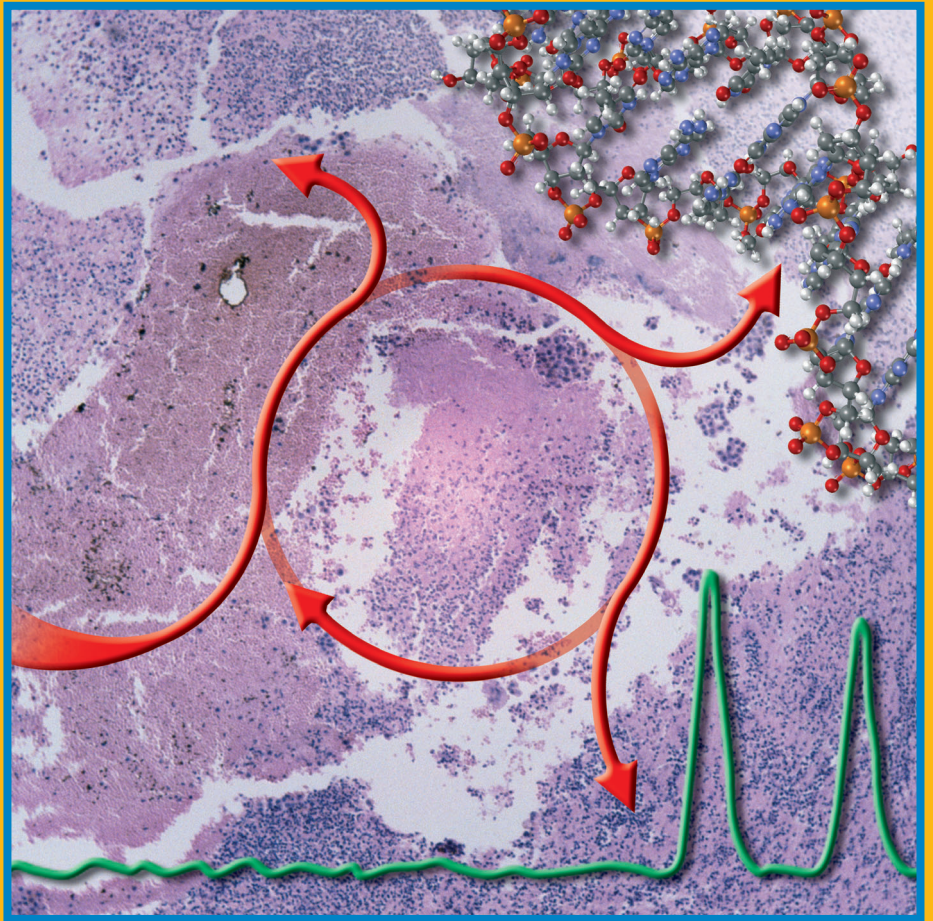


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## Research Article

# Fractionation of polyphenol-enriched apple juice extracts to identify constituents with cancer chemopreventive potential

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Apples and apple juices are widely consumed and rich sources of phytochemicals. The aim of the present study was to determine which apple constituents contribute to potential chemopreventive activities, using a bioactivity-directed approach. A polyphenol-enriched apple juice extract was fractionated by various techniques. Extract and fractions were tested in a series of test systems indicative of cancer preventive potential. These test systems measured antioxidant effects, modulation of carcinogen metabolism, anti-inflammatory and antihormonal activities, and antiproliferative potential. Regression analyses indicated that 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging potential correlated with the sum of low molecular weight (LMW) antioxidants (including chlorogenic acid, flavan-3-ols, and flavonols) and procyanidins, whereas peroxy radicals were more effectively scavenged by LMW compounds than by procyanidins. Quercetin aglycone was identified as a potent Cyp1A inhibitor, whereas phloretin and (–)-epicatechin were the most potent cyclooxygenase 1 (Cox-1) inhibitors. Aromatase and Cyp1A inhibitory potential and cytotoxicity toward HCT116 colon cancer cells increased with increasing content in procyanidins. Overall, apple juice constituents belonging to different structural classes have distinct profiles of biological activity in these *in vitro* test systems. Since carcinogenesis is a complex process, combination of compounds with complementary activities may lead to enhanced preventive effects.

**Keywords:** Apple juice / Aromatase / Epicatechin / Fractionation / Phloridzin

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## 1 Introduction

Advances in our understanding of the carcinogenic process at the cellular and molecular level made over the past few decades have led to the development of a promising new

approach to cancer prevention, termed “chemoprevention” [1]. Chemoprevention aims to halt or reverse the development and progression of precancerous cells through use of nontoxic nutrients and/or pharmacological agents during the time period between tumor initiation and malig-

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**Abbreviations:** AR, aromatase; CI, confidence interval; Cox-1, cyclooxygenase 1; CPC, centrifugal partition chromatography; DP<sub>n</sub>, mean degree of polymerization; DPPH, 1,1-diphenyl-2-picrylhydrazyl;

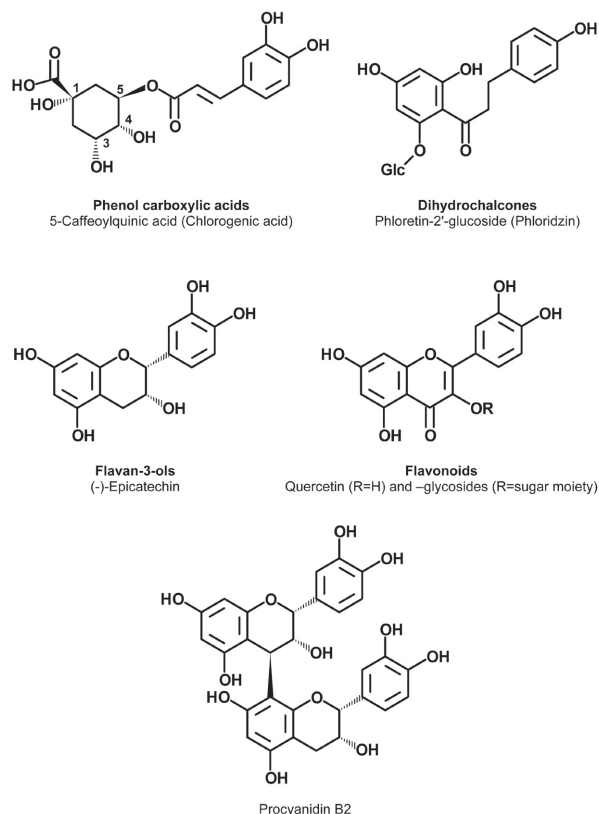
GPC, gel permeation chromatography; IC<sub>50</sub>, halfmaximal inhibitory concentration; LMW, low molecular weight; NP-HPLC, normal-phase HPLC; OR, odds ratio; ORAC, oxygen radical absorbance capacity; QR, NAD(P)H:quinone reductase; RR, relative risk; RT, retention time; TPC, total procyanidin content; X/XO, xanthine/xanthine oxidase assay

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nancy [2]. There is a considerable time frame during which the carcinogenic process could potentially be halted or reversed. Taking this into consideration, the validation and utilization of dietary components, natural products or their synthetic analogs as potential cancer chemopreventive agents in the form of functional foods or nutraceuticals has become an important issue in current health- and cancer-related research.

There are several lines of evidence suggesting that apples and apple juice may be important raw materials for chemopreventive agent development (review in ref. [3]). Apples are the most consumed fruit in Germany, with an average consumption of 21.5 kg *per* household in 2006 (Source and Copyright: Zentrale Markt und Preisberichtsstelle ZMP, Bonn, <http://www.zmp.de>, 04.06.2007), and are a rich source of nutrient as well as non-nutrient components including vitamins. Epidemiologic evidence accumulated over the past years points to the cancer preventive potential of apples, especially for lung and colorectal cancer. As demonstrated in the Nurses' Health Study (NHS) involving 77 000 women, a significant lower risk for lung cancer was observed among the women for increases of 1 serving/day of apples and pears (relative risk (RR) = 0.63; 95% confidence interval (CI) = 0.43–0.91), whereas no effect was seen among men in the Health Professionals Study [4]. In the Zutphen Elderly Study with 728 men, uptake of apples as a source for catechins was nonsignificantly related with reduced lung cancer risk (for a 7.5 mg increase in catechin intake RR = 0.67; 95% CI = 0.38–1.17) [5]. Also, in a large Finnish cohort study with 10 000 men and women, apple intake was strongly associated with a lower risk of lung cancer (RR 0.4; 95% CI = 0.22–0.74) [6]. The results of a case–control study conducted in Hawaii with 528 cases and 528 controls were reported by Le Marchand *et al.* [7]: they found statistically significant inverse associations between lung cancer risk and onions and apples as the main food source of the flavonoid quercetin. The lung cancer odds ratio (OR) for the highest compared with the lowest quartile of intake was 0.5 (95% CI = 0.3–0.9) for onions ( $P$  for trend = 0.001) and 0.6 (95% CI = 0.4–1.0) for apples ( $P$  for trend = 0.03).

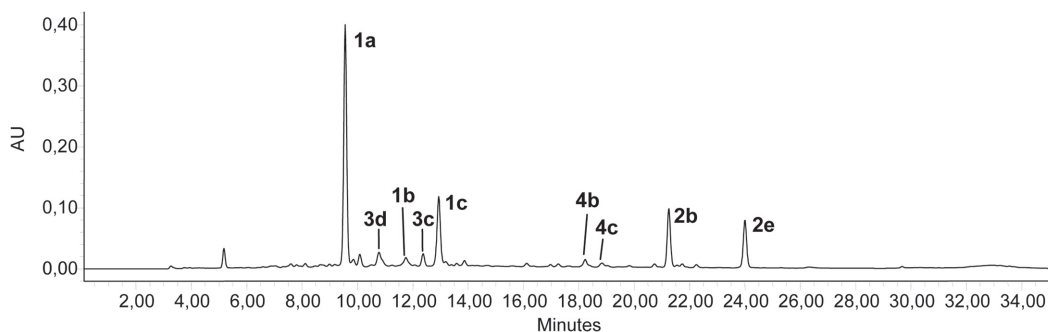
In addition to the strong association with lung cancer prevention, recent publications also indicate preventive effects of apples on colorectal carcinogenesis. Deneo-Pellegrini *et al.* [8] reported that apple consumption was associated with significant dose-dependent reduction in colorectal cancer risk (OR for highest tertile 0.4, 95% CI 0.25–0.66;  $P$  for trend < 0.001) for men and women in a case–control study conducted in Uruguay. Gallus *et al.* analyzed data from multicenter case–control studies conducted in Italy. The OR for consumption of  $\geq 1$  apple/day in comparison with  $\leq 1$  apple/day was significantly reduced for colorectal cancer (0.8, 95% CI = 0.71–0.90) as well as for cancers of the oral cavity, larynx, breast, and ovary [9]. Also, in a large cohort study with 34 000 women (NHS, see above), women



**Figure 1.** Overview of selected typical apple juice components belonging to the structural classes of phenol carboxylic acids, dihydrochalcones, flavan-3-ols (catechins and procyanidins), and flavonoids (quercetin glycosides).

in the highest quintile of apple consumption had an OR of 0.83 (95% CI = 0.7–0.98) for colorectal adenomas compared with women in the lowest quintile of intake ( $P$  for trend = 0.05) [10].

