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Research Article

Comparison of the relative recovery of polyphenolics in two fruit extracts from a model of degradation during digestion and metabolism

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To simulate the effects of digestion and metabolism on the survival of different polyphenolic compounds, extracts of blueberry and apple were deglycosylated by acid hydrolysis, followed by enzymic glucuronidation under neutral conditions, yielding ~5% overall recovery of polyphenolics. The major polyphenolics before and after the treatment were compared, to estimate which species are likely to be present in the intestinal lumen, undegraded and available for absorption, after consumption of the fruit. Whereas blueberry extract consisted predominantly of anthocyanins, epicatechin and caffeoyl quinate esters, the major components of the treated extract were quercetin glucuronides and (unglucuronidated) caffeoyl quinates, with only traces of anthocyanidin derivatives. In apple extract, compositional changes were less marked, but caffeoyl quinates, procyanidins and quercetin were enriched at the expense of caffeic acid, epicatechin and catechin. Hydrophobic compounds like phloretin and quercetin were extensively glucuronidated, whereas caffeic acid and caffeoyl quinate were not. These results suggest that the major polyphenolic components of a fruit are not necessarily the most important contributors to any health benefits because the polyphenolic composition in the intestinal lumen and consequently, in the circulation, may be considerably different.

Keywords: Digestion / Fruit polyphenolics / Glucuronidation / Metabolism / Polyphenolic degradation

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1 Introduction

It is generally accepted that human consumption of fruits and vegetables is very beneficial for health. Fruit juice consumption, for example, has been shown to influence markers of health status beneficially [1, 2]. Polyphenolic phytochemical compounds, such as condensed tannins (*e.g.* procyanidins), flavonoids (*e.g.* quercetin) and phenolic acids (*e.g.* caffeic acid) are of considerable research interest as biologically active food ingredients. Although there is considerable uncertainty about how they function [3], these compounds are popularly believed to be good dietary antioxidants [4] and to have many other beneficial nutritional [5, 6] and pharmacological [7] properties.

Most of the evidence linking the beneficial physiological effects of fruit and vegetables with their constituent polyphenolics is derived from many studies in which purified polyphenolic aglycones show potentially health-enhancing biological activity in cell-based assays *in vitro* [8]. Fruits and vegetables, however, contain hundreds of different compounds, mostly in glycosylated form [9] and the major constitutents of a particular food are not necessarily the ones that get into the circulation at the highest concentrations, or even at all. There are several reasons for this highly complex situation. Glycosylated polyphenolics are thought to have very low bioavailability, apart from those that can be deglycosylated by intestinal glycosidase enzymes [9, 10]. Although most flavonoids appear to be absorbed as aglycones, they are quickly and extensively conjugated by Phase II metabolic enzymes, with glucuronides appearing to be the major human and mammalian metabolites [10–12]. Some *in vitro* studies have been carried out, which indicate that the biological activity of individual polyphenolics *in vivo* may be significantly increased or decreased by glucuronidation. These were inhibition of LDL oxidation in human serum [13], induction of reactive oxygen species in mouse cells [14] and hypertrophy of cultured rat aortic smooth muscle cells [15] (quercetin); release of arachidonic

