Capillary zone electrophoresis of lipoarabinomannan by multi-layered concentration

Pitkänen, Eeva Marja

2022-02

Pitkänen , E M & Sirén , H M M 2022 , ' Capillary zone electrophoresis of lipoarabinomannan by multi-layered concentration ' , Journal of Separation Science , vol. 45 , no. 4 , pp. 945-959 . https://doi.org/10.1002/jssc.202100357

http://hdl.handle.net/10138/356140
https://doi.org/10.1002/jssc.202100357

unspecified
acceptedVersion

Beyond brilliant

Vanquish Neo UHPLC System

- **Beyond discovery,** all-in-one nano-, capillary-, and micro-flow LC for high sensitivity LC-MS workflows
- **Beyond innovation,** accelerating productivity with long-term, trouble-free operation at maximum performance
- **Beyond possibilities,** enabling LC-MS experts and novice users to get high-quality results, every time

Learn more

thermo scientific

Manuscript # jssc.202100357-R2

Capillary zone electrophoresis of lipoarabinomannan by multi-layered concentration

Eeva Marja Pitkänen, MSc & Heli Marja Marita Sirén, Assoc. Professor, Docent, PhD

Department of Chemistry, PO Box 55, FI-00014, University of Helsinki, Helsinki, Finland

Running title: Multi-step concentration of oligosaccharides in CE

Contacting: Corresponding author. e-mail: eevampitkanen12@gmail.com, eeva.m.pitkanen@helsinki.fi; Co-author. e-mail: heli.siren@icloud.com,

Abbreviations: AM – arabinomannan; **APTS** - 1-aminopyrene-3,6,8-trisulfonate; **Ara-1** - arabionose; **Ara-4** – arabinotetraose; **Ara-6** - arabinohexaose; **Ara-8** - arabino-octaose; **LAM** - lipoarabinomannan; **MDA** - malondialdehyde; **Man-1** - mannose, **Man-2** – mannobiose; **Man-3** - mannotriose, **Man-6** – mannohexaose; **MQ**, milli Q water; **WS**, water-stacking.

Keywords: Capillary zone electrophoresis, In-capillary ionization, Multi-layered on-line concentration, Lipoarabinomannan, Oligosaccharides.

ABSTRACT

The present paper describes a capillary zone electrophoresis method which relies on a multi-layered water-alkali solvent stacking with on-line ionization to enhance detection of mannose, arabinose, and their oligosaccharides, which are used as the migration profile standards but are also the distinctive structural components of lipoarabinomannan. Lipoarabinomannan is detected in patients having tuberculosis. The CE method with ionization of the whole saccharides without degradation in alkaline solution inside the capillary is based structural deprotonation of the molecules under ultrahigh pH conditions.

Received: 02/05/2021; Revised: 24/11/2021; Accepted: 09/12/2021
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jssc.202100357.

The validation of the CE parameters revealed that the 15-fold electrolyte - water -injection plug allowed detection of one third lower concentrations than detected without on-line concentration. For the first time, the better detectability was seen especially for highly polymerized, but otherwise poorly ionized, arabino-octaose. The applicability of the method for detecting whole large biological saccharide complexes was confirmed by the glycolipid lipoarabinomannan. For the first time also, the migration of the indestructible lipoarabinomannan was detected together with oligosaccharides used representing the capping units, namely mannose, mannobiose and mannotriose. The myo-inositol-phosphate-lipid unit was seen to migrate separately from the arabinomannan, since it was hydrolyzed from one lipoarabinomannan product under alkaline conditions in CE.

1. Introduction

Mycobacterium tuberculosis, human pathogen has a complex, lipid-rich cell wall composed of polysaccharides, peptidoglycans and lipoarabinomannans (LAMs), the latter consisting of saccharides and a *myo*-inositol- phospholipid unit [1, 2]. The polysaccharide backbone of LAMs has around 33 mannose units as a cyclic mannopyranose and approximately 60 arabinose units as a furanose ring with branched chains either as linear tetra-arabinosides or a branched form of hexa-arabinosides [2-5]. In addition, there are manno-oligosaccharide caps located on the ends of the arabinose units [5-7]. These caps can have either mono-, di- or trimannose units, with the dimannose units being more common [4].

Capillary zone electrophoresis (CZE) gives versatile possibilities to study the whole large biocompounds by modification of their saccharide configurations or manipulation of the separation solution used in the capillary. CZE has been used in characterization of the lipoarabinomannan structure, especially after degradation of LAM identification of the manno-oligosaccharide capping parts as reviewed by Lamari *et al.* [8]. Different approaches

for analyzing saccharides by CZE have been developed e.g., the detection of saccharides reviewed by Paulus and Klockow [9], El Rassi [10], and Mantovani *et al.* [11].

Modification of saccharide molecules with the fluorescent dye, 1-aminopyrene-3,6,8-trisulphonate (APTS), has often been used. The reagent forms ionized molecules by amination of the aldehyde group of monosaccharides. Then, the detection is based on the UV or fluorescence properties of the derivatives. Lately, the yield of oligosaccharide labeling with reductive amination by using APTS was determined to be only ~10% [12]. In the experiments conducted by Monsarrat *et al.* [6], lipoarabinomannan was first degraded in a mild acid solution and then the neutral monosaccharides were freshly ionized during complexation. The quantitatively of the methodology was not doubt in the paper.

In a pre-capillary derivatization procedure, the APTS-labelled monosaccharides were suspended into the aqueous electrolyte solution for CE analysis [13]. They were separated using triethylammonium electrolyte solution (pH below 4) with reversed polarity and detected by laser-induced fluorescence (LIF) at the excitation wavelength of 488 nm and the emission wavelength of 520 nm [13, 14]. LIF detection in CZE was also used in the study of Nigou *et al.* [5]; these researchers characterized the lipoarabinomannan structure, determined the number of capping parts, and the glycosidic linkages present in the LAM molecule. Gilleron *et al.* [14] characterized the capping units of LAM with commercially available coated capillaries as APTS derivatives by LIF detection.

Mostly when CE is used, structural identification is achieved by a combination of CZE and electrospray ionization in MS. Ludwiczak *et al.* [15] combined CE with matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) in the structural elucidation of the lipoarabinomannan and manno-oligosaccharide caps.

Tetraborate complexation has also been used in the determination of saccharides [16]. These anionic saccharide-tetraborate complexes can be detected with direct UV at 195 nm [16].

Furthermore, saccharides have been complexed with copper ion for their detection as copper complexes at the 254 nm wavelength [10]. A third option to separate and detect saccharides by CZE is to apply a very alkaline electrolyte solution i.e., pH above 12 [10]. Under alkaline conditions, monosaccharides undergo both reversible and irreversible transformations such as ionization, mutarotation, enolization, and isomerization with the end products being enediol anions [17, 18]. Ionized complete saccharides, being carbohydrate enediolates, were detected by direct UV absorption at 270 nm as described by Rovio *et al.* [19, 20]. Their electrophoretic mobilities depend on analyte charge, mass, or the ratios, but also on size. Sarazin *et al.* [21] suggested that the direct measurement of whole saccharide molecules in alkaline solutions is due to photo-oxidation of the saccharide compounds under UV light [20]. It has been speculated that in the presence of UV light (i.e., during their detection) hydroxyl radicals or superoxide can oxidize carbohydrates producing malondialdehyde (MDA) or dihydroxyacetone [21, 22]. The UV-absorbing intermediates have been proposed to be malonenolate with the UV absorption maximum at 267 nm [21-23]. Since saccharides for ring structures in water, the ionization may also be due to the -CH_2OH groups in the molecule body at ultra-high pH solvents.

The determination of mono- and oligosaccharides using alkaline solutions with high pH values has been described by several groups. Metsämuuronen *et al.* [24] focused on the xylo-oligosaccharides whereas Hiltunen *et al.* [25] profiled wood-based oligosaccharides such as xylo-, manno-, and cello-oligosaccharides. Lately also, mono-, di-, and trisaccharides as well as cellodextrin oligosaccharides have been studied with CE [26, 27].

