1 Impact of Magnetic assisted Freezing in the physicochemical and
2 functional properties of egg components. Part 2: Egg Yolk

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ABSTRACT

Effects of freezing assisted with magnetic fields (MFs) in a commercial unit Cell Alive System (CAS) at $-50\text{ }^{\circ}\text{C}$, with a static MF only (0% CAS) and with a static MF plus oscillating MF (10% CAS) on egg yolk (Y) was investigated. Y samples were from commercial eggs laid by three hen strains (two of them in fortified cages and the third one free at outdoor). The main goal was to follow the protein thermal denaturation involved in MFs processing. Results showed that freezing treatment was the factor with highest influence. Thermal denaturation enthalpy of Y was markedly affected ($\sim 45\%$ total protein in comparison with Fresh sample) but similar for both MFs processes where 0% CAS was taken as Control freezing treatment. MFs effects were predominantly thermal in nature and constituted the factor with the highest influence on thermal behavior (which run parallel to egg white, as described in Part 1), as well as on the majority of the functional properties exhibited by MFs samples. Rheology and free Sulfhydryl content were studied complementarily to Differential Scanning Calorimetry (DSC) data. Some important functional properties as were also determined. The hen strains-feeding factor did not practically influence the physical or functional behaviors of both untreated and processed Y samples, except in their Color parameters.

Key words

Egg yolk; Magnetic assisted freezing; Physicochemical properties; Functional properties; Protein denaturation; Rheological behavior.

1. Introduction

Eggs are generally considered as tasty, wholesome, and nutritious food. Their protein value is high while their calories and fat content are in moderation. In addition, eggs are easy to digest. They are very good potential sources of fresh materials for health-promoting derivatives, the so called functional foods, as well as an ingredient for the food industry in general. Also they are suitable for nutritional improvement (fortification) of several kinds of foods since they have four major nutritional components: proteins, lipids, all necessary vitamins (except vitamin C),

and minerals. Hens' eggs have been used as a food by human beings since antiquity and used in the food industry because of their nutritional value and organoleptic characteristics, but also and widely by their functional properties. Some of these functional properties include, foaming, thickening, binding, and emulsifying; in addition, the coloring ability and aroma of egg should also be mentioned.

All eggs have two main edible parts: White (or Albumen) (W), and Yolk (Y). White egg proteins have been already dealt with in prior Part 1, **so that** so current section should be restricted to egg yolk (Y). The total dry matter of freshly laid Y is about 52%, of which 65% is fat, 31% is protein, and 4% is composed by carbohydrates, vitamins and minerals. The largest variation of egg yolk dry matter takes place during egg's storage in its shell, due to the transfer of water from the white into the yolk (Guilmineau & Kulozik, 2006; 2007). In other words, as the pH of the W increases during storage, the physical properties of the Y membrane are modified, allowing then a slow diffusion of water and other small molecules from W into Y (Silversides & Budgell, 2004). Egg yolk dry matter is composed of complex-lipids and proteins in a ratio of around 2:1, respectively. In fact, Y is an emulsion of (microscopic particles of highly structural complexity) lipid droplets dispersed in an aqueous protein media, the droplets being of three types: spheres, granules (lipoprotein drops) and profiles (low-density lipoproteins), which have been shown to be chemically constituted by complex mixtures of phospholipids and low density lipoproteins (LDL) (Chang, Powrie, & Fennema, 1977). From another descriptive view, Y is composed by two main fractions: Plasma (80%), which contains LDL and soluble proteins (livetins), and Granula (20%), richer than plasma in protein, like lipovitellins (HDL), phosvitin, and Granular LDL (Ibanoglu & Erçelebi, 2007). LDL is the larger constituent of egg yolk. They have a typical lipoprotein structure, comprising a core of neutral lipids (triglycerides and cholesterol esters) surrounded by an interfacial layer of phospholipids and proteins (LDL apoproteins) (Burley & Vadehra, 1989; Kiosseoglou & Paraskevopoulou, 2005). According to Guilmineau & Kulozig (2007, and references therein) native egg yolk consists of a dispersion of low density lipoproteins (LDL) and insoluble granules in an aqueous solution of soluble glycoproteins (vitelins), where granules consist of a complex of high density lipoproteins (HDL) and a phosphoprotein (phosvitin) bound by phosphocalcic bridges. Egg Yolk is appreciated by its emulsifying properties, of particular value for

the preparation of hot and cold sauces (e.g. mayonnaise, dressings, hollandaise sauce), as well as in the production of biscuits, cakes and ice cream.

