Identification of Phenolic Compounds in Australian-Grown Bell Peppers by Liquid Chromatography Coupled with Electrospray Ionization-Quadrupole-Time-of-Flight-Mass Spectrometry and Estimation of Their Antioxidant Potential

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ABSTRACT: Bell peppers are widely considered as healthy foods that can provide people with various phytochemicals, especially phenolic compounds, which contribute to the antioxidant property of bell peppers. Nevertheless, the acknowledgment of phenolic compounds in bell peppers is still limited. Therefore, this study aimed to determine the phenolic content and the antioxidant potential in pulps and seeds of different bell peppers (green, yellow, and red) by several in vitro assays followed by the characterization and quantification of individual phenolics using liquid chromatography coupled with electrospray ionization-quadrupole-time-of-flight-mass spectrometry (LC-ESI-QTOF-MS/MS) and high-performance liquid chromatography photo-diode array (HPLC-PDA) quantification, respectively. The captured results showed that the pulp of red bell peppers exhibited the highest phenolic content in the total polyphenol content (1.03 ± 0.07 mg GAE/$g_{f.w.}$), total flavonoid content (137.43 ± 6.35 μg QE/$g_{f.w.}$), and total tannin content (0.22 ± 0.01 mg CE/$g_{f.w.}$) as well as the most antioxidant potential in all antioxidant capacity estimation assays including total antioxidant capacity (3.56 ± 0.01 mg AAE/$g_{f.w.}$), 2,2′-diphenyl-1-picrylhydrazyl (0.89 ± 0.01 mg AAE/$g_{f.w.}$), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (1.36 ± 0.12 mg AAE/$g_{f.w.}$), and ferric reducing antioxidant power (0.15 ± 0.01 mg AAE/$g_{f.w.}$). LC-ESI-QTOF-MS/MS isolated and identified a total of 59 phenolic compounds, including flavonoids (21), phenolic acids (20), other phenolic compounds (12), lignans (5), and stilbenes (1) in all samples. According to HPLC-PDA quantification, the seed portions showed a significantly higher amount of phenolic compounds. These findings indicated that the waste of bell peppers can be a potential source of phenolic compounds, which can be utilized as antioxidant ingredients in foods and nutritional products.
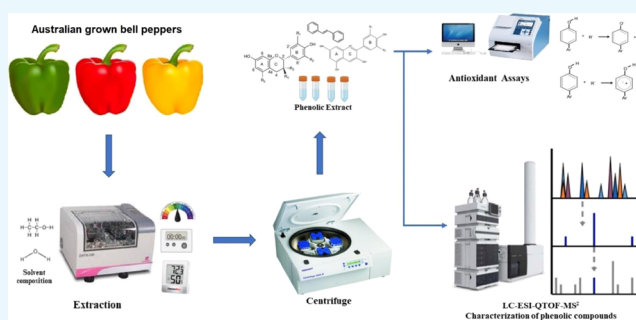
1. INTRODUCTION

Capsicums are typical economical crops belonging to the Solanaceae family and are mainly distributed in tropical and temperate areas.[1] The global production of capsicums was over 11 million tons in 1990 and reached 28 million tons in 2009.[2] According to Australian Bureau of Statistics, the total capsicum (except chilies) production in Australia in 2015 was 38,579 tons and had risen to 49,038 tons in 2017.[3,4] Generally, there are five popular species around the world: *Capsicum annuum* L., *Capsicum baccatum* L., *Capsicum chinese* Jacq., *Capsicum frutescens* L., and *Capsicum pubescens*, among which bell peppers (*Capsicum annuum* L.) are consumed globally as vegetables or processed into different products because of their beautiful color, impressive taste, and nutritional value.[5,6] Bell peppers have many beneficial and pharmacological character-istics such as antioxidative, anti-inflammatory, and anticarcino-genic properties.[7,8] These health-promoting properties are mainly related to the bioactive components including vitamins, carotenoids, and especially phenolic compounds.[9]

Phenolic compounds are the secondary metabolites that are widely found in plants. Generally, phenolic compounds contain one or more hydroxyl groups attached to an aromatic ring including simple types and polymers.[10] These phenolic compounds are normally involved in defense responses in plants such as the insect-repellent property, resistance to ultraviolet interference, and protection against genetic mutations during the physiological activities of plants.[11,12]

Table 1. Summary Table of Phytochemical and Antioxidant Assays in Green, Yellow, and Red Bell Capsicum Fruits[a]

sample	green bell capsicum		yellow bell capsicum		red bell capsicum	
	pulp	seed	pulp	seed	pulp	seed
TPC (mg GAE/$g_{f.w.}$)	0.39 ± 0.02^c	0.43 ± 0.01^c	0.72 ± 0.06^b	0.52 ± 0.02^c	1.03 ± 0.07^a	0.53 ± 0.03^c
TFC (μg QE/$g_{f.w.}$)	30.21 ± 2.36^b	4.98 ± 0.07^c	14.93 ± 0.27^{bc}	3.66 ± 0.05^c	137.43 ± 6.35^a	0.54 ± 0.01^c
TTC (mg CE/$g_{f.w.}$)	0.05 ± 0.00^d	0.11 ± 0.01^{bcd}	0.07 ± 0.00^{cd}	0.13 ± 0.01^b	0.22 ± 0.01^a	0.12 ± 0.00^{bc}
TAC (mg AAE/$g_{f.w.}$)	1.43 ± 0.08^c	0.76 ± 0.00^{de}	2.46 ± 0.03^b	0.88 ± 0.01^d	3.56 ± 0.01^a	0.58 ± 0.03^e
DPPH (mg AAE/$g_{f.w.}$)	0.17 ± 0.01^c	0.12 ± 0.01^c	0.75 ± 0.04^b	0.15 ± 0.00^c	0.89 ± 0.01^a	0.11 ± 0.00^c
ABTS (mg AAE/$g_{f.w.}$)	0.49 ± 0.02^c	0.38 ± 0.01^c	0.96 ± 0.08^b	0.56 ± 0.06^c	1.36 ± 0.12^a	0.56 ± 0.03^c
FRAP (mg AAE/$g_{f.w.}$)	0.02 ± 0.00^c	0.02 ± 0.00^{bc}	0.10 ± 0.00^{ab}	0.04 ± 0.00^{bc}	0.15 ± 0.01^a	0.02 ± 0.00^c

[a]The data are shown as mean $\pm$ standard deviation ($n = 3$); Superscripts a, b, c, d, and e indicate the means in a row with significant difference ($p < 0.05$) using one-way analysis of variance (ANOVA) and Tukey's test. GAE, gallic acid equivalents; QE, quercetin equivalents; CE, catechin equivalents; AAE, ascorbic acid equivalents; total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), total antioxidant content (TAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic) acid (ABTS+) and ferric reducing antioxidant power (FRAP); f.w., fresh weight.

Polyphenolics can be divided into several groups, including phenolic acids, flavonoids, lignans, and stilbenes, which can be extracted using different organic solvents.[13,14] Phenolic compounds exhibit their antioxidant abilities through different mechanisms such as chelating metal ions, scavenging free radicals, and disintegrating peroxides.[15] Commercially, polyphenols are regarded as the supplementary ingredients in human diets because of their superb antioxidant potentials.[16]

Previously, bell peppers were proved to be good sources of phenolic compounds with remarkable antioxidant potential by Blanco-Rios et al.[17] In the present study, polyphenolic contents in bell peppers were evaluated by various in vitro assays such as total polyphenol content (TPC), total flavonoid content (TFC), and total tannin content (TTC), while 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and total antioxidant capacity (TAC) were used to assess the antioxidant potentials.[18,19] However, the colorimetric methods neither isolate nor quantify the individual phenolic compounds. High-performance liquid chromatography coupled with electrospray ionization-quadrupole-time-of-flight-mass spectrometry (LC-ESI-QTOF-MS/MS) and high-performance liquid chromatography photodiode array (HPLC-PDA) quantification are the effective techniques to characterize and quantify various phenolic compounds present in different food materials.[20] Although some phenolic compounds have been identified in bell peppers through high-performance liquid chromatography (HPLC) such as quercetin and luteolin,[6] only a few research studies were related to Australian-grown bell peppers, and the contents and antioxidant capacities of these phenolic compounds in the bell peppers are still unclear.

The objectives of this research were: (a) to extract the polyphenols from different portions of bell peppers, (b) to compare the polyphenol contents and the antioxidant capacities in the bell pepper samples, and (c) to characterize and quantify individual phenolic compounds using LC-ESI-Q-TOF-MS/MS and HPLC-PDA. The outcome of the research could provide a comprehensive analysis of phenolic compounds in Australian-grown bell peppers for further exploitation in the food and pharmaceutical industries.

