# DEVELOPMENT OF A “GREENER” HPLC-UV METHOD FOR THE ANALYSIS OF REDUCING SUGARS IN APPLE JUICE AND INDIGENOUS FRUITS USING ACETONE AS AN ALTERNATIVE SOLVENT

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE (CHEMISTRY)

OF

THE UNIVERSITY OF NAMIBIA

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OCTOBER 2023

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**ABSTRACT**

Chromatographic techniques can be made more environmentally friendly, i.e., “greener”, by a number of different strategies. One approach is to replace the toxic mobile phase solvents such as acetonitrile with greener alternatives. Acetone, in particular, has proven to be a suitable alternative to acetonitrile, since the two solvents have similar physicochemical properties, including solubility, miscibility and viscosity properties. However, due to acetone’s high ultraviolet (UV) cut–off wavelength (330 nm), it normally cannot be used as a mobile phase solvent when performing high performance liquid chromatography (HPLC) analysis with UV detection. In this study, a reversed-phase HPLC–UV method using acetone-containing mobile phase was developed for the determination of reducing sugars in apple juice and two indigenous fruits, *Berchemia discolor* and *Hyphaene petersiana*. Pre-column derivatisation of analytes via reductive amination with *p*-aminobenzoic acid ethyl ester (ABEE) was performed to enable photometric detection at 307 nm. The method was directly compared to a method that utilised acetonitrile-containing mobile phase. Although the detection wavelength of the ABEE derivatives is below the UV cut–off wavelength of acetone, it is high enough above acetone’s absorbance maximum (~280 nm) to enable satisfactory detection of the derivatives. Hence, the method compared well with the acetonitrile method, providing similar resolution and selectivity, as well as sufficient sensitivity to facilitate the quantitation of glucose and fructose in all the fruits and juice investigated in this study. Although the method was only validated in terms of precision, linearity, limit of detection and quantitation but no other aspects such as accuracy, it shows potential to be used as a greener alternative for sugar analysis for laboratories that only have access to HPLC–UV instruments.

## LIST OF CONFERENCE PROCEEDINGS

A. Iyambula, S. Louw, K. Kalili. Development of a "greener" HPLC-UV method for the analysis of reducing sugars in fruit juice and indigenous fruits using acetone as an alternative solvent. Proceedings of the 17th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology. 18-20 May 2022, Ghent, Belgium.

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **%RSD** | Percent relative standard deviation |
| **%v/v** | Percentage volume per volume |
| **µL** | Microliters |
| **µmol** | Micromole |
| **ABEE** | Amino benzoic acid ethyl ester |
| **ACN** | Acetonitrile |
| **AcOH** | Acetic acid |
| **AP** | Aminopyridine |
| **CE** | Capillary electrophoresis |
| **DNA** | Deoxyribonucleic acid |
| **DNPH** | Dinitrophenylhydrazine |
| **EHS** | Environmental, health and safety |
| **ESI** | Electrospray ionization |
| **FID** | Flame ionization detector |
| **FTIR** | Fourier-transform infrared spectroscopy |
| **GAC** | Green analytical chemistry |
| **GC–MS** | Gas chromatography–mass spectrometry |

| | |
|---|---|
| **HILIC** | Hydrophilic interaction liquid chromatography |
| **HPAEC–PAD** | High-performance anion-exchange chromatography with amperometric detection |
| **HPLC–CAD** | High performance liquid chromatography with charged aerosol detection |
| **HPLC–ELSD** | High performance liquid chromatography with evaporative light scattering detection |
| **HPLC–FLD** | High performance liquid chromatography with fluorescence detection |
| **HPLC–UV** | High Performance liquid chromatography with ultraviolet detection |
| **LCA** | Life-cycle assessment |
| **LC–MS** | Liquid chromatography– mass spectrometry |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantification |
| **M** | Molarity |
| ***m/z*** | Mass-to-charge ratio |
| **MALDI** | Matrix laser desorption ionization |
| **mAU** | Milli-absorbance unit |
| **MeOH** | Methanol |

| | |
|---|---|
| **mM** | Millimolar |
| **nm** | Nanometer |
| **NMR** | Nuclear magnetic resonance |
| **$R^2$** | Coefficient of determination |
| **RID** | Refractive index detector |
| **RP–HPLC** | Reversed-phase high performance liquid chromatography |
| **RPLC** | Reversed-phase liquid chromatography |
| **S/N** | Signal-to-noise |
| **SSB** | Sugar sweetened beverages |
| **UHPLC** | Ultra high performance liquid chromatography |
| **UV** | Ultraviolet |
| **UV-vis** | Ultraviolet-visible |

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**ACKNOWLEDGEMENTS**

I would like to express my deepest appreciation to Prof. Stefan Louw for the excellent supervision, guidance, prudent scientific attitude and strong support throughout my BSc and MSc studies. Prof. Louw taught me the basic principles and operation of various HPLC systems and groomed me into becoming an HPLC method developer. I would not have achieved and gained this knowledge without him.

I further extend my acknowledgement to Dr. Kathithileni Kalili who was actively involved in this research by providing constructive suggestions, advice, and technical support and also for her motherly support. A special word of appreciation goes to Prof. Frederic Lynen (Ghent University, Belgium) for the LC–MS analysis. I also want to thank Mrs. Tekla Tjipura for her mentorship.

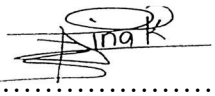
I would like to thank the University of Namibia for the study platform given, the Department of Physics, Chemistry and Material science for the laboratory space and materials used in the research. Further appreciation goes to the Seeding Labs Instrumental Access 2018-19 grant where the HPLC system was obtained. I would like to further acknowledge the Namibian Student Financial Assistance Fund (NSFAF) for funding my studies, I would not have managed without them.

Lastly, I would like to appreciate my family for the great love and support and my son Namulo Wilson for being patient with my studies.

**DECLARATION**

I, Aina Kawiikalelwa Iyambula, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.





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# CHAPTER 1

## 1. INTRODUCTION

### 1.1 Background of the study

One important aspect of method development in chromatography is the assessment of the "greenness" of the method i.e., how environmentally friendly it is. The term 'environmentally friendly' means not harmful to the environment. When developing methods especially those that could be used for routine analysis mostly in quality control laboratories, it is of utmost importance to ensure that the method does not have any negative impact on the environment and the health of the operator. Therefore, many studies in various fields have focused on the topic of green chromatography, which include the use of techniques and methodologies that "reduce or eliminate solvents, reagents, preservatives and other chemical reagents that are hazardous to human health and the environment while enabling faster and efficient analyses without compromising performance criteria" [1]. In the area of liquid chromatography, analysts are interested in developing new green methods or even making the existing methods greener. Acetonitrile is the solvent of choice in liquid chromatography due to its favourable physicochemical properties, however, the solvent is considered toxic due to its high half-life in water and it is less biodegradable. Replacement of acetonitrile is a wise alternative to make analytical methods greener. Green chemistry in the context of solvent replacement gained much attention in 2008 after the shortage of acetonitrile which led to a dramatic increase in the price of acetonitrile. Even today, the cost of acetonitrile is approximately four times higher than that of the commonly used solvents such as methanol [2].

Sweet carbohydrates are normally referred to as sugars and they contribute to sensory properties of fruits and fruit juices [3]. Monosaccharides such as fructose, glucose, galactose and disaccharides such as sucrose and maltose have a sweet taste characteristic [4]. Reducing sugars are defined as sugars that have aldehyde or ketone group in basic solution which allow the sugar to act as a reducing agent [5]. Examples of reducing sugars are glucose, fructose, galactose, maltose and maltotriose. These sugars play a role in various food and beverages as nutrients and enhancing sensory properties [6]. Processed fruit juices contain natural sugars and some sugars may be added to enhance the sweetness. In the field of nutrition, sugar analysis in foodstuffs provides data that can be included on food labels, which aid to monitor the consumption level, and hence regulate diseases such as diabetes. Sugar sweetened beverages (SSB) are associated with type 2 diabetes [7] and cancer risks [8]. Sugars show less susceptibility to changes during processing and storage of foodstuffs compared to other components present in food and beverages such as antioxidants, hence the sugar composition of foodstuffs serves as a reliable quality control tool for authenticity and adulteration of foodstuffs [3].

Indigenous fruits are found in most African countries such Namibia, Angola, Botwana, Tanzania,Ghana, Malawi, South Africa and Zimbabwe, they provide nutritional and health benefits to rural people as they are rich in phytochemicals. *Berchemia discolor* and *Hyphaene petersiana* are two common indigenous fruits mostly harvested in the Northern part of Namibia, both fruits are sources of food, income and are used to make wine as well [9]. The fruits are also used to make value added products like juice, jam and cakes which can stimulate industrial growth, create employment and increase the conservation of indigenous fruits. The carbohydrate content of the indigenous fruits can be used to monitor

the authenticity of the fruits' value-added products. To the best of our knowledge, no study was done to determine the sugar profile of the two indigenous fruits and hence there is a need to be evaluate the sugars present considering their benefits to human lives.

Different analytical techniques are used for qualitative and quantitative analysis of carbohydrates. Spectroscopic techniques such as high resolution nuclear magnetic resonance (NMR) and mass spectrometry are used to provide structural information of complex carbohydrates as well as information on the purity of carbohydrates. MS identifies compounds based on their mass-charge ratio (m/z) where compounds are ionized before entering the MS analyzer. Electrospray ionization (ESI) and matrix laser desorption ionization (MALDI) are the two common ionization methods used for sugar analysis, they enable ionization of carbohydrates that provide molecular ion information [10] . MALDI uses a matrix with analytes that has strong absorption at laser wavelength for ionization. The laser pulse ablates the dried sample and the matrix molecules sublimate into gas phase. The ions are formed through proton transfer and are accelerated by electric field towards mass analyzer. In electrospray ionization, the sample is introduced to a strong electrostatic field through a narrow capillary to form small charged species which are detected by mass spectrometry. The advantage of MALDI is that ionization efficiency is constant with increasing size of neutral carbohydrates while ESI ionization efficiency decreases with increasing molecular size. The limitation of MALDI is that is gives extensive fragmentation due to high internal energy deposited on the ions compared to ESI [10]. ESI ionization method was recently used for the analysis of carbohydrates with both HILIC and RPLC modes [11]. Gas chromatography (GC) is another analytical technique used for carbohydrate analysis, especially low molecular carbohydrates and it

is a helpful technique in identifying unknown carbohydrates. Gas chromatography with mass spectrometry (GC-MS) is often used for carbohydrate identification and gas chromatography with flame ionization (GC-FID) for quantification. Capillary electrophoresis (CE) is another technique used for carbohydrate analysis, charged compound are separated based on their electrophoretic mobility influenced by electric field. However, there are drawbacks associated with this technique such as carbohydrates are neutral, and they lack chromophores. High performance liquid chromatography (HPLC) is the main and popular analytical technique used for carbohydrate analysis. HPLC provide additional advantages over other methods such fast analysis, allow analysis of larger carbohydrates and it is versatile owing to the availability of various detectors, stationary phases which allow analysis to be done in various separation modes such as ion-exchange, ion-exclusion, reversed phase and hydrophilic interaction liquid chromatography (HILIC) mode [12].

The analysis of sugars can be quite challenging because of their intrinsic properties: lack of chromophores, similar polarity and low volatility. The existence of anomers presents another challenge for carbohydrate separation. This has led to the development of different stationary phases for carbohydrate analysis.

Ultraviolet-based detection methods are simple, sensitive, and mostly available as primary detection methods in most laboratories. They allow the of gradient elution which shortens the analysis time thus reducing solvent consumption and cost. Equally important, methods that can be applied to as many sample matrices as possible are needed to cover a broad range of foodstuffs and beverages [13]. Due to an increase in fruit juice production, there is a need to develop cost-effective, sensitive and reliable methods for fruit juice quality control [14].

## 1.2 Statement of the problem

HPLC generates litres of organic waste which might be toxic to the environment and the operators, therefore there is a need to switch to greener or environmentally friendly solvents. Although acetone is classified as a green solvent, its application in liquid chromatography is seldom reported because its high ultraviolet (UV) cut-off has hindered researchers from using this solvent. Another problem is that most of the reported methods for the analysis of sugars have utilized expensive detectors which might not be economical for routine analysis, most especially in small or resource-limited laboratories. There is no perfect method for carbohydrate analysis yet, hence effort to develop simple, robust, reliable and reproducible methods for routine analysis is still an ongoing endeavour in carbohydrate research. Photometric detectors serve as common detectors for all HPLC applications, hence there is a need to develop simple photometric methods for sugar analysis. The use of acetone in reversed-phase liquid chromatography (RPLC) employing UV detectors for carbohydrate analysis has not been attempted. Moreover, the sugar profiles of the two indigenous fruits have not been studied.

