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Article

Bioaccessibility of Apple Polyphenols from Peel and Flesh during Oral Digestion

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ABSTRACT: Health benefits of apple polyphenols for different chronic diseases are postulated. To exert bioactive properties, absorption into the body is required (bioavailability), which is strongly influenced by matrix release (bioaccessibility). For seven apple varieties, in vitro experiments with simulated saliva fluid (SSF) and ex vivo digestion with centrifuged human saliva were conducted. Polyphenol characterization (high-performance liquid chromatography-tandem mass spectrometry) and quantification (high performance liquid chromatography-diode array detection) was related to an aqueous methanolic extraction. A polyphenol release of 63−82% from flesh and 42−58% from peel was estimated. While hydroxycinnamic acid derivatives were released in total, a significant retention was observed for flavanes and flavones. In particular, procyanidins were retained with increasing molecular weight. The data reveal a considerable polyphenol release during the oral digestion; however, differences among the varieties as well as flesh and peel were obvious. Due to negligible differences between both digestion media, the data supported the use of SSF instead of human saliva in further experiments.

KEYWORDS: *phenolic compounds, commercial apple varieties, traditional apple varieties, in vitro oral digestion, ex vivo oral digestion, human saliva, simulated saliva fluid, procyanidins, release*

INTRODUCTION

Apples (*malus domestica* BORKH) are the commercially most important and most consumed fruits in Germany, with a harvest of more than a million tons in 2020[1] and an estimated annual consumption of 21.9 kg in 2019/2020 per capita.[2] Apples are mainly composed of water (85%) and carbohydrates (14%) entailing sugar, mainly fructose, and also considerable quantities of fiber, as well as minor components such as minerals, vitamins (e.g., vitamin C), organic acids, carotenoids, and phenolic compounds.[3,4] The term phenolic compounds includes polyphenols (PP), for example, flavonoids and small molecules containing only one phenolic moiety, such as hydroxycinnamic acids, which are usually not included in the definition of PP. Nevertheless, in this article, the term PP and phenolic compounds are used synonymously for all phenolic compounds present in apples.

Epidemiological studies indicate that a diet rich in vegetables and fruits is associated with a reduced risk of several different chronic diseases.[5,6] It is postulated that these health effects might be related to PP and their antioxidative, anti-inflammatory and antitumor properties.[7−11] Particularly for apples, diverse health benefits are discussed. For example, positive effects on cardiovascular health, diabetes mellitus, and lung cancer are attributed to fiber, vitamins or PP.[3,5,6] Because apples are available throughout the year, they are known to be an important PP source in the western diet.[12] In addition to the health benefits stated above, a reduction of the allergenic potential with regard to the allergen Mal d 1 by PP in apples is proposed.[13]

In all apple varieties, hydroxycinnamic acid derivatives (HZDs), dihydrochalcones, flavanols, flavonols, and anthocyanins are the main PP groups, the latter two only occur in the peel. However, the PP content and profile among apple varieties differ markedly.[14−16] Furthermore, several other factors impacting the PP content besides the variety are known, for example, UV radiation, storage, and growth conditions.[7,17] Nevertheless, it is reported that apple varieties with a high commercial value often exhibit reduced PP contents compared to "old" varieties grown locally in orchards.[18,19] This might be explained by the consumers' demand for sweet apples with slow enzymatic browning. Therefore, breeding is focused on cultivars with a low phenolic content because PP are substrates for the polyphenoloxidase (PPO), an enzyme that oxidizes a number of phenolic substances to highly reactive electrophilic quinones. The latter polymerize in several further reactions forming brown pigments.[20]

To assess possible health benefits of PP, the fraction of absorbed PP in the body (bioavailability) must be taken into consideration.[21] Bioaccessibility describes the amount of PP released from the food matrix, which is available for intestinal absorption.[22] Thus, the bioaccessibility has a strong impact on bioavailability, providing that only PP that are not bound to any food matrix are absorbable.[21]

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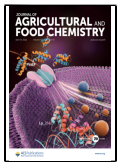

Apples contain a high amount of pectins and are considered as a complex food matrix. Human studies about bioavailability of PP are scarce for different apple products,[23−25] limiting approximations of matrix-dependent bioaccessibility. Furthermore, under in vitro conditions, the oral phase is also often omitted from digestion models studying the bioaccessibility.[22] To the best of our knowledge, only one study is published, which indicates that the oral digestion phase has an effect on the bioaccessibility of PP from apples.[26] Besides a proposed absorption mechanism via the oral mucosa epithelium,[7] released PP are able to react with further matrix components. It is discussed that the interaction of different PP with the apple allergen Mal d 1 plays an important role in the oral allergy syndrome after apple consumption. Various specific investigations of the PP bioaccessibility as well as differences between the individual phenolic structures are missing so far.

A wide range of different in vitro digestion models are used in research, complicating the evaluation of different studies. Therefore, the COST INFOGEST network of scientists combined the digestion models mostly used and made basic recommendations about the setup of static digestion experiments to increase the comparability of future results.[27] However, human saliva contains bacteria of the oral cavity for which deglycosylation of PP is described.[23] To assess the comparability of human saliva and the simulated saliva fluid (SSF) recommended by COST,[27,28] which is considerably simplified compared to human saliva, the release of PP from apples is investigated under in vitro and ex vivo conditions.

MATERIALS AND METHODS

Fruit Material and Preparation. Seven different apple varieties were either donated by A. Siegle from the Obstbauberatung Stuttgart (OBS, Liegenschaftsamt, Stuttgart, Germany; Boskoop, Gewürzluiken, Goldparmäne and Ontario), bought from local supermarkets (LS; Braeburn and Holstein Cox), or from an organic apple farm (OAF; Santana), near Hamburg, Germany. Information on each variety such as supplier, water contents of flesh and peel, as well as proportion of flesh and peel to the whole apple is listed in Table S1 in the Supporting Information. Furthermore, information about the parents and the first mention in the literature is provided for each variety.[29,30]

Apples were stored in the dark at 10 °C for 8 to 21 days until sample preparation. For each variety, 8 to 11 apples were washed, quartered, the stem and carpel were removed, and peeled with a peeler by hand. The weights of peel and flesh were determined individually before freezing the samples in liquid nitrogen immediately. The frozen pieces were freeze-dried for 2 to 4 days using a Labconco Freezone 12 L Console Freeze Dry System (Kansas City, MO). Afterward, the dried peel was milled for 30 s at 10 000 rpm with an IKA Tube Mill Drive (Staufen, Germany), and the flesh was pulverized for 10 s at 10 000 rpm using a Retsch Grindomix GM 200 (Haan, Germany). Powdered samples were homogenized, stored under argon and kept in the dark at room temperature until further use.

Chemicals. All chemicals were of analytical grade. For mass spectrometry (MS), analytic solvents and formic acid were of MS grade. Acetonitrile and methanol were bought from Fisher Scientific (Loughborough, UK). Hydrochloric acid (HCl, 37%) was obtained from Grüssing (Filsum, Germany), and formic acid from Merck (Darmstadt, Germany). Potassium chloride, potassium dihydrogen phosphate, and calcium chloride were bought from Roth (Karlsruhe, Germany). Sodium hydrogen carbonate, magnesia chloride hexahydrate, and ammonium carbonate were acquired from Merck (Darmstadt, Germany). Ultrapure water (ELGA PurLab flex, Veolia Waters, Celle, Germany) was used throughout the experiments.

