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Identification of Fatty acids, Carbohydrates and Organic acids in an Aqueous Extract of Red Watermelon (*Citrullus lanatus*) Rinds by UHPLC-Q Exactive HF Orbitrap

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Abstract

Watermelon is a Cucurbitaceae and a fruit that has great deal of medicinal and nutritional potency. Watermelon is endowed with a lot of nutrients in different proportions such as glucose, fructose, amino acids, fatty acids and vitamins. Supplementing watermelon juice in diet promotes vascular health and World Health Organization (WHO) recommends the intake of the fruits for healthy living. The search for more phytochemicals and nutrients in fruits is a continuous practice especially in the advent of more sensitive spectroscopic equipment. In the current research, rind of commonly consumed red watermelon is investigated for possible nutritional compounds using higher sensitive UHPLC in tandem with Q Exactive HF Orbitrap. The findings revealed the putative occurrence of some carbohydrates (glucose, fructose and galactose), short-chain fatty acids (6,11-dioxododecanoic acid and dihydroxypropoxy-9-oxononanoic acid) and organic acids (citric acid, malic acid and azelaic acid). The results of the research could upgrade the rinds of watermelon and may give rise to considerations on ways to utilize the rinds to serve better purposes such as supplement for human diet or be made part of animal feed like chicken and fish.

Keywords: Red watermelon, rinds, aqueous sample, negative ionization, precursor ions, MS/MS ions and decarboxylation.

Introduction

Citrullus lanatus (watermelon) is a Cucurbitaceae family member and the world's third most popular fruit [1]. Many bioactive substances found in watermelon have been shown to have a range of positive health effects including lowering the risk of obesity, diabetes, and aging-related disorders, as well as reducing the risk of cardiovascular problems [2][3]. It has been found that supplementation of hypertensive patient's diet with watermelon juice enhances the patient vascular heath [4]. Watermelon is rich in sugar, organic acids and amino acids [5]. Watermelon contains low fat-diets with tremendous richness in fibre and carbohydrate and intake of such food is recommended by the World Health Organization

(WHO) as the required diet for everyone for healthy living [6] as carbohydrate supply instant energy for cellular activities, fibre is greatly good for blood cholesterol, that aids in preventing bowel diseases [7]. The presence of numerous phytochemical compounds with pharmaceutical effects including carotenoids, citrulline, and other polyphenolic substances is fundamental to the watermelon's prophylactic and therapeutic effects [8]. Hence, it is becoming a preferred alternative diet. Watermelon has also been shown to be an excellent source of minerals such as potassium, magnesium, calcium, phosphorus, and iron [8][9] and vitamins like thiamine, riboflavin, niacin, and folate [10].

Several scientific researches that reported the chemical composition of watermelon as a nutraceutical fruits concentrated on the normal edible portion which is the pulp. The low sugar rinds are considered non-edible and thus less attention has is given to its nutraceutical worth. It is therefore, the aim of this research to explore the nutraceutical capacity of the rinds of red watermelon cultivars by profiling possible carbohydrates, fatty acids and organic acids using UHPLC-Q Exactive HF Orbitrap. Mechanisms on the fragmentation patterns of some of the profiled compounds will be proposed.

Materials and Methods

Chemicals and reagents

LCMS-grade water, was bought from Merck Millipore (Darmstadt, Germany). An Optima™ LC/MS Grade reagents (Fisher Chemical™) were used for the analysis.

Plant materials and biomass procurement

Matured red watermelon aged 3 months were conventionally grown in a farm at Jender am Hulu, Sepang, Selangor, Malaysia in May 2019. Using tab water followed by distilled water, the fruit was washed and rinsed. After removing the rinds, a blender (Panasonic, 500Ml tumbler. Model No. PSN-MXGM0501) was used to grind the rinds. The ground sample in zip-lock bag was froze at -80^{0}C. The frozen sample was later lyophilised.

Extraction for LCMS analysis

From the lyophilised sample, 5g of was consecutively extracted three times in 100ml Millipore water using ultra-sonication for 30min at less than 40^{0}C. The extract was filtered and collected in a container. The filtrate was then frozen in -80^{0}C refrigerator and later lyophilised. The lyophilised extract was then kept in -80^{0}C until analysis.

