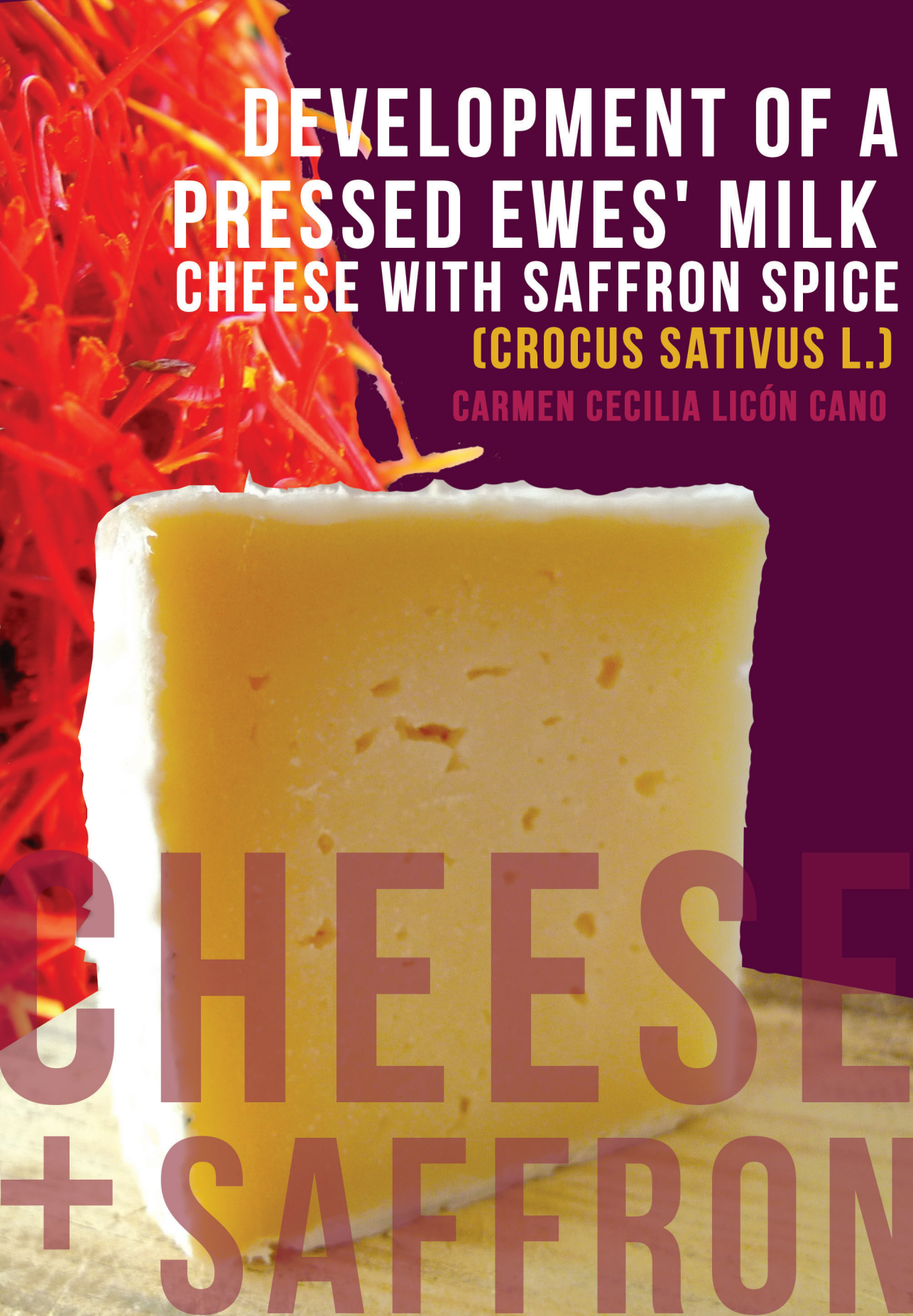
DEVELOPMENT OF A PRESSED EWES' MILK CHEESE WITH SAFFRON SPICE (CROCUS SATIVUS L.)

CARMEN CECILIA LICÓN CANO



UNIVERSIDAD DE CASTILLA-LA MANCHA

ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRÓNOMOS

Departamento de Ciencia y Tecnología Agroforestal y Genética

DOCTORAL THESIS

Development of pressed ewes' milk cheese with saffron spice (*Crocus sativus* L.)

Desarrollo de un queso de leche de oveja con azafrán especia (*Crocus sativus* L.)

Carmen Cecilia Licón Cano

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A dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Memoria presentada como requerimiento para optar al grado de Doctor (Doctorado Internacional)

by

Carmen Cecilia Licón Cano

Advisors: Dra. María Isabel Berruga Fernández
Dr. Manuel Carmona Delgado

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SUMMARY

In Spain, the industry of ewes' milk cheese has a long-established tradition, however, is not competitive due to lack of differentiation among cheese varieties. The objective of this doctoral thesis was to develop a pressed ewes' milk cheese with saffron spice, a product worldwide appreciated for its color, flavor, aroma and healthy benefits, ready to be introduced into the market. This work integrated these two traditional products as an alternative to diversify ewes' milk cheese varieties and increase competitiveness.

To achieve this objective, saffron doses that could promote healthy benefits were determined. Saffron color extraction process in milk and its addition to cheesemaking were standardized, and the potential influence of saffron addition on cheesemaking process was also studied. Color was the parameter more influenced by saffron, showing changes with increasing saffron concentration, ripening time and air exposure, reaching a yellower coloration in cheese.

Finally a new method to isolate, identify and quantify volatiles by headspace sorptive extraction/gas chromatography/mass spectrometry was developed to follow saffron aroma during cheesemaking and in cheeses, and to evaluate their volatile fraction during cheese ripening. Saffron aroma, in terms of safranal, was detected in cheeses changing its volatile fingerprint. Sensory analysis showed that panelists detected color and flavor differences between cheeses with increasing saffron content, showing a preference for cheeses were saffron taste was easily detectable but not predominant and integrated with the ewes' milk cheese characteristic flavor.

As a final result of this doctoral thesis saffron cheeses were developed and due to the involvement of the industry they are currently fabricated and commercialized.

RESUMEN

En España, la industria del queso de leche de oveja tiene una larga tradición, sin embargo no es competitiva debido a la poca variedad de quesos. El objetivo de esta tesis doctoral fue desarrollar un queso de pasta prensada de leche de oveja con azafrán, producto reconocido mundialmente por su color, sabor, aroma y beneficios saludables, listo para ser introducido en el mercado. Este trabajo integra estos dos productos tradicionales como alternativa a la escasa diversidad de los quesos de oveja y poder así incrementar su competitividad.

Para lograr este objetivo se determinaron las dosis de azafrán que pudieran promover beneficios saludables en el consumidor. Se estandarizó el proceso de extracción del color del azafrán en la leche, así como el momento de adición al proceso productivo del queso. También se estudió la posible influencia del azafrán durante el proceso de fabricación. El color fue el parámetro más influenciado por la adición de azafrán mostrando cambios al incrementar la concentración de la especia, al avanzar la maduración y la exposición al aire, observándose quesos con coloraciones más amarillas.

Finalmente se desarrolló un nuevo método para extraer, identificar y cuantificar volátiles mediante espacio de cabeza/cromatografía de gases/espectrometría de masas para el seguimiento del aroma del azafrán durante el proceso productivo y en el producto final, y la evaluación de su fracción volátil durante la maduración. Se detectó la transferencia del aroma del azafrán al queso, en términos de safranal, observándose modificaciones en su perfil aromático. El análisis sensorial demostró que los panelistas detectaron diferencias en el color y sabor de quesos con concentraciones crecientes de azafrán, y mostraron una preferencia hacia quesos en los cuales el sabor de azafrán se detectaba pero no era predominante de manera que se integraba en el sabor característico del queso de oveja.

Como resultado final de esta tesis doctoral se desarrolló un queso con azafrán, y gracias a la implicación de la industria actualmente se produce y comercializa.

CHAPTER 1. JUSTIFICATION

In the Mediterranean basin, ewes' milk and its derivates, especially cheese, are products with a strong traditional background. In Europe during last ten years, production of ewes' milk cheeses has been increasing an average of 2,307 tons/year; nevertheless this increment is not general for all Mediterranean countries: France and Greece has been decreasing its production while Italy and Spain has increased. Moreover, different countries, for example, China and Syria, have been increasing its production in amounts comparable to the increments in all Europe (FAO, 2012). Nowadays, Greece is the country with the first place on producing ewes' milk cheeses, having a wide variety of cheeses, however, in countries like Italy or Spain, most of these cheeses are very similar even between regions: semi-hard or hard, pressed and consumed within 2 to 6 months of ripening. Ewes' milk cheeses are mostly produced on a small local scale and compared with cows' milk cheese industry they are not as competitive. This lack of differentiation makes difficult competition and has generated the need for diversification of the products. One alternative for diversification is to include traditional spices during cheesemaking process, for example saffron, which is also a long-established product in Mediterranean area.

Saffron spice (*Crocus sativus* L.) is the most valuable traditional spices in the world giving not only color to food but also taste and aroma. Castilla-La Mancha saffron is worldwide known for its extraordinary quality. In this region of Spain, in 1999 the first Protected Designation of Origin (PDO) for saffron was created under the name "Azafrán de la Mancha". This PDO covers provinces of Toledo, Cuenca, Ciudad Real and Albacete, the latter highlights for its production quantity (Consejo Regulador Azafrán Mancha, 1999; Mancha, 2008). The special feature about this spice is that flowers only grow up between middle October and beginning of November and traditionally they have to be collected by hand. After, the stigmas are separated from the rest of the flower parts also by hand, increasing the final price of the spice. In 1990, 21,789 kg of saffron were produced in Spain, but from then to year 2005 production started to drastically decrease reaching its lowest

production rate, 820 kg, afterwards it started to grow again and in 2009 reached a production of 1,829 kg. Besides this drop of production, saffron value has been constantly increasing since 1994 to 2011 from 490 to 3,000 euros/kg, respectively (Mancha, 2008; MAGRAMA, 2010). During the last years in Spain, many efforts have been made for promoting saffron consumption in order to recover tradition and its use in cooking.

Saffron has been used as an ingredient in dairy products. The most known among them is an Italian ewes' milk cheese made in Sicily called Piacentinu Ennese, which recently obtained its PDO. However, and despite it has been produced since Roman times, saffron addition to cheesemaking process had not been deeply studied or standardized, and lacks information about attributes provided by saffron to cheese.

This thesis focuses on the standardization of saffron addition during cheesemaking and the characterization of the saffron cheeses. The importance of this work lies in the innovative concept to fit traditional products to modern times by introducing saffron into the ewes' dairy industry which will lead to recover consumption of these two traditional products and cheese differentiation. This knowledge is not only of scientific interest, also the industrial sector, particularly two local small-scale industries in Castilla-La Mancha: FOMAN and Quesería Campo Rus, required the findings for the production of saffron pressed ewes' milk cheese with a standardize process.

CHAPTER 2. OBJECTIVES

The objective of this doctoral thesis was to develop a pressed ewes' milk cheese with saffron, ready to be introduced into the market. In order to achieve this objective it was necessary:

1. To review different bibliographical sources to determine saffron doses normally used on different dishes which could promote healthy effects.
2. To establish the best approach to introduce saffron into cheesemaking based on color extraction in ewes' milk.
3. To study influence of saffron addition during cheesemaking.
4. To establish main physico-chemical and microbiological characteristics of pressed ewes' milk cheeses with saffron.
5. To determine consumer acceptance of the saffron cheeses.
6. To determine color and aroma distribution of saffron in different dairy fractions: cheese, whey, "requesón" and "requesón" whey for which, it was necessary to optimize a specific methodology to analize aroma in cheese.
7. To study the influence of saffron addition in the volatile fraction of cheeses by means of the methodology developed.

OBJETIVOS

El objetivo principal de esta tesis doctoral fue el de desarrollar un queso de leche de oveja de pasta prensada con azafrán listo para ser introducido al mercado. Para lograr este objetivo fue necesario:

1. Revisar diversas fuentes bibliográficas para determinar la cantidad de azafrán que se adiciona normalmente en distintos alimentos y establecer si a esas concentraciones podrían tener un efecto positivo en la salud.
2. Establecer la mejor manera de introducir el azafrán en el proceso de fabricación del queso basado en la extracción de color de azafrán en leche de oveja.
3. Estudiar la influencia de la adición de azafrán en el proceso productivo del queso.
4. Establecer las principales características físico-químicas y microbiológicas del queso con azafrán.
5. Estudiar la aceptación por parte del consumidor de los quesos con azafrán.
6. Determinar el reparto de color y aroma del azafrán en las distintas fracciones lácteas: queso, suero, requesón y suero de requesón; para lo cual fue necesario poner a punto una metodología específica para el análisis de aromas en queso.
7. Estudiar la influencia del azafrán en la fracción volátil de los quesos mediante la metodología desarrollada.

CHAPTER 3. INTRODUCTION

3.1 Ewes' milk

Ewes' milk production is very important in the Mediterranean basin especially for cheese fabrication. Since old ages it has been used because of its nutritional aspects and its better cheese yield compared to cows' or goats' milk. However, in many countries large scale industrialization of the ewes' milk is limited by low volume and season cyclicity of individual milk production (Park *et al.*, 2007). Most of ewes' milk production is intended to cheese fabrication and only a minor part to curd, yogurt or whey cheeses.

In 2010 ewes' milk production in the world was 10,046,507 tons. The first ten producers are shown in Figure 1. These countries produced 73 % of the total production of the world. Spain is the eight producer in the world and the fourth European producer of this type of milk (FAO, 2012).

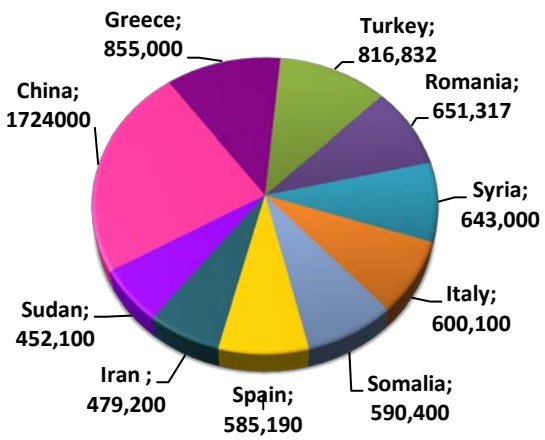


Figure 1. Top ten countries of ewes' milk production (tons) in 2010 (FAO, 2012)

In Spain, Castilla y León and Castilla-La Mancha are the regions which produced more than 90 % of the Spanish production (Figure 2), in which the latter produced the 23 % of the total (MAGRAMA, 2010).

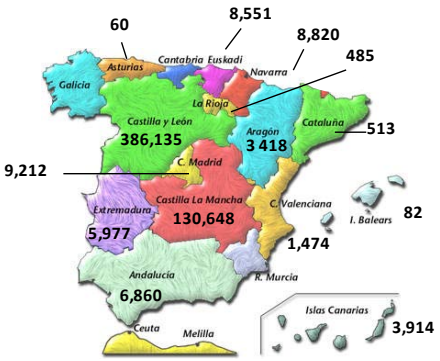


Figure 2. Ewes' milk production in Spain regions (thousand of liters) in 2010 (MAGRAMA, 2012)

3.1.1 Composition

Ewes´, goats´ and cows´ milk have differences on some physico-chemical characteristics, tables 1 and 2 show some physical properties and an average composition of these three types of milk. In Table 1, it can be observed that ewes' milk has higher density, viscosity, titratable acidity and lower refractive index than cows' and goats' milk. Surface tension, freezing point and pH are within the range of cows' milk and viscosity of ewes' milk is much higher than the rest because of its higher fat and protein content (Table 1).

Ewes' milk caseins are richer in calcium and shows better coagulation properties because ß/α_s-casein ratio is higher and coagulation proceeds faster (Park, 2007). Ewes' milk almost doubles the whey protein content, an advantage for whey derivates production among other milk types (Table 2). Immunoglobulins, lactoferrin, transferrin and ferritin are also present in ewes' milk contributing to a

better resistant to microorganisms' growth (Molina *et al.*, 2009). In recent years, ewes' milk proteins have become an important source of some bioactive peptides with properties such as blood pressure regulation, antimicrobial, antithrombotic, antitumoral and antioxidant contributing to a good nutritional value (Park *et al.*, 2007; Recio and López-Expósito, 2008; Molina *et al.*, 2009; Corrêa *et al.*, 2011).

Table 1. Mean values of physical properties of cows', goats' and ewes' milk

Properties	Cow	Goat	Ewe
Specific gravity (density)	1.031	1.034	1.036
Viscosity, C_p	2.00	2.12	3.40
Surface tension (Dynes/cm)	47.2	52.0	46.8
Conductivity (1/Ω*cm)	0.004	0.009	0.004
Refractive index	1.451	1.450	1.349
Freezing point (- °C)	0.55	0.57	0.55
Acidity (lactic acid %)	0.16	0.18	0.23
pH	6.68	6.65	6.68

(Park *et al.*, 2007)

Regarding lipidic fraction, fat globule size of ewes' milk is smaller than in cows' milk (3.30 *vs* 4.55 μm), which is an advantage for digestibility and a more efficient lipid metabolism. Structure and composition of the fat globule membrane is similar in the three species and represents approximately 1 % of total milk fat volume. Levels of short and medium chain fatty acids are significantly higher than in cows' milk, among them capric (C10:00) and caprylic (C8:00) acids are responsible for the characteristic smell and taste of the ewes' and goats' milk and the flavor of the cheeses produced from these milks. Also ewes' milk has the highest quantity of mono unsaturated trans fatty acids, which are associated with risk of coronary heart disease but the higher content of conjugated linoleic acid concentration makes also biological properties of this milk important (Park *et al.*, 2007; Molina *et al.*, 2009).

Lactose is the major carbohydrate present but there are also oligosaccharides, glycopeptides, glycoproteins and nucleotide sugars, unless their functions in ewes' milk have been little studied as well (Park *et al.*, 2007).

Minor fraction of ewes' milk includes minerals and vitamins (1 %) which have not been deeply studied. However it is known that calcium per casein weight is higher than in cows' and goats' milk (Molina *et al.*, 2009).

Table 2. Average composition of cows', goats' and ewes' milk

Composition	Cow	Goat	Ewe
Fat (%)	3.6	3.8	7.9
Non-fat solids (%)	9.0	8.9	12.0
Lactose (%)	4.7	4.1	4.9
Protein (%)	3.2	3.4	6.2
Casein (%)	2.6	2.4	4.2
Albumin, globulin (%)	0.6	0.6	1.0
Non-protein N (%)	0.2	0.4	0.8
Ash (%)	0.7	0.8	0.9
Calories/100 ml	69	70	105

(Park *et al.*, 2007)

One important aspect of milk is that its composition and physico-chemical properties change depending on many factors such as breed, feeding, climate conditions, animal age, animal handling, milking system and lactation stage (Desarzens *et al.*, 1983; Pulina and Nudda, 2002; Coulon *et al.*, 2004). In the particularly case of ewes' milk, lactation stage modifies fat and protein content in a wider range than other milk types. Its fat concentration increases from 4 to 10 % and protein from 5 to 6 % as observed in Figure 3. (Molina et al., 2009). This factor is very important in cheese fabrication because it makes difficult to have a constant level of fat and protein. In big industries, standardization process is a common practice used to solve this problem but in the artisanal sector or small dairies is not

often practiced. Variations in milk composition have also consequences on the sensory properties of milk, especially color, and thus in the final characteristics of the dairy derivates.

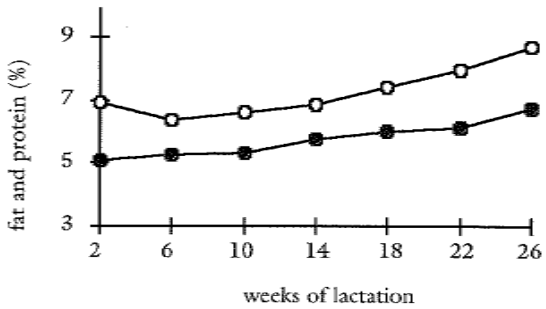


Figure 3. Ewes' milk changes on protein (•) and milk fat (o) content during lactation (Pulina and Nudda, 2002)

Diet of the animals may cause variations on the size or composition of fat and caseins, as well as changes on carotene and riboflavin content. These changes will be reflected on milk color. Milk color is giving mainly by fat and casein which are capable of scatter light. But carotene and riboflavin are also capable of absorb light at several wavelengths in the visible region: ß-carotene absorbs light near 460 nm, while riboflavin absorbs strongly near 470 nm giving to milk yellow and green colorations, respectively (Robinson and Wilvey, 1998; McCarthy and Singh, 2009)

3.2 Ewes' milk cheeses

The production of ewes' milk cheese in the world in 2010 was 692,950 tons, having the second place after cows' milk cheeses. Greece, China, Italy, Syria, France, Spain and Turkey are the principal producers comprising almost 70 % of the world production as observed in Figure 4 (FAO, 2012). These cheeses are produced with a great variety and diverse characteristics; there are fresh, semi-hard, hard, brine ripened, blue-veined cheeses, or in some cases almost liquid.

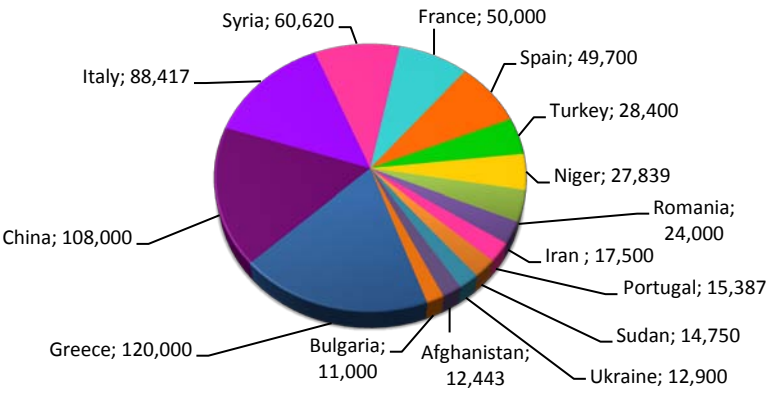


Figure 4. Country production (tons) of ewes' milk cheeses in 2010 (FAO, 2012)

A great part of ewes' milk cheeses, especially in Spain and Italy are made with animal rennet (calf or lamb), uncooked, molded, pressed and ripened for more than 90 days as shown in Figure 5. Many of them belong to a Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI), for example: Pecorino Romano, Fiore Sardo, Zamorano, Roncal, Idiazabal or Manchego (Harbutt, 2010).

The cheeses mentioned have generally a high dry matter content so most of them can be classified as semi-hard or hard cheeses (McSweeney *et al.*, 2004). Its dry matter content varies between 45 and 55 % after manufacturing and during ripening increases as a consequence of water loss, reaching values between 63 and 69 % after six months (Barron *et al.*, 2005b; Pirisi *et al.*, 2011). This water loss will depend on the humidity and temperature of the maturation chamber and on cheese size and shape (Robinson and Wilvey, 1998; Walstra *et al.*, 2001). Fat concentration by dry matter content changes from around 50 % after manufacturing to around 56 % after six months of ripening. Protein by dry matter content ranges between 24 to 36 % (Barron *et al.*, 2005b; Cabezas *et al.*, 2007). pH values after manufacture are normally around 5.2 but can vary between 5.0 and 6.0

during ripening. Values reported for salt by dry matter content ranges from 1.0 to 3.0 % (Fernández-García *et al.*, 2006; Cabezas *et al.*, 2007; Pirisi *et al.*, 2011).

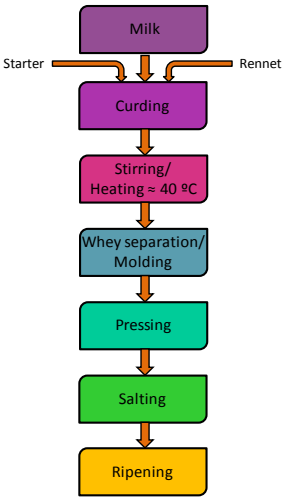


Figure 5. General cheesemaking process for pressed ewes' milk cheeses

3.2.1 Cheese ripening

Cheese ripening is one of the processes that determine the final characteristics of each cheese type. It depends on residual rennet, cheesemaking process, humidity of the curds, temperature and relative humidity in the maturation chamber, time and microorganisms present. During this period a number of biochemical reactions occur divided in three different pathways: glycolysis, lipolysis and proteolysis. They are essential for cheese texture, taste and especially aroma development (Robinson and Wilvey, 1998).

3.2.1.1 Glycolysis

Glycolysis occurs by the microorganisms present in the cheese curd or in the surface, which metabolize lactose to lactic acid and to L- and/or D-lactate and citrate to diacetyl, acetate, acetoin and carbon dioxide. Transformation of lactose to lactic acid occurs mainly in the cheese vat and during pressing which promotes pH decrease to values around 5.2. Lactic acid can be further metabolized depending on the microorganisms present to form lactate, butyrate, formate, acetate or propionate. The former is the precursor of some aroma and flavor compounds in cheese (McSweeney and Fox, 2004).

Most of the citrate of milk is lost in cheese whey and is not metabolized by most strains of *Lactococcus. lactic* subsp. *lactis* or subsp. *cremoris* but is metabolized by some strains of lactococci with the production of diacetyl, acetate, acetoin and carbon dioxide. During ripening of pressed ewes' milk cheeses this pathway is not very marked as it is in Swiss-type or Camembert-type cheeses, thus its contribution to cheese ripening its reduced (Robinson and Wilvey, 1998; Saldo, 2002).

3.2.1.2 Lipolysis

Lipolysis is a biochemical event due to lipases from different sources: naturally present in milk, added with the rennet, produced from starter bacteria, secondary starter microorganisms and/or non-starter lactic acid bacteria, or added by means of exogenous lipase preparations (Collins *et al.*, 2004). The lipid fraction of cheese is primarily composed by triglycerides and low levels of free fatty acids. During ripening, triglycerides may undergo lipolysis which is the hydrolysis of these molecules to free fatty acids and glycerol, mono or diglycerides, and to a lesser extent they can be oxidized. In pressed ewes' milk cheeses, lipolysis is not the predominant phenomenon since lactic acid bacteria are weakly lipolytic in comparison to species such as *Pseudomonas, Acinetobacter and Flavobacterium*. However if the cheese is ripened for an extended period, lactic acid bacteria is

responsible for the liberation of significant levels of free fatty acids (Collins *et al.*, 2004). Later, fatty acids are catabolized by microorganisms' enzymes, especially molds, to form many important flavor and aroma compounds such as methyl ketones. Thermo sensible indigenous lipases in milk are deactivated during pasteurization; therefore lipolysis is more pronounced in raw milk cheeses (Poveda *et al.*, 2000; Fernández-García *et al.*, 2006).

The extent of lipolysis is regularly assessed by determining the concentration of free fatty acids in the cheese which usually increase its concentration during ripening. The most abundant free fatty acids commonly found in Manchego and other ewes' milk cheeses with similar characteristics are acetic (682-1127 mg/kg), palmitic (304-994 mg/kg), oleic (364-986 mg/kg) and myristic (132-443 mg/kg) acids, but also butyric, caproic and caprilic among others are present (Pavia *et al.*, 2000; Poveda *et al.*, 2000; Ballesteros *et al.*, 2006).

3.2.1.3 Proteolysis

Proteolysis may be considered the most complex and important biochemical event in most type of cheeses and particularly in pressed ewes' milk cheeses. During ripening, this pathway is catalysed by proteinases and peptidase from the milk, rennet and microorganisms, having important impact on cheese texture and cheese flavor development. In this process caseins are hydrolyzed to form peptides with lower molecular weight (Upadhyay *et al.*, 2004).

Proteolysis can be divided in two stages. In primary proteolysis, caseins are hydrolyzed by residual coagulant and to a lesser extent by plasmin which results in the formation of large and intermediate-sized peptides. In the secondary proteolysis these peptides are hydrolyzed to free amino acids by the action of microbial peptidases from lactic acid bacteria, molds and yeast (Saldo, 2002). Afterwards, amino acids are catabolized by bacteria present in cheese to form molecules responsible for cheese taste and aroma.

The extent of proteolysis depends on many factors such as concentration of proteolytic enzymes, pH, temperature, salt concentration and moisture content; as a result, proteolytic pathway is unique to a particular cheese variety. In hard pressed ewes' milk cheeses, proteolysis rate is slow compared to other varieties due to their high salt and low moisture content (Upadhyay *et al.*, 2004).

Chymosin is the principal proteinase in traditional rennet used for cheesemaking and most of it is removed in the whey. The residual chymosin plays an important role in the initial proteolysis of caseins, so changes on manufacturing practices, particularly cooking temperature, will affect its activity. Microbial flora restricts some proteolysis products formation because of nutritional requirements; they produce the specific amino acids they need to grow. As a consequence, proteolysis products will strongly depend on the strains present in the cheese (Upadhyay *et al.*, 2004)

The most common non-specific technique to study cheese proteolysis is determination of the soluble nitrogen in different solvents or buffers. Water soluble nitrogen and soluble nitrogen in a solution with pH 4.6 are nitrogen fractions used as indicators of primary casein hydrolysis. Large and intermediate size peptides are soluble on these fractions and the concentration of total nitrogen increase with ripening time, starting from 7 % after manufacture and reaching values of more than 30 % after 240 days (Pavia *et al.*, 1999a; Fallico *et al.*, 2006; Cabezas *et al.*, 2007). Smaller size peptides and free amino acids are soluble in 12 % trichloroacetic acid and in phosphotungstic acid respectively. The former has values from 3.9 to 16.9 while the latter from 0.55 % to almost 10.4 % by 180 days of ripening (Horne *et al.*, 2005; Fallico *et al.*, 2006; Cabezas *et al.*, 2007).

3.2.2 General sensory characteristics

3.2.2.1 Texture

Texture is an important quality attribute of cheeses appreciated by consumers. The International Organization for Standardization defines texture as "all the rheological and structural (geometric and surface) attributes of a product perceptible by means of mechanical, tactile, and, where appropriate, visual and auditory receptors" (ISO, 1992). In cheese, texture measurements are usually done by means of a texturometer using different test such as uniaxial compression, bending, torsion test, texture profile analysis or relaxation test. From all these methods different texture parameters can be obtained which have been correlated with sensory attributes. Table 3 shows a list of textural terms and definitions generally used.

Table 3. Terms usually used in texture analysis of cheese

Term	Definition
Hardness	Force necessary to penetrate the sample with the molar teeth
Firmness	The force required to compress the cheese with the fingers
Fracturability (brittleness)	Breakability of the sample at the first bite
Cohesiveness	Strength of the internal bonds making up the body of the product
Adhesiveness	Work necessary to overcome the attractive forces between the surface of the food and surface of other materials with which the food comes in contact
Gumminess	Energy needed to chew a solid food until it is ready for swallowing
Shortness	Tendency to fracture at small deformation

(modified from Gunasekaran and Mehmet Ak, 2003)

Cheese is essentially a concentrated protein gel where fat and moisture are entrapped. This network structure is critically affected by protein content, fat and water as well as by biochemical activities, promoting a wide variety of cheese

textures (Gunasekaran and Mehmet Ak, 2003). After manufacturing the matrix has a structure consisting on a relatively loose network of clearly recognizable particles. After few hours or days a fusion of these particles leads to the formation of a more homogeneous mass (Walstra *et al.*, 2001; O´Callaghan and Guinee, 2004). Cheese matrix is constantly changing due to partial hydrolysis of the proteins, changes in the water-binding ability of the curd, coalescence of fat globules and changes in pH. These changes are mediated by the residual rennet, microorganisms, their enzymes and changes in mineral equilibrium.

In pressed hard ewes' milk cheeses texture changes occur in two phases. During the first thirty days of ripening, firmness and fracturability decreased as a consequence of cheese matrix softening due to hydrolysis of caseins by the residual rennet (O´Callaghan and Guinee, 2004). After this first month, the cheeses become firmer and more fracturable because the firming effect of water loss is predominant over softening due to proteolysis. Shortness and cohesiveness are constantly decreasing during ripening due to the decrease in the amount of water available for solvation of the protein chains (Pavia *et al.*, 1999b; Juan *et al.*, 2007; Albenzio and Santillo, 2011).

3.2.2.2 Color

Color is one of the most important characteristics in food that determines consumer acceptance. It creates sensory expectation about a determined product even before testing. Color is defined as "the property possessed by an object of producing different sensations on the eye as a result of the way it reflects or emits light", so that is subjective as it depends on light and/or the observer. The most common way to measure color in cheeses is tristimulus colorimetry which includes different color spaces. The most common color space is CIEL*a*b* (Figure 6) consisting on three coordinates: L* coordinate represents lightness or brightness, ranging from 0 to dark colors to 100 to light colors; coordinate a* represents the green/red coloration, negative values of a* means that color is more close to green

while positive values mean that color is more close to red; and coordinate b* is the blue/yellow coordinate, negative values mean that the color has more blue and if values are positive means that the color has more yellow.

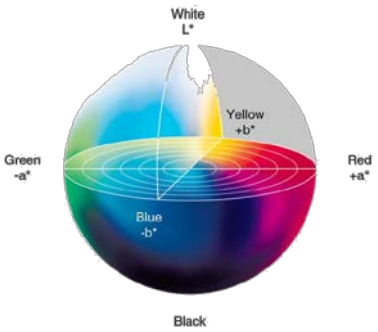


Figure 6. CIEL*a*b* color space

Pressed ewes' milk cheeses normally have a coloration ranging from ivory to pale yellow but this coloration can vary from one variety to another. Color depends on cheese composition which is influenced by milk fat, as well as riboflavin and carotenoid content from the diet of the animals. This have been demonstrated in different studies which concluded that higher carotene content in cheeses, coming from the milk of animals that were fed with mountain grass, resulted to be more yellow than those feed with hay (Jaros and Rohm, 1997; Coulon *et al.*, 2004; Nozière *et al.*, 2006; Hernández-Morales *et al.*, 2010; Todaro *et al.*, 2011).

Studies conducted to evaluate color changes related to cheese composition found that cheeses with less fat and ash content but with more crude protein are less bright, red and yellow (Marchesini *et al.*, 2009). Pastorino *et al.* (2003) found that unsalted cheeses were more opaque than salted cheeses because the former had more open channels with free serum, suggesting that changes on water content in cheeses cause coloration changes as well. This has been confirmed in different studies about color changes during ripening (Rohm and Jaros, 1996; Marchesini *et al.*, 2009; Olson *et al.*, 2011).

As mentioned in section 3.2 of this chapter, dry matter content increased as a consequence of water loss during ripening. In general most authors agree that L* values decrease with ripening, thus cheeses become less bright (Rohm and Jaros, 1996; Marchesini *et al.*, 2009), but changes on a* and especially on b* during maturation have shown different trends depending on the cheese variety. For ewes' and some cows' milk cheeses a decrease on b* coordinate was found while for goats' and hard cows' milk cheeses, b* increased (Rohm and Jaros, 1996; Marchesini *et al.*, 2009; Rinaldi *et al.*, 2010; Sánchez-Macías *et al.*, 2010).

Storage conditions, such as light, packaging and temperature also affect color because they promote degradation of lipids, proteins and vitamins. Studies about this degradation have been conducted in some cheese varieties, especially coloured, such as Cheddar and spreadable cheeses, but little information is known about pressed ewes' milk cheeses. Some authors found that fluorescent light exposure caused decreased in yellowness, redness and lightness over the first days of storage while others mentioned that light increased a* coordinate (Hong et al., 1995a; Kristensen et al., 2001; Juric et al., 2003). Discoloration is caused by oxidation of riboflavin and carotenoids promoted not only by light but also by oxygen. Vacuum packaged Cheddar cheeses exposed to light decreased yellow and red coordinates while Camembert cheeses decreased whiteness (Hong et al., 1995b; Colchin et al., 2001; Rodriguez-Aguilera et al., 2011). No color changes on cheeses packed under modified atmospheres without oxygen and light exposure have been found (Favati et al., 2007; Temiz, 2010). Regarding temperature, a reduced loss of a* and b* values was obtained when storage temperature decreased (Hong et al., 1995a).

3.2.2.3 Taste

Taste is the perceived sense in the tongue and soft palate by non volatile compounds that must make contact with the taste receptors. There are five tastes: sweet, sour, salty, bitter and umami (Figure 7), that in the case of cheese depends on a balance of acid, salts, peptides and free amino acids (Delahunty and Drake, 2004). The taste of fresh cheese curd is bland and slightly sweet due to the residual presence of lactose, after, cheeses can have an acid taste because of the production of lactic acid and its subsequent degradation but this will depend on the concentration of these molecules. Acetic, propanoic and butanoic acid presumably contribute to acidity as well. Salty taste is due to sodium chloride addition during manufacturing and mineral salts of potassium, calcium and magnesium. The apparent saltiness increases with ripening (McSweeney, 1997; Delahunty and Drake, 2004).

Bitter taste of cheese results from the accumulation of hydrophobic peptides formed from proteolysis of caseins, influenced by residual chymosin concentration, microflora and salt content. Bitterness contributes to the desirable final characteristics in mature pressed ewes' milk cheeses but is consider as a defect when it appears at early stages of ripening. Cheese with low salt concentration is very prone to bitterness because the activity of residual coagulant is increased (Smit *et al.*, 2000).

Not all peptides formed by proteolysis contribute to the bitter taste. Large peptides probably do not contribute directly but they do after being hydrolyzed to shorter peptides. Their taste contribution may depend on the terminal amino acids of the peptide chain, as an example, peptides with arginine as a terminal amino acid will have a bitter taste. Figure 7 shows the amino acids different tastes. The hydrolysis of peptides is dependent on the starter culture used in cheese or in the indigenous microflora, so that each microorganism can generate different peptides thus changes on cheese taste (Robinson and Wilvey, 1998).

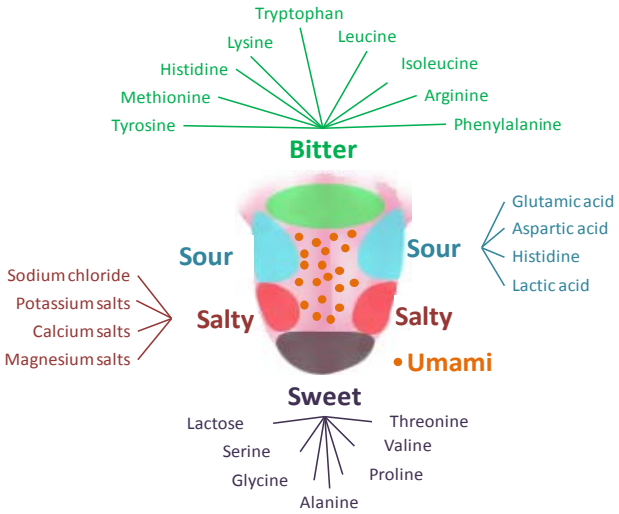


Figure 7. The five tastes and its related compounds in cheese

3.2.2.4 Aroma

Cheese aroma is caused by volatiles capable of being sensed in the nose by the olfactory receptors (Le Quéré, 2004). Cheese aroma formation is mainly dependent on biochemical degradation pathways during cheese ripening mentioned before: glycolysis, lipolysis and proteolysis. Many efforts have been done to characterize volatiles in cheese as a possible tool to determine geographical origin, fraudulent contaminations with other milks, manufacturing season, milk heat treatments, volatile fingerprint and many other potential characteristics. Nevertheless, it has been very difficult to have a unique methodology especially regarding isolation or extraction.

3.2.2.4.1 Methodologies for aroma determination

Most of the aroma compounds are mainly hydrophobic and consequently they tend to concentrate in the cheese fat, therefore, the extraction of these molecules must be suitable for separating them from the fat matrix without artifact generation. For this reason, there have been many studies in order to develop isolation methodologies suitable for cheese avoiding as much as possible sample manipulation (Curioni and Bosset, 2002). Another important issue is the identification of odor active compounds. It has been found that some compounds present in cheese in high quantities do not have a sensory implication on aroma because their odor thresholds are very high, as an example, some ketones have odor thresholds at levels of mg/kg.

Figure 8 shows a resume of methodologies currently used to volatile analysis in ewes' milk cheese, including sample preparation, volatile isolation, separation and identification of compounds.

Starting from sample preparation, cheeses can be directly analyzed or frozen and then analyzed. In most cases the cheese is grated and sometimes dispersed in water. Sample size depends on the isolation methodology ranging from 0.05 g (Januszkiewicz *et al.*, 2008) to 200 g (Milo and Reineccius, 1997).

Classical isolation techniques of volatile analysis, such as distillation, are no longer used so that, they are not included in Figure 8. In this method, the sample was suspended in water and heated or directly heated with steam, having as a result a very dilute aqueous solution. After distillation of this fraction, solvent extraction was performed to concentrate the volatiles. Some of the drawbacks of these methodologies are that highly volatile compounds are poorly recovered, thermally sensitive compounds may disappear, and artifacts may appear. Variations on these techniques such as simultaneous steam distillation with solvent extraction or high vacuum distillation are still used for dairy products but as new methodologies with several advantages have been developed they have been

abandoned (Milo and Reineccius, 1997; Suriyaphan *et al.*, 1999; Le Quéré, 2004; Van Leuven *et al.*, 2008).

Nowadays, the most common isolation techniques are static or dynamic headspace. Here, the volatiles are released in the vapor phase containing the volatiles compounds of the cheese which are further concentrated. Regarding headspace techniques currently used are solid phase micro extraction (SPME), headspace sorptive extraction (HSSE) and purge and trap (P&T). The first two use different fiber materials to adsorb volatiles (Bosset and Gauch, 1993). The most common coatings in SPME are polydimethylsiloxane (PDMS), divinylbenzene (DVB), carboxen (CAR), polyacrylate (PA) and carbowax (CW) or a combination of two or more materials. In HSSE PDMS is the only fiber commercially available at this moment. Between these two techniques SPME is more frequently used. HS-SPME and SBSE/HSSE have been compared using honey or wines concluding that sorptive extraction had concentration capability 40-fold times than SPME (Blasco *et al.*, 2004; Alves *et al.*, 2005; Maggi *et al.*, 2008). HSSE has been only used in the dairy sector for determination of volatiles of "Pesto Genovese" containing Grana Padano cheese and to determine flavor compounds in Bitto cheese (Salvadeo *et al.*, 2007; Panseri *et al.*, 2008). These both techniques need a thermal desorption unit for desorbing the volatiles from the fibers.

Purge and trap is also widely used for aroma determination and has been used in ewes' milk cheeses such as Zamorano, Idiazabal, Manchego and Roncal (Izco and Torre, 2000; Barron *et al.*, 2005b; Barron *et al.*, 2007; Irigoyen *et al.*, 2007). P&T consists on stripping of the volatiles from the cheese, sometimes dispersed in water, with an inert gas and then volatiles are concentrated in a trap prior to analysis. Comparisons between P&T and HS-SPME lead to the conclusion that P&T is more sensitive, showing higher extraction efficiency for compounds with lower boiling points while HS-SPME shows more effectiveness for medium and high boiling point compounds (Mallia *et al.*, 2005; Januszkiewicz *et al.*, 2008).

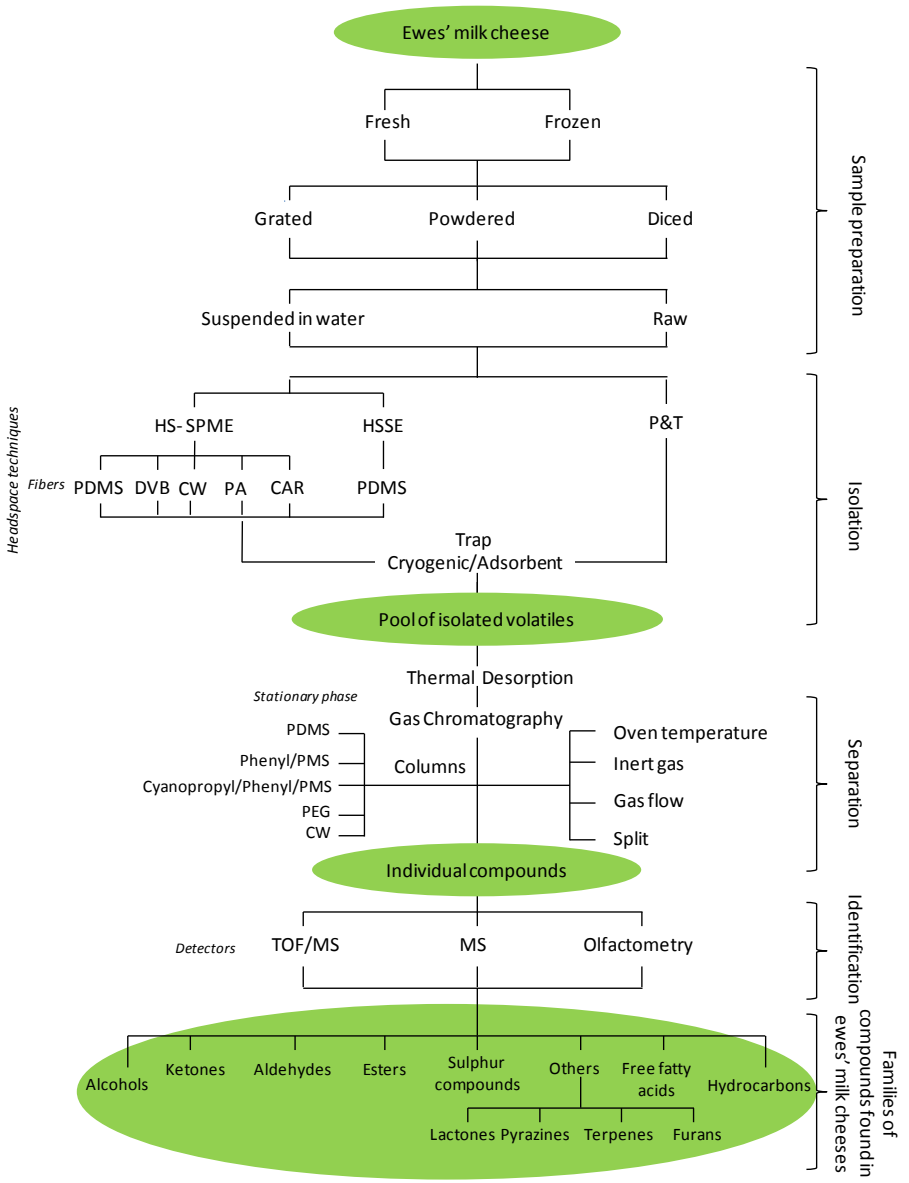


Figure 8. Methodologies currently used for volatile analysis in cheese

HS-SPME:	Headspace solid phase microextraction	PDMS:	Polydymethylsiloxane	DVB:	Divinylbenzene
HSSE:	Headspace sorptive extraction	P&T:	Purge and trap	CAR:	Carboxen
PA:	Polyacrylate	PEG:	Polyethylene glycol	CW:	Carbowax
TOF/MS:	Time of flight mass spectometry	MS:	Mass spectometry		

After isolation, gas chromatography (GC) is universally used to separate volatiles. There are many aspects during GC that can be set from one method to other, for example, the stationary phases of the columns. A wide range of dimensions and phases with different polarities are commercially available. Figure 8 shows stationary phases most commonly use for cheese analysis. Regarding chromatographic conditions, in most cases initial temperatures are between 32 and 40 °C raised to 250 °C. Selection of conditions should be optimized in each particular case since they will depend on the column and the target compounds.

Currently, detection of volatiles is commonly done with mass spectrometry (MS) or time of flight/mass spectrometry (TOF/MS) because these detectors have higher sensitivity compared to others (Gogus et al., 2006). Olfactometry is also used because it allows the identification of odor active compounds between the volatiles found, for example, it has been applied to characterize Gruyere, Manchego, Ragusano, Idiazabal, Cheddar and Piacentinu Ennese (Suriyaphan *et al.*, 1999; Horne *et al.*, 2005; Mallia *et al.*, 2005; Abilleira *et al.*, 2010).

The most common quantification method is to relate areas between internal standard and the target compounds and then calculate an approximate concentration. This method assumes that all compounds detected produce a similar signal in the MS, leading to inaccurate results. Therefore comparison between volatiles obtained with the real standards and calibration curves would offer more solid results. Different quantification methodologies are one of the facts that make difficult comparison between different studies.

