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Chemical quality parameters and anthocyanin pattern of red-fleshed Weirouge apples

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Summary

Red-fleshed 'Weirouge' apples were investigated with respect to their chemical quality parameters including anthocyanins and colour. Anthocyanin concentrations were considerably higher than previously reported for common red-peeled apples. Due to its high malic acid content, the cultivar 'Weirouge' was characterised by a high colour brilliance. Among the anthocyanins, cyanidin-3-maloyl-galactoside and 5-carboxy-pyrano-cyanidin-hexoside not previously found in apples were tentatively identified by HPLC-MS^3. Highest anthocyanin and total phenolics contents were found in the peel corresponding with the respective antioxidant capacities as determined using the FRAP and TEAC assays, respectively.

Introduction

Not only in Europe, apples are one of the most frequently consumed fruits, second most valued after oranges and bananas with respect to health-promoting plant secondary substances (CHUN et al., 2005; VINSON et al., 2001). While it is well established that an increased consumption of fruit and vegetables improves overall well-being, it is both taste and colour that triggers consumers' purchase decision. Whereas apples with red peel are quite common, red-fleshed apples have still not received much attention. It is because of their remarkable anthocyanin content that red-fleshed apples such as 'Purpurroter Cousin', 'Pink Pearl' and also quite recently 'Weirouge' are receiving increasing attention (BUCHTER-WEISBRODT, 2003). In addition to their attractive red colour, pigment degradation and oxidation was reported to be low and assumed to be due to their high acidity (BUCHTER-WEISBRODT, 2003). Moreover, there is a rapidly increasing interest for potential crops for colouring food naturally (STINTZING and CARLE, 2004). Hence, coloured fruits and vegetables are attractive for both fresh fruit consumption and juice processing.

'Weirouge' apples represent the most intensely coloured German cultivar and was bred in Weihenstephan (Germany) primarily for processing purposes and officially registered 1997. However, little is known about its chemical composition. Therefore, the present study aimed to thoroughly investigate colour properties, anthocyanin content and further chemical quality parameters of the peel, the flesh and the whole edible portion of 'Weirouge' apples. The anthocyanin profile was also characterised in detail by HPLC-DAD and mass spectrometric (MS) analyses. Finally, the antioxidant potential was assessed in aqueous extracts by the FRAP and TEAC assays.

Materials and methods

Plant material and extraction

Red-fleshed 'Weirouge' apples from 2005 (Stoppel, Kressbronn, Germany) were washed, the peel dried and the core and stem removed to obtain the edible part of the fruit. At least five apples were separated into flesh and peel, while further fruits were not peeled. After slicing peels, flesh or unpeeled fruits into segments, the samples were immediately frozen in liquid nitrogen and comminuted to obtain a fine powder which was frozen at -80°C until analyses. Exactly five grams of each sample were extracted with aqueous acetone (pH 1 water acidified with trifluoroacetic acid / acetone, 30/70, *v/v*) at a ratio of 1 part solid sample and 2 parts extraction medium. After 60 min extraction under continuous stirring, the solution was passed through a Buchner funnel with a filtering paper (Schleicher-Schuell, Dassel, Germany). The so-obtained filtrate was concentrated *in vacuo* at 28°C until dry and re-dissolved in 5 mL of purified water. The resulting extracts were used for the determination of the total anthocyanin content, the browning index and for objective colour assessment (see below) using a UV-Vis spectrophotometer equipped with UV-WinLab and colour software (Wincol, Perkin-Elmer, Überlingen, Germany). For the assessment of antioxidant capacities, total phenolics content and chemical quality parameters (see below), the samples were extracted with purified water and passed through a filtering paper. Each extraction was repeated twice and the resulting extracts were analysed in duplicate. For the assignment of unknown anthocyanins, the scales from red onions (*Allium cepa* L.) and the peel from blue-coloured Japanese plums (*Prunus salicina* Lindl.) obtained from a local market were homogenised and extracted with aqueous acetone as described above. Aqueous extracts were used for coinjection experiments and mass spectrometric analyses, respectively.

