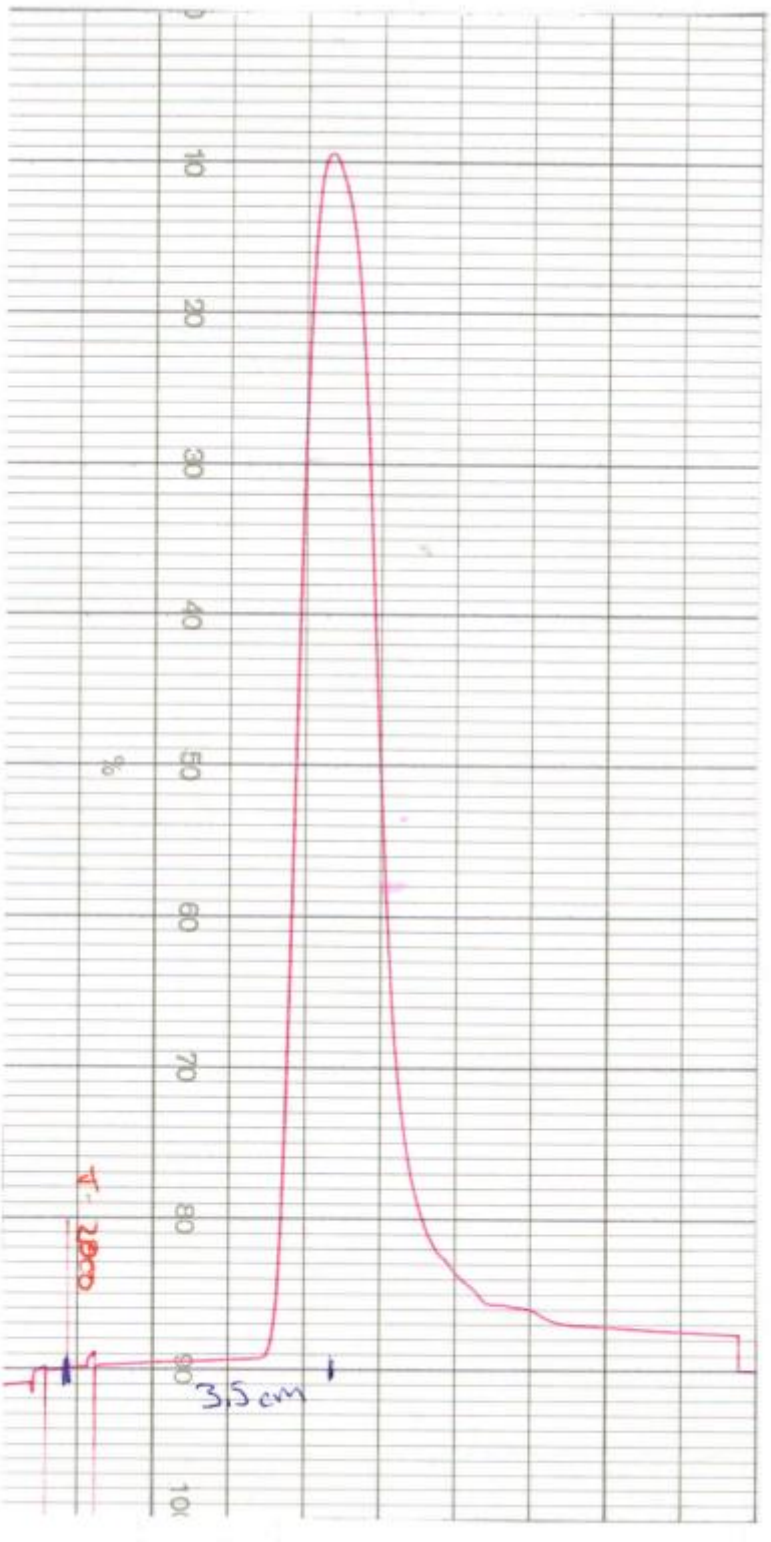
Graph 6.9 – Run of dextran T40 standard



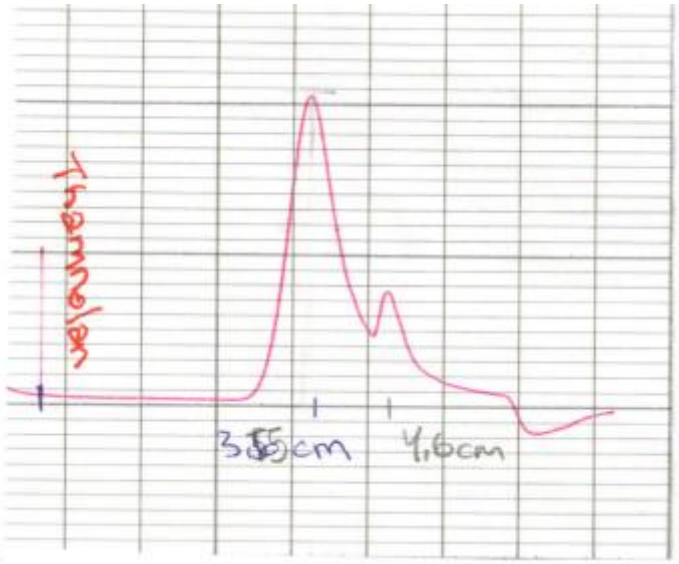
Graph 6.10 – Run of dextran T70 standard



