


## Article

# Comparison and Intercorrelation of Extraction Methods for Polyphenol Content and Antioxidant Capacity of Scab-Resistant Apple Cultivars

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**Abstract:** Polyphenols with antioxidant compounds represent an important group of bioactive substances in apple production. The objectives of this study were firstly to measure four parameters of antioxidant capacity (AC) and polyphenol content (AC-FRAP (Ferric Reducing Ability of Plasma), AC-DPPH (2,2-diphenyl-1-picrylhydrol), AC-TEAC (Trolox Equivalent Antioxidant Capacity) and total polyphenol content (TPC)) of four scab-resistant apple cultivars ('Fujion', 'Gaia', 'Isaaq' and 'Smeralda') using six extraction methods (water control, pectinase, two approaches using ethanol (EtOH-1 and EtOH-2), methanol (MetOH) and methanol + acetone (MetOH + Acetone), and secondly to provide intercorrelations between antioxidant and polyphenol contents of the four resistant apple cultivars under the six extraction methods. Analysis of variance on the four parameters showed a significant ( $p = 0.05$ ) effect for extraction methods and cultivars. TPC showed the highest values among the four parameters in all extraction methods and cultivars compared to the other three measurements. The pectinase extraction method showed the highest TPC values for the four cultivars. The EtOH-2 extraction method showed the lowest AC-FRAP value for all cultivars. The EtOH-2 extraction method showed the highest AC-DPPH values, whereas the control method showed the lowest values for the four cultivars. The AC-TEAC values were generally the lowest, ranging between 10.8 and 40.5 mg TE 100 g<sup>-1</sup> dry matter, and they showed various effects on extraction methods and cultivars. Correlation and regression analyses of 36 pair-variables showed that two pair-variables (TPC vs. AC-FRAP and AC-TEAC vs. AC-DPPH) were significant for all of the six extraction methods and for all cultivars. In conclusion, the extraction method using pectinase enzyme provided the most stable yield of polyphenol content from apple flesh, as confirmed by the examination of four scab-resistant apple cultivars.

**Keywords:** antioxidant capacity; extractable polyphenols; FRAP; DPPH; TEAC; *Venturia inaequalis*

## 1. Introduction

Fruit consumption has been shown to have a positive effect on controlling human chronic diseases originating from an inappropriate diet [1]. A large amount of fiber and

bioactive substances in fruits, including polyphenols, ensure the proper physiological functioning of the human body. In fruits, including apples, these compounds provide primary protection lines against oxidation in human cells and exhibit an effective biological activity in the human body as they help the cytoprotective systems, e.g., [2–6]. In addition, apple consumption enhances the activity of superoxide dismutase and glutathione peroxidase in human blood [7,8]. Polyphenols, as natural organic compounds, contain a phenyl ring attached with hydroxyl groups, e.g., [2,3]. Several polyphenol components have been found in apples, such as flavonoids and phenolic acids, providing the antioxidant activity of the apple fruit, e.g., [9–14]. The main flavonoids measured in apples are anthocyanins, catechins, epicatechins, quercetin glycosides, procyanidins, and phloridzin; and the major phenolic acid compounds of apples are *p*-coumaroylquinic acid and chlorogenic acid, e.g., [10,13]. Several methodologies are used to measure the antioxidant capacity of plant samples, e.g., [15–18] and different methods including Ferric Reducing Ability of Plasma (FRAP), 2,2-diphenyl-1-picrylhydrol (DPPH) and Trolox Equivalent Antioxidant Capacity (TEAC) are applied to measure the antioxidant capacity of the apple fruits, e.g., [15,16]. Apples possess a total antioxidant capacity of ca. 400 mg Trolox equivalent 100 g<sup>−1</sup> dry matter, e.g., [19,20].

The distribution and amount of polyphenol components vary among apple cultivars, e.g., [13]. The antioxidant contents of fruit correlate well with the resistance of apple cultivars to apple scab (*Venturia inaequalis* (Cooke) G. Winter), e.g., [21–23]. For instance, the total polyphenol content of the Hestia scab-resistant cultivars (392 mg GS L<sup>−1</sup>) is 3.6 times greater than the polyphenol content of cultivar (cv.) ‘Gala’ (107 mg GS L<sup>−1</sup>) [23]. In addition, farmers increasingly choose resistant apple cultivars to avoid extensive costs of plant protection and to maintain their apple orchards in a more sustainable production system, such as integrated and organic systems, e.g., [24–28]. Some of the recently bred scab-resistant apple cultivars, such as ‘Gaia’, ‘Isaaq’, ‘Smeralda’, and ‘Fujion’, are of great interest in these sustainable production systems [29], but few studies have been undertaken, and have only partially reported the polyphenol content and antioxidant capacity of these cultivars [30,31].

Antioxidants, mostly formed as polyphenols, are dissolved by means of pectinase enzymes and solvents of methanol, ethanol, acetone, and distilled water, e.g., [30]. In most previous studies, only extractable polyphenol fractions were analyzed; however, the non-extractable polyphenols, present in solid residues, were ignored and are connected to the matrix of the cell wall, e.g., [31–34]. After the first extraction, a second extraction from the residue may dissolve additional amounts of polyphenols; however, the combination of different solvents has rarely been used to dissolve antioxidants from apple fruits.

Although information is available on the polyphenol content and antioxidant capacity of apple cultivars, little research has been undertaken to compare various extraction methods (including solvent combinations) in order to characterize the proportions of these components in scab-resistant apple cultivars. In addition, the intercorrelation of antioxidant capacity and polyphenol contents of resistant apple cultivars among various extraction methods has not been previously investigated. Therefore, the objectives of this study were firstly to measure four parameters of antioxidant capacity and polyphenol content (antioxidant capacity by FRAP, DPPH, and TEAC, in addition to the total polyphenol content (TPC)) of four resistant apple cultivars (‘Fujion’, ‘Gaia’, ‘Isaaq’, and ‘Smeralda’) using six extraction methods (water control, pectinase, two methods using ethanol, methanol and methanol + acetone), and secondly to provide the intercorrelations between the parameters of antioxidant capacity and polyphenol contents of the four resistant apple cultivars under the six extraction methods.

