

**Studies of Oligo- and Polysaccharides  
by MALDI-TOF and ESI-ITMS<sup>n</sup>  
Mass Spectrometry.**

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## Abstract

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This thesis presents methods for analyzing carbohydrates with matrix-assisted laser desorption/ionization time-of-flight, MALDI-TOF, and electrospray ionization ion trap multiple-stage, ESI-IT MS<sup>n</sup>, mass spectrometry, MS.

MALDI-TOF MS was employed to determine chain length distributions of amylopectin, the main constituent of starch. The technique was compared with high-performance anion-exchange chromatography with pulsed amperometric detection, HPAEC-PAD, an established technique for this purpose. Starch from potato, wheat, and waxy maize was debranched with isoamylase and analyzed using both techniques. Similar differences between chain length distributions for amylopectin from different sources were observed with both methods, but MALDI-TOF MS was less reproducible than HPAEC-PAD and overestimated the amount of longer chains. However, MALDI-TOF MS analysis is faster, more sensitive, and provides detailed information on the mass of the unit chains.

Maltoheptaose and three human milk oligosaccharides were derivatized by reductive amination followed by N,N-dimethylation. The carbohydrate derivatives were investigated by MALDI-TOF MS and MALDI post-source decay (PSD) TOF MS. The resulting derivatives have a positive charge localized to the modified reducing end. The approximate detection limit for the resulting maltoheptaose derivative was 50 fmol corresponding to a tenfold increase in sensitivity compared to underivatized oligosaccharides. When the derivatives were analyzed by MALDI-PSD TOF MS the observed fragmentation pattern was dominated by fragment ions retaining the modified reducing terminus, thus simplifying the interpretation of the MS-data.

Saponins from *Quillaja saponaria* Molina with previously identified structures were investigated by ESI-IT MS<sup>n</sup> in both positive and negative ion modes. MS<sup>1</sup>–MS<sup>4</sup> spectra were analyzed showing that structural information can be obtained on both oligosaccharide parts in the saponins from positive ion mode spectra whereas negative ion mode spectra mainly provided information on one of the oligosaccharide parts. Analysis of MS<sup>1</sup>–MS<sup>4</sup> spectra identified useful key fragment ions important for structural elucidation of *Quillaja* saponins. A flowchart involving a stepwise procedure based on the key fragments from MS<sup>1</sup>–MS<sup>3</sup> spectra was constructed for identification of structural elements in the saponin.

**Keywords:** quantification, oligosaccharide sequencing, structure determination

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*I alla fulla fall...*

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## Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals.

- I. Broberg, S., Koch, K., Andersson, R. & Kenne, L. 2000. A comparison between MALDI-TOF mass spectrometry and HPAEC-PAD analysis of debranched starch. *Carbohydrate Polymers* 43, 285-289.
- II. Broberg, S., Broberg, A. & Duus, J.Ø. 2000. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on oligosaccharides derivatized by reductive amination and N,N-dimethylation. *Rapid Communications in Mass Spectrometry* 14, 1801-1805.
- III. Broberg, S., Nord, L.I. & Kenne, L. Oligosaccharide sequences in *Quillaja* saponins by electrospray ionization ion trap multiple-stage mass spectrometry. *Journal of Mass Spectrometry*, in press.
- IV. Broberg, S., Nord, L.I. & Kenne, L. Structure analysis of *Quillaja* saponins by electrospray ionization ion trap multiple-stage mass spectrometry, *manuscript*.

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## Abbreviations

Api	Apiose
Ara	Arabinose
Da	Dalton
DHB	2,5-Dihydroxybenzoic acid
DMBA	N,N-Dimethylbenzylamine
DP	Degree of polymerization
ESI	Electrospray ionization
FAB	Fast atom bombardment
FTICR	Fourier transform ion cyclotron resonance
Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcA	Glucuronic acid
GlcNAc	N-acetyl glucosamine
HPAEC	High-performance anion-exchange chromatography
IT	Ion trap
<i>M</i>	Molecular mass
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS <sup>n</sup>	Multiple-stage mass spectrometry
<i>m/z</i>	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detection
PD	Plasma desorption
PSD	Post-source decay
Rha	Rhamnose
TFA	Trifluoroacetic acid
THAP	2,4,6-Trihydroxyacetophenone
TOF	Time-of-flight
UV	Ultra violet
Xyl	Xylose

# Sammanfattning

## Summary in Swedish

Kolhydrater finns i en mängd former i levande organismer. De grupperas ofta enligt storlek som monosackarider, oligosackarider eller polysackarider. Ordet sackarid kommer från latin (saccharum, socker) som hänförs till den söta smaken hos några av de enkla kolhydraterna. Monosackarider är kolhydrater som inte kan spjälkas upp genom hydrolys till enklare föreningar. Oligosackarider (från grekiskans oligos, några) består av minst två och sällan fler än tio sammanbundna monosackaridenheter. De finns i levande organismer som fria ämnen och även bundna till proteiner och andra organiska föreningar. Polysackarider består av många monosackaridenheter—ibland upp till miljoner. Ofta, men inte alltid, är enheterna identiska. Två av de viktigaste polysackariderna, stärkelse och cellulosa, innehåller sammanbundna enheter av samma monosackarid, glukos. Intresset för att minska användandet av olja som råmaterial för en mängd produkter har ökat intresset för förnyelsebara råvaror. Möjliga användningsområden för polymera kolhydrater har breddats då derivatisering (förändring) kan ge nya material med intressanta egenskaper. Dessa möjligheter har ökat intresset för analysmetoder av polymera kolhydrater. Ett annat ämne där kolhydratanalys har blivit viktigt är i studier av många biologiska processer som t ex enzymatisk aktivitet och cell-cell-interaktioner. Masspektrometri är en av flera metoder för att analysera kolhydrater och glykokonjugat (ämnen som innehåller kolhydrater).

Den här avhandlingen presenterar metoder för att analysera kolhydrater med ”matris-assisterad laser-desorptions/ionisations-tid-för-flygning”- (MALDI-TOF) och elektrospay-”ionisations-jonfälla-fler-stegs”- (ESI-IT MS<sup>n</sup>) masspektrometri (MS).

MALDI-TOF MS användes för att bestämma kedjelängdsfördelningen hos amylopektin, en av stärkelsens främsta beståndsdelar. Tekniken jämfördes med ”högprestanda-anjonbytar-kromatografi med pulsad amperometrisk detektion” (HPAEC-PAD), en teknik som används ofta i detta syfte. Stärkelse från potatis, vete och majs med 97% amylopektin avgränsades med isoamylas och analyserades med bägge teknikerna. Skillnaderna mellan kedjelängdsfördelningen hos amylopektin hos de olika källorna var liknande för båda teknikerna, men MALDI-TOF MS hade sämre reproducerbarhet än HPAEC-PAD och överskattade mängden långa kedjor. MALDI-TOF MS är dock en snabbare och känsligare teknik, samt ger detaljerad information om kedjornas massor.

Maltoheptaos och tre mjölkoligosackarider från människa derivatiserades med reduktiv aminering följt av N,N-dimetylering. Kolhydratderivaten undersöktes med MALDI-TOF MS och MALDI post-source decay (PSD) TOF MS. De resulterande derivaten har en positiv laddning som är lokaliserad vid den modifierade reducerande änden. Den ungefärliga detektionsgränsen för maltoheptaosderivatet var 50 femtomol vilket motsvarar en tiofaldig ökning av känsligheten jämfört med oderivatiserade oligosackarider. När derivaten analyserades med MALDI-PSD TOF MS dominerades fragmenteringsmönstren av fragmentjoner med den modifierade reducerande änden, vilket underlättar tolkningen av MS-data.

Saponiner från *Quillaja saponaria* Molina, vars strukturer har utretts tidigare, undersöktes med ESI-IT MS<sup>n</sup> på både positiva och negativa joner. Analyserade MS<sup>1</sup>–MS<sup>4</sup> spektrum visade att information om strukturen på saponinernas bägge oligosackarider kan fås från positiva joner, medan negativa joner endast ger information om en av oligosackariderna. Vid analys av MS<sup>1</sup>–MS<sup>4</sup> spektrum kunde nyckelfragment som är viktiga för strukturutredningen av *Quillaja*-saponiner identifieras. För att identifiera strukturelementen i saponinen konstruerades ett flödesschema. Detta flödesschema innehåller en stegvis procedur som är baserad på nyckelfragmenten från MS<sup>1</sup>–MS<sup>3</sup> spektrum.



## Introduction

Carbohydrates exist in a variety of shapes in living organisms. They are usually classified as monosaccharides, oligosaccharides or polysaccharides. The term saccharide comes from Latin (saccharum, sugar) and refers to the sweet taste of some simple carbohydrates. Monosaccharides are carbohydrates that cannot be hydrolyzed to simpler compounds. Oligosaccharides (from the Greek oligos, few) contain at least two and generally no more than ten monosaccharide units linked by glycosidic linkages. They exist in living systems as free compounds as well as bound to proteins and other organic compounds. Polysaccharides contain many monosaccharide units—sometimes up to millions (Pérez & Imberty, 1996). Often, but not always, the units are identical. Two of the most important polysaccharides, starch and cellulose, contain linked units of the same monosaccharide, glucose.

The call for decreasing the use of oil as raw material for a diversity of compounds *e.g.* packages and fabrics, has expanded the interest in renewable resources. Possible usage of polymeric carbohydrates is broadened as derivatization can yield novel materials with new, interesting properties (Röper, 1996). These possibilities have increased the interest in methods for analyzing carbohydrate polymers. Another area where carbohydrate analysis has become increasingly important is in studies of many biological processes, such as enzymatic activity and cell-cell interactions (Ernst *et al.*, 2000). Mass spectrometry is one of the techniques that are employed for analysis of carbohydrates and glycoconjugates.

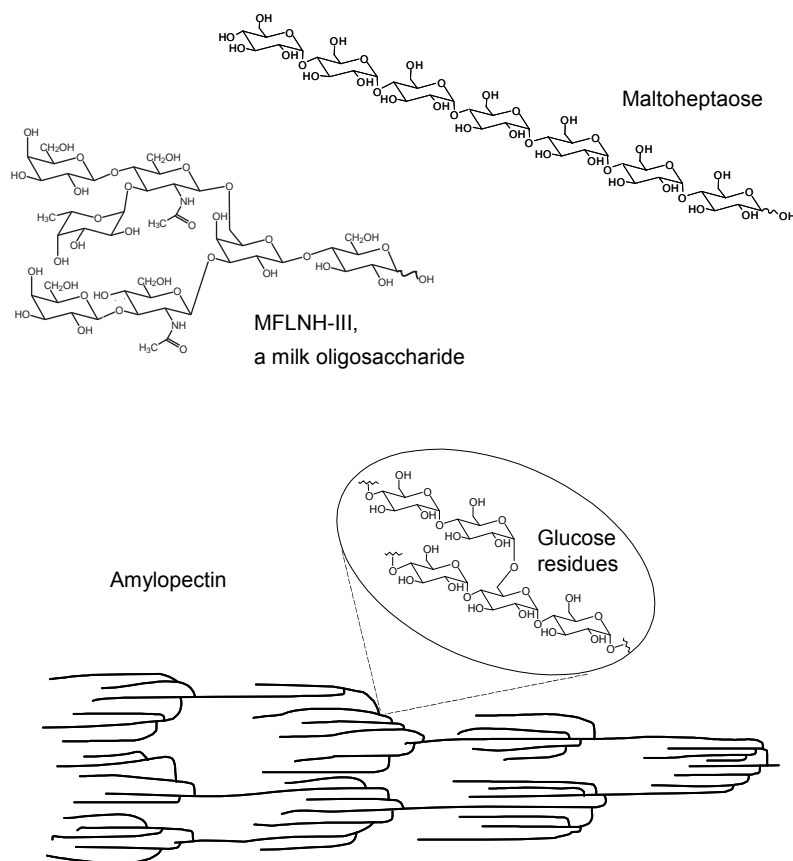
## Aims of the thesis

The goal of this thesis was to develop methods for analysis of carbohydrates or glycoconjugates by mass spectrometric techniques.

