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The Polyphenolic Content of Fruit and Vegetables and their Antioxidant Activities. What Does a Serving Constitute?

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Analysis of the major flavone, flavonol, anthocyanidin and hydroxycinnamic acid constituents (and their glycosides) of onion, tomato, egg plant and apple has been undertaken and the antioxidant activities of the phenolic extracts determined. The major phenolic antioxidant components of egg plant are chlorogenic acid in the flesh and a delphinidin conjugate in the skin. In the case of apple, the major phenolic antioxidants detected are chlorogenic acid, procyanidins/catechin compounds, rutin and phloridzin. Quercetin glycosides are well-known to be the major phenolic components of onion. Assessment of the antioxidant activities of a serving of 100 g fresh weight fruit, vegetable and comparison with previously reported findings for 150 ml beverage (500 ml portion in the case of beer), expressed in μmol Trolox equivalents show that the antioxidant activities of 1 glass (150 ml) red wine ≡ 12 glasses white wine ≡ 2 cups of tea ≡ 4 apples ≡ 5 portions of onion ≡ 5.5 portions egg plant ≡ 3.5 glasses of blackcurrant juice ≡ 3.5 (500 ml) glasses of beer ≡ 7 glasses of orange juice ≡ 20 glasses of apple juice (long life).

Keywords: Hydroxycinnamate, flavonoid composition, polyphenol, total antioxidant activity, TEAC, hplc

INTRODUCTION

There is considerable evidence for a role for the antioxidant constituents of fruit and vegetables in the maintenance of health and disease prevention.[1] In particular, association has been made between intake of high carotenoid-containing fruit and vegetables and protection from certain cancers.[2,3] Recent work is also beginning to highlight the role of the phenolic constituents of the diet, the polyphenols and hydroxycinnamic acids, in contributing to these protective effects. They act as antioxidants by virtue of the free radical scavenging properties of their constituent hydroxyl groups, allowing them to act as reducing agents, hydrogen- or electron-donating agents or singlet oxygen scavengers.[4,5] Studies in *in vitro* systems have shown that the antioxidant activities of flavonoids and their glycosides are higher than that of vitamins C and E and many carotenoids on a molar basis.[6–11] Where the

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antioxidant properties of dietary agents are concerned, there are situations in which knowledge of the total antioxidant potential might be more useful than the individual polyphenolic contents. This work describes analyses of the composition of the phenolic extracts of specific fruit and vegetables in relation to their total antioxidant activities.

EXPERIMENTAL

Chemicals

Methanol and acetonitrile, all hplc grade, were obtained from Rathburn Limited, Walkerburn, Scotland, U.K. All hydroxycinnamic acids, glycosides, anthocyanidins, flavonols and flavones were obtained from Extrasynthese (ZI Lyon Nord, B.P. 62, 69730 Genay, France) and all were hplc grade $\geq$ 98% pure. Elgastat UHP double distilled water (18+ Ω grade) was used in all experiments. *tert*-Butylhydroquinone solution was prepared in 50% methanol/water. Fruits and vegetables were obtained from the local supermarket, during May, New Zealand Royal Gala apples, yellow onions, egg plant and Spanish tomatoes.

Aqueous Methanolic Extraction of Flavonoids from Freeze-dried Fruit and Vegetables

The plant was weighed without the pericarp where necessary and sliced into small cubes. The slices were wrapped in aluminium foil, lyophilised with liquid nitrogen and freeze-dried. The weight of the freeze-dried sample was recorded, and the sample was stored in a desiccator at 20°C until ready for extraction. For extraction, freeze-dried material was weighed (to amounts between 0.5 and 1.5 g) and water (10.0 ml to 0.5 g original weight) and salicylic acid (internal standard range 50–100 μg/ml in methanol depending on vegetable source) added. Methanol is then added in the ratio dry weight extract : water : methanol of 0.5 g : 10 ml : 20 ml and the contents refluxed for 30 min. The methanol was removed by rotary evaporation under vacuum at 40–50°C, and the remaining aqueous extract cooled and filtered before taking aliquots for antioxidant activity measurement and for hplc analysis. The remainder was used for the acid hydrolysis procedure to cleave the glycosides for identification of the aglycones.

