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QUANTITATIVE ANALYSIS OF DIACETYL, PENTANEDIONE AND THEIR PRECURSORS DURING BEER FERMENTATION BY AN ACCURATE GC/MS METHOD

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A GC/MS method previously described for diacetyl was developed for the quanitification of 2,3-pentanedione, and the derivatization procedure was modified for the determination of α-acetohydroxy acid. The reaction of 2,3-pentanedione with 4,5-dichloro-1,2-diaminobenzene produced 6,7-dichloro-2-methyl-3-ethylquinoxaline (DCMEQ), which was extracted with toluene and determined by gas chromatography using a mass selective detector. The formation of DCMEQ was linearly correlated with the 2,3-pentanedione concentration. The method was very simple and sensitive. The detection limit of the 2,3-pentanedione derivative and diacetyl derivative was 0.0007 mg/litre and 0.0002 mg/litre of the toluene extract respectively, and the determination limit was 0.001 mg/litre and 0.0007 mg/litre, respectively. Cautious sample treatment led to a low (10%) and controlled conversion of α-acetohydroxy acids to vicinal diketones. This reproducible percentage of conversion made it possible to determine precisely free vicinal diketones and α-acetohydroxy acids.

The method was applied to the determination of precursors and vicinal diketones concentrations during beer fermentation.

Key Words: *Vicinal diketones, 2-acetohydroxy acids, gas chromatography, mass selective detector, beer.*

Introduction

Diacetyl and 2,3-pentanedione are known to be important flavour compounds of fermented food, such as alcoholic beverages and dairy products, and to be produced by microorganisms during fermentation.

In the brewing industry, the characteristic buttery taste of vicinal diketones has long been a major problem. Human taste thresholds are very low (diacetyl: 0.05–0.1 mg/litre and pentanedione: 1 mg/litre in a lager beer[1,3]), which explains why it is necessary to understand their formation and reduction by yeasts during the brewing process.

Diacetyl and 2,3-pentanedione originate from α-acetolactate and α-acetohydroxybutyrate respectively, through a chemical oxidative decarboxylation. These precursors, which are intermediates in the synthesis pathways of valine, leucine and isoleucine, are produced in the cells, then excreted in the broth where they are converted into vicinal diketones, which are enzymatically reduced by yeasts into acetoin and 3-hydroxy-2-pentanone. To understand the levels and kinetic formation of diketones, it is necessary to study the kinetic formation of the α-acetohydroxy acids by quantifying them during fermentation.

The generally low interest for 2,3-pentanedione is due to its taste threshold (relatively high compared with diacetyl) and to the relative abundance of diacetyl in beer. However, the latter has been estimated[2] to be between 10:1 and 1:10, thus the study of 2,3-pentanedione could also be of prime interest.

The quantitative analysis of diacetyl, 2,3-pentanedione and their precursors during beer fermentation is made difficult by their very low concentrations (less than 0.5 mg/litre for total diketones[2]) and high volatility, the instability of the precursors due to their high sensitivity to temperature and redox potential[6] and the interference with other matrix compounds, such as ethanol and acetoin. Methods for the quantification of diacetyl include colorimetric[8], fluorometric[9,12], enzymic[3] and gas chromatographic[1,10] procedures. Only the latter with an electron-capture detection (GC/EC) makes it possible to quantify the four components[1], with a detection limit of 0.01 mg/litre for diacetyl and 2,3-pentanedione. This method permits the quantification of free vicinal diketones by elimination of air (sample saturation with nitrogen or carbon dioxide) and a gentle heat treatment (35 minutes at 30°C), which lowers the conversion percentage of precursors into diketones. In some experiments, this percentage was lowered to less than 1% by an additional sample adjustment to pH 7.0[7].

For α-acetohydroxy acid quantification, the oxidation of precursors into vicinal diketones was performed in order to determine the total vicinal diketones. The concentrations of the α-acetohydroxy acids were obtained by subtracting the total vicinal diketone concentration (100% oxidation) to the free vicinal diketone concentration (negligible percentage of conversion).

Only total vicinal diketones are usually quantified and one of the reasons for this is the difficulty to stabilize the precursors during free diketone quantification.

Using a very simple and accurate GC/MS method described for the quantification of diacetyl[10], we developed the analysis of pentanedione and we modified the derivatization procedure to enable the quantification of free vicinal diketones with an accurate determination of the percentage of acetohydroxy acid conversion.

