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Liquid Chromatography/Mass Spectroscopy and TLC Analysis for the Identification of Sugars Extracted from the Outer Skin of Almond Fruit (*Prunus dulcis*)

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Abstract

A rapid, sensitive extraction method was developed using the mixture Methanol - Dichloromethane - Water (MDW) (0.3:4:1v/v/v) and MeOH-H_2O phase was assayed for sugar analysis. Photodiode-array detection (DAD) has been used to prove the extracted compound is UV inactive, High-performance liquid chromatography (HPLC) with Evaporative Light Scattering Detector (ELSD) coupled to electrospray ionization mass spectrometric (ESI-MS) detection in the positive ion mode gave MS and MSn fragmentation data which were employed for their structural characterization. The various standard sugars were spotted using the solvent system n-butanol - acetone - diethylamine – water (10:10:2:6, v/v/v/v) in the cellulose layer for TLC analysis which indicated the presence of lactose, sucrose, galactose, xylose. This is the first assay of the sugar profile of the almond fruit outer skin, which can be further developed for characterization and evaluation of their quality with regards to their sugar composition.

Key Words: Sugar extraction; almond fruit; UV inactive; HPLC; ELSD; Separation; LC/M

Introduction

The Almond is a species of tree native to the Middle East. Almond is also the name of the edible and widely cultivated nut of this tree. Within the genus Prunus, it is classified in the subgenus Amygdalus, distinguished from the other subgenera by the corrugated shell (endocarp) surrounding the seed. Almonds grow on a small-to medium- sized tree with a spreading, open canopy, usually 10 to 15 feet in commercial orchards. Leaves are linear or slightly ovate, about 2 to 5 inches in length, with acute tips and finely serrate margins. Overall leaves are smaller and less folded along the midrib than those of its close relative the peach. Almond's fruit is the nut. The entire fruit, including the hull is a drupe; however, the hull dries and splits prior to harvest, revealing what appears to be the pit of the fruit. Botanically, this pit with the kernel inside fits the definition of a nut. Fruiting begins in 3- to 4- year- old trees, with maximal production in 6 to 10 years. Unlike its short-lived cousin the peach, almond trees can produce for 50+ years. Thinning is unnecessary; a high proportion of flowers must set fruits for normal yield [1, 2].

Almonds are one of the few alkaline nuts, and are widely used in ayurvedic medicine to relieve phlegm and coughs and to lubricate the intestines. It is also believed that almonds inhibit the growth of cancer cells [3]. They can also relieve some types of constipation, especially when prepared as a drink by soaking the nuts overnight, removing the peel in the morning, and putting the nuts into a blender with some water. Since almonds are low in lysine, they are an ideal food to combine with legumes in order for the meal to contain complete protein content. They are not ideal to combine with grains as they, too, have low lysine content. Almond milk has been used since ancient times, especially in the Arab world and later in Europe, where it replaced milk on fast days. During the medieval period, almond milk appeared twice as often in English recipes as those of France, despite the fact that almond trees do not grow in England, but they do in France. The well-known European confection marzipan is made by using almond paste, rose water, and sugar. It is said that this recipe dates from the time of the Thirty Years' War [4]. Most of the past research deals the analysis and importance of the nut. The present study is mainly concentrated on the soft fruit portion which is rich in carbohydrates and has wide application in the field of applied chemistry by yielding economical profit.

Method and Material

Selected samples are sliced, dried under vacuum at 60^{0}C for 48 hr and powdered. 100.0 g of raw material was extracted with doubly distilled water 75mL and stirred well with magnetic stirrer for 30'. The resulting syrup was stored at 4˚C in the dark. The syrup was treated with charcoal (coir pith) and agitated for 30' followed by Silica gel (230-400 mesh) packed in a sintered glass crucible for about 2cm thickness connected to suction pump, where rota vapour removed the solvent of the

