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Effect of vegetable coagulant, microbial coagulant and calf rennet on physicochemical, proteolysis, sensory and texture profiles of fresh goats cheese

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Abstract In order to determine the technological suitability of vegetable coagulants for the manufacture of cheeses, two different aqueous extracts from cardoon (*Cynara cardunculus* subsp. *flavescens* and *C. cardunculus* subsp. *cardunculus*) and microbial coagulant (*Mucor miehei*) were compared with calf rennet. Optical sensors showed that the microbial coagulant had the highest clotting time (T_{\max}) whereas both rennet and vegetable coagulants had similar and lower milk clotting times. For most of the physicochemical (Colour CIELab, protein and fat) and textural (cohesiveness and springiness) parameters, no differences were observed between the different enzymes assayed. However, dry matter content, hardness, gumminess and chewiness showed significant differences, higher values being obtained in cheeses made with vegetable coagulants. As regards sensory properties, cheeses made with vegetable coagulants showed less firmness and whiteness but higher bitterness while microbial coagulant and calf rennet showed similar and higher values. No statistically significant differences were found in the soluble nitrogen or non-protein nitrogen fractions between the cheeses. The vegetable coagulants showed higher intensity bands in the Pre- α_s -casein region, which was related to higher α_s -casein proteolytic rates. The use of vegetable coagulants should enable manufacturers to produce fresh cheeses with new textural features, but to improve sensory profile, different technological strategies might be adopted.

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凝乳剂对新鲜羊奶干酪性质的影响

摘要：通过将两种不同刺棘蓟(*Cynara cardunculus* subsp. *flavescens* *Cynara cardunculus* subsp. *cardunculus*)的水浸提物和微生物凝乳剂与小牛凝乳酶进行比较,其目的是为了确定植物凝乳剂用于干酪加工的可行性。光学传感器测定结果表明,微生物凝乳剂的凝固时间最长(T_{max}),而凝乳酶和植物凝乳剂的凝固时间相似,但都低于微生物凝乳剂的凝固时间。对于物化特性(CIE $L^*a^*b^*$ 颜色、蛋白和脂肪)和质地(凝聚力和弹性)特性来说,不同酶对于这些参数的影响差异不显著。然而,不同酶对于干物质含量、硬度、胶粘性和咀嚼性影响显著。用植物凝乳剂加工干酪的参数值较高。对于感官特性来说,用植物凝乳剂加工的干酪具有较低的坚固度和白度,但是苦味较浓。然而,采用微生物凝乳剂和凝乳酶加工的干酪具有较高的坚固度和白度。三类凝乳剂加工干酪的可溶性氮和非蛋白氮差异不显著。植物凝乳剂加工的干酪蛋白经SDS-PAGE分离后在Pre- α s-酪蛋白区域发现有较强的蛋白带。植物凝乳剂使干酪生产生产出新的质地的新鲜羊奶干酪成为可能,但是为了提高这种干酪的感官特性,需要采用特定的加工技术。

Keywords Goat milk · Vegetable coagulant · Proteolysis · Sensory · Texture

关键词 羊奶 · 植物凝乳剂 · 蛋白酶解 · 感官 · 质地

1 Introduction

The development of products with new sensory and textural features is one of the main areas of innovation in cheesemaking. As part of this move, research has focused on the replacement of animal rennet by vegetable coagulant.

Milk coagulation is one of the most important steps in the cheese manufacturing process since it determines the final cheese properties. The difference in protein matrix degradation as a result of the agents used in the clotting process affects the changes that take place in the yield, the cheese texture (elasticity, fragility, adhesiveness, hardness, gumminess and chewiness) and the development of flavours (especially a bitter taste), through the production hydrophobic peptides and the hydrolysis of caseins (Queiroz Macedo et al. 1996).

The most commonly used rennet in this step is animal rennet which contains two enzymes (chymosin and pepsin) that break the Met₁₀₅-Phe₁₀₆ bond of the κ -casein present on the surface of the casein micelles. However, the increase in cheese production, coupled to a diminishing supply of natural animal rennet, is responsible for increases in the demand for alternative milk-coagulating sources. Due to this and a variety of factors (vegetarianism, religious beliefs, etc.), attention is being turned to the use of microbial coagulants and coagulants extracted from plants (Chazara et al. 2007).

Microbial coagulants have been used as animal rennet substitutes because they are easy to produce by fermentation, which allows an unlimited availability and therefore a lower price. In addition, there is no risk of disease transmission from ruminants, and their use is accepted by lacto-vegetarians. The most frequently used microbial coagulants are proteases derived from *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica*, the first of which, *R. Miehei*, having been used as a substitute of animal rennet for almost 40 years (Jacob et al. 2011). *C. parasitica* proteases cleave the Ser₁₀₄-Phe₁₀₅ bond in κ -casein, while *R. miehei* cleaves the Phe₁₀₅-Met₁₀₆ bond. On the other hand, the higher heat stability of the derivatives

obtained from *R. miehei* may be related to excessive proteolysis, with shorter ripening time and bitter cheeses. Coagulants with greater heat stability than calf rennet should be avoided, or, at the very least, the coagulation temperature should be varied to constrain excessive proteolysis (Sousa et al. 2001).