## 2. Materials and Methods

### 2.1. Orchard Site and Sampled Apple Cultivars

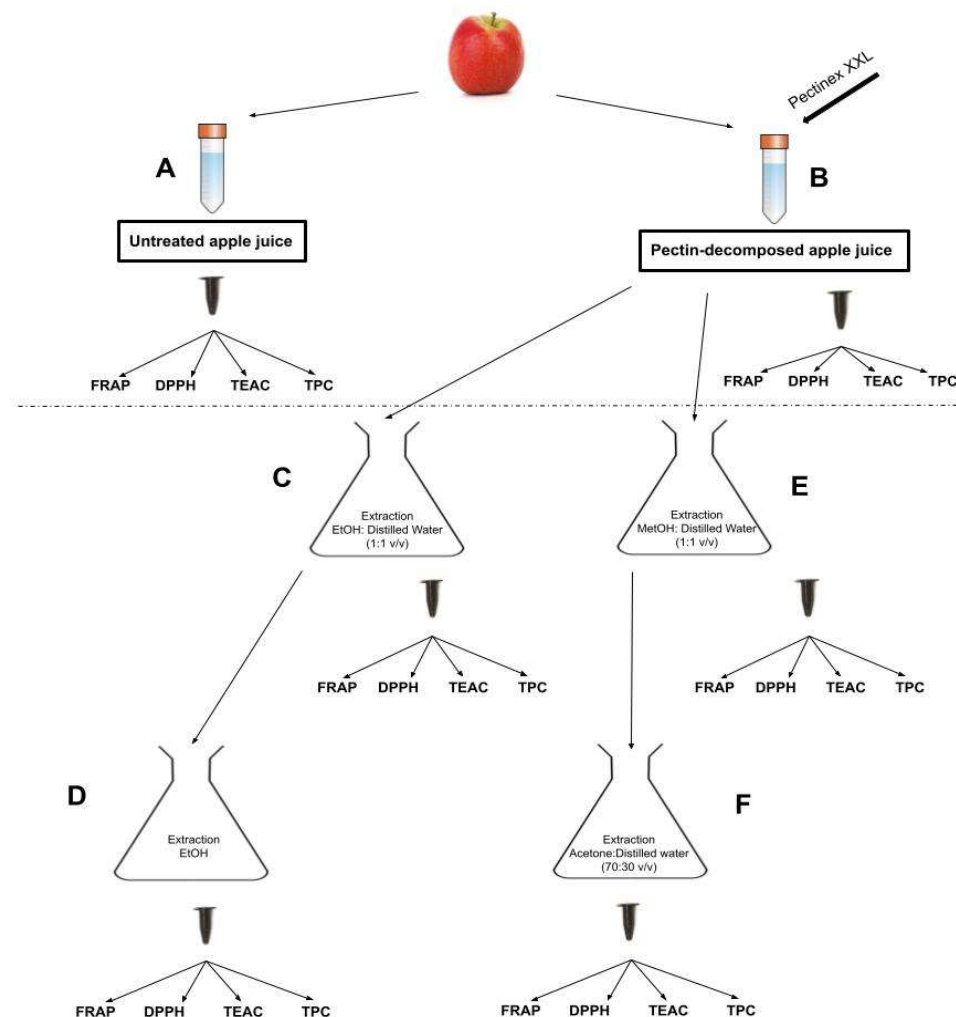
The sampled orchard was established at Derecske in Eastern Hungary, Eastern Europe. The orchard soil type was sandy soil with acidic clay [35]. The orchard was established with

a mixture of apple cultivars. Trees were planted in M9 rootstocks in 2014 and maintained with a slender spindle training system (3000 tree ha<sup>-1</sup>). The orchard has been maintained since 2014 according to the Integrated Fruit Production guidelines.

Four apple cultivars ('Gaia', 'Isaac', 'Smeralda', and 'Fujion') were sampled from the orchard in 2018 at harvest time. All of the four cultivars are resistant to apple scab and cvs. 'Gaia', 'Smeralda', and 'Fujion' are the members of the 'Sweet Resistant' line released by the Consorzio Italiano Vivaisti [29]. For each cultivar, five trees were selected as an experimental unit (4 cultivars × 5 trees). The experimental unit was replicated four times (4 replicates × 4 cultivars × 5 trees). One apple fruit was collected from each of the five selected trees (4 replicates × 4 cultivars × 5 fruits).

## 2.2. Sample Preparation and Extraction Methods

After removal of the core of the fruit, samples were homogenized by grinding in the laboratory and then filled into 50 mL centrifuge tubes (Falcon, VWR, Budapest, Hungary). Then, six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) were prepared, as summarized in Figure 1.



**Figure 1.** Schematic illustration of the preparation of six extraction methods used for determining the total polyphenol content (TPC) and antioxidant content measured by FRAP, DPPH, and TEAC methods. The six extraction methods are (A) Control: untreated apple juice, (B) Pectinase: pectin-decomposed apple juice, (C) EtOH-1: extraction with EtOH: distilled water (1:1 v/v), (D) EtOH-2: extraction with EtOH after EtOH-1, (E) MetOH: extraction with MetOH:distilled water (1:1 v/v), and (F) MetOH + Acetone: extraction with MetOH:distilled water (1:1 v/v) and then extraction with acetone:distilled water (70:30 v/v).

### 2.2.1. Enzymatic Hydrolysis with Pectinase

In the pectinase extraction method (Pectinase), 10 wt. % pectinase solution (Pectinex XXL enzyme, Life Extension Europe B.V., Amsterdam, The Netherlands) was added to the samples in order to obtain a pectin-decomposed apple juice. Samples then were incubated at room temperature for 1 h. Enzymatic hydrolysis was stopped by placing the sample centrifuge tubes in a water bath for 5 min at 100 °C. Homogenized pectin-decomposed apples were transferred to 15 mL centrifuge tubes in order to prepare further extraction methods of ethanol, methanol, and acetone (Figure 1). Each extraction was performed in triplicate.

### 2.2.2. Ethanol Extraction Methods

In extraction 1 (EtOH-1), 5 g of pectin-decomposed samples was added to 5 mL of 96% EtOH:H<sub>2</sub>O (1:1 *v/v*) and placed in an ultrasonic bath for 1 h. Then, the samples were centrifuged at 25 °C, for 10 min, at 4500 rpm (Eppendorf Centrifuge 5810R, OpticsPlanet, Inc., Northbrook, IL, USA), and the supernatants were collected. Each extraction was performed in triplicate.

In extraction 2 (EtOH-2), 5 mL of 96% EtOH was added to each sample residue obtained from extraction 1 (EtOH-1). Then, the solution was mixed with a Vortex shaker. After 1 h of incubation, sample tubes were centrifuged again for 10 min, at 25 °C, at 4500 rpm, and the supernatants were then collected. Each extraction was performed in triplicate.

### 2.2.3. Methanol and Methanol + Acetone Extraction Methods

The extractions were prepared according to Saura-Calixto and Goñi [36]. In the methanol extraction method (MetOH), 5 mL of MetOH:H<sub>2</sub>O (1:1 *v/v*) was added to 5 g of pectin-decomposed samples and placed in an ultrasonic bath for 1 h. The supernatant was then obtained as described for the EtOH-1 extractions. In the methanol + acetone extraction methods (MetOH + Acetone), the remaining sample residue from MetOH was mixed with 5 mL of acetone: H<sub>2</sub>O (70:30 *v/v*), using a Vortex shaker, and then the supernatant was collected as described for the EtOH-1 extractions.

## 2.3. Measures for Polyphenol Content and Antioxidant Capacities

Total polyphenol content (TPC) and antioxidant capacities (measured using FRAP, DPPH, and TEAC) were determined by six extraction methods (Figure 1).

### 2.3.1. Total Polyphenol Content—TPC

Total polyphenol content was determined according to the study of Folin–Ciocalteu [37,38] with a few modifications suggested by Kun-Nemes et al. [34]. The absorbance of the mixtures was determined using a SPECTROstar® Nano device (BMG Labtech, Ortenberg, Germany) at 765 nm. TPC was then expressed as mg gallic acid equivalent (GAE) in 100 g dry matter.

### 2.3.2. Antioxidant Capacity Measured by FRAP

Antioxidant capacity was also determined by the method of Ferric Reducing Ability of Plasma (FRAP) according to the study of Benzie and Strain [39]. The property of antioxidants was based on reducing Fe<sup>3+</sup> (ferric) ions to Fe<sup>2+</sup> (ferrous) ions. The latter form a ferro-tripyridyl triazine (blue complex) with tripyridyl triazine (TPTZ = 2,4,6-tripyridyl-S-triazine), at low pH values. According to the method, prepared mixtures were incubated at 37 °C, and the absorbance was measured after 8 min, at 593 nm. Antioxidant capacity was then expressed as mg ascorbic acid (ASA) equivalent in 100 g dry matter.

### 2.3.3. Antioxidant Capacity Measured by DPPH

Antioxidant capacity by 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazine (DPPH) free radical removal activity was measured according to the

methods of Blois [40] and Brand-Williams et al. [41]. Using a microplate reader, the absorbance was read at 517 nm. DPPH was then expressed as mg Trolox equivalent (TE) in 100 g dry matter.

#### 2.3.4. Antioxidant Capacity Measured by TEAC

Determination of Trolox Equivalent Antioxidant Capacity (TEAC) was performed using the dark green ABTS + cation radical forms, which reacts with the antioxidants contained in the sample. This reaction result in a decrease in the color intensity and fading of the sample according to the study of Stratil et al. [42]. In this study, we used this method with slight modifications as suggested by Kun-Nemes et al. [34]. After incubation of the mixtures for 30 min and at 25 °C, absorbances were measured at 734 nm. TEAC was then expressed in mg Trolox equivalent (TE) in 100 g dry matter according to the studies of Miller et al. [43], Re et al. [44], and Zulueta et al. [45].

