universidad de león

Caracterización tecnológica, química, bioquímica y sensorial del queso de Valdeón con I.G.P. durante la maduración

Technological, chemical, biochemical and sensory characterization of Valdeón cheese with P.G.I. during ripening

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"La posibilidad de realizar un sueño
es lo que hace que la vida sea interesante"
Paulo Coehlo

"Algunas personas quieren que algo ocurra,
otras sueñan con que pasará, otras hacen que suceda"
Michael Jordan

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RESUMEN / SUMMARY

RESUMEN

Durante los últimos años, en España se han llevado a cabo varios estudios de tipificación de quesos que abarcan la práctica totalidad de Denominaciones de Origen Protegidas (D.O.P.) e Indicaciones Geográficas Protegidas (I.G.P.) existentes. Sin embargo, existen algunas variedades de las que sólo se conocen algunos datos, siendo este el caso de la I.G.P. queso de Valdeón.

Esta Memoria de Tesis Doctoral surge con el objetivo principal de caracterizar la I.G.P. Queso de Valdeón estudiándose la influencia que tiene el tiempo de maduración, la estación de elaboración y el tratamiento térmico aplicado a la leche en la calidad final de esta variedad. Para ello, se elaboraron un total de 12 lotes, 8 de ellos a partir de leche pasterizada (2 en cada una de las estaciones del año) y 4 a partir de leche cruda en la Quesería Picos de Europa S.L. (Posada de Valdeón, León, España).

El estudio de la primera parte del objetivo principal, es decir, de la influencia del tiempo de maduración se abordó en cuatro de los artículos de la presente Tesis Doctoral. En primer lugar, se observó que las bacterias acido lácticas, especialmente lactococci, fueron la microbiota predominante durante las primeras etapas de la maduración, siendo gradualmente reemplazadas por los mohos y levaduras. Los recuentos de enterococci y *Enterobacteriaceae* fueron muy bajos o, incluso, no determinados. Esta variedad se caracterizó por un contenido en sólidos totales de 61,80 g/100 g de queso, un ratio sal/humedad de 8,92 g/100 g de humedad, un pH de 6,4-7,6 y una actividad de agua de 0,917. Al final de la maduración, la proteólisis primaria y secundaria fueron muy elevadas lo que dio lugar a una degradación casi total tanto de la αs_1- como de las β-caseínas (aproximadamente el 90%). Asimismo, como consecuencia de este hecho, el perfil de péptidos de los extractos solubles a pH 4,6 mostró una gran complejidad durante toda la maduración y se observó un incremento significativo del contenido en aminoácidos libres, especialmente durante los dos primeros meses, tras los cuales se ralentizó coincidiendo con el importante incremento en aminas biógenas. Al final de la maduración, Glu, Ala, Pro, Tyr, Lys, Asp, Leu y Phe fueron los aminoácidos predominantes, mientras que espermina y tiramina fueron las aminas biógenas mayoritarias. El estudio del contenido en ácidos grasos libres mostró un aumento

significativo de los mismos durante la maduración, alcanzando valores de 6.370,15 mg/100 g de queso a los 120 días. Excepto a los 2 días, los ácidos grasos predominantes fueron el ácido oleico y palmítico. Los ratios de ácidos grasos de cadena corta, media y larga sobre ácidos grasos totales fueron indicativos del tiempo de maduración. Además, el Análisis de Componentes Principales mostró que el ácido oleico, palmítico, butírico y mirístico presentaron la mejor correlación con las variables en función del tiempo de maduración. El estudio de las propiedades reológicas y de textura del queso mostraron que a los 120 días de maduración el queso se caracterizó por valores más elevados de G', G'' y G*, mostrando una disposición más elástica, así como, valores más bajos de fracturabilidad, gomosidad y masticabilidad y valores más altos de adhesividad. Además, a medida que el tiempo de maduración avanzó los quesos se caracterizaron por un descenso en los valores L* y a*. Por último, los atributos sensoriales que más influyeron en la percepción del queso de Valdeón fueron: los sabores salado y picante; los olores agrio y picante; la adhesividad y granulosidad; y la persistencia.

La segunda parte del objetivo principal, la influencia de la estación de elaboración del queso, se afrontó en tres de los artículos que constituyen este trabajo. Los quesos elaborados en verano y otoño presentaron recuentos más bajos de mesófilos totales como respuesta a los valores más elevados de sólidos totales y el ratio sal/humedad y más bajos de actividad de agua. El estudio de las fracciones nitrogenadas reveló una mayor extensión y profundidad de la proteólisis en los quesos de otoño, lo que coincidió con la mayor degradación de las caseínas observadas. Por el contrario, los quesos de primavera fueron los que menor extensión y profundidad de la proteólisis mostraron. Como consecuencia de las variaciones en el desarrollo proteolítico se observaron diferencias significativas tanto en el contenido total como individual de aminoácidos permitiendo caracterizar a los quesos de acuerdo al perfil de aminoácidos. Sin embargo, estas diferencias no se reflejaron en el contenido en aminas biógenas. Por su parte, el perfil de ácidos grasos también estuvo significativamente afectado por la estación de elaboración observándose las mayores concentraciones en los quesos de verano y permitiendo diferenciar los quesos en función de los ratios de ácidos grasos de cadena corta, media y larga sobre ácidos grasos totales. Por otro lado, las réplicas de otoño y verano fueron las que presentaron valores más elevados de los parámetros reológicos estudiados por lo que se caracterizaron por presentar una disposición más elástica con

mayor capacidad para resistir la deformación, así como una masa más firme. Estos resultados coincidieron con lo observado en el estudio del perfil de textura donde estos quesos también fueron los que presentaron valores significativamente más altos de dureza. Por último, los quesos de verano obtuvieron puntuaciones más altas en la valoración sensorial global, si bien no se observaron diferencias significativas entre las diferentes estaciones.

La influencia del tratamiento térmico aplicado a la leche de elaboración sobre las características del queso de Valdeón, tercera parte del objetivo principal de esta Memoria, se desarrolló en tres de los artículos que la componen. En general, los quesos fabricados con leche pasterizada mostraron en relación a los elaborados con leche cruda recuentos microbianos más bajos (5,30 frente a 7,57 unidades log en agar Rogosa), contenido más elevado de sólidos totales (60,87 g/100 g de queso frente a 51,49 g/100 g de queso), mayores ratios de sal/humedad (7,60 frente a 6,57) y pH (7,08 frente a 6,57) y menor actividad de agua (0,937 frente a 0,957). Se observó una mayor extensión de la proteólisis en los quesos de leche pasterizada, mientras que los quesos de leche cruda mostraron mayor profundidad de la misma. Estas diferencias en el desarrollo de la proteólisis se reflejaron en el perfil de aminoácidos, aunque no así en el contenido total de los mismos. Sin embargo, sí se observaron diferencias significativas tanto en el contenido total como individual de aminas biógenas en función del tratamiento térmico aplicado a la leche, especialmente en la concentración de tiramina. De igual modo, los quesos elaborados con leche cruda mostraron concentraciones más altas de ácidos grasos libres si bien los ratios de ácidos grasos de cadena corta, media y larga sobre ácidos grasos totales no se vieron afectados por el tratamiento térmico. Por otro lado, se observó una estructura más elástica con mejor capacidad para resistir la deformación y una matriz más compacta en los lotes elaborados con leche pasterizada, así como, mayores valores de fracturabilidad y dureza. Por último, los quesos elaborados con leche cruda mostraron puntuaciones globales desde el punto de vista sensorial ligeramente superiores.

En el último estudio se realizó la caracterización del peptidoma del queso de Valdeón antes y después de la digestión gastrointestinal. Además, los digeridos fueron comparados con muestras de leche en polvo desnatada pasterizada y digerida en las

mismas condiciones, utilizando una plataforma bioinformática. El perfil peptidómico de los digeridos reveló varias regiones especialmente resistentes a la digestión (entre ellas, β-caseína 60-93, 128-140 y 193-209). Algunas de ellas corresponden a regiones bien conservadas entre especies (humana, vaca, oveja y cabra) e incluyen péptidos con actividad biológica previamente descrita. La gran homología encontrada entre ambos digeridos, el queso y la leche en polvo, sugirió que la digestión gastrointestinal podría acercar el perfil de productos con diferente estado proteolítico. Aunque la mayoría de los péptidos biológicamente activos encontrados en el queso después de la digestión estuvieron también presentes en los digeridos de la leche en polvo, se observaron algunas excepciones que pueden ser atribuidas a la ausencia del péptido precursor correspondiente antes de la digestión.

SUMMARY

In recent years, it has conducted several studies to characterize cheeses in Spain, encompassing almost all Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) currently in existence. However, there are some protected varieties for which only a few studies have been reported such as PGI Valdeón cheese.

This Doctoral Thesis Report arises with the main aim of characterize PGI Valdeón cheese studying the influence of ripening time, season of manufacture and heat treatment applied to milk on the final quality of this variety. For this, 12 batches of Valdeón cheese were elaborated, 8 of them from pasteurized milk (2 of them in each season of the year) and 4 from raw milk in Quesería Picos de Europa S.L. (Posada de Valdeón, León, Spain).

The study of the first part of the main aim, that is, the influence of ripening time was dealt with in four of the papers of this Doctoral Thesis. Firstly, it was found that Lactic acid bacteria, mainly lactococci, were the predominant microbial groups during the early stages of ripening, gradually being replaced by moulds and yeasts. Enterococci and *Enterobacteriaceae* counts were very low or zero. This variety was characterised by a total solids content of 61.80 g per 100 g^{-1} of cheese, a salt/moisture ratio of 8.92 g salt per 100 g^{-1} moisture, a pH of 6.4–7.6 and a water activity of 0.917. At the end of ripening, primary and secondary proteolysis were very high, which resulted in an almost total degradation of αs1- and β-casein. In addition, as consequence of this fact, the peptide profile of the aqueous soluble extracts at pH 4.6 showed great complexity during ripening and it was observed an increase in free amino acids content, especially during the first two months at which a slowing down was observed coinciding with the huge biogenic amines increase. At the end of ripening, Glu, Ala, Pro, Tyr, Lys, Asp, Leu and Phe were the predominant amino acids, while spermine and tyramine were the majority biogenic amines. Free fatty acids content study showed a significantly increase during ripening, reaching values of 6370.15 mg 100 g^{-1} cheese at 120 days. Except at 2 days, oleic and palmitic acids were the predominant free fatty acids. The ratios of short, medium and long chain fatty acids to total FFAs were indicative of ripening time.

Furthermore, the principal components analysis showed that oleic, palmitic, butyric and myristic acids presented the best correlation with the variables as a function of ripening time. The rheological and textural study showed that 120-day-old cheese was characterized by higher G', G'' and G* values, showing a more elastic disposition, as well as, lower fracturability, gumminess and chewiness values and higher adhesiveness values. Moreover, as time increased cheeses were characterized by a decrease in L* and a* values. Finally, the sensory attributes that most influenced the perception of Valdeón cheese were: saltiness and pungenty tastes; sour and pungent odours; adhesiveness and granularity; and taste strength.

The second part of the main aim, the influence of season of manufacture, was dealt with in three of the papers that constitute this work. Cheese elaborated in summer and autumn presented lower total mesophilic counts as result of the high total solids and salt/moisture ratio and low water activity. Nitrogen fractions determination showed a great extent and profound of proteolysis in autumn batches, which agreed with the high casein degradation observed. On the other hand, spring cheeses showed the lowest extent and profound of proteolysis. As consequence of proteolytic variations, it was observed significant differences in both, total and individual free amino acids content allowing characterize cheeses according to amino acids profile. However, these differences were not reflected in biogenic amines content. For its part, free fatty acids profile also was influenced by season of manufacture showing the highest concentration in cheeses made in summer and allowing classified the cheese as function of short, medium and long chain fatty acids to total FFAs ratios. Meanwhile, cheeses made in autumn and summer showed higher rheological parameters values, thus they were characterized by presented a more elastic disposition with better capacity to resisting deformation and a more firmness matrix. These results agreed with those observed in texture profile analysis in which autumn and summer batches presented greater hardness. Finally, cheeses made in summer were given higher overall scores, although no significant differences were observed between cheeses elaborated throughout the year.

The influence of heat treatment applied to milk on Valdeón cheese characteristics third part of the main aim of this Thesis, was dealt with in three papers. In general,

cheeses made from pasteurized milk showed with respect to those elaborated from raw milk lower microbiological counts (5.30 *versus* 7.57 log units in Rogosa agar), higher total solids content (60.87 g 100 g^{-1} of cheese *versus* 51.49 g 100 g^{-1} of cheese), higher salt/moisture ratio (7.60 *versus* 6.57), pH values (7.08 *versus* 6.57) and lower aw (0.937 *versus* 0.957). It was observed greater extent proteolysis in pasteurized-milk cheeses while raw-milk cheeses showed higher deeper proteolysis. These differences in proteolytic activity were reflected in free amino acids profile, although they were not reflected in total amino acids content. However, it was found significant differences in both, total and individual, biogenic amines content, especially in tyramine content. Similarly, cheeses made from raw milk showed higher free fatty acids content although short, medium and long chain fatty acids to total FFAs ratios were not affected by heat treatment applied to milk. On the other hand, it was observed a more elastic with better capacity to resisting deformation and a more compact matrix in batches elaborated from pasteurized milk, as well as, higher fracturability and hardness values. Finally, cheeses made from raw milk showed overall scores slightly higher.

Finally, in the last study the characterization of the peptidome of a Spanish blue cheese, Valdeón, has been conducted before and after gastrointestinal digestion. In addition, the digest were compared to those obtained, in the same conditions, from pasteurized skimmed milk powder using a bioinformatics platform. Peptidomic profiling of digests revealed several regions that are especially resistant to digestion (among them β-casein 60-93, 128-140, and 193-209). Some of them correspond to well-conserved regions between species (human, cow, sheep, and goat) and include peptide sequences with reported bioactivity. The great peptide homology found between both digests, cheese and SMP, suggests that the gastrointestinal digestion could bring closer the profile of products with different proteolytic state. Although most of the biologically active peptides found in cheese after digestion were also present in SMP digest, there were some exceptions that can be attributed to the absence of the relevant precursor peptide before digestion.

1. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. El queso azul

Los quesos azules son una de las variedades más identificables caracterizadas por el crecimiento del moho *Penicillium roqueforti* en su interior (Lawlor *et al*., 2003). La presencia del moho aporta a esta variedad una apariencia diferente y su actividad bioquímica genera un sabor y aroma típico. Además, *P. roqueforti* también produce una maduración más compleja que en otras variedades (Gripon, 1999). Estos quesos se elaboran en varios países y cuentan con importantes ejemplos en Europa como son el queso Roquefort, Gorgonzola o Stilton y en España como Cabrales, Gamonedo o Picón Bejes-Tresviso.

Los quesos de vena azul probablemente se producen desde hace muchísimos años, ya sea de forma deliberada o accidental. Gorgonzola es el primer queso azul que se menciona en la literatura, concretamente en el año 879, mientras que del queso Roquefort no encontramos referencias hasta el año 1070 (Cantor *et al.*, 2004). En España, las primeras referencias corresponden al queso Gamonedo según figura en un escrito de 1641 al Rey Felipe IV, sobre aprovechamiento de pastos. Por su parte, los primeros testimonios de Cabrales datan del siglo XVIII y los encontramos en los escritos de Jovellanos (Reglamento (CE) Nº 510/2006).

Son muchos los métodos de elaboración del queso azul y presentando modificaciones de una variedad a otra, aunque de modo general siguen los pasos que se describen en la Figura 1. La diferencia en el método de elaboración y en cada una de las etapas de producción va a determinar las características finales de los quesos y van a permitir identificar y distinguir unas variedades de queso azul de otras aún siendo todas ellas similares.

1.1.1. Características químicas y fisicoquímicas

La evolución del contenido en sólidos totales en los quesos de vena azul varía en función de las condiciones de maduración seguidas. En aquellos quesos que son

madurados en secaderos durante las primeras etapas de maduración y posteriormente en cuevas naturales, se observa un incremento en el contenido en sólidos totales al inicio de la maduración y un descenso posterior que coincide con su traslado a las cuevas. Esta tendencia se observa en quesos como Cabrales (Alonso *et al.*, 1987) o Picón Bejes-Tresviso (Prieto *et al.*, 2000). Incluso, en algunos casos, los quesos son ahumados antes de ser trasladados a las cuevas naturales como en el queso Gamonedo (González de Llano *et al.*, 1992). Sin embargo, en aquellas otras variedades cuya maduración se realiza íntegramente en cámaras de maduración se observa un aumento del contenido en sólidos totales, especialmente pronunciado al inicio de la misma. Este es el caso de variedades como Gorgonzola (Gobbetti *et al.*, 1997) o Stilton (Madkor *et al.*, 1987b). En cualquiera de los casos, los valores finales observados varían desde 48,7% a 67% (González de Llano *et al.*, 1992; Zarmpoutis *et al.*, 1996; Hayaloglu *et al.*, 2008; Wolf *et al.*, 2011).

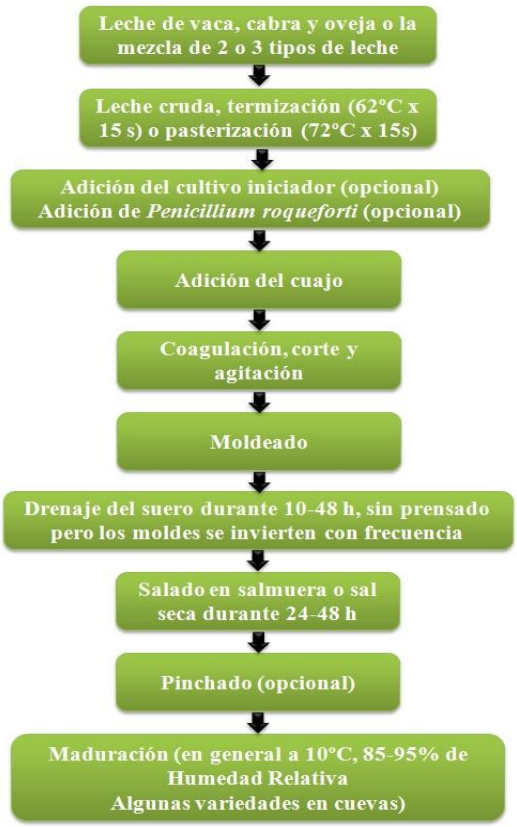

Figura 1. Etapas generales de elaboración del queso azul (Cantor *et al.*, 2004)

El contenido en proteína y grasa es otro parámetro que varía enormemente entre quesos aún siendo variedades similares. Las diferencias observadas son principalmente debidas a diferencias en el ratio grasa/caseína en la leche utilizada para la fabricación (Wolf *et al.*, 2011). El contenido en proteína, expresado sobre extracto seco, varía desde el 34% en Gorgonzola (Seratlic *et al.*, 2011) al 48% en Stilton (Madkor *et al.*, 1987b). Mientras que el contenido en grasa, expresado sobre extracto seco, está comprendido entre el 48% en Gamonedo (González de Llano *et al.*, 1992) y el 63% en Picón Bejes-Tresviso (Prieto *et al.*, 2000). Ambos contenidos permanecen bastante estables a lo largo de la maduración.

La concentración de sal es un parámetro significativo sobre las características microbiológicas y fisicoquímicas de los quesos debido a que regula la actividad de agua, el desarrollo microbiano y la actividad enzimática, especialmente la actividad proteolítica (Wolf *et al.*, 2011). Se han observado importantes variaciones en los niveles de sal en los diferentes quesos de vena azul que van desde el 1,9% en Picón Bejes-Tresviso (Prieto *et al.*, 2000) al 4,9% en Gamonedo (González de Llano *et al.*, 1992). Estas diferencias podrían estar asociadas con los diferentes métodos de salado (Wolf *et al.*, 2011), así como con la intensidad del proceso (Prieto *et al.*, 2000). En cualquier caso, los quesos de vena azul se caracterizan por presentar un incremento del contenido en sal que coincide con el momento del salado y un gradiente de sal mostrando claras diferencias entre el núcleo del queso y la corteza. Los quesos que son salados por inmersión en salmuera o mediante aplicación de sal seca sobre la superficie (como es el caso de la mayoría de variedades azules) requieren de mucho más tiempo para lograr uniformidad en la distribución de la sal que aquellos que son salados tras el corte de la cuajada en la propia cuba (Gobbetti *et al.*, 1997). De este modo, se genera un gradiente durante la maduración de los quesos azules que, sin embargo, a medida que avanza la maduración, decrece obteniéndose al final de la misma valores similares en la corteza y en el centro del queso. Flórez *et al.* (2006a), observaron a los 3 días de maduración del queso Cabrales un gradiente bastante marcado en el contenido en sal (1,90±1,52% NaCl en la superficie y 0,69 ± 0,86% NaCl en el interior), el cual se equilibró claramente al final de la misma (2,35 ± 0,70% NaCl en la superficie y 2,20 ± 0,49% NaCl en el interior).

El pH en los quesos azules decrece rápidamente durante los primeros días de maduración debido a la acción de las bacterias ácido lácticas que degradan la lactosa a ácido láctico mediante la ruta homofermentativa provocando por lo tanto un descenso en el valor de este compuesto. Una acidificación satisfactoria en los quesos azules es especialmente importante. Debe ser lenta pero intensa, ya que si es demasiado rápida la cuajada pierde suero muy rápido y el queso desarrolla una textura muy cerrada que no permite el desarrollo fúngico (López-Díaz *et al.*, 1995). A medida que avanza la maduración se observa un incremento del pH en los quesos azules alcanzando valores de hasta 6,77 en Cabrales (Alonso *et al.*, 1987) o 6,84 en Gorgonzola (Gobbetti *et al.*, 1997). El incremento del pH está probablemente relacionado con el consumo del ácido láctico por parte de los mohos y levaduras y con el proceso proteolítico que tiene lugar durante la maduración y que libera gran cantidad de compuestos alcalinos (aminoácidos y amonio) que contribuyen al aumento del pH en la masa del queso (Fox *et al.*, 1993). La importancia de esta neutralización en los quesos azules radica en la marcada influencia que tiene sobre las características reológicas del queso (Prieto *et al.*, 1999).

Los quesos de vena azul se caracterizan por valores de actividad de agua (aw) al final de la maduración comprendidos entre 0,880 y 0,925 (López-Díaz *et al.*, 1995; Prieto *et al.*, 2000; Flórez *et al.*, 2006a; Flórez *et al.*, 2006b). No sólo la pérdida de humedad, sino también el efecto de la sal, de la lipólisis y de la proteólisis influyen en la actividad de agua en los quesos azules.

1.1.2. Microbiología del queso azul

La maduración de los quesos azules depende de complejas interacciones microbiológicas en un ambiente muy heterogéneo con un importante gradiente de sal y pH. Además, hay grandes diferencias en la estructura del queso que originan variaciones en los niveles de oxígeno y dióxido de carbono. Durante la maduración, las interacciones entre el cultivo iniciador láctico primario y el cultivo iniciador secundario (*P. roqueforti*) determina tanto el tiempo de maduración, como el aroma, la textura y la apariencia del queso final (Van den Tempel & Nielsen, 2000).

Los quesos de vena azul se caracterizan por altos recuentos de microorganismos mesófilos totales durante toda la maduración, alcanzando valores finales que se sitúan en torno a 8 log UFC/g (González de Llano *et al.*, 1992; Gobbetti *et al.*, 1997; Flórez *et al.*, 2006a; Seratlic *et al.*, 2011). Al inicio de la maduración las bacterias ácido lácticas son el grupo microbiano dominante. Durante la coagulación del queso, el pH decrece (como hemos explicado anteriormente) debido principalmente a la actividad metabólica de este grupo de microorganismos (González de Llano *et al.*, 1992). Se ha observado que el crecimiento y producción de ácido láctico por las bacterias termófilas es indispensable para la producción de las condiciones ambientales (bajo pH y ácido láctico como fuente de carbono) necesarias para el rápido crecimiento de *P. roqueforti* (Gobbetti *et al.*, 1997). Además, la producción de gas por parte de las bacterias ácido lácticas y levaduras mediante heterofermentación de la lactosa da lugar a una cuajada abierta, que ayuda a la difusión del oxígeno en la matriz del queso y, por lo tanto, favorece aún más el desarrollo de *P. roqueforti* (Devoyod *et al.*, 1972).

Durante la maduración de la mayoría de quesos de vena azul, se añade una suspensión líquida de conidios de *P. roqueforti* a la leche de partida, o bien los conidios son espolvoreados sobre la cuajada. Tras el desuerado y salado, los quesos son perforados con el fin de favorecer la difusión del oxígeno en el interior del queso y el crecimiento de *P. roqueforti* (Beresford & Williams, 2004). De este modo, la población de mohos en los quesos azules incrementa a partir del día 15 de maduración (González de Llano *et al.*, 1992) y se convierte en el grupo microbiano dominante aproximadamente a los 30 días hasta el final del proceso madurativo como resultado de la fisiología de este grupo que es capaz de crecer bajo condiciones que limitan a otros microorganismos (López-Díaz *et al.*, 1995).

Desde un punto de vista fisiológico, *P. roqueforti* es capaz de crecer en medios con 0,5% de ácido acético, alta concentración de etanol y baja presión de oxígeno. Además, *P. roqueforti* produce conidióforos tuberculados y conidios largos, globosos y lisos. También crece en agar sacarosa creatina y el reverso de sus colonias adquiere una tonalidad negruzca-verde típica en la mayoría de los medios de laboratorio. Sin embargo, esta especie ha sido dividida en *P. roqueforti*, *P. carneum y P. paneum* cuya secuencia de ADNr y su metabolismo secundario difiere (Flórez *et al.*, 2007).

En particular, *P. roqueforti* presenta una temperatura óptima de crecimiento *in vitro* de 20 ± 1 ºC, pH de 6,0 ± 0,1 y entornos con humedad relativa superior a 70 ± 0,1% (Fairclough *et al.*, 2011). Además, su aw óptima de crecimiento es de 0,998 y la mínima de 0,840. La fase lag de crecimiento de *P. roqueforti* (o fase de adaptación) es relativamente estable a aw superiores a 0,920 pero incrementa drásticamente a valores inferiores a 0,920. Esto puede suponer una ventaja en la aplicación de *P. roqueforti* como cultivo iniciador en la maduración del queso azul. Los valores finales de aw permiten a *P. roqueforti* germinar lo más rápidamente posible y crecer durante todas las etapas de elaboración del queso, incluyendo la maduración (Valık *et al.*, 1999). Por otro lado, se ha observado que es capaz de crecer con concentraciones de NaCl que llegan hasta el 4% (Van den Tempel & Nielsen, 2000).

La influencia demostrada del NaCl sobre el crecimiento y, especialmente, la germinación de *P. roqueforti* sugiere que en la elaboración de los quesos azules la cantidad y el tiempo del salado juegan un papel crucial en el desarrollo del moho y, por lo tanto, en el flavor del queso. Concentraciones de sal superiores al 3% inhiben la germinación de los conidios de *P. roqueforti*, aunque la sensibilidad depende de la cepa. Sin embargo, concentraciones del 1% la estimulan. Además, la concentración de sal parece tener también influencia en el crecimiento y el tamaño de las colonias (Godinho & Fox, 1981). El crecimiento de la mayoría de las cepas se ve estimulado a concentraciones superiores al 3,5%, mientras que concentraciones superiores provocan una reducción en el crecimiento (Cantor *et al*., 2004).

P. roqueforti está bien adaptado a crecer en ambientes con bajos niveles de O_2 y altos niveles de CO_2. De hecho, se ha observado que es capaz de crecer en atmósferas con tan sólo un 0,3% de O_2 y un 25% de CO_2 (Van den Tempel & Nielsen, 2000). Sin embargo, el nivel de CO_2 en la atmósfera en la que *P. roqueforti* crece parece afectar a la pigmentación de los conidios así como al modo en que los conidióforos se han producido. Por ejemplo, en una atmósfera con el 5% de O_2 (comúnmente la atmósfera que se encuentra dentro de los quesos azules), los conidióforos surgen de las hifas que crecen sobre o justo debajo de la superficie del medio produciendo una textura aterciopelada uniforme. Cuando niveles más altos de CO_2 están presentes (≥10%)

domina una apariencia “lanosa” o “de algodón” posiblemente debido a la producción de conidióforos que surgen de una masa de hifas aéreas (Fairclough *et al.*, 2011).

1.1.3. Metabolismo de la lactosa en los quesos azules

Debido a que el queso es un producto lácteo fermentado, un factor clave en su elaboración es el metabolismo de la lactosa a ácido láctico mediado por un cultivo de bacterias ácido lácticas seleccionado conocido como cultivo iniciador (McSweeney, 2004). Como ya se ha señalado anteriormente, el grado de acidificación así como la velocidad de la misma, va a influir en las características del queso final, bien sea por repercusión en su textura o bien modificando otras reacciones bioquímicas posteriores como la proteólisis.

La mayoría de la lactosa de la leche se pierde durante el desuerado en el suero como lactosa o como ácido láctico. Sin embargo, un pequeño porcentaje de la misma permanece en la cuajada y será metabolizada rápidamente por el cultivo iniciador a ácido L-láctico durante las primeras etapas de la maduración a un ritmo determinado por la temperatura y la relación sal/humedad (Turner & Thomas, 1980). Este hecho, a su vez, determina que la mayoría del ácido láctico que se detecta al inicio de la maduración en variedades de quesos azules esté en su forma L- (Prieto *et al*., 1999). Además, la conversión de la lactosa a ácido láctico se ve favorecida por la tecnología de elaboración del queso azul: la cuajada es muy húmeda cuando es introducida en los moldes y la ausencia de prensado permite a las BAL acceder a la lactosa residual (Cantor *et al.*, 2004). A los 15 días de maduración, toda la lactosa ha desaparecido en Picón Bejes-Tresviso (Prieto *et al.*, 2000), mientras que en Gamonedo el contenido es de tan sólo un 0,15% (González de Llano *et al.*, 1992).

El ácido láctico producido por este grupo bacteriano es un sustrato importante para una serie de reacciones que tienen lugar durante la maduración del queso (McSweeney, 2004). En los quesos azules, el ácido láctico es metabolizado a CO_2 y H_2O por los mohos y levaduras provocando el incremento del pH del queso como se ha señalado. De hecho, al final de la maduración, el ácido L-láctico ha desaparecido

completamente en Picón Bejes-Tresviso (Prieto *et al.*, 1999), mientras que la forma D-láctico se ha reducido a niveles insignificantes.

1.1.4. Proteólisis en los quesos azules

La proteólisis es el proceso bioquímico primario más complejo e importante que ocurre en los quesos de vena azul durante la maduración (Seratlic *et al.*, 2011). La proteólisis contribuye al ablandamiento de la textura del queso debido a la hidrólisis de la matriz proteica de la cuajada y a la disminución en su aw mediada por los cambios en los enlaces del agua con los nuevos grupos amino y carboxilo formados en la hidrólisis. La proteólisis, además, tiene un efecto directo sobre el sabor debido a la producción de pequeños péptidos y aminoácidos, facilitando la liberación de compuestos sápidos de la matriz del queso y, probablemente más importante, proporcionando aminoácidos libres que son sustrato de una serie de reacciones catabólicas que generan compuestos responsables del sabor (McSweeney, 2004).

En los quesos de vena azul son varias las causas responsables de la extensa proteólisis que experimentan (Tabla I): la actividad de las proteinasas liberadas por los microorganismos pertenecientes al cultivo iniciador y a las bacterias ácido lácticas no pertenecientes al mismo, el cuajo, las proteinasas nativas de la leche y, especialmente, las proteinasas y las exo- y endopeptidasas secretadas por *P. roqueforti* (Zarmpoutis *et al.*, 1996).

Aunque las bacterias ácido lácticas son débilmente proteolíticas, poseen un sistema proteinasa/peptidasa muy amplio capaz de hidrolizar oligopéptidos a pequeños péptidos y aminoácidos (Fox & McSweeney, 1996). Estas bacterias poseen una proteinasa asociada a la membrana celular (Lactocepina, PrtP), oligoendopeptidasas intracelulares (PepO y PepF) y al menos tres aminopeptidasas generales (PepN, PepC y pepG), glutamilaminopeptidasa (PepA), leucilaminopeptidasa (PepL), X-prolildipeptidil-aminopeptidasa (PepX), prolina-iminopeptidasa (PepI), aminopeptidasa P (PepP), prolinasa (PepR), prolidasa (PepQ), dipeptidasas generales (PepV, PepD, PepDA) y tripeptidasa general (PepT), así como un sistema transportador de péptidos y aminoácidos. Este sistema proteolítico es necesario para permitir a las BAL crecer en un

alto número en la leche, la cual contiene sólo una pequeña cantidad de péptidos pequeños y aminoácidos (Sousa *et al.*, 2001). Por su parte, las bacterias ácido lácticas no pertenecientes al cultivo iniciador se convierten en la población bacteriana dominante en los quesos madurados por lo que su sistema proteolítico adquiere relativa importancia en los mismos. La actividad proteolítica de estas bacterias parece que suplementa a la de las bacterias pertenecientes al cultivo iniciador, produciendo péptidos con pesos moleculares similares y aminoácidos libres (Muehlenkamp-Ulate & Warthesen, 1999).

Tabla I. Principales enzimas implicadas en la proteólisis y liberación de aminoácidos durante la maduración del queso azul (Cantor *et al.* , 2004).

Enzima	Especificidad en queso
Plasmina	β-CN y αs_2-CN, tras aminoácido básico (Lys, Arg). Hidroliza β-CN a γ-CN y proteosa-peptonas. Sitios de escisión preferidos: β-CN (28-29, 105-106, 107-108).
Quimosina y otros coagulantes	Hidroliza αs_1-CN a αs_1-CN (f24-199) y αs_1-CN (1-23).
Lactocepina lactocócica	Hidroliza péptidos generados por la ruptura de la caseína por parte de plasmina, cuajo o *P. roqueforti.* Produce diferentes péptidos desde αs_1-CN (f1-23) dependiendo de la cepa.
Peptidasas lactocócicas	Libera aminoácidos de pequeños péptidos. Amplia especificidad aminopeptidasa (p.e. PepN), PepC, PepX y especificidad dipeptidasa.
NSLAB, peptidasas	Contribuye a la liberación de aminoácidos.
***P. roqueforti*, aspartilproteasa**	Hidroliza β-CN preferentemente para producir β-CN (98-209, 30-209, 1-29, 100-209, 1-97/99). Hidroliza αs_1-CN.
***P. roqueforti*, metaloproteasa**	Amplia especificidad.
***P. roqueforti*, serina carboxipeptidasa (extracelular, ácida)**	Libera aminoácidos ácidos, básicos e hidrofóbicos.
***P. roqueforti*, metaloaminopeptidasa (extracelular, alcalina)**	Libera aminoácidos apolares (no después de Gly).
Levaduras	Gran variación entre cepas, desde sin actividad hasta gran actividad proteolítica.

CN, caseína.
NSLAB, bacterias ácido lácticas no pertenecientes al cultivo iniciador.

El cuajo es un extracto obtenido de rumiantes lactantes y está constituido por dos enzimas proteolíticas: la quimosina (EC 3.4.23.4) y, en menor medida, pepsina (EC 3.4.23.1). La principal función de la quimosina en la elaboración del queso es coagular

la leche mediante la hidrólisis específica del enlace Phe_{105}-Met_{106} de la κ-caseína, que es más susceptible a la acción de la quimosina que ningún otro enlace de las proteínas de la leche y da lugar a la coagulación de la misma. La mayor parte del cuajo añadido a la leche se pierde en el suero, aunque una pequeña proporción permanece en la cuajada. La cantidad de estos coagulantes retenidos en la cuajada es fuertemente dependiente del pH establecido en la leche y en la cuajada (Fox & McSweeney, 1996), por lo que dado que el pH aumenta con relativa rapidez en los quesos azules, su acción no parece que tenga mucho peso en la proteólisis de los mismos más allá de su actividad como coagulantes.

Las proteinasas nativas de la leche son principalmente la plasmina y la catepsina D. La plasmina (EC 3.4.21.7) es la proteinasa dominante y su actividad difiere sustancialmente según el tipo de queso. El lugar de escisión primaria de la plasmina sobre la β-caseína son los enlaces Lys_{28}-Lys_{29}, Lys_{105}-Lys_{106} y Lys_{107}-Glu_{108} cuya escisión produce β-CN (f29-209) (γ_1-CN), β –CN (f106-209), (γ_2-CN) y β –CN (f108-209), (γ_3-CN) (Eigel *et al.*, 1984). En los quesos de vena azul, la plasmina en un agente proteolítico importante dado que los valores de pH que se establecen en el queso durante la maduración son cercanos al rango óptimo para su actividad (Prieto *et al.*, 1999). La catepsina D (EC 3.4.23.5) es una aspartil proteinasa ácida que produce el glicomacropéptido κ-CN (f106-169) que también es producido por la acción de la quimosina tras la rotura del enlace Phe_{105}-Phe_{106}. Se han identificado dos lugares de rotura más de la catepsina D sobre la κ-caseína (Leu_{32}-Ser_{33} y Leu_{79}-Ser_{80}). La catepsina D y la quimosina también tienen actividades similares sobre la αs_1-caseína, pero hidroliza la αs_2-caseína de forma muy diferente a la quimosina. Sin embargo, dado que en general la actividad de ambas enzimas es muy similar, resulta difícil cuantificar su contribución en la maduración de aquellas variedades en las que se utiliza cuajo para la coagulación, como es el caso de los quesos azules (Sousa *et al.*, 2001).

P. roqueforti secreta aspartil y metaloproteinasas que han sido bien caracterizadas, incluyendo su especificidad sobre la αs_1- y β-caseínas. También produce proteinasas ácidas intracelulares y exopeptidasas (amino y carboxi) (Sousa *et al.*, 2001). La metaloproteasa es activa a un pH comprendido entre 4,5 y 8,5, siendo su pH óptimo 5,5. Posee una amplia especificidad hidrolizando tanto la αs_1- como la β-caseína (Trieu-Cuot & Gripon, 1982). Es especialmente interesante el hecho de que la metaloproteasa

es capaz de romper el enlace Pro_{90}-Glu_{91} de la β-caseína que no es habitualmente hidrolizado por las proteasas debido al residuo de prolina, y también, al igual que la plasmina, escinde un enlace próximo a Lys_{28}-Lys_{29} (Le Bars & Gripon, 1981; Trieu-Cuot & Gripon, 1982). La aspartil proteasa es estable a pH 3,5-6,0 y tiene dos valores de pH óptimos 3,5 y 5,5, lo cual es explicado por los cambios en la conformación del sustrato. El primer péptido liberado por esta enzima a partir de la αs_1-caseína coincide en punto isoeléctrico y peso molecular con αs_1-CN (f24-199) presentando especificidad similar a la quimosina (Trieu-Cuot & Gripon, 1982). Además, es capaz de hidrolizar la β-caseína en cinco péptidos: β-CN (f98-209), (f30-209), (f1-29), (f100-209) y (f1-97-99) (Le Bars & Gripon, 1981; Trieu-Cuot & Gripon, 1982). Asimismo, *P. roqueforti* posee varias exopeptidasas capaces de escindir estos péptidos formados y una carboxipeptidasa ácida extacelular con una amplia especificidad que libera aminoácidos ácidos, básicos e hidrofóbicos. También cuenta con una metaloaminopeptidasa alcalina extracelular con especificidad por los aminoácidos hidrofóbicos junto con varias peptidasas intracelulares, entre ellas carboxi- y amino-peptidasas alcalinas (Gripon, 1999). Aun teniendo en cuenta en cuenta que tanto la actividad proteolítica como la cantidad de proteasas y peptidasas producidas por *P. roqueforti* varía enormemente entre cepas (Larsen *et al.*, 1998), está considerado que *P. roqueforti* es el principal agente proteolítico en todos los quesos azules (Gripon, 1999).

Como reflejo de la extensa proteólisis que tiene lugar en los quesos azules, se han observado porcentajes de nitrógeno soluble a pH 4,6 que alcanzan valores entre 32,8 y 52% (Zarmpoutis *et al.*, 1996). El ligero incremento de esta fracción al inicio de la maduración se debe fundamentalmente a la actividad proteolítica de la quimosina que está favorecida por el bajo pH y el alto contenido en humedad del queso en esta etapa (Madkor *et al.*, 1987b). Posteriormente, tras la esporulación de *P. roqueforti*, sus proteinasas extracelulares se convierten en dominantes y el rápido aumento del nitrógeno soluble a pH 4,6 se debe principalmente a su acción. Por otro lado, Zarmpoutis *et al.* (1996) observaron como en cinco quesos azules diferentes entre el 77 y el 88% del nitrógeno soluble a pH 4,6 era soluble en ácido tricloroacético (TCA), indicando que la mayoría de este nitrógeno soluble consistía en pequeños péptidos y aminoácidos libres.

Al final de la maduración de los quesos azules se observa una gran degradación tanto de la αs_1- como de la β-caseína, permaneciendo sólo una pequeña parte de ellas intactas (Zarmpoutis *et al.*, 1996). Se ha observado que la hidrólisis de la αs_1-caseína es más rápida que la de la β-caseína (Gobbetti *et al.*, 1997). La fracción αs_1-caseína es inicialmente degradada por la acción del cuajo a αs_1-I-CN [αs_1-CN (f24-199)] la cual, a medida que avanza la maduración, es sustrato de otras enzimas, principalmente aspartilproteasa de *P. roqueforti* o quimosina, originando un incremento de bandas de alta movilidad (fracción integrada por preαs-CN) (Prieto *et al.*, 1999).

Comparado con otro tipo de quesos, la β-caseína es degradada más intensamente. La acción de la plasmina (fundamentalmente cuando el pH del queso se aproxima a su pH óptimo de acción) y de las proteinasas microbianas pueden contribuir a esta degradación, sin embargo, las enzimas secretadas por *P. roqueforti* han mostrado predominancia en la degradación de la β-caseína (Gobbetti *et al.*, 1997). La quimosina no degrada la β-caseína en el queso a niveles destacables y además su actividad decrece a medida que el pH del queso incrementa (Hayaloglu *et al.*, 2008). Trieu-Cuot & Gripon (1982) estudiaron la acción de las metaloproteasas y aspartilproteasas de *P. roqueforti* sobre la αs_1- y β-caseína. Observaron que la endopeptidasa extracelular provocaba una importante degradación de ambas fracciones. Fundamentalmente, la aspartilproteasa rompía tres enlaces, Lys_{29}-Ile_{30}, Lys_{97}-Val_{98} y Lys_{99}-Glu_{100}, liberando los péptidos Ile_{30}-Val_{209}, Val_{98}-Val_{209} y Glu_{100}-Val_{209}, respectivamente. Estos péptidos han sido detectados (junto a otros) en quesos azules como d´Auvergne. La metaloproteasa rompe la β-caseína en los enlaces, Lys_{28}-Lys_{29}, Pro_{90}-Glu_{91} y Glu_{100}-Ala_{101}. Alonso *et al.* (1987), han postulado la acción de estas enzimas en queso Cabrales, junto con la de otra serie de enzimas secretadas por *P. roqueforti* (carboxipeptidasas ácida y alcalina, aminopeptidasas alcalinas, etc.) que son capaces de degradar péptidos dando lugar a una gran cantidad de aminoácidos.

Como resultado de la gran degradación de la β-caseína, al final de la maduración las bandas electroforéticas observadas más importantes son γ_1-caseína [β-CN (f29-209)], γ_2-caseína [β-CN (f106-209)] y γ_3-caseína [β-CN (f108-209)], resultado de la ruptura de esta fracción. La concentración de la γ_2- y γ_3-caseínas es mayor que la de la γ_1-caseína. Además, los electroforetogramas de los quesos de vena azul se caracterizan

por la aparición de dos bandas con movilidad menor que las γ-caseínas, que han sido atribuidas a la degradación de la β-caseína por parte de las proteinasas extracelulares secretadas por *P. roqueforti* y que probablemente coincidan con alguno de los péptidos liberados por la aspartilproteasa descritos anteriormente (Alonso *et al.*, 1987).

Con pocas excepciones, la concentración de todos los aminoácidos incrementa a medida que avanza la maduración, propiedad que permite que sean utilizados como un índice de la misma (Flórez *et al.*, 2006b). Al final de la maduración se alcanzan valores que llegan a los 25,01 mg/g de queso en Gorgonzola (Zarmpoutis *et al.*, 1996), 47,69 mg/g de queso en Cabrales (Flórez *et al.*, 2006b) o 57,32 mg/g de queso en Picón Bejes-Tresviso (Prieto *et al.*, 2000). El alto contenido de aminoácidos al final de la maduración puede ser atribuido a la actividad aminopeptidasa de *P. roqueforti* en este tipo de queso (González de Llano *et al.*, 1991). Independientemente de la concentración final, los quesos de vena azul se caracterizan por tener un patrón final de aminoácidos muy similar. El ácido glutámico, leucina, valina, lisina y fenilalanina son los aminoácidos predominantes, aunque también se detectan en cantidades importantes la tirosina, serina y prolina (Madkor *et al.*, 1987b; González de Llano *et al.*, 1991; Zarmpoutis *et al.*, 1996; Flórez *et al.*, 2006b; Prieto *et al.*, 2000). La alta concentración de leucina, fenilalanina y valina indica la ruptura preferencial de los enlaces peptídicos que generan residuos hidrofóbicos por parte de las enzimas proteolíticas en los quesos de vena azul (Zarmpoutis *et al.*, 1996). Por otra parte, la arginina, responsable del sabor desagradable o amargo, está presente en pequeñas cantidades en estos quesos, al igual que el ácido γ-aminobutírico, un producto de la descarboxilación del ácido glutámico que está presente en cantidades relativamente altas en quesos de baja calidad (González de Llano *et al.*, 1991).

La degradación de proteínas durante la maduración del queso provoca (como hemos visto) la acumulación de aminoácidos que pueden ser convertidos en aminas biógenas debido a la actividad de carboxilasas bacterianas (Innocente & D'agostin, 2002). Debido a la extensa y profunda proteólisis que tiene lugar en los quesos azules, los niveles de aminas biógenas en éstos es superior a la de los quesos no azules (Evans *et al.*, 1988). Asimismo, el contenido total de aminas biógenas aumenta con el tiempo de maduración, aunque se ha observado que los niveles de algunas aminas descienden,

como es el caso de la espermina o la triptamina, o pueden adoptar un comportamiento variable como la putrescina (Komprda *et al.*, 2008). Al final de la maduración, las aminas biógenas mayoritarias en los quesos de vena azul son la tiramina, cadaverina, putrescina e histamina (Novella-Rodríguez *et al.*, 2003; Komprda *et al.*, 2008). El caso de la putrescina (poliamina que puede ser sintetizada alternativamente a partir de la arginina por algunas bacterias) es destacable ya que Novella-Rodríguez *et al.* (2003) la detectaron en las 20 muestras de queso azul que estudiaron, lo cual no ocurrió con ninguno de los otros tipos de queso.

1.1.5. Péptidos bioactivos

Como ya se ha mencionado en el apartado referente a la proteólisis, la degradación de las proteínas libera una serie de péptidos y aminoácidos responsables del flavor directamente o que actúan como precursores de otros compuestos sápidos y aromáticos. Sin embargo, los péptidos liberados de las proteínas de los alimentos durante la fermentación han despertado un especial interés debido a que también pueden afectar a numerosas respuestas fisiológicas en los organismos (Gómez-Ruiz *et al.*, 2002). En los últimos años, extensas evidencias científicas han probado la existencia de péptidos biológicamente activos derivados de las proteínas de los alimentos que pueden tener efectos beneficiosos sobre la salud humana. Estos fragmentos proteicos, conocidos como péptidos bioactivos, pueden ser liberados de sus precursores proteicos inactivos durante la digestión gastrointestinal y/o durante la elaboración de los alimentos (Contreras *et al.*, 2009). Los péptidos bioactivos varían en tamaño desde los 2 a los 50 residuos de aminoácidos y muestran diferentes actividades como antimicrobiana, antioxidante, antitrombótica, antihipertensiva, inmunomoduladora, opioide y actividades antiproliferativas entre otras, afectando a la mayoría de los sistemas del cuerpo: sistema cardiovascular, digestivo, endocrino, inmunitario y nervioso (Hernández-Ledesma *et al.*, 2011).

Hasta la fecha, el grupo de péptidos biológicamente activos producidos durante la fermentación de los productos lácteos más estudiados son los responsables del control de la presión arterial (Fitzgerald & Murray, 2006). La mayoría de los péptidos antihipertensivos derivados de los alimentos actúan mediante la inhibición de la enzima

convertidora de angiotensina (ECA, peptidil-dipeptidasa A; EC 3.4.15.1) (Gómez-Ruiz *et al.*, 2004b). La ECA es uno de los principales reguladores de la presión arterial a través de su acción en dos sistemas corporales. En primer lugar, la ECA forma parte del sistema renina-angiotensina, convirtiendo la angiotensina I en un potente vasoconstrictor, angiotensina II, que también induce la liberación de aldosterona y, por lo tanto, incrementa la concentración de sodio y la presión arterial. La ECA también forma parte del sistema calicreína-cinina ya que hidroliza la bradiquinina, que tiene acción vasodilatadora. Mediante la inhibición de esta enzima, los péptidos bioactivos han mostrado su capacidad para reducir la presión arterial en animales y en estudios clínicos (Hernández-Ledesma *et al.*, 2007). Numerosos estudios han observado que durante la elaboración y, en particular, la maduración del queso, se pueden producir péptidos inhibidores de la ECA (Tabla II).

Tabla II. Ejemplo de péptidos inhibidores de la ECA identificados en diferentes variedades de queso.

Péptido	Secuencia[a]	IC_{50} (μM)[b]	Variedad	Referencia
αs_1-CN f(1–9)	RPKHPIKHQ	13,4	Gouda	1
αs_1-CN f(102-109)	KKYNVPQL	77,1	Manchego	2
αs_2-CN f(205-208)	VRYL	24,1	Manchego	2
β-CN f(60–68)	YPFPGPIPN	14,8	Gouda	1
β-CN f(58–72)	LVYPFPGPINSLPQ	18,0	Crescenza	3
β-CN f(199-204)	VRGPFP	592,0	Manchego	2
β-CN f(47–51)	DKIHP	113,1	Manchego, Mahón, Idiazábal, Roncal, Cabra y Cabrales	4

[a] R: arginina; P: prolina; K: lisina; H: histidina; I: isoleucina; Q: glutamina; Y: tirosina; N: asparagina; V: valina; L: leucina; F: fenilalanina; G: glicina; S: serina y D: ácido aspártico.
[b] Concentración de péptido necesaria para inhibir el 50% de la actividad original de la ECA.
1 Saito *et al.*, 2000; 2 Gómez-Ruiz *et al.*, 2004a; 3 Smacchi & Gobbetti, 1998; 4 Gómez-Ruiz *et al.*, 2006.

Sin embargo, tenemos que tener en cuenta que una vez que estos péptidos son liberados en la elaboración de los alimentos, tienen que ser capaces de resistir la digestión gastrointestinal, ser absorbidos y alcanzar el sistema cardiovascular en su forma activa (Gómez-Ruiz *et al.*, 2004a), o bien, su forma activa puede ser liberada durante la digestión por medio de la acción enzimática. En relación a la importancia de la digestión en la acción fisiológica de los péptidos bioactivos existen numerosos estudios sobre la resistencia a la digestión gastrointestinal de diferentes péptidos con

actividad antihipertensiva, así como su formación por la acción de las enzimas digestivas a partir de secuencias inactivas (Pihlanto-Leppala *et al.*, 1998; Vermeirssen *et al.*, 2003; Gómez-Ruiz *et al.*, 2004a; Quirós *et al.*, 2008; Contreras *et al.*, 2009). Aunque se cree que los péptidos identificados en el queso son rápidamente metabolizados a sus aminoácidos constituyentes, estos estudios han demostrado que varios de ellos son resistentes a los procesos fisiológicos y pueden alcanzar la circulación. Este es el caso de los tripéptidos IPP y VPP, pero también ha sido demostrado en péptidos más largos ricos en prolina como LHLPLP. Este péptido no resiste la simulación gastrointestinal ya que es hidrolizado a su forma activa más corta HLPLP, por peptidasas celulares antes de su transporte a través del epitelio (Hernández-Ledesma *et al.*, 2011). Este es el caso también de las secuencias VRGPGP y LEIVPK que resistieron la simulación de la digestión gastrointestinal en queso Manchego. Es conocido que la presencia de prolina hace que la secuencia aminoacídica sea menos susceptible a la acción de las enzimas proteolíticas (Gómez-Ruiz *et al.*, 2004a). En algunos casos, la forma activa se libera durante el proceso digestivo. Maeno *et al.* (1996) encontraron un potente péptido antihipertensivo *in vivo* (KVLPVP) con inesperadamente baja actividad inhibidora de la ECA *in vitro*, ya que la forma activa fue liberada durante la digestión pancreática de su péptido precursor (KVLPVPQ) mediante la hidrólisis del residuo glutamina. Es por ello, que el estudio del perfil de péptidos con actividad biológica implica en primer lugar la identificación de las secuencias peptídicas del queso, un segundo estudio de la resistencia *in vitro* a la digestión gastrointestinal y un estudio final de su actividad *in vivo*.

Todos estos conocimientos pueden contribuir al desarrollo de productos con beneficios para la salud. Sin embargo, el desarrollo de productos lácteos fermentados (como el queso) con esta propiedad requiere de una comprensión detallada de los mecanismos que subyacen de este efecto más allá de la nutrición básica. En el caso concreto de los péptidos con actividad inhibidora de la ECA, se requiere de un conocimiento exhaustivo de los mecanismos implicados en la interacción entre el centro activo de la enzima y los aminoácidos de esos péptidos, con el fin de producir eficazmente un péptido inhibidor de la ECA y/o un producto fermentado con dichos péptidos (Fitzgerald & Murray, 2006). En este sentido, se están llevando a cabo numerosos estudios para demostrar la estabilidad de los péptidos, su absorción así como

la identificación de la forma activa en el organismo. Al mismo tiempo, avances en las técnicas analíticas capaces de seguir pequeñas cantidades de péptidos o derivados de ellos en matrices complejas y fluidos biológicos están permitiendo desarrollar estos estudios cinéticos en modelos animales y humanos. Por último, avances en nuevas disciplinas como la nutrigenómica y nutrigenética abren nuevas vías para seguir la bioactividad en el organismo mediante la identificación de nuevos y más complejos biomarcadores de exposición y/o actividad. Todos estos avances simultáneamente con el conocimiento de los tecnólogos de alimentos son cruciales para la formulación de productos que garanticen la actividad y biodisponibilidad de los péptidos bioactivos (Hernández-Ledesma *et al.*, 2011).