filtrate. The residue was placed in an air tight glass container covered with 200 ml of boiling 80% ethanol. After simmering for several hours in a steam bath, the container was sealed and stored at room temperature. For the analysis, sample was homogenized in a blender for 3-5'at high speed and then filtered through a Buchner funnel using a vacuum source replicated extraction with 80% EtOH (2 x 50mL) each time and the whole syrup was concentrated. Methanol - Dichloromethane - water (0.3:4:1, v/v/v), Sample tubes fed with the mixture were loosely capped, placed in a water bath for 5S, and left at room temperature for 10'and placed in separating funnel, agitated vigorously by occasional release of pressure, results two phases. The organic phase was discarded which removes the organic impurities and the methanol: water phase was assayed for sugar. The residues were oven-dried at 50°C overnight to remove the residual solvent, and stored at –2° C for analysis.

Instrumentation

The crude mixture was separated into individual components in 26'by reversed phase HPLC on an Adsorbosphere column-NH_2, (250 x 4.6 mm column) using both isocratic and gradient elution with acetonitrile/water and detected using Waters ELSD 2420. In ELSD, the mobile phase is first evaporated. Solid particles remaining from the sample are then carried in the form of a mist into a cell where they are detected by a laser. UV analysis using Agilent 8453 coupled with Diode array detector. LC–MS analysis was performed with LCMSD/ Trap System (Agilent Technologies, 1200 Series) equipped with an electrospray interface. The MS spectra were acquired in positive ion mode. The mobile phase consisted of 0.10% formic acid in hplc grade deionized water (A) (milli-q-water subjected to IR radiation under 3.5 micron filters) and Methanol (B) taken in the stationary phase of Atlantis dc 18 column (50 x 4.6mm - 5μm). The gradient program was as follows: 10% B to 95% B in 4 min, 95% B to 95% B in 1 min, 95% B to 10% B in 0.5 min followed by 10% B in 1.5 min at a flow rate of 1.2 mL min^{-1}. The column oven temperature was kept at 40°C and the injection volume was 2.0 μL. Product mass spectra were recorded in the range of m/z 150-1000. The instrumental parameters were optimized before the run.

Preparation of chromatoplates

Thin layer chromatography was performed for the concentrated separated fraction using Cellulose MN 300 G. The fractions obtained were subjected to one dimensional chromatogram on a cellulose layer plate. Each plate was activated at 110°C prior to use for 10'.

Standard samples

Pure samples D (-) Arabinose, D (-) Ribose, D (+) Xylose, D (+) Galactose, D(+) Glucose, D (+) Mannose, L (-) Sorbose, D (-) Fructose, L (+) Rhamnose, D (+) Sucrose and D (+) Maltose, D (+) Lactose were used as standard.

One – dimensional chromatography

10 mg of each sugar and the separated fractions were dissolved in 1ml of deionised water. 1μL of each sugar solution was applied to the chromatoplate with the micropipette in the usual manner. The chromatoplate was placed in the chamber containing the developing solvent. The solvent system used was n-butanol - acetone - diethylamine - water (10:10:2:6 v/v/v/v). The plates were developed in an almost vertical position at room temperature, covered with lid. [5, 6, 7, 8, 9]. After the elution, plate was dried under warm air. The plate was sprayed with 5% diphenylamine in ethanol, 4% aniline in ethanol and 85% phosphoric acid (5:5:1v/v/v). The plate was heated for 10'at 105°C. While drying coloured spots appear. The R_f values relative to the solvent are reported below.

Result and Discussion

Analysis report showed that the extracted separated components are UV inactive as in Figure 1.