2. RESULTS AND DISCUSSION

2.1. Estimation of Phenolic Compounds in Bell Peppers (TPC, TFC, and TTC).
TPC, TFC, and TTC assays were utilized to evaluate the phenolic content of different

portions of Australian bell peppers. The result is displayed in Table 1, and the TPC of red bell capsicum pulp was significantly higher than that of other samples (1.03 ± 0.07 mg GAE/$g_{fresh\ weight\ (f.w.)}$, $p < 0.05$) followed by yellow bell pepper pulp and red bell pepper seed (0.72 ± 0.06 mg GAE/$g_{f.w.}$ and 0.53 ± 0.03 mg GAE/$g_{f.w.}$, respectively). A previous study by Park et al.[21] also indicated that red capsicums obtained a higher content of polyphenols than other colors of bell capsicums, while the TPC of red bell pulp in our research is less than that of red sweet peppers grown in Kunming, China (from 2 to 5 mg GAE/$g_{f.w.}$).[22] This variation may be related to the accumulation of the ascorbic acid in capsicums,[23] which can also react with Folin−Ciocalteu reagent and may cause an overestimation of TPC values.[24]

Flavonoids were found rich in various vegetables and play an important role in daily diets.[25] Among all the pulp parts, red bell peppers had the highest value of TFC of 137.43 ± 6.35 μg QE/$g_{f.w.}$ ($p < 0.05$), while the TFC of yellow pulp and green pulp had no significant difference. In terms of the seed portions, three bell peppers exhibited a range from 0.54 to 4.98 μg QE/$g_{f.w.}$. Previous research by Sun et al.[26] noticed that red peppers showed a significantly higher flavonoid content than green peppers, which was in agreement with our research. Flavonoid is a kind of pigment to prevent damage from sunlight, which will increase with the exposure time to UV radiation according to Mahdavian et al.[27] Regarding the TTC assay, the red pulp contained the highest content of tannins (0.22 ± 0.01 mg CE/$g_{f.w.}$), which is comparable with the previous study.[28]

2.2. Antioxidant Capacity of Bell Peppers (DPPH, ABTS, FRAP, and TAC).
DPPH, ABTS, FRAP, and TAC assays were four common assays that were used to determine the antioxidant potential of samples through different mechanisms. The results were expressed as milligram ascorbic acid equivalents per gram in fresh weight (mg AAE/$g_{f.w.}$) in Table 1.

The TAC varied dramatically among the three types of bell peppers in this research. The pulp of red bell peppers exhibited the highest antioxidant capacity of 3.56 ± 0.01 mg AAE/$g_{f.w.}$, followed by yellow and green pulps (2.46 ± 0.03 mg AAE/$g_{f.w.}$ and 1.43 ± 0.08 mg AAE/$g_{f.w.}$, respectively). The variation might be explained by the fact that the capsicums were harvested at different ripening stages. The antioxidant activity would be enhanced with the fruit ripening stage because of the accumulation of phytochemicals such as phenolic compounds, ascorbic acid, and carotenoids in the flesh part.[26,29] All of these

Table 2. Correlation Table of Phytochemical and Antioxidant Assays in Different Capsicum Fruits[a]

	TPC	TFC	TTC	DPPH	FRAP	ABTS
TFC	0.841*					
TTC	0.747	0.736				
DPPH	0.920**	0.746	0.448			
FRAP	0.984**	0.846*	0.637	0.958**		
ABTS	0.972**	0.820*	0.617	0.976**	0.985**	
TAC	0.898*	0.866*	0.479	0.965**	0.950**	0.958**

[a]*Indicates that the correlation between two assays is significantly correlated ($p < 0.05$); **indicates that the correlation between two assays is highly correlated ($p < 0.01$); total phenolic content: TPC, total flavonoid content: TFC, total tannin content: TTC, total antioxidant content: TAC, 2,2-diphenyl-1-picrylhydrazyl: DPPH, 2,2-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic) acid: ABTS and ferric reducing antioxidant power: FRAP.

accumulated phytochemicals would contribute to the antioxidant activity of the bell peppers.[30]

According to Table 1, the highest DPPH activity among all the samples was found in the red pepper pulp (0.89 ± 0.01 mg AAE/$g_{f.w.}$), followed by yellow pepper pulp (0.75 ± 0.04 mg AAE/$g_{f.w.}$), while the other portions did not show a significant difference with a range from 0.11 to 0.17 mg AAE/$g_{f.w.}$. Meanwhile, the result of ABTS exhibited a similar trend. In the ABTS assay, red pulp accounted for the highest free radical scavenging capacity (1.36 ± 0.12 mg AAE/$g_{f.w.}$). Our findings were supported by previous studies, which reported the green bell peppers to have a lower free radical scavenging ability than dark-color peppers such as yellow or red bell peppers.[16,21] This variation might be involved in the fact that nutrients are continuously accumulated with mature stages.[31]

FRAP is generally used to determine the metal ion-chelating ability of the food matrix.[32] The highest value of FRAP was found in the pulp of red bell capsicums (0.15 ± 0.01 mg AAE/$g_{f.w.}$), which might be explained by the theory that the metal ion-chelating ability would increase with the maturing stage of peppers as described by Sim and Sil.[33] In short, phenolic and antioxidant properties differ among species and cultivars of the same plants grown in different regions under different climatic conditions, soil characteristics, and cultivation techniques. Moreover, the efficiency of the extraction of phenolics also varies under different extraction conditions, such as the type of solvents, solvent concentration, extraction time, temperature, solvent-to-solid ratio, and so on.[34]

2.3. Correlation of Phenolic Content and Antioxidant Assays. Pearson's correlations between the specific phenolic content (TPC, TFC, and TTC) and antioxidant assays (DPPH, ABTS, FRAP, and TAC) were performed to reveal the relationships between the phenolic content and antioxidant assays. The correlation coefficients are listed in Table 2, and the TPCs of bell peppers were positively correlated with all other assays except TTC (TFC, $r = 0.841$, $p < 0.05$; DPPH, $r = 0.920$, $p < 0.01$; FRAP, $r = 0.984$, $p < 0.01$; ABTS, $r = 0.972$, $p < 0.01$; TAC, $r = 0.898$, $p < 0.05$). In the previous study by Parikh and Patel,[35] the TPC and TFC also exhibited a significant positive correlation in the food matrix ($r = 0.8977$ and $p < 0.05$), while the positive relationships between TPC and DPPH, as well as TPC and ABTS, were also reported by Cortés-Estrada et al.[14] and Alvarez-Parrilla et al.[36] The positive correlation among the TPC and antioxidant assays indicated that the phenolic compounds were the main contributors to the antioxidant capacity of all bell peppers. The positive correlations were also found between total flavonoids with FRAP, ABTS, and TAC ($r = 0.8462$, $p < 0.05$; $r = 0.8201$, $p < 0.05$; $r = 0.8655$, $p < 0.05$, respectively), revealing that the flavonoid compound was one of the primary contributors to

the antioxidant capacities. The phenolic contents in bell peppers exhibited remarkable antioxidant potentials; therefore, it is necessary to characterize the individual polyphenol for a more comprehensive understanding of the phenolic composition.

2.4. LC-ESI-QTOF-MS/MS Characterization of the Phenolic Compounds. Liquid chromatography—mass spectrometry (LC—MS) is a typical method used to identify phytochemicals in fruits and vegetables. In our research, untargeted qualitative analysis of individual phenolic compounds from bell peppers was conducted in both positive and negative ionization modes separately (Supporting Information) followed by the putative identification and characterization approach.[37] Table 3 shows the putative identifications of the phenolic compounds that were tentatively identified based on their theoretical m/z values and isotope distributions (confirmation of the formulas) and MS/MS spectra (tentative confirmation of the structures) using Agilent MassHunter qualitative software and Personal Compound Database and Library (PCDL). Compounds with mass error $< \pm 5$ ppm were selected for further MS/MS identification and characterization purposes. In total, 59 compounds were tentatively isolated from all these three bell peppers, including phenolic acid (20), flavonoids (21), other polyphenols (12), and lignans (6).

2.4.1. Phenolic Acid. Phenolic acids have been identified as vital phenolic compounds in bell peppers.[14] In this research, four subgroups of phenolic acids were identified in bell peppers, including hydroxybenzoic acids, hydroxycinnamic acids, hydroxyphenylpentanoic acids, and hydroxyphenylacetic acids.

