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Identification and quantification of chromophoric substances in barley (*Hordeum vulgare* L.)

The contents of colouring substances in different cultivated varieties of barley (*Hordeum vulgare* L.) were recorded qualitatively and quantitatively to identify which substances contribute to their colour. HPLC was used to quantify the 3-O-glucosides of the anthocyanidins malvidin, peonidin, pelargonidin, cyanidin and delphinidin. Of the varieties examined, only the ancient spring barley variety 'Weihenstephaner Schwarze Nackte' contained anthocyanins, with delphinidin-3-glucoside accounting for the largest proportion. In addition, another substance was detected, which is most likely also an anthocyanin. The total anthocyanin content was at the upper end of the values previously known from black varieties. MALDI-TOF mass spectrometry was used to try to shed more light on the structure of the dark pigments in the outer layers of the black barley grains. The results suggest that the dark substance is a heterooligomer of different, partly phenolic substances. Specifically, the repeated occurrence of a 180 Da monomer could be detected. Since plant melanins are usually synthesised from the phenolic acids found in the plant, this substance could be caffeic acid and would thus be similar to allomelanin from oats (*Avena sativa* L.), which is a homooligomer of *p*-coumaric acid. The development of anthocyanins during malting were recorded photometrically. A clear decrease in the total anthocyanin content could be observed, however, clear weaknesses of the method used to date became apparent.

Descriptors: Anthocyanins, allomelanin, coloured barley, HPLC, MALDI-TOF, polyphenols

1 Introduction

Using several ancient [1] malting barley varieties (*Hordeum vulgare* L.) with external black colouring, analyses were carried out to identify and quantify those pigments responsible for the characteristic colouring.

With the exception of rice, all cereals contain carotenoids, which are responsible for their typical yellow colouring [2]. In addition to the almost exclusively yellow cereals cultivated today, there are also "colourful" varieties with a purple, blue or black colour. In these varieties carotenoids are overlaid by anthocyanins [3], and in some black varieties presumably melanins [4].

https://doi.org/10.23763/BrSc23-12häge

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1.1 Substance groups

Anthocyanins are among the most widespread secondary plant metabolites. They are responsible for the characteristic red to blue colour of numerous fruits such as cherries, currants, grapes, as well as vegetables such as red cabbage and aubergines [5]. The colour probably serves mainly to propagate the plant by attracting animals that pollinate the blossom or eat the flesh, but also to protect it from UV radiation [6].

Anthocyanins are formed in fruits throughout the ripening process with the content continuously increasing until full ripeness. The extent to which they are formed depends on various environmental influences. Increased exposure to sunlight leads to increased synthesis. The same applies to dry locations and saline soils. High temperatures, on the other hand, result in a lower anthocyanin content in most crops [7–11].

Excess nitrogen in the soil can also lead to reduced anthocyanin synthesis [7]. Since slurry or mineral nitrogen fertiliser is often used in conventional barley cultivation [12, 13], this factor must be taken into account, especially if the colouring of the barley is considered to be a valuable product property.

The anthocyanins and anthocyanidins belong to the flavonoids and are all variants of the basic structure shown in Fig. I (supplementary material), which differ in the substituents $R_{1,2}$. In a few, rarely occurring forms, the OH groups located at atoms 5, 7 and 4' can

also be substituted by other groups [14, 15].

The chromophoric aglycone is called anthocyanidin. If it is bound to a sugar, it is called anthocyanin [16]. The sugar bond is necessary for water solubility, which is why hardly any aglyconic anthocyanidins are found in natural substances. In fruits, glucose, galactose, rhamnose and arabinose are the most common sugars [15, 16]. In cereals, glucose is the predominant sugar [17]. If all combinations of sugars and other substituents are added together, more than 600 different anthocyanins have been described to date [18].

Table I (supplementary material) provides an overview of the most important anthocyanidins. The absorbed wavelength and the resulting colour impression refer to the positively charged flavylium form of the anthocyanin, which is only present in acidic environments. The colour is strongly pH-dependent and changes several times across the pH spectrum [15].

Table II (supplementary material) provides an overview of the most common anthocyanins found in plants [19–21]. Not listed in the table is the grape (*Vitis vinifera*). All anthocyanidins listed in Table I (supplementary material) are found in grapes and red wine, mainly as 3-O-glucosides [15]. The composition differs greatly between the different varieties, as well as between regional varieties or cultivars of the same species. For example, *Abdel-Aal* et al. (2006) showed substantial differences in the content and composition of the various anthocyanins between different coloured wheat, barley, rice and maize varieties. For example, the composition of anthocyanins differs in blue and purple wheat (*Triticum aestivum* L.): While delphinidin-3-glyloside accounts for the largest part of the total anthocyanin content in blue varieties at approx. 37 %, mainly pelargonidin-3-glucoside, -rutinoside and peonidin-3-glucoside are found in purple varieties [3].

