Curd-Ripening Evaluation by Flow Injection Analysis of L-Lactic Acid with an Electrochemical Biocell during Mozzarella Cheese Manufacture

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A flow injection analysis procedure was used for the determination of L-lactic acid during the production of mozzarella cheese. The apparatus consists of an electrochemical flow-through wall-jet cell assembled with a platinum sensor covered with the immobilized lactate oxidase enzyme and connected to an amperometer. This system was used to monitor the concentration of L-lactic acid produced by lactose fermentation catalyzed by selected starters during cheese manufacture. Lactate was detected in the range $(5 \times 10^{-6})-(1 \times 10^{-4})$ mol/L with a detection limit of 2×10^{-6} mol/L. L-Lactate has been measured in raw milk and during the manufacture of cow and water buffalo mozzarella cheese. The starter used was a commercially available strain of *Streptococcus thermophilus*. Real time analysis of lactate allowed a control of the curd-ripening evolution at different pasteurization temperatures of the milk. Values of lactic acid were compared with pH variation during the process. This method proved to be more sensitive than the pH measurement procedure for the control of the continuous production of lactic acid particularly near the "stretching point" when very slight pH variations were observed.

Keywords: *L-Lactate biosensor; amperometry; mozzarella cheese; water buffalo and bovine milk; curd and whey; ripening; pasteurization*

INTRODUCTION

In modern cheese manufacture, lactic acid, particularly in the L-form, is the main product of lactose fermentation by specially selected cultures of bacteria. The most important consequences of the acid production in cheese manufacturing are reported by Fox in a recent review (Fox et al., 1990). Mozzarella cheese is one of the most highly valued unripened "pasta filata" cheeses produced in Italy, made from water buffalo or bovine milk. In the manufacture of unripened "pasta filata" cheeses, a rapid acidification of the curd by the starter cultures promotes the transformation of dicalcium paracasein into monocalcium paracasein (Chapman et al., 1981).

The progressive acidification of curd has to be carefully controlled to avoid an unripened curd process which will result in a loss of fat, a yield decrease, and lower manufacturing reproducibility (Altiero et al., 1984). Control of the curd acidification is carried out by pH measurement directly in the curd or by titratable acidity of the whey. This is because lactic acid is mainly produced from lactose within curd particles and is readily transferred into the whey. pH measurement in the curd is considered a more reliable indicator of acid production than whey acidity.

Studies of curd ripening of mozzarella cheese demonstrated that the optimum pH for curd stretching is 4.9 for water buffalo milk (Altiero et al., 1984) and 5.1 for cow milk; these values are close to the isoelectric point of casein. At this pH there is a great increase of the buffer capacity of curd which poses some difficulties in the control of the acid production. In fact, the pH variation near the stretching point is very low. The direct measurement of L-lactic acid in the curd and the whey during the late stages of mozzarella cheese manufacture can solve the problem and allow better control of the lactose fermentation and optimization of the curd-ripening time. The rational cheesemaking practice and the current Italian regulations for soft unripened cheese manufacture (Ministero della Sanità della Repubblica Italiana, 1978) require the pasteurization of milk.

In this work we studied the curd-ripening evolution of water buffalo and bovine milk at different pasteurization temperatures. A commercially available starter of *Streptococcus thermophilus* yielding mainly L-lactic acid was used (Addeo and Coppola, 1983; Hardie, 1986; Fox

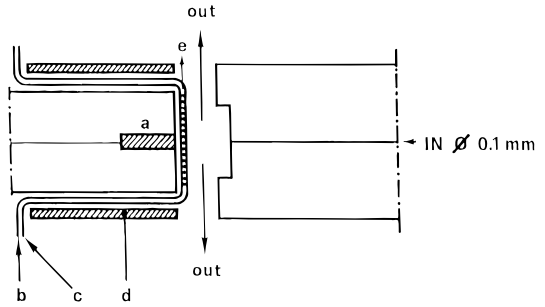

Figure 1. Scheme of wall-jet cell: (a) Pt electrode, (b) cellulose acetate membrane, (c) Immobilon membrane, (d) O-ring, and (e) lactate oxidase.

