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Variation in organic acids content during ripening of Reggianito cheese in air-tight sealed bags

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Reggianito cheese forms were ripened both by the traditional method and by vacuum-packaging the forms in film after initial ageing periods of 30 and 45 days. Nine organic acids (formic, pyruvic, orotic, uric, lactic, acetic, citric, propionic and butyric) and lactose were analyzed each month by high-performance liquid chromatography. Wrapped cheese forms lost less weight than the unwrapped ones. Residual lactose content remained fairly constant during the experiment. Only citric and uric acid contents varied significantly between unwrapped and packaged forms. The level of total organic acids showed an increase along the ripening period, but its composition varied along the process. Initially, lactic acid accounted for 77% of the total, after 10 months propionic and butyric acids constituted 42 and 23% of the total respectively. Each organic acid presented a characteristic pattern of change during ripening. Discriminant analysis classified cheeses according to their age. Stepwise regression analysis allowed estimation of the ripening time of samples according to their organic acids levels.

INTRODUCTION

Cheese ripening involves a series of complex physical, chemical and microbiological changes affecting the principal components of the cheese. Attention has been given to the mechanisms by which cheese curds become distinctively flavoured cheeses, but maturation processes are still less than well understood for most cheeses as a consequence of the heterogeneous nature of the product. There are numerous active microbial enzymes present together with the metabolites; the changes in ripening and maturation depend on the biochemical conditions of the curd, i.e. water activity, pH, oxidation–reduction potential, mineral contents and several other factors such as the ripening temperature, the level and method of salt addition and the nature of the secondary microflora (Law, 1984).

The basic reaction in cheese making is the production of lactic acid by starters. Carbohydrates are fermented via the well-known hexose-diphosphate pathway to pyruvic acid. Lactic acid is then formed from pyruvic acid, which acts here as a hydrogen acceptor, so that the reduced NAD can be reoxidised for a further oxidation of glucose (Adda *et al.*, 1982).

In particular, organic acids contribute to the flavour of most aged cheeses. Bevilacqua and Califano (1992) showed that Port Salut Argentino cheese samples of different ages could be classified solely by the organic acids content.

Moreover, Romano, Provolone and Parmesan cheese have a sharp, peppery 'piccante' flavour primarily due to relatively high levels of short-chain fatty acids, especially butyric (Guinee & Fox, 1987).

Reggianito cheese is the most important hard cheese variety manufactured in and exported from Argentina (about 3500 tons per annum in the last decade). The name Reggianito is given to a domestic Grana cheese, usually used grated as a food ingredient.

For economic and consumer preference reasons, this cheese and its manufacture have evolved somewhat differently than in Italy, although flavour and texture are essentially similar. Argentinian Reggianito is smaller, 25 cm diameter × 15 cm height, about 7 kg versus 30 kg. It has an average fat content of 35-40% and 3.5–4.0% of salt on a dry weight basis.

Quantitative determination of organic acids and lactose could be used to understand microbial metabolism, monitor starter culture activity and follow quality changes during cheese maturation.

Traditionally, Reggianito cheese forms are ripened at 12°C in store-houses and at the end of the ripening period (6 months), because of mould formation, their surfaces are scraped and black wax is applied on to the cheese forms. An alternative procedure involves placing

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the forms in plastic film bags, evacuating, heat shrinking, sealing the bags and then ripening. These new techniques are useful because of the many economic benefits, such as reduced weight losses and fewer handling problems.

The objective of the present work was to investigate the effect of vacuum-packaging on the changes in organic acids content (formic, pyruvic, orotic, uric, lactic, acetic, citric, propionic, and butyric) and lactose during Reggianito cheese aging. Discriminant analysis was applied to high-performance liquid chromatography (HPLC) data to investigate whether classification of this cheese could be done solely by the organic acids, since several authors reported that discriminant analysis allowed classification of other types of cheeses of different ages when considering HPLC profiles. (Pham & Nakai, 1983; Alonso *et al*, 1987; Bevilacqua & Califano, 1992).

MATERIALS AND METHODS

Cheesemaking operations

Twenty-seven cheeses were produced at a local dairy plant (Cabañas y Estancias Santa Rosa S. A.) according to the following procedure: pasteurised milk of 2.6% fat was cooled to 32°C and pumped into a cheese vat. Whey starter (3%) was added, along with 0·2% $CaCl_2$ and 0·035% commercial bovine rennet. Cheeses were produced in one batch to minimize variability between cheese forms.

