

ABSTRACT

Two monosaccharide labeling agents, 1-phenyl-3-methyl-5-pyrazolone (PMP), and 4-(3-Methyl-5-oxo-2-pyrazolin-1-yl) Benzoic Acid (PMPA), were investigated to determine monosaccharide content in palm oil mill effluent waste (POME). Methods published in the literature were optimized to increase efficiency of monosaccharide detection to suit industry needs. The results from PMP derivatization, using pH 8.0 PBS for dilution and microwave irradiation suggest this is the most efficient derivatization method tested in this project. Monosaccharides detected in POME samples were mannose, glucose and xylose.

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INTRODUCTION

The palm oil industry is one of the largest agribusinesses of the country of Malaysia, and is responsible for over 40% of national economic gains.¹ Due to high concentrations of saturated fatty acids in palm oil, the intention of palm oil industry has shifted from providing an edible vegetable oil, to becoming the largest biodiesel supplier to the West.² The waste product from oil extraction has been suggested as a potential health drink, because it contains high concentrations of antioxidants, which have been shown to be beneficial for immune system support.³ In order for the successful transformation of palm oil mill effluent waste to a potentially beneficial and commercially marketable health drink, the quantities of all organic compounds should be known so a quality standard can be set. This project is focused upon the detection and characterization of monosaccharides in palm oil mill effluent (POME) waste. We aim to optimize various techniques published to provide the palm oil mill industry with a rapid, effective method for monosaccharide detection and identification.

Palm Oil Mill Effluent (POME)

As the palm fruit is pressed for oil, the remaining fruit-waste accumulates; the ratio of oil to fruit waste is 1:3 The waste stream is rich in Biological and Chemical Oxygen Demand compounds.⁴ These are organic compounds that either breakdown oxygen (BOD) or generate carbon dioxide (COD). They are hence considered to be environmental hazards, due to their reactive nature. Disposal of POME waste is of great concern for the Malaysian government. Past disposal methods have included dumping the aqueous waste into streams surrounding palm oil mills in Malaysia; this causes environmental problems such as fresh-water stream degradation, and heavy stench in the area. As a result, the Malaysian government is starting to strictly regulate palm oil mill disposal methods.

The pollution from POME has encouraged researchers to find more ecologically compatible disposal methods. Strategies for use and disposal of the waste have included using the waste as a source of bioplastics, using it as a possible renewable energy source,

¹ Economics and Industry Development Division. 2007. Malaysian Palm Oil Board. http://econ.mpob.gov.my/economy/EID_web.htm

² The National Biofuel Policy. March 2006. Malaysian Palm Oil Board. <http://www.mpob.gov.my/html/pdf/Biofuel%20Policy.pdf>

³ Information obtained from conversations with Dr. ChoKyn Rha and Dr. T. G. Sambandan at the Massachusetts Institute of Technology Biomaterials Science and Engineering Laboratory meetings.

⁴ 2007. Malaysian Palm Oil Board. <http://www.mpob.gov.my>

and employing it as composting fertilizer.⁵ However, these studies have yet to yield significant results.

Researchers have observed POME to contain high levels of antioxidants, vitamins, fatty acids, and fruit sugars,⁶ and thus have suggested this waste be developed as a potential health drink. These bioactive compounds are becoming increasingly important in the medical and nutraceutical fields, due to their potential immune system benefit, and anti-tumor properties. Initially, phenolic acids and antioxidants were the main focus of studies looking for health benefits. However, in light of the discovery that polysaccharides are also important in a bioactive sense, a new focus on the quantities, and characterization of the different types and amounts of sugars found in POME has been established.

⁵ Information obtained from conversations with Dr. ChoKyn Rha and Dr. T. G. Sambandan at the Massachusetts Institute of Technology Biomaterials Science and Engineering Laboratory meetings.

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Bioactivity Review

The availability of monosaccharides is inhibited due to the fact that monosaccharides are largely found as polysaccharides or to oligo-protein molecules, and other glycosylated compounds, including glycosylated antioxidants. (Yang et al., 2005) Studies on low grade tea extracts have shown significant antioxidant and hypoglycemic activity. However, when analyzed for antioxidant content, such low grade teas had inadequate concentrations of antioxidants to explain their bioactivity. (Chen et al., 2002). Further investigations led to the discovery that in low grade teas, the hypoglycemic effect was a result of polysaccharide bioactivity.

Tea Polysaccharide Conjugates (TPC) were isolated from low grade green tea. Antioxidant activity of TPC was tested using a deoxyribose assay and lipid peroxidation inhibition assay. Chen and colleagues discovered that TPCs were able to hydroxyl radicals at concentrations of 33 to 167 μ g/mL. TPCs were also shown to inhibit the formation of superoxide radicals. Investigations of the hypoglycemic effect were done in laboratory mice with alloxan induced hyperglycemia and SOD activity. TPCs were given in doses of either 200 or 400 mg/kg for six days. Blood glucose was closely monitored, and daily dosages of TPC continuously reduced blood glucose levels significantly. Blood serum SOD activity also increased with TPC dosages of 400mg/kg. (Chen et al., 2004).

Another study on an ancient Chinese medicinal herb regarded as treatments for a variety of minor ailments was conducted. Experiments on the herb *Angelica senensis* polysaccharides suggested that the plant's sugars supported gastrointestinal tissues and bone marrow cell proliferation. Cyclophosphamide (CY) was injected into laboratory mice, producing a leucopenia myelosuppression. CY also significantly reduced the blood supply and proliferating cell number in both the gastric and duodenal mucosae. Over a 14 day period, daily subcutaneous injections of *Angelica* polysaccharides at 5mg/kg promoted immune system activity, measured as overall health, as well as bone marrow cell regeneration, and significantly increased the number of blood vessel cells in both the gastric and duodenal tissues. (Hui et al., 2006).

Methods of Poly- and Mono-saccharide Analysis Review

To be able to fully investigate the bioactive potential of compounds found in Palm Essence, thus helping the waste product become a commercially marketable health drink, an analysis of the sugar content is crucial. (Yang et al). Because of the large structure of polysaccharides, detection of sugars must be done by monosaccharide analysis.

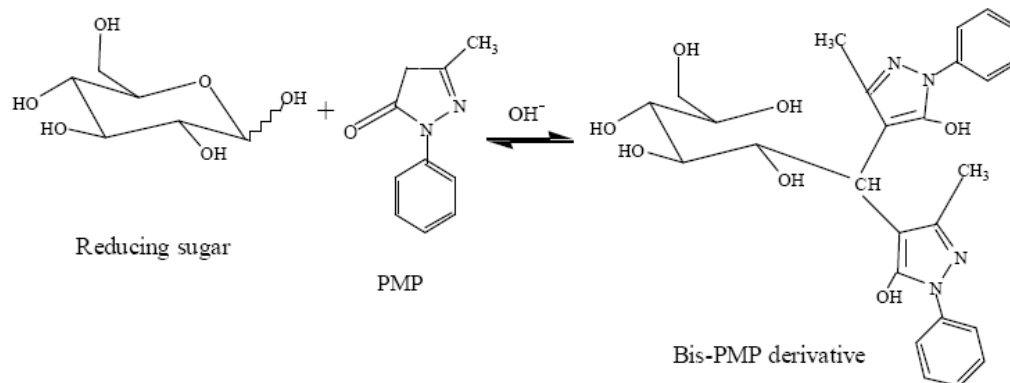
The anthrone test is a specific colorimetric test used for identifying mono- and polysaccharides in plant tissues. Carbohydrate samples are diluted with water and mixed with concentrated H_2SO_4 . Next, samples are mixed in a solution of anthrone crystals dissolved in H_2SO_4 , and are placed in a boiling water bath. The sugar when reacted with H_2SO_4 becomes a hydroxymethyl furfural, and this product reacts with the anthrone reagent to form a blue-green colored complex, which is then read on a spectrophotometer for carbohydrate analysis. Because all carbohydrates do react with the anthrone reagent, this test does not separate or characterize specific sugars present, it is just a total carbohydrate analysis. Therefore, more sophisticated analysis is important for specific determination and high resolution of monosaccharides.

Monosaccharides are simple carbohydrate monomers consisting of a basic similar structure and generally low intrinsic UV spectral activity. (Fu and O'Neil, 1995). Isolation and analysis of these sugars require high resolution separation techniques and sensitive detection techniques for trace amounts of sugar samples. Past methods have included gas and liquid chromatography and more recently Reverse-Phase High Performance Liquid Chromatography (RP-HPLC). New techniques are being continuously developed for pre- or post-column derivatizing techniques to allow UV detection. Derivatization of monosaccharides results in UV-absorbing molecules that aid in the detection sensitivity, allowing for reliable analysis of small carbohydrate sample sizes, and for easier separation due to charges endowed by the derivatization reagents. (Lamari et al., 2003). Currently pre-column derivatization techniques are chosen over post column techniques as pre-column labeling techniques are instrumentally simpler and low detection limits can be reached.

Introduction of labeling agents began in the late 1980's, with Honda and colleagues introducing 1-phenyl-3-methyl-5-pyrazolone (PMP) as a reagent for precolumn derivatization. Since then studies have been carried out to further improve the derivatization methods, and integrate PMP derivatization with monosaccharide analysis of various fruit and plant extracts.

Yang et al., (2005) used PMP derivatization to analyze monosaccharides in *Angelica sinensis* using RP-HPLC, already well known in traditional Chinese medicine as an herb for gastro-intestinal relief, and for a variety of anti-tumor bioactivities. The monosaccharide composition was determined following PMP derivatization as described by Honda et al., 1989. When compared to known standards, polysaccharides present in the samples were found to consist of primarily galacturonic acid, and arabinose. Small amounts of mannose and raffinose were also present.

Although regarded as an efficient and effective labeling agent for reducing mono-, poly- and oligosaccharide detection, PMP derivatization methods require a large excess of reagent used in labeling reactions, and several extractions, which can also result in loss of derivatized product.



Scheme 1: PMP derivatization reaction with reducing aldoses. Product with heat is Bis-PMP derivatives.

Castells et al., 2002 developed a pre-column derivatization for reducing carbohydrates with 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid (PMPA). The structure of PMPA differs from PMP only by a carboxylic group on the aromatic ring. PMPA absorbs at a higher level of UV absorbance than PMP, and is less soluble in water or in hydro-organic solvents at a pH less than 7. This study presented a detailed method of PMPA pre-column derivatization. Separation and extraction methods were simpler, used considerably reduced amounts of reagents and solvents, and reduced the time needed for sample preparation when compared to PMP derivatization.

