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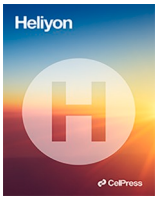

Research article

A global set of barley varieties shows a high diversity in starch structural properties and related gelatinisation characteristics

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ABSTRACT

The gelatinisation temperature and bimodal granule size distribution of barley starch are important characteristics regarding resource efficiency and product quality in the brewing industry. In this work, the diversity in starch amylose content and granule proportions in a set of modern barley varieties (N = 23) was investigated and correlated with their starch gelatinisation behaviour. Milled barley samples had peak starch gelatinisation temperatures ranging from 60.1 to 66.5 °C. Upon separating the barley starch from the non-starch compounds, sample-dependent decreases in starch gelatinisation temperatures were observed, indicating the importance of differences in barley composition. The peak gelatinisation temperatures of milled barley and isolated barley starches were strongly correlated (r = 0.96), indicating that the behaviour of the starch population is strongly reflected in the measurements performed on milled barley. Therefore, we investigated whether amylose content or starch granule size distribution could predict the gelatinisation behaviour of the starches. Broad ranges in the small starch granule volumes (13.9–32.0 v/v%) and amylose contents (18.2–30.7 w/w%) of the barley starches were observed. For the barley samples collected in the north of the USA (N = 8), the small starch granule volumes correlated positively with the peak gelatinisation temperatures of barley starches (r = 0.90, $p < 0.01$). The considerable variation in starch properties described in this work highlights that, besides starch content, starch gelatinisation temperature or granule size distribution might provide brewers with useful information to optimise resource efficiency.

1. Introduction

Annually, over 140 million metric tons of barley is harvested [1], of which approximately 21 % is intended for the malting, brewing and distilling industry [2]. Malting barley varieties have been selected to provide high brewing yields, i.e. a maximum conversion of starch to fermentable sugars, primarily maltose, and dextrin during mashing [3,4]. In producing alcoholic beers, high brewing yields are associated with a high proportion of maltose to dextrin [5]. To provide such high brewing yields, malting barley varieties have been bred to be of a good malting quality. This entails a low base protein content (10–12 dw %) [6,7], a low β-glucan content (2.8–5.1 dw %) [8,9], a high germinative capacity and the possibility to synthesise high concentrations of starch and non-starch-hydrolysing enzymes

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such as protease, β-glucanases and xylanases. A high base starch content (50.1–77.3 dw %) is also considered a primary breeding goal as barley starch is the main source of fermentable sugars in the brewing process [3,4,10–12]. Other starch properties are, however, not the main focus of breeders.

Barley starch consists of supramolecular structures built of glucose-based amylose and amylopectin molecules. Amylose is mostly linear with limited branching and takes up 23–28 % of the barley starch content. Amylopectin is branched and accounts for 72–77 % of barley starch [13–16]. Barley starch shows a bimodal granule size distribution, with large lenticular A-type starch granules (LSG) having reported dimensions of 19.0 μm × 14.9 μm x 7.5 μm and small spherical B-type starch granules (SSG) having a diameter of approximately 3 μm [17].

While in the past, reported SSG proportions in barley ranged from 6.2 to 26.7 on a volume-based percentage (v/v%) [18–22], recently even volume percentages of up to 39 v/v% were reported [17,18]. At this point, it is not fully understood whether changes in the growing environment or breeding led to these higher volume percentages of small starch granules, nor is the impact of small starch granules on the malting and brewing process fully elucidated.

An essential characteristic of starch is the range of temperatures over which the polymer gelatinises. Starch gelatinisation is an irreversible process during which the molecular order and the crystalline structure of the starch granules are lost in the presence of water when a specific temperature is exceeded. For barley starch, this process occurs at temperatures between 50 and 74 °C [23–25]. After gelatinisation, starch is more susceptible to enzymatic hydrolysis by the starch-hydrolysing enzymes, which produce fermentable sugars and dextrins.

Barley starch gelatinisation temperatures were shown to increase by 0.4 °C–3.0 °C during malting [26–28]. During brewing, starch gelatinisation occurs over a broad temperature range. Gelatinised starch granules are predominantly broken down into fermentable sugars and dextrins by the combined action of β-amylase, α-amylase, limit dextrinase and α-glucosidase. These sugars cause an increase in the gelatinisation temperature of the non-gelatinised starch fraction. As a result, the gelatinisation temperature can be increased to above the inactivation temperature of β-amylase, resulting in reduced production of fermentable sugars and, thus, a decrease in the brewhouse efficiency [29,30]. Given the dynamic balance between starch gelatinisation temperatures and enzyme inactivation temperatures, low starch gelatinisation temperatures in barley are generally desired.

Changes in the bimodal starch granule size distribution can affect the barley starch gelatinisation behaviour as SSGs gelatinise at temperatures of, on average, 3 °C higher than LSGs [22,27,31]. Therefore, it can be hypothesised that barley varieties with higher volumes of SSG will have higher overall starch gelatinisation temperatures. At this point in time, a range of SSG proportions in modern industrially-used barley varieties is not documented. Still, it might be broader than generally assumed, considering the recent results of De Schepper et al. (2019) [17]. Besides, contradictory findings have been published about the effect of amylose content on the starch gelatinisation temperature. These can be traced back to differences in the molecular starch structure [32,33]. Therefore, a fresh look at the range in volumes of small starch granules and amylose contents in barley and their effect on starch gelatinisation is needed.

Starch gelatinisation can be affected by the presence of non-starch compounds, such as sugars and minerals native to the barley kernel [27,34,35]. Starch gelatinisation temperatures decrease on average by 2.9 °C upon removing non-starch material from the starch [27,36]. The concentrations of the non-starch materials can differ between barley samples due to varietal effects or differences in the growth conditions. The non-starch material fraction could, therefore, have an important impact on the variability in starch

Table 1
Overview of the collected barley sample set with barley type (2-row/6-row), crop year and growing location.

Variety	Type	Crop year	Growing region
ABI Cardinal	2-row	2021	USA
ABI Eagle	2-row	2021	USA
ND Genesis	2-row	2021	USA
AAC Synergy[A][a]	2-row	2021	USA
ABI 2IM14-8212	2-row	2021	USA
ABI Voyager[A]	2-row	2021	USA
ABI Voyager[B]	2-row	2021	USA
ABI Voyager[C]	2-row	2020	USA
AAC Synergy[B]	2-row	2021	Canada
AAC Synergy[C]	2-row	2021	Canada
Grace	2-row	2021	Uruguay
Explorer	2-row	2021	Uruguay
KWS Fantex	2-row	2021	France
RGT Planet	2-row	2021	France
Laureate	2-row	2021	France
Kadie	2-row	2021	South-Africa
Fandaga	2-row	2021	Ukraine
Overture	2-row	2021	Ukraine
Despina[A]	2-row	2021	Russia
Despina[B]	2-row	2021	Russia
PL-426[A]	6-row	2021	India
PL-426[B]	6-row	2021	India
Leabrook	2-row	2021	Australia

[a] The superscript behind the variety name is used to distinguish between samples of the same genotype based on their, growing region or crop year.

gelatinisation temperatures of barley samples.

Given the above, the aim of this work is to obtain a clear view on the diversity in starch gelatinisation behaviour of industrially relevant barley varieties and to understand if this diversity can be explained by variations in small starch granule volumes and amylose contents or differences in the non-starch barley composition. This study firstly focuses on the variation in the gelatinisation behaviour between the different barley samples in both milled samples and pure isolated starches. Secondly, the ranges in granule and amylose to amylopectin proportions were determined. Lastly, a correlation analysis between the observed differences in starch gelatinisation, the chemical characterisation of the barley composition and the starch properties (amylose content, granule proportions) is performed. These results could provide novel raw material selection criteria for barley breeders and the brewing industry.

