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Phenolic compounds in juices of apple cultivars and their relation to antioxidant activity

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Summary

Apples and their juices are important sources of phenolic compounds in human diet. Using the same juice processing method, we compared phenolic composition as well as antioxidant activity of juices from various table apple cultivars, grown at the same location. Antioxidant activity of apple juices was estimated by application of two assays, ABTS cation radical decolorization and by scavenging of reactive oxygen species (ROS) generated by xanthine/xanthine oxidase ($O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$; superoxide assisted Fenton reaction), as measured by inhibition of ethylene release from KMB (α-Keto-γ-(methylthio)-butyric acid). The apple juices differed in phenolic content and antioxidant activity, where cultivar differences were more relevant than environmental factors. Furthermore, to improve antioxidant performance in the XOD-test, a juice low in phenol content was supplemented with the appropriate amounts of phenols to the level of the best juice indicating that these phenolics contribute to the antioxidant activity of the apple juice. In accordance to literature, the phenolic compounds representing the main antioxidant activity in the apple juices comprise flavan-3-ols and chlorogenic acid.

Abbreviations: KMB, α-keto-γ-methiol-butyric acid; ABTS, azo-bis-benzthiazolinosulfonate; ROS, reactive oxygen species; $OH^{\cdot}$, OH radical; $O_2^{\cdot-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; X, xanthine; XOD, xanthine oxidase; apple cultivars: see Tab. 1.

Introduction

Beside their nutritional value, apples and apple juices promote also health benefits due to their "bioactive" components acting as antioxidants. Vitamins and phenolics represent the predominant compounds with antioxidant activity in apples (Proteggente et al., 2002; Paganga et al., 1999; Bremner, 2000; Francini and Sebastiani, 2013). Polyphenols of apple were also shown to prevent cardiovascular diseases (Koutsos et al., 2015) and they exhibit various physiological functions (Akazome, 2004; Hofseth and Matesic, 2011). Bitsch et al. (2001) proposed apple juice to be suitable as functional food because of its influence on plasmatic antioxidant capacity. A study on pigs could show that a diet supplemented with apples decreased oxidative stress by decreasing malondialdehyde formation in the body and by decreasing DNA damage in the blood cells (Pajk et al., 2006). Phenolics, particularly flavan-3-ols, also play a role in resistance of cultivars against the fungus *Venturia inaequalis* causing the scab disease of apple (Mayr et al., 1995; Treutter et al., 1990). Susceptible and resistant cultivars differ in the flavan-3-ol content of their leaves (Treutter et al., 1990), whereas there was no significant difference in total phenolics as measured by the Folin-test (Picinelli et al., 1995).

The aim of this study was to determine the concentrations of diverse phenolic compounds in scab resistant and susceptible apple cultivars, cultivated at the same location but harvested in two years. Thus, the variation in phenolic content and antioxidant activity of apples, respectively of their juices, harvested within one year, as well as the influence of environmental conditions, comparing the results of the two years. In the lab, juices of these apples were prepared using the same method. The juices were compared for content of phenolics (HPLC) and for antioxidant activity in two selected test systems. The contribution of single phenolic compounds to the antioxidant capacity was estimated with special respect to procyanidins. Furthermore, a low level juice was supplemented with lacking phenolic compounds in order to improve the antioxidant potential. The antioxidant role of polymeric procyanidins was studied after preparation of a polymeric fraction.

Material and methods

Apple fruits were grown in the field of the "Kompetenz-Zentrum Obstbau Bavendorf", South Germany, and were harvested in autumn of the years 2001 and 2002. The weather conditions of the two years can be described as follows: mean temperature (2001: 9.1 °C; 2002: 9.7 °C), rainfall (2001: 1081 mm; 2002: 1187 mm); sun hours (2001: 1771 h; 2002: 1780 h). In spring 2001 the temperatures were higher than in 2002.

The cultivars were divided in two groups: scab susceptible and scab resistant ones. The harvest dates and fruit ripeness parameters are given in Tab. 1. Apple juices were prepared from each 10 fully ripe and healthy fruits on laboratory scale using a mixer. The juices were frozen and stored at -20 °C. Before use in 2003, aliquot amounts of methanol (1 ml/g ice) were added to the frozen juices to prevent enzymatic oxidation during thawing. The diluted juices were homogenised in a cooled (6 °C) ultra sound water bath. The homogenised extracts (1.5 ml) were concentrated to dryness, redissolved in small amounts of methanol (150 µl), centrifuged and then used for HPLC analysis of phenolic compounds. Quantification of phenolic compounds was performed by HPLC with post-column derivatisation using the method described by Treutter et al. (1989).

HPLC determination of phenolics

The HPLC equipment used consists of an autosampler (Gilson-Abimed Modell 231), of two pumps (Kontron Modell 422), and a diode array detector (Bio Tek Kontron 540). For post column derivatisation a further analytical HPLC pump (Gynkotek Modell 300 C) and a VIS-detector (640 nm, Kontron Detektor 432) were used.

The phenolic compounds were separated using chromatographic conditions, which had been optimized for apples, according to Mayr et al. (1995) on a column (250 × 4 mm I.D.) prepacked with Hypersil ODS, 3 µm particle size, following a stepwise gradient using mixtures of 5% formic acid (A) and methanol gradient grade (B) with a flow rate of 0.5 ml/min. The gradient profile used is: 0-5 min, isocratic, 5% B in A; 5-15 min, 5-10% B in A; 15-30 min, isocratic, 10% B in A, 30-50 min, 10-15% B in A, 50-70 min, isocratic, 15% B in A, 70-85 min, 15-20% B in A, 85-95 min, isocratic, 20% B in A, 95-110 min,

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Tab. 1: Properties of apple fruits harvested in 2001 / 2002

