Quantitative analysis methods for sugars

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by

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ABBREVIATIONS

AA  anthranilic acid
ABEE  p-aminobenzoic ethyl ester
API  Atmospheric Pressure Ionization
BGE  Background Electrolyte
CAD  Charged Aerosol Detection
CTAB  cetyltrimethylammonium bromide
C(Z)E  Capillary (Zone) Electrophoresis
DL  Detection Limit
DMP  2,6-dimethoxyphenol
ECL  Electro generated Chemilluminescence
ELSD  Evaporative Light Scattering Detection
ESI  Electrospray Ionization
FID  Flame Ionization Detector
GC  Gas Chromatography
HILIC  Hydrophilic Interaction Liquid Chromatography
HPAEC  High Performance Anion-Exchange Chromatography
HPLC  High Performance Liquid Chromatography
ICH  International Conference on Harmonization
LC  Liquid Chromatography
LIF  Laser Induced Fluorescence
Min  Minutes
MRM  Multiple Reaction Monitoring
MS  Mass Spectrometry
NMP  1-(2-naphthyl)-3-methylpyrazolone
n.r.  Not Reported
PAD  Pulsed Amperometric Detection
PMP  1-phenyl-3-methyl-5-pyrazolone
PMT  Photomultiplier tube
QL  Quantitation Limit
R2  Correlation coefficient
REF  Reference
RI  Refractive Index
RSD  Relative Standard Deviation
SDS  sodium dodecyl sulfate
SIM  Selected Ion Monitoring
S/N  Signal to noise ratio
Temp  Temperature
TMS  TriMethyl Silyl
UV-VIS  Ultra Violet-Visible Spectroscopy
INDEX
Abbreviations ................................................................................................................................................ 3
1 Introduction............................................................................................................................................... 5
2 Liquid Chromatography (LC).................................................................................................................. 6
   2.1 Columns ........................................................................................................................................... 6
   2.2 Detectors in Liquid Chromatography ............................................................................................. 7
      2.2.1 Evaporating Light Scattering Detection (ELSD) ...................................................................... 7
      2.2.2 Refractive Index Detector (RI) ................................................................................................. 9
      2.2.3 Ultra Violet Detection (UV) .................................................................................................. 10
      2.2.4 Mass Spectrometry (MS) ...................................................................................................... 11
      2.2.5 Fluorescence Detection ....................................................................................................... 13
      2.2.6 Charged Aerosol Detection (CAD) ...................................................................................... 14
   2.3 Discussion liquid chromatography ............................................................................................... 15
3 High Performance Anion-exchange Chromatography (HPAEC) ...................................................... 21
   3.1 HPAEC with pulsed amperometric detection (PAD) .................................................................... 21
   3.2 HPAEC with Mass detection ......................................................................................................... 22
   3.3 Discussion high performance anion-exchange chromatography ................................................ 22
4 Gas Chromatography (GC) .................................................................................................................... 25
   4.1 Derivatization ............................................................................................................................ 25
   4.2 Detection ......................................................................................................................................... 25
   4.3 Discussion gas chromatography ................................................................................................... 26
5 Capillary Electrophoresis (CE) ................................................................................................................ 29
   5.1 Detection in Capillary Electrophoresis ......................................................................................... 29
      5.1.1 UV detection after derivatization ......................................................................................... 29
      5.1.2 Indirect UV .......................................................................................................................... 31
      5.1.3 Amperometric/Electrochemical Detection ........................................................................... 32
   5.2 Discussion Capillary Electrophoresis ............................................................................................ 33
6 Conclusion ............................................................................................................................................. 38
References ................................................................................................................................................. 40
1 INTRODUCTION

The separation and quantitative analysis of sugars is challenging for several reasons. To find a proper technique, column and detector for the separation and quantitative detection of sugars is difficult. Liquid chromatography (LC) is most widely used to separate sugars due to the availability. However, traditional reversed phase columns cannot be used for underivatized sugars, as the stationary phase will not provide the required retention and specialized columns are necessary. Detection of sugars faces troubles as their structure contains no chromophores. Detection by UV-VIS, as commonly used in HPLC, is not possible in sugar analysis. Detection techniques such as evaporate light scattering (ELSD), refractive index (RI), Mass spectrometry (MS) can be used but every detector has its own limitations. All liquid chromatography methods in combination with several detectors will be discussed in chapter 2. Another type of liquid chromatography that can be used for sugar analysis is high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD). Anionic separation and PAD detection improves the sensitivity and specificity compared to other LC methods. HPAEC-PAD will be discussed in chapter 3.

Due to their high polarity, hydrophilicity and low volatility, saccharides have to be converted into volatile and stable derivatives, i.e., trimethylsilyl or acetate derivatives, before GC analysis. In general GC methods with FID or MS detection provide a good separation of sugars and a good sensitivity, but require prior steps of reduction and derivation, which are very time consuming and not very practical in routine analysis. The use of GC for sugar separation will be discussed in chapter 4. Beside the LC and GC methods, capillary electrophoresis (CE) can also be used for quantitatively separation of sugars. As in LC detection in CE is the most challenging part. Methods and details are described in chapter 5.

In this review a summary of different techniques used for the separation of sugars is given with a focus on method and validation details (recoveries, detection limits etc.) The articles covered in this review have a focus on mono- and disaccharides like fructose, glucose, sucrose etc.

In the different chapters the possibilities of analysing sugars are summarized and concluded.
2 LIQUID CHROMATOGRAPHY (LC)

Separation and detection of sugars can be done with liquid chromatography using different column types and detectors. In liquid chromatography there are various ways of analysing sugars, a distinguish can be made between columns and detection techniques. Different columns will result in other separation of sugars and results in different validation parameters like detection limits.

2.1 Columns

The stationary phases that can be used for the LC separation of native (underivatized) sugars are cyano- or amino silica-bonded columns, cationic exchange resins (see chapter 3) or hydrophilic stationary phases.

A column packed with hydrogen sulfonated divinyl benzene-styrene copolymer particles can be used for the separation of sugars by ion-exchange chromatography.\textsuperscript{1-2} The addition of acetonitrile (6\%) to the mobile phase (0.045 N H\textsubscript{2}SO\textsubscript{4}) allowed good separation of the desired products within acceptable retention times (< 30 min).\textsuperscript{2}

Hydrophilic interaction liquid chromatography (HILIC) may be described as a variant of normal-phase chromatography, where a hydrophilic bonded stationary phase is used in combination with a mostly organic mobile phase, and gradient elution is usually performed by increasing the water concentration. Separation by HILIC columns has been used to analyse different types of carbohydrates. Karlsson et al. used a HILIC column for the separation of sugars. However, in the short retention time reported (separation within 20 minutes) not all sugars were baseline separated.\textsuperscript{3}

LC analysis of soluble sugars using silica bonded column with amino groups is often reported in literature.\textsuperscript{4-5-6} Reported retention times required for baseline separation vary between 15 min and 60 min, depending on the particle size and eluent. In figure 1 an example is presented of a separation of different sugars with an NH\textsubscript{2} column and detection by ELSD.

![Separation of sugars form plant extracts by LC (Amino column) and ELSD detection](image.png)

Figure 1: Separation of sugars form plant extracts by LC (Amino column) and ELSD detection, reproduced from reference 5.
After derivatization of the sugars for UV or fluorescence detection, separation can be performed on a normal silica based C8 or C18 column, and numerous examples are reported in the literature.\textsuperscript{7,8,9}

Agblevor et al. reported the analysis of biomass sugars on a Prevail column with polymeric beads as stationary phase and found the analysis of the standard sugars on the Prevail had a strong dependence on the composition of the mobile phase. For baseline separation an isocratic run at 15% water and 85% acetonitrile was used.\textsuperscript{10} A chromatogram of the developed method by Agblevor et al. is presented in figure 2.

![Chromatogram](image)

Figure 2: separation of sugars in biomass by a polymeric based column and ELSD detection, reproduced from reference 10.

### 2.2 Detectors in Liquid Chromatography

Commonly used detectors in liquid chromatography are UV, ELSD, RI, CAD, fluorescence and MS. Every detector has its advantages and disadvantages in the detection of sugars. In the paragraph below all detectors will be discussed for the detection of sugars and in table 1 – 5 details of the method are described (page 16-20).

#### 2.2.1 Evaporating Light Scattering Detection (ELSD)

The Evaporating Light Scattering Detector (ELSD) is an universal, nonspecific detector in which the signal intensity is related to the concentration of the analyte in the effluent, but not to its optical characteristics. An ELSD detects all non-volatile solutes after evaporation of the solvent of the column effluent by light dispersion on the solid analyte particles formed. The detector is useful in the quantitative determination of non-chromophoric compounds and can be used in combination with gradients, although attention should be given to the fact that non-volatile salts cannot be used as buffer in the eluent.

A disadvantage of the ELSD is the low reproducibility, a slightly low sensitivity to low molecular weight components and the non-linearity of the detector. Quantification can be done by using a polynomial order or bi-logarithmic calibration line.
The detection of non-chromophoric and non-volatile compounds makes ELSD a suitable technique for
detection of sugars.

Liu et al. developed a method for UV absorbing compounds and sugars in one. Detection was done by UV
and ELSD in parallel. Detection limits reported for the sugars by ELSD, were comparable to the results
with a UV detector for UV-absorbing components in the same samples. They also validated the recovery
and precision and found good accuracy with recovery in the range of 94.2 – 99.9 %. The intra- and inter-
day repeatability of the retention time and the peak area were satisfactory, with %RSD values less than
1.12 %.

