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# Quantitative analysis of malto-oligosaccharides by MALDI-TOF mass spectrometry, capillary electrophoresis and anion exchange chromatography

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**Abstract** The analysis of malto-oligosaccharides by MALDI-TOF mass spectrometry (MS), capillary electrophoresis (CE) and anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is described. Appropriate methods were developed which enabled the resolution of the oligosaccharides and quantification of the peak areas. It could be shown that each technique provided a different distribution profile of the maltodextrins. Using MALDI-TOF MS signals of higher molecular weight oligomers were enhanced while low molecular weight analogues were discriminated. Thus, the response factor depends on the degree of polymerization (DP) of the carbohydrates. Homologues up to DP-15 could be detected. Analysis of the maltodextrins by CE was accomplished by derivatization of the sugars with 4-aminobenzonitrile (ABN) and 8-aminonaphthalene-1,3,6-trisulfonic acid, respectively. By using the latter reagent oligosaccharides up to DP-13 were detected while derivatization with ABN allowed detection up to DP-9. The molecular weight distribution obtained by both approaches were the same. HPAEC-PAD enabled the determination of oligomers up to DP-9. The distribution obtained by this technique showed somewhat lower signals of the small homologues than those found by CE while the opposite held for higher molecular weight compounds. Hydrolysis of the carbohydrates by the derivatization reaction prior to CE analysis, which increased the proportion of low molecular weight homologues, may account for these findings.

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## Introduction

Recent developments of modern analytical methods for both qualitative and quantitative analysis dramatically extended the spectrum for the characterization of complex samples. Among the most exciting innovations in the last decade for this purpose were matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) and capillary electrophoresis (CE). Both techniques are well known for their high resolving power and broad range of application.

After the pioneering work of MALDI-TOF MS by Karas and Hillenkamp [1] in 1988 this technique has evolved into a versatile tool for characterization of natural and synthetic polymers [2–4]. Striking features of this technique are its simplicity of operation, soft ionization with almost no fragmentation and its ability to analyze even big polymers of up to 500 kDa. As an increasing number of applications show, MALDI-TOF MS is particularly attractive for the analysis of oligosaccharides [5, 6]. More recently the extension of MALDI-TOF MS from a strict qualitative tool to a quantitative technique has been investigated in several papers [7–9]. Most authors describe the use of an internal standard to overcome problems of poor shot-to-shot repeatability and local variations in the concentration of the sample components in the crystals. No attempt was made so far, to prove the potential for quantification of homologous oligo- or polymers of this technique. In comparison to chromatography or CE, MALDI-TOF MS provides a number of advantages, for instance, short analysis time, no derivatization of the sugars for detection and only minute amounts of sample needed for analysis.

Modern capillary electrophoresis combines many features of electrophoresis such as high efficiency and broad application range with those of HPLC, for instance automated operation and quantitative analyses of separated zones [10]. Analysis of carbohydrates by CE is difficult because this class of compounds generally lack suitable chromophores for UV detection and ionized functional groups. Borate complexation and indirect detection has

been employed to overcome these problems [11, 12]. In the case of neutral sugars indirect detection is disadvantageous because of low sensitivity of the method at high pH values, where the influence of hydroxyl groups is no longer negligible. Another approach to overcome these problems is the derivatization of neutral sugars prior to analysis. A number of appropriate derivatization agents are described in literature for this purpose [13, 14]. However, pre-column derivatization is limited to aldoses because ketoses are not derivatized or only to a small degree.

Anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) represents a standard technique for the analysis of mono- and oligosaccharides [15, 16]. This technique permits (i) separation of sugars at a strong anion exchange stationary phase because of the weak acidity of carbohydrates at alkaline pH values and (ii) direct detection and quantification of non-derivatized sugars based on their oxidation on the surface of a gold electrode. Both aldoses and ketoses can be analyzed by this technique.

It is the focus of this work to investigate the potential of MALDI-TOF MS for the separation and quantification of malto-oligosaccharides The results obtained are compared and critically evaluated to corresponding data found by CE and HPAEC-PAD. Special emphasis is laid to the determination of the molecular weight distribution of the oligomers and the range of detection with respect to the degree of polymerization (DP).