1.1.6. Lipólisis en los quesos azules

Los lípidos en los alimentos pueden verse sometidos a degradación hidrolítica u oxidativa. Sin embargo, en el queso, los cambios oxidativos son muy limitados debido al bajo potencial de oxidación-reducción (en torno a -250 mV). No obstante, en todos los tipos de quesos, los triglicéridos experimentan hidrólisis por la acción de lipasas que dan lugar a la liberación de ácidos grasos durante la maduración (McSweeney, 2004). Las lipasas que contribuyen a la lipólisis en queso provienen principalmente de tres fuentes: la leche, los microorganismos y algunas preparaciones de cuajo. En el caso de los quesos de vena azul, la extensa lipólisis que tiene lugar está gobernada por el crecimiento y actividad lipolítica de *P. roqueforti* y depende de la cepa utilizada, el tiempo de maduración, la actividad lipolítica residual de la leche, el cultivo iniciador y el cuajo, la eficacia de la homogeneización de la leche, los microorganismos de la superficie del queso, el pH, la temperatura y la concentración de NaCl (Kinsella *et al.*, 1976). *P. roqueforti* produce dos lipasas extracelulares, una lipasa ácida y una alcalina. La lipasa ácida tiene un pH óptimo cercano al predominante en los quesos azules, mientras que la lipasa alcalina muestra la actividad más alta en la grasa de la mantequilla, siendo por tanto superior la actividad de la lipasa ácida (Larsen & Jensen, 1999).

Al final de la maduración, los quesos azules muestran una concentración de ácidos grasos libres muy elevada como resultado de la pronunciada lipólisis que tiene lugar (Tabla III).

Tabla III. Concentración total de ácidos grasos libres en algunas variedades de queso azul.

Variedad	AG libres (mg/Kg)	Referencia
Roquefort	25.969	de la Fuente *et al.* (1993)
Cabrales	33.153	Alonso *et al.* (1987)
Danablu	18.905	Madkor *et al.* (1987a)
Stilton	10.674,5	Madkor *et al.* (1987a)
Picón Bejes-Tresviso	58.355	Prieto *et al.* (2000)
Gamonedo	75.685	González de Llano *et al.* (1992)
Quesos comerciales argentinos	20.300	Wolf *et al.* (2011)

En general, el contenido en ácidos grasos aumenta a medida que avanza la maduración (Madkor *et al.*, 1987a; González de Llano *et al.*, 1992), sin embargo, en algunos quesos se observa un descenso de la concentración de ácidos grasos al final de la misma que ha sido atribuido a su degradación a través de la vía oxidativa a metilcetonas (Prieto *et al.*, 2000). Los ácidos grasos mayoritarios, al final de la maduración, son ácidos grasos de cadena larga: ácido oleico (C18:1), palmítico (C16:0) y mirístico (C14:0) (Madkor *et al.*, 1987a; Prieto *et al.*, 2000; Wolf *et al.*, 2011).

1.1.7. Compuestos volátiles en los quesos azules

Los compuestos volátiles derivan principalmente del catabolismo de la lactosa y de la lipólisis y proteólisis que se producen durante la maduración del queso (Collins *et al.*, 2003). Su presencia varía de unas variedades a otras tanto a nivel cuantitativo como cualitativo, difiriendo su perfil y dando lugar a las características finales que definen una determinada variedad y la distingue del resto. Los compuestos volátiles más importantes en los quesos de vena azul se describen a continuación.

a) Metil cetonas:

El metabolismo de los ácidos grasos por *Penicillium spp*. implica cuatro etapas principales y la vía por la cual se forman las metil cetonas se conoce como β-oxidación

(Figura 2). *P. roqueforti* ha mostrado capacidad para producir metil cetonas cuando se añaden ácidos grasos de cadena larga al medio de cultivo. Sin embargo, los ácidos grasos no son los únicos precursores de metil cetonas en el queso azul. Éstas pueden formarse también a partir de cetoácidos presentes en baja concentración y de forma natural en la grasa de la leche o por oxidación de ácidos grasos monoinsaturados. El ratio de producción de metil cetonas en queso está afectado por la temperatura, pH, estado fisiológico de los mohos y concentración de ácidos grasos. Tanto los conidios como el micelio son capaces de producir alcan-2-onas con un ratio que no es directamente proporcional a los ácidos grasos precursores. Los conidios de *P. roqueforti* son capaces de oxidar ácidos grasos de 2-12 átomos de carbono, siendo el ácido octanoico el sustrato que más rápido convierten. El micelio oxida ácidos grasos con un amplio rango de pH, con un óptimo entre 5 y 7, similar al que hay durante la maduración del queso azul.

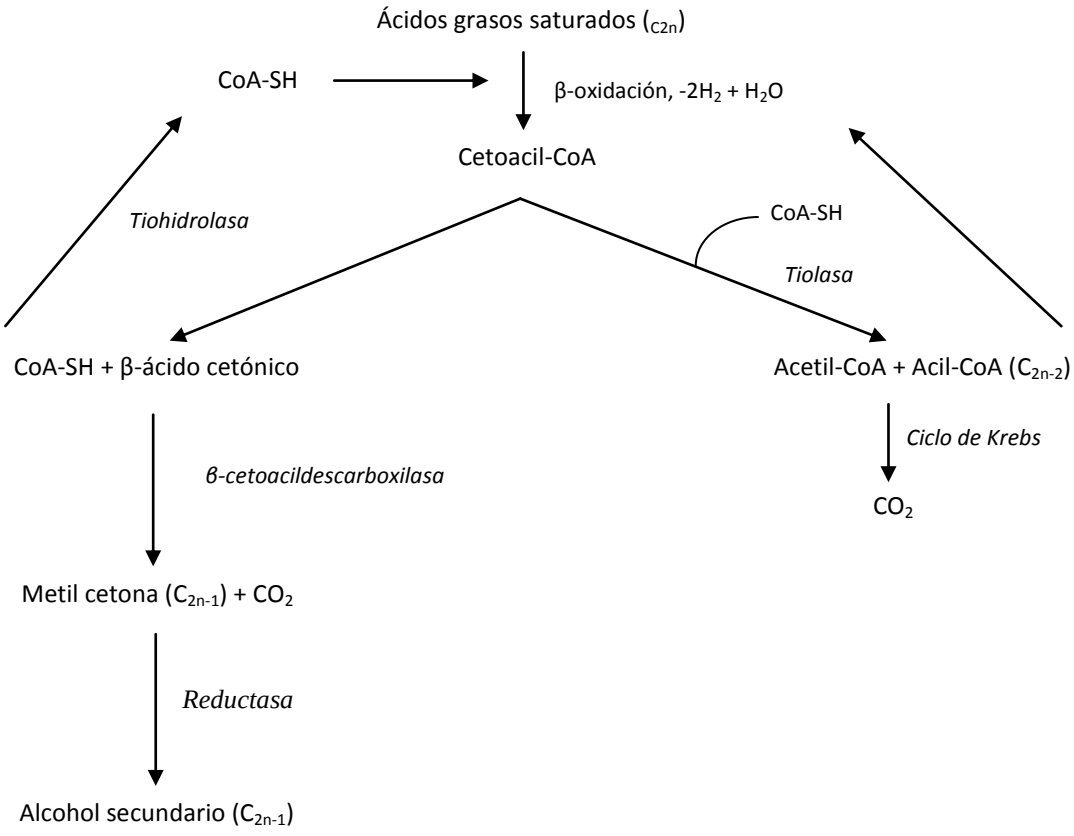

Figura 2. Catabolismo de ácidos grasos por *Penicillium spp*. (McSweeney & Sousa, 2000)

Las cetonas son los principales compuestos del aroma en los quesos de vena azul, representando un porcentaje muy elevado de los mismos. Las cetonas suponen entre un 50 y un 75% del perfil aromático total en Roquefort, Bleu des Causses y Bleu d'Auvergne (Gallois & Langlois, 1990), entre un 47 y un 55% en Gorgonzola (Moio *et al.*, 2000) y entre un 55 y un 75% en Stilton (Gkatzionis *et al.*, 2009). También se ha observado que son los compuestos predominantes en Danish Blue, contribuyendo significativamente al flavor final de este queso (Alewijn *et al.*, 2003). Se considera que las metil cetonas con número impar de átomos de carbono, principalmente 2-heptanona y 2-nonanona, son los principales compuestos responsables del flavor único de los quesos azules (Qian *et al.*, 2002; Frank *et al.*, 2004). Al final de la maduración las cetonas predominantes son la 2-pentanona, 2-heptanona y 2-nonanona (Gallois & Langlois, 1990; Moio *et al.*, 2000; Gkatzionis *et al.*, 2009; Wolf *et al.*, 2011), aunque Alewijn *et al.* (2003) también detectaron 2-undecanona en Danish Blue en altas concentraciones. Sin embargo, hay que tener en cuenta que a lo largo de la maduración de los quesos azules se observa una gran variabilidad en la concentración de metil cetonas como consecuencia de su interconversión a sus correspondientes alcoholes secundarios (Wolf *et al.*, 2011).

b) Alcoholes:

Los alcoholes primarios y secundarios, después de las cetonas, son considerados los compuestos más importantes en el aroma de los quesos madurados con moho. Representan más del 30% de los compuestos volátiles en Gorgonzola (Moio *et al.*, 2000), entre un 10 y un 30% en Stilton (Gkatzionis *et al.*, 2009) y entre el 15 y el 20% en Roquefort (Gallois & Langlois, 1990). Son muchas las vías metabólicas por las cuales se sintetizan alcoholes en el queso. Algunos se pueden formar a partir del metabolismo de la lactosa por la vía de las pentosas fosfato y la vía ácido-mixta, que no solo liberan etanol sino también butan-2,3-diol (Molimard & Spinnler, 1996). Los alcoholes secundarios se pueden formar en queso azul a partir de la reducción enzimática de las metil cetonas por parte de *Penicillium spp.* (Figura 2) (Collins, *et al.*, 2003). Algunos alcoholes pueden derivar de la vía Erhlich a partir del metabolismo de los aminoácidos o de la degradación de los aldehídos. Por último, los ácidos linoleico y linolénico son precursores de compuestos aromáticos de ocho carbonos (Molimard &

Spinnler, 1996). Al final de la maduración el 3-metil butanol es el alcohol predominante en los quesos azules, aunque también se han detectado concentraciones altas de 2-pentanol, 2-heptanol y 2-nonanol (Gallois & Langlois, 1990; Gkatzionis *et al.*, 2009; Wolf *et al.*, 2011). Algunos autores han atribuido a los alcoholes secundarios, en particular al 2-heptanol y al 2-nonanol, el característico aroma de los quesos azules (Molimard & Spinnler, 1996).

c) Ésteres:

La mayoría de los ésteres encontrados en el queso aportan notas frutales y florales. Las notas aromáticas descritas más frecuentemente son piña, plátano, albaricoque, pera, floral, rosa, miel y vino (Molimard & Spinnler, 1996). Los ésteres son constituyentes comunes de la fracción volátil de los quesos y contribuyen de manera considerable al flavor debido a su alta volatilidad a temperatura ambiente y a su bajo umbral de percepción (Hayaloglu *et al.*, 2008). Además, su papel en el queso azul es muy importante ya que contribuyen a atenuar el flavor picante típico de las metil cetonas (Moio *et al.*, 2000). Los ésteres son compuestos muy aromáticos que se forman cuando los ácidos grasos libres reaccionan con alcoholes. Las reacciones de esterificación que dan lugar a su formación tienen lugar entre ácidos grasos de cadena corta y media y alcoholes derivados de la fermentación de la lactosa y del metabolismo de aminoácidos (Collins *et al.*, 2003). En el queso azul los etil ésteres son los compuestos predominantes de esta familia química (Hayaloglu *et al.*, 2008), junto con los metil ésteres (Wolf *et al.*, 2011). En particular, el etil butanoato y etil hexanoato, se han considerado compuestos importantes en los quesos azules (Qian *et al.*, 2002; Frank *et al.*, 2004).

d) Lactonas y compuestos cíclicos:

Las lactonas se caracterizan por sus pronunciadas notas afrutadas (melocotón, albaricoque, coco). Las δ-Lactonas tienen normalmente un umbral de detección más alto que las γ-lactonas (Molimard & Spinnler, 1996). Las lactonas son compuestos cíclicos formados por la esterificación intramolecular de hidroxiácidos grasos, con la pérdida de agua y la formación resultante de una estructura en anillo (Collins *et al.*, 2003). Los ácidos grasos hidroxilados pueden generarse en el catabolismo normal de los ácidos

grasos y también pueden generarse a partir de ácidos grasos insaturados por la acción de lipoxigenasas o hidratasas. *P. roqueforti* puede formar lactonas de 12 átomos de carbono a partir de ácidos grasos saturados de cadena larga (C18:1 y C18:2) (Molimard & Spinnler, 1996).

Las lactonas representan una concentración baja del total del perfil aromático de Roquefort con tan sólo un 10%. Sin embargo, sí que se detectan tanto γ- como δ-lactonas, siendo la γ-dodecalactona la predominante. Las diferencias en el contenido en lactonas entre los diferentes tipos de queso azul están directamente relacionadas con la leche. La cantidad de precursores de lactonas, mayores en la leche de vaca que de oveja, están influidos por varios factores como son la alimentación, estación del año, raza y estado de lactación (Gallois & Langlois, 1990).

e) Aldehídos y otros compuestos:

Los aldehídos se forman a partir de aminoácidos por transaminación, dando lugar a la formación de una imida que puede ser descarboxilada. También se ha propuesto que los aldehídos se forman por degradación de Strecker a partir de aminoácidos (Collins *et al.*, 2003). Esta reacción es simple y puede producirse durante la maduración sin catálisis enzimática. Los aldehídos son compuestos transitorios en el queso ya que rápidamente son transformados en alcoholes o en sus ácidos correspondientes (Molimard & Spinnler, 1996). Los aldehídos más importantes en el queso azul son el acetaldehído y 3-metilbutanal. La importancia de estos compuestos en el queso azul aún no ha sido aclarada. Sin embargo, el 3-metilbutanal se considera como un compuesto aromático activo que se puede formar en el queso azul a partir del catabolismo de la leucina (Wolf *et al.*, 2011).

En los quesos azules se pueden detectar compuestos fenólicos, especialmente *p*-cresol (4-metilfenol) (Frank *et al.*, 2004; Hayaloglu *et al.*, 2008). Este compuesto se forma a partir de la tirosina por degradación atípica de Strecker (Wolf *et al.*, 2011).

Además, el perfil aromático de los quesos azules también se puede caracterizar por la presencia de compuestos sulfurosos, especialmente dimetil disulfuro y dimetil trisulfuro. La degradación de la metionina o de péptidos que contienen metionina durante la maduración del queso produce sulfuro de hidrógeno y metanotiol.

Posteriormente, el metanotiol puede convertirse mediante reacciones de oxidación en dimetil disulfuro y dimetil trisulfuro, produciéndose de este modo un incremento en su concentración final. Estos compuestos son considerados importantes en el flavor de los quesos (Hayaloglu *et al.*, 2008).

1.1.8. Propiedades sensoriales de los quesos azules

El análisis sensorial de los alimentos implica la medida, interpretación y entendimiento de las respuestas humanas a las propiedades de los alimentos percibidas por los sentidos (Karoui *et al.*, 2007). Los atributos sensoriales del queso como el flavor, textura y apariencia juegan un papel importante en la aceptabilidad de los productos influyendo directamente en su éxito en el mercado (González-Martín *et al.*, 2011). Por esta razón, es importante contar con medidas cuantitativas para evaluar las propiedades sensoriales de una manera razonable que permita a la industria alimentaria responder ante las demandas cambiantes de los consumidores y el mercado (de Belie *et al.*, 2003).

La calidad del queso puede medirse directamente mediante métodos sensoriales o indirectamente mediante métodos químicos, mecánicos u ópticos (Karoui *et al.*, 2007). Hoy en día, no existe un método estándar o una guía europea en términos de evaluación sensorial para Denominaciones de Origen Protegidas o Indicaciones Geográficas Protegidas, de manera que cada DOP o IGP decide la manera de cumplir con este requisito legal. En muchos casos, existe una descripción muy genérica de las características sensoriales que el producto debe presentar, pero ninguna alusión a la forma de verificarlo. De hecho, hay una ausencia general de los métodos, entrenamiento y supervisión de los paneles que llevan a cabo el control de la calidad sensorial que aseguren que el producto presenta las características sensoriales que son esperadas. Además, el control de la calidad sensorial a menudo se limita a la comprobación de la ausencia de defectos (Ojeda *et al.*, 2015).

El análisis descriptivo es la herramienta de elección para la diferenciación cualitativa y cuantitativa de los quesos y para explorar y definir las relaciones entre la percepción sensorial e instrumental. En el análisis descriptivo, la selección de los

panelistas, las escalas y el entrenamiento son requisitos fundamentales en el planteamiento inicial. También se requiere un buen lenguaje o léxico de las características sensoriales. El desarrollo de perfiles sensoriales implica el entrenamiento cuidadoso y sistemático de los jueces en la metodología descriptiva durante un largo periodo de tiempo, lo que es costoso. Aunque la importancia del análisis sensorial es incuestionable, este método es muy difícil de implementar para un uso práctico cuando se quiere analizar un gran número de muestras. Es por lo tanto deseable reemplazar la evaluación sensorial por análisis instrumentales más rápidos, simples y baratos (González-Martín *et al.*, 2011).

El flavor es uno de los factores principales que determinan la aceptación de un alimento por los consumidores. El proceso global de la liberación del flavor y su percepción está gobernado por las propiedades de los compuestos del flavor, la naturaleza de la matriz del alimento y las condiciones fisiológicas de la boca, nariz y garganta durante el consumo del alimento. Por otro lado, la interacción sinérgica o antagónica de dos factores como el sabor y el aroma son los responsables de la intensidad global del flavor (González-Tomás *et al.*, 2007). El flavor en el queso proviene de diferentes compuestos (como se ha señalado anteriormente) la mayoría de los cuales se forman durante la maduración. Son muchos los métodos instrumentales que se han utilizado para el estudio de los compuestos responsables del flavor. En algunos de ellos se han utilizado técnicas de espacio de cabeza dinámicos (Valero *et al.*, 2000; Villaseñor *et al.*, 2000). En estos estudios, solamente se detectan los compuestos volátiles mayoritarios. Es de esperar que las moléculas más pesadas tengan una menor recuperación utilizando estas técnicas. Los análisis de espacio de cabeza estáticos conducen a cuantificaciones más reproducibles pero detectan un número muy bajo de compuestos (Partidario *et al.*, 1998). Para quesos de "pasta blanda", las técnicas de destilación a vacío también han sido utilizadas encontrándose gran cantidad de compuestos neutros (Gallois & Langlois, 1990). La extracción-destilación simultánea (Simultaneous Distillation Extraction, SDE) ha sido utilizada para identificar compuestos del flavor por muchos autores (González de Llano *et al.*, 1990). Este método tiende a extraer compuestos de mayor peso molecular que las técnicas de espacio de cabeza dinámicos, a expensas de la pérdida de algunos de los compuestos más volátiles. La micro-extracción en fase sólida (Solid Phase Micro-Extraction,

SPME), es un método relativamente novedoso que se ha utilizado también en algunos casos (Hayaloglu *et al.*, 2008; Wolf *et al.*, 2011). Se pueden establecer las condiciones para extraer tanto compuestos volátiles como ácidos grasos, pero sólo se extrae un intervalo relativamente estrecho de compuestos. Por último, la extracción con disolventes es un método rápido y simple que ha sido utilizado por otros autores. La principal desventaja de este método es la baja concentración de compuestos en el extracto y la pérdida de compuestos altamente volátiles (Alewijn *et al.*, 2003). Son varios los estudios encaminados a caracterizar los compuestos responsables del flavor característico del queso azul (Gallois & Langlois, 1990; González de Llano *et al.*, 1990; de Frutos *et al.*, 1991; Contarini & Toppino, 1995; Moio *et al.*, 2000; Hayaloglu *et al.*, 2008; Wolf *et al.*, 2011) algunos de los cuales además han buscado relacionar los atributos sensoriales del flavor con los compuestos volátiles identificados en el queso (Lawlor *et al.*, 2003).

La textura es otra característica sensorial muy importante, considerada por los consumidores como un factor determinante de la calidad, la elección y la preferencia. En general, la percepción de la textura se define como la manifestación sensorial de la estructura de un alimento y la manera en la que ésta reacciona a las fuerzas aplicadas en las diferentes etapas del consumo del alimento, siendo los sentidos involucrados la vista, el tacto y el oído. Por lo tanto, la percepción de la textura resulta de un proceso dinámico ya que las propiedades físicas de los alimentos cambian continuamente durante su manipulación en la boca (Saint-Eve *et al.*, 2015). El objetivo de los métodos sensoriales es adquirir una impresión de cómo se percibe la textura del queso durante el consumo. Las condiciones de estos estudios son arbitrarias y frecuentemente implican una deformación que da lugar a fractura visual aunque alternativamente, el queso puede ser evaluado por la aplicación de fuerzas o deformaciones que den lugar a fracturas no visibles. En todos los casos, se genera una impresión sensorial y los jueces asignan una puntuación, basada en uno o más criterios (O'Callaghan & Guinee, 2004). Varios intentos se han llevado a cabo para correlacionar las medidas objetivas con los atributos sensoriales de textura en una amplia variedad de quesos (Antoniou *et al.*, 2000).

Aunque son muchos los métodos instrumentales que se han usado para evaluar las propiedades reológicas del queso, éstos pueden dividirse en dos grupos. El primero

de ellos, estaría formado por todos aquellos métodos diseñados para imitar la evaluación sensorial de la textura del queso. En estos ensayos, la muestra es comprimida o penetrada en uno o más mordiscos y, de este modo, se busca simular las acciones de compresión y penetración de los dientes durante la masticación. El método más frecuentemente utilizado es el Análisis de Perfil de Textura (Texture Profile Analysis, TPA) que está diseñado para imitar el movimiento de un diente molar durante la masticación aplicando una doble compresión de mordida (Szczesniak, 1963), aunque también se han utilizado ensayos de corte en los que se mide la resistencia al paso de un cuchillo o alambre a través del queso o ensayos de penetración en los que se mide la fuerza requerida para insertar una sonda (cónica o cilíndrica) a una distancia del queso o alternativamente la profundidad de penetración de la sonda bajo una carga constante en un tiempo dado. El segundo grupo está formado por aquellos métodos instrumentales de los que pueden derivar parámetros reológicos básicos. En este grupo encontramos medidas viscoelásticas lineales que típicamente se llevan a cabo mediante torsión, utilizando reometría oscilatoria. En ellas, se aplica una baja deformación oscilante a la muestra y se mide los esfuerzos resultantes dentro de la misma. Alternativamente, se puede aplicar un pequeño esfuerzo a la muestra y la deformación resultante se mide. Además de la reometría oscilatoria, también pueden utilizarse métodos de deformación (normalmente compresión uniaxial) para obtener parámetros reológicos básicos. Una medida de compresión implica la actuación sobre una muestra rectangular o cilíndrica entre dos platos paralelos. Los ensayos de compresión son más adecuados cuando se aplica una gran tensión de deformación que para una deformación viscoelástica lineal. Esto se debe a que el contacto inicial entre las placas paralelas y la muestra por lo general implica reajustes de la superficie de la muestra debido a su imperfección como resultado de sus características macroestructurales (por ejemplo, venas, grietas o aperturas) y por la dificultad de realizar un corte preciso en la obtención de la muestra (O'Callaghan & Guinee, 2004).

Por último, varios estudios han mostrado que los atributos de apariencia y, especialmente el color, están asociados a los alimentos casi tan frecuentemente como los descriptores de flavor y textura. Es por ello que frecuentemente se han llevado a cabo medidas cuantitativas y cualitativas sensoriales del color con el objetivo de monitorizar los efectos del tratamiento tecnológico en varios quesos tradicionales y

procesados. Generalmente, las propiedades del color se miden utilizando sistemas de coordenadas tridimensionales como las coordenadas CIELAB (Rohm & Jaros, 1997). Este sistema utiliza tres parámetros que son L* que representa la luminosidad (L=0 rendimientos negros y L=1 rendimientos blancos), a* que representa la posición entre el color rojo y verde (valores negativos indican verde mientras que valores positivos indican rojo) y b* que representa la posición entre el amarillo y el azul (valores negativos indican azul y valores positivos indican amarillo). En los quesos de vena azul la percepción del color cobra una importancia más acusada que en otras variedades ya que es una característica distintiva de este tipo de queso debido al crecimiento de *P. roqueforti* en la matriz del mismo que hace que a medida que avance la maduración se acentúen las coloraciones verdosas.

1.2. El queso de Valdeón

La IGP "Queso de Valdeón" es una variedad de vena azul elaborada a partir de leche pasterizada de vaca o una mezcla de leche de vaca con leche de oveja y/o cabra, con un periodo de maduración de 2 meses. La autenticidad de este queso está garantizada desde el año 2003 mediante una Indicación Geográfica Protegida (Reglamento (CE) Nº 135/2004).

La zona de elaboración del queso de Valdeón es la constituida por el término municipal de Posada de Valdeón (León, España). Se encuentra en el Valle de Valdeón, situado en el extremo Noreste de la provincia de León, en el Parque Natural de los Picos de Europa.

Figura 3. IGP Queso de Valdeón

Las primeras referencias escritas de la elaboración del queso de Valdeón datan del siglo XIX, si

bien, se considera que su elaboración se remonta a la época prerromana cuando se utilizaba para ello como materia prima únicamente leche de cabra. En la actualidad se han incorporado nuevas tecnologías a la elaboración del queso de Valdeón como es la utilización de leche pasterizada y se ha diversificado la materia prima con el empleo de leche tanto de vaca y oveja como de cabra. No obstante, siguen plenamente vigentes los principios básicos del proceso de elaboración original.

El queso entero posee forma cilíndrica, con una altura máxima de 15 cm, un diámetro de 25 cm y un peso comprendido entre 0,5 y 3 Kg (aproximadamente 2,4 Kg es lo más habitual). Presenta un porcentaje en materia grasa igual o superior al 45% expresado sobre extracto seco con una humedad mínima del 30%.

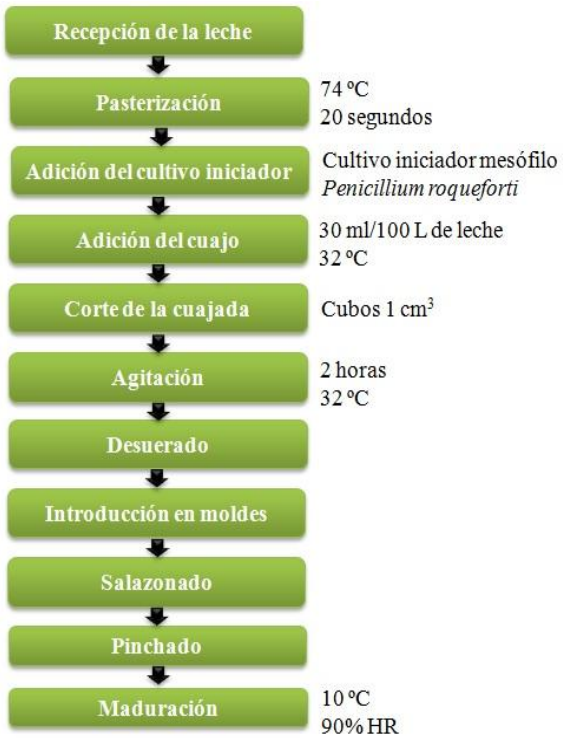

Figura 4. Diagrama de flujo de elaboración de la IGP Queso de Valdeón

La pasta es de color blanco marfil que evoluciona hacia color crema, de intensidad brillante, textura lisa, con numerosas oquedades distribuidas homogéneamente, irregulares, de tamaño variable y de color azul-verdoso, resultado del crecimiento del moho *Penicillium roqueforti* (Figura 3). Su corteza es natural, delgada, blanda, de color amarillento y con tonalidades grisáceas. Presenta un olor ligeramente

ácido o láctico cuando los quesos son semimaduros, muy específico a moho y ligeramente picante. El sabor de esta variedad es intenso, salado y picante, ligeramente ardiente, más acusado cuanto mayor sea el tiempo de maduración.

El proceso de elaboración del queso de Valdeón se resume en la Figura 4 y se describe de forma más detallada en el Capítulo 3 (Material y Métodos), apartado 3.1 (Elaboración del queso).

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2. JUSTIFICACIÓN Y OBJETIVOS

2. JUSTIFICACIÓN Y OBJETIVOS

Durante los últimos años, en España se han llevado a cabo varios estudios de tipificación de quesos tanto elaborados artesanal como industrialmente y que abarcan la práctica totalidad de Denominaciones de Origen Protegidas e Indicaciones Geográficas Protegidas existentes tanto en nuestro país como fuera de él. Sin embargo, existen algunas variedades de las que sólo se conocen algunos datos que aparecen reflejados en los Reglamentos de los Consejos Reguladores, siendo este el caso de la IGP queso de Valdeón.

El queso de Valdeón fue caracterizado en los años 90 en el Departamento de Higiene y Tecnología de los Alimentos de la Facultad de Veterinaria de León, incluyendo el estudio de su composición, fisico-química y, fundamentalmente, su caracterización microbiológica. Sin embargo, el producto que se elabora hoy en día y que está amparado bajo la IGP se elabora exclusivamente de modo industrial difiriendo sustancialmente del queso artesanal tipificado en su momento. La IGP queso de Valdeón no ha sido caracterizada, en especial en lo referente al proceso tecnológico y a las modificaciones en los parámetros químicos, bioquímicos y sensoriales que sufren durante la maduración lo que justifica la presente Tesis Doctoral.

Esta Tesis Doctoral surge con el objetivo principal de caracterizar tecnológica, química, bioquímica y sensorialmente la IGP queso de Valdeón y la influencia que el tiempo de maduración, la época de elaboración y el tratamiento térmico aplicado a la leche van a jugar en la calidad final de esta variedad. Esto permitirá, por un lado, proteger este producto de calidad determinando sus principales atributos que le hacen un alimento original, específico y característico del mercado y, por otro lado, permitirá diferenciar al queso de Valdeón de otros quesos de vena azul, tanto nacionales (Cabrales, Picón Bejes-Tresviso o Gamonedo) como extranjeros (Gorgonzola, Stilton, Roquefort, etc.).

A continuación se describen los objetivos específicos planteados para esta Tesis Doctoral:

1) Determinar la composición química y físico-química de la leche utilizada en la elaboración y el queso.

2) Estudiar la evolución de los principales grupos microbianos presentes en la leche y el queso durante su maduración y la interrelación con los parámetros físico-químicos.

3) Determinar los principales índices de la maduración del queso: glucólisis, proteólisis y lipólisis.

4) Estudiar los quesos desde un punto de vista sensorial: por una parte, empleando un panel de catadores y por otra, realizando un estudio de modo instrumental a través del estudio reológico, análisis del perfil de textura (TPA) y análisis del color mediante colorímetro.

5) Determinar los principales péptidos presentes en el queso y su actividad biológica antes y después de digestión gastrointestinal.

3. MATERIAL Y MÉTODOS

3. MATERIAL Y MÉTODOS

3.1. Elaboración del queso

Para la elaboración de los quesos objeto de nuestro estudio se contó con la colaboración de la Quesería Picos de Europa, S.L. (Posada de Valdeón, León, España). Se elaboraron un total de 12 lotes de acuerdo con el método establecido por el Consejo Regulador de la IGP Queso de Valdeón (Figura 1) (DOUE 2003/C 110/05). El queso se elaboró a partir de una mezcla de leche de vaca y cabra (90% y 10%, respectivamente). Para la fabricación de 8 de los lotes, la leche una vez filtrada fue sometida a un proceso de pasterización a 74 ºC durante 20 segundos. Después, se añadió a la leche a 32 ºC un cultivo iniciador mesófilo comercial (FD-DVS CHN-19, Chr. Hansen S.L., Madrid, España) y un suspensión líquida de esporas (1,6 x 10^5 esporas/mL) de *P. roqueforti* (Biostar, Toledo, España). Después de 2 horas, se añadieron 30 mL de cuajo líquido comercial de ternera (NATUREN liquid 140 S/S, 90% quimosina; 140 ± 5 IMCU/mL; Chr. Hansen S.L., Madrid, España) por cada 100 L de leche. Transcurridas 2 horas, la cuajada se cortó en cubos de un tamaño aproximado de 1 cm^3 y se agitó continuamente a 32 ºC durante aproximadamente 2 horas. Posteriormente, se procedió a un desuerado parcial de la cuajada y a la introducción de la misma en moldes cilíndricos donde permaneció durante 48 horas sin ejercer presión. Transcurrido este tiempo, se retiró la cuajada de los moldes y se aplicó sal seca a la superficie del queso dejándola penetrar durante 5 días. Después, los quesos se sometieron a un proceso de pinchado con el fin de airearlos internamente y promover el crecimiento de *P. roqueforti*. Por último, los quesos se llevaron a cámaras de maduración donde permanecieron a 10 ºC y 90% de humedad relativa durante 4 meses.

De los 8 lotes fabricados con leche pasterizada, dos de ellos se elaboraron en cada una de las estaciones del año, es decir, 2 lotes en verano, 2 lotes en otoño, 2 lotes en invierno y 2 lotes en primavera.

Los 4 lotes restantes se elaboraron a partir de leche cruda siguiendo el mismo método descrito anteriormente, con la única diferencia de que no se les añadió cultivo iniciador mesófilo comercial.

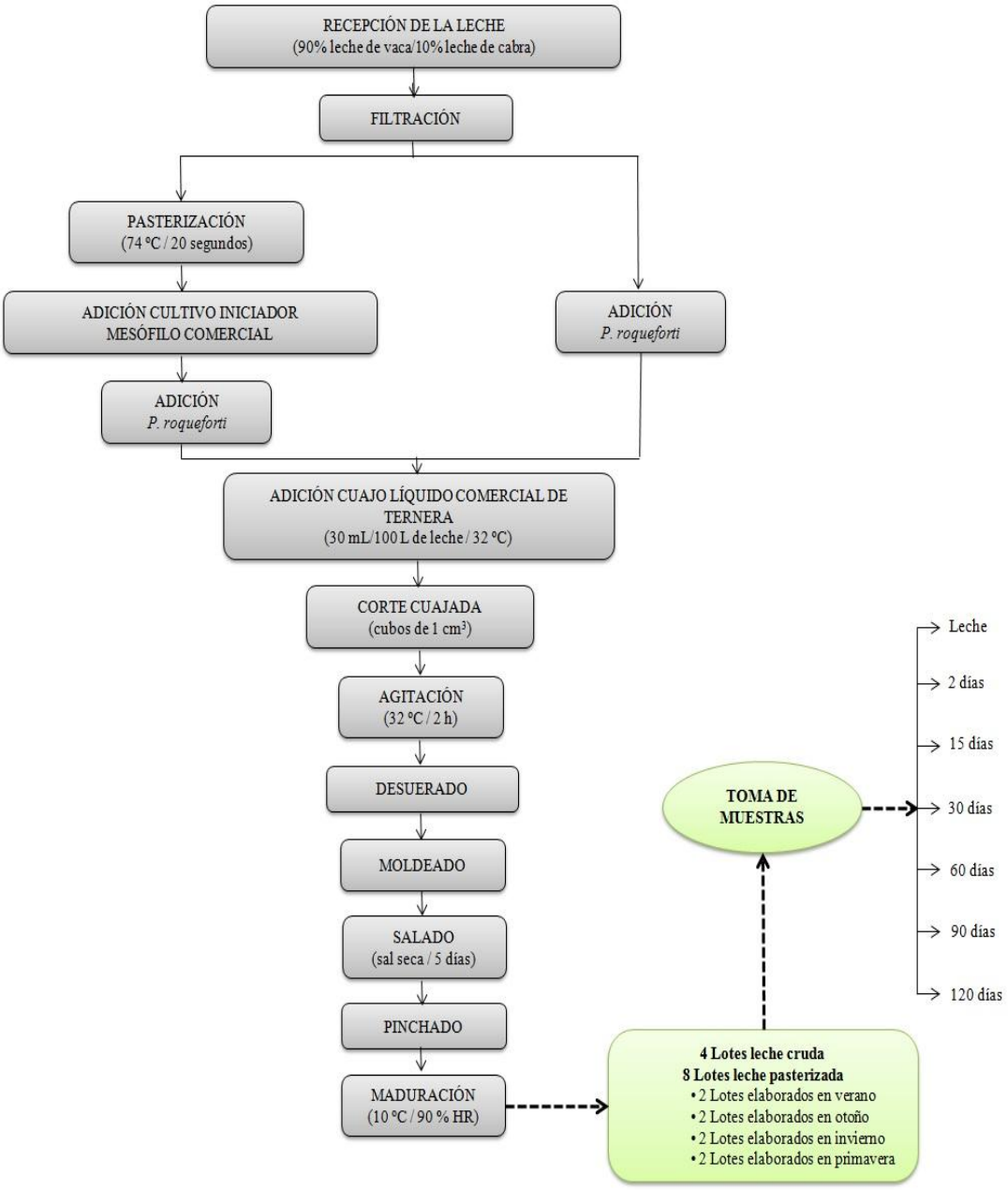

Figura 1. Diagrama de flujo de elaboración y toma de muestras de la IGP queso de Valdeón

De todos los lotes elaborados se tomaron muestras de leche y queso con 2, 15, 30, 60, 90 y 120 días de maduración. Cada muestra de queso consistió en un queso

completo de aproximadamente 2,4 Kg de peso. Todas las muestras fueron trasladadas al laboratorio del Departamento de Higiene y Tecnología (Universidad de León, España) bajo condiciones de refrigeración (por debajo de 5 ºC) y fueron almacenados a congelación (-30 ºC), excepto en el caso de aquellos análisis en que se requirieron las muestras frescas.

3.2. Análisis microbiológicos

Para llevar a cabo los análisis microbiológicos, se homogeneizaron 50 g de cada muestra con 200 mL de una solución estéril de citrato sódico al 2% (p/v) a 40-45 ºC, durante 1 minuto en un Stomacher 400 Lab blender (Seward Medical, Londres, Reino Unido), obteniendo una dilución 1:5, a partir de la cual se prepararon diluciones decimales sucesivas con agua de peptona estéril (Oxoid, Unipath Ltd., Basingstoke, Reino Unido) al 0,1% (p/v), siguiendo las indicaciones dadas por la FIL-IDF en la Norma 122B (1992).

Los análisis microbiológicos realizados fueron los detallados en la Tabla I.

Tabla I. Análisis microbiológicos realizados en el queso de Valdeón.

Grupo microbiano	Medio de cultivo	Temperatura	Tiempo
Aerobios mesófilos	Plate Count Agar (PCA)[1]	30 ºC	48 h
Psicrotrofos totales	Plate Count Agar (PCA)[1]	7 ºC	10 días
Lactococci	M17 Agar[2]	30 ºC	18-24 h
Leuconostoc	MSE Agar[2]	22 ºC	4 días
Lactobacilli	Rogosa Agar[1]	30 ºC	5 días
Micrococcaceae	Mannitol Salt Agar (MSA)[1]	30 ºC	48 h
Enterococci	Kanamycin Aescullin Azide (KAA)[1]	37 ºC	24 h
Enterobacteriaceae	Violet Red Bile Glucose Agar (VRBGA)[1]	37 ºC	18-24 h
Mohos y levaduras	Oxytetracycline Glucose Yeast Extract Agar (OGYEA)[1]	22 ºC	5 días

[1] Oxoid, Unipath Ltd., Basingstoke, Reino Unido.
[2] Biokar, Beauvais, Francia.

Para efectuar los recuentos de todos los grupos microbianos se seleccionaron las placas con un número de colonias comprendido entre 30 y 300, excepto para el recuento de *Enterobacteriaceae* en que se seleccionaron aquellas que contenían entre 15 y 150.

Los recuentos se expresaron en unidades formadoras de colonias por gramo o mililitro (UFC/g o mL).

3.3. Análisis químicos y fisicoquímicos

La determinación de los sólidos totales y del contenido en proteína y en grasa de los quesos se llevó a cabo de acuerdo a las normas 4 (FIL-IDF, 2004), 20-1 (FIL-IDF, 2001) y 222 (FIL-IDF, 2008), respectivamente. El contenido en cloruro sódico se realizó siguiendo la norma 935.43 (AOAC, 1990). El pH fue analizado de acuerdo a la norma 14.022 (AOAC, 1980). Por último, la actividad de agua se determinó instrumentalmente utilizando un equipo AquaLab Dew Point Analyzer CX-2 (Decagon Devices Inc., Pullman, WA, EE.UU.).

3.4. Determinación de los principales índices de maduración del queso

3.4.1. Estudio de la glucólisis

Tanto el contenido en lactosa, como en ácido D- y L-láctico se analizaron utilizando un kit enzimático (R-biopharm, Darmstadt, Alemania).

3.4.2. Estudio de la proteólisis

3.4.2.1. Fracciones nitrogenadas

En primer lugar, se obtuvieron los extractos de nitrógeno soluble a pH 4,6 (NS-pH 4,6) siguiendo el método descrito por Kuchroo & Fox (1982). Para la obtención del extracto de nitrógeno soluble en ácido tricloroacético al 12%, se mezclaron a partes iguales ácido tricloroacético 12% y NS-pH 4,6 y se filtró a través de un filtro Whatman nº 40 (Whatman Biosystems, Maidstone, Reino Unido). Para la obtención del nitrógeno soluble en ácido fosfotungsténico al 5%, se añadieron 35 mL de ácido sulfúrico 3,95 M, 15 mL de ácido fosfotungsténico 33% y 50 mL de NS-pH 4,6 y se filtró a través de un

filtro Whatman nº 40. La determinación del contenido en nitrógeno de las distintas fracciones se llevó a cabo mediante el método Kjeldahl (FIL-IDF 224, 2011) utilizando un sistema de destilación Kjeltec System-1002 y un sistema de digestión 6-1007 Digester (Tecator, Höganäs, Suecia).

3.4.2.2. Electroforesis de las caseínas

Para estudiar la degradación de las caseínas del queso se realizó electroforesis en gel de urea-poliacrilamida de los extractos de NS-pH 4,6, de acuerdo con el método descrito por Andrews (1983) utilizando una unidad Proten II xi Slab Cell (Bio-Rad, Richmond, CA, EE.UU.) alimentada con un equipo LKB2197 Electrofocusing Constant Power Supply (Bromma, Suecia). Las distintas fracciones obtenidas se analizaron utilizando el software TotalLab 1D, nonlinear Dynamix (Newcastle, Reino Unido).

3.4.2.3. Análisis del perfil de péptidos

Los perfiles de péptidos de los extractos solubles a pH 4,6 fueron determinados por UPLC (Ultra Performance Liquid Chromatography) en fase reversa de acuerdo con el método descrito por Sousa *et al*., (2001) con la introducción de algunas modificaciones. Para ello se utilizó un cromatógrafo Waters Acquity UPLC H-Class Core System (Waters Corp., Mildford, MA, EE.UU.). La columna utilizada fue una Acquity UPLC BEH C18 (1,7 µm, 2,1 x 50 nm) (Waters Corp., Mildford, MA, EE.UU.). El flujo se mantuvo a 0,46 mL/min. Las muestras se eluyeron durante 0,37 min con el 100% del solvente A (ácido trifluoroacético 0,1% (v/v) (TFA, grado secuencial, Sigma, St. Louis, MO, EE.UU.) en agua desionizada grado HPLC (Milli-Q system, Waters Corp., Mildford, MA, EE.UU.), después con un gradiente lineal del 20% del solvente B (TFA 0,1% (v/v) en acetonitrilo (grado HPLC, Lab-scan Ltd., Dublín, Irlanda) durante 2,13 min, después con un gradiente lineal del 50% del solvente B durante 7,5 min. Los picos fueron detectados a 214 nm utilizando un detector Waters Acquity UPCL TUV (doble longitud de onda) en interfaz con el software Empower 3 (Waters Corp., Mildford, MA, EE.UU.).

Los datos cromatográficos obtenidos fueron analizados mediante la técnica estadística multivariante descrita por Piraino *et al.*, (2004).

3.4.2.4. Análisis de los aminoácidos libres

La extracción, separación e identificación de los aminoácidos libres se llevó a cabo de acuerdo con el método descrito por Alonso *et al.*, (1994) con algunas modificaciones.

- **Extracción de los aminoácidos libres y derivatización**

Para realizar esta extracción, se homogeneizaron 5 g de queso con 50 mL de ácido perclórico 0,6 N. La mezcla obtenida se centrifugó a 1.575 *g* durante 15 min utilizando una centrífuga Centrifuge 5804R (Eppendorf, Hamburgo, Alemania) y se filtró el sobrenadante a través de un papel Whatman Nº 541 (Whatman, Maidstone, Reino Unido). A continuación, se ajustó el pH a 7,0 ± 0,2 con una solución de hidróxido potásico al 30% (p/v) y se refrigeró a una temperatura de 4 °C durante 12 horas. Transcurrido este tiempo, los extractos obtenidos se filtraron a través de un filtro Millipore de 0,45 µm (Millipore, Molsheim, Francia).

Para la derivatización de las muestras, se tomó una alícuota de 0,4 mL y se procedió a su evaporación en SpeedVac (Savant Plus SC110A, Savant Instruments. Inc, Farmingdale, NY, EE.UU.). Seguidamente, se les añadieron 20 µL de la mezcla que contenía fenilisotiocianato como agente derivatizante. A continuación, se mantuvieron 20 minutos a temperatura ambiente y en oscuridad. Transcurrido este tiempo, se realizó una segunda desecación en Speed-Vac. A las muestras se les añadió 1 mL de disolución diluyente. Seguidamente, se centrifugaron durante 5 minutos a 15.600 *g* en una centrífuga Eppendorf 5414 (Eppendorf, Hamburgo, Alemania) y se filtraron a través de filtros de jeringa Millipore de 0,45 µm de diámetro de poro (Millipore, Molsheim, Francia).

Con el fin de proceder a la cuantificación de los aminoácidos libres mediante el método del estándar externo, se prepararon 25 disoluciones patrón con distintas

concentraciones de aminoácidos. Se realizó el mismo procedimiento de análisis que con las muestras obteniéndose las curvas de calibración para cada aminoácido.

- **Desarrollo cromatográfico**

Para la separación e identificación de los aminoácidos libres se utilizó un cromatógrafo Waters 2695 (Milford, MA, EE.UU.). Para ello, se utilizó una columna de fase reversa C18 Brisa LC2 de Teknokroma (Barcelona, España) (5 µm de diámetro de poro, 4,6 x 250 mm) termostatada a 50 ± 1 ºC. La detección se llevó a cabo en un equipo Waters 2587 (Milford, MA, EE.UU.) de absorción dual a 254 nm. Los aminoácidos libres se separaron utilizando un gradiente de elución lineal de fase móvil A (acetato sódico trihidrato en acetonitrilo calidad HPLC (94:6, v/v)) y fase B (agua/acetonitrilo calidad HPLC (40:60, v/v)). El gradiente empleado se muestra en la Tabla II. El volumen de inyección fue de 20 µL.

Tabla II. Condiciones cromatográficas utilizadas en el análisis de aminoácidos libres.

Tiempo (min)	Flujo (mL/min)	% Fase A	% Fase B
0,90	0	100	0
5,00	0,90	100	0
20,00	0,80	78	22
40,00	0,75	54	46
42,00	0,80	0	100
43,00	1,00	0	100
44,00	1,00	100	0
46,00	1,25	100	0
47,00	0,90	100	0

Los datos obtenidos fueron procesados utilizando el programa informático Millenium32® de Waters ™ (Milford, MA, EE.UU.).

3.4.2.5. Análisis de la concentración de aminas biógenas

La determinación de las aminas biógenas (tiramina, putrescina, cadaverina, espermina, triptamina, feniletilamina, histamina y espermidina) se llevó a cabo siguiendo el método descrito por Moret *et al*., (2005) con algunas modificaciones.

- **Extracción de las aminas biógenas y derivatización**

Se tomaron 4 ± 0,2 g de queso y se sometieron a una doble extracción con ácido clorhídrico 0,4 M en presencia de la disolución de estándar interno (disolución de 1,7-diaminoheptano 0,6 mg/mL (p/v)). El extracto obtenido se llevó a un volumen final de 100 mL con la disolución de ácido clorhídrico 0,4 M.

Para la derivatización de las muestras se tomó 1 mL del extracto obtenido y se añadieron 200 µL de hidróxido de sodio 2 N, 300 µL de una disolución de bicarbonato sódico a saturación y 2 mL de una disolución de cloruro de dansilo (10 mg/mL de acetona (calidad HPLC)). Tras 45 min en un baño de agua a 40 ºC, se añadieron 250 µL de una disolución de hidróxido de amonio al 25% y se mantuvieron a oscuridad 30 min. A continuación, se les añadieron 1,15 mL de acetonitrilo (calidad HPLC) y se sometieron a centrifugación (804 *g*, 5 min, 20 ºC) y filtración con un filtro de jeringa Millipore (Millipore, Molsheim, Francia) de 0,45 µm de diámetro de poro.

Con el fin de determinar el momento de elución de cada una de las aminas biógenas estudiadas, para así, poder identificarlas y cuantificarlas con los cromatogramas obtenidos, se prepararon una serie de disoluciones patrones con distintas concentraciones de aminas. Cada una de ellas se derivatizó del mismo modo que las muestras de queso y se inyectaron en el cromatógrafo para obtener las curvas de calibración para cada una de las aminas objeto de estudio.

- **Desarrollo cromatográfico**

El sistema cromatográfico consistió en un HPLC Waters Alliance (Milford, MA, EE.UU.), equipado con un módulo de separación Waters 2695 conectado a un detector UV/VIS por matriz de fotodiodos Waters 2996 Photodiode Array. La separación de las aminas biógenas se llevó a cabo usando una columna Atlantis Dc18 (5 µm de tamaño de partícula, 150 mm × 4,6 mm D.I.) de Waters Alliance (Milford, MA, EE.UU.) equipada con una precolumna Atlantis Dc18 (5 µm tamaño de partícula, 20 mm × 4,6 mm D.I.) de Waters Alliance (Milford, MA, EE.UU.). El volumen de inyección fue de 20 µL. Las aminas biógenas se separaron utilizando un gradiente de elución lineal (Tabla III) con fase móvil A (acetato de amonio 0,1 M) y B (acetonitrilo, calidad HPLC). La temperatura de la columna se fijó a 40 ± 1 ºC. Los picos fueron detectados a 254 nm y se utilizó el sistema informático Empower versión 2 de Waters™.

Tabla III. Condiciones cromatográficas utilizadas en el análisis de las aminas biógenas.

Tiempo (min)	Flujo (mL/min)	Fase A	Fase B
0	1,0	50,0	50,0
19	1,0	10,0	90,0
20	0,8	0,0	100,0
23	0,1	0,0	100,0
25	0,1	0,0	100,0
30	0,9	0,0	100,0
32	1,0	50,0	50,0

3.4.2.6. Análisis de péptidos con actividad biológica

- **Preparación de las muestras**

Los extractos solubles en agua se obtuvieron siguiendo el procedimiento descrito por Gómez-Ruiz *et al*., (2004). Los extractos obtenidos se ultrafiltraron utilizando membranas Centripore Amino Ultra de 3.000 Da (Millipore Corp., Bedford, MA, EE.UU.). Para la determinación de los caseinofosfopéptidos, se llevó a cabo una etapa de enriquecimiento por precipitación selectiva de acuerdo con el método descrito por Miquel *et al*., (2005). Las muestras se congelaron a -20 ºC hasta el momento del análisis.

- **Simulación de la digestión gastrointestinal**

Se llevó a cabo una hidrólisis en dos etapas de acuerdo a Martos *et al*., (2010) con algunas modificaciones. Para ello, una mezcla de queso y agua (0,5% (p/v)) fue homogeneizada utilizando un Ultraturrax T25 Basic S25N-18G (IKA®, Werke, Alemania). Los hidrolizados se prepararon a partir de este homogeneizado. Las muestras se disolvieron (13 mg/mL de proteína) en fluido gástrico de simulación (SGF, 35 mM NaCl) a pH 2, precalentado a 37 ºC. Para llevar a cabo la primera etapa de digestión *in vitro*, se utilizó pepsina porcina (EC 3.4.23.1, 3.640 unidades/mg, Sigma, St. Louis, MO, EE.UU.) con un ratio enzima/sustrato de 1:20 p/p (182 unidades/mg) a 37 ºC durante 1 h, en presencia de fosfatidilcolina (L-R- fosfatidilcolina, P3841; Sigma, St. Louis, MO, EE.UU.). Las vesículas de fosfatidilcolina (PC) se prepararon disolviendo PC en SGF pH 2 (9,58 mg/mL) y sonicando en hielo (1 minuto 10%, 2

minutos 25%, 2 minutos 40%, 1 minuto 50% y 5 minutos 70% de fuerza), no excediendo bajo ninguna circunstancia los 40 ºC. Posteriormente, se llevó a cabo la digestión duodenal *in vitro* a partir del producto resultante de la digestión gástrica. En primer lugar, se ajustó el pH a 7 utilizando $CaCl_2$ 1 M y Bis-Tris 0,25 M pH 6,5. A continuación, se añadió la solución de sales biliares 0,125 M consistente en la mezcla equimolar de taurocolato y ácido glicodeoxicólico (Sigma, St. Louis, MO, EE.UU.). La mezcla se calentó a 37 ºC durante 15 minutos, añadiéndose previamente, tripsina (EC 232-650-8, Sigma, St. Louis, MO, EE.UU.; 40 unidades/mg proteína), R-quimotripsina (EC 232-671-2, Sigma, St. Louis, MO, EE.UU.; 0,5 unidades/mg proteína), lipasa pancreática porcina (EC 232-619-9, Sigma, St. Louis, MO, EE.UU.; 28,9 unidades/mg proteína), y colipasa (EC 259-490-12, Sigma, St. Louis, MO, EE.UU.; ratio enzima:sustrato 1:895 p/p) diluidas en 35 mM NaCl ajustada a pH 7. La reacción se detuvo añadiendo Pefabloc® SC Fluka 76307 (Sigma, St. Louis, MO, EE.UU.) a concentración final de 1 mM.

- **Análisis mediante RP-HPLC-MS/MS**

El análisis RP-HPLC-MS/MS de los extractos solubles en agua y las digestiones de los quesos se llevó a cabo de acuerdo con el método descrito por Sánchez-Rivera *et al.* (2013) mediante en un sistema HPLC Agilent 1100 System (Agilent Technologies, Waldbronn, Alemania) equipado con una bomba cuaternaria Agilent Series 1100 (Agilent Technologies, Waldbronn, Alemania) y acoplado en línea a una trampa iónica Esquire-3000 (Bruker Daltonik, Bremen, Alemania). Para este experimento se utilizó una columna en fase inversa Xbrige BEH300 C18 (4,6 x 250 mm, 5µm de tamaño de partícula) (Waters Corp., Mildford, MA, EE.UU.). El volumen de inyección fue de 50 µL, y el flujo de 0,8 mL/min. Los peptidos fueron eluidos con un gradiente lineal desde 0 a 45% del solvente B (acetonitrilo/ácido trifluoroacético 0,027%, v/v) y 55% del solvente A (agua/ácido trifluoroacético 0,037%, v/v) en 60 minutos. Se llevaron a cabo dos carreras por cada muestra con el fin de establecer el método con dos "*target mass*" diferentes: 600 *m/z* y 1.200 *m/z*. Los espectros de masas fueron adquiridos en un intervalo entre 100 y 3.000 *m/z*.

