Evaluation of the performance and greenness of analytical methods based on the example of sugar analysis

Master thesis

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Analüütiliste meetodite tulemuslikkuse ja rohelisuse hindamine suhkrute analüüsi näitel

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously been presented for grading.

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Contents

Abbreviations

GAC	green analytical chemistry
GAPI	green analytical procedure index
AGREE	analytical greenness metric approach
NEMI	national environmental methods index
GHS	Globally Harmonized System of Classification and Labeling of Chemicals
PP	penalty points
NFPA	national fire protection association
AGREE	Analytical Greenness Calculator Metric
LCB	lignocellulosic biomass
GHG	greenhouse gases
5-HMF	5-hydroxymethylfurfural
DMF	2,5-dimethylfuran
C5	pentoses
C6	hexoses
$Ca(OH)_2$	lime
HPLC	high-performance liquid chromatography
RI	refractive index detection/refractive index detector
UV	ultraviolet
CE	capillary electrophoresis
NP-HPLC	normal phase HPLC
RP-HPLC	reverse phase HPLC
SEC	size-exclusion chromatography
CZE	capillary zone electrophoresis
CGE	capillary gel electrophoresis
MEKC	micellar electrokinetic capillary chromatography
EOF	electroosmotic flow
LoD	limit of detection
LoQ	limit of quantification
ID	internal diameter
IS	internal standard
IDL	instrumental detection limit
IQL	instrumental quantification limit

RSD	relative standard deviation

Introduction

Fast industrial-scale development is driving a surge in awareness towards sustainable development. The effects of fast economic and industrial growth are now seen in the increase of greenhouse gases, aquatic pollution, and soil contamination. Green chemistry principles were created in order to reduce the ecological impact caused by human development. The principles are being applied in order to find more environmentally friendly alternatives to existing models, concepts, and methods. Green chemistry principles are applied not only to large-scale industries but also to small-scale facilities such as laboratories.

In laboratories, many analyses are completed daily. A lot of methods do not seemingly cause any major impact to nature due to the much smaller volumes of waste generated if compared to industrial-scale projects. However, the issue with the waste that comes from laboratories is that it is much easier to neglect, and that it impacts the environment even more due to the dispersion of the toxic waste produced in such quantities. Greener alternatives are not only beneficial to the environment but also to the laboratory itself. It has been noted that sustainable methods tend to cut energy, solvent, chemical, and equipment costs as well as are more effective than non-green alternatives.

Lignocellulosic biomass is a second-generation source of material that has been recently discovered as an alternative to fossil fuels and first-generation biomasses such as corn starch and sugar beet. Sugars separated from biomass are a vital component required for new-generation fuel, material, chemical, solvent, and food chemical production. That is why their analysis is very important and is focused on by many laboratories.

High-performance liquid chromatography and capillary electrophoresis are both very well-known methods that are used for sugar analysis. Capillary electrophoresis is famous for being a fairly sustainable technique for its requirements of low volumes of chemicals and samples. High-performance liquid chromatography has usually been criticized in terms of greenness for large amounts of organic solvents used for analysis and for long analysis times. However, it can be turned into a greener method as well with the use of purified water as a mobile phase instead of common solvents like acetonitrile. Moreover, the ‘fitness for purpose’ of analytical method is another important feature, which sets preferences for performance characteristics of the method. In case of analysis of sugars in biomass, the high limit of detection in HPLC might be not as crucial as the possibility to avoid excess plastic waste generated by capillary electrophoresis since the latter method often requires sample dilution due to high sugar concentration in biomass samples.

In the following study, a brand new high-performance liquid chromatography method with refractive index detection was developed and validated as an alternative to capillary electrophoresis, developed in previous bachelor thesis study. The principal goal of the project was to compare sustainability or “greenness” and “fitness for purpose” of these two methods, based on carbohydrates analysis from different biomass types. For that, the following tasks were set:

1. To develop and optimize an HPLC separation method with RI detection for analysis of sugars present in biomass;
2. To evaluate and compare performance characteristics of HPLC and CZE methods (peak separation efficiency, reproducibility, accuracy, repeatability, limit of detection, and limit of quantification;
3. To make conclusions on fit-of-purpose of the methods;

4. To evaluate and compare the greenness of the methods by using the greenness metric tool AGREE.

1 Green chemistry

1.1 History of sustainability

The concept of sustainable development has been integrated into nearly every aspect of human activity, from individual choices to large-scale projects. The concept was first introduced in the 18th century in the forest industry but did not attract as much attention. However, initial indifference gave way to later consideration. During the 20th century, the society of developed countries noticed the effects of industrialization on the environment. Pollution of the atmosphere, aquatic ecosystems, and soils became more evident, making this the founding reason for international recognition. [1, 2]

The 20th century was a foundation for many seminars, conferences, and events related to sustainable development. The global acceptance of sustainability gave rise to new areas of research, one of them being "green chemistry". Paul Anastas is credited with pioneering the twelve principles of green chemistry, which later became the foundation for its expanding applications. [1, 2]

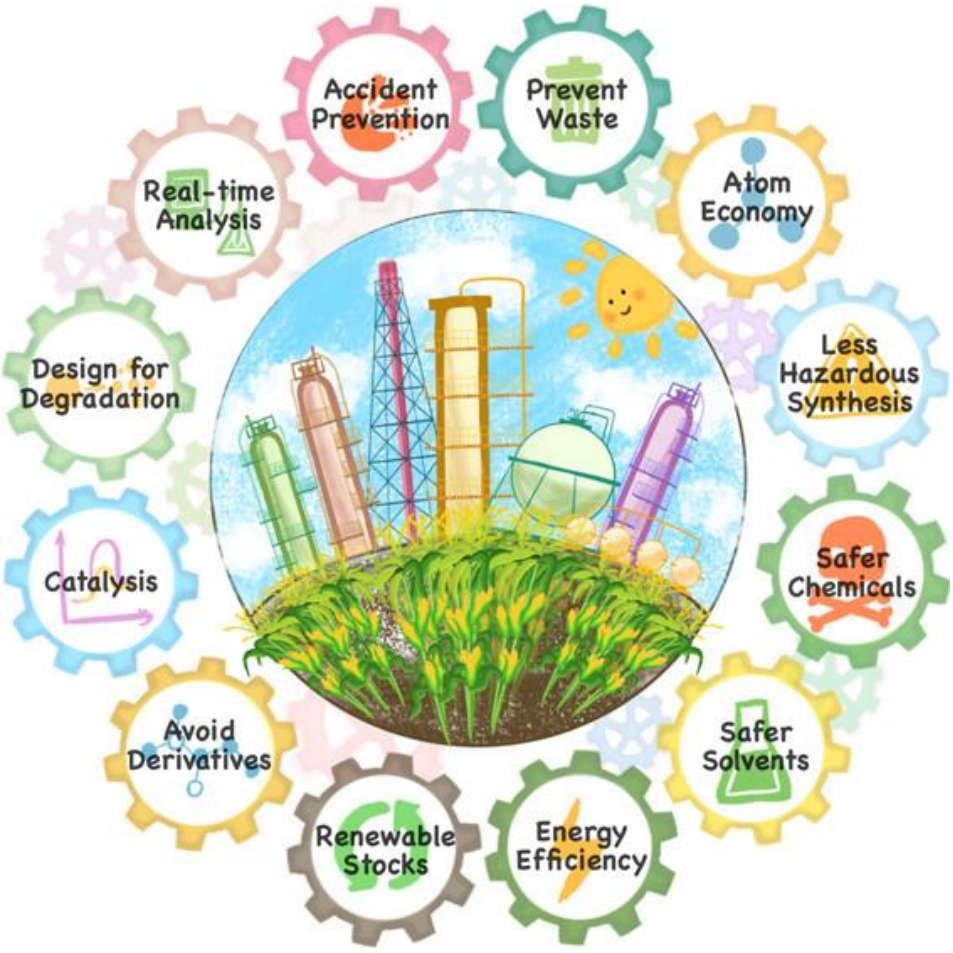


Figure 1. Twelve Principles of Green Chemistry proposed by P. Anastas *[65]*

Green chemistry is a concept widely used across several fields. Its idea revolves around establishing an understanding of the environmental damage one's actions may cause. In addition, it assists in optimizing resource sustainability and providing environmental protection while preserving economic growth. [3, 2] Industries, governments, educational institutions, and technological developers must all consider the environmental, health, and safety issues that may arise due to irresponsible actions. [3] The idea of green chemistry helps alter the attitude and behavior of people in relation to the environment in hopes of achieving sustainability. [2] As such, green chemistry has become one of the primary foci in laboratories as well. [4]

1.2 Green analytical chemistry

The main goal of each industry is to produce a certain product. Any leftover material not incorporated into the final product is considered as waste. Green chemistry allows different principles to be used in order to measure the impact of chemical reactions and waste on the environment. However, a certain problem arises when these principles are applied to analytical chemistry. If industrial processes and synthetic chemistry were to be compared to analytical chemistry, it would be obvious that much less volume of solvents, reagents, and other factors are required for the analysis, which means a lot less waste is produced. [4]

In spite of the lesser scale, waste produced by analytical chemistry may spread further, as such its quantity should be minimized as much as possible. While reduction of the volume of solvents and other factors is one way out, an alternative option is to discover and implement greener alternatives, reduce energy consumption, and miniaturize the analytical procedure itself.

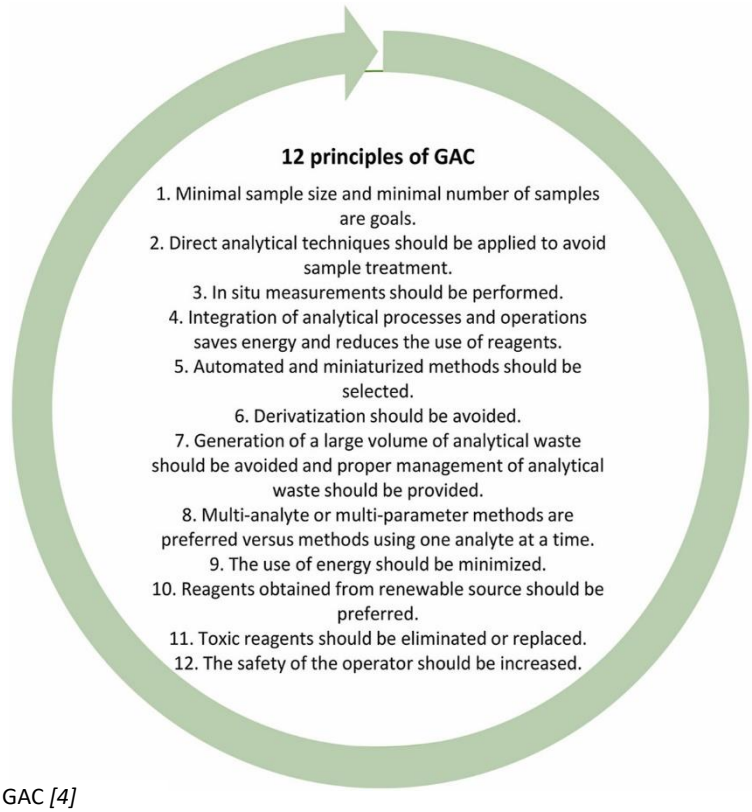


Figure 2. Principles of GAC *[4]*

The twelve principles developed by P. Anastas are not completely applicable to this field, and thus green analytical chemistry (GAC) was derived as a standalone concept. Twelve separate principles were designed for GAC (Figure 2). [4]

These new principles take different parts of the analytical analysis into account and provide a more environmentally friendly alternative for each aspect. [4] They emphasize the importance of safer, milder, and more harmless solvents or refusing their use in general. It is also important to manage energy consumption, avoid derivatization, and choose solvents that originate from renewable sources. [2]

The GAC philosophy has become rather successful in laboratories and academia due to not only preserving the environment but also for various improvements in such areas as cost-effectiveness, effectiveness, speed, and safety. Unlike classical methods, greener alternatives allow us to avoid contact with dangerous solvents that are both harmful to health and very expensive to use. It has also been proven that greener alternatives tend to be more effective and faster, which motivates researchers to pursue the development and modification of analytical methods in academic and industrial laboratories. [2, 5]

1.3 Green analytical chemistry metrics

Metrics are a viable tool that assists in tracking the greenness of analytical methods while also revealing what can be improved in order to make the method more environmentally friendly. [4] Standard green chemistry metrics used in chemical synthesis are inapplicable to analytical

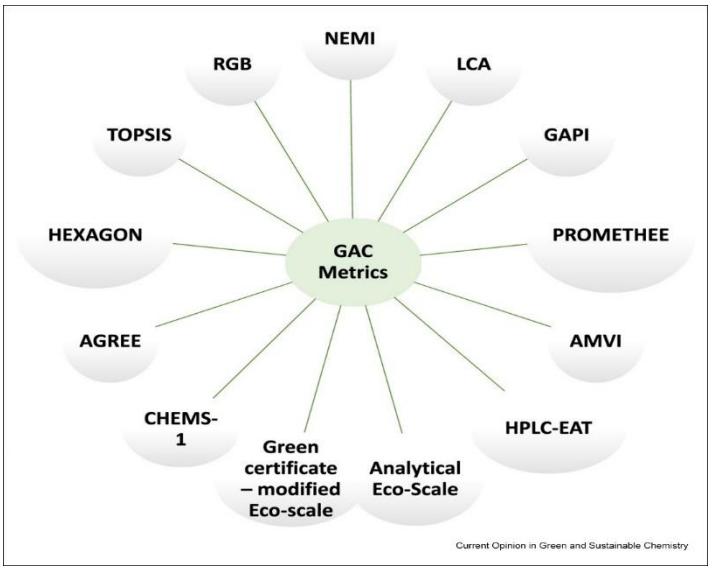


Figure 3. GAC metrics. The abbreviations stand for: HPLC-EAT – High Performance Liquid Chromatography – environmental assessment tools. LCA – life cycle assessment. NEMI – National Environmental assessment tools. AGREE – Analytical Greenness Calculator Metric. PROMETHEE – Preference Ranking Organization Method for Enrichment Evaluations. TOPSIS – Technique for Order of Preference by Similarity to Ideal Solution. *[4, 5]*

chemistry, which (unlike in production) does not always possess a concrete product with a specific mass. That is why metrics specific to GAC were developed. [3, 4, 5] Some of them were designed

for specific analytical methods while certain others were generalized in order to be implementable to a wide range of analytical procedures (Figure 3). [3]

Metrics do not include only energy consumption or solvent volume, but also analyze the sample extraction, preparation, dilution, and treatment. They also focus on the smallest of details like sample size and waste generated from the sample preparation: dilution volume, solvent choice, solvent use, and even disposal. Some of the most widely used and well-known metrics are the Analytical Eco-Scale, GAPI, AGREE, and NEMI. [5]

NEMI (National Environmental Method Index) is the oldest metric tool known. The system consists of a single pictogram that is divided into four separate sections, with each of them representing a specific criterion. The first field corresponds to the lack of hazardous, toxic, and bio-accumulative chemicals used in the analytical method. The second section requires that the chemical used does not fall into the hazardous waste D, F, P, or U listings. The sample pH in the third field must remain within the range of 2-12 to prevent corrosive conditions. In the fourth field, the generated waste amount must not exceed 50 grams. In case one of these conditions is met during the analysis, the corresponding section of the pictogram is colored green. Those criterions which were not met are left uncolored. [3, 4]

NEMI's main advantage stands in its simplicity. The pictogram is easy to use to determine the analyzed procedure's environmental impacts. However, while filling out the pictogram and reading the results is easy, the information gathered from the following metric tool is very broad while the process of checking all the requirements can be time-consuming. Another disadvantage is the pictogram's questionable value in quantitative analysis. Any parameter displayed in the symbol is either above or below a certain value, which makes the metric tool unable to be categorized as semi-quantitative. A major downside is connected to the pictogram preparation process itself, which can turn difficult in case the operator uses multiple non-typical chemicals. To solve the issue of the metric tools' lack of quantitative value, it was suggested to use red, yellow, and green colors to dye the corresponding fields. Red stands for non-sustainable, yellow for moderate, and green for sustainable. [4]

