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A chemometric investigation of the effect of the cheese-making process on contents of biogenic amines in a semi-hard Italian cheese (Toma)

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Abstract

A full 2^3 experimental design was applied to identify conditions of cheesemaking which will minimise the contents of four biogenic amines (putrescine, cadaverine, histamine and tyramine) in cheese. The variables of the study were represented by: (1) pasteurisation of milk, (2) type of bacterial strain and (3) curd temperature. Each experiment was a process of cheesemaking. To evaluate the pure experimental error, each experiment was repeated three times for a total of 24 cheeses. Samples of each cheese, after 30 days of ripening, were analysed for amine contents by HPLC. The conditions for minimising the formation of biogenic amines were different for each amine, confirming that different bacteria and metabolic pathways were involved.

Keywords: Biogenic amines; RP-HPLC; Toma cheese; Experimental design in cheese making

1. Introduction

Biogenic amines are organic bases characterised by their biological activities present in a number of foods, especially red wines and cheese. In particular histamine, tyramine, putrescine and cadaverine are produced during the cheese-ripening process by degradation reactions, which lead to the formation of free aminoacids and afterwards, through specific enzymatic decarboxylation, of amines.

Biogenic amines are recognised as potentially toxic for humans. The toxicity effects are reported to depend on the individual response and on the simultaneous presence of co-factors (e.g. others amines, alchool or pharmaceutical products), which can act in synergy or in antagonism. For example, the toxicity threshold of histamine is lower in the presence of putrescine and cadaverine (Bjeldanes, Schutz, & Morris, 1978; Parrot & Nicot, 1966). Silla Santos (1996) reports a toxicity threshold (100 mg/kg) for histamine while, for tyramine, different toxic thresholds (100 and 800 mg/kg) were reported by ten Brink, Damink, Joosten, and Huis in't Veld (1990). In general, amounts of 1000 mg/kg of total amines are considered dangerous for human health (Taylor, 1985)

The identification (and quantification) of biogenic amines in various kinds of food has received great attention. In dairy products, the main interest is in the different factors involved during the dairy process and the formation of biogenic amines (Abo-Gharbia & El-Sawi, 1999; Antolini, Franciosini, Floridi, & Floridi, 1999; Durlu-Ozkaya, Alichanidis, Litopoulou-Tzanetaki, & Tunail, 1999; Durlu-Ozkaya, Ayhan, & Ozkan, 2000; Fernandez-Garcia, Tomillo, & Nunez, 1999; Fernandez-Garcia, Tomillo, & Nunez, 2000; Gardini et al. 2001; Marino, Maifreni, Moret, & Rondini, 2000; Pattono, Grassi, Civera, & Turi, 2001; Pinho, Ferreira, Mendes, Oliveira, & Ferreira, 2001; Valasamaki, Michaelidou, & Polychroniadou, 1998; Vale & Gloria, 1998; Varlik & Ugur, 2002).

The proteolytic activity of bacteria producing biogenic amines in products is controlled by several process factors and in particular by pH, temperature and NaCl concentration (Pinho et al., 2001); the pasteurisation of the milk and the starter seem to play an important role

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in the formation of amine during the ripening (Ordonez, Ibanez, Torre, & Barcina, 1997).

The present paper presents the results of an investigation of the contents of putrescine, cadaverine, histamine and tyramine in cheese in relation to the variation of three cheesemaking parameters, i.e. the milk pretreatment (raw or pasteurised), the strain of bacteria (mesophilic or thermophilic) and the curd temperature (heated or not heated).