## 1.3 Objectives of the study

The objectives of the study were to:

(a) Develop a RPLC-UV method for the analysis of reducing sugars.

(b) Compare the separation performance of acetone as a "green" alternative mobile phase to acetonitrile in reducing sugar analysis.

(c) Validate and apply the method to the analysis of reducing sugars in apple juice, *Berchemia discolor* and *Hyphaene petersiana* fruit extracts.

## 1.4 Significance of the study

The method developed in this study could potentially be used by quality control laboratories of fruit juice manufacturers already equipped with HPLC-UV instruments. The method would enable manufacturers to perform high throughput analysis of sugars in fruits and fruit juices, hence increasing the laboratories' efficiency. The method would also help with product labelling purposes and identification of possible adulteration. The developed method may serve as a starting point to expand this analytical approach to other applications in the food and beverage industries. In the context of indigenous fruits, the results may help with value-addition to fruits as these fruits can potentially be used to develop potential marketable products such as juice, cakes, etc. This method would be economically beneficial as it would reduce the costs for detoxification or disposal of toxic solvents. Furthermore, the method does not use expensive detectors but common UV detectors that resource-limited laboratories can afford. This study is the first to use acetone in RPLC-UV mode for sugar analysis.

## 1.5 Limitation of the study

The method is limited to reducing sugars only.

# CHAPTER 2

## 2. LITERATURE REVIEW

### 2.1 Sugar analysis in fruits and fruit juices

Sugars serve as a source of energy, and they contribute to the sensory characteristics of fruits and juices. In the fruit juice production sector, sugars are used for quality control to monitor adulteration and ensure fruit juice authenticity. Adulteration of fruit juice involves the addition of corn, cane and beet sugar, cheaper fruit juice concentrates, preservatives, artificial sweeteners and colours [15,16].

Fructose, glucose and sucrose are the major sugars present in fruits and vegetables [17]. Hvizd et al. [18] analysed sugars in various fruit juices such as apple, orange, grape and cranberry juices using hydrophilic interaction liquid chromatography with charged aerosol detector (HILIC–CAD). The results showed that glucose, fructose (**Fig 2.1**) and sucrose are the main sugars present in these fruit juices. Recently, Rodriquez et al. [19] simultaneously analysed organic acids and sugars in various fruit juices using high performance liquid chromatography with refractive index detector (HPLC–RID). The major sugars present in the fruit juices were sucrose, fructose and glucose. The sugar profiles and amounts varied with each juice, with grape juice showing the lowest sucrose concentration. In a recent study by Li [3], the sugar profiles of fruit juices including that of apple juice were determined by HPLC–RID. They found fructose (Fru) and glucose (Glu) in both fruit juices but the ratio of Fru/Glu was unique for each fruit juice. The ratio of Fru/Glu is crucial for the evaluation of fruit juice authenticity.

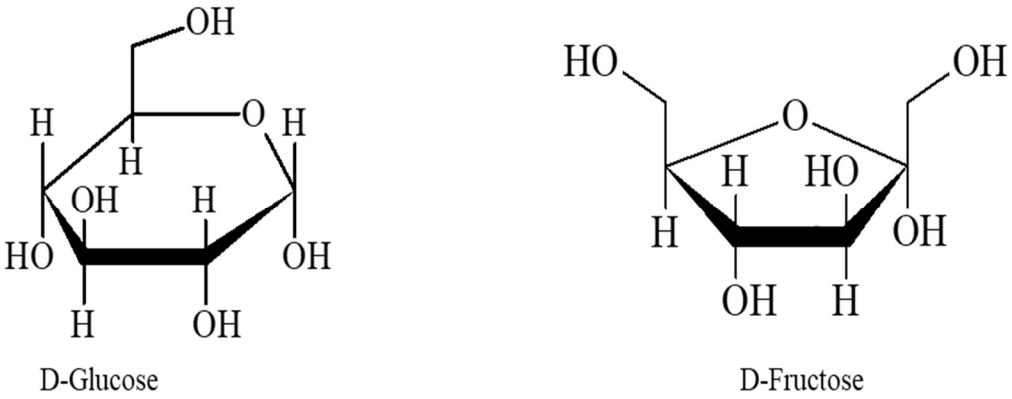


**Fig 2.1** Common reducing sugars found in fruit juices.

### 2.2 Methods for the analysis of sugars

Different analytical methods have been developed for the qualitative and quantitative analysis of sugars in different sample matrices. The method or analytical approach normally depends on the sample matrix and the purpose of analysis. Gas chromatography (GC), either in combination with MS or flame ionisation detector (FID), is one of methods that is used for the analysis of carbohydrates in different matrices such as biological samples [20], food [21] and beverages [22]. GC-MS analysis offers high sensitivity, selectivity and the ability to identify analytes [21]. MS methods provide a broad scope of chemical information on small molecular carbohydrates and are more suitable for qualitative analysis, whilst FID is used more for quantitation, hence detection methods are selected based on the purpose of analysis. Due to low volatility and poor ionisation efficiency of carbohydrates, time-consuming derivatisation and high temperatures are often required to achieve satisfactory analysis in gas chromatography [21,23]. In most cases, silylation, methylation, trifluoroacetylation and acetylation are the most popular derivatisation techniques used for carbohydrates [23]. These derivatisation methods alone are not ideal for reducing sugars due to a variety of isomers formed in the aqueous

solution. These isomers result in numerous peaks on the chromatogram, which makes it difficult to interpret. To solve this, reducing sugars are converted to oximes, where the cyclic hemiacetal is converted into a corresponding open-chain molecule. Consequently, two oximes are produced from each sugar molecule via an oximation reaction (*Z* and *E* configurations) resulting in two chromatographic peaks, thus making the chromatograms less complex [20,21,24].

Capillary electrophoresis (CE) is a powerful technique which offers separation of sugars with high resolution. CE differs from HPLC in the sense that it uses an open tube capillary instead of a chromatographic column. CE offers advantages such as ultra-small sample volumes, low solvent consumption, short analysis time, high-resolution and minimal sample preparation [25]. However, when CE is used with UV detection, derivatisation of sugars is required due to the lack of chromophores which may lead to a decrease in sensitivity and resolution [26].

Sugars can also be quantified using enzymatic methods whereby reactions such as hydrolysis and phosphorylation are used. In these methods, sugar quantities are determined by measuring the pH variation of the sample relative to the amount of $H^+$ and $OH^-$ ions produced during enzymatic reactions, or using the commercial enzyme assay kit to measure absorbance. The enzymatic methods are however difficult and costly, especially when analysing unknown sugar content such as those found in complex natural products [21]. Enzymatic methods are more suitable for known sugars. When analysis of unknown sugar contents is performed, then qualitative techniques such as GC–MS are used to identify the sugars present in the sample before subjecting the sample to enzymatic digestion [21]

Liquid chromatography is the most popular analytical technique for low and high molecular weight carbohydrates due to its high sensitivity, high recoveries, good accuracy and ability to perform both qualitative and quantitative analyses [23,27]. Sugar derivatives are mainly analysed using either ion exchange chromatography, reversed-phase liquid chromatography (RPLC) or hydrophilic interaction liquid chromatography (HILIC). Ion exchange chromatography has been used for sugar analysis in various samples such as proteins and peptides, due to its high sensitivity and selectivity. Anion exchange chromatography with pulsed amperometric detection specifically, was used for the analysis of sugar and sugar alcohols in olive plant extract. The separation was based on the electrostatic interaction of the sugar anion analytes and the positively charged stationary phase [28]. The pH plays an important role in the separation, hence, the pH of the mobile phase is adjusted in order to convert the neutral sugars to their desired sugar ions to achieve separation [29].

RPLC separates carbohydrates on non-polar stationary phase, usually carbon chain bonded to silica surface such as $C_8$ and $C_{18}$ columns. The mobile phase is usually a mixture of water and an organic solvent with or without a buffer to maintain the pH and ionic strength [30]. In this mode, carbohydrates are separated by means of partitioning between the mobile phase and the stationary phase [31].

The most frequently used detectors for liquid chromatography in carbohydrate analysis are the refractive index detector (RID), evaporative light scattering detector (ELSD), ultraviolet-visible (UV-vis) or fluorometric detectors, pulsed amperometric detector (PAD) and charged aerosol detector (CAD) [32]. Refractive index detection is widely used for direct detection of sugars [33], however this method is often characterised by poor sensitivity and it is incompatible with gradient elution [24,34,35]. Refractive index is

temperature sensitive and tend to deviate with change temperature changed hence temperature needs to be carefully maintained during the analysis [4]. Aerosol-based detectors such as the ELSD and CAD have also been used for the analysis of sugars in various matrices [34]. These detectors do not require sample derivatisation, however they are not as common as UV detectors due to their high cost. Moreover, these detectors are both nonlinear, and are restricted to the use of volatile mobile phase only. ELSD has low sensitivity with high limit of detection, whereas CAD provides moderate sensitivity [24,36]. UV is a primary detection method for most HPLC analyses due to its high sensitivity, ability to detect analytes present at low concentrations and it is compatible with gradient elution [37]. UV detection has also been employed in the analysis of sugars, however due to lack of UV chromophores in sugars, this method requires derivatisation of samples for possible detection.

HILIC is also one of the separation modes in HPLC that plays a powerful role in carbohydrate research. In this mode, carbohydrates are separated on a polar stationary phase and water-miscible polar organic solvents such as acetonitrile, aprotic solvents such dioxane and tetrahydrofuran and low molecular alcohols such as ethanol usually containing small amounts of water to create a water enriched layer on the stationary phase. The separation is based on the partition of the analyte between the aqueous layer and the bulk organic mobile phase. The analytes which are more soluble in the organic mobile phase are eluted first. Currently there are wide ranges of polar phases usually bound on organic polymer matrices or silica. Polar phases such as bare silica, cyano bonded, diol bonded, amino bonded, amide bonded, alkylamide and zwitterionic phases are available [38]. The analysis of sugars using these stationary phases is comprehensively discussed

in literature [39]. HILIC has been a powerful mode for the separation of polar compounds such polar peptides and drugs, vitamins, metabolites, polyols, and carbohydrates. HILIC offers an advantage to effectively separate polar compounds which cannot be separated with RPLC mode [38,39]. The mobile phases used are compatible with mass spectrometry making the method even more sensitive [38].

Various researchers have reported the use of HILIC mostly coupled to the universal detectors such as CAD and MS for the analysis of sugars. For example Hutchinson [2] applied HILIC-CAD to the analysis of carbohydrates using an Asahipak $NH_2$P-504E column with the main focus of evaluating alternative solvents to acetonitrile and methanol has also been report. Recently, Pitsch et al. [13] presented the simultaneous analysis of sugars, polyons and ions ($K^+$, $Br^-$, $Cl^-$, $NO^{3-}$and $SO_4^{2-}$) using HILIC–CAD. Hvizd et al. [18] also reported a HILIC–CAD method for the analysis of sugars in fruit juice and honey samples has been reported. The method had a limit of detection of < 10 ng. In this mode, the separation of carbohydrates depends on their nature and interaction with the stationary phase. Fu et al. [40] pointed out that neutral carbohydrates are separated by means of partitioning interaction and ionic carbohydrates are separated by partitioning and electrostatic interactions. The buffer concentration and pH are used to control the ionic interaction between carbohydrate analytes and the stationary phase while temperature is used to alter the chromatographic resolution. Furthermore, the difference in selectivity of the stationary phases shows potential in the design of HILIC/HILIC systems for complex carbohydrate samples. Different HILIC columns are available for carbohydrate analysis and most have been evaluated and shown to have different selectivity and retention behaviors [34,40]. In 2014, Inoue [39] reviewed traditional and novel HILIC columns for carbohydrate analysis. Selection of a suitable column for the optimum separation depends

on the interaction between the stationary and analytes under study, mobile phase, column temperature, the type and buffer concentration, sample solvent, sample matrix, etc. HILIC–MS methods for the analysis of sugars in foodstuffs have also been developed [16,41,42]. HILIC–MS offers an advantage of high selectivity and sensitivity for sugar analysis, however, the HILIC–MS methods are suitable for the analysis of polar or free carbohydrate molecules [43].

One interesting fact about HILIC is its ability to analyse both labelled and unlabelled carbohydrates. Neville et al. [44] reported the analysis of ABEE-labelled oligosaccharide hydrolysate for the identification of glucose units using HILIC–UV. HILIC separates UV chromophore-labelled and fluorophore-labelled carbohydrate based on increasing polarity and the degree of solvation [44].Bawazeer et al. [20] reported the analysis of labelled sugars by reductive amination in biological samples using HILIC–MS. The method was simple with improved separation of sugars in a short time.

