JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Behavior of Myclobutanil, Propiconazole, and Nuarimol Residues during Lager Beer Brewing

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Over a 4 month brewing process, the fate of three fungicides, myclobutanil, propiconazole, and nuarimol, was studied in the spent grain, brewer wort, and final beer product. Only the residual level of myclobutanil after the mashing step was higher than its maximum residue limit (MRL) on barley. A substantial fraction was removed with the spent grain in all cases (26−42%). The half-life times obtained for the fungicides during storage of the spent grains ranged from 82 to 187 days. No significant influence of the boiling stage on the decrease of the fungicide residues was demonstrated. During fermentation, the content reduction varied from 20 to 47%. After the lagering and filtration steps, no significant decrease (<10%) was observed in any of the residues. Finally, during storage of the beer (3 months), the amounts of fungicides fell by 25−50% of their respective concentrations in the finished beer.

KEYWORDS: Beer; brewing processes; fungicide residues; malted barley; spent grain; wort

INTRODUCTION

Beer drinking has been increasing steadily in recent decades even in countries where alcoholic beverages are not traditional. Beer, indeed, has become an international drink, especially among young people. Furthermore, it is now recognized that there may be some health benefits associated with the moderate consumption of beer (*1*). One of the most important factors contributing to the public perception of beer as a "healthy" drink has been the accumulation of studies showing that moderate drinkers have lower death rates from all causes, but especially from cardiovascular-related diseases, than either nondrinkers or heavy drinkers (*2*−*7*). Furthermore, the nutritive aspects of beer, such as the lower content of sugars than most soft drinks, negligible fat content, and vitamin and mineral contents, should not be understated (*8*−*11*).

Four raw materials are required for beer production: barley, hops, water, and yeast. The quality of these raw materials has a decisive influence on the quality of the final product, and knowledge of their properties and of their effects on the process and product provides the basis for their correct handling and processing (*12*).

Barley is the main ingredient for beer production. However, before it is used in the brewery, the barley must first be converted into malt. Unmalted cereals, especially maize and rice, are often used as adjuncts. It is important to note that several pests and diseases can attack these 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. Furthermore, a range of head, root, leaf, and stem diseases may affect barley, depending on climate, environment, and farm history. Some of the more common diseases include crown rot, rust, smut, root rot, net blotch, and nematode infection. Finally, armyworms, cutworms, and mites cause important damage to cereals in some areas (*13*). For this reason, farmers need to protect their crops during silo storage, and pesticides are used to prevent insect infestation. The problem is that traces of these pesticides may remain in the beer produced from the treated ingredients, although the residues may also come from the soil itself or the water used. During the first step (malting), some residues of pesticides having log $K_{ow} > 2$ (as log P) would remain on malt as indicated by some authors (*14*). After mashing and boiling, the pesticides on the malt 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 pesticides, which are often relatively insoluble in water, as occurs with pyrethroid insecticides with high K_{ow} values (*15*). The fourth step in beermaking is fermentation, during which yeasts metabolize sugars into energy, alcohol, carbon dioxide, secondary byproducts, and more yeast. If pesticide residues are present in the fermenting wort, they may cause organoleptic alterations to the finished beer and have toxic effects for the consumer. However, yeasts have been shown to degrade some pesticides, whereas others are removed by surface adsorption. Also important is the degradation of certain pesticides during brewing. The water solubility of some metabolites can be greater than those of their parent compounds as in the case of triadimefon,

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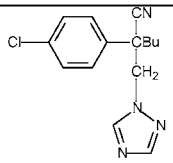
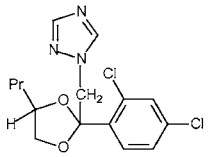
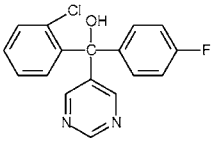
10.1021/jf0505832 CCC: $30.25
Published on Web 10/06/2005

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

Common name	Chemical structure	Molecular formula	Molecular weight	K_{OW} (log P)	Water solubility (mg/L)
Myclobutanil		$C_{15}H_{17}ClN_4$	288.4	2.94	142
Propiconazole (±)		$C_{15}H_{17}Cl_2N_3O_2$	342.2	3.72	100
Nuarimol (±)		$C_{17}H_{12}ClFN_2O$	314.4	3.18	26

triflumizole, carbaryl, or *s*-triazine metabolites, which indicates a higher possibility of carry-over into beer than that for their parent compounds (*16*−*18*).

The malt must not be allowed to act as a transmitter of pesticides that represent a risk for the beer consumer. The health implications of pesticide residues are now well recognized, but their maximum levels in beer are not subject to legislation, at least in Spain, unlike trace element concentration and other parameters (*19*−*21*). With this aim, we have studied the fate of three fungicides commonly used on barley from malt to beer.