This study presents a CZE method with sample multi-stacking for the determination of selected oligosaccharides which were studied as whole molecules by keeping them constant and getting them ionized without derivatization. The oligosaccharides were selected by knowing the LAM structure. LAM was investigated to detect the structural hydrolysis in the

electrophoretic environment in CZE. Mannose and the manno-oligosaccharides used in the study were the similar as the mono-, di-, and tri-mannose caps in the lipoarabinomannan complex. Arabino-oligosaccharides are intended to be analogical with the branched arabinose chains in the structure of the LAM complex. This present paper represents for the first-time the analysis and detection of otherwise very poorly ionized arabino-oligosaccharides. This is also the first time to show that the migration profile of LAM complex can be qualified as a whole structure except the cleavage of *myo*-inositol- phospholipid unit. Further, it was observed that the migration of LAM is due to the ionization of manno-oligosaccharides of the LAM molecule. The work has novelty value in investigation of electrophoretic properties of LAM, although very preliminary research on LAM has already been described by Sirén *et al.* [28].

2. Materials and methods

2.1. Reagents

The basic salts for the preparation of the electrolyte solution, tri-sodium phosphate (Na_3PO_4, purity > 96 %) and potassium hydroxide (KOH, purity > 90 %) were purchased from Sigma-Aldrich Finland Oy (Helsinki, Finland). 1 M and 0.1 M NaOH and 0.1 M HCl were from FF-Chemicals (Haukipudas, Finland). D-mannose and D-(-)-arabinose were from Calbiochem-Merck-Millipore (Espoo, Finland), and D-(+)-xylose was from Sigma-Aldrich of purity > 99% each. 1,4-β-D-mannobiose and 1,4-β-D-mannotriose (purity > 95% both), 1,4-β-D-mannohexaose (purity > 90%), and 1,5-α-L-arabinotetraose, 1,5-α-L-arabinohexaose, and 1,5-α-L-arabino-octaose (purity > 95% each) were all purchased from Megazyme International, Ireland. *myo*-inositol (microbiological quality) was from Merck (Espoo, Finland). Lipoarabinomannan preparation was purified lipoarabinomannan (LAM), NR-14848 (*Mycobacterium tuberculosis,* strain H37Rv) from BEI Resources (NIAID, NIH, USA). Ultra-high-purity water (Milli Q water, Type III water) was obtained from Millipore

water purification system (Millipore, Espoo, Finland, resistivity of 18.2 MΩ·cm at +25 °C). The properties of the mono- and oligosaccharides are presented in Table 1.

2.2 Electrolyte and carbohydrate solutions

Electrolyte solutions were prepared by mixing 120 mM aqueous stock solution of tri-sodium phosphate volumetrically with 1 M aqueous solution of sodium hydroxide and 1 M aqueous stock solution of potassium hydroxide. All electrolyte solutions as well as the stock solutions of tri-sodium phosphate were filtered through 0.45 µm disposable filters (GHP Acrodisc, Pall Life Sciences, Ann Arbor, USA) and degassed in an ultrasonic bath for 15 min prior to further use. The final optimized electrolyte solution used for CZE analyses composed of 12 mM Na_3PO_4, 135 mM NaOH, 112 mM KOH (pH 13.2 at +23 °C).

The stock solutions of 100 µg/mL concentration for the individual mono- and oligosaccharides were prepared in Milli Q water. The solutions were stored at room temperature (+20 °C - +23 °C) and for longer periods at +4 °C. Working solutions of the saccharides were prepared from stock solutions by diluting with Milli Q water. The lyophilized lipoarabinomannan preparation was resuspended with Milli Q water to a 0.5 mg/mL concentration before use and stored at -20 °C.

2.3. Instrumentation

A Hewlett-Packard 3D CE instrument (Agilent, Waldbronn, Germany) equipped with a photodiode array detector (wavelength range 190-600 nm) was used with ChemStation programmes (Agilent) for CZE analyses. Detection of saccharides was done with direct UV at the wavelength of 270 nm with a bandwidth of 10 nm.

Uncoated fused silica capillaries (Biotaq, Inc., Gaithersburg, USA) of inner and outer diameters of 50 µm and 365 µm, respectively, and of capillary lengths from 40 cm to 80 cm were used in method optimization. The final capillary length for the method was 60 cm of total length (L_{tot}) with the effective length (the length from the capillary inlet to the detector,

L_{det}) being 51.5 cm. The new capillaries were conditioned by rinsing with 0.1 M NaOH, Milli Q water, and the electrolyte solution. The conditioning was also repeated each time before new analysis series.

2.4. Electrophoretic conditions

Fresh electrolyte solutions were prepared weekly. Solvent freshness was assured by renewing solutions between each analysis. The optimized electrolyte contained 12 mM Na_3PO_4, 135 mM NaOH, and 112 mM KOH. Before separation analysis, the capillaries were conditioned by flushing with 0.1 M HCl, Milli Q water, 0.1 M NaOH, and Milli Q water, each for 2 min, and with the electrolyte solution for 5 min between injections with a wait time of 0.3 min. At the end of CE analysis, the capillaries were further flushed with Milli Q water.

The electrodes and the capillary ends were left into Milli Q water vials for storage when the sequence analyses were done.

During the preliminary assay of development phase, temperatures of +23 ^{o}C, +27 ^{o}C and +30 ^{o}C were studied. With temperatures higher than +25 ^{o}C, the baseline noise became strong, minimizing resolution. In the final conditions, the temperatures of the capillary cassette, the sample carousel, and the CE laboratory were stabilized at +25 ^{o}C, +24 ^{o}C, and +21 ^{o}C +/- 2 ^{o}C, respectively.

2.5. Solvent and volumes in concentration procedure

The CZE separations were conducted from anode to cathode (the anode in the inlet side). Sample injection was carried out hydrodynamically with the pressure of 50 mbar (0.73 p.s.i.). For the basic alkaline method (subsequently called the Basic method), the samples were injected for 15 s correlating to 21.67 nL, volume which was 2.14 % of the capillary volume. The injection plug length was 11.0 mm (https://sciex.com/ce-features-and-benefits/ce-expert-lite) with the optimized separation voltage of +7.5 kV. In the optimized multi-layered on-line concentration method, i.e., in the Water Stacking method (subsequently referred to as the WS

method) saccharide samples were enriched using on-line concentration and electrical stacking with consecutive injections of 15 times of both Milli Q water and the electrolyte solution at 50 mbar (0.73 p.s.i.) for 5 s (á 7.22 nL); before each sample introduction 108.3 nL water and 108.3 nL of the electrolyte were introduced sequentially: á 7.22 nL (water plug) & á 7.22 nL (electrolyte plug) in a batch for 15 times in the same order). The total volume of the stacking liquids was 216.6 nL corresponding to 18.4 % of the capillary volume. The plug batch was performed after the electrolyte zone which filled the capillary before start of the process. The sample was introduced after the 15-fold dual-plug batch: A sample volume of 86.7 nL was done using 60 s injection time corresponding to 8.55 % of the capillary volume with an injection plug length of 44.04 mm. The ionic strength and pH of the non-ionized water plugs are zero (0 mM) and 7.0, respectively. In electrolyte plugs they are 283 mM and 13.2, respectively. The voltage during the saccharide ionization and compound separation was +7.5 kV.

2.6. Description about the stacking procedure

After sample injection and switching the electricity on, saccharides reached the nearest boarder of the first electrolyte zone in the capillary. The analytes were ionized (partially first, thoroughly later) and then moved through the MQ water zone to another electrolyte zone, etc. When moving from electrolyte zone to another the rest saccharides (which were not yet anions) were ionized by alkaline catalyze. In the analysis start, the MQ water zones were supposed to be promptly separate areas between the electrolyte zones in purpose to reduce movement of ionized saccharides and to make the electrolyte surrounded by water plugs, by being slightly less conductive compared with the main electrolyte. Thus, the electric field was reduced in the plug area, which could be detected from the lower current in the system compared with that in the main analysis. The Figure 1S describes the stacking process (*Supplementary Information*).

When saccharides have no charge in CE, they move with electroosmosis. But in alkaline solution they undergo ionization, although the reaction is slow. Thus, they could enter the deceleration-accelerating zones (correlated with ionization and ionic strength of solvent plugs) before the separation started. When the zones perform the stacking, saccharides are mixed with the main electrolyte. Then, the saccharides were supposed to be totally ionized. It could be detected from their velocity under the applied electric field (7.5 kV in 60 cm capillary). Constant electric field (125 V/cm) was detected from the analysis current since during analysis it was kept stabile at 140 μA. Various kinds of stacking processes are also discussed in detail by Chen et al. [29], but this kind of methodology was not informed before.