En el caso de los caseinofosfopéptidos, los análisis se llevaron a cabo utilizando una columna Mediterranea Sea18 (150 mm x 2,1 mm) (Teknokroma, Barcelona,

España). El volumen de inyección fue de 50 µL y el flujo de 0,2 mL/min. Se utilizó un gradiente lineal desde 0 a 45% de solvente B (acetonitrilo/ácido fórmico 0,1%, v/v) y 55% del solvente A (agua/ácido fórmico 0,1%, v/v) en 120 min. En este caso, los análisis se establecieron a 750 *m/z* y 1.500 *m/z*.

Los datos espectrales se procesaron y transformaron a valores de masas utilizando el programa Data Analysis versión 4.0 (Bruker Daltoniks, Karlsruhe, Alemania). La secuenciación de los péptidos se llevó a cabo utilizando Sequence Editor y MASCOT, utilizando una base de datos que incluía proteínas de leche de vaca y cabra y sus principales variantes genéticas. El programa BioTools (versión 3.2, Bruker Daltoniks, Karlsruhe, Alemania) se empleó para procesar los espectros de espectrometría de masas en tándem (MS/MS) y el análisis comparativo se realizó con la plataforma bioinformática Protein Scape 3.0 (Bruker Daltoniks, Karlsruhe, Alemania).

3.4.2.7. Análisis de la actividad de la plasmina

La actividad de la plasmina se determinó de acuerdo a lo establecido por Richardson & Pearce (1981) utilizando N-succinil-L-Ala-L-Phe-L-Lys 7-amido-4-metil-cumarina (AMC) como sustrato. La actividad plasmina se expresó como unidades de plasmina/g de queso (donde 1 unidad fue definida como la actividad necesaria para liberar 1 nmol de AMC por minuto, bajo las condiciones del análisis).

3.4.3. Estudio de la lipólisis

3.4.3.1. Análisis de los ácidos grasos libres

La extracción, separación e identificación de los ácidos grasos libres (AGLs) se llevó a cabo de acuerdo con el método descrito por de Jong & Badings (1990).

- **Extracción de los ácidos grasos libres**

Para la extracción de los AGLs, se pesó 1 g de queso junto con 3 g de sulfato sódico anhidro y se añadieron 0,3 mL de ácido sulfúrico 2,5 M y 1 mL de la disolución

con los estándares internos (ácido pentanoico [C5:0], ácido nonanoico [C9:0] y ácido heptadecanoico [C17:0] en heptano, 0,5 mg/mL). A continuación, se extrajo la grasa 3 veces añadiendo 3 mL de una mezcla 1:1 de éter dietílico-heptano y centrifugando a 804 *g*, durante 2 minutos a 20 ºC de temperatura utilizando una centrífuga Centrifuge 5804R (Eppendorf, Hamburgo, Alemania). Finalmente, se recogió la fase orgánica y se procedió a la separación de los ácidos grasos libres mediante extracción en fase sólida (SPE) utilizando columnas de polipropileno con relleno de aminopropil Sep-Pak® Vac 3cc (500 mg) (Waters, Milford, MA, EE.UU.).

Se prepararon 8 soluciones estándar con concentraciones crecientes de ácidos grasos (acético [C2:0], butírico [C4:0], caproico [C6:0], caprílico [C8:0], cáprico [C10:0], decenoico [C10:1], láurico [C12:0], mirístico [C14:0], miristoleíco [C14:1], pentadecanoico [C15:0], palmítico [C16:0], palmitoleico [C16:1], esteárico [C18:0], oleico [C18:1], linoleico [C18:2], linolénico [C18:3] y linoleico conjugado [C18:2 conj] (Sigma-Aldrich, St. Louis, MO, EE.UU.)) y concentraciones fijas de estándar internos (pentanoico [C5:0], nonanoico [C9:0] y heptadecanoico [C17:0]) para calcular las curvas de calibración.

- **Desarrollo cromatográfico**

Para la separación e identificación de los AGLs, se utilizó un cromatógrafo Hewlett Packard 6890 Series GC System (Hewlett Packard, Wilmington, DE, EE.UU.) equipado con un inyector automático Hewlett Packard 7683 Series Inyector y un detector Hewlett Packard 5973 Mass Selective Detector. La separación de los ácidos grasos se realizó en una columna Thermo Scientific 30 m x 0,32 mm x 0,50 µm (Thermo Fisher Scientific Inc., Madrid, España). La temperatura del horno fue programada desde 50 ºC hasta alcanzar una temperatura final de 200 ºC a un ritmo de 15 ºC/min y manteniéndose a la temperatura final durante 2 min, para posteriormente incrementar la temperatura hasta 220 ºC con un ritmo de 2 ºC/min manteniéndose durante 5 min. Tanto la temperatura del inyector como del detector fue de 230 ºC. El volumen de inyección fue de 4 µL (Split 10:1). La identificación de los ácidos grasos libres se llevó a cabo por comparación de los tiempos de retención con los de los patrones y posterior confirmación con los espectros de masas de los picos de los ácidos grasos de la base de datos Hewlett Packard Willey 275L Mass Spectral Library. Los

cromatogramas fueron procesados utilizando el programa informático HP G1701BA versión B.01.00 Chemstation Software (Hewlett Packard, Wilmington, DE, EE.UU.).

3.5. Estudio de las propiedades sensoriales

3.5.1. Análisis sensorial mediante panel de catadores

La calidad e intensidad de las características sensoriales de los quesos se evaluó mediante un panel de 20 catadores entrenados del Departamento de Higiene y Tecnología de los Alimentos de la Universidad de León. Los catadores estaban familiarizados con el vocabulario seleccionado y definido para describir los parámetros sensoriales del queso azul (Tabla IV).

Tabla IV. Lista de parámetros sensoriales y su definición (adaptación de Lawlor *et al.*, 2003).

Atributo	Definición
Apariencia	
Intensidad del color	El color del queso desde blanco a naranja (excluyendo los mohos).
Homogeneidad de las oquedades	La regularidad y extensión en que está abierto el interior del queso.
Oquedades azul-verdosas	El grado de enmohecimiento o crecimiento visible del moho en el queso.
Sabor y sensaciones trigeminales	
Salado	Sensación de sabor fundamentalmente típica del cloruro sódico.
Amargo	Sensación de sabor típica de la cafeína y quinina.
Dulce	Sensación de sabor fundamentalmente típica de los azúcares.
Ácido	Fuerte, picante como a cítrico. Sensación de sabor fundamentalmente típica de los ácidos láctico y cítrico.
Astringente	Seco, áspero en la boca. Sensación de desecación, disminución de las sensaciones en la cavidad inferior causando la contracción de los tejidos.
Picante	Sensación penetrante en la boca. Sabor agudo, irritante.
Persistencia	Intensidad o concentración del sabor que va desde soso o sin sabor a sabor concentrado, intenso.
Olor	
Láctico	El olor asociado a los productos lácteos fermentados.
Picante	Sensación penetrante en la cavidad nasal. Olor agudo, irritante.
Mohoso	Combinación de aromas asociados a los mohos. Generalmente son terroso, sucio, rancio, húmedo y ligeramente agrio.
Agrio	Compuestos aromáticos que recuerdan al olor generado por la transpiración de pies.
Afrutado	Mezcla aromática de diferentes frutas.
Textura	
Friabilidad	Tendencia a romperse fácilmente en partículas pequeñas e irregulares.
Adhesividad	Tendencia a resistir la separación de otro material en contacto.
Granulosidad	El grado en que se forman estructuras perceptibles cuando la muestra se rompe.
Firmeza	Desde suave/tierno a duro. Resistencia a la deformación tras la aplicación de una tensión.
Mantecosidad	Graso, como mantequilla, sensación grasa en la boca.

Se llevó a cabo un test descriptivo (ISO, 2005) en el que cada uno de los parámetros fue definido y cuantificado. En total se evaluaron 20 parámetros que fueron agrupados en 4 grandes grupos: olor (láctico, picante, mohoso, agrio y afrutado), sabor y sensaciones trigeminales (salado, amargo, dulce, ácido, astringente, picante y persistencia), textura (friabilidad, adhesividad, granulosidad, firmeza y mantecosidad) y apariencia (color, homogeneidad de las oquedades y color de las oquedades azul-verdosas). Para el análisis de cada uno de los parámetros sensoriales se utilizó la escala hedónica con los siguientes descriptores: nulo, muy débil, débil, moderado, fuerte, muy fuerte y extremadamente fuerte. A cada una de estos descriptores se le otorgó una puntuación de 1 a 7, correspondiendo el 1 con la valoración "nulo" y 7 con "extremadamente fuerte". Por último, a cada queso se le otorgó una puntuación global de 1 a 10.

3.5.2. Análisis del perfil de textura

Los parámetros de textura (fracturabilidad (N), dureza (N), adhesividad (N·s), cohesividad, elasticidad, gomosidad y masticabilidad) fueron determinados con un texturómetro mod. TZ-XT2 (Stable Micro Systems Texturometer, Godalming, Reino Unido), utilizando el análisis instrumental del perfil de textura descrito por Bourne (1982). Con un sacabocados, se obtuvieron 10 réplicas (15 mm de diámetro y 19 mm de altura) para cada queso y cada tiempo de maduración a las que previamente se retiraron 5 mm de la superficie del queso. El análisis se llevó a cabo a una temperatura de 20 ± 2 ºC utilizando para ello un sistema placa-placa con una sonda de acero inoxidable SMS P/75 a una velocidad constante de 0,5 mm/s y compresión de las muestras al 80% mediante dos ciclos de compresión. Los resultados se analizaron utilizando el software Texture Expert (Stable Micro Systems, Godalming, Reino Unido).

3.5.3. Estudio reológico del queso

Las medidas oscilatorias dinámicas fueron realizadas con un Reómetro AR2000ex (TA Instruments Company, New Castle, DE, EE.UU.) utilizando una geometría plato-plato. La placa superior fue de 40 mm de diámetro y arenada para evitar

el deslizamiento. La base consistió en una placa Peltier ajustada a 20 ºC. Con ayuda de un alambre de acero se obtuvieron réplicas de distintas partes del queso de aproximadamente 5 mm de altura y 40 mm de diámetro. El plato superior se bajó hasta estar prácticamente tocando la muestra (2 mm) y se aplicó una fuerza de 1 N para estandarizar todas las mediciones. El rango viscoelástico lineal fue determinado a través de barridos de deformación con frecuencia constante de 6,28 rad/s. Para cada muestra se eligió una constante de deformación para obtener el espectro mecánico como barridos de frecuencia de 0,06 a 628,30 rad/s. Se construyeron gráficas con el módulo de almacenamiento (G´) y el módulo de pérdida (G´´) para caracterizar el comportamiento viscoelástico. Todas las medidas fueron realizadas por triplicado. La integración de los datos se realizó utilizando el software incorporado en el equipo (Rheology Advantage 130 Data Analysis; versión 5.7; New Castle DE, EE.UU.).

3.5.4. Determinación del color

La medida instrumental del color se realizó utilizando un colorímetro de reflectancia Spectrophotometer CM- 700d (Konica Minolta, Osaka, Japón). Lectura en MAV (área de medida/iluminación) y máscara patrón MAV diámetro 8 mm con cristal. El software usado para el tratamiento de los datos fue Color Data Software CM-S100w SpectraMagic TM NX ve. 1.9, Pro USB. (Konica Minolta, Osaka, Japón). Para el análisis del color se tomaron muestras longitudinales del queso de 1 cm de grosor, aproximadamente, exponiendo la superficie interna del queso. Sobre ésta se realizaron 12 medidas en diferentes puntos por duplicado. En todas las determinaciones de color el equipo fue calibrado previamente para el cero y el blanco usando las placas estándar proporcionadas por el fabricante, y utilizando el iluminate D65 y el observador 10º SCI (AMSA, 1991). Los parámetros de color de la escala CIELab estudiados fueron: luminosidad (L*), componente rojo-verde (a*) y componente amarillo-azul (b*).

3.6. Análisis estadístico

El tratamiento estadístico de los resultados se realizó mediante análisis de la varianza (ANOVA) de una vía con el fin de determinar el efecto del tiempo de

maduración, la estación de elaboración y el tratamiento térmico aplicado a la leche sobre las distintas características estudiadas de la IGP Queso de Valdeón. Para ello, se aplicó el test de los mínimos cuadrados (LSD) para un intervalo de confianza del 95% ($p<0,05$). Las correlaciones estadísticas se realizaron mediante el coeficiente de correlación de Pearson. Ambos análisis se llevaron a cabo utilizando el programa informático Statistica versión 6.0 (Statsoft, Tulsa, OK, EE.UU.).

Para los resultados analizados mediante estadística multivariante se realizó Análisis de Componentes Principales utilizando matriz de covarianza y distancia euclídea al cuadrado mediante el programa estadístico Minitab® for Windows release 16.2.2 Minitab, Inc. 2010 (State College, PA, EE.UU.). En el caso del estudio del perfil de péptidos, previo a la aplicación del Análisis de Componentes Principales se llevó a cabo una estandarización de las variables con media cero.

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4. RESULTADOS

Artículo I

Microbiological, physico-chemical and proteolytic changes in a Spanish blue cheese during ripening (Valdeón cheese)

I. Diezhandino, D. Fernández, L. González, P.L.H. McSweeney, J.M. Fresno

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Resumen

El objetivo de este trabajo fue estudiar los cambios microbiológicos, fisico-químicos y proteolíticos durante la maduración del queso de Valdeón. Se elaboraron 8 lotes de queso y se analizaron un total de 48 quesos. Las bacterias ácido lácticas, especialmente lactococci, fueron la microbiota predominante durante las primeras etapas de la maduración, siendo gradualmente reemplazadas por los mohos y levaduras (8 unidades logarítmicas). Los recuentos de enterococci y *Enterobacteriaceae* fueron muy bajos o, incluso, cero. Esta variedad se caracterizó por un contenido en sólidos totales de 61,80 g/100 g de queso, un ratio sal/humedad de 8,92 g/100 g de humedad, un pH de 6,4-7,6 y una actividad de agua de 0,917. Al final de la maduración, la proteólisis primaria y secundaria fueron muy elevadas lo que dio lugar a una degradación casi total tanto de la αs_1- como de la β-caseínas (aproximadamente el 90%). El perfil de péptidos de los extractos solubles a pH 4,6 mostró una gran complejidad durante toda la maduración.

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Microbiological, physico-chemical and proteolytic changes in a Spanish blue cheese during ripening (Valdeón cheese)

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ABSTRACT

The aim of this work was to study the microbiological, physico-chemical and proteolytic changes in Valdeón blue-veined cheese during ripening. Eight replicas of cheese were produced and a total of 48 cheeses were analysed. Lactic acid bacteria, mainly lactococci, were the predominant flora during the early stages of ripening, gradually being replaced by moulds and yeasts (8 log units). Enterococci and *Enterobacteriaceae* counts were very low or zero. This variety was characterised by a total solids content of 61.80 g per 100 g^{-1} of cheese, a salt/moisture ratio of 8.92 g salt per 100 g^{-1} moisture, a pH of 6.4–7.6 and a water activity of 0.917. At the end of ripening, primary and secondary proteolysis were very high, which resulted in an almost total degradation of αs1- and β-casein (approximately 90%). The peptide profile of the aqueous soluble extracts at pH 4.6 showed great complexity during ripening.

1. Introduction

Cheese production in Spain has increased by 22% since 2001, currently accounting for 315,710 tonnes. This upward trend is set to continue in the coming years, unlike other European Union countries where both production and consumption of cheese have stabilised. One of the factors behind this increase has been the emergence of numerous artisanal cheese factories, where many producers process milk from their own farms. In some cases, this has enabled the recovery of traditional varieties, but in others, it has contributed to the loss of the original identity of cheeses.

The cheesemaking tradition in Spain is reflected in the existence of more than 100 different varieties of cheese, many of which are certified as being of distinctive quality through either the Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) certification systems (26 and 2, respectively). These quality designations came into being as strategies to protect original and characteristic regional products from imitation and consequent fraudulent market competition. Differentiation of a cheese granted a quality label involves determination and knowledge of the set of attributes that gives it an original, specific and distinctive product; it is therefore necessary to characterise the cheese. Such characterisation includes several steps, ranging from a study of the raw material from which the cheese is elaborated, through the procedure used to manufacture it, to its main chemical, biochemical and sensory parameters during ripening.

Although various studies have been conducted in recent years to characterise cheeses, encompassing almost all PDOs currently in existence in Europe, some protected cheeses have received very little study. One example of these is the PGI "*Queso de Valdeón*" (Valdeón cheese), for which only a few studies have been reported, related to the microbiota of the artisanal variety (Lopez Diaz, Santos, Gonzalez, Moreno, & Garcia, 1995; Lopez-Diaz, Alonso, Santos, Garcia, & Moreno, 1995). However, to the best of our knowledge, no studies have been conducted of the chemical composition or proteolysis of Valdeón cheese, or of the main biochemical and sensory changes during ripening. Only one study has been published recently about his peptidomic and changes after simulated gastrointestinal digestion (Sánchez-Rivera et al., 2014). This contrasts with other blue-veined cheese varieties, which have been characterised to a greater or lesser extent, such as mould-ripened Civil cheese (Cakmakci et al., 2013), Gorgonzola type-cheese (Seratlic, Miloradovic, Radulovic, & Macej, 2011), Cabrales (Flórez & Mayo, 2006), Picón-Bejes Tresviso (Prieto, Franco, Fresno, Bernardo, & Carballo, 2000) or Gamonedo (González de Llano, Ramos, Rodríguez, Montilla, & Juarez, 1992).

The PGI Valdeón cheese is a blue-veined variety which is manufactured in the municipal region of Valdeón (León, Spain) from a mixture of pasteurised cow's and goat's milk and is ripened for 2 months. It is cylindrical in shape, with a maximum height of 15 cm and a diameter of 25 cm. Although it can weigh between

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0.5 and 3 kg, the most frequent weight is approximately 2.4 kg. The paste is an ivory-white colour with numerous blue-green cavities, evenly distributed due to the growth of moulds of *Penicillium roqueforti*. It has a very characteristic pungent aroma of mould. Unlike the vast majority of Spanish PDO cheeses, PGI Valdeón cheese is mainly marketed in EU countries (75%) and in the United States of America, Malaysia and Japan, providing an indication of its quality and economic potential.

In our opinion, one of the ways to protect these quality products is to conduct a global characterisation in order to determine the main attributes that render them original, specific and distinctive products on the market. Proteolysis plays an important role in the final texture and flavour of the cheese. In blue-veined cheeses, it is associated mainly with the type of fungal flora and its enzymatic activity (Seratlic et al., 2011). In addition, this type of fungal flora would allow differentiate Valdeón cheese from other varieties. Consequently, the aim of our study was to analyse the microbiological, physico-chemical and biochemical changes during the manufacture and ripening of the PGI "Queso de Valdeón", paying particular attention to the degradation of proteins.

2. Materials and methods

2.1. Cheese manufacture and sampling

Eight replicas of Valdeon cheese were manufactured from a mixture of cow's and goat's milk (90% and 10%, respectively) using the standard method established by the Regulatory Board. After it has been filtered, the milk was pasteurised at 74 °C for 20 s. Then, a commercial mesophilic starter culture (FD-DVS CHN-19, Chr. Hansen SL, Madrid, Spain) and a liquid spores suspension (1.6×10^8 spores/ml) of *P. roqueforti* (Biostar, Toledo, Spain) were added to milk at 32 °C; and after 2 h, 30 ml of commercial liquid calf rennet (NATUREN liquid 140 S/S, 90% Chymosin; 140 ± 5 IMCU/ml; Chr. Hansen SL, Madrid, Spain) was added per 100 L of milk. After 60 min, the curd was cut to size of approximately 1 cm^3 cubes. Then the curd was stirred continuously at 32 °C for about 2 h before draining off some of the whey. The curd was placed in cylindrical moulds, but not pressed, where it stayed for 48 h. After this time, the curd was removed from the moulds and dry salt was added to the surface and was left to penetrate for 5 days. Then the cheeses were pierced to promote mould growth. Finally, the cheeses were transferred to a drying room where they remained at 10 °C and 90% relative humidity for 4 months.

Milk and 2-, 15-, 30-, 60-, 90- and 120-day-old cheese samples were taken from each replica. Each sample was made up one of whole cheese (2.4 kg). All samples were taken to the laboratory under refrigeration (below 5 °C) and then stored below freezing (−30 °C), except when the analyses required fresh samples.

2.2. Microbiological analyses

Fifty grams of each sample were homogenised with 200 ml of a sterile solution at 2% (w/v) sodium citrate for 2 min in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Decimal dilutions were prepared by mixing 10 ml with 90 ml of 0.1% (w/v) of sterile peptone water (Oxoid, Unipath Ltd., Basingstoke, UK), according to standard 122B (FIL-IDF, 1992).

Aerobic mesophilic and psychrotrophic bacteria were enumerated on Standard Plate Count Agar (PCA) (Oxoid) after incubation at 30 °C for 48 h and 7 °C for 10 days, respectively. Lactic acid bacteria were determined on three different media: Lactococci on M17 agar (Biokar, Beauvais, France) after incubation at 30 °C for 18–24 h; *Leuconostoc* on MSE agar (Biokar) after incubation at 22 °C for 4 days, and lactobacilli on Rogosa agar (Oxoid) after incubation at 30 °C for 5 days. *Micrococcaceae* were determined on Mannitol Salt Agar (MSA) (Oxoid) after incubation at 30 °C for 48 h; enterococci on Kanamycin Aesculin Azide (KAA) agar (Oxoid) after incubation at 37 °C for 24 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA) (Oxoid) after incubation at 37 °C for 18–24 h; and the moulds and yeasts on Oxytetracycline Glucose Yeast Extract (OGYEA) agar (Oxoid) after incubation at 22 °C for 5 days.

2.3. Physico-chemical and compositional analyses

Total solids, protein and fat contents were determined according to standards 004 (FIL-IDF, 2004), 20-1 (FIL-IDF, 2001) and 221 (FIL-IDF, 2008), respectively. NaCl content was determined according to standard 935.43 (AOAC, 1990). Lactose, D-lactic acid and L-lactic acid contents were determined using a Boehringer Mannheim enzymatic kit (R-biopharm, Roche, Germany). pH was analysed according to standard 14.022 (AOAC, 1980). Water activity (aw) was determined instrumentally using an Aqua Lab Dew Point Analyzer CX-2 (Decagon Devices Inc., Pullman, WA, USA).

2.4. Proteolytic parameters

pH 4.6-soluble nitrogen (pH 4.6-SN) extracts were performed according to Kuchroo and Fox (1982). 12% trichloroacetic acid-soluble nitrogen (TCA-SN) was obtained by mixing equal parts of pH 4.6-SN and TCA 24% and filtering through Whatman No. 40 filter paper (Whatman Biosystems, Maidstone, UK). 5% phosphotungstic acid-soluble nitrogen (PTA-SN) was obtained by adding 35 mL of 3.95 M H_2SO_4 and 15 mL of 33% PTA to 50 ml of the pH4.6-SN extract and filtering through a Whatman No. 40 filter paper. The nitrogen content in all fractions was determined by the macro-Kjeldahl method (FIL-IDF 224, 2011) using a Kjeltec System1002 Distilling Unit and a Digestion System 6 1007 Digester (Tecator).

Urea–polyacrylamide gel electrophoresis (urea–PAGE) of the pH 4.6-insoluble fractions of cheeses was studied following the method described by Shalabi and Fox (1987). Densitometric analysis was performed on the scanned image using gel analysis software using gel analysis software (TotalLab 1D, nonlinear Dynamix, Newcastle-upon-Tyne, UK).

The peptide profiles of pH 4.6-soluble nitrogen extracts of Valdeón cheese were determined by Reverse-Phase Ultra Performance Liquid Chromatography (RP-UPLC) using an Acquity UPLC-H Class (Waters Corp) according to the method described by Sousa and McSweeney (2001). The chromatographic profile was processed using the method described by Piraino, Parente, and McSweeney (2004).

Plasmin activity was determined using a modification of the method described by Richardson and Pearce (1981), using N-succinyl-L-Ala-L-Phe-L-Lys 7-amido-4-methyl-coumarin (AMC) as substrate. Plasmin activity was expressed as plasmin units per g^{-1} cheese (where 1 unit was defined as the activity necessary to release 1 nmol AMC per min under standard assay conditions).

2.5. Statistical analysis

Means with a significant difference were compared by ANOVA/MANOVA analysis with a confidence interval set at 95%. Statistical correlations were carried out by the Pearson's correlation coefficient. Both analyses were carried out using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA). Principal component analysis (PCA) was performed by standardising the variables to zero mean and using a covariance matrix. Statistical analysis was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).

3. Results and discussion

3.1. Microbiological changes during ripening

Changes in the microbial counts are shown in Table 1. All groups underwent an increase in counts during the first 2 days of ripening. This was partly due to the physical retention of microorganisms in curds and partly to microbial growth during coagulation.

The aerobic mesophilic flora counts obtained from the milk were high (5 log units); similar results have been reported by Alegría, González, Díaz and Mayo (2011) for Cabrales cheese made with pasteurised milk. However, they were lower than those observed in other similar varieties of cheese such as Cabrales (Flórez & Mayo, 2006) or Gamonedo (González de Llano et al., 1992). These differences were partly due to pasteurisation of the milk used to manufacture PGI Valdeón cheese and partly due to the strict hygiene during manufacture. The counts of aerobic mesophilic were higher at 2 days of ripening (8 log units) and gradually decreased by the end of the ripening period (approximately 6 log units).

In general, aerobic psychrotrophic bacteria counts were lower than those of total aerobic mesophilic bacteria during ripening, although the difference became smaller as the ripening progressed.

Lactic acid bacteria predominated in the Valdeón cheese during the early stages of ripening. Counts on M17 and MSE presented similar values and behaviour throughout the ripening; the highest counts were obtained in the 2-day-old samples (8 log units) and thereafter gradually decreased to values of 6 logarithmic units. The decline in the counts of lactococci during cheese ripening is due both to the hydrolytic activity of their enzymes (Thomas & Batt, 1969) and to competition with other microbial groups which are better adapted to the conditions which constitute their habitat. Moreover, it could be influenced by the salt concentration and the cheese's salt/moisture ratio. There was a significant negative correlation between salt/moisture ratio and M17 counts ($r = -0.81$) ($p < 0.001$). Meanwhile, *Leuconostoc* produces CO_2 opening the cheese structure and facilitating the introduction of air and, therefore, the development of *P. roqueforti* (Cantor, Van der Tempel, Hansen, & Ardö, 2004).

Lactobacilli counts increased during the first month of ripening, and then remained stable until the end of the process. It should be noted that this stabilisation could be due to the increase in moulds and yeasts counts, which mainly occurred after the first month of ripening. Since lactobacilli are tolerant to hostile environments in the cheese during ripening they were therefore able to grow, reaching final counts that were higher than those of the bacteria belonging to the starter (Fox, McSweeney, & Lynch, 1998). A significant negative correlation was found in the Rogosa counts with regards to levels of aw ($r = -0.44$) ($p < 0.05$) and a significant positive correlation with regards to salt/moisture ratio ($r = 0.47$) ($p < 0.05$), verifying the capacity of lactobacilli to grow in adverse conditions. Lactobacilli play an important role during ripening because they increase the concentration of small peptides, free amino acids and free fatty acids (Albenzio et al., 2001).

Some authors, such as Suzzi et al. (2000) have highlighted the importance of enterococci in cheese ripening. However, their contribution in our study was not very important because KAA counts were quite low throughout ripening, and reduced significantly by the end of it.

Micrococcaceae counts increased significantly during the first 15 days of ripening, after which time they remained constant, with values ranging between 4 and 5 logarithmic units. These values were lower than those reported by González de Llano et al. (1992) for Gamonedo. Nevertheless, this microbial group could play an important role in the ripening of Valdeón cheese because of its important proteolytic and lipolytic activity, contributing to the final texture and flavour of the cheese.

The *Enterobacteriaceae* counts are an excellent indicator of the hygienic quality of a product. Elevated counts from milk and cheese indicate poor hygiene practices during milk collection and cheese manufacture. *Enterobacteriaceae* were not generally detected due to the heat treatment to which the milk had been subjected; however, very low counts were obtained in some cases at 2 and 15 days of ripening, associated to manual surface application of dry salt.

Enterococci, *Micrococcaceae* and *Enterobacteriaceae* counts were found to be much lower than those reported by López et al. (1995) in artisanal Valdeón cheese (6.5 ± 0.3, 7.6 ± 0.5 and 3.9 ± 0.05, respectively). These differences were due to the use of pasteurised milk as well as the strict hygienic conditions during manufacture.

OGYEA counts increased during the first month of ripening, after which time they remained stable at around 8 logarithmic units. The rapid increase in *P. roqueforti* density after salting the cheeses was due, firstly, to pricking the cheeses and secondly, to the values for water activity (0.980–0.940) and salt concentration (1–3%), which were in the optimal range for *P. roqueforti* growth and germination (Godinho & Fox, 1981). Although the increased concentration of NaCl after 30 days of ripening (>3%) led to a reduction in the rate of *P. roqueforti* germination and growth, it also induced sporulation. In consequence, mould counts stabilised after the first month of ripening and the cheese developed a blue-green colour. Also, it was observed a reduction in the growth of a dense mycelium in the cracks and channels produced by pricking. Furthermore, *P. roqueforti* produces methyl ketones, which inhibit excessive growth of mould, and this could be a factor in preventing excessive mould growth in blue-veined cheeses (Girolami & Knight, 1955).

3.2. Changes in the physico-chemical parameters during ripening

Table 2 shows the changes observed in the physico-chemical parameters in the PGI Valdeón cheese during ripening. The total solids content increased significantly ($p < 0.001$) throughout

Table 1
Changes in microbial counts[a] for milk and Valdeón cheese during ripening.

Microbial group	Medium	Milk	2 days	15 days	30 days	60 days	90 days	120 days	
Total aerobic mesophilic	PCAm[b]	4.99 ± 0.52	8.52 ± 0.25	8.15 ± 0.47	7.71 ± 0.72	7.49 ± 0.67	7.72 ± 0.40	6.95 ± 0.58	***
Total aerobic psychrotrophic	PCAp[b]	2.34 ± 2.54	5.57 ± 0.95	6.93 ± 0.76	6.82 ± 0.53	7.18 ± 0.74	7.22 ± 0.87	6.40 ± 0.82	***
Leuconostoc	MSE	4.43 ± 0.55	8.18 ± 0.55	7.63 ± 0.50	7.35 ± 0.55	7.20 ± 0.21	6.82 ± 0.18	6.91 ± 0.38	***
Lactobacilli	ROGOSA	0.29 ± 0.81	4.05 ± 0.93	4.49 ± 1.02	5.67 ± 0.70	5.30 ± 0.95	5.36 ± 0.52	5.01 ± 0.20	***
Lactococci	M17	5.39 ± 1.30	8.89 ± 0.45	7.88 ± 0.74	7.47 ± 1.26	6.70 ± 1.09	6.45 ± 0.62	6.05 ± 0.71	***
Micrococcaceae	MSA	0.00 ± 0.00	2.73 ± 0.86	4.66 ± 1.05	5.19 ± 0.82	5.64 ± 0.99	5.07 ± 1.02	4.69 ± 1.20	***
Enterococci	KAA	0.29 ± 0.83	2.70 ± 1.44	2.92 ± 0.37	3.19 ± 0.91	2.42 ± 1.16	1.89 ± 1.39	1.20 ± 0.58	***
Moulds and yeasts	OGYEA	2.47 ± 1.02	4.20 ± 0.55	5.89 ± 0.13	8.20 ± 0.24	8.59 ± 0.26	8.41 ± 0.04	8.34 ± 0.08	***
Enterobacteriaceae	VRBGA	0.00 ± 0.00	1.28 ± 1.06	0.43 ± 0.55	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	***

Last column shows the significant differences during the ripening. ***$p < 0.001$.
[a] The microbial counts are expressed as log CFU/ml or log CFU/g.
[b] Aerobic mesophilic bacteria counts (*PCAm*). Aerobic psychrotrophic counts (*PCAp*).

Table 2
Evolution of the physico-chemical parameters during ripening of Valdeón cheese.

	2 days	15 days	30 days	60 days	90 days	120 days	
Total solids[a]	51.93 ± 2.32	57.15 ± 2.98	58.61 ± 2.59	60.87 ± 2.79	61.78 ± 2.87	62.74 ± 2.41	***
Protein[b]	34.53 ± 2.33	33.56 ± 1.27	33.82 ± 1.62	33.93 ± 1.73	34.35 ± 1.31	33.98 ± 1.39	NS
Fat[b]	53.62 ± 1.19	53.15 ± 1.10	56.10 ± 2.16	55.50 ± 2.23	56.33 ± 1.09	57.29 ± 1.92	***
Lactose[b]	0.78 ± 0.30	0.18 ± 0.12	0.11 ± 0.10	0.14 ± 0.15	0.09 ± 0.10	0.07 ± 0.10	***
D-lactic acid[b]	0.33 ± 0.19	0.64 ± 0.39	0.36 ± 0.23	0.16 ± 0.14	0.17 ± 0.15	0.10 ± 0.10	***
L-lactic acid[b]	3.24 ± 0.33	2.23 ± 0.51	1.21 ± 0.48	0.49 ± 0.24	0.43 ± 0.23	0.38 ± 0.21	***
NaCl[b]	1.85 ± 0.76	4.68 ± 0.42	4.78 ± 0.30	4.86 ± 0.35	4.78 ± 0.44	5.33 ± 1.05	***
Salt/moisture[c]	2.03 ± 0.87	6.25 ± 0.48	6.78 ± 0.58	7.60 ± 0.96	7.79 ± 1.24	8.92 ± 1.12	***
pH	5.03 ± 0.14	5.19 ± 0.13	5.97 ± 0.27	7.08 ± 0.39	6.83 ± 0.37	6.85 ± 0.41	***
Aw	0.979 ± 0.006	0.956 ± 0.004	0.945 ± 0.007	0.937 ± 0.008	0.928 ± 0.008	0.917 ± 0.006	***

Last column shows the significant differences during the ripening. NS – No significant differences; $^{***}p < 0.001$.
[a] Expressed as g 100 g^{-1} of cheese.
[b] Expressed as g 100 g^{-1} of total solids.
[c] Expressed as g of salt 100 g^{-1} of moisture.

ripening, finally reaching 61.80 ± 2.83 g in 100 g^{-1} of cheese. These values were higher than those reported by other authors for Gorgonzola cheese (Gobbetti, Burzigotti, Smacchi, Corsetti, & De Angelis, 1997; Zarmpoutis, McSweeney, Beechinor, & Fox, 1997), Danablu, Cashel, Chetwynd and Stilton (Zarmpoutis et al., 1997) and Picón Bejes-Tresviso (Prieto et al., 2000), and were similar to those reported for Gamonedo (González de Llano et al., 1992).

Fat and protein contents, expressed as a percentage of total solids, remained constant throughout ripening with values of around 56 g per 100 g^{-1} TS and 33.98 g per 100 g^{-1} TS, respectively. The fat content was very similar to that found in most blue-veined varieties, whereas protein levels were observed to be lower than those reported by other authors for other blue-veined cheeses (Flórez & Mayo, 2006; González de Llano et al., 1992; Prieto et al., 2000; Zarmpoutis et al., 1997).

The initial lactose content (0.78 ± 0.30% of total solids) dropped by 77% during the first 15 days of ripening. This decline was the result of the growth of the lactic acid bacteria from the starter culture during the first stages of ripening. At the start of ripening, L-lactic acid prevailed due to the predominance of LAB belonging to the starter, which transforms lactose, mainly into L-lactic acid (Thomas & Crow, 1983). The positive correlation observed between lactococci counts and L-lactic acid values ($r = 0.85$) ($p < 0.001$) corroborates the major role played by this microbial group in the formation of L-lactic acid. Thereafter, and until 60 days of ripening, a significant decrease in L-lactic acid was observed ($p < 0.001$). This reduction in the L-form was probably due to the development of moulds and yeasts that metabolise it, as well as its conversion into the D-form due to the action of microbial racemases (Prieto et al., 2000).

The concentration of NaCl increased significantly during the first 15 days of ripening, reaching values of 4.7–5 g 100 g^{-1} TS. This increase in the first few days of ripening coincided with the application of dry salt to the cheese. These levels were similar with those reported by other authors for other blue-veined cheeses (Gobbetti et al., 1997; Prieto et al., 2000; Zarmpoutis et al., 1997) The salt/moisture ratio increased significantly ($p < 0.001$) throughout the ripening process, reaching a final value of 8.92 g salt to 100 g^{-1} moisture. These values allow regulation of lactic acid flora and moulds, contributing to the optimal evolution of proteolytic and lipolytic processes.

There was a notable decrease in pH at the beginning of ripening (2 days), which subsequently increased steadily until reaching a maximum at 60 days. At the end of ripening, pH values were between 6.4 and 7.63. These values were slightly higher than those observed in other blue-veined cheeses such as Kuflu (Hayaloglu, Brechany, Deegan, & McSweeney, 2008), Picón Bejes-Tresviso (Prieto et al., 2000) and Gorgonzola, Danablu, Cashel, Stilton and Chetwynd (Zarmpoutis et al., 1997), but similar to those reported by Gobbetti et al. (1997) in Gorgonzola, González de Llano et al. (1992) in Gamonedo and Flórez and Mayo (2006) in Cabrales. This increase in the pH was the result of an intense catabolism of lactic acid and deamination reactions carried out by moulds (Hayaloglu et al., 2008).

The mean water activity values decreased as ripening progressed, from 0.979±.006 to 0.917 ± 0.006 at the end of the ripening period. These values were similar to those observed in other varieties such as Picón Bejes-Tresviso (Prieto et al., 2000) and Cabrales (Flórez & Mayo, 2006).

3.3. Changes in proteolytic parameters during ripening

Fig. 1 shows the evolution of the mean content of the different nitrogen fractions studied throughout ripening of PGI Valdeón cheese. Soluble nitrogen at pH 4.6 (pH 4.6-SN) is a very heterogeneous fraction which is used as an index for the extent of proteolysis. The content of pH 4.6-SN, expressed as a percentage of total nitrogen, increased significantly over the course of the ripening process ($p < 0.001$), reaching final values of 48.52 ± 4.05%. These values were indicative of extensive proteolysis, and were higher than those reported for various Argentinean blue-veined cheeses (Wolf, Perotti, & Zalazar, 2011), but similar to those observed in Stilton, Gorgonzola and Danablu (Zarmpoutis et al., 1997). TCA-SN contains small peptides (2–20 residues) and free amino acids produced by starter and non-starter lactic acid bacteria proteinases and peptidases by hydrolysing intermediate peptides (Sousa, Ardö, & McSweeney, 2001). The average content of TCA-SN, expressed as a percentage of total nitrogen, did not show significant differences during the first 15 days, after which time it increased significantly

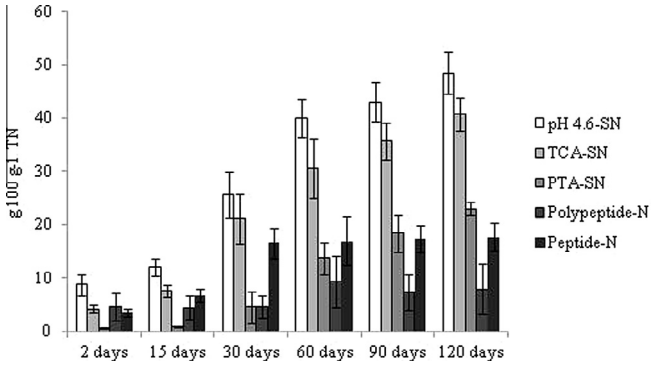

Fig. 1. Changes in soluble nitrogenous components during ripening of Valdeón cheese.

($p < 0.001$) until the end of ripening. When the TCA-SN content was expressed as a percentage of pH 4.6-SN, a value of 84.57% was obtained, equivalent to those observed by other authors (Fernandez-Salguero, Marcos, Alcala, & Esteban, 1989; Zarmpoutis et al., 1997). These results were indicative of the pronounced profound proteolysis in the PGI Valdeón cheese. PTA-SN contains small peptides (<600 Daltons) and free amino acids (McSweeney & Fox, 1993) and is used as an index of amino peptidase activity. The mean PTA-SN content, expressed as a percentage of total nitrogen, followed a pattern identical to the TCA-SN, remaining stable during the first 15 days, after which point it then steadily increased significantly until the end of ripening ($p < 0.001$). The final values obtained (22.94 ± 1.23%) were similar to those reported for other blue-veined cheeses described in the literature (Fernandez-Salguero et al., 1989; Prieto et al., 2000; Zarmpoutis et al., 1997).

Three stages were observed in the evolution of polypeptide-N during ripening: throughout the first month, values remained constant at about 5%; between 30 and 60 days there was a significant increase ($p < 0.05$), and content almost doubled; and finally, during the last 2 months of ripening, content decreased slightly to values close to 8%. During the early stages of ripening, Valdeón cheese showed a balance between primary and secondary proteolysis, as evidenced by the fact that the polypeptide-N was approximately 50% of pH 4.6-SN. However, after 30 days, this value did not exceed 20% of pH 4.6-SN, indicating an imbalance between primary and secondary proteolysis, when the latter predominated as the result of mould growth and the increase in cheese pH.

The mean content of peptide nitrogen increased significantly during the first 30 days of ripening ($p < 0.001$), from 3.51 ± 0.72% to 16.55 ± 2.87%, after which time it remained stable. The decrease in the rate of peptide nitrogen formation after 30 days was accompanied by an increase in the rate of amino acidic nitrogen formation (PTA-SN). This could be caused by the activity of peptidases secreted by *P. roqueforti* after sporulation (mainly alkaline aminopeptidases), which in Valdeón cheese, also occurred at 30 days of ripening. Thus, we found that the peptide-N, expressed as the % TCA-SN, went from more than 80% in the first month of ripening to less than half at the end of it, indicating a strong aminopeptidase activity in this variety of cheese. Between 60 and 90 days after ripening, which is the recommended consumption period for this cheese, a balance was observed between the formation of peptides and their degradation into amino acids.

Changes in the pixel intensity (volume) of the band of the electrophoretical regions in the stained gels of caseins during ripening of Valdeón cheese are shown in Table 3. As can be seen, an extensive degradation of α_{s1}- and β-caseins occurred at the end of ripening (90.22% and 89.84%, respectively). During the first few days of ripening, the degradation of α_{s1}-CN was much more pronounced than in the case of β-CN (40% vs. 15%, respectively). In the initial stages of cheese ripening, the α_{s1}-CN fraction was mainly degraded to α_{s1}-I-CN by the action of the rennet. This strong activity of the rennet was partly caused by the greater amount of coagulant retained in the curds as a result of the manufacture process in Valdeón cheese (it is not pressed), and partly due to pH values (5.0–5.2) and a salt/moisture ratio (<5%) close to the optimal range indicated by Mulvihill and Fox (1980) for chymosin. However, from 30 days until the end of ripening, a very significant decrease was observed in the α_{s1}-CN and β-CN fractions, which was much more pronounced in the case of the latter. The decline in the α_{s1}-CN fraction was accompanied by an increase in low molecular weight peptides in the electropherogram and the almost complete disappearance of the α_{s1}-I-CN fraction, whereas in the case of the β-CN fraction there was an increase in the content of low-mobility products known as γ_1-, γ_2- and γ_3-CN. This was more pronounced in the case of γ_2-CN and γ_3-CN, the concentration of which was double that of γ_1-CN. These results agree with those described by other authors for other varieties of blue-veined cheeses (Fernandez-Salguero et al., 1989; Seratlic et al., 2011; Zarmpoutis et al., 1997).

The increase in the degradation of α_{s1}-CN and β-CN after the first month of ripening coincided with the growth of *P. roqueforti* and the consequent release of proteinases into the cheese, mainly aspartyl proteases, which are very active under the pH conditions established in the cheese (6.0–7.0) (Cantor et al., 2004). The more pronounced slope observed in the degradation of β-CN between the first and second month of ripening could be explained by the additional action of the alkaline protease of the milk, or plasmin, which presents maximum activity at pH values (7.5) which are very close to those observed in Valdeón cheese. However, although higher than in other varieties of cheese (Farkye & Fox, 1990), plasmin activity in Valdeón cheese was very similar throughout ripening, with values from 2.63 ± 0.57 to 3.30 ± 0.49 plasmin units per g^{-1} cheese. This has led us to conclude that the main proteolytic agents during ripening of Valdeón cheese were proteases released by *P. roqueforti*, which was corroborated by the negative correlation observed between the yeast and mould counts on OGYEA and β-CN degradation ($r = -0.76$) ($p < 0.001$), and by the appearance of three bands of low mobility in the electrophoretogram, which Trieu-Cuot and Gripon (1982) have associated with the action of the aspartyl and metalloproteases excreted by *P. roqueforti*.

The RP-UPLC peptide profiles of the pH 4.6-soluble extracts for one of the Valdeón cheese batches are shown in Fig. 2. These chromatograms indicate considerable qualitative and quantitative differences between the cheeses during ripening, as well as a highly complex peptide profile as the result of the pronounced extensive and profound proteolysis in this variety of blue-veined cheese. However, the greater differences between ripening times appear in the retention time interval 2.4–4.5 min. These changes were much more marked after a month of ripening, coinciding with the increase in peptide-N content which, as mentioned earlier, doubled as a result of the activity of peptidases of fungal origin. This strong peptidase activity remained constant in the Valdeón cheese until the end of ripening, although at 90 and 120 days, the degradation of peptides to free amino acids may prevail over the formation of low molecular weight peptides, as it has been observed in other blue-veined cheese varieties (Gobbetti et al., 1997; Prieto et al., 2000). This would explain the reduction in the

Table 3
Changes in the pixel intensity (volume) of the band of the electrophoretical regions in the stained gels of caseins during ripening of Valdeón cheese.

	2 days	15 days	30 days	60 days	90 days	120 days	
γ_2-CN	224.83 ± 46.93	275.30 ± 38.83	591.65 ± 417.75	525.88 ± 111.30	585.36 ± 91.98	640.97 ± 108.29	*
γ_1-CN	167.18 ± 24.18	197.26 ± 20.50	220.77 ± 29.54	285.61 ± 87.67	287.06 ± 15.41	265.49 ± 144.97	**
γ_3-CN	139.20 ± 33.26	175.02 ± 44.74	309.60 ± 122.52	510.59 ± 112.72	484.57 ± 130.65	477.29 ± 86.09	***
B-CN	1400.33 ± 28.11	1377.08 ± 56.76	1188.48 ± 108.17	434.51 ± 67.37	303.37 ± 63.43	142.23 ± 85.71	***
α_{s1}-CN	1139.93 ± 70.99	898.25 ± 158.87	669.79 ± 76.77	266.30 ± 65.44	177.50 ± 48.35	111.53 ± 45.96	***
α_{s1}-I-CN	912.02 ± 185.97	909.98 ± 108.60	555.28 ± 131.28	317.13 ± 58.92	230.41 ± 88.00	69.55 ± 48.34	***

Last column shows the significant differences during the ripening. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

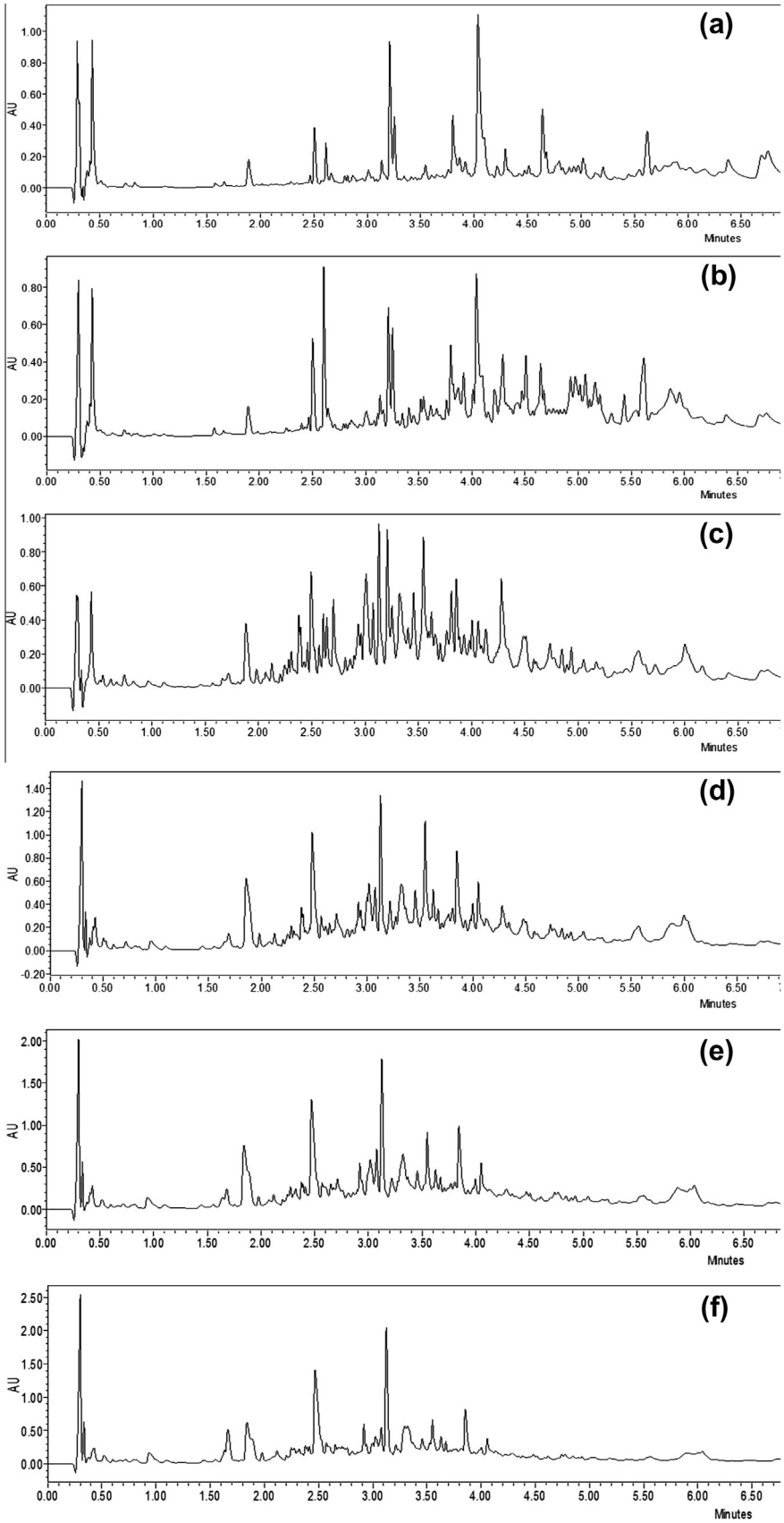

Fig. 2. Reverse-phase UPLC chromatogram of pH 4.6-soluble extract of Valdeón cheese during ripening. (a) 2 days; (b)15 days; (c) 30 days; (d) 60 days; (e) 90 days; (f) 120 days.

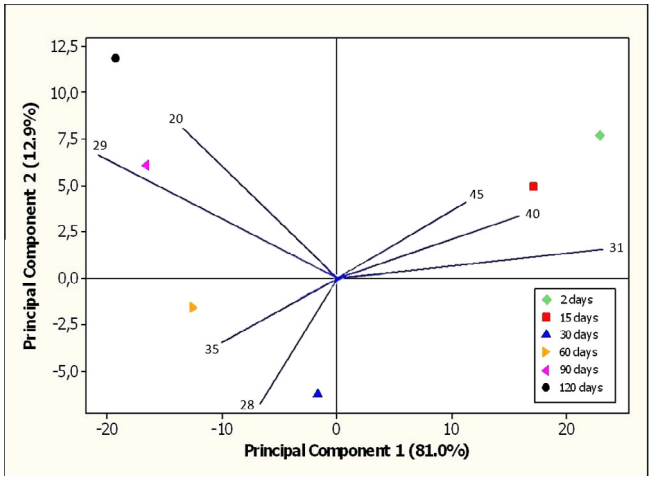

Fig. 3. Score plot of the mean for each ripening time and loading vectors of variables with high loading obtained by principal component analysis of RP-UPLC chromatograms of the pH 4.6-soluble extracts from Valdeón cheese at 2, 15, 30, 60, 90 and 120 days of ripening. The numbers indicate the position of the classes in relation to the total.

complexity of the chromatograms in the last stages of Valdeón cheese ripening.

The score plot obtained from principal component analysis of the peaks height data from RP-UPLC of pH 4.6-soluble extracts from Valdeón cheese is shown in Fig. 3. Principal components (PC) explained 93.9% of the total variation in the peptide profile of the cheeses (81.0% PC1 and 12.9% PC2). The samples were clearly separated according to their age, showing a progression that coincided with the ripening time. Vector loadings indicate that seven main classes (peptides or groups of peptides) accounted for most of the differences (Fig. 3). The high values for class 31 (3.24 min), and to a lesser extent for classes 40 and 45 (4.08 and 4.64 min, respectively), differentiated samples which had been ripened for the least time (2–15 days) from the others. These classes corresponded to the more hydrophobic peptides, since they elute at a higher acetonitrile concentration. By contrast, samples which had been ripened for longer (90 and 120 days) were characterised by high values for classes 20 and 29 (2.44 and 3.08 min), which were associated with the more hydrophilic peptides described earlier. A decrease in the ratio between hydrophobic and hydrophilic peptides over the course of ripening has also been observed in studies of other cheeses (González de Llano, Polo, & Ramos, 1995). Furthermore, the ratio of hydrophobic and hydrophilic peptides in blue-veined cheeses tends to be lower than those observed in other varieties due to the action of exopeptidases, which degrade hydrophobic peptides and release low molecular weight peptides and amino acids (González de Llano et al., 1995).

4. Conclusions

Lactic acid bacteria, mainly lactococci and *Leuconostoc* (6–8 log CFU g^{-1}), were the predominant microbial groups together with mould and yeast (8 log CFU g^{-1}) during ripening of Valdeón cheese. Micrococci and lactobacilli showed high counts at the end of ripening (5 log CFU g^{-1}), while enterococci and *Enterobacteriaceae* levels were very low or zero. The physico-chemical characteristics of Valdeón cheese were very similar to those observed in other European blue-veined cheeses; however, the pH values were slightly higher (around 7.0).

The nitrogenous fractions increased significantly during ripening, and they were indicative of the great primary and secondary proteolysis in this variety of cheese. Urea–PAGE confirmed this intense proteolysis by means of the almost complete degradation of αs1- and β-caseins.

The RP-UPLC peptide profiles of the pH 4.6-soluble extracts showed considerable qualitative and quantitative differences between the cheeses during ripening. However, seven main classes (peptides or groups of peptides) accounted for most of the differences, allowing classify Valdeón cheese according to the ripening time in three groups (2 and 15 days; 30 and 60 days; and 90 and 120 days of ripening).

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Artículo II

Rheological, textural, colour and sensory characteristics of a Spanish blue cheese (Valdeón cheese)

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Resumen

El objetivo de este trabajo fue estudiar las características reológicas, texturales, de color y sensoriales de un queso de vena azul (queso de Valdeón) durante su maduración. Para ello, se elaboraron 8 lotes de queso y se analizaron un total de 48 quesos. Todos los parámetros estudiados fueron afectados por el tiempo de maduración. A los 120 días de maduración el queso se caracterizó por valores más elevados de G', G'' y G*, mostrando una disposición más elástica, así como, valores más bajos de fracturabilidad, gomosidad y masticabilidad y valores más altos de adhesividad. Además, a medida que el tiempo de maduración avanzó los quesos se caracterizaron por un descenso en los valores L* y a*. Por último, los atributos sensoriales que más influyeron en la percepción del queso de Valdeón fueron: los sabores salado y picante; los olores agrio y picante; la adhesividad y granulosidad; y la persistencia.