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acid from human colon cancer cells [16] (epigallocatechin) and oxidative stress-induced cell death (epicatechin) [17], (several flavonoids and phenolic acids; Unpublished results). Total plasma concentrations of different phenolic compounds and their metabolites, reported from a collection of 97 human, single-dose bioavailability studies [18] vary by over two orders of magnitude, between ~0.02 and ~4 μM and the outcome of these studies has only produced data on a small proportion of the hundreds of polyphenolic compounds in a varied diet. The colonic microflora appear to be able to convert polyphenolics that are not absorbed in the small intestine into a range of phenolic acids [19]. These appear to have relatively high bioavailability [11], so their health benefits may be significant, but their structures and composition are unrelated to the phenolic composition of the food from which they were derived. Compounds with high bioavailability are likely to contribute much more to health benefits than those with low bioavailability [20]. Demonstration of biological activity using phenolic aglycones in *in vitro* assays can only indicate the potential, as it inevitably focuses on the major components of the food of interest. The results may mean little without combining them with information on the composition and conjugation of polyphenolics in the circulation, arising from consumption of the food [8]. The latter is almost certainly strongly influenced by many factors including differential degradation or binding to other food components during the digestive process and different rates of absorption by the intestinal cells. Relative rates of absorption can be estimated using intestinal cell monolayers *in vitro* [21] and simulation of digestive processes has also been undertaken [22]. Other factors, however, appear to be of equal or greater importance. Anthocyanins for example, have been shown to be relatively stable during simulated digestion [22] and relatively well absorbed by intestinal cells [23], but their human plasma concentrations are among the lowest measured in bioavailability studies [18]. Oxidative degradation and covalent binding to polymers is an unavoidable side-effect of the antioxidant properties of polyphenolics [4]. Although these processes can be simulated *in vitro* for digestion [22], there are no reports of simulated degradation after absorption and metabolic conjugation. Although human intervention trials and bioavailability studies are by far the best approach to assess the bioavailability and health effects of phytochemicals, they are major undertakings and impractical to carry out for all of the hundreds of plant foods available to be tested. There is an ongoing need to complement *in vitro* bioassay results with alternative sources of information on both the rates of absorption of the tested compounds and their actual availability for absorption.

Here, we demonstrate the use of acid hydrolysis and enzymic glucuronidation to estimate the relative stability of the different polyphenolics in a food during digestion and metabolism and their relative susceptibility to glucuronidation. The information provided is limited, but complementary, to the alternative approaches described above and readily applicable to large numbers of samples.

2 Materials and methods

Uridine-diphosphoglucuronate (UDPGA) was obtained from Nacalai Tesque (Kyoto Japan). Applephenon® apple phenolic extract was obtained from Asahi Breweries (Ibaraki, Japan). Most phenolic standard compounds (catechin, epicatechin, chlorogenic acid, *p*-coumaric acid, ferulic acid, phloridzin, phloretin, rutin) were purchased from Sigma Chemicals (St. Louis, MO) except for quercetin (Acros Organics, Geel, Belgium), caffeic acid (Genzyme Fine Chemicals, Haverhill, Suffolk, UK) and cyanidin-3-galactoside (Polyphenols Laboratories, Sandnes, Norway). All solvents were of HPLC grade; solvents and chemicals not specified above, were obtained from local suppliers.

Blueberry extract was prepared from frozen blueberries purchased from a local supermarket. Blueberries (300 g wet weight) were freeze-dried (Cryodos Benchtop Freeze Dryer, Teltar Industrial S.L., Barcelona, Spain), then extracted by steeping (without grinding or crushing) in methanol containing 0.1% concentrated HCl (250 mL), for 1 h. The extraction was carried out three times and the solvents evaporated under vacuum (Buchi Rotavapor, Flawil, Switzerland). The residue was dissolved in deionised water (100 mL) and applied in three aliquots to a 20 g Strata® C-18 SPE cartridge (Phenomenex, Auckland, New Zealand). Each aliquot was washed with deionised water (30 mL) and phenolic compounds eluted with methanol (30 mL). The three eluates were combined and evaporated under vacuum to yield 1.3 g of extract.

2.1 Determination of total phenolic content by 'Folin' assay

The total phenolic contents of the blueberry and apple extracts and their glucuronidated mixtures were analysed by a minor modification of the Folin–Ciocalteu colorimetric method [24] In summary, the appropriate dilutions of extracts were oxidised with Folin–Ciocalteu reagent and the reaction was neutralised with sodium carbonate. The absorbance of the resulting blue colour was measured at 760 nm after 90 min by a SynergyHT plate reader (BioTek Instruments, Winooski, VT). Catechin was used as the standard, and results were expressed as the mean (in μMol of catechin equivalent *per* gram of dried extract) from three determinations.

