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Urinary Detection of Hydroxycinnamates and Flavonoids in Humans after High Dietary Intake of Fruit

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The determination of the abilities of flavonoids, hydroxycinnamates and phenolics to scavenge free radicals *in vitro* suggests potent combined antioxidant activities of fruits, vegetables, beverages and grains. However, the key question of uptake in humans has only recently been approached consistently. The study described here demonstrates the uptake of hydroxycinnamates, for the first time, and other phenolic components, applying an HPLC method for their detection in the urine of subjects consuming levels of specific fruit equivalent to an approximate intake of 25 mg flavonol glycosides.

Keywords: Ferulic acid, caffeic acid, hydroxycinnamate, flavonoid, absorption, urinary detection

INTRODUCTION

The activities of flavonoids and hydroxycinnamates as antioxidants are well-characterised *in vitro*. Their antioxidant properties relate to the number and nature of the phenolic hydroxyl groups and their arrangement within the phenolic structure. There are several features underlying the efficacy of flavonoids as antioxidants. The free radical scavenging abilities of the phenolic antioxidants depend on their reduction potentials (reviewed in[1] and[2]), and can be assessed through their hydrogen- or electron-donating properties[3]. Their activities as chain-breaking antioxidants, intercepting the propagation of oxidising lipids, additionally depend on the partition coefficients of the flavonoids and their relative lipophilicities and hydrophilicities[4–6].

The inhibition of transition metal-mediated oxidation by flavonoids is influenced by the specific structural arrangement of the phenolic hydroxyl groups facilitating the chelation of iron or copper.[6–10] However, the presence of functional groups of such reducing properties that drive the redox cycling of transition metal ions might promote pro-oxidant effects[11] and such interactions with transition metal ions can result

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in oxidation of the flavonoid molecule, as proposed for the reactions of quercetin and kaempferol with copper ions.[6] Flavonoids and simple phenolics are also effective scavengers of reactive nitrogen species. They have been shown to protect against the nitration of tyrosine induced by peroxynitrite through mechanisms of competitive nitration or electron donation, depending on the specific structural arrangements of the hydroxyl groups within the phenolic structure.[12,13]

Flavonoids and hydroxycinnamates are widely distributed in the plant kingdom, fruits, vegetables, beverages and grains being abundant dietary sources. Epidemiological studies have implicated a role for flavonoids in disease prevention[14,15] but there is limited direct evidence for uptake, absorption, metabolism and excretion of hydroxycinnamates and flavonoids in humans. This study describes a direct method by which phenolics, especially hydroxycinnamates, can be detected in urine and confirms their uptake in subjects consuming high levels of specific fruit.

MATERIALS AND METHODS

Chemicals

Methanol and acetonitrile, all HPLC grade, were obtained from Rathburn Limited, Walkerburn, Scotland, UK. All hydroxycinnamic acids, anthocyanins and flavonoid standards were obtained from Extrasynthese, 69730 Genay, France. Fruits were obtained from a major UK supermarket chain. Elgastat UHP double-distilled water (18 + Ω grade) was used in all experiments. β-Glucosidase was obtained from ICN Biomedicals Inc., Ohio.

Preparation of Standards

Stock solutions of the standards were prepared by dissolving 1–2 mg of sample into either methanol or mobile phase (20% MeOH, 0.1% HCl). Stock solutions were stored at 0–4°C and used within four weeks from the date of preparation after HPLC analysis to check stability.

Extraction of Flavonoids from Freeze-dried Fruit

The fruit was chopped into small pieces, freeze-dried and then lyophilised with liquid nitrogen. The weight of the dried sample was recorded. For aqueous extraction, 0.5–1 g of freeze-dried material was weighed and transferred to a round bottom flask. Water (15 ml), methanol (15 ml) and salicylic acid (internal standard 6 μg/ml) were added. The contents were refluxed for 30 min and filtered using a Whatman no. 4 filter paper. The methanol was removed from the filtrate by rotary evaporation under vacuum, at 40–50°C, and the remaining aqueous extract cooled and filtered prior to taking an aliquot for HPLC analysis. For enzymic treatment, 1 ml of tomato extract was incubated for 1 h at 37°C with 7622 units of β-glucosidase (3811 units/mg).

Supplementation

Six normal healthy volunteers, 2 males and 4 females, age range 22–32 years were supplemented after fasting for 12 h. Each subject consumed a single type of fruit for a 5 h period. One fruit portion was consumed each hour and the portions were calculated to provide in total approximately 25 mg of flavonol glycosides. The fruit chosen and the portions per hour were as follows: 4 British tomatoes (160 g), 6 red cherries (90 g), 25 raspberries (100 g), 25 black grapes (100 g), 6 soft-dried apricots (120 g) and one Red McIntosh apple (160 g). Urine was collected for the duration of the study and for 3 h post-supplementation in order to determine the uptake of the phenolic constituents.

