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A New Method for the Determination of 2-Acetolactate in Dairy Products

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ABSTRACT

Haemin, Fe³⁺ and Cu²⁺ caused significant decarboxylation of 2-acetolactate (ALA) to diacetyl during steam distillation of milk at pH 6.5. In the absence of these compounds, maximum conversion (28%) of ALA to diacetyl occurred at pH 3.5. Addition of 1.5mM CuSO₄ at this pH increased the conversion to 100%. Based on this result, a new method for the determination of ALA was developed, in which interference from pre-formed acetoin was minimised. Comparison of this method with the previous method (Jordan and Cogan, 1995) with which high concentrations of acetoin interfere, was excellent (R²=0.96). Breakdown of ALA to diacetyl in the absence of Cu at pH 0.5 and 6.5 were 0.8 and 2.5% respectively, implying that samples for diacetyl analysis should be adjusted to pH 0.5 before distillation if ALA is also suspected to be present. In quarg (pH 4.5) held at 4°C, ALA was degraded at a rate of 6.9-16.9 µmoles/L/day and from 32-50% of the degraded ALA was converted to diacetyl during extended storage.

1 INTRODUCTION

2 Citrate utilising (Cit⁺) *Lactococcus lactis* subsp. *lactis* produce diacetyl, acetoin and 2-
3 acetolactate (ALA) from citrate in milk fermentations. Diacetyl, although produced in
4 the smallest amounts, is the most important of these compounds as it is responsible for
5 flavour in many fermented dairy products including lactic butter, quarg or buttermilk.

6 ALA is extremely unstable and is easily decarboxylated either oxidatively to diacetyl
7 or non-oxidatively to acetoin (de Man and Pette, 1956). Whether decarboxylation to
8 diacetyl during fermentations occurs enzymatically or chemically is still not clear.

9 Until recently the evidence was in favour of chemical formation but the results of
10 Jordan et al. (1996) provide strong evidence for enzymatic production too. Normally
11 little ALA accumulates in cultures because of the activity of 2-acetolactate
12 decarboxylase (ALD), which is responsible for the production of acetoin from ALA.
13 ALD negative mutants can be selected relatively easily and accumulate ALA which
14 can be converted chemically to diacetyl (Monnet et al., 1997). A naturally occurring
15 ALD negative strain of *Lc. lactis* subsp. *lactis* is used in the NIZO process for lactic
16 butter manufacture and the accumulated ALA is converted chemically to diacetyl by
17 aeration at low pH.

18 A problem arises when the three compounds, ALA, acetoin and diacetyl must
19 be measured in the same sample. ALA is normally measured as acetoin after
20 decarboxylation by heat (Veringa et al. 1984) or acid (Jordan and Cogan, 1988; 1995).
21 After treatment, the samples are assayed for acetoin using the Westerfeld method
22 (1945) and ALA is calculated as the difference in the level of acetoin between the
23 treated and untreated samples. This procedure results in inaccurate measurement of
24 ALA if the level of acetoin in the sample is high compared to that of ALA. Diacetyl is
25 also measured by the Westerfeld procedure but it normally does not interfere with the

1 measurement of acetoin since much smaller amounts of diacetyl are produced than
2 acetoin. Methods for the measurement of diacetyl with which acetoin does not
3 interfere are available e.g. that of Prill and Hammer (1938). Such methods often
4 involve a steam distillation step to separate the diacetyl from the sample (Walsh and
5 Cogan, 1974). When samples which contain ALA are heat-treated, the ALA present in
6 the sample is converted to diacetyl and acetoin which leads to overestimation of both
7 compounds (Veringa et al., 1984). Veringa et al. (1984) and Cronin and Rispin
8 (1996) reported that breakdown of ALA to diacetyl during distillation can be reduced
9 to 2 or 0.2% by adjusting the pH of the samples prior to distillation to 9.0 or 1.0
10 respectively. Gollop et al. (1987) developed a method to measure ALA which
11 involved the oxidative decarboxylation of ALA to diacetyl at 80°C in the presence of
12 Fe^{2+} and Fe^{3+} . The objectives of the present study were to modify the method of
13 Gollop et al (1987) for estimating ALA in dairy products. Preformed acetoin would
14 not interfere with such a method.

