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**Original Research Article**

# Identification of bioactive compounds in red watermelon extract using qualitative liquid chromatography-mass spectrometry

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## Abstract

***Purpose:*** *To qualitatively determine the bioactive compounds in red watermelon fruit extract using LC-MS/MS*
***Methods:*** *Liquid chromatography-mass spectrometry (LC-MS/MS) was used to identify and characterize the bioactive compounds in red watermelon fruit extract, with optimization of chromatographic and MS/MS parameters.*
***Results:*** *The use of total ion chromatogram (TIC) for extraction of fragmentation products from target m/z revealed the retention times for various compounds. Lycopene compounds had retention time (tR) of 1.08 min; retinol (vitamin A) had tR of 1.14 min, while citrulline had tR of 1.23 min. Ascorbic acid (vitamin C) had tR of 1.35 min, while α-tocopherol (vitamin E) had tR of 9.93 min.*
***Conclusion:*** *Using LC-MS/MS analysis, lycopene, citrulline, vitamin A, vitamin C and vitamin E have been identified as primary bioactive compounds in red watermelon pulp extract.*

***Keywords:*** *Red watermelon, Liquid chromatography-mass spectrometry, Bioactives, Lycopene, Citrulline, Retinol, Ascorbic acid, alpha-Tocopherol*



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## INTRODUCTION

Watermelon (*Citrullus lanatus*) which is native to Africa, is a globally cultivated annual vine belonging to the Cucurbitaceae family. With a rapid growth cycle of about 6 months, it exhibits a well-defined growth pattern [1,2]. It contains significant amounts of phenolic compounds, when compared to other fruits. It is also affordable and nutritious, making it accessible to all socio-economic groups [1,2]. Watermelons are readily available, and are usually consumed as fresh fruits. However, due to limited knowledge, there is often a misconception to the effect that watermelons are usually sold as whole fruits without any processing or value-added products.

Lycopene, a carotenoid pigment, is responsible for the red color of watermelons. It offers numerous health benefits. In addition to lycopene, watermelons are known to be rich in vitamins A, C, and E which confer potent antioxidant properties on the fruits [3]. With a water content of about 92 %, watermelon is a low-calorie fruit that provides approximately 30

calories per 100 grams. It is rich in nutrients such as carbohydrates, dietary fiber, vitamins (A, C, B1 and B6); minerals (potassium and magnesium), and antioxidants such as lycopene and β-carotene [1,2]. There is a growing trend in demand for antioxidant-rich food products. These antioxidants neutralize free radicals that contribute to the development of cancer and other chronic diseases such as heart disease and stroke. Watermelon, in particular, is known for its high antioxidant content [4].

Liquid chromatography-mass spectrometry (LC-MS/MS) combines liquid chromatography with tandem mass spectrometry, and it allows for highly sensitive and specific analysis of complex samples. By separating compounds based on their chemical properties and then analyzing them using two stages of mass spectrometry, LC-MS/MS facilitates detailed structural characterization and quantification [5].
In tandem mass spectrometry (MS/MS), a selected precursor ion is fragmented via collision with an inert gas after initial ionization and selection by the first mass analyzer. Analysis of the resultant daughter ions using a second mass analyzer yields a mass spectrum that reveals the structure and composition of the original molecule [6,7]. The selective reaction monitoring (SRM) and multiple reaction monitoring (MRM) are mass spectrometric techniques used for targeted quantification. While SRM involves monitoring a single product ion for each analyte, MRM allows for the simultaneous monitoring of multiple product ions, with increasing throughput and sensitivity [8]. This study was aimed at using LC-MS/MS for qualitative determination of the bioactive compounds in the extract of Setabindo-1 watermelon variety.