MATERIALS AND METHODS

Pesticides and Reagents. Pesticide standards with a purity >97% were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). **Table 1** shows the pesticides used and their main physicochemical characteristics (*22*). Stock standard solutions at 500 μg/mL were prepared by exact weighing and dissolving in 2,2,4-trimethylpentane/toluene (1:1, v/v) and finally stored in the dark at 4 °C. Working standard solutions were prepared freshly by dilution in the same solvent. Solvents for pesticide residue analysis (isooctane, toluene, *n*-hexane, dichloromethane, acetone, and ethyl acetate) were supplied by Scharlau Chemie S.A. (Barcelona, Spain). The Sep-Pak Vac 6 cm^3 (1 g) cartridges (Florisil) used for cleanup were supplied by Waters (Milford, MA). Helium and nitrogen (99.99% purity) were purchased from Abello Linde S.A. (Barcelona, Spain).

Raw Materials. Barley malt [moisture, 4.6%; pH, 6; hot water extract (fine), 80.7; soluble protein, 4.3%; and pesticide residues below detection limit] was obtained from the brewers Estrella de Levante Fábrica de Cerveza S.A. (Murcia, Spain), after the raw grains of two-row spring malting barley (*Hordeum distichum*) had been soaked, allowed to germinate (sprout), heated, and then dried (malting). The same supplier provided maize, rice, and hop pellets (Var. Nugget). Lager yeast, *Saccharomyces carlsbergensis* (Rh), was purchased from Versuchs- und Lehranstalt für Brauerei (Berlin, Germany). The water used in the process [EC, dS m^{-1} at 25 °C, 0.93; pH, 8.22; dissolved solids (mg/L), 771.2; alkalinity (mg/L), 184.3; trihalomethanes (μg/L), 45.2; pesticides below detection limit] was obtained from the municipal network. Routine analyses of the composition of the raw materials were carried out according to European Brewing Convention (EBC) methods (*23*) to assist quality control. The presence of trihalomethanes, originating from the chlorination of surface water, was verified by gas chromatography. GC-MS analysis ensures the absence of pesticides in the water, malt, adjuncts, and hops.

Brewing Process. The malt (200 g), once milled into fine grits to ensure good access of water to the grain particles in the subsequent phase of beer production, was spiked with the standard solution of acetone to get a spiking level of 2 mg/kg. Later, after evaporation of the spiking solvent (3 h), milled malt was thoroughly mixed with 3 volumes of water to yield mash and subjected to the mashing process, an extension of malting with α- and β-amylase enzymes. Boiled, gelatinized starch from milled maize (11 g) and that from rice (63 g) were added as adjuncts during mashing to achieve a higher content of fermentable sugars. At the end of the mashing process (**Figure 1**), 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 bitter tastes, were added and solubilized during wort boiling (90 min) to give beer its characteristic taste and aroma. In addition, wort boiling serves to denature enzymes and other proteins, to sterilize the wort, and to yield a darker liquid, which is an excellent medium for later fermentation. After boiling and clarification, the wort was quickly cooled in preparation for the addition of yeast and subsequent fermentation with brewer's yeast. In this process, yeast metabolizes sugars into energy, alcohol, carbon dioxide, secondary byproducts, and more yeast. For this purpose "lager yeasts" (bottom-fermenting yeast) were added to each fermentation vessel ($n = 3$) containing the oxygenated brewer wort, which was maintained at 15 ± 1 °C for 13 days. At the end of fermentation, the temperature was lowered and the beer matured during 1 week at ~2 °C. Finally, the beer was filtered through a porous plate funnel (10−16 μm) and bottled. **Figure 2** shows the procedure followed for standard beermaking.

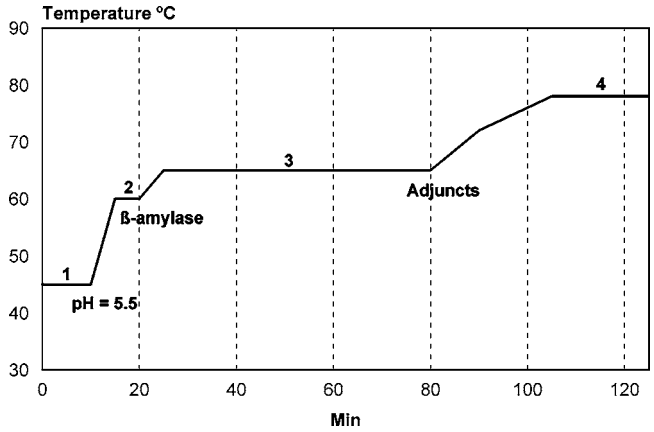


Figure 1. Scheme for the mashing phase: 1, mashing-in; 2, protein pause; 3, sugar pause; 4, mashing-off.

