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Characterization and Quantitation of Low and High Molecular Weight Phenolic Compounds in Apple Seeds

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ABSTRACT: The phenolic constituents of seeds of 12 different apple cultivars were fractionated by sequential extraction with aqueous acetone (30:70, v/v) and ethyl acetate after hexane extraction of the lipids. Low molecular weight phenolic compounds were individually quantitated by RP-HPLC-DAD. The contents of extractable and nonextractable procyanidins were determined by applying RP-HPLC following thiolysis and *n*-butanol/HCl hydrolysis, respectively. As expected, the results revealed marked differences of the ethyl acetate extracts, aqueous acetone extracts, and insoluble residues with regard to contents and mean degrees of polymerization of procyanidins. Total phenolic contents in the defatted apple seed residues ranged between 18.4 and 99.8 mg/g. Phloridzin was the most abundant phenolic compound, representing 79−92% of monomeric polyphenols. Yields of phenolic compounds significantly differed among the cultivars under study, with seeds of cider apples generally being richer in phloridzin and catechins than seeds of dessert apple cultivars. This is the first study presenting comprehensive data on the contents of phenolic compounds in apple seeds comprising extractable and nonextractable procyanidins. Furthermore, the present work points out a strategy for the sustainable and complete exploitation of apple seeds as valuable agro-industrial byproducts, in particular as a rich source of phloridzin and antioxidant flavanols.

KEYWORDS: *phenolic compounds, phloridzin, procyanidins, thiolysis, apple seeds, byproduct valorization*

INTRODUCTION

With a global production of about 71 million tons in 2009, apples are one of the world's most important fruit crops.[1] They are mainly consumed as fresh fruit; however, a large quantity is also industrially processed into juices, ciders, purées, jams, and dried products. In Germany, apple juice is one of the most popular fruit juices. According to estimations, annually up to 300 000 tons of apple pomace arise as solid byproduct from juice extraction.[2] Depending on the processing conditions, apple pomace represents about 25−30% of the fresh fruit weight and is composed of seeds, cores, stems, skin, and parenchyma.[3,4] In the past, apple pomace was mostly used as animal feed[5] or for the more important recovery of apple pectin. During the past decades, however, with epidemiological studies providing evidence for a significantly reduced risk of chronic diseases due to increased intake of fruits and vegetables or products thereof, research has been progressively focused on the recovery of bioactive compounds from apple pomace and other agro-industrial residues.[6]

Phenolic substances, particularly from apples, have experienced intense interest because of their antioxidant properties[7,8] and their putative health-promoting effects.[9−11] Apple polyphenols may be divided into four major classes: hydroxycinnamic acids, dihydrochalcones, flavonols, and flavan-3-ols. The latter are present both in monomeric (epicatechin, catechin) and in oligomeric or even polymeric forms, the so-called procyanidins. Apple procyanidins exclusively consist of monomeric (−)-epicatechin or (+)-catechin units most frequently linked via B-type C4 → C8 or C4 → C6 bonds. Owing to the different constitutive units, their varying number, and the different interflavanoid bond types, the high diversity of procyanidin structures makes their investigation quite challenging. Various methods including photometric tests such as the acid butanol assay and chromatographic methods (RP-HPLC) have been suggested to analyze this heterogeneous class of polyphenols.[12] A common principle of the aforementioned methods is based on the acid-catalyzed depolymerization of procyanidin structures. In this context, thiolytic degradation in combination with a subsequent separation by reversed phase HPLC has been proven to be an efficient tool to characterize high molecular weight procyanidins. In the presence of nucleophilic reagents such as benzyl mercaptan, the extension units of the procyanidins are released as the corresponding benzylthioether adducts, whereas terminal units are liberated as flavan-3-ol monomers. By calculating the proportion of benzylthioether adducts to free flavan-3-ols, estimation of the mean degree of polymerization (DPm) of the investigated procyanidins is possible.[13,14]

Whereas phenolic compounds from apple pomace have been intensively studied,[15−17] data on isolated apple seeds, making up a significant part of the pomace,[18] are rather scarce. The studies reported so far have shown the phenolic pattern of apple seeds to be less versatile than that of the whole fruit. Phloridzin was found to be the predominant low molecular weight phenolic compound of apple seeds.[19,20] In several studies of the past decades phloridzin has been shown to be effective in the adjuvant treatment of diabetes mellitus (type 2), obesity, and stress hyperglycemia due to inhibition of the intestinal absorption and renal reabsorption of glucose.[21]

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Owing to its pharmacological effects and abundant availability, selective recovery of phloridzin from apple seeds appears to be increasingly attractive. Besides phloridzin, phloretin-2′-xyloglucoside, 5-caffeoylquinic acid (chlorogenic acid), *p*-coumaroylquinic acid, (−)-epicatechin, and some quercetin glycosides have also been described in apple seed extracts.[19,20] However, as far as structures and contents of extractable and nonextractable procyanidins of apple seeds are concerned, almost no data are available.

The combined recovery of nutritionally valuable lipids and structurally diverse phenolic compounds as natural food additives appears to be a promising strategy for complete utilization of these byproducts. More detailed information on the phenolic constituents of apple seeds is a prerequisite for establishing economically feasible recovery processes. Therefore, the aim of the present study was the comprehensive investigation of the polyphenolic patterns and the determination of phenolic contents also comprising oligomeric and polymeric procyanidins present in the seeds of 12 apple cultivars after previous recovery of their fatty oils.

MATERIALS AND METHODS

Chemicals. All reagents and solvents were of analytical or HPLC grade and were bought from Merck (Darmstadt, Germany). Benzyl mercaptan and phloridzin were purchased from Fluka (Buchs, Switzerland). (−)-Epicatechin, (+)-catechin, procyanidin B1, procyanidin B2, cyanidin chloride, and quercetin-3-*O*-glycosides of galactose, glucose, and rhamnose were obtained from Extrasynthèse (Lyon, France). *p*-Coumaric acid and phloretin were from Roth (Karlsruhe, Germany). 5-Caffeoylquinic acid (chlorogenic acid) was purchased from Sigma-Aldrich (St. Louis, MO).

Plant Material. Seeds of authenticated apple varieties from the 2009 harvest were used for this study. Ripe apple fruits were provided by different local growers and by the Hohenheim University research station for horticulture (Stuttgart, Germany). The seeds were separated manually from the cores, lyophilized, and finely ground in a laboratory mill (3 × 30 s). For lipid recovery, aliquots of 15 g of the resulting apple seed flours were extracted with *n*-hexane (3 × 100 mL) for 10 min at ambient temperature. A batch of dried seeds of the grape cultivar 'Isabel' was from Aurora Golden Sucos Ltd.a. (São Marcos, Brazil).

Crude Extracts. Approximately 2.5 g of the defatted material was extracted with 50 mL of aqueous acetone (30:70; v/v) by stirring for 15 min at ambient temperature in a centrifuge tube under nitrogen atmosphere. After centrifugation (5 min, 4200 rpm), the aqueous acetone extract was decanted and filtered. This extraction procedure was repeated three times. The combined crude acetone extracts were evaporated under reduced pressure (30 °C, 240 mbar) for complete removal of the organic solvent. The insoluble residue after extraction was lyophilized, weighed, and stored at −20 °C for further investigations of nonextractable procyanidins.

Liquid−Liquid Extraction with Ethyl Acetate. Subsequently, the aqueous crude extract was transferred into a separating funnel and re-extracted with ethyl acetate (4 × 100 mL). The combined ethyl acetate extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dryness (30 °C, 110 mbar). Both the organic and the aqueous phases were lyophilized, weighed, and kept at −20 °C until further analysis. Throughout this work, they are referred to as "ethyl acetate extracts" and "acetone extracts", respectively.

