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Bovine milk composition parameters affecting the ethanol stability

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The objective of the present work was to identify the compositional parameters of raw milk that affected ethanol stability at natural pH when natural milk conditions were not modified. Heat stability, measured as coagulation time (CT), was included in the analysis to verify relation to alcohol test. Statistical models were proposed for alcohol and heat (CT) stabilities. Milk samples of good hygienic quality from dairy farms were classified in two groups according to their alcohol stability. Unstable samples to ethanol (72%, v/v) presented lower values of pH, somatic cells count, casein and non-fat-solids relative to ethanol stable samples (ethanol at 78%, v/v or more); whereas freezing point, chloride, sodium and potassium concentrations were higher in the unstable group. Logistic regression and multiple regression were applied to modelling alcohol and heat stability behaviour respectively. Chloride, potassium, ionic calcium and somatic cell count were included in the alcohol regression model, whereas calcium, phosphorous, urea, pH and ionic calcium were part of CT model. Ionic calcium was the only measured variable that contributed to both models; however coagulation time was noted to be more sensitive to ionic calcium than alcohol. The relation between ionic strength and casein was found to contribute to the alcohol model but not to the CT model. However, the interaction calcium plus magnesium plus phosphorous and casein contributed only to CT model.

Keywords: Ethanol stability, milk composition, bovine milk.

Milk ethanol stability (MES) was defined as the minimum concentration of added aqueous ethanol that gives rise to milk coagulation (Horne & Parker, 1979) and it had been a subject of considerable interest for two main reasons. Firstly, to achieve a better understanding of the factors that controlled micellar stability. Horne (1992) thoroughly discussed newly proposed theories connected to MES and clearly showed that mechanisms involved in it were complex and not totally elucidated. Secondly was the necessity of transferring this knowledge to formulate new dairy products or to extend the shelf life of the existing products such as cream liqueurs or alcoholic beverages (Donnelly & Horne, 1986; Horne & Muir, 1990; Horne, 1992).

There is also a third important reason to explore this subject. MES was used as a simple, cheap, efficient and quick pass-or-fail test to detect milk sourness in many countries. This method is still in use in Argentina, leading to rejection of the batch of milk if clots were formed when 70% (v/v) ethanol solution was added to an equal milk volume. Also the test was applied to predict milk heat stability, because of the necessity to have an easy essay to evaluate this property.

Bacteriological milk quality has improved steadily in Argentinean dairy farms during the last ten years (Taverna & Calvinho, 1999; Taverna et al. 1999) so acidity development cases were rare. However, positive alcohol test results were still occurring causing confusions and good quality milk was rejected. Consequently, lack of reliability of MES at certain seasons was recognised. In effect, during autumn and spring season stability defects were reported in some dairy farms with good milk bacteriological quality (Negri, 2002) with no known reason. Similar behaviour was reported by Donnelly & Horne (1986), who observed that a decrease in MES occurred frequently during winter in Ireland. They suggested high salt balance ratio during late and early lactation as an important contribution to this behaviour.

During the last 20 years important progress had been made in identifying principal factors that affected MES which were considered in the selection of the variables measured in this work. Horne & Parker (1981a) found that

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serum phase components govern the sigmoidal shape and position of the ethanol stability/pH profile. Moreover, these authors (1981a, b) confirmed that among serum phase components the ionic calcium concentration played an important role, a fact previously observed by Davies & White (1958). Salts (calcium, magnesium, phosphorus and citrate) were reported to influence ethanol/pH profile parameters (Donnelly & Horne, 1986; Horne, 1987). Finally, other important variables to ethanol stability previously found were the ionic strength (Horne, 1987) and the pH (Horne & Parker, 1979; Horne, 1992).

All this valuable information was obtained by changing the original micelle equilibrium in some way, e.g. pH or dialysis of milk. Our objective, however, was to determine the composition parameters that affected milk ethanol stability without any external modification to bulk milk samples. MES seasonal variation was used to select two milk groups for the study: stable and unstable to alcohol. Logistic regression was applied to propose a statistical model between MES and the explanatory variables. Logistic regression was a class of lineal model derived from a logarithmic transformation and described the relation between a dichotomous response, provided by the alcohol test, and the set of explanatory or independent variables. Coagulation time (CT) was also measured for every sample and a multiple regression model was applied to determine heat stability (CT) relationship to measured explanatory variables. Finally, variables capable of affecting both heat and ethanol stability were explored.