The Analytical Eco-Scale was developed much later and is currently the most popular tool compared to other metric tools. Unlike NEMI, the idea of the Analytical Eco-Scale revolves around the use of a penalty scale that does not involve a pictogram. There are 100 points in total, and points are subtracted for each parameter that is considered to be harmful for the environment, thus reducing the method's greenness. Points are subtracted for the use of large volumes of toxic reagents and solvents, occupational hazards, energy consumption, the amount of waste produced and how it was managed. Based on the remaining points, the analytical method is evaluated as green, acceptable, or non-acceptable. An ideal green method requires the use solvents or reagents that do not pose a threat to health and the environment; the power consumption must not exceed 0.1kWh; no waste is produced during the process. Methods that employ direct measurements, do not possess sample transport, preservation, processing, or preparation are very few in number. An example of points being assigned is depicted in Figure 4. [3, 4]

Instrument/Process	Energy used	PPs
FTIR, Immunoassay, Spectrofluorometry, Titration, UPLC, UV-VIS spectrometry, hot-plate solvent evaporation (<10 min), needle evaporator, sonicator	<0.1 kWh per sample	0
AAS, GC, ICP-MS, LC	⩽1.5 kWh per sample	1
NMR, GC-MS, LC-MS, XRD, hot-plate solvent evaporation (>2.5 h)	>1.5 kWh per sample	2

Figure 4. List of common instruments and processes, their energy consumption, and calculated penalty points. [4]

The Analytical Eco-Scale is a considerable step forward from NEMI. It provides a quantitative analysis of any method's sustainability as a value that is compared to the perfect score of 100. A myriad of methods can be compared with ease based on their final result. However, just like NEMI, it has its own disadvantages, one of them being the lack of consideration of the type of the pictogram while PP are assigned to a solvent or a chemical. The operator is requested to multiply the number of pictograms that possess the word "danger" or "warning". However, not all chemicals are equal while having the same pictograms. The calculation does not consider the hazard level of the chemical, which makes it impossible to assess the environmental impact. Another drawback is related to the PP assigned to waste management based on quantity and how the operator processed it, yet the nature and the potential hazard the waste may cause are not taken into consideration at all. On a side note, the Analytical Eco-scale results could be made available using an online tool. Even if there are several drawbacks, the Analytical Eco-scale provides quantitative data that involves several steps of analytical method unlike NEMI. [4]

Another well-known tool used for the evaluation of analytical methods is known as the green analytical procedure index (GAPI). The metric uses a pictogram that consists of five pentagrams, which individually assess the environmental footprint of each stage in the analytical process (Figure 5). Each part of the pentagram is colored green, yellow, or red, depending on how it affects the environment. The factors GAPI considers are reagents, procedures, instruments, chemical health hazards and risk to the environment, type of waste and its amount, and power consumption. [4, 6]

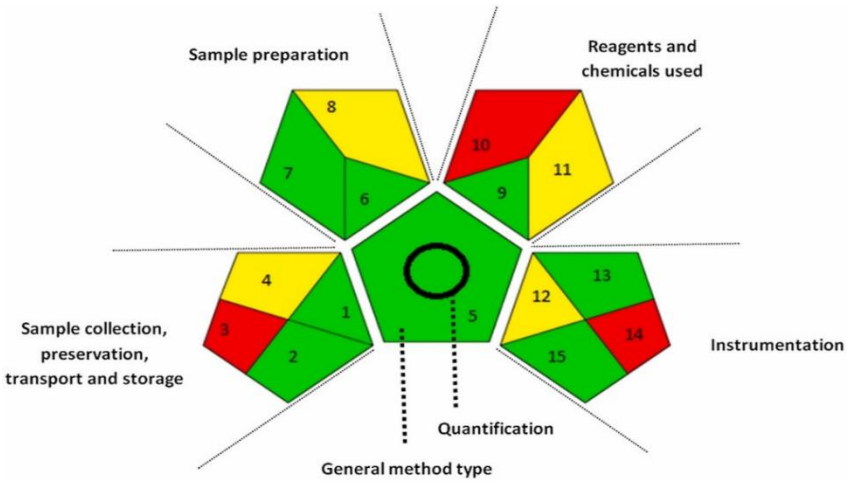


Figure 5. Example of a GAPI diagram. *[4]*

GAPI's compact design allows to compare several methods by greenery at once while also showing the weak point of any analytical process. [4, 6]

The circle placed in the pentagram in the middle has a meaning as well. However, unlike the pentagrams, its purpose differs. The presence of a circle implies the use of the method in both quantitative and qualitative analysis while the lack of it means the method is used only for qualitative analysis. [4]

Compared to the Analytical Eco-Scale, GAPI comprehensively addresses various stages of the analytical process. A glance at the pictogram shows the greenness of the procedure and allows to detect which areas require improvement. However, the method has several limitations. It does not consider the chemicals that are used in the process of synthesizing absorbents or any processes that take place before the extraction. As a general rule, the creation of sorbents and other materials is not as sustainable as their use in the analytical method. Another disadvantage is connected to the volume of the chemicals and waste. Volumes of waste over 10 mL are labeled as red, yet it does not matter whether the value exceeds the threshold by 0.1 mL or 490 mL. [4, 6]

Analytical Greenness Calculator Metric (AGREE) is a relatively new software tool developed in 2020. The software consists of a circular diagram with twelve numbers scattered around the circle, each corresponding to one of the principles of GAC. Each number is colored in a shade of red, yellow, or green, with red meaning that the method does not follow that principle, yellow meaning the principle is followed in moderation while green means the method follows the principle fully. The width of each segment depicts the weight of the principle in the method. Each principle also has a score from 0 to 1, and the final score is depicted in the middle of the diagram. [3, 4, 7]

AGREE's main benefit is that it follows all the rules of GAC. Unlike in other metric tools, color shades are used to evaluate the degree of greenness and assign a score for the compliance to each principle at the same time, providing the operator with better understanding of the level of greenness of their chosen method. However, AGREE shares some of its drawbacks with GAPI: the exact amount of waste and energy are not considered. [4]

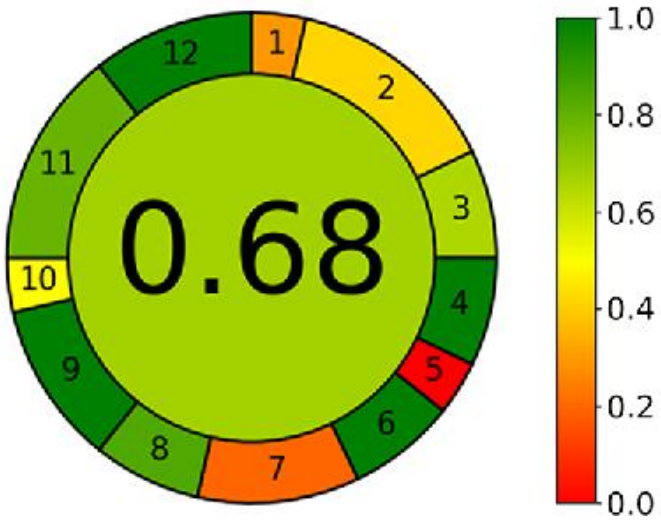


Figure 5. AGREE diagram example. *[3]*

2 Sugars

2.1 Lignocellulosic biomass

The world's growing population and the expansion of industries have caused a surge in fossil fuel consumption. Fossil fuels are relatively easy to acquire, compatible with most modern industrial processes, and are affordable. However, the reserves of this resource are finite and one day will be depleted. Other issues arise in the form greenhouse gases (GHG) that escape into the atmosphere, occurrence of acid rains, and melting glaciers. These reasons gave rise to the development of sustainable alternatives, which would be safe for the environment. Lignocellulosic biomass (LCB) is one such alternative. [8, 9]

LCB is a carbon-neutral renewable raw material that has recently been recognized as a powerful substitute for bioethanol, which is currently one of the most used sources of green energy. [8, 10] Bioethanol is an environmentally friendly fuel substitute since it reduces the amount of GHG produced by approximately 40%. [11] Despite that, its origins may potentially cause an economic imbalance. Bioethanol is produced from valuable food sources such as starch and sugar. By the year 2040, it is estimated that energy demands will rise by 28%, and the usage of bioethanol produced from food stocks will cause a higher demand for food, which in turn will increase its market value. [8, 9] LCB is not produced from food supplies but from forest and inedible crop residues. The replacement of bioethanol with LCB will not only solve the issue of food value inflation but also help process the waste originating from wood, grass, and crop remainders while also decreasing the volume of GHG production by 60%. [11, 12] However, while being a great alternative, LCB processing is an arduous task due to its complex structure. [9]

The main components of LCB are cellulose (35-50%), hemicellulose (20-35%), and lignin (15-20%) along with some residues of pectin, nitrogen, and other inorganic substances. Depending on the source of LCB, the content of each component may vary, with woody crops possessing over 25% of lignin (Figure 7). [13, 14, 15]

	Species	Cellulose [%]	Hemicellulose [%]	Lignin [%]
Hard wood	Poplar	50.8–53.3	26.2–28.7	15.5–16.3
	Oak	40.0–55.0	24.0–40.0	18.0–25.0
Soft wood	Spruce	45.5–49.5	22.9–33.0	27.9–32.0
	Pine	45.0–50.0	25.0–35.0	25.0–35.0
Agricultural waste	Corn stover	35.0–39.6	16.8–35.0	7.0–18.4
	Wheat straw	31.0–44.0	22.0–24.0	16.0–24.0
	Barley straw	33.0–40.0	20.0–35.0	8.0–17.0
	Sugar cane bagasse	26.0–50.0	24.0–34.0	10.0–26.0
Energy crops	Switchgrass	35.0–40.0	25.0–30.0	15.0–20.0

Figure 6. Cellulose, hemicellulose, and lignin content in different LCB sources. *[15]*

Cellulose, hemicellulose, and lignin are firmly intertwined and held together by strong bonds. The covalent and non-covalent bonds create a durable cross-linked three-dimensional polymer network, which is the reason why plant cell walls are very difficult to degrade with different treatments, enzymatic hydrolysis included (Figure 8). [13, 14]

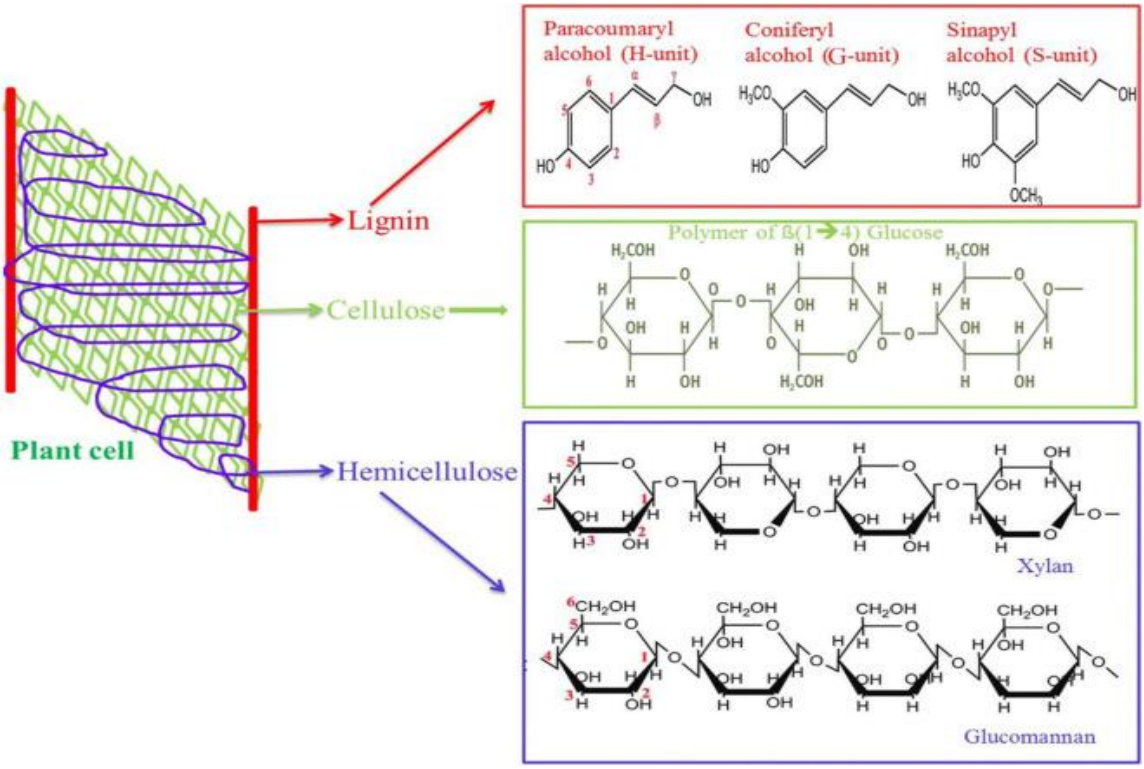


Figure 7. Structure of LCB. *[16]*

Cellulose is the most prevalent and accessible organic polymer within nature. It consists of linear cellobiose molecules where D-glucose molecules are bound together via β–(1→4) glycosidic bonds, making it a great source of fermentable C6-sugars. [14, 17] Hydrogen bonds and Van der Waals interactions create a crystalline structure by binding the cellulose chains, which results in the aggregation of fibers and microfibrils within the plant cell wall. [14, 15]

In contrast to cellulose, hemicellulose exhibits an amorphous, rather than crystalline, structure. It acts as an adhesive, interlinking cellulose fibers both with one another and with lignin. The composition of hemicellulose varies between soft and hard wood. The dominant hemicellulose building block in softwood is glucomannan while hardwood primarily features xylans within its structure. Other building blocks present in hemicellulose are xyloglucan, arabinogalactan, and galactoglycomannan. [8, 15, 16] Hemicellulose also possesses an insignificant amount of the monosaccharide L-fucose. [18] However, seaweed biomasses, such as brown algae, are rich in fucoidans, which fucose can be derived from. [19] Compared to cellulose, hemicellulose is significantly more challenging to pretreat due to its variability in sugar content, which depends on the origin of the biomass. However, when it comes to regioselective chemical and enzymatic modifications, they are easier to use on hemicellulose due to the diversity of sugar molecules, chain length, and the structure of the side chains. [14]

Lignin is the third important component of LCB. It is a polyphenolic polymer, which makes it an ideal natural source of aromatic compounds and polyphenols. Lignin is bound to hemicellulose through

covalent bonds, which is the reason for the toughness and compactness of the plant cell wall. Three major types of monolignols make up its structure: paracoumaryl, coniferyl, and sinapyl alcohols. [14, 15]

2.2 Role of sugars in the industry

At present, sugar production predominantly utilizes first-generation feedstocks, most notably sugarcane, corn starch, and sugar beets in order to synthesize a myriad of chemicals, ingredients, and materials. The conversion methods designed for processing sugars are simple, effective, and possess high yield. However, concerns regarding the sustainability and practicality of first-generation feedstocks have emerged in recent times. [20]

As previously noted, utilizing edible feedstocks for chemical and fuel production carries the potential to disrupt economic balance, leading to price inflation for essential food sources. [8, 20] The sustainability of these methods is questionable as well. Sugarcane and corn are grown at a large scale, which makes forest land conversion into agricultural fields a necessity. As the demands for food and fuel continue to rise, the necessity for expanding agricultural land will likely become increasingly pressing. The expansion in turn will cause common ecological issues such as deforestation and erosion. Fertilizer use will worsen the eutrophication, [20] which is the oversaturation of water with phosphorus and nitrogen. This process is known to cause severe damage to aquatic ecosystems. [21] That is why second-generation LCB feedstocks are now seen as a more viable alternative. [20]

Sugars have been long used by industries to produce first generation biofuels, polymers, and chemicals required for daily needs. Furfural, 5-hydroxymethylfurfural (5-HMF), levulinic acid, xylitol, sorbitol, mannitol, and amino acids are such results of carbohydrate processing. [22] Furfural is an essential chemical often used in oil refining, plastic production, pharmaceutical and agrochemical industries. 5-HMF is an important component used in the creation of biofuels, solvents, fuel additives, plastics, and polymers. Xylitol is another essential product derived from biomass. It serves as an alternative to sugar for non-insulin dependent patients suffering from diabetes. Xylitol is synthesized from xylose via catalytic hydrogenation. [23]

Alcohols, hydrocarbons, and hydrogen are known compounds created by sugar fermentation and reformation. [22] Alcohols like ethanol can be used not only as biofuel, but also as a green source for the production of valued materials such as ethylene and propylene that are used in polymer synthesis. [12, 23] Nylon, a common fabric, can be developed from a furfural that originated from xylose. [12, 22] The majority of the previously mentioned materials can be derived from either storage carbohydrates obtained from sugar crops like sugarcane and sugar beet, or alternatively, from LCB. Notably, LCB also holds the potential for the preparation of not only existing products but also novel ones. [12]