3.2.2.4.2 Ewes' milk cheese aroma

Ewes' milk cheese aroma is very complex and a large variety of compounds has been detected depending on the isolation methodology, stage of ripening, season of the year and animal breed. Most authors agree that compounds present in the volatile fraction of these cheeses belong to common families: alcohols, ketones,

aldehydes, esters, sulfur compounds, free fatty acids, hydrocarbons, lactones, terpenes, furans and pyrazines, as shown in Figure 8. Different pathways are responsible for the formation of these compounds, mainly catabolism of free fatty acids, amino acids, lactate and citrate which are strongly influenced by the specific microorganisms present in the cheese (Figure 9).

Ketones are mainly produced during ripening from partial ß-oxidation of free fatty acids and they are reduced to their corresponding alcohols (Figure 9). Due to their low perception thresholds, methyl ketones have been found as key odorant in surface mold ripened and blue-veined cheeses with fruity, floral and musty notes (Le Quéré, 2004). They are one of the major groups present in ewes' milk cheese depending, on part, of microbiology of the cheeses which at the same time is influenced by thermal treatment of the milk, manufacturing and season of the year. The most common ketones found are 2-propanone, 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone, 2,3-pentanedione and 2,3-butanedione (diacetyl). The latter has buttery and creamy notes and it is described as a contributor to the overall aroma of cheese (Barron *et al*., 2005b; Horne *et al*., 2005; Mallia *et al*., 2005; Massouras *et al*., 2006).

Aldehydes are considered transitory molecules because they are rapidly reduced to alcohols or oxidized to the corresponding acids. They originate from amino acids degradation and unsaturated free fatty acids (Figure 9) (Curioni and Bosset, 2002). Its presence has been identified in ewes' milk cheeses, especially at early stages of ripening, decreasing its concentration with time. The most common are acetaldehyde, propanal, butanal, hexanal, heptanal, 2-propenal, 2-nonenal, 2-methylbutanal and 3-methylbutanal. In addition vanillin has been detected in Piacentinu Ennesse cheeses (Pinho et al., 2003; Horne et al., 2005; Massouras et al., 2006). Curioni and Bosset (2002) mentioned that n-butanal was found as an active odor compound in Manchego cheese while (E)-nonenal in Pecorino. However, in general these compounds are more characteristic of Camembert, Cheddar, Emmental or Grana Padano cheeses.

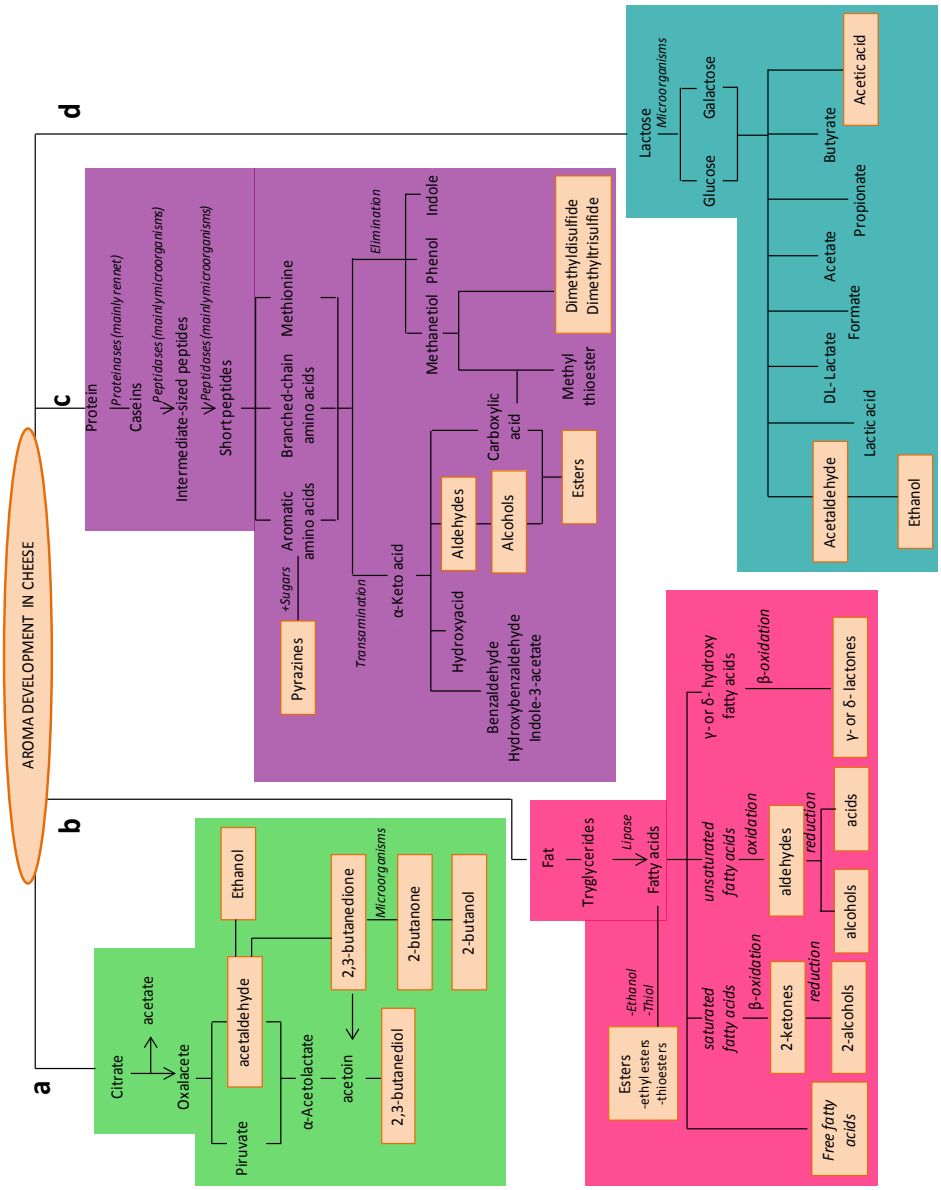


Figure 9. Aroma formation in cheese (modified from McSweeney and Sousa, 2000)

a) citrate metabolism, b)lipolysis and free fatty acid metabolism, c) proteolysis and amino acid catabolism and d) lactose metabolism

Alcohols are formed during ripening as a product of catabolism of free fatty acids by reduction of methyl ketones, lactose metabolism and amino acid metabolism (Figure 9). They have been identified in most ewes' milk cheeses in high quantities and even as a major family in Zamorano, Roncal and Manchego (Izco and Torre, 2000; Fernández-García *et al.*, 2004; Barron *et al.*, 2005b). Primary alcohols have been detected at higher concentrations compared with other alcohols. It has been studied that its concentration changes with ripening and that differences can be found between industrial and artisanal cheeses and even between seasons of the year (Barron *et al.*, 2005a). Ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-pentanol, 2-butoxyethanol and 3-methylbutanol are the most common in Spanish ewes' milk cheeses and Teleme cheese, among them, 2-butanol with chemical and floral notes have been established as odor active compound in Manchego cheese (Barron *et al.*, 2005b; Mallia *et al.*, 2005; Massouras *et al.*, 2006).

Esters, especially ethyl esters, are common cheese volatiles. Esterification reactions occurs between short to medium-chain fatty acids and primary and secondary alcohols derived from lactose fermentation or from amino acid catabolism (Figure 9) (Le Quéré, 2004). These compounds are described as having sweet, fruity and floral notes and, as they have very low perception thresholds, to contribute to the final aroma of cheese. Ethyl ethanoate, ethyl butanoate, ethyl hexanoate and ethyl octanoate have been detected in most ewes' milk cheeses. Barron *et al.* (2007) found that its concentration increased with ripening time and that Idiazabal cheeses with raw milk had more concentration of some esters such as ethyl hexanoate, than pasteurized milk cheeses. They mentioned that these differences are related to a higher esterase activity of lactic acid bacteria in raw milk cheeses. Only ethyl butanoate and hexanoate have been found as active odor compounds in Pecorino cheeses (Curioni and Bosset, 2002).

Acetic acid and free fatty acids are important as well in the volatile fraction of ewes' milk cheeses. The latter are predominant in cheese flavor and also serve as precursors of methyl ketones, alcohols, lactones and esters (Figure 9). Short chain

free fatty acids can originate from lipolysis of milk fat or the breakdown of amino acids (Curioni and Bosset, 2002). Acids constitute one of the main chemical families in Idiazabal cheeses but it is not the case for all ewes' milk cheeses. In general, most ewes' milk cheeses have acetic acid and *n*-butanoic acid, but also propanoic, pentanoic, hexanoic, heptanoic, octanoic, 2-methylpropanoic and 3-methylbutanoic acids have been detected in some cases (Pinho *et al.*, 2003; Barron *et al.*, 2005b). Nevertheless, among all these compounds, butanoic acid with its characteristic rancid cheese-like odor, is more likely to play an important role as demonstrated in Pecorino, Roncal and Manchego cheeses (Curioni and Bosset, 2002; Mallia *et al.*, 2005).

Sulphur compounds play an important role in cheese aroma. They originate from methionine degradation by microorganisms (Figure 9). These compounds are described as having garlic and very ripe cheese odors and their perception threshold is very low, thus they are probably involved in the final aroma, but this fact has only been confirmed for mold-surface and soft smear ripened cheeses (Curioni and Bosset, 2002; Le Quéré, 2004). The most common in ewes' milk cheese are carbondisulphide, methyldisulphide, dimethylsulphide, dimethyldisulphide and dimethyltrisulphide (Izco and Torre, 2000; Fernández-García *et al.*, 2002; Pinho *et al.*, 2003; Fernández-García *et al.*, 2004; Horne *et al.*, 2005).

Hydrocarbons are also found but as minor constituents. They can originate in cheese from animal feed, when the cheeses are smoked or from the wax or coatings. Among them heptane, octane, pentadiene and the aromatic benzene and toluene are the most usual. Most of these compounds have sweet, ethereal and gasoline descriptors. Higher quantities of alkanes have been found in Roncal cheese because of the pasture used for feeding the animals while Manchego cheeses have shown higher concentration of unsaturated hydrocarbons compared to other Spanish ewes' milk cheeses (Fernández-García *et al.*, 2004; Barron *et al.*, 2005a; Barron *et al.*, 2005b).

Other compounds commonly found but in minor fraction are terpenes, pyrazines, furans and lactones. Terpenes as well as unsaturated hydrocarbons can originate in cheese from the different feed sources of the sheep, so that, its presence and concentration depends on the season and the region. In fact, terpenes can be potential markers to identify geographical origin of milk and cheese (Povolo *et al.*, 2007). The most frequent are α-pinene and limonene but borneol, terpinolene, citronellol and terpineol, among many others have been detected as well. They usually have fruity and citric notes (Horne *et al.*, 2005; Barron *et al.*, 2007; Abilleira *et al.*, 2010).

Pyrazines have been claimed as important contributors to cheese flavor in the case of Cheddar and Gruyère but not data about its flavor contribution on ewes' milk cheeses is available. They normally have herbaceous notes and originate from the union of amino acids and sugars, especillay L-valine (Figure 9) (Müller and Rappert, 2010). Some pyrazines found in Piacentinu Enesse cheese are 2,6-dimethyl-3-ethylpyrazine and 2-isopropyl-3-methoxypyrazine (Horne et al., 2005).

Furans (2-ethyl-furan) and lactones (δ-octalactone and γ-decalactone) have been also identified in cheeses but its function as key odorant has not been established. They have sweet and fruity odor descriptors. Other compounds reported in some studies are phenylacetaldehyde, acetophenone and phenol in Zamorano cheese and ethylether and chloroform in Teleme cheeses (Fernández-García *et al.*, 2004; Massouras *et al.*, 2006).

Sometimes, some of these aroma compounds are added to cheese in order to improve quality or to diversify the product. For this purpose many herbs, spices and seeds for example garlic, sage, mint, basil, rosemary and pepper are used. Some of them have also the property to give a different color for example paprika and saffron. As an example of cheeses with flavor and color there are a Swiss-type cows' milk cheese called Lüneberg in Austria, a fermented goats' milk cheese called Bouchon allo Zafferano in Lombardia, an ewes' milk semi-hard cheese named Box in Germany, Cacio allo Zafferano in Italy and the most known

Piacentinu Ennese in Sicily, all these cheeses are made with saffron in a traditional way. In most of the cases they do not have a standardize form or dose to add this spice into the cheesemaking process. A good example is Piacentinu Ennese, which recently obtained the Protected Designation of Origin. In the document that specifies the regulations for the production of this cheese, saffron addition is not very detailed. It is mentioned that "five grams of saffron per one hundred liters of milk, previously homogenized in tempered water, can be added as a maximum dose prior to rennet addition". This statement promotes lack of standardization between batches and factories since more parameters should be controlled. They do not mention if different saffron presentations can be used or how much water is needed to homogenize saffron. Moreover, the regulation mentions that after saffron addition, milk should have a "beautiful yellow color" but they do not point out how much time does saffron need to be completely homogenized (MIPAAF, 2012).

This lack of information makes necessary a deeper study about the behavior and properties of the spice that want to be added to cheese in order to characterized the product.

3.3 Saffron

3.3.1 Definition and production

Saffron spice is the dried stigma of *Crocus sativus* L., including different presentations: in filaments, cut filaments and powder (ISO, 2011). Saffron belongs to the Iris family and is a plant that grows from a corm. From the botanical point of view, a corm is a short, thick shoot with a solid structure. It is similar to an onion, except that is solid and does not have numerous concentric layers (Carmona *et al.*, 2006). These corms have two or three steams and from these one to three flowers are produced. Flowers (Figure 10) have six tepals and from its ovary grow the single

stigma divided in three red filaments which are used as spice because of its taste, aroma and color (Varios, 2006).

Figure 10. Saffron (*Crocus sativus* L.) flower

This spice is the most traditional and most valuable spices in the world, unless there are several proposed origins: from the mountain regions of Asia Minor to Greece, Western Asia, Egypt or Kashmir. Due to saffron´s importance in the various cultures established in the region of the Euphrates River, Mesopotamia could be the true origin of the beginnings of this plant. The origin of the Spanish saffron could be Romanic and afterwards, the Arabians were responsible for its diffusion and promoting its consumption within Europe (Carmona *et al.*, 2006; Caiola and Canini, 2010).

At the present, Iran is the country with the highest saffron production, followed by India, Greece, Spain, Morocco, Italy, Turkey, France and Switzerland, although England, Austria and Germany have maintained its cultivation as a tradition but only producing for their self-demand. Besides, countries like Israel, Japan, Azerbaijan, China or Mexico are also producers but without official data (Carmona et al., 2006).

Mediterranean basin has always played an important role regarding saffron production and trade. From the end of the Middle Ages Spanish saffron was exported because its cultivation was widespread among the national territory. Years later, saffron from "La Mancha" region started to have high demand because of its special high quality (Sánchez Gómez, 2009). Years ago, some saffron dealers

packaged good quality saffron that had not been produced in Spain under the name Mancha thus in 1999 the Protected Designation of Origin named "Azafrán Mancha" was created in order to protect saffron quality and to avoid adulterations and imitations. The word "Mancha" can only be used to designate saffron produced in Toledo, Cuenca, Ciudad Real and Albacete provinces (Figure 11) (DOCE, 2001).

Figure 11. Spanish provinces which produce "Azafrán Mancha"

The special feature about this spice is that flowers only grow between the middle of October and the beginning of November and traditionally they have to be harvested by hand during dawn (Figure 12).

Figure 12. Hand harvesting of saffron in Castilla-La Mancha region

After, the stigmas are manually separated from the rest of the flower parts in a process called "monda" in Castilla-La Mancha region (Figure 13).

Figure 13. Separation of the stigmas called "Monda" in Castilla-La Mancha region

When separation is completed the stigmas are dehydrated which will influence saffron quality and preservation. In Castilla-La Mancha the stigmas are spread over a metallic or silk sieve (Figure 14) and dehydrated from 20 to 45 min using a gas cooker, live coals or an electrical coil, this step is called "tostado" (Consejo Regulador Azafrán Mancha, 1999; Pardo *et al.*, 2002; Carmona *et al.*, 2006). The process is finished when the sample has lost about 80 % of their weight, after being dried at temperatures between 70 and 100 °C for 30 min approximately (Carmona et al., 2005). Depending on flower size and on consequent stigma weight between 100,000 and 300,000 flowers are necessary to obtain one kilogram of saffron (Carmona *et al.*, 2006). The final aspect of the spice is shown in Figure 15.

Figure 14. Saffron dehydration called "tostado" in Castilla-La Mancha region

As a common belief among producers, dehydration process is responsible for the high quality of the saffron from Castilla-La Mancha. It has been demonstrated

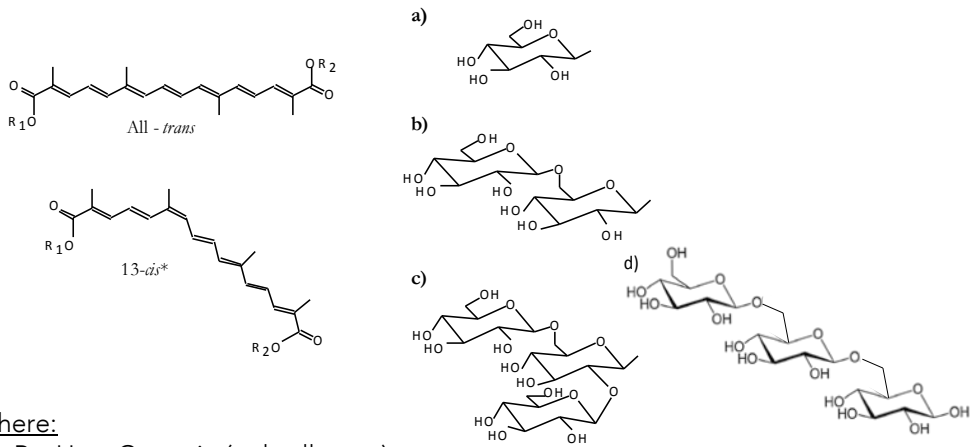
that dehydration at high temperatures increased coloring strength, tone and brightness, as well as aroma development compared with dehydration at ambient temperature (Pardo *et al.*, 2002; Carmona *et al.*, 2005; Carmona *et al.*, 2007b). Moreover, microbiological quality is better compared to sun dried saffron as demonstrated by Cosano *et al.* (2009).

Figure 15. Crocus sativus L. stigma and style after drying

3.3.2 Color, taste and aroma

The molecules responsible for the yellow-red color in saffron are a group of hydrosoluble carotenoids derivates from the 8,8´-diapo-Ψ,Ψ´-carotenedioic acid (known as crocetin) esterified by glucose, gentibiose, neapolitanose or triglucose. They possess an extensive double bond conjugated system so that, several isomers could exist (Figure 16), but generally, the most abundant are all-trans (E) and 13-cis (Z). Crocetin esters are prone to degradation by light, temperature, pH and time following first order kinetics (Alonso *et al.*, 1993a; Orfanou and Tsimidou, 1996; Sánchez *et al.*, 2008), so that many works have been published to establish the best conditions for saffron storage and to avoid loss of coloring strength. These studies concluded that saffron should be stored in dark and low humidity places (Alonso *et al.*, 1993a; Alonso *et al.*, 1993b; Carmona *et al.*, 2005; Bolandi and Ghoddusi, 2006). Different carotenoids have also been found as a minor fraction of the total saffron pigments such as phytoene, phytofluene, tetra-hydrolycopene, β-carotene, ε-carotene, zeaxanthin and lycopene, but their color influence in saffron filaments has not been deeply studied as their concentration is despicable

compared with crocetin esters (Pfander and Schurtenberger, 1982; Castillo et al., 2005; Carmona et al., 2006).



Where:

R_1=R_2=H Crocetin (only all-*trans*)
R_1=H R_2=a Crocetin (β-D-glycosyl) ester
R_1=R_2=a Crocetin Mono-(β-D-gentibiosyl) ester
R_1=H R_2=b Crocetin Di-(β-D-glycosyl) ester
R_1=a R_2=b Crocetin (β-D-glycosyl)-(β-D-gentibiosyl) ester
R_1=R_2=b Crocetin Di-(β-D-gentibiosyl) ester
R_1= b R_2=c Crocetin (D-gentibiosyl)-(β-D-neapolitanosyl) ester
R_1=d R_2=b Crocetin (β-D-triglycosyl)-(β-D-gentibiosyl) ester

(*) In the case of crocetin esters with *cis*–configuration, the position of the substitutes R_1 and R_2 could not be exactly determined in relation to the $C_{13\text{-}14}$ bond.

Figure 16. Crocetin esters structures identified by different authors (modified from Carmona *et al.*, 2006)

Saffron bitter taste has been considered to be given by picrocrocin (4-(ß-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, Figure 17) but this have not been demonstrated. Picrocrocin taste detection threshold has been recently set at 10 mg/L (Sánchez et al., 2010) but in fact, saffron also contains flavonoids such as glycosylated kaempferols that could be involved in saffron bitter taste as well (Carmona *et al.*, 2007a). It has been proved that picrocrocin concentration depends on dehydration process, so that studies relating these two factors have been conducted. Results have lead to the conclusion that picrocrocin

content increases when dehydration temperature increases, besides slow picrocrocin degradation in saffron extracts were observed at temperatures between 20 and 70 °C (Pardo *et al.*, 2002; Del Campo *et al.*, 2010; Sánchez *et al.*, 2011).

Saffron aroma is very complex. Ketones, terpenic aldehydes and over 160 compounds have been involved in saffron aromatic profile, although much of them are believed to be artifacts, nevertheless, safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde, Figure 17) has been considered as the most representative compound (Alonso *et al.*, 1996; Carmona *et al.*, 2007b). The classical theory about safranal formation was proposed by Himeno and Sano (1987) (Figure 17). They suggested that safranal is generated from picrocrocin or from 4-ß-hydroxysafranal promoted by dehydration process because of the temperature reached or by the action of glycosidase. Recently Carmona *et al*. (2006) suggested that during dehydration safranal can be generated by crocetin esters. This theory is supported by the fact that disappearance of picrocrocin in high-temperature induced-aging did not involve the generation of safranal but the exact mechanism of safranal formation from crocetin esters has not been completely established. During saffron storage, safranal content increase changing from spicy and floral notes to vegetable, caramel and citric notes (Maggi et al., 2010).

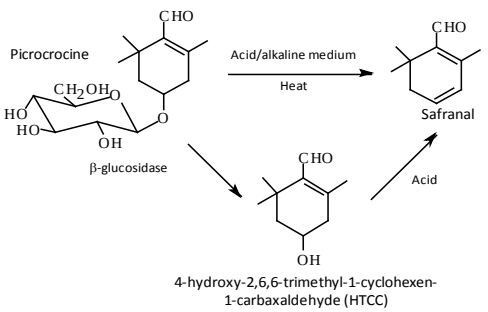


Figure 17. Mechanism of safranal formation from picrocrocin (Himeno and Sano, 1987)

3.3.3 Use and properties

Saffron has been considered over history as a symbol of light, wisdom, spirituality, love, richness and mystery, so that, its use goes back to rituals and religious celebrations in Egypt, Israel and India among other countries (Carmona et al., 2006).

Specialists in ancient dyes state that saffron was known as "the great dye" because since Mesopotamian times it was used to dye clothes, wool, leather and robes. The Phoenicians and Carthaginians used it to dye the veils of the newly married women for its symbolic value as an emblem of fertility (Sánchez Gómez, 2009). In addition, it was used with artistic purposes as saffron pigments has been found in animal prehistoric illustrations (Caiola and Canini, 2010).

The attractive yellow-orange color has also been useful for cosmetics, as an example, Cleopatra used it to dye nails, hair and lips; young Persians as deodorant and Romans in perfumes (Carmona *et al.*, 2006; Sánchez Gómez, 2009; Caiola and Canini, 2010).

Saffron was also appreciated for its healing properties by times of the Greek empire. Dioscorides Pedacio, a medical practitioner of the first century, wrote that saffron was considered as sexual stimulant, anti-inflammatory and as a drunkenness impediment (Pedacio, 1566). Since then saffron has been considered as anodyne, antidepressant, a respiratory decongestant, antispasmodic, aphrodisiac, diaphoretic, emmenagogue, expectorant and sedative (Abdullaev and Espinosa-Aguirre, 2004). During the last decade many reviews have been published summarizing research on this field since this topic is very dynamic and new discoveries are constantly published (Deng *et al.*, 2002; Schmidt *et al.*, 2007; Soeda *et al.*, 2007). During the past decade the activities with more attention have been: antimicrobial, antioxidant, cardiovascular injury, cancer and tumors, antinociceptive, antiinflamatory, premenstrual syndrome, sexual behavior dysfunction and nervous system damage, especially Alzheimer (Akhondzadeh *et al.*, 2010b; Akhondzadeh *et al.*, 2010a).

Several Greeks, Romans and Egyptians historians mentioned that saffron was used as a condiment for beverages and food for the especial bright orange to red color and for its aroma. This use is currently the most common since many traditional dishes such as paella, risotto and bouillabaisse include saffron as an essential ingredient in their recipes. Besides, is also used in breads, puddings, teas and liquors such as the German Gugelhupf, St. Lucia buns in Sweden, Christmas bread in Estonia, candies in Greece, rice pudding in Iran or the Jewish Sabbath bread (Sánchez Gómez, 2009). Moreover there are some dairy products that include saffron in their manufacturing process, such as yogurts and creams but they are more popular in eastern countries like India.

In Europe, the most common use of saffron in dairy products is cheese. These cheeses are made from cows', ewes' and goats' milk and they are semi-hard, hard, fermented or spreadable. Trends of consumer preferences are in the line for food including ingredients with healthy benefits and for products fabricated in their own region or with Protected Designation of Origin (Falguera *et al.*, 2012). Demand for saffron cheeses has a high potential to increase because most of them are fabricated in a traditional way and saffron can contribute as well to diversification of ewes' milk dairy products.

CHAPTER 4. WORK PLAN

This chapter summarizes the work plan followed during the thesis. Figure 18 includes a quick view of the methods used where each color square represents the objectives followed and the sections where the results obtained are present. Nevertheless, the experimental procedure and materials and methods are shown in more detail in the corresponding studies in *Chapter 5. Results* and *Chapter 8. Appendices*.

First of all, a bibliographic revision about saffron doses and related biomedical properties was done following objective 1. Objectives 2 and 3 were achieved by laboratory scale fabrications in in order to establish conditions for saffron addition and if saffron had an influence on cheesemaking. Afterwards, an industry scale cheese fabrication was done and objectives 4 and 5 focused on physico-chemical, microbiological and sensory characterization of saffron cheeses. Following objective 6, a volatile extraction methodology was optimized and saffron color and aroma distribution were analyzed in cheesemaking outputs. Finally in objective 7, volatile characterization of saffron cheeses was carried out.

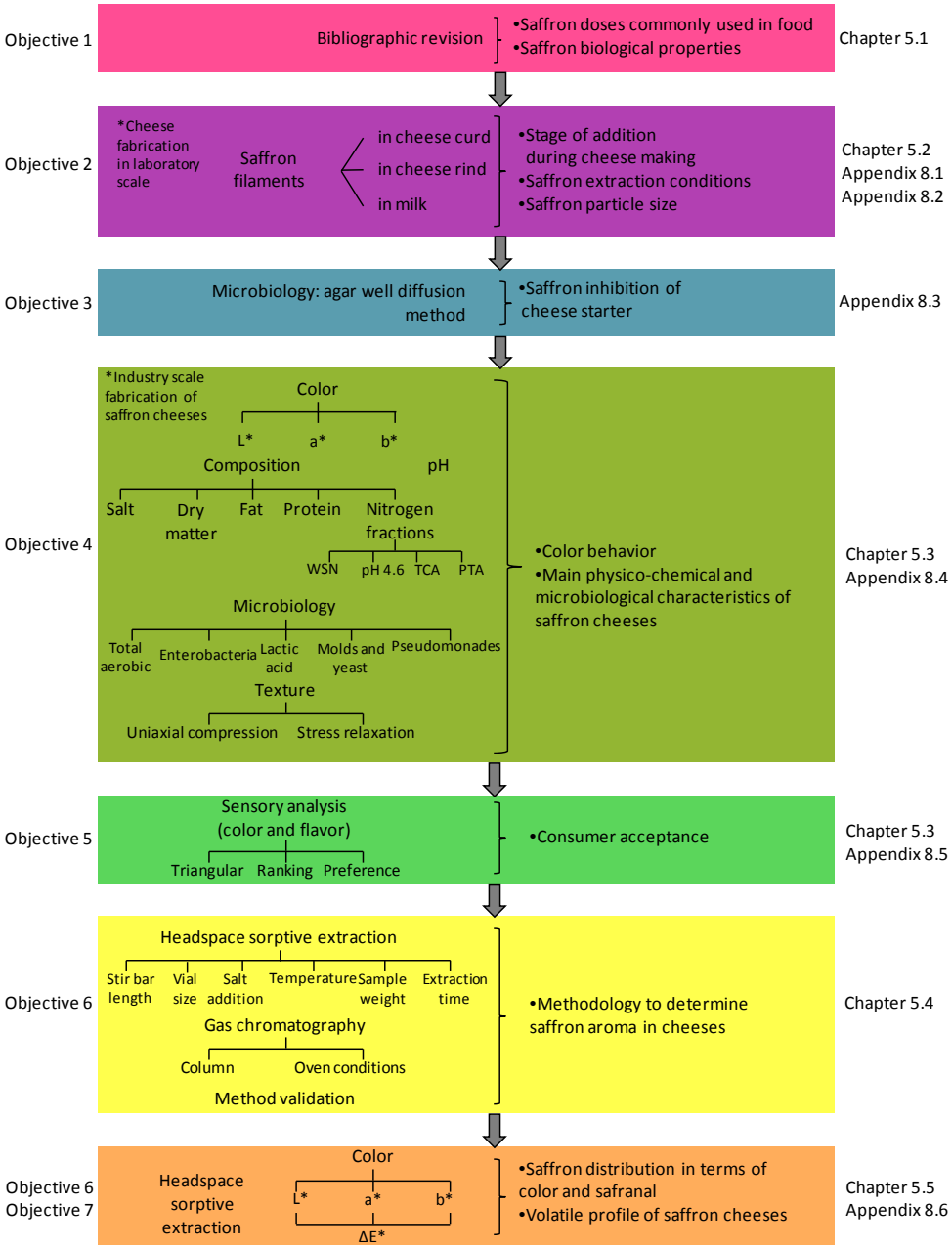


Figure 18. Doctoral thesis work plan

Figure 19 shows the design followed to fabrication of cheese in an industry scale.

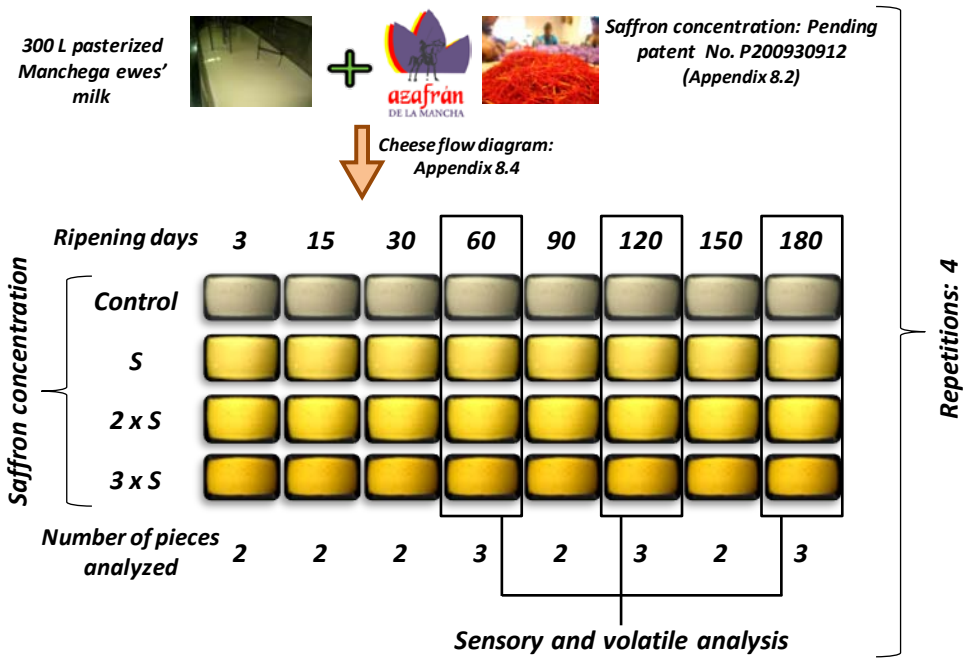


Figure 19. Design for pressed ewes' milk cheese fabrication with three different saffron concentrations, ripening days, repetitions and number of pieces analyzed

Each fabrication consisted on four 300-L vats with different saffron concentrations (Control, S, 2 x S and 3 x S) using pasteurized Manchega ewes' milk. Cheeses were analyzed at days: 3, 15, 30, 60, 90, 120, 150 and 180. Dry matter, fat, protein, nitrogen fractions, pH, color and texture were measured. In addition at days 60, 120 and 180 sensory and volatiles analysis were carried out. Each fabrication was repeated four times.

CHAPTER 5. RESULTS

Results obtained from this doctoral thesis are presented in five sections, corresponding to five scientific papers. Before each publication, general information and a brief description of the article is included. Besides results included in the scientific papers, there are unpublished scientific studies included in appendices 8.1, 8.3 and studies presented in Congresses in appendices 8.5 and 8.6.

5.1 Common saffron doses used in food

5.1.1 Approach

The first objective of this doctoral thesis was to revise saffron quantities commonly used in food in order to establish doses that could be added to ewes' milk cheese. In addition many health benefits were found in saffron.

A revision of the state of the art on these subjects was done and results obtained were published in this scientific paper:

Potential healthy effects of saffron spice (*Crocus sativus* L. stigmas) consumption

Licón, C.C., Carmona, M., Llorens, S., Berruga, M.I. and Alonso, G.L.

Functional Plant Science and Biotechnology 4 (Special Issue 2), 2012, Pages 64-73

ISSN: 1749-0472 **ISBN:** 978-4-903313-67-2

5.1.2 Extended summary

Many studies have been published regarding saffron biological properties. Thus, the first part of the article provides an overview of biological activities of saffron and disease prevention. Also, this work attempted to relate saffron consumption with saffron common doses used in different dishes.

In the review, the studies were divided in seven categories based on the type of disease studied: nervous system damage, cardiovascular injury, cancer and tumors, antinociceptive effects, premenstrual syndrome, sexual behavior, dysfunction and infertility, and other studies. Results showed that biological activity of saffron is based on its great antioxidant ability which allows protecting cells from free radicals.

The studies conducted with patients found that saffron doses ranging from 30 to 200 mg taken daily during 10 days to 22 weeks had influence on improving sexual dysfunction, infertility, Alzheimer, depression and premenstrual syndromes. But most of the works published have tested saffron properties on animals or *in vitro* so human repercussion of these studies is difficult to translate. In this work, doses with potential healthy benefits reported by many authors in animal models were transformed to human doses with an especial formula based on body surface area of a 70 kg person to have a clearer view of doses needed, resulting in a wide range between 1 and more than 4,000,000 mg/person.

Saffron addition to food did not represent any health problem. Only a small number of studies regarding this subject have been published, all of them included saffron allergies but most of them only to pollen or flowers and not to the spice. Only one case of anaphylaxis due to saffron spice has been published. Allergy symptoms, such as asthma or rhinoconjunctivitis have been only attributed to flower handling during saffron harvesting season (Feo *et al.*, 1997; Krautheim and Bircher, 2005). Moreover, microbiological quality of the spice is within European safety regulations as demonstrated by Cosano *et al.* (2009). Potential pathogens such as *Salmonella*, *Staphilococcus aureus*, *Clostridium perfringens* and *Bacillus cereus* were undetectable, incidental or at low prevalence.

The second part of the article shows the minimum perceptible and maximum admissible saffron doses of different dishes such as oils, soups, vegetables, bread, infusions, among others. Dishes use between 75 and 800 mg per liter of food, being oil preparation the product that uses more saffron. Saffron infusions also use high saffron quantities, i.e. a cup of 100-150 ml of saffron infusion is equal to 30 mg of saffron.

Finally, it was observed that saffron properties on the prevention or amelioration of some diseases such as depression, learning behavior problems, seizures and Parkinson can be achieved with saffron addition to food.

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Potential Healthy Effects of Saffron Spice (*Crocus sativus* L. Stigmas) Consumption

Carmen Licón[1] • Manuel Carmona[2] • Silvia Llorens[3] • Maria Isabel Berruga[1] • Gonzalo L. Alonso[2*]

[1] Instituto de Desarrollo Regional, Universidad Castilla-La Mancha, Avenida España s/n E02071 Albacete, Spain
[2] Cátedra de Química Agrícola. E.T.S.I. Agrónomos, Universidad Castilla-La Mancha, Avda. de España, s/n, E02071, Albacete, Spain
[3] Facultad de Medicina, Universidad Castilla-La Mancha, Avda. de España, s/n, E02071, Albacete, Spain

Corresponding author: * Gonzalo.Alonso@uclm.es

ABSTRACT

Saffron (*Crocus sativus* L.), has been used since ancient ages in food for its flavouring, aromatic and colouring properties but also for its biomedical activity. In the past years many efforts have been made in order to demonstrate scientifically the healthy effects attributed to saffron consumption since Dioscorides' time. More than 400 papers have been published in the last decade related to antioxidant properties, cancer, neuronal injury and sedative effect, among others. It has been found that its antioxidant activity is the major responsible for many of the properties that helps to prevent or diminish some diseases. But the majority of these research use animals, making difficult to understand the human application. In this review, a first attempt to translate animal doses to human intake when saffron is included on the diet is carried out, in order to make an estimation of the potential healthy effects in humans.

Keywords: antioxidant properties, *Crocus sativus* L., healthy effects, human equivalent doses, saffron intake
Abbreviations: b.w., body weight; **BSA**, body surface area; **HED**, human equivalent dose; **MI**, myocardial infarction; **PMS**, premenstrual syndrome

CONTENTS

INTRODUCTION

Since ancient ages, spices have placed a major role in cooking, cosmetics, perfumery, global exploration, economics and medicine (Dog 2006). Saffron (*Crocus sativus* L.), is an example of a multi-purpose spice widely used for many centuries. Starting in Mesopotamia, where saffron was used in religious celebrations and for curative purposes; continuing with Phoenicians, where used it to dye cloths, and in ancient Rome, used as a treatment and dye, as well as in perfumes and ointments (Giaccio 2004; Carmona *et al.* 2006). Also used by Cleopatra (69-30 B.C.), it was a cosmetic, phitotherapy and a nail, hair and lips dye. Healing properties of saffron are well known since ancient times, as said by Dioscorides Pedacio, a Greek medical practitioner of the first century, who considered it as sexual stimulant, anti-inflammatory and as a drunkenness impediment. Since then, saffron has been considered as anodyne, antidepressant, a respiratory decongestant, antispasmodic, aphrodisiac, diaphoretic, emmenagogue, expectorant and sedative, among others (Abdullaev and Espinosa-Aguirre 2004).

Recently, research on saffron properties has covered a great interest, demonstrated by the increase of the number of publications in scopus and science direct databases, as shown in **Fig. 1**, where it can be observed the exponential augment, especially from 1996. Approximately, every 2 years, publications duplicate its number, being in 2009 about 5 times more than in 2000. Many reviews have been published in the past recent years (Deng *et al.* 2002; Abdullaev and Espinosa-Aguirre 2004; Schmidt *et al.* 2007; Soeda *et al.* 2007; Kianbakht 2008), but some properties of saffron have been particularly investigated, as seen in **Fig. 2**, where antioxidant, nervous system damage and cancer properties cover a great number of publications, almost 3 to 5 times more than the rest, follow by cardiovascular injury and antinociceptive effects.

The current paper provides an overview of saffron investigations on its biological activity and diseases preven-

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Invited Review

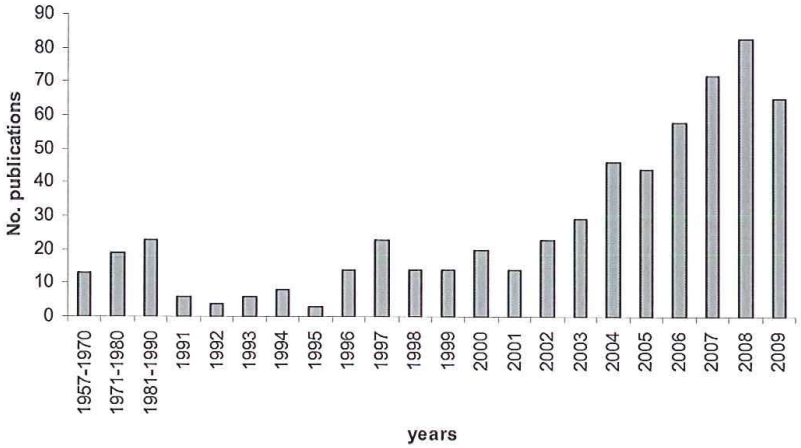


Fig. 1 Biomedical properties of saffron publications during the past years (based on scopus.com and sciencedirect.com).

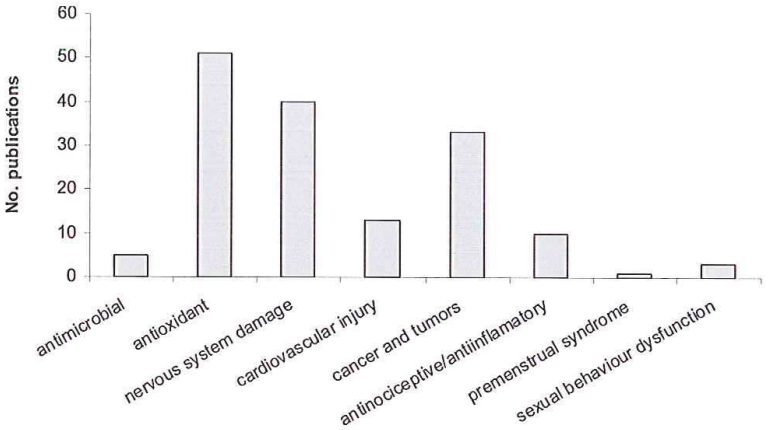


Fig. 2 Principal saffron properties investigated during the past decade (based on scopus.com and sciencedirect.com).

tion during the past decade. In addition, an attempt to relate saffron consumption with its potential healthy benefits when added to different food, so that, the effects found to be effective on animals, will be estimated in humans.

BIOLOGICAL ACTIVITIES OF SAFFRON

The main biological activity of saffron is based on its great antioxidant ability; in fact the antioxidant properties of saffron are well known and have been widely studied since this property is responsible for many of its biomedical attributes. A radical scavenging activity is involved in aging processes, anti-inflammatory, anticancer and wound healing activities, among others (Assimopoulou *et al.* 2005), so many efforts have been made in order to find natural products that posses this property. Assimopoulou *et al.* (2005) suggests that saffron could be used in functional foods, drinks with antioxidant activity and in pharmaceutical and cosmetic preparations, as well as, food supplement with antioxidant properties. Saffron extracts exhibited a remarkable intracellular antioxidant activity. Moreover, the antioxidant efficiency observed in ethanol saffron extracts was equivalent to 116 mg α-tocopherol/g (Chen *et al.* 2008). So that, it can be assumed that this property is responsible for preventing many diseases which mechanisms involve oxidation, such as neurodegenerative injury (Urrutia *et al.* 2007) and cardiovascular diseases, which are described below and injury in kidney or brain tissues caused by ischemia-reperfusion (I/R) (Hosseinzadeh *et al.* 2007b). In addition, treating thermal induced burn wounds with saffron extract cream (20%) result in a significantly increased re-epithelialization that could be explained for the antioxidant effects of this spice (Khorasani *et al.* 2008).

Other important property which converts saffron in a beneficial spice for health is their antimicrobial activity. This one has been studied under different saffron parts; it is well known that many spices such as garlic and basil are antibacterial agents (Low Dog 2006). Ethyl acetate extracts of stigma, stamen and leaves were tested on *Sthaphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli*, *Micrococcus luteus*, *Candida albicans*, *Cladosporium* sp. and *Aspergillus niger*, finding that the leave extract did not show antimicrobial activity at a concentration of 100 mg/ml. The antifungal activity of stigma was higher than stamen; in contrast, the antibacterial activity of stamen was higher than the rest of the parts studied (Vahidi *et al.* 2002).

On the other hand, the anti-*Helicobacter pylori* activity of saffron extracts, safranal and crocin was investigated using aqueous and methanol extracts and four antibiotics as control. All isolates were susceptible to methanol and aqueous saffron extracts, being the minimum inhibitory concentrations of methanol saffron extract, crocin and safranal 677, 26.5 and 16.6 μg/ml, respectively (Nakhaei *et al.* 2008). In other series of studies were determined other antiulcer properties of saffron, suggesting that saffron inhibits gastric acid secretion and stimulates mucus secretion which is a

barrier to prevent damage (Al-Mofleh *et al.* 2006). In this work saffron extracts at 250 mg/kg b.w. produce a significant decrease in the volume of gastric secretion and ulcer index in the animals tested. In addition, Kianbakht and Mozaffari (2009) studied the effects of pretreated rats with saffron extract (25, 100 and 250 mg/kg b.w.), crocin (2.5, 5 and 10 mg/kg b.w.) and safranal (0.25, 2 and 5 ml/kg b.w.), finding that these extracts, prevent gastric lesions, increase lipid peroxidation and decrease glutathione levels induced by indomethacin, effects that are comparable to omeprazole, an inhibitor protons pump, which is used as an antiulcerogenic agent.

These properties of saffron could be applied as possible therapeutic agent for a several diseases as demonstrated by several biomedical studies.

BIOMEDICAL STUDIES WITH SAFFRON

Nervous system damage

1. Neuronal injury

Saffron and its constituents: crocetin glycosides and picrocrocin were demonstrated to cause protective effects on neuronal injury acting as an antioxidant. Crocin, among the rest of the components, results the most potent antioxidant, capable of combating ischemic stress-induced neuron death (Saleem *et al.* 2006; Ochiai *et al.* 2007). In addition, 727.5 mg/kg b.w. of safranal in rats showed protective effects on hippocampal tissue from rats under ischemic conditions, elevating antioxidant capacity of the hippocampus (Hosseinzadeh and Sadeghnia 2005). Ochia *et al.* (2004) suggest that crocin inhibits apoptosis in a model cellular for neuronal differentiation, PC-12 cell line, and combats the serum/glucose deprivation-induced ceramide formation in PC-12 cells by increasing glutathione (GSH) levels and preventing the activation of a pathway for neural cell death.

2. Diabetic neuropathy

Diabetic neuropathy is one of the most frequent complications of diabetes. Vascular and neural diseases are closely related; in fact microvascular dysfunction occurs together with the progression of neural dysfunction. Neuronal ischemia is a well-established characteristic of diabetic neuropathy. The mechanisms of neurotoxicity from high glucose levels are poorly understood, but an increase on reactive oxygen species has been proposed as possible mechanism. Saffron, as antioxidant, can have neuroprotective effects. Saffron extracts and crocin were studied in glucose-induced neurotoxicity, using PC12 cells as a suitable *in vitro* model of diabetic neuropathy, showing that saffron extract (5 and 25 mg/ml) and crocin (10 and 50 μM) could decrease the toxicity caused by glucose, suggesting that saffron and crocin could be potentially useful in diabetic neuropathy treatment (Mousavi *et al.* 2009).

3. Retinal function

Recently, it was published by Maccarone *et al.* (2008) that saffron and carotene extracts (1 mg/kg b.w. /d) as feed supplementation in rats, mitigates retinal damage induced by exposure to continuous bright light (1000 lux) during 24 hrs. They mentioned that the antiapoptotic characteristic of saffron makes it interesting in the treatment of retinal neurodegenerative disease; moreover, it reduces photoreceptor death induced by environmental stresses. In another study using retinal cell cultures from bovine and primate eyes, crocin protected the photoreceptors against blue light or white light-mediated damage in a concentration dependent manner (10–160 μM) (Laabich *et al.* 2006). Finally, saffron can significantly inhibit the elevation of glutamic acid concentration, fact that contributes to neurodegeneration of retina, thus, prevents retina damage (Yang X-G *et al.* 2006). For a different type of retinal malfunction, such as ischemic retinopathy and age-related macular degeneration, which are the leading ocular diseases that cause blindness, it has been studied that crocin analogs increase the blood flow in the retina and choroid and facilitate retinal function recovery, leading to the conclusion that crocin analogs could be used to treat this problem (Xuan *et al.* 1999).

