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A new technological approach for ripening acceleration in cooked cheeses: Homogenization, cooking and washing of the curd

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ABSTRACT

An innovative hard cooked cheese making routine that included the use of homogenization of milk fat, un-pasteurized cheese milk, cooking temperature modification and the inclusion of a curd washing step was studied on lipolysis, proteolysis, composition, volatile compounds profile and sensory attributes of cheeses. Experimental and traditional Reggianito type cheeses were made at pilot scale and analyzed during ripening (45 and 60 days). Ripening acceleration was observed in experimental cheeses based on an increase of lipolysis and proteolysis reactions, which resulted in both a volatile profile characterized by compounds derived from milk fat degradation and the intensification of flavour in the first months of ripening.

1. Introduction

Hard cooked cheeses enzymatically coagulated, such as Reggianito cheese, are characterized by a ripening process that takes long periods, between 6 and 24 months. During this process, complex series of biochemical reactions, involving the metabolism of residual lactose, citrate and lactate, lipolysis, proteolysis, and catabolism of amino acids and free fatty acids are produced. These transformations influence the texture and functionality of cheeses, as well as the flavour profile of each variety (Fox & McSweeney, 2017; McSweeney, 2004).

The conventional strategies used to accelerate cheese ripening are based on the addition of proteases and exogenous lipases (El Soda, 1993; Kheadr, Vuillemard, & El-Deeb, 2002), the use of adjunct cultures to intensify or diversify cheese flavour or the increase of ripening temperature (Khattab, Guirguis, Tawfik, & Farag, 2019). Some of these strategies were successfully employed by our research group for Reggianito type cheeses (Cuffia, Bergamini, Wolf, Hynes, & Perotti, 2019; Peralta, Bergamini, Wolf, Perotti, & Hynes, 2017; Sihufe et al., 2010). However, there are scarce examples of the intervention of cheese making technologies in order to increase the enzymatic reactions performed by native enzymes and coagulant.

The use of cheese milk homogenization is limited to certain varieties in which high levels of lipolysis are desired (e.g. blue cheeses) or when texture is intended to be improved (e.g. low-fat cheeses). Nevertheless, it has been reported that this technological step could cause detrimental effects during cheese making (Zamora, Ferragut, Jaramillo, Guamis, & Trujillo, 2007). As for curd washing and cooking temperature during cheese making, previous studies at lab scale in experimental models and miniature cheeses showed that these parameters influenced plasmin and coagulant activities during storage (Somers & Kelly, 2002; Vélez et al., 2015). However, experiments envolving these technological parameters on cheese makings trials in pilot scale before moving to industrial scale are necessary to validate the results obtained in miniature cheese models or prepared in laboratory conditions, especially if it is wished to demonstrate that the proposed changes are robust or will have some impact on the actual food matrix (Hunter, McNulty, & Banks, 1997). For this, pilot or industrial scale tests and, in addition, sensory evaluations are required.

The focus of the present work was to develop a cheese making routine at pilot scale, intended to accelerate ripening by enhancing the activities of the milk enzymes and coagulant; we selected the following variables to be studied: physical treatment of cream homogenization, un-pasteurized cheese milk, modification of the cooking or scalding temperature, and inclusion of a washing step of the curd during cheese

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making. These changes were aimed at increasing the activities of native enzymes: plasmin and lipoprotein lipase, and coagulant enzyme added and consequently favoring lipolysis, proteolysis and thus volatile compounds development during cheese ripening. The effects of the cheese making routine developed were also studied on the sensory profiles of cheeses.

2. Materials and methods

2.1. Experimental design

The influence of a technological strategy applied in the manufacture of hard cooked cheese was compared in respect to the traditional one. Two processes were carried out in parallel: one followed the traditional technology for Reggianito type cheese: pasteurized milk, cooking temperature at 52 °C without curd washing (T cheeses), another was performed applying a modified technology: fat fraction homogenized, unpasteurized cheese milk, cooking temperature 50 °C with curd washing (E cheeses). Experiences were performed in duplicate.

2.2. Milk pre-treatment

Fig. 1 shows the experimental scheme. A volume of 150 L bulk bovine milk (pH 6.7 ± 0.05) supplied by a nearby dairy plant (Milkaut S.A., Franck, Santa Fe, Argentina) was centrifuged to obtain skim milk (< 0,1% fat) and cream (> 40% fat). These fractions were mixed in order to prepare a cream of 20% fat; one portion was homogenized at 9 MPa/45 °C (Homogenizer 31M-3 TA, Gaulin Corporation, Boston, MA, USA) and the other portion was not homogenized. For milk standardization (50 L, 2.5% fat) for E and T cheeses, the homogenized and unhomogenized creams were blended with skim milk in the appropriate fractions, respectively.

2.3. Cheese making

Cheeses were made in parallel, operating 2 vats of 50 L each. For E cheeses, homogenized milk was heated at 33 °C. For T cheeses, the milk was pasteurized at 63 °C for 20 min and cooled in the vat until 33 °C. In both cases, $CaCl_2$ (Merck, Darmstadt, Germany) was added to a final concentration of 0.014% w/v. Lactic acid (15 g/L)was added to reach the target pH of Reggianito cheese milk (pH 6.3–6.4). Then, the DVS commercial starters *Lactobacillus helveticus* (LH-B02) and *Lactobacillus bulgaricus* (Lb-12) (Chr. Hansen Argentina, Quilmes, Argentina) were added in a concentration enough to reach 10^6 CFU/mL of milk. After that, 0.62 g of coagulant (Maxiren 150, France) were added per vat, previously suspended in 50 mL of distilled water just before use. Once the coagulum acquired the adequate firmness, it was cut until the grains acquired the size of a grain of rice, and the mixture of whey and curd particles was heated under stirring. For both type of cheeses, the heating proceeded until 45 °C at 1 °C/min. In that moment, in E cheeses 30 L of whey was replaced by a lactose solution (4.5% w/v at 45 °C). Then, E and T curds and whey were heated at 50 °C and 52 °C (< 1 °C/min), respectively. Finally, stirring was stopped, the curd went to the bottom and the whey was separated. Curds were put into molds and pressed for 12 h, brined for 3 days in saturated brine at 12 °C. Cheeses of approximately 3.5 kg were obtained and ripened for 60 days at 12 °C and 80% relative humidity.

Samples were taken at 45 and 60 days, according toISO (2008b).

2.4. Analytical determinations

2.4.1. Milk and cream fat content

Total fat analysis was performed in skim milk and cream by Gerber method (Bradley et al., 1992).

