BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

ISSN 1981-6723
Online version

ORIGINAL ARTICLE

Cold mash in brewer wort with low carbohydrate content: a study of the mashing step

Cold mash *na obtenção de mosto cervejeiro com baixo teor de carboidratos: um estudo de brassagem*

Gabriela Dalberto[1]*, João Paulo Niemes[2], Marcos Roberto da Rosa[1]

[1]Universidade Estadual do Centro-Oeste (UNICENTRO), Departamento de Química, Guarapuava/PR - Brasil
[2]Centro Universitário Campo Real, Colegiados de Biomedicina e Engenharia Agronômica, Guarapuava/PR - Brasil

***Corresponding Author:** Gabriela Dalberto, Universidade Estadual do Centro-Oeste (UNICENTRO), Departamento de Química, Alameda Élio Antonio Dalla Vecchia, 838, CEP: 85040-167, Guarapuava/PR – Brasil, e-mail: dalbertogabi@gmail.com

Cite as: Dalberto, G., Niemes, J. P., & Rosa, M. R. (2023). Cold mash in brewer wort with low carbohydrate content: a study of the mashing step. *Brazilian Journal of Food Technology*, *26*, e2023008. https://doi.org/10.1590/1981-6723.00823

Abstract

Cold mash is an innovative and promising brewing technique for obtaining free-alcohol and low-alcohol beers, with a sensory profile similar to traditional beers. While traditional mashing is carried out at high temperatures, between 50 and 75 °C, this technique uses low temperatures, around 10 °C, but with a longer time. This allows more intense extraction of flavors and color from the malt while keeping the concentration of fermentable sugars low, as the enzymatic starch hydrolysis does not occur at its maximum efficiency. A study was performed on the mashing stage of the brewing process using the cold mash technique, with subsequent physicochemical characterization, to evaluate possible changes in these parameters. To obtain beer worts with low concentrations of fermentable sugars, modifications were made to mashing temperature and time, and a two-level factorial design was applied to evaluate the influence of these variables, demonstrating that the main factor mashing time was of great importance in the response. The extract content, related to the concentration of carbohydrates, presented results around 6 °Bx, while the traditional wort has between 11 and 12 °Bx, and the protein content was 0.12 to 0.13%, being below than reported in the literature. For pH, the results were around 6.0, which is above the recommended for brewer's wort, demonstrating that the mashing technique employed promoted changes in this parameter, possibly due to the limited action of the phosphatase enzyme, responsible for lowering the pH through the production of phosphoric acid.

Keywords: Beer; Brewing technique; Enzymatic hydrolysis; Factorial design; Fermentable sugars; Low temperature.

Resumo

Cold mash (brassagem a frio) é uma técnica cervejeira inovadora e que vem se mostrando promissora para a obtenção de cervejas sem álcool e de baixo teor alcoólico, com perfil sensorial semelhante ao de cervejas tradicionais. Enquanto a brassagem tradicional é realizada em temperaturas elevadas, entre 50 e 75 °C, nessa técnica se utilizam baixas temperaturas, em torno de 10 °C, porém com um tempo maior. Isso possibilita uma extração de sabores e coloração do malte com mais intensidade, ao mesmo tempo em que a concentração de açúcares fermentáveis se mantém baixa, pois a etapa de hidrólise enzimática do amido não ocorre em seu máximo de eficiência. Dessa forma, realizou-se um estudo da etapa de brassagem do processo de produção cervejeira empregando a técnica de *cold mash*, com posterior caracterização físico-química, a fim de se avaliarem possíveis mudanças nesses parâmetros. Para se obterem mostos cervejeiros com baixa concentração de açúcares fermentáveis, realizaram-se modificações em relação a temperatura e tempo de brassagem, e aplicou-se um planejamento fatorial em dois níveis a fim de se avaliar a influência dessas variáveis, demonstrando que o fator principal tempo de brassagem teve grande importância na resposta. O parâmetro teor de sólidos solúveis, relacionado à concentração de carboidratos, apresentou valores em torno de 6 ºBx, enquanto o mosto tradicional possui entre 11 e 12 ºBx, e o teor de proteínas situou-se entre 0,12 e 0,13%, estando abaixo do reportado pela literatura. Já para o pH, os resultados se situaram em torno de 6,0, estando acima do recomendado para o mosto cervejeiro, demonstrando que a técnica de brassagem empregada promoveu modificações neste parâmetro, possivelmente pela atuação limitada da enzima fosfatase, responsável pelo abaixamento do pH por meio da produção de ácido fosfórico.

Palavras-chave: Cerveja; Técnica cervejeira; Hidrólise enzimática; Planejamento fatorial; Açúcares fermentáveis; Baixa temperatura.