Preparation of Urine Extract

Urine (1 ml volume) was transferred to a tube containing salicylic acid as internal standard

(final concentration of 10 μg/ml), 100 μl of 3 M HCl and 2.4 ml of methanol added (urine: methanol : acid 28.6 : 68.6 : 2.8). The tubes were vortexed and centrifuged at 800 × g (at 4°C). The supernatant was collected and the methanol removed by rotary evaporation under vacuum at 40°C. The resultant aqueous fraction was filtered using a Flowpore 0.22 μm sterile non-pyrogenic membrane filter directly into an HPLC vial and analysed.

Analysis of Phenolics by Gradient HPLC

HPLC analysis was conducted according to the method of Paganga and Rice-Evans.[16] The HPLC system consisted of an autosampler with Peltier temperature controller, a 626 pump with 600S controller, a Photodiode Array Detector and a software system which controlled all the equipment and carried out data processing. A Nova-Pak C18 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 fixed loop, and the volume injected was 30 μl. Elution (0.5 ml/min) was performed using a solvent system comprising of solvent A (20% methanol in 0.1% hydrochloric acid) and acetonitrile (solvent B) mixed using a linear gradient held at 95% solvent A for 10 min and then decreasing 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. The chromatograms were obtained with detection at both 320 and 520 nm.

RESULTS

Studies on hydroxycinnamate uptake were undertaken through urinary analysis of subjects supplemented with fresh tomatoes and dried apricots. HPLC analysis of the major constituents of the tomato extract, after glucosidase treatment to remove the sugar residues attached to the phenolic hydroxyl groups, is demonstrated in Figure 1 and Table I. The results are consistent with the findings of Macheix *et al.*[17] indicating the presence (post-glucosidase) of caffeic acid, ferulic acid, chlorogenic acid and *p*-coumaric acid, identified from the characteristic retention times under these analytical conditions, relative to that of the internal standard, salicylic acid, and also from the UV/visible spectra, and rutin, observed as the intact rutinoside, since glucosidase does not cleave the link to the disaccharide.[18]

After consumption of the fresh tomato over a period of 5 h, as described in the methods section, and urine collection over this period and the subsequent 3 h, urine was analysed for the presence of the phenolic constituents of the tomato fruit. The HPLC chromatograms prior to and post-supplementation are shown in Figure 2. Comparison of retention times and standard spectra reveals the urinary excretion of the hydroxycinnamates caffeic acid, ferulic acid and *p*-coumaric acid. Chlorogenic acid is not detected but this may have been cleaved to its non-esterified form, caffeic acid, with possible further metabolism. Rutin is expected to be metabolised, via its aglycone quercetin, and either through a variety of lower molecular weight phenolic metabolites[19,20] which would not be observed in the chromatogram on detection at 320 nm or as conjugates such as the glucuronide or sulphate or the methoxylated derivatives, tamarixetin or isorhamnetin. The peak eluting at 16.73 min, closely overlapping with that of ferulic acid, may be a metabolite of rutin.

High dietary intake of the apricot fruit reveals the presence of the hydroxycinnamate, caffeic acid, in the urine (Figure 3), derived presumably from the chlorogenic acid component of the fruit (Figure 3), and a conjugate of naringenin or taxifolin which also reflects the presence of a glycoside of these phenolics in the original fruit. The chemical structures of naringenin and