## Experimental

### Chemicals

All chemicals were purchased at highest quality available. Acetic acid (100%), acetonitrile, 4-aminobenzonitrile (ABN), Dextrin 20, 2,5-dihydroxybenzoic acid (DHB), dimethylsulfoxide, maltose, maltotriose, maltohexose, maltoheptose, methanol, phosphoric acid, sodium cyanoborohydride, sodium hydroxide and anhydrous sodium acetate were purchased from Fluka, Deisenhofen, Germany. 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was obtained from MoBiTec, Göttingen, Germany. Milli-Q water (Millipore) was used for the preparation of the aqueous solutions.

### Instrumental

#### *Capillary electrophoresis*

Experiments were carried out using an HP $^{3D}$CE instrument (Hewlett-Packard, Waldbronn, Germany). All separations were performed in untreated open-tube fused silica capillaries of 70 cm eff. length × 75 μm ID for the separation of the 4-aminobenzonitrile-derivatives and 60 cm × 50 μm ID for the ANTS-derivatives applying a potential of + 20 kV and – 15 kV (reversed mode), respectively. The capillary temperature was maintained constant at 20° C (ABN-derivatives) and 25° C (ANTS-derivatives). Samples were injected by pressure for 2 s at 0.050 Pa excess pressure and detected by UV absorbance at 285 nm (ABN-derivatives) or 235 nm (ANTS-derivatives). Separation was performed in 175 mmol/L borate at pH 10.5 (ABN-derivatives) and 50 mmol/L Tris-phosphate, pH 2.5 (ANTS-derivatives). All solutions were filtered with Sartorius membrane filters (0.45 μm). Peak areas were normalized by dividing the measured area by the migration time of the corresponding peak.

#### *MALDI-TOF mass spectrometry*

A linear laser desorption mass spectrometer, type G2025A Hewlett-Packard, Waldbronn, equipped with a nitrogen laser (337 nm), was used throughout. Malto-oligosaccharides (0.1 mg/mL) were dissolved in water followed by filtration of the solution with Sartorius membrane filters (0.45 μm). 10 μL of this solution were mixed with 10 μL of a 0.1 mol/L 2,5-dihydroxybenzoic acid in acetonitrile. 1 μL of this mixture was transferred to the probe tip of the instrument, where the solvent was evaporated in vacuum. N-acetylgalactosamine was added as an internal standard in a concentration of 0.125 mmol/L to the sample solution. The measured peak areas of the oligosaccharides were normalized relative to the peak area of the internal standard. Ions were detected in the positive mode at an applied voltage of 10 kV and a sampling rate of 5 ns. In general 50 spectra were accumulated. Evaluation of the signals was accomplished by the data acquisition software of the instrument.

#### *Ion exchange chromatography with pulsed amperometric detection*

HPLC analysis was performed with an HPLC instrument DX 300 (Dionex Idstein, Germany,) equipped with a gradient high pressure liquid pump and a pulsed amperometric detector with a gold working electrode and a Ag/AgCl reference electrode. A potential of 0.1 V was applied for 0.5 s followed by a potential of 0.6 V for complete oxidation and removal of all products for 0.1 s and finally a potential of – 0.6 V (0.05 s) for reduction of the oxidized gold surface. A Dionex anion exchange column Carbo Pac PA 100 (250 mm × 4 mm) with guard column was used. 20 mL Dextrin 20 (3 mg/mL in water) were injected on the column. Elution was performed with a mobile phase of 200 mmol/L NaOH and 105 mmol/L sodium acetate. The flow rate was 1 mL/min. All eluent solutions were kept under helium gas to avoid contamination of the alkaline solutions with atmospheric carbondioxide. The separation method is extremely sensitive to even traces of carbonate in the mobile phase.

#### *Derivatization of the oligosaccharides*

Derivatization of the carbohydrates was carried out according to a mechanism of reductive amination with both labeling reagents [17, 18]. For the derivatization with ABN 9.5 mg sodium cyanoborohydride, 57 mg ABN and 48 μL acetic acid are dissolved in 1 mL methanol/water (1 : 1, v/v). 2 mg Dextrin 20 are added to the mixture and heated at 90° C for 1 h, if not otherwise stated. The sample is diluted 1 : 1 (v/v) with running electrolyte prior to analysis. Derivatization of oligosaccharides with ANTS was performed by dissolving 85 mg ANTS in 1 mL of a solution of acetic acid and water (3 : 17, v/v). 2 mg Dextrin 20 were dissolved in 500 μL ANTS solution and 500 μL of a 0.2 mol/L sodium cyanoborohydride in DMSO. The mixture was allowed to react in a sealed vial at a temperature of 40° C for 15 h. Subsequently, the sample was diluted 1 : 1 (v/v) with running buffer and directly analyzed.