For the barleys examined in previous studies, the total contents vary greatly between 8 mg/kg grain weight for black barley and 8 mg/kg grain weight for white barley [22], 77 to 345 mg/kg for blue [3, 23] and 573 to 679 mg/kg for purple varieties [23]. As mentioned at the beginning, some black varieties also contain no anthocyanins at all; in most varieties containing anthocyanin the content is very low. A possible reason for this difference is usually given as the fact that black barley is already sufficiently protected from UV radiation by melanin and therefore does not need an increased anthocyanin content for this purpose [23, 24]. However, this logical theory is somewhat at odds with the data known from other cereals. In rice (*Oryza sativa*), for example, it is the black varieties that contain the most anthocyanins [3].

Melanins are also widespread natural substances (Table III supplementary material), but research to date has mainly focused on the animal eumelanins and pheomelanins, which are responsible, for example, for the colour of human hair, skin and eyes. The exact structure of all melanins is still unclear today.

Plants only possess nitrogen-free melanins, the allomelanins. While eu- and pheomelanins are formed in animals and plants by the enzyme-catalysed oxidation of tyrosine via L-dopa to dopachinone [25], allomelanins are defined only as those mostly nitrogen-free melanins that are not formed in this way [4, 26]. Depending on their provenance, allomelanins therefore differ greatly in molecular structure [27, 28]. According to current knowledge, allomelanins are formed by the polymerisation of various species-specific aromatic compounds, mostly as homopolymers [29]. While naphthalene diols form the precursors of the monomers in most fungi and even plants such as date palms [30–32], the derivatisation and polymerisation of phenolic acids is discussed as being the synthetic pathway of most plant allomelanins. Using black oats (*Avena sativa* L.) as an example, *p*-coumaric acid was identified as a probable monomer of a melanin molecule [33]. In melanin-containing barley, a selection of phenolic acids has recently been named as possible precursors of melanin synthesis, including *p*-coumaric acid [27, 34, 35]. With regard to the molecular structure, it is known that the quinone groups in eu- and pheomelanins are always present in ortho configuration, whereas only meta- and para-quinones are found in allomelanin [30]. Due to the complex nature and the low commercial and medical importance, there is scant detailed research into allomelanins and it is still unclear whether they are a uniform group of substances or whether future findings will justify a division into several groups [30, 36]. According to *Kamei* et al. (1997), allomelains extracted from black sesame and black soybeans suppress tumour growth in in vivo and in vitro experiments on mice [37].

It should be emphasised that allomelanin is not identical to phytomelanin. Although both terms – especially in older literature – are occasionally used synonymously, phytomelanin is a different substance that is not considered to be a melanin [4, 26, 30, 38]. It is considered more likely that several organic substances co-occur in the phytomelanin layer, including polyvinyl alcohols, hydrocarbons and polyacetylenes [39, 40]. The synthesis of the two substances also takes place in fundamentally different ways: While allomelanin is synthesised and accumulated in so-called melanoplasts, which are probably derived from chloroplasts, the formation of phytomelanin occurs extracellularly from the metabolic products of hypodermal cells [40, 41]. Phytomelanin is not found in any cereal species, but only in a small number of plants that can be grouped into a single clade within the *Compositae* family [42–44].

Allomelanin is absolutely insoluble in water as well as organic solvents and therefore plays no role in beverage preparation [28, 45]. Anthocyanins, on the other hand, are highly soluble in water and have a characteristic red colour in an acidic environment, which is responsible for the colour of red wines, for example, and could possibly also be exploited in beer preparation.

1.2 Distribution of coloured substances in the barley grain

All colour-active substances, i.e. carotenoids, anthocyanins and melanins, are mainly found in the husk and aleurone. The endosperm – which makes up the largest part of the grain – is very poor in anthocyanins, melanins are not found there at all. Carotenoids are also present in the endosperm, but most of them are found in the aleurone [41, 46, 47].

The distribution of the dark substances can be seen in Fig. II (supplementary material). Even in the course of malting, which involves

a certain decompartmentalisation of the inner structures, no broader dispersion of the dark colourings can be observed visually. For beer production, the distribution within the grain plays only a subordinate role, since the entire malting barley grain is processed.

Similarly to its function in human skin, melanin serves mainly as sun protection and is therefore found in the outer layers of the barley grain. While animal melanins are spontaneously synthesised when the tissue is exposed to UV radiation [48], there are still no studies on allomelanin as to what extent environmental conditions are involved in melanin production.

The aim of this study was to identify which substances are relevant for the colouring of ancient barley varieties 'Weihenstephaner Schwarze Nackte' and 'Schwarze Criwien' as well as a Syrian landrace barley by using high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionisation/time of flight (MALDI-TOF) mass spectrometry. Further, it was analysed how malting influenced the total anthocyanin content, using a rapid photometric method.

