Purification and Monosaccharide Composition of Polysaccharides Isolated from *Cannabis sativa* Cherry Hemp Root

by
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Abstract

Traditional medicine has used plants to reduce inflammation, tumors, and in some cases the effects of radiation. While these plants tended to be effective, the reason behind their medicinal properties was unknown. There has now been extensive research on polysaccharides in many plants regarding their immunomodulatory effects. Hemp has been shown to have many bioactive properties including anti-anxiety, anti-nausea, anti-arthritic, anti-psychotic, and anti-inflammatory properties. While there has been much research done on the cannabinoids of hemp, there has been no previous research on the polysaccharides. This project focuses on extracting the polysaccharides of hemp roots and determining their monosaccharide composition. The cherry hemp root polysaccharides were extracted using ethanol and water. The crude extract was then purified by anion-exchange chromatography and size exclusion chromatography. The pure polysaccharides were hydrolyzed, and the resulting monosaccharides were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) for HPLC analysis and Trimethylsilyl (TMS) for GC-MS analysis. This data will be used to help predict bioactivity, as certain polysaccharide structures have been shown to be immunostimulatory.

Introduction

Carbohydrates are a very abundant biomolecule made up of primarily carbon, hydrogen, and oxygen. The simplest carbohydrates, monosaccharides, consist of an aldehyde or ketone with multiple hydroxy groups (Nelson 2017). Monosaccharides can be linked through a glycosidic bond to form larger structures. The glycosidic bond is formed between a hydroxy group of one monosaccharide and a carbon from a second monosaccharide (Figure 1).

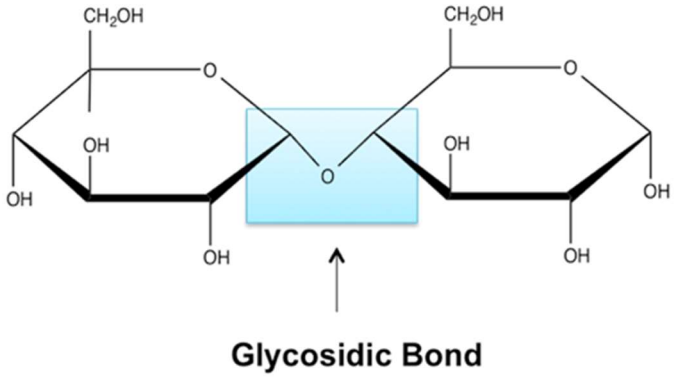


Figure 1. Glycosidic bond between two monosaccharides.

Polysacccharides consist of a large number of monosaccharides linked through glycosidic bonds. Polysaccharides are widely distributed in nature and perform a variety of functions. For example, glycogen and starch are used by animal and plant cells respectively, for energy storage. Other functions of polysaccharides include cellulose acting as a structural element in plants, chitin as a component of the exoskeleton of insects and the cell wall of fungi, heparin as an inhibitor of blood clotting, and peptidoglycan as a component of the cell envelope of gram-positive and gram-negative bacteria (Stick, 2001).

Another important role of carbohydrates is to form glycoconjugates with either lipids or proteins (Taylor and Drickamer, 2006). Rather than the simple monosaccharide composition found in the polysaccharides above, the monosaccharide compositions of glycoconjugates are very complex. These glycoconjugates have a variety of roles in living organisms. These functions include modifying physical properties such as water solubility, acting as structural components, moderating cell-to-cell signaling, and cell-to-cell adhesion.

Polysaccharides in plants also have bioactivity in humans. For example, a study was done on tea (*Camellia sinensis*) and the benefits it has on human health. Immunostimulating activity, anti-diabetic activity, anti-oxidant activity, and anti-tumor activity were all found from the polysaccharides in this tea (Cao, 2013). In another example, the polysaccharides isolated from *Angelica sinensis* (Diels), a traditional Chinese medicine, have been shown to have hematopoietic, anti-inflammatory, antitumor, and antioxidant activities (Jin et al., 2012). In addition, these polysaccharides were shown to have some protective effect against exposure to radiation.

One of the major difficulties of analyzing the monosaccharides found in polysaccharides is the lack of a chromophore in the monosaccharides. This requires that the monosaccharides be derivatized to be detected during HPLC chromatography. One of the most widely used derivatives is the 1-phenyl-3-methyl-5-pyrazolone (PMP) derivative (Stepan & Staudacher, 2011) (Figure 2). This method allows the simultaneous determination of 26 monosaccharides during a single HPLC run. A typical chromatogram is shown below (Figure 3).

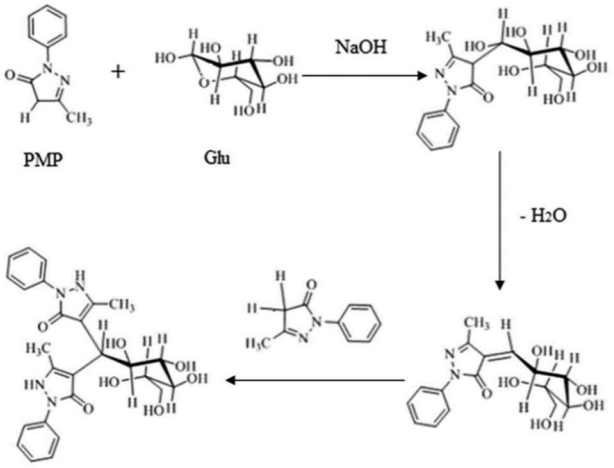
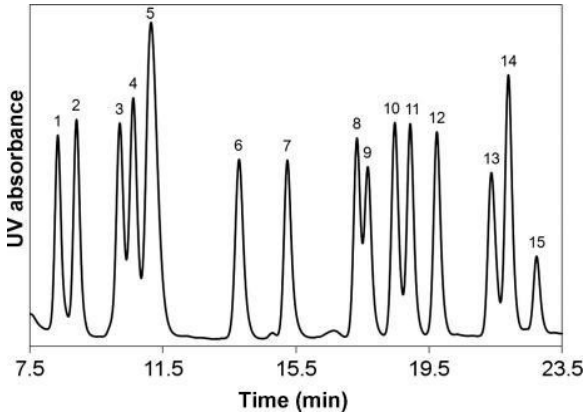


Figure 2. PMP derivatization of glucose (Xian et al., 2015)

Figure 3. Monosaccharides labeled with PMP and separated by reversed phase HPLC. Shown is an application of equal amounts (180 nmol) of Man (1), gulose (2), GlcN (3), ManN (4), Rib (5), GalN (6), galacturonic acid (7), idose (8), Glc (9), 3-O-Me-Glc (10), altrose (11), Xyl (12), Fuc (13), 4-O-Me-Gal (14), and 2-O-Me-Gal (15).



