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# Fingerprinting and characterization of anthocyanins in 94 colored wheat varieties and blue aleurone and purple pericarp wheat crosses

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ABSTRACT

Colored wheat varieties and crosses were analyzed to figure out their anthocyanin profiles, and thus, their potential as health-related food. After method development, the obtained 94 anthocyanin fingerprints allowed the clear differentiation of the blue aleurone and purple pericarp genotypes as well as their breeding lines. The method was trimmed so that the complete analysis of the whole grain flour including sample preparation of up to 20 samples on one plate took less than 3 h (<9 min per sample) and total costs including sample preparation were <1.0 Euro/sample. Sample preparation of the complex wheat matrix was reduced to a minimum (only acidified methanol extraction of the ground whole wheat grain). Separation was well achieved on amino phases with a mixture of ethyl acetate, 2-butanone, water and formic acid. It was superior to the separation on either normal or reversed phases and more robust with regard to intrinsic pH variances of the sample extracts. Pattern recognition of anthocyanins was simply performed by visual detection (the image), a key feature of high-performance thin-layer chromatography. Wheat varieties and crosses with higher anthocyanin contents were easily selectable, and thus, successfully made out. Prominent anthocyanin zones were characterized by electrospray ionization mass spectrometry. Their sugar moiety was characterized via methanolysis and compared with the sugars available freely in the whole wheat grain. The developed profiling is a fast and efficient screening tool with option for quantification or identification on the same HPTLC plate.



## 1. Introduction

Wheat (*Triticum* spp.) is one of the three most important food cereal grains together with maize and rice [1]. The world wheat production for 2014/15 was about 730 million tons [2,3]. Common wheat (*Triticum aestivum*) is economically the most important species. It accounts for the largest share of the global wheat production, whereas durum wheat (*T. durum*) makes up about 5% [4]. Also cultivated, but economically much less important species are spelt or dinkle (*T. spelta*), emmer (*T. dicoccum*) and einkorn (*T. monococcum*). Common wheat is used for baked goods like breads, cakes or cookies, while durum wheat with a harder grain is mostly used for pasta and couscous. [5,6]

Interest in colored cereals has grown in the last decade. Durum wheat owes to carotenoids its yellow pigmentation of the endosperm, while its red, purple or blue grain colors derive from anthocyanins which are located in the aleurone or pericarp. [7,8] The most common anthocyanins found in wheat were based on cyanidin, but compounds containing delphinidin, peonidin or pelargonidin were also found. For the glycosylated molecules, mostly glucose and rutinose were described as sugar components, but also arabinose and galactose. [9–15] Anthocyanins are phytochemicals and belong to the group of flavonoids [16]. While anthocyanins are mostly found in fruit and flower petals, they also occur in vegetables and as mentioned in cereals such as wheat, maize or rice [16–18]. Recent studies have attributed to anthocyanins several health-beneficial properties against, *e.g.,* oxidative stress [17,19–22], cancer [23–26], inflammation [27–29], diabetes and obesity [30]. This makes anthocyanins not only interesting to the food industry as natural colorants (E163), but also as potent bioactive candidates to valorize human diets. Either isolated and added as food additive or added as concentrated extract to products, or naturally present in food, anthocyanin-rich food will contribute to health-related food.

Early analytical methods were limited to the determination of the total anthocyanin concentration [31–33]. In 1970s, first papers described the separation of anthocyanins by high-performance liquid chromatography (HPLC), mostly reversed phase (RP) sepa-

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ration of grapes, grape juice or wine [34–38], but also flower petals [39,40] and cranberries [41]. In the early 1980s, first thin-layer chromatography (TLC) methods of anthocyanins were published. Most reported cellulose [9,42–44] as stationary phase, but also silica gel layers [45]. Samples like wine [45], corn seedlings [42], wheat [9], fruit and fruit juices [43,44,46] were usually analyzed qualitatively. Current analysis has mainly been done via RP-HPLC, allowing not only quantification, but also identification by, *e.g.,* absorption spectra, mass spectra, retention time and standard substances. Especially mass spectrometry (MS) proved to be a helpful tool in the assignment of more complicated anthocyanin representatives via their characteristic fragment ions. [47]