Graph 6.11 – Run of dextran T500 standard

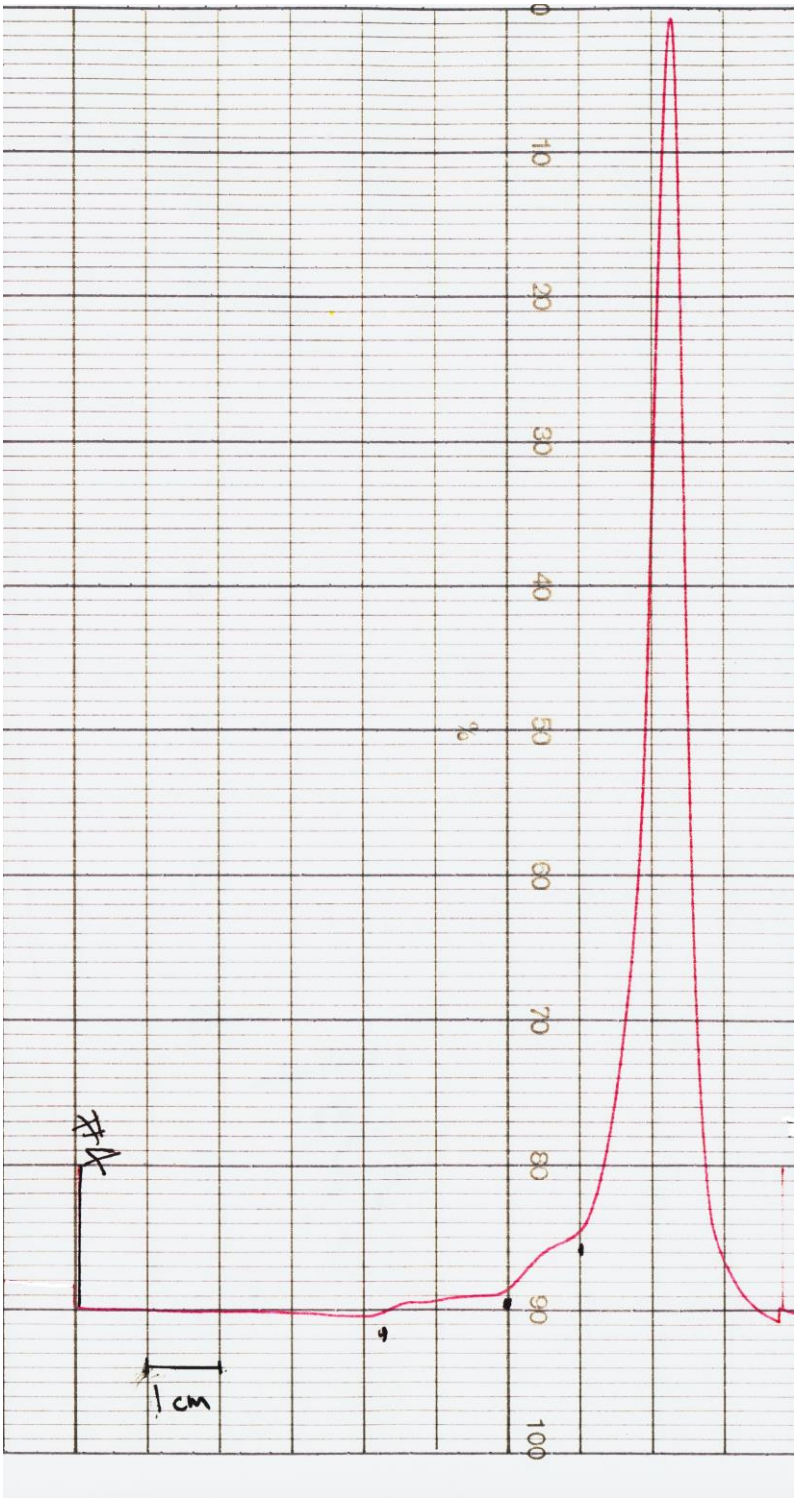


Graph 6.12 – Run of dextran T2000 standard



Graph 6.13 – Run of Fraction III-a-1

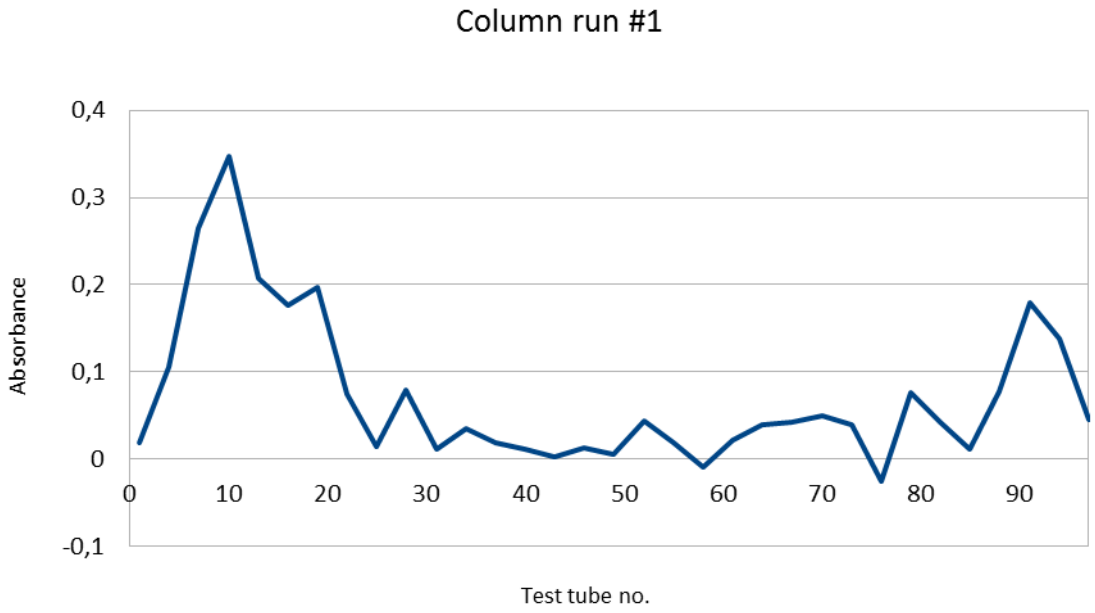
Appendix B



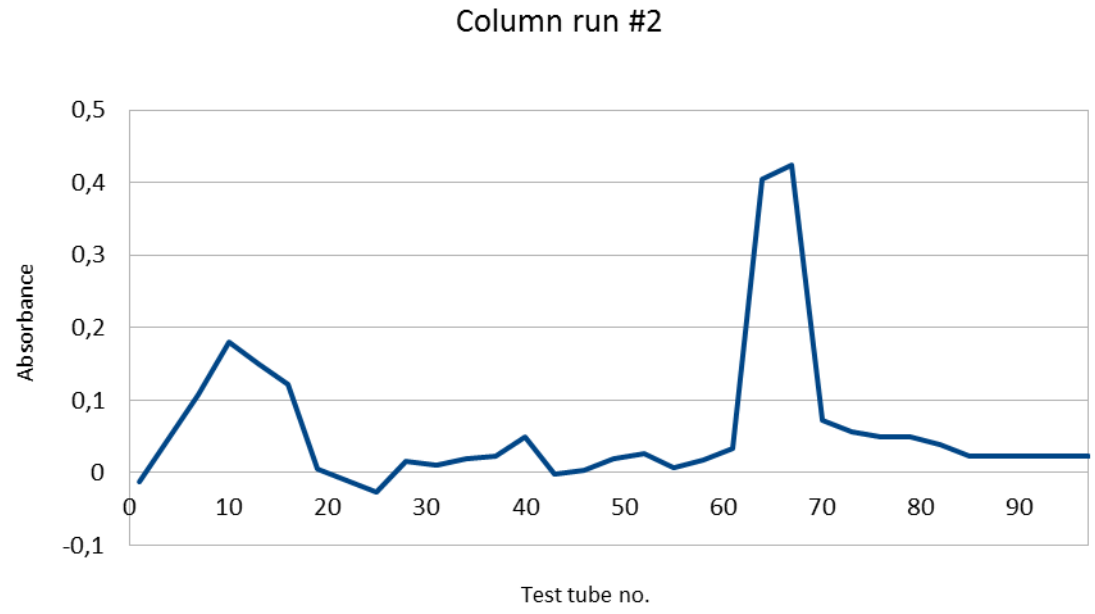
Graph 6.14 – Graph obtained from preparative gel filtration of Fraction III-a, Fraction III-a-1 was collected between minutes 60-70 and lighter polysaccharides follow. The data collector operated at 1 mm/min and therefore 1 column represents 10 minutes of runtime.