PP Standards. Cyanidin-3-glucoside (CYD-glc), (+)catechin (CAT), procyanidins (PC) B1, B2, and C1, and isorhamnetin-3-rutinoside (IRH-rut) were purchased from PhytoLab (Vestenbergsgreuth, Germany). Phlorizin dihydrate (PHL), (−)epicatechin (EC), chlorogenic acid (CA), *p*-coumaric acid (COA), and quercetin-3-glucoside (Q-glc) were acquired from Sigma-Aldrich (Schnelldorf, Germany). Stock solutions of the PP standards were prepared in methanol/water (50/50, v/v) except for CYD-glc, which was dissolved in 0.01% HCl. The concentrations of the stock solutions were quantified using *q*-NMR according to a method published previously.[31] For Q-glc, CYD-glc, and PC C1, *q*-NMR was not successful, therefore, quantification was carried out based on weight.

PP Extraction and Analysis. Extractions were conducted in argon-filled containers in duplicate. An amount of 0.5 g peel or 1.5 g flesh was extracted with 7.5 mL methanol/water (80/20, v/v) containing 0.02% hydrochloric acid. Five glass beads were added to each sample, which were mixed three times for 60 s at 6000 rpm on an IKA Ultra Turax Tube Drive (IKA, Staufen, Germany). The glass beads were removed, and the extraction was continued for further 1.5 h on an IKA shaker (IKA, KS 501 digital, Staufen, Germany) at 100 rpm. After extraction, the tubes were centrifuged (Type Z326 K, Hermle Labortechnik, Wehingen, Germany) at 10 410 rcf at 4 °C for 20 min. The supernatant was removed and stored in the fridge at 5 °C. The residue was re-extracted with fresh extraction solvent for another 1.5 h on the IKA shaker. After centrifugation, the residue was washed with 2.5 mL extraction solvent twice. All supernatants were combined, filtered through 45 μm PTFE syringe filters Ø 2 cm (VWR, West Chester, USA), concentrated (R-215, Büchi, Fawil, Switzerland; bath temperature 30 °C), and adjusted with 0.01% aqueous HCl to a defined volume of 2 mL. The extracts were stored at −20 °C until analysis.

Separation of the PP for characterization and quantification was performed using a 1260 Agilent high-performance liquid chromatography system (Agilent, Santa Clara, CA), equipped with a binary pump (1260 ALS) including a degasser and an autosampler. A C18 Nucleodur Gravity-SB column (150 × 2 mm ID 2 μm (Machery & Nagel, Duren, Germany)) was run at 35 °C and a constant flow rate of 0.2 mL/min. Eluent A consisted of 1/3/96 (v/v/v) and eluent B of 1/90/9 (v/v/v) formic acid/acetonitrile/water. The starting conditions were 95% eluent A and 5% eluent B. Eluent B was raised to 35% in 45 min, then to 60% in 17 min, and to 100% in 2 min, followed by a return to the initial conditions and re-equilibration. The injection volume of samples was 5 μL. Prior to analysis, the samples were diluted 1 + 4 with 0.1% methanolic HCl.

Phenolic structures were characterized with an UV detector (Agilent 1260 Infinity II VWD G7114 A) at 280 nm and a quadrupole-time of flight mass spectrometer (Bruker Impact II, Bruker, Billerica, USA) operated in the positive and negative ionization mode. The samples were ionized in an ESI chamber with nitrogen as the nebulizing gas and a pressure of 2 bar, a flow rate of 8 L/min, and a gas temperature of 250 °C. Ionization energies were −4 and 4 eV, and collision energy for fragmentation was −7 and 7 eV for positive and negative mode, respectively. A scan range of m/z 500−2000 was used. Data evaluation was performed with Bruker Compass Data Analysis 4.4 (Bruker Daltonik, Billerica, USA). Structure assignment was based on in-house phenolic standard compounds and literature data (Table 1).[15,32−35]

Quantification of PP was conducted on a HPLC-DAD System (Agilent 1260 series) equipped with a quaternary pump (G1311B), an autosampler, a degasser, and a diode array detector (1260 DAD VL). Chromatographic conditions were identical to the conditions used for PP characterization. The PP in the samples were quantified using an external calibration. The standard solutions were mixed and diluted with methanol/water (50/50, v/v) or 0.01% for CYD-glc, respectively. Five different calibrant solutions, CYD-glc (1); CAT, EC, PC B1, PC B2 and PC C1 (2); PHL and COU (3); CA (4); Q-glc and ISR-rut (5), were prepared. Calibration ranges for the PP standards are listed in Table 1. If no standard was available, quantification was based on a standard with a similar aglycon and corrected by the corresponding mass correction factor. For the PC oligomers, no correction was required because the increase in absorption per monomer unit and the increase in mass were nearly

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Table 1. Retention Time, Detection Wavelength, Calibration Range, and Mass Spectrometry Data for Compounds Found in the Apple Samples[a]

