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Comparison of the Phenolic Composition of Fruit Juices by Single Step Gradient HPLC Analysis of Multiple Components Versus Multiple Chromatographic Runs Optimised for Individual Families

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After minimal sample preparation, two different HPLC methodologies, one based on a single gradient reversed-phase HPLC step, the other on multiple HPLC runs each optimised for specific components, were used to investigate the composition of flavonoids and phenolic acids in apple and tomato juices. The principal components in apple juice were identified as chlorogenic acid, phloridzin, caffeic acid and *p*-coumaric acid. Tomato juice was found to contain chlorogenic acid, caffeic acid, *p*-coumaric acid, naringenin and rutin. The quantitative estimates of the levels of these compounds, obtained with the two HPLC procedures, were very similar, demonstrating that either method can be used to analyse accurately the phenolic components of apple and tomato juices. Chlorogenic acid in tomato juice was the only component not fully resolved in the single run study and the multiple run analysis prior to enzyme treatment. The single run system of analysis is recommended for the initial investigation of plant phenolics and the multiple run approach for analyses where chromatographic resolution requires improvement.

Keywords: Hydroxycinnamate, flavonoid, apple juice, tomato juice, HPLC

INTRODUCTION

The principal phenolic composition of apple and tomato fruit juices include classes of polyphenolic flavonoids and phenolic acids such as the hydroxycinnamates.[1] In apple juices and apple fruit the primary components have been identified as chlorogenic acid, *p*-coumaric acid, procyanidins (B1, B2 and trimer C1), epicatechin and phloridzin,[2–5] although the levels reported vary significantly between juices produced from different apple cultivars. Since phloridzin and its

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derivatives are virtually restricted to the genus *Malus* and are rarely found in other rosaceous fruits,[6] it has been considered as a marker compound for the adulteration of food products with apple juice.[3,7,8] Differences in phenolic content of juices can arise because of the apple cultivars used to manufacture the juice, the manufacturing process and storage conditions as well the sample preparation and HPLC procedures utilised.[5,9] It has been reported that extraction procedures employing liquid–liquid partition with ethyl acetate result in poor recoveries of flavanols.[3] Several sample preparation and HPLC procedures have been used to assess the total phenolic content of apple juices. However, corresponding studies in tomato juice are very limited and much of the work has focused on the phenolic content of tomato fruit. The major components present in tomato fruit have been identified as chlorogenic acid, rutin and naringenin.[1,10]

Many researchers apply the approach of separate identification of individual families of phenolics when analysing the flavonoid content of complex food matrices. In this study we have compared the composition of fruit juices applying different analytical protocols of a single gradient run HPLC analysis of multiple phenolic components of apple and tomato juices, which is in use at Guy's, with the Glasgow group's multiple HPLC analyses of individual phenolic families using chromatographic conditions that were adjusted to optimise the separation of the compounds under investigation. The study showed that very similar results were obtained from both methodological approaches.

MATERIALS AND METHODS

Juices were purchased from the UK Safeway Supermarket chain. The apple juice was a Safeway own-brand product of concentrated, unsweetened and clear apple juice. A Libby's concentrated tomato juice (Nestlé, Vevey, Switzerland) was used for the tomato juice analysis.

Methanol (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, Peebleshire, UK), hydroxycinnamic acids and flavonoids were obtained from Antioxidant Analysts Suppliers and Consultants (Southamton, UK) and Extrasynthese (ZI Lyon Nord, Genay, France), respectively, salicylic acid was purchased from Fluka Chemicals (Gillingham, UK) and β-glucosidase (from almonds, salt free) from ICN Biomedicals Inc. (Chineham Business Park, Basingstoke, UK) for the single run analyses at Guy's where Elgastat UHP double distilled water (18.2 Ω Grade) was used in all experiments. For the Glasgow analyses, methanol and acetonitrile (HPLC grade) were supplied by Rathburn Chemicals Ltd., all phenolics were purchased from Sigma-Aldrich (Poole, Dorset, UK), β-glucosidase enzyme (from almonds, salt free) was purchased from Sigma-Aldrich. In-house glass distilled water was used in Glasgow.

Standards of chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, phloridzin, phloretin and naringenin were prepared in methanol.

Analysis of Phenolics Using a Single Run HPLC Methodology (Guy's)

Apple juice was prepared with minimal treatment. The analyte mixture consisted of 800 µl apple juice, 100 µl HPLC solvent A (see below) and 100 µl of methanol containing salicylic acid as an internal standard (100 µM final concentration). Samples (30 µl) were injected directly on the HPLC column.

Because it contained particulate matter, tomato juice was extracted with an equal volume of methanol containing 104 µM (final concentration) of the internal standard (as above). Extracts, 30 ml in volume, were centrifuged at 4000 rpm for 15 min at 4°C and the supernatant reduced to 9 ml *in vacuo* at 45–50°C. The extract was made up to a 10 ml volume with water after which 800 µl aliquots were diluted with 200 µl of HPLC solvent A. Triplicate samples, each 30 µl in volume,

were then analysed by gradient reversed-phase HPLC.

The juices were also treated with β-glucosidase (3811 U/mg) to cleave the glycosides. Hydrolysis was performed on 1 ml of apple juice, containing 125 μM (final concentration) internal standard and 1 mg of enzyme by incubation at 37°C for 1 h. Tomato juice extracts, already containing internal standard, were hydrolysed under the same conditions (1 ml extract/1 mg β-glucosidase). All enzyme hydrolysed samples were filtered (Millipore Zymark filter, 0.5 μm) prior to HPLC analysis.