Determination of Low Molecular Weight Phenolics (HPLC-DAD-MS^n). The determination of phenolic compounds of low molecular weight was performed according to a previously developed method[22] with some modifications using an Agilent HPLC series 1100 chromatograph (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1322A degasser, a model G1312A binary gradient pump, a model G1329/G1330A thermo-autosampler, a model G1316A column oven, and a model G1315A diode array detector. The separation was carried out using a Waters (Wexford, Ireland) SunFire C18 column (250 mm × 4.6 mm i.d.; 5 μm particle size) equipped with a Phenomenex guard column SecurityGuard AQ C18, 4 × 2.0 mm (Phenomenex, Aschaffenburg, Germany), operated at 25 °C. The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% acetic acid in water and methanol (30:70, v/v, eluent B). The gradient program was as follows: from 25 to 42% B (28 min), from 42 to 100% B (37 min), 100% B (1 min), from 100 to 25% B (1 min), 25% B (3 min). The flow rate was 0.8 mL/min. The injection volume for all samples was 10 μL. Detection wavelengths were 280 nm (dihydrochalcones, flavan-3-ols), 320 nm (hydroxycinnamic acids), and 370 nm (flavonols). Spectra were recorded from 200 to 600 nm (peak width, 0.2 min; data rate, 1.25/s). Individual compounds were identified by their retention times and UV−vis and mass spectral data.

LC-MS analyses were performed using the same HPLC system as described above connected online to a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an electrospray ionization (ESI) source. The flow rate of nitrogen as drying gas was 9 L/min, and nebulizer pressure was 40 psi. The nebulizer temperature was set at 365 °C. Negative ion mass spectra were recorded in a range of m/z 50−2800.

Individual polyphenolics were quantitated using external calibration with corresponding reference compounds. If standard compounds were not available, quantitation was achieved using the calibration curves of structurally related constituents including a molecular weight correction factor.[23]

Synthesis and Preparative Isolation of (−)-Epicatechin Benzylthioether. Because benzylthioether derivatives resulting from thiolysis were commercially unavailable, these reference compounds had to be prepared in our laboratory. For this purpose, dried seeds of the grape cv. 'Isabel' were finely ground in a laboratory mill (3 × 30 s). After removal of lipid compounds from 15 g of grape seed flour with *n*-hexane (4 × 100 mL), the defatted material was directly used for thiolysis. Approximately 5 g of solid was transferred into a glass flask, and 40 mL of 3.3% (v/v) HCl and 80 mL of 5% (v/v) benzyl mercaptan in methanol were added. Incubation was performed for 30 min under reflux. Subsequently, the thiolysis solution was cooled and then concentrated under reduced pressure, and aliquots (0.8 mL) were subjected to preparative HPLC. Separation of (−)-epicatechin benzylthioether was carried out using a Phenomenex C18 Aqua column (250 mm × 21.2 mm i.d.; particle size, 5 μm; pore size, 125 Å). The HPLC system (Bischoff, Leonberg, Germany) consisted of an LC-CaDI 22-14 system controller, two 2250 solvent delivery HPLC compact pump modules, and an SPD-10AVvp UV−vis detector (Shimadzu, Kyoto, Japan). All data were processed with McDAcq32 Control software (version 2.0) (Bischoff). Eluents were the same as for analytical HPLC. Benzylthioether derivatives were eluted isocratically with 80% eluent B at ambient temperature. The flow rate was 8.0 mL/min. Monitoring was performed at 280 nm. Thioether adducts were identified by comparison with literature data and by HPLC-DAD-MS^n experiments. The chromatographic purity of the isolated (−)-epicatechin benzylthioether adduct was ≥95% (HPLC, λ = 280 nm).

Characterization and Quantitation of High Molecular Weight Phenolics (Procyanidins). *Thiolysis prior to RP-HPLC Analysis.* Thiolytic degradation of high molecular weight flavan-3-ols was performed as previously described[24] with slight modifications. In a glass vial 50 μL of methanolic solutions of the freeze-dried acetone and ethyl acetate extracts, respectively, was mixed with 50 μL of 3.3% (v/v) HCl and 100 μL of 5% (v/v) benzyl mercaptan in methanol. For analysis of the solid residues of the extraction procedure 10−15 mg of the homogenized material was used with 4 times the aforementioned volumes. The vials were sealed, vigorously shaken, and incubated at 50 °C for 10 min. After incubation, the samples were cooled in ice water, membrane-filtered, and transferred to HPLC vials. All incubations were done in duplicate. Samples were stored at −20 °C prior to HPLC analysis as described above. Investigations of epimerization upon thiolysis under the applied conditions were also included. For this purpose, methanolic solutions containing procyanidin B2 reference

Table 1. Contents of Individually Quantitated Low Molecular Weight Phenolic Compounds in the Seeds of Dessert and Cider Apples (Milligrams per Kilogram Defatted DM)

peak	compound	Weinapfel	Brettacher	Welschisner	Maunzenapfel	Boskoop	Champagner Renette	Topaz	Bittenfelder	Bohnapfel	Gewürzluike	Pinova	Royal Gala
7	3-*p*-CouQA[a]	14.4	28.5	10.8	11.6	23.1	7.8	4.4	11.8	30.4	19.7	62.8	48.2
		2.0	*0.0*	*0.6*	*0.6*	*0.3*	*0.0*	*0.3*	*0.8*	*3.6*	*1.3*	*3.8*	*0.8*
8	5-CaQA	974.9	1932.3	556.3	519.9	1304.0	323.9	345.0	824.4	362.0	279.1	643.8	746.1
		64.5	*52.0*	*4.6*	*10.7*	*21.6*	*9.1*	*3.0*	*25.1*	*4.0*	*6.7*	*63.4*	*65.2*
9	4-CaQA[b]	190.7	224.3	165.1	150.9	236.3	96.8	90.6	132.4	141.2	133.2	140.4	126.5
		12.8	*3.3*	*2.7*	*3.4*	*2.3*	*2.2*	*0.3*	*6.9*	*6.3*	*7.3*	*8.7*	*3.6*
	caffeic acid	nd	36.6	nd	nd	nd	6.3	4.7	10.3	nd	nd	15.1	10.9
			0.3				*0.1*	*0.3*	*1.7*			*2.9*	*0.8*
10	3-CaQA[b]	47.3	91.6	44.7	43.3	115.8	24.2	22.3	171.4	153.9	142.3	178.8	162.1
		7.4	*2.9*	*2.3*	*3.3*	*3.9*	*0.4*	*1.1*	*4.6*	*6.9*	*6.1*	*10.9*	*6.3*
11	5-*p*-CouQA[a]	44.8	90.5	40.6	44.0	90.5	36.1	4.1	80.5	60.2	42.0	12.9	38.3
		2.2	*2.1*	*1.1*	*0.5*	*1.0*	*0.7*	*0.2*	*2.7*	*2.1*	*1.7*	*1.9*	*1.1*
12	4-*p*-CouQA[a]	44.6	nd	nd	nd	nd	nd	nd	48.0	nd	nd	nd	nd
		2.1							*1.3*				
1	procyanidin B2	54.6	68.8	53.9	32.5	78.7	tr	tr	16.6	64.0	18.5	54.2	18.5
		2.9	*5.3*	*4.0*	*1.9*	*1.4*			*4.4*	*7.4*	*0.9*	*11.6*	*2.4*
2	epicatechin	51.6	32.5	32.4	28.3	48.7	tr	4.2	16.0	40.1	12.9	45.9	11.4
		0.6	*2.3*	*0.9*	*2.9*	*0.2*		*0.1*	*1.9*	*4.7*	*1.1*	*11.4*	*2.5*
3	3-hydroxyphloridzin[a]	49.3	159.6	107.2	49.0	391.4	48.7	32.1	109.0	231.6	28.4	29.2	137.9
		0.9	*5.0*	*2.7*	*0.6*	*4.7*	*1.6*	*0.2*	*9.3*	*17.2*	*1.9*	*4.6*	*6.5*
4	phloretin-xyloglucoside[a]	178.3	1208.6	807.1	344.1	284.2	314.1	108.0	697.8	1107.7	103.6	1334.3	887.3
		2.1	*32.8*	*7.3*	*15.2*	*6.3*	*7.1*	*3.0*	*40.0*	*94.1*	*3.8*	*113.6*	*37.9*
5	phloridzin	12687.1	22351.8	8190.9	7706.5	21597.1	4133.5	3256.3	8130.6	16457.3	9484.0	11636.2	9354.4
		379.8	*736.2*	*39.3*	*425.8*	*56.1*	*167.7*	*6.9*	*745.1*	*553.5*	*570.1*	*938.2*	*485.2*
6	phloretin	5.7	83.4	7.8	8.2	8.0	4.8	1.8	9.7	34.4	9.6	27.5	5.7
		0.0	*3.6*	*0.4*	*0.4*	*0.5*	*0.1*	*0.4*	*0.6*	*1.4*	*0.0*	*2.2*	*0.2*

