Influence of Fungicide Residues on the Primary Fermentation of Young Lager Beer

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The effect of four sterol biosynthesis-inhibiting fungicides added to the pitching wort on the evolution of several organoleptic parameters during the primary fermentation of young lager beer was assessed. Pyrimidine (nuarimol and fenarimol) and triazole (myclobutanil and propiconazole) fungicides were individually supplied to the pitching wort to obtain a concentration of 1 mg/L. A marked influence in the fermentation rate was observed for the samples with propiconazole residues. From the fourth day onward, the fermentation prematurely ceased (stuck fermentation), and therefore, statistical significant differences were found in fermented extract, alcohol content, fermentable carbohydrates, pH, color, and total polyphenol and flavonoid contents of beer. Myclobutanil residues are only influenced in the total polyphenol and flavonoid contents, while differences in the analyzed parameters were not noticeable for the samples containing nuarimol and fenarimol residues in comparison with the blank sample.

KEYWORDS: Beer; brewing; fungicide residues; lager beer; organoleptic parameters; primary fermentation; wort

INTRODUCTION

Several pests and diseases can attack cereal crops, and good weed control is essential if the crop is to make efficient use of moisture and to prevent weed seeds from contaminating the harvest (*1*). Furthermore, hops have several pests and diseases (*2*). For this reason, farmers need to protect their crops with pesticides. In addition, malting barley in silo storage is often treated with pesticides to prevent insect infestation.

When pesticides are used in or on plant products, residues frequently occur on the raw agricultural commodities. The problem is that pesticide residues may remain in the beer produced from the treated ingredients (*3, 4*). During the first steps (malting, mashing, and boiling), the pesticides on the barley can pass into the wort in different proportions, depending on the process used, although it should be noted that the removal of material in the form of trub and spent grains tends to reduce the level of contaminants, especially pesticides, which are often relatively insoluble in water (*5−11*). Depending upon the stage involved and the physical−chemical properties (mainly K_{OW} [as log *P*] value, water solubility, vapor pressure, and Henry's Law constant) of the residues, differences in their final fate are observed. Therefore, the maltsters should devote special attention to the residues of hydrophobic pesticides with $K_{OW} > 2$ because they can remain on the malt. On the contrary, brewers should control residues of hydrophilic pesticides with $K_{OW} <$

4 because they can be carried over into beer. Thus, the monitoring and surveillance of pesticide residues with K_{OW} values ranging from 2 to 4 (most of them) during the brewing process are essential to obtain a healthy drink.

Also, if pesticide residues are present in the brewer wort, they can alter the normal fermentative process, which causes in certain cases sluggish and even stuck fermentation, although this depends to a great extent on the initial concentrations in the malted barley, on the physical−chemical characteristics of each product, and on the beer making procedure. As a consequence, the organoleptic properties of beer should be modified as in other fermented beverages such as wine (*12, 13*).

Concretely, the four fungicides used in this study are sterol biosynthesis-inhibiting fungicides (SBIs). They inhibit the cytochrome P_{450} monooxygenase, which catalyzes the oxidative C_{14} demethylation of 24-methylenedyhydrolanosterol in the biosynthesis pathway, and are a widely applied class of antifungal agents because of their broad therapeutic window, wide spectrum of activity, and low toxicity (*14*). Some authors suggest that the complex nitrogen composition of the medium may create conditions resembling those responsible for inducing sluggish/stuck fermentation (*15*).

In a previous paper, we studied the fate of propiconazole, myclobutanil, and nuarimol residues during the brewing of lager beer (*9*). The findings show that although a great amount of them (26−42%) is retained on the spent grains after the mashing and boiling stages, residues of their parent concentration in malt

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Table 1. Principal Physical–Chemical Characteristics of the Studied Compounds