Cultivar	Abbre-viations	Harvest date	Fruit diameter [mm]	Flesh firmness [kg/cm²]	Starch [1-10]	Soluble solids [% Brix]	Soluble solids [°Oe]	Acid [mval]	Acid [g/l]	Juice yield [%]
Scab susceptible cultivars										
Summerred	SR1	22.08.01	77	6.8	4.5	12.4	53	16.04	10.75	63.1
	SR2	13.08.02	71	6.8	5.4	11.2	48	15.09	10.11	69.2
Alkmene	Alk1	27.08.01	79	7.8	2.0	12.8	54	13.55	9.08	60.6
	Alk2	23.08.02	73	7.6	2.8	12.8	54	13.25	8.88	67.6
Elstar Elshof	EE1	08.09.01	83	7.3	2.9	13.2	56	16.53	11.08	66.7
	EE2	04.09.02	73	6.4	1.8	10.8	46	11.4	7.64	56.0
Gala Galaxy	GG1	08.09.01	78	9.9	2.6	12.6	54	9.18	6.15	63.4
	GG2	05.09.02	73	8.0	4.0	10.9	46	5.79	3.88	52.0
Jonagored	JG1	02.10.01	84	8.0	6.8	15.4	65	13.86	9.29	72.1
	JG2	18.09.02	85	6.4	7.9	11.7	50	7.27	4.87	52.0
Braeburn Wellburn	Bw1	16.10.01	81	10.9	4.0	13.4	57	17.22	11.54	62.8
	Bw2	22.10.02	81	9.5	5.0	12.1	51	13.38	8.96	73.4
Scab resistant cultivars										
Santana	San1	26.08.01	78	7.5	1.2	12.3	52	18.2	12.19	68.9
	San2	22.08.02	79	7.1	2.0	11.9	51	16.9	11.32	52.9
Gerlinde	Ger1	03.09.01	72	7.1	6.8	14.8	63	15.2	10.18	70.2
	Ger2	29.08.02	70	7.2	9.5	11.4	48	11	7.37	56.9
Rebella	Reb1	14.09.01	70	7.4	4.3	13.7	58	14.85	9.95	59.9
	Reb2	04.09.02	73	6.4	7.8	12.6	54	12.72	8.52	57.0
Rosana	Ros1	20.09.01	84	6.7	2.3	13.2	56	19.39	12.99	75.7
	Ros2	17.09.02	78	6.8	3.2	11.1	47	14.33	9.60	66.0
Ariwa	Ari1	20.09.01	70	8.4	1.9	12.1	51	13.13	8.80	70.1
	Ari2	23.09.02	75	9.5	5.8	13.5	57	12.75	8.54	66.9
Rewena	Rew1	30.09.01	71	6.9	3.6	12.5	53	16.89	11.32	66.0
	Rew2	07.10.02	78	7.4	7.0	12.7	54	14.78	9.90	67.0
Topaz	Top1	03.10.01	78	7.5	5.6	12.6	54	18.13	12.15	75.0
	Top2	24.09.02	78	8.2	2.6	12.8	54	16.15	10.82	64.0
Florina	Flo1	08.10.01	77	8.2	8.3	13.3	57	11.5	7.71	70.4
	Flo2	09.10.02	81	7.3	8.3	12.3	52	9.2	6.16	53.0
Baujade	Bau1	07.11.01	79	10.5	7	14.3	61	17.03	11.41	68.2
	Bau2	24.10.02	76	11.2	7	13.4	57	13.76	9.22	67.8
Goldrush	Gold1	07.11.01	77	9.5	5.8	15.7	67	15.28	10.24	73.1
	Gold2	22.10.02	74	10.1	7	14.3	61	13.85	9.28	71.6

20-25 % B in A, 110-140 min, 25-30 % B in A, 140-160 min, 30-40 % B in A, 160-175 min, 40-50 % B in A, 175-190 min, 50-90 % B in A. Phenolic acids and flavonols were detected at 280 nm whereas the flavan-3-ols were estimated at 640 nm (TREUTTER, 1989; TREUTTER et al., 1994) after post column derivatisation with p-dimethylaminocinnamic aldehyde (DMACA). 6-Methoxyflavone was used as internal standard for quantitative analyses. Reproducibility of the method including extraction and quantification by HPLC is above 90 %.

The individual compounds were identified by retention times and their UV-absorbance spectra via diode array detection and by comparison with standards. These standards were either commercially available from Roth and Sigma (catechin, epicatechin, chlorogenic acid, phloridzin, rutin) or previously isolated from apple and service tree: procyanidins B1, B2, B5, E-B5, C1, phloretin derivatives, hydroxycinnamic acids, quercetin glycosides (MAYR et al., 1995; TREUTTER et al., 1994, ÖLSCHLÄGER et al., 2004).

Fractionation on Sephadex-LH20

For separation of polymeric proanthocyanidins from oligomeric proanthocyanidins and low molecular weight compounds (hydroxycinnamic acids, dihydrochalcones, flavonols) a Sephadex-LH 20 co-

lumn (0.7 cm diameter × 30 cm length) was used. 1.5 ml apple juice was subjected and eluted with methanol. 3 fractions were collected with 10 ml volume each. Fractions were analysed by HPLC. Polymeric proanthocyanidins were estimated as cyanidin equivalents by boiling aliquots after adding a mixture of butanol and concentrated HCl (4:1, v/v) for 20 min.

ABTS-radical decolorization assay

The assay used here is modified, but the principle was described first by MILLER et al. (1993). A number of variations of this assay exist (MILLER et al., 1997; RE et al., 1999; LABRINEA et al., 2004; ARNAO et al., 2001; CANO et al., 2000; ARTS et al., 2004 a, b; KIM et al., 2004; FISCHER et al., 2005). ABTS radicals are quite stable in the absence of a reductant, if the concentration of ABTS is in excess to the ABTS cation radical (CANO et al., 1998, 2000). Interestingly, Trolox performs in this assay ideally concerning dose response effects (velocity and extent of $ABTS^{\cdot+}$ decolorization). In contrast to most phenols tested, the synthetic Trolox (a water soluble vitamin E derivative) showed a straight linear correlation of decreasing ABTS radicals over the whole range. Therefore, Trolox is commonly used as a standard antioxidant to calibrate the assay and antioxidant activity is expressed in TEAC values (TEAC = Trolox equivalent antioxidant concentration). The generation of ABTS radicals from ABTS, however, differ between the various assays, using either acidic manganese dioxide or potassium persulfate, or the radical generator ABAP (2,2´-azinobis(2-amidinopropane) dihydrochloride), or hydrogen peroxide in the presence of heme proteins (pseudoperoxidase, e.g. myoglobin).

In this study ABTS-radicals were produced by reaction of myoglobin with hydrogen peroxide in the presence of ABTS at pH 6.0 and 37 °C (10 mM phosphate pH 6.0, ABTS 1 mM, myoglobin 2.5 µM, H_2O_2 500 µM). Usually 5 ml aliquots were prepared and after a reaction time of 60 min the dark green solution was stored overnight at 4 °C in a refrigerator. Before use, the concentration of ABTS-radicals was checked after dilution (λ_{734nm} = 15 000 M^{-1} cm^{-1}). In general, a 1:10 dilution in 50 mM phosphate pH 7.4 results in an absorbance of about 1.0 at λ = 734 nm. This absorbance was used as startpoint for the decolourisation assays (67 µM $ABTS^{\cdot+}$).

A typical assay contains in 1.0 ml: 50 mM phosphate buffer pH 7.4, $ABTS^{\cdot+}$ 67 µM, and 20 µl of the test solution (20 µl). Decolorization of the assay was measured exactly 5 minutes after starting the reaction with the test solution. For calculation of TEAC values, the fits in the linear range of the received dose-response plots of the different apple juices were compared to the linear equation of Trolox.

