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# Variability in the fermentation rate and colour of young lager beer as influenced by insecticide and herbicide residues

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**Abstract**

The influence of five pesticides, pendimethalin, trifluralin (dinitroaniline herbicides) fenitrothion, malathion, and methidathion (organophosphorus insecticides), on the fermentation of young lager beer was assessed. For this purpose, brewer wort was spiked with the pesticides to obtain a concentration of 1 μg/ml before the pitching with lager yeast (*Saccharomyces carlsbergensis*). The fermentation kinetic was sluggish for the samples treated with fenitrothion, malathion, and trifluralin but increased from the second to the sixth day in the methidathion and pendimethalin treatments in comparison with the blank sample. At the end of fermentation (12 days) statistically significant differences ($p < 0.05$) were found for the extract and attenuation values for the samples treated with fenitrothion and trifluralin. In these cases too, a higher amount of residual sugars (glucose, fructose, maltose and maltotriose) was found in the beer. Significant differences ($p < 0.05$) were also observed for pH and colour of the beer after fermentation among all treated samples. A good quadratic correlation ($R > 0.94$) was found for these parameters in all cases. The total polyphenol content was significantly lower in the fenitrothion and trifluralin treatments.


*Keywords:* Beer colour; Fermentable carbohydrates: fenitrothion, malathion, methidathion, pendimethalin, and trifluralin residues; Lager beer; Primary fermentation

## 1. Introduction

Weeds are responsible for serious loss of yield in barley and their control will usually result in a substantial increase in net income. Weeds compete with small grains for nutrients, water and light, reducing crop yields and grain quality, and yield reduction is directly proportional to the weed population. An integrated weed management programme in small grains will combine cultural, mechanical and chemical weed control practices. On the other hand, damage to barley from insect pests can usually be minimized by cultural practices and the proper use of insecticides. Cultural control should be used wherever possible to reduce the need for insecticides, which, when absolutely necessary, should be applied with caution and only as recommended (Domínguez, 2004; Fincher & Stone, 1993).

In some cases, however, herbicides and insecticides are used in different combinations at many stages of cultivation. Insecticides are also used during postharvest storage. Since the quality of the raw materials used (barley, hops, water and yeasts) has a decisive influence on the quality of a beer, it is important to assess the pollution load of barley and the way in which pesticide residues evolve during malting and brewing. The ingredients used for beer-making must not be allowed to act as a transmitter of unacceptable pollutants that represent a risk for the beer consumer and animals fed by-products (Kunze, 2004).

The problem is that pesticide residues may be transferred from barley to beer, although the residues may also come from the soil itself and the water used, a problem which affects the brewing industry in several countries.

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During the first step (malting), some pesticide residues of $\log K_{ow} > 2$ will remain on the malt (Miyake, Hashimoto, Matsuki, Ono, & Tajima, 2002; Navarro, Pérez, Navarro, & Vela, 2007; Uygun, Özkara, Özbey, & Koksel, 2007). After mashing and boiling, the pesticides in the malt and hops may pass into the wort in different proportions, depending on the process used (Hack, Nitz, & Parlar, 1997; Hengel & Shibamoto, 2002; Miyake et al., 1999; Navarro, Pérez, Navarro, Mena, & Vela, 2006; Navarro, Pérez, Vela, Mena, & Navarro, 2005). Finally, if pesticide residues, especially some fungicides, are present in the brewer wort, they may cause the fermentation to stick, provoking organoleptic alterations in the finished beer and possible toxic effects for the consumer (Jones, Kavanagh, & Clarke, 1988; Navarro, Pérez, Navarro, Mena, & Vela, 2007).

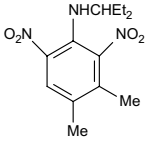
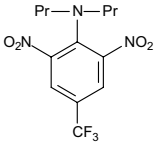
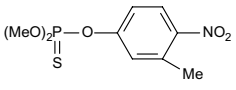
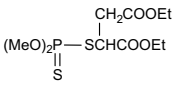
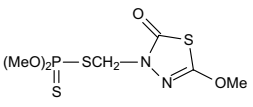
The aim of this study was to assess the influence of two dinitroaniline herbicides (trifluralin and pendimethalin), and three organophosphorus insecticides (malathion, fenitrothion and methidathion), all commonly used in barley cultivation, during the primary fermentation of a young lager beer.