### 2.4. Statistical Analysis

#### 2.4.1. Analyses of Variance—ANOVA

The extraction methods and cultivars were arranged in a randomized experimental design (completely randomized block design—CRBD). The Genstat 5 Release 4.1 (Lawes Agricultural Trust, Rothamsted, UK) program was used for analyses of variance in order to determine the effects of extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) and cultivars ('Gaia', 'Isaaq', 'Smeralda', and 'Fujion') and the two-way interaction of the four measures (TPC, AC-FRAP, AC-DPPH, and AC-TEAC). LSD *t*-tests were then used to show the significant differences of the means among extraction methods and cultivars at  $p = 0.05$  levels.

#### 2.4.2. Correlation and Linear Regression Analyses among Parameters

Relationships among the measures (TPC, AC-FRAP, AC-DPPH, and AC-TEAC) were determined by Pearson's correlation analyses separately for each of the six extraction methods. All possible combinations ( $6 \times 6 = 36$  variable pairs) were used for the analyses. Correlation coefficients were obtained separately for each extraction method using Genstat 5 Release 4.1 (Lawes Agricultural Trust, Rothamsted, UK). Then, linear regression analyses (using the equation of  $f(x) = ax + b$ ) were applied to the best-correlated variable pairs. Then, regression slopes were tested using a *t*-test in order to quantify the significant differences among the extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) at  $p = 0.05$ .

## 3. Results

### 3.1. Total Polyphenol Content (TPC)

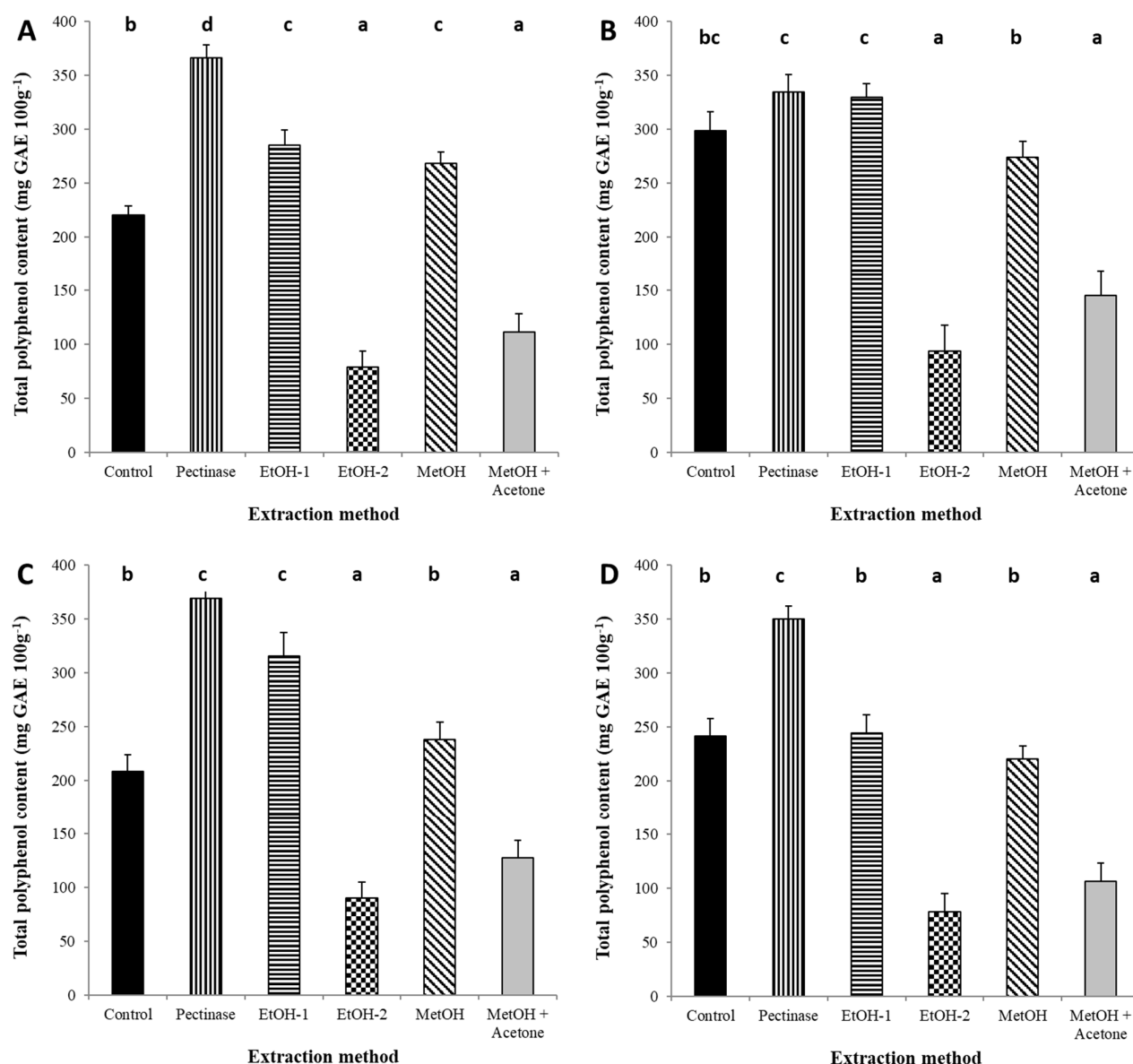
ANOVA showed a significant effect for extraction methods and cultivars at  $p < 0.05$  on the TPC dataset. The two-way interaction was non-significant (Table 1). According to ANOVA, data are presented separately for cultivars and extraction methods (Figure 2).

**Table 1.** Analysis of variance for the effects of extraction method and cultivar on TPC, AC-FRAP, AC-DPPH, and AC-TEAC using six extraction methods and four resistant apple cultivars 'Gaia', 'Isaaq', 'Smeralda', and 'Fujion'. TPC: total polyphenol content, AC-FRAP: antioxidant capacity measured with FRAP method, AC-DPPH: antioxidant capacity measured with DPPH method and AC-TEAC: antioxidant capacity measured with TEAC method.

Variance Sources	df	TPC		AC-FRAP		AC-DPPH		AC-TEAC	
		MS	<i>p</i>	MS	<i>p</i>	MS	<i>p</i>	MS	<i>p</i>
Extraction method (E)	5	2548.8	0.0491	764.2	0.0111	348.5	0.0047	53.4	0.0441
Cultivar (C)	3	42,071	<0.001	1733.4	<0.001	1184.6	<0.001	268.6	<0.001
E × C	15	541.9	0.3528	145.2	0.1831	52.9	0.1063	15.5	0.3148
Error	18	8129.3		2177.6		793.3		3824.1	
Total	23								

df: degrees of freedom. *p*: the probability values associated with the F-tests. MS: mean squares.





E	Control	Pectinase	EtOH-1	EtOH-2	MetOH	MetOH + Acetone
Fujion	a	a	a	a	b	a
Gaia	b	a	b	b	b	c
Isaaq	a	a	b	b	a	b
Smeralda	a	a	a	a	a	a
LSD <sub>0.05</sub>	42.1	ns	43.5	8.3	26.4	14.8

**Figure 2.** The effect of six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) on the values of total polyphenol content (TPC) for four resistant apple cultivars: ‘Fujion’ (A), ‘Gaia’ (B), ‘Isaaq’ (C), and ‘Smeralda’ (D). Data are means of three replicates. For each cultivar, differences among the six extraction methods were based on LSD<sub>0.05</sub> values at  $p = 0.05$ , as LSD<sub>0.05</sub> = 46.5 for cv. ‘Fujion’ (A), 52.5 for cv. ‘Gaia’ (B), 55.6 for cv. ‘Isaaq’ (C), and 44.2 for cv. ‘Smeralda’ (D). In figure (A–D), different letters above each column are significantly different among the extraction methods separated for each cultivar. On the columns, bars indicate standard deviation (SD) values. (E) represents the statistical differences of the TPC values of cultivars among the six extraction methods at  $p = 0.05$ . In figure (E), different letters for each extraction method are significantly different among cultivars ‘Fujion’, ‘Gaia’, ‘Isaaq’, and ‘Smeralda’ at  $p = 0.05$  according to LSD  $t$ -tests. Explanations for Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone are given in Figure 1.