2.2 UDP-glucuronyl transferase (UDPGT) preparation

The enzyme preparation, essentially freeze-dried ovine liver microsomal fraction, was prepared as described previ-

ously [25]. Although this crude preparation undoubtedly contains Phase I hydroxylation enzymes, there is no evidence that they have significant activity after freeze-drying and in the absence of an NADPH regenerating system [26], indicating the near absence of endogenous cofactors for any conjugation enzyme. Detectable modification of polyphenolics by this crude enzyme preparation, other than glucuronidation, is therefore, highly unlikely.

2.3 Acid hydrolytic deglycosylation of fruit extracts

Fruit extracts (25 mg) were heated (100°C/20 min) with water/methanol/concentrated HCl (5:4:1) and aglycones isolated by SPE, essentially as described previously [27]. The isolated yields were 8.6 mg from Blueberry extract and 7.1 mg from Applephenon. Apart from 0.1 mg removed for LCMS analysis, each sample was glucuronidated.

2.4 Glucuronidation procedure

Reactions for LCMS analysis were carried out essentially as described previously [25, 28]. Preparative reactions were carried out in 200 mM Tris buffer (pH 8.0) containing 50 mM sodium glucuronate, 6 mM $CaCl_2$ and 2 mM DTT at a temperature of 32°C (water bath). The reaction mixture (total volume 5 mL, in a 20 mL screw-capped glass vial) contained bovine serum albumen (BSA, 3% w/v) 12 mM UDPGA, and UDPGT (50 mg). Reactions were started by addition of deglycosylated fruit extract (see above) in DMSO (40 uL; 0.8% v/v).

Reactions were terminated after 18 h. EDTA was added to the reaction mixtures to a concentration of 10 mM and the mixture freeze-dried. Blueberry extract was treated for both 18 h. as above and separately, for 3 h. for comparison. The residue was extracted with 3×5 mL of methanol and filtered. The filtrate was dried under vacuum, dissolved in distilled water (approximately 10 mL) and applied to a 20 g C-18 SPE cartridge (Strata, Phenomenex). The cartridge was washed with 30 mL each of water (discarded) and 80:20 methanol/water (evaporated to dryness in a centrifugal concentrator; CentriVap, Labconco, Kansas City, MO). Weight yields for blueberry and apple extracts were 9.9 and 8.1 mg, respectively; total phenolic contents (as catechin equivalents) were 75 and 102 mg/g respectively. Catechin equivalent values for the original fruit extracts were 675 (blueberry) and 741 mg/g (apple).

2.5 HPLC conditions for phenolic analysis

Analyses were carried out as described previously [29], to determine the composition of major components of the treated and untreated fruit extracts. Detector response was calibrated using phenolic standard mixtures with 10 ppm each of catechin, epicatechin, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, phloridzin, phloretin, rutin, quercetin and cyanidin-3-galactoside. LCMS molecular ion and fragmentation information was correlated with HPLC retention times and UV spectra to verify identity and glycosylation of phenolic compounds.

2.6 LCMS analysis procedure

The HPLC standard mixture was analysed by LCMS to characterise the molecular ions and fragmentation of the standards for comparison with unknowns. Anthocyanins and derivatives were identified by LCMS in the ESI positive mode, as described previously [30]. Evidence of identity for anthocyanins was obtained primarily by observation of positive molecular ions (M^+), aglycone fragment ions and characteristic neutral losses (anthocyanins are naturally positively charged under acidic conditions). For example M^+ of malvidin-3-glucoside was observed at *m*/*z* 493 and loss of 162 Da (glucosyl residue) generated the malvidin molecular ion at *m*/*z* 331. Phenolic compounds and glucuronidated derivatives were identified by LCMS in the ESI negative mode, as described previously [29]. Evidence of identity for phenolic compounds, glycoside and glucuronide derivatives was obtained primarily by observation of $(M\text{-}1)^-$ negative molecular ions corresponding to the glucuronide molecular ion, the aglycone fragment ion and the characteristic neutral loss of 176 Da (glucuronyl residue). For example, $(M\text{-}1)^-$ for quercetin glucuronide was observed at *m*/*z* 477 and the quercetin aglycone at *m*/*z* 301, difference 176 Da. The molecular ion, $(M\text{-}1)^-$ for quercetin itself (MW = 302) was also observed at *m*/*z* 301.

