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Food Chemistry 64 (1999) 193–198

Food Chemistry

Freezing low moisture Mozzarella cheese: changes in organic acid content

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Received 11 July 1997; received in revised form and accepted 26 May 1998

Abstract

Vacuum-packaged low moisture Mozzarella cheese samples were ripened both by the traditional method at 4°C and after subjecting the samples to a freeze (−20°C)–thaw (4°C) cycle before ageing. Nine organic acids (formic, pyruvic, orotic, uric, lactic, acetic, citric, propionic and butyric) were analyzed each week by high performance liquid chromatography. There was no significant effect of the freeze–thaw cycle on the variations in organic acids content. They were only affected by ripening time. Each organic acid presented a characteristic pattern of change during ripening. Discriminant analysis classified cheeses according to their age.

Keywords: Cheese freezing; Cheese ripening; Organic acids

1. Introduction

Freezing is a suitable procedure to prolong stability and shelf-life of cheeses, although Fennema (1972) pointed out the lack of unanimity on the extent of damage to cheese caused by freezing. Damage depends on the type of cheese involved (composition, manufacturing procedures), the freezing conditions and the parameters chosen to evaluate the damage. Several authors have studied the changes in texture, melted functional properties and proteolysis of frozen Mozzarella cheese (Dahlstrom, 1978; Diefes, Rizvi, and Bartsh, 1993). Cervantes, Lund, and Olson, (1983) working on low moisture, part-skim Mozzarella cheese, concluded that, after one week of frozen storage, the samples were not significantly affected by freezing or thawing, as assessed by compression, beam bending and sensory evaluation. Bertola, Califano, Bevilacqua, and Zaritzky (1996 a,b) evaluated the effects of: (i) ripening low moisture Mozzarella cheese for various periods before freezing, or completing ageing time after thawing, (ii) fast or slow freezing rates, and (iii) frozen storage on functional properties and proteolysis of cheese. They considered the functional properties of both melted (meltability, apparent viscosity, free oil formation) and unmelted (texture profile analysis) cheese, comparing the results obtained in the freezing experiments with unfrozen cheese loaves of equal ageing time, stored at 4°C (controls). They concluded that Mozzarella could be frozen and then stored at −20°C without quality loss as long as the final product had been aged from 14 to 21 days before being consumed. The freezing rates of this experiment did not affect the obtained results.

Although textural properties of cheese are of paramount importance, equally important is flavour development, because the acceptability of cheese depends on both. The flavour profiles of cheeses are complex and are variety- or type-specific. These profiles are influenced by many substances, e.g. organic acids, sulphur compounds, lactones, methyl ketones and alcohols, phenolic substances (Seitz, 1990; Urbach, 1993). The concentration of free fatty acids, especially the shorter chain ones, is responsible for the characteristic cheese flavour (Singh & Kristoffersen, 1970; Green & Manning, 1982). Organic acids appear because of the hydrolysis of fatty acids, bacterial growth, normal bovine metabolic processes or direct addition of acidulants. No single substance or combination of them possesses a typical cheese flavour. It is believed that typical cheese flavour results from the blending of a variety of specific

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0308-8146/98/$—see front matter
PII: S0308-8146(98)00123-X

individual substances in adequate proportions (Wong, (1974)).

The primary role of the starter culture is to ferment lactose to lactic acid during cheese making. Acid production in the early stages of Mozzarella cheesemaking is largely due to *Streptococcus salivarius*, where as *Lactobacillus bulgaricus* becomes more important toward the end of manufacture (Kindstedt, 1993). In particular, organic acids contribute to the flavour of most aged cheeses. Hough, Califano, Bertola, Bevilacqua, Martinez, Vega, and Zaritzky, (1996), working on Reggianito grating cheese showed that the flavour descriptors: total intensity, cheesy, salty, tongue-tingling, hot and residual intensity were well predicted from organic acids using partial least-squares correlations (PLS). For this type of grating cheese, propionic acid was a good indicator of flavour development. Total aroma intensity was also well correlated by organic acids.