LCMS analysis

The lyophilised sample (100 mg) was reconstituted in 1 mL LCM grade water and filtered through regenerated cellulose (Titan3™, Thermo Scientific™). Using Dionex UltiMate 3000 UHPLC system (Thermo Scientific, MA, USA), 10 uL of the sample was chromatographed on Synchronis C18 column (Thermo Scientific, MA, USA) with a dimension of 2.1 mm ID x 100 mm L x 1.7 um particle size and column temperature was

set at 40°C. A water with formic acid (0.1%) was used as mobile phase A while mobile acetonitrile with formic acid (0.1%) was the mobile phase B. The gradient programme was kept at 0.4 mL/min flow rate. Solvent B was initially runed at 5% for 0 - 2 min, 95% for 2 -12 min, then 95 to 5% for 12 - 13.1 min, and finally with 5% was until 16 min. Q-Exactive HF Orbitrap (Thermo Scientific, MA, USA) was used for MS analysis in negative ionization mode. Molecules with mass range of 60-900 m/z were ionised, sheath gas flow rate of 60 and aux. gas flow rate of 2 and sweep gas flow rate of 1 were maintained. A spray voltage of -3.00kV, capillary temperature was set at 300°C, S-lens RF level of 40 was used and aux. gas heater temperature was set at 370°C.

Result and Discussion

Table 1: Putative compounds identified in aqueous sample of the rind of red watermelon cultivar in negative ionization mode

No	Putative compounds	RT	M-H m/z	Major MS/Ms fragments	Molecular formula	References
	Fatty acids					
1	6,11-dioxododecanoic acid	7.21	227.1285	209.1177, 125.0698, 142. 9919	$C_{12}H_{20}O_4$	[11]
2	Dihydroxypropoxy)-9-oxononanoic acid	5.91	261.1342	187.0968, 125.0961	$C_{12}H_{22}O_6$	[11]
3	9-hydroxy-10,12-octadecadienoic acid	10.22	295.2275	277.2169, 171.1018, 195.1363	$C_{18}H_{32}O_3$	[11]
	Carbohydrates					
4	Fructose	1.51	179.0553	59.0129, 71.0128, 89.0233	$C_6H_{12}O_6$	Massbank
5	D-glucose	10.44	179.0553	59.0129, 71.0128, 89.0233	$C_6H_{12}O_6$	Massbank
6	D-galactose	1.48	179.0553	59.0129, 71.0128, 89.0233	$C_6H_{12}O_6$	Massbank
7	Ribose	13.67	149.0458	59.0128, 71.0128, 89.0230	$C_5H_{10}O_5$	Massbank
8	Arabinose	6.81	149.0455	59.0128, 71.0128, 89.0234	$C_5H_{10}O_5$	Massbank
9	Palatinose	12.18	377.0852	215.0321, 179.0552, 89.0234	$C_{12}H_{22}O_{11}$	Massbank
	Organic acids					
10	Malic acid	1.14	133.0133	115.0026, 71.0129, 72.0011	$C_4H_6O_5$	Massbank
11	Azelaic acid	6.06	187.0969	97.0648, 125.0961, 123.0808,	$C_9H_{16}O_4$	Massbank
12	Malonic acid	0.71	103.0025	59.0129	$C_3H_4O_4$	Massbank
13	Aspartic acid	12.32	132.0292	115.0026, 114.0185, 88.0394, 71.0129	$C_4H_7NO_4$	Massbank
14	Pimelic acid	13.53	159.0654	97.0648, 95.092, 115.0753	$C_7H_{12}O_4$	Massbank
15	Citric acid	0.90	191.0190	87.0077, 101.0230, 111.0076	$C_6H_8O_7$	Massbank
16	2-methylcitric acid	2.76	205.0344,	87.0077, 101.0233, 125.0283	$C_7H_{10}O_7$	Massbank

Fatty acids

Three fatty acids were detected at different retention times (RT) in the negatively ionised aqueous sample of the watermelon rind namely 6,11-dioxododecanoic acid, dihydroxypropoxy-9-oxononanoic acid and 9-hydroxy-10,12-octadecadienoic acid as shown in Table 1. Although, long chain fatty acids are non-polar and are usually detected in non-polar solvents however, the fatty acids detected in this research consisted of short hydrocarbon chain with polar hydroxyl groups that possibly rendered the acids polar.