2. Material and methods

2.1. Materials

Twenty-three barley samples were provided by Anheuser-Busch InBev SA/NV (Leuven, Belgium). The sample set consisted of twenty-one two-row barley samples and two six-row barley samples grown in various locations worldwide: the United States of America, South America, Europe, Africa, Asia and Australia. All samples were from the crop year 2021, except one Voyager sample grown in 2020. The different barley varieties and their respective growing location are listed in Table 1.

2.2. Characterisation of the chemical composition of barley

The chemical characterisation of the barley samples was performed using triplicates. The barley samples were milled using a Tecator Cyclotec 1093 (Foss, Hillerod, Denmark) to a particle size below 0.5 mm. The starch content of the milled barley samples was quantified using the Rapid Total Starch method from Megazyme (Bray, Ireland) with some modifications. Half of the sample size and buffer volumes that were described in the procedure were used. Each sample was corrected for its specific glucose content. β-glucan content was determined using the mixed-linkage beta-glucan kit, EBC Method 3.10.1, from Megazyme (Bray, Ireland). Total arabinoxylan content was quantified according to the method of Courtin, Broekaert & Swennen (2009) [38], and Englyst & Cummings (1984) [37], using an Agilent 6890 gas chromatograph (Santa Clara, California, USA) with flame ionisation detector carrying a Supelco SP 2380-column (Bellefonte, PA, USA). The protein content was determined using a VarioMax cube N (Elementar, Hanau, Germany), which quantifies the total nitrogen content of a sample. The total protein content was calculated by multiplying the total nitrogen content by 6.25, the conversion factor specific for barley protein [39]. The sugar content (glucose, fructose, sucrose, maltose and maltotriose) was quantified as described by De Schepper & Courtin (2023) [40], using an ICS-5000 HPAEC-iPAD device equipped with a PA-100 pre-column and a PA-100 analysis column (Thermofisher Scientific, Waltham, USA).

2.3. Isolation of starch granules from barley

Starch was isolated from barley in duplicate as described by De Schepper et al. (2019) [17], with minor adaptations. Two grams of disc-milled barley (setting two, a distance of 0.2 mm between the two discs) was incubated for 15 h at 45 °C in 20 mL of a sodium phosphate buffer (100 mM, pH 8.0) containing 0.1 M EDTA and 100 μL Alcalase (2.5 Anson units/g, Novozymes, Bagsvaerd, Denmark). The alkaline pH combined with the 0.1 M EDTA inhibits the activity of α-amylase during the treatment [17]. After incubation, the starch granules were separated from the barley matrix by washing over a 38 μm sieve using 250 mL of 70 v/v% ethanol. The obtained starch suspension was centrifuged (30 min, 1500 g), and the starch pellet was dried to the air. Residual EDTA was removed from the isolated starch before analysis by washing as described by De Schepper & Courtin (2023) [40].

2.4. Determination of the gelatinisation behaviour of barley starch

Cyclotec-milled barley samples or isolated starches (2.5–4.0 mg) were accurately weighed in an aluminium pan (Hitachi High-Technologies, Tokyo, Japan). Afterwards, deionised water was added, in a 1 to 3 dry matter to water ratio, and the pans were hermetically sealed. The pans were heated in a Q2000 differential scanning calorimetry (DSC) device (TA Instruments, New Castle, USA) from 0 °C to 130 °C using a heating rate of 1 °C/min. This heating rate was chosen as it is relevant for the mashing process, and it allows for a quasi-equilibrium state in which starch gelatinisation is not affected by diffusion-limited conditions [41]. The obtained gelatinisation endotherms were integrated with TA Universal Analysis software to determine the onset temperature (GT_o, °C), peak temperature (GT_p, °C), conclusion temperature (GT_c, °C) and the enthalpy (ΔH, $J/g_{starch(dw)}$) of gelatinisation. The gelatinisation enthalpy was corrected for the starch content of the samples. In the results section, a distinction is made between the gelatinisation temperature (GT) measured in milled barley samples using subscript (B) and the gelatinisation behaviour of starch isolated from barley using subscript (S).

2.5. Quantification of the small and large starch granule volumes using bright-field microscopy

The starch granule proportions of the isolated barley starches were quantified as described by De Schepper et al. (2019), [17]. For each starch sample, 10–20 mg was suspended in 9.4 mL water with 0.5 mL of Lugol (0.33 % (w/v) I_2, 0.68 % (w/v) KI) to stain starch and 0.1 mL of light green to stain residual protein material. After sonication, the samples were dispersed over a counting chamber

(Marienfeld, Lauda-Königshofen, Germany). Ten images were captured per sample using an ECLIPSE 80i epifluorescence microscope (Nikon Inc., New York, USA) carrying a Nikon Digital Sight DS-US camera at a magnification of 100×. ImageJ software was used to determine the area and the smallest and largest linear distance between the edges of the individual granules. Using the dimensions