The HPLC procedure used in the single step gradient protocol has been previously described by Paganga and Rice-Evans[11] and was used here with a small modification to the gradient end-point from 50 to 40 min (see below). HPLC analysis was carried out on a Waters HPLC system with Millenium 32 software (Milford, MASS, USA). The system consisted of a 717 plus autosampler with temperature controller (20°C), a 600 pump with controller and a 996 photodiode array (PDA) detector. All analyses were performed on a 250 × 4.6 mm (i.d.) 4 μm C18 Nova-Pak cartridge column, maintained at a temperature of 30°C. Injections were by means of an autosampler and the volume injected each time was 30 μl. A mobile phase consisting of solvent A (water/methanol/5 N HCl, 80 : 20 : 0.1, v/v) and solvent B (acetonitrile) was employed as a linear gradient at a flow rate of 0.5 ml/min. The elution began at 95% solvent A for 10 min and then decreasing linearly to 50% solvent A at 40 min, back to 95% solvent A at 55 min and held at these conditions for a further 5 min. An injection delay of 10 min was used to ensure re-equilibration of the column before the next injection. Analysis was performed using spectroscopic analysis by PDA detection from 200 to 600 nm and the monitoring wavelength was 320 nm. Peak identification was confirmed by matching sample peak characteristics to those of authentic standards i.e. retention time and spectral integrity, and by spiking with suspected components (post-enzyme hydrolysis samples only).

Analysis of Phenolics Using a Multiple Run HPLC Methodology (Glasgow)

Triplicate, 400 μl volumes of apple juice were spiked with internal standard (final concentration 4 μM salicylic acid or 3 μM esculin) and 25 μl aliquots were then analysed by HPLC. Tomato juice (3 × 400 μl), containing internal standard (final concentration 5 μM salicylic acid or 5 μM morin) was extracted with methanol (300 μl). After 30 min at room temperature the samples were centrifuged at 2000× *g* and the supernatant diluted 2 : 3 with distilled water prior to analysis. For enzyme hydrolysis, 1 ml of apple juice was incubated for 1 h at 37°C with 1 mg of β-glucosidase enzyme (7 U/mg). Prior to the treatment of tomato juice with enzyme the juice was extracted with methanol, centrifuged and the solvent removed from the supernatant *in vacuo*. The dry sample was redissolved in 1 ml distilled water and incubated for 1 h at 37°C with 1 mg of enzyme (7 U/mg).

Samples were analysed using a Shimadzu (Kyoto, Japan) LC-10AD VP series automated liquid chromatograph comprising a SCL-10A VP system controller, two LC-10AD VP pumps, A SIL-10AD VP autoinjector with sample cooler, A CTO-10AC VP column oven operating at 40°C and a Waters 996-PDA detector linked to Millenium 32 software. Reverse-phase separations were carried out using various columns depending on the compounds being investigated. In order to optimise resolution in the isocratic analyses the mobile phase conditions were designed to provide k' values of *ca.* 4–5 for the compounds of interest. Internal standard retention times within each of the multiple run analyses did not exceed a CV of 0.4%.

The hydroxycinnamates, caffeic acid, *p*-coumaric acid and chlorogenic acid of apple and tomato juices were chromatographed on a 250 × 4.6 mm (i.d.) 5 μm ODS Hypersil column (Astmoor, UK)

eluted isocratically at a flow rate of 1 ml/min with 8% acetonitrile in water containing 2.5% acetic acid. The column eluent was directed to the PDA detector and traces monitored at 313 nm. For analysis of these components esculin was used as an internal standard in apple juice while salicylic acid was used in the study of tomato juice.

The analyses of phloridzin in apple juice and naringenin in tomato juice were carried out on a 250 × 4.6 mm (i.d.) 5 μm ODS Hypersil column, eluted isocratically at a flow rate of 1 ml/min with 22.5% acetonitrile in water containing 2.5% acetic acid for phloridzin and with 20% acetonitrile in water containing 3.8% acetic acid for naringenin. The column eluents were monitored at 285 nm and salicylic acid was used as an internal standard for both juices.

Reverse-phase HPLC of rutin in tomato juice used a 150 × 3.0 mm (i.d.) 4 μm Genesis C18 column (Jones Chromatography, Mid-Glamorgan, UK). The mobile phases were water containing 0.5% trifluoroacetic acid (solvent A) and acetonitrile (solvent B). The column was eluted at a flow rate of 0.5 ml/min with a gradient of 15–40% B over 25 min. Traces were monitored at 365 nm and morin was used as an internal standard.

RESULTS AND DISCUSSION

Comparative data are presented to show the levels of phenolic constituents present in fruit juices of the same batch but analysed according to the different methodological approaches. Results obtained from a short run-time HPLC methodology, with chromatographic conditions specific for the different classes of phenolics are compared to results from a method utilising a single chromatographic procedure to quantify all the phenolics present in apple juice and tomato juice.