Antioxidant capacity

The clear filtrates used for the TEAC (Trolox Equivalent Antioxidant Capacity; VAN DEN BERG et al., 1999) and FRAP assays (Ferric Reducing Capacity Antioxidant Power; BENZIE and STRAIN, 1996), respectively. For calibration purposes, the water soluble vitamin E analogue Trolox (Trolox-equivalents) as well as L-ascorbic acid (vitamin C-equivalents) according to KIM and LEE (2004) were used.

Total phenolics content

Total phenolics content were assessed by the Folin-Ciocalteu method (SINGLETON et al., 1999) and the results were expressed either as gallic acid or L-ascorbic acid equivalents.

Chemical quality parameters

Sucrose, glucose, fructose, sorbitol, ascorbic acid, citric acid and malic acid were quantified using enzymatic test kits (r-biopharm, Darmstadt, Germany). Proline and the formol value were determined according to IFU-Methods No. 49 (1983) and 30 (1984), respectively. Total titratable acids were assessed by titration with 0.25 M NaOH until pH 8.1 was reached and expressed as malic acid.

Anthocyanin content, browning index, and objective colour

All samples were analysed in duplicate by UV-Vis spectrophotometry (Perkin Elmer, Überlingen, Germany). Based on absorption measurements covering the range from 380 to 780 nm, objective colour measurements (CIEL*a*b*) were carried out in McIlvaine buffer solutions at pH 3.5. Chromaticity C* [$C^* = (a^{*2}+b^{*2})^{1/2}$] und hue angle h° [$h^\circ = \arctan(b^*/a^*)$] were calculated from a*- and b*-values at D_{65} and an observer angle of 10°. The browning index was assessed

according to GIUSTI and WROLSTAD (2005). Total anthocyanin content determination was based on a pH-differential method and expressed as cyanidin-3-glucoside equivalents (GIUSTI and WROLSTAD, 2005) according to the following formula: c[mg/L]=A*MW*DF/ε_M*d, with A = absorption value, MW = molecular weight of cyanidin 3-glucoside [448 g/mol], DF = dilution factor, ε_M = molar extinction coefficient of cyanidin 3-glucoside at pH 1 [29600 L/mol*cm], and d = pathlength of the cuvette [1 cm]. The contents of individual anthocyanins were calculated considering the relative peak area ratios of the chromatogram at 520 nm after their identification by HPLC-DAD-MS[3].

Anthocyanin pigment assessment by HPLC-DAD and LC-MS^n

Using a Merck LaChrom Elite HPLC System (Merck-Hitachi, Darmstadt, Germany) equipped with an autosampler L-2200, a pump L-2130, a diode array detector L-2450, and a JetStream column oven, anthocyanins were separated on an analytical C18 Sunfire column (250x4.6 mm, 5µm; Waters, Wexford, Ireland) equipped with a C18 pre-column (4 x 3.0 mm i.d., Phenomenex, Torrance, CA, USA) at a constant temperature of 25°C and a flow rate of 1 mL/min. Eluent A was 5% aqueous formic acid, 100% acetonitrile was used as B. Starting isocratically with 100% A for 5 min, linear gradients were followed to 10% B in 20 min, 13% at 40 min, 20% at 44 min, 25% at 50 min, and finally 100% B at 55 min before re-equilibration to initial conditions. Monitoring was performed at 520 nm.

Using the same method, mass spectrometric analyses were carried out on an Agilent Series 1100 HPLC system (Agilent, Waldbronn, Germany) interfaced with a Bruker Model Esquire 3000+ ion trap mass spectro-meter (Bruker, Bremen, Germany) operating in the positive ionisation mode.

Results and discussion

Chemical quality parameters

Chemical quality parameters of the whole edible part, the peel and flesh fractions of red-fleshed 'Weirouge' apples are compiled in Tab. 1. Literature values for apples (SCHERZ and SENSER, 2000) are also listed for comparison.

Glucose, fructose and sorbitol contents are quite low. In contrast, sucrose contents are higher than expected from the literature pointing to a lower invertase activity in 'Weirouge' apples. Also ascorbic acid and proline fell behind the reference data. Since the formol value of 14 exceeded the AIJN value for apple juice ranging from 3 to 10, (AIJN, 1996), proline can be considered a minor free amino acid in 'Weirouge' as reported for other apple varieties (SCHERZ and SENSER, 2000). The high malic acid content is worth noting because literature values were exceeded by 200 %. On the other hand, citric acid concentrations were in the mean range. Exhibiting a sugar acid ratio of 7:1, 'Weirouge' is considered a fairly acidic apple variety (Tab. 1).

