Recovery and characterisation of coloured phenolic preparations from apple seeds

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The aim of this study was to investigate whether complexly constituted phenolic extracts from apple seeds may be utilised for the recovery of natural coloured antioxidant preparations, which might serve as potential food or cosmetic ingredients.

In a first step, the recovery of phenolic compounds was optimised by varying crucial extraction parameters. A single extraction step at 25 °C using an acetone–water mixture (60:40, v/v) and a solid–to-solvent ratio of 1:8 (w/v) for 1 h was found to be appropriate to achieve both high phenolic yields and antioxidant activities.

In a second step, differently produced apple seed extracts and a phloridzin model solution were enzymatically treated by mushroom polyphenol oxidase to investigate the rate of pigment synthesis. Depending on the extraction procedure applied, synthesis rates, pigment yields and colour properties significantly differed. Compared to the phloridzin model solution, extracts recovered from the seeds showed comparable and even better results, thus indicating such preparations to be a promising alternative to synthetic yellow dyes.

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1. Introduction

Apples (Malus domestica Borkh.) belong to the world’s most important fruit crops. In 2009, their global production accounted for about 70 million tonnes (FAOSTAT, 2010). Approximately 70% thereof is marketed as fresh fruit, while 25–30% of the fruits are processed into juices or juice concentrates, ciders, purées, jams and dried products. With an estimated production of 1.4 million tonnes worldwide during 2004–2005, apple juice and concentrates thereof are the most important processed products (Bhushan, Kalia, Sharma, Singh, & Ajuja, 2008). Consequently, large quantities of apple pomace are accumulated each year in the course of apple juice production as agro-industrial by-products. So far, apple pomace has been primarily used as animal feed (Vendruscolo, Albuquerque, & Streit, 2008) and for the recovery of pectins (Hwang, Kim, & Kim, 2008). During recent decades, however, the recovery of phenolic compounds from apple pomace is increasingly gaining importance due to their antioxidant properties (Vanzani, Rossetto, De Marco, Rigo, & Scarpa, 2011; Vieira et al., 2011) and their potential health benefits (Bellion et al., 2010; Veeriah et al., 2006). While this development has led to many studies on phenolic compounds from apple pomace (Cao, Wang, Pei, & Sun, 2009; Lu & Foo, 1997; Wijngaard & Brunton, 2010), data on isolated apple seeds are rather scarce or outdated. The studies reported so far have shown the phenolic profile of apple seeds to be predominated by phloretin 2′-xyloglucoside, 5-cafeoylquinic acid (chlorogenic acid), p-coumarylquinic acid, (−)-epicatechin, some quer cetin glycosides and phloridzin in particular. The latter was found to be the major low-molecular-weight phenolic constituent amounting to 60–90% of apple seed polyphenols (Fromm, Bayha, Carle, & Kammerer, 2012a; Rupasinghe & Kean, 2008; Schieber et al., 2003). In several studies of recent years, phloridzin has been demonstrated to effectively inhibit the intestinal absorption and renal re-absorption of glucose by the inhibition of sodium–glucose co-transporters 1 and 2 (Alvarado & Crane, 1962; Chinard, Taylor, Nolan, & Enns, 1957; Manzano & Williamson, 2010), thus providing a potential means for the adjuvant treatment of diabetes mellitus type 2, obesity and stress hyperglycaemia (Ehrenkranz, Lewis, Kahn, & Roth, 2005). Apart from its pharmacological properties, phloridzin and its aglycone phloretin have been identified as effective antioxidants inhibiting lipid peroxidation (Rezk, Haenen, van der Vijgh, & Bast, 2002; Rupasinghe & Yasmin, 2010).

Colour is one of the most important characteristics of foods and beverages, strongly determining consumers’ acceptance. Since some of the synthetic azo dyes commonly applied in industrial food processing have been associated with attention deficit hyperactivity disorder (ADHD) (McCann et al., 2007), they are now increasingly replaced by natural food colourants. In this context, an interesting characteristic of phloridzin has been reported upon oxidation by polyphenol oxidase (PPO). While phenolic compounds of apple other than dihydrochalcones were oxidised to reddish-brown compounds, oxidation of phloridzin led to the formation...
of yellow products (Durkee & Poapst, 1965) which may be mainly responsible for the colour of apple juices and ciders (Lea, 1984). Hence, a range of studies on the formation and structures of these water-soluble phloridzin oxidation products (POPi) have been conducted, and the formation of dimeric oxidation products was proposed (Goodenough, Kessel, Lea, & Loefert, 1983; Oszmianski & Lee, 1991; Sarapuu & Kheinaru, 1969). However, only recently the structures of a colourless intermediate (POPi) and of a yellow pigment (POPj) (Fig. 1) could be elucidated by mass spectrometric and NMR spectroscopic experiments, revealing them to be monomeric instead of dimeric oxidation products of phloridzin as postulated earlier (Le Guernévé, Sanoner, Drilleau, & Guyot, 2004). In subsequent experiments the kinetics of their formation, their colouring properties and stability depending on pH value and temperature were investigated, indicating the potential use of POP as natural food colourants (Guyot, Serrand, Le Quéré, Sanoner, & Renard, 2007).

