Research Article

Selective bio-availability of phenolic acids from Scottish strawberries

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Scottish strawberries were found to be a rich source of phenolic acids, namely benzoic (1287.95 ± 279.98 mg/kg) and cinnamic (1159.40 ± 233.96 mg/kg) acids, both free and attached to other plant components. Studies suggest a chemopreventative role for such compounds in several major clinical conditions, but the anticipated benefits are likely to be affected by their bio-availability and metabolic fate. In this pilot study, strawberries (750 g) was consumed by four healthy human volunteers (32 ± 6 years). Only the benzoic acids were detected in the plasma. Of these, the major free (gentisic, protocatechuic and *p*-hydroxybenzoic acid) and conjugated (syringic acid) benzoic acids were 26 – 27% recovered in the urine within 5 h. Cinnamic acids were completely undetected in plasma and only trace amounts were found in the urine. Since, the cinnamic acids escaped absorption early in the gastrointestinal tract, their release and/or metabolism is dependant on the host colonic microbiota. Results indicate that there is a high degree of selective absorption of strawberry phenolic acids into the systemic circulation. If selective absorption of phenolic acids is observed with consumption of other plant-based foods, this is likely to have implications for the bioactive role of these compounds in chronic disease prevention.

Keywords: Cardiovascular disease / Colon cancer / Inflammation / Nutrition / Phytochemicals

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1 Introduction

Diets rich in plant-based foods and particularly those containing a high concentration of phenolics are considered to decrease the risk of developing several major clinical conditions [1, 2]. Phenolic acids, an important subgroup of plant metabolites have potential health benefits against chronic diseases such as diabetes, cardiovascular disease (CVD) and certain cancers such as cancer of the colon [3 – 6]. Integral to their prospective chemoprotective properties is their ability to be released from the food matrix and consequential bio-availability [7 – 10]. Bio-availability is often described as the ability of a compound to be absorbed and enter the systemic circulation, but the more important issue is whether it reaches the target tissue and site of physiological action. In certain diseases such as CVD, it may be beneficial to have high levels of potentially protective compounds present in the circulatory system, whereas, chemoprevention of colon cancer may require direct delivery to the gastrointestinal tract. Indeed, data on associations between dietary intake of fruit and vegetables on disease outcome show clear differences in risk reduction for CVD and cancer [11].

Despite the vast amount of studies aiming to confirm the bioactivity of dietary phenolic acids, very few address the accessibility at the site of action and of these studies, most are limited to *in vitro* cell culture work and animal studies [12, 13]. Assessment of bio-availability requires consideration of various factors. Firstly, the food matrix has been shown to have a major effect on systemic availability [12]. Free phenolic acids have the potential to be more easily absorbed in the small intestine and phenolic acids esterified to single sugars and other plant components such as tartaric, quinic or shikimic acid may also be readily absorbed [7 – 10, 14]. Phenolic acids bound to cell wall components such as polysaccharides and lignin are unlikely to be absorbed in the small intestine and therefore will only be available after microbial release and metabolism in the colon [15, 16]. There is also likely to be competition between compounds for absorption, as observed for gastric absorption in rats [13], and with their competitive affinity for a gut monocarboxylic acid transporter [17]. On absorption phenolics can be subject to extensive hydrolysis and oxidation by phase I enzymes and further conjugated and detoxified by the phase II enzymes [18].

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The complexity of phenolic acid absorption and metabolism has added to the problems correlating dietary intake with physiological effects, and also to the difficulty in establishing useful biomarkers [19]. Before any potential health benefits of phenolic acids can be established, it will be necessary to ascertain, whether these compounds are absorbed into the circulatory system early in the gastrointestinal tract, or are available for further metabolism in the colon. Therefore, the purpose of this study was to determine absorption and excretion profiles of dietary phenolic acids following consumption of a well-characterised plant-food source rich in all the major benzoic and cinnamic acids, both free form and bound to other plant components. Of particular interest is the fraction of phenolic acids not absorbed early in the gastrointestinal tract, as their bioavailability will be dependant on the host's colonic microbiota with potential implications for gut health.