Tab. 1: Chemical quality parameters of 'Weirouge' apples [mg/100 g edible portion] [a]

Chemical Quality Parameter	'Weirouge' apple	Apples [b]
Glucose	527 ± 16	1400-2350
Fructose	4255 ± 60	4800-6400
Sucrose	3755 ± 33	540-2780
Sorbitol	324 ± 21	510-580
Ascorbic acid	2.2 ± 0.00	3-25
Citric acid	16.8 ± 0.00	9-30
Malic acid	1271 ± 2.0	270-790
Total titratable acids [as malic acid]	1190 ± 0.0	-[c]
Proline	1.02 ± 0.00	10
Formol value [mL 0.1mol NaOH/100 g]	14.0 ± 0.34	-[c]
Sugar-acid-ratio[d]	7.1	-[c]

[a] mean ± standard deviation
[b] according to SCHERZ and SENSER (2000)
[c] no data available
[d] (glucose + fructose + sucrose)/(citric acid + malic acid)

Colour properties

A browning index of only 4-5% pointed to a low degree of oxidation products in 'Weirouge'. At pH 3.5, the typical pH for a broad range of foods, the L*-, C*-, and h°-values of the peel and flesh only slightly differed. The colour purity C* und the tonality h° were highest in flesh, followed by unpeeled apples and peel fractions, respectively (Tab. 2).

Since colour stability of 'Weirouge' has previously been reported to be high (BUCHTER-WEISBRODT, 2003), it should be examined whether this was due to the high acidity, to the high pigment concentrations or rather to a specific pigment pattern.

Anthocyanin content and pigment pattern

For this purpose, the total anthocyanin content was determined separately in flesh, peel and unpeeled apple samples (Tab. 2) on a weight basis. The anthocyanin content of the peel was 2.5 times higher than in the flesh. It needs to be considered that besides the core amounting to 14.7 %, the edible part consisted of 10.1 % and 75.2 % peel and flesh, respectively. Total anthocyanin yield of the edible fractions reached 10.8 mg/100 g fresh weight thus being in the same range as reported previously for red-fleshed 'Scugog' apples (*Malus pumila* var. *niedzwetzkyana*) with 10.0 mg/100 g (MAZZA and VELIOGLU, 1992). In contrast, anthocyanin concentration was found to be considerably lower in red-peeled apples ranging from 0.2 to 0.8 mg/100 g (VAN DER SLUIS et al., 2001). The pigment contents in 'Weirouge' apples were comparable to those of strawberries (15-35 mg/100 g), red currants (20-60 mg/100 g), red onions (7-21 mg/100 g), and plums (2-25 mg/100 g) (GIUSTI and WROLSTAD, 2005).

Tab. 2: Colour characteristics and anthocyanin contents of 'Weirouge' apples [a]

Parameter	apple with peel	apple flesh	apple peel
Anthocyanin content [mg/100 g]	10.8 ± 0.02	8.1 ± 0.19	20.6 ± 0.57
Browning index [%]	4.3 ± 0.23	4.9 ± 0.85	4.6 ± 0.35
L* (pH 3.5)	74.9 ± 0.52	74.0 ± 0.57	76.1 ± 1.31
C* (pH 3.5)	41.8 ± 1.06	44.4 ± 0.42	39.4 ± 2.89
h° (pH 3.5)	16.5 ± 0.30	18.7 ± 0.66	15.9 ± 0.70

[a] mean ± standard deviation

According to TIMBERLAKE and BRIDLE (1971), cyanidin-3-galactoside is considered the major compound of all apple varieties, followed by cyanidin-3-glucoside, cyanidin-3-arabinoside and cyanidin-3-xyloside. In addition, the authors gave preliminary evidence of acylated structures the nature of which remained obscure. Except for the acylated anthocyanins, those data were later confirmed by MAZZA and VELIOGLU (1992) in red-fleshed and by VRHOSEK et al. (2004) in red-peeled apples, respectively. The predominant anthocyanin of red-fleshed 'Scugog' (*Malus pumila*) apples was cyanidin-3-galactoside (39%), followed by cyanidin-3-glucoside (27%), cyanidin-3-arabinoside (23%), and cyanidin-3-xyloside (11%), respectively (MAZZA and VELIOGLU, 1992). In contrast, the relative amounts of cyanidin-3-glucoside and cyanidin-3-galactoside were found to differ significantly in red-fleshed 'Weirouge' apples as shown in Tab. 3, being similar to those from *M. domestica* varieties. It is thus suspected that fruits from *M. pumila* should be easily distinguishable from those of *M. domestica* by the ratio of cyanidin-3-galactoside and cyanidin-3-glucoside, respectively.