**2.3 Derivatisation reactions for Sugars**

Carbohydrates lack chromophores or fluorophores in nature, hence derivatisation is often necessary when UV and fluorimetric detection methods are being used. Most derivatisation methods used in liquid chromatography alter the carbonyl group of the sugar and introduce a photometric or fluorimetric tag. Photometric and fluorimetric tags can be attached either before introducing the sample into the chromatographic column (pre-column derivatisation) or after the chromatographic column (post-column derivatisation). For carbohydrates, pre-column derivatisation has been used more frequently and various derivatisation reactions have been explored. This method eliminates sample matrix and selective enrichment of derivatives is often achieved [45]. Common derivatisation

reagents for carbohydrates include 2,4-dinitrophenylhydrazine (DNPH) [24], 2-aminopyridine (2AP), 2-aminobenzoic acid (anthranilic acid), *p*-aminobenzoic acid [45,46] and *p*-aminobenzoic acid ethyl ester [31,47–52]. Derivatisation of reducing sugars can be achieved through the formation of hydrazine or pyrazolones and reductive alkylation, however these methods suffer from incomplete derivatisation and formation of side products [24].

The attachment of photometric and fluorimetric tags by reductive amination using an aromatic amine and reducing agents is frequently used. In this method, the derivatisation tag is connected to the reducing end of the monosaccharide. The reaction is normally carried out at low pH so that the acyclic form of the sugar is favoured over the cyclic form. The reaction requires the use of reducing reagents that are stable under acidic conditions such as sodium cyanoborohydride (**Fig 2.2**) [20]. Reductive amination offers uncomplicated reaction procedures and transforms various compounds of interest such as mono- and di-saccharides, amino sugars and uronic acid into sensible detectable fluorescent and photometric derivatives [46]. Derivatisation of sugars with *p*-aminobenzoic ethyl ester (ABEE) has also been investigated [47,48]. ABEE derivatisation offers simplicity, eliminates doublet peaks [47] and it is highly sensitive [50]. Another advantage of ABEE is its dual nature of being both a UV-absorbing chromophore and fluorophore. Derivatisation reduces the polarity of the sugars making RPLC a suitable separation mode for these sugar derivatives [53].

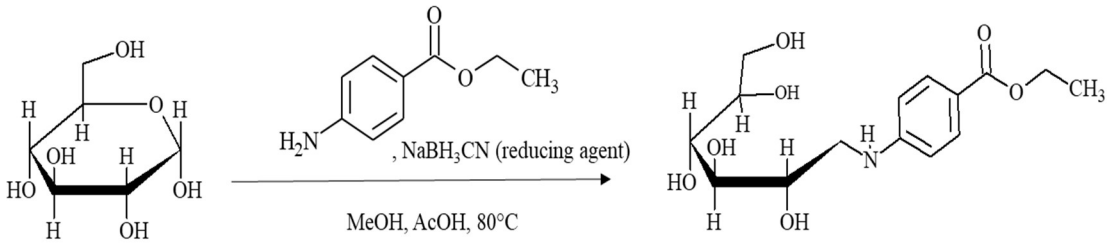


**Fig 2.2** Derivatisation reaction of glucose with ABEE [46].

### 2.4 Green chemistry in HPLC

In green chemistry, analytical chemists aim to make laboratory practices more environmental benign, which includes the use of methods, products, processes, technologies, feedstocks, by-products, solvents and reagents that are less hazardous to human and the environment without compromising performance criteria [54,55]. Green chemistry also aims to prevent and reduce chemical waste, risk of exposure and waste disposal costs. Although the entire laboratory process may not be benign at all stages, it is vital to make some stages greener where possible.

HPLC is the most widely used analytical technique in most chemical laboratories and in quality control laboratories. Besides the benefits of HPLC systems such as fast analysis, high throughput analysis, good sensitivity and high reproducibility, this technique presents threats to the environment and human health as it uses organic solvents which are toxic and generates large quantities of chemical waste especially in routine analysis environments. Therefore, reducing solvent consumption and replacing toxic solvents with benign solvents is highly desirable [56] to achieve a balance between separation performance and eco-friendliness.

One way to achieve this is by using short columns packed with small particles. Short columns require shorter analysis time than longer columns, thus solvent consumption is reduced. Although these columns are often characterised by high back pressures, ultrahigh performance liquid chromatography systems have been introduced to accommodate high back pressures generated by small particles. Moreover, the use of columns packed with small fused-core particles which generate low back pressure is advised.

Another approach of greening LC is using silica-based stationary phase (type-C columns) with unique features such as silica hybrid and ligands compared to the normal silica-based columns. These columns offer fast equilibration time between gradient analyses compared to other commercially available columns, thus, reducing solvent usage in liquid chromatography. They have the ability for dual retention of both polar and non-polar compounds which enables their use in both reversed-phase liquid chromatography and normal-phase liquid chromatography. Another attractive feature of type-C columns is their ability to retain polar compounds at high water content which potentially make liquid chromatography methods green. These stationary phases provide alternatives to greening liquid chromatography and are worth evaluating during method development [57].

**2.5 Alternative solvents in HPLC**

Replacement of toxic solvents with eco-friendly solvents in mobile phases is one approach of enhancing the greenness of the method. The degree of greenness of a solvent is assessed in terms of its environmental, health and safety (EHS) and life-cycle. Environmental aspects include water contamination and air hazard, human safety includes acute and chronic toxicity, irritations, while safety looks at how stable, reactive, flammable and explosive the solvent is. Life-cycle assessment (LCA) focuses on biodegradability,

recyclability, and reusability of a solvent. A green solvent must possess good EHS and LCA properties [58]. Acetonitrile (ACN) is the generic organic solvent in liquid chromatography due to its favourable physicochemical properties such as low UV cut-off. However, the use of acetonitrile in HPLC as a mobile phase is not a greener choice due to its high flammability, volatility, toxicity, high costs of purchase and disposal [54]. Acetonitrile presents chronic and acute toxicity to aquatic life due to its high half-life (2-20 days) in water [1], and therefore is not a solvent of choice from the sustainability point of view.

Methanol is less toxic compared to acetonitrile, although it is not easily biodegradable, it has lower waste disposal costs compared to acetonitrile. Methanol should therefore be chosen over acetonitrile whenever possible [56]. The difference in selectivity of methanol and ACN however hinders the use of methanol as a direct substitute for ACN [59]. Despite the favourable LC properties of acetonitrile and methanol, these solvents are more expensive compared to other commonly used HPLC solvents, hence their use in LC is not economically beneficial. Suggested alternative solvents to acetonitrile include acetone, ethanol, ethyl acetate, *n*-propanol and ethyl lactate [58,60].

Evaluation of alternative benign solvents is pertinent when developing HPLC methods for routine analysis. Acetone is a great alternative mobile phase to acetonitrile because it gives similar separation and selectivity to acetonitrile. In addition to being inexpensive, it has desirable chromatographic properties such as low toxicity, low viscosity, low boiling point which improves ionisation, thus increasing sensitivity in LC–MS. However, acetone is seldom used in LC because it has a high UV cut-off which limits photometric detection. So, the use of acetone is only convenient with universal detectors such as ELSD, RID and CAD. However, these universal detectors also have drawbacks,

for example, RID is not compatible with gradient elution, while CAD and ELSD are not sensitive at low impurity level [59]. Due to its high UV cut-off, using acetone with UV detection in gradient elution results in a rising baseline with increasing acetone content, however this is less of a problem when baseline correction systems are used [61].

Hutchinson and co-workers [2] attempted the use of acetone as an alternative to acetonitrile for carbohydrate analysis in the HILIC mode with corona charged aerosol detection (CAD). Although acetonitrile outperformed acetone, acetone gave satisfactory separation compared to other solvents evaluated such as methanol, ethanol, and isopropanol. In a recent work, Heaton and co-workers [62] illustrated the use of acetone in HILIC coupled to electrospray ionisation (ESI) mass spectrometry for the analysis of small molecules. Interestingly, acetone gave superior signal-to-noise (S/N) ratios, selectivity and reduced analysis time compared to acetonitrile. Although acetonitrile often outperforms acetone, the latter is preferred as it combines superior separation performance with greenness. Furthermore, Hutchinson et al. [2] stated that the combination of benefits offered by green solvents such as economical sustainability, lower toxicity and acceptable separation might outweigh the separation performance compromised by such solvents. In other words, a green solvent with acceptable separation is better than a toxic solvent with outstanding separation performance.

In the rapid communication by Fritz and the co-workers [63], acetone was shown to be a substitute to acetonitrile in peptide analysis: it gave similar separation and reduced analysis time compared to acetonitrile. The author recommended the use of acetone as an

alternative to acetonitrile in liquid chromatography – mass spectrometry (LC–MS) analysis of peptides. Keppel and co-workers [64] presented another successful application of acetone in RPLC employing MS detection for peptide analysis. Another application of acetone in RPLC mode investigated metabolite profiling of complex plant extracts using UV and charged aerosol detection methods [1].

Ethanol is also considered as a greener solvent due to its low toxicity, short life cycle in water, low volatility and its possible synthesis from renewable sources. Ethanol has been used mostly in the field of pharmaceutical analysis to improve the greenness of the methods already developed [65,66]. Although ethanol-water mobile phase has high viscosity [60], it is no longer a challenge when ultrahigh performance liquid chromatography (UHPLC) instrumentation is used or even working at elevated temperatures [54,59]. Applications of ethanol mobile phases are widely reported in literature [65–68].

**2.6 Background information on the indigenous fruits under investigation**

Indigenous fruits play various roles in the well-being of people ranging from food security, health promotion and improving. the economic welfare of rural communities in the developing world [9]. These fruits are native to various African regions such as Namibia, Zimbabwe, Angola, and Botswana. The fruits are sources of vitamins, minerals, and antioxidants. However, they are undervalued because they are unknown in terms of nutritional content, therefore there is a need to evaluate their nutritional content for value addition. There is a lack of scientific knowledge on various indigenous food [9,69], lack of targeted laboratory analyses which can be routinely used for specific quality control tests in case of formal marketing, product identification and development as well as

ensuring the health and safety of consumers [69]. In addition, the sugar profiles of many indigenous fruits are not known.

*Hyphaene petersiana* commonly known as makalani palm and *Berchemia discolor* are two popular indigenous trees in many African countries. In Namibia, the plants are mostly.distributed in the northern part of Namibia such as Omusati, Oshana and Ohangwena regions. The latter (**Fig 2.4 B**)bear fruits which are small drupes of 4-8 mm wide with an average length of 20 mm [70] (**Fig 2.3A**). The pulp contains minerals (K, Ca, Mg, P) [70], high sugar (30%), vitamin C (65 mg/100g) and various phytochemicals such as terpenoids, flavonoids, phenols and cardiac glycosides [71]. The fruits can be consumed fresh or dry [71] and can be fermented to make wine [70–72]. The fruits are used to make nutritious value-added products such as juices, jam and muffins have been made from these fruits. The products had a long shelf-life and scored the sensory preference of 90% [72].

*Hyphaene petersiana* is a single stemmed plant that grows up to 18 m with characteristic fan-shaped leaves of 1.5-2 m long [73] (**Fig 2.4 A**). The fruits are spherical brown nuts, about 50 mm in diameter and single seeded [74] (**Fig 2.3B**). The foliage is used to make different craft objects. The fruits are eaten when fresh or dry, and are used to make a liquor, locally known as ombike in Oshiwambo. The tree is commonly used for wine tapping [73,74]. Moreover, the pulp of the fruits contain carbohydrates (5.33 mg/g), various minerals and phytochemical compounds such as phytates, oxalates, tannins and saponins [74].


**Fig 2.3** Indigenous fruits: (A) *Berchemia discolor* and (B) *Hyphaene petersiana* (Source: Aina Iyambula, Windhoek).


**Fig 2.4** Indigenous plants: (A) *Hyphaene petersiana* and (B) *Berchemia discolor* (Source: Hilde Shaningi, Windhoek).

# CHAPTER 3

# 3. MATERIALS AND METHODS

## 3.1 Chemicals and materials

HPLC-grade acetone, methanol, and acetonitrile (ACN) as well as analytical grade glacial acetic acid and formic acid were obtained from Merck (Germany). Purified water was obtained from a Milli-Q Integral 3 water purification system. The following chemicals were of analytical grade and were purchased from commercial sources: ammonium formate, sodium cyanoborohydride, fructose, glucose, *p*-aminobenzoic acid ethyl ester (ABEE) and chloroform. Filtration was done using a Millipore vacuum pump (model WP6122050) (Merck, Germany), Whatman® filter paper (150 mm) and Millipore Millex-HV Hydrophilic PVDF 0.45 µm pore size syringe filters were purchased from Merck (Germany). The pH was measured with the Thermo Scientific$^{TM}$ Eutech$^{TM}$ pH 6+ pH meter (Singapore).