## 2. Materials and methods

### 2.1. 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 physico-chemical characteristics (Tomlin, 2003). Stock standard solutions of 100 μg/ml were prepared by exact weighing and dissolving in acetone, before storing in the dark at 4 °C. Acetone was supplied by Scharlau Chemie S.A. (Barcelona, Spain). Carboxymethylcellulose/ethylenediamine tetraacetic acid (CMC/EDTA), and green ammonium iron citrate were purchased from Sigma–

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) |
|---|---|---|---|---|---|
| *Pendimethalin*<br>*N*-(1-Ethylpropyl)-2,6-dinitro-3,4-dimethylaniline |  | $C_{13}H_{19}N_3O_4$ | 281 | 5.2 | 0.3 |
| *Trifluralin*<br>2,6-Dinitro-*N*,*N*-dipropyl-4-trifluoromethylaniline |  | $C_{13}H_{16}F_3N_3O_4$ | 335 | 5.1 | 0.2 |
| *Fenitrothion*<br>*O*,*O*-Dimethyl-*O*-(3-methyl-4-nitrophenyl)phosphorothioate |  | $C_9H_{12}NO_5PS$ | 277 | 3.5 | 21 |
| *Malathion*<br>Diethyl-(dimethoxy thioxophosphoryl-thio) succinate |  | $C_{10}H_{19}O_6PS_2$ | 330 | 2.7 | 145 |
| *Methidathion*<br>*S*-(2,3-Dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl)*O*,*O*-dimethylphosphorodithioate |  | $C_6H_{11}N_2O_4PS_3$ | 302 | 2.2 | 200 |

Aldrich Química S.A. (Madrid, Spain). Concentrated ammonia was supplied by Panreac Química S.A. (Barcelona, Spain).

### 2.2. Raw materials

Barley malt (moisture 4.6%, pH 6.0 and pesticides below their 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), heating and then drying (malting). The same supplier provided maize, rice and hop pellets (var Nugget). Lager yeast, *Saccharomyces uvarum (carlsbergensis)* 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, trihalomethanes 45.2 μg/l, and pesticides below detection limit) was obtained from the municipal network. Pesticide residues were analyzed according to the method proposed by Vela, Pérez, Navarro, and Navarro, 2007.

### 2.3. Brewing process

Briefly, the malt (1 kg), once milled into fine grits, was thoroughly mixed with approximately 5 volumes of water to yield a mash, and subjected to mashing as previously published (Navarro et al., 2006). Boiled, gelatinized starch from milled maize (50 g) and rice (300 g) were added as adjuncts during mashing to achieve a higher content of fermentable sugars. At the end of the mashing process, sweet wort and spent grains were separated by filtration. In the next step (wort boiling, 90 min), hop pellets the source of bitter tastes, were added twice (0.5 g, 30 and 80 min).

After wort boiling and cooling, 4.75 l of brewer wort (pH 5.7) were obtained. Before pitching, the pH was adjusted to 5.3 using phosphoric acid because many important brewing processes proceed better or more quickly at a lower pH. The brewhouse yield obtained (amount of extract produced related to the amount of grist used) after wort boiling was 62.7%. On pitching, the hydrometer measured 13.2% (g extract per 100 ml at 20 °C). At this time, wort samples (250 ml) were individually spiked ($n = 3$) with 250 μg of each of the pesticides, except pendimethalin and trifluralin, in which case 50 μg were added because of their low solubility in water. After evaporating of the spiking solvent (3 h), lager yeasts (bottom-fermenting yeast) were added ($10–15 \times 10^6$ cells/ml) to each fermentation vessel ($n = 3$) containing the oxygenated pitching wort at $10 \pm 1$ °C. All the studied compounds can be transferred from malt to wort, although in different proportions, as we have previously demonstrated (Navarro et al., 2006).

Following Kunze, 2004, five control points were differentiated in the fermentation management: (1) when the process begins (*initial*), (2) the fine bubble foam becomes deeper (*low kräusen*), (3) fermentation enters its most intensive stage (*high kräusen*), (4) fermentation becomes less vigorous and the foam looks browner (*kräusen collapsing*), and (5) the rate of fermentation continues to decrease and finally forms a dirty brown layer (*collapsed foam*).