Egg Yolk (Y) is susceptible of being treated by the new processing technologies with different aims, mainly microorganisms and enzymes inactivation for a safe consume at short time or extended shelf-life for long storage periods. Among them we are particularly interested in the application of Magnetic Assisted Freezing (MFs) because it is a recently introduced treatment method that as far as we know has not yet been applied to egg products. MFs combines the application of a variety of magnetic fields with some temperatures below the freezing point of the samples, whose commercial development is giving promising results (Xanthakis, Havet, Chevallier, Abadie, & Le-Bail, 2013; Xanthakis, Le-Bail, & Ramaswamy, 2014). Notwithstanding, the number of studies reported in the literature still is very scarce, particularly concerning the MFs effects on the protein denaturation of treated egg substrates. It has an important local interest because this novel technology is currently in a phase of commercial introduction in Spain as in several other countries.

The main goal of present work is to study the effect of MFs treatments, performed in a commercial unit, on several physicochemical and functional properties of separated Egg Yolk (Y) from three classes of commercial eggs laid by three types of hens living in different conditions. Among the physical properties to be studied a major one is the thermal denaturation of respective proteins involved in MFs treatments by Differential Scanning Calorimetry (DSC), mainly complemented by Textural and Rheological analysis. Interesting to note is that rheological properties are among the most important characteristics of food products determining the functional properties, product development strategies and shelf life of the product and are useful for the design of processing.

All these collected data aimed to help understanding the changes that this new freezing technology could induce in the respective physicochemical and functional properties like foaming or gelling. So that, concerning egg components treatment, we could be able to present a general evaluation of the MFs performance in comparison with respective fresh egg components (Part 1 and Part 2).

2. Materials and methods

2.1. Sample preparation

Three different brown egg classes, laid within 7 days, all from *Gallus gallus* hen flocks fed compound-feed were obtained from a local producer. Three hens' strains corresponded to Lohmann Brown (A), Hn Brown (B), and Isa Brown (C) respectively. While A and B hens lived kept in fortified/enriched cages, C hens remained free at outdoor eating grass **in addition** also.

Fresh egg yolk (Y) was manually separated from each egg; chalazae and the rest of spots and defects were then removed from them (Manzocco, Panozzo, & Nicoli, 2013). Not less than 20 eggs from each strain were broken for the preparation of each Y sample batch. The obtained Fresh Y was very gently homogenized by hand and then immediately processed.

2.2. Composition and pH

Compositional data was **were** determined by triplicate according to AOAC methods (1984): Moisture was evaluated by desiccation of ~5 g using an air-forced oven Selecta 2000367 (JP SELECTA, SA, Spain) at 105 °C until a constant weight was reached. Fat was determined by the Soxhlet method with a Soxtec system Tecator AB S-263 21 (Höganäs, Sweden). Protein was determined by the Dumas method with an analyzer LECO TruMac (Leco Corporation, St. Joseph, MI, USA). Ash content was determined with an oven Select Horn (JP SELECTA, SA, Spain) working at 550 °C for 5 h.

The pH was determined at room temperature with a pH-meter (Crison, pH Burette 24 1S) by triplicate.

2.3. MFs treatments

They were performed as described in Part 1 for W samples. Briefly, a freezer (ABI, Corporation Ltd., Chiba, Japan) equipped with both static and oscillating magnetic fields generators (Cell Alive System – CAS) was used. Details about its characterization are given in (Otero, Pérez-Mateos, Rodríguez, & Sanz, 2017). Among possibilities of the freezer, two of them corresponding to the minimum and to the maximum magnetic intensity field were chosen, i.e., one with only static MF (0.14 mT, 0 Hz; 0% CAS) and the other with the static MF plus oscillating MF (1.66 mT, 6Hz; 10% CAS). The temperature of the freezer was programmed to be –50 °C. Both MFs treatments were applied for ~75 min processing time; cooling rates (°C/min) ranged ~0.04 – 0.05 (in the initial step, before the phase change) and ~0.02 – 0.03 (in the final step, after the phase change), with the higher and lower values corresponding to 0% CAS and 10% CAS respectively.