A chromatogram of the separation of the sugars by Liu et al. is presented in figure 3. Sharma et al. also validated an LC-ELSD method according to the ICH guidelines and found comparable recoveries in the range of 94.1 – 99.9 %. The precision results showed the low values of intra- and inter-
day % RSD of the retention times and peak areas < 1%. Also Bhandari et al. validated the method and
found recoveries of 93.1 – 98.6 % and showed precision results of < 2% of intra- and inter-day % RSD of
retention times and peak areas. The reported RSD values of Bhandari et al. (< 2 %) are slightly higher
compared to the RSD values of Sharma et al. (< 1 %) and Liu et al. (< 1.12 %). Bhandari et al. used an
extraction step for the sugars and took this extraction into the validation data which can cause a higher
% RSD.

The lowest detection limits with the detection of ELSD were reported by Agblevor et al. The separation
was performed on a Prevail carbohydrate ES column. The reported detection limits are a factor 10 lower
compared to the other references.

The combination of LC and ELSD is reported in several other articles. In table 1 (page 16) these
applications are reported with their specific performance parameters.
2.2.2 Refractive Index Detector (RI)

A RI detector detects components based on the refraction of light in solution. When components are eluting from the column, the composition change in the analytical and reference cell is recorded, which changes its photorefractive level. As a result, the amount of light received by the light-receiving section changes, showing a peak which can be detected. Any component in the eluent can be detected which make RI suitable for sugar detection.

The response of RI detection results in linear calibration curves. One of the disadvantages of RI detection is the temperature sensitivity, small fluctuations in temperature results in high variations. RI detection cannot be used in combination with gradients which makes method development limited.

Chavez-Servin et al., Castellari et al. and Barreira et al. all reported validated methods for sugar separation with the use of RI detection. The detection limits are in the same order (see Table 2), although different columns were used for the separation.2-6-11

Castellari et al. validated the method and found a precision of <1% and a recovery of 99.3% for fructose and 99.6% for fructose. They also validated the difference between direct injection and injection after sample clean up. No significant difference was observed.2 Barreira et al. determined the precision of the method by repeatability (intra-day) and intermediate precision (inter-day). The intra-day precision was found to be <1.4% and the intra-day precision <3.6%. The recovery was determined by spiking experiments and are between 88.5 and 99.8%.6 Chavez-Servin et al. found repeatability results of less than 1% and reproducibility of less than 7.0%. Recoveries in all sugars were between 93 and 113%.11

Lopes et al. described a HPLC-RI method with the use of a chiral column.12 The method allows the carbohydrate identification and determination of the absolute configuration (d or l) and simultaneously also determine the configuration of the anomeric centre (α or β) of the monosaccharide. They also studied the effect of the column temperature on the separation.12 The elution profiles of the sugars are depended on column temperature. The change was not uniform for all monosaccharides: for example, when the temperature was increased, the elution profile of lyxose became worse, while for glucose the best elution profile was achieved at 40 °C. Finally 25 °C was chosen as reasonable temperature for all sugars.12 No complete validation was performed of this method.

Liquid chromatography in combination with RI detection is reported in literature. A few applications of sugar separations are summarized in the table below, table 2 (page 17). However more HPLC-RI methods are reported in literature, in which no validation parameters are reported.12-13-14
2.2.3 Ultra Violet Detection (UV)

In liquid chromatography UV/UV-VIS detectors are frequently used to measure components showing an absorption spectrum in the ultraviolet or visible region. An UV detector is equipped with a deuterium lamp (D2 lamp) as a light source and has a light ranging from 190 to 380 nm. As a lot of components exhibit a chromophoric group, so a broad range of substances can be detected. Sensitivity of the detection depends on the component. However the lack of chromophores in the structure prevents the use of UV detection for native sugars. Therefore, the derivatization of sugars is necessary to apply UV detection. The reagents 1-phenyl-3-methyl-5-pyrazolone (PMP) and p-aminobenzoic ethyl ester are the most popular labels that react with reducing carbohydrates under mild conditions. 7-8

![Chemical structure of glucose derivatized with p-aminobenzoic ethyl ester](image)

Figure 4: Labelling reaction of glucose with p-aminobenzoic ethyl ester, reproduced from reference 8

Lv et al. used 1-Phenyl-3-methyl-5-pyrazolone as derivatization reagent and reported recoveries of 94.6 – 108 %. 7 Blanco Gomis et al. used p-aminobenzoic ethyl ester as derivatization reagent to detect the desired sugars and reported recoveries between 90 – 120 %. 8

Lv et al. performed a full validation of the method as described by the ICH guidelines. They reported RSD % values for intra- and inter-day precision of less than 2.4 % for the retention time and less than 4.5 for the peak areas. 7 Blanco-Gomis et al. determined the repeatability as less than 1.6% and the inter-day reproducibility as less than 5%. 8

The reported detection limits of both references are in the nanogram range, which is significant lower compared to the reported detection limits by ELSD and RI. Low detection limits can be interesting in food industry, for example to detect the use of apple juice in the cider making process. 8 An example of the separation of sugars in tea extracts by a c18 column and detection by UV after derivatization with PMP is presented in figure 5. Ten different sugars are separated in 35 minutes.
Separation of sugars using derivatization and UV detection is reported in several applications, as presented in table 3 (page 18).

### 2.2.4 Mass Spectrometry (MS)

Over the past decade, the development of LC/MS methods dedicated to the analysis of sugars and monosaccharide’s in particular, has led to significant advances in terms of sensitivity and specificity while maintaining speed and simplicity of implementation.\(^\text{15}\)

The principle of mass spectrometry is ionizing chemical compounds to generate charged molecules or molecule fragments. The ions are detected. The signal is processed into the spectra of the relative abundance of ions as a function of the mass to charge ratio.

Mass spectrometry can be used in combination with liquid chromatography for the analysis of sugars. Sugar ionization in atmospheric pressure ionization (API) type sources represented the main challenge because of their low efficiency of ionization in negative mode directly related to their low acidic character. However using MS information about chemical structures can be generated which can be very useful in development phases.

Ricochon et al. described an analysis method for the separation and detection of sugars in orange juice. After calibration and analysing samples they found poor linearity and repeatability. The use of an internal standard did not improve the results. They decided to add 1% chloroform to the eluent to form Cl adducts and analysed the sugars in APCI in negative ion mode \([M+35]^–\) and \([M+37]^–\).\(^\text{15}\) After validation of the method the inter-day precision is determined as less than 0.3% based on peak area.
Hammad et al. used a triple quadrupole MS in MRM (multiple reaction monitoring) modes. They analysed the sugars as their alditol acetate anion adducts \([M+\text{CH}_3\text{CO}_2]^-\) using electro spray ionization in negative ion mode. Although very low detection limits are reported (pg range) the precision is around 5% and the average recovery is around 60% for all sugars. This is expected since it might be that the sugars are not complete re-acetylated with acetic anhydride, or undergo side reaction.\textsuperscript{16} A chromatogram of the separation developed by Hammad et al. is presented in figure 5.

![Chromatogram](image)

**Figure 6: Fast separation of sugars by LC-MS, reproduced from reference 16. (xylose (a), galactose (b), mannose (c), glucose (d), fucose (e), N-acetylgalactosamine (f), N-acetylglucosamine (g) (h and i are non sugar compounds))**

Matias et al. used MS with ESI in full scan modes for identification and SIM (selected ion monitoring) for quantification. Sugars were detected in positive ion mode as their sodium adducts \([M+\text{Na}]^+\). Recoveries of the target sugars ranged from 95.4 – 97.7 % and the reproducibility of the injection was reported as less than 3.4 %.\textsuperscript{17}

Mass spectrometry in combination with liquid chromatography and the separation of sugars is reported in literature. A few applications and validation parameters are presented in table 4 (page 19). More methods were found however without validation parameters.\textsuperscript{17-18}
2.2.5 Fluorescence Detection

Fluorescence detection is also reported in combination with applications for the separation and detections of sugars. In fluorescence substances absorbs light at one wavelength and emit light at another wavelength. Substances have specific wavelengths of light that it absorbs (excitation wavelengths) and emits (emission wavelengths).

Fluorescence detection is suitable for trace analysis due to generally having high sensitivity and selectivity (not detecting impurities). There are not many components that originally emit fluorescence (natural fluorescence). However, after reaction with a fluorescence reagent (derivatization) a lot of compounds can be detected as fluorescent substances. This method makes it possible to measure various components with high sensitivity.

Since sugars contain no fluorophores, derivatization of the sugars is required. Common fluorescent tags used for labelling of the monosaccharides prior to HPLC analysis are: anthranilic acid (AA), 2-aminobenzamide, 2-aminopyridine, phenyl isothiocyanate, 9-fluorenylmethoxycarbonylhydrazine, 7-amino-4-methylcoumarin and 7-amino-1,3-naphthalene-disulphonate. Kakita used benzamidide as derivatization reagent and reported high sensitivity, good linearity and good reproducibility. Racaityte et al. used anthranilic acid (AA) as derivatization reagent.

Racaityte et al. developed 2 different methods for the separation of sugars, HPLC-fluorescence and CZE-LIF (laser induced fluorescence), but only validated the HPLC method. Both methods are suitable for the separation and detection of sugars, each with their advantages and disadvantages. Recoveries between 96.1 and 100.2% were determined. The repeatability was found as less than 4.5% and the intermediate precision as less than 4.3%. A chromatogram of the separation by LC and detection by fluorescence is presented in figure 6.

Figure 7: Separation of sugars by fluorescence detection after derivatization with anthranilic acid, reproduced from reference 9.
Kakita et al. validated the method and found the RSD for the retention time varied between 0.05 – 0.36% and for the fluorescence intensity between 1.14 – 1.62%.\(^\text{19}\)

Sensitive methods with fluorescence detection are available for quantification of sugars and are summarized in the table below, table 5 (page 20). Although low detection limits can be reached, derivatization is always required, which makes the method not suitable for routine analysis.

