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Metabolism of Berry Anthocyanins to Phenolic Acids in Humans

TARJA NURMI,*,† JAAKKO MURSU,† MARINA HEINONEN,‡ ANNA NURMI,§ RAIMO HILTUNEN,§ AND SARI VOUTILAINEN†

Research Institute of Public Health, School of Public Health and Clinical Nutrition, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland, Department of Applied Chemistry and Microbiology, P.O. Box 27, Food Chemistry, FI-00014 University of Helsinki, Finland, and Division of Pharmaceutical Biology, Faculty of Pharmacy, P.O. Box 56, FI-00014 University of Helsinki, Finland

We studied the metabolism of berry anthocyanins to phenolic acids in six human subjects by giving them bilberry−lingonberry purée with and without oat cereals. Purée + cereals contained 1435 μmol of anthocyanins and 339 μmol of phenolic acids. The urinary excretion of measured 18 phenolic acids increased 241 μmol during the 48 h follow-up after the purée + cereals supplementation. The excretion peak of dietary phenolic acids was observed at 4−6 h after the purée + cereals supplementation and 2 h earlier after the supplementation of the purée alone. Homovanillic and vanillic acids were the most abundant metabolites, and they were partly produced from anthocyanins. No gallic acid, a fragmentation product of delphinidin glycosides, was detected, and only a very low amount of malvidin glycosides was possibly metabolized to syringic acid. Although anthocyanins were partly fragmented to phenolic acids, still a large part of metabolites remained unknown.

KEYWORDS: Anthocyanins; metabolism; phenolic acids; urine; human

INTRODUCTION

Berries, like vegetables and fruits, are a part of a healthy diet, and all these contain a wide variety of different flavonoids and phenolic acids (*1*−*3*). Epidemiological evidence on the health effects of berries alone is scarce, but a high intake of vegetables and fruits has been inversely associated with a decreased risk of cardiovascular diseases (CVD) (*4*). Epidemiological evidence of the health effects of flavonoids suggests a slightly reduced risk of CVD and possibly lung cancer (*5*). The most recent findings have reported a reduced risk of stroke (*6*) and lung cancer (*7*) among those having a high intake of flavonoids.

Anthocyanins, a group of flavonoids, are especially abundant in berries, and the most recent estimate of the intake of anthocyanins in Finland has been 47 mg/day in a study population of 2700 Finnish adults (*8*). According to the average consumption of berries (15 kg/year) in Finland, however, the daily intake of anthocyanins has been estimated to be 83 mg/d (*9*). In a clinical trial with a combination of bilberries, lingonberries, chokeberries, and raspberries, a significant increase in HDL, and a significant decrease in blood pressure has been observed, when subjects included in their daily diet approximately 160 g of different berries (*10*). The mean intake of anthocyanins during the duration of the study was 515 mg/d.

To study further the possible mechanisms of action of anthocyanins, it is important to know their metabolism in humans. Recent in vitro studies have reported phenolic acids, such as protocatechuic, syringic, vanillic, and *p*-hydroxybenzoic acids, to be main metabolites of anthocyanins after fecal fermentation (*11*−*15*). The metabolism of cyanidin glucosides to protocatechuic acid has been confirmed in a human study after the ingestion of 1 L of blood orange juice. Protocatechuic acid has been detected in serum but not in urine, and only protocatechuic acid has been analyzed (*16*). Other metabolism studies with anthocyanins in humans have mainly focused on methylation, glucuronidation, and sulfation of anthocyanins (*17*−*19*), or measured intact anthocyanins in plasma and urine (*20*). Fragmentation of anthocyanins to phenolic acids in humans has not been studied in detail. The metabolism of phenolic acids and their metabolites have been studied after the ingestion of foods containing phenolic acids (*21*−*23*) or pure compounds (*24*), but not after the berry meal containing mainly anthocyanins.

We carried out two supplementation trials aimed at studying the metabolism of berry anthocyanins to phenolic acids in humans. The intake of phenolic compounds from the diet was extensively restricted, and altogether 18 phenolic acids were determined. Seven phenolic acids were dietary compounds, while 11 were metabolites. The bilberry−lingonberry purée was administered with and without oat cereals to observe the effect of cereals on the metabolism of berry anthocyanins.

* Corresponding author. Tel: +358 40 355 2965. Fax: +358 17 162936. E-mail: tarja.nurmi@uku.fi.

† University of Kuopio.

‡ Department of Applied Chemistry and Microbiology, University of Helsinki.

§ Division of Pharmaceutical Biology, Faculty of Pharmacy, University of Helsinki.

