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Identification of galloylated propelargonidins and procyanidins in buckwheat grain and quantification of rutin and flavanols from homostylous hybrids originating from *F. esculentum* × *F. homotropicum*

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Abstract

The flavonoid rich grain of buckwheat (*Fagopyrum esculentum* Moench, Fam. Polygonaceae) is of high nutritional value. With the aim to improve its agronomic productivity, cultivars were crossed with the wild species *F. homotropicum* which, however, differs in its flavonoid content. The intention of this work was to determine the flavonoid composition in developed interspecific hybrids and to elucidate the proanthocyanidin structures. Seven compounds were purified from methanol extracts of buckwheat (*Fagopyrum esculentum* Moench) grains by Sephadex LH-20 column chromatography. Beside the procyanidin epicatechin-[4–8]-epicatechin-3-*O*-(3,4)-dimethyl-gallate the following propelargonidins were identified: epiafzelechin-[4–6]-epicatechin, epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin, epiafzelechin-[4–8]-epicatechin-3-*O*-(3,4-dimethyl)-gallate, epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin-3-*O*-(3,4-dimethyl)-gallate, epiafzelechin-[4–8]-epicatechin-3-*O*-4-methyl-gallate and epiafzelechin-[4–8]-epicatechin-*p*-OH-benzoate on the basis of HPLC and LC–MS/MS.

Keywords: *Fagopyrum esculentum* Moench; Identification; Acylated proanthocyanidins; Epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin; Epiafzelechin-[4–6]-epicatechin; Epicatechin-[4–8]-epicatechin-*O*-(3,4-dimethyl)-gallate; Epiafzelechin-[4–8]-epicatechin-*O*-(3,4-dimethyl)-gallate; Epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin-*O*-(3,4-dimethyl)-gallate

1. Introduction

The grain of buckwheat (*Fagopyrum esculentum* Moench, Fam. Polygonaceae) is of high nutritional value and contains a favourable amino acid composition with high levels of lysine, arginine, aspartic acid and tryptophan, D-*chiro*-inositol and other bioactive compounds (Campbell, 1997; Wang et al., 2004; Franke, 1989; Reichling and Horz, 1993; Kötter, 1998). The flavonol glycoside rutin represents the most important bioactive compound which occurs rarely in plants in such high concentrations of up to 8% dry weight in the vegetative parts and up to 1% in the grain (Hagels, 1996). Buckwheat leaves are used as pharmaceutical drug against leg oedema and chronic venous insufficiency because of the antioxidative, anti-inflammatory and anticarcinogenic effect of rutin and its ability to reduce fragility of blood vessels (Ihme et al., 1996; Oomah and Mazza, 1996). Another phenolic class in the grain are the flavanols. Former work on the identification of flavanols in buckwheat seed executed by Watanabe (1998) and Quettier-Deleu et al. (2000) indicated the presence of catechin, epicatechin, epicatechingallate, the procyanidins B2 and B5, (+)-catechin-7-*O*-ß-D-glucopyranoside, (−)-epicatechin-3-*O*-*p*-hydroxybenzoate and (−)-epicatechin-3-*O*-(3,4-

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dimethyl) gallate. It is known that also flavanols possess health-promoting effects, such as antioxidant, anticarcinogenic and anti-inflammatory activity (Kada et al., 1985; da Silva et al., 1991; Mukoda et al., 2001; Natella et al., 2002; Santos-Buelga and Scalbert, 2000; Sun and Ho, 2005; Zern and Fernandez, 2005).

It is for these health reasons that in Japan buckwheat is recognized as a valuable source of so called "functional" food. The consumption of buckwheat surmount the own production and Japan imports buckwheat in great quantities. However, a disadvantage for mass production is the self-incompatibility of the heterostylic flowers which prevents the increase of seed yield and common buckwheat production (Dietrich-Szostak and Olezek, 1999; Wang et al., 2004; Wijngaard and Arendt, 2006). *F. homotropicum*, a wild species collected from China by Ohnishi, 1991, possesses self-compatibility and provides the possibility for improvement of productivity through interspecific hybridization. The quantities of rutin and flavanols, however, differ markedly between the two species. The intention of this work was to determine the content of flavonoids in these newly developed self-pollinating types of buckwheat and to elucidate the proanthocyanidin structures. Thirty-three different hybrids were analyzed for their genetic diversity of phenolic profiles by high performance liquid chromatography (HPLC).

2. Results and discussion

2.1. Identification

The flavanols constitute the main phenolic components whereas the flavonols are represented only by rutin. For identification of unknown proanthocyanidins the crude extract was separated on a Sephadex LH-20 column by elution with ethanol. The obtained fractions C and F were further purified by stepwise elution with methanol. In Fig. 1 the scheme for preparation of phenolics is shown. Fraction F contained compounds **17**, **18**, **22**, **29**, **30**, **31**, **33** and **36** and fraction C that of peak **32**.