## EXPERIMENTAL

### Materials

Acetonitrile and water (OptimaTM LCMS grade from Fisher Chemical) were used as eluent/mobile phase in the chromatographic analysis of the compounds. Formic Acid (98 – 100 % formic acid for analysis; EMSURE® ACS, Reag. Ph Eur) was employed as a modifier of the mobile phase. OptimaTM LCMS methanol from Fisher Chemical, was the solvent used for the watermelon extract sample, while a 0.22 µm PTFE filter was used as the sample filter.

Setabindo-1 watermelon variety was sourced from PT Bisi Internasional tbk, Karangploso, Malang Regency, East Java, Indonesia and it complied with the Indonesian Minister of Agriculture Decree no. 1055/KPTA/TP.240/12/97.

### Preparation of watermelon extract

A crude extract was obtained by macerating chopped red watermelon flesh in a 2:1:1 volume ratio of n-hexane : acetone : 96 % ethanol for 48 h, followed by filtration and rotary evaporation.

### Chromatographic conditions

Analysis was performed using a Thermo Vanquish UHPLC system (2-µL injection volume) equipped with a Hypersil Gold C18 column (100 x 2.1 mm, 1.9 µm particle size) at 40 °C (column) and 5 °C (sample). A gradient system comprising a 20:80 volume ratio of 0.1 % formic acid in water and acetonitrile, was used at a flow rate of 200 µL/min.

| Retention time (min) | 0.1% formic acid in water (%) | Acetonitrile (%) |
|---|---|---|
| 0 | 20 | 80 |
| 7 | 0 | 100 |
| 9 | 0 | 100 |
| 10 | 20 | 80 |
| 15 | 20 | 80 |

### Optimization of MS/MS

A Thermo TSQ Fortis triple quadrupole mass spectrometer (QqQ) with heated electrospray ionization (H-ESI) in positive polarity mode (3500 V spray voltage; nitrogen gas flow rate of 15 L/min) and selective reaction monitoring (SRM) was used. The target molecules and their m/z values were: citrulline (176.10 {M+H}+), lycopene (537.5 {M+H}+), ascorbic acid (177.03 {M+H}+), α-tocopherol (431.4 {M+H}+), and retinol (269.2 {M-$H_2O$}+). Collision energy ranged from 10 - 30 eV.

### Sample preparation

Watermelon extract samples (10 mg) were dissolved in 10 mL MeOH. The resultant solution was sonicated for 15 min, left at room temperature, and then filtered into vials using a 0.22 µm PTFE membrane.

## RESULTS

### Data from LC-MS/MS analysis of watermelon compounds

As indicated in Figure 1, the Total Ion Chromatogram (TIC) obtained showed several peaks from which the TIC was extracted to produce fragmentation products. From the target

m/z obtained, the $t_R$ for lycopene was 1.08 min, while the $t_R$ for retinol was 1.14 min. The $t_R$ values for citrulline, ascorbic acid and α-tocopherol were 1.23, 1.35 and 9.93 min, respectively.

**Dominant componds identified in sample using LC-MS/MS**

Citrulline was identified from the ionization result of m/z 176.10. The daughter ion confirmed m/z 113.10 and m/z 159.07. In line with the MS NIST library, the results confirmed that the m/z fragmentation characteristic indicated the presence of citrulline (Figure 2).

Lycopene was identified from the ionization result of m/z 537.50. The confirmed ion fragments were m/z 144.80 and m/z 69.10. In line with the MS NIST library, the results confirmed that the m/z fragmentation characteristics were indicative of presence of lycopene (Figure 3).

Vitamin A or retinol, was identified through the precursor ion at m/z 269.20. The presence of fragment ions at m/z 107.00 and m/z 93.00 further confirmed this identification, in line with the fragmentation pattern of retinol found in the NIST MS library (Figure 4).

Figure 5 illustrates the identification of vitamin C (ascorbic acid). The precursor ion was observed at m/z 177.03, and the characteristic fragment ions at m/z 141.01 and m/z 95.01 validated its identity, in agreement with the fragmentation pattern of ascorbic acid recorded in the NIST MS library.

