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Liquid chromatography-mass spectrometry (LC-MS) method for the determination of sugars in fresh pomegranate fruit juices

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ABSTRACT

Pomegranate is an excellent source of many nutrients able to contribute to a healthy diet. Taif is the first leading Pomegranate producers in Saudi Arabia. To our knowledge, there has been no research on the Taif Pomegranate fruit juices. In this study, qualitative and quantitative identification of sugars (fructose, glucose and sucrose) was carried out for four different pomegranates origins commercially available in Taif province, KSA such as (Abha, Egyptian, Yamani 1, Yamani 2) using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) technique. The LC-MS method optimized in term of the kind of column used mobile phase, and the flow rate, in addition to the parameters for mass spectrometry. The results showed that the concentrations of fructose and glucose in the analyzed samples were between (0.162-0.306 gm/100 gm) and (0.132-0.300 gm/100 gm) respectively. The sucrose was not detected. In conclusion, the authors recommend these methods for analysis of sugars concerning the food and human health safety.

Key words: Taif pomegranate, LC-MS, pomegranate fruit juices, fructose, glucose, sucrose.

INTRODUCTION

The separation and quantitative analysis of sugars is challenging for several reasons. To find a proper technique, column, mobile phases and detector for the separation and quantitative detection of sugars is challenging. Liquid chromatography (LC) is most widely used to separate sugars due to the availability. However traditional reversed phase columns cannot be used for underivatized sugars, as the stationary phase will not provide the required retention and specialized columns are necessary. Detection of sugars faces troubles as their structure contains no chromophores. Detection by UV-VIS, as commonly used in HPLC, is not possible in sugar analysis. Detection techniques such as evaporate light scattering (ELSD), refractive index (RI), Mass spectrometry (MS) can be used but every detector has its own limitations [1].

Spectroscopic techniques, separation techniques, and their multiple combinations are the main tools used to analyze carbohydrates in foods. Glucose, fructose and sucrose are the most abundant carbohydrates present in fruit juices, so in this literature survey we have a focus and measurements on mono- and disaccharides like fructose, glucose, sucrose in same food and pomegranate fruit juice. Most of the reported methods for determination of sugars are chromatographic ones. They include Gas Chromatography (GC), liquid chromatography (LC), high performance liquid chromatography (HPLC) and electrophoresis (EC). High performance liquid chromatography is now the main analytical technique used for the analysis of carbohydrates. Due to their poor volatility, the analysis of carbohydrates has been traditionally carried out by High performance liquid chromatography. High performance liquid chromatography has become the preferred method for quantitating simple sugars in a variety of food products. It is the most appropriate technique for accuracy, precision and practicality for nutritional labeling purposes. HPLC often

offers direct injection of a sample with little pretreatment and simple interpretation of chromatograms, separation and detection of sugars can be done with liquid chromatography using different column types and detectors. In liquid chromatography there are various ways of analysing sugars, a distinguish can be made between columns and detection techniques. Different columns will result in other separation of sugars and results in different validation parameters Liquid chromatography-mass spectrometry (LC- MS) is a powerful analytical chemistry technique, used for many applications, that combines the physical separation capabilities of high performance liquid chromatography with the mass analysis capabilities of mass spectrometry. Mass spectrometry offers major advantages due to its high sensitivity and mass selectivity [2].

Over the past decade, the development of combination LC and Mass spectrometry (MS) methods dedicated to the analysis of sugars and monosaccharides in particular, has led to significant advances in terms of sensitivity and specificity while maintaining speed and simplicity of implementation. Highly sensitive, quick and simple determination method for mono and disaccharide using the combination of liquid chromatography with mass spectrometry. The separation performed with a hydrophilic amino interaction column applying the method for determination of sugars in orange juice sample [3]. Determination of sugar compounds in atmospheric aerosols by liquid chromatography combined with positive electrospray ionization mass spectrometry was reported, use of a polymer-based amino analytical column with mobile phase consisting of 20% 10 mM aqueous ammonium acetate, 8% methanol, and 72% water was found to provide abundant $[M+NH_4]^+$ adduct ions when coupled with electrospray ionization [4].

The improvement of LC-MS method that achieves both chromatographic separation and good MS sensitivity, iodine attachment was used to improve the sensitivity of glucose measurement by LC/MS. After sample preparation, glucose was separated by normal phase chromatography, followed by an ionization by I^- attachment prior to MS by post-column addition of methanolic solution of iodoform. Iodine is capable of forming an anionic adduct with neutral monosaccharides in negative ion mode electrospray mass spectrometry. Quasi- molecular ions $[M + I]^-$ of glucose was achieved [5]. In the other hands method uses chloride attachment by post column addition of chloroform and LC-ESI-MS negative mode equipped with amino carbohydrate column and acetonitrile water as isocratic mobile phase in atmospheric aerosols samples for the quantification and identification of sugars and sugar alcohols [6]. The development and application of an on-line liquid chromatography-mass spectrometry (LC-MS) method using hydrophilic interaction chromatography (HILIC) coupled to negative ion mode electrospray ionization ion trap mass spectrometry (ESI-MS) for the analysis of highly polar carbohydrate which are generally very difficult to retain on traditional RP-LC columns were described [7].

Quantification of monosaccharides using LC-MS equipped with amino- bonded silica phase column applying electrospray ionization in negative mode [8].The second approach is based on small cation attachment to carbohydrates a simple, rapid, sensitive and specific LC-ESI-MS methods under the positive ionization mode. Glucose, fructose and sucrose were determined [9]. MS with ESI in full scan modes for identification and SIM (selected ion monitoring) for quantification Sugars were detected in positive ion mode as their sodium adducts $[M+Na]^+$. Recoveries of the target sugars ranged from 95.4 – 97.7 % and the reproducibility of the injection was reported as less than 3.4 % [10]. Sensitive method based on high-performance liquid chromatography method of pre-column derivatization with 1-phenyl-3-methyl-5-pyrazolone coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) has been established for the analysis of the monosaccharide composition of polysaccharides. the mixture of monosaccharides has been well separated by a reverse-phase HPLC and detected by on-line ESI-MS method The mobile phase of elution system was chosen as acetonitrile (solvent A) and 20 mM aqueous ammonium acetate (solvent B) (pH 3.0) with Zorbax XDB-C18 column [11,12].

The refractive index detector responds to the different in the refractive index of the column effluent as it passes thought floe cell. Glucose, fructose and sucrose were determined in pomegranate fruit maturation by HPLC using a refractive index detector (RID), on a 30 cm Shim pack LC-NH2 column.The other method validated method for sugar separation with the use of RI detection was reported by [13,14]. HPLC-RI method use with the chiral column. The method separated the carbohydrate identification and determination of the monosaccharide. The researchers also studied the effect of the column temperature on the separation. The elution profiles of the sugars are showed the separation depended on column temperature [15]. High-performance liquid chromatography combined with the refractive index detector. The chromatographic separation was achieved for sugars using a Eurospher 100-5 NH_2 column using an isocratic elution with acetonitrile/water (70:30, v/v) at a flow rate of 1.0 ml/min. All the compounds were separated in 16 min [16].

HPLC–RI technique was used for Determination of Fructose, Glucose and Sucrose in Taif Grape juice equipped

with sugar Pack1 column [17]. Juices made from fruits of Tunisian accessions of pomegranate were studied for their organic acids, sugars, and anthocyanin contents, using high performance liquid chromatography sugars, fructose and glucose were most present in pomegranate juice. Sugars were analyzed by the HPLC equipped with a refractive index detector and a µBondapak-NH_2 column (30 cm× 3.9 mm i.d., Waters, Milford, MA, USA) using acetonitrile/water (85:15, v/v) as the mobile phase [18**].**

Sugars in Six pomegranate (*Punica granatum* L.) cultivars of Spanish pomegranate juice were analysed on the same HPLC equipment equipped with a refractive index detector. Sugars analysis was performed on a NH_2 column (30 cm ×3.9 mm i.d., Waters, Milford, MA, USA) using acetonitrile/water (85:15, v/v) as the mobile phase [19].