Highlights

- Use of cold mash technique to produce beer wort with low-sugar content
- Extract content between 5.00 and 6.80 ºBx, while traditional worts have 11.00 to 12.00 ºBx
- Cold mash allows greater extraction of color for the beer due to the longer mashing time

1 Introduction

The search for controlled alcohol consumption and healthier habits makes the market for alcohol-free and low-alcohol beers expanding, where it is possible to perceive that this branch represents a great commercialization potential (Adamenko et al., 2020; Pilarski & Gerogiorgis, 2020). This decrease in alcohol intake is mainly caused by the growing awareness of the population (Francesco et al., 2018; Gernat et al., 2020; Mateo-Gallego et al., 2020; Katainen et al., 2023).

Moderate consumption of beer can bring benefits, reducing the risk of cardiovascular diseases, diabetes, and neurodegenerative diseases, and also contribute to the intake of nutrients, as beer is composed of proteins, vitamins, minerals, and carbohydrates (Gaetano et al., 2016; Grao-Cruces et al., 2022; Kang et al., 2023). When it comes to beers with a lower alcohol concentration, these effects can be enhanced, as their consumption is not limited to just small daily portions (Staub et al., 2022).

Among the beers with lower amounts of alcohol, there is the free-alcohol beer (FAB), which is characterized, according to Brazilian legislation, by an alcohol content of no more than 0.5% alcohol by volume (ABV), and the low-alcohol beer (LAB), with an amount equal to or less than 2.5% ABV of alcohol in its composition (Brasil, 2009). These limits vary for each country, and for most of them, for FAB it is less than 0.5% and for LAB the upper limit is 1.2% ABV (Branyik et al., 2012; American Society of Brewing Chemists, 2018).

As it is considered a very sensitive drink, any change in the beer production process will cause modifications in its sensory profile, and a lower ethanol content will significantly influence the quality of the beverage (Szollosi et al., 2016; Ledley et al., 2023a). Most of the processes developed so far negatively modify the physical and sensory aspects of the beer, resulting in a drink that does not resemble a traditional beer, or demand technologies with high value, impacting the selling price. These are possible causes for its low acceptance and lower consumption (Morado, 2009; Silva et al., 2009; Branyik et al., 2012).

About the production processes, there are different ways to produce low-alcohol beer and free-alcohol beers. The biological method of interrupting the fermentation to produce less alcohol by yeasts, and the physical method of removing alcohol molecules from a beer that was produced and fermented in the traditional way, through physicochemical techniques, such as vacuum distillation (Morado, 2009; Buglass, 2011; Branyik et al., 2012). In addition, the use of yeasts that do not ferment maltose, the carbohydrate in the highest concentration in brewing wort, also stands out as a way to obtain minimal amounts of alcohol in beer (Johansson et al., 2021; Roca-Mesa et al., 2022).

In the beer production process, the stage called mashing is of great importance, since it is at this stage that fermentable sugars are obtained, through the action of enzymes present in the malt, which break down the starch into smaller units (Rosa & Afonso, 2015; Gschaedler, 2017; Ledley et al., 2023a;). It is possible to promote modifications to reach the desirable concentration of these sugars. A slower extraction is suitable for the production of beer with a lower alcohol content, and this can be achieved using an innovative method called cold mash (Dalberto et al., 2021; Aredes et al., 2023; Ledley et al., 2023a).

The cold mashing technique has the objective of extracting color from malts in a more intense way, as well as leaving them with more mouthfeel, at the same time a smaller amount of fermentable sugars is obtained since extraction is slower due to the low temperature (Dalberto et al., 2021). Along with the other characteristics resulting from cold mash, this technique can be used to produce low-alcohol beers and free-alcohol beers, maintaining the sensorial aspects of traditional beers. Less extraction of fermentable sugars will result in lower alcohol content, while the other characteristics of traditional beer, such as color and mouthfeel, will be similar (Dalberto et al., 2021).

Modifications in the mashing stage can influence the composition of the brewer's wort, concerning carbohydrate, protein, and mineral content (Buglass, 2011). A study was performed regarding the impacts that the use of the cold mash technique causes in the brewer's wort, through physicochemical characterization to monitor the mashing stage. To attest to the significance of the study, statistical tools were applied to the results obtained. This research represents a practice of great importance so that the final drink has adequate characteristics and results in a standardized and sensorially pleasant product for consumers.

In addition, there is a shortage in the commercialization of free-alcohol and low-alcohol beers with a profile of flavors and aromas like traditional Pilsner beers, which have color, bitterness, and mouthfeel similar to this style (Bellut & Arendt, 2019). Based on the previously mentioned information, this study aims to open up space for the development of this innovative product, by carrying out the application, study, and optimization of cold mash, seeking new processes to obtain beers with a high-quality standard.

2 Materials and methods

2.1 Brewing wort production

In this research, only the study of the mashing stage was conducted, to evaluate its behavior when using cold mashing. The brewing process was not continued with the use of hops, fermentation, and maturation stages, as this study will be carried out in future stages.