2 Materials and methods

2.1 General reagents

Standard phenolic acids and general laboratory reagents were purchased form (Sigma/Aldrich; Gillingham, England). Strawberries (*Fragaria ananassa*; Alva) were grown in Tayside (Scotland).

2.2 Extraction and analysis of phenolics acids from strawberries

The strawberries were washed, weighed and stored at –80°C. They were then lyophilised (Heto Lab Equipment, Allerød, Denmark) and the moisture loss recorded. They were freeze-milled (Spex 6700, Edison, USA) and the powder stored in a desiccator prior to extraction. Samples (2 g dry weight; $n = 4$) were suspended in water (100 cm^3), in which the pH was reduced to pH 2 with HCl (6 mol/dm^3). Extracted into ethyl acetate (EtOAc; 50 cm^3) and separated the layers by centrifugation (15 min; 5000 rpm). The extraction was repeated three times and the EtOAc extracts combined. The organic layer was left to stand over sodium sulphate (anhydrous) for 1 h and filtered. The combined organic layers were then evaporated to dryness under reduced pressure at temperature not exceeding 40°C and stored in a desiccator prior to analysis by HPLC. The pH of the aqueous fraction was increased to pH 7 with sodium hydroxide (4 mol/dm^3). Sodium hydroxide (4 mol/dm^3) was added to give a final concentration of 1 mol/dm^3 and the sample stirred at room temperature for 4 h under nitrogen. The pH was reduced to pH 2 with HCl (6 mol/dm^3) and the samples extracted into EtOAc (50 $cm^3 \times 3$) and processed as described above. The pH of the aqueous fraction was then increased to pH 7 with NaOH (4 mol/dm^3). HCl (10 mol/dm^3) was added to give a final concentration of 2 mol/dm^3 and the sample incubated with stirring at 95°C for 30 min. Cooled and extracted with EtOAc (50 $cm^3 \times 3$) and processed as described above. The extracts were then re-dissolved in methanol and filtered through a 0.2 μm PVDF membrane. Separation of the phenolic compounds was by HPLC (Spectra SYSTEM, Thermo Fisher Scientific, UK) using AcCN and TFA (0.05% v/v; pH 2.3) and employing gradient elution: involved 11 – 14% AcCN (35 min), 14 – 50% AcCN (5 min), 50% AcCN (10 min) and 50 – 11% AcCN (5 min). Detection was at 215 and 280 nm and the metabolites were quantified by postextraction internal standardisation (4-hydroxtacetovanillone) with reference to their relative retention times ($t_{R'}$) and use of response factors calculated from pure compounds. The LODs for quantification were lower than 100 ng/dm^3. The identity of the phenolic acids was also confirmed by LC-MS (Finnigan MAT 900; Bremen, Germany) (t_R and molecular ion).

2.3 Dietary intervention study

Four volunteers (two male and two female) were recruited with a mean age of 32 ± 6 years. The volunteers had a normal BMI, were not taking any medication and normally consumed a western-style diet. No food or drink other than water was consumed 12 h prior to commencing the study. The volunteers provided a urine sample, which was not the first sample of the morning and the volume and weight was recorded. Blood samples (9 cm^3; LH PST^{TM}II) were also taken, immediately centrifuged (2500 rpm, 15 min, 4°C) and stored at –80°C. The volunteers were given a 750 g portion of strawberries, which they consumed within 30 min and the weight of any remaining strawberries recorded and accounted for when calculating total recoveries. Urine and blood samples were provided (as detailed above) at 1, 3 and 5 h after consumption. This study was approved by the North of Scotland Research Ethics Service (NoSRES) and by the Rowett Research Institute Human Studies Management Committee.

2.4 Extraction and analysis of phenolics acids in urine and plasma

The phenolic acids in the urine (5 cm^3) and plasma (1 cm^3) samples were extracted and quantified as described above (Section 2.2). The final volumes and concentration of internal standard were adjusted accordingly to be within the LODs of the method.