The method is based on the reaction of 2,3-pentanedione with 4,5-dichloro-1,2-diaminobenzene (DCDB) to form 6,7-dichloro-2-methyl-3-ethylquinoxaline (DCMEQ) (Fig. 1). The amount of DCMEQ formed is proportional to the concentration of 2,3-pentanedione present in the sample. DCMEQ is extracted in toluene and quantified in nearly the same way as DCDMQ (diacetyl derivate)[10].

This accurate method permits physiological studies on the synthesis of diacetyl, 2,3-pentanedione and their precursors, as well as their kinetics of production and reduction by yeast. It is also well suited for the quality control of beer during fermentation.

Materials and Methods

Chemicals

Toluene (GC grade) was purchased from Carlo Erba (Nanterre-France). 4,5-dichloro-1,2-diaminobenzene (DCDB), 2,3-butanedione (diacetyl), 2,3-pentanedione, ethyl 2-acetoxy-2-methyl-acetoacetate and dodecane were obtained from Sigma Aldrich (St Quentin Fallavier-France). The industrial wort was

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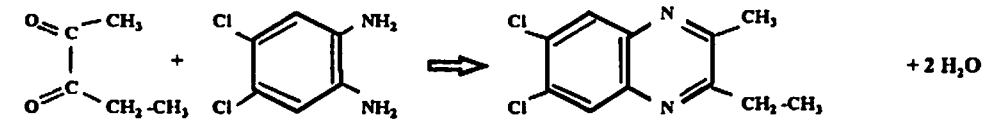

FIG. 1. Derivatization reaction used for the quantitative analysis of pentanedione in beer.

supplied by IFBM (Institut Français de Brasserie Malterie-Vandoeuvre Les Nancy-France).

Preparation of α-acetolactate

Ethyl 2-acetoxy-2-methyl-acetoacetate was transformed to α-acetolactate, ethanol and acetate by addition of two equivalents of NaOH. The saponification reaction was performed at 20°C for 30 min. Reaction yield was determined by the accurate quantification of the ethanol and acetate produced (enzymic kits—Boehringer Mannheim—France SA, Meylan). The mean reaction yield was 97%±2%.

Dilutions were then performed in the wort previously saturated at 4°C with CO_2.

GC/MS apparatus and analytical conditions

GC/MS analyses were performed using a Hewlett Packard 6890 gas chromatograph interfaced with a Hewlett Packard 5972A series mass selective detector. The GC separation was carried out on a Hewlett Packard 5 MS methyl siloxane column (30 m×0.25 µm, 0.25 µm film thickness) using helium as a carrier gas (mean velocity: 47 cm/s; flow rate: 5.2 ml/min). The following GC temperature program was used: injector temperature 250°C; initial oven temperature: 70°C; initial holding time: 1 min 30 s; temperature increment rate: 25°C/min up to 200°C followed by 10°C/min up to 270°C. The retention time observed for dodecane (internal standard), DCDMQ and DCMEQ under the separation conditions used was 4.73, 7.9 and 8.4 min, respectively. Detection was performed using selective ion monitoring. The MS source temperature was maintained at 280°C. For quantification of DCDMQ and DCMEQ, ions 74, 109, 144, 185, 226 and 240 were monitored for retention time 6 to 13 min. For quantification of dodecane, ions 43, 57, 71, 85 and 170 were monitored for retention time 4 to 6 min. Data were processed on a HP vectra XM series 3 employing HP MS chemstation software. Quantification was accomplished by referring to calibration curves obtained from the analysis of known amounts of diacetyl and 2,3-pentanedione added to wort. DCDMQ or DCMEQ peak area ratios to the internal standard were used for calibration and quantification.

Determination of diacetyl, 2,3-pentanedione and precursors in beer

Sample preparation

Sample preparations are shown in Figure 2. The fermentation broth was directly filtered through a kieselghur filter during sampling (the filter was adapted to the sampling valve) in a cooled flask swept with a constant flow of CO_2.

For the determination of free vicinal diketones, 5 mL of the sample was adjusted to pH 7.0 by rapidly adding a (1N) NaOH solution. Derivatization was then performed.

Determination of vicinal diketone precursors was carried out by a complete oxidation to quantify the total diacetyl or 2,3-pentanedione potential. 5 mL samples were shaken vigorously in the presence of air after adding 75 µl of 10 mM FeSO4 and FeC13 solution[4]. Oxidation was performed at 80°C for 10 minutes. After cooling, the sample was derivatized. Oxidation performance was tested using a synthetic α-acetolactate solution diluted in water or wort.

Derivatization

0.5 ml of 20 mM DCDB in 1 M HCl solution was added to 5 ml of known concentrations of diacetyl and 2,3-pentanedione for calibration or fermentation sample. The reaction mixture was heated at 30°C for 5 min.