As regards plant coagulants, although a wide variety of plant-derived proteases is available for milk coagulation, their excessive proteolytic nature reduces cheese yield and increases the perception of bitter tastes, making its use more difficult for cheese production (Lo Piero et al. 2002). Nevertheless, the aqueous extract obtained from the flowers of *Cynara cardunculus*, which has been used for years in artisanal cheese making, especially in Mediterranean countries, southern Europe and western Africa, provides the desired effect. Spain and Portugal have a great variety of cheeses that use *Cynara* sp. as a plant coagulant (Tejada et al. 2008) particularly in the case of sheep milk, although it also gives good results with goat milk. Plant extracts have the ability to hydrolyse the κ -casein, leading to curd formation, and they are also the main enzymes responsible for β -casein hydrolysis (Roseiro et al. 2003). The flowers of *Cynara* sp. contain large amounts of proteolytic enzymes which are responsible for its clotting activity. Two of these enzymes, which are denominated cyprosinos or cynarases, have been purified and characterised, one (cyprosin A) similar to chymosin in activity and specificity, while the other (cyprosin B) is similar to pepsin (Verissimo et al. 1995). The advantages of using plant proteases is that such natural enzymes can be eaten by vegetarians and also may be certified as Kosher and Halal (Pino et al. 2009). The aim and the novelty of this study was to test and compare the effect of different coagulants (one microbial and two vegetable coagulants) on the physicochemical, textural, sensory and proteolytic parameters of a fresh goats cheese, in order to determine its technological suitability for goats cheese production.

2 Materials and methods

2.1 Milk

The milk treatment carried out was the same as that described in Rovira et al. (2011). Fifty litres of fresh goats milk were provided by the Veterinary Faculty experimental farm of the University of Murcia. The average fat, protein and dry matter content of milk of each day of manufacture (analysed by the providers) were 5.38 ± 0.01 , 3.72 ± 0.05 and $9.16 \pm 0.03\%$, respectively ($n=3$).

2.2 Coagulants

Dried samples from wild *C. cardunculus* subsp. *flavescens* species and the cultivated variety of *C. cardunculus* subsp. *cardunculus*, selected in the School of Agricultural Engineers (ETSIA) of Madrid were provided by the Agri-Food Technology Institute (INTAEX), Badajoz.

The extraction of vegetable coagulant from both wild and cultivated species was carried out by suspending 10 g of flowers, previously ground in 90 mL of water for 30 min. The resulting extract was filtered through a cheese cloth and centrifuged at

4,000 rpm for 10 min. The supernatant was filtered through filter paper and kept at 4 °C until use (never more than 2 days).

The microbial coagulant (50 L NG RENIPLUS, Caglio Star España S.A., Murcia) corresponded to a thermolabile enzyme obtained by *M. miehei* fermentation, with a clotting activity of 750 IMCU.mL⁻¹.

The enzymatic composition and milk-clotting activity of liquid calf rennet were provided by Caglio Star S.A. (Cieza, Murcia, Spain). The sample had 80% chymosin and a total milk-clotting activity of 180 IMCU.mL⁻¹.

2.3 Cheesemaking

Pasteurised Murciano–Granadina goats milk was used to manufacture the cheeses at the Food Technology Pilot Plant of the University of Murcia in a double-zero vat (Type 10 L, Pierre Guerin Technologies, Mauze, France). Two sensors were used, the CoAguLite™ (CL) sensor (model 5, Reflectronics Inc., Lexington, KY) (Payne et al. 1993), and a large field-of-view (LFV) sensor (prototype, University Kentucky, Lexington, KY). The sensor configuration details and their coupling to the cheese vat were the same as those described by Rovira et al. (2011). The LFV sensor response at 990 nm was used in this study and the light backscatter ratio for the LFV sensor (R_{LFV}) was obtained as described by Fagan et al. (2008).

Ten litres of pasteurised cooled goats milk were tempered for 10 min until a constant temperature of 33 °C was reached. Stirring slowly, 4 mL of CaCl₂ (Chr. Hansen, France) at a concentration of 510 g.L⁻¹ were added. After reaching the same temperature, the different rennet/coagulants were added to the milk (animal liquid rennet (*A*), *C. cardunculus* subsp. *cardunculus* (*V*₁), *C. cardunculus* subsp. *flavescens* (*V*₂) and microbial coagulant (*M*). The coagulants were standardised to have the same milk clotting activity (56 IMCU.L⁻¹ of milk) in standard milk as described by IDF (1997). The total coagulating strength was adjusted to 80% chymosin rennet clotting time, on a standard milk substrate (milk powder for rennet testing/25 kg. Batch: S1-3 02-552. Chr. Hansen, Denmark), using the Berridge method (IDF 1997). T_{max} is an optical parameter derived from CL, which indicates the time from the addition of the enzyme to the maximum of the first derivative, which is useful for the prediction of milk clotting time. The cutting time (T_{cut}) was determined by multiplying T_{max} by $\beta=2.5$, as described by Fagan et al. (2007a).

After curd cutting (20 s), the curd grain was worked out for 5 min followed by a curd pitching point of 5 min. This was carried out twice and was followed by a final stirring for 5 min. The curd was placed in circular moulds (95×60 mm, internal diameter and height, respectively) with a cheese cloth, pressed (1 atm for 10 min) and brined (17°Be for 20 min). This cheesemaking procedure was repeated three times on alternate days. Four cheeses of 300–400 g were obtained from each vat, which were frozen immediately after brining at -18 °C until analysis (except samples used for sensory and texture analysis) in the freezing chamber of the pilot plant at the University of Murcia.

2.4 Physicochemical analysis

Fat content was measured in duplicate by Gerber's method (ISO 3433:1975). The moisture content was determined by oven drying the cheese samples (3±0.1 g) at

105 °C to constant weight according to IDF (1982) Standard 4A. The protein content of the cheese was calculated according to the Kjeldhal method (IDF 1964). The distillation was carried out in a Buchi automatic distiller (323, Switzerland). The measurement was completed using a 20-Metrohm automatic titrator (702 SM Titrino, Switzerland). The moisture-in-non-fat (MNFS) content was calculated by the formula: $MNFS\% = M \times 100 / 100 - F$, where F is percent fat of cheese and M is percent moisture of cheese. Cheese colour was measured using a CR-200/08 Chroma Meter II (Minolta Ltd., Milton Keynes, UK), and the results were expressed as CIELab values (a^* , b^* and lightness (L^*)).