Acid Hydrolysis of Aqueous Extract Samples

The aqueous sample (2.0 ml), containing added *tert*-butylhydroquinone as antioxidant, was incubated with hydrochloric acid at a final concentration of 1.2 M in final volume of 4 ml.[12] Oxygen was removed from the solution by bubbling nitrogen and the tubes were immediately sealed before placing on a heating block at 80°C. The time of hydrolysis depended on the source of the sample, and aliquots were removed at appropriate time intervals and placed at 0°C. The sample was filtered using an ICN flowpore membrane filter (0.22 μm) prior to hplc analysis.

Total Antioxidant Potential and Total Phenolic Composition

The total antioxidant activity (TAA) of fruit and vegetable extracts was estimated using the ABTS radical cation method.[13] This spectrophotometric technique measures the relative ability of the antioxidants present in an extract to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) generated in the aqueous phase of a mixture and is based on the reduction of the blue-green $ABTS^{\bullet+}$ radical by hydrogen-donating antioxidants. The end point is the suppression of the characteristic long wavelength absorption spectrum of $ABTS^{\bullet+}$ measured specifically at the peak at 734 nm. The assay is standardised with the synthetic antioxidant Trolox (6-hydroxy-2,5,8-tetra metyl chroman-2-carboxylic acid, Hoffman-La Roche), the water-soluble vitamin E analogue (using a range of dilutions with final concentrations from 0–21 μM). $ABTS^{\bullet+}$ is

generated by the interaction of ABTS with ferrylmyoglobin radical species, using metmyoglobin (2.5 μM), H_2O_2 (375 μM) and ABTS (150 μM) (final concentrations). The antioxidant components of the extract reduce $ABTS^{\bullet+}$ as it is formed during the assay, as detected by the suppression of the absorption of $ABTS^{\bullet+}$, to an extent and on a timescale dependent on the antioxidant activity of the sample under investigation. The same results were obtained for the tomato extract using the pre-formed $ABTS^{\bullet+}$. Absorbance measurements are made using a Cobas Fara centrifugal analyser (Roche Diagnostic Systems, P.O. Box 8, Welwyn Garden City, Herts AL7 3AY) 3.25 min after the initiation of the reaction. Dose–response curves were derived using a logit/log 4 plot. Samples were analysed in triplicate and the results were expressed as μmol of Trolox equivalents per mg dry weight, wet weight or phenol content. Total phenol was measured colorimetrically with Folin-Ciocalteu reagent[14] using gallic acid as a calibration standard. The molar antioxidant activity of pure hydroxycinnamates and flavonoids was determined as the Trolox Equivalent Antioxidant Activity Capacity (TEAC) value.[13]

Analysis of Phenolics by Gradient HPLC

The Waters hplc system consisted of an autosampler with Peltier temperature controller, a 626 pump with 600S controller, a Photodiode Array Detector and Millennium software system which controlled all the equipment and carried out data processing. The column, a Nova-Pak C^{18} column (4.6 × 250 mm) with a 4 μm particle size was used and the temperature maintained by the column oven set at 30°C. The injection was by means of an autosampler, with a 100 μL fixed loop and the volume injected was 30 μL. Elution (0.5 ml/min) was performed using a solvent system comprising solvent A (20% methanol in 0.1% hydrochloric acid) and acetonitrile mixed using a linear gradient held at 95% solvent A for 10 min and then decreasing linearly to 50% solvent A at 50 min, back to 95% solvent A at 55 min and held at these conditions for a further 5 min. There was a 10 min delay before the next injection to ensure re-equilibration of the column. Authentic standard solutions were prepared as described below and analysed as precision controls by being randomly placed with the samples. The chromatograms were obtained according to the retention time of each fraction with detection at both 280 and 350 nm. Peak identification of each component was effected post-run using spectroscopic analysis by photodiode array detection from 200–600 nm.

Preparation of HPLC Standards

Stock solutions of the standards were prepared by dissolving 1–2 mg of sample into either methanol or mobile phase solvent A (20% MeOH and 0.1% HCl) (1 ml). Stock solutions were stored at 0–4°C and used within four weeks after checking for stability spectrophotometrically. Anthocyanidins were prepared freshly. Standard mixtures of concentrations range 0–100 μg/ml were prepared in solvent A and aliquoted into hplc vial (non-hydrolysed sample).