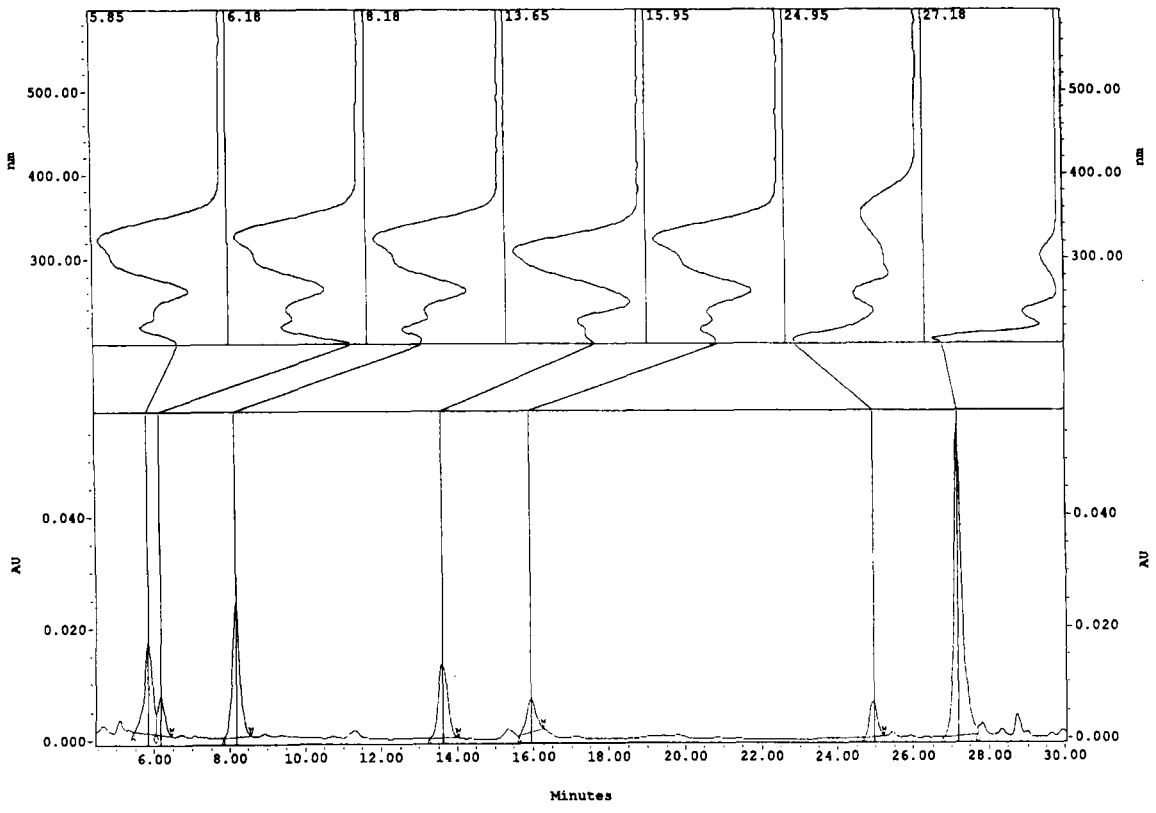

FIGURE 1 HPLC analysis of the major constituents of the tomato extract, after glucosidase treatment.

TABLE I Components identified from the characteristic retention time (min) under these analytical conditions, relative to that of the internal standard, salicylic acid, as well as from the UV/visible spectrum

Compound	Retention time (min)
Chlorogenic acid	5.85, 6.18
Caffeic acid	8.18
p-coumaric acid	13.65
Ferulic acid	15.95
Internal standard	27.18
Rutin	24.95

taxifolin are similar and, as anticipated, it is difficult to distinguish them through their spectra and retention times without applying LC-MS. Studies of urinary excretion of anthocyanidin conjugates were undertaken in subjects consuming high levels of raspberries and apples, both containing anthocyanins related to the anthocyanidin, cyanidin.[17] Diode array detection of the eluting peaks is undertaken at 520 nm, the region of the spectrum characteristic for the detection of the highly coloured anthocyanidins and their conjugates. Analysis of the raspberry extract reveals three anthocyanin components, as shown in Figure 4 retention times 10.37, 9.07 min with an intense band at 6.8 min suggestive of co-elution. The spectroscopic profiles indicate cyanidin-3-glycosides.[21]. Post-supplementation with raspberries and with red apples, two peaks with identical retention times, 42–43 min and 43–44 min, are detected in both volunteers, indicating the urinary excretion of the anthocyanidin conjugates.