2.4.2. Cheese composition and microbial counts

At both sampling days, moisture (ISO, 2004) and pH (Bradley et al., 1992) were determined. Fat and protein contents were assessed at the end of ripening by Gerber-Van Gulik method (ISO, 2008a) and macro-Kjeldahl method (ISO, 2011), respectively.

Thermophilic lactobacilli populations were determined at 45 and 60 days by plating cheese sample dilutions on skim milk agar and counting colonies after 48 h of incubation at 43 °C.

2.4.3. Cheese proteolysis

2.4.3.1. Nitrogen fractions. Nitrogen content by the macro-Kjeldahl method (ISO, 2011) was performed in the fractions of the cheese soluble extract at pH 4.6 (SN), at 12% (w/v) trichoroacetic acid (SN-TCA) and at 2.5% (w/v) phosphotungstic acid (SN-PTA) (Bergamini, Hynes, & Zalazar, 2006; Gripon, Dezmazeaud, Le Bars, & Bergere, 1975). Results were expressed as percentage of total nitrogen (SN/TN). Fractions were assessed at 60 days of ripening, except for SN, which was determined at both sampling times.

2.4.3.2. Electrophoresis. The insoluble residue at pH 4.6 from 60 days' samples was analyzed by Urea-PAGE in a Mini-Protean II cube (BioRad Laboratories, California, USA) by Andrews (1983) method, with a concentration of acrylamide of 7.5% (Hynes, Delacroix-Buchet, Meinardi, & Zalazar, 1999). Proteins were stained by Coomasie Blue G-250.

2.4.4. Plasmin and plasminogen activities

Plasmin and plasminogen activities were determined on cheeses at 45 and 60 days as Richardson and Pearce (1981). This method is based on the cleavage of a non-fluorescent substrate, Nsuccinyl- L-alanyl-L-phenylalanil-L-lysyl-7-amido-4-methyl coumarin (Sigma, St. Louis, MO, USA) by plasmin, to yield a fluorescent product: 7-amino-4-methyl coumarin (AMC). Plasminogen activity was measured by difference after its activation with urokinase (EC 3.4.21.73, Sigma, St. Louis, MO, USA). Fluorescence intensity was monitored for 30 min at 5 min intervals on a fluorescence spectrophotometer (Hitachi F-2000, Japan) with excitation and emission wavelengths of 380 and 460 nm, respectively. In order to compare the different cheeses, the activity values were normalized to the dry matter content of the cheeses. Plasmin and plasminogen activities were defined as nmol of AMC released per gram (of dry matter) per minute at pH 7.5 and 25 °C.

2.4.5. Lipolysis

The concentrations of FFA were determined on cheeses at 45 and 60 days. Extraction of fat matter, isolation of FFA, derivatization to ethyl esters and quantification of FFA were performed as published in Perotti, Bernal, Meinardi, and Zalazar (2005), with some modifications. A PerkinElmer model GC-9000 series gas chromatograph (PerkinElmer Corp., Waltham, MA, USA) equipped with a flame ionization detector (FID) and with a split/splitless injector was utilized. Ethyl esters FFA were separated on a fused silica capillary column (60 m × 0.25 mm; HP-INNOWax, Agilent J&W, USA) coated with a bonded polyethylene glycol stationary phase (0.25 μm layer thickness); carrier gas H_2 flow at 2 mL/min; 1 μL injection; split mode injection at 1:50 splitting ratio; injector and detector temperatures at 250 and 300 °C, respectively; oven temperatures running from 75 °C (1.5 min) up to 150 °C (10 min) at 8 °C/min, then increased to a final temperature of 245 °C (15 min) at 10 °C/min. Resultswere expressed as μmol of FFA per 100 g of fat.

2.4.6. Volatile compounds

Volatile compounds in 45 and 60 days old cheeses were determined by SPME-GC-FID/MS. The procedure and analysis conditions were essentially the same that those described previously (Vélez, Hynes, Meinardi, Wolf, & Perotti, 2017). All analyses were performed in duplicate. Results were expressed in percentages of chemical groups of compounds and in arbitrary units of areas.