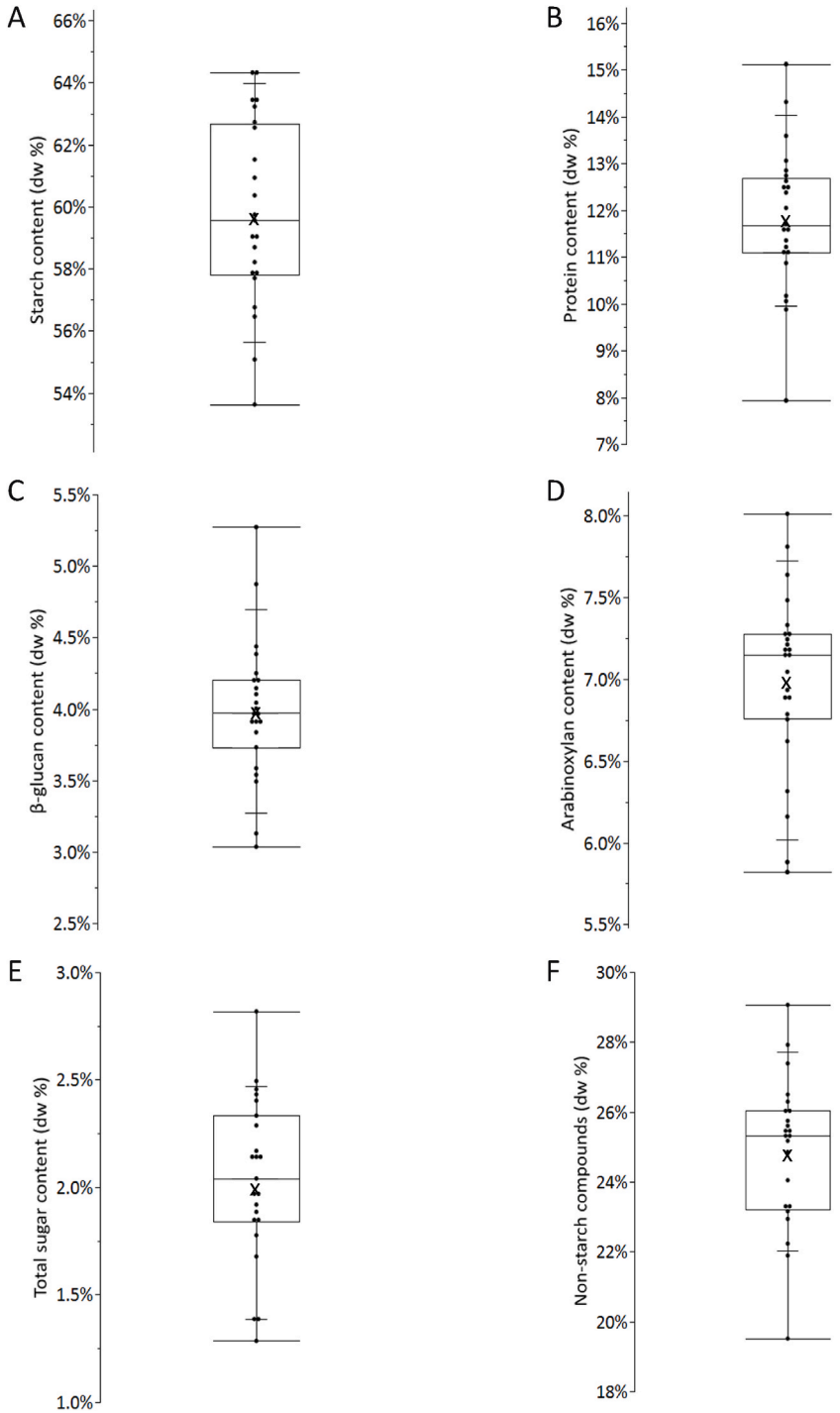

Fig. 1. Boxplot representations of the chemical composition of 23 modern barley varieties on dry weight basis with **A)** the starch content, **B)** the protein content, **C)** the β-glucan content **D)** the arabinoxylan content **E)** the total sugar content and **F)** the sum of all quantified non-starch materials. The boxplots visualise the spread of the data points while identifying the median and the lowest and highest data values, which are indicated by the lower and the upper whiskers. The median is indicated by the black middle line of the box, while the average of the population is indicated by an "x". The values plotted are the result of a biological triplicate measurement.

obtained via image analysis, the small and large starch granules on number basis (n/n%) and volume basis (v/v%) were determined as described by De Schepper et al. (2019), [17]. An aspect ratio of 0.44 was used to calculate the third dimension of the large starch granules, and a cut-off value of 10 μm in diameter was applied to distinguish between small and large starch granules. The average volume of individual small starch granules (V_{SSG}; μm^3) and the proportion of small starch granules on a total starch basis (SSG; v/v%) are reported. The same values were calculated for large granules.

2.6. Quantification of the amylose and amylopectin content in starch

The amylose and amylopectin content of the isolated barley starch samples was quantified according to the method of Ahmed et al. (2019) [33], with some minor adaptations. Here, starch polymers were debranched to linear glucose chains with an isoamylase (≥10 mega units/mg protein, Sigma-Aldrich) treatment. After debranching, the linear chains derived from amylose and amylopectin were separated based on their hydrodynamic radius using a size-exclusion high-performance liquid chromatography (SE-HPLC) system equipped with a Refractive Index Detector and a GRAM 1000 and GRAM 100 column coupled in sequence (PSS, Agilent, Amherst, USA). The system was calibrated using linear pullulan standards of known molecular weight (0.342–805 kDA) (PPS-Polymer) to convert the elution volumes to a hydrodynamic radius. Via the Mark-Houwink equation, the hydrodynamic radius was converted to a degree of polymerisation. Chains with a DP between 2 and 100 are considered to be of amylopectin origin, while chains with a DP > 100 are considered to derive from amylose [33].

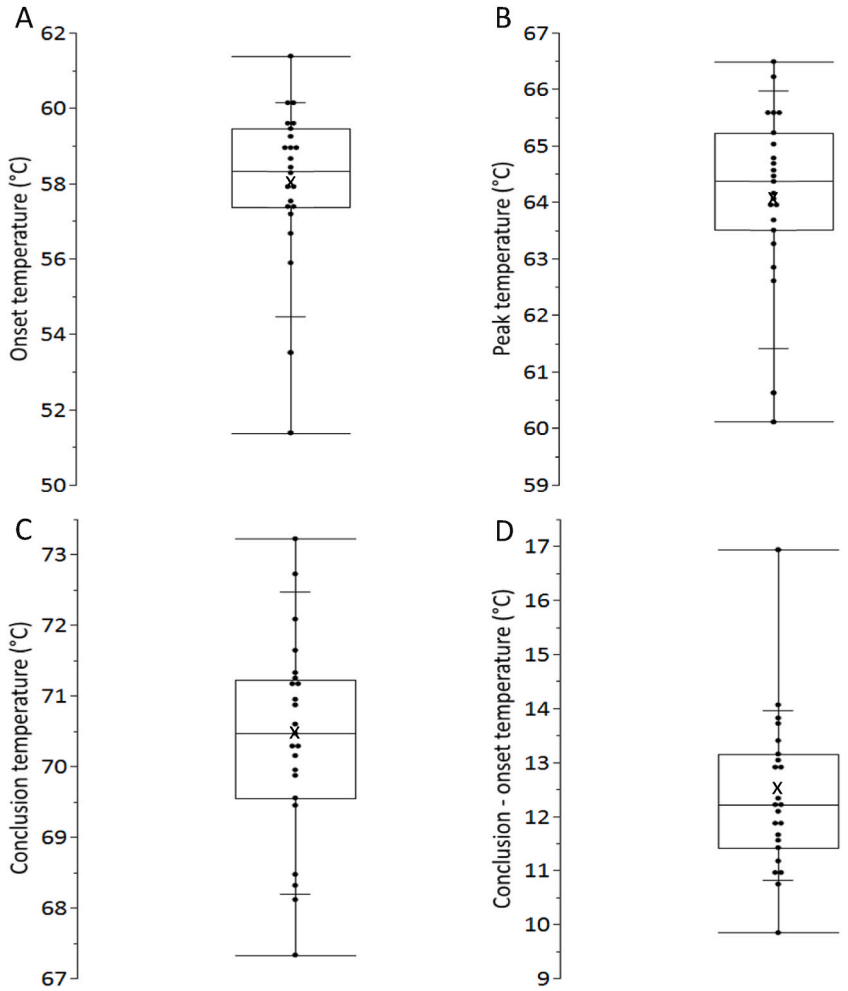

Fig. 2. Boxplot representation of the starch gelatinisation behaviour measured for 23 barley milled samples with A) the onset temperature of gelatinisation **B)** the peak temperature **C)** the conclusion temperature and **D)** the peak width of the gelatinisation endotherm, conclusion – onset temperature. The boxplots visualise the spread of the data points while identifying the median and the lowest and highest data values, which are indicated by the lower and the upper whiskers. The median is indicated by the black middle line of the box, while the average of the population is indicated with "x". The values plotted are the result of a biological triplicate measurement.

2.7. Statistical analysis

JMP Pro 15 Software (SAS Institute Inc., Cary, USA) was used for the statistical analysis of the collected data. F-tests were used to determine overall significant differences ($p < 0.05$). Upon observation of significant differences, an additional Tukey-Kramer post-hoc test was used to compare the mean values further. Multivariate Pearson correlation analysis was performed to quantify Pearson correlation coefficients (r) and the correlation probabilities (p).

3. Results

3.1. Characterisation of the barley composition

The chemical composition of the 23 barley samples, gathered for this study, is provided in Fig. 1. For several varieties, two (Despina and PL-426) or three (Voyager and Synergy) samples were present in the sample set. These were grown in different regions or crop years. A comparison between samples of the same genotype could provide suggestions on the impact of crop year and environmental conditions.