KMB oxidation to ethylene by ROS formed via Xanthine(X)/ Xanthine Oxidase (XOD)

The X/XOD system is applied to produce free radicals ($O_2^{\cdot-}$, $^{\cdot}OH$) beside the main products H_2O_2 and uric acid. About 20 % of the electrons from xanthine are used by XOD for the monovalent reduction of molecular oxygen (FRIDOVICH, 1970). Under our conditions $^{\cdot}OH$ radicals are indirect products formed by the side reaction of Fe^{2+}-ions with H_2O_2 (Fenton reaction). $O_2^{\cdot-}$ is necessary to reduce Fe^{3+}-ions into Fe^{2+}-ions. Trace amounts of Fe^{3+}-ions are present in the buffer solution as well as in the XOD suspension, which also contains EDTA, an enhancer of the Fenton reaction. $^{\cdot}OH$-radicals oxidize α-keto-γ-methiol-butyric acid (KMB) under formation of ethylene, similar as described for methional (BEAUCHAMP and FRIDOVICH, 1970). Under our conditions, XOD converted the given xanthine into uric acid within 20-25 minutes. Immediately after starting the reaction by addition of XOD, the probes were gastight sealed with rubber stops and incubated for 30 min at 37 °C in a gently shaking waterbath in the dark. After that, aliquots of 1 ml of the headspace gas of the reaction tubes were withdrawn with gastight syringes and stored up to GC-quantification of ethylene, sticked into a rubber mat. A Varian CX3300 gas chromatograph was used, equipped with a Varian Al_2O_3 column (60 cm × 1/8 inch). A typical assay contains in 2.0 ml: 100 mM phosphate buffer pH 7.4, 1 mM KMB, 500 µM xanthine, xanthine oxidase 0.04 units/ml, various diluted apple juice 100 µl/ml (solved in water – free of methanol).

Activity of XOD was quantified by HPLC analysis of xanthine and uric acid using a Waters HPLC, consisting of a 600-pump, 717-autosampler and 996-PDA detector, column Merck Cartridge 125 × 4 mm, Lichrospher® 60 RP-select B with precolumn 4 × 4 mm, temperature 35 °C, eluent NaH_2PO_4 50 mM isocratic for 8 min, and methanol (grad. grade) for the washing step of the column after each injection (total time, 20 min including re-equilibration to NaH_2PO_4 50 mM); flow rate 1 ml/min, sample volume 20 µl. Retention times: xanthine 3.0 min, uric acid 4.0 min. For XOD-activity determination the XOD-reaction was performed in the presence or absence of the investigated compound. The enzyme reaction was started with XOD and stopped after exactly 10 min by addition of 50 µl HCl10M. Influence of the extracts on XOD-activity was expressed by comparing amounts of uric acid produced as well as xanthine consumed with the basic reaction in the absence of antioxidants. An assay contains in a final volume of 1.00 ml: 100 mM phosphate buffer pH 7.4, 500 µM xanthine, xanthine oxidase 0.04 units/ml, and amounts of tested sample (apple juice or standard compound).

Results

Phenolic composition of cultivars

Phenolic profiles of the juices prepared from various apple cultivars did not differ in quality. The main hydroxycinnamic acid (HCA) was identified as n-chlorogenic acid (5-caffeoylquinic acid). Several minor peaks with similar UV-absorbance spectra were also calculated as chlorogenic acid and combined together into one group of hydroxycinnamic acids (HCA). Their concentrations (Tab. 2) ranged from 94.6 mg/L (Rem2) to 492.8 mg/L (Eco1). An exceptionally high level was found in Baujade (Bau1) with 747.0 mg/L. More than 50 % of the hydroxycinammic acids are represented by chlorogenic acid which coincides with literature (TREUTTER, 2001; KAHLE et al., 2005). Only the cultivars Alkmene, Elstar Elshof, Remura and Florina produced less.

Since the separation of flavanols and HCA is not sufficient, the total HCA level may be somewhat overestimated by flavanol impurities. The flavanols, however, were quantified more precisely by using selective post column derivatisation with DMACA and detection at 640 nm. This resulted in pure peaks which were identified as catechin, epicatechin, and the procyanidins B1, B2, B5 and C1. These known procyanidins together with some unidentified peaks at 640 nm were calculated as procyanidin B2. The most prominent flavanols in the juices were the monomeric epicatechin and its dimeric and trimeric derivatives procyanidin B2 and procyanidin C1.

The total flavanol concentrations (Tab. 2) covered a broad range from 0.3 mg/L (SR2) to 337.6 mg/L (Rub2), again with the extreme 955.8 mg/L in Baujade (Bau1). The latter value fits into the group of cider apples which often contain much more procyanidins (GUYOT et al., 2003). The majority of our juices have flavanol concentrations as reported in literature from commercial juices and for table apple juices (TREUTTER, 2001; KAHLE et al., 2005).

It may be noted that those cultivars with total flavanols below 10 mg/L, such as Summerred, Santana, Gerlinde, Regunda, Ecolette, exhibited relatively high hydroxycinnamic acid concentrations. By that, the total phenolics are not so much reduced.

Those peaks showing the typical UV-absorbance of phloridzin were calculated as phloridzin and summed up to give the total dihydrochalcone value. All flavonols were calculated as rutin. The concentration of these minor components were between 4.6 mg/L (Alk1)

Tab. 2: Phenol content (mg/L) of apple juices (apples harvested in 2001 / 2002) estimated by HPLC analysis