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Table 1. Quantified Phenolic Compounds in the Study Meal

purée (50 g)	mg/50 g	μmol/50 g	major analytes
anthocyanins	650	1435	575 μmol delphinidin, 401 μmol cyanidin, 202 μmol petunidin, 183 μmol malvidin, and 74 μmol peonidin glycosides[a]
catechins and procyanidins	44	116	catechin, epicatechin, procyanidin B-1, B-2, B-4[b]
flavonols	37	61	not identified[c]
phenolic acids	60	287	chlorogenic, protocatechuic, *p*-coumaric, caffeic and gallic acid
total in purée	791	1899	
oat cereals (50 g)	**mg/50 g**	**μmol/50 g**	**major analytes**
catechins and procyanidins [a]	4	10	epicatechin, procyanidin B-2
phenolic acids	12	51	ferulic, sinapinic, chlorogenic and *p*-coumaric acid
total in cereals	16	61	

[a] Quantified anthocyanins were glucosides, galactosides, arabinosides, and anthocyanidins. [b] B-1 = epicatechin-(4β → 8)-catechin; B-2 = epicatechin-(4β → 8)-epicatechin; and B-4 = catechin-(4α → 8)-epicatechin. [c] Flavonols were quantified with quercetin-3-*O*-rutinoside (rutin), but not identified. Value includes all analytes in the purée with the flavonol spectrum.

MATERIALS AND METHDODS

Study Subjects, Meals, and Design. Six healthy, omnivorous subjects (5 males and 1 female) of mean age 28 ± 3.2 years were recruited, and they participated in two supplementation studies. The first study meal (purée + cereals) consisted of 50 g of oat cereals and 50 g of berry purée containing bilberries (*Vaccinium myrtillus* L.) and lingonberries (*Vaccinium vitis-idaea* L.) in a ratio of 3:1 (w/w). Bilberries and lingonberries are commonly consumed and commercially the most important wild berries in Finland (*25*), and they were therefore used as the anthocyanin source in this study. The purée was prepared by crushing the berries and then concentrating (removing water) until the amount of anthocyanins was at the minimum 600 mg/meal. The second meal was 50 g of berry purée alone. Phenolic compounds quantified in the purée and oat cereals were anthocyanins, catechins, procyanidins, flavonols, and phenolic acids (**Table 1**). Anthocyanins (*2*), flavonols (*26*), catechins, procyanidins (*27*), and phenolic acids (*23*) were analyzed as has been described earlier.

The study subjects refrained from all vegetable foods and drinks for five days prior to both supplementation studies and continued the restricted diet during the 48 h follow-up. The restricted diet consisted mainly of unflavored dairy products, meat, fish, and eggs, and refined wheat flour products and rice. The second supplementation study was carried out two months after the first one. In both supplementation studies, blood samples were taken prior to and 0.5, 1.5, 3, 6, 8, 12, 24, 30, 36, and 48 h after the meal. Plasma was separated and samples were stored at −70 °C until analyzed. Urine samples (total volumes) were collected during the preceding 24 h (baseline), and at 0−2, 2−4, 4−6, 6−8, 8−12, 12−24, 24−30, 30−36, and 36−48 h after the meal. Volume of the urine samples was determined, hydrochloric acid was added to the urine samples for anthocyanin analyses, and aliquots were stored at −70 °C until analyzed. The study protocol was approved by the Research Ethics Committee, Hospital District of Northern Savo.

Measurements. Plasma anthocyanins were extracted from plasma after plasma proteins were precipitated with 10% trifluoroacetic acid. The supernatant was separated with centrifugation and transferred into a Sep-Pak C18 (Waters, Bedford, MA) solid phase extraction cartridge. Urinary anthocyanins were also extracted with a Sep-Pak C18 solid phase extraction cartridge. HPLC analyses of plasma and urinary anthocyanins were carried out as described before (*28*). The HPLC system consisted of a Waters 2690 separations module, a model 996 diode array detector, and Millennium 32 software. The column was Nova-Pak C18 (150 × 3.9 mm, 4 μm, Waters, Millipore, Bedford, MA) equipped with a C18 guard column. Anthocyanins were quantified as cyanidin-3-*O*-glucosides at 520 nm.

Urinary phenolic acids were determined with HPLC using coulometric electrode array detection (ESA Inc. Chelmsford, MA) as described earlier (*23*). Briefly, samples were hydrolyzed overnight at 37 °C with *H. pomatia* enzyme mixture (BioSepra, France), cool samples were acidified with 10 μL of 6 M hydrochloric acid, and analytes were extracted twice with diethyl ether (Riedel-de-Haën, Seelze, Germany). Combined extracts were evaporated, and the dry residue was dissolved in methanol (LabScan, Dublin, Ireland). Samples were diluted with the mobile phase prior to the HPLC run. A reagent blank was prepared in each assay, and recovery values of the analytes were determined with the standards processed like samples. In total, 18 different phenolic acids, including benzoic, phenylacetic, 3-phenylpropionic, and cinnamic acid derivatives, were measured. Specific compounds purchased from Sigma-Aldrigh were *p*-hydroxybenzoic, protocatechuic, vanillic, syringic, gallic, 3,4-dihydroxyphenylacetic, *m*-hydroxyphenylacetic, homovanillic, 3,4-dihydroxyphenylpropionic, *m*-hydroxyphenylpropionic, dihydroferulic, dihydroisoferulic, caffeic, ferulic, sinapinic, isoferulic, *p*-coumaric, and *m*-coumaric acids. Phenolic acids were divided into dietary and metabolic compounds according to their appearance in foods (*29*), and excretion patterns observed earlier (*23*). Dietary compounds were protocatechuic, syringic, gallic, caffeic, ferulic, *p*-coumaric, and sinapinic acids, and the others were considered as metabolites. All phenolic acids were also analyzed from the purée and cereals to confirm that metabolites were not given for the subjects in the study meals. The urinary excretion of phenolic acids was divided by the duration of collection to obtain the excretion rate in μmol/h and to equalize collection periods of a different duration. The total increase in the urinary excretion of a certain phenolic acid was calculated by summing up the excretion of the first and the second follow-up days (48 h), and then subtracting the excretion of the preceding 24 h twice to obtain the total increase without the baseline excretion.