### 2.2.6 Charged Aerosol Detection (CAD)

Charged Aerosol Detection (CAD) is a recent developed detector based on the detection of scattered particles, like in ELSD. The eluent coming from the LC is nebulized using a flow of nitrogen and the resulting aerosol is transported through a drift tube where the volatile components and solvents are evaporated. The dried particle stream is charged with a corona discharge needle, resulting in an electrical charge measured by an electrometer. Like ELSD, the CAD is mass-dependent and is called universal.\(^\text{20}\)

Aerosol detectors are well-suited to detect all non-volatile analytes, regardless of whether these analytes contain a chromophore. These properties make aerosol detectors suitable for the detection of sugars.\(^\text{21}\)

A performance evaluation between ELSD and CAD is reported by Vervoort et al. Fitting of the exponential factor in the calibration equation showed that for the chosen concentration interval the linearity of the CAD is much better than that of the ELSD. In general the CAD is more sensitive than ELSD, the peak shape is little worse in CAD (higher tailing factor) and the repeatability of both detectors is still around a factor 2 worse compared to UV detection.\(^\text{20}\)

Applications of sugars separations are reported, however without validation parameters.\(^\text{21-22}\)
2.3 Discussion liquid chromatography

Different types of liquid chromatography columns are available for the separation of sugars. In all cases separation of sugars is challenging and method development is required to optimize separation in reasonable retention times. After separation, detection is also difficult as sugars contain no chromophores to analyse them with UV, what mostly is done in LC.

The use of ELSD in combination with the separation of sugars has been reported by different authors. With the use of different columns for the separation, detection limits in the same order were found (in the order of 0.1 – 1 µg per injection). A recovery of 93 – 99.9 % was reported and an intra- and inter-day precision as % RSD of the retention time and peak are < 2 %.

A recent developed detector for the analysis of sugars is the CAD detector; however the performance is comparable to the ELSD detector.

Detection of sugars by RI was reported in the first applications of the analysis of sugars. Detection limits are in the same range as reported in ELSD applications. Lopes et al. did some method development by varying the column temperature on a chiral column and RI detection. Castellari et al. found excellent recoveries (>99.3%) and precision results <1%. However other authors reported lower recoveries (between 89 – 113%) and higher precision values.

Mass spectrometry is also an option for the detection of sugars. Several applications are reported where sugars were analysed as their sodium adduct, acetate adduct or chloride adduct. Compared to the detection limits of other detectors, the lowest limits were reported by Hammad et al. using a quadrupole MS, detection limits in pg range per injection are reported. Although very low detection limits are reported (pg range) the precision is around 5% and the average recovery is around 60% for all sugars. Comparable precision values are found in other applications.

For detection by UV a derivatization reagent is required. Applications are reported where 1-Phenyl-3-methyl-5-pyrazolone and p-aminobenzoic ethyl ester is used as derivatization reagent, with recoveries between 90 – 120 %. The reported detection limits of both references are in the nanogram range, which is significant lower compared to the reported detection limits by ELSD and RI. The reported values for precision are <2.4% for intra-day precision and <5% for inter-day precision.

Different derivatization reagents are applicable for detection by fluorescence. Low detection limits are reported with the use of anthranilic acid and benzamidide as derivatization reagent. The detection limits of sugar separation with the use of a fluorescence detector are significant lower compared to the ELSD and RI detection, however a derivatization step is always required.
## Table 1: Overview of LC-ELSD methods for the analysis of sugars.

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min(^a))</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biorad HPX-87H 250 mm x 4.6 mm, 5 µm</td>
<td>Eluent: Isocratic 0.00025 M H(_2)SO(_4) Flow: 0.6 ml/min, Temp: 40°C Injection volume: 30 µl Total analysis time: 20 min</td>
<td>Glucose (8.7) Xylose (9.3) Arabinose (10.2)</td>
<td>34 – 514 53 – 802 20 – 305</td>
<td>0.9979 0.9987 0.9975</td>
<td>2.25 2.59 5.89</td>
<td>0.37 0.29 0.44</td>
<td>1.14 0.89 1.32</td>
<td>1</td>
</tr>
<tr>
<td>Prevail carbohydrate ES column 250 x 4.6 mm packed with 5 µm spherical polymer beads</td>
<td>Eluent: Isocratic Acetonitrile (85%) water (15%) Flow: 1.0 ml/min Injection volume: 5 µl Total analysis time: 40 min</td>
<td>Arabinose Xylose Fructose Mannose Galactose Glucose</td>
<td>1.5 – 50 mg/ml All &gt; 0.98 n.r.</td>
<td>n.r. - 0.024 - 0.085 0.050</td>
<td>n.r. 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zorbax-NH2 column 250 mm x 4.6 mm, 5 µm</td>
<td>Eluent: Gradient starting at 86% ACN, held for 10 min, then shifting ACN linearly to 88% over 20 min, 78% over 30 min, 74% over 40 min, 86% over 50 min and re-equilibrated for 10 min Flow: 0.5 ml/min Temp: 25°C Injection volume: 30 µl Total analysis time: 60 min</td>
<td>Rhamnose Xylose Xylitol Arabinose Glucose Mannitol Sucrose Cellophane α-lactose</td>
<td>671.6 – 5100 355.6 – 2700 165.9 – 1260 329.2 – 2500 268.6 – 2040 165.9 – 1260 86.9 – 660 71.1 – 540 79.0 – 600</td>
<td>0.995 0.992 0.973 0.976 0.988 0.992 0.977 0.999 0.959</td>
<td>2.28 1.05 0.59 0.90 0.45 0.31 0.11 0.08 0.32</td>
<td>1.11 0.56 1.11 1.33 1.17 1.13 0.34 0.76 0.39</td>
<td>3.35 1.68 3.36 4.03 3.35 4.03 1.03 2.30 1.19</td>
<td>4</td>
</tr>
<tr>
<td>Zorbax-NH2 column 250 mm x 4.6 mm, 5 µm</td>
<td>Eluent: Isocratic acetonitrile : water (78:22, v/v) Flow: 0.5 ml/min Temp: 20°C Total analysis time: 40 min</td>
<td>Xylose Xylitol Mannitol Glucose Sucrose</td>
<td>100 – 1000 100 – 1000 40 – 400 100 – 1000 100 – 1000</td>
<td>0.997 0.998 0.997 0.999 0.999</td>
<td>1.12 1.56 2.13 1.98 1.23</td>
<td>0.98 0.76 0.67 0.56 0.56</td>
<td>2.95 2.35 2.02 1.68 1.68</td>
<td>5</td>
</tr>
<tr>
<td>Carbamoyl-silica HILIC column, TSKgel Amide-80, 250 mm x 4.6 mm, 5 µm</td>
<td>Eluent: Isocratic 82% acetonitrile, in 5mM ammonium formate (adjusted to pH 5.5 with formic acid) Flow: 1.0 ml/min, Temp: 60°C Injection volume: 50 µl Total analysis time: 20 min</td>
<td>Fucose Mannose Galactose</td>
<td>n.r. 1.00 0.99 1.00</td>
<td>4 4 4</td>
<td>0.3 0.3 0.3</td>
<td>n.r.</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) If reported

n.r. Not reported in article
Table 2: Overview of LC-RI methods for the analysis of sugars

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer carbohydrates column</td>
<td>Column 250 mm x 4.6 mm, 5µm and an NH2 pre-column 13 mm x 3 mm</td>
<td>Fructose (5.8)</td>
<td>0.5 – 10</td>
<td>0.998</td>
<td>0.78</td>
<td>3.4</td>
<td>5.4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Eluent: Isocratic acetonitrile–water (75:25)</td>
<td>Glucose (6.8)</td>
<td>0.5 – 10</td>
<td>0.998</td>
<td>-</td>
<td>2.6</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flow: 1.8 ml/min</td>
<td>Galactose (7.4)</td>
<td>0.5 – 10</td>
<td>0.978</td>
<td>-</td>
<td>1.2</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temp: 25°C</td>
<td>Sucrose (9.8)</td>
<td>0.5 – 10</td>
<td>0.999</td>
<td>0.99</td>
<td>3.2</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Injection volume: 20 µl</td>
<td>Lactulose (11.7)</td>
<td>0.25 – 3</td>
<td>0.999</td>
<td>2.91</td>
<td>1.0</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total analysis time: 15 min</td>
<td>Lactose (13.7)</td>
<td>2 – 15</td>
<td>0.998</td>
<td>0.46</td>
<td>5.0</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Aminex HPX-87H 300 x 7.8 mm</td>
<td>Column 300 x 4.6 mm with a pre-column 30 x 4.6 mm</td>
<td>Glucose (12.5)</td>
<td>0.5 – 20</td>
<td>0.999</td>
<td>1.41</td>
<td>0.2</td>
<td>n.r.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Eluent: Isocratic 0.003 – 0.05 NH2SO4 with 6% acetonitrile (v/v).</td>
<td>Fructose (13.4)</td>
<td>0.5 – 20</td>
<td>0.999</td>
<td>1.22</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flow: 0.5 ml/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temp: 45°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Injection volume: 20 µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total analysis time: 25 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eurospher 100-5 NH2 column</td>
<td>4.6 x 250 mm, 5 µm</td>
<td>Fructose (5.97)</td>
<td>0.2 – 24</td>
<td>0.9999</td>
<td>0.27</td>
<td>1.0</td>
<td>3.6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Eluent: Isocratic acetonitrile/water, 7:3 (v/v)</td>
<td>Glucose (6.36)</td>
<td>0.3 – 24</td>
<td>0.9999</td>
<td>0.26</td>
<td>1.6</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flow: 1.0 ml/min</td>
<td>Sucrose (7.41)</td>
<td>0.2 – 24</td>
<td>0.9999</td>
<td>0.33</td>
<td>1.2</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temp: 35°C</td>
<td>Raffinose (10.75)</td>
<td>0.3 – 24</td>
<td>0.9991</td>
<td>0.36</td>
<td>1.8</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Injection volume: 20 µl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total analysis time: 15 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> If reported
n.r. Not reported in article
Table 3: Overview of LC-UV methods for the analysis of sugars

