Proximate Analysis and Estimation of Flavan-3-ol Compounds Present in Grape Seed Extract by HPLC Method

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***Abstract*- Grape seeds are produced as waste products of the winery and grape juice-making industries. The United States Food and Drug Administration (FDA) recognized grape seeds as safe food. Flavan-3ols are grouped under the polyphenols, which are present in various fruits naturally. The flavan-3-ol compounds present in the grape seeds have potential effects on several diseases, like endocrine disorders, cancer and diabetes mellitus. Proximate estimation is used for analyzing moisture, total fat, carbohydrate, total phenols and flavonoids. High Performance Liquid Chromatography (HPLC) is a widely used and convenient method that helps to separate compounds and helps to "identify compounds" using various detection systems. The separation was achieved using a C18 analytical column and isocratic elution was carried out. The mobile phase is made of acetonitrile-formic acid (15:85). A 275 nm wavelength was set for the detection. The grape seeds were extracted using acetone.**

***Keywords*: Grape seeds, flavan-3-ol, proximate analysis, HPLC, isocratic elution.**

I. INTRODUCTION

Grapes are one of the most highly consumed fruits in the world. All parts of the grape, including the seed, skin, and flesh, possess various health benefits. Grapes have a wide history in winemaking and traditional medicine. During ancient Greek and Roman civilizations, grapes were used in winemaking **(En-Qin-Xia *et al.*, 2010).** About 50%–75% of grapes are processed to produce wine and juice after cultivation. About 20%–30% of grapes produce **'Pomace'** (which is solid waste obtained from pressing the fruit and contains skin, seeds, and pulp) **(Source: Food Waste to Valuable Resources, 2020**).

The pomace is underutilised, and it is a rich source of phenolic compounds and antioxidants **(Chandrasekar Venkitasamy *et al.*, 2019).** Grape seeds are the industrial byproducts of the winemaking process and the seeds remain an inexpensive and cheapest source of antioxidants. The United States Food and Drug Administration (FDA) recognised grape seed and skin extract as safe due to the compounds present in them, which help protect humans from various diseases **(Charradi K *et al.*, 2013).** Grape seeds are produced as a waste product of the winery and grape juice-making industries. The grape seeds contain **"lipid, protein, carbohydrate, and 5-8% polyphenols"** as per the variety. Polyphenols in the grape seeds mainly **"flavonoids"** including "epicatechin and more highly polymerised procyanidins".

Figure 1- Grape seeds

The grape seed extract has various health benefits, including preventing premature ageing, disease, and decay because it is the richest source of **antioxidants.** A scientific study showed that the antioxidant power of the proanthocyanidins is **'twenty times'** greater than that of Vitamin E, and the grape seed extract provides many health benefits due to its antioxidant effect. Its bond with collagen helps to **"promote youthful skin, cell death, elasticity of the skin, and**

skin flexibility". Some studies proved that proanthocyanidins also possess various health benefits, including protect the body from "sun damage, helping to improve vision, improving the flexibility of joints, arteries, and body tissues such as the heart, and also helping to improve the blood circulation in the body tissues by strengthening the capillaries, arteries, and veins", **(John Shi *et al.,* 2003).**

Grapes are considered to be one of the oldest ancient fruit-bearing plants. In early morphological studies of grape seeds, there were two main morphotypes, which consist of **"pear-shaped"** or **"oblong to ovoid seeds",** that corresponded, respectively, to the taxonomic group. The cultivated type of seed is larger in dimensions than the wild species. The seeds of cultivated grapes generally have **'elongated, pear-shaped seeds with a long beak'.** The wild grapes have shorter seeds, **(Rivera, D. *et al.,* 2007).** The epidermis and the outer integument contained the most phenolic compounds. It belongs to the family **"vitaceae".** The grape seeds are a complex matrix that is abundant in valuable compounds, **(Cadot Y *et al.,* 2006).**

Flavan-3-ols have been found to have various health benefits by acting as anticarcinogenic, antimicrobial, neuroprotective, antioxidant, antiviral, and cardioprotective agents, **(Patrica M. Aron and James A. Kennedy, 2008).** High Performance Liquid Chromatography (HPLC) is a widely used and convenient method that helps to separate compounds and **"identify compounds"** using various detection systems. This HPLC method was developed for qualitative as well as quantitative analysis of compounds **(Daigle, D. J and Conkerton, E. J, 1988).** HPLC is mainly used for the separation of biochemical compounds. The compounds present in the sample are separated by the physical and chemical interactions happening between the samples and substances and by the stationary phase. The component travels slower when it has **"more affinity"** for the absorbent. By contrast, the component travels faster and has **"less affinity"** for the stationary phase. The HPLC is combined with a PDA detector. **(Ramole Rina *et al.,* 2021)**

The objective of the present study is to quantitatively determine the amount of catechins present in the grape seed extract and its potential health benefits to humans.

