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Technological Characterization of Experimental Natural Rennets Pastes


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Four types of lamb rennet pastes were characterized according to the sort of abomasa and the treatment it received: full fresh abomasa (FFA), full dried abomasa (FDA), empty dried abomasa (EDA), and empty frozen abomasa (EFA). Those rennet pastes were studied by means of different technological parameters (milk clotting time, chymosin content, lipase activity, and aptitude against milk coagulation). The highest level of clotting activity (391.20 IMCU/g) corresponded to the rennet EFA whereas the lowest (172.87 IMCU/g) was found in EFA. The chymosin content of the pastes classified them as rennet extracts, the highest chymosin level (80.46%) being found in the paste from FDA and the lowest (71.10%) being obtained from FFA. The highest level of lipase activity (10.57 U/g) was found in EDA whereas the lowest (1.46 U/g) was in paste FDA. Milk coagulation aptitude was studied through the use of a near infra-red radiation dispersion sensor. The highest value of $R_{max}$ (0.049 min$^{-1}$) found in the rennet paste made with EDA indicated a higher level of casein hydrolysis and greater aggregation speed. For the same rennet at $t_{max}$ value of 6.1 min was established lower than for other rennet preparations which reflected the fact that this rennet hydrolyzed 80% of the casein in a shorter period of time. It is concluded that the best natural lamb rennet paste of this work was FDA due to it combines appropriate proteolytic and lipolytic activities although it does not reach the maximum values for each technological parameters analyzed.

Key Words: natural rennet paste, NIR dispersion sensor, chymosin, clotting, lipase activity

INTRODUCTION

Currently, the use of bovine rennet to obtain mature cheeses is a common practice in cheese-making worldwide, whereas the use of natural paste rennets was common for a long time. The latter allowed to obtain different products with more accurate sensory properties although over time paste rennets have become less common due to the intensive use of liquid bovine rennet (Ferrandini, 2006). Consumers sensory preferences has changed in the last years, and now they look for products with different and diverse organoleptic sensations which resembles the taste and flavor of products of our ancestors (Marino, 2004), so it leads to the conclusion that there is a rosy future for this variety of dairy products.

In order to get this objective there is a variety of technological procedures, which can be applied to the cheese manufacture, may be one of the cheapest one can be the replacement of the type of rennet used. The main objective of the rennet is to coagulate the milk by chymosin proteolytic activity (the main component together with pepsin in liquid rennets). Paste rennet contains chymosin as coagulant enzyme, pepsins and gastricsins as proteases and pregastric, gastric enzymes as lipases (Piredda and Addis, 2003). So lipolytic activity distinguishes paste rennets from commercial liquid rennets. The lipase activity in paste rennets can arise from micro-organisms (mold, yeast, and/or bacteria) and/or from animal tissues, both the abomassum and/or

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the pregastric region (tongue, pharynx, epiglottis, and esophagus).

Animal pregastric lipases mainly release short-chain fatty acids, whereas microbial lipases release longer chain fatty acids (Harboe, 1994). A large increase in total free fatty acids (FFAs) when milk fat is used as a substrate is not an indicator of the presence of pregastric lipase in the paste rennet used, but a figure of over 65% short-chain FFAs (C<sub>14</sub>–C<sub>16</sub>), especially butyric acid, is a reliable indication of its presence (Svensson et al., 2006).

Rennet pastes include both proteolytic and lipolytic enzymes so the replacement of liquid rennet to paste one may have a significant incidence in final sensory cheese properties mainly due to the production of short (<12 carbon atoms) and long (>12 carbon atoms) FFAs in it. In addition, these short FFAs promote the formation of other volatile compounds like methyl ketones and alkanes, lactole, aliphatic, and aromatic esters that influence the taste and flavor of cheese (Chávarri, 1999) while long-chain FFAs are considered to play a minor role in cheese flavor due to their high perception thresholds (Collins et al., 2003).

Bustamante (2002) believes that there is a future in the use of pregastric lipases in many varieties of cheese other than those in which they are currently used, such as Provolone, Romano and other varieties like Feta, as well as, to a lesser degree, in tangy Cheddar, Manchego, Samso, Ras, Romi, Domiati, Kopanisti, blue cheeses, etc.

According to the results published by Piredda and Addis (2003), there are numerous past instances of lamb paste rennet being used in the production of traditional PDO cheeses in the Mediterranean area, for example Idiazabal and Roncial in Spain, Feta in Greece and Pecorino Romano, Fiore Sardo and Provolone Valpadana in Italy. In Greece, people from many towns prefer cheese like Feta and Kefalotyri with intense flavor so that is why they are manufactured using lamb rennet paste (Anifantakis, 1976).