Structural carbohydrates derived from LCB can be transformed into hydrocarbons for diesel and jet fuels. Brand new fuel was discovered in the form of 2,5-dimethylfuran (DMF), the physicochemical properties of which are far more similar to that of gasoline compared to ethanol. [12, 24] DMF could be a potential solution to the low fuel economy of ethanol-gasoline blends and the issue of the blend being easily separated into phases when contaminated by water. [12]

Valuable materials can also be produced from LCB sugars with the use of microorganisms. Microbial bioconversion is an effective method in synthetizing ethanol, butanol, isobutanol, glycerol, lactic acid, acrylic acid, and acetone. [12, 23] Conventional butanol is derived from molasses with the use of anaerobic bacteria, yet newer methods were discovered that allow butanol to be produced from LCB by E. coli. [12] These standard chemicals are widely used in areas such as organic synthesis, food production, feeding stuffs, perfume industries, paint production, material synthesis, and pharmaceutical industries. [23]

2.3 Structure of sugars derived from lignocellulosic biomass and their importance

Carbohydrates are considered to be the most widespread biomolecule in nature. Other common names for carbohydrates include saccharides or simply sugars. Sugars with one monomer are known as monosaccharides. Two, three monosaccharides bound together form disaccharides and trisaccharides respectively. Many monosaccharides form large molecules known as polysaccharides. All monosaccharides possess some similar characteristics: insolubility in organic solvents, solubility in water, a sweet taste, and the common formula $(CH_2O)_n$. [25]

Natural sugars usually possess three carbon atoms and at least one chiral carbon atom [25], an atom that does not match its mirror image. [26] Sugars are named D-sugars when the hydroxyl group of the chiral atom is facing the right side of the molecule while L-sugars have the hydroxyl group facing the left side of the molecule. A large majority of natural sugars are D-sugars. [25]

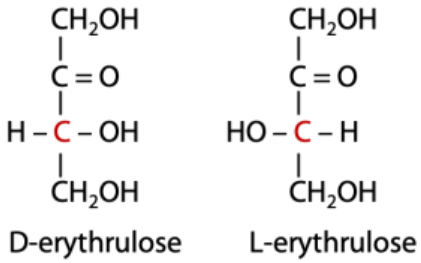


Figure 8. D- and L-sugar examples. *[25]*

Sugars can be divided by the number of carbon atoms (trioses, pentoses, hexoses) and into aldoses or ketoses by their chemical structure. The presence of hydroxyl and carbonyl groups is the reason why sugars can form hemiacetal and hemiketal groups. The process can occur at an intramolecular scale, resulting in familiar cyclic sugar structures. Sugar cycles are able to further react with other sugars or compounds to create larger molecules by forming glycosidic (C-O) or glycosylic (C-N) bonds via condensation. Most natural carbohydrates are found in the form of branching chains of monosaccharide building blocks that were joined together by strong glycosidic bonds. [25]

The breakdown of LCB yields valuable products like oligosaccharides and monosaccharides through the cleavage of glycosidic bonds. [22] Polysaccharides cellulose and hemicellulose are hydrolyzed, resulting in the formation of disaccharides, pentoses (C5), and hexoses (C6). Celluloses are hydrolyzed into cellobioses, which in turn are cleaved into glucose. [8, 16] Hemicelluloses are broken down into xyloglucans, arabinogalactans, galactloglycomannans, and xylans, with the concentration of each depending on the origin of the biomass. [8, 15] Those compounds are further degraded into C5 sugars D-xylose and L-arabinose and C6 sugars D-glucose, D-galactose, and D-mannose (Figure 10). [8, 16, 22]

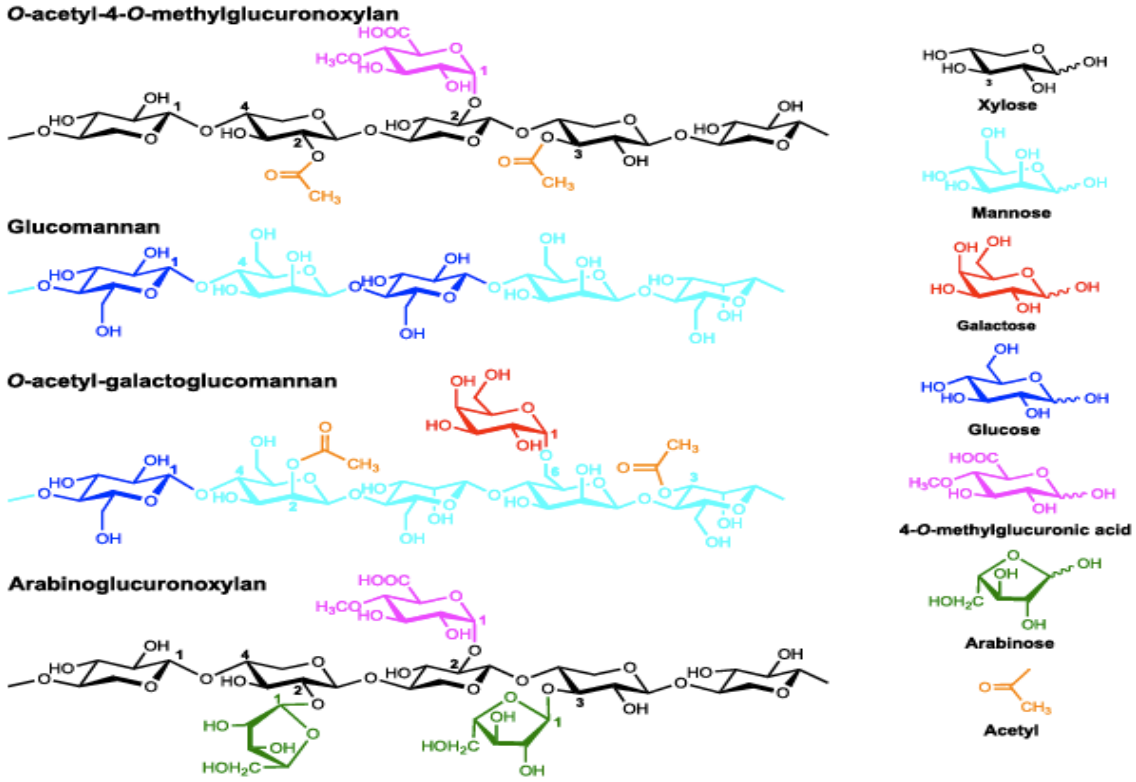


Figure 9. Hemicellulose components and their building blocks. *[22]*

Small amounts of the sugar L-fucose are also produced from the hydrolysis of hemicellulose. [19] With the help of dehydration, hexoses can be further degraded into important compounds such as 5-HMF and furfural (Figure 10). [22]

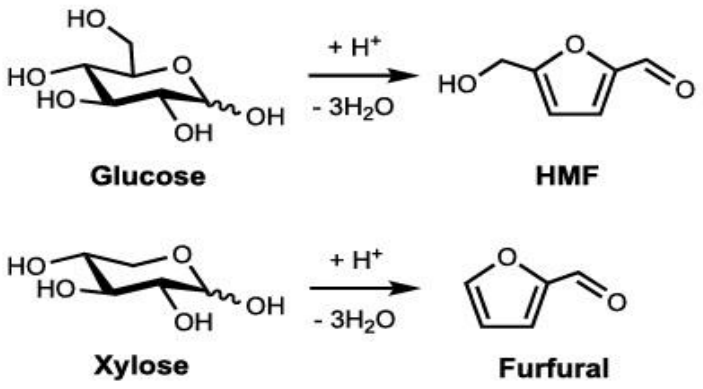


Figure 10. Dehydration of glucose and xylose to 5-HMF and furfural respectively. *[22]*

3 Analytical methods for sugar analysis

3.1 Basics of high-performance liquid chromatography

HPLC is a widely used chromatographic separation method used in both analytical chemistry and biochemistry. It is mostly used to quantify, qualify, and even purify individual components of a sample. A basic HPLC setup consists of a column that possesses a packing material known as a stationary phase, a pump that pushes the mobile phase through said column, and a detector that registers the retention times of the analyte's components (Figure 13). The sample is injected in a small volume into the mobile phase and interacts with the stationary phase based on its chemical or physical properties. There are many different types of HPLC, and the key difference between them lies in the specific phase system used for separation in each analysis. [27]

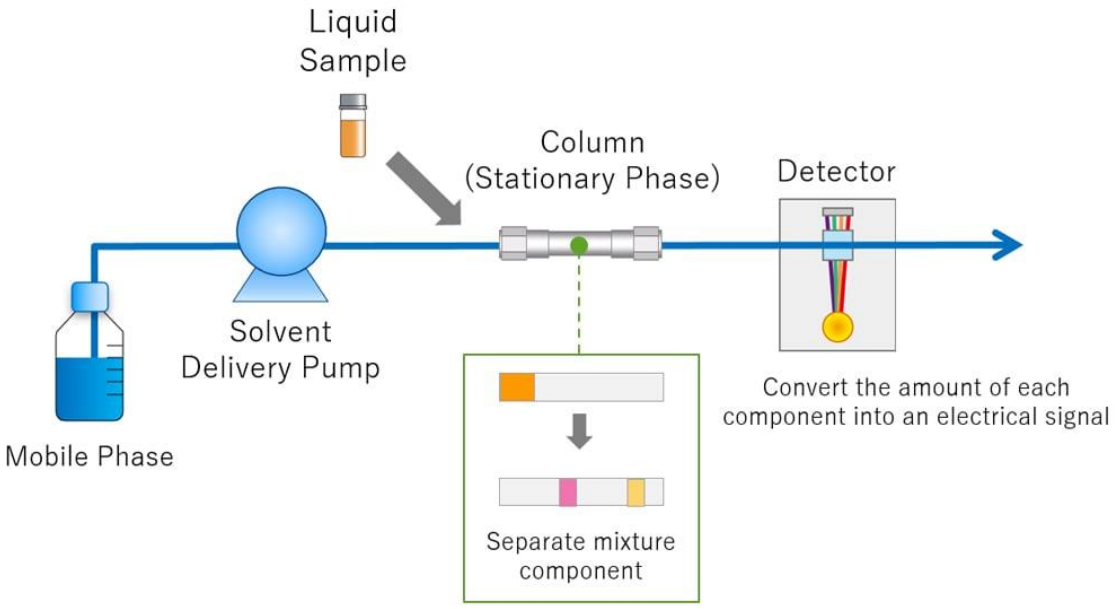


Figure 11. Basic HPLC setup. *[28]*

Normal Phase HPLC (NP-HPLC) separates the components of an analyte based on polarity. A polar stationary phase and a non-polar mobile phase are used in this method. If the compound analyzed is polar, it is retained by the stationary phase while non-polar molecules do not interact with the column. Adsorption of the molecules strongly depends on the polarity of the analyte. More polar molecules create stronger interactions with the column's resin, lengthening the retention time. [27] Typical columns used for the following type of separation contain alumina or silica. [29]

Reversed Phase HPLC (RP-HPLC) is the polar opposite of NP-HPLC. The setup consists of a non-polar stationary phase and a slightly polar mobile phase. RP-HPLC separates components based on their attraction to the non-polar stationary phase of the column. This attraction, called a hydrophobic interaction, occurs between the non-polar analytes and the non-polar resin, while the polar mobile phase promotes movement through the column. [27] The columns used in RP-HPLC usually contain alkyl, aliphatic or phenyl bonded stationary phases. [29]

Size-exclusion HPLC (SEC) is a completely different method. Unlike the previously mentioned separation modes, SEC mainly separates compounds based on the particle size. It has also been used for determining the structure of certain biological molecules such as proteins and amino acids. [27]

Different elution modes are available in HPLC. [29] Isocratic elution employs a mobile phase with a fixed composition. As a result, the total amount of analyte within the system is preserved, constantly distributing itself between the mobile and stationary phases. Gradient elution operates differently. The mobile phase gradually becomes stronger throughout the run. More of the eluent is eluted from the column and into the mobile phase, resulting in a higher total amount of analyte present. [30] Gradient elution is seen as superior to isocratic for various reasons. It offers a significant advantage when aiming to separate compounds completely, without requiring an unreasonable amount of time. Fast separation is achieved without compromising the resolution of earlier eluting peaks or causing excessive broadening of those eluted at the end of the analysis. [29] A major drawback of gradient elution is its incompatibility with certain detectors, such as RID, which is considered as a universal detector used in HPLC. [31] Another disadvantage is the need for more sophisticated and expensive equipment. This, in turn, leads to higher maintenance costs. Using high-speed gradient elution with low-quality apparatus will result in less reproducible and inaccurate results. [29]

Column choice in high-performance liquid chromatography

HPLC columns are precisely manufactured straight tubes of defined length and internal diameter. These columns house the stationary phase, a specialized resin crucial for the separation process, and are usually made of stainless steel, glass, or polymers. For optimal separation, column dimensions play a crucial role. Column lengths can vary up to 25 cm while internal diameters typically range up to 5 mm. [32] Choosing the correct column for analysis can prove to be a difficult task. Several parameters must be taken into account, such as the separation mode, column dimension, and particle size. [33]

NP-HPLC, RP-HPLC, and SEC all have different column types, the choice of which will be the first step. Dimensions are the next step. If the main goals are short analysis time and reduced solvent use, a short column should be chosen. For higher peak resolution, longer columns are the optimal choice. Column width is another parameter that must be considered as not only does it affect separation efficiency, but the pressure under which the analysis is conducted is increased for narrower columns. Pressure must not exceed the recommended settings for the HPLC system in use. [33] Particle size is another major parameter worth consideration. The use of smaller particles in a stationary phase improves peak resolution and efficiency but increases the pressure. Pore size must be taken into consideration as well as it is a way of controlling the retention of compounds. Small pores have a larger surface area, which increases the analysis time. [33]

HPLC columns can also be chosen by the packing type in the stationary phase. Columns come packed with a variety of resins, with silica-based and polymer-based materials being the most widely used. Both have their advantages and disadvantages. [34] Silica packaging has been considered universal as it is usable in RP-HPLC, NP-HPLC, and SEC. It is capable of binding several compounds to the column. Its main disadvantage is its narrow pH operation range (from 2 to 8), and the fast-aging process. [35] Polymer packaging consists of mostly polystyrene-divinylbenzene or polymethacrylate. Its main advantages are its tolerance towards any pH, stronger hydrophobic

properties, and strong selectivity towards large molecules such as sugars, proteins, and DNA. [34, 35]

Polymeric columns are one of the most used column types in HPLC-RID sugar analysis. From this class, C18 columns are seen as universal, but they are not as effective for sugar analysis since di- and monosaccharides do not retain well. SEC columns are known to be very effective in sugar analysis as well due to their ability to separate molecules by size without an additional derivatization step. The separation mechanism of SEC is based on the difference in the molecular size of the components. The SEC column is filled with a gel with particle sizes typically in the range of 3-10 µm. Larger molecules do not diffuse into the gel pores and elute from the column first with the eluent, while smaller molecules diffuse fully and elute last. Medium-sized molecules diffuse partially. [36]

Refractive Index Detector

RID is a universal detector that is capable of responding to all compounds. [37] RID is a very straightforward and easy detector to use. The work principle is based on the refractive index. The detector consists of a glass cell that is separated into two compartments: the sample cell and the reference cell (Figure 14). [38]

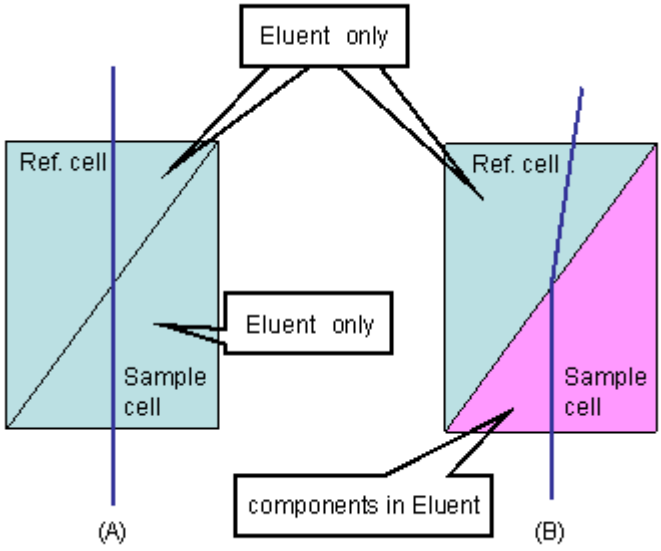


Figure 12. Structure of refractive index detector. *[38]*

Firstly, the reference cell is filled by the mobile phase for a certain amount of time. During the analysis, the mobile phase passes the sample cell. A beam passes both cells at once. When sample components reach the sample cell, the beam will be bent due to the refractive index of the reference medium and the sample present in the mobile phase. The change in the angle of the beam is measured and translated into a signal. [38, 39]