II. MATERIALS AND METHODS

Procurement of raw materials

Whole black grapes were purchased from the local fruit shop. Fruits were washed, and seeds were removed. The seeds were shadow-dried for 7 consecutive days. After complete drying, the seeds were ground into a fine powder using a mortar and pestle.

Obtaining grape seed extract

20 g of powder was weighed using an electronic weighing machine. **(Infra digi-IN series)**. The powder was macerated for 12 hours at room temperature three times using **480 ml** of 100 mM acetate buffer water/acetone (30:70 v/v). The preparation of acetate buffer involves 1.544 g of sodium acetate and 0.7 ml of glacial acetic acid dissolved in 48 ml of H_2O (water), then adding 112 ml of acetone. All the extracted samples were collected in the brown bottle.

Proximate analysis of grape seed extract

Under the proximate analysis, parameters like moisture, pH, ash, carbohydrate, total fat, total phenolic compounds and total flavonoids were analysed.

Moisture: In the testing of food, the determination of moisture is the most important and widely used parameter. Since the amount of dry matter in the food is inversely related to the amount of moisture content it contains, the moisture content of the food varies widely. It follows the principle of heating the sample under specified conditions, and the loss of weight is used to calculate the moisture content of the sample.

- Took a clean dry petri dish.
- Dried the petri dish at 110℃ for 1 hour. Cover the dish, cooled and weighed.
- Weighed accurately 5g of sample accurately and took the sample in a petri dish.
- Spread 5g of sample uniformly in the petri dish layer.
- Weigh as quickly as possible to avoid the loss of moisture.
- Remove the lid and dry the sample at the atmospheric pressure of the hot air oven.
- Maintained a temperature of 110℃.
- After drying, replaced the lid, cooled the sample in a desiccator and reweighed it.
- Reheat the sample if necessary until the consecutive weighing does not vary more than 3-5 mg.

pH : pH is the measure of **"hydrogen ion concentration",** a measure of the acidity or alkalinity of the solution.

- Accurately weighed sodium acetate and glacial acetic acid.
- Dissolved 0.772 g of sodium acetate and 0.35 ml of glacial acetic acid in 48 ml of H_2O (water).

- Then mixed the water mixture with 56 ml of acetone.
- The buffer solution was made.
- The equipment was calibrated and the electrode was inserted into the sample beaker.

Ash: The principle of ash is to burn off the organic matter and determine the inorganic matter remaining. Heating is carried out in two stages: first, removing the water present, charring the sample thoroughly, and finally, ashing at 550 °C in a muffle furnace.

- The crucible and lid are first placed in the furnace at 550 °C overnight to ensure that impurities on the surface of the crucibles are burned off. Using the desiccator, cool the sample for 30 minutes.
- Weigh the crucible and lid.
- Weighed about 5g of sample into the crucible.
- Heated at 550 °C overnight. Cooled down in the desiccator.
- Weigh the ash with the crucible and lid.

Carbohydrate : The carbohydrate estimation follows the Anthrone method.

- In a boiling tube, weigh 100 mg of the sample.
- Hydrolyze by keeping it in a boiling water bath for 3 hours with 5 ml of 2.5 N Hcl at a cool temperature.
- Neutralise the sample with solid sodium carbonate until the effervescence stops.
- By using distilled water, make up the volume to 100 ml and centrifuge it.
- Collect the supernatant in a test tube and take 0.5 ml and 1 ml aliquots for further analysis.
- Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the working standard '0' as blank.
- Make up the volume to 1 ml in all the tubes used in the experiment.
- Then add 4 ml of anthrone reagent.
- In a boiling water bath, heat the test tube for 8 minutes.
- Cool rapidly and read the colour at 630 nm.
- Draw a standard graph by plotting the concentration of the standard on the X-axis and various absorbances on the Y-axis.

Total fat : Total fat content was determined by Soxhlet extraction methods.

- Dried samples (2g) were extracted with petroleum ether in a Soxhlet extraction apparatus for 6–8 hours in the preweighed round bottom flask.
- The extract containing fat and petroleum ether was evaporated over a boiling water bath, dried in an oven at a low temperature, and weighed.
- The differences in the weight of the round bottom flask represented the ether extract (fat content) present in the sample.

Total phenolic compounds : Phenolic compounds present in grape seeds possess great antioxidant activity.

