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Phenolic Profiling of Five Different Australian Grown Apples

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Abstract: Apples (*Malus domestica*) are one of the most widely grown and consumed fruits in the world that contain abundant phenolic compounds that possess remarkable antioxidant potential. The current study characterised phenolic compounds from five different varieties of Australian grown apples (Royal Gala, Pink Lady, Red Delicious, Fuji and Smitten) using LC-ESI-QTOF-MS/MS and quantified through HPLC-PDA. The phenolic content and antioxidant potential were determined using various assays. Red Delicious had the highest total phenolic (121.78 ± 3.45 mg/g fw) and total flavonoid content (101.23 ± 3.75 mg/g fw) among the five apple samples. In LC-ESI-QTOF-MS/MS analysis, a total of 97 different phenolic compounds were characterised in five apple samples, including Royal Gala (37), Pink Lady (54), Red Delicious (17), Fuji (67) and Smitten (46). In the HPLC quantification, phenolic acid (chlorogenic acid, 15.69 ± 0.09 mg/g fw) and flavonoid (quercetin, 18.96 ± 0.08 mg/g fw) were most abundant in Royal Gala. The obtained results highlight the importance of Australian apple varieties as a rich source of functional compounds with potential bioactivity.

Keywords: apple; royal gala; pink lady; red delicious; smitten; fuji; phenolic compounds; antioxidant activity; LC-ESI-QTOF-MS/MS; HPLC

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1. Introduction

Apples (*Malus domestica*) are widely grown and consumed fruits. In 2018, apple production across the globe was 86 million tonnes, mainly from China, America and New Zealand, whereas the apple production in Australia was over 2.6 million tonnes [1]. Apples are usually supplied to the market in the form of fresh fruit or processed products, including dried apples, apple cider, apple juice and sauce [2]. Apples are enriched with bioactives compounds [3], vitamins (water and fat soluble) and minerals like calcium, potassium and phosphorus [4]. These compounds are required by the human body to perform various functions like strengthening of the bones, building muscles, filtering out waste [3], and have positive health benefits against several chronic diseases, including type 2 diabetes, asthma and rheumatoid arthritis [5].

The varieties of apples are due to the difference of agroclimatic regions and zones, cultivation practices, nutritional composition and sensory characteristics [6]. Royal Gala, one of the variety of apples having bright shiny red colour, with stripes ranging from straw yellow to amber orange, has a sensory profile that is sweet, soft, crunchy and slightly acidic [7,8]. Pink Lady is a variety that has been originated from a cross between 'Golden Delicious' and 'Lady Williams', known for its sweet taste, firmness and possesses a scald-free surface [6]. A consumer panel in New Zealand appreciated the Pink Lady variety for its dense flesh, excellent crispness, juiciness, good sugar-acid balance and sweet flavour [9]. The Red Delicious variety when compared to the previous two varieties has a darker crimson red surface with traces of yellow and orange [10]. The physical

characteristics of Red Delicious is an elongated form with a thick peel, grainy and tender with a melting texture, usually exhibiting small but evident humps on the skin surface [11]. While different varieties exhibit different appearances, taste and shapes, apples have one common characteristics, which are the high concentrations of phenolic compounds that exhibit high antioxidant potential [12].

Phenolic compounds are important plant secondary metabolites which exhibit excellent abilities to reduce and eliminate free radicals thereby providing antioxidant and anti-lipid peroxidation properties [13,14]. The phenolic compounds exhibiting antioxidation potential have made the food and nutrition market interested in phenolic compounds, thus replacing the existing chemical anti-oxidation ingredients in food to increase the nutritional value and health benefits [14]. One of the polyphenol mechanisms is the removal of free radicals by supplying hydrogen atoms or separate electrons from the phenol group and eliminating related enzymes, thereby preventing the production of free radicals and their intermediate products [15]. Additionally, phenolic compounds can react with metal ions to inactivate the Fenton reaction [16]. The antioxidant potential are often determined by using a series of different in vitro spectrophotometric-based assays including the total antioxidant capacity (TAC), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, the ferric reducing ability of plasma (FRAP) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) [17].

Liquid chromatography coupled with mass spectrometry (LC-ESI-QTOF-MS/MS) is an effective tool used for the identification and characterisation of phenolic compounds. High pressure liquid chromatography (HPLC) combined with photodiode array detector (HPLC-PDA) is used for the quantification of the phenolics [18,19]. According to a previous study, few phenolic compounds have been identified in apples through HPLC and LC-ESI-QTOF-MS analysis including flavanols (catechin), dihydrochalcones (chlorogenic acid), phenolic acids and anthocyanins [20].

Although there are many studies that have isolated and identified phenolic compounds in different apples, only a few have focused on Australian grown apples. The novelty of this study will encourage the Australian producers to utilise the low-grade produce of the apples to a better use as it is rich in phenolics, since premature or overripe fruits compromise the quality and do not meet the standards of the supermarkets. Therefore, in the current research we extracted phenolics from five popular varieties of Australian grown apples (Royal Gala, Pink Lady, Red Delicious, Fuji and Smitten) and estimated their antioxidant potential. The outcome of the current research will add adequate information on the phenolics and antioxidant potential of Australian grown apples for their further application in the food, nutraceutical and pharmaceutical industries.

2. Materials and Methods

2.1. Chemicals and Reagents

The chemicals used for the extraction and characterisation were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemicals used for phenolic estimation and antioxidant assays were procured from Sigma-Aldrich (St. Louis, MO, USA) including ferric (III) chloride anhydrous, 50% acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), acetonitrile, catechin, ascorbic acid, vanillin, aluminium chloride hexahydrate, 2,2′-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonate), potassium persulfate and Folin-Ciocalteu´s phenol. The standards for HPLC including protocatechuic acid, epicatechin, gallic acid, epicatechin gallate, caffeic acid, quercetin, chlorogenic acid, *p*-hydroxybenzoic acid and kaempferol were procured from Sigma-Aldrich (Castle Hill, NSW, Australia). Ammonium molybdate and sodium acetate hydrated were procured from Sigma-Aldrich (Castle Hill, NSW, Australia). Moreover, 99% ethanol was procured from Thermo Fisher (Waltham, MA, USA), and 98% sulfuric acid was purchased from RCI Labscan Ltd. (Rongmuang, Thailand).

2.2. Sample Preparation and Extraction

Australian grown apple varieties (Royal Gala, Pink Lady, Red Delicious, Fuji and Smitten) were bought from a local market in Melbourne, VIC, Australia. All the samples were fully matured and ripen before harvested, transported and distributed to the local retailers within 2–3 days using refrigerated trucks. The apple peels were removed by a peeler and the core was separated to obtain the pulp. Subsequently, the pulps were blended into a slurry using a blender. 5 g of slurry samples were macerated in 20 mL of 70% ethanol (*w*/*v*) by slightly modifying the protocol of our earlier published study of Gu et al. [21]. The slurry samples were homogenised to prepare the sample extracts of the apples in a homogeniser at 10,000 rpm for 30 s. The homogenised extract samples were incubated in a shaking incubator at 120 rpm, 4 °C for 12 h. The samples were centrifuged for 15 min at 5000 rpm (4 °C). A syringe filter was used to filter the extracts used for LC-ESI-QTOF-MS/MS and HPLC-PDA studies and the samples were stored at −20 °C for further analysis.

2.3. Estimation of Phenolic Compounds and Antioxidant Assays

The estimation of phenolic compounds present in the samples and their potential antioxidant activities were analysed following our previously published protocols of Tang et al. [22] and Wang et al. [23].

2.3.1. Determination of Total Phenolic Content (TPC)

The spectrophotometric method of Yunfeng et al. [24] was used for the determination of TPC with some modifications. For this, 25 µL of the apple extract with 200 µL water and 25 µL Folin–Ciocalteu reagent solution were added to 96-well plates. The reaction mixture was incubated for 5 min (25 °C). Then, 5 µL of 10% sodium carbonate was added to the reaction mixture and incubated for 60 min in the dark at room temperature. The absorbance of the reaction mixture was measured at 765 nm using spectrophotometer. The standard used was gallic acid (0–200 µg/mL) to construct the standard curve and the values of TPC was expressed in mg of gallic acid equivalent per gram of sample (mg GAE/g of sample) (fw).