## 3.2 Instrumentation

### 3.2.1 HPLC analysis

Chromatographic analyses were performed using an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, degasser, auto-sampler, and variable wavelength UV-vis detector. The UV detection wavelength was set at 307 nm for all analysis. The RPLC analyses were performed on a Perkin Elmer Brownlee Analytical $C_{18}$ 150 mm × 4.6 mm I.D. column packed with 3 µm particles. For the HILIC analyses, a Waters XBridge Amide 4.6 mm × 100 mm column with 3.5 µm particles was used. The column temperature for both separation modes was maintained at

40°C, using a Perkin Elmer Flexar oven. An injection volume of 5 µL was used and the mobile phase flow rate was 1 mL/min. Agilent Chemstation software (Rev. B.03.01) was used for instrument control and data acquisition. Mobile phase A consisted of 5 mM ammonium formate buffer at pH 3 in 5% organic modifier and mobile phase B consisted of 5 mM ammonium formate buffer at pH 3 in 95% of the organic modifier.

### 3.2.2 LC–MS analysis

The LC–MS analyses were performed on a Thermo Scientific™ Vanquish™ UHPLC coupled to an Orbitrap Q Exactive™ Plus MS (Thermo Fisher Scientific, Germany). A method adapted from the optimised isocratic acetonitrile-containing mobile phase method was used for the LC–MS experiments. A Kinetex XB-$C_{18}$ (150 mm × 2.1 mm I.D) column packed with 2.6 µm particles was used at a flow rate of 0.2 mL/min, a column temperature of 30°C and with the following mobile phase gradient: 8%B, hold 20 min, 8 – 100%B in 5 min. and hold 4.5 min. All analyses were performed using ESI in negative ion mode ($ESI^-$), with the source temperature maintained at 300°C and an electrospray capillary voltage of 3000 V. A mass range of *m/z* 200 – 2000 was scanned. Xcalibur™ software (version 4.4.) was used for instrument control and data acquisition, while FreeStyle™ software (version 1.7) was used for data processing.

### 3.3 Derivatisation procedure

The sugars were derivatised according to the procedures reported by Gomis et al. [47]. A volume of 400 µL of a 1.4 M aqueous solution of $NaBH_3CN$, 400 µL of glacial acetic acid and 2 mL of 0.6 M ABEE in methanol was added to 5 mL of each standard (2 mM) and sample solution. The mixture was heated at 80 °C for 10 min. After cooling to room temperature, 2 mL of water was added. The resulting solution was subsequently washed

three times with 4 mL of chloroform to remove excess ABEE. The aqueous phase was filtered with a 0.45 μm PVDF filter prior to HPLC analysis.

### 3.4 Sample and standard preparation

A working standard mixture of the ABEE derivatives for preliminary experiments was prepared by mixing 10 μL of each of the derivatised 2 mM stock solution of glucose, maltose, maltotriose, lactose, and diluted with the derivatised 2 mM fructose stock solution in an HPLC vial to 1 mL. The glucose and fructose standard mixture was prepared by mixing 10 μL of glucose (0.02 mM) and 990 μL (1.98 mM) of fructose stock solutions. The 100% apple juice (5 mL) was diluted (see section 3.6) and derivatised. The indigenous fruits, *Hyphaene petersiana* palm fruits and *Berchemia discolor* berries, were bought from a local market. The fruit samples were prepared by homogenising about 5 g of the fruit pulp in 50 mL of Milli-Q water. The solution was allowed to stir at room temperature for 24 hours. The solution was vacuum filtered, and 5 mL of the filtrate was diluted (see section 3.6) and subjected to the derivatisation procedure. The resulting solution was filtered through a 0.45 μm PVDF filter prior to HPLC analysis. The samples were stored in the freezer to prevent microbial growth.

### 3.5 Method validation

#### 3.5.1 Linearity, limit of detection and limit of quantitation

The detector linearity was determined by injecting a series of standard solutions in the range of 1 to 30 mM. The linearity curve was constructed by plotting the detector response against concentration. The limit of detection (LOD) of each sugar derivative was determined using signal-to-noise ratio method by injecting a series of diluted standards

[19]. The limit of quantitation (LOQ) was determined by finding the lowest amount of analyte above the LOD (LOQ = 3.3×LOD) that can be reproducibly quantified [75].

### 3.5.2 Precision

To assess the precision of the method, the samples were prepared in triplicate and two injections were made from each sample. The method precision was expressed as percent relative standard deviation (%RSD) to measure the closeness of the replicate data. The instrument reproducibility was assessed by injecting standards at different concentrations within the same day (intra-day) and between days (inter-day).

$\%RSD = \frac{SD}{\bar{x}} \times 100$, where SD is the standard deviation of response and $\bar{x}$ is the average peak area of each sugar [47].

### 3.6 Quantification of sugars in fruit juice and indigenous fruits

A calibration curve was constructed using the external standard method. The calibration curve of glucose was constructed using 6 data points in the range of 1 to 10 mM and that of fructose covered 4 data points in the range of 6 to 24 mM. The concentration ranges of each analyte that fall within the linear range of detection were chosen to construct the calibration curves. The apple juice was diluted 20 times, berry samples were diluted 10 times and palm fruit samples were diluted 2.5 times. The dilutions were based on a number of preliminary experiments performed with undiluted samples. The standard solutions and the diluted samples were derivatised and analysed by HPLC on the same day. All solutions were analysed in triplicate and the average peak areas were used for subsequent calculations. The calibration curves were constructed by plotting the average peak areas versus the concentrations of the sugars. The coefficient of determination ($R^2$) of each

calibration curve was measured using linear regression analysis. The curve equation was used to determine the concentration of glucose and fructose in the samples.

# CHAPTER 4

# 4. RESULTS AND DISCUSSION

## 4.1 RPLC method development

The comparison of acetone and acetonitrile organic modifiers for the analysis of ABEE-sugar derivatives was performed by first developing a reference method with acetonitrile. Preliminary experiments revealed that the derivatives eluted relatively early when analysed using RPLC (results not shown). Therefore, a method that utilises a low organic content mobile phase (95A:5B) was evaluated (**Fig 4.1**), where mobile phase A consisted of 5 mM ammonium formate buffer at pH 3 in 5% ACN and mobile phase B had 5 mM ammonium formate buffer at pH 3 in 95% of ACN. In this analysis, two peaks were observed for the ABEE-fructose derivative and only one peak for the ABEE-glucose (confirmed by LC–MS, see later). Similar observations were also made in the studies of Bawazeer et al. [20] and Ho Ko *et al*. [76]. Ho Ko et al. [76] observed two ABEE- fructose peaks which was due to the formation of diastereomers. In the study of Bawazeer et al. [20], $^{2}H_{5}$-aniline labelled fructose that was synthesised via reductive amination was also separated into two peaks using HILIC mode due to the presence of a pair of diastereomers. Unlike aldoses, the reductive amination of ketoses results in the formation of an additional stereocenter, hence forming two diastereomers (**Fig 4.2**), which can be separated by regular chromatographic methods. Furthermore, low sensitivity was observed for ABEE-fructose compared to ABEE-glucose, an observation that was also reported previously [76]. It is assumed that this is due to aldoses being more reactive than ketoses during reductive amination with ABEE [76].

Subsequently, the method was optimised to ensure complete separation of the ABEE derivatives of a series of sugars. A mobile phase consisting of 92%A and 8%B gave the best separation, although there is an unused separation space observed in the chromatogram (**Fig 4.3A**). Increasing the organic content above 8% resulted in the co-elution of the ABEE- fructose peaks. After the optimisation of the reference method with acetonitrile, the alternative method with acetone-containing mobile phase was optimised. A mobile phase based on 93%A and 7%B composition (where mobile phase A consisted of 5 mM ammonium formate buffer at pH 3 in 5% acetone and mobile phase B had 5 mM ammonium formate buffer at pH 3 in 95% of acetone) was found to provide separation performance and retention factors similar to those obtained with the acetonitrile mobile phase (**Fig 4.3B**). The results show that acetone is a suitable direct substitute of acetonitrile as both methods yielded similar separation and selectivity. Although acetone is generally regarded as being incompatible with UV detection due to its UV cut-off of 330 nm, these findings revealed that it can be used for the analysis of ABEE-sugar derivatives. This is made possible because the absorbance maximum of these derivatives is 307 nm, which is high enough above acetone's UV absorbance maximum of 280 nm.

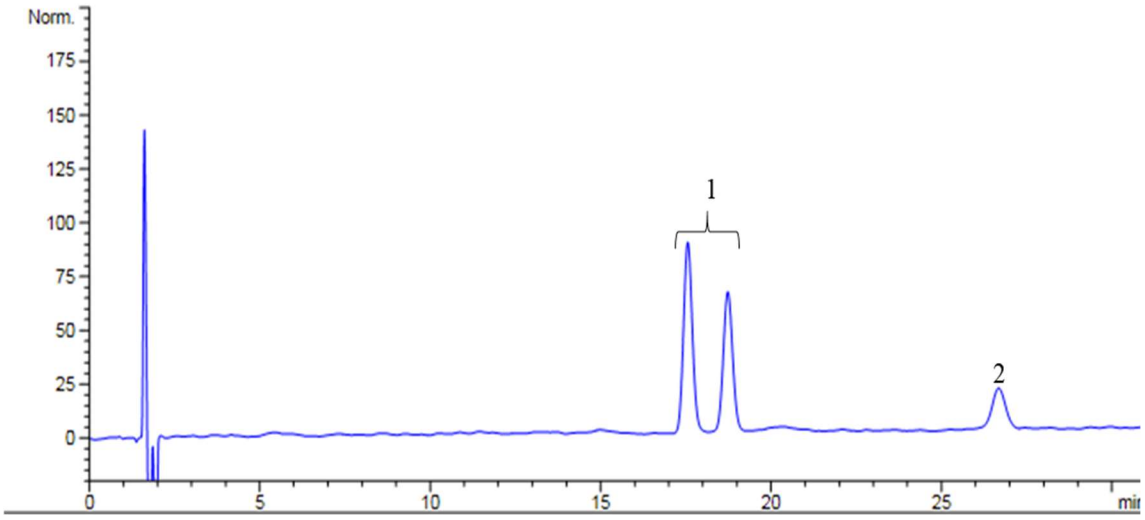


**Fig 4.1** Separation of standard ABEE- fructose and glucose derivatives using the acetonitrile-containing mobile phase (mobile phase composition 95%A:5%B). Compound: (1) fructose, (2) glucose.