Ho Ko and colleagues (2005) introduced *p*-Amino benzoic Acid Ethyl Ester (ABEE) as a pre-column derivatization reagent. Previous pre-column derivatizing agents could only label reducing aldoses, thus fructose and other non reducing sugars were yet to be analyzed in fruit extracts. The use of ABEE allowed detection of all sugars, including fructose. Yogurt and orange juice samples were tested, and were shown to largely consist of fructose, glucose, and galactose. (Ho Ko et al., 2005)

Derivatization procedures are often time consuming; many reactions require over 2 hours for thermal heating of samples. Thus, time can be a rate limiting step, when considered for industrial or commercial use. The use of microwave irradiation has been reported to be a viable alternative. A thorough study of organic compound synthesis by microwave oven heating was conducted by Gedye et al., 1987. Results showed that organic synthesis occurred up to 1240 times faster using microwave irradiation when compared to conventional heating techniques. Sample volumes, heating rates, sample size and other factors were considered, and the use of microwave ovens for organic synthesis was supported. The use of microwaves allows homogenous heat waves to be directed deep

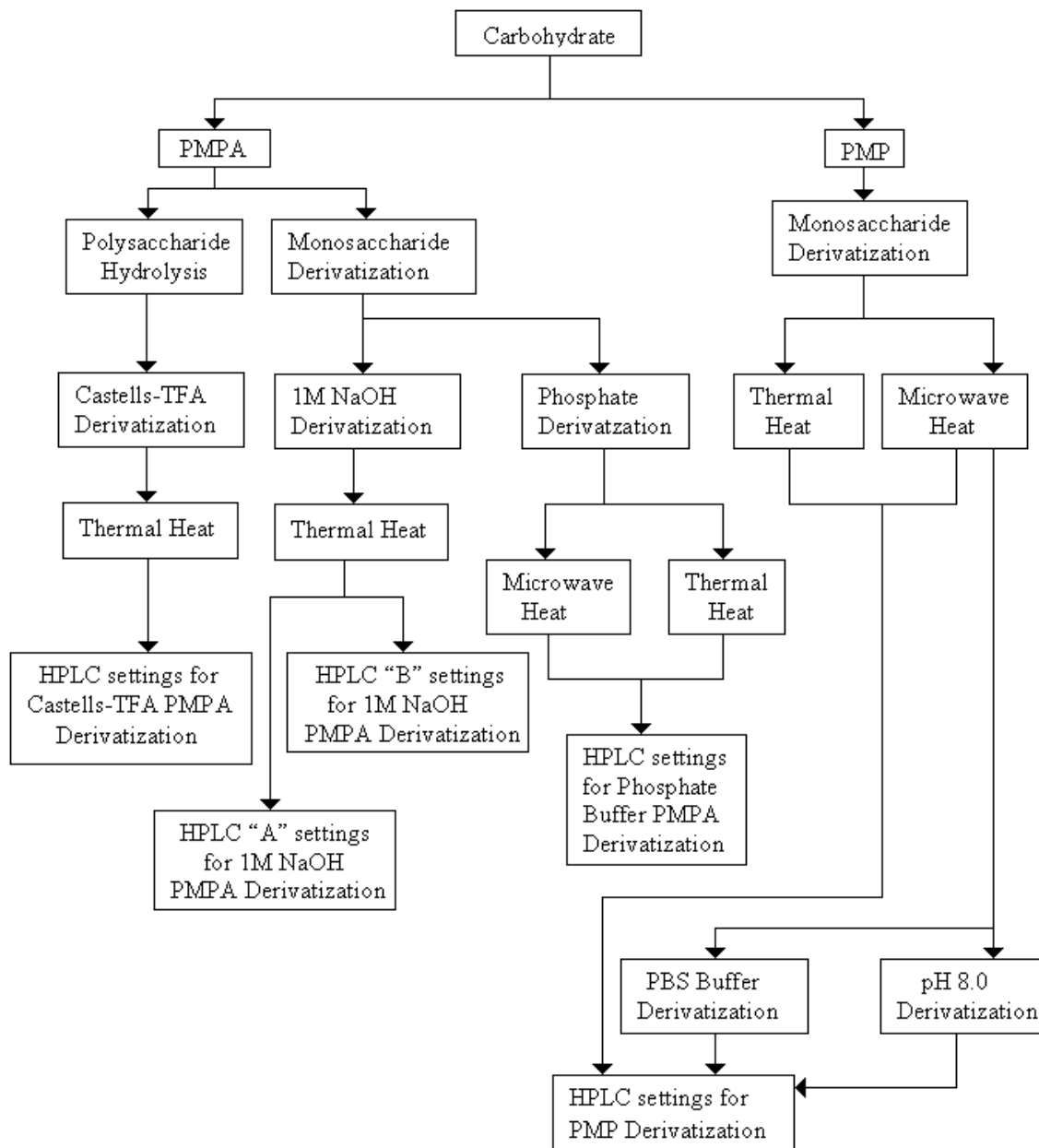
into the sample matrix, rather than heating from the surface as in conventional thermal practices (Acquistucci et al., 1996). This in turn alters the rate of chemical reactions, produces higher yields, and better products. (Saxena et al., 2005). The direct localization of heat allows for significant reduction in time allotted for heating of samples, and also reduces the amount of reagents used. Panfili et al. (2000) developed a rapid assay for choline in a variety of foods, suitable for commercial use using microwave irradiation during the hydrolysis procedure which drastically reduced heating time from 3+ hours to 5 minutes.

Labeling agents require strict conditions for optimum reaction products. Stability of pH during the derivatization procedures is crucial; reactive pH can lead to reverse reactions, leaving no UV-labeled sugar derivatives for analysis. Perhaps due to the fact that hydrogen bonds affect polarity and stability of molecular conformation, results from a study by Yamamoto and Rokushika showed that using HPLC, the retention of PMP aldoses depended on the pH of the eluent. The use of sodium hydroxide in derivatizing solutions raises pH to the optimum levels; however it is very reactive and affects the chirality of sugars. NaOH also produces oxidative breakdown of dye, resulting in overestimates of derivatized product. Thus, limited use of NaOH should be considered.

Project Objectives

This project will examine the use of microwave irradiation to reduce time spent during derivatization procedures. The results from the study may benefit the palm oil industry in using a rapid monosaccharide analysis technique, further supporting the marketability of POME. Optimization of derivatization methods including the use of phosphate buffers instead of NaOH as a pH stabilizer will also be investigated.

MATERIALS AND METHODS



Scheme 2: Flow chart of the various preparation procedures of monosaccharide labeling agents and determination of HPLC parameter settings for accurate analysis.

Hydrolysis and Castells et. al., 2002 Derivatization

Ten mono-, di-, and poly-saccharide standards (galactose, mannose, dextrose, xylose, lactose, sucrose, stachyose, glucose, amylopectin, and fructose) were carefully weighed out to 0.020g each. Palm Essence freeze-dried samples were also weighed out to 0.020g. 5mL of fresh 4M Trifluoroacetic acid (TFA) was added to each, and the samples incubated at 100° C. overnight. The next morning, samples were dried down to a powder in a vacuum chamber.

Following hydrolysis, sugar standards and samples were derivatized by methods published by Castells et al., 2002. Carbohydrate standards and Palm Essence sample were diluted with rdH₂O to contain between 10-120nm of sugar in solution. 50µL of diluted carbohydrate solution was added to 1mL of fresh 0.15M 4-(3-Methyl-5-oxo-2-pyrazolin-1-yl) Benzoic Acid (PMPA). Samples were incubated at 70° C. for 1.5 hours. After samples were cooled to room temperature, 150µL of 2M HCl was added to each. Samples were then centrifuged for 5 min. at 2190 x g. Supernatant was collected and prepared for HPLC injection.

Preparation of PMPA

PMPA crystals (0.441g) were dissolved in 0.25M NaOH in 50:50 MeOH:rdH₂O and brought to final volume of 10mL with 50:50 MeOH:rdH₂O. In our experiments, it produced a grainy milk-white solution. (**Castells-TFA PMPA**)

In an alternative method, PMPA crystals (0.441g) were dissolved in 7mL pure MeOH, and 1M NaOH added dropwise until solution was completely dissolved, yielding a translucent orange solution. (**1M NaOH PMPA**)

The final PMPA preparation method tested, dissolved crystals (0.441g) in 500µL pure MeOH, 500µL Na₂HPO₄ (pH 8.2), and 250µL 1M NaOH. This also produced a translucent orange solution. (**Phosphate PMPA**).

HPLC System

A Hitachi® High-Performance Liquid Chromatography system was used. Pump was L-7100 series, Autosampler was a L-7200 series, and Diode Array Detector was L-7450 series. Column used was a C-18 YMC Combiscreen ODS (50 X 4.6) 5µm, 120Å.

HPLC Settings for Castells-TFA PMPA Derivatization

Solvents used were 0.1M ammonium acetate (solvent A) and acetonitrile (ACN; solvent B) in a linear solvent gradient of 0 min. 45%A/55%B until reaching 0%A/100%B at 55 minutes. Flow rate was set to 0.5mL/min, sample injection volume of 20µL, and UV detector set to 245nm.

HPLC Settings for 1M NaOH PMPA Derivatization - A

Flow rate and injection volume were same as above. UV detection was set to 271nm. Solvent A was 0.05% H₃PO₄, and solvent B was 5% ACN diluted in a phosphate buffer. Run time was 35 min. Parameter settings: linear gradient at 0 minutes consisting of 80%A/20%B, isocratic gradient at 25 minutes of 75%A/5%B until 35 minutes where solvents were again at 80%A/20%B.

HPLC Settings for 1M NaOH PMPA Derivatization - B

Flow rate, injection volume and solvent composition were identical to method A above. The gradient was altered to an isocratic gradient for 5 minutes consisting of 90%A/10%B, then step down to 80%A/20%B at 5 minutes and run as a linear gradient until 35 minutes, where solvents were again returned to original settings.

HPLC Settings for Phosphate Buffer PMPA

Flow rate and injection volume same settings as above. UV detection again set to 271nm. Same solvents used. Gradient changed to isocratic gradient at 0 min 85%A/15%B, step gradient at 1 min 85%A/15%B, step gradient at 2 min 80%A/20%B, then run linearly until 35 minutes reaching 40%A/60%B, then solvents were returned to original settings.

HPLC Settings for PMP Derivatization

Flow rate, injection volume was same as other settings. UV detector was set to 245nm. Solvents used were 0.1M ammonium acetate (NH₂OAC; solvent A), and ACN solvent B. Gradient was set to a linear gradient at 0 minute consisting of 45%A/55%B until a 55 minute run time 0%A/100%B, held at an isocratic gradient for 5 minutes, then solvents were returned to initial settings.

Common Procedures for all Derivatization methods tested

0.050g of each monosaccharide was used as a standard. Palm Essence liquid samples were diluted either 1:5 or 1:10. Prior to acidification with 2M HCl, all samples and standards mixed with either PMPA or 1-phenyl-3-methyl-5-pyrazolone (PMP) were heated at 70°C. for 1.5-2 hours. After separation methods, such as HCl acidification or chloroform extraction, all samples and standards, in any method tested were centrifuged for 5 min. at 2190 x g.

1M NaOH PMPA Derivatization method

Instead of dissolving the 0.050g monosaccharides in rdH₂O, standards/samples were dissolved in 5mL of 0.25M NaOH. 50µL of these standards were then diluted with an additional 400µL of 0.25M NaOH and 50µL of this solution was added to 500µL of PMPA solution as above. 175µL of 2M HCl was added after standards/samples were heated as stated above. Centrifuge procedure/settings same as above. 150µL of supernatant was diluted to 300µL with rdH₂O.

PMP Derivatization method – Standard Thermal Heat

Glucose, galactose, xylose, mannose and glucuronic acid standards (50mg each) were diluted with 5mL of 0.25M NaOH. Palm Essence Liquid samples were diluted to 1:5, and 1:10 with dH₂O. 50µL of standards or sample were added to 100µL of 0.1M PMP reagent and 200µL of dH₂O. After heating, 50µL of 0.6M HCl was added to cooled samples. 600µL of chloroform was added to each sample, samples were vortexed twice for 10 seconds, and the organic layer was carefully removed. Another 600µL of chloroform was added. 100µL centrifuged supernatant was collected and diluted with 200µL rdH₂O for HPLC analysis;

Microwave Irradiation; Used for Phosphate buffer PMPA Derivatization, and PMP Microwave methods

Samples, prepared as described for each derivatizing procedure, were irradiated with microwaves for 2 minutes at half power. (Microwave high power full wattage: 700W; Haier brand, 0.7ft³, 10 power level settings.) At one minute intervals, samples were mixed by vortex for 5 sec. Samples follow post-thermal heat methods as described above.