4. Alzheimer's disease

Alzheimer's disease is the most common form of dementia among people over 65 years old which is characterized by cognitive impairment and memory deterioration, promoted by deposition of amyloid β-peptide (Aβ) fibrils that is caused by oxidation. Thus, to identify agents inhibiting the pathogenesis of Alzheimer's disease, the antioxidant properties of *C. sativus* were examined on Aβ fibrils and compared with that of tomato and carrot by Papandreou *et al.* (2006). The results showed that saffron extracts at concentrations of 300 and 600 μg/ml had twice the antioxidant activity than tomato and carrot extracts. In addition, *C. sativus* stigmas extract significantly inhibited the formation of amyloid fibrils in a concentration and time-dependent manner. In conclusion, the study resulted to demonstrate that saffron extract has antioxidant and antiamyloidogenic activity; as a result, it has a positive effect on cognitive function, indicating that saffron may be valuable for prevention or delay of Alzheimer's disease. Recently, a clinical trial with 54 patients of 55 years old or older, with mild-to-moderate Alzheimer's disease, using a saffron capsule of 30 mg/day, provides preliminary evidence of saffron possible therapeutic effect (Akhondzadeh *et al.* 2010).

5. Parkinson's disease

Parkinson's disease is a terminal, progressive neurodegenerative disorder. A cure has not been developed yet, so many efforts for relief the symptoms have been done. The causes of the disease are marked by generation of excessive free radicals but the exact mechanism is still unclear (Ahmad AS *et al.* 2005; Ahmad M *et al.* 2005; von Bohlen und Halbach *et al.* 2005). The neuromodulatory effects of crocetin (75 μg/kg b.w.) were studied resulting in a neuronal protection from a catecholaminergic neurotoxin that causes loss of cells in the substantia nigra (Ahmad AS *et al.* 2005), mechanism that could be helpful for reducing Parkinson.

6. Seizures

Since traditional medicine, saffron has been used as anticonvulsant agent, but its mechanisms of action deserve further study. Seizures are produced when neurons are activated in an unusually synchronous manner, disturbing the balance between excitation and inhibition and altering several classic neurotransmitters systems such as the glycine, glutamatergic and GABAergic (Engelborghs *et al.* 2000). Depressant effects on the central nervous system are at least partly responsible for inhibiting the alterations mentioned above. Given that the current therapeutic treatment using antiepileptic drugs is associated with side-effects, plants, such as saffron, would be helpful in this treatment, as shown by Hosseinzadeh and Khosravan (2002), who found that ethanolic (0.2-2.0 g/kg b.w.) and aqueous (0.08-0.80 g/kg b.w.) saffron extracts increased the latency of convulsions induced by pentylenetetrazol (PTZ), a popular chemoconvulsant, in a dose-dependent manner and decreased the duration of tonic seizures caused by electroshock. Safranal (0.15-0.35 mg/kg b.w.) showed anticonvulsant behaviour as well, in PTZ-induced seizures (Hosseinzadeh and Talebzadeh 2005). Besides, Hosseinzadeh and Sadeghnia (2007) studied deeply these properties of safranal showing that peripheral administration of safranal (72.75, 145.5 and 291 mg/kg b.w.) exerts a dose dependent decrease in minimal clonic seizure (MCS) induced by PTZ and first generalized tonic-clonic seizures (GTCS). The exact mechanisms of saffron action are unclear yet.

7. Learning behaviour

Several studies have reported that saffron extracts and two of its main ingredients crocin and crocetin, improved memory and learning skills in ethanol-induced learning behavior impairments in mice and rats (Sugiura *et al.* 1994; Abe *et al.* 1999; Abe and Saito 2000), suggesting that oral administration of saffron may be useful as treatment for neurodegenerative disorders and related memory impairment. Recently, rats treated with 30 and 60 mg/kg b.w. of saffron extracts were capable of discriminate between familiar and novel objects (Pitsikas and Sakellaridis 2006), finding the enhancing effects of crocetin esters on memory and its implication in the mechanisms underlying recognition and spatial memory (Pitsikas *et al.* 2007).

8. Anxiety

The traditional therapeutic potential of crocetin esters in anxiety was investigated using a light/dark chamber test in rats. The results showed that crocin at 50 mg/kg b.w. reduced the anxiety of animals but the mechanism that might account for this effect was not determined (Pitsikas *et al.* 2008). In addition, the anxiolytic and hypnotic effects of saffron (56, 80, 320 and 560 mg/kg b.w.) and safranal (0.15 and 0.35 ml/kg b.w.) were similar to diazepam, which is used in pharmacology as a sedative. Safranal was confirmed as anxiolytic in a dose-dependent manner (Hosseinzadeh and Noraei 2009).

9. Sedative/relaxant

The sedative effects of saffron are well known since traditional medicine, and it was confirm by Boskabady and Aslani (2006). Aqueous-ethanolic saffron extract (0.15-0.6%g) and safranal (0.15-0.60 ml containing 0.2 mg/ml solution) showed a potent relaxant effect that is comparable or even higher than theophylline, a relaxing drug. To corroborate the mechanism of action, another study was published, suggesting that relaxation is due to saffron stimulatory effects on β-adrenergic receptors being superior to its agonist's available (Nemati *et al.* 2008). β-adrenoceptors agonists, such as saffron, stimulate the liver, kidneys, increase heart rate and heart contractility rate (Boskabady *et al.* 2008), vasodilatation due to petals (Fatehi *et al.* 2003) and bronchodilation, to which can be attributed the proven antitussive effect of safranal and ethanolic extracts of saffron stigma (Hosseinzadeh and Ghenaati 2006). Relaxant properties of saffron could be useful for treating different conditions described below.

10. Depression

Herbal treatments, including saffron, as antidepressant agents have been widely studied. There is strong evidence that, stigmas, petals, safranal and crocetin esters of saffron exert an antidepressant activity. Since a few years ago, efforts has been made by some research groups, especially in Iran, in order to know the doses of the different extracts that can be useful to treat this disorder. It was observed during 6 weeks 30 patients, that if saffron (30 mg/day) is compared with imipramine (100 mg/day), a antidepressant drug, saffron could be of therapeutic benefit in the treatment to mild to moderate depression (Akhondzadeh *et al.* 2004). As well as safranal (0.15-0.5 mg/kg b.w.) and crocin (50-600 mg/kg b.w.), that were proved to be effective on mice (Karimi *et al.* 2001). Fluoxetine activity, which is a common drug used for treating this disorder, can be compared with aqueous and ethanolic saffron extracts and with kaempferol obtained from saffron petals (Hosseinzadeh *et al.* 2004, 2007a). In the same way, the effect of kaempferol has been studied in 40 depressed patients (between 18 and 55 years) concluding that a treatment of 30 mg/day of a petal extract during 8 weeks and 30 mg/day of a stigma extract during 6 weeks can be helpful for treating this condition (Noorbala *et al.* 2004, 2005; Moshiri *et al.* 2006; Akhondzadeh *et al.* 2007). Finally, Akhondzadeh *et al.* (2008) concluded that, being petals less expensive than stigmas and exerting the same activity could represent a new alternative treatment.

Cardiovascular injury

1. Atherosclerosis

Hyperlipidemia is characterized for abnormal levels of lipids or lipoproteins in the blood stream causing thickness of the arteries' wall leading to a cardiovascular disease named atherosclerosis. Since several efforts have been made in order to know more about this mechanism and its prevention, the possibility of using antioxidants, such as crocin, as an inhibitor of this disease has been evaluated. There is evidence that crocin (25, 50 and 100 mg/kg b.w.) decrease greatly the content of cholesterol, triglyceride and density lipoprotein in blood and increase the content of high density lipoprotein (He *et al.* 2005; Xu *et al.* 2005). Moreover, thiobarbituric acid reactive substances decrease and plasma lipid levels remain unchanged in high lipid diet rabbits (Zheng *et al.* 2006). Sheng *et al.* (2006) confirmed that crocin (25, 50 and 100 mg/kg b.w.) significantly reduced serum triglyceride, total cholesterol, LDL cholesterol and VLDL cholesterol. In the same way, crocin suppressed the absorption of fat and cholesterol. In addition, crocetin can prevent the adhesion of leukocyte to bovine endothelial cells (BEC), which is important because adhesion and migration of leukocyte to endothelial cells is one of the early key steps in the atherosclerosis. This activity may be related to the antioxidant properties of saffron and protection for mitochondrion (Xiang *et al.* 2006). Furthermore, Sheng *et al.* (2006) found that the hypolipidemic effect of crocin was due to its inhibition of pancreatic lipase activity, being this enzyme the key to digestion and absorption of fat, so much effort has been directed to search an inhibitor. Crocin doses from 0.1 to 10,000 μg/ml, result in a dose-dependent, reversible inhibition of lipase that was more potent than the inhibition of gastric lipase (Sheng *et al.* 2006). Recently, another study revealed that saffron had superior hypolipidemic effect than crocin (Asdaq *et al.* 2009).

2. Myocardial infarction

Myocardial infarction (MI) is an acute condition of necrosis of the myocardium that occurs as a result of imbalance between myocardial demand and coronary blood supply. It is well established that reactive oxygen species have been implicated in the pathophysiology of MI and antioxidants suppress its formation. Therefore, the effects of crocin in cardiotoxicity isoproterenol induced were studied. Crocin at 20 mg/kg b.w./day, administered during 21 days, significantly modulated hemodynamic and antioxidant derangements, suggesting a cardioprotective effect through modulation of oxidative stress in such a way that maintains the redox status of the cell (Goyal *et al.* 2010; Joukar *et al.* 2010). In adittion, crocetin has beneficial effects on blocking inflammatory cascades caused by hemorrhage/resuscitation on cardiac injury at doses of 50 mg/kg b.w. (Yan *et al.* 2010).

3. Peripheral vascular diseases

It has been reported that the platelet-rich thrombi are the indispensable sources of thromboembolic complications, such as atherosclerosis, heart attacks, strokes, and peripheral vascular diseases. Therefore, inhibition of platelet functions represents a promising approach for the prevention and treatment of cardiovascular diseases, such as thrombosis. Crocetin effects on platelet activity and thrombosis formation were demonstrated showing a dose-dependent inhibition of platelet aggregation and significantly attenuation of dense granule release, as well as, prolonged the occlusive time in electrical stimulation-induced carotid arterial throm-

bosis. These findings suggest that the favourable impacts of crocetin on platelet activity and thrombosis formation may be related to the inhibition of Ca_2 elevation in stimulated platelets (Yang *et al.* 2008). In accordance with these results, other study using blood from healthy volunteers evaluated the inhibitory activity of saffron extract on human platelets, confirming a dose-dependent inhibition (Jessie and Krishnakantha 2005).

4. Insulin resistance

Insulin resistance is a condition in which normal levels of insulin are inadequate to produce a normal insulin response, situation that is linked to genetic and environmental factors, causing hyperinsulinemia, hypertension, dyslipidemia and being one of the principal factors for developing Diabetes mellitus type 2, which may lead in a cardiovascular disease. Crocetin at doses of 20 mg/kg b.w. and specially 40 mg/kg b.w. is capable of attenuate the development of insulin resistance and the abnormalities mentioned above, as well as, restoring free fatty acid metabolism disorders, which may explain the biochemical and nutritional basis of its inhibitory action (Xi *et al.* 2007).

Cancer and tumours

Chemoprevention is defined as the use of natural or synthetic agents to prevent of block the development of cancer. The chemopreventive and antitumoral potential properties of saffron and several other spices against cancer have been extensively studied during the last decade, proposing different hypotheses for the mode of action of its constituents. The cytotoxic effect of saffron extract (200-2000 µg/ml) was evaluated by Tavakkol-Afshari *et al.* (2008) in HepG2 and HeLa malignant cell lines, resulting in a decrease of viability of malignant cells in a concentration and time-dependent manner, fact confirmed by Feizzadeh *et al.* (2008). Saffron doses inducing 50% cell growth inhibition (IC50) values against HeLa and HepG2 were determined as 800 and 950 µg/ml after 48 hrs, respectively. It was concluded that saffron can cause cell death in which apoptosis or programmed cell death plays an important role (Tavakkol-Afshari *et al.* 2008). The cytotoxic and antitumor properties of saffron petals have been also studied, being the IC50 values of stigma and petal extract against tumour, 5.3 and 10.8 mg/ml (Hosseinzadeh *et al.* 2005), respectively. On the other hand, the genotoxic potential of anti-tumour drugs limits their efficacy in the treatment of cancers, so a study was designed to ascertain the chemoprotective potential of saffron against the genotoxicity of cisplatin, cyclophosphamide and mitomycin, three well known antitumor drugs. Saffron doses of 20, 40 and 80 mg/kg b.w. significantly inhibited the cellular DNA damage induced by the antitumor drugs, suggesting that saffron could be an adjuvant in chemotherapeutic applications (Premkumar *et al.* 2006).

1. Skin cancer

Skin carcinogenesis is a malignant growth of the epidermis that could be caused by UV-A and UV-B- radiation that generate free radicals in the cells. It was found that a saffron infusion (200 mg/kg b.w./d) has a beneficial action when given before and after the induction of skin carcinogenesis. Saffron ingestion inhibited the formation of skin papillomas and simultaneously reduced their size, fact that at least in part, may be due to the induction of cellular defense systems (Das *et al.* 2010).

2. Pancreatic cancer

Pancreatic cancer accounts for a high mortality rate because it has a very poor prognosis, so new therapeutic alternatives are really needed. Given saffron antitumour activity *in vitro* and *in vivo*, the proliferation of pancreatic adenocarcinoma cells is significantly inhibited due to a crocetin treatment (4 mg/kg b.w. /d) in mice. Also, pancreatic cancer growth was also significantly inhibited because of crocetin oral treatment (Dhar *et al.* 2009).

3. Breast cancer

Crocus sativus and different types of *Crocus taxa,* endemic in Greece, containing hydrophilic carotenoids show a dose-dependent inhibitory effect on breast cancer cells proliferation, attributing this effect to crocin contents in saffron (Chryssanthi *et al.* 2007). Some authors (Bathaie *et al.* 2007; Kanakis *et al.* 2007a, 2007b) mentioned that saffron carotenoids interact with DNA and induce some conformational changes on it, having crocetin the most potential.

4. Lung cancer

This type of cancer is the leading cause of cancer related mortality worldwide; so many efforts have been done in order to reduce it. A treatment of 20 mg/kg b.w. of crocetin dissolved in dimethyl sulphoxide was administered in mice, resulting in a reversion of the pathological changes observed in cancerous animals proving the antitumor ability of this compound (Magesh *et al.* 2006).

5. Colorectal cancer

Saffron inhibition on three colorectal cancer cell lines (HCT-116, SW-480 and HT-29) was studied, finding a dose-dependent inhibition of malignant cells growth, being crocin the major responsible of this activity. Moreover, crocin did not affect normal cells growth (Aung *et al.* 2007).

Antinociceptive effects

The antinociceptive, a well known property of saffron, due to their content of flavonoids, tannins, anthocyanins, alkaloids and saponins which was confirmed using safranal at doses between 0.1 and 0.5 ml/kg b.w. (Hosseinzadeh and Shariaty 2007). However, the mechanism responsible remains to be investigated (Hosseinzadeh and Younesi 2002).

Premenstrual syndrome

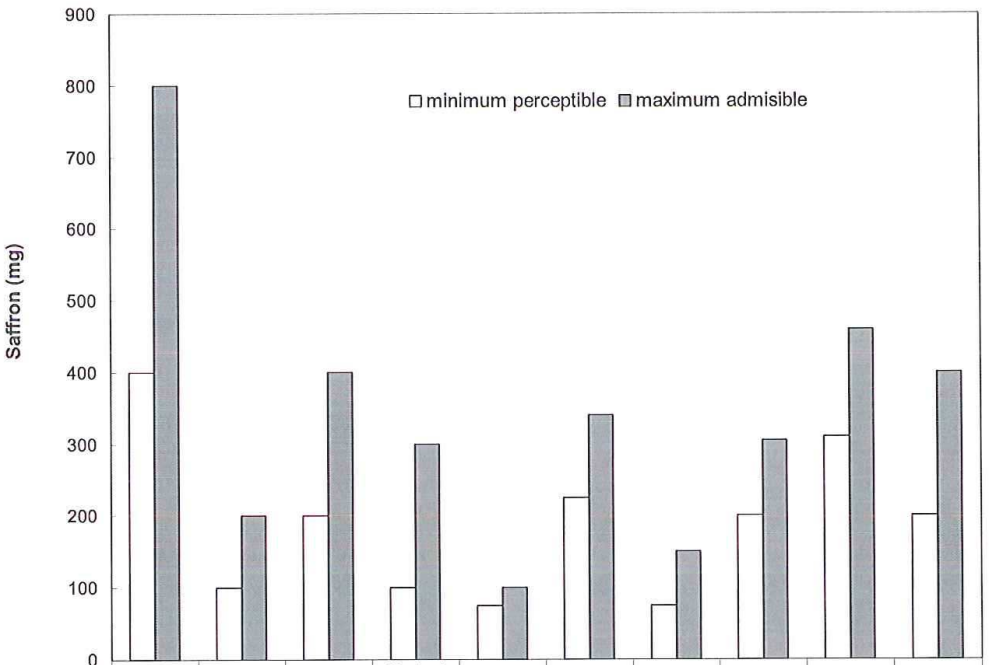
Premenstrual syndromes (PMS) are among the most common health problems reported by women of reproductive age characterised by emotional, behavioural and physical symptoms. There is an overlap between symptoms of depression and those associated with PMS, so saffron also resulted to be effective on treating this syndrome. Women between 20 and 45 years received 15 mg of saffron capsule twice a day, resulting in a relief of several symptoms. Even if the study is in line with previous reports, further research in this area is needed, because it is the first clinical trial done (Agha-Hosseini *et al.* 2008).

Sexual behaviour dysfunction and infertility

Sexual dysfunction is a serious medical and social symptom that occurs in 10-52% of men and 25-63% of women (Porst 2004). Since the available drugs and treatments for these problems have limited efficacy or side-effects, a series of plants, such as saffron, have been proved to have aphrodisiac effects. This fact is confirmed in a study using saffron extracts (80, 160 and 320 mg/kg b.w.) and crocin (100, 200 and 400 mg/kg b.w.), resulting an activity compared to sildenafil, an phosphodiesterase inhibitor, commonly used for treating erectile dysfunction, unlike safranal that showed a vasodilator effect (Hosseinzadeh *et al.* 2008). Recently, a pilot study was published, conducted with twenty male patients with erectile dysfunction, in which 200 mg of dried saffron stigma were taken orally during ten days every morning. After this period of time there was a statistically significant improvement on sexual function with increased number and duration of erectile events (Shamsa *et al.* 2009).

Table 1 Biomedical properties of saffron studied using patients.

Properties	References	Doses (saffron mg)	Frequency
Sexual behaviour dysfunction and infertility	Shamsa *et al.* 2008	200	Daily, 10 days
	Heidary *et al.* 2008	50	3 times a week, 12 weeks
Alzheimer	Akhondzadeh *et al.* 2010	30	Daily, 22 weeks
Depression	Akhondzadeh *et al.* 2004; Noorbala *et al.* 2004, 2005; Moshiri *et al.* 2006; Akhondzadeh Basti *et al.* 2007	30	Daily, 6-weeks
	Akhondzadeh Basti *et al.* 2008	30	Daily, 8-weeks
PMS	Agha-Hosseini *et al.* 2008	30	Daily, 8-weeks



	Oils	Fish soup	Meat soup	Mashed vegetables	Stew	Bread	Rice	Croissant	Pasta	Infussion
minimum perceptible	400	100	200	100	75	225	75	200	310	200
maximum admisible	800	200	400	300	100	340	150	305	460	400

Fig. 3 Minimum and maximum admissible saffron doses in different dishes (mg/L). Based on but modified from Verdú Cantó 2009.

Aside, the effects of saffron on men with idiopathic infertility was proved to be effective, based on an intake of 50 mg of saffron 3 times a week during 3 months, but further research are needed (Heidary *et al.* 2008).

Other studies

More biological applications of saffron and its constituents have been studied such as encephalomyelitis (Ghazavi *et al.* 2009), the hormone changes in pituitary-testis axis of mice (Nazem *et al.* 2009), possible fertility improvement (Ai *et al.* 2009), as a treatment for hemorrhagic shock (Yang R *et al.* 2006), the effects on the fetal development of mice (Golalipour *et al.* 2008), the efficacy against pneumonia (Mannan *et al.* 2006), pancreas-protective effects of saffron ethanolic extracts (Mohajeri *et al.* 2009), protective effects against nephrotoxicity (Boroushaki and Sadeghnia 2009), tyrosinase inhibitory activity (Li and Wu 2002), morphine dependence inhibition (Sahraei *et al.* 2008), among others, but further study needs to be done, in order to know more about the mechanisms of action of saffron and its constituents.

SAFFRON INTAKE AND HUMAN EQUIVALENT DOSES TRANSLATION

Many reviews have been published in the past recent years (Deng *et al.* 2002; Abdullaev and Espinosa-Aguirre 2004; Schmidt *et al.* 2007; Soeda *et al.* 2007; Kianbakht 2008) in order to synthesize saffron properties and its related research, but it is difficult to understand the human repercussion of these studies because most of them use animal doses that are not directly related neither to human doses nor saffron consumption. Studies using patients, principally Iranian, are very few, as presented in **Table 1**, treating problems related to sexual behaviour and depression, principally. For treating sexual behaviour, the studies used 50 and 200 mg during several weeks, in order to increase the number and duration of erectile events. In depression studies, they use unique doses, independently of the body weight of the patient, being 30 mg daily of saffron during 6 or 8 weeks the most common dose used for depression that cause an improvement.

The 30 mg dose per day can easily be attained eating saffron in different food dishes, as shown in **Fig. 3**, which presents minimum perceptible and maximum admissible doses of saffron (mg) per litre of different dishes such as soups, rice, pasta and pastry products, including a saffron

Table 2 K_m factors of different species for conversion of animal doses to human equivalent doses based on BSA.

Species	Weight (kg)	BSA (m^2)	K_m factor
Adult Human	60	1.6	37
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Mouse	0.02	0.007	3

FDA Draft Guidelines 2002

infusion. Most of them can be prepared using between 300 and 500 mg maximum of saffron per litre of food, being oil preparation the product that use more saffron, but its concentration is diluted taking into account that oil is used to prepare a wide variety of dishes and not eat alone. Saffron infusion represents a good way to increase saffron consumption because it is drink directly, without quantity restrictions. A cup of 100-150 ml can contain the active compounds corresponding to the mentioned dose of 30 mg of saffron per day.

In order to compare animal doses used in the majority of the studies with possible human doses, it is necessary to transform these quantities using the body surface area (BSA) normalization, because of converting the safe starting dose based on body weight alone, can result an inappropriate comparison between studies because of the lack of correlations for oxygen utilization, caloric expenditure, blood volume, circulating plasma proteins and renal functions between various mammalian species and differently sized members of the same species, including humans (Reagan-Shaw *et al.* 2007). In this study the recommendation of the U.S. Food and Drug Administration of using BSA normalization has been employed for the purpose to calculate the hypothetical human equivalent doses (HED), parameter used on initial clinical trials in healthy adult volunteers.

The customary approach for calculation of BSA uses the Du Bois height-weight formula: BSA (m2) equals body weight (kg b.w.)$^{0.425}$ multiplied by height (cm)$^{0.725}$ multiplied by 0.007184, has been re-evaluated in similar forms with updated constants, however scientific evidence does not favour one alternative formula over another (Sawyer

Table 3 Human equivalent doses calculated for the different saffron animal studies.

Effects	Reference	Animal	Saffron product	Frequency	Saffron equivalent doses (mg/kg b.w.)[a]	HED (mg/person)[b]
Biological activities						
Antioxidant	Hosseinzadeh *et al.* 2007b	Rats	Ethanolic extract	Mono dose	5 - 80	57 - 908
			Crocin		172 - 1379	1 957 - 15 657
			Safranal		14 505 - 72 523	164 646 - 823 229
Ulcers	Al-Mofleh *et al.* 2006	Rats	Extract	Mono dose	250	2 838
	Kianbakht and Mozaffari 2009	Rats	Extract		25 - 250	284 - 2 838
			Crocin		8 - 35	88 - 391
			Safranal		362 613 - 725	4 116 144 - 8 232
					225	286
Nervous system damage						
Neuronal injury	Hosseinzadeh and Sadeghnia 2005	Mice	Safranal	Mono dose	109 234	1 239 956
Retinal function	Maccarone *et al.* 2008	Rats	Extract	Mono dose	1	11
Parkinson	Ahmad M *et al.* 2005	Rats	Crocin	Daily, 7 days	0.3	3
Seizures	Hosseinzadeh and Khosravan 2002	Mice	Ethanolic extract	Mono dose	0.2 - 2	1 - 11
			Aqueous extract		0.1 - 0.8	0.45–5
	Hosseinzadeh and Talebzadeh 2005	Mice	Safranal		21 757 - 50 766	123 484 - 288 130
	Hosseinzadeh and Sadeghnia 2007	Rats	Safranal		10 923 - 43 694	123 996 - 495 982
Learning behaviour	Pitsikas and Sakellaridis 2006	Rats	Extract	Mono dose	30 - 60	341 - 681
	Pitsikas *et al.* 2007	Rats	Crocin	Daily	52 - 103	587 - 1 174
Anxiety	Pitsikas *et al.* 2008	Rats	Crocin	Mono dose	172	1 957
	Hosseinzadeh and Noraei 2009	Mice	Extract		56 - 560	318 - 3 178
			Safranal		21757 - 50 766	123 484 - 288 130
Sedative/relaxant	Hosseinzadeh and Ghenaati 2006	Guinea pigs	Ethanolic extract	Mono dose	100 - 800	1 514 - 12 108
			Safranal		36 261 - 108 784	548 819 - 1 646 457
Depression	Karimi *et al.* 2001	Mice	Safranal	Mono dose	23 - 75	128 - 426
			Crocin		172 - 2 079	979 - 11 743
	Hosseinzadeh *et al.* 2004	Mice	Aqueous extract		160 - 320	908 - 1 816
			Ethanolic extract		200 - 800	1 135 - 4 541
Cardiovascular injury						
Atherosclerosis	Sheng *et al.* 2006	Rats	Crocin	Daily, 10 days	86 - 1 250	979 - 14 189
	Asqad *et al.* 2009	Rats	Extract	Daily, 5 days	25 - 100	284 - 1 135
Myocardial infarction	Goyal *et al.* 2009	Rats	Crocin	Daily, 21 days	69	783
	Yan *et al.* 2010	Rats	Crocin		172	1957
Pheripheral vascular diseases	Yang *et al.* 2008	Rats	Crocin	Daily, 2 days	86 - 625	979 - 7 095
Insulin resistance	Xi *et al.* 2007	Rats	Crocin	Daily, 8 weeks	69 - 138	783 - 1 566
Cancer and tumours						
Cancer and tumours	Premkumar *et al.* 2006	Mice	Extract	Daily, 5 days	20 - 80	114 - 454
Skin cancer	Das *et al.* 2009	Mice	Extract	Daily, 7 days	200	1 135
Pancreatic cancer	Dhar *et al.* 2009	Mice	Crocin	Daily, 30 days	14	78
Lung cancer	Magesh *et al.* 2006	Mice	Crocin	Daily, 4 weeks	69	391
Antinociceptive effects						
Antinociceptive	Hosseinzadeh and Shariaty 2007	Mice	Safranal	Mono dose	14 505 - 72 522	82 323 - 411 614
Sexual behaviour dysfunction						
Sexual behaviour dysfunction	Hosseinzadeh *et al.* 2008	Rats	Extract	Mono dose	80 - 320	908 - 3 632

[a] Doses were converted to mg/kg b.w. of saffron equivalent, taking into account a saffron humidity of 9%, 0.66% safranal content and 32% on dry basis of crocetin content
[b] HED were calculated using K_m factors based on BSA. The final HED was multiplied by a body weight of 70 kg

and Ratain 2001; Wang and Hihara 2004; Verbraeclen *et al.* 2006; Reagan-Shaw *et al.* 2007). BSA is often represented in mg/m2 and can be translated to human equivalent doses (HED) in mg/kg b.w. according to this formula: HED (mg/kg) equals to animal dose (mg/kg) multiplied by animal Km/ human Km (Reagan-Shaw *et al.* 2007) using factors named K_m factors, for the different species summarized on **Table 2**.

This study pretends to calculate a tentative HED from the animal doses of the different saffron studies and link it to saffron consumption in different dishes. This work it is a first attempt to suggest a tentative reference for human doses that can not be taken lightly, because of the lack of pharmacokinetics studies in the bibliography and other very important data such as LD_{10} values, bioavailability, absorption and elimination kinetics of the saffron compounds in humans. Human equivalent doses for the different saffron properties are shown in **Table 3**, calculations are based on the range of doses proved on each animal study that did not cause toxicity and exerted a noticeable effect. Doses were converted to mg/kg b.w. of saffron equivalent, taking into account an average saffron moisture of 9% (Carmona *et al.* 2006), up to 0.66% safranal content (Maggi *et al.* 2009) and up to 32% crocetin ester content (Sánchez *et al.* 2009). Differences between aqueous and ethanolic extracts were not considered. The final human dose was multiply by a body weight of 70 kg.

From **Table 3**, it can be observed that some of these doses are really approachable for adults, such as antioxidant activity (57-908 mg), depression (128-426 mg) and learning behaviour (341-681), being seizures (1-11 mg) and Parkinson the diseases that needs less saffron doses for its prevention or amelioration. In the other hand, studies which used safranal, show doses that can not be achieved by just eating dishes with saffron, taking into account that safranal content in saffron represents a 0.66% of its composition and in the studies the majority of safranal source was a standard of high purity.

Saffron intake in food, as shown by data presented in this work, could represent a good way to achieve different biological effects and taken daily as a habitude, could represent a preventing method for many diseases, specially taken as an infusion.

CONCLUSION

Saffron has been investigated during years in a wide variety of different biological effects. A big part of these effects are achieved by its antioxidant properties, since it is responsible for many chemical reactions that have effects on preventing many diseases, such cardiovascular and neuronal injury, among others. It is recommended further investigation and clinical trials because of deficiencies on some studies conducted and the lack of pharmacokinetics studies in order to have a better correlation between animal and human doses. Saffron consumption in food could represent a good source for preventing many diseases. Doses of clinical trials made in human patients can be achieved by consuming saffron in food, especially as an infusion.

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5.2 Optimization of saffron addition for cheese fabrication

5.2.1 Approach

After having saffron doses that can be added to ewes' milk cheese, four cheese fabrications at laboratory scale were manufactured intended to determine how saffron could be added: directly in the curd during molding, saffron added to milk before rennet, saffron added to milk before rennet and in the rind, and saffron previously extracted in milk and added before rennet. Results from these fabrications are presented in Appendix 8.1.

It was decided that the best way to add saffron to cheese was making a prior extraction directly in the milk. This methodology can also be used for fabrication of different dairy products and not only to cheese. An experimental design was established to test different extraction factors: temperature, time and saffron concentration. Moreover, milk fat content was also considered because as mentioned in *Chapter 3,* ewes' milk fat composition has marked changes during lactation.

Results obtained from this study were patented and published after. The patent No. P200930912 (2009) is pending and is presented in Appendix 8.2. It includes the saffron doses in cheese and its extraction conditions in ewes' milk. The exact saffron concentration and extraction process use during cheese fabrication is not mentioned in this doctoral thesis to protect industry ownership of this information. Results were published in the following scientific paper:

Preliminary study of saffron (*Crocus sativus* L. stigmas) color extraction in a dairy matrix

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5.2.2 Extended summary

The objective of this work was to study saffron extraction conditions in terms of color in ewes' milk. The parameters tested were: time (20, 40 and 60 minutes), temperature (37, 50 and 70 °C), saffron concentration (2, 4, 6, 8, 10 mg/ml) and milk fat (1.6, 6 and 9 %). Results from the color obtained from the saffron milk extracts were expressed in terms of CIEL*a*b* and CIEL*C*h color spaces.

Results showed that saffron color extraction was primarily influenced by saffron concentration and milk fat content, only slight changes were observed with temperature and extraction time was not significant.

Increasing saffron concentration resulted on increasing red colorations but decreasing on brightness, yellow, tone and saturation. Coordinate a* was the coordinate more influenced by changes on this parameter.

Saffron milk extracts showed L* values ranging from 57 to 74, coordinate a* had values between 11 and 33 and coordinate b* between 71 and 84. Chroma (C*) values were between 79 and 86 and hue values (h) ranged from 64 to 81. All these values are translated in vivid yellow-red colorations, as observed in Figure 20.

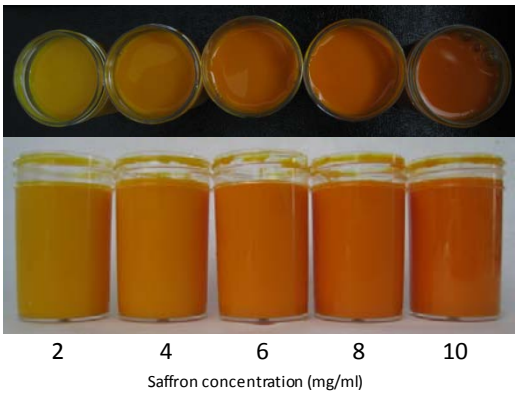

Figure 20. Saffron milk extracts on ewes' milk cheese with 1.6 % milk fat

Milk fat influence resulted to be opposite of saffron concentration because as milk fat was increased, extracts were more bright and yellow and hue increased, while red and saturation decreased. Coordinates L* and a* were the most influenced.

Temperature had less accused effect on saffron milk extracts. As increasing extraction temperature the extracts were slightly brighter and yellower while less red. Nevertheless, it was necessary to increase or decrease temperature in 12 to 20 °C in order to see color changes. Extraction time was not significant for any color coordinate, however is important because it has been demonstrated that saffron degradation depends on time as well.

As a conclusion, it was decided that the saffron extraction in milk should be carried out between 37 and 70 ° C during 20 minutes, considering that changes on milk fat content and saffron concentration would lead to different coloration in the extracts.

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Preliminary study of saffron (*Crocus sativus* L. stigmas) color extraction in a dairy matrix

C.C. Licón [a], M. Carmona [b], R. Rubio [a], A. Molina [a], M.I. Berruga [a,*]

[a] Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR, Universidad de Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain
[b] Albacete Science and Technology Park, Campus Universitario, 02071 Albacete, Spain

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ABSTRACT

Saffron spice has been used for decades as an ingredient in many dairy products but changes in its coloring properties related to milk characteristics have not been paid appropriate attention. Saffron color was studied in ewes' milk at different fat levels and saffron concentrations using tristimulus colorimetry. In order to evaluate saffron extraction, different temperatures and extraction times were tested. Color changes were demonstrated to be statistically significant when increasing the fat content in milk, as well as saffron concentration. The higher milk fat content, turned the extracts brighter and yellower, while less red and vivid, opposite to results obtained by increasing saffron concentration. Extraction time was not significant for color extraction. Milk extracts resulted slightly brighter and yellower when increasing temperature, probably due to crocetin esters degradation or isomerization from *trans* to *cis* configuration. Temperatures between 37 and 70 °C are recommended to avoid structural changes in milk or saffron. Color changes could be due to interactions mediated by phospholipids between milk fat globules and crocetin esters, as well as minor saffron carotenoids.

1. Introduction

Color is one of the main food characteristics influencing consumer preferences and it is also a tool for evaluating food freshness and quality. Colorants have been used as additives since ancient times to make food more attractive and even healthier [1,2].

Saffron spice, the dried stigma of *Crocus sativus* L., has been appreciated since Mesopotamian times up to the present time not only for its biological, aromatic and flavoring properties, but particularly due to its color. Saffron color and its coloring strength ($E^{1\%}_{1\,cm}$ 440 nm) are the two most important factors determining the quality of the spice [3–6]. The compounds responsible for the yellowish red hues of this spice are mainly glicosilated esters of dicarboxilic acid named crocetin (commonly known as crocins). These compounds are water soluble due to a saccharide link with glucose, gentibiose or neapolitanose [1]. Nevertheless, different carotenoids have also been found as a minor fraction of the total pigments such as phytoene, phytofluene, tetra-hydrolycopene, β-carotene, ξ-carotene, zeaxanthin and lycopene, but their color influence in saffron filaments has not been deeply studied as they are despicable compared with crocetin esters presence [6–8].

Nowadays, the techniques used to determine the main quality characteristics of saffron, including the coloring strength, are measured by UV–Vis spectrometry at 440 nm, diluting a saffron sample in water. Besides, HPLC is also used to find adulterations and to analyze saffron components, i.e. crocetins esters, picrocrocine and kaempferol. The later includes several extraction steps using solvents in some cases [9]. A colorimetric reflection method has been found to have a linear correlation between the chromatic parameters measured and coloring strength in saffron powder and it was considered a useful tool for saffron quality control [4,10,11].

Several Mediterranean countries use saffron as part of many traditional dishes such as risotto, paella, bouillabaisse, as well as an ingredient in pastries, puddings, liquors or sauces [1]. Also, some cheeses include saffron in their process, but their usage is very limited because they are locally produced in a small scale. They vary in form, weight and ripening period. The most known saffron cheese is the Piacentinu Ennese, which is an ewes' milk hard cheese from Sicily with a Protected Designation of Origin. In addition, there are more ewes' milk cheeses, such as semi-hard Box cheese in Germany and Cacio allo Zafferano hard cheese in Italy. Besides, there is a Swiss-type cows' milk cheese called Lüneberg in Austria and a fermented goats' milk cheese called Bouchon allo Zafferano in Lombardia. Moreover, in Italy, there is a spreadable cheese, which

* Corresponding author. Tel.: +34 967 59 9200x2615; fax: +34 967 599238.
E-mail addresses: carmencecilia.licon@uclm.es (C.C. Licón), manuel.carmona@uclm.es (M. Carmona), raquel.rubio@uclm.es (R. Rubio), ana.molina@uclm.es (A. Molina), mariaisabel.berruga@uclm.es (M.I. Berruga).

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includes saffron. All of these products are artisanal and not widespread because its production is scarce.

However, adding saffron to milk is more complex than adding it to water, because of fat content and casein colloidal suspension. In addition, physico-chemical composition of milk is variable and mainly influenced by breed, feeding, milking system or lactation stage [12]. Particularly in ewes' milk, fat and protein are susceptible to modify their concentration during lactation stages by increasing from 4 to 10% and from 4.8% to 6%, respectively [13], having the highest fat content, compared to goat (3.8%) and cow (3.6%) [14]. Standardization, which involves skimming or blending skim and whole milk, is a common practice used in dairy industry to obtain the same fat to protein ratio during the whole lactation period. Nevertheless, sheep milk industries are often small scales factories and they do not practice standardization, which highlight the importance of studying the composition factor [15]. Since colorant addition is a critical factor, it is necessary to better understand saffron solubility and color extraction in milk, because both properties will possibly be affected by fat and protein variability.

Saffron color extraction techniques focus mostly on methanolic, ethanolic or aqueous solutions [6,16,17]; however, saffron behavior using a milk matrix has not been studied yet. In order to optimize extraction conditions leading to improvements on the fabrication of dairy products, saffron color extraction in ewes' milk by tristimulus colorimetry was studied.

2. Materials and methods

2.1. Ewes' milk

Three 16-L batches of ewes' milk with different milk fat contents were used. Batch A, commercial semi-skimmed UHT ewes' milk, had a fat content of 1.6% (w/v) (Gaza, Zamora, Spain); batch B, obtained from the experimental farm of Instituto Técnico Agronómico Provincial had a 6.0% of fat content (Diputación de Albacete, Spain) and batch C, from the Experimental farm of Universidad de Castilla-La Mancha, had a fat content of 9.0% (Albacete, Spain). Milk from batches B and C were pasteurized at 72 °C during 20 s. The milk was kept at 4 °C no more than 24 h up to the time when analysis was performed.

Dry matter and protein content of the three milk batches were obtained using a MilkoScan analyzer (Foss, Hillerod, Denmark).

2.2. Saffron extraction

165 g Spanish saffron spice (*C. sativus* L.) from the 2007 harvest of the Protected Designation of Origin "Azafrán de la Mancha" was used. Saffron spice was grounded and characterized according to ISO 3632 Technical specification [5]. Its quality characteristics include moisture and volatile matter content, bitterness ($E_{1\ cm}^{1\%}$ 257 nm) aroma (safranal; $E_{1\ cm}^{1\%}$ 330 nm) and coloring strength ($E_{1\ cm}^{1\%}$ 440 nm). Different powder saffron concentrations were weighted using an analytical scale (Ohaus, AV4101, United States) and were dissolved in each batch of milk. The mixture was prepared in milk following the specification of the pending patent No. P200930912.

2.3. Experimental design

The experiment consists on a multilevel factorial design based on the percentage of milk fat (1.6, 6.0 and 9.0%); temperature of the saffron color extraction (37 ± 4, 50 ± 4 and 70 ± 4 °C); extraction time (20, 40 and 60 min) and saffron concentration (2, 4, 6, 8 and 10 mg mL^{-1}), giving a total of 135 extractions. All treatments were done by triplicate.

2.4. Color measurement

In order to follow saffron spice color extraction in ewes' milk, reflected color was measured at 20, 40 and 60 min using a Minolta CR-400 colorimeter (Minolta Camera Co., Osaka, Japan) with a CR-a33f cone and a calibrated white plate (Minolta 11333110) with $Y = 93.1$, $x = 0.3160$ and $y = 0.3323$. D65 illuminant and an angle vision of 10° were used. CIE L*, a^* and b^* coordinates were obtained from the milk and the saffron milk extracts. L^* corresponds to brightness, a^* value to the red−green component and b^* value represents the yellow−blue component. The color measurement was made in transparent polystyrene 60 mL bottles (Deltalab, Spain), that contained 50 mL of the sample, introducing the colorimeter 2 mm in the liquid and using a white background. Duplicate measurements for every sample at each sampling time were obtained. These values were used for the calculation of hue angle (h = arc tangent [b^*/a^*]) and chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$], expressing intensity and color saturation respectively.

2.5. Statistical analysis

When evaluating composition and color differences between milk batches, analysis of variance (ANOVA; $P < 0.05$) was performed using Statgraphics Plus 5.1. Besides, to know the effect of milk fat content and saffron concentration in the saffron milk extracts, Tukey's test at a significance level of $P < 0.05$ was used to determine differences between the factors studied in each case. A General Linear Model (GLM) was performed to determine the effects of saffron concentration, milk fat, temperature, extraction time and the interactions between these factors on L^*, a^*, b^*, $C^*$and h coordinates.

3. Results

Nowadays, a technique capable of determining crocetin esters from saffron in a complex matrix, such as milk, has not been developed. Available techniques for saffron-milk analysis using HPLC which include several extraction steps, i.e. milk skimming, solvent addition and washing, are not suitable for this case because possible resulting changes in the structure of saffron−milk complex. Due to the former reasons, tristimulus colorimetry was used to establish the capability of ewes' milk for saffron color extraction.

The raw materials used in this study were saffron spice and commercially available ewes' milk which include a wide range of milk fat contents. Table 1 presents the characterization of these two materials, including milk composition and color of the three batches of milk used according to the fat content. On the other hand saffron spice; moisture, volatile content, coloring strength at $E_{1\ cm}^{1\%}$ 257 nm, $E_{1\ cm}^{1\%}$ 330 nm, $E_{1\ cm}^{1\%}$ 440 nm and quality categories according to ISO 3632/TS are presented [5].

Milk fat had a significant effect ($P = 0.000$) on dry matter, reaching a higher value as milk fat was increased and protein presented a slight increase between the three milk batches. Regarding to color, higher percentage of milk fat increased L^* and a^* coordinates, while decreasing b^* values. Coordinates a^* and b^* of batch C resulted to be statistically different ($P \leq 0.004$) from the rest of milk batches, while L^* was different in batch A (Table 1).

Saffron spice characterization resulted as follows; dry matter and volatile content were below the maximum allowed for saffron spice (Table 1), coloring strength at all wavelengths resulted to be above the minimum value specified for category I.

In order to study the influence of saffron concentration and milk fat content on the color coordinates of the saffron milk extracts, color values obtained at 37 °C during an extraction time of 20 min were taken as a reference (Table 2). The values of all color

Table 1
Milk and saffron spice characterization.

	Parameter	Milk Fat (%, w/v)			ANOVA
		1.6 (A)	6.0 (B)	9.0 (C)	
Milk	Dry Matter (%, w/v)	13.45 ± 0.01^{x}	18.72 ± 0.41^{y}	20.51 ± 0.15^{z}	0.000
	Protein Content (%, w/v)	5.95 ± 0.37	6.19 ± 0.55	6.32 ± 0.09	0.267
	L^*	85.03 ± 0.85^{x}	87.28 ± 0.70^{y}	88.34 ± 0.95^{y}	0.002
	a^*	-3.58 ± 0.13^{x}	-3.54 ± 0.20^{x}	-2.83 ± 0.35^{y}	0.004
	b^*	7.10 ± 0.24^{y}	6.96 ± 0.37^{y}	5.48 ± 0.73^{x}	0.003
		Saffron Spice Studied	Categories[a]		
			I	II	III
Saffron	Moisture and Volatile Content % máx	10.02 ± 0.16	12	12	12
	$E^{1\%}_{1\ cm}$ 257 nm Dry Basis, min	104.00 ± 12.72	70	55	40
	$E^{1\%}_{1\ cm}$ 330 nm Dry Basis, min	38.50 ± 0.05	20	20	20
	Dry Basis, max		50	50	50
	$E^{1\%}_{1\ cm}$ 440 nm Dry Basis, min	265.00 ± 4.24	190	150	100

^{x,y,z}, different letters within rows mean significant differences ($P < 0.05$).
[a] According to ISO/TS 3632-1 (2003).

coordinates for the studied saffron milk extracts were in the positive axis of the CIEL*a*b* chart. Values of L^* coordinate ranged between 57 and 74, coordinate a^* had values between 11 and 33, thus red colorations. Coordinate b^* had values from 71 to 84, showing yellow colorations. Regarding to chroma (C^*), the values ranged from 79 to 86, approaching to vivid color. Hue values (h) obtained were between 64 and 81, i.e. tones which oscillate from red to yellow.

