Molecular Nutrition & Food Research
www.mnf-journal.com

Phenolic Metabolites in the Urine and Plasma of Healthy Men After Acute Intake of Purple Potato Extract Rich in Methoxysubstituted Monoacylated Anthocyanins

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Scope: Structurally stable acylated anthocyanins have potential in various food applications but the effects of acylation and methoxysubstitution on anthocyanin metabolism are poorly understood. This is the first study thoroughly investigating phenolic metabolites, their time-wise changes, and pharmacokinetics following an acute intake of methoxysubstituted monoacylated anthocyanins.

Methods and Results: Healthy male volunteers ($n = 17$) consumed a yellow potato meal with and without purple potato extract rich in acylated anthocyanins (152 mg) and hydroxycinnamic acid conjugates (140 mg). Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) is used for identification and quantification of metabolites from serially collected urine and plasma. While the parent anthocyanins are not detected, 28 phenolic metabolites from urine and 14 from plasma are quantified, including hydroxybenzoic and hydroxycinnamic acids and protocatechuic acid sulfates and glucuronides; three (catechol, gallic acid-4-*O*-glucuronide, and 2-methoxybenzoic acid) are detected for the first time after anthocyanin-rich food. Urinary hippuric acid is the most abundant with an increase of 139 μM mM^{-1} creatinine after the treatment. A large additional set of tentatively identified phenolic metabolites are detected. Late urinary peak time values suggest colonic degradation.

Conclusion: Acylated anthocyanins are more bioavailable than earlier reported after extensive degradation in human and/or colonial metabolism to phenolic metabolites, which may be further conjugated and demethylated.

1. Introduction

Anthocyanins, the red-purple colorants of berries and fruits, are hypoglycemic and decrease the risk of type II diabetes.[1,2] While the health properties of dietary components are typically linked to bioavailability, the bioavailability of anthocyanins is extremely low and affected by their vast structural diversity. For example, the urinary recovery of pure ^{13}C-labeled cyanidin-3-*O*-glucoside was less than 2%,[3] and 0.03% for bilberry anthocyanins.[4] Acylation of anthocyanins, i.e., linking one or more organic acids to the glycosyl moiety, decreases the bioavailability even further. The mainly diacylated cyanidin diglucosides of red cabbage are recovered four times less, whereas the monoacylated cyanidin xylosides of purple carrots are recovered 14 times less than the nonacylated anthocyanins of these foods.[5,6] Also, acylated anthocyanins are acutely hypoglycemic as detected with both cooked purple potatoes[7] and their extract[8] (99% acylated anthocyanins). Therefore, the health effects of anthocyanins may be mediated by their more bioavailable metabolites.[9,10]

Acylated anthocyanins have more potential for food industrial applications than nonacylated ones due to their enhanced structural stability[11] and they are common in everyday diets as part of pigmented vegetables and tubers.[5,6,8,12,13] However, their degradation in humans has not been studied extensively previously. Earlier clinical trials investigating the metabolites of anthocyanins have focused mainly on the pure common nonacylated anthocyanin glycoside, cyanidin-3-*O*-glucoside,[3,14] foods rich in nonacylated anthocyanins, such as various berries,[4,15–23] and to some extent on foods with a mix of nonacylated and acylated anthocyanins, such as grapes.[24,25] One study demonstrated that an acute intake of purple potatoes rich in acylated anthocyanins leads to the elevation of the total concentration of phenolics in biofluids of healthy volunteers ($n = 5$). Only a few of the phenolics were tentatively identified due to the lack of standard compounds, and the study did not include

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DOI: 10.1002/mnfr.202000898

a control arm.[26] Altogether, these papers suggest that in addition to possible minor absorption, nonacylated anthocyanins undergo fission of the heterocyclic C-ring leading to phenolic metabolites, such as hydroxybenzoic acids, phenyl acetic acids, phenyl propionic acids, hydroxycinnamic acids, phenyl alcohols, phenyl aldehydes, and hippuric acids. After entering enterohepatic circulation, the metabolites may be further conjugated in the phase II metabolism. The in vivo degradation of acylated anthocyanins, however, is still not understood.

The aim of this study was to investigate the postprandial structural changes of the ingested purple potato extract (PPE) from *Solanum tuberosum* L. 'Synkeä Sakari' rich in methoxysubstituted anthocyanin glucosides with a rutinosyl moiety acylated to a caffeic, coumaric, or ferulic acid as our previous clinical trials gave evidence that the potatoes and their extract decrease postprandial glycemia and insulinemia.[7,8] Here, the phenolic metabolites were determined from the collected urine and plasma samples to clarify the molecular structures potentially affecting the observed health effects. The acylated anthocyanins were served as an extract free from potato matrix instead of comparing two potato varieties in order to remove the effect of compositional differences in phenolic compounds, starch, and monosaccharides on the postprandial state and metabolites. According to our knowledge, this is the first study to screen, identify, and quantify the phenolic metabolites of acylated anthocyanins from serially collected urine and plasma samples of humans.

2. Experimental Section

2.1. Solvents

For solid-phase extraction, formic acid (reagent grade, ≥95.0%) and methanol (HPLC grade, ≥99.8%) were obtained from Sigma-Aldrich (St Louis, MO). For LC-MS analyses, LC-MS grade acetonitrile (Thermo Scientific, Rockford, IL, for anthocyanins and CHROMASOLV, Sigma Aldrich, St Louis, MO, for other phenolics), MS-grade formic acid (98%, Honeywell, Muskegon, MI), and UPLC/MS-grade glacial acetic acid (Biosolve B.V., Dieuze, France) were purchased. Ammonium acetate for HPLC buffer preparation (HPLC-grade, ≥99.0%) was obtained from Sigma-Aldrich (St Louis, MO). Water used was Milli-Q grade (Millipore, Burlington, MA).

2.2. Standard Compounds

Anthocyanin standards (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, pelargonidin-3-*O*-glucoside, pelargonidin-3-*O*-rutinoside, and malvidin-3-*O*-glucoside) were purchased from Extrasynthese (Genay, France). Caffeine-(trimethyl-d_9) (99 atom-% D, later caffeine-d_9), catechol, 4-hydroxybenzaldehyde, 3-*O*-methyl-gallate, methyl vanillate, phloroglucinol, phloroglucinaldehyde, pyrogallol, quercetin, quercetin-3-*O*-rutinoside, protocatechuic aldehyde, taxifolin, as well as caffeic, chlorogenic, ferulic, gallic, hippuric, homoprotocatechuic, homovanillic, 3-hydroxybenzoic, 4-hydroxybenzoic, 5-hydroxyferulic, 4-hydroxyphenylacetic, isovanillic, 2-methoxybenzoic, 3-methyl hippuric, 4-methyl hippuric, *p*-coumaric, protocatechuic, sinapic, syringic, *trans*-cinnamic, and vanillic acids from Sigma-Aldrich (St Louis, MO). Methyl-3,4-dihydroxybenzoate was purchased from Alfa Aesar, Haverhill, MA. Gallic acid-3-*O*-glucuronide, gallic acid-4-*O*-glucuronide, isoferulic acid-3-*O*-glucuronide, protocatechuic acid-3-*O*-glucuronide, protocatechuic acid-4-*O*-glucuronide, protocatechuic acid-3-*O*-sulfate, and protocatechuic acid-4-*O*-sulfate were kindly synthesized by Dr Paul Needs at The Quadram Institute Biosciences, Norwich, UK.

