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Master thesis

Isolation, Purification and Quantification of Sedoheptulose from *Sedum maximum*

Submitted by

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Abstract

Very recently, a human enzyme that converts sedoheptulose into sedoheptulose-7-phosphate has been detected which thus indicates the bioavailability of sedoheptulose for carbohydrate metabolism. Since then there has been a high interest to perform *in vitro* and *in vivo* investigations in order to understand the role of sedoheptulose on human carbohydrate metabolism. Due to a lack of commercially available sedoheptulose in gram-scale, the aim of this thesis was to explore approaches to isolate and purify the sugar from a plant known for its high sedoheptulose content, the *Sedum* species. This resulted in a highly diversified topic: Different analytical methods were used to determine the sugar content and purity of various samples, a purification procedure in lab scale was established and a pre-test concerning the development of a process for the extraction in large scale was performed.

For the identification of unknown compounds and comparison of different samples concerning their purity, nuclear magnetic resonance spectroscopy was used. The first screening of sedoheptulose concentrations and the determination of the extraction efficiency of lyophilized leaves of *Sedum maximum* were performed *via* liquid chromatography coupled to mass spectrometry, whereas for accurate quantification of sedoheptulose anion exchange chromatography coupled to pulsed amperometric detection was used.

Quantitative analysis of sedoheptulose in the leaves of *Sedum maximum* revealed that there was a significant change in sedoheptulose concentrations over the growing season reaching its peak in the mid of June. However, the content during a day did not vary substantially. Moreover, it was proven that the composition of the extract concerning the amount of organic acids and other contaminants in comparison to sedoheptulose was influenced by growing conditions. The measurement of an extract of the stems revealed slightly lower sedoheptulose concentrations but an impressive purity.

The preparative purification of the extract was performed by removal of organic acids with an anion exchange resin followed by protein precipitation with ethanol. Per-O-acetylation allowed purification *via* column chromatography on silica gel. After deprotection, the sedoheptulose was lyophilized. Furthermore, several other attempts for final purification were tested. The purification resulted in several grams of rather pure sedoheptulose that enabled important cell culture-based investigations at the Medical University of Vienna.

Kurzfassung

Kürzlich wurde beim Menschen ein Enzym beschrieben, das Sedoheptulose in Sedoheptulose-7-phosphat umwandelt, und dadurch die Verfügbarkeit von Sedoheptulose für den Kohlenhydrat-Stoffwechsel nachgewiesen. Daraus resultiert nunmehr ein erhebliches biomedizinisches Interesse an *in vitro* und *in vivo* Untersuchungen über die Rolle der Sedoheptulose im humanen Kohlenhydratstoffwechsel. Sedoheptulose ist jedoch kommerziell in größeren Mengen nicht erhältlich. Ziel dieser Arbeit war daher Wege zur Isolierung und Aufreinigung aus einer Pflanze, die für ihren hohen Sedoheptulose-Gehalt bekannt ist – der Fetthenne – zu etablieren. Dies ergab ein sehr breit gefächertes Thema: Verschiedene analytische Methoden wurden für die Bestimmung des Zuckergehalts und der Reinheit verschiedener Proben eingesetzt, eine Methode zur Aufreinigung im Labormaßstab wurde etabliert und ein Vorversuch für die Isolierung im Großmaßstab wurde durchgeführt.

Zur Identifizierung unbekannter Verbindungen und zum Vergleich verschiedener Proben bezüglich ihrer Reinheit, kam die Kernspinresonanz-Spektroskopie zum Einsatz. Für ein erstes Screening von Sedoheptulose-Konzentrationen und die Bestimmung der Extraktionseffizienz von lyophilisierten Blättern von *Sedum maximum* wurde Flüssigchromatographie gekoppelt mit Massenspektrometrie eingesetzt, während für die exakte Quantifizierung von Sedoheptulose Anionenaustausch-Chromatographie mit gepulster amperometrischer Detektion verwendet wurde.

Die quantitativen Bestimmungen von Sedoheptulose in den Blättern von *Sedum maximum* zeigten einen signifikanten Unterschied der Sedoheptulose-Konzentration über die Wachstumsperiode, wobei das Maximum Mitte Juni erreicht wurde. Innerhalb eines Tages waren die Schwankungen jedoch gering. Außerdem wurde gezeigt, dass die Wachstumsbedingungen einen großen Einfluss auf die Zusammensetzung des Extraktes in Bezug auf die enthaltene Menge an organischen Säuren und anderen Verunreinigungen hatten. Die Messung eines Extraktes aus den Stämmen dieser Pflanze ergab zwar eine etwas niedrigere Konzentration an Sedoheptulose, dafür aber mit einer eindrucksvollen Reinheit.

Für die präparative Reinigung des Extrakts wurde nach Abtrennung der organischen Säuren mit einem Anionenaustauscherharz eine Proteinfällung mit Ethanol durchgeführt. Per-O-Acetylierung ermöglichte eine Aufreinigung über Säulenchromatographie mit Kieselgel. Die nach dem Entschützen erhaltene Sedoheptulose wurde abschließend lyophilisiert. Außerdem wurden verschiedene weitere Versuche zur Feinreinigung getestet. Die präparative Reinigung ergab einige Gramm fast reiner Sedoheptulose, wodurch wichtige Zellkulturversuche an der Medizinischen Universität Wien ermöglicht wurden.

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Abbreviations

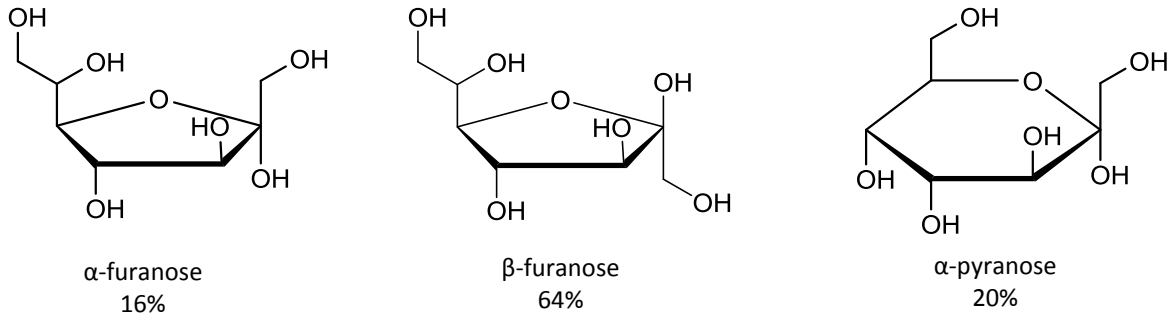
Ac_2O	Acetic anhydride
ACN	Acetonitrile
ATP	Adenosine triphosphate
CARKL	Carbohydrate kinase-like
$CDCl_3$	Deuterated chloroform
CTNS	Nephropathic cystinosis
D_2O	Deuterium oxide
DMAP	4-*N*,*N*-dimethylaminopyridine
EtOH	Ethanol
GC	Gas chromatography
H_2O	Water
HCl	Hydrochloric acid
HCOOH	Formic acid
HILIC	Hydrophilic interaction chromatography
HPAEC	High performance anion exchange chromatography
IC	Ion chromatography
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharide
MeOH	Methanol
MPLC	Medium pressure liquid chromatography
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
NaOMe	Sodium methoxide
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detection
PPP	Pentose phosphate pathway
RSD	Relative standard deviation
TLC	Thin layer chromatography
TOF	Time-of-flight

1 Introduction

1.1 Sedoheptulose and its phosphates

Almost a century ago, considerable amounts of a free non-fermentative reducing sugar were discovered in an aqueous extract of *Sedum spectabile*. Chemical analysis revealed that it was a new heptose and it was thus named sedoheptose (La Forge and Hudson, 1917). Today, this monosaccharide with seven carbon atoms is called sedoheptulose as referring to its keto-structure with a structural formula of $C_7H_{14}O_7$ and the molecular weight of 210.18 g·mol^{-1}.

According to Ceusters et al., sedoheptulose is present in three different tautomeric forms in deuterium oxide (D_2O) (Ceusters et al., 2013) as shown in Scheme 1.



Scheme 1: Tautomeric forms of sedoheptulose present in D_2O

1.1.1 Occurrence of sedoheptulose in food

Sedoheptulose (D-*altro*-heptulose) belongs to the group of naturally occurring higher-carbon sugars and has thus been part of our nutrition. Already in 1972, sedoheptulose was identified in several tropical fruits such as mango and papaya (Ogata et al., 1972). In 2009, when studying minor carbohydrates in carrots, Soria et al. discovered the presence of sedoheptulose in carrots at concentrations between 1.4-24.6 mg·g^{-1} dry weight (Soria et al., 2009) with a water content of about 90% of fresh carrots (Souci et al., 2008). In the same year, several other fruits and vegetables were tested for their sedoheptulose concentration and about 1 µmol·g^{-1} was found in apples, apricots and tomatoes and about 7 µmol·g^{-1} in carrots (Kardon et al., 2008), corresponding to 0.2 mg·g^{-1} and 1.5 mg·g^{-1}, respectively.

Another naturally occuring heptose is the C4 epimer of sedoheptulose, namely mannoheptulose that is present in avocados (La Forge, 1917). Mannoheptulose is stimulating gluconeogenesis in the liver and thus causes hyperglycemia (Paulsen, 1968) whereas no diabetogenic effect of sedoheptulose has been found (Simon et al., 1961).

1.1.2 Relation of sedoheptulose to sedoheptulose-7-phosphate and sedoheptulose-1,7-bisphosphate

The role of the mono- and bisphosphate esters of sedoheptulose has been documented (Benson et al., 1952; Calvin, 1962) and is shown in Figure 1, whereas the occurrence and function of the free monosaccharide have hardly been elucidated but are suspected to serve for carbon storage and cryo-protection in plants (Häfliger et al., 1999; Kull, 1965). It seems that sedoheptulose is produced by a phosphatase from sedoheptulose-7-phosphate (Ceusters et al., 2013). In 1951 it was suggested that "sedoheptulose may be liberated enzymatically from its phosphates during the killing of the plant" (Benson et al., 1951).

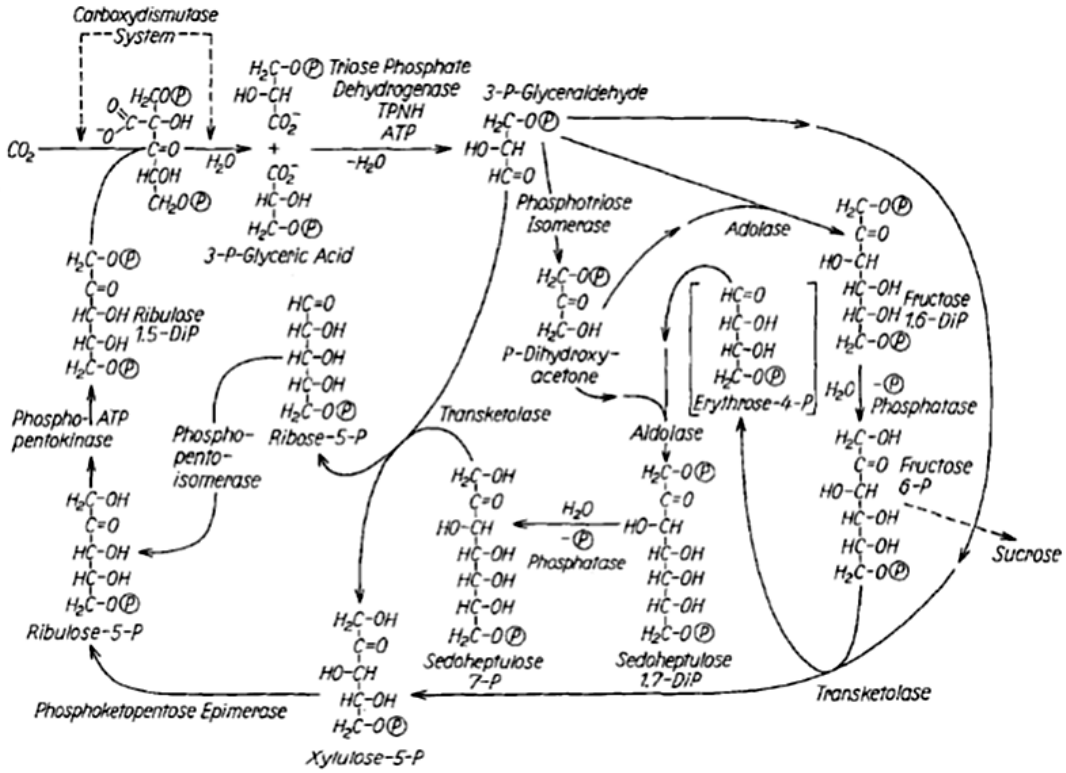


Figure 1: The photosynthetic Carbon Cycle for CO_2 fixation (Calvin, 1962)

Sedoheptulose phosphates occur as intermediates during photosynthesis. Sedoheptulose-1,7-bisphosphate is formed from erythrose-4-phosphate and dihydroxyacetone-phosphate and is further transformed into sedoheptulose-7-phosphate. The latter is then converted with glyceraldehyde-3-phosphate into ribose-5-P and xylulose-5-P which are finally yielding ribulose-5-P that serves as carbon dioxide acceptor (Calvin, 1962; Webber, 1963).

1.1.3 Chemical reactions of sedoheptulose

When sedoheptulose is heated under mild acid conditions, a non-reducing anhydro derivative (sedosan = sedoheptulosan = 2,7-anhydro-β-D-*altro*-heptulopyranose) is formed (La Forge and Hudson, 1917). When the anhydro-sedoheptulose is heated with dilute acid, about 20%

sedoheptulose are present in equilibrium (La Forge and Hudson, 1917; La Forge, 1920). A more exact study was performed in 1956, where it was shown that the anhydride content varies between 91% at 20 °C and 84.5% at 80 °C. Furthermore, another anhydride namely 2,7-anhydro-β-D-*altro*-heptulofuranose was identified at about 2% in the equilibrium (Richtmyer and Pratt, 1956).

In 2008 it was shown that heating of a 200 mM solution of commercially available sedoheptulosan in the presence of 0.05% perchloric acid for 2 h at 100 °C resulted in the formation of about 25 mM sedoheptulose (Kardon et al., 2008). Sedoheptulose anhydride monohydrate is commercially available at reasonable prices.

The reduction of sedoheptulose forms two heptitols named α-sedoheptitol and β-sedoheptitol with α-sedoheptitol being identical to volemite/volemitol (Forge, 1920; La Forge and Hudson, 1917).

Volemitol was found in concentrations up to 25% of the dry weight in leaves of the genus *Primula* and seems there to be important for physiological functions since a NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) dependent ketose reductase (called sedoheptulose reductase) that reduces sedoheptulose to volemitol was identified in these plants. Furthermore, sedoheptulose was present up to 18% of the dry weight and therefore the consistent co-occurrence of sedoheptulose and volemitol in *Primula* was described (Häfliger et al., 1999).

1.2 The history of CARKL and its physiological relevance

Carbohydrate kinases are enzymes that phosphorylate carbohydrates after entering the cells in order to inhibit any return through the cell membrane and to dispose them for further metabolism (Worley et al., 1995). In 2000, Touchman et al. discovered a new gene similar to the genes encoding the carbohydrate kinases already known. Since similarity was rather weak and the substrate still unknown, it was named CARKL (carbohydrate kinase-like) and it was predicted to be encoding a carbohydrate kinase. The discovery of CARKL occurred during studies of cystinosis (Touchman et al., 2000), an inborn error of the transport of cystine out of the cell leading to an accumulation of cystine in all organs and tissues (Nesterova and Gahl, 2013). Cystinosis is caused by a mutation of the nephropathic cystinosis (CTNS) gene and approximately half of all patients are suffering from a 57-kb deletion that also affects the adjacent CARKL gene (Touchman et al., 2000; Wamelink et al., 2008). Elevated levels of sedoheptulose as well as erythritol in the urine of these patients have been observed, thus suggesting CARKL to be a sedoheptulose kinase (Kardon et al., 2008; Wamelink et al., 2008).

In 2012, a collaborative effort of the University of Natural Resources and Life Sciences, Vienna and the Medical University of Vienna definitely identified CARKL as a sedoheptulose kinase converting sedoheptulose into sedoheptulose-7-phosphate and thus providing a link between glycolysis and the

pentose phosphate pathway (Haschemi et al., 2012). Therefore, it was shown for the first time that free sedoheptulose provides an accessible carbon source for carbohydrate metabolism (Nagy and Haschemi, 2013).

Figure 2 shows the primary carbohydrate metabolism in humans and the position where sedoheptulose can be introduced.