### 2.4. Temperature and pH measurements

A digital Testo 110 termometer with ±0.1 °C resolution (Testo GmbH, Lenzkirch, Germany) and GLP 22 pH-meter, 0.001 pH and 0.1 mV (Crison Instruments S.A., Barcelona, Spain) were used for temperature and pH measurements, respectively.

### 2.5. Analysis of colour and total polyphenols

The colour and total polyphenol content of beer were analyzed according to the European Brewery Convention Analysis Committee (EBC, 1998). The colour (EBC units) of the wort and beer was measured at a wavelength of exactly 430 nm in a 10 mm cell (EBC methods 8.5 and 9.6), while for total polyphenols the sample was treated with a solution of CMC/EDTA and then with ferric ions in alkaline solution. The absorbance of the red-coloured solution was measured at 600 nm (EBC method 9.11). Measurements were carried out with a Helios Gamma UV–Vis spectrophotometer (ThermoScientific, Inc., Waltham, MA).

### 2.6. Analysis of fermentable carbohydrates

After centrifugation of the wort samples at 15,000*g* for 5 min to remove particles, 2 ml of supernatant were filtered through a syringe filter (nylon, 0.45 μm pore size). After filtering, 20 μl were injected into the HPLC system, which 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, MA). Data were collected and integrated by Empower Software. The operating conditions were as follows: a 25 cm × 4.6 mm ID Supelcosil™ LC-$NH_2$, 5 μm particles column (Supelco, Bellefonte, PA), acetonitrile/water (75:25, v/v) as 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 the carbohydrates was based on calibration curves obtained after addition of known amounts of carbohydrates (0.1–50 g/l).

### 2.7. Statistical analysis

SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) was used for the one way analysis of variance (ANOVA) procedure. When significant differences were found, the least significant difference (LSD) test was used to determine the differences between treatments. A value of $p < 0.05$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Progress of temperature and specific gravity during fermentation

Primary fermentation was started by pitching the wort (i.e., the adding yeast to the wort). Immediately before this addition of yeast the wort is known as pitching wort and after the yeast has been added the mixture is already referred to as young beer. A very important factor in fermentation is the control of temperature and fermentation time. The evolution of temperature during the different phases of primary fermentation is shown in Fig. 1, while fermentation time was 10–12 days in all cases. Traditional lager fermentations are conducted at temperatures ranging from about 7 to 14 °C. The fermentation temperature in a fermentor is usually allowed to rise spontaneously to a few degrees above the pitching temperature. In our case, the pitching temperature was raised (10 °C) to start the fermentation more quickly. As a consequence of the heat released during fermentation, the temperature of the young beer increased to reach a maximum of 14–16 °C in the *high kräusen* stage with the exception of the sample spiked with fenitrothion, when the maximum temperature reached was 13 °C. Some brewers prefer cold fermented beer with a maximum temperature of 10 °C because fewer by-products are produced, especially higher alcohols and esters.

As can be seen in Fig. 2, where the evolution of specific gravity is shown for blank and treated samples, a noticeable influence of the treatments in the fermentation rate was observed from the second day onwards. Indeed, two different behaviours can be observed in comparison with the blank sample. For samples treated with methidathion and pendimethalin, the fermentative kinetic was quicker from days 2 to 6, probably due to the rapid assimilation of nitrogen present in these compounds by the yeasts. On

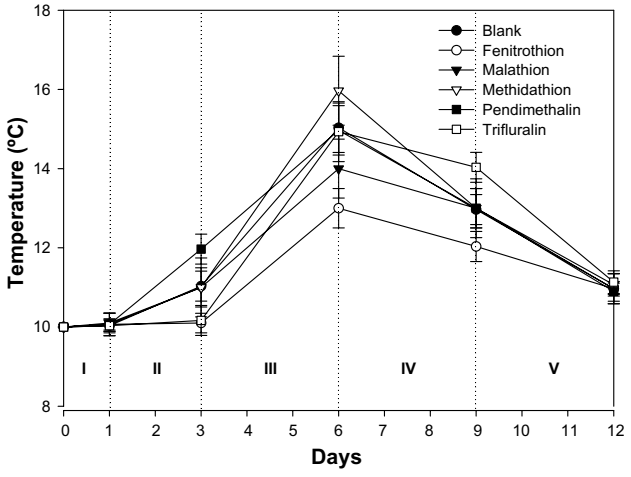



Fig. 1. Evolution of temperature ($n = 3$) during primary fermentation (I: initial, II: low kräusen, III: high kräusen, IV: kräusen collapsing, and V: collapsed foam) for blank and treated samples (error bars are 95% confidence intervals).