Figure 1 shows the chromatographic separation, with diode array detection at 280 nm, of a mixture of standards consisting of hydroxycinnamic acid derivatives (chlorogenic acid, caffeic acid, *p*-coumaric acid and ferulic acid), flavones (luteolin and apigenin), flavonols (kaempferol and quercetin), the flavone glycoside, rutin, anthocyanidins (malvidin, delphinidin and cyanidin), catechins (epicatechin and catechin) and dihydrochalcones (phloridzin and phloretin). The identification is confirmed by the spectrum of each eluant scanned from 200 to 600 nm applying photodiode array detection. The hplc profiles demonstrate the clear separation of all the phenolics, with the exception of the chalcone glycoside phloridzin and the anthocyanin malvidin which co-elute. This does not constitute a problem as hydrolysed plant extracts would be comparing malvidin and phloretin and

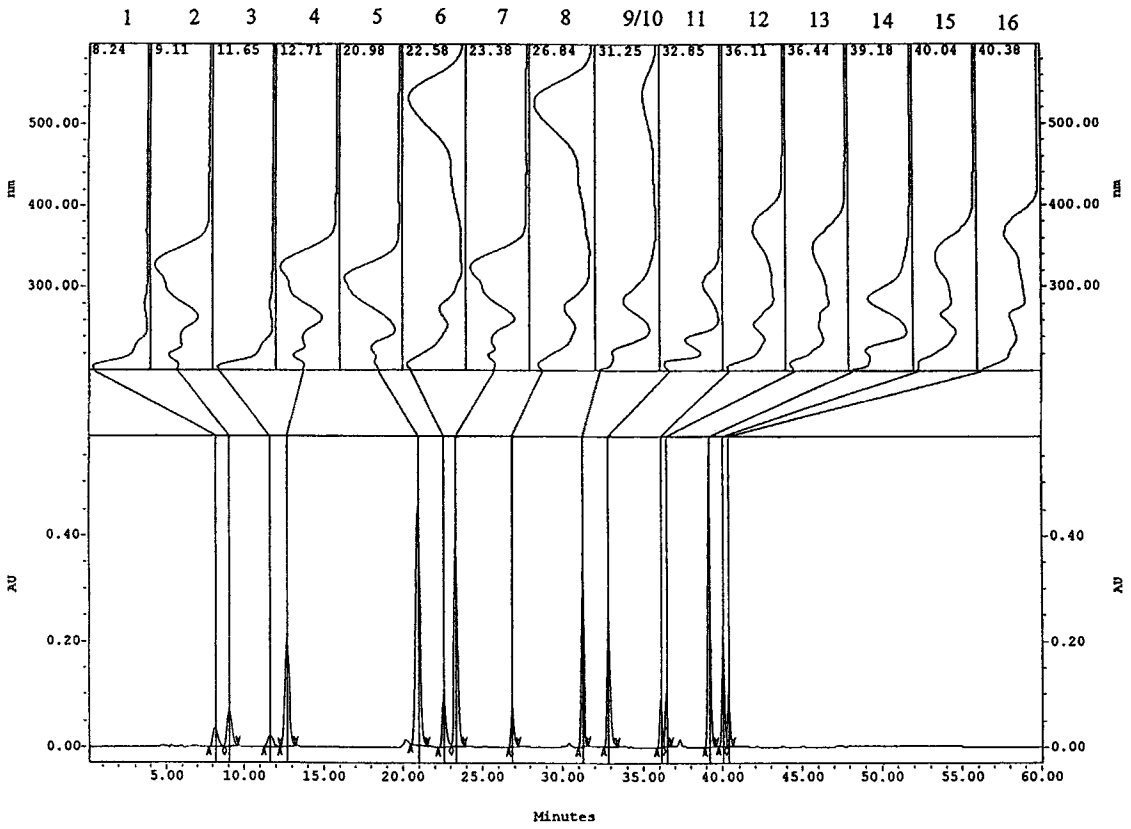

FIGURE 1 The chromatogram of combined standards derived at 280 nm using a gradient elution reverse phase hplc with diode array detection: (1) catechin; (2) chlorogenic acid; (3) epicatechin; (4) caffeic acid; (5) *p*-coumaric acid; (6) delphinidin; (7) ferulic acid; (8) cyanidin; (9) phloridzin; (10) malvidin; (11) salicylic acid (internal standard); (12) quercetin; (13) luteolin; (14) phloretin; (15) apigenin; and (16) kaempferol. Aliquots of standards 30 µL (within a 100 µL fixed loop) were injected onto a Nova-Pak C^{18} column (4.6 × 250 mm) with a 4 µm particle size and eluted with solvent A (20% methanol in 0.1% hydrochloric acid) and acetonitrile. The gradient was as follows: for the initial 10 min, the mixture was 95% solvent A and 5% acetonitrile, then solvent A was decreased linearly to 50% at 50 min, back to 95% solvent A at 55 min and held for a further 5 min at solvent A. The flow rate was 0.5 ml/min.

non-hydrolysed malvidin glycoside and phloridzin, which would be resolved (in a situation in which the latter might coexist). Retention times are shown in Table I.