2.4.1.1. Hydroxybenzoic and Hydroxyphenylpentanoic Acid Derivatives. Compound **1** was found in the seed and pulp of yellow peppers in both positive and negative modes with observed $[M - H]^-$ m/z at 331.0674, RT = 6.866. According to the fragmentations, compound **1** suffered a neutral loss of 162 Da and a further loss of CO_2 molecules (42 Da), resulting in the product ion at m/z 169 and m/z 125, respectively. These two fragments were reported to be the identical loss of galloyl glucose,[38] which was previously characterized from berries, such as blackcurrants, instead of *capsicum* sp.[39] Compound **2** with $[M + H]^+$ m/z at 185.0444 was only detected in the seed of green bell pepper and characterized as 4-O-methylgallic acid according to the product ion at 170 and 142 m/z, corresponding to the loss of CH_3 (15 Da) and ($CH_3 + CO$) (43 Da), respectively.[40] Gallic acid and its derivatives 4-O-methylgallic acid are reported to be the main hydroxybenzoic compounds isolated from bell peppers with remarkable antioxidant capacity.[11] 2-Hydroxybenzoic acid (compound **3** at $[M - H]^-$ m/z 137.0247) was discovered in the seeds of red and yellow bell peppers. The identity was

Table 3. Characterization of Phenolic Compounds in Bell Peppers Using LC-ESI-QTOF-MS/MS[b]

no.	proposed compounds	molecular formula	RT (min)	ionization (ESI⁺/ESI⁻)	molecular weight	theoretical (m/z)	observed (m/z)	mass error (ppm)	MS/MS product ions	bell pepper samples
phenolic acid										
hydroxybenzoic acids										
1	galloyl glucose	$C_{13}H_{16}O_{10}$	6.866	[M − H]⁻[b]	332.0743	331.067	331.0674	1.2	169, 125	YCS, YCP[a]
2	4-O-methylgallic acid	$C_8H_8O_5$	12.938	[M + H]⁺	184.0372	185.0445	185.0444	−0.5	170, 142	GCS
3	2-hydroxybenzoic acid	$C_7H_6O_3$	23.646	[M − H]⁻	138.0317	137.0244	137.0247	2.2	93	YCS, RCS[a]
hydroxycinnamic acids										
4	caffeic acid	$C_9H_8O_4$	4.575	[M − H]⁻	180.0423	179.035	179.0353	1.7	151, 143, 133	RCS
5	p-coumaroyl malic acid	$C_{13}H_{12}O_7$	5.953	[M − H]⁻[b]	280.0583	279.051	279.0514	1.4	163	GCS, YCS, RCS[a]
6	1,2,2′-triferuloylgentiobiose	$C_{42}H_{46}O_{20}$	9.548	[M − H]⁻	870.2582	869.2509	869.2533	2.8	693, 517	YCS
7	caffeic acid 3-O-glucuronide	$C_{15}H_{16}O_{10}$	12.082	[M − H]⁻[b]	356.0743	355.0670	355.0659	−3.1	179	YCP, RCP[a]
8	cinnamic acid	$C_9H_8O_2$	12.430	[M + H]⁻[b]	148.0524	149.0597	149.0603	4.0	103	YCP, GCP, RCP, YCS, RCS[a]
9	caffeoyl glucose	$C_{15}H_{18}O_9$	18.809	[M − H]⁻	342.0951	341.0878	341.0892	4.1	179, 161	GCS, YCS, RCS[a]
10	3-feruloylquinic acid	$C_{17}H_{20}O_9$	20.847	[M − H]⁻[b]	368.1107	367.1034	367.1025	−2.5	298, 288, 192, 191	GCS, YCS, RCS, RCP, YCP[a]
11	3-caffeoylquinic acid	$C_{16}H_{18}O_9$	24.757	[M − H]⁻	354.0951	353.0878	353.0891	3.7	253, 190, 144	GCS, YCS[a]
12	ferulic acid 4-O-glucoside	$C_{16}H_{20}O_9$	29.312	[M − H]⁻	356.1107	355.1034	355.1043	2.5	193, 178, 149, 134	GCS, YCS, GCP[a]
13	1,5-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	31.035	[M − H]⁻	516.1268	515.1195	515.1208	2.5	353, 335, 191, 179	GCS, YCS[a]
14	rosmarinic acid	$C_{18}H_{16}O_8$	39.746	[M − H]⁻[b]	360.0845	359.0772	359.0773	0.3	179	GCS
15	isoferulic acid	$C_{10}H_{10}O_4$	42.847	[M − H]⁻[b]	194.0579	193.0506	193.0506	0.0	178, 149, 134	GCS, YCS, RCS[a]
16	cinnamoyl glucose	$C_{15}H_{18}O_7$	60.985	[M − H]⁻[b]	310.1053	309.0980	309.0965	−4.9	147, 131, 103	GCS
hydroxyphenylacetic acids										
17	3,4-dihydroxyphenylacetic acid	$C_8H_8O_4$	27.904	[M − H]⁻[b]	168.0423	167.0350	167.0346	−2.4	149, 123	GCS, YCS, RCS, GCP[a]
18	2-hydroxy-2-phenylacetic acid	$C_8H_8O_3$	36.105	[M − H]⁻	152.0473	151.0400	151.0407	4.6	136, 92	GCS
hydroxyphenylpentanoic acids										
19	3-hydroxy-3-(3-hydroxyphenyl) propionic acid	$C_9H_{10}O_4$	12.842	[M − H]⁻	182.0579	181.0506	181.0509	1.6	163, 135, 119	RCS
20	dihydrocaffeic acid 3-O-glucuronide	$C_{15}H_{18}O_{10}$	20.796	[M − H]⁻[b]	358.0900	357.0827	357.0828	0.3	181	GCP, YCP, RCP, GCS[a]
flavonoids										
flavanols										
21	(−)-epigallocatechin	$C_{15}H_{14}O_7$	10.622	[M − H]⁻[b]	306.0740	305.0667	305.0659	−2.6	261, 219	YCP, GCP, RCS, RCP[a]
22	4″-O-methylepigallocatechin 3-O-gallate	$C_{23}H_{20}O_{11}$	11.410	[M − H]⁻	472.1006	471.0933	471.0950	4.0	319, 169	YCS
23	(+)-catechin 3-O-gallate	$C_{22}H_{18}O_{10}$	22.306	[M − H]⁻[b]	442.0900	441.0827	441.0805	−5.0	289, 169, 125	RCS
24	4′-O-methyl-(−)-epigallocatechin 7-O-glucuronide	$C_{22}H_{24}O_{13}$	25.999	[M − H]⁻	496.1217	495.1144	495.1163	3.8	415, 313	YCS
25	(−)-epicatechin	$C_{15}H_{14}O_6$	31.118	[M − H]⁻[b]	290.0790	289.0717	289.0717	0.0	245, 205, 179	GCS, YCS, RCS, YCP[a]
26	procyanidin trimer C1	$C_{45}H_{38}O_{18}$	33.736	[M − H]⁻	866.2058	865.1985	865.1990	0.6	739, 713, 695	YCS
flavonols										
27	quercetin 3-O-glucosyl-xyloside	$C_{26}H_{28}O_{16}$	15.395	[M − H]⁻[b]	596.1377	595.1304	595.1299	−0.8	265, 138, 116	GCP, YCP, RCP[a]
28	3-methoxysinensetin	$C_{21}H_{22}O_8$	16.528	[M + H]⁺[b]	402.1315	403.1388	403.1395	1.7	388, 373, 355, 327	GCS
29	myricetin 3-O-glucoside	$C_{21}H_{20}O_{13}$	34.017	[M − H]⁻[b]	480.0904	479.0831	479.0833	0.4	317	RCP
flavanones										
30	naringin 4′-O-glucoside	$C_{33}H_{42}O_{19}$	12.481	[M − H]⁻	742.2320	741.2247	741.2273	3.5	433, 271	GCS, RCS[a]
31	7,4′-dihydroxyflavone	$C_{15}H_{10}O_4$	13.972	[M + H]⁺	254.0579	255.0652	255.0641	−4.3	227, 199, 171	YCS
32	apigenin 7-O-glucuronide	$C_{21}H_{18}O_{11}$	15.812	[M + H]⁺[b]	446.0849	447.0922	447.0930	1.8	271, 253	YCS
33	apigenin 6,8-di-C-glucoside	$C_{27}H_{30}O_{15}$	32.261	[M − H]⁻[b]	594.1585	593.1512	593.1505	−1.2	503, 473	GCS, RCS, GCP[a]
isoflavonoids										
34	6″-O-acetyldaidzin	$C_{23}H_{22}O_{10}$	4.413	[M − H]⁻[b]	458.1213	457.1140	457.1125	−3.3	221	RCP
35	6″-O-acetylglycitin	$C_{24}H_{24}O_{11}$	7.378	[M + H]⁺	488.1319	489.1392	489.1387	−1.0	285, 270	YCP, GCP[a]