The starter consisted mainly of *Lactobacillus bulgaricus, L. helveticus* and *L. fermenti*. To a lesser extent it also contained *Streptococcus lactis, L. plantarum, Propionibacterium shermanii* and *P. globosum*.

When the curd was sufficiently firm, it was cut and held at 50°C for a given time. Afterwards, the curd was moulded, and whey was drained by pressing. The drained cheeses were placed in brine for 10 days, and they were stored in the curing room (15-17°C and 85–90% relative humidity).

Experimental Conditions

Three cheeses were analysed before ripening, at the beginning of the experiment. Nine cheeses were ripened in the traditional way, turning them periodically. They were considered as a control group (T). The remaining forms were divided in two groups. Eight cheeses were wrapped with BK1 film (Grace Argentina) after 30 days of ripening (W30). The remaining seven cheeses were wrapped after ageing for 45 days (W45).

In every case, the plastic bags were evacuated, heat shrunk and sealed. All the cheese forms were left to ripen in the same cold chamber and one cheese from each group was analysed every month over an 8 month period.

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}.

Sample preparation

About 100 g of a representative cheese sample was ground (A-10 Analytical Mill, Tekmar, USA) and homogenised from each cheese. Fifty ml of 0·009 N H_2SO_4 (mobile phase) was added to 7g of ground cheese and extracted for 1 h with agitation on a shaker (Rolco S.R.L.) 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 with a 25 μl Hamilton syringe (Hamilton Co., Reno, NV, USA). Duplicate analyses were performed on all samples.

HPLC analysis

A liquid chromatograph (Waters Associates, Milford, MA, USA) U6K injector; 6000 A solvent delivery system; 450 variable wavelength detector and differential refractometer R401 in series; and Data Module M730 was operated 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^{-1} a cation-exchange (Aminex HPX-87 H Bio-Rad Laboratories, Richmond, CA, USA) column at 65°C; UV detector at 214 or 280 nm.

Lactose and citric-orotic acids coeluted under listed chromatographic conditions. The same happened with uric-formic acids. Thus both orotic and uric acids were determined at 280 nm where lactose, citric and formic did not absorb. As the refractive index, and UV–visible detectors sensed both lactose and citric acid in an additive manner, the mixture was resolved using both detectors. An external standard method (Bevilacqua & Califano, 1989) was used.

Moisture content

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

Statistical analysis

Two-way multivariate analysis of variance (MANOVA) and post-hoc multiple comparisons test were carried out in order to study the effect of both the packaging procedures and the ripening time on the organic acid content. For simultaneous pairwise comparisons Tukey's test was chosen since these simultaneous procedures are designed to maintain the overall protection level themselves (Wilkinson, 1990). Differences in means and F tests were considered significant when the computed probabilities were less than 0·05.

Discriminant analysis was applied to the matrix of data (organic acids and lactose content), regardless of the packaging procedure and 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 visualisation, 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., Evenston, IL, USA).

RESULTS AND DISCUSSION

Visible mould was not observed in any of the packaged cheeses.

Initial water content of the samples was 40·6%, which decreased during ripening. Forms left unwrapped (T) reached 32·1% after 255 days of storage and 25·1% at the end of the experiment (290 days). Cheese forms wrapped in plastic film, both W30 and W45 groups, lost significantly less moisture than the T group, reaching values of 33·6% and 29·7% after 255 and 290 days, respectively (Fig. 1) ($s_{\bar{x}}$ = 0·15%). All the groups fulfilled the Argentinian regulation in terms of water content after 6 months (27–35%).

Lactose content was not significantly affected either by the ripening period or the type of wrapping (T, W30 or W45), presenting a mean value of 4·9 mg g^{-1} cheese ($s_{\bar{x}}$ = 0·3 mg g^{-1}). The residual concentration of lactose depends, among other factors, upon metabolic activity of the lactic acid bacteria, the amount of lactose removed during cheese manufacturing and the NaCl concentration of cheese (Olson, 1990).