The identification of these flavanols was based on their chromatographic behaviour, their UV-absorbance, their reaction with the diagnostic DMACA-reagent (ratio between the areas of the peaks recorded at 640 and 280 nm (A_{640}/A_{280}) (Treutter et al., 1994), and on mass spectroscopy. Furthermore, acidic (HCl) and enzymatic (tannase) hydrolyses were executed. Table 1 summarizes the characterisation of the isolated compounds. The common flavanols catechin, epicatechin, the procyanidins B2 and B5 as well as epicatechin-3-*O*-gallate were recovered and confirmed in the crude extract in comparison to authentic standards (compounds **13**, **14**, **15**, **16**, **22**, respectively). Epicatechin 3-*O*-(3,4-dimethyl)-gallate (**32**) previously iden-

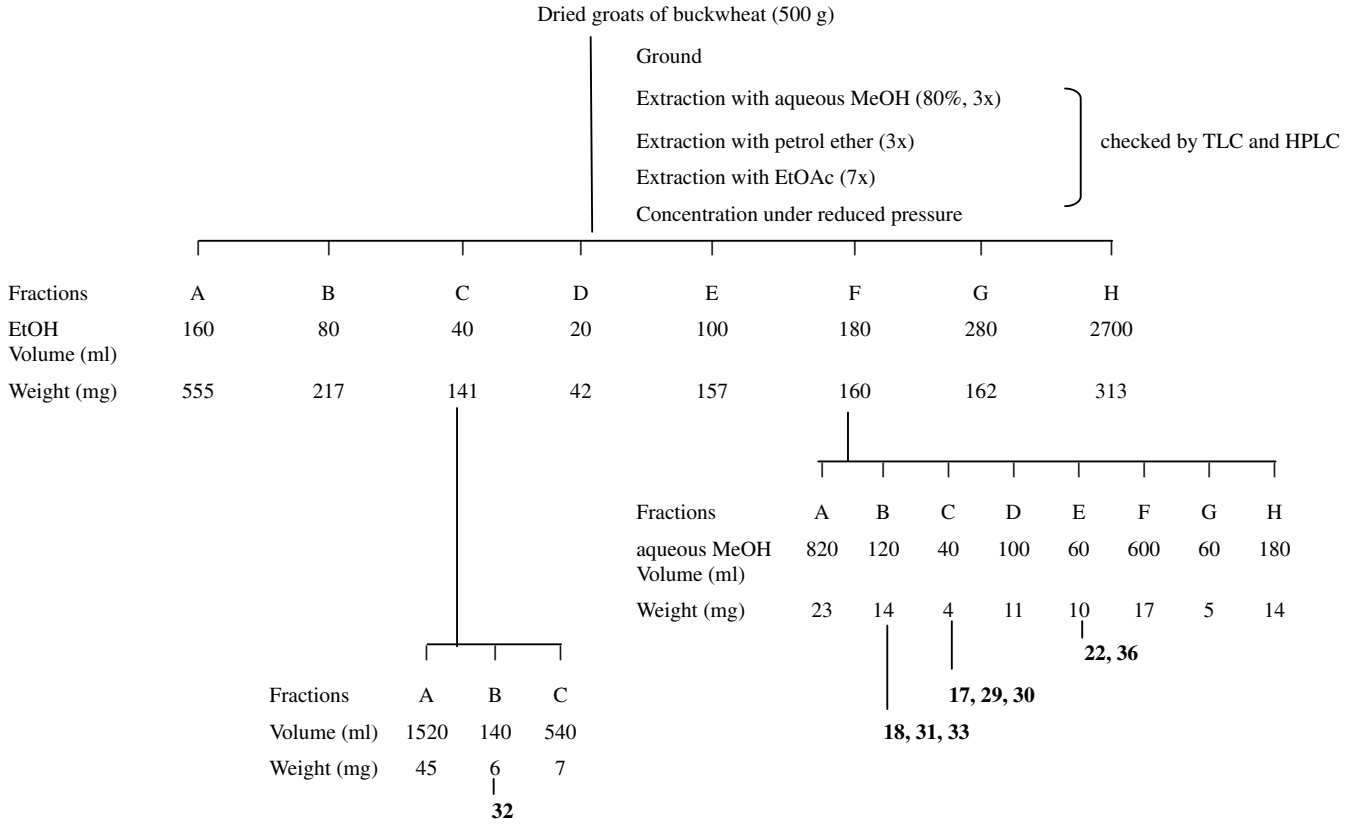

Fig. 1. Scheme for preparation of phenolic compounds from buckwheat seeds, fractions, volumes (ml) and yields (mg) after separation by Sephadex LH-20 column chromatography.