RID is often used for sugar analysis as carbohydrates do not possess chromophores that are necessary for UV detection. Using RID helps avoid an unnecessary sample preparation step that would consist of either ionizing or adding the respective chromophores for UV detection. [40]

3.2 Basics of capillary electrophoresis

CE is a technique that utilizes an electric field to separate charged molecules based on their electrophoretic mobility. A basic CE apparatus consists of a high-voltage power source, a sample injector, a capillary, a detector, and an integrator or computer. In certain devices a thermostat is included since the temperature affects the viscosity of the buffer solution running through the tube. [41, 42]

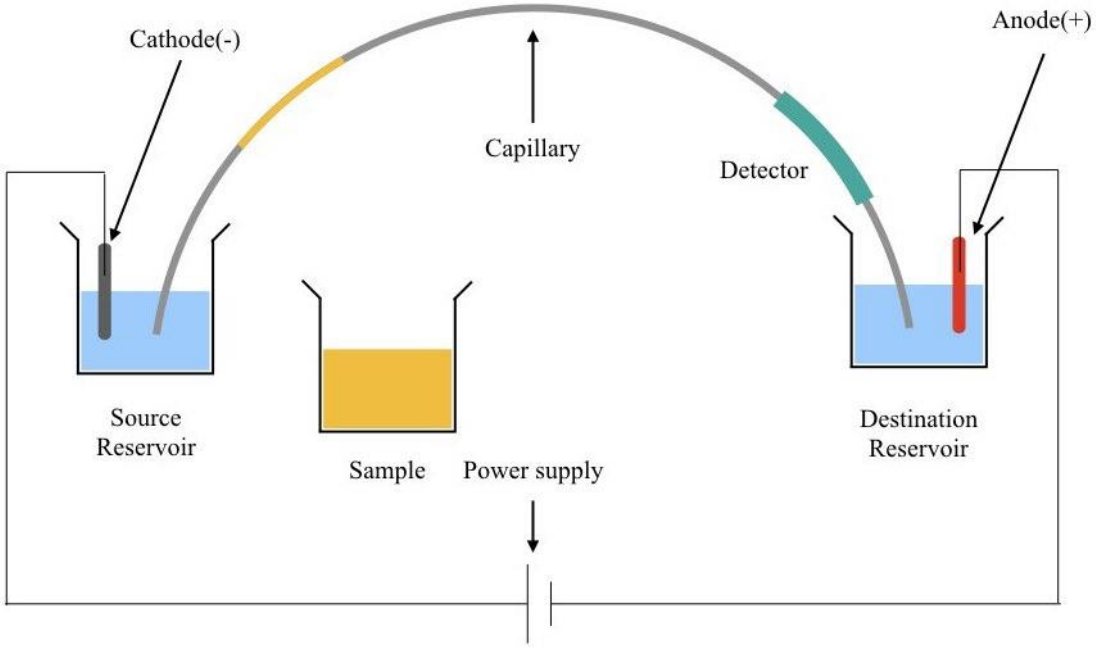


Figure 13. Basic capillary electrophoresis setup. [43]

CE possesses a diverse range of variations, their typing based on the principle of separation. Capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MEKC) are three of the most well-known CE variations, with CZE being the most commonly used method. [41, 44]

CGE and CZE are very similar. Like CZE, the separation is performed in a capillary. However, in the following method, the capillary is filled with a gel that works similarly to a sieve. The separation of molecules is based on their molecular weight. When voltage is applied, the molecules start migrating through the gel. Smaller molecules pass much easier than their larger counterparts. [41, 44, 45]

MEKC is a combination of chromatography and CE. Surfactant micelles are added to an electrolyte solution. Molecules with no charge are separated between the liquid phase and the micelle core while charged molecules migrate based on their charge-to-mass ratio. [45]

CZE bases its separation technique on the difference in electrophoretic mobility of the sample's components. In simple terms, CZE uses a tiny tube filled with a liquid solution (buffer) to separate the analyte. During a CE analysis, each side of the power supply is connected to two electrodes: the cathode and the anode. The side of the capillary where the sample is introduced is added to the

vessel where the anode is located while the other side is added to the vial that is connected to the cathode. These components migrate from the positively charged anode towards the negatively charged cathode, where the detector is positioned to analyze them. Light from the detector passes a small capillary window, an area where the capillary is transparent, in order to measure the absorbance of the sample. [41, 42]

Two important parameters affect the separation of the analyte. Components are separated thanks to different velocities achieved in the capillary when an electric field is applied. The speed is directly proportional to electrophoretic mobility (EP), which depends on the charge (q) and radius (r) of the molecules as well as the viscosity of the buffer: [41, 45]

$$\mu_{EP} = \frac{q}{6\pi\eta r}$$

The real speed the components travel at is directly proportional to the strength of the electrical field (E):

$$v = \mu_{EP} E$$

The following relationship explains why the strength of the electric field influences the migration of the components, meaning: the stronger the electric field, the faster the molecules travel through the capillary. [45]

EOF is the second important parameter of CE. It occurs when the voltage is applied to the capillary. The basis of its work involves the structure of the capillary as well which has silanol groups on its surface. [41]

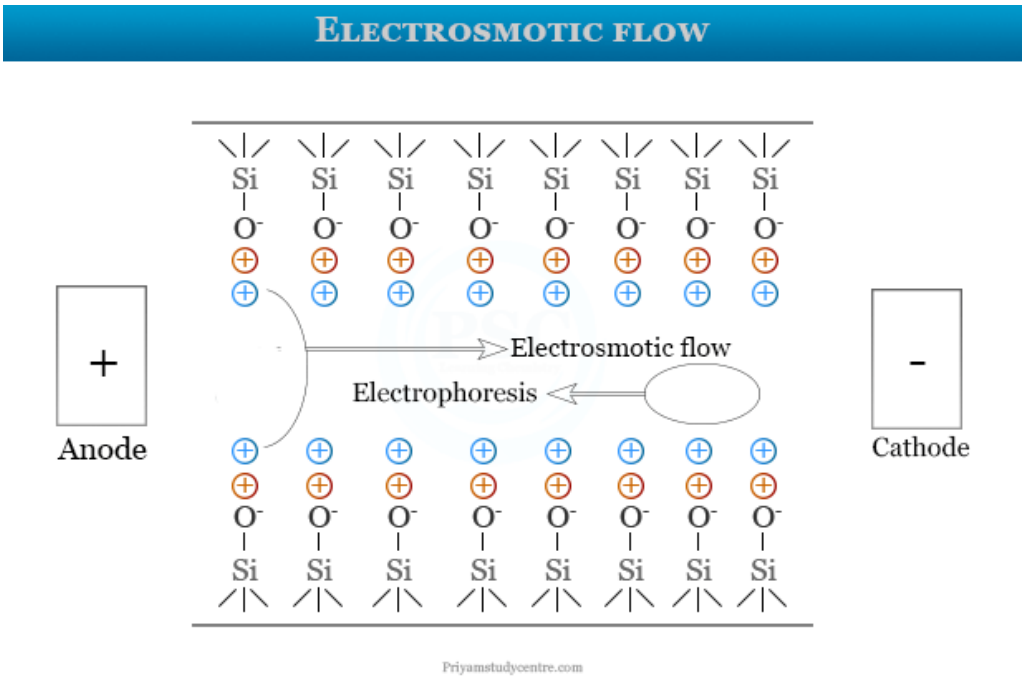


Figure 16. Capillary wall structure and EOF flow in CE. *[70]*

Before first use of a capillary, a preparation step is required. Capillaries undergo a reactivation step to modify the surface silanol groups. This modification is crucial for achieving successful separations and a consistent electroosmotic flow (EOF). Activation is seen as the ionization of the hydroxide

groups present on the surface (Figure 17). Capillaries are often rinsed by a strong alkaline solution in order to preserve the surface density of the silanol groups. [45, 41]

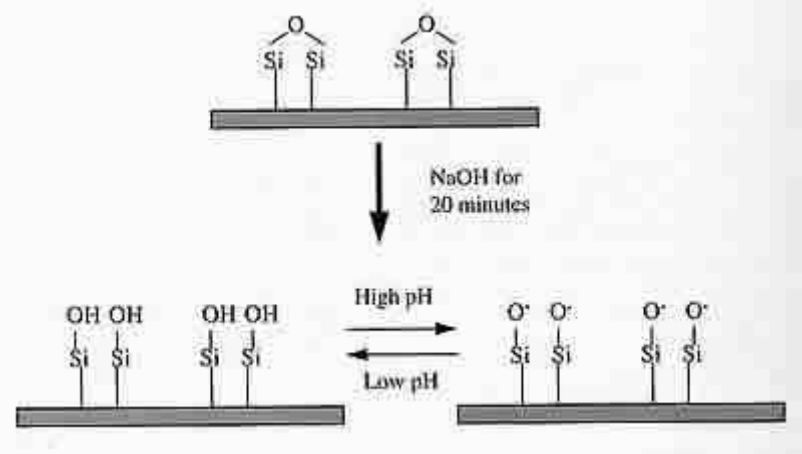


Figure 17. Ionization process of silanol groups during first pretreatment. *[71]*

Ionization occurs in a similar manner during the analysis phase as well. Due to the deprotonation of the silanol groups, the capillary wall gains a negative charge, creating a double layer of cations that are attracted to it. Within the double layer, the innermost layer remains fixed in place. In contrast, the outer layer is mobile and can flow freely along the length of the capillary. The applied electric field causes the free cations to move toward the cathode creating a powerful bulk flow. EOF can be calculated with the following equation: [41, 45]

$$\mu_{EOF} = \frac{\varepsilon}{4\pi\eta} E\zeta$$

where ε is the dielectric constant of the buffer, E is the strength of the electric field, ζ is the zeta potential of the double layer, and η is the viscosity of the buffer solution. A lower viscosity, a higher pH, and a larger ζ potential between the ion layers of the capillary wall guarantee a stronger EOF. [41] EOF is one of the strengths of CE when it is compared to HPLC. Due to the movement of the buffer being more uniform, there is no parabolic flow widening compared to HPLC. As a result, there is no band broadening that is a common issue in HPLC columns (Figure 18). [45]

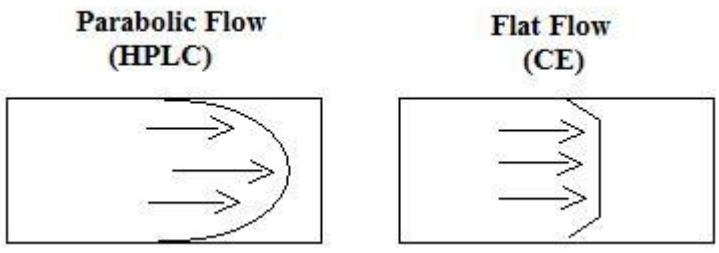


Figure 18. HPLC and CE flow difference. *[41]*

4 Performance parameters of analytical method

In order to guarantee reliable results, particularly during method development, analytical methods are being validated by estimating diferent performance characteristics. Analytical methods are assessed using various metrics, not only to evaluate their individual effectiveness but also to compare multiple techniques. This comparative evaluation is essential for selecting the optimal method. [31, 46]

In the following work, several parameters were estimated during the validation process for HPLC and CE methods. Peak resolution, instrumental detection (IDL) and quantification (IQL) limits, limits of detection (LoD) and quantification (LoQ) of the analytical procedure, calibration linearity, repeatability, and reproducibility, systematic error, and matrix effect, were evaluated for both of these methods.

Peak resolution generally means the ability to separate two peaks. Resolution is measured to evaluate the effectiveness of separation. The higher the resolution, the more effective the separation process is. Peak resolution can be calculated with the following formula:

$$R = \frac{(t_{Rb} - t_{Ra})}{0.85 \times (w_{0,5b} + w_{0,5a})} \quad [1]$$

where t_{Rb} and t_{Ra} are retention times of the compared peaks, and w1 and w2 is ½ of the width of each peak. [47]

IDL is a parameter that depicts the lowest concentration of the analyte required by the instrument to distinguish the sample's signal from the background noise. IDL can be measured in several ways, one of them being ten consecutive measurements of a blank solution. [48] The formula for IDL is the following:

$$IDL = 3 \times s \quad [2]$$

where s is the standard deviation of parallel measurements of blank solution. [31]

Meanwhile, IQL is the variable that shows the lowest concentration of the analyte that can be quantified by the instrument. [48] The following formula is used to calculate IQL:

$$IQL = 5 \times s \quad [3]$$

where s is the standard deviation of parallel measurements of blank solution. [31]

While IDL and IQL are parameters that evaluate the instrument, LoD is a value that depicts the amount of analyte that can be detected by the chosen analytical technique. However, the precise concentration cannot be measured. [49] For evaluating limits of quantification, LoQ is used. The formula to calculate LoD and LoQ takes in the same parameters: mass of the sample and sample dilution. LoD and LoQ were expressed in mass percentage calculated as follows:

$$LOD\ (LOQ)[\%] = \frac{IDL(IQL) \times V}{m * 10000} \quad [4]$$

where *V* is the sample volume (ml), and *m* is the sample mass used for analysis (g). [50]

Any analytical method may possess errors that affect the results. Errors can be divided into systematic and random errors. Random errors happen unpredictably during sample preparation or measurement and are not related to the efficiency of the method. Random error is estimated by standard deviation of parallel measurements. Repeatability and reproducibility are paramaters that describe random error of the analytical procedure and used to evaluate the precision of an analytical technique. [31] A method is considered repeatable if performing the analysis multiple times in one setting produces the same results, making it credible. Reproducibility evaluates the variability of the measurement results if independent experiments are made during longer period of time (several months), performed using the same method and steps. [51]

Systematic errors are an issue that persists due to a faulty method or equipment, and they can affect the method's accuracy and the reliability of the results. Statistical t tests can also be used to analyze the statistical significance of the result. [52, 53]

Matrix effect is a parameter that estimates systematic error analytical methods. It is known as a manifestation of interference caused by the sample's matrix or its own characteristics. Matrix effects can pose a significant challenge as they affect the reliability and performance of the chosen method. Some matrix effects (such as an irrelevant part of the sample coeluting with the analyte) are easily noticeable and can be improved by better cleanup, improved chromatographical method or a more selective detector. More subtle matrix effects may be discovered during method validation. One way of avoiding subtle matrix effects is by using a calibration (for instrumental methods) made with standard mix prepared in sample matrix. [54, 55, 56]

For evaluating matrix effects, a spiking experiment could be applied, where certain amount of standard solution of analyte is added to the sample under study and the peak area ratio is calculated. It allows to determine whether the amount added to the sample (theoretical) corresponds to the result achieved via the chosen analytical method (practical). The ratio can be calculated with the following equation: [56, 57]

$$ME(\%) = \frac{\bar{x}' - \bar{x}}{x_{spike}} \times 100\% \text{ [5]}$$

where $\bar{x}'$ – the mean value of the spiked sample (g/L); $\bar{x}$ – the mean value of the non-spiked sample (g/L); x_{spike} – the added (spiked) concentration (g/L)

If the result is greater than 100% means that the signal is enhanced, or sample is contaminated while a value below 100% means that the signal is suppressed, or analyte was lost during analysis. [60]

A systematic error can be calculated with the following equation:

$$E = 100\% - ME \text{ [6]}$$

The t value of such a test can be calculated with the following equation:

$$t = \frac{|x_{spike} - \bar{x}| \times \sqrt{n}}{s} \text{ [7]}$$

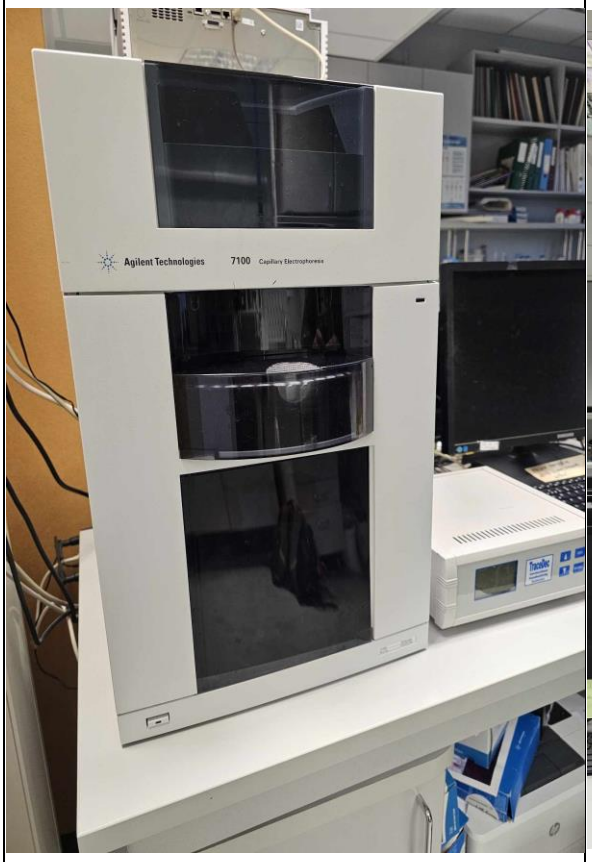
where x_{spike} — concentration of the spike (g/L); $\bar{x}$ – the mean value of the spiked sample; n – number of parallels; s – standard deviation of the spiked sample parallels.