				(M + H)$^+$				(M-H)$^-$				
polyphenols	RT [min]	detection wavelength [nm]	calibration range [mg/L]	expected mass	error [mDa]	fragments		expected mass	error [mDa]	fragments		refs
anthocyanins												
cyanidin-glucoside[b]	12.6	520	10−197	449.1084	2.0	287.0541		447.0927	0.1	285.0386	*284.0323*	*d*,15,32,34
hydroxycinnamic acids												
***p*-coumaric acid**	21.6	320	1−47	163.1395	−1.3	119.0474		165.0552	2.8	147.0420	119.0475	*d*
hydroxycinnamic acid derivatives												
coumaroylquinic acid 1	11.5	320		339.1080	2.1	147.0430	119.0481	337.0923	−0.3	163.0405	173.0456	15,32−35
coumaroylquinic acid 2	16.6	320		339.1080	1.7	147.0428	119.0478	337.0923	−0.6	163.0410	173.0462	15,32−35
coumaroylquinic acid 3	19.0	320		339.1080	2.6	147.0430	119.0485	337.0923	−0.8	163.0406	173.0462	15,32−35
chlorogenic acid	13.5	320	23−910	355.1029	15.2	163.0378	117.0323	353.0873	−0.5	191.0569		*d*,15,32−35
unknown hydroxycinnamic acid derivative[c]	17.4	320		355.1029	−6.9	163.0378	117.0323	353.0873	−0.3	191.0569		
dihydrochalcones glycosides												
phlorizin	34.9	280	5−187	437.1448[c]		275.0751		435.1291	−0.5	273.0769	167.0356	*d*,15,32−35
phloretin-pentosyl-hexoside 1	3.0	280		569.1870	2.2	275.0901		567.1714	−1.0	273.0768	167.0356	15,32,34,35
phloretin-pentosyl-hexoside 2	31.5	280		569.1870	1.5	275.0901		567.1714	−0.8	273.0768	167.0357	15,32,34,35
phloretin-pentosyl-hexoside 3	32.3	280		569.1870	2.2	275.0908		567.1714	−0.8	273.0767	167.0360	15,32,34,35
phloretin-hexosyl-hexoside	29.4	280		599.1976	1.3	275.0902	169.0475	597.1819	−2.0	273.0780	167.0356	32
flavanols												
(+)catechin	12.3	280	2−62	291.0869	8.1	139.0378	123.0431	289.0712	−0.5	245.0823		*d*,15,32−34
(−)epicatechin	17.8	280	10−401	291.0869	2.1	139.0379	123.0430	289.0712	−0.6	245.0822		*d*,15,32−34
procyanidin B1	9.4	280	4−155	579.1503	2.6	289.0694	291.0849	577.1346	−0.4	289.0718	125.0242	*d*,15,32−34
procyanidin B2	15.7	280	5−193	579.1503	2.9	289.0696	291.0850	577.1346	−0.7	289.0717	125.0249	*d*,15,32−34
procyanidin C1	20.6	280	2−100	867.2136	3.2	579.1479	289.0693	865.1980	−0.2	289.3196	125.0252	*d*,15,32,34
procyanidin trimer 1	15.4	280		867.2136	3.2	579.1473	289.0690	865.1980	0.6	289.0718	125.0252	15,32,34
procyanidin trimer 2	22.8	280		867.2136	3.7	579.1484	289.0692	865.1980	−0.8	289.0721	125.0252	15,32,34
continuation of flavanols												
procyanidin tetramer 1	16.6	280		1155.2764	3.3	579.1473	289.0686	1153.2612	−1.4	289.0719	125.0252	15, 32
procyanidin tetramer 2	17.4	280		1155.2764	3.4	577.1331	289.0694	1153.2612	−0.8	289.0721	125.0250	15, 32
procyanidin tetramer 3	22.0	280		1155.2764	3.0	579.1481	577.1300	1153.2612	0.3	289.0707	125.0252	15, 32
procyanidin pentamer 1	19.3	280		722.1741[c]	2.3			720.1548[c]	−1.2	125.0253		15
procyanidin pentamer 2	22.5	280		722.1741[c]	3.0	289.0698		720.1548[c]	−0.8	289.0721	125.0251	15
procyanidin pentamer 3	23.4	280		722.1741[c]	2.3	289.0699		720.1548[c]	−0.6	289.0689	125.0263	15
procyanidin pentamer 4	24.5	280						720.1548[c]	−0.3	125.0257		15
procyanidin hexamer 1	23.9	280		866.2059	3.6	577.1306	289.0701	864.1902	−0.9	289.0723	125.0249	15
procyanidin hexamer 2	24.7	280		866.2059	3.2	577.1336	289.0692	864.1902	−0.5	289.0719	125.0251	15
procyanidin heptamer	25.3	280						1008.2219	3.4	289.0714	125.0249	15
flavonols												
quercetin-hexoside 1	27.9	370		465.1033	1.1	303.0471		463.0878	−0.1	301.0339	300.0273	15, 32−35
quercetin-glucoside[b]	28.6	370	3−103	465.1033	2.0	303.0486		463.0878	−0.2	301.0343	300.0274	*d* 15, 32, 35,
quercetin-pentoside 1	29.9	370		435.0927	1.7	303.0192		433.0771	0.0	301.0346	300.0275	15, 32−35
quercetin-pentoside 2	31.5	370		435.0927	1.5	303.0490		433.0771	−0.2	301.0340	300.0277	15, 32−35

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Table 1. continued

polyphenols	RT [min]	detection wavelength [nm]	calibration range [mg/L]	(M + H)$^+$			(M-H)$^-$			refs
				expected mass	error [mDa]	fragments	expected mass	error [mDa]	fragments	
flavonols										
quercetin-pentoside 3	32.0	370		435.0927	2.1	303.0488	433.0771	0.0	301.0347 300.0273	15, 32−35
quercetin-pentoside 4	30.6	370		435.0927	2.2	303.0485	433.0771	0.0	301.0344 300.0272	15, 32−35
quercetin-deoxyhexosyl-hexoside	27.6	370		611.1612	2.3	303.0486 465.1008	609.1456	0.0	301.0338 300.0276	15, 32−35
quercetin-deoxyhexoside	32.3	370		449.1068	1.5	303.0488	447.0927	−0.6	301.0347 300.0282	15, 32−35
quercetin[f]	42.0	370		303.0505	2.2		301.0348	−0.6	151.0041	33−35
isorhamnetin-rutinoside	32.2	370	1−39	625.1769	5.5	317.0625 479.1143	623.1612	0.3	315.0503 300.0269	[d] 34,
isorhamnetin-hexoside	33.2	370		479.1190	2.6	317.0640 302.0403	477.1033	0.8	243.0310 271.0246	32, 34
isorhamnetin-pentoside 1	36.0	370		449.1084	2.3	357.8623 347.7467	447.0927	−0.2	243.0301 271.0248	32, 34
isorhamnetin-pentoside 2	37.0	370					447.0927	0.0	243.0294 271.0238	32, 34

[a]Compounds used as standards are marked in bold letters. [b]Glucosides were used as standards; due to a similar retention time to galactosides, it is not possible to distinguish between both compounds. [c]Double charged. [d]In-house measurements. [e]Standard addition with neo- and cryptochlorogenic acid was inconclusive. [f]Most likely an artifact of sample preparation.

equal.[31] Flavanols and dihydrochalcones were quantified at 280 nm, hydroxycinnamic acid derivatives at 320 nm, flavonols at 370 nm, and anthocyanins at 520 nm. Data evaluation was performed with Open Lab ChemStation CDS edition C01.07 SR3 (Agilent Technologies). In some samples, coelutions of CAT and CYD-hexoside (CYD-hex), PC tetramer 1 and a coumaroylquinic acid (COUM), PC tetramer 2 and an unknown HZD, two phloretin-pentosyl-hexosides (PHL-pent-hex) and quercetin-pentosides (Q-pent), as well as PHL-pent-hex and a Q-deoxyhexoside (Q-dohex) were observed. When coelution occurred, only one substance showed an absorption at a wavelength of 280 nm and was quantified at this wavelength. The other compound was quantified by subtracting the area of the quantified compound at 280 nm. For quantification of the total PP content, all PP were summed up.

Simulation of Oral Digestion. Oral digestion experiments under in vitro conditions were performed with SSF.[27] For the ex vivo oral digestion, human saliva from two healthy females (age 25 and 23) was collected in the morning before breakfast and brushing teeth. The saliva was centrifuged at 10 410 rcf for 10 min, and the supernatant was used for the experiments. It was required by the ethic commission of the University of Stuttgart that each proband used only her own saliva for experiments. Therefore, differences in PP release do not only represent inter person differences in saliva but also the inter person differences in sample preparation.

SSF was prepared according to the COST protocol.[27,28] Because apples have a low starch content, no α-amylase was added. All fluids for the oral digestion were prewarmed to 37 °C in an incubator (Binder 9010−0323, Tuttlingen, Germany). 1.5 g of freeze-dried flesh or 0.5 g peel was rehydrated to its mean original water content (7.82 mL for flesh, 1.93 mL for peel), then SSF or saliva was added in a 1 + 1 ratio based on the weight of the freeze-dried sample. Samples were homogenized for 5 s, incubated for 2 min at 37 °C, and cooled immediately on ice before they were centrifuged for 30 s at 4 °C and 10 410 rcf. The supernatants were filtered through 45 μm PTFE syringe filters and diluted 1 + 4 with 0.1% methanolic HCl and stored at −20 °C until use. Quantification was performed as described previously. Characterization of the PP was based on the retention times in the respective methanolic extract. The ratio of quantified PP in the oral phase samples and in the extraction with methanol/water was calculated. All oral digestion samples were prepared in duplicate.