For the analysis of anthocyanins in the wheat grain, only one TLC method was found that analyzed the pericarp and coleoptiles of purple wheat in 1972 [9]. Though spectroscopic methods for the determination of the total anthocyanin concentration were also employed [13,14,48,49], (HP)LC seems to be the method of choice [10–15,49]. Recently in 2013, Syed Jaafar *et al.* investigated anthocyanins of blue and purple aleurone wheat crosses by HPLC-DAD [49]. The single anthocyanins were not described or identified, but the total anthocyanin content of different varieties and breeding lines was compared.

The objective of this study was to develop a fast, streamlined and robust HPTLC method for wheat anthocyanins of the same sample set, as used for HPLC-DAD [49]. The HPTLC method was meant as a supporting tool, delivering fast results on the anthocyanin pattern at comparably low costs. It should support an easy visual differentiation between the various genotypes by their anthocyanin pattern, and a clear distinction between breeding lines with low and high anthocyanin content, as the food producing industry is interested in anthocyanin-rich food ingredients for creating health–related food. Such a second orthogonal and independent method should also verify the previously obtained HPLC results [49], as for large sample sets the robustness of the column was an issue, despite the employment of accelerated solvent extraction. Hence, it was also questioned whether a reduced sample preparation is possible for HPTLC, known to be compatible to high matrix loads.

## 2. Material and methods

### 2.1. Reagents

The following anthocyanins were obtained as chloride salts: cyanidin (Cn), delphinidin (Dp), malvidin (Mv), peonidin (Pn), all ABCR, Karlsruhe, Germany, pelargonidin (Pg) and pelargonidin-3-glucoside (Pg-3-glc), both Sigma–Aldrich, Steinheim, Germany, cyanidin-3-glucoside (Cn-3-glc), delphinidin-3-glucoside (Dp-3-glc), malvidin-3-glucoside (Mv-3-glc), and peonidin-3-glucoside (*Pn*-3-glc), all PhytoLab, Vestenbergsgreuth, Germany, and malvidin-3.5-diglucoside (Mv-3.5-diglc) and natural product reagent A (diphenylboric acid-2-aminoethylester), Carl Roth, Karlsruhe, Germany. HPTLC plates silica gel 60 $F_{254}$ (20 cm × 10 cm), HPTLC plates silica gel 60 $NH_2$ $F_{254}$ s, HPTLC plates silica gel 60 RP–18 W (10 cm × 10 cm), HPTLC plates RP-18 $WF_{254}$ s (20 cm × 10 cm), potassium hexocyanoferrat (III), zinc sulphate heptahydrate and ninhydrin were provided by Merck (Darmstadt, Germany). Ethanol, methanol (both ≥99.9%), hydrochloric acid (≥32%), formic acid (>98%), phosphoric acid (85%), sulfuric acid (96%), all per analysis grade, sodium hydroxide and 4-methoxybenzaldehyd (anisaldehyde) were obtained from Carl Roth. Acetone (≥99.5%), acetonitrile (≥99.9%), diphenylamine, basic bismuth carbonate (Riedel de Haën), acetic acid (99–100%), 2-butanone, 2-propyl acetate, pyridine (>99%), aluminum chloride, fast blue salt B and aniline were obtained from Fluka Sigma Aldrich. 1-propanol (per analysis grade) was obtained from ACROS Organics (Geel, Belgium). Methanol for MS and ethyl acetate (>99%) were obtained from VWR, Darmstadt, Germany. 1-Butanol was from Alfa Aesar, Karlsruhe, Germany. Bi-distilled water was generated using a Heraeus Destamat Bi-18E (Thermo Fisher Scientific, Schwerte, Germany). A set of HPTLC plates silica gel 60 $NH_2$ $F_{254}$ s were prewashed with 15% formic acid in methanol (development up to about 90 mm), followed by drying at 120 °C on the TLC Plate Heater (CAMAG, Muttenz, Switzerland) for 10 min.