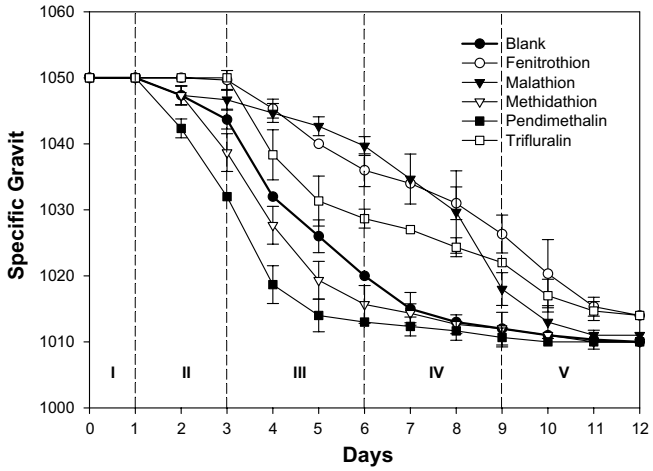



Fig. 2. Progress of specific gravity ($n = 3$) *vs* time during fermentation phases (I: initial, II: low kräusen, III: high kräusen, IV: kräusen collapsing, and V: collapsed foam) for blank and treated samples (error bars are 95% confidence intervals).

the other hand, the fermentation rate of the samples treated with fenitrothion, malathion and trifluralin was slower (sluggish fermentation) than in the blank sample. The main difference between both dinitroaniline herbicides is the presence of the trifluoromethyl moiety in the case of trifluralin, this functional group being able to act on the growth and availability of the yeasts. Trifluralin is stable to hydrolysis within pH range 3–9 but its degradation under anaerobic conditions is faster than under aerobic conditions. It is likely that the increased rate of nitroreduction is important in this respect (Roberts, 1998). As regards the organophosphorus insecticides, fenitrothion is a phosphorothioate compound, and is more toxic than malathion and methidathion (phosphorodithioate compounds). Besides, the major biotransformation pathway of fenitrothion involves oxidative desulfuration to the oxon analogue (fenitrooxon), which is more toxic that the parent compound (Gallo & Lawryk, 1991; Roberts & Hutson, 1999). At the end of fermentation (12 days) the mean values of specific gravity for blank sample and those treated with malathion, methidathion and pendimethalin were significantly different ($p < 0.05$) from those determined in the samples with residues of fenitrothion, and trifluralin.

Slow (sluggish) or stuck (incomplete, arrested) fermentation is one of the main problems that can arise during alcoholic fermentation. By definition, a stuck fermentation is a fermentation that has stopped before all the available sugar in the must has been converted to alcohol and $CO_2$. The serious dangers arising from the premature arrest of alcoholic fermentation are well known. Generally, residual sugar in beer and wine is a dangerous and undesirable condition. If sugar is still present, bacteria may multiply and increase volatile acidity. Sometimes, fermentation resumes after the fermented beverage is bottled, and the yeast produces unsightly sediment in the bottle. The main causes of stuck and sluggish fermentation are a restriction of nutri-

ents, a decrease in oxygen availability, exposure to high temperatures, low pH values, accumulation of ethanol during the must fermentation by yeasts, the brewing practices followed, and the presence of toxic substances like pesticide residues (Bisson, 1999).

### 3.2. Evolution of extract, attenuation and alcohol content

In the technical literature, the sugar content of a wort/beer is typically expressed as extract in degrees Plato (°*P*, specific gravity as the weight of extract in a 100 g solution at 17.5 °C). One degree Plato corresponds to a 1% by weight sugar solution of sucrose. For other sugars, the actual percent weight will be slightly higher. Sucrose produces the heaviest solution from a given mass of sugar. The Plato tables express conversion from specific gravity (SG) to degrees Plato ($°P = \mathrm{SG} - 1/0.004$).