Since the aforementioned studies were conducted with pure chemicals in model solutions, the aim of the present study was to investigate whether complexly constituted phenolic extracts derived from apple seeds are suitable for the recovery of coloured antioxidant preparations, which may serve as potential food or cosmetic ingredients. In a first step, the recovery of phenolic compounds should be optimised by investigating the effects of varying extraction parameters on the contents and antioxidant activities of apple seed extracts. In a second step, different extracts and a phloridzin model solution should be enzymatically oxidised using mushroom PPO to evaluate the efficiency of POPj pigment synthesis.

2. Material and methods

2.1. Chemicals

If not stated otherwise, all reagents and solvents were of analytical or HPLC grade and were bought from VWR (Darmstadt, Germany). Ethanol (96%) was obtained from Nedalco Alcohol (Heilbronn, Germany). Phloridzin (purity > 95%) was obtained from Extrasynthèse (Genay, France). 5-Caffeoylquinic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Acetate fractions were dried with anhydrous sodium sulfate and filtered and washing of the residue, organic solvents were removed under reduced pressure (T = 40 °C). The recovered aqueous phenolic extracts were stored at −20 °C until further analyses.

2.2. Plant material

For most experiments, dried apple seeds kindly provided by Herbstreith & Fox (Neuenheim, Germany) were used. They were recovered from industrial dried apple pomace by sieving and manual separation from other pomace constituents. For some experiments freeze-dried apple seeds of different cider and dessert apple cultivars (harvest 2008) were used. ‘Ripe apple fruits (‘Idared’, ‘Jonagold’, ‘Roter Ziegler’, ‘Boskoop’) were obtained from Hohenheim University research station for horticulture (Stuttgart, Germany), local growers and from the local market.

2.3. Optimisation of phenolic compound extraction from apple seeds

2.3.1. Standard extraction protocol

Seeds obtained from industrial apple pomace were ground continuously for 30 s using a laboratory mill and exhaustively extracted with n-hexane to remove lipophilic constituents. Approx. 2.5 g of the defatted apple seed flour were extracted for 2 h at 25 °C using aqueous acetone (30:70, v/v; solid-to-solvent ratio = 1:8, w/v). To prevent oxidation of phenolic compounds, all extractions were carried out under nitrogen atmosphere. Since preliminary tests had shown stirring to be most effective, this procedure was used throughout instead of shaking or sonication. After filtration and washing of the residue, organic solvents were removed under reduced pressure (T < 30 °C). The recovered aqueous phenolic extracts were stored at −20 °C until further analyses.

2.3.2. Variation of extraction parameters

To optimise the extraction procedure for polyphenols from apple seeds, crucial parameters of the standard extraction protocol, such as type and proportions of solvents (ethanol, methanol, acetone) in water, solid-to-solvent ratios (1:8, 1:16, 1:24, v/v), extraction time (1, 2, 3, 4, 8, 24 h) and temperature (0, 25, 42 °C) were varied. Moreover, the influence of different grinding techniques on the phenolic contents of the resulting apple seed extracts (ASEs) was investigated. For this purpose, continuous grinding (‘30 s’) and successive grinding cycles with intermediate mixing (‘15 s + 15 s; ‘60 s + 30 s’) using a laboratory mill (model A 10, IKA, Staufen, Germany) were applied. For a better comparability the average particle size distributions of the apple seed flours obtained were determined by sieving analysis. Additionally, a sieving mill (model ZM 1, Retsch, Haan, Germany) with a mesh size of 0.5 mm was used for grinding (‘0.5 mm’). Polyphenolic extracts were obtained after an extraction time of 1 h applying the standard extraction protocol.

Finally, the influence of the sample material itself was studied by extracting apple seed flours from different authenticated apple cultivars with aqueous acetone (30:70, v/v) for 1 h. Additionally, seed coats were manually separated from the seeds derived from industrial apple pomace. Both seed coats and de-hulled seeds (embryos) were separately analysed for their phenolic contents.

2.4. Enzymatic oxidation of phenolic compounds in extracts derived from apple seeds

2.4.1. Recovery of phenolic extracts

Defatted seeds from industrial apple pomace were ground (5 × 10 s) with the aforementioned laboratory mill and extracted with the respective solvents for 1 h at ambient temperature under nitrogen atmosphere (solid-to-solvent ratio = 1:8, w/v). After filtration and centrifugation, respectively, the resulting extracts were evaporated to dryness (T < 30 °C), and the residues were redissolved in phosphate buffer (pH 6.5, 100 mM). To assure comparable phloridzin concentrations in each of the samples, the extracts were diluted appropriately with phosphate buffer according to HPLC results. For the recovery of extracts A, B and C, aqueous acetone (30:70, v/v) was used as solvent. Whereas extract A was directly subjected to enzymatic oxidation after removal of the organic solvent, extracts B and C were obtained by subsequent liquid–liquid extraction of the corresponding aqueous crude extracts with ethyl acetate at pH 7 and 1.5, respectively. The ethyl acetate fractions were dried with anhydrous sodium sulfate and filtered. Extract D was recovered using aqueous ethanol (50:50,
v/v), while pure water was used for the extraction of phenolic compounds to obtain extract E.

