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The genotypic variation of the antioxidant potential of different tomato varieties

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Abstract
There is increasing interest in the ability of diets rich in polyphenols to modulate age-related diseases and promote healthy ageing. We have conducted a pilot experiment with eight tomato varieties to correlate the total antioxidant capacity of the tomato variants with the specific constituent flavonoids present. A strong correlation was observed with the flavonol rhamnoglucoside rutin but not with other flavonoids, such as naringenin chalcone, or hydroxycinnamates, such as chlorogenic, which are also present in the tomato. To test the rigor of this correlation a second study was undertaken with a further 37 tomato varieties selected for low, medium and high rutin levels. We show that the flavonol rutin contributes to the greatest extent to the antioxidant capacity of tomatoes and suggest that this flavonoid may be a useful target for up-regulation in tomatoes in order to improve their antioxidant status.

Keywords: *Antioxidant, flavonoid, tomato, rutin, TEAC, MS*

Introduction

There is a growing interest in the production of food plants with increased amounts of flavonoids because of their potential health benefits [1]. Flavonoids are a diverse group of phenolic secondary metabolites that occur naturally in plants and therefore form an integral component of the human diet. Many of the compounds belonging to this group are potent antioxidants *in vitro* and epidemiological studies suggest a direct correlation between high flavonoid intake and decreased risk of cardiovascular disease [1–4], cancer [3,5] and other age-related diseases [6]. Consequently, enhancement of flavonoid biosynthesis in chosen crops, such as tomatoes, is likely to increase human consumption of flavonoids that have the potential to benefit human health.

Flavonoids are the most abundant polyphenols in the human diet and are divided into six main classes based on the degree of oxidation of the C-ring, the hydroxylation pattern of the ring-structure and the substitution in the 3-position: flavanols (e.g. epicatechin), flavonols (e.g. quercetin), flavones (e.g. luteolin), flavanones (e.g. naringenin), isoflavones (e.g. genistein) and anthocyanidins (e.g. cyanidin) [7]. A large number of *in vitro* studies have characterised flavonoids as powerful antioxidants capable to efficient

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scavenging of both reactive oxygen and reactive nitrogen species. Structurally important features which define this antioxidant activity are the hydroxylation pattern, in particular a 3,4-dihydroxy catechol structure in the B-ring and the presence of 2,3 unsaturaturation in conjugation with a 4-oxo-function in the C-ring. The antioxidant efficacy of flavonoids has been described for the protection against oxidative damage to a variety of cellular biomolecules. For example, flavonoids inhibit the oxidation of low density lipoprotein [8,9] and DNA [10]. In addition, flavonoids are effective scavengers of reactive nitrogen species in the form of peroxynitrite [11,12] and limit dopamine oxidation mediated by peroxynitrite in a structure-dependent way involving oxidation or nitration of the flavonoid ring system [13]. Furthermore, their antioxidant properties have also been attributed to their abilities to chelate transition metal ions [14,15] and on their potential to quench singlet oxygen [16].

Among the most prominent flavonoids in tomato fruit are the flavanone naringenin and its chalcone, and the flavonol rutin, quercetin rhamnoglucoside [17–21]. Although most of these flavonoids are located in the peel of the fruit, recent protocols have been designed to increase flavonoid levels in tomato flesh by the use of maize transcription factor genes LC and C1 in the fruit of genetically modified tomato plants [22–24]. In one study, manipulation of a single biosynthetic enzyme, chalcone isomerase resulted in an increase in total fruit flavonols of up to 78-fold [25]. In the same study chalcone synthase and flavonol synthase transgenes were found to act synergistically to up-regulate flavonol biosynthesis significantly in tomato flesh tissues. Whilst increasing flavonoid content in tomato fruit may seem to be beneficial to health, in order to maximise the antioxidant potential it is necessary to obtain a clear idea of which major phenolic families present in tomatoes influence the total antioxidant potential to the greatest extent. In this study we have measured the total antioxidant capacity of a variety of tomato variants and examined the correlation with the specific constituent flavonoids present. We show that the flavonol rutin contributes to the greatest extent to the antioxidant capacity of tomatoes and suggest that this flavonoid may be a useful target for up-regulation in tomatoes in order to improve their antioxidant status.

Experimental

Materials

Chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, rutin, naringenin, naringenin-3-O-glucoside and kaempferol-3-O-rutinoside were purchased from Extrasynthese (Genay Cedex, France). Elgastat UHP double distilled water (18.2 MΩ grade) was used throughout the study. HPLC specialised solvents, acetonitrile and methanol, were purchased from Rathburn (Walkerburn, UK) and HPLC columns from Waters (Watford, Herts., UK). β-Glucosidase was sourced from sweet almonds and obtained from ICN Biomedicals, Oh, USA). All other reagents used were of analytical grade and obtained from the Sigma Chemical Company (Poole, Dorset, UK).

Study tomatoes

The tomato varieties selected for the pilot study were: two varieties of Micro Tomato (MT) and Micro Tomato (28/04/03), T832, Lyc 31, Lyc 434, Lyc 1325, Lyc 1375 and Lyc 1919. Following the pilot investigation a further 37 varieties were specifically selected for their rutin content in order to assess statistical correlation with the antioxidant potential. These tomatoes had previously been analysed for their polyphenol contents and were chosen in order to provide samples with low, medium and particularly high rutin levels. All the tomato varieties tested have been obtained through the Gatersleben Genebank (http//www.ipk-gatersleben.de) and all lines constitute normal genetic variation meaning that they represent plants produced by normal breeding methods (not genetic engineering methods). The dry weight (DW) of the tomatoes was determined by lyophilisation of fruits by standard freeze-drying techniques (Labconco Console Freeze-Dry System).

Extraction procedure

Polyphenols, hydroxycinnamates, flavonoids and their glycosides were extracted from lyophilised tomato by repeated aqueous methanol extractions. A measure of 1 ml of methanol/water (2:1) was added to 100 mg of freeze-dried tomato and samples were mixed continuously for 30 min using a vibrating agitator at room temperature. Following extraction samples were microfuged at 10,000 rpm for 5 min at 4°C. Supernatants were collected and the pellets were re-extracted a further two times with the above protocol. Following the final extraction all supernatants were adjusted to a 3 ml final volume by addition of the aqueous methanol mixture. Samples were purged with nitrogen and protected from light throughout the extraction.