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ABSTRACT

The aim of this work was to study the rheological, textural, colour and sensory characteristics of Valdeón blue-veined cheese during ripening. Eight batches of cheese were manufactured and 48 cheeses were analysed. All parameters studied were affected by ripening time. 120-day-old cheese was characterized by higher G′, G″ and G* values, showing a more elastic disposition, as well as, lower fracturability, gumminess and chewiness values and higher adhesiveness values. Moreover, as time increased cheeses were characterized by a decrease in L * and a * values. Finally, the sensory attributes that most influenced the perception of Valdeón cheese were: saltiness and pungenty; sour and pungent odours; adhesiveness and granularity; and taste strength.

1. Introduction

Cheese has a very complex structure that leads to differences, even within the same variety, as a result both of compositional factors and the changes that these undergo during ripening. It is well-known that pH has the greatest effect on the textural properties of cheese, due to its action on mineral solubilisation and casein dissociation from casein micelles (Pastorino, Hansen, & McMahon, 2003). Others important factors are the ratio of intact casein to moisture (Lawrence, Creamer, & Gilles, 1987), the manufacture techniques and storage conditions (Juan, Trujillo, Guamis, Buffa, & Ferragut, 2007).

Blue veined cheeses are characterised by the growth of *Penicillium roqueforti* in fissures throughout the cheese matrix, and are one of the most easily identifiable types of cheese. During ripening, blue veined cheeses undergo extensive proteolysis and lipolysis resulting in the development of odour, flavour, appearance and texture (Lawlor, Delahunty, Sheehan, & Wilkinson, 2003). The degree of proteolysis in blue cheeses is particularly high due to fungal proteases action, in addition to other enzymes such as chymosin, plasmin and non-starter microorganisms. These proteolytic enzymes play a vital role in the development of changes in the texture, due to hydrolysis of the casein matrix of the curd and through a decrease in the water activity of the curd due to changes in water binding by the new carboxylic acid and amino groups formed on the hydrolysis. They also contribute to the development of flavour, and possibly off-flavour (e.g. bitterness), due to the formation of peptides and amino acids which, in turn, act as substrates for secondary catabolic reactions (e.g. deamination, decarboxylation, transamination, desulphurisation, catabolism of phenylalanine, tyrosine and tryptophan, and amino acid reactions with other compounds), and they are involved in changes in the cheese matrix that allow the release of sapid compounds during chewing (Fox & McSweeney, 1996). Lipolysis is also particularly high in blue veined cheeses. Lipolysis leads to the release of free fatty acids (FFA), which besides having a direct impact on the flavour of the cheese, act as precursors for a number of catabolic reactions that cause the release of other flavour compounds, especially *n*-methyl ketones (McSweeney & Sousa, 2000).

In addition, the growth of *P. roqueforti* in blue veined cheeses produces a colour change in the matrix that varies according to ripening time and the physical and chemical conditions suitable for its germination and growth. The change in colour of blue veined cheeses while they are still on sale is a source of concern for the dairy industry (Fairclough, Cliffe, & Knapper, 2011), because colour is one of the main sensory properties that determine consumer acceptance and purchase of a product (Wadhwani & McMahon, 2012). Furthermore, consumers associate the colour of blue

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veined cheese with its flavour. The presence of a large amount of dark blue veins is usually associated with a stronger flavour in cheese, whereas a change in colour from blue to brown veins is often thought to denote over-ripening or even a defective cheese (Fairclough et al., 2011). These perceptions could lead to consumer rejection, with the consequent economic losses.

Therefore, it is very important to study the sensory parameters that define the product, using both an instrumental approach as well as tasting panels, in order to determine its acceptability to consumers. The aim of this study was to evaluate the rheological, textural, colour and sensory characteristics that define Valdeón cheese, and how these evolve during ripening, in order to determine the optimum time for consumption.

2. Materials and methods

2.1. Cheese manufacture

Eight batches of Valdeón cheese were manufactured according to the method described by Diezhandino, Fernández, González, McSweeney, and Fresno (2015). Calcium chloride (0.2 g l^{-1}) was added to pasteurized mixture of cow's and goat's milk (74 °C for 20 s). Starter cultures used were mesophilic starter culture (FD-DVS CHN-19, Chr. Hansen SL, Madrid, Spain) and a liquid spores suspension (1.6 x 10^8 spores ml^{-1}) of *P. roqueforti* (Biostar, Toledo, Spain). After half an hour, 30 ml of liquid calf rennet (NATUREN liquid 140 S/S, 90% Chymosin; 140 ± 5 IMCU ml^{-1}; Chr. Hansen SL, Madrid, Spain) was added per 100 l of milk, coagulation occurring in 60 min. Then the curd was cut to 1 cm^3 cubes and when it reached the desired consistency, the whey was drained off, and the curd was transferred to cylindrical moulds for 2 days without pressing. After this time, the curd was removed from the molds, salted dry on surface and was pierced to promote mould growth. Finally, the cheeses were transferred to a drying room where they remained at 10 °C and 90% relative humidity for 4 months. Cheese samples were taken from each batch at 2, 15, 30, 60, 90 and 120 days, each sample consisting of a whole cheese (2.4 kg).

2.2. Dynamic oscillatory testing

Dynamic oscillatory measurements were performed with an AR2000ex Rheometer (TA Instruments Company, New Castle, DE, USA) using plate–plate geometry. The upper plate was 40 mm in diameter and was made of sandblasted steel to prevent slippage. The base consisted of a Peltier plate set at 20 °C. The force applied was uniformly set at 1 N for all samples, which were cut to a thickness of 5 mm using a steel wire, and fitted to 40 mm-diameter. The upper plate was lowered until almost in contact with the sample (2 mm). The linear viscoelastic range (LVR) was determined by taking amplitude strain sweep tests at a constant angular frequency of 6.28 rad s^{-1}. This was used to choose the optimum % strain rate in each sample and was then applied to obtain the mechanical spectrum with frequency sweeps between 0.06 and 628.30 rad s^{-1}. The storage modulus (G') and loss modulus (G'') were used to construct graphs to characterise the viscoelastic behaviour. All measurements were performed in triplicate. Data integration was performed using the software incorporated into the equipment, namely Rheology Advantage Data Analysis, version 5.7 (New Castle, DE, USA).

2.3. Texture profile analysis

The texture parameters (fracturability (N), hardness (N), adhesiveness (N.s), cohesiveness, springiness, gumminess and chewiness) were determined using a Stable Micro Systems Texturometer (Godalming, UK), mod. TZ-XT2, by carrying out the texture profile analysis (TPA) described by Bourne (1982). Ten replicas (15 mm in diameter and 19 mm in height) per cheese and ripening time were obtained, after removing a 0.5 cm layer from the surface of the cheese. The test was performed at room temperature (20° ± 2° C) using a plate–plate sensor system with a stainless SMS P/75 probe (relative size of the plate to the sample 5:1) at a constant rate of 0.5 mm s^{-1}, and compressing the samples to 80% using two compression cycles. The results were analysed using the Texture Expert software (Stable Micro Systems) (Godalming, UK).

2.4. Colour analysis

Colour analysis was carried out using a reflectance colorimeter Spectrophotometer CM- 700d (Konica Minolta, Osaka, Japan). The MAV (measurement/illumination area) and MAV mask pattern were read with 8 mm diameter glass. Illuminant D65 was used with an illumination angle of 10°. Longitudinal cheese samples measuring 1 cm thick were taken by duplicate, exposing the internal surface of the cheese, and 12 measurements were carried out at different points of the exposed surface. The CIELab colour space parameters studied were: lightness (L*), the red–green dimension (a*) and the yellow-blue dimension (b*). The results obtained were interpreted using the software package Color Data Software CM-S100w SpectraMagic TM NX ve. 1.9, Pro USB (Konica Minolta, Osaka, Japan).

2.5. Sensory analysis

The quality and intensity of the sensory properties of the cheeses were evaluated by a panel of 20 trained tasters at Food Hygiene and Technology Department of the University of León. Prior to sensory analysis, tasters were trained with different foods and substances that simulated the sensory characteristics in several sessions of 1 h in duration, and then the same training was carried out with cheese. The panellists were acquainted with a selected and defined vocabulary to describe the sensory parameters of blue cheese (Table 1). A descriptive test (ISO, 2005) was carried out in which every parameter was defined and quantified. Panellists assessed a total of 20 parameters, divided into 4 main groups: odour (lactic, pungent, mouldy, sour and fruity), taste and trigeminal sensations (saltiness, bitterness, sweetness, acidity, astringency, pungenty and taste strength), texture (crumbliness, adhesiveness, granularity, firmness and buttery) and appearance (colour intensity, homogenous distribution of the veins and blue–green colour of the veins). To analyse each of the sensory parameters, a hedonic scale was used containing the following descriptors: non-existent, very weak, weak, moderate, strong, very strong and extremely strong. Each of these descriptors was scored on a scale of 1–7, where 1 indicated “non-existent” and 7 “extremely strong”. Lastly, each cheese was given a score on a scale of 1–10 for overall impression.

2.6. Statistical analysis

An ANOVA/MANOVA analysis was carried out to compare means with a significant difference, setting a confidence interval of 95%. Statistical correlations were performed by means of Pearson's correlation coefficient. Both analyses were carried out using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA).

A Principal Component Analysis (PCA) was applied to sensory results using a covariance matrix. The statistical analysis was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).

Table 1
List of sensory parameters and their definitions[a].

Attribute	Definition
Appearance	
Colour intensity	The colour of cheese ranging from white to orange (excluding the mould).
Eyes homogeneity	The regularity and extent to which the interior of the cheese is open.
Blue–green eyes	The degree of mouldiness/visible mould growth in the cheese structure.
Flavour	
Saltiness	Fundamental taste sensation of which sodium chloride is typical.
Bitterness	Chemical-like, disprin, asprin. Taste sensations of which caffeine and quinine are typical.
Sweetness	Fundamental taste sensation of which sucrose is typical.
Acidity	Sour, tangy, citrus-like, the fundamental taste sensations of which lactic acids and citric acids are typical.
Astringent	Mouth drying, harsh. The complex of drying, puckering and shrinking sensations in the lower cavity causing contraction of the body tissue.
Pungenty	Physically penetrating sensation in the mouth. Sharp smelling, irritant.
Taste strength	Intensity or concentration of flavour, ranging from bland or tasteless to a concentrated intense flavour.
Odour	
Lactic	The smell associated with milky products.
Pungent odour	Physically penetrating sensation in the nasal cavity. Sharp smelling, irritant.
Mouldy	The combination of aromatics associated with moulds. They are usually earthy, dirty, stale, musty and slightly sour.
Sour	The aromatics reminiscent of perspiration generated foot odour.
Fruity	The aromaticblend of different fruity identities.
Texture	
Crumbliness	The tendency to break down easily into small, irregular shaped particles.
Adhesiveness	The tendency to resist separation from another material it contacts.
Granularity	The extent to which granular structures are formed as the sample breaks down, perceived in the second half of chewing.
Firmnes	Ranging from soft to firm. High resistance to deformation by applied stress.
Buttery	Buttery, fatty, greasy mouth-feel of any kind.

[a] Modified from Lawlor et al. (2003).

3. Results and discussion

3.1. Dynamic oscillatory measurements

The linear viscoelastic range (LVR) was determined for all the batches by means of dynamic strain sweeps, and then a constant strain sweep was selected for all of them to carry out the frequency sweeps. Fig. 1 shows an example of how the LVR was determined for the 2 day old Valdeón cheese sample.

The frequency sweep revealed that both G' and G'' increased significantly during ripening (Fig. 2), which indicated that at 120 days of ripening, the cheeses presented a more elastic structure with a better capacity for resisting deformation (Xiong & Kinsella, 1991). The results were similar to those described by Farahani, Ezzatpanah, and Abbasi (2014) for an Iranian ewe's milk cheese. During Valdeón cheese ripening, moisture/protein ratio decreased from 2.68 to 1.75 (Diezhandino et al., 2015), leading to an increase in total protein and a reduction of the total volume of the matrix, with a consequent increase in cheese firmness (Madadlou, Khosroshahi, & Mousavi, 2005).

For all ripening times, G′ values were higher than those for G″ showing that the elastic component dominated to the viscoelasticity (Juan et al., 2007; Subramanian & Gunasekaran, 1997) which indicated the predominating solid character of cheeses, as has been reported by other authors for other cheese varieties (Farahani, Ezzatpanah, & Abbasi, 2014 Juan, Ferragut, Guamis, Buffa, & Trujillo, 2004; Kahyaoglu & Kaya, 2003). In addition, the viscoelastic nature of Valdeón cheese was reflected by the frequency dependence of G' and G'' modulus (0.06–628.30 rad s^{-1}) (Dimitreli & Thomareis, 2008; Drake, Gerard, Truong, & Daubert, 1999).

The tan δ, which is defined as the ratio between G'' and G', is a measure that relates the viscous and elastic properties of a viscoelastic material (Bayarri, González-Tomás, & Costell, 2009). The tan δ for all Valdeón samples was between 0.23 and 0.27 (Table 2). This indicated strong viscoelastic solid character of the cheeses (Luyten, Vliet, & Walstra, 1991). Significant differences ($P < 0.05$) were found in tan δ during ripening. However, those differences were very small between 2 and 120 days of ripening (0.25 and 0.23, respectively) due to the storage and loss modulus both changed during ripening in such a way that tan δ remained almost constant. This result is explained by the simultaneous and opposing effects of decrease in water, increase of pH and increase in proteolysis (Dewettinck, Deroo, Messens, & Huyghebaert, 1999).

G^* describes the overall resistance to deformation of a material that is considered an elastic solid, and is expressed in Pa (Dimitreli & Thomareis, 2008). As Table 2 shows, this parameter increased significantly ($p < 0.001$) in Valdeón cheese during ripening, except from 15 to 30 days. This exception at 30-day-old cheese was due to mould growth occurred during the second two weeks of ripening. This led to a significant increase in the rate of proteolysis of the cheese matrix, which in turn caused a reduction in the resistance of the cheese to deformation and an increase in flow capacity; thus, during this stage of ripening, the cheese behaved more as a viscous liquid than as an elastic solid. However, after the first month of ripening, the effect of proteolysis was offset by moisture loss, which caused an increase in G^* as has previously been described by other authors (Dimitreli & Thomareis, 2008; Pereira, Bennett, Hemar, & Campanella, 2001).

3.2. Texture profile analysis

Table 3 shows the means for the texture parameters studied in Valdeón cheese during ripening. Values for hardness, fracturability, gumminess and chewiness showed a very similar behaviour during ripening: these values increased during the first two weeks, then decreased significantly ($p < 0.001$) until the end of the first month, and remained constant thereafter.

Low hardness and fracturability values in the 2 day-old cheese could be related to the reduction in colloidal calcium phosphate content which could occur under the pH conditions (≈4.7) established during coagulation of the milk. Pastorino et al. (2003) reported a reduction in protein–protein interactions and consequently a weaker protein matrix, due to an increase in the proportion of soluble calcium in the cheese, from 45% to 75%, as a result of a fall in pH levels from 5.3 to 4.7. However, the significant increase that occurred in the hardness and fracturability of Valdeón

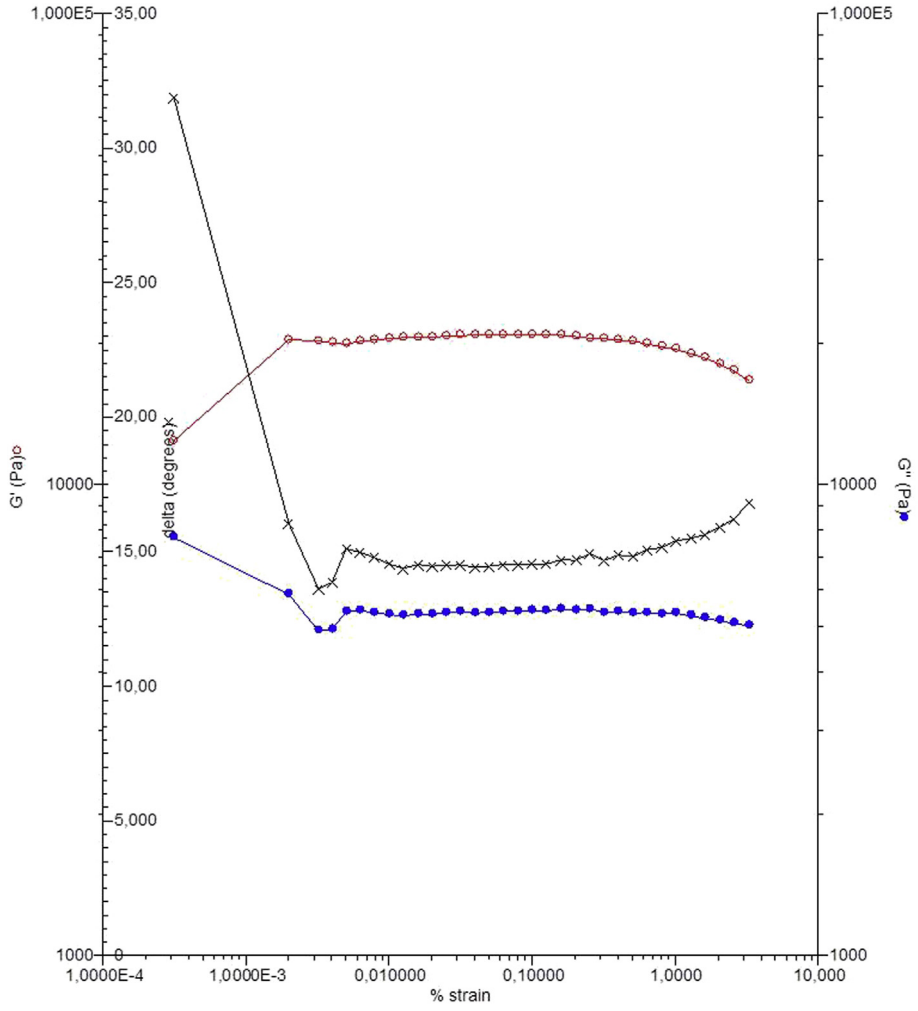

Fig. 1. Strain sweep of Valdeón cheese at an angular frequency of 6.28 rad s^{-1} at day 2. Determination of the Linear Viscoelastic Range (LVR).

cheese at 15 days was caused by two factors: salting and the pH of the cheese. Salt/moisture ratio tripled during the first two weeks of ripening, from 2.03 to 6.25 g NaCl per 100 g^{-1} of moisture (Diezhandino et al., 2015). This produced a significant increase in the ionic strength of the cheese, until similar values to those described by Pastorino et al. (2003), resulting in a reduction in protein solubility due to a salting-out phenomenon. Under these conditions, a greater number of protein–protein interactions are established, allowed for the lower level of electrostatic repulsion between the proteins due to pH values (5.1) very close to the isoelectric point of the caseins in the cheese matrix (Diezhandino et al., 2015), thereby increasing the firmness and hardness of the cheese according to the previously described results about the evolution of G* parameter.

After one month of ripening, there was a significant decrease ($p < 0.001$) in the hardness, fracturability, gumminess and chewiness values of the Valdeón cheese samples, which was mainly associated with the extent and depth of cheese proteolysis due to the development of fungal flora. Proteinases and peptidases released by *P. roqueforti* induced significant hydrolysis of the caseins, releasing low molecular weight compounds with a greater water holding capacity (Diezhandino et al., 2015). This increased proteolysis could be allowed by the low levels of colloidal calcium phosphate typical of these blue veined varieties, since this could reduce protein cross-linking, thereby exposing more protein surface area to proteases (O'Mahony, Lucey, & McSweeney, 2005; Upreti, Metzger, & Hayes, 2006). Moreover, because of the deep proteolysis occurring in this cheese variety, low molecular weight compounds are released that contribute to an increase in pH to values close to 7.0 (Diezhandino et al., 2015). This increase in pH results in an increase in the ionised carboxyl groups (the pKa of glutamic and aspartic acid is 4.25 and 3.86, respectively) and a higher repulsion between proteins, with the consequent weakening of the protein matrix, reducing its firmness (Marchesseau, Gastaldi, Lagaude, & Cuq, 1997). This phenomenon was confirmed in the Valdeón cheese samples by the negative correlation observed between pH and fracturability ($r = -0.87$; $p < 0.05$) and hardness ($r = -0.56$; $p < 0.05$).

Adhesiveness and cohesiveness values showed an opposite behaviour during Valdeón cheese ripening. Adhesiveness increased significantly ($p < 0.01$) during the first two months, but remained constant thereafter, whereas cohesiveness decreased significantly ($p < 0.001$) until near the end of the ripening period. According to Pastorino et al. (2003), the lower elasticity and cohesiveness of Valdeón cheese is due to an increase in ionic strength caused by the addition of salt, together with high levels of calcium solubilisation of caseins induced by the low pH values established during coagulation. A high negative correlation was observed in the Valdeón

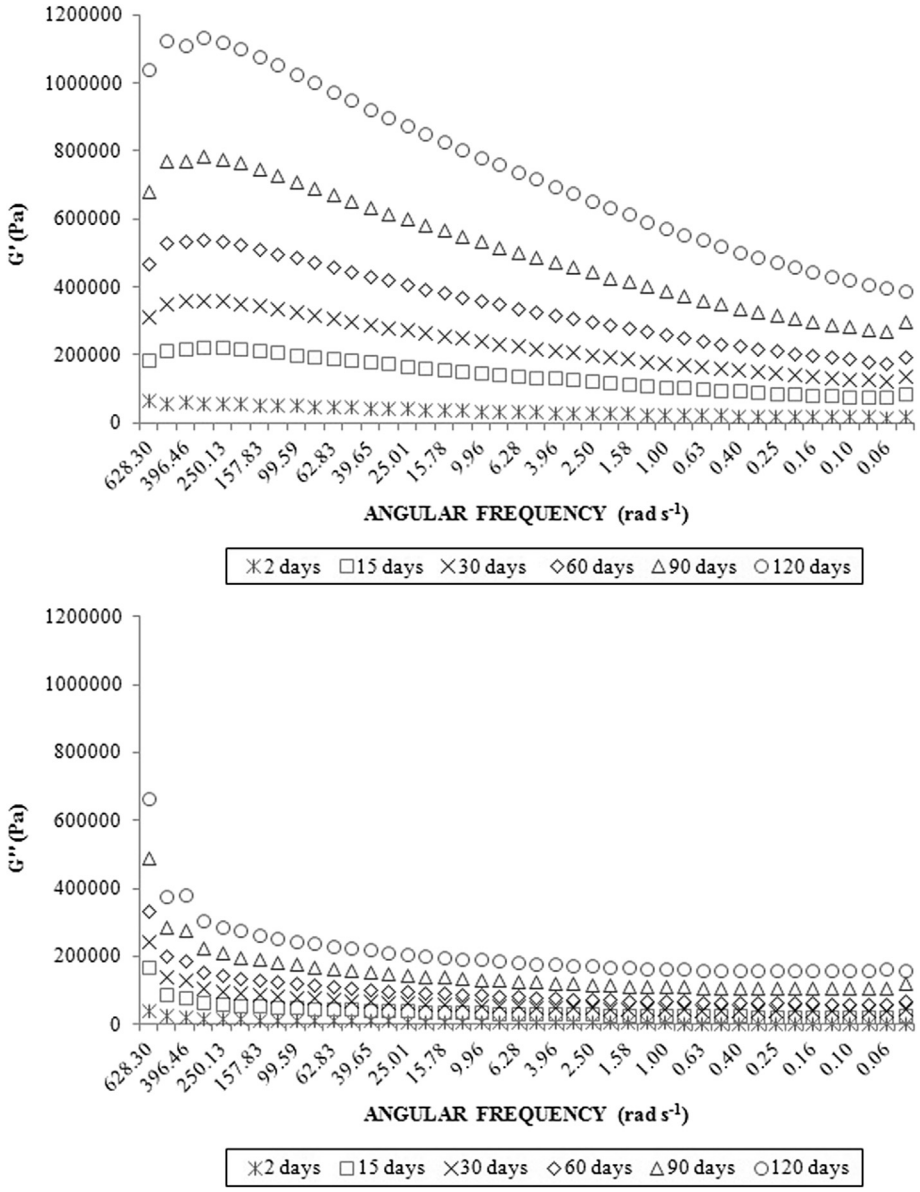

Fig. 2. Angular frequency dependence of an elastic modulus (G') and the viscous modulus (G'') during ripening of Valdeón cheese.

Table 2
Means ± SD of elastic (G') and viscous (G'') components, tan δ and shear modulus (G^*) of 6.28 rad s^{-1} of Valdeón cheese during ripening.

	Ripening time (days)						ANOVA
	2	15	30	60	90	120	
G' (MPa)	37.93 ± 16.05	108.39 ± 80.19	84.74 ± 19.12	112.47 ± 56.01	163.16 ± 90.84	234.04 ± 156.12	***
G'' (MPa)	9.57 ± 4.19	25.61 ± 21.37	21.17 ± 5.80	29.84 ± 15.09	38.86 ± 20.45	53.46 ± 35.37	***
tan δ	0.25 ± 0.02	0.23 ± 0.02	0.25 ± 0.03	0.27 ± 0.02	0.24 ± 0.03	0.23 ± 0.02	*
G^* (MPa)	39.12 ± 16.58	111.41 ± 82.96	87.37 ± 19.86	116.39 ± 57.99	167.76 ± 93.05	240.13 ± 160.10	***

Last column shows the significant differences during ripening. *$p < 0.05$; ***$p < 0.001$.

cheese samples between pH and elasticity ($r = -0.91$; $p < 0.05$) and cohesiveness ($r = -0.89$; $p < 0.05$), and between the salt/moisture ratio and elasticity ($r = -0.95$; $p < 0.01$) and cohesiveness ($r = -0.96$; $p < 0.01$). However, the increase in pH that occurred in the cheese during ripening due to extensive proteolysis contributed to an increase in protein interactions with water and other non-protein compounds, resulting in a more swollen, hydrated protein matrix, which together with the high fat content, helped increase adhesiveness.

Lastly, gumminess and chewiness values were highest in the cheeses at 15 days, subsequently showing a decreasing trend ($p < 0.001$) during ripening, falling by around 60% for gumminess

Table 3
Means ± SD of texture profile analysis parameters fracturability, hardness, adhesiveness, cohesiveness, springiness, gumminess and chewiness for Valdeón cheese during ripening.

	Ripening time (days)						ANOVA
	2	15	30	60	90	120	
Fracturability	11.77 ± 3.6	17.82 ± 4.73	9.33 ± 2.12	5.51 ± 2.10	7.33 ± 2.84	5.08 ± 1.75	***
Hardness	55.87 ± 16.28	85.37 ± 22.45	56.06 ± 10.50	52.36 ± 17.87	58.14 ± 22.88	55.00 ± 13.00	***
Adhesiveness	−1.33 ± 0.59	−3.32 ± 0.90	−6.53 ± 2.51	−8.79 ± 2.26	−10.02 ± 3.26	−8.75 ± 2.15	***
Cohesiveness	0.17 ± 0.02	0.13 ± 0.01	0.11 ± 0.01	0.10 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	***
Springiness	0.47 ± 0.11	0.37 ± 0.03	0.28 ± 0.03	0.26 ± 0.03	0.23 ± 0.04	0.24 ± 0.03	***
Gumminess	9.20 ± 2.05	11.50 ± 4.09	6.10 ± 1.41	5.31 ± 1.96	4.64 ± 1.76	4.30 ± 1.21	***
Chewiness	4.31 ± 1.07	4.24 ± 1.42	1.78 ± 0.45	1.42 ± 0.59	1.07 ± 0.39	1.04 ± 0.40	***

Last column shows the significant differences during ripening. ***$p < 0.001$.

and 75% for chewiness. This behaviour was determined by two physical and chemical factors: pH ($r = -0.90$; $p < 0.05$) and fat content ($r = -0.95$; $p < 0.01$).

3.3. Colour analysis

Table 4 shows the evolution of the colour parameters studied. Lightness values (L*) decreased significantly ($p < 0.001$) during the first two months of ripening, then subsequently remained constant until the end of ripening. Similar results have been described for pressed sheep's cheese (Picon, Alonso, Van Welly, & Nuñez, 2013), for mature goat's cheese (Fresno & Alvarez, 2012) and for mature lactic goat's cheese (Aydemir & Dervisoglu, 2010), and have been attributed to the concentration of the cheese components as a consequence of dehydration of the same during ripening. Indeed, we observed a positive correlation between values for lightness and moisture ($r = 0.90$; $p < 0.05$), confirming the existence of a relationship between these parameters.

The a* values were similar or slightly lower than those reported by other authors for Kulek cheese (Aydemir & Dervisoglu, 2010) and Minas Frescal (Magenis et al., 2014), although in these cases, the values observed were attributed to the presence of riboflavin. However, the a* values in Kulek cheese presented an opposite trend to those in Valdeón cheese, since they reduced during ripening, probably due to oxidation of riboflavin. On the other hand, a particularly pronounced decrease in a* values was observed ($p < 0.001$) after one month of ripening, corresponding to the germination and growth of *P. roqueforti* during this period (Diezhandino et al., 2015), causing the cheese to develop a stronger green colouration as ripening progressed. This was confirmed by the high correlation observed ($r = -0.94$; $p < 0.01$) between a* value and mould counts in OGYEA.

The b* values showed a slight tendency towards yellowish colouration, similar to those described for Majorero cheese by Fresno and Alvarez (2012), but lower than those reported by Aydemir & Dervisoglu, 2010 and Magenis et al. (2014) for other fresh or ripened cheeses. These low b* values led to a predominance of greenish over yellowish colouration, helping to establish a greater contrast between the mould veins and the whitish matrix. This colour contrast is highly valued by consumers as an indication of the sensory quality of Valdeón cheese.

3.4. Sensory analysis

A Principal Component Analysis (PCA) was applied to all Valdeón cheese samples in order to obtain a more simplified overview of the relationship between the sensory properties evaluated. Two principal components (PC1 and PC2) were selected, which together accounted for 93.2% of total variance (71.6% and 21.6%, respectively). The sensory properties that best correlated with PC1 were: pungenty flavour, pungent and sour odour, adhesiveness, graininess and persistence. PC2 was associated with: adhesiveness, creaminess, and bitterness, salty and pungenty flavours (Fig. 3b). None of the sensory properties related to appearance were associated with PC1 and PC2. This is in agreement with the results obtained from the instrumental analysis of colour, where the most significant changes occurred during the first month of ripening in the Valdeón cheese samples, subsequently maintaining similar values during the rest of the ripening period. This was due to the fact that germination and growth of *P. roqueforti* mainly occurred during the first 30 days (Diezhandino et al., 2015), following which both the colour and the characteristics of the veins in the cheese matrix did not undergo any further significant changes.

The score plots for all the samples on the first and second components (Fig. 3a) allowed classifying Valdeón cheese samples according to their ripening time. Samples which had been ripened for the least time (30 days) were characterised by a bitter flavour that differentiated them from the rest. The higher scores given for this parameter in the 30 day old cheese samples could be related to the increase in peptidase activity observed during the early stages of ripening, which could lead to a greater accumulation of the hydrophobic peptides with a small molecular size that are responsible for bitter flavour in cheese (Lemieux & Simard, 1992). In a previous study by Diezhandino et al. (2015), a reduction was observed in the ratio of hydrophobic/hydrophilic peptides in Valdeón cheese during ripening.

Table 4
Means ± SD for colour parameters of Valdeón cheese during ripening.

	Ripening time (days)						ANOVA
	2	15	30	60	90	120	
L*	89.05 ± 2.37	88.84 ± 3.18	77.89 ± 8.67	69.43 ± 8.81	71.48 ± 8.76	69.53 ± 7.68	***
a*	−0.29 ± 0.39	−0.17 ± 0.65	−2.33 ± 1.43	−2.74 ± 1.01	−2.42 ± 1.09	−2.17 ± 1.00	***
b*	11.95 ± 1.98	14.20 ± 2.80	12.07 ± 2.99	11.42 ± 2.94	11.52 ± 3.21	11.19 ± 3.04	***

Last column shows the significant differences during ripening. ***$p < 0.001$.

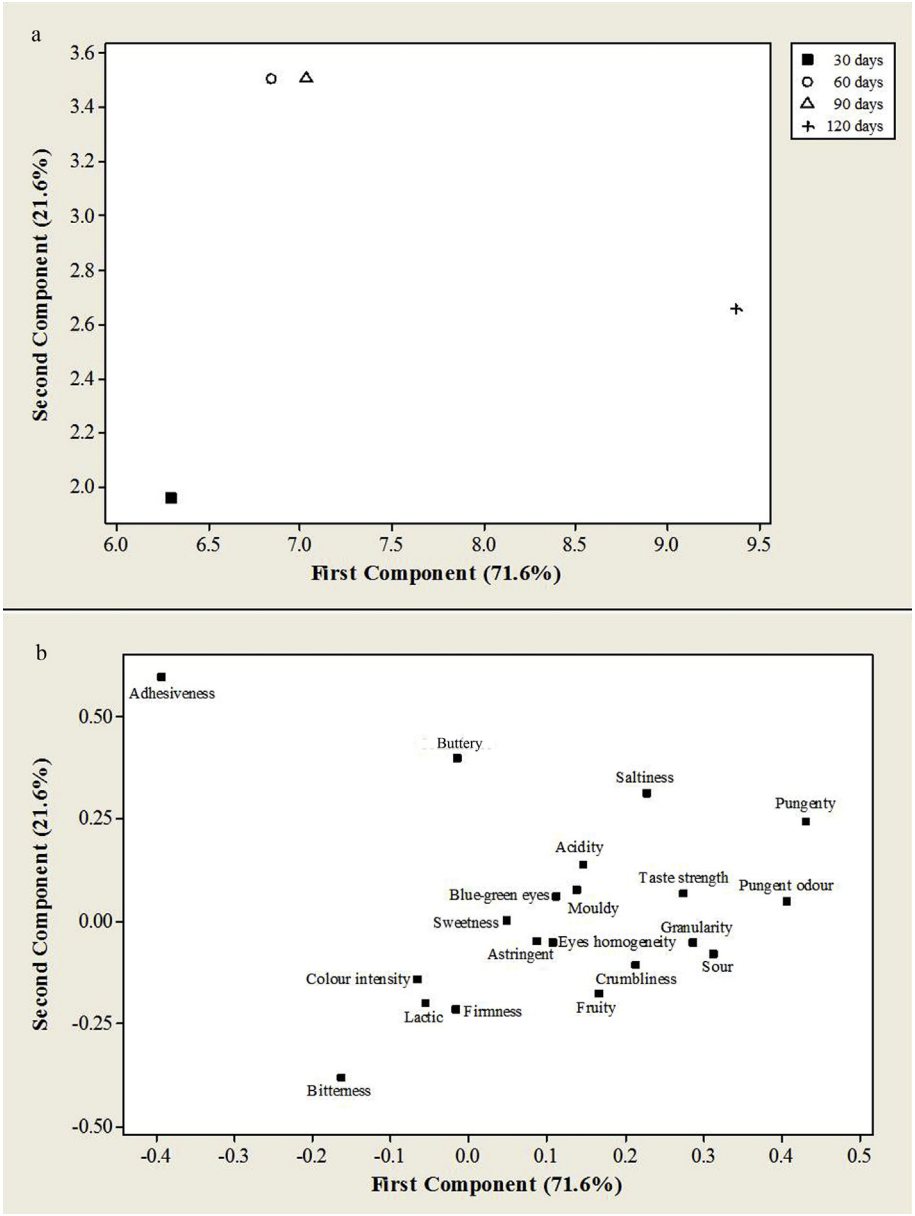

Fig. 3. Score plot of the mean for each ripening time (a) and loading vectors of sensorial attributes (b) obtained by principal component analysis from Valdeón cheese at 2, 15, 30, 60, 90 and 120 days of ripening.

The 60 and 90 day old cheeses were found to be very similar to each other and were characterised by the textural properties of buttery and adhesiveness. These results are related to the greater extent and depth of proteolysis that occurred in the cheese after one month of ripening, together with an optimal percentage of moisture for the development of these properties. The trend shown by the values for adhesiveness and buttery correlated with that described in our instrumental analysis of adhesiveness.

Cheese samples at 120 days of ripening were characterised by a greater number of sensory properties, and were associated with pungenty and salty flavours, pungent and sour odours, high persistence in both the mouth and nose and a grainy texture. The pungent odour could be associated with the increase in lipolysis that this variety of cheese undergoes during ripening, which leads to an accumulation of high concentrations of free fatty acids with less than 10 carbon atoms, which are characterised by their volatile and irritant nature. These cheeses were also characterised by a grainy texture, which was due to the increase in dry matter that occurs in this cheese during ripening. The increase in a sour odour at the end of ripening is associated with an increase in the decomposition of amino acids in the final stages of cheese ripening, during which volatile short chain acids are generated by oxidative processes of aldehydes released during the oxidative deamination of amino acids, as noted by Hemme, Bouillanne, Metro, and Desmazeud (1982). Blue veined cheeses are characterised by the high concentrations of salt necessary to allow the germination and growth of spores of *P. roqueforti* (Guinee & Fox, 1996). Tasters gave high scores for salinity, and after 30 days of ripening, salinity scores increased significantly ($p < 0.001$), reaching the highest values (4.74) obtained with regard to the rest of the flavour properties evaluated in Valdeón cheese.

Lastly, the scores given by the panel of tasters reflecting their overall impression of the Valdeón cheese samples increased significantly ($p < 0.05$) during the first 3 months of ripening, going

from 5.82 at 30 days to 7.16 at 90 days. The absence of any further increase in overall impression scores during the last month (score of 7.11 at 120 days of ripening) was associated by the tasters with the emergence of some defects during the sensory analysis of the cheese: an excessively salty flavour, an undesirable sensation of pungency in the nose and mouth and an odour of ammonia.

4. Conclusions

Valdeón cheese was classified as a viscoelastic solid which presents a more elastic structure as ripening progresses. G* increased during ripening, except from 15 to 30 days, showing an increase in the resistance to deformation as ripening time advanced.

The TPA showed that the highest values for the parameters of hardness, fracturability, gumminess and chewiness were attained during the first two weeks of ripening, and subsequently decreased until the end of the ripening process.

This variety was characterised by a reduction in lightness (L*) in the first two months of ripening. Values for a* decreased during ripening, although this reduction was more pronounced at 30 days, coinciding with the germination and growth of *P. roqueforti* and therefore with the acquisition of a greenish colour. Meanwhile, values for b* indicated a slightly yellowish colouration.

The PCA showed that the properties that most influenced the sensory analysis of Valdeón cheese, and which therefore characterise this variety, were saltiness and pungency, pungent and sour odours, adhesiveness and granularity, and taste strength.

According to the panel of tasters, 90 days was considered the optimum ripening period for the consumption of Valdeón cheese.

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Artículo III

Microbiological, chemical, textural and sensory changes in a Spanish blue cheese (Valdeón cheese) made in different seasons

I. Diezhandino, D. Fernández, M.E. Tornadijo, P.L.H. McSweeney, B. Prieto, J.M. Fresno

En preparación para su publicación

Resumen

La IGP queso de Valdeón es una variedad de vena azul que está elaborada en el término municipal de Posada de Valdeón (León, España). El objetivo de este trabajo fue determinar las características del queso de Valdeón elaborado en las diferentes estaciones del año (verano, otoño, invierno y primavera). Para ello se elaboraron 8 réplicas de queso, dos en cada una de las estaciones. Los quesos elaborados en verano y otoño presentaron recuentos más bajos en PCAm como respuesta a los valores más elevados de sólidos totales y del ratio sal/humedad y más bajos de actividad de agua. El estudio de las fracciones nitrogenadas reveló una mayor extensión y profundidad de la proteólisis en los quesos de otoño, lo que coincidió con la mayor degradación de de las caseínas observada. Por el contrario, los quesos de primavera fueron los que menos extensión y profundidad de la proteólisis mostraron. Por otro lado, las réplicas de otoño y verano fueron las que presentaron valores más elevados de los parámetros reológicos estudiados por lo que se caracterizaron por presentar una disposición más elástica con mayor capacidad para resistir la deformación, así como una masa más firme. Estos resultados coincidieron con lo observado en el estudio del perfil de textura donde los lotes de verano y otoño presentaron mayor dureza. Por último, los quesos de verano presentaron puntuaciones más altas en la valoración sensorial global, si bien no aparecieron diferencias significativas entre los quesos elaborados en las distintas estaciones.

Microbiological, chemical, textural and sensory changes in a Spanish blue cheese (Valdeón cheese) made in different seasons

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Abstract

PGI Valdeón cheese is a blue-veined variety elaborated in the municipal region of Valdeón (León, Spain). The aim of this work was determined the characteristics of Valdeón cheese elaborated at different seasons (summer, autumn, winter and spring). Eight batches were made, two in each season. Cheese elaborated in summer and autumn presented lower PCAm counts as result of the high total solids and salt/moisture values and low water activity. Nitrogen fractions determination showed a great extent and profound of proteolysis in autumn batches, which agreed with the high casein degradation observed. On the other hand, spring cheeses showed the lowest extent and profound of proteolysis. Cheese made in autumn and summer showed higher rheological parameters values, thus they were characterized by presented a more elastic disposition with better capacity to resisting deformation and a more firmness matrix

These results agreed with those observed in texture profile analysis in which autumn and summer batches presented greater hardness. Finally, cheeses made in summer were given higher overall scores, although no significant differences were observed between cheeses elaborated throughout the year.

KEYWORDS: Blue-veined cheese; Season; Microbiology; Proteolysis; Rheology; Sensory

1. Introduction

Blue cheese is a type of soft cheese characterised by the growth of *Penicillium roqueforti* in fissures throughout the matrix (Wolf *et al.*, 2011). During ripening, blue-type cheeses undergo extensive proteolysis and lipolysis resulting in odour, flavour, appearance and texture development (Lawlor *et al.*, 2003). PGI Valdeón cheese is a blue-veined variety elaborated in the municipal region of Valdeón (León, Spain). Traditionally, this cheese was manufactured from raw goat´s milk (occasionally mixed with cow´s milk) during spring and summer and cheeses were ripened in natural caves (López-Díaz *et al.*, 1995). However, nowadays, Valdeón cheese is elaborated from a mixture of pasteurized cow´s, ewe´s and goat´s milk throughout all the year.

Milk composition and milk microbiology depend on different factors such as the breed, lactation, feeding, environment conditions and milking systems, among others. Therefore, and although it was used milk from the same farms during cheese manufacture, it exists a great variability in milk composition during the year which could results in changes in the

final cheese. In fact, keep cheese stability throughout the year is a problem which many cheese producers are facing, especially in countries like New Zealand, Australia, Ireland and United Kingdom (Coker *et al.*, 2005). Although this variability in milk composition is not a problem in Spain where the cattle raising is predominantly intensive, in the case of certain cheeses such as Valdeón cheese, there is concern about the standardization of the manufacture process. This uncertainty is due to, firstly, milk comes from small farms and, therefore, the potential variability is high, and secondly, because the tradition of cheese production over all the year is relatively recent.

Consequently, the aim of this work was study the Valdeón cheese elaborated in the different seasons in order to quantify if there are significant differences throughout the year in the microbialogical, physico-chemical and proteolytic parameters; as well as, quantify textural and sensorial characteristics, and finally, determine if

the manufacturing process is conveniently standardized.

2. Material and methods

2.1. Cheese manufacture and sampling

Eight batches of Valdeón cheese were elaborated according to the method described by Diezhandino *et al*. (2015) in the different seasons of the year: 2 in summer, 2 in autumn, 2 in winter and 2 in spring. It was used a commercial mesophilic starter culture (FD-DVS CHN-19, Chr. Hansen SL, Madrid, Spain) and a liquid spores suspension of *P. roqueforti* (Biostar, Toledo, Spain). 60-days-old cheese sample was taken from each batch (habitual date of cheese consumption). Each sample consisted in a whole cheese (2.4 Kg).

2.2. Physicochemical analysis

Total solids, fat and protein contents were determined according to the standard FIL-IDF methods (FIL-IDF, 2004, FIL-IDF, 2001 and FIL-IDF, 2008, respectively). NaCl and pH determination was carried out in accordance with AOAC methods (AOAC, 1990 and AOAC, 1980, respectively). Lactose and D- and L-lactic acids were determined using a enzymatic kit (R-biopharm, Darmstadt, Germany). Finally, the water activity (aw) was measured using an Aqualab Dew Point Analyzer CX-2 (DECAGON Devices Inc., Pullman, WA, USA).

2.3.Microbiological analysis

Aerobic mesophilic and psychrotrophic bacteria were enumerated on Plate Count Agar (PCA) (Oxoid, Unipath Ltd., Basingstoke, UK) after 48 h of incubation at 30 ºC and 10 days at 7 ºC, respectively; lactococci were determined on M17 agar (Biokar, Beauvais, France). All plates were incubated at 30 ºC for 18-24 h; *Leuconostoc* were scored on MSE agar (Biokar) after 4 days of incubation at 22 ºC; lactobacilli were growth on Rogosa agar (Oxoid) and enumerated after 5 days at 30 ºC; Manitol Salt Agar (MSA) (Oxoid) was used to enumerate *Micrococcaceae* after 48 h of incubation at 30 ºC; enterococci were determined on Kanamycin Aesculin Azide (KAA) agar (Oxoid) after incubation at 37 ºC for 24 h; *Enterobacteriaceae* were scored after 24 h at 37 ºC in Violet Red Bile Glucose Agar (VRBGA) (Oxoid); finally, moulds and yeasts were plated on the surface of Oxytetracycline

Glucose Yeast Extract (OGYE) agar (Oxoid) and incubated at 22 ºC for 5 days.

2.4.Proteolytic parameters

The procedure of Kuchroo & Fox (1982) was followed for the extraction of pH 4.6 soluble nitrogen (pH 4.6-SN), 12% trichloroacetic acid-soluble nitrogen (TCA-SN) and 5% phosphotungstic acid-soluble nitrogen (PTA-SN). The nitrogen content of each fraction was determined by the macro-Kjeldahl method, as described by the FIL-IDF 224 (FIL-IDF, 2011) standard, using a Kjeltec System-1002 Distilling Unit and a Digestion System 6 1007 Digester (Tecator, Höganäs, Sweden).

The casein degradation of the pH 4.6-insoluble fractions of cheese was studied using urea–polyacrylamide gel electrophoresis (urea–PAGE) according to the method described by Shalabi & Fox (1987). For the identification and quantification of the casein fractions, it was used the software TotalLab 1D, nonlinear, Dynamix (Newcastle-upon-tyne, UK).

The pH 4.6-soluble fractions were analysed by Reverse-Phase Ultra Performance Liquid Chromatography (RP-UPLC) using an Acquity UPLC-H Class (Waters Corp., Mildford, MA, USA) following the method described by Sousa & McSweeney (2001) in order to obtain the peptide profile. The chromatograms were processed according to Piraino *et al.* (2004).

Finally, plasmin activity was determined following the method described by Richardson & Pearce (1981).

2.5 Dynamic oscillatory testing

Dynamic oscillatory testing was performed at 20 ºC according to Diezhandino *et al.* (2016) using an AR2000 ex Rheometer (TA instruments Company, New Castle, DE, USA). Parallel plate geometry (40 mm diameter, sandblasted steel to prevent slippage) was used. The force applied was uniformly set at 1 N for all samples. Strain sweep test were made at a constant frequency of 6.28 rad s-1 to determine the linear viscoelasticity zone. Frequency sweeps tests were then performed (within the linear viscoelastic region) from 0.06 to 628.30 rad s^{-1}. To study how manufacture season influenced the viscoelastic properties of the cheeses, a comparison was made of storage modulus (G'), loss modulus (G''), shear modulus (G*) and loss angle (tan δ) at 6.28 rad s^{-1}. Data

integration was performed using the software incorporated into the equipment, namely Rheology Advantage Data Analysis, version 5.7 (New Castle, DE, USA). All measurements were performed in triplicate.

2.6 Texture Profile Analysis

Texture profile analysis (TPA) was performed using a Texturometer mod. TZ-XT2 (Stable Micro Systems, Godalming, UK) according to Bourne (1982). The test conditions were: 20 ± 2 ºC room temperature; two consecutive cycles of 80% compression; constant speed of 0.5 mm s^{-1} and plate-plate sensor system with a stainless SMS P/75 probe. The texture variables determined were: fracturability (N), hardness (N), adhesiveness (N.s), cohesiveness, springiness, gumminess and chewiness.

2.7 Colour measurement

A reflectance colorimeter Spectrophotometer CM- 700d 146 (Konica Minolta, Osaka, Japan) was used to measure the colour of the samples. Measurements were taken on different points of the surface of cheeses. Twelve consecutive measures were taken for every cheese. The MAV (measurement/illumination area) and MAV mask pattern were read with 8 mm diameter glass. Illuminant D65 was used with an illumination angle of 10º. CIELab: L*, a* and b* values were read from each sample. Data were processed using the software package Color Data Software CM-S100w SpectraMagic TM NX ve. 1.9, Pro USB (Konica Minolta, Osaka, Japan).

2.8 Sensory analysis

Sensory attributes were determined by 20 trained panellists at the Food Hygiene and Technology Department of the University of León. Prior to analysis panellists were trained according to Diezhandino *et al.* (2016). A sensory analysis scoring sheet was developed specically for this cheese, taking the following attributes into account: lactic, pungent, mouldy, sour and fruity (odour analysis), saltiness, bitterness, sweetness, acidity, astringency, pungenty and taste strength (taste and trigeminal sensations analysis), crumbliness, adhesiveness, granularity, firmness and buttery (texture analysis) and colour intensity, homogenous distribution of the veins and blue-green

colour of the veins (appearance analysis). Intensity of each attribute was score on a scale from 1 (non-existent) to 7 (extremely strong). Finally, each cheese was given a score on a scale from 1 to 10 for overall impression.

2.9.Statistical Analysis

An ANOVA/MANOVA analysis was carried out to compare means with a significant difference, setting a confidence interval of 95%. Statistical correlations were performed by means of Pearson`s correlation coefficient. Statistica® for Windows version 8.0, StatSoft, Inc. 2007 177 (Tulsa, OK, USA) was used for both statistical analysis of the results. Principal component analysis (PCA) was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA) by standardising the variables to zero mean and using a covariance matrix.

3. Results and discussion

3.1.Microbiological and physico-chemical changes

All physico-chemical parameters studied showed significant differences according to manufacturing season (Table 1), except NaCl content. In general, the cheeses elaborated in summer differed the most from the rest of seasons. These cheeses were characterized for higher total solids, protein, salt/moisture ratio and pH values, as well as, lower D- and L-lactic acids content and aw. On the contrary, batches made in spring showed opposite physico-chemical characteristics, with higher D- and L-lactic acids content and aw, and lower protein, fat, S/M and pH values. Cheeses elaborated in autumn and winter showed physic-chemical characteristics intermediate between summer and spring batches.

Microbiological counts showed significant differences between cheeses elaborated in the different seasons (Table 2), except counts on PCAp, OGYEA and VRBGA. PCAm counts were significant lower in batches made in summer and autumn (6.98 ± 0.25 and 7.07 ± 0.64 log units, respectively), probably as a consequence of the physico-chemical characteristics in these batches which restricted microbiological growth, with higher salt/moisture (8.51 ± 0.66 and 7.96 ± 0.90 g per 100 g^{-1} of moisture, respectively) and total solids values (62.48 ± 2.11and 61.66 ± 1.51 g per 100

g^{-1} of cheese, respectively) and lower aw (0.928 ± 0.005 and 0.936 ± 0.003, respectively). Instead, cheeses made in spring presented the highest PCAm counts (8.45 ± 0.44 log units).

Lactic acid bacteria also showed significant differences. Batches elaborated in summer presented the highest MSE counts and the lowest M17 counts (7.09 ± 0.28 and 4.65 ± 0.34 log units, respectively). The lower counts on M17 were associated with the higher salt content and S/M ratio which stimulated lactococci autolysis (Bie & Sjöström, 1975).

It highlighted the differences found in counts on MSA and KAA. Cheeses made in autumn presented higher counts in both microbial groups (4.92 ± 0.51 and 3.95 ± 0.46 g per 100 g^{-1} of cheese, respectively), while cheese elaborated in winter and spring showed the lowest counts. *Micrococcaceae* and enterococci can play an important role in cheese ripening (Bhowmik & Marth, 1990; Suzzi *et al.*, 2000), so these differences found could result in different sensory characteristics in the final cheese.

No significant differences were found in counts on OGYEA due to the use of *P. roqueforti* in the manufacture of Valdeón cheese as secondary starter culture, resulting in a great homogeneity in this group. On the other hand, independently of manufacture season, *Enterobacteriaceae* were not detected which demonstrated that excellent hygienic conditions were maintained during Valdeón cheese manufacturing.

3.2.Proteolytic changes

The evolution of the different nitrogen fractions studied in Valdeón cheese made at different seasons is showed in Table 3. Soluble nitrogen at pH 4.6 (pH 4.6-SN), expressed as a percentage of total nitrogen, presented significant differences according to manufacture season ($p < 0.05$). Cheeses elaborated in autumn and winter showed the highest values and, therefore, a greater extent of proteolysis. However, and independently of season, in all cases the values were very high (39.98 ± 3.51 g 100 g^{-1} TN on average) due to the important proteolysis that occurs in this variety (Diezhandino *et al.*, 2015). TCA-SN, expressed as a percentage of total nitrogen, also presented significant differences ($p < 0.05$). If TCA-SN is expressed as a percentage of pH 4.6-SN, we found the highest values in summer and autumn cheeses (83.46% and

80.43%, respectively), while spring and winter cheeses showed the lowest values (73.33% and 69.28%, respectively). Cheeses elaborated in summer showed the highest values of PTA-SN, expressed as a percentage of total nitrogen, followed by autumn batches. The TCA-SN and PTA-SN results pointed that the profound of proteolysis was higher in summer and autumn. Finally, no significant differences were observed in polypeptide-N and peptide-N.

Table 3 also showed the changes in the pixel intensity (volume) of the bands of the electrophoretical regions in the strained gels of caseins. All fractions showed significant differences according to manufacture season. In general, cheese made in summer and autumn presented greater caseins degradation while batches elaborated in winter and, specially, spring presented lower caseins degradation. Since counts on OGYEA were very similar throughout the year, the differences observed in the pattern of caseins degradation do not seem to be a consequence of the activity of proteases released by *P. roqueforti*. α_{s1}-CN is degraded by the action of rennet during the early stages of ripening. The low pH and S/M ratio observed in cheese elaborated in spring explained why the α_{s1}-CN degradation is lower in them. In the rest of batches the pH was close to optimum pH described for rennet by Mulvihill & Fox (1980). On the other hand, the activity of the alkaline protease of the milk or plasmin showed significant differences between the batches. Cheeses made in autumn and summer presented the highest activity (3.85 ± 0.59 plasmin units g^{-1} and 3.45 ± 1.72 plasmin units g^{-1}, respectively) while the lowest activity was observed in cheeses elaborated in spring and winter (3.24 ± 0.22 plasmin units g^{-1} y 2.67 ± 0.58 plasmin units g^{-1}, respectively). Therefore, the greatest β-CN degradation found in summer and autumn was explained by the high activity of plasmin.