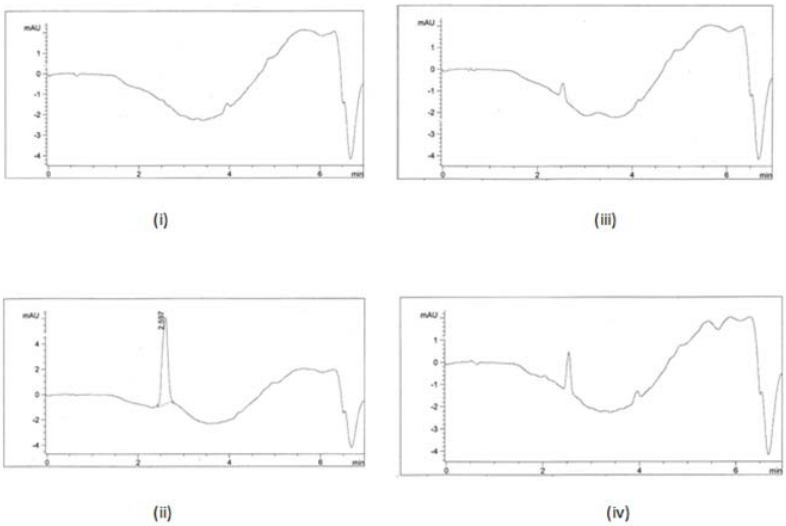

Figure 1: UV inactive spectrum of the Separated Fractions

The Mass Spectrum detector gave the following spectrum of fraction1 at 0.636 and 0.666 min, fraction2 at 0.525 and 0.702min, fraction3 at 0.606 and 2.637min, fraction4 at 0.595 and 2.576min. The MS report recorded at the appropriate time as per MSD for Fraction1 scanned between the time period 0.507:0.600min gave m/z values 126.9, 163.0, 343.2, 360.0, 365.0, 374.0 and 0.600 : 0.878 min gave m/z values 126.9, 163.0, 342.2, 365.0, 365.0, 375.1. Fraction2 scanned between the time periods 0.480: 0.546 min gave m/z values 115.1, 145.1, 175.9, 279.2, 312.1, 366.0, 365.0, 707.2 and 0.573: 0.812 min gave m/z values 111.2, 145.1, 279.2, 312.1, 360.0, 365.0, 707.2. Fraction3 scanned between the time period 0.507: 0.798 min and 2.495: 2.760min gave m/z values 112.9, 145.1, 163.0, 180.1, 198.0, 360.0 and 112.1. Fraction4 scanned between the time period 0.520 : 0.745 and 2.508 : 2.667 gave m/z values 111.2, 145.1, 150.1, 272.9, 305.1, 326.1, 327.1, 331.0 and 112.2, 145.1, 278.9, 312.1 respectively which gives a conclusion that these masses corresponds to Hexose, pentose and disaccharides whose masses are 180.1, 150.1 and 342.2 respectively depicted in Figure-2-5.