2.5 Nitrogen fractions

The nitrogen fraction content of the cheeses was determined following the method described by Ardö (1999). A 0.5 mol.L⁻¹ citrate solution was used for Kjeldahl analysis of total nitrogen (TN) and soluble nitrogen (SN) compounds at pH 4.4. Soluble non-protein nitrogen (NPN) in 12% trichloroacetic acid (TCA) was analysed from the pH 4.4-SN fraction. Each analysis was carried out in triplicate.

2.6 Urea-PAGE, SDS-PAGE and capillary zone electrophoresis

2.6.1 Isolation of caseins

Caseins were obtained according to a method adapted from Pirisi et al. (2007). Cheese samples (5 g) were dispersed in 25 mL of 0.4 mol.L⁻¹ sodium citrate buffer at pH 8 at room temperature and homogenised for 30 s with a Stomacher. Isoelectric caseins were obtained by precipitation of cheese suspensions by adding 1 mol.L⁻¹ HCl to reach pH 4.6, followed by centrifugation at 3,000×g for 10 min. The supernatant was discarded and the precipitate was washed three times with water adjusted to pH 4.6 with 1 mol.L⁻¹ HCl and freeze-dried until analysis.

2.6.2 Urea-PAGE electrophoresis

The pH 4.6-insoluble fraction was dissolved in a 9-mol.L⁻¹ urea solution containing 2-mercaptoethanol (10 mL.L⁻¹) and analysed in a Mini-Protean III cube (BioRad, Hercules, CA) by urea-polyacrylamide gel electrophoresis (urea-PAGE) at pH 8.6, according to the procedure described by Chianese et al. (1992), with a 12% concentration of acrylamide. Proteins were stained with Coomassie blue G-250.

2.6.3 SDS-PAGE electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the Laemmli (1970) technique with 15% polyacrylamide gel (4% stacking gel, 0.75 mm thick) using a Mini-Protean III Cell electrophoresis unit (BioRad Laboratories, Hercules, CA). Caseins were denatured at 100 °C for 5 min in Tris–HCl, pH 6.8, containing 1% SDS (*w/v*) and 4% 2-mercaptoethanol. Immediately after loading, gels were run at 200 V until the leading bands were approximately 1 cm from the bottom of the gel. Gels were immediately removed from the plates and

placed in a fixative solution of 40% methanol (v/v) and 10% TCA (w/v) for 12 h. Proteins were stained with Coomassie Blue G-250 and then destained in a solution containing 40% methanol (v/v) and 10% acetic acid (v/v). The casein degradation was studied by computerised densitometry for the scanned image (Zeineh 1D, American Applied Biotechnology, San Diego, CA).

2.6.4 Capillary zone electrophoresis

Sample and electrophoresis buffers were prepared according to Feligini et al. (2005). Freeze-dried casein samples for capillary zone electrophoresis (CZE) were prepared according to Clément et al. (2006) by dissolving in the sample buffer (1:100, w/w). The suspension was incubated for 1 h at room temperature and occasionally shaken. Before CZE analysis, buffers and samples were filtered through 0.22 μm syringe filters (Millex-GV, Millipore Corporation, Bedford, MA). CZE was carried out using a Beckman P/ACE MDQ system (Beckman Coulter, Inc. Fullerton, CA) with an uncoated fused-silica capillary column (Capillary Tubing, 338472, Beckman Coulter, Inc. Fullerton, CA), 570 mm total length \times 50 μm ID \times 375 μm OD. The distance between the detection window and the outlet was 70 mm, resulting in an effective capillary length of 500 mm. Migrations were run at 30 $^{\circ}\text{C}$ with a constant voltage of 20 kV and intensity of 70 μA . Sample solutions were injected at 0.5 psi (1 psi=6,894.76 Pa) for 15 s, and detection was performed at 214 nm. Levels of α_{s1} -, β_{1} -, β_{2} -, p- κ - and Pre- α_s -casein were determined in triplicate samples and expressed as Corrected Peak Areas (value obtained by dividing the integrated peak area by migration time).

2.7 Texture analysis

Texture profile analysis (TPA) was carried out using a texture analyser QTS-25 (Brookfield CNS Farnell, Borehamwood, Hertfordshire, England) equipped with a load cell of 25 kg and Texture Pro V. 2.1 software. For TPA, four cube shaped samples (3 \times 3 \times 3 cm) were cut from a rindless cheese, wrapped in aluminium foil and equilibrated at 20 \pm 0.5 $^{\circ}\text{C}$ for 3 h before testing. The cubes were placed in the centre of the plate with the help of a ruler. The test conditions were: 20 $^{\circ}\text{C}$ room temperature; two consecutive cycles of 50% compression; cross-head at a constant speed of 30 mm.min⁻¹ and a trigger point of 0.05 N. The texture variables, hardness (expressed as N), gumminess (expressed as N), chewiness (expressed as N*mm), cohesiveness (adimensional) and springiness (expressed as mm), were calculated as described by Bourne (1978).

2.8 Sensory analysis

The sensory attributes were determined 1 day after cheese manufacture by 25 trained panellists, which were specifically trained in this subject. In Table 1, the sensory sheet is described and the anchor points of the 10-point scale are detailed.

Each cheese was split into two parts, which were labelled using different numbers of randomly chosen digits. One was divided into wedges of approximately 1-cm thickness. The other whole half was used for the visual phase. Unsalted crackers, green apples and mineral water were served to remove any aftertaste between samples.