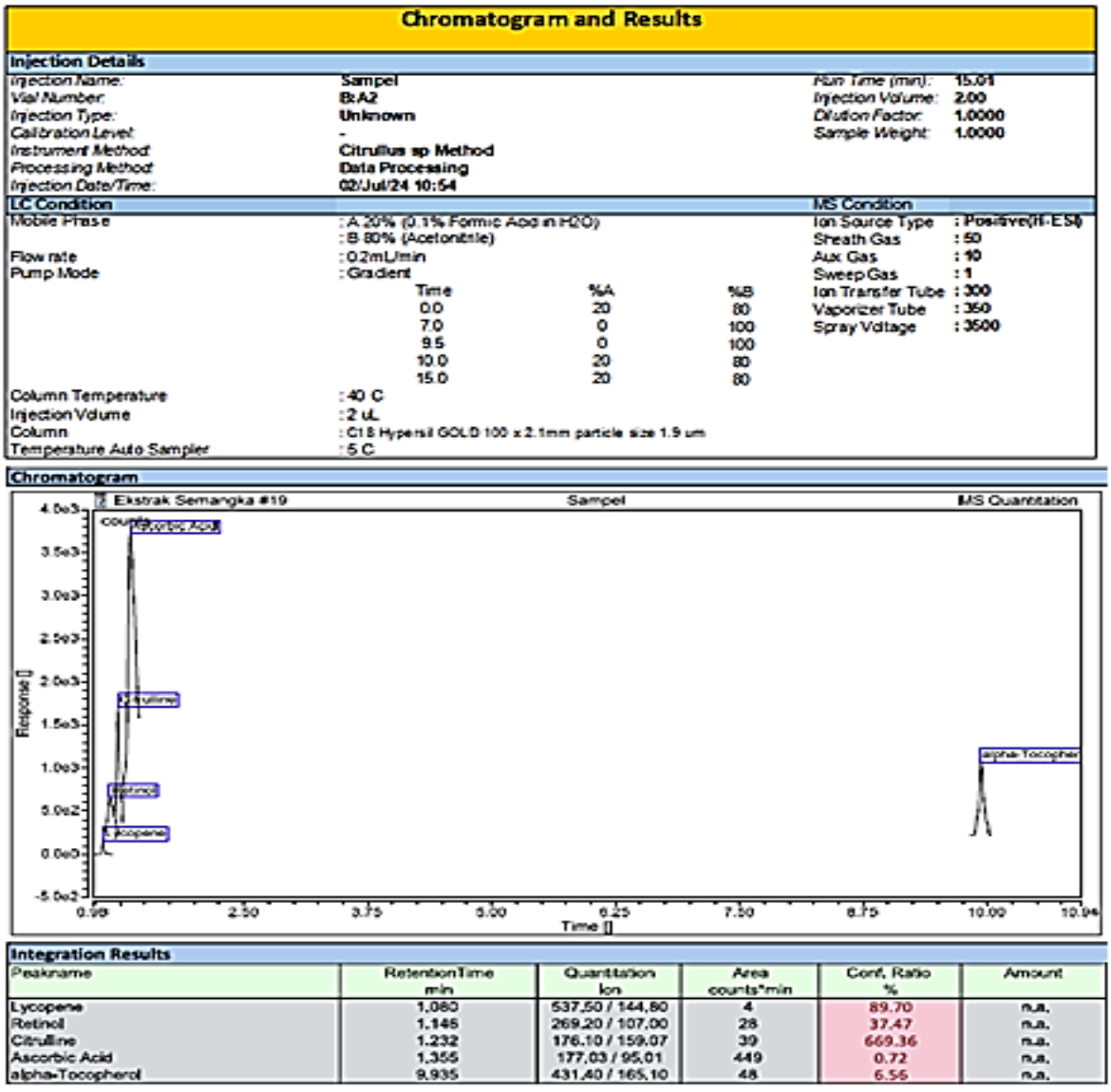


**Figure 1:** Total ion chromatogram obtained from LC-MS/MS

Figure 6 shows the identification of vitamin E (alpha-tocopherol). The precursor ion was detected at m/z 431.40, and the characteristic fragment ions at m/z 165.10 and m/z 121.00 confirmed its identity. This is in agreement with the fragmentation pattern of alpha-tocopherol documented in the NIST MS library.