Figure 1a showed the score plot obtained from principal components analysis of peptide profile from pH 4.6-soluble extracts. Principal components (PC) explained 81.44% of the total variation (64.26% PC1 and 17.18% PC2). The samples were separated according to manufacture season. However, an exception appeared in the cheeses elaborated in summer due to the great differences observed between the

duplicates. These results agreed with the great variability found in this season in all the studies conducted in the present work resulting in high standard deviations. The great differences were observed in the retention time from 1.8 to 3.3 minutes. Vector loadings showed that 6 main classes (peptides or groups of peptides) explained mainly the differences observed (Figure 1b). All cheeses, independently of manufacture season, showed a high hydrophilic/hydrophobic ratio which agree with the ripening time of the cheeses and with the great proteolysis that occurs in this variety.

3.3.Rheology and texture analysis

All parameters studied showed significant differences according to manufacturing season, except tan δ values (Table 4). Cheeses made in autumn, following by cheeses made in summer, presented the highest G´, G´´ and G* values. Therefore, these cheeses were characterized by a more elastic structure with a better capacity for resisting deformation (Xiong & Kinsella, 1991). On the other hand, no significant differences were observed in the TPA, apart from hardness and adhesiveness parameters (Table 4). The greatest differences were observed between batches elaborated in autumn (high hardness and adhesiveness values) and winter (low hardness and adhesiveness values). These results agreed to those observed in rheology analysis. In fact, it was observed a positive correlation ($p < 0.05$) between hardness values and G´, G´´ and G* parameters. The highest values observed in all parameters in batches elaborated in autumn and summer were due to three main factors: low water content, high salt/moisture ratio and high pH values. Firstly, when water content decrease, protein dehydration occurs, resulting in a lower degree of freedom movement of protein molecules and therefore a firmer cheese matrix (Fresno & Álvarez, 2012). In addition, the lowest water content results in high S/M ratio. According to Pastorino *et al.* (2003), at high ionic strength, the solubility of proteins frequently decreases, and proteins come out of solution which causes an increase in hardness and adhesiveness. On the other hand, Watkinson *et al.* (2001) reported that as pH increased the firmness of the cheese increased. In addition, the highest adhesiveness observed in autumn cheeses could be

related with its highest fat content. Finally, the highest profound of proteolysis showed in summer cheeses resulted in lower G´, G´´, G* and hardness compared with autumn cheeses, as well as, the profound of proteolysis also explained the slightly differences observed between winter and spring batches in the rheology and texture studies.

3.4.Colour analysis

The evolution of the colour parameters studied is showed in Table 5. Significant differences were observed in L* and b* values. Cheeses made in spring presented the highest differences regarding the rest of cheeses in both parameters. These batches were characterized by highest L* values and lowest b* values. This behaviour could be related with the highest water content observed in spring cheeses, resulting in high luminosity and yellowish colouration.

However, no significant differences were observed in a* values which was due to the similar growth of *P. roqueforti* (no significant differences in OGYEA counts).

3.5.Sensory analysis

Figure 2 shows the results obtained in sensory analysis of Valdeón cheese at 60 days. No significant differences were observed in any of the attributes studied, except in bitterness and sweetness. In the case of appearance the great homogeneity observed in the blue-green eyes and eyes homogeneity attributes agreed to the results obtained in a* values in the instrumental colour analysis. On the contrary, no significant correlation was observed between instrumental (TPA) and sensory texture analysis. As regards taste parameters, cheese elaborated in summer showed the greatest differences compared with the rest of batches. These results were associated with the highest profound of proteolysis observed in these cheeses which result in a low hydrophobic/hydrophilic ratio (Lemieux & Simard, 1992). On the other hand, cheeses made in winter differed from the rest in sweetness (low scores). These results were related with the higher saltiness, astringent and acidity scores obtained in winter cheeses which could do the sweetness less noticeable. Finally, the overall score did not show significant differences between the different manufacturing seasons. However, cheeses elaborated in summer

presented the highest overall scores (7.22) while winter cheeses showed the lowest (6.44).

4. Conclusions

Valdeón cheese manufacturing in the different seasons of the year resulting in microbiological, physico-chemical and proteolytic changes. The highest differences appeared between cheeses elaborated in autumn and summer which were very similar and cheeses made in winter and spring. However, textural and sensory characteristics were just slightly affected by the manufacturing season. Only significant differences were observed in hardness and adhesiveness, as well as, bitterness and sweetness. Finally, no significant differences were observed in overall scores and, therefore, from a sensory point of view, cheeses were found to be very similar.

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Table 1. Physico-chemical characteristics in Valdeón cheese at 60 days of ripening.

	Summer	Autumn	Winter	Spring	ANOVA
Total solids[1]	62.48±2.11^{a}	61.66±1.51^{ab}	58.02±0.72^{c}	59.38±2.78^{bc}	*
Protein[2]	35.55±1.12^{a}	33.65±1.42^{b}	34.62±1.27^{ab}	32.93±0.98^{b}	*
Fat[2]	54.82±1.09^{a}	58.81±2.07^{b}	55.37±2.03^{a}	54.73± 0.42^{a}	**
Lactose[2]	0.14±0.03^{a}	0.34±0.06^{b}	0.00±0.00^{c}	0.08±0.01^{ac}	**
D-lactic acid[2]	0.06±0.04^{a}	0.22±0.01^{b}	0.09±0.03^{a}	0.27 ±0.06^{b}	*
L-lactic acid[2]	0.17±0.01^{a}	0.47±0.1 ^{b}	0.59±0.06^{b}	0.72±0.20^{b}	*
NaCl[2]	5.09±0.06^{a}	4.94±0.44^{a}	4.92±0.34^{a}	4.64±0.31^{a}	NS
Salt/moisture	8.51±0.66^{a}	7.96±0.90^{a}	6.80±0.49^{b}	6.77±0.34^{b}	**
pH	7.30±0.04^{a}	7.27±0.23^{a}	7.12±0.26^{ab}	6.60±0.40^{b}	*
aw	0.928±0.005^{a}	0.936±0.003^{ab}	0.937±0.006^{ab}	0.946±0.008^{b}	*

[1]Expressed as g 100 g^{-1} of cheese.
[2]Expressed as g 100 g^{-1} of total solids.
Means in the same row with the same superscript do not differ significantly (P > 0.05).
Last column show the significant differences. NS- No significant differences; * p<0.05; **p<0.01.

Table 2. Changes in microbial counts[1] in Valdeón cheese at 60 days of ripening.

	Summer	Autumn	Winter	Spring	ANOVA
PCAm[2]	6.98±0.25^{a}	7.07±0.64^{a}	7.46±0.10^{ab}	8.45±0.44^{b}	*
PCAp[2]	6.81±0.01^{a}	6.32±0.87^{a}	7.75±0.03^{a}	7.86±0.97^{a}	NS
MSE	7.09±0.28^{a}	6.97±0.16^{a}	5.38±0.19^{b}	5.38±0.41^{b}	**
ROGOSA	4.55±0.30^{a}	5.65±0.08^{b}	4.51±0.43^{a}	6.49±0.41^{b}	*
M17	4.65±0.34^{a}	6.26±0.60^{b}	4.70±0.43^{a}	6.20±0.36^{b}	*
MSA	3.80±0.03^{a}	4.92±0.51^{b}	3.13±0.24^{a}	3.44±0.26^{a}	*
KAA	2.70±0.01^{a}	3.95±0.46^{b}	1.63±0.25^{c}	1.41±0.19^{c}	**
OGYEA	8.60±0.10^{a}	8.43±0.06^{a}	8.95±0.85^{a}	8.39±0.30^{a}	NS
VRBGA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	NS

[1]The microbial counts are expressed as log CFU Ml^{-1} or log CFU g^{-1}.
[2]Aerobic mesophilic bacteria counts (*PCAm*). Aerobic psychrotrophic counts (*PCAp*).
Means in the same row with the same superscript do not differ significantly (P > 0.05).
Last column shows the significant differences. NS- No significant differences; * p<0.05; **p<0.01.

Table 3. Changes in soluble nitrogenous components and in the pixel intensity (volume) of the band of the electrophoretical regions in the stained gels of caseins of Valdeón cheese at 60 days of ripening.

	Summer	Autumn	Winter	Spring	ANOVA
pH 4.6-SN	37.49±2.60^{a}	43.44±0.01^{b}	42.52±1.81^{b}	36.48±2.33^{a}	**
TCA-SN	31.29±1.81^{a}	34.94±5.50^{a}	29.46±10.72^{ab}	26.75±0.06^{b}	*
PTA-SN	15.60±2.86^{a}	16.15±2.00^{a}	13.07±1.57^{ab}	9.74±0.84^{a}	*
Polypeptide-SN	6.19±0.79^{a}	8.51±5.51^{a}	13.06±8.91^{a}	9.73±2.40^{a}	NS
Peptide-SN	15.69±1.05^{a}	18.80±7.50^{a}	16.39±9.15^{a}	17.00±0.77^{a}	NS
γ_2-CN	425.84±46.54^{a}	462.11±44.38^{a}	529.40±50.88^{a}	680.18±67.73^{b}	*
γ_1-CN	196.89 ±23.26^{a}	324.55±42.57^{b}	231.66 ±7.21^{a}	389.34±34.92^{b}	**
γ_3-CN	362.81 ±51.58^{a}	533.81±10.15^{bc}	509.70±49.29^{b}	636.06±50.73^{c}	*
β-CN	475.50 ±7.95^{ab}	412.24±10.15^{b}	493.96±7.13^{a}	565.95±3.88^{a}	*
α_{s1}-CN	229.02 ±8.92^{a}	242.52±1.26^{ab}	282.06 ±4.54^{ab}	350.31±48.11^{b}	*
αs1-I-CN	232.04 ±7.42^{a}	348.52±14.99^{b}	363.30±57.95^{b}	324.64±39.85^{ab}	*

Means in the same row with the same superscript do not differ significantly (P > 0.05).
Last column shows the significant differences. NS- No significant differences; * p<0.05; **p<0.01.

Table 4. Means ± SD of rheological and texture profile analysis parameters of Valdeón cheese at 60 days ripening.

	Summer	Autumn	Winter	Spring	ANOVA
G' (MPa)	126.47±10.42^{a}	143.93±0.53^{b}	52.66±4.37^{c}	109.92±3.16^{d}	**
G'' (MPa)	31.65±3.35^{a}	36.42±0.88^{a}	13.93±0.23^{b}	30.47±4.18^{a}	*
G* (MPa)	130.39±10.94^{a}	148.48±0.75^{b}	54.49±4.16^{c}	114.11±4.15^{a}	**
Tan δ	0.25±0.01^{a}	0.24±0.01^{a}	0.27±0.03^{a}	0.29±0.02^{a}	NS
Fracturability (N)	4.46 ±2.27^{a}	6.77±3,33^{a}	5.22 ±2.78^{a}	5.58 ±1.21^{a}	NS
Hardness (N)	56.67±12.05^{ab}	69.21±4.50^{a}	36.31±19.44^{b}	47.27±22.79^{ab}	*
Adhesiveness (N.s)	-8.88 ±2.19^{ab}	-11.69±0.15^{a}	-6.49 ±0.13^{b}	-8.10 ±1.60^{ab}	*
Cohesiveness	0.09 ±0.02^{a}	0.11±0.00^{a}	0.12 ±0.01^{a}	0.09 ±0.01^{a}	NS
Springiness	0.27 ±0.01^{a}	0.27 ±0.01^{a}	0.27 ±0.08^{a}	0.25±0.01^{a}	NS
Gumminess	4.95±0.37^{a}	7.41 ±0.80^{a}	4.43 ±2.71^{a}	4.46 ±2.60^{a}	NS
Chewiness	1.35 ±0.10^{a}	1.94 ±0.10^{a}	1.29 ±1.11^{a}	1.10±0.62^{a}	NS

Means in the same row with the same superscript do not differ significantly (P > 0.05).
Last column shows the significant differences. NS- No significant differences; * p<0.05; **p<0.01.

Table 5. Means ± SD for colour parameters of Valdeón cheese at 60 days of ripening.

	Summer	Autumn	Winter	Spring	ANOVA
L*	71.14 ± 4.96^{ab}	65.47 ± 9.38^{a}	70.65 ± 6.69^{a}	75.20 ± 8.19^{b}	**
a*	-2.39 ± 0.30^{ab}	-1.92 ± 0.50^{a}	-2.52 ± 0.77^{b}	-2.53 ± 0.95^{b}	ND
b*	10.63 ± 1.67^{ac}	12.10 ± 3.18^{ab}	12.54 ± 2.13^{b}	9.73 ± 2.61^{c}	**

Means in the same row with the same superscript do not differ significantly (P > 0.05).
Last column shows the significant differences. NS- No significant differences; **p<0.01.

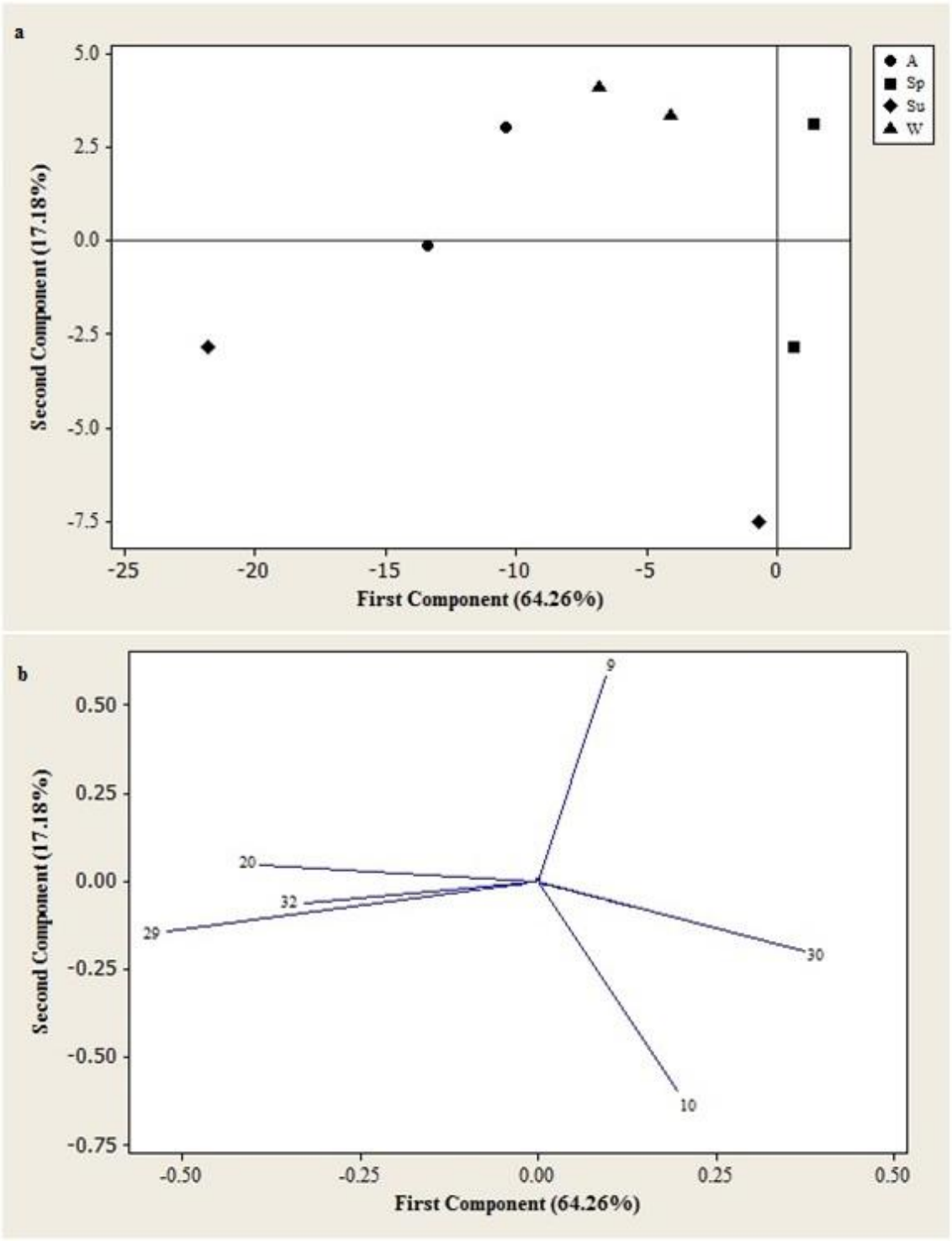

Figure 1. Score plot (a) and loading vectors (b) of variables with high loading obtained by principal component analysis of reverse phase UPLC chromatograms of the pH 4.6-soluble extracts from Valdeón cheese at 60 days of ripening.

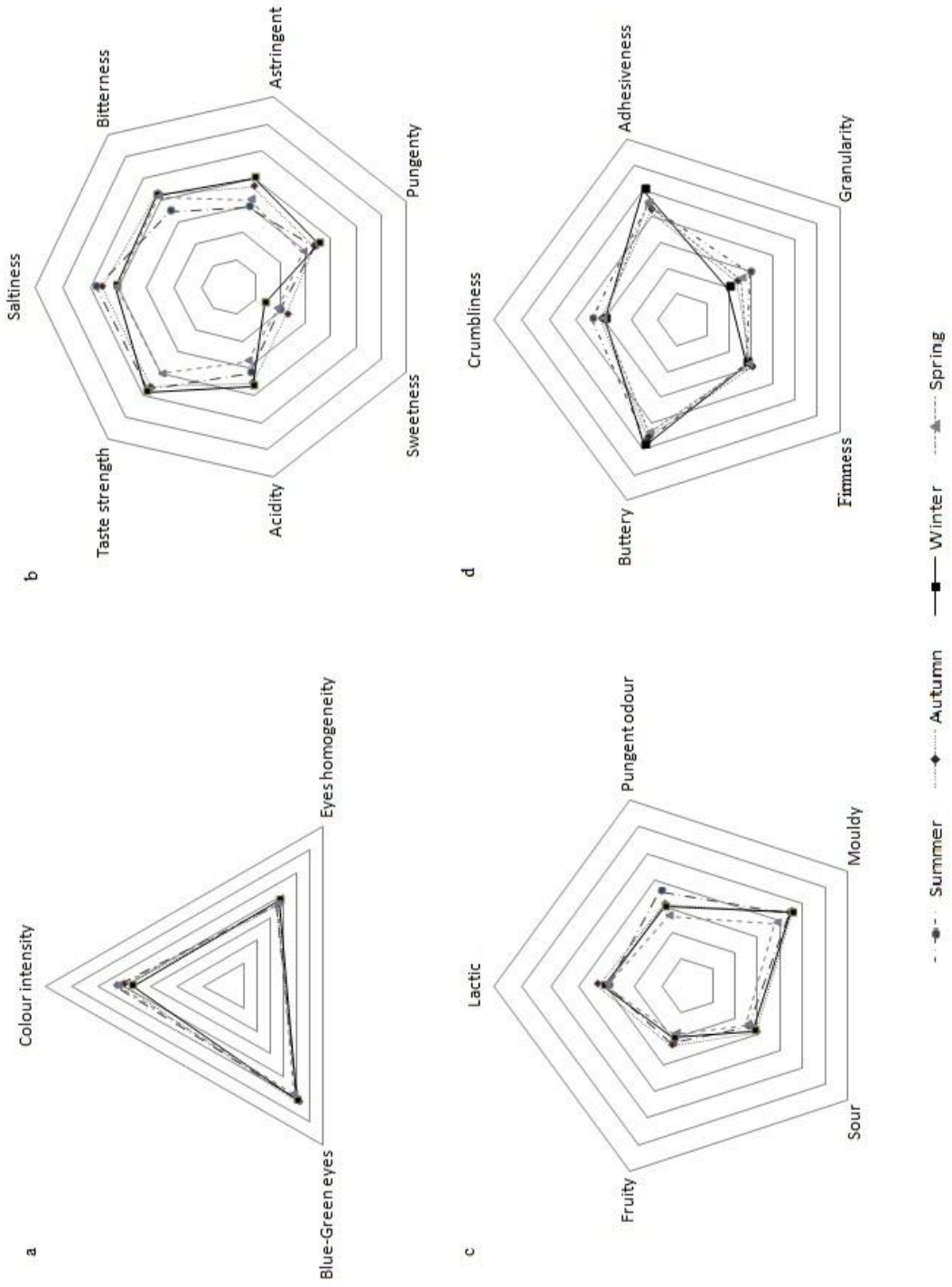

Figure 2. Sensory scores: (a) appearance, (b) taste, (c) odour and (d) texture of Valdeón cheese at 60 days of ripening.

Artículo IV

Effect of milk pasteurization on the microbiological, chemical, textural and sensory characteristics of a Spanish blue cheese (Valdeón cheese)

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En preparación para su publicación

Resumen

El objetivo de este trabajo fue determinar y comparar las diferentes características del queso de Valdeón elaborado con leche pasterizada y leche cruda con el objetivo de establecer las mejores condiciones de fabricación. Para ello, se elaboraron 8 lotes a partir de leche pasterizada y 4 lotes a partir de leche cruda. En general, los quesos fabricados con leche pasterizada mostraron recuentos microbianos más bajos (5,30 frente a 7,57 unidades log en agar Rogosa), contenido más elevado de sólidos totales (60,87 g/100 g de queso frente a 51,49 g/100 g de queso), mayores valores de sal/humedad (7,60 frente a 6,57) y pH (7,08 frente a 6,57) y menor aw (0,937 frente a 0,957). Se observó una mayor extensión de la proteólisis en los quesos de leche pasterizada, mientras que los quesos de leche cruda mostraron mayor profundidad de la misma. Por otro lado, se observó una estructura más elástica con mejor capacidad para resistir la deformación y una matriz más compacta en los lotes elaborados con leche pasterizada, así como, mayores valores de fracturabilidad y dureza. Por último, los quesos elaborados con leche cruda mostraron puntuaciones globales ligeramente superiores.

Effect of milk pasteurization on the microbiological, chemical, textural and sensory characteristics of a Spanish blue cheese (Valdeón cheese)

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Abstract

The aim of this work was to determine and compare the different characteristics of Valdeón cheese elaborated from pasteurized and raw milk in order to establish the best manufacturing conditions. For that, 8 batches from pasteurized milk and 4 batches from raw milk were made. In general, cheeses made from pasteurized milk showed lower microbiological counts (e.g. 5.30 *versus* 7.57 log units in Rogosa agar), higher total solids content (60.87 g 100 g^{-1} of cheese *versus* 51.49 g 100 g^{-1} of cheese), higher salt/moisture (7.60 *versus* 6.57) and pH values (7.08 *versus* 6.57) and lower aw (0.937 *versus* 0.957). It was observed greater extent proteolysis in pasteurized-milk cheeses while raw-milk cheeses showed higher deeper proteolysis. On the other hand, it was observed a more elastic structure with better capacity to resisting deformation and a more compact matrix in batches elaborated from pasteurized milk, as well as, higher fracturability and hardness values. Finally, cheeses made from raw milk showed overall scores slightly higher.

KEYWORDS: Blue-veined cheese; Pasteurization; Microbiology; Proteolysis; Texture; Sensory properties

1. Introduction

Valdeón cheese is a blue-veined variety manufactured in the municipal region of Valdeón (León, Spain). The authenticity of this cheese is guaranteed since 2003 by a Protected Geographical

Indication (PGI).Traditionally, this cheese was made from raw cow's milk and/or goat's milk during spring and summer. However, nowadays, the traditional production methods have been replaced with industrial processes using pasteurized milk and producing the cheese throughout all the year.

For hygienic reasons, most cheeses produced in Europe are elaborated from pasteurized milk; however, raw milk cheeses represent a significant proportion of cheeses. It is known that milk pasteurization impacts on chemical changes during cheese ripening, and finally, on sensory characteristics (aroma, flavour and texture). But, its effect on the cheese characteristics largely depends on the type of variety and on the technology involved (Grappin, & Beuvier, 1997). Modifications in milk induced by pasteurizations include endogenous enzymes denaturation, slight whey protein denaturation and their interaction with caseins and elimination or reduction of endogenous microorganisms, including non-starter lactic acid bacteria (NSLAB) (McSweeney et al., 1993). For this reason, it is important to know the modifications that pasteurization can induce on final cheese characteristics. The aim of this work was to determine and compare the differences in microbiological, physico-chemical and proteolytic parameters of Valdeón cheese made from pasteurized and raw milk and their impact on rheological, textural, colour and sensory characteristics, in order to verify the relevance of producing Valdeón cheese from raw milk.

2. Material and Methods

2.1. Cheese manufacture and sampling

Twelve batches of Valdeón cheese were manufactured following the method described by Diezhandino et al. (2015). All of them were made from a mixture of cow's and goat's milk (90% and 10%, respectively). Eight of the batches were elaborated from pasteurized milk using a commercial mesophilic starter culture (FD-DVS CHN-19, Chr Hansen SL, Madrid, Spain) and a liquid spores suspension (1.6×10^8 spores mL^{-1}) of *P.roqueforti* (Biostar, Toledo, Spain). The other four batches were made from raw milk using only a liquid spores suspension of *P. roqueforti*. A sample from each batch was taken at 60 days of ripening

(habitual consumption date) to carry out the analysis. Each sample was made up one of whole cheese (2.4 Kg).

2.2. Microbiological, physico-chemical and proteolytic analysis

For carrying out microbiological assays, aerobic mesophilic (30 ºC for 48 h) and psychrotrophic (7 ºC for 10 days) bacteria were determined on Plate Count Agar (PCA) (Oxoid, Unipath Ltd., Basingstoke, UK), *Lactococci* (30 ºC for 18-24 h) on M17 agar (Biokar, Beauvais, France), *Leuconostoc* (22 ºC for 4 days) on MSE agar (Biokar), *Lactobacilli* (30 ºC for 5 days) on ROGOSA agar (Oxoid), *Micrococcaceae* (30 ºC for 48 h) on Manitol Salt Agar (MSA) (Oxoid), *enterococci* (37 ºC for 24 h) on Kanamycin Aesculin Azide (KAA) agar (Oxoid), *Enterobacteriaceae* (37 ºC for 18-24 h) on Violet Red Bile Glucose Agar (VRBGA) (Oxoid) and, finally, moulds and yeasts (22 ºC for 5 days) on Oxytetracycline Glucose Yeast Extract (OGYE) agar (Oxoid).Total solids, protein and fat contents were determined following the methods described by standards 004 (FIL-IDF, 2004), 20-1 (FIL-IDF, 2001) and 221 (FIL-IDF, 2008), respectively. NaCl content and pH were determined according to standard 935.43 (AOAC, 1990) and 14.022 (AOAC, 1980). Lactose, D-lactic acid and L-lactic acid were analysed using an enzymatic kit (R-biopharm, Darmstadt, Germany). Finally, water activity (aw) was determined using an Aqualab Dew Point Analyzer CX-2 (Decagon devices Inc., Pullman, WA, USA).

pH 4.6 soluble nitrogen (pH 4.6-SN), 12% trichloroacetic acid-soluble nitrogen (TCA-SN) and 5% phosphotungstic acid-soluble nitrogen (PTA-SN) were prepared following the method described by Kuchroo & Fox (1982) and they was determined using a macro-Kjeldahl method (FIL-IDF 224, 2011). It was used a Kjeltec System-1002 Distilling Unit and a Digestion System-6-1007 Digester (Tecator, Höganäs, Sweden). The pH 4.6 insoluble fractions of the cheeses were analysed by urea-polyacrylamide gel electrophoresis according to Shalabi & Fox (1987). Then, the different fractions were quantified using the gel analysis software TotalLab 1D, nonlinear Dynamix (Newcastle-upon-tyne, UK). The pH 4.6-SN fractions of the cheeses were analysed by reverse-phase high-performance liquid chromatography

(RP-HPLC) following the method described by Sousa & McSweeney (2001) with some modification. The chromatographic profiles were processed according to Piraino et al. (2004). Finally, plasmine activity was performed using a modification of the method described by Richardson & Pearce (1981).

2.3. Rheological and textural analysis

Dynamic oscillatory analysis was performed using an AR2000ex Reometer (TA Instruments Company, New Castle, DE, USA). Storage modulus (G'), loss modulus (G''), phase angle tangent (tan δ) and shear modulus (G*) were determined under frequency rate from 0.06 to 628.30 rad s^{-1} in a linear viscoelastic region (previously determined taking amplitude strain sweep tests at a constant angular frequency of 6.28 rad s^{-1}). All measurements were conducted at 20ºC using a plate-plate geometry. The upper plate was 40 mm in diameter and sandblasted to prevent slippage. Using a steel wire, disk of 5 mm in height and 40 mm in diameter were removed from each cheese by triplicate. The force applied was uniformly set at 1 N for all samples. Data integration was carried out using the software incorporated into the equipment, namely Rheology Advantage 130 Data Analysis, version 5.7 (New Castle, DE, USA).

Texture Profile Analysis (TPA) was carried out according to Bourne (1982) using a Stable Micro Systems Texturometer mod. TZ-XT2 (Godalming, UK). Ten replicas (15 mm in diameter and 19 mm in height) were obtained from each cheese, after removing 0.5 cm layer from the surface. The test was performed compressing the samples to 80% using two compression cycles at a constant rate of 0.5 mm s^{-1} and at room temperature (20 ± 2 ºC). It was used a plate-plate sensor system with a stainless SMS P/75 probe. It was obtained fracturability (N), hardness (N), adhesiveness (N.s), cohesiveness, springiness, gumminess and chewiness parameters.

2.4. Colour analysis

Colours were recorded using a Spectrophotometer CM-700 d (Konica Minolta, Osaka, Japan).

The L* (corresponding to dark/light), a* (red/green) and b*(yellow/blue) were determined according to CIELab. The

MAV (measurement/illumination area) and MAV mask pattern were read with 8 mm diameter glass. It was used an illuminant D65 and an illumination angle of 10º. Each colour test was performed on the internal surface cheese by duplicate and 12 measurements were carried out at different points of the surface. The results were analysed using a Color Data software CM-S100w SpectraMagic TM NX ve. 1.9, Pro USB (Konica Minolta).

2.5.Sensory analysis

Sensory analysis was determined using a panel of 20 trained tasters at Food Hygiene and Technology Department of the University of León. Twenty sensory attributes, five for odour (lactic, pungent, mouldy, sour and fruity), seven for taste (saltiness, bitterness, sweetness, acidity, astringency, pungenty and taste strength), five for texture (crumbliness, adhesiveness, granularity, firmness and buttery) and three for appearance (colour intensity, homogenous distribution of the veins and blue-green colour of the veins) were scored on a scale from 1 to 7. Finally, each cheese was given a score on a scale from 1 to 10 for overall impression.

2.6.Statistical analysis

An ANOVA/MANOVA analysis was used to compare means with a significant difference, setting a confidence interval of 95%. Statistical correlations were performed by means of Pearson`s correlation coefficient. Both analyses were carried out using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA). Principal Component Analysis (PCA) was performed by standardising the variables to zero mean and using a covariance matrix. Statistical analysis was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).

3. Results and Discussion

3.1.Changes in microbiological and physic-chemical analysis

In general, all groups studied showed higher counts in the batches elaborated from raw milk (Table 1), except counts on MSA and OGYEA. These cheeses presented suitable physico-chemical conditions with lower salt/moisture ratio (6.57 ± 0.21) and higher aw values (0.957 ± 0.001) (Table 2) which

resulted in slightly higher lactic acid bacteria counts, specially *Lactobacilli* ($p < 0.05$), enterococci ($p < 0.05$) and *Enterobacteriaceae* ($p < 0.001$). Counts on PCAm, OGYEA and Rogosa in cheeses made from raw milk were similar to those reported by López-Díaz et al. (1995) in artisanal Valdeón cheese, while counts on MSA and MSE were lower.

The *Enterobacteriaceae* counts are an excellent indicator of the hygienic quality of a product. In general, *Enterobacteriaceae* were not detected in pasteurized milk cheeses due to the heat treatment. However, the counts were high in raw milk cheeses, although similar than those reported by other authors in similar varieties as Cabrales (Flórez & Mayo, 2006; Flórez et al., 2006).

Finally, counts on OGYEA were significant lower in cheese elaborated from raw milk ($p < 0.05$). These results were due to the use of starter culture during cheese manufacture with pasteurized milk which helped to lead cheese ripening, as well as, the interactions between the different microbial groups, while the no use of starter culture in raw milk cheeses caused a worse control of these interactions. Although, there is controversy regarding to the effect of BAL on growth and sporulation of *P. roqueforti*, it is clear that the interactions are strain-specific. For this reason, it is very important to choose an adequate starter culture according to its specific properties in order to promote positive interactions (Hansen et al., 1997). On the other hand, the use of raw milk could allow the growth of contaminant microorganisms, as *Geotrichum candidum*, which can cause inhibition of growth and sporulation of *P. roqueforti* (Cantor et al., 2004).

Cheeses made from raw milk showed significant lower total solids and lactose content, as well as, lower S/M ratio values. On the other hand, these cheeses presented significant higher D-lactic acid content. No significant differences were observed in protein, fat, NaCl and L-lactic acid contents.

pH values presented significant differences ($p < 0.05$). Cheeses elaborated from raw milk showed lower values, due to the lower growth of mould and, therefore, lower acid lactic catabolism (Hayaloglu et al., 2008).

Finally, cheeses made from pasteurized milk showed significant lower aw values ($p < 0.05$). This effect could be

due to a greater extent of primary proteolysis in these cheeses. One of the proteolysis effects is the breakage of peptide bonds releasing two new charged groups (NH_3^+/COO^-) which compete for water, reducing the "free" water content (O'Mahony et al., 2005).

3.2.Proteolytic changes

The average values obtained for the different nitrogen fractions studied, expressed as percentage of total nitrogen, are shown in Table 3. Soluble nitrogen at pH 4.6 (pH 4.6-SN) presented significant differences according to heat treatment ($p < 0.001$). The content of pH 4.6-SN was higher in cheeses made from pasteurized milk (15% approximately). These results indicated a greater extent of proteolysis in pasteurized-milk cheese according to the results obtained in caseins degradation (as we discussed latter). No significant differences were observed in TCA-SN content, expressed as percentage of total nitrogen. However, if we expressed this fraction as a percentage of pH 4.6-SN, it was observed higher content in raw milk cheeses (91.22%) than pasteurized milk cheeses (76.56%) indicating a greater deeper of proteolysis. PTA-SN content, expressed as percentage of total nitrogen, showed a similar behaviour than TCA-SN. These results agree with those reported in similar varieties in which it was observed greater secondary proteolysis in cheese elaborated from raw milk as a consequence of higher aminopeptidase activity of bacteria from milk (Grappin, & Beuvier, 1997).

Polypeptide-N showed significant differences ($p < 0.05$) according to heat treatment. When the polypeptide-N was expressed as percentage of pH 4.6-SN, it was much higher in pasteurized milk cheeses (23.43% *versus* 8.78%) indicating a lower prevalence of secondary proteolysis. Finally, no significant differences were observed in peptide-N content.

The differences observed in nitrogen fractions agreed with those observed in the casein degradation study (Table 3). Cheese elaborated from pasteurized milk showed a greater α_{s1}-CN, α_{s1}-I-CN y β-CN degradation which indicated higher formation of low molecular weight peptides and, therefore, a greater extent of proteolysis. It is known that plasmin hydrolyses β-CN to γ-CN and pasteurization increases plasmin activity due to inactivation of plasmin inhibitors

(Farkye & Imafidon, 1995) and/or denaturation of inhibitor(s) of plasminogen activator(s) (Bastian, & Brown, 1996). In fact, the results obtained in the determination of plasmin activity corroborated this affirmation; due to the plasmin activity was high in pasteurized milk cheese (3.30 ± 0.49 plasmin units g^{-1} *versus* 1.23 ± 0.06 plasmin units g^{-1}).

On the other hand, the higher pH observed in pasteurized milk cheeses (pH = 7.08) also promoted the greater plasmin activity, because its value was close to the optimum pH of this enzyme (pH ≈ 7.5). However, it cannot be dismissed the proteases action released by *P. roqueforti*, which have been described as main proteolytic agent in blue-veined cheeses (Diezhandino et al., 2015). The high growth of *P. roqueforti* in pasteurized milk batches could explain why in these batches the α_{s1}-CN and β-CN degradation was higher.

Figure 1a showed the score plot obtained from principal components analysis of the peptide profile of pH 4.6-soluble extracts from Valdeón cheese elaborated from pasteurized and raw milk obtained from RP-UPLC. Principal Components (PC) explained 76.7% of the total variation in the peptide profile of the cheeses (49.6% PC1 and 27.1% PC2). Samples were separated according to heat treatment of milk. The greater differences between samples were observed in the retention time from 1.7 to 3.3 minutes. Vectorial loadings showed that seven main classes (peptides or groups of peptides) explained the most of differences observed (Figure 1b). Cheeses made from raw milk (at right of PC1) were characterized by classes 6, 30 and 31 (1.7 and 3.2 min), while cheeses elaborated from pasteurized milk were positioned at the left of PC1 and they were characterized classes 20, 28, 29 and 32 (2.4, 3.0, 3.1 and 3.3 min, respectively). All cheeses, independently of heat treatment, presented a high hydrophilic/hydrophobic ratio, that is, with predominance of first. These agree with ripening time of samples (60 days) and with the notable proteolysis that occurs in this variety resulting in hydrophobic peptides degradation and releasing low molecular height peptides and amino acids. However, cheeses made from raw milk showed a slight higher hydrophilic/hydrophobic ratio due to they were characterized by class 6 which corresponded to the more

hydrophilic peptides since it elute at a lower acetonitrile concentration.

3.3.Rheology and Texture Analysis

The main values obtained in the dynamic oscillatory test of Valdeón cheese are presented in Table 4. Both G' and G'' were significant higher ($p < 0.05$ and $p < 0.01$, respectively) in cheese made from pasteurized milk being characterized, therefore, by presenting a more elastic structure with a better capacity for resisting deformation (Xiong & Kinsella, 1991). However, it was not observed significant differences in tan δ values between the two treatments. It was due to the interactions of different factors resulting in similar final values. Cheese made from pasteurized milk showed lower water content and higher pH values. These two parameters cause a reduction in tan δ. On the contrary, those cheeses elaborated from pasteurized milk showed higher extent of proteolysis which increases this parameter (Luyten, 1988; Visser, 1991). Lastly, cheeses made from pasteurized milk showed significant higher G* values ($p < 0.05$) due to the lower water content which result in a much firmer cheese matrix (firming effect) (Juan et al., 2007).

Table 4 also shows the results obtained in the Texture Profile Analysis of Valdeón cheese. TPA revealed significant differences ($p < 0.05$) in fracturability, hardness, cohesiveness and springiness. The higher fracturability and hardness values observed in pasteurized milk cheeses agree with the results obtained in the dynamic oscillatory test. In fact, it was found a positive correlation between those two textural attributes and G* values ($r = 0.94$ and $r = 0.92$, respectively) ($p < 0.05$). This behaviour was due to the lower water content observed in pasteurized milk cheeses. Water molecules within the three-dimensional protein matrix weaken the network structure, so that, when the water content decreases, the consistency of the matrix protein increases, producing harder products (Dimitreli & Thomareis, 2007). These results agree with those reported by Gaya et al. (1990) in Manchego cheese produced from pasteurized and raw milk.

Cohesiveness and springiness values were significant lower in pasteurized milk cheeses. These results were related to its higher fat and lower water

contents. Creamer & Olson (1982) associated the decrease in cohesiveness with the loss of elastic structural elements and the decrease in water content for protein solvation. In fact, Fresno & Álvarez (2012) observed a negative correlation between fat content and cohesiveness while Gwartney et al. (2002) observed a decrease in springiness values with increasing in fat content.

3.4. Colour Analysis

Table 5 shows results of colour analysis of cheeses. All parameters studied showed significant differences between cheese made from pasteurized and raw milk. L* values were significant lower in pasteurized milk batches due to the higher total solids content, being observed a negative correlation between L* values and total solids ($r = -0.99$) ($p < 0.01$). These results agree with those reported by Buffa et al. (2001) in goat's cheese. a* values were significant lower ($P < 0.001$) in cheese produced from pasteurized milk due to the higher *P. roqueforti* growth. This caused a stronger green coloration. Finally, b* values were significant higher ($p < 0.05$) in cheeses made from raw milk showing a higher predominance of yellowish colouration.

3.5. Sensory Analysis

Figure 2 shows the results obtained in the sensory analysis of Valdeón cheese. As regards appearance, the score given to blue-green colour of the veins was significant higher ($p < 0.05$) in cheeses elaborated from pasteurized milk due to the higher growth of *P. roqueforti*. In fact, it was observed a positive correlation between this attribute and OGYEA counts ($r = 0.88$) ($p < 0.05$). These results agree with those observed in the instrumental measurement of colour.

In the case of taste, cheese elaborated from raw milk showed significant lower scores ($p < 0.05$) for bitterness and astringency and higher ($p < 0.05$) for sweetness. In the case of bitterness, the differences observed were related to the higher extent of proteolysis found in the cheeses made from pasteurized milk which caused a greater accumulation of hydrophobic peptides with a small molecular size that are responsible for bitterness (Lemieux & Simard, 1992) which agreed with the results obtained in the peptide profile analysis. These, in addition, caused a lower perception of

sweet taste. For the odour parameters, only sour odour showed significant differences ($p < 0.05$) between both treatments. Cheese elaborated from raw milk showed higher scores for this attribute, which was due to the higher accumulation of short chain volatile acids released in the oxidative deamination of amino acids (Hemme et al., 1982) due to the higher deeper of proteolysis found in these cheeses.

As regards texture, cheeses made from pasteurized milk showed firmness scores significant higher ($p < 0.05$) which agree with dynamic oscillatory and TPA results. In fact, it was observed a positive correlation between these results and G* values obtained in the dynamic oscillatory test ($r = 0.99$) ($p < 0.05$) and hardness and fracturability values obtained in TPA ($r = 0.96$ and $r = 0.99$, respectively) ($p < 0.05$). It was very interesting the negative correlation observed between hardness values (TPA) and buttery ($r = -0.97$) ($p < 0.05$), which coincided with the results found by other authors (Adhikari et al., 2003; García et al., 2015). These results showed a good correlation between instrumental and sensory analysis.

Finally, although it was not observed significant differences in the overall impression, cheese elaborated from raw milk showed scores slightly higher (7.56) than cheeses made from pasteurized milk (6.83), which showed a slightly taster preference for raw milk.

4. Conclusions

The use of raw milk in Valdeón cheese resulted in significant changes in most of the microbiological and physico-chemical parameters studied compared to cheeses elaborated from pasteurized milk. These changes produced lower extent of proteolysis and higher scores in sensory analysis overall due to the lower hardness and fracturability, as well as, lower bitterness and higher buttery. However, the use of raw milk in Valdeón cheese implies, firstly, to maximize the hygienic conditions during production and, on the other hand, to use a mesophilic starter culture together with *P. roqueforti*. Thereby, it would be obtained a better control of fermentation process decreasing the contaminant microorganism counts and promoting the optimal growth of *P. roqueforti*, and, in the end, contributing to develop unique and specific sensory characteristics regarding to other blue-veined varieties.

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Table 1. Changes in microbial counts[1] in Valdeón cheese made from pasteurised and raw milk.

	Pasteurized	Raw	ANOVA
PCAm[2]	7.49±0.67	8.40±0.78	NS
PCAp[2]	7.18±0.74	8.44 ±0.00	NS
MSE	6.20±0.91	6.36±0.21	NS
ROGOSA	5.30±0.95	7.57±0.46	*
M17	5.45±0.90	6.36±0.48	NS
MSA	3.82±0.76	3.37 ±0.32	NS
KAA	2.42±1.12	6.30±0.78	*
OGYEA	8.59±0.26	7.88±0.10	*
VRBGA	0.00±0.00	5.66±0.23	***

[1]The microbial counts are expressed as log CFU mL^{-1} or log CFU g^{-1}.
[2]Aerobic mesophilic bacteria counts (*PCAm*). Aerobic psychrotrophic counts (*PCAp*).
Last column shows the significant differences. NS – no significant differences; * $p < 0.05$; *** $p < 0.001$.

Table 2. Physico-chemical characteristics in Valdeón cheese made from pasteurised and raw milk.

	Pasteurized	Raw	ANOVA
Total solids[1]	60.87±2.79	51.49±0.12	*
Protein[2]	33.93±1.73	36.59±0.85	NS
Fat[2]	55.50±2.23	51.70±0.91	NS
Lactose[2]	0.14±0.02	ND	*
D-lactic acid[2]	0.16±0.03	0.39±0.07	*
L-lactic acid[2]	0.49±0.24	0.56±0.12	NS
NaCl[2]	5.06±0.35	6.19±0.45	NS
Salt/moisture	7.87±0.07	6.57±0.21	*
pH	7.08±0.39	6.57±0.06	*
aw	0.937±0.01	0.957±0.01	*

[1]Expressed as g 100 g^{-1} of cheese.
[2]Expressed as g 100 g^{-1} of total solids.
Last column shows the significant differences. NS – no significant differences; * $p < 0.05$
ND: Not detected

Table 3. Changes in soluble nitrogenous components and in the pixel intensity (volume) of the band of the electrophoretical regions in the stained gels of caseins of Valdeón cheese made from pasteurised and raw milk.

	Pasteurized	Raw	ANOVA
pH 4.6-SN	39.98±0.78	33.72±3.68	***
TCA-SN	30.61±0.87	30.76±3.30	NS
PTA-SN	13.65±0.39	11.69±0.49	NS
Polypeptide-SN	9.37±1.65	2.96±0.37	*
Peptide-SN	16.97±0.48	19.06±2.81	NS
γ_2-CN	525.88±110.61	383.52±34.80	NS
γ_1-CN	285.61±84.30	273.51±6.32	NS
γ_3-CN	510.59±109.63	511.46±45.85	NS
β-CN	469.72±94.06	735.13±47.58	**
αs_1-CN	266.30±70.78	329.94±85.25	NS
αs1-I-CN	317.13±61.01	746.58±100.01	**

Last column shows the significant differences. NS – no significant differences; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4. Means ± SD of rheological and texture profile analysis parameters of Valdeón cheese made from pasteurised and raw milk.

	Pasteurized	Raw	ANOVA
G' (MPa)	108.24±36.90	43.88±9.56	*
G'' (MPa)	28.12±9.30	11.86±2.42	**
G* (MPa)	111.87±38.02	45.46±9.86	*
Tan δ	0.26±0.02	0.27±0.01	NS
Fracturability (N)	5.51±2.10	1.43±0.01	*
Hardness (N)	52.36±17.87	28.94±4.69	*
Adhesiveness (N.s)	-8.79±2.26	-8.53±0.77	NS
Cohesiveness	0.10±0.02	0.17±0.06	*
Springiness	0.26±0.03	0.35±0.02	*
Gumminess	5.31±1.96	4.95±2.68	NS
Chewiness	1.42±0.59	1.81±1.06	NS

Last column shows the significant differences. NS – no significant differences; * $p < 0.05$;** $p < 0.01$.

Table 5. Means ± SD for colour parameters of Valdeón cheese made from pasteurised and raw milk.

	Pasteurised	Raw	ANOVA
L*	71.39 ± 8.04[a]	77.1 5 ± 7.34[b]	**
a*	-2.40 ± 0.77[a]	-1.53 ± 1.12[b]	***
b*	11.21 ± 2.69[a]	12.64 ± 3.02[b]	*

Last column shows the significant differences. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

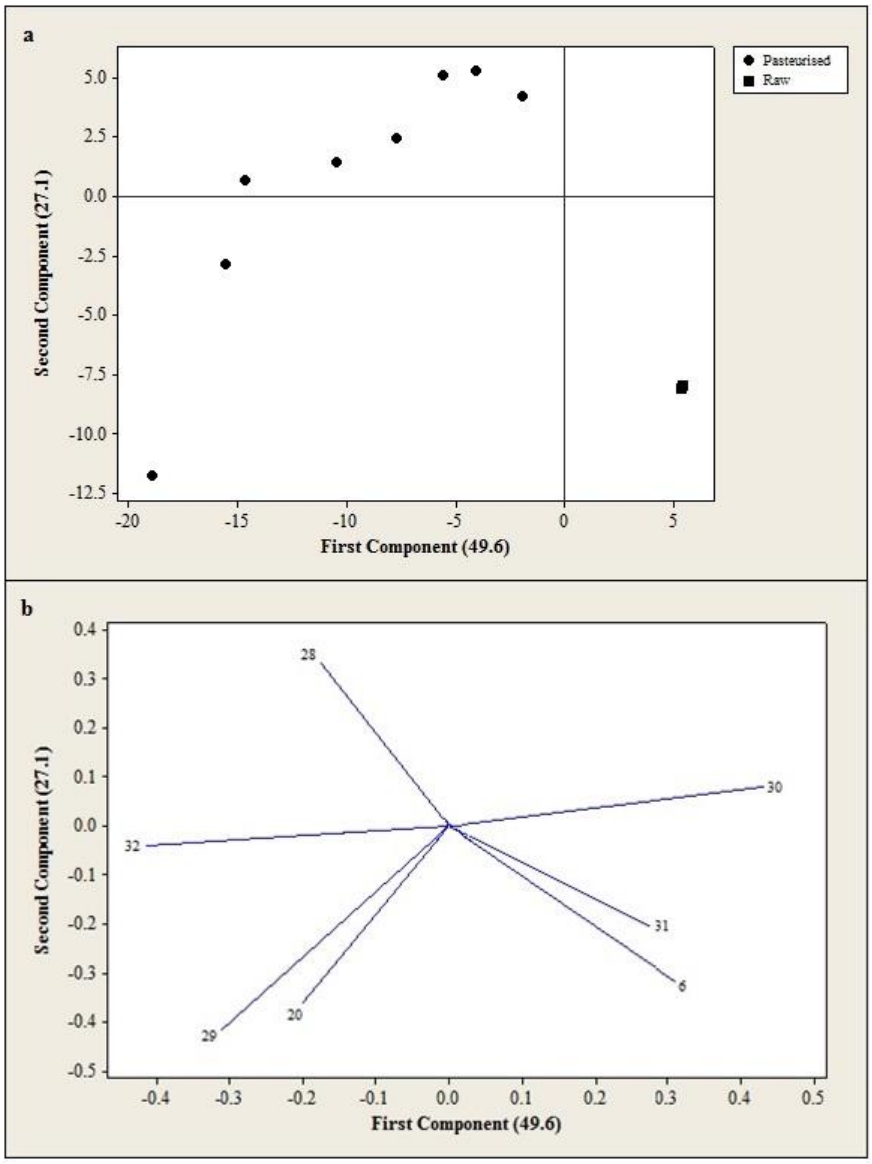

Figure 1. Score plot (a) and loading vectors (b) of variables with high loading obtained by principal component analysis of reverse phase UPLC chromatograms of the pH 4.6-soluble extracts from Valdeón cheese made from pasteurized and raw milk.

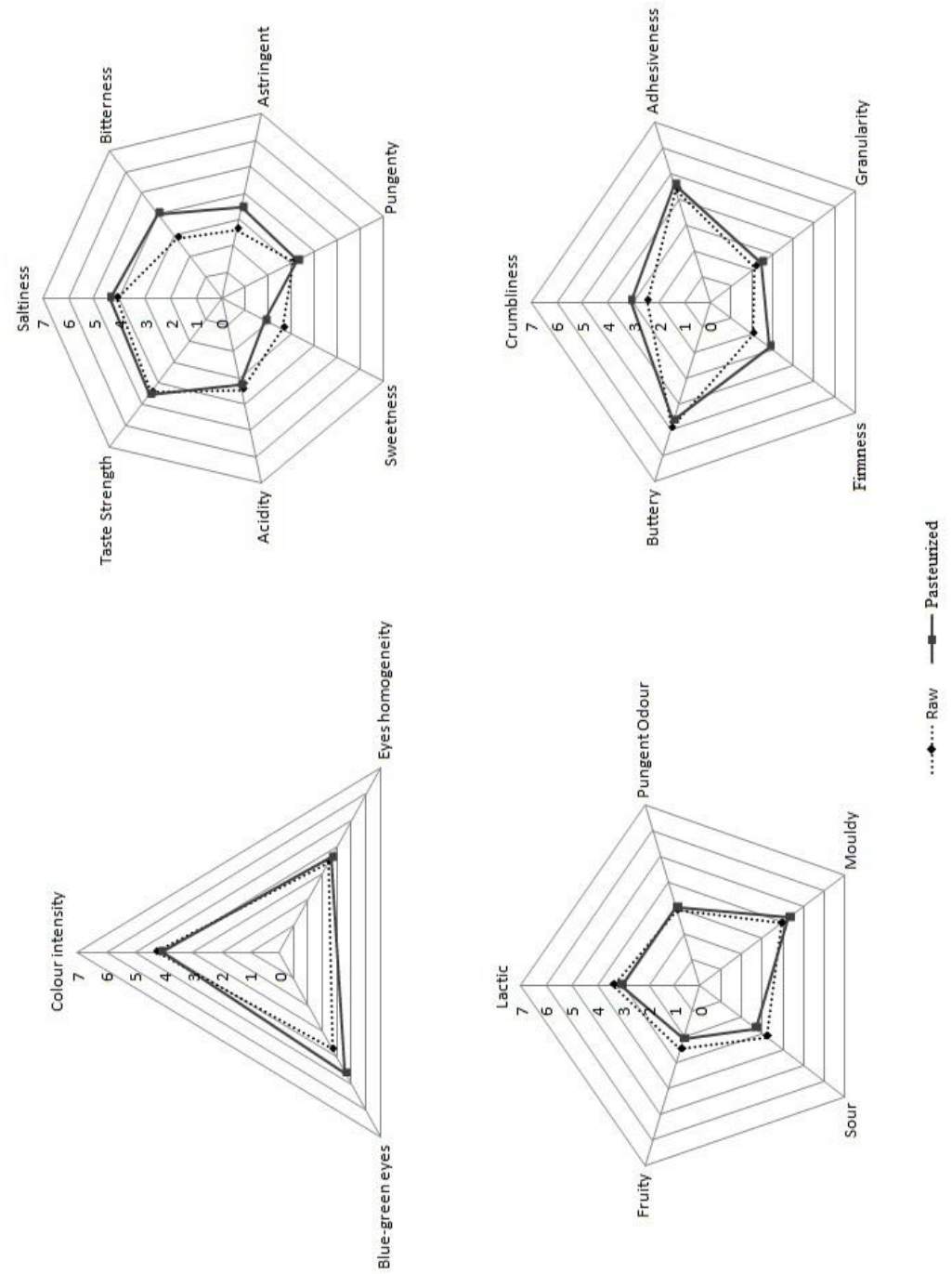

Figure 2. Sensory scores: (a) appearance, (b) taste, (c) odour and (d) texture of Valdeón cheese made from pasteurized and raw milk.