2.3.2. Determination of Total Flavonoids Content (TFC)

The Total Flavonoids Content (TFC) was determined by improvising the aluminium protocol described in Rajurkar and Hande [25]. For this, 80 µL of the apple extract with 120 µL of 50 g/L sodium acetate solution and 80 µL of 2% aluminium chloride were added into the 96-well plate subsequently incubate the reaction mixture at 25 °C for 2.5 h. The absorbance was measured at 440 nm. Quercetin calibration curve (0–50 µg/mL) was constructed and TFC was expressed in quercetin equivalent (mg QE/g fw).

2.3.3. Determination of Total Tannin Content (TTC)

The vanillin-sulfuric acid method with some modifications of Mesfin and Won Hee [26] was used to determine TTC. 25 µL of the apple extract was added to 25 µL of 32% sulfuric acid and 150 µL of 4% vanillin solution in the 96-well plate. The reaction mixture was incubated for 15 min at 25 °C. The absorbance was measured at 500 nm and expressed in mg of catechin equivalent per g of sample weight (mg CE/g fw) based on a calibration curve with concentration from 0–1000 µg/mL.

2.3.4. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH method was used to determine the free radical scavenging activity [27]. For this, 40 µL of DPPH methanolic solution (0.1 mM) and 40 µL of extract were added into the 96-well plate. The reaction mixture was shaken vigorously and incubated for 30 min at 25 °C. The absorbance was measured at 517 nm. The standard used was ascorbic acid to construct the standard curve (0 to 50 µg/mL). The obtained values were expressed in mg of ascorbic acid equivalent per gram (mg AAE/g) (fw).

2.3.5. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing ability was assessed by modifying the FRAP method of Faiza et al. [28]. The FRAP solution was prepared at the ratio of 10:1:1, 300 mM sodium acetate solution, 20 mM Fe [III] solution and 10 mM TRTZ. 20 µL of the apple extract and 280 µL of FRAP dye solution added to the 96-well plate. The reaction mixture was incubated for 10 min at 37 °C. The absorbance was measured at 593 nm. The ascorbic acid standard curve (0–150 µg/mL) was constructed and the values obtained were expressed in mg of ascorbic acid equivalent per gram of sample (mg AAE/g fw).

2.3.6. 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Assay

In ABTS assay, the free radical scavenging activity of the apple samples were determined by following the protocol as in Rajurkar and Hande [25]. First, 88 µL of 140 mM potassium persulfate and 5 mL of 7 mM ABTS solution were mixed to form the $ABTS^+$ stock solution and incubated for 16 h in a dark area. 290 µL of prepared diluted ABTS solution was mixed with 10 µL of extract. Subsequently, incubation of the reaction mixture in the dark area for 6 min (25 °C). The absorbance was measured at 734 nm. The standard curve used to calculate the antioxidant potential was of ascorbic acid (0 to 150 µg/mL). The values were expressed in ascorbic acid equivalents (mg AAE/g) of sample.

2.3.7. Total Antioxidant Capacity (TAC)

The phosphomolybdate [29] method was used to determine the TAC. The formulation for phosphomolybdate reagent was 0.6 M sulphuric acid, 0.004 M ammonium molybdate and 0.028 M sodium phosphate. Then, 260 µL phosphomolybdate reagent was mixed with 40 µL extracts in the 96-well plate. The incubation of the reaction mixture was at 95 °C for 10 min. The absorbance was read at 695 nm after the reaction mixture cools down to room temperature. Ascorbic acid standard curve (0–200 µg/mL) constructed to determine the values of TAC and expressed in mg ascorbic acid equivalents (AAE) per gram (fw).

2.4. LC-ESI-QTOF-MS/MS Analysis of Phenolic Compounds

The identification and characterisation of phenolics in five varieties of apples were conducted using LC-ESI-QTOF-MS/MS and following the protocol described in Suleria et al. [18]. The separation of compounds was carried out through LC column 250 × 4.6 mm, 4 µm with column temperature at 25 °C. The HPLC buffers were sonicated at room temperature for 10 min. The binary solvent delivery system was used as follows: Mobile phase A: 2% acetic acid and 98% water; Mobile phase B: acetonitrile, water and acetic acid (50:49.5:0.5, $v/v/v$). The injected sample volume was 6 µL and the flow rate was at 0.8 mL/min. The program set was carried out as following: 0 min (10% B), 20 min (25% B), 30 min (35% B), 40 min (40% B), 70 min (55% B), 75 min (80% B), 77 min (100% B), 79 min (100% B), 82–85 min (isocratic 10% B). Negative and positive modes were performed for peak identification. Nitrogen gas was used as a nebulizer and drying gas at 45 psi, temperature at 300 °C with the flow rate of 5 L/min. The range of mass spectra were 50–1300 amu. Agilent LC-ESI-QTOF-MS/MS Mass Hunter workstation software (Qualitative Analysis, version B.03.01, Agilent, Santa Clara, CA, USA) was used for data acquisition and analysis.

2.5. HPLC-PDA Analysis

The HPLC-PDA analysis of polyphenols in apples was carried out using Agilent 1200 series HPLC [30,31]. The volume of the injected sample was 20 µL. 280 nm, 320 nm and 370 nm were the wavelengths used for detection. The column and the conditions used were as followed in LC-ESI-QTOF-MS/MS analysis. The wavelengths were used for the identification of hydroxybenzoic acids, hydroxycinnamic acids and flavanol group, respectively. The acquisition of the data and analysis were carried out using Agilent LC-ESI-QTOF-MS/MS Mass Hunter workstation software (Qualitative Analysis, version B.03.01, Agilent, Santa Clara, CA, USA).

2.6. Statistical Analysis

The experiments were performed in triplicates (n = 3) and the data was expressed in mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant differences (HSD) multiple rank test were performed to see the significant difference between the phenolic compounds and antioxidant activities at $p < 0.05$.

3. Results and Discussion

3.1. Phenolic Compound Estimation (TPC, TFC and TTC)

The Folin–Ciocalteu's reagent method determined the total phenolic content in the apple extracts and were expressed as gallic acid equivalents (GAE/g fw) as shown in Table 1. Red Delicious apple showed the highest TPC with 121.78 ± 3.45 mg GAE/g and significantly higher than other samples ($p < 0.05$). The total polyphenol content of five different varieties of apples were in the order of Red Delicious > Royal Gala > Fuji > Pink Lady > Smitten. According to the study of Ting et al. [32], Praveen et al. [33] and Almeida et al. [34], Red Delicious had more phenolic content than Gala, Fuji and Pink Lady, which is consistent to the result of our study. Almeida et al. [34] reported that Fuji apple contains 14.7 ± 0.4 mg (GAE)/g and Ting et al. [32] study showed that Fuji has 489.59 ± 4.21 mg (GAE)/g, the difference in the phenolic content might be due to the geographical location, soil nutrients, growth period and harvest season [35]. Additionally, due to the lack of research on Smitten apple variety, there is no valid data for Smitten for comparison.

Flavonoids have attracted a lot of attention due to their strong antioxidant activity [36]. In TFC, Red Delicious apple had the highest flavonoid content of 101.23 ± 3.75 mg QE/g and the lowest flavonoid content was present in Smitten. In a previous study, TFC of Red Delicious (98 mg QE/g) and Royal Gala (89 mg QE/g) were similar to that of our apple samples [37]. In another study, the values of total flavonoid content of Fuji apple (108 mg QE/g) was reported more than our value which may be due to the difference of varieties or solvent extraction ratio [38]. The TTC in our selected apples ranged between 4.65 ± 0.03 to 2.17 ± 0.05 mg CE/g. Fuji apple showed higher level of tannin content followed by Pink Lady, Smitten, Royal Gala and Red Delicious. Previously, the total tannin content of different varieties ranged from 0.75 mg CE/g to 14.79 mg CE/g, which is consistent with our results [39]. Overall, the variety of Red Delicious had the highest content of TPC and TFC and Fuji variety had a high content of TTC.

Table 1. Phenolic content and antioxidant potential in five varieties of apples.