PMP Calibration Curve – PBS Buffer

Glucose, mannose, xylose, galactose and glucuronic acid standards (50mg each) were diluted to 10mL with Phosphate Buffer, 1X (PBS) pH 7.2, to have a 5mg/mL dilution factor. Standards were further diluted to 0.125mg/mL, 0.250mg/mL, 0.375mg/mL, and 0.500mg/mL with PBS buffer. 50 μ L of each dilution was added to clean glass Pyrex tubes, 250 μ L of PBS buffer was added, and 100 μ L of 0.1M PMP solution was added. Palm Essence samples (50 μ L) were added directly to Pyrex tubes without dilution. Samples were heated following the Microwave procedure, and acidified following same procedures as described.

PMP Calibration Curve – pH 8.0 solution

Mannose, glucuronic acid, galactose, xylose and fucose (50mg each) were diluted with 10mL rdH₂O. Samples were then diluted to contain 0.100mg/mL, 0.200mg/mL, 0.300mg/mL, 0.400mg/mL, 0.500mg/mL, and 0.600mg/mL with PBS buffer. 20 μ L of each dilution sample and 50 μ L of Palm Essence Sample was added to 250 μ L of pH 8.0 solution. 100 μ L of PMP reagent was added, samples were vortexed and followed the microwave procedure, and acidified following same procedures as described.

RESULTS

Hydrolysis and Castells et. al., 2002 Derivatization

The ten hydrolyzed standards and a palm essence sample were injected into the HPLC column, and separated using the Castells-TFA+PMPA HPLC settings. Chromatograms obtained showed almost no resolution for many standards. The glucose standard shown in Figure 1 appeared not to be retained in the column. Amylopectin, trehalose, and raffinose did show some retention (results not shown), however, since the monosaccharide standards didn't separate well, the method was not deemed to be useful.

Interestingly, the Palm Essence sample did result in a number of separable peaks. The dye peak showed at 5 minutes, and other peaks emerged at 7, 11, 14, 16, 21, 27, 34 and 38 minutes (See Figure 2), however in the absence of standard chromatograms, these peaks could not be interpreted.

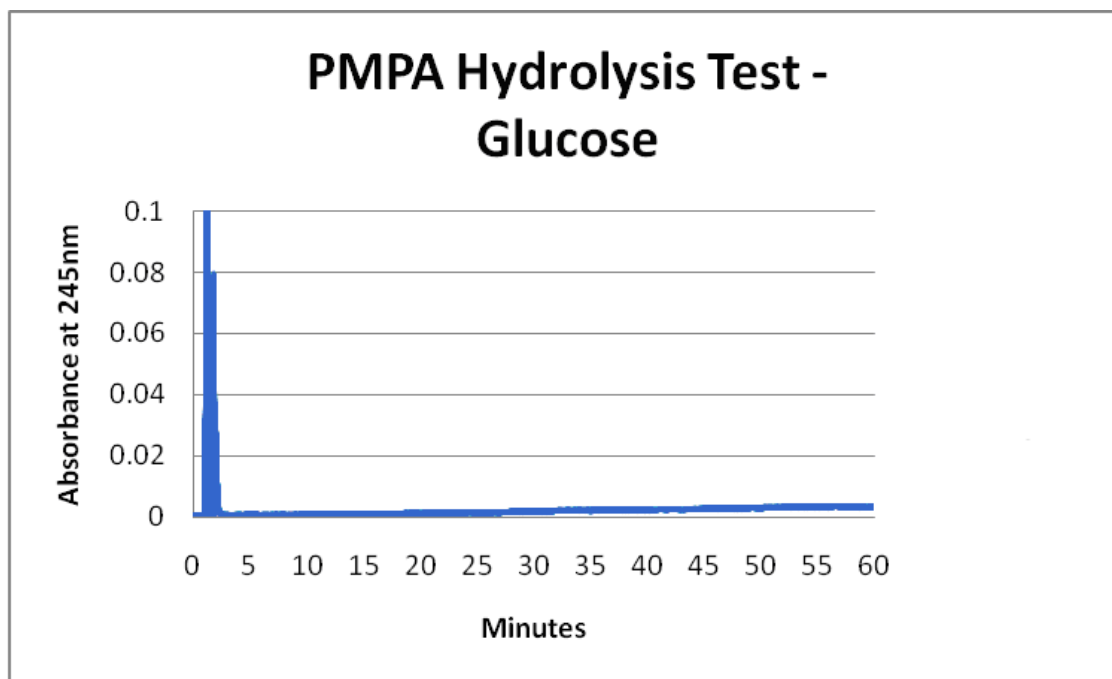


Figure 1: Glucose standard – Castells et al., 2002 Derivatization. A glucose sample derivatized following the method of Castells et al, was injected into the C18 RP-HPLC column and separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate (solvent A) and a acetonitrile (solvent B). (See Methods and Materials section.)

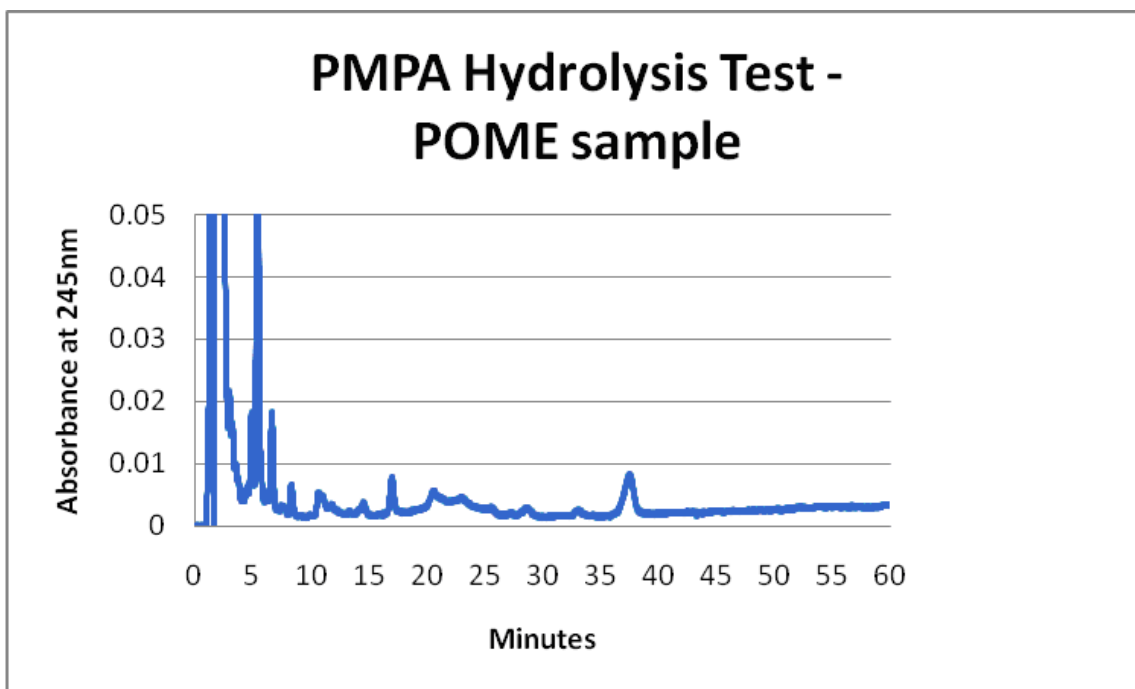


Figure 2: Palm Essence Castells et al., 2002 Derivatization. A POME sample derivatized following the method of Castells et al, was injected into the C18 RP-HPLC column and separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were {a} 0.1M ammonium acetate (solvent A) and {a - check this on all of your figure captions} acetonitrile (solvent B) {Make sure you have (solvent A) and (solvent b) consistently throughout}. (See Methods and Materials section.) *Note: peaks before the 5 minute dye peak are just remnants in the column prior to injection of sample. Seen in every chromatogram.

1M NaOH PMPA

In order to determine whether the hydrolysis procedure or the preparation method for derivatization of carbohydrates was the cause of the analytical problem, monosaccharide standards were derivatized with the PMPA labeling reagent without hydrolysis. The first preparation method of PMPA crystals to make the labeling reagent did not fully dissolve the crystals. The PMPA crystals were still separate from solution in the Castells method, thus changes were made to the reagent preparation to fully dissolve them. Monosaccharides were derivatized following the 1M NaOH PMPA derivatization method and were injected into the HPLC column, following HPLC settings for this method. Chromatogram results showed that sugars were better retained in the system. Prior to running a mixture of sugar standards, single runs for each sugar: glucose, galactose, glucuronic acid, xylose and mannose, were run to define specific retention times (results not shown.) Retention times were as follows: mannose eluted at 7 minutes, glucuronic acid at 12 minutes, glucose at 14 minutes, galactose at 16 minutes, and xylose at 18 minutes. When the mixture was run, the chromatogram revealed most sugars eluted

together, resulting in a large extended peak between 10-16 minutes. Because the standard retention times were previously determined, glucose, glucuronic acid and galactose were considered as the large peak beginning at 10 minutes, and extending until 16 minutes. A small mannose peak was visible at 7 minutes, and a xylose peak at 18 minutes (Figure 3).

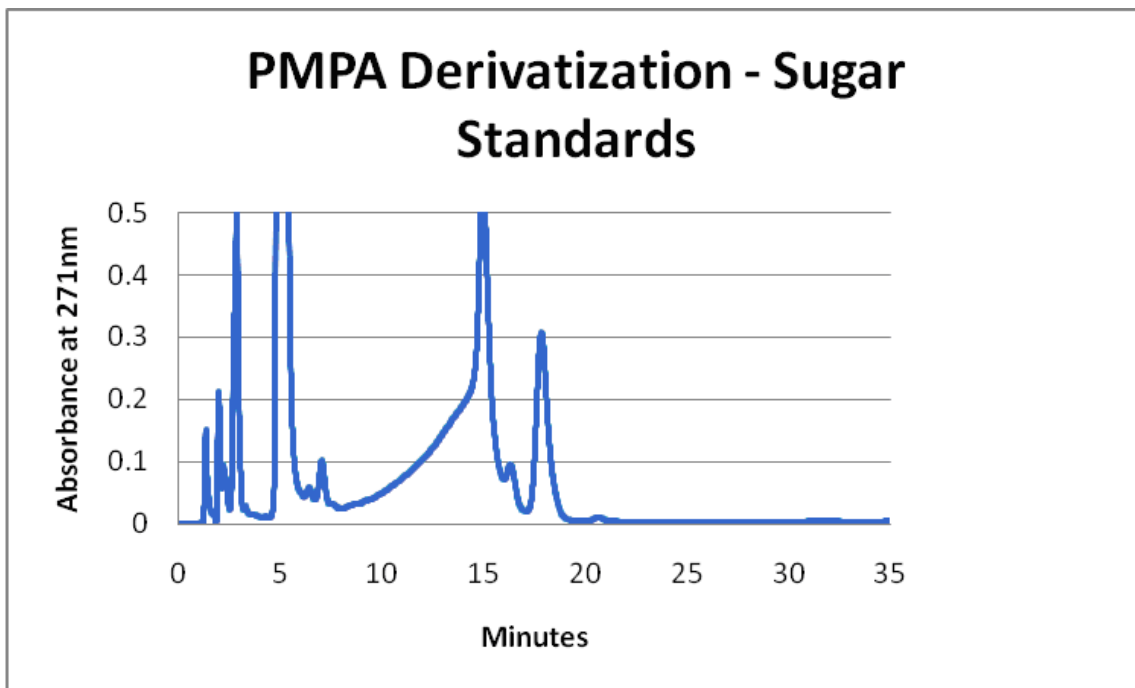


Figure 3: Sugar Standards Mixture. A sugar standard mixture, derivatized following the 1M NaOH method, was injected into the C18 RP-HPLC column and separated using a 25 minute linear gradient from 80%A/20%B to 75%A/25%B. Solvents used were a 0.05% H_3PO_4 Solvent A, and a 5%ACN/ PO_4 Solvent B. (See Methods and Materials Section.)

