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Identification of (Poly)phenolic Compounds in Concord Grape Juice and Their Metabolites in Human Plasma and Urine after Juice Consumption

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ABSTRACT: Analysis of Concord grape juice by HPLC with ESI-MS^n, PDA, and fluorescence detection resulted in the identification and quantification of 60 flavonoids and related phenolic compounds, which were present at an overall concentration of 1508 ± 31 μmol/L. A total of 25 anthocyanins were detected, which were mono- and di-*O*-glucosides, *O*-acetylglucosides, *O*-*p*-coumaroyl-*O*-diglucosides, and *O*-*p*-coumaroylglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin. The anthocyanins represented 46% of the total phenolic content of the juice (680 μmol/L). Tartaric esters of hydroxycinnamic acids, namely, *trans*-caftaric and *trans*-coutaric acids, and to a lesser extent *trans*-fertaric acid accounted for 29% of the phenolic content, with a total concentration of 444 μmol/L, of which 85% comprised *trans*-caftaric acid. Free hydroxycinnamic acids were also quantified but contributed to <1% of the total phenolic content (8.4 μmol/L). The other groups of polyphenolic compounds present in the juice, accounting for 24% of the total, comprised monomeric and oligomeric units of (epi)catechin and (epi)gallocatechin (248 μmol/L), flavonols (76 μmol/L), gallic acid (51 μmol/L), and *trans*-resveratrol (1.5 μmol/L). The bioavailability of the (poly)phenolic compounds in 350 mL of juice was investigated following acute intake by healthy volunteers. Plasma and urine were collected over 0−24 h and analyzed for parent compounds and metabolites. In total, 41 compounds, principally metabolites, were identified.

KEYWORDS: *Vitis labrusca*, Concord purple grape juice, (poly)phenolic constituents, acute ingestion, human plasma and urinary metabolites

INTRODUCTION

Concord grapes belong to the *Vitis labrusca* vines, which are cultivated principally in North America. Concord grapes were developed by Ephraim Wales Bull in 1849, near the Massachusetts village of Concord. They are used to produce an unfermented grape juice, which was originally processed by Dr. Welch and used for communion in local churches.[1] The juice is produced through the hot press method, which involves adding a pectolytic enzyme and heating the crushed grapes, which enhances the extraction of color from the skins into the juice.[2] Successive pasteurizations also ensure the inactivation of yeasts, preventing fermentation of sugars in the grape.

Concord grape juice contains high levels of antioxidants compared to many other commercial fruit juices and drinks, probably as a consequence of the juice containing high concentrations of an unusual combination of anthocyanins, tartarate esters of hydroxycinnamates, and flavan-3-ols.[3] There is also evidence that consumption of Concord grape juice can have potential protective effects on health. Consumption of 5−10 mL/kg body weight of purple grape juice by healthy volunteers for 7−14 days resulted in an improvement in blood vessel endothelial function by increased platelet-derived nitric oxide (NO) production,[4] reduced platelet aggregation,[5] improved flow-mediated dilation,[6] decreased LDL oxidation,[7] and potentially reduced risk of cardiovascular events. When consumed for 6−8 weeks Concord grape juice has been shown to induce improved cognitive function in aged rats.[8] Improved memory function was demonstrated in a recent study in older adult humans with memory decline, but not dementia, who consumed Concord grape juice at a daily dose of 6−9 mL/kg body weight for 12 weeks.[9]

The aim of the current study was to apply HPLC-MS^n methodology to generate a more detailed profile of the polyphenolic and phenolic constituents of Concord grape juice and to gain information on the bioavailability and metabolic fate of these compounds by measuring the parent compounds and their metabolites in plasma and urine after acute consumption of 350 mL of juice by healthy human volunteers.

MATERIALS AND METHODS

Chemicals. Procyanidins B1 and B2, *p*-coumaric acid, ethyl gallate, 3-(3′-hydroxyphenyl)propionic acid (dihydrocoumaric acid), and

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Table 1. HPLC and MS Conditions Used for the Detection of (Poly)phenolic Compounds and Their Metabolites

compound	mobile phase[a]	gradient	column	MS sheath/aux gas (unit/min)	MS capillary temperature (°C)	source voltage (kV)
anthocyanins (juice, urine, plasma)	1% FA in MeOH	10−45%, 60 min	RP-Max	60/40	300	2.00
hydroxycinnamic acids and esters (juice, urine, plasma)	1% FA in MeOH 0.5% AA in MeOH	10−45%, 60 min 5−40%, 60 min	RP-Max Polar-RP	80/60	300	3.00
monomeric and dimeric flavan-3-ols (juice, urine)	0.1% FA in MeOH	10−45%, 60 min 15−50%, 60 min	RP-Max	60/60	315	2.00 (10 eV source fragmentation)
oligomeric flavan-3-ols (juice)	2% AA in ACN (A) 2% AA in aqueous MeOH (95%) (B)	7%−7%−37.6%−100%−100%−7%−7% of B for 0−3−60−63−70−76−86 min	Develosil Diol 100 Å	60/60	275	3.00
flavonols (juice, urine)	0.1% FA in MeOH	20−40%, 60 min 20−60%, 60 min	RP-Max	60/60	315	2.00 (10 eV source fragmentation)
hydroxybenzoic acids (gallic acid) and stilbenes (*trans*-resveratrol) (juice)	0.1% FA in MeOH	10−45%, 60 min	RP-Max	60/60	315	2.00 (10 eV source fragmentation)

[a] AA, acetic acid; ACN, acetonitrile; FA, formic acid; MeOH, methanol.

quercetin-3-*O*-glucoside were purchased from Fluka (Sigma-Aldrich Co. Ltd., Dorset, U.K.). Ferulic acid, (−)-gallocatechin, (−)-epicatechin, (+)-catechin, and *trans*-resveratrol were obtained from Sigma-Aldrich Co. Ltd. (−)-Epigallocatechin and (−)-epicatechin-3-*O*-gallate were purchased from Apin Chemicals Ltd. (Abingdon, U.K.). Caffeic acid and malvidin-3,5-*O*-diglucoside were from AASC Ltd. (Southampton, U.K.). Quercetin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, and myricetin were purchased from Extrasynthese (Lyon, France), and delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside were obtained from PhytoLab Gmbh & Co. KG (Germany). Cyanidin-3-*O*-sambubioside-5-*O*-glucoside was purchased from Polyphenols (Sandnes, Norway). Feruloylglycine was a gift from Professor Takao Yokota, Teikyo University, Utsunomiya, Japan. Acetonitrile, acetone, and formic acid of HPLC grade were purchased from Fisher Scientific Ltd. (Loughborough, Leicestershire, U.K.), methanol was purchased from Rathburn Chemicals (Walkerburn, Scotland), and acetic acid was purchased from WWR International Ltd. (Poole, Dorset, U.K.).