Fig. 1A shows that the starch content ranged from 53.6 dw% for Synergy^{B} to 64.3 dw% for Grace ($\overline{X} = 59.8$ dw%) and was significantly different among the samples ($p < 0.05$). These starch concentrations are in line with what is to be expected for two-row barley samples [11,12,42]. The starch content of PL-426^{A} and PL-426^{B} was 55.1 dw% and 56.5 dw%, respectively, and was lower than the population average, which is in line with literature on six-row barley varieties [42]. Synergy^{B} (53.6 dw%) differed significantly from both Synergy^{C} (62.6 dw%) and Synergy^{A} (60.4 dw%).

The protein content (Fig. 1B) ranged from 7.9 (Kadie) to 15.1 dw% (Synergy^{B}), with a population average of 11.8 dw%. The protein content was negatively correlated ($r = -0.66$, $p < 0.05$) with starch content, consistent with the findings of Holtekjølen et al. (2006), [11]. Significant differences were observed between samples of the same genotype for Voyager and Synergy.

The β-glucan content ranged from 3.0 dw% for Grace to 5.3 dw% for Voyager^{A} (Fig. 1C), while the total arabinoxylan content (Fig. 1D) ranged from 5.8 dw% for Overture to 8.0 dw% for Cardinal. The range found for both non-starch polysaccharides corresponds to the literature [11,43,44]. The β-glucan content of Voyager^{A} significantly differed from the other two Voyager samples. Besides, the PL-426 samples were also significantly different from each other in their β-glucan content. No significant differences were found between the varieties of the same genotype in the arabinoxylan content. The β-glucan content was positively correlated with protein content ($r = 0.50$, $p < 0.05$) and was negatively associated with the starch content ($r = -0.60$, $p < 0.05$), similar to what was observed by Holtekjølen et al. (2006), [11].

The fermentable sugars determined in this work are glucose, fructose, sucrose, maltose and maltotriose. The sum of these sugars ranged from 1.3 dw% for PL-426^{B} to 2.8 dw% for Voyager^{C}. (Fig. 1E). Voyager^{C} was grown in 2020 and had a significantly higher sugar content than the Voyager samples from 2021, which might result from residual enzymatic activity upon storage [45]. Lastly, the sum of all quantified non-starch materials ranged from 19.5 dw% for Kadie to 29.1 dw% for Synergy^{B} ($\overline{X} = 24.9$ dw%) (Fig. 1F). The concentrations of the individual components for each barley sample can be found in Supplementary Table A1.

3.2. The gelatinisation behaviour of starch in milled barley samples

To shed light on the variation in starch gelatinisation behaviour between different industrially used barley varieties, the starch gelatinisation temperatures were quantified in milled barley using DSC. Fig. 2 provides a boxplot representation of $GT_{o(B)}$, $GT_{p(B)}$, $GT_{c(B)}$ and the peak width ($GT_{c(B)} - GT_{o(B)}$) of the gelatinisation endotherms for the 23 barley samples. The gelatinisation data for the individual milled samples can be found in Supplementary Table A2.

For $GT_{o(B)}$ (Fig. 2A), a population average of 58.0 °C was observed with significant differences between the samples ($p < 0.05$). The onset of gelatinisation ranged from 51.4 °C for Leabrook (Australia) to 61.4 °C for Fandaga (Ukraine). Besides Leabrook, Kadie also had a low $GT_{o(B)}$ (53.5 °C) compared to the rest of the barley sample set. No significant differences were observed in the $GT_{o(B)}$ for the barley samples of the same genotype (Voyager, Despina, Synergy and PL-426).

The $GT_{p(B)}$ (Fig. 2B), often considered as an average temperature for gelatinisation of the whole starch population, ranged from 60.1 °C to 66.5 °C ($\overline{X} = 64.1$ °C). Significant differences were observed between the samples ($p < 0.05$). Like $GT_{o(B)}$, Leabrook and Kadie had the lowest peak gelatinisation temperature of the entire sample set. It was further observed that the $GT_{p(B)}$ of the two Despina samples and the two PL-426 samples, grown in Russia and India, respectively, did not differ significantly from each other. In contrast, Synergy^{B} and Synergy^{C} samples, grown in Canada, differed in $GT_{p(B)}$ with 1.4 °C. The same observation can be made for Voyager^{C} and Voyager^{B}.

When heating a starch population in excess water, gelatinisation is not completed until the temperature passes the $GT_{c(B)}$ (Fig. 2C). On average, the $GT_{c(B)}$ was 70.4 °C for the sample set, with a range from 68.3 °C for Leabrook to 73.2 °C for Voyager^{B}. $GT_{c(B)}$ differed significantly between the samples ($p < 0.05$), and similar to $GT_{o(B)}$ and $GT_{p(B)}$, Kadie and Leabrook had the lowest gelatinisation temperature of the sample set. No significant differences were observed between the same genotypes. $GT_{o(B)}$, $GT_{p(B)}$, $GT_{c(B)}$ were positively correlated with each other with an r-value and p-value above 0.75 and below 0.001, respectively, for all three parameters.

The width of the gelatinisation endotherm can be determined by subtracting $GT_{o(B)}$ from $GT_{c(B)}$ (Fig. 2D). $GT_{c(B)}$ and $GT_{o(B)}$ are determined via extrapolations of the slopes of the gelatinisation endotherm. Their value, therefore, does not match the actual start and end of the gelatinisation endotherm. $GT_{c(B)} - GT_{o(B)}$ is therefore considered as a measure for the heterogeneity of the starch

gelatinisation population rather than the temperature range needed to achieve complete gelatinisation [27]. $GT_{c(B)}$ - $GT_{o(B)}$ ranges from 9.8 °C for Fandaga to 16.9 °C for Leabrook ($\overline{X}$ = 12.3 °C) and significant differences were observed between the samples ($p < 0.05$).

The enthalpy of gelatinisation is a measure of the amount of energy needed to gelatinise the starch and is correlated to the crystallinity of the starch [46,47]. $\Delta H_{(B)}$ was determined by integration of the gelatinisation endotherm and corrected for the starch content of the barley samples (Supplementary Table A2). Here, a range of 7.5 $J/g_{starch\ dw}$ for Synergy^{B} to 14.4 $J/g_{starch\ dw}$ for Overture was observed. The variation in the gelatinisation enthalpy can be due to differences in intrinsic starch properties or the presence of macromolecules such as lipids and proteins [48]. However, this was not reflected in the data. No significant differences were observed between barley samples from the same genotype.

A large variation in the gelatinisation behaviour between the different barley samples was observed, in line with the literature [27,49]. The observed variation can be caused by compositional differences in the barley matrix, differences in starch amylose content and differences in the bimodal granule distribution of the starch population [11,27,32]. To assess the impact of these compositional features and differences in intrinsic starch properties on the gelatinisation behaviour, starch was first isolated from the barley matrix.

