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DETERMINATION CARBOHYDRATES IN PINE BARK SAMPLE BY CAPILLARY ELECTROPHORESIS

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The topic of the thesis focuses on the identification and quantification of carbohydrate in Pine Bark sample by Capillary Electrophoresis. The first objective of this thesis is research, establish and evaluate the key factors which influence the analysis process. Based on the main factors, the best analytical parameters are researched to produce high accuracy and efficiency result. The second main goal is determinate and quantify monosaccharides in the available extracted Pine Bark sample by the capillary electrophoresis equipment.

There are six monosaccharides that are predicted in Pine Bark samples: Glucose, Arabinose, Galactose, Mannose, Fructose and Xylose. Five sugars appeared in extracted bark sample with different amounts consists of Arabinose, Galactose, Fructose, Glucose and Xylose. Analytical procedures were performed by capillary electrophoresis with UV detector at wavelength 270 nm. The monosaccharides move through the electro osmotic flow (EOF) of the electrolyte solution which contains 130 mM NaOH 1M: 36 mM Na_2HPO_4 pH 12.65 (±0.02). In particular, the flow of EOF and the analyte are influenced by the voltage of 20 kV at 20 ° C. The migration time of all carbohydrates between 11 and 14 minutes with the RSDs is 2%. For quantification, the range of amount for calibration is 10 – 60 ppm. Pine Bark sample was extracted with hot water or ethanol, then sample was filtered and diluted 1:5 and 1:10 with de-ionized water before analysis by Capillary Electrophoresis. All the calculation of quantification was done automatically and described on the printed report.

Key words
Carbohydrates, Hemicellulose, Pine Bark, Capillary Electrophoresis, Analytical Chemistry.

TABLE OF CONTENTS

1 INTRODUCTION

Wood is a valuable resource with many applications in various fields such as pulp, construction, decoration and so on. Based on that, the wood industry is now continuing to research and development with extensive applications to other industries. According to experts, the wood industry is a promising development for the world economy in general and Finland in particular.

In the wood industry today, research on the chemical composition of wood is taking priority than others. The main chemical constituents of wood include cellulose, hemicellulose, lignin and extractives. Therefore, the sugar content in the wood is quite large and usually separated by extraction method. However, a large amount of carbohydrate in wood effects directly to several it's applications. At present, many projects are being launched primarily for studying methods of quantifying sugars after extraction. From there, the projects continue to test new methods in the extraction process to reduce or increase the amount of sugar in different application objectives.

In chemical analysis, capillary electrophoresis (CE) is considered a new and limited method. However, in the specific field of qualitative and quantification of carbohydrates, this is an extremely popular method with high efficiency. Together with the ion chromatography method, both methods are considered to be leading techniques due to their sensitivity and accuracy in the identification and quantification of molecules. The CE instrument is quite sensitive, and the detection level is also higher than the IC (Ion Chromatography). As a result, CE has a small deviation of molecular concentrations after the analysis process and produce accurate results.

The main topic of this thesis studies and develop the identification and qualification of monosaccharides by Capillary Electrophoresis. The main purpose of this thesis work is testing, evaluate and determine the best conditions of the electrolyte buffer, temperature, voltage and current, which is main factors on analyzing process, for the CE instrument to work and produce the most accurate results possible. In addition, the construction and set up of the calibration table in the CE control system is also an important goal to complete. The practice experiment of this thesis does the quantification of the available extracted Pine Bark sample. The discussion will summarize the assessments of the completed objectives.

2 THEORIES

2.1 The general structure and chemical composition of Pine.

2.1.1 The general structure

Pine is the name which represents for any tree belonging the largest family of conifers, Pinaceae. They usually grow up in northern temperature regions and have great commercial value. Basically, each pine divided into three main parts: the crown, the stem and the roots. Crown at the top which consists of branched and leaves to filter dust from the air and supply the food for a tree. Roots mission is absorbing and storing water and nutrients from the soil. The stem has four layers play an important role to transport foods which is heartwood, sapwood, cambium and bark.

The major chemical composition of pine is based on the common constituents of woody biomasses: cellulose, hemicellulose and lignin. The pine tree has the average values of moisture content approximate 40 -50% with total mass, remain part is dry wood. In addition, polysaccharides such as the cellulose and hemicellulose are supposed to account almost two-thirds of the dry wood mass. In Pine tree, the literature shows that these compounds (cellulose and hemicellulose) are only accounted 70% of the total chemical composition of a tree. Besides, a wide variety of non-structured or low-molecular-mass compounds could be found in a minor part which called extractives (water-soluble organics and inorganics). In common process, extraction technique can be used to separate those polysaccharides by using polar or non-polar solvent. (Räisänen and Athanassiadis, 31 January 2013.)

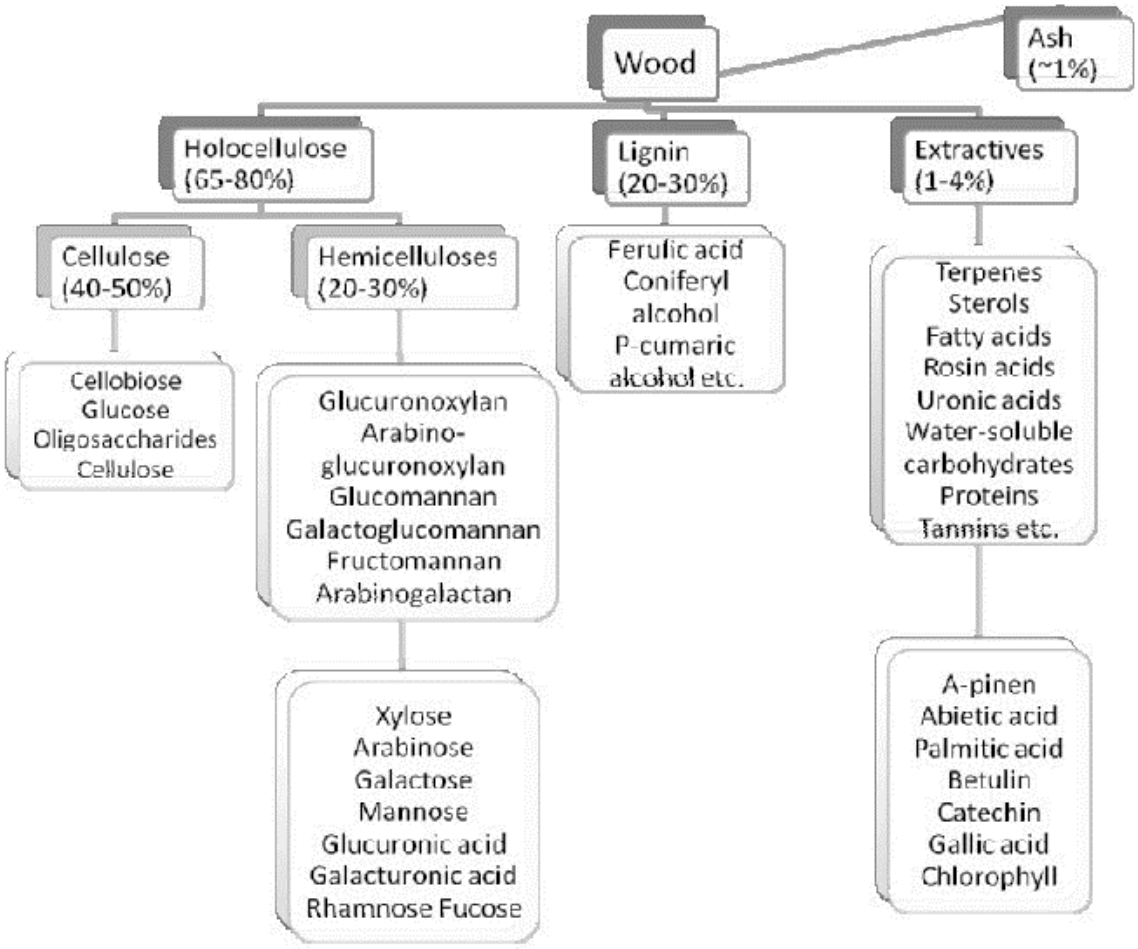


FIGURE 1: Chemical compositions of the pine wood (adapted from Azarov et al. 1999, p. 184).

Figure 1 describes the main constituents and its amount in softwood and hardwood in basic. The monomer sugars come from holocellulose part after some chemical treatment (extraction, degradation and so on). The difference between hardwood and softwood is hemicellulose composition and percentage. Softwood almost forms by hexatomic carbohydrate while pentatomic carbohydrate represents for hardwood. In Pine case, the main chemical structure has less holocellulose (65%) as well extractive (< 5) and more lignin (30%) while hardwood is opposite (Azarov et al. 1999).

Pine is known as an symbolize of softwood with their character is the thick and scaly bark. Therefore, the percentage of cellulose and hemicellulose in this layer is larger than other tree types. In fact, those compounds in Pine bark which accounts for a large percentage while comparing with

others. Besides, Pine bark contains more extractives and other constitutes such as condensed tannins and suberin. For example, in Scott Pine, 22 % and 8% of the total dry stem tree weight, which are cellulose and hemicellulose respectively contain in the bark. (Räisänen and Athanassiadis, 31 January 2013.).

2.1.2 Chemical composition of Pine bark

Cellulose is the most common natural polymer in the world. It is formed by multiple of β -D-glucopyranose units which are linked by β-(1 → 4)-glucosidic bonds. These links can be destroyed then decompose cellulose into single units. In Pine bark case, it is believed that in holocellulose, cellulose content is predominant. Figure 2 shows the structure of cellulose as well its glucose units linked by β-(1 → 4)-glucosidic bond which presents by the red line (M. Rowell; Pettersen; and A. Tshabalala, 2013, p.35)

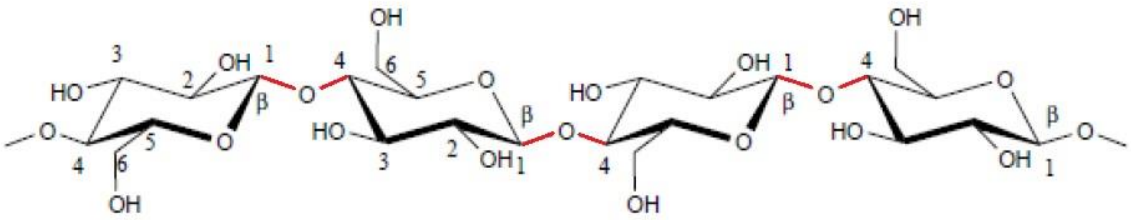
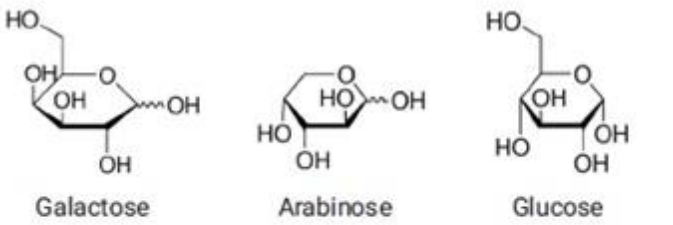


FIGURE 2: Chemical structure of cellulose. (adapted from Laine 2005, p. 15)

In general, hemicellulose in pine bark consists group polysaccharide polymers which containing mainly the sugars D-xylo-pyranose, D-glucopyranose, D-galactopyranose, L-arabinofuranose, D-mannopyranose, D-glucopy-ranosyluronic acid, and D-galactopyranosyluronic acid with minor amounts of other sugars. According to the literature reviews, the structure of hemicellulose in bark can be figured out by first determination of monomer units of those mono-sugars: D-glucose, D-mannose, D-xylose, L-arabinose and D-galactose. The chemical structure of those carbohydrates is shown in Figure 3. (Sjöström 1981, p. 60). In Pine or softwood case, it is believed that hemicellulose contains more mannose and glucose units whereas xylose is the main constituent in hardwood. Besides, acetyl- and methyl-substituted group exist inside the softwood



as well.