Apples are a rich source of phenolic constituents, which are distributed in the pulp, skin, and seeds [11]. The total polyphenol content of apples represents about 0.01–1% of the fresh weight. Content and composition in phenolic compounds vary strongly in dependence of the apple variety, area of cultivation, and year of harvest [12–14]. Main structural classes include phenol carboxylic acid derivatives, catechins and di-, tri-, and oligomeric procyanidins, dihydrochalcones, and flavonoid glycosides (Figs. 1, 2) [15–22]. Theodoratou *et al.* [23] compared the intake of the six major classes of flavonoids, including flavan-3-ols, procyanidins and flavonols, as well as of selected single molecules, including quercetin and (–)-epicatechin, with the risk of colorectal cancer in a large prospective case–control study in Scotland including 1456 incident cases and 1456 population-based controls. Apple consumption was included as a source for flavan-3-ols, procyanidins, and (–)-epicatechin as a single substance, based on data by Kyle and Duthie [24]. Overall, flavonol uptake in the highest



**Figure 2.** HPLC chromatogram of apple juice extract AS02 before fractionation (detection at 280 nm). Major components were identified as chlorogenic acid (1a), 4-coumaroylquinic acid (1c), phloretin-2'-xyloglucoside (2b), and phloridzin (2e). For further peak identification and number code see Table 1.

quartile of intake compared with the lowest quartile was associated with a 27% reduction in colorectal cancer risk (uptake >37 mg/day; adjusted OR 0.73, 95% CI 0.59–0.9, *P* for trend 0.012), 32% for quercetin (uptake >23 mg/day; OR 0.68, 95% CI 0.55–0.84, *P* for trend 0.001), 32% for catechin (uptake >9 mg/day; OR 0.68, 95% CI 0.55–0.83, *P* for trend <0.0005), 26% for epicatechin (uptake >33 mg/day; OR 0.74, 95% CI 0.6–0.9, *P* for trend 0.019) and 22% for procyanidins (uptake >45 mg/day; QR 0.78, 95% CI 0.63–0.96, *P* for trend 0.031).

Apple juice extracts and constituents have been reported to possess a variety of biological activities in cell culture and animal models (reviewed in ref. [3]). These include antioxidant properties and reduction of oxidative stress [25–27], suppression of the induction of phase 1 enzyme Cyp1A1 activity [28], modulation of signal transduction pathways [29–31], reduction of polyamine biosynthesis [29] and of epidermal growth factor receptor kinase and protein kinase C activity [31–33], decrease of cholesterol esterification and lipoprotein secretion [34], prevention of hydrogen peroxide-induced inhibition of gap-junctional intercellular communication [35], as well as induction of apoptosis in various colon cancer cell lines [33, 36].

At present it is not completely understood to what extent the various constituents of apple juice contribute to the potential chemopreventive activities observed with complex apple juice extracts. Therefore, we have fractionated phenolic apple juice extracts and tested them in a series of *in vitro* bioassays indicative of cancer chemopreventive potential *in vivo*. Here we summarize the antioxidant activities of the extracts as well as of fractions and subfractions, their influence on the metabolic activation and detoxification of carcinogens by inhibition of phase 1 Cyp1A activity and induction of phase 2 NAD(P)H:quinone reductase (QR) activity, inhibition of cyclooxygenase 1 (Cox-1), and aromatase (AR; Cyp19) activity as well as antiproliferative properties in a human colon cancer cell line. If applicable, activities were attributed to previously identified compounds.

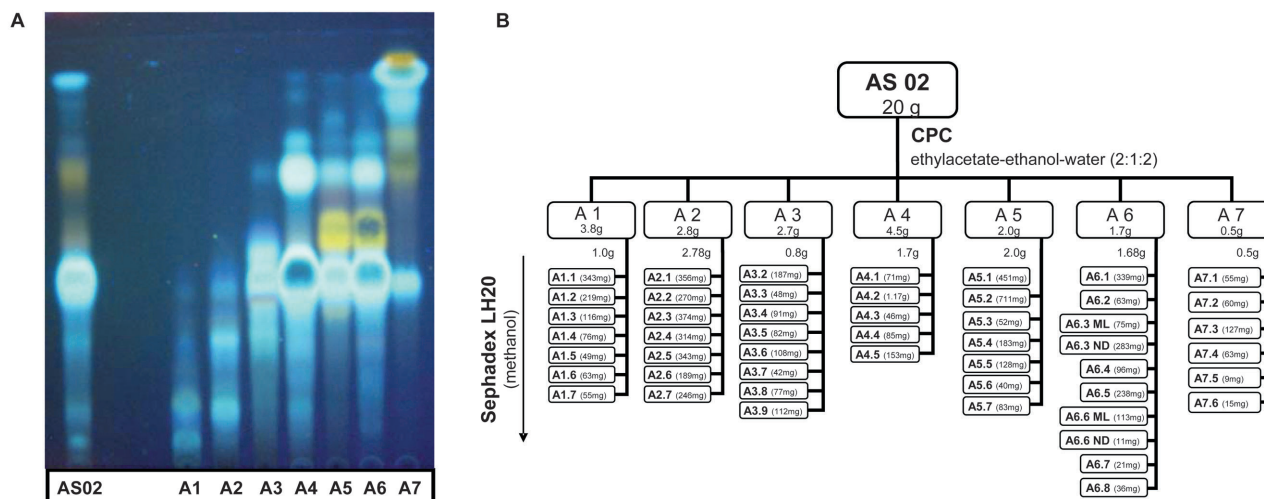
## 2 Materials and methods

### 2.1 Chemicals

All cell culture media and supplements were obtained from Invitrogen (Eggenstein, Germany). Fetal calf serum was provided by PAA Laboratories (Pasching, Austria). 3-Cyano-7-ethoxycoumarin (CEC), and 3-cyano-7-hydroxycoumarin (CHC) were purchased from Molecular Probes (Möbitec, Goettingen, Germany). The reference compounds (+)-catechin, (–)-epicatechin, 5-caffeoylquinic acid (chlorogenic acid), caffeic acid, phloretin-2'-glucoside (phloridzin), phloretin, and quercetin were purchased from Sigma-Aldrich (Deisenhofen, Germany). Quercetin-3-galactoside (hyperosid), quercetin-3-glucoside (isoquercitrin), and quercetin-3-rhamnoside (quercitrin) were obtained from Carl Roth (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich. All organic solvents were of analytical grade quality and purchased from E. Merck (Darmstadt, Germany). Water was double distilled. 4-Coumaroylquinic acid, *p*-coumaric acid, ferulic acid, procyanidin B<sub>1</sub> and B<sub>2</sub>, procyanidin C<sub>1</sub>, phloretin-2'-xyloglucoside, hydroxyphloretin-2'-xyloglucoside, quercetin-3-arabinofuranoside and -pyranoside, quercetin-3-rutinoside (rutin), and quercetin-3-xyloside (reynoutrin) were isolated by column chromatography (as described below) and preparative HPLC and identified based on their UV and mass spectra as well as <sup>1</sup>H- and <sup>13</sup>C-NMR spectra. A detailed description of the instrumentation, methodology, and structure assignment is not the aim of the present study and is given in Zessner [37]. All other compounds were tentatively identified based on their online UV- and mass spectra obtained by HPLC with DAD and LC-MS analyses (methodology as in ref. [38]).

### 2.2 Preparation of apple juice and apple juice extract AS02

Natural cloudy apple juice, which is commercially available, was produced at the Geisenheim Research Center



**Figure 3.** (A) Thin layer chromatogram of AS02 and seven fractions obtained by CPC under UV light (366 nm) after detection with Naturstoffreagenz A. (B) Fractionation scheme of AS02 including fraction yields.

from mixed varieties of apples (20% mixed table apples, 25% Topaz, 17.5% Bohnapfel, 22.5% Winterrambur, 15% Bittenfelder). The fruits were crushed in a hammer mill (Bellmer, Niefern, Germany) and extracted in an HP-L 200 horizontal press (Bucher, Niederweningen, Switzerland). The resulting juice was centrifuged with a separator (SA R 3036; Westfalia, Oelde, Germany) and finally pasteurized (85°C, 30 s). Polyphenols from 100 L juice were retained on 5 L adsorber resin XAD 16P (Rohm and Haas, PA, USA) packed into a Pharmacia BPG chromatography column (100 × 10 cm) equilibrated with ten bed volumes of distilled water. Water-soluble constituents such as sugars, organic acids, and minerals were removed with six bed volumes of distilled water. Afterwards, polyphenolic compounds were eluted with three bed volumes of 96% ethanol. The polyphenolic eluate was concentrated by evaporation using a rotavapor, reconstituted in double-distilled water, and subsequently freeze-dried to yield extract AS02. Polyphenol analysis of the original extract was performed after dissolution in 10% methanol. Analytical quantitative determination of apple juice constituents was performed on a 1090 HPLC/DAD system (Hewlett-Packard, Böblingen, Germany) equipped with an RP 250 × 4.6 mm Aqua 5 μm C18 column and protected with a 4 × 3 mm C18 ODS security guard (Phenomenex, Aschaffenburg, Germany). Gradient elution was applied with an ACN–acetic acid gradient according to a previously published protocol [39]. Detection wavelengths were 280, 320, and 360 nm. Quantification was carried out using peak areas from external calibration curves.