[a]Calculated according to ref 23. [b]Calculated as 5-caffeoylquinic acid; peak, peak in Figure 1; *p*-CouQA, *p*-coumaroylquinic acid; CaQA, caffeoylquinic acid; nd, not detected; tr, trace; DM, dry matter. Italic values correspond to standard deviations of mean values (n = 2).

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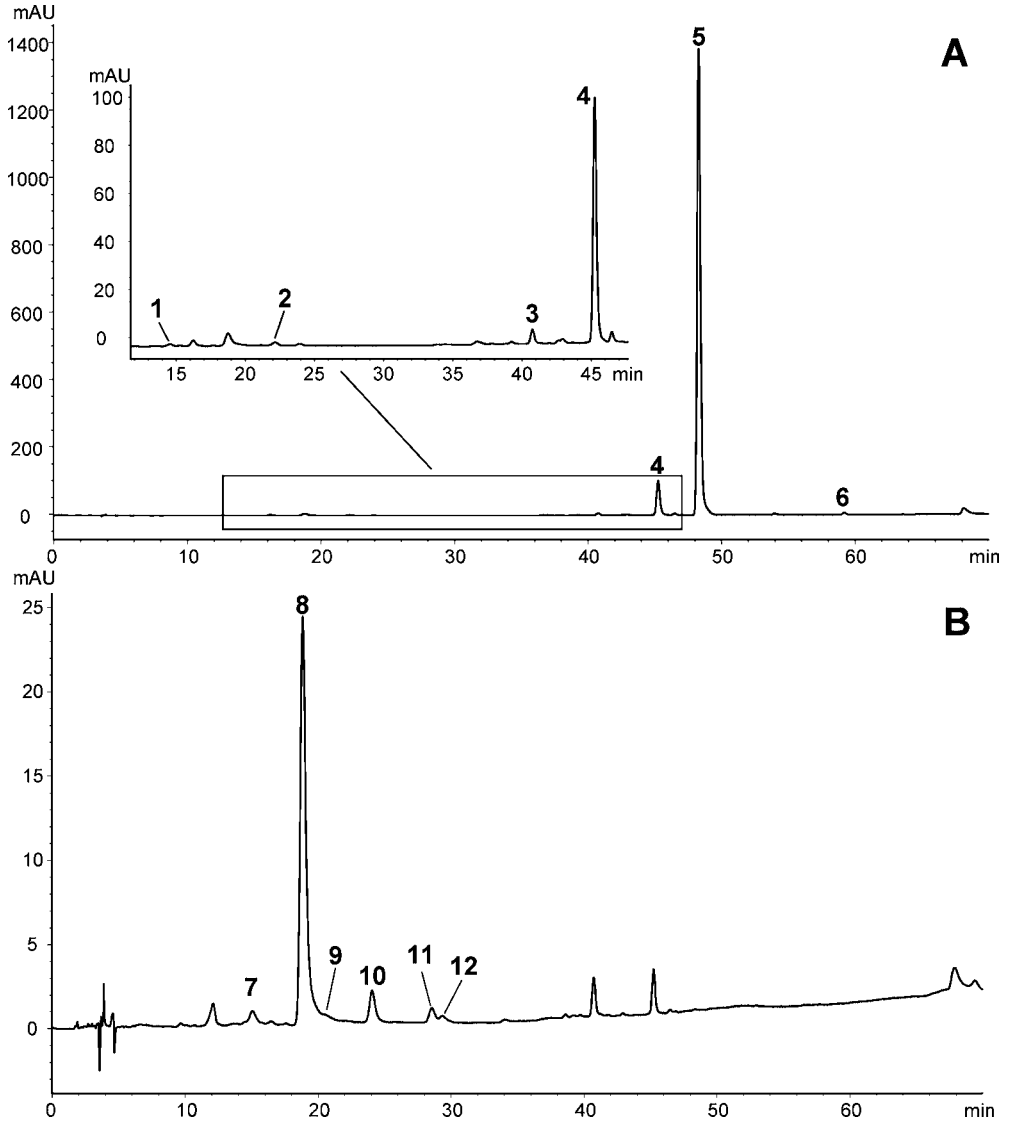

Figure 1. HPLC chromatograms of ethyl acetate and acetone extracts at detection wavelengths of 280 nm (A) and 320 nm (B), respectively. For peak assignment, see Table 1.

compound were subjected to thiolytic degradation as described above, and the conversion rate of (−)-epicatechin to (+)-catechin was calculated.

Contents of high molecular weight flavan-3-ols were determined as the sum of the monomeric constitutive units (−)-epicatechin and (+)-catechin originating from terminal or extension units after thiolytic degradation.

The mean degree of polymerization (DPm) was obtained by calculating the molar ratio of both extension and terminal flavan-3-ol units to terminal units as previously described.[13] The initial contents of (−)-epicatechin and (+)-catechin of each sample were determined separately and subtracted from those after thiolysis experiments to correct for their contribution to the content and the DPm of flavan-3-ols.

n-Butanol/HCl Hydrolysis. Contents of extractable flavan-3-ols were determined according to a previous study.[24] Briefly, 100 μL of methanolic solutions of the dried ethyl acetate and acetone extracts, respectively, was incubated with 2.5 mL of *n*-butanol/HCl reagent (95:5, v/v) and with 100 μL of iron reagent (2%, w/v, solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl) in derivatization tubes for 40 min at 95 °C. For the determination of nonextractable flavan-3-ols approximately 40 mg of the solid residue was incubated with 20 mL of *n*-butanol/HCl reagent and 800 μL of iron reagent for 210 min. After cooling in ice water and membrane filtration, absorption was measured at λ_{max} = 550 nm. All determinations were performed using a Biotek microplate spectrophotometer (Biotek Instruments, Power Wave XS, Bad Friedrichshall, Germany) equipped with Gen5 software (ver. 1.04.5). Derivatizations were performed in duplicate. Total flavan-3-ol contents were quantitated using cyanidin chloride as reference compound.