The obtained effect increasing saffron concentration for some coordinates was opposite to obtained results increasing milk fat. L^*, b^* and h values decreased as saffron concentration was increased but increased as milk fat was augmented. In an opposite way, while a^* coordinate increased its value with saffron concentration, milk fat content decreased it. In the case of coordinate C^*, its values increased as milk fat was increased and the effect with saffron concentration was irregular. Extracts with 6 and 9% of fat showed

Table 2
CIE L^*, a^*, b^*, $C^*$and h coordinates on saffron extracts at 37 °C and 20 min

Color Coordinates	Saffron Concentration (mg mL^{-1})	Milk Fat (%, w/v)			ANOVA
		1.6 (A)	6.0 (B)	9.0 (C)	
L^*	2	$69.77 \pm 1.83^{d,x}$	$71.78 \pm 1.80^{d,x}$	$74.51 \pm 0.65^{c,y}$	0.000
	4	$64.98 \pm 0.72^{c,x}$	$66.53 \pm 1.72^{c,x}$	$70.93 \pm 0.97^{b,y}$	0.000
	6	$60.68 \pm 0.70^{b,x}$	$63.92 \pm 1.91b^{c,y}$	$69.16 \pm 1.92^{b,z}$	0.000
	8	$59.37 \pm 2.87^{ab,x}$	$61.98 \pm 1.16^{ab,xy}$	$64.63 \pm 0.73^{a,y}$	0.001
	10	$57.78 \pm 0.76^{a,x}$	$60.46 \pm 0.94^{a,y}$	$62.86 \pm 1.31^{a,z}$	0.000
	ANOVA	0.000	0.000	0.000	
a^*	2	$14.98 \pm 1.26^{a,y}$	$14.13 \pm 1.08^{a,y}$	$11.32 \pm 1.33^{a,x}$	0.000
	4	$23.35 \pm 1.85^{b,y}$	$22.34 \pm 0.76^{b,y}$	$17.95 \pm 1.16^{b,x}$	0.000
	6	$28.47 \pm 0.64^{c,z}$	$26.56 \pm 0.71^{c,y}$	$23.06 \pm 1.67^{c,x}$	0.000
	8	$31.05 \pm 1.18^{d,y}$	$29.59 \pm 0.72^{d,xy}$	$29.33 \pm 1.23^{d,x}$	0.029
	10	$33.97 \pm 0.83^{e,z}$	$32.39 \pm 0.45^{e,y}$	$30.71 \pm 1.04^{d,x}$	0.000
	ANOVA	0.000	0.000	0.000	
b^*	2	78.66 ± 2.86^{c}	78.72 ± 4.05	81.96 ± 0.71^{b}	0.110
	4	$77.80 \pm 1.62^{bc,x}$	77.87 ± 3.14^{x}	$83.07 \pm 2.67^{b,y}$	0.004
	6	$75.54 \pm 0.94^{bc,x}$	76.68 ± 4.57^{x}	$84.34 \pm 0.24^{b,y}$	0.000
	8	$74.57 \pm 3.46^{ab,x}$	75.81 ± 2.00^{xy}	$78.38 \pm 1.48^{a,y}$	0.048
	10	$71.44 \pm 1.38^{a,x}$	75.47 ± 0.99^{y}	$76.70 \pm 2.58^{a,y}$	0.000
	ANOVA	0.000	0.385	0.000	
C^*	2	79.35 ± 2.85^{a}	80.06 ± 2.82	81.85 ± 1.84^{a}	0.246
	4	$84.16 \pm 1.55^{b,xy}$	82.67 ± 2.42^{x}	$86.10 \pm 1.89^{b,y}$	0.030
	6	81.75 ± 1.44^{ab}	83.15 ± 2.74	85.98 ± 3.77^{b}	0.056
	8	$81.49 \pm 1.52^{ab,x}$	81.63 ± 3.19^{x}	$84.99 \pm 1.19^{ab,y}$	0.021
	10	$79.20 \pm 1.41^{a,x}$	83.06 ± 0.89^{y}	$84.27 \pm 1.06^{ab,y}$	0.000
	ANOVA	0.000	0.214	0.015	
h	2	$79.22 \pm 0.83^{e,x}$	$79.79 \pm 1.18^{e,x}$	$81.87 \pm 0.51^{d,y}$	0.000
	4	$73.31 \pm 1.08^{d,x}$	$73.96 \pm 1.07^{d,x}$	$77.82 \pm 0.43^{d,y}$	0.000
	6	$69.35 \pm 0.36^{c,x}$	$70.86 \pm 0.91^{c,y}$	$73.76 \pm 1.20^{c,z}$	0.000
	8	$67.35 \pm 1.59^{b,x}$	$68.67 \pm 0.91^{b,xy}$	$69.49 \pm 0.91^{b,y}$	0.022
	10	$64.57 \pm 0.31^{a,x}$	$66.77 \pm 0.23^{a,y}$	$68.17 \pm 0.54^{a,z}$	0.000
	ANOVA	0.000	0.000	0.000	

^{a,b,c,d,e}, different letters within columns mean significant differences ($P < 0.05$).
^{x,y,z}, different letters within rows mean significant differences ($P < 0.05$).

higher values with higher saffron concentration, meanwhile values of the extracts with 1.6% of milk fat increased with a saffron concentration of 4 mg mL^{-1} decreasing then when higher concentrations were tested (Table 2).

In order to evaluate the influence on the color coordinates of each variable tested (saffron concentration, milk fat, temperature and extraction time), a General Linear Model was performed. Equations obtained by the GLM are shown in Table 3. Saffron concentration and milk fat content showed significant differences in all color evaluated coordinates. Temperature only affected L^*, a^* and h coordinates, while extraction time did not exerted any influence on color.

It can be observed from Table 3 that all models resulted statistically significant ($P = 0.000$). The correlation coefficient (R^2) for the coordinates L^*, a^* and h were adequate by being closer to 100%, however b^* and C^* coordinates presented lower R^2 values than 100%; consequently they could not be as properly estimated as the other coordinates. Low standard error of the estimation (S.E.E) values corresponds with the higher R^2 values. Coordinates L^*, b^*, h and C^* were significantly reduced ($P < 0.001$) as saffron concentration increased, contrary to the former behavior, values of coordinate a^* increased while saffron concentration increased ($P < 0.001$). The most affected coordinate by saffron concentration was a^* implied by its coefficient (2.11), leading to redder extracts. According to GLM equation, it is required a slight increase of 0.47 mg mL^{-1} of saffron spice to increase one a^* unit, comparing to 0.69 and 2.43 mg mL^{-1} to decrease one L^* and b^* unit, respectively.

GLM coefficients also demonstrated that values of coordinates L^*, b^* and h increased as milk fat was increased ($P < 0.001$), while a^* and C^* decreased ($P < 0.05$). The most affected coordinates by milk fat variation were L^* and a^* as shown by its coefficients (3.26 and −1.93). This means that milk with higher fat content makes the extracts brighter; an increase of 0.3% of milk fat is sufficient to rise one L^* unit. Also the extracts are less red, being enough 0.5% of milk fat increase to drop one unit of a^* coordinate.

The above mentioned behavior confirms the saffron concentration and the milk fat effects displayed by the ANOVA analysis on the color coordinates, except for coordinate C^* which resulted to have an opposite effect when all factors were included on its analysis.

Temperature had a less accused effect than saffron concentration or milk fat content on the saffron milk extracts, showing a coefficient lower than 0.1. Significant positive effect on L^* and h values ($P < 0.001$) and a negative effect on coordinate a^* ($P < 0.001$) were obtained. According to GLM predictions, every increment of 12 and 16 °C, increases one unit the L^* and h values respectively; an increment of 20 °C, decrease one unit the a^* coordinate values. Extraction time had no influence on any of the coordinates studied.

Interaction between saffron concentration and milk fat was positive for coordinates a^* and C^*. Nevertheless, the obtained coefficients were less than 0.1, similar to those showed by temperature.

4. Discussion

Results showed that ewes' milk color was influenced by fat content, particularly with a fat content of 9.0%, which is characteristic of the end of lactation period, showing a brighter but lesser red and yellow color than the rest of the milk batches. L^* coordinate results are in accordance to Popov-Raljic, Lakic, Lalicic-Petronijevic, Barac and Skimic [18] who mentioned that a panel of sensory analysis perceived that milk fat provides a positive effect on brightness, finding whole cow milk brighter than semi-skim milk. Regarding to coordinate b^*, the results obtained in ewes' milk, differs from Frost, Dijksterhuis and Martens [19] who observed in cow milk that panelists perceived a yellower milk color as fat was increased. These differences could be caused by the chemical differences between cow and ewes' milk, since cow milk generally contains more carotenes than ewes' milk [20]. Moreover, protein content also increase with fat content, but this increment was not statistically significant; nevertheless, casein is responsible for the white color of milk. More protein has a direct influence on more disperse molecules in milk, especially caseins micelles, thus increasing brightness [21].

Saffron spice resulted to be quality grade I, which is the best quality that can be obtained for saffron spice, according to ISO 3632 [5]. Saffron quality is a very important factor to take also into account when carrying out extractions. It was demonstrated that saffron from different quality grades, origins and dehydration process, showed different values for CIEL*a^*b^* color coordinates in water solutions and saffron filaments [10,11]. Using saffron spice with different characteristics or quality grades will lead to different color results in any extract.

Regarding to saffron milk extracts, the GLM showed that the fat content of ewes' milk as well as saffron concentration in the saffron milk extracts resulted to have an influence in all of the studied color coordinates ($P < 0.001$; Table 3).

Coordinates a^* and b^* were demonstrated to have a different behavior in ewes' milk than saffron milk extracts when fat content is increased. In the saffron milk extracts, values of coordinate a^* decreased and b^* increased, while in ewes' milk these behavior is opposite (Tables 1 and 3). Brightness (L^*) was the most influenced variable in the saffron milk extracts by milk fat effect (Table 3), increasing its values as milk fat was augmented. These differences confirm saffron influence in the saffron milk extracts.

Table 3
Factor and interactions affecting CIEL*a^*b^*, CIEL*C^*h coordinates in saffron extractions using ewes' milk.

Model[a]	Color Coordinates				
	L^*	a^*	b^*	h	C^*
R^2	88.86	90.18	18.14	90.91	13.80
S.E.E[b]	1.64	2.28	3.92	1.56	3.72
P	0.000	0.000	0.000	0.000	0.000
Constant	63.17 ± 0.88	18.00 ± 0.78	76.19 ± 0.60	78.24 ± 0.47	82.01 ± 0.63
Saffron Concentration[c]	−1.44 ± 0.02^{***b}	2.11 ± 0.11***	−0.41 ± 0.05***	- 1.69 ± 0.02***	−0.49 ± 0.12***
Milk Fat[c]	3.26 ± 0.28***	−1.93 ± 0.23***	0.98 ± 0.24***	0.49 ± 0.07***	−0.20 ± 0.1*
Temperature[c]	0.08 ± 0.02***	−0.05 ± 0.01***	–	0.06 ± 0.01***	–
Time[c]	–	–	–	–	–
Saffron Concentration * Milk Fat	–	0.09 ± 0.03**	–	–	0.08 ± 0.01*

[a] Significance levels for each factors are indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. The model shows interactions that were significant at least for one coordinate.

[b] S.E.E. standard error of the estimation.

[c] Saffron concentration was between 2 and 10 mg mL^{-1}, milk fat between 1.6 and 9% of milk fat, extraction temperatures were between 37 and 70 °C, and extraction time between 20 and 60 min.

Regarding the effect of saffron concentration in saffron milk extracts, L^* and b^* values decreased despite of fat content, while a^* values increased. These results are in agreement with Satyanarayana et al. [22]. They studied bixin solutions, a carotenoid that gives orange–red coloration, soluble in medium polar solvents and widely used in dairy matrix, concluding that purity and concentration of this carotenoids is essential to color shades in food [22,23], as resulted saffron concentration in this study.

Values of the color coordinates of batches A and B belong to the same group when the saffron milk extracts contained 2 and 4 mg mL^{-1}, except for C^* which the behavior was irregular and presented the smallest prediction value. As saffron concentration increased, values of coordinates L^*, a^* and h, were separated into different groups depending on fat content, which corresponded to batches A, B and C, e.g. saffron concentration above 4 mg mL^{-1} makes the extracts different between fat levels. This was confirmed by the interaction between saffron concentration and milk fat, unless it resulted significant only for coordinates a^* and C^* (Table 3). This interaction could indicate a synergy between milk fat and higher saffron concentration to obtain changes in the color of saffron milk extracts, especially for coordinate a^*.

Temperature had a moderate effect on the observed color coordinates during extraction, increasing L^* and hue values thus making the extracts slightly brighter and yellower. In saffron, crocetin esters are mainly present in a *trans* configuration but could possibly be isomerized to *cis* configuration by temperature. These structural changes modify the UV–Vis spectra (440 nm vs 435 nm) between these two isomers varying the coloration as well [1].

When extractions were carried out at 37 °C small slightly colored saffron filaments could be observed. On the other hand at 70 °C a foam film was formed possibly due to proteins and fat lumps floating over the surface, it made difficult the colorimeter measurements, even though the color of the saffron milk extracts were in all cases homogeneous. Some studies suggest that carotenoids, such as crocetin esters, could be degraded because of temperature treatments. Thermal degradation of these compounds has been studied suggesting that coloring strength is better extracted at room temperatures [6,24]. In the same way, Sánchez et al. [6] suggests to use temperatures between 30 and 70 °C, taking into account extraction time. Based on the previous discussion, the obtained results and the observation of the extracts, it is agreed and recommended to use temperatures above 37 °C and below 70 °C, in order to prevent problems derived from foam formation, fat lumps and avoidance of changes in the structure of proteins, milk fat and lactose. Some authors have demonstrated that heat treatment application to milk molecules cause denaturalization, isomerization, lactose degradation, non-enzymatic browning reactions, changes in the structure and compound formation such as lactulose and hidroximetilfurfural [2,25]. In general, hard cheeses use temperatures between 37 and 55 °C. These temperatures are in accordance to the temperatures selected in the study. Nevertheless, spreadable cheeses need higher temperatures, so this fact should be considered, as well as the different operations of the dairy industry, such as homogenization, pasteurization, fermentation, among others.

Extraction time was not significant for any of the color coordinates studied; nevertheless, some authors [6,24,26] concluded that large periods of extraction time cause loss of coloring strength, as well as saturation. Sánchez et al. [6] indicated that crocetin esters are degraded after 56 h at 30 °C, or after 23 h at 50 and 70 °C. As extraction time in this study did not influenced the color of the extracts, and degradation of crocetin esters is dependant of time and temperature, extraction time of 20 min is considered adequate for milk saffron extracts, an important finding for the dairy industry as long extraction periods are not necessary to achieve coloration.

The different behavior of color coordinates in ewes' milk and saffron milk extracts according to the fat content could be explained by different reasons. More milk fat content has a direct consequence in the dry matter content implying the percentage of water is lower. Crocetin esters present in saffron are water soluble substances that give reddish or yellow color to water depending on the used concentration, this fact has not been properly studied in aqueous extracts. In this matrix, as saffron concentration is increased a^* values increased while b^* values decreased. Water content is lower due to higher fat, water could concentrate crocetin esters in the aqueous phase causing a^* values to increase and b^* values to decrease however, the behavior is opposite, a^* values decreased and b^* values increased, suggesting an interaction between saffron color compounds and milk components. Interactions between fat globules and crocetin esters could be influencing color changes in saffron milk extracts. This interaction could be mediated by the phospholipids and the amphiphilic proteins that cover the fat globules and avoid coalescence of fat in the milk serum [27]. Phospholipids represent about 60% and 40%, in whole and skim milk respectively, of the fat globule membrane, having two charged groups in the molecule, thus giving polar properties. The phosphate group present in the molecule could be interacting with any of the sugars present in crocetin esters [28].

Besides, saffron carotenoids, such as β-carotene, ξ-carotene, zeaxanthin and lycopene, which its influence on color has been despised in saffron studies because it represents a minor fraction, could be interacting also with the fat globules, since they are liposoluble compounds.

Even more, it has been demonstrated that some milk proteins are capable of forming unions with carotenoids and vitamins, especially β-lactoglobulin and bovine serum albumin. They can bind a variety of small molecules since the amphiphilic structure of most milk proteins confers excellent surface properties [29–31]. The complex is based on hydrophobic interaction between proteins and carotenoids and it was demonstrated that polar groups of some carotenoids were involved in the binding [32].

Consequently, it can be assumed that many mechanisms of interaction exist between saffron and the milk components (proteins, water, fat, etc); further research regarding milk–saffron interaction is needed to find out which milk and saffron components are involved and which types of interactions are formed.

5. Conclusions

Saffron color extraction in milk depends on several factors such as saffron quality, origin and milk characteristics. Increasing saffron concentration in the milk extracts resulted in a less bright and yellow, more red (a^*) and paled (C^*) extracts, starting with yellow hues and going to red hues (h). Milk fat exerted a marked influence on the extracts, meaning that ewes' milk with more fat would result on brighter and yellower, less red and vivid extracts with a yellower hue. Increasing temperature affected saffron color extraction, resulting on brighter (L^*) and yellower (b^*) extracts. Extraction time did not influence the extracts' color. Ewes' milk was demonstrated as an appropriate matrix for saffron color extraction. Saffron color extraction in milk could be due to interactions between milk fat, proteins, water and crocetin esters, but also between hydrophobic carotenoids in saffron, but further research in needed to gain better understanding.

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5.3 Main physico-chemical and microbiological characteristics of pressed ewes' milk cheeses with saffron and its consumer acceptance

5.3.1 Approach

Before large scale cheese fabrications, an experiment to determine if saffron was able to inhibit lactic acid bacteria necessary to cheese fabrication was done using three saffron doses. This experiment is presented in Appendix 8.3. As a result, saffron inhibition was not observed at any saffron concentration.

For large scale cheese production, four cheese fabrications were done using three different saffron concentrations and a control, as shown in *Chapter 4*. The fabrication process is shown in Appendix 8.4, as well as fabrication parameters (pH, temperature and color).

Main composition, microbiology, texture and color of cheeses were analyzed during ripening for six months. Triangular, ranking and sensory analyses were also carried out. The following paper is based on the characterization of these pressed ewes' milk cheeses and its consumer acceptance.

Chemical, microbiological, textural, color and sensory characteristics of pressed ewes' milk cheeses with saffron (*Crocus sativus* L.) during ripening

Licón, C.C., Carmona, M., Molina, A. and Berruga, M.I.

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5.3.2 Extended summary

The first effect of saffron addition was that saffron cheeses needed approximate one hour more than control cheeses to reach a pH values around 5.2 during pressing, even that lactic acid bacteria inhibition was not found *in vitro* assays. This was probably because control cheeses showed slightly higher total and lactic acid bacteria counts showing a possible antimicrobial properties of saffron against these bacteria. This fact resulted to have influence on several factors: slightly higher pH values and dry matter content, lower salt content and lower values of some nitrogen fractions in saffron cheeses. Texture was affected by saffron addition as well; control cheeses fractured more easily and were less deformable and elastic than saffron cheeses.

As expected, color was the parameter that had evident changes as saffron concentration was increased. Cheeses with more saffron were less bright, more red and especially more yellow. Moreover, it was observed that cheese color was also influenced by air exposure, resulting on less bright and red and more yellow cheeses. This fact is important because it has to be considered when commercializing the product.

Other remarkable result was that compositional and microbiological differences between control and saffron cheeses were less accused with ripening, so that, by the end of the period studied, 180 days, no differences were found. Even so, color and textural differences were still observed.

Sensory analysis was done at three stages of ripening: 60, 120 and 180 days. Color and flavor attributes were analyzed by separate in order to avoid color influences on flavor perception. Results showed that color and flavor differences between control and cheeses with the lowest saffron concentration were noticeable for consumers at all stages of ripening studied. When saffron cheeses were compared between each other, color differences were evident but as cheeses were more ripened, these differences were less marked.

Preference test between control and saffron cheeses (Appendix 8.5) showed that panelist did not have a significant preference for a specific color. Nevertheless at two months of ripening assessors preferred the color of the cheeses with lower saffron concentration. Regarding flavor preference the only significant difference was found at month four when the lowest saffron concentration was preferred. From this results, it can be inferred that consumers were able to notice if cheeses had saffron or not but they preferred cheeses less intense in terms of color.

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Chemical, microbiological, textural, color, and sensory characteristics of pressed ewe milk cheeses with saffron (*Crocus sativus* L.) during ripening

C. C. Licón,* M. Carmona,† A. Molina,* and M. I. Berruga*[1]
*Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR, Universidad de Castilla-La Mancha, Campus Universitario 02071, Albacete, Spain
†Albacete Science and Technology Park, Campus Universitario 02071, Albacete, Spain

ABSTRACT

Adding saffron to dairy products represents an innovative practice to introduce them to niche markets. This paper represents a contribution to this field, as few studies have evaluated the influence of this spice on general aspects and ripening parameters of cheese. In this work, pasteurized ewe milk pressed cheeses with saffron were made to study compositional, microbiological, color, textural, and sensory characteristics in relation to saffron concentration and ripening time. The main changes were observed on sensory characteristics and color. In addition compositional, textural, and microbiological changes could be observed; among them, saffron cheeses were firmer and more elastic but less prone to fracture. A remarkable result that could lead to further studies is that saffron addition slightly slowed down growth of total and lactic acid bacteria. This fact caused slightly lower pH decrease rate during pressing and, as a consequence, lower salt and water content. Compositional differences were not evident by the end of the ripening period.
Key words: ewe milk cheese, saffron color, cheese ripening, texture

INTRODUCTION

Sheep milk production is of high importance in Mediterranean countries, especially Greece, Italy, and Spain, where cheese is one of the main food products in the diet. In Spain, 99% of ewe milk production is converted into cheese, either pure or mixed with other types of milk. In 2010, 44,800 tons of pure ewe milk cheeses were produced, a figure which represents only 14.83% of the total cheese production in Spain (FENIL, 2011). Ewe milk cheese is mostly produced on a small local scale, so most of these cheeses are not as competitive as cow milk cheeses. This small industry must coexist with bigger ones and introduction into niche markets is mandatory to remain competitive (Dubeuf et al., 2010). Innovation in cheese has been in part driven by addition of several ingredients, such as colorants, spices, leaves, flavor, and aroma agents. A common practice consists in adding a natural yellow to orange color to some cheeses using spices such as annatto, which is used in Cheddar and Mimolette manufacturing, among others (Harbutt, 2010). The extraction of annatto has been widely studied (Preston and Rickard, 1980; Chisté et al., 2011) in terms of application to different matrixes (Berset and Marty, 1986; Calvo and Salvador, 2000), color stability (Shumaker and Wendorff, 1998; Martley and Michel, 2001), and degradation (de O. Rios et al., 2005).

Although in other cheeses different spices are used to obtain a yellow to orange color similar to paprika and saffron, studies concerning the coloring influence of these ingredients on different cheese attributes are scarce. In the particular case of Piacentinu Ennese, which is one of the most well-known saffron cheeses, there are studies regarding physicochemical differences between artisanal and industrial cheeses and between cheeses produced with saffron from different origins (Horne et al., 2005; Carpino et al., 2008). Nevertheless, no studies have evaluated the influence of saffron on the ripening process.

The first attempt to study saffron behavior in a dairy matrix has recently been published (Licón et al., 2012). This work evidenced the influence of milk composition, namely fat, on the final color of saffron milk extracts. Moreover, a simple and short extraction method was proposed, optimizing its addition to cheesemaking. This fact is important because saffron remains an expensive spice, mostly because it is harvested by hand. Besides, compounds responsible for saffron color are highly water-soluble carotenoids, known as crocetin esters, and with milk being an emulsion, a study regarding saffron behavior in the milk was mandatory.

Saffron not only gives color to food but also flavor and aroma, a rare combination for a single spice. Saffron's characteristic bitter taste is given by picrocrocin, whereas the aroma mainly comes from safranal (Car-

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[1]Corresponding author: mariaisabel.berruga@uclm.es

mona et al., 2006). In addition, evidence has been presented by Licón et al. (2010) concerning the biomedical properties of saffron in humans that could help with medical problems such as Alzheimer's disease, depression, sexual behavior dysfunction, and infertility.

To diversify ewe milk cheeses and promote consumption of both traditional products, this study attempts to determine whether saffron addition alters the main compositional, microbiological, textural, and sensory characteristics in pure ewe milk cheese and its possible influence on the ripening process.

MATERIALS AND METHODS

Experimental Design

Four different batches of pressed ewe milk cheese were made. Each one consisted in making 4 vats of cheese (300 L) from the same milk tank. These 4 vats included the control vat and 3 vats with saffron added. Saffron addition to the first vat was labeled **S**, because its exact concentration is protected by a pending patent (Berruga Fernández et al., 2009). The second vat, referred to as 2 × S, had 2 times the S concentration, and the third vat contained 3 times the S concentration (3 × S).

Nineteen pieces (~3 kg each) were taken from each vat and ripened. Eight ripening stages were fixed at 3, 15, 30, 60, 90, 120, 150, and 180 d for analysis and 2 cheese pieces at each stage were used. The cheese pieces were divided for their analysis as follows: the microbiological sample was obtained from the internal part of the first piece of cheese and after that, cheeses were cut in halves. From the 4 halves, 2 halves were used for texture (one half from each piece), one half for composition, a quarter of a piece for pH, and the last quarter for color determination. In addition, on d 60, 120, and 180, an additional third piece was used for sensory analysis.

Cheesemaking

Cheeses were manufactured at a local factory (Quesería Campo Rus, Cuenca, Spain), using Manchega breed ewe milk from their own supply. The milk composition (g/100 g) had an average DM content**[AU1: +/– standard deviations?]** of 19.43 ± 0.54, fat content of 7.66 ± 0.38, and protein content of 6.19 ± 0.17. The total bacterial count was 5.91 log cfu/mL. Each vat used 300 L of pasteurized (72°C, 20 s) milk. Saffron addition was done after milk pasteurization, according to the pending patent (Berruga Fernández et al., 2009). For cheese manufacturing, a starter culture containing *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* biovar diacetylactis, and *Streptococcus thermophilus* was added (CHOOZIT MA4001; Danisco, Sassenage, France) at 5 Danisco culture units (DCU)/100 L. Milk was held at 30°C during 20 min, adding 0.025% (vol/vol) of $CaCl_2$ and 0.01% (vol/vol) of lysozyme. Commercial rennet was used for coagulation (chymosin:pepsin, 94:6) at 0.023% (vol/vol). Thirty minutes later the curd was cut into 8- to 10-mm cubes and heated (37°C) and stirred for 45 min before whey separation. Curd was molded and pressed using a pneumatic press (1 bar**[AU2: Please convert to an SI unit (e.g., Pa).]**) for the amount of time needed for the pH to reach 5.2, which was between 4 and 5 h. Pieces of cheese weighing approximately 3 kg were obtained. Cheeses were placed in brine (18% NaCl wt/vol) for 18 h at 9°C. After that, they were kept in a cold chamber (9°C) for 48 h and then were ripened in a maturation chamber at 11 ± 1°C and relative humidity**[AU3: Please confirm that RH represents relative humidity.]** 85% for 180 d. Surface molds were removed when necessary.

Physicochemical Profile

Two centimeters of rind were removed from the cheeses prior to performing analyses. After that, cheese was grated to a uniform grain size (Moulinex, Lyon, France). A pH meter Crison GPL 22 (Crison, Barcelona, Spain) was used for pH determination using a Crison 5232 probe. Dry matter content was determined by drying the sample to a constant weight at 102°C following the International Dairy Federation standard (IDF, 1982). Fat content was determined by the Gerber-Van Gulik method according to the International Organization for Standardization (**ISO**, 2008). Total nitrogen was measured using the Kjeldahl method (AOAC, 1998) and protein was obtained multiplying by a factor of 6.38. Sodium chloride content was determined by using an infrared analyzer FoodScan (Foss Electric A/S, Hillerød, Denmark). Cheese nitrogen fractions were obtained according to IDF (1991). Water-soluble nitrogen (**WSN**), soluble nitrogen at pH 4.6 (**SNpH4.6**), soluble nitrogen in 12% TCA, and soluble nitrogen in 5% phosphotungstic acid were obtained. Soluble nitrogen for these fractions was determined by the Kjeldahl method (AOAC, 1998). All determinations were done in duplicate.

Microbiology

To perform microbial analyses, samples were obtained from the internal part of the cheese with a cheese sampling tool. Two centimeters of rind was removed and cheese samples of 10 g were obtained. Samples were

homogenized with 90 mL of sterile 0.1% (wt/vol) peptone water in a masticator (IUL SA, Barcelona, Spain) for 60 s. Decimal dilution of the homogenates was prepared with 0.1% (wt/vol) peptone water (Scharlau, Barcelona, Spain) and seeded in the corresponding medium in duplicate using a Eddy Jet spiral plater (IUL SA). Total aerobic bacterial counts were performed on plate count agar (PCA; Panreac Química S.L.U., Barcelona, Spain) after incubation at 32°C for 48 h. Lactic acid bacteria were plated on M17 agar (Biokar Diagnostics, Barcelona, Spain) with incubation at 37°C for 48 h. Violet red bile agar with glucose was used for enterobacteria incubation (VRBG; Biokar Diagnostics) at 37°C for 24 h. Molds and yeasts were seeded in rose bengal agar (RB; Scharlau) and incubated at 25°C for 120 h. *Pseudomonas* was grown in cetrimide agar (base) with a cephaloridine Fucidin cetrimide (CFC) selective supplement (Biokar Diagnostics) incubated at 25°C for 120 h. Plates ranging from 30 to 300 cfu were selected for counting. A Countermat Flash (UIL SA) was used for this purpose. Counts were expressed as common logarithm of colony-forming units per gram of sample.

Color Measurement

Tristimulus colorimetry [Commission Internationale d'Éclairage (**CIE**) L*a*b* color space, where L* represents luminance and ranges from 0 for black to 100 for white, a* represents the color's position between red/magenta and green, and b* represents the color's position between yellow and blue], was used to measure cheese color. A Minolta Colorimeter CR-400 (Minolta, Osaka, Japan) with an illuminant D65 and a 10° observer was used. Calibration was done using a Minolta reference white plate 11333110 with Y = 93.1, x = 0.3160, and y = 0.3323.**[AU4: Please define Y, x, and y.]** Color was directly measured in the transversal surface of the cheese, immediately after cutting the cheese in halves. Three measurements were obtained for each piece of cheese. After that, a cheese section was exposed to air at ambient temperature (~21°C). Color was determined at 30 and 60 min after cutting.

Rheological Determinations

Uniaxial Compression. Before analysis, 2 cm of rind were removed. Cubes of 25 × 25 × 25 mm were obtained using a cheese blocker (Boska Holland BV, Bodegraven, the Netherlands) from each cheese half. Cubes were kept at room temperature (~21°C) for 30 min before analysis. Uniaxial compression was carried out according to Pavia et al. (1999). Cubes previously lubricated with glycerin to avoid friction effects were compressed to 80% of their original height at a constant temperature using a TA-TX2 texture analyzer (Stable Micro Systems Ltd., Surrey, UK) with a 245 N load cell and a crosshead speed of 1.3 mm/s. The analysis was carried out in 6 cubes for each half portion. True stress and true strain values were calculated according to Calzada and Peleg (1978).

Stress Relaxation. Samples were obtained in the same way as for uniaxial compression test (12 cubes lubricated with glycerin). The test was carried at a crosshead speed of 3.3 mm/s with a 245 N load cell to a 10% compression for 2 min (TA-TX2 texture analyzer; Stable Micro Systems Ltd., Godalming, UK). The stress-relaxation curves were fit to a linear way based on a Maxwell model and rearranged according to Peleg (1979). From the equations obtained, e and r values were calculated (Juan et al., 2007). The e-values represent the equilibrium residual values of normalized relaxation stress when $t \rightarrow \infty$.**[AU5: Does t represent time?]** The term r is related to an elastic behavior for any material.

Sensory Analysis

Sensory evaluation, regarding color and flavor, consisted of triangular and ranking tests carried out on different days. Tests were done at 60, 120, and 180 d of ripening. To avoid color influence on flavor, color and flavor tests were done separately in both tests (ISO, 2004). Thirty-four untrained panelists between 20 and 60 yr old from the University of Castilla-La Mancha (Albacete, Spain) were selected for the study. All panelists met the following criteria: cheese consumers, healthy, and not subject to food allergies. Sixteen out of the total were males and 18 were females. Tasting sessions were conducted in an illuminated, odor-free, and aerated room at room temperature. Cheese samples were sliced in triangles (~0.5-cm width), presented on white plates and coded with a random 3-digit number. The order in which the samples were given to the panelists in all sessions was randomized. Flavor tests were carried out using a red light to avoid color effect on flavor perception. Panelists were instructed to rinse their mouth with water and eat apple pieces between sampling. Session times were approximately 20 min each.

Triangular test was intended to determine if consumers were able to discriminate between control and saffron cheeses. Control and cheeses with the lowest saffron concentration (S) were used. Panelists were asked to try 3 cheese samples and identify which sample was different.

The aim of the ranking test was to establish if consumers were able to classify cheeses from the lowest to

the highest saffron concentration, regarding color and flavor. For this purpose the 3 saffron cheeses were used.

Statistical Analysis

An ANOVA was performed using the SPSS version 17.0 statistical package (SPSS Inc., Chicago, IL) to determine the effects of saffron concentration and ripening time on each parameter studied ($P < 0.05$). The Tukey test at a significance level of $P < 0.05$ was used to determine differences between means for concentrations and ripening days. In addition, a general linear model (**GLM**) was performed to determine the effects of saffron concentration, ripening time and air exposure and the interactions between these parameters on L*, a*, and b* coordinates. Triangular test results interpretation was based on the ISO (2004) method. Ranking test was analyzed according to Friedman test based on the χ^2 distribution. Differences between saffron concentrations were established using a least significant difference formula based on the *t*-test (Lawless and Heymann, 2010).

RESULTS

Chemical Analysis

Tables 1 and 2 show the physicochemical parameters and nitrogen fractions of the studied cheeses (control and saffron cheeses) at different ripening stages. The pH of the cheeses after salting was around 5.2. No significant differences were found in pH values at any stage of ripening due to saffron addition, even though control cheeses had slightly lower pH values. During ripening, changes in pH values were not significant with the exception of 2 × S cheeses.

Regarding compositional profile, control cheeses showed lower ($P \leq 0.05$) DM values at d 3 and lower protein values at d 60 and 150. No significant differences were found in fat content due to saffron addition (Table 1) but lower salt content was observed ($P \leq 0.05$). The evolution of these parameters during ripening was similar for all types of cheeses. Dry matter increased ($P \leq 0.01$) during ripening; as a consequence of this, water loss and fat and protein content slightly increased with ripening time and salt content significantly increased ($P \leq 0.01$).

Regarding nitrogen fractions (Table 2), differences between control and saffron cheeses were not constant along ripening; however, at d 60, they were more accused**[AU6: What do you mean by they were more accused?]**. In general, control cheeses showed higher values for WSN, SNpH4.6, and 12% TCA fractions. The phosphotungstic acid fraction did not differ significantly between cheeses. These differences were less evident by the end of ripening period. All nitrogen fractions increased constantly ($P \leq 0.05$) during ripening.

Microbiology

No significant differences were found between control and saffron cheeses for enterobacteria, pseudomonae, yeasts, and molds (data not shown); however, total and lactic acid bacteria counts of control cheeses were slightly higher (Figure 1). These differences were only significant for specific days ($P \leq 0.01$). All microbiological groups studied dropped their counts during the ripening period ($P \leq 0.01$; data not shown). After manufacturing, the average total counts were between 8.5 and 9.3 log cfu/g and decreased to 7.0 log cfu/g at 180 d of ripening. Lactic acid bacteria were the predominant group, as expected, because cheeses were made from pasteurized milk. The counts ranged from 9.5 log cfu/g at 3 d to 7.2 log cfu/g after 180 d. A decrease in enterobacteria counts during ripening was observed. At d 3, cheeses had average counts of 2.3 log cfu/g but after 90 d they were absent (data not shown). Moreover, pseudomonads were absent starting from 15 d in all cheeses (data not shown). Yeasts and molds also decreased during ripening but their decreases were not as marked as the rest of the microorganisms; counts started at 2.0 log cfu/g and decreased to 0.7 log cfu/g (data not shown).

Color

Mean values and standard deviation obtained for CIE L*a*b* coordinates are shown in Table 3. In general, changes in L*, a*, and b* coordinates were observed as saffron concentration was increased. The L* coordinate values decreased with saffron addition after d 3 ($P \leq 0.05$); thus, saffron cheeses were less bright than control ones. This coordinate also decreased with ripening time ($P \leq 0.05$). This drop was more marked for saffron cheeses than for control ones.

Values of coordinate a* were negative for all cheeses. A remarkable result is that control cheeses showed similar a* values as 3 × S cheeses. In addition, as saffron concentration was decreased, a* values became more negative ($P \leq 0.001$), thus less red. Although changes during ripening showed significant differences ($P \leq 0.05$), except for 2 × S cheeses, these changes were not very marked.

Coordinate b* was the most influenced by saffron addition. Values increased with saffron concentration; as a result, saffron cheeses were more yellow ($P \leq 0.001$) than control cheeses. This increment was very marked between concentrations: control b* values were around

Table 1. Mean values ± standard deviation for pH and chemical composition from control and saffron cheeses

		Ripening time (d)								
Parameter	Cheese[1]	3	15	30	60	90	120	150	180	*P*-value
pH	Control	5.12 ± 0.01	5.24 ± 0.28	5.18 ± 0.09	5.19 ± 0.08	5.23 ± 0.17	5.17 ± 0.18	5.24 ± 0.06	5.19 ± 0.15	NS
	S	5.24 ± 0.13	5.30 ± 0.12	5.33 ± 0.14	5.27 ± 0.14	5.28 ± 0.15	5.24 ± 0.14	5.19 ± 0.17	5.23 ± 0.16	NS
	2 × S	5.24 ± 0.08^{ab}	5.32 ± 0.08^{b}	5.32 ± 0.11^{ab}	5.25 ± 0.05^{ab}	5.26 ± 0.13^{ab}	5.21 ± 0.13^{ab}	5.18 ± 0.13^{ab}	5.16 ± 0.06^{a}	*
	3 × S	5.22 ± 0.14	5.30 ± 0.14	5.40 ± 0.17	5.24 ± 0.11	5.24 ± 0.16	5.23 ± 0.12	5.18 ± 0.11	5.24 ± 0.11	NS
	P-value[2]	NS	NS	NS	NS	NS	NS	NS	NS	
DM (g/100 g)	Control	52.93 ± 0.47^{a,x}	57.57 ± 3.15^{ab}	59.72 ± 3.03^{abc}	61.79 ± 4.07^{bcd}	63.55 ± 2.41^{bcd}	65.47 ± 3.53^{cd}	64.83 ± 2.29^{bcd}	67.80 ± 3.53^{d}	**
	S	58.17 ± 2.55^{a,y}	61.39 ± 2.31^{abc}	60.45 ± 2.74^{ab}	63.14 ± 2.98^{bcd}	63.95 ± 2.63^{bcd}	65.84 ± 2.52^{d}	65.64 ± 2.48^{cd}	67.48 ± 2.84^{d}	***
	2 × S	57.86 ± 1.08^{a,y}	60.20 ± 3.15^{ab}	59.92 ± 2.69^{ab}	62.32 ± 2.27^{bc}	63.63 ± 2.66^{abc}	64.98 ± 3.09^{cd}	64.81 ± 1.41^{cd}	67.11 ± 2.51^{d}	***
	3 × S	57.55 ± 3.44^{a,y}	59.72 ± 1.21^{ab}	60.50 ± 3.24^{abc}	61.65 ± 2.02^{bcd}	62.68 ± 1.72^{bcd}	63.96 ± 2.65^{cde}	64.62 ± 2.10^{de}	66.70 ± 2.45^{e}	***
	P-value	*	NS	NS	NS	NS	NS	NS	NS	
Fat (g/100 g)	Control	33.53 ± 0.03	33.38 ± 3.95	35.50 ± 0.71	35.50 ± 4.34	35.25 ± 2.66	35.88 ± 2.81	35.13 ± 0.63	35.38 ± 1.38	NS
	S	32.72 ± 3.52	33.75 ± 2.38	33.81 ± 2.67	34.06 ± 2.69	34.31 ± 2.79	36.06 ± 3.03	34.82 ± 1.98	35.38 ± 2.25	NS
	2 × S	32.71 ± 2.36	32.88 ± 3.53	33.13 ± 2.50	33.69 ± 1.73	33.81 ± 2.50	34.00 ± 2.73	34.56 ± 0.62	34.25 ± 2.27	NS
	3 × S	33.42 ± 3.62	32.75 ± 3.30	33.69 ± 2.39	32.75 ± 1.44	34.06 ± 1.99	36.06 ± 2.16	34.38 ± 1.27	35.13 ± 2.36	NS
	P-value	NS	NS	NS	NS	NS	NS	NS	NS	
Protein (g/100 g)	Control	20.14 ± 0.72	21.11 ± 1.65	21.51 ± 0.09	20.72 ± 0.19^{x}	21.65 ± 1.67	22.93 ± 2.36	23.12 ± 0.79^{x}	22.98 ± 0.55	NS
	S	23.39 ± 1.16	22.67 ± 1.88	23.01 ± 0.51	23.25 ± 0.86^{y}	23.95 ± 2.03	24.04 ± 1.10	25.02 ± 1.04^{y}	24.50 ± 0.75	NS
	2 × S	23.01 ± 1.04	23.11 ± 1.17	22.86 ± 0.84	23.31 ± 0.70^{y}	23.79 ± 1.25	24.77 ± 0.84	24.64 ± 0.62^{xy}	24.74 ± 2.25	NS
	3 × S	21.94 ± 1.76	23.03 ± 2.08	23.15 ± 0.77	22.69 ± 0.49^{y}	23.55 ± 2.24	23.97 ± 0.38	23.58 ± 0.97^{xy}	25.06 ± 0.46	NS
	P-value	NS	NS	NS	**	NS	NS	*	NS	
Salt (g/100 g)	Control	1.37 ± 0.03^{a}	1.60 ± 0.11^{ab,y}	1.79 ± 0.02^{bc,y}	1.96 ± 0.03^{bc,y}	2.11 ± 0.15^{c,y}	2.07 ± 0.22^{c,y}	2.09 ± 0.18^{c,y}	2.13 ± 0.27^{c,y}	***
	S	1.01 ± 0.25^{a}	1.30 ± 0.11^{b,x}	1.61 ± 0.06^{c,y}	1.63 ± 0.17^{c,x}	1.80 ± 0.01^{c,x}	1.77 ± 0.09^{c,xy}	1.70 ± 0.03^{c,x}	1.72 ± 0.13^{c,x}	***
	2 × S	0.93 ± 0.26^{a}	1.37 ± 0.10^{b,x}	1.43 ± 0.15^{b,x}	1.62 ± 0.18^{b,x}	1.65 ± 0.12^{b,x}	1.55 ± 0.15^{b,x}	1.61 ± 0.14^{b,x}	1.63 ± 0.21^{b,x}	***
	3 × S	0.78 ± 0.46^{a}	1.38 ± 0.21^{b,xy}	1.63 ± 0.14^{b,y}	1.70 ± 0.22^{b,xy}	1.76 ± 0.01^{b,x}	1.54 ± 0.15^{b,x}	1.81 ± 0.13^{b,x}	1.70 ± 0.06^{b,x}	***
	P-value	NS	*	***	*	***	***	***	**	

[a–e]Means within a row with different superscripts differ ($P < 0.05$).

[x–z]Means within a column with different superscripts differ ($P < 0.05$).**[AU11: 2 not found in table. Change to x,y?]**

[1]S = a specific concentration of saffron added to the cheese (Berruga Fernández et al., 2009); 2 × S = twice the concentration of S; 3 × S = 3 times the concentration of S.

[2]Significance differences between saffron concentration are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Table 2. Mean values ± standard deviation for nitrogen fractions from control and saffron cheeses

		Ripening time (d)								
Parameter[1]	Cheese[2]	3	15	30	60	90	120	150	180	*P*-value
WSN/TN (g/100 g)	Control	$7.37 \pm 0.23^{a,z}$	7.95 ± 1.58^{a}	10.41 ± 0.08^{ab}	$17.91 \pm 0.04^{c,y}$	15.85 ± 2.84^{bc}	$18.11 \pm 2.18^{c,y}$	20.60 ± 3.21^{c}	19.87 ± 2.53^{c}	***
	S	$5.42 \pm 0.70^{a,x}$	7.77 ± 0.86^{ab}	9.48 ± 0.64^{bc}	$12.16 \pm 2.00^{cd,x}$	13.99 ± 2.54^{de}	$14.64 \pm 1.74^{de,x}$	16.90 ± 2.67^{ef}	20.57 ± 0.42^{f}	***
	2 × S	$6.59 \pm 0.27^{a,y}$	6.65 ± 1.28^{a}	9.24 ± 0.38^{ab}	$10.61 \pm 0.37^{bc,x}$	13.15 ± 0.59^{cd}	$14.73 \pm 0.36^{d,\ x}$	18.19 ± 1.01^{e}	18.74 ± 2.81^{e}	***
	3 × S	$6.21 \pm 0.04^{a,xy}$	6.57 ± 1.41^{a}	9.39 ± 0.55^{a}	$13.27 \pm 1.06^{b,x}$	15.41 ± 2.06^{b}	$15.77 \pm 0.35^{bc,xy}$	19.90 ± 2.28^{d}	19.41 ± 2.55^{cd}	***
	P-value[3]	**	NS	NS	***	NS	*	NS	NS	
SNpH4.6/TN (g/100 g)	Control	$7.74 \pm 0.18^{a,y}$	8.87 ± 2.23^{a}	$11.10 \pm 0.25^{ab,y}$	$16.21 \pm 0.15^{cd,y}$	14.18 ± 1.12^{bc}	$17.81 \pm 1.82^{cde,y}$	20.15 ± 1.28^{de}	21.26 ± 0.76^{e}	***
	S	$5.23 \pm 0.16^{a,x}$	8.63 ± 2.36^{ab}	$9.51 \pm 0.93^{ab,x}$	$11.64 \pm 0.73^{bc,xy}$	12.66 ± 3.79^{bcd}	$14.35 \pm 2.22^{bcd,x}$	16.50 ± 3.63^{cd}	18.03 ± 3.35^{d}	***
	2 × S	$5.51 \pm 1.46^{a,xy}$	7.01 ± 1.53^{ab}	$9.30 \pm 0.33^{ab,x}$	$11.45 \pm 2.76^{bc,x}$	11.82 ± 1.08^{bc}	$14.93 \pm 0.16^{cd,xy}$	19.68 ± 2.47^{d}	20.29 ± 4.83^{d}	***
	3 × S	$5.47 \pm 0.93^{a,xy}$	9.32 ± 4.90^{ab}	$9.48 \pm 0.57^{ab,x}$	$13.19 \pm 1.45^{bc,xy}$	13.83 ± 3.45^{bcd}	$16.19 \pm 0.28^{cd,xy}$	19.36 ± 3.05^{cd}	19.66 ± 3.07^{d}	***
	P-value	*	NS	*	*	NS	*	NS	NS	
12% TCA/TN (g/100 g)	Control	$7.51 \pm 0.13^{a,y}$	7.06 ± 2.79^{a}	6.97 ± 0.02^{a}	$13.77 \pm 0.01^{bc,y}$	10.66 ± 1.29^{ab}	12.53 ± 1.32^{bc}	14.40 ± 0.37^{bc}	15.64 ± 0.26^{c}	***
	S	$6.07 \pm 0.73^{ab,x}$	5.62 ± 0.59^{a}	5.45 ± 1.07^{a}	$9.14 \pm 0.39^{abc,x}$	9.47 ± 2.50^{abc}	9.11 ± 3.79^{abc}	11.31 ± 3.37^{bc}	13.52 ± 2.84^{c}	***
	2 × S	$6.80 \pm 0.32^{ab,xy}$	5.02 ± 1.47^{a}	5.06 ± 0.57^{a}	$8.96 \pm 2.38^{bc,x}$	8.54 ± 0.53^{bc}	9.91 ± 0.36^{bc}	11.14 ± 1.56^{cd}	13.42 ± 2.30^{d}	***
	3 × S	$6.01 \pm 0.51^{a,x}$	4.80 ± 1.48^{a}	5.41 ± 0.79^{a}	$10.11 \pm 1.35^{b,xy}$	10.09 ± 1.65^{b}	10.83 ± 0.92^{b}	13.12 ± 2.69^{b}	13.15 ± 2.33^{b}	***
	P-value	*	NS	NS	*	NS	NS	NS	NS	
PTA/TN (g/100 g)	Control	1.37 ± 0.03^{a}	1.50 ± 0.02^{a}	1.77 ± 0.06^{a}	$2.94 \pm 0.10^{ab,y}$	3.07 ± 0.91^{ab}	3.50 ± 1.05^{ab}	4.25 ± 0.78^{b}	4.42 ± 0.19^{b}	**
	S	2.34 ± 1.48^{a}	1.73 ± 0.07^{a}	1.91 ± 0.32^{a}	$2.10 \pm 0.47^{a,x}$	2.65 ± 0.45^{a}	3.35 ± 0.32^{ab}	3.12 ± 1.20^{ab}	4.98 ± 0.90^{b}	***
	2 × S	1.42 ± 0.56^{a}	1.80 ± 0.13^{ab}	1.93 ± 0.62^{abc}	$1.99 \pm 0.07^{abc,x}$	2.30 ± 0.29^{abc}	3.00 ± 0.13^{c}	2.87 ± 0.74^{bc}	4.65 ± 0.26^{d}	***
	3 × S	1.74 ± 1.05^{ab}	1.59 ± 0.07^{a}	1.69 ± 0.31^{ab}	$1.91 \pm 0.08^{ab,x}$	2.32 ± 0.32^{ab}	2.78 ± 0.36^{b}	2.59 ± 0.36^{ab}	4.48 ± 0.17^{c}	***
	P-value	NS	NS	NS	**	NS	NS	NS	NS	

[a–f]Means within a row with different superscripts differ ($P < 0.05$).

[x–z]Means within a column with different superscripts differ ($P < 0.05$).**[AU12: 2 not found in table—remove?]**

[1]WSN/TN = water-soluble nitrogen/total nitrogen; SNpH4.6 = soluble nitrogen at pH 4.6; 12% TCA = soluble nitrogen at 12% TCA; PTA = phosphotungstic acid-soluble nitrogen.

[2]S = a specific concentration of saffron added to the cheese (Berruga Fernández et al., 2009); 2 × S = twice the concentration of S; 3 × S = 3 times the concentration of S.