Sample Stability. During experiments, the formation of two storage products in samples diluted with methanol was observed. To verify the formation of these products due to storage, the samples of the flesh from the varieties Braeburn, Boskoop, and Gewürzluiken were diluted as previously described, 1 + 4 with acidified methanol. The samples were analyzed every 3 to 4 days while stored at room temperature in the dark.

Statistical Analysis. For statistical analysis, Excel 2016 with the add-in Real Statistics (release 7.6)[36] was used. Data were checked for normality and homogeneity of variance by Shapiro−Wilk and Levene's test, respectively. Either one-way analysis of variance (ANOVA, α = 0.05) or Kruskal−Wallis (α = 0.05) was used to test for statistical significances between groups. If the test was significant, Tukey's test or Dunn's test was applied as post-hoc test.

RESULTS

PP Content and Profiles of the Different Apple Varieties. The PP contents in the flesh and peel differed among the apple varieties. In the flesh, 112−604 mg PP in 100 g dry weight (DW, 17−127 mg/100 g fresh weight, FW) was determined. In the peel, the content ranged between 378 and 1224 mg PP per 100 g DW (68−285 mg/100 g FW). The PP content in the dried peel was 2.64 to 4.45 times higher than in the dried flesh (Table 2, Figure 1). However, it must be kept in mind that the peel is only a small part of the whole fruit. Depending on the diameter of the fruit, the ratio of peel to flesh (FW) was between 0.15:1 and 0.23:1. Based on the water content and the ratio of peel and flesh, the PP content of 100 g

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Table 2. Total Phenolic Content of Different Apple Cultivars[a]

variety	PP flesh DW (mg/100 g DW)	PP peel DW (mg/100 g DW)	PP flesh FW (mg/100 g FW)	PP peel FW (mg/100 g FW)	ratio mass peel to flesh (FW)	ratio PP peel to flesh (FW)	calculated PP for cored apple (mg/100 g FW)[b]
Boskoop	354 ± 12	773 ± 19	72 ± 2	193 ± 5	0.15:1	2.91:1	90 ± 3
Braeburn	192 ± 2	631 ± 35	27 ± 0	120 ± 7	0.17:1	4.45:1	41 ± 1
Gewürzluiken	604 ± 25	1152 ± 49	127 ± 5	334 ± 14	0.17:1	2.64:1	158 ± 6
Goldparmäne	476 ± 6	1224 ± 42	86 ± 1	285 ± 10	0.23:1	3.33:1	123 ± 2
Holstein Cox	239 ± 1	535 ± 3	38 ± 0	104 ± 1	0.19:1	2.69:1	49 ± 0
Ontario	327 ± 0	904 ± 78	49 ± 0	172 ± 15	0.15:1	3.50:1	65 ± 2
Santana	112 ± 2	378 ± 5	17 ± 0	68 ± 1	0.20:1	4.06:1	25 ± 0

[a]PP, polyphenol content, mean ± average deviation (n = 2), analysis was performed in duplicate; therefore, the test for variance homogeneity and normality is not feasible. Due to a level of significance of 0.046 (Kruskal–Wallis test), post-hoc tests are not convincing. [b]Content is calculated based on the data for the water content and the ratio of peel and flesh. DW, dry weight; FW, fresh weight.

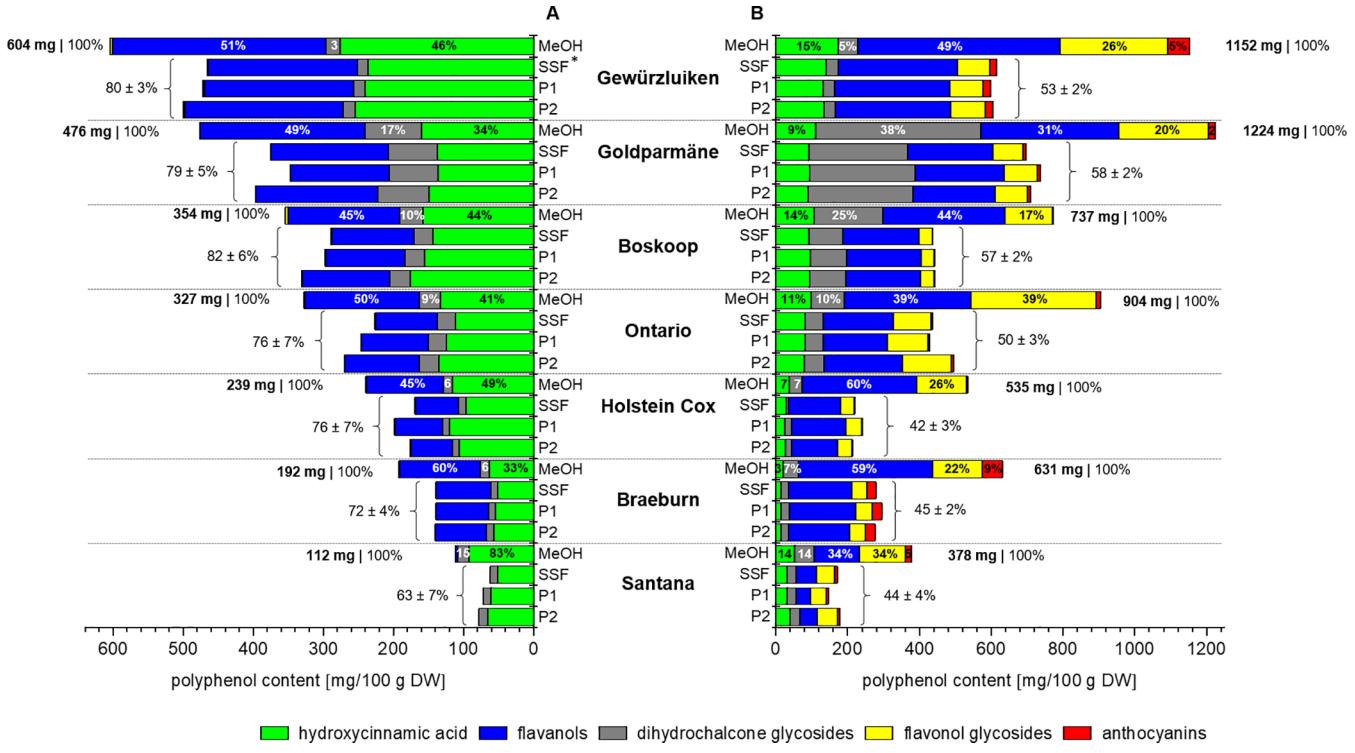

Figure 1. Phenolic profile in flesh (A) and peel (B) in aqueous methanolic extraction (MeOH), SSF according to COST protocol (SSF),[27] and saliva of proband 1 (P1) and 2 (P2). The axis scale for the phenolic content differs by a factor of 2 between the graphs for flesh and peel. The total phenolic content and the ratio of the phenolic groups are included in MeOH extraction. The average release [%] in oral media (n = 6, SSF and saliva, standard of the population) is calculated based on the phenolic content in the MeOH extraction. No significant differences were observed between SSF and human saliva (P1, P2) of each variety and tissue, values below 2% are not shown. *n = 1.

cored apple was calculated. The calculated PP contents ranged between 25 and 158 mg/100 g FW.