2.3. Ethics

The study protocol was accepted by the Ethical Committee of the Hospital District of Southwest Finland (ETMK:93/1801:2016) and registered at clinicaltrials.gov as NCT02940080. Each study subject provided written informed consent.

2.4. Study Design

Seventeen healthy men aged between 18 and 45 years consumed 350 g of steam-cooked yellow potato mash with (study meal) and without (control meal) PPE solution rich in acylated anthocyanins as a breakfast after a 12-h fast in a cross-over, single-blinded, and randomized study described previously. The PPE and yellow potato mash were carefully characterized for nutrient composition, sugars, organic acids, anthocyanins, and other phenolic compounds.[8] For 48 h before and 24 h after consuming a test meal, the participants followed a study diet composed of foods and drinks low in flavonoids and dietary fiber. Total voids of urine were collected at fasting state, then pooled at 0–4, 4–8, 8–12, and 12–24 h postprandially. Urine samples (154 in total) were stored as aliquots at −80 °C containing 1/0.2 V/V trifluoroacetic acid (0.44 M). Venous blood samples (268 in total) were collected into lithium-heparin tubes at fasting state, and then 20, 40, 60, 90, 120, 180, and 240 min postprandially. Blood samples were centrifuged for 15 min, 1500 × *g*, to collect plasma, and then acidified with 1/0.2 V/V trifluoroacetic acid (0.44 M) and stored at −80 °C until analyses.

2.5. Dosage Information

The PPE was extracted from purple potatoes (*Solanum tuberosum* L. 'Synkeä Sakari') as described previously using aqueous ethanol acidified with acetic acid and purified with Amberlite XAD-7HP adsorbent.[27] The PPE was used instead of potatoes as such to remove the effect of the compositional differences of potato varieties. The PPE was carefully characterized earlier,[8] providing 152 mg of anthocyanins and 140 mg hydroxycinnamic acid conjugates per meal corresponding to 0.48 kg of fresh purple potatoes. The methoxysubstituted petunidin and peonidin derivatives dominated with minor amounts of cyanidin, malvidin, delphinidin, and pelargonidin. The anthocyanidins were linked to a glucose and a rutinose (a disaccharide of a rhamnose and a glucose), and 99% of the anthocyanins were monoacylated with a caffeic, ferulic, or coumaric acid. Petunidin-coumaroyl-rutinoside-glucoside represented 60% of the anthocyanins (**Figure 1**). The hydroxycinnamic acid derivatives in the PPE were chlorogenic acid (84%), caffeic acid (6%), and cryptochlorogenic acid (3%). Both meals contained 0.7 mg of flavonol glycosides and 4.5 mg of hydroxycinnamic acids from the yellow potatoes.[8] The amount of anthocyanins was based on the previous clinical trial.[7]

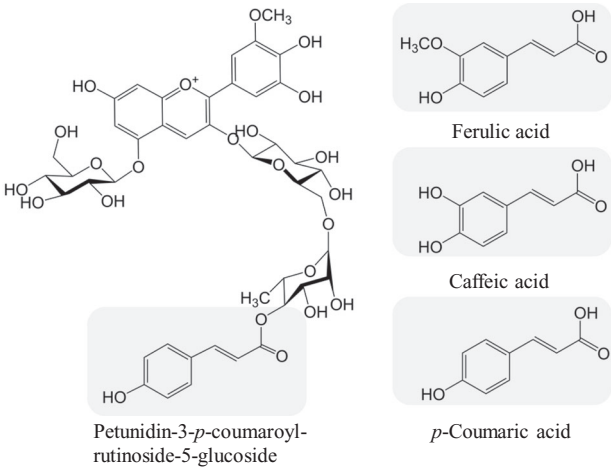

Figure 1. The major anthocyanin of purple potatoes, petunidin-coumaroyl-rutinoside-glucoside, and the acyl groups of purple potato anthocyanins: caffeic, *p*-coumaric, and ferulic acids.[8] The suggested positions of the acyl and glycosyl groups and the isomer of the coumaric acid are based on a previous nuclear magnetic resonance spectroscopy (NMR) characterization study.[48]

2.6. Sample Randomization

The samples of each volunteer were processed and analyzed as a set of 10 (urine) and 16 (plasma) samples. Within these sample sets, the samples were paired according to the sampling time point with randomized order of the study visit. The order of these pairs was randomized within the set, and the order of the sample sets of the volunteers were randomized. The *randperm* function in Matlab (The Mathworks, Cambridge, UK) was used with the default setting of Mersenne Twister.

2.7. Solid-phase Extraction

The modified extraction method[28] with the chosen StrataX (6 mL, 500 mg) solid-phase cartridges was verified by comparing the analytical responses of matrix-matched blank samples spiked with selected compounds before and after solid-phase extraction (extraction recoveries, Supporting Information S1 and S2). Samples were extracted in a room with yellow light to protect the light-sensitive anthocyanins. The SPE cartridges were activated and stabilized using one load volume of 0.25% formic acid in methanol followed by a load volume of 0.1% formic acid in water. Thawed samples were diluted 2/1 with 0.1% formic acid and spiked with 10 µL of 400 mM a mid-polar and mid-range responder, taxifolin, as an internal SPE process control standard. The cartridges were washed twice with a load volume of 2% methanol in 0.1% formic acid for urine, and 0.1% formic acid for plasma. The cartridges were dried under vacuum for 30 min. Samples were eluted with 5 mL 0.25% formic acid in methanol after 10 min of soaking, followed by an additional 2 mL rinse.

The samples were evaporated to a volume of about 200 µL and reconstituted with 200 µL of 0.1% formic acid. As an internal volume control standard, 10 µL of caffeine-d_9 (200 mM) was added. The samples were shaken after which urine samples were spun for 10 s and plasma samples centrifuged 10 000 × *g* for 10 min at 4 °C. For anthocyanin analyses, 200 µL of the supernatant was acidified with 10 µL of strong formic acid. All samples were stored in −80 °C. Prior to analyses, the samples were thawed, shaken carefully, and centrifuged for 10 000 × *g* for 5 min at 4 °C. Matrix-matched positive (urine) and standard-spiked (urine, plasma) quality control samples were processed daily along with the analytical samples.