Table 1 Description of sensory attributes

Attribute	Description	Anchor points		
		1	5	10
Appearance				
Whiteness	Visual sensation varying from white to black	Dark grey	Light yellow	White
Texture				
Coarseness	Grainy surface detected by manual handling	“Granny Smith” apple	Bottom face of biscuit	Section of shortbread
Humidity	Presence of water on cheese surface detected by manual handling	Nutshell	Inner orange peel	Cut of “Granny Smith” apple
Elasticity	Cheese deformability detected by manual handling	Raw carrot	Green olive stuffed with pepper	Cocktail sausage
Firmness	Force required to break the cheese by tooth	Melted cheese	Cocktail sausage	Cooked parrots
Adhesiveness	Adhesion properties of cheese in mouth	Cooked egg white	Cooked egg yolk	Melted cheese
Odour				
Goaty	Goat smell perceptible with olfactory organ	Absence	Medium intensity	High intensity
Vegetal odour	Vegetal smell perceptible with olfactory organ	Absence	Medium intensity	High intensity
Taste				
Goat milk	Oral sensation related with goat milk	Absence	Medium intensity	High intensity
Bitter taste	Oral sensation related with bitter products	Absence	Medium intensity	High intensity
Salty taste	Oral sensation produced by salt	Absence	Medium intensity	High intensity
Overall score	Final score of cheese related with all attributes	Absence	Medium intensity	High intensity

2.9 Statistical analysis

Statistical treatment of the data was performed using SPSS v15.0 (2006, SPSS Ibérica 165 S.L.U. Madrid, Spain).

3 Results

3.1 CL and LFV profile

Figure 1 shows three graphs, the CL (Fig. 1a) and LFV (Fig. 1b) reflectance profiles obtained during the coagulation, and the reflectance profile of the LFV sensor

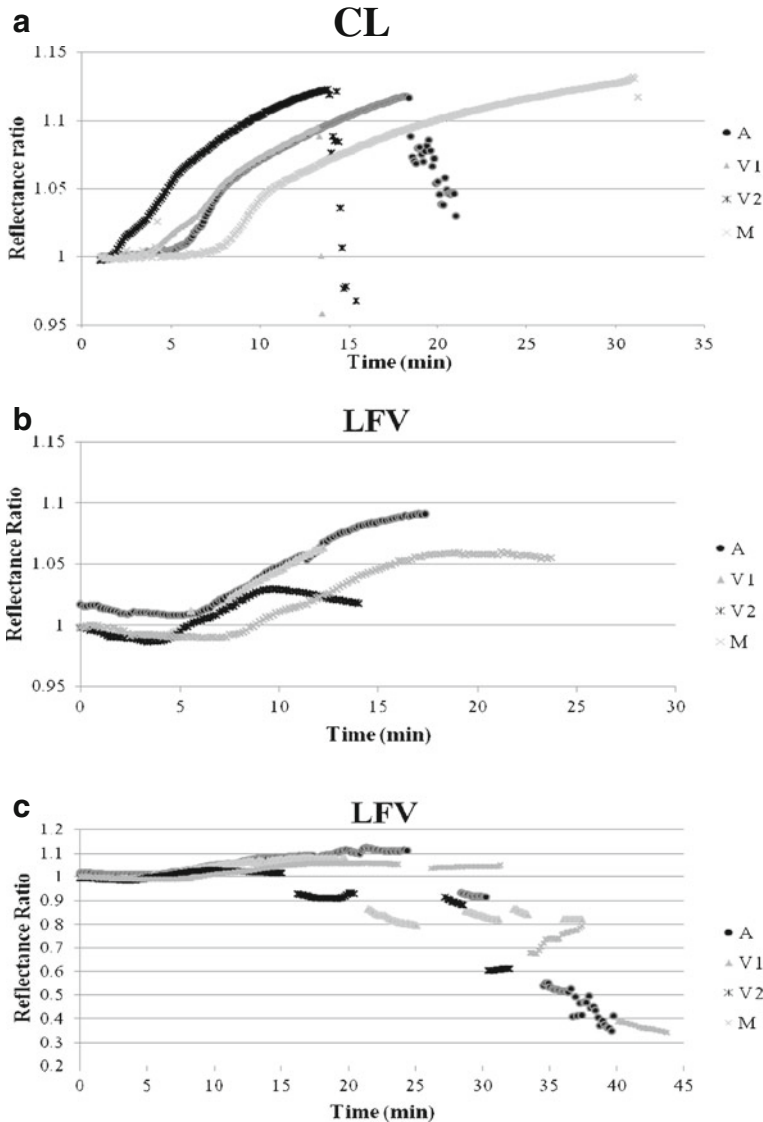


Fig. 1 CL and LFV profile during coagulation and syneresis of four types of coagulant. *A* animal rennet, *M* microbial coagulant, *V*₁ *C. cardunculus* subsp. *cardunculus*, *V*₂ *C. cardunculus* subsp. *flavescens*

obtained during coagulation and syneresis (Fig. 1c). During coagulation, the reflectance ratio of CL (Fig. 1a) increased until cutting time. The same profile can be observed in Fig. 1b, where the ratio obtained by the LFV sensor reflected a sigmoidal increase during clotting. The response of the LFV (Fig. 1b) and CL (Fig. 1a) sensors was similar during coagulation, as the response curve increased in both, although the slope in Fig. 1a is more pronounced, which indicates a lower sensitivity of the LFV sensor. During syneresis, the sensor data for CL showed a high level of scatter, preventing proper monitoring, while the LFV sensor was able to monitor coagulation

and syneresis. During the latter step (syneresis) the reflectance ratio decreased, as can be seen in Fig. 1c. Qualitative results obtained by the sensors (CL and LFV) showed that the microbial coagulant (*M*) had the highest clotting time. Calf rennet (*A*) and vegetable coagulant (*V*₁) provided similar clotting processes, whereas vegetable coagulant (*V*₂) showed a different behaviour in each of the sensors. Quantitative results of T_{\max} are shown in Table 2, which indicated that (*M*) had the highest value (12.20 min) and (*V*₂) the lowest (4.87 min), while (*V*₁) and (*A*) had intermediates values with 6.50 and 7.00 min, respectively.