Statistical Analysis. All experiments were performed in duplicate. Significant differences (α = 0.05) were determined using the Tukey test for different independent samples. Data evaluation was performed with SAS software package (SAS Institute, Cary, NC, software version 9.1).

RESULTS AND DISCUSSION

Recovery of Apple Seed Oil. For the recovery of the lipid fraction, freeze-dried apple seeds were finely ground and successively extracted with *n*-hexane at ambient temperature at laboratory scale to mimic a cold-pressing process. Oil contents of the apple seeds ranged from 16.1% ('Boskoop') to 26.5% ('Champagner Renette'), showing significant differences among the investigated apple cultivars. Consistent with our previous findings,[25] the typical dessert apple cultivars ('Pinova' and

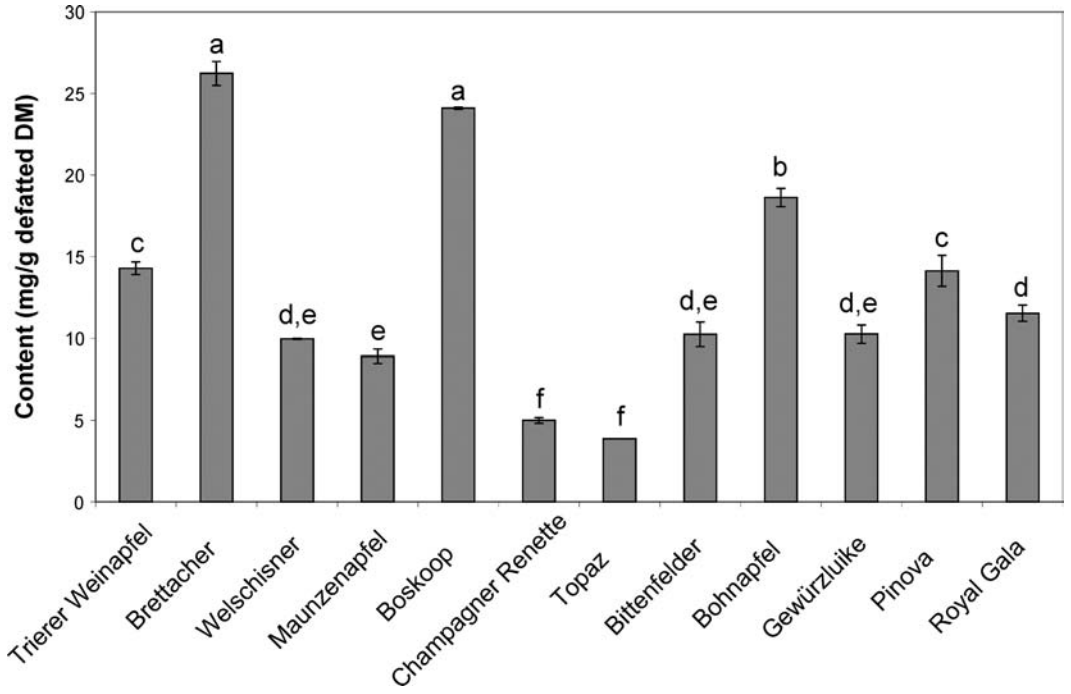

Figure 2. Total contents of low molecular weight phenolic compounds (mg/g defatted DM) in apple seeds of different cultivars. Bars represent the sum of individually quantitated compounds. Identical letters indicate that samples are not significantly different.

'Topaz') exhibited higher lipid contents than cider apple cultivars such as 'Boskoop' and 'Rheinischer Bohnapfel'.

Low Molecular Weight Phenolic Constituents. Phenolic compounds were recovered from the defatted residues of apple seeds by sequential extractions with aqueous acetone (30:70; v/v) and ethyl acetate. Total phenolic contents were calculated as the sum of individually quantitated polyphenols in both the freeze-dried acetone and ethyl acetate extracts (Table 1). Typical chromatograms of the acetone extract (B) and the ethyl acetate extract (A) obtained by RP-HPLC at different detection wavelengths are presented in Figure 1. Subsequent extraction of the crude acetone extracts with ethyl acetate resulted in the accumulation of 90−96% of the monomeric polyphenols in the organic phase. Major constituents of the ethyl acetate extracts were phloridzin, 5-caffeoylquinic acid (chlorogenic acid), and phloretin-2′-xyloglucoside, whereas the aqueous phase after liquid−liquid extraction ("acetone extract") mainly consisted of phenolic acids and phloretin-2′-xyloglucoside. Because additional acidification may lead to hydrolysis of the labile interflavanoid bonds of flavan-3-ols, extraction was performed at native pH ∼3. Hence, depending on their pK_a values, phenolic acids were partially deprotonated. These anionic forms were not extracted with ethyl acetate because of their higher polarity. This also applied to the more polar disaccharidic phloretin-2′-xyloglucoside, which was also detected in appreciable amounts in the freeze-dried acetone extracts.

Additionally, minor phenolic constituents were assigned to phloretin- and hydroxycinnamic acid derivatives. Although present in negligible amounts, quercetin 3-*O*-glucoside, quercetin-3-*O*-galactoside, and quercetin-3-*O*-rhamnoside were also detected in our samples as reported in previous studies of byproducts derived from apple.[19,20,26] Their negligible contents might be due to the fact that the seeds of the present study were not separated from apple pomace as in the aforementioned studies. Thus, "cross-contamination" with additional flavonol glycosides from peels was avoided.

Consistent with previous findings, the phenolic profile of apple seeds was less diverse than that of the edible parts of the apple fruit with only a few compounds prevailing (see Table 1). Phloridzin was the most abundant phenolic compound of the investigated apple seeds under study, accounting for 79% (cv. 'Bittenfelder') to 92% (cv. 'Gewürzluike') of the quantitated phenolics with low molecular weight. Other major compounds were 5-caffeoylquinic acid (chlorogenic acid) and phloretin-2′-xyloglucoside, making up 2−9 and 1−9% of low molecular weight phenolics, respectively. The highest contents of chlorogenic acid were found in the seeds of the cv. 'Topaz', whereas cv. 'Rheinischer Bohnapfel' exhibited only minor amounts. For phloretin-2′-xyloglucoside, content in cv. 'Trierer Weinapfel' was lowest, whereas cv. 'Pinova' showed the highest value. (−)-Epicatechin and procyanidin B2 were only minor phenolic constituents amounting to a maximum of only 0.4% ('Trierer Weinapfel') and 0.5% ('Welschisner') of low molecular weight phenolics, respectively.

The total contents of low molecular weight polyphenols significantly differed between the seeds of the different cultivars under investigation (Figure 2). Ranging from 3.9 to 26.3 mg/g, the total content of monomeric phenolics was approximately 10-fold higher for cv. 'Brettacher' than for cv. 'Topaz'. This is in good agreement with a previous work[27] reporting a comparable range for phloridzin, the major phenolic constituent, in seeds of different apple varieties from Brazil. These findings were underpinned by other data reporting amounts of low molecular weight phenolics in apple seeds comparable to the content of the cv. 'Topaz' in our study.[20,24] Because all of our samples originated from the same geographical area and were harvested at full maturity, climatic or maturity effects should be negligible. Thus, as already observed for polyphenols of other apple tissues,[28−30] it seems that their contents in the seeds are subject to a high degree of genetic variation.