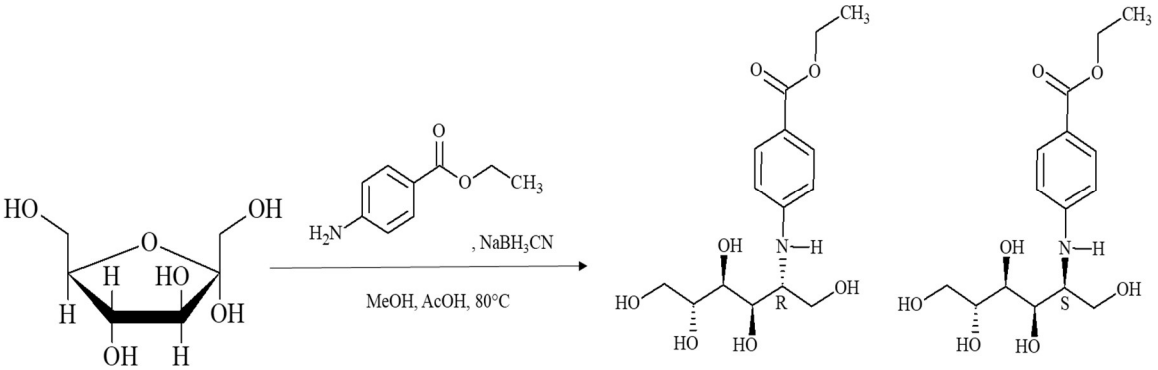


**Fig 4.2** Derivatisation reaction of fructose with ABEE.

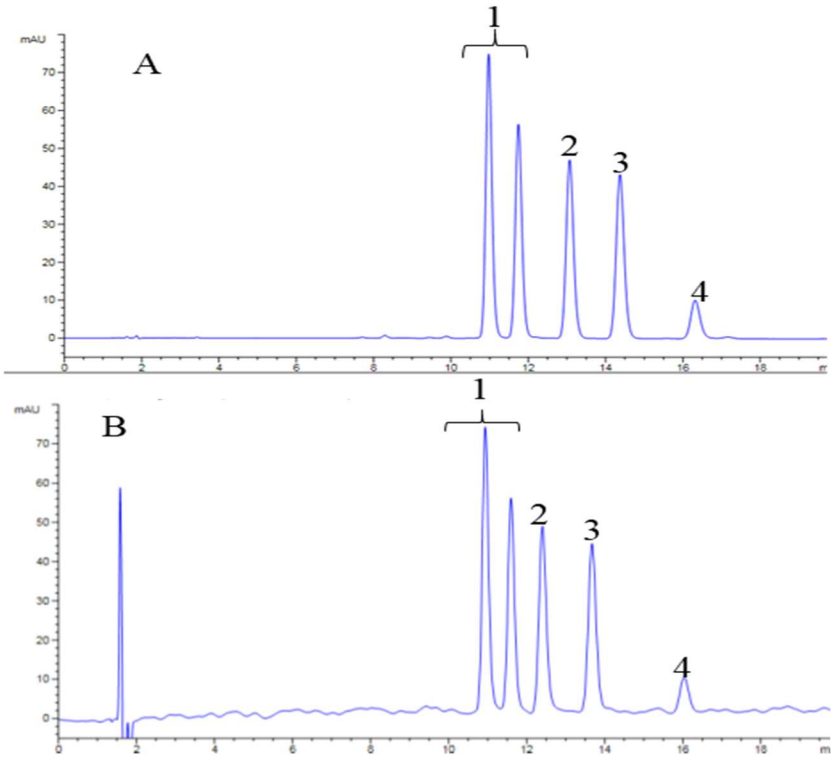


**Fig 4.3** (A) Separation of a working standard mixture of ABEE-sugar derivatives using mobile phase containing acetonitrile (mobile phase composition 92%A:8%B). (B) Separation of a working standard ABEE-sugar derivatives using mobile phase containing acetone (mobile phase composition 93%A:7%B). Compounds: ABEE derivatives of (1) fructose, (2) maltotriose, (3) maltose and (4) glucose.

## 4.2 Development of an acetone method for the analysis of sugar derivatives in fruit juice

Apple juice was chosen as a model sample for the development of an acetone-containing mobile phase method for the separation of ABEE derivatives of glucose and fructose. Gradient analysis is used to significantly reduce HPLC analysis times, thus gradient methods using both acetonitrile and acetone were developed. Firstly, a gradient starting with 0-20% mobile phase B (organic modifier) over 10 min was evaluated for each of the

organic modifiers. The gradient methods resulted in reduced analysis times compared to those yielded by the isocratic methods (**Fig 4.4**). Since the compounds of interest eluted after 10 min, the gradient method was further optimised to eliminate the unused separation space and shorten the analysis time. This was done by narrowing the gradient to 20-30% mobile phase B over 5 min. When using this method, the analytes eluted within 5 min (**Fig 4.5**).

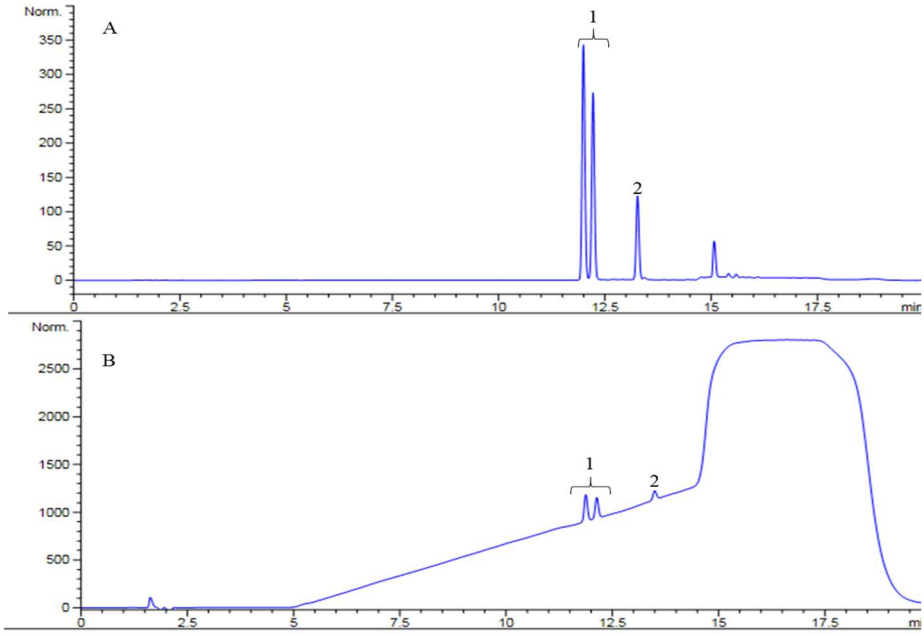


**Fig 4.4** Gradient separation of ABEE sugar derivatives in apple juice using mobile phase containing (A) acetonitrile and (B) acetone. Mobile phase gradient: 0 – 20 %B in 10 min. Compounds: ABEE derivatives of (1) fructose and (2) glucose.

However, although the methods resulted in a significant reduction in retention time, the analytes of interest could not be separated from the sample matrix (**Fig 4.5**). Moreover, the gradient method with acetone resulted in a prominent baseline drift. Acetone is known to cause a rise in baseline with increased organic content in gradient mode with UV detection, therefore, gradient correction tools are normally employed [61].

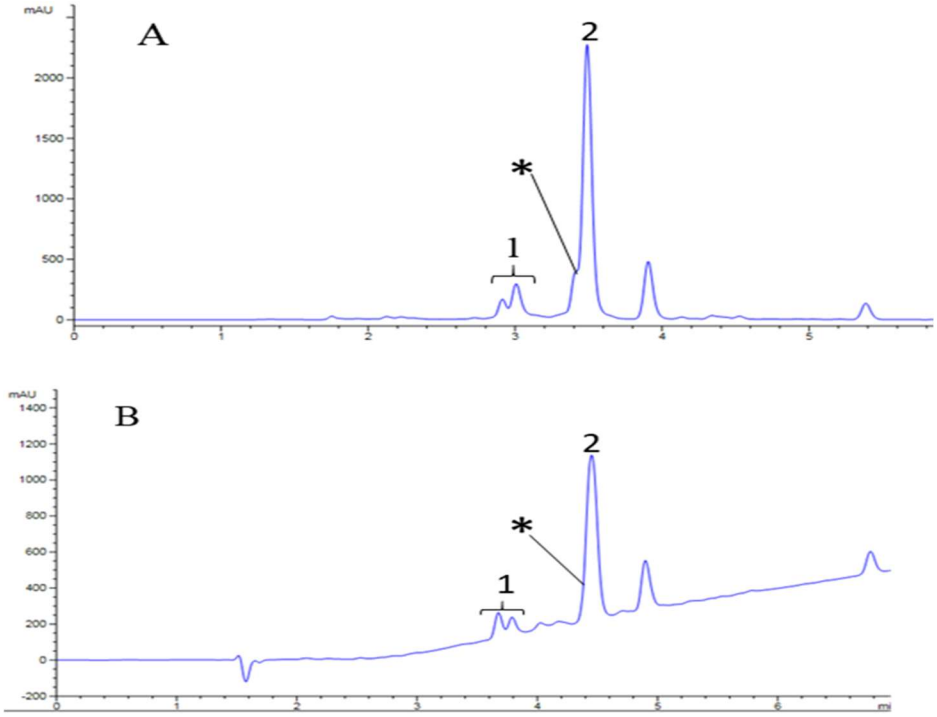


**Fig 4.5** Gradient separation of ABEE sugar derivatives in apple juice using mobile phase containing (A) acetonitrile and (B) acetone. Mobile phase gradient: 20 – 30%B in 5 min. Compounds: ABEE derivatives of (1) fructose and (2) glucose. *Sample constituent.

In an effort to improve separation and eliminate the rising baseline associated with acetone, methods based on the previously developed isocratic methods were used instead. This was done by starting with the isocratic section and subsequently increasing the organic content to 100% after the elution of analytes in order to flush out the later eluting compounds from the column. First, the acetonitrile containing mobile phase method was applied to the analysis of apple juice (**Fig 4.6A**). However, when the same approach was used with the original isocratic acetone containing mobile phase method, co-elution of glucose and a sample constituent occurred. Therefore, the acetone containing mobile

phase method was further optimised by reducing the mobile phase B content to 3% in order to resolve the two peaks (**Fig 4.6B**).

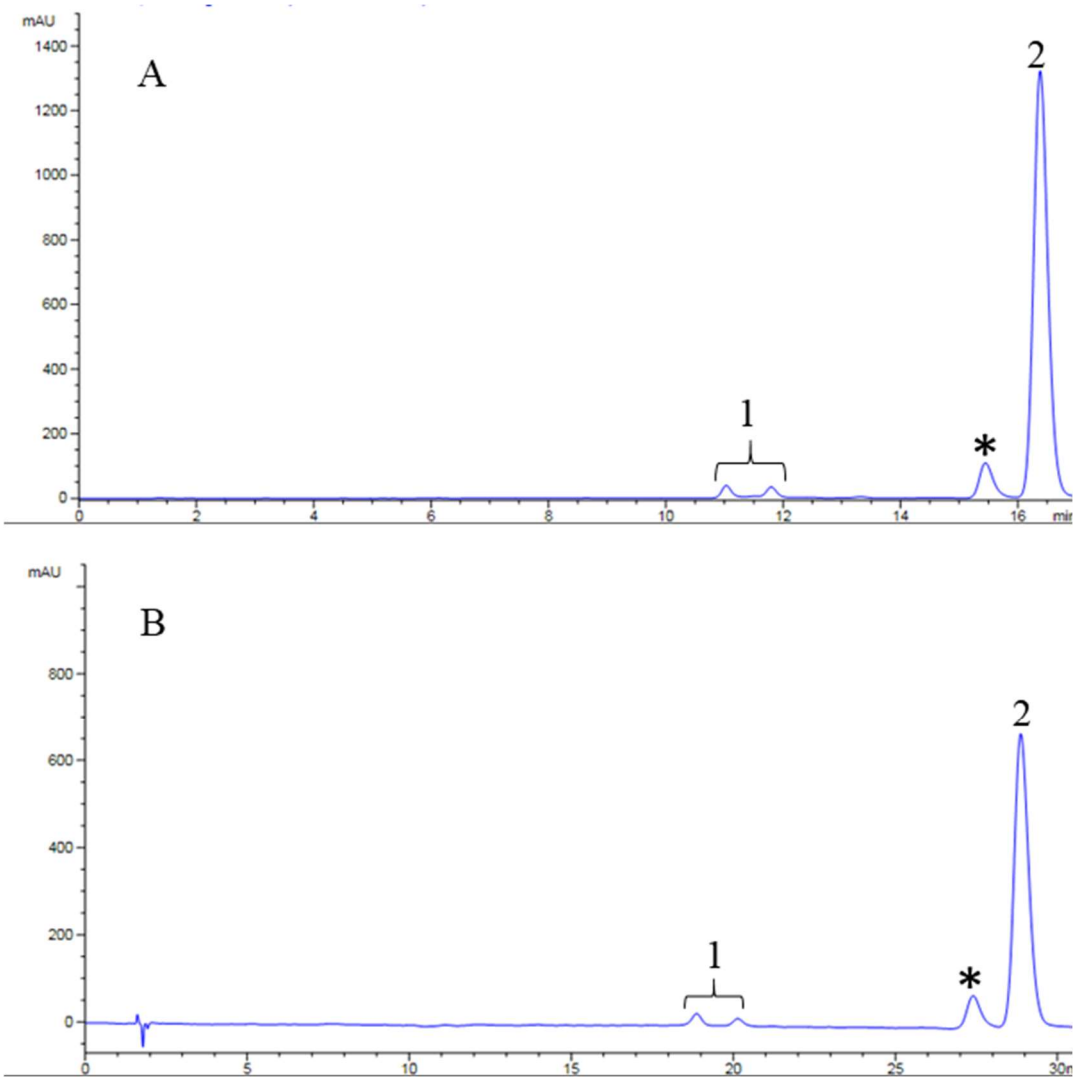


**Fig 4.6** Isocratic separation of ABEE sugar derivatives in apple juice using mobile phase containing (A) acetonitrile (mobile phase composition 92%A:8%B) and (B) acetone (mobile phase composition 97%A:3%B). Compounds: ABEE derivatives of (1) fructose and (2) glucose. *Sample constituent.