[3]Significance differences between saffron concentration are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Table 3. Mean values ± standard deviation for Commission Internationale d'Éclairage (CIE) L*a*b* coordinates from control and saffron cheeses[1]

Color coordinate	Cheese[2]	Ripening time (d) 3	15	30	60	90	120	150	180	*P*-value
L*	Control	86.73 ± 0.70^{e}	86.34 ± 2.39^{c,y}	84.68 ± 1.06^{bc,y}	83.96 ± 0.78^{bc,y}	82.24 ± 2.07^{ab,y}	81.39 ± 2.12^{ab,y}	79.99 ± 0.38^{a}	80.00 ± 0.80^{a,y}	***
	S	86.52 ± 0.85^{e}	82.03 ± 0.66^{a,x}	81.39 ± 1.20^{cd,x}	80.69 ± 1.22^{bcd,x}	79.64 ± 1.44^{abcd,x}	78.60 ± 2.07^{abc,xy}	77.39 ± 3.20^{a}	77.89 ± 2.16^{ab,xy}	***
	2 × S	85.21 ± 1.63^{g}	80.90 ± 0.98^{cd,x}	81.03 ± 1.28^{d,x}	79.56 ± 1.36^{bcd,x}	78.41 ± 1.47^{abc,x}	77.71 ± 2.18^{ab,x}	77.07 ± 1.93^{ab}	76.89 ± 1.81^{a,x}	***
	3 × S	86.81 ± 1.01^{e}	80.85 ± 0.93^{d,x}	80.43 ± 0.83^{d,x}	79.96 ± 0.70^{d,x}	79.03 ± 0.75^{bcd,x}	78.07 ± 1.30^{abc,x}	77.07 ± 2.23^{ab}	76.60 ± 1.54^{a,x}	***
	P-value[2]	NS	***	***	***	***	*	NS	*	
a*	Control	−3.72 ± 0.02^{ab,z}	−2.95 ± 0.29^{b,z}	−2.92 ± 0.26^{b,z}	−3.13 ± 0.34^{ab,z}	−3.17 ± 0.20^{ab,z}	−3.28 ± 0.24^{ab,z}	−3.31 ± 0.18^{ab,z}	−3.33 ± 0.29^{ab,z}	*
	S	−4.99 ± 0.15^{ab,x}	−5.29 ± 0.18^{a,w}	−4.97 ± 0.10^{ab,x}	−4.96 ± 0.18^{ab,x}	−4.89 ± 0.18^{b,x}	−4.97 ± 0.21^{ab,x}	−5.00 ± 0.28^{ab,x}	−4.85 ± 0.30^{b,x}	**
	2 × S	−4.41 ± 0.16^{y}	−4.57 ± 0.18^{x}	−4.00 ± 1.02^{y}	−4.05 ± 0.40^{y}	−4.06 ± 0.36^{y}	−4.14 ± 0.27^{y}	−4.12 ± 0.14^{y}	−3.89 ± 0.32^{y}	NS
	3 × S	−3.80 ± 0.22^{a,z}	−3.81 ± 0.13^{a,y}	−3.46 ± 0.22^{ab,yz}	−3.39 ± 0.25^{bc,z}	−3.27 ± 0.24^{bc,z}	−3.31 ± 0.37^{bc,z}	−3.06 ± 0.22^{c,z}	−3.09 ± 0.30^{bc,z}	***
	P-value	***	***	***	***	***	***	***	***	
b*	Control	14.45 ± 0.07^{w}	12.03 ± 0.71^{w}	12.14 ± 0.77^{w}	13.05 ± 1.23^{w}	12.84 ± 1.33^{w}	12.79 ± 0.97^{w}	13.19 ± 0.91^{w}	13.50 ± 1.49^{w}	NS
	S	33.20 ± 0.81^{a,x}	35.35 ± 1.25^{b,x}	35.40 ± 0.83^{b,x}	35.34 ± 0.70^{b,x}	35.14 ± 0.94^{b,x}	35.71 ± 0.92^{b,x}	34.60 ± 0.87^{b,x}	35.00 ± 1.20^{ab,x}	***
	2 × S	41.51 ± 2.28^{b,y}	46.14 ± 0.72^{b,y}	45.64 ± 1.13^{b,y}	47.21 ± 2.88^{b,y}	46.79 ± 1.86^{b,y}	46.58 ± 1.11^{b,y}	45.85 ± 1.25^{b,y}	44.82 ± 1.05^{b,y}	***
	3 × S	47.15 ± 1.30^{a,z}	52.80 ± 1.18^{c,z}	51.56 ± 0.70^{bc,z}	52.36 ± 0.79^{bc,z}	52.03 ± 1.03^{bc,z}	52.25 ± 0.81^{bc,z}	52.00 ± 0.59^{bc,z}	51.10 ± 1.09^{b,z}	***
	P-value	***	***	***	***	***	***	***	***	

[a–c]Means within a row with different superscripts differ ($P < 0.05$).
[w–z]Means within a column with different superscripts differ ($P < 0.05$).
[1]L* = luminance (ranges from 0 for black to 100 for white); a* = a color's position between red/magenta and green; b* = a color's position between yellow and blue.
[2]S = a specific concentration of saffron added to the cheese (Berruga Fernández et al., 2009); 2 × S = twice the concentration of S; 3 × S = 3 times the concentration of S.
[3]Significance differences between saffron concentration are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Figure 1. Mean values of total bacteria (A) and lactic acid bacteria (B) counts in control cheese (□), specific concentration of saffron-added cheese (S; Berruga Fernández et al., 2009; ■), 2 × S (twice the concentration of S) cheese (○) and 3 × S (3 times the concentration of S) cheese (●) as a function of ripening time. Significance levels for each ripening time are indicated as follows: $^{*}P < 0.05$ and $^{***}P < 0.001$. Samples marked by different letters (a and b) differed significantly.

14, whereas saffron cheeses values ranged between 33 and 51 (Table 3). Control cheeses did not show differences during the ripening period, whereas saffron cheeses only showed significant differences ($P \leq 0.05$) in the first days.

Regarding the effect of air exposure on color, GLM was done to establish if saffron concentration, ripening time, and air exposure affected these characteristics. Results are shown in Table 4. The models obtained by the GLM for the CIE L*a*b* coordinates and the 3 factors studied were significant ($P < 0.001$), except for ripening on b*. Interactions between factors were not statistically significant for any coordinate (data not shown). The coefficient of determination for coordinate

Table 4. General linear model for Commission Internationale d'Éclairage (CIE) L*a*b* coordinates in control and saffron cheeses in terms of saffron concentration, ripening time, and air exposure[1]

	Color coordinate		
Model[2]	L*	a*	b*
R^2	65.766	34.723	88.491
SEE[3]	2.799	0.700	4.370
P-value	0.000	0.000	0.000
Constant	86.290	−5.033	19.747
Saffron concentration[4]	−0.892***	0.424***	11.675***
Ripening time[5]	−0.055***	0.002***	
Air exposure[6]	−0.072***	−0.009***	0.078***

[1]L* = luminance (ranges from 0 for black to 100 for white); a* = a color's position between red/magenta and green; b* = a color's position between yellow and blue.

[2]Significance levels for each factor: ***$P < 0.001$.

[3]Standard error of the estimation.

[4]Saffron concentration was between 0 and 3 × S, where 3 × S = 3 times the concentration of a specific concentration of saffron (S) added to the cheese (Berruga Fernández et al., 2009).

[5]Ripening time was between 3 and 180 d.

[6]Air exposure was between 0 and 60 min.

b* was adequate by being closer to 90%. However coordinate L* and a* presented lower coefficients of determination; consequently, they could not be as properly estimated as b*. Exposure time was statistically significant ($P < 0.001$) for all color coordinates, confirming the visually observed color changes in control and saffron cheeses, but its effect was not as marked as saffron concentration. Cheeses resulted less bright and red and more yellow as exposure time was increased.

Rheological Determinations

Uniaxial Compression. Fracture stress and fracture strain obtained from the stress curves of control and saffron cheeses are shown in Figure 2A and 2B. Saffron addition and ripening time were factors that influenced ($P \leq 0.05$) changes in these parameters. A low fracture stress value indicates a greater fracturability. Control cheeses fractured more easily than saffron cheeses, whereas S cheeses showed the highest fracture stress values. For control cheeses, fracture stress values slightly decreased until d 30 ($P \leq 0.05$), but after that period, the values started to increase until d 180 ($P \leq 0.01$). Saffron cheeses also increased fracture stress with time ($P \leq 0.01$).

Fracture strain describes the deformability of the cheese; high numerical values indicate higher deformability. Fracture strain decreased with ripening time ($P \leq 0.01$), meaning that cheeses were more elastic at the beginning of ripening period. Control cheeses were less elastic than saffron cheeses but as the ripening period lengthened, these differences were less accused**[AU7: What do you mean by differences being less accused?]**. Control cheeses showed the lowest deformability values.

Stress Relaxation. This test was done to study the viscoelastic response of cheese during ripening. Values for e and r were obtained, as explained in the Materials and Methods section. Values of r (s^{-1}) equal to zero correspond to a more elastic solid, whereas values near 1 indicate viscous behavior. Results are shown in Figure 2C and 2D. After manufacturing, cheese 3 × S showed higher e-values that were different ($P \leq 0.01$) from the rest of the cheeses. Between d 15 and 60, no differences were found. After d 60, saffron addition had an influence on e-values, showing differences ($P \leq 0.001$) between saffron concentrations (i.e., as saffron concentration was increased, cheeses became more elastic). Values for r were not affected by saffron addition, except on d 3 when control cheeses had higher values ($P \leq 0.026$). Regarding ripening time, both parameters showed opposite behavior: whereas e decreased with ripening time, r increased, showing that ripening time influences elastic properties, resulting in less elastic cheeses at d 180.

Sensory Analysis

The purpose of the triangular test was to study if consumers were able to make differences between saffron and non-saffron cheeses during ripening. To accomplish the objective, cheeses with the lowest saffron concentration were given to consumers to be compared with the control. Color differences between the control and saffron cheeses were noticeable to consumers ($P \leq 0.05$) at all stages where sensory evaluation was conducted (60, 120, and 180 d). Flavor discrimination was

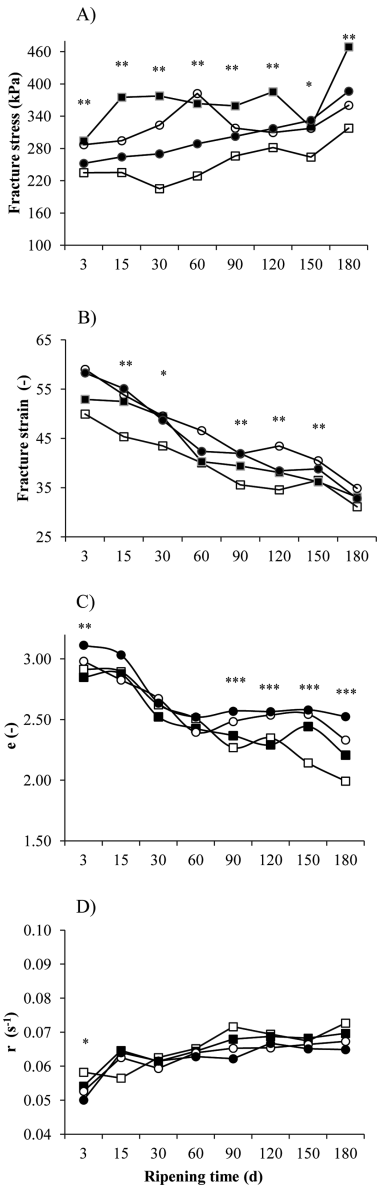

Figure 2. Mean values of fracture stress (A), fracture strain (B), equilibrium residual values of normalized relaxation stress when t → ∞ **[AU13: Does t represent time?]**(e; C), and elastic behavior (r; D) in control cheese (□), specific concentration of saffron-added cheese (S; Berruga Fernández et al., 2009; ■), 2 × S (twice the concentration of S) cheese (○), and 3 × S (3 times the concentration of S) cheese (●) as a function of ripening time. Significance levels for each ripening time are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

also significant ($P \leq 0.05$) until the end of the ripening period.

The ranking test was carried out to know if consumers were able to rank from the lowest to the highest saffron concentration for color and flavor attributes. Consumers were able to order saffron cheeses from the less yellow to the more yellow color at the 3 stages of ripening tested ($P \leq 0.001$). Moreover, cheeses resulted significant different between them.**[AU8: The meaning of this sentence is unclear. Please clarify.]** Flavor ordering was more difficult than color ordering. At 60 d of ripening, flavor differences were statistically significant ($P \leq 0.001$), meaning that consumers were able to order the cheeses from the lowest to the highest saffron flavor. After this time, flavor differences between cheeses, although they were significant ($P \leq 0.05$), were less evident: only S cheeses were significant different from 3 × S cheeses.

DISCUSSION

Most of the compositional parameters tested did not show significant differences between control and saffron cheeses, with the exception of salt content and nitrogen fractions. Nevertheless, certain effects due to saffron addition can be observed, especially regarding bacteria counts, texture, and color.

Control cheeses presented slightly higher total and lactic acid bacteria counts than saffron cheeses, which probably caused a faster lactic acid production during pressing and, thus, lower pH values. Moreover, control cheeses presented higher WSN, SNpH4.6, and 12% TCA values than saffron cheeses, especially at 60 d of ripening, as a possible consequence of slightly higher lactic acid bacteria enzymatic activity. Results suggest that saffron could be slowing down the growth of these bacterial groups. Nevertheless, no studies, to our knowledge, have been published concerning the influence of saffron on lactic acid bacteria. A few studies have focused on the antimicrobial properties of saffron, confirming its antimicrobial properties in different bacteria [e.g., *Micrococcus*, *Staphylococcus*, *Escherichia coli*, *Salmonella*, *Helicobacter pylori*, and fungi (e.g., *Candida*, *Aspergillus*, and *Cladosporium*)], but only moderate activity has been found (Vahidi et al., 2002; Kamble and Patil, 2007; Sekine et al., 2007; Nakhaei et al., 2008; Pintado et al., 2011). Further research is needed to confirm saffron's effect on lactic acid bacteria found in saffron cheeses.

Regarding composition, DM content values in control cheeses were slightly lower, especially at d 3, whereas salt concentrations were significantly higher. As mentioned previously, faster lactic acid production could be taking place in control cheeses, which is directly related

to pressing time. Pressing time is, in part, responsible for whey drainage and is dependent, in the case of some pressed cheeses, on pH. In control cheeses, pH decreased faster, so the pressing time was reduced from 5 to 4 h, resulting in higher water content at d 3. This higher water content could increase the capacity of exchange between whey retained in the curd and salt, thus causing higher salt content in control cheeses. Higher salt content also stimulates proteolytic activity of rennet (Guinee and Fox, 2004); consequently, it might be causing the faster proteolysis rate at the beginning of ripening in control cheeses.

These differences among control and saffron cheeses regarding salt content, DM content, and proteolysis could be the origin of the textural differences found, as texture is developed by many interrelated factors, including the casein matrix, as well as fat, water, and pH. Texture results showed that control cheeses were firmer than saffron cheeses, which is given by the lower fracture stress values. Several authors have reported that firmness of cheese increases with decreasing water content during ripening, thus causing a loss of elastic structural elements (Juan et al., 2007). As mentioned above, control cheeses showed slightly lower DM content than saffron cheeses, explaining the firmness differences between them. Control cheeses were less deformable ($P \leq 0.05$) and elastic than saffron cheeses because they had less fracture strain and higher r values, especially at the beginning of ripening. At d 30, an inflection point in fracture stress of control cheeses was observed, as reported in other ewe milk cheeses (Pavia et al., 1999; Bertolino et al., 2011). This could be explained by the conversion of fresh curd into a mature curd at this stage or ripening, which is influenced by pH (O'Callaghan and Guinee, 2004). Watkinson et al. (2001) found a positive correlation between pH and fracture strain, confirmed by our results, as control cheeses had lower pH values.

Regarding ripening in general terms, control and saffron cheeses presented similar physicochemical values than those reported for pressed ewe milk cheeses (Pavia et al., 2000; Ballesteros et al., 2006; Cabezas et al., 2007). Dry matter, fat, protein and salt content increased during ripening, similar to changes found by Pavia et al. (1999). Nitrogen fractions in control and saffron cheeses were similar to those reported before; nevertheless, the values increased more slowly with ripening time, showing a slower rate of degradation of caseins (Guamis et al., 1997; Ballesteros et al., 2006; Cabezas et al., 2007). Our results showed that rennet was more active than starter bacteria, because the SNpH4.6 fraction had higher values than the 12% TCA fraction (i.e., soluble peptides produced by rennet were not hydrolyzed by bacterial peptidases at the same rate as they were produced; Gobbetti, 2004).

As expected, color was the parameter that had evident changes, as saffron concentration increased because of the coloring properties of saffron. Color data for control cheeses proved to be in line with published results for hard cheeses (Rohm and Jaros, 1996), with the only exception that values for coordinate a* in control cheeses were lower than those obtained by other authors in similar cheeses (Pavia et al., 1999). Cheese storage time and exposure to light have previously been studied in processed cheeses where L* values decreased with storage time (Kristensen et al., 2001). This decrease was found to change linearly with time as a consequence of a browning reaction, probably nonenzymatic, leading to loss of brightness. Values of coordinate b* for saffron cheeses were similar to Cheddar cheese shreds colored with annatto, whereas a* was lower and L* higher, which translates into a more yellow than red color (Colchin et al., 2001). Saffron color mainly evolves due to glycosylated esters of dicarboxylic acid, named crocetin esters, resulting in the yellowish red hues of the spice, so that increasing saffron concentration was reflected by coordinate b* differences between saffron cheeses.

Coordinate L* decreased with ripening time; the major changes in coordinates a* and b* were between d 3 and 15 and after this period remained almost constant. Our results agreed with studies on Emmental cheeses where L* decreased, b* increased, and a* did not show a definite trend throughout ripening (Rohm and Jaros, 1996). These changes could be attributed to loss of water and, thus, fat concentration during ripening, especially changes in b*, as the influence of fat on increasing yellow coloration of cheeses has been demonstrated (Rohm and Jaros, 1996).

Crocetin esters have been intensively studied regarding their coloring properties, but the influence on color of different liposoluble carotenoids that are also present in saffron, such as β-carotene, ξ-carotene, zeaxanthin, and lycopene, have not been thoroughly studied (Carmona et al., 2006). These molecules are prone to degradation due to light exposure, thermal treatment and acidic environment, having a direct impact on saffron coloring properties (Sánchez et al., 2008). Cheeses were exposed to air for 60 min, showing color changes, turning less bright, red, and more yellow as exposure time was increased. This loss of red coloration could be due to oxidation of liposoluble carotenoids present in saffron, whereas increments in coordinate b* could be due to a concentration of crocetin esters due to loss of water on the surface. Previous studies found that decreasing water content in saffron favored the preservation of crocetin esters (Alonso et al., 1993) and avoided loss of yellow coloration.

Sensory evaluation showed color and flavor differences between cheeses. Sensory color differences proved to be in line with the colorimetric parameters obtained, as yellow coloration was the most affected by saffron concentration and was visually evident. During sessions, some panelists commented that saffron flavor was present in cheeses during the early stage of ripening, but as the ripening period increased, this flavor was masked by the development of the characteristic cheesy flavor. Moreover, they thought that the cheese flavor was enhanced by saffron addition. This may explain the fact that flavor differences between cheeses were less evident as ripening time was increased. It is well known that saffron has been used since ancient times for its flavor properties and as a flavor enhancer, although this property has not been studied (Carmona et al., 2006); further research could be useful for this field of study.

CONCLUSIONS

Color was the main aspect modified by the use of saffron on pressed ewe milk cheeses. Saffron cheeses were less bright, less red, and more yellow than the control. Cheese color was also affected by air exposure, making it appear less bright, less red, and yellower with greater exposure time. Sensory analysis revealed that saffron modifies the color and flavor of cheeses and differences between saffron concentrations were perceived. The total and lactic acid bacteria counts were lower in saffron cheeses, suggesting that saffron could be slightly slowing down bacterial growth, causing a smaller pH decrease during pressing and, thus, increasing pressing time and DM content after salting. Saffron cheeses showed lower salt content and were firmer and more elastic. Slight differences in proteolysis rate were observed, although these differences were not evident by the end of the period studied. This work offers, for the first time, new insights about saffron influence on a pressed ewe milk cheese, revealing the need for further studies regarding antimicrobial activity and its properties as a flavor enhancer. This cheese is also an important approach to the diversity of traditional products.

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5.4 Method for volatile analysis of pressed ewes' milk cheese

5.4.1 Approach

After physico-chemical, microbial and sensory analysis of saffron cheeses, characterization in terms of aroma was left. A method using headspace sorptive extraction (HSSE) and gas chromatography/mass spectrometry (GC/MS) was developed for pressed ewes' milk cheeses in order to be able to identify and quantify saffron aroma in cheese. Optimization of the methodology was published on the following paper:

Optimization of headspace sorptive extraction for the analysis of volatiles in pressed ewes' milk cheese

Licón, C.C., Hurtado de Mendoza, J., Maggi, L., Berruga, M.I. Martín Aranda, R.M. and Carmona, M.

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5.4.2 Extended summary

Standards of compounds commonly present in ewes' milk cheeses were used to carry out method optimization. This optimization started with column and chromatographic program selection. The column Elite-Volatiles with a special phase for volatile organic compounds was selected because it showed better resolution values and more symmetric peaks than Elite-5 column. Chromatographic program was set at 40 °C, raised to 240 °C at 5 °C/min because it showed better resolution than programs started at 35 °C or slower temperature increment rates.

A self-made insert and a commercial insert developed by Gerstel were compared as stir bar holder systems resulting that the commercial insert allowed better adsorption of low polarity compounds, such as alcohols and ketones, showing greater peak areas. It was decided to use the commercial insert as holding system.

Regarding volatile extraction methodology, different extraction temperatures were tested, concluding that 45 °C was suitable for most of the compounds present in cheeses, especially for alcohols, ketones, aldehydes and esters. Salt addition was negative for some compounds and the rest were not influenced, as a result salt addition was discarded. The two stir bar sizes commercially available were tested concluding that the larger bar, 2 cm long, could adsorb better most of the compounds so it was selected. Vials with different volume capacity, 20 and 50 mL, were used to study the effect of headspace volume. Only three compounds were affected by vial size. Bigger vials were selected because it allowed testing a wider range of sample weights. The vials selected had a headspace volume of 25 mL.

After setting the above conditions, different sample weights and extraction times were tested. A sample weight of 10 grams was selected because it showed good detection and quantification limits for most compounds. Extraction time was set at 4 hours because some ketones, which are very important in the volatile fraction or ewes' milk cheeses, showed higher concentration with this extraction time.

Method validation showed good linearity between 0.1 and 100 µg/kg, with correlation coefficients higher than 0.98 for all analytes. Recovery rates obtained were between 57.9 and 119.7 % with precision values below 30 % in most of the compounds. Quantification limits were lower than 150 ng/kg and detection limits were between 5.9 and 37.5 ng/kg, thus allowing identification and quantification of volatiles at ng/kg levels.

Commercial ewes' milk cheeses were analyzed after having all extraction and chromatographic parameters established. Results confirmed the capability of the method to characterize in detailed (more than 50 compounds) the volatile fraction of pressed ewes' milk cheese.

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Optimization of headspace sorptive extraction for the analysis of volatiles in pressed ewes' milk cheese

Carmen C. Licón [a,1], Jorge Hurtado de Mendoza [b,1], Luana Maggi [c], María Isabel Berruga [a], Rosa María Martín Aranda [d], Manuel Carmona [c,e,*]

[a] Calidad de Leche y Derivados Lácteos, Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR, Universidad de Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain
[b] Sailab, Argenters, 5 Ed. I. Bajos D, Parc tecnológic del Vallès, 08290 Cerdanyola del Vallès, Spain
[c] Cátedra de Química Agrícola, E.T.S.I. Agrónomos, Universidad Castilla-La Mancha, Campus Universitario, 02071 Albacete, Spain
[d] Departamento de Química Inorgánica y Química Técnica, Facultad de Ciencias (UNED), Paseo Senda del Rey 9, 28040 Madrid, Spain
[e] Albacete Science and Technology Park, Campus Universitario, 02071 Albacete, Spain

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ABSTRACT

An analytical method was developed for simultaneous determination of 50 volatiles in pressed ewe cheese using headspace sorptive extraction coupled to a thermal desorption and gas chromatography mass spectrometry. Method optimization was carried out in terms of chromatographic columns and conditions, stir bar size and holder, vial volume, salt content, sample weight, extraction temperature and time. The proposed method was validated showing good results in linearity (>0.98), precision (9–34%), recovery (58–120%), limit of detection (6–38 ng kg^{-1}) and limit of quantitation (75–150 ng kg^{-1}). The present method was also applied for the analysis of volatiles in ewes' milk cheese samples.

1. Introduction

Pressed ewes' milk cheeses have a wide range of volatile compounds including primary and secondary alcohols, *n*-acids, methyl and ethyl ketones, methyl and ethyl esters, aldehydes, alkanes, unsaturated and aromatic hydrocarbons and terpenes (Barron et al., 2005; Fernández-García, Carbonell, & Nuñez, 2002). The analysis of pressed cheese volatiles is complex. Most pressed cheeses have a high fat and protein content (Fox & McSweeney, 2004), which tends to concentrate volatiles in the fat matrix, thus making analysis difficult due to the lipophilic character of the aroma compounds (Le Quéré, 2004). In addition, factors such as cheese variety, origin and ripening process result in a wide variety of aromatic profiles and hinder the comparison of techniques and the application of certain extraction conditions.

Traditional extraction methods for cheese volatiles, such as steam or vacuum distillation, are nowadays almost abandoned (Moio & Addeo, 1998; Vandeweghe & Reineccius, 1990). Their drawbacks include the poor recovery rate of highly volatile compounds, the disappearance of thermally sensitive compounds and the formation of artifacts (Le Quéré, 2004).

Modern extraction methods used for volatiles analysis include pre-concentration solvent free techniques, as static (S-HS) and dynamic headspace (D-HS), which includes a purge and trap system (P&T) that can either be in on-line or off-line mode. D-HS has mainly been used in the determination of volatile fractions of Spanish ewes' milk cheeses such as Zamorano, Idiazabal, Manchego and Roncal (Barron et al., 2005, 2007; Irigoyen, Ortigosa, Juansaras, Oneca, & Torre, 2007; Izco & Torre, 2000; Muñoz, Ortigosa, Torre, & Izco, 2003). Valero, Villaseñor, Sanz, and Martínez Castro (2000) found that on-line P&T presented higher sensitivity against off-line D-HS, although the latter allowed to analyze the most retained and polar compounds, such as free fatty acids (FFA) and other medium volatility components.

Headspace solid-phase micro extraction (HS-SPME) is another technique currently used for a great variety of cheeses (Condurso, Verzera, Romeo, Ziino, & Conte, 2008; Januszkiewicz, Sabik, Azarnia, & Lee, 2008; Mallia, Fernández-García, & Bosset, 2005; Pinho, Ferreira, & Ferreira, 2003a), as it is solvent free, easy to use, relatively fast and sufficiently sensitive. When P&T and HS-SPME are compared, the first is more sensitive and shows higher extraction efficiency for compounds with lower boiling points (bp), whereas HS-SPME is more effective for medium and high bp compounds, such as fatty acids (Januszkiewicz et al., 2008; Mallia et al., 2005). Among the fibres used for SPME, carboxen/polydimethylsiloxane (CAR/PDMS) stands out due to its efficiency

* Corresponding author. Tel.: +34 967 599310; fax: +34 967 599238.
E-mail address: Manuel.Carmona@uclm.es (M. Carmona).
[1] These authors contributed equally to this work.

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of extraction for a greater number of targeted compounds in Cheddar and Terrincho cheeses in comparison with other fibres tested (Januszkiewicz et al., 2008; Pinho, Pérès, & Ferreira, 2003b).

Recently, stir bar sorptive extraction (SBSE), a sorbent PDMS-coated rod, has been introduced and successfully applied to the analysis of odour-active and volatile constituents in human milk (Buettner, 2007) and sulphonamide residues in milk (Huang, Qiu, & Yuan, 2009). More recently, headspace sorptive extraction (HSSE) has been used for the extraction of volatiles and semivolatiles in several matrices, e.g., the analysis of the volatile fraction of "Pesto Genovese" containing Grana Padano cheese (Salvadeo, Boggia, Evangelisti, & Zunin, 2007) and to determine flavour compounds in Bitto cheese (Panseri et al., 2008). Most of these methods have not been optimized and therefore the different parameters of analysis have not been studied in detail. When SPME/HS-SPME and SBSE/HSSE are compared, the SBSE/HSSE concentration capability was 40-fold higher than that presented by SPME/HS-SPME because of the higher fibre phase ratio coating between SBSE and SPME (Maggi, Zalacain, Mazzoleni, Alonso, & Salinas, 2008). Both techniques require an easy sample preparation, are fast, and allow a series of extractions at the same time (Januszkiewicz et al., 2008; Pinho et al., 2003b).

Since the ewes' milk cheese matrix has a high content of fat and proteins that could interfere in the SBSE response, HSSE presents a good alternative for volatile analysis since it does not come into direct contact with the sample. The aim of this work was to develop and optimize a method for the simultaneous determination of 50 volatiles found in pressed ewes' milk cheese using HSSE coupled to thermal desorption (TD) with gas chromatography mass spectrometry (GC/MS). The present method was also applied for the direct analysis of volatiles in cheese samples.

2. Materials and methods

2.1. Standards and solutions

All standards and reagents used were GC grade. The reagents 1-octene (98%), 2,3-pentanedione (97%), 2-nonanone (99%), 3-methyl-1-butanol (99%), heptanal (95%), nonanal (95%), octanal (92%), (R)-(+)-limonene (97%) and dimethyl sulphide (99%) were supplied by Aldrich (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); 2,3-butanedione (99.4%), 1-heptene (99.5%) and *m*-cymene (99%) by Fluka (Sigma–Aldrich); ethyl acetate (99.5%), ethyl hexanoate (99%), ethyl heptanoate (99%), heptanoic acid (99%) and *n*-propyl acetate (99%) by Chem Service (Chem Service Inc., West Chester, PA, USA).

In addition, 1-methylethyl-benzene, *n*-propylbenzene, *o*-xylene, 2-phenylbutane, 2-methyl-2-phenylpropane, 1,2-dichlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene, 2-chlorotoluene and 4-chlorotoluene, contained in EPA Volatile Organic Compounds Mix 1; benzene, bromobenzene, ethylbenzene, *m*-xylene, 1-phenylbutane, naphthalene, *p*-cymene, styrene, toluene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene contained in EPA Volatile Organic Compounds Mix 2; 2-propanone, carbon disulfide, 2-methyl-2-propanol, 2-butanone, 2-hexanone and 4-methyl-2-pentanone in 8260 Mix 5; and 1-butanol, 2-propanol, 1-propanol, 2-methyl-1-propanol, isopropyl ether and methyl-clycopentane, in Qualitative Peak ID Mix. All mixtures were supplied by Supelco (Sigma–Aldrich). *n*-Octane was used as internal standard and was supplied by Supelco (Sigma–Aldrich). Water was purified through a Milli-Q System (Millipore, Bedford, MA, USA). From the previous list, 14 standards were chosen for column selection: 2-propanol, 3-methyl-1-butanol, 2-propanone, 2-butanone, 2,3-pentanedione, 2-nonanone, heptanal, octanal, nonanal, ethyl hexanoate, 1-heptene, 1-octene, *m*-cymene and limonene.

A standard solution of 200 mg kg^{-1} was prepared by adding all target compounds and the four standard mixtures together into methanol. From this solution, different aqueous dilutions were prepared by adding the internal standard (IS; 2.5 μg kg^{-1}) for the calibration curve (0.1–100.0 μg kg^{-1}) in a 50 mL vial (HS volume 25 mL). Each solution was prepared daily.

2.2. Cheese samples

Four pressed ewes' milk cheeses were bought in "Campo Rus", a local factory situated in Santa María del Campo Rus, Cuenca, Spain. These cheeses, made with "Manchega" breed pasteurized milk, weighed approximately 3 kg and were ripened in a maturation chamber at 11 ± 2 °C for 2 months with a relative humidity of 85%. Cheeses were then stored at 5 ± 3 °C until analysis and their sampling was carried out according to ISO 707/IDF 50 (ISO/IDF, 2008). Two centimeters of rind were removed from the cheese and cubes of 25 mm^3 were obtained using a cheese blocker (BOSKA, Bodegraven, Holland). The cubes were grated to a uniform grain size using a 300 g grater (Moulinex, Lyon, France).

Dry matter, protein, fat and salt content were determined with an infrared analyzer FoodScan (FossElectric, Hillerød, Denmark). These parameters were determined in the grated cheese using 90 mm diameter Petri dishes. Each determination was duplicated for each piece of cheese.

2.3. Gas chromatography mass spectrometry equipment and columns

A gas chromatograph (Varian CP-3800, Palo Alto, CA, USA) was equipped with a Saturn 2200 ion trap mass spectrometer (MSD). Two different capillary columns supplied by PerkinElmer (Shelton, CT, USA) were tested: Elite-Volatiles with a special phase for analysis of volatile organic compounds (30 m × 0.25 mm I.D.; 1.4 μm film thickness) and Elite-5 (5% phenyl + 95% dimethylpolysiloxane; 30 m × 0.25 mm I.D.; 0.25 μm film thickness).

Stir bars were thermally desorbed by a Turbo Matrix ATM thermal desorption (TD) system (PerkinElmer). The TD conditions were as follows: oven, transfer line and tube temperature: 220 °C; desorption time 5 min; a Tenax packed trap was used (PerkinElmer): cold temperature −30 °C and desorption temperature: 290 °C (hold for 5 min); He inlet flow 45 mL min^{-1}; He outlet flow 20 mL min^{-1}. The desorption unit was coupled to the GC/MS (Varian).

After column selection, three different chromatographic programs were tested: 1) set at 35 °C (held for 10 min), raised to 240 °C at 10 °C min^{-1} and kept for 5 min; 2) set at 40 °C (held for 10 min), raised to 240 °C at 5 °C min^{-1} and kept for 5 min and 3) set at 40 °C (held for 10 min), raised to 240 °C at 10 °C min^{-1} and standby for 5 min. The MSD temperatures were: transfer line 250 °C, manifold 60 °C and trap 200 °C and He was used as the carrier gas with a constant pressure of 103 kPa. The use of a constant pressure allowed us to obtain a higher flow during the desorption step and a faster desorbing of the target compounds.

2.4. Selection of headspace sorptive extraction conditions

The use of a stir bar holder (Insert for Twister, Gerstel GmbH, Mülheim an der Rhur, Germany) was evaluated. A trial was carried out comparing the Insert with a self-made holder. The holder consisted of a metal wire inserted into the rubber septum of the vials. Headspace vials of 50 mL were used and the working solution (1.0 μg kg^{-1}) mentioned above was added until a fixed HS volume of 25 mL was obtained. A 2 cm stir bar was used and the vials were kept at 45 °C during 4 h of extraction.

Optimization of the different factors studied was carried out sequentially. First, stir bar size, vial volume, extraction temperature and salt content were considered. Two PDMS stir bars 1 and 2 cm long with a film thickness of 0.5 mm were tested (Twister, Gerstel GmbH). The stir bar was suspended in two different headspace vials (20 and 50 mL) using an Insert capped with a PTFE-faced silicone septum. The salt effect was tested using unsalted and salted samples with addition of anhydrous sodium sulphate (Panreac, Barcelona, Spain) at 20%. The effect of extraction temperature on the GC/MS response of analyzed compounds was evaluated at three different temperatures (25, 35 and 45 °C). For these trials, a fixed sample weight (2.0 g for 20 mL vials and 5.0 g for 50 mL vials) and extraction time (4 h) were chosen. Milli-Q water was added to vials in order to obtain a fixed HS volume of 10 mL and 25 mL, respectively. The samples were stirred at 700 rpm.

In a second step sample weight and extraction time were studied. Three different sample weights (2.5, 5.0 and 10.0 g) and four extraction times (1, 2, 4 and 6 h) were tested.

All extractions were done in a Heraeus UB6 oven (Kendro Laboratory Products GmbH, Langenselbold, Germany). After the corresponding period of time, the stir bar was then removed from the sample, rinsed with distilled water and dried with a cellulose tissue, and transferred into a thermal desorption tube for GC/MS analysis. New stir bars were thermally conditioned for 1 h at 300 °C (Tube Conditioner TC2, Müllheim and der Rhun Gerstel GmbH Germany).

2.5. Analytical method validation

Quality parameters of the HSSE-TD-GC/MS method were assessed by spiking grated cheese with the standards (0.1–100.0 μg kg^{-1}). The validation of the proposed procedure was carried out considering the following parameters: limit of detection and limit of quantification (LOD and LOQ, respectively), precision and recovery.

As there is no Certified Reference Material for cheese, four commercial pressed ewes' milk cheeses were considered as blank samples. Before using these cheeses as blanks, they were held at 45 °C for 24 h into an oven Heraeus UB6 (Kendro Laboratory Products GmbH) to eliminate the possible presence of volatiles and then analyzed to check for any interference (signals, peaks, ion traces) in the region of interest. The absence of interferences allowed using these cheeses as blank matrix for the precision and recovery determination.

For a linearity study, calibration curves were established in aqueous solutions by spiking 50 target compounds at seven different concentration levels in a range from 0.1 to 100.0 μg kg^{-1} (HS volume of 25 mL). For each level of concentration three replicates with three different stir bars were analyzed. LOD and LOQ were determined by 3 and 10 signal to noise ratios of a standard solution at a concentration of 0.1 μg kg^{-1}, respectively; the noise was calculated peak-to-peak by the MS Workstation ver. 6.9.1 software (Varian).

Precision was determined by analyzing the spiked cheese blank samples at three levels (0.1; 0.2; 0.3 μg kg^{-1}) in a day (repeatability) and in three different days by two different analysts (reproducibility) and six determinations per concentration. The precision was expressed as a coefficient of variation (CV) at LOQ level. Recovery was calculated at three levels of target compounds (0.1; 0.2; 0.3 μg kg^{-1}) as the ratio of the compound concentration in spiked blank cheeses and in spiked aqueous solution. For all trials, after spiking cheeses with the different levels of target compounds, two hours elapsed before initiating the procedure of extraction to allow the interaction of analytes with the matrix.

2.6. Statistical analysis

A General Linear Model (GLM) was carried out to determine the effects of stir bar size (1 and 2 cm), vial volume (20 and 50 mL), extraction temperature (25, 35 and 45 °C) and salt effect (20%) on compounds concentration using Statgraphics Plus 5.1 (StatPoint Technologies, Warrenton, VA, USA). To check the effect of sample weight (2.5, 5.0 and 10.0 g) and extraction time (1, 2, 4 and 6 h) an analysis of variance ($P \leq 0.05$) was carried out using the SPSS 17.0 version statistical package (SPSS Inc., Chicago, IL, USA). Tukey's test at a significance level of $P \leq 0.05$ was used to determine differences between levels on sample weight and extraction time.

3. Results and discussion

The cheese samples studied showed a pH of 5.3 ± 0.2 along with the following composition (g per 100 g): dry matter, 59.7 ± 0.1; fat in dry matter 50.2 ± 0.1; protein in dry matter, 39.9 ± 0.1; and salt content 1.98 ± 0.02. All the values were within the range of a common semi-hard pressed ewes' milk cheese at 60 days of ripening with the intrinsic characteristics of Manchego cheese (MARM, 2011). In order to propose an analytical method for cheese volatiles, it has to be kept in mind that ewes' milk cheeses present a higher fat content than cheeses from cows' or goats' milk. This factor is important since it influenced the selection of the extraction method.

3.1. Capillary column and chromatographic program selection

For the best capillary column selection, a standard solution of 200 mg kg^{-1} (2 μL) with a split ratio of 50 was injected directly into the chromatograph by a CombiPal autosampler (Varian). An Elite-Volatiles column with a special phase for volatile organic compounds and an Elite-5 (5% phenyl + 95% dimethylpolysiloxane) were tested. To make the column selection easier, 14 standard compounds with the worst chromatographic resolution were selected to carry out these experiments as they well represent the compounds present in ewes' milk cheese, having different boiling points and eluting throughout the chromatographic run. The program used for column selection was set at 40 °C (held for 10 min), raised to 240 °C at 10 °C min^{-1} and maintained for 5 min. Peaks were identified by comparison with retention times and mass spectra of the standards.

2-Propanone was the first compound present in the chromatogram, so was not included in Fig. 1 as it was the reference compound for resolution calculations. Resolution (R_s) between two peaks was calculated as: $R_s = 2(t_{R1} - t_{R2})/(w_1 + w_2)$, where t_{R1} and t_{R2} are the retention times of the two peaks of interest, and w_1 and w_2 are the peak widths measured at the baseline between tangents drawn to the peak sides. Fig. 1a represents the resolution values obtained for the target compounds and their peak width using the two columns tested: Elite-5 and Elite-Volatiles. No relationship was found between resolution values, chemical groups and column used.

Elite-Volatiles showed better resolution values and more symmetric peaks, especially for polar compounds, such as 1-propanol, 2-butanone and 2,3-pentanedione, than those obtained by Elite-5, which presented tailing peaks for this group of analytes. As such, the Elite-Volatiles column was selected for the analysis of ewes' milk cheese volatiles.

Three chromatographic programs were tested and resolution values were obtained for the target compounds, as shown in Fig. 1b. Program 1, starting at 35 °C, showed the lowest resolution values for target compounds, except for 1-heptene, followed by program 3 and better resolution with program 2, which was set at 40 °C and raised to 240 °C at 5 °C min^{-1}.

3.2. Stir bar holder selection

The effect of the holder on the capability of stir bar compound extraction was tested. A self-made insert was used for allowing the

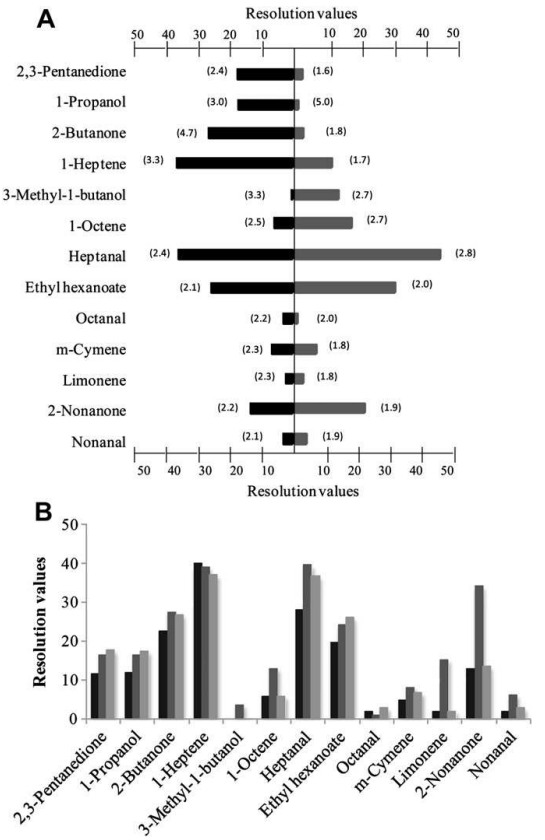

Fig. 1. Panel A: resolution values and peak width (in brackets expressed in mm) of target compounds using Elite-5 (▩) and Elite-Volatiles (■) columns and the following chromatographic program: set at 40 °C, raised to 240 °C at 10 °C min^{-1}. Panel B: resolution values of target compounds using Elite-Volatiles column and three different chromatographic programs: set at 35 °C, raised to 240 °C at 10 °C min^{-1} (■); set at 40 °C, raised to 240 °C at 5 °C min^{-1} (▩); set at 40 °C, raised to 240 °C at 10 °C min^{-1} (▩). Initial set temperatures and final temperatures were held for 10 min and 5 min, respectively, in all cases. Resolution (R_s) between two peaks was calculated as: $R_s = 2(t_{R1} - t_{R2})/(w_1 + w_2)$, where t_{R1} and t_{R2} are the retention times of the two peaks of interest, and w_1 and w_2 are the peak widths measured at the baseline between tangents drawn to the peak sides.

complete exposure of the coated stir bar with the headspace and comparing it with the Insert developed by Gerstel. Using the Insert, the coated stir bar is not in complete exposure to the vapour phase, since it only has an opening in the bottom.

Fig. 2a represents the isolation ability of the two holders for the target compounds. In order to compare the two systems, each compound area was normalized, representing the relative area obtained with each holder. Using the Insert, 38 compounds (76%) presented an area higher than the self-made holder, especially dimethyl sulphide, isopropyl ether, benzene and 2-hexanone. When the self-made holder was used, heptanoic acid and heptanal were not isolated. It seems that there is no relationship between chemical families and the holding system, even though it exists with retention time. The compounds present in the second third of the chromatogram (Fig. 2a) are better isolated with the Insert. On the other hand, the first and the last third showed some

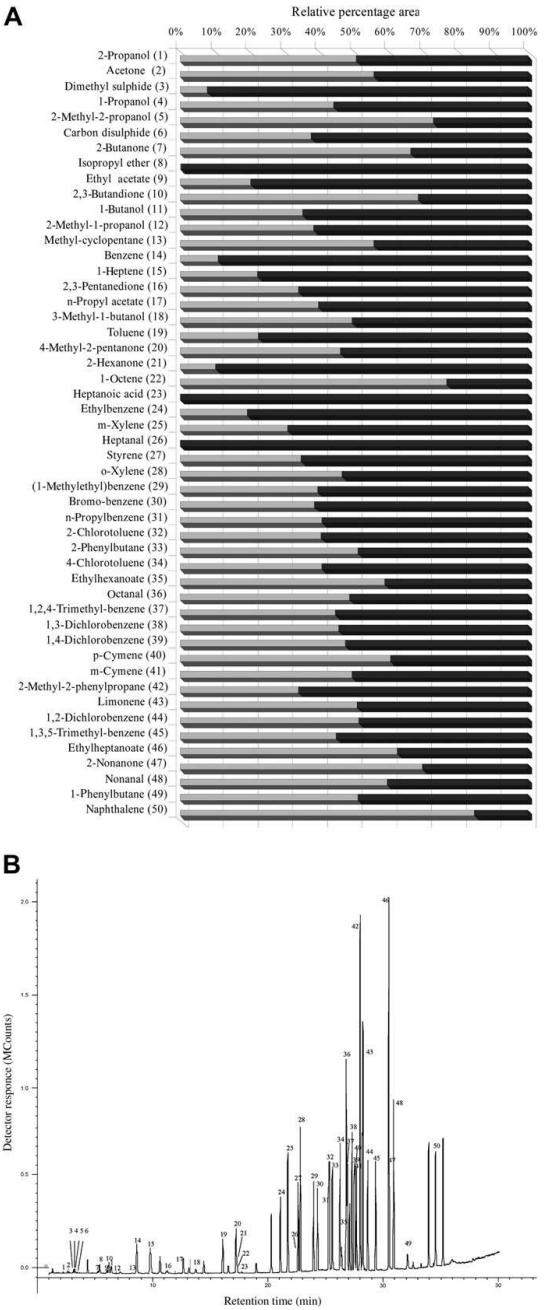

Fig. 2. Panel A: relative percentage area for 50 compounds in a standard solution at 1.0 μg kg^{-1} using stir bar self-made holder (▩) and the Insert holder (■). Relative percentage area was calculated as gas chromatographic (GC) abundance area of each device divided by the sum of the GC abundance for both devices and expressed as percentage. Panel B: Chromatogram for standard solutions at 1.0 μg kg^{-1} using a stir bar Insert (the number of each peak corresponds to the compound name in Panel A).

compounds better isolated with the self-made holder, especially 2-methyl-propanol, 1-octene and naphthalene. Differences between both holding systems could be due to the exposure of the stir bar to the headspace. The Insert, which resembles a small volume vial with a bottom slot, allows the stir bar to be suspended. The Insert could cause a concentration effect while retaining the vapour phase trapped inside the Insert glass. On the other hand, the self-made holder, allowing the complete contact between the stir bar and the vapour phase, leads to the phenomenon known as competitive adsorption where, in this case, higher affinity compounds displace those compounds with lower affinity (David & Sandra, 2007).

Relative presence and compound abundance with respect to the total presence of the compounds also proved important for Insert selection. As observed in Fig. 2b, which presents a chromatogram obtained for the target compounds at 1.0 μg kg^{-1} using the Insert, compounds with a lower polarity showed larger peak areas than highly polar compounds, such as alcohols and ketones. Since an important fraction of the ewes' milk cheese odour profile is represented by alcohols and ketones, which are better adsorbed in the stir bar using the Insert than using the self-made holder, the Insert was chosen for this method.

