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## ORIGINAL ARTICLE

# Multivariate Analysis of the Organic Acids Content of Gouda type Cheese during Ripening

Alicia N. Califano*,[1] and Alicia E. Bevilacqua*,†

*Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CONICET), Facultad de Ciencias Exactas, UNLP, 47 y 116, La Plata 1900, Argentina; and †Departamento de Ingeniería Química, Facultad de Ingeniería, UNLP, Argentina

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Gouda cheeses from a local plant were subjected to different ripening conditions and analyzed. The effects of ripening temperature, type of packaging film and storage period before packaging on the organic acids content of Gouda cheese were studied. A factorial design of $2^3 \times 5$ was used, where the three factors selected in two levels were: (1) time of storage before packaging, 4 and 10 days, (2) ripening temperature, 10 and 20°C, and (3) plastic film (BK1 and BK5; Grace, Argentina). Ripening time was the fourth factor considered; sampling times were 15, 25, 35, 49 and 70 days after production. At the beginning of the experiment (4 days of ripening), mean water content of samples was $44.0 \pm 0.9\%$. At 70 days of ripening, humidities were $39.7 \pm 0.6$ and $40.8 \pm 0.6\%$ for cheeses stored at 20 and 10°C, respectively. Nine organic acids (formic, orotic, uric, lactic, acetic, citric, pyruvic, propionic and butyric) were analyzed in each sample by HPLC. Samples of the same batch with four days of ripening and cheeses traditionally ripened (without packaging) were also studied. None of the factors affected the orotic and uric acid concentrations and butyric acid was not detected in any sample. The remaining acids content increased linearly with ripening time. Increasing storage temperature accelerated the maturation processes. The corresponding $Q_{10}$ for each acid was calculated as the ratio between the rate of increase at 20 and 10°C, obtaining values between 1.2 and 5.8. Three PCA factors interpreted 87% of the total variation in the samples. Discriminant analysis classified cheeses according to their age.



*Key Words*: Gouda cheese; organic acids; cheese ripening; principal component analysis; discriminant analysis.

## INTRODUCTION

Cheese acceptability by consumers depends mainly on its organoleptic properties, with flavor and aroma being two of them. Both properties are attributed to a complex combination of aromatic, volatile and non-volatile chemicals. Flavor profiles of cheeses are influenced by several substances such as organic acids, sulphur compounds, free amino-acids, lactones, methylketones, alcohols, and phenolic substances, etc. (Seitz, 1990; Urbach, 1993).

The basic reaction in cheesemaking is the production of lactic acid from lactose by starters. Carbohydrates are fermented via the hexose-diphosphate pathway to pyruvic acid. Lactic acid is then formed from pyruvic acid (Adda et al., 1982)

[1] To whom correspondence and reprint requests should be addressed. Fax: 54 221 425 4853. E-mail: anc@dalton.quimica.unlp.edu.ar

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A great variety of products such as proteases, peptides, amino-acids, ammoniac, hydrogen sulphide, volatile acids, aldehydes and ketones are accumulated in the cheese mass during ripening. These substances depend on the biochemical complexity of nitrogen substances of cheese, on proteolytic agents and are also generated by several metabolic routes of the degradative ripening process.

Fatty acids are essential elements in flavor and aroma of cheeses. Differences in fatty acids ratios are responsible for the characteristic flavor and aroma of the different cheese varieties. Short-chain fatty acids are associated with the lipolytic activity of bacteria (Green and Manning, 1982; Singh and Kristoffersen, 1970). Hough *et al.* (1996), working on Reggianito grating cheese, showed that propionic acid was a good indicator of flavor development.

Usually, lipolysis leads to the formation of free fatty acids. However, there are acids generated by other means, for example, propionic acid that is formed from lactic acid by propionic bacteria. Intracellular lipases excreted during the death of starter bacteria, for instance with a temperature increase, elevate butyric concentration (Grazier *et al*., 1993). Another possibility is the production of propionic acid from casein amino-acids, which would be an important source of volatile fatty acids (acetic to caproic acids). Moreover, butyric acid production may be originated not only from fats but also through proteolysis and subsequent deamination of caseins. The presence of free fatty acids of short chain like acetic, propionic or butyric acids is mainly related to the intensity of bacterial fermentation that took place during ripening. While free fatty acids of medium or large molecular weight are associated with the lipolysis of fat, the presence of volatile branched chain fatty acids, like isobutyric, isovaleric and isocaproic, supposes an important proteolytic degradation of caseins.