Table 1
Characterisation of isolated flavanols from buckwheat grain

Compound name	No.	*RT* min	λ_{max}	Ratio A_{640}/A_{280}	*m/z* $[M–H]^-$	*m/z* M^2 of $[M–H]^-$	Identified modules (*m/z*)
Monomeric flavanols							
Catechin	**13**	29	278	32.8	289	245; 109	
Epicatechin	**14**	54	278	45.2	289	245; 109	
Galloylated monomers							
Epicatechingallate	**22**	86	276, 300sh	9.3	441	289; 169	Epicatechin (289); Gallic acid (169)
Epicatechin-*O*-3,4-dimethylgallate	**32**	142	268, 300sh	35.5	469	271; 125; 319	Epicatechin 3,4-dimethylgallate
Procyanidins							
Procyanidin B2 = epicatechin-(4–8)-epicatechin	**15**	44			577	407; 425; 289; 125; 161	Epicatechin (289)
Procyanidin B5 = epicatechin-(4–6)-epicatechin	**16**	114	278	34.9	577	289; 407; 125; 123; 109	Epicatechin (289)
Procyanidin B2 dimethylgallate = epicatechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	**31**	106	271, 300sh	11.8	757	605, 125, 289	Epicatechin (289); 3,4-dimethylgallate
Propelargonidins							
Epiafzelechin-(4–6)-epicatechin	**17**	139	276	56.3	561	289; 271; 407; 245; 435; 125	Epicatechin (289); Epiafzelechin (271)
Epiafzelechin-(4–8)-epiafzelechin-*O*-(4–8)-epicatechin	**18**	65	276	43.7	833	289; 271; 164; 543	Epicatechin (289); Epiafzelechin (271); Epiafzelechin-epiafzelechin (543)
Epiafzelechin-(4–8)-epicatechin-*O*-methylgallate	**29**	107	276, 300sh	13.7	727	289; 561; 455	Epicatechin (289); Epiafzelechin-epicatechin (561)
Epiafzelechin-(4–8)-epicatechin-*O*-*p*-hydroxybenzoate	**30**	113	264, 300sh	18.2	682	289; 561; 407; 271	Epicatechin (289); Epiafzelechin (271); Epiafzelechin-epicatechin (561); *p*-hydroxybenzoate
Epiafzelechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	**33**	132	269, 300sh	16.6	741	469; 271; 319; 407	Epiafzelechin (271); Epicatechin-dimethylgallate (469); 3,4-dimethylgallate
Epiafzelechin-(4–8)-epiafzelechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	**36**	147	271, 300sh	17.5	1013	271; 125; 164; 137; 469; 543; 741; 407	Epiafzelechin (271); Epiafzelechin-epiafzelechin (543); Epiafzelechin–epicatechin–dimethylgallate (741); Epicatechin-dimethylgallate (469)

tified by Watanabe (1998) showed a molecular ion at $[M]^-$*m/z* 469. The enzymatic hydrolysis by tannase revealed the presence of epicatechin and (3,4-dimethyl)-gallate which was confirmed by co-chromatography with authentic standards. The mass of compound **32** was found in the fragmentation pattern of compounds **33** and **36** (Fig. 3).

Beside these, several oligomeric propelargonidins and procyanidins were detected. Among those, compounds **29**, **30**, **31**, **33**, **36** are acylated resulting in absorbance spectra with shoulders at 300 nm. Due to the strong UV-absorbance of the acyl moiety they also exhibit a low ratio A_{640}/A_{280} as compared to related compounds. The acyl moieties were identified as (3,4-dimethyl)-gallate or *p*-OH-benzoate by comparison with authentic standards after hydrolysis from the flavanol, and a monomethylgallate was proposed from the mass spectrum of compound **29**.

The propelargonidins (**17**, **18**, **29**, **30**, **33**, **36**) released the anthocyanidin pelargonidin after boiling in hydrochloric acid, thus, identifying epi-/afzelechin as extension unit.

The elution order on rp-HPLC of the proanthocyanidins (Table 1) revealed the exclusive existence of epicatechin and epiafzelechin units with 2,3-*cis* configuration. Their retention times also give information on the interflavan linkage (Correia et al., 2006; Treutter et al., 1994). A meta-analysis of the literature data on the chromatographic behaviour of procyanidins (Fig. 2) shows that proanthocyanidins consisting of epicatechin units or possessing [4–6]-interflavan linkage(s) usually elute later than the corresponding catechin derivatives and [4–8]-linked proanthocyanidins, respectively. As the new compounds eluted later than epicatechin these proanthocyanidins consist exclusively of epicatechin or epiafzelechin units. The dimeric compounds **29**, **30** and **31** are assumed to have [4–8] linkages because of their earlier retention times in comparison to the [4–6]-linked procyanidin B5. Compound **17**, however, showed a longer retention than procyanidin B5, thus pointing possibly to a [4–6]-linkage.

2.2. Quantitative analysis

Eight varieties of *F. esculentum*, a mixed sample of *F. homotropicum* and 33 hybrids from crossings of