2.4.2. Enzymatic synthesis of phloridzin oxidation products

The aforementioned ASEs and a phloridzin model solution, each containing approx. 0.5 mM phloridzin, were incubated in phosphate buffer (pH 6.5) using mushroom PPO (30 U/mL). Incubation experiments were carried out at 30 °C with magnetic stirring (410 rpm) under oxygen exposure (air). For monitoring the formation of POPj, aliquots of the incubation medium (2 mL) were taken and immediately heated for 2 min at 95 °C to stop enzymatic reactions. Subsequently, samples were cooled on ice, membrane-filtered (0.45 μm) and kept at –20 °C until further analysis. In preliminary tests the complete inactivation of PPO under the heating conditions applied was assured.

2.4.3. Colour analysis

Colorimetric measurements were carried out using a Perkin-Elmer Lambda 20 spectrophotometer (Perkin-Elmer, Rodgau-Jügesheim, Germany) equipped with UV WinLab software (version 2.85.04) and WinCol software (version 2.05). L*, a*, b*, chroma \( C^* = \sqrt{(a^*)^2 + (b^*)^2} \) and hue angle \( h = \arctan (b^*/a^*) \) values were calculated from spectra recorded across a range of 380–780 nm using illuminant D65 and a 10° observer angle.

2.5. Determination of phenolic compounds and phloridzin oxidation products (POPi, POPj) by HPLC-DAD-ESI-MS\(^\text{a}\)

HPLC analyses were carried out using an Agilent HPLC series 1100 chromatograph (Agilent, Waldbronn, Germany) equipped with UV WinLab software (version 2.85.04) and WinCol software (version 2.05). L*, a*, b*, chroma \( C^* = \sqrt{(a^*)^2 + (b^*)^2} \) and hue angle \( h = \arctan (b^*/a^*) \) values were calculated from spectra recorded across a range of 380–780 nm using illuminant D65 and a 10° observer angle.

2.6. Synthesis and preparative isolation of phloridzin oxidation products (POPi)

For the synthesis of POPj, a solution of phloridzin (2.6 mM) in phosphate buffer (pH 6.5; 100 mM, 225 mL) was incubated at ambient temperature under oxygen exposure (air) with vigorous stirring in the presence of mushroom PPO (31.104 U) for 24 h. Subsequently, aliquots thereof (2 mL) were incubated in derivatisation tubes at 95 °C for 3 min using a water bath to stop enzymatic reactions. After cooling on ice the aliquots were membrane-filtered (0.45 μm) and subjected to preparative HPLC. Isolation of POPj was carried out using a Phenomenex C18 Aquagel 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-10AVp UV/Vis detector (Shimadzu, Kyoto, Japan). Data were processed with McDaqc32 Control software (version 2.0) (Bischoff). The mobile phase consisted of 2% (v/v) acetic acid in water (elucent A) and 0.5% acetic acid in water and methanol (30:70, v/v, eluent B). Phloridzin oxidation products POPi and POPj were eluted isocratically with 50% eluent B at ambient temperature and a flow rate of 6.0 mL/min. Monitoring was performed at 280 nm. Phloridzin oxidation products were identified by HPLC-DAD-MS\(^\text{a}\) experiments according to mass spectral data previously reported (Le Guernévé et al., 2004). Chromatographic purity of the isolated POPj was ≥ 94% (HPLC, 280 nm).

2.7. Determination of total phenolic contents and of antioxidant activities

All photometric measurements were performed in duplicate with a Biotek microplate spectrophotometer (Power Wave XS, Biotek Instruments, Bad Friedrichshall, Germany) equipped with Gen5 software (Ver. 1.04.5) and 96-well microplate cuvettes.

2.7.1. Folin–Ciocalteu assay

Total phenolic contents (TPCs) of the ASEs were determined applying the Folin–Ciocalteu assay according to Singleton, Orthofer, and Lamuela-Raventos (1999) with some modifications. Briefly, 50 μL of sample were mixed with 60 μL of Folin–Ciocalteu reagent (diluted with water, 1:6, v/v). After incubation for 3 min, 80 μL of sodium carbonate solution (7.5%, w/v in water) were added and thoroughly shaken. Absorption measurements at 720 nm were performed after 60 min of incubation in the dark. Total phenolics are expressed as gallic acid equivalents (mg GAE/g defatted matter).
2.7.2. TEAC assay

Spectrophotometric determination of the antioxidant activity was performed as previously described (Van den Berg, Haenen, van den Berg, & Bast, 1999). For the daily preparation of the ABTS radical reagent, 0.5 mL of ABTS solution (20 mM in phosphate buffer, pH 7.4) and 100 mL of ABAP solution (2.5 mM in phosphate buffer, pH 7.4) were blended and heated for 15 min at 60 °C in a water bath in the dark. The phosphate buffer was prepared by blending 812 mL of a Na2HPO4·2H2O solution (66 mM) with 182 mL of KH2PO4 solution (66 mM) and 25 mL of ascorbic acid. Antioxidant activity was measured by mixing 40 μL of sample with 200 μL of radical solution in microplate cuvettes. Absorption was measured after 6 min at 734 nm.