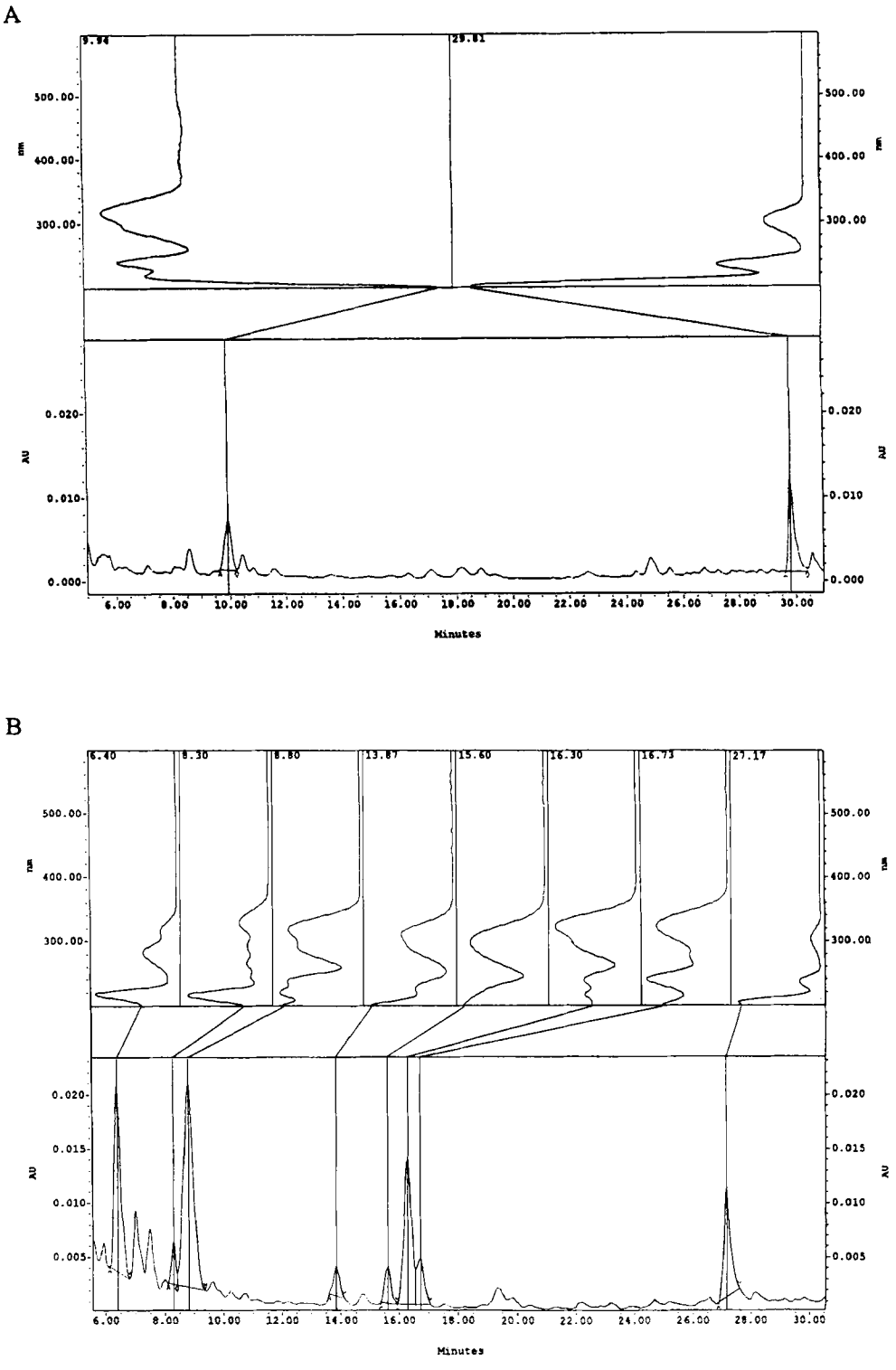

FIGURE 2 HPLC analysis of the major phenolic constituents present in urine prior (A) to and post (B) supplementation.