In the present work, formic, acetic, pyruvic, propionic, uric, orotic, citric, lactic and butyric acids were analyzed by high performance liquid chromatography (HPLC) in low moisture Mozzarella cheese samples ripened at 4°C for different periods and also in samples that were frozen, kept at −20°C for 6 days, thawed and tempered at 4°C to complete the same ageing times. These experiments allowed us to characterize the changes in organic acids content along refrigerated ripening and also to study the effect of freezing on the organic acids composition of low moisture Mozzarella cheese. Discriminant analysis was applied to our HPLC data to investigate whether classification could be done solely by organic acids, since we have used this statistical procedure successfully before to characterize Port Salut Argentino and Reggianito cheeses (Bevilacqua & Califano, 1992; Lombardi, Bevilacqua, & Califano, 1994).

2. Materials and methods

2.1. Experimental conditions

A commercial brand of low moisture Mozzarella produced at a local dairy plant according to standard practices described elsewhere (Bertola et al., 1996a) was used through the experiment. A representative wedge was taken from ten cheese loaves aged for 3 days to analyse the initial organic acid content. Immediately afterwards each loaf was divided into six samples that were immediately vacuum-packaged with BK1 film (EVA/Saran/EVA, Grace Argentina). The gaseous permeability of the film was $pO_2 = 150$ cm^3 day^{-1} m^{-2} atm^{-1} and $pCO_2 = 1000$ cm^3 day^{-1} m^{-2} atm^{-1}. Three samples from each loaf were refrigerated at 4°C for different ageing times (6, 13, 20, 27, 34 or 41 days) while the remaining samples were frozen and kept at −20°C for 6 days. After thawing they were stored at 4°C to reach the desired ripening times.

The refrigerated or frozen procedures were considered as the two levels of one treatment factor (ripening scheme) and ripening time was the second treatment factor. Since six ripening times were chosen, a complete block design would have required 12 samples from each loaf. Thus the assignment of treatments to the samples corresponded to a balanced incomplete block design as shown in Table 1 (Box, Hunter, & Hunter, 1978).

2.2. Sample preparation

About 100 g of a representative cheese sample was ground (A-10 Analytical Mill, Tekmar, USA) and homogenized from each cheese. Fifty millilitres 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, USA) and centrifuged at 7000 × ***g*** for 5 min, according to a modification of the method of 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 was injected. Duplicate analyses were performed on all samples.

2.3. 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

Table 1
Assignment of ageing times (treatments) to the samples according to a balanced incomplete block design. 'Control' refers to the samples aged at 4°C without being subject to the freeze–thaw cycle

Cheese loaf	Control (4°C)	Freeze–thaw cycle
	Ageing time (days)	
A	6, 13, 20	6, 13, 20
B	6, 27, 34	6, 27, 34
C	6, 13, 41	6, 13, 41
D	13, 27, 34	13, 27, 34
E	20, 27, 41	20, 27, 41
F	13, 20, 27	13, 20, 27
G	20, 34, 41	20, 34, 41
H	13, 34, 41	13, 34, 41
I	6, 20, 34	6, 20, 34
J	6, 27, 41	6, 27, 41

vacuum; flow rate, 0.7 ml min^{-1}; 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 & Califano, 1989) was used.

2.4. Moisture content

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

2.5. Statistical analysis

Statistical analyses were carried out on the averages of the duplicate results. Two-way multivariate analysis of variance (MANOVA) and post-hoc multiple comparison tests were carried out to study the effect of both freezing procedures and ripening time on the organic acid content. The treatment averages were adjusted to allow for the fact that not every treatment occurs in every block (Box et al., 1978). For simultaneous pairwise comparisons the Least Significant Difference (LSD) test was chosen. Differences in averages and F tests were considered significant when the computed probabilities were less then 0.05.

To gain insight into the structure of the data set, Principal Components Analysis (PCA) was performed. PCA is a well-known mathematical transformation of the raw data; it is an exploratory technique that indicates relationships among groups of variables and secondly shows relationships between objects (Piggott & Sharman, 1986). 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.

Discriminant analysis (DA) was applied to the data matrix (organic acids) considering each ripening time as a group. To compute the actual discriminant function, 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. For better visualization, the canonical scores were plotted in the discriminant space. The group classification coefficients and constants comprised the Fisher discriminant functions for classifying raw data. They were computed for each group of cheeses.

All statistical procedures were computed using the SYSTAT software (SYSTAT, Inc., Evanston, IL, USA).

3. Results and discussion

Cheese loaves had an average water content of $47.2 \pm 0.3\%$.

Statistical analysis did not show significant differences among the acid contents of the refrigerated and frozen groups, except at certain ageing times where the interaction (ripening scheme × maturing time) was significant.