Juice Analysis. Aliquots of 100% Concord grape juice (Welch Foods Inc., Concord, MA) were taken from a freshly opened bottle stored at 2 °C. Prior to HPLC-MS^n analysis, the juice was centrifuged at 16110*g* for 3 min at 4 °C. Flavan-3-ols, flavonols, and free hydroxycinnamic and hydroxybenzoic acids were analyzed in 20 μL of the undiluted juice, and *trans*-resveratrol was analyzed in 100 μL aliquots, whereas the juice was diluted 20-fold prior to analysis of anthocyanins and tartarate esters. All samples were analyzed in triplicate.

Bioavailability Study. The bioavailability of purple grape juice (poly)phenolic compounds was assessed by acute feeding of 350 mL of 100% Concord juice to healthy volunteers. Three male and five female healthy volunteers (20−40 years of age and a BMI range of 20.7−26.1 kg/m^2) participated in the study. The participants were nonsmokers, not under medication or taking any vitamins or other supplements, and were generally healthy. Prior to the start of the study, participants were asked to follow a diet low in (poly)phenolic compounds for 2 days. This involved avoiding fruits, vegetables, tea, coffee, wine, chocolate, whole grains, and wholemeal food items. The day before the acute juice intake, volunteers collected urine for 24 h. On the morning of the study, volunteers attended the trial unit after an overnight fast and ingested 350 mL of 100% Concord grape juice. Ad libitum water but no other food or drink was allowed for the next 3 h. Blood was sampled at baseline and after 0.5, 1, 2, 3, 4, 6, 8, and 24 h, and urine was collected in batches between 0 and 2 h, between 2 and 8 h, and between 8 and 24 h. Lunch was provided and consisted of a selection of white rolls, ham, margarine spread, cheese, and ready salted crisps. Volunteers returned home after 8 h and remained on the low-(poly)phenol diet and continued to collect urine until the following morning, by means of bottles provided surrounded by frozen blocks and kept in cooler bags. The study protocol was approved by the University of Glasgow Medical Faculty Ethics Committee, and all subjects gave written informed consent.

Extraction of Plasma. Plasma extraction for the analysis of anthocyanins and related metabolites was based on the method of Garcia-Alonso et al. [10] Duplicate 700 μL volumes of plasma acidified with 50% aqueous formic acid, and containing 750 ng of cyanidin-3-*O*-sambubioside-5-*O*-glucoside added as an internal standard was loaded onto a Strata C18 (6 mL/500 mg) SPE cartridge (Phenomenex, Inc., Cheshire, U.K.), preconditioned with 6 mL of methanol acidified with 1% formic acid, followed by 6 mL of acidified water (1% formic acid). The cartridge was then washed with 6 mL of acidified water, after which anthocyanins were eluted with 6 mL of acidified methanol, which was dried for 2 h using a centrifugal vacuum concentrator at 37 °C (SPS SpeedVac, Thermo Savant, Waltham, MA), prior to being freeze-dried overnight (Thermo Savant SuperModulyo). Pellets were resuspended in 150 μL of 1% formic acid containing 10% methanol before being centrifuged in 0.2 μm Micro-Spin eppendorf filter (Alltech Associates Applied Sciences Ltd., Lancashire, U.K.). Volumes of 100 μL were analyzed by HPLC-MS^n. Typical recovery values of the internal standard were on average 70 ± 8% (n = 8).

Table 2. HPLC-MS^2 Identification and Quantification of Anthocyanins in Concord Grape Juice

t_R (min)	anthocyanin[a]	$[M - H]^+$ (m/z)	MS^2 (m/z)	μmol/L[b]
9.1	delphinidin-3,5-*O*-diglucoside*	627	465, 303	19.3 ± 0.6 [a]
12.2	cyanidin-3,5-*O*-diglucoside*	611	449, 287	14.1 ± 0.2 [b]
13.8	petunidin-3,5-*O*-diglucoside*	641	479, 317	12.8 ± 0.6 [c]
17.4	peonidin-3,5-*O*-diglucoside*	625	463, 301	13.2 ± 0.2 [d]
17.5	delphinidin-3-*O*-glucoside	465	303	164.8 ± 3.5 [a]
18.4	malvidin-3,5-*O*-diglucoside	655	493, 331	20.8 ± 0.4 [e]
20.6	cyanidin-3-*O*-glucoside	449	287	71.5 ± 0.1 [b]
23.2	petunidin-3-*O*-glucoside	479	317	61.6 ± 1.1 [c]
26.6	peonidin-3-*O*-glucoside	463	301	19.5 ± 0.2 [d]
28.4	malvidin-3-*O*-glucoside	493	331	23.0 ± 0.1 [f]
34.8	delphinidin-3-*O*-(6″-*O*-acetyl)glucoside*	507	303	21.0 ± 0.3 [a]
38.9	delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside*	773	611, 465, 303	50.7 ± 4.8 [a]
39.1	cyanidin-3-*O*-(6″-*O*-acetyl)glucoside*	491	287	18.2 ± 1.8 [b]
40.6	petunidin-3-*O*-(6″-*O*-acetyl)glucoside*	521	317	11.7 ± 0.2 [c]
42.5	cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside*	757	595, 449, 287	21.6 ± 2.5 [b]
43.1	petunidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside*	787	625, 479, 317	29.1 ± 4.0 [c]
44.3	peonidin-3-*O*-(6″-*O*-acetyl)glucoside*	505	301	4.1 ± 0.2 [d]
45.0	malvidin-3-*O*-(6″-*O*-acetyl)glucoside*	535	331	6.2 ± 0.1 [f]
46.6	malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside*	801	639, 493, 301	25.7 ± 0.1 [e]
46.9	peonidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside*	771	609, 463, 301	7.2 ± 0.2 [d]
48.0	delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside*	611	303	16.6 ± 0.2 [a]
51.6	cyanidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside*	595	287	24.0 ± 1.7 [b]
52.6	petunidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside*	625	317	8.7 ± 0.4 [c]
56.0	peonidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside*	609	301	4.1 ± 0.2 [d]
56.1	malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside*	639	331	10.0 ± 0.9 [f]

[a] *, Tentative identification based on mass spectra as well as relative HPLC retention times and published data. [b]Quantified as [a]delphinidin-3-*O*-glucoside equivalents; [b]cyanidin-3-*O*-glucoside equivalents; [c]petunidin-3-*O*-glucoside equivalents; [d]peonidin-3-*O*-glucoside equivalents; [e]malvidin-3,5-*O*-diglucoside equivalents; and [f]malvidin-3-*O*-glucoside equivalents.