Recently evaporative light-scattering detection (ELSD) has become important in sugar analysis by HPLC, mainly because it allows the use of gradient elution. A high performance liquid chromatographic method with a dynamically modified amino column and evaporative light-scattering detector (ELSD) was established for the direct analysis of the carbohydrates (including fructose, glucose, sucrose, maltose, lactose and raffinose) in some drinks (apple, pineapple and orange juices, grape wine and liquor). A separation column (Zorbax Rx-SIL, 250 mm × 4.6 mm I.D., 5 µm, Hewlett-Packard, USA) which was modified by ethylenediamine and a guard column (Zorbax Rx-SIL, 12.5 mm × 4.6 mm I.D., 5 µm) were used. The mobile phase was a mixture of water and acetonitrile (1:2.6, v/v) containing 0.03% (v/v) ethylenediamine [20].

Separation of monosaccharides by hydrophilic interaction liquid chromatography with evaporative light scattering detection system equipped with TSK gel Amide –80 column and sugars eluted with 82% acetonitrile [21]. A reversed phase liquid chromatography-evaporative light scattering detection, combined with solid phase extraction method was developed for the separation and determination of sugars in tobacco leaf. Separation was performed with a Hypersil NH2 analytical column and the mobile phase 75%:25% acetonitrile-water [22].

In general GC methods [23] with mass spectrometry (MS) or flame ionization detector (FID) detection provide a good separation of sugars and a good sensitivity, but require prior steps of reduction and derivation, which are very time consuming and not very practical in routine analysis . Considering The high diversity of carbohydrates in terms of structure, size, functionality, and low volatility, GC has been mainly used to study mono-, di-, and trisaccharides. The monosaccharide (fructose, glucose) and sucrose caramels determined by gas liquid chromatography–mass spectrometry (GLC–MS) of their trimethylsilyl (TMS) [24]. Although GC provides excellent results for the analysis of food carbohydrates, the usefulness of this technique is limited by the required sample preparation steps and its application to relatively small carbohydrates.

In recent years, due to the higher efficiency, faster separation times, easy operation and low sample volumes required, capillary electrophoresis (CE) has evolved into an interesting. The capabilities of the ligand exchange capillary electrophoresis mode for the determination of sugars (glucose, fructose, and sucrose) in real samples (fruit juices, wines, and drug preparations) were studied [25].

Pomegranate is one of the important commercial fruits in Saudi Arabia and generally very well adapted to the Mediterranean climate.IT is cultivated in the southwestern region (Taif area) Saudi Arabia and in many other Mediterranean countries such as Egypt and very nearby Abha and Yamen. The aim of the present work of this chapter is to study the individual sugars such as (fructose, glucose and sucrose) for cultivars pomegranates under study. The sugars compositions were examined in the pomegranate cultivars using LC-ESI-MS technique equipped with Polaris NH column and acetonitrile :water as mobile phase.

MATERIALS AND METHODS

2.1 Liquid chromatography – Mass Spectrometry (LC-MS) system

Chromatography was performed on a Water Alliance HPLC 2695 (Waters Associates Inc., Milford, MA consisting of a quaternary pump, auto-sampler, column thermostat, and a Water 3100 mass spectrometer quadruple MS. Equipped with a n Electrospray Ionization (ESI) source and a nitrogen gas generator unit, NM30LA-MS Gas Generator from Peak Scientific Instruments Ltd., MA, USA, for nitrogen gas supplying. Comparison of the different Polaris column from the same company with different particle sizes and length for the optimization of sugars (fructose, glucose and sucrose) separation, in this work we used two kinds of analytical column type Polaris NH_2 (50 mm × 4.6 mm) 3 um practical size column, Polaris NH_2 (150 mm × 3 mm) 5 µm piratical size were study for the separation. While a guard pre- column module with NH_2 (10.0 mm × 50 mm) 5 µm Guard insert was used. Various solvent systems were tested for the development of suitable Liquid Chromatography-Mass Spectrometry (LC-MS) method for the analysis of fructose, glucose and sucrose

(individually and in mixture).

The suitability of the solvent system was decided on the basis of separation of sugars completely from the degradation products. Several mobile phases such as (acetonitrile-water with and without formic acid, methanol water, acetonitrile-methanol) at different ration and flow rate were tested. The column was kept at a temperature of 25 ^{O}C. The total flow from the column was 0.3 ml/ minute. The univariate method was used for optimization of the LC-MS system. The concentration of the products were determined from the peak areas under the curve using Maslynx 4.1 software. Centrifuge kind Mikro 22R was supplied from Hettich, Republic of Germany. Water purification system and the ultrasonic bath supplied by Barnstead International.

Before the quantitative and qualitative determination of sugars in the samples, we prepared standard solutions of different sugars: fructose, glucose and sucrose. With those standard solutions of different sugars we made calibration lines for each one of the sugar, which later used for assessing the concentration corresponding to the different peaks in the chromatograms.

2.2 Reagents

Glucose and sucrose were obtained from Techno Pharmachem, Bahadurgarh, India. Fructose from Riedel-deHaen and Fluke respectively. Methanol and acetonitrile HPLC grad from fluka. Three stock standards sugars solutions were prepared. The prepared standard solutions of sugars were stored at 4 ^{O}C. All the samples before injection in the HPLC sonicated for at least 15 minutes in ultrasonic bath to remove air bubbles and passed through filtration desk 0.45 um. Working solution prepared in suitable concentration used for direct determinate were made up in water.

2.2.1 Preparation of standards and solutions for calibration curves

Sucrose, fructose and glucose were separately weight and dissolved in deionized water and acetonitrile to produce a stock standard solution at a final concentration of 1000 µg/ml of each sugar. These stock solutions were used to prepare solution for method validation, mixtures of standards sugars and the calibration curves. The standards solutions were stored at 4 ^{O}C. Calibrations curve were establish at six points mass load of for fructose and glucose 0.3, 0.75, 1.75, 3.0, 6.0 9.0 µg. While the mass load were for sucrose 0.025, 0.063, 0.125, 0.25, 0.5, 0.75 µg. Mixture of standards sugars were papered with final concentration 300 µg/ml for each fructose and glucose, and 25 µg/ ml for sucrose this mixture can be injected for different mass into the column by alternating the size of the injection with the injection syringe.

2.2.2 Sample Preparation

Pomegranate fresh fruit from Taif product, Abha product in KSA and imported pomegranate (Yamani 1&2 and Egyptian) were purchased from local market. The seeds of pomegranate get from the fruit by make a shallow slit at the top the pomegranate around the crown, cut all the way around the top of the rind, creating a shallow circle. Don't push the knife in too deep, or you may burst some seeds. Pull the crown of the pomegranate off to reveal the inner seeds. Turn the pomegranate over. Repeat the process of creating a narrow slit in the rind, cutting a circle around the base of the fruit. Pull the bottom off the fruit. Turn the pomegranate back over. Cuts slits in the sides of the rind, following the lines of the pith, from the top of the fruit to the bottom. Pull the fruit apart; you will now have sections of the fruit with the seeds fully exposed. Gently loosen the seeds from the pith and place them into the clean bowl.

The pomegranate seeds were washed under distilled water then dried. The triplicate fresh pomegranate from each kind were exactly weight (100 gm). The total amounts of the seeds hand squeezed to get pure fresh pomegranate juice. The suspension solution were passed through a stainless steel mesh sieves before filtering by Whatman filter paper using Buchner funnel. The pomegranate juice sample final volume of solution sample 60 ml. Centrifuge and stored in refrigerator. An aliquot of the samples was diluted with purified water to bring the concentration of the samples within the range of the calibration curves. All standards and solutions ready to analysis were passed through the 0.45 um desk filter and sonicated for 15 minutes in ultrasonic bath.

2.2.3 Spike of pomegranate sample

In order to evaluate possible matrix effects and the accuracy validation of the method used in this study. Three 0.5 ml batches of the pomegranate samples were prepared to examine the recovery yield and the validation of the process were applied to determined fructose. One batch was spike with zero concentration of fructose. The second batch was spike with an amount of fructose, so the final concentration of the sample is 100 ug/ml fructose spiked. As well as a standard solution of final concentration of 200 ug/ml was directly injected for

spiking chromatographic analysis. Sugars concentration was measured based on the peak area using Maslynx 4.1 software, for the triplicate injection as mention above. The same manner was carrying out to monitor recovery for the process to determination of glucose.

RESULTS AND DISCUSSION

Sometimes method development in chromatography-mass spectrometry can be challenging and it takes long time to get your task. Screening multiple column and different mobile phases and the parameters of the mass spectrometry going thought univariate optimization method it is taking time, so it is challenging to develop a method for complex mixtures such as pomegranate fruit juice. So we take this task and develop a method for analysis of sugars (fructose, glucose and sucrose) in Taif pomegranate fruit juice and compare with the pomegranate fruit juice in the region. The three sugars under study were successfully separated then identified and determined quantitatively as will see below.