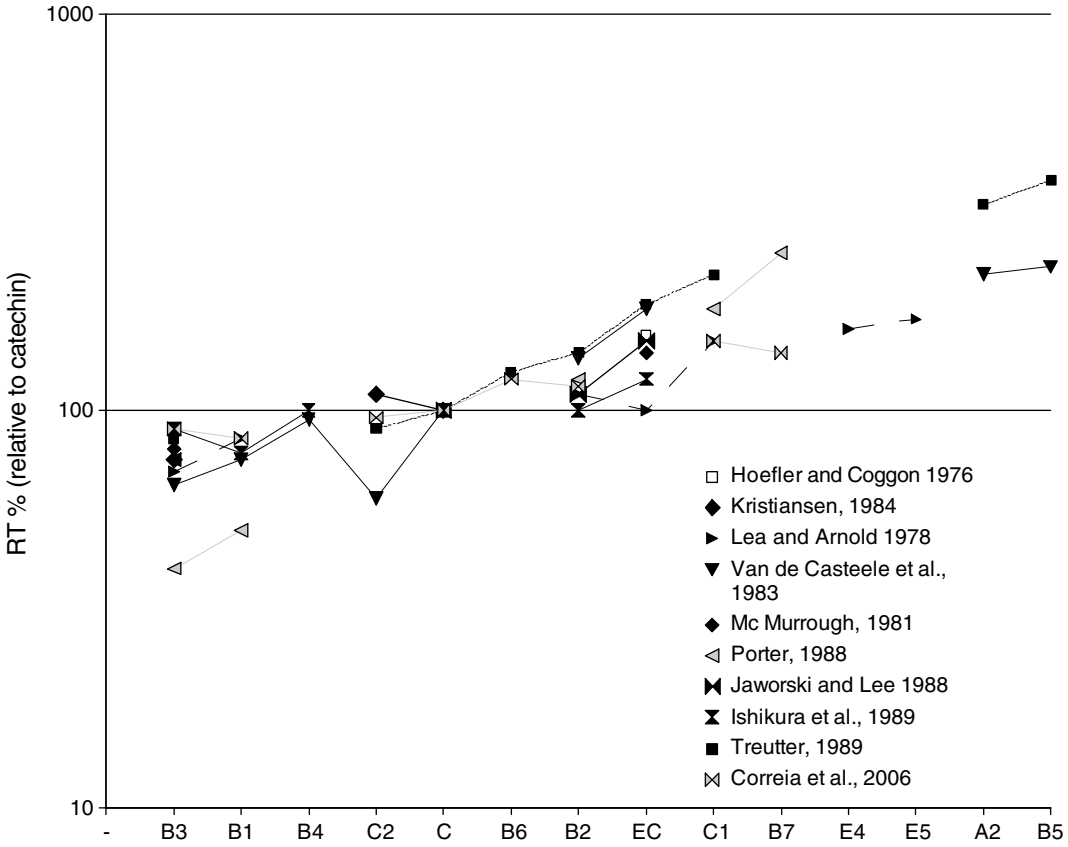

Fig. 2. Relative retention (%) on reversed-phase HPLC of procyanidins (PC) derived from catechin (C) and epicatechin (E) related to catechin (= 100%) based on the literature data Procyanidins: B3: C-(4–8)-C; B1: E-(4–8)-C; B4: C-(4–8)-E; C2: C-(4–8)-C-(4–8)-C; B6: C-(4–6)-C; B2: E-(4–8)-E; C1: E-(4–8)-E-(4–8)-E; B7: E-(4–6)-C; E4: E-(4–8)-E-(4–8)-E-(4–8)-E; E5: E-(4–8)-E-(4–8)-E-(4–8)-E-(4–8)-E; A2: E-(2-O-7, 4–8)-E; B5: E-(4–6)-E. (See above-mentioned references for further information.)

F. esculentum and *F. homotropicum* were analysed for their flavonoid composition. In this comparison of defined buckwheat varieties rutin, total flavanols as well as the content of the identified single procyanidins and propelargonidins were evaluated. That way it was possible to compare different buckwheat lines for their breeding potential to improve the flavonoid content in the grain. The results showed no qualitative but quantitative differences.

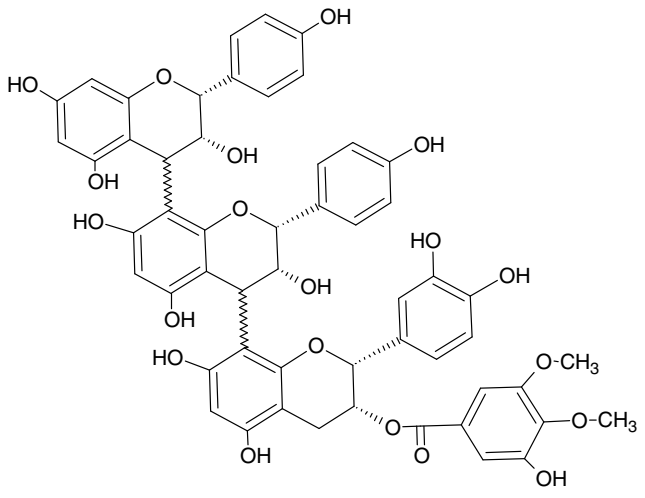

Fig. 3. Trimeric galloylated propelargonidin epiafzelechin-(4.8) epiafzelechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate (**36**) isolated from buckwheat grain.

In Table 2 the results of varieties from *F. esculentum* and *F. homotropicum* were presented. In *F. homotropicum* the flavanol content was three to seven times higher than in *F. esculentum*. The main flavanols from *F. homotropicum* grains were epicatechin, epicatechin-3,4-dimethylgallate, epiafzelechin-[4–8]-epicatechin- 3,4-dimethylgallate and epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin-(3,4-dimethyl)-gallate.

In contrast rutin was represented in higher amounts in *F. esculentum*. The rutin variability was conspicuous in this variety. These oppositional phenolic features of *F. esculentum* and *F. homotropicum* were combined by breeding.