RESULTS AND DISCUSSION

The antioxidant activities of the extracts of fruit and vegetables are presented in Table II. The TAA method, which is based on scavenging of the $ABTS^{\bullet+}$ radical by the combined hydrogen-donating components of the fruit and vegetable extracts, measures the collective antioxidant activity of the extract. On the basis of fresh weight, onion, egg plant and apple are rather similar in their antioxidant activities at 5.8 ± 3.2, 4.9 ± 1.8 and 6.4 ± 2.7 µmol Trolox equivalent/g wet weight, respectively, by contrast with tomato at 1.6 ± 0.6 µmol Trolox equivalent/g wet weight. The lower range of values for tomatoes is consistent with their greater water content in comparison with the other fruit and vegetables. When adjusted for the total phenolic content of the extract, the antioxidant activities are close (6.6 ± 3.6, 6.7 ± 0.6 and 6.2 ± 0.6 and 4.6 ± 1.9 mmol per gram phenol respectively). The relatively low value for the tomato extract may

TABLE I Summary of the retention times and peak areas of standard phenolic components in Figure 1. Chromatography conditions as in the text (* analysed separately)

Component	Concentration (μg/ml)	RT (min)	Mean peak area (mV·s) $n=6$	SD	CV
Catechin	11.2	8.24	314777	5547	1.76
Chlorogenic acid	8.3	9.11	689971	8930	1.29
Epicatechin	5.4	11.65	210697	3339	1.58
Caffeic acid	14.1	12.71	1868404	22271	1.19
p-Coumaric acid	16.6	20.98	4095901	63713	1.56
Deiphinidin	8.2	22.58	665929	5414	0.81
Ferulic acid	9.7	23.38	380992	2214	0.58
Rutin	7.1	25.48	383703	1793	0.61
Cyanidin		26.84	2884853	15423	0.53
Malvidin/*Phloridzin	4.3	31.24	1953811	12294	0.63
Salicylic acid	100	32.51	3308646	35551	1.07
Quercetin	6.5	35.43	407895	5497	1.35
Luteolin	4.8	36.44	503133	13909	2.76
*Naringenin	16.5	38.64	2412477	26317	0.77
Phloretin	6.1	39.18	2845827	22386	0.79
Apigenin	4.47	40.04	910759	1402	0.15
Kaempferol	4.7	40.38	479754	14556	3.03

TABLE II The total antioxidant activity of onion ($n=7$), tomato ($n=6$), apple ($n=7$) and egg plant ($n=7$). Results are expressed as mean values μmol Trolox equivalents ± standard deviation per gram dry weight, per gram wet weight and per gram of phenol. Each fruit or vegetable was weighed, extracted and the TAA of the extract determined in triplicate at three different dilutions within the linearity range of the assay: this procedure was repeated three times in duplicate ($n=3$)

Source	% Dry matter	TAA μmol Trolox equivalents/g dry weight	TAA μmol Trolox equivalents/g wet weight	TAA mmol/g phenol
Onion	10	58.0 ± 10	5.8 ± 3.2	6.1 ± 3.6
Tomato	6.4	25.0 ± 12	1.6 ± 0.6	4.6 ± 1.9
Apple (with seeds and pips)	2.1	32.0 ± 5.7	6.4 ± 2.7	6.2 ± 0.6
Egg plant	6	81.3 ± 18.2	4.9 ± 1.8	6.7 ± 0.6

be due to its high proportion of phenolics with relatively lower antioxidant activities than that of the polyphenols. It should be noted that the method applied to extract the phenolic constituents of the samples does not destroy vitamin C but will not include contributions from the natural low levels of reduced glutathione (which tends to be oxidised during the refluxing stage of the procedure) or from the carotenoids and tocopherols whose lipophilicity precludes extraction into the solvent system applied here.