Metabolites of tartaric acid esters of hydroxycinnamic acids in plasma were extracted and analyzed as described previously by Stalmach et al.[11] Briefly, 450 μL of acidified plasma was deproteinized using acetonitrile (2.5 volume), before an extraction step using methanol (2.5 volume) was carried out. Extracts were dried under a stream of nitrogen at 35 °C and resuspended in 250 μL of the mobile phase containing 10% methanol. Ethyl gallate was used as an internal standard (4 ng/μL), and typical recoveries of 73 ± 3% (n = 8) were achieved. Volumes of 100 μL were analyzed by HPLC-MS^n.

Analysis of Urine. Metabolites of flavan-3-ols, flavonols, and hydroxycinnamic acids in unprocessed urine were analyzed by HPLC-MS^n system. Because of their low levels, anthocyanins were purified and concentrated according to the same process used for plasma, using 2 μg of cyanidin-3-*O*-sambubioside-5-*O*-glucoside as an internal standard, after which 50 μL volumes were analyzed by HPLC-MS^n.

Reversed Phase HPLC-PDA-ESI-MS^n. Concord grape juice (poly)phenolic compounds and metabolites in body fluids were analyzed using a Surveyor HPLC with a PDA detector and a LCQ Duo ion trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific, San Jose, CA). A summary of the mobile phases, gradients, columns, and mass spectrometer conditions is presented in Table 1. Separations of (poly)phenolic compounds and metabolites were performed at 40 °C using a 4 μm Synergi 250 × 4.6 mm i.d. reversed phase column (Phenomenex, Macclesfield, U.K.). Injections were carried out with an autosampler maintained at 4 °C. The mobile phase was pumped at a flow rate of 1 mL/min. The column eluate initially passed through the PDA detector and was then split, with 0.3 mL/min directed to the mass spectrometer with ESI operating in full-scan positive (detection of anthocyanins) or negative ionization mode (m/z 100−1000), data dependent MS^2. The tuning of the mass spectrometer was optimized by infusing diluted Concord grape juice (detection of anthocyanins), a standard of (−)-epicatechin (detection of flavonoids and hydroxybenzoic acids), or a standard of ferulic acid (detection of hydroxycinnamic acids), dissolved in the initial HPLC mobile phase, into the source at a flow rate of 0.3 mL/min, and the collision energy was set at 35%. Post-HPLC, anthocyanins and derivatives were detected and quantified either with the PDA at 520 nm or using selective reaction monitoring (SRM). Mean quantitative data are expressed as micromoles ± SE (n = 3) of available standard equivalents. Other flavonoids and their metabolites were detected and quantified using selected ion monitoring (SIM), and identification was confirmed by mass spectrometry using consecutive reaction monitoring (CRM). Mean quantitative data are expressed as micromoles ± SE (n = 3) of available standard equivalents. Hydroxycinnamic acids and metabolites were detected and quantified using SIM, tartarate esters were quantified using PDA at 325 nm, and identification was confirmed by mass spectrometry using CRM. Mean quantitative data are expressed as micromoles ± SE (n = 3) of available standard equivalents.

Analysis of Procyanidins. Analysis of the procyanidins in the juice with a degree of polymerization of 2 and above was based on a previously described method.[12] Briefly, 10 mL of juice was freeze-dried, and procyanidins were extracted in triplicate with 5 mL of an acetone-based solution (acetone/water/acetic acid, 70:29.5:0.5, v/v/v). After vortexing, the samples were sonicated for 5 min at 50 °C and centrifuged at 2600g for 10 min. Supernatants were collected and passed through a SPE cartridge, Strata SCX (55 μm, 70 Å, 500 mg/3 mL) (Phenomenex,

Table 3. HPLC-MS^2 Identification and Quantification of Flavan-3-ols, Flavonols, Hydroxycinnamic Acids, Hydroxybenzoic Acids, and Stilbenes in Concord Grape Juice

	t_R (min)	compound[a]	$[M - H]^-$ (m/z)	MS^2 (m/z)	μmol/L[b]
hydroxybenzoic acids	5.9	gallic acid	169	125	50.9 ± 2.5 [a]
hydroxycinamic acids	23.2	caffeic acid	179	135	2.0 ± 0.2 [b]
	34.9	*p*-coumaric acid	163	119	5.4 ± 0.2 [c]
	43.0	ferulic acid	193	149, 178, 134	1.1 ± 0.1 [d]
tartarate esters	12.1	*trans*-caftaric acid*	311	149, 179	378.6 ± 12.5 [b]
	17.2	*trans*-coutaric acid*	295	163	53.0 ± 1.8 [c]
	20.5	*trans*-fertaric acid*	325	193	12.4 ± 0.6 [d]
flavan-3-ol monomers	7.6	gallocatechin	305	179, 221, 261, 165, 125	8.0 ± 0.1 [e]
	13.1	epigallocatechin	305	179, 221, 261, 165, 125	3.2 ± 0.4 [f]
	14.1	catechin	289	245, 205	20.4 ± 0.7 [g]
	21.5	epicatechin	289	245, 205	61.8 ± 5.2 [h]
	31.4	epicatechin-3-*O*-gallate	441	289, 331, 193, 169	2.2 ± 0.1 [i]
flavonols	37.1	myricetin-*O*-glycoside*	479	316, 317	18.3 ± 0.3 [j]
	39.9	myricetin-*O*-glucuronide*	493	317	1.2 ± 0.0 [j]
	43.7	luteolin-*O*-glycoside*	447	285	0.2 ± 0.0 [k]
	44.3	quercetin-3-*O*-galactoside	463	301, 300	0.8 ± 0.2 [l]
	45.3	quercetin-3-*O*-glucoside	463	301, 300	22.3 ± 0.3 [m]
	46.5	laricitrin-*O*-glycoside*	493	331, 330	0.7 ± 0.0 [n]
	47.8	quercetin-3-*O*-glucuronide	477	301	30.1 ± 0.6 [o]
	50.3	kaempferol-*O*-galactoside*	447	284, 285	0.3 ± 0.0 [p]
	52.4	kaempferol-3-*O*-glucoside	447	284, 285	1.8 ± 0.0 [p]
	53.5	isorhamnetin-3-*O*-glucoside	477	314, 315	0.6 ± 0.0 [n]
stilbenes	50.2	*trans*-resveratrol	227		1.5 ± 0.0 [q]

[a] *, Tentative identification based on mass spectra as well as relative HPLC retention times and published data. [b] Quantified as [a]gallic acid; [b]caffeic acid equivalents; [c]*p*-coumaric acid equivalents; [d]ferulic acid equivalents; [e](−)-gallocatechin; [f](−)-epigallocatechin; [g](+)-catechin; [h](−)-epicatechin; [i](−)-epicatechin-3-*O*-gallate; myricetin equivalents; [k]luteolin-7-*O*-glucoside equivalents; [l]quercetin-3-*O*-galactoside; [m]quercetin-3-*O*-glucoside; [n]isorhamnetin-3-*O*-glucoside equivalents; [o]quercetin-3-*O*-glucuronide; [P]kaempferol-3-*O*-glucoside equivalents; and [q]*trans*-resveratrol.