2 Materials and Methods

2.1 Melanin

2.1.1 Reagents

The following were used to extract melanin from the husks: hydrochloric acid (HCl 32 %) from ThermoScientific (Schwerte, Germany), NaOH pellets from Carl Roth (Karlsruhe, Germany), ammonium hydroxide solution (BioUltra, 1 M NH_3 in H_2O) from Sigma-Aldrich, trichloromethane (Rotisolv®) from Carl Roth, ethanol (undenatured HPLC grade > 99.9 %) and ethyl acetate from Bernd Kraft. TFA (trifluoroacetic acid > 99.9 %) from Carl Roth was used and DHB (2,5-dihydroxybenzoic acid > 99.0 %) from Sigma-Aldrich as the MALDI matrix.

2.1.2 Samples for melanin analysis

The melanin determination was carried out using two ancient spring barley grain samples: An unmalted barley 'Weihenstephaner Schwarze Nackte' (harvest year 2021) and barley malt of the variety 'Schwarze Criewen' (harvest year 2020). To obtain the husk material, the grains were cut in half lengthwise, boiled in water for approx. 1 h and then the small-stranded interior of the endosperm was carefully scraped out with a scalpel. The resulting shells were dried at room temperature and finely ground (f = 30 Hz, t = 120 s) in the ball mill (MM 400, Retsch, Haan, Germany).

A synthetically produced melanin and a melanin extracted from cuttlefish (*Sepia officinalis*) (Sigma-Aldrich, St. Louis, MO, United States) were used as reference substances for the photometric measurements.

2.1.3 Extraction

The preparation process followed the method developed by *Varga* et al. (2016) of multi-stage separation of all extraneous components: (1) acid hydrolysis to remove carbohydrates and proteins, (2) washing with organic solvents to remove lipids, and (3) precipitation (several times) of phenolic components [33].

A small amount of the ground material was suspended in 6 ml 0.5 N NaOH and then heated in a thermoblock (ϑ = 100 °C, t = 1 h), briefly vortexing every 15 min. The sample material was then transferred to a centrifuge tube. The sample material was rinsed with a few drops of NaOH in order to transfer any residues sticking to the rim. After centrifugation (a = 12850 g, t = 10 min), the supernatant was transferred into a plastic tube and acidified to pH 2.5 with 7 N (coarse) or 1 N (fine) HCl. The acidified supernatant was stored for at least 12 h at room temperature. After storage, centrifugation was repeated (a = 12850 g, t = 10 min) and the supernatant was decanted. To the solid phase 3 ml of 7 N HCl was added and the tube was mixed in the vortex. The contents were then transferred to a glass tube, which was hydrolysed at ϑ = 100 °C, t = 2 h. The cooled suspension was transferred to a plastic tube and centrifuged again using the same parameters. After decanting the supernatant, the solid phase was filtered under suction onto a glass filter paper (GF/B, 55 mm, Whatman, Cytiva, Amersham, United Kingdom) and rinsed with H_2O until neutralisation. The resulting brownish-black material was washed alternately with chloroform, ethyl acetate and ethanol and dried at ϑ = 50 °C in a drying oven.

The dried material was dissolved in 1 N NH_3 in a beaker and treated in an ultrasonic bath for 5 min. The solution obtained was filtered into a beaker using a syringe with a filter attachment and gradually acidified until flocculation at approx. pH 7. The precipitated flocs were suction-filtered onto glass filter paper. The steps described in the last paragraph were carried out a total of three times. The material thus obtained on the glass filter paper was dried and stored in a dry location at room temperature until further analysis.

2.1.4 Spectrophotometric identification

For measurement, the extracted melanin samples and the standards were transferred to 0.1 M NaOH. For this purpose, a small piece of the glass filter paper with the sample was immersed into a test tube with 5 ml solvent and agitated in the vortex until no further decolourisation could be observed on the filter paper. The now dark coloured solvent was decanted into the cuvette.

An arbitrary amount of sample was added to the solvent, as the intention was to obtain a qualitative assessment of the absorbance spectra rather than quantification. In the photometer (DR 6000, Lange), the absorbance was measured in the range 250–800 nm against 0.1 M NaOH as a blank value.

2.1.5 Mass spectrometric characterisation

To prepare the MALDI samples, small pieces were cut from the glass filter papers and immersed in sample vessels containing 1 ml TFA, squeezed out a little and agitated in the vortex mixer. In the process, very small, barely perceptible amounts of melanin detached from the filter paper. The vessels were then briefly centrifuged in a microspin to separate any impurities, e.g. cellulose particles from the filter paper. The melanin standards were dissolved in aqueous 1 M NH_3.

Ammonia or TFA extracts (1 µl) and, after drying, 1 µl of the matrix (20 mg/l DHB in H_2O) was applied to the MALDI target plate and again allowed to dry. A peptide mixture (Peptide Calibration Standard II, Bruker, Billerica, MA, United States) was used as an external calibration standard.