The monosaccharides can also be analyzed using gas chromatography-mass spectrometry (GC-MS). A number of different derivatives have been used with GC-MS, but this project uses the trimethylsilyl (TMS) derivatives (Figure 4). This derivative makes the monosaccharides volatile so that they can be eluted through the gas chromatography column. There is an increased complication in using GC-MS instead of HPLC analysis. The TMS derivative results in multiple tautomeric forms for each

monosaccharide. As a result, the GC-MS chromatogram can have multiple peaks for one sugar, while the HPLC chromatogram only shows one peak per sugar.

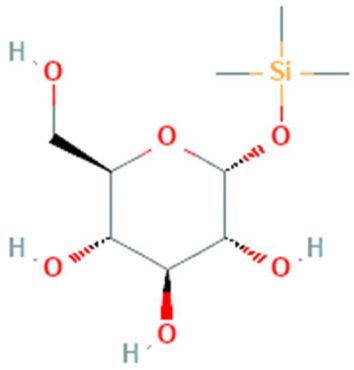


Figure 4. Structure of TMS derivative of glucose (National Center for Biotechnology Information)

Specifically, in this project the focus is on polysaccharides from *Cannabis sativa*. While both hemp and marijuana belong to the plant species *Cannabis sativa,* the difference is that hemp has less than 0.3% tetrahydrocannabinol, or THC (USDA, 2016). THC is the psychoactive component found in *Cannabis sativa*. Hemp has specific properties that have made it useful for production in America. The strong fibers have historically made hemp great to use for production of thread and cloth. In colonial America, hemp was used widely for paper and its use became increasingly important. During World War II, hemp fibers were used for rope and cloth in the factories (Ripley, 2017). Hemp has also demonstrated "potent bioactivities on human health" (Andre, 2016), including anti-anxiety, anti-nausea, anti-arthritic, anti-psychotic, anti-inflammatory, and immunomodulatory properties (Andre, 2016). However, in 1937, the Marijuana Tax Act resulted in the strict control of the cultivation and sale of all types of cannabis including hemp. Consequently, it became increasingly difficult to legally grow hemp, and its production began to fall (Earleywine, 2005). The legal climate for the

cultivation of hemp began to change in 2014, with the passage of the Agricultural Act of 2014 (USDA, 2016). This bill allowed state departments of agriculture and higher education institutions to grow hemp for research purposes. The objective of this study is to analyze the monosaccharaide composition of polysaccharides isolated from *Cannabis sativa*.

Materials and Methods

Extraction of Polysaccharides Present in Hemp Root

Root from cherry hemp (10 g) was extracted with 500 mL of refluxing 95% ethanol for 4 hours. The ethanol extract, containing small molecules, was discarded and the residue was further extracted with 500 mL of refluxing water overnight. The aqueous extract was filtered to remove solid material. The clarified solution was concentrated under reduced pressure in a Rotavapor. To remove protein, the concentrated solution was treated with 20 mL Sevag's reagent (1:4 n-butanol:chloroform) and sonicated for 20 minutes (Sevag et al. 1938). After sonication the solution was centrifuged at 4000*xg* for 10 min. The aqueous layer (upper layer) was transferred to a graduated cylinder and the volume determined. The solution was then transferred to another container and 4 volumes of 95% ethanol was added to precipitate the polysaccharides overnight at 4 ^{o}C. The solution was centrifuged at 4000 rpm, and the ethanol was decanted. The pellet was dissolved in water and lyophilized. The mass of crude polysaccharide was determined and the percent polysaccharide in hemp root calculated. The crude polysaccharide was dissolved in water for further purification.

Purification of Polysaccharides from Hemp Root

Polysaccharides were purified by anion ion-exchange chromatography on an AKTA FPLC system equipped with a HiPrep 16/10 DEAE-Sepharose column. The sample was loaded onto the column and eluted with a step gradient of increasing sodium chloride in water containing 0.0, 0.2, 0.4, 0.6 and 1.0 M sodium chloride at a flow rate of 3 mL/min. As the column was washed, 5 mL fractions were collected. Fractions containing sugar were determined using a phenol sulfuric acid assay (Masuko et al. 2005).

Related fractions containing sugar were pooled, dialyzed, and assayed for purity by high performance size exclusion chromatography (HPSEC) using a TSK Gel PwXL G4000 analytical size exclusion column. The column was eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min. Polysaccharides were detected using a refractive index (RI) detector.

Determination of Monosaccharide Composition Using PMP Derivatives

The polysaccharides were hydrolyzed with 4M trifluoroacetic acid (TFA) for 5 hours at 100 ^{o}C. After hydrolysis of the polysaccharide was complete, the excess TFA was removed with an air pump blowing into the sample in a heated water bath and washed 3 times with 200μL of methanol. Then, 100μL of water, 500 μL of 0.5 M NaOH, and 100 μL of 0.5 M methanolic PMP were added to the vial, and the mixture was heated at 80 ^{o}C for 1 hour. After 1 hour, the reaction mixture was allowed to cool to room temperature and HCl was added to set the pH between 4 and 8. HPLC grade chloroform (500 μL) was added and the sample was vortexed and centrifuged. The upper layer was transferred to an HPLC vial using a glass Pasteur pipet.

The sample was analyzed by reverse phase HPLC. A Dionex UltiMate 3000 liquid chromatography system consisting of a quaternary solvent pump/mixer, temperature-controlled autosampler, temperature-controlled column compartment, and multiwavelength UV-Vis detector was used. Separation was carried out on a Phenomenex Hyperclone C18 (150 x 4.6 mm) column with a flow rate of 0.6 mL/min. The mobile phase consisted of two components (A) 15% acetonitrile in 50mM sodium phosphate pH 6.9 and (B) 40% acetonitrile in 50mM sodium phosphate pH 6.9. At the beginning of the HPLC analysis, (A) was set at 100%, and (B) was set at 0%. After 15 minutes, (A) was decreased to 92% and (B) was increased to 8%. At 50 minutes, (A) was decreased to 80% and (B) was increased to 20%. At 58 minutes, (A) was increased to 100% and (B) was decreased to 0%, and the analysis was continued for an additional 7 minutes for a total of 65 minutes. The column temperature was 30 ^{o}C, the autosampler temperature was 32 ^{o}C and the UV-Vis detector set at 245 nm. Standard samples of various monosaccharides were used to determine the retention times of the PMP derivatives. The retention time of each peak was used to determine the identity of the monosaccharides present.

Determination of Monosaccharide Composition using TMS Derivatives

The polysaccharides were hydrolyzed with 1M methanolic HCl and heated for 16 to 18 hours at 80 ^{o}C. The sample was washed with methanol 3 times to remove excess HCl. Tri-Sil was then added and the sample was heated at 80 ^{o}C for 30 minutes. After derivatization, the excess Tri-Sil was precipitated out in hexane and the sample was vortexed and centrifuged. The supernatant, containing the sample, was filtered using a 0.22 μm syringe filter and dried down to 100 μL using an air pump blowing into the

sample sitting in a heated water bath. The sample was transferred to a GC-MS vial with a glass Pasteur pipette.