Hplc analysis of the phenolic extracts was undertaken and the major phenolic components were identified and quantified (Table III). The major polyphenolic constituents in onion are the quercetin glycosides (retention times (RTs) of 19.90, 30.23 and 32.03 min) (Figure 2). The flavonol glycoside at 30.23 min was identified as quercetin-4′-O-glucoside by spiking the onion extract with authentic standard and by comparison of the spectral characteristics. The concentration of the flavonol glycoside eluting at 19.90 min was 4831 ± 1050 (calculated on the basis of the quercetin-4′-glucoside response) and that of quercetin-4′-O-glucoside was 2460 ± 671 mg/kg dry weight. The identifications of the quercetin glycosides were confirmed by their spectra and substantiated by hydrolysis which yielded

TABLE III Concentrations of flavonoids and hydroxycinnamates in fruit and vegetable extracts (*quercetin glycosides calculated as quercetin-3-glucoside. NM, not measured)

Source	Major phenolic components	RT (min)	Amount (mg/kg dry weight)
Onion			
	*Quercetin glycoside	19.90	4831 ± 1050
	Quercetin-4′-O-glucoside	30.23	2460 ± 671
	*Quercetin glycoside	32.03	NM
Tomato	*p*-Coumaroyl ester	6.64	NM
	Chlorogenic acid	8.31	301 ± 90
	Naringenin	37.44	282 ± 90
	Rutin	25.64	302 ± 25
	Quercetin glycoside	22.11	NM
Apple			
Peeled	Chlorogenic acid	8.65	1505 ± 231
	p-Coumaroyl ester	10.38	241 ± 27
	Rutin	25.50	576 ± 54
	Phloretin glycoside	29.18	306 ± 59
	Phloridzin	31.05	357 ± 63
	Procyanidin/catechin compounds	5.18, 5.91, 6.25, 7.85	1721.2
Egg plant			
Flesh pre-hydrolysis	Not identified	4.84	NM
	Chlorogenic acid	8.58	9477 ± 2078
Skin post-hydrolysis	Delphinidin	22.40	548 ± 154

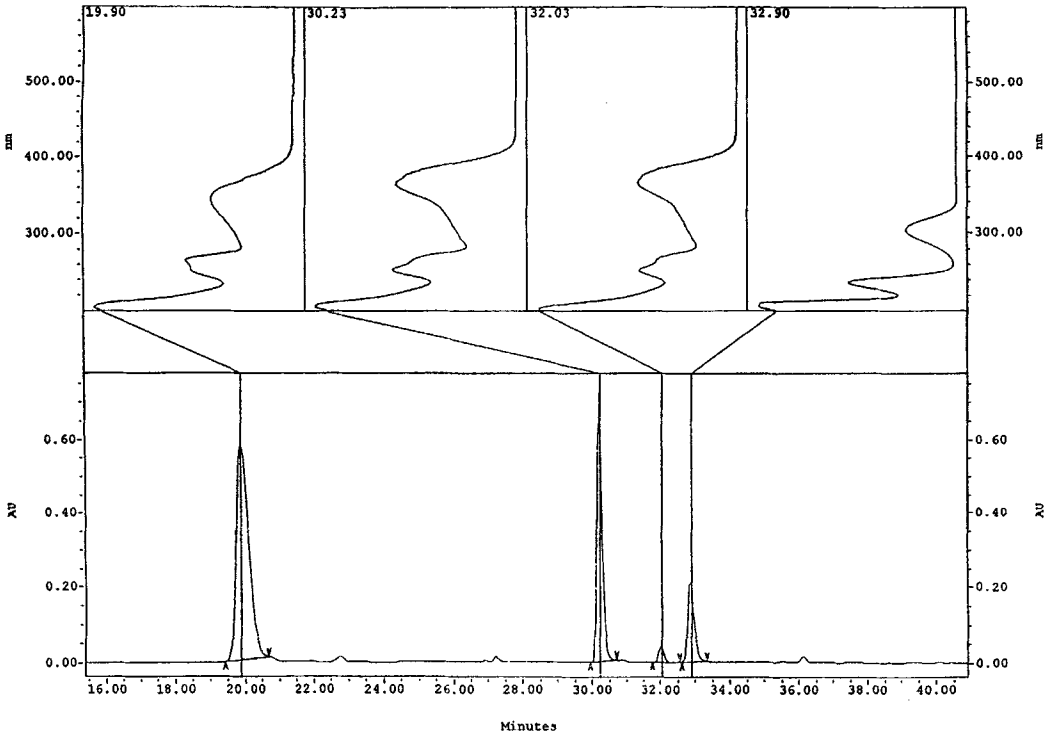

FIGURE 2 The chromatogram and associated spectra of the constituents of onion derived at 280 nm using a gradient elution reverse phase hplc with diode array detection, the peaks at 19.9, 30.23, 32.03 and 32.9 min correspond to a quercetin glycoside, quercetin-4′-O-glucoside, a quercetin glycoside and salicylic acid as internal standard respectively. The hplc conditions are as shown in Figure 1.

