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# Levels and management of glycosidic nitrile production in North American grown barley varieties

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## KEYWORDS

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Glycosidic nitrile (GN) from malted barley has been identified as the primary precursor of ethyl carbamate (urethane) in new-make whiskey. A specific GN, epiheterodendrin (EPH), is responsible. EPH is a type of cyanogenic glycoside, forming toxic hydrogen cyanide in the pathway leading to ethyl carbamate (EC). The presence of EC, a known carcinogen in many fermented foods, is regulated to varying degrees. For whiskey, the Canadian government has mandated a 150 ppb limit, while the US has a voluntary limit of 125 ppb. In Europe, standards call for newly released distilling barleys to be of the non-GN type. North American breeders are just beginning to select for non-GN and options are likely a decade away. This work considers GN levels of common North American varieties and evaluates malting regime variations, including steep out moisture (40 percent and 45 percent), length of germination (two, four, and six days), and temperature (14 °C, 15 °C and 16 °C) to determine impacts on GN. General standards hold that low producers fall under 0.5 g/tonne GN while high producers sit over 1.5 g/tonne. Varieties tested here averaged 0.20 – 1.52 g/tonne across treatments. As expected, management to minimize acrospire growth mitigated GN while all three process variations had significant impacts: ranges being 0.70 – 1.38 g/tonne (germination time), 0.83 – 1.09 g/tonne (temperature) and 0.56 – 0.96 g/tonne (steep out moisture). While this work does not provide a long-term solution, management is a tool while breeding programs develop North Americana non-GN varieties.

## INTRODUCTION

Glycosidic nitrile develops in the living tissues of barley including leaves and shoots (rootlets not included). Cyanogenic glycosides such as GN act as a defense mechanism for plants against pathogens and herbivores [1, 2]. However, in the distilling process GN is enzymatically converted to cyanide which then becomes EC in the presence of copper and ethanol, with heat promoting the conversion. It is known that GN production is under genetic control [3] and markers have been developed to assist breeding programs in selection for the trait, specifically selecting for EPH-null genotypes [4]. These markers were proprietary until recently when made available for purchase through Heriot-Watt University. Access to this tool is an important boon for North American breeders.

Although the malting process typically aims to minimize growth of acrospires (barley's initial shoot), GN

levels are greatly increased in malted barley as compared to unmalted grain, and extended germination times and excessive acrospire growth are associated with high levels of EC precursors [5]. With North American EPH-null varieties years away from regular production and the potential for greater regulation of EC at any time, maltsters and distillers must work together to manage its production in malt spirits. There are two points at which management practices can be employed: pre-distillation considerations of the ingredients to be used, and in the methods used for distillation. Here we discuss best practices from both aspects for management of ethyl carbamate in malted grain spirits.

In 1990 the United Distillers International Research Centre released three articles detailing early understanding of the production of glycosidic nitrile in malting barley, identifying measurable cyanide (MC)

as involved in EC accumulation in whiskey [5-7]. Precisely, Epiheterodendrin (EPH), a glycosidic nitrile, has since been named as the culprit precursor for cyanide production in grain distillation and it is currently indicated as the primary cause of EC in malt-based spirits. GN is a type of cyanogenic glycoside, compounds having the ability to release noxious hydrogen cyanide which is highly toxic to most living organisms. This toxicity is due to hydrogen cyanides' ability to inhibit the electron transport system by binding cytochromes [8] and arresting metabolic activities. This toxicity has been found to offer plants a chemical defense mechanism whereby the chewing action from insects allows hydrolyzation of the glycoside by  $\beta$ -glucosidase. Under normal conditions  $\beta$ -glucosidase is spatially separated by plant tissues [9]. Interestingly, and unlike other cyanogenic plants, no specific  $\beta$ -glucosidase for this reaction is present in barley leaf tissues, meaning the function is not operational [2]. Specific to the distilling process,  $\beta$ -glucosidase has been indicated as present in barley grain endosperms; however, the enzyme is rendered inactive at normal mashing temperatures (145 °F/63 °C) [5], preventing action during fermentation. Hydrolysis is completed when yeast for fermentation is added.  $\beta$ -glucosidase introduced from yeast leads to hydrogen cyanide production