2.8. UHPLC-MS/MS Analyses

Phenolic metabolites and anthocyanins were analyzed using 1290 Infinity UHPLC combined with 6490 Triple Quad mass spectrometer equipped with iFunnel (Agilent Technologies, Santa Clara, CA). The analytes were separated in an ACQUITY UPLC HSS T3 1.8 µm (2.1 × 100 mm, Waters, Milford, MA) column at 35 °C. The injection volume was 5 µL for urine and 1 µL for plasma. The sample rack was cooled to 4 °C. Identification and quantification of the phenolic metabolites and anthocyanins were conducted with comprehensive, targeted multiple reaction monitoring (MRM) panels (Supporting Information S1, S2), which contained the degradants, metabolites and conjugates predicted by the research group and presented in the current literature for nonacylated anthocyanins. Authentic standard compounds (Chapter 2.2) were used in identification whenever available.

For anthocyanins, the gradient consisted of 5% formic acid in water (A) and 5% formic acid in acetonitrile (B) at 0.4 mL min^{-1} followingly: 0–1 min, 5% B; 5 min, 10% B; 30 min, 25% B; 31 min, 95% B; 32 min, 95% B; 32.1 min, 5; % B and 36 min, 5% B. Collision gas temperature was 220 °C, gas flow 14 L min^{-1}, nebulizer pressure 25 psi, capillary voltage 3.5 kV and collision energy 5 eV for phosphatidyl choline and anthocyanidins, and 28 eV for anthocyanins. The retention times for the purple potato anthocyanins were verified using urine spiked with the PPE. For phenolic metabolites, the mobile phase A was 10 mM ammonium acetate in water (pH 5) and B was 10 mM ammonium acetate in acetonitrile (pH 5). The flow rate was 0.4 mL min^{-1}. The concentration of eluent B was 1% for a minute, then at 3 min, 5%; 8 min, 60%; 8.50 min, 99% and 9–12 min, 1%. MRM was used in negative mode except for caffeine-d_9 and phosphatidyl choline. Collision gas temperature was 220 °C, gas flow 14 L h^{-1}, nebulizer 25 psi, and capillary voltage 3500 V. Collision energies were optimized for each compound available. The performance of the mass spectrometer was monitored by analyzing repeatedly a matrix-matched sample containing all available standard compounds.

The data was processed using MassHunter Quantitative Analysis B.06.00 (Agilent Technologies, Santa Clara, CA, USA). The sample volumes were corrected on the basis of the caffeine-d_9 levels in each sample. Then, the collected data was divided into two parts: the quantified metabolite data set, of which identification of the compounds was confirmed using the available standard compounds, and the screened metabolite data set, which was tentatively identified based on the MRM transitions and chromatographic retention. Quantification was performed using matrix-matched external standard curves, and endogenously present target metabolites were subtracted. All quantified metabolites were within the linear range of the standard curves. If a metabolite was detected in the samples of over half of the study volunteers (nine or more), it was interpreted as detected, and further quantified.

2.9. Urinary Creatinine

Urinary creatinine was analyzed in the laboratory of the Hospital District of Southwest Finland using enzymatic methods (Cobas C702 automatic analyzer, Roche Diagnostics GmbH, Mannheim, Germany).

2.10. Pharmacokinetic and Statistical Analyses

For the separate time points of the quantified data set, statistical analyses were performed using R 3.5.1.[29] For normally distributed data (Shapiro–Wilk test), the paired-samples *t*-test was used to compare the differences in the metabolite concentrations between the plasma and urine samples at certain time points after the control and the study meal. The nonparametric counterpart, the Wilcoxon signed rank test, was used otherwise. The significance level was set to 0.05. Additionally, the pharmacokinetic incremental area under the curve (iAUC) values were calculated using the trapezoidal rule, and maximum concentration (C_{max}) and time point at the maximum concentration (t_{max}) were determined as the mean of the maximal values of the individual volunteers. For urine, t_{max} was determined on the basis of the most frequent categorical time period variable.

The screened and tentatively identified data set was visualized as heatmaps using R 3.5.1[29] with gplots version 3.0.1.1[30] and RColorBrewer version 1.1.2.[31] The differences between the volume corrected metabolite areas between the study and control meals were calculated resulting in a variable describing change between the meals in certain time point or period (delta). Due to the large differences in the areas of the screened metabolites, the fasting state value of the delta variable was set to zero by subtracting it from the postprandial values. All compounds were normalized to range [−1, 1] by dividing with the absolute maximum delta of the corresponding compound. The time-wise change of the delta variable of the compounds regarding to the fasting state can be observed, but the change is not comparable between the compounds.

3. Results and Discussion

3.1. Anthocyanins

In urine, five anthocyanin degradants were detected: cyanidin-3-*O*-glucoside, as confirmed with the standard compound, and tentatively identified, malvidin-rutinoside, petunidin-glucoside, peonidin-glucoside, and peonidin-glucuronide (aglycones, **Figure 2**; heatmap **Figure 3**A). The anthocyanin degradants peaked at 8–12 h, and were present in urine samples until 24 h, suggesting enterohepatic circulation of the degradants. These results indicate that the monoacyl group and rutinose are hydrolyzed. Detection of cyanidin-3-*O*-glucoside refers to the high bioavailability of the minor cyanidin-based anthocyanins present in potato or *O*-demethylation of the methoxysubstituted peonidin to cyanidin. Detecting peonidin-glucuronide, a conjugate, indicates phase II metabolism. A subset of plasma samples was analyzed for anthocyanins, but none were detected.