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min$^a$)</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP-C18 column 4.6 mm x 250 mm, 5 µm</td>
<td>Eluent: Gradient acetonitrile and 0.045% KH$_2$PO$_4$ – 0.05% triethylamine buffer (pH 7.0) gradient elution of 90–89–86% B by a linear decrease from 0–15–40 min Flow: 1.0 ml/min Temp: 35°C Injection volume: 20 µl Derivatization with: 1-Phenyl-3-methyl-5-pyrazolone (PMP) Total analysis time: 40 min</td>
<td>Mannose (7.76) Ribose (8.43) Rhamnose (9.68) Glucose (25.66) Xylose (29.18) Galactose (32.30) Arabinose (33.65)</td>
<td>1 - 30</td>
<td>0.999</td>
<td>0.35</td>
<td>3.2E-3</td>
<td>n.r.</td>
<td>7$^b$</td>
</tr>
<tr>
<td>Kromasil C8 200 mm x 2.1mm, 3.5 µm</td>
<td>Eluent: isocratic A (100mM sodium citrate buffer, pH 5.5/THF = 88/12) B (acetonitrile) ratio A/B = 99/1 (v/v %) Flow: 0.15 ml/min Temp: 45°C Injection: 5 µl Derivatization with: p-aminobenzoic ethyl ester (ABEE) Total analysis time: separation of sugars in 35 min, excess derivatization reagent 60 min.</td>
<td>Glucose Galactose Xylose Arabinose Ribose Fucose Rhamnose</td>
<td>2 – 30 µg</td>
<td>0.999</td>
<td>n.r.</td>
<td>5.4 E-4</td>
<td>n.r.</td>
<td>8$^c$</td>
</tr>
</tbody>
</table>

n.r. Not reported in article

$^a$ If reported

$^b$ Derivatization with 1-Phenyl-3-methyl-5-pyrazolone (PMP) and detection at 250 nm. The results show that the recoveries ranged between 94.6% and 108.0% and the RSD values fell within 1.8–4.9%.

$^c$ Derivatization with p-aminobenzoic ethyl ester (ABEE) and detection at 307 nm. The average recoveries obtained, which ranged between 90% and 102%
## Table 4: Overview of LC-MS methods for the analysis of sugars

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMC-Pack Polyamine II 250 mm x 4.6 mm composed of a polymeric support (silica base) with mixed secondary and tertiary amino derivative (particles size are 5 µm and pores size are 12 nm) Pre-column 7.5 mm x 4.6 mm</td>
<td>Eluent: A: 100% water, B: 99% acetonitrile with 1% chloroform Isocratic mode with 75% acetonitrile over 30 min Flow: 1 ml/min Injection volume: 20 µl Total analysis time: 20 min</td>
<td>Glucose 0.3 – 51.2 Sucrose 1.0 – 51.2 Fucose 0.1 – 51.2 Fructose 0.1 – 25.6 Mannose 5.0 – 256 Xylose 1.7 – 256 Galactose 5.0 – 256 Rhamnose 0.3 – 102.4 Arabinose 0.7 – 256</td>
<td>0.3 – 51.2 1.0 – 51.2 0.1 – 51.2 0.1 – 25.6 5.0 – 256 1.7 – 256 5.0 – 256 0.3 – 102.4 0.7 – 256</td>
<td>1.000 n.r. 0.999 0.999 0.997 0.999 0.999 0.997 0.998</td>
<td>0.27 0.11 0.15 0.35 0.25 0.21 0.25 0.13 0.28</td>
<td>1.6 E-3 6 E-3 6 E-3 6 E-3 30 E-3 10 E-3 10 E-3 2 E-3 30 E-3</td>
<td>5 E-3 20 E-3 1.8 E-3 2 E-3 100 E-3 33 E-3 100 E-3 6.8 E-3 13 E-3</td>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypercarb column 50 x 2.1 mm, 3 µm particle size Hypercarb guard column 10 x 2.1 mm, 3 µm particle size</td>
<td>Eluent: A: 100% H2O containing 0.01% ammonium acetate, B: 100% acetonitrile. A gradient of 0% to 15% B in 15 min Flow: 0.15 ml/min Temp: 7°C Injection volume: 20 µl Total analysis time: 5 min</td>
<td>Glucose (2.57) Galactose (2.12) Mannose (2.31) Fucose (2.80) Xylose (2.06)</td>
<td>n.r.</td>
<td>0.9988 0.9991 0.9990 0.9989 0.9814</td>
<td>n.r.</td>
<td>1.5 E-6 2 E-6 10 E-6 10 E-6 10 E-6</td>
<td>16&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Hi plex Ca column 250 x 4.0 mm</td>
<td>Eluent: Isocratic ultra-pure water Flow: 0.2 ml/min Injection volume: 25 µl Total analysis time: 20 min</td>
<td>Glucose (13.1) Fructose (16.8) Sucrose (11.0)</td>
<td>0.1 – 4.5 0.1 – 4.5 0.1 – 4.5</td>
<td>0.999 0.998 0.996</td>
<td>3.0 3.2 3.4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>17&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

n.r. Not reported in article

<sup>a</sup> If reported

<sup>b</sup> Linear ion trap mass spectrometer, LTQ-MS atmospheric pressure chemical ionization (APCI) interface operating in negative ion mode. Detection in full scan mode (with m/z measured from 100 to 500) and in SIM mode

<sup>c</sup>The mass spectrometer was run in negative ion multiple reaction monitoring (MRM) mode. Therefore, the MRM transitions for the aldose monosaccharides used in this study correspond to the [M + CH3CO2]<sup>-</sup> precursor ion yielding the [M - H]<sup>-</sup> product ion, where M corresponds to the mass of alditol sugar (the molecular mass of the aldose _ 2 hydrogens).

<sup>d</sup>The atmospheric pressure (AP)-electrospray ionization (ESI) mass spectrometer was a triple quadrupole Target sugars were determined by LC-ESI-MS operating in the positive ionization mode. Target sugars were identified in the scan mode (m/z 100 - 2000) and quantified in the selected ion monitoring (SIM) mode.
Table 5: Overview of LC-Fluorescence methods for the analysis of sugars.

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSKgel Amide-80 column packed with chemically bonded carbamoyl–silica gel</td>
<td>Eluent: Gradient 37.5 min linear gradient from A acetonitrile–water, (80:20) to B acetonitrile–water, (60:40) Flow: 0.6 ml/min Temp: 80°C Injection volume: 10 µl Derivatization with: benzamidine Total analysis time: 45 min</td>
<td>Ribose Fructose Glucose Maltose</td>
<td>All 0.36E-3 – 0.36</td>
<td>&lt;0.999</td>
<td>3.3E-4 2.7E-4 3.2E-4 6.7E-4</td>
<td>n.r.</td>
<td>19&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C18 reversed phase column: YMC-Pack ODS-A 150 mm x 4.6 mm, 5 µm, Precolumn: C18 4 mm x 3 mm</td>
<td>Mobile phase: A: 50mM sodium acetate buffer pH 4.1 in water, B: 20% A in methanol. Gradient 0–35 min 3% B isocratic, 35–80 min linear gradient 3–8% B, 80–85 min linear gradient 8–9% B, 85–90 min 9% B isocratic, 90–95 min 100% B linear gradient Flow: 0.7 ml/min Temp: 25°C Derivatization with: anthranilic acid Total analysis time: 55 min</td>
<td>Galactose Mannose Fucose</td>
<td>All 0.1 – 4.0</td>
<td>0.999 0.998 0.997</td>
<td>3.0 2.0 4.5</td>
<td>2.3E-3 2.3E-3 2.5E-3</td>
<td>9&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

n.r. Not reported in article
<sup>a</sup> If reported
<sup>b</sup> Derivatization with post column eluent mixed with 1.0M potassium hydroxide solution and benzamidine, each at a flow rate of 0.6 ml/min. Fluorescence intensities of the reaction mixtures were monitored at 288 nm for excitation and 470 nm for emission
<sup>c</sup> Derivatization reaction with anthranilic acid. For detection the following wavelengths were used: excitation 230 nm, emission 425 nm
3 HIGH PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY (HPAEC)

Another type of liquid chromatography is High Performance Anion-Exchange Chromatography (HPAEC) with pulsed amperometric detection (PAD). HPAEC-PAD is often used for the separation of sugars. It requires a dedicated system and special detector. All references make use of a PA type (PA-1/PA-10/PA-20/PA-100/PA-200) of column. The PA column is an anion-exchange column, suitable for the separation of sugars. The choice of the PA column depends on the types of sugars to be separated (mono-, di-, saccharide etc) and the resolution needed for the specific sample matrix.

3.1 HPAEC with pulsed amperometric detection (PAD)

HPAEC-PAD takes advantage of the affinity between the ionized group of sugars at alkaline pH (pH 12) and a pellicular quaternary amine stationary phase. Therefore high resolution and highly selective separation of non-derivatized sugars can be achieved.

PAD has gained prominence as a selective and sensitive technique for the determination of carbohydrates due to the fact that oxidefree detection for aldehyde and alcohol-containing compounds at gold (Au) electrodes in alkaline media (pH 12) can be easily executed. Pulsed amperometric detection uses a triple-step potential waveform to combine amperometric detection with alternating anodic and cathodic polarization pulses to clean and reactivate the electrode surface. The use of Au electrodes has the distinct advantage that detection can be achieved without simultaneous reduction of oxygen (dissolved in the mobile phase).

Detection limits gained with HPAED-PAD detection are in the same range as with LC with UV detection after derivatization and LC with MS detection, in the nanogram per injection range. An example of the separation of sugars by HPAED with PAD detection is presented in figure 8.