3 Results and discussion

3.1 Glucuronidation of fruit extracts

Extracts of apple and blueberry were glucuronidated after acid hydrolysis to remove preexisting glycosylation. The overall yields of the two reactions, determined as catechin equivalents, using the Folin assay, were approximately 5% from each extract. It has been estimated that the proportion of dietary polyphenolics that is absorbed from the intestine is also around 5% [31]. The Folin assay quantifies phenolic hydroxyl groups, so it should detect any degraded, rearranged or polymerised compounds present and should only be slightly affected by the extent of glycosylation. The high losses of polyphenolic material indicate that the bulk of it was degraded into forms that were either insoluble, or bound to protein or other high molecular weight material, preventing isolation from the deglycosylation and glucuronidation reactions using SPE.

The major detectable components of the deglycosylated and glucuronidated mixtures were determined by LCMS and compared with the untreated extract. The chemical and enzymic treatments were originally intended only to syn-

thesise glucuronide mixtures from fruit extracts, but coincidentally, they also qualitatively model the main characteristics of the stomach, intestinal lumen and bloodstream, that are relevant to polyphenolic degradation. These are: low pH, followed by neutral pH, combined with relatively high concentrations of proteins and lipids. The model environment differs considerably in detail from that *in vivo*, but should give a reasonable qualitative comparison of the relative stability of different polyphenolic species. Although the absolute extent of degradation may not be the same as that *in vivo*, the relative extent of degradation of different classes of polyphenolics should be comparable to that *in vivo*.

3.2 Blueberry extract

Blueberry extract was largely composed of anthocyanins, chlorogenic acid (a caffeoyl quinate ester), epicatechin (the major individual component), plus glycosides of ferulic acid and quercetin (Fig. 1A, Table 1). Hydrolytic deglycosylation (Table 1) reduced the proportion of chlorogenic acid (apparently some was hydrolysed to caffeic acid), eliminated epicatechin and enriched the other compounds, particularly anthocyanidins. Anthocyanidins are reportedly especially stable under these acidic deglycosylation conditions [27]. The quinate esters of caffeic acid were the only carbohydrate derivatives to survive the treatment; no glycosidically linked sugars were detected. Some chlorogenic acid (~55%) appeared as a methylated form, (molecular ion at 367 Da, rather than 353 Da), probably a methyl ester of the quinate residue. This was probably an artefact arising from reaction with methanol during the deglycosylation treatment. Glucuronidation of the hydrolysed blueberry extract (Fig. 1B, Table 1) resulted in elimination of nearly all of the anthocyanidins; the only observed species being petunidin glucuronide. The resulting mixture was dominated by chlorogenic acid (mostly converted back to the deesterified form), other caffeic acid derivatives and glucuronides of quercetin (the major glucuronide species), ferulic acid and caffeoyl quinate methyl ester. Caffeic acid was detected, but not its glucuronide.

Anthocyanins can undergo a series of rearrangements under neutral conditions [32], involving relatively reactive intermediates and these rearrangements apparently promoted their almost complete disappearance during the enzymic glucuronidation. The anthocyanidins, or their glucuronides, apparently either degraded during the enzymic reaction, or were converted oxidatively into forms (for example, polymers) that were not isolated from the reactions or were not amenable to LCMS detection. Instability and high losses of anthocyanins during blueberry juice processing has been reported previously [33]. The shorter 3 h. reaction mixture contained no more detectable anthocyanin derivatives than the 18 h. one, so the degradation reactions appear to be relatively rapid and not significantly