Artículo V

Changes in free fatty acid profile during ripening of a Spanish blue veined cheese (Valdeón) made in different seasons and from pasteurized or raw milk

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En preparación para su publicación

Resumen

La lipólisis es un fenómeno muy importante que tiene lugar más extensamente en las variedades de vena azul. El objetivo de este trabajo fue identificar y cuantificar el perfil de ácidos grasos libres en el queso de Valdeón durante la maduración, de acuerdo con la estación de elaboración y el tratamiento térmico aplicado a la leche. La concentración de ácidos grasos libres aumentó significativamente durante la maduración. La cantidad total fue 6370,15 mg/100 g de queso a los 120 días. Excepto a los 2 días, los ácidos grasos predominantes fueron el ácido oleico y palmítico. El perfil de ácidos grasos estuvo significativamente afectado por la estación de elaboración. Las mayores concentraciones se observaron en los quesos de verano. Los quesos de elaborados con leche cruda mostraron concentraciones más elevadas de ácidos grasos. Los ratios de ácidos grasos de cadena corta, media y larga sobre ácidos grasos totales fueron indicativos del tiempo de maduración y estación de elaboración, pero no se vieron afectados por el tratamiento térmico de la leche. El Análisis de Componentes Principales mostró que el ácido oleico, palmítico, butírico y mirístico presentaron la mejor correlación con las variables en función del tiempo de maduración, estación y leche utilizada. Por lo tanto, el tiempo de maduración, la estación y el tratamiento térmico aplicado a la leche fueron factores que ejercieron una importante influencia sobre la concentración y perfil de ácidos grasos libres en el queso de Valdeón.

Changes in free fatty acid profile during ripening of a Spanish blue veined cheese (Valdeón cheese) made in different seasons and from pasteurized or raw milk

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Abstract

BACKGROUND: Lipolysis is a very important phenomena which occurs most extensively in blue veined varieties. The aim of this work was to identify and quantify the profile of free fatty acids (FFA) in Valdeón cheese during ripening, according to the cheesemaking season and heat treatment applied to milk.

RESULTS: the concentration of FFAs significantly increased during ripening. The total amount was 6379.15 mg 100 g^{-1} of cheese at 120 days. Except at 2 days, the predominant FFAs were oleic and palmitic acids,. The profile of FFAs was significantly influenced by the season of manufacture. The highest concentrations were observed in summer cheeses. Raw milk cheeses showed higher concentrations of FFAs. The ratios of short, medium and long chain fatty acids to total FFAs were indicative of ripening time and season of manufacture, but were not influenced by heat treatment of milk. A principal components analysis showed that oleic, palmitic, butyric and myristic acids presented the best correlation with the variables as a function of ripening time, season and milk used.

CONCLUSION: Ripening time, season of manufacture and heat treatment applied to milk were the factors that exercised an important influence upon the concentration and profile of FAAs in Valdeón cheese.

Keywords: Blue veined cheese; lipolysis; seasonal variation; raw milk; pasteurized milk; ripening

1. Introduction

The biochemistry of cheese ripening includes phenomena such as glycolysis, proteolysis and lipolysis. Lipolysis occurs as a result of the presence of lipolytic enzymes that catalyze the hydrolysis of triacylglycerides, the main lipid component of milk. The products of the reaction are free or non-esterified fatty acids, partial glycerides (mono- and diglycerides) and, in some cases, glycerol[1]. The origin of lipases in cheese may be associated with six possible sources: milk, rennet preparation (rennet paste), starter, adjunct starter, non-starter bacteria and, if used, exogenous lipases[2-4].

The extent of lipolysis in blue veined cheese is caused mainly by the growth and lipolytic activity of *Penicillium roqueforti*, and depends on the strain used, the length of ripening time, the degree of residual lipolytic activity in the milk, the starter culture and rennet used, the efficiency of homogenization of the milk used to make the cheese, surface microorganisms, pH, temperature, and salt concentration[5].

The FFAs released as a result of lipolysis (especially short and medium chain FFAs) can remain in the cheese as such, contributing to the organoleptic characteristics of the final product, or can act as precursors of other compounds, specially methyl ketones and secondary alcohols, which also contribute in an important way to the aroma of the ripened product[6]. Although extensive lipolysis could be considered undesirable in most varieties of cheese[7], it makes a positive contribution to flavour in some cheeses, for example, some hard Italian and blue veined varieties, whose characteristic flavour is precisely due to the presence of free fatty acids[1].

The relative proportion of fatty acids in milk fat depends on the animal species, breed, lactation state and feed, and may influence the proportion of FFAs in ripened cheeses, as can weather conditions and the microbiological quality of the raw milk[8,9]. It is reasonable to expect that seasonal patterns of lipolysis will affect the final composition and, therefore, determine the aroma of different varieties of cheese[10]. Similarly, the application of heat treatment, such as pasteurization, during cheesemaking will also determine the pattern of FFAs observed in the ripened cheese, partly due to an improvement in the hygienic quality of

the milk used, and partly due to the almost complete inactivation of lipoprotein lipase (an endogenous milk enzyme)[11].

Valdeón cheese is a traditional blue veined manufactured with pasteurized cow`s milk or a mixture of cow, sheep and/or goat`s milk in the municipality of Posada de Valdeón (León, Spain). The authenticity of this cheese has been guaranteed since 2003 by a Protected Geographical Indication (PGI). Although some aspects of this cheese have been studied recently[12,13], no research has been conducted to date on lipolysis.

In our opinion, the study of lipolysis, and therefore of the content of free fatty acids, is of enormous importance in blue veined cheese varieties due to the vast extent to which it occur, and the positive or negative effect it can have on the final quality of these cheeses. Consequently, the aim of this work was to determine and compare changes in the profile of free fatty acids during ripening of the PGI Valdeón cheese, according to the season of manufacture and the kind of milk used (raw or pasteurized). These data will make it possible to differentiate this variety of traditional blue veined cheese from others, and to establish the best conditions for manufacture and ripening with a view to subsequent sale.

2. Materials and methods

2.1.Cheeses

Twelve replicas of Valdeón cheese were manufactured according to the method described by Diezhandino *et al.* [12]. Eight of them were elaborated from pasteurized milk (90% cow´s milk and 10% goat´s milk), using a commercial mesophilic starter culture (FD-DVS CHN-19, Chr. Hansen SL, Madrid, Spain) and a liquid spore suspension (1.6 x 10^8 spores mL^{-1}) of *P. roqueforti* (Biostar, Toledo, Spain). The other four replicas were elaborated from raw milk (90% cow´s milk and 10% goat´s milk) using only a liquid spore suspension of *P.roqueforti*. The rennet used was commercial liquid calf rennet (NATUREN liquid 140 S/S, 90% Chymosin; 140 ± 5 IMCU ml^{-1}; Chr. Hansen SL, Madrid, Spain). Cheeses were ripened for 4 months in a drying room at 10º C and 90% relative humidity. Cheese samples were taken from each replica at 2, 15, 30, 60, 90 and 120 days. Each sample was made up one of whole cheese (2.4 kg).

2.2.Free fatty acid analysis

The extraction, separation and identification of free fatty acids was carried out according to the method described by De Jong and Bandings[14].

To extract the FFAs, 1 g of cheese was weighed together with 3 g of anhydrous sodium sulfate, and then 0.3 mL of 2.5 M sulfuric acid and 1 mL of internal standard solution (pentanoic acid [C5:0], nonanoic acid [C9:0] and heptadecanoic acid [C17:0] in heptane, 0.5 mg ml^{-1} (w/v)) was added. Next, the fat was extracted by adding 3 ml of a 1:1 ether diethyl-heptane mixture, performing centrifugation at 804 *g* for 2 minutes at a temperature of 20° C, and repeating this process for three times. Finally, the organic phase was collected and the free fatty acids were separated by means of solid phase extraction (SPE) using polypropylene columns containing Sep-Pak® Aminopropyl 3 cc Vac cartridges (500 mg) (Waters, Milford, Ireland).

FFAs were separated and identified using a Hewlett Packard 6890 Series GC System gas chromatograph (Hewlett Packard, Wilmington, DE, USA) equipped with a Hewlett Packard 7683 Series injector and a Hewlett Packard 5973 Mass Selective Detector. Separation of the fatty acids was performed in a Thermo Scientific column measuring 30 m x 0.32 mm x 0.50 µm (Thermo Fisher Scientific Inc., Madrid, Spain). The oven temperature was programmed to start at 50° C and increase at a rate of 15° C min^{-1} until reaching a temperature of 200° C, which was maintained for 2 min. Then, the temperature was increased at a rate of 2° C min^{-1} to 220° C, at which point it was maintained for 5 minutes. The temperature of both the injector and detector was 230° C. Injector volume was 4 µl (Split 10:1).

Eight standard solutions were prepared with increasing concentrations of fatty acids ([C2:0], butyric acid [C4:0], pentanoic acid [C5:0], caproic acid [C6:0], caprylic acid [C8:0], nonanoic acid [C9:0], capric acid [C10:0], decenoic acid [C10:1], lauric acid [C12:0], myristic acid [C14: 0], myristoleic acid [C14:1], palmitic acid [C16:0] pentadecanoic acid [C15:0], palmitoleic acid [C16: 1], heptadecanoic acid [C17:0], stearic acid [C18:0], oleic acid [C18:1], linoleic acid [C18:2] and linolenic acid [C18:3] (Sigma-Aldrich, St. Louis, MO, USA)) and fixed concentrations of internal standards to calculate the calibration curves (Table 1). Identification of free

fatty acids was performed by comparing retention times with those of patterns followed by confirmation against the mass spectra of fatty acid peaks in the Hewlett Packard Willey database 275 L Mass Spectral Library. The chromatograms were processed using HP G1701BA version B.01.00 Chemstation Software (Hewlett Packard, Wilmington, USA).

2.3.Statistics

Means with a significant difference were compared by ANOVA/MANOVA analysis with a confidence interval of 95% using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA). Principal component analysis (PCA) was performed by standardizing the variables to zero mean and using a covariance matrix. Statistical analysis was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).

3. Results and discussion

3.1. Free fatty acid composition

Table 2 shows the mean concentrations of the different FFAs during ripening of the PGI Valdeón cheese manufactured in different seasons of the year and from pasteurized or raw milk. The high standard deviations observed in the table reflect the high variability between different seasons and heat treatments of milk, which has also been observed in other varieties of blue veined cheese[15].

The concentration of most of the studied FFAs increased during ripening, coinciding with the findings of other studies on similar cheeses, such as Gorgonzola[16] or Stilton[17]. In the case of butyric, palmitoleic, stearic and oleic acid, a slight decline was observed at 120 days of ripening, due to the hydrolysis of FFAs to form other compounds such as methyl ketones, alcohols, lactones, aldehydes, etc., resulting in a reduction in their concentration[18]. In particular, methyl ketones are important fatty acid catabolites in blue veined cheeses, due to the action of *P. roqueforti* lipases[5]. Contarini and Toppino[19] and Prieto *et al*.[20] have also observed this effect in Gorgonzola and Picón Bejes-Tresviso, respectively. The total value (C4:0 - C18:3) reached at the end of ripening was 6379.15 mg 100 g^{-1} of cheese, similar to values observed in other varieties of blue veined cheese, such as Gamonedo[21], and higher than those of

others, such as Roquefort[22]. However, the comparison of the results obtained in other studies should be made with caution, since the use of different methods may lead to different values[10].

Oleic (C18: 1) and palmitic (C16: 0) acids predominated at all ripening times studied, except at 2 days. Similar profiles of free fatty acids have been observed by other authors in different varieties of blue veined cheese, such as Roquefort[23], Cabrales[24,25] or Picón Bejes-Tresviso[20].

An ANOVA analysis revealed that both the season of manufacture and heat treatment significantly influenced the majority of the FFAs studied (Table 1). Cheeses made in summer presented the highest total concentration of fatty acids (6657.26 mg 100 g^{-1} of cheese) and, for most of the fatty acids studied, the highest concentrations at the end of ripening, whereas the lowest concentration corresponded to winter cheeses (3509.26 mg 100 g^{-1} cheese) (Figure 1). This trend has also been reported by Chávarri *et al.*[26] in other varieties of pressed cheese, such as Idiazábal cheese. During the early stages of ripening, the total concentration and individual concentration of each of the FFAs studied was found to be lower in cheeses made with raw milk than in those made with pasteurized milk (Figure 2). However, at the end of ripening (120 days), the concentration of FFAs observed in raw milk cheeses was almost three times higher than in pasteurized milk cheeses (13436.66 mg 100 g^{-1} of cheese and 4614.77 mg 100 g^{-1} of cheese, respectively). This effect is probably related to the different activity of lipases released by *P. roqueforti* and their capacity to produce methyl ketones (alkan-2-ones) from FFAs (27). *P. roqueforti* presented more growth in cheeses made with pasteurized milk (Diezhandino, unpublished), which contributed to increased degradation of the FFAs to methyl ketones at the end of ripening, and a deceleration was observed in the increase of total FFA concentration, and even a decrease at 120 days of ripening. On the other hand, the environmental conditions established in the cheese could also have contributed to the FFA profile observed, since the cheeses made with pasteurized milk showed a higher salt content/humidity ratio. Studies carried out on other varieties of blue veined cheese[16] have found that high salt concentrations limit the

production of lipases and probably their activity. Similarly, lipoprotein lipase (LPL), which is a powerful endogenous milk lipase, remains active in cheeses made with raw milk whereas it is denatured in cheeses made from pasteurized milk, as a result of the heat treatment. A 72° C treatment for 15 s almost totally inactivates the enzyme, although it is necessary to reach 78° C for 10 s in order to inactivate it completely[27]. In addition, although lactic acid bacteria (LAB) are less lipolytic than other bacterial species such as Pseudomonas, Acinetobacter and Flavobacterium, they are nevertheless considered responsible for the release of significant levels of FFAs[27] due to the presence of high numbers of LAB in cheese. Cheeses made with raw milk presented significantly higher counts of ROGOSA and KAA than those made with pasteurized milk, in of the order of 2 to 4 logarithmic units, respectively (Diezhandino, unpublished).

Figure 3 shows the evolution of short chain (SCFA), medium chain (MCFA) and long chain fatty acids (LCFA) during ripening, expressed as a ratio of total FAAs. The SCFA/total FFA ratio decreased significantly ($P < 0.05$) at the beginning of ripening and then showed a slight increase to 60 days, remaining stable thereafter until the end of ripening. This decrease in SCFAs during the early stages of ripening was a result of their decomposition to aromatic compounds such as γ - or δ-lactones, alcohols and methyl ketones[28]. The MCFA/total FFA ratio experienced a significant decrease ($P < 0.05$) during the first month of ripening and then remained fairly stable until the end. In contrast, the LCFA/total FAA ratio presented the opposite trend, increasing significantly ($P < 0.05$) during the first month of ripening and then remaining fairly stable. Except at two days of ripening, LCFAs were consistently found to be the predominant fatty acids. It should be noted that LCFAs are thought to play only a minor role in the flavour of cheese due to a high LCFA perception threshold[29], although they can contribute to the development of soapy flavours in some cases. In contrast, the perception threshold for SCFAs and MCFAs is considerably lower and each one gives a characteristic flavour note. Butanoic acid provides “rancid” and “cheesy” flavours. Hexanoic acid has an “pungent”, “blue cheese” flavour note,

and octanoic acid has a "wax", "soap" "goat", "musty", "rancid" and "fruity" flavour note[27].

A mean representation of the same indices separated by season of manufacture and heat treatment of the milk is shown in Figures 4 and 5. Significantly lower values were observed ($P < 0.05$) for the SCFA/total FFAs and MCFA/FFAs ratio in cheeses made in summer (Figures 4A and 4B). In contrast, these same cheeses presented significantly higher values ($P < 0.05$) for the LCFA/total FFAs ratio (Figure 4C). The results of the three indices studied coincided with those observed by Fernández-García *et al.*[10] in another variety of cheese. However, no significant differences were observed between the heat treatments applied to milk in any of the ratios studied (SCFA/total FFAs, MCFA/total FFAs and LCFA/total FFAs). This indicates that although this factor had an impact on the total concentration of free fatty acids in Valdeón cheese, it did not influence the free fatty acid profile.

3.2.Principal component analysis

A principal component analysis (PCA) was performed for mean fatty acid concentrations during ripening of the PGI Valdeón cheese, according to season of manufacture and the heat treatment applied to the milk, in order to determine which fatty acids varied most and to characterize the cheeses accordingly.

Table 3 shows the loading coefficients obtained from the PCA as a function of ripening time. Two principal components (PC1 and PC2) were selected, which together accounted for 99.9% of total variance (99.1% and 0.8%, respectively). The variables that best correlated with PC1 were oleic, palmitic and butyric acids (C18:1, C16:0 and C4:0), while those that correlated best with PC2 were palmitic, myristic and oleic acids (C16:0, C14:0 and C18:1). Figure 6 shows the distribution of samples (2, 15, 30, 60, 90 and 120 days) on the plane formed by the two main components selected. Samples from shorter ripening times (2 and 15 days) were grouped to the left of PC1, indicating that these samples contained lower amounts of C16:0, C18:1 and C4:0. As ripening time advanced, these fatty acids were present in large quanties, with the exception of the 120 day sample which, despite representing the longest ripening time, presented a lower content of these fatty

acids than the 90 day sample. Thus, the 120 day sample appeared in the lower part of the plane, indicating a higher content in C16:0 and C14:0 than in the other samples.

Table 4 shows the loading coefficients obtained in the PCA as a function of the season of manufacture. The selected principal components explained 99.2% of the total variance. Principal component 1 (PC1) explained 96.7% of the total variance observed, and the variables that correlated best with this component were oleic, palmitic and butyric acids (C4:0, C18:1 and C16:0). Meanwhile, principal component 2 (PC2) only explained 2.5% of the variance, and correlated best with palmitic, oleic and butyric acids (C4:0, C16:0 and C18:1). Figure 7 shows the distribution of the variables on the plane. In this case, samples corresponding to winter cheeses appeared on the left of PC1, those manufactured in the summer appeared on the right of PC1, while autumn and spring cheeses were intermediate. This distribution is indicative of a higher content in C16:0, C18:1 and C4:0 in summer.

Finally, Table 5 shows the loading coefficients obtained in the PCA as a function of the heat treatment applied to the milk. The selected principal components (PC1 and PC2) explained 99.9% of the total variance observed (98.1% and 1.8%, respectively). Both axes correlated best with the same fatty acids: oleic, palmitic and myristic acids (C14:0, C18:1 and C16:0). Sample distribution on the plane formed by PC1 and PC2 is shown in Figure 8. Three groups are evident: the first contains short ripening time samples (2 and 15 days for both treatments and 30 days for raw milk samples) that appear on the left of PC1; the second group appears in an intermediate region of PC1 and corresponds to the rest of the samples made with pasteurized milk; and lastly, the third group consists of the rest of the samples made with raw milk. This indicates that with the exception of the sample at 30 days of ripening, cheeses made with raw milk were characterized by having a greater concentration of C18:1, C16:0 and C14:0.

4. Conclusions

Free fatty acids increased during ripening of Valdeón cheese and were influenced by the season of manufacture and heat treatment of the milk used. In

general, the highest concentrations of FFAs corresponded to cheeses made in the summer, reaching 6657.26 mg 100 g^{-1} of cheese. Meanwhile, cheeses made with raw milk presented higher values of total FFAs than those made with pasteurized milk. Except for samples at 2 days of ripening, oleic and palmitic acids were the most abundant fatty acids. Both the final values and the FFA profile were similar to those reported for other varieties of blue veined cheese. The SCFA/total FFA, MCFA/total FFA and LCFA/total FFA ratios were indicative of ripening time and season of manufacture. However, they were not influenced by heat treatment of the milk. A principal components analysis correctly classified the cheeses according to ripening time, divided by season of manufacture and heat treatment of milk, respectively. In all cases, regardless of the effect studied, oleic, palmitic, and to a lesser extent, butyric and myristic acids were the FFAs that presented the best correlation with the variables.

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Table 1. Parameters of calibration standard solutions of FFAs studied: concentration range, equation of calibration curves, correlation coefficients (R2), limit of detection (LOD) and limit of quantification (LOQ).

FAAs	Concentration Range (mg L^{-1})	Equation	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
C4	25 - 750	y = 1.0979x - 0.0182	0.997	0.43	1.45
C6	25 – 750	y = 0.713x + 0.4033	0.993	1.21	4.02
C8	25 – 800	y = 1.1521x – 0.0572	0.998	0.42	1.39
C10	25 – 850	y = 0.9686x – 0.1051	0.998	0.56	1.88
C12	30 – 850	y = 0.7736x + 0.1735	0.996	0.89	2.97
C14	30 – 950	y = 0.7191x + 0.2375	0.997	0.99	3.31
C14:1	30 – 900	y = 0.8274 + 0.1774	0.996	0.82	2.74
C16	30 – 850	y = 0.764x + 0.8585	0.992	1.30	4.33
C16:1	30 – 900	y = 0.7019x + 0.7422	0.994	1.26	4.18
C18:0	30 – 850	y = 0.8442x + 0.8477	0.993	1.10	3.66
C18:1	30 – 900	y = 0.6342x + 0.9672	0.989	1.85	6.16
C18:2	40 – 1100	y = 0.7475x + 1.1192	0.992	1.69	5.62
C18:3	30 - 850	y = 1.1381x + 1,0715	0.991	0.89	2.95

Table 2. Means ± standard deviation of the free fatty acid concentration (mg 100 g^{-1} cheese) found in Valdeón cheese made from pasteurized and raw milk throughout the year and during the ripening.

FFAs	2 days	15 days	30 days	60 days	90 days	120 days	ANOVA		
							A	S	T
C4	5.32±1.97	7.88±2.80	107.05±126.47	423.98±208.06	512.40±195.33	467.87±164.07	***	*	NS
C6	ND	ND	38.60±59.15	229.44±140.40	287.14±133.85	298.17±176.58	***	NS	**
C8	1.18±2.54	1.56±3.32	19.73±20.16	83.15±72.28	101.45±82.01	118.49±110.83	***	***	***
C10	13.36±2.30	17.67±6.68	57.99±43.01	186.22±98.69	223.58±117.10	301.01±229.65	***	***	***
C12	ND	ND	38.12±35.32	139.50±77.82	180.68±110.09	247.82±216.55	***	**	***
C14	ND	6.33±6.46	98.70±85.39	293.84±189.37	392.16±274.71	558.31±529.97	***	*	***
C14:1	ND	ND	9.47±14.36	45.12±25.19	55.72±35.84	87.90± 3.40	***	NS	***
C16	9.89±15.28	38.95±34.94	287.06±237.44	907.72±548.46	1331.45±566.45	1370.54±1117.77	***	*	***
C16:1	ND	ND	29.72 43.69	116.67±67.86	159.92±40.60	126.46±66.80	***	*	*
C18:0	ND	4.90±10.71	36.26±39.00	184.13±80.47	261.72±156.64	248.36±178.92	***	NS	**
C18:1	ND	53.28±81.12	751.25±701.04	2059.58±1337.83	2784.90±1464.98	2380.45±1364.86	***	NS	*
C18:2	ND	ND	14.13±26.24	89.47±56.41	99.05±45.45	114.23±110.62	***	**	**
C18:3	ND	ND	19.00±11.65	41.08±15.14	54.42±21.05	59.54±19.36	***	**	NS

Last column shows the significant differences (A- Age; S – Season; T – Treatment). *NS: no significant differences; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$*

ND: Not detected

Table 3. Factor loading calculated from Principal Component Analysis applied to the FFAs of the Valdeón cheese samples during ripening.

FAAs	PC1	PC2
C4	0.166	0.035
C6	0.1	-0.105
C8	0.036	-0.066
C10	0.081	-0.248
C12	0.069	-0.226
C14	0.15	-0.547
C14:1	0.023	-0.1
C16	0.434	-0.588
C16:1	0.049	0.042
C18:0	0.085	-0.065
C18:1	0.853	0.455
C18:2	0.037	-0.053
C18:3	0.018	-0.022

Table 4. Factor loading calculated from Principal Component Analysis applied to the FFAs of the Valdeón cheese samples made thoughtout the year.

FAAs	PC1	PC2
C4:0	0.172	0.141
C6:0	0.091	0.088
C8:0	0.029	0.01
C10:0	0.07	0.018
C12:0	0.056	0.052
C14:0	0.115	0.173
C14:1	0.018	0.04
C16:0	0.344	0.836
C16:1	0.05	0.075
C18:0	0.064	0.244
C18:1	0.902	-0.407
C18:2	0.033	0.091
C18:3	0.019	0

Table 5. Factor loading calculated from Principal Component Analysis applied to the FFAs of the Valdeón cheese samples made from pasteurized and raw milk.

FAAs	PC1	PC2
C4:0	0.125	0.279
C6:0	0.095	0.031
C8:0	0.045	-0.048
C10:0	0.097	-0.149
C12:0	0.088	-0.165
C14:0	0.207	-0.448
C14:1	0.029	-0.054
C16:0	0.53	-0.619
C16:1	0.041	0.061
C18:0	0.085	0
C18:1	0.788	0.526
C18:2	0.04	-0.04
C18:3	0.014	0.015

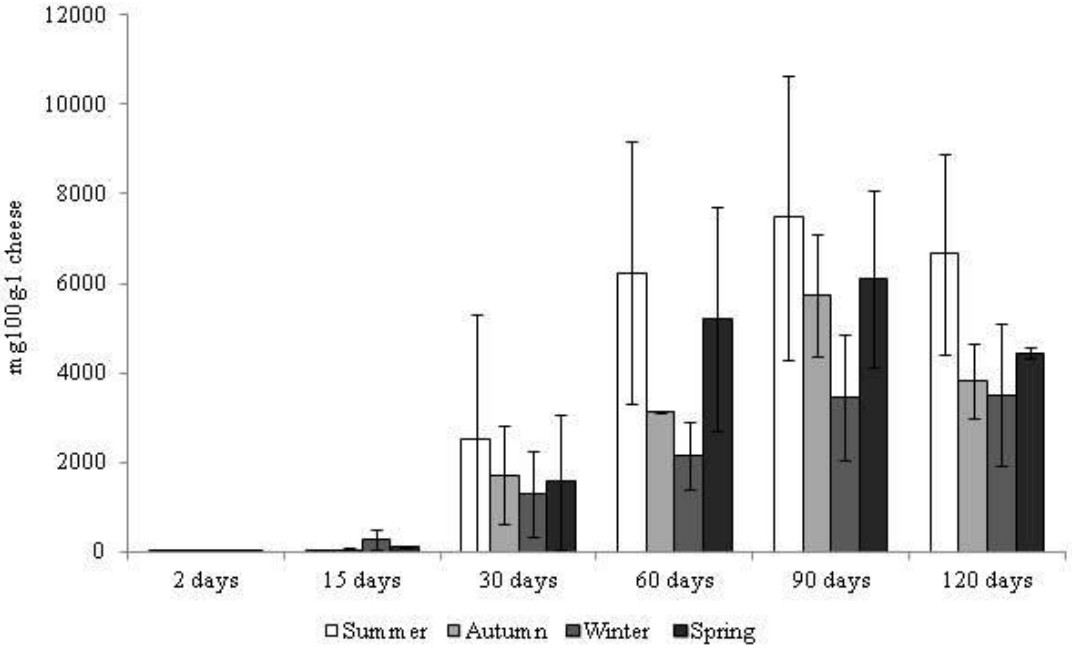

Figure 1. Means±standard deviation of the free fatty acid concentrations found in Valdeón cheese throughout the year.

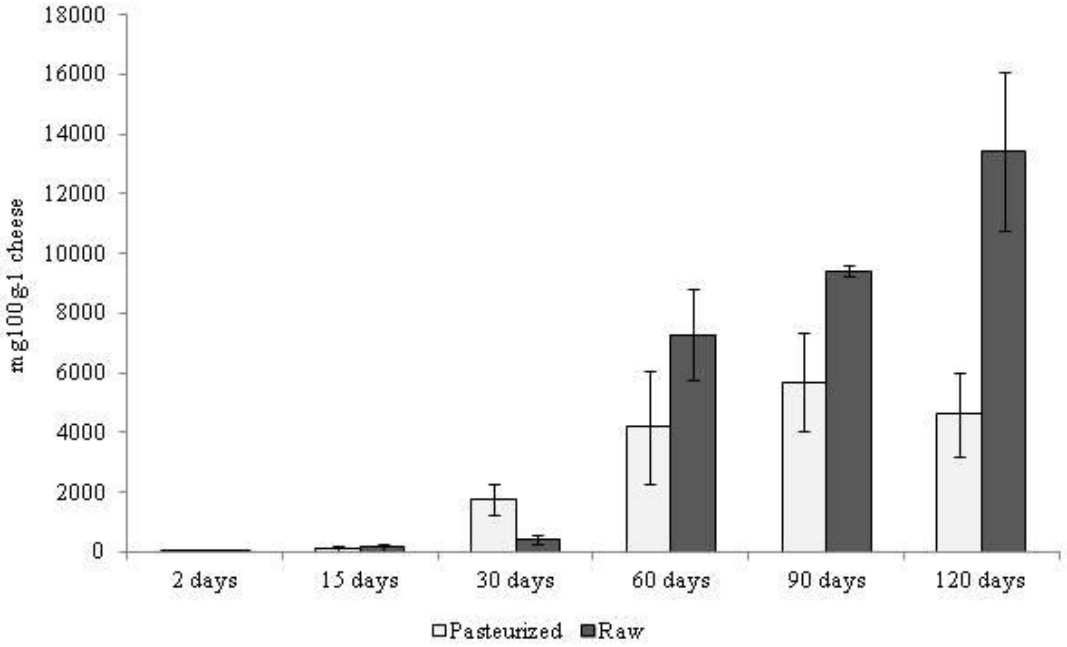

Figure 2. Means±standard deviation of the free fatty acid concentrations found in Valdeón cheese made from raw and pasteurized milk.

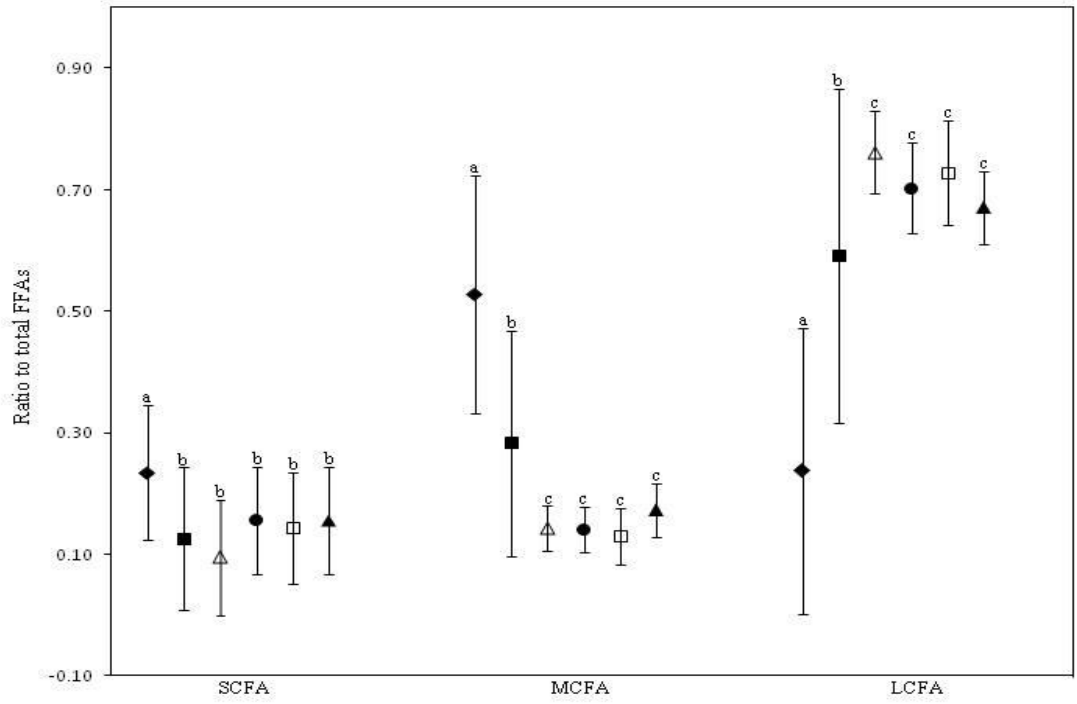

Figure 3. Means±standard deviation of ratios of short, medium and long chain fatty acids (SCFAs, MCFAs and LCFAs, respectively) to the total amount of free fatty acid (FAA) in Valdeón cheese during ripening. Means with the same superscript letters do not differ significantly ($p > 0.05$). 2 days (◆), 15 days (■), 30 days (△), 60 days (●), 90 days (□) and 120 days (▲).

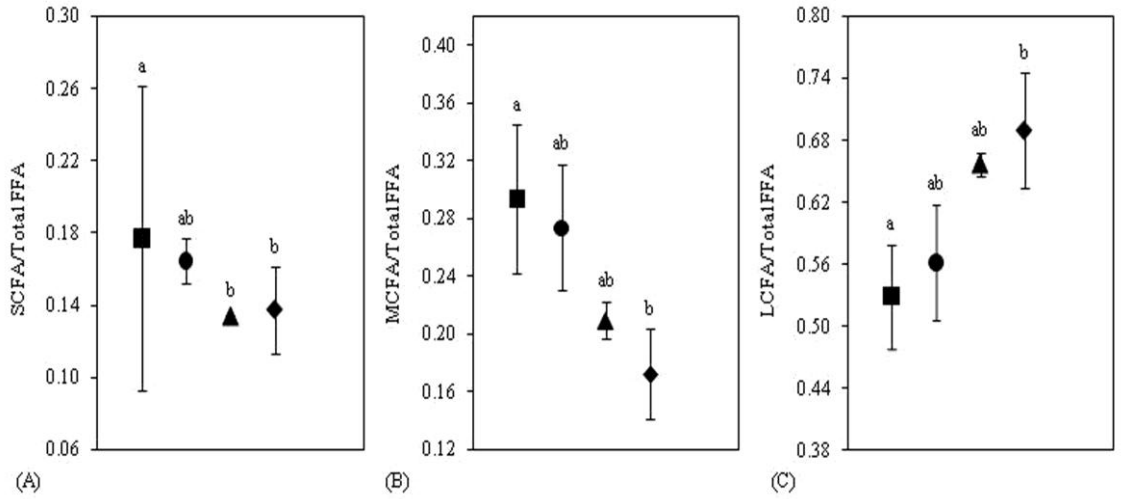

Figure 4. Means±standard deviation of ratios of short, medium and long chain fatty acids (SCFAs, MCFAs and LCFAs, respectively) to the total amount of free fatty acid (FAA) in Valdeón cheese throughout the year. (A) SCFA/total FFA; (B) MCFA/total FFA; (C) LCFA/total FFA. Means with the same superscript letters do not differ significantly ($p > 0.05$). Summer (■), autumn (●), winter (▲) and spring (◆).

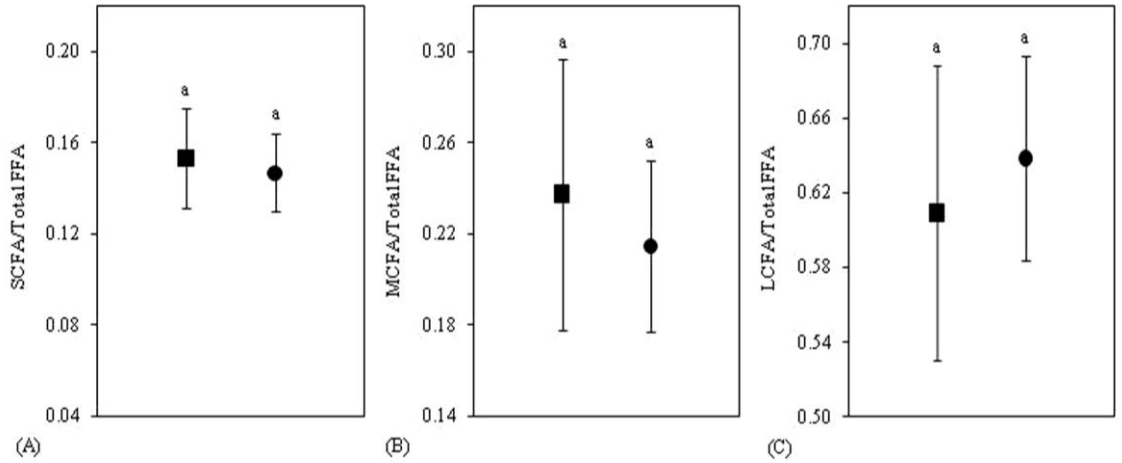

Figure 5. Means±standard deviation of ratios of short, medium and long chain fatty acids (SCFAs, MCFAs and LCFAs, respectively) to the total amount of free fatty acid (FAA) in Valdeón cheese made from pasteurized and raw milk. (A) SCFA/total FFA; (B) MCFA/total FFA; (C) LCFA/total FFA. Means with the same superscript letters do not differ significantly ($p > 0.05$). Pasteurized milk (■) and raw milk (●).

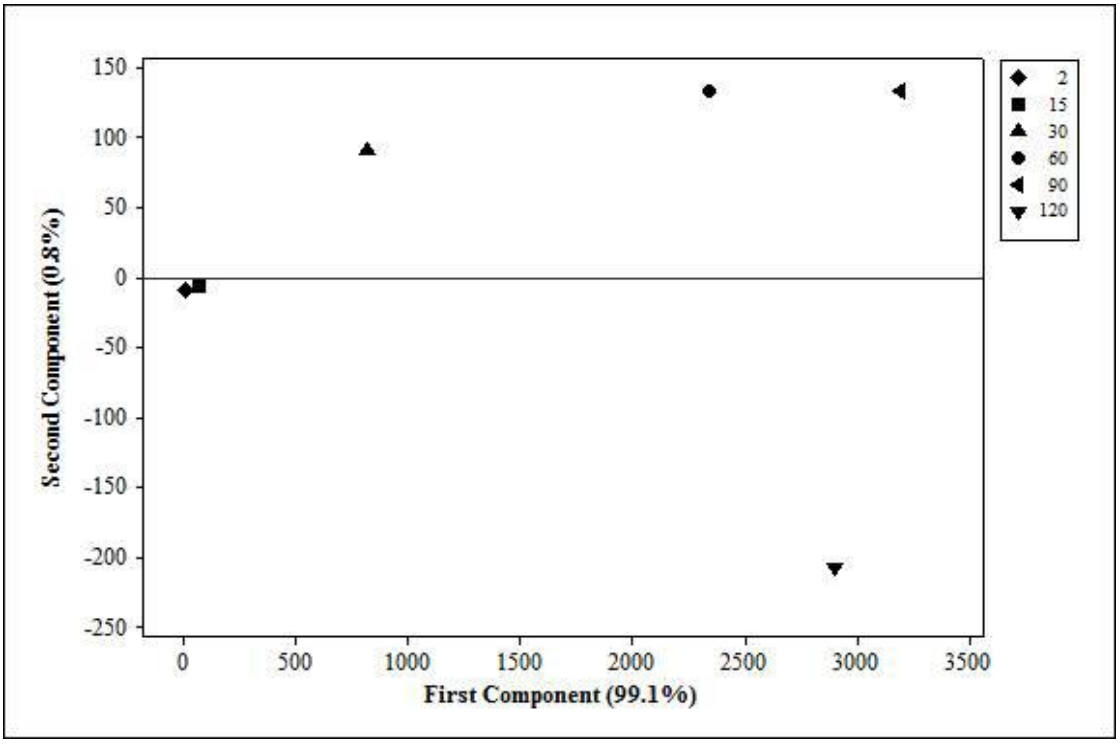

Figure 6. Plotting of Valdeón cheese during ripening on the plane defined by the two principal components obtained by PCA of the FFAs.

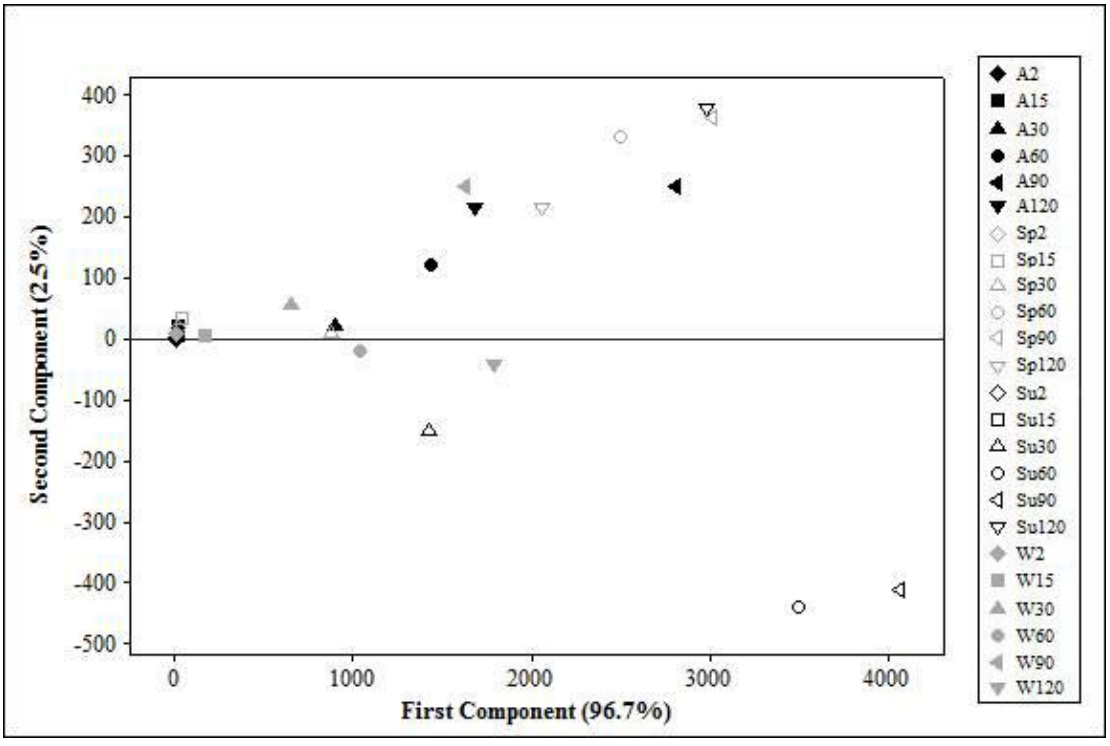

Figure 7. Plotting of Valdeón cheese throughout the year on the plane defined by the two principal components obtained by PCA of the FFAs.

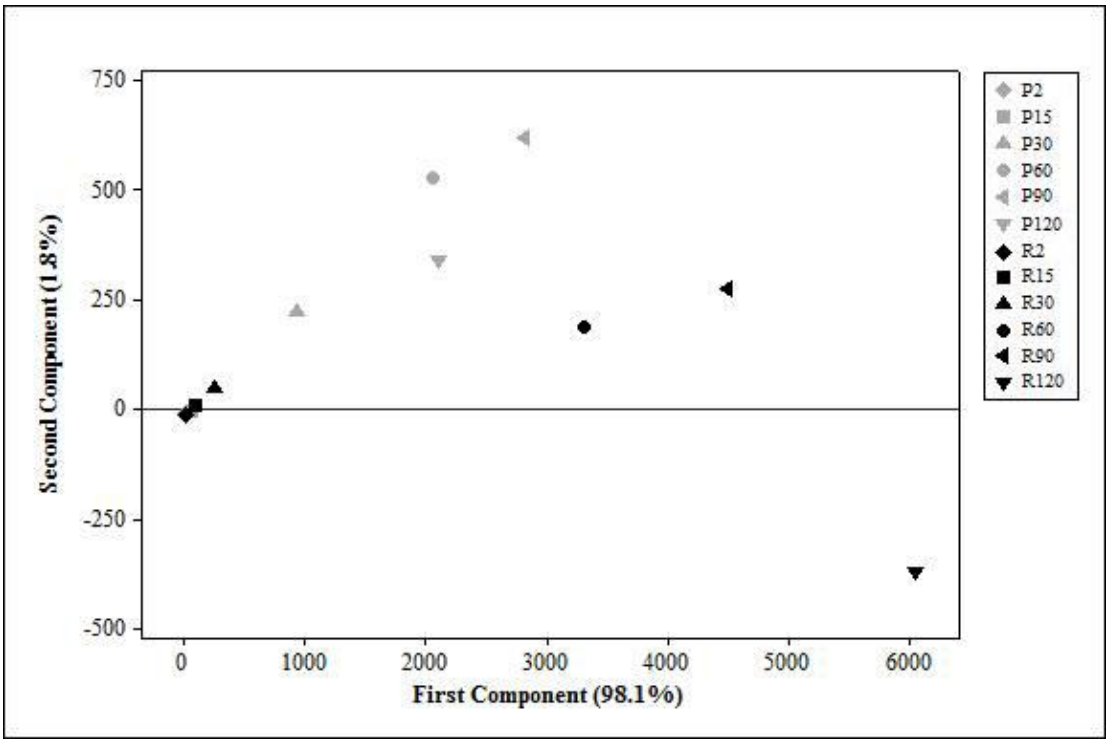

Figure 8. Plotting of Valdeón cheese made from pasteurized and raw milk on the plane defined by the two principal components obtained by PCA of the FFAs.

Artículo VI

Evolution of free amino acids and biogenic amines contents during ripening of a Spanish blue cheese (Valdeón cheese)

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En preparación para su publicación

Resumen

Se estudió el contenido en aminoácidos libres y aminas biógenas durante la maduración del queso de Valdeón elaborado a lo largo del año (verano, otoño, invierno y primavera) y a partir de leche pasterizada y leche cruda. El tiempo de maduración influyó significativamente sobre el contenido en aminoácidos libres, especialmente durante los dos primeros meses tras los cuales este incremento se ralentizó coincidiendo con el importante incremento en aminas biógenas. Al final de la maduración Glu, Ala, Pro, Tyr, Lys, Asp, Leu y Phe dueron los aminoácidos predominantes, mientas que espermina y tiramina fueron las aminas biógenas mayoritarias. Se observaron diferencias significativas entre las distintas estaciones de elaboración tanto en el contenido total como individual de aminoácidos permitiendo caracterizar a los quesos de acuerdo al perfil de aminoácidos. Sin embargo, no se observó efecto sobre el contenido en aminas biógenas. Por último, aunque el tratamiento térmico aplicado a la leche no afectó al contenido total en aminoácidos, sí permitió observar diferencias en el perfil de aminoácidos entre la los quesos elaborados con leche pasterizada y leche cruda. Sin embargo, se observaron diferencias significativas tanto en el contenido total como individual de aminas biógenas en función del tratamiento térmico aplicado a la leche, especialmente en la concentración de tiramina.

Evolution of Free Amino Acids and Biogenic Amines contents during ripening of a Spanish blue cheese (Valdeón cheese)

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Abstract

Free amino acids and biogenic amines (BA) contents were studied during ripening of Valdeón cheese made throughout the year (summer, autumn, winter and spring) and from pasteurized and raw milk. Ripening time significantly influenced free amino acids content, especially during the first two months at which slowdowns increase was observed coinciding with the huge biogenic amines increase. At the end of ripening Glu, Ala, Pro, Tyr, Lys, Asp, Leu y Phe were the predominant amino acids, while spermine and tyramine were the majority BA. There were significant differences between seasons for total and individual amino acids contents allowing characterized the cheeses according to their amino acid profile. However, no seasonal effects were observed on the levels of total BA. Finally, although heat treatment applied to the milk did not have effect on the total amino acid content it allowed observe a different amino acids profile between pasteurized and raw milk. However, it was observed significant differences as total as individual BA contents between pasteurized and raw milk cheeses, especially in tyramine concentration.

KEYWORDS: Blue-veined cheese; biogenic amines; free amino acids; pasteurization; ripening; season.

1. Introduction

Cheeses represent an ideal environment for the formation of proteolytic products, namely, free amino acids and biogenic amines, directly influenced by bacterial activity, pH, salt concentration and, indirectly, by water availability,

storage, temperature and ripening time (Pinho et al., 2001). Proteolysis is the most complex and perhaps the most important biochemical event during ripening of most cheese varieties (Fox et al., 1996), but it is especially important in the case of blue-veined cheeses in which several studies have reported extensive proteolysis compared to other varieties (Zarmpoutis et al., 1996; Gobbetti et al., 1997; Prieto et al., 2000; Wolf et al., 2011; Diezhandino et al., 2015). The enzymes contributing to the complicated proteolysis in Blue cheese originate from the milk, rennet, starter and non-starter bacteria, moulds and yeasts, with the main contribution from the mould culture, *P. roqueforti* (Cantor et al., 2004). Proteolysis plays a vital role in the development of texture as well as flavour. Proteolysis contributes to textural changes of the cheese matrix, due to breakdown of the protein network, decrease in aw through water binding by liberated carboxyl and amino groups and increase in pH, which facilitates the release of sapid compounds during mastication. It contributes directly to flavour and to off-flavour (e.g., bitterness) of cheese through the formation of peptides and free amino acids as well as liberation of substrates (amino acids) for secondary catabolic changes, i.e., transamination, deamination, decarboxylation, desulphuration, catabolism of aromatic amino acids and reactions of amino acids with other compounds (Sousa et al., 2001). Therefore, the decarboxylation of some amino acids in cheese leads to the formation of biogenic amines (BA), non-volatile amines with important physiological effects in humans (Gaya et al., 2005). Many factors contribute to the presence and accumulation of BA, such as availability of free amino acids, pH, aw, salt-in-moisture level, temperature, redox potential, bacterial density, and synergistic effects between microorganisms and, primarily, the presence of microorganisms (Schirone et al., 2011). Hence, large variation has been observed in different varieties and, even, within each variety (Valsamaki et al., 2000). Under normal conditions, exogenous BA ingested with food are rapidly detoxified by the action of amine oxidases, but whenever the detoxification process is disturbed, or the BA concentration in food is very high, BA become toxic metabolites responsible of serious human health problems (Linares et al., 2012).

Thus, knowledge on the levels of BA in cheese is necessary to assess the health hazards arising from consumption of these products. Furthermore, these could be useful as indicators of freshness and hygienic quality of the raw materials and the manufacturing conditions used within cheese manufacture (Pinho et al., 2001). The aim of this work was to research the free amino acid and biogenic amines contents in Valdeon blue-veined cheese, studying the effects of ripening time, season of manufacture and heat treatment of milk.

2. Materials and methods

2.1.Cheeses

Twelve batches of Valdeón cheese were manufactured according to the method described by Diezhandino et al., 2015. Eight of them were made from pasteurized milk (90% cow´s milk and 10% goat´s milk). For that, it was used a commercial mesophilic starter culture (FD-DVS CHN-19, Chr. Hansen SL, Madrid, Spain) and a liquid spores suspension (1.6 x 10^8 spores mL^1) of *P. roqueforti* (Biostar, Toledo, Spain). The other four batches were made from raw milk (90% cow´s milk and 10% goat´s milk) using a liquid spores suspension of *P.roqueforti*. The rennet used was a commercial liquid calf rennet (NATUREN liquid 140 S/S, 90% Chymosin; 140 ± 5 IMCU mL^{-1}; Chr. Hansen SL, Madrid, Spain). Cheeses were ripened for 4 months in a drying room at 10 ºC and 90% relative humidity.

Cheese samples were taken from each batch at 2, 15, 30, 60, 90 and 120 days. Each sample consisted of a whole cheese (2.4 Kg).

2.2.Amino acid analysis

Free amino acids separation, identification and quantification were carried out by reverse-phase high-performed liquid chromatography (RP-HPLC) according to the method described by Alonso et al. (1994) with some modification.

The chromatographic system consisted of an HPLC Waters Alliance (Milford, Massachusetts, U.S.A.), equipped with a Waters 2695 separation module. The separation of amino acids was carried out using a C18 Brisa LC2 column (Teknokroma, Barcelona, Spain) (5µm particle size, 250 mm x 4.6 mm I.D.) thermostated at 50 ºC ±1. The detection was carried out by Waters 2487

(Milford, MA, EEUU) equipment at 254 nm. The volume injected was 20 µL. Free amino acids were separated by using a linear elution gradient with mobile phases A (sodium acetate trihydrate:acetonitrile, 94:6) and B (acetonitrile, HPLC quality:water, 60:40).

2.3.Biogenic amines analysis

Biogenic amines (tyramine, putrescine, cadaverine, spermine, tryptamine, phenylethylamine, histamine and spermidine) extraction, derivation, separation, identification and quantification were carried out following the method described by Combarros-Fuertes et al. (2016).

The chromatographic system consisted of an HPLC Waters Alliance (Milford, Massachusetts, U.S.A.), equipped with a Waters 2695 separation module connected to a Waters 2996 photodiode array detector. The separation of biogenic amines was carried out using a Waters Atlantis Dc18 column (5 µm particle size, 150 mm x 4.6 mm I.D.) equipped with a Waters Atlantis Dc18 guard-column (5 µm particle size, 20 mm x 4.6 mm I.D.). The volume injected was 20 µL. Biogenic amines were separated by using a linear elution gradient with mobile phases A (ammonium acetate 0.1 M) and B (acetonitrile, HPLC quality). The temperature of the column was set at 40 ºC ± 5 ºC. Peaks were detected at 254 nm and the computer package used was Empower Version 2 by Waters™.

Determination of biogenic amines was carried out in the samples of 60, 90 and 120 days of ripening.

2.4.Statistics

Means with a significant difference were compared by ANOVA/MANOVA analysis with a confidence interval set at 95%. Statistical correlations were carried out by the Pearson‘s correlation coefficient. Both analyses were carried out using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA).Principal component analysis (PCA) was performed using a covariance matrix. Statistical analysis was carried out using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).

3. Results and discussion

3.1.Free amino acids analysis

Table 1 shows the evolution of free amino acids content during ripening of Valdeón cheese. In general, the content

of all free amino acids studied increased significantly during ripening, except Eta and Cys contents which remained constant or decreased slightly. However, it was observed a slowing down in the increase of the amino acids content or even, in some cases, a decrease during the last month of ripening. This fact could be related (as discussed latter) to amino acid catabolism by reactions such as decarboxylation, desamination, transamination, among others. These catabolic reactions release compounds responsible for flavour in cheese (Smit et al., 2000).

The free amino acid concentration increased around 8 times during ripening reaching final values of 2290.03 mg 100 g^{-1} TS. These results were due to the important extent and profound proteolysis that occurs in Valdeón cheese, especially after two months of ripening as observed by Diezhandino et al. (2015). In fact, a positive correlation was observed between total free amino acids values and nitrogen fractions studied (pH 4.6-soluble nitrogen, 12% trichloroacetic acid-soluble nitrogen and 5% phosphotungstic acid-soluble nitrogen) by Diezhandino et al. (2015) ($r = 0.99$, 0.99 and 0.96, respectively; $p < 0.05$). Final free amino acids values were lower to those described for other varieties of blue-veined cheese like Picón-Bejes Tresviso (Prieto et al., 2000), although they were higher to those observed in Cabrales (Flórez et al., 2006) and Gamonedo (González de Llano et al., 1991). At the end of ripening, the predominant free amino acids were Glu, Ala, Pro, Tyr, Lys, Asp, Leu and Phe, which represented 76% of the total free amino acids studied. These results agree, with small differences, with those reported by other authors in other similar varieties (Madkor et al., 1987; González de Llano et al., 1991; Zarmpoutis et al., 1996; Prieto et al., 2000). Kabelová et al. (2009) highlighted the preponderance of these amino acids in cheese by their relationship to clearly differentiated flavores: lysine with spicy, proline with sweet and glutamic acid with the salty-umami. Other authors (Zarmpoutis et al., 1996) associated the high leucine and phenylalanine concentration with the preferential cleavage of peptide bonds involving hydrophobic residues by the proteolytic enzymes in blue-veined cheese.