The MALDI-TOF measurement was performed in positive linear mode with 1000 shots per single measurement (f = 1000 Hz) with deflection up to *m/z* > 450 in the Autoflex Speed MALDI mass spectrometer (Bruker, Billerica, MA, United States). For each sample, ten individual measurements from different locations of the target spot were combined.

2.2 Anthocyanins

2.2.1 Reagents

The extraction solvent was methanol (Rotisolv® HPLC Grade, Carl Roth, Karlsruhe, Germany) acidified with 15 % 1 N HCl standard solution (Bernd Kraft, Duisburg, Germany). The eluents used for HPLC were ultrapure water type 1 (eluent A) and methanol (eluent B; Rotisolv® HPLC Grade, Carl Roth), each acidified with 1 % formic acid (98 %, Bernd Kraft, Duisburg, Germany).

The anthocyanin standards used were cyanidin-, pelargonidin- and peonidin-3-glucoside chloride of 95 % purity and delphinidin-3-glucoside chloride of 98 % purity from Cayman Chemical (Ann Harbor, MI, United States) and peonidin-3-glucoside chloride in 95 % purity from Extrasynthese (Genay, France).

2.2.2 Samples for anthocyanin analysis

During the analyses, various malted and unmalted samples were examined. Malting spring barley of the ancient varieties 'Weihenstephaner Schwarze Nackte' and 'Schwarze Criewen', were analysed unmalted, as well as a local Syrian landrace variety that was available from a previous research project and 'Schwarze Pfauengerste' barley. The 'Weihenstephaner Schwarze Nackte' variety was grown under different conditions: The samples came from the different growing areas of Lower Rhine (NR), Kerpen-Buir (KE), Haus Düsse (DU), Bingen (BI) and Kleve (KL). In the Kleve growing area, part of the harvest was treated (b), the other not (nb). In Bingen, the non-irrigated crop (nw) was compared with the irrigated crop (w).

All harvests of the ancient spring barley 'Weihenstephaner Schwarze Nackte' were analysed as raw grain as well as malt. Malting was carried out as a modified MEBAK small malting process using a rotating germination box. Steeping times were observed according to the regulations, but pre-sorting of the barley was omitted given the fact that the raw grain samples were also analysed with unsorted barley. Malting only the whole barley would have falsified the results of the analyses. A kilning programme for Pilsner malt was used.

2.2.3 Extraction

Anthocyanin analysis was performed using two methods, both presented by Abdel-Aal et al. (1999; 2006): Quantification was performed by HPLC/DAD (diode array detector) and a rapid photometric method to approximate the total anthocyanin content (TAC).

For both methods, barley or malt was finely ground in the grain mill (Laboratory Mill 120, Perten Instruments, Stockholm, Sweden) and extracted in acidified methanol. For extraction, the sample containers were agitated for 30 min on the roller mixer (RS-TR05, Phoenix Instrument, Garbsen, Germany) and then centrifuged (Centrifuge 2810 R, Eppendorf, Hamburg, Germany; t = 30 min, a = 17000 g). For the rapid method, the supernatant in the volumetric flask was made up to 50 ml. A further purification step was necessary for the HPLC method in which the supernatants were stored overnight at $\vartheta = -20$ °C and then centrifuged again (t = 30 min, a = 17 000 g, $\vartheta = 0$ °C). The supernatant was used as a sample.

2.2.4 Measurement

For the rapid method, the absorbance at $\lambda = 535$ nm was determined for the sample in the photometer (DR 6000, Lange) and converted to the total anthocyanin content according to the formula TAC = 288.21 × A presented by Abdel-Aal et al. (1999) [49].

HPLC separation was performed using an Agilent Series 1200 separation system equipped with a Phenomenex Luna 5u (100 A, 300 x 3.90 mm, 5 micron) C18 column. The following elution gradients were used: 0–35 min, 5–40 % B; 35–36 min, 40–100 % B; 36–44 min, 100 % B; 44–45 min, 100–5 % B; 45–50 min, 5 % B.

Five calibration solutions were prepared from the anthocyanin standards with 1, 2, 5, 10 and 20 mg/l of each anthocyanin. From these, simple regression lines were set up, on the basis of which the DAD was calibrated before each of the two measurement runs. The measurements were carried out for all anthocyanins at $\lambda = 525$ nm.

2.2.5 Statistical evaluation

The data were examined in a two-factor analysis of variance, whereby each combination of growing region and any additional information available was defined as an expression of the factor level growing conditions. The processing condition (raw vs. malted) was defined as the second, cross-classified factor level. All marginal differences were determined at a confidence level of $1 - \alpha = 90$ %.