As explained in detail in Part 1, Conventional Freezing may not be chosen as Control, because this process is usually performed at selected temperatures but at very high cooling rates, that are not accessible in MF processing, as shown above. 10% CAS was considered current Control MFs treatments (Wowk, 2012).

In the case of DSC and Rheology, the procedure used for analysis was: Fresh Y (YA, YB, YC) samples were prepared in the morning and subsequently subjected to MFs freezing treatment; the frozen samples were then kept in the refrigerator (2 – 4 °C) overnight for thawing and then immediately encapsulated and DSC run or rheological measurement, keeping the operating conditions always at temperatures lower than 20 °C, to avoid undesirable protein denaturation. It was rationalized in Part 1. However, and just in case this way was not suitable to be managed in most of the physicochemical determinations it was followed the same methodology as for W, a total of 72 Y individual 100 mL portions for each CAS condition from the above referred Y (YA, YB, YC) sample batch, were deep-frozen at -80 °C with a Forma - 86C ULT Freezer (Thermo Fisher Scientific, Waltham, MA, U.S.A) at a cooling rate of 1 °C/min. Successively, samples were thawed for two hours at room temperature for daily analytical use. Only determinations like **Emulsifying properties** were affected and, fortunately their results (see corresponding Results headings) seemed not to observe inconsistent values relative to the general study, like in W samples.

2.4. Color

Color was evaluated with a spectrophotometer CM-3500d (Konica Minolta, Tokyo, Japan) managed by the data software CM-S100w SpectraMagic™ using the CIE Lab scale (D65/10°). Objective color CIE-LAB tristimulus values, L* (lightness); a* (redness), and b* (yellowness) parameters of the different Y samples were performed on a glass Petri-dish (40 mm internal diameter) filled up with Y samples. Five measurements were carried out at the dish center and four at radial positions distributed 90 ° apart, and averaged for each sample.

2.5. Free sulfhydryl content (SH)

The concentration of free sulfhydryl groups (SH) of Y samples were determined using the Ellman's reagent (5', 5-dithiobis 2-nitrobenzoic, DTNB) (Sigma Aldrich, 500mg), as reported by Beveridge, Toma, & Nakai (1974), i.e., about 1.5 g sample were solubilized in 10 mL 0.086 M Tris buffer containing 0.09 M glycine plus 4 mM EDTA (10.4 g Tris base, 6.9 g glycine and 1.2 g EDTA per liter, pH 8.0). An aliquot (0.1 mL) of sample solution was then mixed with 2.9 mL 0.5% (w/v) SDS in the same buffer plus 20 µL Ellman's reagent (4 mg/mL in the same buffer). After 15 min reaction at room temperature, the sample was centrifuged for 20 min at 19900×g, and the supernatant used for the absorbance determination at 412 nm in a spectrophotometer Thermo Scientific, Genesys 10S-UV-Vis, using the buffer as a blank. The -SH content was calculated by, $SH = 73.53 \times A_{412} \times D/C$, where A_{412} is the absorbance of the sample, D is the dilution number (10.2), and C is the sample concentration in mg/mL. To this respect, Part 1 contains some derivations taken from Ellman (1952). Measurements, expressed in µM/g, were performed in 6 aliquots and averaged.