Cultivar	Abbrev.	Chlorogenic acid	**Total Hydroxycinnamic acids**	Total Dihydrochalcones	Total Flavonols	Catechin	Epicatechin	Procyanidin B1	Procyanidin B2	Procyanidin B5	Procyanidin C1	**Total Flavanols**	*Total Phenolics*
a) Scab susceptible cultivars													
Summerred	SR 1	187.4	**256.9**	15.4	6.4	0.04	0.12	0.08	0.03	0.01	0.04	**0.8**	*279.4*
	SR 2	106.0	**161.0**	10.8	2.7	0.01	0.00	0.00	0.01	0.01	0.03	**0.3**	*174.7*
Alkmene	Alk 1	43.3	**136.2**	4.6	5.5	3.1	40.2	8.5	64.4	8.6	45.4	**197.8**	*344.1*
	Alk 2	36.9	**138.3**	9.5	7.5	3.0	32.2	8.7	55.4	4.4	42.1	**174.1**	*329.4*
Elstar Elshof	EE 1	88.1	**248.1**	19.3	8.6	7.0	61.8	16.2	74.6	5.5	46.6	**240.0**	*515.9*
	EE 2	35.8	**110.6**	8.5	6.0	2.7	22.0	6.5	25.8	2.3	17.7	**88.8**	*213.9*
Gala Galaxy	GG 1	189.6	**317.6**	11.9	7.4	6.2	38.8	17.1	42.7	2.6	32.9	**157.8**	*494.6*
	GG 2	195.0	**336.6**	49.7	8.3	5.9	29.6	10.5	17.0	2.0	13.1	**85.9**	*480.4*
Jonagored	JG 1	216.0	**360.9**	23.2	15.0	2.4	36.3	9.9	72.3	5.0	52.1	**220.2**	*619.3*
	JG 2	109.6	**172.8**	8.7	10.5	0.9	12.3	3.8	24.7	1.9	15.0	**71.5**	*263.6*
Braeburn Wellburn	BW 1	199.3	**386.2**	13.3	14.1	6.2	53.7	14.6	63.6	5.1	48.7	**223.6**	*637.3*
	BW 2	82.4	**227.0**	7.7	9.9	1.5	10.1	1.3	6.1	0.8	3.5	**25.7**	*270.2*
b) Scab resistant cultivars													
Santana	San 1	154.8	**260.4**	13.4	11.9	0.01	0.03	0.01	0.01	0.01	0.28	**0.6**	*286.3*
	San 2	158.3	**249.4**	15.4	8.2	0.09	0.01	0.03	1.69	0.04	0.02	**2.1**	*275.2*
Ahra	Ahr 1	268.3	**337.4**	20.2	11.0	3.5	39.1	8.7	53.6	3.8	20.9	**156.8**	*525.4*
	Ahr 2	196.4	**297.4**	20.5	17.3	8.9	62.7	18.6	67.4	4.5	30.9	**215.2**	*550.4*
Gerlinde	Ger 1	241.3	**354.4**	14.7	14.0	0.06	0.55	0.05	0.31	0.08	0.56	**2.4**	*385.5*
	Ger 2	178.9	**262.2**	21.2	8.2	0.02	0.16	0.01	0.04	0.02	0.02	**0.6**	*292.2*
Rubinola	Rub 2	297.7	**452.2**	15.1	10.8	16.0	89.8	31.3	106.6	6.2	53.3	**337.6**	*815.6*
	Rub 1	352.0	**481.4**	20.1	15.7	4.6	55.9	18.8	100.3	4.1	46.6	**265.5**	*782.8*
Remura	Rem 1	51.4	**151.4**	6.5	5.6	7.1	52.0	16.9	87.8	5.5	46.9	**246.5**	*410.0*
	Rem 2	31.6	**94.6**	4.8	5.4	4.1	29.8	6.8	35.4	1.7	18.1	**109.4**	*214.2*
Rebella	Reb 1	74.0	**154.0**	18.8	21.9	0.1	3.3	0.6	7.5	0.6	7.0	**24.3**	*219.0*
	Reb 2	58.3	**112.2**	12.9	16.8	0.1	2.4	0.5	2.7	0.3	2.1	**9.7**	*151.7*
Vesna	Ves 1	83.5	**199.8**	9.5	12.5	6.9	44.4	12.8	60.8	4.0	27.1	**172.5**	*394.3*
	Ves 2	166.0	**268.3**	10.9	11.9	7.2	46.1	15.4	65.2	3.5	34.9	**194.3**	*485.2*
Reanda	Rea 1	240.3	**354.1**	12.0	10.0	4.8	48.2	10.0	57.3	3.6	26.1	**167.8**	*544.0*
	Rea 2	174.6	**254.7**	8.0	8.6	3.7	26.5	4.7	24.1	2.2	9.7	**77.5**	*348.8*
Rosana	Ros 1	73.9	**187.3**	11.0	18.1	6.4	55.5	19.7	84.7	6.8	56.4	**265.2**	*481.6*
	Ros 2	48.4	**105.3**	16.9	9.4	2.1	16.7	4.2	16.0	1.5	9.4	**55.7**	*187.2*
Ariwa	Ari 1	154.4	**267.8**	8.6	6.1	2.6	35.8	11.2	61.3	4.7	35.0	**188.8**	*471.3*
	Ari 2	212.8	**314.2**	10.7	10.3	1.6	19.3	4.1	26.8	3.1	21.8	**91.5**	*426.7*
Regunde	Regu 1	364.4	**471.9**	20.4	7.2	0.06	0.34	0.08	0.61	0.03	0.07	**1.4**	*500.8*
	Regu 2	199.9	**280.8**	12.5	5.9	0.06	0.94	0.06	0.32	0.02	0.05	**1.6**	*300.8*
Rewena	Rew 1	223.2	**330.5**	27.7	8.5	6.6	57.4	13.9	80.0	6.3	53.2	**251.4**	*618.1*
	Rew 2	157.3	**254.6**	8.2	11.2	5.0	40.8	8.0	47.3	4.3	37.1	**164.6**	*438.5*
Topaz	Top 1	90.5	**193.3**	8.0	6.7	5.0	48.8	12.8	54.1	4.0	24.3	**168.1**	*376.1*
	Top 2	95.7	**178.0**	5.2	12.2	2.3	29.9	6.9	39.1	3.3	24.7	**118.3**	*313.8*
Ecolette	Eco 1	329.0	**492.8**	19.9	10.8	0.1	2.3	0.10	1.9	0.12	0.14	**5.3**	*528.8*
	Eco 2	195.8	**283.9**	8.8	10.1	0.1	1.4	0.11	0.98	0.15	0.30	**3.2**	*306.0*
Florina	Flo 1	98.1	**297.2**	13.1	8.0	5.6	51.7	15.0	69.0	5.4	49.3	**221.4**	*539.6*
	Flo 2	64.4	**214.4**	11.4	10.4	9.8	77.7	22.5	87.9	7.0	68.6	**325.0**	*561.3*

Cultivar	Abbrev.	Chlorogenic acid	**Total Hydroxycinnamic acids**	Total Dihydrochalcones	Total Flavonols	Catechin	Epicatechin	Procyanidin B1	Procyanidin B2	Procyanidin B5	Procyanidin C1	**Total Flavanols**	*Total Phenolics*
Regine	Regi 1	7.5	**395.1**	22.3	12.1	4.4	53.2	10.2	79.1	4.7	35.3	**212.7**	*642.2*
	Regi 2	216.3	**337.7**	16.0	15.6	4.1	46.6	9.7	66.6	3.5	33.5	**188.0**	*557.3*
Enterprise	Ent 1	268.8	**387.9**	12.2	6.9	10.1	29.7	13.1	30.4	1.8	13.1	**105.8**	*512.9*
	Ent 2	277.2	**375.6**	8.5	8.1	9.1	25.4	12.5	24.6	1.5	15.3	**98.4**	*490.6*
Goldstar	GoSt 1	91.2	**191.5**	10.0	15.8	4.9	32.0	18.2	63.9	3.3	29.9	**173.0**	*390.3*
	GoSt 2	90.1	**180.7**	9.0	17.1	4.6	33.1	21.9	81.4	5.6	41.0	**222.1**	*428.9*
Baujade	Bau 1	445.6	**747.0**	23.0	27.9	19.1	186.4	45.9	285.5	24.5	230.0	**955.8**	*1753.7*
	Bau 2	279.2	**450.2**	24.1	19.9	5.3	57.9	15.9	99.2	8.0	73.5	**317.4**	*811.6*
Goldrush	Gold 1	162.7	**268.8**	18.2	12.1	5.0	50.7	16.4	49.6	4.5	37.3	**185.6**	*484.7*
	Gold 2	134.8	**242.8**	14.2	15.3	2.9	24.3	5.6	17.1	2.4	10.5	**68.8**	*341.2*

and 49.7 mg/L (GG2) for dihydrochalcones and between 2.7 mg/L (SR2) and 27.9 mg/L (Bau1) for flavonols, according to literature (TREUTTER, 2001; KAHLE et al., 2005).