3 Results and discussion

3.1 Melanine

3.1.1 Identification

All extracted samples show extinction patterns with an approximately exponentially decreasing curve between $\lambda = 250$–400 nm (Fig. 1). The extinction of both the synthetic melanin and the two grain samples examined could be mapped as exponential functions with a high coefficient of determination ($R > 0.98$). Since this curve is typical for melanins, the result of this preliminary photometric test may be interpreted as meaning that the extracted black substance

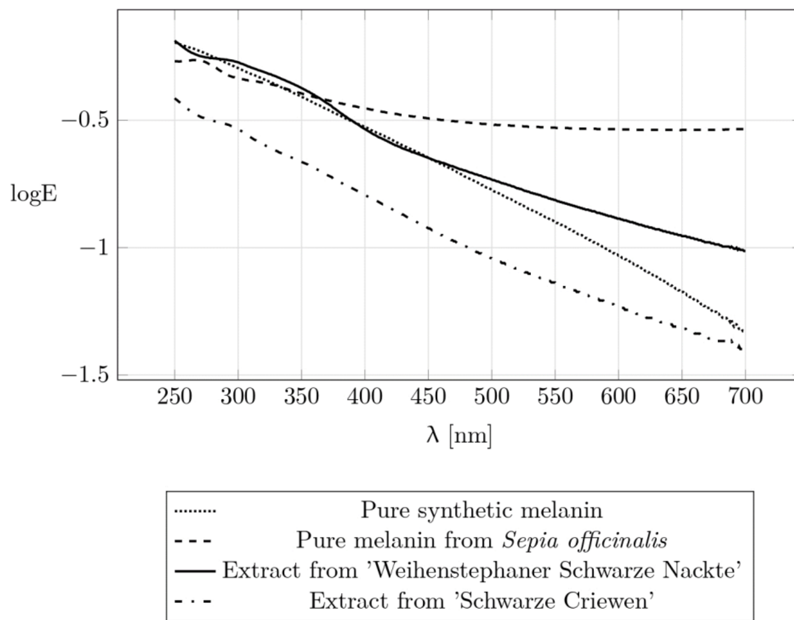

Fig. 1 Extinction of different melanins on a logarithmic scale

is very probably an (allo)melanin.

3.1.2 Structural characterisation

The MALDI-TOF spectra of all investigated samples show a similar pattern: A main peak at m/z 665 is followed by a series of n = 2 – 3 equidistant peaks after each Δm = 178 Da. All peaks are flanked by several minor peaks, which are consistent with sodium and potassium adducts of the same molecule. As the ions are singly charged, Δm/z = 1 corresponds to a molecular mass difference of 1 Da (Fig. 2).

The presence of the main peak at m/z 665 and the repeating peak sequence with mass difference of 178 Da is to be interpreted that the detected substance is present as an oligomeric mixture consisting of a common core group with a differing number or complete absence of a monomer of mass 180 Da. The difference of 2 Da results from the bond within the polymer replacing two hydrogen atoms. This is a markedly different structure from that presented by Varga et al. (2016) as oat allomelanin, which was a homopolymer of *p*-coumaric acid. Based on the assumption that the melanin detected in barley is also composed of phenolic acids, it is hereby hypothesised that this is a heteropolymer with at least n = 1 – 3 caffeic acid units. Caffeic acid is a phenolic acid that is closely related structurally to coumaric acid and has been detected in barley and other cereals [50].

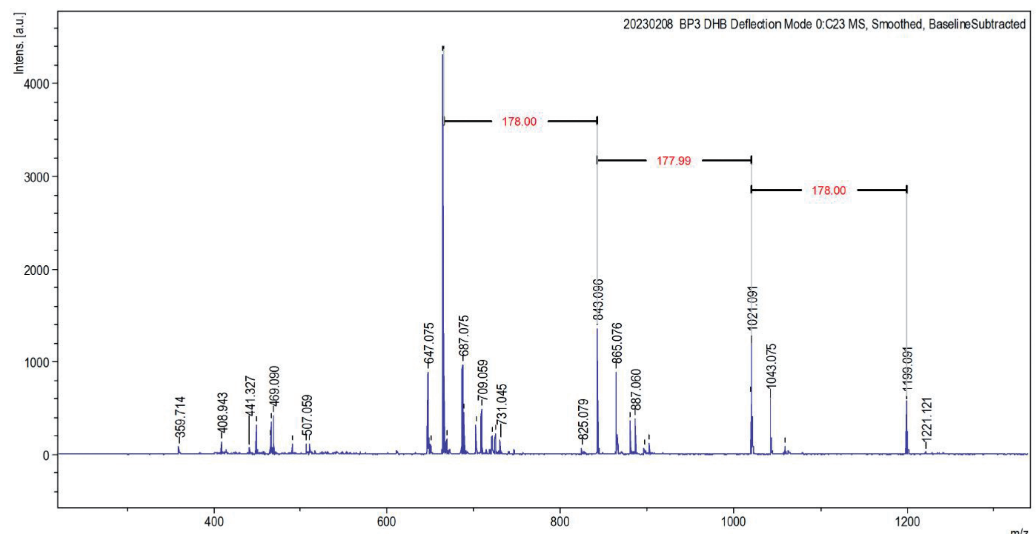

Fig. 2 MALDI-TOF mass spectrum of a barley malt sample base peak at m/z 665

No conclusions can be drawn about the core group structure of the melanin molecule, as no further regularities can be detected in the mass spectra. As the m/z value of the main peak cannot be rationalised based on multiples of the monomeric unit. However, it can be excluded that the substance is a homopolymer. Further work will be required to define the core group structure in detail.