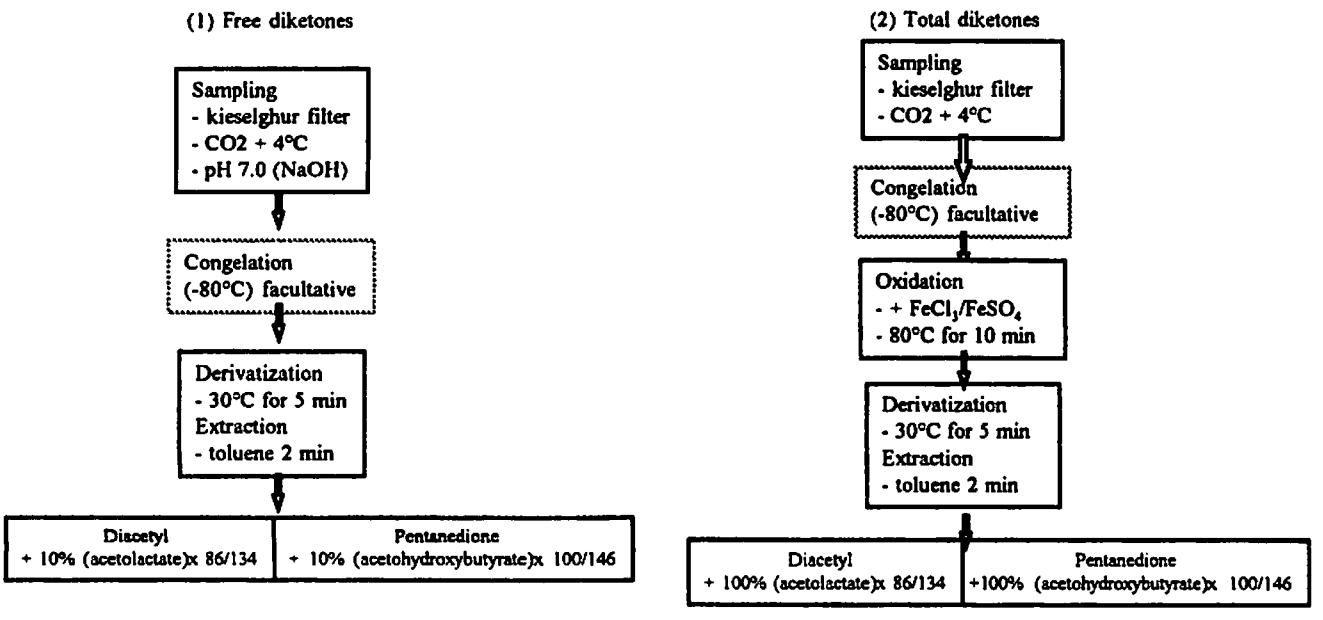

FIG. 2. Schematic diagram of the sample preparation for the analysis of free vicinal diketones (1) and total diketones (2) in fermenting wort.

DCDMQ and DCMEQ were then extracted in 1.5 ml of toluene containing 1 µg/ml of dodecane (C12) as an internal standard. Extraction was performed by slowly rolling the tubes along their length for 120 s which gave reproducible results. 1 µl of the toluene layer was analysed by GC/MS.

Estimation of α-acetolactate oxidation to diacetyl during sampling and derivatization was studied using a synthetic α-acetolactate solution to estimate the percentage of conversion. Moreover, it was used to determine the concentration of precursors.

Linearity, determination limits and reproducibility were determined in water and wort[5]. The detection limit LD was determined from measurements of base line noise. LD= mb+3(σb), where mb is the mean of base line noise and σb is the standard deviation.

The determination limit was obtained from: $L_Q=m+100\ \sigma b/(CV)_Q$, where m is the mean of the measured concentrations of the diacetyl or 2,3-pentanedione close to the detection limit, σb the standard deviation and $(CV)_Q$ the desired coefficient variation (5% in this case).

Fermentation

Fermentations were carried out on a 15-litre reactor (LSL-Biolafitte, Saint-Germain-en Laye). The industrial wort was transferred to the fermentor under sterile conditions.

An industrial lager-type yeast strain, *Saccharomyces uvarum (carlbergensis)*, provided by IFBM (Vandoeuvre-les-Nancy), was used. The wort was inoculated at 10^7 cell/mL and the pressure was maintained at 0.5 bar. Three temperatures were studied: 10°C, 13°C and 16°C. Before inoculation, the wort was aerated up to saturation.