15

16 **MATERIALS AND METHODS**

17

18 **Breakdown of synthetic 2-acetolactate**

19 ALA was obtained by saponification of 2-acetoxy-2-methylacetoacetate (Aldrich
20 Chemical Company, P.O. Box 355, Milwaukee, Wisconsin. 53201 USA) with two
21 equivalents of NaOH for 15 min before use. CuSO_4 , FeCl_3 and haemin (Sigma
22 Chemical Company, Poole, Dorset BH17 7NH, England) were added to reconstituted
23 heat-treated (121°C for 5min) skim milk (RSM) (100g/l), pH 6.5, containing the
24 required amount of ALA. Samples were analysed for ALA and acetoin by the Jordan
25 and Cogan (1995) modification of the Westerfeld (1945) method, and for diacetyl in

the first 10ml of steam distillate by the Walsh and Cogan (1974) modification of the Prill and Hammer (1938) method. The Jordan and Cogan (1995) method for measuring ALA and acetoin does not involve distillation; instead ALA is decarboxylated to acetoin by HCl at 4°C and the ALA concentration is obtained by subtracting the level of acetoin obtained in the absence of HCl from that obtained in its presence. Occasionally acetoin was also measured in the 2nd 10 ml of steam distillate by the Westerfeld (1945) procedure.

Final method for estimating ALA

To 3ml of sample, 5.5 ml of buffer [0.2M citric acid and 0.4 M Na₂HPO₄, mixed in a ratio of 2.56:1] and 1.5ml of 10mM CuSO₄ in the buffer was added. For samples with low diacetyl concentrations, the amount of sample was increased to 9ml and the amount of CuSO₄ buffer solution to 21ml. The final Cu²⁺ concentration was 1.5mM and the pH of the mixture of sample, buffer and CuSO₄ was 3.5. The mixture was steam distilled and the first 10ml of distillate collected for diacetyl determination. Samples were also distilled using water instead of the CuSO₄ buffer solution and adjusting the pH to 0.5 using 5M H₂SO₄. The addition of 700µl of 5M H₂SO₄ to 10 ml of milk or lactic culture reduced the pH from 6.5 and 4.5 respectively to 0.5. The ALA concentration of the sample was calculated as the difference in diacetyl concentrations between the sample distilled at pH 3.5 in the presence of CuSO₄ and the sample distilled at pH 0.5 in the absence of CuSO₄.

Comparison of Westerfeld and CuSO₄ method for ALA determination

Lc. lactis subsp. *lactis* strains 4/25A and 1166M1 and *Lactobacillus casei* 4191M3 were used. Strain 4/25A is a naturally occurring ALD negative mutant while the others

were selected by mutation of their respective parents (Monnet et al. 1997). Strains 1166 and 4/25A were from the DPC collection (Moorepark, Fermoy, Co. Cork, Ireland); while 4191 was from the Centre de Recherche International 'André Gaillard', Yoplaît (Ivry sur Seine, France). Heat treated RSM was inoculated with 10g/l of a fresh culture and incubated at 30°C under static conditions. The cultures were sampled at regular intervals and ALA was determined using both the Westerfeld (1945) method modified by Jordan and Cogan (1995) and the CuSO₄ method.

Quarg

Quarg samples containing different levels of ALA were obtained from the Centre de Recherche International 'André Gaillard', Yoplaît (Ivry sur Seine, France). Triplicate samples were analysed for ALA, using the CuSO₄ method, and diacetyl during storage at 4°C.

RESULTS.

In the Westerfeld procedure, ALA is usually determined as the difference in the acetoin concentrations before and after decarboxylation by heat (Veringa et al. 1984) or acid (Jordan and Cogan, 1988; 1995). Where two compounds are measured by the same procedure, like ALA and acetoin, the CV of ALA is related to the CV acetoin by the following equation (Dagnélie, 1973):

$$CV_{ALA} = CV_{ACETOIN} \sqrt{1 + 2(r + 1) / r^2}$$

where CV_{ALA} and CV_{ACETOIN} are the coefficients of variation of ALA and acetoin respectively and r is the ratio of the concentration of ALA to that of acetoin. This equation can be used to construct a series of lines, relating the CV_{ALA} to the ratio of