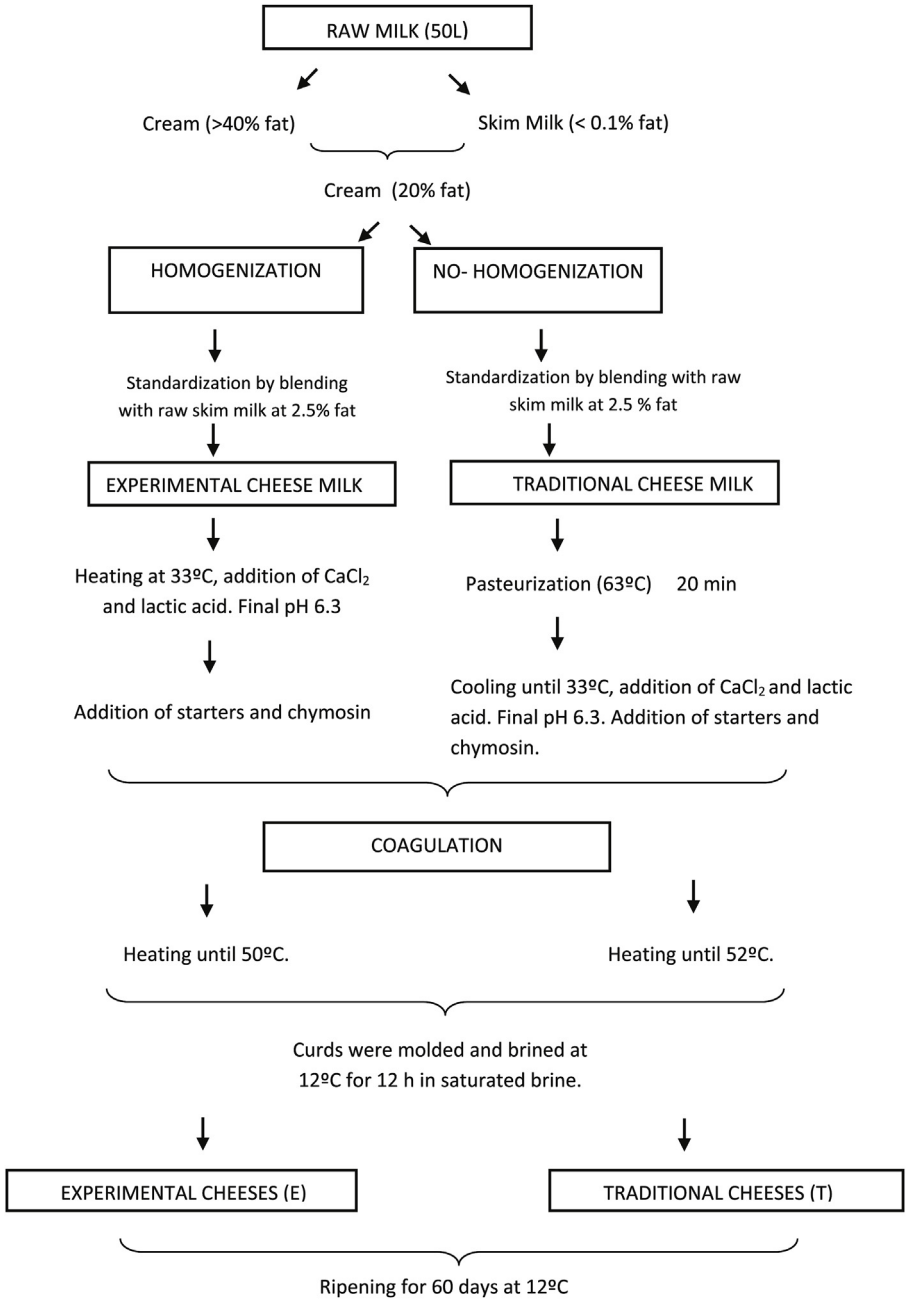

Fig. 1. Schema of cheese making.

2.4.7. Sensory analysis

2.4.7.1. Samples presentation and considerations. The samples were evaluated at 45d and 60d of storage. At the first sample time, the panel did not taste samples, as Argentinean Food Legislation does not allow tasting cheeses made with raw milk (ANMAT, 2014). Indeed, legislation excludes from the obligation to pasteurize the milk if it is destined to the manufacture of cheeses as long as they ripen at a temperature higher than 5 °C during a period greater than or equal to 60 days.

For internal appearance evaluation, the half of whole cheeses, E and T, were used. For aroma, flavour and manual texture evaluation, E and T cheese cubes of 1 cm side were cut from the cheese center, discarding the edges.

2.4.7.2. Trained sensory panel. A panel of 10 assessors was selected and trained following the guidelines of ISO (1993). They all had a minimum of 100 h experience in discrimination and descriptive tests.

Initially assessors generated their individual descriptors and during subsequent sessions the list of descriptors was reduced by consensus, as many of the words had a common sensory stimulus (Stone & Sidel, 1993). Where possible, references were presented to standardize the meaning of descriptors and to help panel uniformity. For 45d cheese samples, 3 training sessions and 2 measurement sessions were necessary. For 60d samples, were necessary 2 training sessions because the flavour attribute was added, and 2 measurement sessions were also needed. In both cases the measurements were performed by duplicate.

Aroma, flavour and manual texture evaluation was carried out in individual booths with controlled temperature and lighting. Internal appearance evaluation, was carried out in a chamber equipped with artificial, daylight-type fluorescent lighting.

2.4.8. Statistical analysis

One-way ANOVA was applied to composition and proteolysis in order to assess differences between the two cheese making technologies. As for enzymatic activities, lipolysis, and volatile compound profiles, two-way ANOVA was performed to study de influence of the technology and ripening time. Tukey test for pairwise comparison at 95% confidence level was employed.

Regarding sensory analysis, for internal appearance, aroma and manual texture, analysis of variance (ANOVA) was applied to the data considering the assessors as a random effect and ripening time * the technology used (E and T cheeses) as fixed effects. For flavour attribute, ANOVA was applied for each descriptor to analyze the effect of the technology used, and considering the assessors as a random effect.

2.5. Results and discussion

2.5.1. Physicochemical composition and microbial counts

In the present work, a new technological approach was compared with the traditional procedure employed in hard cooked cheese making technology. At 60 days, ripening was stopped in order to avoid sensory defects, as E cheeses had already reached high levels of lipolysis at 45 days (see below).

For both times of ripening, the technological changes applied did not influence pH, moisture, fat and protein contents (Table 1). Moreover, no changes were evidenced through ripening time, except for pH, which increased significantly. Values were similar to those found in miniature hard cooked cheeses (Reggianito type) (Vélez, Perotti, Wolf, Hynes, & Zalazar, 2010), except for moisture content, which presented higher values in cheeses from the present study. This result is expected, as cheeses had only two months of ripening.

As for microbial counts a maximum of 8 log CFU/g was reached at 45 days for T and E cheeses (Table 1), and ripening time did not have a significant influence on results ($P > 0.05$).

2.5.2. Plasmin and plasminogen activities and proteolysis

Cheese making technology and ripening time did not have influence on plasmin and plasminogen activities ($P > 0.05$, Table 2). Although the curd was washed in E cheeses, no noticeable changes have been observed. Washing of the curd is a known technological procedure that increases the plasmin activity in cheeses due to the removal of plasminogen activator inhibitors and plasmin inhibitors (Delacroix-Buchet & Fournier, 1992; Somers & Kelly, 2002) Previous results of our research group in minicurds of hard cheeses have shown that washing of the curd increased the enzyme activity (Vélez, Perotti, Candioti, Bergamini, & Hynes, 2016). However, the modifications of the protein network due to the inclusion a greater number of fat globules in the casein matrix due to homogenization could have modified the enzymatic reactions *in situ* (Iucci, Lanciotti, Kelly, & Huppertz, 2008). Several studies have indicated that differences in processing technology influence the micro-structure of cheeses and the biochemistry of ripening (Kelly, Huppertz, & Sheehan, 2008; Lanciotti et al., 2004; Lopez, Maillard, Briard-Bion, Camier, & Hannon, 2006).

Table 1
Physicochemical composition and nitrogen fractions of traditional (T) and experimental (E) cheeses, at 45 and 60 days of ripening.

Parameters	Cheese type	
	Traditional (T)	Experimental (E)
Moisture (%) (45days)	39 ± 1[a]	41.6 ± 0.8[a]
Moisture (%) (60 days)	38 ± 1[a]	41 ± 1[a]
FDM % (60 days)	41 ± 1[a]	43.2 ± 0.5[a]
PDM % (60 days)	48 ± 1[a]	49 ± 2[a]
pH (45days)	5.40 ± 0.02[a]	5.40 ± 0.03[a]
pH (60 days)	5.45 ± 0.04[a]	5.47 ± 0.03[a]
SN pH 4,6/TN (45 days)	11.4 ± 0.9[a]	13 ± 1[b]
SN pH 4,6/TN (60 days)	13.2 ± 0.6[a]	15.6 ± 0.8[b]
SN-TCA/TN (60 days)	10.5 ± 0.7[a]	14 ± 1[b]
SN-PTA/TN (60 days)	5.6 ± 0.5[a]	8.3 ± 0.8[b]
log CFU/g (45 days)	8.07[a] ± 0.1	8.19[a] ± 0.23
log CFU/g (60 days)	7.77[a] ± 0.06	7.79[a] ± 0.26

FDM Fat in dry matter.
PDM Protein in dry matter.
CFU Colony Forming Unit of thermophilic lactobacilli population.
Soluble nitrogen (SN) fractions were expressed as percentages of total nitrogen (TN).
Means in a row with different lowercase letters indicate significant differences.

