Characterisation of emulsion properties and of interface composition in O/W emulsions prepared with hen egg yolk, plasma and granules

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Abstract

Comprehension of hen egg yolk emulsifying properties remains incomplete because competition between its various emulsifiers (proteins and lipoproteins containing phospholipids) has not been clearly elucidated and colloidal interactions between yolk-stabilised oil droplets have not been documented. Recent studies emphasised the interest of the fractionation of yolk into plasma and granules to improve this comprehension. In the present study, we characterised, concurrently, emulsion properties (oil droplet size and stability against creaming) and interface attributes (interfacial concentrations of proteins and phospholipids, SDS-PAGE profiles of adsorbed proteins and zeta potential) in oil-in-water (O/W) emulsions prepared with yolk, plasma and granules. We observed these features at four physicochemical conditions (pH 3.0 or 7.0 and at 0.15 or 0.55 M NaCl). Emulsion properties in emulsions made with yolk or plasma varied similarly as a function of pH and NaCl concentration whereas granules emulsions exhibited distinct properties. Therefore the main contributors to yolk emulsifying properties are to be sought for among plasma constituents (proteinaceous or phospholipids). Since, in plasma emulsions, variations of emulsion stability against creaming correlated exclusively to variations of protein interfacial concentration, a driving contribution of the proteinaceous part of plasma, namely apo-LDL, was hypothesised. In the pH and ionic strength ranges studied, zeta potentials of the interfaces were low, excluding extended electrostatic repulsion between oil droplets. We deduced that steric repulsion is the main interaction opposing to droplet aggregation in food emulsions made with yolk. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Egg yolk; Plasma; Granules; Emulsion; Interface; Colloidal interactions

1. Introduction

Hen egg yolk associates appreciated organoleptic characteristics with very good emulsifying properties so that it has become a key ingredient for the preparation of a large variety of food emulsions. But despite the fact that egg yolk has long been empirically recognised as a ‘reference’ emulsifying ingredient, its emulsifying properties still remain fairly poorly understood, presumably because of its remarkably complex chemical composition. When focussing on the emulsifiers in particular, egg yolk appears as a mixture of several proteins and lipoproteins which include apoproteins and phospholipids. As a result, the properties of oil-in-water (O/W) emulsions made with yolk are conditioned for a part by complex interactions between the numerous emulsifiers.

Fractionation of yolk constituents can help to screen those which effectively contribute to the emulsifying properties, provided that these constituents are recovered undenatured. Egg yolk can be fractionated into plasma and granules by a mild centrifugation without causing any denaturation (McBee & Cotterill, 1979). The supernatant (plasma) which represents 75–81% of the yolk dry matter, accounts for 52–58% of the proteins and for 85% of the phospholipids. The precipitated granules which make the remaining 19–25% of the yolk dry matter, account for 42–48% of the proteins and for 15% of the phospholipids (Anton & Gandemer, 1997; Burley & Cook, 1961; Causier, Matringe & Lorient, 1991; Dyer-Hurdon and Nnanna, 1993; Saari et al., 1964). Plasma is composed of 85% low-density lipoproteins (LDL) and 15% livetins. Granules are made of 70% high-density lipoproteins (HDL), 16% phosphvitin and 12% LDL (McCully, Mok & Common, 1962).

In the recent years, several authors have compared the emulsifying properties of yolk with those of plasma and granules. Comparing the stability of emulsions against creaming and the granulometry of emulsions, Anton (1998), Anton and Gandemer (1997) and Dyer-Hurdon...
and Nnanna (1993), found that emulsions prepared with yolk matched closely those made with plasma whereas emulsions prepared with granules exhibited distinct properties. This suggests that the main contributors to yolk emulsifying properties belong to plasma.