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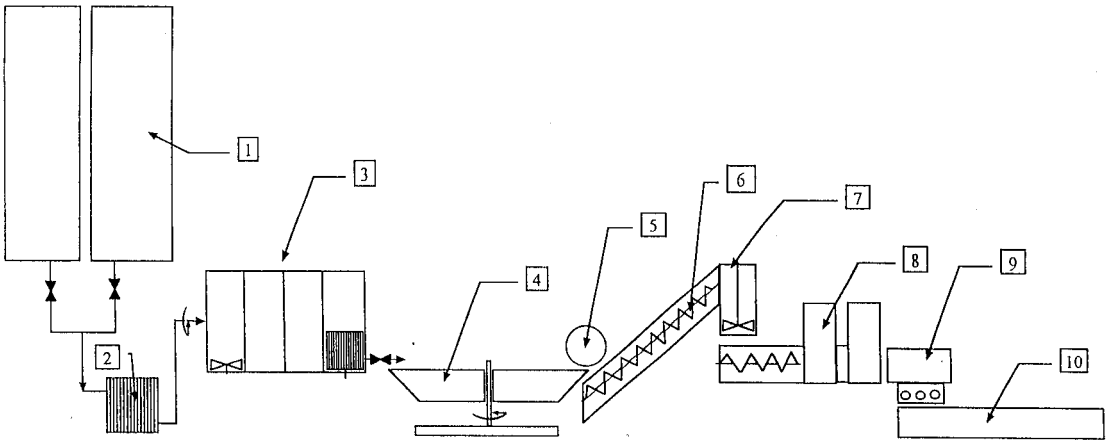

Figure 2. Scheme of the mozzarella cheesemaking plant (capacity 5000 L/h): (1) tank, (2) pasteurizator, (3) polyvalent, (4) ripening pools, (5) curd mincer, (6) cochlea, (7) curd slicer, (8) curd stretching, (9) curd molder, and (10) hardening pools.

et al., 1990). L-Lactic acid was measured using a flow injection analysis (FIA) system consisting of a L-lactate oxidase-based biosensor inserted in a wall-jet flow cell of 10 μL volume. This type of cell has been recently used for the determination of glucose and lactate in biological samples and wines (Palleschi et al., 1989, 1990, 1994). The amperometric measurement, based on a linear relationship between the current produced by the enzymatic reaction and the concentration of lactate, allows the determination of lactate in less than 30 s during the various steps of mozzarella cheese manufacturing. This results in a close control of the lactate fate during curd ripening.

MATERIALS AND METHODS

L-Lactic acid (lithium salt) and lactate oxidase from *Pediococcus* sp. (EC 1.1.3.2.; 30 units mg^{-1}) were purchased from Sigma Chemical Co. (St. Louis, MO). Cellulose acetate membrane (100 Da nominal molecular weight cutoff) was prepared in our laboratory according to a procedure reported in the literature (Mascini et al., 1987). All other reagents were of analytical grade from Farmitalia C. Erba (Milan, Italy). Microporous polycarbonate membrane (0.03 μm) was obtained from Nuclepore (Pleasanton, CA). The Immobilon-AV affinity membrane (0.65 μm pore size, 125 μm thick) was from Millipore (Bedford, MA). The wall-jet cell used was a model 565 from Metrohm (Herisau, Switzerland). This cell was assembled with a working platinum electrode, 1.6 mm diameter (model HF 2013), from BAS (BioAnalytical Systems, Lafayette, IN). Current variations were measured with an ABD (amperometric biosensor detector) from Universal Sensors (Metairie, LA) and recorded with a model 868 AMEL recorder (Milan, Italy). The working buffer was pumped with a Minipuls 3 peristaltic pump from Gilson (Villiers le Bel, France). Samples were injected using a Rheodyne injection valve, model 7125, with a closed loop of 20 μL. pH measurements were performed with a portable pH meter from Hanna (Padova, Italy). Spectrophotometric determination of D- and L-lactic acid on mozzarella samples was carried out using a Boeringher Mannheim kit, no. 1112.821 (Mannheim, Germany).

Electrochemical Biosensor Assembling. The wall-jet cell (Figure 1) was assembled using a three-membrane configuration: the cellulose acetate membrane in contact with the platinum electrode, the enzyme membrane, and a polycarbonate membrane as external. The enzyme was immobilized on the Immobilon membrane according to a procedure reported in the literature (Palleschi et al., 1994).