FIGURE 3: The chemical structure of monosaccharides (adapted from Claudimir L. L. and Fernando S. L., 2018)

Hemicellulose in the Pine bark mainly contains Galactoglucomannans (GGM) which accounts for 65% of total polysaccharides. GGM are formed by mannose, glucose and galactose units with the ratio 3 - 4 : 1: 0.1- 0.2 by β-(1→4)- and α-(1→6)- bonds. The former links mannose and glucose and the second is mannose and galactose bonds. The figure 4 shows the chemical structure of GGM with the red part performs mannose units, green is galactose sugars and the blue one is glucose units. (Per Stenius, 2000, p31-37)

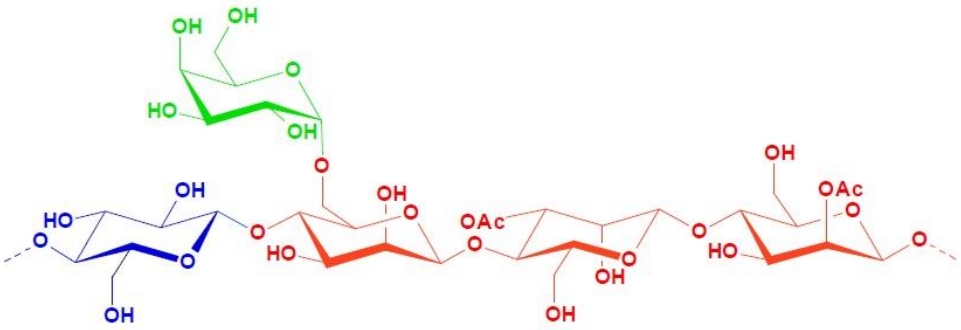


FIGURE 4: Chemical structure of O-acetyl-galactoglucomannan (GGM) compound. (adapted from Jens Krogell, 2015.)

In addition, the other important component is Arabinoglucuronoxylan (AGX) (5 – 10% of the total polysaccharides amounts). AGX has xylose units were linked by the β-(1→4)- bonds on the main branch while α-(1→2)- and α-(1→3) –bonds which link with Glucose and Arabinose units respectively with the main branch. In addition, the ratio between three main component arabinose, glucuronic acid and xylose is 1:2:8. The xylan constituent in softwood differs from hardwood cause without acetyl groups. Besides, the other composition, Arabinogalactan accounts for less than 1% of the total wood dry mass which its main sugars are arabinose and galactose units. (Per Stenius, 2000, p31-37.)

Figure 5 shows the chemical structure of Arabinoglucuronoxylan with the β-(1→4)- bonds is red lines and the α-(1→2)- and α-(1→3) bonds are green and blue line respectively.

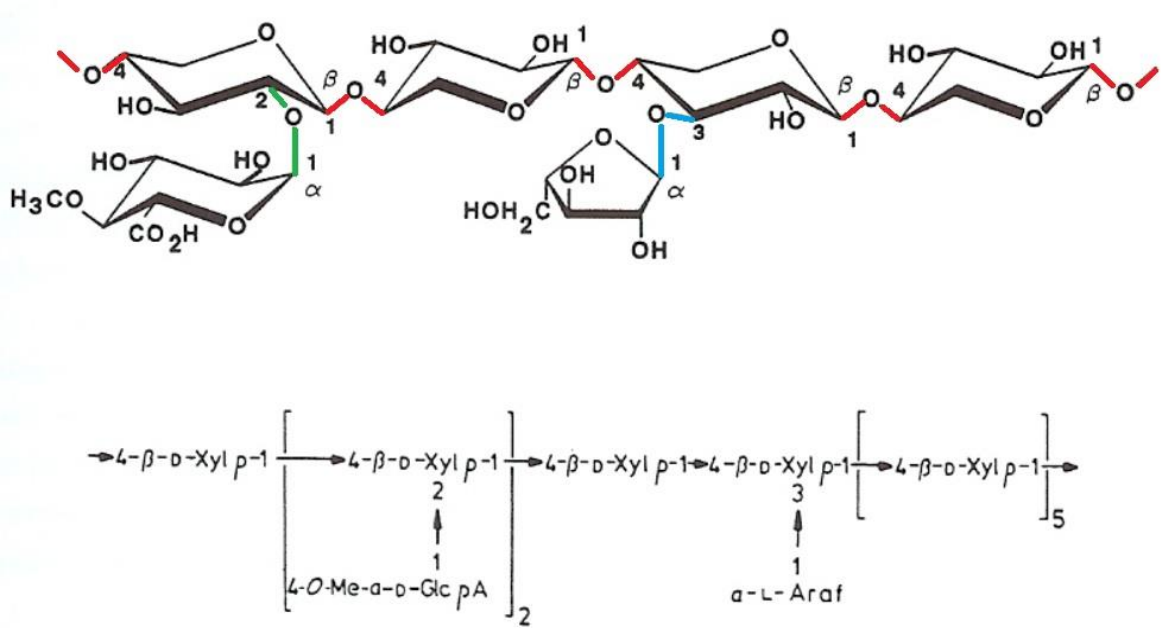


FIGURE 5: The chemical structure of Arabinoglucuronoxylan. (adapted from Per Stenius, 2000, p31-37.)

In Pine bark, xyloglucan has already known as a compound accounted for a small percentage. The figure 6 shown the structure of xyloglucan which contain sugar units and their bonds.

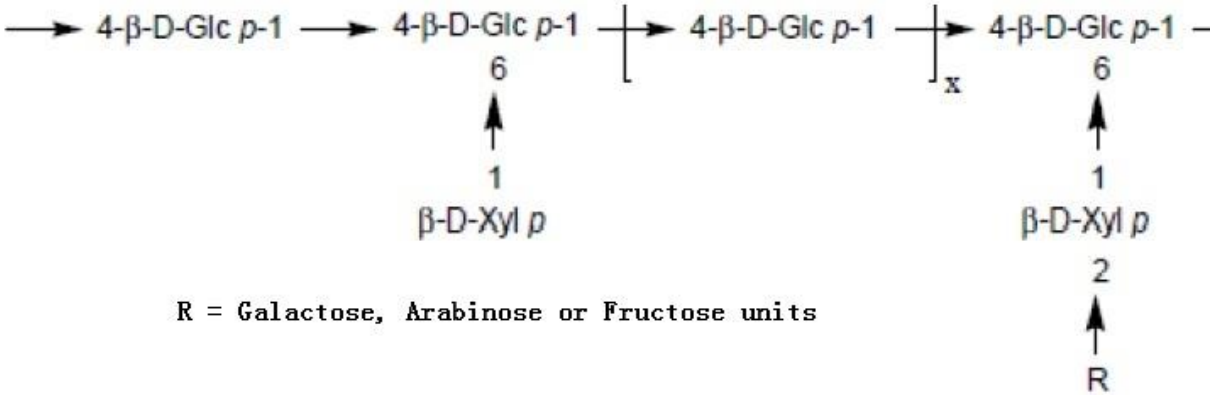


FIGURE 6: Structure of Xyloglucan (adapted from Laine, 2005, p.17)

The xyloglucan is structured by Glucose units linked by β-(1→4)- on the main branch. Furthermore, the side branches have only single xylose units by β -(1→6)- bonds. In some case, the galactose, arabinose or fructose units link to xylose by -(1→6)- bonds. (Gladyshko, 2011)

Lignin and extractives also play an important role in Pine bark operation mechanism. Polysaccharides microfibrils are bonded together by aromatic polymer. Lignin acts as "glue" between cellulose and hemicellulose so it is difficult to extract lignin from bark. In addition, lignin is supposed to be formed by several thousand units (phenylpropanoids) linked together by carbon-carbon and carbon-oxygen bonds. (Laine, 2005).

Besides, extractive or known as natural chemical compounds which soluble in several solvents such as water, methanol, acetone and so on. In addition, extractives of bark Pine consists of free sugars which could be released by hydrolysis in hot water extraction. Its constituents mainly are glucose and fructose. In some case, arabinose is the most abundant compound. (R. Rowell, 2013) Furthermore, Pine bark have larger percentages of extractives than other parts of the tree. Other chemical compounds in extractives are divided into several groups (oil, resin and water-soluble) while using a separating technique. (Laine, 2005.)

Basically, in sapwood, the abundant composition is cellulose while the percentage of hemicellulose, lignin and extractive are lower (42.5%, 28%, 24.5% and 0.2% respectively). It is believed that the lignin would have been linked with carbohydrate by the hydrogen bonding in the sapwood structure. Then, it forms a cellulose-hemicellulose-lignin matrix in the cell wall, a layer

of the sapwood. The sapwood after extracting is supposed to have more glucose carbohydrate than others. (Nacera B., Djamel A., Andrey P. & Stefan W., Mar 2018)

2.2 Extraction process of Pine bark

Currently, several extraction techniques of Pine bark are used frequently and continue researching as well as developing. Some popular and basic methods are hot water, ethanol, hexane and acetone extract. Besides, there are other complicated techniques by combining those ones such as the hot water-hexane (HWH) and hot water-ethanol (HWE) extracts. All methods were carried out with aqueous solutions at distinctive pH, time and temperature. (P Reyes, RT Mendonça, MG Aguayo, J Rodríguez, B Vega, P Fardim, 2013).

Water source is absolutely known as renewable energy. Therefore, it is believed that water as a catalyst in hemicellulose extraction is a promising method at this time as well as in the future. The advantage of the hot water extraction method is the non-toxic catalyst, limited corrosion of equipment than acid-catalyst and cheaper than other methods. Pressurized hot water extraction (HWE) method in this experiment was carried out in the Soxhlet apparatus (Figure 7) with the temperature reaches the solution's boiling point for the best result hemicellulose yield. However, the temperature and pH are possible to adjust highly depending on the material used as well as the product desired. (Krogell J., 2015).

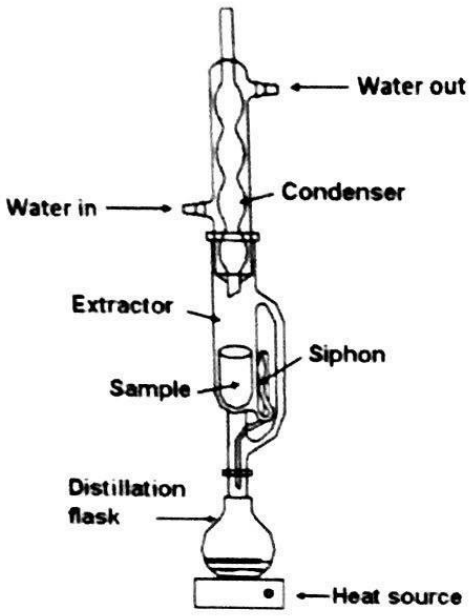


FIGURE 7: Soxhlet extraction apparatus (CentriaTKI,2018)

Monomer sugars of holocellulose in general and hemicellulose in particular, which is separated by hydrolysis phenomenon. In fact, not only carbohydrate but other valuable by-products (acetic acid,

furfural, HMF etc.) also were dissolved by this chemical reaction. (Garotte et al., 2001). The mechanism of chemical hydrolysis is the breakable of glycosidic bonds in polysaccharides by the catalyst then forms the monosaccharides (glucose, mannose, arabinose, xylose and galactose). (Roger M. Rowell, 2013.)

Figure 8 describes the water extraction of cellulose and hemicellulose fraction. The extraction of Pine Bark is divided into three stages. The hemicellulose is separated first by reacts with the solvent applied (hot water and ethanol). The solution contains hemicellulose is filtered and then freeze-drying.