1M NaOH PMPA Derivatization – B HPLC Settings

Because the previous chromatogram (Figure 3) showed sugar standard mixture peaks unable to be fully separated, HPLC gradient changes were made to stretch out peaks and elution times. The HPLC buffer gradient changes, from a 25 minute linear gradient of an 80/20 solvent ratio ending at 75/25 solvent ratio, to a linear gradient for 5 minutes held at 90/10 then held isocratically at 80/20 for 30 minutes, seemed to barely separate monosaccharide standards. The baseline for each chromatogram was hardly definable; many breakdown dye peaks eluted, making it impossible to identify the actual sugar derivative peak. (Figures 4-7). Sugar standards run alone consisted of the same peaks in each chromatogram. The theory of peak extension, or stretching out, seemed to have worked however, as the dye peak, usually eluting at 5 minutes as seen in Figures 2 and 3, was retained extending from 5 minutes to 10 minutes. Sample preparation of standards were prepared in the same manner as samples prepared in Figure 3, and amounts of

PMPA label reagent for sugars were the same as in Figure 2, thus dye concentrations were the same per sample, and injection volume was the same. The xylose standard seemed to show a single peak at 18 minutes, which was not seen in either the galactose or the mannose run, thus possibly defining the xylose retention time at 18 minutes. However, there are a number of much smaller peaks eluting between 18-25 minutes that are also not evident in Figures 4 and 5. (Compare Figure 6 with Figures 4 and 5). A mannose or galactose peak was unable to be determined, as their chromatograms were nearly identical (Figures 4 and 5). The chromatogram of the standard mixtures did show some retained peaks, however due to the absence of monosaccharide standard chromatograms these peaks were also unable to be identified.

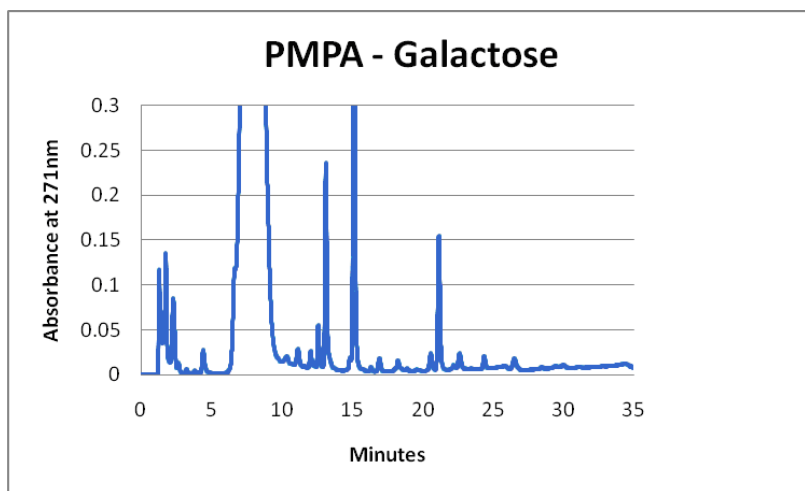


Figure 4: Galactose Standard Chromatogram. A galactose standard was prepared following the 1M NaOH derivatization method, and separated in a C18 RP-HPLC column using the same solvents used as in Figure 3. A 5 minute linear gradient of a 90%A/10%B to 80%A/20%B was run, then held isocratically at 80%A/20%B for 30 minutes. (See Methods and Materials Section.)

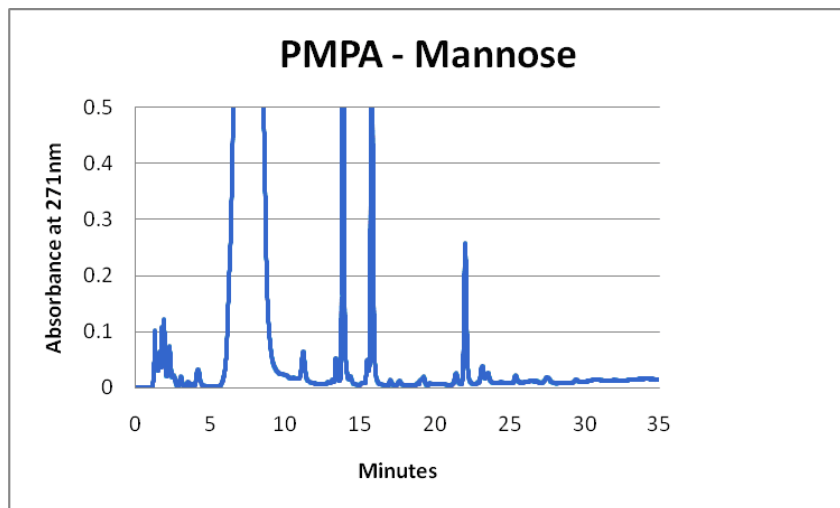


Figure 5: Mannose Standard Chromatogram. A mannose standard was prepared following the 1M NaOH derivatization method, and separated in a C18 RP-HPLC column using the same solvents

used as in Figure 3. A 5 minute linear gradient of a 90%A/10%B to 80%A/20%B was run, then held isocratically at 80%A/20%B for 30 minutes. (See Methods and Materials Section.)

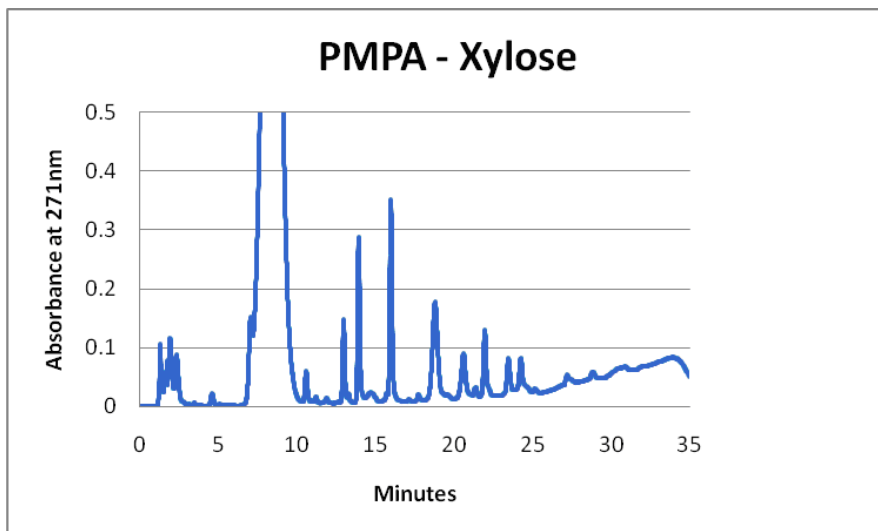


Figure 6: Xylose Standard Chromatogram. A xylose standard was prepared following the 1M NaOH derivatization method, and separated in a C18 RP-HPLC column using the same solvents used as in Figure 3. A 5 minute linear gradient of a 90%A/10%B to 80%A/20%B was run, then held isocratically at 80%A/20%B for 30 minutes. (See Methods and Materials Section.)

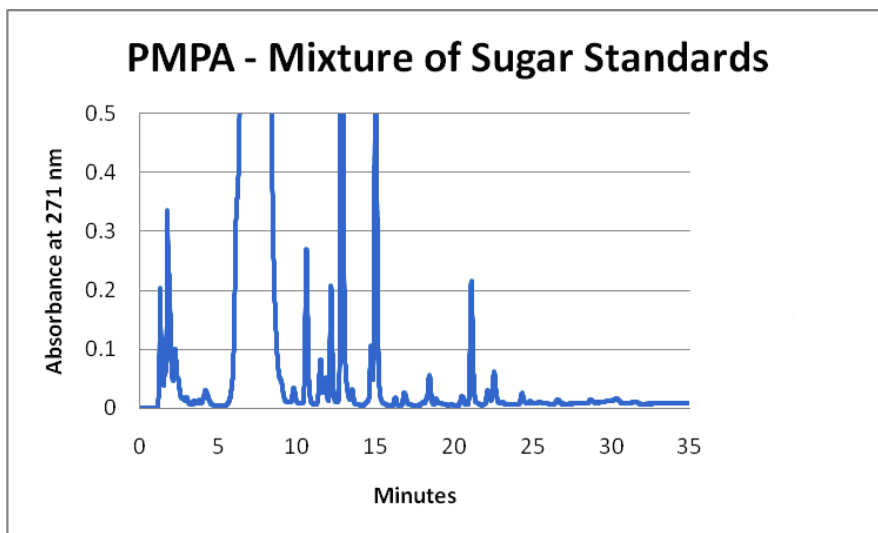


Figure 7: Mix Sugar Standard Chromatogram. A sugar standard mixture sample was prepared following the 1M NaOH derivatization method, and separated in a C18 RP-HPLC column using the same solvents used as in Figure 3. A 5 minute linear gradient of a 90%A/10%B to 80%A/20%B was run, then held isocratically at 80%A/20%B for 30 minutes. (See Methods and Materials Section.)

At this point, PMPA derivatization was abandoned. The ease of the actual derivatization procedure was negated by the complexity of RP-HPLC parameter settings. Thus focus on monosaccharide derivatization and optimization studies was shifted towards the use of 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization.

PMP results

Optimization of monosaccharide analysis following PMP derivatization began with identifying and validating standard thermal heating procedures. Monosaccharide standards were derivatized singly and each injected into the HPLC column. Results defined retention times for each sugar tested: mannose peak showed at 12 minutes, glucuronic acid at 17 minutes, and glucose at 29 minutes. Galactose eluted at 32 minutes and xylose at 34 minutes (Results not shown). Chromatograms obtained from a mixture of the five standards showed good separation. The standard thermal heating mixture displayed good retention of sugars. The dye peak shows at 7.95 minutes. All of the sugars in the sample eluted at times comparable to those of the single standards. (Figure 8).

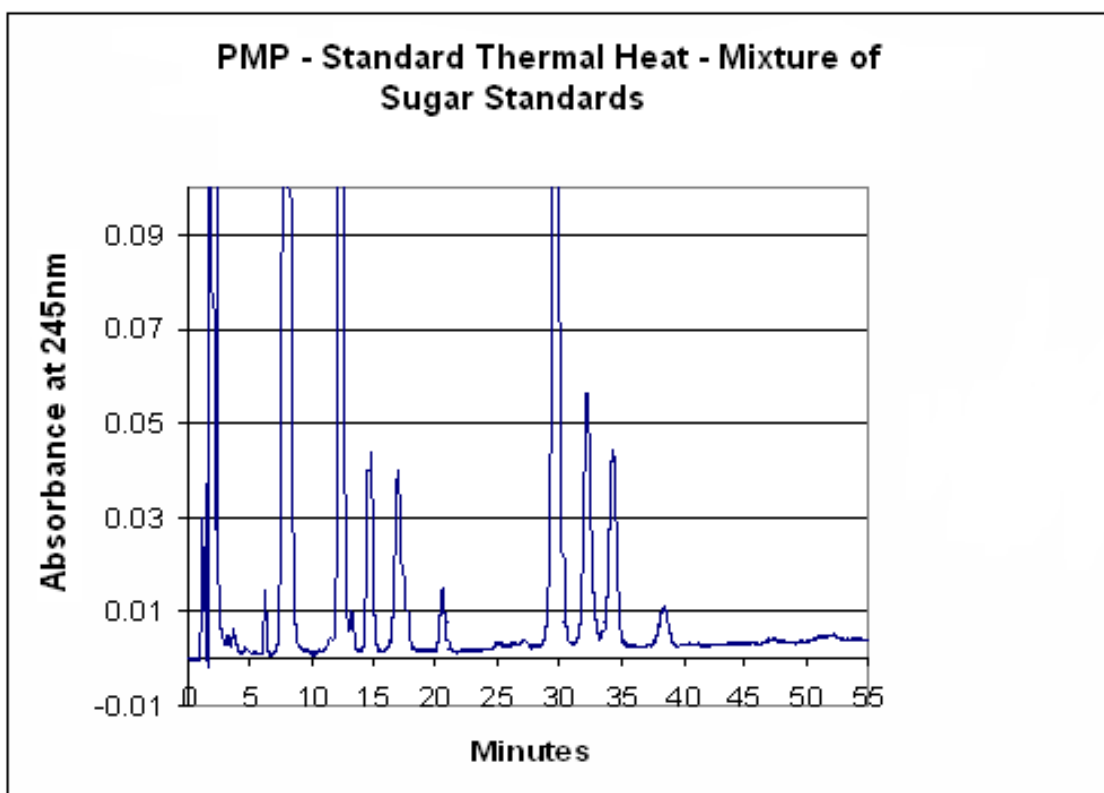


Figure 8: HPLC graph of standard heated mixture of monosaccharide standards. A sugar standard mixture was prepared following the PMP derivatization methods, and injected into the C18 RP-HPLC column separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate solvent (A) and a acetonitrile solvent (B). (See Methods and Materials section.)