- 2 g of sample was weighed and ground well three times with 10 ml of 80% methanol.
- The supernatant was dried in a water bath at 50–60 degrees Celsius.
- Then, the dried supernatant was dissolved in 5 ml of distilled water. 0.5 mL of the sample was pipetted out in test tubes, and the volume was made up to 3 ml with distilled water.
- 5 ml of Follin's water (1:2) reagent was added, and after 3 minutes, 2 ml of a 20% sodium bicarbonate solution was added.
- The content was mixed thoroughly, and the test tubes stood for 30 minutes at room temperature.
- The developed colour was measured at 650 nm. Gallic acid was used for the preparation of standard curves at different concentrations of 10, 20, 30, 40, and 50 µg/mL.
- Total phenol content was expressed as the percentage of gallic acid equivalents per 100 g of fresh sample (mg gallic acid/100 g of dried weight basis).

Total Flavonoids : Flavonoids compounds widely present in grape seed extract.

- The total flavonoid content (TF) of the dried extract was determined according to the colorimetric assay, with a slight modification: a 0.1 mL sample was diluted to 1 mL with 80% methanol.
- At zero time, 0.3 mL of (5% w/v) $NaNO_2$ was added. After 5 minutes, 0.3 mL of (10% w/v) $AlCl_3$ was added.
- The mixture was shaken well, and the mixture was read at 510 nm to determine the absorbance.
- A linear standard calibration curve was prepared using quercetin at different concentrations (20, 40, 60, 80, and 100 µg/mL).
- The total flavonoid compounds are expressed as mg of quercetin in 100 g of dry matter.

HPLC analysis

Reagents and chemicals

All reagents and chemicals were in the analytical range and HPLC grade. The epicatechin was used as a standard. For the mobile phase, acetonitrile (99.9%) and formic acid (0.01) were used. The grape seed extract was run under a degasser to remove any lumps present in the extract for 15 minutes. The sample was prepared with 30:70 v/v acetone, stored in brown glass bottles, and kept at room temperature for further analysis by the HPLC method.

Chromatographic condition

The column was a C18 (250 nm × 4.6 nm) with a particle size of 5 µm. Isocratic elution was carried out for the mobile phase. For the mobile phase, the mixture of solvents A and B (**solvent A-acetonitrile** (99.9%) + water (0.01%) and **solvent B**-formic acid (0.01%) + water (99.9%)) was used and freshly prepared. The solvents were mixed in a ratio of 15:85. The flow rate was expressed as **1 ml/min".** 20 µl of sample was injected, and the run time was fixed for 20 minutes. And the wavelength is determined to be 275 nm.

Validation of the method

Flavan-3-ol is also called **"catechins" (Angela Giovana Batistai *et al.*, 2021).** The result was concluded by considering the peak area and retention time. A standard chromatogram and a sample chromatogram were obtained after the run time of the standard and the sample. These chromatograms help in the quantification of the specific required compound.

III. RESULT

Proximate profile of grape seed extract

The nutrient profile is given in the following table,

Table 1- Proximate profile of grape seed extract

NUTRIENTS	VALUE
Moisture	10.2 %
pH	5.67
Ash	0.89 g/100 g
Carbohydrate	3.8 g/100 g
Total fat	3.68 g/100 g
Total phenolic compounds	2749.5 mg/100 g
Total flavonoids	1867.7 mg/100 g

High Performance Liquid Chromatography

The main aim of this HPLC analysis is to find the amount of **catechin (flavan-3-ol)** present in the grape seed extract. The **standard (epicatechin)** and **sample (grape seed extract)** were run in chromatography. The retained peak was determined by comparing the sample chromatogram with the standard chromatogram and the qualitative and quantitative estimations were done.

STANDARD CHROMATOGRAM:

Table 2 – Summary of the Standard chromatograph

Name	Retention Time	Area	% Area	Height
Epicatechin	7.150	513086	100.00	20750

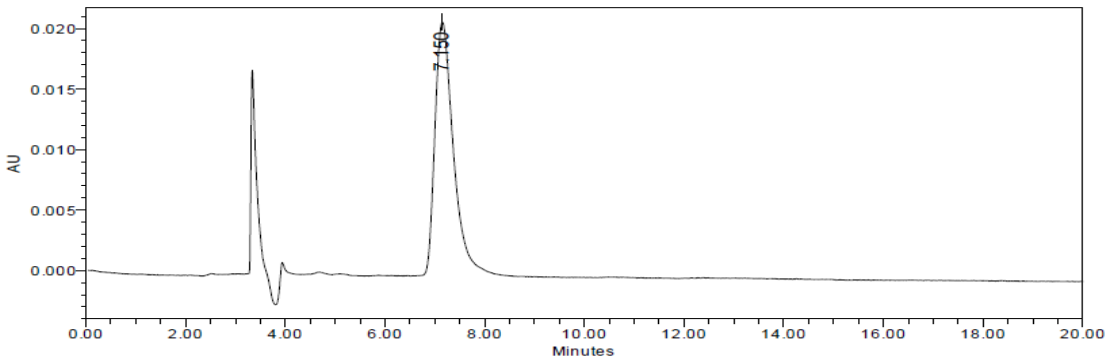

Figure 2 - Standard chromatograph (Epicatechins)