1 ALA:acetoin, at different values of CV_{ACETOIN} . The results (Fig 1) show that there is a
2 significant increase in the CV_{ALA} when the ALA:acetoin ratio is low, which is
3 normally the case in LAB. Under these circumstances, the measurement of ALA is
4 inaccurate. It is generally regarded that the CV for acetoin is ~5%. At this value and
5 at an ALA:acetoin ratio of 0.4, the CV_{ALA} is 21.5%. Many LAB, especially citrate
6 utilizing *Lc. lactis* subsp. *lactis*, produce small amounts (<0.1 mM) of diacetyl and
7 large amounts of acetoin (up to 4mM) in milk, so that a method for measuring ALA
8 based on diacetyl rather than acetoin would be particularly useful. In this case the
9 ALA:diacetyl ratio would be high and estimates of ALA would be accurate.

10 Gollop et al. (1987) proposed a method for the determination of ALA, in
11 which ALA is oxidatively decarboxylated to diacetyl by heating at 80°C for 10min at
12 pH 4.0 in the presence of 0.15 mM each of FeCl_3 and FeSO_4 followed by 'air'
13 distillation at 60°C. When this method was evaluated in samples which were steam
14 distilled at 100°C rather than 'air' distilled at 60°C, the conversion of ALA to diacetyl
15 was low and not directly proportional to the concentration of ALA in the sample (Fig.
16 2). It was, therefore, considered to be unsatisfactory.

17 The effect of pH on the conversion of ALA to diacetyl is shown in Fig.3.
18 Maximum conversion (28%) occurred at pH 3.5 and 0.8% was converted at pH 0.5.
19 At pH 6.5, the normal pH of milk, ~2.5% would be converted. The findings at pH 0.5
20 agree with those of Cronin and Rispin (1996).

21 Various concentrations of Cu^{2+} , Fe^{3+} and haemin were investigated for their
22 ability to transform ALA to diacetyl during steam distillation. The results (Table 1)
23 show that more diacetyl was formed in the presence of Cu^{2+} than Fe^{3+} and significant
24 production also occurred in the presence of haemin. These experiments were carried
25 out in milk at pH 6.5 but the results would probably show the same relativities, if the

1 milk had been adjusted to pH 3.5 prior to distillation, except that conversion to
2 diacetyl would be greater. To find the optimum concentration of CuSO_4 necessary for
3 transformation of ALA, 1mM ALA in RSM was distilled in the presence of a range of
4 CuSO_4 solutions made up in 18.8mM H_2SO_4 . The pH of the mixture of CuSO_4
5 solution and RSM was 3.5. The results (Fig. 4) showed that conversion to diacetyl
6 increased as the CuSO_4 concentration increased and that recovery was 98 % in the
7 presence of 1.5mM CuSO_4 .

8 To measure ALA accurately, it is important to determine the conditions where
9 the breakdown of ALA to diacetyl is minimised, which will give the true diacetyl
10 concentration in the sample, and maximised, which will give the total of ALA plus
11 diacetyl. The actual level of ALA is obtained by difference. Standard curves
12 containing 0.25-4mmol ALA/l were distilled at pH 3.5, in the presence of 1.5mmol
13 CuSO_4 /l (in 18.8mmol H_2SO_4 /l) and, at pH 0.5, in the absence of copper, and the
14 levels of diacetyl and acetoin determined in the 1st and 2nd 10 ml fractions of
15 distillate. The results (Fig 5) show that, ALA was transformed to 102% diacetyl and
16 little or no acetoin at pH 3.5, in the presence of Cu^{2+} , and to 93% of acetoin and little
17 or no diacetyl at pH 0.5 in the absence of Cu^{2+} . Production of diacetyl at pH 0.5 was
18 1.3%. These results imply that diacetyl should be determined at pH 0.5 in the absence
19 of Cu^{2+} and ALA at pH 3.5 in the presence of Cu^{2+} . The figures for ALA should then
20 be corrected for any 'free' diacetyl to obtain the true level of ALA in the sample.

21 The 100 % recovery of diacetyl from ALA in the presence of Cu^{2+} was
22 confirmed by running 10 standard curves and estimating the s.d. about each point (Fig.
23 5). The s.ds. were small indicating that the method is very reproducible. At ALA
24 concentrations >4mM, breakdown rates at pH 3.5 decreased due to incomplete

1 recovery of the large amounts of diacetyl in the first 10ml of steam distillate, which is
2 the fraction used for the determination of diacetyl.