5 Experimental procedure

5.1 Equipment and tools

The following CZE and HPLC apparatus were used in the comparative study (Table 1).

Table 1. HPLC and CE machines

	
Agilent 7100 Detector: UV (wavelength 270 nm) Data processing: Agilent ChemStation	Agilent 1260 Infinity II Detector: RID Data processing: Agilent ChemStation

Automatic pipettes with volumes of 10 µl, 200 µl, 1000 µl, 5 ml, and 10 ml were used for the sample, buffer, and wash solution preparation processes. 1 µl Eppendorf and 10 ml centrifuge tubes were used as storage mediums and dilution vessels.

Hettich EBA 200 S centrifuge and Sanyo Labo Autoclave participated in the sample preparation steps as well.

5.2 Experimental conditions for capillary electrophoresis

For CZE, a quartz capillary was used (Polymicro Technologies, Phoenix, AZ, USA). The parameters of the capillary were the following: length – 80 cm, effective length – 71 cm, inner diameter (ID) – 50 µm. The voltage applied to the capillary was 18 kV. The electric field strength achieved equaled approximately 130-140 µA.

Prior to analysis in CZE, a new capillary was prepared by cutting it to a specific length and creating a window to allow UV light to pass through for detection. The capillary was pretreated by different solutions set in a specific timeframe and order:

1. 30 minutes with 1M NaOH solution
2. 5 minutes with purified distilled water
3. 5 minutes with buffer solution

During analysis, the capillary had to be washed in the following order:

1. 3 minutes with 5% acetic acid
2. 5 minutes with 1M NaOH
3. 3 minutes with purified distilled water
4. 10 minutes with buffer solution

Prewash before every run:

1. 5 minutes with buffer solution

Wash cycle after every run:

1. 3 minutes with 5% acetic acid
2. 5 minutes with 1M NaOH
3. 3 minutes with purified distilled water

The sample was being introduced into the system via a pump at 50 mbar for 10 seconds. The washing cycle was repeated before and after every run in order to wash out the sample particles that may have remained behind, to add more buffer solution to the capillary so that the compounds used in the previous steps would not interfere with the next run. This step is especially vital in case the earlier analyte had a higher concentration compared to the next sample as the remaining particles of the concentrated compound will influence the results.

5.3 Experimental conditions for high-performance liquid chromatography

For HPLC, a SEC column Shodex Sugar SP0810 was used (Resonac Europe GmbH, Munich, Germany). The column had the following parameters: length – 300 mm, counter ion – Pb^{2+}, in-column solvent – H_2O, max usable flowrate – 0.6 ml/min, max pressure – 3 MPa, particle size – 7 µm, ID – 8 mm.

In HPLC, different preparation steps were taken. Before every analysis, the following conditions were met:

1. Flowrate: 0.6 ml/min
2. Detector temperature: 55°C
3. Column temperature: 80°C

Once the detector reached the correct temperature, the purge valve was opened for approximately 5 minutes in order to fill the reference cell with the mobile phase. Purified distilled water was used as the mobile phase. The conditions were based on the recommendations of National Renewable Energy Laboratory (NREL), [58] and the conditions for the use of the column by the manufacturer. The sample was introduced into the system via an autosampler.

5.4 Chemicals

Table 2. Chemicals used in the process.

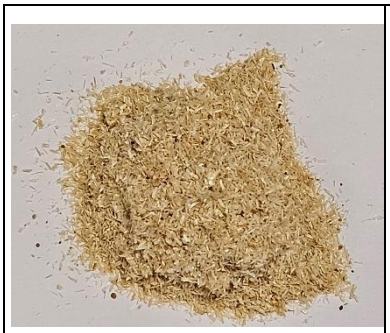
Name of chemical	Area of use	Manufacturer
D-glucose dehydrated	Calibration standard preparation	Fisher Chemical, USA
D-(+)-xylose	Calibration standard preparation	Sigma, USA
D-(+)-galactose	Calibration standard preparation	Fluka, USA
D-(-)-arabinose	Calibration standard preparation	Sigma, USA
D-raffinose pentahydrate	Calibration standard preparation	Sigma, USA
D-(+)-cellobiose	Calibration standard preparation	Fluka, USA
L-fucose	Calibration standard preparation	Alfa Aesar, USA
5-HMF	Calibration standard preparation	Acros Organics, USA
D-(+)-mannose	Calibration standard preparation	Acros Organics, USA
H_2SO_4	Biomass sample preparation	Honeywell, USA
Disodium phosphate	Wash solution preparation	Fisher bioreagents, USA

(Na_2HPO_4)	Buffer solution preparation	
NaOH	Wash solution preparation	Sigma-Aldrich, USA
	Buffer solution preparation	
$CaCO_3$	Biomass sample preparation	Sigma-Aldrich, USA
CH_3COOH	Wash solution preparation	Fluka, USA
Purified distilled water	Sample preparation	Millipore, USA
	Buffer solution preparation	
	Calibration standard preparation	

5.5 Biomass sample preparation procedure

In the comparative study of HPLC and CZE, the following wood and plant biomasses were used: cotton, tangerine, barley, and buckwheat hulls. Barley and tangerine biomasses were pretreated at different parameters, which could result in varying sugar yields. Several barley samples that were analyzed were made at the following temperatures: 150°C, 160°C, 170°C, 180°C, 190°C, 200°C.

Table 3. Biomasses analyzed.

 **Cotton**	 **Buckwheat hulls**	 **Ordinary barley**
 **Barley pretreated at 150°C**	 **Barley pretreated at 160°C**	 **Barley pretreated at 170°C**

		
Barley pretreated at 180°C	**Barley pretreated at 190°C**	**Barley pretreated at 200°C**

Before analysis, the biomass was degraded by sulfuric acid, and cellulose and hemicellulose were hydrolyzed into monomeric sugars (Klason method). The Klason method is the oldest and most popular method used for biomass analysis. While its main goal is the extraction of lignin, it is applicable to sugar analysis as well. The method uses concentrated sulfuric acid to hydrolyze and solubilize carbohydrates present in LCB while lignin is left in the precipitate. A certain amount of lignin, known as soluble lignin, is measured from the solution via UV spectrophotometry while the filtered precipitate is weighed. While being a rather effective method, the main disadvantage of the Klason method is how it possesses many different steps, resulting in a higher probability of mistakes. [59]

The following steps were made in order to ensure the result would be solubilized sugar solutions acceptable for CZE and HPLC analysis:

1. 0.2 mg of biomass was weighed in a beaker.
2. The water bath was preheated to 30°C.
3. 3ml of 73% H_2SO_4 was added to the biomass and mixed gently with a spatula without smearing the beaker walls with the sample. The spatula was left in the beaker. Either action was taken in order to prevent sample loss.
4. The beaker was covered with folium and heated in the water bath at 30°C for approximately 1 hour.
5. The sample was stirred during the heating phase after every 20 minutes to ensure each part of the sample was treated equally.
6. The beakers were removed from the water bath.
7. 72ml of water was added to the samples. The samples were stirred and covered with folium.
8. The beakers with samples were set into an autoclave for further degradation at 121°C for one hour.
9. During the process, the sugars were solubilized while insoluble lignin stayed in the precipitate. The precipitate was removed from the sugar solutions via vacuum filtering.

A strong acid was used in the Klason method to break down the bonds between cellulose, hemicellulose, and lignin, and later solubilize the sugars. As a result, an acidic sample solution was acquired. CZE is a method very sensitive to the pH value of the sample. Solutions with a very low pH will cause a voltage loss during a CZE run. In terms of HPLC, the manufacturer did not recommend using highly acidic solutions in the analysis as it could damage the column. In order to neutralize the samples, the following steps were taken:

1. 0.5 g of solid $CaCO_3$ was weighed.
2. 5 ml of the sample was transferred into a 10 ml centrifuge tube.

3. $CaCO_3$ was added to the tube in parts in order to avoid overflow caused by the neutralization process.
4. The sample was shaken for the removal of excess gas.
5. Gas-free samples were tested with an indicator to determine if the pH was neutral.
6. Ready samples were centrifuged at 5500 RPM for five minutes.
7. Supernatant that contains the analytes was transferred to new 10 ml tubes and centrifuged again at 7000 RPM for five minutes.

The biomass samples chosen for the study were rich in sugars. For CZE, sample dilutions were prepared that would fit the calibration range.

6 Results and Discussion

6.1 Optimization of capillary electrophoresis

Analyzing sugars is known to be a rather difficult process. Sugars lack fluorophores that would make them detectable by a fluorescence detector or any chromophores for them to be able to absorb UV light. However, in strongly alkaline conditions, sugars are ionized and are capable of absorbing UV light. In the following work, a strongly alkaline buffer solution was used in order for the analytes to gain an ionic form (Figure 20). [60]

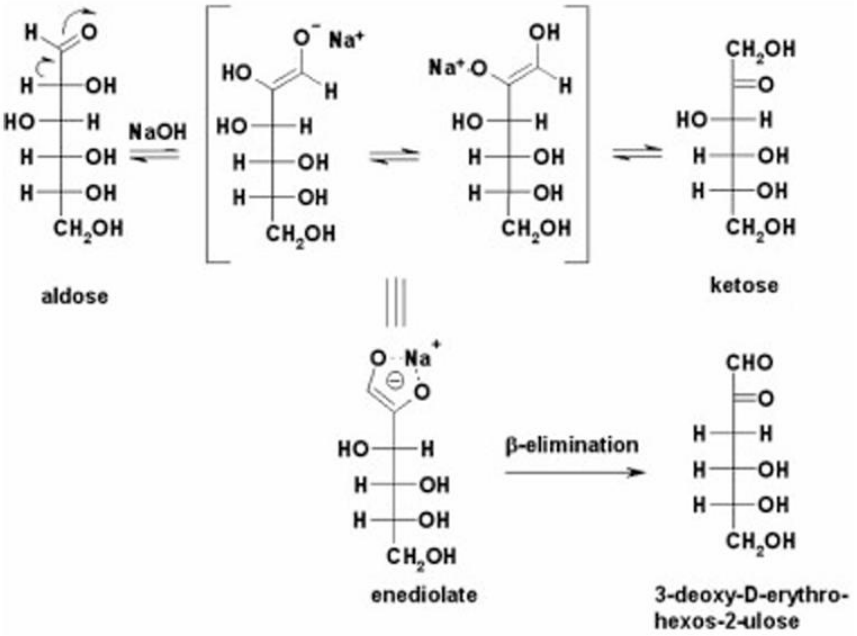


Figure 19. Aldose ionization in strongly alkaline conditions. *[60]*

The CZE method for sugar separation based on their charges was optimized in order to separate seven sugars: fucose, cellobiose, galactose, glucose, mannose, xylose, and arabinose. The furfural 5-HMF was separated as well. Voltage was tested from 18kV to 19.4 kV in order to improve the stability of the analysis compared to prior runs when high voltage would cause baseline fluctuations and a lot of background noise in the chromatogram.

The best results were achieved at the voltage 18kV. No instability was noticed, and the noise was significantly reduced. All the sugars were ionized, but the sample analysis time was sacrificed as a result, increasing from 40 minutes to 50 minutes. However, this time does not include the 14 min required for washing the capillary, making the total analysis time reach 64 min. The results are displayed in Figure 21.

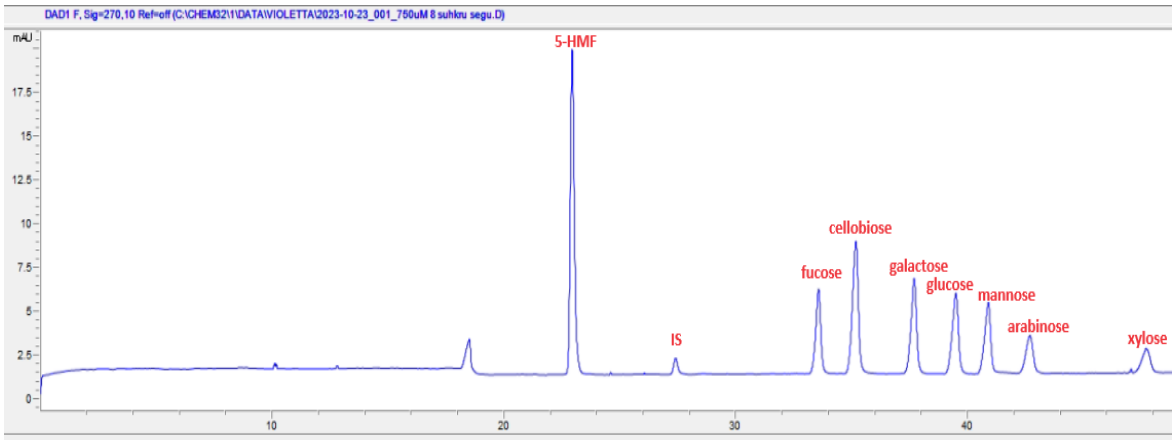


Figure 20. 0.14 g/L sugar standard + IS.

The sugar raffinose, which is not present in biomass, was added to the analysis as an internal standard (IS). The voltage change reduced the likelihood of baseline fluctuations and excess noise, but the addition of an IS to the analyte was vital in order to verify that the peak present belonged to the expected component. In case any fluctuations did occur, the IS and the EOF peak could be used as reference points to compare the sample results with a standard solution and determine the peaks.

6.2 Optimization of high-performance liquid chromatography

The HPLC method was developed as an alternative to CZE to measure higher sugar concentrations without the need for additional dilution. Seven sugars were planned to be separated: fucose, cellobiose, galactose, glucose, mannose, xylose, and arabinose along with the furfural 5-HMF. During development, different parameters were tested, such as column temperature, detector temperature, flow rate, solvent ratio, and injection volume. Column temperatures were tested from 70°C-80°C, flow rate was tested from 0.6 ml/min to 1 ml/min, solvent ratio was tested as pure water and 70:30 water to acetonitrile, injection volumes were tested from 5µL to 10µL. The detector temperature tested was only 55°C as per the column manufacturer's recommendation to keep the temperature value as close to the column temperature as possible.