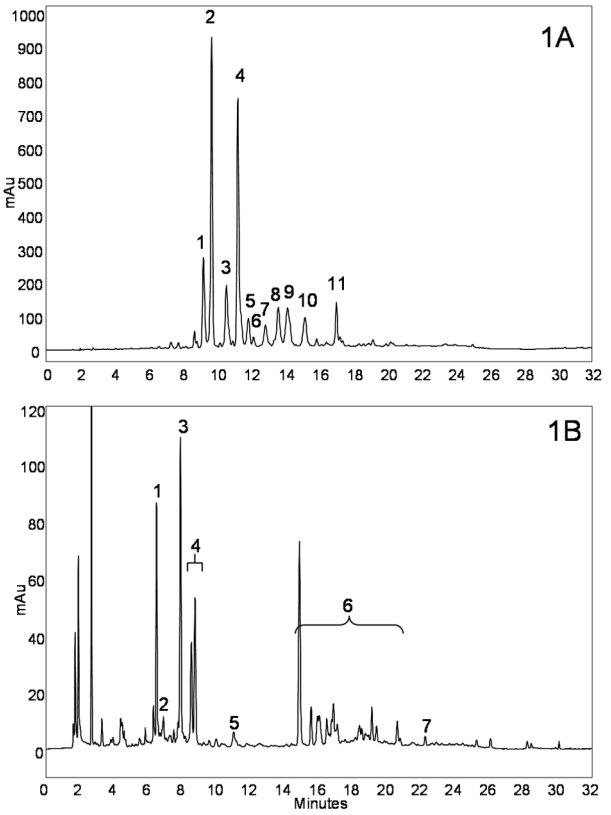

Figure 1. HPLC chromatogram of (A) blueberry extract at 280 nm, (B) hydrolysed/glucuronidated blueberry extract at 280 nm. Identities of major peaks, derived from LCMS analysis: (A) 1 – delphinidin galactoside, 2 – chlorogenic acid, 3 – delphinidin glucoside, 4 – epicatechin (major component)/ delphinidin arabinoside/cyanidin galactoside, 5 – petunidin galactoside 6 – cyanidin galactoside, 7 – petunidin arabinoside, 8 – malvidin/petunidin galactosides, 9 – malvidin glucoside/quercetin hexoside, 10 – malvidin arabinoside, 11 – quercetin hexoside. (B): 1 – chlorogenic acid, 2 – caffeic acid, 3 – caffeoyl quinates, 4 – petunidin glucuronide, 5 – caffeoyl quinate methyl ester, 6 – quercetin glucuronide isomers, 7 – petunidin glucuronide. Unlabelled peaks did not correspond with any compound identified in the original fruit extract (1A), but none was a glucuronide.

inhibited by glucuronidation. In a control experiment (results not shown), blueberry extract was subjected to enzymic glucuronidation without prior deglycosylation. Glucuronidation of anthocyanins was not observed and the proportion of anthocyanins was again reduced to less than 1%. Anthocyanidins do not appear to be significantly less stable than anthocyanins under the glucuronidation reaction conditions.

The enzymic glucuronidation reaction was carried out under very mild conditions, at pH 8.0 close to that in the intestinal lumen and body fluids. Nevertheless, only traces of anthocyanidin glycosides or glucuronides could be detected by LCMS after the enzymic incubation. The observed very low bioavailability of anthocyanins [18]

Table 1. Identified major components of blueberry extract before and after hydrolysis/glucuronidation (approximately percentages determined by HPLC/UV)

Compound	Blueberry extract (%)	Hydrolysed blueberry extract (%)	Glucuronidated + hydrolysed blueberry extract (%)
Chlorogenic acid/caffeoyl quinates	21	9[a)]	23
Caffeic acid	~1	5	2
Epicatechin	50	<1	<1
Ferulic acid	3 (Free and glycosides)	10	5 (Glucuronides)
Quercetin	2 (Hexoside)	8	28 (Glucuronides)
Anthocyanidins	22 (Glycosides)	45	~1 (Glucuronides)

a) 5% Methyl ester, 4% free acid.