Marked differences in phenolic quantity were found between the cultivars and between the two harvest years (Tab. 2). In general, the total phenol concentrations tend to be higher in 2001 than compared to 2002. This was the case in 17 out of the 26 cultivars tested. Five cultivars even showed in 2002 less than 50 % of the concentration in 2001. These were Elstar Elshof, Jonagored, Braeburn Wellburn, Rosana and Baujade. In contrast to these environmentally sensitive cultivars, 9 varieties were not affected by the season and showed only a variation within a range of 10 %: Alkmene, Gala Galaxy, Santana, Ahra, Rubinola, Ariwa, Florina, Enterprise, and Goldstar.

Antioxidant capacity

ABTS-radical decolourisation assay

For tests concerning the scavenging activity towards ABTS-radicals, six apple juices were selected exhibiting extreme differences in their total phenol content. The order of antioxidant activity (Fig. 1) was Bau1 > Bau2 > JG1 > EE2 > BW2 > San1. This does not exactly represent the descending total phenolic patterns, but it fits to the descending order of flavanol amounts of the respective juices (Tab. 2). The calculated TEAC values (mmol/L Trolox) were listed in Tab. 3. In order to identify the contribution of individual phenolics to the antioxidant activity, several standards were tested. Their corresponding TEAC values are listed in Tab. 4. This supports the assumption that flavan-3-ols play a predominant role as antioxidants of the apple juices tested.

Ethylene formation from KMB by X/XOD

Apple juices are potent inhibitors of ethene formation from KMB in the X/XOD test. From a first approach with dilution series of 4 selected juices (Fig. 2), an assay concentration of 0.27 % (V/V) was deduced for further screening of the juices from all cultivars and harvest years (Tab. 1). The corresponding inhibition of ethene formation (in %) from KMB is shown in Fig. 3. The antioxidant activity correlates best with the amount of total flavanols ($r^2 = 0.64$) and procyanidins ($r^2 = 0.65$), followed by hydroxycinnamic acids ($r^2 = 0.57$) and total phenolics ($r^2 = 0.51$). None of the apple juices showed in the examined concentration an inhibition of the enzyme xanthine oxidase, as measured by HPLC of the produced amounts of uric acid and consumed amounts of xanthine (data not shown), compared to the control without apple juice. A series of pure standards and defined phenolic fractions were also tested in the X/XOD assay. Their IC_{50}-values were estimated (Tab. 5) and show the following order: catechin < epicatechin < procyanidin C1 < a polymeric fraction isolated from *Sorbus domestica* < procyanidin B2 < chlorogenic acid < rutin < mixture of C1, E4, B5, E-B5. The dihydrochalcone phloridzin did not show any inhibition. As for the ABTS system, the flavanols were found to be most effective. However, the correlation of the concentration of

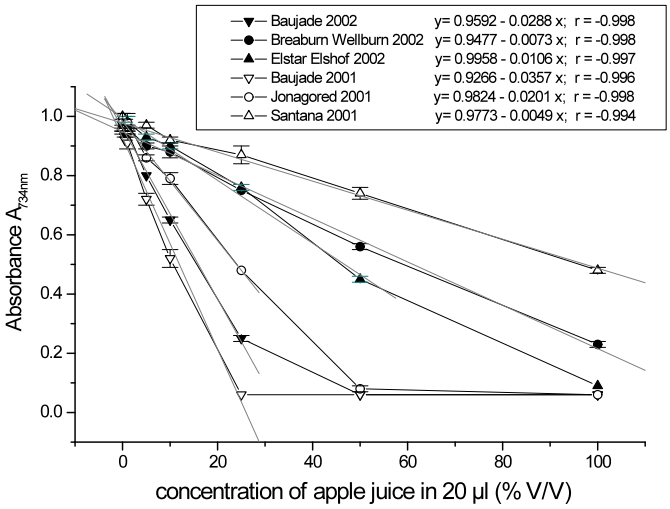

Fig. 1: Extent of reaction of ABTS-cation radicals with diluted apple juices. 20 µl of the appropriate dilution (as indicated on the x-axis) of the apple juice was placed to 980 µl of buffered ABTS-radicals (67 µM). After mixing, remaining absorbance was measured exactly after 5 min of reaction time.

Tab. 3: TEAC values (ABTS radical decolourisation) of selected apple juices.

Apple juice	TEAC (mmol/L)
Baujade 2001	13.3
Baujade 2002	10.8
Jonagored 2001	7.5
Elstar Elshof 2002	3.9
Braeburn Wellburn 2002	2.8
Santana 2001	1.9

Tab. 4: TEAC values (ABTS radical decolourisation) of selected standards.

Standard compound	TEAC (mmol/L)
catechin	3.9
rutin	3.6
procyanidin B2	3.3
procyanidin C1	2.0
chlorogenic acid	1.3

phenolic compounds in the juices and their corresponding antioxidant capacity is not really significant, showing only a tendency. This may be due to the non-linearity of the enzymatic test system in the range of concentrations used (Fig. 4). Another reason for the variation may be the contribution or interference of compounds, which were not quantified in the juices. To get a more detailed view of the contribution of phenolic compounds and their extent to the antioxidant capacity of apple juices, two approaches were performed. In the first experiment, a juice poor in phenolics was supplemented with standards. The second trial focused on the role of polymeric proanthocyanidins so far not detectable by the HPLC system used.

Tab. 5: IC_{50} values (X/XOD mediated ethene release from KMB) of apple juices and selected standards.