2.6. Emulsifying properties

2% Two percent w/v Y solutions in 0.5 M NaCl at pH 6.5 were emulsified with sunflower oil, in the 60:40 ratio, for 1 min at room temperature using a high speed (11000 rpm) mixer (IKA-WERKE T25 BASIC) equipped with a 12 mm diameter head. Emulsifying activity index (EAI), the ability of a protein to form an emulsion that remains unchanged for a particular duration, and emulsion stability index (ESI), the life time of the stable emulsion, were determined using the turbidimetric method of Pearce & Kinsella (1978), with minor modifications made by Tang, Yang, Chen, & Peng (2002). An aliquot of emulsion was taken from the bottom of the homogenized

emulsion immediately (0 min) or 5 min after homogenization, diluted 1:150 with a sodium dodecyl sulfate (SDS) 0.1% (w/v) solution. Absorbance was then measured at 500 nm against a blank (diluting solution) using a spectrophotometer Thermo Scientific, Genesys 10S-UV-Vis. EAI (m²/g) and ESI (min) values were calculated by,

$$\text{EAI} = (2 \times 2.303 \times A_0 \times \text{DF}) / (c \times \varnothing \times (1 - \theta) \times 1000)$$

$$\text{ESI} = (A_0 / (A_0 - A_5)) \times 5$$

Where DF is the dilution factor (150), c is the weight of protein per unit volume of protein aqueous (mg/mL) emulsion, $\varnothing$ is the optical path (0.01 m), θ is the fraction of oil used in the emulsion (0.4), and A_0 and A_5 are the absorbance of the diluted emulsions at 0 and 5 min, respectively. Measurements were performed in 6 replications.

2.7. Rheological measurements

Oscillatory shear measurements were conducted using a Bohlin CVO-100 rheometer (Bohlin Instruments Ltd., Gloucestershire, UK), with cone-plate geometry (cone angle 1°, gap 0.03 mm). Temperature was controlled at 25 °C. Frequency sweep tests were carried out at the frequency range 0.1–10 Hz with a strain of 30%, selected from the linear viscoelastic region (LVE), to evaluate the dynamic rheological properties including storage modulus (G' , Pa) and loss modulus (G'' , Pa). The exponents n' and n'' were calculated from the power law equations, $G' = G_0' \times \omega^{n'}$ and $G'' = G_0'' \times \omega^{n''}$, where ω is the angular frequency (Hz), and G_1' and G_1'' are the storage and loss module respectively, determined at 1 Hz. Measurements were at least by triplicate.

2.8. Differential scanning calorimetry (DSC)

It was determined with a model TA-Q1000 Differential Scanning Calorimeter (TA Instruments, New Castle, DE, USA). In order to facilitate general sampling and, to get water enough for a complete protein thermal denaturation in the Y concentrated component, Y samples were diluted with an aqueous solution of NaCl 1% (w/v) solution, so as to obtain a suspension containing 80% (w/w) pure Y and 20% NaCl 1% (w/v), that were gently stirred manually for homogenization, as recommended by Guilmineau & Kulozik (2007) for other kind of application. Aliquots of approximately 8

– 10 mg (± 0.002 mg) were weighed out using a model ME235S electronic balance (Sartorius, Goettingen, Germany), tightly encapsulated in hermetic aluminum pans and scanned under dry nitrogen (50 mL/min) purge. An empty capsule was used as reference. Samples were heated from 5 to 100 °C at 10 °C/min and a second scan was occasionally run to check for reversible effects. Peak temperatures (T_{peak} , °C) and transition enthalpies (ΔH , J/g_{dm}) by linear baseline integration were calculated and normalized to dry matter content by encapsulated-sample desiccation. Samples were analyzed in triplicate.

2.9. Statistical analysis

One-way analysis of variance (UNIANOVA) was carried out (IBM SPSS Statistics v22.0, Armonk, NY, Inc.) to determine the influence of the source of variation (breeds/freezing treatment/interaction) by taken into account dependent variables with high loading F values and high correlation coefficients ($r^2 > 0.80$). Differences of means between pairs were resolved using confidence intervals with the Bonferroni or Tamhane tests depending on the variance homogeneity. Level of significance was set at $p < 0.05$.