The aim of this research is to technologically characterize different lamb paste rennets in order to determine the most suitable one to be used in cheese production.

**MATERIALS AND METHODS**

**Lamb Rennet Pastes**

In making the different lamb paste rennets, abomasas from 1-month-old lambs Castilla-León was employed by Cuajos Caporal, S.L. (La Cisterniga, Valladolid, Spain). Selected abomasas were transported in refrigerated brine and stored at 4–6 °C. In no case were there more than seven days between slaughter and processing of the abomasas. Figure 1 shows the flow-chart of rennet paste production. Four different paste rennets were made, differentiated by the state of the abomasas and the treatment received: empty frozen abomasus (EFA), empty dried abomasus (EDA), full fresh abomasus (FFA).

The stomachs selected had yellowish, light brown color, which indicated that animals had only been fed milk. After that they were put into refrigerated plastic containers with brine at 4–6 °C and taken to the factory. One part of abomasas was opened and its content rejected (empty abomasas) while the others were kept full with their content (full abomasas). On one hand a part of the empty ones was frozen at approximately −30 °C while the others were air-dried at 30 °C during 2 days in a ventilated room. On the other hand a part of the full stomachs was not treated so they were named as FFAs; in the meanwhile the rest of the full ones were air-dried at 30 °C during 3 days in an air-drier. External fat was eliminated: coagulated milk lumps were not removed and the tissue of at least five stomachs of each group were cut in pieces and ground in a commercial meat grinder, mixed with 23% of salt and ground again to obtain a paste. The stomachs that had any grass inside were discarded.

Four batches of each of these types of rennet were made, each of ~1 kg and were stored in refrigeration in

![Figure 1. Flow diagram of lamb rennet pastes production.](http://example.com/figure1.png)
Methods

Milk-clotting Activity

Milk-clotting activity (MCA) was calculated by determining the clotting time in standard powdered skimmed milk during testing to reduce evaporative cooling. Sample temperature was controlled using a circulating water bath with control accuracy of ±0.01°C. The period of time after milk heating and enzyme addition was normally 20 min. The water bath and milk temperatures were measured before enzyme addition to confirm that they were in thermal equilibrium. The paste rennet extract was added to the milk and stirred thoroughly for 30 s. Reflectance measurements were then taken simultaneously with enzyme addition during the storage time. Additional samples were taken and the evolution of enzymatic activities and technological parameters were studied.

Chymosin Content

The calculation of chymosin content was based on the F-factor, which is the coefficient of the milk-clotting time of the standard milk sample at two different pH values (6.5 and 6.0). This method is based on the incubation at pH 6.0. The chymosin samples were estimated by linear regression of seven mixtures of chymosin standard (98.77% IMCU, powdered rennet) and test sample, powdered rennet, different proportions according to 157:1992.

Lipase Activity

Lipase activity was determined by a method described by Vallet et al. (1992). Tributyrin (Fluka, Madrid, Spain) emulsifying reagent was prepared with 10 mM KH₂PO₄ (pH 6.8) and gum arabic. To prepare the emulsified substrate reagent was sonicated with 50 mL of 0.25% NaCl. The final pH was 6.8 containing 20 mL of emulsified substrate enzyme containing sample. Assays at 35°C and the pH was maintained at 10 mM NaOH. Enzyme blanks were

hermetically-sealed glass containers at 4–6°C for at least 9 months in order to evaluate the loss of enzymatic activity during storage. This storage period is the maximum commercial shelf-life of the product due to the loss of lipase activity after 4–5 months in refrigerated conditions (Bustamante et al., 2000).

Periodically during the storage time, samples were taken and the evolution of enzymatic activities and technological parameters were studied.
Figure 2. Diffuse reflectance ratio versus time and derivatives parameters: $R$ – light scattering profile; $R'$ – first derivative of $R$.

Statistical Methods

The data were analyzed using the general linear model (GLM) procedure of the variable/multivariable Statistix for Windows (7.0, Analytical Software, USA) or the variable/multivariable Statistical Packages for the Social Sciences (SPSS 9.0.1, 1999).

RESULTS AND DISCUSSION

Enzymatic Activities

Milk-clotting Activity

The first aim was to evaluate the influence of each parameter assayed and to determine which of them significantly affected coagulating activity. Each of the components of variation (batch, number within the batch and type of rennet preparation) was studied in relation to clotting activity, using the ANOVA linear model.