3.3. Selection of HSSE extraction conditions

Temperature, salt addition, stir bar size, vial volume, sample weight and extraction time were evaluated using grated ewes' milk cheese samples to set HSSE conditions. Fifty compounds were detected, quantified and analyzed by a General Linear Model (GLM) and ANOVA. Fig. 3 presents the effect of each factor mentioned, but only showing the compounds where at least one factor had an influence on their concentration. A positive effect means that a factor increase also produced a significant ($P < 0.05$) compound concentration increment. On the contrary, a negative effect means that a factor increase produced significant ($P < 0.05$) compound concentration decreased.

3.3.1. Effect of extraction temperature

Theoretically, increasing extraction temperature allows compounds to be released from the cheese matrix (Burbank & Qian, 2005), but in this study only six compounds presented this expected behaviour: 3-methyl-1-butanol, 2-hexanone, heptanal, octanal, ethyl hexanoate and 1-octene (Fig. 3). Aldehydes were the chemical group most affected by the temperature increment, therefore the successful extraction for these compounds is temperature dependent. Other compounds such as 2-propanone and 1-propanol with a very low bp were negatively affected by temperature, as has been reported by Pinho, Ferreira, and Ferreira (2002). The same behaviour was observed for 1-methyletylbenzene, 2-methyl-2-phenylpropane and *m*-cymene. The remaining compounds were not influenced by temperature, meaning that no significant differences were observed in the range tested. An extraction temperature of 45 °C was found to be suitable for most of the compounds, especially some alcohols, ketones, aldehydes and esters. The temperature selected is in accordance with different methods for volatile analysis of cheese since it avoids decomposition (Valero et al., 2000).

3.3.2. Effect of salt addition

In this study salt addition (20% anhydrous sodium sulphate) exerted a negative effect on 14% of the compounds studied (Fig. 3), especially alcohols and ketones, which are very important for the cheese odour profile. Salting was positive only for two compounds (ethyl hexanoate and ethylbenzene). The rest of the analytes were not influenced at a statistically significant level by adding salt. On the other hand, most compounds presented lower concentrations when salt addition was used (data not shown). These results showed that in this particular case, salt addition is not necessary.

This observation could be affected by cheese fat, since its high content in cheese samples may favour non-polar compound fat adsorption, instead of allowing migration to the vapour phase. Also, it has been reported that salt does not improve the extraction efficiency of hydrophobic analytes, e.g., pesticides and polycyclic aromatic hydrocarbons, but instead reduces it (Prieto et al., 2010). According to some authors, salt addition promotes the migration of non-polar compounds to the sample surface, minimizing the interaction with the PDMS stir bar and allows interactions between the solutes and the salt, which reduces the ability of the analytes to move (Garcia-Falcon, Cancho-Grande, & Simal-Gandara, 2004; Zuin, Montero, Bauer, & Popp, 2005).

3.3.3. Effect of stir bar size and vial volume

Two stir bar sizes (1 and 2 cm long) were commercially available, having a volume of 24 and 47 μL PDMS, respectively. Although it is the same PDMS phase, there is no direct relationship between the amount of polymer phase and the type of compounds adsorbed. Fig. 3 shows that 1-propanol, 1-butanol, 2-propanone 1-heptene and styrene increased their concentration values using a stir bar of 2 cm in length. On the contrary, a significantly higher concentration of heptanal, 1-octene and some aromatic hydrocarbons was achieved using a stir bar of 1 cm in length. The rest of the compounds were not significantly influenced by the size of the stir bar used, although most of them presented higher concentrations (data not shown) when using the larger bar, so a PDMS-coated stir bar of 2 cm in length was selected.

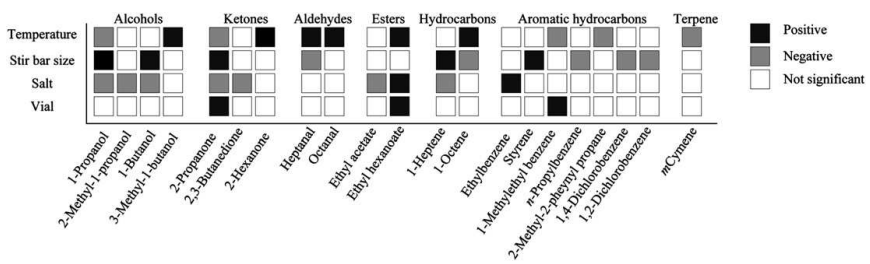

Fig. 3. Effect of temperature, stir bar size, salt addition and vial volume on target compounds concentration. A positive effect (■) means that a factor increase also produced a significant ($P < 0.05$) compound concentration increase. A negative effect (■) means that a factor increase produced a significant ($P < 0.05$) compound concentration decrease. Factor increases not giving a significant effect are denoted by open squares (□).

Vials with different volumes (20 and 50 mL) were used to study the effect of headspace volume. Most of the compounds were not influenced by vial size tested, except for 2-propanone, ethyl hexanoate and 1-methylethyl-benzene. Vials of 50 mL (HS volume of 25 mL) were selected because they could be used to test a wider range of sample weights and could also compensate for the decrease of 2-propanone and 1-methylethyl-benzene due to the extraction temperature selected.

3.3.4. Effect of sample weight and extraction time

A series of analyses were carried out in order to optimize sample weight and extraction time using a PDMS-coated stir bar of 2 cm size in a 50 mL vial at 45 °C with no salt addition. Table 1 shows the analysis of variance for 50 compounds studied and the Tukey's test to establish differences within factors. When increasing sample weight, a uniform release of volatiles was not achieved. It was observed that for four out of six alcohols and five out of seven

Table 1
Effect of sample weight and extraction time on target compounds using analysis of variance.

Compounds	Significance[a]	Sample weight (g)[b]			Significance[a]	Extraction time (h)[b]			
		02.5	05.0	10.0		1	2	4	6
Alcohols									
2-Propanol	0.00	a	a	b	0.36	a	a	a	a
1-Propanol	0.51	a	a	a	0.13	a	a	a	a
2-Methyl-2-propanol	0.15	a	a	a	0.13	a	a	a	a
1-Butanol	0.03	a	ab	b	0.34	a	a	a	a
2-Methyl-1-propanol	0.01	a	a	b	0.19	a	a	a	a
3-Methyl-1-butanol	0.03	a	ab	b	0.00	a	b	bc	c
Ketones									
2-Propanone	0.31	a	a	a	0.00	a	a	b	a
2-Butanone	0.03	a	ab	b	0.03	ab	ab	b	a
2,3-Butanedione	0.01	a	a	b	0.38	a	a	a	a
2,3-Pentanedione	0.53	a	a	a	0.16	a	a	a	a
4-Methyl-2-pentanone	0.02	b	a	b	0.00	a	b	b	b
2-Hexanone	0.00	b	a	b	0.00	a	b	b	b
2-Nonanone	0.01	b	a	b	0.00	a	b	c	c
Aldehydes									
Heptanal	0.00	b	a	b	0.12	a	a	a	a
Octanal	0.44	a	a	a	0.28	a	a	a	a
Nonanal	0.24	a	a	a	0.25	a	a	a	a
Ethers									
Isopropyl ether	0.54	a	a	a	0.46	a	a	a	a
Esters									
Ethyl Acetate	0.01	a	a	b	0.38	a	a	a	a
n-Propyl acetate	0.35	a	a	a	0.60	a	a	a	a
Ethyl hexanoate	0.10	a	a	a	0.17	a	a	a	a
Ethyl heptanoate	0.10	a	a	a	0.00	a	b	c	c
Hydrocarbons									
Methyl cyclopentane	0.09	a	a	a	0.04	b	ab	ab	a
1-Heptene	0.03	b	ab	a	0.07	a	a	a	a
1-Octene	0.53	a	a	a	0.82	a	a	a	a
Aromatic hydrocarbons									
Benzene	0.05	a	a	a	0.05	a	a	a	a
Toluene	0.24	a	a	a	0.04	a	ab	b	ab
Ethylbenzene	0.20	a	a	a	0.00	ab	a	b	c
m-Xylene	0.08	a	a	a	0.00	b	a	ab	c
Styrene	0.00	b	a	b	0.00	a	b	b	c
o-Xylene	0.43	a	a	a	0.01	bc	a	ab	c
1-Methylethyl-benzene	0.63	a	a	a	0.07	a	a	a	a
Bromo-benzene	0.36	a	a	a	0.01	a	a	a	b
n-Propylbenzene	0.50	a	a	a	0.02	a	ab	ab	b
2-Chlorotoluene	0.63	a	a	a	0.06	a	a	a	a
2-Phenylbutane	0.45	a	a	a	0.02	a	b	ab	b
4-Chlorotoluene	0.29	a	a	a	0.35	a	a	a	a
1,2,4-Trimethylbenzene	0.00	b	a	b	0.00	a	b	bc	c
1,3-Dichlorobenzene	0.35	a	a	a	0.42	a	a	a	a
1,4-Dichlorobenzene	0.53	a	a	a	0.54	a	a	a	a
2-Methyl-2-phenylpropane	0.58	a	a	a	0.19	a	a	a	a
1,2-Dichlorobenzene	0.10	a	a	a	0.71	a	a	a	a
1,3,5-Trimethylbenzene	0.46	a	a	a	0.00	a	b	b	b
1-Phenylbutane	0.08	a	a	a	0.05	a	ab	ab	b
Naphthalene	0.56	a	a	a	0.05	a	a	a	a
Terpenes									
p-Cymene	0.49	a	a	a	0.12	a	a	a	a
m-Cymene	0.51	a	a	a	0.16	a	a	a	a
Limonene	0.08	a	a	a	0.01	a	ab	bc	c
Acids									
Heptanoic acid	0.24	a	a	a	0.02	a	ab	b	b
Sulphur compounds									
Dimethyl sulphide	0.13	a	a	a	0.21	a	a	a	a
Carbon disulphide	0.11	a	a	a	0.23	a	a	a	a

[a] Significance was determined by analysis of variance.
[b] Different letters within rows mean significant differences between each sample weight and extraction time ($P < 0.05$).

ketones, concentration was improved as sample size was increased from 2.5 to 10.0 g (Table 1). The compounds most influenced by sample weight were 2-propanol, 2-methyl-1-propanol, 2,3-butanedione, 2-hexanone, 2-nonanone, heptanal and ethyl acetate as indicated by the ANOVA values ($P \leq 0.01$). Two compounds, styrene and 1,2,4-trimethylbenzene with $P \leq 0.01$, showed unusual behaviour as there were no significant differences between 2.5 and 10.0 g, although the concentration decreased when using 5.0 g. The same behaviour was observed for 4-methyl-2-pentanone, 2-hexanone, 2-nonanone and heptanal. Only 1-heptene presented higher values using 2.5 g of sample weight. The rest of the compounds were not influenced by this factor. A sample weight of 10.0 g was selected, thus allowing good detection and quantification limits for most compounds.

Table 2
Validation parameters for the target compounds.

Compounds	Retention time (min)	*r*	LOD[a] (ng/kg)	LOQ[b] (ng/kg)	Precision (%)[c] at LOQ	Recovery (%) at LOQ	S/N peak to peak[d]
Alcohols							
2-Propanol	2.23	0.990	23.1	76.4	15.5	57.9	13
1-Propanol	3.23	0.999	37.5	133.3	27.8	71.2	10
2-Methyl-2-propanol	3.25	0.999	18.8	76.9	28.4	63.1	16
1-Butanol	6.35	1.000	23.1	80.8	25.2	89.1	13
2-Methyl-1-propanol	6.35	0.993	13.7	75.7	33.8	119.5	81
3-Methyl-1-butanol	13.70	0.981	14.3	79.1	21.9	119.7	21
Ketones							
2-Propanone	2.72	0.986	30.0	110.7	34.5	58.1	11
2-Butanone	5.20	0.986	24.1	79.5	27.5	97.3	33
2,3-Butanedione	6.35	0.995	21.5	81.3	16.3	91.2	14
2,3-Pentanedione	11.04	0.985	18.8	78.8	33.4	99.6	16
4-Methyl-2-pentanone	17.27	0.996	20.0	76.0	27.5	64.2	30
2-Hexanone	17.28	0.996	7.9	76.2	16.2	87.9	38
2-Nonanone	30.50	0.987	8.6	79.2	20.2	78.4	35
Aldehydes							
Heptanal	22.50	1.000	20.0	79.3	10.8	81.9	15
Octanal	27.09	1.000	8.4	78.6	21.4	65.2	36
Nonanal	30.92	1.000	6.7	77.8	20.8	69.2	45
Ether							
Isopropyl ether	5.74	0.999	24.0	150.0	29.0	109.5	10
Esters							
Ethyl Acetate	6.35	0.997	26.5	77.6	26.3	59.3	14
n-Propyl acetate	11.86	0.999	20.0	75.9	34.7	117.2	15
Ethyl hexanoate	26.53	1.000	15.0	82.7	15.5	75.2	12
Ethyl heptanoate	30.48	0.999	12.0	82.2	25.9	78.1	25
Hydrocarbons							
Methyl-cyclopentane	8.15	0.987	27.3	93.6	21.7	97.5	11
1-Heptene	9.35	0.986	22.5	126.2	18.3	109.2	10
1-Octene	17.69	0.985	20.0	77.1	17.7	109.2	15
Aromatic hydrocarbons							
Benzene	8.53	1.000	32.9	141.2	14.7	101.5	10
Toluene	16.03	0.981	8.8	79.3	15.9	87.4	34
Ethylbenzene	21.04	1.000	15.8	81.0	11.6	87.6	19
m-Xylene	21.70	1.000	14.4	77.4	15.2	90.3	69
Styrene	22.58	0.998	10.2	78.5	13.3	82.7	58
o-Xylene	22.81	1.000	18.1	79.1	14.8	85.2	13
1-Methylethyl-benzene	23.95	1.000	8.1	77.0	16.2	73.2	37
Bromo-benzene	24.36	0.999	10.4	75.2	20.3	83.8	29
n-Propylbenzene	25.24	0.990	11.1	77.1	16.8	73.5	27
2-Chlorotoluene	25.25	0.991	17.7	79.0	17.2	79.8	17
2-Phenylbutane	25.63	0.998	9.1	80.6	18.6	76.0	33
4-Chlorotoluene	26.16	0.988	12.0	79.7	15.3	73.5	25
1,2,4-Trimethyl-benzene	27.27	0.998	14.3	76.4	9.0	78.7	21
1,3-Dichlorobenzene	27.67	0.986	5.9	79.3	22.5	79.9	51
1,4-Dichlorobenzene	27.68	0.996	7.3	76.4	23.3	82.8	41
2-Methyl-2-phenylpropane	28.17	0.985	16.5	79.5	12.2	80.8	14
1,2-Dichlorobenzene	28.36	0.999	23.1	80.5	24.3	73.5	13
1,3,5-Trimethyl-benzene	29.92	0.998	9.1	79.6	12.7	82.5	33
1-Phenylbutane	31.24	1.000	15.8	75.3	18.8	84.8	19
Naphthalene	34.52	0.998	10.0	88.5	21.3	65.2	15
Terpenes							
p-Cymene	28.16	0.990	10.0	81.9	23.0	79.2	20
m-Cymene	28.17	0.986	9.1	76.1	15.3	86.4	33
Limonene	28.25	0.987	11.6	78.8	18.0	87.2	26
Acid							
Heptanoic acid	17.79	0.987	14.3	80.2	32.7	63.7	21
Sulphur compounds							
Dimethyl sulphide	2.99	0.985	30.0	83.0	32.5	63.1	15
Carbon disulphide	3.44	0.983	21.8	81.9	33.6	75.6	44

[a] LOD: limit of detection.
[b] LOQ: limit of quantification.
[c] Precision showed is referred to within-laboratory reproducibility.
[d] S/N (signal to noise) peak to peak in spiked cheese at LOQ.

Regarding extraction time, different behaviours were observed, depending on the chemical family. Alcohols, except for 3-methyl-1-butanol, aldehydes, ethers, esters with the exception of ethyl heptanoate, and sulphur compounds were not affected by the extraction time used. Ketones presented heterogeneous behaviour; 2-propanone and 2-butanone reached higher concentration values at 4 h of extraction, whereas 4-methyl-2-pentanone and 2-hexanone did not show significant differences between these three extraction times. For 2-nonanone, ethyl heptanoate, 1,2,4-trimethylbenzene and limonene there were no differences between 4 and 6 h, reaching the highest values at these times.

Aromatic hydrocarbons were especially influenced by this factor ($P \leq 0.05$). For some compounds in this family, such as ethylbenzene, *m*-xylene, styrene and *o*-xylene, the highest concentration was reached at 6 h of extraction. In Fig. 2b, it can be observed that the peaks of compounds 2 (2-propanone) and 7 (2-butanone) are low compared with the final part of the chromatogram. Extraction time was set at 4 h, as a good compromise between efficiency and run time for most target compounds.

3.4. Validation method

The proposed method for the analysis of 50 cheese volatiles has been validated (Table 2) in agreement with the criteria of Commission Decision 2002/657/EC (European Commission, 2002). The method showed a good linearity in the range from 0.1 to 100.0 μg kg^{-1} with correlation coefficients higher than 0.98 for all the analytes studied. The recoveries calculated at 0.1 μg kg^{-1} ranged between 57.9 and 119.7% for all target compounds (Table 2). The precision mean values were below 30% except for 2-methyl-1-propanol (33.8%), 2-propanone (34.5%), 2,3-pentanedione (33.4%), *n*-propyl acetate (34.7%), heptanoic acid (32.7%), dimethyl sulphide (32.5%) and carbon disulphide (33.6%). The values of reproducibility obtained for these compounds using HS-SPME by Pinho et al. (2003a,b) are lower than those found with HSSE.

LOQ and LOD provide useful information about validation criteria (Table 2). The LOD values were lower than 38 ng kg^{-1} for all target compounds. The LOQ values were lower than 100 ng kg^{-1} for all analytes except for 1-propanol (133.3 ng kg^{-1}), 2-propanone (110.7 ng kg^{-1}), isopropyl ether (150.0 ng kg^{-1}), 1-heptene (126.2 ng kg^{-1}) and benzene (141.2 ng kg^{-1}). The value of the signal/noise ratio was between 10 and 81 (Table 2). For ethyl hexanoate, heptanoic acid and dimethyl sulphide, LODs of 0.57, 0.54 and 1.61 mg kg^{-1}, and LOQs of 1.29, 1.24 and 3.65 mg kg^{-1}, respectively, have been reported by HS-SPME combined with GC/MS (Januszkiewicz et al., 2008). Similar values of LOQs for acids (3–4 mg kg^{-1}) were obtained by Pinho et al. (2003b) using HS-SPME in combination with GC/MS. No other extraction data have been found in literature. In the case of HSSE, the LOD and LOQ values for ethyl hexanoate were established as 15.0 and 82.7 ng kg^{-1}, respectively. For heptanoic acid, LOD was 14.3 ng kg^{-1} and LOQ was 80.2 ng kg^{-1}, whereas for dimethyl sulphide, LOD and LOQ were 30.0 and 83.0 ng kg^{-1}, respectively. The values of LOD and LOQ obtained by HSSE were almost two orders of magnitude lower than those found with HS-SPME. For the other volatiles studied, no bibliographic references to LOD and LOQ values were found. The results presented in this study confirm that the proposed method is suitable to determine the volatiles in ewes' milk cheese assayed at ng kg^{-1} levels.

3.5. Application to cheese samples

The proposed method was applied to four ewes' milk cheese samples and the volatiles studied are shown in Table 3. In all samples, ketones represented the main constituents of the volatiles (~94% of total volatile content) and the predominant compounds were 2,3-pentanedione (94% of total ketones), followed by 2-hexanone, associated with fruity and floral notes (Molimard & Spinnler, 1996), 2-nonanone, with fruity and musty aroma, and 4-methyl-2-pentanone. Other ketones such as 2,3-butanedione and 2-butanone were detected, and a cheesy note has been assigned to the first, whereas the second was described as butterscotch (Arora, Cormier, & Lee, 1995). Aromatic hydrocarbons represented 1.3% of the total content followed by aliphatic hydrocarbons with 0.8%. The compounds 2-methyl-2-phenylpropane (~23%) and 1-octene (~84%) were the most representative of aromatic and aliphatic hydrocarbons, respectively. Alcohols were 0.9% of total volatile content; 1-butanol comprising 63% of total alcohols followed by 2-propanol, associated with fruity aroma, 2-methyl-1-propanol, related to plastic and bad notes, and 1-propanol, which was considered responsible for sweet and candy descriptors (Arora et al., 1995). Terpenes comprised 0.4% of total volatile content, the predominant being *m*-cymene (~73% of total terpenes) followed

Table 3
Minimum, maximum and standard deviation (expressed as ng kg^{-1}) of volatile compounds studied in four cheese samples analyzed by headspace sorptive extraction/thermal desorption/gas chromatography/mass spectrometry (HSSE/TD/GC/MS).

Compounds	Min ± SD (ng kg^{-1})	Max ± SD (ng kg^{-1})
Alcohols		
2-Propanol	393 ± 151	1078 ± 121
1-Propanol	122 ± 26	252 ± 28
2-Methyl-2-propanol	99 ± 35	202 ± 21
1-Butanol	269 ± 63	4773 ± 609
2-Methyl-1-propanol	159 ± 59	736 ± 165
Ketones		
2-Butanone	256 ± 89	500 ± 135
2,3-Butanedione	272 ± 78	736 ± 165
2,3-Pentanedione	$20.0 \times 10^4 \pm 7.5 \times 10^3$	$97.0 \times 10^4 \pm 16.0 \times 10^4$
4-Methyl-2-pentanone	470 ± 49	577 ± 66
2-Hexanone	471 ± 52	757 ± 109
2-Nonanone	31 ± 11	1032 ± 276
Aldehydes		
Heptanal	123 ± 22	600 ± 63
Octanal	162 ± 58	445 ± 68
Nonanal	332 ± 126	864 ± 96
Ether		
Isopropyl ether	48 ± 13	175 ± 23
Esters		
Ethyl acetate	272 ± 31	736 ± 165
Ethyl hexanoate	28 ± 11	364 ± 41
Ethyl heptanoate	268 ± 30	308 ± 42
Hydrocarbons		
Methyl-cyclopentane	168 ± 63	925 ± 146
1-Octene	292 ± 65	7484 ± 766
Aromatic hydrocarbons		
Benzene	143 ± 31	468 ± 118
Toluene	224 ± 61	424 ± 61
Ethylbenzene	276 ± 28	548 ± 64
Styrene	208 ± 73	436 ± 74
1-Methylethyl-benzene	360 ± 37	722 ± 78
Bromo-benzene	208 ± 68	342 ± 116
n-Propylbenzene	54 ± 18	355 ± 117
2-Chlorotoluene	24 ± 8	100 ± 18
2-Phenylbutane	187 ± 51	997 ± 109
4-Chlorotoluene	220 ± 73	1552 ± 543
1,2,4-Trimethyl-benzene	96 ± 29	1765 ± 296
2-Methyl-2-phenylpropane	383 ± 42	2233 ± 732
1,2-Dichlorobenzene	31 ± 10	221 ± 66
1,3,5-Trimethyl-benzene	477 ± 81	1521 ± 602
1-Phenylbutane	264 ± 84	636 ± 213
Terpenes		
p-Cymene	220 ± 23	480 ± 51
m-Cymene	383 ± 43	2233 ± 232
Limonene	113 ± 32	453 ± 46
Sulphur compounds		
Dimethyl sulphide	272 ± 82	1320 ± 160
Carbon disulphide	3 ± 1	5 ± 2

by limonene, classified as having citrus notes. Aldehydes represented 0.2% of total volatiles, the most abundant was heptanal, identified as soapy (Arora et al., 1995), followed by nonanal and octanal, associated with green and fatty descriptors (Curioni & Bosset, 2002). Esters were 0.1% of total content and ethyl acetate (~50% of total esters) was the more abundant, and related to fruity notes and pineapple, whereas ethyl hexanoate was identified with "young cheese" (Arora et al., 1995). Moreover, sulphur compounds represented 0.1% of total volatile content with dimethyl sulphide being the most abundant (99% of sulphur compounds). Molimard and Spinnler (1996) assigned cabbage notes to dimethyl sulphide, whereas Arora et al. (1995) classified it as a pomegranate aroma. These results confirm that it is possible to characterize the volatile profile of pressed ewes' milk cheese with the proposed method, without fat being a limiting factor.

4. Conclusions

A procedure for the simultaneous determination of 50 volatiles was validated for pressed ewes' milk cheese. The optimum extraction conditions were fixed as: 10.0 g of sample placed into a vial volume of 50 mL, water added to obtain an headspace volume of 25 mL, a stir bar (2 cm) placed into the insert and the sample stirred at 700 rpm for 4 h at 45 °C followed by thermal desorption/gas chromatography/mass spectrometry analysis. An Elite-Volatiles column was selected, and the chromatographic program was set at 40 °C (held for 10 min) raised to 240 °C at 5 °C min^{-1} and maintained for 5 min. Good linearity, precision, recovery and values of LOD and LOQ were obtained for all target compounds. This analytical method was successfully applied to the identification and quantification of volatiles in pressed ewes' milk cheese at ng kg^{-1} levels.

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5.5 Volatile fraction of pressed ewes' milk cheese with saffron

5.5.1 Approach

Reaching this stage of the doctoral thesis, two points were pending: color and aroma carry over during cheesemaking and volatile characterization of saffron cheeses. The first section of this study had as an objective to determine saffron distribution, in terms of color and safranal, during the cheesemaking process (Appendix 8.6). Color and safranal content were determined in cheese, whey, "requesón" (typical Spanish cheese whey) and "requesón" whey. The second section studied the influence of saffron on the volatile fraction of the pressed ewes' milk cheeses fabricated for this doctoral thesis.

Saffron (*Crocus sativus* L.) carryover and its influence on the volatile fraction of pressed ewes' milk cheese during ripening

Licón, C.C., Serrano, J., Librán, C., Carmona, M. and Berruga, M.I.

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5.5.2 Extended summary

Saffron distribution during cheesemaking in terms of color was measured using tristimulus colorimetry. Color differences between control and saffron fabrications were higher in cheese and "requesón" than they were in the liquid matrixes (Appendix 8.6).

Regarding aroma, the extraction methodology optimized in the previous work was used for safranal determination. Saffron used for cheese fabrication had a 3.20 $^{0}/_{000}$ safranal content and corresponded to quality grade A, the best quality for

saffron. Safranal recuperation was about 34 % in the curd; the rest was lost in the whey. From the safranal contained in the whey, around 11 % was retained in the "requesón" and the rest was lost in the "requesón" whey. During the complete process, more than 43 % of safranal was retained in the solid matrixes.

Saffron concentration increments in saffron cheeses did not correspond to safranal increments when analyzing cheese volatile fraction.

Results from determination of volatiles resulted in sixty nine compounds identified. Alcohols, ketones and aldehydes constituted the main chemical families but esters, hydrocarbons, sulphur compounds and terpenes, among others were also identified.

At two months of ripening, the main differences between control and saffron cheeses were higher concentration of heptanal, 1-butanol, 2-methyl-1-propanol, 2-butanol, 1-octene, *p*- and *m*-cymene and some aromatic hydrocarbons while lower concentrations of octanal, nonanal, 4-nonanone, 1-propanol, 3-methyl-1-butanol, ethyl heptanoate, 1-heptene, carbon disulfide and limonene in saffron cheeses.

Differences between saffron cheeses were mainly given by some alcohols and ketones. According to discriminant analysis acetic acid, 2-methyl-1-propanol, toluene, 4-penten-2-ol and 2,3-butanedione were the compounds with more influence on making a discrimination between the lowest saffron concentration and the other two saffron cheeses. 2-pentanone, 2,3-pentanedione, 2-propanol, 2-methyl-1-propanol and 2,3-butanedione were compounds with more influence on the separation of cheeses with the highest saffron concentration from the rest.

Constant differences on 2,3-butanedione (diacetyl) content were observed between control and saffron cheeses and among saffron cheeses. This could be due to the slightly differences found in lactic acid bacteria counts described in section 5.3 of this chapter.

In general, saffron and control cheeses had the same volatile compounds but at different concentration. Results suggest that possible bacteriostatic effect of saffron on lactic acid bacteria and surface molds of cheeses influences the formation of some aroma compounds. However, by the end of ripening differences between saffron and control cheeses were less accused.

Saffron (*Crocus sativus* L.) carryover and its influence on the volatile fraction of pressed ewes' milk cheese

Carmen C. Licón[a], Jessica Serrano[b], Celia Librán[a], Manuel Carmona[bc] and María Isabel Berruga[a]

[a]*Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR, Universidad de Castilla-La Mancha, Campus Universitario 02071, Albacete, Spain.*
[b]*Cátedra de Química Agrícola, ETSIA, Universidad de Castilla-La Mancha, Campus Universitario 02071, Albacete, Spain.*
[c]*Albacete Science and Technology Park, Campus Universitario 02071, Albacete, Spain.*

ABSTRACT

Saffron addition to cheese represents a good alternative to improve flavor and to diversify ewes' milk cheeses, making necessary a characterization of the final product. In this work two objectives were followed: determine safranal distribution in cheesemaking process outputs and the further characterization of the volatile fraction of pressed ewes' milk cheeses manufactured with three different saffron concentrations. Volatiles were isolated by headspace sorptive extraction and identified and quantified by gas chromatography/mass spectrometry. Results showed that safranal retention in cheese was around 32 % which corresponded with the safranal concentrations found in the volatile fraction of the lowest saffron concentration cheeses but not in higher saffron concentrations. More than 68 compounds were identified in cheeses which in general, showed the same volatiles but in some cases, at different concentrations depending on saffron content. Alcohols and ketones were the families most influenced by saffron addition. As a conclusion, saffron influence on the volatile fraction of saffron cheeses was demonstrated being less marked as ripening time increased.

1. Introduction

Ewes' milk cheese industry is very important in the Mediterranean area where a wide variety of cheeses are produced. Manchego, Pecorino Romano and Roquefort, among many others are well-known around the world since they are subjected to high quality standards of a protected designation of origin (PDO) or protected geographical indication (PGI) (Harbutt, 2010). Some of them use additional ingredients in its manufacturing process, such as rosemary, paprika, peppercorns or saffron which changes the characteristics of the cheese, especially flavor and color. These additional ingredients are added in order to improve the quality or to diversify the variety of cheeses.

Flavor is an essential characteristic for cheese, so that during the last few years many efforts have been made to study cheese flavor and to gain knowledge about impact odorants, its formation and changes during ripening. Predominant volatile compounds in ewes' milk cheeses are alcohols, ketones, aldehydes, acids and esters, but hydrocarbons, sulphur compounds and terpenes are also present. Works have been done to characterize the volatile fraction of different cheeses, to identify the geographical origin and to observe seasonal variation and milk heat treatment (Barron, et al., 2007; Fernández-García, Carbonell, & Nuñez, 2002; Fernández-García, Serrano, & Nuñez, 2002; Mallia, Fernández-García, & Olivier Bosset, 2005). Nevertheless when new flavor ingredients are added only few studies focus attention on the characteristics that these agents provide to the final product, for example addition of ethanol to improve ester profile of Swiss-type cheeses or characteristics of Turkish cheeses added with different herbs (Hayaloglu & Fox, 2008; Richoux, Maillard, Kerjean, Lortal, & Thierry, 2008).

One of the most known ewes' milk cheeses with added ingredients is the Piacentinu Ennesse which includes saffron and peppercorns during the cheese making process. Recently in Spain, saffron has been introduced as well for the production of pressed ewes' milk cheeses. Saffron cheeses has been studied in terms of chemical, microbiological and sensory characteristics (Carpino, Rapisarda, Belvedere, & Licitra, 2008; Fallico, et al., 2006; Horne, et al., 2005). Recently studies about the influence of increasing saffron concentration on the composition, microbiology, texture, color and sensory characteristics of pressed ewes' milk cheeses has been conducted (Licón, Carmona, Molina, & Berruga 2012). Results lead to the conclusion that saffron influences color, flavor, salt content and texture. Nevertheless flavor attributes provided by saffron to cheese have not been deeply detailed.

Saffron is the dried stigma of *Crocus sativus* L. used as an ingredient in many traditional and actual dishes. It is one of the few spices that provide color, taste and aroma to food. This spice is one of the most expensive in the market, so that, it would be important to optimize its retention during cheesemaking process since no studies have been carried out on this subject. Saffron aroma is very complex and many studies have focused on determine compounds that contribute to the aroma perception (Alonso, Salinas, Esteban-Infantes, &

[1] Corresponding autor: Tel.: +34 967 599200 ext. 2615; fax: +34 967 599238. *E-mail addres*: MariaIsabel.Berruga@uclm.es

Sánchez-Fernández, 1996; Alonso, Salinas, Sánchez-Fernández, & Garijo, 2001; Carmona et al. 2006). Volatile fraction is mainly formed by ketones and terpenic aldehydes, but safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the major compound present.

During saffron storage, safranal content increase changing from spicy and floral notes to vegetable, caramel and citric (Maggi, et al., 2010). The importance about studying saffron aroma in the final product lies in the fact that saffron aroma depends also on thermal treatment, so that, it is not the same aroma perception when smelling directly the spice than when it has been added to food and has been subjected to further cooking (Carmona, Zalacain, Salinas, & Alonso, 2007).

The principal objectives of this study were (i) to determine saffron distribution, in terms of safranal, during the cheesemaking process; and (ii) to study influence of saffron addition on ewes' milk cheeses volatile fraction during ripening.

2. Materials and methods

2.1 Saffron aroma distribution

Saffron. Spanish saffron spice (*Crocus sativus* L.) from the Protected Designation of Origin "Azafrán de la Mancha" was used. Saffron spice was grounded and characterized according to ISO 3632 Technical specification (2003). Safranal concentration was determined with headspace sorptive extraction (HSSE) according to the methodology of Licón et al. (2012) with slightly modifications: extraction time (2 hrs) and stir bar length (1 cm). Calibration curves with a safranal standard solution (88% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used.

Cheese fabrication. "Manchega" breed ewes' raw milk from a commercial farm (Albacete, Spain) was used. Milk had an average composition (g/100g) of dry matter 16.35 ± 0.10, fat content of 5.21 ± 0.18 and protein content of 5.42 ± 0.11. Two different fabrications were produced by duplicate: one with 1% (w/v) of saffron spice (A) and one with a safranal standard solution (0.23 mg/ml) (B). Before cheese fabrication, saffron/safranal extractions in ewes' milk were carried out according to the pending patent No. P200930912 (Berruga Fernández, et al., 2009). Two liters of milk were used in each fabrication. After extraction, milk was heated in a water bath (30 ºC) and commercial rennet (0.015 g/L) was added. After 30 minutes curd was cut into 8-10 mm cubes and heated to 37 °C during 20 min before whey separation. Curd was placed in perforated plastic molds (6x6x7cm; Busqui, Spain) with an approximate weight of 100 g and pressed by gravity during 2 hours. Meanwhile, cheese whey was stirred at 750 rpm and heated to reach a temperature of 75-80 ºC. After, stirring was stopped and the cheese whey was heated to 90 ºC to allow flocculation of the whey proteins. Proteins were separated from the whey with a perforated spoon to obtain the traditional Spanish whey cheese called Requesón. The Requesón was placed in perforated plastic molds (Busqui, Spain) to cold down during 2 hours. Cheese and Requesón were kept at 2 ºC no more than 18 hours up to the time when analyses were performed.

Safranal determination. Safranal was analyzed on milk extracts, cheese, cheese whey, Requesón and Requesón whey. Safranal isolation was made using HSSE technique and was analyzed by thermal desorption coupled to gas chromatography/mass spectrometry (TD/GC/MS) according to Licón et al. (2012). For liquid samples the extraction time was 2 h using 1 cm length sorptive bars. Safranal was identified by comparison of retention time and spectra with the real standard (88% purity, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). For quantification, calibration curves were done in ewes' milk and cheese by spiking a standard solution of safranal at five different concentration levels in a range from 10 to 160 µg/kg. Ethyl octanoate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as internal standard (1 mg/kg).

Safranal distribution. Results were expressed as percentages. Safranal recovery in the cheese and cheese whey were calculated taking as a reference the milk, so that, their safranal content was divided by safranal content in milk. For Requesón and Requesón whey, the initial safranal concentration was referred as the cheese whey, so that, their safranal content was divided by the safranal content obtained in the cheese whey.

2.2 Determination of cheese volatile fraction

Cheesemaking. Cheeses were manufactured at a local factory ("Quesería CampoRus", Cuenca, Spain) using "Manchega" breed ewes' milk from their own supply. The milk composition (g/100 g) had an average (± standard deviation) DM content of 19.43 ± 0.54, fat content of 7.66 ± 0.38 and protein content of 6.19 ± 0.17. Four vats of 300 L of pasteurized (72 ºC, 20 s) milk were fabricated with three different saffron concentrations (S, 2xS and 3xS) and a control without saffron (C). Saffron concentration and extraction procedure were used according to the pending patent No. P200930912 (Berruga Fernández et al., 2009). For cheese manufacturing, a starter culture containing *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis biovar*. *diacetylactis* and *Streptococcus thermophilus* was added (CHOOZIT MA4001, Danisco, Sassenage, France) at 5 Danisco culture units (DCU)/100 L. Milk was held at 30 °C during 20 min, adding 0.025 % (v/v) of $CaCl_2$ and 0.01 % (vol/vol) of lysozyme. Commercial rennet (BioRen, Hundsbichler GmbH, Unterlangkampfer, Austria) was used for coagulation (chymosine:pepsine,

94:6) at 0.023 % (vol/vol). Thirty minutes later the curd was cut into 8- to 10-mm cubes and heated (37 °C) and stirred during 45 min before whey separation. Curd was molded and pressed using a pneumatic press (100 kPa) for the amount of time needed for the pH to reach 5.2, which was on average 4 h. Pieces of cheese weighing approximately 3 kg were obtained. Cheeses were placed in brine (18 % NaCl w/v) during 18 h at 9 °C. After that, they were kept in a cold chamber (9 ºC) during 48 h and then were ripened in a maturation chamber at 11±1 ºC and RH 85 % during 180 days. Surface molds were removed when necessary. Dry matter, protein, fat and salt content were determined with an infrared analyzer FoodScan (FossElectric A/S, Hillerød, Denmark) by duplicate.

***Aroma analysis*.** The volatile fraction of saffron and control cheeses was analyzed at 2, 4 and 6 months of ripening. Volatiles were isolated by HSSE and analyzed by TD/GC/MS, according to the methodology proposed by Licón et al. (2012). Ethyl octanoate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as internal standard (1 mg/kg). All extractions were done by triplicate. Identification was done with comparison with mass spectra of authentic compounds or mass spectra of NIST 11 Mass Spectra Library (Scientific Instrument Services, NJ, USA). Quantification was carried out with calibration curves with authentic standards or with comparison with internal standard, *n*-octane (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All standards and reagents used were GC grade: 1-Octene (98%), 2,3-pentanedione (97%), 2-nonanone (99%), 3-methyl-1-butanol (99%), heptanal (95%), nonanal (95%), octanal (92%), (R)-(+)-limonene (97%) and dimethyl sulfide (99%) were supplied by Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); 2,3-butanedione (99.4%), 1-heptene (99.5%) and *m*-cymene (99%) by Fluka (Sigma-Aldrich, Steinheim, Germany); ethyl acetate (99.5%), ethyl hexanoate (99%) and ethyl heptanoate (99%) by Chem Service (Chem Service Inc., West Chester, PA, USA). In addition, 1-methylethyl-benzene, *n*-propylbenzene, *o*-xylene, 2-methyl-2-phenylpropane and 2-chlorotoluene contained in EPA Volatile Organic Compounds Mix 1; benzene, ethylbenzene, *m*-xylene, naphthalene, *p*-cymene, styrene and toluene contained in EPA Volatile Organic Compounds Mix 2; 2-propanone, carbon disulfide, 2-methyl-2-propanol, 2-butanone, 2-hexanone and 4-methyl-2-pentanone in 8260 Mix 5; and 1-butanol, 2-propanol, 1-propanol, 2-methyl-1-propanol and isopropyl ether in Qualitative Peak ID Mix. All mixes were supplied by Supelco (Sigma-Aldrich, Steinheim, Germany). Water was purified through a Milli-Q System (Millipore, Bedford, MA, USA).

***Statistical analysis*.** Analysis of variance ($P \leq 0.05$) was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Tukey's test at a significance level of $P \leq 0.05$ was used to determine differences on each volatile between cheeses with different saffron concentration and with different ripening time. Discriminate analysis was performed to ascertain which of the different volatiles were most useful to differentiate between saffron concentrations and ripening time. Wilk's lambda (λ) was used as the statistical selection criterion for the variables.

3. Results and discussion

3.1 Safranal distribution in cheese products

Saffron had a coloring strength of 254.5 ± 3.6, $E^{1\%}_{1cm}$ 257 nm of 96.2 ± 1.3 and $E^{1\%}_{1cm}$ 330 nm of 35.6 ± 0.5. According to ISO 3632 (2003) saffron was category I, the best quality grade that this spice can have. HSSE was selected as the isolation technique to study saffron aroma because it avoids generation of artifacts (Carmona, et al., 2007). Safranal concentration in saffron spice measured by HSSE/GC/MS was 3.20±0.36 mg per g of saffron.

In order to quantify safranal in liquid samples, calibration curves in milk were used. For cheese and Requesón calibration curves were carried out in cheese. During the calibration there were almost no differences between liquid and solid matrixes, since the chromatographic peaks for milk were only 0.15 times higher than the peaks resulted in the cheese.

Safranal distribution (%) in the different steps of the cheesemaking process is shown in Figure 1. No differences were found on the safranal recovery rates between both fabrications on any fraction. Cheese retained 36 and 32 % of safranal in fabrications A and B respectively, while around 69 % was lost in the cheese whey in both fabrications. From this remaining safranal only 10 and 11 % was retained in the Requesón A and B respectively, while the rest was lost in the Requesón whey. Between 43 and 46 % of the safranal added to milk was retained in solid fractions during processing (data not shown).

Safranal retention in cheese could be due to interactions with caseins and/or lipids retained in the cheese matrix since it has been found that both molecules (lipids in a greater extent) have affinity for hydrophobic aroma compounds such as safranal (Kopjar, Andriot, Saint-Eve, Souchon, & Guichard, 2010; Kühn, Zhu, Considine, & Singh, 2007). Interactions of milk protein and fat with different flavor compounds have been deeply studied in order to gain knowledge about perceived flavor in cheese (Han, et al., 2011; Kopjar, et al., 2010; Kühn, et al., 2007; Livney, 2010; Wackerbarth, Stoll, Gebken, Pelters, & Bindrich, 2009). It has been found that caseins, whey proteins and milk lipids can have hydrophobic or covalent interactions with aroma molecules such as 2-nonanone, 1-nonanal and vanillin. These interactions would be strongly dependant on the protein and lipid conformation and the hydrophobicity of the aroma compound (Kühn,

Considine, & Singh, 2008; Piraprez, Hérent, & Collin, 1998).

It has been reported that β-Lactoglobulin and bovine serum albumin (BSA), both present in cheese whey, have higher affinity for different flavor compounds compared with caseins, explaining the higher safranal content in this fraction than in cheese (Kühn, et al., 2008; Li, Grün, & Fernando, 2000). Nevertheless after Requesón fabrication, only around 10 % of safranal was retained even that most of whey proteins are retained in the Requesón. The higher safranal content in the Requesón whey could be related to heat-induced structural changes in whey proteins during fabrication of Requesón, making some peptide chains of the protein unavailable to bind with safranal. Studies about heat treatment influence on the binding properties of flavor compounds with whey proteins have revealed that heat treatment affects flavor binding in a way that higher temperatures and time would lead to less binding properties of the protein (Chobpattana, Jeon, Smith, & Loughin, 2002; Kühn, et al., 2007; Kühn, et al., 2008).

All these possible interactions deserve further study to gain knowledge about saffron and milk components in order to optimize saffron addition to dairy products.

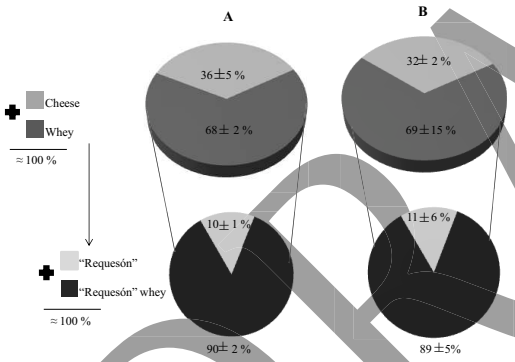

Figure 1. Safranal retention coefficients during cheese making process in cheeses made with saffron spice (A) and safranal standard solution (B)

3.1 *Aroma in saffron cheeses*

General cheese composition. All composition values were within the range of a common semi-hard pressed ewes' milk cheese with the exception of pH that was lower than values reported (Cabezas, Sánchez, Poveda, Seseña, & Palop, 2007). Table 1 shows the compositional parameters of the control and saffron cheeses at the ripening stages studied. Control cheeses showed similar pH values than saffron cheeses ($P \leq 0.05$). In general, this parameter was constant during ripening, which indicates that the cheese fabrication process was adequate. Regarding compositional profile, no significant differences were found in dry matter, fat and protein content due to saffron addition. The evolution of these parameters during ripening was similar for all types of cheeses. Dry matter increased significantly ($P \leq 0.05$) during ripening. Fat and protein by dry matter content remained constant. Control cheeses presented higher salt content than saffron cheeses ($P \leq 0.05$). Although microbiology of cheeses is not presented here, previous results showed that saffron slightly decreased lactic acid bacteria counts which caused one hour decrease during pressing. As a result, water content was higher in control cheeses and promoted higher salt exchange between the brine and the cheese matrix and thus its higher content (C.C. Licón, et al., 2012).

Volatile composition in saffron cheeses. Headspace sorptive extraction followed by GC allowed the separation of 68 peaks corresponding to 69 volatile compounds in the headspace of saffron ewes' milk cheeses during ripening. 4-Methyl-2-pentanone could not be separated from 2-hexanone, so that, one peak was taken as two compounds. Most of the compounds identified have been previously detected in other ewes' milk cheese varieties, but not often quantified with real standards - , Gaya, Medina, & Nuñez, 2004; Horne, et al., 2005; Mallia, et al., 2005). Table 2 reports the mean concentration (μg/kg) of the compounds found, as well as the effects of saffron concentration and ripening on the volatile profile of cheeses. These compounds belonged to different chemical families: aldehydes, ketones, alcohols, esters, ethers, hydrocarbons, aromatic hydrocarbons, acids, sulphur compounds, terpenes and furans.

During ripening notable differences were found in the volatile profile of control and saffron cheeses. Saffron addition also change the volatile profile of cheeses but these differences were less marked as ripening time was increased. Aldehydes, ketones and alcohols constituted the main chemical families in the volatile fraction of control and saffron cheeses as previously found by different authors in ewes' milk cheeses (Barron, et al., 2005; Mallia, et al., 2005).

Aldehydes are very common in cheese and together with ketones, are the major secondary products of oxidation of fatty acids, although they also can be formed from catabolism of amino acids among other pathways (McSweeney & Sousa, 2000). They are considered as transitory compounds because they are rapidly reduced to alcohols or oxidized to the corresponding acids (Le Quéré, 2004; Panseri, et al., 2008). Six aldehydes were detected being octanal and nonanal the most abundant. At 2 m control cheeses showed significant ($P \leq 0.01$) higher concentration of these two aldehydes than saffron cheeses. These volatiles are very common among many kind of cheeses, such as Mozzarela or Grana Padano, giving green-grass and herbaceous notes.