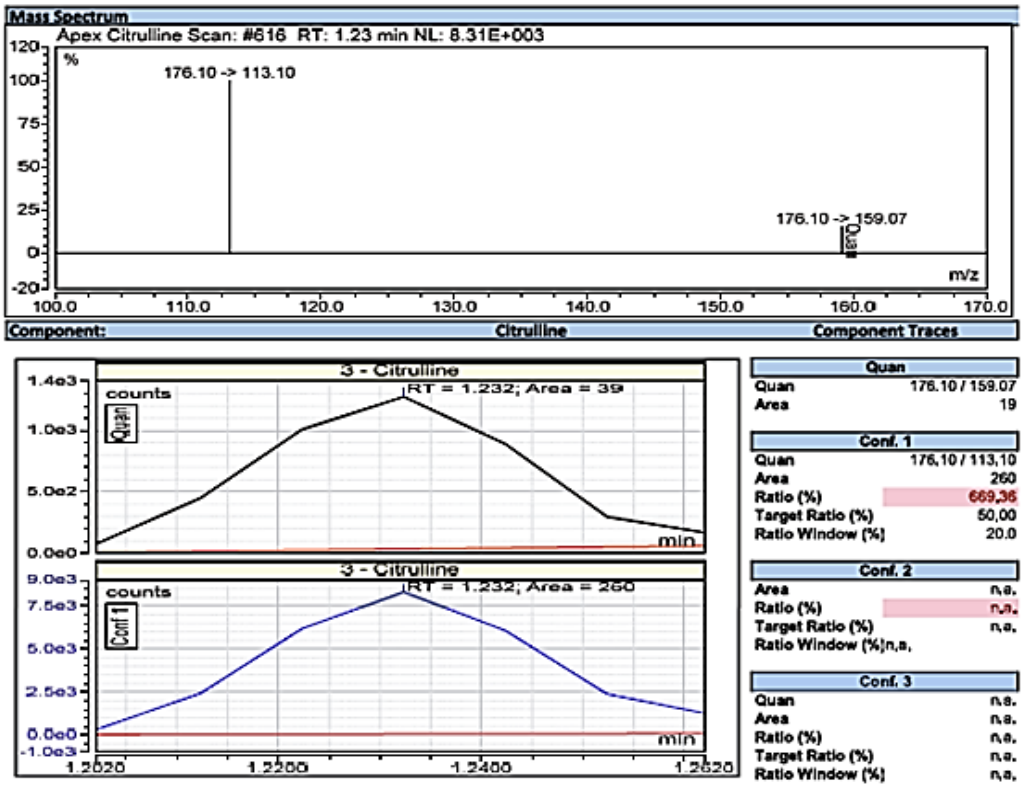


**Figure 2:** Chromatogram for citrulline

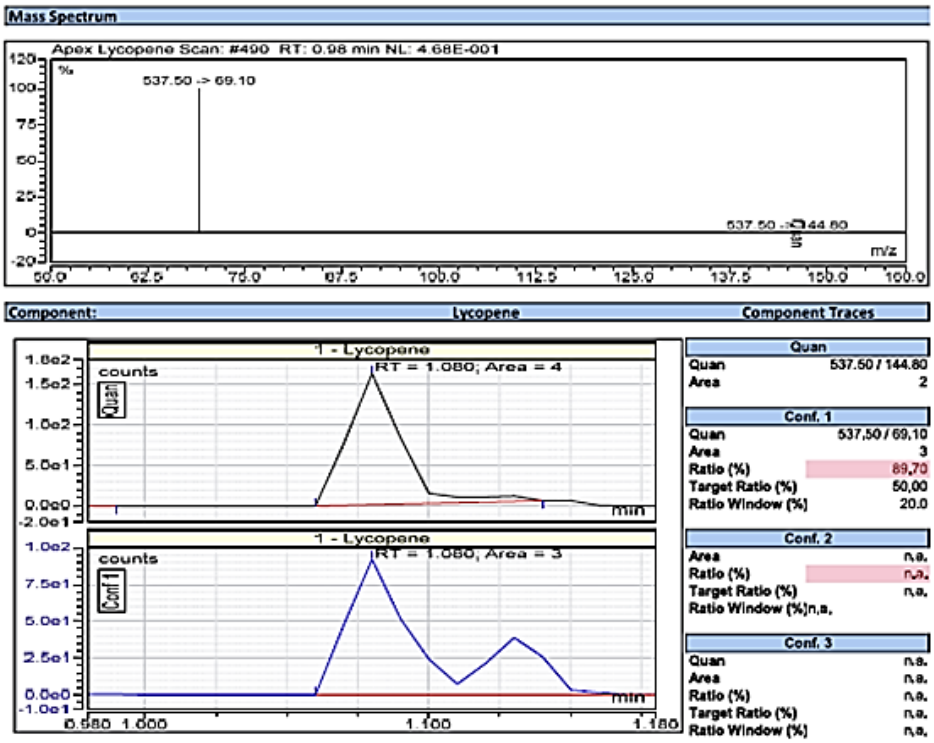


**Figure 3:** Chromatogram for lycopene

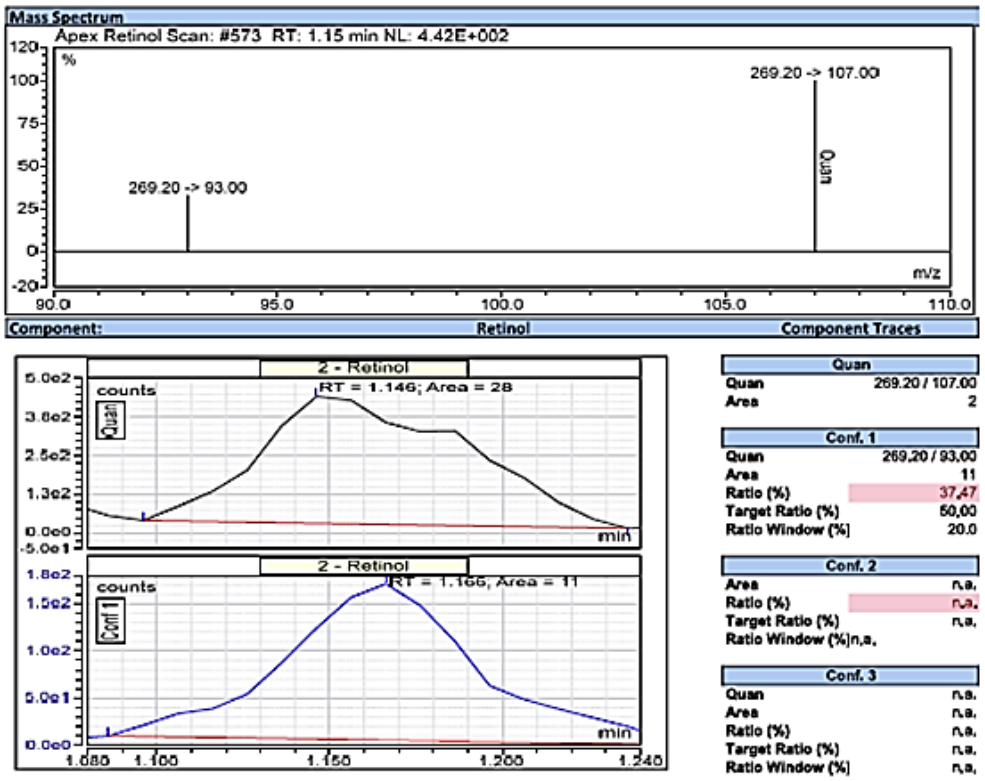


**Figure 4:** Chromatogram of vitamin A (retinol)

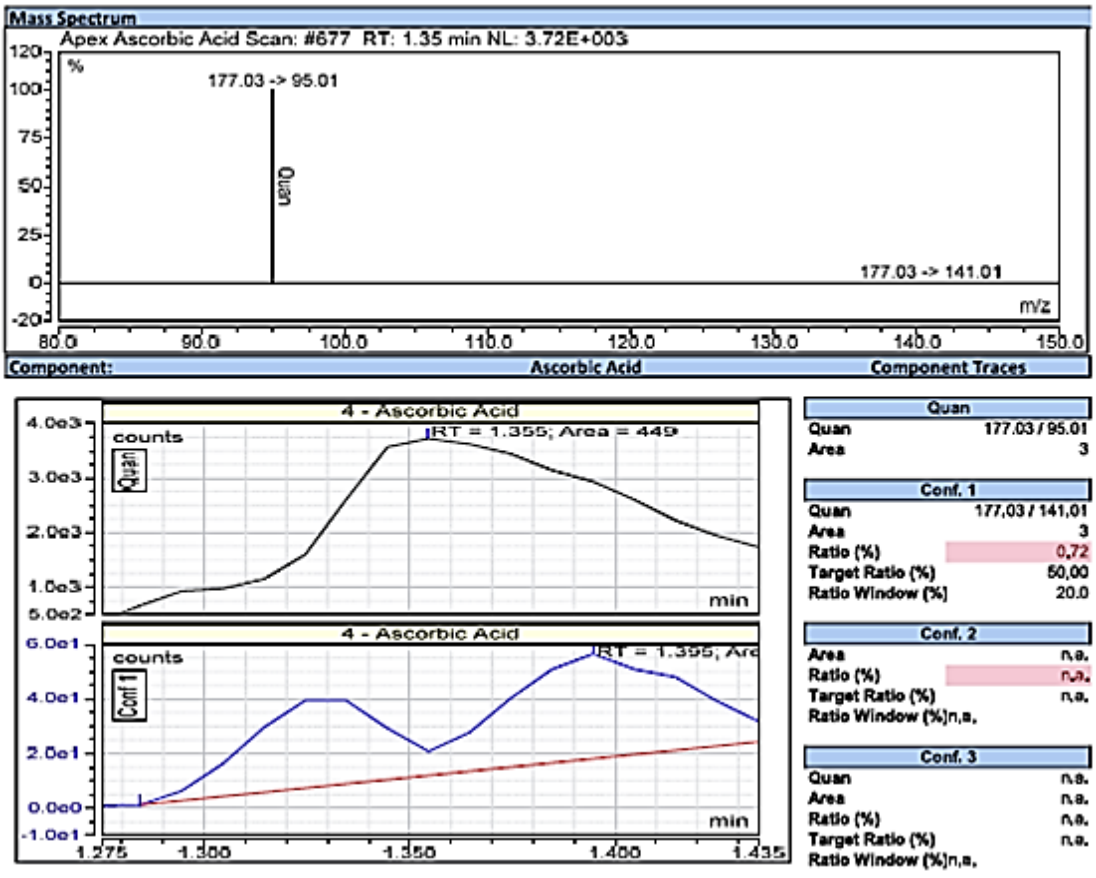


**Figure 5:** Chromatogram of vitamin C (ascorbic acid)

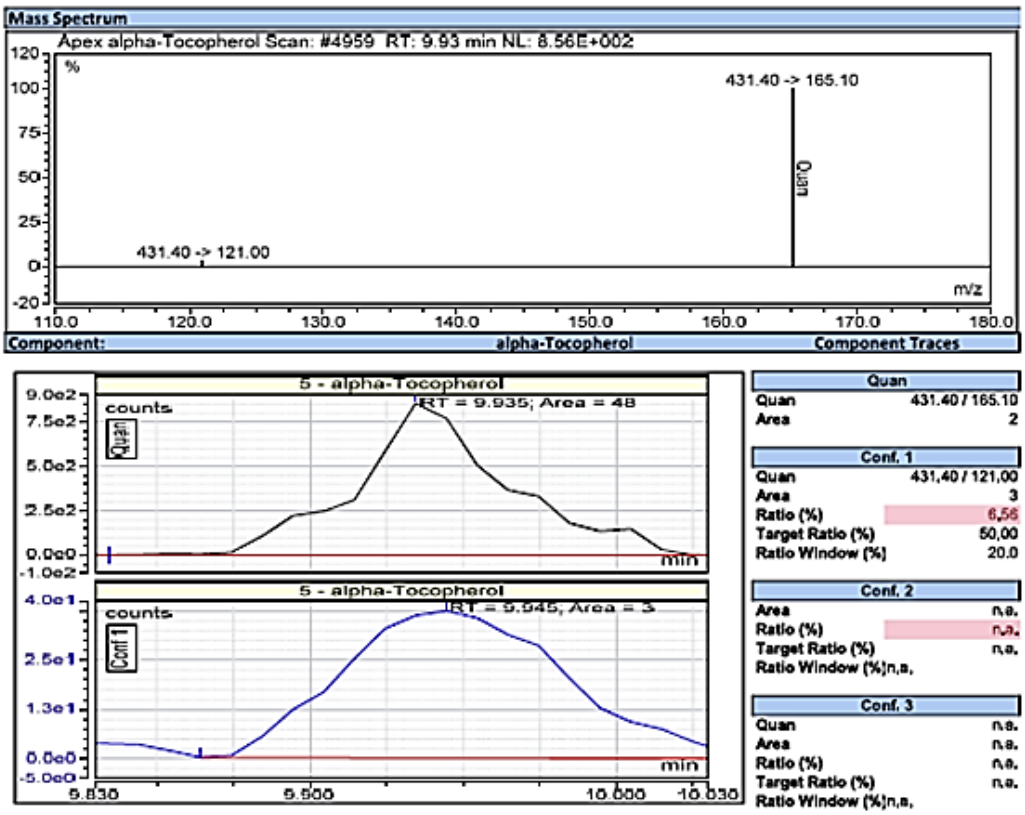


**Figure 6:** Chromatogram of vitamin E (α-tocoferol)

## DISCUSSION

This study used an extract from the watermelon variety Setabindo-1 sourced from PT Bisi Internasional Tbk in Karangploso, Malang Regency, East Java, Indonesia. The bioactive compounds in this specific variety may differ from those reported in the literature. The extract was prepared from watermelon as the feedstock through a liquid-liquid extraction method utilizing a solvent mixture of n-hexane, acetone, and 96 % ethanol in a volume ratio of 2:1:1. Qualitative LC-MS/MS analysis identified the presence of lycopene, citrulline, retinol (vitamin A), ascorbic acid (vitamin C), and alpha-tocopherol (vitamin E). These results are in agreement with data from the U.S. Department of Agriculture (USDA) which indicate that 100 g of watermelon flesh contains 4.532 µg of lycopene, 91.45 g of water, 303 µg of β-carotene, 569 IU of vitamin A, and 78 µg of β-cryptoxanthin, in addition to vitamin C and vitamin E. The findings indicate that lycopene is the predominant bioactive compound in watermelon, thereby emphasizing its potential as a functional food [1,2]. Lycopene is the carotenoid responsible for the red pigmentation of watermelons.