### 2.3 Fractionation of apple juice extract AS02

The initial fractionation of extract AS02 was achieved by centrifugal partition chromatography (CPC). The FCPC

instrument was from Kromaton (Angers, France) and equipped with a 1000 mL rotor, a Varian ProStar Solvent Delivery Module 210, a Rheodyne 3725 injection system (Rheodyne, Coati, USA), and a Varian UV–Vis Detector ProStar 320 (at 280 nm). After thorough optimization, a ternary solvent system of ethyl acetate/ethanol/water (2:1:2) was selected (descending mode, rotor speed 800 U/min, flow rate 7 mL/min). A total of 20 g AS02 was separated in portions of 2–3 g. The eluate was collected and combined according to the chromatographic pattern after TLC on silica gel 60 F<sub>254</sub> (Merck, Darmstadt, Germany) using ethyl acetate/formic acid/acetic acid/water (100:11:11:27) as mobile phase (detection with Naturstoffreagenz A/PEG), to yield seven fractions A1–A7 (summarized in Figs. 3A and B). The seven fractions A1–A7 were further separated by gel permeation chromatography (GPC) on Sephadex LH 20 (Pharmacia, Uppsala, Sweden) equilibrated with methanol and packed into a Kronlab medium pressure glass column ECO (200 × 2.5 cm) using methanol as mobile phase with a constant flow rate of 2–3 mL/min. The eluate was analyzed by TLC as described above and fractions with similar composition were combined, the solvent evaporated and the residue freeze-dried after suspension in water. After fractionation of 0.5–4.5 g of A1–A7, a total of 48 subfractions and two precipitates were obtained with yields of 9 mg (A7.5) to 1170 mg (A4.2), as depicted in Fig. 3B. Fractions were tested for potential cancer chemopreventive activities in eight *in vitro* test systems as described below.

### 2.4 High pressure liquid chromatographic separation of AS02, fractions and subfractions

Fractions and subfractions were separated by RP-HPLC to monitor their polyphenol composition. A quantitative analysis of the apple juice constituents was not performed.

**Table 1.** Overview of identified apple polyphenols

1	Phenol carboxylic acids	2	Dihydrochalcones
<b>1a</b>	5-Caffeoylquinic acid (chlorogenic acid)	<b>2a</b>	3-Hydroxyphloretin-2'-xyloglucoside
<b>1b</b>	Caffeic acid	<b>2b</b>	Phloretin-2'-xyloglucoside
<b>1c</b>	4-Coumaroylquinic acid	<b>2c</b>	3-Hydroxyphloretin (RT 18.1 min) <sup>a)</sup>
<b>1d</b>	<i>p</i> -Coumaric acid	<b>2d</b>	Phloretin glycoside 2 (RT 13.7 min) <sup>a)</sup>
<b>1e</b>	Ferulic acid	<b>2e</b>	Phloretin-2'-glucoside (phloridzin)
<b>1f</b>	Chlorogenic acid 2 (RT 10.9 min) <sup>a)</sup>	<b>2f</b>	Phloretin (aglycone)
<b>1g</b>	Chlorogenic acid 3 (RT 13.1 min) <sup>a)</sup>		
<b>1h</b>	Chlorogenic acid 4 (RT 13.6 min) <sup>a)</sup>		
<b>1i</b>	Chlorogenic acid 5 (RT 15.4 min) <sup>a)</sup>		
<b>1j</b>	<i>cis</i> -Chlorogenic acid <sup>a)</sup>		
3	Flavan-3-ols (catechins and procyanidins)	4	Quercetin glycosides
<b>3a</b>	Procyanidin B <sub>1</sub>	<b>4a</b>	Quercetin-3-rutinoside (rutin)
<b>3b</b>	(+)-Catechin	<b>4b</b>	Quercetin-3-galactoside (hyperoside)
<b>3c</b>	(-)-Epicatechin	<b>4c</b>	Quercetin-3-glucoside (isoquercitrin)
<b>3d</b>	Procyanidin B <sub>2</sub>	<b>4d</b>	Quercetin-3-xyloside (reynoutrin)
<b>3e</b>	Procyanidin C <sub>1</sub>	<b>4e</b>	Quercetin-3-arabinofuranoside
<b>3f</b>	Procyanidin-tetramer <sup>a)</sup>	<b>4f</b>	Quercetin-3-arabinopyranoside
<b>3g</b>	Procyanidin-pentamer <sup>a)</sup>	<b>4g</b>	Quercetin-3-rhamnoside (quercitrin)
<b>3h</b>	Procyanidin oligo- and polymers <sup>a)</sup>	<b>4h</b>	Quercetin (aglycone)

a) Compounds were tentatively assigned to structural classes based on their online UV- and mass spectra obtained by HPLC with DAD and LC-MS analyses as described in ref. [38].

Extract, fractions and subfractions were dissolved in methanol, and 10  $\mu$ L each were separated on a Waters HPLC instrument (Waters, Milford, USA) equipped with a 250  $\times$  4.6 mm Aqua 5  $\mu$ m C18 column (Phenomenex). The solvent system was composed of solvent A (double distilled water containing 0.1% v/v TFA) and solvent B (ACN containing 0.1% v/v TFA). The following gradient system was utilized: 0–25 min: 10–30% B; 25–30 min: 30–75% B; 30–35 min: 75–10% B at a flow rate of 1 mL/min. Detection was at 280 nm. Known compounds were identified by comparison of retention times (RTs) and UV-spectra with reference substances. All identified apple juice components are summarized in Table 1 and number coded according to their structural classes.

### 2.5 Normal-phase (NP) HPLC separation of AS02

AS02 procyanidins were separated by normal-phase HPLC (NP-HPLC) according to Lazarus *et al.* [40] on a 1090 HPLC system (Hewlett-Packard) equipped with a 250  $\times$  4.6 mm Luna-Silica column and protected with a 4  $\times$  3 mm guard column filled with the same material (Phenomenex). The solvent system was composed of solvent A (dichloromethane), solvent B (methanol), and solvent C (50% acetic acid). Procyanidins were eluted with a gradient starting with 14% B; 0–30 min: 14–28.4% B; 30–60 min: 28.4–50% B; 60–65 min: 50–86% B; 65–70 min 100% B, with constant 4% C at a flow rate of 1 mL/min. Elution was monitored by fluorescence detection with excitation at

276 nm and emission detection at 316 nm. Peak assignment was done in comparison with the literature [38, 40].

### 2.6 Total procyanidin content (TPC)

The TPC of AS02 and normal-phase fractions (described below) was determined using the acid butanol assay as described previously [41]. Briefly, 75  $\mu$ L methanolic apple extract solution (approximately 2 mg/mL) was added to 1.4 mL of a solution of 1-butanol/hydrochloric acid (95:5 v/v). The reaction mixture was heated for 2 h at 95°C and then cooled to room temperature. The absorbance was measured at 555 nm on Spectronic Instruments (Rochester, NY, USA) Spectronic Genesys<sup>TM</sup> 2PC UV/Vis spectrophotometer. The TPC was quantified using a purified standard of procyanidins (a mixture of different tetramers of B-type procyanidins), obtained by preparative HPLC as described previously [38].

### 2.7 Mean degree of polymerization (DPM)

To evaluate the DPM of procyanidins the thiolysis method was modified after Guyot *et al.* [42]. Briefly, in a 1.5 mL vial (Trott, Kriftel, Germany), 200  $\mu$ L of a methanolic apple juice extract solution (2 mg/mL) was mixed with 400  $\mu$ L of benzyl mercaptan solution (5% in methanol v/v) acidified with concentrated HCl (1.1% v/v) and sealed with an inert Teflon cap (Trott). The reaction was carried out at 60°C for 30 min to ensure complete degradation of the poly-

mers. The reaction mixture was cooled and stored at  $-20^{\circ}\text{C}$  to minimize epimerization and any side reactions prior to HPLC analysis. The RTs of the thiolysis products (+)-catechin benzylthioether and (–)-epicatechin benzylthioether were determined after thiolytic degradation of a mixture of procyanidins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, and B<sub>4</sub>.

## 2.8 Normal-phase fractionation of apple juice extract AS04

To correlate bioactivities with procyanidin content, 386 mg of apple juice extract AS04 similar to AS02 was separated by normal-phase preparative HPLC over a Knauer Eurospher 100 Si ( $250 \times 16 \text{ mm}$ ,  $5 \mu$ ) column using an elution system similar to the one described above, composed of solvent A (dichloromethane), solvent B (methanol), and solvent C (50% acetic acid) to yield five fractions NP.1–NP.5. Procyanidins were eluted with a gradient starting with 14% B; 0–30 min: 14–28% B; 30–40 min: 28–39% B; 40–54 min: 39–60% B; with constant 4% C. Known low molecular weight compounds (LMW) were quantified by RP-HPLC, and the procyanidin content was determined using the acid butanol assay. Results are summarized in Table 3.

## 2.9 *In vitro* test systems of potential cancer chemopreventive activity

Experimental details of most test systems utilized in this study are summarized in Gerhauser *et al.* [43, 44]. In brief, radical scavenging potential was determined photometrically by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format. Superoxide anion radicals were generated by oxidation of hypoxanthine to uric acid by xanthine oxidase and quantitated by the concomitant reduction of 3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) adjusted to a 96-well microplate format (xanthine/xanthine oxidase assay; X/XO). Oxygen radical absorbance capacity (ORAC) using fluorescein and 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) as a peroxy radical generator was quantified as described in ref. [45]. Inhibition of Cyp1A (EC 1.14.14.1) enzymatic activity and induction of QR (EC 1.6.99.2) in cultured Hepa1c1c7 cells were assayed as described by Crespi *et al.* [46] and Gerhauser *et al.* [47], monitoring the dealkylation of 3-cyano-7-ethoxycoumarin to 3-cyano-7-hydroxycoumarin and the NADPH-dependent menadiol-mediated reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan, respectively. Inhibition of Cox-1 (prostaglandin G/H synthase, EC 1.14.99.1) activity was determined with the system described by Jang *et al.* [48], measuring oxygen consumption during the conversion of arachidonic acid to prostaglandins. Inhibition of AR activity was estimated using human recombinant Cyp19 (EC 1.14.14.1) and *O*-benzylfluorescein benzyl ester as a sub-

strate [49]. Cultivation of the human colon cancer cell line HCT116 and determination of cytotoxicity by sulforhodamine B staining was performed as described earlier [50]. Extract, fractions and subfractions were dissolved in DMSO. Activities were compared with those of 23 pure apple juice constituents, which are commercially available or were isolated during the fractionation. If applicable, activities of fractions were attributed to compounds identified therein. A complete list of results obtained with purified compounds will be published elsewhere (Pan *et al.*, in preparation).