On the other hand, during fermentation the extract is continuously being fermented. Attenuation is defined as the percentage of sugar converted to alcohol and $CO_2$ by the yeast. Apparent attenuation is computed from the measured specific gravity (converted to extract) of the beer, as follows:

$\mathrm{AA} = 1 - \mathrm{AE}/\mathrm{OE}$,
AE = apparent extract of beer, as measured by a hydrometer,
OE = original extract of the wort.

However, the specific gravity of the beer is depressed by the lower specific gravity of alcohol (0.8, approximately), so the measured apparent extract is smaller than the real extract (sugars remaining in the finished beer). Real extract, RE, can be measured by dealcoholizing the beer (typically by boiling gently), adding distilled water back to the original volume, and then taking the specific gravity. Or, we can use Balling's approximation (Thomas, 2007):

$$\mathrm{RE} = 0.18 * \mathrm{OE} + 0.82 * \mathrm{AE},$$

with this approximation, we can compute an estimation of the real attenuation as

$$\mathrm{RA} = 1 - \mathrm{RE}/\mathrm{OE},$$
$$\mathrm{RA} = 1 - (0.18 * \mathrm{OE} + 0.82 * \mathrm{AE})/\mathrm{OE}.$$

The evolution of extract and degree of attenuation during the different phases of primary fermentation are shown in Tables 2 and 3, respectively.

After fermentation, no significant differences ($p < 0.05$) were found in the extract and attenuation values between the blank sample and those fermented in the presence of malathion, methidathion and pendimethalin but were found for the samples treated with fenitrothion and trifluralin. 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 (% by volume) at the end of fermentation was substantially lower in the sample containing fenitrothion (4.5%) and trifluralin (4.4%) residues than in those spiked with malathion (5.2%), methidathion (5.4%) and pendimethalin (5.4%) while in the blank sample the alcohol content was 5.5%. These results show that fenitrothion and trifluralin residues affect the growth and fermentability of brewer's yeast influencing the fermentative kinetic and, causing sluggish fermentation from the *high kräusen* phase onwards.

Table 2
Evolution of extract (°*P*) during the primary fermentation

| Days | °*P* | | | | | |
|---|---|---|---|---|---|---|
| | Blank | Fenitrothion | Malathion | Methidathion | Pendimethalin | Trifluralin |
| 0 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| 1 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 |
| 3 | 10.7 | 12.5* | 11.9* | 10.0* | 8.8* | 12.5* |
| 6 | 6.4 | 9.6* | 10.4* | 5.3* | 4.9* | 8.2* |
| 9 | 4.7 | 7.8* | 5.9* | 4.7 | 4.5 | 6.8* |
| 12 | 4.3 | 5.1 | 4.5 | 4.3 | 4.3 | 5.1* |

* Significant at $p < 0.05$ level.

Table 3
Evolution of real attenuation (%) during the primary fermentation

| Days | % | | | | | |
|---|---|---|---|---|---|---|
| | Blank | Fenitrothion | Malathion | Methidathion | Pendimethalin | Trifluralin |
| 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | 14.6 | 0.0* | 4.9* | 19.4* | 29.2* | 0.0* |
| 6 | 48.6 | 22.7* | 16.2* | 56.7* | 59.9* | 34.0* |
| 9 | 61.6 | 37.3* | 51.8* | 61.6 | 63.2 | 45.4* |
| 12 | 64.8 | 58.3* | 63.2 | 64.8 | 64.8 | 58.3* |

* Significant at $p < 0.05$ level.

### 3.3. Changes in the content of fermentable carbohydrates during fermentation

The main biochemical route for a yeast cell to produce energy from sugars in the absence of oxygen is called alcoholic fermentation. The sugars in wort are not all fermented equally well. Since yeast has to hydrolyze sugar polymers before it can use them, it always attacks hexoses first. In a normal process, carbohydrates are assimilated by brewer's yeast in the following order: fructose, glucose, maltose, and maltotriose. Sucrose is split into fructose and glucose by the yeast-produce enzyme, invertase, and is not assimilated by yeast as sucrose (Munroe, 2006). Therefore, this sugar is treated at the beginning of fermentation by the yeast.