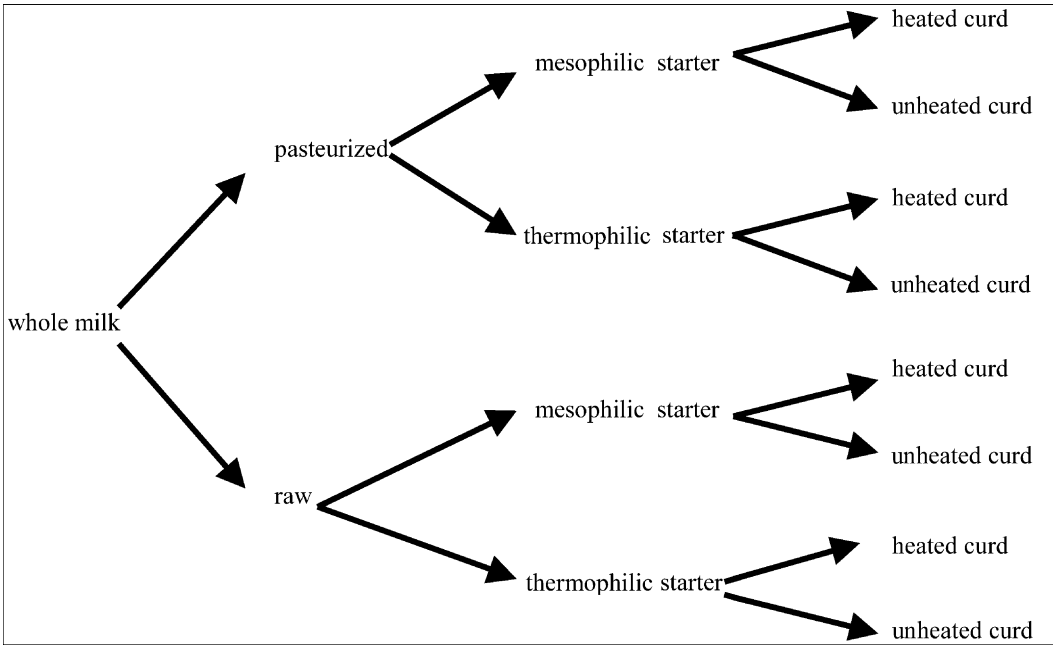
The cheese considered in this investigation is a semihard, whole bovine milk product, generally known in north-west Italy as "Toma". For this purpose, a chemometric study of experimental design was planned in which each experiment corresponds to a full cycle of preparation of cheese.

The amines were determined on samples of each experimental lot of cheese after a period of ripening of 30 days and their content represents the experimental response of the factorial experimental design.

2. Materials and methods

2.1. Materials

Eight experimental lots of cheese were obtained from separate vats (50 l) of bovine whole milk. The different cheesemakings were set as follows:



The coagulation was achieved at 35 °C by means of liquid bovine rennet (1:10 000). In the case of "heated curd", a temperature of 44 °C was applied for 12 min. In all cases, curd was put in cylindrical moulds (25 cm diameter), then salted in a 21% NaCl w/w brine for 12 h and ripened at 10 °C and 85% R.H. for 1 month.

The bacterial cultures were represented by commercial starter whose composition, indicated by the producer was, for mesophilic strains: *Lactococcus lactis* (subspecies *lactis*), *Lactococcus lactis* (subsp *cremoris*), *Lactococcus lactis* (subsp *biovar*), *Lactococcus lactis* (subsp *diacetylactis*) and *Leuconostoc mesenterioides* (subsp *cremoris*) and for thermophilic starter: *Streptococcus thermophilus* and *Lactobacillus delbreuckii* subsp *bulgaricus*.

2.2. Reagents

Ultrapure water, from a Milli-Q system (Millipore), and acetonitrile, HCl 0.1 M and acetone, HPLC grade, were purchased from Merck. Histamine dihydrochloride, tyramine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride and dansylchloride were Sigma-Aldrich reagents.

2.3. Analytical methods

The amine identification and quantification were made by HPLC, after extraction, and dansyl-chloride derivatisation was according to the protocol of Moret and Conte (1996). Ten miligrams of cheese were homogenised with 15 ml of 0.1 M HCl by means of an Ultraturrax macerator at medium speed. After centrifugation (10 000 rpm for 20 min), the sediment was again extracted with 10 ml of 0.1 M HCl and centrifuged. The two supernatant fractions were combined.

To 1.0 ml of the extract, with 1.0 ml of 5% dansychloride solution, in acetone, a saturated solution of

sodium hydrogenocarbonate was added. After 1 h at 40 °C, the extract was dried, recovered with acetonitrile (ACN) (1.0 ml), filtered through cellulose filters (0.20 μm), and 50 μl injected into the chromatographic system. The mobile phase was a water/ACN mixture in the following gradient elution: 0–5 min water/ACN 35:65, 5–20 min water/ACN 25/75. Flow-rate was 0.8 ml/min and UV detection at 254 nm.

2.4. Instruments

An HPLC Lachrom, Merck-Hitachi (Tokyo, Japan), equipped with a quaternary pump L-7100, an UV detector L-7400 and an interface L-7000 were used; the chromatographic column was a Merck LiChrospher 100 RP-18 250×4 mm (5 μm) coupled with a guard column of the same material.