Some enzymes from the starters and others from the secondary flora induced during cheese elaboration are responsible for the chemical changes (Bhownik and Marth, 1988). The heterofermentative metabolism of lactose, by means of a flora different from the starter/secondary flora, may produce formic acid, acetic acid and ethanol (Law, 1984).

Heterofermentative lactobacilli metabolize citrate or induce oxidase enzyme activity; oxidase acts on NADH producing acetic acid, ethanol and other carbonylic compounds (Laleye *et al*., 1990). Citrate consumption may occur by more than one route, for example, some micro-organisms such as *Lactobacillus casei*, *L. plantarum* and *L. brevis* utilize citrate as energy source in the absence of carbohydrates. Citrate is converted to pyruvate and then used to generate the cellular components. Acetic acid produced by citrate consumption may indicate the heterofermentative characteristic of lactose.

Traditionally, Gouda Argentino cheese forms are ripened without packaging in chambers at 10°C for 5–7 days and are turned daily, until color changes of the rind are observed. Cheese is then moved to another chamber at 14–18°C for 40 days, to finish the ageing process. An alternative technique involves wrapping the unmatured cheeses in plastic films before ripening. Thus, cheeses without rind are produced (Fradin, 1984) and undesirable microbial and mold growth are prevented, avoiding the need of brushing and washing the surfaces before applying wax to the finished forms. Ageing the packaged cheese forms implies many economic benefits, such as reduced weight losses and fewer handling problems.

Bertola *et al.* (1998) previously studied ripening Gouda Argentino cheese in plastic films of low gaseous permeability, considering the degree of proteolysis and associated textural changes. They concluded that it was possible to obtain packaged forms with characteristics similar to the traditionally ripened ones. Nonprotein nitrogen and rheological parameters were time- and temperature-dependent. Although textural changes are the major events occurring during curing, equally important is flavor development, for the acceptability of cheese depends on both.

The objective of the present work was to investigate the effect of plastic-film type, ripening time and temperature on the changes in organic acids content (formic, orotic, uric, lactic, acetic, citric, pyruvic, propionic and butyric) during Gouda cheese ageing. Discriminant analysis was applied to our HPLC data to investigate whether classification of cheese forms of different ages could be done solely by organic acids content, as several authors have successfully done in other types of cheeses (Pham and Nakai, 1983; Bevilacqua and Califano, 1992; Lombardi *et al*., 1994; Califano and Bevilacqua, 1999).

## MATERIALS AND METHODS

### *Experimental Conditions*

Gouda cheeses from a local plant (Unión Gandarense, Argentina) were used. Cheeses were produced with pasteurized milk (3% fat, 1.3 g/cm$^3$ density) at 34°C, and starter containing *Streptococcus termophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. Chymosin derived by microbial fermentation was used.

After milk coagulation, curd was cut into 0.5 cm cubes, stirred and heated to 39°C for 25–35 min. Cooking time depended on seasonal characteristics of milk. Cheeses were pre-pressed under whey, cut, molded and pressed for 2.5 h. Salting was carried out by immersing cheeses in brine (8–10°C, pH = 5.2) for 48 h. Then, cheese loaves were stored at 10°C for 4 days and turned daily. At this stage, loaves were brought to our laboratory to begin the experiment.

Cheeses from the same batch were submitted to different ripening conditions according to a $2^3 \times 5$ factorial design, being the two-level factors:

(1) *Time of storage before packaging*: 4 and 10 days at 10°C.
(2) *Ripening temperature*: 10 and 20°C.
(3) *Packaging film*: BK1 and BK5 (Grace, Argentina), with oxygen permeabilities of 175 and 375 cm$^3$/m$^2$ day atm, respectively, and a water vapor permeability of 4 g/cm$^2$ day at 23°C for both films. After the corresponding storage period (4 or 10 days at 10°C), cheeses were vacuum packaged in BK1 or BK5 films; thermal shrinkage at 90°C for 10 s was applied.

Ripening time was the fourth factor considered; sampling was performed at 15, 25, 35, 49 and 70 days after production.

Samples of three loaves from the same batch with 4 days of ageing were analyzed to determine initial conditions.