Materials and Methods

Milk samples

Eighty five bulk milk samples were collected and analysed during autumn and spring, when alcohol problems appeared, during 2001. Milk pools were produced proportionally from morning and evening milking of dairy farms from the Central Dairy Area of Argentina. Random milk pool samples from different farms were considered in the study if they had acceptable bacteriological quality (pH range 6·6–6·8; acidity °D range 14–16; Standard plate count cfu/ml <100 000). Animal feed was not special, rather those commonly used in farms according to the time of the year. Upon receipt, samples were classified immediately in two groups at natural pH: (a) stable when precipitation occurred at ethanol concentration of 78% (v/v) or greater and (b) unstable when precipitation occurred at 72% (v/v) or less. Alcohol test was performed by adding equal volumes of ethanol to the milk. Ethanol concentrations of 72 and 78% (v/v) were selected because these concentrations correspond to pH 6·6 and 6·7 respectively according to Horne & Muir (1990, Fig. 1).

Compositional analysis

The following analyses were performed in duplicate at least for every sample: somatic cell count (SCC) were counted electronically using Fossomatic 5000 (Foss Electric, Hillerød, Denmark) according to International Dairy Federation Standard (IDF; 1995); standard plate count of aerobic mesophilic bacteria (SPC) was measured according to IDF (1991a); milk acidity was determined by titration with NaOH (Instituto Argentino de Racionalización de Materiales, 1976); pH was measured by an Orion Ross® Sure-flow TM electrode and an Orion pH-ISE 710A meter (Beverly, MA, USA); freezing point (°C) was determined according to IDF (1991b) by an Astor 400SE apparatus (Astori, Italy); total nitrogen (TN), non-protein N (NPN) and non-casein N (NCN) were measured using micro-Kjeldahl techniques (IDF, 1964, 1993). These N fractions were then used to calculate total protein ((TN−NPN) × 6·38), casein protein ((TN−NCN) × 6·38) and whey protein ((NCN−NPN) × 6·38). Casein number was expressed as casein/total protein × 100. Fat, lactose, citrate (Cit), non-fat solids (NFS) and urea were analysed using an IR milk analyser (Milkoscan Ft 120, Foss Electric, Hillerød, Denmark; IDF, 1996). Phosphorous (P) and chloride (Cl) concentrations were determined by the phosphomolybdate method (IDF, 1987) and the Mohr's method (Bradley et al. 1992). Calcium (Ca), sodium (Na), magnesium (Mg) and potassium (K) concentration were measured by atomic absorption spectrometry (Perkin-Elmer 5000 spectrometer, Connecticut, USA, 1982). Ionized calcium concentration (Ca^{2+}) was determined using a Phoenix CAL1502 electrode (Houston, USA; Geerts et al. 1983). The heat stability of milk was evaluated as the CT at 140 °C in a stirred temperature-controlled oil bath, measured by a modification of the method proposed by Davies & White (1966) (Negri, 2002). Briefly, the glass tubes with pendulous movement used by Davies & White (1966) were substituted by capillaries, filled with milk, closed using a flame and then introduced into the bath. Capillaries ensured instant heat transfer evenly to the milk volume and CT (min) was determined when clot was detected by simple observation.