Trolox equivalent antioxidant activity (TEAC)

The TEAC assay was performed as previously described in Re *et al.* [26], to determine the H-donating potential of the methanolic extracts of the tomatoes. Solutions of 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) (7 mM) and potassium persulphate (2.45 mM) were mixed and allowed to stand for 18 h in order to generate the

ABTS radical cation ($ABTS^{\cdot+}$). For experiments, the $ABTS^{\cdot+}$ stock solution was diluted with PBS (pH 7.4) and equilibrated at 30°C to an absorbance of 0.70 at 734 nm. Trolox for the standard curve was added to the $ABTS^{\cdot+}$ solution to achieve final concentrations of 1.25, 2.5 and 5 μM. Each extract under investigation, was added to the $ABTS^{\cdot+}$ solution to achieve final concentrations of 0.11, 0.22, 0.33 and 0.44 μg/ml extract and the solutions were mixed and the decline in absorbance (734 nm) followed for 10 min (the reaction was not complete at this time). Appropriate solvent blanks were run for each addition. Calculation of the TEAC value was as detailed in Re *et al.* [26] All TEAC calculations were made using data collected in the linear range. In addition, there was linearity between the amount of extract and the TEAC measurement [For T832 tomato: 0.11 mg/ml = 15.8% inhibition; 0.22 = 32.4; 0.33 = 50.4; 0.44 = 60.4].

Detection of phenolic constituents by HPLC

HPLC analysis was performed to characterise and quantify the identifiable phenolic components: hydroxycinnamates, flavonoids and their glycosides. Extracts were diluted 1:5 with mobile phase A (aqueous methanol (20%) containing HCl (0.1%) before injection of 50 μl onto the HPLC system. Separation of phenolic constituents was achieved using reverse-phase HPLC using a Nova-Pak C18 column (4.6 × 250 mm) (Waters) with 4 μm particle size. A Waters 626 pump and 600-controller system with an auto-injector 717 and a photodiode array detector 996 linked to the Millennium Software system was utilised. The temperature of the column was maintained at 30°C. The mobile phase consisted of: (A) aqueous methanol (20%) containing HCl (0.1%) and (B) acetonitrile-methanol (1:1) and was pumped through the column at 0.6 ml min^{-1}. The following gradient system was used (min/% B): 0/0, 50/100, 60/100, 65/0 for detection of all compounds. The eluant was monitored by photodiode array detection at 320 nm and spectra of products obtained over the 220–600 nm range. Calibration curves of flavonoid and hydroxycinnamates were constructed using authentic standards (0–50 μM) and in each case were found to be linear with correlation coefficients of <0.995. All standards and samples were quantified relative to the internal standard, 3,4-dimethoxy-hydroxycinnamic acid, which also acted as a LC retention time marker. The presence of phenolic glucosides in the tomato extracts was established by treatment with β-glucosidase. Extracts were lyophilised to remove methanol prior to their incubation with enzyme (1000 units/ml) for 120 min; 37°C in a 0.1 M sodium acetate buffer, pH 5.0. In addition, were subjected to LC-MS/MS in order to ascribe the presence of glucosides and other glycosides. Total phenolic levels in the tomatoes were assessed using the Folin-Ciocaulteu method as described previously [17].

Characterisation of phenolic constituents by LC-MS/MS

Mass spectrometry was undertaken to characterise and establish the identity of the phenolic constituents. LC/MS/MS was performed using a ThermoFinnigan (San Jose, CA) LCQ Deca XP instrument. For electrospray ionisation (ESI), a 50 mm × 2.0 mm C18 Luna column (Phenomenex, Macclesfield, UK) column with the following gradient (0.1 ml/min) was used (A: 0.1% formic acid in water, B: Acetonitrile): 10 min 100% A, 50 min 75% A, 59 min 50% A, 60 min 100% A, 70 min 100% A. For atmospheric pressure chemical ionisation (APCI), a 250 mm × 5 mm C18 column (Waters, Hertsford, UK) with the same gradient as above but a flow rate of 0.700 ml/min. The mass spectrometer was tuned with rutin and naringenin as standard. Quantification of naringenin was achieved by ESI with an isocratic method (30% A, 70% B, 0.100 ml/min) and hesperetin as internal standard. Hesperetin and naringenin were detected by selected reaction monitoring (SRM; 303 → 177 for Hesperetin, 273 → 147 for Naringenin).

Results

TEAC measurements for antioxidant activity of the plot study tomato extracts

The TEAC assay is designed for analysis of the chemical assessment of the reducing properties of antioxidants through their hydrogen-donating abilities. The TEAC values of each methanolic tomato extract are listed in Table I. Both varieties of MT had the highest antioxidant capacity relative to Trolox (66 ± 1.9 and 57 ± 0.7) whereas the T 832 only achieved half this value (33 ± 0.5). The order of

Table I. TEAC (mean ± SD; $n = 4$) of selected tomato extracts

Tomato	TEAC (mmol Trolox/kg Tomato DW)
Micro Tomato	66 ± 1.9
Micro Tomato (28/04/03)	57 ± 0.7
T 832	33 ± 0.5
Lyc 31	51 ± 1.7
Lyc 434	52 ± 2.7
Lyc 1325	44 ± 0.3
Lyc 1375	37 ± 1.5
Lyc 1919	37 ± 0.3

Methanolic extracts of each tomato was prepared as described in the "Experimental" section. Briefly, lyophilised tomato (100 mg) was extracted three times using methanol/water (2:1) and antioxidant capacity was assessed relative to Trolox.

antioxidant potential of the aqueous methanol extracts was: MT > MT (28/04/03) > Lyc 434 = Lyc 31 > Lyc 1325 > Lyc 1375 = Lyc 1919 > T 832.