quercetin. The concentration of quercetin determined after acid hydrolysis was 2382 ± 454 mg/kg dry weight or 255 mg/kg fresh weight. The intra-assay coefficient of variance was less than 5.3% ($n = 10$). Other minor glycosides of quercetin were also detected, but at levels less than 1% of the two major quercetin glycosides. The total level of quercetin detected in onion was approximately half that of the 5076 mg/kg dry weight reported by Hertog *et al.*[12] and within the range 104–1260 mg/kg fresh weight reported by Starke *et al.*[15] Rhodes *et al.*[16] reported levels of total flavonol concentrations of 918, 711 and 803 mg/kg fresh weight in red, pink and brown onion.

The phenolic identified in egg plant was identified exclusively as chlorogenic acid at 9477 ± 2078 mg/kg dry weight, being detected at RT = 8.58 min (Figure 3). Other studies have detected chlorogenic acid (5′-caffeoyl quinone) as well as the 3′- and 4′-derivatives, in addition to the 5′-*p*-coumaroyl quinic ester.[17] The latter was not detected in the egg plant flesh in this study. The major phenolic constituent of skin, post hydrolysis, was essentially an anthocyanidin identified as delphinidin (548 ± 54 mg/kg dry weight).

The flesh of apple has been reported by others to contain chlorogenic acid, catechin, epicatechin, gallocatechin, epigallocatechin and a series of biflavans (procyanidins A_1A_2, B_1, B_2, B_3).[18] Analysis and identification of the major constituents of apple flesh (Figure 4) applying this method also identified chlorogenic acid (1505 ± 231 mg/kg dry weight) as the major phenolic and a combined content of catechin/epicatechin/ procyanidin related compounds (1721.2 mg/kg dry weight) as the major flavonoids, characterised from the UV spectra of their eluting peaks. The