Fig. 3 shows the evolution of fermentable carbohydrates during fermentation for both blank and treated samples.

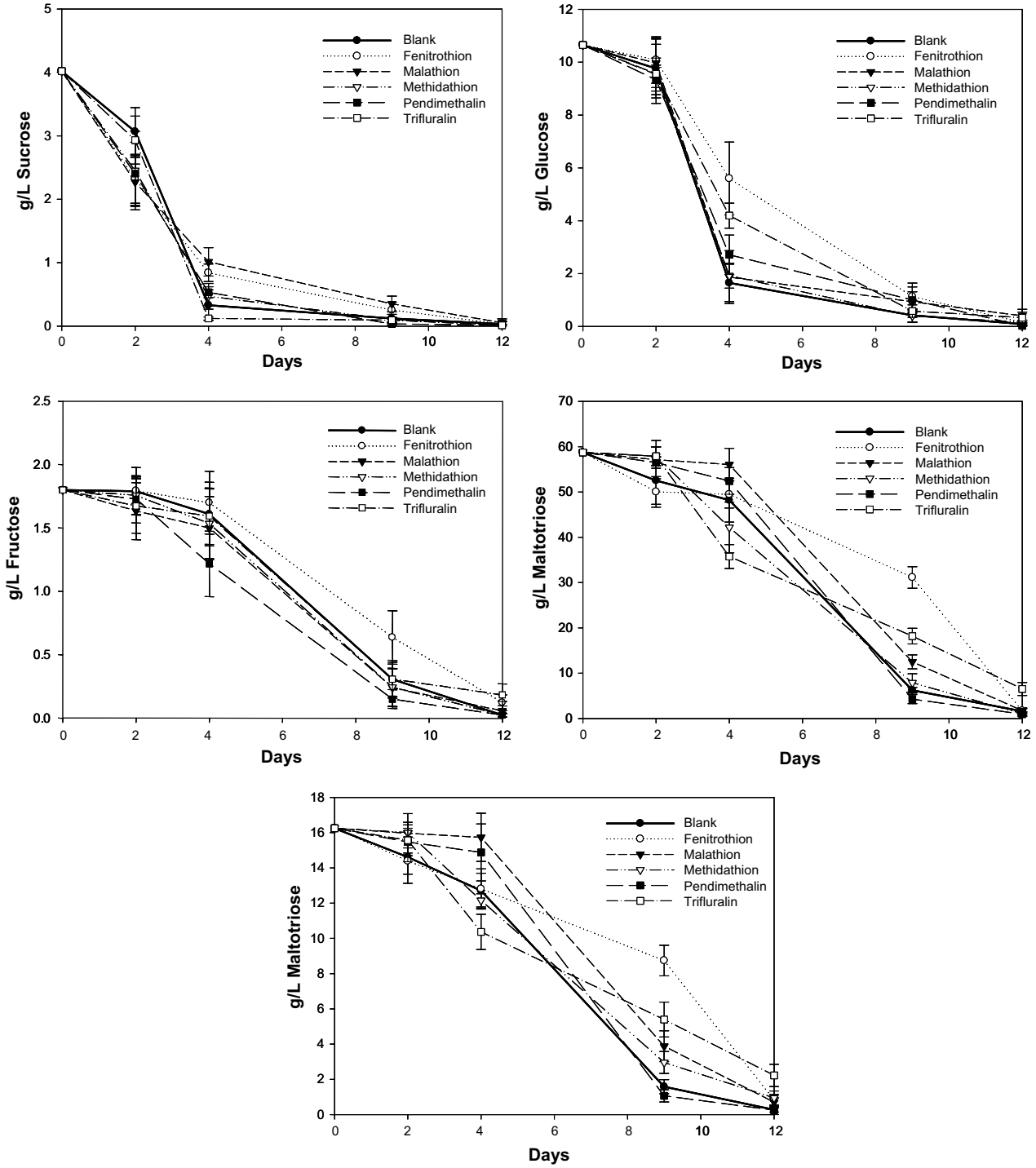



Fig. 3. Changes in sugars content ($n = 3$) *vs* time during fermentation for blank and samples treated with pesticides (error bars are 95% confidence intervals).