3 Results and discussion

3.1 Fruit selection and analysis

To study phenolic acid absorption and excretion patterns it was necessary to select a plant-based food, which contained representative compounds from both the benzoic and cin-

Table 1. Phenolic acid content of Strawberries

Phenolic acid IUPAC name	Synonym	t_R (min)	M^+ (amu)	Phenolic acid free (mg/kg)	Phenolic acid conjugated (mg/kg)
3,4,5-Trihydroxybenzoic acid	gallic acid	4.46	169.00	0.21 ± 0.08	181.85 ± 62.11
3,4-Dihydroxybenzoic acid	protocatechuic acid	6.72	153.10	7.03 ± 1.10	67.79 ± 22.28
4-Hydroxybenzoic acid	p-hydroxybenzoic acid	10.40	137.00	188.93 ± 21.86	649.27 ± 105.18
2,5-Diydroxybenzoic acid	gentisic acid	11.58	153.10	n.d.	45.29 ± 23.41
3-(3,4-Dihydroxyphenyl)acrylic acid	caffeic acid	12.85	179.00	21.72 ± 2.78	127.04 ± 7.70
4-Hydroxy-3-methoxybenzoic acid	vanillic acid	13.45	167.00	n.d.	68.28 ± 25.09
4-Hydroxy-3,5-dimethoxybenzoic acid	syringic acid	15.37	197.00	2.66 ± 0.24	3.12 ± 2.33
3-(4-Hydroxyphenyl)acrylic acid	p-coumaric acid	22.21	163.10	7.24 ± 1.45	642.28 ± 104.03
3-(4-Hydroxy-3-methoxyphenyl)-acrylic acid	ferulic acid	29.23	193.10	36.18 ± 5.80	57.36 ± 21.58
3-(4-Hydroxy-3,5-dimethoxy-phenyl)acrylic acid	sinapic acid	31.71	223.10	162.74 ± 20.58	104.84 ± 70.03
2-Hydroxybenzoic acid	salicylic acid	33.71	137.00	n.d.	73.55 ± 16.30

Conjugated phenolic acids are a summation of both the alkali- and acid-labile fractions. Values are specified on a dry weight basis in mg/kg (moisture content = 90.9%) and are given as mean ± SDs ($n = 4$). Not detected = n.d.; Retention times = t_R and molecular ions = M^+.

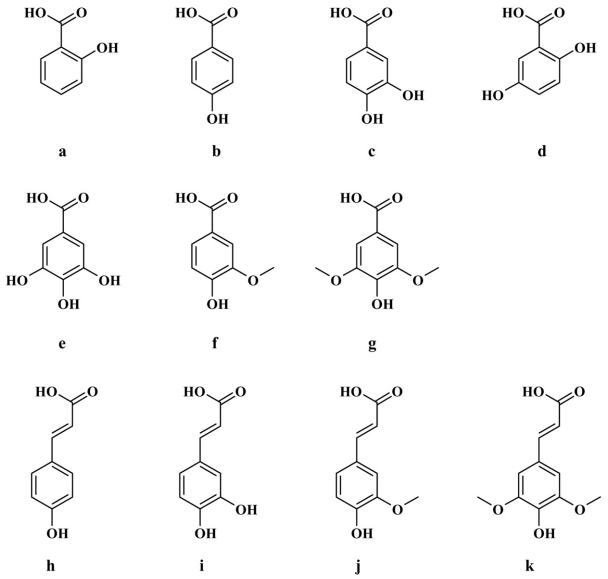

Figure 1. Structures of the predominant benzoic (C6C1; a – g) and cinnamic acids (C6C3; h – k) found in commonly consumed and locally produced Scottish fruits. Salicylic acid (a), *p*-hydroxybenzoic acid (b), protocatechuic acid (c), gentisic acid (d), gallic acid (e), vanillic acid (f), syringic acid (g), *p*-coumaric acid (h), caffeic acid (i), ferulic acid (j) and sinapic acid (k).