The sample weights (kg ± RSD, $n = 3$) obtained after the beermaking processes were as follows: mashing, 0.09 ± 3.33 for spent

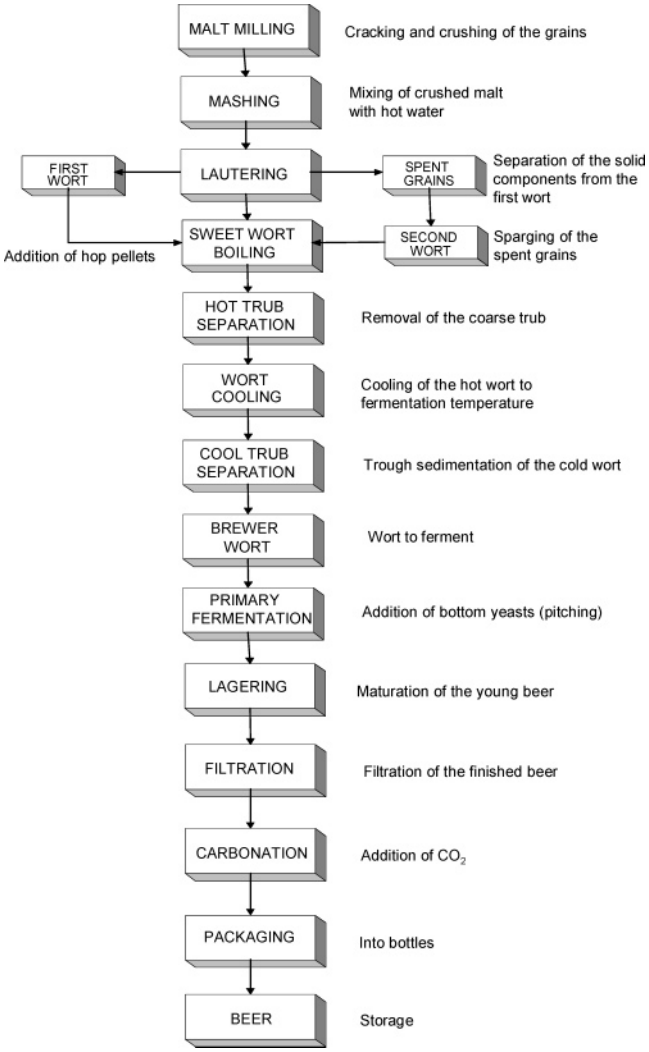


Figure 2. Scheme of the principal stages of the brewing process.

grain (as dry weight) and 1.09 ± 3.11 for sweet wort; and boiling, 1.05 ± 0.66 for brewer wort.

Pesticide Analysis. *Gas Chromatography−ECD System*. A Hewlett-Packard 5890 series II gas chromatograph equipped with an electron capture detector (ECD), a 7673 autosampler (Hewlett-Packard), and a split−splitless injector connected to an HP ChemStation (Hewlett-Packard) was used. The Equity-5 (30 m × 0.25 mm × 0.25 μm film thickness) fused silica capillary column contained 5% diphenyl, 95% dimethyl polysiloxane (Supelco, Bellefonte, PA). The operating temperatures were as follows: injection port, 250 °C; detector, 300 °C; column oven, initial 70 °C, hold for 1 min, programmed at 10 °C/min (from 70 to 160 °C), hold for 2 min; programmed at 5 °C/min (from 160 to 250 °C), hold for 0 min; programmed at 30 °C/min (from 250 to 280 °C); and hold for 4 min at 280 °C for a total run time of 35 min. The sample (2 μL) was injected in the splitless mode (0.75 min). Nitrogen at 1.2 and 30 mL/min was used as carrier and makeup gas, respectively. In those conditions, the compounds were identified by their retention times. Quantitation was achieved by linear regression against calibration standards (2−3000 ng/mL) and using the GC ChemStation software.

Gas Chromatography−MSD System. A Finnigan Trace Ultra GC (Thermo Electron Corp.) with a split−splitless injector and an AS2000 autosampler and connected by a transfer line to a Finnigan Polaris Q ion trap MS (Thermo Electron Corp.) was used. Updated Dell ChemStation application with Xcalibur software was used to control and automate many features of the GC-ITMS system. The Rtx-5MS (Restek) fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness) contained 5% diphenyl, 95% dimethyl polysiloxane as liquid phase. The injector and MS transfer line were operated at 230 and 275 °C, respectively. The IT operation conditions were as follows: acquisition mode sequential FS (50−400 *m*/*z*) and SIM mode (exact mass ± 0.4 amu); multiplier, 1788; ion source temperature, 230 °C; emission current, 250 μamp; AGC 50. The carrier gas was He at 1.2 mL min^{-1}. The sample (2 μL) was injected in the splitless mode (60 s), and the oven temperature was programmed as specified previously for GC-ECD system. Confirmation of the fungicides was based on the retention time of the target ion and on the three qualifier-to-target ion ratios. The qualifier-to-target ion percentage was then determined by dividing the abundance of the selected qualifier ion (Q) by the target ion (T) and multiplying by 100.