In Table 3 the results of the 33 different hybrids were presented. The genotypes were sorted with regard to their total flavanol content and divided into 4 groups. In group 1 a quarter of the analysed buckwheat lines was listed showing a low flavanol content ranging from 43 to 60 mg/100 g DW. The mean rutin value was found at 10 mg/100 g DW. For half of the hybrids (group 2) flavanol contents between 72 and 110 mg/100 g DW were found. The variability of the rutin contents was high but no hybrids with high rutin amounts were found. Particu-

Table 2
Content of rutin and flavanols (mg/100 g DW) in grains of *F. esculentum* varieties and *F. homotropicum*

	Catechin	Epicatechin	Epicatechingallate	Epicatechin-3-*O*-dimethylgallate	Procyanidin B2	Procyanidin B5	Epiafzelechin-(4–6)-epicatechin	Epiafzelechin-(4–8)-epicatechin-*p*-OH-benzoate	Epiafzelechin-(4–8)-epicatechin-methylgallate	Epiafzelechin-(4–8)-epicatechin-(3,4-dimethyl)-gallate	Epicatechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	Epiafzelechin-(4–8)-epicatechin	Epiafzelechin-(4–8)-epiafzelechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	Other flavanols	Total flavanols	Rutin
	13	**14**	**22**	**32**	**15**	**16**	**17**	**30**	**29**	**33**	**31**	**18**	**36**			**11**
F. esculentum																
Hruszowska	0.6	2.4	0.8	0.4	0.4	0.4	0.3	tr	0.1	2.8	0.2	tr	1.3	5.6	15.4	5.4
Sun Rutin	1.8	2.3	0.6	0.6	0.3	0.5	0.4	0.1	0.1	2.5	0.1	tr	1.4	5.6	16.3	6.6
Pulanska/PA054	1.6	5.3	0.7	0.9	0.4	0.5	0.7	0.1	0.2	3.2	0.1	tr	1.3	6.3	21.2	7.5
Sobano	6.6	6.6	0.4	0.4	1.3	0.4	0.3	0.1	0.2	1.7	0.1	–	0.8	7.3	26.2	8.4
Ballada	0.7	7.4	1.1	0.6	0.7	0.7	0.7	0.1	0.3	3.6	0.2	0.1	1.4	9.1	26.7	6.2
Demetra	1.7	6.8	2.2	0.1	0.8	0.9	0.8	0.1	0.3	5.4	0.6	0.4	1.2	8.7	30.0	10.5
Sumchanka Sun	1.8	11.0	1.3	1.1	1.1	1.1	0.9	0.2	0.3	5.2	0.3	0.1	2.5	11.6	38.4	6.5
Irmenka	1.5	8.8	1.9	0.8	0.8	0.7	0.8	0.9	0.2	5.7	0.4	0.2	3.4	14.5	40.6	10.9
F. homotropicum	0.9	22.6	3.9	10.8	1.6	2.5	3.0	0.7	0.6	16.7	1.6	0.5	10.6	38.5	113.9	1.1

larly in this group the hybrids of the crossing "no name × *F. homotropicum*" presented the highest concentrations of the epicatechin- (3,4-dimethyl)-gallate.

In group 3 the 6 different buckwheat genotypes showed the highest flavanol contents between 123 and 220 mg/100 g DW. The propelargonidin epiafzelechin-[4–8]-epicatechin-*O*-dimethylgallate was determined in high amounts in "Kora × [Sumchanka × *F. homotropicum*]". The trimeric propelargonidin epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin-(3,4-dimethyl)- gallate showed the highest concentration in "Kazanka × [Sumchanka × *F. homotropicum*]".

In group 4 the hybrids with the highest rutin contents were arranged. Pulanska/PA054 × *F. homotropicum* and Sun Rutin × [Sumchanka × *F. homotropicum*] showed promising conditions for a future selection of individuals with high amounts of this interesting flavonol glycoside.

The results generally indicated higher total flavanol than rutin contents in the buckwheat seed. Compared with the self-incompatible varieties of *F. esculentum* the hybridization with *F. homotropicum* favoured increased flavanol content in the progeny. Against it the rutin content was not enhanced except for the two hybrids "Pulanska/PA054 × *F. homotropicum*" and "Sun Rutin × [Sumchanka × *F. homotropicum*]".

The presence of galloylated flavan 3-ols and in particular of propelargonidins as major components has not been reported for other crops and compared with oat and barley buckwheat seeds showed higher antioxidant activity (Watanabe, 1998; Quettier-Deleu et al., 2000; Holasova et al., 2002). Breeding for higher concentrations of rutin and flavanols may lead to even healthier buckwheat varieties.

3. Experimental

3.1. Plant material

For the identification of phenolic compounds a pooled sample of commercially available buckwheat grains (*F. esculentum* Moench; unknown variety) was used. The seeds used for the quantification were collected from hybrids grown in a greenhouse with a minimum temperature of 22 °C and with light supplement to a 16/8 h day/night photoperiod. All plants were harvested separately for flavonoid analysis.

3.2. Extraction for HPLC analysis

A pooled sample of hulled buckwheat seed was ground in a mortar or a ball mill, depending on the available amount. The extraction of phenolic compounds was performed by adding 500 µl aqueous methanol ($MeOH/H_2O$, 80/20, v/v), containing flavone ($c = 0.02$ mg/ml) as an internal standard, to 100 mg dry powder for 30 min in a cooled ultrasound water bath (7 °C).