Antioxidant Assays	Royal Gala	Pink Lady	Red Delicious	Fuji	Smitten
TPC (mg GAE/g)	104.21 ± 3.10 ^{b}	94.23 ± 2.24 ^{c}	121.78 ± 3.45 ^{a}	102.26 ± 2.14 ^{b}	83.98 ± 1.05 ^{d}
TFC (mg QE/g)	93.73 ± 1.10 ^{b}	81.23 ± 2.25 ^{d}	101.23 ± 3.75 ^{a}	87.26 ± 1.54 ^{c}	72.19 ± 1.75 ^{e}
TTC (mg CE/g)	3.45 ± 0.09 ^{d}	4.25 ± 0.01 ^{b}	2.17 ± 0.05 ^{e}	4.65 ± 0.03 ^{a}	3.95 ± 0.08 ^{c}
DPPH (mg AAE/g)	3.39 ± 0.05 ^{b}	2.56 ± 0.03 ^{c}	3.53 ± 0.07 ^{a}	1.98 ± 0.01 ^{d}	1.17 ± 0.02 ^{e}
FRAP (mg AAE/g)	4.12 ± 0.07 ^{b}	3.15 ± 0.12 ^{c}	4.42 ± 0.01 ^{a}	2.12 ± 0.04 ^{d}	2.15 ± 0.02 ^{d}
ABTS (mg AAE/g)	3.22 ± 0.12 ^{a}	2.94 ± 0.01 ^{b}	3.24 ± 0.09 ^{a}	1.87 ± 0.10 ^{c}	1.49 ± 0.09 ^{d}
TAC (mg AAE/g)	2.68 ± 0.09 ^{b}	2.19 ± 0.11 ^{c}	3.12 ± 0.01 ^{a}	1.96 ± 0.08 ^{d}	1.32 ± 0.01 ^{e}

All values are expressed as the mean ± SD and performed in triplicates. Different letters (a, b, c, d, e) within the same column are significantly different ($p < 0.05$) from each other. The five varieties of apples are reported based on fresh weight. CE (catechin equivalents), QE (quercetin equivalents), GAE (gallic acid equivalents), AAE (ascorbic acid equivalents). TFC (total flavonoids content), TPC (total phenolic content), TTC (total tannins content), FRAP (ferric reducing ability of plasma), DPPH (2,2′-diphenyl-1-picrylhydrazyl), TAC (total antioxidant capacity), ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid).

3.2. Antioxidant Activities (DPPH, FRAP, ABTS and TAC)

The antioxidant potential of five varieties of apple samples were estimated by four assays including DPPH, FRAP, ABTS and TAC assays, and the antioxidant activities were expressed in ascorbic acid (AAE) per gram (fw) as mentioned in Table 1.

In the DPPH assay, the free radical scavenging activity is determined which is attributed to the phenolic compounds [40]. The apple varieties in the current study varied from 1.17 to 3.53 mg AAE/g. Red Delicious had the highest antioxidant potential followed by Royal Gala, Pink Lady, Fuji and Smitten. Previous studies reported that antioxidant potential for over ten varieties of apples ranged from 0.26 to 9.30 mg AAE/g [41,42]. The values of Fuji and Red Delicious apples are slightly higher than ours which might be because of the cultivar, location, maturity and storage of apples which may change the concentration of antioxidant potential [43].

FRAP assay can provide comprehensive information about the antioxidant activities of five varieties of apples since various antioxidant assays can help us to understand the antioxidant properties of apples better [44]. In FRAP assay, the electron transfer method was used to measure the capacity to reduce Fe^{3+} to Fe^{2+} [20]. The FRAP values were significantly different ($p < 0.05$) from 2.12 ± 0.04 mg AAE/g to 4.42 ± 0.01 mg AAE/g among the apple varieties. The highest FRAP capacity was recorded in Red Delicious, followed by Royal Gala, Pink Lady, Fuji, and Smitten.

In the ABTS assay, the antiradical scavenging activities were determined based of the hydrogen atom donating tendency of polyphenols [40]. The highest antioxidant ability was demonstrated in the order of Red Delicious > Royal Gala > Pink Lady > Fuji > Smitten. Upon comparison with the previous studies' Royal Gala and Fuji showed higher antioxidant ability than the previous reported values [41,42]. The reason might be because of the cultivar, location, maturity and storage of apples which may change the concentration of antioxidant potential [43]. In the TAC assay, the mechanism very similar to FRAP where reduction of molybdenum (VI) to molybdenum (V) in the presence of phenolics. In the current study, Red Delicious had the highest total antioxidant followed by Royal Gala, Pink Lady, Fuji and Smitten. Previously Khanizadeh et al.'s [35] study showed the values ranging from 0.323 to 1.246 mg AAE/g and the values were lower than our study. A difference in the concentration might be because of the difference between cultivars, location, harvesting time and maturity of samples [6].

3.3. Correlation between Phenolic Compounds and Antioxidant Activities

The correlation between the polyphenols and antioxidant activities was performed with a Pearson's correlation test (Table 2). TPC shows a strong positive correlation with TFC with $r^2 = 0.975$, $p \leq 0.01$, this indicates that TFC contributes largely to the total phenolic content. Additionally, TPC was strongly correlated with TAC with r^2 value of 0.920 ($p \leq 0.05$). A previous study by Vasantha Rupasinghe and Clegg [45] reported a similar correlation between TPC and TAC.

Table 2. Correlation coefficients (r^2) between phenolic contents and antioxidant assays.

Variables	TPC	TFC	TTC	DPPH	ABTS	FRAP
TFC	0.975 **					
TTC	−0.736	−0.702				
DPPH	0.832	0.903 *	−0.685			
ABTS	0.754	0.815	−0.830	0.952 **		
FRAP	0.681	0.756	−0.614	0.961 **	0.938 **	
TAC	0.920 *	0.952 **	−0.751	0.980 **	0.931 *	0.912 *

** Significant correlation with $p \leq 0.01$; * Significant correlation with $p \leq 0.05$.

TFC had a significantly strong correlation with DPPH and TAC with r^2 value of 0.903 ($p \leq 0.01$) and 0.952 ($p \leq 0.05$) respectively indicating that flavonoids were one of the significant contributors for the antioxidant activities. The results confirm with the previous studies of Maleeha et al. [46] and Ruiz-Torralba et al. [47], on phenolic compounds contributing towards antioxidant potential. A non-significant correlation were observed between TTC and antioxidant assays indicating the contribution of tannins to antioxidant activity is limited, which confirms with Kam et al. [48] study.

The correlation among the antioxidant assays had strong correlation with each other. Significant positive correlation was observed between DPPH with ABTS, FRAP and TAC (r^2 = 0.952, r^2 = 0.961, and r^2 = 0.980, $p \leq 0.01$). The correlation displayed in our study was similar to Kriengsak et al. [49], where a high correlation was observed between the four assays. Similarly, ABTS was observed to have high significant correlation with FRAP and TAC with r^2 = 0.938, $p \leq 0.01$ and r^2 = 0.931 ($p \leq 0.05$), respectively. On the other hand, FRAP was correlated with TAC with r^2 = 0. 912 ($p \leq 0.05$).

Overall, phenolic compounds were highly correlated with antioxidant assays, which indicated that both classes of phenolic compounds including phenolic acids and flavonoids have strong antioxidant potential. The four antioxidants' assays were strongly correlated with each other.

3.4. Phenolic Compounds Profile by LC-MS/MS Analysis

LC- MS/MS has been a useful and reliable tool for identification and characterisation of phenolics in several plant samples. Qualitative analyses of phenolics from five varieties of apples (Royal Gala, Pink Lady, Red Delicious, Fuji and Smitten) were achieved using mass spectrometry in both negative and positive modes of ionisation (ESI^-/ESI^+). The compounds in the apples were identified based on their precursor ions and MS spectra. The basis for the compounds to be further analysed were the PCDL library score more than 80 and mass error < 5 ppm (Table 3). In our current study, 97 different phenolic compounds were characterised in five apple samples, including 27 phenolic acids, 52 flavonoids, 5 lignans and 13 other polyphenols.

Table 3. Identification and characterisation of polyphenols in apples by using LC-ESI-QTOF-MS/MS.