Common and chemical (IUPAC) name	Chemical structure	Molecular formula	Molecular weight	Log K_{ow}	Water solubility (mg/L)
Propiconazole (±)-1-([2-(2,4-Dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl)-1H-1,2,4-triazole		$C_{15}H_{17}Cl_2N_3O_2$	342.2	3.72	100
Myclobutanil 2-(4-chloro-phenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-hexanetrile		$C_{15}H_{17}ClN_4$	288.8	2.94	142
Fenarimol (±)-(2-Chlorophenyl)(4-chlorophenyl)-pyrimidin-5-yl)methanol		$C_{17}H_{12}Cl_2N_2O$	331.2	3.69	14
Nuarimol (±)-(2-Chlorophenyl)(4-fluorophenyl)-pyrimidin-5-yl)methanol		$C_{17}H_{12}ClFN_2O$	314.7	3.18	26

(4−8%) are present in the brewer wort. Other data show that the carryover of fenarimol in red wines varied from 48 to 60% from the pressed must until the finished wine (*16*).

For this reason, the objective of this study was to asses the influence of pyrimidine and triazole fungicides, chemically distinct but inhibiting sterol biosynthesis through a common mode of action (*17*), on several organoleptic characteristics during the fermentation of young lager beer.

MATERIALS AND METHODS

Pesticides and Reagents. Pesticide standards with a purity higher than 98% were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). **Table 1** shows the pesticides used and their main physicochemical characteristics (*18*). Stock standard solutions of 100 µg/mL were prepared by exact weighing and dissolving in acetone and finally stored in the dark at 4 °C. The solvents acetone and isooctane were supplied by Scharlau Chemie S.A. (Barcelona, Spain). Carboxymethyl cellulose/ethylenediamine tetraacetic acid (CMC/EDTA), green ammonium iron citrate, and *p*-dimethylamnocinnamaldehyde were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Hydrochloric acid and concentrated ammonia were supplied from Panreac Química S.A. (Barcelona, Spain).

Raw Materials. Barley malt (moisture 4.6%, pH 6.0, and pesticides below detection limit) was obtained from the brewer Estrella de Levante Fábrica de Cerveza S.A. (Murcia, Spain), after soaking the raw grains of two-row spring malting barley (*Hordeum distichum*), allowing them to germinate (sprout), heat, and then dry (malting). The same supplier provided maize, rice, and hop pellets (Var Nugget). Lager yeast, *Saccharomyces carlbergensis* (Rh), was purchased from Versuchs-und Lehranstalt für Brauerei (Berlin, Germany). The water used in the process (EC 0.93 dS/m at 25 °C, pH 8.22, DOC 1.42 mg/L, alkalinity 184.3 mg/L, trialomethanes 45.2 µg/L, and pesticides below detection limit) was obtained from the municipal network.

Brewing Process. The malt (800 g), once milled into fine grits to ensure good access of water to the grain particles in the subsequent phase of beer production, was thoroughly mixed with approximately 5 volumes of water to yield mash and subjected to mashing as previously published (*9*). Boiled, gelatinized starch from milled maize (45 g) and rice (250 g) was added as an adjunct during mashing to achieve a higher content of fermentable sugars. At the end of the mashing process, soluble substances and residual solid particles were separated by filtration into sweet wort and spent grains, respectively. In the next step, hop pellets (0.5 g twice, 30 and 80 min), the source of the bitter taste, were added during wort boiling (90 min). After boiling and clarification, the brewer wort (3.8 L) was quickly cooled in preparation for the addition of yeast and subsequent fermentation with brewer's yeast. At this time, wort samples (300 mL) were individually spiked ($n = 3$) with 300 µg of each one of the fungicides. After evaporation of the spiking solvent (3 h), lager yeasts (bottom fermenting yeast) were added (10×10^6 to− 15×10^6 cells/mL) to each fermentation vessel ($n = 3$) containing the oxygenated pitching wort, which was maintained at 10 ± 1 °C for 13 days. In fermentation management, five control points were differentiated according to Kunze (*19*): (i) when the process begins (initial), (ii) the fine bubble foam becomes deeper (low krausen), (iii) fermentation has entered its most intensive stage (high krausen), (iv) the fermentation has become less vigorous, and the foam looks browner (krausen collapsing), and (v) the rate of fermentation continues to decrease and finally forms a dirty brown layer (collapsed foam). **Figure 1** shows the procedure followed for standard beer making.