Table 3. continued

no.	proposed compounds	molecular formula	RT (min)	ionization (ESI⁺/ESI⁻)	molecular weight	theoretical (m/z)	observed (m/z)	mass error (ppm)	MS/MS product ions	bell pepper samples
36	6″-O-malonyldaidzin	$C_{24}H_{22}O_{12}$	16.246	[M + H]⁻[b]	502.1111	503.1184	503.1200	3.2	255	YCS
37	3′-hydroxygenistein	$C_{15}H_{10}O_6$	31.357	[M + H]⁺	286.0477	287.0550	287.0540	−3.5	269, 259	GCS
38	2′-hydroxyformononetin	$C_{16}H_{12}O_5$	37.872	[M + H]⁺	284.0685	285.0758	285.0767	3.2	270, 253, 229, 225	GCP
39	formononetin 7-O-glucuronide	$C_{22}H_{20}O_{10}$	41.953	[M − H]⁻	444.1056	443.0983	443.0976	−1.6	267, 252	YCS, GCS, RCS[a]
40	3-hydroxyphloretin 2′-O-glucoside	$C_{21}H_{24}O_{11}$	42.383	[M − H]⁻	452.1319	451.1246	451.1237	−2.0	289, 273	GCS, RCS[a]
41	dihydromyricetin 3-O-rhamnoside	$C_{21}H_{22}O_{12}$	45.349	[M − H]⁻	466.1111	465.1038	465.1036	−0.4	301	GCS
other polyphenols										
hydroxybenzaldehydes										
42	p-anisaldehyde	$C_8H_8O_2$	29.312	[M + H]⁺[b]	136.0524	135.0451	135.0452	0.7	122, 109	YCS, RCP, GCP, YCP[a]
curcuminoids										
43	Demethoxycurcumin	$C_{20}H_{18}O_5$	76.809	[M − H]⁻	338.1154	337.1081	337.1087	1.8	217	RCS, GCS[a]
furanocoumarins										
44	isopimpinellin	$C_{13}H_{10}O_5$	4.478	[M + H]⁺[b]	246.0528	247.0601	247.0605	1.6	232, 217, 205, 203	RCS
45	2,3-dihydroxy-1-guaiacylpropanone	$C_{10}H_{12}O_5$	9.879	[M − H]⁻[b]	212.0685	211.0612	211.0602	−4.7	167, 123, 105, 93	YCS
46	2-hydroxy-4-methoxyacetophenone 5-sulfate	$C_9H_{10}O_7S$	12.891	[M − H]⁻	262.0147	261.0074	261.0078	1.5	181, 97	RCS
47	coumarin	$C_9H_6O_2$	15.875	[M + H]⁺	146.0368	147.0441	147.0447	4.1	103, 91	YCP
48	esculin	$C_{15}H_{16}O_9$	82.047	[M + H]⁺	340.0794	341.0867	341.087	0.9	179, 151	GCP, RCP[a]
tyrosols										
49	3,4-DHPEA-AC	$C_{10}H_{12}O_4$	9.515	[M − H]⁻	196.0736	195.0663	195.0667	2.1	135	GCS, RCS[a]
50	demethyloleuropein	$C_{24}H_{30}O_{13}$	23.100	[M − H]⁻	526.1686	525.1613	525.1621	1.5	495	GCS, YCS[a]
51	3,4-DHPEA-EDA	$C_{17}H_{20}O_6$	23.564	[M − H]⁻	320.1260	319.1187	319.1189	0.6	275, 195	YCS, RCS[a]
52	hydroxytyrosol 4-O-glucoside	$C_{14}H_{20}O_8$	43.474	[M − H]⁻	316.1158	315.1085	315.1072	−4.1	153, 123	RCS
53	lithospermic acid	$C_{27}H_{22}O_{12}$	31.035	[M − H]⁻	538.1111	537.1038	537.1047	1.7	493, 339, 295	GCS, YCS[a]
lignans										
lignans										
54	enterolactone	$C_{18}H_{18}O_4$	4.234	[M + H]⁺[b]	298.1205	299.1278	299.1279	0.3	281, 187, 165	GCS
55	schisanhenol	$C_{23}H_{30}O_6$	22.684	[M + H]⁺	402.2042	403.2115	403.2100	−3.7	385, 354, 331	GCP[a]
56	schisandrin C	$C_{22}H_{24}O_6$	32.682	[M + H]⁺[b]	384.1573	385.1646	385.1651	1.3	370, 315, 300	GCS, RCS[a]
57	todolactol A	$C_{20}H_{24}O_7$	41.489	[M − H]⁺[b]	376.1522	375.1449	375.1438	3.4	313, 137	GCS, YCS, RCS, RCP[a]
58	matairesinol	$C_{20}H_{22}O_6$	61.187	[M − H]⁻	358.1416	357.1343	357.1337	−1.7	342, 327, 313, 221	YCS
stilbenes										
stilbenes										
59	resveratrol 3-O-glucoside	$C_{20}H_{22}O_8$	42.864	[M − H]⁻	390.1315	389.1242	389.1228	−3.6	227	GCS, YCS, RCS[a]

[a]Compound was detected in more than one bell pepper samples, and data presented in this table are from the asterisk sample. [b]Compounds were detected in both negative [M − H]⁻ and positive [M + H]⁺ modes of ionization while only single-mode data were presented.

confirmed by its product ion at m/z 93, corresponding to the loss of CO_2 (44 Da).[41] 2-Hydroxybenzoic acid is a kind of phenolic compound abundant in fruits, vegetables, and herbs.[42] To the best of our knowledge, this is the first time that the presence of 2-hydroxybenzoic acid in bell peppers is reported.

Compound **19** is a derivative of hydroxyphenylpentanoic acids putatively characterized in the ESI⁻ mode with observed m/z at 181.0509 in the seed of red bell peppers. The distinctive fragments were observed at m/z 163, 135, and 119 which are related to the loss of H_2O, HCOOH, and H_2O plus CO_2, respectively.[43] 3-Hydroxy-3-(3-hydroxyphenyl) propionic acid has been captured from the hops and juniper berry,[44] while this is the first time to characterize it from capsicum spp.

2.4.1.2. Hydroxycinnamic Acid Derivatives. Hydroxycinnamic acids exist in foods in either free form or bound to other polymers,[45] and a total of 13 hydroxycinnamic acids were tentatively characterized in the extracts of bell peppers. Caffeic acid (compound **4**) with [M − H]⁻ m/z at 179.0353 was identified from the seed of red bell peppers, and the product ions were noticed at 151, 143, and 133, because of the loss of CO (28 Da), two H_2O (36 Da) molecules, and HCOOH (46 Da), respectively.[46] Caffeic acid has been successfully isolated from various fruits and vegetables, such as bell peppers, apple, kiwi fruits, pear, and guava with remarkable antioxidant potential through HPLC experiments.[17,45,47,48]

The precursor of 1,2,2′-triferuloylgentiobiose (compound **6**) was at [M − H]⁻ m/z 869.2533, providing fragment ions at m/z 693 ($C_{32}H_{38}O_{17}$) and m/z 517 ($C_{22}H_{30}O_{14}$), corresponding

to the loss of one feruloyl unit ($C_{10}H_8O_3$) and two feruloyl units, respectively.[49] 1,2,2′-Triferuloylgentiobiose is a derivative of ferulic acid which has been identified in broccoli cultivars.[50] To the best of our knowledge, this is the first time that 1,2,2′-triferuloylgentiobiose is discovered in bell peppers.