Watermelons also contain a range of carotenoids that contribute to the variations in the flesh colors of the fruits. These carotenoids serve as important functional components and micronutrients. The compositions and concentrations of carotenoids are crucial in evaluating the quality of watermelons: most of the β-carotene and lycopene are found in the red-fleshed varieties. Lycopene comprises the largest portion of total carotenoids, making up 84 to 97 %. Both lycopene and vitamin C are potent antioxidants known for their numerous health benefits. In particular, lycopene has the potential to offer protection against several chronic diseases such as cancer, diabetes, and cardiovascular conditions [2,9].

The capacity to scavenge singlet oxygen is essential for the protective action of antioxidants. Due to the production of free radicals and their interaction with macromolecules, the oxidation of proteins, lipids, and DNA is a significant contributor to metabolic disorders. Lycopene exerts protective effect against pathogenic infections. Among its antioxidant functions, it has been reported that lycopene protects white blood cell DNA from oxidative damage [2]. Lycopene prevents atherosclerosis through several mechanisms, viz: prevention of endothelial damage, modulation of lipid metabolism, inhibition of LDL oxidation, reduction of the rate of production of reactive oxygen species (ROS)

*in vitro*, reduction of inflammatory responses, inhibition of foam cell formation, and suppression of smooth muscle cell proliferation [10,11].

In addition to lycopene, the red watermelon extract contains vitamin A, vitamin C, vitamin E, and citrulline. Vitamin A plays a crucial role in regulating the metabolism of carbohydrates, lipids, and proteins [12]. Vitamin A is a potent natural antioxidant that helps maintain healthy skin and hair by promoting the growth of new elastin and collagen fibers. Vitamin A supplementation is widely recognized as an effective strategy for preventing tooth decay and lung cancer. Vitamin C is essential for combating free radical-induced damage, as it scavenges oxygen free radicals and enhances the immune system. Additionally, vitamin C is a coenzyme needed for the production of healthy collagen [1,2,13]. Vitamin E has both antioxidant and anti-inflammatory properties. It prevents endothelial dysfunction, and it reduces inflammation and biomarkers of oxidative stress. Furthermore, vitamin E lowers levels of intercellular adhesion molecule-1 [13,14].

Citrulline is a biologically significant amino acid that plays an important role in digestion in mammals. Over the last 40 years, citrulline has been recognized as a transition metabolite which has attracted the interest of researchers due to its implications in human health. Watermelon serves as a rich source of citrulline, and its consumption elevates the levels of L-citrulline and L-arginine in the plasma. L-Citrulline is a crucial substrate for synthesis of nitric oxide [2,15–17]. Moreover, citrulline functions as a vasodilator and an antioxidant, and it is utilized in humans for the regulation of nitric oxide levels [2,16–18]. It has been demonstrated that, among its other roles, citrulline substantially affectes skeletal muscle strength, diabetes, immunology, and neurology [2].

### Limitations of this study

A major limitation in this study is that only qualitative analysis was conducted. There was no quantitative analysis. Therefore, the results obtained may not reflect the actual quantity of each identified bioactive compound.

## CONCLUSION

The major bioactive compounds identified in the qualitative LC-MS/MS analysis of the extract of Setabindo-1 variety of red watermelon were lycopene, citrulline, vitamin A, vitamin C, and vitamin E. These compounds possess potent antioxidant properties.

## DECLARATIONS

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The authors are grateful to members of the Biomedical Laboratory, Faculty of Medicine, University of Muhammadiyah Malang, Pharmacology Laboratory, Faculty of Medicine, University of Brawijaya Malang, Materia Medica Batu Herbal Laboratory, Indonesia Healthy Animal Laboratory, PT Bisi Internasional Tbk, and all parties who assisted in the implementation of this study.

### *Ethical approval*

This study was approved by the Faculty of Medicine, Universitas Muhammadiyah Malang (no. E.5.a/181/KEPK-UMM/VI/2024).

### *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Mochamad Bahrudin: Conceptualization, Methodology, Software, Data curation, Writing-Original draft preparation. Asra Al Fauzi, Paulus Sugianto: Supervision, Validation, Writing-Reviewing and Editing. All authors have read and approved the manuscript for publication.

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