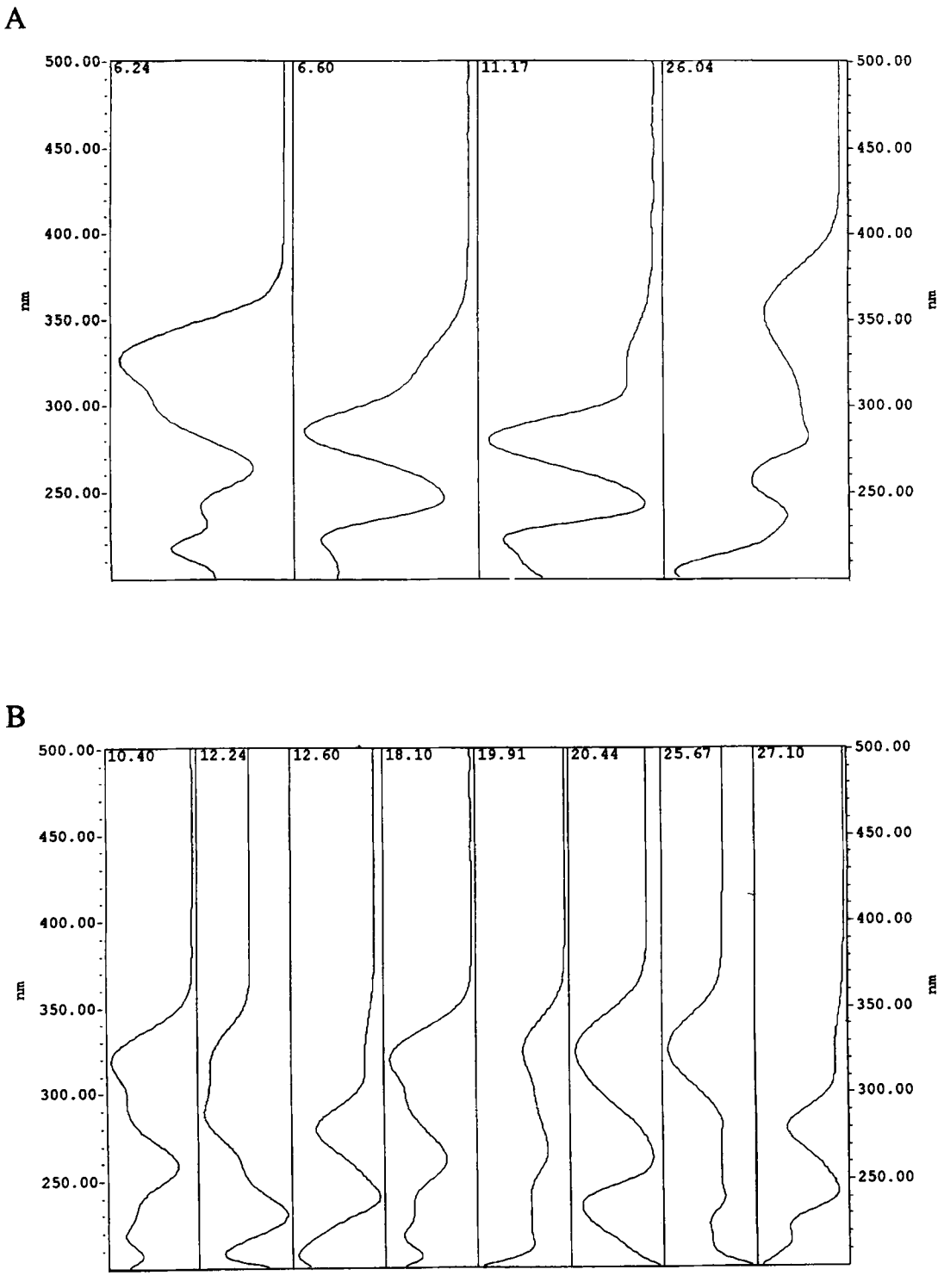

FIGURE 3 Absorption spectra of the major phenolic constituents present in apricot (A) and urine (B) post-supplementation, recorded on HPLC analysis by diode array detection.

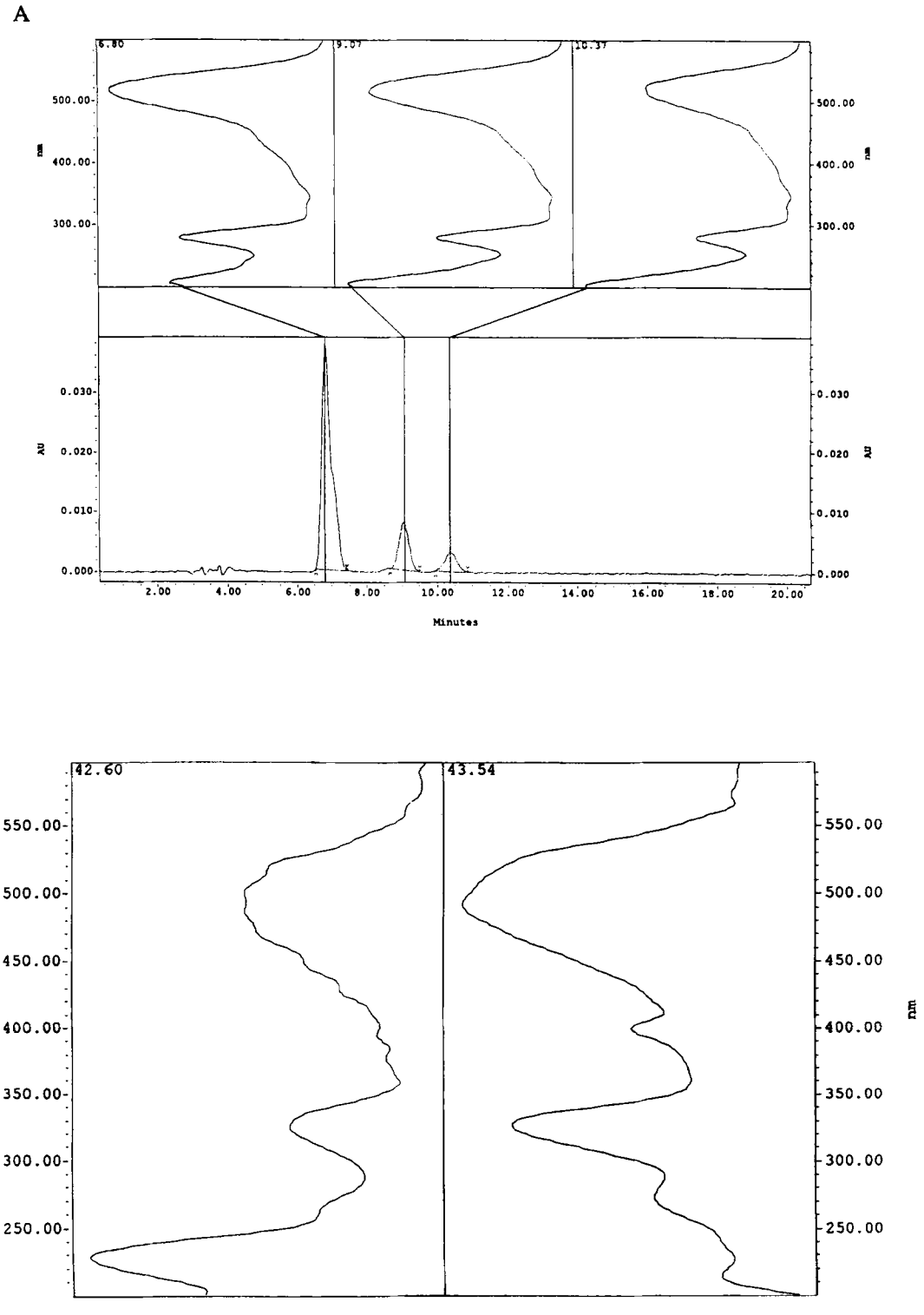

FIGURE 4(A) and (B)

