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Method Validation and Determination of Total Antioxidants, Polyphenols, Flavonoids, and Vitamin C Contents in the Yemeni Grapes

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Abstract

The quality of grapes is an important issue for nutritional value and content of antioxidants, which are essential for human health. Yemen is Famous for growing different types of grapes in large quantities. This study aims to validate the analysis methods of determination of total antioxidants (TAC), polyphenols (TPC), flavonoids (TFC), and vitamin C (Vit. C) contents, in terms of linearity, repeatability, accuracy, and limits of detection (LOD) and quantification (LOQ), for measuring TAC, TPC, TFC, and Vit. C in grape samples, with good linearity (R^2) are 0.9968, 0.9967, 0.9971, and 0.9966, exhibited high repeatability(% RSD) are ranged (1.452 – 4.836%), (0.720 – 3.240%), (0.766 - 6.001%), and (0.539-2.976%), also high sensitivity (LOD = 0.279, 0.243, 0.288, and 76.376 ppm), LOQ = (0.928, 0.809, 0.961, and 245.588 ppm), and the high accuracy (%R) (80.80-95.00%), (91.44-104.35%), (92.37-101.87), and (88.89-104.65%), respectively. The analysis results of fourteen Yemeni grape samples indicated that the highest value of TAC for a Black grape sample was 6755.3 mg AAE/kg, while the lowest value for the Asmi grape sample was 1621.7 mg AAE/kg. For the TPC, the highest value was 4718.3 mg GAE/kg for the Black grape whereas the lowest content was 1382.3 mg GAE/kg for the Asmi grape. TFC ranged from 341.8 for the Black grape to 32.0 mg QE /kg for the Asmi grape—lastly, Vit. C ranged from 2418.1mg AAE/kg Black grape to 766.5 mg AAE/kg for Asmi grape. This study indicated that Yemeni grape varieties exhibit high TAC, TPC, TFC, and Vit. C contents, which can be used as a natural source of antioxidants.

Article Info

Keywords:
Yemeni Grapes, Antioxidants, Polyphenols, Flavonoids, Vitamin C, Method Validation

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1. INTRODUCTION

Antioxidant molecules, ions, or relatively stable radicals are capable of delaying, inhibiting, or preventing the oxidation of biomolecules like proteins, lipids, carbohydrates, and DNA [1–3]. Antioxidants may scavenge the free radicals or break the chain reaction due to their redox properties [4, 5], and prevent the damage by these free radicals [6]. An essential component of antioxidants is vital in maintaining excellent health [7]. Nutrition experts nowadays are interested in natural antioxidants produced by food factories and consumed by people because they maintain human health and have rupeudic value. Several secondary metabolites such as polyphenols, flavonoids, and, vitamin C are consumed as part of our daily fruit and vegetable diet [8]. Fruits and vegetables are good sources of antioxidant compounds [9], and the antioxidant activity in fruits is mainly due to the presence of phenols, and flavonoids [10]. Interest in these compounds has intensified in recent years as a result of their health benefits and their association with a reduced risk for many diseases like cancer and cardiovascular disease associated with daily consumption of antioxidant substances present in fruits and vegetables [11–13]. The grape, an economic plant with good agricultural characteristics, is one of the most important

Figure 1. Different Types of Yemeni Grapes

fruit commodities [14]. Fresh grape is rich in nutrients (dietary fiber, minerals, etc.) and bioactive compounds such as phenolic acids, flavonoids, isoflavonoids, thiols, carotenoids, ascorbic acid, tocopherols, etc. [15, 16]. Research has proven that grapes are a good source of antioxidants. [17], as grapes are among the fruits that contain the highest percentage of phenolic content of substances [18].

Yemen grows twenty different cultivars are grown in different areas. Raziki, Bayad, Black, Aasmi, and Gubari grape types are among the most grown cultivars in Yemen. Areas with cool, mild weather are ideal for growing grapes. The most famous are the areas around Sana'a, including Bani Hashish (30km east of Sana'a), one of the most fertile areas for growing grapes. Bani al-Harith and Wadi Dhahr (north of Sana'a, Khawlan (southeast) of Sana'a, and Saadah governorate in northern Yemen, where Sana'a has 80% of grape cultivation. All Yemeni cultivars are table grapes and part of their yield is dried for raisin production [19]. Different types of Yemeni Grapes (Figure 1).