Analysis of Color, Total Polyphenols, and Flavonoids. The color (EBC units) of the beer was measured at a wavelength of exactly 430 nm in a 10 mm cell (EBC method 8.5). The bitter substances were extracted from acidified beer with isooctane. After centrifugation, the absorbance of the isooctane layer was measured at 275 nm (EBC method 9.8). The determination of total polyphenols in beer was realized by treatment of the sample with a solution of CMC/EDTA and posterior reaction with ferric ions in alkaline solution. The absorbance of the red solution was measured at 600 nm (EBC method 9.11). Finally, determination of flavonoids was carried out by spectrophotometry (640 nm) after addition of the chromogen solution under acidic conditions (EBC method 9.12) (*20*).

Analysis of Fermentable Carbohydrates. After centrifugation of the wort samples at 15 000*g* for 5 min to remove particles, 2 mL of the supernatant was filtered through a syringe filter (nylon, 0.45 µm pore size). After filtering, 20 µL was injected into the HPLC system. The apparatus consisted of a Waters 501 liquid chromatography pump equipped with a Rheodyne injector (Millipore Co., Wellesley, MA) and a Waters 410 Differential Refractometer (Millipore Co., Wellesley,
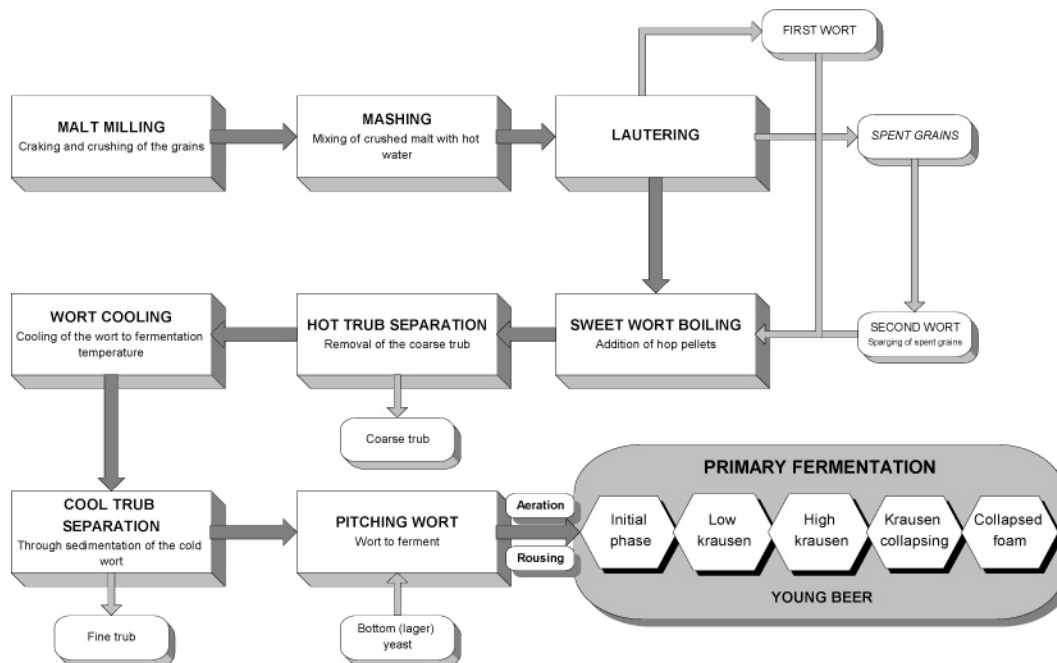
Figure 1. Diagram illustrating phases for the brewing process.

MA). Data were collected and integrated by Empower Software. The operating conditions were as follows: a 25 cm × 4.6 mm i.d. Supelcosil LC-NH$_2$, 5 μm particle column (Supelco, Bellefonte, PA) with acetonitrile/water (75:25) as the mobile phase at a flow rate of 1 mL/ min and room temperature. In these conditions, the retention times of carbohydrates were 3.81, 4.22, 5.30, 6.31, and 9.65 min for fructose, glucose, sucrose, maltose, and maltotriose, respectively. Quantitation of carbohydrates was based on calibration curves obtained after the addition of known amounts of carbohydrates (0.1−50 g/L).