Chromatograms illustrating the total phenolic content of apple and tomato juice from the single step gradient HPLC analysis (Guy's) are illustrated in Figures 1 and 2 while quantitative estimates of the components detected are presented in Tables I and II. Using this procedure the major phenolics in apple juice were identified as chlorogenic acid (R_t 9.9 min), caffeic acid (R_t 14.2 min), *p*-coumaric acid (R_t 24.6 min), ferulic acid (R_t 28.6 min), phloridzin (R_t 38.0 min) and

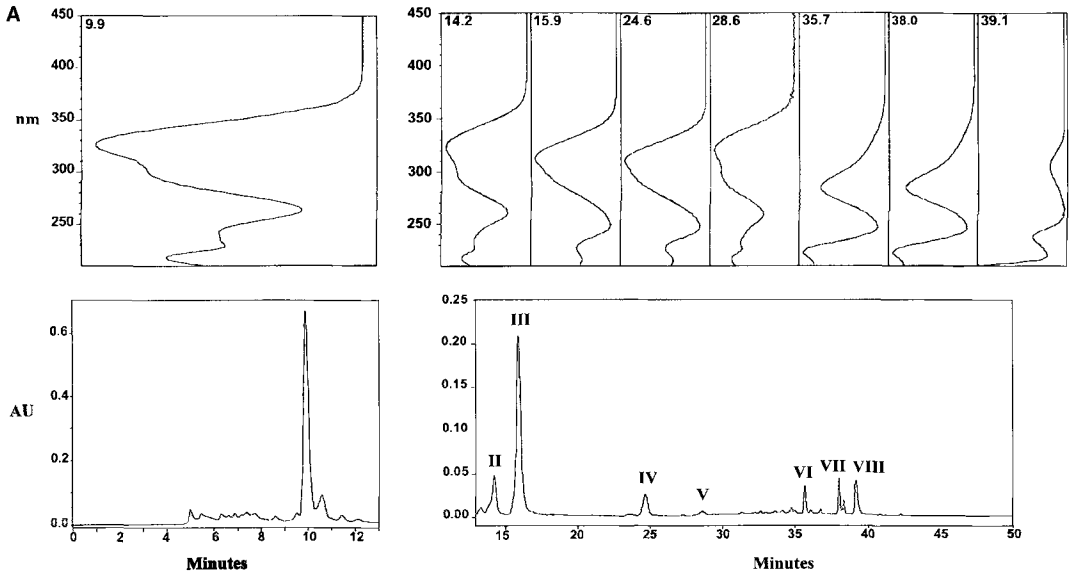

FIGURE 1(A)

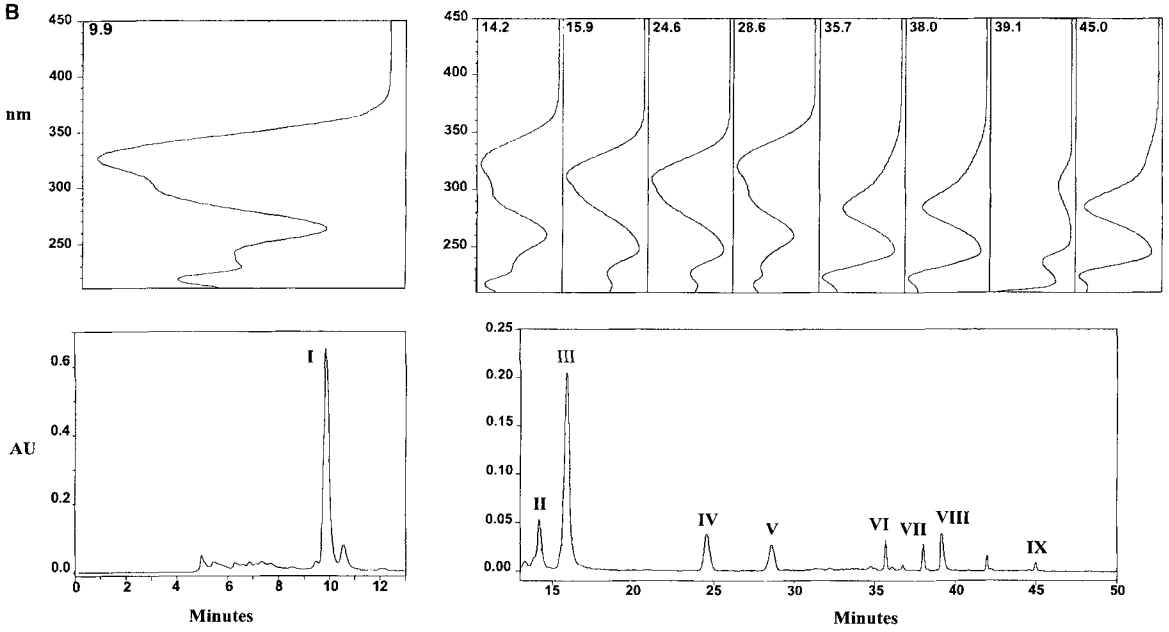

FIGURE 1(B)

FIGURE 1 Single step gradient reverse-phase HPLC analysis of the phenolic composition of apple juice before (A) and after (B) enzyme hydrolysis with β-glucosidase. A: peaks eluting at 9.9, 14.2, 15.9, 24.6, 28.6, 35.7, 38.0 and 39.2 min correspond to I – chlorogenic acid, II – caffeic acid, III – *p*-coumaryl quinic acid, IV – *p*-coumaric acid, V – ferulic acid, VI – phloretin xyloglucoside, VII – phloridzin and VIII – salicylic acid (internal standard). B: eluting peaks are the same as presented in A, with an additional peak at 45.0 min, corresponding to IX – phloretin. Identification of peaks was made by reference to retention times, spectral determination and spiking with authentic standards. Analysis was performed using spectroscopic analysis by PDA detection from 200 to 600 nm and the monitoring wavelength was 320 nm.