## 3 Results

### 3.1 Initial characterization of extract AS02

The AS02 extract represents the typical phenolic composition of common apple juice. As depicted in Fig. 2, nine components were detected at 280 nm as single peaks and quantified by comparison with reference standard compounds. Chlorogenic acid was identified as the major component (1a, 185.5 mg/g), followed by 4-coumaroylquinic acid (1c, 77.3 mg/g), phloretin-2'-xyloglucoside (2b, 69.5 mg/g), and phloridzin (2e, 27.9 mg/g). Minor peaks were assigned to (–)-epicatechin (3c, 19.2 mg/g), the epicatechin dimer procyanidin B<sub>2</sub> (3d, 15.2 mg/g), caffeic acid (1b, 4.8 mg/g), and two quercetin glycosides, quercetin-3-galactoside (4b, 0.8 mg/g), and quercetin-3-glucoside (4c, 1.4 mg/g). Overall, 411.2 mg per 1 g AS02 could be attributed to known LMW apple components.

Extract AS02 was tested in a series of *in vitro* test systems with mechanisms relevant for cancer chemopreventive activity *in vivo*. Three test systems analyzed the potential to scavenge reactive radicals. As summarized in Table 2 (first line), AS02 was a good scavenger of DPPH radicals, superoxide anion radicals (X/XO system), and peroxy radicals (ORAC), with halfmaximal scavenging concentrations (SC<sub>50</sub>) of 10.7, 23.4  $\mu\text{g/mL}$ , and 2.2 ORAC units at a test concentration of 0.4  $\mu\text{g/mL}$ , respectively. AS02 was also found to inhibit cytochrome P450 1A (Cyp1A), a phase 1 enzyme involved in the metabolic activation of carcinogens, with a halfmaximal inhibitory concentration (IC<sub>50</sub>) of 12.3  $\mu\text{g/mL}$ . Concomitantly, it was able to induce the activity of QR, which was selected as a marker enzyme for induction of phase 2 detoxifying enzymes, by 25% at a concentration of 20  $\mu\text{g/mL}$ , and doubled the specific activity of QR (CD value) at a concentration of 112  $\mu\text{g/mL}$  (not shown). Anti-inflammatory potential was assessed by inhibiting the activity of sheep seminal vesicle Cox-1. AS02 was 69% inhibitory at a test concentration of 400  $\mu\text{g/mL}$ . In addition, AS02 was able to inhibit the activity of AR, a key enzyme for the generation of estrogens from testosterone. We determined an IC<sub>50</sub> value of 11.5  $\mu\text{g/mL}$ . AS02 also possessed potential to inhibit the proliferation of HCT116 human colon cancer cells (Tox). The IC<sub>50</sub> value was determined as 44.3  $\mu\text{g/mL}$ .

**Table 2.** Summary of potential cancer chemopreventive activities of AS02, fractions A1, A2, and subfractions thereof

	DPPH <sup>a)</sup> (IC <sub>50</sub> in µg/mL)	X/XO (IC <sub>50</sub> in µg/mL)	ORAC (units)	Cyp1A (IC <sub>50</sub> in µg/mL)	QR (fold induction at 20 µg/mL)	Cox-1 (% inhibition at 400 µg/mL)	AR (IC <sub>50</sub> in µg/mL)	Tox (IC <sub>50</sub> in µg/mL)
<b>AS02</b>	10.7	23.4	2.2	12.3	1.25	<b>69</b>	11.5	44.3
<b>A1</b>	10.3	24.3	1.0	38.8	1.1	36	13	>200
<b>A1.1</b>	>10 (8)	>20 (24)	0.5	>20	1.3	4	>20 (10)	>200
<b>A1.2</b>	>10 (18)	>20 (32)	0.9	>20	1.2	3	>20 (9)	>200
<b>A1.3</b>	>10 (35)	>20 (43)	1.0	>20	1.1	14	>20 (12)	152.9
<b>A1.4</b>	<b>5.6</b>	>20 (42)	<b>2.0</b>	>20	1.2	35	>20 (31)	68.8
<b>A1.5</b>	<b>7.3</b>	17	<b>2.3</b>	>20	1.3	<b>51</b>	11.7	68.0
<b>A1.6</b>	<b>3.5</b>	11	<b>2.7</b>	14	1.1	<b>51</b>	<b>2.9</b>	65.3
<b>A1.7</b>	<b>2.9</b>	<b>8</b>	<b>2.9</b>	<b>4.2</b>	1.4	<b>73</b>	<b>2.9</b>	52.8
<b>A2</b>	9.1	12.6	1.5	25	1.2	<b>46</b>	<b>8.2</b>	149.0
<b>A2.1</b>	>50 (19)	>50 (30)	0.4	>50 (11)	<b>2.1</b>	16	>50 (49)	>200
<b>A2.2</b>	>50 (19)	>50 (31)	0.4	>50 (11)	<b>1.5</b>	14	>50 (20)	>200
<b>A2.3</b>	42.4	20.4	1.1	>50 (18)	<b>1.7</b>	17	35	197.4
<b>A2.4</b>	19.6	11.3	1.5	>50 (33)	<b>1.5</b>	26	23.4	104.1
<b>A2.5</b>	17.4	<b>9.4</b>	1.5	>50 (41)	1.4	42	11.4	54.3
<b>A2.6</b>	9.3	<b>6.4</b>	1.7	30.5	1.3	62	<b>4.3</b>	35.3
<b>A2.7</b>	10.9	<b>8</b>	1.6	14.4	1.7	64	<b>2.6</b>	<b>24.3</b>

DPPH, DPPH scavenging (SC<sub>50</sub> in mg/mL); X/XO, O<sub>2</sub><sup>-•</sup>-scavenging (SC<sub>50</sub> in µg/mL); ORAC, ROO<sup>-•</sup>-scavenging (ORAC units at a final concentration of 0.4 µg/mL in comparison with 1 µM Trolox); Cyp1A, Cyp1A inhibition (IC<sub>50</sub> in µg/mL); QR, QR induction (fold induction at 20 µg/mL); Cox-1, Cox-1 inhibition (% inhibition at 400 µg/mL); AR, aromatase (Cyp19) inhibition (IC<sub>50</sub> in µg/mL); Tox, inhibition of HCT116 cell proliferation (IC<sub>50</sub> in µg/mL). Values in parenthesis indicate the percentage of inhibition at the respective concentration. Most potent activities are highlighted in bold print.

a) Test systems.

### 3.2 Fractionation by CPC

The apple juice extract AS02 was fractionated in two dimensions. First, AS02 was separated into seven subfractions by CPC as described in Section 2. The fractionation pattern visualized by TLC (Fig. 3A) clearly demonstrated that the chosen CPC system separated the mixture of compounds based on polarity. Polar compounds (indicated by low *R<sub>f</sub>* values) eluted in earlier fractions than apolar constituents. A fractionation scheme with fraction yields is given in Fig. 3B.

Bioassay testing revealed that selected CPC fractions contributed by varying degrees to the observed activities of AS02. Detailed results and major components of the seven CPC fractions will be described and discussed with their respective subfractions below. In summary, DPPH scavenging potential was concentrated in fractions A3–A5, whereas the most potent superoxide anion radical scavengers were eluted in fractions A3 and A4. In contrast, highest ORAC values indicative for peroxyl radical scavenging were determined with fractions A4–A6. Cyp1A inhibitory potential was clearly concentrated in the least polar fraction A7, whereas none of the fractions potently induced QR activity. Still, fraction A7 was most active with 1.6-fold induction at a concentration of 20 µg/mL. Fraction A3 was most potent in inhibiting AR activity, while anti-inflammatory potential was clearly clustered in fractions A5 and A6. Based on these results, GPC on Sephadex

LH20 was selected as the second dimension of fractionation to separate structural classes of components with similar polarity.

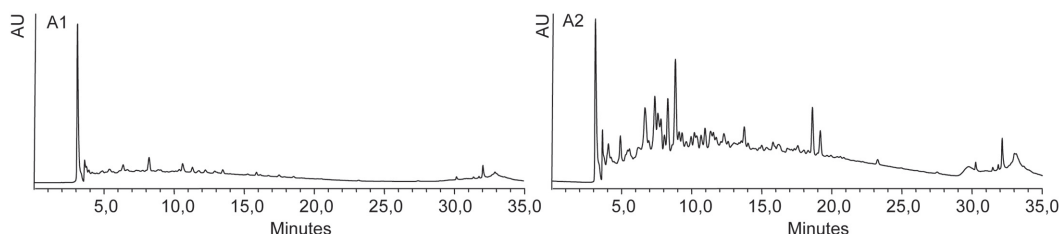
### 3.3 Subfractionation by GPC

#### 3.3.1 Fractionation of A1 and A2

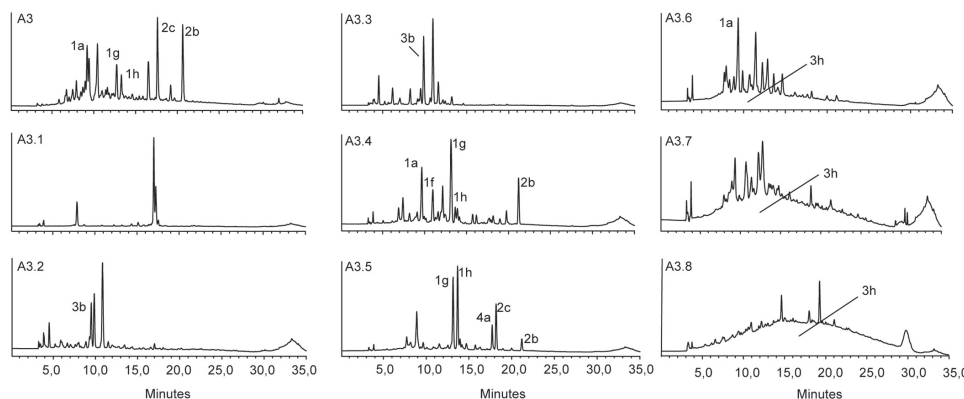
As shown in Fig. 3A, fractions A1 and A2 mainly contained very polar substances which showed a diffuse blue fluorescence after TLC separation. No known apple juice component could be attributed to any of the peaks detected after HPLC separation (Fig. 4). Carbohydrate analyses indicated a sugar content of 32.3 and 23%, respectively, after acid hydrolysis (data not shown). This indicated that a substantial amount of carbohydrates was bound to polyphenols and only detectable after treatment under hydrolyzing conditions.