During fermentations, diacetyl, 2,3-pentanedione and their precursors were quantified to demonstrate the suitability of the method.

Results and Discussion

Performance of the method

The chromatographic separation of the 2,3-pentanedione derivative from the diacetyl derivative is effective and DCMEQ has a unique mass spectrum with relative abundance (Fig. 3) which differed from that of DCDMQ, thus permitting the use of selective ion monitoring. This detection with selective ion monitoring improves the method sensitivity.

The results reported in Table I demonstrate the performance of the GC/MS method for the analysis of diacetyl and 2,3-pentanedione in fermented wort. This method is characterized by its very low determination limit and low analytical error. When 2,3-pentanedione was analysed in the range of 0.001–1 mg/litre in water or wort, the peak-area ratio (y) was directly proportional to the 2,3-pentanedione concentration and the slope of standard curves did not vary with the matrix used (Fig. 4).

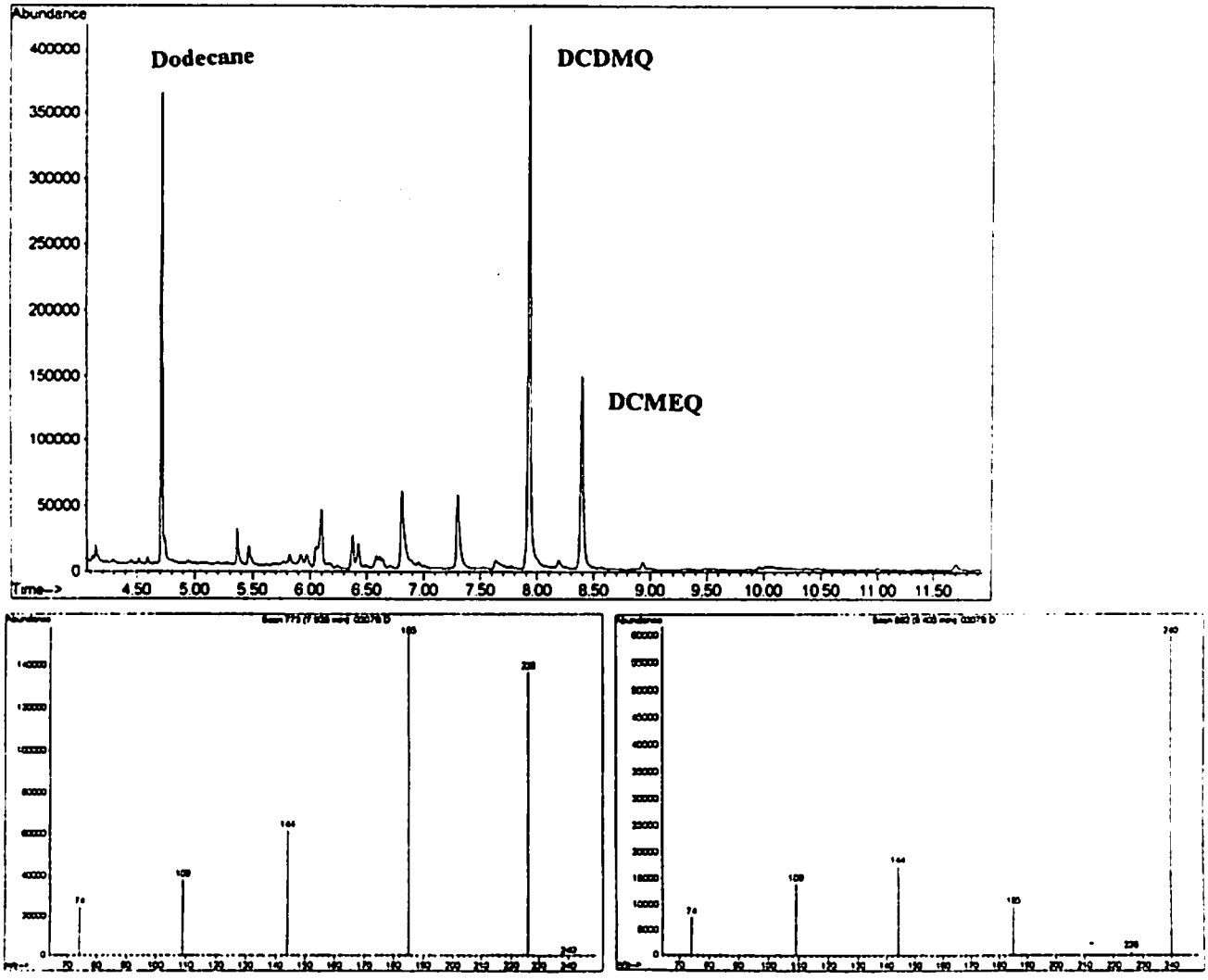

FIG. 3. Total ion chromatogram (SIM mode) of the diacetyl derivative (DCDMQ) and the pentanedione derivative (DCMEQ) extracted from fermenting wort. Mass spectra of DCDMQ (fragment used for selective ion monitoring) with relative abundance in parentheses: 74 (13), 109 (20), 144 (30), 185 (100), 226 (97), 240 (0). Mass spectra of DCMEQ (fragment used for selective ion monitring) with relative abundance in parentheses: 74 (14), 109 (25), 144 (30), 185 (16), 225 (6), 240 (100).

TABLE I. Performance of the GC/MS method for the quantification of diacetyl and pentanedione

	Diacetyl	Pentanedione
nb of measurements	6	6
Detection limit (mg/litre)	0.0002	0.0007