No.	Proposed Compounds	Molecular Formula	RT (min)	Ionization (ESI^+/ESI^-)	Molecular Weight	Theoretical (*m*/*z*)	Observed (*m*/*z*)	Error (ppm)	MS^2 Product Ions	Samples
				Phenolic acid						
				Hydroxybenzoic acids						
1	Gallic acid 4-*O*-glucoside	$C_{13}H_{16}O_{10}$	6.866	$[M-H]^-$	332.0743	331.0670	331.0674	1.2	169, 125	RG
2	Protocatechuic acid 4-*O*-glucoside	$C_{13}H_{16}O_9$	7.379	** $[M-H]^-$	316.0794	315.0721	315.0718	−1.0	153	RD, F, * RG, S, PL
3	2-Hydroxybenzoic acid	$C_7H_6O_3$	7.608	** $[M-H]^-$	138.0317	137.0244	137.0242	−1.5	93	PL, * RD, RG, S, F
4	3-*O*-Methylgallic acid	$C_8H_8O_5$	12.930	$[M+H]^+$	184.0372	185.0445	185.0452	3.8	170, 142	F, * PL
5	2,3-Dihydroxybenzoic acid	$C_7H_6O_4$	15.580	$[M-H]^-$	154.0266	153.0193	153.0196	2.0	109	RG, * PL, F
				Hydroxycinnamic acids						
6	m-Coumaric acid	$C_9H_8O_3$	5.256	** $[M-H]^-$	164.0473	163.04	163.0393	−4.3	119	S,* RD, RG, PL, F
7	Caffeic acid	$C_9H_8O_4$	5.898	$[M+H]^+$	180.0423	181.0496	181.0494	−1.1	143, 133	S
8	*p*-Coumaroyl tartaric acid	$C_{13}H_{12}O_8$	8.632	$[M-H]^-$	296.0532	295.0459	295.0468	3.1	115	F
9	Cinnamic acid	$C_9H_8O_2$	9.314	** $[M-H]^-$	148.0524	147.0451	147.0449	−1.4	103	RG, * RD, F
10	3-Caffeoylquinic acid	$C_{16}H_{18}O_9$	12.979	** $[M-H]^-$	354.0951	353.0878	353.088	0.6	253, 190, 144	PL, S, * RG, F
11	3- *p*-Coumaroylquinic acid	$C_{16}H_{18}O_8$	18.131	** $[M-H]^-$	338.1002	337.0929	337.0924	−1.5	265, 173, 162	PL,* RG, F, S
12	*p*-Coumaric acid 4-*O*-glucoside	$C_{15}H_{18}O_8$	20.881	$[M-H]^-$	326.1002	325.0929	325.0925	−1.2	163	PL,* RG, F
13	Rosmarinic acid	$C_{18}H_{16}O_8$	22.273	$[M-H]^-$	360.0845	359.0772	359.0755	−4.7	179	* PL, F
14	Caffeic acid 3-*O*-glucuronide	$C_{15}H_{16}O_{10}$	22.737	$[M-H]^-$	356.0743	355.067	355.0677	2.0	179	PL
15	Ferulic acid	$C_{10}H_{10}O_4$	23.366	** $[M-H]^-$	194.0579	193.0506	193.0505	−0.5	178, 149, 134	S,* PL, F
16	Caffeoyl glucose	$C_{15}H_{18}O_9$	24.244	$[M-H]^-$	342.0951	341.0878	341.0886	2.3	179, 161	RD,* PL
17	Ferulic acid 4-*O*-glucuronide	$C_{16}H_{18}O_{10}$	25.785	$[M-H]^-$	370.09	369.0827	369.0814	−3.5	193	* PL, F
18	1-Sinapoyl-2,2′-diferuloylgentiobiose	$C_{43}H_{48}O_{21}$	26.763	$[M-H]^-$	900.2688	899.2615	899.2579	−4.0	613, 201	PL
19	Sinapic acid	$C_{11}H_{12}O_5$	30.185	** $[M-H]^-$	224.0685	223.0612	223.0603	−4.0	205, 163	* F, PL, S
20	3-Feruloylquinic acid	$C_{17}H_{20}O_9$	33.605	** $[M-H]^-$	368.1107	367.1034	367.1019	−4.1	298, 288, 192, 191	* RG, F
21	1,2,2′-Triferuloylgentiobiose	$C_{42}H_{46}O_{20}$	34.101	$[M-H]^-$	870.2582	869.2509	869.2498	−1.3	693, 517	S
22	Ferulic acid 4-*O*-glucoside	$C_{16}H_{20}O_9$	35.526	** $[M-H]^-$	356.1107	355.1034	355.1039	1.4	193, 178, 149, 134	* PL, RG, S, F
23	*p*-Coumaroyl malic acid	$C_{13}H_{12}O_7$	41.506	$[M-H]^-$	280.0583	279.051	279.0524	5.0	163, 119	S

Table 3. *Cont.*

No.	Proposed Compounds	Molecular Formula	RT (min)	Ionization (ESI^+/ESI^-)	Molecular Weight	Theoretical (*m*/*z*)	Observed (*m*/*z*)	Error (ppm)	MS^2 Product Ions	Samples
				Hydroxyphenylacetic acids						
24	2-Hydroxy-2-phenylacetic acid	$C_8H_8O_3$	31.517	** $[M-H]^-$	152.0473	151.04	151.0402	1.3	136, 92	PL
25	3,4-Dihydroxyphenylacetic acid	$C_8H_8O_4$	20.749	** $[M-H]^-$	168.0423	167.035	167.0343	−4.2	149, 123	* RG, PL, F
				Hydroxyphenylpropanoic acids						
26	Dihydroferulic acid 4-sulfate	$C_{10}H_{12}O_7S$	4.076	$[M-H]^-$	276.0304	275.0231	275.0229	−0.7	195, 151, 177	F
27	Dihydroferulic acid 4-*O*-glucuronide	$C_{16}H_{20}O_{10}$	6.866	$[M-H]^-$	372.1056	371.0983	371.0986	0.8	195	* RG, PL
				Flavonoids Anthocyanins						
28	Cyanidin 3-*O*-diglucoside-5-*O*-glucoside	$C_{33}H_{41}O_{21}$	21.567	$[M+H]^+$	773.214	774.2213	774.2216	0.4	610, 464	S
29	Cyanidin 3-*O*-(6″-p-coumaroyl-glucoside)	$C_{30}H_{27}O_{13}$	22.205	** $[M+H]^+$	595.1452	596.1525	596.1553	4.7	287	RG,* PL
30	Peonidin 3-*O*-sambubioside-5-*O*-glucoside	$C_{33}H_{41}O_{20}$	22.561	** $[M+H]^+$	757.2191	758.2264	758.2228	−4.7	595, 449, 287	* S, F
31	Cyanidin 3,5-*O*-diglucoside	$C_{27}H_{31}O_{16}$	37.067	** $[M+H]^+$	611.1612	612.1685	612.1693	1.3	449, 287	* F, S, PL
32	Delphinidin 3-*O*-xyloside	$C_{20}H_{19}O_{11}$	37.212	$[M+H]^+$	435.0927	436.1	436.0996	−0.9	303	PL
33	Delphinidin 3-*O*-glucosyl-glucoside	$C_{27}H_{31}O_{17}$	37.232	** $[M+H]^+$	627.1561	628.1634	628.1648	2.2	465, 303	F
34	Cyanidin 3-*O*-(2-*O*-(6-*O*-(E)-caffeoyl-D glucoside)-D-glucoside)-5-*O*-D-glucoside	$C_{43}H_{49}O_{24}$	38.918	$[M+H]^+$	949.2614	950.2687	950.2679	−0.8	787, 463, 301	RG
35	Delphinidin 3-*O*-galactoside	$C_{21}H_{21}O_{12}$	45.301	** $[M-H]^-$	465.1033	464.096	464.0964	0.9	303	S, F,* PL
				Dihydrochalcones						
36	3-Hydroxyphloretin 2′-*O*-glucoside	$C_{21}H_{24}O_{11}$	24.659	$[M-H]^-$	452.1319	451.1246	451.1249	0.7	289, 273	* PL, RG, F, S
37	3-Hydroxyphloretin 2′-*O*-xylosyl-glucoside	$C_{26}H_{32}O_{15}$	37.564	$[M-H]^-$	584.1741	583.1668	583.1665	−0.5	289	RG
38	Phloridzin	$C_{21}H_{24}O_{10}$	51.613	** $[M-H]^-$	436.1369	435.1296	435.1284	−2.8	273	* RG, PL, S, F