Atmospheric pressure ionization is regarded as a "soft" ionization process. This results in a mass spectrum typically dominated by a single ion that corresponds in protonated the positive ion mode $[M+H]^+$ or deprotonated in the corresponds negative ion mode $[M-H]^-$, for example, a compound with molecular weight of 200 Da in the positive ion mode will result in a spectrum with a " base peak" at 201 mass to charge ratio (m/z). In negative ion mode (dependent on the chemistry of the compound), the spectrum will be dominated by an ion at 199 m/z). Hence, the selective ion record (SIR) mass spectrum rang (100 – 420 m/z) for our ions of interest; Fructose, glucose and sucrose at 179.00 Da, 179.00 Da and 341.00 Da respectively.

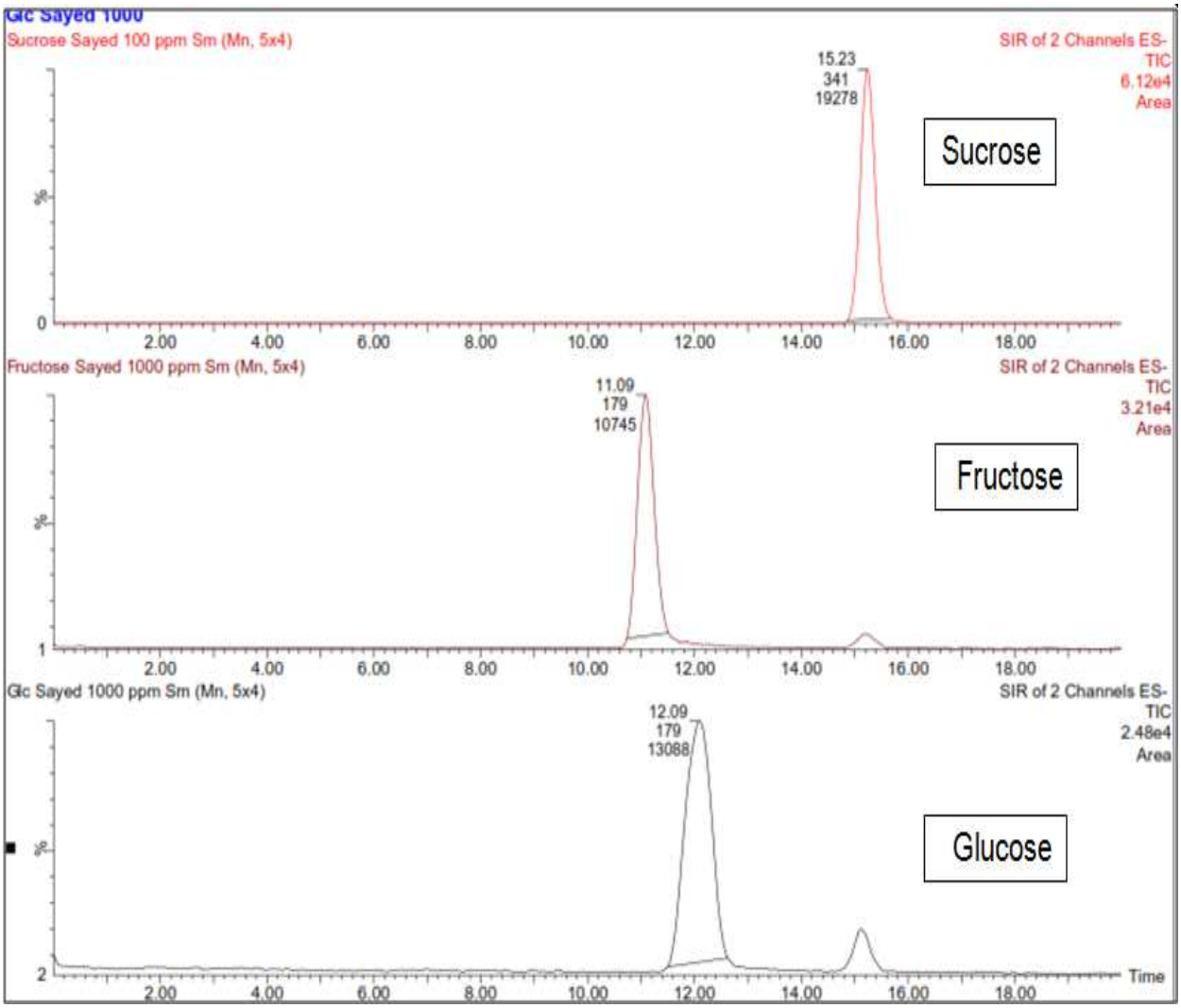


Figure (1): Chromatograms of standards fructose, glucose and sucrose, with Polaris NH_2 150 mm × 3.0 mm × 5 µm particle size column using LC-ESI- MS system, at optimum conditions

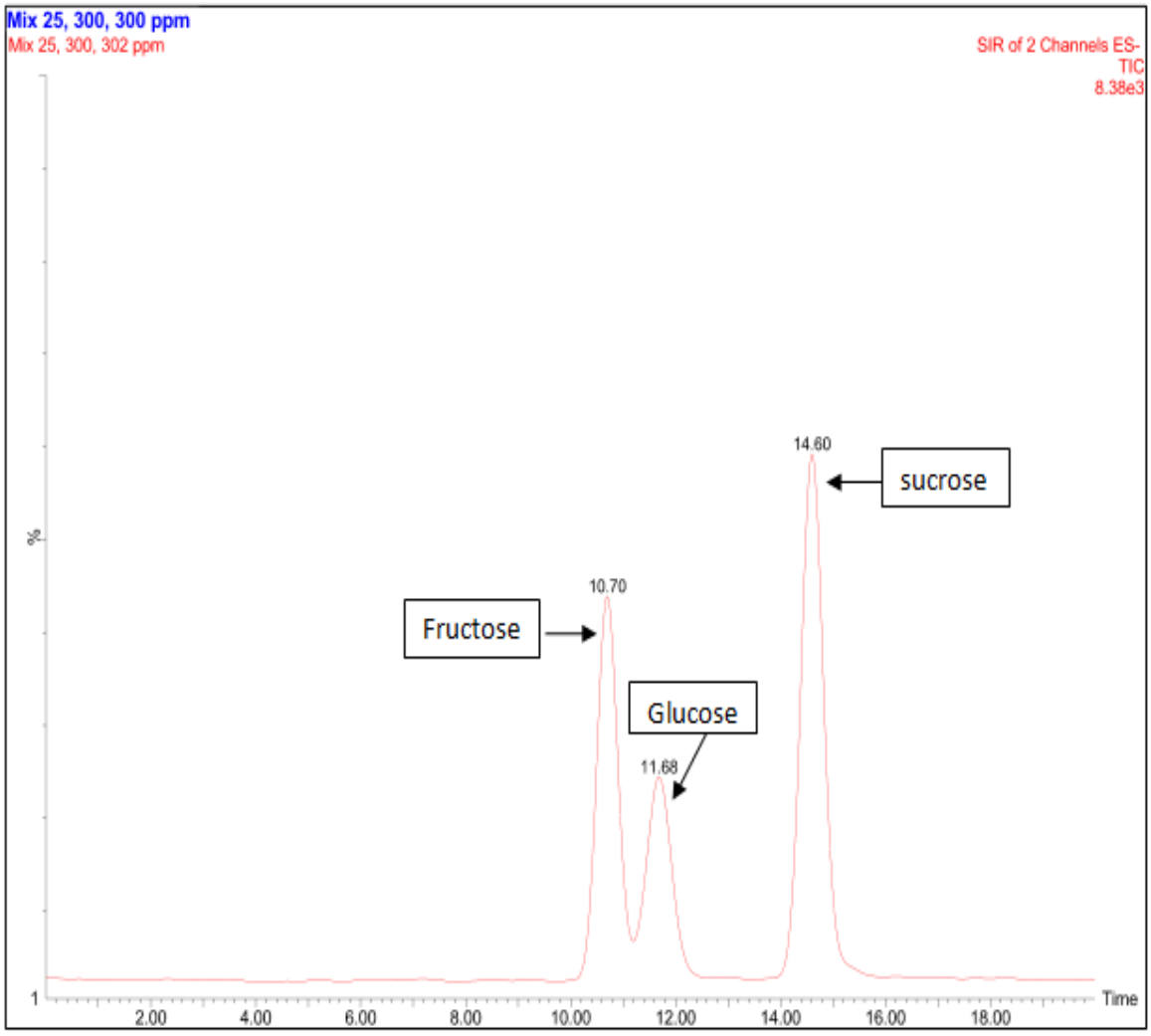


Figure (2): Separation chromatograms for mixture of fructose, glucose and sucrose with Polaris NH_2 150 mm × 3.0 mm × 5 µm particle size column using LC-ESI- MS system at optimum conditions