3.2 Cheese physicochemical parameters

No statistically significant differences ($P > 0.05$) were determined for the fat content, the protein content or the MNFS content between cheeses made with different types of rennet and coagulant (Table 2). However, the results provided on a dry matter basis show that (*M*) and (*V*₂) are different from each other, while (*A*) and (*V*₁) were statistically identical to (*M*) and (*V*₂). Table 2 also shows the results of the soluble nitrogen fractions of each cheese type used to analyse proteolysis. It can be seen that no significant differences ($P > 0.05$) were found for the SN/TN and NPN/TN ratios for any of the cheese samples.

3.3 Electrophoretic patterns of caseins

Urea-PAGE electropherograms of the water insoluble extract from cheese samples manufactured with different coagulants and rennets were compared (Fig. 2a). Three areas of electrophoretic bands (from higher to lower molecular weight) were identified: β -s, α _s- and Pre- α _s-casein (products from α _s-casein degradation). Qualitative differences were observed between the cheeses made with (*V*₁) and (*V*₂) and the cheeses made with non-vegetable coagulants, their electropherograms showing higher intensity bands in the Pre- α _s-casein region.

Table 2 Mean values and standard deviation of optical parameter T_{\max} ($n=3$) and physicochemical parameters ($n=12$) of the different cheeses

	<i>A</i>	<i>M</i>	<i>V</i> ₁	<i>V</i> ₂
T_{\max}	7.00 ^b ±0.10	12.20 ^a ±0.20	6.5 ^{b,c} ±1.51	4.87 ^c ±0.12
Fat	21.95 ^a ±0.44	21.83 ^a ±1.49	22.82 ^a ±1.17	22.66 ^a ±0.79
Dry matter	42.81 ^{a,b} ±2.25	41.52 ^b ±1.96	43.27 ^{a,b} ±1.61	44.45 ^a ±1.18
MNFS	73.27 ^a ±2.55	74.81 ^a ±1.78	73.50 ^a ±1.54	71.83 ^a ±1.73
Protein	14.77 ^a ±1.05	15.05 ^a ±0.51	14.77 ^a ±0.38	14.62 ^a ±0.20
SN/TN	7.90 ^a ±2.36	6.54 ^a ±1.39	7.59 ^a ±3.00	6.19 ^a ±3.06
NPN/TN	1.60 ^a ±0.79	1.44 ^a ±1.23	2.94 ^a ±1.50	3.25 ^a ±1.06

Different letters (a–c) in the same row indicate statistically significant differences (Tukey's test $P < 0.05$).

A animal rennet, *M* microbial coagulant, *V*₁ *Cynara cardunculus* subsp. *cardunculus*, *V*₂ *C. cardunculus* subsp. *flavescens*, MNFS moisture in non-fat substance, SN soluble nitrogen, TN total nitrogen, NPN non-protein nitrogen

Fig. 2 Urea-polyacrylamide (a) and SDS-polyacrylamide (b) gel electrophoresis of pH 4.6 insoluble fraction of cheese samples. *A* animal rennet, *M* microbial coagulant, *V₁* *C. cardunculus* subsp. *cardunculus*, *V₂* *C. cardunculus* subsp. *flavescens*, β -CN β -casein, α_s -CN α_s -casein, *Pre- α_s -CN* pre- α_s -casein (products from α_s -casein degradation), α_{s2} -CN α_{s2} -casein, α_{s1} -CN α_{s1} -casein, γ -CN γ -casein (products from β -casein degradation), *p- κ -CN* para- κ -casein

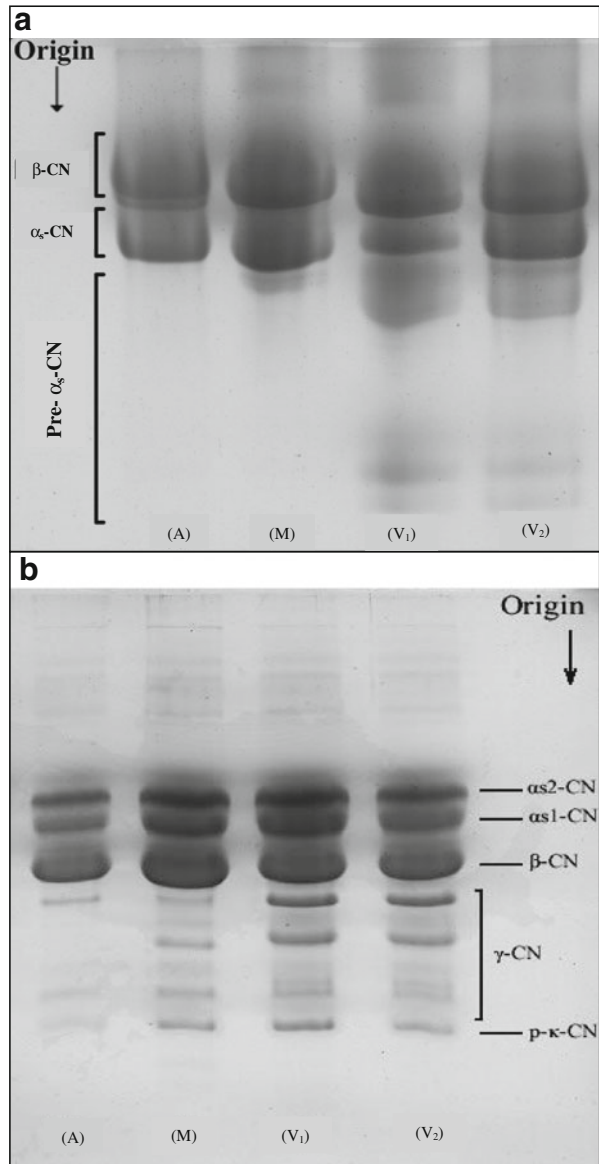


Figure 2b shows the SDS electrophoretic patterns of the cheese samples in order to identify the β -casein degradation products. Qualitative differences were observed in the γ -casein region. Samples (*V₁*) and (*V₂*) clearly showed higher intensity γ peptides than the (*A*) and (*M*) samples.