Statistical Methods. Statistical analyses were carried out with the SPSS statistical software version 14.0 for Windows (SPSS, Chicago, IL) using the nonparametric Wilcoxon test for the dependent samples, and $P < 0.05$ was regarded as statistically significant.

RESULTS

Plasma Anthocyanins. The ingested amount of anthocyanins was 1435 μmol (650 mg), and anthocyanins were detected in plasma from 1.5 to 6 h. The maximum concentration of plasma anthocyanins was observed 3 h after the purée + cereals supplementation, and the concentration of total anthocyanins was 149 nmol/L. After the purée alone, the maximum concentration was observed 1.5 h after the meal, and the concentration was 138 nmol/L. The urinary excretion of anthocyanins was not determined in detail, but two urine samples at 2−4 h were analyzed to verify that the excretion of ingested anthocyanins was low and that it was less than 0.01% of the ingested amount after the purée + cereals supplementation.

Dietary and Metabolic Phenolic Acids after the Purée + Cereals Supplementation and Purée Alone. The total baseline excretion of urinary phenolic acids was 115 ± 26 μmol/day during the preceding 24 h before the purée + cereals supplementation, and 191 ± 102 μmol/day before the purée alone. The excretion of dietary phenolic acids was significantly higher at 2−4 h ($P = 0.046$) after the purée + cereals supplementation, and at 30−36 h ($P = 0.046$) after the supplementation of the purée alone (**Figure 1A**). The peak excretion of dietary phenolic

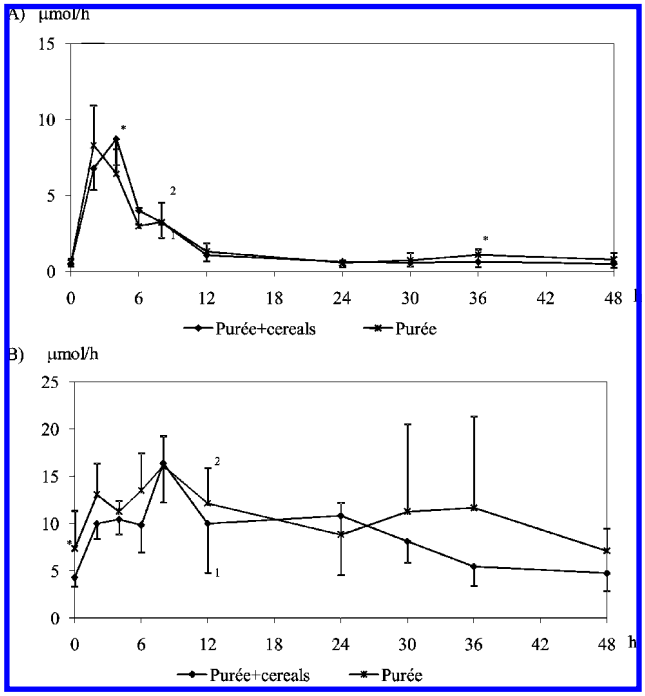

Figure 1. Urinary excretion of dietary and metabolic phenolic acids. (**A**) Dietary phenolic acids. (**B**) Phenolic acid metabolites. [1] SD bars for purée + cereals downward. [2] SD bars for purée upward. * Significant difference.

acids was postponed because of the oat cereals. The baseline excretion of metabolites differed significantly ($P = 0.046$), and the peak occurred at 6−8 h after both supplementations (**Figure 1B**). No effect of oat cereals was observed on the excretion of metabolites, as was observed for the dietary phenolic acids. Total increase in the excretion of dietary phenolic acids was 13.5% of the ingested amount (**Table 2**). The urinary excretion of dietary phenolic acids was 10.5, 18.0, and 8.7% of the total excretion during the preceding, first, and second follow-up days, respectively. The ingested amount of phenolic acids was 339 and anthocyanins 1435 μmol, while the total increase in the excretion of phenolic acids was 241 μmol.