Bioassay analyses revealed that after GPC, especially later eluting subfractions of A1, *i.e.*, A1.5–A1.7, were associated with potent activities in all test systems except QR induction (Table 2). Substances responsible for these activities have not been identified so far. Subfractionation of A2 revealed a similar pattern. NMR analyses indicated aromatic signals in late eluting subfractions. These signals were attributed to oligo- and polymeric procyanidins, which were not separated by the utilized RP-HPLC system. Interestingly, hitherto unidentified compounds with QR-inducing potential were particularly clustered in early eluting





**Figure 4.** RP-HPLC separation of fractions A1 (left) and A2 (right) (detection at 280 nm). Details see Section 2.



	DPPH <sup>a</sup> (IC <sub>50</sub> in µg/ml)	X/XO (IC <sub>50</sub> in µg/ml)	ORAC (units)	Cyp1A (IC <sub>50</sub> in µg/ml)	QR (fold ind. at 20 µg/ml)	Cox-1 (% inhib. at 400 µg/ml)	AR (IC <sub>50</sub> in µg/ml)	Tox (IC <sub>50</sub> in µg/ml)
<b>A3</b>	<b>6.5</b>	<b>9.3</b>	<b>2.0</b>	27.8	1.1	<b>58</b>	<b>5.9</b>	147.3
<b>A3.1</b>	>10 (14)	>20 (22)	0.4	>20	1.9	18	13.8	>200
<b>A3.2</b>	>10 (19)	>20 (18)	1.3	>20	1.2	12	>20 (16)	>200
<b>A3.3</b>	>10 (18)	>20 (24)	<b>2.1</b>	>20	1.3	18	>20 (16)	>200
<b>A3.4</b>	>10 (38)	>20 (43)	<b>2.7</b>	>20	1.3	23	16.7	135.1
<b>A3.5</b>	<b>6.8</b>	>20 (48)	<b>2.4</b>	>20	1.3	33	>20 (13)	96.0
<b>A3.6</b>	<b>4.8</b>	12.8	<b>2.7</b>	13.8	1.1	<b>51</b>	<b>5.9</b>	65.8
<b>A3.7</b>	<b>4.7</b>	19	<b>2.3</b>	>20	1.2	<b>73</b>	<b>4.2</b>	63.0
<b>A3.8</b>	<b>4.2</b>	11.8	1.9	<b>8.8</b>	1.1	<b>59</b>	<b>2.1</b>	<b>40.6</b>

<sup>a</sup>Test systems as described in footnote Table 2

**Figure 5.** (upper) RP-HPLC separation of A3 and subfractions (detection at 280 nm). (lower) Summary of chemopreventive activities.

subfractions A2.1–A2.3, which were mostly devoid of other activities. Current analyses indicate that these effects might be due to terpenoid structures.

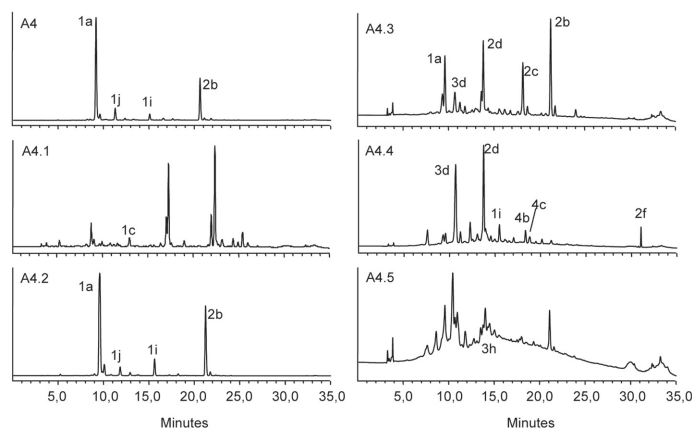
### 3.3.2 Fractionation of A3

Fraction A3 was composed of a mix of different classes of compounds. Several phenol carboxylic acids (1a, 1g, 1h) and dihydrochalcones (2b and 2c) were detectable by HPLC (Fig. 5). Early eluting fractions were mainly composed of unknown structures. Only (+)-catechin (3b) was identified in subfractions A3.2 and A3.3. However, these fractions were not active in our set of bioassays at the chosen concentration limits. Elution of chlorogenic acid (1a) and isomers thereof (1f, 1g, 1h) as well as of phloretin-

2'-xyloglucoside (2b) and 3-hydroxyphloretin (2c) was clustered in intermediate eluting fractions A3.4 and A3.5. These fractions demonstrated antioxidant activities with DPPH- and good peroxy scavenging potential, with increasing ORAC values up to 2.7, which were attributed to chlorogenic acid (1a) and its isomers (1f, 1g, 1h). Fractions A3.6–A3.8 were characterized by a typical broad procyanidin peak (3h) and showed high activities in almost all test systems including antioxidant potential and inhibition of the enzyme activities of Cyp1A, Cox-1, and AR.

### 3.3.3 Fractionation of A4

Fraction A4 mainly contained chlorogenic acid (1a) and phloretin-2'-xyloglucoside (2b) (Fig. 6). A very similar



	DPPH <sup>a</sup> (IC <sub>50</sub> in µg/ml)	X/XO (IC <sub>50</sub> in µg/ml)	ORAC (units)	Cyp1A (IC <sub>50</sub> in µg/ml)	QR (fold ind. at 20 µg/ml)	Cox-1 (% inhib. at 400 µg/ml)	AR (IC <sub>50</sub> in µg/ml)	Tox (IC <sub>50</sub> in µg/ml)
<b>A4</b>	<b>6.5</b>	<b>9.6</b>	<b>2.7</b>	>40	1.1	<b>52</b>	15.8	181.8
<b>A4.1</b>	>10 (15)	>20 (6)	1.0	>20	1.1	1	>20 (25)	>200
<b>A4.2</b>	<b>10</b>	>20 (48)	<b>2.3</b>	>20	1.1	14	>20 (23)	133.3
<b>A4.3</b>	<b>4.1</b>	14.8	<b>3.1</b>	<b>10.4</b>	1.3	<b>51</b>	14.5	87.8
<b>A4.4</b>	<b>3.9</b>	12.6	<b>3.5</b>	<b>9.5</b>	1.3	<b>95</b>	<b>6.7</b>	66.7
<b>A4.5</b>	<b>2.5</b>	<b>8.8</b>	<b>2.7</b>	<b>4.4</b>	1.3	<b>57</b>	<b>2.7</b>	56.0

<sup>a</sup>Test systems as described in footnote Table 2

**Figure 6.** (upper) RP-HPLC separation of A4 and subfractions (detection at 280 nm); (lower) Summary of chemopreventive activities.

HPLC chromatogram was obtained with subfraction A4.2, but in contrast to A4, this fraction was only active in the DPPH and ORAC assay, indicating that components other than the two major compounds 1a and 2b may play a more important role for biological activity of A4 in the additional test systems. Bioactivities increased with later eluting fractions. Subfraction A4.4 demonstrated pronounced Cox-1 inhibitory potential. This may be attributed to the elution of phloretin (2f), the aglycon of phloridzin, which inhibits Cox-1 activity with an IC<sub>50</sub> value of 18.1 µM (4.9 µg/mL). Fraction A4.5 showed good biological activities due to the presence of procyanidins (3h).

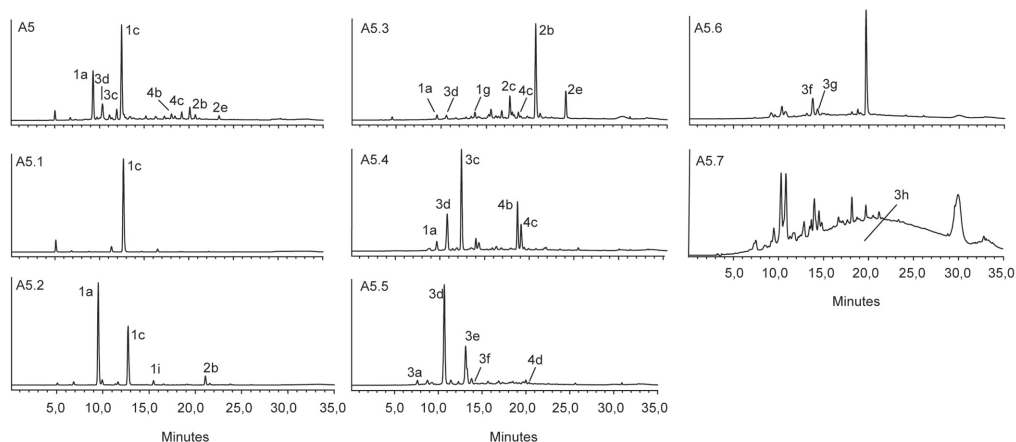
### 3.3.4 Fractionation of A5

The HPLC chromatogram of fraction A5 indicated the presence of all four major structural classes of apple juice components, *i. e.*, phenol carboxylic acids in the form of chlorogenic acid (1a) and 4-coumaroylquinic acid (1c), the dihydrochalcones phloretin-2'-xyloglucoside (2b) and phloridzin (2e), (-)-epicatechin (3c) as a flavan-3-ol and its dimer procyanidin B<sub>2</sub> (3d), as well as the flavonoids quercetin-3-galactoside (4b) and quercetin-3-glucoside (4c) (Fig. 7). Fraction A5 represented one of the fractions with pronounced anti-inflammatory activity; therefore we were particularly interested whether this activity could be assigned to one specific class of compounds. Similar to the subfractionation of other AS02 fractions, early eluting subfractions

did not reveal interesting profiles of bioactivities. In fractions A5.1–A5.3, mainly phenol carboxylic acids and dihydrochalcones were detected. A5.4 showed good antioxidant activity. Also, anti-inflammatory potential was clearly detected in this fraction and resulted in 95% inhibition of Cox-1 activity at a concentration of 400 µg/mL. The major peak of A5.4 could be assigned to (-)-epicatechin (3c), which was identified as a potent Cox-1 inhibitor from apple juice, with an IC<sub>50</sub> value of 7.5 µM (2.2 µg/mL), in agreement with published literature [51]. Other compounds identified were procyanidin B<sub>2</sub> (3d), quercetin-3-galactoside (4b), and quercetin-3-glucoside (4c), which may contribute to the anti-inflammatory activity. Fraction A5.5 was characterized by the presence of procyanidins B<sub>1</sub> (3a) and B<sub>2</sub> (3d), procyanidin tri- and tetramer (3e and 3f), and the flavonoid quercetin-3-xyloside (4d). Antioxidant activity as well as the potential to inhibit AR activity increased with increasing fraction numbers. In fraction A5.6, two procyanidins, the tetramer (3f) and the pentamer (3g) were detected together with a hitherto unidentified flavonoid structure. Fraction A5.7 was among the most potent subfractions, but beside the broad procyanidin peak, no other peaks could be clearly assigned to known compounds.