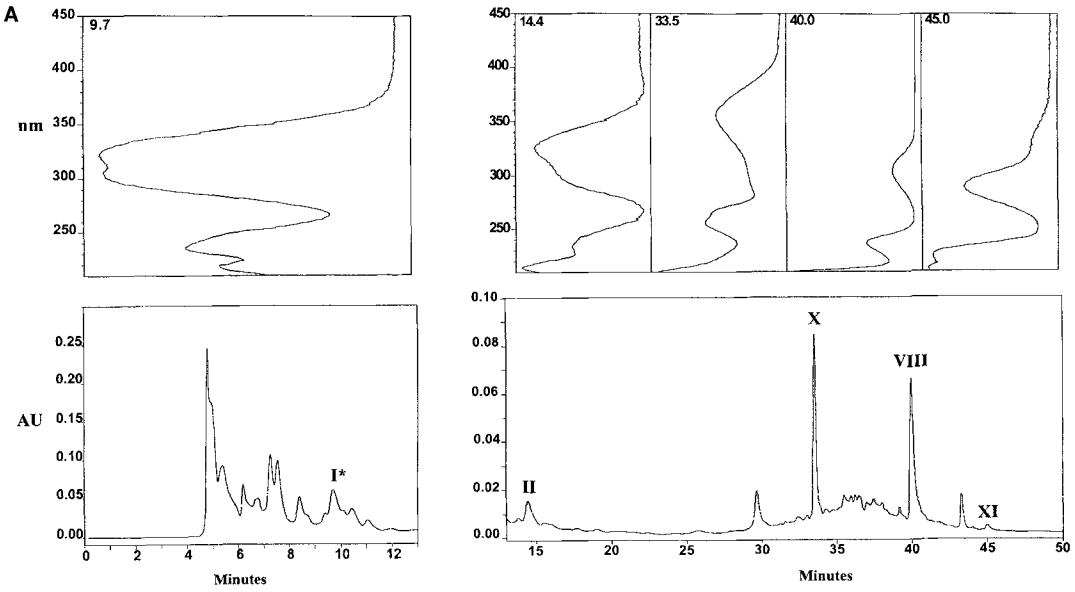

FIGURE 2(A)

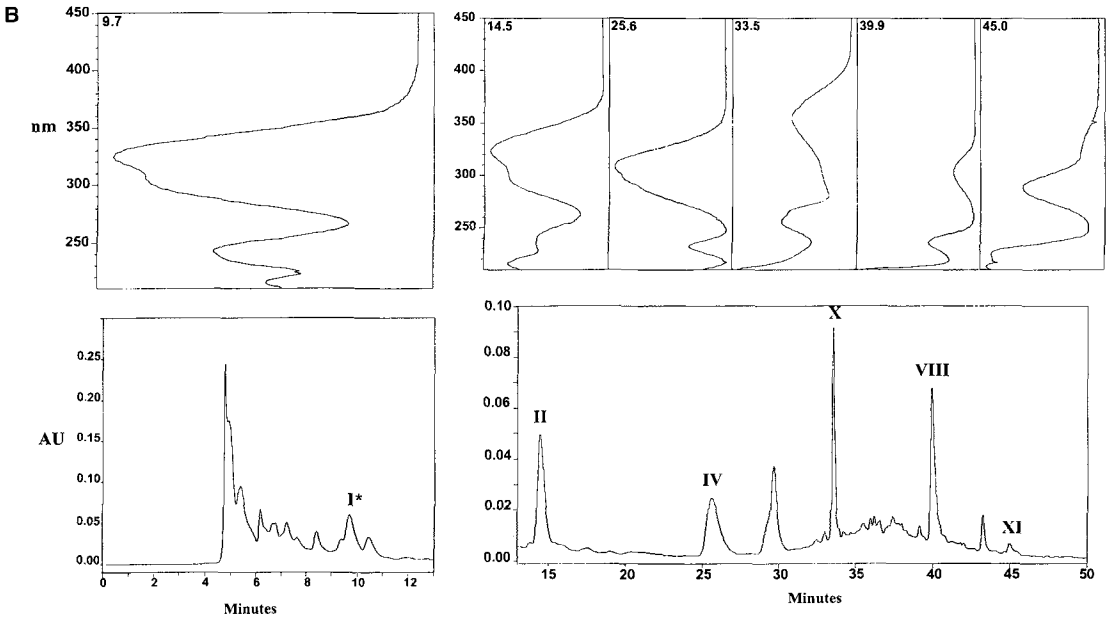

FIGURE 2(B)

FIGURE 2 Single step gradient reverse-phase HPLC analysis of the phenolic composition of tomato juice before (A) and after (B) enzyme hydrolysis with β-glucosidase. A: peaks eluting at 9.7, 14.4, 33.5, 40.0 and 45.0 min correspond to I – chlorogenic acid (*co-eluting peaks), II – caffeic acid, X – rutin, VIII – salicylic acid (internal standard) and XI – naringenin. B: eluting peaks are the same as presented in A, with an additional peak at 25.6 min, corresponding to IV – *p*-coumaric acid. Identification of peaks was made by reference to retention times, spectral determination and spiking with authentic standards. Analysis was performed using spectroscopic analysis by PDA detection from 200 to 600 nm and the monitoring wavelength was 320 nm.