2.5. Chemometric treatment

Experimental design provides a useful tool for analysing the effect of experimental factors. In the present study a 2-level full factorial design was employed to investigate the effect of three experimental factors on the amounts of biogenic amines formed in the final product. The factors examined were: the milk pre-treatment (raw or pasteurised), the starter bacteria (mesophilic or thermophilic) and the treatment of the curd (heated or unheated curd). The two level full factorial design allows the study of the effect of the principal factors and of their interactions on the investigated response (the amounts of amines formed). The number of experiments required is 2^p, p being the number of investigated factors. These experiments correspond to all the possible combinations of the two levels (usually indicated with + and −) of the considered factors. Factorial design theory has been widely described elsewhere (Box, Hunter, & Hunter, 1978; Carlson, 1992; Deming & Morgan, 1993).

According to a 2^3 full factorial design, eight cheeses were prepared. Each cheesemaking, that corresponds to a complete process of production, was replicated three times in order to obtain an estimation of the experimental uncertainty.

The three levels of each experimental factors are:

1. milk type (MILK): raw milk (indicated in the experimental design with the sign −) and pasteurised milk (indicated with +);
2. ripening strain (STRAIN): thermophilic starter bacteria (indicated with −) or mesophilic starter bacteria (indicated with +);
3. the curd treatment (CURD): unheated (indicated with −) or heated (indicated with +).

The eight experiments are reported in Table 1 together with the experimental responses.

The average concentrations were used to calculate the ordinary least squares (OLS) regression models that relate the content "y" of the amines to the experimental factors and to their interactions.

The significant effects were evaluated by a t test where each regression coefficient was compared with the standard error multiplied for the proper t value.

The general form of the model investigated is:

$$y = b_0 + b_1 \cdot \mathrm{MILK} + b_2 \cdot \mathrm{STRAIN} + b_3 \cdot \mathrm{CURD} + b_{12} \cdot \mathrm{MILK} \cdot \mathrm{STRAIN} + b_{13} \cdot \mathrm{MILK} \cdot \mathrm{CURD} + b_{23} \cdot \mathrm{STRAIN} \cdot \mathrm{CURD} + b_{123} \cdot \mathrm{MILK} \cdot \mathrm{STRAIN} \cdot \mathrm{CURD}$$

where the coefficients b_i measure the effect on the experimental response y of the corresponding variable and of their interactions.

For each amine, the experimental errors obtained in the different experiments were pooled to obtain a

Table 1
Average concentrations of the biogenic amines, standard deviations and total amounts of amines (mg/kg) obtained in the eight experiments of the full factorial design (n.d. not detectable)

	MILK	STRAIN	CURD	Cadaverine	Histamine	Putrescine	Tyramine	Total
EXP 1	−	−	−	220±14	23±1	330±19	111±8	686
EXP 2	+	−	−	136±32	n.d.	93±10	35±4	264
EXP 3	−	+	−	333±4	15±2	270±28	108±6	723
EXP 4	+	+	−	83±10	67±1	119±5	11±3	213
EXP 5	−	−	+	400±16	29±2	530±58	110±13	1076
EXP 6	+	−	+	221±5	2±3	90±10	n.d.	313
EXP 7	−	+	+	480±14	9±7	180±24	54±7	723
EXP 8	+	+	+	134±6	n.d.	47±4	14±1	195

MILK:	−raw milk	+ pasteurised milk
RIPENING STRAIN:	−thermophilic bacteria	+ mesophilic bacteria
CURD:	−without heating	+ with heating

general stimate of the experimental error and the pooled variance was calculated

$$s_{pe}^2 = \frac{\sum_{i=1,N} \left(\nu_i \cdot s_i^2\right)}{\sum_{i=1,N} \nu_i}$$

where ν_i is the number of degrees of freedom of each estimate of the experimental error s_i^2 of the ith experiment, which is equal to the number of replicates minus 1 ($\nu = 2$ for each s_i^2).

3. Results and discussion

Table 1 summarises the outline of experiments and the average concentrations of the biogenic amines detected at the end of the first month of ripening. These results, obtained from the three replicated experiments, are shown together with the corresponding experimental errors. A good reproducibility was observed within each group of three replicates.

The importance of the principal factor effects is highlighted by Table 2. In bold are the values of the effect for every factor. The higher the value, the more important is the factor in affecting the amine content. The "plus" or "minus" sign indicates an increase or a decrease of the amine contents when the considered factor is changed.

The factor MILK has the most relevant effect. The use of raw milk leads to a higher content of all the amines. This effect is more important for cadaverine and less important for tyramine and histamine.