The molecular ions of the fatty acids are likely generated by the removal of acidic hydrogen from carboxylic acid group of the fatty acids' molecules to form carboxylate ions (deprotonation) before the carboxylate ions (precursor ions) fragment into smaller ions in a pattern that is peculiar to a specific fatty acid.

The fragmentation pattern produced by the precursor ion m/z 261.1342 at RT 5.91s (Figure 1a) was proposed for dihydroxypropoxy-9-oxononanoic acid. The major peak at m/z 187 could be a result of cleavage between ethereal oxygen and carbon of the ion m/z 261.1342 while the peak at m/z 125.0962 possibly emanated from carbon-carbon cleavage and subsequent neutral loss of hydrogen. Another compound was detected at RT 10.22s whose molecules was ionized in negative mode to precursor ions m/z 295.2275 fragment MS/MS ions were attributed to 9-hydroxy-10,12-octadecadienoic acid. Two major peaks appeared in the compound's spectrum at m/z 277.1269 and m/z 171.1019 as shown in Figure 1b. The peak at m/z 277.1269 was due to fragment ion (triene carboxylate ion) which possibly emanated from the precursor as a result of neutral loss of water molecules ion while the peak at m/z 171.1019 is a result of cleavage between the sp^3 bearing the -OH group and one of the double bonded carbons.

