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Changes in organic acids during ripening of Port Salut Argentino cheese

A. E. Bevilacqua

Centro de Investigación y Desarrollo en Criotecnologia de Alimentos, Fac. Ingenieria, La Plata University, Argentina

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A. N. Califano*

Centro de Investigación y Desarrollo en Criotecnologia de Alimentos, Fac. Ciencias Exactas, La Plata University, 47 y 116, La Plata, 1900, Argentina

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Nine organic acids (formic, pyruvic, orotic, uric, lactic, acetic, citric, propionic and butyric) from 24 Port Salut Argentino cheese samples of different ages were analysed by high performance liquid chromatography. The level of total organic acids showed little change during the first 31 days, but it then increased rapidly up to the end of ripening. Lactic acid accounted for about 90% of the total organic acids content up to 31 days. Afterwards, the rapid increase in butyric acid level was responsible for the rise in total organic acids concentration. Each organic acid presented a characteristic pattern of change during ripening. Stepwise discriminant analysis classified cheeses according to their age. Discriminant functions were calculated for classifying unknown samples from their organic acids contents. Stepwise regression analysis allowed estimation of the ripening time of samples according to their organic acid levels.

INTRODUCTION

Cheese ripening is characterized by a series of complex physical, chemical and microbiological changes affecting the principal components of the cheese. It is well known that organic acid information is important in understanding metabolism and quality of milk products. The acids appear as a result of the hydrolysis of fatty acids, normal bovine metabolic processes, bacterial growth, or direct addition as acidulants. They are believed to contribute to the flavour of most aged cheeses (Adda *et al.*, 1982). Quantitative determinations of these acids in dairy products is important to flavour studies, for nutritional reasons and as an indicator of bacterial activity.

Port Salut Argentino cheese is the most important cheese variety manufactured in Argentina (Anon, 1982). Proteolysis and related textural changes during its ripening have been studied (Bertola *et al.*, 1989). In the present work, formic, acetic, pyruvic, propionic, uric, orotic, citric, lactic and butyric acids were analysed by reverse phase high performance liquid chromatography (HPLC) in Port Salut Argentino cheese samples during ripening. Both stepwise regression and discriminant analysis were applied to our HPLC data to investigate whether classification of this cheese could be done solely on the organic acids, since Pham & Nakai (1983) reported that stepwise discriminant analysis allowed classification of Cheddar cheese samples of different ages when considering HPLC profiles of water-soluble fractions. More recently, Alonso *et al.* (1987) used this statistical procedure to characterize frozen Cabrales cheese.

Manufacturing procedures differ between Port Salut Argentino producers: usually these are made from 3·0% fat milk and calcium chloride (0·025%); a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and 0·02% rennet extract are added to heat-treated milk (33°C). When the curd is sufficiently firm, it is simply cut, washed and moulded, and whey is drained by pressing under brine solution. Moulded cheese is

* To whom correspondence should be addressed.

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stored at 30°C until a pH of 5·2 to 5·3 is reached. It is then salted by immersing the moulds in brine solution for 4 h and stored in drying chambers, at 10°C, air relative humidity 58–60%. After 5 days it is wrapped with ethyl vinyl acetate (EVA) type film and stored at 10°C for ripening. This cheese is creamy white, soft (but firm to cut), slightly acid in flavour with a delicate aroma, and melts well under high heat.

MATERIALS AND METHODS

Sample preparation

A commercial brand of Port Salut Argentino cheese was used throughout the experiment. Twenty-four cheese samples were individually packaged in EVA–EVA film immediately after the drying storage, and stored at 10°C for different periods. The gaseous permeability of the film was PO_2 = 3732 cm^3 day^{-1} m^{-2} atm^{-1} and PCO_2 = 17012 cm^3 day^{-1} m^{-2} atm^{-1}.

At each established time, four packages were taken and analysed. A 7·0 g sample was added to 50·0 ml of buffer–acetonitrile mobile phase (0·5% (w/v) $(NH_4)_2PO_4$–0·2% (v/v) acetonitrile, at pH 2·24 with H_3PO_4), homogenized, extracted for 1 h and centrifuged at 7000 × g for 5 min. The supernatant was filtered once through filter paper and twice through a 0·45 μm membrane filter (Sartorius SM 11606); 10 μl was injected with a 25 μl Hamilton syringe (Hamilton Co., Reno, NV). Duplicate analyses were performed on all samples.