Table 2. Retention Times (R_t), Target and Qualifier Ions (T, Q_1, Q_2, and Q_3), and Percentage of Qualifier-to-Target Ratios (Q_1/T, Q_2/T, and Q_3/T) of the Fungicides

	R_t (min)		target and qualifier ions			
fungicide	GC-ECD	GC-MSD	T	Q_1 (Q_1/T %)	Q_2 (Q_2/T %)	Q_3 (Q_3/T %)
myclobutanil	25.06	24.76	179	150 (45.2)	82 (41.4)	181 (34.1)
propiconazole I	27.69	27.13	173	259 (97.1)	69 (65.6)	175 (65.0)
propiconazole I	27.91	27.32	173	259 (94.5)	69 (63.4)	175 (62.6)
nuarimol I	28.30	27.60	107	139 (76.1)	235 (72.3)	203 (64.2)
nuarimol II	28.47	27.79	107	139 (75.8)	235 (69.4)	203 (62.5)

Extraction Procedures. *Wort and Beer*. Wort or beer samples (20 mL) were placed in a 100 mL glass flask with hermetic closing, with 40 mL of *n*-hexane + dichloromethane (1+1 by volume) and 2 g of anhydrous NaCl. The flasks were introduced into an ultrasonic bath (Ultrasons 613, Selecta) with distilled water for 10 min, and the liquid was passed through phase separator paper (Albet 302, 15 cm ∅). Finally, the organic phase was concentrated by rotary vacuum evaporation (35 °C) to dryness and redissolved in 10 mL of iso-octane + toluene (1+1, v/v) and injected into the GC systems in the conditions specified previously.

Malt and Spent Grain. Samples (5 g) were homogenized at 8000 rpm for 5 min in a high-speed electric mixer (Polytron-Aggregate, Kinematica) with 10 mL of distilled water and 20 mL of *n*-hexane. After this time, 20 mL of dichloromethane was added, and the mixture was centrifuged for 10 min at 3000 rpm. The liquid phase was quantitatively transferred to a glass funnel provided with a phase separator paper. After filtration, the organic phase was concentrated through rotary vacuum evaporation to dryness and redissolved in 10 mL of *n*-hexane. In the cleanup step, the column was washed with *n*-hexane (10 mL), and the compounds were eluted from the cartridge (Florisil) with 10 mL of *n*-hexane + ethyl acetate (20+80, v/v). Finally, the eluates were evaporated and the residue was dissolved in isooctane + toluene, as in the case of wort and beer.

Recovery Assays. Untreated samples of malted barley, spent grain (dry weight), wort, and beer were spiked by adding 60−80 μL of acetone containing a known amount of each pesticide to 5 g of solid material or 20 mL of wort and beer. The solvent was evaporated at room temperature for 3 h, and the samples were then homogenized in an electric mixer or ultrasonic bath, depending on whether the sample was solid or liquid, for 5 min and subsequently stored at room temperature in the darkness for 20 min prior to extraction according to the above procedure. Samples were spiked at levels ranging from 10 to 2000 ng/g, and the recovery assays were replicated five times.

RESULTS AND DISCUSSION

Analytical Determination. In all cases, quantitation was achieved by GC-ECD due to its higher sensitivity. The temperature program elutes the fungicides from 25 to 30 min (225−245 °C, respectively). Fungicides were identified by their R_t values and their qualifier-to-target abundance ratios, as listed in **Table 2**. Isomers of propiconazole and nuarimol are not well distinguished by GC-MS. The criteria for their identification were determined on the basis of the retention times and the target-to-qualifier ion ratios using their respective *m*/*z*. Linear calibration curves were obtained for all of the pesticides in

different concentration ranges (2−3000 ng/mL). The correlation coefficients derived from the linear regressions were >0.999, with strong correlation between concentration and area for all of the studied compounds. The interday repeatability was calculated using the relative standard deviation (RSD) for 10 successive injections of a mixture of the fungicides and was in the range of 3.8−6.3%. Detection limits (LODs) for GC-ECD were calculated using a signal-to-noise ratio of 3 for all of the investigated compounds and were 2, 2.5, and 0.25 pg for myclobutanil, propiconazole, and nuarimol, respectively.

Fungicide recovery from spiked solid samples varied from 81.2 to 107.4% with RSD from 4.2 to 8.7% and a mean recovery in excess of 89% in all cases. In wort and beer, the recoveries from spiked samples ranged between of 92.3 and 103.2% with a RSD from 3.7 to 7.2%. The slopes of the calibration graphs with the standards directly prepared in 2,2,4-trimethylpentane/toluene (1:1, v/v) and the standard addition calibration graphs obtained from the different samples were similar, confirming the absence of any matrix effect.

The limit of quantitation (LOQ) was defined as the lowest fortification level attempted. For malt and spent grain, LOQs calculated according to the optimized extraction procedure and GC-ECD system were 2, 2.5, and 0.25 ng/g for myclobutanil, propiconazole, and nuarimol, respectively. In wort and beer, the values for the same compounds were 0.5, 0.62, and 0.06 ng/mL.

Therefore, the analytical methodology used, rapid and reliable, allows the correct determination of the studied compounds at levels well below the maximum residue limits (MRLs) established for barley by Spanish and European legislation (0.02, 0.05, and 0.20 mg/kg for myclobutanil, propiconazole, and nuarimol, respectively).