Identification and quantification of phenolics

The methanolic extracts of the tomatoes were initially analysed by HPLC with photodiode array detection to identify and quantify the amounts of flavonoids, hydroxycinnamates and their glycosides Figure 1, MT as example). Chlorogenic acid [RT: ~9.3 min], rutin (quercetin-3-O-rutinside) [RT: ~35.7 min], kaempferol-3-O-rutinoside [RT: ~38.4 min] and chalconaringenin/naringenin [RT: ~50.2 min] were identified in all tomato extracts and were confirmed by retention time of standards, spectral matching and spiking samples with authentic standards. There was co-elution of naringenin with its chalcone, although selective spectral analysis of the peak at 50.2 min revealed the presence of both naringenin (Spectra recorded at 50.0 min) and chalconaringenin (spectra recorded at 50.25 min). In all tomatoes, except for T832, the major phenolic compounds present were chalconaringenin/naringenin.

The presence of all phenolic compounds was confirmed by mass spectrometry using SRM of their most abundant product ions (normally the aglycone). Figure 2 shows the product ion spectra for the compounds identified by mass spectrometry. Rutin (Figure 2A, $[M + H^+]^+$ ion at *m/z* 611) shows an intense signal for the aglycone (quercetin, $[M + H^+]^+$ ion at *m/z* 303) and the loss of rhamnose (*m/z* 465). Naringenin (Figure 2B, $[M + H^+]^+$ ion at *m/z* 273) showed two major fragments at *m/z* 147 (which could be assigned to a $^{1,4}B^+$-ion) and 153 (the $^{1,3}A^+$ ion). Kaempferol-3-O-rutinoside (Figure 2C, $[M + H^+]^+$ ion at *m/z* 595) showed a similar fragmentation pattern to rutin, with an intense aglycone ($[M + H^+]^+$ ion at *m/z* 287) and the loss of rhamnose (*m/z* 449).

LC-MS/MS was also undertaken to identify other phenolic components that could not be confirmed by LC-PDA, due to the absence of authentic standards. The quercetin-hexoside (Figure 2D, $[M + H^+]^+$ ion at *m/z* 465) shows an abundant product ion for the aglycone quercetin, but no other intense fragments. Naringenin-glucoside (Figure 2E, $[M + H^+]^+$ ion at *m/z* 435) was also be identified by its mass and its major fragment, the aglycone (*m/z* 273). Dihydrokaempferol-hexoside (Figure 2F, $[M + H^+]^+$ ion at *m/z* 451) was identified by its major fragment, the dihydrokaempferol aglycone (*m/z* 289).[22,23] The latter compound was found to be the compound running at retention time

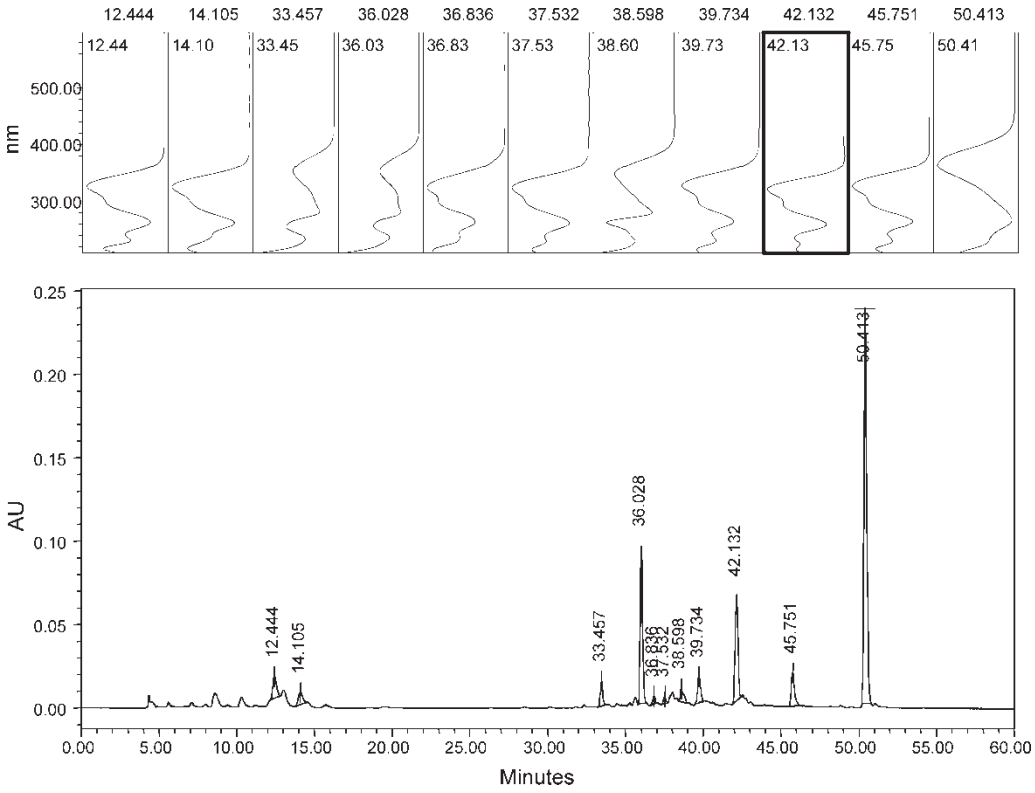

Figure 1. Representative HPLC with photodiode array detection chromatogram for the methanolic tomato extracts. Spectra were monitored over the range 200–700 nm and all peaks in the plots represented were derived at 320 nm. Phenolics were determined by co-elution with authentic standards and were identified as follows: chlorogenic acid (~9.3 min), rutin (~35.7 min), kaempferol-3-O-rutinoside (~38.4 min) and chalconaringenin/naringenin (~50.2 min). Example given is that of micro tomato.