3.1.3 Identification and quantification of individual anthocyanins

The anthocyanin standards eluted between t_R = 25 and t_R = 33 min in the same order as reported in several previous publications: Delphinidin-, cyanidin-, pelargonidin-, peonidin-, malvidin-3-glucoside. While no anthocyanins were found in the ancient spring barley varieties 'Schwarze Criewen', 'Schwarze Pfauengerste' or the Syrian variety, all five target substances were detected in the different samples of the variety 'Weihenstephaner Schwarze Nackte', but in varying composition and partly only in some of the samples.

In all samples, by far the largest proportion was accounted for by delphinidin-3-glucoside, which is in line with previous publications on black and blue barley varieties. In contrast, cyanidin-, peonidin- and malvidin-3-glucoside were detected as the main components in purple varieties. With the exception of malvidin-3-glucoside, these substances were also found in all unmalted samples. Pelargonidin-3-glucoside, on the other hand, was only detected in a small amount in a single sample.

The significant and strong decrease of almost all anthocyanins in the malt samples compared to the unmalted grain is striking (Table 1, see page 102): The total amount of quantifiable anthocyanins was between 12 and 66 % lower than their initial amount. While delphinidin-3-glucoside decreased significantly and cyanidin-, pelargonidin- and malvidin-3-glucoside dropped below the detection limit in some cases, malvidin-3-glucoside increased significantly in

Table 1 Anthocyanin content HPLC [mg/kg], rounded mean values of triple determination

Growing conditions	Anthocyanin content in mg/kg					
	Dp	Cy	Pg	Pn	Mv	∑*
Lower Rhine 2021						
Raw grain	64.74	10.19	n/d	8.90	n/d	83.88
Malt	19.33	3.61	n/d	4.49	n/d	27.48
Kleve 2022, n.t.						
Raw grain	25.04	3.56	3.02	2.65	0.97	35.30
Malt	21.95	n/d	n/d	1.87	6.74	30.55
Kleve 2022, t.						
Raw grain	23.19	3.31	n/d	6.89	1.14	34.56
Malt	16.15	n/d	n/d	1.29	9.63	27.07
Haus Düsse 2022						
Raw grain	19.53	2.73	n/d	5.10	3.74	31.15
Malt	6.98	n/d	n/d	n/d	7.22	14.20
Bingen 2022, n.i.						
Raw grain	16.69	3.62	n/d	3.67	n/d	24.03
Malt	9.23	n/d	n/d	n/d	0.24	9.47
Bingen 2022, i.						
Raw grain	14.88	2.07	n/d	1.92	n/d	18.90
Malt	9.67	n/d	n/d	n/d	n/d	9.67
LSD	0.74	0.32	0.34	0.48	0.69	

*Sum of quantifiable anthocyanins. Others were detected in all samples.

Dp: Delphinidin-3-glucoside, Cy: Cyanidin-3-glucoside, Pg: Pelargonidin-3-glucoside,

Pn: Peonidin-3-glucoside, Mv: Malvidin-3-glucoside.

n.t.: not treated; t.: treated; n.i.: not irrigated; i.: irrigated

n/d: not detected

LSD: Least significant difference ($\alpha = 0.1$)

all samples in which it was already present in the unmalted state. The reason for this is unclear, but it is conceivable that malvidin is initially present in the grain as a colourless precursor and is only converted into the colour-active anthocyanin configuration during malting. The conversion of colourless anthocyanogens into anthocyanins is influenced by the effects of heat and acid and could therefore possibly also be specifically controlled in a well-honed malting process [51].

The fact that the majority of anthocyanins, in contrast, strongly decrease during malting contradicts previous assumptions that malting does not influence the anthocyanin composition.

In all chromatograms, another peak was found after malvidin-3-glucoside (t_R = 37 min). Since this substance elutes very quickly after the known anthocyanins and absorbs light in the same wavelength range, it can be assumed that this is another anthocyanin. The precise anthocyanin could not be determined without considerable additional effort; nor would even an approximate quantification be possible without a calibration standard. For this reason, it was not possible to carry out HPLC quantification of the total anthocyanins.

It is possible that the additional anthocyanin is a malonyl or succinyl glucoside of the anthocyanidins mentioned, which usually elute after the anthocyanins determined here. Anthocyanins based on other anthocyanidins from barley, however, are not yet known [3].