The emulsifying properties of plasma constituents, i.e. LDL and livetins, have been evaluated in comparison with other food emulsifiers, particularly proteins. Mizutani and Nakamura (1984) showed that LDL are better emulsifiers than bovine serum albumin. Aluko, Keeratiurai and Mine (1998) showed that LDL are more surface active than whey proteins, and Mine and Keeratiurai (2000) showed that they were also more surface active than caseins. Concurrently, livetins have revealed poor adsorption capacity at the oil–water interface when they are emulsified together with milk caseins or yolk lipoproteins (Shenton, 1979). This would ascertain the major contribution of LDL to plasma emulsifying properties and therefore to yolk emulsifying properties.

A comprehensive scheme was generally accepted to account for the driving influence of LDL, as it was first described by Kiosseoglou and Sherman (1983a–c). Plasma constituents (LDL and livetins) are remarkably soluble in the common pH range of food emulsions and whatever NaCl concentration (Cook & Martin, 1969). The soluble plasma constituents can adsorb efficiently at the oil–water interface and LDL displace competitively livetins from the interface. Different techniques, such as monitoring of adsorption kinetics (Kiosseoglou and Sherman, 1983b), treatment with protease (Mizutani & Nakamura, 1985; Shenton, 1979), have revealed that the protein part of LDL, apo-LDL, is essential. Kiosseoglou and Sherman (1983a–c) hypothesised that LDL are disrupted during the adsorption process, thus liberating apoproteins and phospholipids. Mine (1998a) reported results which support this hypothesis, namely by showing that the PC/PE ratios in the adsorbed film could be different from that in the original LDL. Apo-LDL would adsorb more readily than other soluble proteins due to their high hydrophobicity and to their flexible structure.

Contrary to plasma, specific conditions are required to have granules proteins soluble, namely high NaCl concentration (Causseret et al., 1991). Granules aggregates have poor surface activity and they do not adsorb efficiently when they compete with soluble proteins. But when the physicochemical conditions do allow granules solubilisation, solubilised HDL could provide additional emulsifiers and favour wider interface area coverage. Phosvitin have been shown to be easily displaced from the interface by yolk lipoproteins (Hunt, 1992; Shenton, 1979). Consequently it should not be expected to contribute much to yolk emulsifying properties.

However, in a recent study, Mine (1998b) found that granules proteins and especially apo-HDL are the main proteins in the interfacial film in O/W emulsions whereas several polypeptides constitutive of apo-LDL were absent. He reported the same observation at both pH 3.0 and 7.0. Based on these findings, the author minimised the aforementioned contribution of LDL and stated that HDL were the main contributors to yolk emulsifying properties. Consequently, these findings raised again the question of the identification of the constituents governing yolk emulsifying properties. This latter study however concentrated on the composition of the interface and the results were not confirmed by direct measurement of emulsion properties.

The present study aims to screen the constituents of yolk which govern its emulsifying properties and to investigate the colloidal interactions between oil droplets. The latter have not been much documented so far; they may provide complementary information to understand the aggregation properties in emulsion made with yolk. To achieve these purposes, we combined assessment of the emulsion properties and the characterisation of the composition of the interfaces in emulsions prepared with plain egg yolk, plasma and granules. Concerning emulsions properties, we determined the oil droplet sizes and the stability to creaming. We then characterised, in the same emulsions, the oil–water interfaces in terms of interfacial concentrations of proteins and phospholipids, electrophoretic profiles of adsorbed and unadsorbed proteins and finally zeta potentials. We measured these features at different pH and NaCl concentrations likely to occur in food emulsions in order to observe the responses to variations of physicochemical conditions of the aqueous phase.