### 3.3.5 Fractionation of A6

Fraction A6 was dominated by the presence of the dihydrochalcone glucoside phloridzin (2e) (Fig. 8). This frac-



	DPPH <sup>a</sup> (IC <sub>50</sub> in µg/ml)	X/XO (IC <sub>50</sub> in µg/ml)	ORAC (units)	Cyp1A (IC <sub>50</sub> in µg/ml)	QR (fold ind. at 20 µg/ml)	Cox-1 (% inhib. at 400 µg/ml)	AR (IC <sub>50</sub> in µg/ml)	Tox (IC <sub>50</sub> in µg/ml)
<b>A5</b>	<b>6.0</b>	<b>12.4</b>	<b>3.1</b>	26.5	1.1	<b>85</b>	16.9	143.3
<b>A5.1</b>	>50 (27)	>50 (15)	1.5	>50 (26)	1.4	9	>50 (15)	>200
<b>A5.2</b>	17.2	12.2	<b>2.1</b>	>50 (24)	1.3	8	>50 (17)	132.6
<b>A5.3</b>	12.6	<b>7.9</b>	1.9	22.3	1.7	27	n.m.	61.8
<b>A5.4</b>	<b>8.9</b>	<b>6.7</b>	<b>2.5</b>	22.3	1.5	<b>96</b>	21.6	117.4
<b>A5.5</b>	<b>7.7</b>	<b>5.4</b>	<b>2.5</b>	23.5	1.4	<b>65</b>	11.2	54.8
<b>A5.6</b>	<b>7.3</b>	<b>7</b>	1.7	18.1	1.6	46	<b>4</b>	<b>25.5</b>
<b>A5.7</b>	<b>6.9</b>	<b>5.4</b>	1.5	<b>8.7</b>	1.3	41	<b>2.2</b>	<b>12.5</b>

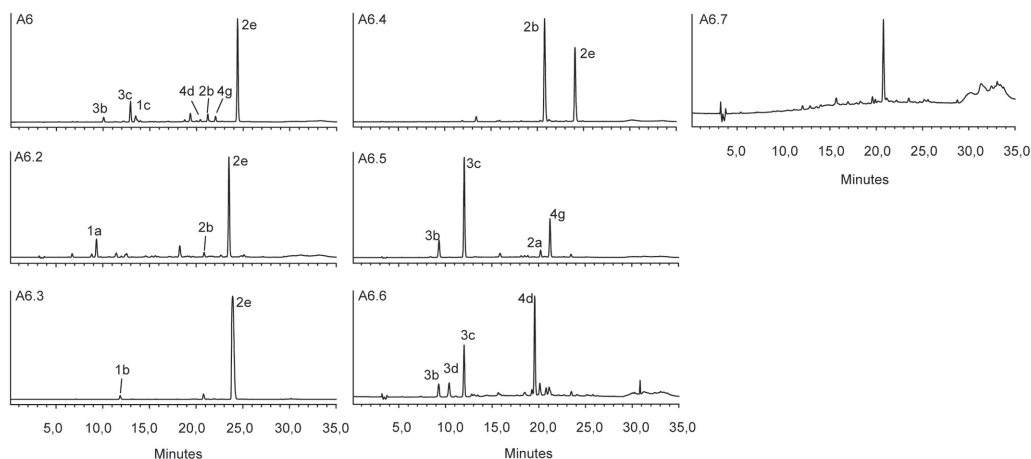
<sup>a</sup>Test systems as described in footnote Table 2; n.m. not measurable because of high background fluorescence

**Figure 7.** (upper) RP-HPLC separation of A5 and subfractions (detection at 280 nm). (lower) Summary of chemopreventive activities.

tion as well as all subfractions was extremely potent as peroxy radical scavenging in the ORAC assay with 2.5–3.6 ORAC units, whereas DPPH scavenging increased with later eluting fractions. Fractions A6.2 and A6.3 contained mostly phloridzin (2e). In fraction A6.4, phloridzin (2e) was coeluted with phloretin-2'-xyloglucoside (2b), two of the most characteristic apple juice components. Interestingly, the composition of fraction A6.5 was very different from that of fraction A6.4 and contained (+)-catechin (3b) and (–)-epicatechin (3c) as well as hydroxyphloretin-2'-xyloglucoside (2a) and quercetin-3-rhamnoside (quercitrin, 4g). Similar to fraction A5.4, fraction A6.5 was extremely potent in inhibiting Cox-1 activity, beside good antioxidant activity in all three test systems. The Cox-1 inhibitory potential was assigned to (–)-epicatechin (3c), whereas the other compounds may contribute to the additional activities. Good antioxidant and anti-inflammatory effects of fraction A6.6 were again attributed to the presence of the catechin derivatives 3b–3d. The quercetin-3-xyloside (reynoutrin, 4d) might account for the AR inhibitory activity of A6.6. Fraction A6.7 mainly contained procyanidins together with one unidentified medium-polar structure with an RT of 21 min.

### 3.3.6 Fractionation of A7

Fraction A7 contained a mix of phenol carboxylic acids and nonpolar dihydrochalcones, which were eluted very late, or not at all, from the CPC rotor and migrated to the solvent front after TCL separation (Fig. 3A). Caffeic acid (1b) was identified as the major component, accompanied by *p*-coumaric acid (1d) and traces of chlorogenic acid (1a), 4-coumaroylquinic acid (1c), and ferulic acid (1e) (Fig. 9). In addition, phloridzin (2e) and considerable amounts of the aglycone phloretin (2f) were detected. Subfractions A7.2 and A7.3 possessed very good peroxy radical scavenging activity with 3.7 and 3.6 ORAC units, respectively. These were attributed to the presence of phenol carboxylic acids in A7.2, and their combination with phloridzin (2e) in A7.3. Potential to scavenge DPPH and superoxide anion radicals was observed with all fractions and increased with later elution. Of note, we measured very strong Cyp1A inhibitory activity in fractions A7.5 and A7.6. Although none of the peaks of A7.5 was assigned to known structures, quercetin aglycone (4h) was identified in fraction A7.6 (chromatogram not shown). Quercetin was by far the most potent inhibitor of Cyp1A activity with an IC<sub>50</sub> value of 0.026 µM (0.008 µg/mL) [44]. The aglycones phloretin (2f) and quer-



	DPPH <sup>a</sup> (IC <sub>50</sub> in µg/ml)	X/XO (IC <sub>50</sub> in µg/ml)	ORAC (units)	Cyp1A (IC <sub>50</sub> in µg/ml)	QR (fold ind. at 20 µg/ml)	Cox-1 (% inhib. at 400 µg/ml)	AR (IC <sub>50</sub> in µg/ml)	Tox (IC <sub>50</sub> in µg/ml)
<b>A6</b>	<b>9.1</b>	16.6	<b>2.9</b>	17.4	1.1	<b>92</b>	12.5	>200
<b>A6.2</b>	12.5	12.7	<b>2.9</b>	18.3	1.7	33	21.2	92.6
<b>A6.3</b>	16.8	11.5	<b>3.1</b>	42.9	1.1	30	10.3	>200
<b>A6.4</b>	<b>7.8</b>	13	<b>3.3</b>	27.7	1.1	26	n.m.	137.6
<b>A6.5</b>	<b>4.6</b>	<b>4.7</b>	<b>3.6</b>	14.4	1.2	<b>96</b>	>20 (46)	107.7
<b>A6.6</b>	<b>4.8</b>	<b>6</b>	<b>3</b>	10.8	1.3	<b>93</b>	<b>5.5</b>	71.7
<b>A6.7</b>	<b>4.3</b>	14.6	<b>2.5</b>	<b>9.2</b>	1.5	<b>75</b>	<b>2.2</b>	<b>28.4</b>

<sup>a</sup>Test systems as described in footnote Table 2; n.m. not measurable because of high background fluorescence

**Figure 8.** (upper) RP-HPLC separation of A6 and subfractions (detection at 280 nm). (lower) Summary of chemopreventive activities.

etin (4h) also contributed to the pronounced induction of QR activity, inhibition of Cox-1 activity, and increasing toxicity of the later eluting subfractions A7.4–A7.6.

### 3.4 The role of procyanidins

#### 3.4.1 Analysis and fractionation by NP-HPLC

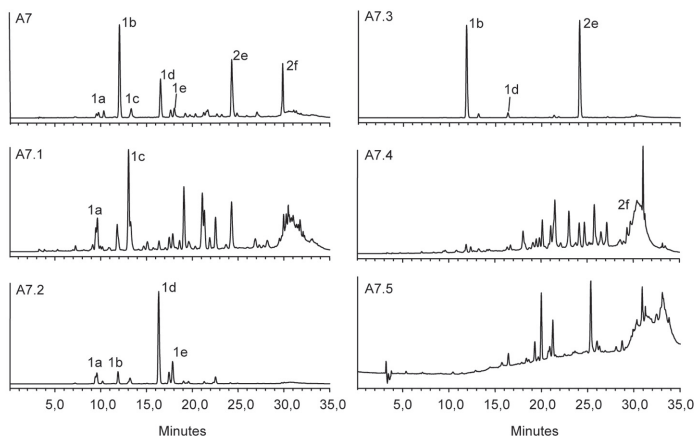
The bioassay results obtained with fractions and subfractions of AS02 indicated that procyanidins may contribute to the observed biological effects, although they were not properly detectable. Therefore, AS02 was analyzed by NP-HPLC, which allows separation of procyanidins according to their degree of polymerization. As shown in Fig. 10, procyanidins up to hexamers were separated and detectable by fluorescence detection (elution of polymeric procyanidin peak not shown). We determined a TPC of AS02 of  $42.4 \pm 1.3\%$  using the acid butanol assay. The DPM measured by thiolysis was  $5.8 \pm 0.1$ .

