

# Volatile Compounds in Cider: Inoculation Time and Fermentation Temperature Effects

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## ABSTRACT

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A cider fermentation at laboratory scale with controlled inoculation with selected yeasts and malolactic bacteria was performed. The composition of the major volatile compounds with low boiling points (<145°C) was determined by gas chromatography throughout the fermentation process, under the different operating conditions of inoculation time of the microorganisms (simultaneous or sequential) and fermentation temperature (15°C or 22°C). Temperature had a more important effect on the levels of certain volatile compounds when the simultaneous inoculation method was used. It was observed that when fermentation temperature increased to 22°C, using the simultaneous method, the final concentrations of ethyl acetate and some of the higher alcohols decreased, while others maintained similar levels. In the sequential inoculation model, after completion of the alcoholic fermentation at 15°C, an increase in the temperature of the malolactic fermentation (15°C to 22°C) showed no statistically significant differences in the profiles of the volatile compounds tested. Thus, malolactic fermentation could be conducted at 22°C, favouring malic acid degradation, without losses in the major volatile compounds, in relation to the levels measured at the lower temperature. Considering the traditionally recognized preference for low alcoholic fermentation temperatures in cider making, the results allow for the optimisation of the operating conditions.

**Key words:** Alcoholic fermentation, cider, malolactic fermentation, volatile compounds.

## INTRODUCTION

Cider production is a complex process in which two important events take place: consumption of sugars by yeasts yielding ethanol and CO<sub>2</sub>, and decarboxylation of malic acid into lactic acid and CO<sub>2</sub>, by malolactic bacteria. The so-called malolactic fermentation is a desirable process since it reduces acidity, improves the organoleptic quality of the product and contributes to its microbial stabilization. In traditional and industrial cider making, no external sources of malolactic bacteria are added to the juice, so spontaneous fermentation generally occurs slowly and is difficult to control. To avoid these problems, the

use of selected pure culture starters is of interest for industrial production to provide uniformity in the final product. The use of indigenous malolactic strains as starter cultures has been reported to allow better adaptation to juices and to yield better results in malic acid degradation<sup>3,8</sup>. Not only the selected strains conducting the process, or the properties of the musts, but also operating conditions such as inoculation method and fermentation temperature, can affect the quality of cider flavour<sup>9</sup>. The effect of temperature on flavour development in cider is generally associated with microbial activity, although a rising temperature could also promote physical loss of low boiling point volatiles<sup>15</sup>.

The most important volatile compounds formed during fermentation that affect the organoleptic characteristics of cider are higher alcohols, esters and carbonyl compounds. These are mainly produced by yeast metabolism. It has been reported that the production of flavour components in cider depends to a great extent on the yeast species employed<sup>10</sup>. Although in winemaking the effects of malolactic fermentation on the sensory attributes of wine was once a matter of controversy, now it is generally agreed that there is enhanced quality in those that have undergone malolactic fermentation. During this process, several volatile compounds can be formed or their concentrations altered. Since compounds produced by malolactic bacteria that could influence the sensory characteristics of wine are not generally present in large concentrations, the development of unique aromas and flavours results from the complex interactions between yeast and bacteria metabolites, influenced by fermentation temperature<sup>4</sup>. But the effect of a particular malolactic strain also needs to be tested. The effect of different added malolactic bacteria strains on the volatile composition of wines and also on their sensory properties has been reported<sup>4,12</sup>.

In this context, and regarding cider production, the contribution of a specific malolactic strain to the volatile composition of cider, the interactions with a particular yeast strain under different operating conditions (inoculation method and temperature fermentation) are of interest from a practical point of view.

A selected strain of indigenous malolactic bacteria (a *Lactobacillus hilgardii* strain) was inoculated at different stages of the process. At the beginning, together with the yeast inoculum (simultaneous method), and once alcoholic fermentation was completed (sequential method). In addition, two different fermentation temperatures were examined (15°C and 22°C). Comparison of the evolution of a number of major volatile compounds in ciders obtained under these different conditions was performed.