In paper I the possibility for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) quantification of debranched amylopectins from different starches was evaluated in comparison with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis, which is an established method for this purpose. In paper II oligosaccharides were derivatized in a novel fashion in order to increase the sensitivity for MALDI-TOF investigations and also to simplify the interpretation of recorded MS/MS data. In paper III and IV previously identified saponins from *Quillaja saponaria* Molina were studied with electrospray ionization ion trap multiple stage MS (ESI-IT MS<sup>n</sup>) in order to obtain a procedure for rapid structure analysis of the two oligosaccharide parts of the *Quillaja* saponins.

# Carbohydrates

Carbohydrates constitute a multifaceted group of compounds (Scheme 1) with molecular masses up to millions of Da for amylopectins in starch. The possibility of substitution at different positions in the monomeric units makes the primary structure of linear carbohydrates more complex than polymers built up of amino acids, *i.e.* proteins, that only have the well-defined amide bond forming their linear structure. Another complexity within the linear structures rises from the possibility of  $\alpha$ - or  $\beta$ -configuration at the anomeric carbon of the monomeric units. For example,  $\alpha$ -amylose in starch consists of  $\alpha(1\rightarrow4)$ -linked glucose residues while cellulose in wood contains  $\beta(1\rightarrow4)$ -linked glucose residues. Their different stereochemistry of the glycosidic linkage gives  $\alpha$ -amylose and cellulose totally different properties. The monomeric units can also be in the shape of five- or six-membered rings and have D- or L-configuration. In addition the carbohydrates can be branched. A monomeric unit can be linked to more than two other units, giving numerous possibilities for positional isomerism. The complexity of carbohydrates makes the structure analysis complicated.



Scheme 1

Depending on the type of carbohydrate, the analytical procedure differs. Carbohydrates are typically analyzed with chromatographic, spectroscopic, and spectrometric methods such as gel filtration, HPAEC-PAD, gas chromatography (GC), nuclear magnetic resonance (NMR) and different MS techniques with or without prior derivatization. Polysaccharides are often depolymerized into smaller parts, oligo- or monosaccharides, prior to analysis. Depolymerization can be done with for example acidic hydrolysis or enzymatic cleavage. Monosaccharides are typically analyzed with GC after suitable derivatization. Oligosaccharides are often analyzed with NMR and MS in parallel with GC analysis of the derivatized monosaccharides obtained by degradation. The GC analysis gives information on which components are present in the oligosaccharide while NMR is utilized for linkage, sequence and conformation analysis. Mass spectrometry gives precise mass information of the analyte and its fragments, which subsequently can give sequence and to some extent linkage information along with knowledge on whether the monosaccharides are *e.g.* pentoses or hexoses. MS is a faster and more sensitive technique than NMR.

## Analytical methods

### Mass spectrometry

Mass spectrometry is the analysis of ions based on their mass-to-charge ratio ( $m/z$ ). The first instrument, a mass spectrograph, was built by Thomson (Thomson, 1913). The instrumental set-up varies but always includes an *ion source* that converts the analyte molecules into ions and a *mass analyzer* for analysis of the ions. In some instruments it is possible to fragment selected ions (precursor ions) and subsequently analyze the fragment ions, MS/MS. The mass analyzers have three important characteristics, mass accuracy, mass range and mass resolution. The molecular mass is in this thesis denoted as  $M$ . Mass accuracy is the  $m/z$  measurement error and the mass range is the range of  $m/z$  ratios amenable to analysis by a given analyzer. The mass resolution is often defined as  $M/\Delta M$  where  $\Delta M$  is the width of a peak at a specified height (often half maximum).

#### *Ion sources*

Electron impact (EI) was invented by Dempster (Dempster, 1918) and was further developed into chemical ionization (CI) in the 1960's (Munson & Field, 1966). EI is routinely utilized as ion source for small organic molecules and CI provides a slightly softer technique to be used if less fragmentation is desired. Their drawback is the limited mass range due to the extensive fragmentation. Fast atom bombardment (FAB) ionization was invented in 1981 by Barber and co-workers (Barber *et al.*, 1981) as an even softer ionization method having a mass range of 2000–17000 Da depending on the sensitivity required. The first method that was able to ionize high-molecular mass molecules such as proteins was plasma

desorption (PD) in 1982 (Håkansson *et al.*, 1982), based on the method by Macfarlane and co-workers (MacFarlane & Torgerson, 1976).

In the eighties Fenn and co-workers (Yamashita & Fenn, 1984) developed electrospray ionization (ESI) as a technique to ionize intact large molecules in solution. The nature of ESI often turns especially high mass analytes into ions of differing charge states leading to spectra with numerous peaks from the same analyte. This makes it possible to analyze high mass ions in mass analyzers with a low  $m/z$  limit. Multiple peaks from each analyte complicate the analysis of mixtures. However, ESI has the advantage of being compatible with liquid chromatography and capillary electrophoresis systems so mixtures can be separated prior to MS analysis.

During the same period Karas and Hillenkamp (Karas *et al.*, 1987) discovered matrix assisted laser desorption/ionization (MALDI) independently of Tanaka and co-workers (Tanaka *et al.*, 1987). Tanaka was able to obtain protein molecular ions with masses of typically 25000 Da with a matrix made of an ultra fine metal powder mixed with glycerol when Karas and Hillenkamp investigated the polypeptide mellitin, 2843 Da, and the oligosaccharide stachyose, 666 Da. It is however the type of matrix that Karas and Hillenkamp used, a UV-light absorbing organic compound, that is the basis for most of the now existing MALDI applications (Karas & Hillenkamp, 1988).

### *Mass analyzers*

The earliest mass analyzers separated ions with a magnetic field. In order to improve the resolution the magnetic analyzer was later coupled to an electrostatic analyzer forming a double-focusing instrument (Mattauch & Herzog, 1934). These instruments have since then been constructed in a variety of set-ups, fulfilling different requirements and still excel in elemental composition analysis thanks to the excellent resolution. Comisarow and Marshall developed the first Fourier transform ion cyclotron resonance (FTICR) analyzer in 1974 (Comisarow & Marschall, 1974) and today the FTICR analyzers have the highest mass resolution as well as the highest mass accuracy of the existing mass analyzers.

The time-of-flight (TOF) mass analyzers were introduced in the 1940's (Cameron & Eggers, 1948; Stephens, 1946) and the combination with the new, pulsed ionization techniques MALDI and PD in the 1980's increased the potential of the TOF analyzers. The electrostatic mirror developed by Mamyrin and co-workers 1973 (Mamyrin *et al.*, 1973) was introduced as reflectors in TOF instruments in the 1990's and improved the mass resolution dramatically (further explanation below).

In the early 1950's Paul and co-workers invented the quadrupole mass filter and the quadrupole ion trap (IT) (Paul & Steinwedel, 1953). The quadrupole mass filter is now both utilized as a stand-alone mass analyzer, often coupled to an on-line separation technique, and as a mass filter in multi-stage mass spectrometers *e.g.* Q-TOF (Q as in quadrupole) analyzers. The ion trap offers the possibility of successive mass spectrometric steps within one mass analyzer, MS<sup>n</sup>.

## MS techniques applied in this thesis

### MALDI-TOF MS

MALDI-TOF MS can be used to measure the molecular mass of carbohydrates up to  $10^6$  Da, as shown by Garozzo *et al.* on fractionated and permethylated dextran standards (Garozzo *et al.*, 1995). The ionization/desorption of biomolecules in MALDI requires, as the name implies, a matrix. The analyte is embedded in the matrix which absorbs the UV-light emitted from a laser, often a nitrogen laser with 337 nm wavelength, thereby aiding the analyte to desorb in intact form. A fraction of the analyte molecules is ionized (Quist *et al.*, 1994). Details on the ionization mechanism are unknown. Different matrices suite different biomolecules and for carbohydrates the most frequently used matrix is 2,5-dihydroxybenzoic acid (DHB) (Strupat *et al.*, 1991). Other useful matrices are 2,4,6-trihydroxyacetophenone (THAP) (Papac *et al.*, 1996; Pieles *et al.*, 1993) for sialylated oligosaccharides, DHB with an addition of 1-hydroxyisoquinoline (Mohr *et al.*, 1995) give results with good reproducibility for maltose chains and  $\alpha$ -cyano-4-hydroxy-cinnamic acid (HCCA) (Beavis *et al.*, 1992) are sometimes used to get more fragmentation in post-source decay (PSD) MS on oligosaccharides (Mo *et al.*, 1998). Since MALDI is a pulsed ion source it is often coupled to a discontinuous mass analyzer such as a TOF-analyzer. In a MALDI-TOF instrument the ions formed in the ion source are accelerated in an electric field and thereafter propagate through a field free region before reaching the detector. When entering the field free region all ions of the same charge state have obtained approximately the same kinetic energy, which is defined by the acceleration voltage. Hence, low mass ions will travel faster and hit the detector before heavier ions (Figure 1). A TOF spectrum is obtained by measuring the time elapsed from the laser pulse to the detection of the various ions having propagated through the field free region and by measuring the intensity of the detector signal at each time. Summation of TOF-spectra from several laser shots is typically performed. A mass spectrum can be obtained by converting the TOF to  $m/z$  using the fundamental relations between these quantities via a calibration procedure. This is the principle for the linear MALDI-TOF instrument that was used in paper I.

To improve resolution MALDI-TOF instruments are often equipped with a *delayed extraction* (Kovtoun, 1997; Wiley & McLaren, 1955) facility. When delayed extraction is applied, the acceleration voltage is switched on some hundred nanoseconds after the laser pulse has hit the sample. The delayed extraction procedure compensates for the difference in the time of ion formation and for the energy dispersion originating from the initial ion velocity distribution. Reflector MALDI-TOF instruments are equipped with an *electrostatic mirror* (Mamyrin *et al.*, 1973) to improve the resolution. The ions of identical mass produced in the ion source have not exactly the same kinetic energy when entering the field free region due to differences in initial kinetic energy prior to acceleration. This leads to a broadening of the peaks and thus decreased resolution. The ions with higher initial kinetic energy will have a higher velocity and therefore a shorter flight-time in the flight tube. They will, however, spend a longer time in the electrostatic mirror as they have more energy and thus penetrate deeper into the

electrostatic mirror (Figure 2). Consequently an ion with lower initial kinetic energy will hit the detector simultaneously (if the parameters are set correctly) as an ion with higher initial kinetic energy. This results in a narrowing of the peaks. A reflector MALDI-TOF instrument with a delayed extraction facility was employed in paper II.