Three cheeses were stored at 10°C for 8 days and then placed in ripening chamber at 16°C and 70–80% relative humidity for 20 days without packaging. At this time, they were waxed and ripened under traditional unpacked conditions for another 20 days at 10°C. After this time they were also analyzed. Usually, after painting, storage life of these cheeses is 90 days maximum if they are kept between 8 and 12°C.

In all cases, analyzed samples were obtained discarding external and central parts of the cheese.

### *Sample Preparation*

About 100 g of a representative cheese sample was ground (A-10 Analytical Mill, Tekmar, U.S.A.) and homogenized from each cheese. Fifty milliliters of 0.009 N

$H_2SO_4$ (mobile phase) was added to 7 g of ground cheese and extracted for 1 h with agitation on a shaker (Model 75, Burrell Scientific, Pittsburgh, PA, U.S.A.) and centrifuged at 7000 ***g*** for 5 min, according to a modification of the method by Bevilacqua and Califano (1989). The supernatant was filtered once through filter paper and twice through a 0.45 μm membrane filter (Millipore Waters Associates, SM N11306); 10 μl were injected. Two samples were analyzed for all cheese loaves.

*HPLC Analysis*

A Waters liquid chromatograph (Waters Associates, Milford, MA) was equipped with a model 717 autosampler, a model 600 controller, a photo-diode array UV–Vis detector (model 996), a column oven built in our Institute and a Data Module M730. The UV detector was set at 214 and 280 nm.

According to Bouzas *et al.* (1991), operating conditions were: mobile phase, 0.009 N $H_2SO_4$, filtered through 0.2 μm membrane filters (Millipore Waters Associates SM N11306) and degassed by sonication under vacuum; flow rate, 0.7 mL/min; and column temperature 65°C. A cation exchange (Aminex HPX — 87 H) column was used (Bio-Rad Laboratories, Richmond, CA).

Citric–orotic acids were not completely resolved under listed chromatographic conditions. The same happened with uric–formic acids. Thus, both orotic and uric acids were determined at 280 nm where citric and formic did not absorb and the mixture was resolved using both wavelength absorbances in an additive manner. An external standard method (Bevilacqua and Califano, 1989) was used.

*Moisture Content*

Samples were dried in a vacuum oven at 60°C to constant weight.

*Statistical Analysis*

Statistical analyses were carried out on the averages of the duplicate results. Two-way analysis of variance (ANOVA) and *post-hoc* multiple comparisons tests were carried out to study the effect of both freezing procedures and ripening time on the organic acid content. For simultaneous pair-wise comparisons, least significant difference (LSD) test was chosen. Difference in averages and F tests (ANOVA) were considered significant when the computed probabilities were less than 0.05 ($P < 0.05$). The ANOVA used was without replication.

Principal components analysis (PCA) was used to obtain a graphical description of the effects identified by the ANOVA. PCA is a well-known mathematical transformation of the raw data, an exploratory technique that indicates relationships among groups of variables and secondly shows relationships between objects (Pigott and Sharman, 1986). In this study, the data matrix can be visualized as describing a multi-dimensional space, with one dimension (variable) for each acid and each sample (object) can be represented as a point in space.

PCA generates a set of new orthogonal variables, the principal components, linear combinations of the original variables, so that the maximal amount of variance contained in the original data set is concentrated in the first principal components, reducing the number of variables. The successive linear combinations are extracted in such a way that they are uncorrelated with each other and account for successively smaller amounts of the total variation. In the present paper, the criteria of eigenvalue equal 1.0 have been used as the cut-off point. The component loadings are the correlations between the original and PCA transformed variables. These loadings

achieve a maximum value of one (or minus one) when the principal component and original variable are completely correlated. Once the number of principal components is determined, the sample scores are calculated for each component. These scores represent the sample in the new principal components space. The derived scores would then be used in later analyses in place of the original responses (Dillon and Goldstein, 1984a).

Discriminant analysis (DA) is a statistical technique for classifying individuals or objects into mutually exclusive and exhaustive groups on the basis of a set of independent variables. DA involves deriving linear combinations of the independent variables that will discriminate between the *a priori* defined groups in such a way that the misclassification error rates are minimized. This is accomplished by maximizing the between-group variance relative to the within-group variance. This methodology can be thought of in terms of a rather simple "scoring system" that assigns to each object in the sample a score that is essentially a weighted average of the object's values on the set of independent variables. Once a score is determined, it can be transformed into an *a posteriori* probability that gives the likelihood of the object belonging to each of the groups responses (Dillon and Goldstein, 1984b).