which later reacts with ethanol in the presence of copper to produce EC. Table 1 provides clarifying definitions for the various compounds to consider in this discussion. As GN is a product of plant leaf and shoot tissues it makes sense that levels are below the detectable limit in unmalted grain, but increase readily with malt processing as this inevitably allows the growth of the grains initial shoot, or acrospire. Various publications reference measurement of potential for EC production via quantification of MC after addition of yeast or  $\beta$ -glucosidase. MC is a measure of total cyanide including hydrogen cyanide, copper cyanide complexes, and cyanohydrins. Other reservoirs of measurable cyanide include lactonitrile, free cyanate, and thiocyanate [10]. Cook, *et al.* [5] methodically characterized MC levels in germinating barley to be a specific component of acrospires and indicated the impact of variety along with a negative correlation with corn size. The effect of corn size makes sense as the ratio of acrospire to other grain tissues would decrease with larger corns. That work also found positive correlations with grain moisture content during germination, germination temperatures, air ventilation, germination time and usage of gibberellic acid. Crop year and growing location were indicated to not have an impact on barley GN levels.

Malted barley plays a key role in the production of malt whiskey, where it is the primary ingredient, but also in other distilled spirits utilizing malted barley for enzymatic conversion of starches from adjunct grains such as corn, wheat, rye and more. Distillers working exclusively with unmalted cereal grains and exogenous enzymes bypass the potential for production of EC due to GN. However, with the rise of the craft spirit industry, emergence of American single malt whiskeys, and distillers incorporating unique ingredients, it is important to note there are other potential sources of GN aside from malted barley. For example, sorghum, sugar cane and stone fruits contribute to increased risk for EC production [12, 13].

EPH is considered the only cyanide releasing source present in barley [14], making its presence in varieties of great importance to the distilling process. It is arguably the main concern for whiskey distillers when considering EC production, however other potential precursory sources have been proposed. In wine, management of nitrogen and Fe<sup>II,III</sup> fertility levels has been indicated as an important EC control point, however these compounds do not distill and therefore

**TABLE 1** Definitions of common terms in the discussion of GN and EC.

COMPOUND	DESCRIPTION
Glycosidic Nitrile(GN)	Term used to describe a chemical compound that contains a cyanide functional group covalently bonded to a sugar. Naturally found in many plants, the cyanogenic aspects act in plant defense mechanisms against pathogens and herbivores.
Cyanogenic Glycosides	A class of secondary metabolites found in plants that release hydrogen cyanide gas when exposed to the hydrolyzing enzymes $\beta$ -glycosidases [11].
Epiheterodendrin (EPH)	A specific glycosidic nitrile (GN) and type of cyanogenic glycoside synthesized in barley seeds during germination. Culprit precursor for cyanide production in grain distillation, currently indicated as the primary cause of EC in malt-based spirits. Markers have been developed to select for varieties which are inactive for the EPH gene.
Measurable Cyanide (MC)	Early investigations of EC formation generally measured precursors as measurable cyanide. Modern understanding has specifically identified EPH as the source.
Ethyl Carbamate (EC)	AKA urethane, a known carcinogen contained in many fermented foods. Its presence in consumer products has become regulated in various countries.