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## MATERIALS AND METHODS

### Microorganisms

A commercial active-dry yeast strain of *Saccharomyces cerevisiae* subsp. *bayanus* (strain Pasteur Institute, Paris, 1969, "Champagne", supplied by Novo Ferment, Switzerland) was used. The malolactic bacteria was isolated in the cellar of the cider industry Escanciador, S.A. (Villaviciosa, Asturias, Spain), and was identified as a *Lb. hilgardii* strain by PCR amplification of 16S rDNA using the eubacterial universal pair of primers 63f and 1387r, as described by Marchesi et al.<sup>13</sup>, followed by sequencing and database comparison (BLAST searching).

### Experimental conditions

Concentrated apple juice (bright, enzymatically treated), supplied by an industrial cider factory, was reconstituted with distilled water (1:6), to a final density of ~1060 g/litre. The juice was sterilized in a tangential flow filtration device (Filtron Omegacell 150™, Millipore, Bedford, MA, USA) connected to a peristaltic pump, using polyethersulfone membranes (0.33 µm pore diameter). Fermentations (4 L) were carried out in pre-sterilized 250 mL Erlenmeyer flasks, containing 100 mL of the culture medium. The flasks were placed in an orbital shaker (New Brunswick, G25) (100 rpm) at the assay temperature.

Yeast (active-dried preparation) was rehydrated in sterile apple juice and grown under aerobic conditions at 250 rpm and 28°C, for 18 h. The apple must was then inoculated with yeast to a final concentration of 10<sup>6</sup> cfu/mL.

Malolactic bacteria were grown in apple juice, prepared as previously described, and supplemented with 0.5% (w/v) yeast extract and incubated at 30°C for 6 days without shaking, due to the microaerophilic nature of this bacteria. To start the malolactic transformation, a high cell inoculum was used. It was adjusted to 10<sup>7</sup> cfu/mL in the fermentation medium (must or cider just after alcoholic fermentation, in each of the experiments). In sequential inoculation fermentations, once the alcoholic fermentation was completed as a result of yeast sugar metabolism at 15°C (specific gravity reached ~1005), the malolactic bacteria inoculum was added to the flasks and the flasks were incubated under the assay temperatures and same conditions.

### Sample preparation and analytical methods

Volatile compounds with boiling points lower than 145°C were analysed using a gas chromatograph<sup>14</sup> (GC-14B, Shimadzu, Kyoto, Japan) equipped with a FID detector and an auto injector (AOC-20i, Shimadzu, Kyoto, Japan), fitted with a Supelcowax 10 (Supelco, Bellefonte, PA, USA) column (60 m × 0.25 mm i.d., phase thickness 0.25 µm). Chromatographic conditions were as follows: initial temperature 40°C for 10 min; programme rate 4°C/min to 80°C; 80°C for 10 min; programme rate 35°C/min; final temperature 200°C for 15 min. Finally, column temperature was equilibrated at the initial temperature for 20 min until the next injection. Injector and detector temperature were 200°C and 230°C, respectively; the carrier gas was He at 150 kPa; volume injected, 5 µL. Samples were directly injected after membrane filtration.

Relative standard deviation in duplicate analysis was less than 6%. Analytical grade compounds were used as standards: ethyl acetate, ethanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, (Sigma and Merck). Quantification was performed according to an external standard method. Data obtained were registered and processed in a Chromatopac C-R6A (Shimadzu, Kyoto, Japan), based on peak area measurements.

Two-sample comparisons were performed with statistical software (Statgraphics Plus 3.11).