To obtain the wort using the cold mash technique, the process began with the grinding of 200 g L^{-1} of Pilsner malt and 10 g L^{-1} of Caraamber malt in a manual grain crusher, to expose the interior of malt grains. Then, mineral water was added to the crushed malt, initiating the process called cold mash, using previously determined times and temperatures (Rosa & Afonso, 2015; Dalberto et al., 2021), which will be presented in Table 1.

The resulting wort was filtered and then boiled (at approximately 95 °C) for one hour. Then, a new filtration was performed to eliminate the hot trub, consisting mainly of denatured proteins (Rosa & Afonso, 2015).

2.2 Optimization study

To evaluate the influence of temperature and mashing time variables on the production of brewer's wort using the cold mash technique, a two-level factorial design was applied. The planning tests were performed in triplicates. The variables studied and the levels evaluated are shown in Table 1.

Table 1. Factors and levels of fractional factorial design 2^2 to optimize the mashing stage in the production process of low-alcohol and free-alcohol beers.

Factors	Levels	
	(-)	(+)
Mashing Temperature (A)	7 °C	9 °C
Mashing Time (B)	12 hours	24 hours

The effects of the factors in the factorial design were calculated using Equation 1, where R+ and R- are the differences in the means of the levels (+) and (-), respectively, of the factors involved.

$$E_f = (R^+) - (R^-) \tag{1}$$

The effects of the variables on the physicochemical parameters of low-alcohol and free-alcohol beers were tested for statistical significance at the 95% confidence level by calculating the standard error and Pareto's graph using Minitab v. software. 16.2.2.

2.3 Physical-chemical characterization

The wort samples were analyzed using the physicochemical parameters of extract content, pH, and total acidity, using the international methods proposed by the American Society of Brewing Chemists (ASBC) (American Society of Brewing Chemists, 2018). The pH was determined using a digital pH meter after calibration with pH 4.0 and 7.0 standards. Extract content was determined from a refractometer with a Brix ABBE BIOBRIX degree scale, at 25 °C. The protein content of the brewing wort was determined based on the biuret method (Carvalho & Jong, 2002).

The color of the wort was determined according to Analytica-EBC method 8.3, where the color analysis was performed in a UV spectrometer at 430 nm using a quartz cuvette (Equation 2) (Brunelli et al., 2014).

$$EBC \text{ color} = Ab_{430nm} \times 25 \tag{2}$$

Ab is the absorbance at 430 nm wavelength, and EBC is the unit of measure of beer color.

3 Results and discussion

The study of the mashing step was characterized using the physicochemical parameters to verify if the standards determined by legislation and official guides were followed. These analyses were important to monitor the possible changes presented in cold mash compared to information reported in the literature for traditional hot mash.

To evaluate the influence of the temperature and time in the mashing stage, a 2^2 factorial design was used. The average effects calculated from the 2^2 factorial design are shown in Table 2.

Table 2. Calculated average effects and standard errors applied in the optimization of the mashing step for factorial design 2^2.

Effects	estimate ± standard deviation[a,b]				
	Extract content (°Bx)	pH	Total acidity (mEq L^{-1})	Color (EBC)	Protein content (%)
Global average	6.03 ± 0.03	6.16 ± 0.03	81.30 ± 1.25	11.00 ± 0.40	0.12 ± 0.01
Main effect					
Mashing temperature (A)	0.75 ± 0.06	-0.08 ± 0.06	2.50 ± 2.50	1.00 ± 0.80	-0.00 ± 0.02
Mashing time (B)	1.05 ± 0.06	0.08 ± 0.06	12.50 ± 2.50	2.50 ± 0.80	0.01 ± 0.02
Two-factor interaction effect					
A x B	-0.25 ± 0.06	0.08 ± 0.06	-7.50 ± 2.50	-1.00 ± 0.80	-0.00 ± 0.02

[a]Standard error of effects was calculated from the standard deviations. [b]Effects greater than 0.17 for extract content and pH; 6.94 for total acidity; 2.22 for color and 0.02 for protein content are significant at the 95% confidence level.

Brewing wort is mainly composed of carbohydrates, in the form of fermentable and non-fermentable sugars, which together with other minor substances present in the solution form the extract content. This parameter makes it possible to determine the approximate amount of alcohol that the beer will have at the end of the process (Gschaedler, 2017).

For a traditional beer, with about 5% ABV, the value that must be obtained for extract content is approximately 12 °Bx. To obtain low-alcohol (2.5%) or free-alcohol (0.5%) beers, the results for this analysis should be close to 5 °Bx (Branyik et al., 2012; American Society of Brewing Chemists, 2018). The results obtained for extract content are shown in Table 3.

Table 3. Average values for the physicochemical parameter of extract content and their standard deviations obtained by factorial design (2^2).