Methylated Phenolic Acids. The highest increase in the urinary excretion of phenolic acids was observed for the methylated metabolic compounds (**Table 2** and **Figure 2A**). The excretion of homovanillic acid was higher at the baseline of the purée study ($P = 0.028$), and in both studies, the excretion of homovanillic acid increased from 2 to 8 h and then decreased back to the baseline value in 36 h after the purée + cereals supplementation. Vanillic acid was excreted in two peaks during the first 12 h after both meals, and the third peak was observed at 12−24 h when the excretion of homovanillic acid decreased. The excretion of dihydroferulic acid increased more rapidly after the supplementation of the purée alone being higher at 0−2 h ($P = 0.028$), and the excretion also continued higher at 24−30 h ($P = 0.028$) and 36−48 h ($P = 0.046$). The excretion of other methylated phenolic acid metabolites was much lower than that of dihydroferulic acid, but the slightly increased excretion of isoferulic and dihydroisoferulic acid was observed during the first 24 h (**Figure 2B**). The urinary excretion of ferulic acid increased rapidly after the supplementation and was higher at 2−4 h after the purée + cereals ($P = 0.028$) supplementation in contrast to the purée alone. One third of the ingested ferulic acid was excreted as such.

Hydroxylated Dietary Phenolic Acids. Caffeic acid was excreted during the first 12 h, and the total increase in the excretion of caffeic acid was approximately 10% of the ingested amount (**Table 2**). The peak excretion of caffeic acid was higher after the purée + cereals supplementation, and was still higher at 4−6 h ($P = 0.028$) after the meal in contrast to the purée alone. The peak excretion of protocatechuic and *p*-coumaric acid occurred at the same periods as that of caffeic acid (**Figure 3A**). Twenty-nine percent of the ingested protocatechuic acid and 8% of *p*-coumaric acid were excreted as such. The urinary excretion of protocatechuic acid was significantly higher after the supplementation of the purée alone at 8−12, 30−36, and 36−48 h ($P = 0.028$, 0.028 and 0.046) respectively. The urinary excretion of *p*-coumaric acid was higher at the baseline ($P = 0.046$) and at 12−24 h after the purée + cereals supplementation ($P = 0.046$). *m*-Coumaric acid was not detected in any of the samples.

Hydroxylated Phenolic Acid Metabolites. The urinary excretion of 3,4-dihydroxyphenylpropionic acid increased slowly up to 8 h after the purée + cereals supplementation, and decreased back to the baseline value in 36 h (**Figure 3B**). The urinary excretion of 3,4-dihydroxyphenylpropionic acid increased more rapidly after the supplementation of the purée alone, but otherwise, the urinary excretion of 3,4-dihydroxyphenylpropionic and acetic acids did not differ. The urinary excretion of *m*-hydroxyphenylpropionic acid increased slightly at 0−2 h (**Figure 3C**), but the clear increase in the excretion rate was seen 8 h after the purée + cereals supplementation, after which the excretion rate was constant up to 24 h. The urinary excretion of *m*-hydroxyphenylacetic acid had a profile similar to that of *m*-hydroxyphenylpropionic acid, but acetic acid was more abundant. The excretion of *p*-hydroxybenzoic acid was significantly higher at the baseline ($P = 0.046$), at 0−2 h ($P = 0.028$), and at 2−4 h ($P = 0.028$) after the supplementation of the purée alone. The excretion of *p*-hydroxybenzoic acid was equal from 8 to 24 h, while significantly higher excretion was again observed at 24−30 h ($P = 0.028$) and 36−48 h ($P = 0.046$) after the supplementation of the purée alone.

Other Phenolic Acids. The excreted amounts of different phenolic acids were low in contrast to the ingested amounts of anthocyanins. Although delphinidin glycosides were the most abundant phenolic compounds in the purée, no gallic acid was detected, and only a very low amount of syringic acid, possible metabolite of malvidin, was excreted (**Table 2**). Suggested metabolic pathways for anthocyanins and phenolic acids are presented together to show the possible metabolic pathways (**Figure 4**).

DISCUSSION

In the present study, the urinary excretion of 18 different phenolic acids was determined after the supplementation of bilberry−lingonberry purée + cereals and purée alone in order to study the fragmentation of anthocyanins to phenolic acids. The excretion of phenolic acids increased after the supplementation, but not so much that the determined phenolic acids could be considered to be the main metabolites of the ingested anthocyanins in humans.

Anthocyanins. In two recent studies with different doses of strawberries the urinary excretion of anthocyanins has been shown to be dose dependent (*30*, *31*), but the percentage recoveries in urine have been as low as before, varying from less than 1 up to 5% of the ingested amount of anthocyanins (*17*). In our study, plasma anthocyanin concentrations were 138−149 nmol/L, while in the other studies the observed values have mainly been below 100 nmol/L, also after doses as high