2.7. Determination of limit of detection, limit of quantification and regression equations

In the Basic method, the eight concentrations 7.5, 10, 15, 20, 30, 40, 60 and 80 μg/mL were used to calculate the calibration range for all other saccharides except Ara-8 (Table 1). Ara-8 was quantified by using the seven concentrations, i.e., 10, 15, 20, 30, 60, 80 and 120 μg/mL. Four replicates of each saccharide level were tested except for five replicates for Man-1 (Table 1) and three for Ara-8. In the WS method, an eight-point calibration was created from 2.5, 3.75, 5.0, 7.5, 10, 15, 20, and 30 μg/mL concentrations for the saccharides Man-1, Man-2, Man-3, Man-6, Ara-1, and Ara-4 (Table 1). In addition, seven-point calibration curves were used for the oligosaccharides Ara-6 (Table 1) and Ara-8 (3.75, 5.0, 7.5, 10, 15, 20 and 30 μg/mL). Four replicates of each saccharide level were used except for five replicates for Man-2 and Ara-1. (More information in *Supplementary Information*).

2.8 Quantity and reliability of the method

The determinations of the lowest calibration-based detectable amounts of the analytes in the method**,** the lowest concentrations in samples when injected, i.e., method limits of detection (MDL), the lowest calibration-based quantitative amounts by the method, i.e., limits of

quantifications (LOQ), the regression equations, and repeatability according to Ref. [30] are presented in *Supplementary Information*.

3. Results and discussion

Several publications describe about ionization of neutral saccharides in a highly alkaline electrolyte in CE [19, 20, 25, 27, 28, 31]. In the present study, several electrolyte solutions were tested to obtain ionized saccharides and to lower their concentrations for quantification. Saccharides were chosen to correlate the functionality of lipoarabinomannan (LAM) structure, which contains the arabino- and manno-oligosaccharides. A multi-layered preconcentration procedure was developed for on-line concentration of the analytes during electrophoresis procedure.

3.1. Optimization of the electrolyte solution

Based on the findings of Rovio *et al*. [18, 19] rather high concentrations of NaOH were beneficial for ionization of saccharides. Thus, a 135 mM concentration of NaOH was chosen to decrease mobility (a viscose and high-conducting solution), to improve resolution (for allowing the process for ionization), and to enrich saccharides in multi-layered concentration procedure. However, in contrast to the results of Rovio *et al.* [18], we found the concentration of KOH to increase the resolution of the studied saccharides, and the peaks were sharper with larger peak areas (Table 1S in the *Supplementary Information*). Similar findings were reported by Hiltunen *et al*. [25]. In turn, Zhao *et al.* [25] stated that the increased pH of the electrolyte solution mostly affected the separation and resolution of the saccharides. By using the optimized concentrations, i.e., 12 mM Na_3PO_4, 135 mM NaOH, and 112 mM KOH in the electrolyte solution, the peaks in the electropherograms were acceptably sharp using both +10 kV and +7.5 kV voltages (Figures 1 and **2**). By evaluating the data with concentration titration for each saccharide, the peak widths were narrow in all cases (Worth noticing: Nearly equal values also with the highest concentration).

3.2. Optimization of the separation voltage

During method development, the separation voltage was first kept constant at +10 kV. Then, the ionic strength and the pH of the electrolyte solution were studied. The separation voltage was as low as possible for moderate current with minor Joule heat formation in the capillary. In turn, the increased heat affected negatively on the separation of the analytes by decreasing the detectability. It was analogical to the published data of Mayer [32] on the negative effects generated from heat formation when he informed that the repeatability of the analyses is better by lowering the separation voltage. Zhao *et al.* [26] showed that the separation of the glucose, mannose, xylose, fructose, ribose, arabinose, and galactose monosaccharides, the sucrose and lactose disaccharides, and the *myo*-inositol polyol could be improved when the voltage was decreased from +16 kV to +8 kV. In the present work with large saccharide oligomers, the effect of voltage was detected in peak widths and areas, but not significantly in peak heights (*Supplementary Information:* Figure 2S, Table 1S).

To decrease the Joule heat and the current, different separation voltages were examined using mono and oligosaccharides of both mannose and arabinose at two concentrations (20 µg/mL and 60 µg/mL). With the separation voltage of +7.5 kV, the current was 140 µA; this was at an acceptable level regarding the Joule heat phenomenon. The voltage of +7.5 kV also increased the analytes´ intensities (see Fig. S-2 in the *Supplementary Information*). It seemed that the lower voltage had a greater effect on mannose saccharides than arabinose saccharides. This could be due to the differences in the ring structures of the carbohydrates; mannose is a hexose being more easily ionized than arabinose with a pentose ring. The conditions also favored ionization of arabinose.

3.3. Optimization of the on-line concentration and stacking

Sample stacking is a straightforward and very frequently used method to increase sensitivity in CZE by concentrating ions in sample solutions [29, 36-37]. The electrophoretic mobility of

an ion in a solution having a low conductivity is faster than that in a high conductivity solution. The sample is often diluted with a low conductivity solution or with pure water, while the electrolyte solution is prepared to have high ionic strength and high conductivity [29, 34-36].

Low ionic strength of Milli Q water was chosen as the sample diluent. In addition, MQ-water and the alkaline electrolyte solution pressurized as the two adjacent zones in the capillary were used to obtain great differences in pH, ionic strength, and conductivity. When a positive voltage was applied, a discontinuous electrolyte field was formed and weakly acidic compounds, as saccharides became focused on the boundary of the pH zone created by the sample plug and electrolyte [34]. At the same time, the pH-mediated sample stacking took place (*Supplementary Information;* Figure 3S).

We compared the on-line stacking behavior by using 5, 10, and 15 times repeated consecutive injections with plugs of MQ-water and the electrolyte for the adjacent zones. The results were compared to those obtained with the non-stacking method, i.e., without a solvent plug, by detecting the effect with mono- and oligosaccharides of mannose and arabinose (see Fig. 4S in the *Supplementary Information*). The concentration effect with sequential 15 times executed consecutive solvent zones were made of 0 mM and 283 mM concentrations. The adjacent plug composition was beneficial for both Ara-1 and arabino-oligosaccharides, especially at low concentrations, e.g., increasing peak areas of arabinoses by 50 %. The advantage of the pre-concentration was that the changes in pH and ionic strength not only favored the ionization of the hydroxyl groups of arabinoses but also created stacking boundaries for low-concentrated analytes. Lower and higher conductivities and pH differences between the plugs resulted in the formation of multiple adjacent layers with low and high conductivity zones. The system was very effective for both ionization and stacking of arabinose compounds. The formation could be detected with the mobility of

electroosmosis which was 8 min faster in the water-electrolyte layering than in the Basic method. Therefore, the multi-layered on-line concentration performed by 15 times plugin with water mediated stacking was chosen in the optimized CZE method.

3.4. Optimization of time in the pressurized sample introduction

First, the time of 15 seconds and the injection pressure of 50 mbar (0.73 p.s.i.) were used during the method development, when +7.5 kV was used for creating the electrical field in CZE. Later, the effects of injection times of 30, 45, and 60 s with 50 mbar (0.73 p.s.i.) were also studied using two concentrations of mannose and arabinose including their oligosaccharides. The common trend was that peak areas of the analytes increased when the sample injection time was increased from 15 s to 60 s (see Fig. S-4 in the *Supplementary Information*). However, the trend was not linear due to the slow ionization process and differences in the masses and charges of the saccharides. It was observed that a larger sample volume favored manno-oligosaccharides more than arabino-oligosaccharides due to poorer ionization properties of arabinose in the alkaline solution. Because of the observations and based on the optimization results, the injection time of 60 s was chosen for the CZE method.

3.5. Comparison of sensitivities obtained by the two methods

The efficiency of the primary CZE method (Basic method) and the optimized multi-layered on-line concentration method were compared using 20 µg/mL and 60 µg/mL concentrations of the studied saccharides as a mixture and separately the behavior of LAM with the concentration of 200 µg/mL. The lowest effect of concentration increase was noticed with Man-6; it was 3.8 times and 2.0 times enhanced at 20 µg/mL and 60 µg/mL, respectively. Mostly, the increase was between 3.4 and 10.0 when the multi-layered boundaries were used to focus the saccharide zones. The multi-layered on-line concentration method enables introduction of rather large sample volumes to capillary, which was especially beneficial

when there were low concentrations of analytes (Figure 5S in the *Supplementary Information*) and when real patient samples would be studied.