Cheshire, U.K.), following preconditioning of the cartridge with distilled water. Five microliters of the collected samples was then injected into an HPLC system equipped with a fluorescence detector (FP-920, Jasco (U.K.) Ltd.) and linked to a mass spectrometer. Separation was achieved using a Develosil Diol 100 Å (250 × 4.6 mm, 5 μm) (Phenomenex, Cheshire, UK). The mobile phase consisted of acidified acetonitrile and acidified aqueous methanol, and chromatographic conditions were used as previously described.[12] Following separation, procyanidins were detected and quantified using a fluorometer (excitation and emission wavelengths at 230 and 320 nm), and identification was confirmed by mass spectrometry in full-scan negative ionization (m/z 100−2000), data dependent MS. Mean quantitative data are expressed as micromoles ± SE (n = 3) of (−)-epicatechin equivalents.

RESULTS

Analysis of Juice Anthocyanins. HPLC-PDA-MS^n analysis detected 25 anthocyanins in the Concord grape juice. Identification of individual compounds was based on the m/z of the positively charged molecular ion ($[M - H]^+$) and the MS^2 fragmentation, retention time of commercially available standards, absorbance spectra, and elution order, which depends upon the anthocyanidin aglycone and the attached sugar, as previously described.[13−17] The anthocyanin content of the juice, in agreement with that previously published,[17] is presented in Table 2. The anthocyanins consisted of 3-*O*-glucosides ($[M - 162]^+$), 3,5-*O*-diglucosides ($[M - 162 - 324]^+$), 3-*O*-(6″-*O*-acetyl)glucosides ($[M - 204]^+$), 3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucosides ($[M - 162 - 308 - 470]^+$), and 3-*O*-(6″-*O*-*p*-coumaroyl)glucosides ($[M - 308]^+$) of delphinidin (m/z 303), cyanidin (m/z 287), petunidin (m/z 317), peonidin (m/z 301), and malvidin (m/z 331). Quantification of anthocyanins was carried out using SRM, as appropriate separation of all the individual peaks could not be achieved.

The main compounds were delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside, accounting for ca. 50% of the total anthocyanin content (Table 2). Delphinidin-3-*O*-glucoside alone accounted for a quarter of the total amount (165 ± 4 μmol/L). Monoglucosides accounted for 50% of the total anthocyanins (340 ± 2 μmol/L), and glycosylated, acetylated, and *p*-coumaroyl delphinidin represented 40% (272 ± 6 μmol/L).

Analysis of Juice Hydroxycinnamic and Hydroxybenzoic Acids. Information on the identification and quantification of hydroxycinnamates and hydroxybenzoic acids in Concord grape

Table 4. Diol-HPLC-MS Identification and Fluorescence Quantification of Procyanidins in Concord Grape Juice[a]

t_R (min)	procyanidin	$[M - H]^-$ (m/z)	μmol/L
9.7	(epi)C−(epi)C dimer	577	69.7 ± 2.6
12.3	(epi)C−(epi)GC dimer	593	12.8 ± 0.4
14.3	(epi)C−(epi)Cg dimer	729	1.3 ± 0.1
16.8	(epi)C−(epi)C−(epi)C trimer	865	26.6 ± 0.9
19.9	(epi)C−(epi)C−(epi)GC trimer	881	7.8 ± 0.2
24.8	(epi)C−(epi)C−(epi)C−(epi)C tetramer	1153	14.0 ± 0.6
27.7	(epi)C−(epi)C−(epi)C−(epi)GC tetramer	1169	6.0 ± 0.2
31.5	(epi)C−(epi)C−(epi)C−(epi)C−(epi)C pentamer	1441	5.4 ± 0.2
33.9	(epi)C−(epi)C−(epi)C−(epi)C−(epi)GC pentamer	1457	3.3 ± 0.1
37.0	(epi)C−(epi)C−(epi)C−(epi)C−(epi)C−(epi)C hexamer	1729	3.5 ± 0.1
38.9	(epi)C−(epi)C−(epi)C−(epi)C−(epi)C−(epi)GC hexamer	1745	0.9 ± 0.0
42.4	(epi)C−(epi)C−(epi)C−(epi)C−(epi)GC−(epi)GC hexamer	1761	0.9 ± 0.1

[a] Tentative identifications based on the retention time of cocoa procyanidins made of (epi)catechin units, as previously reported;[12] quantification in (−)epicatechin equivalents (fluorescence detection).

juice is presented in Table 3. The sole hydroxybenzoate was gallic acid at a concentration of 51 ± 3 μmol/L. Identification of free hydroxycinnamic acids was confirmed by injection of standards, and the total concentration of caffeic, *p*-coumaric, and ferulic acids reached 8.4 ± 0.4 μmol/L. In addition, tartaric acid esters of hydroxycinnamic acids were also identified, mainly as *trans*-caftaric, *trans*-coutaric, and *trans*-fertaric acids, according to their absorbance and mass spectra.[18] The main tartaric ester in the juice was caftaric acid (379 ± 12 μmol/L), followed by coutaric acid (53 ± 2 μmol/L) and fertaric acid (12 ± 1 μmol/L) (Table 3).

Analysis of Juice Flavan-3-ols. Reversed phase HPLC separated five flavan-3-ol monomers, which were identified by cochromatography and MS^2 (Table 3). Epicatechin (reverse phase HPLC does not separate (+) and (−)-flavan-3-ols, so the enantiomers are not specified) was found in highest amounts followed by catechin, gallocatechin, epigallocatechin, and epicatechin-3-*O*-gallate at respective concentrations of 62 ± 5, 20 ± 1, 8.0 ± 0.1, 3.2 ± 0.4, and 2.2 ± 0.1 μmol/L. Procyanidins B1 and B2 were also analyzed by reversed phase, as the diol column does not distinguish between individual oligomeric compounds. Concentrations of 12.0 ± 0.7 and 25.3 ± 0.7 μmol/L were quantified for procyanidins B1 and B2, respectively (Table 3).