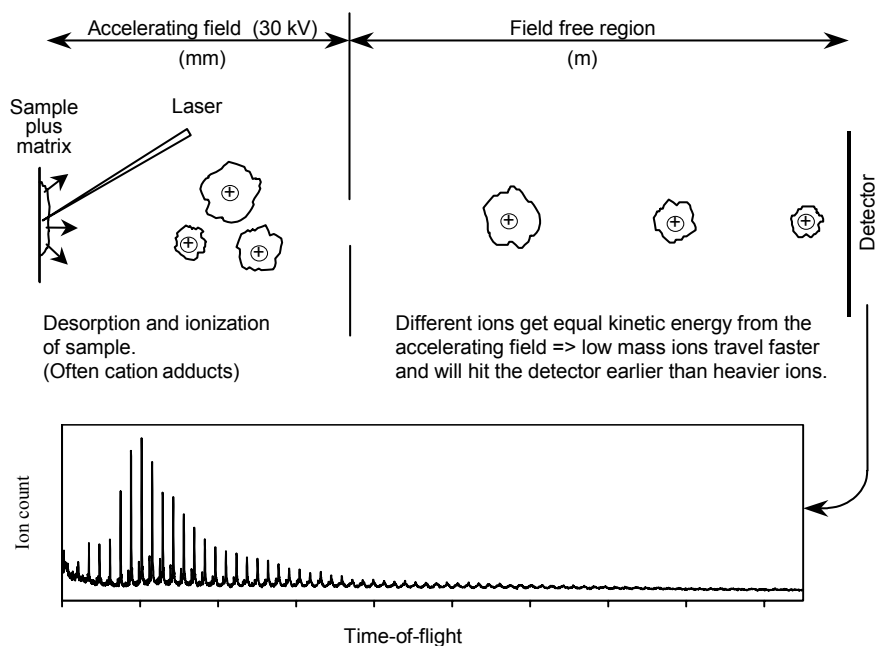
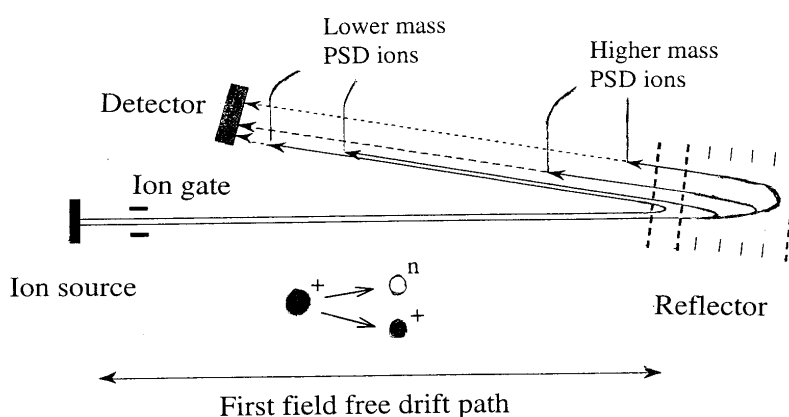


Figure 1. Principle of MALDI-TOF MS

### MALDI PSD TOF MS

Structural information on carbohydrates can be obtained by MS by detection of fragment ions originating from an oligosaccharide. Typically, a *precursor ion* is isolated in a first mass spectrometric step and after fragmentation the resulting fragment ions are monitored in a subsequent mass spectrometric step, i.e. MS/MS. The addition of a reflector in a MALDI-TOF instrument does not only increase the resolution but also enables MS/MS analysis. When MALDI-TOF MS is employed at least two fundamentally different methods for studying fragmentation can be used. In-source decay fragmentation can be studied also with a linear TOF system, but this technique does not involve selection of a precursor ion and furthermore ions in the lower mass range may be disturbed by the matrix ions. If a reflector TOF instrument equipped with a deflection electrode as a timed ion gate (Figure 2) is used, MS/MS experiments can be performed. Ions of different  $m/z$  will reach the ion gate at different times. By applying a voltage that results in a deflection of all ions except ions of the desired  $m/z$  value, the ions of interest can be selected to enter the first field free region. Fragment ions formed by spontaneous fragmentation of the precursor ion in the first field free drift path, post-source decay, can thus be monitored (Spengler *et al.*, 1992; Spengler *et al.*, 1995). Fragment ions of differing masses, formed from the same precursor ion, will have

the same velocity as their precursor ion, but will have different kinetic energies because of their differing masses. Fragment ions are then discriminated according to their mass in the reflector, since larger fragment ions (with higher kinetic energy) penetrate deeper into the reflector than smaller fragment ions and will reach the detector later. By step-wise changing the voltage of the electrostatic mirror fragment ions of different  $m/z$  can be analyzed. MALDI-PSD TOF MS was employed in paper II in this thesis.



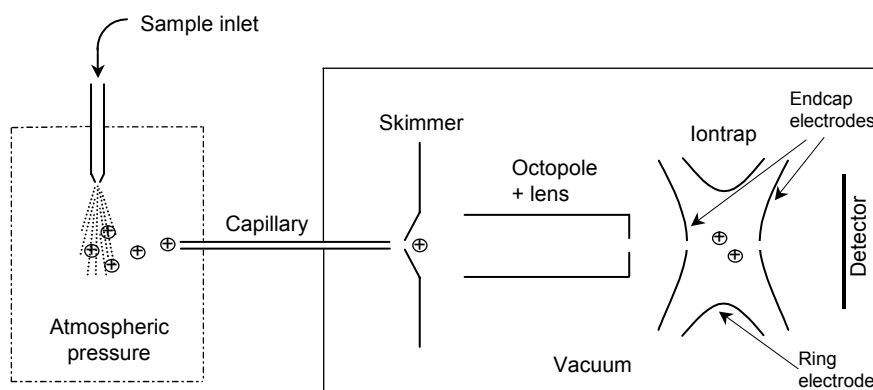
**Figure 2.** Principle of MALDI-PSD TOF MS.

### *ESI-IT MS*

Electrospraying of charged droplets can occur when a liquid at atmospheric pressure is passed at low flow-rate through the end of a narrow tube placed in an electric field. The field induces a charge accumulation at the liquid surface that will break to form highly charged droplets. As the solvent evaporates, the droplets rupture and yield smaller and smaller droplets until gas phase ions are produced. The process is aided with a heated gas. The electric field is arranged so that the charged droplets will enter a capillary (Figure 3). The motion of the droplets through the capillary is determined by an electric field along the capillary. The pressure gradient along the capillary provides additional evaporation of solvent and bare ions enter through the skimmers into the ion trap.

The ion trap consists of a ring electrode between two endcap electrodes (Figure 3). The internal surface shape of these three electrodes follows a three dimensional nearly hyperbolic profile. A high RF voltage is applied to the ring, while the endcaps are held at ground. The oscillating potential difference established between the ring and the endcap electrodes forms a quadrupolar field. Depending on the level of the RF voltage, the field can trap ions of a particular mass range. The ions entering from the external ion source are cooled by collisions with an inert gas inside the ion trap to extract energy from the ion beam to avoid the ions

from passing through the ion trap and out of the exit endcap. A scan sequence is applied to acquire the mass spectrum. It starts with a clearance of the ion trap and continues with accumulation when the ions are trapped in the RF field using a low quadrupolar amplitude and cooling with the inert gas. During the subsequent mass analysis the field strength is increased to progressively eject ions of increasing  $m/z$  values out of the trap by passing through the exit endcap.



**Figure 3.** Principle of ESI-IT MS.

### ***ESI-IT multiple-stage MS***

The quadrupole ion trap has the possibility of *time-dependent* multiple-stage MS (abbreviated  $MS^n$  where  $n$  refers to the number of successive MS steps), instead of the *space-dependent* MS/MS offered by mass analyzers in a series. This gives theoretically infinite number of MS cycles, only limited by the amount of ions. An MS/MS scan starts as an MS scan with a clearance of the ion trap and accumulation of the ions. Subsequently the precursor ion is isolated by ejection of all other ions. An additional voltage is applied to facilitate collisions between the precursor ions and the inert gas, which cause fragmentation of the precursor ions. With the trap now storing fragment ions the RF drive level/field can be ramped to produce a mass spectrum, or an additional stage of  $MS^n$  isolation and fragmentation can be initiated.

The quadrupole ion trap was employed in paper III and IV because of its  $MS^n$  facility.

### **Chromatography**

Carbohydrate analysis often involves various chromatographic methods such as gel filtration, affinity chromatography, ion-exchange chromatography etc. for separation. The eluate containing different saccharides can be monitored with for example refractive index detection, electrochemical detection or mass spectrometry. The techniques HPAEC-PAD and gel filtration were used in paper I, but will not be further described here.



# Comparison between MALDI-TOF MS and HPAEC-PAD analysis of debranched starch (Paper I)

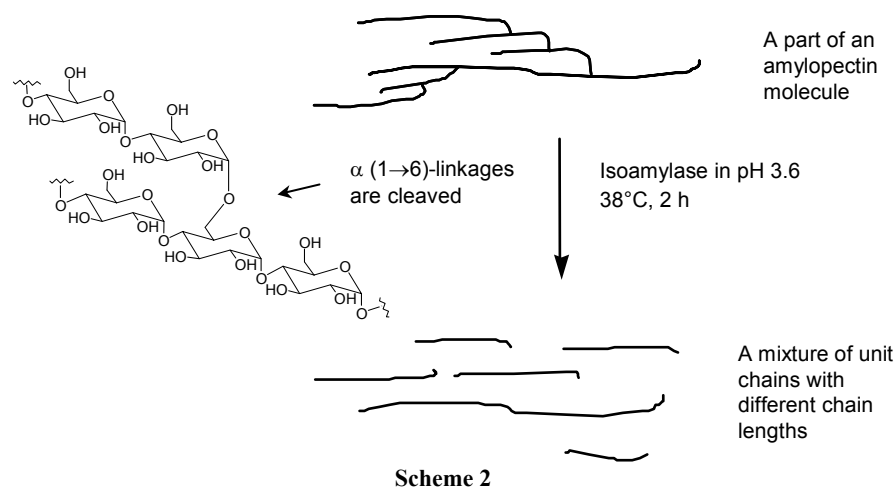
## Starch

Starch is a major storage polysaccharide of green plants and the second most abundant carbohydrate in plants, next to cellulose. It consists of two major components, amylopectin and amylose, both containing  $\alpha(1\rightarrow4)$ -linked D-glucose units. Amylopectin is highly branched through  $\alpha(1\rightarrow6)$ -linkages, while amylose is mainly a linear molecule. The degree of polymerization (DP), i.e. the number of glucose units, of amylopectin is 6 000–600 000 (Aberle *et al.*, 1994; Banks & Greenwood, 1975) and the molecular mass ranges from  $10^6$  to  $10^8$  Da, rendering it the position of being one of the largest naturally occurring molecules (Pérez & Imbert, 1996). The branching frequency is around 5% (Manners, 1989) and the branch points are not randomly distributed (Bertoft & Avall, 1992; Hizukuri *et al.*, 1989). Amylose molecules are significantly smaller with a DP of 500–6 000 and a molecular mass of  $10^5$ – $10^6$  Da (Hizukuri *et al.*, 1981). The ratio between amylopectin and amylose differs between starches, but a typical value is 75% amylopectin and 25% amylose. Waxy maize is an example of a genotype with almost exclusively amylopectin (>97%) and there also exist genotypes with increased amylose content (high-amylose starches).