3.6. Validation of the methods

The Basic method and the multi-layered on-line concentration method (WS) were validated and calibrated using mono- and oligosaccharides of mannose and arabinose. For the purpose, the method limit of detection (MLD) and the limit of quantification (LOQ) for all analytes were studied. Furthermore, the concentration calibration lines with the correlation coefficients (R^2) were determined for each of the saccharides. The MLD (the lowest concentrations of the analytes in samples from which injected and studied by the methods and the LOQ (the experimentally measured amounts of the analytes measured with concentration calibration were used for quantitative control. In addition, the lowest calibration-based quantitative amount of each analyte studied by the method and the lowest calibration-based detection amount of the analytes studied by the method were measured. The respective equations as a function of the concentrations are presented as average values of 4-5 repetitions for each saccharide (*Supplementary Information*). The repeatability of the methods was determined using mixtures of mannose saccharides.

3.6.1. Method limit of detection, limit of quantification and regression equations

The MLD values and those of the lowest calibration-based quantitative amount of each analyte obtained with the Basic method for Man-1 and Ara-1 were 7.5 µg/mL. However, the corresponding values with the lowest calibration-based quantitative amount were 3.2 µg/mL and 1.1 µg/mL, respectively. Results obtained by the Basic method are like the published values [20, 25] nevertheless of the differences between the electrolyte solutions used in the other publications compared to the electrolyte used in this study (12 mM Na_3PO_4, 135 mM NaOH and 112 mM KOH). Hiltunen *et al.* [25] and Rovio *et al.* [20] utilized a different composition in their alkaline electrolyte solutions. Rovio *et al.* [20] used 130 mM NaOH and

36 mM Na_2HPO_4 (pH of 12.6) whereas Hiltunen *et al.* [25] used a more complex solution composed of 35 mM Na_3PO_4, 35 mM K_3PO_4, 90 mM NaOH and 65 mM KOH for separation of five xylo-, three manno-, and five cello-oligosaccharides.

Zhao *et al*. [26] has used two different electrolyte solutions at different pH values. With the electrolyte solution used by Hiltunen *et al.* [25] at a voltage of +14 kV, Zhao *et al*. [26] estimated the LOD and LOQ values for mannose as 3 and 5 µg/mL, respectively. When they used the electrolyte solution prepared only from sodium hydroxide (130 mM) with pH of 13.0 and separating voltage of +10 kV, the LOD and LOQ values for Man-1 were slightly lower being 2 and 4 µg/mL, respectively. They could not separate fructose and Ara-1 at pH 12.6 but succeeded to do their separation at pH 13.0 with the LOD and LOQ values for arabinose 2 and 4 µg/mL, respectively. These are in good agreement with the results obtained with the Basic method for Ara-1, since the LOD and LOQ concentrations were 1.1 µg/mL and 3.8 µg/mL, respectively. However, the conditions in Basic method did not support the easy ionization of arabino-octaose (Ara-8). Therefore, in the WS method its MDL and LOQ values were much higher, being 10.0 µg/mL and 53.6 µg/mL, respectively. In the electropherograms, the peaks of Ara-8 were wide due to low reaction kinetics and low-speed migration (Tables 2S and 3S in the *Supplementary Information*).

Both the MDL and LOQ values of the manno-oligosaccharides studied with the WS method are approximately three times lower than measured with the Basic method. The MLD values of the arabinose oligosaccharides with the WS ranged from 0.5 to 3.0 µg/mL i.e., clearly lower than the values obtained with the Basic method. The LOQ values with the WS method ranged from 1.6 to 10.0 µg/mL. To obtain better identification the studied oligosaccharides needed multi-layered stacking to promote their ionization for concentrated anion zones.

With the Basic method, the concentration response curves showed good linearity for all the other saccharides except for Ara-8 (the correlation coefficient, R^2 – value, was less than

0.950). With the WS method, the concentration response curves also displayed good linearity with the correlation coefficients (R^2) greater than 0.950 for all the saccharides (see Table 4S in the *Supplementary Information*).

When manno-oligosaccharides were studied from wood [24, 25], the LOD and LOQ values for Man-4 were 3.8 µg/mL and 12.8 µg/mL with migration the time of 59.0 min, for Man-3 values were 3.1 and 10.3 µg/mL with the migration time of 63.4 min, and for Man-2 values were 4.4 and 14.5 µg/mL with the migration time of 72.3 min. The values are at similar magnitude as calculated with our Basic method, although the effective length of the capillary was 86 cm instead of 51.5 cm and the CE instruments were from different manufacturers. In the present study, with the WS method the same manno-oligomers migrated within 28.3 min, and with the validated Basic method they moved within 62 min including the corresponding monomer. In the work reported in ref. [24] Man-2 migrated very late within 72 min. To compare the recognition of Man-3 it appeared 2.4 time more intensively with the WS method than with the method used in ref. [24]. On the contrary, Man-2 appeared in the profile as a more intensive peak when the performance of the two Basic methods were compared. Other results are compiled in **Table 2**.

3.6.2. Migration and effective mobilities

The migration times (t_R) and effective mobilities (μ_e) of the saccharide analytes obtained are presented in **Table 3**. The effective mobility was used instead of the migration time to show the electrophoretic repeatability of the analytes by considering the electro-osmotic flow which correlated with the changes during analyses. The effective mobility, μ_e, was calculated using Equation (1) [31].

Man-6 which has the highest ionization ability (the highest negative charge) and the highest molar mass of the manno-oligosaccharides having cis diol on a six-membered ring (at the C2 and C3 positions), migrated the most rapidly [24], followed by Man-3, Man-2, and Man-1

based on their smaller ratios. The results also showed that Man-1 migrated faster than Ara-1, which originate from hexoses that migrate before pentoses in alkaline solutions, as also shown by Rovio *et al.* [19, 20]. In contrast to the migration order of mannose saccharides, Ara-1 displayed the shortest migration time of all the analyzed arabino-oligosaccharides. In fact, there are hardly any differences between the electrophoretic mobilities of the arabino-oligosaccharides. The studied arabino-oligosaccharides are structurally so close to each other that even with the WS method, separation was not possible. This is probably due to the presence of the pentose ring which means that the arabino-oligosaccharides possess poorer ionization properties. Thus, these oligosaccharides cannot be separated from each other under the conditions of either the Basic or the WS method with the experimental parameters chosen for LAM determination in this study (Table 3).

All the migration times of the analyzed saccharides obtained with the WS method were shortened and approximately halved as compared to the Basic method (Table 3). The RSD % values obtained with the WS method were rather high but still at an acceptable level (RSD less than 10%). The higher RSD % values were due to the discontinuity of the conductivity ranges in solutions because of the multi-layered on-line concentration process, i.e., the water-stacking of the WS method.

3.6.3. Repeatability

The repeatability of the methods was evaluated using a mixture of four mannose saccharides (Man-1, Man-2, Man-3 and Man-6) at two concentrations with four-fold concentration differences. With the Basic method, the mixtures of mannose saccharides were analyzed at concentrations of 7.5 µg/mL and 30 µg/mL. With the WS method, the analyzed concentrations of the mixtures were 5 µg/mL and 20 µg/mL. The results are presented in the Table 3S in the *Supplementary Information*.

The RSD % values of mobilities with the Basic method were determined by either with five repetitions per vial or with five separate vials. The all RSD% were below 1 % at both concentration levels of the saccharides. The standard deviations were lower at concentrations of 30 µg/mL nevertheless of the repetitions or separate vials in the measurement. The only exception was Man-2, where the RSD % of mobilities was small at concentration of 7.5 µg/mL with five separate vials used for sample introduction in the measurements.

With the WS method the RSD % values of mobilities were overall higher than with the Basic method. With the other saccharides except Man-6, the RSD % values were at the highest 2.9 %. With Man-6 the RSD % was randomly high (13.0 %) at concentration of 20 µg/mL measured with four repetitions per vial compared to the concentration of 5 µg/mL where the RSD % was 3.2. The RSD % values were not clearly better determined by either with repetitions per vial or with separate vials as was already seen with the Basic method. The RSD % values obtained with both methods were good although in the WS method, when the discontinued conductivity zone to the multi-layered on-line concentration steps were used (*Supplementary Information*).

3.7. Migration profiles of the lipoarabinomannan compared to its structural components.

The optimized method parameters on the migration of the lipoarabinomannan preparation resulted in larger peak areas also for LAM (in the *Supplementary Information*). The migration profiles of LAM, *myo*-inositol phospholipid, and mixture of mannose saccharides with the Basic and WS methods are shown in Figure 2.