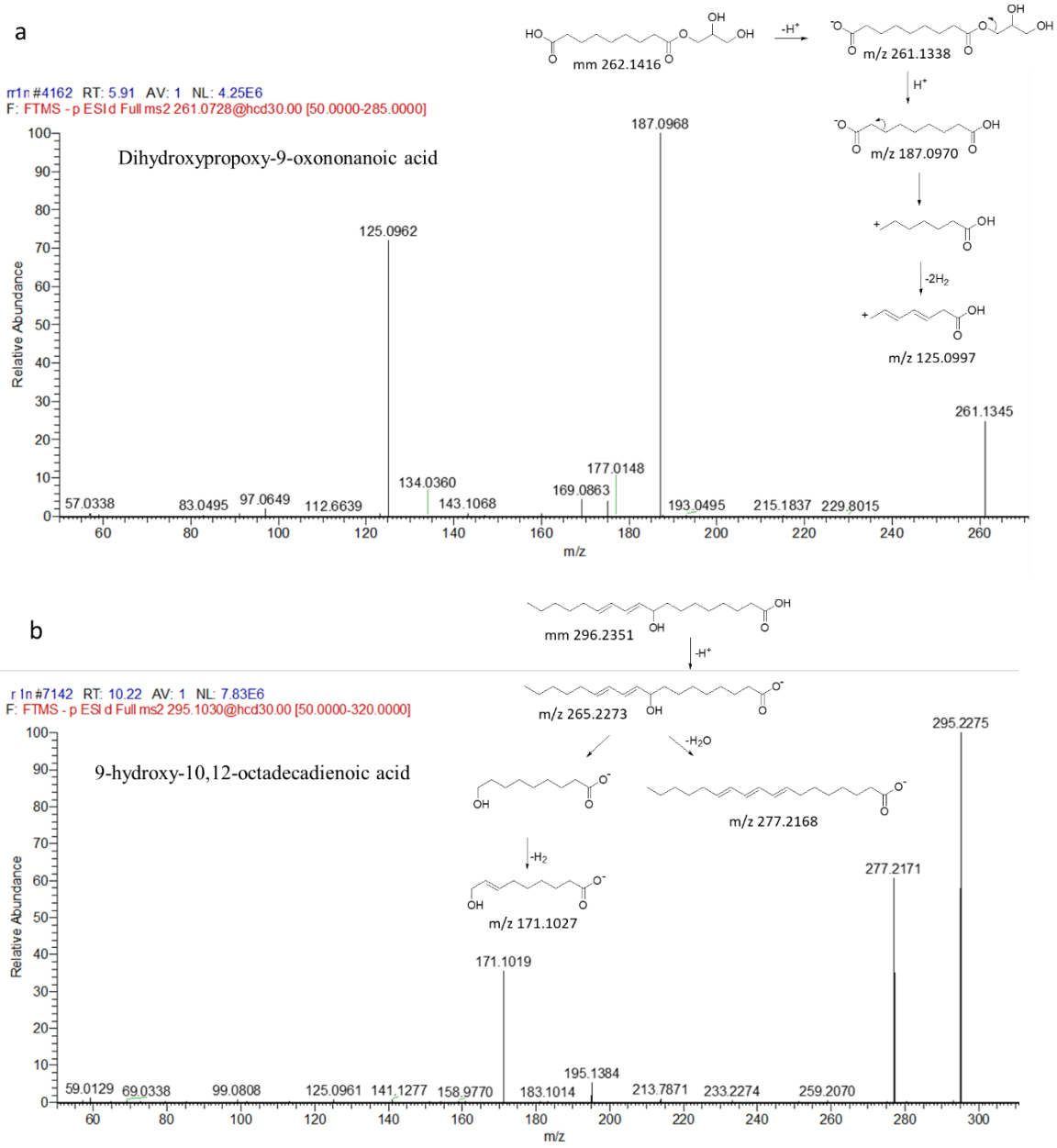


Figure 1: Proposed MS/MS spectra and fragmentation mechanisms showing major MS/MS ions of a. Dihydroxypropoxy-9-oxononanoic acid b. 9-hydroxy-10,12-octadecadienoic acid

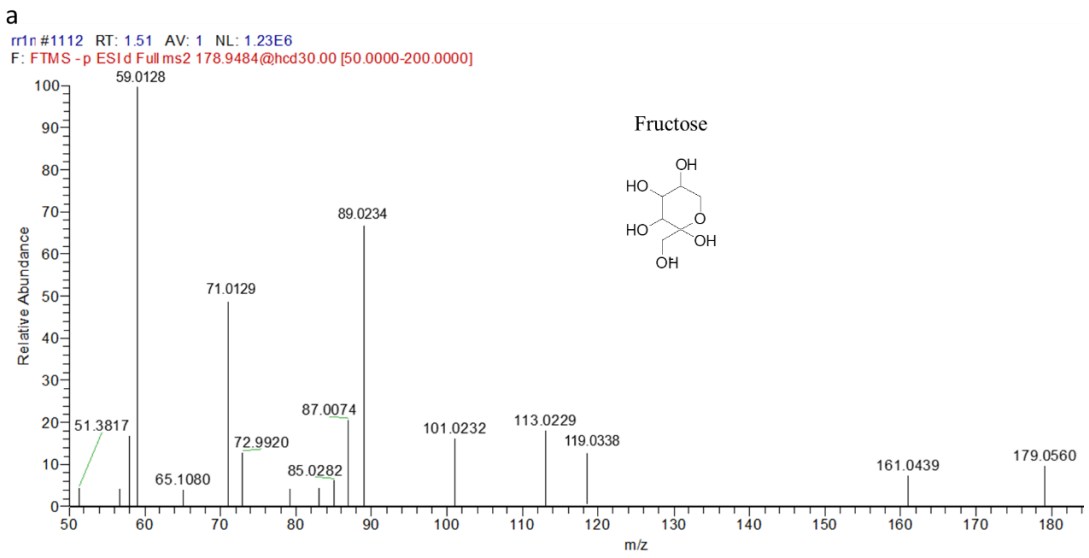
Monosaccharides

Fructose, glucose, galactose, ribose and arabinose were among the few monosaccharides putatively identified in this negatively ionized aqueous sample of red watermelon rind cultivar. Chromatographic resolution of monosaccharides in complex mixtures is always a difficult task owing to the structural similarity and the number of stereoisomers in each group [12]. These factors

mainly obstructed a fully-achieved quantitative and time-resolved analysis of lower monosaccharides to hexoses and even longer-chained members [12]. However, in this research, analysis is based on the exact mass-to-charge ratio of the precursor ions (amu) and relative percentage abundance or intensities of the MS/MS fragment ions of the compound as provided in the library. Available data showed that glucose, fructose and galactose produced almost the same types of MS/MS ions that are varied in intensities.