## RESULTS AND DISCUSSION

Completion of malolactic fermentation was confirmed in each experiment and malic acid consumption was monitored by enzymatic assay (Boehringer Mannheim, Mannheim, Germany). With sequential inoculation, malolactic fermentation was completed in 25 days at 15°C and 16 days at 22°C, from the beginning of the process. With simultaneous inoculation, 30 and 9 days were required at 15°C and 22°C, respectively. Specific gravity was monitored and alcoholic fermentation was completed in 11 days at 15°C when yeasts were inoculated first. With simultaneous inoculation, fermentation required 14 days at 15°C, and 4 days at 22°C (data not shown).

### Effect of temperature with sequential inoculation of microorganisms

Volatile compounds were analysed throughout the fermentation process for each temperature, and the evolution of their concentrations was compared. Profiles obtained for ethyl acetate (Fig. 1), ethanol (Fig. 2), 1-propanol (Fig. 3), 2-methyl-1-propanol (Fig. 4), 1-butanol (Fig. 5), 2-methyl-1-butanol (Fig. 6) and 3-methyl-1-butanol (Fig. 7) are shown.

Ethanol is an important flavour component and a product of the Embden-Meyerhof-Parnas glycolytic pathway whose main function is the production of energy. This pathway also gives rise to pyruvic acid, a precursor to acetic acid and ethyl acetate and also for the synthesis of higher keto acids. These acids, derived from sugars or

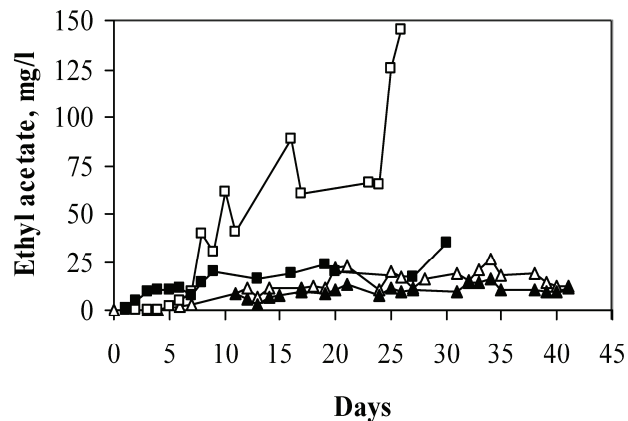
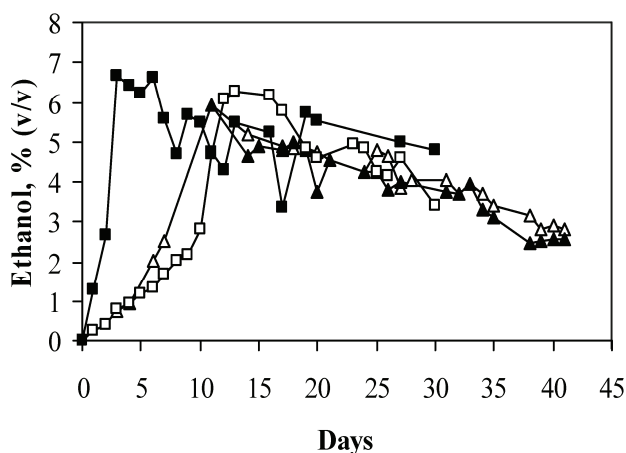


Fig. 1. Comparison of ethyl acetate evolution when sequential (△▲) and simultaneous (□■) inoculation was performed at 15°C (open symbols) and at 22°C (solid symbols).