![Figure 8: Analysis of sugars in biomass by HPAED-PAD on a PA-20 column, reproduced from reference 26. (1) fucose (I.S.), (2) sucrose, (3) arabinose, (4) galactose, (5) glucose, (6) xylose, (7) mannose, (8) fructose (9) lactose (I.S.), (10) cellobiose, and (11) maltose)](image)

Sevcik et al. reported analytical accuracy of the spiked recoveries which typically ranged from 84 to 98%. The inter-day precision for the retention time was determined and found to be lower than 3%. 26
Raessler et al. tested the stability of the retention times after subsequent injections (at the same day) and found RSD <1%.24

3.2 HPAEC with Mass detection

Beside PAD detection, MS detection can be used in combination with HPAEC although this is a technological challenge. Typical alkali acetate and hydroxide eluents are not compatible with atmospheric pressure ionization (API) due to their non-volatility and high conductance. Bruggink et al. created a system with a desalting device installed between the column and the MS. The desalter converts the alkali hydroxide and acetate into water and acetic acid continuously exchanging the alkali cations by hydronium ions using a selective cation exchange membrane and a regenerant.25 Bruggink et al. created a HPAED-MS system where quantification was done in SIM mode. In this case MS was only used for structure elucidation. In the validation of both detectors it appeared that the detection limits of MS were 10 times higher compared to PAD detection.25 The sugars separated by PAD and MS detection are presented in figure 9, the differences in sensitivity of the detectors is clearly visible.

Figure 9: Separation of glucose (1), fructose (2) and sucrose (3) by HPAED chromatography with PAD and MS detection, reproduced from reference 25.

3.3 Discussion high performance anion-exchange chromatography

High performance anion-exchange chromatography with PAD detection is a sufficient way of separating and detecting sugars. Different type of columns can be used to optimize the separation. The correlation coefficient of the calibration curves is in all reported references >0.99.

Detection limits reported with HPAED-PAD are in the nanogram range and are comparable to LC with UV or fluorescence detection after derivatization or LC with MS detection.

Bruggink et al.25 developed a system to use MS as detection in combination with HPAEC. They also reported impressive detection limits with PAD detection; however they are the builder and owner of the HPAEC system.

In table 6 HPAEC methods and validation parameters are presented. More HPAEC methods are reported in literature, without validation parameters.27-28
Table 6: Overview of HPAEC-PAD (MS) methods for the analysis of sugars

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CarboPac PA-10 250 x 4.6 mm Guard column Carbo Pac PG-10</td>
<td>Eluent: 18 mM NaOH Flow: 1 ml/min Temp: 40 °C Detection: PAD Ag/AgCl reference electrode, Au working electrode Injection volume: 25 µl Total analysis time: 26 min</td>
<td>Inositol Sorbitol Mannitol Arabinose Galactose Glucose Sucrose Fructose Raffinose</td>
<td>0.5 – 10</td>
<td>0.9988 0.9993 0.9998 0.9995 0.9998 0.9994 0.9993 0.9966 0.9991</td>
<td>n.r.</td>
<td>0.09E-3 0.08E-3 0.04E-3 0.22E-3 0.15E-3 0.25E-3 0.24E-3 0.57E-3 0.28E-3</td>
<td>0.25E-3 0.2E-3 0.1E-3 0.55E-3 0.38E-3 0.63E-3 0.6E-3 0.7E-3</td>
<td>24</td>
</tr>
<tr>
<td>CarboPac PA-1 anion exchange column 250 x 4 mm. Guard column PA-1 50 x 4 mm</td>
<td>Eluent: Gradient with 300 mmol/l both sodium acetate and sodium hydroxide and water Flow: 1 ml/min Detection: PAD Au working electrode Injection volume: 50 µl Total analysis time: 40 min</td>
<td>Glucose (18.1) Mannose (19.5) Fructose</td>
<td>0.18 - 18</td>
<td>0.9998 0.9998 0.9996</td>
<td>n.r.</td>
<td>9E-3 9E-3 10E-3</td>
<td>n.r.</td>
<td>23</td>
</tr>
<tr>
<td>CarboPac PA-20 150 x 3 mm Guard column CarboPac PA-20 30 x 3 mm</td>
<td>Eluent: 1.0 mM NaOH (isocratic) Flow: 0.5 ml/min Temp: 40 °C Detection: Au electrode with Ag/AgCl as reference Injection volume: 10 µl Total analysis time: 15 min</td>
<td>Sucrose (5.76) Arabinose (6.14) Galactose (7.76) Glucose (8.58) Xylose (10.15) Mannose (10.53) Fructose (12.87)</td>
<td>0.22 – 1.720 0.02 – 0.22 0.03 – 0.24 0.46 – 3.71 0.03 – 0.25 0.02 – 0.19 0.27 – 2.23</td>
<td>0.9995 0.9953 0.9969 0.9996 0.9978 0.9997 0.9977</td>
<td>n.r.</td>
<td>0.12E-3 0.09E-3 0.11E-3 0.12E-3 0.11E-3 0.22E-3 0.25E-3</td>
<td>0.39E-3 0.29E-3 0.38E-3 0.38E-3 0.32E-3 0.72E-3 0.80E-3</td>
<td>26</td>
</tr>
<tr>
<td>Column</td>
<td>Method details</td>
<td>Sugars (retention time min$^a$)</td>
<td>Linear range (µg/ml)</td>
<td>R2</td>
<td>RSD %</td>
<td>DL (µg)</td>
<td>QL (µg)</td>
<td>Ref</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>CarboPac PA-200 3 x 250 mm with a CarboPac PA-200 3 x 50 mm guard column</td>
<td>Eluent: 60 mM NaOH Flow: 0.5 ml/min Temp: 30 °C Detection: Ag/AgCl reference electrode and Au working electrode and MS detection with electrospray ionisation Total analysis time: 6 min</td>
<td>Glucose Fructose Sucrose</td>
<td>n.r.</td>
<td>0.9999</td>
<td>n.r.</td>
<td>0.02E-3</td>
<td>0.21E-3</td>
<td>25</td>
</tr>
</tbody>
</table>

n.r. Not reported in article

$^a$ If reported

$^b$ Detection limits of Mass detection in SIM (selected ion monitoring) mode.
GAS CHROMATOGRAPHY (GC)

Beside liquid chromatography, gas chromatography is a good alternative for the analysis of sugars. Compared to liquid chromatography the main advantage of GC is much higher separation power. In GC many sugars can be detected, even chiral components, in relative short retention times. Although the resolution in GC is much better compared to other techniques, a derivatization step is always required which can be seen as a drawback.

4.1 Derivatization

Due to their high polarity, hydrophilicity and low volatility, all sugars needs to be converted into volatilizable and stable derivatives prior to the GC or GC-MS analysis. Preparation of carbohydrate derivatives for GC and GC–MS present several difficulties. First, there is a high number of functional groups in the molecule (about one in each carbon atom, most of them being hydroxyls). Secondly, the presence of different tautomeric forms in solution gives rise to complex chromatograms. Finally, the lability of some molecules and in certain cases, the steric hindrance must be considered.

Due to the relatively low volatility of carbohydrates, GC analysis is limited to derivatized sugars of low molecular weight, mainly mono-, di- and tri-saccharides. Classical derivatization methods consist in the substitution of the polar groups of carbohydrates in order to increase their volatility. Methyl ethers, acetates, trifluoroacetates and trimethylsilyl ethers are the most common derivatives used for carbohydrate determination. The good volatility and stability characteristics of the derivatives formed make trimethylsilyl (TMS) ethers the most popular derivatives applied to GC analysis of saccharides which are reported in the references in table 6.

Medeiros et al. determined the recoveries of derivative sugars, which is between 68 and 118 % for different sugars at different levels. In the review of Ruiz-Matute et al. a summary of the most common derivatization methods used for sugar analysis, including advantages and disadvantages, is presented.

4.2 Detection

After derivatization detection can be done by FID (flame ionisation detector) and MS (mass spectrometry). The FID detection is based on the detection of ions formed during combustion of organic compounds in a hydrogen flame. The ions formed are proportional to the concentration of the organic species in the gas stream. FID is a suitable detector for the detection of sugars. Beside FID detection also MS detection is a proper detector for sugars analysis. Mass detection can be used in both full scan and in single ion mode. Medeiros et al. reported detection limits in the range of 0.1-0.3 ng, also Gomez-Gonzalez et al. detection limits in the low nanogram range. The detection limits of Gomez-Gonzalez et al. are slightly lower compared to the limits reported by Medeiros et al. This can be explained by the differences in MS detection, Medeiros et al. quantified the sugars in TIC (total ion current) mode, while Gomez-Gonzales et al. used SRM (single reaction monitoring).
Medeiros et al. determined the reproducibility of the analytical procedure through the relative standard deviation of replicate measurement. The RSD values ranged from 5.5 – 9.3%. Fernandez-Artigas et al. reported a RSD range of 3.8 – 5.6% for the precision study. Fuzfai et al. reported an average RSD of 3.3%.

A chromatogram of the separation of different trimethylsilylated sugars in gum is presented in figure 10.

**Figure 10:** Chromatogram of trimethylsilylated sugars in gum arabic analysed by GC-MS, reproduced from reference 33.

### 4.3 Discussion gas chromatography

The main advantage of using gas chromatography is the high resolution in relative short retention times, although derivatization of the sugars is always required. The reported detection limits are the nanogram range, comparable to HPAED-PAD or LC with derivatized sugars for UV or fluorescence.

In GC different types of derivatization reagent are available, methylation, acetates and silylation. In all reported references the trimethylsilyl ether is used, the good volatility and stability characteristics of the derivatives formed make trimethylsilyl (TMS) ethers the most popular derivatives applied to GC analysis of saccharides. Most reported methods used MS as detection, due to the extra information obtained with MS. Not many applications could be found for quantitative sugars separation and detection by GC-MS or GC-FID. This could be due to the low recovery values reported (68 and 118 %), relative high precision results (around 5%) and the extra work of the derivatization. The use of GC-MS to identify sugars is mainly used in the reported references.