Removal and Transference of Residues. As a general rule, ∼200 g of grain is used to produce 1 L of wort at 12 °Brix, although this amount varies according to whether a higher or lower alcoholic content is desired. Any residues present in the grain, even if completely transferred to the beer, should, therefore, undergo dilution by a factor of 5. Taking into account the low solubility of most pesticide residues in water and their tendency to be easily adsorbed on the suspended matter, as in winemaking, the presence of residues in beer should be very low (*24*).

In our case, we took different samples (spent grain, sweet and brewer wort, and beer) from mashing to 4 months later to study the behavior of pesticide residues during the different elaboration steps. The residual concentrations found during this time are shown in **Table 3**. For propiconazole and nuarimol, the residual levels found after the mashing step (sweet wort) were below their respective MRLs established by Spanish legislation for barley (*25*, *26*), contrary to the case of myclobutanil, the residual value of which at this time was higher than its MRL (0.02 mg/kg). The residue levels found for this compound were ∼0.02 mg/kg after 83 days (during the storage period), indicating the great stability of the compound.

Bearing in mind the mean yields obtained after the beermaking procedures of mashing and boiling (brewing process), the total residual amount (micrograms) present in each of the brewing flasks was calculated, as shown in **Table 4**, with the aim of ascertaining any changes in fungicide residue levels during the process. The total amount present in the malt (100%) was considered to be the starting point to study the disappearance of the fungicides. As shown in **Table 4**, the amounts remaining in the matured and filtered beer do not exceed 3.5%, even in the most unfavorable case (nuarimol).

Table 3. Residual Levels Found in the Different Control Stages ($n = 3$)

	mean ± RSD (μg/kg)		
stage	myclobutanil	propiconazole	nuarimol
malt	2000 ± 20	1971 ± 10	1999 ± 8
spent grain (as dw)	1648 ± 14	1772 ± 11	1099 ± 3
sweet wort	33 ± 12	17 ± 12	24 ± 4
brewer wort	31 ± 9	16 ± 8	23 ± 6
wort, 3 days	30 ± 20	11 ± 13	23 ± 9
wort, 5 days	27 ± 17	11 ± 9	22 ± 13
wort, 7 days	25 ± 2	9 ± 8	20 ± 9
wort, 9 days	25 ± 13	8 ± 2	19 ± 11
young beer, 11 days	24 ± 8	8 ± 7	19 ± 10
finished beer, 23 days[a]	25 ± 9	8 ± 12	17 ± 19
beer, 53 days	23 ± 6	8 ± 2	16 ± 14
beer, 83 days	19 ± 20	6 ± 12	15 ± 8
beer, 113 days	13 ± 7	6 ± 23	14 ± 10

[a] Matured and filtered beer.

Table 4. Mean Amount of Pesticide in the Whole Weight or Volume of Sample for Each Control Stage and Percentage Remaining

	myclobutanil		propiconazole		nuarimol	
stage	μg	%	μg	%	μg	%
malt	400.32	100.00	394.24	100.00	398.00	100.00
spent grain	153.83	38.43	165.36	41.95	102.60	25.78
sweet wort	35.86	8.95	15.90	4.03	23.80	5.97
brewer wort	33.00	8.25	15.00	3.80	23.00	5.70
young beer	26.00	6.50	8.00	2.03	19.00	4.77
finished beer	26.00	6.50	8.00	2.03	17.00	4.30
stored beer	13.00	3.25	6.00	1.52	14.00	3.52

The residual amount of myclobutanil in sweet wort (after mashing) was close to 9%, whereas the amount present in spent grain was 38%, which indicates that during maceration, 53% of the compound was degraded. There is limited published information available on the metabolism of myclobutanil. The compound is stable in the face of hydrolysis, but decomposes in aqueous solutions after exposure to light. Its DT_{50} is 222 days in sterile water and 15−22 days in pond water (*22*, *27*). Minimal degradation during boiling was observed (8%). The same behavior was observed during primary fermentation (11 days) when 8% of the product was degraded. Finally, the filtration and maturation processes lessen the content of the compound by 50%, probably by surface adsorption.

For propiconazole, the residual level in sweet wort, once separated from the spent grain, was ∼16% of the parent concentration in malt, whereas 42% was retained in the spent grain. Therefore, 42% of the fungicide residue was eliminated during the process. According to the bibliographical data, the main degradation pathways for this compound involve hydroxylation of the propyl side chain and the dioxolane ring and finally the formation of 1,2,4-triazole (*27*). Nonsignificant losses (6%) were observed for this compound during boiling because it is stable up to 320 °C, whereas its concentration fell by 47% with respect to the level measured in the brewer wort during fermentation. Similar findings were found by Hengel and Shibamoto (*38*) working with tebuconazole ($K_{OW} = 3.70$), another triazole fungicide. Finally, after maturation and filtration, 25% of the amount was eliminated in the young beer.