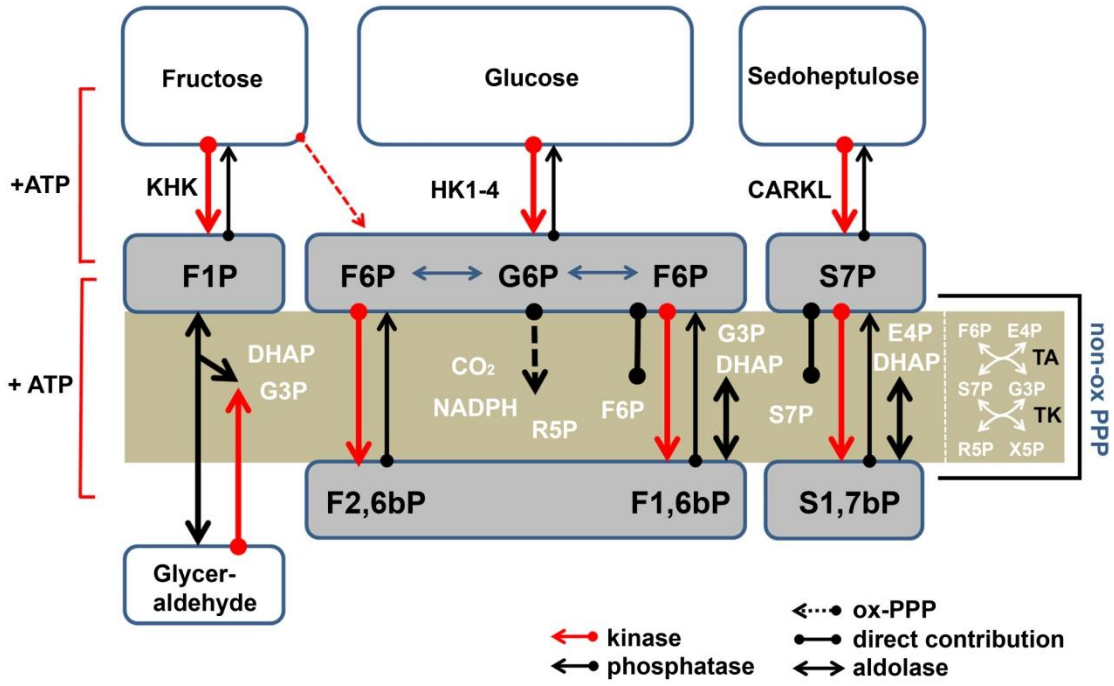


Figure 2: Regulation of primary carbohydrate metabolism by phosphorylation and non-linear carbohydrate phosphate interconversions (Nagy and Haschemi, 2013)

In order to comply with the energetic demand of the cells, anabolic and catabolic metabolism have to be balanced by an interplay between carbohydrate, fat and amino acid metabolism and oxygen consumption. By sharing several intermediates, the two major pathways of primary carbohydrate metabolism – glycolysis and the pentose phosphate pathway (PPP) – are strongly linked to each other. Glycolysis converts glucose into pyruvate yielding ATP (adenosine triphosphate) and NADH (reduced form of nicotinamide adenine dinucleotide). The PPP can be divided into two branches: The oxidative branch generates NADPH and ribulose-5-phosphate (Wood, 1985), two important precursors for anabolic pathways, whereas the non-oxidative branch (also regenerative branch) interconverts the sugar-phosphates by its two main enzymes transaldolase and transketolase (Koolman and Röhm, 2005) providing a substrate bridge to glycolysis. Within this non-oxidative branch of the PPP, sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate are generated from ribose-5-phosphate and xylulose-5-phosphate by a transketolase activity and from fructose-6-phosphate and erythrose-4-phosphate by a transaldolase (Kruger and von Schaewen, 2003). The existence of sedoheptulose-7-phosphate and its role as intermediate in the PPP have already been

known for a long time, but now with the discovery of the sedoheptulose kinase a new possibility of sedoheptulose-7-phosphate formation was introduced. Compared to glycolysis, the regulation of the PPP is not well known yet. Nevertheless, CARKL could be one of the enzymes regulating the various reversible reactions of the non-oxidative branch of the PPP by sedoheptulose-7-phosphate supply and hence could be rate-limiting for primary carbon metabolism (Nagy and Haschemi, 2013).

Since the specific modulation of glycolytic energy flux was found to be crucial for macrophage activation and polarization, CARKL seems thus to influence immune cell development. CARKL mRNA (messenger ribonucleic acid) is repressed upon LPS (lipopolysaccharide) stimulation *in vitro* and *in vivo* in mice and humans and this downregulation seems to be necessary for proper M1 polarization (Haschemi et al., 2012). Therefore, high CARKL expression appears to repress the "pro-inflammatory'' macrophage phenotype.

As observed after activation by LPS, an increase in extracellular acidification rates and a reduction of oxygen consumption rates (as signals for macrophage activation) were also observed upon knockdown of the CARKL gene. After macrophage activation, the expression of CARKL was found to be repressed in macrophages and monocytes in both mice and humans *in vivo* and *in vitro* (Haschemi et al., 2012).

Furthermore, CARKL was found to be responsible for the repression of the LPS induced tumor necrosis factor α secretion and catalyzes a reaction in the PPP that refocuses the cellular metabolism to a high redox state upon physiological or artificial downregulation (Haschemi et al., 2012).

All these findings may provide new possibilities for medical treatment of chronic diseases.

1.3 *Sedum maximum* - "Große Fetthenne"

Sedum maximum (also *Hylotelephium telephium* ssp. *maximum* or *Sedum telephium* ssp. *maximum*) and also the former mentioned *Sedum spectabile* belong to the family of Crassulaceae with *Sedum* representing the largest genus with more than 400 varieties (Eggli, 2003; Hanelt and Institute of Plant Genetics and Crop Plant Research, 2001).

This family of leaf-succulents is spread nearly all over the world but is predominantly found in the northern hemisphere. The term succulence is derived from the Latin word *succus* meaning juice and describes the water reservoir in the plants that is responsible for the independency from water supply during dry periods (Eggli, 2008). In Austria, the vernacular name of *Sedum maximum* is "Große Fetthenne" referring to the thick leaves originating from this leaf-succulence.

The name Crassulaceae is derived from the most important physiological adaptation in succulent plant metabolism, the Crassulaceaen Acid Metabolism (CAM) (Hart and Eggli, 1995). This metabolic pathway enables the plant to keep their stomas closed during the day in order to reduce evaporation

of water. However, opened stomas are necessary for carbon dioxide uptake and hence for growth. CAM offers a solution to that challenge: During the night, carbon dioxide is absorbed and accumulated as malic acid. In the morning, the stomas are closed and carbon dioxide is separated from the malic acid by light energy (Eggli, 2008).

In 1933, the concentrations of malic acid and sedoheptulose were investigated in *Sedum praealtum* (see Figure 3). It was discovered that the concentrations of this two substances were changing indirect proportionally and that the sedoheptulose content in detached leaves showed a several-fold change during a day with the highest level at 6 p.m. whereas the aldose content remained almost constant (Bennet-Clark, 1933).

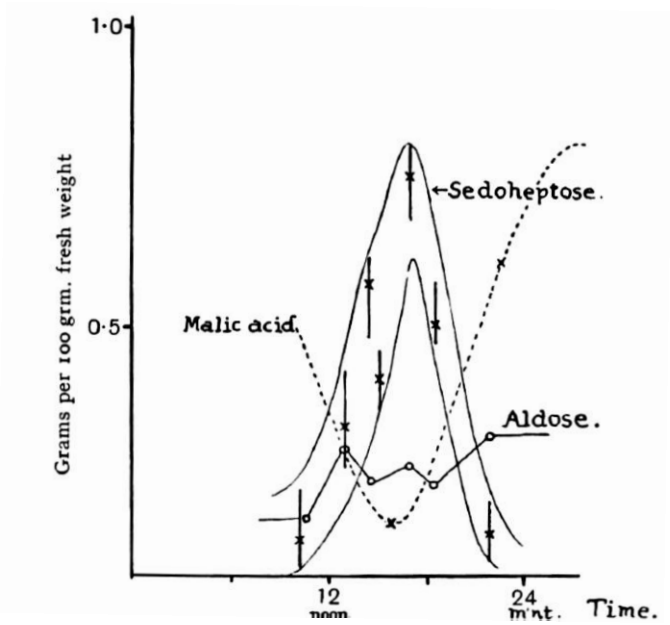


Figure 3: Diurnal fluctuations of malic acid and sedoheptulose in detached leaves of *Sedum praealtum* (Bennet-Clark, 1933)

Concerning the sedoheptulose content in the plants, different information is available. However, no data specifically for *Sedum maximum* has been found. The malate concentration is reported to be dependent on water supply in *Sedum telephium* (Smirnoff, 1996). Therefore, watering could also influence the sedoheptulose content.

As mentioned before, sedoheptulose was first discovered in *Sedum spectabile* (La Forge and Hudson, 1917) and due to its high concentration in this plant, *Sedum spectabile* was used as source of sedoheptulose in several previous publications (Ceusters et al., 2013; Haschemi et al., 2012; La Forge and Hudson, 1917; La Forge, 1920; Wamelink et al., 2008).

1.4 Isolation and purification of sedoheptulose from leaves of *Sedum sp*.

For many studies performed with sedoheptulose, sedoheptulose was purified from the leaves from *Sedum spectabile* based on a protocol from 1917 (La Forge and Hudson, 1917). The purification procedures included aqueous extractions from the leaves followed by ion exchange resins (Ceusters et al., 2013). Glucose was partly removed enzymatically using glucose oxidase followed by an anion exchange resin for the removal of the resulting glucuronic acid (Ceusters et al., 2013). Furthermore, activated charcoal and protein precipitation with ethanol were used (Haschemi et al., 2012; Schmidt et al., 1998).

The method used in the framework of this thesis to purify a larger amount of sedoheptulose from an aqueous extract was derived from previously used protocols (Haschemi et al., 2012; Schmidt et al., 1998) using an anion exchange resin and protein removal with ethanol. However, usage of activated charcoal was omitted. Instead, the purification was performed by using routine methods of organic chemistry such as per-O-acetylation, preparative column chromatography with silica gel and deacetylation in the end. Several other procedures for further purification were tested and implemented.

Alternatives to the isolation of sedoheptulose from plants, have been provided by chemical synthesis from D-allose (Hricovíniová-Bíliková and Petruš, 1999) and by a biotechnological approach performed in *Bacillus subtilis* mutants deficient in transketolase, which are able to produce sedoheptulose from glucose (Yokota, 1993). Furthermore, sedoheptulose-7-phosphate has already been prepared in multi-gram scale by enzymatic synthesis from β-hydroxypyruvate and D-ribose-5-phosphate (Charmantray et al., 2009).

1.5 Analysis of sedoheptulose

For the measurement of polar compounds such as sugars, different analytical methods are available. In previous studies, sedoheptulose has mostly been quantified *via* gas chromatography coupled to mass spectrometry (GC-MS) (Ogata et al., 1972; Soria et al., 2009). Additionally, paper chromatography has been used for the detection of several keto-sugars (Ogata et al., 1972). These two methods have in common that prior to analysis derivatization is necessary since sugars are neither volatile nor UV-active. Already in 1953, the colorimetric determination of heptoses has been established (Dische, 1953). For GC-MS measurements, the derivatization is mostly performed *via* trimethylsilylation (Ogata et al., 1972; Soria et al., 2009). Due to its high separating capacity and sensitivity, GC-MS can be used for isomer separation and for low analyte concentrations. Furthermore, enzymatic assays with sedoheptulose kinase have been used for the quantification of sedoheptulose (Kardon et al., 2008).

Pulsed amperometric detection (PAD) following ion chromatography (IC) has been established as a powerful technique for the direct detection of carbohydrates (Zook and William, 1995) and has been successfully applied for the separation and quantification of sedoheptulose (Ceusters et al., 2013). Due to their weak acid character, carbohydrates can be ionized at pH values greater than 12 and are thus separable *via* high performance anion exchange chromatography (HPAEC).

The possibilities for detection of carbohydrates after anion exchange chromatography are limited since carbohydrates are, as mentioned before, not UV active and refractive index detection is not suitable for gradient systems. Pulsed amperometric detection enables direct quantification of carbohydrates without any prior derivatization and is not hampered by IC gradients. The analytes are oxidized on a gold electrode resulting in a current that is monitored. Different potentials are applied for oxidation, reduction and reoxidation of the electrode. For different analytes different potentials are necessary thus HPAEC-PAD is quite selective and sensitive for carbohydrates when using the appropriate detection voltage.

Although no application especially for sedoheptulose has been published until now, liquid chromatography coupled to mass spectrometry (LC-MS) *via* electrospray ionization (ESI) has become the most widespread application in metabolomics (Gika et al., 2014) and is also suitable for sedoheptulose analysis.

Due to non-volatile mobile phase components, ion chromatography cannot be directly coupled to ESI. However, it is possible to remove e.g. sodium hydroxide with an ion suppressor between LC and MS. In practice, reversed-phase (RP) chromatography and hydrophilic interaction chromatography (HILIC) are used for separation. The latter one was applied in this work.

Last but not least, nuclear magnetic resonance (NMR) spectroscopy has many applications in metabolomics (Kim et al., 2010; Verpoorte et al., 2007) and emerged to be the most frequently used analytical technique for the observation of the purification since sample preparation is quite simple. Furthermore, NMR offers an overview of the impurities proportional to the substance of interest (in this case sedoheptulose). Therefore, the achievement of a purification step could be immediately monitored and also the different batches of leaves could be easily compared. Commercial sedoheptulose standard served as a reference. Since for all these comparative measurements only ^{1}H NMR was required, the analysis was also very rapid.

1.6 Scope of the work

Since the identification of CARKL as a sedoheptulose kinase by a group from the Medical University of Vienna [in cooperation with the University of Natural Resources and Life Sciences Vienna (BOKU)], the necessity for appropriate analytical methods to quantify sedoheptulose arose. Furthermore, due to a lack of commercially available sedoheptulose in gram scale, larger amounts of this sugar should be prepared.

Sedoheptulose should be purified from the leaves of *Sedum maximum* in order to provide material for further experiments concerning the impact of sedoheptulose on human metabolism. Therefore, a final purification protocol for gram amounts should be established.

The change in sedoheptulose concentrations in the leaves over the growing season and within one day should be studied in order to enable the harvest at the optimal time in the following years. Therefore, an extraction protocol for the sugar from the leaves had to be established prior to quantitative analysis.

Furthermore, the composition of different aqueous extracts should be compared and contaminants in the aqueous extract from the leaves should be identified.

In parallel, first test experiments to support large-scale manufacture were performed.

2 Material and Methods

2.1 Solvents and chemicals

All chemicals were purchased from Merck, Sigma-Aldrich, VWR and Fluka and used as delivered if not indicated otherwise.

2.1.1 Sedoheptulose standard

As already stated, there was no sedoheptulose commercially available in the beginning of this work. Fortunately, there was some sedoheptulose available from a former purification at the group of organic chemistry at BOKU. The sample was dissolved in D_2O and after proton NMR measurement lyophilized and weighed. This sample (2.5 mg) was dissolved in LC-MS grade water (1 mL) and used as standard in the first measurements to enable identification and a rough quantification of sedoheptulose.

D-Sedoheptulose became commercially available at Sigma-Aldrich in the beginning of June 2013. One package of 10 mg was ordered for more than 200 Euro as reference material. Purity was specified with ≥ 95% and the storage temperature between 2-8 °C. The brown material was dissolved in D_2O and transferred to an NMR tube for proton NMR measurement. Afterwards it was pipetted into a weighted small glass vial and the NMR tube was rinsed with UHQ water. After lyophilizing, the exact mass was determined for usage as external standard for final quantification.

2.1.2 Solutions

0.1 M Sodium methoxide (NaOMe) solution

230 mg solid sodium were dissolved in dry methanol (100 mL) in a fume-hood

4% Sodium hydroxide (NaOH) solution

20 g sodium hydroxide pellets were dissolved in tap water (500 mL)

6% Hydrochloric acid (HCl) solution

68 mL hydrochloric acid (37%) were filled up with tap water (to 500 mL)

2.2 Plant material

The *Sedum maximum* plants used in this study were provided by the Medical University of Vienna (Dr. Haschemi). Additionally, two different concentrated plant extracts derived from the leaves (termed extract A and B), which originated from different planting conditions, were provided for purification and analysis.

2.3 General organic chemistry methods

2.3.1 Liquid chromatography

Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated glass plates (Merck). For detection of substances, the TLC plates were dipped in anisaldehyde-H_2SO_4 reagent (see Stahl and Kaltenbach, 1961) and heated to about 250 °C on a hot plate.

Column chromatography for purification was performed on silica gel 60 (230-400 mesh, Merck).

2.3.2 Regeneration of Dowex 50 (H^+)

The used resin was treated with 3 M HCl (about 6 times the volume of the resin) and afterwards washed with UHQ water until neutral.

2.3.3 Measurement of optical rotation

For the determination of the optical rotation of sedoheptulose in water a polarimeter from Perkin-Elmer 243 B with a sodium-vapor lamp was used. The temperature was set to 20 °C with a water bath. Calibration to zero was performed with the empty cuvette. 9.5 mg of the purest sedoheptulose available (Sedo_Ether2/1) were dissolved in 1.0 mL HPLC grade water, resulting in a concentration of 9.5 $mg \cdot mL^{-1}$. This solution was filled into the cuvette such that no air bubbles were present.

2.4 Sugar extraction from plants

2.4.1 Sample preparation for analysis

2.4.1.1 Lyophilization

Each week three plants were delivered to BOKU at 5 pm. The transporting conditions could not be monitored. As shown in Figure 4, one stem of each plant was then cut, washed with water and dried with paper towels. The leaves were removed from the stem, cut into pieces with scissors, weighed in into 250 mL round bottom flasks and frozen. The frozen samples were lyophilized with a Christ Beta 1-8LD instrument overnight. The dried weighted leaves were transferred into falcon tubes and stored at -80 °C until further extraction.

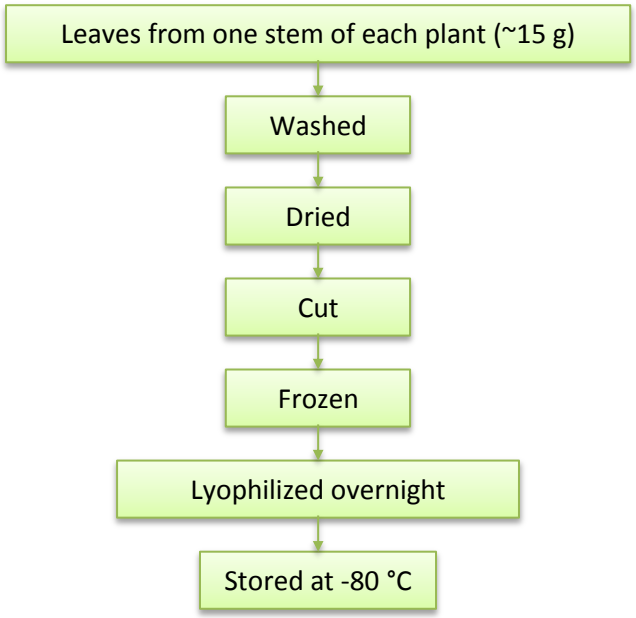


Figure 4: Flow chart of lyophilization

2.4.1.2 Determination of the extraction efficiency of lyophilized leaves

Four different extraction procedures were tested in order to test the extraction efficiency as shown in Figure 5. About 1 g of the lyophilized leaves was ground with a porcelain mortar. After weighing in aliquots of 100 mg, some samples were further ground with liquid nitrogen. Then either 5 mL water or 5 mL ammonium acetate (10 mM, pH 7) were added and vortexed. After 30 min the samples were centrifuged at 4000 rcf. The insoluble residue was extracted a second time with 5 mL water or ammonium acetate overnight. For protein precipitation 300 µL sample were pipetted into 1200 µL of preheated (50 °C) ethanol (EtOH) and vortexed. After centrifugation at 20000 rcf, the supernatant was filtered over 0.2 µm nylon filters and diluted to concentrations of about 10 µM in 50% acetonitrile (ACN) for analysis *via* liquid chromatography coupled to mass spectrometry (LC-MS).