Apple juice	IC_{50} (% V/V)
Regunde 2001	0.4
Gerlinde 2002	0.6
Enterprise 2001	0.3
Baujade 2002	0.2
Standard compound	IC_{50} (µmol/L)
catechin	4.5
epicatechin	8.0
procyanidin C1	10.0
procyanidin B2	15.0
chlorogenic acid	16.0
rutin	19.0

Supplementation experiment

The apple juices of Elstar Elshof 2002 (EE2) and of Baujade 2002 (Bau 2) differed markedly (app. factor 10) in amounts of chlorogenic acid and of flavanols, represented by catechin (Tab. 6). In the X/XOD test final concentrations of 0.27% apple juice were used in the assay, which means that the difference between Bau 2 and EE 2 was 2.93 µM for chlorogenic acid and 7.43 µM for flavanols, respectively. To elucidate whether these components are the relevant ones responsible for the inhibition of ethene formation from KMB in the X/XOD test, the missing amounts of chlorogenic acid and/or the flavanol monomer catechin were added to EE2 (0.27%) in the assay. Fig. 5 illustrates the effects on the antioxidant performance in the X/XOD assay of EE2 by adapting the chlorogenic acid or the flavanol level or both of EE2 to the level of Bau2. Supplementation with chlorogenic acid alone improved the antioxidant activity only marginally, whereas addition of catechin highly increased antioxidant action of EE2. Surprisingly, chlorogenic acid was more active in combination with catechin. Although the activity of Bau1 could not be achieved completely by EE2 supplemented in this way, we can deduce from the

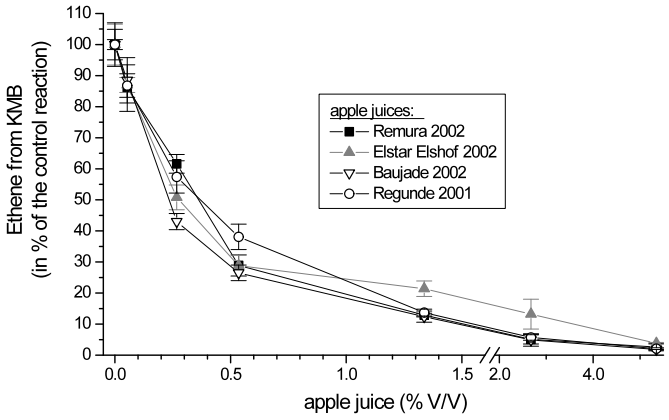

Fig. 2: Dose response plots of of apple juices determined in the X/XOD/KMB test. The higher the antioxidant action of the apple juice, the lower is the amount of liberated ethylene from KMB. In a final aqueous (phosphate buffered 100 mM, pH 7.4) reaction volume of 2.0 ml, xanthine 500 µM is converted into uric acid by 40 mU/ml XOD in gas tight sealed reaction tubes (12 ml). 1 ml gas aliquots of their headspace were quantified by GC. Ethylene is liberated from KMB via the superoxide assisted Fenton reaction, producing OH radicals. In the basic reaction without apple juice the amount of formed ethene from KMB was approx. 7 ± 0.35 nmol (=100%), accumulated in 30 min at 37 °C.

experiment that the flavanols of the apple juices are mainly responsible for the antioxidant activity in the X/XOD test. However, it should be noted that the catechin supplement may somewhat overestimate the contribution of flavanols to the antioxidant capacity of the juice, since catechin has a higher effect than epicatechin and its derivatives (Tab. 5) in this system.

Preparation and antioxidant activity of a polymeric fraction of proanthocyanidins

Due to their diversity (eluting as a broad unspecific increase of the baseline) and weak UV-absorbing properties, polymeric proanthocyanidins are not sufficiently separated and detected by HPLC (SHOJI et al., 2006). They react also only weakly with DMACA (TREUTTER, 1989). Butanol/HCl treatment in the heat (boiling for 20 min) greatly enhances photometric detection by conversion of the polymers into coloured anthocyanidins. The latter can be quantified by absorbance at 540 nm, expressed as cyanidin equivalents. We found some evidence that the difference between supplemented EE2 and Bau1 may be due to polymeric proanthocyanidins. To elucidate the contribution of polymeric procyanidins to antioxidant capacity, Bau2 apple juice (we assumed to contain significant amounts of polymeric proanthocyanidins) was separated into fractions by Sephadex column chromatography. The obtained fractions where analyzed by HPLC and by photometry after boiling in butanol/HCl. Antioxidant activity of the fractions was also determined in the X/XOD test. Three fractions were received by Sephadex column separation of Bau2. Fraction1, containing HCAs and catechins, exerted approximately 90% of the antioxidant activity of the original apple juice Bau2, as shown in Fig. 6. Oligomeric proanthocyanidins and catechins represent mainly fraction2 and showed lower but still significant antioxidant activity, followed by fraction3. However, no signals were detected by HPLC analysis of fraction3, although it was positive in the Butanol/HCl assay for proanthocyanidins (Tab. 7). So we can deduce from this experiment that fraction3 contains polymeric proanthocyanidins performing antioxidant activity. Thus, polymeric proanthocyanidins certainly contribute partly to the antioxidant performance of the apple juice in the X/XOD test.

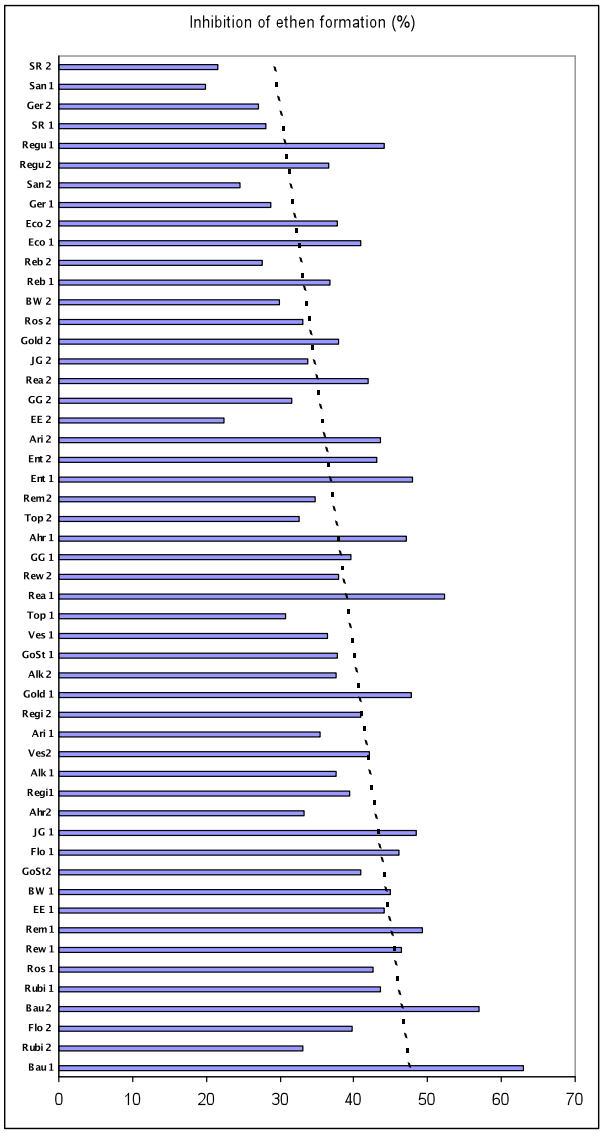

Fig. 3: Inhibition of ethene formation from KMB by apple juices (final concentration in the assay 0.27 % V/V) in the X/XOD test (100 % ethene formation from X/XOD/KMB: 8.5 ± 0.8 nmol). The samples are arranged in the order of increasing "Total Flavanol concentration". The dotted line marks the trend of increasing antioxidant activity with $r^2 = 0.65$.