Determination limit (mg/litre)	0.0007	0.001
Standard error[a]	1.7%	1.5%

[a]observed in water and wort at 0.1 mg/litre (m=0.11 mg/litre, σ_{n-1}=0.0017) and 1 mg/litre (m=1.02 mg/litre, σ_{n-1}=0.017).

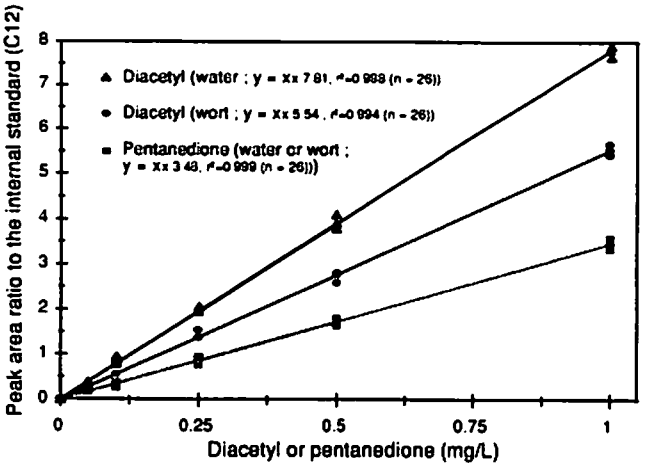

FIG. 4. Standard curves for the GC/MS quantification of diacetyl and pentanedione. Diacetyl or pentanedione were added to water or wort and derivatized with DCDB. Derivatives were quantified by GC/selective ion monitoring.

For diacetyl the matrix has a significant influence, as noticed by Martineau *et al.*[10] (Fig. 4). The slope of standard curves varies with the matrix used and consequently the determination limit obtained in water was lower than in wort (0.0005 mg/L in water[10] and 0.0007 mg/L in wort (Table I)). Unfortunately, the nature or origin of the interference are not known.

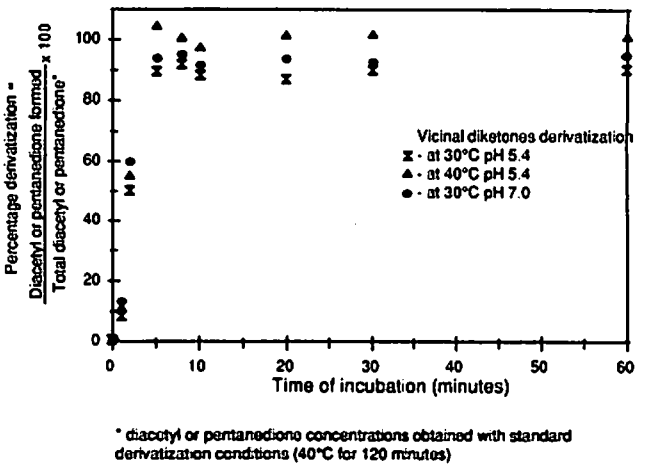

FIG. 5. Effect of temperature and pH on the derivatization kinetics of 0.25 mg/litre pentanedione or diacetyl in wort (initial wort pH=5.4; wort pH was adjusted at 7.0 by (1N) NaOH).

For fermentation studied, the standard curve (diacetyl+2,3-pentanedione) has to be performed in the same wort as that of the fermentation.

This method does not require sample distillation or extraction, which avoids the loss of compounds during sample preparation. Moreover, the total analysis times are significantly shorter (15 min for free vicinal diketones, i.e. 5 min derivatization and 10 min GC/MS analysis, and 25 min for total vicinal diketones, +10 min oxidative treatment), than that of the GC/EC analysis where the times required are 45 min and 2 h 15 min for free vicinal diketones and total vicinal diketones, respectively[1].