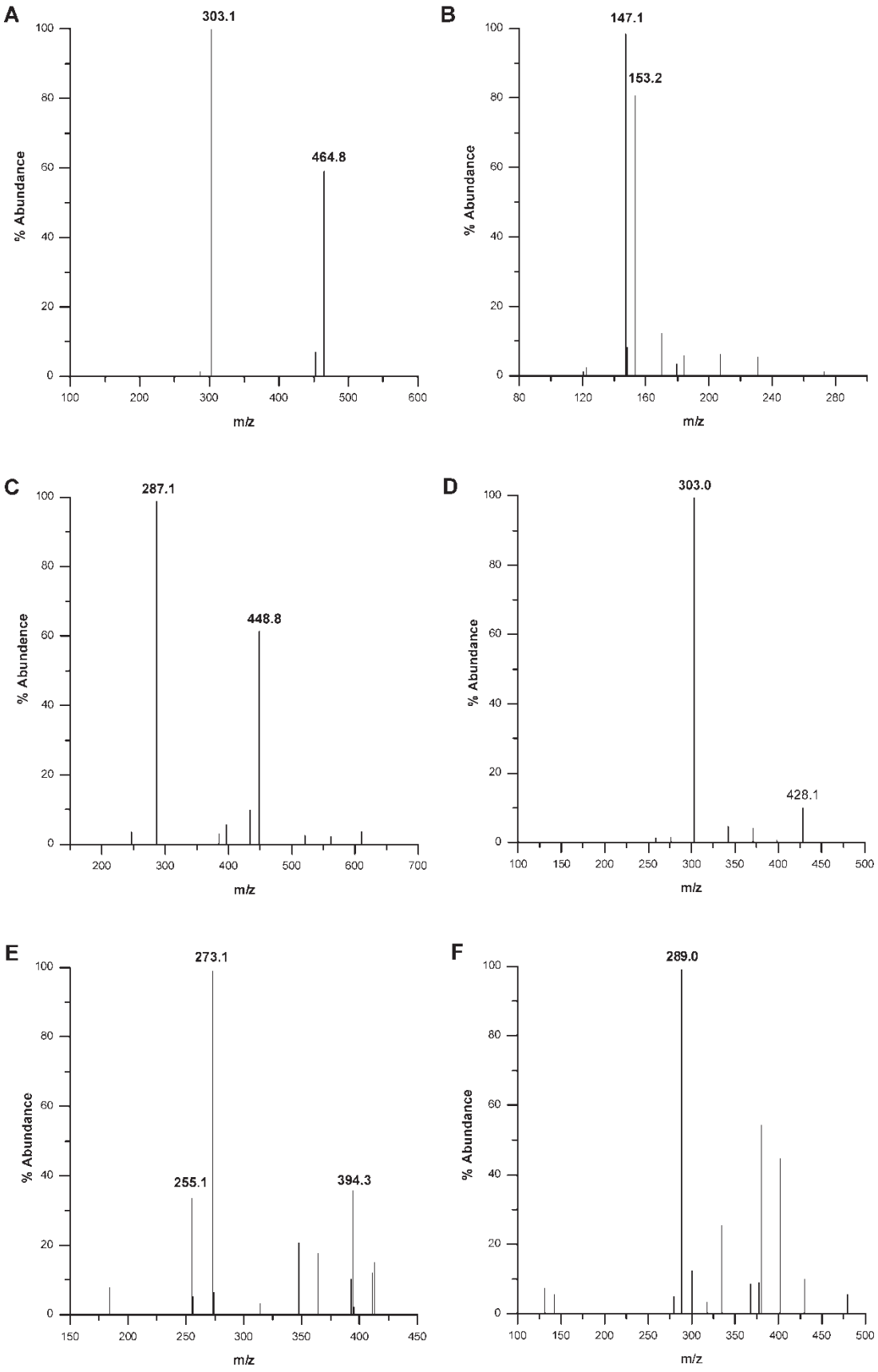

Figure 2. Product ion spectra for identified compounds (A) Rutin (MS/MS of *m/z* 611), (B) Naringenin (MS/MS of *m/z* 273), (C) Kaempferol-rutinoside (MS/MS of *m/z* 595), (D) Quercetin-hexoside (MS/MS of *m/z* 465), (E) Naringenin-glucoside (MS/MS of *m/z* 435) and (F) Dihydrokaempferol-hexoside (MS/MS of *m/z* 451).

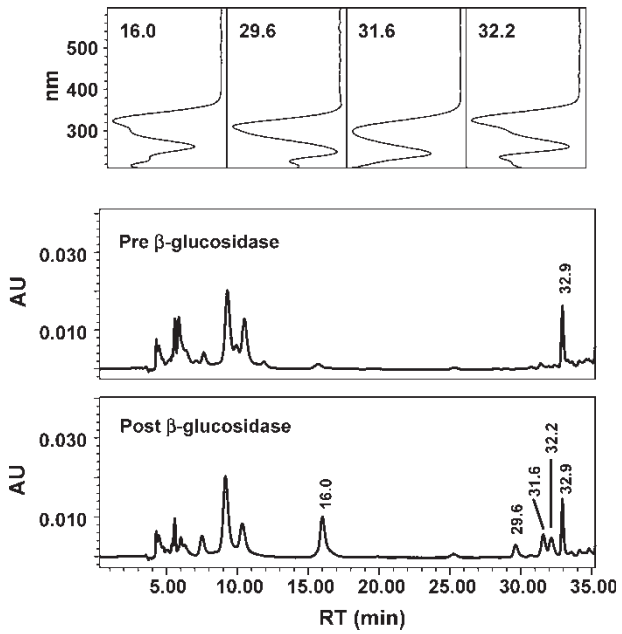

Figure 3. Representative HPLC with photodiode array detection chromatograms for tomato extracts pre- and post- β-glucosidase treatment. Phenolics were determined post enzyme treatment (3800 units/ml; 2 h; 37°C) by co-elution with authentic standards and were identified as follows: caffeic acid (~16 min), p-coumaric acid (~29.6 min) and ferulic acid (~32.2 min). In addition, one other hydroxycinnamate was detected which thus far remains unidentified.

~32.9 min on the LC-PDA and was a significant constituent in most tomato extracts.

β-Glucosidase treatment of the tomato extracts led to the appearance of four new peaks, the retention time and spectra of which suggested that they were derived from hydroxycinnamate glucosides (Figure 3; Micro Tom as example). Following enzyme treatment (3800 unit/ml; 2 h; 37°C), caffeic acid (RT: ~16 min), p-coumaric acid (RT: ~29.6 min) and ferulic acid (RT: ~32.2 min) as well as one other hydroxycinnamate (RT: ~ 31.6 min) appeared. The presence of these phenolics, which was confirmed by co-elution (as above), spectral matching and spiking with authentic standards, indicates the presence of polar hydroxycinnamate glucosides, which appear to run at about 5 min on our LC system (Figure 3). The peak at 32.9 min was present before and after enzyme treatment.