Compounds **5**, **9**, and **15** were three hydroxycinnamic acids only isolated from seeds of bell peppers. Compound **5** detected in both modes with observed $[M − H]^−$ m/z at 279.0514 was inferred as *p*-coumaroyl malic acid according to the fragment at m/z 163, corresponding to the loss of malic acid ($C_4H_4O_4$), and it was previously identified in the powder of tomatoes.[51,52] Caffeoyl glucose (compound **9**) was characterized in the negative mode at m/z 341.0892, in which the MS^2 spectrum showed the product ions at m/z 179 and m/z 161, corresponding to the loss of the hexosyl moiety and further loss of a water molecule.[53] Caffeoyl glucose has been isolated from black currents, tomatoes, eggplants, chili peppers, and bell peppers with superb antioxidant potentials.[45] Compound **15** was tentatively identified as isoferulic acid ($[M − H]^−$ m/z at 193.0506) because of the loss of CH_3, CO_2, and (CH_3 + CO_2), resulting in the major fragment ions at m/z 178, m/z 149, and m/z 134, respectively.[43] The identification of ferulic acid 4-*O*-glucoside (compound **12** with m/z 355.1043 in the ESI$^−$ mode) was achieved by the product ions at m/z 193, m/z 178, m/z 149, and m/z 134, which arose from the loss of $C_6H_{10}O_5$ (162 Da), $C_7H_{13}O_5$ (177), $C_7H_{10}O_7$ (206 Da), and $C_8H_{13}O_7$ (221 Da) from the parent ion, respectively.[54] Isoferulic acid is generally isolated from Chinese propolis that play an important role in scavenging free radicals and was also reported in bell peppers in a previous study.[55,56]

Compounds **11** and **13** were two compounds captured in the seeds of green and yellow bell peppers. 3-Caffeoylquinic acid (compound **11**) was detected in the negative mode with observed m/z at 353.0891, and the fragmentation resulted in the product ions at m/z 253 $[M − H−3H_2O−HCOOH]^−$, m/z 190 $[M − H−3H_2O−C_6H_5O_2]^−$, and m/z 144 $[M − H−H_2O−C_7H_{11}O_6]^−$.[46] 1,5-Dicaffeoylquinic acid was proposed as compound **13**, showing the parent ion at m/z 515.1208 in the ESI$^−$ mode. The MS^2 spectrum showed the product ions at m/z 353, 335, and m/z 191, indicating the possible loss of $C_9H_6O_3$ (162 Da), $C_9H_8O_4$ (180 Da), and $C_{18}H_{12}O_6$ (324 Da) from the parent ion, respectively.[53] Caffeoylquinic acids have been identified in the leaves of red peppers (*capsicum* spp.) as well as *Chrysanthemum coronarium* L. (as known as Tonghao, a kind of edible vegetable) with health-promoting properties.[57,58] Cinnamic acid (compound **8**) was detected in all samples except the seed of green bell peppers, which was found in both ESI$^+$ and ESI$^−$ modes. In the current study, compound **8** with precursor m/z at 149.0603 was expressed in the positive mode of the pulp of red bell peppers, and the typical product ion was at m/z 103, corresponding to the loss of a carboxylic acid moiety unit (46 Da).[54] Cinnamic acid and its derivatives are normally present in fruits and vegetables, including citrus fruits, grape, tea, cocoa, spinach, and celery,[56] while this is the first time that cinnamic acid is reported in bell peppers.

2.4.2. Flavonoids. 2.4.2.1. Flavanols and Flavanone Derivatives.

Compounds **22** and **26** were only observed in the seed of yellow peppers in the negative mode with the precursor at m/z 473.1098 and m/z 865.1990, respectively. Compound **22** was tentatively inferred as 4″-*O*-methylepigallocatechin 3-*O*-gallate by comparing the characteristic fragment ions at m/z 319 and m/z 169, indicating the presence of the galloyl group and methyl-(epi)gallocatechin.[59] Galloylated

catechins have stronger scavenging properties than non-galloylated catechins, and the (−)-epigallocatechin gallate is considered to have the highest ability to prohibit free radicals, which was the first time to be reported in peppers.[60,61] The identity of procyanidin trimer C1 (compound **26**) was confirmed by the major fragments at m/z 739 and m/z 713, corresponding to the loss of 126 Da loss of the heterocyclic ring fission reaction and 152 Da loss of the retro-Diels−Alder reaction, respectively.[62] Compound **25** detected in both modes with observed m/z 289.0717 in the negative mode was identified as (−)-epicatechin found in all the seeds and the pulp of yellow peppers. The product ion at m/z 245 was related to the loss of the CO_2 moiety, while the fragments at m/z 205 and m/z 179 contributed to the loss of a flavonoid A ring (84 Da) and B ring (110 Da), respectively.[41] (−)-Epicatechin has been isolated from cherries, raspberries, and blackberries which was proved to obtain great antioxidant ability.[63] In the present study, epicatechin, catechin, and related derivatives are the main flavanols identified in bell peppers, which is consistent with the previous study.[64]

Compound **30** ($[M − H]^−$, m/z 741.2273) detected in the seeds of green and red bell peppers was tentatively characterized as naringin 4′-*O*-glucoside according to the characteristic fragment ions at m/z 433 and m/z 271, corresponding to the loss of rhamnose plus glucose moieties (308 Da) and rhamnose plus two glucose moieties (470 Da), respectively.[65] Naringin and its derivatives are the typical flavonoids that are mainly captured in citrus plants exhibiting fantastic antioxidant properties in in vitro and in vivo tests.[66]

2.4.2.2. Flavones, Isoflavonoids, Dihydrochalcones, and Dihydroflavonol Derivatives.

7,4′-Dihydroxyflavone (compound **31** with $[M + H]^+$ at m/z 255.0641, RT = 13.972) was only detected from the seed of yellow peppers. The confirmation was achieved by analyzing the product ions at m/z 227, m/z 199, and m/z 171, corresponding to the loss of CO molecules (28 Da), two CO (56 Da) molecules, and three CO (84) molecules from the precursor, respectively.[67] Two apigenin derivatives (compound **32**, apigenin 7-*O*-glucuronide and compound **33**, apigenin 6,7-di-C-glucoside) were also discovered in the peppers. Apigenin 7-*O*-glucuronide ($[M + H]^+$ at m/z 447.0930) was only identified in YCS in the present study, with the product ions at m/z 271 (M + H − 176, loss of a glucuronide moiety) and m/z 253 (M + H − 176, loss of a glucuronide moiety and H_2O), while the confirmation of apigenin 6,8-di-C-glucoside ($[M − H]^−$ at m/z 593.1505) was achieved by comparing the MS/MS spectra at m/z 503 (M − H − 90 Da) and m/z 473 (M − H − 120 Da), resulting from the loss of cross-ring cleavages of the glycoside moiety.[68] Apigenin is a kind of flavone with low toxicity, and it is widespread in the plant kingdom such as onions, celery, and dried parsley, which can help plants to resist the damage of sun light.[69,70] In addition, *O*-glycosylated apigenin was reported to be abundant in bell peppers, which agreed with our research.[71]

Compound **35** ($[M + H]^+$, m/z 489.1387) was captured in the pulp of green and yellow peppers with the product ions at m/z 285 $[M + H−C_2H_2O−C_6H_{10}O_5]^+$ and m/z 270 $[M + H−C_2H_2O−C_6H_{10}O_5−CH_3]^+$; therefore, the compound was speculated as 6″*O*-acetylglycitin.[72] Isoflavonoids are mainly isolated from soybeans, which are commercially utilized as phytoestrogens.[73]