3. Results and discussion

Due to logistic needs, MFs processed samples were fast cooled down and kept at – 80 °C waiting for analysis in most of the determinations. They were then thawed at room temperature immediately before analysis. In the case of DSC and Rheology, because frozen storage at so low temperatures could induce additional protein denaturation on Y samples, thus masking initial processing effects, the procedure was: Fresh egg samples were prepared in the morning and subsequently subjected to freezing treatment; the frozen samples were then kept in the refrigerator (2 – 4 °C) overnight for thawing and then immediately encapsulated and DSC run, keeping the operating conditions always at temperatures lower than 20 °C.

3.1. Composition

Table 1 shows the macro-constituents distribution in native Y samples according to the three egg classes (A, B, C). Concerning Y samples, YA and YB did not differ but in fat content, YB being significantly fattier than YA. YC was significantly the lower in containing water and protein, at the cost of being the fattier one.

3.2. MFs freezing diagrams

Figure 1 records the Time-Temperature typical diagrams of MFs treatment of Y (YA, YB, YC) samples at both processing conditions of 0% CAS and 10% CAS. Y samples showed similar profiles in both MFs processes: a considerable freezing plateau (shorter in 0% CAS) presenting a small sub-cooling particularly in 0% CAS, mainly because their high concentration, and almost no sloped plateau, with asymptotic-like declining (more pronounced in 10% CAS) that makes end point difficult to be appreciated, presumably due to inaccuracy in probe positioning at the sample holder center.

3.3. pH

The influence of the egg lied strains on the pH of Y samples were not significantly different, so that the below data corresponded to the mean values for the three (A, B, C) strains,

Y: Fresh, 6.02 ± 0.38^a ; 0 %, 6.09 ± 0.07^a ; 10 %, 6.14 ± 0.07^a

where the three results did not observe significant differences ($p < 0.05$). This stability was presumably due to the highly stable Y components nature and/or the minor contribution of those being labile to processing. Data were in general accordance with those reported by Severa, Nedomová, & Buchar (2010).

3.4 Color

Since poultry are not capable of carotenoid synthesis, the egg yolk color is originated from feed (Na, Song, Lee, Lee, Lee, & An, 2004). Xanthophyll content of egg mainly accounts for the yellowish color of egg yolk and whole egg (Nys, 2000). Table 2 summarized the Y (YA, YB, YC) tristimulus color parameters (L^* , a^* , b^*) variation as due to both MFs freezing processes (0% and 10%) with respect to untreated (Fresh) samples. Lightness (L^*) increased significantly with MFs treatment ($r^2 = 0.99$) in all strains (YA, YB, YC), with similar increments in both processing conditions. MacDougall (1982) indicated that changes in the lightness of food can be resulted from changes in the configuration of proteins. Concerning a^* , all classes decreased significantly by MFs (the processed samples becoming redder), with significantly

lesser extents in 10% CAS. Relative to b^* , YA and YB showed significantly decreasing ($r^2 = 0.97$) values, with significantly lesser extent in 10% CAS (becoming not as yellower as 0% CAS). However, YC showed significantly increasing with MFs treatment ($r^2 = 0.99$), with significantly lesser extent in 10% (stronger red color when passing from 0% CAS to 10% CAS, i.e., yellow moving towards orange). It was presumably due to a more carotene/carotenoids presence in the diet. Y color was considerably influenced by the laying hen strains and by the carotenoids content of their compound feed but, presumably mainly by the vegetal xanthophylls intake in the case of free laying hen flock.

3.5. SH

Sulfhydryl (SH) groups influence significantly the functional properties of food proteins and play an important role in the formation of specific structures such as protein gels and foams. Figure 2 shows SH results determined in Y (YA, YB, YC) samples at 0% CAS and 10% CAS vs. Fresh, untreated samples. In egg yolk, the high density (HDL) and low density lipoproteins (LDL) have SH groups as free or disulfide bonds. Y Fresh data were ~12% lower than those reported in the bibliography: Nguyen & Burley (1984) investigated the thermal processing of ten fractions of the major lipoproteins of egg yolk, and reported 8.1 $\mu\text{M/g}$ for the summa of all contributions at 20 °C. Badr (2006) reported 7.82 g/kg (presumably 7.82 mM/g, as reasoned in Part 1 for egg white). Yan, Qiao, Gu, Li, Xu, Wang, Reuhs, & Yang (2010) measured ~1.2 $\mu\text{M/g}$, an extremely small value relative to current one. SH increased significantly with freezing ($r^2 = 0.93$). MFs yielded progressive and significantly higher SH amounts in 0% CAS and 10% CAS relative to Fresh sample, as shown by the following average SH values for the three strains in the three cases: 6.86 (Fresh), 8.61 (0% CAS), and 9.12 (10% CAS). Strains did not cause differential contributions except for YC which showed a particular behavior at 10% CAS producing a significantly greater increment in SH; it made this sample the highest in both, within and among treatments. Since YC had the significantly lower protein content (Table 1), it could be that this YC behavior may have a (Isa Brown) hen strain origin.

