ORIGINAL ARTICLE

Detection of Ascorbic acid in red and yellow crimson watermelon juices using RP-HPLC

Wan Mazlina Md Saad[1], Nur Shafinaz Mohamad Salin[1], Nurul Nabilah Akmal Hashim[1], Nurarifah Abd Rahim[1], Fatimah Salim[2,3]

[1] Centre of Medical Laboratory Technology, Faculty of Health Sciences, Universiti Teknologi MARA Cawangan Selangor Kampus Puncak Alam, 42300 Bandar Puncak Alam, Selangor, Malaysia.
[2] Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Universiti Teknologi MARA Cawangan Selangor Kampus Puncak Alam, 42300 Bandar Puncak Alam, Selangor, Malaysia
[3] Centre of Foundation Studies, Universiti Teknologi MARA Cawangan Selangor Kampus Dengkil, 43800 Dengkil, Selangor, Malaysia

ABSTRACT

Introduction: Ascorbic acid (AA) is a significant micronutrient known for its potent antioxidant properties beneficial to health. AA acts as a cofactor in various physiological reactions including improving endovascular function, modifying immunologic and inflammation functions, and aiding in scavenging free radicals. Different methods have been performed to quantify AA in fruits. However, there is limited data for AA analysis in red and yellow crimson watermelon using RP-HPLC, with a simple sample preparation process. Thus, the present study was conducted to detect the quantification of AA in the juices of local red and yellow crimson watermelon (*Citrullus lanatus*) flesh. **Methods:** The detection was carried out using the reversed-phase high-performance liquid chromatography (RP-HPLC) by injecting 20 µl samples into a C-18 column with a temperature set at 25 ℃. The overall analysis was conducted using 0.1 % ortho-phosphoric acid and acetonitrile (95:5, v/v) as mobile phase with a flow rate of 1 mL/min at a detection wavelength of 254 nm. **Results:** The results show that the retention time of AA was eluted at 2.8 min in both juice samples. This method was then validated using linearity (0.9838), limit of detection (LOD) (0.47 µg/mL) and limit of quantification (LOQ) (1.43 µg/mL). The concentration of AA in red and yellow watermelon samples were found at 19.88 µg/mL and 19.05 µg/mL, respectively. **Conclusion:** The present study concludes that AA content in red and yellow crimson watermelon was successfully detected and quantified using the RP-HPLC method. The results were reliable and successfully validated using linearity, LOD and LOQ.
Malaysian Journal of Medicine and Health Sciences (2022) 18(8):263-368. doi:10-268.47836/mjmhs18.8.34

Keywords: Ascorbic acid, watermelon, RP-HPLC

Corresponding Author:
Wan Mazlina Md Saad, PhD
Email: wanmaz755@uitm.edu.my
Tel: +603-32584429

INTRODUCTION

Watermelon or its scientific name *Citrullus lanatus* (Thunb.) Matsum. and Nakai belongs to the Cucurbitaceae family, and is botanically considered fruit (1). Watermelon is renowned as the leading and most popular fruit to be consumed due to its high consumption in tropical countries and humid climates (2). Many researchers and scientists studied watermelon for its pharmacological and therapeutic potentials including antimicrobial, antifungal, hepatoprotective, anti-ulcer genic, anti-diabetic, laxative, antisecretory, anti-prostatic hyperplasia, antioxidant, analgesic, antifungal, anti-hypertensive and anti-inflammatory (3-5).

Watermelon is also known as an ethnomedicinal plant where all the fruit's parts can be used in treating human diseases. The watermelon plant contains primary metabolites, such as carbohydrates, proteins, minerals, vitamins, amino acids and fats. The plant also contains secondary metabolites such as alkaloids, steroids, saponins, glycoside, flavonoids, tannins, phenolic compounds, lycopene, phytofluene, phytoene, beta-carotene and lutein (4). Carotenoids in watermelon give the fruit its flesh colours. Red is due to lycopene, yellow is predominantly due to neoxanthin, and orange

is mainly caused by beta-carotene. These carotenoids have antioxidant capacities in preventing oxidative and tissue damage, reducing the risks of many diseases (5).