Factor			average value ± standard deviation[a]
(A)	**(B)**	**Sample***	**Extract content (°Bx)**
(-)	(-)	B7-12	5.00 ± 0.00
(+)	(-)	B9-12	6.00 ± 0.00
(-)	(+)	B7-24	6.30 ± 0.14
(+)	(+)	B9-24	6.80 ± 0.00

[a]Standard deviation calculated from triplicates of the factorial design tests. *B7: mashing temperature of 7 °C; B9: mashing temperature of 9 °C; 12: mashing time of 12 hours; 24: mashing time of 24 hours.

The analysis of the t-test and Pareto's graph at the 95% confidence level for extract content (Figures 1 and 2, Table 2) showed that all the studied factors are significant, as well as the interaction effect between temperature and mashing time (Figure 3), since calculated $t_{values} > t_{statistic}$ and $p < 0.05$. These factors cannot be analyzed separately, as their effects depend on the levels of other variables.

The data obtained for extract content were between 5.0 and 6.8 °Bx, with the concentration of soluble solids increasing along with the increase in mashing time and mashing temperature. The theoretical alcohol content is in the range of 2.8% and 3.8%.

Previous studies using the cold mash technique have shown that the brewing wort has a higher amount of non-fermentable sugars and a lower amount of fermentable sugars, which is the opposite of what is found for the wort obtained with hot mash (Dalberto et al., 2021). It is expected that the real alcohol content, to be studied in the future, is lower than the theoretical one, and the beers can be classified as low-alcohol content.

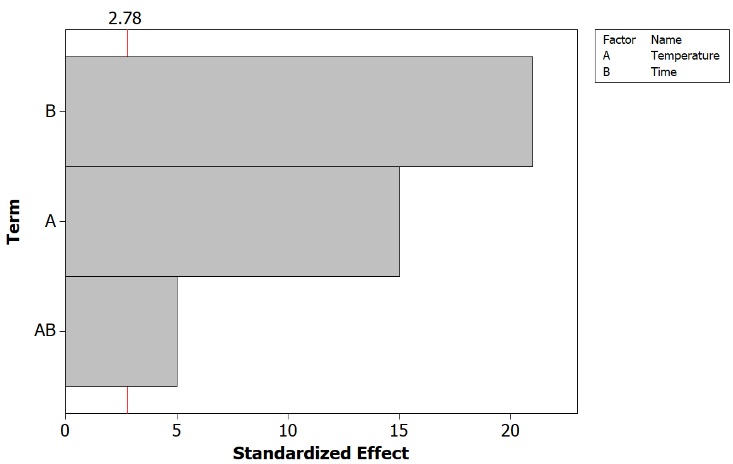

Figure 1. Pareto's graph at 95% confidence level for the standardized effects of factorial design applied to extract content of the wort samples.

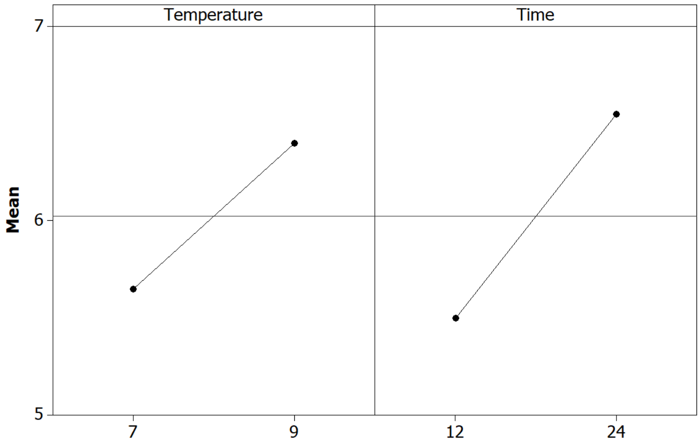

Figure 2. Graph of the significant main effects of mashing time and temperature for extract content.

Among the two main effects studied, it is noted that the mashing time is more important for the analysis of extract content, as there is a greater amplitude between the maximum and minimum points (Figure 2), systematically influencing the response. The two main effects are characterized as synergistic effects, since, by increasing the mashing time from 12 to 24 hours and the mashing temperature from 7 to 9 ºC, there is an increase in the concentration to the content of soluble solids.

The interaction effects of two factors (Figure 3) indicate that the lowest values of extract content, where it is possible to obtain beers with lower amounts of alcohol, are achieved when using the mashing time of 12 hours and the temperature of 7 ºC. These variables result in a lower level of fermentable sugars, as there is a shorter exposure time for starch hydrolysis (Dalberto et al., 2021) and a mild temperature, where the enzymatic action does not reach its maximum efficiency (Rosa & Afonso, 2015), resulting in lower alcohol content after the fermentation step.