3.3. The gelatinisation behaviour of starch isolates of barley

Fig. 3 and Supplementary Table A3 show the gelatinisation behaviour of barley starch in the abscence of non-starch material native to barley. Parameters determined for isolated barley starch are indicated with (S) in subscript. $GT_{o(S)}$ ranged from 53.4 °C to 57.7 °C

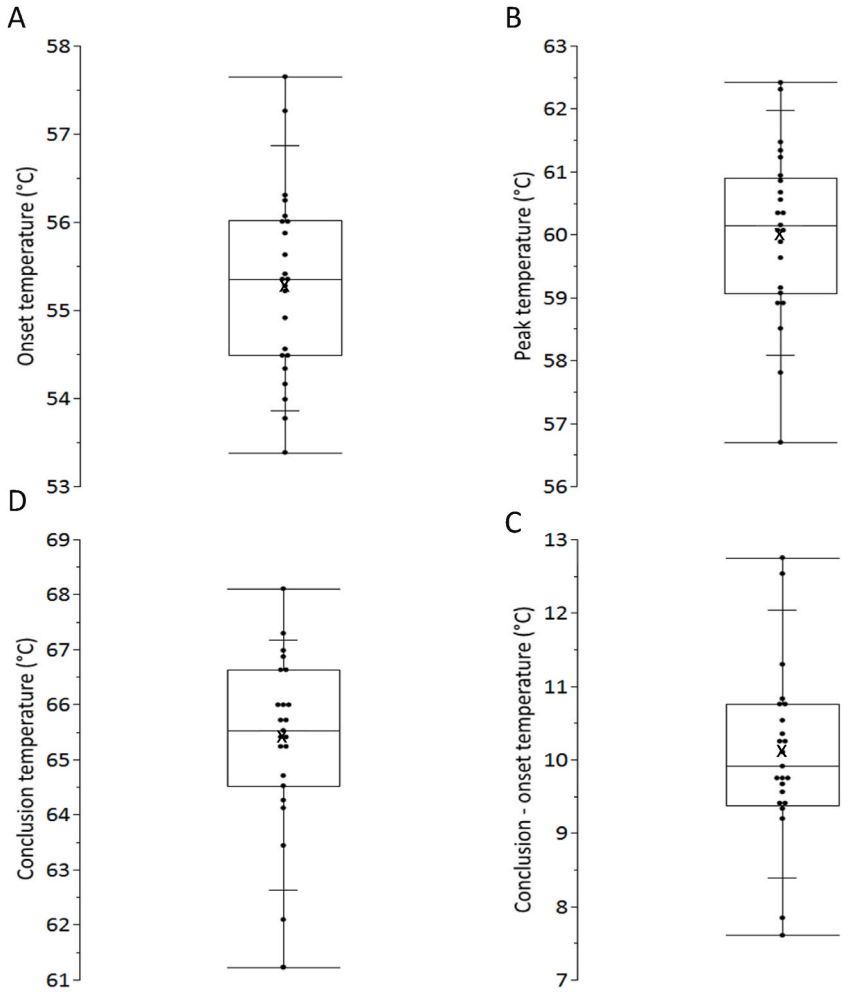

Fig. 3. Boxplot representation of the starch gelatinisation behaviour measured for 23 barley starch samples with A) the onset temperature of gelatinisation, **B)** the peak temperature, **C)** the conclusion temperature, and **D)** the peak width of the gelatinisation endotherm equal to $GT_{c(S)}$ - $GT_{o(S)}$. The boxplots visualise the spread of the data points while identifying the median and the lowest and highest data values, which are indicated by the lower and the upper whiskers. The median is indicated by the black middle line of the box, while the population's average is indicated by "x". The average values plotted are determined based on a duplicate measurement of two starches isolated from the same barley variety, yielding a quadruple measurement per barley sample.

with an average of 55.3 °C. As for the milled samples, the Leabrook variety showed the lowest $GT_{o(S)}$, while Fandaga had the highest $GT_{o(S)}$ of the sample set. $GT_{p(S)}$ ranged from 56.7 to 62.4 °C with a population average of 60.1 °C. Like the milled samples, the isolated barley starches from Fandaga and Overture had the highest peak gelatinisation temperature, while Leabrook and Kadie showed the lowest $GT_{p(S)}$. For $GT_{c(S)}$, a population average of 65.4 °C was measured with a range from 61.2 °C to 68.1 °C. Leabrook and Kadie showed the lowest $GT_{c(S)}$, while the Voyagers of 2021 showed the highest conclusion temperature. Significant differences were observed for $GT_{o(S)}$, $GT_{p(S)}$, $GT_{c(S)}$ ($p < 0.05$), and all three temperatures correlated positively with r-values above 0.70 ($p < 0.05$).

$GT_{c(S)}$ - $GT_{o(S)}$ ranged from 7.6 °C to 12.8 °C with an average value of 10.1 °C. Here, the Kadie sample showed the least heterogeneous starch gelatinisation behaviour, while the Voyager[B] sample displayed the largest heterogeneity. Both Kadie and Leabrook displayed the most heterogeneous gelatinisation behaviour in the presence of the non-starch compounds native to the variety with a temperature range of 14.9 and 16.9 °C, respectively. Here, however, the $GT_{c(S)}$ - $GT_{o(S)}$ equalled 7.6 °C and 7.9 °C for Kadie and Leabrook. Upon comparing the DSC profiles of Kadie and Leabrook barley with the DSC profiles of their respective isolated starches, one can observe that indeed gelatinisation occurs more heterogeneously for barley compared to the isolated starches (**Supplementary graph A.1**). In fact, upon comparing $GT_{c(S)}$ - $GT_{o(S)}$ (Supplementary table A3) with $GT_{c(B)}$ - $GT_{o(B)}$ (Supplementary table A2), it can be observed that the isolated starches gelatinise less heterogeneously. It seems that the non-starch components in the milled barley samples thus induce a more heterogeneous starch gelatinisation. Indeed, several components such as sugars, lipids or proteins interact with the starch granules, altering their intrinsic gelatinisation behaviour [30,48]. We hypothesise that the broadening of the peak could indicate that not all starch granules are affected the same by the presence of these matrix compounds.

The starch gelatinisation enthalpy ranged from 9.5 for Voyager[B] to 12.8 $J/g_{(starch\ (dw))}$ for Overture. Synergy[C] starch had a gelatinisation enthalpy of 11.6 $J/g_{(starch\ (dw))}$, while as a milled barley sample, it had the lowest gelatinisation enthalpy of the sample set (7.5 $J/g_{(starch\ (dw))}$). It thus seems that the presence of non-starch material in the barley matrix may indeed be responsible for the lower observed gelatinisation enthalpy [48].

3.4. Differences in the gelatinisation behaviour between milled barley samples and barley starches

Upon correlating the gelatinisation behaviour of the starch in milled barley samples and their starch isolates, it was observed that the gelatinisation properties $GT_{o(B)}$, $GT_{p(B)}$ and $GT_{c(B)}$ were strongly and significantly ($p < 0.001$) correlated with $GT_{o(S)}$ ($r = 0.76$), $GT_{p(S)}$ ($r = 0.96$) and $GT_{c(S)}$ ($r = 0.84$) respectively. These results indicate that most of the variation in the starch gelatinisation behaviour in milled samples originates from the native starch population. However, in milled samples, higher gelatinisation temperatures were observed than for the isolated starches. In fact, a significant difference of, on average, 2.7 °C, 4.1 °C and 5.1 °C was observed in GT_o, GT_p and GT_c, respectively, between the two sample types and the difference was sample dependent. To illustrate this, the difference between the gelatinisation temperatures observed for barley and isolated starch is shown in Fig. 4 and Supplementary Table A4.