In table 7 applications of analysis of sugars using GC are summarised. More applications can be found however without validation parameters.
### Table 7: Overview of GC methods for the analysis of sugars

| Column | Method details | Sugars (retention time min)  
| (α, β if applicable) | Linear range  
| (µg/ml) | R2 | RSD % | DL (µg) | QL (µg) | Ref |
|-------|--------------|--------------------------------|
| DB5-MS capillary column 30 m x 0.25mm I.D. and film thickness of 0.25 µm | Carrier gas: He  
Constant flow 1.3 mL/min.  
MS source temp: 230°C  
Injection: Split less mode, split less time 30 s.  
Injector temp: 280°C  
Injection volume: 1 µl  
Temp program: Consisted of injection at 65 °C and hold for 2 min, temperature increase of 6 °C/min to 300 °C, followed by an isothermal hold at 300 °C for 15 min  
Total analysis time: 45 min | Glucose (24.53 – 26.02)  
Levoglucosan (21.11)  
Sorbitol (25.33)  
Sucrose (35.33)  
More sugars separated, 4 sugars (groups) validated | 2.0 – 200  
1.6 – 160  
1.9 – 190  
1.2 – 120  
Recovery in range 68-118% | 0.9857  
0.9998  
0.9975  
0.9990 | 7.4  
7.5  
9.3  
5.5 | 0.15E-3  
0.13E-3  
0.36E-3  
0.28E-3 | n.r. | 30b |
| Factor Four fused-silica capillary column  
VF-5 ms, 30 m x 0.25 mm, 0.25 µm | Carrier gas: He  
Constant flow 1.3 mL/min  
Detection: MS  
Injection: split/splitless mode  
Injector temp: 280 °C  
Injection volume: 1µl  
Temp program: initial temperature = 65 °C (held for 2 min), increased at 6 °C/min to 300 °C (held for 30 min)  
Total analysis time: 50 min | Arabinose (19.69, 19.75)  
Xylose (21.56, 22.50)  
Glucose (24.83, 26.75)  
Mannose (22.85)  
Galactose (24.10, 24.98)  
Fructose (23.14, 23.29)  
Sucrose (35.15)  
Rhamnose (19.69, 21.19)  
Xylitol (21.39)  
Mannitol (25.21) | All range 0.1 – 150 | 0.997  
0.998  
0.997  
0.997  
0.997  
0.995  
0.990  
0.994  
0.997  
0.996 | n.r.  
0.076E-3  
0.076E-3  
0.015E-3  
0.30E-3  
0.076E-3  
0.03E-3  
0.015E-3  
0.076E-3  
0.076E-3  
0.076E-3 | 0.25E-3  
0.25E-3  
0.25E-3  
1.0E-3  
0.25E-3  
0.1E-3  
0.25E-3  
0.25E-3  
0.25E-3  
0.25E-3 | 31c |
### Quantitative analysis methods for sugars

<table>
<thead>
<tr>
<th>Column</th>
<th>Method details</th>
<th>Sugars (retention time min(^a)) ((\alpha, \beta) if applicable)</th>
<th>Linear range ((\mu)g/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL ((\mu)g)</th>
<th>QL ((\mu)g)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>The fused silica capillary column C-382 25QC2/SGL-1; 25 m x 0.25 mm I.D., 0.25 mm film thickness coated with 100% dimethyl polysiloxane</td>
<td>Carrier gas: N2  Constant flow 1.5 mL/min  Detection: FID  Injection split ratio: 1:20  Injection temp: 290°C  Detector temp: 300°C  Temp program: Initial temperature was 180 °C, followed by a heating rate of 3°C/min to 280°C. The final temperature was maintained for 23 min  Total analysis time: not reported</td>
<td>Fructose  Glucose  Sucrose  Maltose  Raffinose</td>
<td>n.r.</td>
<td>0.999</td>
<td>5.6</td>
<td>n.r.</td>
<td>n.r.</td>
<td>32(^d)</td>
</tr>
<tr>
<td>30m x 0.25 mm I.D. fused-silica column coated with a 0.25 (\mu)L film of SE-54</td>
<td>Carrier gas: He  Detection: MS  Inlet pressure of 110 kPa  Split-splitless injector  Injection temp: 300°C  Temp program: from 165°C up to 235°C at 2°C/min  Total analysis time: 35 min</td>
<td>Xylose (17.79)  Arabinose (18.92)  Rhamnose (21.42)  Fucose (22.31)  Glucose (27.41)  Mannose (27.63)  Galactose (28.57)</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>33(^e)</td>
</tr>
</tbody>
</table>

n.r. Not reported in article

\(^a\) If reported

\(^b\) The MS was operated in the electron impact mode with an ionization energy of 70 eV. The scan range was set from 50 to 650 Da at 1.27 scan/s. Derivatization performed using N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) and pyridine.

\(^c\) Ion trap mass spectrometer was operated in the electron impact ionization (EI) positive mode, for which the instrumental parameters were set at the following values: filament emission current=80 \(\mu\)A; transfer line, ion trap, and manifold temperatures=280, 200, and 50 °C, respectively. A filament multiplier delay of 6 min was established to prevent instrument damage. The MS/MS step was carried out by collision induced dissociation (CID) in non-resonant excitation mode. Derivatization to obtain the trimethylsilyl derivatives using 100 \(\mu\)L of BSTFA containing 2% TMCS.

\(^d\) The carbohydrate analysis was based on the preparation of the oxime trimethylsilyl sugar (TMS) ethers. Detection by FID. No full validation is reported.

\(^e\) No validation parameters reported. Derivatization with Trimethylsilylation pyridine (50 \(\mu\)l) and hexamethyldisilazane (100 \(\mu\)l). The operating MS conditions were electron impact ionisation (70eV), scan rate 1 scans 1 over the range m/z 40 650, and source temperature 200 °C, EI in TIC mode.

Suzanne de Goeij
CAPILLARY ELECTROPHORESIS (CE)

Besides liquid and gas chromatography, a number of analytical methods are described using capillary electrophoresis as separation technique for the separation of sugars. CE utilizes an open tubular capillary, which can be rapidly flushed with fresh buffer directly after detection of interesting peaks and be prepared for the next injection. It is a powerful technique concerning the simplicity, short analysis time, efficiency and low sample consumption.

Due to the $pK_a$ of monosaccharide’s being more than 11, monosaccharide’s are negatively charged in strong basic running buffer and can be further separated under a fixed electric field. For improving the separation efficiency, some surface-active agents, such as SDS, tetrahydrofuran (THF), hexadecyltrimethylammonium bromide (CTAB), etc. can be added into the running buffer.

Detection in Capillary Electrophoresis

As well as LC and GC chromatographic methods, detection of sugars in CE is challenging because of the absence of chromophoric groups. In this review the CE separation of sugars is described and compared with three types of detection: UV after derivatization, indirect UV detection and amperometric/electrochemical detection. All different types of detection are discussed and tabulated separately.

UV detection after derivatization

Carbohydrates lack a light-absorbing chromophore, which makes direct UV detection impossible unless a derivatization procedure is involved prior to analysis. The combination of a sensitive functional group such as pyrazolone together with a strong absorption moiety would result in an attractive reagent. The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) with strong UV absorbance at 245 nm is a popular label for the HPLC method that can react with reducing carbohydrates under mild conditions, requiring no acids catalyst and causing no isomerisation. Wang et al. used this reagent for derivatization with a recovery of 94 – 102.5 %.

Beside PMP also 1-(2-naphthyl)-3-methylpyrazolone (NMP) is reported as derivatization reagent. You et al. reported a recovery of 93.9 – 105.1 % using NMP as derivatization reagent. The reaction of NMP with sugars is presented in figure 11.
Quantitative analysis methods for sugars

Figure 11: Derivatization scheme of 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) for sugars, reproduced from reference 38.

Wang et al. tested an increasing concentration of methanol added to the background electrolyte solution, which resulted in increasing resolution of the sugars to be separated. The best separation was achieved at 4% methanol under the condition of 175 mM borate buffer at pH 11.0.\(^\text{39}\)

You et al. validated the method and found good reproducibility with RSD% of the migration time and peak area, respectively, from 0.44 to 0.48 and from 3.2 to 4.8.\(^\text{37}\) Xia et al. also performed reproducibility experiments and the results show that the intra-day reproducibility were less than 0.48% for the migration time and 4.8% for the peak areas, and the inter-day were less than 0.72% for the migration time and 6.3% for the peak areas, indicating that the method precision was satisfactory.\(^\text{38}\) Wang et al. also validated the method and found higher results, the intra-day reproducibility was less than 3.7% for migration time and 4.1% for peak areas, the inter-day results were less than 5.4% for all variables.\(^\text{39}\)

Figure 12: Electropherogram of sugars in food application, detection by UV after derivatization with PMP, reproduced from reference 39. (Peaks: 1. glucosamine, 2. maltose, 3. lactose, 4. xylose, 5. rabinose, 6. glucose, 7. ribose, 8. rhamnose, 9. fucose, 10. galactose, 11. mannose, 12. glucuronic acid, and 13. galacturonic acid.)

Table 8 summarizes a few applications of CE with UV detection after derivatization.
5.1.2 Indirect UV

As discussed previously, direct UV detection of sugars is impossible. However adding an UV active reagent to the background electrolyte makes indirect UV detection possible. A highly alkaline pH condition was used in order to charge carbohydrates negatively and to promote migration towards the anode. Electroosmotic flow was reversed to the direction of the anode by adding cetyltrimethylammonium bromide CTAB to the electrolyte. All references use CTAB in combination with an UV active compound in the BGE. Rizelio et al. selected sorbate as the co-ion because it is a strong chromophore with mobility similar to that of fructose, glucose and sucrose. Gao et al. used 2,6-dimethoxyphenol (DMP) for indirect UV detection as the electrophoretic mobility of DMP matches well with that of monosaccharide’s and DMP has a high extinction coefficient or high UV absorption at the detection wavelength chosen which ensures an enhanced detectability. The limitation of choosing DMP to facilitate indirect UV detection is that DMP is prone to be oxidized under exposure of air or light and the BGE has to be made freshly to maintain a constant pH.