Table 2 shows the free amino acids content of Valdeón cheese at 90 days of ripening made in the different seasons. All individual free amino acids studied, as well as the total concentration, showed significant differences, except Eta, Met, Cys and Trp. In general, these differences appeared between batches elaborated in winter with respect the rest of batches, showing the first higher concentrations.

A Principal Component Analysis (PCA) was performed in order to determine which amino acids varied most according to manufacturing season. Two Principal Component was selected: PC1 and PC2 which explained 98.1% of total variation (88.4% and 9.7%, respectively). Figure 1 shows the position of the samples of Valdeón cheese (summer, autumn, winter and spring) on the plane defined by PC1 and PC2. It was observed three groups: the first group was composed of cheeses made in winter, the second included cheeses elaborated in spring, and finally, the third one was formed by cheeses manufactured in summer and autumn. The variables which best correlated with PC1 were Glu, Asp, Leu and Pro with positive correlation and Ala with negative correlation (Table 3). This component allowed separating winter samples (at right of PC1) from the rest of cheeses, characterized, therefore, by higher Glu, Asp and Leu concentrations, and lower Ala content than those elaborated in summer, autumn and spring. These results agree with those reported by Gaya et al. (2005) in Manchego cheese, where the authors observed that the higher Glu relative percentages (among others) determined the position of winter cheeses completely separated from the rest. It is worth pointing out the high Glu content in cheeses made in winter due to this amino acid supposed more than 20% of total free amino acid concentration. Amino acid transamination is catalysed by aminotransferases and results in the formation of α-ketoacids while the α-ketoacid acceptor, often α-ketoglutarate, is transformed to the corresponding amino acid, Glu (Yvon & Rijnen, 2001). Also, its catabolism can produce Gaba by decarboxylation reactions produced, mainly, by strain of *Lactobacillus* as regulating mechanism of the intracellular pH in acid conditions (Ardö, 2006). The high Gaba concentrations are, generally, associated with worse sensorial

properties in cheese (Gorostiza et al., 2004). However, its content in Valdeón cheese, independently of manufacturing season, was very low (between 0.58% and 1.03% of total free amino acids studied). Meanwhile, the variables which best correlated with PC2 were Gln and Tyr with positive correlation and Ala with negative correlation. This component allowed separated the batches positioned at the left of PC1 (summer, autumn and spring cheeses). Cheeses made in spring (on top) were characterized by higher Gln and Tyr concentrations and lower Ala content than summer and autumn cheeses. Finally, cheese made in summer and autumn (on the bottom of PC2) formed a only group and they were characterized by higher Ala content than spring batches.

No significant differences were observed in free amino acid content of Valdeón cheese elaborated from pasteurized and raw milk. At 90 days of ripening, batches made from pasteurized milk showed 2471.90 mg 100 g^{-1} TS and cheeses elaborated from raw milk 2311.70 mg 100 g^{-1} TS. This fact was associated with the using of *P. roqueforti* as secondary starter culture during cheese making which allowed standardizing the productive process and resulting in similar profound proteolysis. In a previous work conducted by Fresno et al. (2013) about nitrogen fractions study of this cheese, the authors did not observed significant differences in TCA-SN and PTA-SN values between cheese elaborated from pasteurized and raw milk. However, it was observed differences in the amino acids profile. Asn, Ser, Gly, Gaba+Thr, Orn, Asp and Tyr showed significantly higher concentrations in cheeses made from pasteurized milk (Figure 2), while Eta, Cys and Ala contents were significantly higher in cheeses elaborated from raw milk. It highlighted the differences found in Asn since its concentration could be used as pasteurization indicator due to the heat treatment produces the asparaginase inactivation, allowing the accumulation of this amino acid in the cheese (Bullock & Irvine, 1956; Frau et al., 1997) and, therefore, this fact justified that in our study cheeses made from pasteurized milk showed higher Asn content. On the other hand, it should be noted that Orn and Ser were not detected in cheeses elaborated from raw milk. Orn has been found in higher concentration in cheeses made from

cow´s milk than in cheeses elaborated from ewe´s or goat´s milk and its decarboxylation can release putrescine (Linares et al., 2012). For this reason, Orn was not observed in raw milk cheeses since the higher contaminant microorganism content could increase the putrescine formation. In fact, as discussed in the next section, raw milk cheeses showed higher putrescine content than pasteurized milk cheeses.

Finally, Gaba is produced by glutamic decarboxylation by microorganisms, especially *Lactobacillus* strains, as a mechanism of intracellular pH regulation in acid conditions (Ardö, 2006). Its presence has been used as quality indicator in cheese due to its association with the appearance of atypical aromas. However, recently various authors (Sugino et al., 2008; Diana et al., 2014) have studied the Gaba importance from a point of view of its impact on health. Gaba appears to induce hypertension, promotes diuresis, presents tranquilizing effects, is used for treating alcoholism and depression, stimulates immune cells, increases the level of growth hormone in plasma and protein synthesis in the brain, in addition to its possible prevention diabetes. These authors have also described several bioactive effects for Orn as decrease fatigue and promote ammonium secretion.

3.2.Biogenic amines analysis

The evolution of BA contents during ripening of Valdeón cheese (from 60 to 120 ripening days) is shown in Table 4. In general, BA content of cheese can be extremely variable and depends on the type of cheese, the ripening time, the manufactured process and the microorganism present (Schirone et al., 2011). Total BA concentration increased significantly during ripening reaching final values of 1829.48 ± 37.83 mg Kg^{-1} of cheese, which represented an increase of almost 100% with regard to 60-day-old cheese. This important increase in BA concentration coincided with the slowing down in free amino acids formation at 60 days of ripening, because the catabolic reactions using amino acids as substrates increased their activity at two months of ripening. Final BA content was similar to those reported for blue cheese by Komprda et al. (2008), althought it was higher than that observed in non-blue cheeses (Vale & Gloria, 1998; Valsamaki et al., 2000; Renes et al., 2014). Nevertheless, Novella-Rodríguez et al. (2003) found

that blue-cheese presented higher BA variability. At the end of ripening, spermine and tyramine were the predominant BA, which represented 89% of the total BA studied. These results agree with those reported by Komprda et al. (2008) in a Czech blue-vein cheese, in which tyramine was the BA predominant. Furthermore, only tyramine and spermine showed significant differences during ripening, while the rest remained constant or increased slightly their values. Currently, there is no consensus as to what should be the maximum permitted concentration of biogenic amines, whether jointly or individually, in foodstuffs. Most studies have concentrated on histamine and tyramine, as these are the biogenic amines most often associated with food poisoning (Renes et al., 2014). Maximum tolerable limits set for tyramine ranged from 100 to 800 mg Kg^{-1} (Ten Brink et al., 1990) while the maximun level established for histamine by Silla-Santos (1996) was 100 mg Kg^{-1}. Even though, tyramine was one of the majorities BA in Valdeón cheese at the end of ripening, it did not exceed the 800 mg Kg^{-1} of cheese, although its final concentration was greater than 100 mg Kg^{-1} of cheese. However, due to the great differences between people regarding the robustness of their detoxification system and possible synergistic effects of other biogenic amines, the toxicological limit for tyramine is difficult to establish (Komprda et al., 2008). In the case of histamine, its content ranged from 39.30 ± 3.42 to 44.61 ± 0.52 mg Kg^{-1} of cheese at the end of ripening, so it remained below the limits set by Silla-Santos (1996).

Table 5 shows the BA content of Valdeón cheese made throughout the year (at 90 days of ripening). There were not significant differences ($P > 0.05$) in the total BA content with values ranging between 1159.75 ± 60.05 mg Kg^{-1} of cheese in spring and 1581.23 ± 129.17 mg Kg^{-1} of cheese in summer. This great homogeneity in the total BA content was due to no significant differences were observed in the majority BA (spermine and tyramine). These results agree with those described by Gaya et al. (2005) where tyramine levels were not dependent on the season of manufacture. The rest of BA studied, although showed significant differences ($P<0.05$), their trend were very

ambiguous according to the results reported by Komprda et al. (2012).

Figure 3 shows the BA content of Valdeón cheese made from pasteurized and raw milk at 90 days of ripening. BA total concentration of cheeses elaborated from raw milk was almost double that those made from pasteurized milk (2615.09 and 1377.62 mg Kg^{-1} of cheese, respectively). These results agree with those described by other authors for other varieties of cheese (Schneller et al., 1997; Gaya et al., 2005; Combarros-Fuertes et al., 2016). The pasteurization reduces the bacterial load present in milk, including BA producers. Thereby, cheeses elaborated with pasteurized milk use to have lower BA concentrations than those made with raw milk (Linares et al., 2012). Moreover, the cofactor for decarboxylase activity (pyridoxal phosphate) is heat-sensitive, so pasteurization can also contribute to lower amine content values in the cheeses made from pasteurized milk (Novella-Rodríguez et al., 2003). Except phenylethilamine and spermidine, all BA showed significant differences ($p < 0.05$). Only spermine content was higher in cheeses made from pasteurized milk which agreed with the results obtained by Combarros-Fuertes et al. (2016) in Zamorano cheese. It was remarkable, the great difference observed in tyramine content, since in cheeses made from pasteurized milk its content was five times lower than in cheeses elaborated from raw milk (295.36 versus 1478.33 mg Kg^{-1} of cheese). These results were associated with the highest counts in KAA observed in cheeses elaborated from raw milk (6.46 ± 0.14 versus 1.89 ± 0.44 ufc g^{-1}, unpublished results). In fact, there was a significant positive correlation between tyramine and KAA counts ($r = 0.79$) ($p < 0.01$). Although it is difficult to find a correlation between the presences of high concentration of BA in cheese with an increment of a specific group of LAB (Linares et al., 2012), in certain cases this trait can be considered as species-characteristic, such as the production of tyramine in *Enterococcus* (Ladero et al., 2012). Schirone et al. (2012) related the high level of tyramine in Pecorino cheese with the activity of thermoresistant enterococci (usual contaminants of raw milk). However, it cannot be underestimated the role of non-starter lactic acid bacterias such as *Lactobacillus*, specially *Lactobacillus*

plantarum, which have also been associated with high concentration of tyramine in cheese (Valsamaki et al., 2000; Schirone et al., 2012; Combarros-Fuertes et al., 2016). In addition to tyramine, tryptamine, histamine, cadaverine and putrescine contents also were higher in cheeses made from raw milk. *Enterobacteriaceae* would be associated with cadaverine, putrescine, and histamine formation, mainly when a deterioration process occurs in either raw materials or end products (Marino et al., 2003). In fact, in Valdeón cheese the counts in VRBGA were significantly higher in raw-milk (3.22 ± 0.33 CFU g^{-1}) than in pasteurized-milk cheeses in which no counts were detected (unpublished results) and a significant positive correlation was found in the VRBGA counts with regards to levels of cadaverine ($r = 0.99$) ($p < 0.001$), putrescine ($r = 0.99$) ($p < 0.001$) and histamine ($r = 0.83$) ($p < 0.01$).

4. Conclusions

Total free amino acids content were significantly influenced by ripening time and manufacturing season. At the end of ripening the major amino acids were Glu, Ala, Pro, Tyr, Lys, Asp, Leu and Phe. Cheeses made in winter were characterized by high Glu contents, spring cheeses by high Gln contents and, finally, summer and autumn cheeses by their high Ala content. However, heat treatment applied to milk just influenced the amino acid profile.

BA content increased significantly during ripening due to the extensive proteolysis that occurred in blue-veined cheeses. At the end of ripening spermine and tyramine were the predominantly BA. Season of manufacture had no significant effect on the total BA content although slight differences were observed in the profile. However, the trend of these differences was very ambiguous. Pasteurization reduced significantly the total BA content especially tyramine concentration.

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Table 1. Evolution of the amino acids content (mg 100 g^{-1} TS) during ripening of Valdeón cheese.

AA's	2 days	15 days	30 days	60 days	90 days	120 days	
Asp	16.05 ± 6.73^{a}	34.06 ± 14.21^{b}	50.87 ± 18.44^{c}	126.31 ± 89.53^{d}	155.54 ± 81.97^{e}	161.86 ± 49.04^{e}	***
Glu	43.03 ± 14.55^{a}	77.98 ± 24.17^{b}	136.59 ± 54.48^{c}	336.25 ± 147.21^{d}	376.31 ± 112.99^{e}	383.14 ± 91.53^{e}	***
Asn	4.28 ± 1.04^{a}	4.84 ± 1.74^{a}	6.51 ± 2.12^{b}	7.18 ± 1.75^{b}	7.04 ± 2.15^{b}	6.99 ± 2.78^{b}	***
Ser	0.22 ± 0.61^{a}	0.94 ± 1.68^{a}	3.71 ± 4.79^{b}	15.18 ± 4.81^{c}	18.04 ± 7.31^{d}	20.10 ± 3.62^{d}	***
Gln	1.15 ± 1.21^{a}	14.99 ± 4.17^{a}	79.95 ± 25.49^{b}	111.22 ± 42.50^{c}	129.84 ± 58.97^{d}	104.74 ± 33.14^{c}	***
Gly	0.02 ± 0.05^{a}	0.49 ± 0.96^{a}	5.76 ± 1.05^{b}	14.52 ± 6.34^{c}	20.18 ± 9.64^{d}	20.05 ± 7.17^{d}	***
His	0.18 ± 0.46^{a}	4.76 ± 3.55^{b}	12.87 ± 9.58^{c}	44.83 ± 11.17^{d}	59.23 ± 15.65^{e}	62.10 ± 10.78^{e}	***
Cit	3.98 ± 2.80^{a}	6.22 ± 3.06^{a}	14.26 ± 5.46^{b}	18.41 ± 7.00^{c}	24.27 ± 7.60^{d}	31.40 ± 10.80^{e}	***
Arg-Tau	ND	ND	0.71 ± 2.49^{a}	7.94 ± 4.96^{b}	13.18 ± 6.44^{c}	14.48 ± 8.40^{c}	***
Gaba-Thr	1.12 ± 1.94^{a}	1.50 ± 0.88^{a}	8.46 ± 6.34^{b}	12.29 ± 3.82^{c}	16.38 ± 4.89^{d}	16.87 ± 5.57^{d}	***
Ala	34.74 ± 8.23^{a}	38.88 ± 11.05^{a}	125.85 ± 34.16^{b}	204.50 ± 65.33^{c}	198.21 ± 67.17^{c}	190.97 ± 65.80^{c}	***
Pro	21.05 ± 14.31^{a}	42.52 ± 25.54^{b}	77.77 ± 18.24^{c}	181.48 ± 44.96^{d}	227.65 ± 42.70^{e}	299.19 ± 56.39^{f}	***
Eta	8.12 ± 2.71^{ab}	7.91 ± 1.83^{a}	11.63 ± 2.87^{c}	11.57 ± 3.61^{c}	9.72 ± 1.98^{b}	8.47 ± 2.83^{ab}	***
Tyr	27.39 ± 10.04^{a}	46.92 ± 14.81^{a}	150.32 ± 72.83^{b}	222.55 ± 58.10^{cd}	208.92 ± 42.08^{c}	243.52 ± 64.34^{d}	***
Val	7.63 ± 3.13^{b}	13.68 ± 5.93^{b}	30.84 ± 12.64^{c}	64.14 ± 16.26^{d}	80.21 ± 18.06^{e}	83.41 ± 16.17^{e}	***
Met	4.76 ± 1.71^{a}	6.70 ± 2.98^{a}	25.56 ± 8.17^{b}	39.35 ± 6.58^{c}	46.16 ± 6.05^{d}	45.17 ± 10.43^{d}	***
Cys	8.23 ± 0.49^{cd}	7.98 ± 0.46^{bc}	8.86 ± 0.48^{e}	8.60 ± 1.39^{de}	7.81 ± 1.17^{b}	7.01 ± 0.47^{a}	***
Ile	8.64 ± 3.90^{a}	16.81 ± 9.56^{a}	33.14 ± 18.56^{b}	83.46 ± 28.08^{c}	102.69 ± 32.32^{d}	100.93 ± 30.60^{d}	***
Leu	24.00 ± 10.93^{a}	37.05 ± 15.27^{b}	67.26 ± 37.65^{c}	122.33 ± 48.93^{d}	146.72 ± 56.24^{e}	143.44 ± 34.30^{e}	***
Phe	27.65 ± 13.93^{a}	35.78 ± 9.73^{a}	60.72 ± 26.03^{b}	108.35 ± 41.68^{c}	127.14 ± 32.72^{d}	125.99 ± 34.91^{d}	***
Trp	4.87 ± 1.71^{a}	6.38 ± 1.74^{ab}	7.82 ± 3.99^{b}	13.15 ± 5.50^{c}	14.84 ± 7.38^{cd}	15.80 ± 4.64^{d}	***
Orn	12.67 ± 4.88^{a}	18.34 ± 4.61^{b}	12.77 ± 4.95^{a}	16.59 ± 8.04^{b}	18.51 ± 8.49^{b}	17.04 ± 7.52^{b}	*
Lys	20.97 ± 7.73^{a}	46.09 ± 24.18^{b}	102.22 ± 37.66^{c}	191.45 ± 91.28^{d}	207.94 ± 62.51^{d}	187.36 ± 46.50^{d}	***
Total	**280.77 ± 12.38^{a}**	**470.83 ± 20.49^{b}**	**1034.47 ± 46.67^{c}**	**1961.66 ± 89.97^{d}**	**2216.52 ± 97.68^{e}**	**2290.03 ±104.19^{e}**	***

Means in the same row with the same superscript do not differ significantly (P > 0.05).

Last column shows the significant differences during ripening. **: p < 0.05; ***: p < 0.001*

ND: Not detected

Table 2. Free amino acids content (mg 100 g^{-1} TS) of Valdeón cheese made throughout the year at 90 days of ripening.

AA's	Summer	Autumn	Winter	Spring	
Asp	115.04 ±17.49^{a}	130.34 ±55.12^{a}	281.46 ±5.90^{b}	95.33 ±34.74 ^{a}	***
Glu	323.85 ±35.66^{a}	317.65 ±12.97^{a}	558.91 ±28.01^{b}	304.85 ±45.71^{a}	***
Asn	5.96 ±0.96^{ab}	4.98 ±0.47^{a}	9.45 ±1.55^{c}	7.76 ±1.96^{bc}	**
Ser	27.10 ±8.71^{b}	13.91 ±1.54^{a}	16.41 ±2.77^{a}	14.73 ±5.55^{a}	*
Gln	111.74 ±35.89^{a}	104.57 ±5.55^{a}	96.86 ±14.85^{a}	206.17 ±73.07^{b}	**
Gly	23.91 ±7.09^{c}	15.95 ±0.86^{b}	8.88 ±2.65^{a}	31.97 ±2.87^{d}	***
His	44.96 ±6.09^{a}	53.82 ±5.32^{a}	80.75 ±8.40^{b}	57.39 ±12.49^{a}	***
Cit	19.46 ±4.90^{a}	16.74 ±4.60^{a}	27.69 ±2.78^{b}	33.19 ±2.77^{b}	***
Arg-Tau	21.10 ±1.87^{b}	6.03 ±1.37^{a}	16.08 ±2.36^{b}	9.49 ±3.94^{a}	***
Gaba-Thr	20.93 ±6.07^{b}	13.10 ±2.57^{a}	19.18 ±1.17^{b}	12.31 ±0.55^{a}	**
Ala	267.89 ±33.35^{b}	222.68 ±6.11^{ab}	115.91 ±66.21^{c}	186.35 ±23.22^{a}	***
Pro	195.91 ±6.02^{a}	206.37 ±20.24^{a}	289.83 ±26.88^{b}	218.48 ±27.07^{a}	***
Eta	10.21 ±1.37^{a}	9.37 ±2.86^{a}	10.22 ±2.80^{a}	9.05 ±0.50^{a}	NS
Tyr	201.97 ±10.98^{a}	230.02 ±48.34^{ab}	158.22 ±12.44^{c}	245.47 ±19.34^{b}	**
Val	80.41 ±8.32^{b}	66.69 ±9.90^{a}	106.24 ±5.02^{c}	67.50 ±8.66^{a}	***
Met	45.71 ±4.99^{a}	40.81 ±7.76^{a}	46.69 ±3.57^{a}	51.45 ±2.96^{a}	NS
Cys	7.41 ±0.25^{a}	7.40 ±0.29^{a}	8.94 ±2.11^{a}	7.50 ±0.12^{a}	NS
Ile	79.17 ±10.15^{a}	84.23 ±12.96^{a}	152.13 ±12.75^{b}	95.24 ±16.28^{a}	***
Leu	116.15 ±3.36^{ab}	99.26 ±5.99^{a}	236.49 ±24.00^{c}	135.01 ±4.21^{b}	***
Phe	97.31 ±8.01^{a}	110.19 ±10.80^{ab}	174.40 ±24.83^{c}	126.65 ±2.78^{b}	***
Trp	10.42 ±1.89^{a}	13.93 ±0.78^{a}	21.83 ±13.12^{a}	13.18 ±0.35^{a}	NS
Orn	7.99 ±1.76^{c}	16.55 ±1.08^{a}	26.23 ±6.38^{b}	23.28 ±7.26^{ab}	**
Lys	203.06 ± 40.16^{a}	175.12 ±48.27^{a}	282.76 ±67.98^{b}	170.81 ±14.27^{a}	*
Total	**2037.67 ± 10.73^{a}**	**1959.70 ± 217.81^{a}**	**2745.55 ± 59.74^{b}**	**2123.00 ± 124.56^{a}**	*******

Means in the same row with the same superscript do not differ significantly (P > 0.05).

Last column shows the significant differences during ripening. *NS: no significant differences; *: p < 0.05; **: p < 0.01; ***: p < 0.001*

Table 3. Factor loading calculated from Principal Component Analysis applied to the Free amino acids of Valdeón cheese made throughout the year at 90 days of ripening.

AA´s	PC1	PC2
Asp	0.438	-0.153
Glu	0.633	-0.086
Asn	0.008	0.016
Ser	-0.006	-0.059
Gln	-0.120	0.694
Gly	-0.040	0.075
His	0.074	0.074
Cit	0.011	0.107
Arg-Tau	0.010	-0.048
Gaba-Thr	0.010	-0.044
Ala	-0.283	-0.501
Pro	0.215	0.152
Eta	0.002	-0.006
Tyr	-0.177	0.239
Val	0.090	-0.056
Met	0.001	0.053
Cys	0.004	0.001
Ile	0.171	0.115
Leu	0.309	0.194
Phe	0.163	0.192
Trp	0.024	0.013
Orn	0.026	0.092
Lys	0.260	-0.153

Table 4. Biogenic amines (BA) content (mg Kg^{-1} cheese) during ripening of Valdeón cheese.

BA	60 days	90 days	120 days	
Tryptamine	11.63±3.67^{a}	12.44±3.18^{a}	11.97±1.39^{a}	NS
Phenylethylamine	18.33±0.45^{a}	21.63±0.88^{a}	25.51±5.56^{a}	NS
Putrescine	22.27±2.20^{a}	23.44±1.98^{a}	26.40±4.97^{a}	NS
Cadaverine	6.39±1.28^{a}	10.98±6.50^{a}	9.80±1.14^{a}	NS
Histamine	39.30±3.42^{a}	42.55±4.93^{a}	44.61±0.52^{a}	NS
Tyramine	200.37±62.11^{a}	295.36±25.34^{ab}	440.49±60.97^{b}	*
Spermidine	48.63±8.86^{a}	65.88±21.30^{a}	77.25±11.25^{a}	NS
Spermine	645.55±31.83^{a}	905.34±32.92^{b}	1193.45±122.59^{c}	*
Total	**992.46±24.20^{a}**	**1377.62±72.41^{b}**	**1829.48±37.83^{c}**	**

Means in the same row with the same superscript do not differ significantly (P > 0.05).

Last column shows the significant differences during ripening. *NS: no significant differences; *: p < 0.05; **: p < 0.01.*

Table 5. Biogenic amines (BA) content (mg Kg^{-1} cheese) of Valdeón cheese made throughout the year at 90 days of ripening.

BA	Summer	Autumn	Winter	Spring	
Tryptamine	8.19±1.89^{a}	21.19±0.47^{b}	9.14±3.62^{a}	11.24±0.08^{a}	*
Phenylethylamine	13.69±1.93 ^{a}	28.32±0.58^{b}	22.76±2.96^{bc}	21.76±2.38^{c}	*
Putrescine	19.60±0.53^{a}	30.08±2.70^{b}	25.17±2.45^{b}	18.90±0.11^{a}	*
Cadaverine	5.72±1.07^{a}	7.05±1.95^{a}	7.51±1.94^{a}	23.63±2.74^{b}	*
Histamine	30.10±4.88^{a}	48.04±5.97^{b}	44.17±0.31^{b}	47.90±0.02^{b}	*
Tyramine	294.68±22.69^{a}	331.88±68.86^{a}	310.95±68.67^{a}	243.93±22.34^{a}	NS
Spermidine	79.22±5.23^{a}	82.67±1.14^{a}	67.00±8.41^{a}	34.64±11.65^{b}	*
Spermine	1130.03±98.01^{a}	727.20±375.79^{a}	1006.39±184.58^{a}	757.74±75.91^{a}	NS
Total	1581.23±129.17^{a}	1276.43±306.85^{a}	1493.09±248.27^{a}	1159.75±60.05^{a}	**NS**

Means in the same row with the same superscript do not differ significantly (P > 0.05).
Last column shows the significant differences during ripening. *NS: no significant differences; *: p < 0.05.*

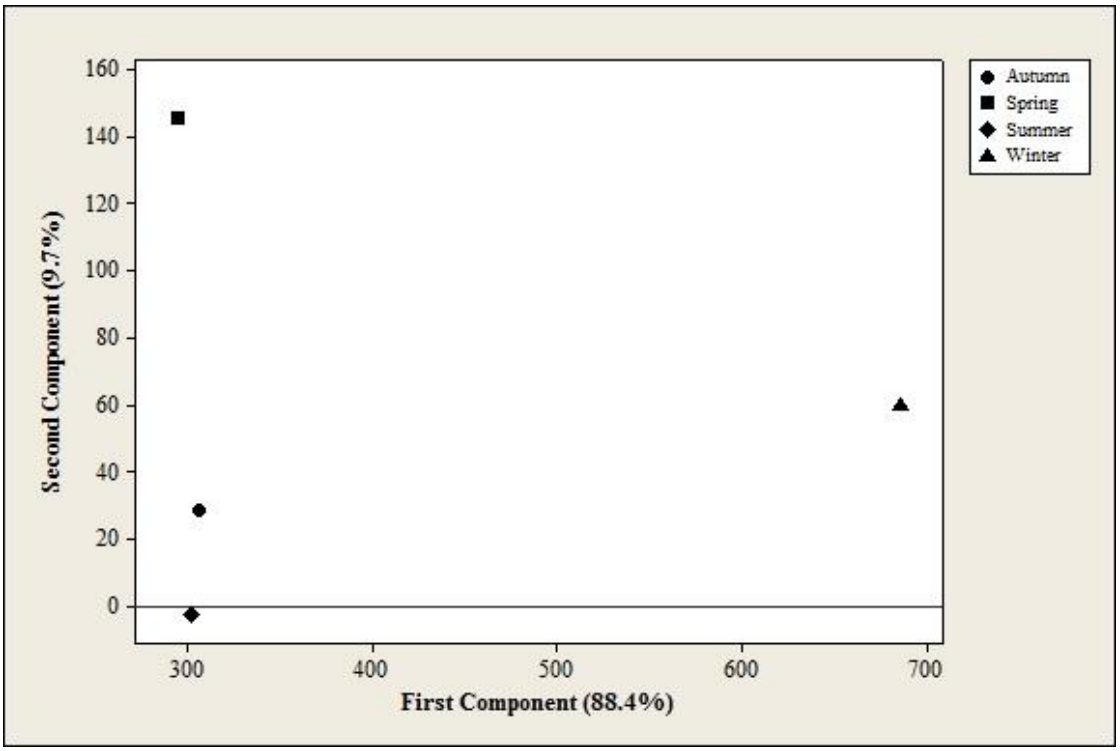

Figure 1. Plotting of Valdeón cheese throughout the year on the plane defined by the two principal components obtained by PCA of the free amino acids.

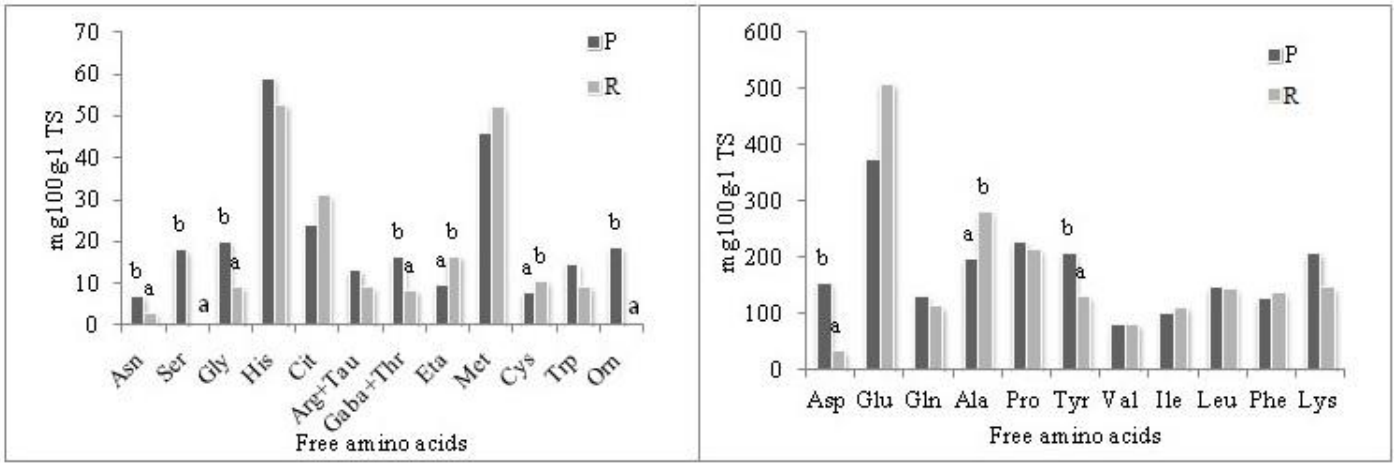

Figure 2. Free amino acids contents (mg 100 g^{-1} TS) found in Valdeón cheese made from pasteurized (P) and raw milk (R) at 90 days of ripening. Means with different superscript differ significantly ($P < 0.05$).

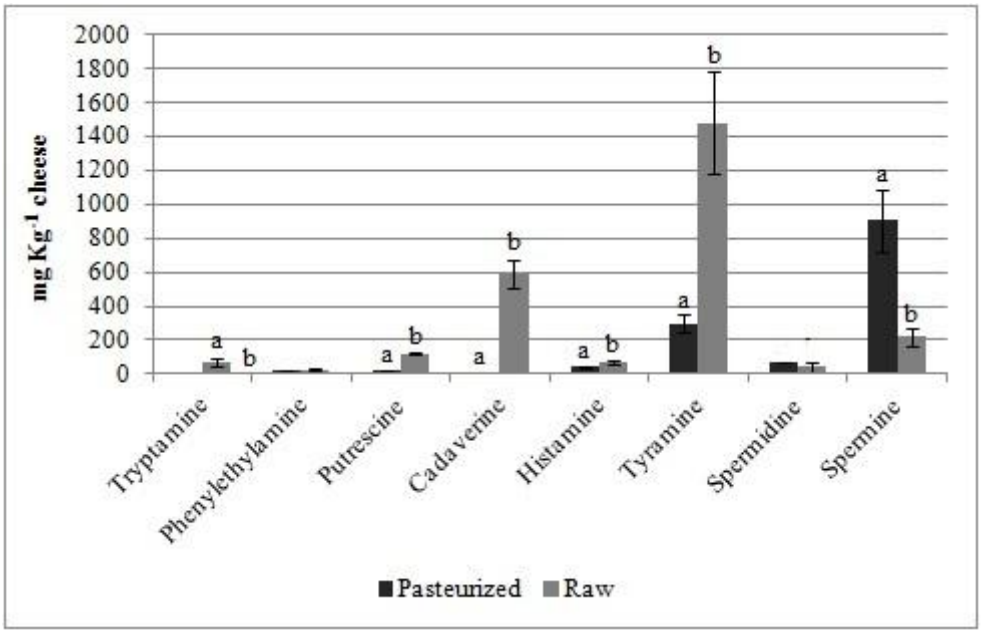

Figure 3. Biogenic amines contents (mg Kg-1 of cheese) found in Valdeón cheese made from pasteurized (P) and raw milk (R) at 90 days of ripening. Means with different superscript differ significantly ($P < 0.05$).

Artículo VII

Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion

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Resumen

Es cada vez más evidente que la digestión puede afectar la actividad biológica del queso mediante la liberación de nuevos péptidos activos a partir de sus precursores o, por el contrario, dando lugar a fragmentos sin actividad. La caracterización del peptidoma de un queso azul español, Valdeón, ha sido llevada a cabo antes y después de la digestión gastrointestinal, y los digeridos han sido comparados con los obtenidos a partir de leche desnatada pasterizada en polvo utilizando una plataforma bioinformática. El perfil peptidómico de los digeridos reveló varias regiones que son especialmente resistentes a la digestión (entre ellas, β-caseína 60-93, 128-140 y 193-209). Algunas de ellas corresponden a regiones bien conservadas entre especies (humana, vaca, oveja y cabra) e incluyen péptidos con actividad biológica previamente descrita. La gran homología encontrada entre ambos digeridos, el queso y la leche en polvo, sugirió que la digestión gastrointestinal podría acercar el perfil de productos con diferente estado proteolítico. Aunque la mayoría de los péptidos biológicamente activos encontrados en el queso después de la digestión estuvieron también presentes en los digeridos de la leche en polvo, se observaron algunas excepciones que pueden ser atribuidas a la ausencia del péptido precursor correspondiente antes de la digestión.

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Research Article

Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion

It is increasingly evident that digestion can affect the biological activity of cheese by the release of new active peptides from their precursors or, on the contrary, giving rise to fragments without activity. The characterization of the peptidome of a Spanish blue cheese, Valdeón, has been conducted before and after gastrointestinal digestion, and the digests have been compared to those obtained from pasteurized skimmed milk powder (SMP) using a bioinformatics platform. Peptidomic profiling of digests revealed several regions that are especially resistant to digestion (among them β-casein 60–93, 128–140, and 193–209). Some of them correspond to well-conserved regions between species (human, cow, sheep, and goat) and include peptide sequences with reported bioactivity. The great peptide homology found between both digests, cheese and SMP, suggests that the gastrointestinal digestion could bring closer the profile of products with different proteolytic state. Although most of the biologically active peptides found in cheese after digestion were also present in SMP digest, there were some exceptions that can be attributed to the absence of the relevant precursor peptide before digestion.

Keywords:
Active peptide / Gastrointestinal digestion / Peptidomics / MS/MS / Valdeón cheese
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Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Peptides in cheese are not only important for the organoleptic characteristics of this product but they might also contribute to its high biological value. An important number of medium and low molecular weight peptides resulting from casein during cheese ripening have been identified. Many of these peptides can interact with receptors at the gastrointestinal tract (opioid receptors), facilitate mineral absorption as the caseinophosphopeptides (CPPs), or be absorbed and reach the blood stream where they can exert a biological effect, for instance, the antihypertensive activity [1,2]. These peptides are released by residual coagulant, indigenous milk enzymes, starter, and nonstarter adventitious microflora; and, sometimes, enzymes from secondary flora (e.g. from *Penicillium* sp. in mould-ripened cheeses) [3]. Upon human ingestion, gastrointestinal enzymes come into play. There are several studies on cheese digestion using human gastrointestinal enzymes under physiological conditions [4–6]. These in vitro simulations are intended to be consistent with the in vivo digestion studies [7, 8]. It is increasingly evident that digestion can affect the biological activity, by the release of new active peptides from their precursors or, on the contrary, giving rise to fragments with less or no activity. This brings up the question about the effect that this physiological process could have on the peptide profile of food matrices containing the same proteins but in a different proteolytic state. In order to shed some light on this issue, we have characterized the peptidome of the Spanish fat, blue-veined cheese. This cheese is made out of cow`s milk or a mixture of cow, sheep, and/or goat's milk, in a region called Valdeón (León, Spain). Its authenticity is guaranteed by a Protected Geographical Indication since 2003. The peptide profile of Valdeón cheese has been checked before and after simulated gastrointestinal digestion. The digestion products have been compared to those obtained from pasteurized skimmed milk powder (SMP), a

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Abbreviations: **ACE**, angiotensin I-converting enzyme; **CPP**, caseinophosphopeptide; **IC_{50}**, the half maximal inhibitory concentration; **SMP**, skimmed milk powder; **WSE**, water-soluble extract

*These authors contributed equally to this work.

matrix where milk proteins are mainly intact, subjected to the same enzymatic digestion.

2 Materials and methods

2.1 Samples

Two samples of different batches of 60 days old cheese, made out of pasteurized cow and goat's milk, were analyzed. One corresponded to the winter cheese production (batch D) and the other to the summer production (batch A), according to the standard methods established by the Regulation Council. The protein in dry matter contents of cheese (Kjeldahl standard method, International Dairy Federation) were 36.2 and 35.7%, respectively. A sample of SMP (protein content was 40.0%) purchased at a local market was also used.

2.2 Preparation of samples

The water-soluble extracts (WSE) of Valdeon cheese were obtained as described by Gómez-Ruiz et al. [4]. Afterwards, the WSEs were ultrafiltrated using membranes Centripore Amino Ultra of 3000 Da (Millipore, MA, USA). The WSE and ultrafiltrates were frozen at –20°C until analysis.

In order to perform CPPs determination, an enrichment step by selective precipitation was carried out, as previously described [9]. Briefly, this was performed by adding $CaCl_2$ at 1% w/v (40 mol $CaCl_2$/mol casein) and ethanol, 99.8% v/v to a final concentration of 50% v/v. The resulting precipitate was collected by centrifugation at 12 000 × *g* for 10 min at 10°C, resolubilized in Milli-Q® water, subsequently freeze-dried and stored at –20°C until use.

2.3 Simulation of the in vitro static gastrointestinal digestion

A representative sample of cheese (1 g) and water were mixed (0.5%, p/v) and homogenized using an ULTRATURRAX T25 Basic S25N-18G (IKA® Werke, Germany). The SMP was reconstituted with water (10%) and homogenized. The simulated gastrointestinal digestions were carried out in triplicate by the method previously described [10]. The samples were dissolved (13 mg/mL of protein) in simulated gastric fluid (35 mM NaCl) at pH 2, preheated at 37°C. Porcine pepsin at an enzyme/substrate ratio of 1:20 w/w (182 units/mg) at 37°C during 1 h, in the presence of phosphatidylcholine (P3841; Sigma) vesicles was used. The in vitro duodenal digestion was carried out on the resulting product from stomach digestion adjusted to pH 7 by using 1M $CaCl_2$, 0.25 M Bis-Tris pH 6.5, and 0.125 M bile salts equimolar mixture of sodium taurocholate (Sigma) and glicodeoxycholic acid (Sigma). Trypsin (EC 232–650–8, Sigma; 40 units/mg protein), Chymotrypsin (EC 232–671–2, Sigma; 0.5 units/mg protein), porcine pancreatic lipase (EC 232–619–9, Sigma; 28.9 units/mg protein), and colipase (EC 259–490–12, Sigma; enzyme: substrate ratio 1:895 w/w) were diluted in 35 mM NaCl adjusted to pH 7 and added to the mixture. The reaction was stopped by adding Pefabloc® SC (Fluka 76307) at 1 mM final concentration.

2.4 SDS-page

Samples were diluted at a concentration of 2 mg/mL of protein in a buffer containing 2% w/v SDS, 62.5 mM Tris HCl pH 6.8, 10% v/v glycerol, 5% v/v β-mercaptoethanol were heated for 5 min at 95°C and loaded on 12% Bis-tris polyacrylamide gels (Criterion_XT, Bio-Rad, CA, USA). Electrophoretic separations were run at 150 V in a Criterion cell using XT-MES running buffer. Gels were stained with Coomassie Blue (BioSafe Coomassie G-250 Stain, Bio-Rad).

2.5 Analysis by reversed phase-HPLC-MS/MS

Reversed phase-HPLC-MS/MS analyses of WSE and digests from cheese and SMP samples were carried out as described by Sánchez-Rivera et al. [11]. The column used was Waters (XBridge™ BEH 300 C18 5 μm, 4.6 × 250 mm; Waters, MA, USA), the injection volume 50 μL, and the flow set at 0.8 mL/min. The peptides were eluted with a linear gradient from 0 to 45% of solvent B (acetonitrile/trifluoroacetic acid 0.027%) and 55% of solvent A (water/trifluoroacetic acid 0.037%) in 60 min. Two runs were performed per digestion triplicate in order to set up the method for different target mass: 600 *m/z* (permeate < 3 kDa, WSE and SMP) and 1200 *m/z* (WSE and SMP). Spectra were recorded over the mass/charge *m/z* range 100–3000.

In the case of samples enriched in CPPs, the analyses were carried out using a Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The injection volume was 50 μL and the flow rate 0.2 mL/min. A linear gradient from 0 to 45% of solvent B (acetonitrile/formic acid 0.1%) and 55% of solvent A (water/formic acid 0.1%) in 120 min was used. In these analyses, the target mass was set at 750 *m/z* and 1500 *m/z*.

Data processing was done by using Data Analysis™ (version 4.0; Bruker Daltoniks, Germany). The peptide sequencing was performed by MASCOT, using a homemade database that includes the cow's and goat's milk proteins and main genetic variants thereof. The matched MS/MS spectra were interpreted by using BioTools version 3.2 and the comparative peptidome analysis by means of the bioinformatics platform Protein Scape 3.0, both from Bruker Daltoniks. Peptides present in at least one replicate have been included in data sets.

3 Results and discussion

3.1 Electrophoretic profile of WSE of the cheeses and SMP

Figure 1A shows the SDS-PAGE separation of Valdeón cheese (batches D and A) and their gastrointestinal simulations in

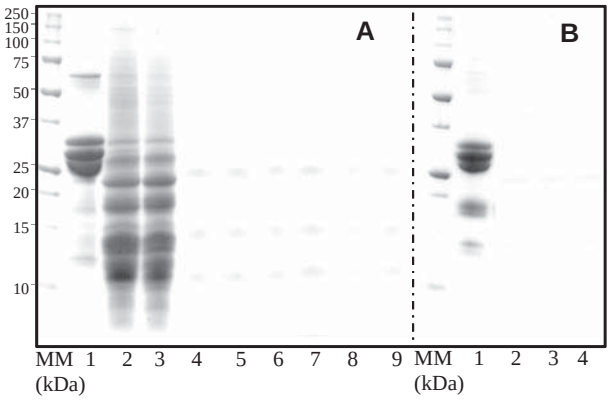

Figure 1. (A) Bis-Tris SDS-PAGE electrophoretic run of commercial casein (lane 1), WSE of Valdeón cheese batch D (lane 2), batch A (lane 3) and digests of cheese in triplicate from batch D (lanes 4–6) and batch A (lanes 7–9). (B) Bis-Tris SDS-PAGE electrophoretic run of SMP control sample before digestion (lane 1), digests of SMP in triplicate (lanes 2–4). MM = molecular marker.

triplicate. Figure 1B corresponds to PAGE-SDS of SMP digest in triplicate. Despite the extensive proteolysis that naturally occurs in Valdeón cheese, as a blue-mould cheese, there were still bands corresponding to caseins in the cheese before digestion (WSE). Similar protein patterns could be observed for both batches. After gastrointestinal digestion these bands were barely visible. The bands that remained detectable with MWs of approximately 24, 14, and 12 kDa were also found after the electrophoretic separation of the gastrointestinal fluid in a blank (i.e. digestion without sample, data not shown) and therefore, they could correspond to the enzymes employed or fragments thereof, since the MW of trypsin and chymotrypsin are 23.3 and 24 kDa, respectively.

3.2 Peptide profile of Valdeón cheese

The identified peptides (121) in the total WSE and the 3 kDa fraction of the two different cheese batches before digestion are listed in Supporting Information Table 1. Peptides identified in at least one batch have been included, since 75% of the peptides were found in both. Those peptides originating from the most abundant casein fractions (β- and α_{s1}-casein, from cow, and/or goat) dominated the cheese peptidome although a few peptides derived from α_{s2}-casein and κ-casein were also detected. The analysis of the 3 kDa permeate fraction allowed to narrow the identification of 64 peptides between 500–1000 Da, that contained less than 7 residues. The blue cheese undergoes extensive proteolysis due to the action of enzymes from different origins as mentioned before, with the main contribution from the mould culture, *Penicillium roqueforti*. This leads to the larger number of different peptides produced in blue cheese compared to semihard cheeses [12].

Figure 2 shows the peptides released from β- and α_{s1}-casein, where bovine and/or caprine origin is indicated. A distributed pattern of peptides was found within the β-casein sequence, covering most of its sequence (78% protein coverage) (Fig. 2A). The N-terminal part of the protein, where the phosphorylated serines are located, was source of a high amount of peptides, whose detection was enhanced by the selective precipitation employed. A total of 19 CPPs were identified from β-casein. Among these, 5 contained more than one phosphorylated serine and the other 13 were identified in the region comprised between the residues 30–46 of the β-casein sequence, and were monophosphorylated. From these, four arose from the 29–30 cleavage site (f30–36/37/39/42), which is characteristic of aspartyl-proteinases action, secreted by *P. roquefortii* [13, 14]. In accordance to our results, numerous phosphorylated peptides from the N-terminal region of β-casein had been also reported in Parmiggiano cheese [15]. Similarly, various peptides from the C-terminal region of this protein have been found in Manchego [4], Parmiggiano [15], and Cheddar cheeses [16], among others. Some other regions that gave rise to a notable number of peptides were the ones containing residues 60–70, 80–90, 110–120, 130–140, and the C-terminal part of the β-casein sequence. Some of the identified regions include previously reported biologically active sequences like opioid peptides: fragment (f) 60–66 (β-casomorphin 7) and f114–119 (neocasomorphin) [17] or angiotensin I-converting enzyme (ACE) inhibitors, like f47–52 (DKIHPF), which had been previously found in Manchego cheese [4, 18]. Other identified peptides, like β-casein f169–175 had been previously reported to show low in vitro ACE-inhibitory activity, whose half maximal inhibitory concentration ($IC_{50} > 1000$) was high, but it exerted a high antihypertensive activity in spontaneously hypertensive rats after oral administration [19].

The sequence coverage of α_{s1}-casein reached 52% (Fig. 2B). Among others, 17 CPPs were identified of which four of them arose from region 109–118 and were monophosphorylated. CPPs containing two or more phosphorylated residues were also found, as it is the case of f62–73. Likewise, 12 of the CPPs from α_{s1}-casein were diphosphorylated and belonged to the domain comprised between residues 39 and 55. In complex peptide mixtures the detection of phosphorylated forms is impaired, since the nonphosphorylated peptides are more easily ionized. To overcome this difficulty, instead of a selective precipitation, other authors have conducted a selective isolation on TiO_2 micro-columns when analyzing complex samples from milk proteins digestion [20]. On the other hand, the region comprising the residues from 83 to 91 was also source of a noteworthy number of peptides, 8 identified sequences. Similarly, in Manchego cheese, numerous peptides between residues 83 and 95 were found [4], while in Parmiggiano cheese, peptides α_{s1}-casein f80–114, f83–114, and f85–114 were identified [15]. Among the identified peptides from α_{s1}-casein, the sequence f157–164, with ACE-inhibitory activity (IC_{50} 98 μM), had been previously found in fermented casein with different lactic acid starters, and after hydrolysis with pepsin and trypsin [21].

Figure 2. Identified peptides in Valdeón cheese before digestion from (A) β-casein and (B) α_{S1}-casein. Peptides belonging exclusively to goat's milk are represented at the bottom, differing residues squared. Sequences in bold: peptides unique from cow's milk. *S*: phosphorylated serine.

Table 1. Identified peptides with reported bioactivity found before and/or after simulated digestion of Valdeón cheese

Fragment	Before digestion	After digestion	Activity
α_{s1}-Casein f(143–149)[a)]		AYFYPEL	Antihypertensive [34]/Mucin production [35]
α_{s1}-Casein f(144–149)[a)]		YFYPEL[c)]	Antioxidant [31, 33]/Mucin production [35]
α_{s1}-Casein f(157–164)[a) b)]	DAYPSGAW	DAYPSGAW[c)]	IACE [21]
β-Casein f(47–52)[a) b)]	DKIHPF	DKIHPF	IACE [4, 18]
β-Casein f(60–68)[a)]	YPFPGPIPN		IACE/Antihypertensive [48]
β-Casein f(98–105)[a)]		VKEAMAPK[c)]	IACE [32]
β-Casein f(108–113)[a) b)]		EMPFPK[c)]	IACE [21]
β-Casein f(114–119)[a) b)]	YPVEPF	YPVEPF[c)]	Opioid [17]
β-Casein f(133–138)[a) b)]		LHLPLP	Antihypertensive [29]
β-Casein f(133–139)[a) b)]		LHLPLPL[c)]	IACE [29]
β-Casein f(132–140)[a)]		NLHLPLPLL	IACE [30]
β-Casein f(130–140)[a)]		VENLHLPLPLL	IACE [30]
β-Casein f(169–175)[a) b)]	KVLPVPQ		Antihypertensive [19]
β-Casein f(193–198)[a)]/f(191–196)[b)]		YQEPVL	IACE [48]
κ-Casein f(18–24)[a)]		FSDKIAK	Antibacterial, IACE [37]

a) Peptide sequence is present only in cow's milk.
b) Peptide sequence is present in goat's milk.
c) Found also in skim milk powder digests.

3.3 Identification of peptides after simulation of gastrointestinal digestion of Valdeón cheese

A total of 139 peptides were found after Valdeón cheese digestion (Supporting Information Table 2). There was not a great difference in the number of peptides found before digestion (121 peptides), due to the proteolysis naturally occurring in this cheese [22–24]. However, a careful analysis, using a bioinformatics platform (ProteinScape), revealed some differences in the peptide profile before and after gastrointestinal digestion. A total of 28 peptides found in the digested cheese samples were already present before digestion that demonstrates their resistance to the gastrointestinal enzymes, and resulted in a total peptide homology of 12.1%. Indicative examples of resistant peptides to digestion from β-casein are GPFPIIV [f203–209 (cow's milk)/f201–207 (goat's milk)], YPVEPF f114–119, and DKIHPF f47–52. The last sequence has been often found after simulated digestion of different dairy products, including in vivo studies [8]. However, the two first peptides have been found only in cheese and not in simulated digests of other dairy products, which could indicate that they are generated by the fermentation process [4, 25, 26]. Regarding the α_{s1}-casein resistant peptides, the sequences f83–89 (KEDVPSE), f84–90 (EDVPSER), and f83–90 (KEDVPSER) survived digestion. These results are in agreement with previous reports that found several peptides comprised between residues 80–91 in casein subjected to simulated gastrointestinal digestion [20]. Moreover, α_{s1}-casein f80–88 (HIQKEDVPS), f80–89 (HIQKEDVPSE), f80–90 (HIQKEDVPSER), f80–91 (HIQKEDVPSERY), and f81–89 (IQKEDVPSE) have been identified in the jejunum of healthy humans who ingested casein [8]. Another resistant sequence corresponded to an ACE-inhibitory peptide, f157–164 DAYPSGAW, which had been also detected in an in vivo study of the evacuation of casein peptides in calf stomach [27].

In addition, new peptides formed during digestion were identified. An example of new sequences generated during digestion is the β-casein f133–138 (LHLPLP), a potent antihypertensive peptide [28], whose precursor peptide f128–138, was present in cheese before digestion (Table 1). Therefore, it is important to highlight that the active form of the antihypertensive peptide, which produced a decrease in the systolic blood pressure of spontaneously hypertensive rats of 25.3 mm of Hg [29], was released during cheese-simulated digestion but not during SMP digestion. Another new peptide arose from digestion, β-casein f132–140, NLHLPLPLL, had been previously identified in sodium caseinate hydrolysates produced by *Lb. helveticus* NCC2765, and described as ACE-inhibitor, showing an IC_{50} value of 15 μM [30]. Both peptides belong to a highly conserved β-casein region in different mammals, which has led to numerous and different combination of peptides that have been found in digests from human milk [31] and cows' milk proteins both in vitro [20, 26] and in vivo [8], by using different MS combinations: nanoLC-QTOF, HPLC-ion trap, or MALDI-TOF. Another new released sequence from β-casein was f98–105, EAMAPK, previously described as antioxidant [32]. Similarly, the β-casein f108–113, EMPFPK, to whom ACE-inhibitory activity has been attributed [21], was only identified after digestion (Table 1). Furthermore, this peptide has been also found in human effluents after ingestion of casein [8]. From the α_{s1}-casein sequence, peptides f144–149 (AYFYPEL) and f143–149 (YFYPEL) were generated during digestion. Both peptides have been previously found to exert antioxidant activity [31, 33], and mucin secretory activity in HT29-MTX cells [35]. In addition, the second one has demonstrated potent antihypertensive activity [34] (Table 1). Both peptides are known to be released after hydrolysis of casein with pepsin [36]. The sequence f143–149 (AYFYPEL) has been also found in human [7] and calf stomach [27] during in vivo

digestion studies. Although before simulated digestion, no peptides were found beyond position 164, after digestion at least 10 fragments from the C-terminal part of the protein were identified. The formation of these peptides might be produced by the action of gastric pepsin, since α_{s1}-casein f165–169 has been found in calf stomach after casein ingestion [27]. κ-Casein gave rise to newly released peptides during in vitro digestion, as for instance, f18–24. This peptide shows ACE-inhibitory activity (IC_{50} 113.6 μM) and antibacterial activity [37].

A total of 39 CPPs could be identified in cheese digests. The majority of these peptides (30) were monophosphorylated. Similar results were reported in digestion studies of Beaufort cheese [6] and casein [20]. From these identified CPPs, 16 arose from β-casein, 14 of which were monophosphorylated and belonged to the region corresponding to residues 30–46. Moreover, eight of these peptides survived digestion, since they were already present before digestion (f30–37/42, f32–37/42/43, and f33–37/42/43). Interestingly, the common trait of these eight peptides after digestion is the presence of ^{30}Ile ^{32}Lys, and ^{33}Phe at the N-terminal ending but the absence of ^{31}Glu. Moreover, the digestion process led to the formation of four additional peptides from this domain containing N-terminal Phe^{33} (f33–39/44/45/46), which can be generated by pepsin [38]. Some identified CPPs showed two or more phosphorylated residues. Among them, the peptides β-casein f7–25 and also α_{s2}-casein f5–15, displayed the cluster sequence SpSpSpEE, which could provide mineral-binding properties [39]. α_{s1}-Casein gave rise to 14 CPPs after digestion, seven of them being diphosphorylated, while the other seven were monophosphorylated, and belonged to the domains 40–55 and 108–119, respectively. No CPPs were found from α_{s1}-casein 60–70, where the phosphorylated cluster is located. Despite the sequence C- or N-terminal changes caused by the digestive enzymes activity, no change in the phosphorylated residues was observed for the identified CPPs upon cheese digestion.