## Results and discussion

In this study a mixture of malto-oligosaccharides (Dextrin 20) was chosen as model mixture for a number of reasons. First, the molecular weight distribution of Dextrin 20 ranges from glucose as the monomer to approx. DP-15 to DP-20. Second, pure malto-oligosaccharides, which are commercially available, allow to spike the mixture with defined concentrations of these compounds. Finally, malto-oligosaccharides can easily be analyzed by all three

techniques. According to a substance data sheet of the supplier the composition of Dextrin 20 is as follows: monosaccharides 2.3%, disaccharides 7.9%, trisaccharides 9.6%, tetrasaccharides 6.2% and penta- and higher oligosaccharides 74.0%.

## MALDI-TOF MS

Direct quantification of sample components in MALDI-TOF MS is difficult to be accomplished because signal intensity depends on a number of factors such as laser energy, variations of the embedding of sample molecules in the matrix crystals and spectra collection. The addition of an internal standard to the sample is an appropriate approach to improve shot-to-shot reproducibility. However, the determination of a polymer distribution should be possible if the homologues of the polymers are uniformly dissolved and mixed in the matrix solution. In the case of maltodextrins all oligomers are well soluble in water and water/acetonitrile mixtures, which were used for the investigations. Suitable homogeneity of the co-crystals of matrix and sample molecules, which represents an important prerequisite for effective desorption and ionization, was achieved in order to perform such an analysis. Carbohydrates show a high affinity to alkali metal ions such as sodium or potassium in MALDI-TOF MS [8]. Therefore, all sugars were detected in the form of sodium or potassium adducts, even though these ions are present only in traces. Figure 1 shows a representative mass spectrum of Dextrin 20. Oligomers could be detected from DP-2 to DP-15 with a maximal signal at DP-6. The major peak of each doublet of peaks represents the sodium adduct while the smaller signal is the potassium adduct. Evaluation of peak area and peak height of the sodium adducts gave similar results, although peak area evaluation was more reproducible. If defined concentrations of individual oligosaccharides (DP-2, DP-3, DP-6 and DP-7) are spiked

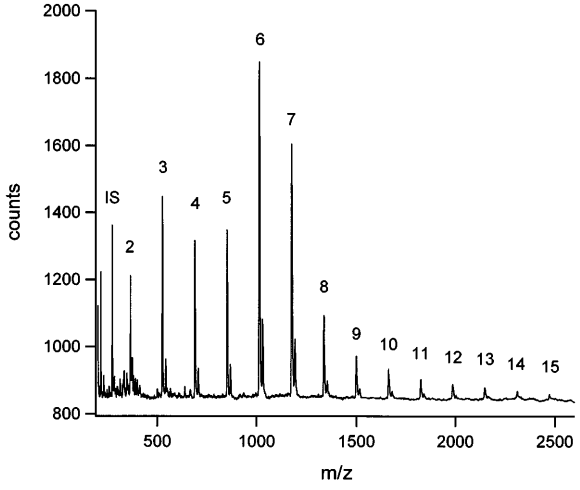


**Fig. 1** MALDI-TOF mass spectrum of Dextrin 20. Numbers indicate the DP of the corresponding malto-oligomer, IS: internal standard

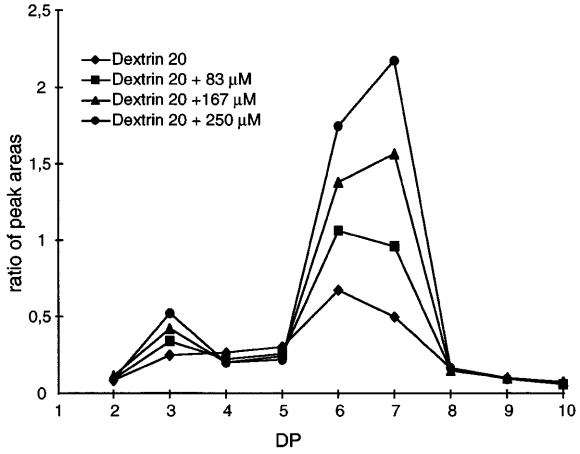


**Fig. 2** Influence of the addition of defined concentrations of DP-2, DP-3, DP-6 and DP-7 on the peak areas of Dextrin 20 measured by MALDI-TOF MS. Peak areas were normalized relative to the peak area of the internal standard

to Dextrin 20, increases in the corresponding signal intensities could be measured. This is shown in Fig. 2. A closer look to Fig. 2 shows that the signal response of maltose and maltotriose is much smaller than the response of maltohexose and maltoheptose. It is obvious that the mass sensitivity in MALDI-TOF MS depends on the molecular weight of the analytes. Therefore, the peak profile as given in the spectrum of Fig. 1 cannot be related directly to the molecular weight distribution.