3 At pH 3.5, standard curves for acetoin (0.25 to 4 mM) in RSM in the presence
4 and absence of CuSO₄ were superimposable, indicating that Cu²⁺ did not catalyse the
5 conversion of acetoin to diacetyl (data not shown). This result implies that acetoin
6 does not interfere with the measurement of ALA by the CuSO₄ method.

7 Cu₂SO₄/H₂SO₄ is a strong acid and significant variation in pH in mixtures of it
8 and sample could result, depending on the buffer capacity of the sample. Changing the
9 pH by ±0.5 of a pH unit from pH 3.5 in the presence of 1.5mM CuSO₄ resulted in
10 only 80% recovery of the ALA as diacetyl, whereas a change of ±0.3 of a pH unit
11 resulted in 100 % recovery as diacetyl. These results imply that the pH of the mixture
12 must be carefully controlled. Use of a buffer [0.2 M citric acid and 0.4 M Na₂HPO₄
13 mixed in a ratio of 2.56:1] instead of H₂SO₄ also showed 100% recovery of ALA as
14 diacetyl in the standard curves. The buffer pH was 3.3 and resulted in a pH of 3.5
15 when RSM was mixed with it and the CuSO₄ solution. During growth, acid
16 production by *Lc. lactis* subsp. *lactis* can decrease the pH of milk from 6.5 to ~4.5.
17 Samples taken at various stages during growth in milk when mixed with the
18 CuSO₄/buffer resulted in a pH of 3.5 ±0.05 indicating that the buffer retained the
19 correct pH over the range of pH values expected in a typical milk fermentation.

20 The Westerfeld (1945) method as modified by Jordan and Cogan (1995) is
21 commonly used to measure ALA. To determine how this method compared with the
22 new method three ALD negative mutants, *Lc. lactis* subsp. *lactis* 4/25A and 1166M1
23 and *Lb. casei* 4191M3 were grown in RSM at 30°C and sampled at intervals for ALA
24 using both methods. Figure 6 shows that the correlation between the two methods is
25 very good and indicates that the CuSO₄ method overestimates ALA by 5.7%

1 compared with the Westerfeld method. This is probably due to the inherent inaccuracy
2 in the Westerfeld method when the ALA concentration is low compared to that of
3 acetoin (Fig 1).

4 As a further test of the applicability of the method, four samples of quarg (pH
5 4.5) were analysed for diacetyl and ALA, using the CuSO_4 method, during storage at
6 4°C . The differences between replicate samples was $< 6\%$ for both compounds. For
7 practical reasons it was not possible to measure the levels at day 0 nor ALA by the
8 Westerfeld (1945) procedure as the acetoin concentration was 3mM and the
9 ALA:acetoin ratio was too low to give reliable results (Fig. 1). An inverse relationship
10 was observed between the disappearance of ALA and appearance of diacetyl during
11 storage (Fig. 7). The rates of ALA degradation in samples A, B, C and D were 6.9,
12 14.6, 11.5 and 16.9 $\mu\text{moles/L/day}$ respectively. Regression of the three diacetyl values
13 on the three ALA values in each sample gave R^2 values of >0.98 and conversion rates
14 of 44, 37, 50 and 32% for samples A, B, C and D respectively.

16 DISCUSSION

17 The results in this paper show that ALA can be effectively measured as diacetyl after
18 distillation in the presence of Cu^{2+} at pH 3.5. Diacetyl itself should be measured in
19 samples adjusted to pH 0.5 and the ALA concentrations corrected accordingly. The
20 mechanism of the Cu or Fe catalysed formation of diacetyl from ALA is not clear.
21 Gollop et al (1987) thought that a complex between the enediol, formed after
22 decarboxylation of the ALA, and a $\text{Cu}^{2+}\text{-O}_2$ complex was involved. ALA can also be
23 measured as acetoin by the Westerfeld method (1945) before and after
24 decarboxylation with HCl (Jordan and Cogan, 1995). The Westerfeld method does not
25 measure ALA concentrations satisfactorily when the ALA concentration is low

1 compared to acetoin, which is commonly the case in many LAB. In citrate utilising
2 lactococci, diacetyl is normally produced in small concentrations (<0.1mM) compared
3 to acetoin which is produced in large concentrations (up to 4mM). In this case, low
4 relative errors during the measurement of acetoin result in high relative errors in
5 determining ALA because of the fact that ALA is calculated as a difference (Fig. 1).
6 The relationship between the CuSO₄ and Westerfeld methods for the determination of
7 ALA was good but the Cu₂SO₄ method is considered to be the better method because
8 small amounts of 'free' diacetyl will not influence the measurement of ALA as diacetyl
9 to any great extent. In this case, the ratio of ALA:diacetyl would be high and would
10 not affect the CV (Fig 1).