To better correlate potential chemopreventive activities with procyanidin content, extract AS04 similar to AS02 was fractionated by preparative NP-HPLC into five fractions NP.1–NP.5 with yields of 8 mg (2.1%, NP.1), 101.1 mg (26.3%, NP.2), 17.9 mg (4.6%, NP.3), 102.5 mg

(26.6%, NP.4), and 21.9 mg (5.7%, NP.5). About 30% could not be recovered. The composition of LMW compounds, TPC, and DPM of fractions NP.1–NP.5 are summarized in Table 3 (upper). With respect to LMW compounds, only 2% of NP.1 could be identified, and the fraction was free of procyanidins. NP.2 contained large quantities of chlorogenic acid (1a), phloridzin (2e), and 4-coumaroylquinic acid (1c), which accounted for about 90% of the fraction. The remaining 10% was composed of procyanidins (7%), quercetin-glycosides (1.6%), and traces of catechin monomers (0.6%). NP.3 was enriched in phloretin-2'-xyloglucoside (2b, 48.4%), in addition to about 27% procyanidins. Fraction NP.4 contained 74% procyanidins, whereas NP.5 was composed to 100% of procyanidins, with a DPM of 7.9.

#### 3.4.2 Bioactivity testing of extract AS04 and fractions NP.1–NP.5

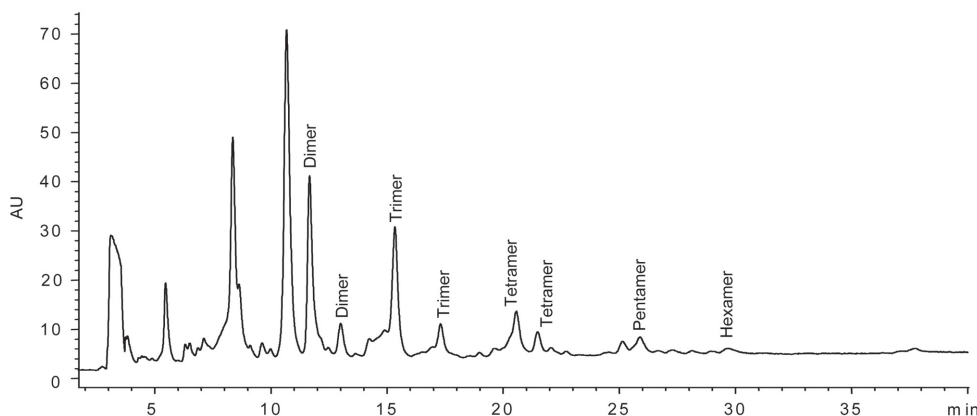
Extract AS04 and fractions NP.1–NP.5 were tested in the set of bioassays as described before. The results are summarized in Table 3 (lower). The activities of AS04 were similar to those of AS02, except lower induction of QR activity (AS04 CD >250 µg/mL vs. AS02 CD = 112 µg/mL), and stronger inhibition of AR activity (IC<sub>50</sub> AS04



	DPPH <sup>a</sup> (IC <sub>50</sub> in µg/ml)	X/XO (IC <sub>50</sub> in µg/ml)	ORAC (units)	Cyp1A (IC <sub>50</sub> in µg/ml)	QR (fold ind. at 20 µg/ml)	Cox-1 (% inhib. at 400 µg/ml)	AR (IC <sub>50</sub> in µg/ml)	Tox (IC <sub>50</sub> in µg/ml)
<b>A7</b>	<b>8.9</b>	15.9	<b>2.3</b>	<b>5.7</b>	1.6	35	10.7	198.9
<b>A7.1</b>	24.1	27.8	1.9	<b>8.1</b>	1.5	<b>53</b>	>20 (44)	n.d.
<b>A7.2</b>	<b>10.2</b>	<b>10.8</b>	<b>3.7</b>	<b>10.1</b>	1.4	47	>20 (43)	133.0
<b>A7.3</b>	<b>7.8</b>	<b>4.5</b>	<b>3.6</b>	<b>8.0</b>	1.5	35	15.5	82.6
<b>A7.4</b>	<b>4.7</b>	<b>6.6</b>	<b>2.4</b>	<b>8.1</b>	<b>2.5</b>	<b>76</b>	n.m.	<b>29.0</b>
<b>A7.5</b>	<b>5.3</b>	<b>7.7</b>	1.7	<b>0.07</b>	<b>2.3</b>	<b>79</b>	n.m.	<b>41.1</b>
<b>A7.6</b>	<b>5.1</b>	<b>1.4</b>	<b>3.5</b>	<b>0.018</b>	tox.	<b>94</b>	n.m.	<b>14.5</b>

<sup>a</sup>Test systems as described in footnote Table 2; n.m. not measurable because of high background fluorescence; tox. toxic at a concentration of 20 µg/ml.

**Figure 9.** (upper) RP-HPLC separation of A7 and subfractions (detection at 280 nm). (lower) Summary of chemopreventive activities.



**Figure 10.** NP-HPLC separation of AS02. Procyanidins were detected by fluorescence detection with excitation at 278 nm and emission detection at 312 nm.

5.9 µg/mL vs. AS02 11.5 µg/mL). Of all AS04 fractions, fraction NP.3 enriched in phloretin-2'-xyloglucoside (2b) was least active. With increasing amounts of procyanidins, potential to scavenge DPPH radicals and to inhibit AR activity clearly increased. In contrast, peroxy radical scavenging potential in the ORAC assay declined from fraction NP.2–NP.5. Cyp1A was about equally inhibited by fraction

NP.1, NP.2, NP.4, and NP.5. This was attributed to the very active quercetin (4h) and its glycosides (4d, 4g) in fractions NP.1 and NP.2, and to procyanidins in fractions NP.4 and NP.5. Cox-1 activity was most strongly inhibited by fraction NP.1, but active constituents have not been identified so far. Interestingly, fraction NP.5 with the highest content in procyanidins and the highest DPM was less inhibitory than

**Table 3.** Composition of NP-HPLC fractions NP.1–NP.5 and summary of their bioassay results

mg/g		NP.1	NP.2	NP.3	NP.4	NP.5
<b>1a</b>	Chlorogenic acid	11.3	697.9	37.4	3.4	n.d.
<b>1c</b>	4-Coumaroyl quinic acid	1.5	73.7	n.d.	n.d.	n.d.
<b>1d</b>	<i>p</i> -Coumaric acid	6.4	n.d.	n.d.	n.d.	n.d.
<b>2a</b>	3-Hydroxyphloretin-2'-xyloglucoside	n.d.	n.d.	22.5	11.7	n.d.
<b>2b</b>	Phloretin-2'-xyloglucoside	n.d.	13.0	484.0	42.7	n.d.
<b>2e</b>	Phloridzin	n.d.	129.6	39.0	3.3	n.d.
<b>2e<sup>#</sup></b>	3-Hydroxyphloridzin	n.d.	1.8	6.2	1.8	n.d.
<b>2f</b>	Phloretin (aglycone)	0.5	n.d.	n.d.	n.d.	n.d.
<b>3a</b>	Procyanidin B <sub>1</sub>	n.d.	n.d.	12.4	n.d.	n.d.
<b>3b</b>	(+)-Catechin	n.d.	1.5	n.d.	n.d.	n.d.
<b>3c</b>	(-)-Epicatechin	n.d.	4.5	n.d.	n.d.	n.d.
<b>3d</b>	Procyanidin B <sub>2</sub>	n.d.	n.d.	28.4	n.d.	n.d.
<b>4a</b>	Quercetin-3-rutinoside	n.d.	n.d.	11.7	n.d.	n.d.
<b>4b</b>	Quercetin-3-galactoside	n.d.	4.4	n.d.	n.d.	n.d.
<b>4c</b>	Quercetin-3-glucoside	n.d.	1.5	1.0	n.d.	n.d.
<b>4d</b>	Quercetin-3-xyloside	n.d.	4.5	4.5	n.d.	n.d.
<b>4g</b>	Quercetin-3-rhamnoside	n.d.	5.2	1.6	n.d.	n.d.
<b>4h</b>	Quercetin	0.6	0.7	n.d.	n.d.	n.d.
Procyanidins (acid butanol assay)		0	70.2	268.2	739.2	1030.7
Total		20.4	1008.7	877.0	802.0	1030.7
DPm		0	3.1	3.4	4.6	7.9

Bioassay results	AS04	NP.1	NP.2	NP.3	NP.4	NP.5
DPPH <sup>a)</sup> (IC <sub>50</sub> in µg/mL)	<b>8.7</b>	20.9	15.1	24.3	13.6	<b>9.1</b>
X/XO (IC <sub>50</sub> in µg/mL)	17.1	30.4	23.2	43.7	24.3	24.2
ORAC <sup>b)</sup> (units)	<b>3.9</b>	<b>3</b>	<b>3.8</b>	<b>3.7</b>	2.2	1.5
Cyp1A (IC <sub>50</sub> in µg/mL)	11.5	<b>6.2</b>	10.9	46	10.8	10.7
QR <sup>c)</sup> (CD in µg/mL)	>250	41	>50	>50	42.2	35.3
Cox-1 <sup>d)</sup> (% inhibition)	5	41	12	<b>30</b>	<b>33</b>	11
AR (IC <sub>50</sub> in µg/mL)	<b>5.9</b>	>50	>50	>50	<b>7.6</b>	<b>2.5</b>

n.d., not detectable.

a) Test systems as described in footnote Table 2 with the following modifications:

b) Tested at a concentration of 1 µg/mL instead of 0.4 µg/mL. ORAC value of AS04 at 0.4 µg/mL: 2.4.

c) Concentration required to double the specific activity of quinone reductase (CD) was determined instead of the fold induction at 20 µg/mL.