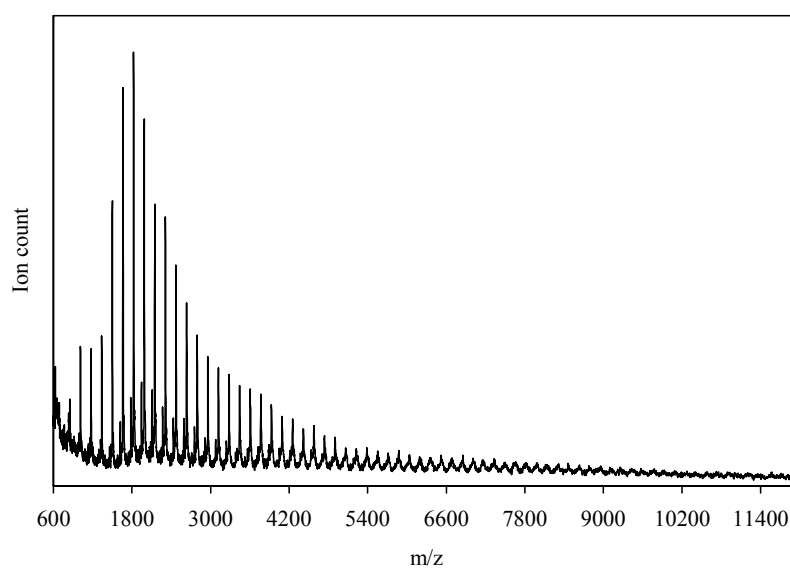
Starch is widely used in technical industries for a variety of products such as paper, glue/adhesives, concrete, pharmaceuticals, clothing etc. Understanding the composition and chemical structure of different starches is crucial for proper applications of starch in industry. There are many parameters to study in starch, but the focus in paper II was on the chain length distribution of amylopectin, which can be analyzed after debranching the amylopectin. We compared MALDI-TOF MS with HPAEC-PAD analysis, which is an established method for chain length distribution. The advantage of MALDI-TOF compared to HPAEC-PAD analysis is the speed and the possibility to obtain mass information. We also wanted to investigate if MALDI-TOF MS could be applied without prior removal of the amylose.

## MALDI-TOF MS versus HPAEC-PAD analysis

Isolated amylopectin from potato and wheat starch was debranched with isoamylase, a debranching enzyme that cleaves the  $\alpha(1\rightarrow6)$ -linkage in the branch points, leaving the  $\alpha(1\rightarrow4)$ -linkages intact. The resulting mixture contained the different chains which have differing chain lengths (Scheme 2). Every population of chains with a certain length is defined as a unit chain.

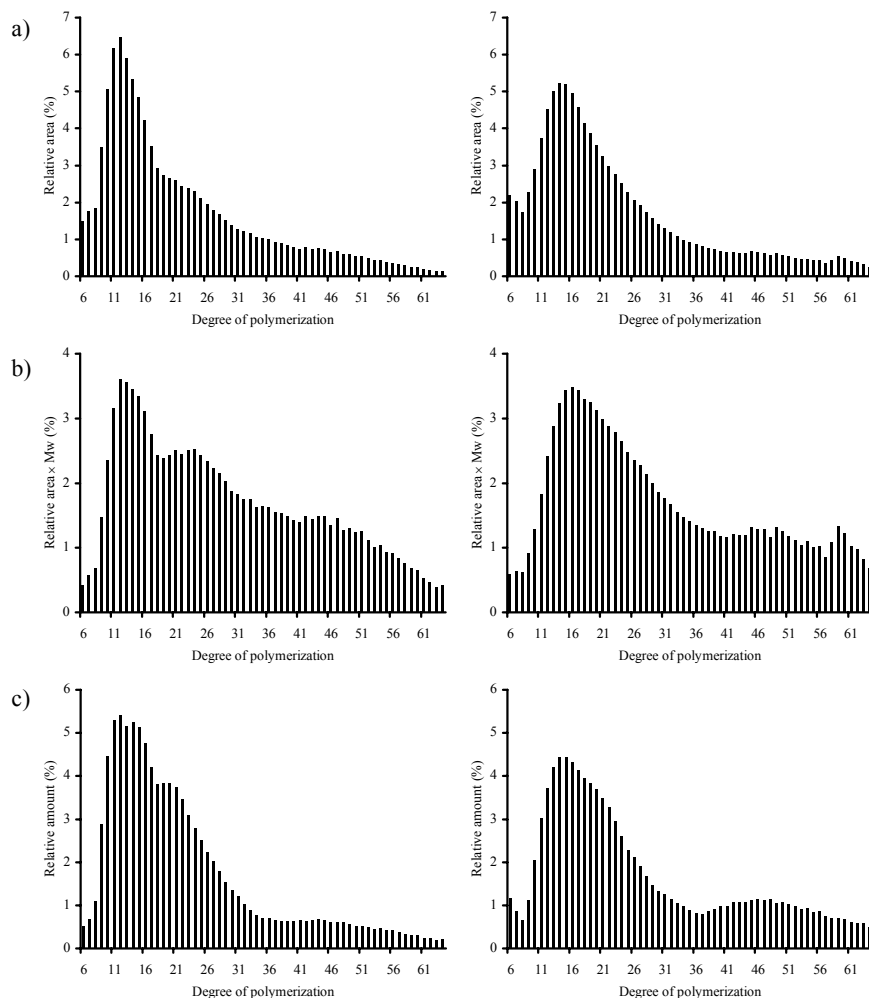


The chain length distribution was determined using HPAEC-PAD and corrections for the different detector responses for the individual unit chains were made (Koch *et al.*, 1998). The material was simultaneously analyzed by MALDI-TOF MS with 2,4,6-trihydroxyacetophenone (THAP) as matrix (Figure 4).



**Figure 4.** MALDI-TOF mass spectrum of debranched wheat amylopectin.

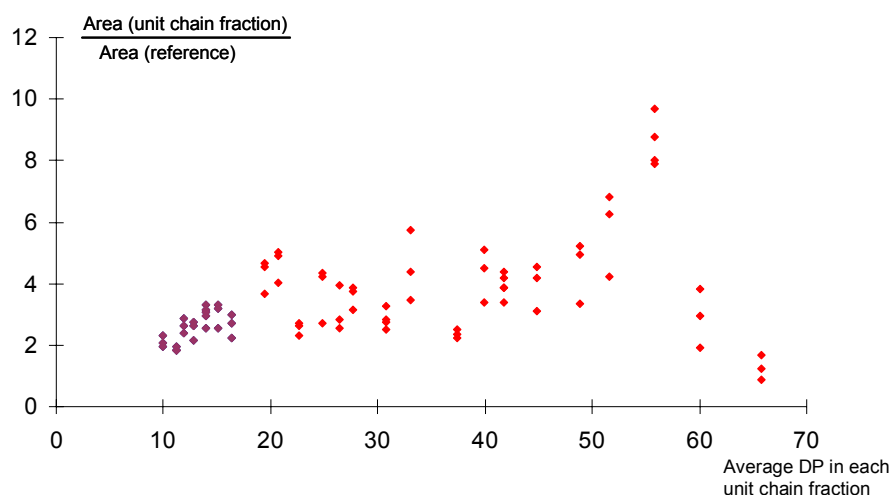
Similar characteristic differences between chain length distribution for amylopectin from the different sources were observed with both methods (Figure 5 a-c). However, MALDI-TOF MS seemed to overestimate chains of DP over 21 when comparisons were made on a relative weight basis.



**Figure 5.** Bar graphs showing the chain length distribution of wheat (left) and potato (right) amylopectin a) on a relative area basis obtained by MALDI-TOF MS; b) on a relative weight basis obtained by MALDI-TOF MS. Each peak area was multiplied with the molecular weight of the corresponding chain; c) on a relative amount basis obtained by HPAEC-PAD (calibrated).

An attempt to quantify the results from the MALDI-TOF experiments was made in a similar way as had been performed with the HPAEC-PAD (Koch *et al.*, 1998). To investigate the relation between the MALDI-TOF detection response and DP, waxy maize was debranched with isoamylase and fractionated on a Bio-Gel P-6 gel filtration column to obtain unit chain fractions. The average DP for each unit chain fraction was calculated and the concentrations were determined (with the aid of glucose content determination) and thereafter diluted with water to equal concentration. Each unit chain fraction was then mixed with similar amount of maltoheptaose for reference. The results showed that the variation in response for DP over 16 was large (Figure 6). Thus, it was not possible to make reliable

corrections for the fact that the signal may be overestimated for long unit chains. Small additional peaks were detected at  $m/z$  twice as high as those for the molecules in the unit fractions. This may indicate possible aggregation of chains, which could partly explain the overestimation of long unit chains. Chains longer than DP 80 have not yet been detected with HPEAC-PAD and if the amylose part of starch would be included in the separation, the longer chains could disturb the chromatography. Consequently the amylose part is often excluded prior to HPAEC-PAD analysis. Whole starch from potato and wheat was now debranched with isoamylase and analyzed with MALDI-TOF MS to investigate if the longer chains would disturb the ionization/desorption part of the mass spectrometry. No significant differences between the unit chain length profiles for isolated amylopectin and whole starch samples, respectively, were found. Hence, in contrast to HPEAC-PAD analysis the MALDI-TOF MS technique can be used for chain length distribution analysis of amylopectin without prior separation of the amylose fraction. Furthermore, MALDI-TOF MS provides detailed information on the molecular mass of the unit chains and analysis of a debranched starch sample is completed in a few minutes compared to HPAEC-PAD analysis that takes about 1.5 h.



**Figure 6.** Graph showing the ratios of the areas from each unit chain fraction divided by the areas from the reference. The unit chain fractions were obtained from waxy maize.

## Outlook

Improving the sample preparation for MALDI-TOF MS might increase the reproducibility and thus facilitate determination of response relation. The use of a MALDI-TOF MS with better resolution, *e.g.* with delayed extraction, would probably improve the possibility to calculate the detector response for different chain lengths. MALDI-TOF MS was in this study utilized for determination of amylopectin chain length profiles, but other oligo- and polysaccharides can be determined with the same technique after developing an appropriate methodology.