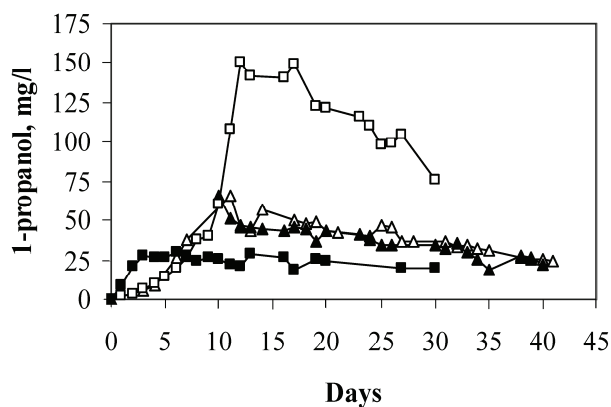
amino acids when present in excess, are the key compounds in the production of the majority of alcohols, carbonyls, acids and esters. Carbonyl compounds are derived from decarboxylation and reduction, while oxidation gives rise to some of the alcohols and acids. Among esters, one of the most significant compounds that affect flavour in fermented beverages is ethyl acetate. As ethanol is the dominant alcohol in cider, ethyl acetate, produced from acetyl-CoA and ethanol, is the most abundant ester.

As expected, the highest levels of the volatile compounds were obtained during alcoholic fermentation as a result of yeast metabolism. The only exception was ethyl acetate (Fig. 1), which showed an increase during the malolactic fermentation.

Ethanol levels (Fig. 2) were clearly diminished during malolactic fermentation at the assay temperatures. Apart from chemical conversions during maturation, as acetaldehyde and/or ester formation, this reduction could also be due to a lack of total anaerobiosis conditions in the fermentation flasks.



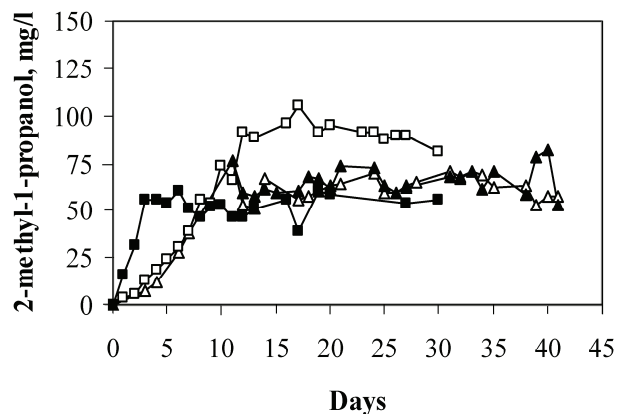
**Fig. 2.** Ethanol production with sequential inoculation of microorganisms (15°C ( $\Delta$ ) and 22°C ( $\blacktriangle$ ); values obtained during alcoholic fermentation at 15°C are shown. With the simultaneous method, at 15°C ( $\square$ ) and 22°C ( $\blacksquare$ ).



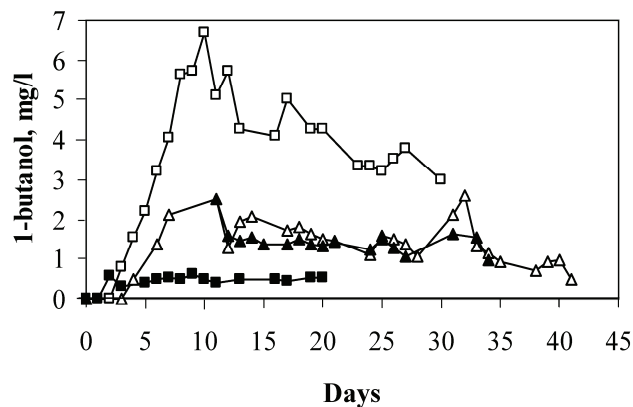
**Fig. 3.** The 1-propanol profiles in the sequential inoculation method at both temperatures (15°C ( $\Delta$ ), for alcoholic and malolactic fermentation, and 22°C ( $\blacktriangle$ ) for malolactic fermentation). With simultaneous inoculation, at 15°C ( $\square$ ) and 22°C ( $\blacksquare$ ).