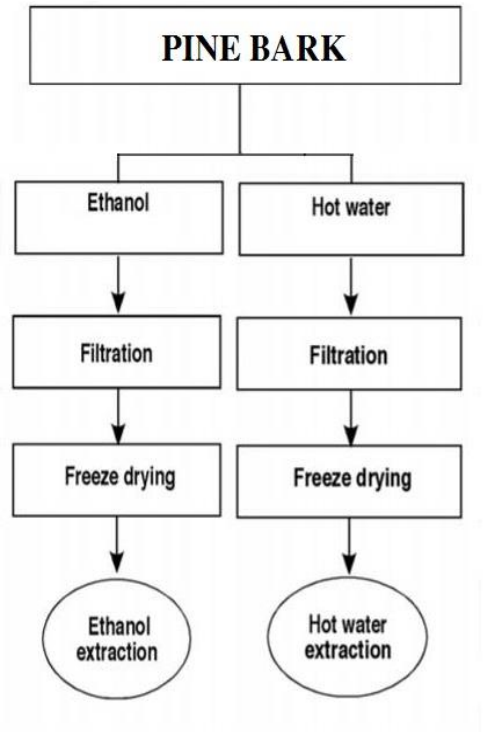


FIGURE 8: Four Stages of Extraction process. (adapted from Roger M. Rowell, 2013.)

The mechanism of HWE method is explained simplify by three sections. First of all, water flows in the secondary cell wall to approach exactly the place hemicellulose located. Then, hemicellulose is dissolved from the complicated matrix of wood structure to the aqueous phase. Finally, the hemicellulose in water phase is diffused from the wood into the water outside and prepare for the isolation or utilization. (Roger M. Rowell, 2013.)

Isolation and utilization hemicellulose from water solution is carried out by several methods such as are ethanol precipitation, filtration, film evaporation. According to Krogell, filtration and ethanol precipitation are the most common methods for this section treatment of hemicellulose. Insides that, the advantage of filtration is cheap in industrial scale while ethanol precipitation method is the opposite. However, the product from ethanol precipitation has the better quality of hemicellulose whereas some impurity remains in solution after filtrate. (Roger M. Rowell, 2013.)

2.3 Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) is a technique of analysis ions based on their different capacity of moving rate (mainly by their charge) in electrolyte solution (pH buffer), under the influence of the high applied voltage, a certain E (electromagnetic) and the electro-osmotic flow (EOF). (Daniel C. Harris, 2007)

2.3.1 Configuration of the CE instrument

Analyzer equipment employed in this work is Hewlett-Packard HP3DCE. Basically, CE system is shown in figure 9 which consists of the following components:

Separated capillaries are usually made of silicon (the most common capillary), Teflon or PEEK, with an outer diameter (OD) of 365 μm, an inner diameter (ID) of 10 to 150 μm (most commonly 50 μm). Total capillary length can be from 10 to 100 cm (usually 60 cm). During electrophoresis, the capillary is filled with a buffer solution. This work was tested on capillary 56 cm x 50 μm. (Hewlett Packard, France, 2012)

Electrode Buffer: Used to create an electrophoresis environment, which occurs when high pressure is applied to the capillaries. Noted that the two vials at the two ends of the capillary have the equal volume. (Hewlett Packard, France, 2012)

High voltage power supplies typically range from 5 to 30 kV, which is applied to the capillary to produce a large electric field for the electrolyte. For analyzing the cation, the positive voltage is

used and on the contrary, to analyze the anions, the negative current is applied at both ends of the voltage. (Hewlett Packard, France, 2012)

Detector: The detector and signal detector of the analyte after capillary electrophoresis are usually located at the end (near the end or end) of the capillary depending on the type of capillary. There are many types of sensors used for different purposes. In this experiment, the UV-Vis sensor was used. (Hewlett Packard, France, 2012)

The UV-VIS detector is the most commonly used detector used to detect quantifiable compounds that absorb light at wavelengths in the UV-VIS. The UV-VIS detector consists of two types: the first uses monochromatic rays, detects and quantifies individual substances; The second type uses a diode array, detecting multiple substances simultaneously, each absorbing a certain wavelength. (Hewlett Packard, France, 2012)

Controls system: Usually the computer uses the appropriate software, to record, display and process the analysis results.

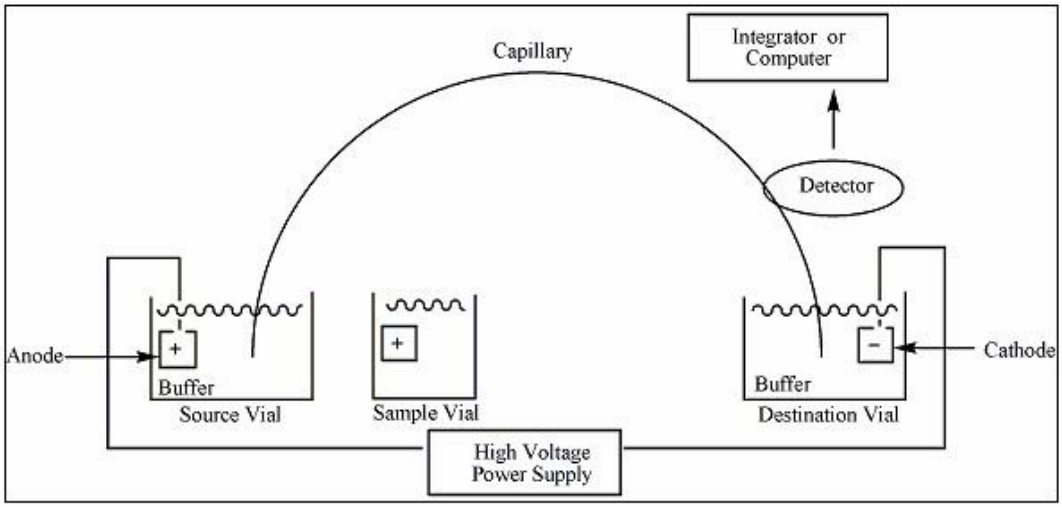


FIGURE 9: Capillary electrophoresis system (adapted from Hewlett Packard, France, 2012)

2.3.2 Principle

Electrophoretic Mobility, μ: is defined as the migration rate of ions (cm/s) per unit electric field strength (usually V/cm) of a charged particle in electrophoresis. (Abhilasha Shourie and Shilpa P. Chapadgaonkar, 2015, p.184). The greater voltage, the faster migration rate of ions. (Hewlett Packard, France, 2012)

Electro-Osmotic Flow (EOF) is formed while a high voltage applied into capillary filled up electrolyte solvent, which has a convenient condition for the ionization of molecules and has the pH based on their pKa. (Altria, Kevin D., 1995.). Figure 10 describes clearly the structure layers inside the capillary which is working with the buffer. While the electrolyte solution is placed inside the silicate capillary under the pH greater than 2.5, the negative charge silanoate group (SiO-) is formed and create a layer in the capillary wall. Then, it attached the positive charge cation of the electrolyte solution, which forms a stable inner layer and one outer layer. The outer layer moves freely inside the capillary and starts to be pulled in the cathode (-) when the electric field is applied. While the pH value is increased, the EOF flows faster and reach the maximum peak at very high pH value. (Hewlett Packard, France, 2012)

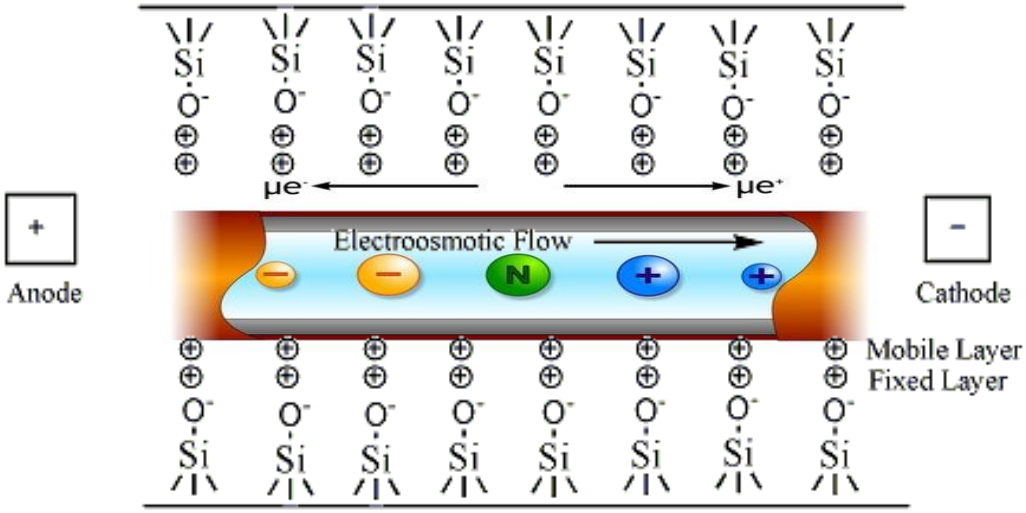


FIGURE 10: The structure of layers inside the capillary while the electrolyte buffer is applied (adapted from Skoog, D.A.; Holler, F.J.; Crouch, 2007.)

Electro-active mechanism*:* Under the effect of Electric Field Force (EFF) and Electro-Osmotic Flow (EOF), ions have differences in electrophoretic mobility, will move to the electrode at different velocity and then separate from each other. In addition, their volume, size and flexibility also effect to the electrophoretic mobility. Simulation of CE instrument operation is described in the figure 11. (Hewlett Packard, France, 2012)

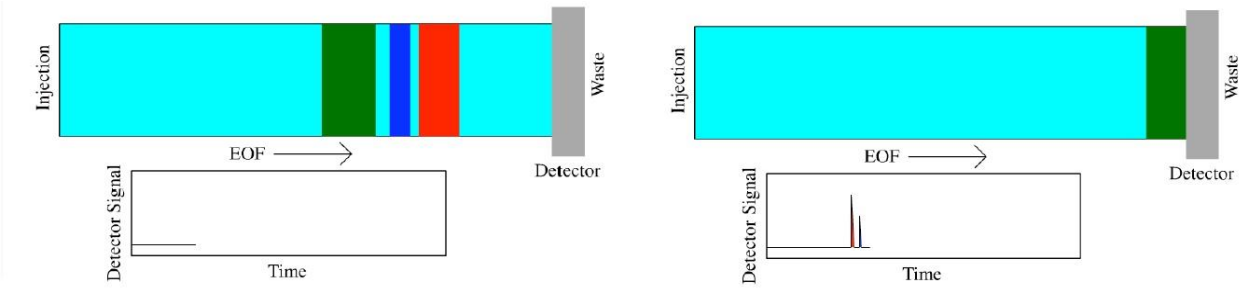


FIGURE 11: CE instrument operation. (adapted from Geraldine Leonard, 2016.).

Three blue, green and red regions flow under the EOF inside the capillary from the left to the detector. While a region moves through the detector, its migration time is recorded on the electropherograms as a peak. The height of this peak represents for the absorption of the ion while the peak's area performs its amount. (Geraldine Leonard, 2016.).

Due to Electro-Osmotic Flow (EOF), buffer compounds move to the cathode at a rate of up to 2 mm / s which depends on the pH, viscosity of the medium and is usually greater than the rate of ion mobility. In the CE, at the beginning, the cation was attached toward negative electrode and the anion is affected by positive electrode. However, while the electrolyte solution is applied, all ions move from the anode towards the cathode normally, but in some case, it is reserve flow. The smaller size (charge) of ions, the faster migration rate. In addition, the negative charge ions move slower than the positive one. (Geraldine Leonard, 2016.).

Depending on the purpose of analyzing anion or cations, the voltage applied to the electrodes is different. In the cationic analysis case, the positive voltage is used. Thus, the positive ions will shift faster, the neutral ions will move according to the velocity of the EOF while the negative ions

will move slowly. Then, those ions move through the detector by different migration time, which are detected and recorded as electrophysiological data. (Hewlett Packard, France, 2012)

In this work purpose which is separating carbohydrate case, the positive voltage is applied with the buffer alkaline condition (pH > 12) as EOF because the hydroxyl group is weak acids, which is ionized in this solution.