namic acid classes (Fig. 1) [20]. For consideration of the food matrix, it was also important that these phenolic acids were present in both the free form and bound to other plant components. Strawberries were analysed and found to contain similar amounts of benzoic and cinnamic acids both free (198.84 ± 23.28 mg/kg[1] for benzoic acids and 227.88 ± 30.61 mg/kg[1] for cinnamic acids) and esterified (1089.11 ± 256.70 mg/kg for benzoic acids and 931.52 ± 203.35 mg/kg for cinnamic acids) to other plant components (Table 1). Previous studies mainly concerning methanol soluble cinnamic acids from strawberries also found *p*-hydroxycinnamic acid to be a major component and both caffeic acid and ferulic acid have been reported [21, 22]. The esterified phenolic acid fraction contained all of the commonly observed benzoic and cinnamic acids with structurally analogous *p*-hydroxybenzoic acid *p*-hydroxycinnamic acid being most and equally abundant (649.27 ± 105.18 mg/kg and 642.28 ± 104.03 mg/kg, respectively) as reported previously [23].

3.2 Absorption of phenolic acids

Prior to consuming the strawberries, the only free phenolic acid detected in the plasma was protocatechuic acid (Table 2). This was detected for one volunteer only at a low level (2.35 ± 4.70 μg/L). Syringic, vanillic and *p*-hydroxybenzoic acids were also detected at low levels (1.14 ± 2.28, 10.66 ± 21.31 and 8.90 ± 14.60 μg/L, respectively) as conjugated moieties. It is likely that these low levels of benzoic acids detected in the plasma could have entered the circulatory system form the gut *via* the hepatic portal system and could originate from foods consumed prior to the fasting period. Within 5 h of strawberry consumption, the only phenolic acids detected in the plasma were benzoic acid and their derivatives. No free or bound cinnamic acids were detected at either 1, 3 or 5 h after consumption (Fig. 2, Table 2). A total benzoic acid plasma concentration were found to be highest at 1 h, with gentisic acid (110.11 ± 100.52 μg/L) being the major component in the free fraction. In strawberries, gentisic acid was only present esterified to another plant component and therefore, must have been rapidly de-conjugated in the blood. Protocate-

Table 2. Phenolic acid concentration of plasma

	Concentration (μg/L)			
	0 h	1 h	3 h	5 h
Phenolic acids (free)				
Gallic acid	n.d.	n.d.	n.d.	n.d.
Protocatechuic acid	2.35 ± 4.70	17.31 ± 16.24	8.53 ± 10.07	4.15 ± 8.30
p-Hydroxybenzoic acid	n.d.	29.85 ± 22.09	21.12 ± 12.93	6.37 ± 5.23
Vanillic acid	n.d.	0.57 ± 1.14	4.78 ± 2.66	0.18 ± 0.36
Syringic acid	n.d.	n.d.	n.d.	n.d.
Salicylic acid	n.d.	n.d.	n.d.	n.d.
Gentisic acid	n.d.	110.11 ± 100.52	76.88 ± 42.80	72.87 ± 38.47
p-Coumaric acid	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.d.	n.d.	n.d.	n.d.
Sinapic acid	n.d.	n.d.	n.d.	n.d.
Caffeic acid	n.d.	n.d.	n.d.	n.d.
Phenolic acids (conjugated)				
Gallic acid	n.d.	n.d.	n.d.	n.d.
Protocatechuic acid	n.d.	1.96 ± 3.92	13.33 ± 26.66	4.25 ± 8.51
p-Hydroxybenzoic acid	8.90 ± 14.60	69.60 ± 69.55	34.85 ± 11.35	7.96 ± 9.25
Vanillic acid	10.66 ± 21.31	19.86 ± 29.66	25.93 ± 18.16	20.54 ± 12.16
Syringic acid	1.14 ± 2.28	72.90 ± 88.37	56.15 ± 65.95	41.71 ± 60.42
Salicylic acid	n.d.	n.d.	n.d.	n.d.
Gentisic acid	n.d.	52.55 ± 84.33	117.65 ± 39.63	72.89 ± 47.11
p-Coumaric acid	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.d.	n.d.	n.d.	n.d.
Sinapic acid	n.d.	n.d.	n.d.	n.d.
Caffeic acid	n.d.	n.d.	n.d.	n.d.

Values are given as mean ± SDs ($n = 4$). Not detected = n.d.