Mozzarella Cheese Production. Four lots of mozzarella cheese from water buffalo and cow milk were produced in the dairy farm Pomella SpA (Frosinone, Italy). The scheme of the plant (capacity of about 5000 L/h) is shown in Figure 2. The experimental conditions were the following: A, water buffalo milk pasteurized at 65 °C for 40 s; B, water buffalo milk pasteurized at 68 °C for 40 s; C, cow milk pasteurized at 65 °C for 40 s; D, cow milk pasteurized at 68 °C for 40 s; 200 L of milk was used for each trial and was manufactured according to the technological parameters reported in Figure 3. The milk was directly inoculated with a commercially available starter of *S. thermophilus* from Sacco-Cadorago (Como, Italy). The manufacture and sampling times are reported in Table 1.

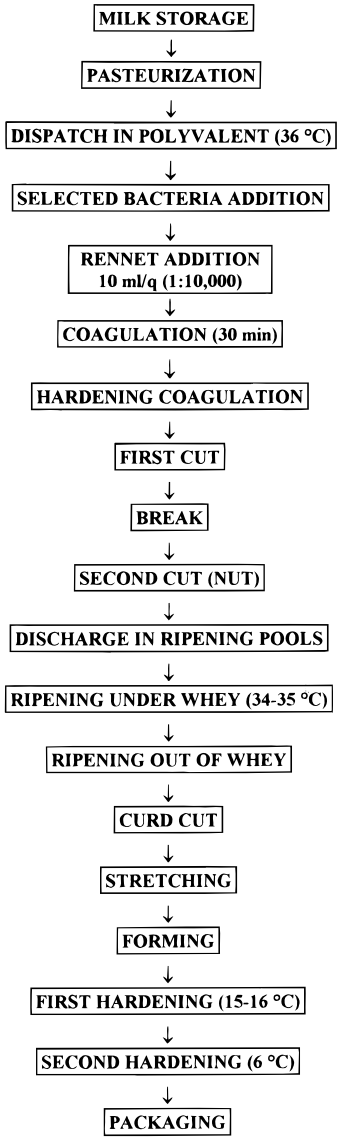

Figure 3. Scheme of mozzarella cheese manufacture.

Table 1. Sampling Times for L-Lactate and pH Analyses in Milk (M), Whey (W), and Curd (C)

ref	milk	pasteurization temp (°C)	dispatch in polyvalent (h), M	discharge in ripening pools (h), W/C	control (h), W/C							stretching (h), C
					1	2	3	4	5	6	7	
A	water buffalo	65	22.30	0.30	1.00	1.30	2.00	2.30	3.00	3.30	4.00	4.00
B	water buffalo	68	23.00	1.00	1.30	2.00	2.30	3.00	3.30	4.00		4.00
C	cow	65	24.00	2.00	2.30	3.00	3.30	4.00	4.30	5.00		5.00
D	cow	68	0.30	2.30	3.30	4.30	5.00					5.00

Table 2. Cow and Water Buffalo Milk Analytical Parameters

	cow milk	water buffalo milk
titratable acidity (°SH)	7.0	8.0
pH	6.6	6.4
antibiotics (UI)	none	none
fat (%)	3.6	8.3
dry matter (%)	12.6	18.8

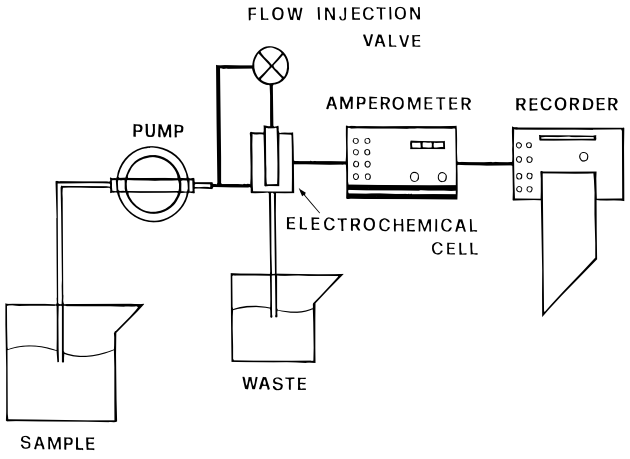

Figure 4. Scheme of flow system.