2.3.3 Advantages, disadvantages and applications

The use of capillary has many advantages while comparing with other analytical methods. The first thing is CE technique has a simple structure as well as compact form. Then, the cost of the CE method is cheaper than others. Second, the working process is fast, automatically and control a whole time by a computer system, which is convenient for technicians. All the analyzing work is done with a very small amount of sample and other required chemicals. Furthermore, the result of CE is high quality, effective and resolution. (Frazier, Richard A, et al.2007.)

On the other hand, there are still disadvantages occurred while using CE technique. Basically, this method is worked for analyzing ionized-molecules. Therefore, the neutral substances could be unidentified. Another important weaker point of CE, while comparing to other techniques, which is Joule heating. In this case, the high voltage is applied to increase the speeds of EOF, but the buffer solution is evaporated by the heat. This phenomenon may destroy and denature the molecules. In addition, CE technique is extremely sensitive when analyzing ions. In some case, the recording from the buffer solution would have much noise and effect to the peak identification of ions in the electrograph. Furthermore, the detector could identify strange ion and present some unknown peak which gives the incorrect result. (Daniel C. Harris, 2007).

CE method has many applications in industrial technology fields such as food nutrient, wood (analyzing carbohydrates), biochemistry forensic (DNA profiling, Protein identification), metal industry (inorganic anion and metal cation), cosmetic and pharmacies (analysis of anti-HIV drugs). Because of the sensitively of this technique, food industrial application is focused on researching and developing the most. The infinite possibilities and benefits of carboxylic acid analysis in wine and carbohydrates in yoghurt, milk and fruit juices of the CE method are highly appreciated by

experts. This shows that the potential of this analytical technique is considerable and is being studied more in the future. (Daniel C. Harris, 2007).

2.4 Quantification of carbohydrates

In analytical chemistry, a substance in unknown concentration sample is quantified by a calibration curve or known well as a standard curve. The calibration curve represents for the analytical signal of the instrument. This curve is built on the range of concentration close to the substance's expected amount in the sample. Basically, the data on the curve would fit on the straight line, which is described by the linear equation: y = mx + yo. The concentration of a substance in sample is calculated based on the x value in this equation. In some case, the calibration curve is described by the quadratic equation. (Daniel C. Harris, 2007)

Limit of detection (LoD) is defined as the minimum ions amount which can be detected in the test sample. Limit of quantitation (LoQ) – the lowest concentration of ions which is detected automatically with an acceptable repeatability as well as available relative standard deviation (RSDs). Both could be determined by the calibration curve or based on the calculated ratio between the peak and the noise from electropherogram. (Daniel C. Harris, 2007)

In quantification, the relative standard deviation (RSDs) value plays an important role in the calculated result. In some situation, this factor effects directly to the accurate measurement of the analytical instrument or technique. The lowest the RSDs, the higher the accuracy. RSDs is a descriptive statistics value used to measure relative volatility of a set of data with different mean values. This factor is calculated by dividing the standard deviation to the mean, which is performed by the equation (Daniel C. Harris, 2007)

$$S = \sqrt{\frac{\Sigma (x-X)^2}{N-1}} \qquad \text{(Daniel C. Harris, 2007)} \qquad (1)$$

$$RSD\ (\%) = \frac{S * 100}{X} \qquad \text{(Daniel C. Harris, 2007)} \qquad (2)$$

Where, x is mean values, N represents for the count of mean values, S is Standard Deviation value and X presents mean of the data.

4 PRACTICAL EXPERIMENT

This practical work aims to research and develop the determination of mono-saccharide in Pine bark sample by capillary zone electrophoresis method (CZE). This part describes the most suitable condition parameters about voltage and temperature to identify the signal peak of six carbohydrates.

4.1 Chemicals and Reagents

Table 1 shows all of chemicals and reagents which were used in the experiment for determination of the monosaccharides in Bark sample. The 10 % Acetic acid is prepared by diluting the 99% concentrated Acetic acid. The water is used in this work is de-ionized water, which was supplied by the purification system (Millipore UHQ) of Centria Research and Development.

Compound	**Brand Production**	**State**
D-(+)-xylose 99%	Acros Organic Co. - USA	Solid
D-(+)-galactose 99%	Acros Organic Co. - USA	Solid
D-(+)-mannose 99%	Acros Organic Co. - USA	Solid
D-(+)-glucose 99%	CentriaTKI - Finland	Solid
D-(+)-fructose 99%	CentriaTKI - Finland	Solid
D-(-)-Arabinose 99%	Alfa Aesar, GmBH - Germany	Solid
Disodium hydrogen phosphate dihydrate $Na_2HPO_4.2H_2O$	Merck - Germany	Solid
Acetic Acid 10% CH_3COOH	Merck - Germany	Liquid
Sodium hydroxide NaOH 1M	Fluka – Switzerland	Liquid
Hydrochloride Acid HCl 1M	Fluka – Switzerland	Liquid
De-ionized water	CentriaTKI - Finland	Liquid

TABLE 1: Materials of the analyzing carbohydrate in Pine Bark experiment.

4.2 Preparing carbohydrate stock samples and Electrolyte solution

The 500 mM $Na_2HPO_4.2H_2O$ was prepared by weighing 4.472g and diluted to 50ml de-ionized water. Then, 1.3 ml NaOH 1M and 0.72ml $Na_2HPO_4.2H_2O$ 500 mM was added into 10ml volumetric flask. The de-ionized water was fill up to the mark. The final electrolyte solution contains 130 mM NaOH and 36 mM $Na_2HPO_4.2H_2O$. The electrolyte solution was held on a table at the room temperature about 30 minutes before pH was adjusted.

Then, the solution was adjusted to pH 12.65 ($\pm$ 0.02) by drops of hydrochloride acid HCl 1M. The pH value is measured with 826 pH mobile Metrohm device at exact temperature 20^{o}C. After that, the solution is filtrated by 0.2µm Syringe Filter (Phenex-RC). Then, the solution is added into the vial by 1.8 cm of height. The electrolyte buffer is prepared daily for analyzing work. Noticed that both of buffer vials should have the same level of liquid for analyzing.

The 1000ppm stock solution of each carbohydrate was prepared into de-ionized water. After that, the standard of each carbohydrate is diluted by de-ionized water, which is calibrated at the concentration range of 10 – 60 ppm.

Compound	**Molecular formula**	**MW(g/mol)**	**Concentrate**	**pKa**
Arabinose	$C_5H_{10}O_5$	150.1	1000 ppm	12.43
Fructose	$C_6H_{12}O_6$	180.2		12.06
Galactose	$C_6H_{12}O_6$	180.2		12.35
Glucose	$C_6H_{12}O_6$	180.2		12.35
Mannose	$C_6H_{12}O_6$	180.2		12.08
Xylose	$C_5H_{10}O_5$	150.1		12.29

TABLE 2: Molecular formulas, molar masses, pKa values of six carbohydrates researched.

50mg of each carbohydrate was weighed and diluted to 50 ml of de-ionized water, which form 1000 ppm stock solution of each sugar. The stocks should be stored in the fridge at 4^{o}C. Fresh solution should be prepared after every two weeks. The carbohydrate is calibrated at range concentration of 10-60 ppm was prepared by diluting 1000 ppm standard in de-ionized water.

Firstly, 1 ml of 1000ppm standard was added into 10 ml volumetric flask and fill the water up to the mark. Then, 1 ml of diluted solution and 1.5 ml of electrolyte buffer were added into 10 ml volumetric flask to make 10 ppm standard sample. (3 ml and 6 ml respectively for 30 ppm and 60 ppm standard sample). The calibration sample is stability by this way. Then, the stocks solution is added into vials by 1.8cm of height. All the diluted solution which placed at the room temperature for 20 minutes. Calibration work should be done once a week and the new stocks must be prepared for this case.

4.3 Preparing Pine Bark sample

5 – 10 g of dry basic bark sample is weighed in extraction thimble. Then, it is diluted with 50ml de-ionized water and transferred into the 250 ml flask. Then, a small amount of water or ethanol is added to the extraction solution. The solution is extracted by the Soxhlet apparatus at 45^{o}C, 80^{o}C or 100 ^{o}C for 60 minutes. After that, the extracted solution is filtered by vacuum filtration. The extracted sample after filtrated which had light yellow color. The extracted sample is freezed or stored in the fridge at 2^{o}C.

The extracted sample is diluted by ratio 1:2, 1:5 or 1:10 (v/v) by de-ionized water. After that, the diluted solution is filtered again by 0.2 µm Syringe Filter (Phenex-RC). The electrolyte buffer is noticed that impossible to add in the sample while analyzing by CE. Then, the sample is added into a vial by 1.8 cm of height. Each of sample is analyzed three times. The figure 12 extracted sample before and after filtration

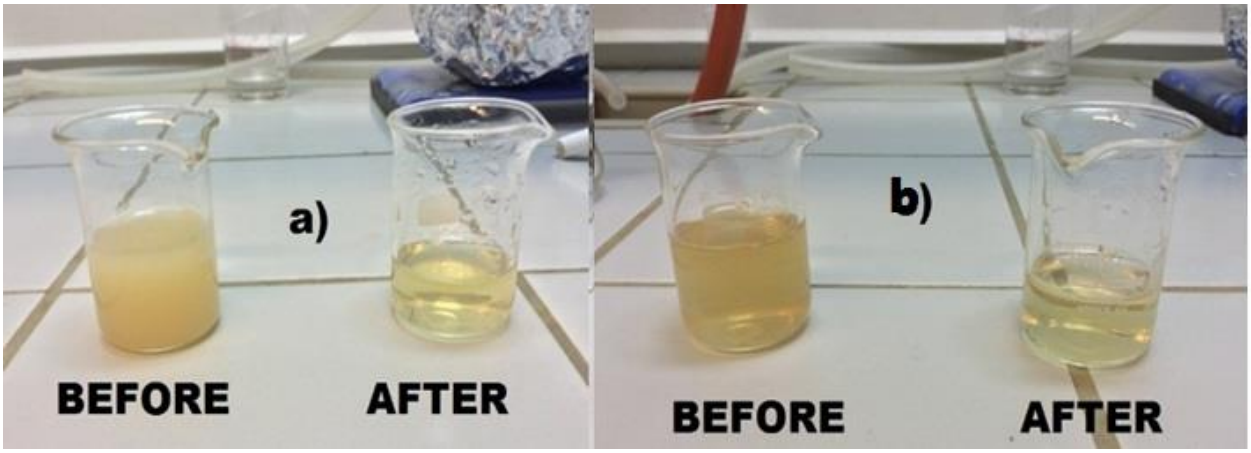


FIGURE 12: The extracted sample before and after filtrated by 0.2µm Syringe. a) ~5g extracted by ethanol. b) ~5g extracted by hot water.:

4.4 Capillary Electrophoresis parameters

The analyzing carbohydrate in Pine bark for this work which was performed by instrument $HP^{3D}CE$ with the UV detector. The direction UV detector was set up at wavelength 270 nm and a bandwidth at 10nm. In this experiment, the dimension of capillary is 56 cm x 50 µm. the temperature was stable at 20^{o}C. The voltage applied is 20 kV for the separation.

Complete flushing of the capillary is necessary done once a week with deionized water, hydrochloric acid, water, sodium hydroxide, water by order within 5 min each turn. Then, the pre-conditioning of CE is set up with rinsing 0.1 M sodium hydroxide (10 min), de-ionized water (10 min) and the buffer solution (15 min) daily. Between each run, the capillary was rinsed with de-ionized water (4 min), 10% Acetic acid (3 min), de-ionized water (3 min) and buffer (4 min).

The injection starts with electrolyte buffer which Voltage 16 kV is applied, then sample is injected with pressure 35 mbar for 10 seconds and the electrolyte solution by the same pressure in 10 seconds. The working process of sample lasts 15 minutes. The current could be oscillated from 180 to 190 µA and the power is approximate 3.5 W.