After testing, the following conditions provided the best separation results:

1. Flow rate: 0.6 ml/min
2. Column temperature: 80°C
3. Detector temperature: 55°C
4. Solvent: pure water 1:1
5. Injection volume: 5µL

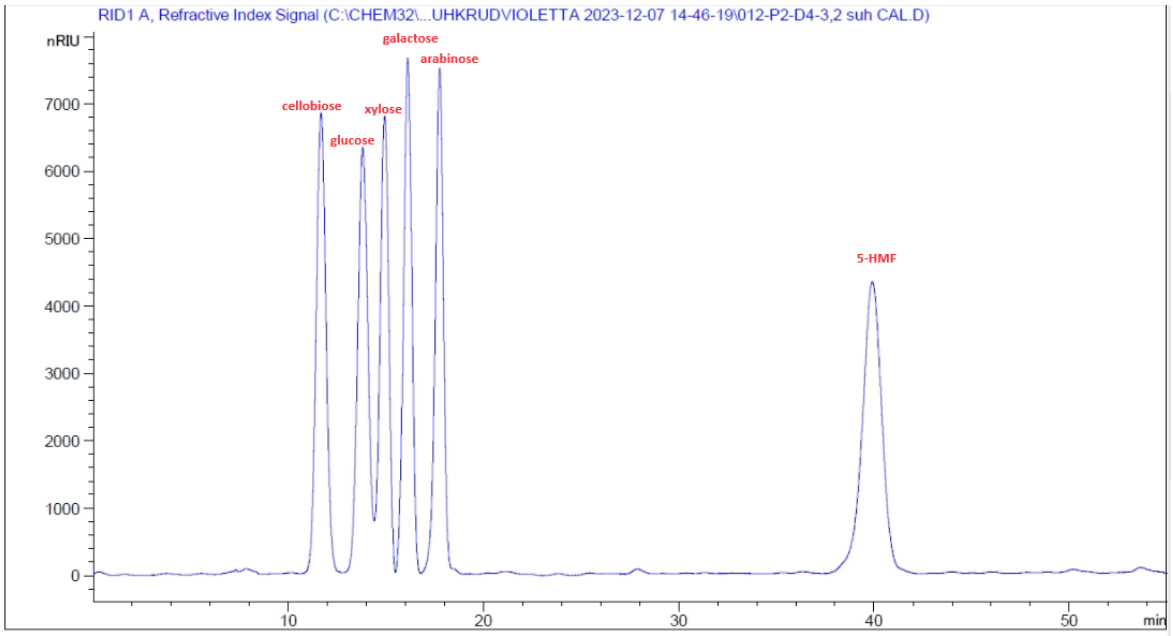


Figure 21. 3.2 g/L sample of 5 sugars and 5-HMF

The optimized method allowed to separate cellobiose, glucose, xylose, galactose, arabinose and 5-HMF. Fucose and mannose could not be separated and were achieved as a merged peak. Since their concentrations in biomass are below the LOD of chromatographic method they were removed from the calibration standard mix for HPLC. The result of separation of 5 sugars and 5-HMF are shown in Figure 22.

6.3 Analytical performance of methods

Calibration and instrumental detection and quantification limits

Calibration solutions of different concentrations were prepared in order to create the linear calibration curves and later evaluate the linearity of the methods. For CE method the concentrations of sugars used were: 0.01 g/L, 0.02 g/L, 0.05 g/L, 0.09 g/L, 0.14 g/L, and 0.18 g/L. Each concentration was measured thrice for accuracy (Table 4). The correlation coefficients (R^2) were above 0.99 for all sugars, which indicates that more than 99% of variability in data is covered by the linear calibration model.

Table 4. Calibration curves, instrumental detection and quantification limits of CE method

Analyte	Calibration range, g/L	Equation	R^2	IDL, g/L	IQL, g/L
5-HMF	0.01-0.18 g/L	y = 1900.4x - 0.2201	0,9989	0.001	0.003
Fucose	0.01-0.14 g/L	y = 192.05x + 1.7507	0.9912	0.003	0.005
Cellobiose	0.01-0.14 g/L	y = 482.93x + 1.0094	0.9921	0.006	0.010
Galactose	0.01-0.14 g/L	y = 351.47x + 1.6063	0.9950	0.003	0.006
Glucose	0.01-0.14 g/L	y = 368.31x + 0.5599	0.9961	0.003	0.006
Mannose	0.01-0.14 g/L	y = 0.0576x + 1.7297	0.9978	0.002	0.003

Arabinose	0.01-0.14 g/L	y = 210.16x + 2.2921	0.9904	0.001	0.003
Xylose	0.01-0.14 g/L	y = 156.96x + 1.6878	0.9922	0.004	0.007

The concentrations used for calibration in HPLC were: 0.2 g/L, 0.4 g/L, 0.8 g/L, 1.6 g/L, 3.2 g/L, 6.4 g/L. Each concentration was measured in triplicate. The linear calibration model was also used, and correlation coefficients (R^2) were above 0.99 (Table 5).

Table 5. Calibration curves, quantification, and detection limits

Analyte	**Calibration range**	**Equation**	**R^2**	**IDL, g/L**	**IQL, g/L**
Cellobiose	0.2-6.4 g/L	y = 76335x – 3153.3	0.9997	0.08	0.21
Glucose	0.2-6.4 g/L	y = 74656x – 2409.4	0.9996	0.09	0.23
Xylose	0.2-6.4 g/L	y = 68403x – 5393.2	0.9994	0.13	0.32
Galactose	0.2-6.4 g/L	y = 73603x – 3464.6	0.9992	0.16	0.40
Arabinose	0.2-6.4 g/L	y = 79741x – 9414.1	0.9921	0.18	0.46
5-HMF	0.2-6.4 g/L	y = 95564x – 5356.5	0.9993	0.22	0.55

Quantification and detection limits of the procedures

LoD and LoQ were calculated for both methods by using equation [4] and expressed in mass % per dry weight of biomass. It is worth noting that both LoD and LoQ values of CZE are much lower than that of HPLC, meaning that CZE is capable of detecting and quantifying the analytes at much lower concentrations.

Table 6. Comparison of quantification and detection limits of two methods

	LoD, dw%		**LoQ, dw%**	
Analyte	**CZE**	**HPLC**	**CZE**	**HPLC**
5-HMF	0.04	5.74	0.04	19.1
Fucose	0.11	-	0.18	-
Cellobiose	0.21	2.16	0.35	7.2
Galactose	0.11	4.17	0.21	13.9
Glucose	0.11	2.46	0.21	8.2
Mannose	0.07	-	0.11	-
Arabinose	0.04	4.81	0.04	16.0
Xylose	0.14	3.38	0.25	11.3

High-performance liquid chromatography and capillary electrophoresis peak resolution comparison

HPLC and CE peak resolution was calculated with equation [1]. In general, good peak resolution is shown by the R value exceeding 1%. If the value is below 1%, the resolution is poor, meaning the peaks are not completely separated. [61] The results shown in the table below (Table 7.) describe how the separation in CZE is better than in HPLC. CZE was capable of separating all 8 analytes while

HPLC was able to fully separate only cellobiose and glucose, galactose and arabinose, and arabinose and 5-HMF.

Table 7. Peak resolution for CE and HPLC

CZE		HPLC	
Analyte	**R%**	**Analyte**	**R%**
5-HMF/fucose	6.3	Cellobiose/Glucose	1.79
fucose/cellobiose	1.0	Glucose/Xylose	0.85
cellobiose/galactose	1.9	Xylose/Galactose	0.76
galactose/glucose	1.5	Galactose/Arabinose	1.05
glucose/mannose	1.1	Arabinose/5-HMF	5.85
mannose/arabinose	1.3		
arabinose/xylose	4.0		

Repeatability and reproducibility evaluation

The repeatability of the CZE method was evaluated by repeated experiments with a single sample for the entire day. A 0.05 g/L standard was analyzed six times in one day. Repeatability was evaluated via relative standard deviation (RSD). Reproducibility was evaluated in a similar manner with a similar solution, but the solution was prepared anew in the same conditions every time. The analysis was conducted for two weeks six times.

HPLC method repeatability was evaluated by repeated experiments with a single sample for the entire day. A 1.6 g/L standard was analyzed six times in one day. Repeatability was evaluated with the help of relative standard deviation (RSD). Reproducibility was evaluated in a similar manner with a similar solution, but the solution was prepared anew in the same conditions every time. The analysis was conducted for two weeks six times.

Table 8. Repeatability RSD

	CZE		HPLC	
Analyte	**Repeatability, RSD, %**	**Reproducibility RSD, %**	**Repeatability, RSD, %**	**Reproducibility RSD, %**
5-HMF	1.6	1.7	0.2	0.7
Fucose	5.0	8.1	-	-
Cellobiose	3.3	4.3	0.3	0.7
Galactose	3.8	5.9	0.3	0.4
Glucose	3.0	5.9	0.3	0.4
Mannose	2.2	6.8	-	-
Arabinose	3.1	9.0	0.3	0.4
Xylose	8.7	13.7	0.3	0.6

Matrix effect and systematic error

The matrix effect was calculated via equation [4] for both methods while a two-tailed t-test was performed to evaluate the statistical significance of the systematic error in both HPLC and CE (Equation 7). To evaluate the significance of systematic error a t-test was used. A null hypothesis was set that there is no systematic error present in both HPLC and CE. $\alpha = 0.05$ with confidence level 95% was adopted for this analysis. Calculated t-value was compared to critical one in Table 9. [53]

A cotton biomass sample was used for this test. The sample was analyzed six times. From Table 13 (Appendix 6) a critical t value was found to be 2.571 at $\alpha = 0.05$ and 5 degrees of freedom.

Table 9. Matrix effect and systematic error for HPLC and CZE

	CZE			HPLC		
Analyte	**Matrix effect, ME %**	**Systematic error, %**	**t value**	**Matrix effect, ME %**	**Systematic error, %**	**t value**
5-HMF	97.5	2.5	2.1	99.6	0.4	2.2
Fucose	97.4	2.6	5.3	-	-	-
Cellobiose	95.8	4.2	3.1	98.3	1.7	2.1
Galactose	97.9	2.1	5.7	99.2	0.8	2.5
Glucose	98.0	2.0	4.1	99.6	0.4	2.2
Mannose	97.9	2.1	4.8	-	-	-
Arabinose	97.6	2.4	4.3	99.5	0.5	2.3
Xylose	83.3	16.7	5.6	99.5	0.5	2.3

Data presented in Table 9 demonstrates that CE method has statistically significant systematic error for the most of sugars (t>tcrit), while HPLC t-values were below the critical t value, which means that HPLC method provides no significant systematic error.

Biomass sample results

A total of twelve biomasses were analyzed during this study. A total of twenty-two samples were hydrolyzed with the Klason method. Different barley types were analyzed via HPLC only as a separate study.

Table 10. Biomass results for HPLC

	Concentration, dw %						
Biomass	**cellobiose**	**glycose**	**xylose**	**galactose**	**arabinose**	**5-HMF**	**TOTAL, dw %**
Barley 150 °C	<7.2	46.6 (±7.0)	24.8(±3.7)	<13.9	<16.0	<19.1	71.4(±10.7)
Barley 160 °C	<7.2	46.1 (±6.9)	25.2(±3.8)	<13.9	<16.0	<19.1	71.4(±10.7)
Barley 170 °C	<7.2	48.1 (±7.2)	26.2(±3.9)	<13.9	<16.0	<19.1	74.3(±11.1)
Barley 180 °C	<7.2	47.3(±7.1)	14.5(±2.2)	<13.9	<16.0	<19.1	57.8(±9.3)
Barley 190 °C	<7.2	51.0(±7.7)	13.8(±2.1)	<13.9	<16.0	<19.1	60.8(±9.8)

Barley 200 °C	<7.2	50.2(±7.5)	11.8(±1.8)	<13.9	<16.0	<19.1	59.0(±9.3)
Barley untreated	<7.2	47.4(±7.1)	25.9(±3.9)	<13.9	<16.0	<19.1	73.3(±11.0)
Buckwheat hulls	<7.2	26.3(±3.9)	17.6(±2.6)	<13.9	<16.0	<19.1	43.9(±6.4)
Tangerine hydrated	<7.2	27.2(±4.1)	<11.3	<13.9	<16.0	<19.1	27.2(±4.1)
Tangerine + hesperidin	<7.2	27.5(±4.1)	<11.3	<13.9	<16.0	<19.1	27.5(±4.1)
Tangerine + carbonate	<7.2	24.9(±3.7)	<11.3	<13.9	<16.0	<19.1	24.9(±3.7)
Cotton	<7.2	44.2(±6.6)	12.0(±1.8)	<13.9	<16.0	<19.1	56.2(±8.4)

Table 11. Biomass results for CZE

Concentration, dw %									
Biomass	5-HMF	fucose	cellobiose	galactose	glycose	mannose	arabinose	xylose	**TOTAL dw %**
Buckwheat hulls	<0.04	<0.18	<0.35	0.7(±0.1)	27.8(±3.6)	<0.11	1.2(±0.2)	21.9(±2.9)	51.7(±6.8)
Tangerine hydrated	1.1(±0.2)	<0.18	<0.35	6.7(±0.9)	7.1(±0.9)	1.5(±0.2)	1.3(±0.2)	1.3(±0.2)	19.0(±2.6)
Tangerine + hesperidin	1.6(±0.3)	1.3(±0.2)	4.4(±0.6)	8.9(±1.2)	8.6(±1.1)	2.2(±0.3)	2.0(±0.3)	1.8(±0.2)	30.9(±4.5)
Tangerine + carbonate	2.1(±0.1)	2.7(±0.4)	5.9(±0.8)	11.0(±1.4)	11.0(±1.4)	3.0(±0.4)	2.4(±0.3)	2.3(±0.3)	40.4(±5.1)
Cotton	0.1(±0.1)	<0.18	<0.35	0.57(±0.1)	37.44(±4.9)	1.59(±0.2)	0.1(±0.2)	12.4(±1.6)	42.3(±7.1)

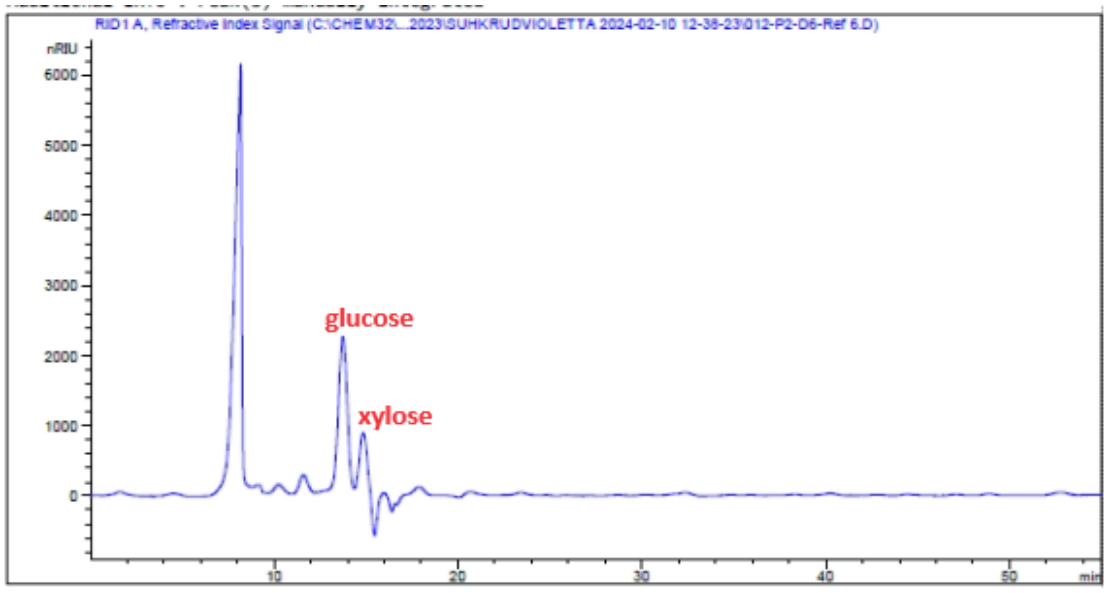


Figure 22. Example chromatogram of cotton analysis via HPLC. Sample: parallel nr 6. Dilution - 0x.

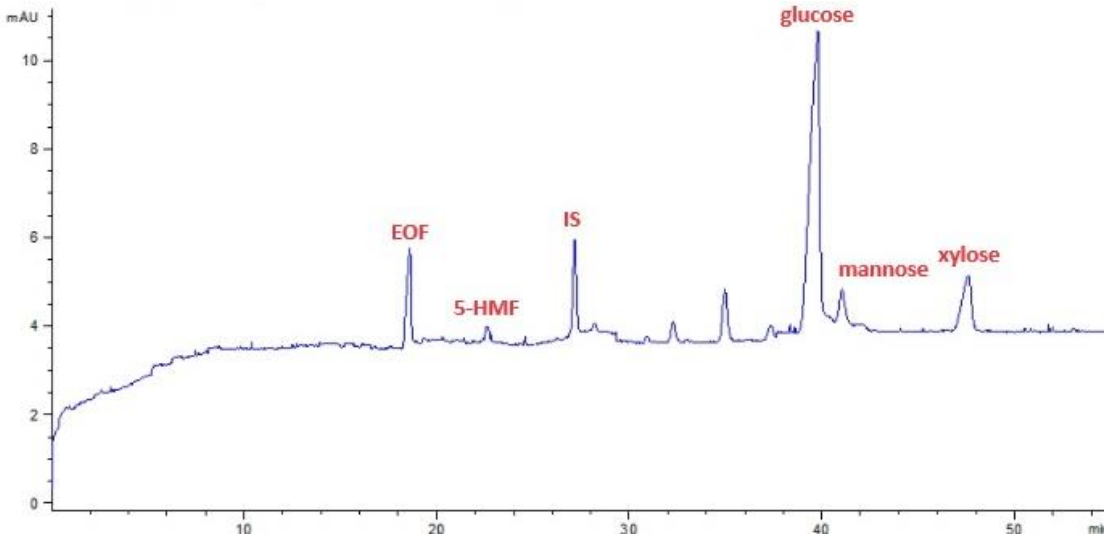


Figure 23. Example chromatogram of cotton analyzed via CZE. Sample: parallel nr 6. Dilution: 0x. IS – raffinose.