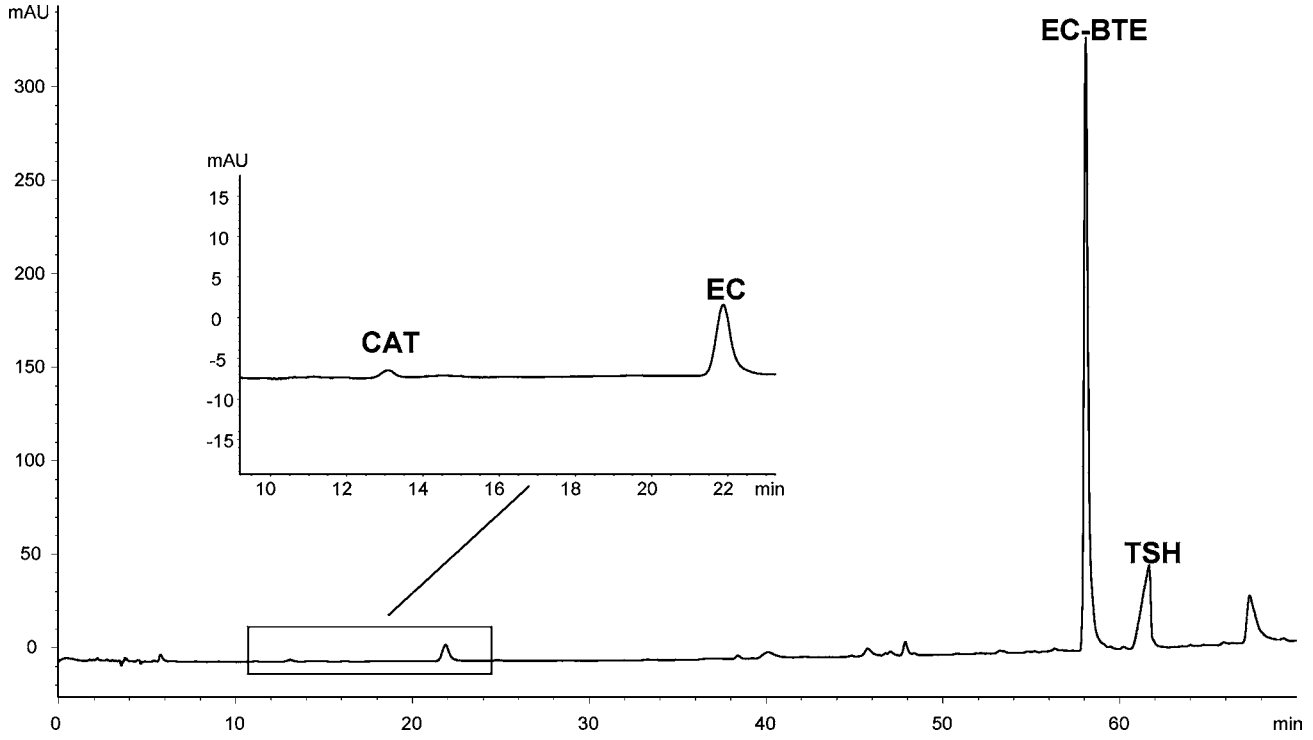

Figure 3. Chromatogram of apple procyanidins (acetone extract) after thiolysis at a detection wavelength of 280 nm. CAT, (+)-catechin; EC, (−)-epicatechin; EC-BTE, (−)-epicatechin benzylthioether; TSH, benzyl mercaptan excess.

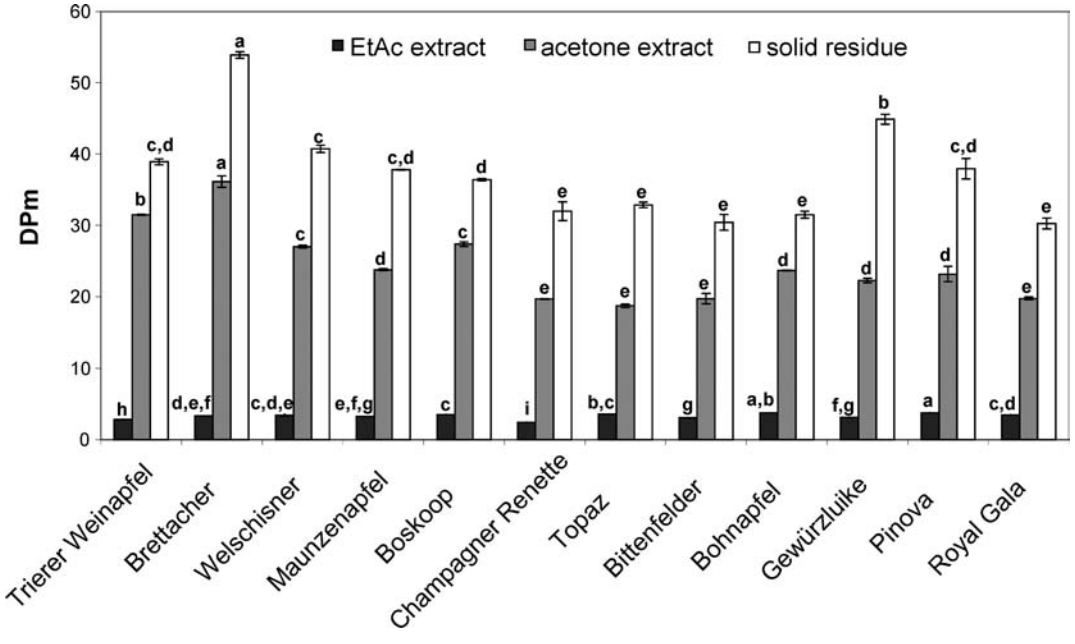

Figure 4. Mean degree of polymerization (DPm) of procyanidins of apple seed extracts from different cultivars. Identical letters within one type of bars indicate that samples do not significantly differ. Bars represent the mean ± standard deviation (n = 2).

High Molecular Weight Phenolics (Procyanidins). Characterization and quantitation of oligomeric and polymeric procyanidins (PCs) was achieved by RP-HPLC following thiolysis of the freeze-dried ethyl acetate and acetone extracts. Because polymeric PCs tend to form stable complexes with other polymeric substances of insoluble plant cell walls such as proteins or polysaccharides, their extraction is often incomplete.[31,32] Consequently, for the determination of total flavan-3-ol contents, both extractable and nonextractable PCs need to be considered. Therefore, the solid residue after extraction with aqueous acetone (30:70, v/v) was further submitted to thiolysis prior to HPLC analysis. Figure 3 shows a representative chromatogram obtained after thiolysis. As demonstrated by the chromatogram, upon thiolysis of the different samples only (+)-catechin, (−)-epicatechin, and (−)-epicatechin benzylthioether could be detected. Consequently, flavan-3-ols in apple seeds exclusively consisted of (+)-catechin and (−)-epicatechin subunits, the latter being highly preponderant.