There were similarities in the electropherograms of the LAM analyte obtained with the studied methods. The LAM peaks were quite wide due to the heterogeneity of the LAM molecular complex. The migration profile of (lipo)arabinomannan was located between that of Man-3 and Man-2 (Figure 2). We postulate that the electrophoretic migration of LAM is based on the mannose caps of the molecule. In the LAM molecule, hydrophilic mannose caps

are located on the outer surface of the molecule and are free to react when there are alkaline electrolyte conditions. Mannosaccharides become ionized under alkaline conditions and produce a negative charge on the LAM molecule. Although the most common capping units are the di-mannose units [4], also the mono- and tri-mannose units can be involved in the ionization and therefore also in the migration of the lipoarabinomannan. Furthermore, the mannose units in the structure of the mannan backbone can also become ionized. Nigou *et al.* [4] reported that there are approximately seven mannose caps per molecule of *M. tuberculosis* strain H37Rv which was the strain examined in this study.

The lipoarabinomannan molecular complex is labile in alkaline conditions [38] with the *myo*-inositol- phospholipid part of the molecule being released. The electrolyte solution used in the present CZE methods seemed to promote this degradation. Based on the electropherogram, **Fig. 2b**, peak 5 could be the released *myo*-inositol- phospholipid complex. This complex migrates faster than pure *myo*-inositol **(Fig. 2c**) due to the higher molecular weight of this lipid-containing complex. The main peak 6 in **Fig. 2b** was arabinomannan (AM). The multi-layered on-line concentration in the WS method meant that the *myo*-inositol- phospholipid complex overlapped during electro-osmosis (**Fig. 2e**).

3.8. Migration profiles of the arabinose saccharides

The migration of lipoarabinomannan seemed to be based on the ionization of the mannose units rather than on the arabinose units. All the studied arabinose saccharides migrated more slowly than lipoarabinomannan (WS method, Figure 2 e) and mannose saccharides (WS method, Figure 2 d). The electropherograms of arabinose saccharides obtained with the WS method are presented in Figure 3. The profile of the mixture made of Ara - 1, Ara -4, and Ara - 6 (Figure 3 a) indicates that only Ara - 1 could be separated from the rest of the arabino - oligosaccharides. In case of mannoses, the migration order was the largest first and the smallest last; but in case of arabinoses, they moved in the reversed order by the arrangement

of Ara - 1, Ara -4, Ara -6, and Ara - 8 (Figures 3 b - e). Most probably, the reason is the arabinose units which are as furanose rings and then the hydrolysis step is restricted. Moreover, the separation of large arabinoses is reduced by the viscose electrolyte and the WS method. The electropherograms of arabinose saccharides obtained with the WS method are presented in **Fig. 4**. The profile of the mixture made of Ara-1, Ara-4, and Ara-6 (**Fig. 4a**) indicates that only Ara-1 could be separated from the studied arabinose oligosaccharides while Ara-4 and Ara-6 migrated as a single peak.

4. Concluding remarks

The presented multi-layered on-line concentration with 15-fold water-electrolyte plug combination in stacking revealed increased sensitivity for both mannose and arabinose, and the corresponding oligosaccharides. Lipoarabinomannan a glycolipid, was used as a representative of a complex biological carbohydrate containing molecule to show the applicability of the developed multi-layered concentration method in CZE using UV detection. The method itself was based on the ionization of carbohydrates in an alkaline environment.

This study showed for the first time that the migration of LAM seemed to be due to the migration of the mannose capping units of the molecule rather than the arabinose units of the molecule structure. The alkaline lability of the molecule was observed during the CZE, the *myo*-inositol- phospholipid unit of LAM migrated separately from the arabinomannan under high alkaline conditions in the study. The very preliminary results of the migration of LAM confirm that the multi-layered on-line concentration could be applicable for detecting large biological molecular complexes consisting of saccharides.

Acknowledgments: Docent Dr. Tamara Tuuminen is acknowledged for the inspiring research idea of analyzing lipoarabinomannans using capillary zone electrophoresis. Dr. Ewen McDonald is thanked for linguistic revision of the manuscript.

Funding: This work was supported by the following private Finnish foundations: The Tuberculosis Association of the University of Tampere, the Nummela Sanatorium Association, the Research Foundation of the Pulmonary Diseases and the Research Foundation of Clinical Chemistry. The funding sources was not involved in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Conflict of interest statement: None. We declare that we have no financial relationships or personal relationships with any commercial entity or person that has any interest in the subject of this paper.

References

[1] Brennan P.J., Nikaido H., The envelope of mycobacteria. *Annu. Rev. Biochem.* 1995, *64*, 29-63.

[2] Chatterjee D., Khoo K.H., Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects: a mini review. *Glycobiology* 1998, *8*, 113-120.

[3] Mishra A.K., Driessen N.N., Appelmelk B.J., Besra G.S., Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction: a review. *FEMS Microbiol Rev.* 2011, *35*, 1126-1157.

[4] Nigou J., Gilleron M., Puzo G., Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 2003, *85*, 153-166.

[5] Nigou J., Vercellone A., Puzo, G., New structural insights into the molecular deciphering of mycobacterial lipoglycan binding to C-type lectins: Lipoarabinomannan glycoform

characterization and quantification by CE at the subnanomole level. *J. Mol. Biol*. 2000, *299*, 1353-1362.

[6] Monsarrat B., Brando T., Condouret P., Nigou, J., Puzo, G., Characterization of mannooligosaccharide caps in mycobacterial lipoarabinomannan by capillary electrophoresis/electrospray mass spectrometry. *Glycobiology* 1999, *9*, 335-342.

[7] Petzold, C.J., Stanton, L.H., Leary, J.A., Structural characterization of lipoarabinomannans from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* by ESI mass spectrometry. *J. Am. Soc. Mass Spectrom.* 2005, *16*, 1109-1116.

[8] Lamari, F.N., Gioldassi, X.M., Mitropoulou, T.N., Karamanos, N.K., Structure analysis of lipoglycans and lipoglycan-derived carbohydrates by capillary electrophoresis and mass spectrometry: a review. *Biomed. Chromatogr.* 2002, *16*, 116-126.

[9] Paulus, A., Klockow, A., Detection of carbohydrates in capillary electrophoresis: a review. *J. Chromatogr A* 1996, *720*, 353-376.

[10] El Rassi, Z., Recent developments in capillary electrophoresis and capillary electrochromatography of carbohydrates species: a review, *Electrophoresis* 1999, *20*, 3134-3144.

[11] Mantovani V., Galeotti, F., Maccari, F., Volpi, N., Recent advances in capillary electrophoresis separation of monosaccharides, oligosaccharides, and polysaccharides: a review. *Electrophoresis* 2018, *39*, 179-189.

[12] Krenkova J., Dusa F., Cmelik R., Comparison of oligosaccharide labeling employing reductive amination and hydrazone formation chemistries. *Electrophoresis* 2020, *41*, 684-690.

[13] Decout A., Silva-Gomes S., Drocourt D., Blattes E., Rivière M., Prandi J., Larrouy-Maumus G., Caminade A.M., Hamasur B., Källenius G., Kaur D., Dobos K.M., Lucas M., Sutcliffe I.C., Besra G.S., Appelmelk B.J., Gilleron M., Jackson M., Vercellone A., Tiraby

G., Nigou J., Deciphering the molecular basis of mycobacteria and lipoglycan recognition by the C-type lectin Dectin-2. *Science Reports* 2018, *8,* 1-11.

[14] Gilleron M., Bala L., Brando T., Vercellone A., Puzo G., *Mycobacterium tuberculosis* H37Rv parietal and cellular Lipoarabinomannans. *J. Bio. Chem.* 2000, *275*, 677-684.

[15] Ludwiczak P., Brando T., Monsarrat B., Puzo, G., Structural characterization of *Mycobacterium tuberculosis* lipoarabinomannans by the combination of capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* 2001, *73*, 2323-2330.

[16] Hoffstetter-Kuhn S., Paulu A., Gassmann E., Widmer H.M., Influence of borate complexation on the electrophoretic behavior of carbohydrates in capillary electrophoresis. *Anal. Chem.* 1991, *63*, 1541-1547.

[17] Yang B.Y., Montgomery R., Alkaline degradation of fructofuranosides. *Carbohydr. Res.* 1996, *280*, 47-57.