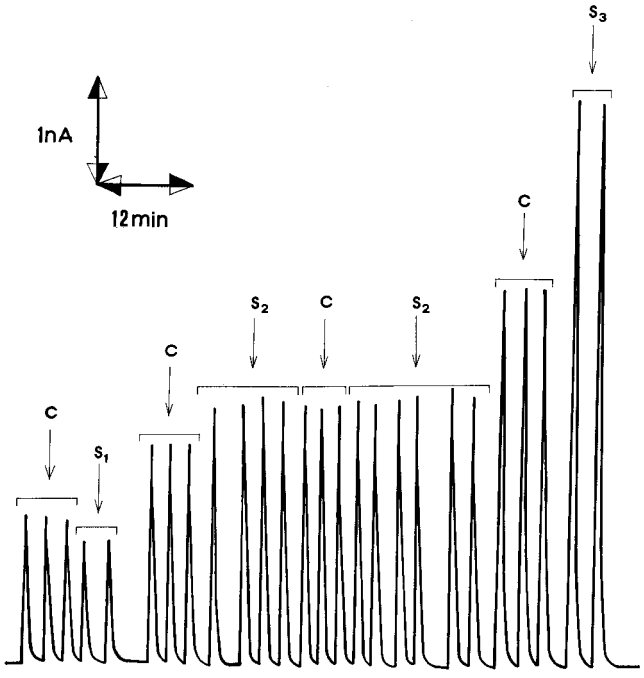

Figure 5. Reproducibility of L-lactate standards (S) and samples (C) in FIA using a L-lactate probe assembled in wall-jet cell.

Other analyses carried out on water buffalo and cow milk samples are reported in Table 2.

Procedures. A scheme of the flow system is reported in Figure 4. A 0.1 M phosphate buffer solution (pH 7.0) was pumped through the electrochemical cell by the peristaltic pump until the current reached a steady state. Samples were loaded into the loop of the Rheodyne valve and injected into the flow stream. Peak heights were related to the concentration of lactate in the sample.

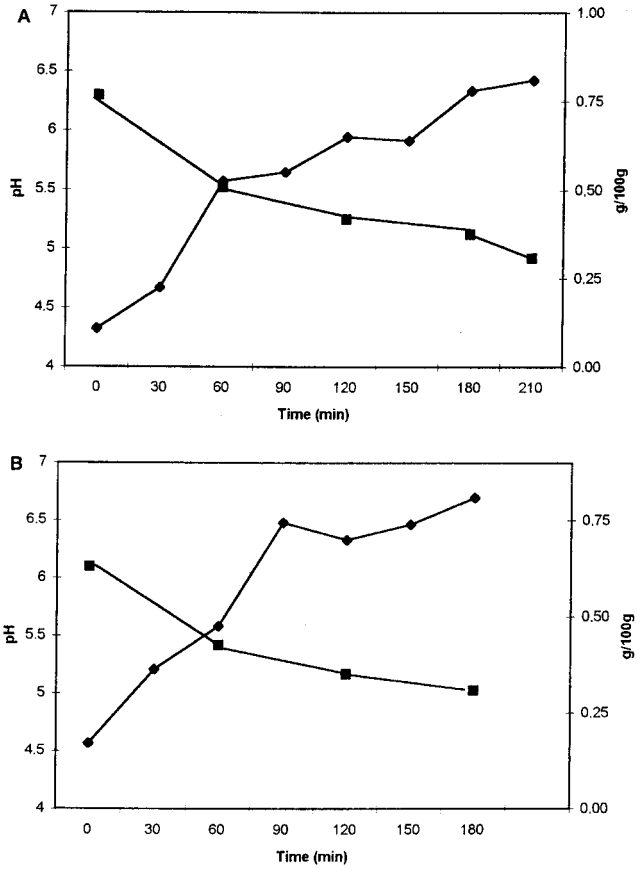

Figure 6. pH (■) and L-lactate (◆) evolution during curd ripening in the manufacture of water buffalo mozzarella cheese with different pasteurization temperatures of the milk: (A) 65 °C and (B) 68 °C.

Samples for the analysis were prepared according to the following procedure: For curd−whey analysis, 100 g of curd was weighed and pressed (in a homemade tool at about 2 kg/cm^2 for 3 min) to obtain whey for the analysis. Whey was diluted in the range 1:100−1:2000 with buffer and immediately processed. Whey analysis was carried out by dilution of whey samples. pH measurements in curd were performed by insertion of the pH probe directly into the curd.