5 RESULS

5.1 Identification of monosaccharides

Six of carbohydrates were analyzed under the standard condition (20kV and 20°C) and the electrolyte solution consists of 130 mM NaOH and 36 mM Na_2HPO_4, pH 12.65 (± 0.02).

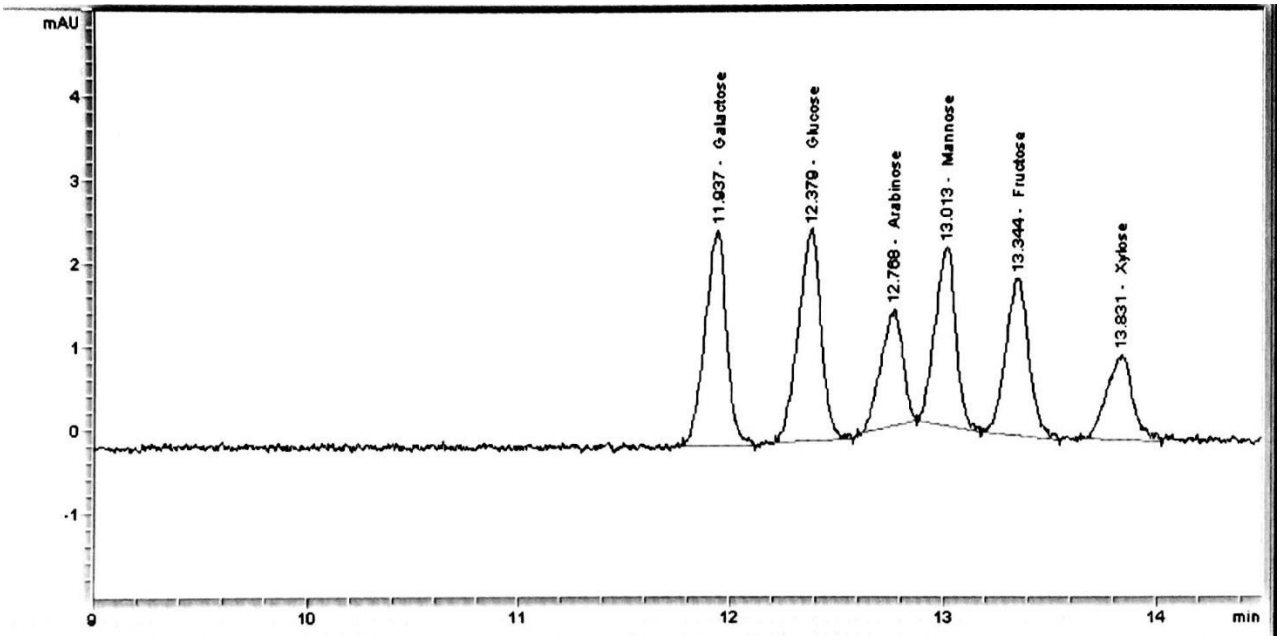


FIGURE 13: The electropherogram of carbohydrates 60 mg/l, which analyzed under the EOF pH 12.65, was detected at wave length 270 nm and the bandwidth is 10.

The migration of carbohydrate standards are shown in the figure 13 by order: Galactose, Glucose, Arabinose, Mannose, Fructose and Xylose. The migration times are listed on the top of each peak. The amount of each carbohydrate (or sugar) is 60ppm (mg/l).

5.2 Calibration and Limit of Quantitation.

All carbohydrates which was analyzed in this work would have their individual calibration graph. The electrolyte solution for all calibration of six sugar have the concentration of composition which corrects with the standard condition. The details of migration time (min), amount of standard (mg/l), Peak area, linearity equation, quadratic equation and coefficient (R^2) is presented clearly and totally in the table and figure below. Table 3 and figure 14 show the values in Galactose calibration. The range concentration of Galactose standard is 10 – 60 ppm.

Migration Time	Average MT	RSD (%)	Compound	Amt (mg/l)	Area
11.901	11.920	0.13	Galactose	10	4.197
11.924				30	11.357
11.937				60	19.230

TABLE 3: The amount and peak area at 11.920 minutes of Galactose.

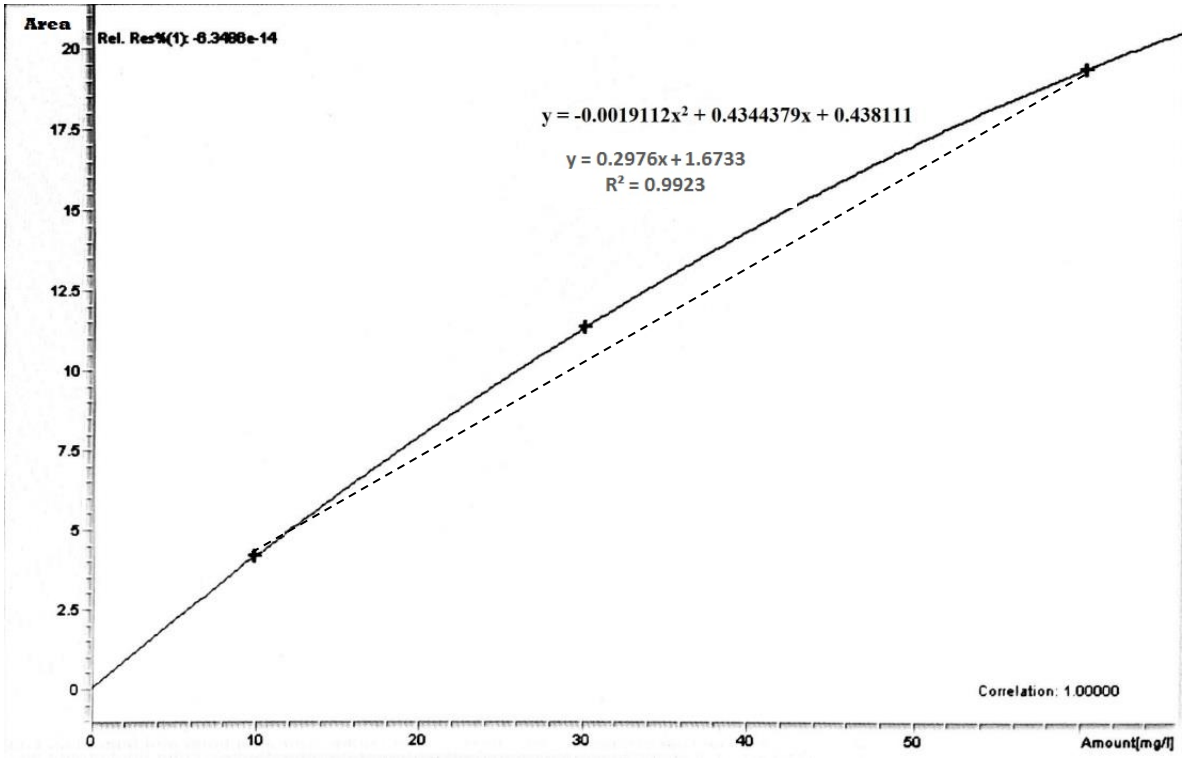


FIGURE 14: The linearity and coefficient of Galactose analyzing.

Table 4 and figure 15 show the values in Glucose calibration. The range concentration of Glucose standard is 10 – 60 ppm.

Migration Time	Average MT	RSD (%)	Compound	Amt (mg/l)	Area
12.325	12.354	0.17	Glucose	10	3.959
12.358				30	11.528
12.379				60	19.077

TABLE 4: The amount and peak area at 12.354 minutes of Glucose.

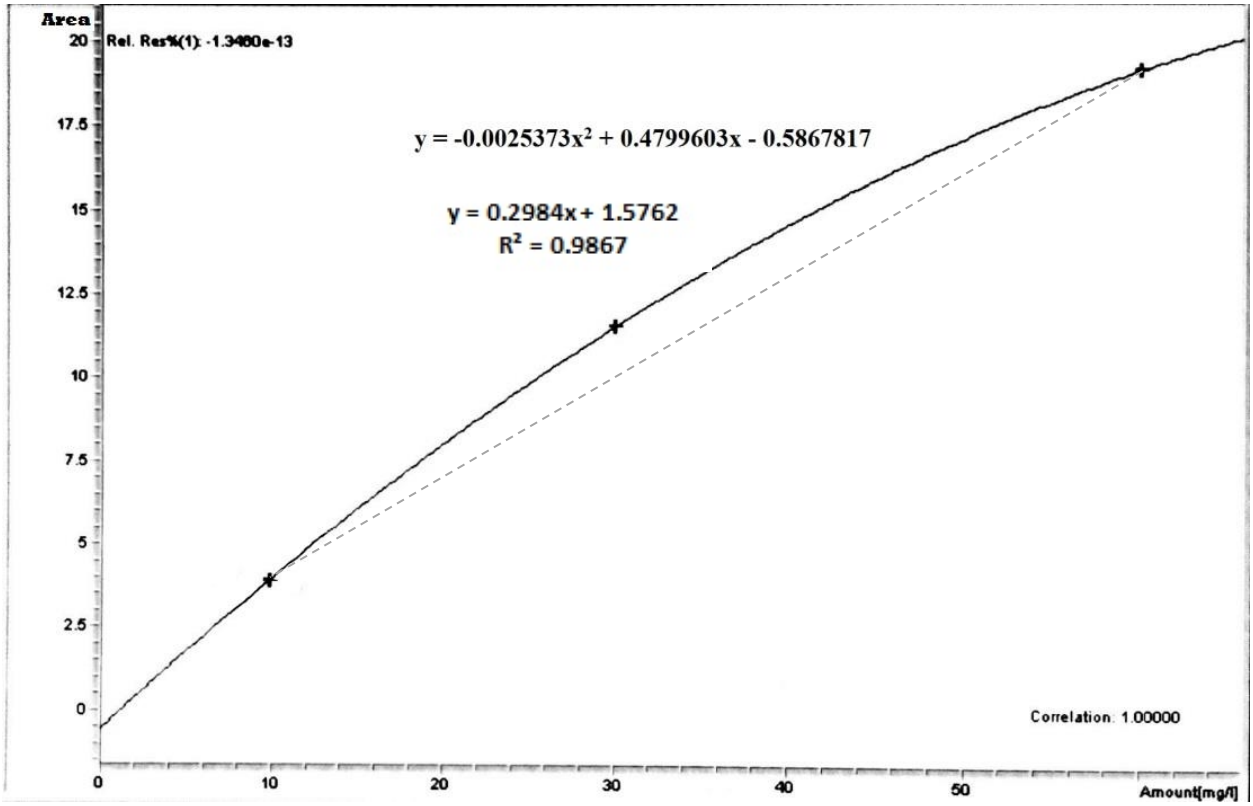


FIGURE 15: The calibration curve, linearity and coefficient of Glucose analyzing.

Table 5 and figure 16 show the values in Arabinose calibration. The range concentration of Arabinose standard is 10 – 60 ppm.

Migration Time	Average MT	RSD (%)	Compound	Amt (mg/l)	Area
12.777	12.778	0.06	Arabinose	10	2.796
12.789				30	5.178
12.768				60	11.822

TABLE 5: The amount and peak area at 12.778 minutes of Arabinose.