This is the first clinical study to detect degradants of monoacylated anthocyanins of purple potatoes in human urine;[26] fragments of cyanidin-derived mono- and diacylated anthocyanins of red cabbage have been detected in earlier clinical trials.[5,32] The nonacylated monoglycosylated anthocyanins are deglycosylated after ingestion and extensively glucuronidated and methylated, and also sulfated in minor amounts,[22,28] leading to 91% of the total anthocyanins appearing as conjugates of aglycones in urine.[22] On the contrary, our results show that the absorbed potato acylated anthocyanins seem to be resistant for deglycosylation. Nonacylated anthocyanin conjugates have been detected after a meal of red cabbage rich in acylated anthocyanins,[5,32] but according to our knowledge, this is the first time glucuronidation is reported for acylated anthocyanins derived from purple potatoes.[26]

Acylation decreases the bioavailability of anthocyanins[5,6] possibly due to the steric hindrance of several glycosyl and acyl moieties against the gastric absorption mechanisms as detected in vitro.[33,34] Selective structure-dependent absorption of anthocyanins has been suggested,[12] but understanding the dependence requires more investigations as contradictory results have been presented. Previous clinical interventions which fed purple potatoes[26] and Concord grape juice[24] (38% of total anthocyanins were monoacylated, mainly delphinidin, petunidin, and malvidin diglucosides) to healthy volunteers either did not detect acylated anthocyanins[24,26] or any anthocyanins at all[26] in human biofluids. Other clinical interventions have detected selected, structurally different (B-ring, acylation pattern, sugar moiety) acylated anthocyanins of red cabbage (mono- and diacylated cyanidin diglucosides),[5,32] purple carrot (monoacylated cyanidin xylosides),[6] and purple sweet potatoes (diacylated cyanidin and peonidin sophorosides)[12,13] in human plasma and/or urine, and in addition, of eggplant (monoacylated delphinidin rutinosides) in rats.[35] As our methodology was optimized with nonacylated anthocyanins and the level of acylated anthocyanins in biological fluids is low due to their extremely poor bioavailability, more studies are needed to confirm our results.

The B-ring substitution and sugar moieties of the ingested anthocyanins affect the bioavailability in vivo.[4,22,36] In humans, increased hydroxylation of B-ring (delphinidin, cyanidin) may increase the bioavailability of nonacylated anthocyanins[36] but it should be noted that anthocyanidins are readily interconverted to each other in phase I and II reactions via demethylation, methylation, hydroxylation, and dihydroxylation reactions in vivo. For example, the methylation of nonacylated cyanidin to peonidin has been well-recognized,[14,22,35] whereas malvidin may be demethylated to petunidin.[22] The anthocyanidin may also affect the degree of enzymatic conjugation; for malvidin, it may be decreased but for the slightly smaller petunidin, the conjugation is similar compared to cyanidin glycosides.[22] In future, studies feeding pure labeled anthocyanins are ideal for further structural comparisons.

3.2. Quantified Phenolic Metabolites

From urine, 28 phenolic metabolites were identified and quantified (Figure 2, **Figure 4**A, **Table 1**, Supporting Information S3, S5). The mean maximum concentrations (C_{max}) varied from 2 nM mM^{-1} creatinine of quercetin to 290 μM mM^{-1} creatinine of hippuric acid, and the t_{max} values of the detected metabolites were late (4–8, 8–12 h). Of the urinary metabolites, 19 were

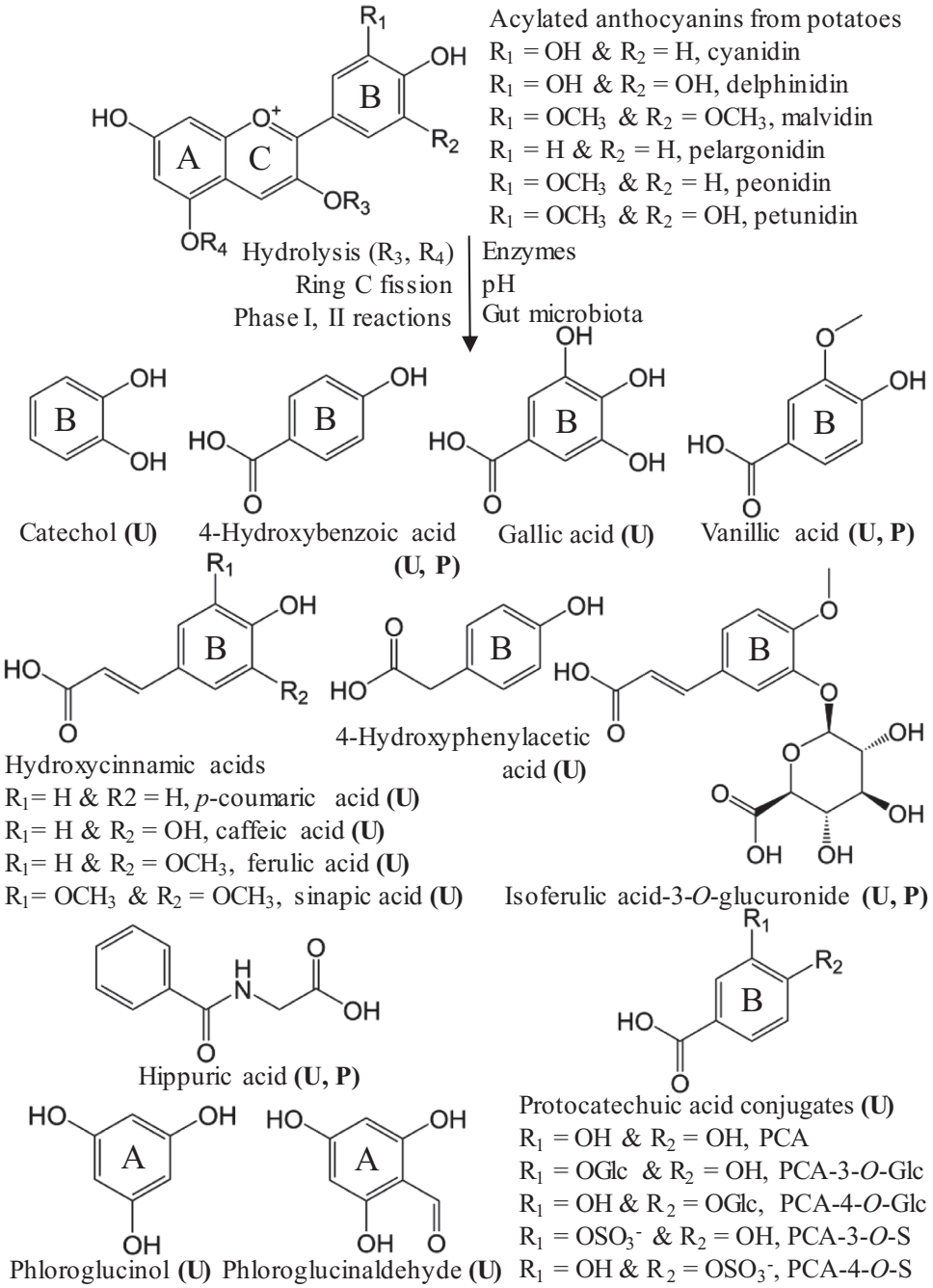

Figure 2. Identified phenolic metabolites detected in urine (U) and plasma (P) after a meal of purple potato extract rich in acylated anthocyanins and their suggested origin. PCA, protocatechuic acid; glc, glucuronide; s, sulfate. R_3 and R_4 may carry a glycoside of which R_3 may be acylated with a hydroxycinnamic acid. The values are presented in the Supporting Information S3 and S4.