The results obtained showed that the variables ‘batch’ and ‘type of rennet preparation’ significantly influenced ($p < 0.05$) the milk clotting activity, as did the co-variable ‘day of storage’, whereas the number of the sample within a batch had no significant influence ($p > 0.05$), and is therefore eliminated from the ANOVA statistical model.

The variable ‘storage day of the rennet’ is taken as a co-variable in order to reduce the use of degrees of freedom. Table 1 compares the relationships between ‘milk-clotting activity’ and the different batches and types of rennet preparation.

The greatest level of milk clotting activity was related to the paste rennet made from EDA, followed by FDA with statistically significant ($p < 0.05$) differences between the two.

The lowest level of clotting activity was that of EFA rennet, due to the fact that the freezing process reduced the coagulating activity in the final product, according to the results of López (1993). No significant difference was found between this type of rennet preparation and the activity found in the FFA rennet.

From the data in Table 1 it can be concluded that there were significant differences between the different batches only in EFA rennet. This may be caused by the lack of homogeneity in the paste, which is typical of these types of rennet.

The evolution of the milk clotting time of all the lamb rennet pastes during the storage period is shown in Figure 3. Looking at the variation in milk-clotting activity of the different types of rennet preparation for each batch analyzed during the storage period, the reductions in clotting activity found in all the pastes at the start of storage is at odds with the work of Bustamante et al. (2000), in which it was found that in lamb paste rennet (from the Lucha breed) clotting activity increased after 60 days of refrigeration at 4°C, and then it remained constant for 1 year.

On the other hand, the increase in clotting activity which was found in all types of rennet preparations towards the end of the storage period may be due to the fact that chymosin and pepsin are secreted aszymogens which are activated auto-catalytically at pH lower than 4.0. In paste rennets there is a more pronounced increase in coagulating activity as time goes on, because pastes made from EDA have a pH value of ~5.0, delaying the activation of thezymogens.

The highest levels of clotting activity of all types of paste rennet preparations, both at the start and at the end of storage, were in pastes made from empty
Figure 3. Evolution of milk-clotting activity along the storage (a) EDA; (b) FDA; (c) EFA; (d) FFA (•) Batch 1, (■) Batch 2, (△) Batch 3, (×) Batch 4.

Abomasal dried according to the traditional method (EDA). The lowest level found was in batch 3, and the highest in batch 1. These values remained constant throughout storage. This type of rennet has pH ~5.0 and is activated more slowly.

Values lower than 270 IMCU/g were found for the four batches of the type of rennet made from fresh full abomasum (FFA), the highest level being that of batch 4 at the end of storage and the lowest that of batch 1 throughout storage. The paste rennet in batch 4 showed a peculiar behavior at 4 months of storage, its clotting activity almost coinciding with batch 4 at the same time. These results are most likely due to the high level of heterogeneity of these rennets.

FFA paste rennet does not present a very marked increase in clotting activity over time because the zymogens are activated practically right at the start, since the abomasum used in the preparation of the paste have pH values between 4.0 and 4.3.

The variation in the clotting activity of the four batches of EFA rennets was similar throughout storage. This type of rennet exhibits the lowest level of clotting activity of all those studied. The freezing process undergone by the abomasum before transformation negatively affects the coagulating activity of the paste rennet obtained, as other researchers have also found (Lopez, 1993). This reduction in clotting activity is probably due to a denaturalization of the coagulating enzymes during freezing.

**Chymosin Content**

For this parameter the same statistical criteria as with milk-clotting activity were used to relate experimental values for the independent variables studied with storage time being taken as a co-variable.

According to the statistical values found, none of the sources of variation significantly affected the chymosin content (p > 0.05). However, storage time influenced chymosin content in the experimental paste rennets studied (p < 0.01).

As there were no differences among the variables studied, simple variance analysis was used to study the relation between the chymosin percentage of the different paste rennet preparations and the day of storage (Table 2).

According to the results, for the EDA rennet preparation the chymosin percentage did not vary during storage, although there was a significant reduction in activity after 103 days due to the aforementioned heterogeneity of the pastes. The average value found in EDA rennet was 75.62%, similar to that found by Irigoyen et al. in 2001 (77.6%) in a similar rennet used for making Roncal cheese. Bustamante et al. (2000) found chymosin values between 73% and 80% in rennet preparations from Lacha sheep.