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Table 2. Proportions (Percent) of Constitutive Units of Procyanidins in the Ethyl Acetate Extracts (A), Acetone Extracts (B), and Solid Extraction Residues (C) of Apple Seeds[a]

extract		Weinapfel	Brettacher	Welschisner	Maunzenapfel	Boskoop	Champagner Renette	Topaz	Bittenfelder	Bohnapfel	Gewürzluike	Pinova	Royal Gala
A	CATt	6.1efg	5.8fg	5.2g	7.5efg	5.8efg	27.2a	13.1bc	11.9bc	8.8de	14.5b	8.5def	11.4cd
		0.06	*0.09*	*0.10*	*0.12*	*0.18*	*0.07*	*0.49*	*0.75*	*1.17*	*0.31*	*1.26*	*1.76*
A	ECt	29.1a	24.3b	23.7b	23.1b	22.4b	14.2d	24.3b	18.36c	15.7cd	17.4c	15.9cd	16.5cd
		0.47	*0.22*	*1.28*	*0.05*	*0.16*	*0.24*	*0.03*	*0.90*	*0.88*	*0.81*	*1.20*	*0.12*
A	ECe	64.9de	69.9bc	71.1bc	69.4bc	71.7abc	58.6f	62.7e	69.8bc	75.4a	68.1cd	75.6a	72.1ab
		0.40	*0.31*	*1.18*	*0.08*	*0.03*	*0.31*	*0.52*	*1.66*	*2.04*	*0.50*	*0.05*	*1.65*
B	CATt	0.6ef	0.5f	0.7e	1.1d	0.7e	1.6b	2.1a	1.7b	1.6	1.5b	1.3c	1.6b
		0.00	*0.02*	*0.02*	*0.04*	*0.00*	*0.06*	*0.06*	*0.07*	*0.02*	*0.01*	*0.08*	*0.03*
B	ECt	2.6d	2.31e	3.0c	3.1bc	3.0c	3.5a	3.3ab	3.4a	2.6d	3.0c	3.0c	3.5a
		0.01	*0.04*	*0.04*	*0.01*	*0.04*	*0.05*	*0.01*	*0.12*	*0.02*	*0.08*	*0.13*	*0.02*
B	ECe	96.8b	97.2a	96.3c	95.8d	96.3c	94.9e	94.7e	94.9e	95.8d	95.5d	95.7d	94.9e
		0.01	*0.06*	*0.02*	*0.02*	*0.05*	*0.01*	*0.07*	*0.19*	*0.01*	*0.07*	*0.20*	*0.05*
C	CATt	0.8bc	0.4f	0.8bc	0.8bc	0.8bc	0.9b	1.5a	0.7cde	0.5def	0.5ef	0.7bcd	0.8bc
		0.02	*0.01*	*0.02*	*0.02*	*0.04*	*0.05*	*0.18*	*0.02*	*0.02*	*0.02*	*0.06*	*0.05*
C	ECt	1.8cde	1.4f	1.7def	1.8cde	2.0bc	2.2b	1.6ef	2.6a	2.7a	1.8cde	1.9bcd	2.5a
		0.01	*0.02*	*0.05*	*0.02*	*0.03*	*0.08*	*0.14*	*0.10*	*0.07*	*0.01*	*0.16*	*0.03*
C	ECe	97.4cd	98.2a	97.5bc	97.4cd	97.3d	96.9e	96.9e	96.7e	96.8e	97.8b	97.4cd	96.7e
		0.03	*0.02*	*0.03*	*0.00*	*0.01*	*0.13*	*0.04*	*0.12*	*0.05*	*0.03*	*0.10*	*0.08*

[a]Italic values correspond to standard deviations (n = 2). Values within one line with the same letter are not significantly different. CATt, (+)-catechin from terminal units; ECt, (−)-epicatechin from terminal units; ECe, (−)-epicatechin from extension units.

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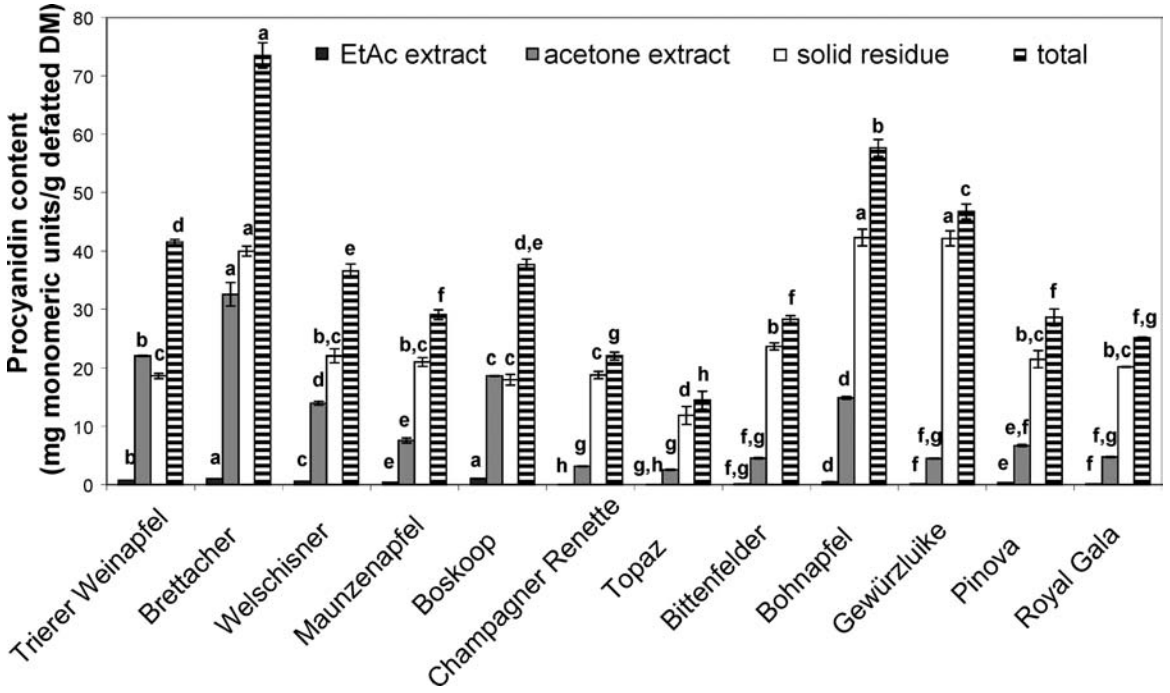

Figure 5. Procyanidin contents (mg monomeric units/g defatted DM) in apple seeds from different cultivars quantitated using thiolysis prior to RP-HPLC. Identical letters within one type of bars indicate that samples do not significantly differ. Bars represent the mean ± standard deviation (n = 2).

Additional experiments with procyanidin B2 reference compound indicated that approximately 4% of (−)-epicatechin was converted to (+)-catechin upon thiolysis, which had to be considered in the evaluation of thiolyzed samples. By calculating the molar ratio of these constitutive units, determination of the DPm as shown in Figure 4 and further structural characterization of the PCs in apple seeds according to their individual proportions were feasible (see Table 2).

The DPm of the PCs in the ethyl acetate extracts of apple seeds ranged from 2.4 to 3.8, suggesting their preferential occurrence as oligomeric flavan-3-ols. Due to the limited polarity of the solvent, polymeric PCs were distinctively discriminated by the extraction procedure. The highest DPm was determined for cv. 'Pinova', whereas cv. 'Champagner Renette' showed the lowest value. Statistical analysis revealed DPm of the ethyl acetate extracts to be comparable for the seeds of the investigated cultivars. This might be ascribed to a leveling effect of ethyl acetate on the extraction of oligomeric PCs, because a similar DPm (3.1) was reported for grape seed extracts using ethyl acetate.[33] Thus, slightly differing DPm values could be explained rather by methodical conditions than by varietal differences. With regard to the structure of the PCs, (+)-catechin was exclusively found as a terminal constitutive unit, accounting for 5.2−27.2% of total PCs in the ethyl acetate extracts. Hence, some cultivars, in particular 'Champagner Renette', showed a relatively high proportion of terminal (+)-catechin units, being much higher than already reported for seeds and other tissues.[24] (−)-Epicatechin contributed to 14.2−29.0 and 58.6−75.6% of the terminal and extension subunits of the PCs in the ethyl acetate extracts, respectively.