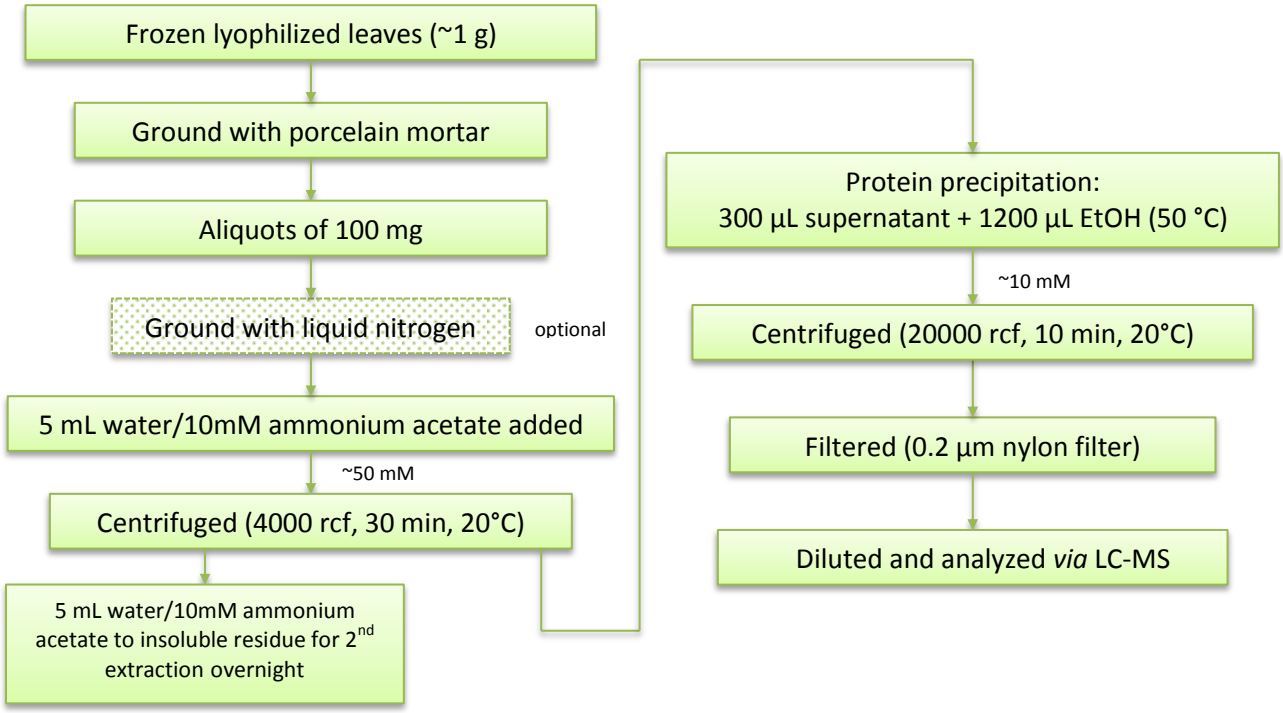


Figure 5: Flow-chart for the examination of extraction efficiency

For the final protocol slight changes were performed in order to facilitate handling and reduce possibilities of errors. Therefore, only half of the volume was used for protein precipitation to reduce pipetting steps and enable the use of 200 µL pipette tips that facilitate pipetting of the ethanolic solutions compared to the large tips. The repeatability was checked with three replicates.

Furthermore, removal of EtOH was necessary for analysis *via* anion exchange chromatography and filtration was not performed before an additional dilution step.

2.4.1.3 Final extraction protocol for lyophilized leaves

As can be seen in Figure 6, the lyophilized leaves were ground in a porcelain mortar. 100 mg of this powder were dissolved in 5 mL water and mixed on a vortex. After 30 min, the samples were centrifuged at 4000 rcf with a Hermle Z323 centrifuge for 30 min. For protein precipitation, 150 µL of the supernatant were pipetted into 600 µL EtOH (50 °C), vortexed and then centrifuged with a table centrifuge MIKRO 200R from Hettich Zentrifugen at 20000 rcf for 10 min. Since the content of organic solvent should be as low as possible for anion exchange chromatography, 100 µL of the supernatant were mixed with 200 µL water and dried under reduced pressure with a SpeedVac concentrator Savant SPD121P coupled to a refrigerated vapor pump Savant RVT400 from Thermo Scientific and a rotary vane vacuum pump from Vacuubrand GmbH for EtOH removal. The residue was then dissolved in 1 mL water, filtered over 0.2 µm PVDF 13 mm syringe filters and finally diluted to the appropriate concentration for measurement. This was about 100 µM for the anion exchange chromatography.

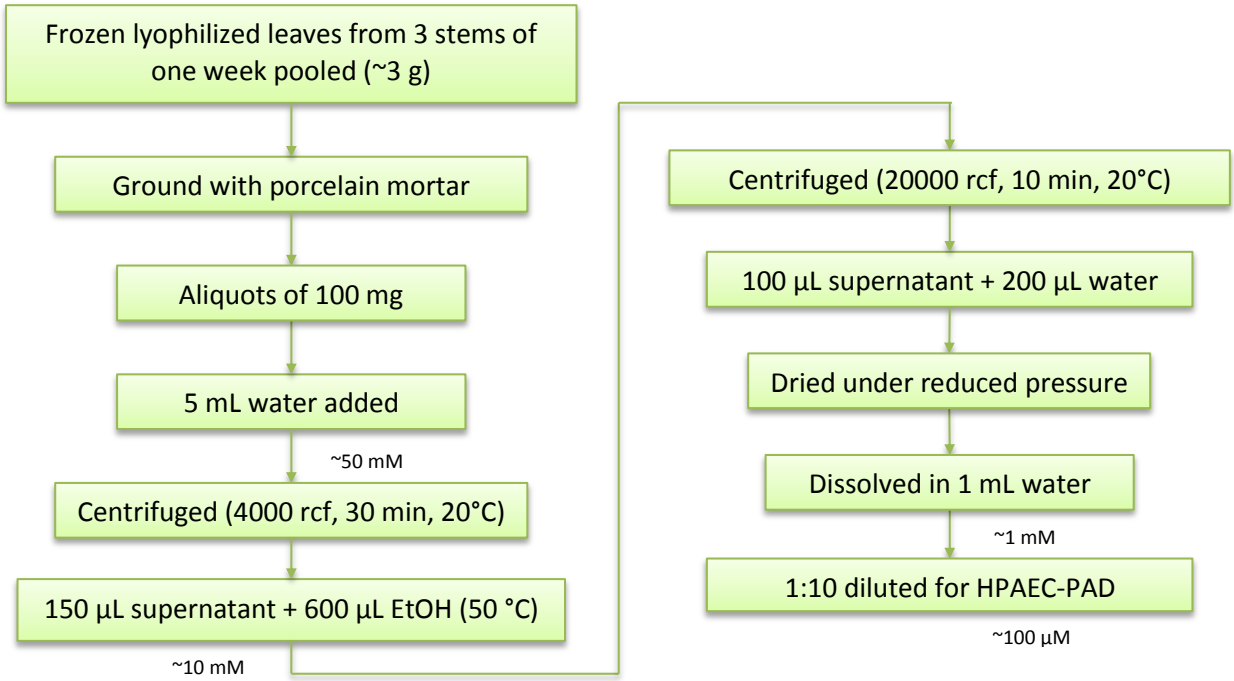


Figure 6: Flow-chart of the final extraction procedure for analysis

2.4.2 First attempt of isolation of larger amounts of sedoheptulose

A first attempt for large scale extraction was carried out in the Food Technology Laboratory at BOKU.

45 kg of frozen leaves (from the same growing conditions as the leaves for concentrate A) provided by the Medical University of Vienna were cut with a large food processor (Alexanderwerk) and mixed with 45 L of water. After 2 hours of thawing, this mixture was further ground in two steps in a wet grinding mill (Stephan Microcut) with 0.5 mm followed by 0.2 mm. In an agitator vessel with jacket, steam was added, boiled for 30 min and afterwards cooled to 60 °C. This mixture was pumped into a decanter (Sharples), centrifuged in a semi-continuous chamber bowl separator (Gea Westfalia Separator Group) and then frozen at -30 °C.

This boiled extract was used after centrifugation for the tests of different anion exchange resins and was then kept for further processing.

2.5 Preparative purification of sedoheptulose

Several steps were carried out to purify 700 mL of the extract B provided by the Medical University of Vienna in two batches. After anion exchange and protein precipitation, per-O-acetylation was carried out to enable the removal of other polar metabolites by washing with saturated aqueous $NaHCO_3$. Subsequent column chromatography was performed in order to separate the acetylated glucose from the acetylated sedoheptulose. After deacetylation, some further purification procedures were tested. Figure 7 gives an overview over the purification steps that are described in detail afterwards.

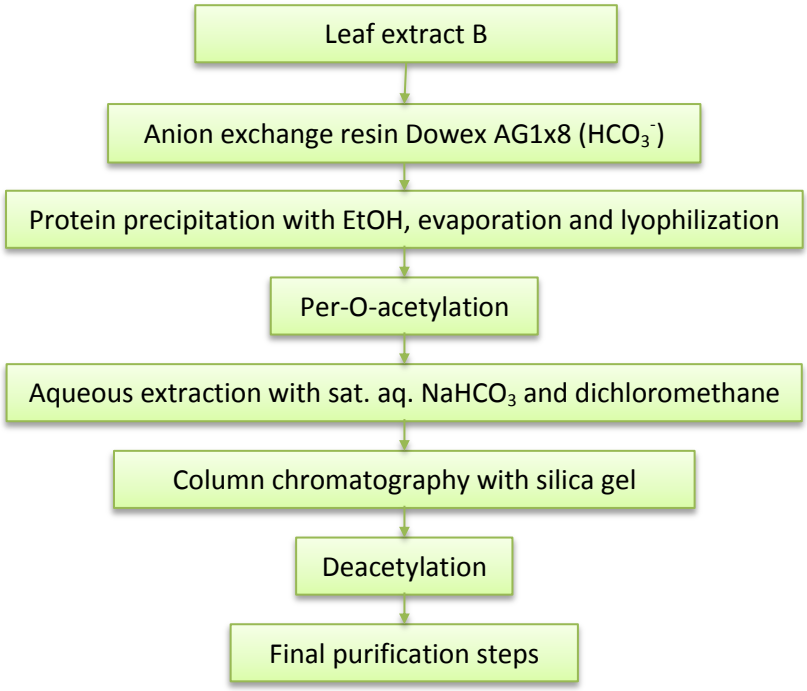


Figure 7: Flow-chart of the purification steps

2.5.1 Anion exchange resin for the removal of organic acids

As already reported earlier (Wamelink et al., 2008) and approved by NMR measurement, the plant extract contained considerable amounts of organic acids. In order to remove them, an anion exchange resin should be used. Negatively charged substances should bind to the resin whereas the sedoheptulose without any charged groups at neutral or slightly acidic pH does not interact with the resin and should elute from the column without any retention.

2.5.1.1 Testing of different anion exchange resins

In order to provide a food safe product, different food grade ion exchange resins were tested after consulting Lanxess, a company producing those food grade resins. Lewatit S 6368A and Lewatit S 4528 from Lanxess were tested.

According to the product information sheet, Lewatit S 6368A is a strongly basic macroporous anion exchange resin and should be suitable for the removal of acid and simultaneous decolorization of e.g. sugar solutions in its hydroxide form and for the decolorization only in its chloride form. It was delivered in the chloride form. On inquiry, information concerning the pre-use treatment for food applications has been available. As described, 75 g of the resin were soaked in potable water, then washed, treated with 500 mL 4% NaOH followed by water, 500 mL 6% HCl followed by water and again 500 mL 4% NaOH and water until the flow-through reached pH 7.

Lewatit S 4528, a weak basic anion exchanger, was already delivered in the hydroxide form and was tested without any pre-treatment.

This resulted in three different resins to be tried:

- Lewatit S 6368A in hydroxide form
- Lewatit S 6368A in chloride form
- Lewatit S 4528 in hydroxide form

The boiled plant extract produced at BOKU was used after centrifugation at 8000 rcf. 20 mL (~ 10 mM sedoheptulose) were mixed with 5 g of each resin and slowly stirred in a beaker for about 20 min. After filtration, the extract was lyophilized for NMR measurement.

Unfortunately, no satisfying results concerning the removal of organic acids were achieved.

Therefore, the anion exchange resin Dowex AG1x8 (HCO_3^-) used in the working group of organic chemistry for several years was tested. Since this resulted in the complete removal of the organic acids, it was used for the purification of 700 mL of concentrated leaf extract B (~ 18 °Brix) despite the fact that no food grade status could then be given to the product.

2.5.1.2 Use of Dowex AG1x8 (HCO_3^-) for purification in lab scale

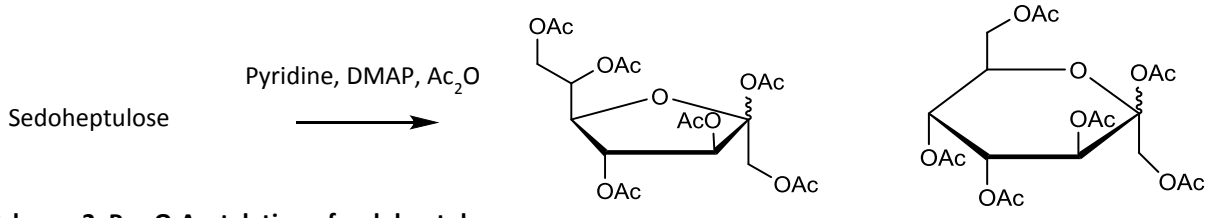
About 1.2 L Dowex AG1x8 anion exchange resin in HCO_3^- form were filled into a column with a diameter of 4.5 cm and a length of about 1 m (bed height: 75 cm) and washed with water. In two batches 700 mL (300 + 400 mL) of the concentrated leaf extract B (provided by the Medical University of Vienna) were loaded onto the column, eluted with water and collected afterwards. For the second batch, the column was filled with new resin.

2.5.2 Protein precipitation with EtOH, evaporation and lyophilizing

The liquid after ion exchange of the first batch was poured into 96% ethanol (200 mL of eluate into 800 mL EtOH = mixed 1:4), stirred for 30 minutes at 4 °C and then centrifuged with 20000 rcf for 15 min. The supernatant was concentrated *in vacuo* in a 2 L flask to about 20 mL and then transferred to a 100 mL flask and further concentrated to a viscous syrup. Afterwards, the syrup was lyophilized for complete water removal. Until the next step, the sample was kept under high vacuum. The same procedure was done with the second batch.

2.5.3 Per-O-acetylation and extraction with sat. aq. $NaHCO_3$ and dichloromethane

As shown in Scheme 2, per-O-acetylation of sedoheptulose was achieved by treatment of the powdered sample with acetic anhydride (Ac_2O), pyridine and 4-*N*,*N*-dimethylaminopyridine (DMAP) as catalyst (Hoefle et al., 1978).



Scheme 2: Per-O-Acetylation of sedoheptulose

In order to find out whether acetylation worked, the whole procedure was tested with a small amount of the sample. Batch 1 and Batch 2 were then treated in the same way.

2.5.3.1 Pre-test

224 mg of Batch 1 after protein precipitation were acetylated. 10 mL of dry pyridine (SeccoSolv®) and a spatula tip of DMAP were added to the flask with the sample and a magnetic bar. Then 3 mL of dry acetic anhydride were added dropwise. The pipette and other tools that came into contact with acetic anhydride were immediately rinsed with methanol (MeOH) or EtOH. Progress of acetylation was monitored *via* TLC with toluene:ethyl acetate (1:1) and per-O-acetylated glucose as a reference. After stirring overnight at room temperature with a drying tube filled with $CaSO_4$ granulate (Drierite), 16 mL of dry MeOH were added to quench the remaining acetic anhydride. After 15 min of stirring, the solution was co-evaporated with toluene three to five times.

For solvent extraction, the acetylated sample was dissolved in 80 mL dichloromethane, and 120 mL saturated $NaHCO_3$ solution was added. Mixing had to be done carefully because of the formation of CO_2 due to the still remaining acids. The organic phase was removed and the aqueous phase was extracted two more times with 10 mL of dichloromethane each. Then the aqueous phase was removed and discarded. The organic phase was once more extracted with 30 mL saturated $NaHCO_3$ and the aqueous one again two times with dichloromethane. The organic phase containing the per-O-acetylated sedoheptulose was then dried with $MgSO_4$, filtered and concentrated *in vacuo*.

2.5.3.2 Batch 1

11.3 g of the dry crumbled sample were mixed with 100 mL of dry pyridine and a spatula tip of DMAP. Then, 50 mL dry acetic anhydride were mixed with 50 mL pyridine and added dropwise while cooling the flask with ice water. It was stirred overnight at room temperature. When checking *via* TLC in the morning there was still a small spot beneath the acetylated sedoheptulose. Therefore, another 10 mL of acetic anhydride were added and stirred for four more hours. TLC showed no change and the reaction was stopped by adding 25 mL of dry MeOH with cooling and stirring for 15 min followed by evaporation as described before.

The remaining syrup was dissolved in about 100 mL of dichloromethane and solvent extraction with about 200 mL of saturated $NaHCO_3$ solution was carried out as before. Then the organic phase was

dried with $MgSO_4$ and additionally some dry silica gel was added to remove some color. After filtering, the organic phase was concentrated.

2.5.3.3 Batch 2

16.7 g of the dry crumbled sample were mixed with 100 mL of dry pyridine and a spatula tip of DMAP. Then, 75 mL dry acetic anhydride were mixed with 50 mL pyridine and added dropwise while cooling the flask with ice water. It was stirred overnight at room temperature. Then the same procedure as for Batch 1 was performed with the difference that 30 mL of dry MeOH were used to stop the reaction.