**FIGURE 1** Control points for GN production and EC formation throughout the whiskey process.

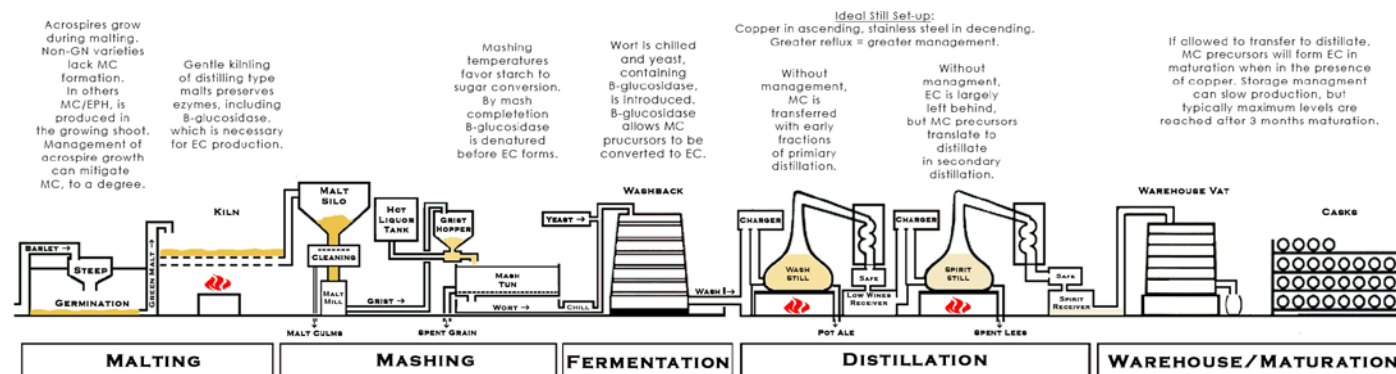


Figure adapted from Elmaghraby [20].

are not of issue for distilled spirits [10]. Riffkin, *et al.* [15] demonstrated that one source of the cyanate precursor is the oxidation of amino acids by sodium hypochlorite, a strong oxidizer which has been used as a cleaning agent in distilleries and as a biocide/fungicide in the treatment of distillery grains. Riffkin, Wilson and Bringham [16] also distilled bovine serum albumin in a laboratory copper alembic still and showed that ethyl carbamate was detected in the distillate at 38 ppb (0.04 g/tonne) after 72 hours elapsed time, demonstrating that amino acids from diverse sources could provide a source of cyanide precursors, albeit in seemingly very low proportions compared to MC. Amino acid sources in malt increase to a point as the malting process progresses. Total protein is modified to soluble protein and free amino nitrogen (FAN). Conscious maltsters will

work to manage degree of modification which will stabilize both acrospire growth and FAN production. Additionally, commercial fermentations typically utilize the majority of wort FAN prior to distillation as FAN is a primary source of yeast nutrition and FAN remaining in beer has been linked to off-flavor production [17].

These alternative sources for EC production point to the need for each distiller to intimately understand their raw material as well as cleaning and production practices. Figure 1 details the process of whiskey production from maturing through maturation, highlighting key points for EC production control, including raw ingredient selection and copper placement within the distillation process. Careful raw material selections combined with best distilling practices will execute the greatest control of EC.

**TABLE 2** Malting barley varieties commercially grown in North America and assessed in this study’s initial malt survey.

VARIETY	HEAD TYPE	PLANTING TYPE	BREEDER
AAC Synergy	Two	Spring	Agriculture & Agrifood Canada
AC Metcalfe	Two	Spring	Agriculture & Agrifood Canada
Newdale	Two	Spring	Agriculture & Agrifood Canada
CDC Copeland	Two	Spring	University of Saskatchewan
Conlon	Two	Spring	North Dakota State University
Pinnacle	Two	Spring	North Dakota State University
LCS Genie	Two	Spring	Limagrain Cereal Seeds
LCS Odyssey	Two	Spring	Limagrain Cereal Seeds
Full Pint	Two	Spring	Oregon State University
Endeavor	Two	Winter	USDA – ARS (Aberdeen, ID)
LCS Calypso	Two	Winter	Limagrain Cereal Seeds
LCS Violetta	Two	Winter	Limagrain Cereal Seeds
Thoroughbred	Six	Winter	Virginia Tech

## MATERIALS AND METHODS

### SURVEY OF GN LEVELS IN COMMONLY GROWN NORTH AMERICAN VARIETIES

A study was conducted consisting of seventy-eight malt samples representing different varieties of barley collected from commercial and craft malthouses in North America. Malts were assessed for GN content as described later in this document. Varieties considered are listed in Table 2.