3.1.4 Total anthocyanin content

Using the rapid photometric method, it was first possible to confirm the finding that ancient spring barley 'Weihenstephaner Schwarze Nackte' was the only variety examined that contained anthocyanins; the other samples showed no local extinction maximum in the typical range between 520–550 nm (Fig. 3).

In the samples of ancient spring barley 'Weihenstephaner Schwarze Nackte', the photometrically determined total anthocyanin contents could be compared both between the individual cultivation areas and between grain and malt. Significant differences were found between all the cereals grown under different conditions, which coincided with the values of the different anthocyanins determined by HPLC. In contrast, the photometrically determined data on anthocyanins in malt must be viewed with caution: Here, decreases of varying extents were observed, in some cases even an increase in anthocyanins. The precise HPLC results do not provide a basis for this correlation. Rather, the reason for the apparent increase in anthocyanins can be inferred by closely examining the extinction graphs: The extinction of the samples is increased in the entire range below $\lambda < 500$ nm, which is probably due to the carotenoids absorbing at low wavelengths: The content of carotenoids, especially xanthophylls, increases markedly in the course of malting [52]. If the y-shift of the entire spectra is taken into account, the increase of the local maximum in the red region is much smaller.

Although Abdel-Aal's method depicts the contents in the raw grain similarly to the HPLC results, the values in the malt analysis are clearly distorted. Since the contents occurring in the malt are of particular interest for a possible use of anthocyanin-containing barley for beer production, it would be desirable to develop an independent photometric method for approximating the total anthocyanin content in barley malt. For this, all anthocyanins would first have to be quantified by HPLC and then a factor determined for conversion into the extinction. It might be useful to include a second wavelength in the yellow range in addition to the anthocyanin peak in order to quantify and calculate the increase in carotenoids.

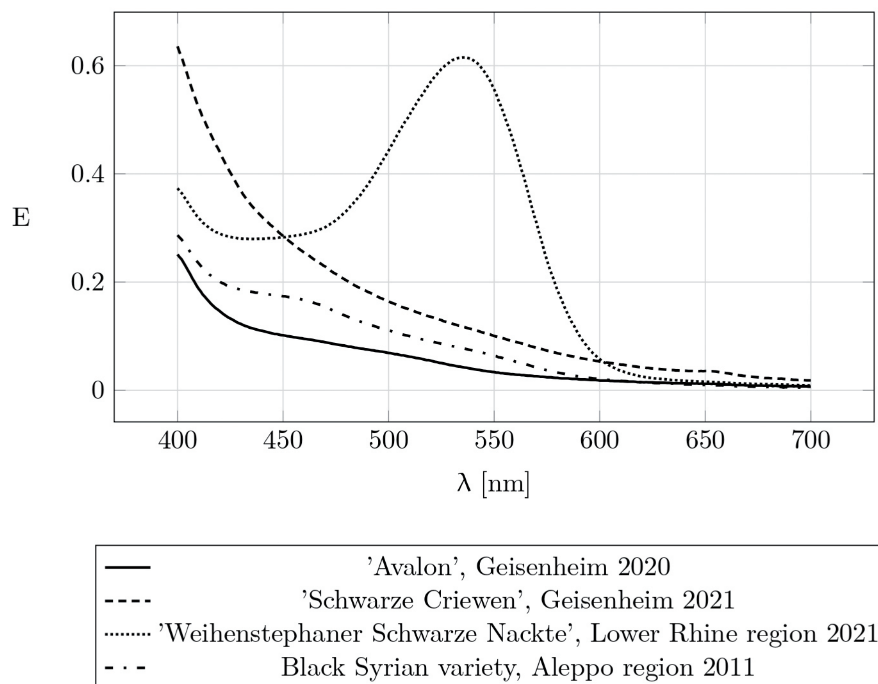

Fig. 3 Extinction patterns of different barley varieties: The colour intensity of the extracts differs strongly according to the variety. Only the variety 'Weihenstephaner Schwarze Nackte' has a peak at λ ≈ 535 nm, which is typical for anthocyanins

4 Conclusion

The substances responsible for the colouring of dark barley are allomelanins and various anthocyanins. Using MALDI-TOF mass spectrometry, it was possible to gain a small insight into the hitherto little-researched structure of the melanins, which suggests that the melanins of different barley origins are similar, but fundamentally different from those found in oats. Specifically, they appear to be heteropolymers that are partly formed from caffeic acid monomers.

Using HPLC analyses, it was possible to confirm the findings that anthocyanins are present in some barley varieties, of which delphinidin-3-glucoside represents by far the largest proportion. Furthermore, the 3-O-glucosides of cyanidin, pelargonidin, peonidin and malvidin were detected in several, but not all samples. Contrary to earlier publications, a clear decrease in most anthocyanins could be detected during grain malting. Malvidin-3-glucoside was the only anthocyanin that actually increased during malting, possibly due to synthesis from precursors promoted by heat.