For the factor STRAIN, the use of mesophilic bacteria seems to slightly favour the formation of cadaverine (positive sign) but not the formation of putrescine, histamine or tyramine.

The CURD factor effect is significant only for cadaverine and tyramine but with opposite sign. Heating of the curd increases the formation of cadaverine and decreases the amount of tyramine.

The previous observations deal with the principal factor effects but, as can be seen from the matrix plot, a correlation among histamine, tyramine and putrescine is observed while no correlation exists between cadaverine and the other amines. The interaction factor effects describe the simultaneous variation that the effects exert on the system in a synergistic or antagonistic way. So, for example, the negative signs of all the principal effects in the tyramine OLS regression model and the positive signs of all the relevant interactions indicate an antagonistic effect for this amine.

To further investigate the interaction effects, two-way tables were constructed. This statistical treatment allows

Table 2
OLS regression models relating the biogenic amine amounts **y** to the experimental variables

Cadaverine	**y = 251−107** MILK + **6** STRAIN + **57** CURD−**41** MILK×STRAIN−**23** MILK×CURD−**8** STRAIN×CURD
Histamine	**y = 10−9** MILK−**4** STRAIN + **4** MILK×STRAIN−**2** STRAIN×CURD
Putrescine	**y = 207−120** MILK−**54** STRAIN + **50** MILK×STRAIN-**24** MILK×CURD−**46** STRAIN×CURD + **28** MILK×STRAIN×CURD
Tyramine	**y = 56−40** MILK−**9** STRAIN−**11** CURD + **6** MILK×STRAIN + **11** MILK×STRAIN×CURD

Table 3
Two-way tables illustrating MILK–STRAIN interaction for the four analytes

		Cadaverine		**Histamine**		**Putrescine**		**Tyramine**	
STRAIN	Mesophilic	406	108	12	33	225	83	81	12
	Thermophilic	310	179	26	1	430	91	110	17
		Raw	Pasteurised	Raw	Pasteurised	Raw	Pasteurised	Raw	Pasteurised
		MILK		**MILK**		**MILK**		**MILK**	

Table 4
Two-way tables illustrating MILK–CURD and STARTER–CURD interactions for cadaverine and putrescine

		Cadaverine		**Cadaverine**		**Putrescine**		**Putrescine**	
CURD	Heating	440	178	310	307	355	68	310	113
	No Heating	276	109	311	208	300	106	211	194
		Raw	Pasteurised	Thermoph.	Mesoph.	Raw	Pasteurised	Thermoph.	Mesoph.
		MILK		**STRAIN**		**MILK**		**STRAIN**	

the evaluation of the effect of one factor when at the same time another factor is changing. Tables 3 and 4 are constructed by averaging the response of each couple of combinations of the variables, while the other is kept constant.

In Table 3, the interactions MILK×STRAIN, in the rows, are indicated, i.e. the values linked to the milk, while the columns show the values linked to the starter bacteria. In the bottom left quadrant is the average of the response of the experiments characterised by the MILK value (−) and the STRAIN value (−). Moving from this quadrant to the bottom right the variation induced by MILK is considered for a constant value of STRAIN. The decrease in the response of 131 mg/kg means that the use of pasteurised milk, in the presence of thermophilic strain, inhibits the formation of cadaverine.

Of course the same interpretation can be obtained when this two-way table is analysed in the opposite direction.

When raw milk is used, the change from thermophilic to mesophilic bacteria causes an increase of cadaverine content of 93 mg/kg while, for pasteurised milk, the change from thermophilic to mesophilic bacteria causes a decrease of the amine content of 71 mg/kg. In this case, the effect of the interaction is larger, since the STRAIN effect changes sign, as a function of the milk used. In other words, in the case of raw milk, the use of thermophilic bacteria is associated with a lower content of cadaverine and a lower content is obtained when mesophilic bacteria are added to pasteurised milk.

For histamine, when raw milk is used, the change from thermophilic to mesophilic strain causes a decrease in its amount.

Putrescine is lowered by the use of mesophilic bacteria with raw milk, while the type of strain does not affect the amount formed in the presence of pasteurised milk. Similar conclusions can be drawn for tyramine, with smaller variations in concentration. In summary, the amounts of histamine, putrescine and tyramine suggest a synergistic effect between milk and starter strains.