Statistical analysis

Mean values, confidence intervals, mean comparison by Student's *t*-test, logistic and multiple regression were calculated using SAS (1989). Significance of $P<0{\cdot}05$ was used in every analysis. Logistic and multiple regression were done on a data base of 26 stable and 26 unstable sample results. Decrease in data number occurred because methods eliminated uncompleted rows. The response variable in the Logistic Regression Model was a discrete variable: unstable or stable milk samples to alcohol. The log odds, that was $\ln[p/(1-p)]$ where p was the probability of being part of one alcohol classification, was the function chosen to model alcohol effect. The logistic regression model fitted the log odds to explanatory variables by a linear function, Hosmer & Lemeshow goodness of fit test and Wald χ^2's parameter were used to evaluate the procedure of fitting and individual effect each variable had in the logistic model, respectively. Results of the model were predicted probability

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Table 1. Mean values (X), sample size (N) and confidence interval (CI) of the parameters measured to characterise stable and unstable milk samples with respect to ethanol stability

	Ethanol-stable samples			Ethanol-unstable samples		
Parameters	X	CI 95%	N	X	CI 95%	N
Acidity (°D)	14·1	13·9–14·3	50	14·2	13·9–14·5	28
pH*	6·71	6·70–6·72	57	6·68	6·67–6·69	28
SPC ($\times 10^{-3}$ cfu/ml)	25	15–39	54	30	15–58	27
SCC ($\times 10^{-3}$ cell/ml)*	372	324–427	52	284	233–346	26
Freezing point (°C)*	0·518	0·515–0·521	49	0·523	0·520–0·526	19
NFS (g/l)*	87·4	86·8–88·0	56	86·0	85·1–86·9	27
Fat (g/l)	36·4	35·6–36·8	56	35·9	34·5–37·3	28
Lactose (g/l)	47·2	46·9–47·5	56	46·7	46·2–47·2	28
Protein (g/l)	33·2	32·8–33·6	44	32·4	31·7–33·1	26
Casein (g/l)*	24·7	24·3–25·1	44	23·8	22·3–25·3	25
Whey protein (g/l)	6·3	6·1–6·5	43	6·2	6·0–6·4	25
NPN (g/l)	0·35	0·34–0·36	46	0·36	0·34–0·38	27
Urea (g/l)	0·37	0·35–0·39	56	0·38	0·34–0·42	28
Citrate (g/l)	1·31	1·28–1·34	56	1·32	1·27–1·37	28
P (g/l)	0·95	0·93–0·97	55	0·94	0·94–0·95	28
Cl (g/l)*	1·45	1·42–1·48	54	1·61	1·54–1·68	28
Ca (g/l)	1·11	1·08–1·14	52	1·15	1·09–1·23	28
Mg (g/l)	0·11	0·10–0·11	53	0·12	0·11–0·13	28
Na (g/l)*	0·45	0·42–0·48	53	0·52	0·47–0·57	27
K (g/l)*	1·49	1·46–1·52	53	1·55	1·50–1·60	28
Ca^{2+} (ppm)	74·10	66·45–81·75	48	88·71	73·67–103·73	28
CT (min)*	23·8	22·3–25·3	52	19·9	17·2–22·6	28

*Values were significantly different ($P<0·05$)

of an improved outcome after the inverse transformation of log odds were done. Multiple regression method was applied to CT. This statistical model goodness of fit was evaluated considering the determination coefficient (R^2) and the model percentage of deviation (%D) with respect to experimental values. The latter was obtained by Eq. (1) as proposed by Heldman (1974):

$$\%D = \sqrt{\frac{\sum_{i=1}^{N} ((Tv - Ev)/Ev)_i^2}{N-1}} \times 100 \qquad (1)$$

where Tv is the theoretical value obtained when statistical models were applied, Ev the experimental value and N the number of observations.

Results and Discussion

Mean values, number of determinations and confidence intervals of every measured variable are presented in Table 1. Results obtained were similar to those reported in others studies in the same dairy area (Taverna & Coulon, 2000; Taverna et al. 2001a–d).

Variables which presented significant difference between the two groups of milk were also indicated in Table 1. Low casein concentration was one of the factors that characterized unstable samples, together with the fact that total protein concentration in both groups was equal, it was concluded that unstable group had a lower casein number (73·45%). Important differences between groups were due to mineral elements; among them Cl, Na and K presented higher values in the unstable group than in the stable group. Mastitis can be disregarded as the cause of these differences in salt concentrations since the unstable group had a lower mean SCC value. Horne & Parker (1981b) proposed a mechanism for ethanol-induced precipitation in which the dielectric strength of the micelle medium played an important role. Ionic strength, given by Cl, K and Na, affected that property. In effect, ionic strength increase reduced the dielectric constant of the medium weakening the energy barrier that prevents coagulation. Horne & Parker (1981a) observed that the addition of NaCl decreased ethanol stability.