All values obtained for SN pH 4,6/TN were comparable with those reported for Reggianito cheeses made at pilot scale and Reggianito commercial cheeses (Candioti et al., 2002; E. R.; Hynes, Bergamini, Suárez, & Zalazar, 2003; Wolf, Perotti, Bernal, & Zalazar, 2010) and to those found in miniature cheeses (Vélez et al., 2010). Besides, this index was higher in E samples, at both times of ripening. This increase could be correlated with a higher coagulant activity due to the lower cooking temperature applied during making of this type of cheese, as the intensity band of the αs1-I peptide observed in the electrophoretogram was more intense in E cheeses, indicating a higher production. Fig. 2 shows, as an example (one replicate), the electrophoresis profiles for E and T cheeses at both times of ripening.

In the analysis of the nitrogen fractions, the SN-TCA/TN and the SN-PTA/TN values were also higher for E cheeses than in T cheeses, indicating a deeper secondary proteolysis in E versus T cheeses. This effect could be due to the fact that raw milk was employed in E cheeses, as similar results have been reported (Bachmann et al., 2011; Grappin & Beuvier, 1997). Indeed, the differentiate contribution of the non starter lactic acid bacteria (NSLAB) in the formation of small and medium peptides and free amino acids in raw milk cheeses versus those of pasteurized milk cheeses has been identified as one of the causes for the greater proteolysis and peptidolysis in cheeses (Albenzio et al., 2001; McSweeney, 2004).

2.5.3. Lipolysis

The free fatty acids (FFA) profiles of all cheeses are shown in Table 3. Palmitic and myristic acids were the most abundant saturated fatty acids, and oleic acid was the major unsaturated fatty acid. This is expected, as they are the main constituents of cow milk triacylglycerides (MacGibbon & Taylor, 2006). Besides, FFA pattern was similar to that found for Reggianito cheeses made at pilot scale and at industrial scale previously (Perotti et al., 2005; Wolf et al., 2010).

Regardless of the ripening time, the individual concentrations of all FFA were higher in E cheeses. According to our knowledge, there is scarce information about the effect of low homogenization on the FFA concentrations during cheese ripening. Some authors reported an increase in the lipolysis degree (calculated as the sum of the individual concentrations) after employing a homogenization step in cheese milk (Brito, Manríquez, Molina & Pinto, 2003; Deegan et al., 2013; Michalski et al., 2004). Lipolysis levels found in E cheeses were similar to our previous work (Vélez et al., 2017) in miniature Reggianito-type cheeses made with homogenized cream (20% fat, the same as present work), while the values for T cheeses were lower than control cheeses reported in that publication, probably because raw cheese milk was employed in that case. In fact, FFA values from T cheeses were comparable to miniature Reggianito-type cheeses made with heated milk (65 °C/20 min) by Vélez et al. (2010), with some differences probably regarding to the smaller scale (5L) and higher ripening time (90 days in that work).

Through the two sample times studied, all FFA concentrations remained without changes, except for C4:0 which decreased ($p < 0.05$) in T and E cheeses. In our previous work (Vélez et al., 2017), we described that the majority of FFA concentrations in cheeses made with homogenized cream increased from 3 to 45 days, but then the levels remained constant up to 90 days of ripening. As for control cheeses made with raw milk, in that work we found a steady increase of FFA

Table 2
Plasmin and plasminogen activities of traditional (T) and experimental (E) cheeses, at 45 and 60 days of ripening.

Technology	Traditional (T)		Experimental (E)		Significance of the effects		
Ripening Time	45d	60d	45d	60d	F1	F2	2 F1xF2
Plasmin	8.57 ± 0.80	10.99 ± 0.95	3.77 ± 0.20	4.10 ± 0.05	NS	NS	NS
Plasminogen	40 ± 10	37.7 ± 80	14 ± 3	394 ± 10	NS	NS	NS

NS, No Significant.
F1 = Cheese making technology applied; F2 = Ripening Time.
*P < 0.05.
Values are means ± SD.

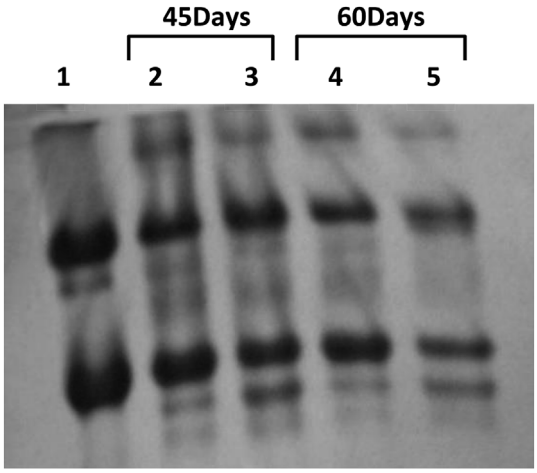

Fig. 2. Urea-PAGE electrophoretograms of traditional (T) and experimental (E) cheeses, at 45 and 60 days of ripening. Lane 1: standard of caseinate; lanes 2 and 3: traditional and experimental (T and E) cheeses at 45 days, lanes 4 and 5: traditional and experimental (T and E) cheeses at 60 days.

from 3 to 90 days. The increasing trend from the early beginning of storage up to the end has been widely reported for the majority of cheese varieties, showing that fat is accessible to the action of the enzymes present in the food matrix throughout the maturation (Atasoy & Türkoğlu, 2009; Hickey, Kilcawley, Beresford, & Wilkinson, 2007; Malacarne et al., 2009; O'Mahony, Sheehan, Delahunty, & McSweeney, 2006; Voigt, Chevalier, Qian, & Kelly, 2010; Werner, Nielsen, Ardö, Rage, & Antila, 1993). As for Reggianito cheeses made by the traditional way a few detailed studies with intermediate sample times during cheese ripening have been reported. Sihufe et al. (2007) analyzed the lipolysis in commercial Reggianito cheeses matured at 12 °C during 2, 4 and 6 months. They found that the FFA concentrations had an increasing trend until 4 months of ripening, generally remaining without changes during the last 2 months of ripening. More recently, Ceruti, Zorrilla, Sabbag, Costa, and Sihufe (2015) also studied commercial Reggianito cheeses ripened at 12 °C and sampled at intermediate times (2, 4 and 6 months) finding in this case no influence of the time of ripening on FFA profiles.