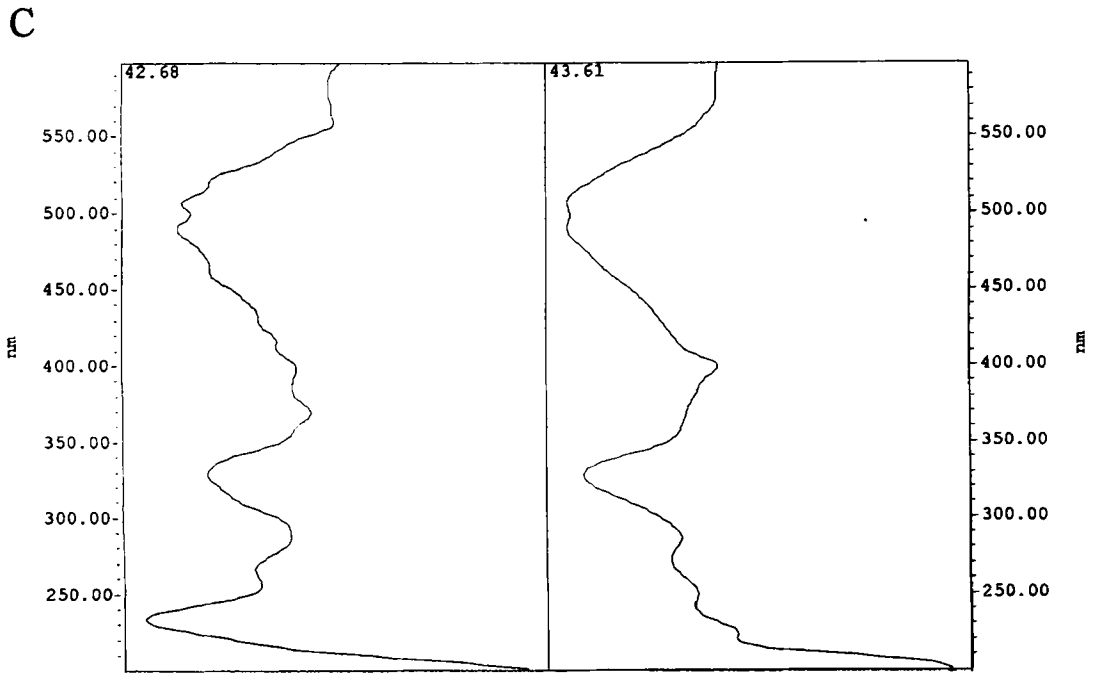

FIGURE 4(C)

FIGURE 4 HPLC analysis of the raspberry (A) and absorption spectra of anthocyanidin derivatives in urine post-supplementation with raspberries (B) and red apples (C). Recorded on HPLC analysis by diode array detection.

DISCUSSION

The antioxidant function of flavonoids and phenolic compounds *in vivo* will depend on the ways in which they are metabolised in the gastrointestinal tract and the structures of the resulting metabolites. Conjugation with glucuronide or with sulphate is generally considered to be the most common final step in the metabolism of intact flavonoids.

This is the first report of the identification of hydroxycinnamates, rich constituents of fruit, in the urine after high level of fruit intake. Free ferulic, caffeic and *p*-coumaric acids were detected in the urine, presumably due to the high levels ingested from the intake of fruit administered. It should be emphasised that this level of intake of fruit over a 5-hour period is high and shows uptake and excretion to be quite fast. While extrapolation of these results to excretion from uptake from a standard diet will not necessarily be direct, the results clearly confirm uptake of these phenolics from the diet.

A number of early studies hypothesised that flavonoids would not enter the circulation, either as the natural glycosides or as the aglycone hydrolysis products[22] but would be cleaved by the enzymes of the intestinal bacteria at the central heterocyclic ring, generating products with no antioxidant activity.[23–25] However, over the last twelve months attention has been increasingly concentrated on the detection of excreted dietary polyphenolics in humans *in vivo*. Ingestion of 500 g of broccoli, rich in kaempferol and quercetin, daily for 12 days by two volunteers revealed the presence of kaempferol conjugates in the urine, detected as free kaempferol on HPLC analysis of the hydrolysed urine and

confirmed by LC-MS analysis.[26] This procedure failed to detect quercetin, suggesting that the uptake of dietary quercetin from this source is limited in some way compared to that of kaempferol, or that quercetin is more extensively metabolised during or after absorption.