Ca^{2+} of both groups presented similar concentration to previous reports for raw milk at natural pH: values of 81·2–92·2 ppm were recorded by Geerts et al. (1983), 89·78 ppm at 4 °C by Muldoon & Liska (1969) and 56·11–100·2 ppm by Demott (1968). Ca^{2+} concentration was significantly different between groups when $P<0·1$. The addition of Ca^{2+} was proved to increase ethanol unstability (Horne & Parker, 1981a; Horne, 1987; Horne & Muir, 1990) and according to results of Table 1 same tendency was verified. Additionally, Ca^{2+} presented correlation (correlation coefficient $r=-0·6$) with respect to CT only. Thus, Ca^{2+} increment caused CT reduction which was in agreement with previous works (Fox & Morrissey, 1977; Singh & Fox, 1987a, b; Singh & Creamer, 1992; Jeurnink & De Kruif, 1995; Le Ray et al. 1998; Aoki et al. 1999; Le Greät & Gaucheron, 1999).

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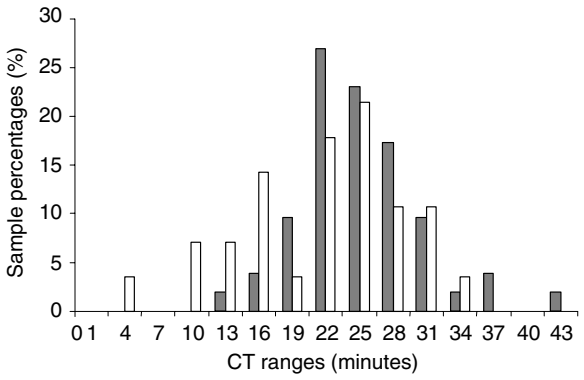

Fig. 1. Percentage frequency distribution of coagulation time values at 140 °C from (■) ethanol-stable and (□) unstable milk sample groups.

Difference in NFS was attributed to casein concentration variation between groups (r=0·54) and the difference in the freezing point was related to minerals, particularly to the sum of Na, K and Cl (r=0·44). pH results presented similar behaviour to that found by Horne & Parker (1979) and by Horne & Muir (1990) when they verified that ethanol stability increased when pH was raised artificially.

CT mean values, measured using capillary method, were in agreement with those reported by other authors using the standard method. Davies & White (1966) and Morrissey et al. (1981) obtained CT mean value and confidence interval equal to 19·67±0·73 and 18·23±3·20 min from milk samples of individual cows respectively, whereas Holt et al. (1978) obtained CT mean value 20·15±3·06 min from silo milk.

CT mean values between groups were different, showing that the unstable group had less heat stability. However, Fig. 1 helps to explain why ethanol stability still was not a reliable test to predict heat behaviour. According to frequency distribution (Fig. 1), 94·23% of the stable samples together with 82·14% of the unstable samples presented CT values between 34 and 13 min. So, there was a large range of intersection between CT values of both groups, proving that there was not a biunivocal correspondence between both stabilities. In fact, according to frequency distribution (Fig. 1) the higher percentages of stable samples presented CT values between 22 and 25 min, coinciding with unstable behaviour. The results confirmed that alcohol test was not a good heat stability predictor, which was widely known (Horne & Parker, 1979; Horne & Muir, 1990).

Another aim of this study was to obtain the relationships between alcohol stability and CT and the explanatory variables. Obtained models and goodness of fit are shown in Table 2. Each statistical model was fitted using 52 observations.

The odds obtained after transformation (odds=exp[logit(θ_i)]) indicated the chance a sample had to be classified as either stable or unstable. Odds classification limit was one; then, every predicted value less than limit value indicated that sample was alcohol stable while values higher than one indicate unstable samples.