Table 3
Content of rutin and flavanols (mg/100 g DW) in grains of *F. esculentum* x *F. homotropicum* hybrids

	Catechin	Epicatechin	Epicatechingallate	Epicatehin-3-*O*-dimethylgallate	Procyanidin B2	Procyanidin B5	Epiafzelechin-(4–6)-epicatechin	Epiafzelechin-(4–8)-epicatechin-*p*-OH-benzoate	Epiafzelechin-(4–8)-epicatechin-methylgallate	Epiafzelechin-(4–8)-epicatechin-(3,4-dimethyl)-gallate	Epicatechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	Epiafzelechin-(4–8)-epicatechin-(4–8)-epicatechin	Epiafzelechin-(4–8)-epiafzelechin-(4–8)-epicatechin-*O*-(3,4-dimethyl)-gallate	Other flavanols	Total flavanols	Rutin
	13	**14**	**22**	**32**	**15**	**16**	**17**	**30**	**29**	**33**	**31**	**18**	**36**			**11**
Group 1																
Sumchanka Sun × *F. homotropicum*	0.7	2.6	1.2	1.5	0.5	0.4	0.4	0.2	0.05	5.2	0.2	0.1	2.6	7.8	23.4	5.4
Chirminskaya × [Sumchanka × *F. homotropicum*]	1.5	5.9	1.5	3.4	0.9	1.3	0.4	0.5	0.1	8.5	0.5	0.2	5.3	13.6	43.3	8.4
Skorospelaja × *F. homotropicum*	1.3	11.0	1.9	0.1	1.2	1.4	0.9	2.5	0.5	2.4	1.3	0.6	0.2	19.8	45.1	3.6
Pavle Komar × [Sumchanka × *F. homotropicum*]	0.5	11.9	2.6	2.0	1.1	0.7	0.8	0.4	0.1	6.0	0.7	0.2	3.7	15.5	46.1	13.1
Ukrainka × *F. homotropicum*	2.9	13.0	2.9	0.8	0.8	0.8	0.9	3.1	0.4	2.7	1.1	1.0	2.1	23.9	46.1	13.4
Kitawase × [Sumchanka × *F. homotropicum*]	0.8	29.8	4.0	0.7	1.2	2.8	2.3	1.0	0.2	10.2	1.0	0.9	5.5	9.7	46.2	12.1
Kazanski × [Sumchanka × *F. homotropicum*]	1.6	11.3	2.5	3.4	0.8	1.2	0.9	0.4	0.1	10.7	0.7	0.2	5.1	17.0	55.9	14.9
Dozdik × [Sumchanka x *F. homotropicum*]	0.9	18.7	2.2	1.6	1.6	1.1	0.3	4.6	0.1	2.9	0.5	0.6	0.1	24.4	59.5	12.1
Luba × [Sumchanka × *F. homotropicum*]	6.9	14.8	3.2	1.6	1.4	0.5	1.3	0.4	0.1	4.7	0.9	0.3	2.9	21.1	59.9	1.5
Group 2																
Kalaminsky × [Sumchanka × *F. homotropicum*]	1.5	19.6	3.3	3.5	1.7	1.9	0.9	0.5	0.1	11.0	0.8	0.5	5.2	22.3	72.5	4.4
Dicul × [Sumchanka × *F. homotropicum*]	3.4	22.4	4.2	3.7	2.2	2.3	1.4	1.9	3.1	8.7	2.3	0.5	4.7	10.0	72.8	13.0
Siva × *F. homotropicum*	5.1	22.6	3.0	2.3	1.4	1.1	1.2	1.2	0.3	6.8	1.7	0.3	4.1	22.6	73.7	2.2
La Harpe × [Sumchanka × *F. homotropicum*]	1.3	15.3	3.2	2.6	1.2	1.8	1.9	1.4	0.1	9.4	1.5	1.4	6.1	26.7	73.9	16.2
Mancan × *F. homotropicum*	0.5	16.4	3.5	5.6	1.0	1.2	1.6	1.9	0.1	10.1	1.3	0.8	6.1	25.6	75.6	6.7
Manisoba × [Sumchanka × *F. homotropicum*]	2.5	32.4	3.8	2.9	1.4	1.0	1.7	0.6	0.4	6.7	1.6	0.6	3.7	20.6	79.9	9.3
Koghan × [Sumchanka × *F. homotropicum*]	6.0	24.4	4.4	2.4	1.3	0.5	2.0	1.1	0.2	7.4	1.5	0.3	4.7	25.8	81.9	4.5
Harkönen × *F. homotropicum*	7.8	21.5	4.1	3.4	1.4	1.9	2.0	1.7	0.2	8.7	0.9	0.5	5.3	25.7	85.0	3.0
FAG 80/84 × [Sumchanka × *F. homotropicum*]	0.2	27.8	5.4	2.5	1.7	0.8	0.4	1.9	3.9	3.4	2.7	0.7	0.3	38.9	90.4	10.3
Demetra × [Sumchanka × *F. homotropicum*]	1.8	31.7	4.3	2.9	2.5	2.4	2.0	1.4	2.5	5.9	2.0	0.6	3.0	33.2	96.3	16.8
No name × [Sumchanka × *F. homotropicum*]	7.7	25.7	4.3	5.3	1.8	2.6	1.6	0.7	0.1	12.4	0.8	0.7	5.8	27.0	96.6	11.2
FAG 83 var. emarginatum × *F. homotropicum*	8.3	32.6	3.8	2.5	1.2	0.7	0.6	6.9	0.1	4.9	0.5	0.3	0.1	35.4	98.0	2.3
Rutina Smith Kline × [Sumchanka × *F. homotropicum*]	7.2	24.3	4.7	3.2	1.7	1.6	1.8	1.0	0.4	12.9	2.3	2.7	5.9	30.8	100.5	8.7