2.7.3. FRAP assay

Antioxidant activity was determined according to a method previously reported (Benzie & Strain, 1996). FRAP reagent was prepared daily by blending 2.5 mL of TPTZ solution (10 mM in 40 mM HCl), 2.5 mL aqueous FeCl3 solution (20 mM) and 25 mL of an acetate buffer (300 mM, pH 3.6). Sample aliquots (20 μL) were mixed with 150 μL of FRAP reagent, and absorption was measured after 4 min at 593 nm.

Aqueous Trolox solutions were used for calibration of both the TEAC and FRAP assay. Antioxidant activities were calculated as μmol Trolox antioxidant equivalents (TAE)/g defatted matter.

2.8. Statistical analysis

All experiments were performed at least in duplicate. Significant differences (α = 0.05) were determined using the Tukey or Tukey–Kramer test for different independent samples. Data evaluation was performed with the SAS software package (Software Version 9.1; SAS Institute, Cary, NC).

3. Results and discussion

3.1. Optimisation of polyphenol extraction from apple seeds

The effects of varying crucial extraction parameters on the efficiency of polyphenol extraction from apple seed residues were assessed by means of Folin–Ciocalteu, TEAC and FRAP assays. Some of the extracts were additionally analysed by RP-HPLC.

3.1.1. Extraction solvents

In general, polyphenol recovery was strongly determined by the solvent chosen for extraction of the plant material. Fig. 2a illustrates the influence of varying acetone/water ratios on the total phenolic contents (TPCs) of the extracts obtained. Maximum TPCs amounting to 2.99 and 2.89 mg GAE/g were found for 60% and 70% acetone, respectively, whereas the extraction with 100% acetone significantly from 2.96 to 3.46 mg GAE/g. TPC gains were less pronounced, only amounting to 17% and 14%, when extraction times of 1 and 2 h, respectively, were applied. Again, extraction time only slightly improved polyphenol extraction yields by 12% at maximum from 2.96 to 3.32 mg GAE/g.

3.1.2. Extraction time and solid-to-solvent ratio

Extraction time and solid-to-solvent ratio are also known to influence extraction yields. As illustrated by Fig. 2c the effect of extraction time on TPCs of the ASEs was not significant. When extending extraction times up to 24 h, TPCs remained almost constant, amounting between 3.00 (1 h) and 3.28 mg GAE/g (8 h). Similar observations applied to the corresponding antioxidant activities, ranging from 5.54 to 7.29 μmol TAE/g and from 42.47 to 46.53 μmol TAE/g for the FRAP and TEAC assays, respectively. Consequently, a minimum extraction time of 1 h was considered most suitable. In Fig. 2d TPCs of ASEs obtained using different solid-to-solvent ratios in combination with different extraction times are presented. With decreasing solid-to-solvent ratios from 1.8 to 1:24 TPCs were augmented for each of the extraction periods. As shown for the extraction over 3 h, TPCs increased most significantly by 25% from 2.77 to 3.46 mg GAE/g. TPC gains were less pronounced, only amounting to 17% and 14%, when extraction times of 1 and 2 h, respectively, were applied. Again, extraction time only slightly improved polyphenol extraction yields by 12% at maximum from 2.96 to 3.32 mg GAE/g at a solid-to-solvent ratio of 1:16 (w/v). Thus, in agreement with a previous report (Al-Farsi & Lee, 2008), decreasing the solid-to-solvent ratio proved to be more relevant than extension of extraction time.

3.1.3. Temperature

Temperature may have great influence not only on the solubility of a solute, but also on its stability. In our experiments the effect of different temperatures on extraction yields was investigated. Since acetone–water was shown to be most efficient, this extraction medium was used in subsequent experiments. Due to the low boiling point of acetone (56 °C), temperature effects were evaluated between 0 and 42 °C. As illustrated by the results for TPCs (Fig. 2e), extraction was most efficient at ambient temperature (25 °C), yielding a significantly higher TPC value (2.86 mg GAE/g) than for lower and higher temperatures (0 °C: 2.52 mg GAE/g; 42 °C: 2.32 mg GAE/g). These findings correlated with the results of the TEAC and FRAP assays, since the extract obtained at 25 °C
exhibited highest antioxidant activities. Thus, low temperatures obviously negatively affected the solubility and extraction of phenolics, whereas elevated extraction temperature appeared to be associated with progressive oxidative losses. Furthermore, due to solvent evaporation at elevated temperatures, the acetone-to-water ratio may have changed which might have negatively affected extraction yields, as reported earlier (Durling et al., 2007).

3.1.4. Effect of sample comminution

Since extraction yields are strongly dependent on the particle size of the sample material, and consequently on the comminution procedure applied, different grinding protocols, such as continuous grinding (‘30 s’) and successive grinding cycles with intermediate mixing (‘15 s + 15 s’; ‘60 s + 30 s’) were studied using a laboratory mill. Alternatively, a sieving mill (‘0.5 mm’) was used. Fig. 2f summarises the TPCs of the resulting extracts. With values of 3.31 and 3.07 mg GAE/g, highest TPCs were determined when grinding apple seeds for ‘60 s + 30 s’ and ‘30 s’, respectively. This is in agreement with their average particle size distribution showing high proportions of particles with minimum size <0.1 mm (6.6% and 17.1%, respectively). Subsequent grinding for 15 s with intermediate mixing (‘15 s + 15 s’) resulted in a significantly lower TPC of the extract (2.66 mg GAE/g), although 83.2% of the particles ranged between 0.315 and 1.0 mm. Interestingly, particle sizes of the latter sample were not below 0.1 mm, thus confirming this particle size to be of utmost importance for efficient polyphenol extraction. Using a sieving mill, TPC of the corresponding extract amounted to 2.76 mg GAE/g. HPLC analysis revealed an appreciable phloridzin content of 2.96 mg/g, thus indicating the sieving mill to be an alternative to the laboratory mill. However, it is worth mentioning that during grinding with the sieving mill a marked raise of temperature was observed, which might have favoured oxidative