Statistical Analysis. SigmaStat 3.10 for Windows (Systat Software, Inc., San Jose, CA) was used for One Way Analysis of Variance (ANOVA). To isolate the group or groups that differs from the others, a multiple comparison procedure was used (Holm−Sidak method).

RESULTS AND DISCUSSION

Brewhouse Yield. At the end of the wort boiling, when cooled, 3.8 L of brewer wort was obtained. After boiling, the pH value was 5.8. Before pitching, the pH was adjusted to 5.3 by use of phosphoric acid because of many important processes proceeding better or more quickly at a lower pH. On pitching, the hydrometer measured 13.2% (g of extract per 100 mL at 20 °C). To measure the brewhouse yield (Y_{bh}), the amount of extract produced was related to the amount of grist used (*19*). The value was calculated from

$$Y_{bh} = \text{extract mass (g)} \times 100/\text{grist mass (g)}$$

The yield obtained after wort boiling was 60.7%. The brewhouse yield depends on several factors such as the raw materials, the brewhouse equipment, the mash process, the lautering operations, and the overall operations methods. Other causes different from the malt quality have been pointed out. Thus, carbonate- and sulfate-rich water reduces the yield. Also, soft water, as a result of its pH lowering nature, improves enzyme activity and thus the yield, but this is not our case. Other causes, such as laboratory equipment, control the mash and lautering processes, and in a general way, the development of the overall operating methods could be pointed as responsible for low yields.

Progress of Specific Gravity. A marked influence in the fermentation rate was observed (see **Figure 2** where the
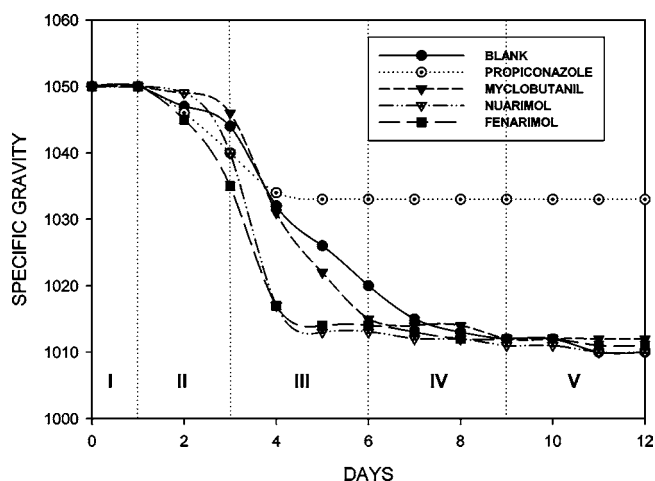

Figure 2. Evolution of specific gravity ($n = 3$) vs time during fermentation phases (I: initial; II: low krausen; III: high krausen; IV: krausen collapsing; and V: collapsed foam) for blank and treated samples.

evolution of specific gravity with time is shown for both blank and treated samples). As can be seen, from the fourth day onward, the fermentation prematurely ceases (stuck fermentation, i.e., the premature termination of fermentation before all fermentable sugars have been metabolized) in the samples with propiconazole residues as compared with the blank. This finding is important because residues of this compound can pass from barley and malt to brewer wort as some authors have shown (*7, 9*). In the case of the blank sample and that with myclobutanil residues, the evolution of specific gravity is similar. For samples treated with fenarimol and nuarimol residues, the fermentative kinetics is quicker from days 2 to 6, probably due to the rapid assimilation of nitrogen by the yeasts.

Evolution of Temperature, Degree Plato, Fermented Extract, and Alcohol Content. Primary fermentation was started by pitching of the wort (i.e., the addition of the yeast to the wort). Immediately before the addition of yeast, the wort is called pitching wort, and this, immediately after the yeast addition, is already referred to as young beer. For yeast growing, it is fundamental to provide the yeast with an appropriate oxygen