The Pectinase extraction method showed the highest TPC value (369.2 mg GAE 100 g<sup>-1</sup>) for cv. 'Isaaq', whereas the lowest TPC value was 78.2 mg GAE 100 g<sup>-1</sup> in the EtOH-2 extraction method for cv. 'Smeralda' (Figure 2A–D).

Each of the four cultivars showed significantly lower mean TPC values in the methods of EtOH-2 and MetOH + Acetone than the control method at  $p < 0.05$  (Figure 2A–D) when extraction methods were compared. In the case of cv. 'Fujion', TPC values in the methods of Pectinase, EtOH-1, and MetOH were significantly higher at  $p < 0.05$  than the control method (Figure 2A), whereas in the case of cv. 'Gaia', TPC values in the methods of Pectinase, EtOH-1, and MetOH were not significantly different from the control method (Figure 2B). In the case of cvs. 'Isaaq', TPC values in the methods of Pectinase and EtOH-1 were significantly higher at  $p < 0.05$  than in the control method, whereas the MetOH method was not significantly different from the control method (Figure 2C). In the case of cv. 'Smeralda', TPC values in the method of Pectinase were significantly higher at  $p < 0.05$  compared to the control method, whereas the methods of EtOH-1 and MetOH were not significantly different from the control method (Figure 2D).

TPC values in the control method were significantly higher in cv. 'Gaia' than the those of the other three cultivars at  $p < 0.05$  when cultivars were compared. TPC values in the Pectinase method were non-significant among the four cultivars. In the case of cvs. 'Fujion' and 'Smeralda', the TPC values in the methods of EtOH-1 and EtOH-2 were significantly different from those of cvs. 'Gaia' and 'Isaaq' at  $p < 0.05$ . The TPC values of the method of MetOH were non-significant for cvs. 'Fujion' and 'Gaia', whereas these values were significantly different from the TPC values of cvs. 'Isaaq' and 'Smeralda' at  $p < 0.05$ . The TPC values of the method MetOH + Acetone were significantly different among cvs. 'Smeralda', 'Isaaq', and 'Gaia' at  $p < 0.05$ , whereas the TPC values of the method MetOH + Acetone were non-significantly different between cvs. 'Fujion' and 'Smeralda'.

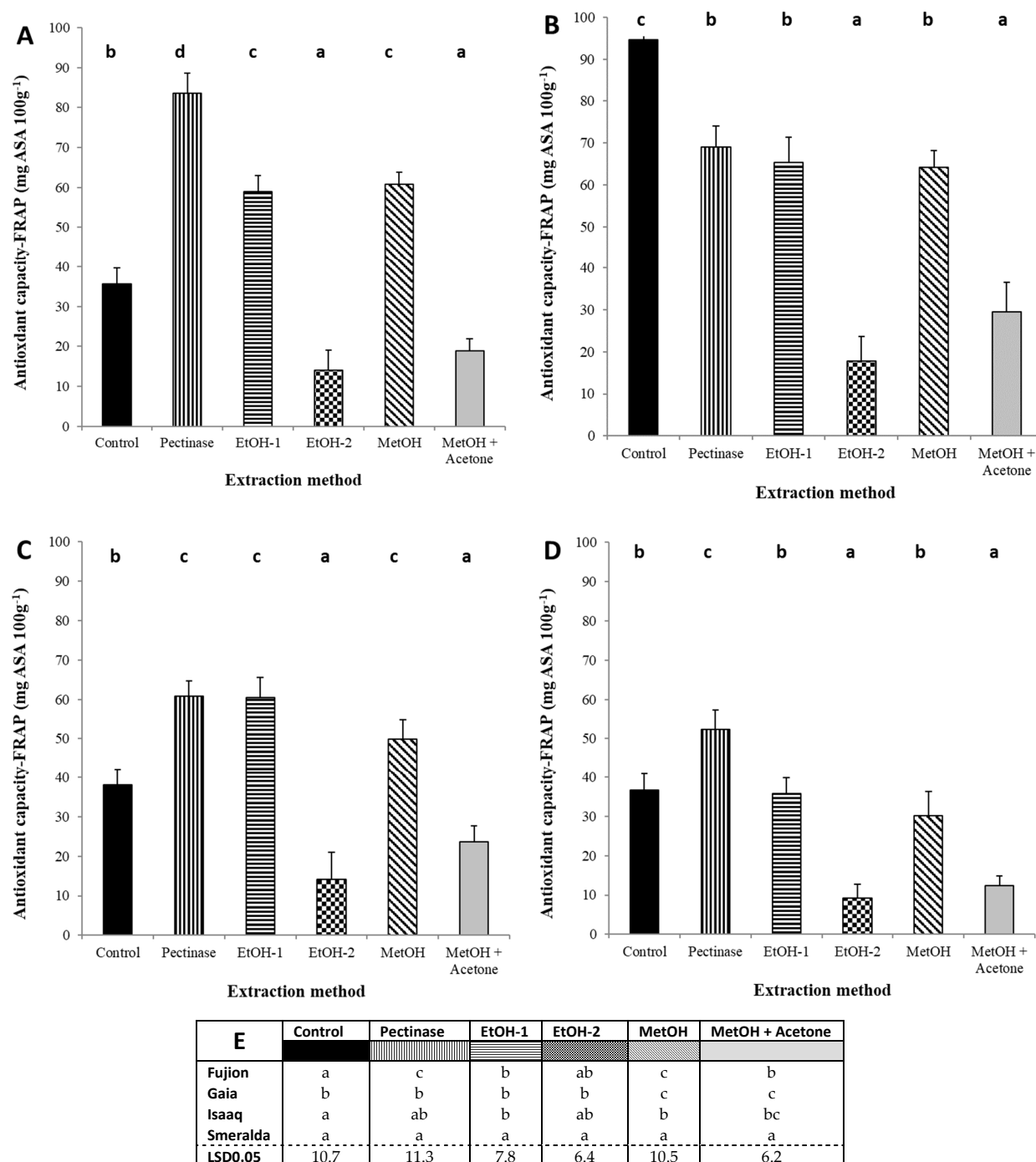
### 3.2. Antioxidant Capacity Measured with Ferric Reducing Ability of Plasma (AC-FRAP)

There was a significant effect of extraction methods and cultivars at  $p < 0.05$  according to the ANOVA prepared on the AC-FRAP dataset. The two-way interaction between the extraction method and cultivar was non-significant (Table 1). According to ANOVA, data are presented separately for cultivars and extraction methods (Figure 3).

The highest AC-FRAP value was 94.7 mg ASA 100 g<sup>-1</sup> in the control extraction method for cv. 'Gaia', whereas the lowest was 9.28 mg GAE 100 g<sup>-1</sup> in the EtOH-2 extraction method for cv. 'Smeralda' (Figure 3A–D). The EtOH-2 extraction method showed the lowest AC-FRAP value for all the four resistant apple cultivars (Figure 3A–D).

Comparing the extraction methods, all of the four cultivars showed significantly lower mean AC-FRAP values in the methods of EtOH-2 and MetOH + Acetone compared to the control method at  $p < 0.05$  (Figure 3A–D). In case of cvs. 'Fujion' and 'Isaaq', AC-FRAP values in the methods of Pectinase, EtOH-1, and MetOH were significantly higher than the those of control method at  $p < 0.05$  (Figure 3A,C). In the case of cv. 'Gaia', AC-FRAP values in all extraction methods were significantly lower at  $p < 0.05$  than those of the control method (Figure 3B). In the case of cv. 'Smeralda', AC-FRAP values in the method of Pectinase were significantly higher at  $p < 0.05$  than those of the control method, whereas the methods of EtOH-1 and MetOH were not significantly different from the control method (Figure 3D).