The derivatized sample was analyzed by GC-MS. The initial temperature was 80 ^{o}C and the initial time was 2 minutes. After 2 minutes, the temperature increased at a rate of 20 ^{o}C/min up to 140 ^{o}C, 2 ^{o}C/min up to 200 ^{o}C, and 30 ^{o}C/min up to 250 ^{o}C. Standard samples of 8 different monosaccharides were used to determine the retention times and mass spectra of the TMS derivatives. The retention times and mass spectra were used to determine the identity of the monosaccharides present.

Results and Discussion

The phenol sulfuric acid assay showed five fractions eluted from the ion exchange column (Figure 5). The first and fifth fraction did not contain enough sugar to perform further analysis. Therefore, all purity and composition analysis were done on fractions HR2-4.

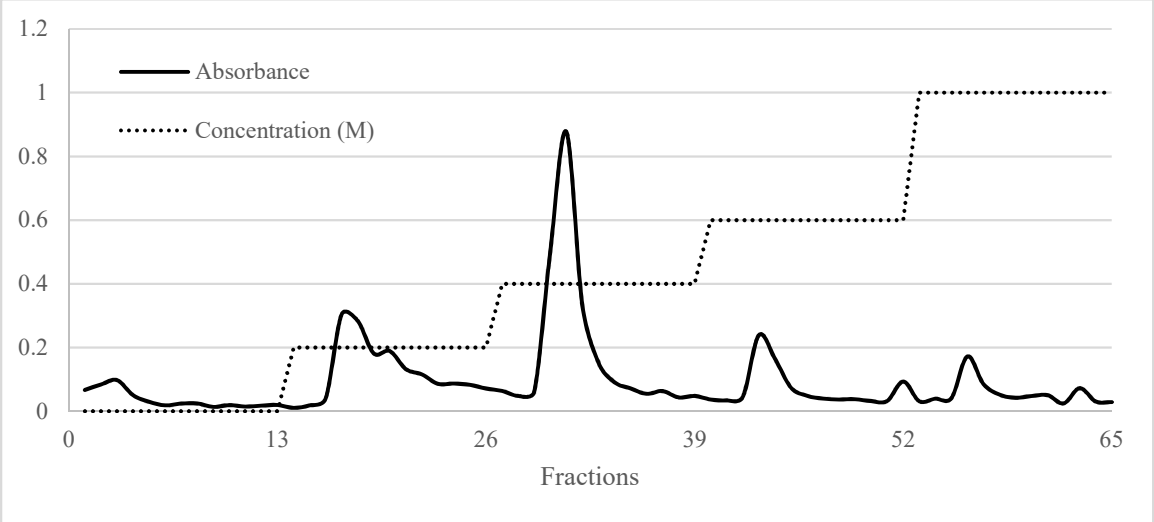


Figure 5. Polysaccharide fractions using anion exchange chromatography

The size exclusion analysis showed very impure fractions (Appendix I). HR2 had an intermediate amount of material, but also consisted of multiple peaks. HR3 was the purest fraction of the three analyzed. It contained the most material and consisted of one major peak. HR4 had very little material.

A series of dextran standards with known molecular weights were used to form a calibration curve. The retention time was plotted versus the log of the molecular weight resulting in the equation shown below ($r^2 = 0.9373$):

$$y = -1.8878x + 20.352$$

where y is retention time (min) and x is the log of the molecular weight. The molecular weights of each peak were determined using the calibration curve (Table 1). Some of the peaks have molecular weights outside of the range of calibration.

PEAK	1	2	3	4	5	6
HR2	*205368.2	168.4229	31.44201	*2.968299	---	---
HR3	*157226.2	174.4869	*4.793873	---	---	---
HR4	*319367.5	*131739.5	*6167.74	119.259	18.31663	*2.183962

*outside of calibration range

Table 1. Molecular weights of each compound in fractions HR2-4 in kDa

There is no data based on the PMP derivatives. The derivatization was performed multiple times, but the chromatogram did not show any peaks. This was true for both the samples and the standards. As a result, all of the data reported here is based on the TMS derivatives that were analyzed by GC-MS.

A series of 8 standards were derivatized, and the retention times were used to determine the monosaccharides present in each of the HR fractions (Appendix II). For sugars with similar retention times, the mass spectra were compared. The areas of the

peaks were used to determine the percentages of each monosaccharide present in each fraction (Table 2). Since there was not a comprehensive set of standards used, some of the chromatograms have peaks that are not assigned to a sugar. The data also lacks some of the peaks of select sugars. This is likely due to the concentration difference of the monosaccharides between the standards and the sample.

	HR2 RUN 1	HR2 RUN 2	HR3 RUN 1	HR3 RUN 2	HR4 RUN 1	HR4 RUN 2
ARABINOSE	11.06	12.05	10.37	10.32	7.01	7.32
GLUCOSE	---	---	---	---	---	---
XYLOSE	33.99	33.37	---	---	---	---
MANNOSE	2.40	7.65	5.12	5.14	6.15	6.05
RHAMNOSE	8.08	7.66	13.68	13.32	25.28	22.93
GLUCURONIC ACID	---	---	---	1.93	---	1.18
GALACTURONIC ACID	---	---	46.78	39.96	38.61	38.84
GALACTOSE	21.13	21.93	17.99	19.14	17.73	17.65
TOTAL	**76.66**	**82.57**	**93.94**	**89.81**	**94.78**	**93.97**

Table 2. Percent monosaccharide composition of hemp root fractions

HR2 contained the monosaccharides arabinose, xylose, mannose, rhamnose, and galactose. HR3 contained a major peak outside of the calibration range with a molecular weight of about 4.8 kDa. The monosaccharides arabinose, mannose, rhamnose, galacturonic acid, and galactose were found in HR3. HR4 contained multiple small peaks, but only two were within the calibration range with molecular weights of 119.3 kDa and 18.3 kDa. The monosaccharides found in HR4 were arabinose, mannose, rhamnose, galacturonic acid, and galactose. There may be traces of glucuronic acid in both HR3 and HR4.

In the future, there will be more material extracted for analysis. The new material and current material will be further purified by size exclusion chromatography. The pure polysaccharides can then be tested for biological activity.