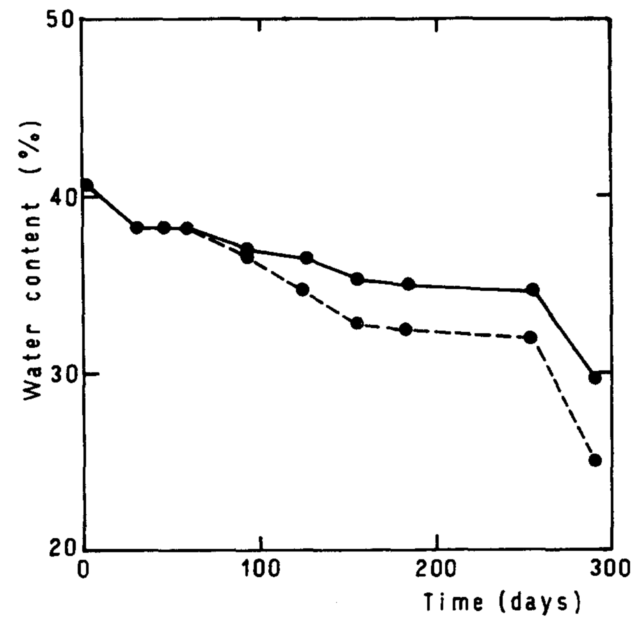

Fig. 1. Water content (%) of Reggianito cheese at different stages of ripening. (– – – –) Unwrapped cheese forms (T); (——) cheese forms wrapped in plastic film after 30 and 45 days of ripening (W30 and W45, respectively).

Average concentration of total organic acids in the cheese form showed an overall increase along the different stages of ripening (Fig. 2), but the type of wrapping did not affect these results. Lactic acid concentration initially accounted for about 77% 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 lactic acid acting to inhibit contaminants (i.e. coliforms) (Scott, 1985). After ageing for 3 months, lactic acid represented 58% of the total acids, and 34% after ripening for 255 days. Butyric and propionic acids represented 16% and 1·4%, respectively, of the initial total organic acid concentration. After ripening for 6 months, they accounted for 26% and 37% of the total, and at the end of the experiment propionic acid constituted 42% of the total. Butyric, propionic and orotic acid concentrations rose as ripening progressed, although at different rates. Both orotic and butyric remained fairly constant after ageing for 4 months (Fig. 3(a)), Propionibacteria produced propionic and acetic salts through propionate fermentation which involves a complex double cycle via pyruvate (Law, 1984). Furthermore, as the milk globule membrane had been destroyed during the process, fat was probably attacked by the rennet lipases, which released fatty acids, such as butyric from triglycerides (Caboni *et al.*, 1990). Arnold *et al* (1975) found a direct relationship between the flavour intensity of commercial Romano cheese and butyric acid content.

Formic acid was formed during the first 46 days of ripening, decreasing gradually thereafter. Thomas (1985) showed that, when the sugar supply is limited, a large proportion of sugar is fermented to formate, acetate and ethanol by homofermentative lactic streptococci. Acetic acid showed irregular changes with a maximum content around 2 months of ageing and a minimum after maturing for 6 months (Fig. 3(b)).

Lactic acid content presented a maximum plateau

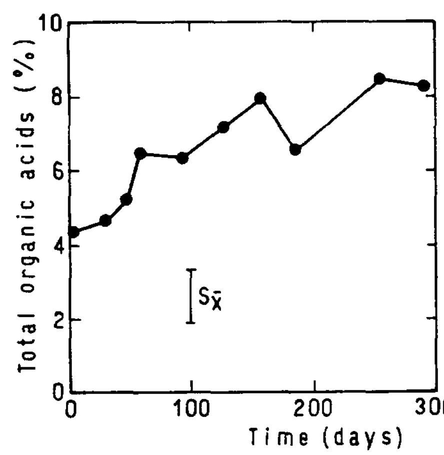

Fig. 2. Changes in the total organic acid content (%) of Reggianito cheese during ripening. Bar indicates the corresponding standard error of the means ($s_{\bar{x}}$).