The following results depict that HPLC was only capable of identifying highly concentrated sugars in the biomass: xylose and glucose. No other analytes were detected, making CZE more sensitive towards small concentrations. The comparison of the results of cotton however showed that HPLC is much closer to the real value of the biomass while buckwheat hulls were analyzed roughly in the same manner by both methods. As a result, HPLC can be used to measure concentrated sugars in order to avoid sample dilution, which is a necessity in CZE.

6.4 Greenness evaluation with AGREE calculator

Figure 24. AGREE greenness evaluation metric tool results. Left – CZE. Right – HPLC.

Both method's greenness was evaluated via the AGREE calculator which is based on the twelve principles of GAC. The first step was sample pretreatment, which is the same for both methods and involved the use of the Klason method for biomass sample degradation and sample neutralization

with the help of lime. As the sample is not analyzed directly and requires many preliminary steps, the first condition is labeled red.

The second step was sample amount, which is around 0.2 ml for both methods. The score is shown as green for both methods. Device positioning is also the same for both methods, which is atline – the sample is analyzed by a standalone instrument nearby, [62] and is scored as orange due to not being green.

In sample preparation stages, CZE scored poorer due to an additional step: sample dilution. Other perquisite steps are the same for both methods. In automation, CZE scored a bit poorer due to a requirement to sometimes empty the buffer solution vials in order to avoid overflow, adding a manual step to the process. In HPLC, no such step is required. In terms of derivatization, HPLC has no derivatization steps while CZE does possess a derivatization step in the form of sugar ionization in a sodium hydroxide buffer. However, the metric tool does not consider sodium hydroxide to be a dangerous chemical in this step, so both methods are green in terms of this parameter.

Due to the requirement of dilution, CZE scores poorer in the seventh evaluation due to the increased amount of plastic waste generated in the process. Both methods also scored a bit poorly when it comes to the number of analytes and run times. HPLC is capable of detecting only 6 analytes while CZE detects 8 analytes. The run times are very similar for both methods.

Energy consumption is slightly better for HPLC. The average consumption for HPLC is 6.3 kWh while it's 6.9 kWh for CZE per day [63], meaning both methods are not demanding in terms of energy requirements.

The reagents used in both methods are inorganic compounds. In HPLC only sulfuric acid is used in sample preparation while CZE uses both sulfuric acid in sample preparation and sodium hydroxide as a buffer solution. Neither compound is considered organic, which makes both methods slightly less green. In the case of toxicity, neither method employs toxic reagents. However, operator's safety can be put at risk due to the fact that both sulfuric acid and sodium hydroxide are corrosive compounds. The other threats that the metric tool offers to select are toxicity to aquatic organisms, bioaccumulation, persistence, flammability, oxidization, and explosiveness, which neither of the compounds possesses.

6.5 Analysis of results

Table 12. Comparison of results for HPLC and CZE. Starting with peak resolution, the results are shown as average of all sugars.

Parameter	HPLC	CZE
Number of sugars separated	6	8
Analysis time, min	55	64
Peak resolution, %	2.06	2.44
IDL, g/L	0.14	0.003
IQL, g/L	0.36	0.005

LoD, mw%	3.78	0.095
LoQ, mw%	12.62	0.14
Repeatability, RSD%	0.28	3.83
Reproducibility, RSD%	0.32	6.92
Matrix effect, %	99.3	95.7
Systematic error, %	0.7	4.3
t value	2.3	4.4
Greenness score	0.65	0.56

The comparative study results are shown in Table 12. CZE is still more effective at separating all analytes, having successfully separated all seven sugars and the furfural 5-HMF. In case of HPLC, arabinose, mannose, and fucose were not separated from one another.

If CZE is more effective when it comes to separation, then HPLC separates all compounds at a faster rate, requiring only 45 minutes for one analysis. The average analysis time for CZE is 64 minutes, with the mandatory washing procedure included in the analysis time.

Peak resolution has proven to be better for CZE. The peaks are more defined and do not overlap, which is not completely true for HPLC as the peaks are very close together, and the glucose and xylose peaks are slightly merged. The resolution calculation showed that xylose and glucose are separated very poorly, with the resolution value being lower than 1. The average resolution for all analytes was higher for CZE as well.

CZE has proven to remain the more sensitive method out of the two techniques, being capable of detecting sugars at concentration 0.003 g/L, and quantifying them at concentration 0.005 g/L. HPLC however is capable of detecting sugars at a much higher concentration of 0.14 g/L and quantifying them at 0.36 g/L. The LoD and LoQ for CZE are also considerably lower, making the method about 100 times more sensitive to the analyte. At the same time, in the case of analysis of biomass, high LoQ values of HPLC are advantageous at eliminating additional dilution of samples.

In terms of repeatability and reproducibility, HPLC has proven to be more accurate. The RSD of the repeatability of HPLC was 0.28% and reproducibility was 0.32%, meaning there was only a slight difference in the results. In comparison, the results for CZE were less repeatable and reproducible. The RSD for repeatability was 3.83% and the reproducibility was 6.92% respectively, which means that there is a bigger variation in the results.

Matrix effect and systematic error were also evaluated for both methods. Overall, HPLC has the least matrix effect, with an overall systematic error of 0.7%, and the average t-value of the analytes is less than 2.517, meaning that there is no statistically significant systematic error present, proving the null hypothesis set for this method. In the case of CZE, the matrix effect is evident, with an overall systematic error of 4.3%, and notably, xylose possessed the highest error at 16.7%. The average t-value was 4.3, which is more than 2.517 and means that the null hypothesis cannot be accepted, and there is a systematic error present.

The greenness score for HPLC was 0.65 and for CZE — 0.56, which shows that the newly developed HPLC RID method has proven to be greener than CZE. However, it is worth noting that the majority of issues involving both methods come from the fact that the sample preparation step is the least green aspect of the analysis. The greenness of both methods would have been higher if a more sustainable alternative to the Klason Method had been found.

7 Conclusion

Nowadays, a lot of effort is dedicated to the development of green analytical chemistry. Green chemistry has become a major topic due to the evident impact of human activity on nature. Sustainable chemistry not only is a benefit to the environment but to the chemists themselves as greener alternatives tend to be more efficient and less demanding in terms of funding, safety, and energy. Biomass is a green alternative to fossil fuels and possesses great potential as an efficient source of materials and fuels, yet methods for analyzing and characterizing biomass must be improved accordingly as well. HPLC and capillary electrophoresis are two very well-known methods for the analysis of sugars present in biomass. Capillary electrophoresis is known to be more sustainable due to its low solvent usage and sample volumes compared to a traditional HPLC method. However, HPLC is known to be more stable and faster when it comes to sugar analysis.

In the following study, a brand new high-performance liquid chromatography method with refractive index detection was developed and validated as an alternative to capillary electrophoresis, developed in previous bachelor thesis study. The principal goal of the project was to compare sustainability or "greenness" and "fitness for purpose" of these two methods, based on carbohydrates analysis from different biomass types.

For that, important performance characteristics such as peak separation efficiency, reproducibility, accuracy, repeatability, limit of detection, and limit of quantification were estimated and compared for both methods. Statistical tests were performed to check the reliability of the results. Both methods were tested using a greenness metric tool AGREE to evaluate their greenness. The optimization results depicted that the best HPLC separation was achieved at detector temperature 55°C, column temperature 80°C, sample injection volume 5µL, and flow rate 0.6 ml/min. Cellobiose, glucose, galactose, 5-HMF, and xylose were fully separated while arabinose, mannose, and fucose emerged as a single peak. The run time of the analysis was 45 minutes. In order to determine the reliability of the new HPLC method, several parameters were used. Calibration curves were calculated to determine the linearity of the calibration.

Instrumental detection and quantification limits, and limits of detection and quantification of the analysis procedure were also estimated. The average IDL of all analytes was approximately 0.1 g/L, IQL was 0.4 g/L, LoD was 3.8 dw%, and LoQ was 12.6 dw%. The average repeatability was 0.28% and reproducibility was 0.32%. The same parameters measured for capillary electrophoresis were IDL 0.003 g/L, IQL 0.005 g/L, LoD was 0.095 dw% and LoQ was 0.14 dw%. Compared to HPLC, capillary electrophoresis was more sensitive to lower concentrations of the analytes. However, the repeatability and reproducibility of capillary electrophoresis have proven to be less promising: repeatability was 3.8% for all analytes while reproducibility was 6.8%, making the following method less reliable. The systematic error evaluation has also proven that there is a systematic error present in the data received with the analysis by capillary electrophoresis, which further proves it to be less accurate compared to HPLC.

The newly developed HPLC method was capable of separating and detecting main analytes present in biomass samples. It has proven to be far more stable, accurate, repeatable, and reproducible than CE and therefore its application to analysis of biomass samples with high sugar content was more reasonable.

Both methods were evaluated for sustainability by the AGREE metric tool. HPLC has proven to be a slightly greener alternative due to the lack of dilution and extra sample preparation steps. However,

it was concluded that one of the main sources of unsustainability for both methods is the biomass sample preparation step, which involves the use of strong acids and many steps including dissolution, precipitation, drying, weighting and many others.

All in all, the following thesis could prove as a starting point for further optimization of the developed method to make it more sensitive and sustainable for the environment.

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Abstract

As industries expand, there is a growing recognition of the need for sustainable development, which was the basis for the creation of the twelve principles of green chemistry. Analytical laboratories seem to impact nature less due to the smaller volumes of waste generated in the process of an analysis. Though the waste is less concentrated, its elusiveness and rapid dispersion can pose a serious threat to the environment. Greener methods benefit not only the environment but the laboratory itself as it has been proven that sustainable methods cut the costs related to analysis and improve efficiency.

Lignocellulosic biomass is a promising new source of materials that offers a green alternative to fossil fuels and first-generation biofuels. Sugars derived from lignocellulosic biomass are the focal point of many laboratories worldwide due to being a major component of many important materials and compounds sought by society.

High-performance liquid chromatography and capillary electrophoresis are both very well-known methods that are used for sugar analysis. While capillary electrophoresis is famous for using less chemicals and having low sample volumes, high-performance liquid chromatography has proven to be more stable and efficient.

In the following study, a brand new high-performance liquid chromatography method with refractive index detection was developed as a greener and more efficient alternative to a previously developed capillary electrophoresis method. Important performance parameters were calculated for both methods in order to evaluate their greenness, efficiency, and reproducibility.

Annotatsioon

Tööstuste laienemisel kasvab ka arusaam jätkusuutlikust arengust, mis oli põhjuseks luua kaksteist rohelise keemia printsiipi. Ühest küljest, analüütilised laborid ei koorma loodust nii palju kui tööstused kuna tekitatakse vähem jäätmeid. Teisest küljest, need jäätmed laotuvad laiali suurema kiirusega ning neid on palju raskem jälgida. Rohelised meetodid on kasuks mitte ainult loodusele vaid ka laboritele, sest on tõestatud, et võttes kasutusele rohelised meetodid vähenevad kulud ja pareneb analüüsi efektiivsus.

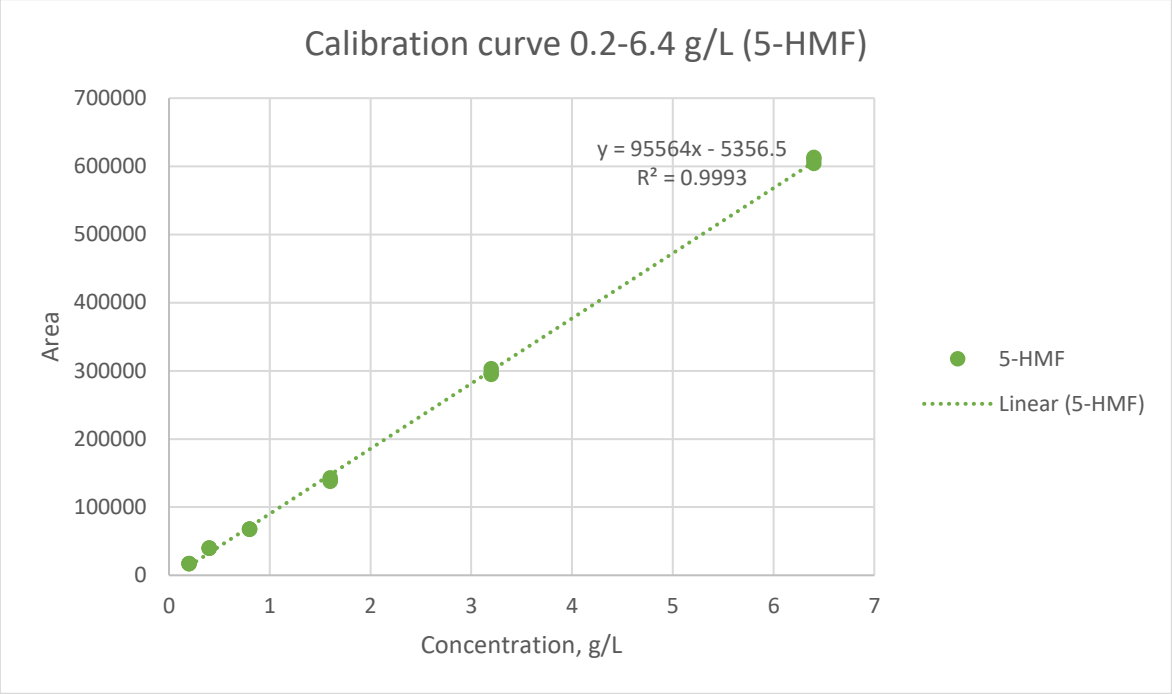
Lignotselluloosne biomass on uus lubav väärtuslikke materjalide allikas, mis on ka roheliseks alternatiiviks esimese põlvkonna biokütustele. Biomassist eraldatud suhkrud on tähelepanu keskpunktiks laboritele üle maailma, sest paljude ühiskonnale oluliste materjalide komponente saab kätte just suhkrute ümbertöötlemisel.

Kõrgefektiivne vedelikkromatograafia ja kapillaarelektroforees on tuntud meetodid suhkrute analüüsiks. Kapillaarelektroforees on kuulus sellepärast, et antud meetodi analüüsiks vajaliku proovi ja solventide maht on väga madal. Kõrgefektiivne vedelikkromatograafia on aga palju stabiilsem ja efektiivsem.