Appendix C

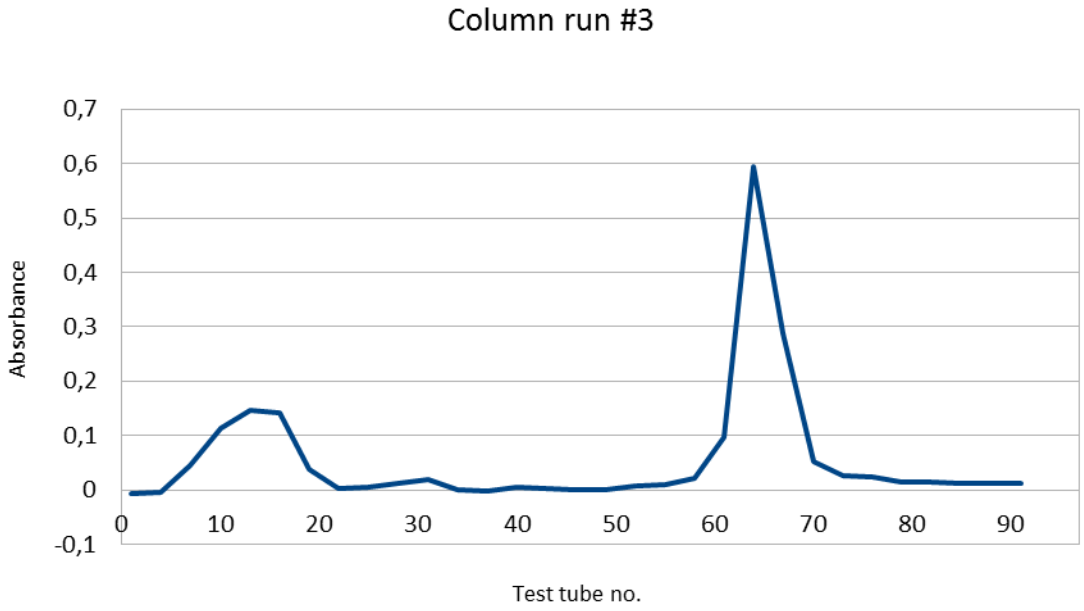
Graphs obtained by spectroscopic measurements after each ion exchange column run and a phenol-sulfuric acid test. They show absorbance measured at 360 nm.