Monosaccharide standards heated using microwave irradiation were also prepared and injected into the HPLC column, and separated using the same method as above.

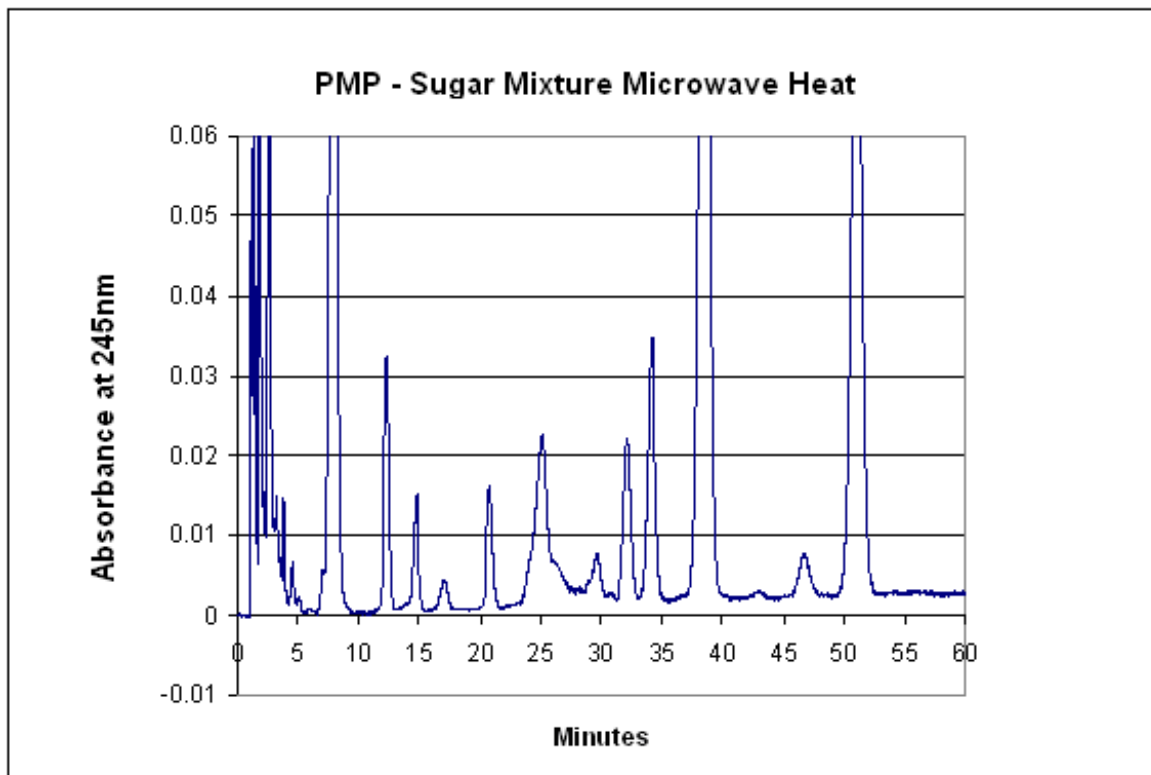


Figure 9: HPLC graph of microwave irradiated mixture of monosaccharide standards. A sugar standard mixture was prepared following the PMP derivatization methods with microwave heat, and injected into the C18 RP-HPLC column separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate solvent A and a acetonitrile solvent B. (See Methods and Materials section.)

The standards following microwave heating procedures had the same retention times as samples heated under conventional practices. Again, single runs of each monosaccharide (not shown) were completed to define retention times, and were used to identify peaks separated in the sugar standard mixture sample with microwave heat. The dye peak elutes again at 8 minutes, and the first sugar, mannose elutes again at 12 minutes. A small amount of glucuronic acid was retained in the sample injection, showing at 17 minutes. At 25 minutes, there is a large peak, the source of which was unable to be determined. Again, a small amount of glucose was detected at 29 minutes. At 32 and 34 minutes respectively, galactose and xylose were again separated. (Figure 9) Through comparison to previous results, we have shown that the change in heating methods does not affect detection parameters or retention times. However, this microwave method seems to produce much dye breakdown, with two large peaks at 38 and 52 minutes.

POME samples were then prepared following thermal heat and microwave irradiation procedures and injected onto the HPLC column following parameter settings same as above. Chromatograms obtained during this run showed very good separation for the thermal heat sample (Figure 10). Galactose was not detected in the POME-PMP derivatized sample, as no peak emerging at 32 minutes was observed. Peaks eluting at 12, 29 and 34 minutes corresponded to mannose, glucose and xylose.

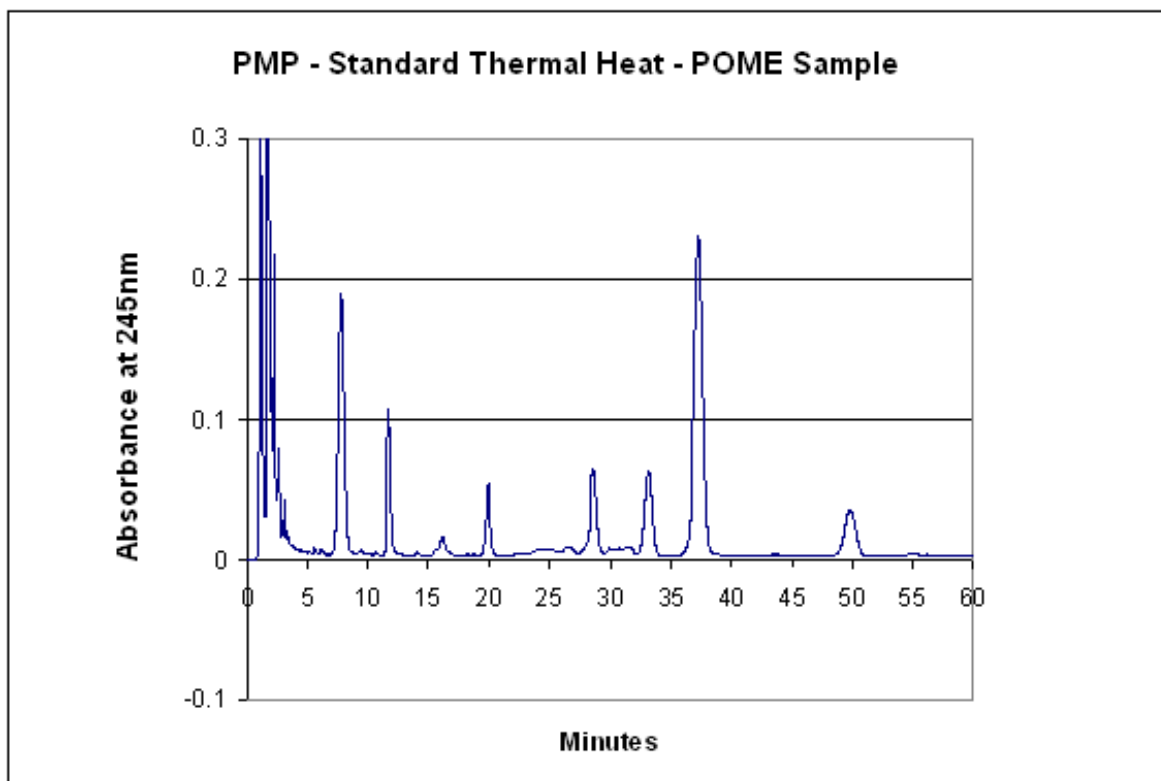


Figure 10: Chromatogram of POME sample derivatized with PMP reagent. A POME sample was prepared following the PMP derivatization methods, and injected into the C18 RP-HPLC column separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate solvent A and a acetonitrile solvent B. (See Methods and Materials section.)

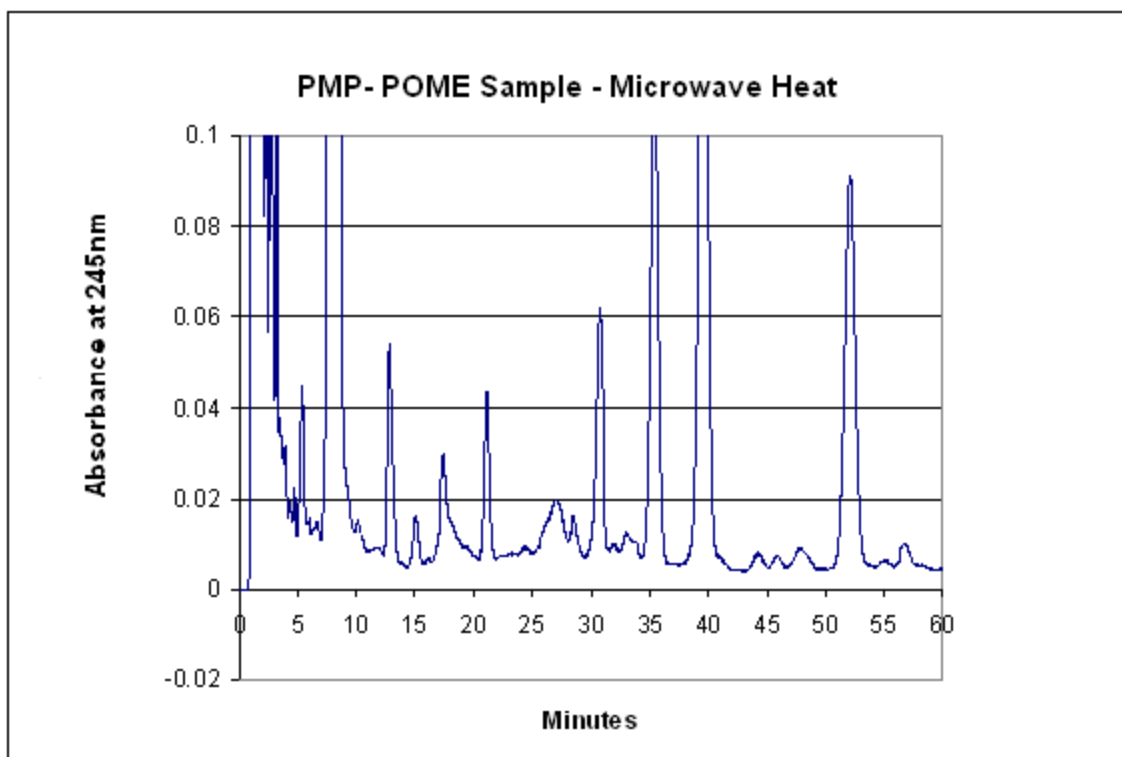


Figure 11: Chromatogram of Palm Essence sample derivatized with PMP microwave. A sugar standard mixture was prepared following the PMP derivatization methods with microwave heat, and injected into the C18 RP-HPLC column separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate solvent A and a acetonitrile solvent B. (See Methods and Materials section.)