Table 2. Urinary Excretion of Phenolic Acids after the Purée + Cereals Meal

	urinary excretion μmol (mean ± sd)			
dietary phenolic acids (amount in the study meal μmol [a])	preceding 24 h (baseline)	0−24 h after the meal	24−48 h after the meal	total increase in excretion
protocatechuic acid (40)	1.5 ± 0.7	13.1 ± 2.3	1.4 ± 0.8	11.5
syringic acid	0.7 ± 0.3	3.3 ± 0.4	0.7 ± 0.2	2.6
caffeic acid (104[b])	0.8 ± 0.5	12.6 ± 1.8	1.2 ± 0.3	12.2
ferulic acid (29)	8.6 ± 3.9	18.4 ± 5.6	8.6 ± 4.1	9.8
p-coumaric acid (123)	0.6 ± 0.4	9.9 ± 1.5	1.2 ± 2.0	9.9
total (339)	12.2 ± 3.8	57.2 ± 8.6	13.1 ± 5.5	45.9
metabolites	preceding 24 h	0−24 h after the meal	24−48 h after the meal	total increase in excretion
3,4-dihydroxyphenylpropionic acid	0.0 ± 0.0	16.4 ± 8.9	3.7 ± 3.7	20.1
m-hydroxyphenylpropionic acid	2.5 ± 2.0	9.7 ± 6.4	10.6 ± 6.5	15.4
dihydroferulic acid	2.7 ± 2.2	10.3 ± 6.6	3.9 ± 6.1	8.8
isoferulic acid	0.8 ± 0.9	4.4 ± 2.9	0.3 ± 0.3	3.2
dihydroisoferulic acid	0.4 ± 1.0	1.4 ± 1.3	0.6 ± 0.6	1.1
3,4-dihydroxyphenylacetic acid	12.9 ± 3.6	23.6 ± 3.8	14.0 ± 3.6	11.7
m-hydroxyphenylacetic acid	13.9 ± 7.0	27.7 ± 16.3	35.8 ± 16.7	35.7
homovanillic acid	29.6 ± 2.2	63.2 ± 16.1	34.4 ± 6.8	38.5
vanillic acid	21.8 ± 18.6	77.6 ± 101.2	16.4 ± 17.8	50.3
p-hydroxybenzoic acid	18.5 ± 4.4	28.9 ± 11.4	18.5 ± 6.7	10.3
total	103.1 ± 23.6	263.1 ± 100.1	138.3 ± 43.7	195.1

[a] In addition to these, the purée + cereals meal contained 26 μmol of gallic acid and 17 μmol of sinapinic acid. [b] The sum of chlorogenic and caffeic acid is given as molar amount of caffeic acid.

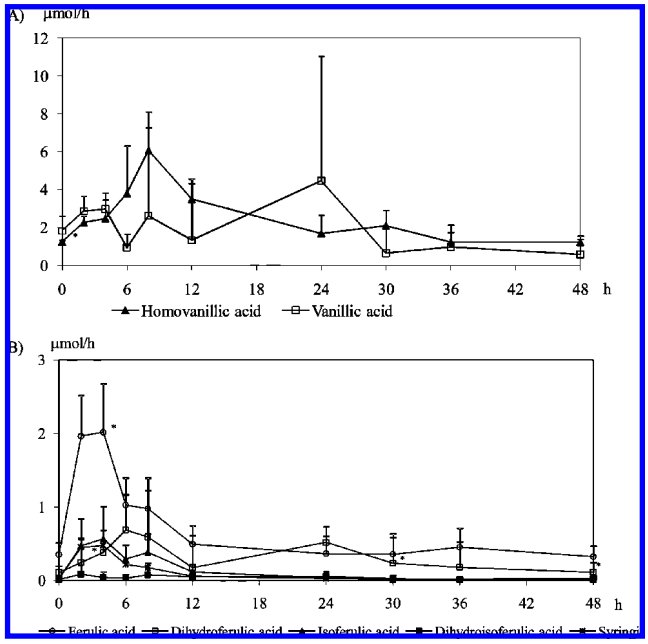

Figure 2. Urinary excretion of methylated phenolic acids after purée + cereals. (**A**) Major metabolites. (**B**) Dietary ferulic and syringic acid, and minor metabolites. * Significantly different from the purée alone. See details from the results of methylated phenolic acids.

as those in our study (*32*). Although anthocyanins have been reported to be absorbed (*33*), at least in rats, it is not likely that one can detect anthocyanins or anthocyanidin glucuronides and sulfates in high concentrations in plasma because of the instability of anthocyanins at neutral pH (*34*). The reported concentrations included all analytes with an anthocyanidin skeleton, and therefore, they represented the total anthocyanins in plasma. The measured nmol/L concentrations confirmed that anthocyanins were really metabolized.

Baseline Urinary Excretion of Phenolic Acids. The total baseline urinary excretion of phenolic acids prior to the purée + cereals supplentation was 115 ± 26 μmol/d, while in another study after the similarly restricted diet, a baseline excretion of 95 ± 11 μmol/d has been observed (*23*). In the present study, the baseline excretion was 20 μmol/d higher, but also five new phenolic acids were included in the analyses. Two abundant phenolic acids in urine after the restricted diet were homovanillic and 3,4-dihydroxyphenylacetic acids. They are partly produced from the necessary neurotransmitters, catechol amines (*35*), and therefore, their excretion was relatively high. Only ferulic acid of the dietary phenolic acids was excreted at the baseline over 1 μmol/d. That was probably due to the ferulic acid present in the refined cereal products that were allowed to be consumed during the dietary restriction period.