There is a study included three types of grapes (Aasmi, Raziqi, and Aswad) to evaluate the antioxidant capacity, total phenolic and flavonoid contents and to evaluate the changes in the activities of some antioxidant enzymes catalase, peroxidase, and polyphenol oxidase Peel and flesh [20].

For there is no previous study to estimate antioxidants in the whole fruit of Yemeni grapes, the purpose of this study is to validate the methods of antioxidants, polyphenols, flavonoids, and vitamin C. Also, the estimation of TA, TP, TF, and Vit. C contents in whole fruit from fourteen (Black, Aasmi, Raziki, and Zaiton) Yemeni grapes samples.

2. MATERIALS AND METHODS

2.1. Chemicals and Solvents

All chemicals and reagents used were pure analytical grade. Standards: ascorbic acid 99.7%, gallic acid 99.8%, quercetin 99.9% were purchased from BDH, potassium iodide 99%, iodine 99.5%, and sodium acetate 99.8% were obtained from (BDH, UK), sodium carbonate 99.5% was obtained from (Pspark Scientific), aluminum chloride 99.7%, 2.2-dipyridyl 99.5% were from Pspark Scientific, ferric chloride hexahydrate 99% was from Merck. Scharlau (Spain) provided 99% of the methanol, 99.5% acetone, and, 96% ethanol.

2.2. Spectrophotometric Assays:

Spectrophotometric assays were performed using a spectrophotometer (Specord 200, Analytikjena, Germany) to determine the TAC, TPC, and TFC.

2.3. Standard Solutions:

Stock standard solutions (1000–5000 ppm) of ascorbic acid, gallic acid, and quercetin were prepared. The intermediate and, the working solutions were prepared by diluting the solutions appropriately.

2.4. Grapes Samples Collection:

Different types of Yemeni grapes were collected from different cultivation areas in Sana'a (Khawlan, Bani Hashish) and Saadah governorates. The samples include; Black, Rizki, Aasmi, and Zaiton Grapes. The samples were collected during the summer of 2023. All samples were transferred to the laboratory. Subsequently, the samples were washed, and then homogenized using an electrical mixer, and analyzed immediately.

2.5. Spiking of Graps Samples:

For the method validation, the grapes samples were spiked with known amounts of standard solutions and kept at 4°C overnight before the analysis [21].

0.5g of homogenized grape sample was weighed and extracted with 10 ml of solvent mixture (acetone: ethanol: Deionized water (DIW)) in the ratio (1:1:1) by shaking for 10 mins, then the sample was centrifuged for 5 mins. At 3500 rpm. The extract will be used for the analysis of TAC, TPC, and TFC.

1.0 g of grapes was extracted using 40 mL of DIW for 15 mins. The vitamin C in the extract was determined using the iodine redox titration method [22].

2.6. Determination of Antioxidants in Grapes Samples

2.6.1. etermination of TAC:

Determination of total antioxidant content TAC was performed according to the methods described by Othman et al., Sacchi et al., and Santana et al., [23–25] with some modifications using ferric (III), 2.2-bipyridyl reducing content (FBRC). 200 µL of grapes extract was added to a 10 mL measuring flask. Then, 2 mL of acetate buffer (pH 4), 1.5 mL $FeCl_3$ (0.01M), and 1.5 mL of 2.2-dipyridyl solution (0.1%w/v) was added, and the solution was made up to 10 mL with deionized water, mixed well, and placed in a water bath at 50 ◦C for 30 min. After cooling, the absorbance was measured at 520 nm using a reagent

as a blank. The concentration of TAC in the samples was correlated with the calibration curve of ascorbic acid. The results were expressed as (mg AAE/kg).

2.6.2. Determination of TPC:

Determination of Total Polyphenol Content TPC was determined using the method published by Singleton et al., with some modifications [26]. By adding 200 μL of grapes extract into a 10 mL measuring flask, 3.6 mL of deionized water and 0.4 mL of Folin–Ciocalteau reagent was added, the mixture was shaken and left for 5 mins, and 4 mL of 7%w/v sodium carbonate solution was added. The solution was completed to 10 mL with deionized water and incubated in a water bath at 50 ^{o}C for 20 min. TPC was measured at λ_{max} 750 nm against a reagent blank. The TPC concentration was correlated with the calibration curve of gallic acid. The results were expressed as (mg GAE/kg).