## Derivatization of oligosaccharides for analysis by mass spectrometry (Paper II)

Mass spectrometry can among other techniques be used for analysis of oligosaccharides. Derivatizations, such as methylation, acetylation or reductive amination, are often used to increase the sensitivity or to change the fragmentation characteristics. When using MALDI-TOF MS, underivatized oligosaccharides can often be analyzed on sample amounts down to low picomole level on the target. After different derivatizations with for example 2-aminopyridine (Okamoto *et al.*, 1997), Girard's Reagent T (Naven & Harvey, 1996), 4-aminobenzoic acid-2-(diethylamino)ethyl ester (Takao *et al.*, 1996), or an aminooxyacetyl peptide (NH<sub>2</sub>-O-CH<sub>2</sub>CO-KLEEQRPERVKG) (Zhao *et al.*, 1997), the detection limit can be lowered, down to the low femtomole level.

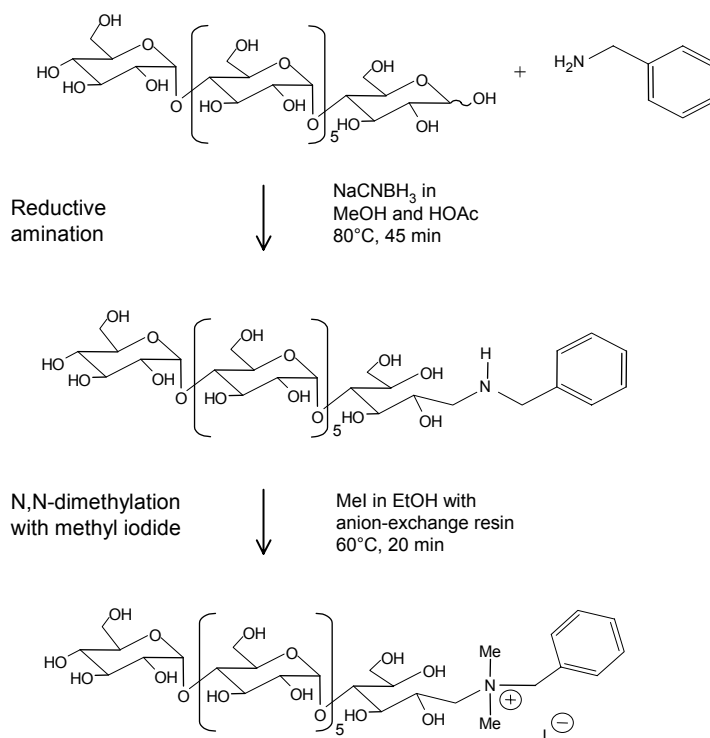
The aim of the study in paper II was to derivatize carbohydrates to obtain compounds suitable for MS analysis. The desired carbohydrate derivatives should be amenable to MS analysis at low sample amounts as well as give informative and simple fragment ion mass spectra. The latter objective can be achieved by charge remote fragmentation, a phenomenon discussed by Jensen *et al.* (Jensen *et al.*, 1985) referring to fragmentation of an ion where the cleavage is not necessarily located in the vicinity of the charge. In paper II a carbohydrate derivative is presented, which is formed by reductive amination with benzylamine followed by N,N-dimethylation with methyl iodide. The derivative (hereafter called DMBA-derivative) has a fixed positive charge at the modified reducing terminus. Initially the procedure was examined on maltoheptaose and subsequently three human milk oligosaccharides were derivatized.

### Derivatization and mass spectrometry

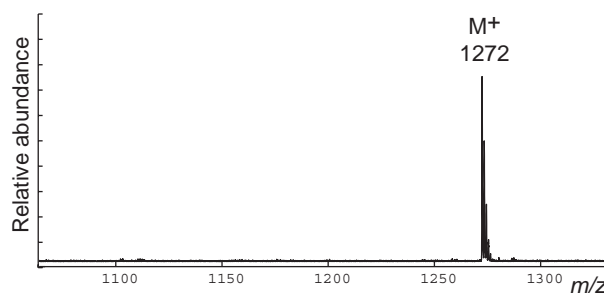
Maltoheptaose was reductively aminated with benzylamine followed by an N,N-dimethylation with methyl iodide to achieve a fixed positive charge at the modified reducing terminus (Scheme 3).

The reductive amination of maltoheptaose with benzylamine and the subsequent N,N-dimethylation with methyl iodide in the presence of an anion exchanger resulted in almost exclusively the desired product (Figure 7). The DMBA-derivatization prior to MALDI-TOF MS led to a tenfold increase in sensitivity compared to the underivatized maltoheptaose (Figure 8). To compare with published results obtained by other groups, maltoheptaose was also derivatized with 2-aminopyridine (Okamoto *et al.*, 1997) and Girard's Reagent T (Naven & Harvey, 1996) and these derivatives showed only a two and a half-fold and a fivefold increase, respectively (Figure 2 in paper II). The matrix used for the MALDI-TOF analysis was 2,5-dihydroxybenzoic acid (DHB) with an addition of 0.1% trifluoroacetic acid (TFA).

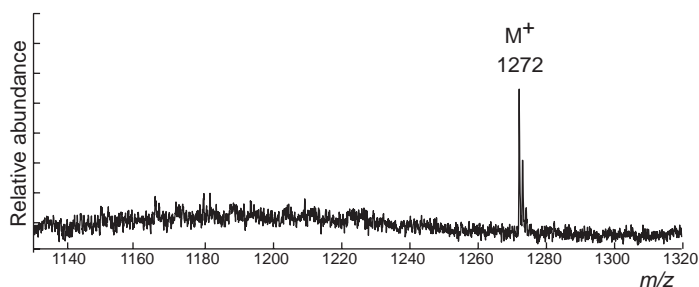
To investigate if more complex oligosaccharides could be derivatized using the same methodology, three human milk oligosaccharides were derivatized. LNF-I, LND-I and MFLNH-III, containing both fucose and N-acetyl-glucosamine residues, were all derivatized giving similar results as maltoheptaose (Figure 1 in paper II).



**Scheme 3.**

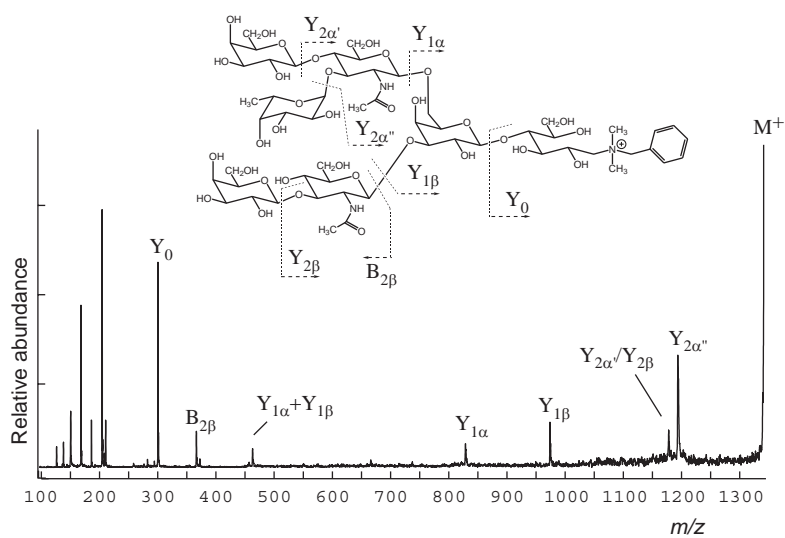


**Figure 7.** MALDI-TOF mass spectrum of DMBA-derivative of maltoheptaose.



**Figure 8.** MALDI-TOF mass spectrum of DMBA-derivative of maltoheptaose. Approximately 50 fmol was loaded onto the sample probe.

The DMBA-derivatives also proved to be amenable to MALDI-PSD TOF analysis. The PSD-spectrum for the DMBA-derivative of MFLNH-III is shown in Figure 9. The dominating fragment ions in mass spectra of the DMBA-derivatives belong to the Y-series (Domon & Costello, 1988), but also X-series fragments are observed in the PSD-spectrum of DMBA-maltoheptaose (Figure 3 in paper II). Both the Y- and X-series of fragments retain the modified reducing terminus and since there is a pre-formed positive charge at the modified reducing end of the analyte, these fragments will probably be important in any MS/MS-analysis of this type of oligosaccharide derivative. MS/MS spectra of underivatized oligosaccharides typically show fragments from both the reducing end (X-, Y- and Z-series) and the non-reducing end (A-, B- and C-series) (Domon & Costello, 1988) which complicate the interpretation.



**Figure 9.** MALDI-PSD TOF mass spectrum of the DMBA-derivative of MFLNH-III.

## Outlook

It would be interesting to analyze these derivatives with other MS techniques with MS/MS facility, *e.g.* FAB magnetic sector MS/MS and ESI-IT MS<sup>n</sup>, to observe similarities and differences between the techniques in the ability to determine structures of oligosaccharides. FAB magnetic sector employs the possibility of controlling fragmentation of the ions by introducing a collision gas after the precursor ion selection and ESI-IT MS<sup>n</sup> can regulate the fragmentation by changing the fragmentation amplitude. ESI-IT MS<sup>n</sup> also has the advantage of more than two consecutive MS steps.



## ESI-IT MS<sup>n</sup> on *Quillaja* saponins (Paper III and IV)

### Saponins

Saponins are a group of steroidal and triterpenoid glycosides which have the ability to lower the surface tension of aqueous solutions (Samuelsson, 1992). The word saponin refers to the foaming properties in aqueous solution, similar to those of a soap solution. Saponins are used technically, *e.g.* as wetting agents in the photographic industry and as wetting agents in agriculture (San Martín & Briones, 1999).

### *Quillaja* saponins

The bark of the *Quillaja saponaria* Molina (Rosaceae) tree, native to Chile, Peru and Bolivia, contains a mixture of triterpenoid saponins. These saponins are used as additives in food products, cosmetics and in pharmaceutical products (San Martín & Briones, 1999). *Quillaja* saponins upregulate the immune response and are therefore used as adjuvants with vaccines (Kensil, 1996). The application of these saponins in humans calls for detailed knowledge about the different saponin structures in the purified bark extract used.

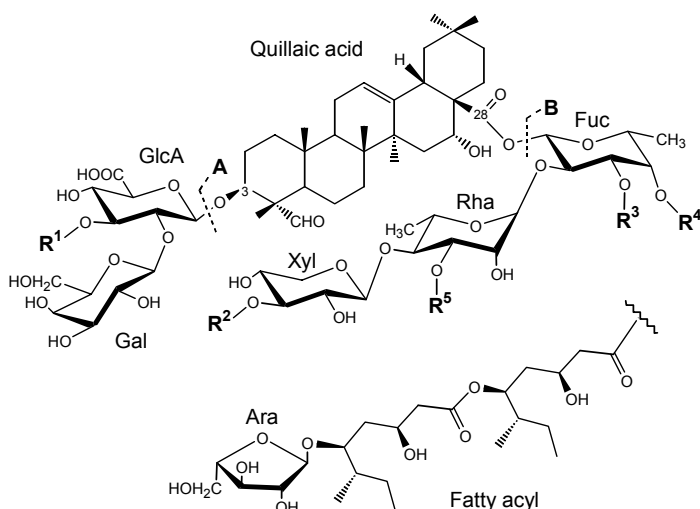
It is both complicated and time-consuming to isolate and completely describe structures of *Quillaja* saponins. Several chromatographic steps and combinations of chemical and spectroscopic techniques are involved and it is therefore not practical to structurally characterize all minor components. The basic structure reported for most *Quillaja* saponins is the triterpene quillaic acid substituted at C-3 with a di- or trisaccharide and at C-28 with a complex oligosaccharide (Figure 10).