HPLC analysis

A Waters liquid chomatograph (Waters Associates, Milford, MA) was equipped with a model U6K injector fitted with a 20 μl sample loop, a Model 6000A solvent delivery system, a Model 450 variable wavelength detector and a Data Module M730. The detector was set at 214 nm.

According to Bevilacqua & Califano (1989), operating conditions were: mobile phase, aqueous 0·5% (w/v) $(NH_4)_2PO_4$–0·2% (v/v) acetonitrile (at pH 2·24 with (H_3PO_4); flow rate, 1·2 ml min^{-1}; ambient column temperature; and chart speed, 1 cm min^{-1}. A reverse phase Beckman C8 (250 × 4·6 mm), ultrasphere-octyl column with a 5 μm particle diameter was used. The mobile phase was prepared by dissolving analytical grade $(NH_4)_2PO_4$ in distilled water, HPLC grade acetonitrile and H_3PO_4. HPLC grade reagents were used as standards (Sigma Chemical Co., St. Louis, MO). Solvents were degassed under vacuum. Both solvents and standard solutions were filtered through 0·2 and 0·45 μm membrane filters (Sartorius SM 11607, SM 11606), respectively. Quantitation was based on the external standard method.

Moisture content

Samples were dried in a vacuum oven at 80°C to constant weight.

Data treatment

Stepwise regression analysis and stepwise discriminant analysis were carried out with the BMDP program (BMDP, 1989), using a Micro-Vax 2 computer.

The stepwise regression module sequentially adds (and/or deletes) a variable to an initial linear model and tests at each stage to see if the added variable is significant or not. At each stage in the computation, a decision is made as to whether to terminate the computer run with the current regression equation or to move to the next stage.

The stepwise discriminant analysis module seeks to describe the subspace that discriminates best between classes, i.e. that separates the objects in one class from the objects in the other classes as well as possible. This procedure works iteratively, including variables into a subset one at a time on the basis of maximizing the ratio of between to within group variation. Discriminant functions are calculated, which permit samples to be allocated to one of the groups.

RESULTS AND DISCUSSION

The water content of cheese samples was 51–52% throughout the experiment.

A typical high performance liquid chromatogram of Port Salut Argentino cheese is shown in Fig. 1. This study has shown great variability among samples, but some overall comments might be made. The values for total organic acids at different stages of ripening (Fig. 2) showed little change up to 31 days, but they showed a marked increase at the end of the ripening period (40 days). Lactic acid concentration accounted for about 90% of the total organic acid content up to 31 days. Afterwards, the rapid increase in butyric acid concentration was responsible for higher total organic acid content. Lactic acid content decreased 15% between 10 and 31 days, exhibiting a 20% increase towards the end of the experiment (Fig. 3(a)). Acetic, butyric and propionic acid concentrations rose as ripening progressed up to 16 days, remained fairly constant between 16 and 31 days, and increased again, although at different rates, thereafter (Fig. 3(b)).

The rise of acetic and butyric acids might be explained by butyric acid fermentation, which produces them at the expense of glucose. Propionic acid fermentation may account for the production of the corresponding acid plus acetic acid, at the expense of lactic acid. Butyric and propionic acid fermentations were the result of the presence of an abundant secondary micro-