AA exists as two enantiomers, commonly denoted as 'L' for levo and 'D' for dextro, due to their different interaction with polarized light. However, the most common form that occurs naturally in food is L-ascorbic acid (L-AA), also popularly known as vitamin C. It is a water-soluble vitamin that is essential in the human diet and has proven to be a good source of antioxidants (6). AA is a diprotic acid due to an enediol group in its heterocyclic lactone ring with a molecular formula of $C_6H_8O_6$. The IUPAC name is (*R*)-5-((*S*)-1,2-dihydroxyethyl)-3,4-dihydroxyfuran-2(*5H*)-one and the common name is 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol (7), with polar and hydrophilic compound characteristics. Dehydroascorbic acid (DHAA) compound is the first oxidation product of AA and these two compounds can form a reversible redox couple reaction (8).

The Association of Official Agricultural Chemists (AOAC) applies the titration method of AA with 2,6-dichloroindophenol in an acidic solution as the official method for detecting vitamin C in fruit juices. However, detection is limited to low concentrations of DHAA (9). Nevertheless, this method is not chemically specific for AA detection (10). Additionally, the red colour of the reagent may interfere with the quantification of AA in watermelon, especially of the red type.

Currently, there is limited data on the standard preparation and analytical method for the analysis of AA in different food samples and matrices, including the red and yellow watermelon juices. In addition, the analysis of the targeted compound using HPLC has been widely applied due to its sensitivity and specificity (11). Therefore, this study was carried out to determine AA content in red and yellow flesh juice of *C. lanatus* using RP-HPLC.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile (MeCN) (Amresco, Selangor Darul Ehsan Malaysia), Methanol (MeOH) and Ethanol, (RCI Labscan, Bangkok Thailand), Acetone, Orthophosphoric acid, Acetic acid, Formic acid, Glacial Acetic acid and Ethyl acetate (Merck Milipore, Darmstadt, Germany), HPLC Analytical Grade of L-Ascorbic acid Standard (Sigma Aldrich).

Sample collection

Matured red and yellow fleshes watermelon (*Citrullus lanatus*) fruit with a range weight of 1.5-3.0 kg were obtained from the local farm in Selangor Fruit Valley (SFV), Bestari Jaya, Selangor, Malaysia. Species identification of watermelon was performed by the Forest Research Institute Malaysia (FRIM).

Sample preparation

The sample was processed according to procedures reported by Hernandez et al. (2006), Tarrago-Trani et al. (2012) and Spнola et al. (2014) with slight modifications (10-12). Watermelon flesh was cut into smaller pieces prior to homogenization in a HESSTAR blender in the dark and kept in ice. The homogenized mixture was thoroughly mixed and weighed at 2 g into a 50 mL centrifuge tube. Approximately, 2.5 mL of the extraction solution consisting of 3 % OPA and 8 % acetic acid, was added into a tube containing the weighed sample, and centrifuged at 4 ℃ (6 000 rpm) for 20 minutes. Following centrifugation, the resulting supernatant was decanted into another tube, and kept on ice. The centrifugation procedure was repeated in triplicate to ensure that AA was entirely extracted from the watermelon fruit sample. The resulting supernatants were combined and thoroughly mixed. The extract was filtered using a 0.45-µm polyvinylidene membrane and later stored at 4 ℃ until analysis. All processes were performed in the dark with the least exposure to air in order to minimize the oxidation of AA in the fruit samples during extraction.

Standard preparation

The standard AA solution (10mg/mL) was prepared by diluting 100 mg of AA standard in 10 mL of pure distilled water. The AA solution was stored in an amber glass- bottle with a stopper. The solution was diluted in distilled water to prepare 1 mg/mL of the standard solution. For RP-HPLC analysis, different standard solution concentrations were prepared by diluting the AA stock solution into 0.1 % OPA : MeCN.

RP-HPLC Analysis

RP-HPLC analysis was carried out using Agilent 1220 Infinity LC (Germany) instrument equipped with a gradient pump (model G4288B), variable wavelength detector (VWD), and operated with the ChemStation™ software. Approximately, 20 µL of the sample was injected into a ZORBAX Eclipse XDB-C18 analytical column (5 µm, 4.6 mm x 250 mm) with a temperature set at 25 ℃ and mobile phase of 0.1 % OPA:MeCN (95:5, v/v). The flow rate of RP-HPLC analysis was set at 1 mL/min. The separated AA in the column was later being detected using a variable wavelength detector (VWD) at 254 nm.

Method validation

The validation method was achieved by referring to the guidelines from The International Council for Harmonization (ICH) and Gangrude et al. (2020) (13).

Linearity

The calibration for the optimized method was carried out by injecting varied concentrations of AA standard ranged from 100 to 500 µg/mL into RP-HPLC column. A calibration curve was then plotted for standard AA against peak area. The linearity was then assessed by the regression equation and correlation coefficient (R^2).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

From the calibration curve obtained, the sensitivity of LOD and LOQ were then calculated using the equations below.