Procyanidins with a degree of polymerization of >3 do not chromatograph satisfactorily on HPLC C_{18} supports. The procyanidins were, therefore, also analyzed by HPLC-MS using a diol support with the partially identified peaks, which separate procyanidins according to their molecular masses, being quantified by fluorometry in (−)-epicatechin equivalents, due to a lack of available standards (Table 4). Compounds with a negative molecular ion, $[M - H]^-$, at m/z 577 865, 1153, 1441, and 1729 were identified as dimeric, trimeric, tetrameric, pentameric, and hexameric procyanidins of (epi)catechin, whereas peaks with a $[M - H]^-$ at m/z 593, 881, 1169, 1457, and 1745 contain one (epi)gallocatechin unit, corresponding to the addition of an extra hydroxyl group of 16 amu.[19] A $[M - H]^-$ at m/z 729 and its subsequent MS^2 fragmentation are consistent with a dimeric procyanidin containing one unit of (epi)catechin and one unit of (epi)catechin-3-*O*-gallate.[20] A compound with $[M - H]^-$ at m/z 1761 was putatively identified as a hexamer containing two (epi)gallocatechin units (+32 amu)[19] (Table 4). The main procyanidins in the juice were the dimers and trimers of (epi)catechin units (Table 4).

Analysis of Juice Flavonols. A total of 10 flavonol compounds were identified and quantified (Table 3). Myricetin conjugates yielded MS^2 fragments at m/z 316, corresponding with the $[M - H]^-$ of authentic myricetin. The loss of 162 amu ($[M - 162]^-$) corresponds to the cleavage of a sugar moiety, and maximum absorbance at 350 and 255 nm confirms the presence of a glycosylated flavonol. The component with a retention time of 37.1 min was therefore identified as a myricetin-*O*-glycoside. The MS^2 fragmentation of the peak at 39.9 min yielded m/z 317, a loss of 176 amu from m/z 493, corresponding to a glucuronic acid. MS^3 fragmentation produced ions at m/z 179 and 151, matching that of a standard of myricetin. The peak was therefore partially identified as a myricetin-*O*-glucuronide. The peak eluting at 43.7 min had a $[M - H]^-$ of m/z 447, yielding m/z 285, characterized by the loss of a sugar moiety. The MS^3 fragmentation of m/z 285 produced similar ions to those of a standard of luteolin, thus identifying the peak as a luteolin-*O*-glycoside. The next two eluting peaks both had a $[M - H]^-$ at m/z 463, with MS^2 fragments at m/z 301 and 300 and retention times matching those of standards of quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside. The peak eluting at 46.5 min had a $[M - H]^-$ at m/z 493 and produced MS^2 fragments at m/z 331 and 330 corresponding to a standard of laricitrin (3-*O*-methylmyricetin). The loss of 162 amu putatively identifies this compound as a laricitrin-*O*-glycoside. The MS spectrum and λ_{max} (350, 255 nm) of the 47.8 min peak corresponded to those of quercetin-3-*O*-glucuronide obtained from a green bean extract. The peaks at 50.3 and 52.4 min both had a $[M - H]^-$ at m/z 447, with MS^2 ions at m/z 284 and 285. The later eluting peak cochromatographed with and had the same mass spectral pattern as kaempferol-3-*O*-glucoside. The peak eluting at 50.3 min was putatively identified as kaempferol-3-*O*-galactoside, due to its earlier retention time compared to the 3-glucoside. Finally, the peak at 53.5 min was identified as isorhamnetin-3-*O*-glucoside on the basis of its properties matching those of an authentic standard.

The concentration of flavonols in the Concord grape juice was 76 ± 1 μmol/L with the main components being quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucoside, followed by the myricetin-*O*-glycoside. The other flavonols were present in only small quantities (Table 3).

Analysis of Juice Stilbenes. *trans*-Resveratrol was identified in the juice by cochromatography with an authentic standard, and its concentration amounted to 1.5 ± 0.0 μmol/L.