In agreement with earlier reports on both red-fleshed and red-peeled fruits (MAZZA and VELIOGLU, 1992; TIMBERLAKE and BRIDLE, 1971; VRHOSEK et al., 2004; WU and PRIOR 2005a), the dominating anthocyanin in 'Weirouge' apples was cyanidin-3-galactoside (**1**) accompanied by cyanidin-3-glucoside (**2**), cyanidin-3-arabinoside (**4**), cyanidin-7-arabinoside (**8**), cyanidin-3-xyloside (**9**) and peonidin-3-galactoside (**5**) which were assigned by comparison of specific mass spectrometric, UV-Vis and retention time data from the literature (WU and PRIOR, 2005a,b) and an own data bank (Tab. 3, Fig. 1). Additionally, cyanidin-3-maloyl-galactoside (**6**) was found in apple for the first time. Furthermore, a cyanidin structure with a pentose moiety (**10**) and a cyanidin structure (**3**) with an identical mass as (**6**) were detected. Due to its short retention time and specific fragmentation pattern, (**3**) was assigned to a cyanidin-diglycosidic structure. While the presence of cyanidin (**11**) in fruits has earlier been considered an artefact of sample clean-up, anthocyanidins have also been reported as genuine compounds from beans only recently (MACZ-POP et al., 2006). It is worth mentioning that the predominance of malic acid is also reflected by the acylation pattern. A similar relationship was also found to apply to blackberry being rich in oxalic acid (SCHERZ and SENSER, 2000a) and exhibiting an oxalyl-anthocyanin at the same time (STINTZING et al., 2002). For closer assignment of the maloyl-derivative, extracts from blue-peeled plums (WU and PRIOR, 2005a) previously reported to contain cyanidin-3-maloylglucoside were analysed by HPLC-DAD-MS^3. Cyanidin-3-maloyl-glucoside was detected at a retention time of 32.6 min with an absorption maximum at 520 nm. Its specific mass spectral data matched exactly those of (**6**). Due to its higher polarity, (**6**) was tentatively assigned as cyanidin-3-maloyl-galactoside. From a biosynthetic standpoint, the positive quantitative correlation between the glycosides and the respective maloyl-derivative as reported previously (WU and PRIOR, 2005a) is plausible. Interestingly, a further compound (**7**) exhibiting identical spectroscopic properties as reported for 5-carboxy-pyranocyanidin-glucoside in red onion scales (FOSSEN and ANDERSEN, 2003) was detected. However, closer assignment and clarification if this was the glucoside or the galactoside derivative was impossible since freshly prepared red onion extracts were devoid of this compound (data not shown). Such pyranoanthocyanidin-type structures have first been found in wines (e.g., FULCRAND et al., 1996; BAKKER et al., 1997), later in fruit juices (HILLEBRAND et al., 2004; REIN et al., 2005; SCHWARZ et al., 2004) and only very recently as genuine fruit compounds (ANDERSEN et al., 2005; FOSSEN and ANDERSEN, 2003).

Total phenolics content and antioxidant capacity

The antioxidant capacities of aqueous extracts from red-fleshed apples with peel, flesh and peel fractions, respectively, are shown in Tab. 4. Following the same trend, absolute values for FRAP and TEAC varied considerably. This can be ascribed to different assay conditions and diverse reaction mechanisms as previously reported (PRIOR and CAO, 1999; HALVORSEN et al., 2002; PRIOR et al., 2005; RECHNER et al., 1997). The peel exhibited the highest values and the lowest activities were found in the flesh. These findings reflect an accumulation of UV-absorbing colourless and coloured phenolics in the peel substantiating their photoprotective effect (EDREVA, 2005). Considering the suggestion