According to the MS spectra, compound **37** was captured at $[M + H]^+$ m/z 287.0540, which could be putatively identified as 3′-hydroxygenistein. The product ions were observed at m/z
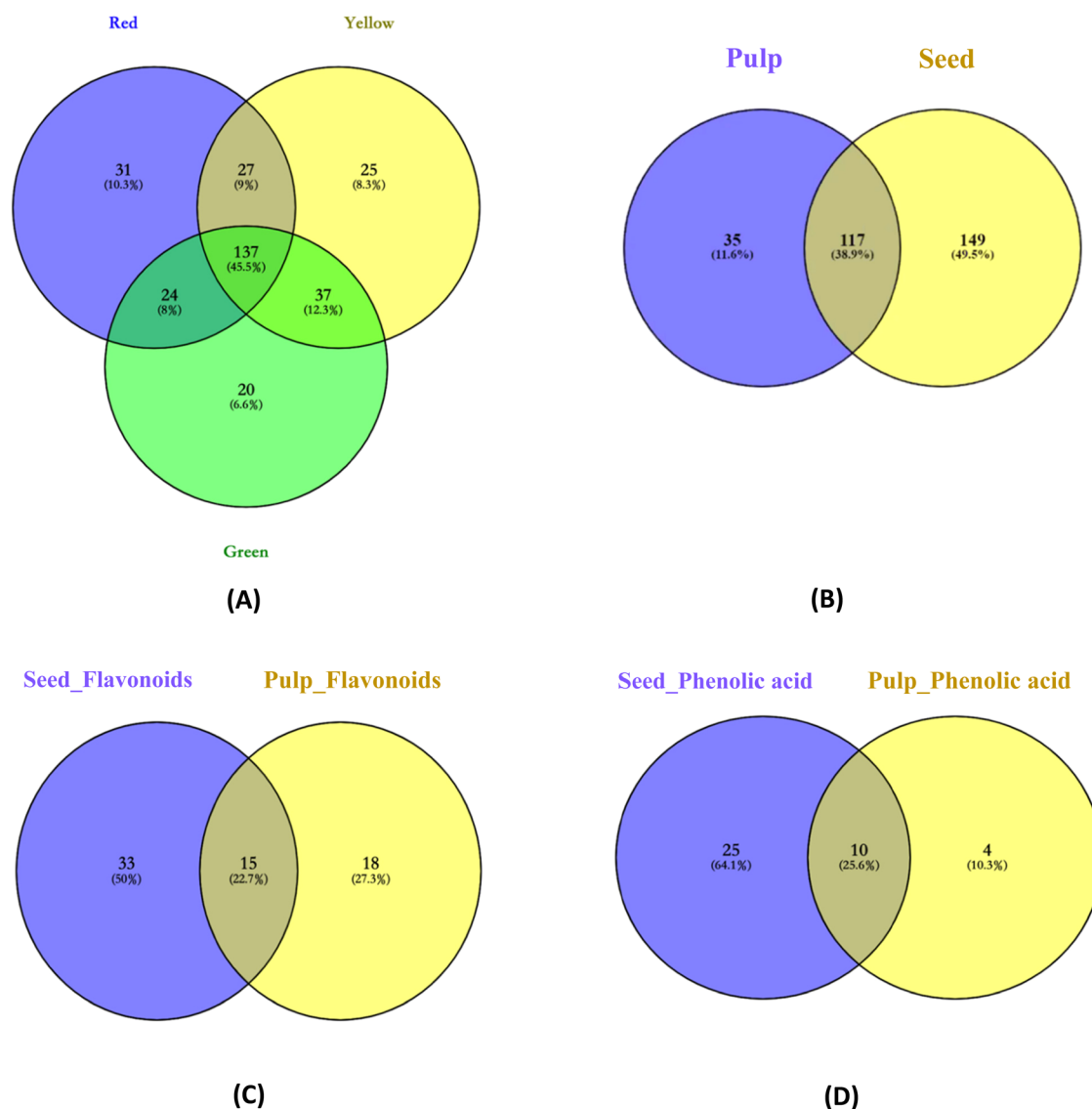
Figure 1. Venn diagram of phenolic compounds present in different colors of bell peppers. (A) Relations of total phenolic compounds present in three colors of Australian bell peppers; (B) relations of phenolic compounds existing in different parts (pulp and seeds); (C) distribution of flavonoids compounds in different parts of bell peppers (pulp and seeds); (D) distribution of phenolic acids in different parts of bell peppers (pulp and seeds).

269 and m/z 259, representing the loss of H_2O (18 Da) and CO (28 Da) from the precursor, respectively.[74] This compound is normally identified from fermented soybean products,[75] and to the best of our knowledge, this is the first time to characterize this compound from bell peppers. Compound **39** with observed $[M - H]^-$ m/z at 443.0976 was inferred as formononetin 7-O-glucuronide because of the main fragments at m/z 267 and m/z 252, resulting from a glucose unit and further loss of CH_3 from the parent ion.[76]

Compound **40** (3-hydroxyphloretin 2′-O-glucoside) was the only dihydrochalcone derivative discovered in the present research, and it was captured in the seeds of green and red bell peppers with observed m/z 451.1237 in the ESI^- mode. According to the MS^2 analysis, two fragments were observed at m/z 289 and m/z 273, corresponding to the loss of the hexosyl moiety and phloretin, respectively.[77] Previously, 3-hydroxy-phloretin 2′-O-glucoside has been isolated from the flesh of apples; however, this is the first time that this compound was

identified in bell peppers through LC−MS/MS to the best of our knowledge.[77]

Dihydromyricetin 3-O-rhamnoside (compound **41**, m/z 465.1036, ESI^-) was the only dihydroflavonols isolated from the seeds of green peppers, and the product ion at m/z 301 generated by cleavage of the rhamnoside group (loss of 164 Da) allowed the further identification of this compound.[78]

2.4.3. Other Polyphenols. Twelve other polyphenols, which were categorized into hydroxybenzaldehydes, curcuminoids, furanocoumarins, hydroxybenzoketones, hydroxycoumarins, tyrosols, and other phenolic compounds, were found in the peppers.

Compound **43** was found in the seeds of green and red bell peppers and was tentatively characterized as demethoxycurcumin with the observed precursor at $[M - H]^-$ m/z 337.1087. In the MS/MS fragmentation, the characteristic ion was observed at 217, corresponding to the loss of C_8H_8O (120 Da).[79] Demethoxycurcumin is a valuable compound mainly isolated from traditional Chinese medicines Gandou and

turmeric, and the antioxidant capacity of demethoxycurcumin is remarkable, especially in preventing the unsaturated fatty acid such as linoleic acid from oxidation.[79−81]

Compound **47** (coumarin with $[M + H]^+$ m/z at 147.0447, RT = 15.875) and compound **48** (esculin with $[M + H]^+$ m/z at 341.087, RT = 82.047) were two hydroxycoumarins that were only found from the pulp of bell peppers in the ESI$^+$ mode. The characterization of coumarin was supported by its MS^2 spectrum, which generated the fragment ions at m/z 103 because of a typical loss of CO_2 and the fragment ion at m/z 91 because of the loss of two CO units. Coumarin has been identified in *Capsicum annuum* before, with considerable antioxidant capacity.[82] In the MS/MS spectrum of compound **48**, the product ions were found at m/z 179 and m/z 151 because of the loss of hexose and the sequential loss of CO, respectively, allowing the confirmation of esculin.[83]

A total of four tyrosols were isolated from the bell peppers, and all of them were only captured from the seeds of the bell peppers. Compound **49** ($[M − H]^−$ at m/z 195.0667) was inferred as 3,4-DHPEA-AC because the major fragment was found at m/z 135 corresponding to the cleavage of $C_2H_4O_2$ (60 Da).[84] Compound **51** was tentatively characterized as 3,4-DHPEA-EDA with the ESI$^−$ mode at m/z 319.1189. In the MS/MS analysis, the product ions were detected at m/z 275 $[M − H−CH_2CHO]^−$ and m/z 195 $[M − H−C_5H_6(CHO)_2]^−$.[85] Compound **52** at m/z 315.1072 in the ESI$^−$ mode, which yielded the main product ions at m/z 153 and m/z 123, corresponding to the loss of a hexose moiety and a further loss of CH_2O group, respectively, was characterized as hydroxytyrosol 4-O-glucoside.[86] Compound **53** showing precursor ion $[M − H]^−$ at m/z 537.1047 was confirmed as lithospermic acid found in the seeds of green and yellow peppers based on the MS^2 fragmentation at m/z 493 (loss of CO_2), m/z 339 (loss of a danshensu (DSS)), and 295 (loss of a DSS and CO_2).[87]

2.4.4. Lignans and Stilbenes. A total of six lignans were identified in bell peppers. Compound **55** detected in the ESI$^+$ mode at m/z 403.2100 was inferred as schisanhenol based on the product ions at m/z 385 $[M + H−H_2O]^+$, m/z 354 $[M + H−H_2O−OCH_3]^+$, and m/z 331 $[M + H−H_2O−C_4H_6]^+$.[88] Compounds **56**, **58**, and **59** were three compounds that were isolated from the seeds of peppers only. Schisandrin C (compound **56**, m/z 385.1651) was profiled in the positive mode, while the major fragments were identified at m/z 370, m/z 315, and m/z 300, corresponding to the loss of CH_3, C_5H_{10}, and CH_3 plus C_5H_{10}, respectively, from the precursor.[88] Compound **58** was only identified in the seeds of yellow bell peppers with the ESI$^−$ mode at m/z 357.1377, and the main fragments were observed at m/z 342 $[M − H−CH_3]^−$, m/z 327 $[M − H−C_2H_6]^−$, m/z 313 $[M − H−CO_2]^−$, and m/z 221 $[M − H−C_8H_8O_2]^−$, allowing the confirmation as matairesinol.[53] Compound **59** (resveratrol 3-O-glucoside) was the only stilbenes identified in all the seeds of bell peppers with the negative mode at m/z 389.1228. The confirmation was achieved by the MS/MS fragmentation that resulted in the product ion at m/z 227 (loss of 162 Da), indicating the presence of a glucose monosaccharide side group.[89] The presence of resveratrol in *Capsicum* spp. has been proved in the previous study by applying the HPLC technique and standards, and the bell pepper exhibited a significantly higher value than the other species.[64]

The screening and profiling of the phenolic compounds provide an overall idea of antioxidation compounds in three colors of Australian bell peppers. Some of them have already been proved to obtain strong antioxidation properties, illustrating that bell peppers could be a good source of phenolic compounds engaged in the food, feed, cosmetics, and medicinal industries.

2.5. Distribution of Phenolic Compound—Venn Diagram. The LC-ESI/QTOF-MS/MS experiment scoped the individual compound among bell peppers; however, the difference of phenolic compounds between bell peppers required a better analysis. The Venn diagrams summarized the distribution of phenolic compounds in bell pepper varieties and the difference between seeds and pulps (Figure 1).