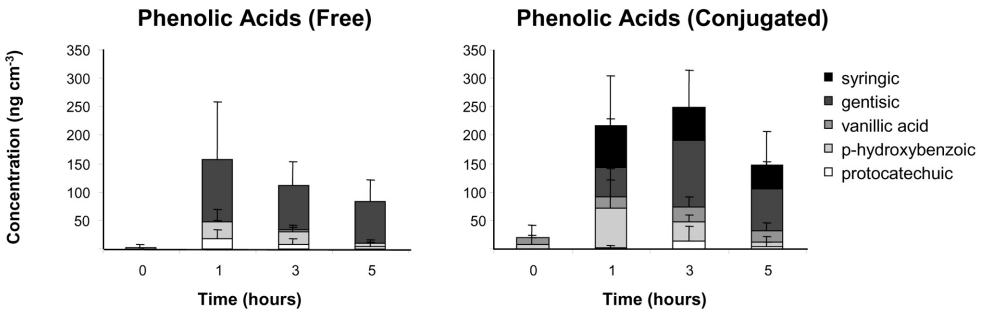

Figure 2. Phenolic acid concentration (mg/L) in plasma. Data are given as mean ± SDs ($n = 4$).

chuic and vanillic acid were also detected as free phenolic acids 1 h after consumption. Again, it appears that these acids are being de-conjugated, as vanillic is not available as a free acid in strawberries and protocatechuic acid is present only in low concentration. It is impossible to say whether the *p*-hydroxybenzoic acid detected had been de-conjugated, as this is a major free phenolic acid in strawberries. At 3 and 5 h after consumption all four free phenolic acids were still detected, but the concentrations of all decreased with time. Syringic acid was not detected in the free phenolic acid fraction, but was found to be the predominant conjugated acid in plasma 1 h after consumption (72.90 ± 88.37 μg/L). In strawberries this acid is found to be both free and esterified and therefore, must be conjugated on absorption. Protocatechuic, vanillic, gentisic and *p*-hydroxybenzoic acid were also all detected as conjugates. With the exception of *p*-hydroxybenzoic acid, maximum concentrations were achieved 3 h after consumption. It appears that when the benzoic acids are free and/or de-conjugated after absorption, their concentrations were measured to be at their highest 1 h after consumption. Whereas benzoic acids found to be in their conjugated form in the plasma mostly achieve maximum measured concentration at 3 h. For these benzoic acids it is difficult to say whether

Table 3. Concentration of phenolic acids in urine

	Concentration (mg/L)		
	1 h	3 h	5 h
Phenolic acids (free)			
Gallic acid	0.04 ± 0.05	0.04 ± 0.03	0.07 ± 0.01
Protocatechuic acid	0.74 ± 0.90	0.35 ± 0.68	0.72 ± 0.11
p-Hydroxybenzoic acid	3.14 ± 4.88	8.38 ± 11.19	10.43 ± 2.90
Vanillic acid	0.07 ± 0.07	n.d.	1.03 ± 0.03
Syringic acid	0.06 ± 0.09	0.01 ± 0.02	0.10 ± 0.03
Salicylic acid	0.10 ± 0.14	0.13 ± 0.07	0.16 ± 0.05
Gentisic acid	0.19 ± 0.28	0.33 ± 0.29	1.01 ± 0.18
p-Coumaric acid	0.82 ± 0.72	0.99 ± 0.34	0.52 ± 0.11
Ferulic acid	n.d.	n.d.	0.03 ± 0.02
Sinapic acid	n.d.	n.d.	0.25 ± 0.08
Caffeic acid	0.32 ± 0.38	n.d.	0.12 ± 0.06

Values are given as mean ± SDs ($n = 4$). Not detected = n.d.