Evidence for absorption of constituents of *Gingko biloba* extract, mainly flavonol glycosides in the plant,[27] after oral administration of a single bolus to six human volunteers, was sought through urinary and plasma analysis,[28] Flavonoid metabolites were detected in the urine in the form of substituted benzoic acids, accounting for less than 30% of the flavonoids administered. No hydroxyphenylacetic acid or hydroxyphenylpropionic acid derivatives were observed suggesting that a more extensive metabolism had occurred, compared with previously published reports on rats supplemented with *Gingko biloba*.

In the course of investigating the underlying detrimental features of the interactions between grapefruit juice and specific drugs, the renal excretion of naringin (naringenin 7-glucoside), naringenin and its glucuronides has been investigated after administration of grapefruit juice to six healthy adults. Grapefruit juice (20 ml or 621 μmol/l naringin) per kg body weight were consumed.[29] Naringenin (and not naringin) glucuronides were detected in the urine after this dosage, suggesting that cleavage of the sugar moiety by glycosidase from intestinal bacteria, as suggested for flavonoids by Havsteen,[30] is an early step in the metabolism of naringin. It has also been proposed that glycosidic linkages are less stable than, for example, glucuronide linkages, and might not withstand the acidic environment of the stomach.[31] A more recent study[32] has provided further evidence for absorption of naringin and hesperidin (hesperetin glucoside) from the gastrointestinal tract after oral administration of pure compound, citrus juice or whole grapefruit, although it is not clear whether they are absorbed as glycosides or as the aglycones after cleavage of the glycosidic linkages.

The uptake of catechin, a major constituent of red wine, and its gallate forms, major components of green tea, have been well-studied as have their urinary excretion. As long ago as 1971, Das[33] reported *m*-hydroxyphenylpropionic acid as the major urinary metabolite of catechin. Administration of radiolabelled 3-O-methylcatechin to three human volunteers revealed 46% in the urine in the form of glucuronides and sulphates within 2 h of administration.[34] More recently, studies investigating the excretion of the constituents of decaffeinated green tea, mainly epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate identified the two former flavanols in the sulphated form in the urine of the four supplemented volunteers but not those of their gallate esters.[35]

The study described here adds to the growing weight of evidence for the bioavailability of hydroxycinnamates and flavonoids. Evidence for the bioactivity of these phenolics *in vivo* should now be sought to support the notion that their antioxidant properties contribute to their proposed role in health protection and disease prevention.

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References

[1] S.V. Jovanovic, S. Steenken, M.G. Simic and Y. Hara (1998) Antioxidant properties of flavonoids: reduction potentials and electron transfer reactions of flavonoid radicals. In: *Flavonoids in Health and Disease* (Eds. C. Rice-Evans and L. Packer) Marcel Dekker, New York, pp. 137–161.

[2] S.V. Jovanovic, S. Steenken, M. Rosic, B. Marjanovic and M.G. Simic (1994) Flavonoids as antioxidants. *Journal of the American Chemical Society* **116**, 4846–4851.

[3] C. Rice-Evans, N.J. Miller and G. Paganga (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine* **20**, 933–956.

[4] H. Chimi, J. Cillard, P. Cillard and M. Rahmani (1991) Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. *Journal of the American Oil Chemical Society* **68**, 307–312.

[5] N. Salah, N.J. Miller, G. Paganga, L. Tijburg, G.P. Bolwell and C.A. Rice-Evans (1995) Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics* **322**, 339–346.

[6] J. Brown, H. Khodr, R. Hider and C. Rice-Evans (1998) Structural-dependence of flavonoid interactions with copper ions: implications for their antioxidant properties. *Biochemical Journal* **330**, 1173–1178.

[7] M. Thompson and C.R. Williams (1976) Stability of flavonoid complexes of copper(II) and flavonoid antioxidant activity. *Analytica Chimica Acta* **85**, 375–381.

[8] I. Afanas'ev, O.I. Dorozhko, A.V. Brodskii, A. Kostyuk and A.L. Potapovitch (1989) Chelating and free radical scavenging mechanisms of inhibitory action of rutin and qquercetin in lipid peroxidation, *Biochemical Pharmacology* **38**, 1763–1769.

[9] G. Paganga, H. Al-Hashim, B. Scott, O. Aruoma, R. Hider, B. Halliwell and C. Rice-Evans (1996) Mechanisms of the antioxidant activities of quercetin and catechin. *Redox Report* **2**, 359–364.

[10] I. Morel, G. Lescoat, P. Cogrel, O. Sergent, N. Pasdeloup, P. Brissot, P. Cillard and J. Cillard (1993) Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. *Biochemical Pharmacology* **45**, 13–19.

[11] B. Halliwell (1996) Vitamin C: antioxidant or pro-oxidant *in vivo*. *Free Radical Research* **25**, 439–454.