The pattern observed for individual concentrations of FFA was reflected in the degree of lipolysis (total free fatty acids TFFA, mg/kg cheese). At 45 and 60 days, mean values were higher in E than in T cheeses ($p < 0.05$), and they did not change through time. Values obtained for E cheeses are higher than those obtained in matured Reggianito cheeses (Perotti et al., 2005; Wolf et al., 2010). Values found in T cheeses were moderate and within the ranges 1100–3000 mg/kg reported for commercial cheeses and cheeses elaborated at pilot scale: (Ceruti et al., 2015; Perotti, Bernal, Wolf, & Zalazar, 2008; Sihufe et al., 2007; Wolf et al., 2010).

As published previously (Brito, Manríquez A, Molina C, & Pinto C, 2003; Michalski et al., 2004; Vélez et al., 2017), the homogenization step performed increased the percentage of fat hydrolyzed. In the present work, at 45 days, T and E cheeses had 1 and 8% fat hydrolyzed,

Table 3
Free fatty acids profiles (μmol/100 g fat) of traditional (T) and experimental (E) cheeses, at 45 and 60 days of ripening.

Technology	Traditional (T)		Experimental (E)		Significance of the effects		
Ripening Time	45d	60d	45d	60d	F1	F2	F1xF2
$C_{4:0}$	516^{BC} ± 50	190^{BD} ± 23	1562^{AC} ± 125	1200^{AD} ± 101	*	*	NS
$C_{6:0}$	40^{B} ± 10	14^{B} ± 3	377^{A} ± 80	394^{A} ± 10	*	NS	NS
$C_{8:0}$	28^{B} ± 5	14^{B} ± 2	273^{A} ± 50	264^{A} ± 40	*	NS	NS
$C_{10:0}$	59^{B} ± 25	43^{B} ± 6	608^{A} ± 130	577^{A} ± 35	*	NS	NS
$C_{12:0}$	60^{B} ± 30	59^{B} ± 11	463^{A} ± 100	409^{A} ± 35	*	NS	NS
$C_{14:0}$	661^{B} ± 130	640^{B} ± 153	7494^{A} ± 400	5805^{A} ± 300	*	NS	NS
$C_{16:0}$	1357^{B} ± 151	1135^{B} ± 110	12289^{A} ± 360	8690^{A} ± 200	*	NS	NS
$C_{18:0}$	399^{B} ± 50	328^{B} ± 16	2646^{A} ± 150	1782^{A} ± 125	*	NS	NS
$C_{18:1}$	1191^{B} ± 101	1178^{B} ± 14	6583^{A} ± 200	4586^{A} ± 150	*	NS	NS
$C_{18:2}$	181^{B} ± 80	260^{B} ± 34	958^{A} ± 110	804^{A} ± 80	*	NS	NS
TFFA	4491^{B} ± 633	3860^{B} ± 329	33252^{A} ± 500	24510^{A} ± 350	*	NS	NS
%SCFFA	13.02^{a} ± 0.37	5.65^{b} ± 0.02	6.66^{b} ± 1.77	7.83^{b} ± 1.77	*	*	*
%MCFFA	2.59 ± 0.87	2.66 ± 0.66	3.21 ± 0.89	4.15 ± 0.89	NS	NS	NS
%LCFFA	84.39^{a} ± 0.50	91.69^{a} ± 0.67	90.13^{b} ± 2.67	88.02^{b} ± 2.67	NS	NS	*

NS, No Significant.
F1 = Cheese Making Technology; F2 = Ripening Time.
^{A,B} Values within rows with different superscripts differ significantly ($P < 0.05$) for F1.
^{C,D} Values within rows with different superscripts differ significantly ($P < 0.05$) for F2.
^{a,b} Values within rows with different superscripts differ significantly ($P < 0{\cdot}05$), when F1 × F2 is significant.
*P < 0.05.
Values are means ± SD.

Table 4
Percentages of chemical groups of volatile compounds.

Technology	Traditional (T)		Experimental (E)		Significance of the effects		
Ripening Time	45 d	60 d	45 d	60 d	F1	F2	F1xF2
Ketones	25.98 ± 0.15^{b}	17.30 ± 0.06^{c}	34.91 ± 0.02^{a}	9.18 ± 0.73^{d}	NS	*	*
Aldehydes	10.20 ± 1.54^{a}	6.55 ± 0.11^{b}	1.69 ± 0.04^{c}	3.41 ± 0.61^{bc}	*	NS	*
Alcohols	23.78 ± 0.91^{AD}	29.23 ± 2.03^{AC}	8.48 ± 0.18^{BD}	17.37 ± 2.98^{BC}	*	*	NS
Esters	5.36 ± 0.4^{b}	9.64 ± 0.02^{a}	2.53 ± 0.04^{d}	4.50 ± 0.23^{c}	*	*	*
Acids	34.67 ± 2.34^{BD}	37.28 ± 2.19^{BC}	52.39 ± 0.09^{AD}	65.54 ± 4.55^{AC}	*	*	NS

F1: Technology applied during cheese manufacture.
F2: Ripening time.
A,B, Values within rows with different superscripts differ significantly ($P < 0.05$) for F1.
C,D, Values within rows with different superscripts differ significantly ($P < 0.05$) for F2.
a,b, Values within rows with different superscripts differ significantly ($P < 0.05$), when F1xF2 significant.
* indicates significant effect at $P < 0.05$, NS indicates not significant.

respectively. These values remained without changes up to 60 days, showing that the increased fat accessibility due to homogenization enhanced lipolysis reactions. E lipolysis values were comparable to those found in mould ripened cheeses such as Camembert or Danablue, in which a homogenization step is applied in cheese milk (Ardö, 2011; Collins, McSweeney, & Wilkinson, 2003).

FFA were classified in short (SCFFA), medium (MCFFA) and large (LCFFA) and the group percentages were calculated (Table 3) in order to assess whether certain FFA were generated preferentially. For SCFFAA and LCFFA, the interaction between the technology applied for cheese manufacture and ripening time was significant. T cheeses at 45 days presented the highest % SCFA and the percentages of E cheeses at both ripening times were similar to T cheeses sampled at 60 days. Results were in concordance with % LCFFA, as T cheeses at 45 days presented the lowest values.

2.5.4. Volatile compounds

Forty-two compounds were identified in cheese samples: 8 ketones, 5 aldehydes, 13 alcohols, 6 esters and 10 acids. The percentages of the different chemical classes calculated in relation to the total of peak areas are shown in Table 4.