TABLE I *Levels of phenolic components in apple juice analysed by HPLC in a multiple run analysis and a single run analysis*

Juice sample	Component (mg/l ± SD)								
	Chlorogenic acid	Caffeic acid		*p*-Coumaric acid		Ferulic acid		Phloridzin	Phloretin
	Pre/post-hydr.	Pre-hydr.	Post-hydr.	Pre-hydr.	Post-hydr.	Pre-hydr.	Post-hydr.	Pre-hydr.	Post-hydr.
Multiple run analysis									
A1	83.5 ± 1.5	2.8 ± 0.1	2.4′	1.4 ± 0.1	2.4′	*	*	12.2 ± 0.05	*
A2	79.8 ± 1.6	2.4 ± 0.3	2.9′	1.4 ± 0.04	2.5′	*	*	12.4 ± 0.1	*
A3	69.3 ± 0.9	4.0 ± 0.2	2.7′	2.1 ± 0.03	3.1′	*	*	13.3 ± 0.03	*
A4	94.5 ± 1.2	1.4 ± 0.2	1.7′	1.8 ± 0.06	2.1′	*	*	9.7 ± 0.03	*
Single run analysis									
A1	89.3 ± 3.8	2.8 ± 0.05	3.5 ± 0.2	1.4 ± 0.1	2.9 ± 0.2	0.5 ± 0.03	2.9 ± 0.1	12.9 ± 0.7	1.2 ± 0.2
A2	86.5 ± 4.3	2.8 ± 0.1	3.4 ± 0.1	1.4 ± 0.1	2.9 ± 0.1	0.5 ± 0.03	2.7 ± 0.1	13.0 ± 0.8	1.5 ± 0.4
A3	85.4 ± 3.3	3.8 ± 0.5	4.3′	2.4 ± 1.4	3.2′	0.5′	2.9′	13.8 ± 1.2	2.7′
A4	102.4 ± 4.4	1.9′	2.6′	1.0 ± 0.3	1.2′	0.3′	2.7′	10.4 ± 1.2	2.3′

Replicates are all ≥ 3, except for those superscripted ($n = 2$). Post-hydr. = post-enzyme hydrolysis; * = not analysed.

phloretin (R_t 45 min) (post-enzyme treatment, Figure 1B). In tomato juice the principal components were chlorogenic acid (R_t 9.7 min), caffeic acid (R_t 14.4 min), *p*-coumaric acid (R_t 25.6 min) (post-enzyme treatment, Figure 2B), rutin (R_t 33.5 min) and naringenin (R_t 45.0 min). Recoveries were calculated from the post-hydrolysis samples in a range 85–115% for all components.

TABLE II Levels of phenolic components in tomato juice analysed by HPLC in a multiple run analysis and a single run analysis

Juice sample	Component (mg/l ± SD)						
	Chlorogenic acid	Caffeic acid		*p*-Coumaric acid		Rutin	Naringenin
	Pre/post-hydr.	Pre-hydr.	Post-hydr.	Pre-hydr.	Post-hydr.	Pre/post-hydr.	Pre/post-hydr.
Multiple run analysis							
A1	6.0 ± 0.01	0.8 ± 0.01	4.3 ± 0.02	0.2 ± 0.01	3.0 ± 0.1	20.2 ± 0.3	1.1 ± 0.1
A2	6.4 ± 0.3	0.8 ± 0.03	4.4 ± 0.3	0.2 ± 0.01	3.1 ± 0.2	19.8 ± 0.3	1.1 ± 0.1
A3	10.9 ± 0.4	1.1 ± 0.04	6.9 ± 0.1	0.2 ± 0	4.9 ± 0.2	31.4 ± 0.6	9.2 ± 0.2
A4	8.9 ± 0.2	1.1 ± 0.1	5.7 ± 0.2	0.1 ± 0	4.0 ± 0.1	31.4 ± 0.5	10.5 ± 0
Single run analysis							
A1	7.8 ± 0.2	1.5 ± 0.05	6.6 ± 0.1	n.d.	6.0 ± 0.1	25.5 ± 0.2	3.2 ± 0.3
A2	6.7 ± 0.1	1.4 ± 0.04	6.2 ± 0.1	n.d.	5.3 ± 0.1	22.8 ± 0.2	2.6 ± 0.2
A3	9.2 ± 0.8	1.2 ± 0.1	7.6 ± 0.1	n.d.	7.1 ± 0.1	30.6 ± 0.7	9.6 ± 1.0
A4	10.6 ± 0.1	1.5 ± 0.1	8.7 ± 0.1	n.d.	7.8 ± 0.1	35.8 ± 0.4	8.4 ± 0.2

Replicates are all ≥ 3. Post-hydr. = post-enzyme hydrolysis; n.d. = not detected.

Figures 3 and 4 show the chromatograms obtained in the analysis of apple and tomato juices using multiple HPLC runs (Glasgow). The major phenolics in apple juice were chlorogenic acid, caffeic acid, *p*-coumaric acid and phloridzin. Chlorogenic acid, caffeic acid and *p*-coumaric acid were also detected in tomato juice. Recoveries for each component were calculated on the basis of the internal standard and were in the range 95–110%. By altering the chromatographic conditions the presence of rutin and naringenin were demonstrated in tomato juice. The quantitative estimates of the phenolics detected in apple and tomato juices by the multi-step HPLC analysis are presented in Tables I and II.