Table 3. *Cont.*

No.	Proposed Compounds	Molecular Formula	RT (min)	Ionization (ESI^+/ESI^-)	Molecular Weight	Theoretical (*m*/*z*)	Observed (*m*/*z*)	Error (ppm)	MS^2 Product Ions	Samples
					Dihydroflavonols					
39	Dihydromyricetin 3-*O*-rhamnoside	$C_{21}H_{22}O_{12}$	23.549	** $[M-H]^-$	466.1111	465.1038	465.1031	−1.5	301	RG, F,* PL, F, PL
40	Dihydroquercetin 3-*O*-rhamnoside	$C_{21}H_{22}O_{11}$	32.081	** $[M-H]^-$	450.1162	449.1089	449.1081	−1.8	303	S,* PL
41	Dihydroquercetin	$C_{15}H_{12}O_7$	38.674	** $[M-H]^-$	304.0583	303.051	303.0518	2.6	285, 275, 151	S,* PL
					Flavanols					
42	(+)-Gallocatechin	$C_{15}H_{14}O_7$	4.494	** $[M-H]^-$	306.074	305.0667	305.068	4.3	261, 219	S, PL, F,* RD
43	(+)-Gallocatechin 3-*O*-gallate	$C_{22}H_{18}O_{11}$	11.106	$[M-H]^-$	458.0849	457.0776	457.0781	1.1	305, 169	F,* S
44	Procyanidin dimer B1	$C_{30}H_{26}O_{12}$	21.362	** $[M-H]^-$	578.1424	577.1351	577.1333	−3.1	451	* PL, RG, S, F
45	(+)-Catechin 3-*O*-gallate	$C_{22}H_{18}O_{10}$	22.306	** $[M-H]^-$	442.09	441.0827	441.0805	−5.0	289, 169, 125	* PL, F
46	(+)-Catechin	$C_{15}H_{14}O_6$	26.597	** $[M-H]^-$	290.079	289.0717	289.0706	−3.8	245, 205, 179	* RG, S, PL, F
47	4′-*O*-Methyl-(-)-epigallocatechin 7-*O*-glucuronide	$C_{22}H_{24}O_{13}$	27.607	$[M-H]^-$	496.1217	495.1144	495.116	3.2	451, 313	RG,* PL, F
48	Procyanidin trimer C1	$C_{45}H_{38}O_{18}$	28.966	** $[M-H]^-$	866.2058	865.1985	865.1961	−2.8	739, 713, 695	* RG, S, PL, F
49	Cinnamtannin A2	$C_{60}H_{50}O_{24}$	35.444	** $[M-H]^-$	1154.269	1153.2617	1153.263	1.1	739	RG,* PL, F
50	Prodelphinidin dimer B3	$C_{30}H_{26}O_{14}$	67.792	** $[M+H]^+$	610.1323	611.1396	611.1407	1.8	469, 311, 291	PL,* F
					Flavanones					
51	Hesperetin 3′,7-*O*-diglucuronide	$C_{28}H_{30}O_{18}$	21.163	** $[M-H]^-$	654.1432	653.1359	653.1361	0.3	477, 301, 286, 242	S,* PL
52	6-Prenylnaringenin	$C_{20}H_{20}O_5$	35.742	$[M+H]^+$	340.1311	341.1384	341.1375	−2.6	323, 137	F
53	Narirutin	$C_{27}H_{32}O_{14}$	38.326	$[M-H]^-$	580.1792	579.1719	579.171	−1.6	271	RG
54	Hesperetin 3′-*O*-glucuronide	$C_{22}H_{22}O_{12}$	52.421	** $[M+H]^+$	478.1111	479.1184	479.1199	3.1	301, 175, 113, 85	RD, RG, PL,* F
					Flavones					
55	Apigenin 7-*O*-apiosyl-glucoside	$C_{26}H_{28}O_{14}$	14.031	** $[M+H]^+$	564.1479	565.1552	565.1552	0.0	296	PL,* S
56	Apigenin 7-*O*-glucuronide	$C_{21}H_{18}O_{11}$	15.812	** $[M+H]^+$	446.0849	447.0922	447.093	1.8	271, 253	* PL, S
57	7,4′-Dihydroxyflavone	$C_{15}H_{10}O_4$	18.251	$[M+H]^+$	254.0579	255.0652	255.0643	−3.5	227, 199, 171	F
58	Cirsilineol	$C_{18}H_{16}O_7$	26.744	** $[M+H]^+$	344.0896	345.0969	345.0962	−2.0	330, 312, 297, 284	* PL, RD
59	Apigenin 6,8-di-C-glucoside	$C_{27}H_{30}O_{15}$	43.578	** $[M-H]^-$	594.1585	593.1512	593.1527	2.5	503, 473	PL, S,* RG, F
60	6-Hydroxyluteolin 7-*O*-rhamnoside	$C_{21}H_{20}O_{11}$	46.758	** $[M-H]^-$	448.1006	447.0933	447.0928	−1.1	301	* RG, PL, RD, S, F
61	Chrysoeriol 7-*O*-glucoside	$C_{22}H_{22}O_{11}$	54.226	** $[M+H]^+$	462.1162	463.1235	463.1255	4.3	445, 427, 409, 381	RG, PL,* F

Table 3. *Cont.*

No.	Proposed Compounds	Molecular Formula	RT (min)	Ionization (ESI^+/ESI^-)	Molecular Weight	Theoretical (*m*/*z*)	Observed (*m*/*z*)	Error (ppm)	MS^2 Product Ions	Samples
					Flavonols					
62	Myricetin 3-*O*-galactoside	$C_{21}H_{20}O_{13}$	19.288	$[M-H]^-$	480.0904	479.0831	479.081	−4.4	317	RD
63	Quercetin 3-*O*-glucosyl-xyloside	$C_{26}H_{28}O_{16}$	21.146	$[M-H]^-$	596.1377	595.1304	595.1291	−2.2	265, 138, 116	PL
64	Quercetin 3-*O*-xylosyl-rutinoside	$C_{32}H_{38}O_{20}$	23.124	** $[M+H]^+$	742.1956	743.2029	743.2022	−0.9	479, 317	F,* S
65	Kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside	$C_{33}H_{40}O_{20}$	24.867	** $[M-H]^-$	756.2113	755.204	755.2068	3.7	285	RG,* F
66	Kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside	$C_{33}H_{40}O_{19}$	25.198	** $[M-H]^-$	740.2164	739.2091	739.2115	3.2	593, 447, 285	S,* F
67	Kaempferol 3-*O*-xylosyl-glucoside	$C_{26}H_{28}O_{15}$	28.135	** $[M+H]^+$	580.1428	581.1501	581.1479	−3.8		* PL, RG, F
68	Kaempferol 3,7-*O*-diglucoside	$C_{27}H_{30}O_{16}$	37.879	** $[M-H]^-$	610.1534	609.1461	609.1451	−1.6	447, 285	* RG, S
69	Myricetin 3-*O*-rhamnoside	$C_{21}H_{20}O_{12}$	39.996	** $[M-H]^-$	464.0955	463.0882	463.0862	−4.3	317	* RD, RG, S
70	Quercetin 3-*O*-xylosyl-glucuronide	$C_{26}H_{26}O_{17}$	43.207	$[M+H]^+$	610.117	611.1243	611.1255	2.0	479, 303, 285, 239	F,* PL
71	Quercetin 3-*O*-arabinoside	$C_{20}H_{18}O_{11}$	45.665	** $[M-H]^-$	434.0849	433.0776	433.0781	1.2	301	* RG, S
					Isoflavonoids					
72	6″-*O*-Malonylglycitin	$C_{25}H_{24}O_{13}$	7.256	$[M+H]^+$	532.1217	533.129	533.1286	−0.8	285, 270, 253	S
73	6″-*O*-Malonyldaidzin	$C_{24}H_{22}O_{12}$	16.246	$[M+H]^+$	502.1111	503.1184	503.12	3.2	255	F
74	Dihydrobiochanin A	$C_{16}H_{14}O_5$	22.255	$[M+H]^+$	286.0841	287.0914	287.0925	3.8	269, 203, 201, 175	F,* PL
75	Violanone	$C_{17}H_{16}O_6$	24.926	$[M+H]^+$	316.0947	317.102	317.1016	−1.3	300, 285, 135	F
76	3′-Hydroxygenistein	$C_{15}H_{10}O_6$	27.116	$[M+H]^+$	286.0477	287.055	287.0547	−1.0	269, 259	* S, F
77	Formononetin 7-*O*-glucuronide	$C_{22}H_{20}O_{10}$	42.45	** $[M-H]^-$	444.1056	443.0983	443.0973	−2.3	267, 252	* S, F
78	5,6,7,3′,4′-Pentahydroxyisoflavone	$C_{15}H_{10}O_7$	42.893	** $[M+H]^+$	302.0427	303.05	303.0487	−4.3	285, 257	* PL, S, RD, RG, F
79	6″-*O*-Malonylgenistin	$C_{24}H_{22}O_{13}$	64.297	** $[M+H]^+$	518.106	519.1133	519.1157	4.6	271	* F, S