3.1 Ionization and mass spectrometry conditions:

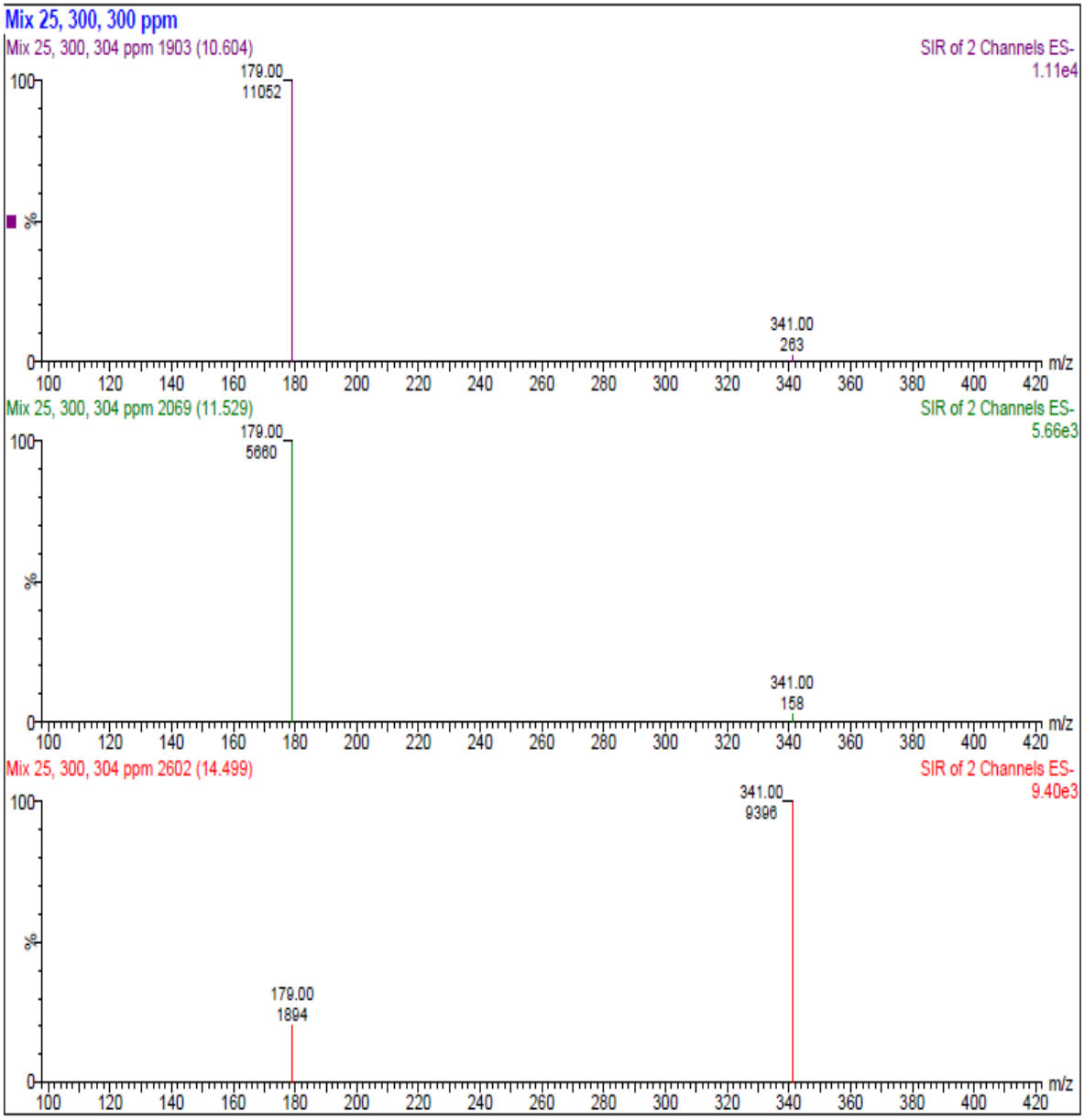
Direct infusion method using ESI-MS (waters 3100) in negative ion mode was performed to get optimum condition for detection fructose, glucose and sucrose ions. The analytical conditions for injection include the injection of sugars standards (1µg/ml) directly to the ion source by means of a syringe pump at flow rate (0.02 ml/ min) for ten minutes.

Fructose, glucose and sucrose compounds are deprotonated to form $[M-H]^-$ ions with the molecular ions recording at 179.00 , 179.00 and 341.00 Da respectively in the negative electrospray ionization (ESI) mode.

The ionization efficiency improved by optimization parameters were tested for their ability in promoting the signal for the mass spectrometry (MS). For the purpose of identifying abundant ions that are characteristic of the sugars analysis, we first studied MS patterns by directly infusing sugars standards solution at a level of 1 ug/ml into the ESI at a flow rate of 20 µl/minutes.

The sugars standards were tested with two organic solvent (methanol and acetonitrile) with water. The deprotonated ion $[M-H]^-$ were found to be dominant in the respective ESI mass spectra in acetonitrile solvent. During method development, the tuning parameters for the ESI source were varied to obtained the maximum $[M-H]^-$ ion intensity for the sugars compounds. The starting conditions were the default conditions of the MS. The resulting individual tune was found dramatically different for the cone voltage. The following tuning parameters were found to be optimal conditions for the suite for the sugars compounds: capillary voltage, 3.0 kV; cone voltage, 20 V; extractor voltage, 2V; RF lens, 0.3V; source temperature, 110^{O}C; desolvation temperature, 450^{O}C;

desolvation gas flow, 500 L/hr; cone gas flow, 50 L/hr. The overall ionization efficiency to form $[M-H]^-$ ions for the sugars ion molecular can be seen basis peak at mass 179.00 m/z. 179.00 m/z and 314.00 m/z .The cone voltage at 50 Volts has the lowest ionization efficiency among the test for the optimal conditions.



Figures (3):selective ion recording (SIR) spectrum of fructose, glucose and sucrose using LC-ESI-MS negative mode technique. Range: 100- 420 m/z, Column: Polaris NH_2 (150 mm × 3 mm) 5 µm particle size. Mobile phase isocratic (75 acetonitrile:25 water V/V)

3.2 Chromatographic information

3.2.1 Mobile phase solvent evaluation

Various solvent system were tried for the development of suitable LC-MS method for the analysis of fructose, glucose and sucrose individually and mixture. The suitability of the solvent system was decided on the basis of separation of sugars completely from the degradation products. Several mobile phases such as organic solvent, methanol: water and acetonitrile: water with and without formic acid were tested in addition to methanol: acetonitrile solvent at different ratio. In the process of method development was found that the solvent system composition of acetonitrile: water (75:25 V/V) at flow rate of 300 µl/ minute and the standards the samples gave sharp symmetry peaks at a good precise and accurate retention time of 11.09, 12.09 and 15.23 minutes for fructose, glucose and sucrose respectively as can be seen in figure (1) & (2). Optimization the quality of mobile phase solvent can contribute to improving the chromatographic or mass spectroscopic properties of

the analyte as well as the overall detection limits of instrument system. The favorable results were obtained meet our expectation which gave an acceptable peak get sharp symmetrical peak with good resolution for the mixture. Isocratic elution was optimized and carried out in a mobile phase as the isocratic mode consisting of acetonitrile: water (75:25 V/V) thought this study.