With the exception of the chalconaringenin/naringenin, quantification of the identified phenolics in the tomato extracts was achieved by the assessment of "area-under-curve" of each peak relative to the internal standard and comparison with calibration curves of flavonoid and hydroxycinnamates (0–100 μM). Levels of chlorogenic acid were relatively similar in all tomatoes (range: 60–259 μg/g tomato) except for that of the Lyc 1325 (Figure 4A), which had levels of 1115 ± 67 μg/g tomato. The T832 tomato had the lowest level of the rutinoside of kaempferol (Figure 4B) and was also relatively low in rutin (Figure 4C). The Lyc 1375 tomato was also low in these three phenolics. Both MTs had by for the highest levels of rutin (Figure 4C). Chalconaringenin/naringenin levels were quantified by LC-MS/MS with hesperetin as internal standard. As both compounds co-eluted and showed the same fragmentation (with m/z 147 as major product ion), they were quantified together. The concentration was determined using the ratios of the area under the curve of naringenin/chalconaringenin and hesperetin (SRM 307 → 177) and a calibration line. The levels for Naringenin/Chalconaringenin varied between 200 and 3000 μg/g tomato, with T832 having the lowest level and Lyc 434 the highest. Levels of the measured hydroxycinnamate glucosides (Figure 5) were lower than that of the flavonoids and chlorogenic acid. The MTs had the highest amounts of caffeic acid glucosides but amongst the lowest amounts of p-coumaric acid glucosides (Figure 5A and B). Generally, the T832 tomato had relatively high levels of hydroxycinnamate glucosides.

Correlation of polyphenolic content with TEAC

In order to access which phenolic constituents contribute to the total antioxidant potential of the tomatoes, correlations were drawn between individual components and the TEAC value (Figure 6). Interestingly, only rutin showed a significant correlation with the TEAC value having an R^2 of 0.732 ($p = 0.006$). In contrast, levels of chlorogenic acid (Figure 6B) and chalconaringenin/-naringenin (Figure 6C) did not correlate with TEAC value of the tomatoes, $R^2 = 0.004$ and 0.231, respectively. The levels of all other quantified phenolics/phenolic glycosides did not correlate with the TEAC value. In addition, there were good correlations of the TEAC value with total phenolics (Figure 6E and F). Rutin levels were identified as having the best correlation with antioxidant capacity. To strengthen this observation a further study was carried out utilising 37 different tomato varieties that were selected according to low, medium and high rutin levels. Following extraction these were analysed specifically for rutin levels and their TEAC value was determined. Figure 7A shows that there is a very good correlation between tomato rutin levels and the TEAC value of the extracts ($R^2 = 0.494, p < 0.001$). Spiking experiments were performed to access the robustness the correlation between rutin levels and TEAC values. Five tomato varieties were reanalysed for their TEAC value and rutin levels (Figure 7B, open symbols) and after spiking with known rutin amounts their TEAC's assessed. Spiking with rutin increased the TEAC value of the extracts in line with the correlation curve calculated for all 37 varieties (Figure 7B, closed symbols). The results from these experiments also