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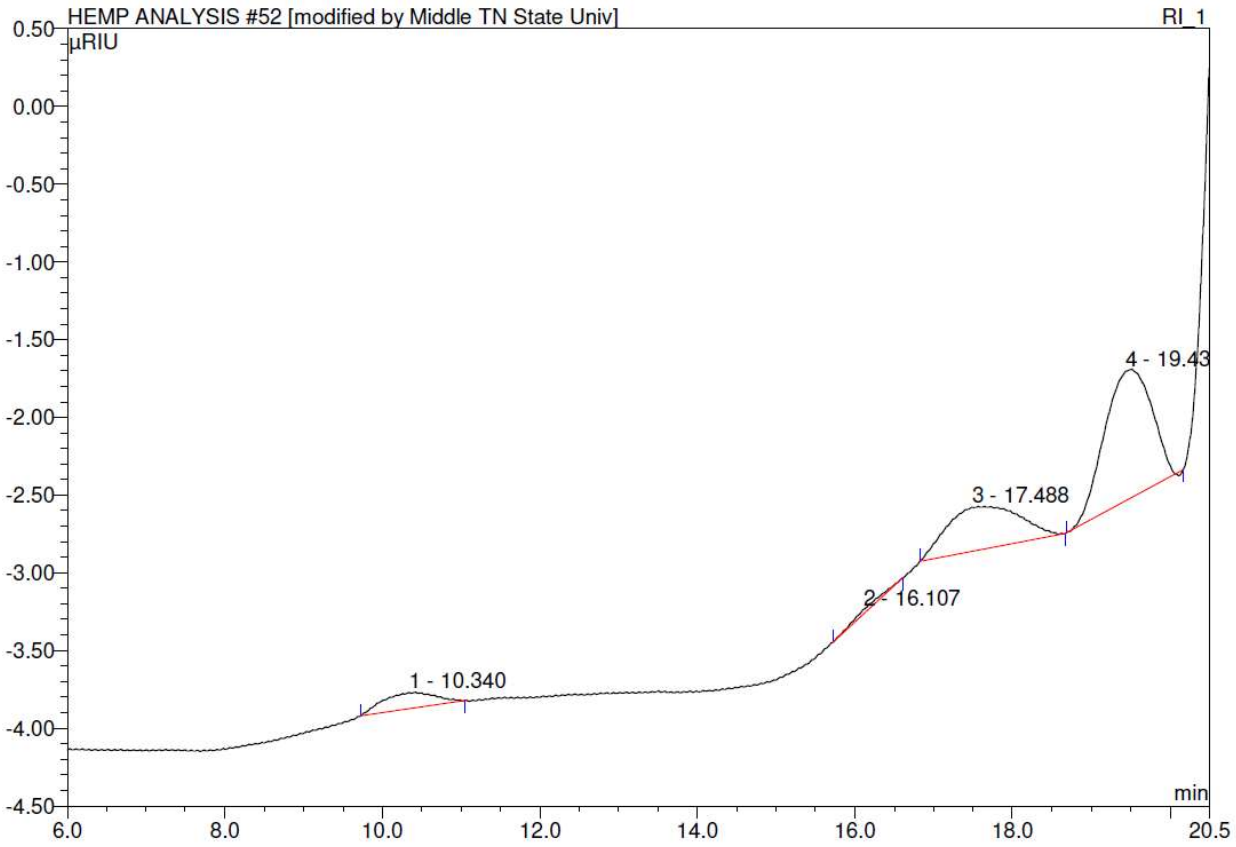
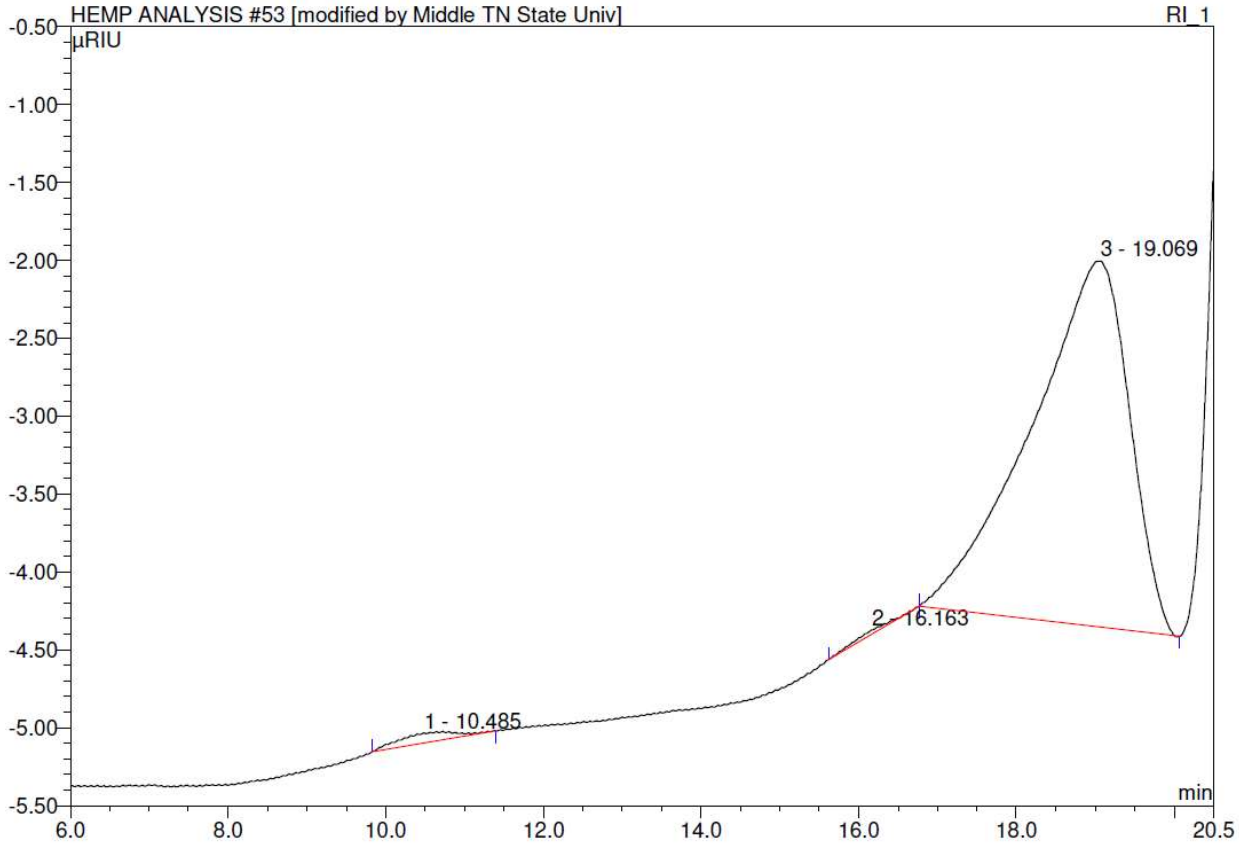
Appendix I. Chromatograms from the Refractive Index Detector