Microwave samples produced results comparable to the standard thermal heat sample. The dye peak was slightly larger in the POME-PMP microwave sample than in the conventional heat sample. The sugars in this chromatogram were again mannose at 12 minutes, glucose at 29-30 minutes, and xylose last at 34-35 minutes. Again, it was noted that the dye break down at 39 and 54 minutes were detected in the POME microwave sample.

Further validation of this project lead to developing a calibration of the sugar standards. Known sugar concentrations in equal increments, 0.004ug/mL, 0.008ug/mL, 0.010ug/mL 0.020ug/mL, were prepared for derivatization and injection into HPLC column. Previous microwave results suggested that because of the intense irradiation, dye breakdown increased. Also noted was that the previous method required higher concentrations, in relation to sample size, of NaOH. Required for slightly basic pH stability during derivatization, NaOH also reacted quickly with the dye. Thus preparation of calibration sugar standards used first a pH 7.2 Phosphate Buffered Saline (PBS) and then the same PBS with a pH adjusted to 8.0, to reduce breakdown dye detection in the run. This buffer provides pH stability eliminating any unnecessary use of NaOH. The POME sample prepared using the PBS resulted in a cleaner chromatogram. Break down of dye,

especially at retention times 38 and 54 minutes were not detected, thus clearing up many uninterpreted break down peaks. Sugar detection still remained the same, with retention times in accordance with above samples. (Figure 12)

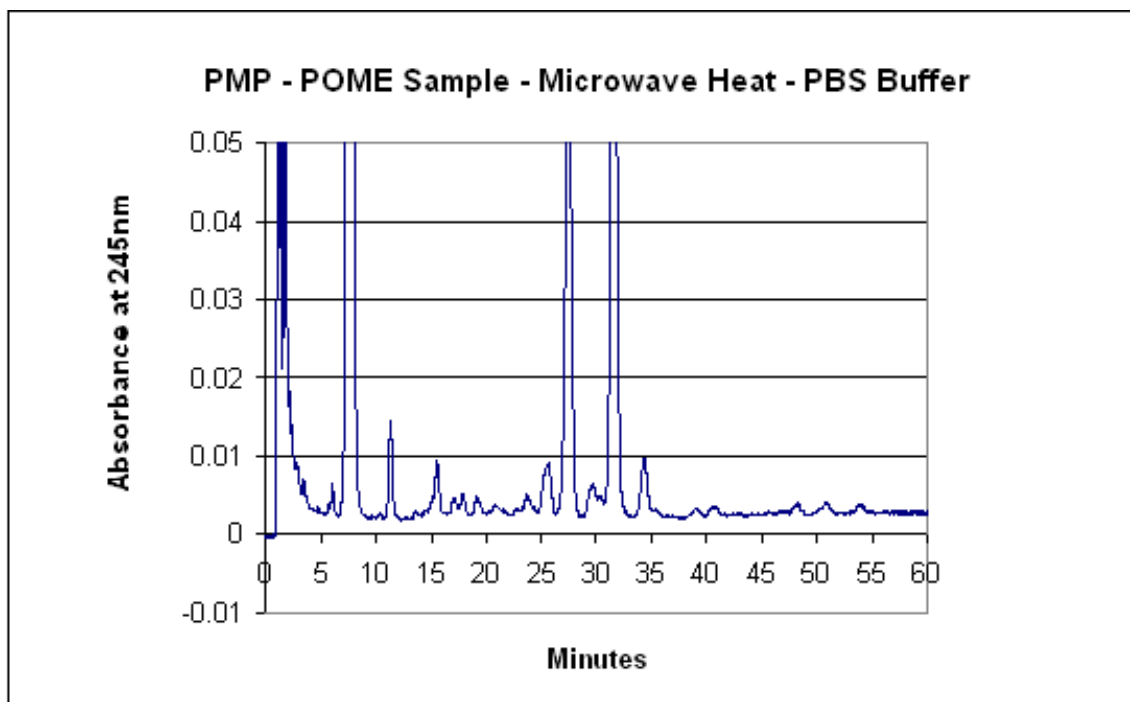


Figure 12: Chromatogram of POME sample made with PBS buffer. A POME sample was prepared following the PMP derivatization methods with microwave heat and use of PBS buffer for pH stability, and injected into the C18 RP-HPLC column separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate solvent A and an acetonitrile solvent B. (See Methods and Materials section.)

Although PBS was shown to reduce dye breakdown, the pH still was just under optimal conditions as interpreted from the papers obtained for this project. Thus, an adjusted pH 8.0 phosphate buffer solution was also used to see if resolution was better. Retention times were defined (results not shown) and separation of sugars in a standard mixture resulted in sugar elution similar to previous tests. Chromatographic results show that the use of this buffer helped maintain optimal pH conditions for the label to react with the monosaccharides, as seen by the clean separation and detection of sugars from the HPLC run. As seen in Figure 13, because the sample amount prepared for analysis was very small, sugar peaks detected were also small. However, when compared to the dye peak run alone comparison with the dye peak run shows the sugar peaks emerging at specific times are specific sugars and not dye breakdown products. (Results not shown)

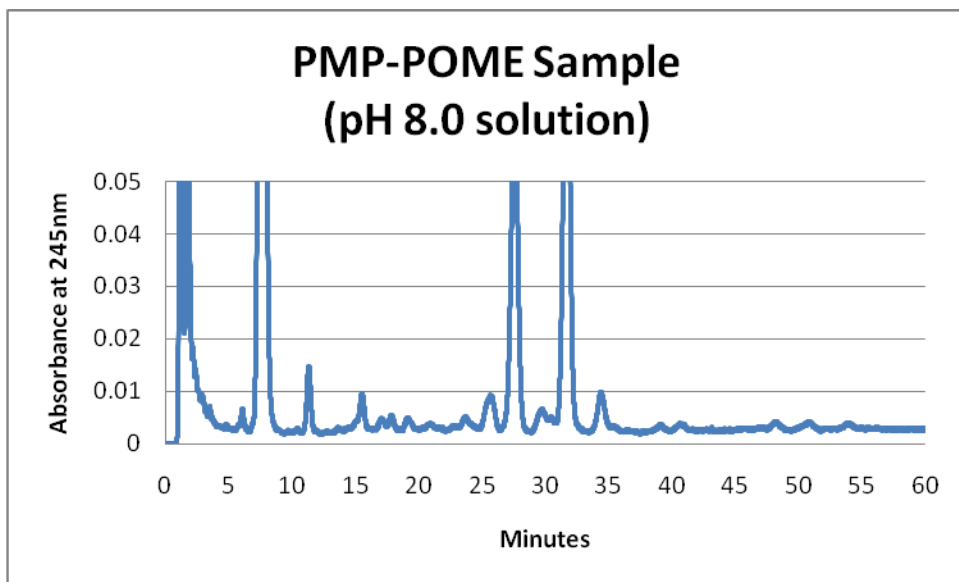


Figure 13: Chromatogram of POME sample made with pH 8.0 buffer. A POME sample was prepared following the PMP derivatization methods with microwave heat and use of PBS buffer for pH stability, and injected into the C18 RP-HPLC column separated using a 60 minute linear gradient from 45%A/55%B to 0%A/100%B. Solvents used were a 0.1M ammonium acetate (solvent A) and a acetonitrile (solvent B). (See Methods and Materials section.)

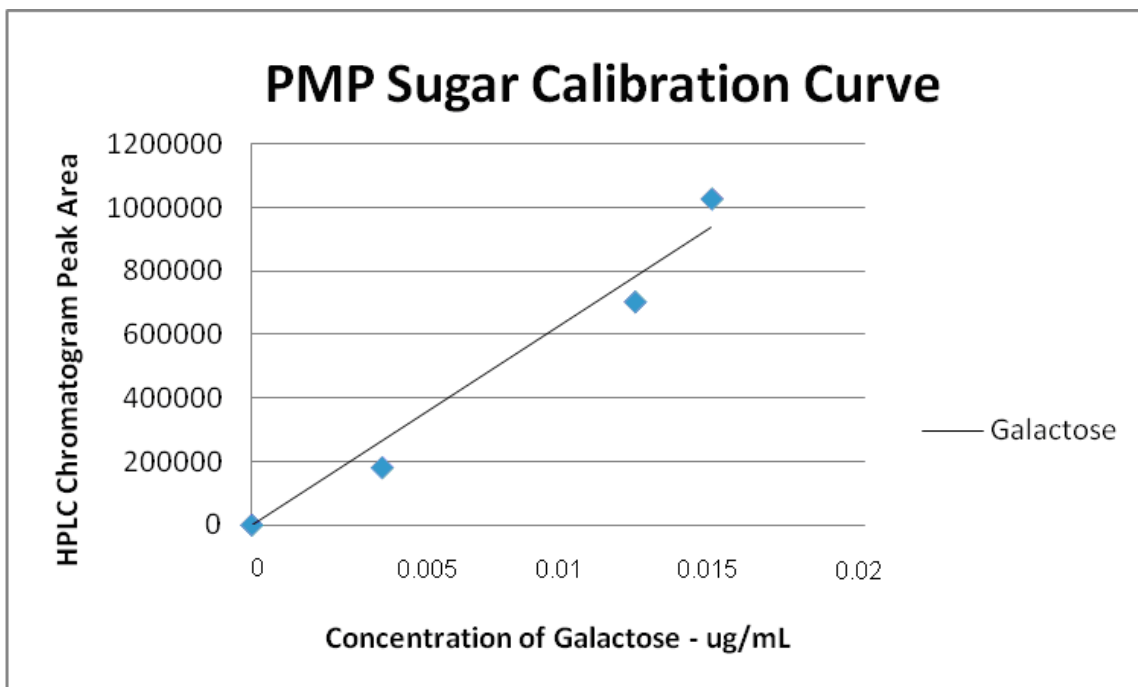


Figure 14: Calibration Curves for Galactose.

The first set of calibration curves and POME sample were prepared with no internal standard. Known monosaccharide concentrations of the standards were observed to correlate to peak area; higher sugar concentrations resulted in larger peak area. The data from the calibration study resulted in some linearity, however the actual data points show some scatter from the resulting trendline (Figure 14). Sugar calibration curves may reach a limit of concentration as peak areas reaching over 500,000 seem not to fully correlate to concentration (Results not shown).