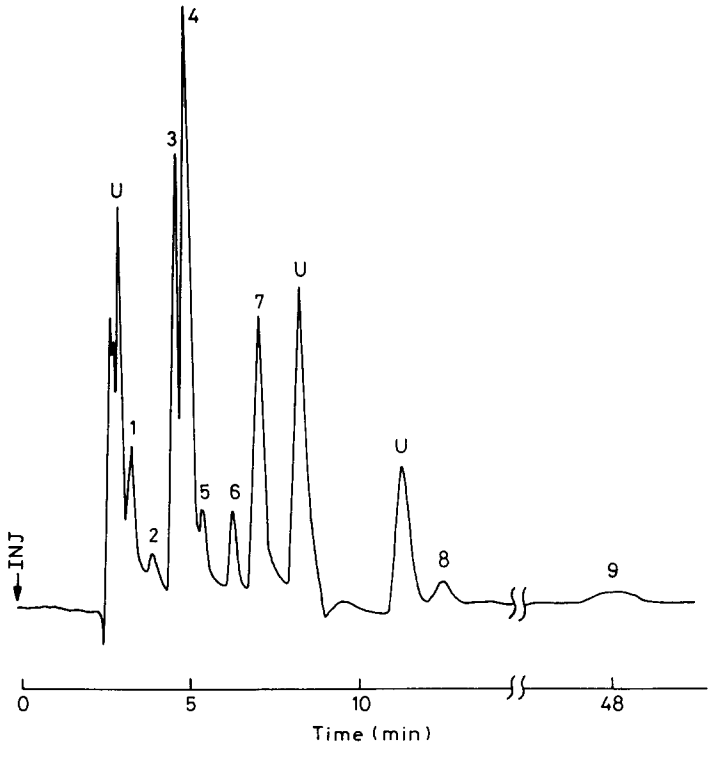

Fig. 1. Typical high performance liquid chromatogram of Port Salut Argentino cheese (16 days of ripening). The numbers correspond to the following acids: 1, formic; 2, pyruvic; 3, orotic; 4, lactic; 5, acetic; 6, uric; 7, citric; 8, propionic; 9, butyric; U, unidentified peak.

flora (Esponda *et al.*, 1983). Orotic, uric and citric acid contents decreased gradually between 16 and 31 days, remaining stable at the last stage of storage (Fig. 3(c)). Citrate can be used as a substrate by the starter to produce pyruvic and acetic acids (Adda *et al.*, 1982). Formic acid concentration increased gradually throughout the ripening period (Fig. 3(b)), as was expected due to the presence of *S. thermophilus* and *L. bulgaricus* (Thomas, 1985). Pyruvic acid showed irregular changes, presenting a minimum plateau between 24 and 31 days (Fig. 3(a)). Pyruvate can be used by lactic acid bacteria to produce diacetyl, according to a mechanism which is pH and oxygen dependent (Collins, 1972; Dwivedi, 1973).

In general, the level of individual organic acids remained constant between 16 and 31 days. Bertola *et al.* (1989) reported a similar lag-phase while studying protein breakdown by lactic microflora.

It is notable that this kind of cheese is used commercially before it is 40 days of age since consumers tend to reject it afterwards because of 'too acid flavour'.

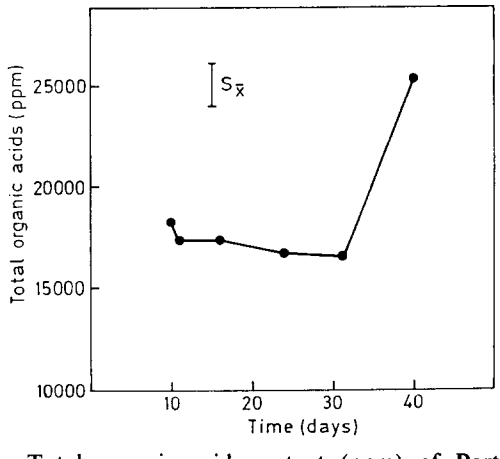

Fig. 2. Total organic acid content (ppm) of Port Salut Argentino cheese at different stages of ripening.

Statistical analysis

Nine acids of 24 samples and their age score were the information for the BMDP program. Samples were discriminated in seven groups (Table 1). Distribution of group means and canonical plots of all samples are shown in Fig. 4. The actual percentage of correct prediction estimated was 75%, when tested with six cheese samples not included in the data set, from which the discriminant function was derived. Uric and butyric acids were selected by the program to be the most effective for discrimination.