## Capillary electrophoresis

Most carbohydrates lack chromophoric groups which makes their detection in capillary electrophoresis with an UV detector a difficult task. Pre-column derivatization is the method of choice to add a chromophore and/or an ionic functionality to the carbohydrate molecule [13]. In this study two different labeling reagents were used. 4-Aminobenzonitrile forms stable derivatives with high electrophoretic mobilities of the sugars. Separation was performed in a borate buffer at pH 10.5 where the sugars migrated as their negatively charged borate complexes. Figure 3a shows the separation of Dextrin 20 as 4-aminobenzonitrile-derivatives. Glucose has the highest migration velocity and migrates as an anion into the opposite direction to the electroosmotic flow. Thus, it is detected as the last sample constituent at about 32 min. While the low molecular weight sugars are excellently resolved in this system, resolution steadily decreases with increasing DP of the carbohydrates. Thus, the number of oligomers which can be separated is limited. Here, homologues up to DP 10 could be detected.

Derivatization of the malto-oligosaccharides with ANTS is particularly attractive because the resulting derivatives are threefold negatively charged over a broad pH range and possess an excellent chromophore for UV or fluorescence detection. Analysis of the ANTS-sugar de-

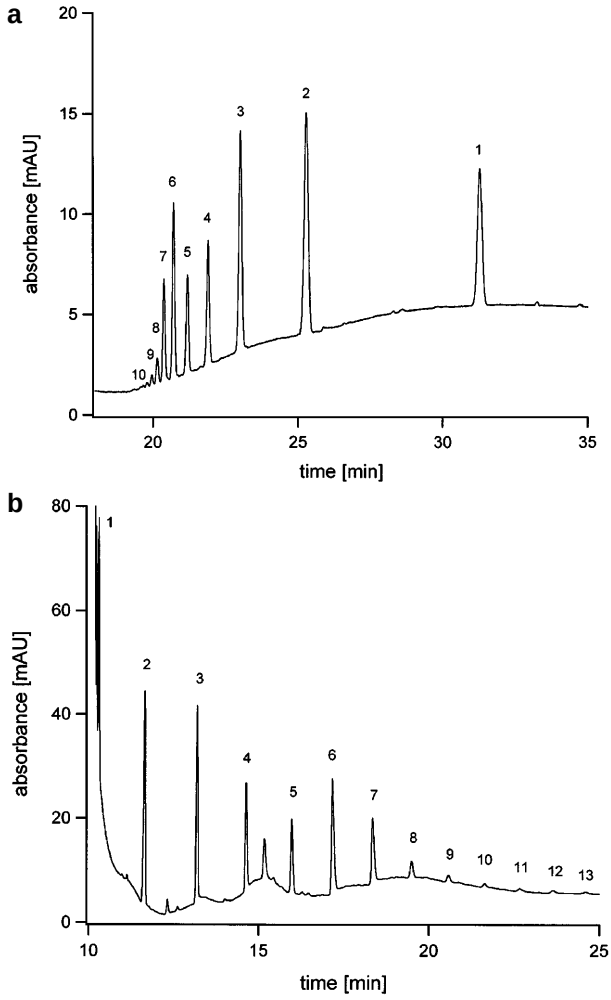


**Fig. 3 a, b** Electropherogram of Dextrin 20 after derivatization with **a** ABN and **b** ANTS. Analysis of ANTS-oligosaccharides with reversed voltage. Peak numbers indicate the DP of the corresponding malto-oligomer

rivatives was carried out at pH 2.5 where the electroosmotic flow is almost zero and the analytes migrate towards the anode. Figure 3b shows the separation of Dextrin 20. The monosaccharide migrates fastest because of the absence of the electroosmotic flow and elutes at the descending slope of the peak of the derivatization agent. As clearly seen in Fig. 3b, resolution of the oligosaccharide derivatives keeps fairly constant and is independent from the DP. Maltooligosaccharides up to DP-13 could be detected. Thus, this method is well suited for the analysis of higher molecular weight oligosaccharides.