Table 1. Mean values ± standard deviation for pH and chemical composition of control and saffron cheeses

Parameters	Ripening (months)	Control	S	2xS	3xS	ANOVA
pH	2	5.19±0.08	5.27±0.14	5.25±0.05	5.24±0.11	NS
	4	5.17±0.18	5.24±0.14	5.21±0.13	5.23±0.12	NS
	6	5.19±0.15	5.23±0.16	5.16±0.06	5.24±0.11	NS
	ANOVA	NS	NS	NS	NS	
Dry matter (g/ 100)	2	64.74±0.17^{a}	66.88±0.62^{a}	65.46±1.09^{a}	64.24±0.01^{a}	NS
	4	68.51±0.55^{b}	67.44±0.16^{ab}	68.91±0.42^{b}	67.98±0.53^{b}	NS
	6	70.84±0.40^{c}	71.13±1.60^{b}	71.43±0.39^{c}	70.52±0.05^{c}	NS
	ANOVA	***	**	**	**	
Fat/Dry Matter (g/ 100)	2	53.96±0.11	53.93±0.01	53.55±0.38	53.83±0.15	NS
	4	54.10±0.19	54.32±0.14	53.32±1.88	53.73±0.05	NS
	6	53.48±0.34	54.02±0.17	53.33±1.70	53.98±0.54	NS
	ANOVA	NS	NS	NS	NS	
Protein/Dry Matter (g/ 100)	2	35.22±0.81	34.03±0.69	34.94±0.81	35.13±1.32	NS
	4	33.77±1.35	35.02±2.36	35.11±0.01	35.48±1.12	NS
	6	32.92±0.88	33.6±0.92	32.21±1.38	35.48±0.77	NS
	ANOVA	NS	NS	NS	NS	
NaCl (g/ 100)	2	1.94±0.01^{z}	1.51±0.01^{xy}	1.48±0.03^{x}	1.63±0.06^{y}	***
	4	1.88±0.03^{y}	1.72±0.11^{xy}	1.42±0.03^{x}	1.43±0.14^{x}	*
	6	1.91±0.09^{z}	1.62±0.01^{xy}	1.44±0.03^{x}	1.74±0.01^{yz}	**
	ANOVA	NS	NS	NS	NS	

^{a,b,c} different letters within the same column mean significant differences ($P \leq 0.05$).
^{x,y,z} different letters within the same row mean significant differences ($P \leq 0.05$).

Hexanal and 3-methyl butanal were only present at 6 m of ripening in all cheeses. The latter has been identified as a potent odorant in Camembert and aged Cheddar giving at low concentrations fruity odors (Curioni & Bosset, 2002). In Manchego cheese, this compound has been identified as floral or soapy but its odor intensity was weak (Mallia, et al., 2005).

Safranal is a terpenic aldehyde representing the major fraction of saffron aroma. This molecule was present in saffron cheeses increasing its concentration by the end of the ripening, as a probable consequence of loss of water. As expected, 3xS cheeses showed higher safranal concentration than the rest of saffron cheeses ($P \leq 0.001$), however 2xS did not showed higher safranal concentration than S, except at 6 m. Safranal represented a minor fraction of total aroma of cheeses, nevertheless, previous studies reveled flavor differences among control and saffron cheese and between saffron cheeses (C.C. Licón, et al., 2012). This compound gives floral, sweet and with time, pungent notes but its threshold has not been yet studied in dairy products.

Ketones are mainly produced during ripening from partial β-oxidation of free fatty acids and they are reduced to their corresponding alcohols - , Gaya, et al., 2004). Nine ketones were detected, most of them were methyl ketones which has been previously reported as the most common found in ewes' milk cheeses (Barron, et al., 2005). For control cheeses, 4-nonanone was the most abundant ketone present decreasing its concentration during ripening, while in saffron cheeses its concentration was constant. For saffron cheeses, 4-methyl-2-pentanone and 2-propanone represented the major ketone fraction at 2 m of ripening and its behavior during ripening did not show a specific trend. 2-nonanone was also present increasing its concentration with time in all cheeses. Changes in concentration of 2-pentanone and 2-heptanone during ripening were contrary for control and saffron cheeses. The former decreased its concentration while the latter increased it with ripening time ($P \leq 0.05$). 2,3-butanedione (diacetyl), an important compound found in cheeses described as having buttery notes with a strong odor intensity is formed by citrate metabolism and is latter reduced to 2,3-butanediol (Mallia, et al., 2005). Its presence depends in great part of the bacterial strains present in cheese. Previous data showed that control cheeses had slightly higher bacterial counts than saffron (C.C. Licón, et al., 2012) which could correspond with higher diacetyl concentrations in control. These authors observed that increasing saffron concentration, caused a slightly lower bacterial counts, corresponding with lower diacetyl concentration. During ripening, diacetyl decreased in control and S cheeses, remained almost constant in 2xS cheeses and increased in 3xS. This differences among saffron concentration could be due to the equilibrium between its production and reduction to 2,3-butanediol that in the case of 3xS as having slightly lower bacterial activity, its reduction could be slower than its production.

Generally, at the low redox potential of cheese, aldehydes and ketones are mostly reduced to alcohols (Curioni & Bosset, 2002). Alcohols represented more than 20% of the total volatile fraction of cheeses. Sixteen molecules could be identified where 1-propanol, 2-propanol and 3-methyl-1-butanol were concentration in control cheeses while increased in saffron cheeses. 3-mehtyl-1-butanol decreased in control and S and increased in 2xS and 3xS, this compound is likely to play an important role in the aroma of these cheeses because

it has an odor threshold of 300 μg/kg in water, values lower than the ones present in our samples (Curioni & Bosset, 2002). 2-propanol increased from month 2 to month 4 and then decreased in all cheeses. During ripening 2-butanol increased in control and S cheeses while its behavior was not clear in 2xS and 3xS. This compound is a reduction product from diacetyl which also showed different behavior depending on saffron concentration, which suggests that, these differences could be influenced by cheese microorganisms differences as well. Among other alcohols present, 1-pentanol, 2-pentanol and 2-heptanol were only present in saffron cheeses at 6 m of ripening. These three alcohols have been described as weak odor active compounds for Manchego cheese with chemical, floral and mushroom descriptors (Mallia, et al., 2005).

Esters are common constituents of cheese that are produced as a reaction of acids with primary and secondary alcohols; the most common are ethyl esters, related to sweet, fruity and floral notes with a very low olfactory threshold. They are also correlated with the growth of lactic acid bacteria and *Micrococcaceae* (Bertolino, Dolci, Giordano, Rolle, & Zeppa, 2011; Panseri, et al., 2008). Six esters were found in the volatile fraction of saffron cheeses showing differences between saffron and control cheeses. Ethyl hexanoate and ethyl heptanoate represented the most abundant esters in the volatile fraction of cheeses, especially at 2 m of ripening where saffron cheeses showed lower concentrations ($P \leq 0.01$). A negative correlation between saffron concentration and ethyl hexanoate was observed but this correlation was positive with ripening time ($P \leq 0.05$), probably as a concentration effect due to loss of water. In contrast, ethyl heptanoate did not show a constant correlation with saffron concentration and during ripening.

Acids, especially short chain free fatty acids are important, and in some cases, predominant components of the flavor of most of ripened cheeses and they serve as precursors of methyl ketones, alcohols, lactones and esters (Curioni & Bosset, 2002). In this study only two acids were detected, acetic and butanoic acid. Butanoic acid was only detected at 6 months while acetic acid constantly increased its concentration during ripening in control cheeses ($P \leq 0.05$) whereas in saffron cheeses decreased from 2 to 4 months and then increased. During cheese ripening, acids containing four or more carbon atoms may originate from lipolysis of milk fat, while acetic acid is originate from lactose metabolism. In cheeses made from pasteurized milk lipolysis is not as marked as in cheeses made from raw milk because starter cultures are weakly lipolytic, explaining the low content of butanoic acid in these cheeses since they were made from pasteurized milk (Curioni & Bosset, 2002). However, acetic acid presence does not correspond to growth of lactic acid bacteria, since it would be expected lower concentrations in 3xS cheeses as they showed slightly lower counts of lactic acid bacteria (C.C. Licón, et al., 2012).

Sulphur compounds originate from methionine degradation and play an important role in the flavor of cheese. Three sulphur compounds were detected in control and saffron cheeses increasing its concentration with ripening, especially with 4 months. The most abundant was carbon disulfide with lower concentration in saffron cheeses at 2 and 4 months but higher at 6 months ($P \leq 0.05$). During ripening, control and S cheeses increased its concentration from month 2 to month 4 and then decreased while in 2xS and 3xS constantly increased. Dimethyl sulfide was present in saffron cheeses after 4 months, while dimethyl sulphone was only detected at 6 months, contrary to control cheeses where these two compounds were detected at all ripening stages. These compounds have sulfurous, garlic, cabbage and hot milk odor descriptors and their perception thresholds are very low, thus they probably contribute to the final aroma of cheese especially of control (Curioni & Bosset, 2002).

Terpenes are transferred to cheese from the ewes' forage and their presence is especially important in cheeses manufactured in alpine regions (Bugaud, Buchin, Hauwuy, & Coulon, 2001). Four terpenes were detected in control and saffron samples, representing a high portion of the total volatile fraction (data not shown), as previously observed in other ewes' milk cheeses such as Idiazabal, Manchego and Zamorano

- , Carbonell, et al., 2004). *p*-cymene and *m*-cymene showed significant differences between control and saffron cheeses, especially at 2 months were these two compounds were not detected in control. Moreover, a negative correlation with saffron concentration was observed. At early stages of ripening, limonene was presented in control cheeses at higher levels ($P \leq 0.05$) than in saffron cheeses, but its concentration decreased to the extent that saffron cheeses showed significant higher levels ($P \leq 0.05$) after 6 months. These differences are remarkable since limonene has been reported as secondary metabolite of some strains of *Penicillium* spp., molds that are the most abundant in the rind of semi-hard ewes' milk cheeses (Börjesson, Stöllman, & Schnürer, 1992; Freitas & Malcata, 2000; Kure, Skaar, & Brendehaug, 2004; Pasanen, Lappalainen, & Pasanen, 1996). In this study, molds in the surface of the saffron cheeses were not characterized but limonene behavior suggests that saffron could be favoring the presence of some strains or slowing down the growth of others, as saffron has a moderate activity against some molds and yeast strains (Sekine, Sugano, Majid, & Fujii, 2007). This fact deserves further study to deep knowledge about saffron antifungal activities.

Ethers, hydrocarbons, aromatic hydrocarbons, 2-pentyl-furan and ionone represented a minor fraction on the volatile profile of cheeses. The most abundant compounds in these families were 1-heptene, 1-octene, *n*-butylbenzene and 2-methyl-2-pheynylpropane, they

have a high odor thresholds in the range of mg/kg, so it is possible that they do not contribute to cheese aroma but they could serve as precursors for the formation of other aromatic compounds (Arora, Cormier, & Lee, 1995). It has been suggested that these compounds come from grass and may be produced by degradation of carotenes - , Gaya, et al., 2004). 2-pentyl-furan was only found at 2 months of ripening although this family of compounds is not often found in cheeses. Nevertheless, they are known to be powerful flavor compounds having pleasant taste and odor and can contribute to cheese aroma (Curioni & Bosset, 2002). Ionone (α or β) was not detected until 4 months of ripening, probably as a concentration effect. This compound is commonly found in essential oils of some aromatic plants giving floral, sweet and berry fruity notes but its presence is not frequent in cheese (Goodner, Mahattanatawee, Plotto, Sotomayor, & Jordán, 2006).

***Discriminant analyses*.** As shown in Table 2, most volatile compounds were present in all cheeses, but at different concentrations independently of saffron presence. Discriminant analyses were done to answer three questions regarding saffron and its addition to milk for ewes' milk cheese production.

The first point was to identify differences between control and saffron cheeses besides safranal presence, which were evident according to function 1 of the discriminant analysis (Figure 2). From the analysis three functions explaining 100% of the variance were obtained, which the first component explained 96.3% of the variance. Compounds with more weight in function 1 were mainly alcohols and ketones, among them the higher coefficient factors were: 4-nonanone (14.33), 2,3-butanedione (10.35), 2-butanol (6.63), 2,3-butanediol (5.57), benzene (4.01), 3-methyl-1-butanol (-7.77) and acetic acid (-7.83). Results are related with significant lower concentration in saffron cheeses of 4-nonanone, 2,3-butanedione, 2,3-butanediol, and 3-methyl-1-butanol at one of the three stages of ripening, while is also related to higher acetic acid and benzene concentration as saffron concentration was increased (Table 2). Some of these compounds, especially diacetyl and acetic acid, are strongly related to starter activity so its high weight on determining differences between control and saffron cheeses confirms the observations previously described by Licón et al. (2012) about saffron bacteriostatic effect on the starter.

To establish which compounds could explain differences between saffron cheeses, a second discriminant analysis, excluding the control, was carried out where two functions were obtained, the first one explaining 88.6% of the variance. In this analysis (Figure 3) there is a clear separation between S cheeses and the rest according to function 1. Compounds with higher coefficients were acetic acid (21.18), 2-methyl-1-propanol (18.61), toluene (10.97), 4-penten-2-ol (9.29) and 2,3-butanedione (-14.69). These compounds, except for acetic acid, showed similar concentration values between 2xS and 3xS cheeses. Once again, diacetyl and acetic acid show an important role to discriminate between cheeses. Function 2 explained 11.4% of the variance but cheeses did not showed a clear separation, although 3xS were slightly separate from the rest. 2-pentanone (7.63), 2,3-pentanedione (8.26), 2-methyl-1-propanol (5.28), 2-propanol (-10.92) and 2,3-butanedione (-7.49) were the compounds with higher coefficients in function 2, probably because some of them showed higher concentration in 3xS cheeses at some extent of ripening (Table 2). It can be observed that, as in the first discriminant analysis, most of these compounds were alcohols and ketones, which share mainly two common formation pathways: β-oxidation of free fatty acids and glycolysis, depending primarily on microorganisms, especially molds and lactic acid bacteria enzymes. Cheeses for this study were fabricated from pasteurized milk, so that, the molds and lactic acid bacteria present should be the same in all cases. Once again differences between control and saffron cheeses on the volatiles mentioned above confirm that differences on the surface molds and lactic acid bacteria growth have a strong influence on the volatile profile of saffron cheeses.

The last objective of this work was to study volatile changes in saffron cheeses during ripening. Two functions were obtained explaining 86.4 and 13.6 % of variance, respectively, allowing a clear separation between different ripening times according to both functions (Figure 4). Once again, the compounds showing higher coefficient values were mainly alcohols and ketones. For function 1, compounds with higher positive coefficients were 1-butanol (27.68) and 2-methyl-2-propanol (9.20), both compounds showed higher concentration at six months of ripening. 2-Methyl-1-propanol (-26.04) and benzene (-15.12) were the volatiles with higher negative coefficients. In 3xS cheeses, the former was only detected at 6 months of ripening, while the latter was present at higher concentrations. According to function 2, cheeses with 4 months of ripening were slightly more separated than the rest which 2-propanol (-12.09), 2-methyl-2-propanol (8.76), 2-pentanone (-8.47) and 2-methyl-1-propanol (-10.83) showed higher coefficients. According to both functions, dispersion between samples at 6 months of ripening was lower than dispersion for samples at 2 and 4 months, confirming that differences between saffron cheeses decreased by the end of ripening.

4. Conclusions

At the conditions used for this study saffron recuperation, in terms of safranal, during ewes' milk cheese and "Requesón" manufacture was around 46 % while the rest was lost in the "Requesón" whey. Our results showed that safranal could have more affinity with caseins than with whey proteins, as a consequence

of its denaturation due to the high temperatures used for fabrication of "Requesón". All cheeses contained approximately the same volatiles but at different concentrations, where alcohols and ketones were the volatile groups most influenced by saffron addition. Diacetyl, acetic acid and limonene behavior was a key to confirm that the slight bacteriostatic effect of saffron on lactic acid bacteria and probably on some molds strains, could have a direct influence on the volatile fingerprint of cheeses, fact that deservs further study to gain knowledge about this subject.

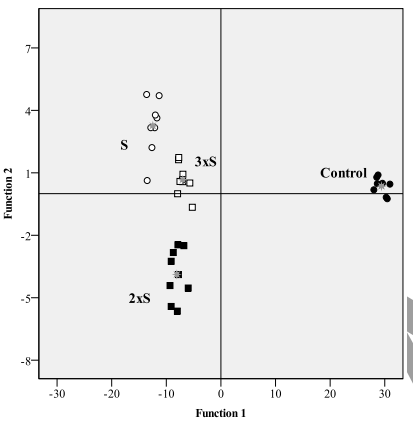

Figure 2. Plot of sample distribution using two canonical discriminant functions according to saffron concentration: control (●), S (○), 2xS (■), 3xS (□) and group centroid (✸).

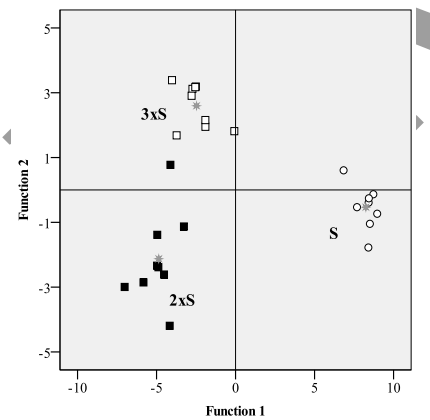

Figure 3. Plot of sample distribution using two canonical discriminant functions according to saffron concentration excluding control: S (○), 2xS (■), 3xS (□) and group centroid (✸).

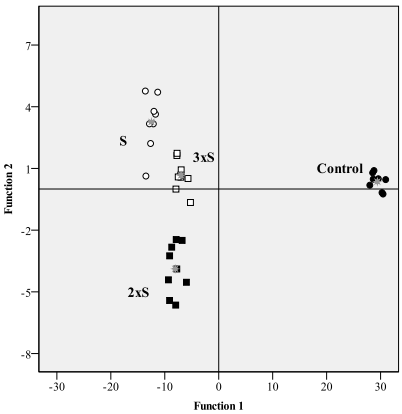

Figure 4. Plot of sample distribution using two canonical discriminant functions according to ripening time excluding control: 2 m (○), 4 m (□), 6 m (■) and group centroid (✸).

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Table 2. Mean values (μg/kg) of volatiles found in control and saffron cheeses at each ripening time

Compunds	Id[1]/C[2]	C			S			2xS			3xS			Saffron effect[4]		
		2m	4m	6m	2m	4m	6m	2m	4m	6m	2m	4m	6m	2m	4m	6m
Aldehydes																
Hexanal	L/IS	-[3]	-	111	-	-	391	-	-	203	-	-	610			**
Heptanal	S/CC	-	1720	1819	765	840	1454	696	242	218	929[b]	318[a]	-		*	
Octanal	S/CC	78675[b]	14712[a]	4039[a]	9459[ab]	16110[b]	-	9478	8568	16097	14502	3962	14084	***		
Nonanal	S/CC	247576[b]	22983[a]	8397[a]	-	9625[b]	1629[a]	-	8216	10052	1922	7585	360	**		
3-Methyl-butanal	L/IS	-	-	20	-	-	98	-	-	22	-	-	49			***
Safranal	S/CC	-	-	-	34	53	47	31[a]	24[a]	127[b]	62[a]	86[a]	186[b]	***	*	**
Ketones																
2-Propanone	S/CC	-	1934	2552	1465	4951	3957	584	285	312	3237	2663	-			
2-Butanone	S/CC	-	4210[b]	551[a]	89[a]	3565[b]	714[a]	298	2172	96	3666	1827	655			
2-Pentanone	L/IS	1087	1063	662	250[a]	655[a]	1956[b]	203[a]	290[a]	1102[b]	603[a]	362[a]	4310[b]	**	***	**
4-Methyl-2-pentanone + 2-Hexanone	S/CC	2929[b]	13599[c]	385[a]	3922[a]	15195[b]	-	1993	180	2673	3958	197	3100	*	***	
2-Heptanone	L/IS	1975[b]	465[a]	415[a]	78[a]	131[a]	516[b]	78[a]	-	401[b]	126[a]	-	2068[b]	***	***	*
2-Nonanone	S/CC	5137[a]	9026[ab]	10442[b]	1903[a]	6644[a]	4523[ab]	1740	1190	5214	1859	591	4268	***	**	
4-Nonanone	L/IS	286680[b]	13966[a]	1970[a]	1815	15512	1741	307	389	104	1867	308	-	***		
2,3-Butanedione	S/CC	-	7373	3870	-	1510[b]	569[a]	226	229	222	89	222	524		***	
2,3-Pentanedione	S/CC	134	-	4040	-	-	2471	2892	-	-	6683	-	-	*		
Alcohols																
1-Propanol	S/CC	18050	19765	5227	2818	10053	10896	1617	2959	1657	1498	7417	9597	***		
1-Butanol	S/CC	8	286	377	184	249	29	56[a]	107[a]	451[b]	231	172	4142			
1-Pentanol	S/CC	37	17	19	-	-	37	-	-	12	-	-	77	***		**
2-Methyl-1-propanol	S/CC	-	17732	-	2383[a]	17038[b]	-	514[a]	855[b]	-	2557	628	23463	**	***	
3-Methyl-1-butanol	S/CC	203879[a]	12766[a]	3350[a]	4045	4273	1850	3074	6448	7235	2712[a]	5688[b]	-	***	***	
2-Propanol	S/CC	696[a]	19874[b]	13011[b]	1202[a]	9666[c]	5531[b]	275	5475	2068	745	3041	2855	***	***	***
2-Butanol	S/CC	302	4332	14926	3634	811	10325	6	33	-	6380	49	-			
2-Pentanol	L/IS	-	-	141	-	-	182	-	-	85	-	-	338			*
2-Heptanol	L/IS	74[b]	23[a]	34[a]	-	-	35	-	-	15	-	-	71	***	***	*
2-Methyl-2-propanol	S/CC	56	118	47	11	119	78	5[a]	34[b]	98[c]	11[a]	38[a]	149[b]	***	***	
3-Methyl-2-butanol	S/CC	-	2663	1259	1286	1805	4739	2699	5910	3199	5723[b]	5471[b]	3[a]			
4-Penten-2-ol	L/IS	-	496	301	50[a]	125[a]	284[b]	15	595	51	41	375	173	**		**
2-Nonen-1-ol	L/IS	284	62	61	-	42	145	-	-	154	190	-	265	**	***	
2,3-Butanediol	S/CC	788	253	992	49[a]	111[a]	869[b]	135[a]	-	610[b]	130[a]	-	1901[b]	***		*
2-Methyl-cyclopentanol	S/CC	38	6674	9923	6068	13475	5314	3385	844	2969	4891[b]	327[a]	1[a]	*	**	
Phenol	L/IS	-	-	15	-	-	48	-	-	35	-	-	27			

Continued Table 2. Mean values (μg/kg) of volatiles found in control and saffron cheeses at each ripening time

Compounds	Id[1]/C[2]	C			S			2xS			3xS			Saffron effect[4]		
		2m	4m	6m	2m	4m	6m	2m	4m	6m	2m	4m	6m	2m	4m	6m
Esters																
Methyl hexanoate	L/IS	-	45	45	22[a]	28[a]	104[b]	20[a]	-	62[b]	30[a]	-	165[b]	***	***	
Ethyl butanoate	L/IS	-	130	195	-	14[a]	106[b]	-	-	22	-	-	83		***	***
Ethyl butyrate	L/IS	-	97	89	24[a]	65[a]	219[b]	24[a]	-	106[b]	44[a]	-	342[b]	*	***	*
Ethyl hexanoate	S/CC	414[a]	3043[b]	3692[b]	71[a]	629[b]	604[b]	-	133[a]	326[b]	86[a]	145[b]	-	**	***	***
Ethyl heptanoate	S/CC	336901[b]	3374[a]	7234[a]	-	2016	3302	-	2134	-	2118	-	2491	***		
Propyl ethanoate	S/CC	61	66	127	-	91	114	-	62	47	-	-	-			
Acids																
Acetic acid	S/CC	159[a]	780[ab]	1281[b]	818[a]	288[a]	3292[b]	287[a]	128[a]	1285[b]	1454	-	3058		***	
Butanoic acid	S/CC	-	-	122	-	-	44	-	-	102	-	-	191			*
Sulphur compounds																
Carbon disulfide	S/CC	9188[a]	30312[b]	4986[a]	1321[a]	17549[b]	5546[a]	1399[a]	5672[b]	5925[b]	1798[a]	5204[ab]	6447[b]	***	***	
Dimethyl sulfide	S/CC	504	576	132	-	746	349	-	218	527	-	100	268	***		
Dimethyl sulfone	L/IS	37	20	19	-	-	42	-	-	18	-	-	58	***		**
Terpenes																
Limonene	S/CC	108878[b]	10723[a]	3801[a]	2563	2701	2998	4778	4431	8804	5495[a]	3040[a]	78757[b]	***		***
p-Cymene	S/CC	-	24703	15726	4764[a]	15240[b]	13706[b]	3848[b]	432[a]	14628[c]	5321[b]	308[a]	-	***	***	
m-Cymene	S/CC	-	36580	33199	7039[a]	22514[b]	20345[b]	5662[b]	642[a]	21650[c]	7821[b]	422[a]	14092[c]	***	***	***
α-Pinene	L/IS	-	64	59	24[a]	28[a]	90[b]	35[a]	-	51[b]	50[a]	-	144[b]	***	***	
Ethers																
Methyl-tert-butyl-ether	S/CC	-	7	5	3[a]	9[b]	7[ab]	6	3	5	6	1	2		**	
Ethyl-tert-butyl-ether	S/CC	4[a]	21[b]	6[a]	3	10	14	4	9	2	4[a]	11[b]	8[b]			***
Tert-amyl-methyl-ether	S/CC	1	5	4	1	2	1	1	1	1	2	2	-			
Isopropyl-ether	S/CC	-	1252	1055	473	418	1208	-	1447	1496	791	1880	2215			
Hydrocarbons																
n-Pentane	L/IS	-	-	19	-	-	56	-	-	15	-	-	51			
2,6,7-Trymethyl-decane	L/IS	-	548[b]	294[a]	1057[b]	309[a]	707[b]	1158[b]	691[ab]	400[a]	1425[b]	441[a]	1074[ab]	***		
Dichloromethane	L/IS	-	40	-	-	28	-	-	40	-	-	33	-			
1-Heptene	S/CC	50287[b]	8318[a]	4473[a]	2060	4422	9267	-	4344[a]	11943[b]	1996[a]	4169[b]	-	***		*
1-Octene	S/CC	498	1045	22129	11602	8477	2305	6697[b]	747[a]	1356[a]	8675	1246	3100	*		*

Continued Table 2. Mean values (µg/kg) of volatiles found in control and saffron cheeses at each ripening time

Compounds	Id[1]/C[2]	C			S			2xS			3xS			Saffron effect[4]		
		2m	4m	6m	2m	4m	6m	2m	4m	6m	2m	4m	6m	2m	4m	6m
Aromatic hydrocarbons																
Benzene	S/CC	-	26[b]	6[a]	10	8	9	14	32	11	14	13	34			
Ethylbenzene	S/CC	472[b]	941[c]	4[a]	239[a]	883[b]	14[a]	248	231	116	239[b]	187[b]	1[a]	***	***	
n-Propylbenzene	S/CC	17[a]	112[b]	31[a]	20	27	35	26[a]	29[a]	72[b]	36	20	21		***	
n-Butylbenzene	S/CC	1908	4971	1923	1168	2138	1448	1599	1336	2276	619[a]	746[a]	11440[b]			*
1-Methylethyl-benzene	S/CC	52	31	22	30	35	52	28	51	23	35	62	9801			
1-Methylpropyl-benzene	S/CC	2687[c]	326[b]	24[a]	70	97	291	53[a]	35[a]	340[b]	69[ab]	34[a]	234[b]	***	***	
2-Methyl-2-phenylpropane	S/CC	-	24689	15717	4762[a]	15232[b]	13698[b]	3846[b]	432[a]	14620[c]	5318[b]	309[a]	20876[c]	***	***	
Toluene	S/CC	-	2775	-	388[a]	2606[b]	11[a]	262[a]	-	585[b]	400	518	-	***	***	*
2-Chlorotoluene	S/CC	10	48	46	2	6	22	26	33	16	26	14	50			
Naphthalene	S/CC	34[a]	586[b]	319[ab]	153	436	181	184	123	349	137	149	3558		*	
Styrene	S/CC	11	122	45	224	189	327	136[b]	45[a]	26[a]	189[a]	114[a]	1125[b]	***		***
Others																
2-pentyl-furan	L/IS	34	-	-	13	-	-	21	-	-	24	-	-			
Ionone	L/IS	-	43	51	-	35[a]	142[b]	-	333[b]	68[a]	-	181	292		***	

[1]Identificaction method: S=comparison with mass spectra of authentic compounds and L=comparison with mass spectra of NIST Library (Scientific Instrument Services, NJ, USA)

[2]Quantification method: CC=calibrated with authentic compounds and IS=comparison with internal standard.

[3]Not detected in any of the 3 samples.

[4]Differences between control and saffron cheeses within the same ripening time: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

[a,b,c] Means followed by a different superscript within the same cheese type were significantly ($P \leq 0.05$) different.

CHAPTER 6. DISCUSSION

Results from this doctoral thesis demonstrated that saffron addition to pressed ewes' milk cheeses is an interesting alternative to diversify both traditional Spanish products. Influence of saffron addition on ewes' milk cheeses can be outlined in two points: 1) healthy benefits and 2) cheesemaking and final product characteristics, including the technological parameters and saffron color and aroma transference.

6.1 Saffron healthy benefits

In different dishes such as soups, rice, desserts or infusions, saffron doses ranged from 75 to 800 mg of saffron/liter or kg of food, depending on the matrix and the desire characteristics of the final product. Bibliographic revision about saffron biological properties showed that doses previously mentioned are not enough to exert most of the biological activities proved, since doses ranging from 0.1 to more than 725,000 mg of saffron/kg of human body weight are necessary in most cases (Chapter 5.1). However, a regular consumption of food including saffron, especially saffron infusions could be useful for those properties that require less saffron quantities. As an example, including between 0.45 to 11 mg of saffron in the diet of a 70 kg person, could be helpful for retinal function, Parkinson, seizures and pancreatic cancer (Hosseinzadeh and Khosravan, 2002; Ahmad *et al.*, 2005; Hosseinzadeh and Talebzadeh, 2005; Maccarone *et al.*, 2008; Dhar *et al.*, 2009). These quantities can be added to highly consumed products, for example cheese, that according to the European Food Safety Authority (2011), European intake of this product is around 20 to 70 g/person/day, portions that can easily reach quantities mentioned above.

Saffron addition is safe from the microbiological point of view since studies on saffron from different origins showed the absence of pathogens and an excellent microbiological quality, especially Spanish and Italian saffron (Cosano *et al.*, 2009).

Rather, saffron has shown a moderate antibacterial and fungicidal activity against *Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli, Helicobacter pylori, Candida albicans, Aspergillus niger, Salmonella* sp., and *Cladospourium* sp. (Vahidi *et al.*, 2002; Kamble and Patil, 2007; Sekine *et al.*, 2007; Nakhaei *et al.*, 2008; Pintado *et al.*, 2011) which are human pathogens and cause health problems.

6.2 Saffron influence on cheesemaking and the final product

6.2.1 Technological aspects

Saffron addition into cheesemaking by means of saffron extraction in milk was an adequate method since it exploited saffron color. Extraction time was established at 20 minutes, not interfering with normal operations in a cheese factory.

The first influence of saffron addition to cheesemaking was observed during pressing. Saffron cheeses had a longer pressing time (≈1 h) as pH decreased slower compared to control (Appendix 8.4). This delay caused differences on initial dry matter content and initial pH. This fact could decrease the capacity of exchange between whey retained in the curd and salt thus decreasing salt content in saffron cheeses compared to control. Delaying pH drop during pressing was probably due to slower production of lactic acid. This theory is supported by the fact that in the final product, the total and lactic acid bacteria counts were slightly lower in saffron cheeses (Chapter 5.3). From these results, it can also be inferred that saffron had a slightly bacteriostatic effect, although antimicrobial effect of saffron against lactic acid bacteria was not found (Appendix 8.3). This slight bacteriostatic effect of saffron was not observed for the rest of microorganisms studied: enterobacteria, pseudomonas, molds and yeasts.

The bacteriostatic effect on the starter growth caused further differences between saffron and control cheeses; such as proteolysis rate and texture. Color and aroma of the cheeses were the aspects more influenced by saffron so that; they are going to be discussed by separate in the following sections.

Saffron cheeses showed lower values of some of the nitrogen fractions studied: water soluble nitrogen, soluble nitrogen at pH 4.6 and soluble nitrogen in 12 % of trichloroacetic acid. Water soluble nitrogen is related to casein hydrolysis while the other two fractions with soluble peptides. Soluble peptides in the pH 4.6 fraction are principally evolved by rennet activity while starter is primarily responsible for the formation of soluble peptides in 12 % of trichloroacetic acid. During ripening saffron cheeses showed higher values of the former fraction suggesting that soluble peptides produced by rennet were not hydrolyzed by bacterial peptidases at the same rate as they were produced.

Differences on texture parameters were also observed as a probable consequence of differences on salt content, dry matter and proteolysis. Saffron cheeses were firmer and more deformable and elastic than control. Firmness differences could be due to the higher dry matter content in saffron cheeses at the beginning of ripening as observed by Juan *et al*. (2007). A positive correlation has been observed between pH values and deformation by Watkinson and coworkers (2001), confirmed by our results since saffron cheeses were more deformable showing slightly higher pH values than control.

All differences found did affect the final quality of saffron cheeses. By the end of the study, control and saffron cheeses showed similar compositional values.

6.2.2 Color

In the course of this thesis, saffron color in ewes' milk and ewes' milk cheese was measured by tristimulus colorimetry. This technique is simple and allows evaluation of the contribution of color from crocetin esters in complex matrixes such as milk and cheese without solvent extraction, which could promote changes in the structure of saffron-milk or saffron-cheese complexes.

Cheese fabrication on laboratory scale revealed that the best approach to add saffron into the cheesemaking process was to make a prior extraction in milk. Direct saffron addition to the curd caused loss of filaments in the whey and a non uniform cheese color in the curd (Appendix 8.1). Although color addition to dairy products is a common practice, there were no studies about colorant extraction directly in milk or an optimized procedure, unless extraction in other matrixes is common among other colorants used. For example, annatto, one of the most popular colorant in dairy products is extracted from seeds using oil, steam or solvents and then is added to the cheese as a color extract, and saffron is also extracted in water before addition to milk to fabricate Piacentinu Ennese cheese (Preston and Rickard, 1980; MIPAAF, 2011).

After testing several factors for saffron color extraction directly in ewes' milk, particle size was an important parameter. From the two particle sizes tested it was observed that after 60 minutes of extraction, the bigger particle size was still visible while the smaller one was dissolved after 20 minutes of extraction (Appendix 8.1). An important factor during extraction of some compounds in different spices is particle size, which resulted to have a similar behavior in saffron than in other spices such as paprika, celery seeds and cinnamon (Ramesh *et al.*, 2001; Rafajlovska *et al.*, 2007; Kuang *et al.*, 2011; Sowbhagya *et al.*, 2011).

Adequate conditions for extracting saffron color in milk were temperatures between 37 and 70 °C during 20 min (Chapter 5.2). Saffron concentration resulted to be the most important factor influencing color. Increasing saffron concentration

(from 2 to 10 mg saffron/ml milk) decreased brightness and yellowness but increased redness in the extracts.

Different milk fat contents and indirectly, protein content, characteristic of industrial production of ewes' milk were tested to study possible differences on the color of the extracts. Changes in the color coordinates with increasing fat were different in plain milk than in the saffron milk extracts. Ewes' milk without saffron increased brightness and redness while decreased yellowness contrary to saffron milk extracts which increased yellowness. These results lead to the conclusion that interactions between saffron and milk components could be influencing color extraction. Fat in milk is present in globules surrounded by a milk fat globule membrane made up of proteins, phospholipids and other miscellaneous compounds (Gallier *et al.*, 2010). Phospholipids have two charged groups in their molecules having polar properties, thus the phosphate group present in the molecule could be interacting with any of the sugars present in crocetin esters (Taylor and MacGibbon, 2011). On the other hand, milk proteins are also capable of forming unions with different molecules, such as carotenoids and vitamins involving hydrophobic unions (Talbott, 2002; Wackerbarth *et al.*, 2009; Livney, 2010), so that, they can be contributing as well to the color behavior found in the saffron-milk extracts. Further studies are necessary to deep knowledge about this subject.

Regarding saffron color distribution during cheesemaking (Appendix 8.6), liquid matrixes (whey and "requesón" whey) showed lower color retention than solid matrixes (cheese and "requesón"), among them, color differences were higher in cheese than they were in "requesón".

Regarding color in the final product, saffron cheeses had a bright yellow color that decreased on brightness and increased on redness and yellowness with more saffron (Chapter 5.3). Yellow coordinate was the most influenced parameter by saffron addition as increments were very marked between concentrations. During ripening brightness decreased while red and yellow coordinates remained almost

constant after 15 days of fabrication. Changes on coloration in the surface of cut cheeses were also observed, as a result, color changes during one hour of air exposure were followed. Results showed that air exposure affected significantly all color parameters, turning the cheeses less bright and especially more yellow. Initially, it was considered that increasing yellowness could be due to a concentration effect of the crocetin esters as a result of water loss and exudation of the surface during exposure. Nevertheless, only around 0.1 % of water is lost in one hour and so this theory was discarded (data not shown). It is also possible that the color of the sample experienced a bathochromic effect, commonly known as red shift, meaning that there is a displacement in the wave length of maximum absorbance of a determined molecule and thus the perceived coloration of the sample tends to orange-red. It should be pointed out that saffron liposoluble carotenoids are very likely to be present in the cheeses, so that, these color changes could be due to union between carotenoids and proteins since bathochromic effects has been found when carotenoids, and even crocetin, are linked to proteins (Krawczyk and Britton, 2001; Zsila *et al.*, 2002). Another theory is that synergic effects can occur between saffron and milk components, as demonstrated by Serrano-Díaz (2011) in aqueous saffron solutions between picrocrocin and crocetin esters. This shift is probably mediated by oxygen since it has been found that sodium salts of *trans*-crocetin enhances transport of oxygen in blood (Singer *et al.*, 2000). This finding is very interesting and deserves further study in order to determine if crocetin esters/protein complex or oxygen is in fact responsible for this color changes or both create a synergic effect.

Visually, color was the only characteristics that revealed differences between cheeses with different saffron concentrations. Sensory analysis showed that assessors were able to distinguish between cheeses with the lowest saffron concentration from cheeses without saffron and between different saffron concentrations, differences that continued to be evident during ripening. Panelists tended to prefer the color of the lowest saffron concentration, suggesting that consumers prefer lighter, less red and less yellow color (Chapter 5.3 and Appendix 8.5).

6.2.2 Aroma

The developed methodology for volatile extraction of pressed ewes' milk cheeses is very simple since grating is the only sample manipulation. Besides, high fat and protein content are not critical factors, so it can be used for all cheese varieties. The method was adequate to isolate, identify and quantify more than 50 volatiles normally present in cheese at concentrations of ng/kg with recoveries between 57 and 120 % and precision below 30 %. Mean detection limits were lower than 38 ng/kg while quantification limits were lower than 100 ng/kg (Chapter 5.4). The calibration of more than 50 compounds in this work is valuable since only few works validate volatiles as most of them make quantification based on the internal standard. Until now, headspace sorptive extraction has been only used in the dairy sector in "Pesto Genovese" containing Grana Padano (Salvadeo *et al.*, 2007) and to determine flavor compounds in Bitto cheese (Panseri *et al.*, 2008).

Saffron aroma distribution during cheesemaking and characterization of the volatile fraction of saffron cheeses were carried out using the proposed methodology. This was an important parameter as one quality attribute of the cheese is to exhibit the characteristic note of the spice, which is mainly due to safranal. Saffron aroma distribution, in terms of safranal, (Chapter 5.5), was around 43 % in cheese and "requesón", while the rest was lost in the "requesón" whey. Retention of safranal in these solid fractions could be mediated by proteins and fat, as both molecules have been demonstrated to interact with aromatic compounds (Piraprez *et al.*, 1998; Kühn *et al.*, 2008). Previous works have proved that aroma compounds can form hydrophobic unions with caseins, whey proteins and lipids, which may be enhanced by increasing hydrophobicity and presence of double bonds in the aromatic molecule. This factor relies on the protein and lipid conformation (Piraprez *et al.*, 1998; Kühn *et al.*, 2006, 2008; Kopjar *et al.*, 2010). It would be expected a higher retention of safranal in the solid matrixes since safranal is a non polar molecule and only partially water soluble, but around 57 % of the safranal is lost in the "requesón" whey. The composition of this last fraction has not been precisely determined but it is known that lactose and vitamins such as

riboflavin are still present. These molecules could be mediating interactions between safranal and water contained in "requesón" whey but no available data supporting this fact has been found. All these interactions deserve further study since it would be important to demonstrate union between saffron and milk components in order to improve potential applications of these complexes as food additives, or to improve sensory qualities of the dairy derivates.

Regarding characterization of saffron cheeses according to saffron concentration (Chapter 5.5), besides safranal content, all cheeses had the same volatiles but in some cases, at different concentration. These differences were less accused by the end of ripening. Results from volatile characterization showed that some compounds sharing common formation pathways such as citrate metabolism or amino acid catabolism (both depending on microorganisms and proteolysis, respectively), were present in saffron cheeses at lower concentrations than in control. This information agreed from one side with the slightly bacteriostatic effect of saffron on lactic acid bacteria, and from the other side with the slower proteolysis rate in saffron cheeses. Lactic acid bacteria is responsible for the formation of compounds such as diacetyl and its further reduction to 2,3-butanediol and 2-butanol, that in this case was slower with higher saffron concentration. In the other hand the slower proteolysis ratio corresponded with lower concentrations, for example, of 3-methyl-1-butanol and carbon disulfide formed from leucine and methionine catabolism, respectively. Other remarkable result was the lower presence of limonene, which has been found as a secondary metabolite of fungi, such as *Penicillium brevicompactum* and *P. roqueforti*, which are commonly found in the rind of cheeses (Börjesson *et al.*, 1992; Kure *et al.*, 2004). Surface molds were not identified on this study and unless any effect was observed on the growth of internal molds due to saffron addition (Chapter 5.3), differences on limonene concentration suggests that saffron could be slowing down growth of some surface mold strains, and thus other strains could be growing faster. This fact deserves further study as possible saffron influence on bacteria and molds growth could be useful for diversifying cheese volatile fingerprint.

Regarding flavor sensory analysis of saffron cheeses, results showed that panelists were able to distinguish the flavor of cheeses with different saffron concentrations and a tendency to prefer the flavor of the lowest saffron concentration cheeses was manifested by panelists (Appendix 8.5). These differences less marked by the end of the study (Chapter 5.3), as a probable consequence of the homogenization or the volatile fingerprint observed (Chapter 5.5). Lower saffron concentration cheeses showed higher concentration of some volatiles for example: 3-methyl-1-butanal, 2-nonanone, 2-propanol, 2-butanol and ethyl butanoate which have fruity, floral and sweet descriptors, heptanal with herbaceous notes, toluene related to nutty descriptors, *p*- and *m*- cymene to fresh and citrus notes and finally diacetyl with caramel and buttery notes. Most of them have been described as key odorant in some cheeses so panelist could prefer higher presence of some of these notes.

Some panelist mentioned that they perceived saffron flavor in cheeses during the early stages of ripening, but through ripening this flavor was masked by the development of the characteristic cheesy flavor and even more, assessors thought that cheese flavor was enhanced by saffron addition. It is well known that saffron is a flavor enhancer (Carmona *et al.*, 2006) but this property has not been considered in this study, so further research could be useful. It would be interesting to determine if tendency to prefer the lowest saffron concentration in cheeses is related to higher presence of the aromatic compounds mentioned, or in contrast, panelists preferred these cheeses as a direct influence of saffron aroma.

Findings obtained from this doctoral thesis allowed to gain knowledge about saffron as a cheese ingredient and results can be extrapolated to food in general. Standardization of the process for saffron extraction in milk, saffron addittion to cheesemaking and characterization of saffron cheeses was very helpful for obtaining a good quality and consistent product. These aspects were also fundamental for the two dairy industries involved in this project and allowed fabrication and commercialization of cheeses in an industry scale. Currently, around 300 kg of saffron cheeses with the lowest saffron concentration are

fabricated per month. Saffron cheese is distributed in countries outside Spain, such as Unites States, Canada, México, Arab Emirates and Germany. Although introducing and increasing marketing is a difficult and slow task, this product has a good acceptance among consumers and a great potential in niche markets and restoratorion allowing a constant improving on the sales field.

CHAPTER 7. CONCLUSIONS

The main conclusions obtained from this doctoral thesis were:

1. Saffron dosis normally present in food are between 75 and 800 mg of saffron per liter or kg of food. At these doses saffron addittion is a good way to prevent or ameliorate some diseases, thus it should included systematically in the diet.

2. The best approach to include saffron into the cheesemaking process was to make a previous extraction in milk and add it to the cheese vat after the starter and before rennet, taking into account milk fat content as it has a direct influence on saffron milk extract color. Color extraction was better extracted at temperatures between 37 and 70 °C during 20 minutes.

3. Saffron addition decreased total and lactic acid bacteria growth. This caused a slower pH decrease during pressing, so that, pressing time increased approximately one hour. Saffron cheeses also showed higher dry matter values, lower salt content and differences on proteolysis rate and texture.

4. Color was the main parameter that showed differences between cheeses with and whitout saffron. This parameter depended on ripening, saffron concentration and air exposure. During ripening, cheeses were losing brightness. Increasing saffron concentration also caused loss of brightness but red and yellow indexes increased. Air exposure decreaced. With air exposure cheeses become less bright and noticeably more yellow.

5. In sensory analysis panelists detected color differences between cheeses with and without saffron and between different saffron concentrations, differences that were evident during ripening. Regarding flavor, differences were also perceptible but they were less marked as ripening time increased. A tendency among panelists to prefer cheeses with the lowest saffron concentration was observed, probably as they were less yellow.

6. A methodoly to analyse volatile fraction of cheese by GC/MS with a previous isolation of volaties by headspace sorptive extraction was developed. Isolation conditions with a stir bar of 2 cm, suspended in the headspace by the manufacturer commercial insert were established: 10 g of grated cheese placed into a 50 mL vial, water addittion to obtain a headspace of 25 mL, stirring the sample at 700 rpm for 4 h at 45 °C. Regarding gas chromatography, an Elite-Volatiles column and a temperature program starting at 40 °C (held for 10 min) raised to 240 °C at 5 °C/min and mantained for 5 min were selected. More than 50 compounds were identified and quantified with linearity of more than 0.98, precision between 9 and 34 %, recovery between 58 and 120 %, detection limits between 6 and 38 ng/kg and quantification limits between 75 and 150 ng/kg.

7. Saffron distribution in terms of color and aroma during cheesemaking was obtained. In solid fraction, cheese and "requesón", color retention was higher. While around 43 % of safranal was retained in the same fractions.

8. Characterization of volatile fraction of saffron cheeses showed that alcohols and ketones were the volatile families most influenced by saffron addittion. In general all cheeses had the same volatiles but at different concentrations. These differences were less evident through ripening. Variations of some volatiles can be related with saffron bacteriostatic effect on lactic acid bacteria and its possible influence on the surface molds.

The studies included in this doctoral thesis allowed reaching the main objective: develop a pressed ewes' milk cheese with saffron ready to be introduced into the market. Due to the strong involvement of the cheese industries who participated in the project, saffron cheeses are currently fabricated and commercialized.

CONCLUSIONES

Las conclusiones más importantes de esta tesis doctoral son:

1. Las dosis de azafrán que se utilizan normalmente en la preparación de alimentos se encuentran entre 75 y 800 mg de azafrán por litro o kg de comida. A estas dosis, la adición de azafrán a los alimentos es una buena manera de prevenir o mejorar ciertas enfermedades por lo que debería incluirse de forma sistemática en la dieta.

2. La mejor manera de adicionar azafrán durante el proceso de fabricación del queso fue mediante una extracción previa en leche y su adición posterior a la cuba quesera después de los cultivos iniciadores y antes del cuajo, teniendo en cuenta el contenido graso de la leche, ya que afecta el color del extracto. Las mejores condiciones de extracción de color se obtuvieron con temperaturas entre 37 y 70 °C durante 20 minutos.

3. La adición de azafrán redujo el crecimiento de bacterias totales y lácticas en los quesos. A su vez, esto causó que el descenso de pH durante el prensado fuera más lento, por lo que el tiempo de prensado se incrementó aproximadamente una hora. Los quesos con azafrán también mostraron valores mayores de extracto seco, menor contenido en sal y una modificación en la velocidad de proteólisis y la textura.