In the acetone extracts of the seeds, (+)-catechin comprised between 0.5 and 2.1% of the terminal units of PCs, whereas corresponding (−)-epicatechin proportions ranged from 2.3 to 3.5%. (−)-Epicatechin extension units represented 94.7−97.2% of the constitutive units. The DPm of PCs in the acetone extracts markedly differed for the seeds of the different apple cultivars. Highest values were determined for the seeds of cv. 'Brettacher' (36.1) and lowest for cv. 'Topaz' (18.7). The DPm values reported in the current study exceeded those of the aforementioned study also dealing with apple seed phenolics.[24] Because methodical differences between the latter and our work were marginal, the observed differences may be mainly due to different varieties and cultivation areas, also going along with differences in climatic conditions.

As is apparent from Figure 4, characterization of the PCs in the insoluble extraction residues showed significantly differing DPm values ranging between 30.3 (cv. 'Royal Gala') and 53.9 (cv. 'Brettacher'). Thus, as expected, the nonextractable PCs of apple seeds mainly consisted of highly polymerized flavan-3-ol units. The proportions of the terminal units (+)-catechin and (−)-epicatechin in the insoluble PCs were slightly lower than for the acetone extracts, ranging from 0.4 to 1.5% and from 1.4 to 2.7%, respectively. To some extent this observation may be explained by the higher polymerization degrees of the nonextractable PCs; 96.7−98.2% of their total units were made up by (−)-epicatechin originating from extension units.

In accordance with numerous studies on products derived from apples,[13,14,24] (−)-epicatechin was the predominant subunit of the procyanidins present in the seeds.

Quantitation of PCs Using Thiolysis prior to RP-HPLC. PC contents were determined by the addition of the amounts of the individually quantitated terminal monomers ((−)-epicatechin or (+)-catechin) and (−)-epicatechin corresponding to extension units after thiolytic degradation. Total PC contents were calculated as the sum of PCs in the ethyl acetate extracts and in the acetone extracts including the solid extraction residues (Figure 5). Analogous to our findings on DPm, PC contents also varied markedly between the seeds of different apple cultivars.

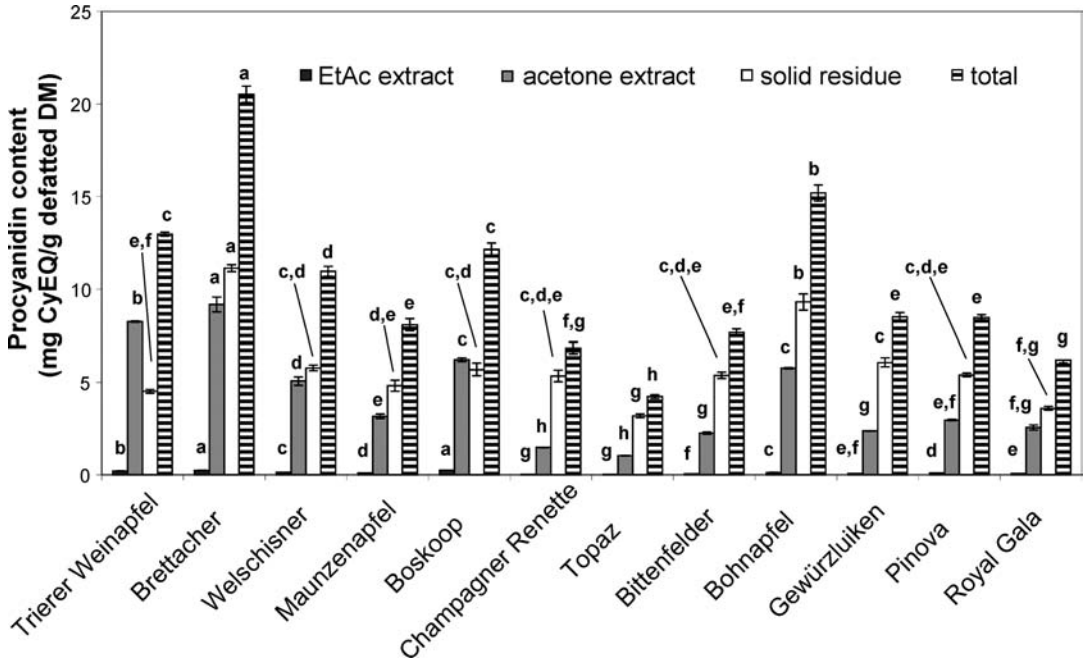

Figure 6. Procyanidin contents (mg CyEQ/g defatted DM) in apple seeds from different cultivars following *n*-butanol/HCl hydrolysis. Identical letters within one type of bars indicate that samples do not significantly differ. Bars represent the mean ± standard deviation (n = 2).

Table 3. Total Phenolic Contents of Apple Seeds of Different Cultivars

	extractable phenolics (mg/g defatted DM)					
cultivar	non-PCs[b]	oligomeric PCs[a] (DPm < 3.9)	polymeric PCs[a] (DPm > 18.7)	total	nonextractable PCs[a] (mg/g defatted DM)	total (mg/g defatted DM)
Trierer Weinapfel	14.29 ± 0.39	0.79 ± 0.00	22.04 ± 0.03	37.12 ± 0.39	18.64 ± 0.44	55.76 ± 0.59
Brettacher	26.24 ± 0.74	1.02 ± 0.02	32.55 ± 2.00	59.80 ± 2.14	39.96 ± 0.86	99.76 ± 2.30
Welschisner	9.96 ± 0.04	0.55 ± 0.02	13.97 ± 0.34	24.48 ± 0.34	22.04 ± 1.14	46.52 ± 1.19
Maunzenapfel	8.91 ± 0.43	0.40 ± 0.02	7.59 ± 0.42	16.89 ± 0.60	21.05 ± 0.75	37.94 ± 0.96
Boskoop	24.10 ± 0.06	1.05 ± 0.01	18.62 ± 0.11	43.77 ± 0.13	17.98 ± 0.93	61.75 ± 0.94
Champagner Renette	5.00 ± 0.17	0.03 ± 0.00	3.16 ± 0.09	8.19 ± 0.19	18.79 ± 0.61	26.98 ± 0.64
Topaz	3.87 ± 0.01	0.07 ± 0.00	2.55 ± 0.09	6.49 ± 0.09	11.88 ± 1.54	18.38 ± 1.54
Bittenfelder	10.24 ± 0.75	0.12 ± 0.01	4.55 ± 0.09	14.92 ± 0.75	23.61 ± 0.58	38.53 ± 0.95
Bohnapfel	18.62 ± 0.56	0.47 ± 0.02	14.90 ± 0.26	34.00 ± 0.62	42.27 ± 1.44	76.27 ± 1.57
Gewürzluike	10.25 ± 0.57	0.13 ± 0.01	4.48 ± 0.08	14.87 ± 0.58	42.14 ± 1.29	57.01 ± 1.41
Pinova	14.13 ± 0.95	0.38 ± 0.00	6.70 ± 0.18	21.21 ± 0.96	21.49 ± 1.43	42.70 ± 1.73
Royal Gala	11.53 ± 0.49	0.15 ± 0.02	4.72 ± 0.10	16.40 ± 0.50	20.20 ± 0.07	36.61 ± 0.51

[a]Sum of individually quantitated monomers by RP-HPLC following thiolysis. [b]Sum of individual phenolic constituents with low molecular weight, procyanidin B2 contents not considered; DM, dry matter; PCs, procyanidins; DPm, mean degree of polymerization. Results are expressed as the mean ± standard deviation (n = 2).