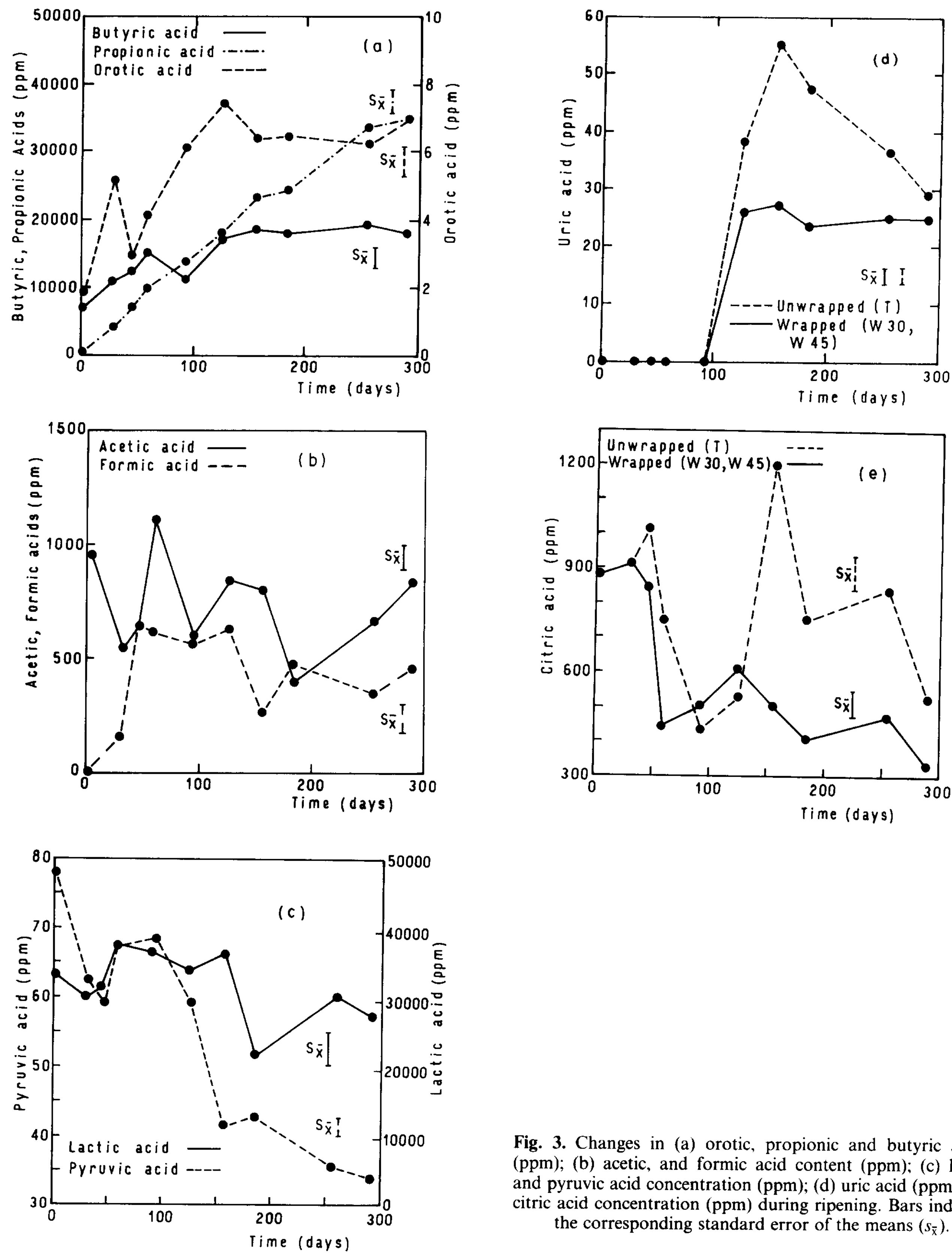

Fig. 3. Changes in (a) orotic, propionic and butyric acids (ppm); (b) acetic, and formic acid content (ppm); (c) lactic and pyruvic acid concentration (ppm); (d) uric acid (ppm); (e) citric acid concentration (ppm) during ripening. Bars indicate the corresponding standard error of the means ($s_{\bar{x}}$).

between 2 and 5 months of ageing, a minimum around 6 months, increasing towards the end of the experiment. Pyruvic acid content decreased along the ageing process (Fig. 3(c)). Pyruvate is readily formed through the glycolytic pathway, but it also acts as substrate of several metabolic reactions.

Statistical analysis did not show significant differences among the wrapped and unwrapped groups (T, W30 and W45) for formic, butyric, propionic, orotic, pyruvic, acetic and lactic acids at a given time. On the other hand, uric and citric acid contents presented significant differences between the unwrapped cheese forms (T) and the samples packaged in plastic film (W30 and W45), although the two wrapped groups did not differ between themselves. Uric acid was not detected during the first 3 months of storage, rising rapidly afterwards. The packaged forms (W30 and W45) reached a maximum at about 4 months of ripening, remaining fairly constant thereafter. The forms left unwrapped (T) increased their uric concentration up to 4 months, reaching a maximum that doubled the amounts in the W30 and W45 groups, and decreased rapidly at the last stage of storage (Fig. 3(d)). Citric acid content diminished sharply between 46 and 59 days of ripening. Afterwards it fluctuated, with a slight tendency to decrease (Fig. 3(e)). This decrease might be explained by citrate metabolism. Although metabolism of citrate is important only in mesophilic cultures, Bottazzi and Dellaglio (1967) and Groux (1973) reported that several strains of *Str. thermophilus* produced traces of diacetyl in milk, inferring that citrate might have been metabolized.