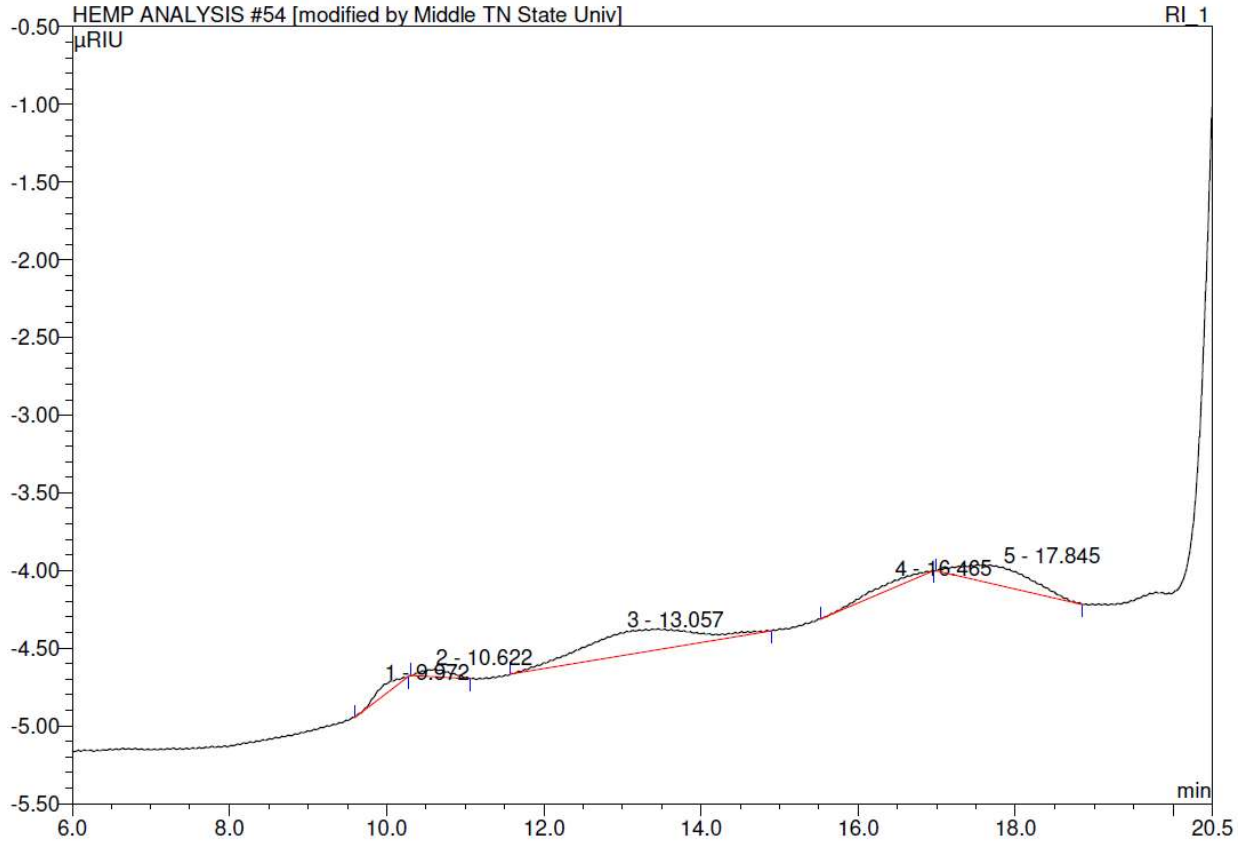
Fraction HR2



Fraction HR3

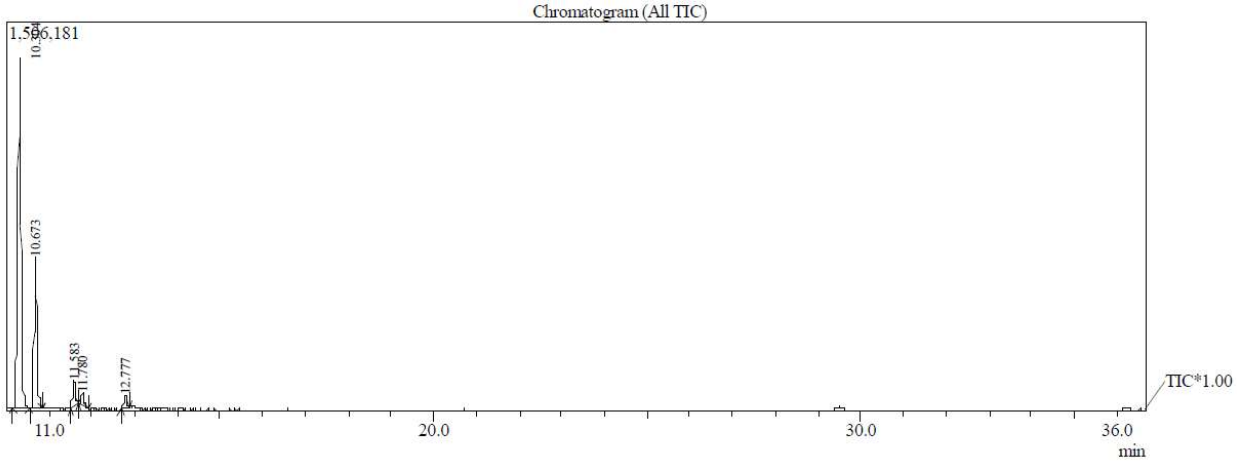


Fraction HR4

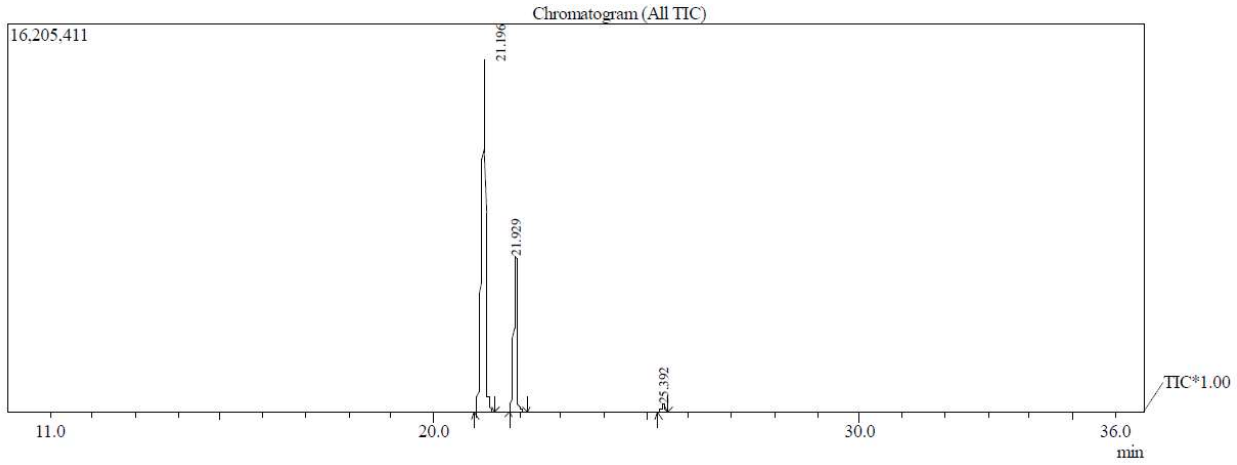


Appendix II. Chromatograms from the GC-MS analysis