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Table 2. Evolution of Temperature (°C), Degree Plato (°*P*), and Degree of Attenuation (*V*, %) during Primary Fermentation (*n* = 3)

fermentation phase	mean ± RSD														
	blank			fenarimol			myclobutanil			nuarimol			propiconazole		
	T	°*P*	*V*	*T*	°*P*	*V*	*T*	°*P*	*V*	*T*	°*P*	*V*	*T*	°*P*	*V*
I[a]	10 ± 2.3	12.2 ± 1.5	1 ± 4.1	10 ± 3.3	11.7 ± 2.2	1 ± 3.4	10 ± 3.2	12.8 ± 1.9	0 ± 0.6	10 ± 2.3	11.4 ± 3.5	2 ± 2.2	10 ± 1.7	12.0 ± 2.5	1 ± 1.1
II	11 ± 3.3	11.5 ± 2.1	8 ± 2.2	12 ± 4.1	9.1 ± 2.4	21 ± 3.5	12 ± 2.3	12.0 ± 4.5	5 ± 1.6	13 ± 3.4	10.4 ± 4.5	24 ± 3.3	11 ± 1.4	10.4 ± 2.6	11 ± 2.2
III	15 ± 4.6	5.2 ± 2.2	39 ± 1.5	15 ± 2.3	3.6 ± 3.4	45 ± 4.2	14 ± 3.5	3.9 ± 3.5	45 ± 2.4	16 ± 2.3	3.4 ± 3.6	46 ± 3.1	11 ± 2.6	8.6 ± 3.1	24 ± 3.3
IV	13 ± 3.4	3.1 ± 1.7	45 ± 1.6	14 ± 2.1	3.1 ± 3.1	49 ± 2.5	12 ± 3.6	3.1 ± 3.7	48 ± 2.2	14 ± 4.2	2.8 ± 3.2	49 ± 2.6	10 ± 3.2	8.4 ± 2.8	25 ± 2.3
V	11 ± 2.1	2.6 ± 3.1	47 ± 3.4	11 ± 1.4	2.8 ± 2.4	50 ± 3.2	9 ± 2.8	3.1 ± 2.8	49 ± 3.1	11 ± 2.5	2.6 ± 2.5	50 ± 3.2	9 ± 3.3	8.4 ± 3.7	25 ± 2.5

[a] I: initial; II: low krausen; III: high krausen; IV: krausen collapsing; and V: collapsed foam.

content in the wort to initiate the multiplication and fermentation steps. For this, aeration (process to provide oxygen) and rousing (yeasts mixing) are of great importance for a correct fermentation management. An inadequate aeration can have undesirable consequences such as longer fermentation time, defective secondary fermentation, problem with beer quality, etc.

The evolution of temperature, degree Plato (°*P*, specific gravity as the weight of extract in a 100 g solution at 17.5 °C), and degree of attenuation (*V*, the extent of conversion to alcohol) during the different phases of primary fermentation are shown in **Table 2**. Very important in fermentation management is the control of temperature and fermentation time. Fermentation time was 12 days except in the case of the propiconazole assay, where significant differences were observed regarding the blank. Although the pitching temperature is normally 5−6 °C, in our case, it was raised (10−12 °C) to start the fermentation more quickly. As a consequence of the heat released during fermentation, the temperature of the wort was increased until a maximum of 14−16 °C in the high krausen stage with the exception of the sample spiked with propiconazole when the maximum temperature was 11 °C. The brewers prefer cold fermented beer with a maximum temperature of 10 °C because of less byproducts, mainly higher alcohols and esters. On the other hand, during fermentation, the extract is continuously being fermented. No significant differences (*P* < 0.05) were found for the *V* value between the blank sample and those fermented in the presence of myclobutanil, fenarimol, and nuarimol residues (47−50), while for the sample treated with propiconazole, the value of *V* was approximately half (25) that in the other assays. The same behavior can be observed for (°*P*). The final value (8.4) is approximately 3 times higher than in the other cases (2.6−3.1). The difference between the extract content of the pitching wort and that of the beer at any time point is called the fermented extract. As a consequence, the alcohol content at the end of fermentation was sensibly lower in the sample containing propiconazole residues (1.4%) than in those spiked with myclobutanil (5%), nuarimol (5.2%), and fenarimol (5.4%), while in the blank sample, the alcohol content was 5.5%. These results show that propiconazole strongly affects the growth and fermentability of brewer's yeast, influencing the fermentative kinetics and causing stuck fermentation from the high krausen phase.