The precursor ion m/z 179.0560 in the MS spectrum below (figure 2a) produced the MS/MS ions whose peaks' intensities were attributed to fructose m/z 179.0561(Massbank: MoNA034819) [13]. The precursor ion as expected generated a peak with 100% intensity at m/z 59.0128 (Figure 2a) which is characteristic of fructose. Besides, fragment ion m/z 161.0439 may be due to the loss of water molecule (18amu) by the precursor ion all other MMS/MS fragment ions' occurrence was initiated by the ring's opening through bond dissociation between cyclic oxygen and carbon (Figure 2b).

a



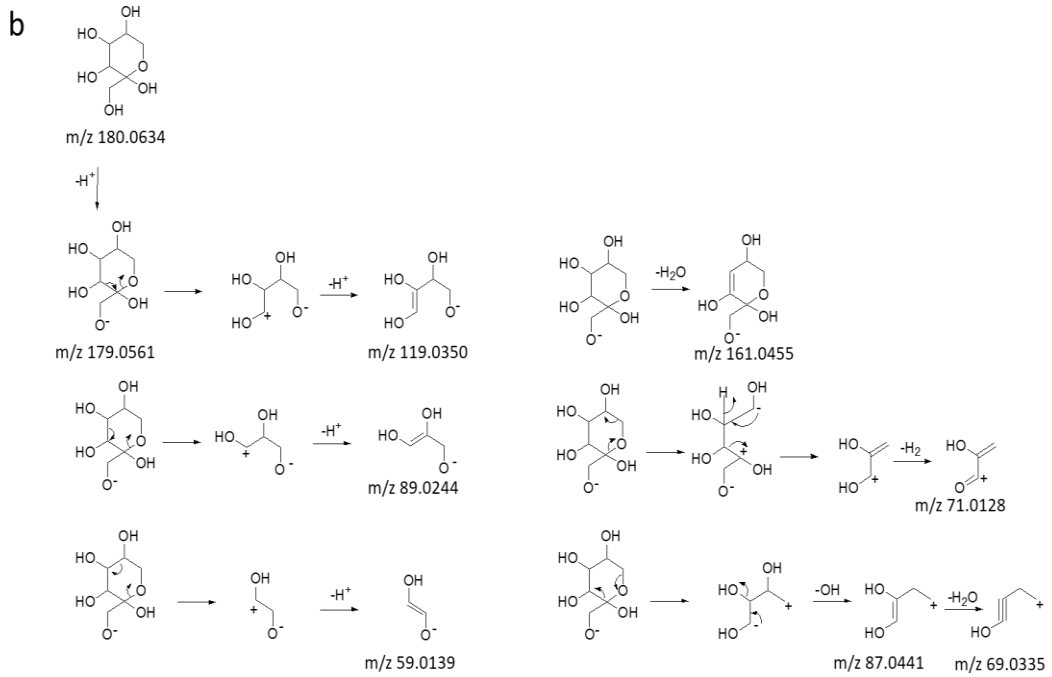


Figure 2: MS/MS spectrum and mechanism of the fragmentation pattern of fructose

Glucose and galactose were putatively identified by comparing their major MS/MS ion's peak intensities with the peak intensities of their spectral data deposited in the MS library. The proposed MS/MS spectrum of glucose revealed that a fragment ion at m/z 69.0234 reached an intensity of 100% which corresponded with the library-provided glucose spectrum (Massbank: MoNA038462). Similarly, the 100% peak intensity reached by MS/MS ion at m/z 71.0129 depicted in the proposed galactose MS/MS spectrum (Figure 3c) agreed with the galactose spectrum (Massbank: MoNA038462).