Nevertheless, the standard curves for diacetyl and 2,3-pentanedione have to be realized in each wort or medium studied.

Precursor quantification

As the standard conditions for derivatization[10] (40°C for 120 min) are incompatible with the low stability of vicinal diketone precursors (sensitivity to heat treatment, acidic pH medium and the presence of oxygen), alternative conditions for derivatization were developed, in order to avoid the oxidative decarboxylation of coexisting α-acetohydroxyacids.

TABLE II. Percentage of α-acetolactate conversion into diacetyl in wort of different pH wort during different treatments

	Wort without pH adjustment (pH 5.4)		Wort pH 7.0 (adjustment with (1N) NaOH)	
Treatment applied to the α-acetolactate solution[a]	Diacetyl determined	% conversion (CV, n)*	Diacetyl determined	% conversion (CV, n)*
Derivatization at 40°C for 2 h	0.168	38% CF=3%, n=8	0.142	32% CV=2%, n=6
Derivatization at 30°C for 5 min	0.115	26% CV=1.5%, n=6	0.046	**10.4% CV=0.8%, n=6**
Oxidation at 80°C for 10 min and derivatization at 30°C for 5 min	0.381	86% CV=1.2%, n=6		
Oxidation at 80°C for 10 min with $FeCl_3/FeSO_4$ and derivatization at 30°C for 5 min	0.452	**102% CV=0.3%, n=6**	0.270	61% CV=1.5%, n=6

[a]0.68 mg/L synthetic α-acetolactate (0.443 eq. diacetyl) solution
*n=number of measurements.