### MANAGEMENT OF GN

Micro malting trials were conducted on both low and high GN producing varieties as well as an unknown modern release (Buzz) from the MSU breeding program to investigate the effect of germination, moisture content, temperature and time on the level of GN in the resulting

malts, as well as potential interactive effects.

Barley grown in trials at the Montana State University Post Farm in Bozeman Montana in 2018 was sourced for five common malting varieties: Hockett, Metcalfe, Odyssey, Buzz and Synergy. Barley was malted in three replicates for each variety in each treatment according to a standard MSU malting protocol with Custom Laboratory Products (Milton Keynes, U.K.) steep/germ tanks and a kiln. Samples of barley (120 g), plumped over a 6/64" sieve, were loaded into round steeping cages (19.05 cm diam. x 12.7 cm tall), with four quadrants. Each steep tank accommodated four cages, allowing 16 samples to be malted simultaneously. Typically target steep out moisture is 45 percent. A control line (Genie) was included in every run to ensure uniformity of malting between runs. The basic regime consisted of a 48-hour steep, in which grain was continually maintained at 15 °C and underwent a multi-steep program with a steep/rest pattern of 10-hour steep, 18-hour rest, six-hour steep, 10-hour rest, and four-hour steep, with an average target moisture of 45 percent. Germination consisted of 96 hours at a constant 15 °C. Throughout steeping and germination, humidity was maintained at greater than 98 percent and agitation consisted of five minutes of cage turning at 0.61 RPM in every 30-minute period. Aeration with moist air through the grain occurred for one out of every 10 minutes. After germination, samples were kilned via forced air in the CLP kiln over a 24-hour period consisting of 12 hours at 60 °C, six hours at 65 °C, two hours at 75 °C, and three hours at 85 °C. Upon completion, samples contained on average 4.0 percent moisture and were manually

de-culmed. Alterations to the program were made to assess regime change effects on glycosidic nitrile. Variations included steep out moisture at both 40 percent and 45 percent, time in germination (two days, four days, and six days), and malting temperature (14 °C, 15 °C, 16 °C). Due to limited time and resources a fully factorial evaluation of all malt regime combinations was not possible.

### GLYCOSIDIC NITRILE ANALYSIS

GN levels in malt were measured following Method 4.21 of the European Brewing Convention. Briefly, malted barley samples were ground in a Buhler disk mill to pass through a 1.5mm screen. The grist was suspended in a buffered solution containing beta-glucosidase and incubated at 60 °C for one hour with intermittent stirring. The resulting mash was distilled, the distillate was assayed for cyanide by reaction with Chloramine-T, and absorbance was measured with a spectrophotometer at 590 nm. A standard curve of KCN was prepared and used to convert absorbance to g/tonne of GN.

### STATISTICAL ANALYSIS

Three main factors were tested for impact on GN levels: steep-out moisture, time in germination, and malting temperature. Because the experimental design was not full factorial, the impact of each factor was determined in a specific subset of data to reduce unintended variability. Examining the impact of germination time was examined in 15 °C and 45 percent steep-out moisture. The impact of temperature was examined for four days of germination and 45 percent steep-out moisture. The impact of varied

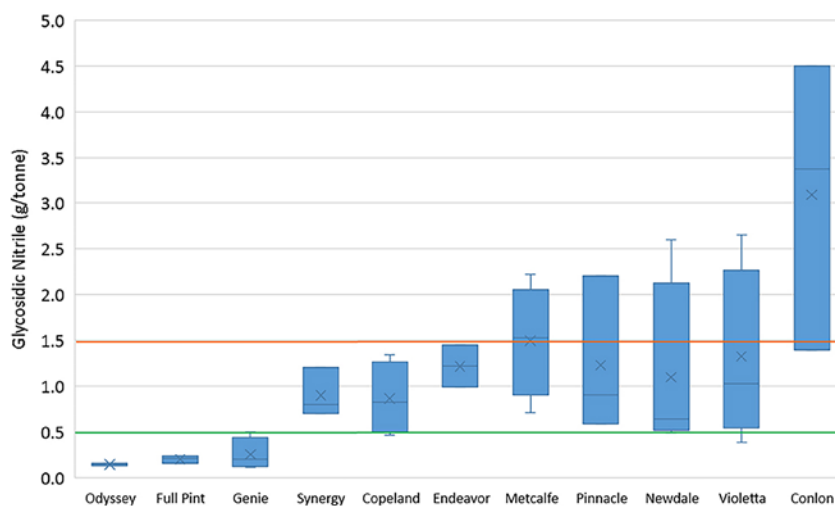
steep out moisture was examined for four days of germination and 15 °C. Response variables GN per tonne,  $\beta$ -glucans, and soluble protein were each examined using univariate linear models. Three models were produced: steep out moisture by variety, time in germination by variety, and temperature by variety. Model assumptions were examined using diagnostics plots. Significant interactions were observed for all models. Post-hoc means comparisons were made based on these models using Tukey's tests with  $p = 0.05$ .