Regarding nuarimol, 68% of the initial amount was eliminated during the mashing phase, because 26 and 6% was retained in spent grain and sweet wort, respectively. The major degradation reactions observed in plant/soil surfaces and water include hydroxylation of the phenyl groups, oxidation of the carbinol

carbon atom, dehalogenation, and carbinol dehydroxylation (*27*). During boiling, only 5% of the amount present in the sweet wort was removed, whereas 39% of nuarimol residues were eliminated between brewer wort and the finished beer. Bibliographical data indicate that the elimination of another pyrimidinil carbinol fungicide (fenarimol), analogous to nuarimol, varies from 40 to 52% in red wines from the pressed must until the finished wine, similar to those values obtained by us (*28*). Finally, loss during storage (3 months) was <20%.

The colloidal stability of the beer during maturation is greatly improved due to the precipitation of protein−tannin complexes, which are only sparingly soluble at low temperature. The development of such a haze is normally avoided by using different stabilizing methods in the finished beer, which removes further amounts of proteinaceous matter and/or tannins, and subsequent filtration to ensure a brilliant look and long shelf life of the product (*12*). Similar or better haze stability can be obtained by the use of proanthocyanidin-free malt (*29*). The interaction between tannins and hordein, a starchy matter contained in barley, is based on the formation of hydrogen bonds, which form more rapidly at lower temperatures (*12*). Stabilization involves removing the tannins and/or the hordein from the beer. A similar mechanism could be proposed for the removal of pesticide residues during brewing. In this respect, different stabilizers or fining agents, such as the synthetic insoluble polymer called polyvinylpolypyrrolidone (PVPP), which closely resembles polyproline, can be used to reduce the content of residues in the finished product. PVPP possesses a carbonyl group neighboring a proton-free nitrogen atom, which may be considered to be a very potent site for the formation of hydrogen bonds (*30*). Alternatively, hordein and pesticide residues may be removed by treatment with specific bentonite, activated carbon, silica gels, which bind haze-forming polypeptides but have little effect on foam-promoting substances, hydrogels with a moisture content of >50%, or xerogels (dry gels) with a 5% water content (*12*). Fining agents are commonly used throughout the winemaking process to clarify and stabilize must and wine physically and chemically and to remove various compounds such as pesticide residues that can affect the organoleptic and health-associated properties of the wines (*31*).

The bibliographical information available concerning changes in pesticide residues during brewing mainly refers to hops. Only a few papers have studied the fate and removal of pesticides from barley to beer, unlike in the case of wine (*32*−*35*). One of the critical points for quality assurance in beermaking is to check residues of pesticides with K_{OW} values ranging from 2 to 4. Some findings indicate that pesticide residues on barley having $K_{OW} > 2$ would remain on malt (*14*). It has been found that the carry-over of pesticide residues into wort and beer depends on their K_{OW} values. Thus, the percentage remaining of glyphosate ($K_{OW} < -3.2$) in wort and beer is >90% (*15*). Carbaryl undergoes a reduction of ∼90% during the brewing process through its conversion into 1-naphthol (*16*). On the other hand, most of the pesticides having a high K_{OW} such as pyrethroid or organochlorine pesticides undergo a drastic reduction during brewing, so that they are absent in the finished beer or detectable in only negligible concentrations (*15*, *24*, *36*).

Influence of Beermaking Procedures. The percentages of fungicide residues remaining after the beermaking phases are shown in **Figures 3** and **4**. During mashing, the soluble substances (sugars, amino acids, and peptides) produced in malting and mashing are extracted into the liquid fraction (sweet wort), which is then separated from the residual solid particles (spent grains). Although different separation methods exist, the most common is lautering, in which grain particles form a filter medium through which wort is run off, carefully applying raking and pressure differentials to optimize flow. Additional water (sparge) is used to ensure the efficient leaching of wort from solid particles (*37*). At the end of the mashing phase, the remaining percentages were below 10% of the amount recorded in malt, propiconazole showing the greatest decrease (to 4%). On the contrary, the retained amounts on spent grain were relatively high (38, 42, and 26% for myclobutanil, propiconazole, and nuarimol, respectively, all of the compounds having $K_{OW} > 2$). Similar behavior was observed for atrazine and terbuthylazine during mashing, when 70 and 90%, respectively, were adsorbed on spent grain (*17*). In a general way, adsorption affinity depends on the polarity of the compounds: the more polar the pesticide, the lower the adsorbed amount (*38*). It is necessary to bear in mind that maceration of the malt and adjuncts produces a great quantity of suspended matter, which could adsorb the pesticide residues, and, if the recorded levels allow it, the spent grains could be used as animal feeds, implying a commercial use for this byproduct. Supposing that the percentages retained on the mash filter grains are similar for a certain range of concentrations (≤2 mg/kg), the maximum concentrations on malted barley would be 0.02, 0.06, and 0.36 mg/kg for myclobutanil, propiconazole, and nuarimol, respec-