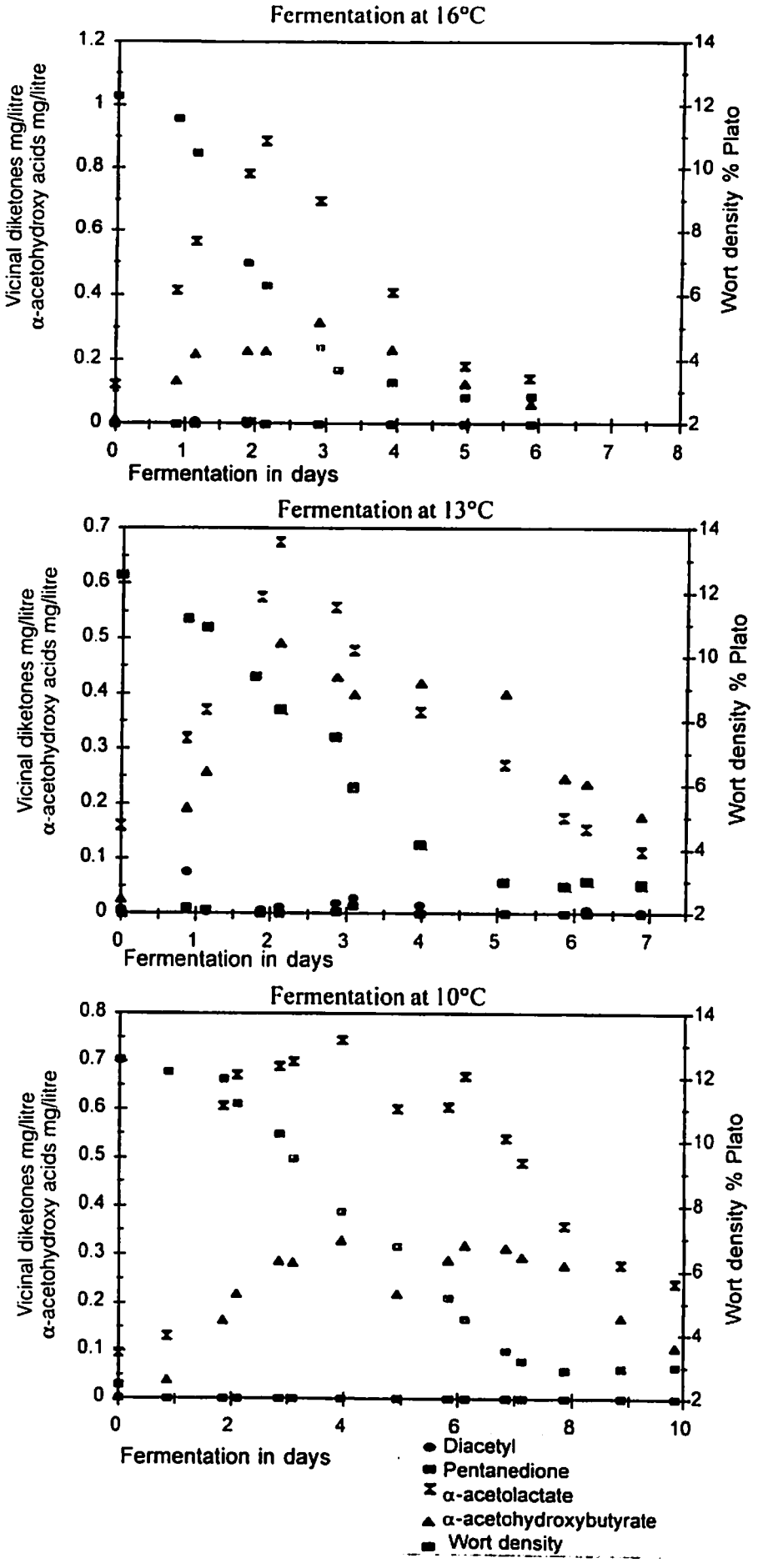

FIG. 6. Changes in vicinal diketone and the α-acetohydroxy acid contents during three main fermentations at different temperatures.

Accordingly, the derivatization kinetics at two temperatures (standard 40°C and 30°C) were studied in order to reduce the heat treatment and consequently the precursor conversion, and at two pH values (wort unmodified pH and pH 7.0). At pH 7.0, acetolactate and aceto-hydroxybutyrate were demonstrated to be stable[7]. Furthermore, sampling of the fermented wort and preparation of the acetolactate were performed in the presence of CO_2 to avoid oxidation by air.

Firstly, the derivatization kinetics were studied at various times (2 min to 2 h) at the reference temperature of 40°C and at 30°C with an aliquot of samples containing 0.25 mg/litre of diacetyl and 2,3-pentanedione (Fig. 5). The derivatization was completed after incubating for 5 min. The quantified amount of diacetyl or 2,3-pentanedione at 30°C was 10% lower than that at 40°C. The sensitivity was still satisfactory under these conditions (see above).

Secondly, the effect of pH on derivatization was studied (Fig. 5). In a wort at pH 7.0 and 30°C, a percentage of diacetyl or pentanedione derivatives only 5% lower than at pH 5.4 and 40°C was obtained. The performance of derivatization at 30°C and pH 7.0 is totally satisfactory.

For free vicinal diketone quantification, a standard curve was prepared under these conditions.

To confirm the positive effect of these conditions on precursor stability, synthetic α-acetolactate solutions were derivatised under various conditions and quantified the diacetyl produced (Table II).

Under these new conditions (30°C, 5 min, pH 7.0), the conversion percentage of acetolactate to diacetyl was 10% with good reproducibility (CV=0.8%). This result was confirmed with samples frozen for 2 weeks (−80°C, pH previously adjusted to 7.0) and with acetolactate solution concentrations up to 1 mg/litre (at 1 mg/litre:CV=2%, 13 measurements). A significantly lower percentage of conversion was found for higher concentrations (at 50 mg/litre synthetic α-acetolactate solution, 0.5% of conversion, CV=3%, 9 measurements).

In beer, α-acetohydroxy acid concentrations are generally lower than 1 mg/litre[11] and a 10% of conversion in vicinal free diketone quantification was applied.

The results described previously[7] (1% conversion at pH 7.0 and 20°C) were tested for acetolactate solution concentration at 1.6 mg/litre (1 mg/litre equivalent diacetyl) and with an additional distillation step.

As the standard oxidation treatment was time-consuming (60°C for 90 minutes), the duration of treatment (10 min) was reduced by employing a higher treatment temperature (80°C) and the addition of an oxidative solution ($FeCl_3$/$FeSO_4$). This solution and the thermal conditions have already been described as the most efficient for α-acetohydroxy acid conversion into vicinal diketones[4].

The performance of oxidative treatment performance was studied using the same synthetic acetolactate solutions as described previously (Table II). 100% conversion was obtained using a $FeCl_3$/$FeSO_4$ oxidative solution as with the standard treatment. In addition, it was confirmed that, under these oxidative conditions, the reduced diacetyl product (acetoin) was not oxidized to diacetyl (results not shown).

For quantification of acetohydroxybutyrate, the results obtained with acetolactate (percentage of conversion with and without oxidation) were used because the behaviour of this compound has often been described to be the same as that of acetolactate[7].