Comparisons of MFs data were not available because no prior studies have been reported in the literature.

3.6. EAI and ESI

Emulsifying Activity Index (EAI, m^2/g) results of Y (YA, YB, YC) samples are shown in Figure 3a as a function of untreated (Fresh) and MFs processed at 0% CAS and 10% CAS. EAI data were slightly but significantly higher in MFs frozen samples, with no significant differences between both 0% CAS and 10% CAS processes ($r^2 = 0.62$). It seemed that any difference with respect to fresh samples may be essentially thermal in nature. Current results of Fresh Y were in line with those ($\sim 25 \text{ m}^2/\text{g}$) reported by Yan, Qiao, Gu, Li, Xu, Wang, Reuhs, & Yang (2010) for fresh egg yolk.

Emulsion Stability Index (ESI, min) results of Y (YA, YB, YC) samples are shown in Figure 3b as a function of untreated (Fresh) and MFs processed at 0% CAS and 10% CAS. ESI data were considerable and significantly higher in MFs frozen samples ($r^2 = 0.85$) with no significant differences between both treatments except for YB strain. Current values for Fresh samples were slightly higher, in the line of the reported one ($\sim 6 \text{ min}$) by Yan, Qiao, Gu, Li, Xu, Wang, Reuhs, & Yang (2010) for fresh egg yolk. There are other prior studies dealing with emulsifying properties of egg yolk and/or its components but their judging indexes (droplets mean diameter, creaming rate, influence of temperature, etc.; see, for instance, Le Denmat, Anton, & Gandemer, 1999) did not enable comparisons.

The emulsifying components in egg yolk are phospholipids, lipoproteins and proteins (Mine, 1998). In general, the two main components, the plasma (LDL) and granules (HDL), are qualified to participate with competition in emulsifying activities. However, environmental conditions (especially pH and ionic strength) are key factors in determining the structural status of LDL and HDL and also competition between plasma and granule proteins for interface adsorption (Anton, 2013). Extracted LDL from hen egg yolk, due to its amphipathic character and high structural flexibility, are now confirmed as the principal contributor to emulsifying properties of yolk, by forming a film at the interface between oil and water (Martinet, Beaumal, Dalgalarrodo, & Anton, 2002). These authors confirmed that only a small amount of the LDL phospholipids takes part in the adsorption at the oil-water interface and that the protein part of LDL played an essential role.

These results initially appeared somewhat contradictory: It will be demonstrated later on by DSC that Fresh egg yolk processing by MFs will yield protein denaturation with not significant differences between both 0% CAS and 10% CAS. And, it has been well established that denatured, aggregated proteins leads to their insolubility, which

is a big drawback to achieve a good emulsifying property of egg yolk (Octavio Rodiles-López, et al., 2008), since they could not adsorb at the interface.

On the other hand, the formation of disulfide bonds in the oil-water interface can lead to emulsion stability increment (Wu, Xiong, & Chen, 2011). Concerning this, it should be noted that Figure 2b showed that free SH groups increased significantly in MFs processed Y (YA, YB, YC) samples; i.e., MFs lead to the exposing of buried SH groups. Consequently, this increase in the emulsions stability could be related to the S-S bonds or S-SH exchange.