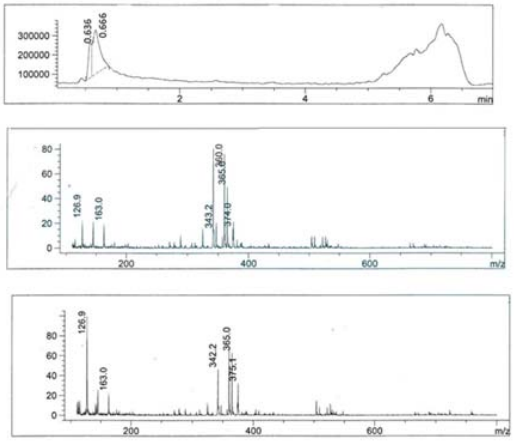

Figure 2: Mass report of Separated Fraction 1

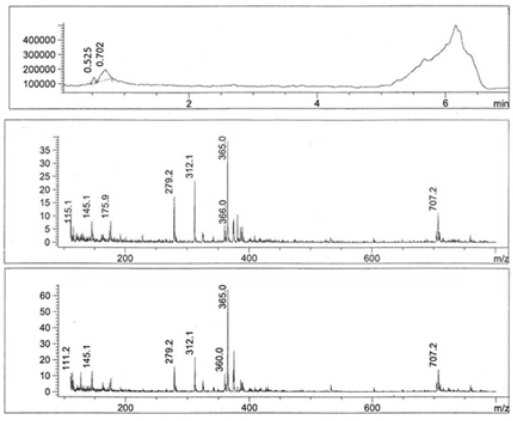

Figure 3: Mass report of Separated Fraction 2

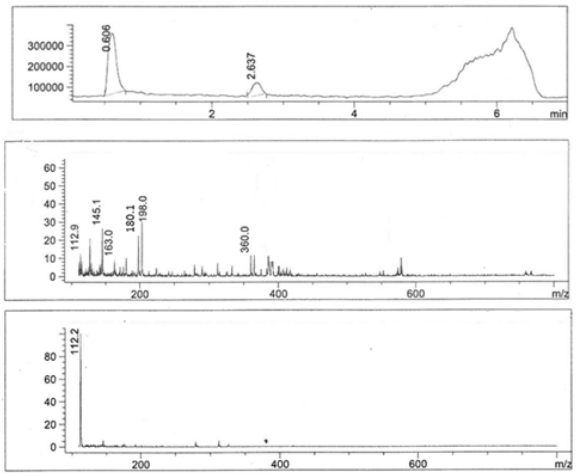

Figure 4: Mass report of Separated Fraction 3

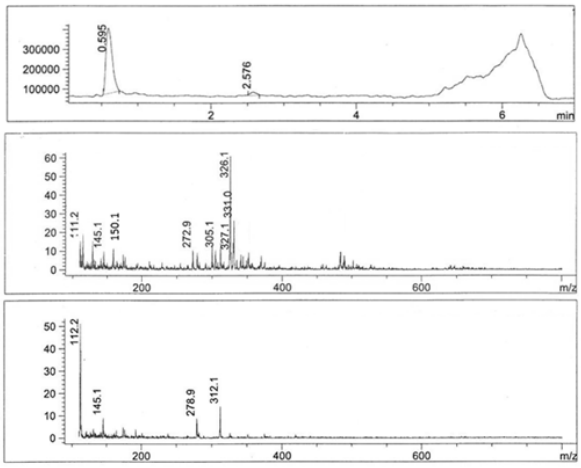

Figure 5: Mass report of Separated Fraction 4

Thin layer chromatographic analysis report

Four separated and purified sample fractions are spotted in the cellulose layer and the eluted species were mentioned as F 1, F 2, F 3, F 4 in the chromatogram shown in Figure 6. The fractions obtained were found to be matching with four standard sugars. R_f value for the analytical grade samples shown in Table 1.

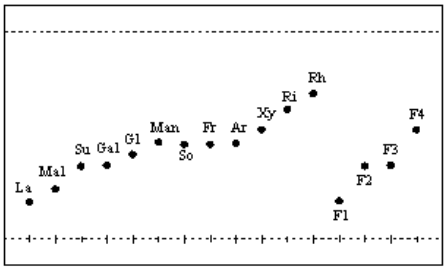

Figure 6: Developed thin layer chromatogram over a cellulose layer, (La – Lactose, So – Sorbose, Ar- Arabinose, Rh – Rhamnose, Ri – Ribose, Xy-Xylose, Gal – Galactose, Gl - Glucose, Man – Mannose, Fr - Fructose, Su – Sucrose and Mal –Maltose).

Table 1. R_f values matching of the analytical standard samples and the separated samples

Sugars	R_f (Scale of R_f =1)	Fraction matching
Lactose	0.18	F1
Maltose	0.24	-
Sucrose	0.35	F2
Galactose	0.36	F3
Glucose	0.41	-
Mannose	0.47	-
Sorbose	0.46	-
Fructose	0.46	-
Arabinose	0.46	-
Xylose	0.53	F4
Ribose	0.63	-
Rhamnose	0.70	-

Conclusion

The quantity of the discarded portion is very high; therefore, because of disposal problems the household solid wastes are of greater importance. A fruitful and economic industrial application was applied in the current work. Based on the above studies, a rapid method for the extraction of water soluble sugar has been developed. The mixture Methanol-Dichloromethane-Water gives better results as compared with Methanol-Chloroform-Water [10]. HPLC has proven to be more selective than conventional wet methods; additionally, HPLC allows individual quantification of several individual sugars in a single chromatographic run. Mass and TLC analysis gives accurate confirmation for the presence of lactose, galactose, sucrose and xylose which were extracted from the outer skin of Almond fruit (*Prunus dulcis*).

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