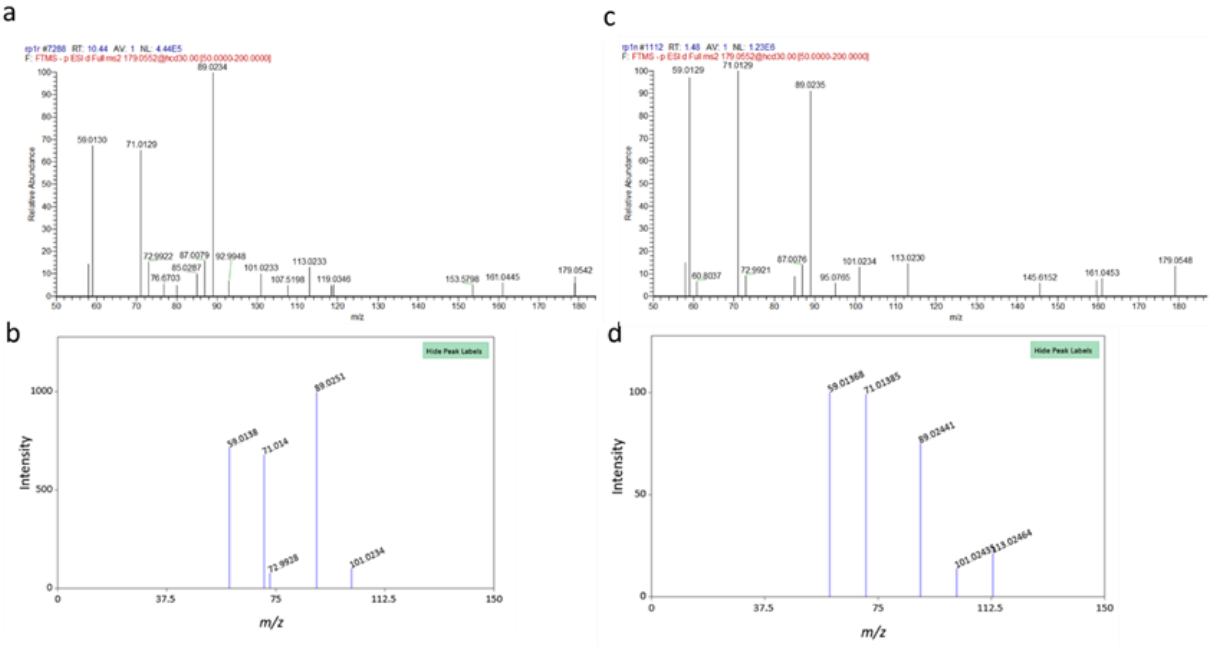


Figure **Error! No text of specified style in document.**: MS/MS ions' peak intensities in a. A proposed spectrum of D-glucose b. A proposed spectrum of D-galactose c. A reference spectrum of D-galactose (Massbank: MoNA038462). d. A reference spectrum of D-galactose (Massbank: MoNA038462)

Organic acids

Organic acids are a group of chemicals that contain one or more carboxylic groups. In fruits, analysis of organic acids is useful in investigating the authenticity of fruit juices [14]. Besides their antimicrobial activities, reports show that organic acids enhance the digestibility of proteins and amino acids and also reduce the level of ammonia and biogenic ammine [15] which were known to be toxic. Through their acidification, they were also reported to enhance the activities of digestive enzymes and improve the secretion of the pancreas [16]. As a nutraceutical fruit, watermelon was reported to have a total content of about 3% organic acids [17].

In this research, the detection of some organic acids was successful in the aqueous sample of watermelon rind in negative ionization mode. Malic acid, citric acid, malonic acid and azelaic acid were putatively detected at RTs 0.90 min, 1.14 min, 0.71min and 6.06 min respectively. It is observed from the result that the shorter the hydrocarbon chain of the acid and the greater the number of carboxylic acid group the acid has, the more polar is the acid and the earlier the acid is eluted from the sample.