Discriminant analysis was applied to the data matrix (organic acids) considering each combination of (ripening time × temperature) as a group. To compute the actual discriminant functions, scores, and classification probabilities, the null hypothesis that the groups were equivalent was tested. Because the effects involved a categorical variable, the Mahalanobis distance and posterior probabilities were calculated. These distances were computed in the discriminant space itself. The closer a case was to a particular group's location in that space the more likely it was that it belonged to that group. The probability of group membership was computed from these distances by the code. The group classification coefficients and constants comprised the Fisher discriminant functions for classifying raw data. They were computed for each group of cheeses. For better visualization, the canonical scores were plotted in the discriminant space.

All statistical procedures were computed using the SYSTAT software (SYSTAT, Inc., Evenston, IL, U.S.A.).

## RESULTS AND DISCUSSION

Water content values of cheeses ripened at 20°C were lower than in cheeses ripened at 10°C due to the temperature dependence of the water diffusion coefficient. At the beginning of the experiment (4 days of ripening), mean water content of samples was $44.0 \pm 0.9\%$. At 70 days of ripening, humidities were $39.7 \pm 0.6$ and $40.8 \pm 0.6\%$ for cheeses stored at 20 and at 10°C, respectively (Fig. 1). Samples stored at the higher temperature showed some condensed water on the packaging film. Water content values were within the allowed range (36.0–44.0%) for this type of cheese according to the local regulations (Código Alimentario Argentino, 1996). There was no significant effect of the time of storage before packaging or the type of film on the water content of the loaves.

Butyric acid was not detected in any of the samples analyzed. Orotic and uric acid concentrations were not significantly affected by any of the factors studied, presenting a mean value of 3.1 and 3.7 ppm, respectively.

Neither the time of storage before packaging nor the packaging film presented a significant effect on the content of the six remaining acids (formic, lactic, acetic, citric and propionic). The concentration of each acid raised with ripening time, thus average concentration of total organic acids in the cheese forms showed an overall increase

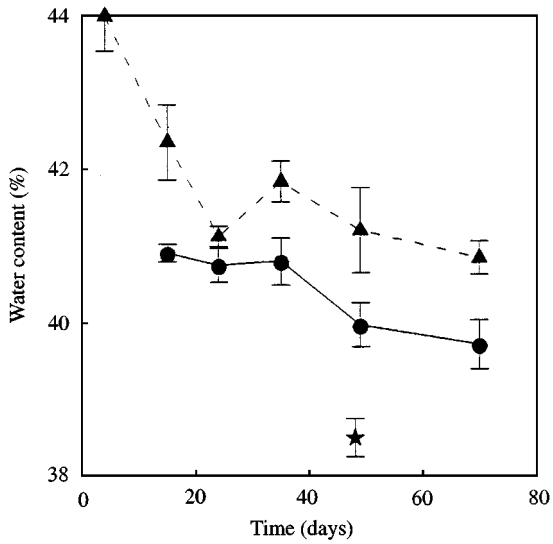


FIGURE 1. Effect of ripening time on water content for both temperatures. (▲) 10°C; (●) 20°C; ★ ripened unpackaged (traditional). Bars indicate S.E. of the mean.

along the different stages of ripening. Higher temperatures accelerated the rate of increase. Application of elevated ripening temperatures is one of the known procedures to obtain an accelerated ripening of cheese (Law, 1980; Fedrick and Dulley, 1984). After ageing for 70 days, the total organic acids content at 20°C was 44% greater than at 10°C. The temperature dependence varied with each acid, since different metabolic processes were involved. The corresponding $Q_{10}$ for each acid was calculated as the ratio between the rate of increase at 10 and 20°C. The obtained $Q_{10}$ values were: formic, 1.2; acetic, 1.5; pyruvic, 2.1; lactic, 3.1; citric acid, 3.5; and propionic, 5.8.