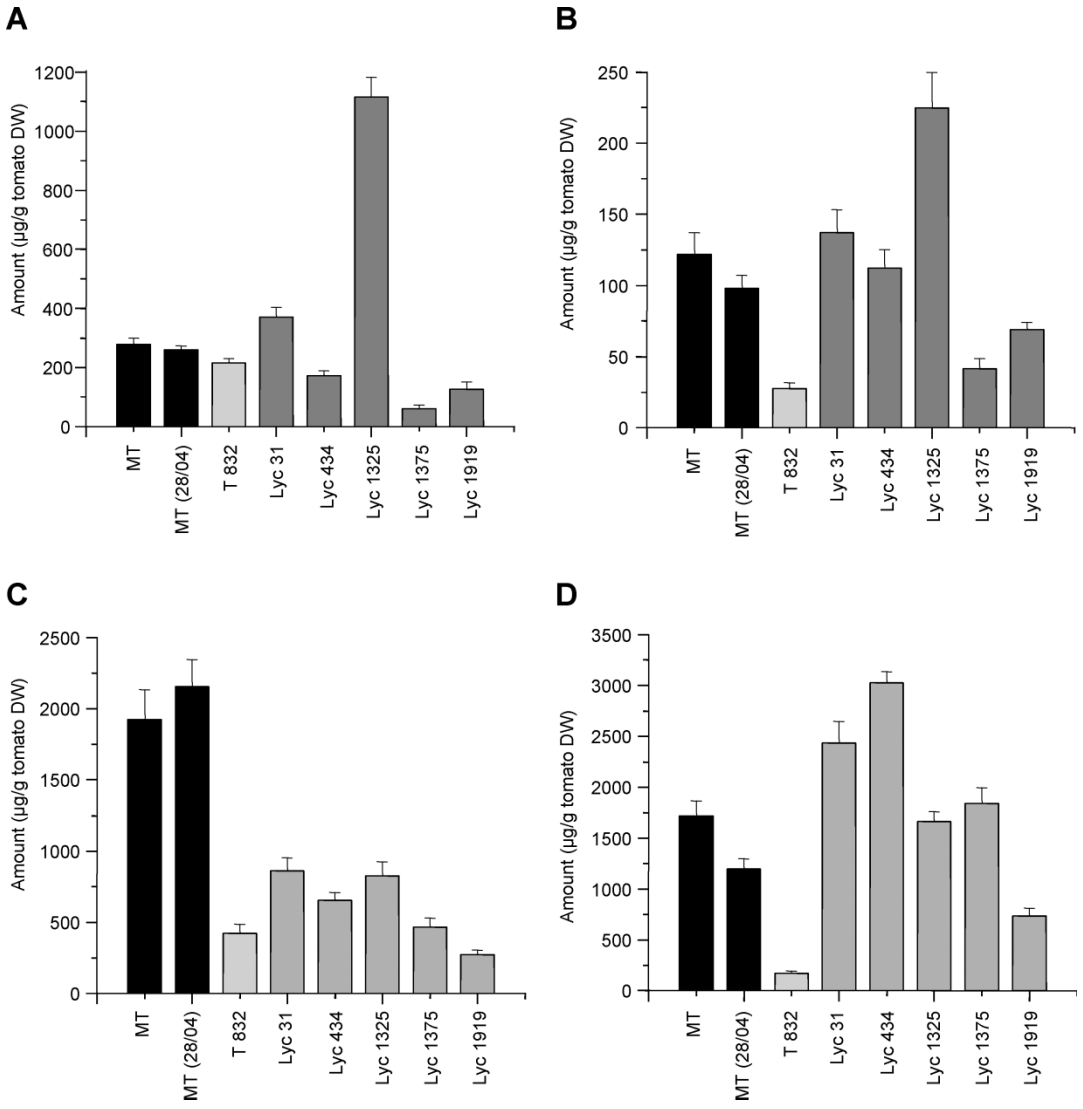

Figure 4. Levels of phenolics in the various tomato varieties. Chlorogenic acid (A), kaempferol-3-O-rutinoside (B) and rutin (C) were all quantified relative to authentic standard compounds by analysis of peaks extracted at 320 nm. LC-PDA quantification was made relative to the internal standard 3,4-dimethoxy cinnamic acid. Chalconaringenin/naringenin (D) was quantified by LC-MS/MS analysis by measurement of the $m/z = 147$ fragment with hesperetin as an internal standard. The results presented are mean ± SD, of four separate measurements.

provided evidence that the total antioxidant potential of extracted tomato extracts persists following prolonged storage (3 months).

Discussion

There is increasing interest in the ability of diets rich in polyphenols to modulate age-related diseases and promote healthy ageing. In particular, diets rich in flavonoids have been linked to reductions in age-related neurodegenerative and cardiovascular diseases and cancer progression. How precisely these actions are mediated remain unclear. However, there is strong evidence to suggest that flavonoids may be capable of reducing the oxidative stress and damage associated with these diseases. Consequently there has been great interest in producing plant products with increased flavonoid content. This has been achieved using genetic modification, where it was possible to generate several tomato lines with significantly altered flavonoid content by manipulating several key enzymatic steps in the tomato flavonoid biosynthetic pathway [22–24]. Most notably an up to 78-fold increase in total fruit flavonols was achieved through ectopic expression of a single biosynthetic enzyme, chalcone isomerase [25]. In addition, chalcone synthase and flavonol synthase transgenes were found to act synergistically to up-regulate flavonol biosynthesis significantly in tomato flesh tissues. Furthermore, the expression of maize transcription factor genes LC and C1 in the fruit of genetically modified tomato plants efficiently up-regulated the flavonoid pathway in tomato fruit flesh, resulting in high levels of the flavonol kaempferol and, to a lesser extent,

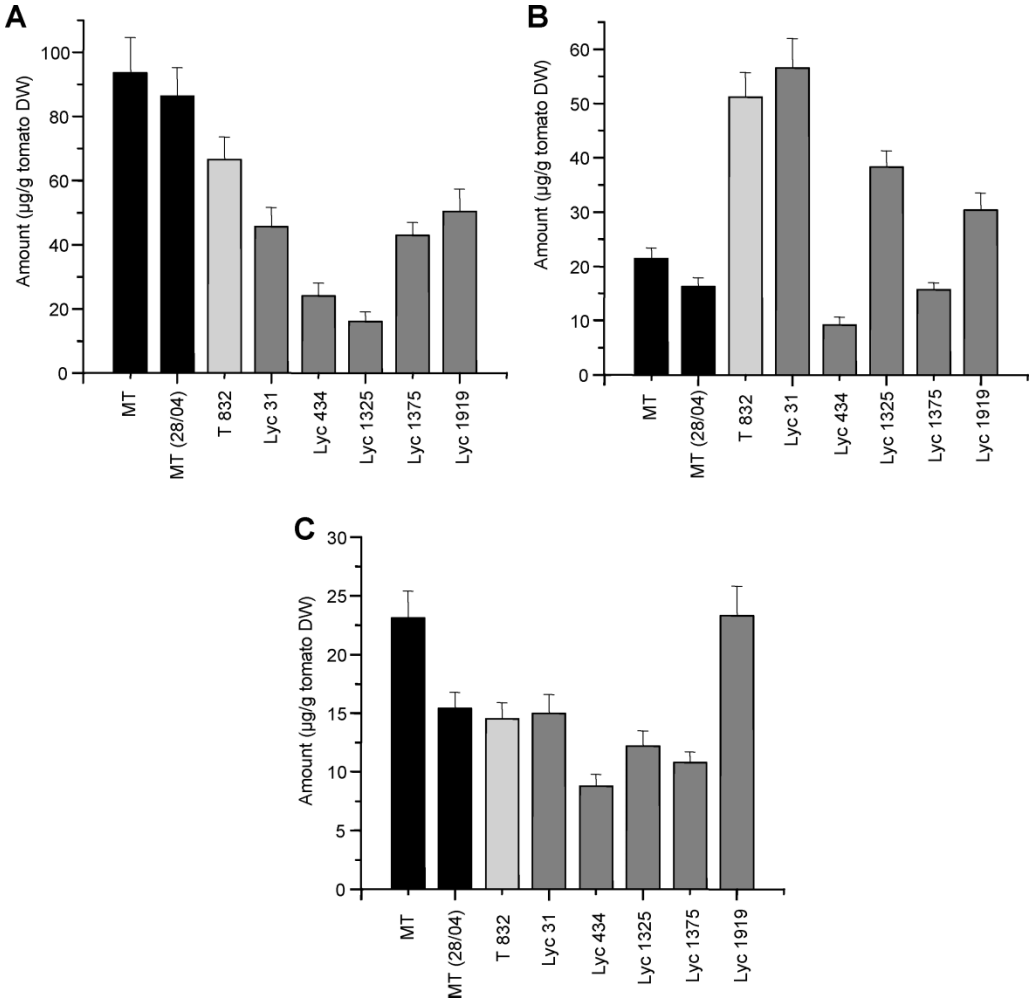

Figure 5. Levels of hydroxycinnamate glucosides in the various tomato varieties. Quantification of caffeic acid (A), p-coumaric acid (B) and ferulic acid (C) were quantified relative to authentic standard compounds following β-glucosidase treatment (3800 units/ml; 2 h; 37°C). Analysis of peak areas was made on peaks extracted at 320 nm and all quantification was made relative to the internal standard 3,4-dimethoxy cinnamic acid. The results presented are mean ± SD, of four separate measurements.

the flavanone naringenin in their flesh, a tissue that normally does not produce any flavonoids [24].