2. Materials and methods

2.1. Materials

Fresh hen eggs were manually broken, the yolks were carefully rolled on a filter paper (Whatman) to remove albumen and chalazas adhering to the vitellin membrane and this membrane was punctured to flow out unspoiled egg yolk. The egg yolks were pooled and gently homogenised at 4°C. A portion was set apart to prepare plain egg yolk solutions while the remainder was fractionated into plasma and granules according to the method described by McBeé and Cotterill (1979). In this view, yolk was diluted (1:1 w/w) in an isotonic saline solution (0.17 M NaCl) and stirred for 1 h before centrifugation at 10,000 g for 45 min at 10°C. Precipitated granules obtained from the first centrifugation were washed with a 0.17 M NaCl solution and collected. The supernatant (plasma) was poured out and centrifuged again for complete removal of granules. The protein contents of plain yolk, plasma and granules were assayed according to the modified Lowry method described by Markwell, Hass, Bieber and Tolbert (1978). We determined protein concentrations of 170 mg/g in yolk, 58 mg/g in plasma and 281 mg/g in granules. With each of the three yolk products we prepared four different solutions...
combining two pH (3.0 and 7.0) and two NaCl concentrations (0.15 and 0.55 M). The protein concentration was standardised at 25 mg/ml. Glycine (50 mM) and imidazole (50 mM) were used to buffer the pH 3.0 and 7.0, respectively.

A single batch of commercial sunflower oil was used to prepare the emulsions. Prior to emulsion preparation, the oil was washed of polar components: the oil was diluted (1:1 v/v) in hexane and eluted on a silica column to extract polar lipids. Hexane was then evaporated from the eluted neutral lipid fraction and the purified sunflower oil was stored in a dark flask at 4°C until the preparation of the emulsion. Analysis with gas chromatography revealed that we extracted nearly 2% of polar lipids from the crude sunflower oil, mainly mono- and diglycerides. After washing, the purified sunflower oil did not hold quantifiable amount of polar lipids.

2.2. Determination of protein solubility

The protein concentration was first adjusted to 1 mg/ml with an appropriate buffer solution (same pH and NaCl concentration as the original solution). An aliquot of the dilution was withdrawn for protein assay and the remainder was centrifuged at 10,000 g for 20 min at 10°C. The protein content of the supernatant was assayed and the protein solubility was determined as the ratio:

\[ \frac{\text{protein in the supernatant}}{\text{protein in the initial dilution}} \times 100 \]

2.3. Preparation of the emulsion

Oil-in-water emulsions were prepared with the yolk products solutions and the purified sunflower oil. A constant oil volume fraction of 0.30 was chosen. Emulsion premixes were prepared using the rotor stator system Polytron PT3000 (Kinematica, Littau, Switzerland) equipped with a 12 mm head working at 20,000 rpm for 30 s. Homogenisation of the coarse emulsions was then achieved with a two-stage high pressure valve homogeniser (Stansted Fluid Power, Stansted, UK). The first and second valves applied 200 and 25 bar, respectively. The emulsion (40 ml) was left to re-circulate in the homogeniser for 3 min at a rate of flow of 120 ml/min.

2.4. Determination of oil droplet size distribution

Right after emulsification, 2 ml of the emulsion was diluted in 23 ml of a sodium dodecyl sulphate (SDS) solution (SDS 1%, Tris–HCl 50 mM, pH 8.0). This dilution ensures complete deflocculation of the oil droplets. The oil droplet size distribution was estimated by laser light scattering using a Malvern Mastersizer 3600 (Malvern Instruments, Malvern, UK). The system was equipped with a lens of 45 mm focal length and the manufacturer’s presentation code 0503 was selected to take into account the refractive index of the oil. The volume-surface mean diameter of the oil droplets size distribution (d32 in μm) and their specific surface area (m²/ml of oil) were recorded.