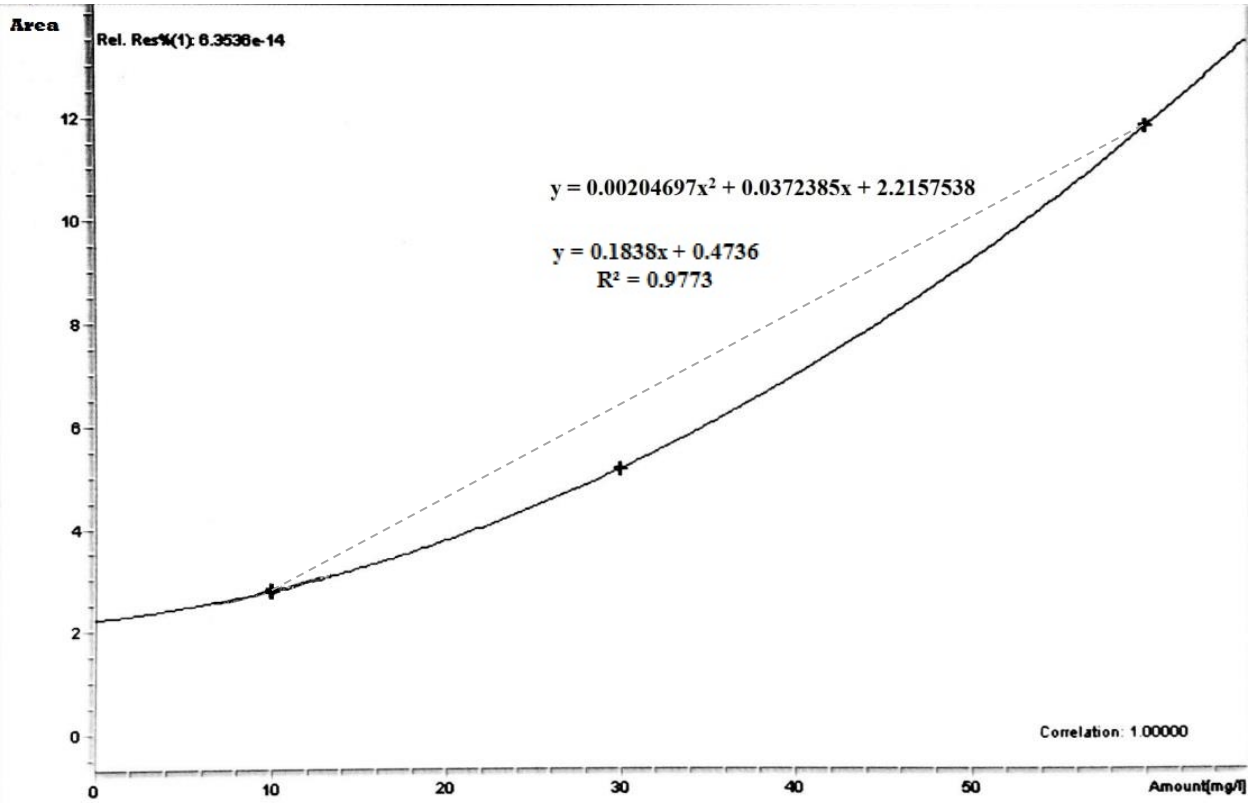


FIGURE 16: The calibration curve, linearity and coefficient of Arabinose analyzing.

Table 6 and figure 17 show the values in Mannose calibration. The range concentration of Mannose standard is 10 – 60 ppm.

Migration Time	Average MT	RSD (%)	Compound	Amt (mg/l)	Area
12.923	12.972	0.28	Mannose	10	3.845
12.980				30	7.481
13.013				60	16.089

TABLE 6: The amount and peak area at 12.972 minutes of Mannose.

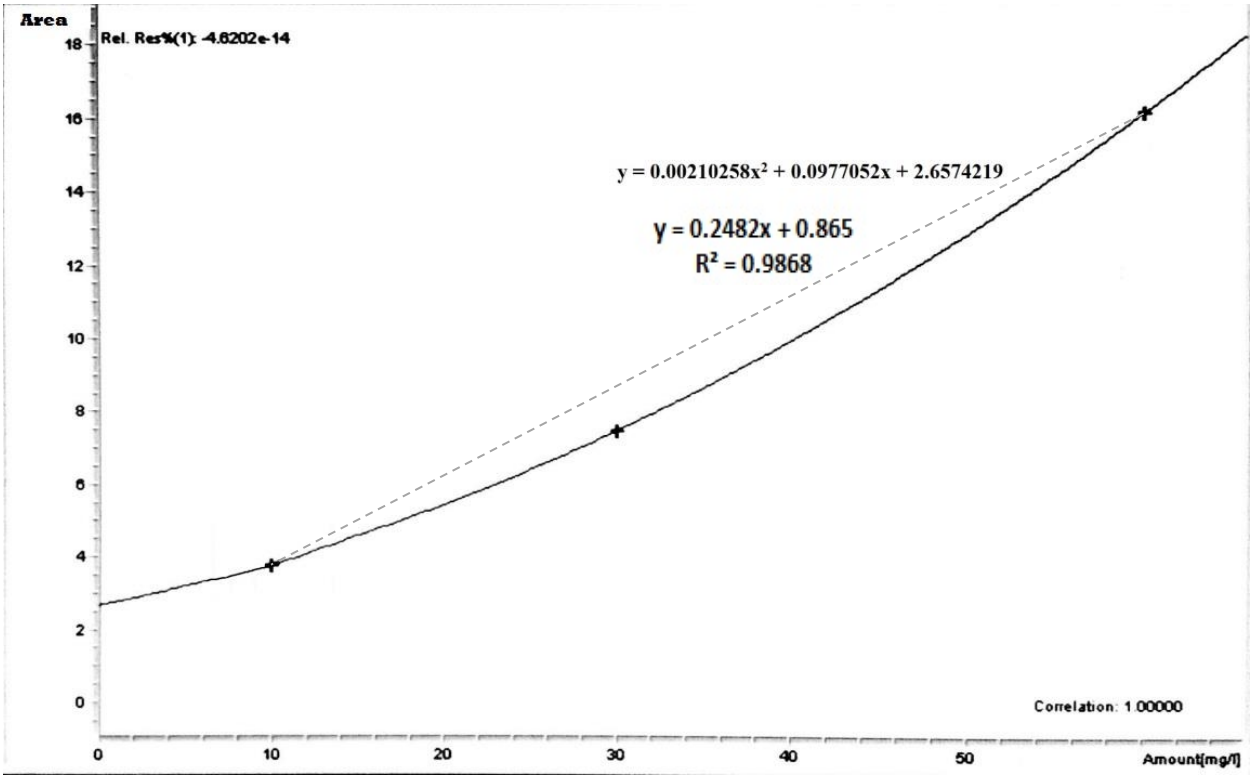


FIGURE 17: The calibration curve, linearity and coefficient of Mannose analyzing.

Table 7 and figure 18 show the values in Fructose calibration. The range concentration of Fructose standard is 10 – 60 ppm.

Migration Time	Average MT	RSD (%)	Compound	Amt (mg/l)	Area
13.327	13.339	0.06	Fructose	10	3.588
13.346				30	8.995
13.344				60	14.352

TABLE 7: The amount and peak area at 13.339 minutes of Fructose.

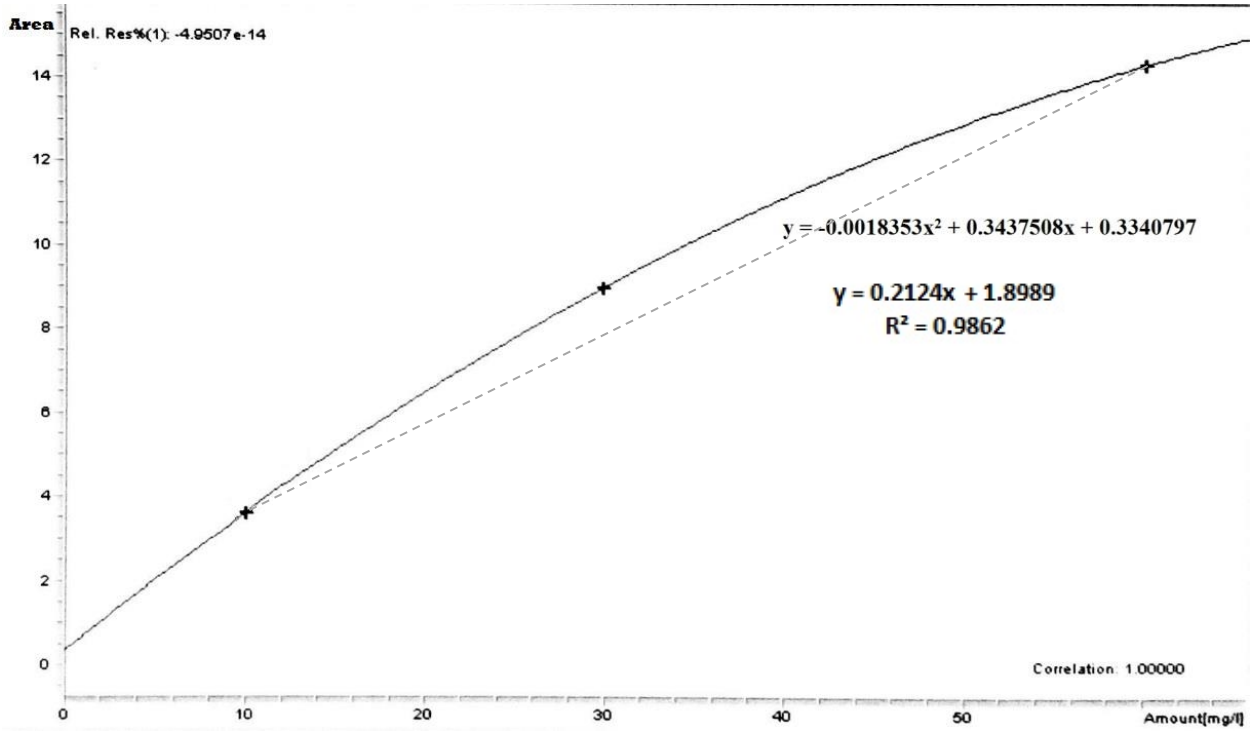


FIGURE 18: The calibration curve, linearity and coefficient of Fructose analyzing

Table 8 and figure 19 show the values in Fructose calibration. The range concentration of Xylose standard is 10 – 60 ppm.

Migration Time	Average MT	RSD (%)	Compound	Amt (mg/l)	Area
13.870	13.854	0.12	Xylose	10	2.564
13.861				30	6.578
13.831				60	9.533

TABLE 8: The amount and peak area at 13.854 minutes of Xylose.

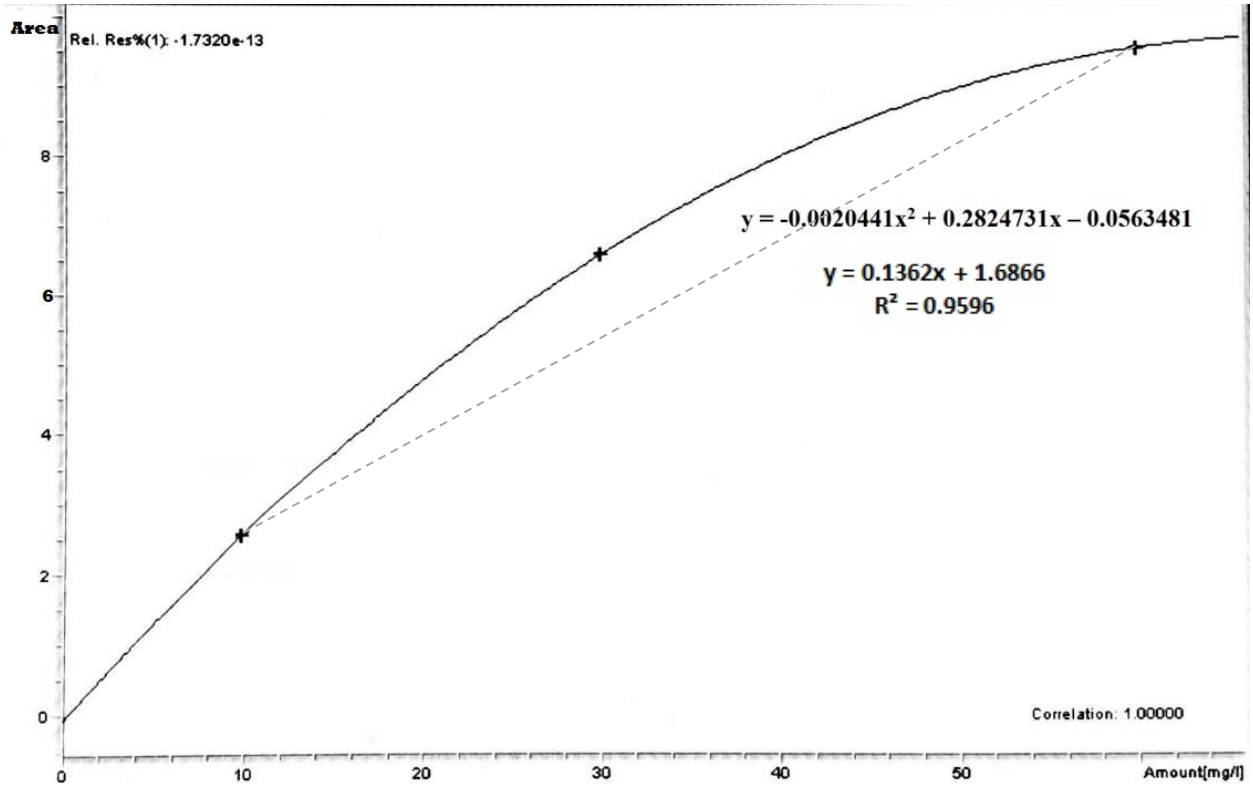


FIGURE 19: The calibration curve, linearity and coefficient of Xylose analyzing.