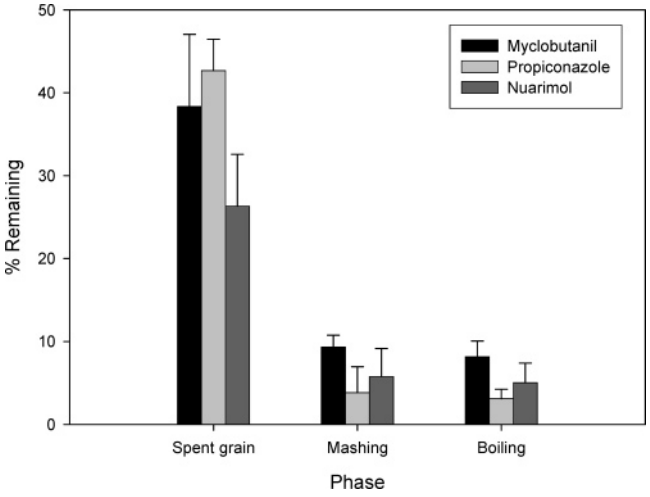


Figure 3. Percentage of fungicide residues remaining after mashing and boiling of the wort; error bars are 95% confidence intervals.

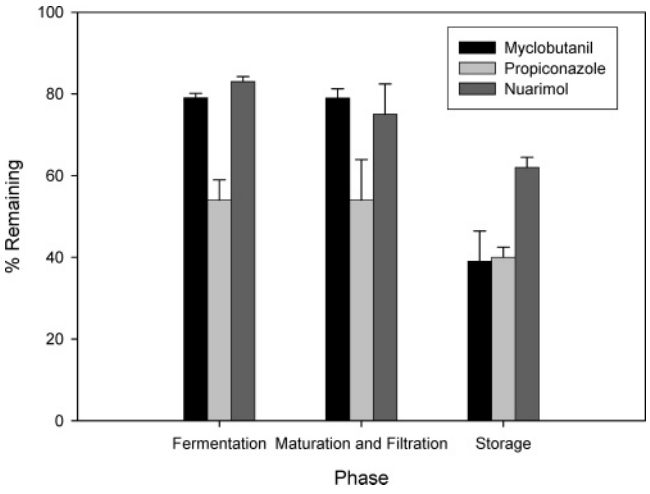


Figure 4. Percentage of fungicide residues remaining after fermentation of the wort and maturation and storage of the beer; error bars are 95% confidence intervals.

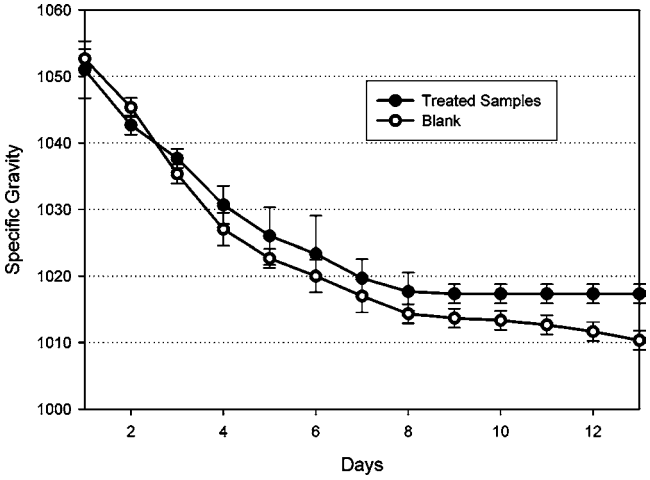


Figure 5. Evolution of specific gravity during fermentation ($n = 3$); error bars are 95% confidence intervals.

tively, to remain below the respective MRLs, taking into account the percentage in the spent grains once separated from the sweet wort. On the other hand, as we can see from **Figure 3**, a very small decrease was observed in the residual content after the wort had been boiled, which points to the stability of the three compounds at temperatures >100 °C.

With regard to the influence of the fermentative process on the elimination of the studied fungicides (**Figure 4**), there was a significant reduction in the case of propiconazole (47% of the content recorded in brewer wort) but much less for the other two compounds (~20%). On the other hand, a marked influence in the fermentation rate was observed (see **Figure 5**, where the evolution of specific gravity with time is shown for both blank and treated samples). As can be seen, from the third day onward, there is a significant decrease in the fermentation rate in the samples with fungicide residues compared with the blank (sluggish fermentation), and after 8 days, the fermentation prematurely ceases (stuck fermentation; i.e., the premature termination of fermentation before all fermentable sugars have been metabolized). The three compounds are systemic fungicides, and they interfere with ergosterol biosynthesis by inhibiting the demethylation of steroids. Ergosterol is a crystalline sterol, $C_{28}H_{43}OH$, synthesized by yeast from sugars or derived from ergot and converted into vitamin D_2 when exposed to ultraviolet radiation. Azoles target the ergosterol biosynthetic enzyme, lanosterol 14α-demethylase, and are a widely applied class of antifungal agents because of their broad therapeutic window, wide spectrum of activity, and low toxicity (*39*). The results obtained in our laboratory (unpublished data) have demonstrated that some triazole fungicides, such as propiconazole, strongly affect the growth and fermentability of brewer's yeast, influencing the fermentative kinetic and, depending on the dose, causing stuck fermentation.