2.6.3. Determination of TFC:

Salomon et al. [27], described a method for determined the TFC. By diluting 3 mL of grape extract in a 10 mL measuring flask with 2 mL of 2%w/v of aluminum chloride. The volume was made up to 10 mL with a solvent (ethanol: water) (2:1). After 10 min, absorbance was measured at 430 nm against a reagent blank. The analysis was performed in triplicate, and the results were expressed as (mg QE/kg)

2.6.4. Determination of Vitamin C:

Vitamin C was determined using redox titration, the method described by Satpathy et al. [22]. The sample solution was pipetted into a 250mL conical flask, and 150mL of distilled water was added to it, followed by a 3mL starch indicator solution. Then the sample solution was titrated with 0.005mol L-1 iodine solution. The titration's endpoint was identified as the first permanent trace of a dark blue-black color due to the starch- iodide complex. The analysis was performed in triplicate, and the results were expressed as (mg AAE/kg).

2.7. Methods Validation

The applicability of the methods for the quantitative determination of TAA, TPC, TFC, and vit. C in grape samples was verified by performing analytical validation by the analysis series of spiked grapes samples with ascorbic acid, gallic acid, and quercetin standard solutions in triplicate, and the absorbance or the volume was used to plot a standard calibration curve. The linearity regression equation, correlation coefficient (R2), precision as relative standard deviation (%RSD), limit of detection (LOD), limit of quantification (LOQ), and recovery (%R) were calculated from the calibration curve according to the previously published papers [21, 27–32].

3. RESULTS AND DISCUSSION

3.1. Method Validation for (TAC) in Black Grapes

3.1.1. Linearity, Precision, Accuracy, LOD and LOQ:

To calculate the analysis method linearity, precision (repeatability), and accuracy (recovery) for black grape samples were spiked with different concentrations of ascorbic acid ranging from 0.5 to 5 ppm. The spiked samples were left for 24 hours; after that, the samples were extracted and measured. The linearity, repeatability, and recovery results are summarized in (Table 1). The calibration curve plotted the concentrations against the absorbance as shown in (Figure 2), the obtained result showed that the curve of black grape samples has good linearity over the range of concentration study, with correlation coefficients R^2 0.9968, high repeatability %RSD values ranged (1.452%– 4.836%), the recovery of ascorbic acid were in the range of (80.80%– 95.00%), which briefly indicates the proposed method has acceptable recovery for the analysis of TAC in grape, and the LOD, LOQ values were 0.278 and 0.928 ppm, respectively.

3.2. Method Validation for TPC in Black Grapes

3.2.1. Linearity, Precision, Accuracy, LOD and LOQ:

To calculate the analysis methods linearity, repeatability, and recovery of Black grape samples were spiked with different concentrations of gallic acid ranging from 2 to 8 ppm. The spiked samples were left for 24 hours, after that, the samples were extracted and measured. The linearity, repeatability, and recovery results for the Black grape sample were summarized in (Table 2). The calibration curve plotted the concentrations against the absorbance as shown in (Figure 3). The obtained results showed that the curve of Black Grape samples has good linearity over the range of concentration study, with correlation coefficients (R^2) 0.9967, high repeatability %RSD values ranged (0.720% – 3.240%), the recovery of quercetin were in the range of 91.44%–104.35%, which briefly indicates the proposed method has acceptable recovery for the analysis of TAC in grape, the LOD, and, LOQ values of Black Grape sample were 0.243 and 0.809 ppm, respectively.

3.3. Method Validation for (TFC) in Grapes Samples

3.3.1. Linearity, Precision, Accuracy, LOD and LOQ:

To calculate the analysis method linearity, repeatability, and recovery for the black grape samples were spiked with different concentrations of quercetin ranging from 2 to10 ppm. The spiked samples were left for 24 hours, af-

Table 1. Method Validation for Total Antioxidant Content (TAC)

Spiked Conc. of Ascorbic Acid (ppm)	Ave. Abs. of STD, (n=3)	Abs. of Unspiked Sample	Ave. of Spiked Sample, (n=3)	Abs. of Difference	SD	%RSD	LOD, ppm	LOQ, ppm	%R
0.50	0.080	0.392	0.457	0.065	0.012	2.626	0.279	0.928	80.80
1.00	0.145	0.392	0.517	0.125	0.025	4.836			86.00
2.00	0.295	0.392	0.647	0.255	0.021	3.246			86.30
3.00	0.437	0.392	0.807	0.415	0.015	1.859			95.00
4.00	0.553	0.392	0.903	0.511	0.021	2.326			92.40
5.00	0.677	0.392	1.033	0.641	0.015	1.452			94.80