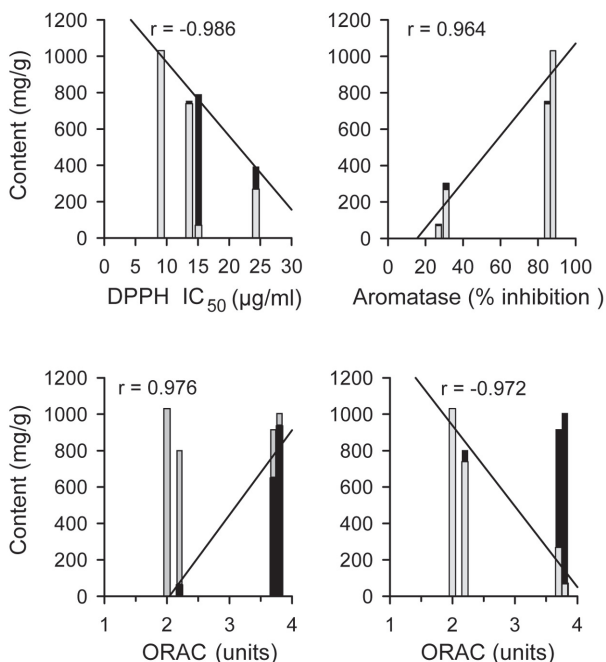
d) Tested at a final concentration of 100 µg/mL instead of 400 µg/mL. Cox-1 inhibition of AS04 at 400 mg/mL = 62%.

fractions NP.3 and NP.4, which contained procyanidins with an intermediate DPm of 3.4 and 4.6 in addition to LMW compounds.

### 3.4.3 Correlation of bioassay results with LMW compounds and TPC

To further investigate the contribution of LMW compounds and procyanidins to the observed biological effects, amounts of LMW compounds active in a particular test system and of procyanidins of fractions NP.2–NP.5 were plotted *versus* activities in the DPPH, ORAC and AR assay (from Table 3). Fraction NP.1 was omitted since components have not been identified yet. Correlation coefficients were determined by linear regression analyses. As indicated in Fig. 11, IC<sub>50</sub> values in the DPPH assay were linearly correlated with the sum of LMW compounds (black) and TPC (gray), and we determined a correlation coefficient  $r = 0.986$  (upper left). When the analyses were limited to

the content of either LMW compounds active in the DPPH assay (1a, 1c, 3b, 3c, 4a–4d, 4g–4h) or to TPC (including 3a, 3d), correlation coefficients were calculated as  $r = 0.117$  and  $0.683$ , respectively (data not shown). The percentage of inhibition of AR enzymatic activity at a test concentration of 50 µg/mL was linearly correlated with the sum of LMW compounds and TPC of fractions NP.2–NP.5, with a correlation coefficient of  $r = 0.964$  (upper right). Since the fraction of LMW compounds with inhibitory activity in the AR assay is very small (2f, 4c, 4d, 4h), AR inhibition can almost exclusively be attributed to the procyanidin fraction. An interesting pattern of correlation between the contents of the fractions and ORAC units was observed. Fraction NP.5, which exclusively contained procyanidins, resulted in the lowest peroxy radical scavenging potential with an ORAC<sub>ROO</sub> value of 2 units at a test concentration of 1 µg/mL. With increasing portions of LMW compounds with potential to scavenge peroxy radicals (1a, 1c,



**Figure 11.** Correlation of DPPH radical scavenging (upper left), percentage of AR inhibition at a test concentration of 50 µg/mL (upper right), and peroxy radical scavenging in the ORAC test (lower left: vs. LMW, lower right: vs. TPC), with the content in LMW compounds active in the particular test system (black portion of stacked bar) and/or TPC (gray portion) of fractions NP.2–NP.5. Regression analyses to determine correlation coefficients  $r$  were performed with Sigma Stat 2.0.

1d, 2b, 2e, 2e<sup>#</sup>, 2f, 3a–3d, 4a–4d, 4g, 4h), ORAC values increased, indicating that LMW compounds such as chlorogenic acid (1a) play a more important role than oligomeric procyanidins in scavenging peroxy radicals in the ORAC assay. We determined a linear correlation with  $r = 0.976$  for LMW compounds (lower left), and consequently an inverse correlation with  $r = 0.972$  for TPC (lower right), whereas other than in the DPPH assay, the sum of LMW compounds and TPC was not correlated with peroxy scavenging potential ( $r = 0.192$ ).

## 4 Discussion

We have fractionated a polyphenolic extract of apple juice to identify components with potential cancer chemopreventive activities. The extract was separated into seven fractions and further subfractionated into 48 subfractions and two precipitates. As shown in Fig. 3A, CPC split the apple juice components according to polarity. Further separation was achieved by GPC on Sephadex LH20. Additional fractionation of apple juice extract with normal phase chromatography provided five fractions with increasing content in procyanidins.

Bioassay testing of all fractions and subfractions allows the following conclusions:

(i) DPPH- and superoxide anion (X/XO assay) radical scavenging often increased with decreasing polarity. Low  $IC_{50}$  values were observed with late eluting subfractions containing flavonoids (flavan-3-ols and quercetin-glycosides) and procyanidins. Importantly however,  $IC_{50}$  values were better correlated with the sum of procyanidins and LMW compounds active in the DPPH assay than with the content in either LMW compounds or procyanidins alone.

(ii) Known apple juice components of the main four structural classes present in apple juice, *i. e.*, phenol carboxylic acids, dihydrochalcones, flavan-3-ols and procyanidins, and quercetin-glycosides, all possessed good potential to scavenge peroxy radicals in the ORAC assay. Correlation analyses indicate that overall, LMW compounds may play a more important role than oligomeric procyanidins.

(iii) Potent inhibition of Cyp1A activity was associated with late eluting fractions which contained procyanidins. In addition, the aglycone quercetin (4h) was identified as a potent Cyp1A inhibitor with an  $IC_{50}$  value of 0.026 µM (0.008 µg/mL) [44]. Interestingly, quercetin was so potent that it may have contributed to the inhibitory effects of some fractions when present in trace amounts without even being detectable.

(iv) Potential to induce QR activity was mainly distributed between two early eluting fractions A2.1 and A3.1 and two late eluting fractions A7.6 and A7.7. Current further fractionations indicate that the early eluted fractions contain some aroma components of apple juice which may account for the observed activity. QR inducing activity of the late eluting fraction is most likely associated with the elution of quercetin aglycone (4h).

(v) Our results indicate that various compounds in apple juice contribute to anti-inflammatory effects. Strongest inhibition of Cox-1 activity with >90% inhibition at a concentration of 400 µg/mL was determined for intermediate eluting subfractions A4.4, A5.4, A6.5/A6.6, and fraction A7.6. Potent Cox-1 inhibition of A5.4 and A6.5/A6.6 was attributed to (–)-epicatechin (3c) with an  $IC_{50}$  value of 7.5 µM (2.2 µg/mL), whereas the activity of the nonpolar fraction A7.6 was associated with the elution of quercetin (4h). Anti-inflammatory potential was also associated with late eluting subfractions containing procyanidins. Notably, fractionation of procyanidins by NP-HPLC indicated that fractions NP.3 and NP.4 with an intermediate DPm were more potent in inhibiting Cox-1 activity than fraction NP.5 with the highest DPm and the highest TPC. This observation may point against a nonspecific inhibition of Cox-1 activity due to procyanidin–protein interactions.

(vi) Inhibition of AR activity was strongly correlated with increasing content in procyanidins, as indicated in Fig. 11. Presently we cannot rule out that these effects are due to nonspecific protein binding.

(vii) Procyanidins were identified as a very interesting class of apple juice components, since all late eluting sub-fractions containing procyanidins demonstrated activity in all test systems except QR induction (also compare a recent review in ref. [52]).

(viii) Correlation analyses demonstrate that procyanidins alone or in combination with LMW compounds contribute to the measured potential cancer chemopreventive activities.

Several earlier publications have identified procyanidins as an important class of apple (juice) constituents. Apple procyanidins were separated by various chromatographic techniques, including thiolysis-HPLC, high-speed counter current chromatography, size-exclusion chromatography and normal-phase chromatography as in the present study [42, 53–56]. Recent literature also points to potential cancer preventive activity of oligomeric procyanidins from apple skin, lentils, almonds, grape seed, and pine bark, especially antioxidant and radical scavenging activity [57–61], as well as cytotoxic effects and induction of apoptosis in colon and other cancer cell lines [29, 36, 58, 60]. Barth *et al.* [62] compared the effects of clear and cloudy apple juice in a rat model for colon carcinogenesis using dimethylhydrazine (DMH) as a carcinogen. After intervention for 8 wk, cloudy apple juice was more potent in inhibiting carcinogen-induced epithelial cell proliferation and DNA-damage than clear apple juice. Also, cloudy apple juice reduced the number of aberrant crypt foci as a preneoplastic marker for colon carcinogenesis by 28%, whereas clear apple juice was ineffective. These results may be explained by a higher content in procyanidins in cloudy apple juice than in clear juice, as shown by Oszmianski *et al.* [61] and Hümmer *et al.*, who recently determined that cloud particles responsible for the turbidity of cloudy apple juice contained up to 60% of oligomeric procyanidins (personal communication). Also, Gosse *et al.* [29] demonstrated that a procyanidin-enriched fraction from apples at a very low concentration (0.01% in drinking water) significantly reduced the number of aberrant crypt foci in the azoxymethane-induced rat carcinogenesis model by 50%, whereas a fraction containing monomeric flavonoids was ineffective.

Until recently it was basically impossible to analyze whether procyanidins contribute to the cancer preventive effects of fruit and vegetables in humans, due to a lack of data about their concentrations in common foods and estimates of normal consumption. Four years ago, Gu *et al.* [63] published a database based on NP-HPLC analyses with procyanidin levels in common and infant food from the US. Highest levels were detected in various berries, grains, beans, nuts, red wine, and dark chocolate [63]. Comprehensive nutrient databases will certainly help to define the role of procyanidin consumption in cancer prevention in humans.

Apples and apple juice are an integral part of the human diet and are consumed by a majority of the population including children. Apples are a rich source of polyphenolic

compounds of various structural classes, which possess a broad spectrum of potential cancer chemopreventive activities. Overall, our study and others suggest that apples and especially cloudy apple juice should be further investigated as part of a cancer prevention strategy in humans.

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