Changes of Sugars during Fermentation. The most important process to transform wort into beer is fermentation of the sugars in the wort to ethanol and carbon dioxide by enzymes in the yeast. **Figures 3−7** show the evolution of fermentable carbohydrates during fermentation for both blank and treated samples. The concentration of sugars in the pitching wort was in the following order: maltose > maltotriose > glucose > sucrose > fructose. No significant differences were observed between the blank sample and those treated with myclobutanil, nuarimol, and fenarimol residues, while in the case of the propiconazole assay, a delay in the glucose consumption was
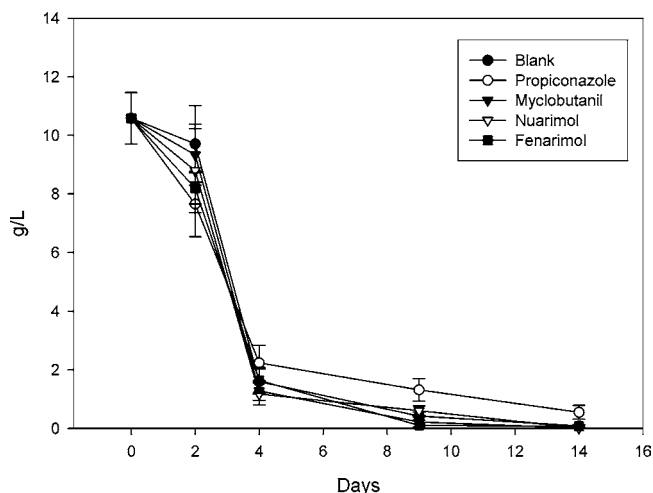

Figure 3. Change in glucose content (*n* = 3) vs time during fermentation for blank and samples treated with fungicides (error bars are 95% confidence intervals).
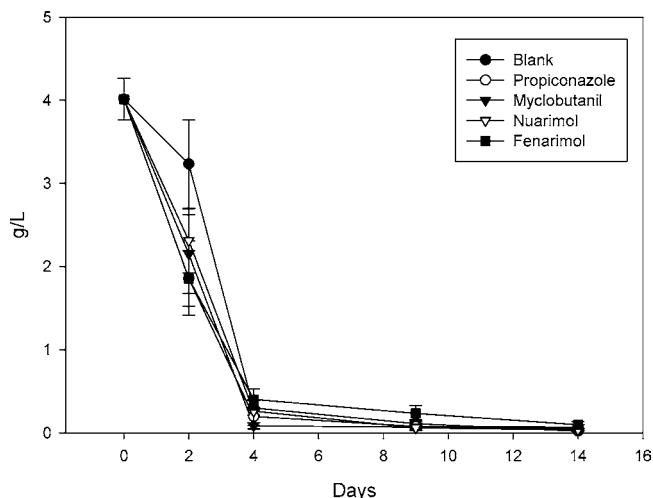

Figure 4. Change in sucrose content (*n* = 3) vs time during fermentation for blank and samples treated with fungicides (error bars are 95% confidence intervals).