## Ion exchange chromatography with pulsed amperometric detection

Anion exchange chromatography with pulsed amperometric detection allows the analysis of carbohydrates without derivatization. Figure 4 shows the chromatogram of Dextrin 20, which was obtained by using this technique. It is obvious that oligosaccharides with low DP's are excellently detected while it is more difficult to determine higher molecular weight homologues. Oligomers could be detected up to DP-9. It should be noted that higher oligosaccharides can be detected when a gradient elution system is used. Although the same functional groups of the sugars are utilized for the electrochemical detection, the response factors of the different sugars are not identical. Plots of various concentrations of glucose, maltose, maltohexose and maltoheptose versus the corresponding peak areas point out that the response factor increases with increasing DP. Table 1 provides response factors calculated from the slopes of linearity measurements. Five concentrations in the range of 20 µg/g to 220 µg/g were prepared to study the response factor. For each concentration the average of two analytical experiments was used for the calculation of the response factors.

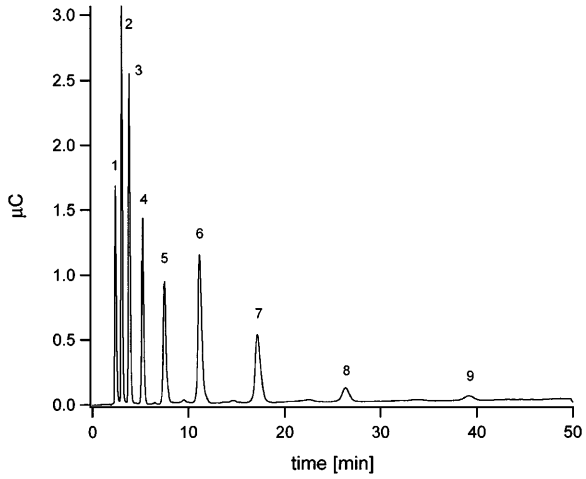


**Fig. 4** Chromatogram of Dextrin 20 obtained by HPAEC-PAD. Peak numbers indicate the DP of the corresponding oligomer

**Table 1** Response factors of selected sugars calculated from the slopes of linearity measurement in HPAEC-PAD

| Sugar | Response factor (in arbitrary units) |
|---|---|
| Glucose | 1 |
| Maltose | 1.13 |
| Maltohexose | 2.81 |
| Maltoheptose | 3.29 |

## Discussion of the results

Clearly, MALDI-TOF MS shows the highest efficiency and peak resolution of all three techniques evaluated. In addition, this technique allowed to analyze oligomers up to DP-15. However, glucose, which was found in high proportions by both other techniques, could hardly be detected. In contrast to CE and HPAEC-PAD the highest signal was found for maltohexose. Examination of the response factors by analyzing a sample of equal concentrations of DP-2, DP-3, DP-6 and DP-7 with MALDI-TOF

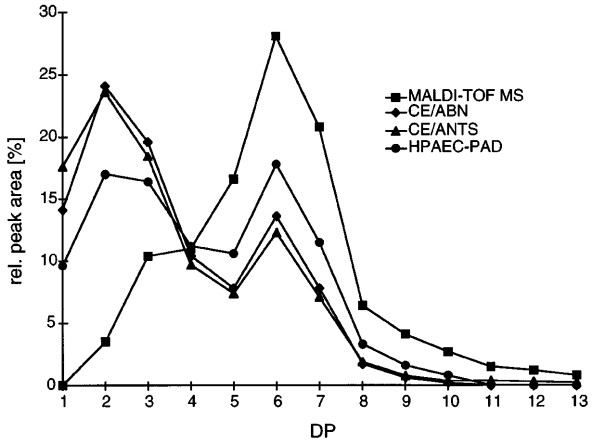


**Fig. 5** Plots of the relative peak areas of Dextrin 20 versus the corresponding DPs, measured by four different techniques. The relative peak area is the proportion of the total area of all sugars expressed in per cent

MS pointed out, that the response factor increases with the molecular weight of the analytes. Thus, the highest response was found for DP-7. Obviously, desorption and ionization is more effective for bigger oligosaccharides than for smaller homologues which are discriminated. This finding holds at least for those oligomers used in these studies. Figure 5 shows plots of the relative peak areas of oligosaccharides of Dextrin 20 obtained by MALDI-TOF MS, CE and HPAEC-PAD. Each technique produces a different distribution profile. In the lower molecular weight range (DP-1 to DP-4) capillary electrophoresis shows the higher signals, while from DP-5 on highest responses are measured by MALDI-TOF MS. Responses in HPAEC-PAD also depend on the DP of the sugars. Here, higher response factors are calculated for maltose than for glucose. Thus it can be assumed that the response factors of higher homologues also depend on the molecular weight.