### 4. 3 Analysis of sugars with HILIC

The analysis of sugar derivatives with HILIC was evaluated in an attempt to develop a method with a shorter analysis time than the RPLC method. Since HILIC uses a lot of the organic solvent as compared to reversed-phase liquid chromatography, the use of alternative non-toxic solvents is even more relevant for HILIC methods. First, a mixture of sugars was analysed using an acetonitrile-containing mobile phase with a composition of 10%A:90%B (where mobile phase A consisted of 5 mM ammonium formate buffer at pH 3 in 5% ACN and mobile phase B had 5 mM ammonium formate buffer at pH 3 in 95% of ACN). The disaccharides (maltose and lactose) could be separated from the rest of the analytes, but all the monosaccharides (glucose, fructose, and maltose) co-eluted with each other (**Fig 4.7**). In an attempt to resolve the monosaccharides, the mobile phase aqueous content was reduced to 5% in order to increase the retention of the analytes. Hence, the analysis was repeated using 100% mobile phase B which increased the retention of the monosaccharides, but they still co-eluted with each other (**Fig 4.8**). Although HILIC is the method of choice for the analysis of sugars [43], the polarity of the ABEE sugar derivatives is reduced, thus, the derivatised monosaccharides are poorly retained and separation could not be achieved with this mode. Since the HILIC separation of the monosaccharides could not be achieved using the acetonitrile-containing mobile phase, acetone-containing mobile phase was not evaluated. Subsequently, the optimised RPLC method was used for all the remaining experiments.

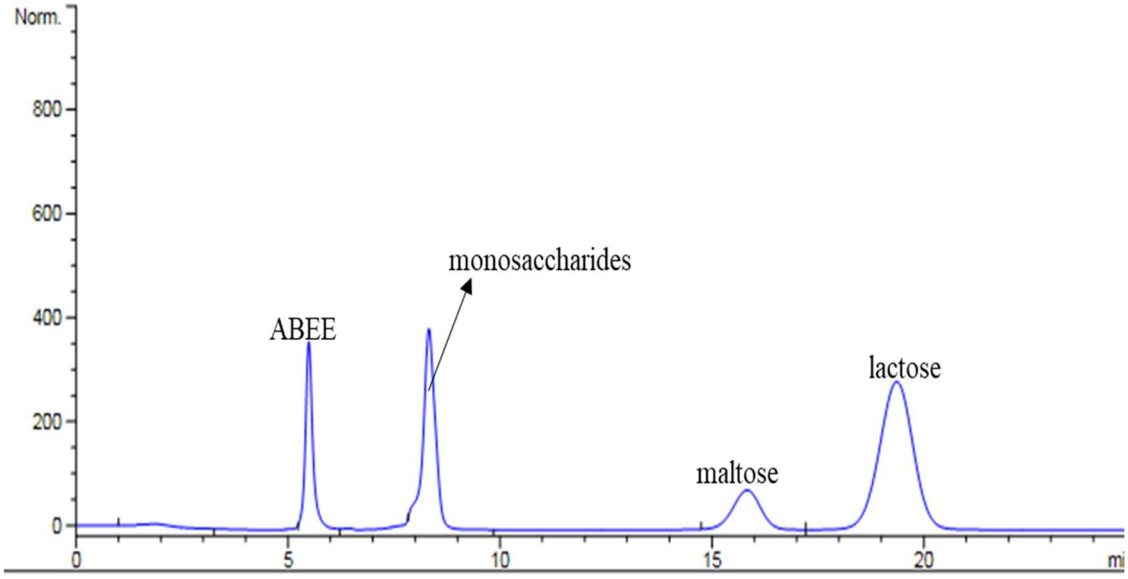


**Fig. 4.7** Isocratic HILIC separation of standard ABEE sugar derivatives using acetonitrile-containing mobile phase (mobile phase composition 10%A:90%B).

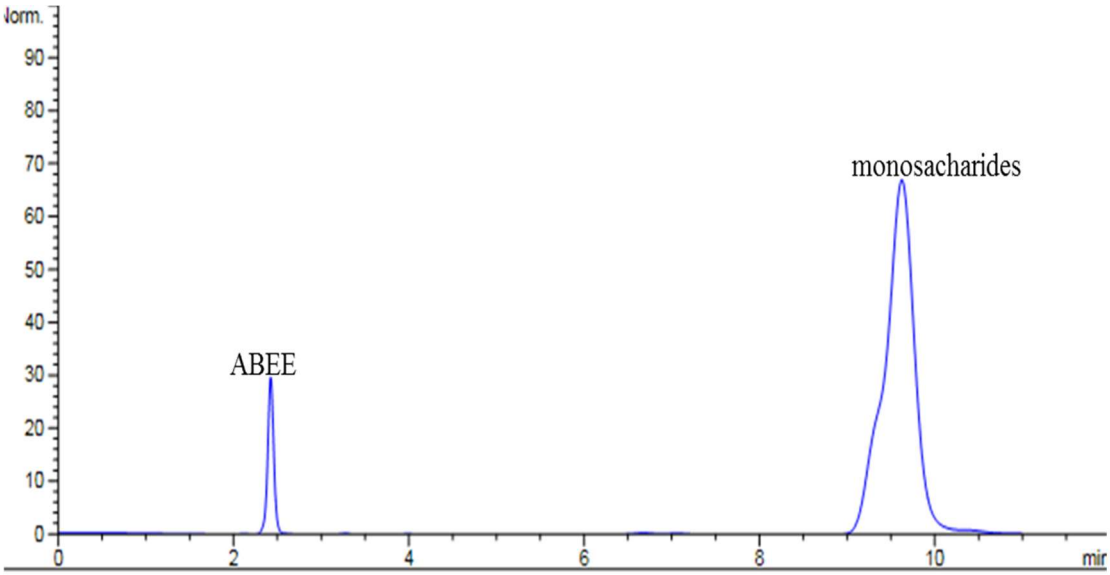


**Fig 4.8** HILIC separation of the standard ABEE with 100% mobile phase B of the acetonitrile-containing mobile.

### 4.4 Qualitative analysis of sugars in indigenous fruits

The developed acetone-containing mobile phase RPLC method was applied to the analysis of reducing sugars in two indigenous fruits, *Berchemia discolor* (berries) and *Hyphaene petersiana* (palm fruits) in order to study their sugar profiles. Broad ABEE-glucose peaks were observed in the preliminary analysis (using acetonitrile-containing mobile phase) of undiluted fruit samples (**Fig 4.9**) and therefore the samples were diluted prior to all subsequent experiments. Using the optimised acetone method for the analysis of the diluted samples, glucose and fructose were successfully separated from all other sample constituents (**Fig 4.10** and **Fig 4.11**).

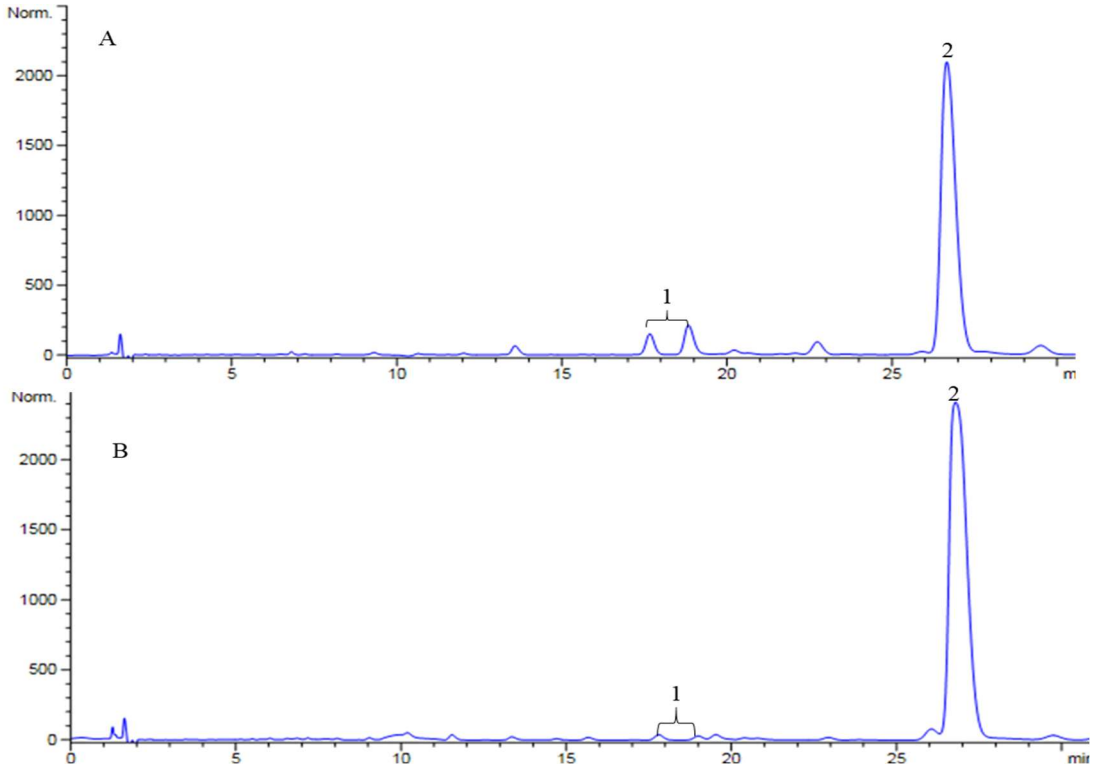


**Fig 4.9** Chromatograms of undiluted derivatised (A) berry and (B) palm fruit samples analysed using acetonitrile-containing mobile phase (mobile phase composition 95%A:5%B). Compound: (1) fructose, and (2) glucose.

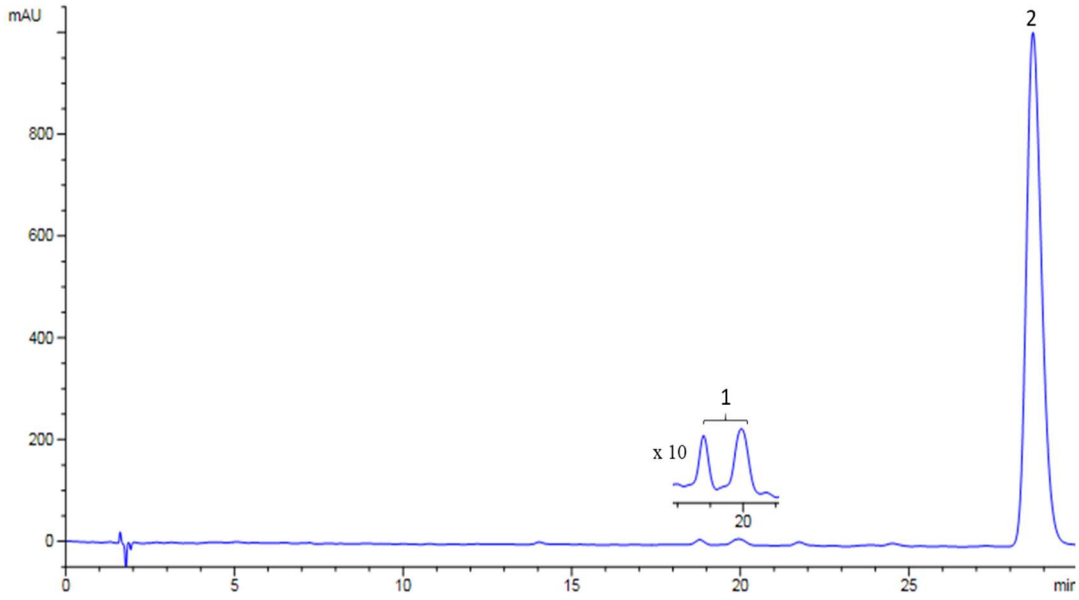


**Fig 4.10** Isocratic separation of ABEE sugar derivatives in *Berchemia discolor* berries, using mobile phase containing acetone (mobile phase composition 97%A:3%B). Compounds: ABEE derivatives of (1) fructose and (2) glucose.