Lactic acid concentration initially accounted for about 89% of the total organic acid content since the primary purpose of a dairy starter culture is to produce lactic acid from lactose at a high rate in the early stages. Formation of lactic acid is essential for proper manufacturing, flavor development, normal ripening, and good keeping quality (Wong, 1974). The relative initial contribution of citric, acetic and propionic acid was 5, 3.3 and 1.8%, respectively. At the end of the experiment (70 days), ageing at 20°C has nearly doubled the total acid content and the relative contributions of each acid have been modified (lactic = 73%; citric = 7.8%; propionic = 9.8%; acetic = 5.7%). Ripening at 10°C has only increased the total organic acid concentration about 27% (lactic = 81%; citric = 6.3%; acetic = 6.1%; propionic = 3.3%).

The production of lactic, citric, acetic and pyruvic acids linearly increases with time and temperature (Fig. 2(a)–(d)). Formic acid content also rose linearly with time at 20°C, while at 10°C it decreased between 49 and 70 days (Fig. 2 (e)). Increases in formic and acetic acid content may be due to the homofermentative action of *S. termophilus* which produces formate, acetate and ethanol (Thomas, 1985), and the subsequent decrease of formic acid by the reversible reaction with acetyl-coA (Marth, 1974). The effect of rising the storage temperature on propionic acid content is remarkable (Fig. 2(f)). At 20°C propionic acid concentration increased exponentially during the ripening process.

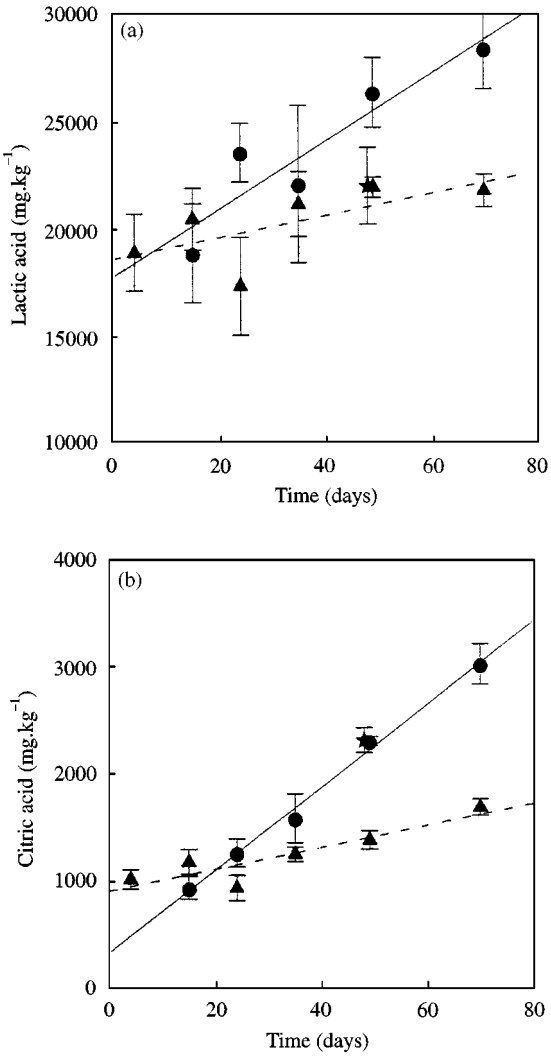


FIGURE 2. Changes in (a) lactic acid; (b) citric; (c) acetic; (d) pyruvic; (e) formic; (f) propionic concentration (ppm) during ripening. (▲) 10°C; (●) 20°C; ★ ripened unpackaged (traditional). Bars indicate standard error of the mean.

Results from PCA of the eight organic acids detected showed three interpretable factors that described about 86% of the total variation in the 41 samples analyzed (about 58, 17 and 11%, respectively). Loadings of the variables for factor 1, 2 and 3 are shown in Table 1. Factor 1 was heavily loaded on citric, pyruvic, acetic, propionic and lactic acids while factor 2 was mainly an orotic/uric factor. Factor 3 did not show a clear picture, with many acids having moderate loadings. Sample scores were calculated and an ANOVA of the sample scores of PCA factor 1 showed that both temperature and ripening time had a significant effect on the calculated scores, while neither the time of storage before packaging nor the packaging film affected the results. Figure 3 strongly indicates a linear correlation between PCA factor 1 and ripening time. The slopes of the scores versus time curves were estimated for 20 and