The difference between $GT_{o(B)}$ and $GT_{o(S)}$, further referred to as ΔGT_o, was on average 2.7 °C, with extremes of −2.0 °C and 4.9 °C for Leabrook and Despina[B]. Leabrook and Kadie were the only two samples where the presence of the non-starch compounds led to a decrease in the onset gelatinisation temperature (Supplementary Figure A1). The ΔGT_p was, on average, 4.1 °C with the lowest value for Kadie (2.8 °C) and the highest for Despina[B] (4.9 °C). The difference in conclusion temperatures, ΔGT_c, was on average 5.1 °C, with

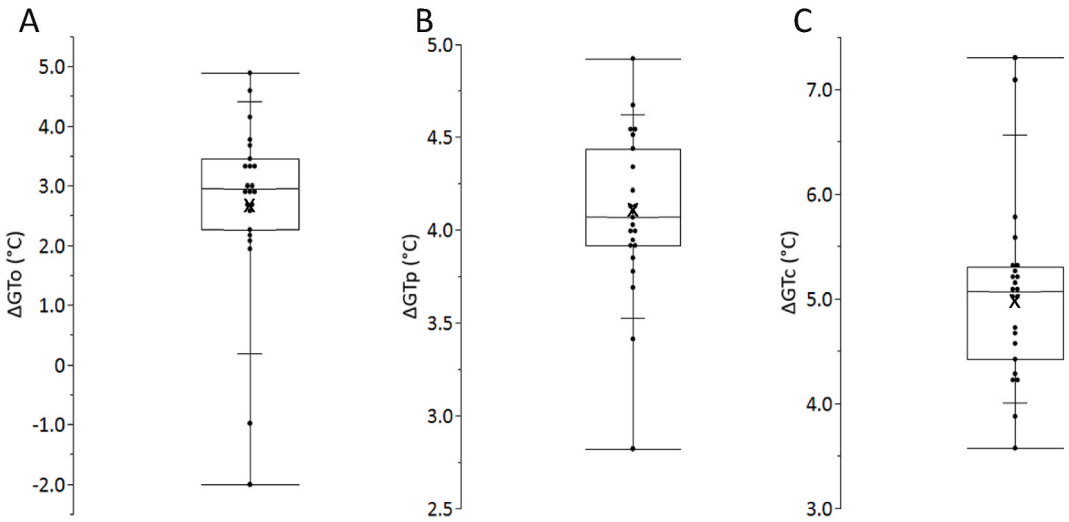

Fig. 4. A boxplot representation of the decrease in the gelatinisation temperature upon removing the non-starch material from the barley starch with A) ΔGT_o equal to the difference in the onset temperature measured in milled samples and the isolated barley starch ($GT_{o(B)} - GT_{o(S)}$) **B)** ΔGT_p equal to the difference in the peak temperature measured in milled samples and the isolated barley starch ($GT_{p(B)} - GT_{p(S)}$) and **C)** ΔGT_c equal to the difference in conclusion temperature measured in milled barley samples and isolated barley starch ($GT_{c(B)}$ - $GT_{c(S)}$). The boxplots visualise the spread of the data points while identifying the median and the lowest and highest data values, which are indicated by the lower and the upper whiskers. The median is indicated by the black middle line of the box, while the average of the population is indicated by "x".

the lowest difference for Voyager^{A} (3.6 °C) and the highest for 2IM14-8212 (7.3 °C).

Based on the results above, the distinct decrease in starch gelatinisation temperature upon removing the non-starch material on the starch gelatinisation behaviour suggests that the composition of the non-starch fraction might be important when selecting for low gelatinisation temperatures in barley. However, given the strong correlation between the gelatinisation behaviour measured in milled barley samples and isolated starches, we investigated if intrinsic starch properties such as amylose and granule proportions could be a driving factor behind differences in the starch gelatinisation temperatures within the sample set.

3.5. Amylose and amylopectin proportions in barley starch

The total amylose content is provided in Fig. 5 and Supplementary Table A5. A broad range in the amylose content was observed, going from 18.2 % for PL-426^{A} to 30.7 % for 2IM14–8212 ($\overline{X} = 24.7$ %). Significant differences were observed between the samples ($p < 0.05$), and the obtained amylose and amylopectin values were in line with the literature [14,32]. Synergy^{B} differed significantly from the other Synergy samples in the set, and Voyager^{A} differed significantly from Voyager^{C}. The other samples from the same genotype (PL-426 and Despina) did not differ significantly from each other.

3.6. Barley starch granule proportions

The granule properties of the different isolated starches are shown in Table 2. The bimodal size distribution of barley starch granules can be quantified in two ways, namely on a number basis (n/n%) and a volume basis (v/v%). On a number basis, a narrow range of 96.2–98.4 n/n% with a population average of 97.4 n/n% was observed for the small starch granules. These values are similar to those reported in the literature for barley starch [17,50]. Number-based percentages are often less relevant for industrial purposes as they cannot be easily related to starch mass. Therefore, volume-based percentages are more appropriate in this context. The volume proportions of SSG differed significantly between the samples ($p < 0.05$) with a broad range from 13.9 for Kadie to 32.0 v/v% for Despina^{B} ($\overline{X} = 23.2$ v/v%). The observed volume-based percentages of the small starch granules are lower and more diverse than those described in the work of De Schepper et al. (2019) [17], where values from 32 to 39 v/v% were reported.

Variation in the volume-based percentages can be due to the amount of SSG (n/n%) and the volume of the individual granules (V_{SSG}). The average volume of the individual SSG and LSG was, therefore, determined. For the SSG, an average granule volume of 13.5 μm^3 was observed with a range from 10.4 μm^3 for Kadie to 16.8 μm^3 for Despina^{B}. For the LSG, a range from 1416 μm^3 for Voyager^{C} to 3232 μm^3 for Synergy^{A} ($\overline{X} = 2141$ μm^3) was observed. Significant differences were present between the samples for both granule types ($p < 0.05$). The small starch granule volumes were positively correlated with the average volume of the small starch granules ($r = 0.52$, $p < 0.05$) and the SSG on a number basis ($r = 0.57$, $p < 0.05$). It thus seems that higher SSG proportions in barley can be due to the formation of more small starch granules during grain filling and to the granules being larger. However, these two parameters do not

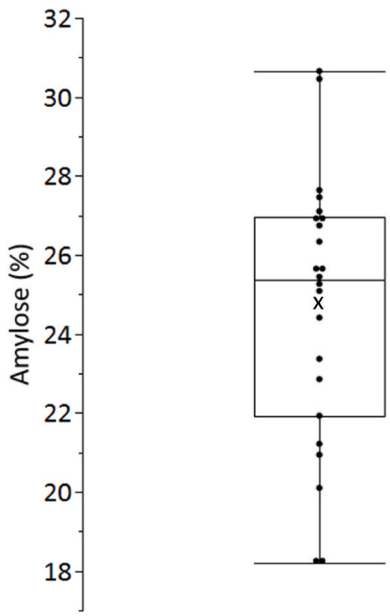

Fig. 5. A boxplot representation of the amylose content for 23 barley starches. The boxplots visualise the spread of the data points while identifying the median and the lowest and highest data values, which are indicated by the lower and the upper whiskers. The median is indicated by the black middle line of the box, while the average of the population is indicated by "x". The average values plotted are determined based on a duplicate measurement of two starches isolated from the same barley variety, yielding a quadruple measurement per barley sample, which includes two technical and two biological replicates.

Table 2
The starch granule proportions for 23 barley starch samples. For each sample, the small starch granule proportion on a number basis (n/n%) and volume basis (v/v%) is provided, as well as the average volume of the small and large starch granules. Standard deviations are determined based on a duplicate measurement of two starches isolated from the same barley variety, yielding a quadruple measurement per barley sample, which includes two technical and two biological replicates. Values within one column are significantly different ($p < 0.05$) when they do not share the same uppercase letters.