It stands out that the varieties with the highest PP content, Gewürzluiken and Goldparmäne, are varieties that are not commercially important and are usually not found in a German supermarket, whereas the commercially important varieties such as Braeburn and Holstein Cox showed lower levels of PP. Boskoop was an exception. This variety had a relatively high PP content and is available in German supermarkets during autumn and early winter. However, Boskoop apples are usually used for cooking and baking and not for fresh consumption.

All PP found in the different apple varieties and their quantified content in the dry mass are provided in the Supporting Information (Tables S2 and S3). The phenolic profiles of the flesh samples were comparable among cultivars and contained 33–49% HZDs, 45–60% flavanols, 3–17% dihydrochalcone glycosides, and traces (≤2%) of flavonol glycosides. An exception was Santana, which contained only 2% flavanols (Figure 1) and 83% HZDs. An outstanding high amount of dihydrochalcone glycosides was found in Goldparmäne with 17%, whereas these compounds were especially low in Gewürzluiken (3%). All found dihydrochalcones were glycosides of phloretin. The main hydroxycinnamic acid derivate in all apples was chlorogenic acid (CA). Roughly 20–25% of all flavanols in the flesh were the monomers CAT and EC. Exceptions here were on the one side Ontario with just 15% monomers and on the other side Santana with >60% monomers. The most prominent PC in all samples was the dimer B2 (Figure 2).

The PP profiles of flesh and peel differed significantly. Flavonols with 17–39% and anthocyanins with 2–9% were exclusively found in the peel (Figure 1), which is in accordance with the literature data.[13] With a proportion of 3–15%, HZDs were less prominent in the PP composition compared to the

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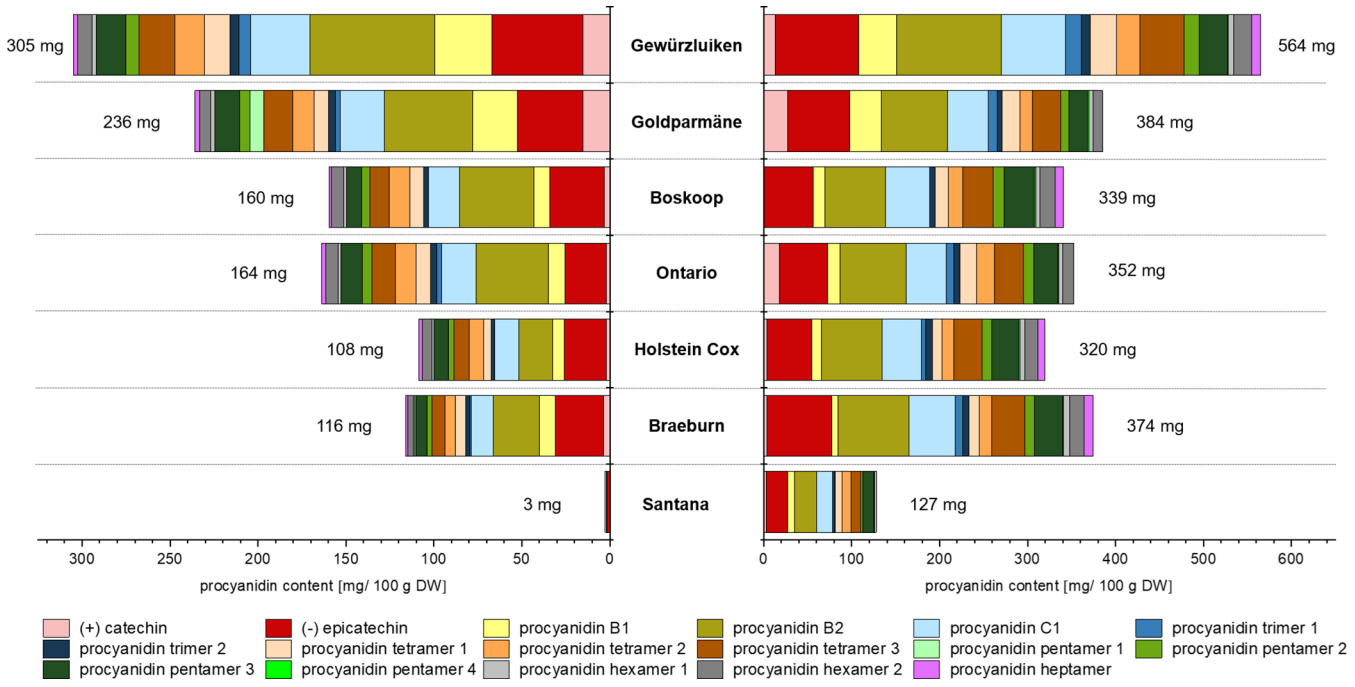

Figure 2. Distribution of different procyanidins in flesh (A) and peel (B) from methanolic extraction. The axis scale of the procyanidin content differs by a factor of 2 between the graphs for flesh and peel.

flesh. Flavanols (31−60%) were the most important group in the peel, and the amount of dihydrochalcone glycosides differed significantly among the varieties (5−38%). Compared to other apples investigated, Braeburn and Holstein Cox showed low contents of both dihydrochalcones and HZDs (below 8%). On the contrary, Goldparmäne und Boskoop exhibited high dihydrochalcone contents of 38% and 25% in the peel, respectively. The individual phenolic structures found in peel and flesh were similar. In most cases, flavonol glycosides were quercetin glycosides. Only one cyanidin hexoside was detected in significant amounts, according to the literature, most likely cyanidin galactoside.[19] Because the anthocyanin and flavonol contents strongly depend on UV radiation, the contents were more variable than for other PP structures.[17]

In apple flesh and peel, flavanols were the most diverse flavonoids, regarding individual structures, molecular weights, and contents in apples (Figure 2). With the extraction method used, a heptamer (PC 7) consisting of seven monomer (catechin or epicatechin) units was the oligomer with the highest molecular weight (MW). However, it should be kept in mind that even longer PCs might be present in the apples, which were not extractable and/or analyzable with the methods optimized for monomeric structures. With increasing MW, it was more likely that the PCs were double charged in the ESI source.[15] The isotope pattern allowed to distinguish between single- and double-charged PCs and therefore to decide if, for example, a hexamer or a trimer was detected. In the flesh, the flavanol contents ranged between 109 and 305 mg per 100 g DW and in the peel between 320 and 564 mg/100 g DW. In Santana, the flavanol content was exceedingly low with 3 mg/100 DW and 127 mg/100 g DW in flesh and peel, respectively. The most important flavanols in flesh were EC (14−23%, 60% in Santana), followed by PC B2 (16−27%) and PC C1 (11−24%). In peel and flesh, the same PC compounds were found in roughly the same ratio.

PP Release during the Oral Digestion Phase. Neither in flesh nor in peel, significant differences between the three media used to simulate oral digestion ex vivo and in vitro (saliva from proband 1 and 2 and SSF) were observed (Figure 1). The bioavailability of the PP, individual and summarized into groups, is provided in the Supporting Information (Table S4 for peel, Table S5 for flesh).

Depending on the apple variety, 63−82% PP were released from flesh based on the PP content of the methanolic extract (Figure 1). With 63%, the average release was remarkably lower for Santana than for other apples. Because the PP content of Santana was low, it might be possible that some PP released during oral digestion were below the limit of detection and quantification and therefore the amount of free PP might be underestimated. Of course, the absolute PP release was higher from peel than from flesh; however, the bioaccessibility of PP from the peel was significantly reduced (42−58%) compared to flesh.