[12] A.S. Pannala, R. Razaq, B. Halliwell, S. Singh and C. Rice-Evans (1998) Inhibition of peroxynitrite-dependent nitration by hydroxycinnamates: nitration or electron donation? *Free Radical Biology and Medicine* **24**, 594–606.

[13] A.S. Pannala, C.A. Rice-Evans, B. Halliwell and S. Singh (1997) Inhibition of peroxynitrite-mediated nitration by catechin polyphenols. *Biochemical Biophysical Research Communications* **232**, 164–168.

[14] M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman, M.B. Katan and D. Kromhout (1993) Dietary flavonoids, antioxidant vitamins and coronary heart disease. The Zutphen elderly study. *The Lancet* **342**, 1007–1011.

[15] P. Knecht, R. Jarvinen, A. Reunanen and J. Maatela (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *British Medical Journal* **312**, 478–481.

[16] G. Paganga and C.A. Rice-Evans (1997) The identification of flavonoids as glycosides in plasma. *FEBS Letters* **401**, 78–82.

[17] J. Macheix, A. Fleuriet and J. Billot (1990) *Fruit Phenolics*, CRC Press, Boca Raton.

[18] J. Harborne (1964) Plant Polyphenols-XIV. Characterization of flavonoid glycosides by acidic and enzymic hydrolyses. *Phytochemistry* **4**, 107–120.

[19] L. Griffiths (1982) Mammalian metabolism of flavonoids. In: *The Flavonoids: Advances in Research* (Eds. J. Harborne and T. Mabry) Chapman and Hall, London, pp. 681–718.

[20] J. Kuhnau (1976) The Flavonoids. A class of semi-essential food components: their role in human nutrition. *World Review of Nutrition and Dietetics* **24**, 117–191.

[21] D. Strack and V. Wray (1989) Anthocyanins. In: *Methods in Plant Biochemistry* Vol 1 (Eds. P.M. Dey and J. Harborne) Academic Press, London, pp. 325–356.

[22] W.G. Clark and E.M. Mackay (1950) The absorption and excretion of rutin and related flavonoid substances. *Journal of the American Medical Association* **143**, 1411–1455.

[23] A.M. Hackett (1996) The metabolism of flavonoid compounds in mammals. In: *Plant Flavonoids in Biology and Medicine* (Eds. V. Cody, E. Middleton and J. Harborne) Alan Liss, New York, pp. 177–194.

[24] A.R.S. Ibrahim and Y.J. Abul-Hajj (1990) Microbiological transformation of flavone and isoflavone. *Xenobiotica* **20**, 363–373.

[25] S. Baba, T. Furuta, M. Fujioka and T. Goramaru (1983) Studies on drug metabolism by use of isotopes. XXVII Urinary metabolites of rutin in rats and the role of intestinal microflora in the metabolism of rutin. *Journal of Pharmaceutical Science* **72**, 1155–1158.

[26] S.E. Nielsen, M. Kall, U. Justesen, A. Schou and L.O. Dragsted (1997) Human absorption and excretion of flavonoids after broccoli consumption. *Cancer Letters* **114**, 173–174.

[27] P.G. Pietta, P.L. Mauri, A. Bruno and A. Rava (1991) Identification of flavonoids from *Gingko biloba* L., Anthemis-Nobilis L. and Equisetum-Arvense L. by high performance liquid chromatography with diode array detection. *Journal of Chromatography* **553**, 233–237.

[28] P.G. Pietta, C. Gardana and P.L. Mauri (1997) Identification of *Gingko biloba* flavonol metabolites after oral administration in humans. *Journal of Chromatography B* **693**, 249–255.

[29] U. Fuhr and A.L. Kummert (1995) The fate of naringin in humans: a key to grapefruit juice–drug interactions. *Clinical Pharmacology and Therapeutics* **58**, 365–373.

[30] B. Havsteen (1983) Flavonoids, a class of natural products of high pharmacological potency. *Biochemical Pharmacology* **32**, 1141–1148.

[31] K.R. Markham (1989) Flavones, flavonols and their glycosides. In: *Methods in Plant Biochemistry* (Eds. P.M. Dey and J.B. Harborne) Academic Press, London, pp. 197–235.

[32] B. Ameer, R.A. Weintraub, J.V. Johnson, R.A. Yost and R. L. Rousseff (1996) Flavanone absorption after naringin, hesperidin and citrus administration. *Clinical Pharmacology and Therapeutics* **60**, 34–40.

[33] N.P. Das (1971) Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. *Biochemical Pharmacology* **20**, 3435–3445.

[34] A.M. Hackett, L.A. Griffiths and M. Wermeille (1985) The quantitative disposition of 3-O-methyl-[U-^{14}C] in man following oral administration. *Xenobiotica* **15**, 907–914.

[35] M.-J. Lee, Z.-Y. Yang, H. Li, L. Chen, Y. Sun, S. Gobbo, D.A. Balentine and C.S. Yang (1995) Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiology, Biomarkers and Prevention* **4**, 33393–399.