4. El color fue el parámetro que mostró más diferencias entre quesos con y sin azafrán. Este parámetro dependió de la maduración, la concentración de azafrán y la exposición al aire. Durante la maduración los quesos fueron perdiendo luminosidad, algo que también ocurrió al aumentar la concentración de azafrán en los quesos. El incrementó de azafrán en los quesos causó también que el índice de rojo y el índice de amarillo aumentaran. La exposición al aire decreció la luminosidad de los quesos e incrementó considerablemente el índice de amarillo.

5. En el análisis sensorial los panelistas detectaron diferencias de color entre los quesos con y sin azafrán y entre quesos con distintas concentraciones de azafrán, diferencias que se mantuvieron durante la maduración. En lo que respecta al sabor, las diferencias también fueron perceptibles pero menos marcadas al aumentar la maduración. Los catadores tuvieron una tendencia hacia los quesos con menor contenido de azafrán probablemente porque eran menos amarillos.

6. Se desarrolló un método de análisis de volátiles en queso mediante CG/MS con aislamiento previo de los volátiles mediante una barra agitadora adsorbente. Las condiciones de aislamiento con la barra agitadora de 2 cm de largo, suspendida en el espacio de cabeza por medio del inserto comercial del fabricante fueron las siguientes: 10 gramos de queso rallado colocados en un vial de 50 mL, adición de agua hasta obtener un espacio de cabeza de 25 mL, con agitación a 700 rpm durante 4 h a 45 °C. En lo que respecta a las condiciones cromatográficas, se eligió una columna "Elite-Volatiles" y un programa de temperatura que inició a 40 °C (mantenida durante 10 min), aumentó hasta 240 °C a 5 °C/min y se mantuvo durante 5 min. Más de 50 compuestos fueron identificados y cuantificados con una linealidad de más de 0.98, una precisión de entre 9 y 34 %, una recuperación de entre 58 y 120 %, un límite de detección de entre 6 y 38 ng/kg y un límite de cuantificación de entre 75 y 150 ng/kg.

7. Se obtuvo la distribución del azafrán, en términos de color y aroma durante el proceso completo de transformación de la leche. En las fracciones sólidas, queso y requesón, se retuvo más color que en las fracciones líquidas. Mientras que alrededor del 43 % de safranal se retuvo durante el proceso.

8. La caracterización de la fracción volátil de los quesos demostró que los alcoholes y cetonas fueron las familias volátiles más influidas por la presencia de azafrán. Aunque todos los quesos presentaron en general los mismos compuestos, estaban presentes en distintas concentraciones. Las diferencias fueron menos marcadas con la maduración. Las variaciones en la concentración de volátiles puede estar relacionada con el efecto bacteriostático del azafrán sobre las

bacterias lácticas y con el posible efecto sobre los hongos de la superficie del queso.

Los trabajos incluidos en esta tesis doctoral permitieron alcanzar el objetivo propuesto: desarrollar un queso de leche de oveja de pasta prensada con azafrán listo para ser introducido en el mercado. Gracias a la fuerte implicación de las empresas que participaron en el proyecto, la fabricación a escala industrial y la comercialización de los mismos se está llevando a cabo actualmente.

CHAPTER 8. APPENDICES

8.1 Preliminary studies for saffron addition and extraction

8.1.1 Approach

Laboratory scale cheese fabrications were made in order to study different forms of saffron addition: 1) saffron addition directly in the curd during molding, 2) saffron addition in the milk before rennet addition, 3) saffron addition in the milk before rennet addition and in the rind by means of oil and fat commonly used and 4) saffron previously extracted in milk and added before rennet. Results present in this section have not been published.

8.1.2 Results

Cheeses were fabricated from 10-L of pasteurized Manchega ewes' milk from the Experimental farm of the University of Castilla-La Mancha (Albacete, Spain) following the diagram of Appendix 8.4.

The first trial consisted on adding saffron stigmas in the curd during molding, as shown in Figure 21, resulted to be a time consuming operation that in day-by-day activities in a cheese industry was not feasible. Moreover, it was very difficult to clean the molds and the rest of the material needed for cheese fabrication.

Figure 21. Cheeses with saffron added during molding

Adding saffron stigmas before rennet addition resulted in loss of many filaments in the whey and cheeses showed yellow-orange spots around the visible filaments as it can be observed in Figure 22.

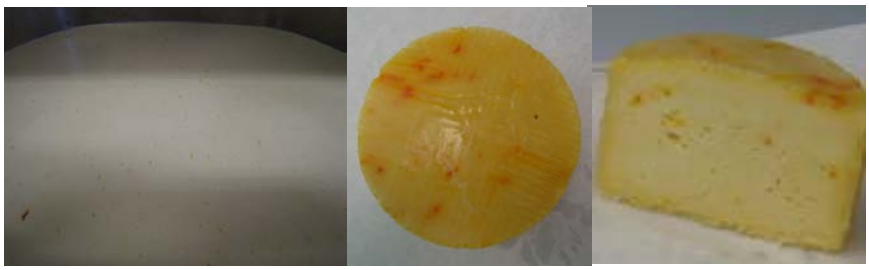

Figure 22. Curd and cheese with saffron stigmas added before curding

The third fabrication was made adding saffron before rennet and adding stigmas to the rind of the cheeses by means of olive oil or fat usually used to cover cheeses. Stigmas did not show improvement to cheese color and this process was time consuming as well, making it very difficult to practice in the cheese industry. Cheeses obtained from this trial are shown in Figure 23.

Figure 23. Cheeses with the stigma in the curd and rind

Finally, other cheese vat was made with crashed saffron filaments previously dissolved in milk and added to the cheese vat after the starter and before rennet. Cheeses obtained from this fabrication had a more uniform color (Figure 24).

Figure 24. Cheese obtained with a previous saffron extraction

From these preliminary trials it was observed that saffron particle size was important in order to allow a complete extraction and to decrease losses in the cheese whey. Two particle sizes were tested before the final design of the extraction conditions was established. Saffron was grinded and sieved through a 500 µm mesh to obtain two particle sizes: the first one, 50 % of the powder passed through this mesh while in the second one, 95 % of the powder passed through.

One gram of saffron was added to 500 mL of ewes' milk and was extracted during 60 minutes at 37 °C. It can be observed in Figure 25 that saffron with bigger particle size was not completely extracted while the smaller one was almost not visible.

Particle size 1
50 % passed through 500 µm mesh

Particle size 2
95 % passed through 500 µm mesh

Figure 25. Saffron with different particle size extracted in milk at 37 °C during 60

8.2 Saffron extraction procedure

8.2.1 Approach

This appendix includes the translation of the abstract of the pending patent No. P200930912 named "Ewes' milk cheese with saffron procedure and cheese obtained from this method".

Procedimiento de elaboración de queso de oveja con azafrán y queso obtenido mediante dicho procedimiento

Berruga, M.I., **Licón, C.C.**, Carmona, M., Molina, A., Román, M., Olivares, V., Olivares, F. and Olivares, S.

Patent No: P200930912

Country: Spain

8.2.2 Summary

Ewes' milk cheese with saffron fabrication method including milk reception, previous milk treatments, curding, molding, pressing, aeration and ripening in which before curding, a saffron extraction is made adding powder saffron into a milk volume in order to obtain a saffron extract in milk. Ewes' milk cheese with saffron obtained from this procedure having a fat/dry matter content > 0.3.

Ewes' milk cheese with saffron is characterized by > 40 % of dry matter, fat/dry matter content ratio > 0.3 and protein/dry matter ratio > 0.3.

MINISTERIO DE INDUSTRIA, TURISMO Y COMERCIO

Oficina Española de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P200930912	
Fecha de recepción:	27 octubre 2009, 16:03 (CET)	
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Su referencia:	Isabel Carvajal	
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Número de solicitantes:	3	
País:	ES	
Título:	Procedimiento de elaboración de queso de oveja con azafrán y queso obtenido mediante dicho procedimiento.	
Documentos enviados:	Descripcion.pdf (6 p.) Reivindicaciones.pdf (2 p.) Resumen.pdf (1 p.) POWATT.pdf (1 p.) OTRO-1.pdf (1 p.) OTRO-2.pdf (1 p.) FEERCPT-1.pdf (1 p.) OTRO-3.pdf (1 p.)	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
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Resumen

Procedimiento de elaboración de queso de oveja con azafrán, que comprende las etapas de recepción, tratamiento previo de la leche destinada a queso, cuajado, moldeado, prensado, oreo y maduración del queso, en el cual, antes del cuajado de la leche, se realiza una etapa de extracción de azafrán en la cual se añade y se mezcla azafrán molido con un volumen de leche, con el fin de obtener un extracto de azafrán en leche. Queso de oveja con azafrán obtenido mediante dicho procedimiento que presenta una relación grasa/extracto seco > 0,3.

Reivindicaciones

1. Procedimiento de elaboración de queso de oveja con azafrán a partir de leche de oveja, que comprende recepción, tratamiento previo de la leche destinada a queso, cuajado, moldeado, prensado, oreo y maduración del queso, **caracterizado** porque se lleva a cabo, antes del cuajado de la leche, una etapa de extracción de azafrán en la cual se añade y se mezcla azafrán molido con un volumen de leche, según una proporción azafrán/leche comprendida entre 1 y 48 g de azafrán por litro de leche, a una temperatura entre 46 y 54°C, para obtener un extracto de azafrán en leche.

2. Procedimiento según la reivindicación 1 caracterizado porque la extracción se realiza con agitación a 350 rpm, durante 20 minutos.

3. Procedimiento según la reivindicación 1 caracterizado porque se incorpora el extracto de azafrán en leche 5 minutos antes de iniciar el cuajado.

4. Queso de oveja con azafrán obtenido según el procedimiento definido en la reivindicación 1, caracterizado porque presenta un extracto seco $\geq$ 40%, una relación proteína/extracto seco > 0,3 y una relación de grasa/extracto seco > 0,3.

8.3 Saffron inhibition of lactic acid bacteria

8.3.1 Approach

Saffron antimicrobial properties on different bacteria have been demonstrated. Three saffron concentrations were tested to know if saffron was able to inhibit the growth of lactic acid bacteria used as a starter for cheese fabrication. Results from this work have not been published.

8.3.2 Results

The antimicrobial activity was performed by agar well diffusion method according to the experimental design of Althaus *et al.* (2009). The starter CHOOZIT MA4001 (Danisco, Sassenage, France) which includes *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis biovar. diacetylactis* and *Streptococcus thermophilus,* was used. Starter (0.0155 g) was dissolved in 100 mL nutrient broth (Panreac, Barcelona, Spain). Petri dishes of 90 mm diameter were filled with 10 mL of Agar MRS (Scharlau, Barcelona, Spain) inoculated with 8.5 x 10^6 or 8.5 x 10^4 cfu/mL of the starter and allowed to solidify. Four wells of 14 mm of diameter were aseptically bored into each plate and filled with 200 µL of three different doses of saffron (0.5, 5.0 and 50 mg) dissolved in 50 mL of commercially available UHT semi skimmed ewes' milk (Gaza, Zamora, Spain). The remaining hole was filled with semi skimmed ewes' milk as a control as shown in Figure 26.

Figure 26. Petri dishes with different saffron concentrations in MRS agar applying the well diffused method to evaluate inhibition of lactic acid bacteria

After 30 min of diffusion the plates were incubated at 37 °C for 48 h in aerobic conditions. The evaluation of antimicrobial activity was carried out by measuring in triplicate the inhibition zone including the diameter of the hole itself with a digital caliper (range 0-150 mm, accuracy ±0.01 mm, VWR, Barcelona, Spain).

As a result, no inhibition zones were observed in any of the starter concentrations or saffron doses, as seen in Figure 27.

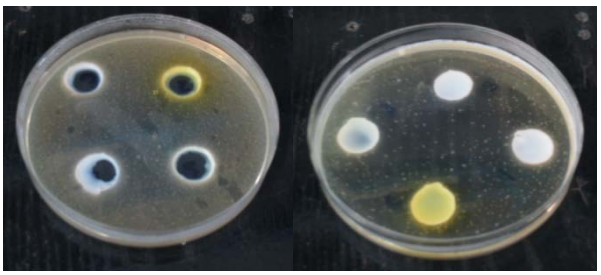

Figure 27. Petri dishes with starter after incubation

8.4 Flow diagram of ewes' milk cheese fabrication and parameters

8.4.1 Approach

Fabrication parameters during ewes' milk cheese fabrication were followed in order to determine possible interferences of saffron addition during cheesemaking. The cheese fabrication process used for pressed ewes' milk cheese with saffron manufacturing is shown in Figure 28.

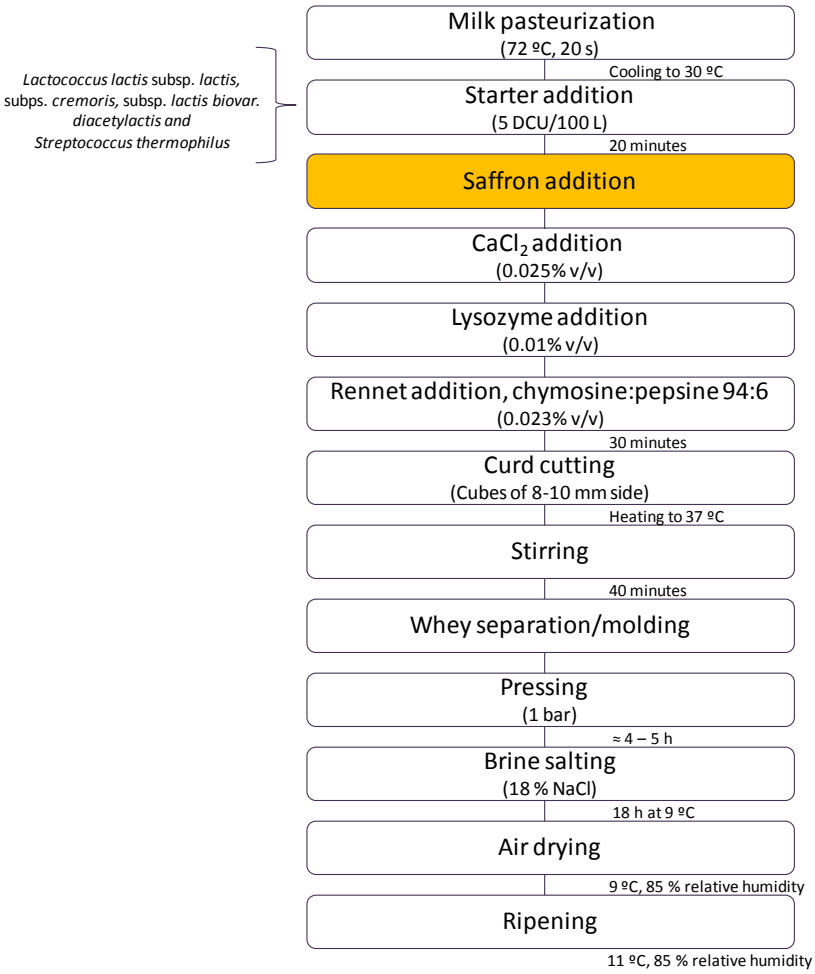

Figure 28. Fabrication process of pressed ewes' milk cheese with saffron

8.4.2 Results

During cheese fabrication, pH and temperature were registered to verify the process. Figures 29 and 30 show the average pH and temperatures obtained for saffron cheeses in the four fabrications. The values obtained were within a typical process with the exception of pressing time that was increased approximately one hour for saffron cheeses compared with control (Figure 29).

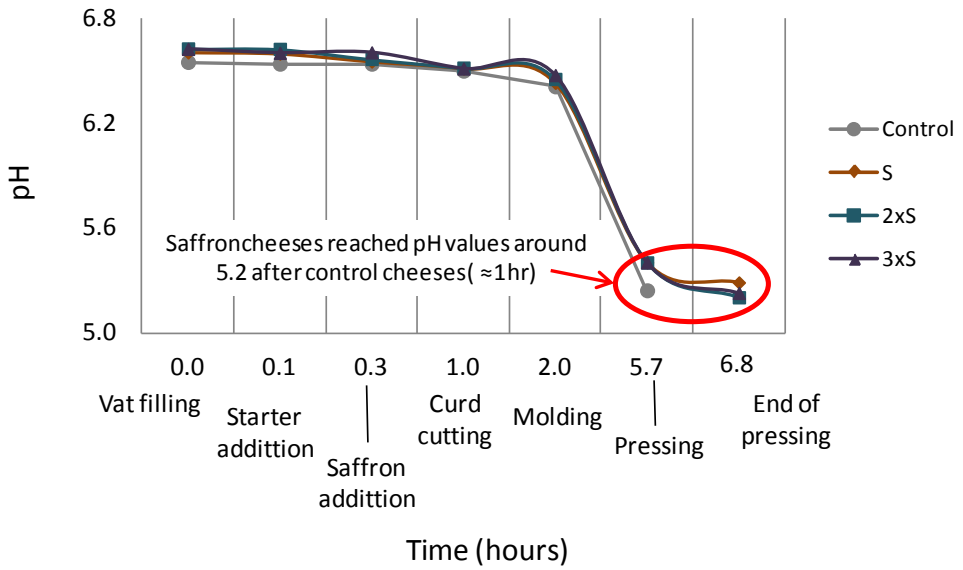

Figure 29. Average pH values during saffron and control cheese fabrication

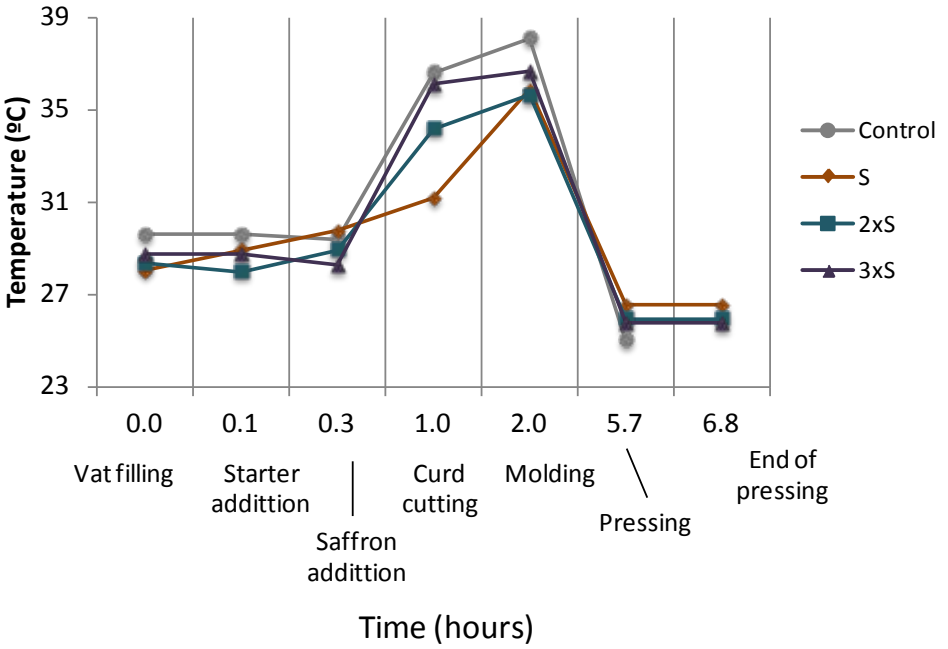

Figure 30. Average temperature during saffron and control cheese fabrication

Color coordinates in CIEL*a*b* space were also recorded for milk, milk with saffron (except for control cheese fabrication) and whey. Figure 31 shows that brightness differences between saffron milk and whey compared with control are not marked but whey with saffron is less bright. Coordinate b* increased with increasing saffron concentration while a* decreased in milk with saffron and whey.

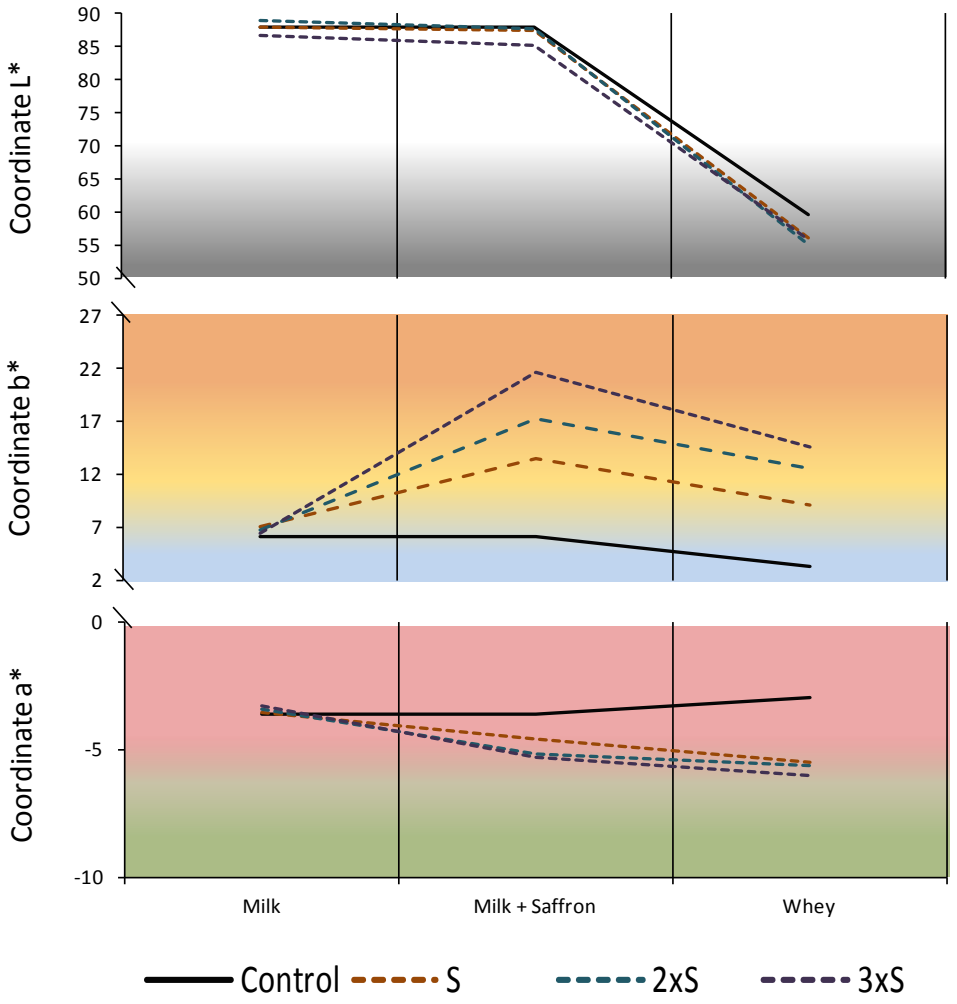

Figure 31. CIEL*a*b* coordinates for milk, milk with saffron and whey derived from cheese fabrication

8.5 Preference test

8.5.1 Approach

The objective of this communication was to study color and flavor preferences of one of the saffron concentrations used during cheese fabrication by means of sensory analysis. Results were presented in 2010 in a national Congress:

Evaluación sensorial de queso de oveja con azafrán

Licón, C., Lozoya, S., Molina, A., y Berruga, M.I.

XXXV Congreso de la Sociedad Española de Ovinotecnia y Caprinotecnia

Valladolid, Spain

ISBN: 978-84-938243-0-3

Pages: 399-404

8.5.2 Summary

When consumers compared ewes' milk cheeses with and without saffron, they were able to distinguish color and flavor. Panelists were able to rank from the lowest to the highest saffron concentration although differences were less accused as ripening was increased. In general, no significant differences were obtained for one of the saffron concentrations but assessors tend to preferred cheeses with less saffron concentration as they were less yellow.

EVALUACIÓN SENSORIAL DE QUESO DE OVEJA CON AZAFRÁN

SENSORY EVALUATION OF SAFFRON EWE'S CHEESE

LICÓN, C.; LOZOYA, S.; MOLINA, A. y BERRUGA, M.I.*

Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR; Campus Universitario s/n, 02071, Albacete. Universidad de Castilla-La Mancha

**mariaisabel.berruga@uclm.es*

RESUMEN

El empleo de azafrán en la elaboración de queso de oveja podría ser una alternativa para aumentar el valor económico de ambas materias primas: leche y azafrán. En este trabajo se han realizado 3 tipos de pruebas sensoriales (triangular, de ordenamiento y de preferencia) para evaluar el color y sabor de quesos de oveja con azafrán, a tres concentraciones de la especia diferentes. En general, los consumidores fueron capaces de distinguir el queso con azafrán de uno sin esta especia, al tiempo que mostraron una mayor preferencia por el queso con menor concentración de azafrán y con una coloración amarilla más ligera.

Palabras clave: *queso de oveja, azafrán, evaluación sensorial.*

SUMMARY

Saffron use in ewe's cheese production could represent an alternative to increase the economic value of both raw materials: milk and saffron. Three different sensory tests were made in this work (triangular, ordering and preference) in order to evaluate the color and flavor of the saffron cheeses at three different saffron concentrations. In general, consumers were capable of discriminate between cheese with and without saffron, as well as, the majority of them showed a preference for the cheese with less saffron concentration and a lighter color.

Key words: *ewe cheese, sensory evaluation, saffron.*

Introducción

España ocupa el cuarto lugar a nivel europeo en producción de leche de oveja, con un total de 410,000 toneladas en el año 2007 (FAO, 2010), destinando el 99,9% de esta producción a la elaboración de queso de oveja, ya sea mezcla o puro de oveja. En Castilla-La Mancha, la producción de queso Manchego representa el 42% de la producción nacional de quesos con DOP, (MAPA, 2009) por lo que surge la necesidad de dar un valor agregado al resto de los quesos que no cuentan con esta denominación, valor que se puede lograr añadiendo especias como el romero, entre otras.

El azafrán, es un producto nacional con una gran importancia, que cuenta con una DOP denominada Azafrán Mancha, que podría representar una buena oportunidad para la elaboración de queso de oveja, siempre y cuando satisfaga las preferencias de los consumi-

dores, dicha aceptación se puede medir llevando a cabo una evaluación sensorial del nuevo producto. La evaluación sensorial es utilizada en diversos tipos de industrias, de la cual se puede obtener, medir, analizar e interpretar las reacciones a determinadas características del alimento, tal y como son percibidas por los sentidos de la vista, olfato, gusto, tacto y oído (Barcino Angulo, 2001), siendo de especial importancia para el control y mejora de la calidad de los alimentos. El objetivo de este trabajo es el de analizar mediante pruebas sensoriales el color y sabor de quesos elaborados con distintas concentraciones de azafrán para determinar la preferencia del consumidor sobre un queso de oveja adicionado con azafrán.

Materiales y métodos

Se elaboraron 9 cubas de queso de 300 L de leche de oveja de raza Manchega pasterizada (72ºC, 20 seg.), con tres concentraciones de azafrán distintas (CI, C2 y C3, siendo C2 y C3 el doble y triple de la concentración CI, respectivamente), siguiendo la preparación de la patente en trámite No. P200930912 y 2 cubas de queso sin azafrán como control. Para su elaboración se adicionó un cultivo iniciador (CHOOZIT MA4001, Danisco, Sassenage, Francia) a la dosis de 5 DCU/100 L. La leche se mantuvo a 30ºC durante 20 min., adicionándose 0,025% (v/v) de $CaCl_2$ y 0,01% (v/v) de lisozima. Para la coagulación se utilizó cuajo comercial (quimosina:pepsina, 94:6) a la dosis de 0,023% (v/v). Treinta minutos después se realizó el corte de la cuajada en cubos de 8-10 mm, a continuación se calentó a 37ºC durante 45 min. antes del desuerado. La cuajada se moldeó y prensó en una prensa neumática (1 bar) durante el tiempo necesario hasta alcanzar un pH de 5,2. Los quesos se introdujeron en salmuera (18%) durante 18 h a 9ºC. Todos los quesos se almacenaron en cámara de maduración a 11±1ºC y HR 85% hasta su análisis a los 2, 4 y 6 meses de maduración.

La medición de pH se llevó a cabo con un pHmetro Crison Mod. GPL 22 con un electrodo de penetración (CRISON, España). El extracto seco, proteína y grasa se determinaron mediante un analizador de infrarrojo cercano NIRS FoodScan (FOSS, Dinamarca). Los análisis se realizaron por duplicado en cada pieza de queso. Para la lectura se eliminó 1 cm de corteza, se trituró el queso durante 20 segundos en una picadora Moulinex y se colocó en placas de Petri. Los resultados se analizaron mediante un análisis de varianza (ANOVA) con un nivel de confianza del 95% con el paquete estadístico SPSS 17.0.

Para el análisis sensorial se llevaron a cabo tres pruebas que evaluaron las características de color y sabor del queso, con paneles de estudiantes, docentes y no docentes de la UCLM, no entrenados, cuyas edades oscilaron entre los 20 y 60 años, todas ellas de acuerdo a la metodología propuesta por Anzaldúa-Morales (1994): 1) *prueba triangular*: para determinar si el consumidor es capaz de distinguir un queso con azafrán de uno puro de oveja, se enfrentaron a los 2 meses de maduración quesos control sin azafrán con quesos preparados con la concentración más baja de azafrán empleada en el estudio (CI), en esta prueba participaron 55 catadores; 2) *prueba de ordenamiento*: para determinar si los consumidores distinguen entre las distintas concentraciones de azafrán se utilizaron muestras de las tres concentraciones (CI, C2 y C3) con 26 catadores a los 2 meses de maduración y con 34 a los 6 meses; y 3) *prueba de preferencia*: para determinar la concentración de mayor aceptabilidad se compararon las tres concentraciones entre sí comparándolas por parejas, participando 49 catadores a los 2 meses de maduración, 34 a los 4 meses y 36 a los 6 meses. Todas las pruebas se llevaron a cabo en una sala iluminada, aireada y libre de olores extraños. Las pruebas de sabor se realizaron con lámparas con luz roja para eliminar el efecto del color sobre la percepción del sabor. Los resultados se analizaron mediante las ta-

blas de significancia que corresponden a cada prueba (Anzaldúa-Morales, 1994).

Resultados y discusión

En la tabla 1 se muestra la composición a lo largo de la maduración de los quesos elaborados con azafrán comparados con el queso control, y en general no hubo diferencias significativas entre las distintas variedades de queso. Se observó una evolución a lo largo de la maduración típica de un queso de oveja, ya que conforme aumentaba el tiempo de maduración, los quesos iban perdiendo humedad y, por lo tanto, aumentando el porcentaje de extracto seco y en consecuencia el de proteína y grasa (Cabezas et al., 2007).

La prueba triangular, se llevó a cabo con la finalidad de determinar si los consumidores eran capaces de diferenciar entre un queso de oveja sin azafrán y un queso con la concentración de azafrán más baja utilizada (CI). De los 55 catadores participantes, 42 personas fueron capaces de diferenciar el azafrán en el queso ($P< 0,01$), indicando 28 de ellos una diferencia moderada; por otro lado, 54 fueron capaces de diferenciar el color de los mismos, mencionando que había mucha diferencia entre las muestras.

En las pruebas de ordenamiento se busca que el catador sea capaz de ordenar de manera ascendente o descendiente una serie de muestras ordenadas de manera aleatoria, bajo una característica específica. En éste caso a los 2 meses de maduración los 26 catadores participantes fueron capaces de ordenar de manera ascendente el color de los quesos con las tres concentraciones de azafrán ($P< 0,05$). Veintidós de veintiséis panelistas distinguieron por su sabor el aumento de azafrán en el queso. ($P< 0,05$). A los 6 meses de maduración, 32 de 34 catadores fueron capaces de ordenar el color correctamente ($P<0,05$), sin embargo menos del 50% (16 de 34; NS), fueron capaces de ordenar el sabor, por lo que se intuye que conforme maduran los quesos las diferencias de sabor entre éstos son menos perceptibles para el consumidor.

Tabla 1. pH y composición de extracto seco, materia grasa y proteína en quesos sin azafrán (Control) y quesos con distintas concentraciones de azafrán (CI, C2 y C3) (n=6).

Maduración (días)	Control	pH			
		CI	C2	C3	ANOVA
60 d	5,26±0,01	5,30±0,15	5,24±0,06	5,26±0,12	NS
120 d	5,23±0,29	5,22±0,10	5,25±0,14	5,21±0,09	NS
180 d	5,20±0,09	5,18±0,09	5,15±0,03	5,15±0,06	NS
ANOVA	NS	NS	NS	NS	
Maduración (días)	**Control**	**Extracto Seco (g/100 g)**			
		CI	C2	C3	ANOVA
60 d	$59,66\pm0,08^{a,X}$	$62,36\pm2,10^{b}$	$61,98\pm1,22^{b,x}$	$61,19\pm0,83^{b,x}$	NS
120 d	$64,51\pm0,11^{y}$	65,10±3,60	$63,71\pm2,13^{x}$	$63,62\pm3,07^{xy}$	NS
180 d	$66,60\pm0,00^{z}$	65,65±1,40	$66,25\pm0,41^{y}$	$65,88\pm0,28^{y}$	NS
ANOVA	***	NS	**	**	
Maduración (días)	**Control**	**Materia Grasa/Extracto seco (g/100g)**			
		CI	C2	C3	ANOVA
60 d	$50,16\pm0,03^{a,y}$	$51,89\pm0,57^{b}$	$52,04\pm0,85^{b}$	$51,24\pm0,30^{ab}$	**
120 d	$49,32\pm0,16^{x}$	56,60±1,64	50,75±2,60	53,95±3,18	NS
180 d	$51,32\pm0,00^{z}$	51,05±0,66	51,44±0,42	50,76±0,14	NS
ANOVA	***	NS	NS	NS	
Maduración (días)	**Control**	**Proteína/Extracto seco (g/100g)**			
		CI	C2	C3	ANOVA
60 d	$39,90\pm0,03^{x}$	40,28±0,73	$40,23\pm1,14^{x}$	40,77±0,88	NS
120 d	$40,06\pm0,07^{b,x}$	$39,48\pm0,23^{a,}$	$43,03\pm0,05^{d,y}$	$40,85\pm0,02^{c}$	***
180 d	$40,65\pm0,00^{y}$	40,12±0,07	$39,58\pm0,30^{x}$	39,65±0,90	NS
ANOVA	***	NS	**	NS	

*(p≤0,05), ** (p≤0,01), *** (p≤0,001)

x,y,z, valores en la misma columna con diferente superíndice son diferentes significativamente (p<0,05).

a,b,c , valores en la misma fila con diferente superíndice son diferentes significativamente (p<0,05).

En la tabla 2 se muestran los resultados de las pruebas de preferencia del sabor a lo largo a los 2, 4 y 6 meses de maduración, incluidos el número de catadores de cada etapa del análisis. A partir de la tabla, podemos observar que no existe una preferencia significativa por ninguna de las concentraciones, con excepción del mes 4 en que los catadores prefirieron el queso de concentración CI contra el queso de concentración C2. Respecto al color del mismo (Tabla 3), tampoco existe en la mayoría de los casos una preferencia significativa por un color determinado; sin embargo, a los 2 meses de maduración los consumidores prefirieron el color del queso con menor concentración de azafrán sobre el resto de con-

centraciones, al igual que en el mes 6, que se prefirió el color del queso C1 sobre el color del queso C3.

Conclusiones

Al comparar un queso de oveja con un queso al azafrán, los panelistas participantes en el estudio fueron capaces de distinguir visualmente la presencia de azafrán y de detectar su sabor. Tampoco tuvieron dificultades a la hora de distinguir visualmente entre quesos con distintos niveles de esta especia, aunque manifestaron mayores dificultades a la hora de apreciar en boca estos niveles, sobre todo a medida que aumentaba la maduración. Al realizar pruebas de preferencia, en general los panelistas prefieren quesos que contenga menor cantidad de azafrán y colores amarillos más ligeros.

Tabla 2. **Prueba de preferencia del sabor de los quesos con azafrán a lo largo de la maduración.**

Meses de maduración (catadores)	C1/C2		C2/C3		C1/C3	
	C1	C2	C2	C3	C1	C3
2 m (49)	18	30	23	24	22	26
Significancia*	NS		NS		NS	
4 m (34)	24	10	18	16	16	18
Significancia	0.05		NS		NS	
6 m (36)	21	15	19	17	23	13
Significancia	NS		NS		NS	

*La significancia se obtuvo de Anzaldúa-Morales, 1994.

Tabla 3. **Prueba de preferencia del color de los quesos con azafrán a lo largo de la maduración.**

Meses de maduración (catadores)	C1/C2		C2/C3		C1/C3	
	C1	C2	C2	C3	C1	C3
2 m (49)	32	17	27	21	35	13
Significancia*	0.05		NS		0.01	
4 m (34)	17	17	17	16	20	14
Significancia	NS		NS		NS	
6 m (36)	23	13	24	12	27	9
Significancia	NS		NS		0.01	

*La significancia se obtuvo de Anzaldúa-Morales, 1994.

Agradecimientos

Este trabajo ha sido financiado por Proyecto de Investigación PBI08-0245-8453 y la beca predoctoral de C. Licón de la JJCC Castilla-La Mancha. Los autores agradecen la colaboración de las empresas lácteas Quesera Campo Rus (Cuenca) y FOMAN (Albacete).

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8.6 Saffron color and aroma transference during fabrication of ewes' milk dairy products

8.6.1 Approach

The aim of this study was to determine saffron distribution, in terms of color and aroma, during the fabrication of cheese and "requesón" from ewes' milk. Color was measured by tristimulus colorimetry and aroma by gas chromatography/mass spectrometry in cheese, whey, "requesón" and "requesón" whey. Results from this work will be presented in a national Congress in September 2012:

Transferencia de color y aroma del azafrán en la elaboración de derivados lácteos de leche de oveja

Molero, J., **Licón, C.,** Serrano, J., Carmona, M., Molina, A. y Berruga, M.I.

XXXVII Congreso de la Sociedad Española de Ovinotecnia y Caprinotecnia

Ciudad Real, Spain

8.6.1 Summary

Milk and cheese with saffron showed a less bright, less red and more yellow color compared to control while whey, "requesón" and "requesón" whey were brighter, more yellow and less red. Color differences between saffron and non-saffron fabrications were higher in cheese and "requesón" than in whey or "requesón" whey. Around 46 % of saffron aroma, in terms of safranal, was retained in the cheese and "requesón" while the rest was lost in the "requesón" whey. This fact could be interesting to new applications of the latter fraction as an additive in different products for example beverages.

TRANSFERENCIA DE COLOR Y AROMA DEL AZAFRÁN EN LA ELABORACIÓN DE DERIVADOS LÁCTEOS DE LECHE DE OVEJA

MOLERO, J.[a]; LICÓN, C.[a]; SERRANO, J.[a]; CARMONA, M.[b]; MOLINA, A.[a] y BERRUGA, M.I.[a]*
[a]Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA-IDR
[b]Parque Científico y Tecnológico de Albacete
Campus Universitario s/n, 02071, Albacete. Universidad de Castilla-La Mancha
*mariaisabel.berruga@uclm.es

RESUMEN
El empleo de azafrán en la elaboración de queso y requesón de oveja representa una alternativa para aumentar el valor económico de productos lácteos tradicionales de Castilla La-Mancha. El objetivo de este estudio fue la determinación del grado de transferencia, en términos de color y aroma, del azafrán durante el proceso de producción de queso y requesón de leche de oveja. Los resultados mostraron que al adicionar azafrán el rendimiento quesero fue mayor. Las matrices sólidas presentaron un diferencial de color mayor que las líquidas, sin embargo, la distribución de safranal fue mayor en las matrices líquidas.
Palabras clave: leche de oveja, aromas, color, azafrán, queso

INTRODUCCIÓN
El color de un alimento es uno de los principales aspectos que consideran los consumidores cuando eligen un producto, por ello, la adición de colorantes a la comida ha sido una práctica habitual utilizada desde la antigüedad. El azafrán (Crocus *sativus* L) es una de las especias utilizadas con este fin además de transmitir también sabor y aroma a los alimentos. Los compuestos responsables del color del azafrán son carotenoides solubles en agua llamados ésteres de crocetina mientras que el safranal es una de las sustancias principales responsables del aroma característico del azafrán (Carmona y col. 2006). Recientemente se ha desarrollado un método de extracción de azafrán en leche que permite el mejor aprovechamiento de la especia para la fabricación de derivados lácteos (Licón y col. 2012a). Sin embargo es necesario conocer su distribución durante el proceso productivo, ya que esta especie es una de las más caras del mundo por lo es importante optimizar la adición de la misma.

Este trabajo tiene como finalidad conocer como se distribuye el azafrán, en términos de color y aroma (safranal), durante el proceso de elaboración de queso y requesón de leche de oveja.

MATERIALES Y MÉTODOS
La fabricación de queso y requesón a escala laboratorio consistió en una elaboración (A) sin adición de azafrán y una elaboración (B) adicionando un 1% (p/v) de azafrán. Cada una se realizó por duplicado. Para ello, se utilizaron 2L de leche cruda de oveja con una composición de 5,21% de grasa, 5,42% de proteína y 16,35% de sólidos totales. La leche se calentó hasta 30 ºC adicionando cuajo comercial 0,0022% (v/v).Treinta minutos después se realizó el corte de la cuajada en cubos de 8-10 mm, a continuación se calentó a 37ºC durante 20 min antes del desuerado. La cuajada se moldeó en moldes de plástico perforadas (6x6x7cm) con un peso aproximado de 100 g (Busqui, España) y se prensó por gravedad durante 2 horas. El suero obtenido de la elaboración del queso se calentó con agitación constante hasta 80-85 ºC, llegada esta temperatura se interrumpió la agitación hasta que se alcanzaron los 90 ºC. Las proteínas

que floculadas se recogieron de la superficie con la ayuda de una cuchara perforada y se moldearon en las mismas condiciones que el queso. El queso y requesón se orearon a 4 ºC durante 24 horas.

Los rendimiento de queso y requesón se calcularon como la relación del peso de cada producto obtenido respecto al volumen de leche y suero empleados (%). La composición de la leche cruda de oveja se midió mediante un analizador NIRS (MilkoScan, FOSS, Dinamarca). Las mediciones de color y aroma se llevaron a cabo en la leche, cuajada, suero, requesón y suero de requesón. El color se determinó con un colorímetro Minolta CR-400, con iluminante D65 y observador de 10º (Osaka, Japón). Se obtuvieron las coordenadas L*, a* y b* y se calculó la diferencia de color de acuerdo a la siguiente fórmula: $\Delta E^* = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$. La concentración de safranal se midió por extracción en espacio de cabeza estático (HSSE) y se identificó y cuantificó por cromatografía de gases/espectrometría de masas (GC/MS) de acuerdo a la metodología dessarrollada por Licón y col. (2012b). Se llevaron a cabo modificaciones para las muestras líquidas que consistieron en un tiempo de extracción de 2 hrs y en una barra adsorbente de 1 cm. Todos los análisis se llevaron a cabo por duplicado. Los resultados se analizaron mediante un análisis de varianza (ANOVA) con un nivel de confianza del 95% con el paquete estadístico SPSS 15.0.

RESULTADOS Y DISCUSIÓN

En la tabla 1 se muestran los rendimientos obtenidos para el queso y requesón. No hubo diferencias significativas entre los rendimientos obtenidos sin embargo, los rendimientos fueron mayores para el queso elaborado con azafrán. Dichos valores se aproximan a los rendimientos que se obtienen en la elaboración de queso blanco y requesones italianos, respectivamente (Farkye, 2004).

Tabla 1. Efecto de la adición de azafrán sobre el rendimiento

Rendimiento (%)	Queso	Requesón
Control	21,54±0,05	5,11±0,17
Azafrán	23,12±1,12	5,08±0,42
ANOVA	NS	NS

La tabla 2 muestra las coordenadas de color y el diferencial de color de las fracciones analizadas con y sin azafrán. La leche y el queso con azafrán mostraron un color menos brillante, menos rojo y más amarillo mientras que el suero de queso, requesón y suero de requesón fueron más brillantes, más amarillos y menos rojos. El diferencial de color fue mayor en el queso y en el requesón que en los respectivos sueros. Las sustancias responsables del color amarillo-anaranjado que imparte el azafrán son un grupo de carotenoides solubles en agua llamados ésteres de crocetina. Dichos carotenoides podrían estar interaccionando con las caseínas del queso y las proteínas del suero en el requesón y por tanto ser las responsables de la gran diferencia encontrada entre los valores de la coordenada b* del control y de los productos con azafrán. Se ha comprobado que las proteínas presentes en la leche son capaces de unir una amplia variedad de moléculas (Livney 2010).

Tabla 2. Coordenadas L*, a* y b* y diferencia de color (ΔE*)

PRODUCTO		CONTROL	AZAFRÁN	ANOVA	Δ E
LECHE	L	87,97±0,74	85,13±1,92	*	27,84±5,49
	a	-3,63±0,19	-5,80±1,21	**	
	b	6,16±1,61	33,72±2,85	*	
QUESO	L	84,32±0,11	77,94±1,80	NS	39,98±0,01
	a	-2,65±0,71	-0,88±0,99	***	
	b	12,63±0,18	52,01±0,25	*	
SUERO DE QUESO	L	59,73±5,41	62,69±8,23	***	12,63±0,50
	a	-2,95±1,69	-5,16±1,20	***	
	b	3,29±1,38	15,19±2,23	*	
REQUESÓN	L	82,16±0,08	84,63±0,92	NS	23,62±0,39
	a	-1,37±0,14	-4,78±0,11	NS	
	b	9,24±0,28	32,48±0,41	*	
SUERO DE REQUESÓN	L	58,05±0,58	62,85±6,55	*	22,61±4,27
	a	-5,20±0,11	-7,81±2,46	NS	
	b	2,72±0,46	23,31±6,66	***	

*P≤0,05, **P≤0,01, ***P≤0,001

La distribución de azafrán en la producción de queso y requesón con azafrán se muestra en la figura 1. En el queso se retuvo una media de 36% del safranal inicial mientras que en el requesón solo una media de 10% del safranal presente en el suero. Las matrices líquidas presentaron mayor cantidad de safranal que las matrices sólidas observándose una mayor recuperación en el suero de requesón. La interacción entre compuestos aromáticos y proteínas, grasas y azúcares de la leche ha sido ampliamente estudiada. Se ha demostrado que la unión entre compuestos del aroma y las proteínas, tanto caseínas y proteínas del suero, se llevan a cabo mediante uniones hidrófobas. Por otro lado, la presencia de safranal en el suero y suero requesón puede ser atribuida a la parcial solubilización del mismo en el agua por medio de otras moléculas presentes como por ejemplo la lactosa o la riboflavina (Kopjar y col. 2010)

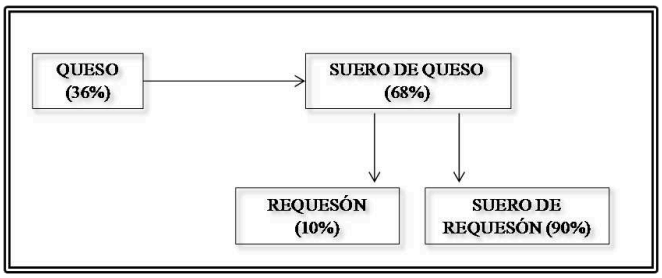

Figura 1. Porcentaje medio de recuperación de safranal

En este estudio, del total del safranal presente en la leche se retiene el 46% en las matrices sólidas mientras que el 54% se pierde en el suero de requesón, por lo que su aprovechamiento como aditivo para bebidas podría representar el aprovechamiento del mismo ya que el suero de requesón es una matriz rica en lactosa y sales, aunque su composición no ha sido ampliamente estudiada.

CONCLUSIONES

La adición de azafrán en la elaboración de queso aumentó el rendimiento del mismo. Los quesos con azafrán son menos brillantes, menos rojos y más amarillos que

los quesos sin azafrán mientras que los requesones son más brillantes y amarillos y menos rojos. La transferencia de color de azafrán es mayor en el queso y en el requesón que en las fracciones líquidas, mientras que la recuperación de safranal fue mayor en los sueros. De las fracciones sólidas, el queso presentó una mayor recuperación de safranal que el requesón. La presencia de safranal en el suero de requesón puede representar una alternativa para su uso como ingrediente en bebidas.

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FABRICATION OF EWES' MILK DAIRY PRODUCTS WITH SAFFRON: COLOR AND AROMA TRANSFERENCE

ABSTRACT

Saffron addittion to cheese and whey cheeses represents an alternative to increase economical value of these traditional products in Castilla-La Mancha. The objective of this work was to determine saffron distribution, in terms of color and aroma, during the fabrication of cheese and requesón cheese from ewes' milk. Results showed that cheese yield of saffron cheeses was higher. Solid matrixes with saffron had higher color differences than liquid matrixes, nevertheless, aroma distribution was higher in the liquid matrixes.

Key words: ewes' milk, aroma, color, saffron, cheese

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