Differences in the release were obvious among the PP groups (Table 3). In the flesh, a release of 87 ± 12% HZDs and 81 ± 7% dihydrochalcones based on their content in the methanolic extracts was determined. In contrast, the release of flavanols was significantly reduced (66 ± 7%). The release of flavonols is not discussed because they were only present in traces in the methanolic extracts. The differences in bioaccessibility of the PP in the flesh lead to a PP profile with an increased proportion of dihydrochalcones and HZDs and reduced flavanols in the oral digestion samples. Furthermore, it indicates that the phenolic profile had an impact on the overall bioaccessibility. For example, an apple with a high percentage of dihydrochalcones and HZDs in the flesh had a higher overall PP release than an apple rich in flavanols.

In the peel, the release was reduced for all phenolic structures. The bioaccessibility was the highest for HZDs (77 ± 8%), followed by dihydrochalcones and flavanols with 54 ±

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Table 3. Bioaccessibility of Phenolics from Flesh and Peel Based on the Content Found in the Methanolic Extraction[1]

phenolic groups	bioaccessibility from the flesh (%)	bioaccessibility from the peel (%)
hydroxycinnamic acid derivatives	87[a] ± 12	77[a] ± 8
dihydrochalcones	81[a] ± 7	54[b] ± 5
flavanols	66[1,b]±7	52[bc] ± 9
flavonols		33[c] ± 4
anthocyanins		42[c] ± 6

[1]The data do not contain the values for Santana due to a low flavanol content, resulting in concentrations below the limit of quantification in the oral phases. Mean ± standard deviation of the population (n = 21) is shown. Significant differences in the same column are indicated by different letters. Test for significance was done by one-way ANOVA, followed by a Tuckey's test (α = 0.05) for the flesh and Kruskal–Wallis, followed by Dunn's test (α = 0.05) for the peel.

5% and 52 ± 9%, respectively. With 42 ± 6% and 33 ± 4%, anthocyanins and flavonols were the phenolics with the lowest bioaccessibility. However, it must be kept in mind that the anthocyanin concentration in the samples was relatively low and sometimes coelution of CAT and the CYD-hex occurred, thus the calculated value might include a considerable error. The results indicated that the proportion of HZDs and dihydrochalcones increased, while flavonols and anthocyanins decreased during oral digestion with respect to the methanolic extracts. Because flavonols are present in relevant amounts in the peel (methanolic extracts 17–39%), the reduced overall bioaccessibility from the peel might be explained by different polysaccharide compositions but also by the generally low release of flavonols as well.

A closer look at flavanols showed a general trend in peel and flesh to a decreased bioaccessibility with an increasing number of subunits (MW) (Figure 3). However, a marked difference among the apple varieties was observed (Figures S6 and S7). As noted for other phenolics, the release of PCs was poorer for peel than for flesh. The high variance for CAT might be explained by generally low concentrations, close to the limit of quantification, and its coelution with CYD-hex in some samples. Both PC hexamers, the heptamer, pentamers 1 and

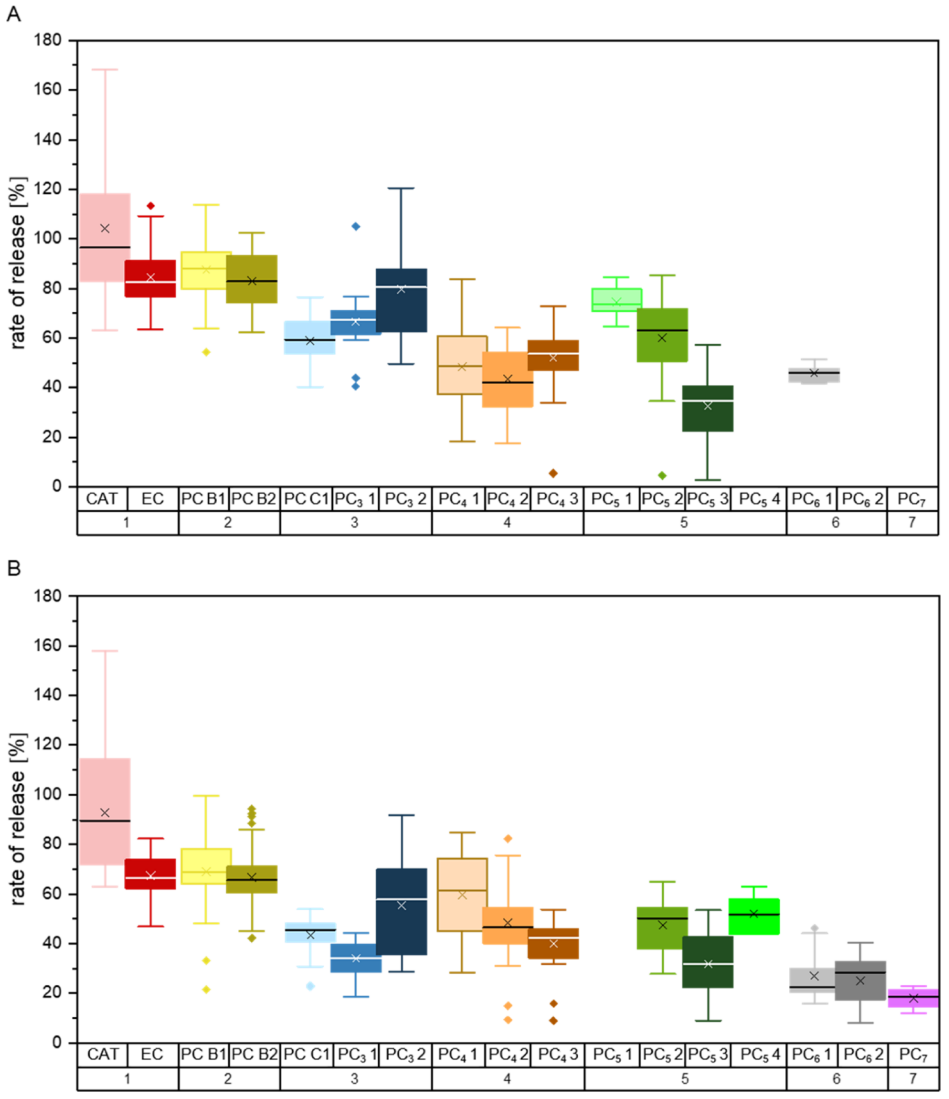

Figure 3. Release [%] of individual flavanols according to their monomer units (1–7) over all apple varieties from flesh (A) and peel (B) in digestion media (saliva, SSF) based on methanolic extracts. Samples without detectable compounds are not included. In Boskoop and Braeburn, catechin is absent due to low values and therefore high errors. Detailed data, individual for each apple variety, are provided in Figures S6 and S7.