11 Kaneko et al. (1987, 1990) have reported, that growing Cit⁺ *Lactococcus lactis*
12 subsp. *lactis* in RSM in the presence of metal ions (Cu²⁺, Fe²⁺, Fe³⁺, and Mo⁶⁺) or
13 haemin increased the production of diacetyl during growth, with Cu²⁺ being the most
14 effective. The results of our study show that if ALA is produced by a culture it can be
15 converted to diacetyl at a high rate by heat in the presence of metal ions or haemin,
16 haemin and Cu²⁺ being more effective than Fe²⁺. The results of Kaneko et al.(1987,
17 1990) could be due to an artefact of the method of analysis used by them (i.e. head
18 space analysis after heating at 80°C for 30 min) which resulted in oxidation of the
19 ALA to diacetyl during analysis rather than a true effect of the metal ions or haemin
20 on diacetyl synthase formation. Unfortunately, Kaneko et al. (1987, 1990) did not
21 report whether their strain produced ALA.

22 In cultured dairy products such as lactic butter, buttermilk and quarg, 1-5 mg
23 of diacetyl/L is required to give organoleptically acceptable products. It is important to
24 be able to detect the levels of ALA in these products post-manufacture in order to
25 determine the potential of the product to develop diacetyl during storage, particularly

1 where ALD negative mutants, are used. Monnet et al. (1994) have shown that the rate
2 of degradation of ALA decreases with decrease in temperature. However, the present
3 results show that even when quarg was stored at 4°C, a significant proportion (32-
4 50%) of the ALA is spontaneously decarboxylated to diacetyl during storage over
5 extended periods.

6
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9
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1 **Table 1.** Decarboxylation of 2-acetolactate to diacetyl in RSM at pH 6.5 during
2 steam distillation^a

Additive	Concentration	n	Decarboxylation	sd
	mM		n	
Haemin	0.01	5	9.3	1.8
	0.10	4	26.7	4.2
CuSO ₄	0.10	1	21.1	
	0.20	3	22.7	3.9
	2.00	1	67.3	
FeCl ₃	0.20	3	11.3	0.9
Control		4	2.5	0.3

3 ^a The initial concentration of ALA was 1.2mM

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5 Figure 1. Modelling the effect of the ratio of 2-acetolactate to acetoin on the
6 coefficient of variation of 2-acetolactate as determined by the Westerfeld (1945)
7 procedure. The CVs of acetoin used are shown in the legend.

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12 Figure 2. Production of diacetyl from 2-acetolactate during steam distillation of milk
13 at pH 4.0 using the method of Gollop et al (1987).

14

Figure 3. Effect of pH on the production of diacetyl from 2-acetolactate in milk during steam distillation. The initial level of 2-acetolactate was 1 mM.

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4 Figure 4. Effect of CuSO₄ on the decarboxylation of 2-acetolactate to diacetyl in milk
5 during steam distillation at pH 3.5. The initial concentration of ALA was 1mM.

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5 Figure 5. Decarboxylation of 2-acetolactate to diacetyl (closed symbols) and acetoin
6 (open symbols) at pH 3.5 in the presence of CuSO_4 (squares) and at pH 0.5 in the
7 absence of CuSO_4 (circles). The regression equations for diacetyl production at pH 3.5
8 and acetoin production at pH 0.5 are shown. For clarity, the trend lines for diacetyl
9 production at pH 0.5 and acetoin production at pH 3.5 are omitted.

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6 Figure 6. Regression of the CuSO₄ method for measuring 2-acetolactate on the
7 Westerfeld method. The regression equation is shown.

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4 Figure 7. Changes in the concentrations of diacetyl (closed symbols) and 2-
5 acetolactate (open symbols) in 4 samples of quarg during storage at 4°C.

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