Nine acids and lactose of 27 cheeses and their age score were the information for the discriminant analysis module of the SYSTAT program. The different packagings tested were not considered since their effect was not significant for most of the compounds analysed. Samples were discriminated into nine groups according to their ripening time. Canonical plots of all samples are shown in Fig. 4. It is clear that in this figure, T, W30 and W45 cheeses of each age group appeared closely located, thus confirming the hypothesis that the packaged forms did not differ from the unpackaged ones in their behaviour with respect to their acid and lactose content. Because ripening is a continuous phenomenon, groups may not be separated clearly. Groups I and J, 255 and 290 days, respectively, are not discriminated by the program, as T(J) was assigned to group I. Figure 4 shows that four subsets may be considered according to their acid content: (1) cheese forms just after coming out of the brine (A); (2) young cheeses, 31 to 93 days (B–E), characterized for an increase in propionic, orotic, butyric and formic acid contents and a decrease in pyruvic and citric concentrations; (3) middle-aged cheeses, 126 to 156 days (F–G), which corresponds to a period where propionic, butyric and orotic concentrations increased and pyruvic acid decreased, while the remaining acids remained fairly constant except uric acid which was abruptly generated; (4) old cheeses, 184–290 days (H–J), samples of high total organic acid concentration, mainly because of the increase in propionic, acetic and lactic acid contents.

Propionic, pyruvic, lactic and citric 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:

$$t = 6{\cdot}0 \times 10^{-3}\,[\mathrm{Pr}] - 0{\cdot}91\,[\mathrm{P}] - 2{\cdot}0 \times 10^{-3}\,[\mathrm{L}] - 4{\cdot}1 \times 10^{-2}\,[\mathrm{C}] + 160$$

where: t is ripening time (days); [Pr] is propionic acid concentration (ppm); [P] is pyruvic acid concentration (ppm); [L] is lactic acid concentration (ppm); and [C] citric acid concentration (ppm).

The correlation coefficient was 0·974.

To conclude, it might be said that (i) packaged forms lost less weight than the unwrapped ones and did not present surface-mould growing; (ii) the period that W groups were left to ripen before packaging did not affect the results of the experiment; (iii) only citric and uric acid contents varied significantly between unwrapped and packaged forms; (iv) from the viewpoint of organic acid composition alone, wrapped and unwrapped forms did not differ significantly. The same conclusions were reached by Hough *et al.* (1992) through a sensory descriptive analysis study and by Gianuzzi *et al.* (1992) working on microbiological changes during ripening of Reggianito cheese. Both studies were conducted on the same cheese forms used in the present work.

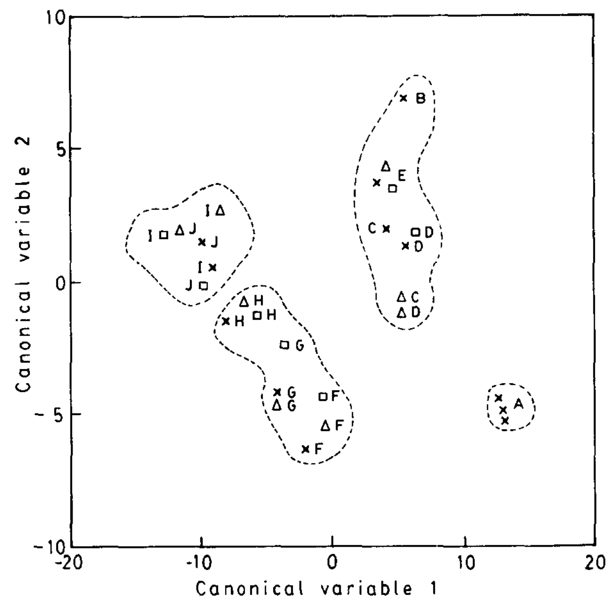

Fig. 4. Canonical plot of 27 Reggianito cheese samples. (X) Indicates unwrapped cheese forms (T); (Δ) cheese forms wrapped in plastic film after 30 days (W30); (□) cheese forms wrapped in plastic film after 45 days (W45). Letters indicate ripening time: A, 0 days; B, 31 days; C, 46 days; D, 59 days; E, 93 days; F, 126 days; G, 156 days; H, 184 days; I, 255 days; J, 290 days.

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