observed after 4 days (**Figure 3**). No significant differences were observed between the blank and the other samples, although assimilation of sucrose was something slower in the blank sample during the first 48 h (**Figure 4**). Fructose assimilation follows a different behavior from glucose and sucrose. Samples with nuarimol and fenarimol (pyrimidine fungicides) residues consume this sugar more quickly than those with propiconazole and myclobutanil (triazole fungicides) residues. The slowest assimilation corresponds to the blank sample. In all cases, the higher consumption occurs from 24 to 216 h (**Figure 5**). Maltose exhibits a similar behavior, although the biggest consumption
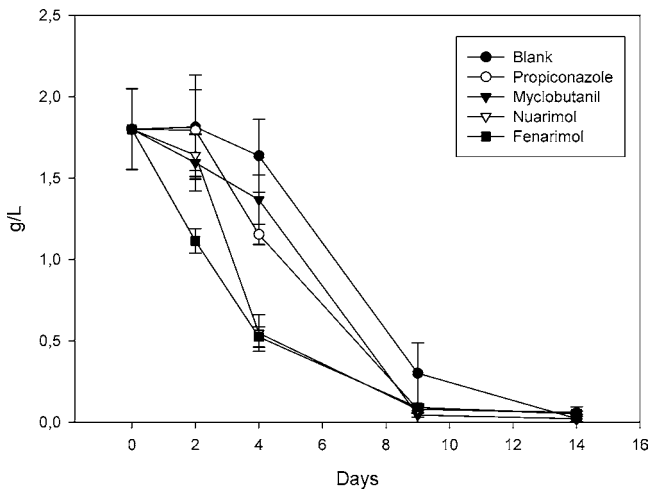
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Figure 5. Change in fructose content ($n = 3$) vs time during fermentation for blank and samples treated with fungicides (error bars are 95% confidence intervals).
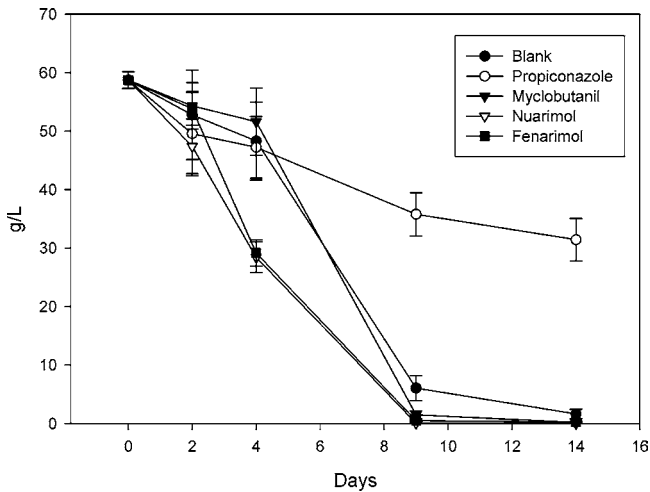

Figure 7. Change in maltotriose content ($n = 3$) vs time during fermentation for blank and samples treated with fungicides (error bars are 95% confidence intervals).

Figure 6. Change in maltose content ($n = 3$) vs time during fermentation for blank and samples treated with fungicides (error bars are 95% confidence intervals).

Color (EBC units)

Figure 8. Correlation between pH and color values ($n = 3$) of beer during fermentation.

takes place between 96 and 216 h, during the main fermentation. It is important to remark that after the fourth day, the consumption of this sugar is decreased drastically by the yeasts in the sample with propiconazole residues, which is logical bearing in mind that fermentation was stopped at this time (**Figure 6**). Finally, maltotriose is the last sugar assimilated by the yeasts. No significant differences were observed when comparing the behavior of the blank sample and those with residues of nuarimol and fenarimol. On the other hand, triazole fungicides, especially propiconazole, have a marked influence on the assimilation of this sugar by the yeasts (**Figure 7**).