Table 3. *Cont.*

No.	Proposed Compounds	Molecular Formula	RT (min)	Ionization (ESI^+/ESI^-)	Molecular Weight	Theoretical (*m*/*z*)	Observed (*m*/*z*)	Error (ppm)	MS^2 Product Ions	Samples
					Lignans					
80	Enterolactone	$C_{18}H_{18}O_4$	4.234	$[M+H]^+$	298.1205	299.1278	299.1279	0.3	281, 187, 165	PL
81	7-Hydroxymatairesinol	$C_{20}H_{22}O_7$	47.587	$[M-H]^-$	374.1366	373.1293	373.1283	−2.7	343, 313,	S, F,* RG
82	Schisandrin C	$C_{22}H_{24}O_6$	59.344	$[M+H]^+$	384.1573	385.1646	385.1663	4.4	370, 315, 300	S,* F
83	Secoisolariciresinol-sesquilignan	$C_{30}H_{38}O_{10}$	59.607	$[M-H]^-$	558.2465	557.2392	557.2387	−0.9	539, 521, 509, 361	F
84	Schisandrol B	$C_{23}H_{28}O_7$	63.253	$[M+H]^+$	416.1835	417.1908	417.1929	5.0	224, 193, 165	F
					Other polyphenols					
					Curcuminoids					
85	Demethoxycurcumin	$C_{20}H_{18}O_5$	81.976	$[M-H]^-$	338.1154	337.1081	337.108	−0.3	217	RD
					Furanocoumarins					
86	Isopimpinellin	$C_{13}H_{10}O_5$	4.478	$[M+H]^+$	246.0528	247.0601	247.0605	1.6	232, 217, 205, 203	* RD, F
					Hydroxybenzaldehydes					
87	*p*-Anisaldehyde	$C_8H_8O_2$	26.251	** $[M+H]^+$	136.0524	137.0597	137.0596	−0.7	122, 109	PL,* F, S
88	4-Hydroxybenzaldehyde	$C_7H_6O_2$	44.568	** $[M-H]^-$	122.0368	121.0295	121.0301	5.0	77	S, F,* RD
					Hydroxycoumarins					
89	Coumarin	$C_9H_6O_2$	25.364	* $[M-H]^-$	146.0368	145.0295	145.0302	4.8	103, 91	F
					Hydroxyphenylpropenes					
90	2-Methoxy-5-prop-1-enylphenol	$C_{10}H_{12}O_2$	25.903	$[M+H]^+$	164.0837	165.091	165.0906	−2.4	149, 137, 133, 124	F
					Other polyphenols					
91	Salvianolic acid C	$C_{26}H_{20}O_{10}$	9.665	$[M-H]^-$	492.1056	491.0983	491.0963	−4.1	311, 267, 249	S
92	Salvianolic acid B	$C_{36}H_{30}O_{16}$	28.598	$[M-H]^-$	718.1534	717.1461	717.1436	−3.5	519, 339, 321, 295	RD
					Phenolic terpenes					
93	Rosmanol	$C_{20}H_{26}O_5$	22.23	$[M+H]^+$	346.178	347.1853	347.1844	−2.6	301, 241, 231	S
94	Carnosic acid	$C_{20}H_{28}O_4$	80.419	** $[M-H]^-$	332.1988	331.1915	331.1905	−3.0	287, 269	* RD, F
					Tyrosols					
95	Hydroxytyrosol 4-*O*-glucoside	$C_{14}H_{20}O_8$	14.338	** $[M-H]^-$	316.1158	315.1085	315.109	1.6	153, 123	F,* PL
96	3,4-DHPEA-AC	$C_{10}H_{12}O_4$	25.537	** $[M-H]^-$	196.0736	195.0663	195.0658	−2.6	135	* PL, F, S
97	Demethyloleuropein	$C_{24}H_{30}O_{13}$	51.646	* $[M-H]^-$	526.1686	525.1613	525.1599	−2.7	495	* RG, F

* Data presented in the table are from the sample indicated with an asterisk; ** Compounds were detected in both negative $[M-H]^-$ and positive $[M+H]^+$ mode of ionization while only single mode data was presented. Apple samples mentioned in abbreviations are Royal Gala "RG"; Red Delicious "RD"; Fuji "F"; Smitten "S"; Pink Lady "PL".

3.4.1. Phenolic Acids

In our research, 27 phenolic acids including hydroxyphenylacetic acids (2), hydroxycinnamic acids (18), hydroxybenzoic acids (5), and hydroxyphenylpropanoic acids (2) were identified and characterised in five varieties of apples.

Compound **1** was tentatively characterised as protocatechuic acid 4-*O*-glucoside present in negative mode of ionisation and identified in Royal Gala, Red Delicious and Fuji apples. The compound had precursor ion at *m/z* 315.0718 and on further MS/MS analysis showed product ions at *m/z* 125 (loss of CO_2, 44 Da) and *m/z* 169 (loss of hexosyl moiety, 162 Da) [50]. In previous study of Gu et al. [21] reported tentatively characterised protocatechuic acid 4-*O*-glucoside from fresh apples. Compound **12** (($[M-H]^-$ *m/z* at 325.0925) was tentatively characterised as *p*-Coumaric acid 4-*O*-glucoside based on the product ions at *m/z* 163, due to the loss of hexosyl moiety (162 Da) from the precursor ions [50]. Identified in Pink Lady, Royal Gala and Fuji apples.

Compound **7** was tentatively characterised as caffeic acid in Smitten variety based on the precursor ion at $[M+H]^+$ at *m/z* 181.0494 and confirmed based on the MS^2 fragmentation with product ions at m/z 143 (loss of two water molecules, 36 Da) and m/z 133 (loss of HCOOH, 46 Da) [51]. Compound 15 was observed in Smitten, Pink Lady and Fuji and tentatively characterised as ferulic acid based on the precursor ion at ($[M-H]^-$ at m/z 193.0505. Upon further MS/MS analysis, the product ions at m/z 178 (loss of CH_3, 15 Da), m/z 149 (loss of CO_2, 44 Da) and m/z 134 (loss of CH_3-CO_2, 59 Da) confirmed the compound [52]. Compounds **19** (($[M-H]^-$ m/z at 223.0603) identified in Fuji, Pink Lady and Smitten apples. MS/MS analysis confirmed the compound as sinapic acid by fragments at m/z 205 and m/z 163 due to the consecutive loss of H_2O and 2CHO from the precursor ion respectively [53]. Previously, Lee et al. [54] reported the presence of caffeic acid, ferulic acid and sinapic acid in apples. Caffeic acid abundantly present in both pulp and peel [54]. Other phenolic compounds to our best knowledge were first time detected in Australian grown apples.

3.4.2. Flavonoids

A total of 52 Flavonoids were identified in the five apple samples including anthocyanins (8), dihydrochalcones (3), dihydroflavonols (3), flavanols (9), flavones (4), flavanones (7), flavonols (10), and Isoflavonoids (8).