FAG 43/87 Silverhull × [Sumchanka × *F. homotropicum*]	2.0	42.6	4.4	3.4	1.9	2.4	2.5	0.9	0.2	9.5	1.4	0.6	3.9	27.5	103.1	10.4			
No name × *F. homotropicum*	9.5	31.1	5.5	0.2	2.6	2.4	1.9	13.1	1.1	3.2	0.7	0.8	0.8	31.4	104.1	4.7			
Hruszowska × *F. homotropicum*	0.6	35.1	5.5	2.4	3.2	1.5	2.0	2.6	0.6	10.3	2.2	1.2	5.7	18.8	109.7	1.7			
Group 3																			
NSL83124 × *F. homotropicum*	4.2	45.8	2.8	10.7	4.4	6.5	2.8	0.9	0.2	12.1	0.6	1.1	5.1	25.8	122.9	4.7			
Tankinskaya var. *emarginatum* × *F. homotropicum*	3.4	35.5	7.8	5.1	3.4	3.6	2.1	14.7	1.6	7.1	0.7	0.5	4.3	35.9	126.0	5.7			
Kazanka × [Sumchanka × *F. homotropicum*]	0.5	16.9	3.3	5.0	0.6	1.0	2.3	1.8	2.1	10.0	0.2	4.1	6.2	72.8	126.7	5.6			
Khazanckaya × [Sumchanka × *F. homotropicum*]	5.3	63.5	5.3	5.0	4.5	1.8	3.6	–	0.9	4.6	0.8	1.2	1.4	31.3	128.9	3.4			
Kora × [Sumchanka × *F. homotropicum*]	1.4	44.2	6.0	9.7	2.8	4.1	3.0	1.8	0.2	14.5	1.7	0.6	5.7	41.8	137.3	4.6			
Sobano × [Ballada × *F. homotropicum*]	1.7	78.9	32.2	1.0	12.6	2.5	4.0	4.9	3.5	1.0	1.8	11.4	0.9	36.8	219.7	20.9			
Group 4																			
Pulanska/PA054 × *F. homotropicum*	2.7	5.2	1.1	2.5	0.5	0.8	0.6	0.5	0.1	7.3	0.5	0.4	5.3	13.9	41.3	48.0			
Sun Rutin × [Sumchanka × *F. homotropicum*]	1.3	19.8	2.4	7.3	1.5	2.5	1.2	0.4	0.2	11.5	0.8	0.1	5.9	24.1	73.8	31.0			

After centrifugation, the supernatant was evaporated, the residue re-dissolved in 100 µl methanol (80%) and 10 µl were injected for HPLC analysis.

3.3. Extraction of seeds and preparative fractionation of phenolic compounds

The procedure for the isolation of phenolic compounds from buckwheat seeds is shown in Fig. 1. A mixed sample of dry dehulled buckwheat seeds (500 g) was homogenised in an Ultraturrax with 1500 ml aqueous MeOH (80%) and 3 times extracted in an ultrasonic water bath (30 min at 7 °C). After centrifugation for 20 min at 10,000 rpm and 4 °C the clear supernatants were combined and evaporated in vacuo, diluted with H_2O and extracted 3 times (2 min) with petrol ether (4200 ml in total) at a ratio of 1:1 (v:v). The aqueous/methanolic phase was reduced to 1400 ml and the phenolic compounds were separated from the sugars by extraction 7 times with ethyl acetate (2800 ml in total). Both the combined ethyl acetate extracts and the aqueous phase were evaporated to dryness, resuspended in water and lyophilised. Each extraction step was monitored by TLC and HPLC for phenolic compounds. Fractionation of phenolic compounds was performed with Sephadex LH-20 according to Thompson et al. (1972). For this the lyophilised ethyl acetate extract was redissolved in ethanol (EtOH) and subjected onto a glass column (30 × 300 mm i.d.) packed with Sephadex LH-20. The phenolic compounds were eluted with 4.5 l EtOH which were collected in tubes of 20 ml which were grouped according to their phenolic content. Eight fractions (A–H) were obtained from the ethanol eluate and lyophilised. Fractions C and F were separated again on a Sephadex LH-20 column (20 × 360 mm i.d.) by stepwise elution with methanol/water. Fraction C was redissolved in H_2O and separated with the following gradient: 250 ml H_2O, 100 ml 10% MeOH, 300 ml 20% MeOH, 300 ml 30% MeOH, 300 ml 40% MeOH, 300 ml 50% MeOH. Fraction F was redissolved in 15% MeOH and separated with the following gradient: 300 ml 15% MeOH, 200 ml 20% MeOH, 200 ml 30% MeOH, 200 ml 40% MeOH, 200 ml 50% MeOH, 200 ml 60% MeOH, 200 ml 70% MeOH and 200 ml 80% MeOH. Similar fractions were combined, evaporated to dryness, resuspended in water and lyophilised.