The precursor ion m/z 133.0133 produced a fragmentation pattern that is consistent with malic acid. The top peak fragment ion m/z 115.0026 was likely due to neutral loss of water from the compound precursor ion (m/z 133.0133-18amu) [18] while the second top fragment ion peak at m/z 71.0129 was due to possible loss of carbon dioxide from the ion m/z 115.0026. Direct decarboxylation of the precursor ion likely produced carbon dioxide and the ion m/z 89.0244 which could be dehydrogenated to the ion m/z 87.0088 close to it (Figure 3).

Citric acid is tricarboxylic acid thus; decarboxylation may be the leading process of its fragmentation. Citric acid produced its top peak with 100% intensity at m/z 111.0077. Removal of one of the carboxylate groups likely formed the peak at m/z 147. 0299 and the removal of two molecules of water from fragment ion m/z 147.0299 possibly led to the appearance of the top peak at m/z 111.0077 (m/z 147. 0299-2x18amu) (Figure 3.14). The second top peak that occurred at m/z 87.0077 may be due to the removal of two carboxylate groups followed by deprotonation while the fragment ion peak at m/z 59.0129 may be due to the removal of three carboxylate groups from the precursor ion. The only peak that outstands (100% intensity) in the spectrum of compound with molecular ion m/z 103.0025 detected at RT 0.71min is the one occurring at m/z 59.0129 and is specific for malonic acid.

Malonic acid consists of two carboxylic groups linked by methylene group (-CH_2-) and the peak at m/z 59.0129 was perhaps due to the removal of one of its carboxylate groups. Azelaic acid which was not even reported in watermelon. However, in this research, compound with molecular ion (precursor ion) m/z 187.0969 was putatively detected as azelaic acid. The precursor ion fragment and produced a peak with 100% intensity peak at m/z 125.0962 which may be due to the removal of carbon dioxide and water molecules. The peak at m/z 97.0648 was likely due to the removal of two carboxylate groups and the subsequent loss of hydrogen molecules.

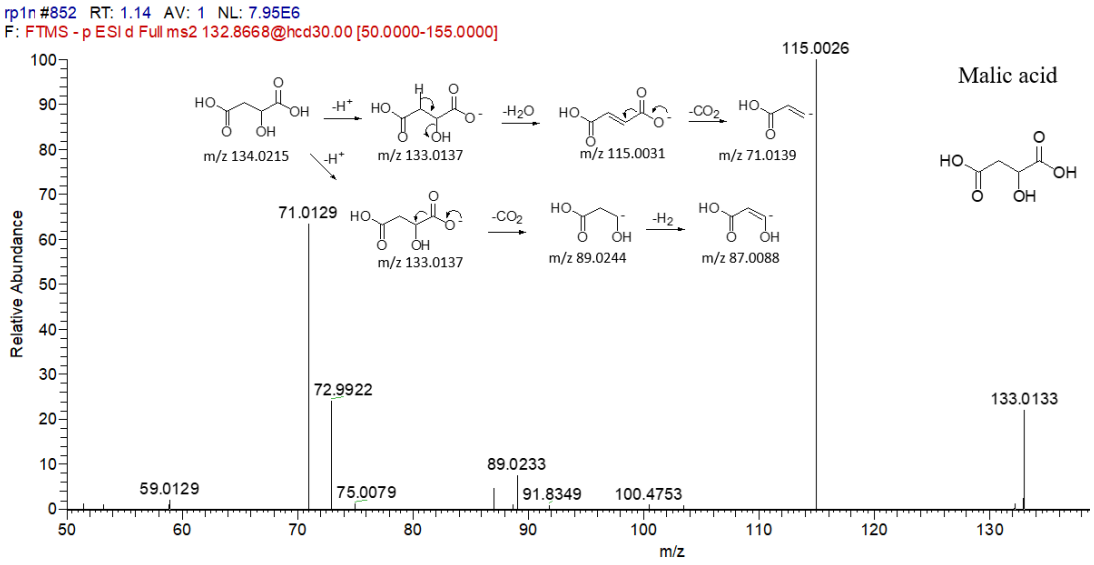


Figure 4: Proposed MS/MS spectrum of malic acid with the mechanism of its fragmentation