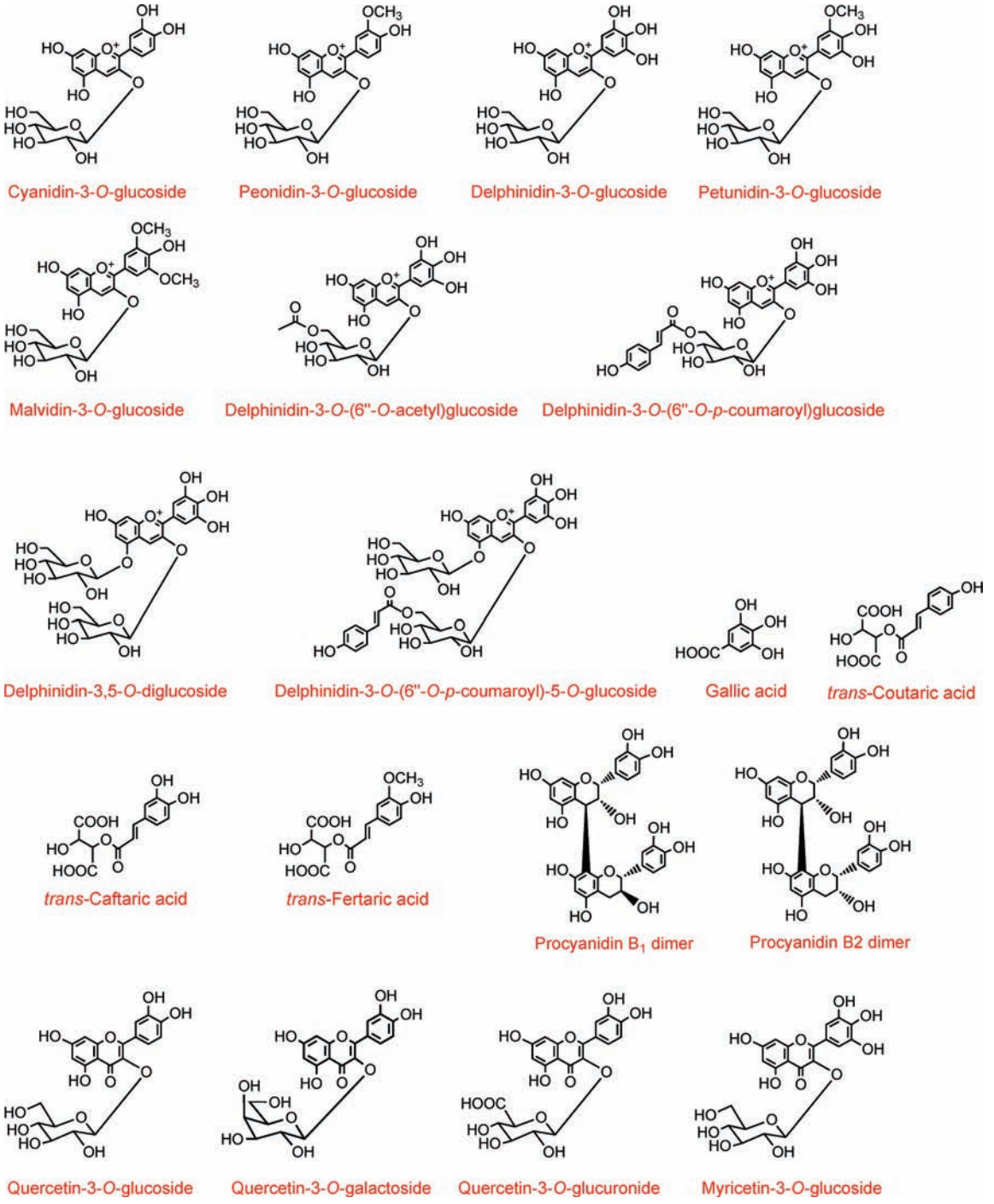

Figure 1. Structures of key flavonoids and phenolic compounds identified in Concord grape juice.

Structures of the (poly)phenolic compounds contained in 100% Concord grape juice are shown in Figure 1. The total content of (poly)phenolic compounds amounted to 1508 ± 31 μmol/L (Table 5). The main group of compounds in the juice was the anthocyanins (680 ± 17 μmol/L), followed by the tartarate esters of hydroxycinnamic acids (444 ± 15 μmol/L). The lower concentrations of flavan-3-ol monomers, procyanidins flavonols, gallic acid, free hydroxycinnamic acids, and *trans*-resveratrol accounted for 25% of the total (poly)phenol content.

Analysis of Plasma and Urine. Following acute intake of 350 mL of Concord grape juice, a total of 41 metabolites and polyphenolic compound derivatives were identified in the plasma and urine samples collected from volunteers over 24 h post-ingestion (Table 6).

Ten flavan-3-ol metabolites of (epi)catechin and (epi)-gallocatechin were detected in urine. Peaks M1−M10 were partially identified as a complex array of glucuronide, sulfate, and methyl metabolites of (epi)catechin and (epi)gallocatechin, which have been detected in previous studies following the consumption of green tea[21,22] and a polyphenol-rich drink.[23]

Peak M11 had a $[M - H]^-$ at m/z 559, yielding MS^2 and MS^3 fragments at m/z 479 and m/z 316 and 317, respectively, with a

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Table 5. (Poly)phenolic Content of a 350 mL Serving of Concord Grape Juice

compound	μmol/L	μmol/350 mL	% of total composition
anthocyanins	680 ± 17	238 ± 6	46
tartarate esters of hydroxycinnamic acids	444 ± 15	155 ± 5	29
oligomeric flavan-3-ols	152 ± 5	53 ± 2	10
monomeric flavan-3-ols	95 ± 6	33 ± 2	6
flavonols	76 ± 1	27 ± 1	5
hydroxybenzoic acids	51 ± 3	18 ± 1	3
free hydroxycinnamic acids	8.4 ± 0.4	2.9 ± 0.1	1
stillbenes	1.5 ± 0.0	0.5 ± 0.0	<1
total	**1508 ± 31**	**528 ± 11**	**100**

loss of 80 amu corresponding to a sulfate ion. MS^2 and MS^3 fragments corresponded to those of the myricetin-*O*-glycoside, detected in the juice (Table 3). This compound, which was detected in urine, was therefore identified as a myricetin-*O*-glycoside-*O*-sulfate. It was the only flavonol metabolite to be detected in the body fluids.

Peaks M12−M16 had the same MS fragment patterns and HPLC retention times as delphinidin-3-*O*-glucoside (M12), cyanidin-3-*O*-glucoside (M13), petunidin-3-*O*-glucoside (M14), peonidin-3-*O*-glucoside (M15), and malvidin-3-*O*-glucoside (M16), all of which occurred in Concord grape juice (Table 2). All of these anthocyanin glucosides were present in urine, and only delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside, two of the main juice anthocyanins, were detected in plasma. An additional nine metabolites of anthocyanins were also identified, principally in urine. Peaks M17 and M18 both had a $[M - H]^-$ at m/z 479, which produced an ion at m/z 303 upon MS^2 fragmentation. This loss of 176 amu corresponds to cleavage of a glucuronic acid moiety. Peaks M17 and M18 were therefore identified as delphinidin-*O*-glucuronides. A similar m/z 176 loss was also observed the $[M - H]^+$ at m/z 463 (peak M19), m/z 493 (peaks M20 and M21), m/z 477 (peaks M22 and M23), and m/z 507 (peaks M24 and M25), yielding MS^2 fragments at m/z 287 (cyanidin), m/z 317 (petunidin), m/z 301 (peonidin), and m/z 331 (malvidin). Peaks M17−M25 were all identified as anthocyanidin-*O*-glucuronides as indicated in Table 6.

Peaks M26−M28, M30, M31, M33, M34, M36−38, M40, and M41 have all been previously identified against authentic standards and were detected in urine and/or plasma samples of volunteers following a single intake of coffee containing chlorogenic acids[11,24] (Table 6; Figure 2). These comprise a wide array of free, sulfated, and glucuronidated caffeic, ferulic, isoferulic, dihydrocaffeic, and dihydroferulic acids and feruloylglycine. In addition to these 12 metabolites, another 4 compounds were detected in plasma and urine samples. Peak M29 had a $[M - H]^-$ at m/z 245, yielding a MS^2 fragment at m/z 165 (loss of 80 amu, cleavage of a sulfate moiety) and MS^3 fragments at m/z 121 and 119, corresponding to a standard of dihydrocoumaric acid (aka 3-(3′-hydroxyphenyl)propionic acid). Peak M29 was therefore partially identified as a dihydrocoumaric acid-*O*-sulfate. Peak M32 was identified as a coumaric acid-*O*-sulfate, with a $[M - H]^-$ at m/z 243 yielding a daughter fragment at m/z 163 with an 80 amu loss, and a subsequent MS^3 ion at m/z 119, corresponding to the fragmentation pattern of a standard of coumaric acid. It was not possible to discriminate between the para, ortho, or meta positions of the hydroxyl substitution of the phenyl group. Peak M35 had a similar MS fragmentation profile and HPLC retention time as a standard of dihydrocoumaric acid. Finally, the fragmentation pattern and retention time of peak M39 corresponded to those of a standard of *p*-coumaric acid (Table 6).