Lactic acid concentration initially (3 days) accounted for about 79.3% 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. The glycolysis of lactose to lactic acid requires about 14 enzymatic steps. Formation of lactic acid is essential for proper manufacture, flavour development, normal ripening,and good keeping quality (Wong, 1974). Ageing time affected lactic acid content and the interaction (ageing time × ripening scheme) was also significant because of the differences in behaviour found between 20 and 34 days of ripening [Fig. 1(a)]. The concentration increased at 20 days, decreasing 1 week later in the frozen samples. In the refrigerated controls the maximum appeared at 27 days; the concentration decreased sharply at 34 days, reaching the same values of the frozen cheese at 41 days, where it accounted for 72.3% of the total organic acid concentration. An analogous trend was observed during ripening of Reggianito cheese in air-tight sealed bags (Lombardi et al., 1994). Uric and formic acids also presented a similar pattern of changes, with a maximum between 20 and 27 days, but the acid contents of the refrigerated group did not differ from the frozen samples [Fig. 1(b)]. The increase in formic acid concentration might be explained by the presence of *S. salivarius* ssp. *thermophilus* which also produces formic acid from lactose (Thomas, 1985), and its subsequent decrease by the reversible reaction with acetyl-CoA (Marth, 1974).

Citric acid initially (3 days) represented 9.1% of the total (1370 mg kg^{-1}). It sharply rose in the following 3 days of ripening, nearly doubling its content. The frozen samples presented a maximum in citric concentration at 13 days of ageing with a slight tendency to decrease at longer maturing times, although the differences were not statistically significant. The concentration in the refrigerated controls raised at 20 days and was minimum after maturing for 34 days [Fig. 1(c)]. Citrate is involved in the Krebs or citric acid cycle where it acts both as substrate and product.