During the maturation phase (lagering), the temperature was lowered and the beer was maintained at ~2 °C for 1 week, which favors assimilation of the off-flavor component, diacetyl, by yeast cells. Finally, the beer was filtered and bottled. No significant decreases in the residual levels were observed in any case during this process, as is shown in **Figure 4**. Only nuarimol decreased its concentration (by 10%) with regard to the young beer. Finally, after the storage period (3 months), the concentration of myclobutanil fell sharply (50%), whereas the decreases observed in the other compounds were less pronounced, being <25% of the amount in the finished beer.

Table 5. Residual Levels of the Fungicide Residues during Storage of the Spent Grains ($n = 3$)

	mean ± RSD (mg/kg)		
days	myclobutanil	propiconazole	nuarimol
0	1.65 ± 6.27	1.77 ± 2.57	1.10 ± 4.78
15	1.49 ± 5.56	1.65 ± 9.59	1.03 ± 3.54
30	1.14 ± 8.46	1.49 ± 3.32	0.91 ± 7.98
60	0.86 ± 3.67	1.38 ± 6.98	0.80 ± 6.41
90	0.77 ± 6.90	1.23 ± 2.65	0.76 ± 5.55

Table 6. Dissipation Parameters for the Fungicide Residues during Storage of the Spent Grains

parameter	myclobutanil	propiconazole	nuarimol
K (days^{-1})	0.0085	0.0037	0.0041
r	0.966	0.987	0.963
R_0	1.58	1.73	1.07
$t_{1/2}$ (days)	82	187	169
t_{MRL}[a] (days)	515	958	408

[a] Necessary time to reach the MRL.

In conclusion, we may affirm that the decrease of pesticide residues, and even their removal, will depend to a great extent on the initial concentrations in the malted barley, on the physical−chemical characteristics of each product, and on the beermaking procedure. Therefore, it is very unlikely that after an appropriate elaboration process, the remaining levels of pesticides will be harmful to health.

Dissipation of Fungicide Residues from Spent Grain. The mash filter grains are nutritionally similar to brewer's grains; however, they differ substantially in appearance because the malted barley used is ground rather than left whole prior to the brewing process. This grinding helps extraction of more liquor (wort), and the resultant grains are therefore higher in dry matter. A moist byproduct from the brewing industry, made up of spent grains, is widely fed to ruminant animals, used as a buffer or as a forage or concentrate replacer. High in digestible fiber and good-quality protein but low in starch, the spent grain is quite undegradeable due to the heat process during manufacture. It is ideal for mixing with other forage rations to simulate dry matter and an excellent feed for cattle and sheep. However, mash filter grains possess several limiting factors because they have a higher dry matter content than traditional brewer's grains, and feed rates should be slightly lower to provide the same level of nutrients. Other uses, including as human food or for producing thermic energy, have also been suggested (*40*).

Therefore, although the nutritional potential of the spent grains for animals has been demonstrated, it is important to ascertain the pollution load of the same and how any fungicide residues evolve during storage. **Table 5** shows the fungicide residue values obtained during storage of the mash filter grains at ambient temperature.

Several models (*41*, *42*) are used to describe the decay of pesticides in different matrices. Probably the most commonly used model is the following equation, where R_t is the residue at time t, R_0 is the residue at time zero, and K is the rate constant (*43*, *44*).

$$\ln R_t = \ln R_0 - Kt \quad (1)$$

To know the dissipation rate of residues in the spent grains, the experimental data were fitted to the above model. The study began when the mash filter grains were dried (1 day after the mash was filtered) and concluded after the storage time. Five

sampling points (0, 15, 30, 60, and 95 days) were used to calculate the statistical parameters according to the usual first-order kinetics equation (eq 1). **Table 6** shows the values derived from the fit. In all cases, there was a good linear correlation between ln R_t and time ($r \geq 0.96$). Additionally, the differences between the analytical and theoretical concentration calculated (R_0) at 0 days are quite small (0.07, 0.04, and 0.03 for myclobutanil, propiconazole, and nuarimol, respectively), which indicates that the model is valid.

According to the calculated values for the constant rate (K) and half-life time, the following dissipation rate was observed: myclobutanil > nuarimol > propiconazole. The necessary times to reach their respective MRLs were 408, 515, and 958 days for nuarimol, myclobutanil, and propiconazole, respectively, which indicates a high persistence level and minimum degradation for the three compounds, especially in the case of propiconazole.

ACKNOWLEDGMENT

We thank the brewers Estrella de Levante and Fábrica de Cerveza for technical help with this research.

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Received for review March 15, 2005. Revised manuscript received September 8, 2005. Accepted September 9, 2005. We thank the Ministerio de Educación y Ciencia of Spain (Project AGL2002-03560) for financial assistance.

JF0505832