The linear equation of six carbohydrates have the curve shape which is presented by the quadratic equation. Furthermore, all the coefficient values are 1. The limit of quantitation (LoQ) for all carbohydrates was 6 mg/l under the pressure injection 50mbar within 10 seconds of stock sample.

5.3 Quantification of Pine bark sample

The figure 20 presents the electropherogram of the carbohydrates in the Pine Bark after extraction in hot water for 60 minutes at 80ºC. The carbohydrate peaks are detected and identified automatically, which are based on the calibration table by the CE control system. The amount of each carbohydrate was calculated by the quadratic equation then recorded on the report document.

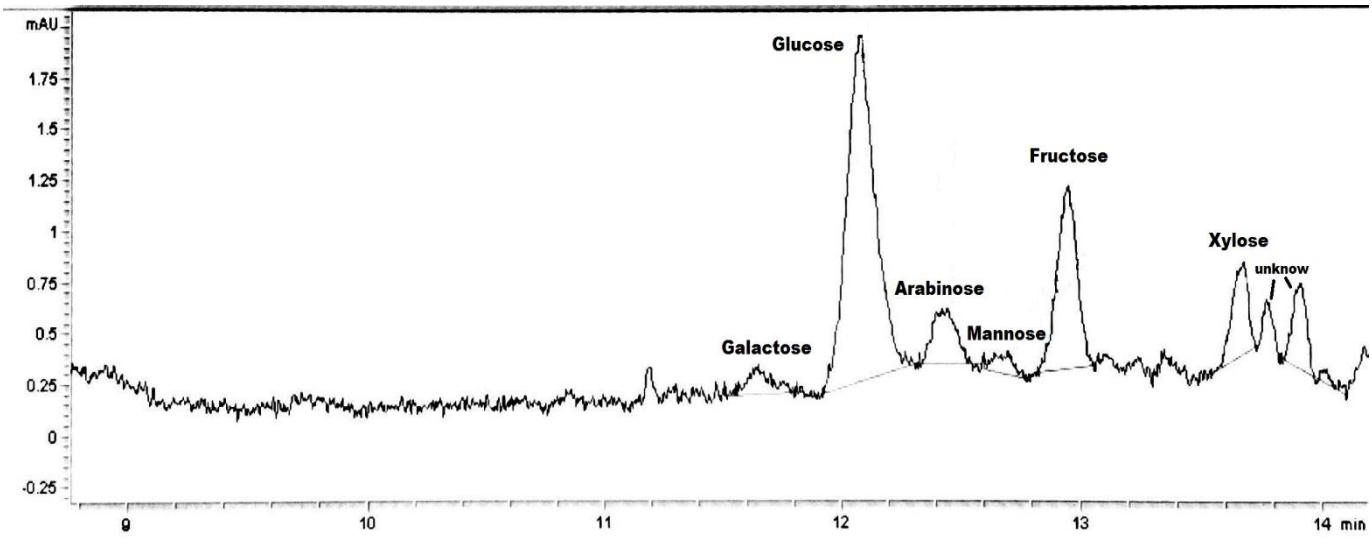


FIGURE 20: Peak electropherogram of carbohydrate in Pine Bark sample extracted by hot water.

The samples which are used in this work are Pine bark after extractives. Two types of samples were analyzed in this work, one was ethanol whereas another was hot water extract. Soxhlet instruction was used in this experiment for the extraction. In addition, the ratio of dilution chosen is 1 ml : 2 ml.

The results are shown from the tables 9 to 12 below, which consist the identification, the amounts of individual and total sugars. average relative standard deviation (RSDs), temperature, time of extraction and the total weight of dry sample Pine bark. All the samples were analyzed three times.

Table 9 presents all the standard condition of extracting process for the sample. Then, the analyzing result of Pine bark sample was extracted by hot water, which shown on table 10.

Name	**Temperature (ºC)**	**Time (min)**	**Mass of dry bark (g)**	**Volume (ml)**
SP12	80	60	5.3276	50
SP13	100	60	5.3417	50

TABLE 9: The parameters in extraction process by hot water of Pine bark sample (Riitta, CentriaTKI, 2018)

The amount: ppm (mg/l). (-) = not detected . The RSD is calculated by equation (1) & (2)								
Name	**Galactose**	**Glucose**	**Arabinose**	**Mannose**	**Fructose**	**Xylose**	**Total**	**RSDs (%)**
SP12 (1 : 2)	2.62	58.13	12.48	-	31.44	5.46	110.13	4.82
	1.92	54.63	11.5	-	35.62	5.96	109.63	
	2.35	49.36	9.75	-	33.25	6.12	100.83	
SP13 (1 : 2)	1.52	35.37	7.86	-	14.42	6.54	65.71	8.2
	1.64	29.53	5.41	-	16.15	6.27	59	
	1.34	26.93	4.12	-	16.91	6.77	56.07	

TABLE 10: The value of sugars in Pine bark sample after extraction in hot water. The ratio diluted which is attached under the name of sample.

Table 11 presents all the standard condition of extracting process for the sample. Then, table 12 shows the analyzing result of Pine bark sample was extracted by ethanol.

Name	**Temperature (ºC)**	**Time (min)**	**Mass of dry bark (g)**	**Volume (ml)**
SP2	80	60	5.2994	50
SP3	100	60	5.3390	50

TABLE 11: The condition in extraction process by ethanol of Pine bark sample (Riitta, CentriaTKI, 2018)

The amount: ppm (mg/l). (-) = not detected . The RSD is calculated by equation (1) & (2)								
Name	**Galactose**	**Glucose**	**Arabinose**	**Mannose**	**Fructose**	**Xylose**	**Total**	**RSDs (%)**
SP2 (1 : 2)	-	13.92	4.92	-	2.16	42.49	63.49	5.4
	-	14.32	5.12	-	3.02	48.33	70.79	
	-	18.22	6.21	-	2.58	40.67	67.68	
SP3 (1 : 2)	-	12.41	-	-	3.19	48.91	64.51	9.45
	-	14.53	-	-	3.52	42.35	60.4	
	-	11.33	-	-	3.95	38.32	53.6	

TABLE 12: The value of sugars in Pine bark sample after extraction in ethanol. The ratio diluted which is attached under the name of sample.

The Figure 21 shows the comparison between two sample after extraction in hot water and ethanol in the same temperature 80ºC. All carbohydrates average concentration (mg/l) was presented by columns to figure out their difference.

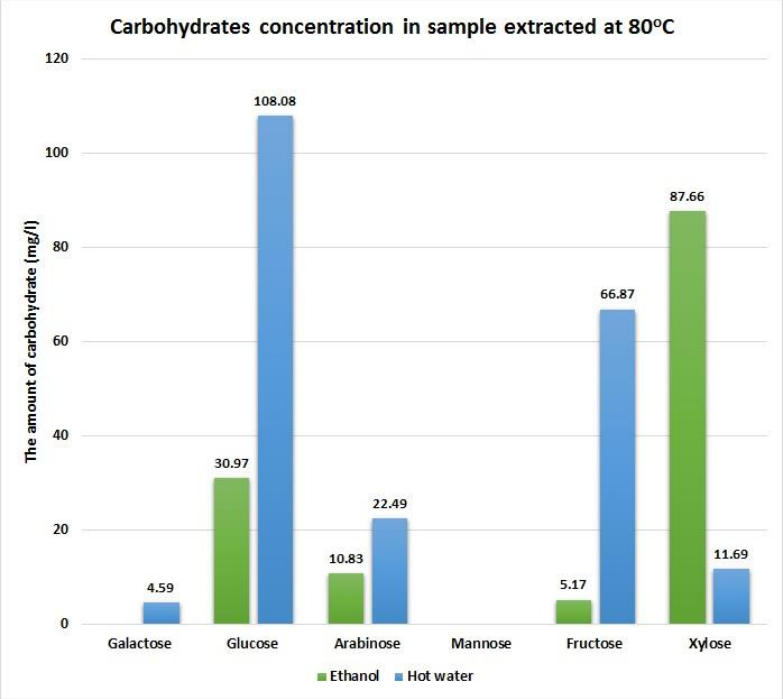


FIGURE 21: The average amount of carbohydrates in sample after extraction at 80°C

The Figure 22 shows the comparison between two sample after extraction in hot water and ethanol in the same temperature 100°C. All carbohydrates average concentration (mg/l) was presented by columns to figure out their difference in the easy way.

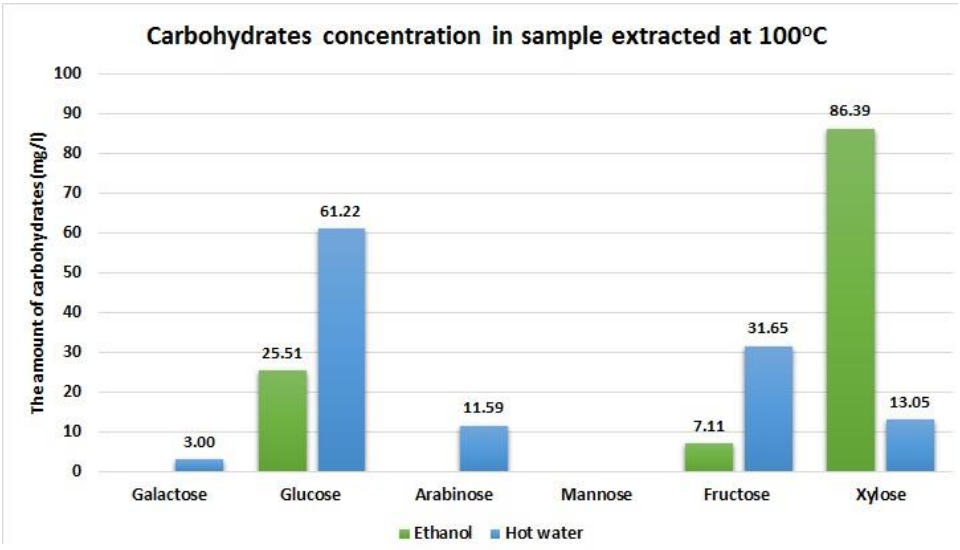


FIGURE 22: The average amount of sugars in sample after extraction at 100°C

6 DISCUSSIONS

6.1 Analyzing carbohydrate procedure

Based on the theory, initially, five carbohydrates were expected to be contained in the Pine bark after extraction. Glucose is the substance that is supposed to be most abundant in the sample. However, after several analyzing tests of IC (Ione Chromatography), Fructose was predicted to exist in the sample through the report. Therefore, Fructose is the sixth carbohydrate added in the standard sample group of the CE analysis. Finally, the standard carbohydrate group analyzed in this work includes Glucose, Arabinose, Mannose, Galactose, Xylose and Fructose.