### Structure analysis of saponins from *Quillaja saponaria* Molina by ESI-IT MS<sup>n</sup>

Most of the *Quillaja* saponins are composed of combinations of certain common structural elements that can be recognized without complete characterization and this has previously been utilized for structural classification of *Quillaja* saponins by monomer mapping using accurate mass data (van Setten *et al.*, 1995) and by multivariate analysis of <sup>1</sup>H NMR spectra (Nord *et al.*, 2001). There is nevertheless a need for rapid and sensitive methods for structure characterization of saponin components in extracts and other preparations containing *Quillaja* saponins. *Quillaja* saponins have previously been investigated by nanoelectrospray ion trap multiple-stage tandem mass spectrometry (van Setten *et al.*, 1998; van Setten *et al.*, 2000) as a pre-NMR tool in the structure elucidation.

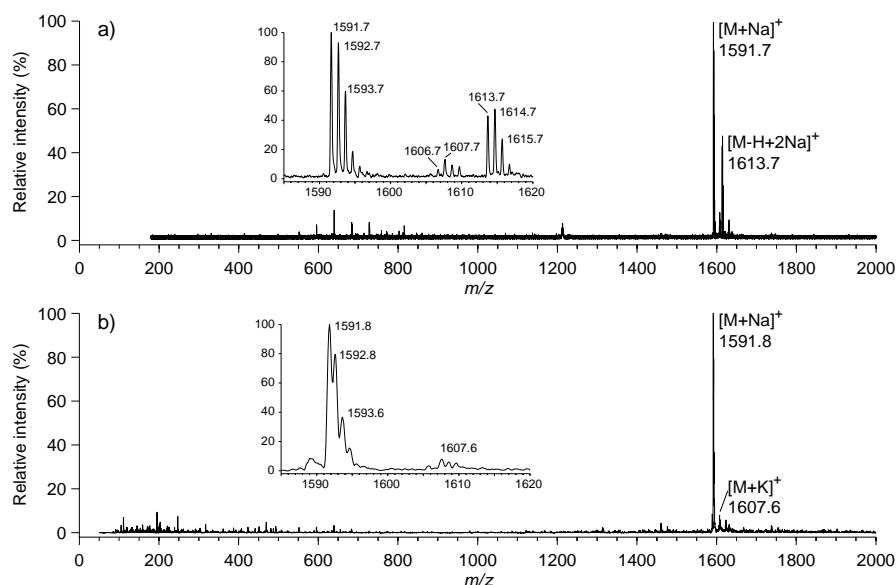
In paper III and IV the analysis of a set of *Quillaja* saponins by ESI-IT MS<sup>n</sup> is described. The investigated saponins have previously been isolated (Guo *et al.*, 2000a; Guo & Kenne, 2000b; Nord & Kenne, 1999; Nyberg *et al.*, 2003; Nyberg *et al.*, 2000) and their structures (Figure 10) determined by MALDI-TOF MS,

NMR and chemical methods. The numbering of the compounds is preserved from the original publications. MS spectra of compound **6** by MALDI-TOF MS and ESI-IT MS<sup>1</sup> are shown in Figure 11. The mass resolution is better in MALDI-TOF MS than in ESI-IT MS<sup>n</sup>, but the possibility of fragmentation in successive MS steps is restricted to ESI-IT MS<sup>n</sup>. Hence, the experiments were performed on the ESI-IT MS with the opportunity to perform MS<sup>n</sup> experiments.



Compound	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	Mass
<b>4</b>	H	H	Rha	Acetyl	H	1436.65
<b>5</b>	Rha	H	Rha	Acetyl	H	1582.70
<b>6</b>	Xyl	H	Rha	Acetyl	H	1568.69
<b>7</b>	Rha	Api	Rha	Acetyl	H	1714.75
<b>8</b>	Xyl	Api	Rha	Acetyl	H	1700.73
<b>9</b>	Rha	Xyl	Rha	Acetyl	H	1714.75
<b>10</b>	Xyl	Xyl	Rha	Acetyl	H	1700.73
<b>11a,b</b>	Rha, Xyl	H	Glc	Acetyl	H	1598.70, 1584.68
<b>16a,b</b>	Rha, Xyl	H	Glc6OAc	Acetyl	Glc	1802.76, 1788.75
<b>17a,b</b>	Rha, Xyl	H	Rha	Acetyl	Glc	1744.76, 1730.74
<b>B1</b>	Rha	H	H	Fatty acyl	Glc	2032.95
<b>B1a</b>	Rha	H	Fatty acyl	H	Glc	2032.95
<b>B2</b>	Xyl	H	H	Fatty acyl	Glc	2018.93
<b>B3</b>	Rha	Api	H	Fatty acyl	Glc	2164.99
<b>B5</b>	Rha	Xyl	H	Fatty acyl	Glc	2164.99
<b>S1</b>	Rha	H	H	Fatty acyl	H	1870.90
<b>S1a</b>	Rha	H	Fatty acyl	H	H	1870.90
<b>S3</b>	Rha	Xyl	H	Fatty acyl	H	2002.94
<b>S5</b>	Rha	Api	H	Fatty acyl	H	2002.94
<b>S6</b>	Xyl	Api	H	Fatty acyl	H	1988.92

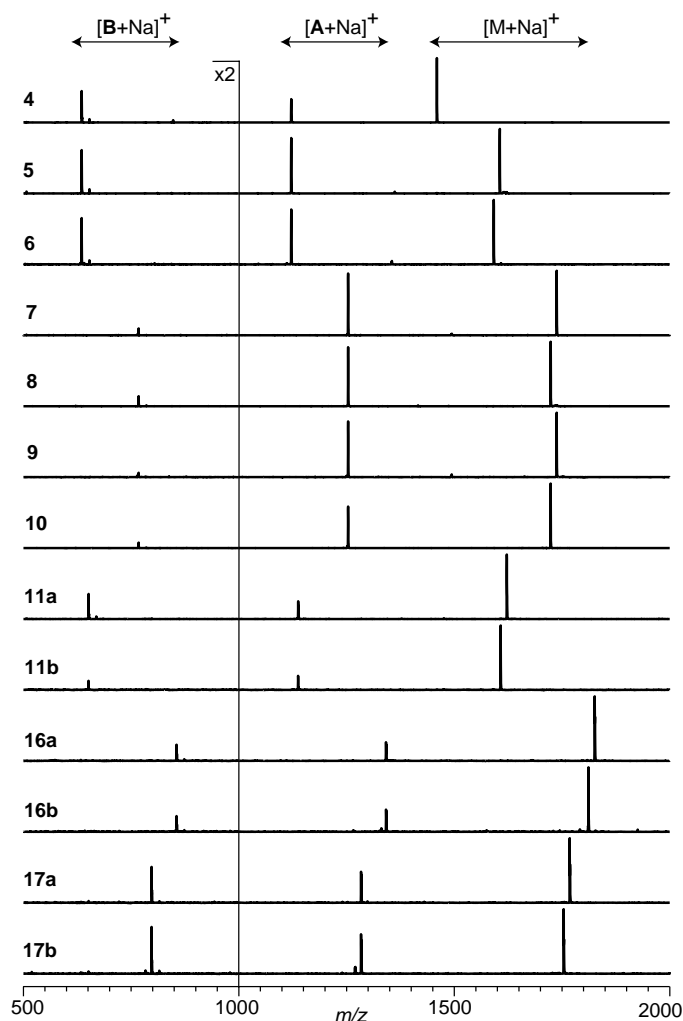
**Figure 10.** Structures of the investigated *Quillaja* saponins with the glycosidic cleavages giving the **A** and **B** fragments. The numbering of the R-substituents differs from that in paper III, but is consistent with the numbering in paper IV.



**Figure 11.** (a) MALDI-TOF and b) ESI-IT mass spectra of compound **6**. The mass spectra are dominated by  $[M+Na]^+$  and  $[M-H+2Na]^+$  in (a) and by  $[M+Na]^+$  in (b) and exhibit negligible fragmentation.

### Paper III

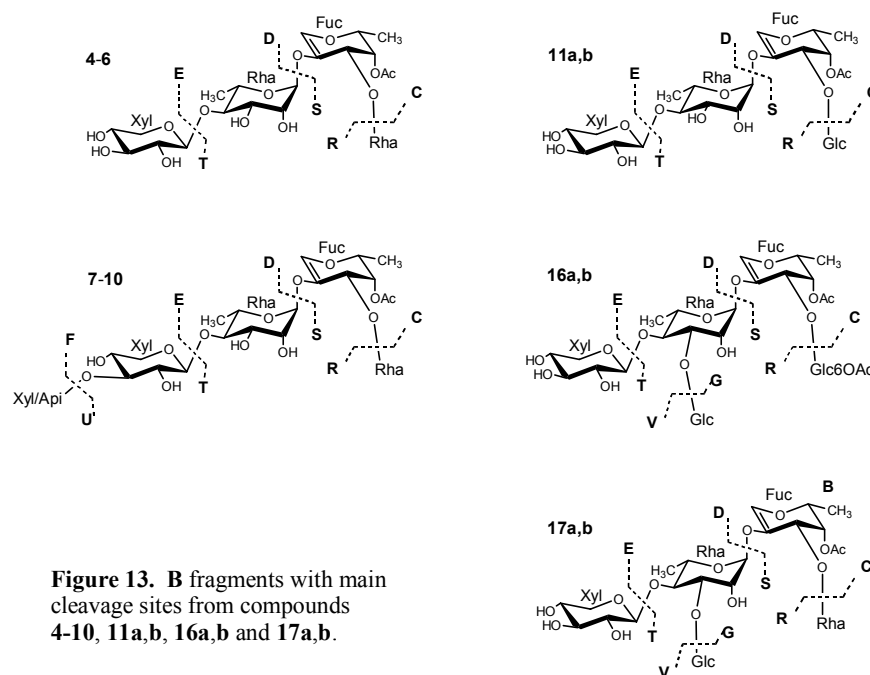
Compounds **4–10**, **11a,b**, **16a,b** and **17a,b** were investigated in both positive and negative ion mode  $MS^1$ – $MS^4$  in order to improve knowledge of the fragmentation pathways. The ions observed in positive ion mode  $MS^1$  spectra were singly charged  $[M+Na]^+$  ions that were consistent with the expected masses. Figure 10 shows the calculated monoisotopic mass of each compound. For seven of the samples spectra showed only one component, whereas for three of the samples two components with a mass difference of 14 Da were observed (**11a,b**, **16a,b** and **17a,b**) corresponding to the mass difference between the rhamnose and the xylose residues.  $MS^2$  analysis in the positive ion mode was performed on the  $[M+Na]^+$  ion in samples containing one component, **4–10**, and on each  $[M+Na]^+$  ion of samples **11a,b**, **16a,b** and **17a,b**. The mass difference between the  $[M+Na]^+$  and the **A** fragment ions in the  $MS^2$  spectra (Figure 12) reflected the different C-3 oligosaccharides in the compounds. The  $MS^2$  spectra of **4–6** showed identical masses for the respective **A** and **B** fragments, consistent with the identical structure of the C-28 oligosaccharide for these compounds (Figure 10). The **A** and **B** fragment ions in the  $MS^2$  spectra of **7–10** were observed at 132 higher  $m/z$  values than in those of **4–6**, as expected, since they have an additional pentose in the C-28 oligosaccharide. The **A** and **B** fragment ions for **11a**, **11b**, **16a**, **16b**, **17a** and **17b** were consistent with the monosaccharide substituents given in Figure 10.