Similar results were obtained with both HPLC methodologies. Chlorogenic acid was the major component of the apple juice under investigation, followed by phloridzin, caffeic acid and *p*-coumaric acid (Table I). Treatment with β-glucosidase revealed small increases in the concentrations of caffeic acid and *p*-coumaric acid. The study by single run analysis also showed ferulic acid in the pre-enzyme hydrolysis treatment and recorded an average 7-fold increase in the aglycone following enzyme treatment. Rutin was the principal phenolic component in the tomato juice, followed by chlorogenic acid, caffeic acid, *p*-coumaric acid and naringenin (Table II). Enzymic hydrolysis of the tomato juice extracts resulted in increased levels of caffeic acid and *p*-coumaric acid using both methodologies. Overall, the samples analysed after enzyme hydrolysis showed an average 5-fold increase in caffeic acid. Although low levels of *p*-coumaric acid were detected by multiple run analysis prior to enzyme hydrolysis, it was only detected by single run analysis following β-glucosidase treatment. Enzymic hydrolysis of the tomato juice extracts resulted in levels of *p*-coumaric acid of between 3 and 9 mg/l. The resolution of the chlorogenic acid peak at R_t 9.7 min in the single run analysis (Figure 2A and B) was unsatisfactory due to the presence of co-eluting products. Using the multiple HPLC runs, baseline resolution was achieved for all components in apple and tomato juice with the exception of the chlorogenic acid peak in tomato juice before enzyme hydrolysis which eluted close to an earlier eluting minor peak. However, even in this instance, resolution remained close to baseline (see peak I, Figure 3C).

The presence of ferulic acid, as far as we are aware, has not been reported in apple fruit *per se*, but small or trace amounts have been detected in apple juice.[8,12] In this present study, ferulic acid was only identified in the single run analysis. This discrepancy may reflect the full investigative nature of the single run analysis that enabled the