2.5.4 Column chromatography with silica gel

Different solvents and solvent ratios for column chromatography on silica gel were tested on TLC, resulting in diethyl ether:hexane (5:1) to be the best one for separation of per-O-acetylated sedoheptulose from the per-O-acetylated glucose also present in small amounts. Since using several liters of diethyl ether for one batch would have been neither cost efficient nor very safe, a more common solvent system with toluene and ethyl acetate in a ratio of 3:2 was used for the purification of the two batches. Diethyl ether:hexane was only used for the final purification of one fraction.

2.5.4.1 Pre-test

The acetylated sedoheptulose from the pre-test before was purified on a small silica gel column. Sedoheptulose containing fractions were identified *via* TLC, pooled and concentrated *in vacuo*.

2.5.4.2 MPLC

For the large batches, a MPLC system with a Besta pump and a Büchi Fraction Collector C660 was used with a column with a diameter of 6 cm. The column was filled with 450 g silica gel resulting in a bed height of about 30 cm. About 4.5 L solvent (toluene:ethyl acetate 3:2) were needed for each column. The column was flushed with the solvent at a flow rate of 80 $mL \cdot min^{-1}$ until all air bubbles were removed. In order to reduce solvent consumption, the solvent was pumped back to the solvent bottle after the column. The per-O-acetylated sedoheptulose batches were dissolved in the solvent system and loaded onto the column. The flow rate was set to 80 $mL \cdot min^{-1}$. After the first 500 mL of solvent had been eluted, 100 mL fractions were collected in 250 mL beakers. Afterwards, the fractions were checked *via* TLC (toluene:ethyl acetate 3:2), pooled as to remove the glucose content and concentrated *in vacuo*. Figure 8 gives an overview over the different batches and indicates the labeling of the resulting batches.

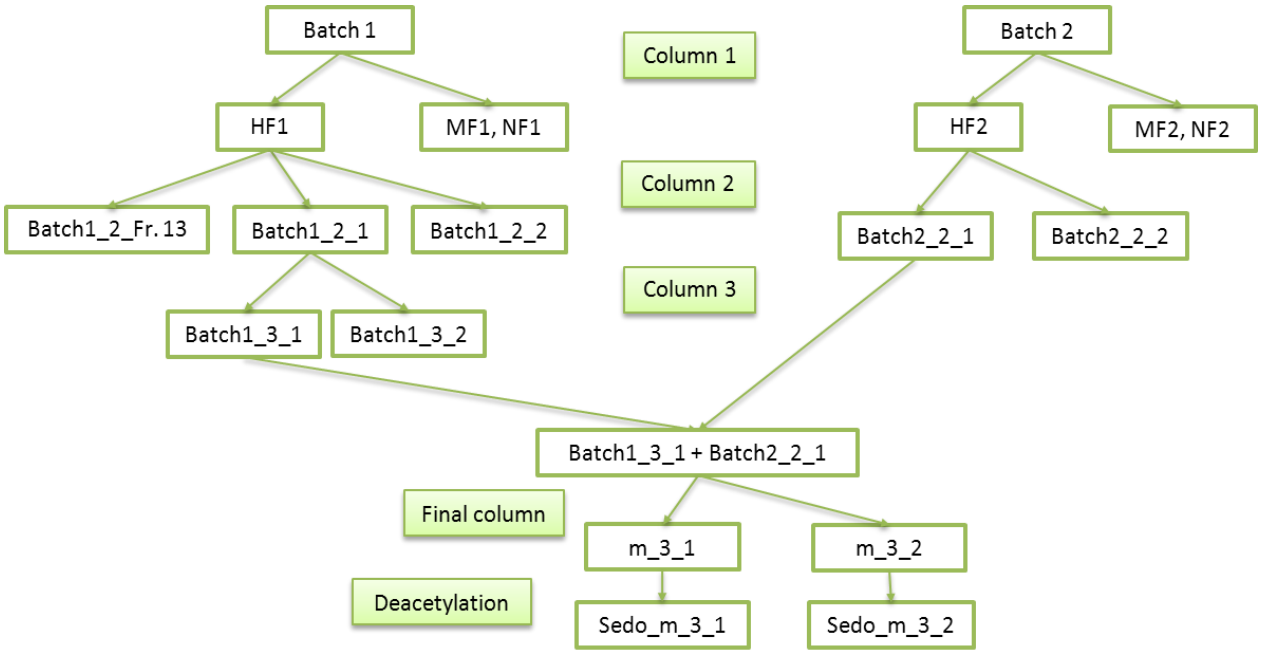


Figure 8: Overview over the batches resulting from column chromatographies. Columns were filled with silica gel and eluted with toluene:ethyl acetate (3:2), batches after deacetylation were labeled with Sedo_xxx.

Column 1

In the first separation step, the two batches were loaded onto separate columns and after elution pooled as follows (see Table 1 and Table 2).

Table 1: Pooling and labeling of the fractions from Batch 1 after the first column

Name of the pooled fraction	Number of fractions
MF1 (“Mischfraktion”, mixed fraction)	4, 10 - 13
HF1 (“Hauptfraktion”, main fraction)	5 - 9
NF1 (“Nachfraktion”, late fraction)	14 - 18

Table 2: Pooling and labeling of the fractions from Batch 2 after the first column

Name of the pooled fraction	Number of fractions
MF2 (“Mischfraktion”, mixed fraction)	6
HF2 (“Hauptfraktion”, main fraction)	7 - 14
NF2 (“Nachfraktion”, late fraction)	15 - 22

In Figure 9 several spots can be seen. The one at the top (A) is the acetylated glucose as identified with a pure standard of per-O-acetylated glucose, followed by not completely separated isomers of acetylated sedoheptulose (furanose and pyranose each in α and β form: B, C). The fractions with smallest R_f values (D) were neither identified nor used and are still available for further studies (NF1 and NF2).

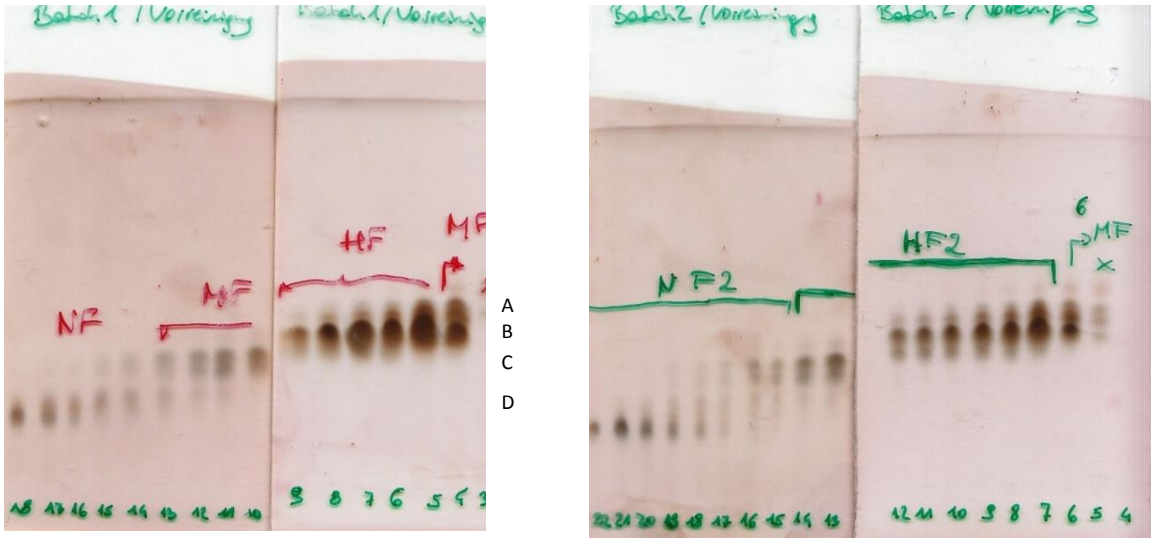


Figure 9: TLC after the first column of Batch 1 and Batch 2

Column 2

The main fractions HF1 (~5 g) and HF2 (~8 g) from the first column were each loaded onto a second column. Eluted fractions were checked *via* TLC (Figure 10) and pooled as follows (see Table 3 and Table 4):

Table 3: Pooling and labeling of the fractions from HF1 after the second column

Name of the pooled fraction	Number of fractions
Added to MF1	7
Batch 1 column 2 Fr. 1 (Batch1_2_1)	8 - 12
Fr. 13 (purest fraction for column with diethyl ether)	13
Batch 1 column 2 Fr. 2 (Batch1_2_2)	14 - 18

Table 4: Pooling and labeling of the fractions from HF2 after the second column

Name of the pooled fraction	Number of fractions
Added to MF2	5
Batch 2 column 2 Fr. 1 (Batch2_2_1)	6 - 13
Batch 2 column 2 Fr. 2 (Batch2_2_2)	14 - 18

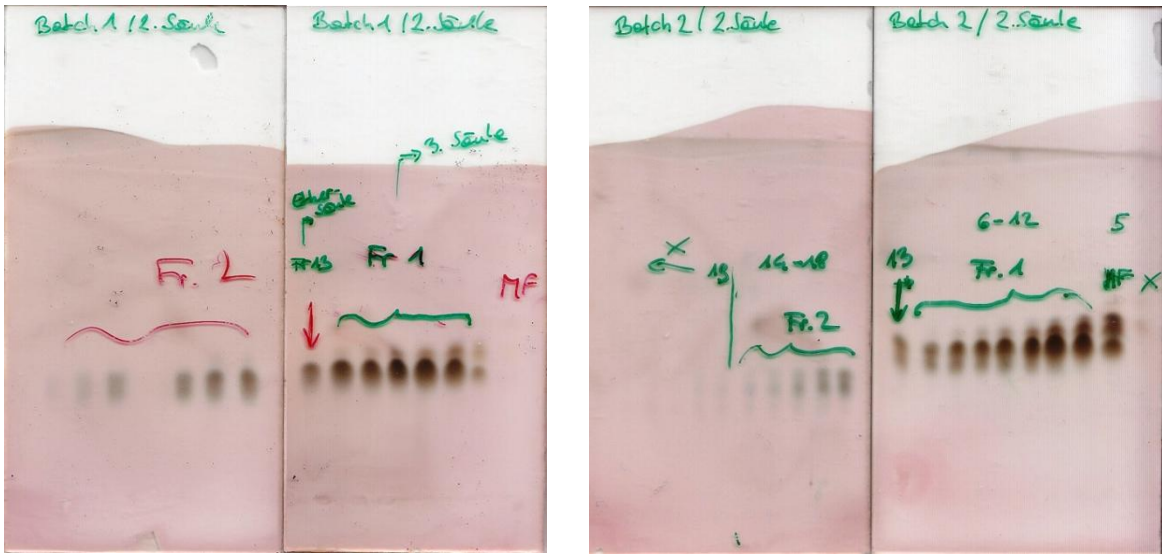


Figure 10: TLC after the second column of the main fraction from Batch 1 and Batch 2

Column 3

Batch1_2_1 was loaded onto a third column. Eluted fractions were checked *via* TLC (see Figure 11, left side) and pooled as shown in Table 5.

Table 5: Pooling and labeling of the fractions from Batch1_2_1 after the third column

Name of the pooled fraction	Number of fractions
Batch1_3_1 → added to Batch2_2_1	8 - 11
Batch1_3_2	12 - 18

The final column was performed with Batch 2_2_1 combined with Batch1_3_1. Eluted fractions (see Figure 11, right side) were pooled as shown in Table 6.

Table 6: Pooling and labeling of the fractions from Batch2_2_1 combined with Batch1_3_1 after the final column

Name of the pooled fraction	Number of fractions
Batch1+2_3_1 (m_3_1)	9 - 11
Batch1+2_3_2 (m_3_2)	12 - 21

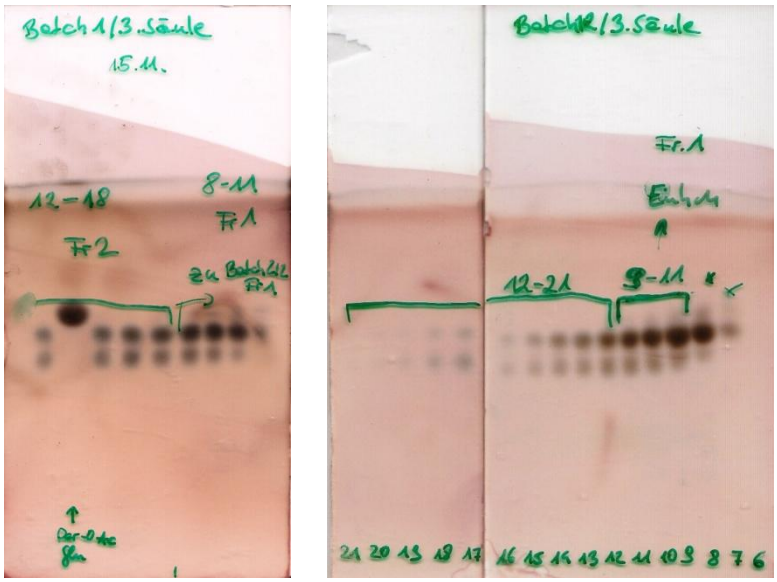


Figure 11: TLC after the third column of Batch 1 and the final column of Batch 2_2_1 combined with Batch1_3_1

2.5.4.3 Final purification of one fraction (~ 280 mg) with diethyl ether:hexane (5:1)

One of the purest fractions, resulting from the column chromatographies described before, was Batch1_2_Fr.13. In order to completely purify this fraction, a column chromatography with diethyl ether:hexane (5:1) as solvent system was performed. Therefore, a column with a water jacket was filled with about 60 g of dissolved silica gel and rinsed with the solvent. Batch1_2_Fr.13 was dissolved in the solvent, loaded onto the column and eluted. 2 mL fractions were collected with a fraction collector Retriever II from ISCO. The different fractions were checked *via* TLC (see Figure 12) and pooled as shown in Table 7:

Table 7: Pooling and labeling of fractions after the column with diethyl ether for final purification

Name of the pooled fraction	Number of fractions
Ether F1	39 - 44
Ether MF	45 - 64
Ether F2	65 - 73

As can be seen in Figure 12, no more acetylated glucose (A) was present in the fractions and a separation between the two acetylated sedoheptulose spots (B and C) was possible.

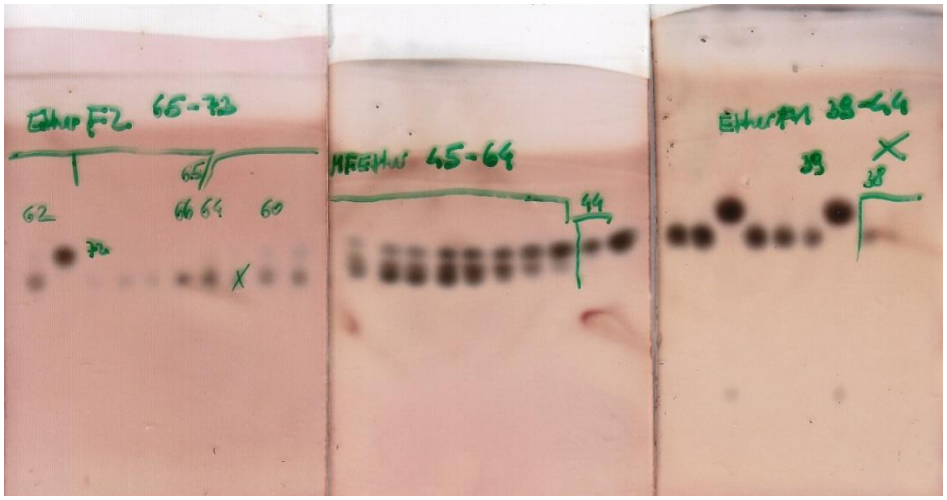


Figure 12: TLC after the separation with diethyl ether, large spots: per-O-acetylated glucose (A) as reference

2.5.5 Deacetylation by transesterification (Zemplén de-O-acetylation)

The sample was dissolved in dry methanol and 0.1 M sodium methoxide solution (NaOMe) was added until the pH was about 8 to 9. The pH was checked several times and in case that the pH decreased, additional sodium methoxide solution was added to keep the pH at 8 to 9. The progress of the reaction was controlled *via* TLC (MeOH:chloroform:water = 10:10:3) and carried on for three hours. To quench the reaction the cation exchange resin Dowex 50 (H^+) was used until the pH was 7. The resin was filtered off and the filtrate was concentrated *in vacuo*. The product was checked by proton NMR measurement.

2.5.6 Additional steps for final purification

In order to further improve the purity, several different options were tested. The following Figure 13 shows which batches were used and indicates the final labeling.