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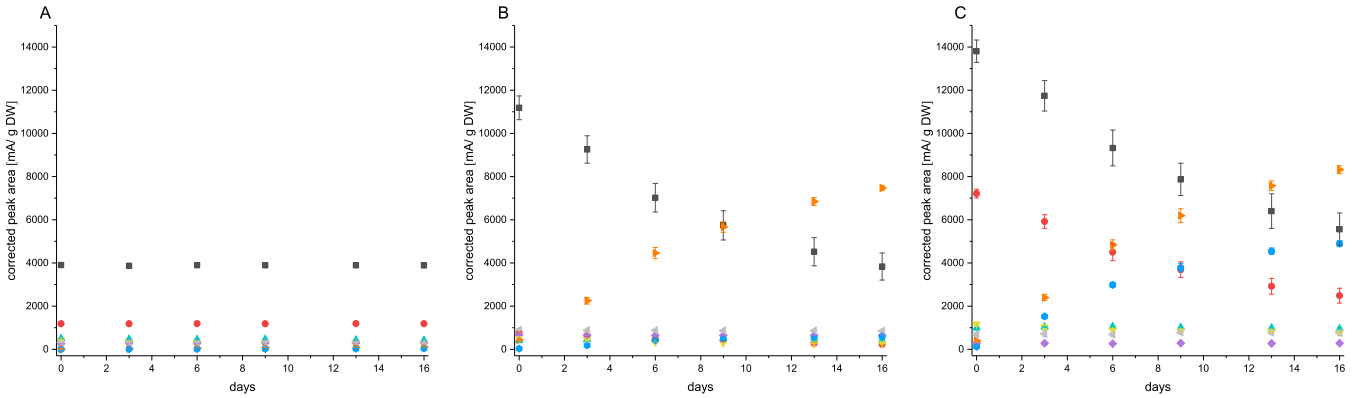

Figure 4. Decay of CA (black), COUM (red), EC (turquoise), PC B2 (yellow), PHL-pent-hex (purple), and PHL (gray), and formation of storage products 1 (orange) and 2 (blue) in the flesh of Braeburn (A), Boskoop (B), and Gewürzluiken (C). Experiments were performed in duplicate, and mean ± average deviation (n = 2) is shown.

4, as well as tetramer 3 were only found in part of the SSF and saliva samples.

Sample Stability. Subsequent to the oral digestion, saliva samples and SSF were diluted 1 + 4 with methanol, analyzed, and stored in a freezer at −20 °C. Data evaluation showed two compounds, at 24.3 min (m/z 369.1181 (positive mode) and 367.1057 (negative mode), product 1) and 25.8 min (m/z 353.1129 (positive mode) and 353.1229 (negative mode), product 2) in the samples (fragmentation for mother ions is listed in Table S8), which could not be identified as typical phenolic compounds routinely found in apples. Measurements 6 to 9 months later revealed a significant increase in these compounds, interpreting both as products due to oxidation and storage. Similar products have been detected in the stored aqueous extracts obtained from the aqueous methanolic extraction, but to a lesser extent. The amount of formed storage products, estimated on the area at 320 nm, differed between the samples. Furthermore, a decrease of CA and COUM contents in some apples was observed.

To understand the impact of oxidation during the oral phase and sample storage, aqueous extracts of the methanolic extraction of the flesh of Boskoop, Braeburn, and Gewürzluiken were diluted with methanol and stored at room temperature. The decay of CA and COUM and the formation of products were monitored. The products formed exhibited absorption maxima at 328 and 312 nm with a generally broad absorption range from 265 to 380 and 260 to 360 nm for products 1 and 2, respectively. The absorption spectra of the two products are provided in Table S8 in the Supporting Information, illustrating that an absorption typical for browning products is missing. In Figure 4, the stability of the main PP, CA, COUM, PC B2, EC, PHL, and PHL-pent-hex, in the stored samples is shown. In Boskoop, Braeburn, and Gewürzluiken, the contents of EC, PHL, and PHL-pent-hex were stable over the experiment time (16 days), while PC B2 decreased slightly. In Gewürzluiken, the apple variety with the highest CA and COUM contents, a substantial decrease of both phenolics and a prominent product formation (products 1 and 2) were observed. In Boskoop, only the CA content was markedly reduced while product 1 was formed. In contrast to these varieties with high phenolic contents, only negligible amounts of both products were formed in Braeburn, even though CA and COUM were present in the sample.

To better illustrate the correlation between the decay of CA and COUM and the progressive formation of the products 1 and 2 in Gewürzluiken and between CA and product 1 in Boskoop (Figure 4), the relative peak areas versus time was plotted (see Figure 5). The decrease of CA and COUM as well as the increase of products 1 and 2 were comparable among compounds and both apples. In Boskoop, only CA decreased and product 1 was formed, whereas in Gewürzluiken CA and COUM diminished and products 1 and 2 increased. This might indicate a correlation between reduction of CA and formation of product 1 and decay of COUM and formation of product 2. Surprisingly, CA and COUM were stable in Braeburn.

DISCUSSION

PP Content and Profile in the Different Apple Varieties. The PP content differed significantly among the apple varieties (Table 2). It is striking that the commercial

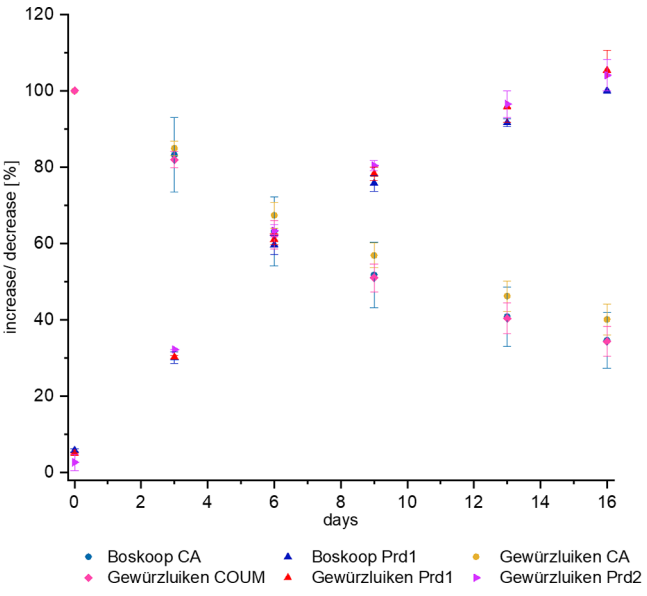

Figure 5. Similarity between CA and COUM decay and formation of the respective storage products (Prd 1, 2) for Boskoop and Gewürzluiken. Experiments were performed in duplicate, and mean ± average deviation (n = 2) is shown. The decrease is calculated based on the area at the start of experiment, and the increase of the product is based on the absolute area at day 16. Data for Braeburn are not shown, due to minimal changes (Figure 4).