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Division, %RSD=Relative Standard Division, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

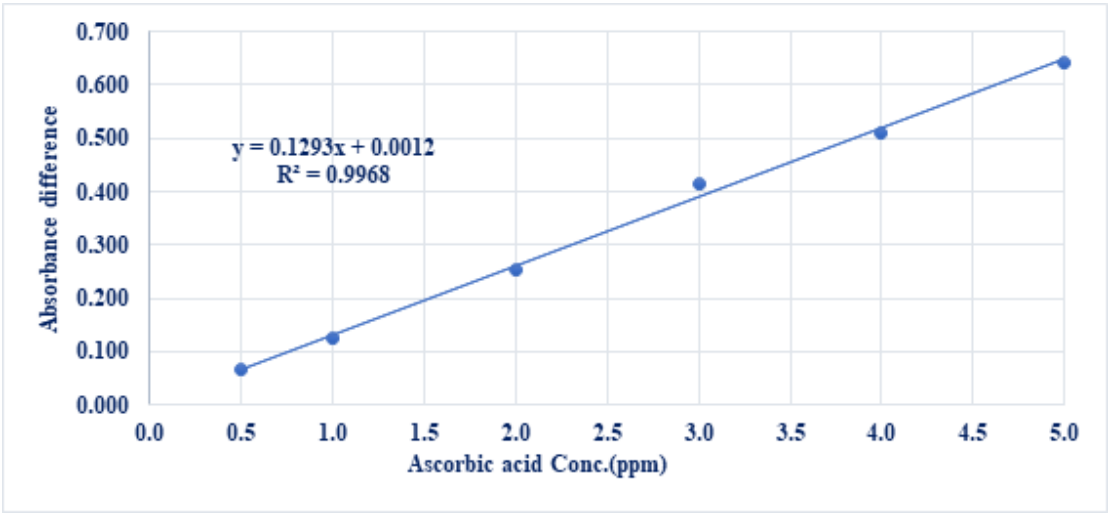

Figure 2. Method Linearity of TAC Determination

Table 2. Method Validation for Total Polyphenol Content (TPC)

Spiked Conc. of Gallic Acid (ppm)	Ave. Abs. of STD, (n=3)	Ave. of Spiked Sample, (n=3)	Absorbance of Difference	SD	Calculated Conc.	%RSD	LOD, ppm	LOQ, ppm	%R
0.00	0.000	0.233	0.000	0.008		3.240	0.243	0.809	
2.00	0.121	0.343	0.110	0.008	1.829	2.372			91.44
3.00	0.192	0.433	0.200	0.012	3.130	2.885			104.35
4.00	0.270	0.494	0.261	0.012	3.867	2.335			96.67
6.00	0.393	0.616	0.383	0.010	5.852	1.648			97.54
8.00	0.510	0.765	0.532	0.006	8.340	0.720			104.25

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Division, %RSD=Relative Standard Division, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

ter that, the samples were extracted and measured. The linearity repeatability, LOD, LOQ, and recovery results for each type were summarized in (Table 3). The calibration curve plots the concentrations against the absorbance, as shown in (Figure 4). The obtained results showed that the curve of Black Grape samples has good linearity over the range of concentration study, with correlation coefficients (R^2) 0.9971, high repeatability %RSD values ranged (0.766 %– 6.001%), the recovery of quercetin were in the range of 92.37%– 101.87%, which briefly