3.4 Comparison of peptides released after simulation of gastrointestinal digestion of Valdeón cheese and skimmed milk powder

In order to assess if the same peptides can be generated with the ingestion of foods with a very different proteolytic state, the peptide profile of the digested cheese was compared with that found after gastrointestinal digestion of SMP (Supporting Information Table 3). The analysis revealed a difference in the number of peptides found in SMP (84 peptides) and cheese (122 peptides, leaving out those from goat origin). Taking into account that no undigested casein was found neither in Valdeón cheese nor SMP (Fig. 1), this difference was attributed to the high grade of proteolysis of Valdeón cheese. The proteolysis in cheese creates a large number of cleavage sites exposed to enzymes leading to the generation of a higher number of different peptides. Despite of this, good sequence coverage was reached in the peptide analysis of both, Valdeón cheese and SMP digests. In the case of β-casein, protein coverage was slightly higher in cheese (87%) than in milk powder (80.4%).

The exhaustive comparative analysis revealed differences in peptide identity between the SMP and cheese digests. From the casein fraction, 36 peptides were found to be common for both matrices, and a total peptide homology of 19% was achieved. β-Casein contributed the most to this issue, since 25 of the common peptides were generated from this protein. In contrast, as described above, the percentage of homology in cheese (before and after digestion) was 12.1%. The higher peptide homology between digested cheese and the SMP digest suggests that the digestion process reduces the differences in the peptidome of both matrices. Figures 3 and 4 show the peptides that have arisen from β-casein and α_{s1}-casein, respectively, after digestion of Valdeón cheese and SMP. In β-casein, various peptides belonging to the region 128–140 displayed C-terminal Leu residues, which could be due to the chymotrypsin activity in accordance to previous studies [40]. This domain seemed to be particularly resistant to hydrolysis during cheese ripening or digestion. For instance, the nonactive fragments f133–139 (LHLPLPL) and f134–139 (HLPLPL) were found in both digested matrices. Dupont et al. [41] also reported the resistance of this region and the presence of those two peptides after in vitro digestion of β-casein. Although the high number of peptides from this highly conserved region is remarkable, the potent antihypertensive peptide, LHLPLP f133–138 could not be found in milk digests. It seems that the presence of a certain precursor peptide is needed for the release of the active peptide during digestion [42]. However, recently this peptide was identified in the jejunum of humans who consumed casein [8]. It remains to be investigated if this peptide could be released in vivo in a sufficient amount to exert an antihypertensive effect, since in most clinical studies where milk or fermented milk is used as control, no antihypertensive effects were observed [43–45]. Similarly, bovine α_{s1}-casein f144–149 (YFYPEL), whose antioxidant activity has been cited above, was identified in SMP together with the related fragment 145–149 (FYPEL), but the antihypertensive sequence f143–149 (AYFYPEL) was not found. Nevertheless, the last sequence was found in human gastric samples after ingestion of milk or yogurt [7]. It would be of great interest to know the amount of these peptides after human digestion to correlate their activity with their presence in gastrointestinal effluents.

Other peptides that were found in both digests are the β-casein sequences VKEAMAPK f98–105, EMPFPK f108–113, reported as ACE-inhibitors [21, 33] and YPVEPF f114–119, reported as opioid [17]. The β-casein region 106–119, that gave rise to the latter peptides, together with the domains 60–93 and 133–140 seem to be very resistant to gastrointestinal digestion in both SMP and cheese, probably due to the presence of Pro in their sequences [46]. These results are in agreement with those found in casein fractions and β-casein digests [20, 41]. Moreover, the resistance of the phosphorylated regions of caseins to digestion has been proved in these

Figure 3. Identified peptides in Valdeón cheese (A) and SMP (B) from β-casein after digestion. Peptides belonging exclusively to goat's milk are represented at the bottom, differing residues squared. *S*: phosphorylated serine.

Figure 4. Identified peptides in Valdeón cheese (A) and SMP (B) arising from α_{S1}-casein after digestion. Peptides belonging exclusively to goat's milk are represented at the bottom, differing residues squared. *S*: phosphorylated serine.

samples. In Valdeón cheese, the β-casein region 30–46 appeared to be highly resistant, leading to the formation of 14 CPPs in digests. Picariello et al. [20] also reported the presence of numerous CPPs from this region and specifically found the sequences f33–42 and f33–43 after in vitro digestion of casein. In SMP, a total of 18 CPPs were identified. Ten of them were found in β-casein, only three of those being monophosphorylated. However, the phosphorylated region 5–25 appeared to be more resistant to digestion, giving rise to seven peptides, among them, two sequences containing the phosphoseryl cluster, i.e. f12–25 and f6–25. The phosphoseryl cluster was conserved in both SMP f12–25, f6–25, and cheese f7–25, as a common trait resulting from digestion. This is consistent with the presence of β-casein f7–25, f2–25, and f1–24 in lumen contents of rats perfused with β-casein f1–25 [47]. This behavior could help to elucidate and better understand the cleavage patterns and breakdown of milk proteins and peptide release during the gastrointestinal process in the small intestine.

4 Concluding remarks

The peptide profile of Valdeón cheese showed the presence of some ACE inhibitory and opioid peptides. After gastrointestinal simulation, a higher number of bioactive peptides, including antihypertensive, antioxidant, intestinal mucin-secretor, and antibacterial were found. However, not all these sequences could be detected after digestion of SMP. The exceptions could be due to peptide precursor differences that result in a distinct peptide profile after digestion. On the other hand, the peptidomic profiling of digests reveals several regions that are especially resistant to gastrointestinal digestion in SMP and cheese, mainly in β-casein 60–93, 128–140, and 193–209 but also in β-casein 30–46, and α_{s1}-casein 40–55 and 109–118, where the phosphoserines are located. Indeed, no changes in the phorphorylated residues were observed after simulated digestion of cheese. Some of these resistant regions are well conserved between species in dairy proteins. Interestingly, numerous sequences with reported biological activity belong to them. The higher peptide homology between digested cheese and the SMP digest compared to that found in cheese before and after digestion, confirms that the digestion process could bring closer the profile of certain regions, even if they have different grade of proteolysis before digestion. The remaining differences in the cleavage pattern during digestion could also have other health implications, like metabolic or toxicological ones (exposure of potential epitopes). Kinetics of peptide release could provide further information in this sense.

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5. DISCUSIÓN GENERAL

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5.1. Influencia del tiempo de maduración en las características de la IGP queso de Valdeón

Una parte importante de la presente Memoria de Tesis Doctoral estuvo encaminada a la caracterización del queso de Valdeón durante la maduración desde un punto de vista microbiológico, fisico-químico, proteolítico, lipolítico y sensorial. El queso de Valdeón se caracterizó por presentar recuentos de bacterias aerobias mesófilas totales elevados coincidiendo con otras variedades similares (Alegría *et al.*, 2011). En general, al inicio de la maduración predominaron las bacterias ácido lácticas pertenecientes al cultivo iniciador (lactococci y *Leuconostoc*), cuyos recuentos más elevados correspondieron a los dos días. Los recuentos de lactobacilli aumentaron durante el primer mes de maduración como consecuencia de su capacidad para tolerar ambientes hostiles (Fox *et al.*, 1998). A partir del mes de maduración y hasta el final de la misma, los mohos y levaduras dominaron los recuentos microbianos. El rápido incremento de la densidad de *Penicillium roqueforti* que se observó tras el salado del queso se debió a su incorporación como cultivo iniciador secundario y a que los valores de actividad de agua (aw) y la concentración de sal se situaron en el rango óptimo para su crecimiento y germinación (Godinho & Fox, 1981). El incremento de la concentración de NaCl tras los 30 días de maduración (>3%) dio lugar a una reducción de la tasa de germinación y crecimiento de *P. roqueforti*, aunque también indujo su esporulación. Esto tuvo como resultado la estabilización de los recuentos de mohos tras el primer mes de maduración.

No parece que enterococci juegue un papel importante en la maduración del queso de Valdeón dado los bajos recuentos que se observaron. Sin embargo y pese a que los recuentos de *Micrococcaceae* fueron inferiores a los descritos en otros quesos azules (González de Llano *et al.*, 1992), sí que se consideró que este grupo pudo jugar un papel importante en la maduración debido a su importante actividad proteolítica y lipolítica, contribuyendo a la textura y flavor final. En general, *Enterobacteriaceae* no

fueron detectadas debido al tratamiento de pasterización y a las excelentes condiciones higiénico-sanitarias.

El contenido en sólidos totales del queso de Valdeón aumentó a lo largo de la maduración alcanzando valores superiores a los descritos en otros quesos similares (Alonso *et al.*, 1987; González de Llano *et al.*, 1992; Zarmpoutis *et al.*, 1996; Gobbetti *et al.*, 1997). Tanto el contenido en grasa como en proteína, expresados como porcentaje de sólidos totales, permanecieron sin variaciones a lo largo de la maduración. Mientras que el contenido en grasa fue similar, el de proteína resultó ser inferior al final de la maduración al de otras variedades similares (Flórez & Mayo, 2006; González de Llano *et al.*, 1992; Prieto *et al.*, 2000).

Como resultado del desarrollo de las bacterias ácido lácticas, la lactosa se redujo considerablemente durante las primeras etapas de la maduración, produciéndose un incremento del ácido L-láctico y, por lo tanto, un descenso del pH. Al final de la misma se observó asimismo un descenso tanto ácido D- como L-láctico como consecuencia de la actividad de los mohos y levaduras (Prieto *et al.*, 2000), lo que junto a las reacciones de desaminación produjo el aumento del pH alcanzándose valores similares al de otras variedades de queso azul (Hayaloglu *et al.*, 2008; Prieto *et al.*, 2000). Por otra parte, se observó un incremento en la concentración de sal especialmente pronunciado al inicio de la maduración coincidiendo con la aplicación de sal seca en el queso. Los niveles observados coincidieron con los descritos en otros quesos azules (Zarmpoutis *et al.*, 1996; Gobbetti *et al.*, 1997; Prieto *et al.*, 2000). Como consecuencia del incremento de la concentración de sal y el descenso de la humedad, se observó un incremento de la relación sal/humedad durante la maduración del queso de Valdeón. Además, los valores de aw disminuyeron a lo largo de la maduración situándose en valores similares a los descritos en Picón Bejes-Tresviso (Prieto *et al.*, 2000) y Cabrales (Flórez & Mayo, 2006).

El queso de Valdeón mostró una gran extensión y profundidad de la proteólisis. Este hecho se reflejó, en primer lugar, en los altos valores observados de NS-pH 4,6, NS-TCA y NS-PTA. Al final de la maduración los valores obtenidos en estos tres índices fueron similares a otros quesos de vena azul (Fernández-Salguero *et al.*, 1989; Zarmpoutis *et al.*, 1996). Durante las primeras etapas de la maduración se observó un

equilibrio entre la proteólisis primaria y secundaria como demostró el hecho de que el nitrógeno polipeptídico representara el 50% del NS-pH 4,6. Sin embargo, a partir del mes de maduración predominó la proteólisis secundaria El contenido medio de nitrógeno peptídico aumentó durante los primeros 30 días de maduración para a partir de este momento permanecer estable. Este comportamiento se atribuyó principalmente a la actividad de las peptidasas secretadas por *P.roqueforti* tras su esporulación (fundamentalmente aminopeptidasas alcalinas).

En segundo lugar, la gran proteólisis que tuvo lugar en el queso de Valdeón se reflejó en la extensa degradación tanto de la αs_1- como de la β-caseína al final de la maduración. Durante los primeros días de la maduración, la degradación de la αs_1-CN fue mucho más marcada como consecuencia de la acción del cuajo sobre ella. El descenso de la fracción αs_1-CN vino marcado por un incremento de péptidos de bajo peso molecular y la casi desaparición de la fracción αs_1-I-CN; mientras en el caso de la fracción β-CN se tradujo en un aumento del contenido en productos de baja movilidad conocidos como γ_1-, γ_2- y γ_3-CN. Estos resultados concuerdan con los descritos por otros autores para otras variedades de quesos de vena azul (Fernández-Salguero *et al.*, 1989; Zarmpoutis *et al.*, 1996; Seratlic *et al.*, 2011). El incremento en la degradación de la αs_1-CN y β-CN a partir del mes de maduración coincidió con el desarrollo del *P. roqueforti* del queso y la consiguiente liberación de proteinasas al medio, fundamentalmente aspartil proteasas, las cuales son muy activas bajo las condiciones de pH que se instauraron en el queso (Cantor *et al.*, 2004). La mayor pendiente observada en la degradación de la β-CN entre el primer y el segundo mes de maduración podría venir explicada por la acción adicional de la plasmina. Sin embargo, su actividad en el queso de Valdeón, aunque mayor que en otras variedades de queso (Farkye & Fox, 1990), fue muy similar durante toda la maduración. Ello nos llevó a concluir que los principales agentes proteolíticos durante la maduración del queso de Valdeón fueron las proteasas liberadas por *P. roqueforti*.

Por otro lado, los cromatogramas obtenidos en el estudio del perfil de péptidos mostraron una gran complejidad. Las diferencias entre los mismos fueron mucho más acusadas al mes de maduración debido a la actividad de las peptidasas secretadas por *P. roqueforti* y se mantuvo hasta el final de la misma. Si bien, a los 90 y 120 días la

degradación de los péptidos a aminoácidos libres prevaleció sobre la formación de péptidos de bajo peso molecular. Ello se tradujo en una menor complejidad de los cromatogramas en las últimas fases de la maduración del queso de Valdeón. El análisis de componentes principales mostró que al inicio de la maduración el queso se caracterizó por presentar mayor concentración de péptidos hidrofóbicos, mientras que al final de la misma prevalecieron los péptidos más hidrofílicos. El ratio de péptidos hidrofóbicos e hidrofílicos en los quesos azules tiende a ser menor que el observado en otro tipo de variedades debido a la acción de las exopeptidasas que degradan los péptidos hidrofóbicos y liberan péptidos de bajo peso molecular y aminoácidos (González de Llano *et al.*, 1995).

Como cabría esperar, el contenido en aminoácidos libres aumentó a medida que avanzó el tiempo de maduración. Sin embargo, se observó una ralentización o incluso, en el caso de algunos aminoácidos, un descenso de su contenido en el último mes de maduración probablemente debido a su catabolismo coincidiendo con el aumento de la concentración de aminas biógenas que se observó con el aumento del tiempo. El contenido final en aminoácidos libres fue inferior al descrito para variedades similares como Picón-Bejes Tresviso (Prieto *et al.*, 2000), aunque superior a otras como Cabrales (Flórez *et al.*, 2006) y Gamonedo (González de Llano *et al.*, 1991). Al final de la maduración los aminoácidos libres predominantes fueron ácido glutámico (Glu), alanina (Ala), prolina (Pro), tirosina (Tyr), ácido aspártico (Asp), leucina (Leu) y fenilalanina (Phe) lo que coincide, con pequeñas diferencias, con los resultados obtenidos por otros autores en otras variedades de queso azul (Madkor *et al*., 1987a; González de Llano *et al.*, 1991; Zarmpoutis *et al.*, 1996; Prieto *et al.*, 2000). Por su parte, la concentración final de aminas biógenas fue similar a la observada por Komprda *et al.* (2008) en queso azul, aunque superior a la descrita para otras variedades de queso (Vale & Gloria, 1998; Valsamaki *et al.*, 2000; Renes *et al.*, 2014). Al final de la maduración, espermina y tiramina fueron las aminas biógenas predominante siendo, además, las únicas que presentaron diferencias significativas en su contenido a lo largo de la misma.

El queso de Valdeón también mostró una importante lipólisis a lo largo de la maduración. La concentración de la mayoría de los ácidos grasos libres estudiados experimentó un aumento a medida que avanzó la misma coincidiendo con lo observado

en otros quesos similares como Gorgonzola (Gobbetti *et al.*, 1997) o Stilton (Madkor *et al.*, 1987b). En el caso de algunos ácidos grasos libres, se observó un ligero descenso de su contenido a los 120 días de maduración como consecuencia de su hidrólisis y la consiguiente formación de compuestos como metil cetonas, alcoholes, aldehídos etc. Este comportamiento también fue observado por Contarini & Toppino (1995) y Prieto *et al.* (2000) en Gorgonzola y Picón Bejes-Tresviso, respectivamente. El contenido total observado al final de la maduración fue similar al descrito en otras variedades similares como Gamonedo (González de Llano *et al.*, 1992) y superior al descrito en Roquefort (Woo *et al.*, 1984). Excepto a los 2 días de maduración, los ácidos grasos predominantes en el queso de Valdeón fueron el ácido oleico y el ácido palmítico, coincidiendo con los resultados observados en Roquefort (Vanbelle *et al.*, 1978), Cabrales (Alonso *et al.*, 1987; de la Fuente *et al.*, 1993) y Picón Bejes-Tresviso (Prieto *et al.*, 2000).

El ratio ácidos grasos de cadena corta/ácidos grasos libres totales, experimentó un descenso significativo durante las primeras etapas de la maduración como resultado de su descomposición a compuestos aromáticos como γ- o δ-lactonas, alcoholes o metil cetonas (Laskaridis *et al.*, 2013). Posteriormente, a los 60 días de maduración mostraron un ligero aumento, para permanecer estables hasta el final de la misma. Por su parte el ratio ácidos grasos de cadena media/ácidos grasos libres totales descendió significativamente durante el primer mes de maduración y posteriormente permaneció constante. El ratio ácidos grasos de cadena larga/ácidos grasos totales mostró una tendencia opuesta a la de los ácidos grasos de cadena media. Con la excepción de los 2 días de maduración, los ácidos grasos de cadena larga fueron los predominantes.

El análisis de componentes principales permitió separar las muestras en función del tiempo de maduración. Los ácidos butírico, mirístico, palmítico y oleico fueron los que mejor correlación mostraron. Las muestras de 2 y 15 días de maduración se caracterizaron por presentar un contenido más bajo de ácido palmítico, oleico y butírico. A medida que aumentó el tiempo de maduración, estos ácidos grasos estuvieron presentes en mayor cantidad, con la excepción de los quesos de 120 días de maduración cuyo contenido fue inferior al mostrado por las muestras de 90 días. Además, los quesos

de 120 días de maduración se caracterizaron por presentar valores más elevados de ácido mirístico.

Al igual que otras variedades (Kahyaoglu & Kaya, 2003; Juan *et al.*, 2004; Farahani *et al.*, 2014), el queso de Valdeón se caracterizó por ser un sólido viscoelástico, ya que durante toda la maduración los valores del módulo elástico (G') fueron superiores a los del módulo viscoso (G''), y ambos módulos fueron dependientes de la frecuencia (Dimitreli & Thomareis, 2008). Sin embargo, al final de la maduración los quesos presentaron una disposición estructural más elástica con una mejor capacidad para resistir la deformación que en las primeras etapas de la misma, coincidiendo con los estudios llevados a cabo por Farahani (2014). Los valores mostrados por la Tan δ durante toda la maduración indicaron el carácter sólidos viscoelástico de los quesos (Luyten *et al.*, 1991). Se observó un descenso significativo del valor de este parámetro al final de la maduración, si bien, las diferencias observadas fueron muy pequeñas debido al efecto simultáneo y opuesto del descenso en el contenido en agua, incremento del pH e incremento de la proteólisis (Dewettinck *et al.*, 1999). El tiempo de maduración también dio lugar a un aumento de los valores de G* debido a que la reducción del contenido en humedad contrarrestó el efecto de la proteólisis como ha sido descrito por otros autores (Pereira *et al.*, 2001; Dimitreli & Thomareis, 2008).

El queso de Valdeón se caracterizó por bajos valores de dureza y fracturabilidad en las etapas iniciales de la maduración probablemente como consecuencia de la reducción del contenido en fosfato cálcico coloidal que se produciría bajo las condiciones de pH instauradas durante la coagulación de la leche. Sin embargo, posteriormente se produjo un aumento de los valores de ambos parámetros provocado por la aplicación de sal seca y el aumento del pH, teniendo lugar un aumento de la fuerza iónica en el queso hasta unos valores próximos a los señalados por Pastorino *et al.* (2003). Esto dio lugar a una reducción en la solubilidad de las proteínas debido a un fenómeno de "*salting-out*", incrementando de este modo la firmeza y dureza del queso. A partir del primer mes de maduración, los quesos exhibieron un descenso en los valores de dureza y fracturabilidad lo cual se debió principalmente a la extensión y profundidad de la proteólisis de la masa (Marchesseau *et al.*, 1997).

De igual modo, el queso de Valdeón se caracterizó por presentar un descenso en los valores de cohesividad a medida que avanzó la maduración y un incremento en los de adhesividad. El incremento de la fuerza iónica originado por la adición de sal conjuntamente con los altos niveles de solubilización del calcio de las caseínas provocados por los bajos valores de pH instaurados durante la coagulación serían los factores responsables de la menor elasticidad y cohesividad del queso (Pastorino *et al.*, 2003). Por otro lado, el incremento del pH producido en el queso durante la maduración contribuyó a incrementar las interacciones de las proteínas con el agua y otros compuestos no proteicos dando lugar a una matriz proteica más hidratada e hinchada, lo que unido al alto contenido en materia grasa provocaron un incremento en la adhesividad. La gomosidad y masticabilidad mostraron valores máximos en el queso de Valdeón al inicio de la maduración (15 días) y posteriormente se redujeron progresivamente como consecuencia del aumento del pH, la proteólisis y el aumento de la materia grasa.

Se observó una reducción en los valores de luminosidad durante los primeros meses de maduración del queso de Valdeón como consecuencia del aumento del extracto seco. Este comportamiento también fue observado en otras variedades de queso (Aydemir & Dervisoglu, 2010; Fresno & Alvarez, 2012; Picon *et al.*, 2013). Los valores de a* descendieron a medida que avanzó la maduración, siendo este descenso especialmente pronunciado a partir del mes correspondiendo con el crecimiento y germinación de *P. roqueforti*, y por lo tanto, con la adquisición de una coloración más verde en el queso. Los valores de b* mostraron una ligera tendencia hacia coloraciones amarillentas con valores muy similares a los descritos para el queso Majorero por Fresno & Álvarez (2012) aunque inferiores a los señalados para otros quesos frescos o madurados por Aydemir & Dervisoglu (2010) y Magenis *et al.* (2014).

Por último, se sometió el queso de Valdeón a cata mediante un panel de catadores entrenados observándose que durante las primeras etapas de la maduración (30 días) se caracterizó por un sabor amargo, lo que se relacionó con el incremento de la actividad peptidasa observada durante estas etapas y que conllevó a una mayor acumulación de péptidos hidrofóbicos de pequeño tamaño molecular responsables del carácter amargo del queso (Lemieux & Simard, 1992). Los quesos de 60 y 90 días

resultaron ser muy similares entre sí y se caracterizaron por lo atributos de textura adhesividad y mantecosidad. Estos resultados estarían relacionados con la mayor extensión y profundidad de la proteólisis que se produjo en el queso a partir del mes de maduración, unido a unos porcentajes de humedad óptimos para el desarrollo de estas características. Las muestras de queso de 120 días de maduración se caracterizaron por un mayor número de atributos sensoriales. Resultaron ser quesos asociados a sabores picante y salado; aromas agrio y picante; gran persistencia tanto en boca como en nariz; y una textura granulosa. El aroma picante podría estar asociado al incremento de la lipólisis que sufre esta variedad durante la maduración dando lugar a una acumulación de altas concentraciones de ácidos grasos libres de menos de 10 carbonos, los cuales se caracterizan por su carácter volátil e irritante. Estos quesos se caracterizaron por una textura granulosa como consecuencia del aumento del extracto seco que se produjo con el tiempo de maduración. El incremento del aroma agrio al final de la maduración está relacionado con el incremento de las reacciones de descomposición de los aminoácidos en las fases finales de la maduración del queso, durante las que se genera ácidos de cadena corta volátiles a través de procesos oxidativos de aldehídos liberados durante la desaminación oxidativa de los aminoácidos (Hemme *et al.*, 1982). Por otra parte, los quesos de vena azul se caracterizan por presentar altas concentraciones de sal para poder facilitar la germinación y crecimiento de esporas de *P. roqueforti* (Guinee & Fox, 2004). Finalmente, los quesos que mejor valoración global obtuvieron por parte de los catadores fueron los correspondientes a los 90 días de maduración, estableciéndose esta etapa como la óptima para su consumo.

5.2. Influencia de la estación de elaboración en las características de la IGP queso de Valdeón

Otra parte de esta Tesis Doctoral se centró en el estudio y comparación de las características del queso de Valdeón elaborado en las diferentes estaciones del año con el fin de determinar el grado de estandarización del proceso productivo. Todos los parámetros físico-químicos estudiados estuvieron influenciados por la estación de elaboración, excepto el contenido en NaCl. Los quesos elaborados en verano presentaron las características físico-químicas que más difirieron del resto de estaciones.

En general, estos quesos se caracterizaron por presentar los valores más altos de sólidos totales, proteína, ratio sal/humedad y pH, así como valores más bajos de los ácidos D- y L-láctico y aw. Los lotes elaborados en primavera se caracterizaron por mostrar un comportamiento contrario, siendo invierno y otoño quesos con unas características intermedias.

La estación de elaboración repercutió sobre las características microbiológicas del queso afectando a los recuentos de todos los grupos microbianos estudiados, a excepción de PCAp, OGYEA y VRBGA. Los recuentos de mesófilos aerobios totales fueron inferiores en los lotes elaborados en verano y otoño como consecuencia de las condiciones físico-químicas que se instauraron en ellos con valores más elevados de sal/humedad y extracto seco y menores de aw. Por el contrario, los lotes elaborados en primavera mostraron los valores más elevados.

Pese a la utilización de cultivo iniciador, se observaron diferencias en los recuentos de bacterias ácido lácticas lo que se atribuyó a las características físico-químicas de los quesos. Sin embargo, se observó una gran homogeneidad en los recuentos en OGYEA debido a la utilización de *P. roqueforti* como cultivo iniciador secundario.

Fueron destacables las diferencias observadas en los recuentos en MSA y KAA ya que pueden afectar a la maduración dando lugar a diferencias en las características sensoriales finales. Los quesos elaborados en otoño mostraron los recuentos más elevados mientras que los fabricados en invierno y primavera los más bajos.

La extensión y profundidad de la proteólisis observada estuvo influenciada por la estación de elaboración del queso. Los lotes elaborados en otoño e invierno mostraron una mayor extensión de la misma con valores más elevados de NS-pH 4,6. Sin embargo, la profundidad de la proteólisis fue mayor en los lotes elaborados en verano y otoño, con valores más altos de NS-TCA expresado como porcentaje de NS-pH 4,6 y NS-PTA. Estos resultados coincidieron con la degradación de las caseínas, donde la mayor degradación se observó en los lotes de otoño y verano. Este comportamiento se debió, por un lado, a que los valores de pH y del ratio sal/humedad de los lotes de primavera se alejaron más que el resto al rango óptimo de actuación del

cuajo sobre la fracción αs_1-CN y, por otro, a que la actividad de la plasmina fue mayor en los lotes de verano y otoño, explicando de este modo la mayor degradación de la β-CN. Estas diferencias en el grado de proteólisis de los quesos se reflejaron también en el perfil de péptidos lo que permitió separar y clasificar los quesos en función de la estación de elaboración.

Como consecuencia de las diferencias observadas en la extensión y profundidad de la proteólisis, tanto el contenido total como individual de aminoácidos libres, a excepción de etanolamina (Eta), metionina (Met), cisteína (Cys) y triptófano (Trp), mostraron diferencias a lo largo del año. El análisis de componentes principales permitió separar las muestras en función de la estación de elaboración e identificar qué aminoácidos libres variaron más. Se observaron tres grupos. El primero de ellos estuvo integrado por los quesos elaborados en invierno que se caracterizaron por altos contenidos en Glu, Asp y Leu y menor concentración de Ala. Estos resultados coincidieron con los observados por Gaya *et al.* (2005) en queso Manchego. El segundo de ellos lo formaron los quesos elaborados en primavera que se caracterizaron por mayor contenido en glutamina (Gln) y Tyr. Por último, el tercer grupo lo compusieron los lotes elaborados en otoño y verano que se caracterizaron por presentar un mayor contenido en Ala que el resto de los lotes.

Sin embargo, no se observaron diferencias significativas en el contenido total en aminas biógenas pese a las diferencias observadas en el resto de índices de las proteólisis. Esta gran homogeneidad observada se debió a que las aminas biógenas mayoritarias en todas las estaciones fueron espermina y tiramina, y ninguna de ellas presentó diferencias en función de la estación de elaboración. Estos resultados coinciden con los descritos por Gaya *et al.* (2005). El resto de aminas biógenas estudiadas aunque mostraron diferencias su tendencia fue muy ambigua de acuerdo con Komprda *et al.* (2012).

La estación de elaboración también afectó a la lipólisis y, por lo tanto, al contenido de la mayoría de ácidos grasos libres estudiados, así como a la concentración final. Los quesos elaborados en verano presentaron el mayor contenido total, así como de la mayoría de los ácidos grasos libres, mientras que los quesos elaborados en inviernos mostraron los valores más bajos. Estos resultados coinciden con los

observados por Chávarri *et al.* (1999) en queso Idiazabal. Además, los quesos elaborados en verano presentaron valores significativamente más bajos de ácidos grasos de cadena corta y ácidos grasos de cadena media y valores más altos de ácidos grasos de cadena larga, todos ellos expresados como ratio sobre el contenido en ácidos grasos libres totales, coincidiendo con los resultados obtenidos por Fernández-García *et al.* (2006) en otra variedad de queso. El análisis de componentes principales permitió separar a los quesos en función de la estación de elaboración siendo los ácidos butírico, palmítico y oleico fueron los que mejor correlación presentaron.

En cuanto a las propiedades reológicas, la estación de elaboración repercutió sobre todos los parámetros reológicos estudiados, excepto sobre la Tan δ. Los quesos elaborados en otoño, seguidos por los de verano, mostraron valores más elevados de G', G'' y G* caracterizándose por lo tanto por presentar una disposición estructural más elástica con mejor capacidad para resistir la deformación (Xiong & Kinsella, 1991). Sin embargo, únicamente la dureza y la adhesividad mostraron diferencias significativas en el análisis del perfil de textura con los valores más elevados presentados por los lotes elaborados en otoño y los más bajos por los fabricados en invierno. Los valores más altos en todos estos parámetros observados en los lotes de otoño y verano se asociaron con su menor contenido en agua, mayor ratio sal/humedad y menor pH (Fresno & Álvarez, 2012) así como su mayor contenido en grasa (Watkinson *et al.*, 2001).

Por otro lado, los quesos elaborados en primavera se caracterizaron por valores más elevados de L* y más bajos de b* lo que se asoció con el mayor contenido en humedad que presentaron dando lugar a una mayor luminosidad y predominio de las tonalidades amarillentas. Los valores de a* fueron muy similares en todos los casos como resultado del crecimiento homogéneo de *P. roqueforti* a lo largo del año.

Por último, la estación de elaboración no tuvo influencia sobre ninguno de los atributos sensoriales estudiados, a excepción de los sabores amargo y dulce, observándose una gran homogeneidad en todas las puntuaciones otorgadas por los panelistas. Los quesos elaborados en verano mostraron las mayores diferencias en el sabor amargo respecto al resto de estaciones. Estos resultados se relacionaron con la mayor profundidad de la proteólisis observada en ellos que dio lugar a un menor ratio péptidos hidrofóbicos/hidrofílicos y, por lo tanto, a una menor percepción del sabor

amargo (Lemieux & Simard, 1992). Por el contrario, los lotes elaborados en invierno mostraron puntuaciones más bajas en cuanto al sabor dulce debido a las altas puntuaciones obtenidas en los sabores salado, astringente y ácido provocando una menor percepción del dulce. Por último, no se observaron diferencias en las puntuaciones globales entre las distintas estaciones.

5.3. Influencia del tratamiento térmico aplicado a la leche en las características de la IGP queso de Valdeón

Para determinar las diferencias en las características del queso de Valdeón elaborado con leche pasterizada y leche cruda, durante parte de esta Tesis Doctoral se llevó a cabo un estudio de caracterización en el que se compararon ambos tratamientos. En él se observó que los quesos elaborados con leche cruda se caracterizaron por presentar recuentos superiores en todos los grupos microbianos estudiados (excepto en MSA y OGYEA) debido a que las condiciones físico-químicas que se instauraron en ellos fueron más favorables que en los quesos elaborados con leche pasterizada, con valores más bajos en el ratio sal/humedad y más altos de aw. Los recuentos en PCAm, OGYEA y Rogosa en estos quesos fueron similares a los observados en el queso de Valdeón artesanal (López-Díaz *et al.*, 1995). En los quesos elaborados con leche cruda los recuentos en *Enterobacteriaceae* fueron elevados aunque similares a los observados en otras variedades similares como Cabrales (Flórez & Mayo, 2006; Flórez *et al.*, 2006), mientras que en los elaborados con leche pasterizada no fueron detectados debido al tratamiento térmico aplicado a la leche. Los recuentos en OGYEA fueron más altos en los quesos elaborados con leche pasterizada debido, por un lado, a que la utilización de cultivo iniciador durante su fabricación contribuyó a dirigir la maduración de los mismos, así como las interacciones con los distintos grupos microbianos, y por otra, a que el empleo de leche cruda facilitó la aparición de microbiota contaminante que puede afectar al crecimiento y esporulación de *P. roqueforti* (Cantor *et al.*, 2004).

Los quesos elaborados con leche cruda se caracterizaron por presentar valores más bajos de sólidos totales, lactosa y del ratio sal/humedad, así como, valores más elevados de ácido D-láctico y aw. Además, como consecuencia del menor crecimiento en estos quesos de mohos y levaduras y, por lo tanto, de una menor intensidad del

catabolismo del ácido láctico, los valores de pH fueron menores en los quesos fabricados con leche pasterizada.

Aunque en cualquiera de los dos tratamientos la proteólisis fue muy elevada, sí que se observaron diferencias en la extensión y profundidad de la misma entre ambos. Los quesos elaborados con leche pasterizada se caracterizaron por presentar una mayor extensión de la proteólisis con valores más elevados de NS-pH 4,6 y mayor degradación de las fracciones α_{s1}-CN, α_{s1}-I-CN y β-CN. Esto se debió al mayor crecimiento y esporulación de *P. roqueforti* observados en ellos, ya que *P. roqueforti* ha sido descrito como el principal agente proteolítico en los quesos de vena azul. Asimismo, la pasterización incrementa la actividad de la plasmina (Bastian & Brown, 1996) lo que dio lugar a una mayor actividad de la misma cuando la leche se pasterizó y, por lo tanto, mayor hidrólisis de la β-CN. Sin embargo, la profundidad de la proteólisis fue mayor en los quesos fabricados con leche cruda lo que dio lugar a valores más elevados de NS-TCA y NS-PTA y más bajos de N-polipeptídico, todos ellos expresados como porcentaje del NS-pH 4,6. Esta tendencia coincide con los resultados obtenidos en otras variedades de queso similares (Grappin & Beuvier, 1997). Además, el tratamiento térmico repercutió en el perfil peptídico observado en los quesos lo que permitió diferenciarlos en función de la leche utilizada en su elaboración. Aunque en todos los casos se observó un ratio elevado de péptidos hidrofílicos/hidrofóbicos, los quesos elaborados con leche cruda mostraron un ratio ligeramente superior.

Pese a las diferencias observadas tanto en la extensión como en la profundidad de la proteólisis entre ambos tratamientos, no se observaron diferencias en el contenido final de aminoácidos libres. Este hecho se atribuyó a la utilización de *P. roqueforti* como cultivo iniciador secundario dando lugar la estandarización del proceso productivo. Sin embargo, sí aparecieron diferencias significativas en el perfil de aminoácidos, siendo el contenido de asparagina (Asn), serina (Ser), glicina (Gly), ácido γ-aminobutírico + treonina (Gaba+Thr), ornitina (Orn), Asp y Tyr mayor en los quesos elaborados con leche pasterizada y el contenido en Eta, Cys y Ala menor. Cabe destacar las diferencias encontradas en la concentración de Asn ya que ésta puede ser utilizada como indicador de la pasterización debido a que el tratamiento térmico produce la inactivación de la asparaginasa permitiendo la acumulación de este aminoácido (Frau *et*

al., 1997). Además, la concentración de aminas biógenas en los quesos elaborados con leche cruda fue superior a la de los quesos elaborados con leche pasterizada coincidiendo con lo observado en otras variedades (Schneller *et al.*, 1997; Gaya *et al.*, 2005; Combarros-Fuertes *et al.*, 2016). Estos resultados se debieron, por un lado, a la reducción bacteriana producida por la pasterización y, por otro, a que el cofactor para la actividad descarboxilasa (piridoxal fosfato) es termolábil, por lo que la pasterización contribuye a reducir el contenido en aminas biógenas (Novella-Rodríguez *et al.*, 2003). Únicamente la espermina presentó concentraciones más altas en los quesos elaborados con leche pasterizada coincidiendo con los resultados obtenidos por Combarros-Fuertes *et al.* (2016) en queso Zamorano. Destaca la gran diferencia observada en el contenido en tiramina entre ambos tratamientos, siendo muy superior en el caso de los quesos elaborados con leche cruda. Estos resultados se asociaron a los mayores recuentos en KAA (Ladero *et al.*, 2012; Schirone *et al.*, 2012) y *Lactobacillus* (Valsamaki *et al.*, 2000; Schirone *et al.*, 2012; Combarros-Fuertes *et al.*, 2016) observados en estos quesos. El contenido en triptamina, histamina, cadaverina y putrescina también fue superior en los quesos elaborados con leche cruda. En este caso, el mayor contenido se asoció con los mayores recuentos de *Enterobacteriaceae* (Marino *et al.*, 2003).

Al igual que en el desarrollo de la proteólisis, el tratamiento térmico aplicado a la leche también influyó sobre la lipólisis y el contenido en ácidos grasos libres de los quesos. Al inicio de la maduración los quesos elaborados con leche pasterizada mostraron un contenido total más elevado, sin embargo, al final de la misma el contenido alcanzado en los quesos elaborados con leche cruda fue mucho mayor. Esta tendencia se asoció con la diferente actividad de las lipasas liberadas por *P. roqueforti* y su actividad para producir metil cetonas, a la inactivación de la lipasa nativa de la leche (lipoprotein lipasa) como resultado del tratamiento térmico y a los mayores recuentos alcanzados por las bacterias acido lácticas en los quesos elaborados con leche cruda (Collins *et al.*, 2003). Sin embargo, no se observaron diferencias significativas en los ratios ácidos grasos de cadena corta, media y larga expresados sobre el contenido en ácidos grasos totales lo que indicó que aunque el tratamiento térmico repercutió sobre el contenido total, no influyó en el perfil de ácidos grasos libres. El análisis de componentes principales permitió determinar que los ácidos oleico, palmítico y mirístico fueron los que más variaron en función de la leche de elaboración de los

quesos. Con excepción de los 30 días de maduración, los quesos elaborados con leche cruda se caracterizaron por tener mayor concentración de estos tres ácidos grasos.

El estudio de las propiedades sensoriales del queso, tanto instrumental como sensorial, reveló diferencias entre los quesos elaborados con leche pasterizada y cruda. Los primeros se caracterizaron por presentar valores más elevados de G', G'', G*, dureza y fracturabilidad, lo que se tradujo en una disposición estructural más elástica con mejor capacidad para resistir la deformación (Xiong & Kinsella, 1991) así como una matriz más firme. Este comportamiento se debió al menor contenido en agua mostrado por estos quesos (Dimitreli & Thomareis, 2008; Juan *et al.*, 2007). Al mismo tiempo, los valores de cohesividad y elasticidad fueron significativamente más bajos en los quesos elaborados con leche pasterizada lo que se relacionó con el mayor contenido en grasa y menor contenido en agua (Creamer & Olson, 1982; Gwartney *et al.*, 2002; Fresno & Álvarez, 2012).

Por otro lado, los lotes elaborados con leche pasterizada se caracterizaron por presentar valores significativamente más bajos tanto de L*, como de a* y b* debido al mayor contenido en sólidos totales y mayor crecimiento de *P. roqueforti* que provocaron una menor luminosidad, mayor predominio de las coloraciones verdes y menor de las coloraciones amarillentas.

Para finalizar, el tratamiento térmico aplicado a la leche de elaboración influyó sobre algunos de los atributos sensoriales evaluados. Los quesos elaborados con leche pasterizada mostraron mayores puntuaciones en el atributo venas azul-verdosas coincidiendo con los resultados obtenidos en el análisis instrumental del color y con el mayor crecimiento de *P. roqueforti* en estos quesos. En el caso del sabor, los quesos elaborados con leche cruda mostraron puntuaciones más bajas para los atributos amargor y astringencia y más elevados en el caso del sabor dulce. El mayor amargor percibido por los panelistas para el atributo amargor se debió a la acumulación de péptidos hidrofóbicos responsables del mismo (Lemieux & Simard, 1992) que, como se vio en el estudio del perfil de péptidos, fue mayor en estos quesos. Este hecho, además, dio lugar a una menor percepción del sabor dulce. En cuanto al olor, los quesos elaborados con leche cruda presentaron puntuaciones más elevadas para el atributo agrio debido a la mayor acumulación de ácidos volátiles de cadena corta liberados

durante la desaminación oxidativa de los aminoácidos (Hemme *et al.*, 1982) dado que la profundidad de la proteólisis fue mayor en estos quesos. En el caso de la textura, coincidiendo con el análisis oscilatorio dinámico y de perfil de textura, los quesos elaborados con leche pasterizada recibieron puntuaciones más elevada para el atributo firmeza. Por último, no se observaron diferencias significativas en cuanto a las puntuaciones globales otorgadas por los panelistas aunque los quesos elaborados con leche cruda recibieron puntuaciones ligeramente superiores.

5.4. Presencia de péptidos bioactivos en la IGP queso de Valdeón antes y después de la simulación de digestión gastrointestinal

Finalmente, la última parte de la presente Memoria de Tesis Doctoral se centró en el estudio del perfil de péptidos del queso de Valdeón antes y tras simulación de la digestión gastrointestinal. Además, los digeridos obtenidos fueron comparados con leche desnatada en polvo pasterizada digerida bajo las mismas condiciones. Se observó que la extensa proteólisis que tiene lugar en el queso de Valdeón dio lugar a una gran cantidad de péptidos originados principalmente de las fracciones caseínicas más abundantes (β- y αs_1-caseína, de vaca y/o cabra) aunque algunos pocos derivaron de la αs_2- y κ-caseína.

La secuencia β-caseína mostró un patrón distribuido de péptidos. Al igual que en otras variedades, la región N-terminal (donde se localizan las serinas fosforiladas) fue fuente de una gran cantidad de péptidos fosforilados. De estos, cuatro de ellos surgieron del punto de escisión 29-30, característico de la acción de la aspartil proteasa secretada por *P. roqueforti* (Le Bars & Gripon, 1981). Algunos de los péptidos identificados derivados de la β-caseína incluyeron secuencias biológicamente activas como péptidos opioides (f60-66; f114-119) (Jinsmaa & Yoshikawa, 1999) o inhibidores de la enzima convertidora de angiotensina I (ACE) (f47-52) (Gómez-Ruiz *et al.*, 2004; Gómez-Ruiz *et al.*, 2006).

La secuencia αs_1-caseína mostró un patrón menos distribuido que la β-caseína. La región comprendida entre el aminoácido 83 al 91 fue fuente de numerosos péptidos coincidiendo con lo observado en queso Manchego (Gómez-Ruiz *et al.*, 2004). De entre

los péptidos identificados, destacó la secuencia f157-164 cuya actividad inhibitoria de la ACE ha sido encontrada en caseína fermentada con diferentes cultivos iniciadores acidolácticos, y tras hidrólisis con pepsina y tripsina (Pihlanto-Leppala *et al.*, 1998).

Con el objetivo de determinar el efecto de la digestión gastrointestinal en el perfil de péptidos y en su actividad biológica, se llevó a cabo una digestión gastrointestinal *in vitro* del queso. No se observaron grandes diferencias en el número de péptidos encontrados aunque sí que existieron diferencias en el perfil. Únicamente 28 de los péptidos encontrados tras la digestión aparecían también antes de ella lo que mostró su resistencia a las enzimas gastrointestinales. Algunos de ellos han sido encontrados tras simulación de la digestión de diferentes productos lácteos (Boutrou *et al.*, 2013), mientras que otros únicamente se han observado en queso lo que podría indicar que se han generado durante el proceso fermentativo (Gómez-Ruiz *et al.*, 2004; Qureshi *et al.*, 2013; Sadat-Mekmene *et al.*, 2013). Algunos de estos péptidos capaces de resistir la digestión son compuestos biológicamente activos como es el caso del péptido inhibidor de la ACE (f157-164 DAYPASGAW) (Yvon & Pelissier, 1987).

Además, durante la digestión se formaron nuevos péptidos. Un ejemplo de nueva secuencia generada es la β-caseína f133-138 (LHPLPLP), un potente péptido antihipertensivo (Quirós *et al.*, 2007), cuyo péptido precursor f128-138 está presente en el queso antes de la simulación de la digestión. Por lo tanto, es un aspecto muy interesante que la forma activa de un péptido antihipertensivo que produce un descenso en la presión sanguínea de ratas hipertensas espontáneas se libere durante la simulación de la digestión del queso. Otro péptido liberado durante la digestión, β-caseína f132-140, NLHLPLPLL, ha sido previamente descrito como inhibidor de la ACE (Robert *et al.*, 2004). Del mismo modo, la β-caseína f108-113, EMPFPK, a la que se le ha atribuido actividad inhibitoria de la ACE (Pihlanto-Leppala *et al.*, 1998), únicamente se encontró tras la digestión. A partir de la αs_1-caseína, se generaron durante la digestión gastrointestinal los péptidos f144-149 (AYFYPEL) y f143-149 (YFYPEL). Ambos ejercen actividad antioxidante (Hernández-Ledesma *et al.*, 2002) y actividad secretora de mucina en células HT29-MTX (Martínez-Maqueda *et al.*, 2013) . Asimismo, el segundo de ellos, ha mostrado una potente actividad antihipertensiva (Contreras *et al.*, 2009). La κ-caseína también dio lugar a la liberación de nuevos péptidos como la

fracción f18-24 que muestra actividad inhibitoria de la ACE y actividad antibacteriana (López-Expósito *et al.*, 2006).

5.5. Referencias

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6. CONCLUSIONES
CONCLUSIONS

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PRIMERA. Las bacterias ácido lácticas, especialmente lactococos y *Leuconostoc*, fueron el grupo microbiano predominante al inicio de la maduración siendo gradualmente reemplazado por los mohos y levaduras. Las micrococáceas y los lactobacilos mostraron altos recuentos al final de la maduración, mientras que los recuentos de enterococos y *Enterobacteriaceae* fueron muy bajos o, incluso, no detectados.

SEGUNDA. Las características fisico-químicas del queso de Valdeón fueron muy similares a las observadas en otros quesos de vena azul europeos, aunque los valores de pH fueron ligeramente superiores.

TERCERA. El queso de Valdeón mostró una gran extensión y profundidad de la proteólisis a lo largo de la maduración lo que dio lugar a unos altos valores de las fracciones nitrogenadas, a la casi total degradación de las αs_1- y β-caseínas, a la aparición de considerables diferencias, tanto cualitativas como cuantitativas, en el perfil de péptidos, así como, al incremento de los aminoácidos y aminas biógenas. Al final de la maduración, los aminoácidos mayoritarios fueron Glu, Ala, Pro, Tyr, Lys, Asp, Leu y Phe, mientras que las aminas biógenas más abundantes fueron espermina y tiramina.

CUARTA. El contenido en ácidos grasos del queso de Valdeón se vio influenciado por el tiempo de maduración observándose su aumento como resultado de la gran lipólisis que tuvo lugar, alcanzando valores finales similares a otras variedades de queso azul. Los ácidos grasos mayoritarios al final de la misma fueron el ácido oleico y palmítico.

QUINTA. El queso de Valdeón se clasificó como un sólido viscoelástico ya que presentó una estructura más elástica a medida que avanza la maduración, así como mayor resistencia a la deformación. Además, los valores más elevados de dureza, fracturabilidad, gomosidad y masticabilidad coincidieron con las dos semanas de maduración, a partir de las cuales descendieron paulatinamente hasta el final de la misma.

SEXTA. Esta variedad se caracterizó por un descenso en la luminosidad (L*) en los primeros dos meses de maduración. Los valores de a* igualmente disminuyeron, aunque el descenso más pronunciado se produjo a los 30 días coincidiendo con el crecimiento y esporulación de *P. roqueforti* y, por lo tanto, con la adquisición de coloraciones verdes. Por su parte, los valores de b* fueron indicativos de una ligera coloración amarillenta.

SEPTIMA. Las atributos sensoriales que más influyeron y que, por lo tanto, caracterizaron esta variedad fueron los sabores salado y picante; los olores picante y agrio; los atributos de textura adhesividad y granulosidad; y la persistencia. De acuerdo al panel de catadores, el tiempo considerado óptimo para el consumo del queso de Valdeón fueron los 90 días de maduración.

OCTAVA. La estación de elaboración influyó en las características microbiológicas y fisico-químicas del queso de Valdeón, así como en la proteólisis y lipólisis. En general, las mayores diferencias se observaron entre los quesos fabricados en otoño y verano con respecto a los elaborados en invierno y primavera. Sin embargo, estas diferencias apenas repercutieron sobre las características de textura y sensoriales del queso. Además, no fueron detectadas diferencias significativas en la valoración global de los quesos por el panel de catadores mostrando una gran homogeneidad desde el punto de vista sensorial entre todos ellos.

NOVENA. La utilización de leche cruda durante la elaboración del queso de Valdeón influyó sobre la mayoría de los parámetros microbiológicos y fisico-químicos. Estos cambios se tradujeron en una menor extensión y mayor profundidad de la proteólisis, un mayor grado de la lipólisis, así como mayores puntuaciones en el análisis sensorial debido a la menor dureza y fracturabilidad, así como menor amargor y mayor mantecosidad. Sin embargo, el uso de leche cruda implicaría, en primer lugar, maximizar las condiciones higiénicas del proceso y, en segundo, utilizar un cultivo iniciador mesófilo junto con *P. roqueforti*. De esta manera, se conseguiría un mejor control del proceso fermentativo reduciendo los recuentos de la microbiota alterante y facilitando el óptimo crecimiento del moho *P. roqueforti*, contribuyendo a la adquisición de unas características sensoriales únicas y específicas con respecto a otras variedades de quesos de vena azul.

DÉCIMA. El perfil de péptidos de queso de Valdeón mostró la presencia de algunos de ellos con actividad opioide e inhibidora de la enzima convertidora de angiotensina I. Tras la simulación de la digestión gastrointestinal se observó un mayor número de péptidos bioactivos, incluyendo péptidos antihipertensivos, antioxidantes, secretores de mucina intestinal y antibacterianos. Además, el perfil peptidómico del queso de Valdeón reveló varias regiones que son especialmente resistentes a la digestión gastrointestinal en leche en polvo desnatada y en queso, algunas de las cuales son regiones bien conservadas en las proteínas lácteas de distintas especies y poseen actividad biológica previamente descrita.

CONCLUSIONS

FIRST. Lactic acid bacteria, mainly lactococci and *Leuconostoc*, were the predominant microbial groups during the early stages of ripening, gradually being replaced by moulds and yeast. Micrococci and lactobacilli showed high counts at the end of ripening, while enterococci and *Enterobacteriaceae* levels were very low or zero.

SECOND. The physic-chemical characteristics of Valdeón cheese were very similar to those observed in other European blue-veined cheeses; however, the pH values were slightly higher.

THIRD. Valdeón cheese showed a great extent and profound of proteolysis during ripening which resulted in high nitrogenous fractions values, the almost complete degradation of αs1- and β-caseins, the presence of considerable, qualitative and quantitative, differences between peptide profiles, as well as, the increase in free amino acids and biogenic amines contents. At the end of ripening the major amino acids were Glu, Ala, Pro, Tyr, Lys, Asp, Leu and Phe, while the major biogenic amines were espermine and tyramine.

FOURTH. Free fatty acids content of Valdeón cheese was influenced by ripening time showing their increase as result of the great lipolysis that occurred, reaching final values similar than other blue-veined varieties. The major free fatty acids at the end of ripening were oleic and palmitic acids.

FIFTH. Valdeón cheese was classified as a viscoelastic solid which presents a more elastic structure as ripening progresses, as well as, an increase in the resistance to deformation. In addition, the highest values for hardness, fracturability, gumminess and chewiness were attained during the first two weeks of ripening and subsequently decreased until the end of ripening process.

SIXTH. This variety was characterized by a reduction in lightness (L*) in the first two months of ripening. Values for a* decreased during ripening, although this reduction was more pronounced at 30 days, coinciding with the germination and growth of *P. roqueforti* and therefore with the acquisition of a greenish colour. Meanwhile, values for b* indicated a slightly yellowish colouration.

SEVENTH. Sensory attributes that most influenced and that, therefore, characterized this variety were saltiness and pungency, pungent and sour odours, adhesiveness and granularity, and taste strength. According to the panel of tasters, 90 days was considered the optimum ripening period for the consumption of Valdeón cheese.

EIGHTH. Season of manufacture influenced the microbial and physic-chemical characteristics of Valdeón cheese, as well as, proteolysis and lipolysis. In general, the highest differences appeared between cheeses elaborated in autumn and summer with respect to cheeses made in winter and spring. However, textural and sensory characteristics were just slightly affected by these differences. In addition, no significant differences were observed in overall scores and, therefore, from a sensory point of view, cheeses were found to be very similar.

NINTH. The use of raw milk in Valdeón cheese manufacturing resulted in significant changes in most of the microbiological and physico-chemical parameters studied. These changes produced lower extent and higher profound of proteolysis, a high lipolysis, as well as, higher scores in sensory analysis overall due to the lower hardness and fracturability, as well as, lower bitterness and higher buttery. However, the use of raw milk in Valdeón cheese implies, firstly, to maximize the hygienic conditions during production and, on the other hand, to use a mesophilic starter culture together with *P. roqueforti*. Thereby, it would be obtained a better control of fermentation process decreasing the contaminant microorganism counts and promoting the optimal growth of *P. roqueforti*, and, in the end, contributing to develop unique and specific sensory characteristics regarding to other blue-veined varieties.

TENTH. The peptide profile of Valdeón cheese showed the presence of some opioid and angiotensin I-converting enzyme inhibitors peptides. After gastrointestinal digestion simulation, a higher number of bioactive peptides, including antihypertensive, antioxidant, intestinal mucin-secretor, and antibacterial were found. On the other hand, the peptidomic profiling of digests reveals several regions that are especially resistant to gastrointestinal digestion in skimmed milk powder and cheese, some of them are well conserved between species in dairy proteins and have biological activity previously reported.