Overall, acids, ketones and alcohols were the groups prevalent in the volatile fraction of cheeses. Regardless the type of cheese and ripening time, acids constituted the main chemical group in all cheeses. For acids and alcohols, both technology and the ripening time had a significant effect, and interaction effects were not observed. The highest percentages of acids and alcohols were reached in E and T cheeses, respectively, and their proportions increased during ripening. For ketones, aldehydes and esters, interaction effects were observed. Ketones and aldehydes percentages were the highest in E and T cheeses at 45 days, respectively. As for ester group, the highest values were detected in T cheeses.

Volatile compounds in cheeses have different origins. They can derive mainly from glycolysis, lipolysis and proteolysis and the secondary reactions involving their catabolic products lactate/citrate, free amino acids and free fatty acids, respectively (Boscaini, van Ruth, Biasioli, Gasperi, & Mark, 2003). Besides, several compounds can originate from more than one metabolic pathway.

In grana-type cheeses, lipolysis and free fatty acid catabolism are important biochemical events that occur during ripening. Enzymes with lipolytic activity can act on triglycerides and release linear carboxylic acids with more than 4 atom carbon (Curioni & Bosset, 2002). In particular, short and medium-chain fatty acids (C_4 to C_{12}) are important contributors to grana-type cheese flavour (Qian & Reineccius, 2002, 2003; Barbieri et al., 1994; Moio & Addeo, 1998). In previous studies, the acidic fraction of grana type cheeses such as Parmigiano Reggiano, Grana Padano and Reggianito represented between 32 and 38% of the total compounds (Wolf & Perotti, 2013).

On the other hand, fatty acids act as precursors of other volatile compounds such as methyl ketones, alcohols, lactones, aldehydes and esters (Collins et al., 2003). Methyl ketones are typical volatile compounds formed by β-oxidation of free fatty acids to β-ketoacids and their subsequently decarboxylation to methyl ketones with the loss of one carbon atom (Molimard & Spinnler, 1996). The presence of some aldehydes and alcohols in cheeses is also related to degradation of milk fat. Straight chain aldehydes like hexanal and heptanal are major secondary products of an autooxidation process of unsaturated fatty acids via hydroperoxides (Barbieri et al., 1994). On the other hand, secondary alcohols such as 2-propanol, 2-pentanol, 2-heptanol, etc., and linear-chain primary alcohols like 1-propanol, 1-butanol and 1-pentanol, are produced by reduction of their corresponding methyl ketones and aldehydes, respectively, through the activity of lactic acid bacteria dehydrogenases or by chemical reactions through the decrease of redox potential (Buchin et al., 1998). Esters are typical compounds derived from milk fat. Ethyl esters, mainly, ethyl acetate, ethyl butanoate and ethyl hexanoate have been reported as important contributors to fruity notes in hard grana-type cheeses (Boscaini et al., 2003; Curioni & Bosset, 2002). The biosynthesis of flavour-active esters in dairy systems proceeds through two enzymatic mechanisms: esterification and alcoholysis (Liu, Holland, & Crow, 2004). Esterases and lipases provided by LAB can catalyze the synthesis of esters in culture medium (Abeijón Mukdsi, Medina, Alvarez, & González, 2009) and under cheese ripening conditions (Richoux, Maillard, Kerjean, Lortal, & Thierry, 2008).

In our study, approximately the half of the volatile compounds identified in all cheeses (twenty-three volatile compounds) can be derived from lipolysis and fatty acid degradation (Table 5). They represented between 53 and 87% of the total area of compounds and thus, this fact reveals the important contribution of lipolysis and fatty acid catabolism to the production of volatile compounds in these cheeses. For this reason, in the present study only those compounds derived from lipolysis process were discussed.

For the majority of volatile compounds, the interaction effect was significant; thus, the two factors studied influenced on the cheese volatile profiles.

Among ketones group, diacetyl + 2-pentanone (unresolved peak) and 2-heptanone were the most abundant (taking into account the peak area values); they ranged from 50 to 85% of total ketones in the analyzed cheeses. With exception of 2-hexanone, the levels of methyl ketones were higher in E cheeses at 45 days and decreased throughout ripening. In T cheeses were recorded the lowest levels, which remained unchanged.

As for aldehydes, heptanal was detected only at 60 days and no differences were found between both types of cheeses.

The most representative alcohols were 2-propanol, 1-propanol, 2-pentanol and 1-butanol. For 1- and 2-propanol, higher values were found in E and T cheeses respectively, and their levels increased with

Table 5
Volatile compounds (peak area values x10^3) of traditional (T) and experimental (E) cheeses, at 45 and 60 days of ripening.

Technology (F1)	Traditional (T)		Experimental (E)		Significance of the effects		
Ripening Time F2)	45d	60d	45d	60d	F1	F2	F1x F2
Ketones							
2-propanone	37.8 ± 1.6^{b}	42.9 ± 1.6^{b}	87.0 ± 4.2^{a}	38.5 ± 6.2^{b}	*	*	*
Diacetyl + 2-pentanone	349.6 ± 22.8^{b}	217.2 ± 6.3^{c}	2458.2 ± 41.9^{a}	199.7 ± 29.0^{c}	*	*	*
2-hexanone	78.8 ± 11.9^{b}	75.8 ± 6.1^{b}	90.1 ± 5.6^{b}	132.1 ± 16.5^{a}	*	NS	*
2-heptanone	540.7 ± 70.6^{b}	501.6 ± 11.7^{b}	1149.1 ± 15.2^{a}	337.4 ± 30.5^{c}	*	*	*
2-nonanone	88.6 ± 17.1^{bc}	112.6 ± 7.8^{b}	306.1 ± 13.7^{a}	43.1 ± 6.6^{c}	*	*	*
Aldehydes							
Heptanal	n.d^{AD}	17.5 ± 2.1^{AC}	n.d^{BD}	14.0 ± 1.8^{BC}	*	*	NS
Alcohols							
2-propanol	54.8 ± 2.4^{AD}	61.5 ± 6.4^{AC}	12.0 ± 2.1^{BD}	37.3 ± 7.9^{BC}	*	*	NS
1-propanol	90.7 ± 7.8^{BD}	123.4 ± 10.5^{BC}	129.9 ± 4.9^{AD}	197.3 ± 21.0^{AC}	*	*	NS
2-pentanol	57.9 ± 4.6^{c}	99.7 ± 3.3^{b}	157.0 ± 5.1^{a}	95.3 ± 2.9^{b}	*	*	*
1-butanol	53.3 ± 3.4^{b}	58.2 ± 7.4^{b}	60.1 ± 3.0^{b}	128.4 ± 12.5^{a}	*	*	*
1-pentanol	35.3 ± 4.5^{a}	11.0 ± 0.3^{b}	10.8 ± 1.4^{b}	19.0 ± 1.0^{b}	*	*	*
2-heptanol	5.2 ± 1.4^{d}	15.5 ± 0.9^{c}	38.1 ± 0.4^{a}	34.8 ± 2.6^{b}	*	NS	*
1-hexanol	9.0 ± 0.7^{c}	6.0 ± 1.3^{d}	16.0 ± 1.1^{b}	37.2 ± 1.1^{a}	*	*	*
1-heptanol	n.d^{b}	26.8 ± 1.4^{a}	17.9 ± 0.4^{a}	21.2 ± 4.3^{a}	*	*	NS
Esters							
Ethyl butanoate	139.8 ± 16.4^{c}	341.6 ± 3.2^{a}	150.0 ± 5.2^{c}	249.7 ± 21.5^{b}	*	*	*
Ethyl hexanoate	11.6 ± 2.3b	98.7 ± 6.2a	118.7 ± 3.3a	119.2 ± 13.3a	*	*	*
Isoamyl butanoate	n.d^{D}	8.4 ± 0.6a^{C}	n.d^{D}	7.6 ± 0.4^{C}	NS	*	NS
Ethyl octanoate	n.d^{c}	11.6 ± 0.4^{b}	n.d^{c}	22.1 ± 2.9^{a}	*	*	*
Acids							
Butanoic	737.6 ± 137.6^{BD}	1407.6 ± 91.5^{BC}	3001.9 ± 8.9^{AD}	3624.8 ± 41.3^{AC}	*	*	NS
Hexanoic	240.1 ± 14.5^{B}	369.7 ± 25.6^{B}	2078.3 ± 89.8^{A}	2222.6 ± 98.6^{A}	*	NS	NS
Octanoic	68.7 ± 13.2^{B}	99.0 ± 6.2^{B}	506.4 ± 21.6^{A}	526.1 ± 72.5^{A}	*	NS	NS
Decanoic	44.3 ± 10.6^{c}	49.2 ± 1.9^{c}	205.6 ± 5.4^{b}	327.1 ± 20.3^{a}	*	*	*
Dodecanoic	12.0 ± 2.8^{c}	11.3 ± 0.3^{c}	24.6 ± 1.8^{b}	33.5 ± 5.9^{a}	*	*	*