Comparing the cultivars, AC-FRAP values in the control for extraction method were significantly higher for cv. 'Gaia' than those of the other three cultivars at  $p < 0.05$ . AC-FRAP values in the Pectinase method were significantly higher for cvs. 'Fujion' and 'Gaia' compared to cv. 'Smeralda'. In the case of cvs. 'Fujion', 'Gaia', and 'Isaaq', the AC-FRAP values were significantly higher in the methods of EtOH-1, MetOH, and MetOH + Acetone than for cv. 'Smeralda'. The AC-FRAP values of cv. 'Gaia' were significantly higher in the method of EtOH-2 than for cv. 'Smeralda'.



**Figure 3.** The effect of six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) on the values of antioxidant capacity measured with the FRAP method (AC-FRAP) for four resistant apple cultivars: ‘Fujion’ (A), ‘Gaia’ (B), ‘Isaaq’ (C), and ‘Smeralda’ (D). Data are means of three replicates. For each cultivar, differences among the six extraction methods were based on  $LSD_{0.05}$  values at  $p = 0.05$ , as  $LSD_{0.05} = 10.5$  for cv. ‘Fujion’ (A),  $11.9$  for cv. ‘Gaia’ (B),  $9.8$  for cv. ‘Isaaq’ (C), and  $6.7$  for cv. ‘Smeralda’ (D). In figure (A–D), different letters above each column are significantly different among the extraction methods separated for each cultivar. On the columns, bars indicate standard deviation (SD) values. Figure (E) represents the statistical differences of the AC-FRAP values of cultivars among the six extraction methods at  $p = 0.05$ . In figure (E), different letters for each extraction method are significantly different among cultivars ‘Fujion’, ‘Gaia’, ‘Isaaq’, and ‘Smeralda’ at  $p = 0.05$  according to  $LSD$   $t$ -tests. Explanations for Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone are given in Figure 1.



### 3.3. Antioxidant Capacity Measured with DPPH (AC-DPPH)

Extraction methods and cultivars showed a significant effect at  $p < 0.05$  according to ANOVA, prepared on the AC-DPPH dataset. The two-way interaction between extraction method and cultivar was non-significant (Table 1). According to ANOVA, data are presented separately for cultivars and extraction methods (Figure 4).

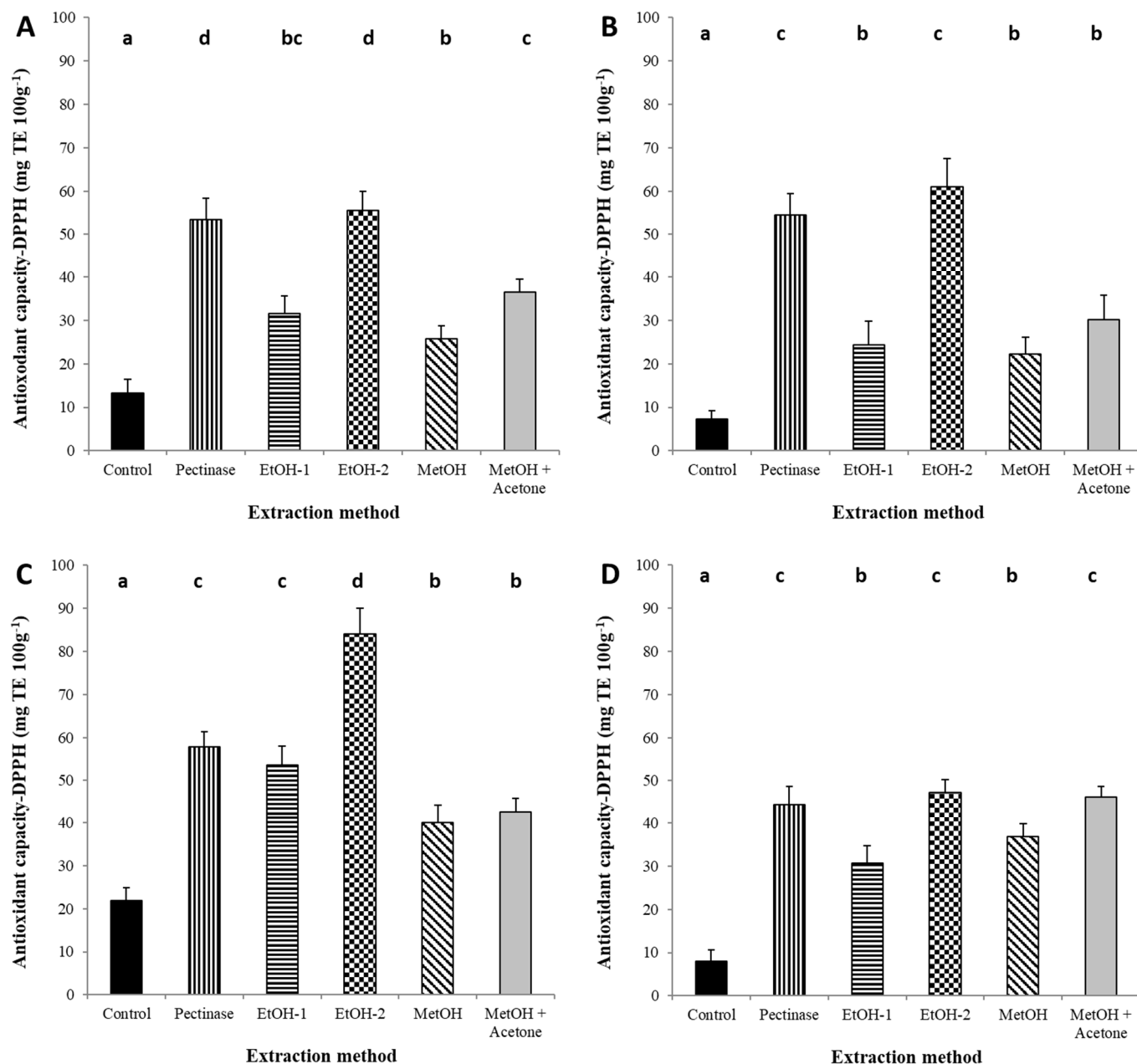


Figure 4. Cont.

E	Control	Pectinase	EtOH-1	EtOH-2	MetOH	MetOH + Acetone
Fujion	b	a	a	ab	a	ab
Gaia	a	a	a	b	a	a
Isaaq	c	a	b	c	b	bc
Smeralda	ab	a	b	a	b	c
LSD0.05	6.2	ns	7.4	8.5	6.1	7.1

**Figure 4.** The effect of six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) on the values of antioxidant capacity measured with the DPPH method (AC-DPPH) for four resistant apple cultivars: ‘Fujion’ (A), ‘Gaia’ (B), ‘Isaaq’ (C), and ‘Smeralda’ (D). Data are means of three replicates. For each cultivar, differences among the six extraction methods were based on  $LSD_{0.05}$  values at  $p = 0.05$ , as  $LSD_{0.05} = 7.5$  for cv. ‘Fujion’ (A), 8.1 for cv. ‘Gaia’ (B), 8.8 for cv. ‘Isaaq’ (C), and 6.2 for cv. ‘Smeralda’ (D). In figure (A–D), different letters above each column are significantly different among the extraction methods separated for each cultivar. On the columns, bars indicate standard deviation (SD) values. Figure (E) represents the statistical differences of the AC-DPPH values of cultivars among the six extraction methods at  $p = 0.05$ . In figure (E), different letters for each extraction method are significantly different among cultivars ‘Fujion’, ‘Gaia’, ‘Isaaq’, and ‘Smeralda’ at  $p = 0.05$  according to LSD  $t$ -tests. Explanations for Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone are given in Figure 1.