Fig. 2. Effects of varying crucial extraction parameters (aqueous acetone (a), aqueous methanol or ethanol (b), extraction time (c), solid-to-solvent ratio (w/v) (d), temperature (e), comminution (f), sample material (g)) on total phenolic contents (mg GAE/g defatted matter) of extracts recovered from apple seeds. Bars represent means of at least two determinations ± standard error. Identical letters indicate that values are not significantly different.
polyphenol losses and aggregation of lipid-containing particles. Consequently, in order to optimise grinding of apple seeds, cooling is a prerequisite. For the extract obtained from apple seed flour ‘60 s + 30 s’, antioxidant activities of 7.25 μmol TAE/g (FRAP) and 46.47 μmol TAE/g (TEAC) were determined, thus exhibiting markedly higher antioxidant activity than the remaining samples, which had comparable antioxidant properties independent of the grinding procedure.

3.1.5. Sample material

Sample material itself may also play an important role for extraction yields. While there are several studies dealing with the phenolic contents of apple fruits showing cultivar-dependent differences (Keller, Streker, Arnold, Schieber, & Carle, 2001; Tsao, Yang, Young, & Zhou, 2003), data on apple seeds are scarce. In order to fill this gap, seed flours of different apple cultivars, seed coats and de-hulled seeds should be analysed for their phenolic contents and antioxidant activities. As apparent from Fig. 2g, TPCs of the seeds of the different cultivars significantly differed. Highest contents were determined for seeds of the cvs. ‘Boskoop’ and ‘Roter Ziegler’, which contained 16.19 and 9.36 mg GAE/g, respectively. In contrast, seeds of cv. ‘Jonagold’ exhibited a much lower TPC, only accounting for 18% of the phenolic content of cv. ‘Boskoop’. In accordance with the respective pulps and peels, seeds of cider apple cultivars contain higher levels of polyphenols than those of dessert apples. As observed for TPCs, phloridzin contents also differed significantly among the seeds of different cultivars, ranging between 2.60 and 16.41 mg/g for the cvs. ‘Jonagold’ and ‘Boskoop’.

Investigations into different apple seed tissues revealed the seed coats to exhibit a TPC of 8.10 mg GAE/g, whereas only 0.20 mg GAE/g was found for the seeds. Taking into account the average proportion of the seed coats (~37%), coat phenolics accounted for 96% of TPC of the whole seeds. Consequently, seed polyphenols are almost exclusively deposited in the seed coat tissue, while the fat-containing embryos are mostly devoid of such polar compounds. Since some of the cultivars, in particular ‘Boskoop’, exhibited a significantly lower seed coat-to-embryo ratio, the distribution of phenolics appears to be an additional reason for the observed quantitative differences. These results were corroborated by HPLC, revealing a negligible phloridzin content (0.10 mg/g) in the embryos. Interestingly, the phloridzin level of the seed coat (4.92 mg/g) was approx. half the amount determined by the Folin–Ciocalteau assay. The observed discrepancy may be ascribed to the fact that monomeric, oligomeric and polymeric phenolics are assessed by the photometric assay, while only low-molecular-weight phenolics, particularly phloridzin, are covered by RP-HPLC. Antioxidative activities of the respective ASES showed good correlation with their TPCs, with cv. ‘Boskoop’ exhibiting the highest TEAC and FRAP values, whereas cv. ‘Jonagold’ showed the opposite.

3.2. Identification of apple seed phenolics by HPLC-DAD-MS^n

Identification of individual phenolic constituents was achieved by comparing their retention times, UV/Vis and mass spectral data with those of reference compounds. Fig. 3 shows typical chromatograms recorded at different wavelengths. Retention times, UV/Vis and mass spectral data as well as peak assignments are specified in Table 1. At 280 nm a hydroxybenzoic acid, flavan-3-ols and most of all compounds exhibiting dihydrochalcone structures were detected. Compound 1 revealed an [M–H]^– ion at m/z 153 producing a fragment ion at m/z 109 in MS^n experiments, thus indicating the loss of a carboxylic function. According to its UV/Vis and mass spectra including its retention time, compound 1 was identified as protocatechuic acid. Among the flavan-3-ols, compounds 2 and 3 were identified as procyanidin B2 and (–)-epicatechin, respectively. In a ‘Boskoop’ seed extract, however, an additional compound (4) occurred, showing UV/Vis spectral data typical for procyanidins and an [M–H]^– ion at m/z 865. In MS^n and MS^n experiments fragment ions previously reported for a procyanidin trimer of the B-type were obtained (Verardo, Bonoli, Marconi, & Caboni, 2008; Xiao et al., 2008). Reports on flavan-3-ols in apple seeds are quite contradictory. Whereas some authors identified (–)-epicatechin and procyanidin B2 (Schieber et al., 2003) others detected (–)-epicatechin and (+)-catechin (Rupasinghe & Kean, 2008) or even reported an absence of flavan-3-ols (Lu & Foo, 1998). These discrepancies may be explained by different analytical methods and differing raw materials.