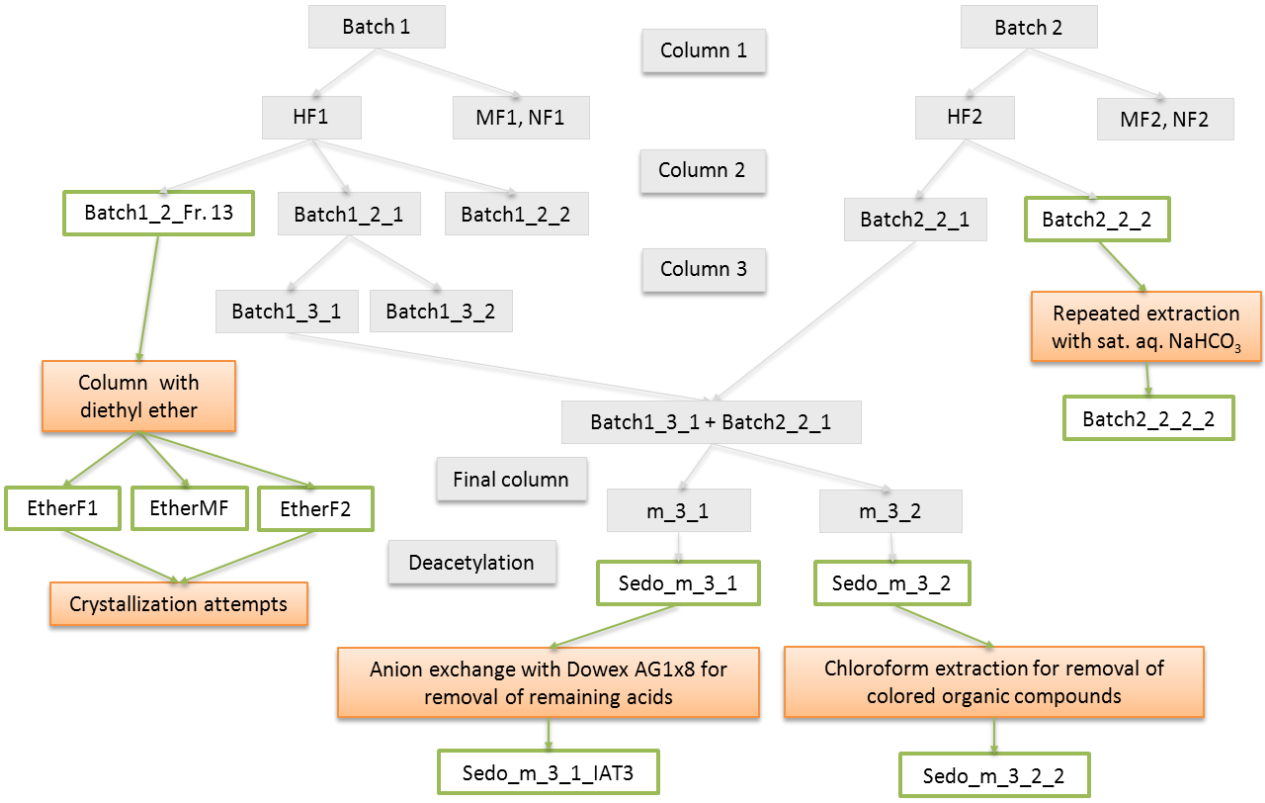


Figure 13: Additional purification steps tested (orange)

2.5.6.1 Repeated extraction of sedoheptulose acetate with sat. aq. $NaHCO_3$ after column chromatography

Batch2_2_2 (in the acetylated form) was dissolved in dichloromethane and once again washed with sat. aq. $NaHCO_3$. The yellow colored aqueous phase was washed three times with dichloromethane. The only slightly yellow colored organic phase was dried with $MgSO_4$, filtered and concentrated *in vacuo*.

2.5.6.2 Lyophilization for removal of methanol

In order to completely remove methanol after the deacetylation step, the samples were twice dissolved in water (for inj.) and lyophilized. This step was partly combined with the two following purification steps for which lyophilizing was also necessary.

2.5.6.3 Chloroform extraction for removal of colored organic compounds

Batch Sedo_m_3_2 was dissolved in approx. 30 mL water (for inj.) in a 100 mL shaped flask with a magnetic stirrer. About 2 mL of chloroform were added and the sample was extracted for 3-4 min under vigorous stirring. The chloroform phase was removed with a Pasteur pipette. Afterwards, 2 mL of chloroform were added and the whole procedure was repeated five times. The chloroform phase

was removed and pressurized air was used to remove residual chloroform. The sample was then frozen and lyophilized.

2.5.6.4 Anion exchange with Dowex AG1x8 for removal of remaining acids

The anion exchange resin Dowex AG1x8 was used again to try whether it was possible to remove further contaminants. About 40 mL of the resin were washed with UHQ water and water for inj.

Pre-test

About 0.5 mL of the washed resin were placed in a small glass frit and washed with about 1 mL of D_2O. 17.5 mg of Sedo_m_3_1 dissolved in 0.6 mL D_2O (from the NMR sample) were loaded on the resin and rinsed with D_2O. The pH of the filtrate was checked with indicator paper. This solution was then immediately measured *via* NMR.

Application to batch Sedo_m_3_1

The method from the pre-test was then applied to the whole batch. About 30 mL of the resin were put in a glass frit to form a resin with a height of 2 cm. After washing again with water for inj., the sample was dissolved in 20 mL water for inj. and was transferred to the resin and rinsed with water for inj. The pH was checked and appeared to be 9-10. Therefore, a cation exchange resin in H^+ form was added to the filtrate under stirring until pH was 7. The suspension was filtered and the filtrate was then frozen and lyophilized. Since after lyophilizing the pH was again alkaline, the procedure was repeated.

2.5.6.5 Crystallization attempts

No crystallization of sedoheptulose has been reported yet. Nevertheless, crystallization would offer the finest method for final purification. Since free sedoheptulose is not present in just one form (Ceusters et al., 2013), it was tested whether crystallization of the per-O-acetylated sedoheptulose was possible. Therefore, the purest fractions from the column with diethyl ether were taken and different solvents and solvent mixtures (ethanol, diethyl ether, hexane, ethyl acetate, dichloromethane) were tested.

2.6 Analytical methods

Depending on the purpose, different analytical methods were used within the framework of this thesis. For the identification of unknown compounds and comparison of different samples concerning their purity, ^{1}H NMR was used. The first screening of sedoheptulose concentrations and the determination of the extraction efficiency was performed *via* LC-MS whereas for further quantification HPAEC-PAD was used. Furthermore, LC-MS was used to verify contaminants identified by NMR spectroscopy.

2.6.1 Nuclear Magnetic Resonance spectroscopy (NMR)

Sample preparation for NMR is quite simple. For aqueous sedoheptulose samples lyophilization followed by dissolving in D_2O (99.8 atom % ^2H from Merck) and for per-O-acetylated samples drying and dissolving in $CDCl_3$ (99.8 atom % ^2H from Euriso-Top) was performed. NMR spectra of these solutions were recorded on a Bruker Avance III 600 instrument at 27 °C.

For all comparative measurements ^1H NMR was performed whereas for structural elucidation of impurities COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), HMBC (heteronuclear multiple bond coherence) and APT (attached proton test) spectra were recorded. Furthermore, a ^{31}P NMR for checking the phosphate level was performed.

Some spectra showed a rather intensive signal at approximately 4.7 ppm resulting from insufficient water suppression during NMR recording that can be ignored when comparing spectra. Furthermore, slight shifts in ppm values are caused by small variations in pH values of the samples.

2.6.2 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

In the context of this thesis, an established method especially for monosaccharides from the group of Nanoglycobiology, Institute of Biologically Inspired Materials, Department of Nanobiotechnology at BOKU was used. This group already used HPAEC-PAD for the identification of the glycan structure of a heptose-containing S-layer glycoprotein in 1995 (Kosma et al., 1995). Since then many improvements concerning the detection – particularly the waveform of the applied voltage – have arisen and have been implemented.

A Dionex ICS-3000 metal-free system with a CarboPac® PA1 column (250x4 mm) and a CarboPac® PA1 guard column both from Dionex were used at a flow rate of 1 $mL \cdot min^{-1}$. The column oven temperature was set to 25 °C.

For eluent preparation 50% sodium hydroxide (NaOH) solution from J.T. Baker was used to prepare a 200 mM and 50 mM NaOH solution with high quality water. It is very important not to prepare the solution from sodium hydroxide pellets because they are coated with a thin layer of sodium carbonate. Since carbonate concentration is critical in carbohydrate analysis, all eluents have to be degassed and kept blanketed under helium during the measurement. Furthermore, preparation of eluents has always to be kept consistent (Thermo Fisher Scientific Inc., 2014a).

Elution was carried out isocratically with 16 mM NaOH for the first 20 min. Then a linear gradient to 100 mM NaOH was applied from 20 to 40 min followed by an increase over two minutes to 200 mM hold until 47 min. The starting condition with 16 mM NaOH was reached again at 49 min and kept until 70 min for equilibration (see Table 8).

Table 8: Gradient for elution for HPAEC

Time [min]	NaOH [mM]
0 - 20	16
20 - 40	16 → 100
40 - 42	100 → 200
42 - 47	200
47 - 49	200 → 16
49 - 70	16

Pulsed amperometric detection was carried out using the recommended parameters from the technical note of the manufacturer as listed in Table 9. The resulting waveform is illustrated in Figure 14.

Table 9: Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector (Thermo Fisher Scientific Inc., 2014b)

Time [s]	Potential [V]	Integration
0.00	+ 0.1	
0.20	+ 0.1	Start
0.40	+ 0.1	End
0.41	- 2.0	
0.42	- 2.0	
0.43	+ 0.6	
0.44	- 0.1	
0.50	- 0.1	

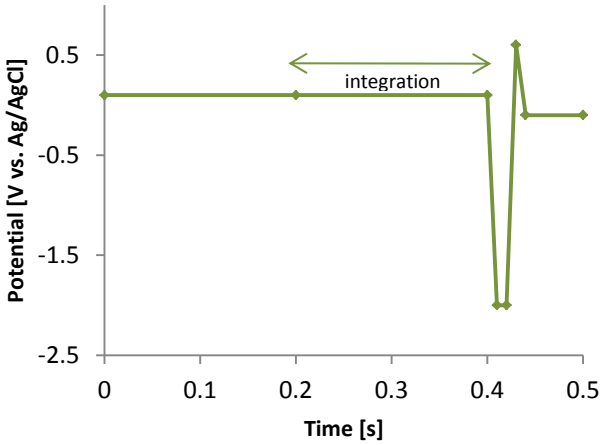


Figure 14: Waveform of potential for pulsed amperometric detection

The signals were integrated between 0.20 and 0.40 s and evaluated with Chromeleon™ software.

Aqueous samples were prepared as shown in Figure 6. External calibration was performed using the sedoheptulose standard from Sigma-Aldrich and a standard addition experiment with three different spikes was carried out in order to eliminate the risk of any matrix influence from the leaf extract.

2.6.3 Liquid Chromatography coupled to Time-of-Flight Mass Spectrometry (LC-TOF-MS)

In an Agilent 1200 series HPLC with a CTC autosampler two different columns (Unison UK-Amino 250x2 mm from U.S. Impact and XBridge Amide 150x2.1 mm from Waters) especially designed for sugar separation were tested with HILIC conditions according to their description. Since no specific information about the separation of glucose from sedoheptulose was found, different gradients for the mobile phase were tested. Although both columns showed retention of sedoheptulose, no baseline separation of sedoheptulose from glucose was achieved. Nevertheless, detection *via* mass spectrometry was possible due to the different masses of the analytes.

The detection was performed on an Agilent 6210 Series Time-of-Flight (TOF) mass spectrometer with ESI and TOF parameters given in Table 10. Data evaluation was performed by using Agilent Mass Hunter Qualitative and Quantitative Analysis software. Quantification was based on the peak area in the extracted ion chromatogram (extraction width = 0.005 *m/z*). Sedoheptulose was observed as its negative ion $[M-H]^-$ with 209.0667 *m/z.*

The Unison UK-Amino column was used with HILIC conditions at a flow rate of 0.3 $mL \cdot min^{-1}$. The temperature was set to 65 °C and the mobile phase consisted of acetonitrile, water and 0.1% formic acid.

Eluent A: 98.9% water/1% ACN/0.1% formic acid
Eluent B: 98.9% ACN/1% water/0.1% formic acid

The final gradient started with 75% B decreasing to 70% B until 5 min. Afterwards, B was decreased to 40% and increased to 90% for cleaning followed by equilibration to starting conditions again. ESI and TOF parameters are listed in Table 10.

Table 10: ESI and TOF parameters for quantification of sedoheptulose

Ion polarity	negative
Gas temperature	350
Drying gas	11 $L \cdot min^{-1}$
Nebulizer	30 psig
VCap	3500 V
Fragmentor	120 V
Skimmer	60 V
OCT 1 RF Vpp	250 V

3 Results and Discussion

3.1 Analytical results

3.1.1 Sedoheptulose standard

The ^{1}H NMR spectrum of the commercially available sedoheptulose received from Sigma-Aldrich is shown in Figure 15. There was a large signal at about 1.8 ppm that was identified as sodium acetate and accounts for approximately 28% on a molar basis. Considering the different molecular weights resulted in calculated 87% (w/w) sedoheptulose content. Thus, the specified purity of ≥ 95% was not reached. Since the same signal was detected in the purified batches, this standard seems to have also been obtained *via* sedoheptulose acetates.

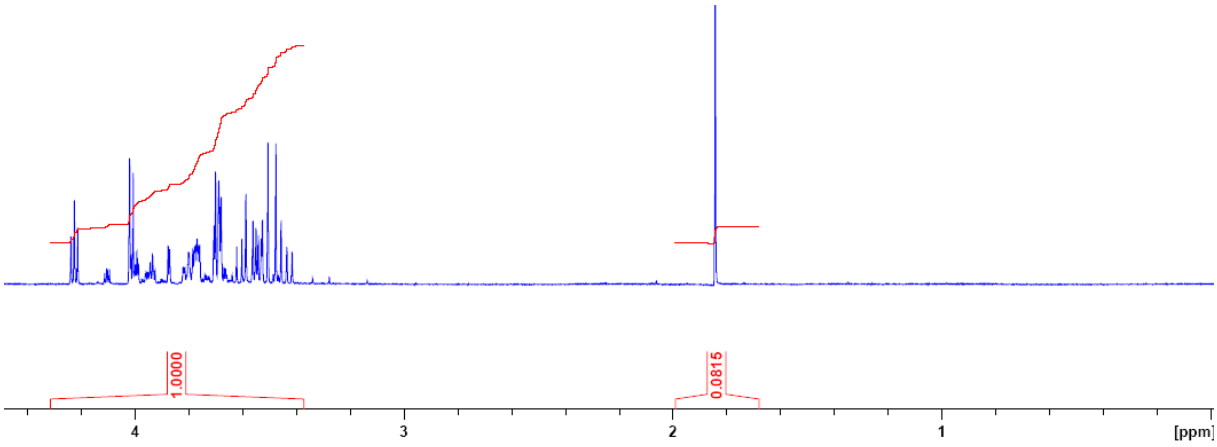


Figure 15: ^{1}H NMR spectrum of the sedoheptulose received from Sigma-Aldrich with a specified purity of 95%

Due to the lack of a pure standard of sedoheptulose, accurate absolute quantification was not possible but fortunately also not immediately needed. Within the scope of this thesis, the comparison of the different samples concerning extraction efficiency and purification steps was the main challenge, while absolute levels were only interesting for an estimation of the possible total content of sedoheptulose of the plants.

All concentration values given in the following were obtained considering a sedoheptulose standard purity of 95% as given in the certificate. Accordingly, the values obtained in this study are not absolute concentration values but should be conceived as method based relative values. This concept was fit for purpose for the intended study.

3.1.2 Sample preparation for quantitative analysis

Different extraction protocols were tested on lyophilized leaves. Lyophilization worked well resulting in a constant dry weight of leaves of 6.6 ± 0.7% (N=30). For comparison, the dry weight of stems was measured only once resulting in a dry weight of 16.6%. The rather low value of leaf dry matter was to be expected due to the leaf-succulence characteristics of *Sedum maximum*.

As can be readily observed in Figure 16, LC-MS data revealed that there were no significant differences in the investigated extraction methods. Sedoheptulose was quantified by external calibration using the previously described standard. All concentration values were normalized to the water extraction. The use of liquid nitrogen did not significantly improve the extraction efficiency yielding 97 ± 2% (N=3). Hence, the extraction could be simplified to an aqueous extraction of the ground lyophilized leaves. Repeated extraction overnight yielded only about 1/10 of the concentration of the first extraction. As a consequence, it was decided that one extraction step was sufficient for quantitative determination and that repeated extractions could be discarded.

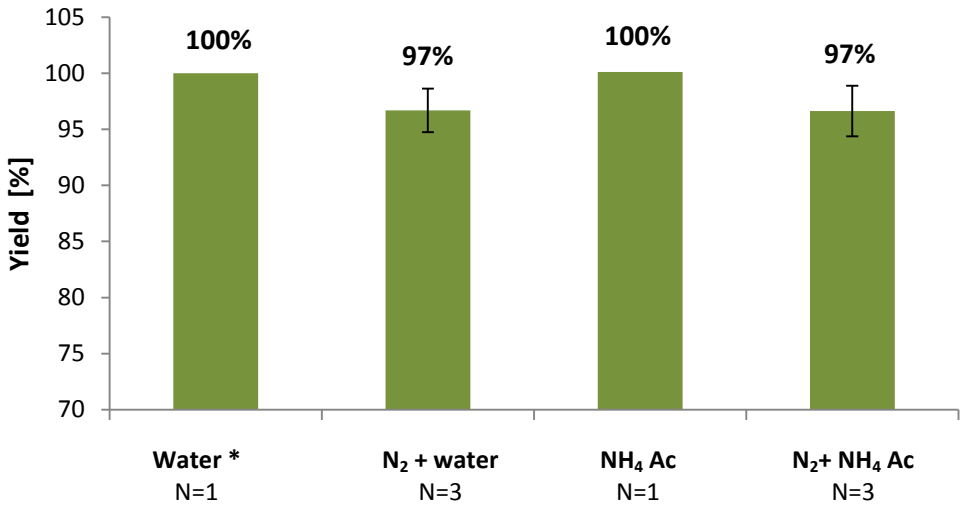


Figure 16: Comparison of different extraction procedures for lyophilized leaves. Error bars denote standard deviations of the triplicates. *Water without liquid nitrogen set to 100%

The finally implemented extraction procedure is given in 2.4.1.3. At this point is has to be mentioned that it worked very well for nearly all samples. However, the leaves of week 29 had to be lyophilized a second time since grinding with the porcelain mortar was not possible due to some residual moisture. Furthermore, it was observed that the leaves harvested in August (calendar week 31 and for diurnal variation) showed starting gel formation about half an hour after the ground lyophilized leaves had been mixed with water. Therefore, in future studies more attention should be paid on rapid reprocessing of all samples.

3.1.3 Measurement of sedoheptulose concentrations by HPAEC-PAD

After testing the extraction efficiency with LC-MS, all lyophilized leaf samples were measured with anion exchange chromatography and electrochemical detection. The applied method provides a very good separation of glucose, fructose and sedoheptulose as shown in Figure 17.