Since it was difficult to determine casein levels based on the optical density of electrophoretic bands, CZE was used as the quantitative technique. The corrected peak areas for the residual *p- κ -*, α_{s1} -, β_1 -, β_2 - and *Pre- α_s -*casein analysed by CZE are reported in Table 3. Significantly higher amount of hydrolysis products (*p- κ -* and *Pre-*

Table 3 Means values and standard deviation ($n=12$) of corrected peak areas (value obtained dividing the integrated peak area by migration time) for the residual p- κ -, α_{s1} -, β_1 -, β_2 - and pre- α_s -casein analysed by capillary zone electrophoresis

	<i>A</i>	<i>M</i>	<i>V</i> ₁	<i>V</i> ₂
p- κ casein	3.29 ^a ±0.24	3.74 ^a ±0.21	4.91 ^b ±0.83	4.86 ^b ±0.71
α_{s1} -casein	8.07 ^a ±0.67	8.01 ^a ±0.78	7.21 ^b ±0.66	6.71 ^c ±0.92
β_1 -casein	18.90 ^a ±1.12	18.33 ^a ±1.32	9.69 ^b ±1.27	11.65 ^c ±1.34
β_2 -casein	24.13 ^a ±2.89	20.61 ^b ±2.98	13.25 ^c ±1.49	15.98 ^d ±1.89
Pre- α_s -casein	1.31 ^a ±0.16	2.21 ^b ±0.23	4.20 ^c ±0.31	4.32 ^c ±0.86

Different letters in the same row indicate statistically significant differences (Tukey's test $P<0.05$)

A animal rennet, *M* microbial coagulant, *V*₁ *Cynara cardunculus* subsp. *cardunculus*, *V*₂ *C. cardunculus* subsp. *flavescens*

α_s -casein) were found in cheeses made with (*V*₁) and (*V*₂) than those made with microbial coagulant (*M*), although all of these values were higher than those found when calf rennet (*A*) was used. The values of β_1 - and β_2 -casein showed significant differences ($P<0.05$), being lower in cheeses made with vegetable coagulants (*V*₁ and *V*₂) than in those made with (*A*) and (*M*).

3.4 Colour, texture and sensory profile

Table 4 shows the average values for the cheese colour measurements. There were no statistically significant differences ($P>0.05$) in any of the colour attributes between the cheeses studied. Table 4 also displays the average values for the texture parameters determined by instrumental analysis. Cohesiveness and springiness showed no significant differences ($P<0.05$) between the different cheeses, while

Table 4 Mean values and standard deviation ($n=12$) of cheese colour and texture parameters

	<i>A</i>	<i>M</i>	<i>V</i> ₁	<i>V</i> ₂
Colour				
L*	89.47 ^a ±1.00	87.82 ^a ±2.12	87.95 ^a ±2.22	89.42 ^a ±1.63
a*	4.77 ^a ±0.22	5.05 ^a ±0.08	5.20 ^a ±0.30	4.80 ^a ±0.10
b*	2.53 ^a ±0.55	3.46 ^a ±0.82	3.66 ^a ±0.84	2.31 ^a ±0.73
Texture				
Hardness	5.84 ^c ±0.90	5.79 ^c ±1.10	9.51 ^a ±1.98	7.99 ^b ±0.77
Gumminess	1.80 ^c ±0.16	1.95 ^{b,c} ±0.44	2.91 ^a ±0.59	2.40 ^b ±0.48
Chewiness	19.98 ^c ±2.04	21.72 ^{b,c} ±5.06	33.28 ^a ±7.22	27.14 ^b ±6.23
Cohesiveness	0.31 ^a ±0.05	0.34 ^a ±0.03	0.31 ^a ±0.03	0.30 ^a ±0.06
Springiness	11.08 ^a ±0.51	11.12 ^a ±0.47	11.42 ^a ±0.41	11.25 ^a ±0.53

Different letters in the same row indicate statistically significant differences (Tukey's test $P<0.05$)

A animal rennet, *M* microbial coagulant, *V*₁ *Cynara cardunculus* subsp. *cardunculus*, *V*₂ *C. cardunculus* subsp. *flavescens*

differences ($P < 0.05$) were found in hardness, chewiness and gumminess. As regards hardness, cheeses made with microbial coagulant (M) and animal rennet (A), which showed no significant differences between them, had the lowest values, while the cheeses made with vegetable coagulants (V_1 and V_2) were significantly different from each other. The highest value of hardness corresponded to the cheese made with vegetable coagulant (V_1). Gumminess divided the cheeses into three groups, those with the highest values (V_1), another formed by (V_2) cheeses and the last one formed by cheeses made with (M) and (A), which showed the lowest values. Three groups were also established for chewiness: the first with cheeses produced with (V_1) which showed the highest value, the second corresponding to the cheeses made with the microbial coagulant (M) and the third to the cheeses made with (A), (M) and (V_2).