SAMPLE CHROMATOGRAM:

Table 3 – Summary of the Sample chromatograph

Name	Retention Time	Area	% Area	Height
Unknown compound	3.686	56141117	84.24	2677631
Unknown compound	4.754	9846344	14.77	302114
Epicatechin	6.963	655991	0.98	14702

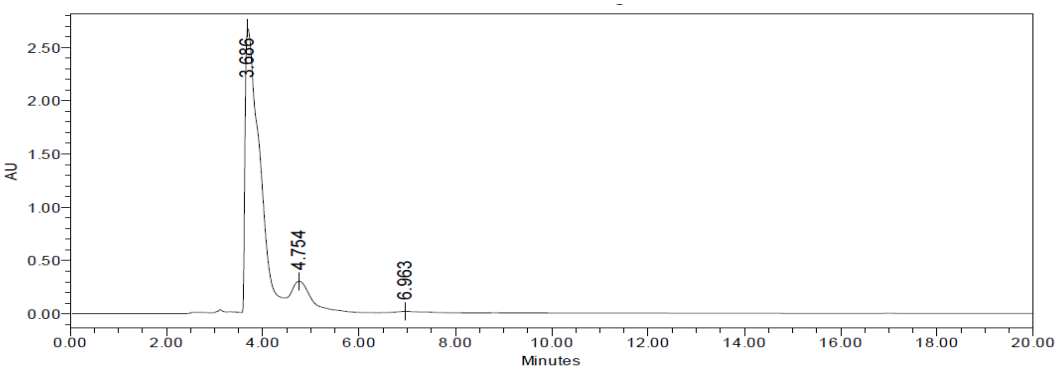

Figure 3 - Sample chromatogram (grape seed extract)

AREA GRAPH:

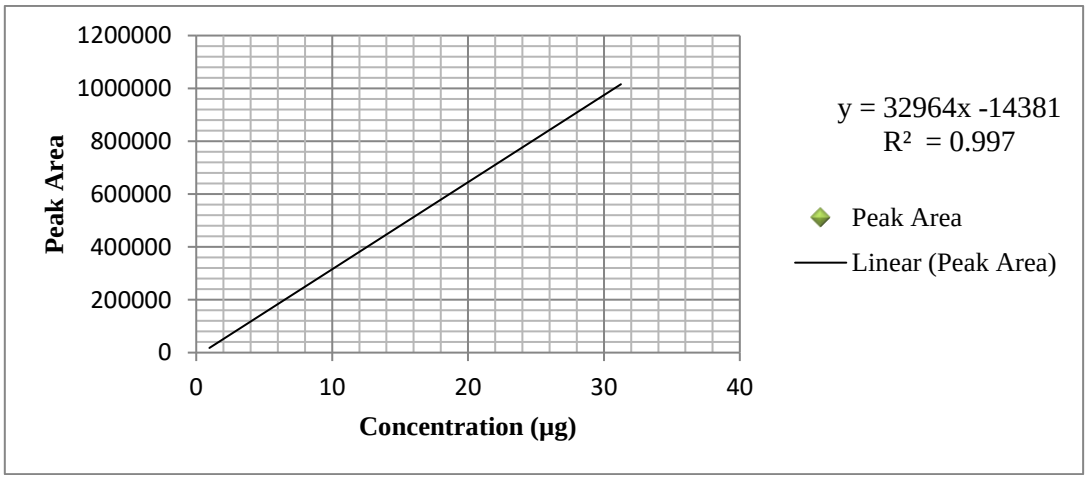

Figure 4 – Area graph of epicatechin

For the grape seed extract, the concentration of the flavonoid compound **epicatechin** was identified as **"48.816 mg/480 ml of extract made from 20 g of dried seed powder".**

IV. CONCLUSION

Under this study, the proximate analysis or nutrient profile of the sample was estimated using simple procedures and can be applied to various samples. The HPLC method was also validated using the isocratic elution using acetonitrile (15%), formic acid (85%), and a 275 nm wavelength. The method facilitated was not time-consuming or vague. The method was precise, acceptable, and accurate. The quantification of flavan-3-ol present in the grape seed extract was successfully done. As per various studies the flavan-3-ol possess various health benefits to humans, including **"improve estrogen activity in females, reduce the proliferation of cancer cells, improve the skin health and also protect the human body from various diseases caused by oxidative stress".** This study insisted that the grape seed extract is the great source of antioxidants because it contains **flavan-3-ols.**

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