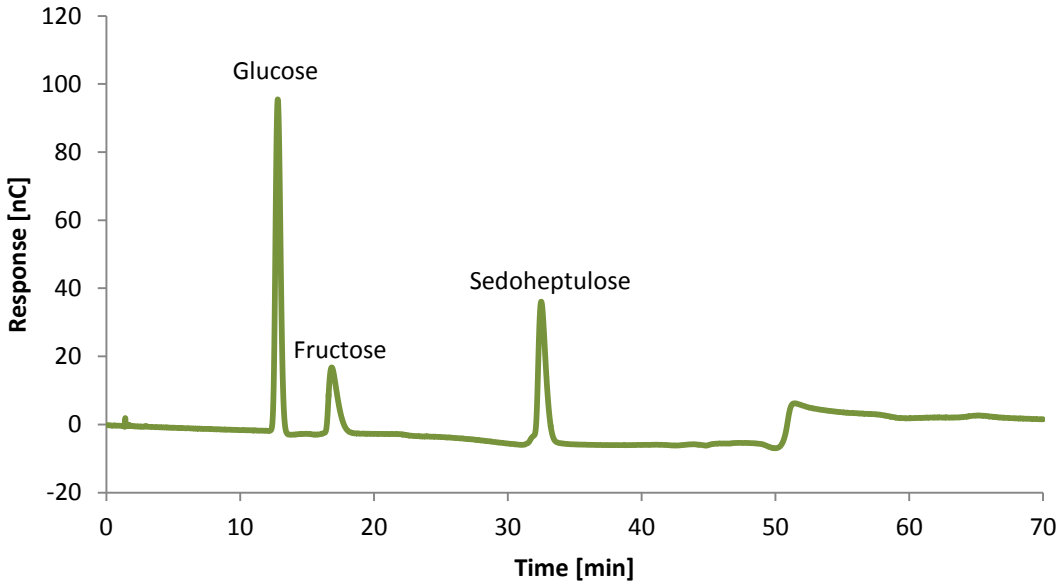


Figure 17: HPAEC-PAD chromatogram of a 100 µM mixed aqueous standard of glucose, fructose and sedoheptulose

The chromatogram of one aqueous sample of week 24 is shown in Figure 18. The peak for sedoheptulose equals the one of the standard and a small peak for glucose can be seen confirming the presence of glucose.

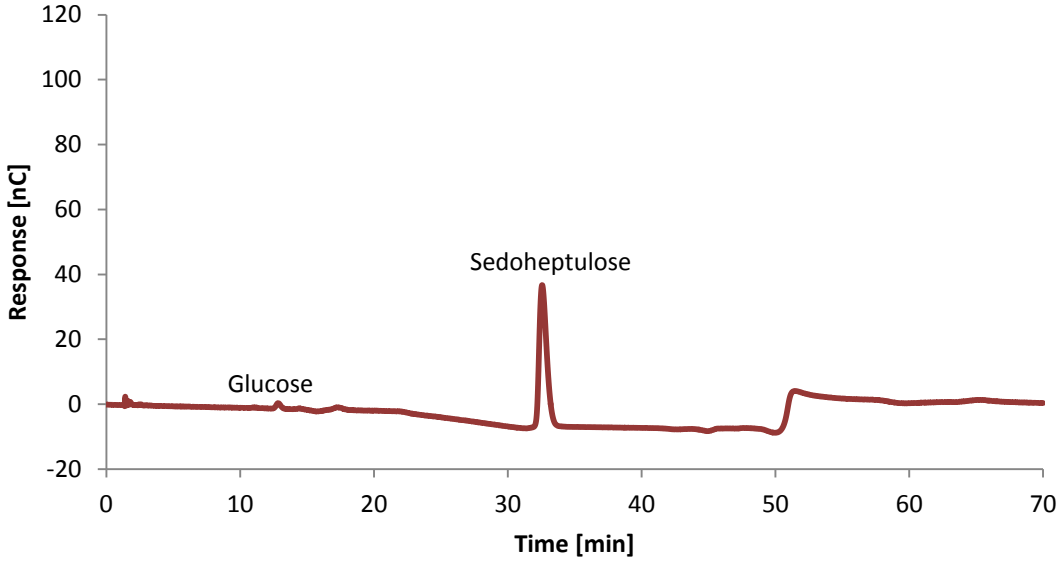


Figure 18: HPAEC-PAD chromatogram of an aqueous extract of lyophilized leaves (week 24)

Measurement repeatability was determined with five repetitive injections and resulted in an excellent relative standard deviation (RSD) of 1% (N=5). To estimate procedure repeatability, three replicates (100 mg) of the lyophilized leaves were extracted resulting in an RSD of 3% (N=3) hence proving that the sample was homogenous and the procedure repeatable. Calculation of the limit of detection (LOD) was performed with the 3σ criterion quantifying the baseline noise to the peak height of a standard. Typical LODs were in the low µM range (≤ 3 µM). Accordingly, the limit of quantification (LOQ) for sedoheptulose ranged at 10 µM. External calibration was performed with aqueous standards between 10 and 400 µM representing the linear working range. Since all samples were available in very high concentrations, dilutions to reach about 100 µM were used.

3.1.3.1 Standard addition experiment

In order to evaluate the matrix influence of the sample on the sedoheptulose concentration measured, a standard addition was performed. One sample was spiked with three different concentrations (50, 100 and 200 µM). The resulting curve is shown in Figure 19.

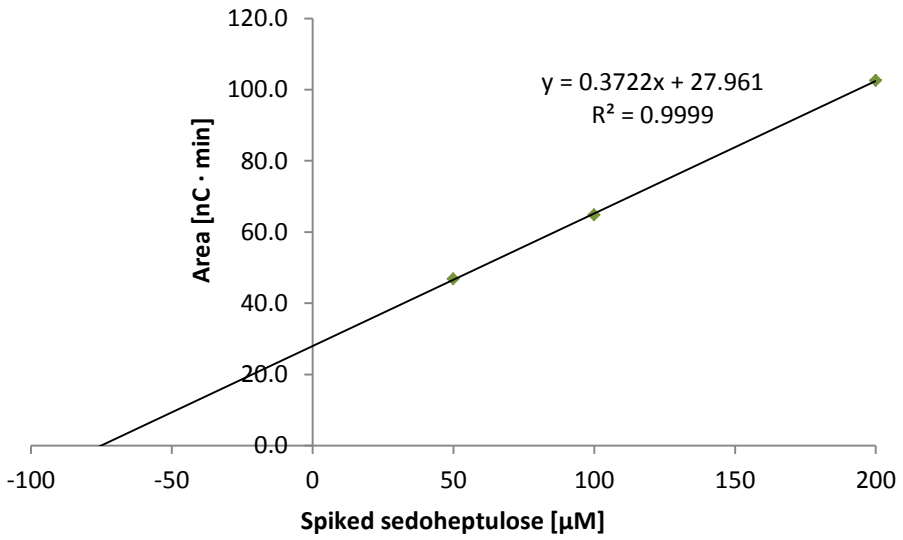


Figure 19: Standard addition with three spikes (50, 100 and 200 µM sedoheptulose) of one sample (week 21)

The slope of the standard addition curve agrees with the one from the external calibration curve. The sedoheptulose concentration calculated from the standard addition experiment was 75.1 µM for the sample, respectively 39.3% (g per 100 g dry weight) compared to 40.9% obtained by external calibration. Thus, there are no matrix influences on the detectable sedoheptulose concentration and external calibration using aqueous standards was a valid quantification concept.

3.1.3.2 Weekly samples

For the weekly samples, the leaves of the three different stems per week were pooled and then extracted three times. However, leaves of only every second or third week were analyzed since this

was sufficient to get the information whether there was a change in sedoheptulose concentration over the weeks or not. This reduction of the number of analyzed samples was also necessary because of the long run time for one sample of 70 min.

Figure 20 shows the calibration curve for sedoheptulose and Figure 21 shows the results for the relative changes in sedoheptulose concentration over the growing season. The data of week 29 were not considered for the comparison because sample preparation was slightly different. With an RSD of 19% for all values except week 29, a significant change in sedoheptulose concentration over the weeks was observed. Starting in the beginning of May (week 19) the concentration reached its peak in the mid of June (week 24) and afterwards decreased significantly.

Nevertheless, the optimal period for harvest is approximately in the mid of June but seems not to be restricted to only a few days since only minor differences in the concentrations between week 21 and week 26 have been observed.

The leaves of week 29 had to be lyophilized a second time before extraction. Maybe this resulted in a better extraction however, since extraction efficiency for the used protocol was really good, the influence should not be that pronounced. Another explanation for the higher sedoheptulose content in week 29 could be that from this week on the plants were located on a terrace at BOKU in spite of being delivered every week. They were extremely exposed to the sun with a wall behind reflecting the sun light.

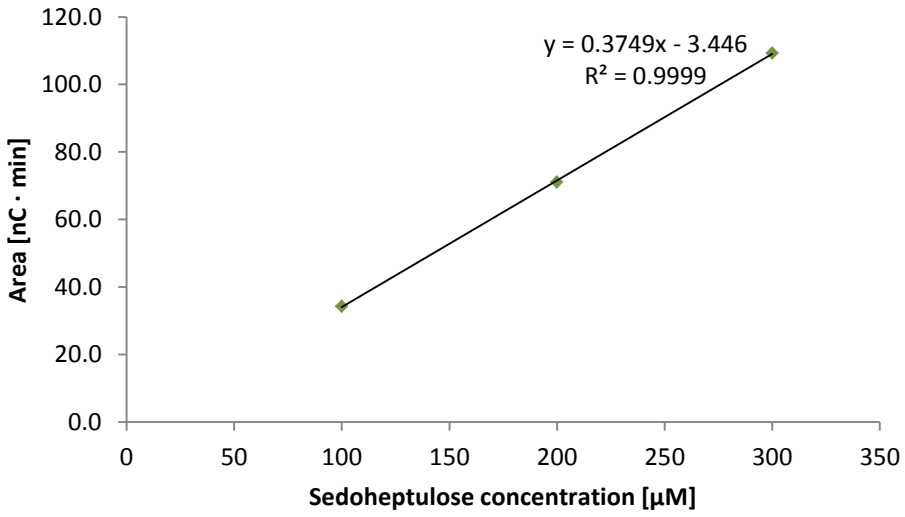


Figure 20: Calibration curve for the calculation of the sedoheptulose concentrations of the weekly samples

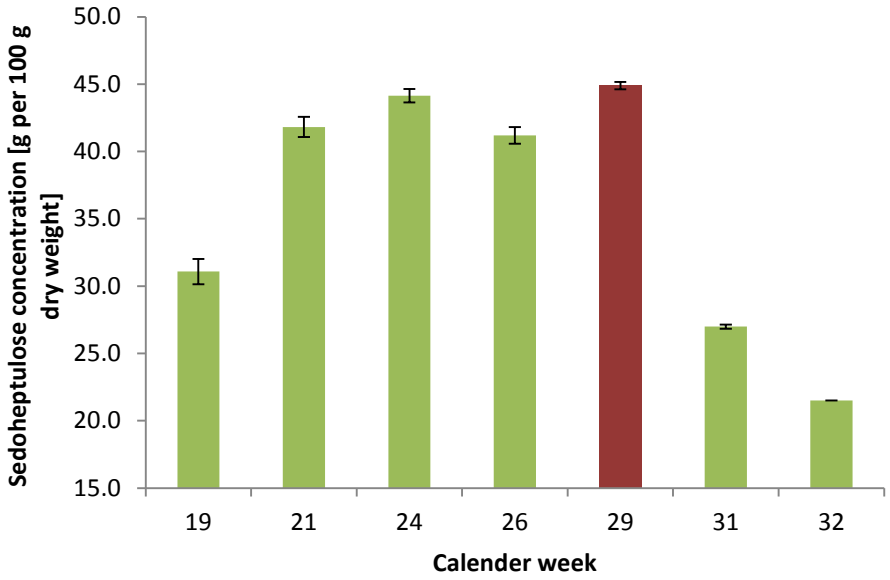


Figure 21: Variations in sedoheptulose concentration over the weeks (N=3). The data represent the mean value of the three extraction replicates with the standard deviation represented by the error bars.

3.1.3.3 Diurnal variations of the sedoheptulose content in the leaves of Sedum maximum

After determination of the influence of the growing season on the sedoheptulose concentration, the variations within one day were studied. Therefore, five samples during one day were analyzed resulting in an RSD of 11%. When comparing this value with the RSDs for measurement (1%) and procedure repeatability (3%) there would be a significant difference. However, as shown in Figure 23 there are only minor variations between 11 am and 5 pm and only in the morning the concentration is slightly lower. Therefore, the time of harvest within one day seems not to be that critical regarding the amount of sedoheptulose that can be obtained and it will not be worth the effort considering those small variations in planning the harvest time.

The external calibration curve for quantification is shown in Figure 22.

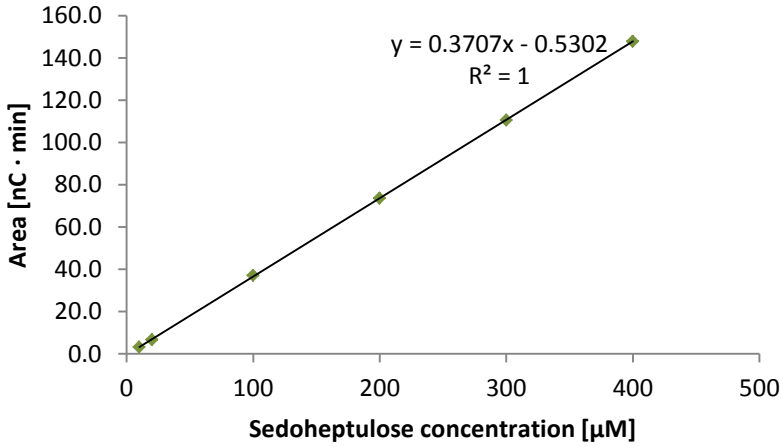


Figure 22: Calibration curve for the calculation of sedoheptulose concentrations in day samples

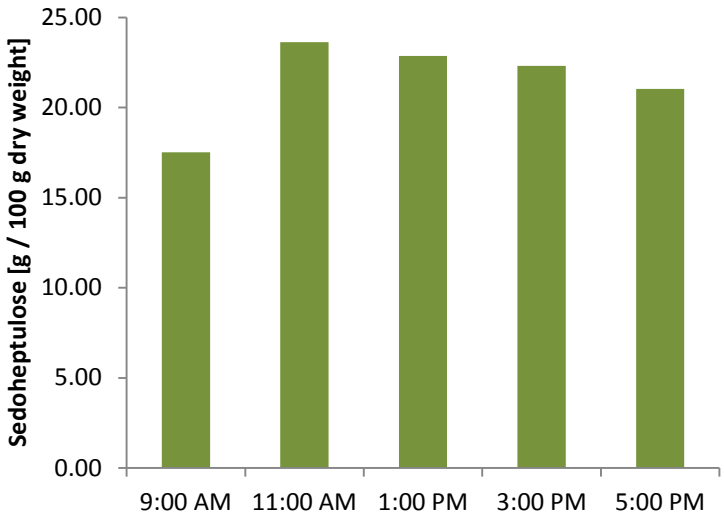


Figure 23: Diurnal variation of the sedoheptulose concentration between 9 am and 5 pm on Aug. 6th 2013, N=1

3.1.3.4 Sedoheptulose concentration in stems

In order to find out whether stems could also be used for sugar extraction, the lyophilized stems of calendar week 29 were also analyzed. Sample preparation was performed exactly as for the leaves. The only difference was that grinding did not give the same fine powder as for the leaves. Some larger solid pieces remained. This could be the reason for a higher standard deviation. The concentration was with 33.8 ± 2.3% (N=3) lower than that of the leaves of the same week with 44.1 ± 0.5%. However, there were considerable amounts of sedoheptulose in the stems. Thus, the stems could be processed together with the leaves providing a device is used for crushing that also manages breakage of the stems.

3.1.3.5 Important note on measurement stability

The HPAEC-PAD system needed a very long time for equilibration which was monitored by quality control standards. Under fully equilibrated conditions, measurement repeatability was very good with an RSD of 1% (N=5).

Besides equilibration time, eluent preparation is very critical when HPAEC routine analysis is performed. In Figure 24 two calibration curves of sedoheptulose, received from two independent measurements with different eluent batches, are shown. It can be observed that they severely differ in their slope, i.e. the sensitivity of the method.

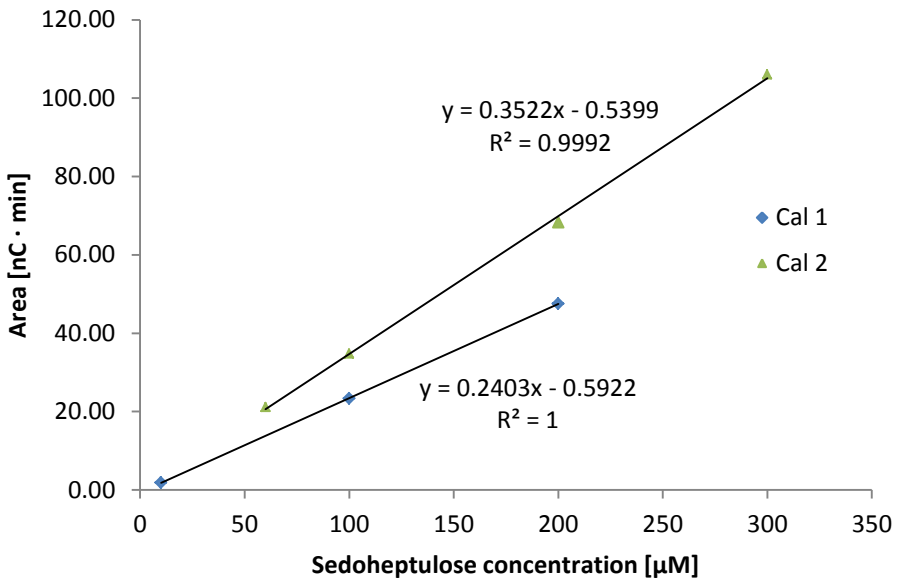


Figure 24: Calibration curves of sedoheptulose with separately prepared eluents

However, refilling of eluents cannot be avoided due to the long run time and high flow rate of 1 mL·min^{-1}. Therefore, it is inevitable to make a new calibration after refilling eluents. This effect could be due to different carbonate concentrations resulting e.g. from slightly different degassing conditions. As provided in the technical note from the manufacturer (Thermo Fisher Scientific Inc., 2014a), carbonate contamination has to be minimized and eluent preparation has to be kept the same every time. Carbonate can strongly bind to the column and thus interferes with carbohydrate binding. Therefore, it is extremely important to measure a new calibration curve after refilling eluents and samples of one series should be measured within one run if possible. Above all, eluent preparation should be kept as similar as possible.