3.7. Rheology

Similar to WB, YB (for the sake of brevity) showed $G' > G''$ but with much smaller differences (indicating much weaker gel-like behavior) and stronger dependence on frequency (Figure 4a) than WB. Y (YA, YB, YC) samples showed significant differences between freezing treatments ($r^2 = 0.89$) for G' (Figure 4b), although remained non-significant for G'' (Figure 4c) module ($r^2 = 0.54$). The results of G' and G'' determined at 1Hz showed that Fresh YA in comparison to YB and YC had the highest G' (Fig. 4b) and G'' values (Fig. 4c). MFs freezing at 0% CAS and 10% CAS led to noticeable decrease in G' , although significant differences were only found for YA and YB (It seemed that the big YC dispersion may be a main point). No significant differences between both treatments were observed in any of the three strains. Regarding G'' , only YA showed significant decrease with MFs freezing, while YB and YC were practically unaffected. It should be mentioned that the YC brand, which showed the lower G'' value in the Fresh state, registered a very slight ($p < 0.05$) increase upon 10% CAS freezing. These results could initially indicate that gelation of egg yolk, which is a common and undesirable problem for the egg industry, did not occur under MFs processing. The real situation is not so as explained latter on when dealing with DSC results. Rheology seemed not to be a good tool to judge egg yolk denaturation. Both module (G' and G'') results were able to fit the power law model with $r^2 > 0.96$ in all cases. The power law exponent n' resulting from the mechanical spectra of G' is related to structural stability: the higher the n' values, the higher the instability of the matrix against frequency changes. By examining the results of n' for Y (YA, YB, YC) in Figure 4d, freezing under MFs induced considerable destabilizing effects of the yolk structure as denoted by the

significant increase in n' values. Both 0% CAS and 10% CAS treatments produced similar instabilities, except again for YC, where static plus oscillating MFs apparently was somewhat less detrimental. According to Telis-Romero et al. (2006), egg yolk showed a pseudoplastic and temperature dependent rheological behavior. Singh, Sharma, & Ramaswamy (2015) concluded that egg yolk behaved as a thixotropic fluid.

3.8. DSC

Figure 5a shows typical normalized DSC traces of Egg Yolk Y (YA, YB, YC) of untreated (Fresh, solid line) and MFs processed samples at $-50\text{ }^{\circ}\text{C}$ and 0% CAS (Dash line) or 10% CAS (Dot line). Fresh sample showed a small, rounded main endotherm at $T_{\text{peak}} \sim 85.5\text{ }^{\circ}\text{C}$, overlapped with a preceding, small event starting at $\sim 60\text{ }^{\circ}\text{C}$. MFs samples showed similar but smoother profiles. Y processed samples not only did not exhibited any visible appearance of protein denaturation/aggregation, gelling effects (contrary to W) but they retained a high fluidity for a long time, which may lead to erroneous conclusions. This peculiar fact has been widely dealt with in mechanism studies (out of present scope) on native egg yolks of any source (highly complex structured systems; see Introduction), and reported by several authors (see, for example: Au, Acevedo, Horner, & Wang (2015) and references therein).

Figure 5b shows the statistic evaluation of the normalized ΔH (J/g_{dm}) data of untreated (Fresh) and MFs frozen (0% CAS and 10% CAS) samples. Fresh YA and YB yielded similar results, while YC was significantly lower in correspondence with its significantly smaller protein content (Table 1). Both MFs treatments showed a significant ΔH reduction ($r^2 = 0.82$) of $\sim 45\%$ relative to untreated, with no significant differences between both treatments neither among strains. Similar to W, Fresh Y calorimetric studies have been found in the literature by reporting consistent DSC traces without endothermic thermal data quantification. Several studies have also appeared reporting consistent DSC traces (W and/or Y) with peak temperatures but without any enthalpy data associated, or by separated peak integration without integral enthalpy data (Sing & Ramaswamy, 2014), even with (in our opinion) apparently erroneous baselines construction yielding undervalued integration results

(Németh, Harváth, Drobecz, Friedrich, Pásztor-Huszár, & Balla, 2010). We did not find any thermal denaturation enthalpy data for Fresh Y but that reported by Yan, Qiao, Gu, Li, Xu, Wang, Reuhs, & Yang (2010) who gave 1.190 ± 0.007 J/g for an endothermic event starting at 71.90 ± 0.09 °C and ending at 87.86 ± 0.19 °C that, from a theoretical point of view, may be transformed into 6 – 7 J/g_{dm}, somewhat higher but of the same order of magnitude that current results. DSC data were consistent with current protein solubility, and SH determinations shown before. No prior data on MFs freezing was found for comparison.