elevated statistically significantly after the study meal compared to the control meal. Hippuric acid reached an increase of 139 µM mM^{-1} creatinine from the control levels after the study meal (p = 0.026 and 0.006 at 4–8 and 8–12 h). Monomethoxysubstituted vanillic acid (p = 0.002 and 0.001 at 0–4 and 4–8 h, respectively) and dimethoxysubstituted sinapic acid (p = 0.000 at 4–8 h) were detected. Other elevated abundant metabolites were hydroxycinnamic acids, including caffeic acid (p = 0.006 and 0.003 at 4–8 and 8–12 h), p-coumaric acid (p = 0.014 at 4–8 h), and ferulic acid (p = 0.041, <0.001, and 0.010 at 0–4, 4–8, and 8–12 h). Dihydroxysubstituted protocatechuic acid was increased (p = 0.003, 0.003, and 0.001 at 0–4, 4–8, and 8–12 h, respectively). Furthermore, its 3- and 4-sulfates and glucuronides were detected, of which protocatechuic acid-4-O-glucuronide, -3-O-sulfate and -4-O-sulfate were increased (p = 0.008, 0.017, 0.025 at 0–4 , 4–8, and 8–12 h; p = 0.020, 0.001, and 0.010 at 0–4,

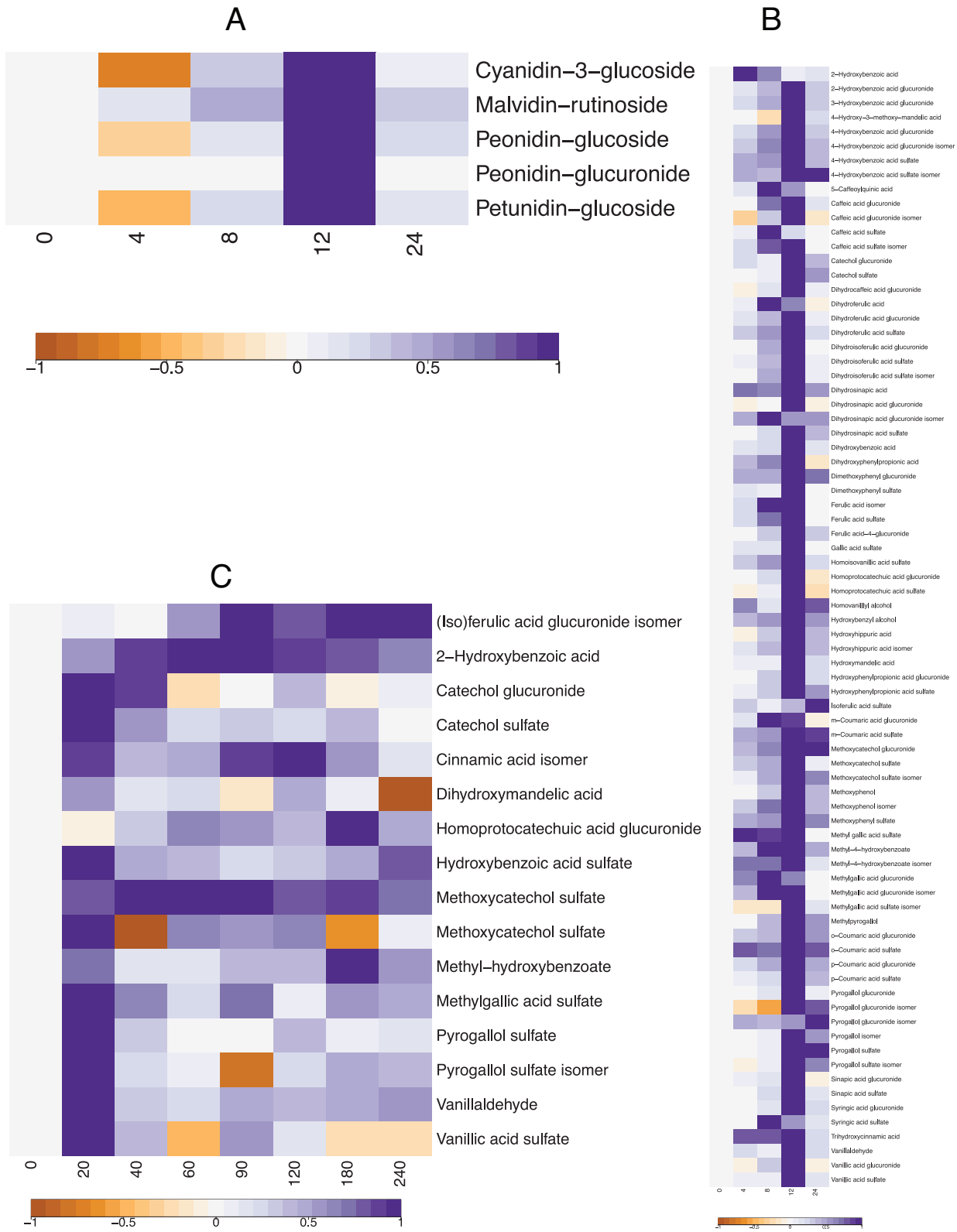

Figure 3. Screened A) anthocyanins in urine, B) phenolic metabolites in urine, and C) phenolic metabolites in plasma of 17 healthy men after a potato meal with (study meal) or without (control meal) anthocyanin-rich purple potato extract. Tentative identifications are based on the MRM transition and chromatographic retention. The heatmaps describe change between the samples collected after the study and control meal at certain time points standardized to the range of [−1, 1]. Purple color indicates that larger amount of the metabolites were detected after the study meal, whereas orange indicates the opposite.