Methylated Phenolic Acids. The most abundant metabolites in urine were methylated phenolic acids, and their excretion was also highest prior to the supplementations. Although all of the ingested ferulic acid would have been metabolized to homovanillic and vanillic acids, still approximately 60 μmol had some other parent molecules, which in our study were very likely anthocyanins (**Figure 4**). A third of the ingested ferulic acid was excreted as such. Almost the same amount was metabolized to dihydroferulic acid, and the rest of ferulic acid was converted to homovanillic and vanillic acid. It is very likely that a part of malvidin glycosides were also metabolized to homovanillic and vanillic acids via demethoxylation. That conclusion was supported by the fact that the urinary excretion of direct cleavage products of malvidin, such as syringic or sinapinic acids, was low or nonexistent. The variation between the subjects (**Figure 2A**) in the excretion of vanillic acid at 12−24 h after the pureé + cereals supplementation was high due to one subject excreting only vanillic acid and no homovanillic acid at all. Even without that one subject, vanillic acid would have been among the three most abundant metabolites. Isoferulic acid has earlier been reported to be a metabolite of caffeic acid (*21, 22*), which was the possible metabolic pathway in our study too. However, isoferulic acid could be a metabolite of cyanidin glycosides after a cleavage of the C-ring and methylation of the fragment. Methylated anthocyanins were unlikely the parent compounds of isoferulic acid because several metabolic reactions, partly occurring in the colon, would have been needed before the fast excretion during the first follow-up hours. The berry purée + cereals did not contain any isoferulic acid, and therefore, it had to be a metabolite, although the excretion profile was similar to those observed for the dietary phenolic acids (**Figure 2B**).

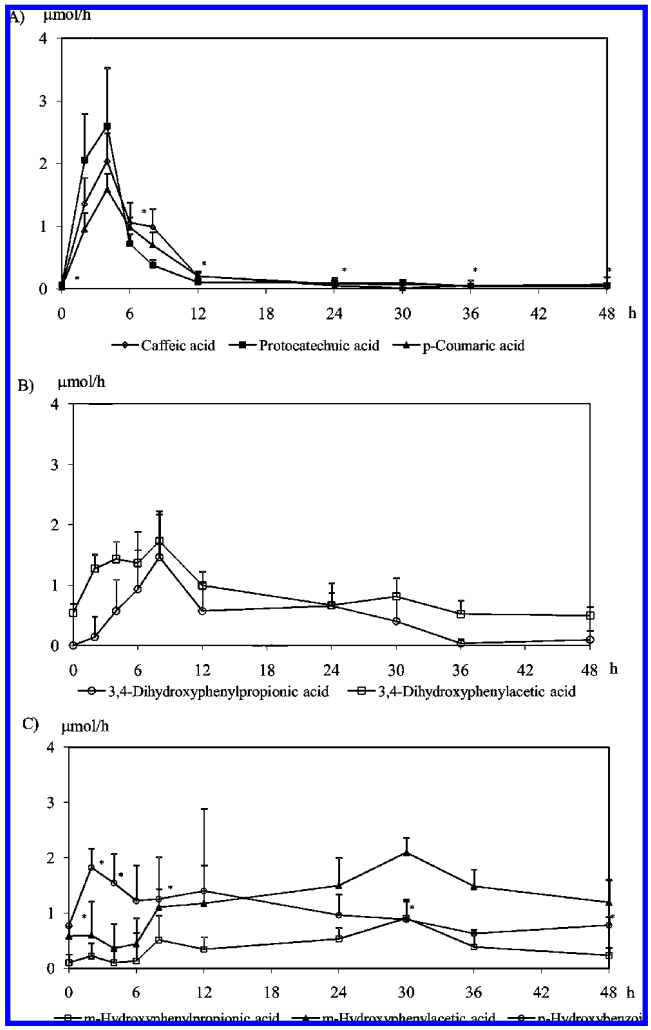

Figure 3. Urinary excretion of di- and monohydroxylated phenolic acids after purée + cereals. (**A**) Dietary phenolic acids. (**B**) Dihydroxy substituted phenolic acid metabolites. (**C**) Monohydroxy substituted phenolic acid metabolites. * Significantly different from the purée alone. See details from the results of hydroxylated phenolic acid.