Decrease of the pH Value and Color of Beer during Fermentation. The pH value at the beginning of fermentation was 5.3, falling substantially during fermentation in all cases. The pH fell particularly in the initial and logarithmic growth phases until 4.3–4.6. This could be due to the formation of organic acids formed mainly by yeast from the amino acids present in wort, the use of primary phosphate ions by the yeasts, the uptake of ammonium ions by the yeasts, and the uptake of potassium ions by the yeasts and release of hydrogen ions into the beer (*19*). The mean pH values at the end of the fermentation were 4.1 for the blank sample and 3.0, 3.7, 3.8, and 3.9 for those containing residues of propiconazole, myclobutanil, fe-
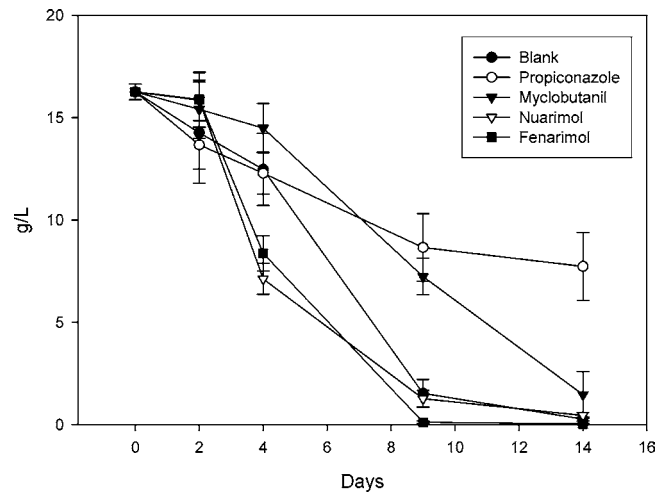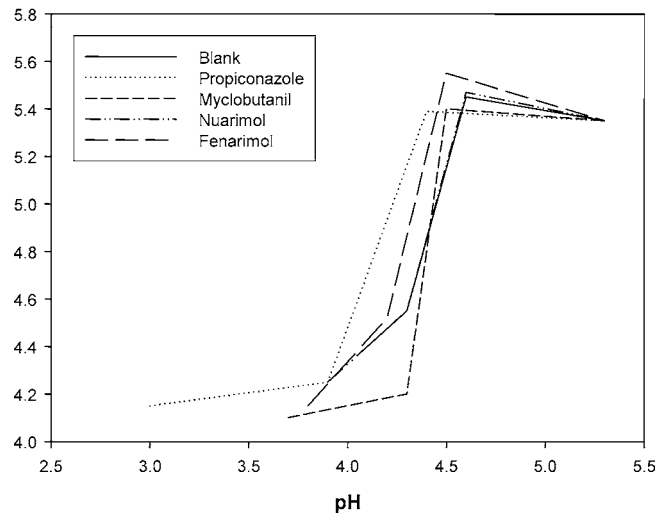
narimol, and nuarimol. Also, in this case, the presence of propiconazole alters sensibly the final quality of the beer. This finding is important because pH values below 4.0 cause an acidic beer taste, and acidification by microbial infections during fermentation must be avoided.

Figure 8 shows the correlation between pH and color during fermentation. At the beginning of the process, 5.35 EBC units were recorded. Although a slight increase after 2 days of fermentation can be observed in all cases, the color of the beer fell about 1–1.5 EBC units during fermentation, possibly due to the decoloration of some substances caused by the drop in pH and absorption of highly colored compounds in the yeast cells or precipitation in the vessel bottom (*19*).

Bitter Substances and Total Polyphenol and Flavonoid Contents. Table 3 shows the values for bitterness, total polyphenol, and flavonoid values found at the end of fermentation. As a result of a decrease in pH during fermentation, a number of colloidally dissolved bitter substances and polyphenols can precipitate as surface active compounds on the CO_2 bubbles in the foam head or as a result of adsorption on the yeast cells (*19*). As consequence of their low solubility at a pH below 5 and temperatures lower than 10 °C, the α-acids were not isomerized during the boiling of the wort precipitate. For

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Table 3. Bitter Substances, Total Polyphenol, and Flavonoid Contents in Beer ($n = 3$)

parameter	blank	nuarimol	fenarimol	myclobutanil	propiconazole
			mean ± RSD		
bitterness units	BDL[a]	BDL	BDL	BDL	BDL
total polyphenols (mg/L)	59.0 ± 2.6	61.2 ± 4.6	60.7 ± 4.4	33.6 ± 4.1	17.2 ± 5.7
flavonoids (mg/L)	35.2 ± 3.5	36.8 ± 6.7	35.8 ± 2.9	33.8 ± 3.8	16.8 ± 6.6

[a] BDL: below detection limit.

this reason, the values of bitterness are below detection limits in all cases. Regarding total polyphenol and flavonoid contents found after fermentation, significant differences ($P < 0.05$) were observed between the samples containing residues of triazole fungicides and the others, especially in the case of propiconazole due to the stuck fermentation after 4 days.

Bearing in mind the previous discussion, if the pitching wort contains SBIs, especially triazole compounds, it is important to use fining agents such as activated charcoal, bentonite, or PVPP to eliminate or at least reduce the SBI concentration in the wort since it can alter the quality of the beer. Some results obtained by us (unpublished data) confirm that the use of activated charcoal and bentonite considerably reduce the level of these compounds in the wort. Concretely, more than 80% of myclobutanil and propiconazole residues can be removed.

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