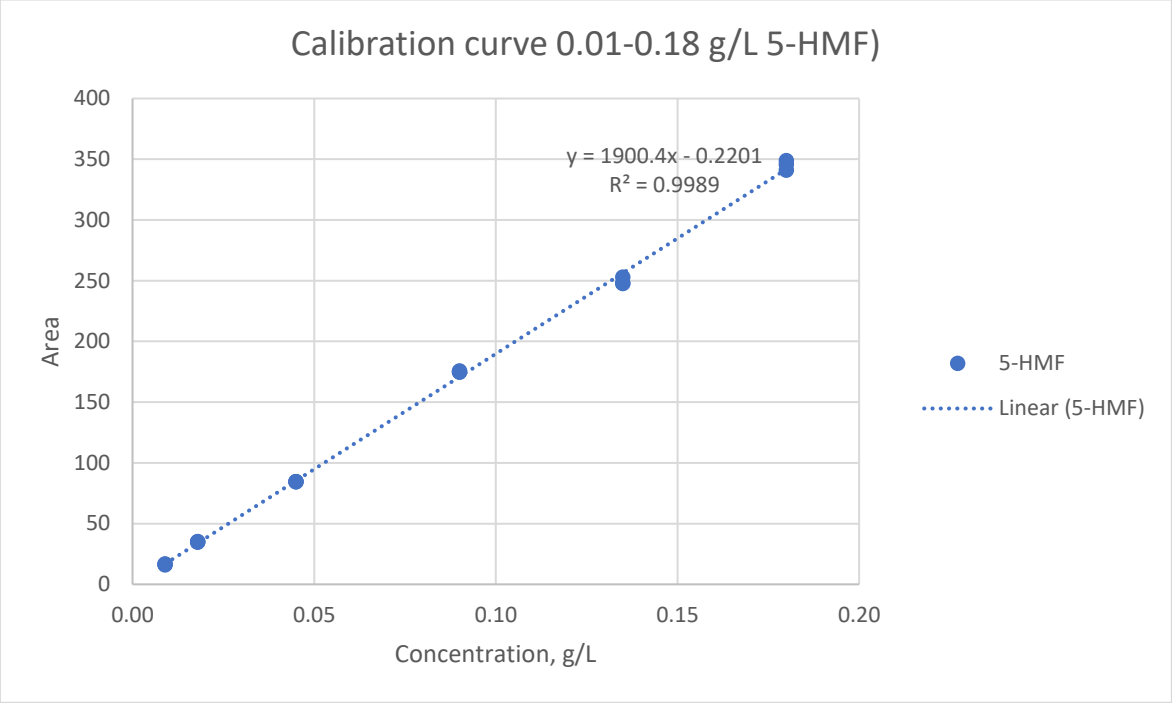
Antud töö eesmärgiks on arendada uus kõrgefektiivse vedelikkromatograafia meetod refraktiivse indeksi detektoriga, mis oleks asenduseks eelnevalt arendatud kapillaarelektroforeesi meetodile. Katsete käigus arvutati välja olulised tulemuslikkuse näitajad ning võrreldi mõlemad meetodid omavahel nende rohelisuse, efektiivsuse ja korratavuse hindamiseks.

Appendices

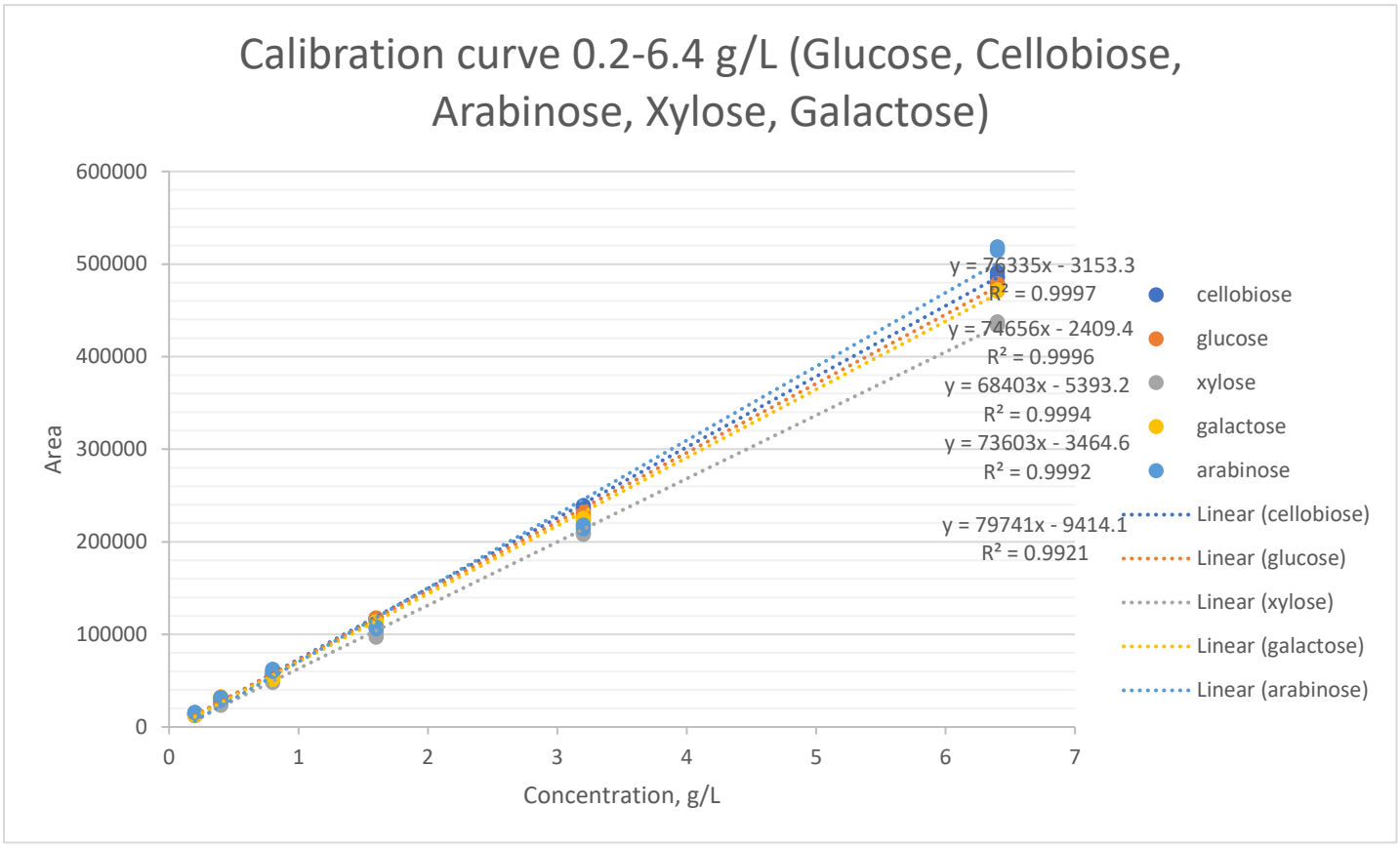
Appendix 1 5-HMF calibration curve for HPLC



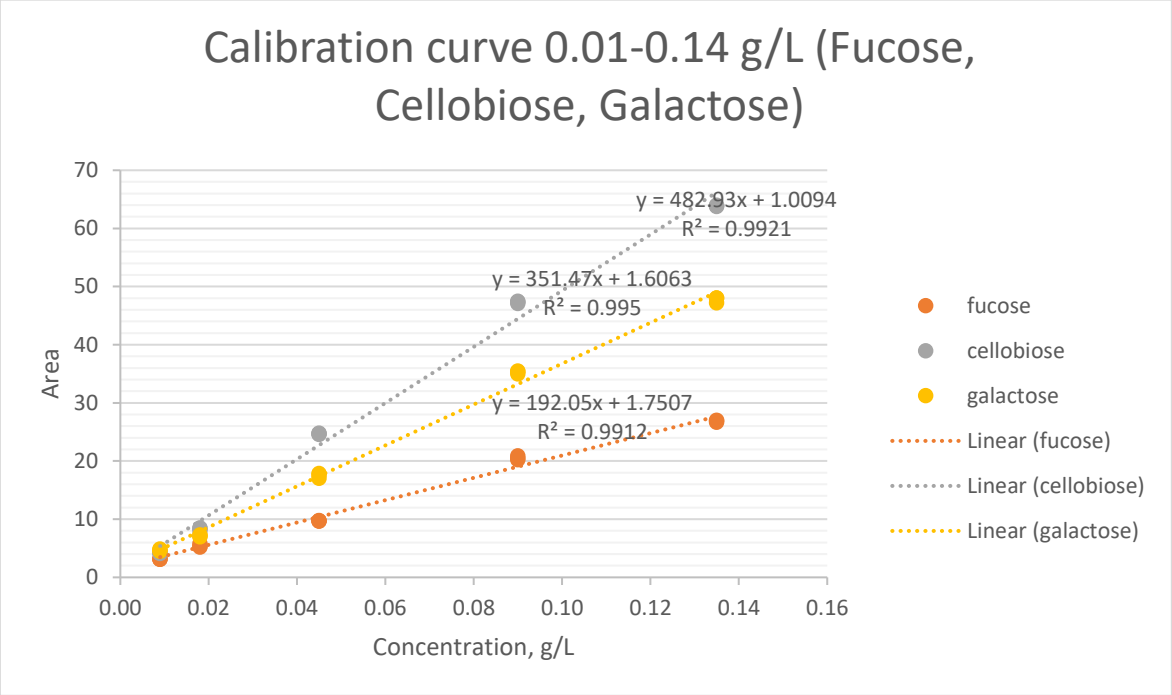
Appendix 2 5-HMF calibration curve for CE



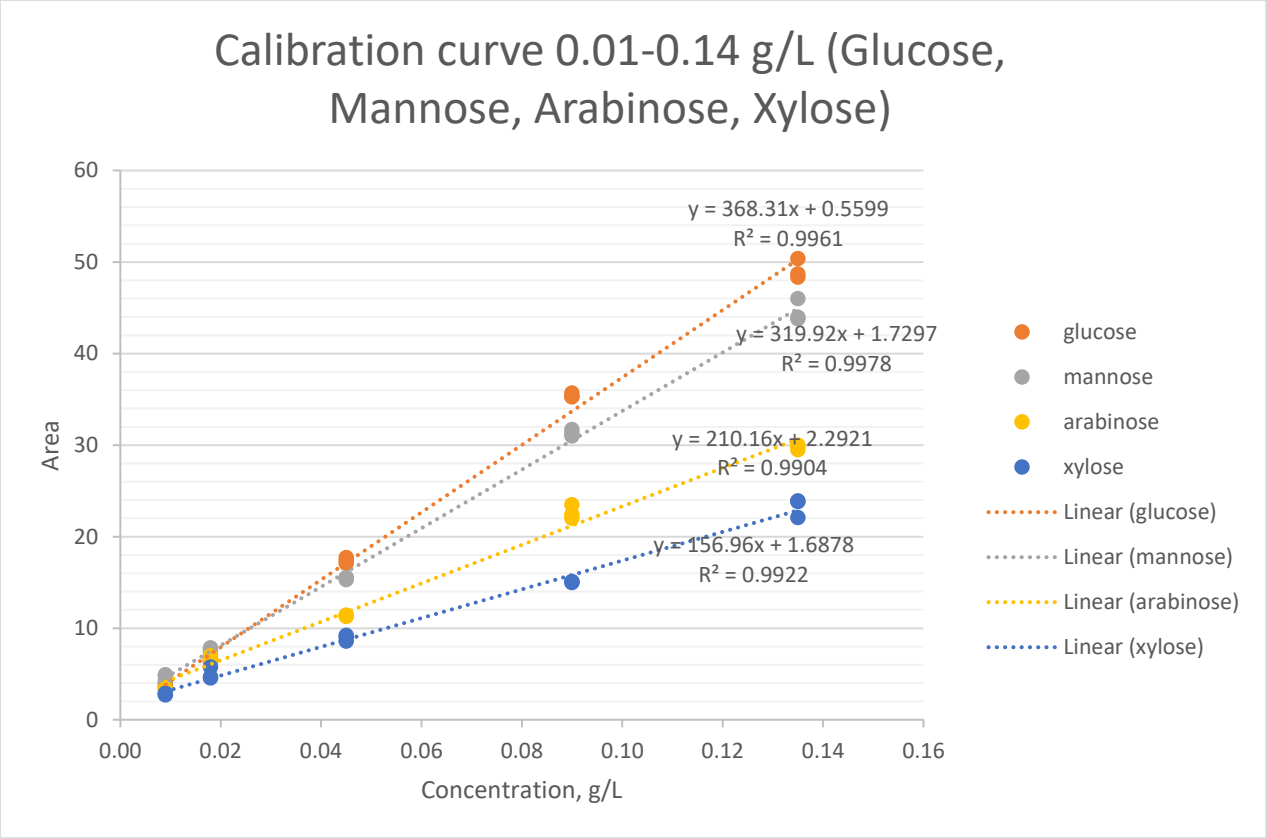
Appendix 3 Glucose, cellobiose, arabinose, xylose, galactose calibration curve for HPLC



Appendix 4 Fucose, cellobiose, and galactose calibration curve for CE



Appendix 5 Glucose, mannose, arabinose, and xylose calibration curve for CE



Appendix 6 Student's t table

Table 13. Student's t table *[64]*

cum. prob	$t_{.50}$	$t_{.75}$	$t_{.80}$	$t_{.85}$	$t_{.90}$	$t_{.95}$	$t_{.975}$	$t_{.99}$	$t_{.995}$	$t_{.999}$	$t_{.9995}$
one-tail	0.50	0.25	0.20	0.15	0.10	0.05	0.025	0.01	0.005	0.001	0.0005
two-tails	1.00	0.50	0.40	0.30	0.20	0.10	0.05	0.02	0.01	0.002	0.001
df											
1	0.000	1.000	1.376	1.963	3.078	6.314	12.71	31.82	63.66	318.31	636.62
2	0.000	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925	22.327	31.599
3	0.000	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.841	10.215	12.924
4	0.000	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4.604	7.173	8.610
5	0.000	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4.032	5.893	6.869
6	0.000	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3.707	5.208	5.959
7	0.000	0.711	0.896	1.119	1.415	1.895	2.365	2.998	3.499	4.785	5.408
8	0.000	0.706	0.889	1.108	1.397	1.860	2.306	2.896	3.355	4.501	5.041
9	0.000	0.703	0.883	1.100	1.383	1.833	2.262	2.821	3.250	4.297	4.781
10	0.000	0.700	0.879	1.093	1.372	1.812	2.228	2.764	3.169	4.144	4.587
11	0.000	0.697	0.876	1.088	1.363	1.796	2.201	2.718	3.106	4.025	4.437
12	0.000	0.695	0.873	1.083	1.356	1.782	2.179	2.681	3.055	3.930	4.318
13	0.000	0.694	0.870	1.079	1.350	1.771	2.160	2.650	3.012	3.852	4.221
14	0.000	0.692	0.868	1.076	1.345	1.761	2.145	2.624	2.977	3.787	4.140
15	0.000	0.691	0.866	1.074	1.341	1.753	2.131	2.602	2.947	3.733	4.073
16	0.000	0.690	0.865	1.071	1.337	1.746	2.120	2.583	2.921	3.686	4.015
17	0.000	0.689	0.863	1.069	1.333	1.740	2.110	2.567	2.898	3.646	3.965
18	0.000	0.688	0.862	1.067	1.330	1.734	2.101	2.552	2.878	3.610	3.922
19	0.000	0.688	0.861	1.066	1.328	1.729	2.093	2.539	2.861	3.579	3.883
20	0.000	0.687	0.860	1.064	1.325	1.725	2.086	2.528	2.845	3.552	3.850
21	0.000	0.686	0.859	1.063	1.323	1.721	2.080	2.518	2.831	3.527	3.819
22	0.000	0.686	0.858	1.061	1.321	1.717	2.074	2.508	2.819	3.505	3.792
23	0.000	0.685	0.858	1.060	1.319	1.714	2.069	2.500	2.807	3.485	3.768
24	0.000	0.685	0.857	1.059	1.318	1.711	2.064	2.492	2.797	3.467	3.745
25	0.000	0.684	0.856	1.058	1.316	1.708	2.060	2.485	2.787	3.450	3.725
26	0.000	0.684	0.856	1.058	1.315	1.706	2.056	2.479	2.779	3.435	3.707
27	0.000	0.684	0.855	1.057	1.314	1.703	2.052	2.473	2.771	3.421	3.690
28	0.000	0.683	0.855	1.056	1.313	1.701	2.048	2.467	2.763	3.408	3.674
29	0.000	0.683	0.854	1.055	1.311	1.699	2.045	2.462	2.756	3.396	3.659
30	0.000	0.683	0.854	1.055	1.310	1.697	2.042	2.457	2.750	3.385	3.646
40	0.000	0.681	0.851	1.050	1.303	1.684	2.021	2.423	2.704	3.307	3.551
60	0.000	0.679	0.848	1.045	1.296	1.671	2.000	2.390	2.660	3.232	3.460
80	0.000	0.678	0.846	1.043	1.292	1.664	1.990	2.374	2.639	3.195	3.416
100	0.000	0.677	0.845	1.042	1.290	1.660	1.984	2.364	2.626	3.174	3.390
1000	0.000	0.675	0.842	1.037	1.282	1.646	1.962	2.330	2.581	3.098	3.300
z	0.000	0.674	0.842	1.036	1.282	1.645	1.960	2.326	2.576	3.090	3.291
	0%	50%	60%	70%	80%	90%	95%	98%	99%	99.8%	99.9%
	Confidence Level										

Annex
to Rector's directive No 1-8/17 of 7 April 2020

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