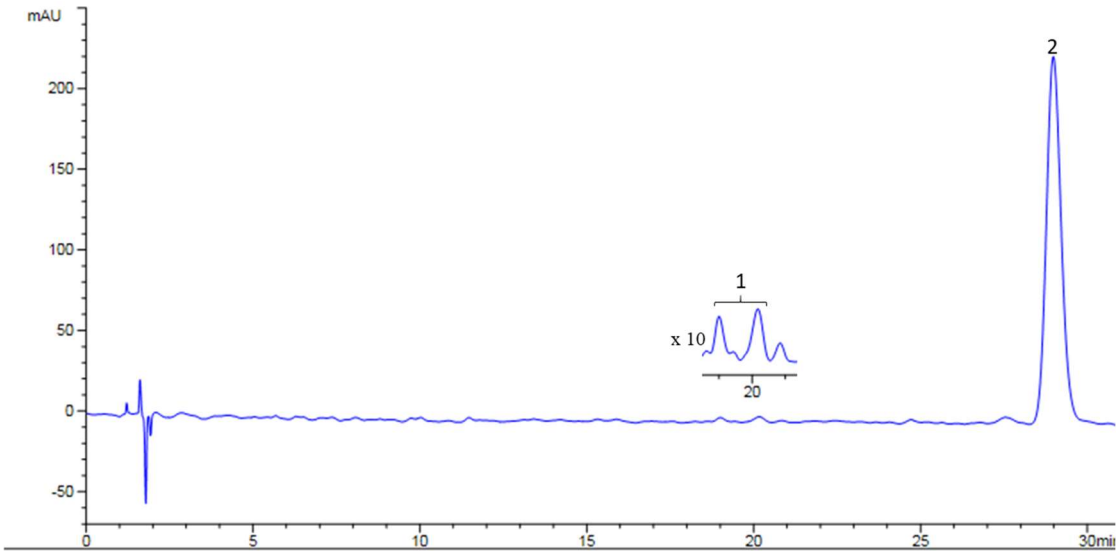


**Fig 4.11** Isocratic separation of ABEE sugar derivatives in *Hyphaene petersiana* fruits, using mobile phase containing acetone (mobile phase composition 97%A:3%B). Compounds: ABEE derivatives of (1) fructose and (2) glucose.

Subsequently, the derivatised samples and standards were analysed using LC-MS (**Fig 4.12**) in order to confirm the identities of the sugar derivatives detected in the samples. Based on the studies done on the fragmentation of ABEE-oligosaccharides [10], we were able to confidently confirm the identities of the sugars investigated in this study. The $ESI^-$ mass spectra of the ABEE sugar derivatives in the standard solutions (**Fig 4.13**) and apple juice samples (**Fig 4.14**) (the spectra of the other samples appear in Appendix 1) were found to be identical, with the base peak observed at *m/z* 328, which corresponds to the $[M-H]^-$ ions of ABEE-glucose and ABEE-fructose. The elemental composition of the $[M-H]^-$ ions, $C_{15}H_{22}NO_7$, was calculated from their experimentally determined accurate mass, 328.1399 Da and therefore the molecular formulae of the neutral sugar molecules is $C_{15}H_{23}NO_7$. Peaks observed at *m/z* 374 and 657 were also common to the standard and sample spectra. The peak at *m/z* 374 corresponds to the formate adduct $[M + HCO_2]^-$,

while the peak at *m/z* 657 corresponds to dimers $[2M-H]^-$ of ABEE-glucose and ABEE-fructose. Moreover, the peak at m/z 310 in the samples is a result of loss of water from the deprotonated molecule, i.e. the $[M-H-H_2O]^-$ ion.

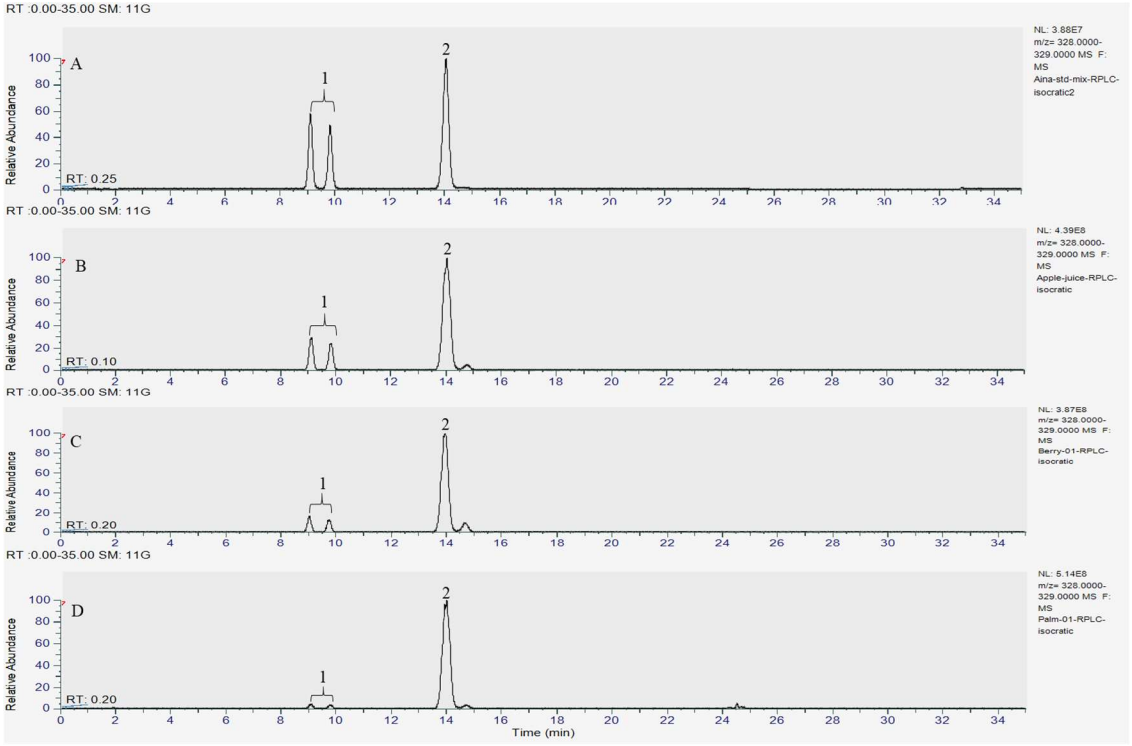


**Fig 4.12** Extracted ion chromatograms (*m/z* 328) of the RPLC–MS analysis of (A) derivatised sugar standards, (B) apple juice, (C) berry and (D) palm fruit samples. Compounds: ABEE derivatives of (1) fructose and (2) glucose.

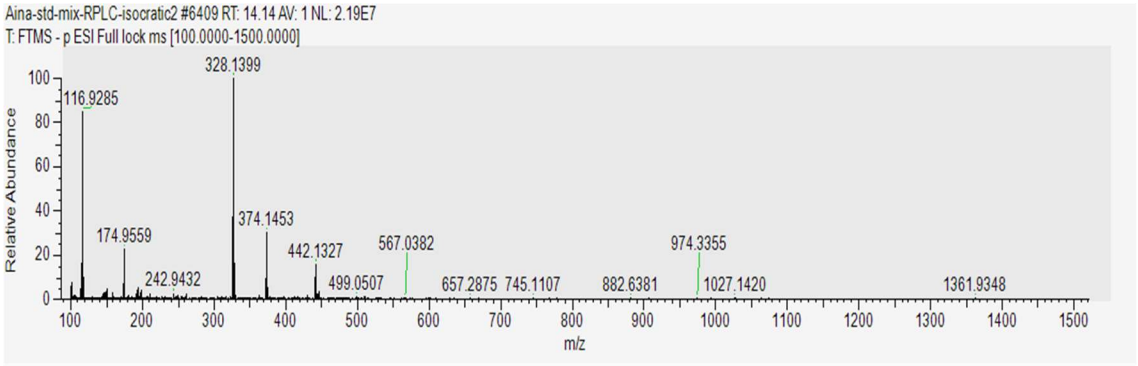


**Fig 4.13** ESI$^-$ mass spectrum of the ABEE-glucose standard.

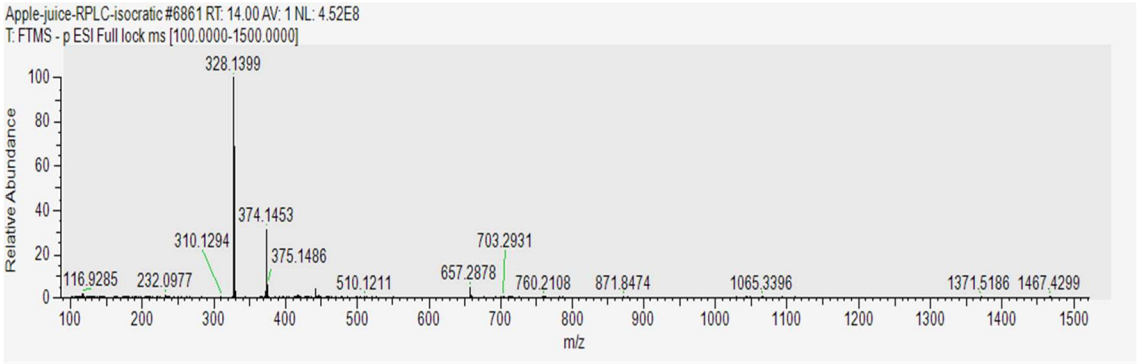


**Fig 4.14** ESI$^-$ mass spectrum of the ABEE-glucose in apple juice.

### 4.5 Method validation and application

To assess the method reproducibility, samples were prepared in triplicate and analysed in triplicate. The method showed good precision with %RSD in the range of 1.57– 9.3% for both intra- and inter-day analyses, 1.57-2.52 and 8.5-9.3 respectively (**Table 4.1**). The linearity of the developed acetone method was verified by analysing ABEE-fructose in the range of 6-24 mM (**Fig 4.15**) and ABEE-glucose in the range of 1-30 mM (**Fig 4.16**) in triplicate. The calibration curves for ABEE-glucose and ABEE-fructose showed a good correlation between the peak area and standard sugar concentration with $R^2 > 0.99$. The LOD and LOQ for ABEE-glucose were found to be 0.01 mM and 0.1 mM, respectively and for ABEE-fructose they are 1 mM and 3 mM, respectively.

**Table 4.1** Quantitative parameters of the method.

| Compound | Linear range (mM) | Coefficient of determination ($r^2$) | LOQ (mM) | LOD (mM) | Intraday precision (%) | Interday precision (%) |
|---|---|---|---|---|---|---|
| Fructose | 6-24 | 0.9913 | 1.00 | 3.00 | 2.52 | 9.30 |
| Glucose | 1-30 | 0.9927 | 0.10 | 0.01 | 1.57 | 8.50 |

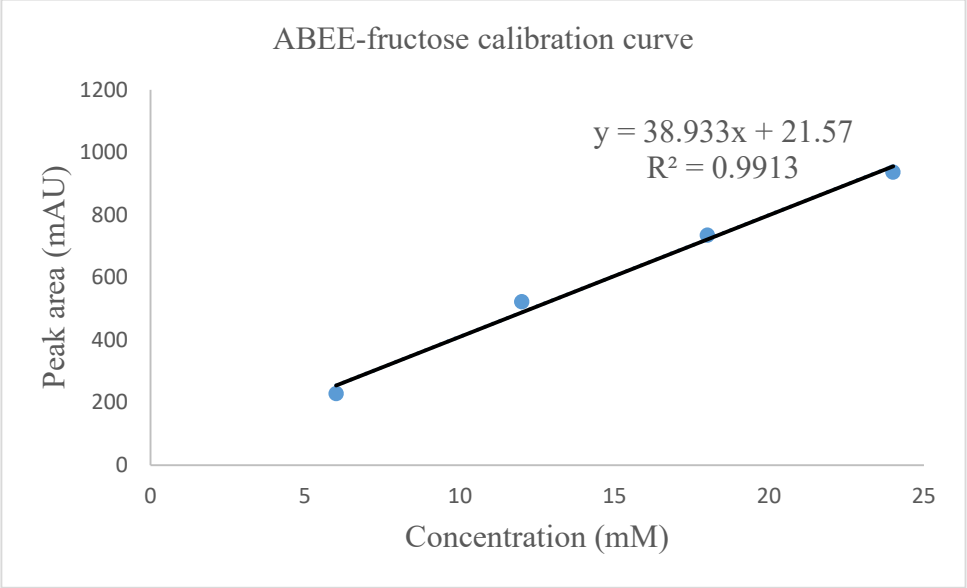


**Fig 4.15** Standard calibration curve of ABEE-fructose.

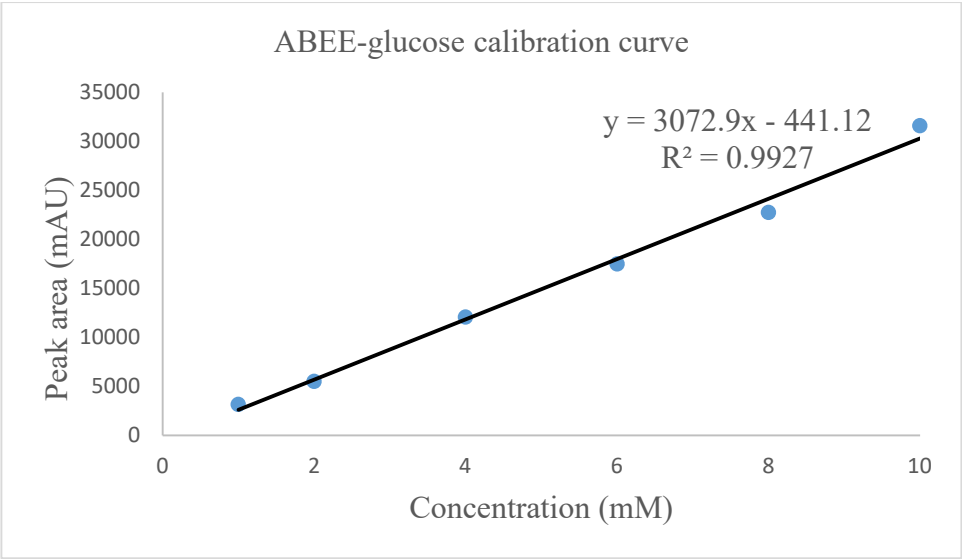


**Fig 4.16** Standard calibration curve of ABEE-glucose.

Using the validated method, fructose and glucose could be quantitatively determined in the apple juice, berries, and palm fruit (**Table 4.2**). The results showed expected amounts of glucose and fructose in the apple juice. Apple juice is expected to have carbohydrate content ranging from 1-4 g/100 mL glucose and 5-8 g/100 mL fructose [77]. The values obtained also agree with the amounts of glucose (1.452-1.509 g/100 mL) and fructose (6.080-7.369 g/100 mL) previously obtained in apple juice at different ripening stages [15].