The concentration of sugars in the pitching wort was in the following order: maltose, maltotriose, glucose, sucrose, and fructose. The main consumption of glucose occurred from day 2 to day 4. At the end of fermentation, significant differences ($p < 0.05$) were observed for samples treated with malathion (0.4 g/l) and trifluralin (0.32 g/l) compared with the blank sample (0.1 g/l). In the case of fructose degradation was less pronounced since this sugar was consumed by the yeast mainly from day 2 to day 9. The final values showed significant differences ($p < 0.05$) for samples containing fenitrothion and trifluralin residues. Sucrose was mainly metabolized during the first four days of fermentation, after which time the process slowed down and no significant differences were observed in any case at the end of fermentation. Finally, the evolution of maltose and maltotriose was very similar in all the assays as can be seen in

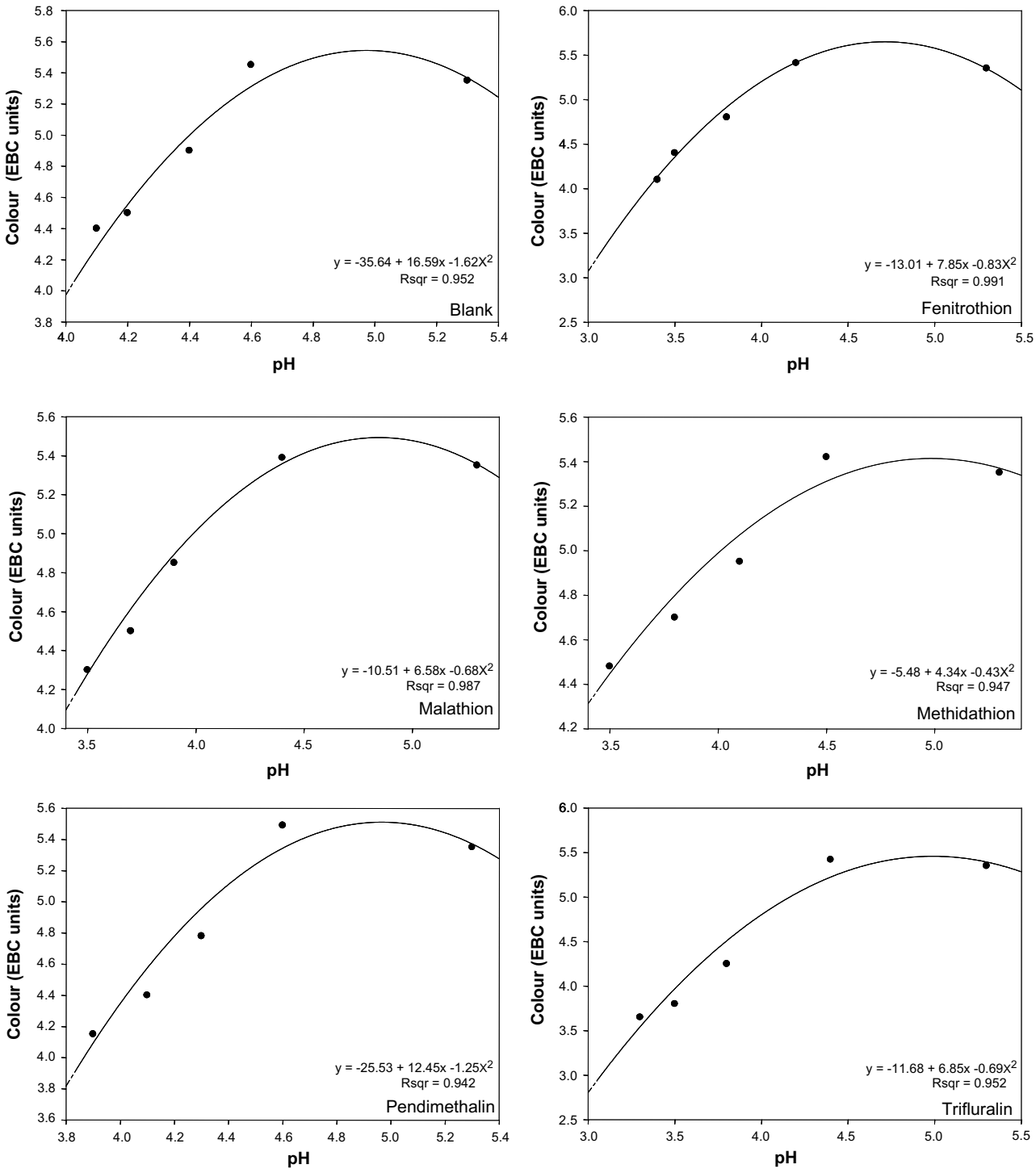



Fig. 4. Correlation between pH and colour for blank and treated samples.

Fig. 3. In the case of maltose, significant differences ($p < 0.05$) were evident after 12 days of fermentation for the samples treated with methidathion (1.1 g/l), pendimethalin (0.9 g/l), and trifluralin (6.5 g/l) compared with the blank sample (1.6 g/l). For maltotriose, the final values of the samples treated with fenitrothion (0.8 g/l), malathion (0.7 g/l), methidathion (0.9 g/l), and trifluralin (2.2 g/l) were significantly higher than that observed for the blank sample (0.3 g/l).

### 3.4. Decrease in the pH value and beer colour during fermentation

The pH value fell substantially from its initial value of 5.3 during fermentation in all cases, particularly in the initial and logarithmic growth phases reaching 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, and the consumption of buffering compounds such as basic amino acids and primary phosphates (Kunze, 2004; Munroe, 2006). The mean pH values at the end of the fermentation were 4.1 for the blank sample and 3.9, 3.6, 3.5, 3.4, and 3.3 for those containing residues of pendimethalin, methidathion, malathion, fenitrothion, and trifluralin, respectively. Significant differences were observed in all cases for the treated samples in comparison with the blank sample. This finding is important because pH values below 4.0 cause an acidic beer taste as result, mainly of microbial infections during fermentation, and should therefore be avoided.