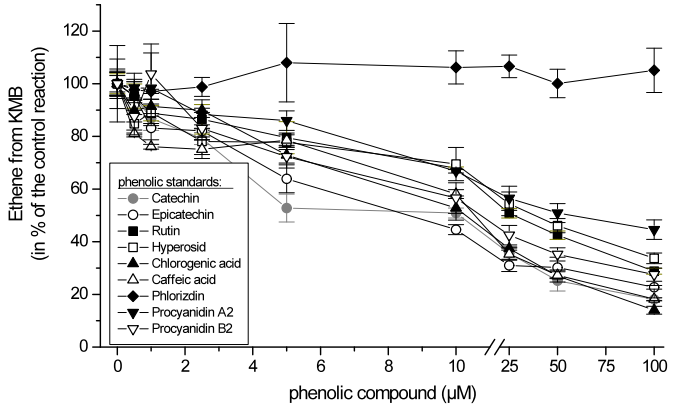

Fig. 4 : Dose response plots of standards determined in the X/XOD/KMB test. Conditions see captions of Fig. 2.

Tab. 6: Flavan-3-ol and chlorogenic acid content of apple juices from EE2 and Bau2

apple variety	in 100 % apple juice	
	chlorogenic acid (mM)	all flavanols, calculated as catechin (mM)
Bau2	1.27	3.28
EE2	0.10	0.31

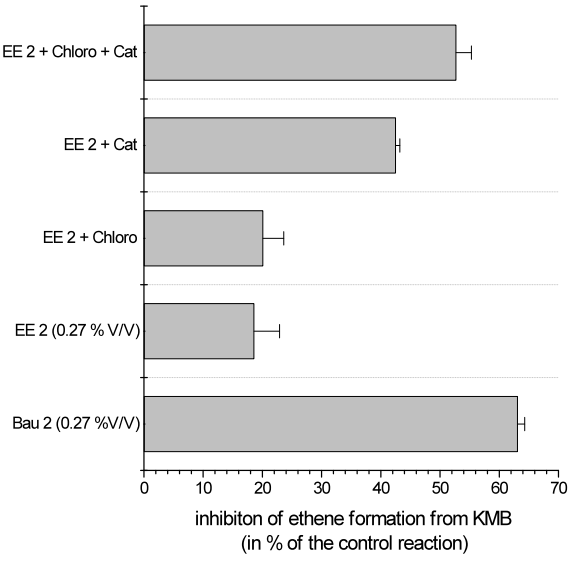

Fig. 5: Baujade 2002 (Bau2) and Elstar Elshof 2002 (EE2) were compared in the X/XOD/KMB test (apple juice: 0.27 %V/V final concentration) for their antioxidant activity. Whether their difference in antioxidant activity could be related to missing amounts of chlorogenic acid and catechins of EE2 (see Tab. 6) was checked by appropriate supplementation of EE2 with either chlorogenic acid or catechin or both.

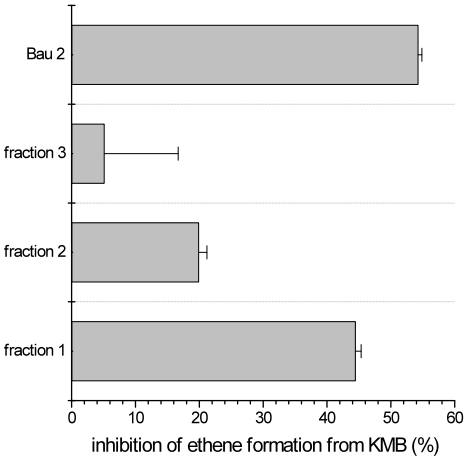

Fig. 6: Three fractions of the apple juice "Baujade 2002" (Bau2), received by Sephadex column chromatography, were analyzed in the X/XOD/KMB test. The antioxidant activity is plotted as inhibition of ethene formation from KMB (in % of the control reaction (100 %, producing 8.7 ± 0.4 nmol ethene) and compared to Bau2 (0.27 %V/V final concentration). Fraction1 mainly contained HCAs and catechins, fraction2 oligomeric catechins and flavanols whereas fraction3 consisted of polymeric flavanols. The fractions were also analyzed by HPLC and the Butanol/HCl method for proanthocyanidins (Tab. 7).

Tab. 7: Proanthocyanidin (PAC) content of Bau2 and corresponding sephadex fractions, estimated as cyanidin equivalents by absorbance at 540 nm after boiling in Butanol/HCl for 20 min

compound	PAC (mmol/L)
Bau2	0.83
fraction 1	0.41
fraction 2	0.29
fraction 3	0.15

Discussion

Apples are one of the major fruits frequently consumed in Germany both as fresh or processed fruits. By this, apples represent a major source of phenolics in human nutrition. Although it is commonly accepted that apples are good for health, the magic ingredient of apple has not be found so far. Nevertheless, the biological impact of apple, similar to that of other fruits, may be largely due to the presence of antioxidants, where phenolics are considered to be more important than vitamin C (EBERHARDT et al., 2000). In general, five major phenolic groups are found in various apple cultivars: hydroxycinnamic acids, flavan-3-ols (monomeric catechins and procyanidins), anthocyanins, flavonols, and dihydrochalcones. The extraction of phenolic compounds by a domestic juicer, as reported in this study, is not exhaustive. Therefore, the values do not show the absolute content of the fruits. However, the values obtained are in the range of those published for apple juices, as reviewed by TREUTTER (2001). During juice making (pressing, centrifugation) the easily extractable phenolic compounds from the fruit flesh are obtained whereas those in the apple peel surely remain in the residue. This is confirmed by the general phenolic profile in our juices, closely resembling those of apple fruit flesh with hydroxycinnamic acids and flavan-3-ols as the predominating groups (TSAO et al., 2003, 2005; VAN DER SLUIS, 2001). Seasonal sensitive or insensitive apples were also described by LATA et al. (2005). Seasonal related differences may be due to the variable light and temperature regimes. By that, at least apple skin phenolics (AWAD et al., 2000; AWAD and DE JAGER, 2002) were affected. This variability of concentrations of individual phenolics or groups of compounds in our study is not related to the resistance potential of the cultivars against the apple scab *Venturia inaequalis*, which could be speculated since phenolic compounds are involved in scab defence (TREUTTER et al., 1990; PICINELLI et al., 1995). Furthermore, there is no relationship of juice phenolics to fruit ripeness parameters, such as flesh firmness, starch degradation, soluble solids or acid content. That is remarkable because of the generally accepted dependency of biosynthesis on accumulation of phenylpropanoids and flavonoids to the availabiltity of carbohydrates, which was found for apple leaves (RUEHMANN and TREUTTER, 2003; STRISSEL et al., 2005; TREUTTER 2005). It is furthermore noticeable that early ripening varieties such as 'Alkmene' and 'Gerlinde' are not devoid of phenolics. Despite of their short periods of fruit development, the partitioning of primary metabolites towards the secondary metabolism is sufficient for the accumulation of phenolics in the fruits of the respective cultivars. It may be assumed that there are independent genetic and environmentally affected regulators of phenolic metabolism. This assumption is confirmed by the observation that some cultivars show big differences between the two years whilst others exhibit similar phenolic patterns when comparing the two harvest years 2001 and 2002. The weather conditions of the two years with respect to temperature, rainfall and sun hours showed only slight differences.