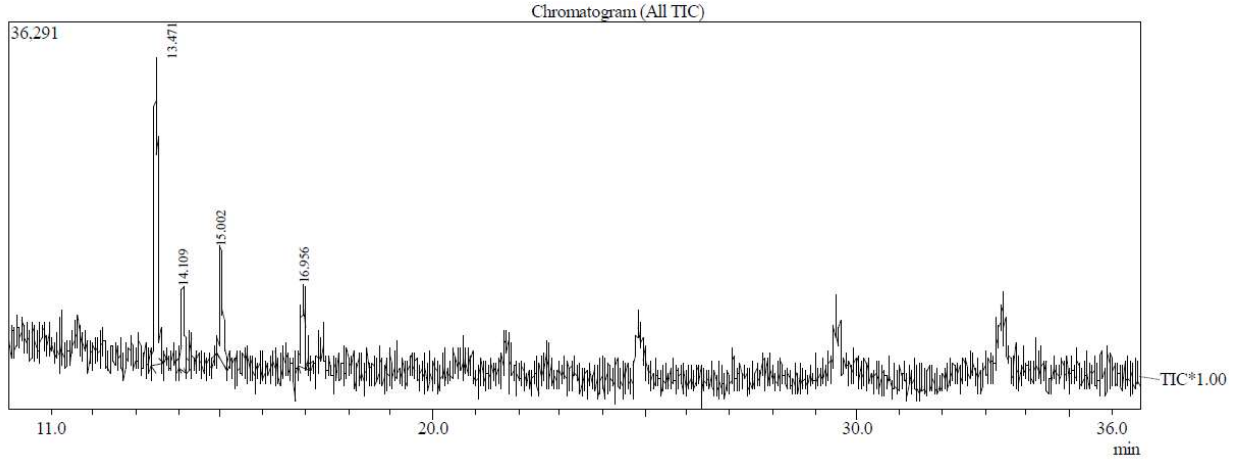
ARABINOSE



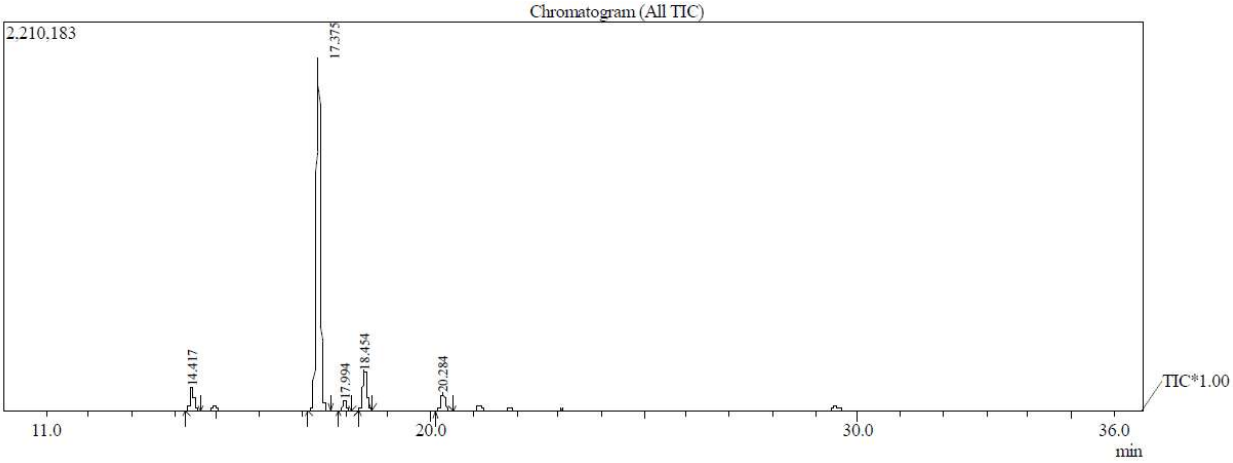
GLUCOSE



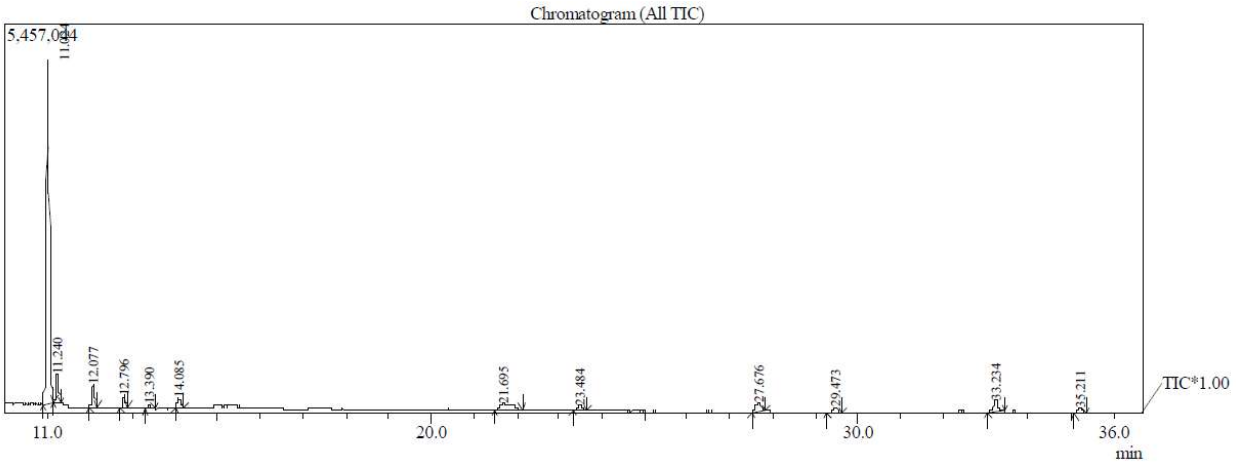
XYLOSE



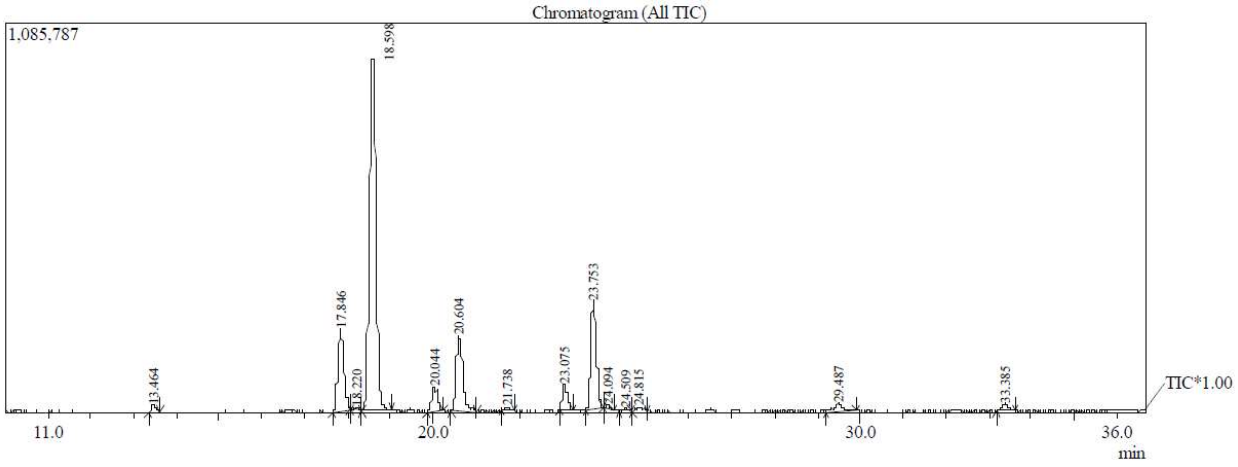
MANNOSE



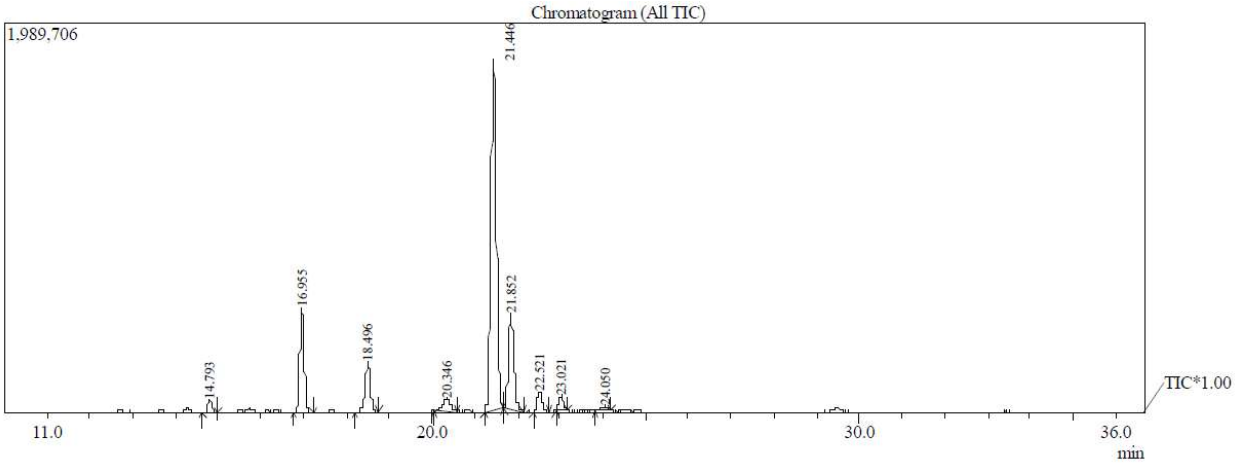