## RESULTS

### GN PRODUCTION LEVELS OF BARLEY VARIETIES CURRENTLY GROWN IN NORTH AMERICA

Levels of GN have been successfully lowered in UK barley varieties through selective breeding, however little is known about the levels in

**FIGURE 2** Levels of GN in North American commercial and craft malts surveyed.



Seventy eight malt samples representing different varieties of barley were collected from commercial and craft malthouses across North America and evaluated for GN levels. Here boxplots represent the range for the 11 commonly grown varieties represented in the survey. The green line at 0.5g/tonne represents the threshold under which lines would be considered non-GN producing, while the orange line at 1.5g/tonne represents the threshold at which lines falling above would be considered high GN producers.

North American barley varieties. A goal of this work was to establish baseline levels of GN for barley varieties commonly grown in North America. Barley varieties are typically categorized according to their propensity to produce GN with three categories established: EPH-null genotype: <0.5g/tonne, Low-producer: 0.5-1.5 g/tonne, High-Producer: >1.5g/tonne.

Table 2 lists the cultivars evaluated. The collection includes spring, winter, two and six row varieties that are commonly used in commercial and craft malt operations across the country. Figure 2 displays the measured GN for each variety, determined as described in the methods.

#### MANAGEMENT OF GN CONTENT OF NA VARIETIES DURING MALTING

Steep out moisture, time in germination and germination temperature were tested to determine impact on GN production in a selection of two-row malting barleys, Figure 3. Time in germination allows metabolic processes to progress allowing acrospire growth, which correlates with increases in GN. This effect can be seen with GN-producing cultivars having increasing GN values with progressive days of germination. Increasing germination time from two days to six days increased GN per tonne for Hockett, Metcalfe, and Synergy ( $p < 0.05$ , Tukey's test). The increase from two days to four days was sufficient to detect an increase in Buzz ( $p < 0.05$ , Tukey's test). However, Synergy, Metcalfe, Buzz and Hockett are all over the threshold limit of 0.5 g/tonne with only two days of germination (treatment averages being 1.19, 1.05 and 1.43 g/tonne respectively), while Hockett is the least offensive, having an average rate of 0.71 g/tonne. The only variety maintaining acceptable levels and not impacted by germination time is Odyssey, which due to lack of GN production remains stable throughout with all