Table 2. Identified components of Applephenon before and after hydrolysis/glucuronidation (approximately percentages determined by HPLC/UV)

Compound	Applephenon (%)	Hydrolysed Applephenon (%)	Glucuronidated + hydrolysed Applephenon (%)
Catechin	4	4	<1
Epicatechin	11	3	<1
Chlorogenic acid/caffeoyl quinate	20	14[a)]	29
Caffeic acid	7	10	2
p-Coumaric acid	7 (Free and derivatives)	3	14 (Free and derivatives)
Quercetin	3 (Free and glycosides)	7	9 Glucuronides
Phloretin	8 (Free and glycosides)	11	9 Glucuronides
Procyanidins	~36	~31	~46
Cyanidin	ND	2[b)]	ND

ND: Not detected.
a) 8% Methyl ester, 6% free acid.
b) Probably from hydrolysis of procyanidins.

could be readily explained by extensive nonenzymic degradation, polymerisation and/or binding to major food components, such as proteins or polysaccharides, before intestinal absorption can occur. The relative rate of absorption of anthocyanins may be as high as, or higher than other polyphenolics, but their real lumenal concentrations could be very much lower than would be expected from their high concentrations in many fruits.

3.3 Apple extract

Applephenon was mainly composed of catechin, epicatechin, chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin glycosides, phloridzin, phloretin and procyanidins (Fig. 2A, Table 2). The deglycosylation treatment made only minor changes to the proportions of most components, apart from reducing *p*-coumaric acid from 7 to 3% and epicatechin from 11 to 3% (Table 2). A small amount of cyanidin was produced, presumably from acid hydrolysis of procyanidins. This did not, however, survive the subsequent glucuronidation. Glucuronidation of the deglycosylated extract (Fig. 2B, Table 2), noticeably enriched unglucuronidated chlorogenic acid/caffeoyl quinates, *p*-coumaric acid derivatives, procyanidins and quercetin glucuronides, at the expense of catechin, epicatechin and (unglucuronidated) caffeic acid. The chemical and enzymic modification of Applephenon clearly did enrich the more stable species, but the compositional changes were more modest than those observed with blueberry extract.

In principle, glucuronide mixtures produced from fruit extracts, as described here, should be useful for *in vitro* bioassay testing, to simulate the likely effects of the metabolites of bioavailable polyphenolics obtainable from consumption of the fruit. The very low purity (*i. e.* polyphenolic content) and difficulty of fully characterising the complex glucuronide mixtures, however, would complicate any results obtained. It would be more useful to use the approach described here, or if possible, an *in vivo* bioavailability study, to estimate the major circulating metabolites, then synthesise them and assay them individually. Glucuronidation of individual compounds gives much higher yields and much more certainty about composition, thus allowing meaningful bioassay testing [34]. It may be appropriate, however, to bioassay the native forms of compounds which are resistant to glucuronidation, such as catechins, chlorogenic and caffeic acids as they may be present at more sig-