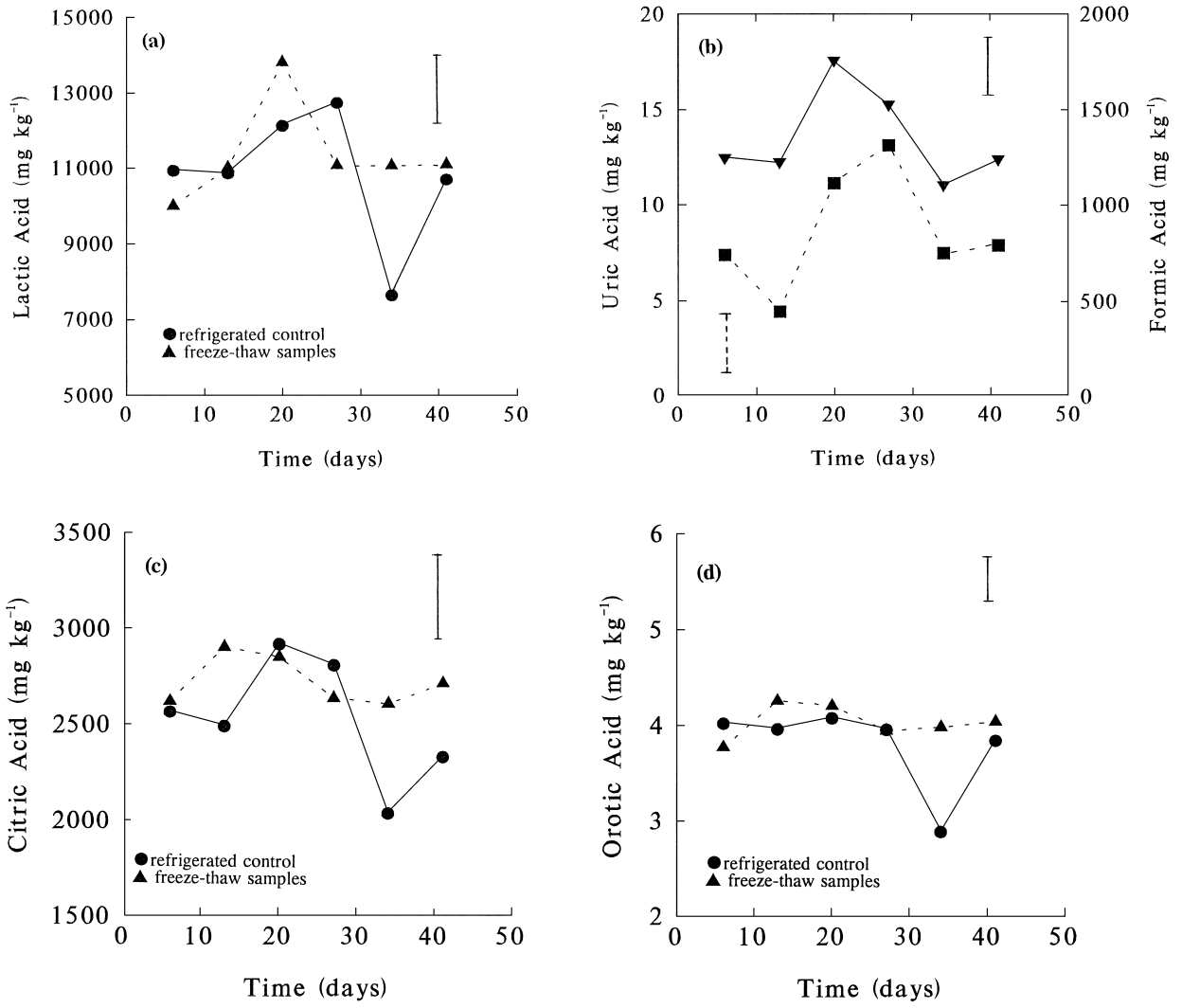

Fig. 1. Changes in (a) lactic acid (mg kg^{-1}); (b) uric (■) and formic (▼) (mg kg^{-1}); (c) citric (mg kg^{-1}); (d) orotic acid concentration (mg kg^{-1}) during ripening. Bars indicate the corresponding least significant difference (LSD) for each acid ($p < 0.05$).

Pyruvic acid content decreased 80% between the third and sixth days of ripening, reaching and average concentration of 36 ± 9 mg kg^{-1} that did not change significantly thereafter. Pyruvate is readily formed through the glycolytic pathway, but it also acts as substrate of several metabolic reactions such as the formation of formic acid, ethanol and diacetyl, acetoin and 2,3-butylene glycol (Marth, 1974). Orotic acid content did not significantly change during ripening, except for the refrigerated group aged for 34 days [Fig. 1(d)]. Acetic and propionic acids were not detected as was expected, because the starter consisted mainly of *S. salivarius* ssp. *thermophilus* which is a homofermentative microorganism. Butyric acid only appeared in a few samples, in a random pattern, never exceeding 700 mg kg^{-1}, which suggests the presence of non starter bacteria.

Results from PCA of the seven organic acids showed three interpretable factors that described about 82% of the total variation in the 60 samples (about 47, 23 and 12%, respectively). Loadings of the variables for factor 1 and 2 are shown in Fig. 2 after Varimax rotation. Factor 1 was heavily loaded on lactic, orotic, citric and formic acids while factor 2 was mainly a butyric/uric factor. Factor 3 did not show a clear picture, with many acids having moderate loadings (Fig. 3). The objects (samples) were plotted in a three-dimensional space which can be visualized as a three-dimensional projection of the original multi-dimensional space. Inspection of sample scores in Fig. 4 showed that there is no clear-cut distinction between the refrigerated and frozen groups. Furthermore, by applying an ANOVA to the sample scores of each PCA component, only ripening time produced a significant effect, while frozen the samples did not affect the results.

Seven acids and their age scores were the information for the discriminant analysis module of the SYSTAT

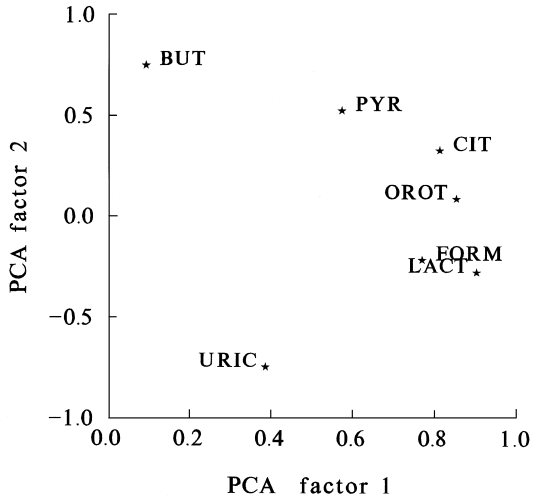

Fig. 2. Loadings of the seven organic acids detected (abbreviated using upper case letters) on the first and second principal components.