DISCUSSION

This is the first publication reporting in detail the identity of 60 flavonoids and phenolic compounds in Concord grape juice. Key structures are illustrated in Figure 1. The main group of compounds in the juice was the anthocyanins (680 ± 17 μmol/L), present as mono- and diglucosides, with acetyl and *p*-coumaroyl moieties of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Figure 1). The anthocyanin profile of Concord grape juice corresponds to that previously described,[17] with delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside being the major anthocyanins in Concord grape, unlike grape juice or wine produced from *Vitis vinifera*, in which the predominant anthocyanin is malvidin-3-*O*-glucoside.[13] The presence of pelargonidin-3-*O*-glucoside in Concord grape has also been reported,[17] but it was not detected in the current study despite a limit of detection of 0.5 ng (ca. 1 pmol) for all five anthocyanin monoglucosides. Overall, the anthocyanins accounted for 46% of the total (poly)phenolic content of the juice (Table 5).

Tartaric acid esters of hydroxycinnamates, namely, caftaric acid, coutaric acid, and, to a lesser extent, fertaric acid, were present in the juice and accounted for 29% of the total (poly)phenolic compounds. Cultivars of *V. labrusca* tend to have a higher content of these compounds, which occur mainly as the "trans" form, than *V. vinifera*.[25] Values obtained in this study for *trans*-coutaric acid (53 μmol/L) are similar to those previously reported in Concord grape (41 μmol/L),[25] although significantly less *trans*-caftaric acid was detected in the juice than the concentration previously reported (379 vs 715 μmol/L). Caftaric acid is readily oxidized during the crushing process in the presence of grape polyphenol oxidase, oxygen, and glutathione,[26,27] which could explain the variable levels of caftaric acid.

The third group of polyphenolic compounds identified in the grape juice was the procyanidins, which accounted for only 10% of the total (poly)phenols. The main procyanidins were (epi)catechin dimers, accounting for ca. 46% of the total procyanidin content. Although standards of (−)-epicatechin and procyanidins B1 and B2 yielded the same response in the fluorometer detector (data not shown), the presence of an (epi)gallocatechin and/or gallic acid unit suppresses fluorescence,[28] which, in this case, results in an underestimation of the total level of procyanidins. In the absence of reference compounds, there are, however, no methods currently available to accurately quantify oligomeric procyanidins.[29,30] The profile and content of monomeric flavan-3-ols in the Concord grape juice (95 ± 6 μmol/L) are similar to those described in red wine, although no procyanidins larger than trimers were quantified.[31] The concentration of flavan-3-ols varies with the pressing method (hot or cold) and with the pasteurization process, with hot-pressed and pasteurized juice containing more flavan-3-ols compared to unpasteurized, cold-pressed juice.[32] The Concord grape juice tested was prepared using the hot-press method, providing its rich and deep purple color, and was subsequently pasteurized. Levels reported previously

Table 6. HPLC and Mass Spectral Characteristics and Identity of Human Urinary Metabolites Obtained After an Acute Intake of 350 mL of Concord Grape Juice

peak	metabolite (*isomer*)[a]	t_R (min)	$[M - H]^-$ (m/z)[b]	MS^2	MS^3	location[c]
	flavan-3-ols					
M1	*O*-methyl-(epi)catechin-*O*-glucuronide*	16.7	479	303	285, 259, 244, 235, 219	U
M2	*O*-methyl-(epi)gallocatechin-*O*-sulfate	16.9	399	319	301, 275, 260, 235, 233, 165	U
M3	(−)-epicatechin-*O*-glucuronide*	18.8	465	289	245, 205	U
M4	(−)-epicatechin-*O*-sulfate (*1*)*	21.2	369	289	245, 205	U
M5	(−)-epicatechin-*O*-sulfate (*2*)*	27.0	369	289	245, 205	U
M6	*O*-methyl-(epi)catechin-*O*-sulfate (*1*)*	25.4	383	303	285, 259, 244, 235, 219	U
M7	*O*-methyl-(epi)catechin-*O*-sulfate (*2*)*	27.8	383	303	285, 259, 244, 235, 219	U
M8	*O*-methyl-(epi)catechin-*O*-sulfate (*3*)*	31.8	383	303	285, 259, 244, 235, 219	U
M9	*O*-methyl-(epi)catechin-*O*-sulfate (*4*)*	34.2	383	303	285, 259, 244, 235, 219	U
M10	*O*-methyl-(epi)catechin-*O*-sulfate (5)*	36.2	383	303	285, 259, 244, 235, 219, 137	U
	flavonol and anthocyanins					
M11	myricetin-*O*-glycoside-*O*-sulfate*	30.9	559	479	317	U
M12	delphinidin-3-*O*-glucoside	17.4	465^+	303		U, P
M13	cyanidin-3-*O*-glucoside	20.6	449^+	287		U
M14	petunidin-3-*O*-glucoside	23.2	479^+	317		U, P
M15	peonidin-3-*O*-glucoside	26.7	463^+	301		U
M16	malvidin-3-*O*-glucoside	28.4	493^+	331		U
M17	delphinidin-*O*-glucuronide (*1*)*	7.3	479^+	303		U, P
M18	delphinidin-*O*-glucuronide (*2*)*	9.7	479^+	303		U, P (traces)
M19	cyanidin-*O*-glucuronide*	14.9	463^+	287		U, P (traces)
M20	petunidin-*O*-glucuronide (*1*)*	12.8	493^+	317		U, P
M21	petunidin-*O*-glucuronide (*2*)*	17.3	493^+	317		U, P
M22	peonidin-*O*-glucuronide (*1*)*	28.3	477^+	301		U, P (traces)
M23	peonidin-*O*-glucuronide (*2*)*	29.4	477^+	301		U, P (traces)
M24	malvidin-*O*-glucuronide (*1*)*	20.9	507^+	331		U, P (traces)
M25	malvidin-*O*-glucuronide (*2*)*	29.8	507^+	331		U, P (traces)
	hydroxycinnamates					
M26	dihydrocaffeic acid-3′-*O*-sulfate	11.1	261	181	137	U, P
M27	caffeic acid-4′-*O*-sulfate	13.2	259	179	135	U
M28	caffeic acid-3′-*O*-sulfate	14.3	259	179	135	U, P
M29	dihydrocoumaric acid-*O*-sulfate*	14.3	245	165	121, 119	U, P
M30	dihydroferulic acid-4′-*O*-sulfate	14.5	275	195	177, 151, 136, 135, 123, 119	U, P
M31	dihydrocaffeic acid	14.6	181	137		U
M32	coumaric acid-*O*-sulfate*	16.3	243	163	119	U
M33	ferulic acid-4′-*O*-sulfate	18.0	273	193	178, 149, 134	U, P
M34	isoferulic acid-3′-*O*-sulfate	21.5	273	193	178	U
M35	dihydrocoumaric acid	25.6	165	121, 119		U, P
M36	feruloylglycine	26.2	250	206, 191, 177, 149, 100		U
M37	isoferulic acid-3′-*O*-glucuronide	27.5	369	193	178	U
M38	dihydroferulic acid	29.9	195	177, 151, 136, 135, 123, 119		U
M39	*p*-coumaric acid	30.7	163	119		U, P
M40	caffeic acid	31.4	179	135		P (traces)
M41	ferulic acid	53.0	193	178, 149, 134		P