LOD = (3 x standard deviation of response) / slope of calibration curve **Eq. 1**

LOQ = (10 x standard deviation of response) / slope of calibration curve **Eq. 2**

Quantification of AA in Watermelon

The concentration of AA in watermelon (*C. lanatus*) and its percentage content in 1 mL of the sample extract were calculated using the equations below.

$$\text{Concentration of analyte} = \frac{\text{Peak area of analyte + y - intercept of calibration curve}}{\text{Slope of calibration curve}} \quad \textbf{Eq.3}$$

$$\text{AA content \%} = \frac{A_1 \times W_2 \times P}{A_2 \times W_1} \quad \textbf{Eq.4}$$

Where, A_1= Peak area of sample solution, A_2= Peak area of standard solution, W_1= Weight in g of sample, W_2= Weight in g of standard, P= Purity of standard AA

RESULT

Identification of Ascorbic Acid in *C. lanatus.*

The chromatogram of AA in standard and watermelon samples were presented in Figure 1 and the retention time (RT) was tabulated in Table I. The retention time for AA peak standard was compared to the watermelon samples. The AA peak was detected at retention times of 2.792 min, 2.796 min, and 2.798 min, respectively for the standard, red watermelon juice and yellow watermelon juice (Figure 1). The peak area and height of the AA standard, *C. lanatus* red fleshed and yellow fleshed juice extracts are presented in Table 1. The current data was compared with previous studies reported that retention time for ascorbic acid ranges 3-4 minutes (14-16).

Table I: RP-HPLC Analysis of Ascorbic acid

Sample	Ascorbic acid		
	Height (mAU)	Area (mAU*s)	Retention time (min)
Ascorbic acid standard	1227.122	5408.493	2.792
Red crimson water-melon	7.770	33.481	2.796
Yellow crimson water-melon	2.271	9.778	2.798

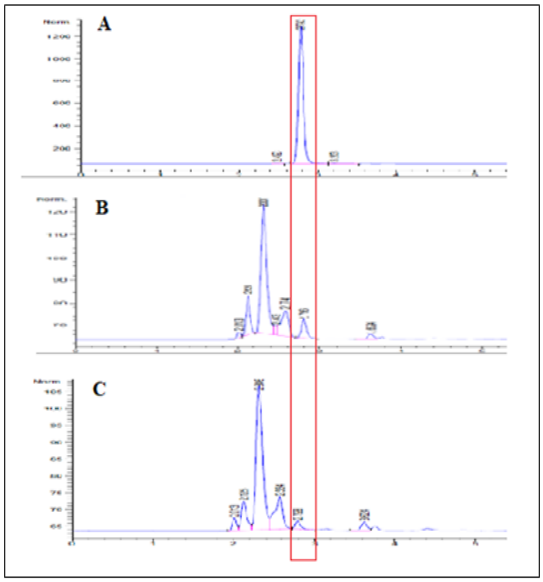

Figure 1: Chromatogram of ascorbic acid standard (A), red flesh water melon (B) anf yellow flesh watermelon (C)

Method validation

Table II tabulated the validation analysis for the detection of AA in red and yellow watermelon juice. The derived equation was y = 28.494x + 533.02, where x represents concentration in µg/mL and y represents the RP-HPLC peak area with a correlation coefficient (R^2) of 0.9838. The R2 value was compared to previous studies, which reported values within range of 0.97 - 0.991 (10, 17). The Limits of detection (LOD) and limits of quantitation (LOQ) were calculated at 0.47 µg/mL and 1.43 µg/mL respectively and demonstrated that the RP-HPLC method able to quantify AA at low concentration (Table II).

Table II : Calibration data of the reported RP-HPLC method

Com-pound	Regression equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Ascorbic acid	y = 28.494x + 533.02	0.9838	100-500	0.47	1.43

DISCUSSION

Identification of Ascorbic Acid in *C. lanatus.*

The current study demonstrated the identification of AA in red and yellow watermelon juice using the RP-HPLC method. The results showed that the analytical method used gave faster detection of AA (less than 3 minutes) through a non-polar stationary phase (C18). A study by Hu et al. (2012) describes the faster elution of a polar compound because of their weak interaction with the non-polar column's particle. Hence, it's hardly adsorbed and retains at a shorter time (14). Thus, this study presented a faster peak elution of AA in the standard and the juices.