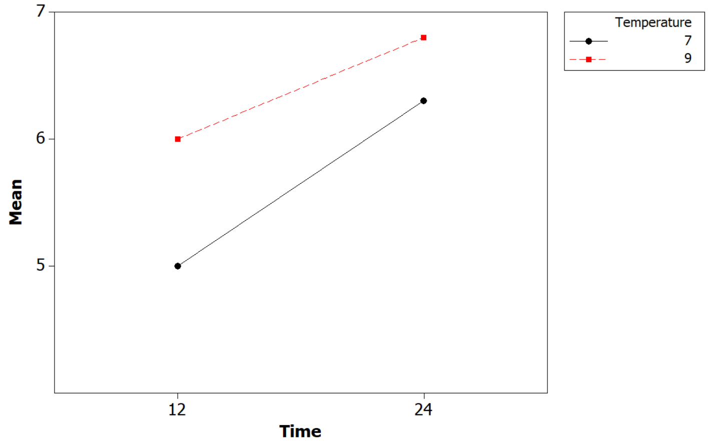

Figure 3. Graph of the interaction effects between mashing temperature and time for extract content.

Even using the highest temperature for mashing, but associated with the shortest time, 12 hours, the value obtained for the concentration of the extract was 6.0 ºBx, demonstrating that time is a more important parameter than the temperature to obtain lower alcohol contents.

Controlling the pH of the brewing wort is of great importance, as the performance of the enzymes responsible for starch hydrolysis will depend on this parameter (Perim et al., 2013). The pH range in which the α-amylase and β-amylase enzymes reach their maximum efficiency is between 5.4 and 5.8. After fermentation, it tends to decrease to values of 4.0 and 5.0, as during this phase various organic acids are formed (Brunelli et al., 2014; Francesco et al., 2018). The results obtained for the wort pH are shown in Table 4.

Table 4. Average values for the physicochemical parameter of pH and their standard deviations obtained by factorial design (2^2).

Factors			average value ± standard deviation[a]
(A)	**(B)**	**Sample***	**pH**
(-)	(-)	B7-12	6.20 ± 0.14
(+)	(-)	B9-12	6.05 ± 0.00
(-)	(+)	B7-24	6.20 ± 0.14
(+)	(+)	B9-24	6.20 ± 0.00

[a]Standard deviation calculated from triplicates of the factorial design tests. *B7: mashing temperature of 7 °C; B9: mashing temperature of 9 °C; 12: mashing time of 12 hours; 24: mashing time of 24 hours.

The pH of the brewer's wort ranged from 6.05 to 6.20 (Table 4). Studies conducted by Kutkoski et al. (2019) showed a pH between 5.66 and 6.07, these data being above those reported in the literature for traditional wort (Brunelli et al., 2014; Kutkoski et al., 2019). These higher pH values contribute to extracting undesirable compounds from the malt husk, such as higher levels of tannins, which can impact the colloidal and sensory stability of the beer through increased astringency (Bortoli et al., 2013).

Among the enzymes that act in the mashing stage, phosphatase is one of those responsible for lowering the pH of the wort, as they decompose organic phosphates, producing phosphoric acid (Pilarski & Gerogiorgis, 2020; Rosa & Afonso, 2015; Francesco et al., 2018). Since in the cold mash technique, no heating is performed during the mashing stage, each enzyme does not act in its optimal temperature and pH range, and this can be one of the causes of high values for the wort pH. This behavior may also explain the lower concentration of sugars found in the wort when this technique is used, since the action of the main enzymes responsible for starch hydrolysis, which will result in fermentable sugars, is also modified (Brunelli et al., 2014). It is necessary to correct the pH of the wort before starting mashing when using the cold mash technique, to avoid the extraction of undesirable compounds that can be metabolized by the yeasts in the fermentation stage and converted into off-flavors (Rosa & Afonso, 2015).

It is possible that the longer mashing time results in greater extraction of phenolic compounds from malt grains, such as phenolic acids and tannins, which are also responsible for the increase in the pH value. Regarding sensory characteristics, tannins result in astringent flavors, which cause the sensation of a drier drink (Quifer-Rada et al., 2015; Silva et al., 2021).

Analysis of the t-test and Pareto's graph at the 95% confidence level for pH (Figure 4) demonstrates that none of the factors studied, temperature and mashing time, are significant, since the calculated $t_{values} < t_{statistic}$ and $p > 0.05$. There was no significant interaction effect between the studied variables (Table 2). It is possible to optimize each factor in a univariate way since the effects for the pH parameter do not depend on the levels of the studied factors.

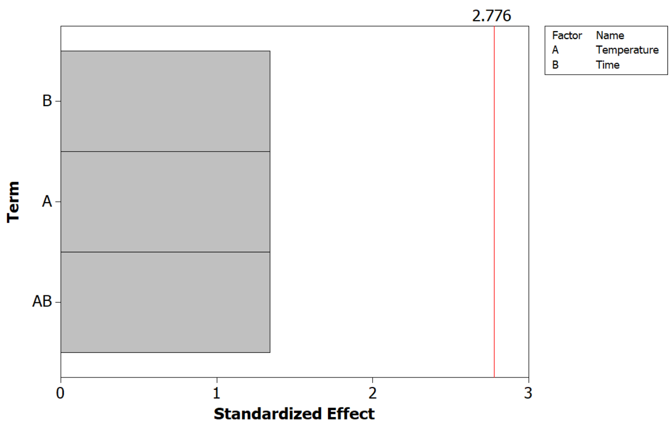

Figure 4. Pareto's graph at 95% confidence level for the standardized effects of factorial design applied to pH of the wort samples.