### 2.2. Standard solutions

Anthocyanidins and anthocyanins were separately dissolved in 0.5% hydrochloric acid (32%) in methanol. The individual stock solutions were pipetted together into a standard mixture containing all 11 anthocyanins and anthocyanidins according to [47] or three anthocyanins at a comparable concentration (Mv-3.5-diglc 24 ng/μL, Mv-3-glc 16 ng/μL and *Pn*-3-glc 11 ng/μL), as Cn-3-glc and Dp-3-glc were consumed and purchase was expensive. Sugars (60 mg each) were dissolved together, as 3- or 4-sugar mixture, in 10 mL methanol and water (1:1, *V/V*) and diluted accordingly (120 μg/mL). Two standard mixtures of methylated derivatives of galactose, xylose, rhamnose, galacturonic acid and fructose (Mix 1) as well as glucose, mannose, arabinose, fucose and glucuronic acid (Mix 2) were prepared (150 ng/μL each, except for fructose with 450 ng/μL) [50,51]. Note that galacturonic acid, glucose, fucose and fructose are hardly seen in the mixture, as by mistake only one third was taken. All solutions were stored in the dark at −20 °C.

### 2.3. Sample preparation

Wheat grains were harvested in 2011, milled into whole grain flour and obtained from the University of Natural Resources and Life Science, BUKO, Vienna, Austria (Table S-1) [49]. The 94 samples were stored at −20 °C until use. For anthocyanin and sugar analyses, 100 mg of the sample was suspended in 1.5 mL acidified methanol (0.5% hydrochloric acid, 32%), while for fingerprint analysis 300 mg and for MS recording 450 mg sample were taken. The suspensions were vortexed, ultrasonicated (30 min) and centrifuged (5 min, 11600 × *g*). The clear supernatant was transferred in a 1.5 mL glass vial and stored in a freezer at −20 °C.

For analysis of the methylated sugar derivatives, about 200 mg of the wheat samples no. 15 and 61 (Table S-1) were suspended in 1.5 mL methanolic hydrochloric acid (2 mol/L), vortexed, vigorously shaken (3 min) and centrifuged (5 min, 11600 × *g*). Each supernatant (1.2 mL) was transferred in a reaction tube and put in an oven (100 °C, 4 h) for methanolysis [50,51]. After cooling down, pyridine (50 μL) was added.

### 2.4. HPTLC methods

The wheat extracts (25 μL) were sprayed on bandwise with the Automatic TLC Sampler 4 (ATS 4, CAMAG) using band lengths of 7.0 mm (up to 15.0 mm for overlapped application), 8.9-mm track distance and 8.0-mm distance from the lower edge. In the Automatic Developing Chamber 2 (ADC2, CAMAG), anthocyanin fingerprinting was performed on prewashed HPTLC plates silica gel 60 $NH_2$ $F_{254}$ s with a mixture of ethyl acetate – 2-butanone – formic acid – water (7:3:1:1.5; *V/V/V/V*) up to 60 mm from the lower plate edge. Relative humidity (≤2%, via molecular sieve for 5.0 min) was adjusted, plate conditioning time was set to 0.5 min and drying time after chromatography was 5.0 min.

Separation of the sugar and methylated sugar components on HPTLC plates silica gel 60 $F_{254}$ in the ADC2 with a mixture of 1-butanol – *i*-propanol – acetic acid – 2% boric acid solution (6:14:1:3; *V/V/V/V*) [52] and *i*-propyl acetate – ethyl acetate – methanol – water (5:4:1:0.1, *V/V/V/V*) [50,51], respectively. The migration dis-