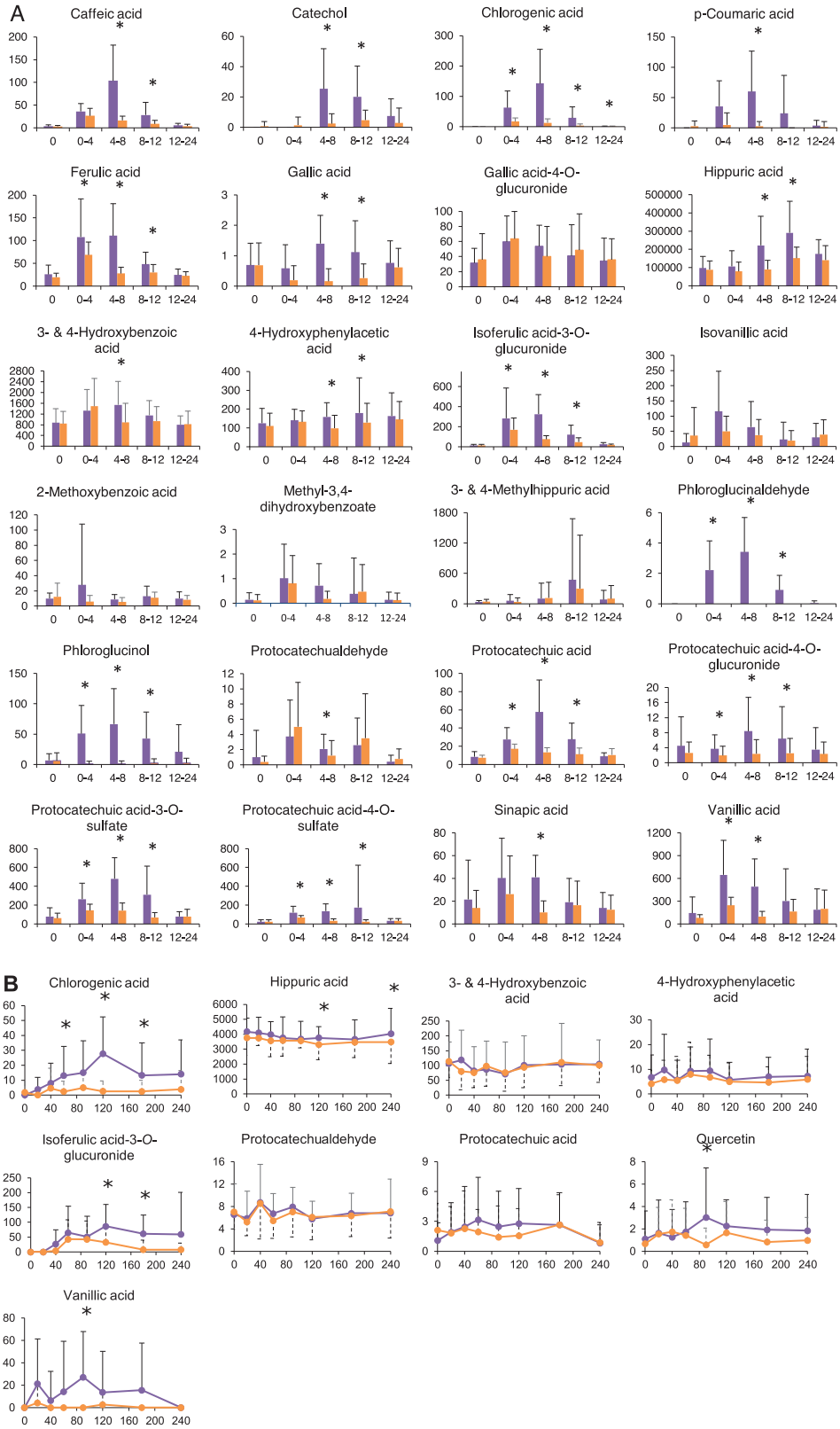

Figure 4. The phenolic metabolites identified and quantified in A) urine and B) plasma of 17 healthy men using corresponding standard compounds. Values are given as concentrations (nM mM^{-1} creatinine, urine and nM, plasma) with standard deviations in the fasting state (0-point) and postprandially (Figure A, h; Figure B, min). The purple plots represent the treatment values whereas the orange plots represent the control values. The between-meal significant differences ($p < 0.05$) are shown with asterisks. Supporting information S5 and S6 contain the *p*-values.

Table 1. The urinary pharmacokinetic parameters of the phenolic metabolites after an acute intake of a purple potato extract rich in methoxysubstituted, monoacylated anthocyanins.

	n	C_{max} [nM mM^{-1} creatinine]	SD	t_{max} [h]	Detected earlier in
Caffeic acid	17	93.6	71.0	4–8	[16,17,19,21]
Catechol	13	41.7	17.4	4–8	
Chlorogenic acid[a]	17	131.8	99.9	4–8	[19]
p-Coumaric acid	14	85.0	73.9	8–12	[16–19,21,24,28]
Ferulic acid	17	119.9	67.5	4–8	[3,14–18,21,23,28]
Gallic acid	14	1.8	0.8	4–8	[17,21]
Gallic acid-4-*O*-glucuronide	17	68.6	40.4	0–4	
Hippuric acid	17	288 692.8	166 291.8	8–12	[3,15,17,19,21,23,26,28]
Homoprotocatechuic acid[b]	14	26.6	19.6	4–8	[3,14,16–19,21,23,28]
Homovanillic acid[c]	17	538.1	437.5	4–8	[16–19,21,23,25,28]
4-Hydroxybenzaldehyde	17	200.0	212.6	8–12	[3,17,19,21]
3- & 4-Hydroxybenzoic acid	17	1791.7	840.4	4–8	[3,16–19,21,23,28]
4-Hydoxyphenylacetic acid[d]	17	266.3	170.4	0–4	[3,14,17,19,21]
Isoferulic acid-3-*O*-glucuronide	17	345.6	255.8	4–8	[17,19,23,24]
Isovanillic acid	15	140.2	117.5	0–4	[3,14,17,19,21]
2-Methoxybenzoic acid[e]	16	34.7	73.5	8–12	
Methyl-3,4-dihydroxybenzoate	12	1.7	1.9	4–8	[3,14,21]
3- & 4-Methylhippuric acid	14	560.4	1273.3	8–12	[28]
Phloroglucinaldehyde	16	3.5	1.9	4–8	[3,18,28]
Phloroglucinol	15	86.4	49.9	0–4	[21]
Protocatechualdehyde	17	5.9	5.0	8–12	[3,19,21]
Protocatechuic acid	17	53.8	32.3	4–8	[3,14–19,21,23]
Protocatechuic acid-4-*O*-glucuronide	15	8.8	9.1	4–8	[3,14,18,28]
Protocatechuic acid-3-*O*-sulfate	17	482.3	250.6	4–8	[3,14,18,21,28]
Protocatechuic acid-4-*O*-sulfate	17	239.4	418.3	0–4	[3,14,18,21,28]
Quercetin[a]	17	1.5	1.9	4–8	[17,19]
Sinapic acid	17	53.7	36.1	4–8	[17–19,28]
Vanillic acid	17	676.7	412.9	0–4	[14,16–19,21,23,25,28]

The values are presented as mean with standard deviations (SD) detected in *n* study participants with the exception of the categorical t_{max}, which is presented on the basis of the highest frequency. C_{max}, the mean of the maximum metabolite concentrations of the volunteers; t_{max} was determined on the basis of the highest frequency of the categorical time period variable; [a] Chlorogenic acid and quercetin are not likely anthocyanin metabolites but are derived from the meals; Quantified with [b]protocatechuic acid; [c]vanillic acid; [d]caffeic acid; [e]gallic acid.