Hydroxylated Dietary Phenolic Acids and *p*-Hydroxybenzoic Acid. In human subjects, protocatechuic acid has been shown to be a major metabolite of cyanidin glucosides in plasma (*16*), but it has not been detected in urine. Although protocatechuic acid was a possible metabolite of cyanidin glycosides, according to the urinary excretion profile in our study, protocatechuic acid was more likely ingested in the berry purée and then excreted like the other dietary phenolic acids (**Figure 3A**). In our study, approximately 29% of the ingested protocatechuic acid was traced; therefore, more than being a metabolite itself, protocatechuic acid was metabolized, for example, to vanillic or *p*-hydroxybenzoic acid. *p*-Hydroxybenzoic acid has been a major metabolite of rosmarinic acid (*23*), which indicates that caffeic acid, a fragment of rosmarinic acid, could be metabolized to *p*-hydroxybenzoic acid as well. After the single dose of oregano extract, *p*-hydroxybenzoic acid was excreted in 8 h (*23*). In the present study, the urinary excretion of *p*-hydroxybenzoic acid was still elevated after 30 h of follow-up, which indicated that it was produced from different anthocyanins and phenolic acids first in the small intestine and later in the colon. *p*-Dehydroxylation has been reported to be preferential (*36*), and therefore, *p*-hydroxylated phenolic acids have not been considered to be important metabolites of phenolic compounds. Continuous excretion of *p*-hydroxybenzoic acid in our study proved that some amount of *p*-hydroxylated metabolites was produced in the colon, or in other organs. The total increase in the excretion of *p*-hydroxybenzoic acid was small, and therefore, it was not the main metabolite of anthocyanins. In addition to anthocyanins, catechins (*37*) and protocatechuic (*38*), caffeic (*23*), and *p*-coumaric acids (*22*) were also possible parent molecules for *p*-hydroxybenzoic acid. All dietary phenolic acids had very similar excretion profiles and even the total increase in the excretion was approximately 10 μmol for each compound, although the ingested amounts varied from 29 (ferulic acid) to 123 (*p*-coumaric acid) μmol. According to these results, it was justified to divide phenolic acids to dietary compounds and metabolites. Oat cereals probably affected gastric emptying time, which was proven by the postponed peak excretion of dietary phenolic acids after the purée + cereals supplementation in our study. The absorption of phenolic compounds was not affected by the cereals because the excretion rates were equal after both supplementations. Cereals have been shown to affect the gastric emptying time (*39*, *40*), but the effects of viscous fiber have been small on the absorption of nutrients (*41*).

Hydroxyphenylpropionic and Acetic Acids. The simplest metabolic pathway for anthocyanins to produce 3,4-dihydroxyphenylpropionic and phenylacetic acids was the cleavage of the C-ring and oxidation of cyanidin glycosides. Another possible source of 3,4-dihydroxyphenylpropionic and acetic acid was caffeic acid. The hydrogenation of the caffeic acid double bond to produce 3,4-dihydroxyphenylpropionic acid as well as the decarboxylation and β-oxidation needed to convert caffeic acid to 3,4-dihydroxyphenylacetic acid occurs in the colon (*38*), meaning that 3,4-dihydroxyphenylpropionic and acetic acids produced from caffeic acid were excreted at the earliest at 8−12 h after the supplementation, when the excretion rates were already decreased. According to these excretion profiles, it was concluded that part of 3,4-dihydroxyphenylpropionic and acetic acids were produced from anthocyanins. The cleavage of the anthocyanin C-ring seemed to occur already in the small intestine in contrast to that reported, for example, for quercetin (*42*, *43*). Both 3,4-dihydroxyphenylpropionic and acetic acids were very likely metabolized further to *m*-hydroxyphenylpropionic and acetic acids, which were excreted at the highest rates after the excretion of 3,4-dihydroxyphenylpropionic and acetic acids began to decrease.

Other Phenolic Acids. The main anthocyanins in the purée were delphinidin glycosides, and the purée contained also gallic acid. Therefore, at least some amount of gallic acid was expected to be excreted in urine, but none was detected. Syringic acid was a minor metabolite excreted during the first 8 h of the follow up (**Figure 2B**), which indicated that it was produced in the small intestine, for example, from malvidin. However, it cannot be excluded that gallic acid present in the purée was partly methylated to syringic acid, as has been suggested before (*23*). Sinapinic acid, which was present in the oat cereals, could be another parent molecule for syringic acid after a β-oxidation of the acid chain. That usually occurs in the colon (*38*), but the excretion of syringic acid was higher at 2−4 h after the purée + cereals supplementation, which was before a colonic metabolite could be detected in urine. Therefore malvidin glycosides and gallic acid seemed to be the most probable sources of syringic acid. Because syringic acid was not present in the study meal, it must be a metabolite, although the urinary excretion profile showed the behavior of a dietary compound.

Strengths and Weaknesses of the Study. Strengths of this study were the extensively restricted diet, long follow-up time,