Tab. 3: HPLC-DAD and mass spectrometric data for anthocyanins from 'Weirouge' apples

	Anthocyanin[a]	R_t	$\lambda_{vis\text{-}max}$	A_{440} / $A_{vis\text{-}max}$	*m/z*	MS^2 *m/z*	MS^3 *m/z*	Anthocyanin contents [mg/kg]		
		[min]	[nm]	[%]	$[M]^+$	$[M]^+$	$[M]^+$	unpeeled apple	apple flesh	apple peel
1	Cyd-3-gal	25.6	516	34	449	287	287	92.57	67.65	171.91
2	Cyd-3-glc	27.2	515	37	449	287	287	0.65	0.57	1.32
3	Cyd-pent-rha[c]	28.6	517	37	565, 419	287	-[b]	0.58	0.66	0.99
4	Cyd-3-ara	28.8	516	33	419	287	287	2.41	1.53	5.48
5	Peo-3-gal	29.7	-[b]	-[b]	463	301	286	0.15	0.18	0.36
6	Cyd-3-maloyl-gal[c]	31.3	514	48	565, 449	287	-[b]	0.43	0.44	0.92
7	5-Carboxy-Pyrano-cyd-hex[c]	32.6	503	45	517	355	355, 327	0.88	0.89	1.83
8	Cyd-7-ara	34.2	516	36	419	287	287, 217	1.83	1.28	6.13
9	Cyd-3-xyl	35.7	517	33	419	287	287	8.04	7.31	13.79
10	Cyd-pent	37.3	514	34	419	287	287	0.28	0.22	1.70
11	Cyanidin	41.4	522	36	287	287	-[b]	0.67	0.23	1.48

[a] Cyd: cyanidin; Peo: peonidin; gal: galactoside; glc: glucoside; ara: arabinoside; rha: rhamnosid; xyl: xyloside; hex: hexosid; pent: pentosid
[b] not detectable
[c] tentatively identified

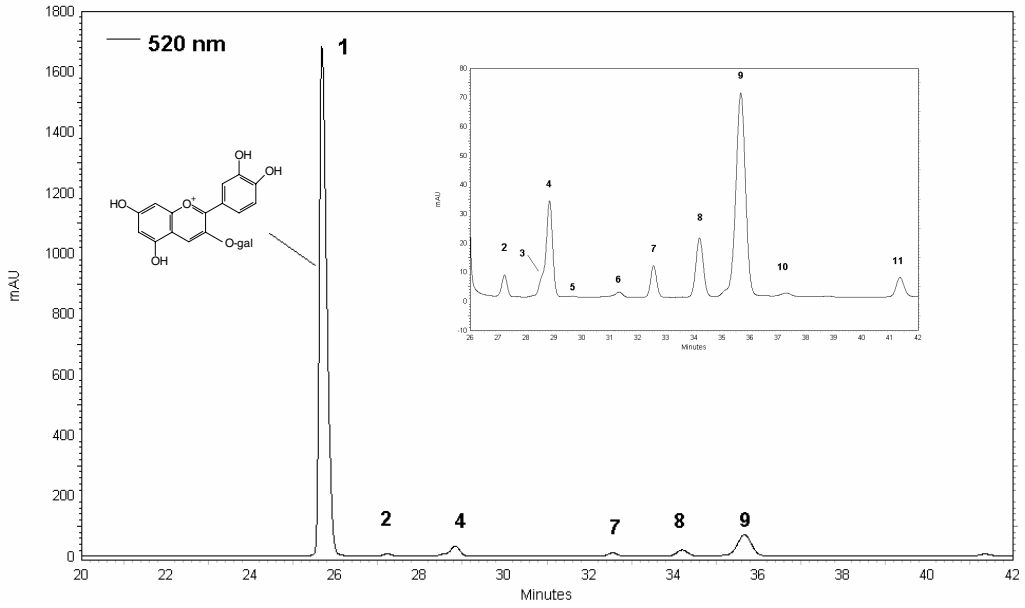

Fig. 1: Chromatogram obtained from unpeeled red fleshed 'Weirouge' apples (Peak assignment is given in Tab. 3).