[18] Coca M., García M.T., González G., Peña M., García J.A., Study of coloured components formed in sugar beet processing. *Food Chem.* 2004, *86*, 421-433.

[19] Rovio S., Yli-Kauhaluoma J., Sirén H., Determination of neutral carbohydrates by CZE with direct UV detection. *Electrophoresis* 2007, *28*, 3129-3135.

[20] Rovio S., Simolin H., Koljonen K., Sirén H., Determination of monosaccharide composition in plant fiber materials by capillary zone electrophoresis. *J. Chromatogr. A* 2008, *1185*, 139-144.

[21] Sarazin C., Delaunay N., Costanza C., Eudes V., Mallet J.M., Gareil P., New Avenue for mid-UV-range detection of underivatized carbohydrates and amino acids in capillary electrophoresis. *Anal. Chem.* 2011, *83*, 7381-7387.

[22] Oliver J.D., Rosser A.A., Fellows C.M., Guillaneuf Y., Clement J.L., Gaborieau M., Castignolles P., Understanding and improving direct UV detection of monosaccharides and disaccharides in free solution capillary electrophoresis. *Anal. Chim. Acta* 2014, *809*, 183-193.

[23] Schmid T., Himmelsbach M., Oliver J.D., Gaborieau M., Castignolles P., Buchberger W., Investigation of photochemical reactions of saccharides during direct ultraviolet absorbance detection in capillary electrophoresis. *J. Chromatogr. A* 2015, *1388*, 259-266.

[24] Metsämuuronen S., Lyytikäinen K., Backfolk K., Sirén H., Determination of xylo-oligosaccharides in enzymatically hydrolysed pulp by liquid chromatography and capillary electrophoresis. *Cellulose* 2013, *20*, 1121-1133.

[25] Hiltunen S., Sirén H., Heiskanen I., Backfolk K., Capillary electrophoretic profiling of wood-based oligosaccharides. *Cellulose* 2016, *23*, 3331-3340.

[26] Zhao L., Chanon A.M., Chattopadhyay N., Dami I.E., Blakeslee J.J., Quantification of carbohydrates in grape tissues using capillary zone electrophoresis. *Front. Plant Sci.* 2016, *7*, 1-14.

[27] Alinat E., Jemmali S., Delaunay N., Archer X., Gareil P., Analysis of underivatized cellodextrin oligosaccharides by capillary electrophoresis with direct photochemically induced UV-detection. *Electrophoresis* 2015, *36,* 1555-1563.

[28] Sirén H., Savolainen L.E., Tuuminen T., Capillary electrophoresis as a method to determine underivatized urinary lipoarabinomannans, a biomarker of active tuberculosis caused by *Mycobacterium tuberculosis*. *J. Sep. Sci.* 2016, *39*, 2853-2861.

[29] Chen Y., Lü W., Chen X., Teng M., Review of recent developments of on-line sample stacking techniques and their application in capillary electrophoresis. Cent. Eur. J. Chem. 2012, 10, 611-638.

[30] Sung H.S., Laborde-Kummer E., Gaudin K., Dubost J.P., Development and validation of a capillary electrophoresis method for the determination of sulfate in effervescent tablets. *Eur. J. Pharm. Biopharm.* 2006, *64*, 33-37.

[31] Soga T., Heiger D.N., Simultaneous determination of monosaccharides in glycoproteins by capillary electrophoresis. *Anal. Chem.* 1998, *261*, 73-78.

[32] Sarazin C., Delaunay N., Costanza C., Eudes V., Gareil P., Vial J., On the use of response surface strategy to elucidate the electrophoretic migration of carbohydrates and optimize their separation. *J. Sep. Sci.* 2012, *35*, 1351-1358.

[33] Mayer B.X., How to increase precision in capillary electrophoresis: a review. *J. Chromatogr. A* 2001, *907*, 21-37.

[34] Lin C.H., Kaneta T., On-line sample concentration technique in capillary electrophoresis: Velocity gradient techniques and sample concentration techniques for biomolecules: a review. *Electrophoresis* 2004, *25*, 4058-4073.

[35] Wu X.Z., New approaches to sample preparation for capillary electrophoresis. *Trends Anal. Chem.* 2003, *22*, 48-58.

[36] Simonet B.M., Ríos A., Valcárcel M., Enhancing sensitivity in capillary electrophoresis. *Trends Anal. Chem.* 2003, *22*, 605-614.

[37] Veraart J.R., Lingeman H., Brinkman U.A.Th., Coupling of biological sample handling and capillary electrophoresis: a review. *J. Chromatogr. A* 1999, *856*, 483-514.

[38] Hunter, S.W., Brennan, P.J., Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of *Mycobacterium tuberculosis*. *J. Biol. Chem.* 1990, *265*, 9272-9279.

Figure 1. Structure of lipoarabinomannan (LAM).

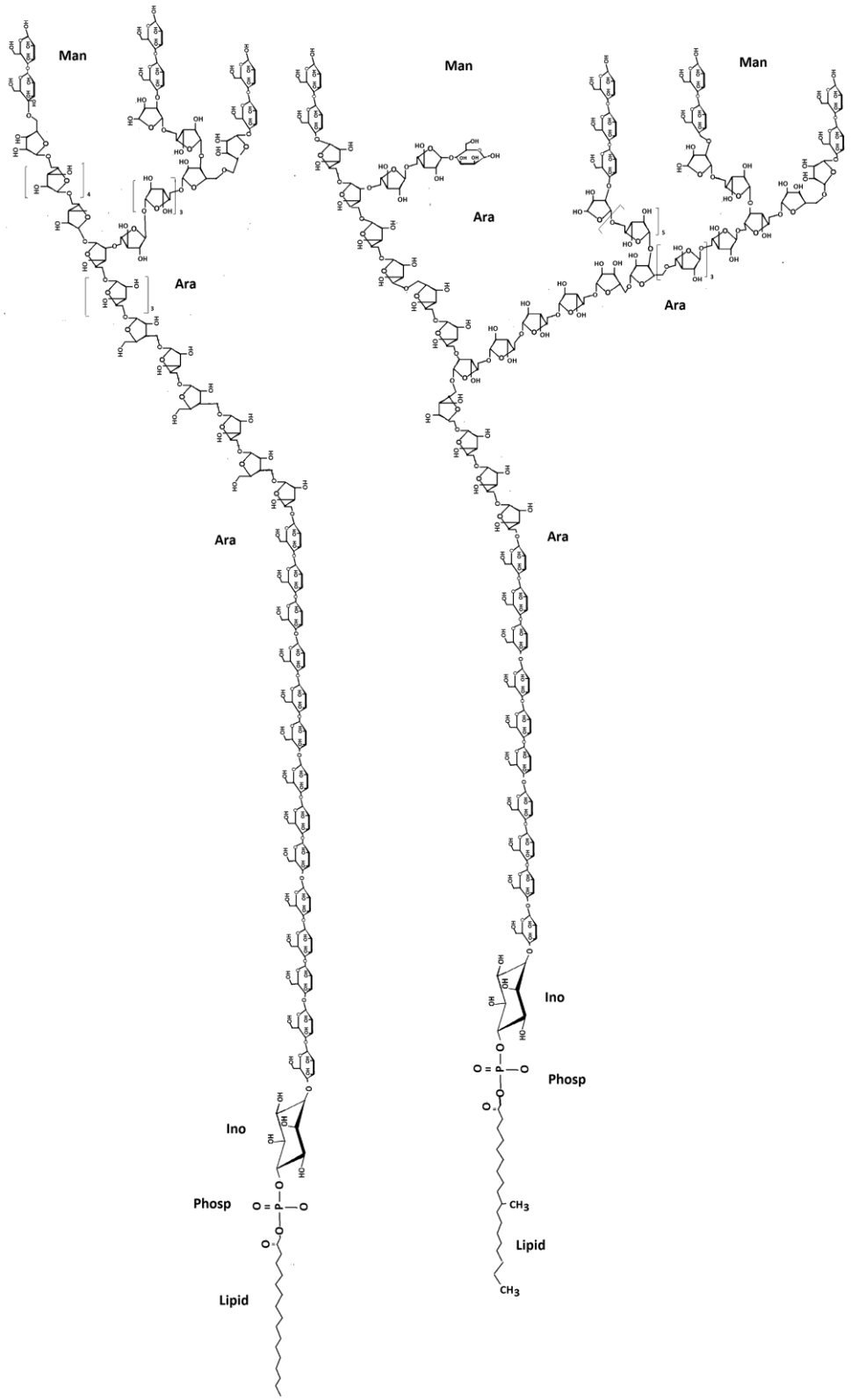


Figure 2. Resolution of saccharides with the final electrolyte solution. Peak identities: (1) Man-6, (2) Man-3, (3) Man-1, (4) Ara-1 and (5) Xyl-1. The final electrolyte solution was 135 mM NaOH, 12 mM Na_3PO_4 with 112 mM KOH (pH 13.21, *I* 319 mM). The capillary was conditioned with acidic and basic solutions. The concentration of each saccharide in the mixture was 20 µg/mL. Separation conditions: capillary 51.5/60 cm (L_{det}/ L_{tot}), separation voltage +10 kV with injection pressure and time of 50 mbar and 15 s, measuring wavelength 270 nm, separation temperature +25 °C.