Table 5 shows the sensory profiles. As regards appearance, the parameter whiteness pointed to significant differences ($P < 0.05$) between the cheeses made with vegetable coagulants and those made with animal rennet and microbial coagulant. Coarseness, humidity, elasticity and adhesiveness (texture-related sensory parameters), showed no significant differences ($P < 0.05$). As regards firmness, the trained panellists identified differences between cheeses obtained with (A) and (M) and those made with vegetable coagulants (V_1 and V_2), these last two showing the lowest values, with no differences between them. Odour-related (goat odour, vegetable odour) showed no significant differences ($P > 0.05$). As regards the

Table 5 Mean values and standard deviation ($n=75$) of sensory attributes

	A	M	V_1	V_2
Appearance				
Whiteness	6.96 ^a ±1.41	7.11 ^a ±1.55	5.82 ^b ±1.76	5.52 ^b ±1.76
Texture				
Coarseness	3.26 ^a ±1.85	2.78 ^a ±1.77	3.22 ^a ±1.57	3.27 ^a ±1.67
Humidity	6.24 ^a ±1.74	6.50 ^a ±1.97	6.47 ^a ±1.87	6.40 ^a ±1.68
Elasticity	6.31 ^a ±1.65	6.04 ^a ±1.80	6.00 ^a ±2.05	6.05 ^a ±1.99
Firmness	4.70 ^a ±1.47	4.57 ^a ±1.56	3.43 ^b ±1.55	3.45 ^b ±1.64
Adhesiveness	3.44 ^a ±1.55	3.17 ^a ±1.48	3.52 ^a ±1.79	3.75 ^a ±1.57
Odour				
Goaty	2.98 ^a ±1.92	2.98 ^a ±1.97	2.91 ^a ±1.95	2.92 ^a ±1.86
Vegetal odour	1.90 ^a ±1.42	1.74 ^a ±1.27	1.94 ^a ±1.36	2.29 ^a ±1.78
Taste				
Goaty	3.41 ^a ±2.08	3.31 ^a ±1.95	3.15 ^a ±2.08	2.93 ^a ±1.70
Bitter	1.69 ^a ±1.26	1.94 ^a ±1.68	2.98 ^b ±2.00	3.27 ^b ±2.20
Salty	2.85 ^a ±1.73	3.22 ^a ±1.83	3.41 ^a ±1.94	3.04 ^a ±1.91
Overall score	6.38 ^a ±1.44	5.90 ^a ±1.51	4.88 ^b ±1.42	4.71 ^b ±1.61

Different letters in the same row indicate statistically significant differences (Tukey's test $P < 0.05$)

A animal rennet, M microbial coagulant, V_1 *Cynara cardunculus* subsp. *cardunculus*, V_2 *C. cardunculus* subsp. *flavescens*

taste-related parameters, there were no significant differences for goaty and salty tastes with any of the coagulants used. The bitter taste showed significant differences ($P < 0.05$) between cheeses made with vegetable coagulants and those made with animal rennet and microbial coagulant. The overall score divided the cheeses into two groups: those made with microbial coagulant and animal rennet, which enjoyed higher overall acceptance, and those made with vegetable coagulant.

4 Discussion

4.1 Influence of the different agents during cheesemaking

The increase in the reflectance ratio value during coagulation by both sensors (CL and LFV) is mainly related to casein aggregation, which form larger particles and produces an increase in light reflectance. The more pronounced slope in the CL sensor during coagulation indicates a better response by the sensor and possibly due to the fact that the LFV sensor used as was a prototype sensor. However, both sensors showed a similar behaviour, indicating that LFV sensor is sensitive to chemical reactions occurred during milk coagulation (Fagan et al. 2007a). Therefore, the different behaviour shown by the two sensors during (V_2) coagulation was unexpected and contradicted the observations of the above authors.

As regards the differences in the T_{\max} values observed in this study (Table 2), although milk clotting times were adjusted for standard milk by the Berridge method, the CL sensor showed values that differed from those obtained using the Berridge method, as also described by Castillo et al. (2002). These authors indicated that the values of T_{\max} are higher for the microbial coagulant than for animal rennet due to the higher degree of non-specific proteolysis obtained with the microbial coagulant, results that agree with those shown in Table 2. The lower T_{\max} values obtained by plant coagulants (V_1 and V_2) may be related to higher proteolytic activity, as was concluded by Macedo and Malcata (1997) and can be observed in Table 3. The different milk clotting times shown in Fig. 2 may be related to different rates of hydrolysis, depending on the different specificity of the coagulants used in each type of cheese. The results agree with those of Castillo et al. (2002) who found that animal rennet led to a faster increase in reflectance due to a higher hydrolysis rate and higher specific hydrolysis.

During syneresis, the highly erratic behaviour of CL made it difficult to define a response trend (Fagan et al. 2007a) and it was necessary to use the LFV sensor to monitor this process, demonstrating its advantage of being able to obtain a reflectance ratio during syneresis with low data dispersion. Fagan et al. (2007b) compared both sensors and noted that the LFV response is related with micellar casein aggregation and curd firmness during coagulation, which would explain the sharp increase of the ratio during coagulation and the changes in curd moisture and fat content in whey during syneresis. Note that the LFV sensor showed a high potential for monitoring both coagulation and syneresis (Fagan et al. 2007a; Rovira et al. 2011).

4.2 Effect of the different agents on cheese properties

In order to study the use of vegetable and microbial coagulants as an alternative to calf rennet for fresh goats cheese production, it is necessary to confirm their effect on cheese physicochemical properties. Pino et al. (2009) found no significant differences ($P > 0.05$) between cheeses made with calf rennet and plant coagulant concerning moisture and protein values. As shown in Table 2, our protein content results agree with those reported by Pino et al. (2009). The differences observed in dry matter content between microbial (M) and vegetable (V_2) coagulants (Table 2) can be explained by the three-dimensional structure of the curd, which would lead to variations in water retention, as mentioned by Sanjuán et al. (2002). In addition, the lack of significant differences in MNFS indicates that moisture differences are influenced by the fat content of the cheese.