Data from the determination of total acidity are shown in Table 5. This parameter refers to the total titratable organic acids in beer. This analysis indicates microbial contamination in beer, due to the excess of acetic acid, and it is also possible to observe high values for the total acidity when only barley malt is used, instead of using adjuncts or wheat malt (Li & Liu, 2015; Alves et al., 2020). The determination of the total acidity of the brewing wort was carried out in this research, where there is little data in the literature for this parameter in this stage of the beer production process.

Table 5. Average values for the physicochemical parameter of total acidity and their standard deviations obtained by factorial design (2^2).

Factors			average value ± standard deviation[a]
(A)	**(B)**	**Samples***	**Total acidity (mEq L^{-1})**
(-)	(-)	B7-12	70.00 ± 0.00
(+)	(-)	B9-12	80.00 ± 0.00
(-)	(+)	B7-24	90.00 ± 0.00
(+)	(+)	B9-24	85.00 ± 7.00

[a]Standard deviation calculated from triplicates of the factorial design tests. *B7: mashing temperature of 7 °C; B9: mashing temperature of 9 °C; 12: mashing time of 12 hours; 24: mashing time of 24 hours.

Among the samples in this study, an increase in the total acidity values is observed when the mashing temperature is increased, from 7 to 9 °C, as well as when the mashing time is increased, from 12 to 24 hours. The data obtained for the wort varied between 70.00 and 90.00 mEq L^{-1}, while Kutkoski et al. (2019) obtained a maximum acidity value of 24.60 mEq L^{-1} using hot mash, indicating that the different mashing techniques, cold and hot, influenced this parameter. As the total acidity values tend to decrease after the fermentation stage, it is possible to obtain results close to those reported in the literature for the finished beer (Kutkoski et al., 2019).

Analysis of the t-test and Pareto's graph at the 95% confidence level for total acidity (Figure 5, Table 2) showed that the mashing time factor is significant, as well as the interaction effect between temperature and time, for the calculated $t_{values} > t_{statistic}$ and $p < 0.05$.

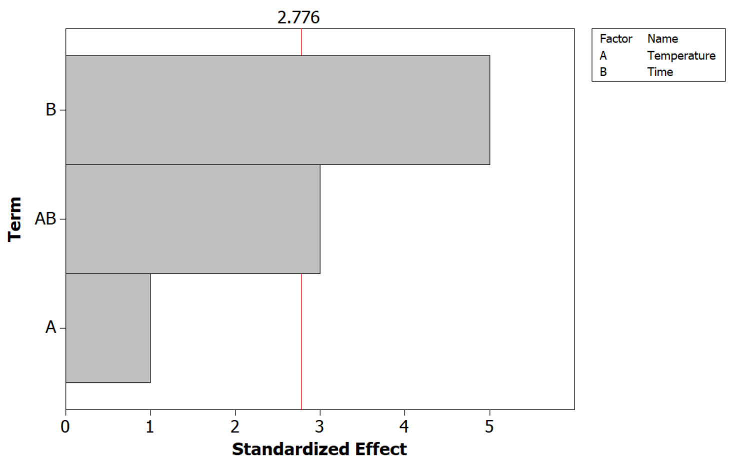

Figure 5. Pareto's graph at 95% confidence level for the standardized effects of factorial design applied to total acidity of the wort samples.

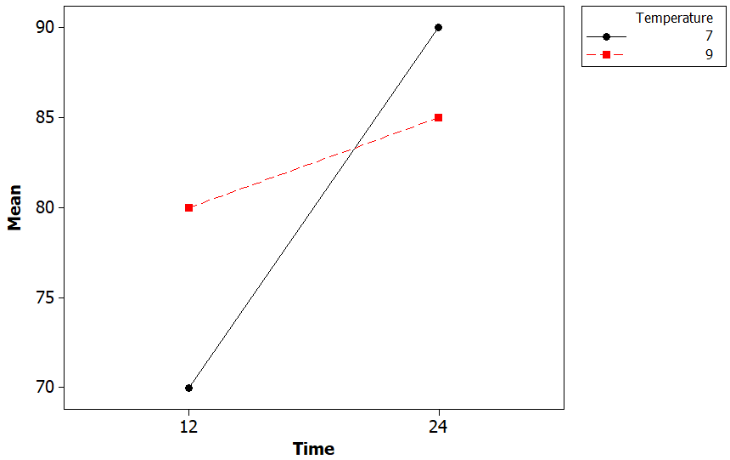

Figure 6. Graph of the interaction effects between mashing temperature and time for total acidity.