The berries had higher fructose and glucose concentrations than the palm fruits. This is expected since the berries taste sweeter than the palm fruits. Moreover, the Fru/Glu ratio of the berries is high compared to the palm fruits which could explain the variation in sweetness of these two fruits. In all the samples, the fructose concentration was predominantly higher than that of glucose.

**Table 4.2** Concentrations of fructose and glucose in apple juice (g/100 mL), *Berchemia discolor* berries (mg/g) and *Hyphaene petersiana* palm fruits (mg/g).

| | Fructose | | | Glucose | | |
|---|---|---|---|---|---|---|
| Sample | Average concentration | Average Injection Repeatability (%RSD) | Reproducibility (%RSD) | Average Concentration | Average Injection Repeatability (%RSD) | Reproducibility (%RSD) |
| Apple juice | 6.1 | 1.4 | 9.6 | 2.9 | 0.9 | 6.6 |
| Berry | 319.9 | 0.5 | 3.6 | 217.6 | 0.4 | 2.5 |
| Palm fruit | 44.5 | 2.9 | 7.0 | 41.6 | 0.3 | 8.9 |

# CHAPTER 5

## 5. CONCLUSIONS

The separation of sugar derivatives using two mobile phases containing different organic solvents was achieved. In spite of the high UV cut-off for acetone, it was proven that acetone could be used as an alternative solvent to acetonitrile with photometric detection for sugar analysis. Although sample derivatisation is not considered to be a green approach, the use of a non-toxic mobile phase solvent enhanced the method greenness and allowed the use of a simple and economical UV detector. This method merits its application in quality control laboratories most especially resource-limited laboratories. The method was applicable to both fruits and fruit juices and could potentially be used to analyse sugars in other sample matrices. Acetone gave similar separation and selectivity to acetonitrile. Although acetone is generally not compatible with photometric detectors, it was possible to achieve satisfactory separation of these derivatives at 307 nm because they absorb above the acetone UV absorbance maximum of 280 nm. The two reducing sugars, fructose and glucose, were present in all samples and their identities were confirmed with LC–MS. This is the first time that the detailed sugar profile of *Berchemia discolor* berries and *Hyphaene petersiana* palm fruits have been determined. In conclusion, the developed RPLC–UV method was able to separate the ABEE-sugars using the acetone containing mobile phase.

# CHAPTER 6

## 6. RECOMMENDATIONS

Although the method is not targeting the non-reducing sugars, the authors recommend that hydrolysis of non-reducing sugars is done prior to the analysis so that the total sugar content of the samples can be quantified. Furthermore, more validation experiments such as accuracy and matrix effect determinations should be performed. The analysis of sugar derivatives with HILIC–UV should be further explored by evaluating different stationary phases, performing gradient analysis and analysis conditions such as temperature. Last but equally important, other green solvents should also be evaluated for the analysis of these sugar derivatives.

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## Appendix 1: Research permission letter

**CENTRE FOR RESEARCH SERVICES**
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University of Namibia, Private Bag 13301, Windhoek, Namibia
340 Mandume Ndemufayo Avenue, Pioneers Park, Office F223 - Block, Second Floor
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UNAM
UNIVERSITY OF NAMIBIA

**RESEARCH PERMISSION LETTER**

**Date: 31/10/2022**

**Student Name:** AINA IYAMBULA

**Student Number:** 201606028

**Programme:** Masters of Science (Chemistry)

**Approved Research Title:** Development of An HPLC Method Using RPLC And HILIC Coupled in Series for The Simultaneous Analysis of Sugars and Phenolic Compounds in Beer

**TO WHOM IT MAY CONCERN**

I hereby confirm that the above-mentioned student is registered at the University of Namibia for the programme indicated. The proposed study met all the requirements as stipulated in the University guidelines and has been approved by the relevant committees.

The proposal adheres to ethical principles as per attached Ethical Clearance Certificate. Permission is hereby granted to carry out the research as described in the approved proposal.

Best Regards

**Dr. AEE Shikongo**
**Head: Postgraduate Support Services**
**Tel: +264 61 206 3129**
**E-mail: aeshikongo@unam.na**

CENTRE FOR RESEARCH SERVICES
Office of the Director
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## Appendix 2: Ethical clearance

UNAM

# ETHICAL CLEARANCE CERTIFICATE

**Ethical Clearance Reference Number: SOS-0010 Date: 25 October 2021**

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

**Title of Project:** DEVELOPMENT OF AN HPLC METHOD USING RPLC AND HILIC COUPLED IN SERIES FOR THE SIMULTANEOUS ANALYSIS OF SUGARS AND PHENOLIC COMPOUNDS IN BEER

**Student:** AINA IYAMBULA

**Student Number:** 201606028

**Supervisor(s):** DR STEFAN LOUW (UNIVERSITY OF NAMIBIA)

**Centre for Research Services**
Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee
4. The ethics committee retains the right to:

i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.

Dr. Zivayi Chiguvare (Chairperson Ethics Committee)

Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)

## Appendix 3: ESI$^-$ Mass Spectra

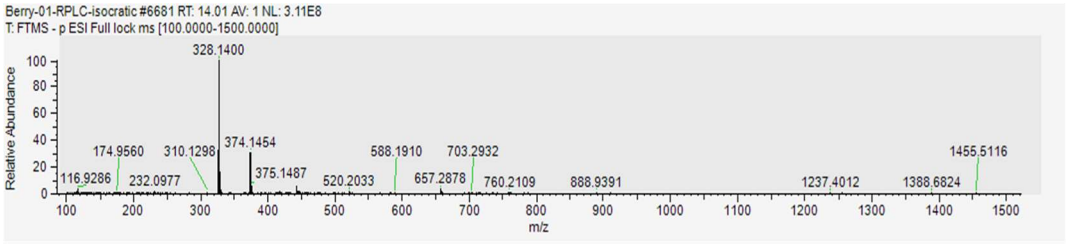


Fig A1a: ESI$^-$ mass spectrum of ABEE-glucose in berry fruits.

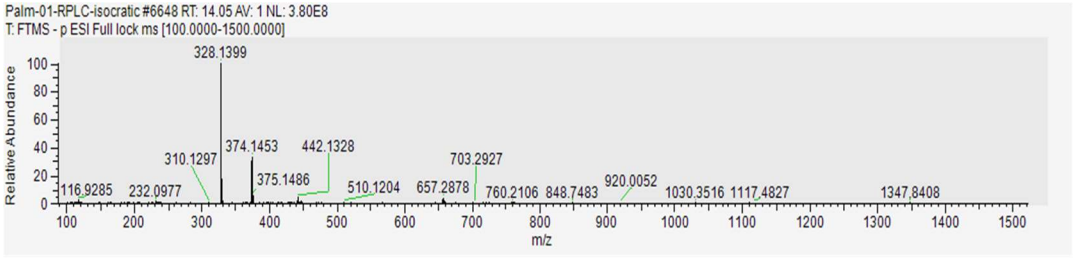


Fig A1b: ESI$^-$ mass spectrum of ABEE-glucose in palm fruits.

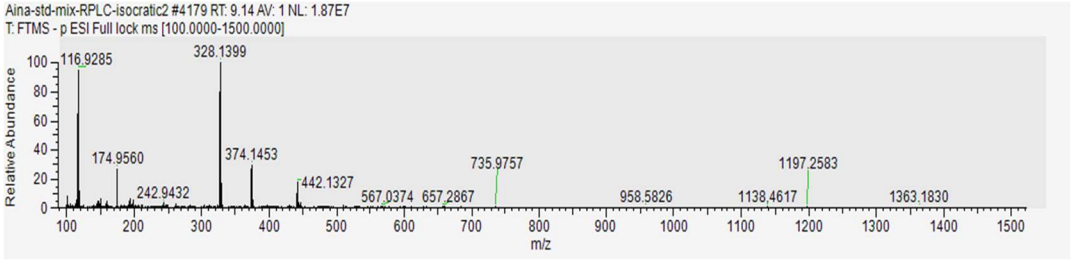


Fig A1c: ESI$^-$ mass spectrum of standard ABEE-fructose.

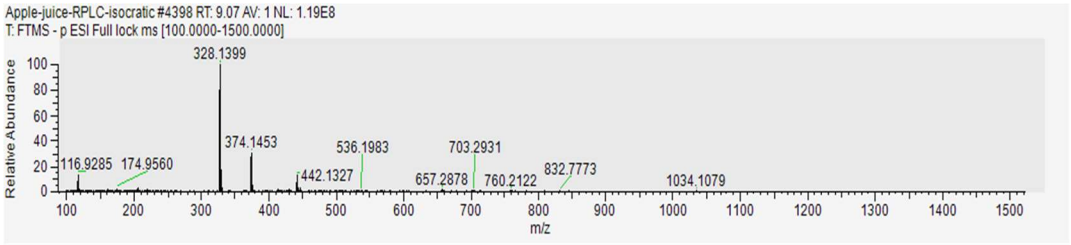


Fig A1d: ESI$^-$ mass spectrum of ABEE-fructose in apple juice.

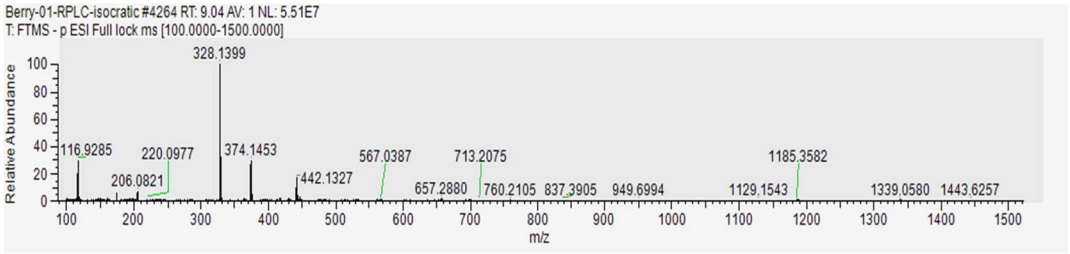


Fig A1e: ESI$^-$ mass spectrum of ABEE-fructose in berry fruits.

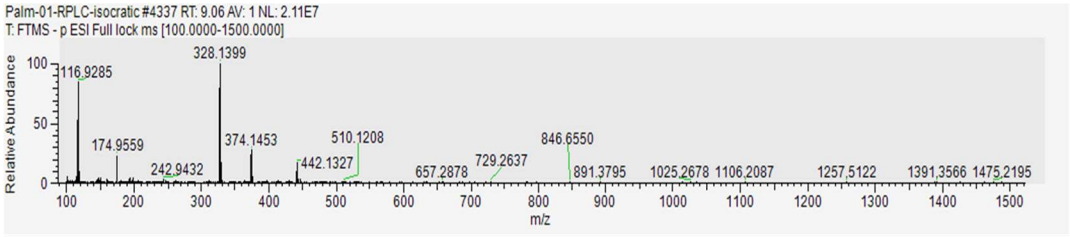


Fig A1f: ESI$^-$ mass spectrum of ABEE-fructose in palm fruit.