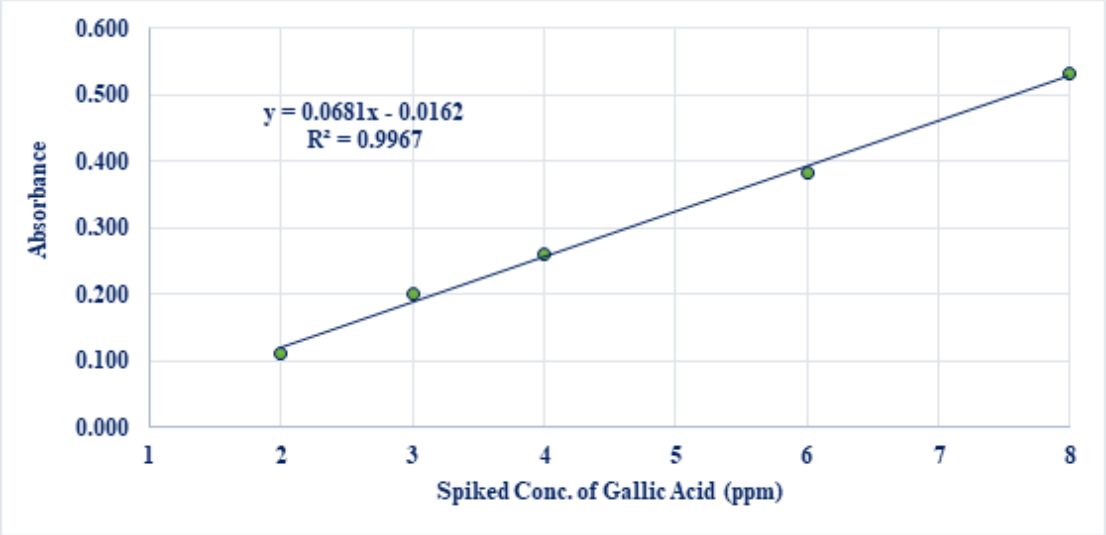

Figure 3. Method Linearity of TPC Determination

Table 3. Method Validation for Total Flavonoids Content (TFC)

Spiked Conc. of Quercetin (ppm)	Ave. Abs. of STD, (n=3)	Ave. of Spiked Sample, (n=3)	Abs. of Differ-ence	SD	%RSD	LOD, ppm	LOQ, ppm	%R
0	0.000	0.144	0	0.009	6.001	0.288	0.961	
2	0.121	0.221	0.077	0.009	3.854			92.37
4	0.192	0.312	0.168	0.006	2.002			97.11
6	0.270	0.400	0.256	0.006	1.378			98.48
8	0.393	0.503	0.360	0.013	2.516			98.99
10	0.510	0.617	0.473	0.005	0.766			101.87

Conc.= Concentration, Abs.=Absorbance, Ave.=Average, STD=Standard, SD=Standard Division, %RSD=Relative Standard Division, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

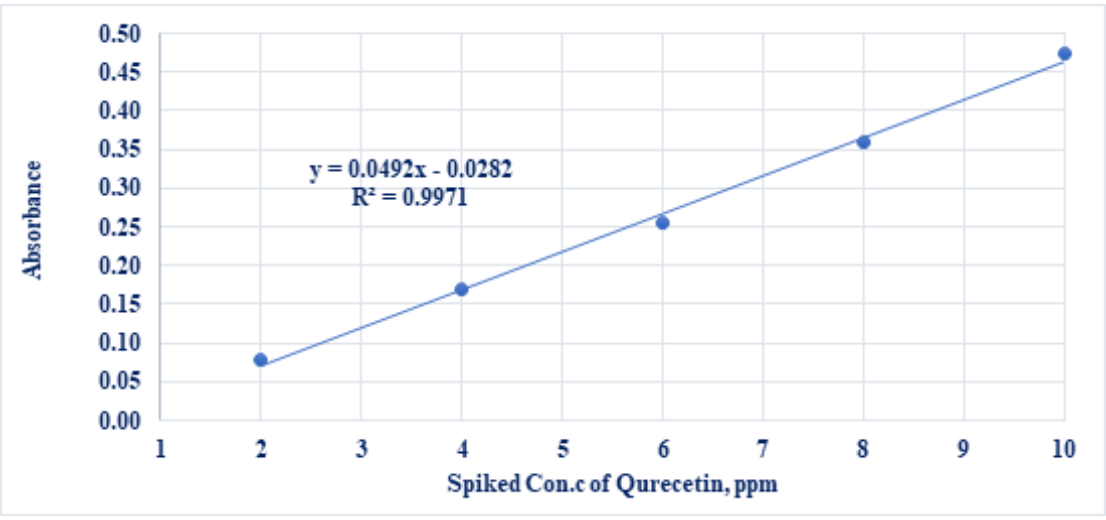

Figure 4. Method Linearity of TFC Determination.

indicates the proposed method has acceptable recovery for the analysis of TAC in grape, the LOD, and LOQ values of black grape sample was 0.288 and 0.961 ppm, respectively.