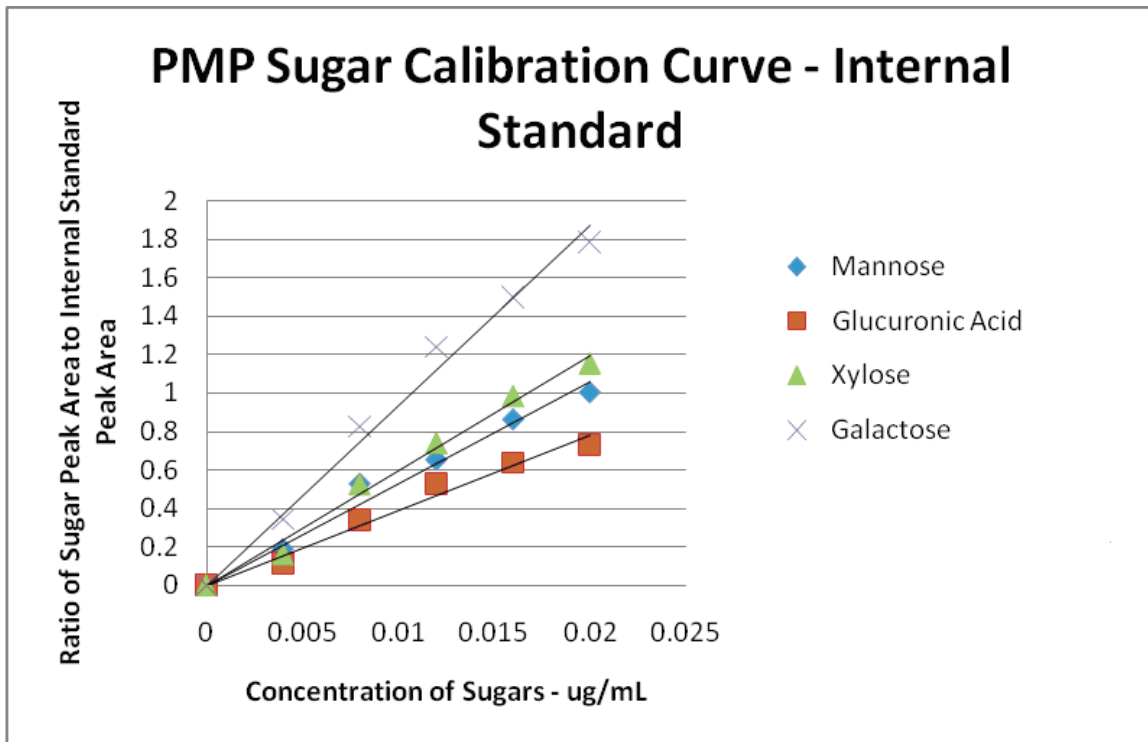


Figure 15: Calibration Curves for sugar standards, with Internal fucose standard.

Calibration curves of sugar standards and the POME sample were then developed in relation to an internal fucose standard. The reason for the internal standard was to provide a ratio of unknown [sugar] peak area to the known concentration of the internal standard that was not used as a sugar standard. This allows for more accurate sugar quantification. The internal standard would also eliminate any discrepancies in peak detection limits, as observed in the previous calibration run. Results from this run showed better linearity (Figure 15).

Palm Essence Samples

50 μ L of POME sample was analyzed directly without dilution. The three determined sugar peaks observed in the chromatogram were xylose, mannose and glucuronic acid. (Figure 13) The following table displays the resulting sugar concentrations for the POME sample, according to the calibration curve equations obtained from internal standard run.

Determined Sugar Concentrations in POME Sample

Mannose	8.5 x 10⁻⁴ mg/mL
Glucuronic Acid (Glucose)	1.03 x 10⁻² mg/mL
Xylose	1.285 x 10⁻² mg/mL

Table 1: Estimated sugar concentrations of free monosaccharides in Palm Essence sample tested.

Microwave results PMPA preliminary data

Preparation of monosaccharide standards and POME was completed following microwave irradiation methods as described. A preparation of a sugar standard mixture sample consisting of glucose, galactose, glucuronic acid, xylose and mannose was tested. Actual sugar quantification was not completed, as since no single labeled monosaccharide was run to define monosaccharide retention time. Several unidentified peaks were observed, in addition to the dye and dye breakdown peaks (Dye only run not shown). The peaks which eluted in the chromatogram emerged at the same time as the retention times for the monosaccharide standards, as compared with previous data, and HPLC chromatograms obtained from the Castells et al., 2002 paper. Separation of sugars was concentrated within the first 15 minutes of the run. The POME sample monosaccharides also seemed to be separated, however in the absence of monosaccharide chromatograms, peaks were not identified.

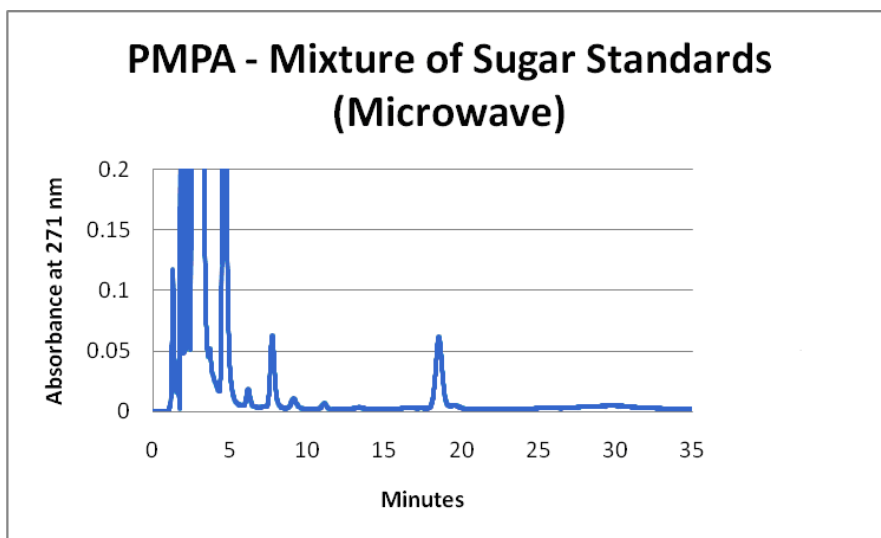


Figure 16: Microwave test with PMPA reagent. A mixture of sugar standard sample was prepared and injected into the C18 RP-HPLC column, and separated following an isocratic gradient for 2 minutes held at 85%A/15%B, then a linear gradient from 2-35 minutes from 85%A/15%B to 40%A/60%B. Solvents used were a 0.05% H_3PO_4 (Solvent A) and a 5%ACN/ PO_4 (Solvent B) (See Methods and Materials Section).

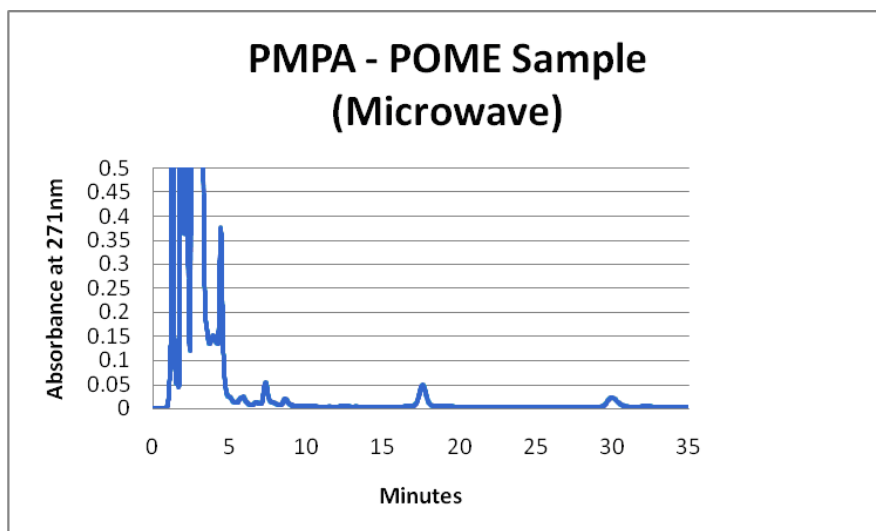


Figure 17: Microwave test of PMPA derivatization with POME. A POME sample was prepared and injected into the C18 RP-HPLC column, and separated following an isocratic gradient for 2 minutes held at 85%A/15%B, then a linear gradient from 2-35 minutes from 85%A/15%B to 40%A/60%B. Solvents used were a 0.05% H_3PO_4 (Solvent A), and a 5%ACN/ PO_4 (Solvent B) (See Methods and Materials Section).

Sugar levels, as seen in Figures 16 and 17, were barely detected by RP-HPLC. In consideration that the sample in regards to PMPA preparation and dervatization methodology was in fault, preparation of samples used NaH_2PO_4 solution, with pH adjusted to 8.0. This was to reduce NaOH concentrations used, as well as maintain a more stable pH. Chromatographic results show better separation, however because monosaccharide retention times were yet to be determined, peaks are still undefined. (Figure 18)

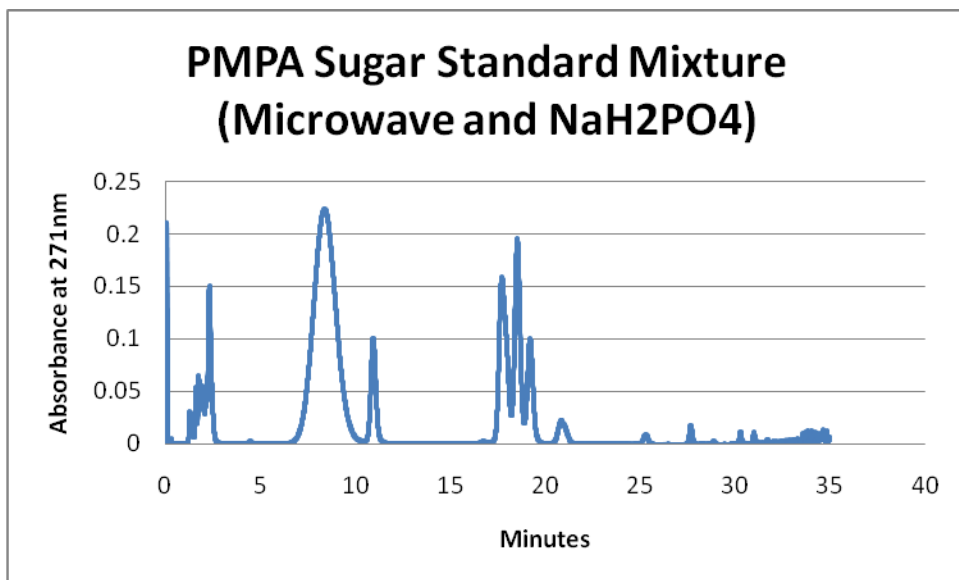


Figure 18: Microwave test of PMPA derivatization with POME and NaH₂PO₄. A POME sample was prepared and injected into the C18 RP-HPLC column, and separated following an isocratic gradient for 2 minutes held at 85%A/15%B, then a linear gradient from 2-35 minutes from 85%A/15%B to 40%A/60%B. Solvents used were a 0.05% H₃PO₄ (Solvent A), and a 5% ACN/PO₄ (Solvent B) (See Methods and Materials Section).

DISCUSSION

Using two different carbohydrate labeling agents, this project sought to improve carbohydrate analysis in palm fruit extracts for industrial or commercial use. Project work began with analysis of palm fruit extracts following published methods of Yang et al., 2005, and Castells et al., 2002. Since primary results suggested derivatization was partial to none, further improvements in methodology and HPLC settings were carried out. The use of microwave irradiation was explored as an alternative to standard thermal heating procedures. The final results obtained from this project suggest the use of PMP, a pH 8.0 solution for dilution of carbohydrates, and microwave irradiation to analyze monosaccharides in Palm Oil Mill Effluent (POME) waste is optimal.

PMPA

Carbohydrate analysis is a daunting task as derivatization procedures often require specific reactive conditions in order for optimal results to be obtained (Yang et al., 2005). Published methods described in papers seemed to be vague and incomplete in their description of PMPA crystal dissolving, at least as was attempted in this project. Dissolving methods stated the use of more MeOH in the solution, however because the crystals didn't dissolve, the next trial involved 1M NaOH added drop-wise for complete dissolving. As mentioned in the paper, the pH range of the solution needed to be within 7.8-8.2 for complete crystal dissolving. Although the paper did use NaOH solution at correct pH, the dissolving method didn't work. This might have been due to instability of pH. Although widely used for raising pH levels, NaOH is highly reactive, and is relatively instable in terms of pH. This reactivity could be seen specifically in microwave irradiation methods. Significant breakdown and oxidization of the labeling agent were detected after HPLC analysis. In addition, it was noted that NaOH may change chirality of carbohydrates. Therefore, the use of PBS buffer, and the pH 8.0 solution were explored in an effort to reduce addition of NaOH. Results suggest better stability during derivatization reactions as HPLC chromatograms displayed cleaner separation. Further investigations into dissolving methods requiring even less NaOH should be considered.