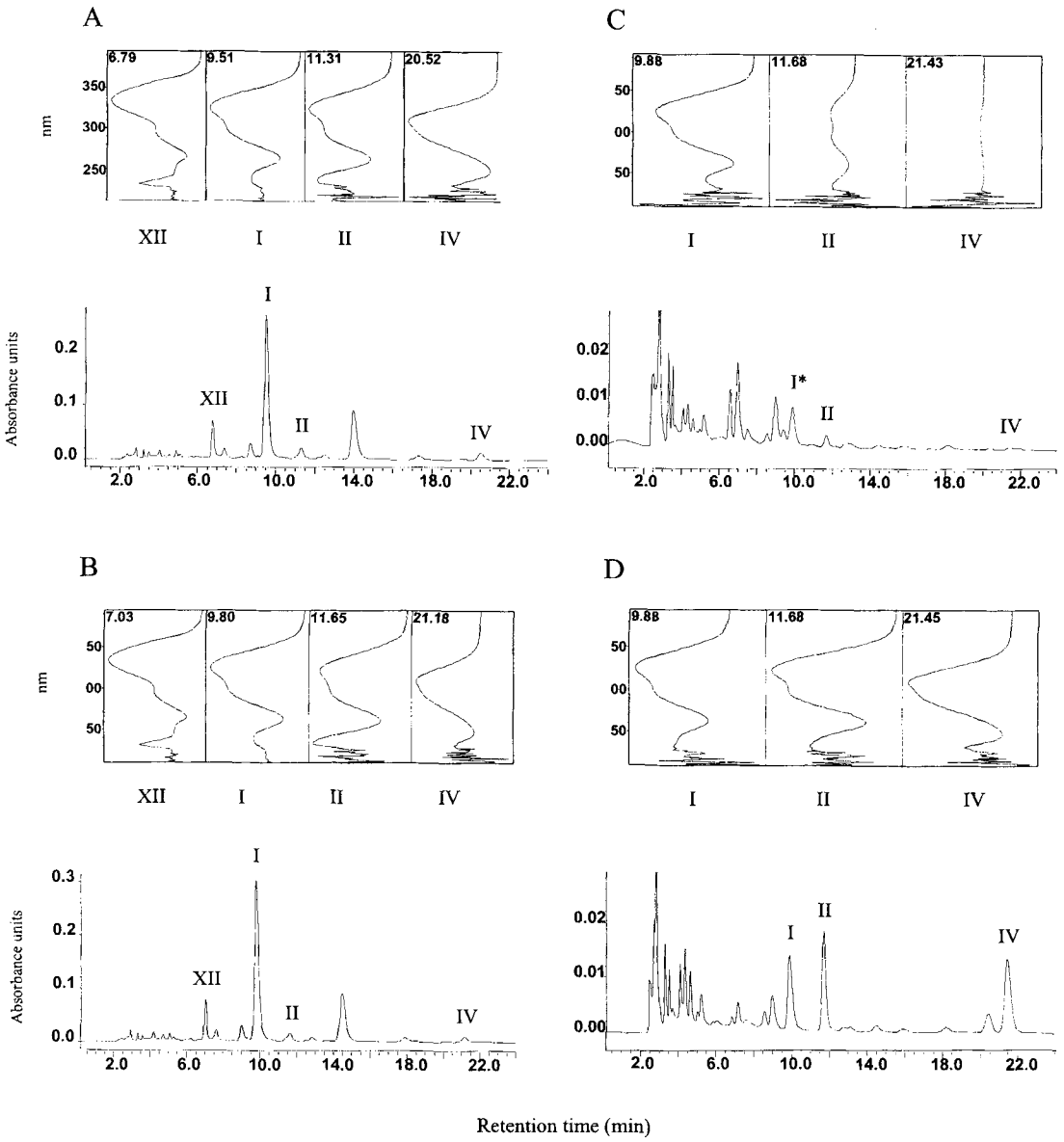

FIGURE 3 Isocratic reverse-phase HPLC analysis of hydroxycinnamates in apple and tomato juice before and after enzymatic hydrolysis with β-glucosidase. A: apple juice prior to hydrolysis. B: apple juice after hydrolysis. C: tomato juice before hydrolysis. D: tomato juice after hydrolysis. In all chromatograms, I – chlorogenic acid (*co-eluting peaks in C), II – caffeic acid and IV – *p*-coumaric acid. Esculin (XII) used as an internal standard with apple juice, while salicylic acid was the internal standard with tomato juice. The salicylic acid peak at R_t 30.0 min is not included in C and D. Identification of peaks based on retention times, matching absorbance spectra and co-chromatography with authentic standards.

majority of phenolic components to be detected, whereas the application of the multiple run technique requires a knowledge of the compounds likely to be present in order to optimise the chromatographic conditions for resolution of the individual families.

The single analytical method also provisionally identified two further phenolic constituents of

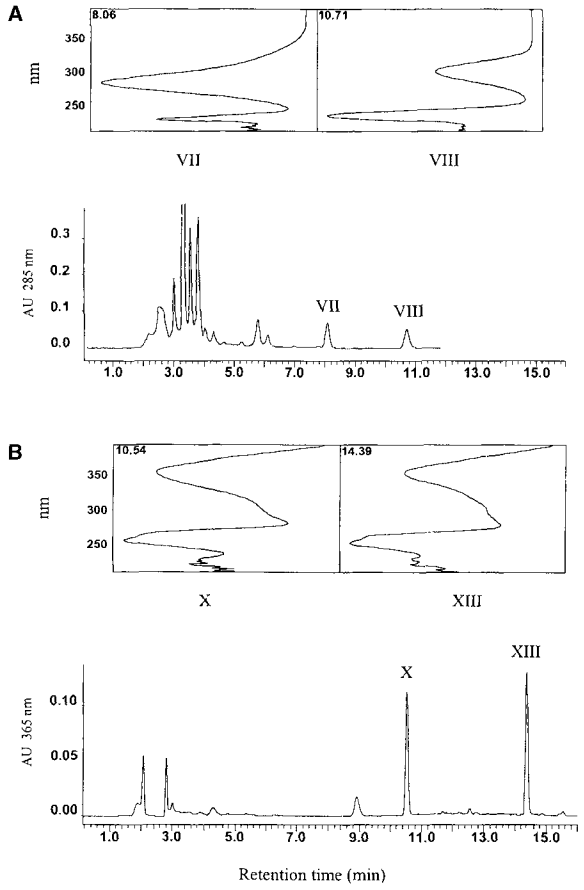

FIGURE 4 Reverse-phase HPLC analysis of phloridzin in apple juice (A) and rutin in tomato juice (B). A: Peak VII is phloridzin and salicylic acid (VIII) was used as the internal standard. B: Peak X is rutin and morin (XIII) was used as the internal standard. Identification of peaks based on retention times, matching of absorbance spectra and co-chromatography with authentic standards.

apple juice. Peaks eluting at R_t 15.9 min and R_t 38.0 min were identified by their spectral and retention time characteristics as the quinic acid ester of *p*-coumaric acid and the xyloglucoside of phloretin (Figure 1). Standards of these compounds were not available but their quantification was achieved based on the absorption of *p*-coumaric acid and phloretin, respectively (personal communication C.R.-E.: Dr. A. Lea and Dr. M. Clifford). The average concentrations of *p*-coumaryl quinic acid and phloretin xyloglucoside in the 4 bottles of apple juice analysed were 34.6 ± 1.0 and 16.5 ± 1.0 mg/l, respectively.

Overall, the levels of phenolics present in apple juice (Table I) and tomato juice (Table II) batches were very similar between the two methodologies. This was the case for almost all components although there was some variation in the estimates of the levels of chlorogenic acid in apple juice batches A3 and A4 and in the concentration of *p*-coumaric acid in tomato juice after treatment with β-glucosidase. Within batch variation was minimal. This consistency in the level of phenolics was also maintained between the two methodologies used. Thus, phloridzin in apple juice was consistent using the separate methodologies (multiple run = 12.3 mg/l and single run = 12.9 mg/l). Likewise, levels of rutin and naringenin in tomato juice were consistent within the batches, using the two HPLC methodologies. Batches of tomato juice also varied in composition between 22 and 32 mg/l for rutin and 2–9 mg/l for naringenin. These differences may reflect processing variation or differences in the phenolic levels of the fruit used to produce the juice. Such variation has been found for chlorogenic acid range values among the pulp of four apple varieties, including Golden Delicious (29–57 mg/kg), Green Reineta (266–357 mg/kg), and Red Delicious (63–106 mg/kg) and Granny Smith (28–71 mg/kg).[13]

The identification of rutin as the major phenolic component in tomato juice was in agreement with a study on tomato fruit.[10] Based on a conversion from the dry weight quoted (302 mg/kg)[10] to fresh weight (*ca.* 92% water), 24 mg/kg of rutin was present in the tomato fruit. This level was within the range of the concentrations obtained for the tomato juice batches (Table II). The minor constituents of chlorogenic acid, caffeic acid and *p*-coumaric acid and the flavanone, naringenin were also present in the tomato juice. However, Paganga *et al.*[10] recorded higher levels of chlorogenic (24 mg/kg fw) and naringenin (23 mg/kg fw) in tomato fruit when compared to the levels in the juices studies here. The ratio of the

levels in the fruit phenolics of chlorogenic acid, naringenin and rutin was 1 : 1 : 1 but in this present study the ratio was 1 : 3 : 0.05 (bottles A1 and A2) and 1 : 3 : 1 (bottles A3 and A4).