Whilst it appears favourable to increase flavonoid and hydroxycinnamate levels generally in tomatoes, commercially it is of greater importance to identify the most interesting flavonoids in terms of those with the greatest potential to increase antioxidant potential in tomato fruits. In this study we show that rutin levels in tomato fruits are the main determinant for increased total antioxidant activity. Increases in rutin and total polyphenol levels correlated well with measures of total antioxidant activity, whereas the correlations with other polyphenolic components such as naringenin were less convincing. Therefore, specific targeting the flavonol synthetic pathways in tomatoes may prove useful in increasing antioxidant potential of tomatoes. As an alternative to transgenics, we suggest the use of gene bank accessions as a rich source for selecting desired traits to be used in further breeding.

However, it should be stressed that increasing rutin levels in tomatoes in order to provide greater antioxidant protection *in vivo* only makes sense if rutin is absorbed intact and as efficiently as other flavonoid compounds present in the tomato (e.g. naringenin). This is not the case with flavonoids with the highest antioxidant potential being subject to the greatest degree of metabolic modifications that alter their "classical" antioxidant nature and reduce their total absorption [27,28]. Rutin will be subject to extensive phase I deglycosylation and phase II metabolism of the resulting aglycone to yield glucuronides, sulphates and O-methylated forms during transfer across the small intestine [28] and then again in the liver. In addition, further transformation has been reported in the colon where the enzymes of the gut microflora degrade flavonoids to simple phenolic acids, which may also be absorbed and subsequently further metabolised in the liver. These circulating metabolites of flavonoids, such as glucuronides or O-methylated forms, and intracellular metabolites, for example flavonoid-GSH adducts, have a reduced ability to donate hydrogen [29] and are

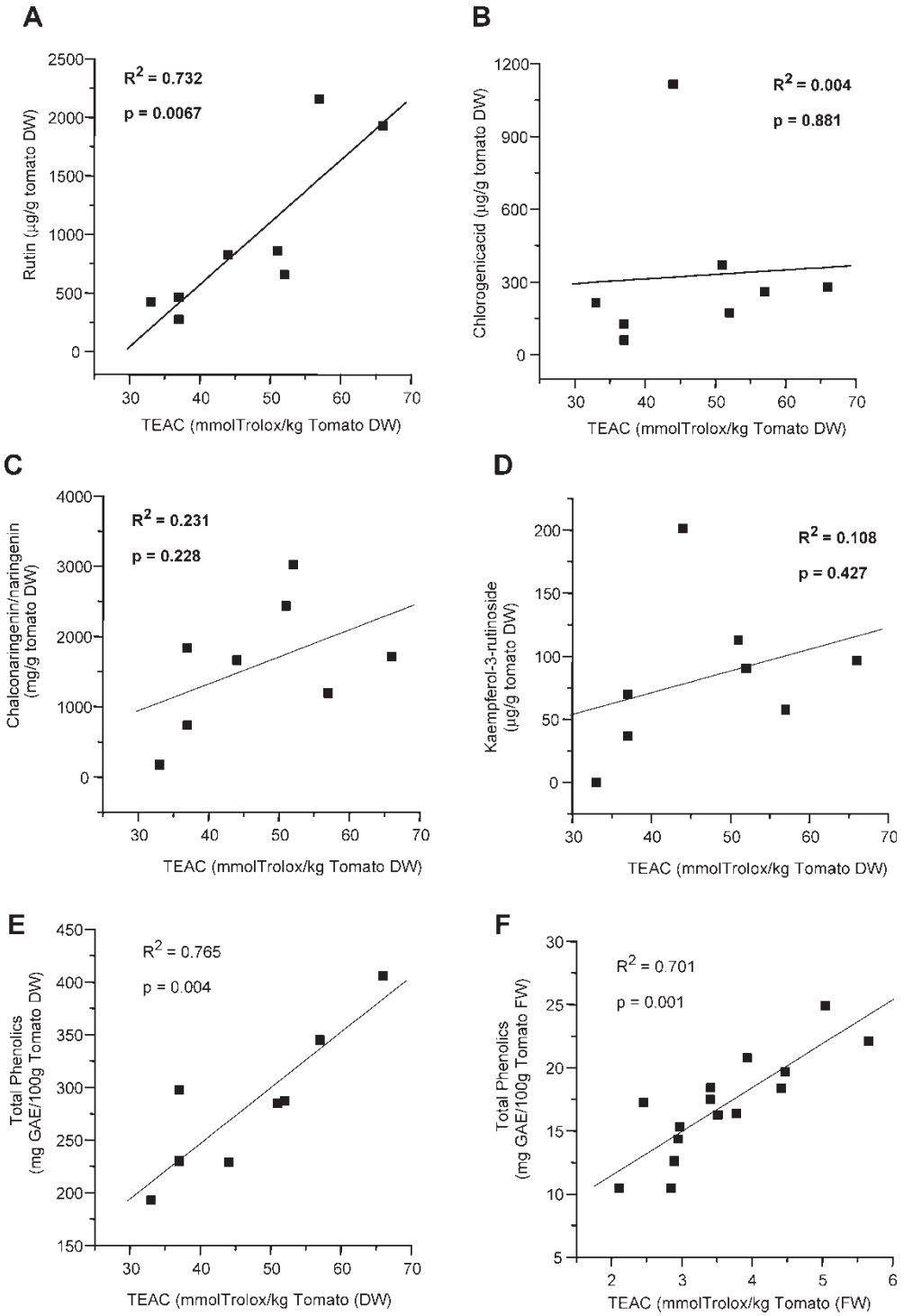

Figure 6. Correlation between levels of (A) rutin; (B) chlorogenic acid; (C) chalconaringenin/naringenin; (D) kaempferol-3-rutinoside; (E) total phenolics (DW tomatoes); (F) total phenolics (selected FW tomatoes) and the TEAC value. Amounts of chlorogenic acid, kaempferol-3-rutinoside and rutin were assessed by HPLC-PDA and naringenin by LC-MS/MS (see Fig. 3). Total phenolics were assessed as described in the "Materials and Methods" section using gallic acid as standard. The correlation coefficients (r^2) and the corresponding significance values (p) are indicated on each plot.