Seven linear discriminant functions were calculated for each group of cheeses (Table 1). Commercial samples of Port Salut Argentino cheese were used as unknown samples to verify the functions. By substituting acid contents of an unknown sample into these equations, its discriminant scores were calculated. The

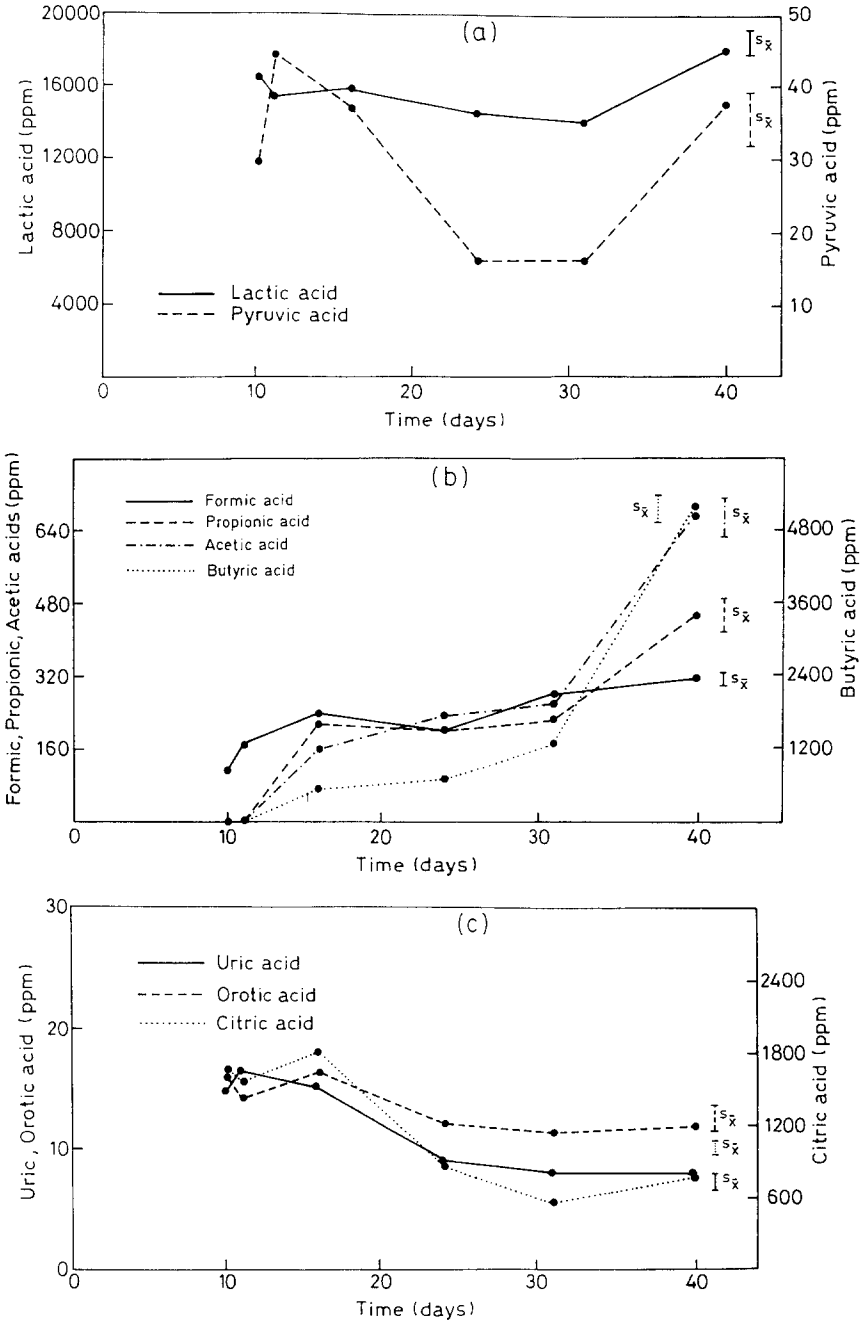

Fig 3. (a) Changes in lactic and pyruvic acid concentrations (ppm) during ripening; (b) accumulation of formic, acetic, propionic and butyric acids (ppm) during ripening; (c) changes in uric, orotic and citric acid concentrations (ppm) during ripening. Bars indicate the corresponding standard error of the means ($s_{\bar{x}}$).

unknown case was assigned to the group with the largest discriminant score. For better visualization of the location of the unknown sample within the original data set, its canonical variables were also calculated (Fig. 4).