Soga et al. selected 2,6-pyridinedicarboxylic acid (PDC) for this work based on its excellent capacity for anion analysis by indirect UV detection. An electropherogram of the separation done by Soga et al. is presented in figure 13.

In all cases similar results were obtained with respect to separation of sugars and detection limits.

Figure 13: Determination of sugars in glycoproteins by capillary electrophoresis with indirect UV detection, reproduced from reference 42.

Gao et al. determined the repeatability and found RSD values of less than 2.2% for migration time and less than 2.5% for peak height. Rizelio et al. reported RSD values of the migration time and peak are all below 5%. Soga et al. determined the RSD% for migration time < 0.23% and for peak area < 2.7%.

In table 9 applications are summarized using CE and indirect UV detection.

Suzanne de Goeij
5.1.3 Amperometric/Electrochemical Detection

Another option in CE detection is electrochemical detection, especially amperometric detection. This detection technique is suitable for analysis of sugars because the electro active hydroxyl groups in carbohydrates can be catalytically oxidized to produce significant current responses on the surface of metal electrodes such as copper and nickel disk electrodes. This is reported in several papers.\textsuperscript{43-44-45}

Carbohydrates are not considered electro-active compounds under normal amperometric conditions at the surface of carbon electrodes. Luo et al. have developed several electrode materials for the catalytic oxidation of carbohydrates at constant applied potentials; one of these electrodes is copper electrode.\textsuperscript{47} All reported references selected copper disk electrode acting as working electrode for the determination of the sugars.\textsuperscript{43-44-45}

In order to select a proper potential applied to the working electrode, Hu et al. performed hydrodynamic voltammograms for three sugars. The response of the three sugars was monitored after separation at different applied potentials. Considering the detection sensitivity of the studied analyzes and the baseline noise, Hu et al. choose a potential of 0.65 V (vs. Ag/AgCl, 3 mol/l KCl) for detection.\textsuperscript{43}

In all cases NaOH is used as background electrolyte. The alkaline conditions also satisfied the pH requirements for the proper performance of the Cu microelectrode in the electro-catalytic detection of sugars. Yang et al. tested the NaOH concentration from 30 to 150 mmol/L. Increasing the NaOH concentration resulted in better separation of the desired sugars however, also the noise level increased by increasing NaOH concentration. Finally 120 mmol/L was selected as separation electrolyte.\textsuperscript{44} Coa et al. and Hu et al. selected 50 mmol/L NaOH concentration as running buffer to obtain satisfactory separation in relative short analysis time.\textsuperscript{43-45} An example of the separation of the sugars by Yang et al. is presented in figure 14.

![Electropherogram of sugars by CE with amperometric detection](image)

Figure 14: Electropherogram of sugars by CE with amperometric detection, reproduced from reference 44. (1: fucose, 2: galactose, 3: glucose, 4: rhamnose, 5: arabinose, 6: fructose and 7: xylose)
Hu et al. and Yang et al. were able to separate their interested sugars within 30 minutes were Coa et al. managed the separation of the desired sugars within 15 minutes.

Hu et al. validated the method on precision and found the precisions of migration time and peak current (in terms of relative standard deviation(RSD)) were 0.17 - 0.24% and 2.32- 2.81%, respectively. Yang et al. found the RSD of the migration time <1% and the RSD of the peak area was <5%.

Beside amperometric detection using a copper electrode, applications are reported tris(2,2′-bipyridyl)ruthenium(II) [Ru(bpy)3^{3+}]-based electrogenerated chemiluminescence (ECL) detection. Li et al. used in the CE a Pt working electrode (1.0 mm in diameter), a Pt wire counter electrode (1 mm in diameter) and a Ag/AgCl reference electrode in a conventional three-electrode system. Controlled potential electrolysis for Ru(bpy)3^{2+} oxidation was carried out with an electrochemical analyser. The ECL light was captured by a photomultiplier tube (PMT). Derivatization is still required; Li et al. used 2-diethylaminoethanethiol (DEAET) as a derivatization reagent.

Table 10 represents a few applications of CE using amperometric and electrochemical detection.

### 5.2 Discussion Capillary Electrophoresis

CE is a good alternative for the separation of sugars. In relative short retention times (within ~ 30 minutes) separation of different sugars can be achieved. For the optimization of the required separation of the sugars different capillaries can be used. Due to the $pK_a$ of monosaccharides being more than 11, monosaccharides are negatively charged in strong basic running buffer, like borate or NaOH buffer. Playing around with the concentration of the running buffer can improve the separation.

After separation a few possible detection methods are available; UV after derivatization, indirect UV and electrochemical detection. UV after derivatization always requires extra work where the recovery need to be taken into account. Reported recoveries are >95%. Indirect UV results in significant higher detection limits compared to UV with derivatization and electrochemical detection. Advantage of indirect UV is that limited extra work is required.

All detectors reported repeatability results of the migration time and peak are <5%, mostly <1% for migration time. Reproducibility results reported are <6%.
Table 8: Overview of CE methods using UV detection for the analysis of sugars

<table>
<thead>
<tr>
<th>Capillary</th>
<th>Method details</th>
<th>Sugars (migration time min(^a))</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>fused-silica capillary: Total length 48.5 cm, effective length 40 cm (50 µm-inner diameter)</td>
<td>BGE: Running borate buffer pH 9.46 Temp: 20°C Voltage: 22 kV Injection: 10 s at 50 mbar DAD detection: 251 nm Derivatization with 1-(2-naphthyl)-3-methylpyrazolone (NMP) Total analysis time: 20 min</td>
<td>Xylose (12.80) Arabinose(13.20) Glucose (13.33) Rhamnose (13.64) Mannose (14.48) Fucose (14.71) Galactose (15.24)</td>
<td>All sugars 0.05 – 18 All together &gt;0.9980</td>
<td>All in range 0.44 – 0.48</td>
<td>All sugars &lt; 0.02 µg/ml</td>
<td>n.r.</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Total length 58.5 cm x 50 µm I.D., (50 cm effective length)</td>
<td>BGE: borate buffer (55 mM) pH 9.46 with phosphoric acid (1.0 M) Temp: 20°C Voltage: 22 kV Injection: atmospherically at 10 s DAD detection: 254 nm Derivatization with 1-naphthyl-3-methyl-5-pyrazolone Total analysis time: 20 min</td>
<td>Xylose (11.46) Arabinose(11.79) Glucose (11.89) Rhamnose (11.99) Mannose (12.85) Fucose (13.04) Galactose (13.52)</td>
<td>All sugars 1 – 45 Recovery all sugars 93.9 – 105.1 %</td>
<td>0.9996</td>
<td>0.9997</td>
<td>0.9991</td>
<td>0.9980</td>
<td>0.9989</td>
</tr>
<tr>
<td>Fused silica Internal diameter of 50 µm, 58.5 cm in total length (effective length 48.5 cm)</td>
<td>BGE: 175 mM borate buffer containing 4% methanol at pH 11 Temp: 25°C Voltage: 15 kV Injection: 0.5 psi for 5 s Detection wavelength: 245 nm Derivatization with 1-phenyl-3-methyl-5-pyrazolone Total analysis time: 30 min</td>
<td>Maltose (18.86) Lactose (22.39) Xylose (23.21) Arabinose (24.62) Glucose (26.10) Ribose (27.79) Rhamnose (29.73) Fucose (31.30) Galactose (33.11) Mannose (34.69)</td>
<td>All sugars 0.5 – 25 Recovery all sugars 94.0 – 102.5 %</td>
<td>0.9948</td>
<td>0.9985</td>
<td>0.9902</td>
<td>0.9908</td>
<td>0.9915</td>
</tr>
</tbody>
</table>

n.r. Not reported in article \(^a\) If reported
Table 9: Overview of CE methods using indirect UV detection for the analysis of sugars.

<table>
<thead>
<tr>
<th>Capillary</th>
<th>Method details</th>
<th>Sugars (migration time min(^a))</th>
<th>Linear range (µg/ml)</th>
<th>R2</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyamide-coated fused silica capillary; 115 cm length (105 cm to detector) 50 µm I.D., 375 µm O.D.</td>
<td>BGE: 2,6-dimethoxyphenol (DMP) 8 mM, cetyltrimethylammonium bromide (CTAB) 1.5 mM pH 12.0 Temp: Ambient Voltage: 18 kV Injection: hydrodynamic pressure at 50 mbar for 1 s On-column UV detection at 224 nm Total analysis time: 60 min</td>
<td>Glucose Galactose Rhamnose Fucose Xylose Mannose</td>
<td>n.r.</td>
<td>0.9985</td>
<td>2.7</td>
<td>4.9</td>
<td>n.r.</td>
<td>40</td>
</tr>
<tr>
<td>Uncoated fused-silica 60.0 cm total length, 8.5 cm effective length, 50 µm I.D. and 375 µm O.D.</td>
<td>BGE: 20 mmol/L sorbic acid, 0.2 mmol/L CTAB, 40 mmol/L NaOH at pH 12.2 Temp: 25°C Voltage: 25 kV Injection: hydrodynamic at 50 mbar for 3 s UV detection at 254 nm, reference at 360 nm Total analysis time: 3 min</td>
<td>Fructose Glucose Sucrose</td>
<td>180 – 3603 180 – 3603 171 – 1172</td>
<td>0.9993</td>
<td>0.69</td>
<td>26</td>
<td>26</td>
<td>88</td>
</tr>
<tr>
<td>Fused silica 50 µm i.d. x 80.5 cm total length (72 cm effective length)</td>
<td>BGE: 20 mM 2,6-pyridinedicarboxylic acid (PDC) and 0.5 mM cetyltrimethylammonium bromide (CTAB) at pH 12.1 Temp: 20°C Voltage: 25 kV Injection: 50 mbar for 6.0 s DAD detection at 350 nm and reference at 275 nm Total analysis time: 25 min</td>
<td>Ribose Mannose Xylose Glucose Galactose Fucose</td>
<td>All 20 – 200</td>
<td>1.0000</td>
<td>0.02</td>
<td>8.3</td>
<td>n.r.</td>
<td>42</td>
</tr>
</tbody>
</table>

n.r. Not reported in article

\(^a\) If reported
Table 10: Overview of CE methods using amperometric/electrochemical detection for the analysis of sugars.