An efficient peak of AA with a high resolution and sensitivity was obtained from the chromatogram using the C-18 column paired with a variable wavelength detector (VWD) at 254 nm (Figure 1). This is in agreement with Tian et al. (2020) which demonstrated a precise elution peak at 4 minutes using C-18 and VWD (18). AA was previously reported to highly absorb UV at a wavelength of 254-265 nm (19-20). Hence, the detection of VWD at 254 nm paired with the C-18 column was performed and demonstrated a high resolution of AA with eliminated noise. In addition, a flow rate of 1 mL/min 0.1 % OPA : MeCN (95:5, v/v) showed the optimum ratio composition of mobile phase with improved separation and detection of AA in watermelon juice samples (21).

Method Validation.

A calibration equation was constructed to demonstrate linearity by plotting the calibration curve of the area against concentration of AA standard (µg/mL) at five different concentrations (100, 200, 300, 400 and 500 µg/mL). Linear regression showed a good positive correlation between these two parameters. The derived equation for AA was y = 28.494x + 533.02, with a correlation coefficient (R2) of 0.9838. The R2 value was compared to Spнola et al. (2014) with 0.97, and indicated that the method for determination of AA in red and yellow watermelon juice was linear and reproducible (10).

Limits of detection (LOD) and limits of quantitation (LOQ) were determined from the calibration curve for the method sensitivity test. The LOD and LOQ were calculated at 0.47 µg/mL and 1.43 µg/mL, respectively (Table 2). The LOD and LOQ were within the range reported from a previous study ranging from 1.2 Ч 10-3 µg/mL to 7.2 µg/mL (10). This indicated a sensitive method to detect low concentrations of an analyte (14). Therefore, this study demonstrated that the method used was sensitive and able to detect low AA concentrations in the juice samples. As for the selectivity of the method, the presence of other compounds, as seen in the chromatogram of juices did not cause any interferences of the AA peak. A sharp and well-resolved peak was generated under the tested RP-HPLC conditions. Hence, this study showed that the method used was sufficiently selective and specific for AA determination in watermelon juice samples (22).

Ascorbic Acid Content in *C. lanatus* Juice.

The calculated concentration of AA compound in the red local watermelon juice was 19.88 µg/mL with 0.00006 % of AA content while 19.05 µg/mL in yellow flesh watermelon contributed to 0.00002 % of AA. This data indicated that approximately 2 mg of AA is present in every 100 mL of *C. lanatus* juice. However, these values are lower than the data reported by Newze et al. (2015) (4.08 mg/100 ml) by iodometric titration method, suggesting natural rapid degradation of the unstable AA during sample preparation (23). AA is sensitive towards environmental exposure. Therefore, studies involving vitamin C extraction and determination should ensure maximum AA preservation.

The consumption of watermelon provides a significant amount of vitamin C, thus can be a good source of vitamin C for daily consumption. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) suggested the daily requirement for vitamin C in adults at about 70 mg per day, for good immunity. Moreover, AA able to protect against the oxidation of low-density lipoproteins (LDL) by neutralizing the free radicals and other reactive oxygen species (ROS). This preventive mechanism can help prevent atherosclerosis and cardiovascular diseases (24). Mason et al. (2019) reported that supplementation of vitamin C in an individual with type 2 diabetes, can improve glycaemic and control blood pressure (25). Hence, an adequate daily intake of AA through watermelon consumption may help protect against inflammation-associated diseases.

CONCLUSION

In conclusion, AA identification using a specific RP-HPLC method showed high sensitivity and selectivity capable of detecting a low concentration. Both red and yellow watermelon crimson provide equivalent amount of vitamin C (19-20 µg/ml) with slightly higher content in red watermelon. The amount of AA detected in this famous fruit demonstrated potential as a source of energy and immune booster while having good antioxidants properties. More studies on the nutrient profile of local fruits and their health benefits are essential to provide information to the public on these affordable and accessible food source. This would help increase

the annual demand of local fruits, while concurrently improve the Malaysian economy, specifically the agriculture sector.

ACKNOWLEDGEMENTS

A special acknowledgement to Ministry of Higher Education, Malaysia, through Lestari Grant Scheme and Institute of Research Management and Innovation (IRMI), Universiti Teknologi MARA for the funding with reference no: 600- IRMI5/3/LESTARI.(047/2019), Mr Effendy from the Selangor Fruit Valley, Selangor for providing the watermelon samples; Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Centre of Medical Laboratory Technology, Centre of Postgraduate Study, Faculty of Health Sciences, Universiti Teknologi MARA, Selangor Branch, Puncak Alam Campus.

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