The biosynthesis of higher alcohols is considered to be linked to amino acid metabolism. Higher alcohols are formed as by-products of both anabolic (Genevois and Lafon pathway) and catabolic metabolism (Ehrlich pathway)<sup>7</sup>, and allow the re-equilibration of the redox balance involving NAD<sup>+</sup>/NADH cofactors. The compound 1-propanol may be formed from the carbon skeleton corresponding to the amino acid threonine by transamination<sup>5</sup>. The compound 2-methyl-1-propanol is produced from valine, while 2-methyl and 3-methyl-butanol are derived from isoleucine and leucine, respectively<sup>2</sup>. The higher alcohols are quantitatively the major volatile components in cider and probably contribute more to the flavour of cider than to beer, where they are present in much lower amounts. The level of higher alcohols seems to be influenced by temperature during fermentation<sup>16</sup>.

As for ethanol levels, 1-propanol (Fig. 3) and 1-butanol (Fig. 5) concentrations diminished during malolactic fermentation at the assay temperatures employed. This may be due to conversions during maturation. The compounds



**Fig. 4.** Evolution of 2-methyl-1-propanol with sequential inoculation (at 15°C ( $\Delta$ ) and 22°C ( $\blacktriangle$ ), including detected levels during alcoholic fermentation at 15°C); values obtained using simultaneous inoculation, at 15°C ( $\square$ ) and 22°C ( $\blacksquare$ ).



**Fig. 5.** Comparison of 1-butanol concentrations reached in the sequential model at the assay temperatures, 15°C ( $\Delta$ ) and 22°C ( $\blacktriangle$ ), showing alcoholic fermentation at 15°C ( $\Delta$ ), for 11 days. In the simultaneous model, at 15°C ( $\square$ ) and 22°C ( $\blacksquare$ ).

2-methyl-1 propanol (Fig. 4) and 2- and 3-methyl-butanol (Fig. 6 and 7) were maintained at a constant level during malolactic fermentation.

Very similar profiles were obtained for the ester, ethanol and higher alcohols tested at both temperatures, indicating that the range of temperature of the malolactic fermentation assayed did not have a significant influence on the levels of these compounds (there were no statistically significant differences at the 95% confidence level).

### Effect of temperature with simultaneous inoculation of microorganisms

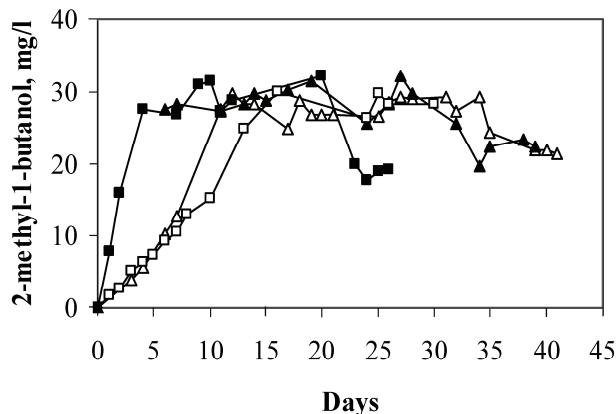
Volatile compounds were monitored at both fermentation temperature assayed during the process and the results obtained were compared (Fig. 1 to 7).

More differences could be observed in the profiles of the compounds analysed at the two temperatures tested, when the microorganisms were inoculated simultaneously in apple juice. Interactions between microorganisms, as well as changes in yeast metabolism as a consequence of different temperatures during alcoholic fermentation, were expected in this case.

Ethyl acetate, (Fig. 1) reached higher concentrations at 15°C than at 22°C showing maximum levels at the middle and final stages of the malolactic fermentation<sup>3</sup>, related to high levels of acetic acid formed (data not shown). It has been reported that ethyl acetate can negatively affect wine aroma when levels are over 200 mg/litre<sup>1</sup>.

Maximum ethanol content was obtained earlier at 22°C (Fig. 2), in accordance with earlier sugar consumption by yeast, than at 15°C (data not shown). At both temperatures, a diminution in the concentrations was detected, as observed when sequential inoculation was performed, but once again, similar values were monitored at the temperatures tested.