Table 2. Optimal variable and parameters value set to alcohol and coagulation time statistical models

Dependent variable	Parameter ± SE	Parameters of fit
logit(θ_i)		
Intercept	−37·41±19·69	Goodness of fit=3·68,
log(SCC_i)	−4·19±2·05	P=0·88
Cl_i	205·60±60·99	
K_i	170·20±71·97	
Ca_i^{2+}	485·10±217·2	
CT_i		
Intercept	−463·10±125·91	R^2=0·72,
pH_i	72·91±18·35	D (%)=20·8
$Urea_i$	210·51±60·78	
P_i	171·45±66·67	
Ca_i	−178·31±36·78	
Ca_i^{2+}	−878·44±170·80	

i=1,2,...,n samples; Ca^{2+} units, g/100 ml; SE, standard error; R^2, determination coefficient; %D, the model percentage of deviation with respect to experimental values

Contribution of Cl and K to ethanol stability was expected since they modified ionic strength and through it charge interactions with the micelles. However, Na, which was part of the milk ionic strength, was not included in the model; it may play an alternative role in ethanol stability or present a non-linear behaviour and further studies should be carried. SCC contribution to alcohol stability was not clear since it did not present significant correlation to any of the measured variables in this study. A hypothesis was to consider it as another component of the serum phase which, in general terms, was known to participate in alcohol stability (Horne & Parker, 1981a).

According to result (Table 2), the only variable that contributed to both stabilities was ionic calcium. Moreover, an increase of Ca^{2+} concentration produced a decrease in both stabilities. These results were in agreement with previous reports. Horne & Muir (1990), reviewing the advances in the subject, mentioned that Ca^{2+} may be assigned a dominant role in ethanol stability. The negative influence on CT was widely proved as mentioned above. However, sensitivity of each stability to Ca^{2+} should be considered to be different. In effect, according to Wald κ^2 parameter, Ca^{2+} presented third order of significance (4·99) whereas Cl (11·36) and K (5·60) were placed in first and second order respectively. Partial determination coefficients of CT model were selected to carry out the same analysis for heat stability. In this case, Ca^{2+} presented the highest coefficient (R^2=0·36), second place corresponded to Ca (R^2=0·19), third to pH (R^2=0·08), then to urea (R^2=0·05) and finally to P (R^2=0·04). Obtained results suggested that heat stability showed more sensitivity to Ca^{2+} than ethanol stability.

Every variable included in the CT model was already known to influence heat stability (Fox & Morrissey, 1977; Van Boekel et al. 1989; Singh & Creamer, 1992; Chavez

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et al. 2002). Moreover, positive effect of pH, urea and P with respect to CT stability together with negative effects of Ca and Ca^{2+} were in concordance with published works cited in this paper. It should be added that Mg may have the same behaviour as Ca since they were highly correlated ($r=0{\cdot}689$, $P=1\times10^{-4}$).

Last point of this study was to consider mineral contribution to both stabilities since they influenced micelle equilibrium. Thus, minerals were divided into two groups according to their interaction with micelle systems: first group included those minerals that influence the environment surrounding micelles (ionic strength; Eq. 2) and second group included those integral to the micelle (micelle structure; Eq.3).

$$V1=(\mathrm{Cl}+\mathrm{Na}+\mathrm{K})/\mathrm{casein} \quad (2)$$

$$V2=(\mathrm{Mg}+\mathrm{Ca}+\mathrm{P})/\mathrm{casein} \quad (3)$$

In order to explore the participation of $V1$ and $V2$ in each stability, these relationships were included in the data base and new statistical models were obtained. Methods applied were the same as those used to determined model proposed in Table 2. As a result, $V1$ was selected as an optimal variable of the new alcohol stability model (goodness of fit 5·23, $P=0{\cdot}81$) whereas $V2$ was not part of it. However, $V2$ was included in the new CT model ($R^2=0{\cdot}68$; $D=22{\cdot}67\%$) but $V1$ was not. This allowed the proposal that variation in ionic strength was more important to alcohol stability than to heat stability; whereas ions (Ca, Mg and P) that formed part of the micelle structure showed the opposite behaviour.

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