Fig. 4 shows the correlation between pH and colour during fermentation. In all cases a good non-linear regression (quadratic) was observed with $R$ values ranging from 0.94 to 0.99. At the beginning of the process 5.4 EBC units were recorded. Although a slight increase after two days of fermentation can be observed in all cases, the colour of the beer fell about 1–1.7 EBC units during fermentation, possibly due to the decolouration of some substances caused by the drop in pH, and absorption of highly coloured compounds in the yeast cells or precipitation in the vessel bottom (Kunze, 2004). Also, in this case, the treated samples differed significantly ($p < 0.05$) from the blank sample. Finally, with regard to the total polyphenol content (Table 4) after fermentation, significant differences ($p < 0.05$) were observed for the samples containing residues of fenitrothion and trifluralin with respect to the control.

Table 4
Total polyphenol content in beer ($n = 3$)

| Treatment | mg/l ± SD |
|---|---|
| Blank | 59 ± 2.4 |
| Fenitrothion | 51 ± 2.5* |
| Malathion | 57 ± 3.4 |
| Methidathion | 60 ± 3.0 |
| Pendimethalin | 61 ± 4.3 |
| Trifluralin | 55 ± 3.3* |

* Significant at $p < 0.05$ level.

## 4. Conclusions

If present in the malt, pesticide residues may be transferred to brewer wort although the extent of the process will strongly depend on their log $K_{OW}$, as has been demonstrated by several authors. The findings of this work show that fenitrothion, malathion and trifluralin residues affect the growth and fermentability of brewer's yeast, influencing the fermentative kinetic and, causing sluggish fermentation from the *high kräusen* phase onwards. On the other hand, the presence of methidathion and pendimethalin residues accelerated the fermentation rate during the *low* and *high kräusen* phases. A higher amount of residual sugars (glucose, fructose, maltose and maltotriose) was found after fermentation in the samples treated with fenitrothion and trifluralin. Therefore, significant differences in the extract and percentage of sugars converted to alcohol and $CO_2$ (attenuation) were found in the beers. Finally, the pH and beer colour of the treatments differed significantly from the blank sample.

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