RESULTS AND DISCUSSION

The reactions involved in the analysis of L-lactic acid are the following:

$$\text{lactate} + O_2 + H_2O \rightarrow \text{pyruvate} + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

Reaction 1 is catalyzed by the enzyme lactate oxidase. The hydrogen peroxide produced by the enzymatic reaction is oxidized at the Pt electrode, and the output current is correlated to the concentration of lactate present in the sample.

Table 3. Features of the Lactate Biosensor Cell Used for the Analysis of Lactate in Whey and Reproducibility of Lactate Measurements Expressed as Relative Standard Deviation (RSD%)

detection limit (mol/L)		linearity range (mol/L)		RSD %	
min	max	from	to	lactate (10^{-4} mol/L), mean of 6 determinations	sample of whey, mean of 3 determinations
5×10^{-7}	1×10^{-3}	1×10^{-6}	7×10^{-4}	1.25	1.40

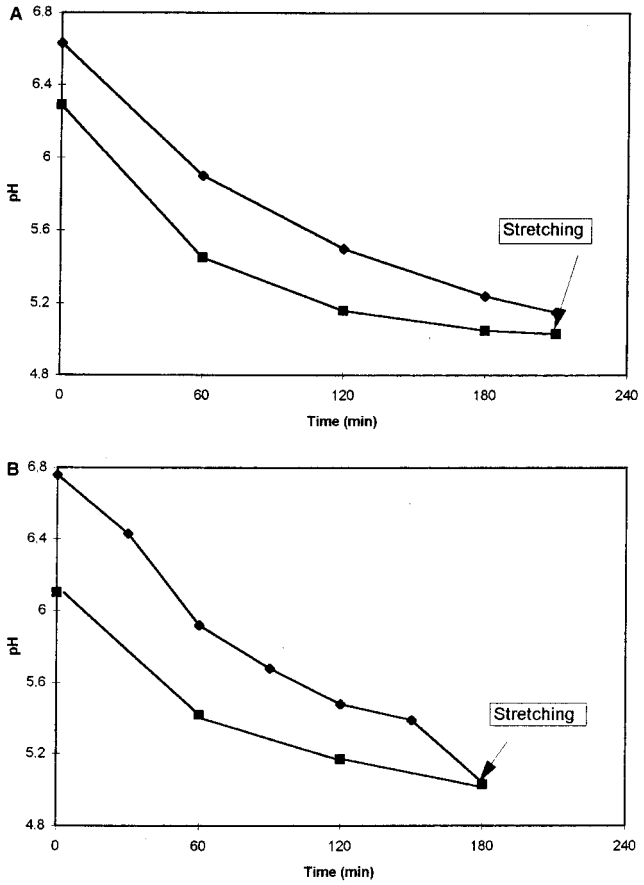

Figure 7. pH evolution in curd (■) and whey (◆) during ripening in the manufacture of water buffalo mozzarella cheese with different pasteurization temperatures of the milk: (A) 65 °C and (B) 68 °C.

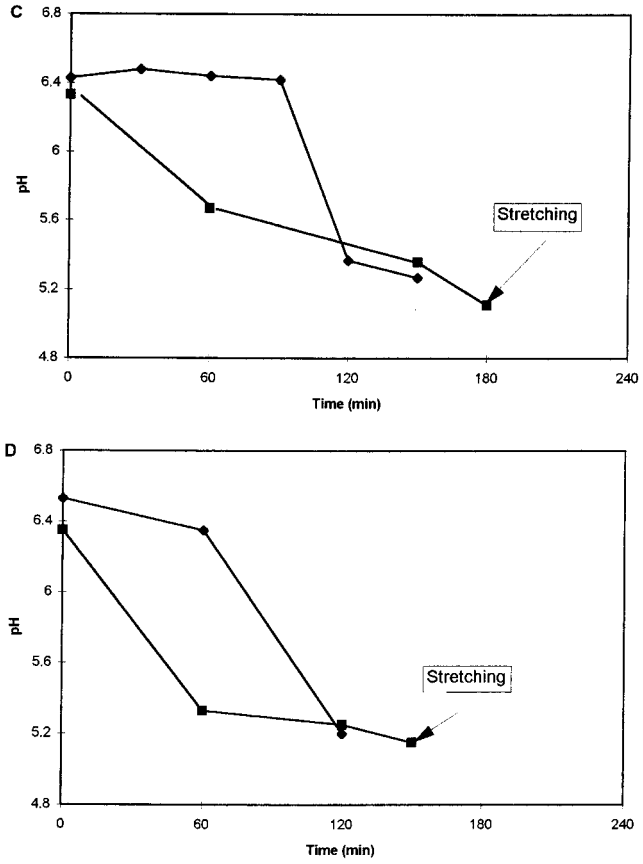

Figure 8. pH evolution in curd (■) and whey (◆) during ripening in the manufacture of cow mozzarella cheese with different pasteurization temperatures of the milk: (C) 65 °C and (D) 68 °C.