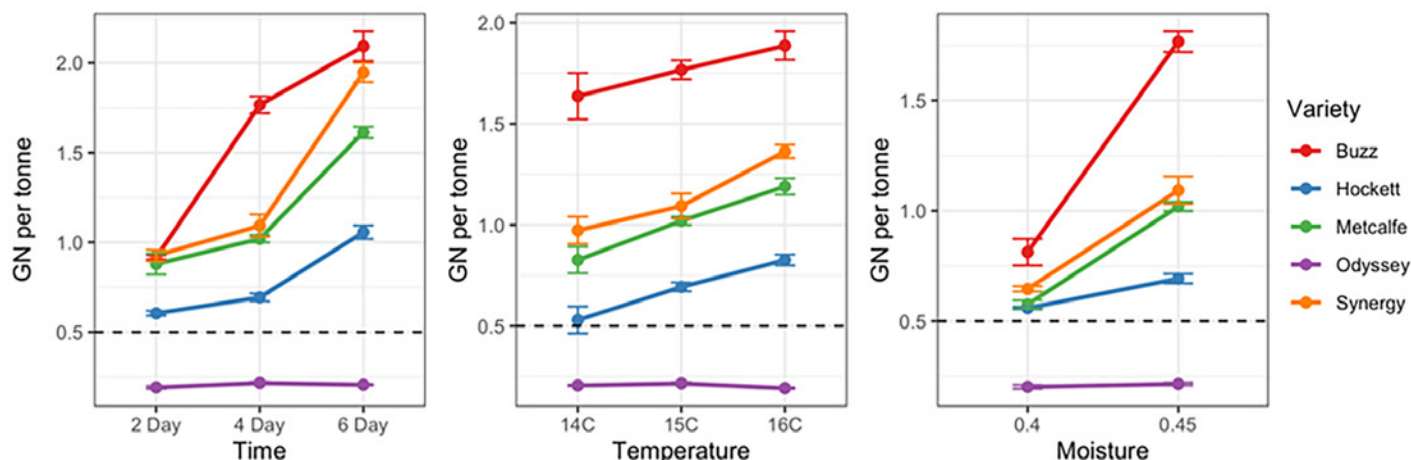
points well below the accepted threshold of 0.5 g/tonne and resulting in an average rate of 0.18 g/tonne GN.

#### IMPACT OF VARIED MALTING PARAMETERS ON GLYCOSIDIC NITRILE LEVELS:

Metabolic processes are favored to a point with increased temperature. Increases of temperature were also found to have negative impacts with elevated levels of GN production (Figure 3). An increase in temperature from 14 °C to 16 °C increased GN per tonne in Synergy, Odyssey and Metcalfe ( $p < 0.05$ , Tukey's test). Varietal rankings remain as compared to time in germination, with Odyssey maintaining low levels (average = 0.21 g/tonne), Hockett having mid-range GN levels (average = 0.68 g/tonne), and Synergy, Metcalfe and Buzz again producing moderate to high levels of the EC precursor (averages = 1.14, 1.01, and 1.76 g/tonne GN respectively). Buzz in particular shows signs of high GN production with all three treatments above the 1.50 g/tonne threshold.

Step-out moisture is a critical control parameter during the malting process. Sufficient hydration is necessary for barley embryo health and is required for moisture dispersion across the endosperm, allowing hydrolytic movement and action of enzymes central to the modification process. Depending on the desired malt style, maltsters target 42 to 45 percent moisture at steep-out. In the interest of elucidating control for GN with this metric here we tested 40 percent and 45 percent steep-out moistures. Higher steep-out moisture was found to contribute to higher GN levels in the malt. Elevated moistures did not impact Odyssey (Figure 3). The remaining varieties have levels above the EPH-null genotype threshold even at 40 percent steep-out moisture and show significant GN increase at 45 percent ( $p < 0.05$ , Tukey's test), although Hockett's increase was marginal.

**FIGURE 3** Germination time, temperature, and steep out moisture impact on Glycosidic Nitrile levels.



Impact of varied malting parameters on glycosidic nitrile levels. Time references time spent in germination, temperature variations were applied during germination and moisture percent references grain moisture at steep out. Error bars indicate the standard error of the mean (n = 3).

Buzz was impacted to the greatest extent, with the 45 percent moisture treatment being the only of the group to surpass the 1.50 g/tonne threshold at 1.77 g/tonne GN.

To provide context with respect to the level of modification,  $\beta$ -glucan and soluble protein levels for selected treatments are provided, Figure 4. Samples measured for this reference point all had 45 percent moisture at steep out and were held at 15 °C throughout germination. It is clear that Odyssey is again unique, having low generation of soluble protein and overall low  $\beta$ -glucan.