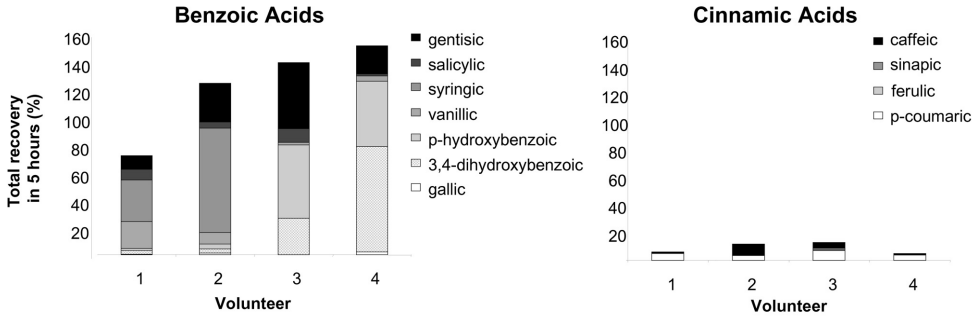

Figure 3. Recovery (% of total consumed) of benzoic and cinnamic acids for each of the volunteers within 5 h of strawberry consumption.

conjugation occurs after absorption as they are present bound to other plant components in strawberries and may have been absorbed as simple esters.

3.3 Excretion of phenolic acids

In an attempt to quantify the total amount of phenolic acids absorbed and excreted, total urine was collected at periods of 1, 3 and 5 h following strawberry consumption. Again the benzoic acids derivatives were the predominant phenolic acids recovered in the urine (Fig. 3, Table 3). Gallic and salicylic were not detected in the plasma and only 1 and 6%, respectively of the total amount consumed was recovered in the urine. Vanillic acid, which was present in the plasma at low concentration in the free form, also had a low recovery in the urine (8%). However, the major free phenolic acids in plasma; gentisic, *p*-hydroxybenzoic and protocatechuic acid were 26–27% recovered. Syringic acid, which was only available in the plasma as a conjugate was also 26% recovered in the urine. With the exception of gentisic acid (69% detected in the conjugated form), the majority of phenolic acids detected in the urine were unconjugated. Trace amounts of the cinnamic acids; namely caffeic and *p*-coumaric were also recovered in the urine (4 and 5%, respectively). Since cinnamic acids were not detected in the plasma, it is possible that these low levels could have been excreted *via* the hepatic portal system from foods consumed prior to the fasting period.

4 Concluding remarks

Strawberries provided a plant-based food source rich in both benzoic and cinnamic acids, free and bound to other plant components. After consumption of strawberries, only the benzoic acids were detected in the plasma, suggesting a high degree of absorption selectivity. The major free benzoic acids (gentisic, protocatechuic and *p*-hydroxybenzoic acid) and the major conjugated acid (syringic acid) detected in the plasma were 26–27% recovered in the urine within 5 h after consumption. These recoveries are relatively high considering that the concentration of benzoic acids attached to other plant components was greater than five-fold more than that of free benzoic acids and such rapid absorption and excretion suggests a potential detoxification mechanism. The cinnamic acids were not detected in the plasma,

although trace amounts, namely *p*-coumaric acid and caffeic acid were detected in the urine. It appears that the bulk of the cinnamic acid fraction, irrespective of whether they were free or bound to other plant components escapes absorption in the small intestine and is available for release and metabolism in the colon. Although, these cinnamic acid derivatives and their metabolites have the potential to be protective in diseases of the gastrointestinal tract, bioavailabilty will be largely dependant on the host's microbiota. Microbial metabolites produced in the colon may also enter the circulatory system *via* the hepatic portal system. Concentrations of these compounds in the plasma are likely to be low, as observed for the baseline plasma samples taken prior to strawberry consumption. It was interesting to note that all of the compounds detected in this fasted plasma were also benzoic acids. It is possible that when less competition for absorption occurs as in the case of consumption of a plant-based food, particularly rich in a single cinnamic acid (*e.g*. caffeic acid in coffee) that these cinnamic acids could be absorbed in the small intestine [24]. Many studies report potential health benefits associated with phenolic compounds form soft fruits such as strawberries [25 – 27], which contain a wide range of phenolic compounds [23, 28, 29]. Consumption of a diet rich in plant-based foods will also provide a variety of phenolic acids. It is likely that this will result in selective absorption and excretion of phenolic acids based on their structural characteristics, which will have a major effect on their bio-availability and consequential bioactivity.

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