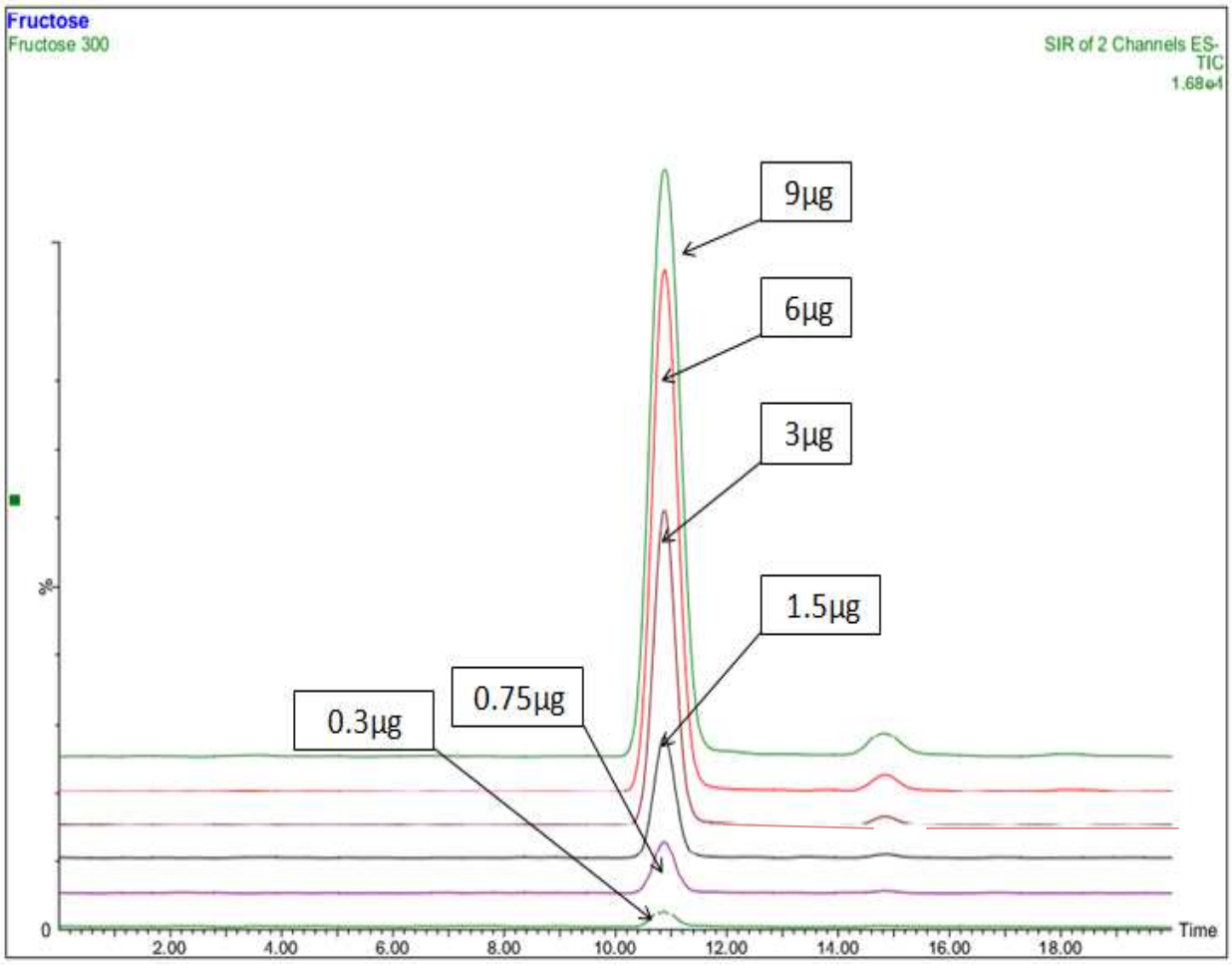


Figure (4): Overlay chromatograms for individual fructose with Polaris NH_2

150 mm × 3.0 mm × 5 µm particle size column using LC-ESI- MS system at optimum conditions

3.2.2 Column selection

It has often been stated that the column is the heart of the liquid chromatography. Choice of the wrong column and mobile phase conditions for the sample at hand can trivialize all the advantages of expensive, sophisticated instrumentation and data systems in a laboratory. Two types of analytical liquid chromatography columns are used for the test of the separation of sugars (fructose, glucose and sucrose) from the same company (Polaris NH_2 150 mm × 3 mm × 5 um, and Polaris NH_2 50 mm × 4.6 mm × 3µm) . In all cases separation of sugars is challenging and methods development is required to optimize separation in reasonable retention time.

In order to select the proper column for the analysis, the column length and particle size taken into consideration. The column length enables selective separation of the analyte. The longer column, the greater in the retention time. The chromatographic separation was performed using a column coupled on series with guard column with NH_2 (10.0 mm × 50 mm) 5 µm at the front. Separation was achieved using a mobile phase consisting of acetonitrile: water at flow rate of 300 ul/min. The column was maintained at ambient temperature with injection volume of 20 ul and run time was 20 minutes. A blank chromatogram was recorded before the studies. So Polaris NH_2 15.0 mm × 3 mm × 5 µm was chosen as a column for separation of fructose, glucose and sucrose thought this study.

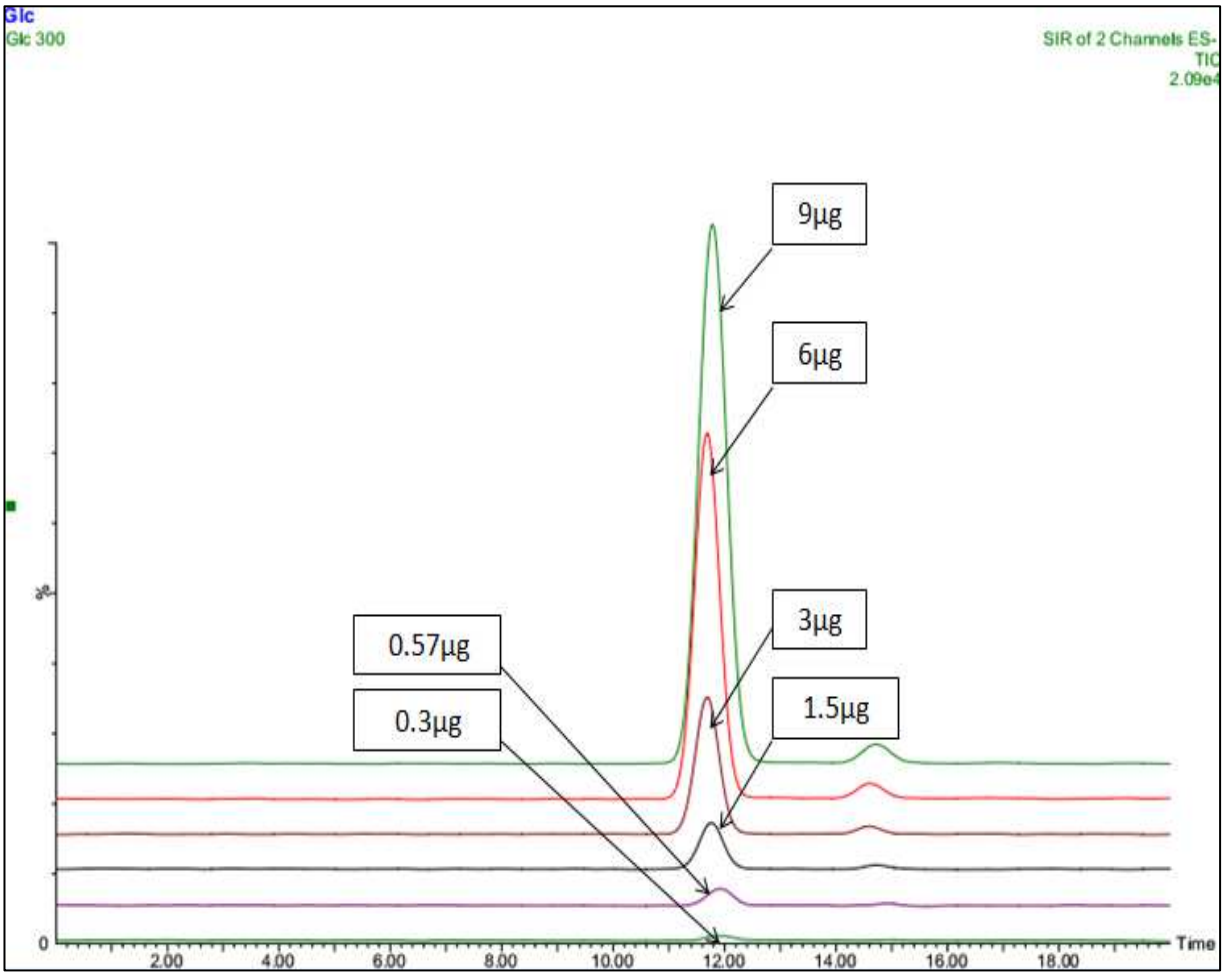


Figure (5): Overlay chromatograms for individual glucose with Polaris NH_2 150 mm × 3.0 mm × 5 µm particle size column using LC-ESI- MS system

3.2.3 Chromatographic system Performance

Optimization of chromatographic conditions cannot be made independently from that of the MS component. When one considers the low level nature of the analytes present in the pomegranates fruit juice and the co-existence of hundreds of other compounds, some degree of separation is desired before ionization and MS detection to minimize signal suppression and other interferences from the bulk matrix of the sample. Before the quantitative and qualitative determination of sugars in the samples, we prepared standard solutions of different sugars: sucrose, glucose and fructose. With those standard solutions of different sugars we made calibration lines for each one of the sugar, which later used for assessing the concentration corresponding to the different peaks in the chromatograms.