CURD shows relevant interactions with MILK and STRAIN only for cadaverine and putrescine. The two-way table calculated for these analytes (Table 4) leads to the following:

- When raw milk is used, changing from unheated to heated curd causes an increase of the cadaverine concentration from 276 to 440 mg/kg, while with pasteurised milk the variation is only 69 mg/kg. The best cheesemaking conditions seem to correspond to the use of pasteurised milk without heating the curd
- When thermophilic bacteria are used as starter, the variation from no heating to heating of the curd does not cause an increase of cadaverine while, when mesophilic bacteria are used, the variation is 99 mg/kg. With unheated curd, the change from thermophilic bacteria to mesophilic bacteria causes a decrease in cadaverine concentration of 103 mg/kg while, when the curd is heated, a decrease of 3 mg/kg is observed.

Table 4 provides the following with regard to putrescine:

- When raw milk is used, heating the curd causes an increase of 55 mg/kg while, when pasteurised

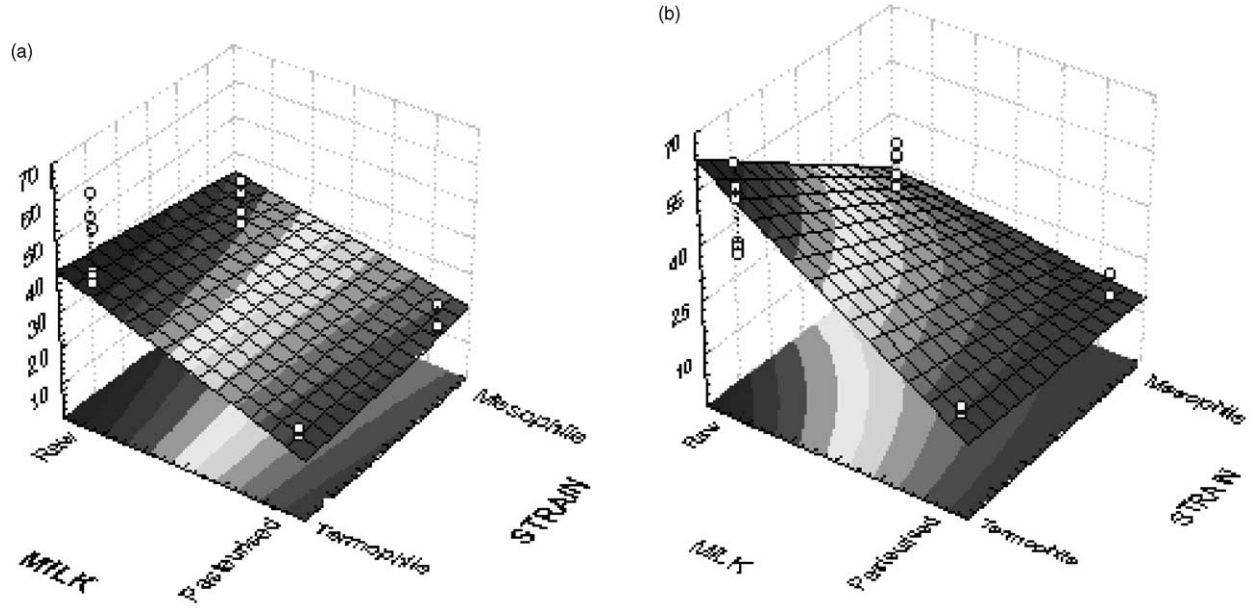


Fig. 1. (a) 3D plot for putrescine (mg/kg) of MILK and STRAIN as a function of the response when CURD unheated (−) (b) 3D plot for putrescine (mg/kg) of MILK and STRAIN as a function of the response when CURD is heated (+).