Table 4. Method Validation for Vit. C

Spiked Conc. of Ascorbic Acid (ppm)	Ave. Iodine Volume STD, mL (n=3)	Ave. of Spiked Sample, (n=3)	Volume of Difference	SD	%RSD	LOD, ppm	LOQ, ppm	%R
0	0.000	2.567	0	0.076	2.976	76.376	254.588	
250	0.150	2.700	0.077	0.050	1.852			88.89
500	0.280	2.833	0.168	0.015	0.539			95.24
750	0.430	3.017	0.256	0.058	1.914			104.65
1000	0.580	3.150	0.360	0.030	0.952			100.57
1500	0.840	3.417	0.473	0.035	1.028			101.19

Conc.= Concentration, Ave.=Average, STD=Standard, SD=Standard Division, %RSD=Relative Standard Division, LOD=Limit of Detection, LOQ=Limit of Quantification, %R=Recovery Percentage.

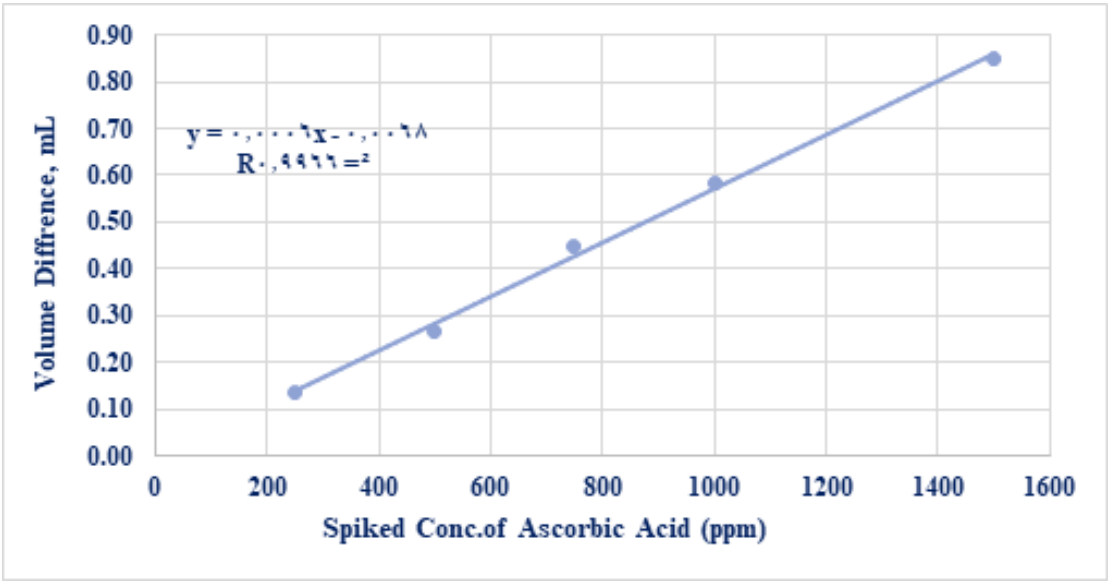

Figure 5. Method Linearity of Vitamin C Determination

3.4. Method Validation of Vitamin C Concentration by Titration:

To calculate the method linearity, repeatability, and recovery for the Black Grape samples were spiked with different concentrations of vit. C ranging from 250 to 1500 ppm. The spiked samples were left for 24 hours, after that, the samples were extracted and measured. The results are summarized in Table 4. The calibration curve plots the concentrations against the iodin consumed volume (Figure 5). The curve of black grape samples has good linearity over the range of concentration study, with correlation coefficients (R^2) 0.9966, high repeatability %RSD values ranging (0.539%– 2.976%), the recovery of vit. C were in the range of 88.89%–104.65%, which briefly indicates the proposed method has acceptable recovery for the analysis of TAC in grape, and the LOD, and LOQ values of the black grape sample were 76.376 and 254.588 ppm, respectively.

3.5. Real Samples Analysis Results:

The optimized method was applied to analyze the TAC, TPC, TFC, and vit. C of Yemeni grape samples. Fourteen Yemeni grape samples were analyzed to estimate TAC, TPC, TFC, and vit. C contents. Samples of different types of grapes were collected from Sana'a (Khawlan, Bani Hashish) and Saadah governorates. The locations, cultivars code of collected samples, and the obtained results of the analysis of TAC, TPC, TFC, and Vit. C. are summarized in Table 5 and represented in Figure 6. For 14 grape samples (5 Black, 5 Raziki, 2 Asmi, and 2 Zaiton).