Anthocyanin contents were detected in the ancient spring barley variety 'Weihenstephaner Schwarze Nackte' that are at the upper end of the values known to date for black varieties [3, 22, 23]. In contrast, no anthocyanins were found in the other varieties examined. Therefore, it makes sense to take a closer look at the "black barley" category, which has so far been commonly used for categorisation [49, 53–55] and to distinguish those varieties with and without anthocyanins.

The rapid photometric method also provided solid results for the examination of black malting barley, but its suitability for malt analysis must be questioned. It may be possible to develop a rapid method specifically designed for malt in order to quickly and reliably determine the total anthocyanin content of this raw material, which is particularly important for quality aspects of beer.

Acknowledgement

Thank you to Felix Baumgartner for his support during the small malting trials at Geisenheim University. The authors would also like to thank the North Rhine-Westphalia Chamber of Agriculture, Plant Genetic Resources for providing the seeds of the dark spring barley of ancient origin.

Funding

The work was financially supported by the EU-funded project "Sustainable crop rotation expansion through summer cereals in water-sensitive areas using the example of ancient cereal varieties for baking and brewing" (korn B). This involves funding from the European Agricultural Fund for Rural Development.

Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Received 31 August 2023, accepted 27 September 2023

Supplementary material

Table I Most common anthocyanidins

Name	R_1	R_2	λ_{max} [nm]	Colour impression
Pelargonidin	H	H	520	orange-red
Cyanidin	OH	H	535	orange-red
Delphinidin	OH	OH	545	purple
Peonidin	OCH_3	H	515	orange-red
Petunidin	OCH_3	OH	525	purple
Malvidin	OCH_3	OCH_3	525	purple

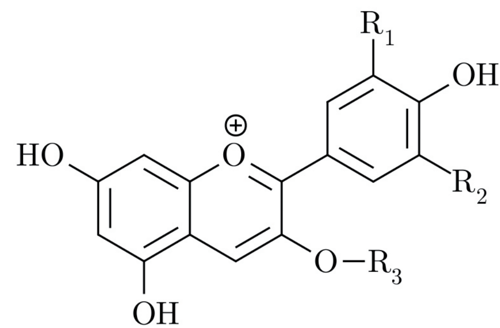


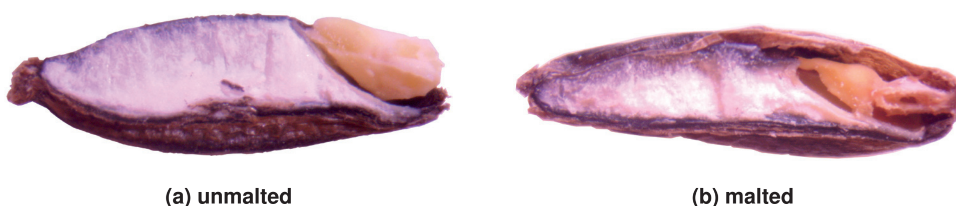
Fig. I R_3 is always a sugar molecule in anthocyanins and an H atom in anthocyanidins. The individual anthocyanidins differ from each other in the substituents $R_{1,2}$

Table II Important anthocyanins and their occurrence in nature

Name (systematic)	Name (trivial)	Occurrence (examples)	References
Cyanidin-3-glucoside	Chrysanthemin, Kuromanin	Cherry, red cabbage, variegated maize, purple barley, apple	[3, 54, 56–58]
Cyanidin-3-galactoside	Ideain	Apple, pear, *Crataegus* spp.	[19, 58]
Cyanidin-3-rutinoside	Antirrhinin	Apricot, plum	[59, 60]
Malvidin-3-glucoside	Oenin	Blueberry, kidney bean	[61, 62]
Malvidin-3-galactoside	Primulin	Blueberry, cranberry	[63, 64]
Peonidine-3-glucoside	–	purple wheat, blueberry	[57]
Pelargonidin-3-glucoside	Callistephin	Blueberry, raspberry, strawberry, colourful maize, red rice	[3, 57, 62]
Delphinidin-3-glucoside	Myrtillin	Blackcurrant, black and blue barley, blueberry, blood orange, kidney bean, aubergine	[54, 65, 66]

Table III Dark chromophores in natural substances and living organisms

Substance class	(presumed) structure	Occurrence (selection)	References
Eumelanin	5,6-Dihydroxyindole copolymer	Dark fur, skin, feathers, eyes	[67, 68]
Phaeomelanin	Benzothiazine copolymer	Red fur, mucous membranes	[68]
Allomelanin	Polymer or oligomer of phenolic acids	*Hordeum vulgare*, *Oryza sativa*, numerous fungal species	[33, 69, 70]
Phytomelanin	Polyvinyl alcohols, hydrocarbons	Tough seed coat in *Helianthus* spp. and other composite plants	[71, 72]
Melanoidins	Glycosylamines	Maillard products in coffee, bread, roasted malt	[73–75]



(a) unmalted (b) malted

Fig. II Distribution of the dark substances in the barley grain (10x magnified photograph)