The compounds 1-propanol, 2-methyl-1-propanol and 1-butanol also reached higher concentrations at 15°C than at 22°C ( $P < 0.05$ , indicating significant differences between the two means). The highest concentrations corresponded to the final of alcoholic fermentation at each temperature. A reduction in the concentrations of 1-propanol and 1-butanol at 15°C was also observed.



**Fig. 6.** Profiles of the 2-methyl-1-butanol content at 15°C (open symbols) and at 22°C (solid symbols), with sequential (triangles) and simultaneous (squares) inoculation.

For 2-methyl-1-butanol and 3-methyl-1-butanol, the maximum levels were achieved earlier at 22°C than at 15°C, corresponding to the final stages of alcoholic fermentation at each temperature, but no significant differences ( $P > 0.05$ ) were observed between the profiles obtained.

Regarding winemaking, it has been published<sup>6</sup> that generally, any factor that increases the speed of fermentation in wine, such as an increase in the fermentation temperature, simultaneously causes a reduction in ester production and an increase in higher alcohol formation. Under the experimental conditions used, it was found that when the fermentation temperature increased, the final concentration of ethyl acetate and some of the higher alcohols decreased (for 1-propanol, 2-methyl-1-propanol and 1-butanol), while 2- and 3-methyl-1-butanol kept similar patterns, as well as ethanol. In a previous study<sup>11</sup> it has been reported that an increase in cider fermentation temperature (from 8°C to 18°C) induced an increase in the amount of 2-methyl-1-propanol and 2- and 3-methyl-butanol and a decrease in 1-propanol.

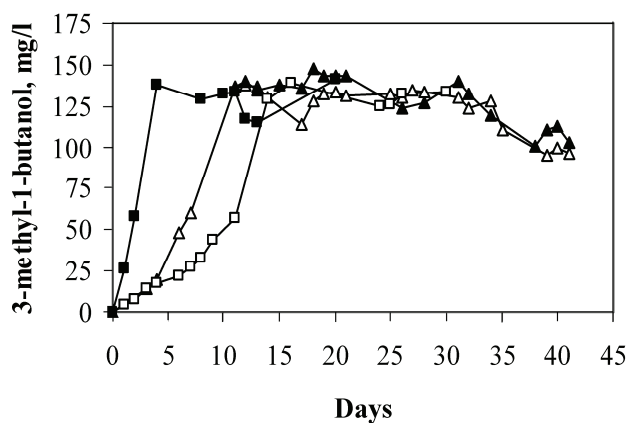
### Effect of the inoculation method on the volatile composition during alcoholic fermentation

For ethyl acetate formation, the inoculation method appeared to have a detectable effect: at 15°C, larger concentrations were reached when simultaneous inoculation was performed (Fig. 1). At 22°C, no significant differences were observed.

In addition, the inoculation method also affected the levels for 1-propanol, 2-methyl-1-propanol and 1-butanol. At 15°C, higher concentrations were obtained with simultaneous inoculation, but lower than at 22°C when using the sequential model.

## CONCLUSIONS

In this study, the effect of a particular malolactic strain on the composition of major volatile compounds in cider was studied, and the influence of different fermentation conditions and the interactions with a commercial yeast strain were compared.



**Fig. 7.** Evolution of 3-methyl-1-butanol at the two assayed temperatures (at 15°C (open symbols) and at 22°C (solid symbols)) in the sequential (triangles) and simultaneous (squares) model.