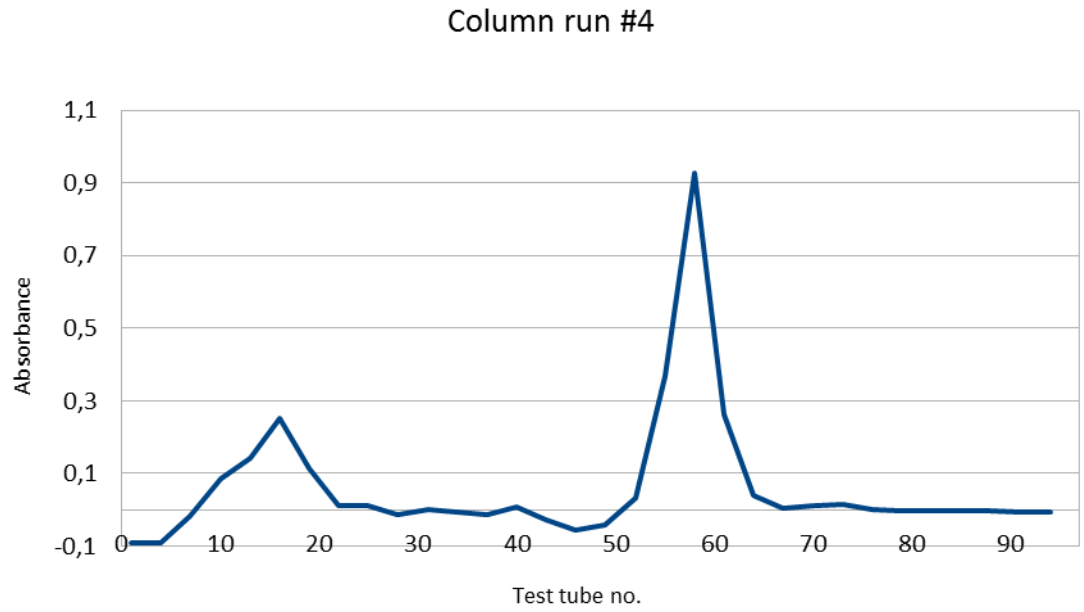
Graph 6.15 – Ion exchange column run #1. Fraction III-a was collected from test tubes #1-31 and Fraction III-b was collected from test tubes #85-97.



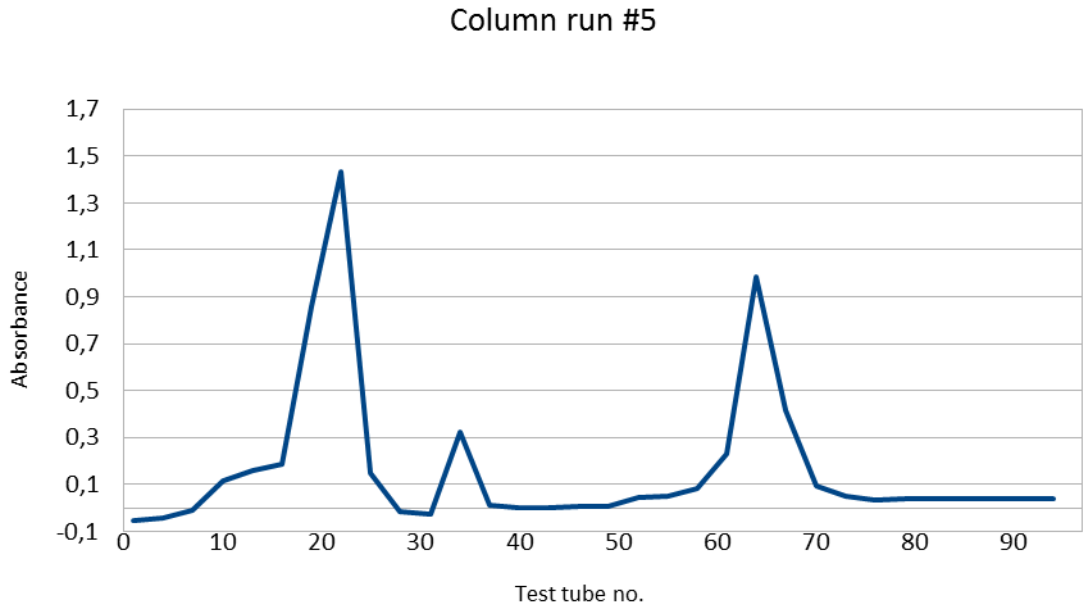
Graph 6.16 – Ion exchange column run #2. Fraction III-a was collected from test tubes #1-19 and Fraction III-b was collected from test tubes #61-73



Graph 6.17 – Ion exchange column run #3. Fraction III-a was collected from test tubes #7-19 and Fraction III-b was collected from test tubes #58-70



Graph 6.18 – Ion exchange column run #4. Fraction III-a was collected from test tubes #7-22 and Fraction III-b was collected from test tubes #52-64



Graph 6.19 – Ion exchange column run #5. Fraction III-a was collected from test tubes #10-22 and Fraction III-b was collected from test tubes #61-70