The features of the lactate sensors have been defined in previous works (Mascini et al., 1988; Palleschi et al., 1989, 1990, 1994). The sensor was calibrated at different flow rates by injection of aliquots of lactate in the range $(1 \times 10^{-5})-(5 \times 10^{-4})$ mol/L. Good sensitivity was obtained using flow rates between 0.2 and 1 mL/min in 0.1 M phosphate buffer (pH 7.0) with the highest response at 0.2 mL/min. Although relatively slow, this flow rate allowed a sampling rate of 3 min and was then selected for further experiments. Analytical features of the lactate biocell are reported in Table 3. Measurements of lactate standard solutions and whey samples are shown in Figure 5. The response of the probe was very fast and highly reproducible (CV = 2%). After more than 100 analyses, the probe still retained its initial sensitivity.

Lactate and pH Analysis during Mozzarella Cheese Manufacturing. Figure 6 shows the variation of pH and of L-lactic acid content of curd during the ripening of water buffalo mozzarella cheese. A rapid increase in L-lactic acid content during the first stages of manufacture was followed by a constant, but less pronounced, increase. pH decreased initially and then leveled off at values around 5. This is due to the buffer capacity of whey in the curd which (because of casein and soluble phosphate and citrate) increases considerably below pH 5.5 (Fox et al., 1990). As a consequence a loss in the significance of the pH measurement occurs. The measurement of lactate is not influenced by this parameter.

One interference could be the decrease of the enzyme activity at pH below 5.5, but it must be considered that in the analysis of lactate by FIA the sample (20 μL) has to be diluted with a buffer solution to maintain the sensor response within the linear range, whose upper limit is defined by the apparent Michaelis−Menten constant (K_m) of the immobilized enzyme. So the measurement is always carried out at the same pH of the buffer (7.0). Figures 7 and 8 show pH variations in curd and whey during ripening in the manufacture of the four lots of mozzarella cheese. It can be observed that, at the "stretching point", pH values for both the curd and the whey were almost pH 5, which is considered the optimum value (Coppola et al., 1990).

The production of L-lactic acid has been also monitored (Figures 9 and 10). The evolution of lactate, in the water buffalo curd (Figure 9), has the same trend as that in the whey, though lactate in curd exhibited a more erratic profile. This experiment was carried out at two different milk pasteurization temperatures (65 and 68 °C). A higher concentration of lactate in the curd

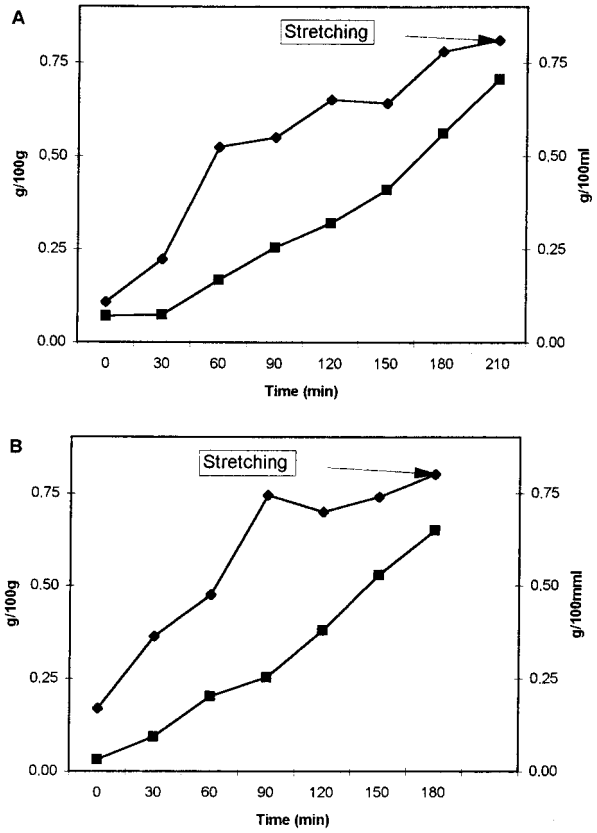

Figure 9. L-Lactate evolution in curd (◆) and whey (■) during ripening in the manufacture of water buffalo mozzarella cheese with different pasteurization temperatures of the milk: (A) 65 °C and (B) 68 °C.