Peak areas in arbitrary units (values are means ± standard deviation).
F1: Technology applied during cheese manufacture.
F2: Ripening Time.
A,B, Values within rows with different superscripts differ significantly ($P < 0.05$) for F1.
C,D, Values within rows with different superscripts differ significantly ($P < 0.05$) for F2.
a,b, Values within rows with different superscripts differ significantly ($P < 0.05$), when F1xF2 significant.
* indicates significant effect at $P < 0.05$, NS indicates not significant.

Table 6
Descriptors and references used for Sensory Profile of hard cheeses.

Descriptor	Reference (scale value reach by consensus)
Appearance internal	
Color intensity	Hard cheese elaborated with pasteurized milk and ripening by 4 months (6) a
Color intensity of edge	Hard cheese elaborated with pasteurized milk and ripening by 4 months (8)
Aroma	
Total intensity	Hard cheese elaborated with pasteurized milk and ripening by 4 months (6)
Milky-cream	Hard cheese elaborated with pasteurized milk and ripening by 4 months (0)
Acid	Hard cheese elaborated with pasteurized milk and ripening by 4 months (0)
Hot	Hard cheese elaborated with pasteurized milk and ripening by 4 months (6)
Flavor	
Total intensity	Hard cheese elaborated with pasteurized milk and ripening by 5 months (7)a
Milky-cream	Hard cheese elaborated with pasteurized milk and ripening by 5 months (1)
Acid	Hard cheese elaborated with pasteurized milk and ripening by 4 months (0)
Whey milk/sour milk	Hard cheese elaborated with raw milk and ripening by 3 months (2) [b]
Hot	Hard cheese elaborated with pasteurized milk and ripening by 5 months (7)
Salty	Hard cheese elaborated with pasteurized milk and ripening by 5 months (7)
After taste	Hard cheese elaborated with pasteurized milk and ripening by 5 months (6)
Manual texture	
Hardness	Hard cheese elaborated with pasteurized milk and ripening by 4 months (7)
Elasticity	Hard cheese elaborated with pasteurized milk and ripening by 4 months (2)
Fracturability	Hard cheese elaborated with pasteurized milk and ripening by 4 months (7)
Oily	Hard cheese elaborated with pasteurized milk and ripening by 4 months (7)
Gummies	Hard cheese elaborated with pasteurized milk and ripening by 4 months (1)

[a]Escuela Agrotécnica Salesiana, 25 de Mayo, Buenos Aires, Argentina.
[b] Luberriaga S.A., Gral. Viamonte, Buenos Aires, Argentina.

Table 7
Average sensory scores given by the panel for technology used (E and T cheese) effect. Anova F-probability values for samples cheese are also indicated.

Attribute/descriptor	T cheese	E cheese	Anova F-probability
Internal appearance			
Color intensity	4.1	3.0	< .001
Color intensity of edge	5.5	4.0	< .001
Aroma			
Total intensity	5.2	6.6	0.002
Milky-cream	3.4	1.6	0.016
Acid	0.3	1.3	0.055
Hot	2.8	5.0	0.006
Manual texture			
Hardness	5.9	5.3	0.167
Elasticity	3.0	3.0	0.488
Fracturability	5.8	6.0	0.459
Oily	6.6	6.8	0.347
Gummies	2.7	2.5	0.599

ripening time. 2-pentanol and 1-butanol reached the highest levels in E cheeses at 45 and 60 days, respectively. In addition, 2-heptanol and 1-hexanol had higher area values in E cheeses.

Among esters, ethyl butanoate and ethyl hexanoate were the most important from quantitative viewpoint. At 45 days, ethyl hexanoate reached the highest level in E cheeses which remained constant through 60 days; on the contrary, a marked increased was observed during ripening in T cheeses, reaching at 60 days the same values of E cheeses. The amounts of ethyl butanoate were similar in both types of cheeses at 45 days; then, an increase was detected during ripening, reaching the highest levels in T cheeses.

Table 8
Average flavour attribute given by the panel for traditional (T) and experimental (E) cheeses, at 60 days of ripening. Fisher-LSD values are also indicated.

Descriptors	Flavor		
	T	E	Fisher-LSD
Total intensity	5.4	7.6	1.0
Acid	0.2	1.3	0.9
Milky-cream	4.1	1.0	1.3
Whey milk/sour milk	0.4	2.3	1.6
Hot	4.2	7.0	0.9
Salty	4.7	6.9	0.7
After taste	4.5	6.7	0.6

In relation to acids, butanoic, hexanoic and octanoic were the most abundant. The formation of these compounds was accelerated by the modified technology applied. In fact, higher values were already found at 45 days in E cheeses which were even higher than those detected in T cheeses at 60 days.