The chymosin content of the FDA rennet preparation is similar to that of the EDA rennet preparation throughout storage, with a higher average chymosin value (80.46%).
Table 2. Chymosin content of different rennet pastes throughout storage.

<table>
<thead>
<tr>
<th>Rennet paste</th>
<th>Days of storage</th>
<th>Chymosin (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDA</td>
<td>54</td>
<td>82.31 ± 16.51 a</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>82.48 ± 10.99 a</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>59.46 ± 7.34 b</td>
</tr>
<tr>
<td></td>
<td>122</td>
<td>78.29 ± 3.19 a</td>
</tr>
<tr>
<td>FFA</td>
<td>71</td>
<td>77.35 ± 11.49 a</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>83.72 ± 8.08 a</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>69.89 ± 8.76 ab</td>
</tr>
<tr>
<td></td>
<td>144</td>
<td>53.43 ± 17.00 b</td>
</tr>
<tr>
<td>EFA</td>
<td>68</td>
<td>80.16 ± 13.98 a</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>78.78 ± 10.17 ab</td>
</tr>
<tr>
<td></td>
<td>117</td>
<td>69.70 ± 19.57 ab</td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>59.11 ± 13.21 b</td>
</tr>
<tr>
<td>FDA</td>
<td>12</td>
<td>76.53 ± 12.01 b</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>93.21 ± 7.17 a</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>69.36 ± 5.00 b</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>80.72 ± 6.29 ab</td>
</tr>
</tbody>
</table>

Means followed by the same letter in the same column and for each type of rennet preparation are not significantly different (p<0.05).

For the FFA rennet preparation, there was a significant reduction in chymosin content at the end of storage, as with the EFA rennet preparation. Both preparations had lower average values than the other paste rennets (71.10 and 71.94% respectively). In another ovine paste rennet (Irigoyen et al. 2001) found that the chymosin content was below 54.0%.

The wide standard deviation found in the samples analyzed is due to the heterogeneity of the different paste rennet samples within one batch.

As for the variation observed as a result of the heterogeneity of the samples during the determination of chymosin content in this experiment, there was no possible to adjust the other indexes to statistical analysis. It was found that milk temperature affected only the first significant level lower than the results of Castillo (2001), the effect of the independent variables parameters, which evaluate behavior of casein gel formation.

The mean $R_{\text{max}}$ values calcculated for EDA and the FFA and EFA rennets, the former having the highest value, is related to casein hydrolysis speed (Castillo, 2001). In this study, we found that the $R_{\text{max}}$ parameter is related to casein hydrolysis speed.

Table 3. Lipase activity in the different rennet pastes.

<table>
<thead>
<tr>
<th>Rennet pastes</th>
<th>Lipase activity (U/g rennet paste)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDA</td>
<td>10.57 ± 2.82 a</td>
</tr>
<tr>
<td>FFA</td>
<td>4.57 ± 2.19 b</td>
</tr>
<tr>
<td>EFA</td>
<td>9.23 ± 3.06 a</td>
</tr>
<tr>
<td>FDA</td>
<td>1.46 ± 0.63 c</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different (p<0.05).

Table 4. Analysis of variance and F-statistic for dependent variables (temperature as co-variable).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>$R_{\text{max}}$</th>
<th>$R_{\text{max}}$</th>
<th>$R_{\text{cut}}$</th>
<th>$F_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>3</td>
<td>2.49</td>
<td>0.13</td>
<td>0.54</td>
<td>0.96</td>
</tr>
<tr>
<td>Replication</td>
<td>1</td>
<td>0.36</td>
<td>0.97</td>
<td>4.33*</td>
<td>0.18</td>
</tr>
<tr>
<td>Rennet pastes</td>
<td>3</td>
<td>9.62**</td>
<td>2.13</td>
<td>0.74</td>
<td>15.31**</td>
</tr>
<tr>
<td>Co-variable</td>
<td>Temperature</td>
<td>p = 0.145</td>
<td>0.4847</td>
<td>0.0379</td>
<td>0.2043</td>
</tr>
</tbody>
</table>

DF: degrees of freedom; F: F-value; p: significance. * significant at 93%, ** significant at 99%.

Rennert Paste Coagulation

Figure 2 shows the typical chart of diffuse reflectance ratio versus time and derivatives parameters. The values of the aptitude indicators against coagulation, determined by the diffuse reflectance sensor, were compared statistically with each of the independent variables assayed. In order to do so, the values for $F$ and significance $p$ were calculated (Table 4).