The TAC ranged from 1621.7±250.3 for AG-1 to 6755.3±500.3 mg AAE/kg for BG-5, The TAC values for black, Raziki, Aasmi, and Zaiton varied from 4568.3±254.2 to 6755.3±500.3 mg AAE/kg fw, 2220.2±196.0 to 3782.7±161.6, 1621.7±250.3 to 1873.9±90.5, and 2208.8±112.4 to 2324.7±131.9 mg AAE/kg fw, respectively. As can be seen, black grapes

Table 5. TAC, TPC, TFC, and Vit. C of Grapes Real Samples Results

No.	Cultivars Code	Location	Average ±SD			
			TAC mg AAE/kg fw	TPC mg GAE/kg fw	TFC mg QE/kg fw	Vit. c mg AAE/kg fw
1	BG-1	Khawlan-Alhisn	4568.3±254.2	2979.6±193.4	185.2±5.7	2418.1±117.3
2	BG-2	Khawlan-Buni Bahlul	5894.5±180.8	3308.2±123.1	303.5±4.8	1714.3±48.1
3	BG-3	Khawlan-Almakhrafiu	5782.6±117.0	3591.1±84.5	260.1±7.3	1638.0±50.4
4	BG-4	Saadah-Auyr	4643.5±258.1	2447.2±87.5	188.9±6.5	1888.9±73.0
5	BG-5	Khawlan-Buni Bahlul	6755.3±500.3	4718.3±115.6	341.8±11.3	1033.3±64.2
6	RG-1	Khawlan-Sahaam	2220.2±196.0	1466.8±80.0	111.3±4.7	882.4±35.3
7	RG-2	Khawlan-Alhisn Alabyad	2505.2±114.1	1501.9±136.6	97.3±3.8	1195.9±29.4
8	RG-3	Khawlan-Darab easkar	3639.8±245.4	1972.4±207.9	289.0±22.7	1316.5±41.1
9	RG-4	Khawlan-Alzahruh	3782.7±161.6	2627.4±227.1	209.6±6.2	843±31.2
10	RG-2	Khawlan-Aljueara	2322.8±130.0	1705.5±85.9	223.2±14.4	941.2±65.1
11	AG-1	Bani Hashish-Qae alsalahi	1621.7±250.3	1455.5±228.9	32.0±4.3	766.5±40.3
12	AG-2	Bani Hashish-Qaeralsalahi	1873.9±90.5	1382.3±140.9	62.4±6.8	1333.6±48.5
13	ZG-1	Bani Hashish-Wadi-rijam	2324.7±131.9	2132.5±151.7	122.8±6.9	1333.6±39.1
14	ZG-2	Bani Hashish-Alqarduh	2208.8±112.4	2082.9±49.8	120.0±2.6	1335.4±36.6
BG=Black Grape, RG=Raziki Grape, AG=Asmi Grape, ZG=Zaiton Grape, fw= fresh weight						