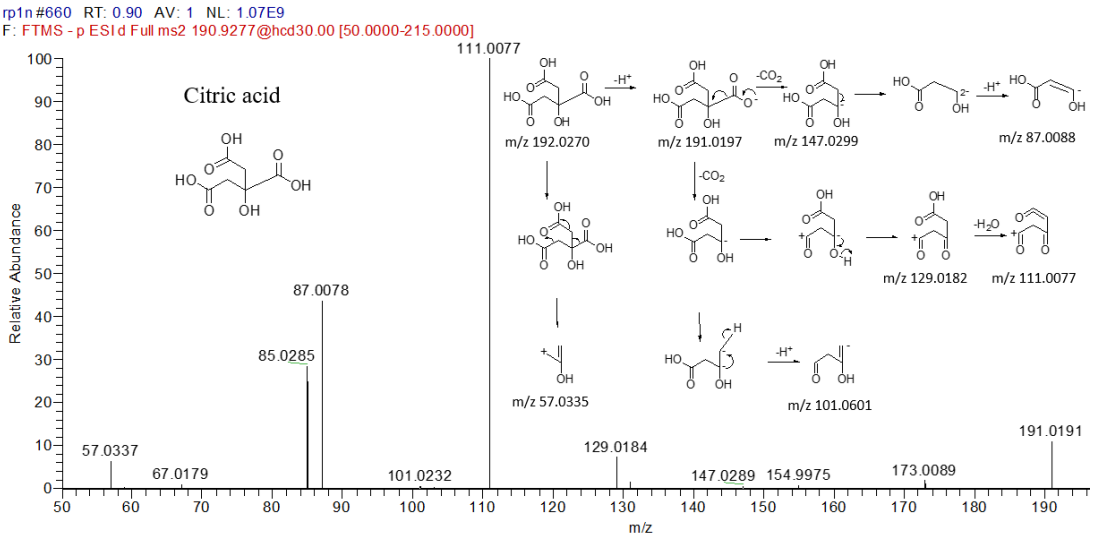


Figure 5: Proposed MS/MS spectrum of citric acid with the mechanism of its fragmentation

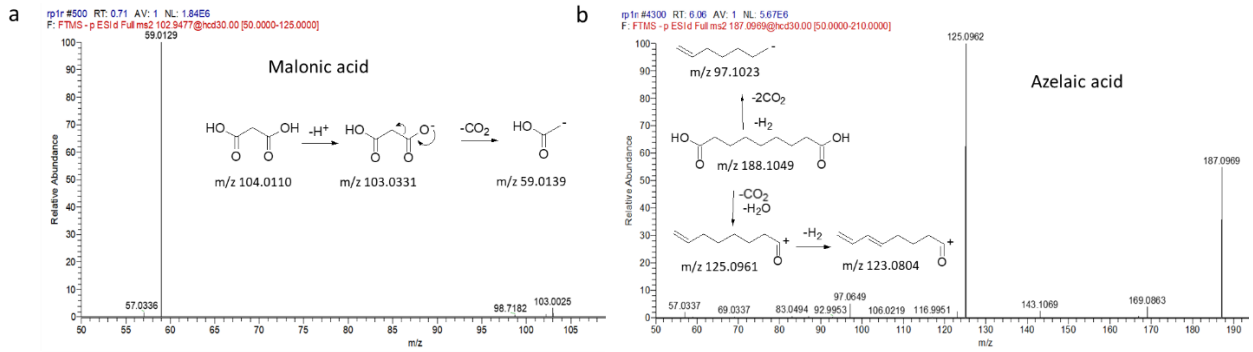


Figure 6: Proposed MS/MS spectra of malonic acid and azelaic acid with the mechanisms for their fragmentation patterns**.**

Conclusion

The findings of the research portrayed the nutritional potential of red watermelon rind. Although, the rind is non-sugary, the result shows that rinds contain certain types of carbohydrates as glucose, fructose and galactose were putatively identified in the extract. The experimental MS/MS ions spectral data obtained of the compounds detected in the research could provide additional information for the identification of same compounds in other sample matrices. It is concluded that watermelon rind could be worthy of consumption by human or could be made parts of animal feeds.

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