RHAMNOSE



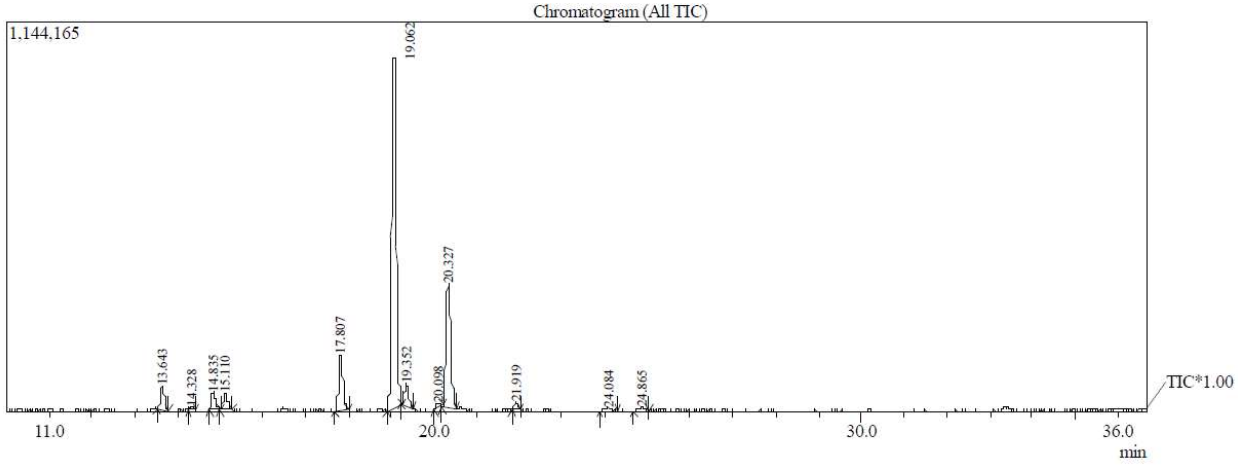
GLUCURONIC ACID



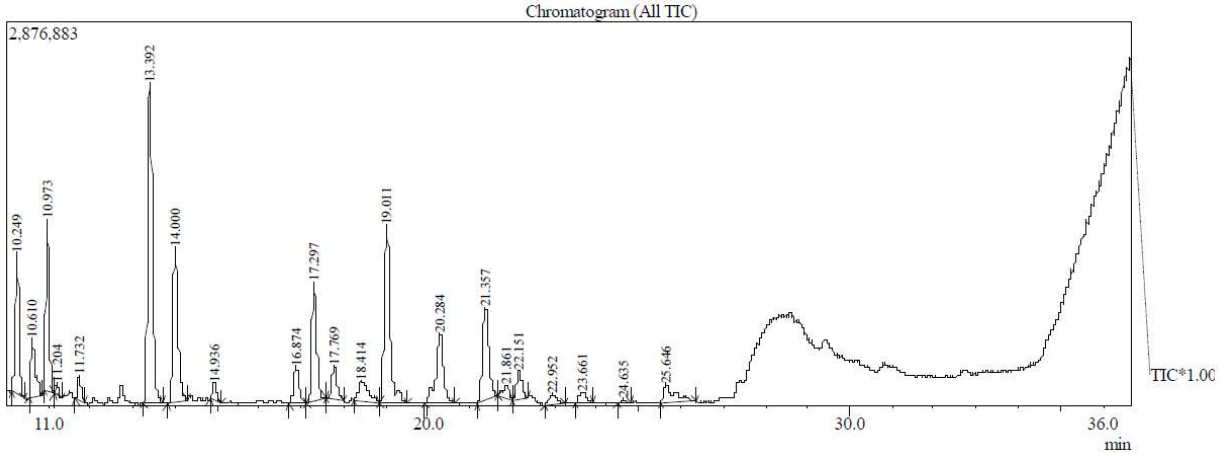
GALACTURONIC ACID



GALACTOSE



HR2 RUN 1



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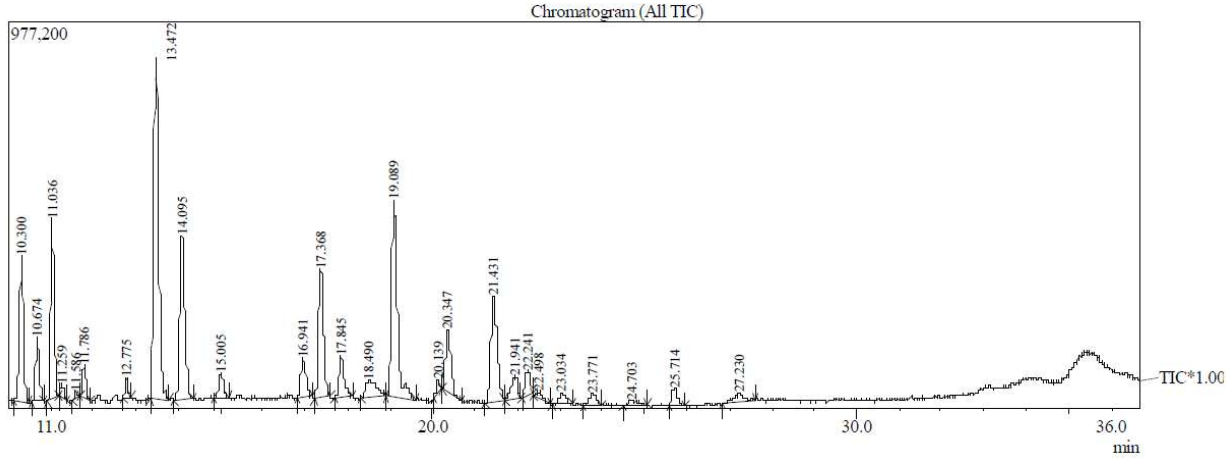