Peak identification was based on the retention times t_R, Identification of the three sugars were confirmed with known standards injected individually through the LC-ESI-MS system and the retention time were for fructose (t_R =11.09 ± 0.1 minutes, glucose (t_R = 12.09 ± 0.11 minutes) and sucrose (t_R = 15.23 ± 0.01 minutes) as shown in figure (1) in addition to the selective ion record (SIR) for the negative ion mode as shown in figure (3) (line spectrums). Overlay chromatograms of fructose, glucose and sucrose illustrated into figure (4), figure (5) and figure (6) respectively which demonstrate highly reproducibility of the method under study and the retention time were highly precise. The chromatographic profiles showed very good separation of the three sugars figure (2) and fructose was the first eluent and the glucose the second and sucrose the last this agreed with the theory of the column that we are used [26]. In the chromatographic analysis, with 5 um NH_2 150 mm × 3.0 mm column, each run was performed in about 15 minutes with great resolution. Figure (3) shows the line spectrum for the LC-ESI-MS chromatograms for fructose, glucose and sucrose monitoring ions (with SIR mode) at 179.0, 179.0 and 341.0 m/z. It demonstrates that the specificity of the system eliminates the need for complete separation of the individual compounds of different molecular weight. The retention times were found to be extremely stable, varying with in ±0.08 minutes. As a result the retention time are still useful for verifying the presence/ absence of analytes of different molecular weight.

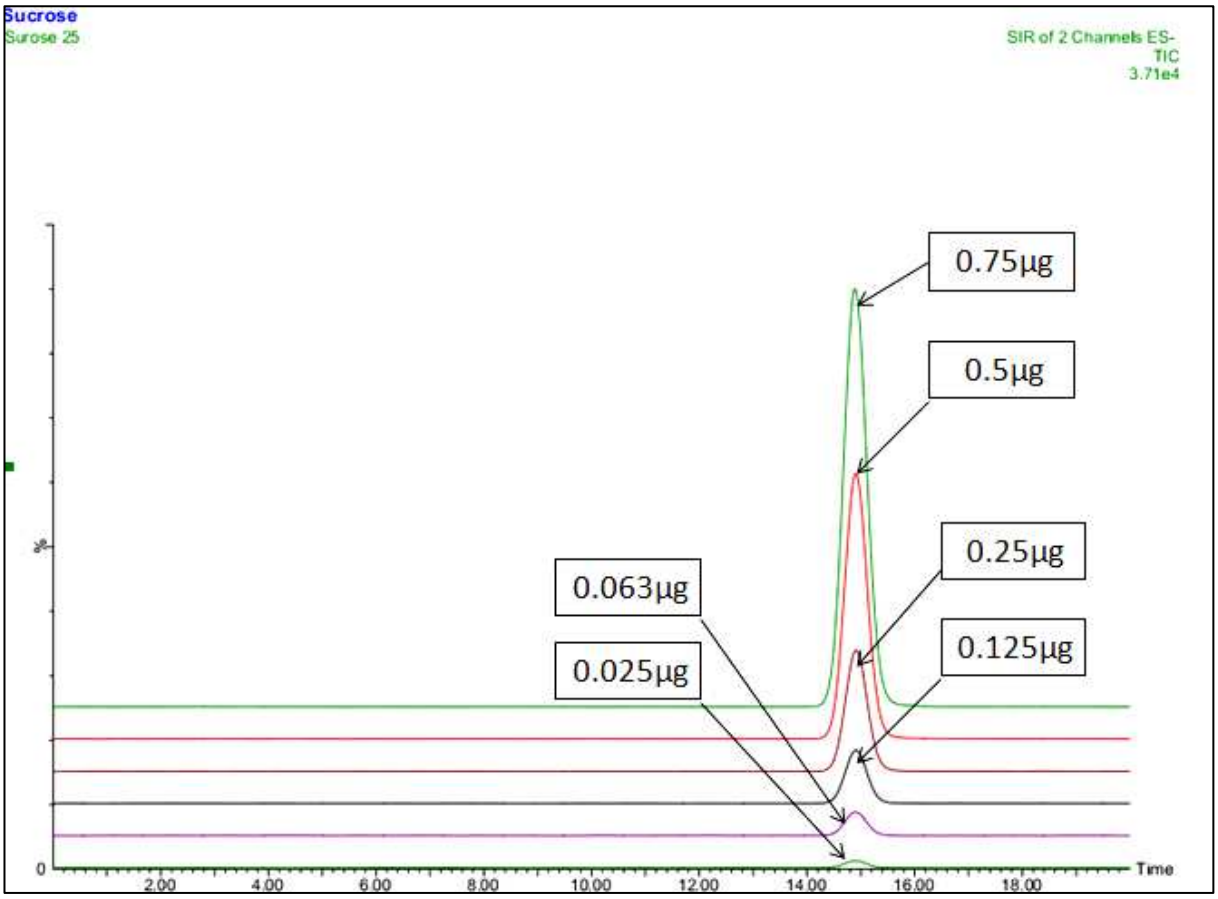


Figure (6): Overlay chromatograms for individual sucrose with Polaris NH_2 150 mm × 3.0 mm × 5 µm particle size column using LC-ESI- MS system

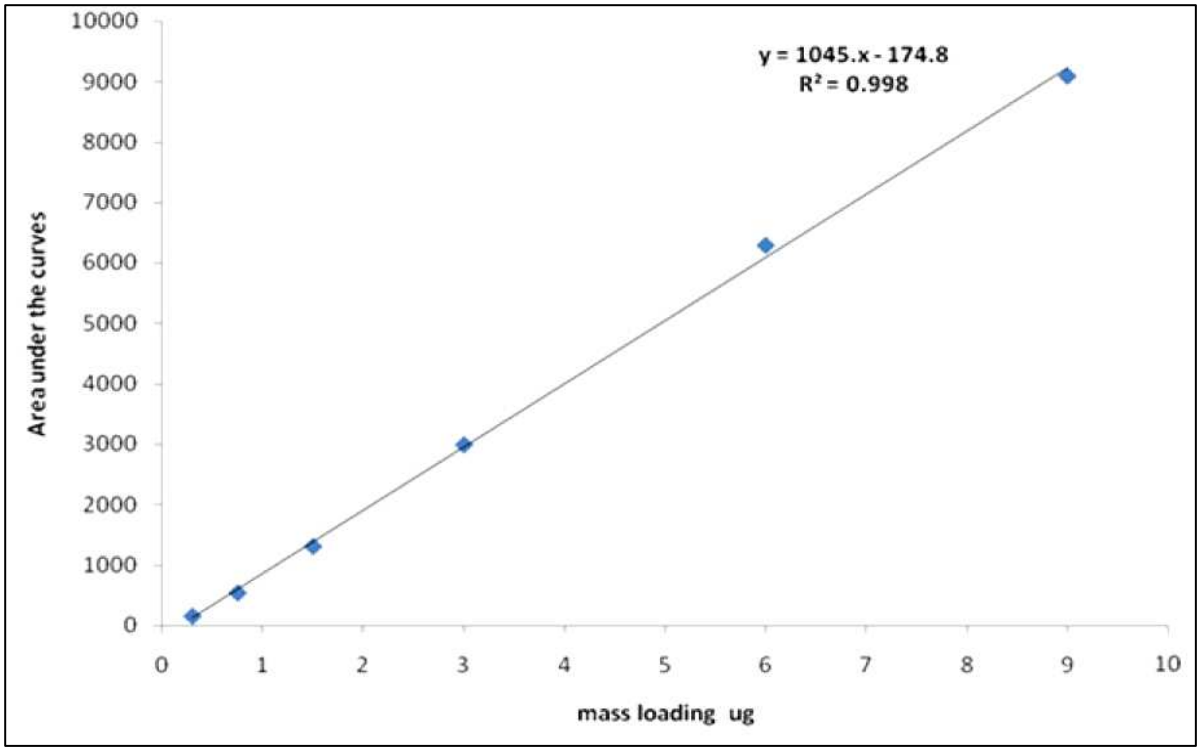


Figure (7): Calibration curve of individual fructose at optimum conditions using LC-ESI-MS system with Polaris NH_2 150 mm × 3 mm × 5 µm particle size column