[a] *, Tentative identification based on mass spectra. [b] +, molecular ion (m/z) identification in positive ionization mode. [c] P, plasma; U, urine.

for (+)-catechin and (−)-epicatechin for hot-pressed, pasteurized Concord juice ranged from 20.0 to 36.8 μmol/L (20.4 and 61.8 μmol/L for (+)-catechin and (−)-epicatechin, respectively, in our study) and from 20.9 to 41.7 μmol/L for procyanidins B1 and B2 (12.0 and 25.3 μmol/L for procyanidins B1 and B2, respectively, in our study).[32]

The remaining 9% of (poly)phenols consisted of flavonols, gallic acid, free hydroxycinnamic acids, and *trans*-resveratrol (Table 5). The total content of polyphenolic compounds amounted to 1508 ± 31 μmol/L of the juice.

Following an intake of 350 mL of Concord grape juice by healthy volunteers, absorption of various polyphenolic

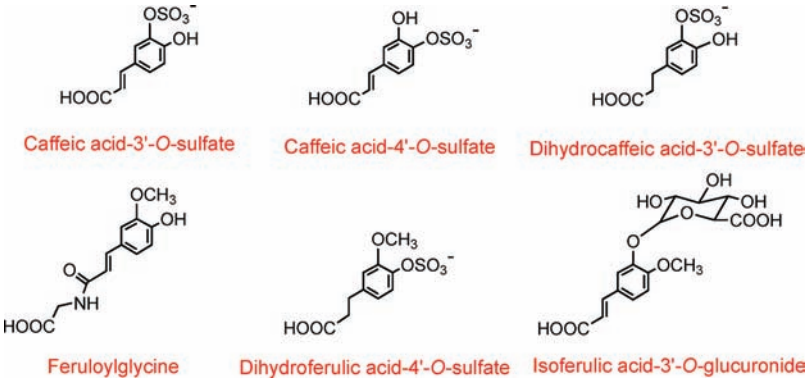

Figure 2. Structures of selected conjugates of tartarate esters of hydroxycinnamic acid metabolites identified in human plasma and/or urine after acute ingestion of 350 mL of Concord grape juice.

compounds resulted in the detection of a wide array of metabolites and derivatives in plasma and urine samples (Table 6). Structures of the identified bioavailable metabolites are presented in Figure 2. Sulfated, glucuronidated, and methylated (epi)catechin, and only one methyl-(epi)gallocatechin-sulfate, were detected in urine. The urinary profile of flavan-3-ol metabolites resembled that of metabolites reported in earlier studies.[22,33−35] These metabolites, however, did not accumulate in detectable quantities in plasma in the present study, probably due to the low amounts ingested (33 μmol) compared to the flavan-3-ol intakes in the earlier studies with green tea (ca. 600 μmol)[21,22] and a polyphenol-rich drink (362 μmol).[36,37]

Ingestion of 238 μmol of anthocyanins contained in the Concord grape juice resulted in their absorption and metabolism and could be detected in both plasma and urine. Only 3-*O*-glucoside anthocyanins could be detected in plasma and urine samples. Anthocyanin-3-glycosides are absorbed and excreted intact, although their bioavailability appears to be low.[10,38] Previous studies have reported that both the type of aglycone and the moiety attached affect their bioavailability.[39−42] Nonetheless, extensive methylation and glucuronidation of anthocyanins upon ingestion and absorption do occur,[43−46] which is consistent with the presence of anthocyanin glucuronides in plasma and urine detected in this study (Table 6).

Metabolism of tartarate esters of hydroxycinnamic acids resulted in the detection of a wide array of methyl, sulfate, and glucuronide metabolites of phenolic acids, whereas no intact *trans*-caftaric acid, *trans*-coutaric acid, or *trans*-fertaric acid could be detected in either plasma or urine after ingestion of 155 μmol of these compounds (Table 6). There is currently only limited information available on the absorption and metabolism of tartaric esters of hydroxycinnamic acids. Following intake of a single serving of white wine containing 162 μmol of tartaric esters, free, glucuronidated, and sulfated caffeic, ferulic, and *p*-coumaric acids were present in the plasma of humans, but no intact esters were detected,[47] which is in accordance with the data obtained in the current study. Plasma and urinary profiles of tartaric ester metabolites also resembled those obtained following intake of coffee containing chlorogenic acids, which are quinic acid esters of hydroxycinnamic acids.[11,24]

No intact or phase II metabolites of procyanidins could be detected in plasma or urine. Administration of procyanidin dimers and trimers to rats by gavage (1.8 mmol/kg of body weight) resulted in the circulation of dimers and trimers in plasma, at a maximum concentration of 2.5 nmol/L after 1 h,[48] suggesting an extremely low bioavailability despite high levels ingested. Oral administration of [^{14}C]procyanidin B2 by rats revealed a urinary excretion of 60% of the dose ingested,[49] which comprised microbial catabolites resulting from a colonic metabolism, with hydroxyphenylvaleric acids and hydroxyphenylpropionic acids being detected following ingestion of procyanidins.[50,51]

This paper has concentrated on the detailed analysis and identification of phenolic metabolites in Concord grape juice, plasma, and urine. Now that the main metabolites have been established, subsequent publications will consider the in vivo metabolism process of Concord grape juice (poly)phenolic compounds, emphasizing the role of the gastrointestinal tract on their metabolism and further degradation by bacteria in the colon.

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NOTE ADDED AFTER ASAP PUBLICATION

In the original ASAP publication of August 12, 2011, some compound names in Table 2 and the first paragraph under Results were in error. These have been corrected in the ASAP publication of August 17, 2011.