Reported levels of phenolics in apple juice vary greatly and have been shown to be in part due the variety differences in the apples[2,3,13] and the processing method used to produce the juice.[5,9,12] HPLC protocols used in the analysis of fruit phenolics are generally very similar in the use of PDA detection, reverse-phase columns and gradient elution with an acidic mobile phase. In part, however, a significant variation in phenolic content can be realised as a direct result of sample preparation steps prior to HPLC analysis. This is particularly evident in the use of diethyl ether and/or ethyl acetate liquid–liquid extraction methods. Vallés *et al.*[3] used an ethyl acetate extraction of apple juice and analysed the neutral phenolic compounds in the organic layer by HPLC. They reported low recovery of flavonol glycosides (30%). Likewise, a diethyl ether and ethyl acetate extraction resulted in very low levels of chlorogenic acid (2.5–9.1 mg/l)[8] that is *ca.* 70-fold lower than levels (300 mg/l) found in apple juice when analysed using a direct injection method[4] and also significantly lower than levels found in apple fruit pulp[10,13] and peel.[13] The less hydrophilic dihydrochalcone, phloridzin, was recorded at a range of 13.8–26.9 mg/l and was similar to levels found in the direct injection study.[4] Therefore purification steps should avoid the use of ethyl acetate to ensure a maximum recovery of the flavonols and esterified hydroxycinnamates (i.e. chlorogenic acid). Methanolic extraction of apple and tomato fruit[10,13] has also been successfully used to recover total phenolic components.

A high level of chlorogenic acid is consistent with the findings of other studies on apple juice phenolics which showed this hydroxycinnamate to be a major component.[4,14] Delage *et al.*[2] reported this to be the case in four single cultivar apple juices, except for Judor, where phloridzin (12 mg/l) was present in a higher concentration than chlorogenic acid (7 mg/l). In addition, of the four juices studied, the juice prepared from Kermerrien cultivar had the highest level of chlorogenic acid at 249 mg/l. This represented 35-fold higher than the level in juice from Judor apples. However, Delage *et al.*[2] did use a sample preparation method using an ethyl acetate extraction prior to HPLC. Therefore, the levels of chlorogenic acid, although high for some of the apple juices, are likely to have been underestimated. In another study of the pulp of a further four apple cultivars, the major component was chlorogenic acid except for a higher level of catechin in Granny Smith.[13] Here again there were large cultivar differences in chlorogenic acid content, which ranged from 50 to 300 mg/kg. In addition, the apple peel was analysed and found to contain additional components of rutin and very high levels of procyanadin B2 (100–400 mg/kg). New Zealand Royal Gala apples have been shown to have high levels of chlorogenic acid (120 mg/kg fw) and naringenin (46 mg/kg fw) isolated from the pulp.[10]

In this present study it was not known what apple cultivar was used to produce the juice, or whether it consisted of a blend. Neither procyanadins nor rutin were detected. This may be a function of the use of different cultivars and/or processing methods. Spanos *et al.*[9] have shown that different processing treatments will effect the levels of phenolic constituents. Heat treatment was shown to protect phenolics in the juice from polyphenol oxidase, while enzymic clarification caused hydrolysis of conjugated hydroxycinnamates and long-term storage lowered the concentration of procyanadins. Part of this analysis was undertaken on Granny Smith apples and it was clear that these fruit had a much lower level of phenolic constituents than reported by Escarpa and González[13] on the same variety. This discrepancy could be due to the fact that the apples in the Spanos *et al.*[9] study were stored for 3 and 9 months to compare the effect of short and long periods of storage on phenolic content. But no control group of fresh juice was assayed.

The present study has shown that by utilising minimal pre-HPLC extraction procedures, the total phenolic content of apple juice and tomato juice can be analysed readily. Consistent results have been obtained in the two laboratories using different HPLC approaches of single runs of multiple components or multiple runs of individual components, although the chlorogenic acid was not resolved appropriately in tomato juice extract for the single run approach and to a lesser degree in the pre-hydrolysis samples of the multiple run analysis. The method of direct injection of clear juice, as in the case of apple juice used here and elsewhere[4] is recommended, whereas the more viscous and particulate tomato juice requires extraction with methanol.

The outcome of these analytical investigations suggest that routine analyses can be undertaken more rapidly and accurate results obtained using the single-step gradient HPLC system. However, it is important to recognise that this will be dependent on the plant extract. For example, there may be conditions in which the multiple run approach will need to be applied to constituents eluting at specific regions of the chromatogram where co-elution occurs. Under these circumstances, the multiple run for individual families of compounds, using a variety of solvent systems, would circumvent this problem. Our recommendation is that the initial approach to studying plant phenolic constituents would be to apply the single run system, and to use the multiple run approach where chromatographic resolution requires improvement.

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