Compound **31** (Cyanidin 3,5-*O*-diglucoside) and compound **33** (Delphinidin 3-*O*-glucosyl-glucoside) were both detected in the positive mode of ionization with the precursor ions at m/z 612.1693 and m/z 628.1648, respectively. The MS/MS experiment allowed the further identification of these compounds based on the peaks after removal of the sugar moieties for both compounds [55].

Compound **36** and compound **37** were tentatively characterised as 3-hydroxyphloretin 2′-*O*-glucoside and 3-hydroxyphloretin 2′-*O*-xylosyl-glucoside present in negative mode of ionisation with precursor ions at m/z 451.1249 and m/z 583.1665, respectively. 3-hydroxyphloretin 2′-*O*-glucoside was confirmed by fragment ions at m/z 289 [M-H-glucoside] and m/z 273 [M-H-phloretin aglycon] [56] identified in Pink Lady, Royal Gala, Fuji and Smitten apples. Whereas, 3-Hydroxyphloretin 2′-*O*-xylosyl-glucoside was identified by fragment ions at m/z 289, due to the loss of xylosyl-glucoside disaccharide (132 + 162 Da) [57] observed in Royal Gala apples. Phloridzin (compound **38)** with precursor ion at [($[M-H]^-$, m/z 435.1284], and confirmed by product ions at m/z 273 due to the loss of glucoside (162 Da) [58] identified in Pink Lady, Royal Gala, Fuji and Smitten apples. Kelebek et al. [58] reported the presence of phloridzin in apples.

Three flavanols derivatives (Compound **44**, **46**, **48**) were all detected in four samples including Pink Lady, Royal Gala, Fuji and Smitten apples. Compound **44**, **46**, **48** with negative mode of ionisation with precursor ions at m/z 577.1333, m/z 289.0706 and m/z 865.1961 were tentatively characterised as procyanidin dimer B1, (+)-catechin and procyanidin trimer C1 respectively. The compound procyanidin trimer C1 was confirmed by product ions at m/z 739, m/z 713 and m/z 695, due to the loss of heterocyclic ring fission

(HRF) reaction (126 Da), loss of retro-Diels-Alder (RDA) (152 Da) and loss of H_2O [59]. While the loss of phloroglucinol (126 Da) from the precursor ion confirmed the presence of procyanidin dimer B1 [60]. Whereas, (+)-catechin compound confirmed based on the fragment ions at m/z 245, m/z 205 and m/z 179, due to corresponding loss of CO_2 (44 Da), flavonoid A ring (84 Da) and flavonoid B ring (110 Da) from the precursor ion, respectively [50]. Previously Nicoli et al. [61] reported the presence of (+)-catechin in apple varieties. (+)-catechin has a positive health benefit including scavenging free radicals, delaying aging and benefitting the intestinal microbes [62].

Compound **51** (hesperetin 3′,7-*O*-diglucuronide) and compound **53** (narirutin) were found both in negative ionization modes based on the precusor ions at m/z 653.1361 and m/z 579.1710, respectively. Compound **51** was confirmed by the product ion at m/z 477 [M-H-glucuronide, loss of 176 Da], m/z 301 [M-H-2 glucuronide, loss of 352 Da], m/z 286 [M-H-2glucuronide-CH_3, loss of 367 Da] and m/z 242 [M-H-2glucuronide-OCH_2-CHO] [63], while compound **53** was confirmed by loss of neohesperidose moiety (308 Da) [64] from the precursor ion. In our study compound **51** was identified in Smitten and Pink Lady whereas compound **53** was identified in Royal Gala and Red Delicious. To our best knowledge it was first time detected in Australian grown apples.

Apigenin 7-*O*-glucuronide (Compound **56**) and cirsilineol (compound **58**) were tentatively characterised in negative mode of ionisation at m/z 447.0930 and m/z 345.0962, respectively. The MS/MS analysis confirmed the compound **56** at product ions m/z 271 due to the corresponding loss of glucuronide (176 Da) and loss of glucuronide and m/z 253 due to the loss of H_2O-CH_2O (194 Da) from the precursor ion [65]. The presence of cirsilineol was confirmed by the product ions at m/z 330 [M+H-CH_3], m/z 312 [M+H-CH_3-H_2O], m/z 297 [M+H-2CH_3-H_2O] and m/z 284 [M+H-CH_3-H_2O-CO] [66]. According to previous reports, compounds have been characterised in several plants including Ocimum species [66].

Compound **62** (Myricetin 3-*O*-galactoside with ($[M-H]^-$ m/z at 479.081) identified in Red Delicious and compound **63** (Quercetin 3-*O*-glucosyl-xyloside with ($[M-H]^-$ m/z at 595.1291) identified in Pink Lady were only detected in the negative ionization mode, and identified according to the fragment peaks at m/z 317 [M-H-glucoside, loss of 162 Da] [67] and m/z 265 [M-H-glucose-xylose, loss of 330 Da] [51], respectively. Compound **65, 66** and **68** present in the negative mode of ionisation were identified as kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside, kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside and kaempferol 3,7-*O*-diglucoside according to the ($[M-H]^-$ at m/z 755.2068, m/z 739.2115 and m/z 609.1451, respectively Kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside exhibited the product ions at m/z 285, corresponding to the loss of the sugar units from the precursor ion [68]. The presence of kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside was confirmed by the product ions at m/z 593 [M-H-$C_6H_{10}O_4$], m/z 447 [M-H-2$C_6H_{10}O_4$], and m/z 285 [M-H-2$C_6H_{10}O_4$-$C_6H_{10}O_5$] [69]. Whereas, kaempferol 3,7-*O*-diglucoside exhibited the product ions at m/z 447 and m/z 285, corresponding to the loss of glucoside and consecutive loss of glucoside from the parent ion [70]. It worth noted that these compounds were first time detected in Australian grown apple samples to the best of our knowledge.

Compound **73** and **75** detected in positive mode were identified as 6″-*O*-Malonyldaidzin and violanone with precursor ion at m/z 503.1200 and m/z 317.1016, respectively. 6″-*O*-Malonyldaidzin was confirmed by the product ion at m/z 255 [71], corresponding to the loss of malonyl-glucoside from precursor, while the compound violanone was confirmed by the intensive peaks at m/z 300 [M+H-CH_3, loss of 15 Da], m/z 285 [M+H-2CH_3, loss of 30 Da] and m/z 135 [M+H-$C_{10}H_{12}O_3$] [72]. Previously, several studies had discovered the existence of the above isoflavonoids in fruits [71,73–76].

3.4.3. Lignans

Compound **82** (Schisandrin C) was detected only in the positive ionization mode with precursor ions at m/z 385.1663. The fragmentation peaks confirmed the compound

schisantherin C based on product ions at m/z 370 [M+H-CH_3OH], m/z 315 [M+H-C_5H_{10}] and m/z 300 [M+H-CH_3-C_5H_{10}] [77].

3.4.4. Other Polyphenols

In other polyphenols, curcuminoids (1), furanocoumarins (1), hydroxybenzaldehydes (2), hydroxycoumarins (1), hydroxyphenylpropenes (1), phenolic terpenes (2), tyrosols (3) and other polyphenols (2), while tyrosols was the dominant subclass were identified in apple samples.

Compound **88** was tentatively characterised as 4-hydroxybenzaldehyde based on the precursor ion at ($[M-H]^-$ at m/z 121.0301 and confirmed based on the MS^2 fragmentation, which exhibited the loss of CO_2 (44 Da) from the precursor, resulting in the product ion at m/z 77 [78]. Rosmanol (compound **93**) was found in positive modes, and tentatively characterised according to the precursors $[M+H]^+$ at m/z 347.1844. In the MS^2 experiment, peaks at m/z 301 (loss of H_2O) and m/z 231(loss of CO_2) achieved the identification of coumarin [79]. Meanwhile, compound **94** (carnosic acid with ($[M-H]^-$ at m/z 331.1905) was confirmed by the fragments at m/z 287 and m/z 296, resulting from the loss of CO_2 and further loss of H_2O from the precursor [80]. To best of our knowledge, this is the first time it has been detected in apple samples.