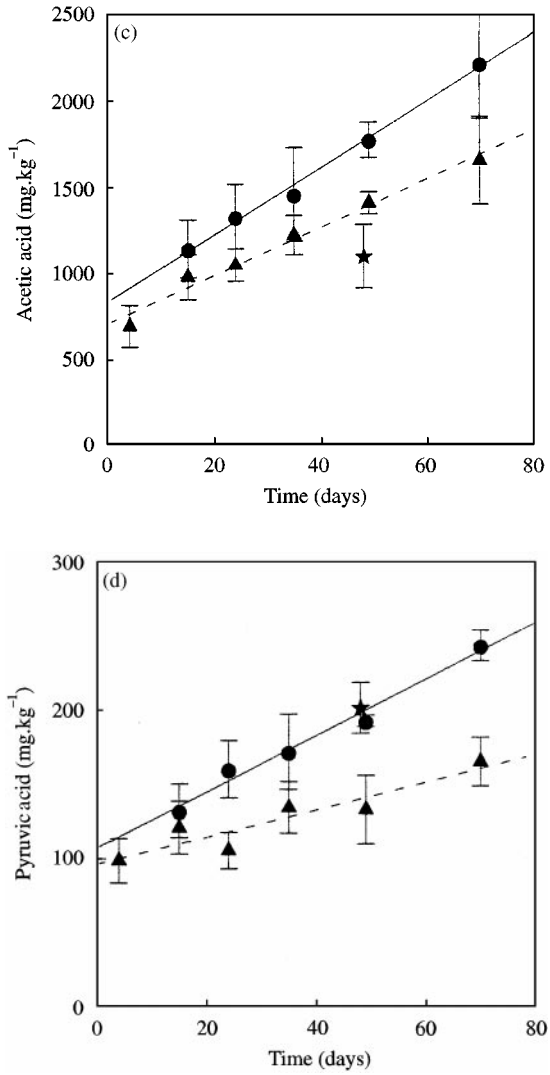


FIGURE 2. Changes in (a) lactic acid; (b) citric; (c) acetic; (d) pyruvic; (e) formic; (f) propionic concentration (ppm) during ripening. (▲) 10°C; (●) 20°C; ★ ripened unpackaged (traditional). Bars indicate standard error of the mean.

10°C. As they represented an overall rate of formation of the organic acids at each temperature, the quotient of both rates estimated the $Q_{10}$ ($Q_{10} \cong 2.5$).

Six organic acids (lactic, citric, propionic, acetic, formic and pyruvic) were the information for the discriminant analysis module of the SYSTAT program. The main difference between DA and PCA is that DA calculates factors which discriminate best between groups of samples, while PCA calculates factors which explain the greatest proportion of variance (Piggott, 1982). Each time–temperature combination was considered as a group, the type of film and time before packaging were not considered since their effect was not significant. The variables correctly classified 80% of the samples.

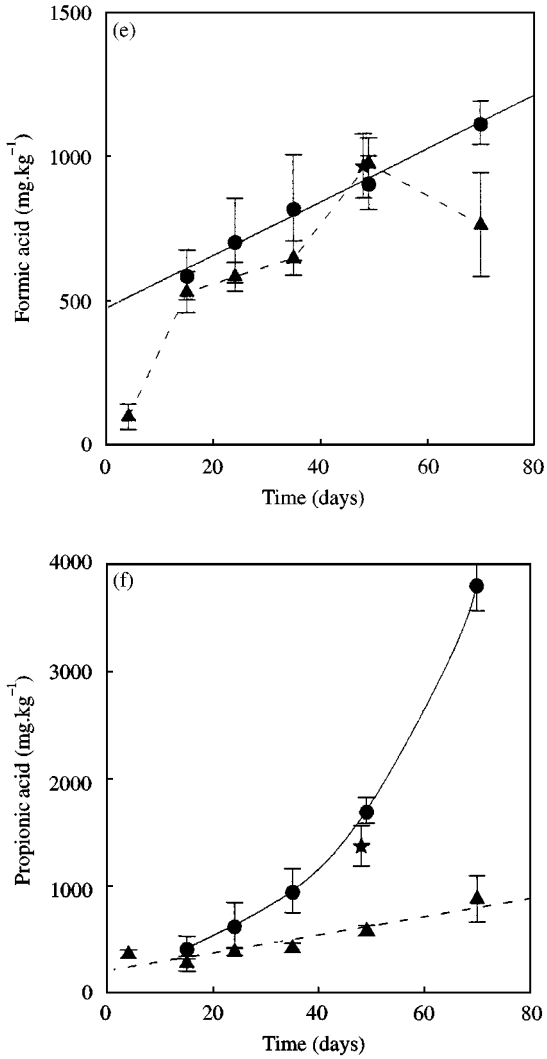


FIGURE 2. Continued.