The statistical study relating the values of the independent variables $R_{\text{max}}$, $R_{\text{cut}}$, $R_{\text{cut}}$, and $F_{\text{max}}$ to the independent variables batch number within batch. Differences between the lipase activity of EDA and FFA paste rennets, both with significantly higher values for lipase activity (10.57 and 9.23 U/g, respectively) than FFA (4.57 U/g) and EFA (9.62 U/g) rennets (Table 3). Bastante (2002) analyzed various lamb paste rennets from different traditional cheese-making facilities at different times of the year and found highly variable levels of lipase activity between 0.11 and 5.84 U/g, some of them being similar to the value found in the FFA rennet as mentioned in this study.

Other authors have found lower values (1.07 U/g) of lipase activity in lamb paste rennets (Chavarrí, 1999), while in hygienized bovine rennet paste Castillo et al. (2001) found so very lower values (0.79 U/g).
Table 5. Effect of replication and rennet paste on $R_{\text{max}}, R_{\text{cut}}$, and $T_{\text{max}}$.

<table>
<thead>
<tr>
<th>Replication</th>
<th>$R_{\text{max}}$ (min$^{-1}$)</th>
<th>$R_{\text{cut}}$ (min)</th>
<th>$T_{\text{max}}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.271 ± 0.325 b</td>
<td>1.451 ± 0.043 a</td>
<td>No effect</td>
</tr>
<tr>
<td>2</td>
<td>No effect</td>
<td>1.451 ± 0.043 a</td>
<td>No effect</td>
</tr>
<tr>
<td>Rennet</td>
<td>EDA 0.049 ± 0.004 a</td>
<td>No effect</td>
<td>6.1 ± 0.1 b</td>
</tr>
<tr>
<td></td>
<td>FFA 0.040 ± 0.003 a</td>
<td>12.7 ± 2.1 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EFA 0.041 ± 0.005 b</td>
<td>11.6 ± 3.6 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FDA 0.044 ± 0.002 ab</td>
<td>9.7 ± 1.0 a</td>
<td></td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different ($p<0.05$).

The EDA rennet preparation had a greater speed of casein hydrolysis than the FFA and EFA rennets. For the dependent variable $T_{\text{max}}$, time in which ~80% of the $\kappa$-casein is hydrolyzed, it was observed that EDA needed significantly less time than the other rennet preparations in order to achieve the same effect; in other words, clotting time would be shorter. This was expected since EDA rennet had significantly ($p<0.05$) higher milk-clotting activity (Table 1).

Selection of Rennet Paste

The main purpose in selecting lamb rennet paste is to set up a production method that permits the recovery of traditional flavors in cheeses, which are currently made using commercial bovine rennet.

In order to select the experimental paste rennet to be used in the production of a given cheese, the parameters that determine the technological adaptability of the rennet preparation must be evaluated, and checks must be made to ensure that the microbiological requirements established by the quality norms are met (Presidencia del Gobierno, 1996).

The choice of paste rennet to be used in cheese production is fundamentally made by looking for a balance between lipolytic and clotting activity. As there were no significant differences in chymosin content among the rennets assayed, the paste rennet with the optimal balance is the one derived from FFA. As shown in Table 6, the paste rennet chosen had a chymosin content of 71.1%, clotting activity of 177 IMCU/g and lipase activity of 4.57 U/g. In lamb paste rennets, Barzaghi et al. (1997) and Bustamante et al. (2000) have found average chymosin values similar to those found here in FFA rennet (73.6 and 76.5%, respectively). As for clotting activity, Bustamante et al. (2000) have found values between 155 and 363 IMCU/g whereas Barzaghi et al. (1997) have found in average similar values to those in this study, both in lamb paste rennet.

On the other hand, Bustamante et al. (2000) have found lipase activity scores between 3.7 and 26.9 U/g in various lamb paste rennets, although Barzaghi et al. (1997) found lower values (1.65 and 1.85 U/g) than those of this study.

Addis et al. (2005) prepared ovine paste rennet by slaughtering suckling lambs in which milk-clotting activity of 95 IMCU/g and 81.69% chymosin were found. In Feta cheese, Moutzou et al. (2004) used artisinally-made liquid bovine and lamb rennet with 70% chymosin, and found very low levels of clotting activity and lipase (31 IMCU/mL and 1.67 U/mL, respectively).

There is no doubt that the production of cheeses with this lamb paste rennet (FFA) would contribute to the development of new types of commercially viable cheese with interesting flavors.

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