Dyes and Pigments 92 (2012) 1355–1360

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

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

ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form
26 September 2011
Accepted 28 September 2011
Available online 5 October 2011

Keywords:
Ewes' milk
Saffron spice
CIEL*a*b*
CIEL*C*h
Crocetin esters
Milk composition

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).

0143-7208/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2011.09.022

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.

Acknowledgments

The work was financially supported by the Consejería de Educación y Ciencia of the JCCM with the research project PBI08-0245-8453 and C. Licón grant.

References

[1] Carmona M, Zalacain Aramburu A, Alonso Díaz-Marta G. El color, sabor y aroma del azafrán especia. Albacete: Altabán Ediciones; 2006.
[2] Zulueta A, Esteve ML, Frígola A. Carotenoids and color of fruit juice and milk beverage mixtures. J Food Sci 2007;72(9):457–63.
[3] Licon C, Carmona M, Llorens S, Berruga MI, Alonso GL. Potential healthy effects of saffron spice (*Crocus sativus* L. stigmas) consumption. Func Plant Sci Biotech 2010;4(2):64–73.
[4] Alonso GL, Sánchez-Fernández MA, Sáez JR, Zalacain A, Salinas MR. Evaluation of the color of Spanish saffron using tristimulus colorimetry. Ital J Food Sci 2003;15(2):249–58.
[5] ISO. Technical specification. Saffron (*Crocus sativus* L.) Part 1. (Specification). Part 2. (Test methods). Geneva, Switzerland: International Organization of Standarization; 2003.
[6] Sánchez AM, Carmona M, Ordoudi SA, Tsimidou MZ, Alonso GL. Kinetics of individual crocetin ester degradation in aqueous extracts of saffron (*Crocus sativus* L.) upon thermal treatment in the dark. J Agric Food Chem 2008;56(5):1627–37.
[7] Pfander H, Schurtenberger H. Biosynthesis of C20-carotenoids in *Crocus sativus*. Phytochemistry 1982;21(5):1039–42.
[8] Castillo R, Fernández JA, Gómez-Gómez L. Implications of carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of *Crocus sativus* and its closer relatives. Plant Physiol 2005;139(2):674–89.
[9] Various. Azafrán en Europa. Problemas y estratégias para valorizar la calidad y mejorar la competitividad. Unión Eurcpea; 2006.
[10] Pardo JE, Zalacain A, Carmona M, Loćpez E, Alvarruiz A, Alonso GL. Influence of the type of dehydration process on the sensory properties of saffron spice. Ital J Food Sci 2002;14(4):413–21.
[11] Yadollahi A, Shojaei ZA, Farahnaky A. Study of colouring, aromatic strength and bitterness of saffron (*Crocus sativus* L.) cultivated in the UK. Acta Hortic; 2007:455–61.
[12] Pulina G, Nudda A. Milk production. Dairy sheep feeding and nutrition. Bologna: Editorial Avenue Media; 2002. p. 11–27.
[13] Molina MP, Berruga MI, Molina A. Calidad de leche de oveja en ovinotecnia. In: Sañudo C, Cerero R, editors. Producción y economía en la especie ovina. Zaragoza: Prensas Universitarias de Zaragoza; 2009. p. 355–67.
[14] Park YW, Juárez M, Ramos M, Haenlein GFW. Physico-chemical characteristics of goat and sheep milk. Small Ruminant Res 2007;68(1–2):88–113.
[15] Varman AH, Sutherland JP. Milk and milk products: technology, chemistry and microbiology. New York: Aspen Publishers; 2001.
[16] Mohajeri SA, Hosseinzadeh H, Keyhanfar F, Aghamohammadian J. Extraction of crocin from saffron (*Crocus sativus*) using molecularly imprinted polymer solid-phase extraction. J Sep Sci 2010;33(15):2302–9.
[17] Sánchez AM, Carmona M, Prodanov M, Alonso GL. Effect of centrifugal ultrafiltration on the composition of aqueous extracts of saffron spice (*Crocus sativus* L.). J Agric Food Chem 2008;56(16):7293–301.
[18] Popov-Raljić JV, Lakić NS, Laličić-Petronijević JG, Barać MB, Sikimić VM. Color changes of UHT milk during storage. Sensors 2008;8(9):5961–74.
[19] Frøst MB, Dijksterhuis G, Martens M. Sensory perception of fat in milk. Food Qual Prefer 2001;12(5–7):327–36.
[20] Jandal JM. Comparative aspects of goat and sheep milk. Small Ruminant Res 1996;22(2):177–85.
[21] Fox PF. History of dairy chemistry. In: Fuquay JW, Fox PF, McSweeney PLH, editors. Encyclopedia of dairy sciences. 2nd ed. London, UK: Academic Press; 2011. p. 18–25.
[22] Satyanarayana A, Rao PP, Rao DG. Influence of source and quality on the color characteristics of annatto dyes and formulations. LWT Food Sci Technol 2010;43(9):1456–60.
[23] Chisté RC, Benassi MT, Mercadante AZ. Effect of solvent type on the extractability of bioactive compounds, antioxidant capacity and colour properties of natural annatto extracts. Int J Food Sci Tech 2011;46(9):1863–70.
[24] Orfanou O, Tsimidou M. Evaluation of the colouring strength of saffron spice by UV–Vis spectrometry. Food Chem 1996;57(3):463–9.
[25] Pereda J, Ferragut V, Quevedo JM, Guamis B, Trujillo AJ. Heat damage evaluation in ultra-high pressure homogenized milk. Food Hydrocolloid 2009;23(7):1974–9.
[26] Ogawa I, Yamano H, Miyagawa K. Application of deaerated water in extraction of colorants from dyer's saffron florets. J Appl Polym Sci 1999;74:1701–4.
[27] Goff D. Dairy chemistry and physics. Dairy science and technology education. Guelph: University of Guelp; 2009.
[28] Taylor MW, MacGibbon AKH. Milk lipids. General characteristics. In: Fuquay JW, Fox PF, McSweeney PLH, editors. Encyclopedia of dairy sciences. 2nd ed. London, UK: Academic Press; 2011. p. 349–54.
[29] Wackerbarth H, Stoll T, Gebken S, Pelters C, Bindrich U. Carotenoid–protein interaction as an approach for the formulation of functional food emulsions. Food Res Int 2009;42(9):1254–8.
[30] Livney YD. Milk proteins as vehicles for bioactives. Curr Opin Colloid Interface Sci 2010;15(1–2):73–83.
[31] Talbott L. Process for the uniform coloration of cheese; 2002. United States of America.
[32] Petitpas I, Grüne T, Bhattacharya AA, Curry S. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. J Mol Biol 2001;314(5):955–60.