**Figure 12.** Positive ion mode MS<sup>2</sup> spectra of compounds **4–10**, **11a,b**, **16a,b**, and **17a,b**. Observed  $m/z$  ranges for the  $[M+Na]^+$  ions as well as the **A** and **B** fragment ions are indicated.

The **B** fragment ions for all compounds were selected and analyzed by positive MS<sup>3</sup>, and the key fragments (Figure 13) formed are presented in Table 1. The C-28 oligosaccharide is identical in compounds **4–6** and consequently the masses for the fragment ions are the same. The **C** and **S** fragment ions for compounds **7–10** have masses that are 132 Da higher than the corresponding ions for compounds **4–6**. This reflects the additional pentose ( $R^2$ -substituent, Figure 10) in compounds **7–10**. The **D** and **E** fragment ions for compounds **11a** and **11b** are 16 Da higher compared to those from **4–6**, and this is explained by the  $R^3$ -substituent that is a glucose residue instead of the rhamnose for compounds **4–6**. The  $R^3$ - and  $R^5$ -positions in compounds **16a** and **16b** are substituted by a 6-O-acetylated glucose and a glucose, respectively. This is reflected by expected changes in the masses of

the C, D, E, and S fragment ions compared to corresponding ions of 4–6. For compounds 17a and 17b the R<sup>5</sup>-position is substituted by a glucose residue and consequently the masses of the C, E, and S fragment ions are increased by 162 Da compared to corresponding ions of 4–6.



**Figure 13.** B fragments with main cleavage sites from compounds 4-10, 11a,b, 16a,b and 17a,b.

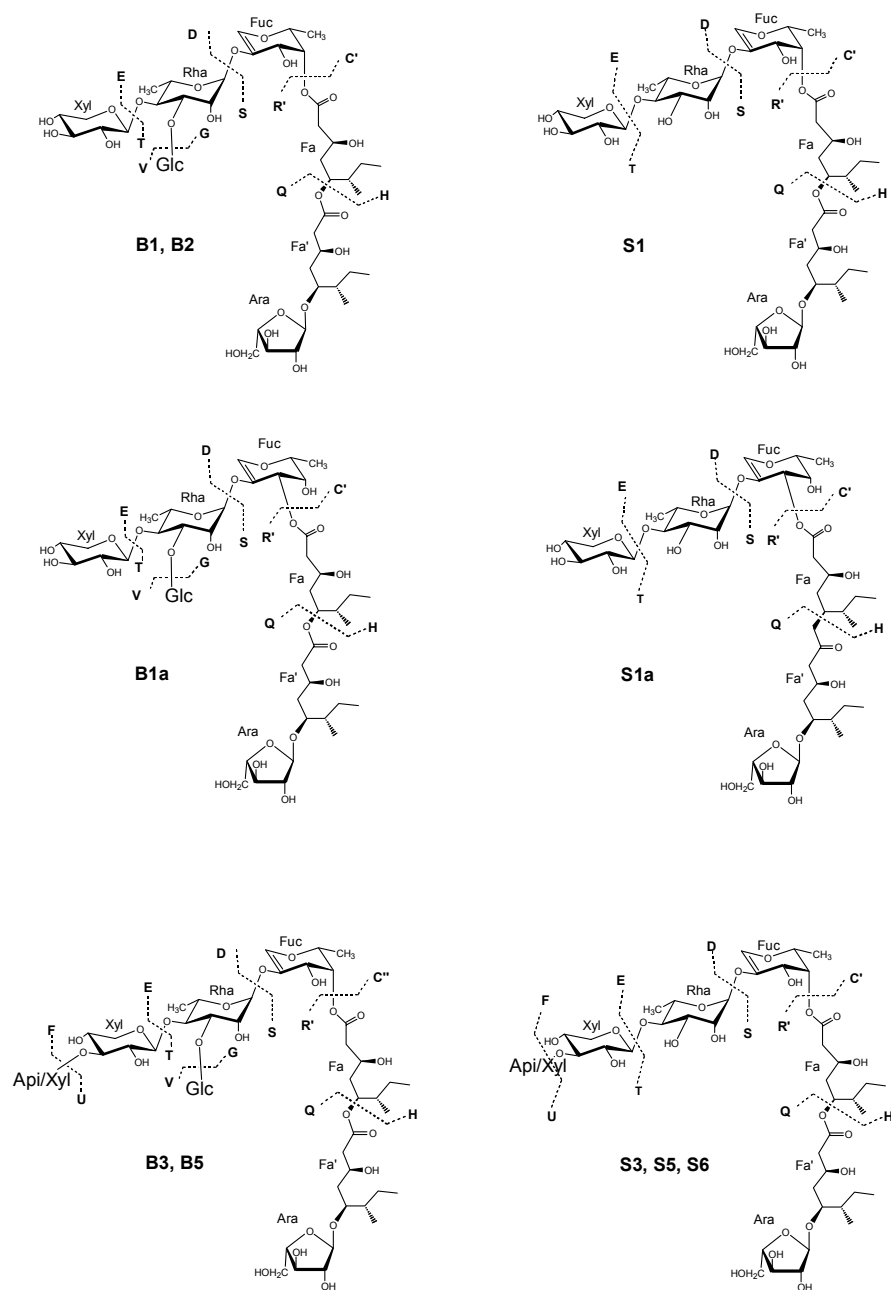
**Table 1.** Key fragment ions observed in positive ESI-IT MS<sup>2-3</sup> spectra of *Quillaja* saponin components

	Observed ions <sup>a</sup> ; [X+Na] <sup>+</sup>														
Compound	A	B	C	D	E	F	G	S	C-S	C-T	C-U	C-V	S-T	S-U	S-V <sup>b</sup>
4-6	1121	635	489	357	503			301	- <sup>b</sup>	357			-		
7-10	1253	767	621	357	503	635		433	-	357	489		-	301	
11a,b	1137	651	489	373	519			301	-	357			-		
16a,b	1341	855	651	415	723		693	463	-	-		-	331		283
17a,b	1283	797	651	357	665		635	463	-	519		489	331		283

<sup>a</sup> See Fig. 13 for structure of the fragment ions. Given masses of ions include sodium.

<sup>b</sup> Not observed.

Positive MS<sup>4</sup> analysis gave information on the fragmentation pathways, but added no information for the structural elucidation. Negative ion mode MS<sup>1</sup>–MS<sup>4</sup> analyses were performed on all compounds. They supplied information on the fragmentation pathways in negative ion mode and confirmed the structures of the C-3 oligosaccharides. Consequently, it is enough to perform positive ion mode MS<sup>1</sup>–MS<sup>3</sup> experiments to obtain the structural information on both the C-3 and C-28 oligosaccharide.



**Figure 14.** B fragments with main cleavage sites from compounds **B1a**, **B1–B3**, **B5**, **S1a**, **S1**, **S3**, **S5**, and **S6**.

## Paper IV

Two classes of *Quillaja* saponins were studied, the *B-series* substituted with a glycosyl at the R<sup>5</sup> position and the *S-series* without this glycosyl group. Compounds **B1a**, **B1–B3**, **B5**, **S1a**, **S1**, **S3**, **S5**, and **S6** (Figure 10) were investigated in positive MS<sup>1</sup>–MS<sup>3</sup>. The ions observed in MS<sup>1</sup> spectra were all singly charged [M+Na]<sup>+</sup> ions that were consistent with the expected masses. MS<sup>2</sup> analysis was performed with the [M+Na]<sup>+</sup> ions as precursor ions and the mass difference between the [M+Na]<sup>+</sup> and the **A** fragment ions in the MS<sup>2</sup> spectra reflected the different C-3 oligosaccharides in the compounds. The **A** and **B** fragment ions for the compounds were consistent with the monosaccharide substituents in the C-28 oligosaccharide given in Figure 10.

The **B** fragment ions for all compounds were selected and analyzed by positive MS<sup>3</sup>, and the key fragments (Figure 14) formed are presented in Table 2. The substituents in the C-28 oligosaccharide are the same in compounds **B1**, **B1a** and **B2**, a glucose and a fatty acyl, and consequently the masses for the fragment ions are the same. The **H** and **S** fragment ions for compounds **B3** and **B5** have masses that are 132 Da higher than the corresponding ions for compounds **B1**, **B1a** and **B2**. This reflects the additional pentose (R<sup>2</sup>-substituent, Figure 10) in compounds **B3** and **B5**. The **H** and **S** fragment ions for compounds **S1** and **S1a** are 162 Da lower compared to those from **B1a**, **B1** and **B2**. This reflects the structural difference at the R<sup>5</sup> position since they all have a fatty acyl. The additional pentose (xylose or apiose) at R<sup>2</sup> in **S3**, **S5** and **S6** is manifested by the 132 Da higher **H** and **S** fragment ions compared to **S1** and **S1a**. For **B1a** and **S1a** where the fatty acyl is in the fucosyl O-3 the R<sup>2</sup>-fragments at *m/z* 517 have relative intensities of almost 100 (Table 1 in paper IV), compared to less than 30 for the compounds **B1–B3**, **B5**, **S1**, **S3**, **S5**, and **S6** where the fatty acyl is in the fucosyl O-4.

**Table 2.** Key fragment ions observed in positive ESI-IT MS<sup>2–3</sup> spectra of *Quillaja* saponin components

Compound	Observed ions <sup>a</sup> ; [X+Na] <sup>+</sup>																Calc.	
	A	B	C'	D	E	F	G	H	Q	R'	S	H-S	H-T	H-U	H-V	S-T		S-U
B1a, B1, B2	1571	1085	591	645	953		923	763	345	517	463	323	631		-	331		484, 484, 470
B3, B5		1703	1217	723	645	<sup>b</sup>	1085	1055	895	345	517	595	-	631	763	733	-	463 484
S1a, S1		1409	923	429	645	791		601	345	517	301	323	469			-		484
S3, S5, S6		1541	1055	561	645	-	923		733	345	517	433	323	469	601	-	-	484, 484, 470

<sup>a</sup> See Fig. 14 for structure of the fragment ions. Given masses of ions include sodium.