2.5. Determination of emulsion stability against creaming

We assessed the stability of the emulsions to creaming after accelerated ageing. Aliquots (13 ml) of the emulsions were poured into a 15 ml centrifugation tube and left to age for 24 h at ambient temperature (20–22°C). The tubes were then centrifuged at 2000 g for 30 min at 10°C. After centrifugation, we recorded both the volume of the creamed emulsion at the top and the volume of the translucent aqueous phase underneath. From these values, we calculated a stability index as follows:

\[ \text{Stability} = \frac{\text{creamed emulsion vol.}}{\text{creamed emulsion vol.} + \text{aqueous phase vol.}} \times 100 \]

2.6. Determination of zeta potential of the oil droplets

Zeta potentials were measured by a capillary electrophoresis technique coupled with laser light scattering using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). The oil droplets are placed in an electric field and the apparatus allows accurate determination of the drift velocity of these droplets from the Doppler shift of the light scattered by the moving droplets. The measures were conducted at a constant temperature of 25°C. The emulsions were diluted into buffer solutions corresponding to their continuous phase (same pH and NaCl concentration) to a final disperse phase fraction of approximately 5 × 10⁻⁶. The dielectric constant of all the solutions was assumed to be 79.

2.7. Determination of the interfacial concentration of protein and phospholipid

We adapted the method described by Patton and Huston (1986) to separate the oil droplets from the aqueous phase of the emulsions. The emulsions (2 ml) were diluted with an equal volume of a concentrated sucrose solution (500 mg/ml) without changing the pH and NaCl concentration. This dilution was then deposited carefully with a syringe at the bottom of a 15 ml centrifugation tube containing 10 ml of a buffer solution corresponding to the aqueous phase of the emulsion (same pH and NaCl concentration). The tubes were then centrifuged at 3000 g for 2 h at 10°C. After the centrifugation, three distinct phases could be observed in the tubes: (i) the creamed oil droplets at the top; (ii) the intermediate clear buffer solution; (iii) and the translucent yellow aqueous phase of the emulsion at the bottom. The tubes were frozen at −20°C and cut so as to separate the three phases. Both protein and phospholipids concentrations were determined in each of the three phases. Protein was assayed according to the modified
Lowry method described by Markwell et al. (1978) and phospholipids were quantified according to the method described by Bartlett (1959). Based on the amounts brought by the yolk products in the initial 2 ml of emulsions, we checked that we recovered at least 90% of the total protein and phospholipid. Protein and phospholipid of the creamed emulsion were attributed to the adsorbed constituents but there were also those in the buffer solution which were assumed to come from very small droplets unable to cream till the top of the tube. The concentrations of adsorbed protein and phospholipid were referred to the volume of oil before calculating their interfacial concentrations as follows:

\[
\text{interfacial concentration} = \frac{\text{concentration per volume of oil (mg/ml of oil)}}{\text{specific surface area (m}^2/\text{ml of oil)}}
\]

2.8. Protein composition of the interfacial film

The protein composition of the interfacial film in the emulsions was investigated by SDS-PAGE. The samples were first diluted (1:1 v/v) in a dissociation buffer consisting of a 0.5 M Tris–HCl pH 6.8, 0.05% bromophenol blue, 10% glycerol, 10% β-mercaptoethanol, 4% SDS solution, and heated in boiling water for 5 min. Electrophoreses were run on polyacrylamide gels (stacking: 3.5% and resolving: 9%) with a migration buffer consisting of a Tris–HCl 0.1 M pH 8.25, tricine 0.1 M, SDS 0.1% solution. The proteins were first stained with a Coomassie blue solution with added aluminium nitrate according to the method described by Ito, Abe and Adachi (1983) to stain acidic phosphoproteins (Coomassie blue 0.05%, ethanol 25%, acetic acid 10%, triton 1%, aluminium nitrate 0.1 M). The usual staining procedure was then applied (Coomassie blue 0.05%, ethanol 25%, acetic acid 10%) to get a complete staining of yolk proteins. The gels were destained in a solution containing acetic acid (7%), ethanol (40%) and water (53%). Molecular weights were calibrated with reference to the following calibration kit: phosphorylase b (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da) and α-lactalbumin (14,400 Da). The gels were scanned on an imaging densitometer Biorad GS710 and the \( R_i \) and percentage ratio of each band were determined with the Quantity one 4.1 software (Biorad, Ivry-sur-Seine, France).