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varieties (Braeburn, Santana, and Holstein Cox) were the cultivars with the lowest overall PP content. This is in accordance with observations in literature,[18,19] reporting lower PP contents in "newer" apple varieties. This might be explained by the demand of consumers for varieties with non-astringent, sweet tasting, and slow-browning properties, which led to the breeding and cultivation of varieties with a reduced amount of PP for the market. A 2.6 to 4.5 times higher PP content is determined in the peel than in the flesh, which is in the range of two to six reported in the literature.[12]

Diverse extraction and quantification methods are used in the literature, which makes it problematic to differentiate the PP content among different studies and apple cultivars. Furthermore, a lot of studies do not compare between peel and flesh, and the results are either reported as FW or DW complicating a comparison even further. We quantified PP contents of 112−604 mg/100 g DW (17−127 mg/100 g FW) and 378−1224 mg/100 g DW (68−334 mg/100 g FW) in pulp and peel, respectively (Table 2). Kschonsek et al. reported considerable lower PP contents for flesh (10−42 mg/100 g DW) and peel (100−495 mg/100 g DW).[18] In contrast, Jakobek et al. determined significantly higher PP contents with 265−686 mg/100 g FW and 559−1400 mg/100 g FW in flesh and peel, respectively.[15] In another study, a PP content of 52−272 mg/100 g DW[16] without distinguishing between peel and pulp is reported. Most studies cited determined significantly higher flavanol contents amounting to over 80% of total PP.[15,16,19] However, in these investigations, particular attention was paid to PC quantification applying respective methods. Therefore, it is relatively likely that the PC content in our work is underestimated. It is well documented that PCs with higher molecular weights are often not extracted completely with water-organic solvents without an additional hydrolysis step to release these PP from the residue.[37,38]

The content of dihydrochalcones we found is comparable to those reported in previous literature.[16,18,19] HZD contents are similar to the data reported by Vrhovsek et al. for whole apples but in a lower range compared to those reported by other groups.[15,16,19] The flavonol content of the peel quantified by us and Jakobek et al. is also comparable.[15] The lower flavonol content reported by Vronshek et al. and Wojdyło et al. is not surprising because both works do not distinguish between flesh and peel, and the flavonols are only present in the peel.[16,19] All data for PP in apples illustrate that the individual PP structures are similar among the apple varieties investigated and that they only differ in their PP content and profile.[15,16,18,19]

Bioaccessibility of the PP from Flesh and Peel. A release of 63−82% of total PP from the flesh and 42−58% from the peel was observed during oral digestion in vitro and ex vivo. It seems that the bioaccessibility of PP from varieties with less PP is slightly reduced compared to varieties with higher PP amounts. This might be due to the fact that for some phenolics, the limit of quantification was reached. In addition, variety of specific phenolic profiles had an impact on the amount of released PP because high contents of HZDs increased while flavanols and flavonols decreased the overall release. This might also explain the reduced release of PP from the peel compared to the flesh. Nevertheless, differences in the polysaccharide composition among flesh and peel might also have had an impact.

The high release of PP from flesh and peel during oral digestion is surprising. To the best of our knowledge, only Tenore et al. published data about bioaccessibility in the oral digestion phase of PHL, Q-rutinosid, and PCs from apples.[26] With a release of 35% for PCs and Q-rutinosid and 27% for PHL, the bioaccessibility was significantly lower than our data. In contrast to our findings, no significant differences in the release were observed for flesh and peel and among the varieties investigated. The dissimilarities between our findings and the study by Tenore et al. might be explained by the different composition of the simulated saliva. Furthermore, the high degree of milling of our samples influenced the release of PP positively, indicating an important impact of grinding apples carefully during consumption. In general, the process of freeze-drying might also effect the release of PP in the short aqueous oral digestion phase. For a deeper understanding of the release in vivo, chewing experiments with fresh apples are required.

No substantial deviations (3−7% flesh, 2−4% peel) in PP release were observed between the SSF and human saliva of two probands, indicating no significant differences in the saliva and its microbial composition (Figure 1). Minor variations might be explained by the fact that each proband was only allowed to work with her own saliva during sample preparation. The high degree of comparability between SSF and human saliva allows the substitution of human saliva by SSF, greatly simplifying sample preparation because the ethical concerns about the use of biofluids by a third person are avoided.

The formation of complexes between PCs and prolin-rich saliva proteins is well documented in literature.[39] Because the bioavailability of the PCs is comparable between the in vitro digestion with SSF and ex vivo using human saliva, it is unlikely that the formation of these complexes is responsible for the reduced bioaccessibility observed. The main reason for the lower release in the oral phase is the reduced extractability of PCs from the apple matrix, already discussed for the methanol water extraction. Most likely, this effect is even more pronounced for aqueous fluids such as saliva or SSF. The formation of aglycones due to breaking of β-glyosidic bonds is not observed in the saliva samples. This is in accordance with the literature, reporting degradation of PP to aglycons only in non-centrifuged saliva.[23]

Sample Stability. Samples obtained from in vitro or ex vivo digestion are not stable at room temperature or storage at −20 °C despite subsequent dilution with methanol. It can be speculated if remaining PPO in the extract is responsible for the formation of the two products. In the literature, a higher PPO activity is reported for Boskoop than for Braeburn.[40] However, at 80% methanol content, a still active PPO would be surprising.

The products formed do not absorb light at 420 nm (spectra provided in S7 in the Supporting Information) as known for browning products, and m/z is too low for typical dimerization products. Therefore, we believe that products 1 and 2 were stable oxidation products of CA and COUM. It is reported that some (enzymatic) oxidation products of CA exhibit absorption maxima at or near 325 nm.[41]

The formed products can be used as markers for CA and COUM decay. Products 1 and 2 were only minor compounds (signal area below 5% compared to CA and COUM) in all the samples measured immediately after simulated digestion, effecting CA and COUM quantification only slightly. It remains unclear if the formation of the products already took place in the oral digestion or was an effect of sample analysis,

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but the data indicate that sample storage, even at low temperature, should be avoided.

The data indicate a significant release of apple PP during oral digestion, which might be already absorbed by the oral mucosa epithelium. Thus, PP might reach specific tissues and organs without undergoing intestinal digestion. Bioaccessibility differs between flesh and peel. However, despite a reduced PP release from the apple peel, the study supports the consumption of fresh apples including peel due to an increased PP content and diverse phenolic profile. Furthermore, the importance of thorough chewing, which increases cell decompartmentation and retention time in the oral cavity and decreases the particle size for gastrointestinal digestion, is pointed out. The data support replacing centrifuged human saliva by SSF, which simplifies in vitro digestion experiments. For further studies regarding the impact of mucosal cells on saliva and also to investigate more apple varieties, samples should be analyzed subsequent to the simulated oral phase to avoid decay of CA and COUM and the formation of artifacts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c08130.

S1: Supplier, water content, ratio of peel and pulp to the whole apple without core, ancestry, and year and country of the first description of the different apple varieties used; S2: Quantified PP and PP groups in the peel of the different apple varieties; S3: Quantified PP and PP groups in the flesh of the different apple varieties; S6: Release of the procyanidins (PC) from the peel of the different cultivars in percent [%] based on their content in the methanolic extraction against the number of monomer units per procyanidin; S7: Release of the PC in percent [%] from the flesh of the different cultivars based on their content in the methanolic extraction against the number of monomer units per procyanidin and S8: UV−Vis and mass spectrometry data of the two formed oxidation products during the 16-day storage experiment (PDF)

S4: Quantified PP and PP groups in the samples of the simulated oral digestion in the peel of the different apple varieties (PDF)

S5: Quantified PP and PP groups in the samples of the simulated oral digestion in the flesh of the different apple varieties (PDF)

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Notes

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■ ABBREVIATIONS

CA, chlorogenic acid; CAT, catechin; COA, coumaric acid; COUM, coumaroylquinic acid; CYD, cyanidin; DW, dry weight; dohex, deoxyhexoside; EC, epicatechin; glc, glucoside; FW, fresh weight; HCl, hydrochloric acid; hex, hexoside; HZD, unknown hydroxycinnamic acid derivative; IHR-rut, isorhamnetin-rutinoside; MW, molecular weight; P, proband; PC, procyanidin; pent, pentoside; PHL, phlorizin; PHL-pent-hex, phloretin-pentosyl-hexoside; PP, polyphenol; Prd, product; Q, quercetin; SSF, SSF

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