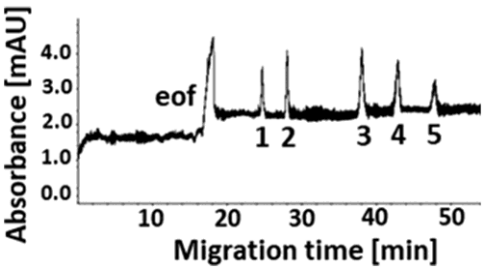


Figure 3. Separation of the mannose saccharides and LAM. (a) Mannose saccharides each of 20 µg/mL in the mixture separated with the Basic method, (b) LAM concentration of ~200 µg/mL with the Basic method, and (c) *myo*-inositol concentration of 15 µg/mL with the Basic method, (d) mannose saccharides each of 20 µg/mL in the mixture with the WS method, (e) LAM concentration of 20 µg/mL with the WS method. Peak identities: (1) Man-6, (2) Man-3, (3) Man-2, (4) Man-1, (5) assumed to be the *myo*-inositol- phospholipid complex of LAM; (6) AM; (7) *myo*-inositol. Analytical conditions: capillary 51.5/60 cm (L_{det}/ L_{tot}), electrolyte solution composed of 12 mM Na_3PO_4, 135 mM NaOH and 112 mM KOH (pH 13.2 at +23 ^{o}C), separation voltage +7.5 kV, measuring wavelength 270 nm, separation temperature +25 ^{o}C. Basic method: sample injection 50 mbar, 15 s with no on-line concentration. WS method: sample injection 50 mbar, 60 s and 15 times multi-layered on-line concentration.

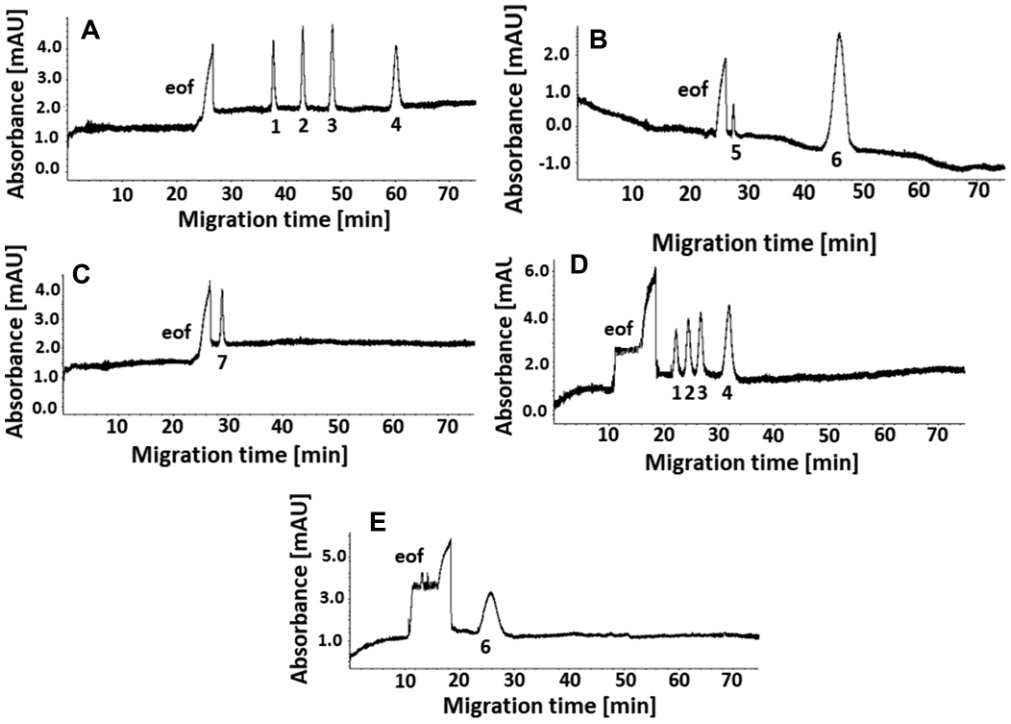


Figure 4. Migration profiles of the studied arabinose saccharides with the WS method. (a) Arabinose saccharides (Ara-1, Ara-4, Ara-8) as a mixture, (b) arabinose c) arabinotetraose, (d) arabinohexaose, (e) arabino-octaose. Peak identities: (1) Ara-1, (2) mixture of Ara-4 and Ara-8, (3) Ara-4, (4) Ara-6, (5) Ara-8. Concentrations of Ara-1, Ara-4 and Ara-8 in a mixture 20 µg/mL each; Ara-1, Ara-4, Ara-8 were 20 µg/mL and Ara-6 was 15 µg/mL. Analytical conditions: capillary 51.5/60 cm (L_{det}/L_{tot}), electrolyte solution 12 mM Na_3PO_4, 135 mM NaOH and 112 mM KOH (pH 13.2 at +23 °C), separation voltage +7.5 kV, measuring wavelength 270 nm, separation temperature +25 °C. WS method: sample injection 50 mbar, 60 s and 15 times multi-layered on-line concentration.

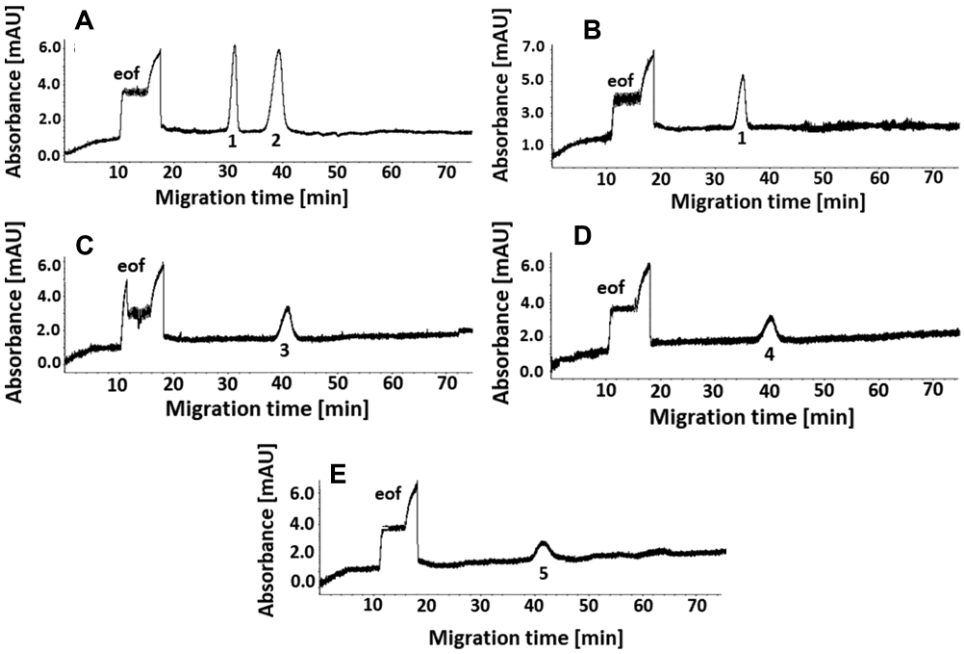
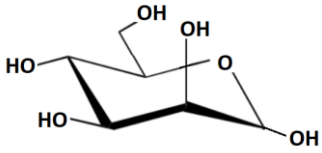
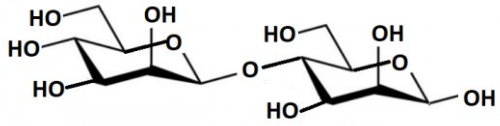
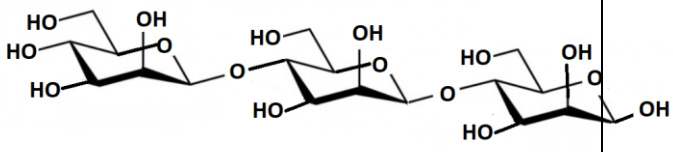
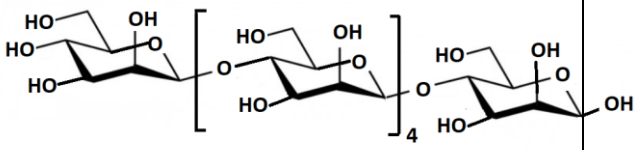
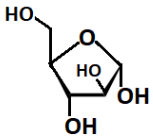
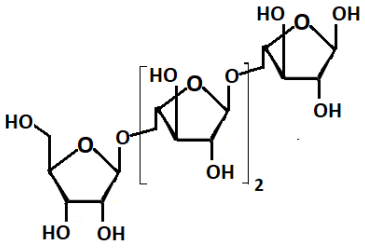
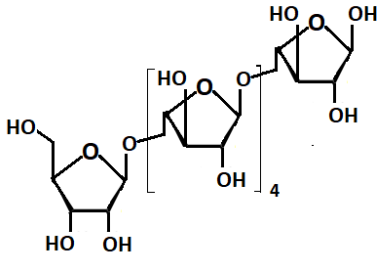
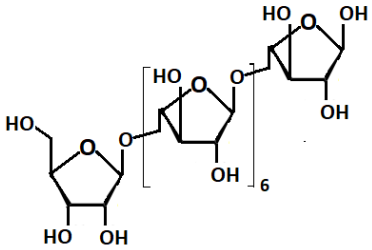
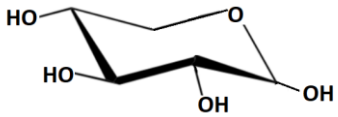
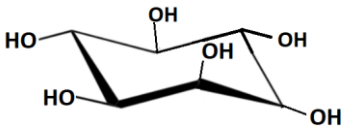