2.9. Statistical analysis

Three replicates were made for emulsion stability, oil droplet size, protein solubility, zeta potential, interfacial concentration of protein and phospholipids. For these parameters, the results were subjected to a one-way analysis of variance using Statgraphics software (Statistical Graphics Corporation, Rockville, MD). Confidence intervals were set at 95% (\( p < 0.05 \)). The analysis of the profiles of the adsorbed proteins was done on a single electrophoresis.
3. Results

3.1. Determination of protein solubility

In plasma solutions, proteins were found to be completely soluble whatever the pH and NaCl concentration (Fig. 1). On the contrary, protein solubility in granules solutions exhibited important variations. Granules proteins were poorly soluble at pH 3.0 whatever the NaCl concentration (7.0% on average); they were only partially soluble at pH 7.0 and 0.15 M NaCl (29.6%) and complete solubilisation was achieved at pH 7.0 and 0.55 M NaCl. The protein solubility in yolk solutions was between those in plasma and granules, reflecting the relative contribution of each fraction to the total protein content of yolk, i.e. approximately 55% of yolk proteins recovered in plasma and 45% in granules.
3.2. Characterisation of emulsions properties

Fig. 2 shows the results of emulsion stability against creaming. In our experimental conditions (steady oil volume fraction of 0.30, initial protein concentration of 25 mg/ml in the aqueous phase and homogenisation at 200 bar), the stability of emulsions against creaming was influenced mainly by the pH of the aqueous phase. With plasma, emulsion stability was found to be maximal at pH 3.0 whatever the NaCl concentration (93% on average at pH 3.0 versus 66% at pH 7.0). The NaCl concentration had no direct effect on emulsion stability with plasma since the average stability at 0.15 or 0.55 M was the same. The higher NaCl concentration induced only partial levelling of the emulsion stability results, with slightly reduced stability at pH 3.0 (91% at 0.55 M NaCl versus 96% at 0.15 M NaCl) and moderate enhancement at pH 7.0 (69% at 0.55 M NaCl versus 64% at 0.15 M NaCl).

Emulsions made with granules exhibited opposite sensitivity to pH and NaCl concentration. The solution with pH 7.0 favoured maximum stability to creaming whatever the NaCl concentration (78% on average at pH 7.0 versus 42% at pH 3.0) and higher NaCl concentration resulted in better stability at pH 7.0 (87% at pH 7.0 and 0.55 M NaCl versus 70% at pH 7.0 and 0.15 M NaCl).

Emulsions made with plain egg yolk responded like those made with plasma to variations of pH and NaCl concentration, with a slightly lower beneficial effect of pH 3.0 (21% increase on average in emulsions prepared with egg yolk compared to 27% in emulsions prepared with plasma). Note that at pH 7.0, granules provided better emulsion stability than plasma. However, this was not recovered in plain egg yolk since the latter behaved like plasma.

The oil droplet size in the emulsions was evaluated by the volume-surface mean diameter \( d_{3.2} \) (Fig. 3). Emulsions made with plasma or yolk presented similar and constant \( d_{3.2} \) whatever the pH or NaCl concentration, ranging between 0.41 and 0.48 \( \mu \text{m} \). Emulsions made with granules at pH 7.0 were comparable to those made with plasma or yolk. However at pH 3.0, emulsions made with granules exhibited much larger oil droplets with \( d_{3.2} \) comprised between 1.68 and 1.72 \( \mu \text{m} \). The NaCl concentration had no influence on the oil droplet size.