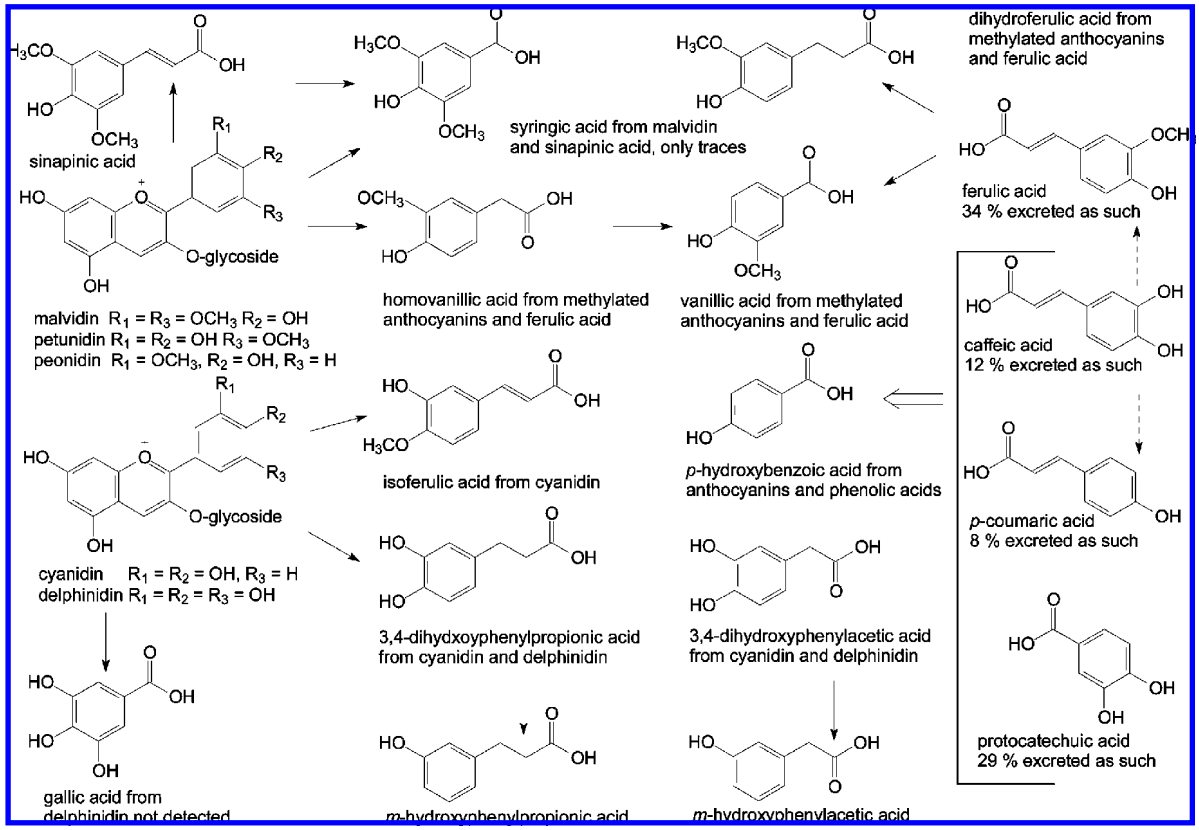

Figure 4. Metabolites of anthocyanins and phenolic acids in humans after purée + cereals.

frequent sampling combined with the total urine collection, and double supplementation. The extensively restricted diet guaranteed that all of the observed changes were due to the phenolic compounds present in the study meals. The remarkable baseline urinary excretion of phenolic acids revealed that it was not possible to completely eliminate their excretion by dietary restriction. Therefore, a baseline excretion always needs to be measured if the metabolism of phenolic acids is studied. The 48-h follow-up time was shown to be sufficient to study the fragmentation of anthocyanins and phenolic acid metabolism because the excretion of all phenolic acids, except *m*-hydroxyphenylacetic acid, was returned to the baseline value or close to that at the end of the follow-up time. The frequent sampling and total urine collection enabled us to compare the amount of metabolites to the ingested amount of phenolic compounds. Double supplementation showed that the metabolism of administered phenolic compounds was repeatable. That study design also revealed that the interpretation of metabolism was very sensitive to disturbances caused by dietary intake. The baseline urinary excretion of phenolic acids doubled prior to the purée supplementation when the study subjects made two exceptions to the restricted diet. The observed difference was due to the increased excretion of vanillic, protocatechuic, and *p*-hydroxybenzoic acids. Vanillic aldehyde obtained from a vanilla flavored protein drink was oxidized to vanillic acid and caused a five times higher excretion in one subject in contrast to that observed after the purée + cereals supplementation. The urinary excretion of protocatechuic and *p*-hydroxybenzoic acid increased because of the coffee that one subject consumed prior to the purée supplementation. Because of the excessive urinary excretion of a few metabolites, the detailed metabolism of phenolic acids was presented from the study with purée + cereals supplementation.

Because fragmentation of anthocyanins to phenolic acids was not as complete as was expected, one drawback of the study was that fecal samples were not collected, and therefore, defecated anthocyanin and phenolic acid fractions remained unknown. In one animal study, roughly estimated fecal output of anthocyanins after berry extract diets ranged from 10 to 25% of the ingested amount (*44*), but in vitro anthocyanins have completely decomposed in 2 h during fecal fermentation (*11*). The fecal excretion of phenolic acids have been shown to be the highest for phenylpropionic, phenylacetic, and benzoic acids (*45*), and high also for *m*-hydroxyphenylpropionic acid (*45*, *46*), which was one of the metabolites in our study. In general, the reported concentrations of phenolic acids in feces have been so high that definitely part of the metabolites in our study was defecated.

Conclusions. In our study, we measured for the first time a wide variety of different phenolic acids in human urine to determine the fragmentation of anthocyanins to phenolic acids in human. Although 18 different phenolic acids were determined, the urinary excretion increased less than what was the ingested amount of phenolic acids alone. The most abundant metabolites were methylated phenolic acids, which were partly produced from anthocyanins. A postponed peak urinary excretion of dietary phenolic acids was observed after the purée + cereals supplementation, in contrast to the purée alone, but the excretion of metabolites had similar profiles during the first follow-up hours. Although fragmentation of anthocyanins to phenolic acids might occur to some extent, still a large part of the anthocyanin metabolites remained unknown.

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