LEE et al. (2003) investigated the contribution of phenolic compounds to apple antioxidant capacity using methanol extracts and the ABTS assay. In their study, the quercetin glucosides had the highest proportion, both in amount and in antioxidant activity, followed by epicatechin and procyanidin B2. The dihydrochalcone phloretin and chlorogenic acid showed comparably weak activities. In another study (IMEH et al., 2002), apple fruits from 31 varieties were harvested from the same growing area, the same age and the same stage of ripeness in order to compare vitamin C content, phenol content and their relation to the antioxidant activity (ABTS assay). They found that antioxidant activity correlated to total phenols better as measured by the Folin-Ciocalteu test ($r^2 = 0.5331$) than with the phenol content quantified by HPLC ($r^2 = 0.30$). This is not surprising, because the Folin test is by itself a kind of antioxidant test system, utilising the reduction potential of polyphenolics and other compounds in a chemical reaction. In our study we found the best correlation for the flavan-3-ol and procyanidin content ($r^2 = 0.65$) of the investigated apple juices, concerning antioxidant activity in the XOD test. Total phenol content by HPLC correlated to antioxidant activity only with $r^2 = 0.51$. In a study on four apple cultivars van der SLUIS et al. (2001) found the highest antioxidant activity in the cultivar showing the highest flavonoid concentration. Nevertheless, no general correlation could be found between flavonoid concentration and antioxidant capacity. In a further analysis of 9 apple cultivars (IMEH et al., 2002), differences in their antioxidant acitivity were shown in the FRAP test (ferric reducing antioxidant power – this test is rather strange, because in vivo reduction of free ferric ions is correlated to oxidative stress. Thus, metal ions are kept safely bound to proteins, and if free, the ferroxidase caeruloplasmin prevents Fe^{2+} mediated Fenton reactions). Again, neither convincing relationships to total phenols (Folin-Ciocalteu-assay) nor to free and conjugated phenol fractions could be established. This is consistent with our findings. However, our experiments clearly show that flavan-3-ols contributed most to the antioxidant potential of the tested apple juices, whereas chlorogenic acid, dominating quantitatively, did only play a subordinate role. This is contradictory to the proposal of MILLER and RICE-EVANS (1997), who stated that chlorogenic acid and phloridzin are important antioxidants in apple juices. Thus, the test system applied to measure antioxidant activity is of importance for the derived results.

In this study, two model systems have been selected for the measurement of antioxidant activity of apple juices. On the one hand, the popular and simple TEAC assay (TEAC = Trolox equivalent antioxidant concentration) was used, quantifying the decrease of preformed dark blue green ABTS cation radicals, induced by antioxidant solutions as scavengers and/or reducing agents. Thereby ABTS radicals are converted into colourless products (ABTS and others). The advantage of the assay is the good differentiation between phenols, which is certainly based on the restricted reactivity of the ABTS radical, attributing it a weak oxidant. Therefore, we applied on the other hand oxidation of xanthine (X) by xanthine oxidase (XOD) as a source of reactive oxygen species (ROS: superoxide, hydrogen peroxide, OH radical), which is of relevance in vivo. Furthermore, the ability to decrease XOD activity, an oxidative stress related enzyme, could also be checked in this system as a possible mode of antioxidant action. The number of adjacent OH-groups is one of the important determinants of antioxidant activity, as observed by BORS and MICHEL (2002). One critical structural constraint for optimal antioxidant potential is the presence of a catechol group (BORS and MICHEL, 2002). Structure function relationships are not always working, as for example in the ABTS assay: p-coumaric acid (RICE-EVANS et al., 1996), with only one phenolic OH-group, is a much stronger antioxidant than caffeic acid (with the catechol group). Moreover, activity of the caffeic catecholic group is enhanced by methoxylation (ferulic acid). Ascorbic acid (enediol structure) is surely underestimated in the ABTS assay, performing like Trolox with only one phenolic group. Thus, besides the reduction potential, dimerization and disproportionation reactions of the oxidized antioxidants play a crucial role, superimposed by the stereochemical affinity of reductant (antioxidant) and free radical,

defining reaction kinetics in the in vitro test systems. Nevertheless, the main apple phenolics chlorogenic acid and the derivatives of epicatechin and quercetin show the structural feature of the catecholic group. Other prerequisites for flavonoids are a 2,3-double bond and 3- and 5-OH-groups adjacent to the 4-keto-structure (BORS and MICHEL, 2002; RICE-EVANS et al., 1996). This is fulfilled by apple flavonols. However, their concentrations are quite low in the juices. Thus, they do not substantially contribute to the antioxidant potential of apple juices.

The results of our study indicate the flavan-3-ols and chlorogenic acid as the major antioxidants in apple juice. Compared to the fresh apple fruits (skin), flavonols (especially quercetin and its glycosylated derivatives) play a minor role in apple juices due to their low content. Antioxidant activity of apple juices varied as well as the content of phenols varied in quantity, but no strong correlation could be established. However, especially the catechins and procyanidins were found to be highly active in both in vitro test systems, ABTS decolorization and the X/XOD catalyzed formation of ethylene from KMB. As reported by EBERHARDT et al. (2000), the prominent Vitamin C (ascorbic acid) is only a minor contributor to the antioxidant activity of apple juice, where the "bioactive" polyphenols play the major role, probably exhibiting health promoting effects. From the results of this study it cannot be decided which apple variety is the most recommendable for the consumer. The antioxidant effect in vitro gives a general insight into the capacity a fruit has. However, this represents only a small portion of its health beneficiary effect since the transcriptional regulation of polyphenols may additionally stimulate further antioxidant systems in the human body (VALCO et al. 2007; JUNG et al., 2009; UPADHYAY and DIXIT, 2015

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