Furthermore, retention times can differ between runs with newly prepared eluents due to different composition and slight changes in pressure. Since the retention time is the most important parameter for identification, comparison with the standard is essential.

Relief for those problems is produced by automatic eluent generation systems, where only water has to be refilled and capillary systems that reduce the flow rate from 1 mL·min^{-1} to 10 µL·min^{-1} and thus the eluent consumption by a factor of 100. Those capillary systems may also provide a valuable tool for processing larger sample sizes.

3.1.4 Search for contaminants

Via NMR measurement (see Figure 25) glucose, malic acid, isocitric acid, γ-aminobutyric acid (GABA) and alanine besides other minor components could be identified in the crude extracts of the leaves. ^{1}H NMR chemical shifts were compared with literature data (Allouche et al., 2007; Verpoorte et al., 2007) paying attention to slight changes due to different pH conditions. The sample was of pH 4.5 whereas literature data are given for pH 6 or 7.

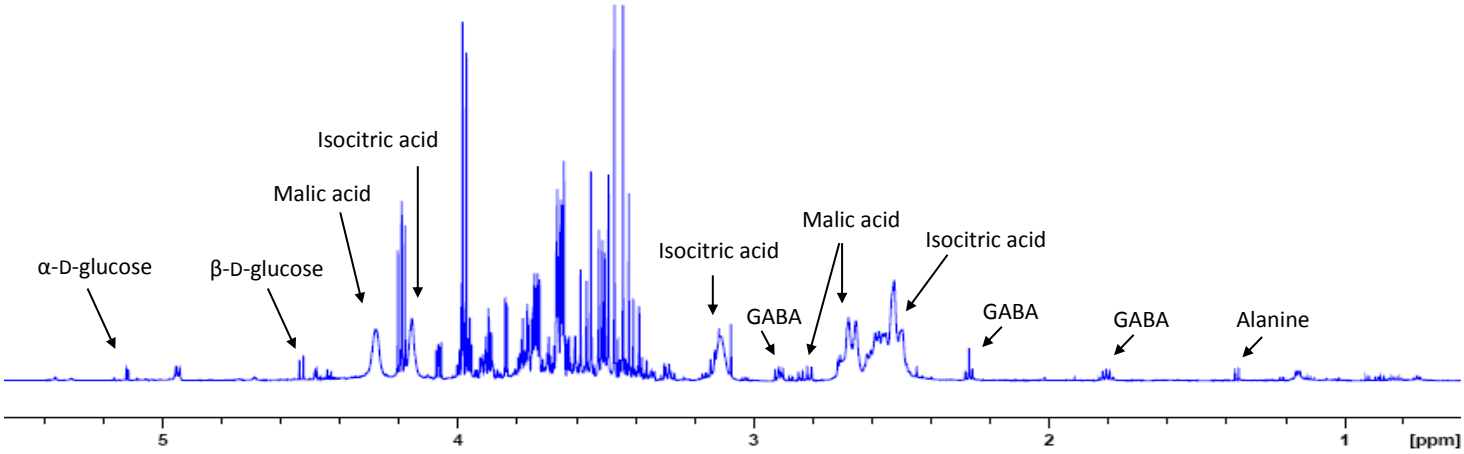


Figure 25: ^{1}H NMR spectrum of the primary extract of leaves (extract B) with the main impurities labeled

^{31}P NMR data of the extract B showed that phosphate levels were very low (data not shown).

Furthermore, the identified compounds were confirmed by TOF-MS measurements. The observed *m/z* ratios are listed in Table 11.

Table 11: Observed m/z (± 0.005) of the contaminants by TOF-MS

Substance	Observed *m/z*	
	$[M-H]^-$	$[M+H]^+$
Glucose	179.0561	
Malic acid	133.0142	
Isocitric acid	191.0197	
GABA		104.0706
Alanine		90.0550

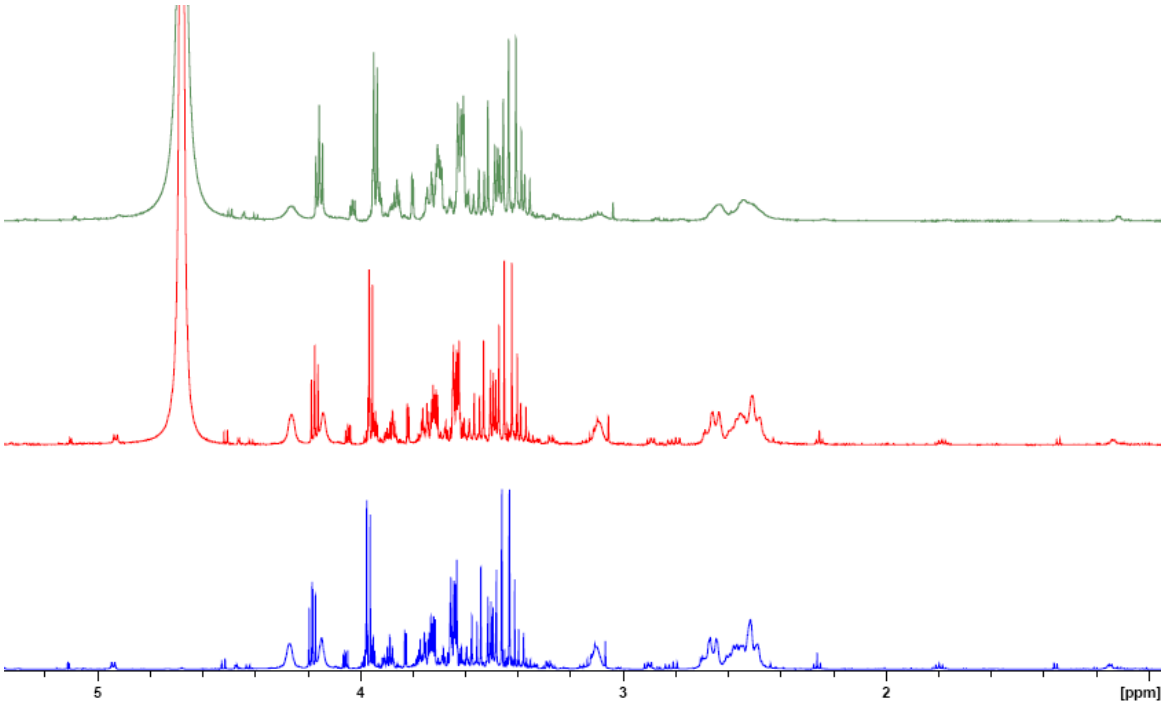


Figure 26: Comparison of ^{1}H NMR spectra of the final crude concentrates.
Extract A (green), extract B stored (red), extract B (blue)

As shown in Figure 26, the two extracts ("B" and "B stored"), which originated from the same growing region, were almost of the same composition. This proved that storage at -30 °C for over two months did not have any influence on the sedoheptulose or any other component. The extract A, which was prepared from the leaves of differently cultivated plants, contained a much higher relative amount of sedoheptulose, hence growing conditions were obviously influencing the composition. Although the plants for extract B were harvested about three weeks later than for extract A, the differences will most probably be rather due to growing conditions than due to harvest time since the sedoheptulose content did not vary that significantly within three weeks (see Figure 21).

Composition of the stems

In order to make sure that the composition of the extract derived from stems is not completely different to that from the leaves, a proton NMR was measured. Figure 27 shows the comparison of the extract from lyophilized stems, the extract B (from leaves) and the commercially available sedoheptulose revealing an impressive purity of the extract from stems. It has to be noted that this sample was an extract of stems only from calendar week 29.

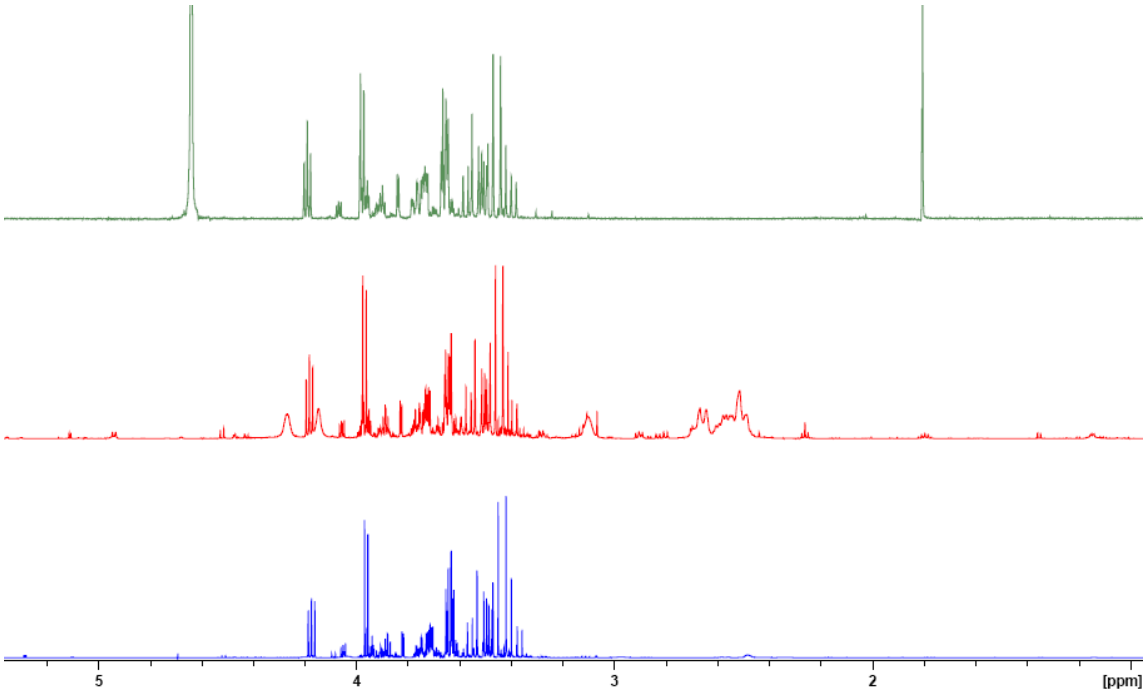


Figure 27: ^{1}H NMR spectra of sedoheptulose from Sigma-Aldrich (green) compared with the extract B from leaves (red) and the extract from lyophilized stems (blue).

3.2 Results of the purification in lab-scale

The purification of sedoheptulose by acetylation, as performed in the framework of this thesis, was suitable for purification in lab scale and provided enough sedoheptulose for a couple of cell-culture experiments. However, up-scaling is definitely not possible since the application to a 700 mL batch already involved enormous volumes of solvents.

3.2.1 Anion exchange and protein precipitation

The suitability of the different anion exchange resins was tested with small amounts in beakers resulting in Dowex AG1x8 to be the ideal one (see Figure 28) for the nearly complete removal of organic acids.

When 300 and 400 mL of the concentrated leaf extract B were applied to the resin, enormous formation of gas was observed. Since the anion exchange resin was present in HCO_3^- form and there was quite a high amount of organic acids present in the extract, the formation of CO_2 was expected though not in that dimension. The resin bed completely broke and horizontal layers of gas disrupted and slowed down the flow.

Nevertheless, nearly complete decolorization was achieved because the colored components got stuck to the resin.

Furthermore, partial removal of proteins occurred in the first batch due to precipitation on the column whereas for the second batch no precipitation was observed.

The most crucial point when using an anion exchange resin was the change in pH since sugars are not stable at high pH values and undergo alkaline degradation. Additionally, sugars get negatively charged at high pH values (> 12) and could therefore also bind to the resin.

Protein precipitation with EtOH worked well and after centrifugation, the liquid could be easily decanted from a colorless precipitate. The sedoheptulose containing solution was then concentrated *in vacuo* to give a yellow to ocher concentrate without any problems.

For further purifications, protein precipitation prior to the anion exchange resin could be tested.

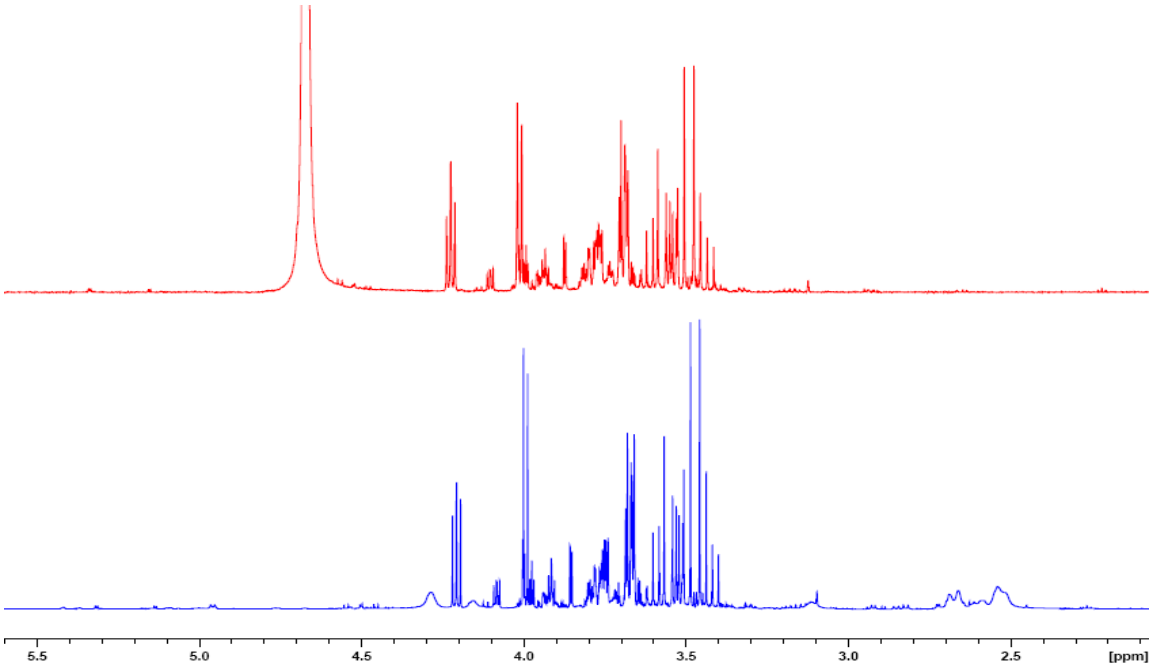


Figure 28: Comparison of the ^{1}H NMR spectra before (blue) and after (red) Dowex AG1x8 anion exchange resin

3.2.2 Acetylation and column chromatography

Dissolving the dry sample in dry pyridine was very slow and only progressed when acetic anhydride was added and acetylation proceeded. Besides that, it worked without any problems and the reaction was completed after stirring overnight at room temperature. For a small amount the reaction was continued but no further conversion into product could be detected *via* TLC with toluene:ethyl acetate (3:2).

Acetylation resulted in four different sedoheptulose acetate isomers, namely α-furanose, β-furanose, α-pyranose and β-pyranose. By using the column eluted with diethyl ether it was possible to separate two pairs of them as can be seen in the proton NMR spectra in Figure 29. The sum of the lower two spectra corresponds to the material that had been loaded on the column (green). Unfortunately, the two separated fractions still consist of two isomers (one furanose and one pyranose) in a ratio of about 2:1. Thus, crystallization was not possible although acetates are normally easy to crystallize.

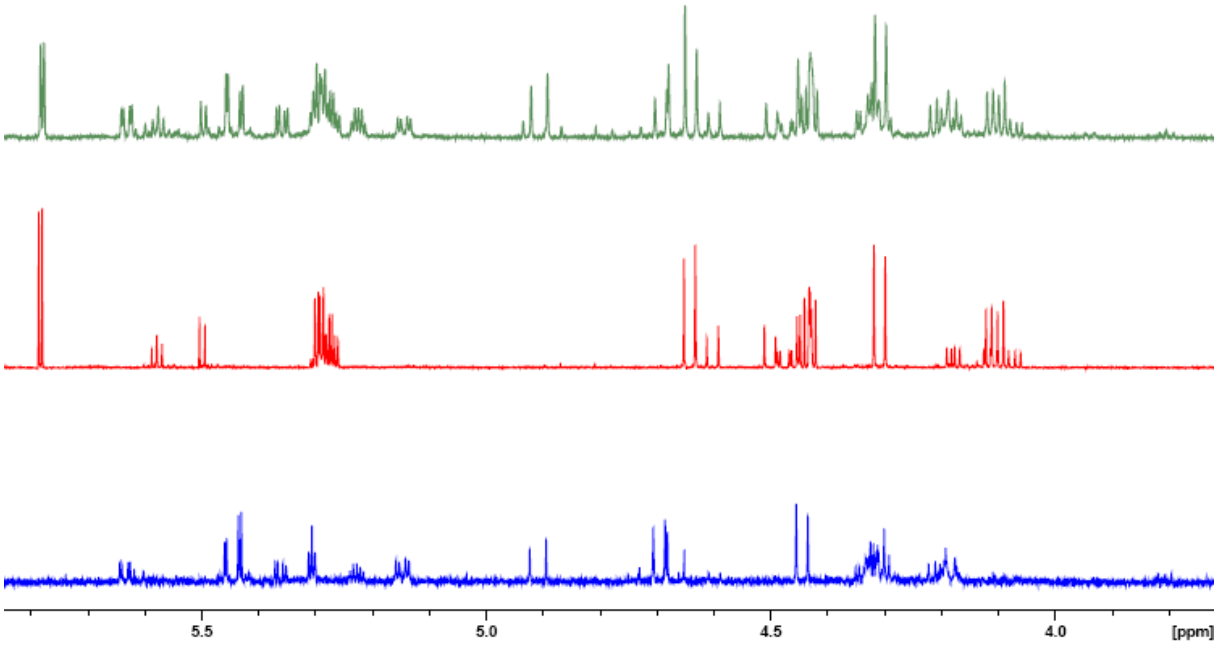


Figure 29: ^{1}H NMR spectra of per-O-acetylated sedoheptulose before (green) and after separation with diethyl ether, first fraction EtherF1 (red), later fraction EtherF2 (blue)

The purification of about 10 g of the per-O-acetylated sedoheptulose by column chromatography was costly in time and material. The pooling of fractions was always a compromise between separation from glucose and yield of sedoheptulose since complete separation was not possible. That was also the reason why several columns were necessary. Combined with high amounts of sample, several kilograms of silica gel and several dozen liters of solvents were necessary. Besides of that, the MPLC system was working really fine and one column separation could be done in about two hours (excluding the time needed for evaporation of the solvent).