TABLE 1

Component loadings of the variables considered in the PCA analysis, for factors 1, 2 and 3

| Variable | PCA factor 1 | PCA factor 2 | PCA factor 3 |
|---|---|---|---|
| Citric acid | 0.930 | 0.034 | − 0.205 |
| Orotic acid | 0.173 | 0.801 | 0.530 |
| Pyruvic acid | 0.916 | 0.036 | − 0.048 |
| Lactic acid | 0.853 | 0.252 | 0.175 |
| Formic acid | 0.794 | − 0.137 | 0.127 |
| Uric acid | 0.062 | 0.731 | − 0.648 |
| Acetic acid | 0.892 | − 0.160 | 0.144 |
| Propionic acid | 0.876 | − 0.243 | − 0.224 |

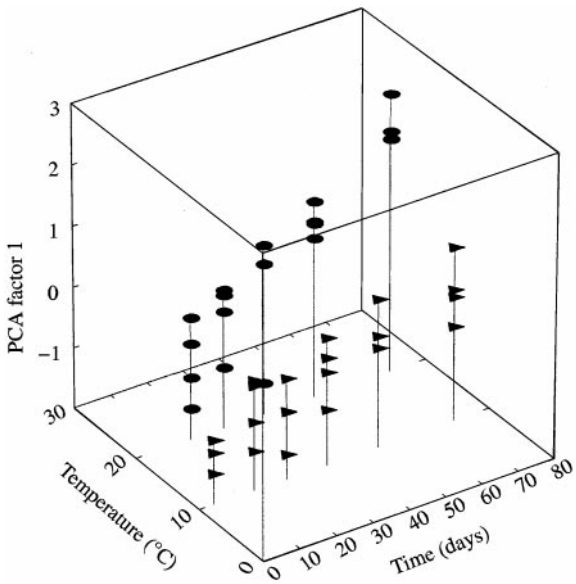


FIGURE 3. Samples scores on the first principal component (PCA factor 1) as a function of temperature and ageing time. (▲) 10°C; (●) 20°C

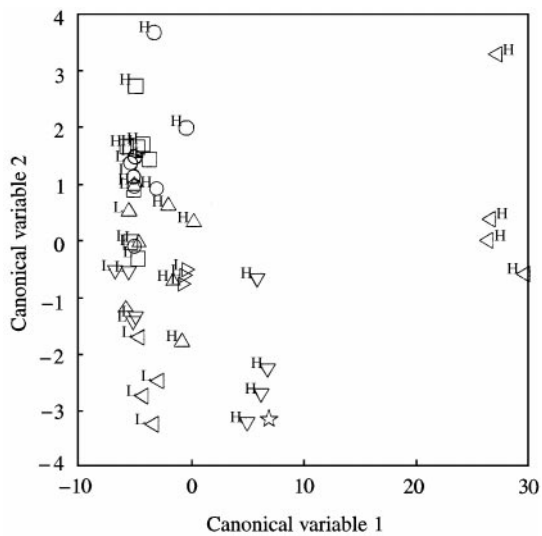


FIGURE 4. Canonical plot of Gouda cheese samples. Letters indicate ripening temperature: H, 20°C; L, 10°C. Symbols indicate ageing time: ▷, 5 days; □, 15 days; ○, 24 days; △, 35 days; ▽, 49 days; ◁, 70 days; ★ ripened unpackaged (traditional).

Canonical plots of all samples are shown in Figure 4. From Figure 4, it is clear that cheeses of each age–temperature group appeared closely located. Those ripened for 49 days at 20°C and for 70 days at both temperatures were distinctly separated from the rest. The average of canonical variables 1 and 2 for the samples that were ripened

according to the traditional procedure appears close to the high temperature-49 days group. For samples aged for 15 days, the two groups, 10 and 20°C, lay close to each other. Due to the scale employed, it is more difficult to observe the separation between the other groups. As ripening time increases, the storage temperature effect becomes more noticeable, and groups tend to appear more distant.

To conclude, it might be said that it is possible to ripe this type of cheese, wrapped in plastic film under vacuum, obtaining a product of good quality, similar to those aged by traditional procedures.

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