less effective scavengers of reactive oxygen and nitrogen species relative to their parent aglycone form. Indeed, studies have indicated that although such conjugates and metabolites may participate directly in plasma antioxidant reactions and by scavenging reactive oxygen and nitrogen species in the circulation their effectiveness is reduced relative to their parent aglycones [30–34].

Current interest in the elucidation of the potential mechanism of action of flavonoids *in vivo* has centred

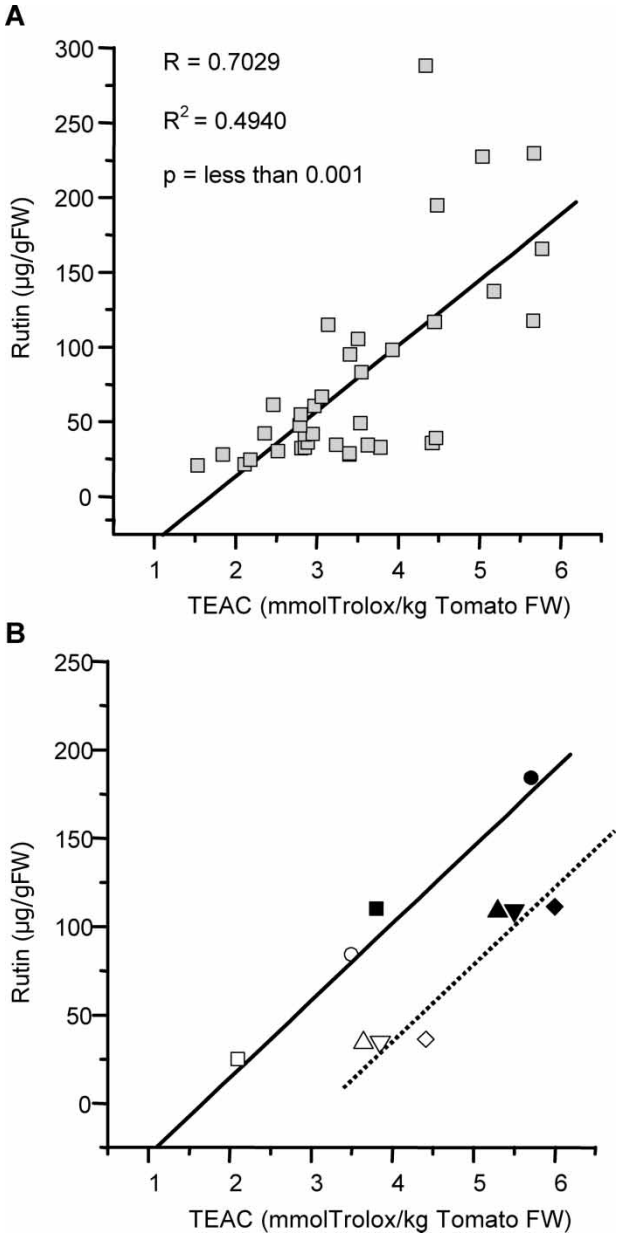

Figure 7. Correlation between levels of rutin and the TEAC value. Plot A: Amounts of rutin in 37 different tomato lines were assessed by HPLC-PDA. The correlation coefficients (r^2) and the corresponding significance values (p) are indicated. Plot B: Increase in the TEAC of five tomatoes spiked with known amounts of rutin. Tomato sample (open symbols); Spiked sample (closed symbols). Samples are plotted with the correlation line obtained in Figure 7A (solid line). Spiked samples off this line increased in a manner parallel to the correlation this line (dashed line).

on the bioactivity of these phase I and II metabolites and have indicated that metabolites may also mediate oxidative-stress induced cellular damage. For example, epicatechin and one of its major *in vivo* metabolites, 3′-O-methyl epicatechin, have been shown to elicit strong cytoprotective effects against oxidative stress in fibroblasts and neurons [29,35,36]. Importantly, the cytoprotective action of these compounds was found to involve both the inhibition caspase-3 activation [36,37] and activation of pro-apoptotic MAPK proteins [37]. In particular, both epicatechin and 3′-O-methyl-epicatechin acted to protect neurons against oxLDL-induced activation of JNK, c-jun and pro-caspase-3. It is now thought that flavonoids and their metabolites are likely to exert modulatory effects in cells independent of classical antioxidant capacity through selective actions at different components of a number of protein kinase and lipid kinase signalling cascades such as phosphoinositide 3-kinase (PI 3-kinase), Akt/PKB, tyrosine kinases, protein kinase C (PKC) and mitogen-activated protein kinase (MAP kinases) [37–43]. Inhibitory or stimulatory actions at these pathways are likely to profoundly affect cellular function by altering the phosphorylation state of target molecules and/or by modulating gene expression.

Increased intake of flavonoids lead to an increased accumulation of both flavonoids and their metabolites in the circulation. However, this is unlikely to mean that the plasma antioxidant status is altered significantly even if greater amounts of rutin and it derivative metabolites are present in the circulation. Indeed, flavonoids are unlikely to express beneficial action *in vivo* through out-competing antioxidants such as ascorbate, which are present at much higher concentrations. More likely is the possibility that flavonol metabolites derived from increased rutin intake may act favourably by exerting effects on specific signalling pathways highlighted above.

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