<sup>b</sup> Not observed.

### *Structure analysis procedure (paper III and IV)*

The key fragments obtained from positive MS<sup>1</sup>–MS<sup>3</sup> corresponded to certain structural elements of the saponin structures. These elements contain the different structural variations in both the C-3 and the C-28 oligosaccharide and were used for the construction of a flowchart in paper III. The flowchart provides a structure analysis procedure based on the positive MS<sup>1</sup>–MS<sup>3</sup> experiments. In paper IV the flowchart was extended (Figure 15) to cover the 23 *Quillaja* saponins investigated.

The procedure starts by subtracting the  $m/z$  value for [A+Na]<sup>+</sup> from the  $m/z$  value for [M+Na]<sup>+</sup> in the MS<sup>2</sup> spectrum to identify the R<sup>1</sup>-substituent in the C-3 oligosaccharide. The basic structure of the C-3 oligosaccharide is a galactose  $\alpha(1\rightarrow2)$ -linked to the glucuronic acid residue, 338 Da. The R<sup>1</sup>-substituent in the 3-position of the glucuronic acid residue can either be a hydrogen, a xylose or a rhamnose having the extra molecular masses 0, 132 and 146 Da, respectively. The mass difference between [M+Na]<sup>+</sup> and [A+Na]<sup>+</sup> is either 338, 470 or 484 Da reflecting the C-3 oligosaccharide.

The MS<sup>3</sup> spectrum from the precursor ion [B+Na]<sup>+</sup> is then investigated to determine the substituents in the C-28 oligosaccharide. The R<sup>3</sup>- and R<sup>4</sup>-substituents on the modified fucose can be obtained from the **D** fragments. The different [D+Na]<sup>+</sup> ions have  $m/z$  645, 415, 373 or 357. **D** fragments with  $m/z$  645 originate from the **B**- and **S**-type of saponins containing a fatty acyl. The position of the fatty acyl in the B- and S-type of saponins can be determined by the abundance of the R<sup>2</sup>-fragment. For **B1a** and **S1a** where fucosyl O-3 is substituted by the fatty acyl the R<sup>2</sup>-fragments at  $m/z$  517 have relative intensities of almost 100, compared to less than 30 for the compounds **B1–B3**, **B5**, **S1**, **S3**, **S5**, and **S6**. If the compound is a B- or S-type of saponins the procedure continues with determining the R<sup>5</sup>-substituent by investigating whether the **S** fragment contains a glucose or not. If there are ions at  $m/z$  463 or 595 there is a glucose in the R<sup>5</sup> position, hence the saponin is of B-type, **B1a**, **B1–B3** or **B5**. Otherwise the R<sup>5</sup> position is unsubstituted. The R<sup>2</sup>-substituent is then determined by investigating whether the **S** fragment contains a pentose or not. If there are ions at  $m/z$  433 or 595 there is a pentose in the R<sup>2</sup> position, as for compounds **B3**, **B5**, **S3**, **S5**, and **S6**, otherwise it is unsubstituted. The **S** fragment is a very important key fragment since it corresponds to the oligosaccharide at the C-2 in the fucosyl residue. For **B1**, **B1a**, and **B2** the  $m/z$  463 reflects the basic C-3 disaccharide and an additional glucose and for **B3** and **B5** the  $m/z$  595 the basic disaccharide and additional glucose and pentose residues. For **S1** and **S1a** the  $m/z$  301 reflects the disaccharide of the basic structure and for **S3**, **S5**, and **S6** the  $m/z$  433 the basic disaccharide and an additional pentose. If there is no peak at  $m/z$  645 there is an acetyl in the R<sup>4</sup> position instead of a fatty acyl at the modified fucosyl, and the **D** fragment reflects the substituent at the R<sup>3</sup> position. For **16a** and **16b** the  $m/z$  415 reflects the modified fucosyl with an acetyl and an O-acetylated glucose, for **17a** and **17b** the  $m/z$  373 the modified fucosyl with an acetyl and an glucose and for **4–10** and **11a** and **11b** the  $m/z$  357 the modified fucosyl with an acetyl and a rhamnose. Then the peaks corresponding to the **S** fragment are studied as for the B- and S-type of saponins. If the ions at  $m/z$  463 are abundant there is a glucose in the R<sup>5</sup> position, as for **16a**, **16b**, **17a** or **17b**, otherwise it is unsubstituted. If there are ions at  $m/z$

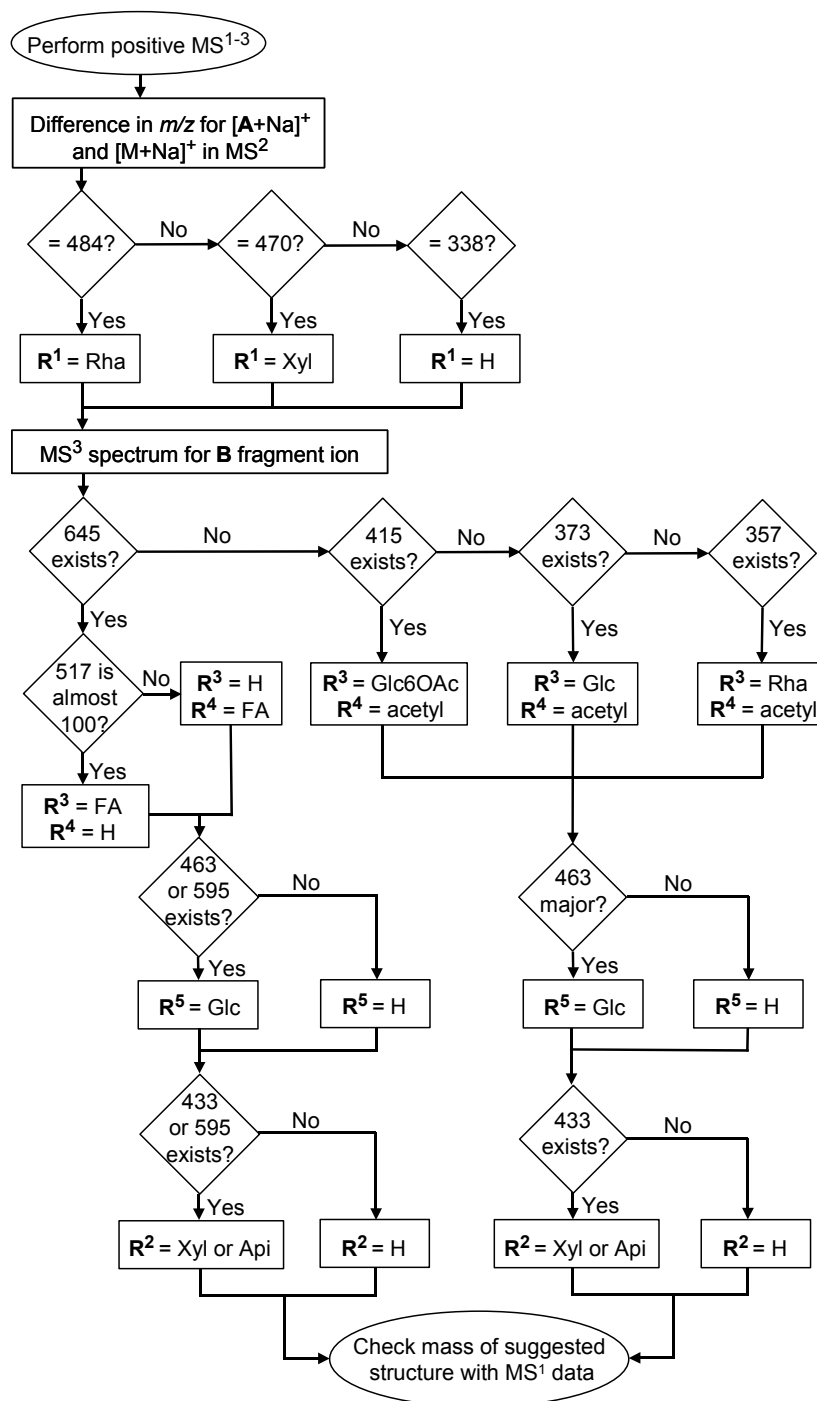


433 there is a pentose in the R<sup>2</sup> position, as for compounds **7–10**, otherwise it is unsubstituted

Finally the mass of the suggested structure is compared with the molecular mass obtained from the MS<sup>1</sup> spectrum for verification.

## Outlook

The flowchart procedure is verified for any of the 23 saponins investigated, but the procedure can also indicate the structure of unknown compounds of the same kind. This work is a part of a larger study intended to develop methods for automated identification of *Quillaja* saponins by means of ESI-IT MS<sup>n</sup> in combination with multivariate analysis of spectra.



**Figure 15.** Flowchart for structure analysis of *Quillaja* saponin components with positive MS<sup>1</sup>–MS<sup>3</sup> obtained after investigation of compounds 4–10, 11a,b, 16a,b, 17a,b, B1a, B1–B3, B5, S1a, S1, S3, S5, and S6. FA = fatty acyl

## Concluding remarks

Paper I-IV have shown some opportunities of mass spectrometry for analysis of carbohydrates and glycoconjugates.

MALDI-TOF MS can be utilized for studying the chain length distribution for debranched amylopectin from different sources. It is a faster technique than HPAEC-PAD and can be applied without prior removal of the amylose. However, the technique is found to be less reproducible than HPAEC-PAD for this purpose and it also overestimated the presence of longer unit chains and it was difficult at this point to determine a relation between the MALDI-TOF detection response and DP of the unit chains.

The two-step derivatization procedure presented in paper II results in carbohydrate derivatives that can be analyzed by MALDI-TOF MS at high sensitivity. Compared to underivatized material the DMBA-derivative of maltoheptaose showed a tenfold increase in sensitivity. The oligosaccharide derivative has a fixed positive charge at the modified reducing terminus. This feature probably enhances the sensitivity of the analysis but also affects the fragmentation pattern observed when the derivative is analyzed with MALDI-PSD TOF MS and presumably with other MS/MS-techniques. Due to the localized positive charge the fragment ion mass spectrum is dominated by fragments retaining the modified reducing terminus, which simplifies the interpretation of the MS/MS-data.

A method to structurally characterize the components in fractions from *Quillaja saponaria* Molina by ESI-IT MS<sup>n</sup> on several non-derivatized saponin components has been presented. A structure analysis procedure was formed based on a series of positive MS<sup>1</sup>–MS<sup>3</sup> experiments. To simplify the analysis, a flowchart was constructed containing a stepwise procedure aiming to determine the structure of the key fragments. In this way structural elements containing varying terminal monosaccharides or a C-18 fatty acyl as substituents in the investigated *Quillaja* saponins were identified. Peak intensity ratios in MS<sup>3</sup> spectra were found to be correlated to structural features of the investigated saponins and are therefore of value for identification of terminal monosaccharide residues.

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