Compounds **95** and **96** detected in negative mode were detected as hydroxytyrosol 4-*O*-glucoside and 3,4-DHPEA-AC, precursor ion at m/z 315.1090 and m/z 195.0658, respectively. On further analysis, hydroxytyrosol 4-*O*-glucoside was confirmed by the product ions at m/z 153 and m/z 123, corresponding to the loss of glucoside (162 Da) and glucoside-CH_2O (192 Da) from the precursor ion, respectively [78] and 3,4-DHPEA-AC was confirmed by the product ions at m/z 135 [M-H-$C_2H_4O_2$] [81].

Compounds **91** and **92** were found in negative ionization mode and identified as salvianolic acid C and salvianolic acid B with precursor ions at m/z 491.0963 and m/z 717.1436, respectively. Salvianolic acid C was confirmed by the product ion at m/z 311 [M-H-caffeic acid], m/z 267 [M-H-caffeic-CO_2] and m/z 249 [M-H-CO_2-H_2O][82], while salvianolic acid B was confirmed by the intensive peaks at m/z 519 [M-H-Danshensu, loss of 198 Da], m/z 339 [M-H-Danshesu-caffeic acid, loss of 378], m/z 321 [M-H-2 × Danshensu, loss of 396 Da] and m/z 295 [M-H-Danshensu-caffeic acid-CO_2, loss of 422 Da][82]. Previously, both compounds were detected in *Salvia miltiorrhiza* [83]. Salvianolic acid, known for its antioxidant potential, can effectively remove oxygen free radicals in the human body. This compound is one of the natural products with the strongest antioxidant effect [84]. However, these compounds have been discovered for the first time in apple varieties to the best of our knowledge.

3.5. Quantitative Analysis of Phenolic Compounds by HPLC-PDA

The most effective way of quantification of phenolic compounds is by HPLC-PDA analysis [85]. In our study, 10 phenolic compounds (mainly phenolic acids and flavonoids) were chosen to be quantified since it is difficult to complete the qualification of all the identified compounds. Since a few compounds have too low UV absorption to be detected, the content of phenolic compounds in five apple samples are shown in Table 4.

In phenolic acids, chlorogenic acid, *p*-hydroxybenzoic acid and caffeic acid were the major phenolic acids in Royal Gala, while Pink Lady contained high content in chlorogenic acid, *p*-hydroxybenzoic acid and protocatechuic acid. It was observed that Red Delicious had highest content in caffeic acid when compared to other samples. Caffeic acid, chlorogenic acid and protocatechuic acid were detected in Fuji. Whereas Smitten apples had gallic acid and *p*-hydroxybenzoic acid, these compounds were not observed in Fuji.

According to previous studies, chlorogenic acid and caffeic acid have been identified and quantified in several apple cultivars [86,87]. While Soares et al.'s [88] study indicated that apples, including gala, showed a low concentration of gallic acid and *p*-hydroxybenzoic acid, only few studies focused on identification of Fuji. Hence, further studies are required to analyse the quantitation of Fuji and Smitten.

Table 4. Quantitative analysis in phenolic compounds of five kinds of apple samples.

No.	Compound Name	Molecular Formula	RT (min)	Royal Gala (mg/g)	Pink Lady (mg/g)	Red Delicious (mg/g)	Fuji (mg/g)	Smitten (mg/g)	Phenolic Class
1	Gallic acid	$C_7H_6O_5$	6.836	2.34 ± 0.06^{c}	1.23 ± 0.05^{d}	4.56 ± 0.09^{a}	-	3.25 ± 0.07^{b}	Phenolic acids
2	Protocatechuic acid	$C_7H_6O_4$	12.569	3.69 ± 0.07^{b}	4.59 ± 0.08^{a}	1.25 ± 0.05^{d}	2.59 ± 0.07^{c}	-	Phenolic acids
3	*p*-Hydroxybenzoic acid	$C_7H_6O_3$	20.24	4.6 ± 0.08^{b}	6.37 ± 0.09^{a}	2.13 ± 0.06^{c}	-	1.29 ± 0.05^{d}	Phenolic acids
4	Chlorogenic acid	$C_{16}H_{18}O_9$	20.579	11.25 ± 0.07^{b}	15.69 ± 0.09^{a}	4.59 ± 0.06^{c}	3.18 ± 0.05^{d}	1.24 ± 0.05^{e}	Phenolic acids
5	Caffeic acid	$C_9H_8O_4$	25.001	4.56 ± 0.06^{c}	2.14 ± 0.05^{e}	10.25 ± 0.09^{a}	5.69 ± 0.07^{b}	3.69 ± 0.05^{d}	Phenolic acids
6	Catechin	$C_{15}H_{14}O_6$	19.704	15.64 ± 0.08^{b}	10.25 ± 0.08^{c}	3.68 ± 0.05^{e}	18.61 ± 0.09^{a}	4.59 ± 0.07^{d}	Flavonoids
7	Epicatechin	$C_{15}H_{14}O_6$	24.961	7.13 ± 0.08^{a}	2.14 ± 0.06^{b}	2.14 ± 0.05^{b}	2.39 ± 0.06^{b}	7.59 ± 0.09^{a}	Flavonoids
8	Epicatechin gallate	$C_{22}H_{18}O_{10}$	38.015	3.21 ± 0.07^{a}	0.26 ± 0.02^{c}	-	1.21 ± 0.05^{b}	3.67 ± 0.07^{a}	Flavonoids
9	Quercetin	$C_{15}H_{10}O_7$	70.098	18.96 ± 0.08^{b}	7.45 ± 0.06^{d}	19.67 ± 0.09^{a}	4.98 ± 0.05^{e}	14.79 ± 0.07^{c}	Flavonoids
10	Kaempferol	$C_{15}H_{10}O_6$	80.347	14.25 ± 0.09^{a}	3.69 ± 0.05^{e}	9.67 ± 0.07^{c}	11.59 ± 0.08^{b}	6.97 ± 0.07^{d}	Flavonoids

Experiments performed in triplicates are expressed as the mean ± SD. Means followed by different letters (a, b, c, d, e) within the same column are significantly different ($p < 0.05$) from each other. Data of five kinds of apples are reported (fw).

In flavonoids, a total of four flavonoids (catechin, epicatechin, quercetin, kaempferol) were detected among five apple samples. In general, Fuji was detected the highest catechin content while Red Delicious was the lowest. In contrast, the highest quercetin was detected in Red Delicious while Fuji contained the lowest quercetin. Epicatechin was detected in Royal Gala and Smitten the compounds were 7.13 $\pm$ 0.08 mg/g and 7.59 $\pm$ 0.09 mg/g respectively. Smitten contained the highest Kaempferol (14.25 $\pm$ 0.09 mg/g) among five samples. Compound epicatechin gallate was negligible in all the samples.

Previous studies showed that catechin and quercetin are main flavonoids that contribute to the antioxidant potential of apples [61,89]. Previously reported that epicatechin and kaempferol have been successfully synthesised and characterised [90,91]. However, to the best of our knowledge epicatechin gallate was not detected in apples hence more further studies are needed to verify the detection of this flavonoids.

In conclusion, Royal Gala, Red Delicious and Smitten had abundant quercetin content. Pink Lady had a high concentration of compounds including chlorogenic acid and catechin. Fuji had most abundant amount kaempferol and catechin content among five samples. Finally, phenolic acids were more abundant in Pink Lady and Royal Gala while flavonoids were more abundant in Royal Gala, which is consistent with the previous study.

4. Conclusions

In conclusion, various methods have been successfully utilized for the determination, characterisation, and quantitation of phenolic compounds among five different varieties of Australian grown apples. In phenolic compound estimation, Red Delicious showed higher TPC, TFC, DPPH, FRAP, ABTS and TAC values than other apple samples while Fuji exhibited the highest TTC value. The correlation between flavonoids and phenolic acids exhibited a major contribution towards the antioxidant activities of apples. The LC-ESI-QTOF-MS/MS qualification identified a total of 97 different phenolic compounds in five apple samples, including phenolic acids, flavonoids, lignans, other polyphenols and stilbenes. 10 phenolic compounds were quantification through HPLC-PDA based on the difference of UV spectra and retention times. The analysis showed that phenolic acids were more abundant in Pink Lady and Royal Gala whereas flavonoids were more abundant in Royal Gala.

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