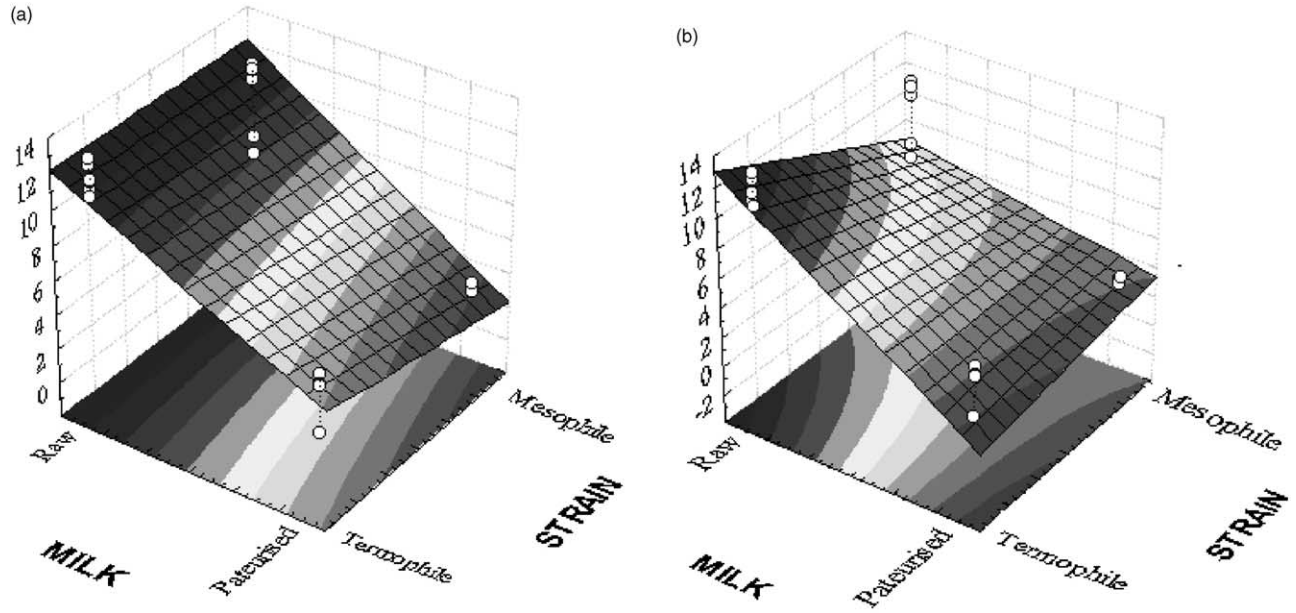


Fig. 2. (a) 3D plot for tyramine (mg/kg) of MILK and STRAIN as a function of the response when CURD is not heated (−). (b) 3D plot of tyramine (mg/kg) of MILK and STRAIN as a function of the response when CURD is heated (+).

milk is used, a decrease of 38 mg/kg is observed. The interaction is caused by the opposite effect observed when changing the curd for different milk settings. The global effect of curd was found to be insignificant, because there is a compensation between the effects observed for the two different milk levels. The best cheese-making conditions, with respect to putrescine, seem to be the use of pasteurised milk, together with heating of the curd. Without heating, changing from raw to pasteurised milk causes a decrease of 194 mg/kg while, when the curd is heated the decrease is 287 mg/kg. The interaction originates from the major effect of the milk factor observed when the curd is heated. Heating the curd causes a decrease of the amine concentration of 81 mg/kg when mesophilic bacteria are added while, with thermophilic bacteria, an increase of 99 mg/kg is observed. The relevant interaction is caused by the opposite effect of curd factor under the two different conditions.

The models reported in Table 2 also show that the interactions of the three factors are relevant for tyramine and putrescine. The procedure used to resolve this interaction effect is similar to the one employed for the interaction of two factors, but a three-way table must be constructed in which the experiment reported in Table 1 as (−, −, −) correspond to the origin of the axes. The effect can be better understood by the aid of the contour plot reported in Figs. 1a, b and 2a, b, where a two factor interaction (MILK×STRAIN) is built for each level of CURD. Fig. 1a and b represent the 3D plots showing the variables MILK and STRAIN for putrescine as functions, respectively, of the response obtained when the variable CURD is not heated (−) and heated (+). As can be seen, the shape and trend are different and in particular for heated curd the lowest amount of putrescine is obtained for both the strains with pasteurised milk. When heated curd is used we also observe a lower amount of amine with raw milk and mesophile strains. Similar results can be observed for tyramine, but the value of the effects is smaller.

4. Conclusions

The chemometric analysis has proved to be a useful tool for the comprehension of the importance of the interactions among single variables in the cheese-making process.

The heat treatment of the milk seems to play a key role in the determination of the amounts of all biogenic amines. This is not surprising when considering that many decarboxylating bacteria are killed by pasteurisation.

Other authors (e.g. Ordonez et al., 1997) have reported the same conclusion. However, in our study a significant role of the starter cultures and curd temperature has also been demonstrated.

Considering the total amount of biogenic amines, the best cheese-making conditions seem to be those of experiment 8 (Table 1), when pasteurised milk, mesophilic starters and heated curd are used.

Undoubtedly, further studies are needed in order to evaluate how the biogenic amine amounts change during ripening and to better understand the specific role of the native bacterial strains of milk, and especially their relationship with added starter strains.

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