Variety	SSG (n/n%)	SSG (v/v%)	V_{SSG} (μm^3)	V_{LSG} (μm^3)
ABI Cardinal	97.2 ± 0.1^{ABCDE}	18.5 ± 1.4^{CDEF}	11.8 ± 0.5^{FGHI}	$2036.5 \pm 287.5^{BCDEFG}$
ABI Eagle	96.5 ± 0.4^{BCDE}	17.2 ± 0.7^{DEF}	11.2 ± 0.1^{HI}	1785.1 ± 189.4^{CDEFG}
ND Genesis	96.4 ± 0.1^{CDE}	15.8 ± 1.1^{EF}	13.6 ± 0.1^{BCDEFGH}	$2171.9 \pm 159.7^{BCDEFGH}$
AAC Synergy[Aa]	98.0 ± 0.0^{AB}	19.3 ± 1.0^{CDEF}	13.8 ± 0.2^{BCDEFGH}	3231.9 ± 67.1^{A}
ABI 2IM14-8212	98.0 ± 0.1^{AB}	27.3 ± 0.3^{ABC}	11.8 ± 0.9^{EFGHI}	1686.8 ± 281.5^{DEFG}
ABI Voyager[A]	97.7 ± 0.0^{ABCDE}	27.1 ± 0.8^{ABC}	15.3 ± 0.1^{ABCD}	$2015.4 \pm 155.7^{BCDEFG}$
ABI Voyager[B]	97.2 ± 0.3^{ABCDE}	25.8 ± 1.4^{ABCD}	14.0 ± 1.1^{ABCDEFGH}	1531.7 ± 9.9^{FG}
ABI Voyager[C]	96.2 ± 0.6^{DE}	24.8 ± 2.7^{ABCDE}	13.1 ± 1.1^{BCDEFGHI}	1416.5 ± 1.0^{G}
AAC Synergy[B]	97.9 ± 1.1^{ABC}	19.25 ± 0.1^{CDEF}	14.2 ± 0.4^{ABCDEF}	2738.0 ± 76.0^{AB}
AAC Synergy[C]	98.2 ± 0.1^{A}	23.9 ± 1.7^{ABCDE}	12.6 ± 0.5^{EFGHI}	2657.9 ± 338.0^{ABC}
Grace	97.8 ± 0.2^{ABCD}	32.0 ± 2.6^{A}	16.1 ± 0.5^{AB}	1757.3 ± 86.3^{DEFG}
Explorer	97.5 ± 0.2^{ABCDE}	25.6 ± 1.3^{ABCD}	12.8 ± 0.5^{CDEFGHI}	1704.7 ± 98.4^{DEFG}
KWS Fantex	97.4 ± 0.2^{ABCDE}	24.7 ± 2.8^{ABCDE}	14.8 ± 0.8^{ABCDE}	2084.0 ± 8.6^{BCDEFG}
RGT Planet	97.3 ± 0.4^{ABCDE}	22.1 ± 1.0^{BCDEF}	15.7 ± 0.7^{ABC}	2517.7 ± 199.8^{ABCD}
Laureate	97.3 ± 0.2^{ABCDE}	21.4 ± 1.3^{CDEF}	15.3 ± 0.2^{ABCD}	2271.3 ± 42.3^{BCDEFG}
Kadie	96.7 ± 0.2^{BCDE}	13.9 ± 0.8^{F}	10.4 ± 0.3^{I}	$2263.9 \pm 216.5^{BCDEFG}$
Fandaga	96.3 ± 0.1^{DE}	16.0 ± 0.7^{EF}	12.2 ± 0.1^{EFGHI}	2531.3 ± 122.3^{ABCD}
Overture	98.5 ± 0.1^{A}	24.8 ± 4.8^{ABCDE}	12.7 ± 0.0^{DEFGHI}	$2348.8 \pm 756.1^{ABCDEF}$
Despina[A]	97.5 ± 0.7^{ABCDE}	30.9 ± 3.7^{AB}	13.6 ± 1.9^{BCDEFGH}	2517.1 ± 143.2^{ABCD}
Despina[B]	97.9 ± 0.0^{ABC}	32.0 ± 1.0^{A}	16.8 ± 0.9^{A}	2464.1 ± 301.6^{ABCDE}
PL-426[A]	97.4 ± 0.7^{ABCDE}	24.0 ± 2.5^{ABCDE}	12.6 ± 1.2^{EFGHI}	1895.9 ± 36.5^{BCDEFG}
PL-426[B]	97.0 ± 0.2^{ABCDE}	22.6 ± 4.3^{BCDEF}	14.7 ± 0.7^{ABCDEF}	$2040.5 \pm 175.7^{BCDEFG}$
Leabrook	97.6 ± 0.1^{ABCDE}	25.5 ± 4.5^{ABCD}	11.7 ± 0.8^{GHI}	1586.8 ± 33.1^{EFG}

[a] The superscript behind the variety name is used to distinguish between samples of the same genotype based on their growing region or crop year.

explain all variations in SSG (v/v%) between the samples.

4. General discussion

The malting and brewing industries are global industries which utilised over 50 barley varieties between 2020 and 2022 [51–55]. Hence, the industry constantly faces large variations in the raw material, yet a uniform end-product of high quality is always desired. Therefore, the malting and brewing industry needs to be able to cope with the high variability in the raw material. Below, suggestions on the importance of barley starch structural properties and its gelatinisation behaviour in the context of brewhouse efficiency are discussed.

The 23 analysed barley samples display a large variability in i) chemical composition, ii) starch gelatinisation behaviour and iii) starch structural properties such as the bimodal granule size distribution and amylose content. This variation results from the combination of different genotypes and environmental conditions experienced during grain filling by the barley crop [56–58]. While differences between samples from the same genotype were observed for the listed characteristics, no conclusions could be drawn on the impact of genotype, crop year or environmental conditions using the current sample set. Regardless of the barley variety however, brewers often start mashing in at 65 °C as it is believed that starch will efficiently gelatinise and be hydrolysed at this temperature [59]. In this work, a range from 60.1 to 66.5 °C was observed in GT_p. Typical mashing-in temperatures are thus lower than the GT_p of several samples in the set. Besides, all samples analysed in this work had a $GT_{c(B)}$ above 67.3 °C, well above the mash-in temperature of 65 °C. It is further known that during malting and mashing, the starch gelatinisation temperatures increase [26–29,36]. It can thus be expected that the GT_p in malt starch, upon mashing, increases above 65 °C. Meanwhile, at 65 °C, β-amylase starts to undergo inactivation. Upon increasing the mashing temperature, the non-gelatinised starch fraction further gelatinises. B-amylase is, however, rapidly inactivated at higher temperatures and can therefore not convert this residual starch fraction to maltose, leading to potential losses in resource efficiency [29,30]. Given the expected increase in the starch gelatinisation temperature during malting and brewing, the selection of barley with lower GTs is needed.

When taking a closer look at the variation in gelatinisation behaviour between the samples, the results show that this variation could be due to i) the presence of non-starch material and ii) starch structural properties. Fig. 4 showed a distinct decrease in gelatinisation behaviour upon removal of the non-starch material from the starches. This suggests that the non-starch fraction might play a role in the gelatinisation behaviour of starch. A weak but significant positive correlation between the sum of all non-starch materials and $GT_{o(B)}$, $GT_{p(B)}$ and $GT_{c(B)}$ ($r = 0.41$, $r = 0.49$, $r = 0.58$, $p < 0.05$) was observed. Although the correlation was weak, this suggests that the total concentration of non-starch material plays a role in the gelatinisation behaviour of starch. The composition of the non-starch fraction might also impact the starch gelatinisation behaviour. Indeed, a weak positive correlation was further uncovered between the protein content and $GT_{p(B)}$ and $GT_{c(B)}$ ($r = 0.43$, $r = 0.42$, $p < 0.05$). This correlation can be explained via starch-protein interactions in the milled barley samples. The non-soluble protein matrix around the starch granules might prevent the

starch granules from swelling, which could result in a higher starch gelatinisation temperature [60]. It should be noted that the correlations described above do not provide direct proof of the impact of the presence of non-starch material on the barley starch gelatinisation temperature. Future research should, therefore, focus on establishing a causal relationship between non-starch materials in barley and malt and the gelatinisation behaviour of starch.