A total of 301 phenolic compounds were identified in bell pepper samples, while 137 (45.5%) common phenolic compounds were identified in all samples. Most exclusive compounds were found in red bell peppers (10.3%) followed by yellow (8.3%) and green (6.6%) peppers sequentially. Previous studies also suggested that red bell peppers exhibited a higher phenolic content as compared to yellow and green peppers.[16,90]

Figure 1B showed that pulp and seed portions shared 117 (38.9%) common phenolic compounds. Remarkably, a total of 149 (49.5%) were found exclusively in seeds, while 35 unique compounds were discovered in pulps. The same trend was also observed in previous research by Sayin,[91] which indicated that the seed portions of bell peppers obtain a higher content of phenolic compounds than the pulp portion. The distributions of flavonoids and phenolic acids in seed and pulp are shown in Figure 1C,D; according to the figures, seeds of bell peppers had higher amounts of both flavonoids and phenolic acid, which accounted for 50 and 64.1%, respectively.

Overall, the seeds had higher flavonoids, phenolic acids, and total polyphenols than the pulp portion, which is in agreement with previous research.[11] Although the seed of bell peppers is regarded as food waste, it presents the potential of being utilized in the food and pharmaceutical industries because of the phenolic compounds and antioxidant capacity.

2.6. Heat Map and Hierarchical Clustering Phenolic Compound Analysis. A heat map (Figure 2) was conducted along with hierarchical clusters for further analyzing HPLC-PDA-quantified phenolic compounds in bell peppers. A total of 10 phenolic compounds consisting of five phenolic acids and five flavonoids were quantified. The color represents the average content of phenolic compounds of samples. Correlation was used as the distance measure for determining the similarity between samples, and the axis of the map showed the similarity among the samples.

In the heat map, the samples were clustered in BP-1 (including YCS and YCP) and BP-2 (including GCS, RCS, GCP, and RCP) because they have a high level of difference in phenolic contents between the groups. Among group BP-2, the phenolic contents of GCS were closer to RCS, while GCP and RCP have more similar phenolic quantities.

The phenolic compounds were clustered into three groups (PC-1, PC-2, and PC-3) and several subgroups based on the concentration similarity in the samples. Gallic acid and syringic acid composed the PC-1 cluster, both of which exhibited the highest concentration in YCS and YCP, and the lowest in GCP and RCP. The PC-2 cluster consisted of caffeic acid and catechin, showing the highest concentration in GCS and RCS. A previous study also concluded that the highest concentration of catechin was found in red capsicum seeds, which was in agreement with our finding.[64] Six targeted phenolic com-
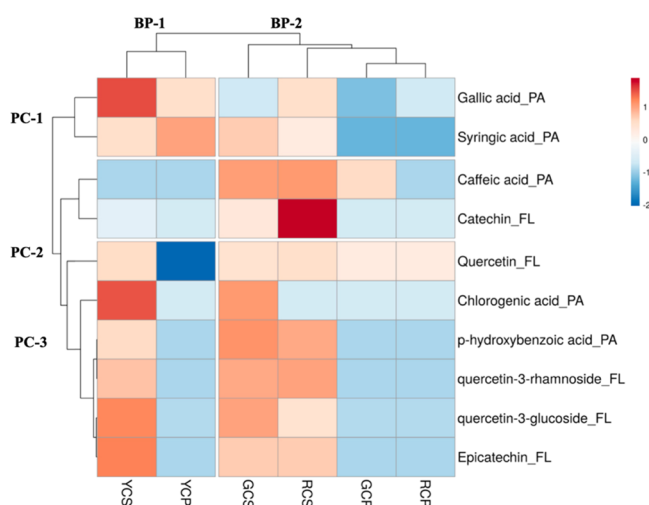
Figure 2. Heatmap showing the distribution of target phenolic compounds and the concentration of these compounds. More the red color is the higher concentration of the target phenolic compounds; more the blue color is the lower the concentration of the target phenolic compounds. BP 1−2: bell pepper cluster; PC 1−3: phenolic acid cluster; PA-phenolic acid; FL-flavonoid. Seeds of green bell peppers "GCS"; pulp of green bell peppers "GCP"; pulp of yellow bell peppers "YCS"; pulp of yellow bell peppers "YCP"; seeds of red bell peppers "RCS"; pulp of red bell peppers "RCP".

pounds formed the cluster PC-3, including two phenolic acids (chlorogenic acid and *p*-hydroxybenzoic acid) and four flavonoids (quercetin, quercetin-3-rhamnoside, quercetin-3-glucoside, and epicatechin). The concentration of several phenolic contents in Jalapeño (*Capsicum annuum*) was determined using HPLC in a previous study conducted by Sandoval-Castro et al.,[92] and they demonstrated that phenolic acids mainly existed in the seeds of the bell peppers, which was consistent with our findings. In terms of flavonoids, epicatechin, quercetin-3-glucoside, and quercetin-3-rhamnoside were found abundant in seeds of all bell peppers. Our findings were supported by the previous studies, which found that quercetin-3-glucoside primarily existed in red peppers (*Capsicum annuum* L.) and the catechin was accumulated in the seed of *Capsicum annuum* L.[33,93]

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents. All the chemicals and reagents were of analytical grade and produced by Sigma-Aldrich (Castle Hill, NSW, Australia). Ethanol and analytical-grade acetonitrile were produced by Merck KGaA (Darmstadt, Germany). The standards for antioxidant assays were purchased from Sigma-Aldrich (St. Louis, MO, USA), including gallic acid, quercetin, catechin, and L-ascorbic acid. Folin and Ciocalteu's phenol, aluminum chloride hexahydrate, vanillin, DPPH, ferric(III) chloride anhydrous, and ABTS, were produced by Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate anhydrous was from Gilman Australia (SA, Australia), and sodium acetate hydrated was from Ajax Finechem (VIC, Australia). Sulfuric acid 98% was produced by RCI Labscan (Rongmuang, Thailand). In terms of the mobile phases of the LC−MS/MS experiment, acetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) and acetonitrile was from LiChrosolv (Darmstadt, Germany). Analytical-grade standards for HPLC-PDA quantification analysis were purchased from Sigma-Aldrich (St. Louis, MO,

USA) including catechin, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*-glucoside, quercetin, kaempferol, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, and ferulic acid.

3.2. Sample Preparation. Three types of Australian-grown bell peppers (green, red, and yellow) were collected from a local market in August 2019 (Kensington VIC, Australia). The fresh bell peppers were separated into seeds and pulps. The pulp parts were blended to slurry separately with a 1.5 L blender (Russell Hobbs Classic, mode DZ-1613, Melbourne, VIC, Australia). The slurry was kept at −20 °C for further analysis. The seed part was separated followed by dehydration at −45 °C/50 MPa using a Dynavac engineering FD3 Freeze Drier (Belmont, W.A., Australia) and an Edwards RV12 oil-sealed rotary vane pump (Bolton, England). After lyophilization, the seeds were ground into powder and stored at −20 °C for further analysis.

3.3. Extraction of Phenolic Compounds. The extracts were prepared based on a previous study[94] with some modifications. The samples were homogenized with an Ultra-Turrax T25 Homogenizer (IKA, Staufen, Germany) at 10,000 rpm for 30 s. After that, the samples were extracted in 80% ethanol and were incubated in a ZWYR-240 incubator shaker (Labwit, Ashwood, VIC, Australia) with 120 rpm at 4 °C for 12 h followed by the centrifugation (Hettich Refrigerated Centrifuge) at 10,000 rpm for 15 min. The supernatant was collected and stored at −20 °C for further analysis. All the results were gained based on fresh weight. For HPLC and LC−MS experiments, the extracts were purified using a 0.22 μm syringe filter (Thermo Fisher Scientific Inc., Waltham, MA, USA).

3.4. Phenolic Compound Estimation and Antioxidant Assays. TPC, TFC, and TTC assays were performed to estimate the phenolic contents of the samples, while the antioxidant potentials were determined through DPPH, ABTS, FRAP, and TAC assays. The absorbance was measured using a Multiskan Go microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.4.1. Determination of TPC. The TPC was estimated by following the Folin−Ciocalteu method with modifications.[19] Folin−Ciocalteu reagent (25 μL), Milli-Q water (200 μL), and 25 μL of standard/extracts were added into a 96-well plate (Costar, Corning, NY, USA) for triplicate and incubated for 5 min at room temperature. Then, 10% (w/w) sodium carbonate (25 μL) was added and stored in a dark environment for 1 h at room temperature. The absorbance was read at 765 nm against a blank using a Multiskan Go microplate photometer. The calibration curve of the standard reagent (gallic acid, ranging from 0 to 200 μg/mL) was plotted by converting the absorbance. The TPC values were calculated with the standard curve, and the results were presented as mg of gallic acid equivalents per gram ± standard error (mg GAE/g ± SE) based on the fresh weight (f.w.).