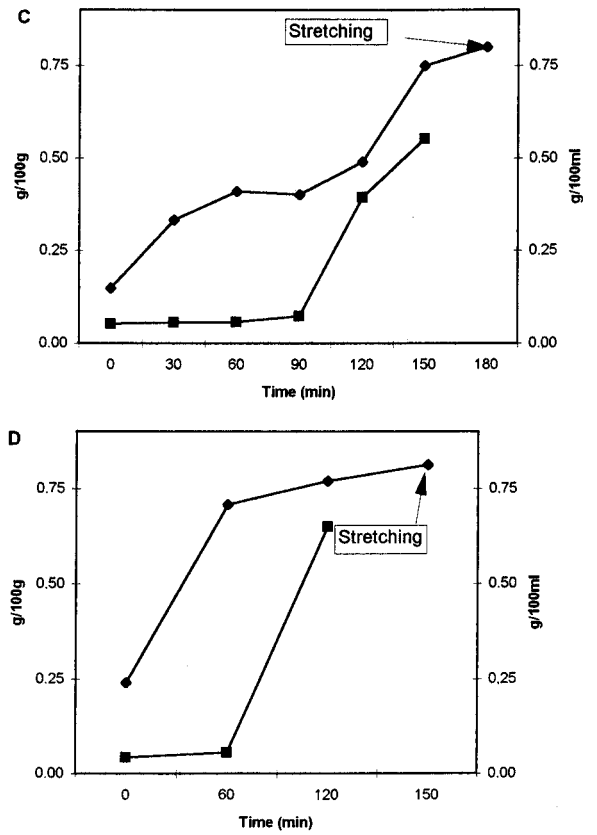

Figure 10. L-lactate evolution in curd (◆) and whey (■) during ripening in the manufacture of cow mozzarella cheese with different pasteurization temperatures of the milk: (C) 65 °C and (D) 68 °C.

than that in the whey was observed at both temperatures. When cow milk was used, a sharp increase of lactate in the curd was observed in the first stages, while this occurred later in the whey. This was consistent with the pH variations measured (Figure 8). Initially, lactate appears to be retained inside the curd particles with later release.

A parameter which can prove useful for the definition of protocols of cheesemaking is the amount of lactic acid in the cheese at the end of curd ripening (stretching point). This was about 0.8 g % for all types of mozzarella cheese. This value for L-lactic acid has been confirmed by measurement with a spectrophotometric procedure. No D-lactic acid was found in the samples. Moreover, in order to test the accuracy of our method, four samples of curd were analyzed with the biosensor and a reference procedure. Results are reported in Table 4 where it is shown that the relative error ranges from 1.2% to 6.6%.

Both the water buffalo and cow milk mozzarella cheeses showed faster ripening times after pasteurization at 68 °C. This could be explained by lower competition of thermophilic bacteria still present in the milk after heating. Furthermore, higher pasteurization temperatures favor the release of more milk components which act as bioactivators of lactic acid bacteria growth (Jago et al., 1959; Reiter, 1973).

Table 4. Comparative Investigation of Lactic Acid Concentrations in Curd Samples

sample no.	H_2O_2 probe lactate (g/100 g)	spectrophotometric lactate (g/100 g)	rel error (%)
1	0.81	0.86	6.2
2	0.76	0.81	6.6
3	0.81	0.84	3.5
4	0.80	0.79	1.2

[a] The results shown are based on biosensor procedure and the reference spectrophotometric procedure.

CONCLUSIONS

Real time L-lactic acid measurement by FIA can be suitable for the control of the mozzarella cheese curd-ripening time. The sensitivity of the measurement and the ease of procedure can lead to a better evaluation of the stretching point, improving the manufacturing quality. This procedure can also be applied to the improvement of strains with high acid-producing ability in order to reduce the manufacturing time and for the selection of multiple-strain starters to obtain constant performances and sanitary production of mozzarella cheese.

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