As regards the nitrogen fractions, the proportion of total soluble nitrogen (SN/TN) reflects the extent of proteolysis due to casein hydrolysis through the action of coagulants and milk proteases present at the start of ripening (Tejada et al. 2008). Pino et al. (2009) found statistically higher levels of SN in cheese made with plant coagulant than in cheese made with calf rennet after two days of ripening, which is contrary to our results (Table 2). They also found no significant differences ($P > 0.05$) in NPN and explained that the production of low-molecular weight nitrogen compounds depends on the microbial enzymes present rather than on the coagulants used, which is consistent with the results of the present work. However, the fact that no differences were observed in NPN and SN in this study may be related to the high standard deviations obtained in some of the measurements. The values of SN/TN (Table 2) were contradictory to the results achieved on casein degradation (Table 3).

The differences observed between the bands of each of the agents compared (Fig. 2) were expected and are consistent with the findings of Lawrence et al. (1987), who suggested that these differences occur because breakpoints on casein molecules are specific to each type of coagulant. From Fig. 2a, it can be seen that cheeses made with vegetable coagulants (V_1 and V_2) showed the highest electrophoretic mobility in the Pre- α_s -casein degradation product region. These components result from the hydrolysis of α_s -caseins by milk plasmin, coagulants and animal rennets (Larsen et al. 1998; Fernández-Salguero and Sanjuán 1999), so that the vegetable coagulants are related with higher α_s -casein proteolytic rates. The electrophoresis patterns of the cheese samples shown in Fig. 2b suggests that cheeses manufactured with (V_1) and (V_2) coagulant have a higher β -casein proteolysis rate than cheeses manufactured with (A) and (M) coagulants, as mentioned by Sousa et al. (2001), which may be related with the higher bitterness perceived (Mendiola, 2000).

The results in Table 3 indicate that more κ - and α_{s1} -casein were hydrolysed by the vegetable coagulants (V_1 and V_2), which showed higher proteolytic activity. These results coincide with those found by Sousa et al. (2001) who indicated that plant proteases hydrolyse more α_{s1} -peptide bonds than animal rennet casein. The lack of significant differences found in α_{s1} -casein between (A) and (M) agree with Lawrence et al. (1987), who indicated that microbial rennets broke α_s -casein at similar rates to chymosin. The data obtained for β -caseins can be explained by the higher proteolytic activity of plant coagulants on β -caseins, as observed by Galán et al. (2008). The

differences in β_2 -caseins between (*A*) and (*M*) cheeses agree with Lawrence et al. (1987) who indicated that β -casein was more hydrolysed by *M. miehei* than by calf rennet in Cheddar cheese. The results shown in Table 3 agree with those published by Macedo and Malcata (1997) who found that an aqueous extract of *C. cardunculus* contained higher proteolytic activity than chymosin in la Serra cheese.

4.3 Influence of the different agents on cheese features

One important feature of cheese is its texture profile, which in this study was significantly influenced by the different agents used. In Majorero cheese, Calvo et al. (2007) found that hardness and firmness significantly increased as a consequence of the low moisture content, which may be related with our results regarding hardness (Table 2). The differences found between the hardness in the texture analysis and firmness in the sensory analysis were unexpected because, as indicated by Benedito et al. (2000), sensory and instrumental hardness usually correlate very well, as they found in Mahon cheese. The significant differences observed in cheese gumminess and chewiness may be related to differences in the moisture content, as described by Pinto et al. (2011). The different agents used for cheesemaking did not influence cheese springiness or cohesiveness.

As regards colour, the type of coagulant used did not influence the final cheese colour measured instrumentally. Whiteness, determined by sensory analysis, differed significantly between the cheeses made with vegetable and other coagulants. However, as seen in Table 4, no significant differences were determined in the L value, which is related with lightness. This may have been because the CIELab parameters were measured in the central part of the cheeses, while the piece considered for the sensory test had rind, and its colour could have influenced the panellists. The results for bitterness indicated that plant extracts imparted a more bitter taste. These findings highlight the effect of cardoon extracts on the flavour of cheeses. Mendiola (2000) argued that the hydrolysis products of β -casein produced a slightly bitter taste and, as seen in Table 3, the β_1 - and β_2 -casein contents were significantly lower in cheeses made with vegetable coagulant: the greater the degree of hydrolysis the greater the bitter taste. Finally, it should be noted that the use of plant coagulants would result in cheeses with lower consumer acceptance, as confirmed by the significant differences obtained for the overall score.

5 Conclusions

Cheeses made with different coagulants produced differences in the graphs obtained by means of optical sensors (CL and LFV sensors) since the degree of hydrolysis, micellar aggregation and curd hardness depended on the type of coagulant used. In this study, vegetable coagulants showed lower milk clotting time than the corresponding for microbial coagulant, which stated their better technological suitability of the vegetable coagulant as an alternative to animal rennet. The fat, MNFS and protein contents of the cheeses were not influenced by the type of coagulant, although differences were found in the dry matter content, the highest values corresponding to the cheeses made with vegetable coagulants. Nitrogen fractions

and CIELab colour parameters in fresh cheese were not affected by the type of coagulant used. Cheeses made with vegetable coagulant showed greater hardness, gumminess and chewiness than those made with calf rennet and microbial coagulant. Finally, the sensory panel found that cheeses made with vegetable coagulants were darker, bitterer and less firm than those made with animal rennet or microbial coagulant. From the results of this study, it can be concluded that although vegetable coagulants may be used as an alternative to animal rennet, new technological strategies might be adopted to improve sensory results, such as increased ripening time and the use of starters.

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