Because ripening is a continuous phenomenon, groups may not be separated clearly. Groups A and B, 10 and 11 days, respectively, are not discriminated by the program. Figure 4 shows that three subsets may be considered according to their acid content: (1) young or fresh cheese, between 10 and 16 days of ripening, characterized for a decrease in uric, orotic and citric

Table 1. Linear discriminant functions computed ([*U*] = uric acid concentration and [*B*] = butyric acid concentration, both expressed in ppm).

Class	Ripening time (days)	Discriminant function
A	10	−16·68 + 1·99 [*U*] − 0·0032 [*B*]
B	11	−19·70 + 2·18 [*U*] − 0·0035 [*B*]
C	16	−15·11 + 1·84 [*U*] − 0·0018 [*B*]
D	24	− 6·33 + 1·06 [*U*] − 0·00076 [*B*]
E	31	− 5·18 + 0·774 [*U*] + 0·0005 [*B*]
F	40	−17·30 − 0·00884 [*U*] + 0·00643 [*B*]

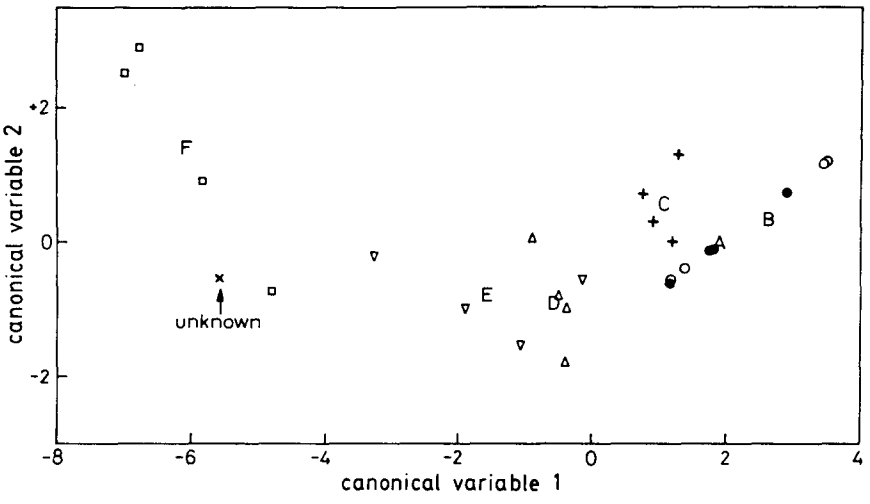

Fig. 4. Canonical plot of 24 Port Salut Argentino cheese samples. Letters indicate mean values of the class, symbols indicate the location of the samples: A, ●, 10 days; B, ○, 11 days; C, X, 16 days; D, Δ, 24 days; E, ▽, 31 days; F, □, 40 days.

acid contents and a small relative increase in propionic, formic, acetic and butyric acid concentrations; (2) middle-aged cheese, 24 to 31 days, which corresponds to a period where the individual concentration of organic acids remains fairly constant (lag-phase); (3) old cheese (40 days), samples of high total organic acid concentrations, mainly because of the increase in lactic, butyric, propionic and formic acid contents.

Formic, orotic, lactic, uric and butyric acid concentrations were found to be significant predictors of the ripening time by stepwise regression analysis of the organic acid data, when using a multiple variable linear model. The estimated regression equation was:

$$t = 0{\cdot}0369\,[F] - 0{\cdot}389\,[O] + 0{\cdot}678 \times 10^{-3}\,[L] - 1{\cdot}3\,[U] + 0{\cdot}204 \times 10^{-2}\,[B] + 21{\cdot}5$$

where: t = ripening time (days);
[F] = formic acid concentration (ppm);
[O] = orotic acid concentration (ppm);
[L] = lactic acid concentration (ppm);
[U] = uric acid concentration (ppm);
[B] = butyric acid concentration (ppm).

The correlation coefficient was 0·9603 and the relative standard error was 15%.

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