3.4. High-performance liquid chromatography (HPLC)

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

The phenolic compounds were separated on a column (250 × 4 mm I.D.) prepacked with Hypersil ODS, 3 µm particle size, following a stepwise gradient using mixtures of solvent A (formic acid, 5% in water) and solvent B (meth-

anol, gradient grade) from 95:5, v/v to 10:90, v/v with a flow rate of 0.5 ml/min (Treutter et al., 1994). The gradient profile (% B in A) used was: 0–5 min, isocratic, 5% B; 5–15 min, 5–10% B; 15–30 min, isocratic, 10% B; 30–50 min, 10–15% B; 50–70 min, isocratic, 15% B; 70–85 min, 15–20% B; 85–95 min, isocratic, 20% B; 95–110 min, 20–25% B; 110–140 min, 25–30% B; 140–160 min, 30–40% B; 160–175 min, 40–50% B, 175–190 min, 50–90% B.

Rutin was detected at 360 nm, whereas the flavanols were estimated at 640 nm after post-column derivatization with *p*-dimethyl-aminocinnamic aldehyde (DMACA; Treutter, 1989; Treutter et al., 1994).

3.5. RP–HPLC–MS/MS

Samples were analyzed using a Shimadzu LC-10 A Series (Schimadzu, Hannover) automated liquid chromatograph comprising an SCL-10Avp system controller, two LC-10 A vp pumps, an SIL-10AD vp autoinjector with sample cooler, a CTO-10AC vp column oven, and an SPD-10A vp UV–vis detector. Reverse-phase separations were carried out at 30 °C using a 50 × 2.0 mm i.d., 4 μm Phenomenex Synergy Fusion-RP 18 column. The mobile phase consisted of (A) water – acetic acid (99.9/0.1; v/v) and (B) methanol – acetic acid (99.9/0.1; v/v). The gradient was 0–17 min, 20–100% B linear. The eluting stream (0.2 ml/min) from the HPLC apparatus was introduced into the mass spectrometer to obtain the MS and MS/MS data. All analyses were executed using a Turbo Ionspray source in the negative mode and using as capillary voltage: −4200 V; nebulizer gas, curtain gas, drying gas and collisions gas: N_2; entrance potential: 10 V; focusing potential: −330 V. The declustering potential was −50 V and the collisions energy −52 V. Full scan data acquisition was performed by scanning from *m*/*z* 50 to 2000 amu in profile mode at a cycle time of 3 s with a step size of 0.1 amu and a pause between each scan of 3 ms.

3.6. Quantitative analysis

The known compounds were quantified based on response factors obtained from their calibration curves using the internal standard method. For that, flavone was added to the extraction solvent at a concentration of 0.1 ml. The response factors for rutin (quantified at 360 nm; response factor 2.23×10^{-5}), catechin (quantified at 640 nm; response factor 0.13×10^{-5}), epicatechin (quantified at 640 nm; response factor 0.09×10^{-5}) and procyanidin B2 (quantified at 640 nm; response factor 0.18×10^{-5}) were calculated from authentic compounds. The flavanols epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin, epiafzelechin-[4–6]-epicatechin, epicatechin-[4–8]-epicatechin-*O*-3,4-dimethylgallate, epiafzelechin-[4–8]-epicatechin-*O*-(3,4-dimethyl)-gallate, epiafzelechin-[4–8]-epiafzelechin-[4–8]-epicatechin-*O*-(3,4-dimethyl)-gallate, epiafzelechin-[4–8]-epicatechin-methylgallate and epiafzelechin-[4–8]-epicatechin-benzoate were quantified as epicatechin.

3.7. Peak identification

The peaks were identified according to their UV-absorbance, their chromatographic behaviour on reversed phase chromatography (HPLC) and thin layer chromatography in comparison to authentic standards, enzymatic hydrolysis by tannase and LC–MS/MS data.

The following standards were available from Roth (Karlsruhe): catechin, epicatechin, rutin. The procyanidin B2 was previously isolated from service tree (*Sorbus domestica* L.) fruit (Ölschläger et al., 2004). Procyanidin B5 was isolated from apple leaves (Treutter et al., 1994).

3.7.1. Acid hydrolysis

Acid hydrolysis was performed by adding 100 μl 0.1 M HCl to 100 μl of the sample which was then incubated for 15 min in a boiling water bath. The phenolic compounds were extracted from this solution with an equal volume of EtOAC.

3.7.2. Enzymatic hydrolysis

The samples containing 0.2–1 mg of the compound of interest were evaporated to dryness under vacuum. The residue was diluted with 300 μl of natrium acetate buffer 0.1 M (pH 4.6) and incubated with 2 mg of the enzyme tannase (braunschweiger biotechnologie) in a waterbath at 37 °C for 20 h. After extraction with ethyl acetate (3 × with each 300 μl) the ethyl acetate phase was evaporated to dryness, redissolved in 100 μl MeOH and injected onto the HPLC system.

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