In the ethyl acetate extracts contents were low, ranging from 0.03 mg/g (cv. 'Champagner Renette') to 1.04 mg/g (cv. 'Boskoop'). In contrast, much higher amounts were determined in the acetone extracts, showing a broad range from 2.55 to 32.54 mg/g flavan-3-ol monomers. Lowest contents were observed for the cv. 'Topaz', whereas cv. 'Brettacher' exhibited the highest contents.

With the exception of cv. 'Trierer Weinapfel' and cv. 'Boskoop', the highest PC yields were quantitated in the insoluble residues after aqueous-acetone extraction. For most of the cultivars under investigation the contents of nonextractable PCs were up to 10-fold (cv. 'Gewürzluike') higher than that of the extractable ones. As apparent from comparison of their DPm with those of the flavan-3-ols in the acetone extracts (see Figure 4), the higher PC contents in the residues could be ascribed only to some extent to their larger structures. Thus, there must have been not only structural but also marked quantitative discrepancies with regard to PC compositions of the different type of samples.

Apple seed residues of cv. 'Rheinischer Bohnapfel' exhibited highest amounts of insoluble PCs (42.3 mg/g), whereas their content in cv. 'Topaz' was lowest (11.9 mg/g). With proportions varying from 45% for cv. 'Trierer Weinapfel' to 90% for cv. 'Gewürzluike', this type of PC made up a significant part of the total PCs of apple seeds. This is in accordance with literature data referring to an underestimation of total polyphenols due to high quantities of nonextractable proanthocyanidins of various fruits and vegetables.[32] With the nonextractable portion of PCs taken into account, their total contents ranged from 14.5 to 73.5 mg/g for seeds of cv. 'Topaz' and cv. 'Brettacher', respectively.

Estimation of PC Contents Applying *n*-Butanol/HCl Hydrolysis. Additionally, PC contents of apple seeds were estimated by *n*-butanol/HCl hydrolysis. The contents of the different extracts and solid residues are expressed in cyanidin equivalents of defatted dry matter (mg CyEQ/g DM; Figure 6). By comparison of the PC yields determined by thiolysis prior to RP-HPLC with those obtained by *n*-butanol/HCl hydrolysis, it becomes evident that the latter were markedly lower. Because only extension units are converted into the colored anthocyanidins upon *n*-butanol/HCl hydrolysis, underestimation of PCs when using this method is obvious. Consequently, lower DPm values are associated with increasing underestimation. Moreover, side reactions may lead to the formation of polymeric phlobaphenes, resulting in diminished anthocyanidin concentrations. Similar findings were reported in a previous study.[24]

Nevertheless, PC contents determined by this spectrophotometrical method showed the same trends as observed by thiolysis and subsequent HPLC determination. Contents of oligomeric PCs in the ethyl acetate extracts ranged from 0.02 to 0.24 mg CyEQ/g for the seeds of cv. 'Topaz' and cv. 'Boskoop', respectively. As far as the acetone extracts were concerned, highest yields of extractable PCs were found in seeds of cv. 'Brettacher' (9.2 mg/g), whereas cv. 'Topaz' exhibited the lowest contents (1.04 mg/g). Again, with contents ranging from 3.2 mg/g (cv. 'Topaz') to 11.1 mg/g (cv. 'Brettacher'), nonextractable PCs contributed to a high extent to total PC contents. Total contents of apple seed PCs varied from 4.2 to 20.5 mg/g for the cultivars under study, being highest for cv. 'Brettacher' and lowest for cv. 'Topaz'.

Total Contents of Phenolic Constituents Determined by RP-HPLC. Total contents of phenolic constituents, calculated as the sum of individually quantitated low molecular weight phenolics and monomeric equivalents of both extractable and nonextractable PCs after thiolysis, are summarized in Table 3.

Taking into account the amounts of nonextractable PCs, total phenolic contents of the investigated apple seeds ranged from 18.38 mg/g (cv. 'Topaz') to 99.76 mg/g (cv. 'Brettacher'), indicating seeds of cider apples (e.g., cv. 'Boskoop' and cv. 'Brettacher') to generally exhibit higher amounts of phenolic compounds than dessert apples (e.g., cv. 'Topaz' and cv. 'Royal Gala'). Nonextractable PCs accounted for 29−74% of total phenolics. Interestingly, the proportions of extractable polyphenols were also clearly higher for the seeds of the cider cv. 'Brettacher', cv. 'Boskoop', and cv. 'Trierer Weinapfel', whereas they were almost equal and even lower for the remaining cultivars, with cv. 'Gewürzluike' showing the lowest relative amount (26%) of extractable phenolic compounds. Although their direct extraction is not feasible, nonextractable PCs represent an important fraction of phenolic constituents in apple seeds. Very recently, it has been shown that nonextractable polymeric PCs may be effectively released as oligomers or monomers from cranberry pomace by alkaline hydrolysis.[34] Therefore, alkaline treatment may be a useful means for the recovery of PCs bound to insoluble cell wall material of apple seeds.

The contents of extractable polyphenols without degrading pretreatment were in the range from 6.5 mg/g (cv. 'Topaz') to 59.8 mg/g (cv. 'Brettacher'). Extractable polymeric PCs accounted for 29−59%, whereas low molecular weight phenolics, in particular phloridzin, represented between 39 and 70% of the extractable phenolic fraction. According to findings on the antioxidant capacities of edible part fractions from cv. 'Weirouge' apples, phenolic seed constituents as an additional fraction might also contribute to a significant degree to the overall antioxidant activity of products derived from apples depending on the production process.[35] Therefore, detailed investigations of the antioxidant properties of extracts derived from apple seeds are the subject of our current work.

Comparison of our results on oil yields and phenolic contents of apple seeds revealed an inverse correlation for seeds of cider and dessert apple cultivars. The higher the oil yields in the seeds of different varieties, the lower were their phenolic contents in this study. Analogous to findings on the edible proportions of dessert and cider apples,[30] seeds of the cider apple cultivars were richer in phenolic compounds. Inversely, seeds of dessert apple varieties were very recently shown to exhibit generally higher oil contents than those of cider apples.[25]

Apart from being an interesting source of fatty oil, the results presented in our study demonstrate apple seed residues to be a rich source of phenolic compounds, making their complete recovery worthwhile. Total polyphenol contents significantly differed for the seeds of different cultivars, with seeds of cider apples containing considerably higher amounts. Sequential extraction with aqueous acetone (70:30, v/v) and ethyl acetate provided a simple means of selective fractionation and recovery of low molecular weight phenolics, particularly of phloridzin, and highly polymerized procyanidins of apple seeds, respectively. The so-obtained fractions enriched in such potential health-promoting constituents may be utilized further as functional ingredients in feed and foods. In this context, systematic application of resin adsorption technology would prove to be a very effective tool for further purification as well as for the selective enrichment of target compounds, for example, phloridzin, thus allowing the production of tailor-made phenolic preparations as outlined in a recent study.[36] In conclusion, recovery of highly unsaturated lipids under mild process conditions and subsequent extraction and fractionation of phenolics from the defatted apple seed residues may be a promising strategy for an economical and exhaustive exploitation of these widely available agro-industrial by-products.

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ABBREVIATIONS USED

cv., cultivar; PC(s), procyanidin(s); EtAc, ethyl acetate; DM, dry matter; DPm, mean degree of polymerization; CyEQ, cyanidin equivalents.

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