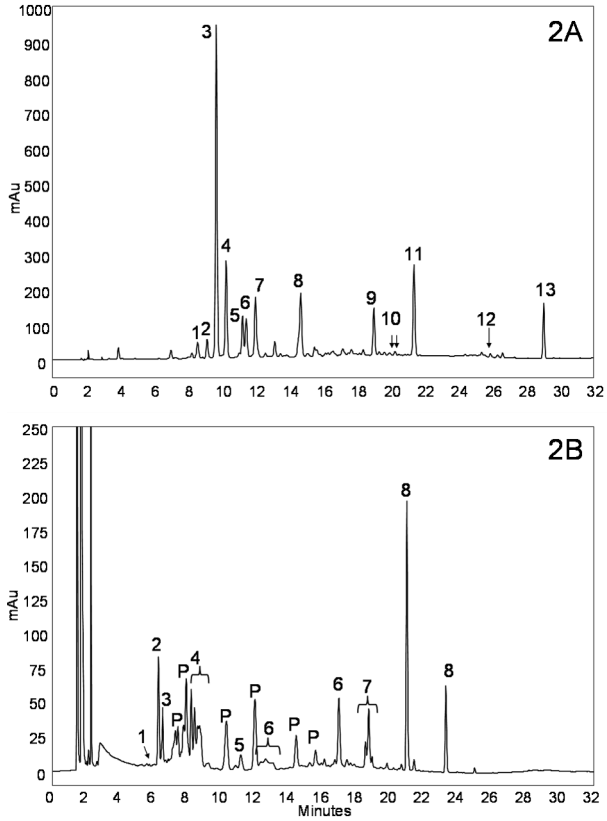

Figure 2. HPLC chromatogram of (A) Applephenon apple extract at 280 nm, (B) hydrolysed/glucuronidated apple extract at 280 nm. Identities of major peaks, derived from LCMS: (A) 1 – a procyanidin, 2 – catechin, 3 – chlorogenic acid, 4 – caffeic acid, 5 – epicatechin, 6 – a procyanidin, 7 – *p*-coumaric acid glycoside, 8 – *p*-coumaric acid, 9 – phloretin pentoside, 10 – quercetin glycosides, 11 – phloridzin (phloretin glucoside), 12 – quercetin, 13 – phloretin. Unidentified peaks between 12 and 27 min were identified as procyanidins. (B): 1 – catechin, 2 – chlorogenic acid, 3 – caffeic acid, 4 – caffeoyl quinates, 5 – caffeoyl quinate methyl ester, 6 – *p*-coumaric acid derivatives, 7 – quercetin glucuronide isomers, phloretin glucuronides, P – procyanidins. *p*-coumaric acid derivatives ionised weakly in the LCMS analysis and the derivative could not be identified, but did not appear to be glucuronyl.

nificant concentrations *in vivo*, in unglucuronidated form, than compounds like quercetin or phloretin.

Procyanidin (catechin oligomer) glucuronides were not detected in this study, although their presence cannot be ruled out, because of the heterogeneous composition of procyanidins. They might be expected to be similar to catechins in being relatively resistant to glucuronidation. Catechins were found to be glucuronidated *in vitro*, but only in low yields [34]. The proportion of procyanidins in apple extract was increased moderately by the treatments used here, so they should be readily available for absorption *in vivo*.

The results from this study may question the relevance of the strong research interest [35] in anthocyanins. Blueberry extract appears to contain approximately three times more non-anthocyanin polyphenolics than anthocyanins. Losses of anthocyanins were particularly high, under the mild conditions of the enzymic reaction and may be similar in the intestinal lumen and after absorption, in good agreement with their exceptionally low reported maximum physiological concentration of 0.03 μM [18]. Health benefits of blueberry consumption are at least as likely to be related to other components, for example, chlorogenic acid. Chlorogenic acid was a major component of both fruit extracts used in this study and also occurs at relatively high concentrations in many plant foods, especially coffee and other berries and apples [31], thus making it a probable major phenolic component of a normal diet. A high intake, coupled with high availability for absorption and resistance to glucuronidation, may make chlorogenic acid a potentially important bioactive dietary polyphenolic.

4 Concluding remarks

This study has provided additional evidence that polyphenolic phytochemicals differ radically in both their ability to survive the rigors of treatments that model digestion and metabolism and in their susceptibility to glucuronidation, the major mammalian conjugative metabolic process. Anthocyanins and catechins stood out as being much less stable than other compounds tested. Relatively polar compounds, such as chlorogenic and caffeic acids, were resistant to glucuronidation compared with quercetin and phloretin. This further reinforces the caveat on *in vitro* bioassay testing of phytochemicals. That is, bioassay results can, at best, only indicate the potential, unless applied to the forms of food components that are demonstrably present in the circulation at concentrations similar to those giving significant activity in the bioassay. The experimental approach proposed here (like any *in vitro* approach) has many limitations compared with a human or animal bioavailability study, but is applicable to screening of many phytochemical extracts using a fraction of the resources required for a rigorous bioavailability study. This approach should at least indicate which polyphenolic components of a fruit are less likely to be physiologically important.

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