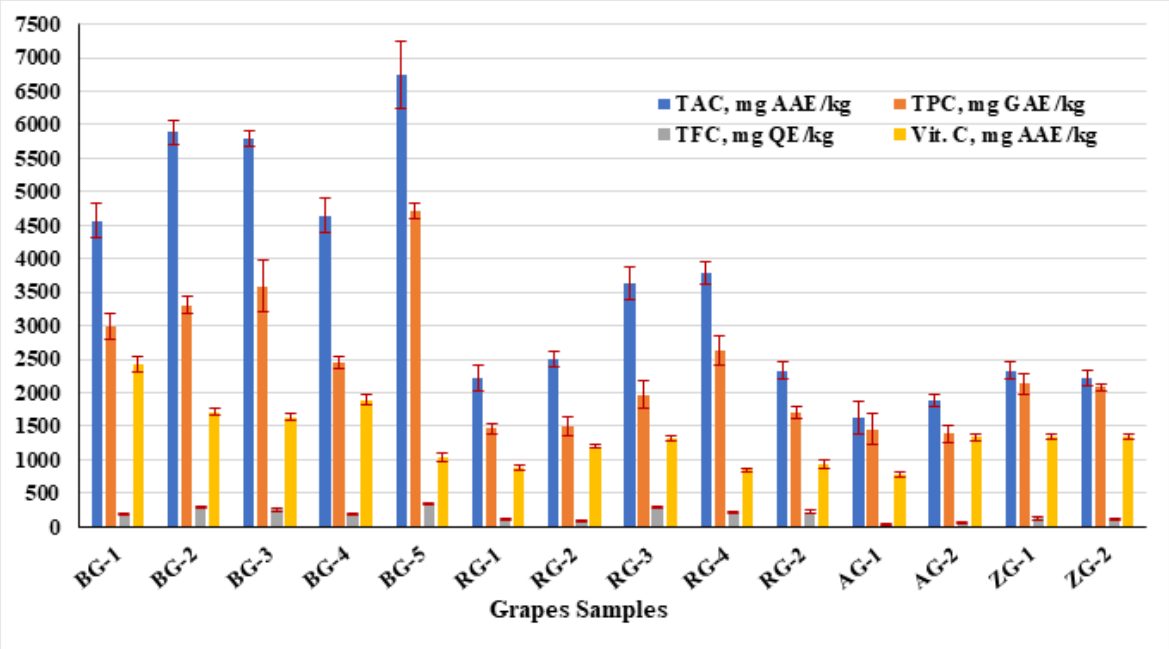

Figure 6. TAC, TPC, TFC, and Vit. C of Grapes Real Samples Results

had greater TAC values than the other grape varieties. In comparision, the study conducted by Nile et al. (2015), concluded that the antioxidant activities of 20 different grape varieties were ranged from 791±2.9 to 1970 ±2.2 mg/kg by FRAP assay [33], which is lower than the results obtained in this study.

TPC varied significantly in black grape (BG-5) found to have the highest total phenolic content at 4718.3±115.6 mg GAE/kg fw, whereas, the asmi grape (AG-2) has the lowest at 1382.3±140.9 mg GAE/kg fw (Table 5). Eshghi et al. (2014) reported that TPC in 35 grapevine (Vitis vinifera L.) cultivars grown in Fars province (Iran) were between 60.77 and 975.68 mg/ kg fw [14], while, Liu et al. (2018), found the TPC from 30 grape varieties had values ranged from 294 to 1407 mg GAE/kg fw [17], which are lower than the results obtained in this study. Similer results were reported by Kök et al. (2017), for TPC of eight different grape varieties, the concentration ranged from 3145.5 to 1036.2 mg GAE/ kg fw [34], Also, TPC in five Algerian table grape varieties were

ranged from 1210 ± 0.004 to 3040±0.006 mg GAE/kg fw as reported by Derradji-Benmeziane et al. (2014) [35]. Postharvest processing conditions, environmental factors, and genotypes all influenced the composition of phenolic compounds in grape fruit [36, 37]. Plants use phenolic compounds as defense mechanisms to fend off oxygen species and shield cells and molecules from harm [37]. The TFC in grapes from four different varieties (black, raziki, asmi, and zaiton) has varied ranges from 185.2±5.7 to 341.8±11.3, 97.3±3.8 to 289.0±22.7, 32.0±4.3 to 62.4±6.8, and 120.0±2.6 to 122.8±6.9 mg QE/kg fw, respectively, according to this study. The black grape (BG-5) had the highest total flavonoid content at 341.8±11.3 mg QE/kg fw, while the asmi grape (AG-1) had the lowest at 32.0±4.3 mg QE/kg fw. In a study conducted by Liu et al. (2018) the TFC for 30 grape varieties ranged from 82 to 132 mg QE/kg fw [17]. While, Benmeziane et al. (2014),reported the TFC for five tested grapes varied between 400 ± 0.001 and 1090 ± 0.004 mg CE/kg [35]. These concluded that the values obtained are in agood agreement with the values obtained in previous studies.

Vitamin C content in this study varied significantly, with the black grape (BG-1) having the greatest value at 2418.1±117.3 mg GAE/kg fw and the asmi grape (AG-1) having the lowest at 766.5±40.3 (Table 5). Vitamin C in five Algerian table grape varieties ranged from 123.3 ± 0.001 to 308.0 ± 49.8 mg AAE/L [35]. Eshghi et al. (2014) in a study conducted that Vitamin C in 35 grapevine cultivars grown in Fars province (Iran) ranged from 37.9 to 98.3 mg AAE/L fw [14].

4. CONCLUSION

Yemen produces huge amounts of different types of grapes every year. Therefore, using Yemeni grapes as a source of antioxidant substances in the pharmaceutical, cosmetics, and biopesticide industries is very important. In this study, we validate the analysis methods of TAC, TPC, TFC, and Vit. C which showed high repeatability, good linearity, high sensitivity, and high accuracy. The real sample results of varied cultivars showed high values of antioxidants. The grapes in our study showed substantial antioxidant activity. Therefore, we recommend increasing the consumption of these fruits in our diet.

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