According to their elution order and mass spectral behaviour, peaks 6, 7, 8 were assigned to the dihydrochalcones 3-hydroxyphlorizin (m/z 451), phloretin 2’-O-xylolglucoside (m/z 567) and phloridzin (m/z 435), respectively. Upon collision-induced dissociation (CID), compounds 7 and 8 generated a fragment at m/z 273, indicating the loss of their saccharide moieties and the release of the phloretin aglycone. Compound 6, however, provided an ion at m/z 289, indicating the presence of an additional hydroxyl group (A = 16 Da). A similar fragmentation pattern has been previously reported in positive ionisation mode (Alonso-Salces et al., 2004). Eluting shortly before compound 6, compound 5 was detected, providing an [M–H]^– ion at m/z 597. Its fragmentation pathway was almost identical to that of phloridzin. Furthermore, fragments at m/z 435 (MS^n) and m/z 273 (MS^n) were generated. The formation of these fragments was ascribed to two successive losses of a hexose moiety (162 Da each). Moreover, the UV/Vis spectrum of this compound showed maxima at 280 and 230 nm, thus being similar to a previously reported unknown dihydrochalcone or flavanone structure (Lu & Foo, 1998). Based on these findings, compound 5 was concluded to represent a phloretin dihexoside. Compound 9 was identified as 5-cafeoylquinic acid by its UV/Vis spectral data and elution behaviour. This was corroborated by the fragmentation pattern, revealing an [M–H]^– ion at m/z 353 and a fragment at m/z 191 in the MS^n experiment. Two compounds (10, 11), which sequentially eluted at 18.5 and 19.2 min, showed similar UV/Vis spectra and an identical [M–H]^– ion at m/z 337, indicating the occurrence of p-coumaroylquinic acid isomers. According to published data (Clifford, Johnston, Knight, & Kuhnert, 2003), peaks 10 and 11 were assigned to the 4- and 5-isomers of p-coumaroylquinic acid, producing MS^n fragments at m/z 173 and 191, respectively. The occurrence of chlorogenic acid and p-coumaroylquinic acid in apple seeds is consistent with previous reports (Lu & Foo, 1998; Schieber et al., 2003). In the present study, however, 4- and 5-p-coumaroyl-quinic acid isomers were additionally detected in apple seeds.

Compounds 12–16 showed UV/Vis spectral data characteristic of flavonols. Each of these compounds provided an [M–H]^– ion at m/z 301 in MS^n experiments, indicating the occurrence of quercetin. In subsequent MS^n experiments, they showed different fragmentation patterns due to differing saccharide moieties. For compounds 12 and 13, a loss of a hexose moiety (162 Da) was observed. By comparison of their retention times with those of authentic standards, compounds 12 and 13 were assigned to quercetin 3-O-galactoside and quercetin 3-O-glucoside, respectively. According to a loss of 146 Da, compound 16 was identified as quercetin 3-O-rhamnoside. This assignment was corroborated by the comparison of its retention time with that of the corresponding reference compound. The loss of 132 Da for compounds 14 and 15 upon fragmentation indicated these substances to be quercetin pento-sides. Due to the lack of authentic standards, compounds 14 and 15 were tentatively identified as quercetin 3-O-xylloside and quercetin 3-O-arabinoside, respectively, according to their elution behaviour on a comparable stationary phase as previously reported (Schieber, Hilt, Conrad, Beifuss, & Carle, 2002).
3.3 Enzymatic oxidation of apple seed extracts

3.3.1 Analysis of the incubation media by HPLC

Syntheses of the pigment POPj upon oxidation of various ASEs and a phloridzin model solution were monitored by HPLC over a period of up to 32 h (Fig. 4a). Although the study was focused on the synthesis of POPj, changes of relevant educts and intermediates were also recorded during incubation (Fig. 4b–e), thus providing additional information on the enzymatic reaction.

3.3.1.1 General findings. According to Fig. 4a, the ASEs (A–E) and the phloridzin model solution (PMS) significantly differed, both with regard to product formation kinetics and maximum yields of POPj. Most distinct increases of POPj contents were observed during the first 2 h (except for extract E), while only minor changes were observed thereafter. This is in accordance with previous findings, demonstrating the conversion of POPj into POPi to be very slow due to low affinity of mushroom PPO towards POPi (Guyot et al., 2007). Samples also showed considerable differences with regard to the conversion of phloridzin, phloretin 2'-xyloglucoside, chlorogenic acid and POPi (Fig. 4b–e). Upon oxidation, phloridzin was converted, with POPi being simultaneously formed and accumulated to different extents (see Fig. 4e). POPj synthesis was probably further hindered by increasingly formed oxidation products of phenolic compounds, which were shown to significantly inhibit PPO activity (Le Bourvellec, Le Quéré, Sanoner, Drilleau, & Guyot, 2004). This might be the reason why POPi was incompletely converted into POPj.