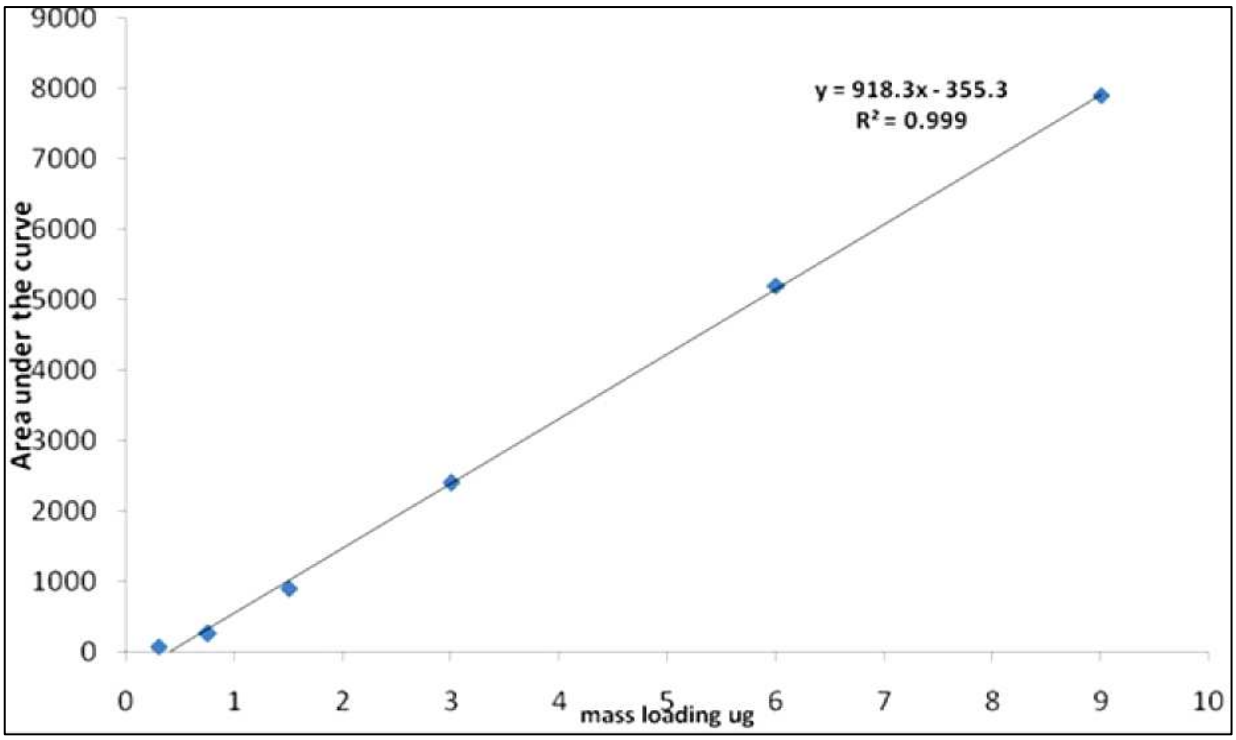


Figure (8): Calibration curve of individual glucose at optimum conditions using LC-ESI-MS system with Polaris NH_2 150 mm × 3 mm × 5 µm particle size column

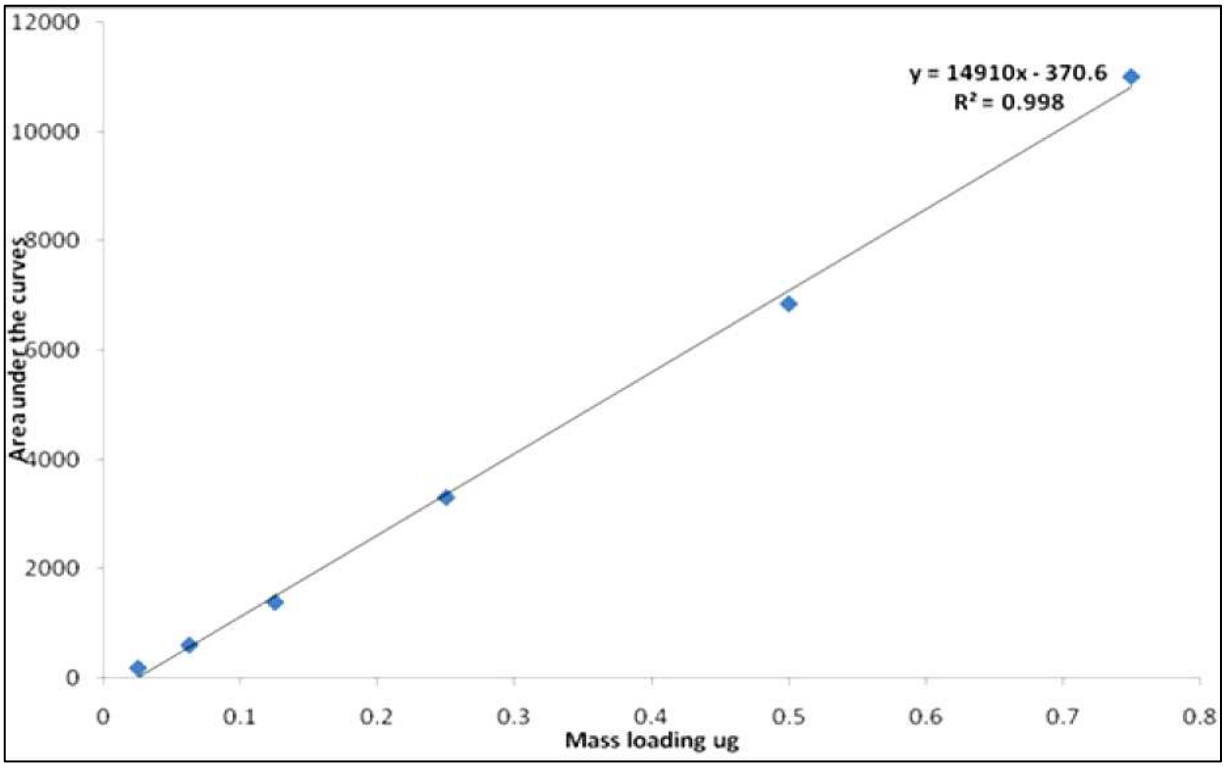


Figure (9): Calibration curve of individual sucrose at optimum conditions using LC-ESI-MS system with Polaris NH_2 150 mm × 3 mm × 5 µm particle size column

3.3 Linearity and detection

The calibration curves for fructose, glucose and sucrose were shown in figure (7), figure (8) and figure (9) which were correlation coefficient for their lines 0.998, 0.999 and 0.998 respectively. Calibration curves were established by plotting peak area of the sugars (fructose, glucose and sucrose) under study versus the analyte mass loading at the optimum conditions. Calibration linearity for fructose, glucose and sucrose were investigated by making the replicate injections of each standard prepared at six different concentrations. It is the calibration curve fructose, glucose and sucrose the excellent linearity was achieved throughout the range from (0.3-9 µg) mass load for fructose and glucose. The range from (0.025-0.75 µg) for sucrose. The linearity equation of fructose, glucose and sucrose as described below. The external standard method was used to establish calibration curve and quantify fructose, glucose and sucrose in Taif , Abha, Egyptian, Yamani 1 and Yamani 2 pomegranate fresh fruit juice.