4–8, and 8–12 h; $p = 0.003$, 0.002, and <0.001 at 0–4, 4–8, and 8–12 h, respectively). Protocatechuic acid-3-*O*-glucuronide was detected but not quantified due to co-elution.

Of the 14 phenolic metabolites detected and quantified from plasma (Figure 2, Figure 4B, **Table 2**, Supporting Information S4 and S6), five were statistically significantly elevated after the study meal compared to the control meal. Chlorogenic acid was increased at 60, 120, and 180 min postprandially ($p = 0.024$, <0.001, and 0.022, respectively), hippuric acid at 0, 120, and 240 min ($p = 0.039$, 0.001, 0.038), isoferulic acid-3-*O*-glucuronide at 120 and 180 min ($p = 0.018$ and 0.013), quercetin at 90 min ($p = 0.038$), and vanillic acid at 90 min ($p = 0.036$). The C_{max} ranged between 2 nM of methyl-3,4-dihydroxybenzoate and 4700 nM of hippuric acid, whereas most of the t_{max} values were between 1 and 2 h. Plasma contained fewer metabolites compared to urine, to which the kidneys concentrate metabolites for excretion. Late-rise metabolites, however, were not detected from plasma due to the 4-h postprandial sampling.

These results show that the acylated anthocyanins of purple potatoes are degraded into phenolic metabolites in humans. *O*-Demethylation of the metabolites may occur as the potato anthocyanins are mainly methoxysubstituted, but dihydroxylated catechol and protocatechuic acid and its conjugates were abundant in urine. Detecting conjugated phenolic acids indicates phase II metabolism as suggested in studies regarding berry anthocyanins.[17–19,28] Hippuric acid, the major metabolite of potato anthocyanins, is another phase II metabolite formed by the liver cells from benzoic acid and glycine, and is a recognized biomarker for consumption of polyphenol-rich berries and fruits.[37,38] However, the levels of hippuric acid are affected also by endogenous, such as protein, metabolism. Both urine and plasma contained *p*-coumaric acid, and in urine, also ferulic acid and caffeic acid were abundant; they might be originated from the A- and B-rings and the acyl groups of the parent anthocyanins. *p*-Coumaric acid and caffeic acid were both also present in minor amounts in the ingested PPE, and

Table 2. The plasma pharmacokinetic parameters of the phenolic metabolites after an acute intake of a purple potato extract rich in methoxysubstituted, monoacylated anthocyanins.

	n	C_{max} [nM]	SD	t_{max} [h]	SD	$iAUC_{0\text{-}4}$ [nM*h L^{-1}]	SD	Detected earlier in
Caffeic acid	17	27.1	18.9	1.7	1.2	14.4	12.3	[17,19,24]
Chlorogenic acid[a]	16	36.4	24.0	2.5	0.8	59.9	53.9	[17,19]
Hippuric acid	17	4649.2	1293.3	1.7	1.6	370.6	611.6	[3,15,17,19,21,28]
4-Hydroxybenzaldehyde	17	264.2	122.7	1.2	1.0	40.9	42.8	[3,17–19,21,28]
3- & 4-Hydroxybenzoic acid	17	214.3	123.7	1.4	1.3	163.7	175.8	[17,19,21,28]
4-Hydroxyphenylacetic acid[b]	12	23.1	17.0	1.3	1.2	12.8	13.0	[17,19,21]
Isoferulic acid-3-*O*-glucuronide	15	161.2	130.2	2.2	1.2	240.6	173.8	[17,19,23]
Methyl-3,4-dihydroxybenzoate	17	1.8	1.1	1.7	1.4	1.6	1.7	[3,14,18,21,28]
Phloroglucinaldehyde	12	13.3	7.0	1.6	1.3	14.5	17.7	[4,18,21,28]
Protocatechualdehyde	17	12.3	6.5	1.1	1.1	9.0	9.6	[18,19,21,28]
Protocatechuic acid	16	7.9	2.9	1.8	1.3	7.0	6.9	[3,4,14,15,17–19,21,28]
Protocatechuic acid-3-*O*-sulfate	16	15.4	8.2	1.6	0.9	13.6	15.8	[3,14,18,28]
Quercetin[a]	15	6.4	4.0	1.8	1.1	7.3	7.5	[17]
Vanillic acid	12	91.5	38.4	1.4	0.7	76.0	71.6	[3,4,14,18,19,28]

The values are presented as mean with standard deviations (SD) detected in *n* study participants. C_{max}, the mean of the maximum metabolite concentrations of the volunteers; t_{max}, mean of the time point when C_{max} was observed; iAUC, incremental area under the curve calculated using the trapezoidal rule; [a] Chlorogenic acid and quercetin are not likely anthocyanin metabolites but are derived from the meals; [b] Quantified with caffeic acid.

chlorogenic acid, abundant in the PPE, may partially break down to caffeic acid. Ferulic acid may be formed from caffeic acid via *O*-methylation.[39] Chlorogenic acid and quercetin are most likely derived from the PPE and yellow potatoes[8] and are not metabolites of anthocyanins.

Under physiological conditions, nonacylated anthocyanins are rapidly hydrolyzed leading to the formation of an unstable α-diketone which, without the protecting glycoside, is subjected to degradation leading to a phenolic acid (expected B-ring metabolite) and aldehyde (expected A-ring metabolite).[40] Thereafter, the phenolic metabolites may be conjugated as detected with labeled cyanidin-3-*O*-glucoside in vivo.[3,14] Acylated anthocyanins, on the other hand, are structurally more stable than nonacylated anthocyanins as the acyl groups protect from the nucleophilic attack of water, and in comparison to hydroxysubstituted anthocyanins, methoxysubstituted are less reactive. Even 72% of the applied acylated anthocyanins of potatoes (but only 45% of the cyanidin-based, acylated anthocyanins of carrot) may reach the colonic vessel in an in vitro gastrointestinal model.[41] In colon, acylated anthocyanins may be degraded by human gut microbiota as detected in vitro with mono- and diacylated pelargonidin sophoroside glucosides.[40] This is substantiated by our in vivo results: in addition to late t_{max} times, urinary protocatechuic acid and phloroglucinaldehyde, the known colonic metabolites of cyanidin-3-*O*-glucoside,[42] were detected. The proposed degradation mechanism occurs first by enzymatic hydrolysis of the acyl and glycosyl groups followed by enzymatic and/or spontaneous C-ring fission of the unstable anthocyanidin leading to phenolic metabolites, which may be then subjected to further phase I and II reactions (Figure 2).