## DISCUSSION

Varieties show differences in the production of GN that are also dependent on the malting regime. Of the five lines tested, Buzz was found to be the least suitable for use if a maltster is interested in maintaining low GN levels. Synergy and Metcalfe, having more moderate production levels of GN while also maintaining reasonable levels of  $\beta$ -glucan and soluble protein at shorter germination times, could be candidate varieties for maltsters and distillers unable to use the few non-GN varieties currently grown in North America: Genie, Odyssey, and Full Pint. Low levels of GN were obtainable using Hockett, however, this variety is known to be slow modifying. Utilizing Hockett and managing for low GN would likely produce a malt that is not favorable when looked at in the light of overall malt quality as parameters such as  $\beta$ -glucan would likely be undermodified.

It is highly likely that there are measurable interactions between the treatments tested here, i.e. combining lower temperature with shorter germination will have complementary effects to further lower GN production. Due to time and space constraints we were unable to test a fully factorial experiment to understand these potentially beneficial interactions. In addition, the malting control measures assessed here are common broad-stroke avenues for controlling level of modification while other management options may have utility, such as restricted ventilation, pressure treatments (i.e. wet casting), and use of abscisic acid [5]. Another interesting approach for producing low-GN malts but not explored here is the potential for

**FIGURE 4** Soluble Protein &  $\beta$ -glucan levels of selected barley varieties under median trial malting conditions.

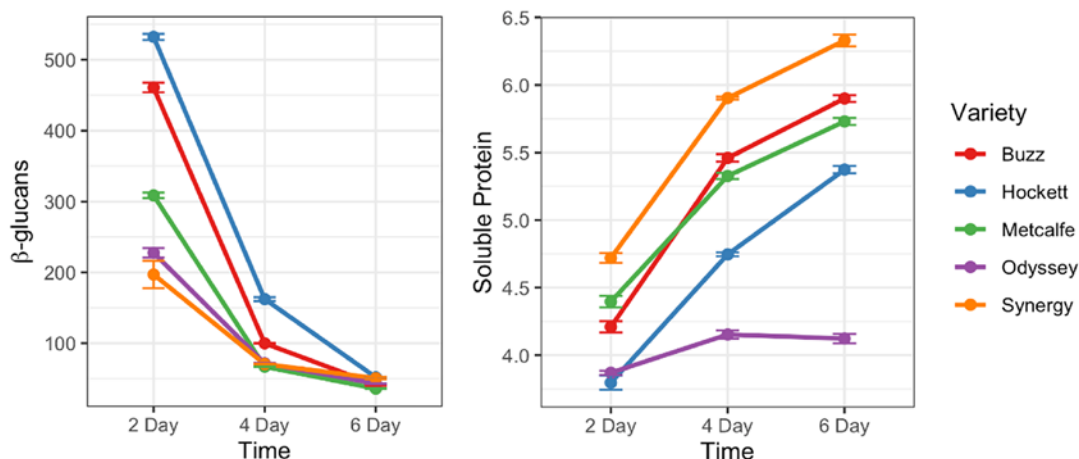


Illustration of grain modification for selected varieties under standard conditions of 45% moisture at steep out, and 15°C in germination. Error bars indicate the standard error of the mean (n = 3).

malting hull-less barleys as their acrospires are largely removed in the de-culming process post malting, resulting in very low levels in the cleaned finished malt. As every system is unique and the variables are many, maltsters using GN-producing varieties who desire lower GN levels in malt can use the information provided here as a starting point for dialing in their own procedures. Utilizing lab assessment of GN levels to determine what practices will yield the best overall results will be a key metric.

## CONCLUSION

North American breeding programs such as Montana State University have started making crosses to integrate the EPH-null trait into locally adapted varieties, while also utilizing tools such as the EPH KASP marker (available through Heriot-Watt) to streamline the selection process. However, ubiquitous availability of non-GN lines in North America is likely still a decade away. Management of GN and EC production in distilled whiskeys will be a collaborative effort between maltsters and distillers and will require both communication and education to accomplish. The information provided here is designed to be an informative starting point. Maltsters not having access to non-GN varieties but wanting to effect maximized control over GN production will find that GN quantification is an important tool for developing best practices within their specific production system.

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