The graph for the two-factor interaction effect (Figure 6) between temperature and time showed that the highest total acidity value is obtained when mashing is performed at 7 ºC and for 24 hours, and the lowest value at 7 ºC and 12 hours. The mashing time factor exerts a great influence on this parameter, and the data to be obtained after the fermentation stage, which will be carried out in the future, must be considered, to be able to choose the most appropriate condition to obtain FABs and LABs that are sensorially balanced in terms of total acidity.

The color parameter of beers is important for the composition of the physical aspect of the drink, and is determined using the EBC (European Brewery Convention) scale of units as a standard (American Society of Brewing Chemists, 2018).

In this study, the same method was used to determine the color of brewing worts, where the result would provide an adequate approximation for the color of the finished beer, since the fermentation process results in a small decrease in color (Buglass, 2011). It was desired to obtain higher color values for the wort, compared to the values normally obtained for Pilsner-style beers, to ensure that the final product has the appropriate color. The results obtained for the color determination are shown in Table 6.

The color value for sample B7-12, of 9 EBC, corresponds to the yellow straw color, and for samples B9-12, B7-24, B9-24, which resulted in 11 and 12 EBC, the color values correspond to the golden color (Brunelli et al., 2014).

Table 6. Average values for the physicochemical parameter of color and their standard deviations obtained by factorial design (2^2).

Factors			average value ± standard deviation[a]
(A)	**(B)**	**Sample***	**Color (EBC)**
(-)	(-)	B7-12	9.00 ± 0.70
(+)	(-)	B9-12	11.00 ± 0.70
(-)	(+)	B7-24	12.00 ± 1.40
(+)	(+)	B9-24	12.00 ± 1.40

[a]Standard deviation calculated from triplicates of the factorial design tests. *B7: mashing temperature of 7 ºC; B9: mashing temperature of 9 ºC; 12: mashing time of 12 hours; 24: mashing time of 24 hours.

Analysis of the t-test and the Pareto's graph at the 95% confidence level for the color parameter (Figure 7) showed that the main factor mashing time is significant, as the calculated $t_{values} > t_{statistic}$ and $p < 0.05$. There was no significant interaction effect between the two variables studied in the evaluation of the color of the brewer's worts (Table 2).

The color of the musts has an increase in its value when the mashing time is increased, from 12 to 24 hours. This happens because the color is related to the malt (Rosa & Afonso, 2015), which is present in mashing from the beginning of the process, therefore, the 24-hour time allows a greater extraction of color, and also of flavors, for the wort.

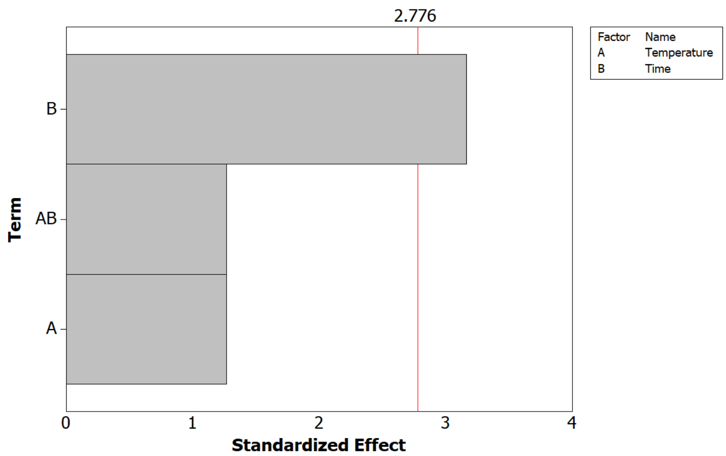

Figure 7. Pareto's graph at 95% confidence level for the standardized effects of factorial design applied to color of the wort samples.

In addition to the malt, there is also an intensification of the color during the wort boiling step, as the Maillard reaction is propitiated, which occurs between a protein or an amino acid and a reducing sugar, such as glucose and maltose, resulting in the formation of brown colored compounds called melanoidins (Buglass, 2011). To obtain a brewing wort with a higher value for the color parameter, which, consequently, will also have a higher concentration of malted flavors, a mashing time of 24 hours can be determined for the final beer formulation.

Studies conducted by Kutkoski et al. (2019) for the coloring of beers in the maturation phase showed results between 5 and 8 EBC. In this research, colors between 9 and 12 EBC were obtained for the brewer's wort. It is not possible to make a comparison between the wort and the beer in the maturation process, since it is known that the color decreases by approximately 2 units after the fermentation stage. Cold mash may present color between 7 and 10 EBC, evidencing the color gain that this new mashing process will confer on the finished beer (Kutkoski et al., 2019; Dalberto et al., 2021).