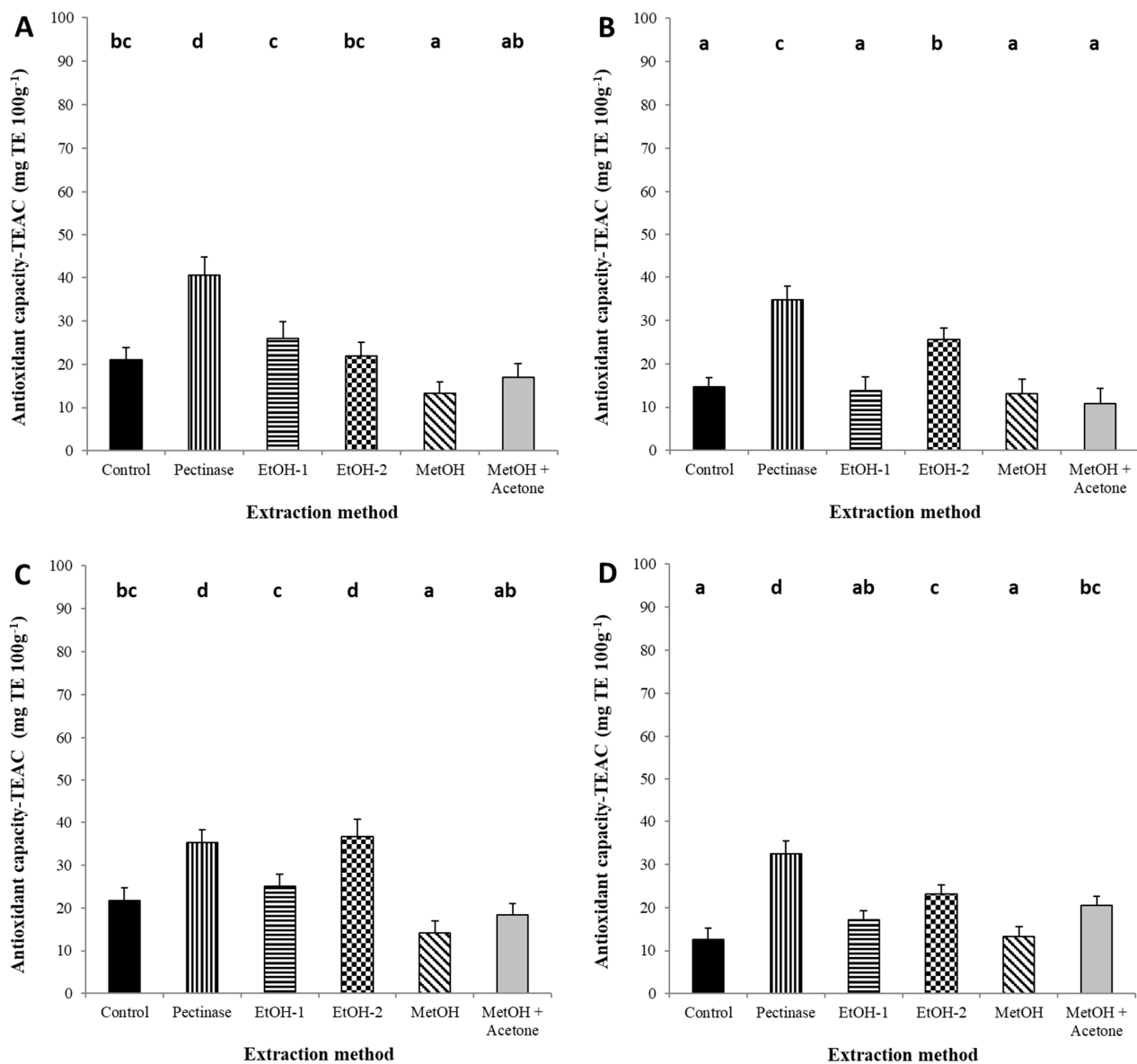
The lowest AC-DPPH value was  $7.23 \text{ mg TE } 100 \text{ g}^{-1}$  in the control extraction method for cv. ‘Gaia’, whereas the highest value was  $84.1 \text{ mg TE } 100 \text{ g}^{-1}$  in the EtOH-2 extraction method for cv. ‘Isaaq’ (Figure 4A–D). The EtOH-2 extraction method showed the highest, whereas the control showed the lowest, AC-DPPH values for all of the four resistant apple cultivars (Figure 4A–D).

All cultivars showed significantly higher mean AC-DPPH values in all extraction methods compared to the control method at  $p < 0.05$  (Figure 4A–D). In the case of cv. ‘Fujion’ and ‘Gaia’, AC-DPPH values in the methods of Pectinase and EtOH-2 were significantly higher at  $p < 0.05$  than those of the extraction methods of EtOH-1, MetOH, and MetOH + Acetone (Figure 4A,B). In the case of cvs. ‘Isaaq’ and ‘Smeralda’, AC-DPPH values in the methods of Pectinase, EtOH-1, and EtOH-2 were not significantly higher than in the methods of MetOH and MetOH + Acetone (Figure 4C,D).

When cultivars were compared, AC-DPPH values in the control method were significantly higher for cvs. ‘Fujion’ and ‘Isaaq’ than cv. ‘Gaia’ at  $p < 0.05$  (Figure 4E). The AC-DPPH value in the Pectinase extraction method was non-significant for the four cultivars (Figure 4E). The AC-DPPH values of the method of EtOH-1 were significantly lower for cvs. ‘Fujion’ and ‘Gaia’ compared to the values of cv. ‘Isaaq’ and ‘Smeralda’ (Figure 4E). The AC-DPPH values of the method of EtOH-2 for cvs. ‘Gaia’ and ‘Isaaq’ were significantly higher than the values of cv. ‘Smeralda’ (Figure 4E). The AC-DPPH values of cvs. ‘Fujion’ and ‘Gaia’ for the method MetOH were significantly lower at  $p < 0.05$  than values of cvs. ‘Isaaq’ and ‘Smeralda’. The AC-DPPH values of cv. ‘Gaia’ for the method MetOH + Acetone were significantly lower at  $p < 0.05$  compared to values of cvs. ‘Isaaq’ and ‘Smeralda’ (Figure 4E).

### 3.4. Antioxidant Capacity Measured with TEAC (AC-TEAC)

The AC-TEAC dataset showed a significant effect for extraction methods and cultivars at  $p < 0.05$  according to ANOVA. The two-way interaction between extraction method and cultivar was non-significant (Table 1). According to ANOVA, data are presented separately for cultivars and extraction methods (Figure 5).



E	Control	Pectinase	EtOH-1	EtOH-2	MetOH	MetOH + Acetone
Fujion	b	a	a	a	a	ab
Gaia	a	a	b	a	a	a
Isaaq	b	a	a	b	a	b
Smeralda	a	a	a	a	a	b
LSD0.05	5.2	8.1	6.5	5.7	ns	6.3

**Figure 5.** The effect of six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) on the values of antioxidant capacity measured with TEAC method (AC-TEAC) for four resistant apple cultivars: ‘Fujion’ (A), ‘Gaia’ (B), ‘Isaaq’ (C), and ‘Smeralda’ (D). Data are means of three replicates. For each cultivar, differences among the six extraction methods were based on LSD0.05 values at  $p = 0.05$ , as LSD0.05 = 6.5 for cv. ‘Fujion’ (A), 6.1 for cv. ‘Gaia’ (B), 5.8 for cv. ‘Isaaq’ (C), and 5.2 for cv. ‘Smeralda’ (D). In figure (A–D), different letters above each column are significantly different among the extraction methods separated for each cultivar. On the columns, bars indicate standard deviation (SD) values. Figure (E) represents the statistical differences of the AC-TEAC values of cultivars among the six extraction methods at  $p = 0.05$ . In figure (E), different letters for each extraction method are significantly different among cultivars ‘Fujion’, ‘Gaia’, ‘Isaaq’, and ‘Smeralda’ at  $p = 0.05$  according to LSD  $t$ -tests. Explanations for Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone are given in Figure 1.