Interestingly, pigment formation started immediately after PPO addition. This is in disagreement to previous studies, reporting the existence of a lag period due to the necessary conversion of the enzyme into its active form (Guyot et al., 2007; Oszmianski & Lee, 1991). This lag phase, however, was significantly shortened by low amounts of o-diphenolic structures, such as chlorogenic acid (Oszmianski & Lee, 1991). Such synergistic effects might explain the missing lag phase in our oxidation experiments when applying more complex ASEs. Considering the model solution, the absence of the lag phase might be ascribed to differing purities of the ASEs.

Fig. 3. HPLC chromatograms of apple seed extracts at detection wavelengths of 280 nm (a), 320 nm (b) and 370 nm (c). For peak assignment see Table 1.
reference compounds used. Fig. 4c illustrates that phloretin 2'-O-
xyloglucoside was also converted by mushroom PPO. Due to its
dihydrochalcone structure, phloretin 2'-O-xyloglucoside is as-
sumed to be similarly oxidised to an analogous pigment. However,
since such a structure could not be detected, it was concluded that POPj derived from phloretin 2'-O-xyloglucoside is as-
sumed to be similarly oxidised to an analogous pigment. However,

3.3.1.2. Extract E. In contrast to the remaining samples, the aqueous extract E showed a completely different behaviour upon oxidation.
POPj was detected only after a lag phase of about 3.5 h, but consid-
erable formation was noted after ~18 h, resulting in the lowest
synthesis rate (0.011 mM/h) and unsatisfactory yield (0.09 mM,
1192 mAU min, 2 h). The observed differences were most likely
due to the extraction conditions applied. Both extracts were obtained
with aqueous acetone, but extract B was further purified by
liquid–liquid extraction using ethyl acetate at pH 7. Hence, ex-
tract B was almost devoid of phenolic acids, while chlorogenic acid
contents of extract A remained virtually constant during incuba-
tion. Moreover, polymeric procyanidins are likely to be efficiently
removed in extract B using ethyl acetate, as recently reported
(Fromm et al., 2012a). The latter are known to significantly inhibit
PPO activity with increasing average degree of polymerisation (Le
Bourvellec et al., 2004). Thus, in contrast to extract A which is sup-
posed to contain highly polymerised procyanidins, the inhibitory
effect of the latter on PPO activity was obviously negligible in ex-
tract B (Fromm et al., 2012a). As demonstrated in a previous study
(Oszmianski & Lee, 1991), the presence of chlorogenic acid must be

table 1
Retention times, UV/Vis spectra and mass spectral characteristics of phenolic compounds in apple seed extracts.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Assignment</th>
<th>rt (min)</th>
<th>λ_{max} (nm)</th>
<th>[M-H]^+ (m/z)</th>
<th>HPLC-ESI(-)-MS^n experiment m/z (% base peak)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protocatechuic acid</td>
<td>6.8</td>
<td>260, 295</td>
<td>153</td>
<td>-MS^2 [153]: 109 (100)</td>
</tr>
<tr>
<td>2</td>
<td>Procyanidin B2</td>
<td>16.2</td>
<td>232, 280</td>
<td>577</td>
<td>-MS^2 [577]: 425 (100), 407 (67), 408 (46), 451 (37), 289 (36)</td>
</tr>
<tr>
<td>3</td>
<td>(-)-Epicatechin</td>
<td>18.4</td>
<td>231, 278</td>
<td>289</td>
<td>-MS^2 [289]: 245 (100), 205 (54), 161 (16), 246 (10), 110 (8)</td>
</tr>
<tr>
<td>4</td>
<td>Procyanidin trimmer</td>
<td>21.6</td>
<td>231, 280</td>
<td>865</td>
<td>-MS^2 [865]: 695 (100), 739 (50), 577 (46), 696 (41), 713 (39)</td>
</tr>
<tr>
<td>5</td>
<td>Phloretin dihexoside</td>
<td>29.5</td>
<td>230, 280</td>
<td>597</td>
<td>-MS^2 [597]: 436 (100), 435 (24), 274 (21), 499 (19)</td>
</tr>
<tr>
<td>6</td>
<td>3-Hydroxyphloridzin</td>
<td>32.9</td>
<td>230, 284</td>
<td>451</td>
<td>-MS^2 [451]: 289 (100), 290 (30), 167 (58), 349 (3)</td>
</tr>
</tbody>
</table>
| 7    | Phloretin 2'-O-
xyloglucoside | 34.7    | 230, 284   | 567          | -MS^2 [567]: 273 (100), 274 (19), 167 (6), 419 (6), 531 (3) |
| 8    | Phloridzin | 38.8    | 231, 284   | 435          | -MS^2 [435]: 273 (100), 274 (14), 272 (3), 167 (2) |
| 9    | 5-O-Caffeoylquinic acid (Chlorogenic acid) | 14.0    | 243, 302sh, 326 | 353          | -MS^2 [353]: 191 (100), 192 (9), 179 (5), 135 (2) |
| 10   | 4-O-p-Coumaroylquinic acid | 18.5    | 232, 311   | 337          | -MS^2 [337]: 173 (100), 174 (8) |
| 11   | 5-O-p-Coumaroylquinic acid | 19.2    | 232, 311   | 337          | -MS^2 [337]: 173 (100), 174 (8) |
| 12   | Quercetin 3-O-galactoside | 31.1    | 255, 354   | 463          | -MS^2 [463]: 301 (100), 309 (39), 302 (4), 381 (4), 308 (4) |
| 13   | Quercetin 3-O-glucoside | 31.9    | 254, 355   | 463          | -MS^2 [463]: 301 (100), 303 (32), 302 (23), 173 (18), 381 (9) |
| 14   | Quercetin 3-C-glucoside | 33.6    | 254, 356   | 433          | -MS^2 [433]: 301 (100), 302 (16), 300 (12), 123 (7), 197 (7) |
| 15   | Quercetin 3-O-
arinobioside | 35.6    | 261, 355   | 433          | -MS^2 [433]: 301 (100), 302 (17), 271 (7), 197 (3) |
| 16   | Quercetin 3-O-
ramnoside | 36.4    | 253, 350   | 447          | -MS^2 [447]: 301 (100), 302 (18), 255 (15), 314 (12) |