Performing sequential inoculation after completion of the alcoholic fermentation at 15°C, and an increase in the temperature of the malolactic fermentation from 15°C to 22°C did not reveal a significant influence on the volatile composition of ciders obtained, at least for the compounds studied. Since major volatile compounds are mainly formed during yeast metabolism, changes in the fermentation temperature during the course of alcoholic fermentation had a stronger influence on the levels of the compounds tested, than once fermentation was completed. Published reports have suggested that the optimal temperature for malolactic fermentation is 20 to 25°C<sup>10</sup>. Thus, malolactic fermentation could be conducted at 22°C, favouring malic acid degradation, without loss of the major volatile compounds tested, in relation to the levels measured at the lower temperature. With simultaneous inoculation, when the fermentation temperature increased to 22°C, the final concentrations of ethyl acetate and some higher alcohols decreased, while others maintained similar levels. By using sequential inoculation (after performing alcoholic fermentation at 15°C, a widely preferred cider making practice) an increase from 15°C to 22°C for the malolactic fermentation did not induce a reduction in the levels of the volatile compounds tested.

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#### REFERENCES

1. Amerine, M. A., Berg, H. W., Kunkee, R.E., Ough, C.S., Singleton, V.L and Webb, A.D., *Technology of Wine Making*. The AVI Publishing Company: Westport, Connecticut, 1982.
2. Berry, D.R. and D.C. Watson., *Yeast Biotechnology*. D.R. Berry, I. Russell and G.G. Stewart, Eds. Allen and Unwin: London, 1987.
3. Davis, C.R., Wibowo, D.J., Eschenbruch, R., Lee, T.H. and Fleet, G.H., Practical implications of malolactic fermentation: a

review. *American Journal of Enology and Viticulture*, 1985, **36**, 290–301.

4. Delaquis, P., Cliff, M., King, M., Girard, B., Hall, J. and Reynolds, A., Effect of two commercial malolactic cultures on the chemical and sensory properties of chancellor wines vinified with different yeast and fermentation temperatures. *American Journal of Enology and Viticulture*, 2000, **51**, 42–48.
5. Derrick, S. and Large, P.L., Activities of the enzymes of the Ehrlich pathway and formation of branched-chain alcohols in *Saccharomyces cerevisiae* and *Candida utilis* grown in continuous culture on valine or ammonium as sole nitrogen source. *Journal of General Microbiology*, 1993, **139**, 2783–2792.
6. Etiévant, P. X., *Volatile Compounds in Foods and Beverages*. H. Maarse, Ed., Marcel Dekker Inc: New York, 1991.
7. Hammond, J., The contribution of yeast to beer flavour. *Brewers Guardian*, 1986, **115**, 27–33.
8. Henick-Kling, T., Sandine, W.E. and Heatherbell, D.A., Evaluation of malolactic bacteria isolated from Oregon wines. *Applied and Environmental Microbiology*, 1989, **55**, 2010–2016.
9. Jarvis, B., Forster, M.J. and Kinsella, W.P., Factors affecting the development of cider flavour. *Journal of Applied Bacteriology Symp. Suppl.*, 1995, **79**, 5S–18S.
10. Lafon-Lafourcade, S., *Biotechnology*. Vol. V, G. Reed, Ed., Verlag-Chemie: Heidelberg, 1983.
11. Leguerinel, I., Cleret, J.J., Bourgeois, C. and Mafart, P., Yeast strain and the formation of flavour components in cider. *Journal of the Institute of Brewing*, 1988, **96**, 391–395.
12. Maicas, S., Gil, J.V., Pardo, I. and Ferrer, S., Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Research International*, 1999, **32**, 491–496.
13. Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. and Wade, W.G., Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*, 1998, **64**, 795–799.
14. Parrondo, J., García, L.A. and Díaz, M., Production of an alcoholic beverage by fermentation of whey permeate with *Kluyveromyces fragilis* II: aroma composition. *Journal of the Institute of Brewing*, 2000, **106**, 377–382.
15. Scott, J.A. and Swanffield, C.H., Observations on the influence of temperature, dissolved oxygen and juice source on stored alcoholic cider flavour development. *Food Biotechnology*, 1998, **12**, 13–26.
16. Williams, A.A., Flavour research and the cider industry. *Journal of the Institute of Brewing*, 1974, **80**, 455–470.

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