The proteins present in wort and beer originate mainly from malt grains, and are composed of amino acids. A large part of the proteins is precipitated and eliminated during mashing, boiling and cooling steps. In addition, a part is used by yeasts during the fermentation process, and the residue of proteins and amino acids that remain in the finished beer contributes to the mouthfeel of the drink and the retention of foam, and to the characteristic flavors for certain brewing styles (Buglass, 2011; Gasiński et al., 2023). The results obtained for determining the protein content are shown in Table 7.

Table 7. Average values for the physicochemical parameter of proteins content and their standard deviations obtained by factorial design (2^2).

Factors			average value ± standard deviation[a]
(A)	**(B)**	**Sample[*]**	**Proteins content (%)**
(-)	(-)	B7-12	0.12 ± 0.00
(+)	(-)	B9-12	0.12 ± 0.00
(-)	(+)	B7-24	0.13 ± 0.01
(+)	(+)	B9-24	0.13 ± 0.01

[a]Standard deviation calculated from triplicates of the factorial design tests. *B7: mashing temperature of 7 °C; B9: mashing temperature of 9 °C; 12: mashing time of 12 hours; 24: mashing time of 24 hours.

The data obtained for the protein content in this study were between 0.12 and 0.13%, these values being lower than those presented by Brunelli et al. (2014) for beers prepared with the addition of honey, where it was obtained protein contents between 0.38 and 0.79%. The absence of heating during the mashing step may be the explanation for the low concentration of wort proteins, as the temperature contributes to their extraction from the malt grains and their breakdown into smaller units, by activating enzymes (Brunelli et al., 2014; Niu et al., 2018; Kerr et al., 2019).

Studies conducted by Silva (2005) for the determination of proteins in malt, brewery wort and beer demonstrated that there is a significant decay in the values between the wort and the beer, where part of the protein content is eliminated after the fermentation and maturation stages. So, there is a tendency for the data obtained in this study for proteins to be even smaller for the finished beer. The importance of obtaining low values for the protein content lies in the possibility of analyzing the gluten concentration in these beers, which may be low enough to be characterized as a gluten-free beer, below 20 ppm (Cela et al., 2022; Gasiński et al., 2023; Ledley et al., 2023b), making room for reaching different consumers.

The analysis of the t-test and the Pareto's graph at the 95% confidence level for the protein content (Figure 8) demonstrates that none of the factors studied, temperature and mashing time, are significant, since the calculated $t_{values} < t_{statistic}$ and $p > 0.05$. There was no significant interaction effect between the studied variables (Table 2). It is possible to optimize each factor in a univariate way, since the effects on protein concentration do not depend on the levels of the studied factors.

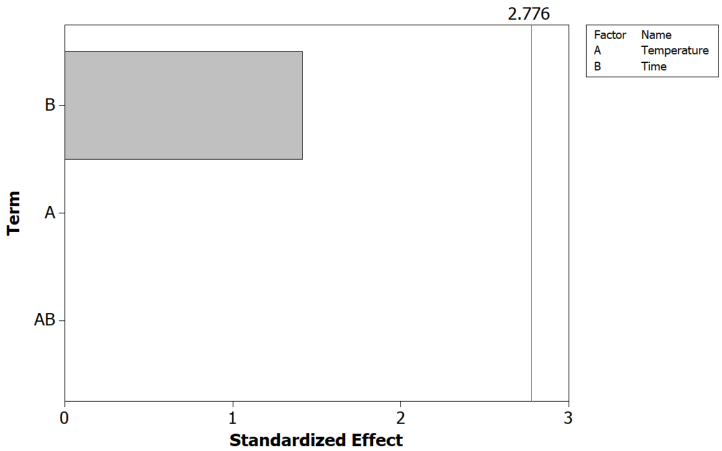

Figure 8. Pareto's graph at 95% confidence level for the standardized effects of factorial design applied to proteins content of the wort samples.

4 Conclusions

The study of the mashing step demonstrated that the use of the cold mash technique was adequate to reach a lower concentration of carbohydrates (extract content) for the brewing wort, and this could make it possible to obtain free-alcohol and low-alcohol beers in future studies. This highlights the innovation present in this mashing technique and the advantages of using it to obtain these characteristics.

The application of a 2^2 factorial design allowed the verification that the mashing time was a more important factor than the temperature, systematically influencing the physicochemical parameters of extract content, total acidity, and color of the wort samples. Among these parameters, only for the color analysis, the results were similar to those of traditional beers obtained. The pH showed values above those reported in the literature for the brewer's wort, which may be a characteristic of the cold mash technique. Concerning the protein content, data obtained were below that found for traditional worts, which may indicate an even lower protein concentration for the finished beer.

Aiming to pay attention to the sensory aspects of subsequent beer samples, the conditions in which the mashing time is longer, 24 hours, may prove to be more appropriate, as there is a greater intensity of color and flavors and malty aromas, due to the longer extraction time of these malt characteristics.

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Funding: None.

Received: Jan. 23, 2023; **Accepted:** Sept. 13, 2023

Associate Editor: Charles W. Isidoro Haminiuk.