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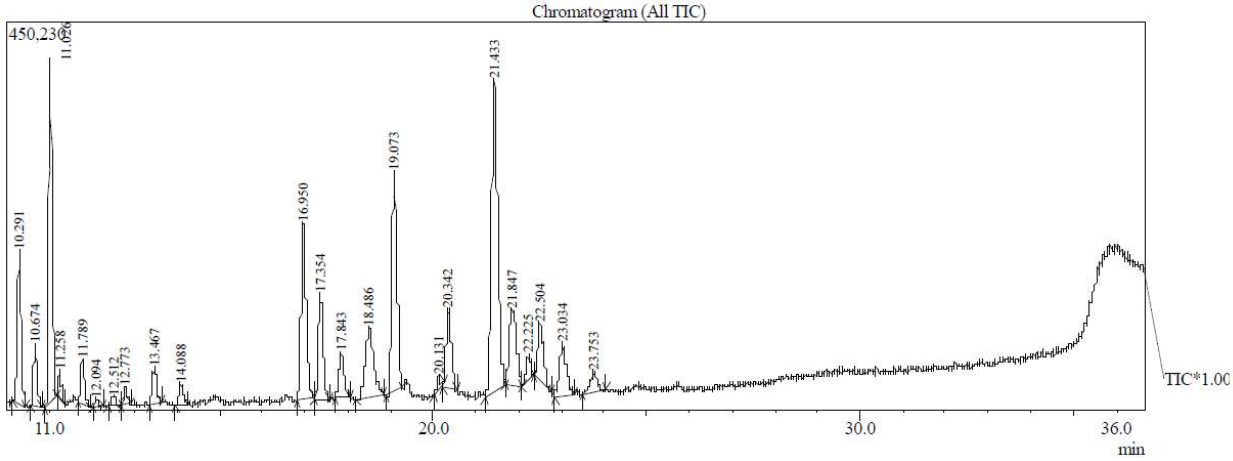
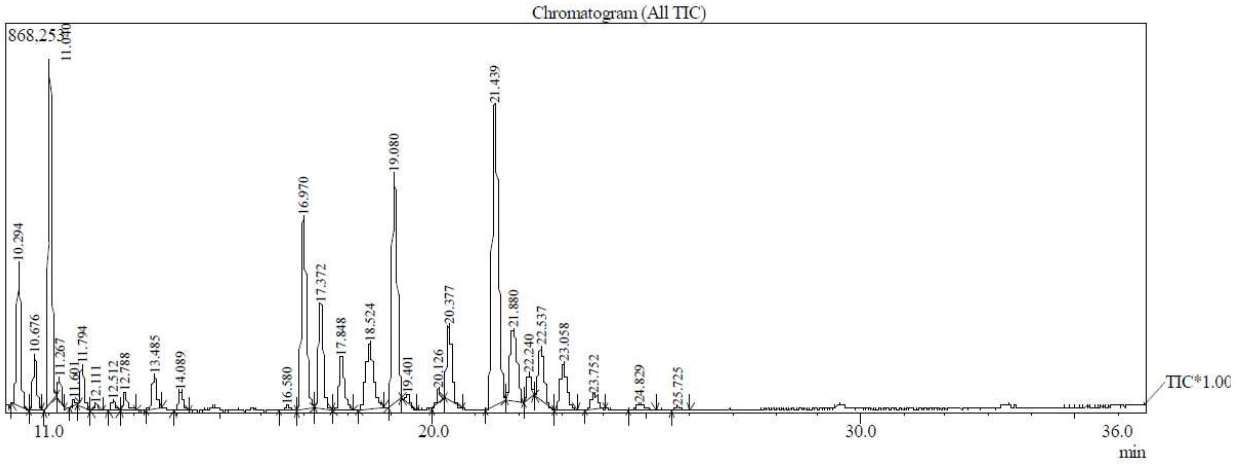
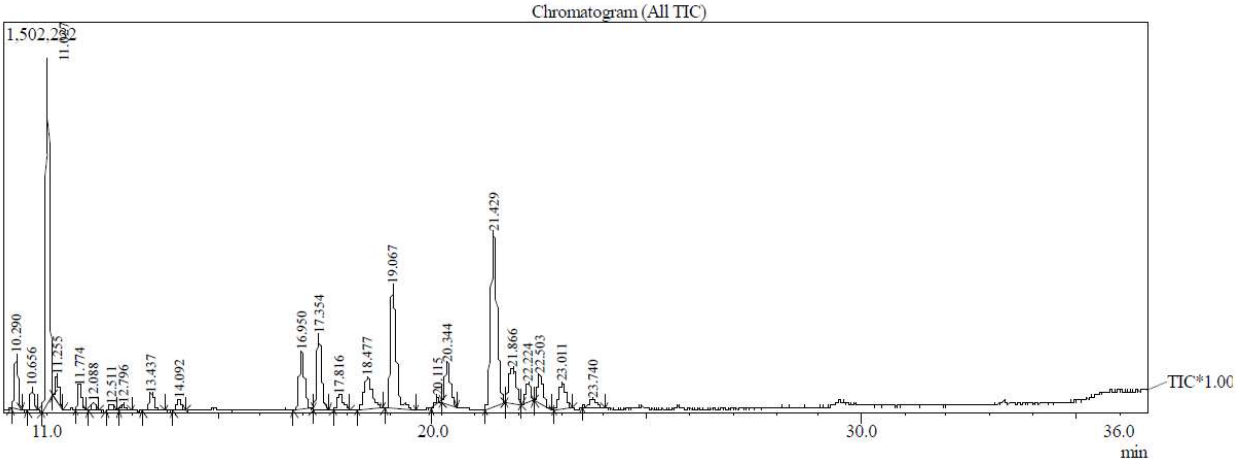


HR3 RUN 2



HR4 RUN 1



HR4 RUN 2