Considering the earlier reported beneficial health effects of purple potatoes[7] and the PPE[8] on human carbohydrate metabolism, acylated anthocyanins may inhibit pancreatic α-amylase[43] and intestinal maltase[44] as detected in vitro. More bioavailable phenolic metabolites may contribute to these health effects; for example, protocatechuic acid has insulin-like properties[45,46] and other phenolic metabolites may modulate carbohydrate metabolism by inhibiting salivary α-amylase, enhancing the intestinal uptake of glucose and downregulating sugar transporters as detected in vitro.[47] More investigations of the physiological role of the phenolic metabolites are required to understand the health effects and underlying molecular mechanisms of anthocyanin-rich foods.

Metabolites not previously reported in clinical trials feeding anthocyanin-rich foods were detected: catechol, gallic acid-4-*O*-glucuronide, and 2-methoxybenzoic acid, of which only catechol was increased statistically significantly in urine after the study meal. The lack of statistical significance may be due to inter-individual variation amongst the volunteers or that the metabolite was derived from the yellow potatoes used both in the control and study meals. Furthermore, phloroglucinol was detected earlier by only one study,[21] which fed raspberries rich in both anthocyanins and other phenolic compounds to healthy human volunteers. Earlier, a meal of purple potatoes led to the tentative identification of homovanillic acid/dihydrocaffeic acid, dihydroferulic acid, (iso)ferulic acid, dihydrocaffeic acid sulfate, and hippuric acid, but the study lacked the control arm.[26] As a next-step suggestion, a trial feeding labelled acylated anthocyanins would further discriminate the potato-derived metabolites from anthocyanin-derived metabolites. Here, large inter-individual variation was detected;[15,17–19] individual metabolism and gut microbiota composition may affect the metabolism of anthocyanins.

3.3. Screening of Tentatively Identified Phenolic Metabolites

A large number of phenolic metabolites and conjugates were detected, tentatively identified and heatmapped (Figure 3B), most of them peaking at 8–12 h. For example, vanillaldehyde

and hydroxyhippuric acids, and isomers of hydroxybenzoic acid, dihydroferulic acid, and dihydrosinapic acid were detected. Sulfates and glucuronides were detected for hydroxycinnamic acid derivatives (coumaric, caffeic, and ferulic acids), benzoic acid derivatives (hydroxybenzoic, vanillic, syringic, and methylgallic acids), phenol derivatives (catechol and pyrogallol), phenylacetic acid derivatives (homoprotocatechuic acid), and phenylpropanoic acids such as dihydro(iso)ferulic and dihydrosinapic acids. From plasma, 16 tentatively identified metabolites were detected (Figure 3C). 2-Hydroxybenzoic acid showed high analytical response compared to the other ones, peaking at 90 min after the study meal. Several sulfate and glucuronide conjugates were detected: catechol glucuronide, catechol sulfate, homoprotocatechuic acid glucuronide, hydroxybenzoic acid sulfate, (iso)ferulic acid glucuronide isomer, two methoxycatechol sulfate isomers, methylgallic acid sulfate, and vanillic acid sulfate.

The screened data set highlights the vast extent of phenolic metabolites after an acute intake of PPE rich in acylated anthocyanins even though the identification was only tentative due to practical challenges. The number of possible degradants and conjugates to be screened was large as the potato anthocyanins contain all six anthocyanidins varying in the acylation pattern, but the availability of commercial standards is limited. Therefore, in addition to our large compound library, the MRM transitions and chromatographic retention were used for identifying the screened dataset as reported earlier.[22] Additionally, the concentration of anthocyanins and phenolic metabolites is low in physiological samples. Here their levels were concentrated using optimized SPE and evaporation, and the samples were analyzed using a modern state-of-the-art mass spectrometer with a targeted MRM approach providing high instrumental sensitivity and capacity to scan numerous transitions without compromising the amount of data points per peak. Regardless of its high sensitivity, a targeted MRM approach is limited to predicted metabolites, whereas an untargeted approach, which is usually performed with high resolution, could lead to the identification of unpredicted metabolites.

4. Concluding Remarks

This report demonstrates that an acute intake of PPE rich in methoxysubstituted anthocyanins monoacylated with caffeic, coumaric and ferulic acids leads to absorption and excretion of only a small number of anthocyanins degradants and an extensive number of spontaneous and/or colonic phenolic metabolites and their phase II conjugates. The rutinose and acyl groups of anthocyanins are hydrolyzed and the methoxysubstituted anthocyanidins may be *O*-demethylated. Novel phenolic metabolites of acylated potato anthocyanins, such as glucuronyl and sulfonyl conjugates of protocatechuic acid, were detected. Three novel phenolic metabolites not reported earlier in clinical trials after a meal rich in anthocyanins, such as purple potatoes, were detected: catechol, gallic acid-4-*O*-glucuronide, and 2-methoxybenzoic acid, of which only catechol was increased statistically significantly in comparison to the control yellow potato meal without the PPE. These results provide new insights on the bioavailability and metabolism of acylated anthocyanins in humans.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This study was funded by the Doctoral Programme of Molecular Life Sciences of the University of Turku Graduate School, Finland, the Orion Research Foundation sr, Finland, ERVA of the City of Turku, Finland, the Raisio Research Foundation, Finland, the Magnus Ehrnrooth Foundation, Finland, the Biotechnology and Biological Sciences Research Council (BBSRC, UK) through an Institute Strategic Programme Grant ("Food Innovation and Health"; Grant No. BB/R012512/1 and its constituent projects BBS/E/F/000PR10343, BBS/E/F/000PR10345 and BBS/E/F/000PR10346) to the Quadram Institute Biosciences and a Norwich Research Park Biosciences Doctoral Training Partnership award to JP (grant number BB/M011216/1), and the Niemi foundation, Finland. The volunteers are thanked for their participation. MD Matti Viitanen is thanked for ethical and medical expertise, Sanna Himanen for blood sampling (the City Hospital of Turku, Finland). Dr Paul Needs is thanked for synthesis of the standard compounds, Dr Shikha Saha for analytical advice and Dr Marianne Defernez for sample randomization (the Quadram Institute Bioscience, UK). Tuomas Jokioja is thanked for his help with R. Dr Jari Heinonen and Professor Tuomo Sainio are thanked for extracting the PPE (the Lappeenranta-Lahti University of Technology LUT, Finland), and Dr Mikko Griinari for potato cultivation.

Author Contribution

J.J., K.L., and B.Y. concepted the study and organized the clinical trial. J.J. and J.P. optimized the analysis method and collected the data under supervision of M.P. and P.K., and J.J. processed the data. J.J., B.Y., P.K., and K.L. participated to funding acquisition and B.Y., P.K., and K.L. provided the resources. J.J. drafted the original manuscript with the help of K.L., and all authors commented on and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

acylated anthocyanins, mass spectrometry, postprandial metabolites, purple-fleshed potatoes, *Solanum tuberosum* L.

Received: September 15, 2020
Revised: January 11, 2021
Published online: March 30, 2021

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