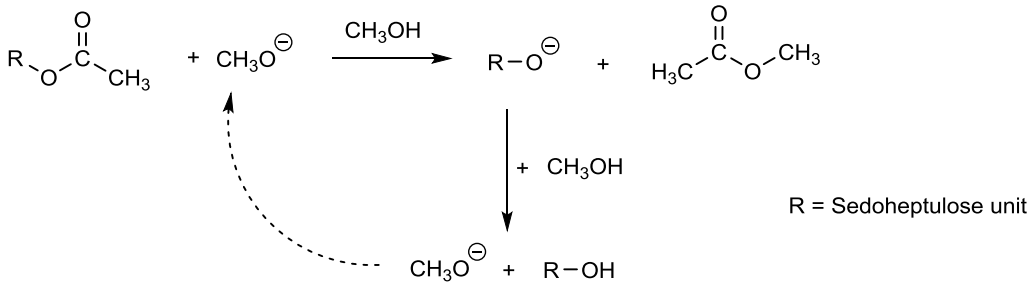
Unfortunately, the most intensely yellow-colored fractions were the ones containing the highest content of sedoheptulose. Therefore, a complete decolorization was not possible at that stage though detection of the fractions with high sedoheptulose content was simplified.

The TLCs with the different fractions and how they were combined were already presented in chapter 2.5.4. Since column chromatography was done several times, many different batches had to be pooled and concentrated *in vacuo* afterwards.

Pooling of the fractions with lower mobility led to a purer product batch than pooling of the first fractions since the glucose acetate elutes slightly before the sedoheptulose acetate. The first fractions are thus more likely to contain some remaining glucose.

3.2.3 Zemplén de-O-acetylation

During the cleavage of the acetate groups (see Scheme 3) the pH was a critical factor. After adding 0.1 M sodium methoxide solution until a pH of 8 to 9 was reached, the pH decreased consistently especially in the beginning of the reaction indicating the release of acetic acid. Therefore, it was important to keep the pH between 8 and 9 and add some more sodium methoxide in case that the pH was too low. Furthermore, it was observed that when the sample was dissolved in dry MeOH, it was only slightly yellow. However, after adding sodium methoxide and pH increase to about 9, the liquid became darker resulting in a brown solution. Perhaps there has been a chromophoric substance present at very low concentrations that is only colored at high pH values but, curiously, the color did not disappear after neutralization. The reason has not been found yet. Furthermore, prolonged reaction times (12 h) also led to an increase of contaminants as minor additional spots on the TLC plate indicated. Apart from that, attention has to be paid in order to prevent formation of sodium acetate. In the final batches different amounts of residual acetate have been found which could be removed by Bio-Gel P2 (fine polyacrylamide beads) *via* size exclusion chromatography in future applications.



Scheme 3: General scheme of the catalytic Zemplén deacetylation

3.2.4 Results of the additional purification steps

3.2.4.1 Repeated extraction of sedoheptulose acetate with sat. aq. $NaHCO_3$ and dichloromethane

Batch2_2_2_2 resulting from the repeated aqueous extraction was the purest one besides the one from the column with diethyl ether. This step was found to be the best applicable one in order to improve the purity without being as critical as the anion exchange resin treatment and thus could be used in further purifications.

3.2.4.2 Chloroform extraction for removal of color and possible organic compounds

When Batch Sedo_m_3_2 was dissolved in water for inj., a brown precipitate occurred but dissolved immediately when chloroform was added. The chloroform phase was yellow colored with a decrease in color intensity after five repeated extractions. Nevertheless, the aqueous phase remained brown and proton NMR spectra showed only little improvements in purity (see Figure 30: Sedo_m_3_2_2).

3.2.4.3 Anion exchange with Dowex AG1x8 for removal of remaining acids

Although it had worked quite fine in the pre-test and using a small amount of resin did not influence the pH but resulted in the nearly complete decolorizing and a slight decrease of acids in the region around 2.1 ppm, the application to the whole batch Sedo_m_3_1 appeared to be problematic. The pH increased to 9 after passing the resin and the eluate was not fully decolorized. Since sugars are not stable at high pH values, the solution had to be neutralized subsequently with an acidic ion exchange resin. Apparently, this step was also a bit tricky because the pH increased again after some time. Therefore, much attention had to be paid to this step and it was not applied to the second batch Sedo_m_3_2. Nevertheless, this step led in part to a decolorization and removal of contaminants, most likely acids (see Figure 30). In addition, the signal at 1.8 ppm could be extremely decreased by this step also when compared to the commercially available sedoheptulose from Sigma-Aldrich.

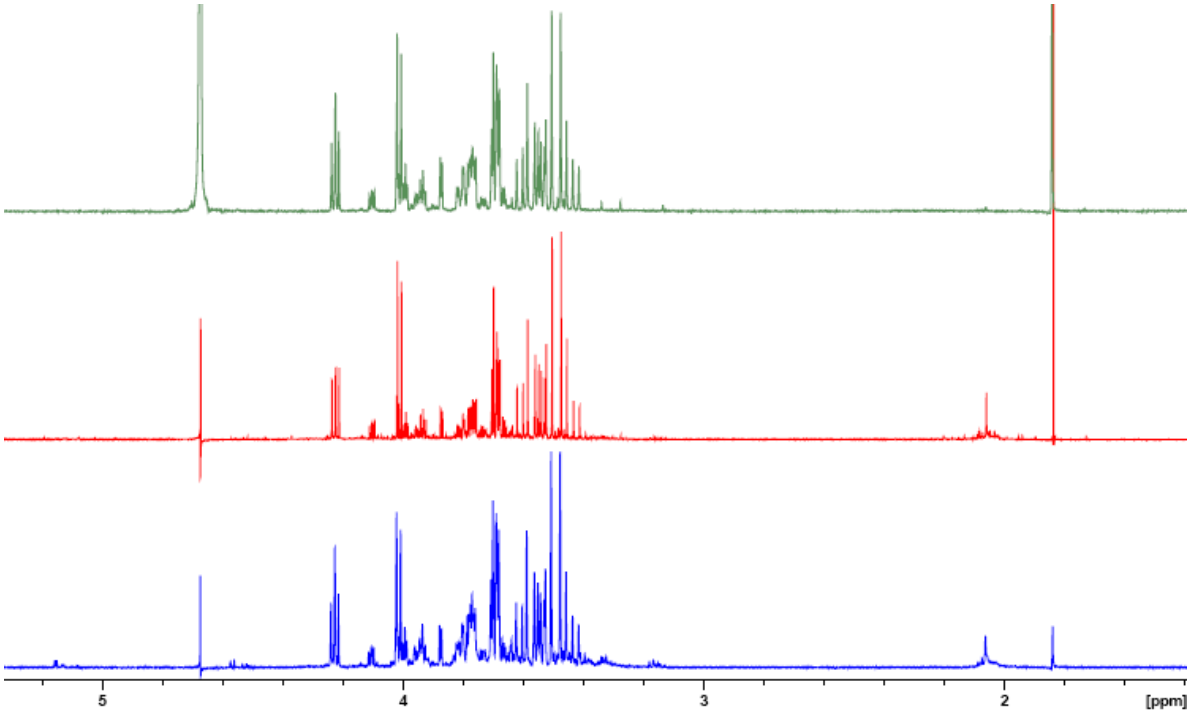


Figure 30: Comparison of ^{1}H NMR spectra of the sedoheptulose from Sigma-Aldrich (green) with Sedo_m_3_2_2 (red) and Sedo_m_3_1_IAT3 (blue)

3.2.4.4 Lyophilizing for removal of methanol from Zemplén reaction

Lyophilizing of these highly concentrated sugar solutions was a time consuming process that required constant cooling with liquid nitrogen or dry ice in order to keep the sample frozen. For the removal of about 50 mL water several days (2-5) were necessary. Methanol was successfully removed by this step, which was quite important since the final sedoheptulose preparation will be used for cell cultures and thus should be free of any toxic substances.

After lyophilizing or foaming on high vacuum, the sedoheptulose was of a dry crumbly consistence and could be easily powdered. Unfortunately, when the flask was opened for a few minutes, the dry powder structure collapsed due to the hygroscopic structure and a thick syrup was formed. It was really sticky and hence quite difficult to handle since it stuck to every surface it came into contact with. Furthermore, weighing was not absolutely exact because it was time dependent.

3.2.4.5 Crystallization attempts

As already described before, proton NMR spectra revealed that even though two spots from the TLC of the sedoheptulose acetates could be separated on the column with diethyl ether (Figure 12), there were still two different isomers in the separated fractions present at ratios of about 1:2 (Figure 29). Thus, crystallization was not possible. The free sedoheptulose is present in three different isomers as described in chapter 1. Thus, it was neither possible to crystallize the per-O-acetylated sedoheptulose nor the free sugar. As reported before, crystallization of free sedoheptulose has not been successful (Webber, 1963). However, crystalline sedoheptulose hexaacetate was produced by Richtmyer and Pratt *via* acetolysis with acetic anhydride and perchloric acid as catalyst of the tetraacetate of sedoheptulosan (Richtmyer and Pratt, 1956).

3.2.5 Rough mass balance of the purification

The concentrated leaf extract B used for the purification in lab-scale had about 18 °Brix. °Brix is the unit of the relative density of a solution and is most commonly used for fruit juices. 1 °Brix refers to a 1% sucrose solution (1 g sucrose in 100 g solution). However, this value is used for an estimation of the total sugar concentration and sucrose is only used as reference substance. Therefore, the concentrate should contain about 18% sugars. If it was only sedoheptulose, this would lead to 126 g sedoheptulose in 700 mL of the concentrated leaf extract (300 mL Batch 1 + 400 mL Batch 2) that were purified. Measurement of the sedoheptulose concentration of the concentrated leaf extract *via* LC-MS and HPAEC revealed a concentration of about 460 mM, hence about 96 $g \cdot L^{-1}$. After the anion exchange and protein precipitation 28 g (11.3 + 16.7 g) dry matter were received. Instead of collecting the fractions after anion exchange in a quantitative way, only the most highly concentrated fractions were used explaining this loss in weight. After acetylation (molar mass of sugars approximately doubles), extraction with sat. aq. $NaHCO_3$ and the first silica gel column 13 g (5 + 8 g) mixed fractions, about 4 g (2 + 2 g) late fractions and 15 g (6 + 9 g) main fractions were received. Hence, 32 g acetylated substances were received in total. Loss of mass was mainly due to removal of water soluble components in the extraction. All further purification steps were only performed with the main fractions whereupon the mass was reduced to about 12 g while purity was increased. Deacetylation finally yielded slightly more than 5 g sedoheptulose in total. The mixed and late fractions are still available in their acetylated form.

3.2.6 Final batches

20.6 mg of Sedo_m_3_2_2 and 24.7 mg of Sedo_m_3_1_IAT3 were put in a small vial as back-up samples and have been stored at BOKU. The remaining 2.54 g of Sedo_m_3_2_2 and 2.55 g of Sedo_m_3_1_IAT3 were handed over to the group of Dr. Haschemi of the Clinical Institute of Medical and Chemical Laboratory Diagnosis, Medical University of Vienna.

Food grade status could not be kept over the whole process since the anion exchange resin was not of food grade status and large centrifuges from the Institute of Biochemistry had to be used.

Furthermore, the 115 mg of Sedo_EtherMF were divided in half. The one half was also given to Medical University of Vienna, the other half was used for final NMR measurements and determination of the optical rotation.

The mixed fractions (~ 5 + 8 g) containing some more glucose are still present in their acetylated form and can be purified by further column chromatography if needed.

3.2.7 Optical rotation

The sedoheptulose solution with a concentration of 9.5 mg·mL^{-1} (0.95 g per 100 mL) was measured over a period of about 60 min. The values for the optical rotation were changing constantly between 0.124 and 0.162. The mean value of 30 measurements over 10 min was used for calculation.

The specific optical rotation is calculated as follows:

$$[\alpha]_D^{20} = \frac{100 \cdot \alpha}{l \cdot c}$$

c … concentration [g·(100 mL)$^{-1}$]

l … length of cuvette [dm]

α … measured rotation

$$[\alpha]_D^{20} = \frac{100 \cdot 0.139}{1 \cdot 0.95} \pm \frac{100 \cdot 0.011}{1 \cdot 0.95} = +14.6 \pm 1.1\,^\circ$$

Richtmyer and Pratt measured the optical rotation of sedoheptulose received from deacetylation of its hexaacetate and found $[\alpha]_D^{20}$ at + 8.2 ° (Richtmyer and Pratt, 1956).

3.3 Results from the pre-test concerning extraction of larger amounts of leaves

The tested procedure with crushing the leaves, mixing with water and then cooking for 30 min resulted in a turbid extract with a fine sediment that was not possible to separate with e.g. filter clothes. The use of a plate filter could be possible but has not been tested yet. Only a small amount was used after centrifugation for the testing of the different anion exchange resins. However, the rest of the boiled extract was not further used and was stored for future experiments.

Nevertheless, as can be seen in Figure 31, the composition of the boiled extract was the same as of the concentrated leaf extract A (same growing conditions) that resulted from an extraction procedure without boiling.

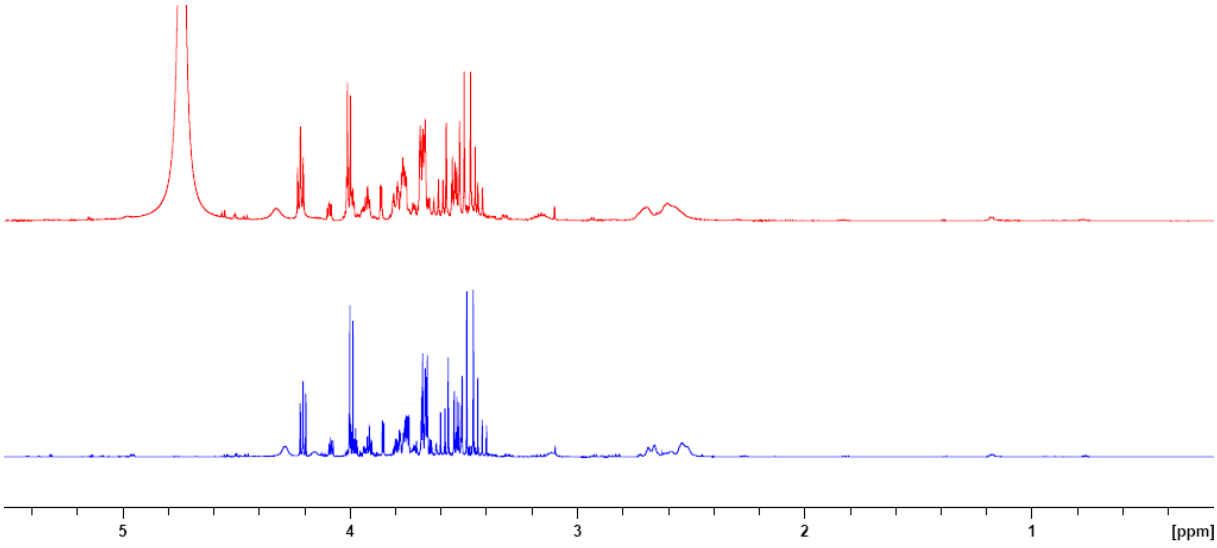


Figure 31: Comparison of the ^{1}H NMR spectra of the boiled extract (blue) and extract A (red)

4 Conclusion

Since the project of isolating sedoheptulose from leaves of *Sedum maximum* only started and thus no data were available, I had to establish almost everything from the beginning. The following points summarize the most important discoveries:

- The dry weight of the leaves was about 7% of the fresh weight. This dry matter consisted of about 40% sedoheptulose. Stems showed a dry matter of about 17% that comprised about 34% sedoheptulose. Therefore, extraction of the stems could be implemented as novel sedoheptulose source.
- A sample preparation procedure for the analysis of the sedoheptulose content in the plants was established. However, formation of gel has to be avoided in sample preparation by rapid reprocessing.
- Anion chromatography with pulsed amperometric detection provided a powerful tool for the accurate quantification of sedoheptulose.
- There was a significant change in sedoheptulose concentrations in the leaves of *Sedum maximum* over the growing season reaching its peak in the mid of June whereas the content during a day did not vary that pronounced.
- The main constituents besides sedoheptulose were: malic and isocitric acid, glucose, GABA, and alanine. Their concentrations were varying depending on growing conditions.
- Application of anion exchange resins for removal of organic acids had to be performed carefully and constant monitoring of pH was necessary since sugars are sensitive to alkaline conditions. Furthermore, due to the high acid content an enormous volume of CO_2 was formed when the resin was used in HCO_3^- form.
- Per-O-acetylation followed by silica gel column chromatography provided a useful though time-consuming tool for the purification in lab scale. Nevertheless, deacetylation was critical regarding the formation of sodium acetate.
- Although boiling had no influence on the composition of the extract, it involved a difficult separation of solid matter. Therefore, extraction procedures without boiling would be beneficial for future purposes.

Certainly, there would have been numerous other aspects to be investigated. Quite evidently (and for me unfortunately), a single master thesis cannot cover all these aspects in detail and so the following points remain for future and ongoing investigations.

- Measurement of sedoheptulose with HPAEC-PAD takes 70 min per run at the moment. Despite the long time necessary for equilibration, the run time might be reduced by application of a sodium acetate gradient. Furthermore, the usage of capillary systems could be useful for processing larger sample sizes.
- Exact measurement of protein concentration still has to be performed and then also an application for the removed protein could be established.
- The purification of larger amounts of plant extract might be performed *via* simulated moving bed (SMB) chromatography. In order to enable subsequent studies *in vivo*, the final processes for large scale isolation and purification of sedoheptulose has to be of food grade.
- Further tests concerning the crystallization could be carried out. In the ideal case, a solvent mixture, where sedoheptulose is mainly present in one form, can be found.
- The development of ^{13}C and ^{14}C labeled sedoheptulose as internal standard as well as for flux analysis would be beneficial.

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