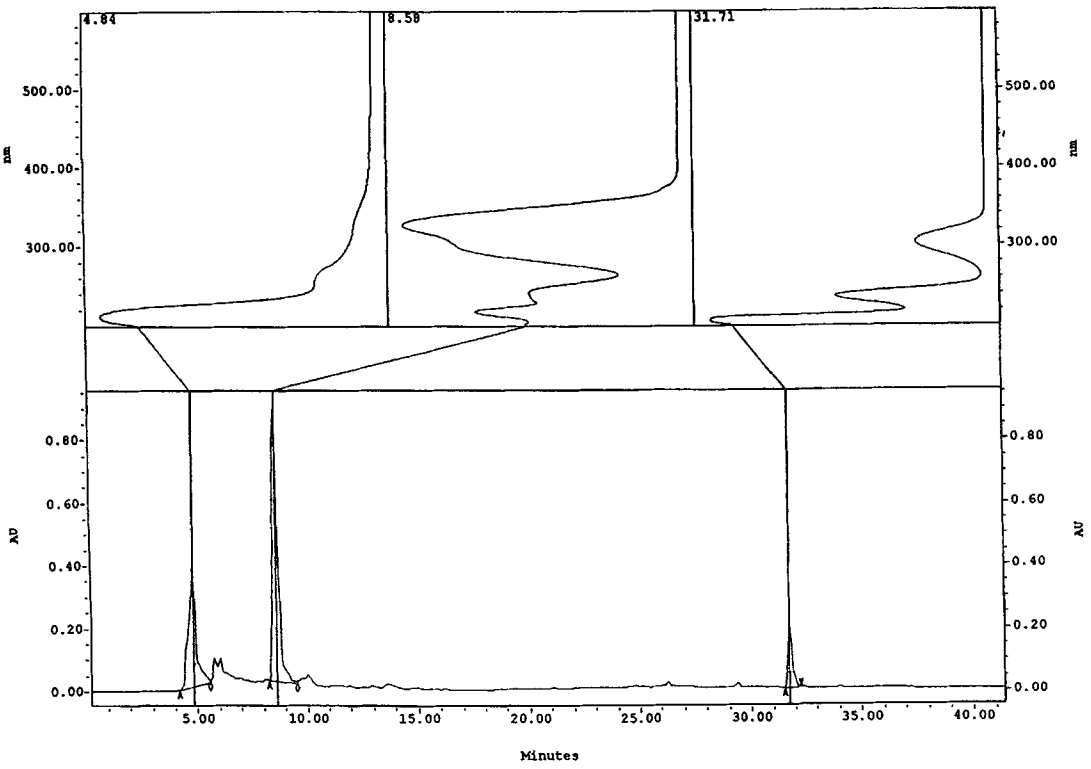

FIGURE 3 The chromatogram and associated spectra of the constituents of egg plant flesh derived at 280 nm using a gradient elution reverse phase hplc with diode array detection. The peaks labelled 8.58 and 31.71 min are chlorogenic acid and the internal standard, salicylic acid, respectively. The early eluting peak at 4.84 min is unidentified.

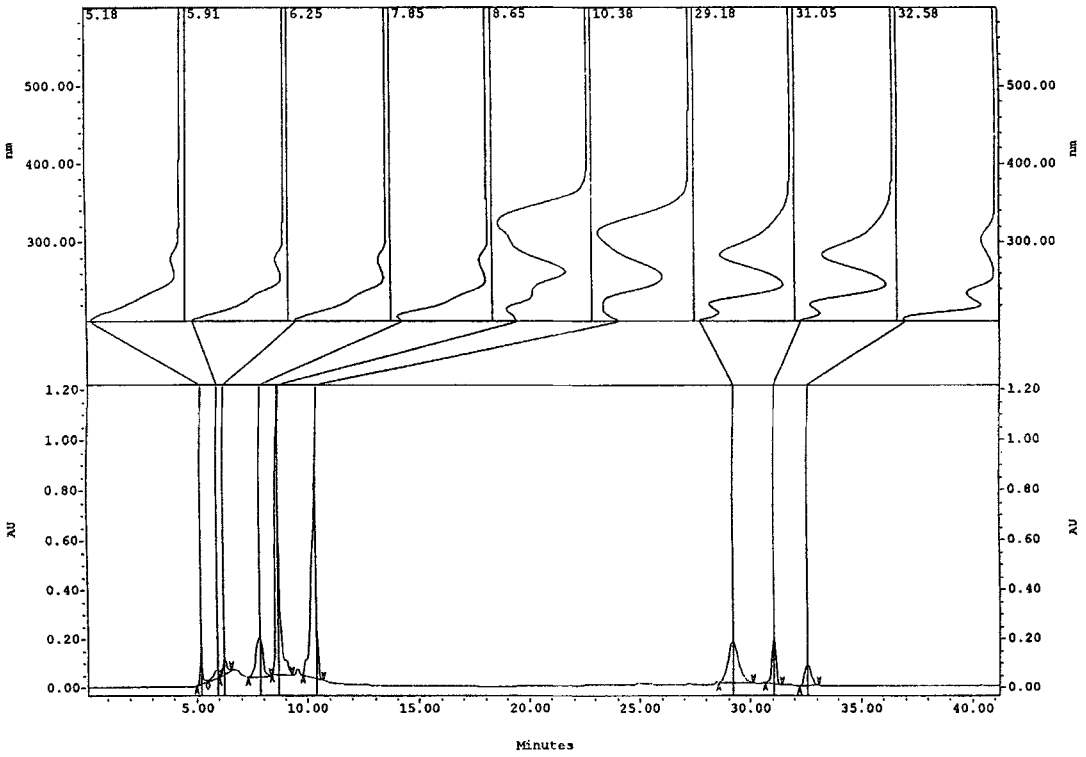

FIGURE 4 The chromatogram and associated spectra of the constituents of apple flesh derived at 280 nm using a gradient elution reverse phase hplc with diode array detection; the peaks eluting at 8.65, 10.38, 29.18, 31.05 and 32.58 min correspond to chlorogenic acid, *p*-coumaroyl ester, phloretin glycoside, phloridzin and salicylic acid (internal standard), respectively. Peaks eluting at 5.18, 5.91, 6.25 and 7.85 min are catechins and procyanidin compounds not specifically identified. The hplc conditions are as shown in Figure 1.