Setting HPLC parameters also required intense troubleshooting; although initial investigations didn't take into exact HPLC settings as described in Castells paper, later analysis using PMPA as a labeling agent used Castells HPLC settings as a base. Gradient of phosphate buffer concentration was gradually changed to extend and retract retention peaks of carbohydrates so as to bring elution of sugars within reliable and reproducible retention times. Although the final results from PMPA derivatization indicate that gradient used for this HPLC run brought all sugars between the 7 to the 25 minute mark on a 35 minute run time, further studies on gradient development may be pursued.

Because the PMP method was mostly resolved, additional tests for PMPA derivatization continued. Microwave irradiation was proven to be an efficient, reliable method for heating purposes, thus radically reducing time required for reaction. If this method were applied to PMPA derivatization, the time required for sample preparation would be reduced, as the dye extraction process is instrumentally simpler with the use of PMPA as a labeling agent. The

preliminary results showed some peak retention and resolution, however further investigation into this method is needed for full, accurate monosaccharide detection.

Hydrolysis

At the time of this writing, only one standard thermal hydrolysis method was able to be tested. Again derived from Castells et al., (2002), the hydrolysis seemed not to provide adequate breakage of the glycosidic bonds which bind monosaccharides to other compounds. Hydrolysis reactions generally require hours of standard thermal heating under acidic conditions, thus allowing bound monosaccharides to become free in solution. Following that procedure, samples are usually dried to complete powder, in order for specific amounts of carbohydrates used for derivatization. To complete this part of the study, experiments should be designed to test concentrations and molarity of the TFA hydrolysis solution. In addition, the use of microwave irradiation for hydrolysis should be considered, as it would drastically reduce time consumed, thus providing rapid analytical techniques that can be employed by commercial and industrial laboratories.

PMP

The methods as described by Yang et al., (2005) and Honda et al., (1989) resulted in adequate separation and detection of free reducing monosaccharides. However, concentrations of labeling agents, as well as excessive amounts of reagent were added for derivatization. Due to finalization of HPLC parameters determined early in the duration of this project, focus was on PMP derivatization of monosaccharides. Hence, optimization of methods was mostly carried out. Final PMP results suggest that preparation of PMP reagent with the pH 8.0 solution maintains a stable slightly basic solution, conducive to the PMP derivatization reaction. Microwave irradiation methods were also optimized, with final microwave times settled at two minutes at half power. Thorough vortexing of samples was determined to be very important during derivatization; because the reaction yields products at a much higher rate, diffusion of labeling agent and sugars will not happen as when left to heat for several hours. Although results suggest optimum levels may have been reached with the completed research, future work determining true optimum pH should be considered.

Palm Oil Mill Effluent waste samples were derivatized with PMP, and free monosaccharide content was determined following the phosphate buffer pH 8.0 – Microwave method as described above. Three main monosaccharides, mannose, glucose and xylose, were present in the samples, at concentrations of 0.00085 mg/mL, 0.0103 mg/mL and 0.01285 mg/mL respectively. In order to complete this study, further investigations into bound monosaccharides of POME samples should be hydrolyzed and made ready for analysis.

Conclusions

Industrial and commercial quality standard tests must be efficient in terms of time consumed in practices. In regards to carbohydrate analysis, the derivatization methods presented in this study were carried out to efficiently reduce time consumed, as well as amounts of solvents and reagents used. PMPA methods, although methodologically simpler in extraction processes still require further optimization. Hence, this study suggests the use of PMP as a derivatizing agent to determine reducing aldoses in POME. Microwave derivatization drastically reduces time spent for reactions, providing a simple, effective technique suitable for a fast pace detection environment.

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APPENDIX A

PHENOLS REVIEW

Antioxidants have been studied as treatments for a variety of long-term illnesses. The concept that oxygen free radicals are damaging to cells helps explain one potential value of antioxidants. More recently it has been proposed that antioxidants, in addition to ridding cells of free radicals, act as catalysts, either as enzymes or co-enzymes, to aid the cell in metabolism, providing the cell with more available energy (Gregg, 1998).

Studies were specifically done on the bioavailability of a common antioxidant, Vitamin C in commercial orange juice. Its effects on prostaglandin levels were observed (Sanchez Moreno et al., 2003) by studying the plasma concentrations of both the prostaglandin and the antioxidant. Sanchez-Moreno et al., showed that consuming 2 glasses of orange juice (500mL) per day reduced the plasma concentrations of the prostaglandin *8-epi*-PGF_{2a}, and increased the plasma concentrations of Vitamin C significantly. This result was more pronounced in smokers, perhaps due to the fact that smoking causes high oxidative stress. This study also showed that at high concentrations of Vitamin C absorption in the gastrointestinal tract favors the interaction between the gastrointestinal acids and the juice. The data implies that the beneficial attributes of high concentrations of vitamin C and the reduced levels of *8-epi*-PGF_{2a} are more pronounced in people with high oxidative stress (Sanchez-Moreno et al., 2003)

MATERIALS AND METHODS

Phenol Calibration Curve Preparation

Prior to beginning the analysis, Gallic Acid (GAE) stock solution (15% Sodium Carbonate solution and 10% Folin Ciocalteu Reagent) was prepared. Phenol calibration standards were prepared by adding 0 μ L, 100 μ L, 200 μ L, 300 μ L and 500 μ L of GAE stock solution to 10mL dH₂O. This dilution series yielded 0, 50, 100, 150 and 250mg/L gallic acid phenol samples for a complete range.

POME Sample Preparation

POME 1 sample was diluted as a 1:5 solution. 2mL of OPP 1 was added to a 10mL volumetric flask, and made to volume with dH₂O.

POME 2 sample was diluted as a 1:10 solution. 1mL of OPP 2 was added to a 10mL volumetric flask and made to volume with dH₂O.

Phenol Calibration Method

400 μ L of each dilution factor and POME sample was added to another 10mL volumetric flask. 2mL of FC reagent was then added, and after 8 minutes, 800 μ L of Na₂CO₃ was added. Samples were vortexed. Two sets of dilutions and OPP samples (A and B – refer to Table 1) were read three times on a spectrophotometer set to 760nm.

RESULTS

The determination of phenolic acids was also completed in this study. Total phenols in GAE equivalents was observed in POME samples diluted 1:5 and 1:10 respectively OPL1 and OPL2. GAE is considered to be the most prevalent and most active phenol in most fruit extracts with high antioxidant concentrations. Results from the spectrophotometer readings support the fact that POME waste is naturally high in antioxidants. (Refer to Tables 1A, 2A and 3A). Efforts in sample hydrolysis should be conducted after monosaccharide detection experiments reveal mannose, xylose and glucuronic acid/glucose sugars present in POME samples. The monosaccharides may be bound to phenol compounds, thus {giving – appearing to have} higher concentrations of antioxidants than are actually present.

Phenol Calibration

The results obtained from the known [phenol] solutions were averaged to determine a calibration curve. The ratio of spectrophotometric values to [phenol] closely followed good linearity, with the equation $y = 0.002x$ (Refer to Figure 1A). Scatter among data samples was also low.

Sample ID	Reading 1 760nm	Reading 2 760 nm	Reading 3 760nm	Average	Standard Deviation
0 – A	0.000	0.000	0.000	0.000	0
50 – A	0.132	0.118	0.135	0.128	0.009074
100 – A	0.262	0.212	0.268	0.247	0.030746
150 - A	0.240	0.256	0.314	0.270	0.038936
250	0.440	0.476	0.493	0.467	0.02062
0 – B	0.000	0.000	0.000	0.000	0
50 – B	0.130	0.142	0.124	0.132	0.009165
100 –B	0.228	0.263	0.219	0.257	0.023245
150 – B	0.232	0.248	0.322	0.267	0.048014
250 –B	0.450	0.434	0.456	0.449	0.011372

Table 1A : List of Sample ID and Spectrophotometric Reading at 760nm. Note: Reading three was completed two hours after the first two readings. FC reagent, GAE stock solution, and Na₂CO₃, along with Palm Essence Samples were left at room temperature.

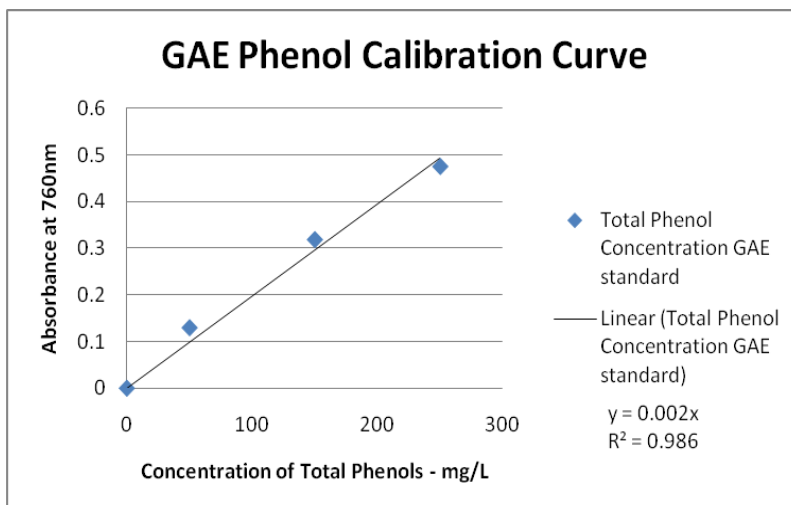


Figure 1A: Calibration Curve of Phenolic Acid using GAE as standard phenol. Equation of line: $y = 0.0019x$.

Determination of GAE Total Phenol in POME

Equation of Line from calibration curve is $Y = 0.0019X$; Y being Absorbance at 760nm, and X being concentration.

POME Sample 1 – diluted 5X

Absorbance at 760nm (Y value)	Concentration of GAE total Phenols (X value)	Multiplied by Dilution Factor – 5X
0.107	56.3	281
0.127	66.84	334
0.207	105.7	525
0.121	63.68	318
0.136	71.57	358
0.195	102.6	513

Table 2A: Absorbance values of POME 1 sample readings and dilution factor following calibration curve line $Y = 0.0019X$. Spectrophotometer set to 760nm, sample read 6 times and averaged together.

From the data presented above, the average total phenol concentration for the POME -1 sample is 388 mg/L +/- 33.

POME Sample 2 – diluted 10X

Absorbance at 760nm (Y value)	Concentration of GAE total Phenols (X value)	Multiplied by Dilution Factor – 10X
0.682	358.9	3589
0.617	324.7	3247
0.535	281.6	2816
0.687	361.6	3616
0.687	361.6	3616
0.543	287.3	2873

Table 3A: Absorbance values of POME 2 sample readings and dilution factor following calibration curve line $Y = 0.0019X$. Spectrophotometer set to 760nm, sample read 6 times and averaged together.

From the data presented above, the average total phenol concentration for the POME – 2 samples, diluted to 10X is 3292mg/L +/- 506.

Phenol Conclusion

Total phenols in terms of a GAE standard were determined in POME as to help with developing a basic idea of antioxidant content. Results showed that high concentrations of antioxidants were present 388 mg/L +/- 33 and 3292mg/L +/- 506 respectively. After determination of sugars present, extraction of main monosaccharides from the sample may also provide altered results in total phenol concentrations, as monosaccharides may be bound to phenolic compounds. Further work in this area should also be considered.

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