The calibration slope, intercepts, the coefficient of determination for standards are list below and on the same figures:

Y=1045 X- 174.8 R^2 0.998 for fructose Y=918.3 X- 355.3 R^2 0.999 for glucose Y=14910 X-370.6 R^2 0.998 for sucrose

Where, Y stands peak area, and x stands for sugars concentration. R^2 stand for correlation coefficient. Hence the determination limit for fructose, glucose and sucrose less than 0.3 µg, 0.3 µg and 0.025 µg respectively. In order to used the method for efficient separation of the sugars under study similar from fruit samples. The standards mixture at (300 ppm fructose, 300 ppm glucose and 25 ppm sucrose) were injected within (1- 30 µl) under the optimum condition, the result indicate that the all the peaks were symmetry in perfect shape, nice peak area as well as the highly reproducibility of the retention time which can be used to analysis of fructose and glucose and sucrose directly with few dilution to save time and solvents. This gives excellent achievement of the separation of the method. Furthermore, retention times were very reproducible.

Recovery checks for fructose and glucose were carried out in two different analysis sessions using Taif pomegranate samples. The recoveries were between 96% -101 %. The recovery checks gave very good average values from triplicate injections. The high recovery values suggest that this LC-MS method of analysis is excellent procedure for analysis of fructose, glucose and sucrose. No significant effects were observed from the matrices on the sugars under study.

3.4 Analysis of fresh pomegranate fruit juices

In this study pomegranate fruit were collected from the local markets of Taif – KSA. It is the mean of the triplicate samples for each kind. We used the method optimized above t o quantify sugars (fructose, glucose and sucrose) in pomegranate fresh juice samples. Fructose and glucose compounds were found in all the five samples and sucrose was not detected in all samples under study. Table (1) reported the means of the samples, It is the mean of the triplicate samples for each kinds and the range of the concentrations for fructose (0.162- 0.306 gm/100 gm) and glucose (0.132-0.300 gm/100 gm) for different brands. The total sugars content was found to be between 0.294 - 0.616 gm/100 gm. The lowest sugars content was found 0.294 gm./ 100 grams for Yaman 2 while the highest was found 0.616 gm./100 gram for Taif brand pomegranate. It can see that the concentration of sugars in pomegranates under study vary from country to country depend on the strong influence of the climate of the place, different environmental place, types of the soil and agricultural procedures on the contents of these elements in which is study. It was useful to note that the ratio between fructose and glucose nearly one.

Several potential peaks are visible on the chromatograms it is far away from the area of interest and it identified it is types for monosaccharide.

Table (1) shows standards deviation for fructose and glucose in all kinds of pomegranate less than 2%. Fructose and glucose concentration in Taif pomegranate were significant different with that measured for Egypt and Yaman 1, Yaman 2 pomegranates. On the other hand, the result was obtained for determination the fructose and glucose were present in all sample in different concentration. These results show the significant different in the concentration of fructose and glucose in Spanish, Tunisian, Oman's and the pomegranates for (Taif, Abha, Egypt, Yaman 1, Yaman 2) under study. In spite of using different techniques for measurement the results agree what they reported that the ratio for fructose and glucose nearly one in pomegranates juice and not detected for sucrose undoubtedly duo to the strong influence of the climate of the place, different environmental place, types of the soil and agricultural procedures on the contents [13, 26-29].

Table (1): composition of sugars in fresh pomegranate fruit juice in 100 grams weight of pomegranate arils

Brands Sugars	Taif-KSA	Abha-KSA	Egyptian	Yamani 1	Yamani 2
Fructose	0.31±0.07 gm	0.27±0.08 gm	0.24±0.04 gm	0.18± 0.04gm	0.16±0.03gm
Glucose	0.30±0.06 gm	0.25±0.08 gm	0.19±0.05 gm	0.12± 0.05gm	0.13 ±0.06gm
Sucrose	Not detected	Not detected	Not detected	Not detected	Not detected

CONCLUSION

An LC-MS method was developed for the determination of fructose, glucose and sucrose presented in pomegranate fruit juice in about 15 minute. The method was simple suitable and rapid procedure no need to derivatization or make attachment reaction for the sugars. This method greatly reduces sample pretreatment. Recoveries for those target compounds were in the range of 96-101 % and the determination limits of less than 0.3 µg for both fructose and glucose 0.025 µg for sucrose were achieved for all test sugar compounds. This achievement done by using Polaris NH_2 150 mm × 3.0 mm × 5 um column. The main advantages of this system were excellent separation in relative short retention times and very good efficiency and low sample and materials consumption.

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REFERENCES

[1] Bob AJ. Liquid chromatography –Mass spectrometry an introduction, Wiley &Sons Inc., NY, USA, (**2006**).
[2] Lioyd R S, Joseph JK, John WD. Introduction to modern liquid chromatography, A John Wiley & Sons, Inc., New Jersey, USA ,3rd Ed, (**2010**).
[3] Ricochon GG, Ricochon CP, Girardin M, Muniglia L. *Journal of Chromatography B*. **2011**; 879:1529-1536.
[4] Wan EC, Yu JZ. *Journal of Chromatography A*. **2006**;24:175-181.
[5] Eduard R, Tomuta V, Stein DT. *Analytica Chimica Acta*. **2007**; 591:155–160.
[6] Eric C.H.Wan and Jianz H. *Journal of Environmental Science and Technology*. **2007**;41: 2459-2466.
[7] Carla A, Larson T, Gilday A,Graham I, Bergström E, Thomas-Oates. J. *Journal Rapid Communications in Mass Spectrometry*. **2008**;22:1399–1407.
[8] Hammad L.A., Saleh MM, Novotny MV, Mechref Y. *Journal of the American Society for Mass Spectrometry*. **2009**;20:1224-1234.
[9] Javier M, Jerónimo G, Luis R, Rafael AB. *Journal Biomass and bioenergy*. 2011;35:2006-**2012**.
[10] Matias J., J. Gonzalez, L. Royano and R.A. Barrena, *Journal Biomass and Bioenergy*. **2011**;35:2006-2012.
[11] Chandraju S., R. Mythily and C. S. Chidan Kumar. *Journal of Chemical and Pharmaceutical Research*. **2011**;3:422-429.
[12] Wu X, Jiang W, Lu J, Yu Y, Wu. *Journal of Food Chemistry*. **2014**;15:976-983.
[13] AL- Maiman SA, Ahmad D. *Journal of Food Chemistry*. **2002**;76: 437-441.
[14] Chavez-Servin JL, Castellote AI, Lopez-Sabater MC. *Journal of Chromatography A*. **2004**; 1043:211-215.
[15] Lopes JF, Gaspar EM. *Journal of Chromatography A*. **2008**;1188:34-43.
[16] Barreira JCM, Pereira JA, Beatriz M, Oliveira PP, Ferreira ICFR. *Journal of Plant Foods for Human Nutrition*. **2010**;65:38-43.
[17] Mahmood S, Mahmmed TA, Salih AB,, El-Sayed SAH. *Archives of Applied Science Research*. **2011**; 3 (6): 488-496.
[18] Néjib H, Mokhtar T, Rania J, Afaf KE, Messaoud M, Pablo M, Francisca H. *International Journal of Food Properties*. **20111**;14:741–757.
[19] Legua P, Melgarejo P, Martínez JJ, Martínez R, Hernández F. *International Journal of Food Properties*. **2012**;15:481–494.
[20] Yang W, Ming-Yu D. Journal of Chromatography A. **2000**; 904:113–117.
[21] Karlsson G, Winge S, Sandberg H. Journal of Chromatography A. **2005**;1092:246-249.
[22] Tao P, Changmin B, Yanjuan X, Guowang X, Zhongyi Y, Yong S, Liming P. Journal of Liquid Chromatography & Related Technologies.**2006**;9:1281-1289.
[23] Cui St.,"*Food Carbohydrates, chemistry, physical, properties, and applications*" CRC Press, Taylor & Francis Group, New York, USA, **2005**;79-81.
[24] Valerie Ratsimba, García Fernández JM, Defaye J, Nigay H, Voilley A. *Journal of Chromatography A*. **1999**; 844:283–293.
[25] Alekseeva AV, Kartsova LA, Kazachishcheva NV. *Journal of Analytical Chemistry*. **2010**;65:202–208.
[26] Vladimir VT, Oliver F. *Analytical Biochemistry*. **2002**; 301:298-307
[27] Dumlu, MU,Gurkan E. *Journal of Medicinal Food*. **2007**;10:392-395,

[28] Salma MZA, Abdulrahman AO, Maryam MA. *Science and Technology*. **2001**;6:39-44.
[29] Walid E, Nizar T, Ma Y, He SH, Ali R, Nazar N. *International Journal of food Engineering.* **2011**;7:1-13.