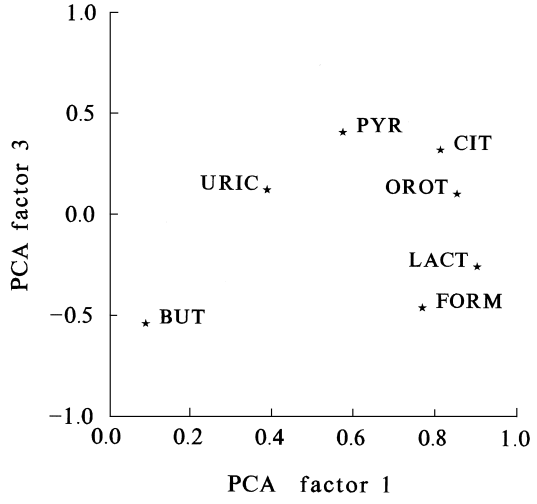

Fig. 3. Loadings of the seven organic acids detected (abbreviated using upper case letters) on the first and third principal components.

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).

The different storing procedures were not considered since their effect was not significant in most cases. Samples were discriminated into three groups according to their ripening time. Group A corresponded to samples aged between 6 and 13 days. Group B comprised samples ripened between 20 and 27 days. Samples matured between 34 and 41 days belonged to group C. Group B corresponds to a period of high lactic, formic, citric and uric content. Canonical plots of all samples are shown in Fig. 5. The variables correctly classified 80% of the samples.

By organic acid composition alone, the Mozzarella samples that were frozen before complete ripening did not differ significantly from the refrigerated control group of the same ageing time.

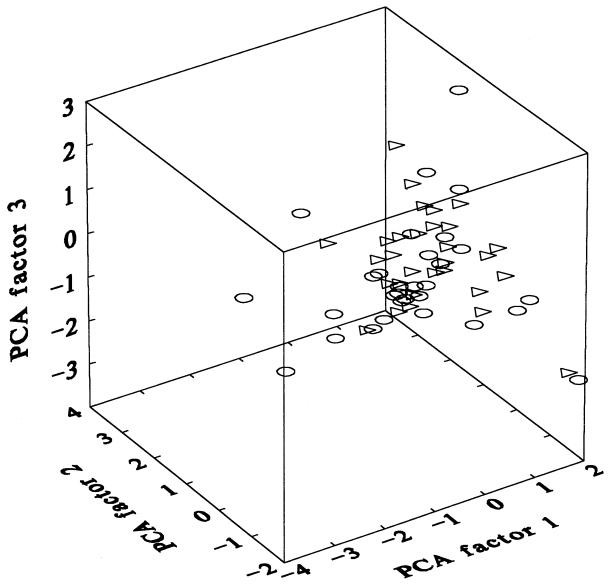

Fig. 4. Sample scores on the first, second and third principal components. ○ corresponds to the refrigerated control; △ indicates samples that were subjected to a freeze–thaw cycle before ageing.

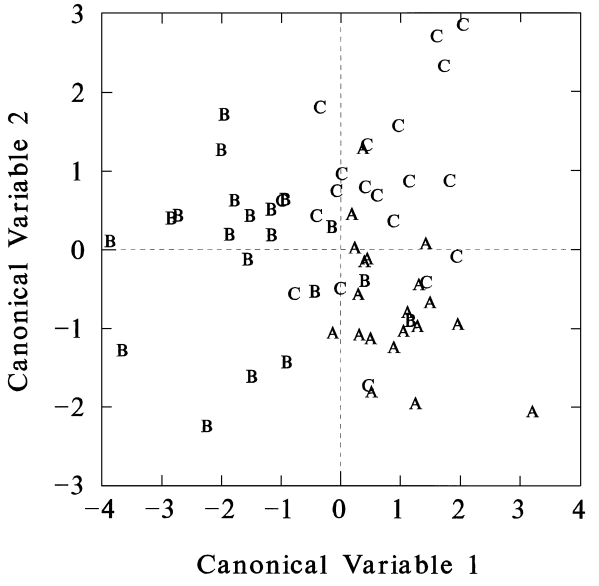

Fig. 5. Canonical plot of 60 Mozzarella cheese samples. Letters indicate ripening time: A, 6–13 days; B, 20–27 days; C, 34–41 days.

Acknowledgements

The authors acknowledge the financial support for this project from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Unión Gandarense Inc. and Grace Argentina for donating cheese samples and plastic films, respectively.

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