of KIM and LEE (2004) and CHUN et al. (2005) the values were expressed as vitamin C-equivalents. The latter always exhibited lower values than their coresponding Trolox-equivalents. HALVORSEN et al. (2001) reported FRAP-values for 'Golden Delicious', 'Granny Smith' and 'Gala' varieties amounting to 0.15 mmol/100 g, 0.51 mmol/100 g, 0.22 mmol/100 g in the edible fruit part. In the present study a value of 0.24 mmol the Trolox-equivalent per 100 g edible portion was found for 'Weirouge'. These differences may be ascribed to the specific phenolic profiles and their particular concentration governed by season, climatic conditions and intraspecific variations (VAN DER SLUIS et al., 2001).

The phenolic compound profile in different apple varieties has been frequently investigated, showing a high variability both from a quantitative and a qualitative perspective (KELLER et al., 2001; MÜLLER and TREUTTER, 2001; RITTER and DIETRICH, 1996; TREUTTER, 2001; TSAO et al., 2003; VAN DER SLUIS et al., 2001; VRHOSEK et al., 2004). Benzoic- and hydroxycinnamic acid derivatives, dihydrochalcones, flavonols, flavan-3-ols, catechin and proanthocyanidin derivatives were detected. The phenolic profile of 'Weirouge' apples has already been well documented with chlorogenic acid (38.02 mg/L), phloretin-2-xylosylglucoside (10.48 mg/L), *p*-coumaroylglucoside (7.29 mg/L) and phloridzin (7.21 mg/L) constituting the major compounds in the juice (MÜLLER and TREUTTER, 2001). Hence, in the present study individual phenolics were not assessed. Instead, total phenolics were quantified by the Folin-Ciocalteu method, considering the contribution of ascorbic acid. Values both expressed as gallic acid and vitamin C-equivalents, respectively, are shown in Tab. 4. The highest antioxidant potential was found in the peel paralleling total phenolics and anthocyanin contents. TSAO et al. (2003) reported comparable gallic acid equivalents of 2012 mg/kg for 'Red Delicious' peel. According to CHUN et al. (2005), the total phenolics content amounted to 118 mg gallic acid per 100 g surmounting those of 'Weirouge' with only 54 mg/100 g. By analogy, values were higher for the vitamin C-equivalent antioxidant capacity of 205 mg/100 g compared to 89 mg/100 g in 'Weirouge' corroborating the well-known positive correlation of phenolics content and antioxidant potential (CHUN et al., 2005; RECHNER et al., 1997; VINSON et al., 1998, 2001).

Conclusion

The present study reports on chemical quality parameters of red-fleshed 'Weirouge' apples including their anthocyanin profile. These data together with previously published phenolic compound patterns

Tab. 4: Antioxidant capacity [mg/kg] of edible part fractions from 'Weirouge' apples (n=6)[a]

Parameter	unpeeled apple	apple flesh	apple peel
TEAC [Trolox-equivalent]	1463 ± 179	911 ± 83	4879 ± 522
FRAP [Trolox-equivalent]	599 ± 63	374 ± 82	1772 ± 237
TEAC [Vitamin C-equivalent]	888 ± 109	563 ± 51	3004 ± 317
FRAP [Vitamin C-equivalent]	403 ± 39	243 ± 56	1166 ± 170
Total phenolics [Gallic acid-equivalent]	538 ± 42 [b]	379 ± 22	1684 ± 184
Total phenolics [Vitamin C-equivalent]	784 ± 59 [b]	540 ± 28	2385 ± 248

[a] mean value ± standard deviation
[b] corrected by the ascorbic acid content of 22 mg

allow to classify red-fleshed apples in comparison to common non-anthocyanic fleshed fruits. Anthocyanins hitherto unknown for apple cultivars could be identified by mass spectrometric detection. The ratio of cyanidin-3-galactoside and cyanidin-3-glucoside is suggested as a tool to differentiate *Malus pumila* from *M. domestica* varieties. The total anthocyanin contents in 'Weirouge' was more than ten times higher compared to other apples. The low browning index and high colour brilliance and stability cannot be ascribed to a particular pigment pattern but rather to the high acidity of the fruit stabilising anthocyanin colour (STINTZING et al., 2002b). In summary, both from a nutritional and technological point of view, red-fleshed apples deserve due attention.

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