Conclusions

Contrarily to egg white (W), Fresh Y samples were significantly changed by MFs processing in most of the above properties. Concerning hen strains, they influenced Y color parameters, particularly those of hens flock living cage-free, and eating grass. Thawing of MFs frozen samples exhibited highly denatured/aggregate egg proteins with great reduction in their functional properties. Similar to W, it can be concluded that both MFs treatments of Y did not present any clear advantage against conventional freezing at the same processing temperature.

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Figure legends

Figure 1. Temperature (°C) vs. time (min) typical diagrams: Egg Yolk (Y), Yellow lines; under MFs treated at -50 °C and 0% CAS (Dash lines) or 10% CAS (Solid lines). Blue lines indicate respective processing temperatures.

Figure 2. Mean values $\pm$ standard deviations of Free Sulfhydryl Content (SH, $\mu\text{M/g}$) of Egg Yolk (Y): YA, $\square$; YB, $\blacksquare$; YC, $\boxtimes$; as a function of processing: Untreated, Fresh; MFs treated at -50 °C and 0% CAS or 10% CAS. Different lowercase letters indicate significant ($p < 0.05$) differences among treatments within a strain. Different uppercase letters indicate significant ($p < 0.05$) differences among strains within a treatment.

Figure 3. Mean values $\pm$ standard deviations of **a)** Emulsion Ability Index (EAI, m^2/mg), and **b)** Emulsion Ability Index (ESI, min) of Egg Yolk (Y): YA, $\square$; YB, $\blacksquare$; YC, $\boxtimes$; as a function of processing: Untreated, Fresh; MFs treated at -50 °C and 0% CAS or 10% CAS. Different lowercase letters indicate significant ($p < 0.05$) differences among treatments within a strain. Different uppercase letters indicate significant ($p < 0.05$) differences among strains within a treatment.

Figure 4. **a)** Frequency sweep test of Egg Yolk (Y) as a function of processing: Untreated, Raw; MFs treated at -50 °C and 0% CAS or 10% CAS. Storage module (G' , Pa), Open symbols; Loss module (G'' , Pa), Solid symbols. Fresh, Triangles; 0% CAS, Squares; 10% CAS, Circles. **b)** Mean values $\pm$ standard deviations of Storage Module (G'_1 , Pa) and, **c)** Loss Module (G''_1 , Pa) at 1 Hz, and **d)** Power law exponent (n' , dimensionless), of Egg Yolk (Y): YA, $\square$; YB, $\blacksquare$; YC, $\boxtimes$; as a function of processing: Untreated, Fresh; MFs treated at -50 °C and 0% CAS or 10% CAS. Different lowercase letters indicate significant ($p < 0.05$) differences among treatments within a strain. Different uppercase letters indicate significant ($p < 0.05$) differences among strains within a treatment.

613 **Figure 5. a)** Normalized typical DSC traces (ΔH , J/g_{dm}) as a function of temperature
614 (°C) of Egg Yolk (Y) as a function of processing: Untreated, Fresh (Solid line); MFs
615 treated at -50 °C and 0% CAS (Dash line) or 10% CAS (Dot line). **b)** Mean values $\pm$
616 standard deviations of Normalized Thermal Denaturation Enthalpy (ΔH , J/g_{dm}) of
617 Egg Yolk (Y): YA, ; YB, ; YC, ; as a function of processing: Untreated, Fresh;
618 MFs treated at -50 °C and 0% or 10% CAS. Different lowercase letters indicate
619 significant ($p < 0.05$) differences among treatments within a strain. Different
620 uppercase letters indicate significant ($p < 0.05$) differences among strains within a
621 treatment.