<table>
<thead>
<tr>
<th>Capillary</th>
<th>Method details</th>
<th>Sugars (migration time min(^a))</th>
<th>Linear range (µg/ml)</th>
<th>(R^2)</th>
<th>RSD %</th>
<th>DL (µg)</th>
<th>QL (µg)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyimide-coated fused silica Capillary 45 cm long, O.D. 360 µm, I.D. 25 µm</td>
<td>BGE: 0.05 mol/l NaOH buffer solution (pH 12.7) Temp: 22°C Voltage: 5 kV Injection: electro-kinetically, applying 5 kV for 10 s Detection potential 0.65 V (vs. Ag/AgCl, 3 mol/l KCl) Total analysis time: 26 min</td>
<td>Sucrose (16.42) Glucose (18.50) Fructose (19.56)</td>
<td>All 0.1 – 100</td>
<td>0.9989</td>
<td>0.17</td>
<td>1.37</td>
<td>n.r.</td>
<td>43(^b)</td>
</tr>
<tr>
<td>Bare fused-silica capillary 60 cm long with 25 µm inner diameter and 360 µm outer diameter</td>
<td>BGE: 120 mmol/L NaOH solution Voltage: 12 kV Injection: Electro-kinetically at 12 kV for 10 s Detection potential 0.65 V Total analysis time: 30 min</td>
<td>Fucose Galactose Glucose Rhamnose Arabinose Fructose Xylose</td>
<td>All sugars 1 – 100</td>
<td>0.998</td>
<td>0.4</td>
<td>0.28</td>
<td>n.r.</td>
<td>44(^c)</td>
</tr>
<tr>
<td>A 60 cm length of 25 µm I.D. and 360 µm O.D. fused silica Capillary</td>
<td>BGE: 50 mmol/l NaOH solution Voltage: 16 kV Injection: 16 kV/6 s Working potential is 0.65 V Total analysis time: 20 min</td>
<td>Sucrose Maltose Glucose Fructose</td>
<td>1 – 500</td>
<td>0.9996</td>
<td>n.r.</td>
<td>0.9</td>
<td>n.r.</td>
<td>45(^d)</td>
</tr>
<tr>
<td>A 25-µm i.d. 90-cm long uncoated fused-silica capillary</td>
<td>BGE: pH 8.3; 30 mmol/L borate containing 20% (v/v) ACN; 100 mmol/L borate detection buffer containing 5.0 mmol/L Ru(bpy)(_3)(^2+) Voltage: 15 kV Injection: 10 kV/8 s Working potential 1.23 V (vs. Ag/AgCl) Total analysis time: 20 min</td>
<td>Xylose Rhamnose Glucose</td>
<td>0.01 – 40</td>
<td>&gt;0.997</td>
<td>&gt;0.97</td>
<td>n.r.</td>
<td>n.r.</td>
<td>46(^e)</td>
</tr>
</tbody>
</table>

n.r. Not reported in article

\(^a\) If reported
The electrochemical cell consisted of a platinum auxiliary electrode, a copper disk working electrode and an Ag/AgCl (3 mol/l KCl) reference electrode.

A three electrode system, which consisted of a Cu disk electrode or diameter 300 µm as working electrode, a saturated calomel electrode (SCE) as reference electrode, and a platinum wire as counter electrode, was used in both electrochemistry and detection experiments.

A copper-disk electrode with 140 µm diameter was employed as the working electrodes.

A Pt working electrode (1.0 mm in diameter), a Pt wire counter electrode (1 mm in diameter) and a Ag/AgCl reference electrode were used in a conventional three-electrode system. Derivatization by trifluoroacetic acid (TFA)/DEAET diethylaminoethanethiol.

Only detection limits of glucose reported.
6 CONCLUSION

The separation and quantitative analysis of sugars is challenging for several reasons. To find a proper technique, column and detector makes separation and detection of quantitative sugars analysis a challenge.

Liquid chromatography is the most popular technique to detect sugars although there are several challenges to overcome. Special columns are on the market for sugars separation, but in all cases method development is still required and not always optimum baseline separation is achieved. Typical analysis times are between 30 – 50 minutes.

Detection of sugars in LC gives several possibilities, ELSD, RI, UV, MS, Fluorescence and CAD, all with their own advantages and disadvantages.

A disadvantage of the ELSD is the low reproducibility, slightly low sensitivity to low molecular weight components and the non-linearity of the detector. An advantage is relative simple use of the detector. The use of ELSD in combination with the separation of sugars has been reported by different authors. With the use of different columns for the separation, detection limits in the same order were found (in the order of 0.1 – 1 µg per injection). A recovery of 93 – 99.9 % was reported and an intra- and inter-day precision as % RSD of the retention time and peak are < 2 %.

The response of RI detection results in linear calibration curves. A disadvantage in RI detection is the temperature sensitivity, small fluctuations in temperature results in high variations. RI detection cannot be used in combination with gradients which makes method development limited. Detection limits of RI are in the same range as ELSD applications. Castellari et al. found excellent recoveries (>99.3%) and precision results <1%. However other authors reported lower recoveries (between 89 – 113%) and higher precision values, repeatability <1.5% and reproducibility <7.0%.

Mass spectrometry is also an option for the detection of sugars. Mass spectrometry for the detection of sugars is difficult due to the low efficiency of ionization related to the low acidic character of sugars. Several applications are reported were sugars were analysed as their sodium adduct, acetate adduct or chloride adduct. Compared to the detection limits of other detectors, the lowest limits were reported by Hammad et al. using a quadrupole MS, detection limits in pg range per injection are reported. Although very low detection limits are reported (pg range) the precision is around 5% and the average recovery is around 60% for all sugars. Comparable precision values are found in other applications. Beside quantification, the use of MS can generate information about chemical structures which can be very useful development phases.

For detection by UV a derivatization reagent is required. Applications are reported where 1-Phenyl-3-methyl-5-pyrazolone and p-aminobenzoic ethyl ester is used as derivatization reagent, with recoveries between 90 – 120 %. The reported detection limits are in the nanogram range, which is significant lower compared to the reported detection limits by ELSD and RI. The reported values for precision are <2.4% for intra-day precision and <5% for inter-day precision.
Different derivatization reagents are applicable for detection by fluorescence. Low detection limits are reported with the use of anthranilic acid\textsuperscript{9} and benzamidide\textsuperscript{19} as derivatization reagent. The detection limits of sugar separation with the use of a fluorescence detector are significant lower compared to the ELSD and RI detection, and comparable to the detection limits found by UV after derivatization, however a derivatization step is always required.

Another type of liquid chromatography is high-performance anion-exchange chromatography with PAD detection. Different types of columns can be used to optimize the separation. The correlation coefficient of the calibration curves is in all reported references >0.99. Detection limits reported with HPAED-PAD are in the nanogram range and are comparable to LC with UV or fluorescence detection after derivatization or LC with MS detection. Bruggink et al\textsuperscript{32} developed a system to use MS as detection. They also reported impressive detection limits with PAD detection; however they are the builder and owner of the HPAEC system.

Besides LC, gas chromatography is an option for the separation and detection of sugars. The main advantage of using gas chromatography is the high resolution in relative short retention times. A disadvantage is that derivatization of the sugars always is required. In all reported references the trimethylsilyl ether is used, the good volatility and stability characteristics of the derivatives formed make trimethylsilyl (TMS) ethers the most popular derivatives applied to GC analysis of saccharides. Not many applications could be found for quantitative sugars separation and detection by GC-MS or GC-FID. This could be due to the low recovery values reported (68 and 118 %), relative high precision results (around 5%) and the extra work of the derivatization. Most reported methods used MS as detection, due to the extra information obtained with MS.

CE is a good alternative for the separation of sugars. It is a powerful technique concerning the simplicity, short analysis time, efficiency and low sample consumption. In relative short retention times (within ~ 30 minutes) separation of different sugars can be achieved. For the optimization of the required separation of the sugars different capillaries can be used. Due to the \(pK_a\) of monosaccharide’s being more than 11, monosaccharide’s are negatively charged in strong basic running buffer, like borate or NaOH buffer. Playing around with the concentration of the running buffer can improve the separation.

After separation a few possible detection methods are available; UV after derivatization, indirect UV and electrochemical detection. UV after derivatization always requires extra work where the recovery needs to be taken into account. Reported recoveries are >95%. Indirect UV results in significant higher detection limits compared to UV with derivatization and electrochemical detection. An advantage of indirect UV is that limited extra work is required. All detectors reported repeatability results of the migration time and peak are <5%, mostly <1% for migration time. Reproducibility results reported are <6%.

Depending on the separation needed, amount of samples to be analysed and the required detection limits different methods can be applied. For traces of sugars in routine analysis in the food industry, a HPAED-PAD can be used. For bulk analysis for research purposes when having lots of samples a LC-ELSD method is sufficient. When traces of sugars are not analyses routinely, LC-UV or CE-UV can be applied. If the sample contains lot of different sugars and high resolution is needed, a GC method is the best option.
REFERENCES


Quantitative analysis methods for sugars


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