The AC-TEAC value was the lowest (10.8 mg TE 100 g<sup>-1</sup>) in the MetOH + Acetone extraction method for cv. 'Gaia', whereas the highest was (40.5 mg TE 100 g<sup>-1</sup>) in the Pectinase extraction method for cv. 'Fujion' (Figure 5A–D).

In the case of cvs. 'Fujion' and 'Isaaq', AC-TEAC values in the method of Pectinase were significantly higher, whereas in the extraction method of MetOH they were lower at  $p < 0.05$  than in the control method (Figure 5A,C). In the case of cv. 'Gaia', AC-TEAC values in the methods of Pectinase and EtOH-2 were significantly higher, whereas all other methods were non-significant compared to the control method (Figure 5B). In the case of cv. 'Smeralda', AC-TEAC values in the methods of Pectinase, EtOH-2, and MetOH + Acetone were significantly higher at  $p < 0.05$  than those of the control method (Figure 5D).

In cvs. 'Fujion' and 'Isaaq', AC-TEAC values were significantly higher in the control method compared to the other two cultivars (Figure 5E). AC-TEAC values in the methods of Pectinase and MetOH were non-significant among the four cultivars. The AC-TEAC values of cv. 'Gaia' in the methods of EtOH-1 were significantly higher compared to the other three cultivars.

The AC-TEAC values of cv. 'Isaaq' in the method of EtOH-2 were significantly higher than the other three cultivars at  $p < 0.05$ . The AC-TEAC values of cv. 'Gaia' in the method MetOH + Acetone were significantly lower than those of cvs. 'Isaaq' and 'Smeralda' at  $p < 0.05$  (Figure 5E).

### 3.5. Relationship among Parameters

#### 3.5.1. Pearson Correlation Analyses

When the dataset of  $6 \times 6$  pair-variables was analyzed for each extraction method, 4, 2, 2, 3, 4 and 4 pair-variables (a total of 19) correlated significantly at  $p = 0.05$  level in the Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone extraction treatments, respectively (Table 2). Among these 19 significant pair-variables, six were correlated negatively and 13 were correlated positively (Table 2).

Among the 19 significant pair-variables, two pair-variables were significant in all of the six extraction methods. These two pair-variables were TPC vs. AC-FRAP and AC-TEAC vs. AC-DPPH, which were correlated positively, indicating a strong connection among the four measurements related to extraction methods (Table 2).

**Table 2.** Pearson's correlation coefficients (r) among 4 measures for six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) on four resistant apple cultivars 'Fujion', 'Gaia', 'Isaaq', and 'Smeralda'. Significant ( $p < 0.05$ ) correlation coefficient values are presented with bold figures in each extraction method. TPC: total polyphenol content, AC-FRAP: antioxidant capacity measured with FRAP method, AC-DPPH: antioxidant capacity measured with DPPH method and AC-TEAC: antioxidant capacity measured with TEAC method. Data were combined for three replicates and cultivars. Explanations for Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone are given in Figure 1.

Control	TPC	AC-FRAP	AC-DPPH
AC-FRAP	<b>0.9330</b>		
AC-DPPH	<b>−0.7808</b>	−0.5136	
AC-TEAC	<b>−0.6862</b>	−0.4063	<b>0.8639</b>
Pectinase	TPC	AC-FRAP	AC-DPPH
AC-FRAP	0.9439		
AC-DPPH	0.3055	0.4507	
AC-TEAC	0.5054	0.1979	0.6575
EtOH-1	TPC	AC-FRAP	AC-DPPH
AC-FRAP	<b>0.9434</b>		
AC-DPPH	0.1818	0.1188	
AC-TEAC	−0.0330	0.1733	<b>0.6597</b>

Table 2. Cont.

Control	TPC	AC-FRAP	AC-DPPH
EtOH-2	TPC	AC-FRAP	AC-DPPH
AC-FRAP	0.8033		
AC-DPPH	0.6667	0.4113	
AC-TEAC	0.5994	0.1854	0.9523
MetOH	TPC	AC-FRAP	AC-DPPH
AC-FRAP	0.9665		
AC-DPPH	−0.9022	−0.7637	
AC-TEAC	−0.4171	−0.1711	0.7658
MetOH + Acetone	TPC	AC-FRAP	AC-DPPH
AC-FRAP	0.9667		
AC-DPPH	−0.6259	−0.64966	
AC-TEAC	−0.8596	−0.8671	0.9608

### 3.5.2. Linear Regression Analyses

The two significant pair-variables are further presented using linear regression analysis, which shows the data of the six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone) with separated symbols (Figure 6). Significant linear relationships were found for the pair-variable of AC-FRAP vs. TPC and AC-DPPH vs. AC-TEAC with  $r$  values of 0.656 and 0.732, and with  $p$  values of 0.048 and 0.025, respectively.

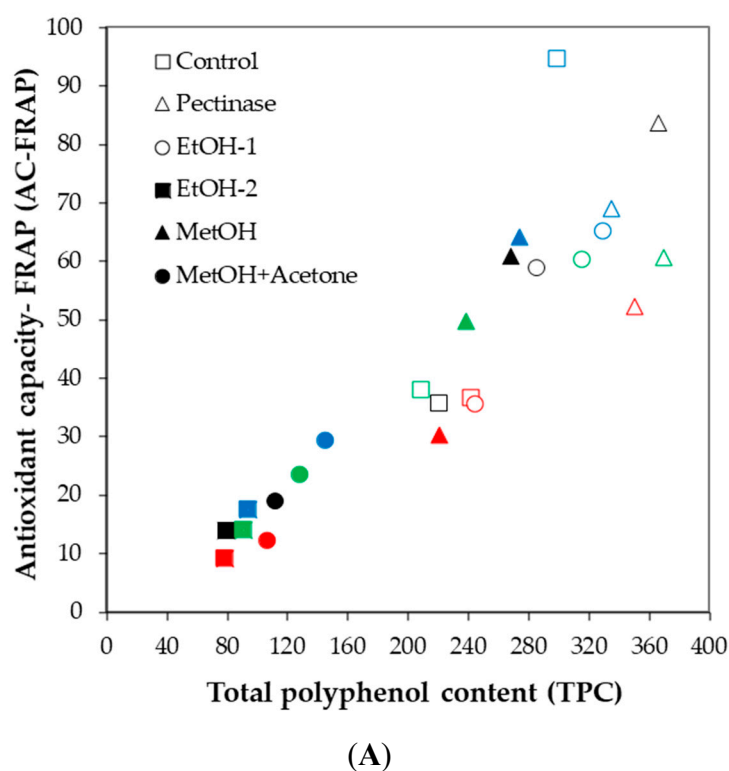
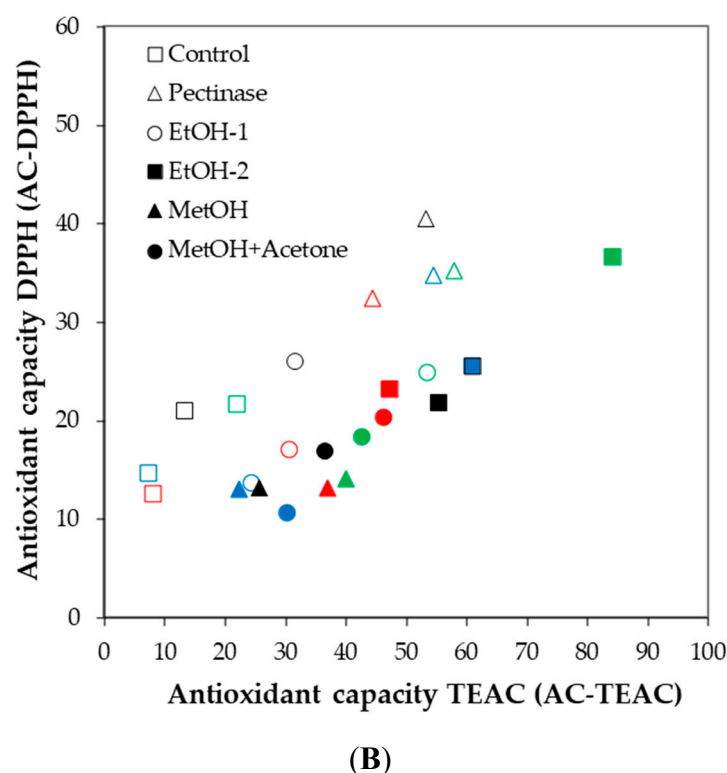


Figure 6. Cont.