There is a strong indication that the high proportion of low molecular weight carbohydrates in capillary electrophoresis is generated by hydrolysis during the derivatization procedure, which takes place under acidic conditions and at elevated temperatures. This was studied in a series of experiments where the derivatization conditions were changed. If no degradation occurs during derivatization procedure, the ratio of peak areas of a low molecular weight homologe, e.g. DP-2, to the peak areas of higher molecular weight homologues, e.g. DP-6, should be unchanged. Table 2 summarizes the data obtained. The higher the temperature or the longer the reaction the higher the ratio of peak areas is. Thus, higher temperatures and longer derivatization times generate higher proportions of derivatized maltose compared to maltohexose. Even under strongest derivatization conditions no quantitative reaction could be observed. Investigations of the reaction product after derivatization with ABN at 120° C for 60 min by MALDI-TOF mass spectrum showed that both non-derivatized and derivatized oligosaccharides were found with almost equal peak intensities (results not shown here).

**Table 2** Comparison of the normalized peak areas of maltose and maltohexose obtained in capillary electrophoresis after derivatization with ABN at different derivatization conditions

| Derivatization condition | Peak area of maltose [mAU] | Peak area of maltohexose [mAU] | Ratio of peak areas (DP2 : DP6) |
|---|---|---|---|
| 90° C/15 min | 0.058 | 0.038 | 1.53 |
| 90° C/30 min | 0.088 | 0.053 | 1.66 |
| 90° C/60 min | 0.113 | 0.060 | 1.88 |
| 120° C/15 min | 0.089 | 0.045 | 1.97 |
| 120° C/30 min | 0.125 | 0.063 | 1.97 |
| 120° C/60 min | 0.165 | 0.069 | 2.39 |

## Conclusions

MALDI-TOF mass spectrometry is unmatched with respect to its simplicity of operation for the analysis of oligosaccharides. In addition this technique is best suited for the detection of higher molecular weight oligosaccharides, which are detected neither by CE nor by HPAEC-PAD. However, determination of the oligomer distribution from a mass spectrum may be critical and necessitates a proper validation of the method and the determination of the individual response factors. Further investigations about the desorption and ionization process are necessary in order to understand the reasons for the observed mass biasing. For instance, multimer formation depending on the carbohydrate concentrations in the co-crystals could be the cause for the mass discrimination. In addition instrumental aspects such as the lensing and detector systems should be optimized for this purpose. Remarkable features of capillary electrophoresis with pre-column derivatization of the carbohydrates are its simplicity and ruggedness. With respect to sensitivity and reproducibility of migration time and peak area CE is comparable to HPLC. However, quantification of oligosaccharides and determination of the oligomer distribution may cause problems because of the derivatization reaction, which was incomplete, and the risk of hydrolysis of higher molecular weight carbohydrates in the derivatization process. Anion exchange chromatography with pulsed amperometric detection does not need any pre-column derivatization steps. But also this technique necessitates a proper investigation of the response factors, which depend on the molecular weight. Disadvantages of HPAEC-PAD are its need for skilled operators and sophisticated equipment with expensive columns and with the need for continuous helium degassing of the mobile phases to prevent dissolution of even traces of atmospheric carbondioxide.

The results obtained by all three techniques demonstrate, that molecular weigth distributions cannot be calculated directly from the relative peak areas. This holds at least for malto-oligosaccharides in the range of DP-1 to DP-15. A proper investigation of the individual response

factors is essential for all techniques. None of the techniques used in this study is the "best" technique. Each one has strong and weak points. Thus, it depends on external factors such as available sample amount, required precision of analysis, analysis time, sample composition, etc., which technique is best suited for the corresponding application. In further studies it will be investigated whether these results can be transfered to other complex carbohydrates such as glycoprotein glycans.

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