All reagents used in the experiment must be placed at room temperature for a while before analysis via the CE. The stabilized temperature gives most accurate results because the detector of the CE instrument is very sensitive. The mobility temperature leads to the deviation of the analysis results such as migration time, amounts or unidentified peak. Samples containing carbohydrates such as standard samples 1000 ppm and Pine bark samples should be stored at 2-4^{o}C. High temperatures affect the chemical structure and undetected by the UV detector in some cases. After 2 weeks, samples will produce inaccurate results and they should be replaced with new.

The pH of the buffer solution was determined based on the pKa value of the monosaccharides analyzed in this experiment. These values are shown in Table 2. In addition, the electrolyte solution used is the phosphate buffer, so pKa of Na_2HPO_4 is also considered together with carbohydrates to give a final value. Because the hydroxyl groups of sugars are separated, which forms the monosaccharides in the alkaline electrolyte environment, the selected pH value will be at 12.6. In this work, the pH value is set to 12.65 with a deviation of 0.02. However, the electrolyte buffer is recommended to adjust accurately at 12.65 because the slightly deviated value also affects the migration time of the molecules. For an example, arabinose migrates faster and overlaps with glucose when pH 12.61 is used. Furthermore, the migration time is also deviated from the calibration table, the peak is unidentified. Like the analysis samples, the electrolyte solution is left to stand at room temperature for a while to have the stable temperature.

Pine Bark sample after extraction will be light yellow solution while the one contains drier bark weight is opaquer than the other. For example, in Figure 12, sample b contained 10 g dry bark, so the color of the solution before and after filtration is darker than the other two samples. Basically, the sample is extracted by ethanol and water look like no significant difference after being filtered by microfilter. The more diluted the bark sample is, the lighter yellow color becomes.

Capillary used in this experiment has a size of 56cm x 50um. Therefore, the solutions to be analyzed must be carefully filtered with 0.2 microfilter to ensure that the precipitates are completely removed, avoiding congestion. The flushing with the five stages mentioned in the procedure, which is recommended be completed once a week. This process kept the capillary clean and balanced at the best level. Between each sample run, 10% acetic acid was used to stabilize the noise, resulting in a clear peak shape and no interference.

The temperature set for sample analysis is at 20 ° C equals to room temperature to minimize the difference in temperature for the samples. Temperature mobility result in inaccurate analysis so this issue should be noted. The applied voltage for this analyzing process is 20 kV, which is supposed to be quite high. In fact, high voltage increases temperature, resulting in large variations in current at each run. However, 20 kV is the best result after several trials with standard carbohydrate samples. When the 20 kV voltage is applied, the ions moved faster, the identified peaks were not overlapped (especially fructose, mannose and arabinose). Compared to other voltage levels, this result fit with the initial requirements and goals of this work. Besides, the inject condition (35 mbar within 10 sec) is suitable for this analyzing work. The overlapped peak appears while increasing the pressure or time injection.

Noticed that the temperature plays an important role in this process. Therefore, it is required to notify and evaluate through the current while the instrument is working. The acceptable current range is 180 - 190 μA. The current is out of this range means the temperature is unstable at that time, which affects to quantification result.

6.2 Identification of monosaccharides

According to Figure 13, the order of the carbohydrates identified in turn is galactose, glucose, arabinose, mannose, fructose and xylose. In theory, mannose is supposed to move faster than arabinose because of its molecular structure. Mannose, galactose and glucose are all hexoses while pentose consists of arabinose and xylose. Pentose sugar moves faster than hexose under the effect of EOF flow. However, the practical experiments in this work shown that arabinose moved faster and even show signs of increasing velocity when the pH is at 12.61. The reasons are predicted come from many different aspects such as the temperature, the voltage or the electrolyte buffer.

In addition, the shape of the preformed peaks for the monosaccharides also differed according to the description of Figure 13. Peaks of galactose, glucose and mannose were quite high, the peak of fructose was at average while arabinose and xylose peaks were rather low. This can be explained by the fact that arabinose and xylose are hexoses, so the movement speed of these molecules compared with the EOF line is rather slow. In addition, the distance between arabinose ions while moving is larger, so it takes a long time move completely through the detector. In result, their peak is short and have a wide bottom. Meanwhile, molecules such as galactose or glucose, ions will move faster than the EOF, so the duration of the move through the detector will be shorter, resulting in a higher and slimmer peak.

6.3 Calibration and detected limits

The calibration curve of the sugar analyzing was performed with selective concentrations of 10 ppm, 30 ppm and 60 ppm. The LOQ is determined to be 6 mg/l, but the identified peak was interference when analyzing carbohydrate at this concentration. Therefore, 10 mg /l was chosen as the lowest acceptable concentration, in order to produce the most accurate results automatically. In the case of 60 mg/l, at this concentration, the arabinose and mannose peaks have closely migration time. One trial at the amount of 80 mg/l showed that the peaks of those substances overlap. Therefore, 60 mg/l is determined to be the maximum concentration of carbohydrate contained in the sample for the best quantification. Therefore, the bark samples should have the

dilution rates which are consistent with the acceptable concentration range in the automatically system.

Standard sample with these amount values are analyzed to build the calibration curve for each carbohydrate. The construction of the curve is based on two main values: migration time and peak area of each substance. In theory, both peak height and peak area are selected to express the calibration curve. However, peak area is more commonly used because it is less affected by factors such as voltage or temperature. Meanwhile, when the temperature fluctuates, the current is out of acceptable range, the peak height will change. In addition, short peaks such as arabinose or xylose, it is much more difficult to set a calibration curve. From there, the linear equation is incorrect, directly affecting the quantitative results.

In practice, the migration time of a substance with three concentrations is different. Therefore, the average migration time and RSD (%) are calculated to give the best result with standard deviation. This average result was performed as the migration time of this sugar molecule in the calibration table. The standard deviation was used to extend the migration time range, which ensures that the molecule is identified by detector when analyzing the real sample. RSD was installed directly in the calibration setting of the CE control system. RSD (%) of the six substances was different so the average and suitable level for all was decided at 2%. All the calibration data of six carbohydrate is recorded as a calibration table which saved in the “SUGAR” method.

6.4 Quantification of Pine Bark sample

The linear equation of the all monosaccharides in this work was expressed by equation $y = ax + b$ with the coefficient (R^2). The amount of sugar molecules is calculated by the quadratic equation, the value of y represents for the areas while x is the amount of sugars. In the CE method, calculations are performed automatically, and the results are displayed in the final report. Noticed that the calibration of six carbohydrates is required to renew once a week because the electrolyte solution would change after each preparation turn.

The bark sample in this CE analyzing process is divided into two types based on the difference of solvent extraction used: hot water and ethanol. In addition, the dilution ratios are 1: 2. Figure 20

describes the identified peaks in a sample bark. In fact, the other samples give the same peaks electropherogram, differing only in the peak area (represents for the amount of carbohydrate). On figure 20, all six carbohydrates appear in the bark sample electropherograms. However, the height of peaks is different. Especially, Galactose and Mannose peaks are too small to be able to recognize automatically. In high temperature, Arabinose is either. Besides, unknown peaks also appear at the 8th and the 14th minutes.

The bark of each tree species has its own characteristics about carbohydrates composition, and for Pine bark, these substances are identified by this thesis work. This is slightly different from the theory that mannose is expected to exist and have a large amount in Pine bark sample, galactose is either. On the contrary, Fructose is a rather unexpected existence with a large amount. However, some of the theoretical references also refer that fructose is contained in hemicellulose part, so this result is quite possible.

The reported results of quantify carbohydrate after extraction in hot water and ethanol is different. In hot water extracted case, the figure 21 shows that the glucose content in pine bark was the largest, which accounted for more than half of total sugar mass, at 60 - 100 mg/l. However, these Pine bark samples contain a very small amount of galactose, at 3 - 4 mg/l. Because the limited of quantitation is 6 mg/l, galactose is impossible to be identified automatically by the CE system. Instead of that, galactose peak area is determined by technician and calculated by CE control system.

On the contrary, according the results in figure 22, the bark sample after extraction in ethanol contain the large amount of xylose monosaccharide, at 85 - 87 mg/l. However, it contains a very small amount of Fructose, at 4 - 5 mg/l. In the case of 100^{o}C, the sample contained only Glucose, Fructose and Xylose. Differences from the two types of samples can be explained by the fact that using two different chemicals for extraction, which destroys different bonds of polysaccharides such as Galactoglucomannans (GGM), Arabinoglucuronoxylan (AGX) and xyloglucan. It could be explained that ethanol solvent destroyed the bonds of AGX stronger and released more xylose units. Whereas, the sample which extracted by hot water contains more glucose units because of the bonds in Xyloglucan were broken. Furthermore, the temperature of extraction process increases, which lead the amount of monosaccharides (or sugars) reduces. this phenomenon occurred in both case (ethanol and hot water).

According to the results, sample after extraction in hot water, which contains more sugars type. In ethanol extraction case, Arabinose and Galactose is completely disappearing in the sample. This indicates that the pine bark extracted from the hot water produced more stable and better quantification results than the ethanol case. In addition, the relative standard deviation (RSDs) of results at 80^{o}C is 5% while it reaches 10% at a higher temperature. It proves that the amount of sugars is more accurately while the sample was extracted at a lower temperature. Therefore, the lower temperature should be chosen on the extraction process.

Those statements are based on the analyzing results obtained only by the Hewlett-Packard HP^{3D}CE method under the parameters and conditions was shown in this report. However, different studies will produce different or even opposite results. The results are confirmed temporarily through this thesis work.

7 CONCLUSSION

In pine bark, carbohydrates exist in the form of three main substances: glucose, arabinose and fructose. Glucose is the main constituent of more than half of the total carbohydrate mass. The carbohydrates were analyzed by the 56 cm x 50 um capillary with the UV detection at wavelength 270 nm. The important parameters of CE method which must be pay an attention includes temperature, voltage and buffer solvent. The temperature for analyzing process is 20^{o}C with the voltage 20 kV. The electrolyte buffer is 130 mM NaOH 1M : 36 mM Na_2HPO_4 with pH 12.65 (±0.02). During the analysis, the temperature is sensitive and mobility, usually due to the influence of voltage. Therefore, the temperature factor should be noted and kept it stable to get the accurate result.

Chemical analysis by Capillary Electrophoresis is being invested and developed by developed countries around the world because of its potential in application fields. This method is applied in the fields of food industry, pharmacy, biochemistry and so on. One of the advantages of the CE method is its ability to analyze samples in large concentrations. This is the advantage that CE technique performs outstanding while compared with other analytical techniques. In some cases, the original sample can be analyzed directly without dilution. The standard deviation of the result reaches the lowest possible level. In CE method, the acceptable concentration could reach 100-200 mg /l.

However, a defect of the CE technique that needs to be improved is the sensitivity of the detector. In addition, the detector recognizes strange molecules that will affect major analytes such as overlapped peak or increase the noise. Then, the result is inaccurate. The only solution is that the pH of the electrolyte buffer must be stable and correct in the deviation in the range of 0.02.

In general, this research presents a basic understanding of capillary electrophoresis (organization, principle, applications) and its parameters applied to analyze as well as quantification amount of sugars in Pine Bark. On the other hands, it provides knowledge of the structure and chemical composition of Pine tree. Capillary Electrophoresis is a great technique which is focused on research and development in the future, especially in Food industry because of the high efficient in carbohydrates analysis.

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