Table 1. The abbreviations, molecular formulas, molecular weights (MW) and chemical structures of selected carbohydrates used in the study.

Saccharide	Abbr.	Molecular formula	MW *) (g/mol)	Chemical structure
D-Mannose	Man-1	$C_6H_{12}O_6$	180.16	
1,4-β-D-Mannobiose	Man-2	$C_{12}H_{22}O_{11}$	342.30	
1,4-β-D-Mannotriose	Man-3	$C_{18}H_{32}O_{16}$	504.44	
1,4-β-D-Mannohexaose	Man-6	$C_{36}H_{62}O_{31}$	990.86	
D-(-)-Arabinose	Ara-1	$C_5H_{10}O_5$	150.13	

1,5-α-L-Arabinotetraose	Ara-4	$C_{20}H_{34}O_{17}$	546.57	
1,5-α-L-Arabinohexaose	Ara-6	$C_{30}H_{50}O_{25}$	810.72	
1,5-α-L-Arabino-octaose	Ara-8	$C_{40}H_{66}O_{33}$	1074.93	
D-(+)-Xylose	Xyl-1	$C_5H_{10}O_5$	150.13	
myo-Inositol	Ino	$C_6H_{12}O_6$	180.16	
Lipoarabinomannan Main functional groups e.g.:	LAM	-	~17300	The chemical structure is in Figure 1.

mannose, arabinose, *myo*-inositol, phosphate and the lipids: palmitic acid and tuberculostearic acid				

*) Molar masses taken from Refs. [2] and [10],

Table 2. Compilation of the method limits of detection (MLD; minimum concentration which can be detected with the method), the limits of quantification (LOQ) calculated based on peak areas and concentrations, and the lowest calibration-based detection amounts. The values are averages from four repeated analyses. (Details explained in *Supplementary Information*.)

	Lowest calibration-based quantitative amount in the method [a,c] **LOQ** **(µg/mL)**	**Method limit** [b] **MLD** **(µg/mL)**	**Lowest calibration-based detection amount in the method** [a] **(µg/mL)**
Analyte	Experimentally measured with calibration (7 or 8-level conc.)	Lowest concentrations in samples when injected and studied by the methods (1 -level conc.)	Experimentally measured with calibration (7 or 8-level conc.
Basic method			
Man-6	17.4	7.5	5.2
Man-3	14.0	7.5	4.2
Man-2	12.7	7.5	3.8

Man-1	10.8	7.5	3.2
Ara-8	53.6	10.0	16.1
Ara-6	14.8	7.5	4.4
Ara-4	15.7	7.5	4.7
Ara-1	3.8	7.5	1.1
WS method			
Man-6	7.4	2.5	2.2
Man-3	4.3	2.5	1.3
Man-2	3.5	2.5	1.0
Man-1	3.6	2.5	1.1
Ara-8	10.0	3.75	3.0
Ara-6	8.2	3.75	2.5
Ara-4	3.3	2.5	1.0
Ara-1	1.6	2.5	0.5

a) Data with 7 or 8 level concentration calibration lines.

b) Data calculated with one concentration.

c) LOQ = 10 * *SD*/*S*

Table 3. Migration times and electrophoretic mobility of mono- and oligosaccharides with the EOF results of each of the analysis. The effective mobility, μ_e, was calculated according to the equations in Ref. [31].

Basic method								
Analyte	Migration time (min) of EOF	RSD (%)	Mobility ($m^2V^{-1}s^{-1}$) of EOF	RSD (%)	Migration time (min) of the analyte	RSD (%)	Mobility ($m^2V^{-1}s^{-1}$) of the analyte	RSD (%)
Man-6	26.6	2.1	$2.58*10^{-8}$	2.0	37.8	2.9	$-7.60*10^{-9}$	1.6
Man-3	26.6	2.0	$2.58*10^{-8}$	1.9	43.3	3.2	$-9.93*10^{-9}$	1.6
Man-2	26.5	2.4	$2.59*10^{-8}$	2.4	48.5	4.2	$-1.17*10^{-8}$	1.3

Man-1	26.8	2.6	$2.56*10^{-8}$	2.6	62.0	4.3	$-1.45*10^{-8}$	2.2
Ara-8	26.5	3.3	$2.59*10^{-8}$	3.4	87.8	8.4	$-1.80*10^{-8}$	1.0
Ara-6	26.7	0.6	$2.57*10^{-8}$	0.6	95.4	1.4	$-1.85*10^{-8}$	0.4
Ara-4	26.6	2.2	$2.58*10^{-8}$	2.3	88.9	5.0	$-1.81*10^{-8}$	1.3
Ara-1	26.7	1.7	$2.57*10^{-8}$	1.6	69.6	2.7	$-1.59*10^{-8}$	1.6
myo-Inositol	26.6	1.3	$2.58*10^{-8}$	1.3	28.8	1.5	$-1.99*10^{-9}$	4.7
Optimized method								
Man-6	19.2	7.5	$3.60*10^{-8}$	7.4	23.1	10.6	$-5.99*10^{-9}$	7.3
Man-3	18.4	5.6	$3.75*10^{-8}$	5.6	23.7	7.7	$-8.27*10^{-9}$	3.2
Man-2	17.7	7.2	$3.90*10^{-8}$	7.7	24.3	10.6	$-1.04*10^{-8}$	3.7
Man-1	17.6	6.4	$3.91*10^{-8}$	6.4	28.3	11.2	$-1.45*10^{-8}$	2.6
Ara-8	18.5	6.2	$3.73*10^{-8}$	6.2	42.7	11.9	$-2.09*10^{-8}$	1.0
Ara-6	18.5	4.8	$3.72*10^{-8}$	4.7	43.3	10.2	$-2.12*10^{-8}$	3.8
Ara-4	17.7	5.1	$3.89*10^{-8}$	5.2	39.0	10.8	$-2.11*10^{-8}$	2.0
Ara-1	18.8	9.7	$3.67*10^{-8}$	7.8	36.1	9.7	$-1.75*10^{-8}$	7.8

The effective mobility, μ_e, was calculated according to the equation: $\mu_e = (L_{det}L_{tot} / t_R V) - (L_{det}L_{tot} / t_{EOF} V)$
($m^2V^{-1}s^{-1}$). Other details are in the Supplementary Information.

The L_{det} and L_{tot} are the effective length of the capillary and the total length of the capillary, respectively. The V is the applied voltage, the t_R is the migration time of the anion and the t_{EOF} is the migration time of the electro-osmotic flow.