Abbreviations: rt, retention time; sh, shoulder.

As apparent from Fig. 4a for the phloridzin model solution, extracts D and C, synthe-
sis rates were highest during the first 1.5 h because the respective
POPj formation rates of 0.492 mM/h, 0.491 mM/h, and 0.404 mM/h,
respectively, were quite similar. However, considerable differences
were observed for pigment yields, with extract D showing highest
accumulation (0.50 mM, 1.75 h). Oxidative treatment of extract C, obtained by an additional liquid–liquid extraction with ethyl
acetate at pH 1.5, also led to the formation of high amounts of POPj (0.39 mM, 1.75 h). Pigment formation was accompanied by a rapid and complete conversion of phloridzin (Fig. 4b) and phloretin 2′-O-xyloglucoside (Fig. 4c) within 15 min. Additionally, POPi was most effectively converted into POPj within the first 2 h, as indicated by the marked decreases of the corresponding peak areas in Fig. 4e.

In contrast, POPj content of the phloridzin model solution was significantly lower (0.16 mM, 2 h), due to a considerable decrease of the synthesis rate caused by the stagnating conversion of POPi (as shown in Fig. 4e) into POPj shortly after adding PPO (~0.5 h). Again, synergistic effects in the presence of chlorogenic acid may have enhanced POPj formation in comparison with the model solution. Interestingly, chlorogenic acid was completely metabolised after 2 h, illustrating improved enzyme activity. This was probably due to the absence of inhibiting polymeric procyanidins in extracts C and D, which were recovered using ethyl acetate and alcohol, respectively. These solvents were shown to exclude polymeric procyanidins from extraction (Fromm et al., 2012a; Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998).

3.3.2. Evaluation of colour properties upon formation of POPj

\( L^* \), \( C^* \) and \( h^* \) values were used to characterise colour changes of the samples upon oxidation with PPO. Most significant changes were observed during the first 6 h of the oxidation experiments (Fig. 5). Within this period, lightness only slightly decreased for the phloridzin model solution as well as for extracts A, B and E, which is reflected by \( L^* \) values still being between 95.6 and 88.1. For extracts C and D, however, the decrease of lightness was more

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**Fig. 4.** Kinetics of POPj (a), phloridzin (b), phloretin 2′-O-xyloglucoside (c), chlorogenic acid (d) and POPi (e) upon oxidation of apple seed extracts A–E and a phloridzin model solution (PMS) after adding mushroom PPO (30 U/mL). Values are means ± standard deviation (\( n = 2 \)).
Enzymatic treatment of ASEs revealed marked differences in POPj pigment formation. Depending on the extraction procedure, synthesis rates and pigment yields signiﬁcantly differed, most likely due to varying POPj inhibition. Compared to the phloridzin model solution, ASEs generally exhibited higher pigment yields and colour brilliance. A simple extraction with aqueous ethanol (50%, v/v) has proven to be the most effective way for the obtention of coloured extracts from apple seeds, resulting in a maximum yield of 0.71 mM POPj (after 31.5 h). Due to its simplicity and the easy handling of an organic solvent having relatively low toxicity, industrial scale-up may be readily performed, thus allowing the comfortable recovery of larger quantities of such colourants. Besides their colouring properties such preparations may also serve as natural antioxidants, since they were demonstrated to exhibit appreciable antioxidant activities (data not shown).

The results of the present study demonstrated that apple seeds might be fully exploited for the recovery of coloured preparations displaying antioxidant activities which may represent an interesting alternative to synthetic dyes. Altogether, the sequential extraction of highly unsaturated fatty oils (Fromm, Bayha, Carle, & Kammner, 2012b) and antioxidant phenolic compounds from the defatted residual seed hulls, in particular of phloridzin as an adjuvant in the treatment of diabetes and for weight control appears to be promising. In addition, the subsequent oxidation of phloridzin derivatives, as demonstrated in this study, may provide a further opportunity to perform a more economical and exhaustive exploitation of by-products arising from apple processing. However, before such preparations may be effectively utilised for dietary purposes, further knowledge not only on their colour stability in different food matrices but most of all on their toxicity is required to rule out any health risks, since apple seeds are also known to contain cyanogenic glycosides.

**Acknowledgment**

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.09.042.

**References**