dihydrochalcone glycosides phloridzin (357 ± 63 mg/kg dry weight) and another phloretin glycoside (306 ± 59) (derived from the core tissue and seeds[19]) were also identified by their peaks and spectral characteristics (Figure 4). The apple skin contained a quercetin glycoside, a glycoside of phloretin, as well as an anthocyanin (characteristic of the red variety of apples studied), which upon acid hydrolysis released 873 ± 344 mg/kg quercetin (RT = 35.31 min), 428 ± 196 mg/kg phloretin (RT = 38.31 min) and cyanidin (964 ± 329 mg/kg dry weight of skin) (RT = 26.1 min) respectively. The quantification of anthocyanidins in apple is not well-documented, although there is a wealth of information on their glycosylated forms.[20] It has been reported that the anthocyanins (mainly cyanidin 3-galactoside) and flavonol glycosides (principally quercetin 3-glucoside, galactoside and arabinoside) are found almost exclusively in apple skin.[21]

The major hydroxycinnamate and polyphenolic components in tomatoes studied are the quinic acid ester of caffeic acid (chlorogenic acid), a quercetin glycoside and naringenin with RTs of 8.31, 25.64 and 37.44 min, respectively. The quercetin glycoside was identified as the rutinoside of quercetin from the retention time and spiking of the sample with pure rutin itself. The recoveries of all spiked samples were in the range 85–102%. The levels of the major components in tomatoes were 301 ± 90 mg/kg dry weight chlorogenic acid, 302 ± 25 mg/kg dry weight rutin and 282 ± 90 mg/kg dry weight naringenin. Quercetin was reported[21] at the low level of 8.0 ± 3.1 mg/kg dry. Naringenin was detected in the tomato sample as the aglycone. According to Wardale[22]

TABLE IV Calculation of the antioxidant activities of 100 g fresh weight portion of fruit, vegetable compared with 150 ml volume beverage (500 ml beer) in μmoles Trolox equivalents

Source	μmol Trolox equivalents/100 g fresh weight portion	μmol Trolox equivalents/150 ml volume beverage	μmol Trolox equivalents/500 ml beer
Apple (peeled)	640		
Tomato	160		
Egg plant	490		
Onion	580		
Red wine (Rioja–Bordeaux)		2100–3400	
White wine		220	
Black tea (0.25%)		1400	
Green tea (0.25%)		1350	
Beer			500–1000
Apple juice (long life)		140	
Orange juice		400	
Blackcurrant juice		800	

naringenin occurs in the free state only, but other studies also show the presence of naringenin glycosides.[23] Naringenin has been reported in cuticles of tomato in the forms of chalconaringenin, naringenin and naringenin-7-glucoside,[23] quantified by ultraviolet spectroscopy.

The antioxidant activities using the $ABTS^{\bullet+}$ radical cation assay can be compared to those derived from the ORAC assay, based on the reduction of the peroxyl radical by the hydrogen donating antioxidant constituents.[24,25] Comparing the antioxidant activity of the vegetables, egg plant and onion, and the fruit, apple and tomato, the results are 81.3, 58.0, 32.0, 25.0 μmol Trolox equivalents/g dry weight (TEAC assay) versus 80, 40, 13.2, 37.8 μmol Trolox equivalents/g dry weight ($ORAC_{ROO}$) corresponding to 4.9, 5.8, 6.4, 1.6 μmol Trolox equivalents/g wet weight (TEAC) versus 3.9, 4.5, 2.2, 1.9 μmol Trolox equivalents/g wet weight ($ORAC_{ROO}$). Considering that different varieties of fruit and vegetables were studied and keeping in mind the contrasting nature of the radicals involved in each methodological approach, peroxyl radicals versus $ABTS^{\bullet+}$ radicals, the wet weight data are reasonably close.

On the basis of these findings the antioxidant activities of the phenolic and vitamin C components of one serving of tomatoes, apples, onions, egg plant (as 100 g fresh weight) can be compared with 150 ml of a range of fresh beverages, red wine and white wine,[26] black tea, green tea[9] and fruit juices[26] (Table IV). The results show that the beverages red wine and teas have the highest antioxidant activity per 150 ml serving in the range 1350–3400, and that a 100 g portion of onion, apple, egg plant lies in the same range as orange juice, blackcurrant juice (150 ml) and 500 ml of beer 400–1000. The antioxidant activities of the phenolic components of a serving of tomatoes (100 g), 150 ml of white wine, apple juice are between 140–220 μmol Trolox equivalents/100 g fresh weight portion of fruit and vegetables, 150 ml portion beverage.

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