Consequently, the acetolactate and diacetyl concentrations and the acetohydroxybutyrate and 2,3-pentanedione concentrations were calculated using the 10% conversion of precursors during free vicinal diketone quantification and the 100% conversion of precursors during total vicinal diketone quantification.

Kinetics of production of vicinal diketones and their precursors during beer fermentation

When the present method was applied to the analysis of fermenting wort, vicinal diketones were not detectable during three different main fermentations, whereas the precursors were found to accumulate during the first two days (corresponding to yeast growth) and to diminish thereafter (Fig. 6). These results were confirmed for ten fermentations performed at different top pressures or pitching rates (results not shown). However, measurable levels of diacetyl were detected during the second fermentation (13°C) after 24 hours. This is probably due to a problem in sample handling (exposure to air).

The difference between our results and those reported previously[2], which showed that vicinal diketone contents reached their maximum values during active fermentation, demonstrates the great influence of sample handling and the analysis. In most of the usual analytical procedures used for vicinal diketones in beer[8,9,12], the α-acetohydroxy acids are partly converted to vicinal diketones either because of low pH of the sample, or exposure to air or high temperature.

These results confirm those obtained previously by off line analysis[7] with cautious sample handling and on line analysis[11], where vicinal diketones were not detected during the main fermentation.

These results were explained[7] by studying the reduction rate of vicinal diketones which was shown to be 10 times as high as their rate of formation with flocculated cells under fermentation conditions. Moreover, it is well known that, for the species *Saccharomyces cerevisiae*[14], the reduction of vicinal diketones involves enzyme catalysis whereas the oxidative decarboxylation of the α-acetohydroxy acids is a chemical reaction.

Conclusion

A new method is proposed to quantify diacetyl, 2,3-pentanedione and the precursors of vicinal diketones in fermenting wort. The GC/MS method has good sensitivity and is currently the most accurate method available. Furthermore, the instability of precursors was taken into account and the percentage of conversion into diketones determined precisely.

Using this method, no vicinal diketones were detected during the main fermentation.

These results indicate that the usually high levels of vicinal diketones obtained during fermentation were handling and analysis artefacts due to the instability of α-acetohydroxy acids.

Nevertheless, the determination of total vicinal diketone levels in the brewing industry is adapted for quality control because the conversion of precursors to vicinal diketones could take place at high levels during the industrial process. It is thus important to determine the potential of vicinal diketones and buttery taste levels.

For physiological studies, the accurate determination of α-acetohydroxy acid formation could be a valuable tool for analysing the influence of wort composition, such as the amino acid content, or fermentation conditions, such as temperature and top pressure, on their metabolic pathways.

References

1. Buckee, G. K. & Mundy, A. P. *Journal of the Institute of Brewing*, 1994, **100**, 247–253.
2. Garcia-Villanova, R. J. & Garcia-Estepa, R. M. *Analitical Bromatologia*, 1987, 297–324.
3. Gibson, T. D., Parker, S. M. & Woodward, J. R. *Enzyme and Microbial Technology*, 1991, **13**, 171–178.
4. Gollop, N., Barak, Z. & Chipman, D. M. *Analytical Biochemistry*, 1987, **160**, 323–331.
5. Hubaux, A. & Vos, G. *Analytical Chemistry*, 1970, **42**, 849–855.
6. Inoue, T., Masuyama, K., Yamamoto, Y., Okada, K. & Kuroiwa, Y. *Journal of Fermentation Technology*, 1968, **47**, 342–344.
7. Inoue, T. & Yamamoto, Y. *Proceedings of the American Society of Brewing Chemists*, 1970, 198–208.
8. Institute of Brewing, *Recommended Methods of Analysis*, Method 8.9.1, 1991, 229.
9. Mariaud, M. & Levillain, P. *Talanta*, 1994, **41**, 75–79.
10. Martineau, B., Acree, T. & Henick-Kling, T. *Biotechnology Techniques*, 1994, **8**, 7–12.

11. Mathis, C., Pons, M. N. & Engasser, J. M. *Analytical Chimica Acta*, 1993, **279**, 59–66.
12. McCarthy, S. L. *American Society of Brewing Chemists*, 1995, **53**, 178–181.
13. Meilgaard, M. C. *Journal of the American Society of Brewing Chemists*, 1980, **38**, 99–107.
14. Yamauchi, Y., Okamoto, T., Murayama, H., Kajino, K., Amikura, T., Hiratsu, H., Nagara, A., Kamiya, T. & Inoue, T. *Journal of Biotechnology*, 1995, **38**, 101–108.