3.4.2. Determination of TFC. Modified aluminum chloride method[95] was used to evaluate the TFC of the bell peppers. Extracts (80 μL) and standard were mixed with 2% (w/v) aluminum chloride ethanolic solution (80 μL) and 120 μL of sodium acetate (50 mg/mL). The mixtures were incubated in darkness at room temperature for 1 h, and the results were measured at 440 nm. The values of TFC were expressed in quercetin equivalent (μg QE/g) in fresh weight, and the concentration of quercetin was ranging from 0−50 μg/mL.

3.4.3. Determination of TTC. The determination of TTC was based on the method developed by Zou, et al.[96] First of all, 25 μL samples and standard were mixed with 150 μL of 4% methanolic vanillin solution (w/v). After that, 25 μL of 32% (v/v) sulfuric acid (diluted with methanol) was added into a 96-well plate and incubated at 25 °C for 15 min. The standard curve was calculated with catechin standard ranging from 0−1000 μg/mL. The absorbance was measured at 500 nm, and the tannin content was calculated according to the calibration curve with the unit of mg CE/g$_{f.w.}$.

3.4.4. DPPH Antioxidant Assay. The free radical scavenging ability was examined by DPPH assay by adopting the previous method[19] with slight modifications. First, 40 μL of extracts/standard and 260 μL of DPPH radical ethanolic solution were mixed in a 96-well plate. Second, the mixtures were incubated for 30 min at room temperature before measuring the absorbance at 517 nm. The standard curve was developed under the concentration of the ascorbic acid solution from 0−50 μg/mL, while the results were calculated according to the equivalent of ascorbic acid and expressed as mg of ascorbic acid equivalents per g (mg AAE/g$_{f.w.}$).

3.4.5. ABTS+ Radical Scavenging Assay. ABTS$^+$ radical cation decolorization was engaged to assess the radical scavenging activity of samples, and the method was previously described by Ma and Dunshea[97] with few modifications. Free radical scavenging capacity was based on the positive charges in the ABTS$^+$ solution. The ABTS$^+$ stock solution was prepared by mixing 88 μL of 140 mM potassium and 5 mL of 7 mM of ABTS solution. After that, the reaction mixture was stored in darkness for 16 h. Ten microliters of prepared extracts/standard would be mixed with 290 μL of ABTS$^+$ solutions. The absorbance was measured at 734 nm after the incubation at room temperature for 6 min. The results were shown with mg ascorbic acid equivalent per gram of fresh samples (AAE/g$_{f.w.}$).

3.4.6. FRAP Assay. The ferric reducing capacity of the compounds was determined through the FRAP method of Benzie, et al. Benzie and Strain[98] with modifications. Twenty microliters of samples/standard were mixed with 280 μL of FRAP reagent, which was produced by mixing 300 mM acetate buffer, 10 mM TPTZ solution, and 20 mM ferric chloride in a ratio of 10:1:1 (v/v/v). The mixture was then incubated for 10 min at 37 °C, and the absorbance was obtained at 593 nm. The standard curve was plotted using ascorbic acid (ranging from 0 to 50 μg/mL), and the results were expressed as the mg AAE/g$_{f.w.}$.

3.4.7. TAC Assay. TAC assay was modified based on the methods of Jan, et al.[18] First, 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate were mixed in a ratio of 1:1:1 (v/v/v) to prepare the phosphomolybdate reagent. Then, 20 μL of samples and standard were mixed with 280 μL of preparation dye solution followed by incubation at 95 °C for 90 min, and the absorbance was observed at 765 nm. The standard curve was constructed with the 0−300 μg/mL ascorbic acids, and the results would be described as mg AAE per fresh gram.

3.5. LC-ESI-QTOF-MS/MS Characterization of Phenolic Compounds. The LC−MS analysis was carried out with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 6520 I Accurate-Mass Q-TOF detector, (Agilent Technologies, Santa Clara, CA, USA) via an ESI and was based on the method of Gu, et al.[95] A Synergi Hydro-RP 80 Å, LC column (250 mm × 4.6 mm, 4

μm) (Phenomenex, Lane Cove, NSW, Australia) was applied to isolate the compounds at room temperature, and the multisampler was set at 10 °C with an injection volume of 6 μL. The composition of mobile phase A consisted of acetic acid and water in a ratio of 0.5:99.5 v/v. The mobile phase B was a mixture with three compounds which were acetonitrile, acetic acid, and water in a ratio of 50:49.5:0.5 (v/v/v). The whole gradient profile was 85 min with a flow rate of 0.8 mL/min and described as follows: 0 min with 10% B, 20 min with 25% B, 30 min with 35% B, 40 min with 40% B, 70 min with 55% B, 75 min with 80% B, 77 min with 100% B, 79 min with 100% B, 82−85 min with isocratic 10% B to equilibrate the column. The pressure of nebulization gas was set at 45 psi with a flow rate of 5 L/min at 300 °C. At the same time, the sheath gas was set at a flow rate of 11 L/min at 250 °C. The mass scan range was from 50−1300 *m/z*. MS/MS analyses were carried out in automatic mode with collision energy (10, 15, and 30 eV) for fragmentation. Peak identification was performed in both positive and negative modes separately while the instrument control, data acquisition, and processing were performed using MassHunter workstation software (Qualitative Analysis, version B.03.01) (Agilent Technologies, Santa Clara, CA, USA).

3.6. HPLC-PDA Quantification Analysis. The phenolic content quantification was carried out using an Agilent 1200 HPLC system attached with a photodiode array detector. The parameters of HPLC were set as the same as those of LC−MS mentioned above except the injection volume was 20 μL. The absorbance of samples was detected under three wavelengths of 280, 320, and 370 nm simultaneously with 1.25 scan/s (peak width = 0.2 min) spectral acquisition rate. The standard curve would be drawn based on the different concentrations of the standards. The data and analysis were carried out with the help of the Agilent LC-ESI-QTOF/MS MassHunter workstation (Qualitative Analysis, version B.03.01) (Agilent Technologies, Santa Clara, CA, USA).

3.7. Statistics Analysis. The results of phenolic compound estimation and antioxidant capacity evaluation were obtained with three parallel experiments (*n* = 3). The results were displayed with mean ± standard deviation. The significance of each result was measured based on one-way ANOVA (*p* < 0.05) with Tukey's honestly significant difference under the help of Minitab Statistical Software for Windows Version 18.0 (Minitab Inc., USA).

4. CONCLUSIONS

Overall, pulps and seeds of yellow, green, and red bell peppers were proved to be abundant in phenolic compounds with considerable antioxidant potential by various in vitro assays. The pulp of red bell peppers exhibited the highest phenolic compound content and antioxidant potential in all the assays including TPC, TFC, TTC, TAC, DPPH, ABTS, and FRAP. The LC−MS also engaged to characterize the individual phenolic compounds in bell peppers, and a total of 59 phenolic compounds were isolated from all parts of bell peppers. Ten phenolic compounds were quantified by HLC-PDA, and the seeds exhibited a relatively higher concentration of most compounds than pulps did. According to the results, different parts of bell peppers could be good sources of phenolic compounds with remarkable antioxidant capacity in the food, cosmetics, pharmaceutical, and nutraceutical industries. In addition, the methodology provided in this study could be an example for other research studies in the area that characterize

phenolic compounds in plants. Future studies, including toxicological and animal studies, can support and boost the commercialization of these compounds.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06532.

> LC-ESI-QTOF-MS/MS basic peak chromatograph for characterization of phenolic compounds of Australian-grown bell peppers; (a) the pulp of green bell peppers in the negative ionization mode; (b) the pulp of red bell peppers in the negative ionization mode; (c) the pulp of yellow bell peppers in the negative ionization mode; (d) the pulp of green bell peppers in the positive ionization mode; (e) the pulp of red bell peppers in the positive ionization mode; (f) the pulp of yellow bell peppers in the positive ionization mode; (g) the seed of green bell peppers in the negative ionization mode; (h) the seed of red bell peppers in the negative ionization mode; (i) the seed of yellow bell peppers in the negative ionization mode; (j) the seed of green bell peppers in the positive ionization mode; (k) the seed of red bell peppers in the positive ionization mode; and (l) the seed of yellow bell peppers in the positive ionization mode (PDF)

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Notes

The authors declare no competing financial interest.

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