Besides the impact of the non-starch fraction, it was further observed that $GT_{o(B)}$, $GT_{p(B)}$ and $GT_{c(B)}$ were strongly and positively correlated with $GT_{o(S)}$, $GT_{p(S)}$ and $GT_{c(S)}$ ($r = 0.76$, $r = 0.96$, $r = 0.85$, $p < 0.0001$). This strong correlation indicates that the gelatinisation behaviour measured in milled barley samples reflects the gelatinisation behaviour of the native starch population, despite the impact of the non-starch fraction. This suggests that the gelatinisation behaviour of the native starch population is a crucial factor to consider. Starch is, however, a complex polymer which shows a bimodal starch granule size distribution in small and large starch granules and consists of amylose and amylopectin molecules [17,28,31,32]. We investigated whether the starch gelatinisation behaviour was associated with the structural properties of the polymer.

No correlation was observed between amylose content and the starch gelatinisation behaviour in this work, which is in accordance with the results of Ahmed et al. (2019) [33], yet contradicts the results of Yu et al. (2018) [32], which reported a positive association. It should be mentioned that the fine structure of the amylose and amylopectin molecules can also impact the gelatinisation behaviour of the starch [61,62]. Multivariate data analysis also showed no correlation between the gelatinisation properties of isolated starch and the proportions of SSG (v/v%), SGG (n/n%), V_{SSG} (μm^3) and V_{LSG} (μm^3). This disproves our hypothesis that a larger proportion of SSG would result in higher overall gelatinisation temperatures. Determining the gelatinisation temperature can thus not serve as an indirect method to estimate granule proportions. In addition, starch content was also not correlated with the amylose content or granule proportions. Thus, selecting barley based on high starch content does not provide assurance on the structural properties of the starch. To ensure high brewing efficiencies, small starch granule volumes, amylose content, starch content and starch gelatinisation behaviour should, therefore, be considered separately during barley selection.

The sample set used in this work is highly variable, with different varieties and growing regions. This genotypical and

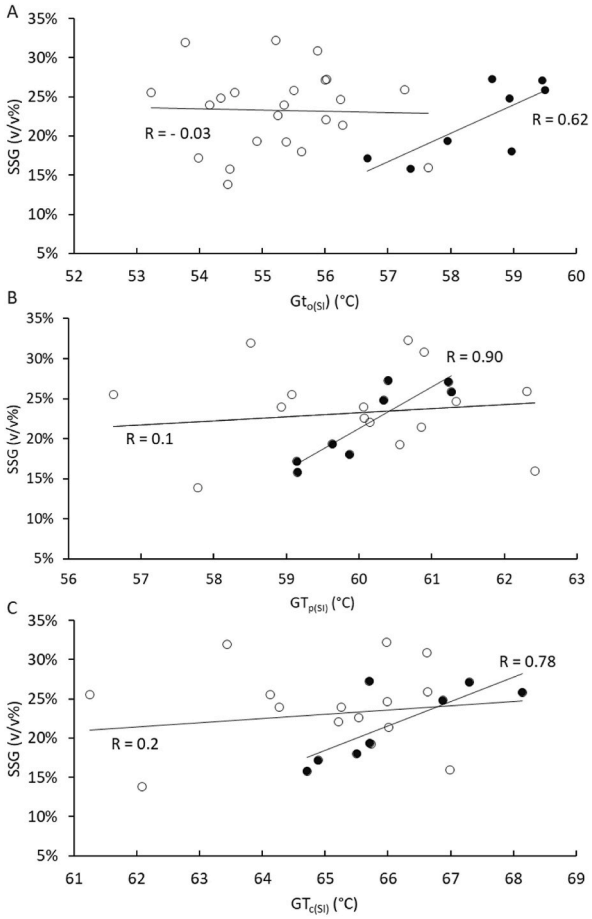

Fig. 6. The relation between the gelatinisation behaviour of barley starch and the volume-based percentages of small starch granules with **A)** the relationship between $GT_{o(S)}$ and SSG (v/v%) **B)** the relationship between $GT_{p(S)}$ and SSG (v/v%) and **C)** the relationship between $GT_{c(S)}$ and SSG (v/v%). The whole sample set is indicated in white dots, while the samples from the USA are indicated in black dots. The respective R-values are provided for the whole sample set as for the samples from the USA.

environmental variability could mask correlations between SSG v/v% and the gelatinisation behaviour of the starch [57,63–65]. To test this hypothesis and exclude the environmental impact in part, a case study on the eight barley samples growing in a similar region in the USA was conducted. In Fig. 6, SSG(v/v%) of this subset are plotted against $GT_{o(S)}$, $GT_{p(S)}$ and $GT_{c(S)}$. Fig. 6 shows no correlation between SSG (v/v%) and the gelatinisation behaviour for the whole data set. The samples cultivated in the USA, however, group together. For these samples, a trend towards significance was observed for $GT_{o(S)}$ and SSG (v/v%) ($r = 0.62$, $p = 0.09$). Furthermore, a strong correlation between SSG (v/v%) and $GT_{p(S)}$ and $GT_{c(S)}$ ($r = 0.90$, $p < 0.01$, $r = 0.78$, $p < 0.05$) was observed. We can thus conclude that SSG proportions could potentially drive the observed gelatinisation temperatures, but also that many more factors need to be considered. Note that barley malt can still contain significant amounts of small starch granules [17] and that they gelatinise at higher temperatures (a 3 °C higher in GT_p than LSG), without taking in the increasing effect of sugars on the starch gelatinisation temperature during mashing into account [22,27]. Recent literature does clearly indicate the negative impact of SSG on brewing yield [22,27,30,66]. Therefore, we advise the malting and brewing industry to quantify granule proportions in barley and malt.

5. Conclusion

Considering the impact of high gelatinisation temperatures on brewing efficiency, the wide range of gelatinisation temperatures in this study points to the importance of starch gelatinisation behaviour as a quality characteristic in the selection of barley for malting and brewing. $GT_{p(B)}$ was weakly correlated with protein content, suggesting that the composition of non-starch material in barley might need to be considered when striving for low starch gelatinisation temperatures. Strong correlations between the GT_o, GT_p, and GT_c in milled barley and isolated starches indicate that the gelatinisation behaviour in milled barley strongly reflects the gelatinisation behaviour of the native starch population. When interested in the gelanisation behaviour upon barley selection, milled barley samples can thus be used and isolation of starch is not needed. However, when information on starch granule proportions and amylose content are further desired, starch has to be isolated.

A high variability in granule and amylose to amylopectin proportions was observed. No correlation between the structural properties of the starches and the gelatinisation behaviour was uncovered. Thus, small starch granule proportions and amylose concentrations cannot be estimated based on the gelatinisation behaviour of the starch when measured with a DSC. To ensure a high resource efficiency, the malting and brewing industry should consider the gelatinisation behaviour of starch, small starch granule proportions and amylose concentrations separately from each other in the selection process of barley.

Future research should focus on a GxE study for the starch gelatinisation behaviour, amylose content and granule proportions in barley to better understand the origin of the higher variability observed in this work. This could help different industries to select their barley crop depending on their specific needs.

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Ethics declarations

Not applicable.

Data availability statement

The data associated to this study has not been deposited into a publicly available repository. The data that has been used is confidential.

CRediT authorship contribution statement

D.R.S. Gielens: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **C.F. De Schepper:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation. **N.A. Langenaeken:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **A. Galant:** Writing – review & editing, Resources, Methodology. **C.M. Courtin:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29662.

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