**Figure 6.** Relationships between 2 significant variable-pairs TPC vs. AC-FRAP (A) and AC-TEAC vs. AC-DPPH (B) for six extraction methods (Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone). Data represent the combination of three replicates. TPC: total polyphenol content, AC-FRAP: antioxidant capacity measured with FRAP method, AC-DPPH: antioxidant capacity measured with DPPH method, and AC-TEAC: antioxidant capacity measured with TEAC method. Explanations for Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone are given in Figure 1. Different colors of symbols refer to cultivars: black: cv. ‘Fujion’, blue: cv. ‘Gaia’, green: cv. ‘Isaaq’, and red: cv. ‘Smeralda’.

However, the slope parameters for the pair-variables showed no differences among the Control, Pectinase, EtOH-1, EtOH-2, MetOH, and MetOH + Acetone extraction methods, and *t*-tests showed *p* values of 0.147 and 0.069 (Figure 6).

#### 4. Discussion

This study demonstrated that values of TPC and three ACs were significantly different among six extraction methods, and the extraction method of pectinase generally showed the highest values of total polyphenol content, whereas EtOH-2 (an ethanol solvent including a second phase ethanol treatment) showed the lowest values of the parameter for all cultivars (Table 1, Figure 2). In solid–liquid extraction methods, solvents of ethanol, methanol, and acetone are widely applied extraction techniques, e.g., [46]; however, results of previous studies widely differ in the efficacy of extraction methods on the polyphenol content and antioxidant capacity of fruits, e.g., [18,46–49]. For instance, the efficiency of water, methanol, and acetone solvents was shown to be significant for the extraction of the soluble polyphenolic compounds [46]. In addition, previous studies aimed to optimize the yield of extracted polyphenolic compounds; for instance, Zardo et al. [47] investigated the ratio of the solvents of ethanol, acetone, and methanol to enhance the extraction of polyphenolic compounds from fruit samples. The study of Wijngaard and Brunton [48] optimized the gained TPC in the extraction methods by acetone and ethanol; they suggested that these two solvents were more preferable than methanol for polyphenolic extraction. The good extraction efficiency of a water–ethanol mixture can be explained because ethanol may weaken the bonds between polyphenolics–protein and polyphenolics–cellulose [49]. In

addition, the study of Hobbi et al. [18] showed that the highest total polyphenol content was measured in a mixture of 65:35 acetone:water (*v/v*) at 60 °C compared to other unmixed solvents (i.e., water, acetone, and ethanol). Our study confirmed a good yield of polyphenol content using the pectinase extraction method, and using the solvents of ethanol and methanol used alone (Figure 2).

TPC values were higher than values of AC-FRAP, AC-DPPH, and AC-TEAC in this study. In addition, values of AC-FRAP and AC-DPPH were generally higher compared to values of AC-TEAC for all extraction methods (Figures 2–5). Several previous studies used FRAP, DPPH, and TAEC methods for determining the antioxidant capacity of plant samples, e.g., [15–18]; however, a standard methodology was not provided, and different extraction methods were applied to measure the antioxidant capacity of apple samples, e.g., [15,16]. In the case of apple, Hobbi et al. [18] determined the antioxidant activity of apple samples based on DPPH methods using acetone, water, and ethanol solvents. The results showed that the radical scavenging activity of DPPH was increased in the order of water extraction < ethanol:water < acetone:water [18]. In the study of Bai et al. [17], the radical scavenging activity of DPPH was 1.4 times more in an acetonic extract than in a methanolic extract. In the case of the FRAP method, Khanizadeh et al. [13] demonstrated that apple fruit samples had high antioxidant activity among various apple cultivars. The study of Tsao et al. [10] found significant correlations between the values of polyphenol content and TEAC, and the study of Gliszczynska-Swiglo and Tyrakowska [50] demonstrated a significant relationship between the values of TPC and ferric-reducing antioxidant power. In addition, our study showed strong intercorrelations among the measured parameters, as significant relationships were found between the pair-variables of AC-DPPH and AC-TEAC, and between TPC and AC-FRAP, in all extraction methods, confirmed by correlation and regression analyses (Table 2 and Figure 6).

The amounts of total polyphenol content and antioxidant capacity differed among resistant apple cultivars and also among extraction methods (Figures 2–5). Several previous studies demonstrated that apple cultivars differed in their total polyphenol content or antioxidant capacity, e.g., [12,13,16,19,22,51–63]. For instance, the study of Vieira et al. [16] showed that the total phenolic content ranged between 128.3 and 212.0 mg GAE 100 g<sup>−1</sup> dry matter among the investigated apple cultivars. Some previous studies also showed that cultivars play major roles in the potential polyphenol content of apple, e.g., [13,54–56]; and therefore, a cultivar has a great influence on the amount of total content of flavanols, phenolics, and anthocyanins, and the antioxidant capacity, e.g., [12,16,57,58]. Vrhovsek et al. [64] and Kschonsek et al. [62] showed that the polyphenol content of the apple flesh in the old apple cultivars was up to 30% higher than in the newly bred cultivars. However, previous studies also demonstrated that the contribution of polyphenols to the total antioxidant efficiency of apples can range between 45 and 80%, depending on cultivars, e.g., [55]. For scab-resistant cultivars, previous studies showed a positive correlation between the antioxidant content of fruit flesh and resistance of apple cultivars to apple scab, e.g., [21–23,59]. This was also confirmed in this study (Figures 2–5). In addition, this study was the first to show that antioxidant capacity of cultivars differed among extraction methods, which was not previously demonstrated for the four selected resistant apple cultivars. However, as many previous studies also noted, the chemical compositions of an apple cultivar may also be dependent on fruit maturity, growing area, agricultural and management practices, storage conditions, and environmental factors, e.g., [9,60,65].

## 5. Conclusions

This study found differences among six extraction methods for polyphenol content and antioxidant capacity of four scab-resistant apple cultivars and the following conclusions were drawn:

- TPC values were higher than the values of AC-FRAP, AC-DPPH, and AC-TEAC. In addition, values of AC-FRAP and AC-DPPH were generally higher than values of AC-

TEAC for all extraction methods. Therefore, the methods used for TPC determination of resistant apples can be recommended over the other three methods.

- Values of TPC, AC-FRAP, AC-DPPH, and AC-TEAC were significantly different among the six extraction methods. Values of TPC were generally the highest in the pectinase method and the lowest in ethanol solvent including a second phase extraction (EtOH-2). Our results suggest that the extraction method using the pectinase enzyme may provide the most stable yield of polyphenol content from samples of apple flesh, as confirmed by the examination of four cultivars.
- This study confirmed that values of TPC, AC-FRAP, AC-DPPH, and AC-TEAC varied among the four resistant cultivars, and demonstrated that the antioxidant capacity of the four cultivars differed among extraction methods. These results suggest that the type of extraction method has to be taken into account when a cultivar is assessed for polyphenol content and/or antioxidant capacity.
- Correlation and regression analyses conducted in this study suggest strong relationships between the pair-variables of TPC vs. AC-FRAP and AC-TEAC vs. AC-DPPH, indicating strong connections among extraction methods and the measured antioxidant capacity values.

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