2.5.5. Sensory analysis

2.5.5.1. Profile development. Assessors obtained eighteen descriptors; they are shown in Table 6 grouped by attributes with their corresponding definitions. Aroma and flavour descriptors are similar to those used by other authors (Heisserer & Chambers IV, 1993; Hough, Martinez, Barbieri, Contarini, & Vega, 1994; Piggot & Mowat, 1991). Manual texture descriptors definitions were done according to Muñoz (1986).

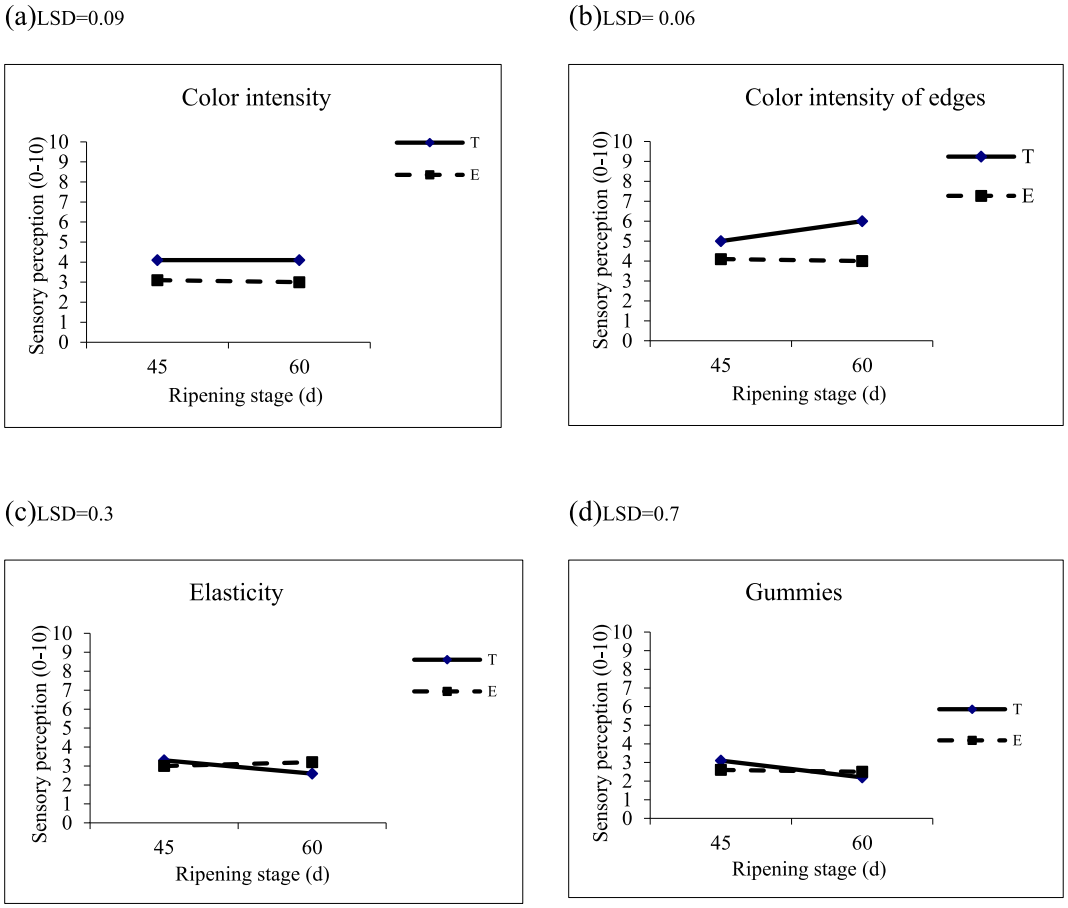

Fig. 3. Sensory perception (0–10) of technology used versus ripening stage (days) for (a) color intensity, (b) color intensity of edges, (c) elasticity and (d) gummies. Interaction Fisher-LSD-values are indicated.

2.5.5.2. Appearance, aroma and manual texture. To the best of our knowledge, there is scarce information regarding the effects of low homogenization on sensorial attributes of cheeses. In particular, studies on hard cooked cheeses have not been performed yet.

Analysis of variance showed that there were significant differences between E and T cheeses (technology used effect) for color intensity, color intensity of edge, total intensity of aroma, milky-cream and hot. Table 7 shows that T cheese sample had more color intensity, color intensity of edge and was more milky-cream while E sample had more total intensity of aroma and hot. The effect of the technology implemented was not evident for manual texture descriptors as well as acid.

Differences found in color could be due to the effective light scattering of the smaller homogenized fat globules. Similar results have been reported in Emmental cheeses (Deegan et al., 2013), fresh cheeses (Zamora, Ferragut, Juan, Guamis, & Trujillo, 2011) and turkish cheeses (Karaman & Akalın, 2013).

Fig. 3 shows the interaction plots: significant interaction effects between the treatment applied and the ripening time were found for color intensity, color intensity of edge, elasticity and gummies; the magnitude-type interaction for color intensity descriptor means that T cheese samples had grater intensity than E cheeses, at both ripening times.

E cheese sample were not affected by ripening time in none of 4 descriptors mentioned above, nevertheless T sample lost elasticity and gummies with the maturation and their edges were darkening. Piggot and Mowat (1991) found that texture of cheddar cheeses was more dependent on cheese making procedure and raw materials than on ripening period. Hough et al. (1994) found similar results for Reggianito grating cheese.

2.5.5.3. Flavour attribute. At 60 days, the technology used influenced significantly all flavour descriptors. Table 8 shows the averages obtained for each type of cheese. E cheese was perceived by the trained panel with greater intensity than T cheese for all evaluated descriptors, with the exception of milky-cream. These results are in concordance with the increased lipolysis found for E cheeses and with the differences found between both types of cheeses for volatile compounds profiles.

3. Conclusions

The present work has allowed us to validate an innovative technology for hard cooked cheese making, which accelerated ripening. The technological approach proposed included the use of raw milk, homogenization of milk fat fraction, reduction of cooking temperature and adding a curd washing stage. An increase of lipolysis and proteolysis reactions was verified. As for volatile compounds, the acid group dominated the profiles of cheeses. Moreover, the formation of some key flavour compounds derived from fat degradation such as 2-propanone, diacetyl + 2-pentanone, 2-heptanone, 2-nonanone, 2-pentanol, ethyl hexanoate and short-chain fatty acids reached the maximum levels at 45 days. These results were in concordance with the sensorial analysis, as an increase of hot taste and an overall intensification of flavour at 45 days of ripening were detected. Overall, the innovative cheese making approach applied accelerated cheese ripening without leading to atypical flavours.

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