The particular properties of emulsions prepared with granules could be closely related to their structure which was altered by pH and NaCl concentration. In the physico-chemical conditions occurring in native egg yolk (pH 6.0–6.5 and low ionic strength), granules constituents, namely HDL and phosvitin, are aggregated via calcium cation (\( \text{Ca}^{2+} \)) bridging between negatively charged phosphoserine residues of the proteins (Causert et al., 1991). These aggregates have diameters ranging from 0.3 to 2 \( \mu \text{m} \) (Burley & Cook, 1961; Saari, Powrie & Fennema, 1964). At pH 3.0, granules were extremely poorly soluble which indicated that they were kept in an aggregated state. At pH 7.0 and 0.15 M NaCl, they were partially solubilised due to limited disruption of phosphocalcic bridges: the monovalent cation \( \text{Na}^+ \) displaced the divalent cation \( \text{Ca}^{2+} \). At pH 7.0 and 0.55 M NaCl, the \( \text{Na}^+ \) cations in large excess could displace completely the \( \text{Ca}^{2+} \) cations, thus allowing complete solubilisation. As it has been previously reported by Aluko and Mine (1998) and Anton, Beaumal and Gandemer (2000), aggregated granules were less surface active than soluble...
granules constituents. The aggregates were particularly inefficient in covering the large interfacial area created during emulsification which resulted in a higher degree of re-coalescence, whereas soluble granular constituents allowed more interfacial area to be covered, thus limiting re-coalescence.

3.3. Characterisation of the interface

The interfacial films formed around oil droplets in the emulsions were characterised for their zeta potentials, for their phospholipid and protein concentrations and for the profile of the adsorbed proteins. Observation of zeta potentials (Fig. 4) showed that the interfacial films in emulsions prepared with yolk, plasma or granules at pH 3.0 and 0.15 M NaCl were quite singular. In these conditions they were found to support charges with rather large magnitudes (+20.8, +21.3 and +17.8 mV for yolk, plasma and granules, respectively). These values allowed surface potentials
which reached or approached the threshold surface potential of 20 mV evaluated by Friberg (1997) to produce effective electrostatic repulsion. In any other pH and NaCl concentration combinations, zeta potentials were quite low in magnitude, not exceeding 10 mV. Note that the interfaces changed from positive charges at pH 3.0 to slightly negative charges at pH 7.0. This is coherent with the variations of yolk proteins charges which can be predicted from their isoelectric points. Apo-LDL isoelectric region is between pH 6.5 and 7.3 (Kojima & Nakamura, 1985; Nakamura, Hayakawa & Sato, 1977), the isoelectric point of livetins varies between pH 4.3 and 5.5 (Ternes, 1989) and the isoelectric point of phosvitin is around pH 4.0 (Ternes, 1989). Apo-HDL have not been characterised precisely but their isoelectric region is supposed to be in an “enlarged” neutral pH region.

The interfacial phospholipid concentration in the emulsions prepared with yolk, plasma and granules varied with pH and NaCl concentration (Fig. 5). In emulsions prepared with plasma, phospholipid concentration at the interface varied with NaCl concentration but not with pH. At the lower NaCl concentration (0.15 M) phospholipids were less concentrated at the interface than at the higher NaCl concentration (0.55 M): 1.02 versus 1.41 mg/m², respectively. On the contrary, in emulsions prepared with granules, the phospholipid concentration was sensitive essentially to pH. At pH 3.0 phospholipid concentration was much higher than at pH 7.0 (1.47 mg/m² on average at pH 3.0 versus 0.55 mg/m² at pH 7.0). Phospholipid concentration was particularly very high in emulsions prepared with granules at pH 3.0 and 0.55 M NaCl (1.89 mg/m²). As was noticed in emulsions prepared with granules, interfacial phospholipid concentration in emulsions prepared with plain egg yolk varied principally with pH. The higher interfacial phospholipid concentration was observed at pH 3.0 (1.38 mg/m² which represents a 34% increase compared to pH 7.0).

Protein interfacial concentration \( (\Gamma) \) in the emulsions prepared with yolk, plasma and granules also varied with pH and NaCl concentration (Fig. 6). In emulsions made with plasma, the pH was found to influence \( \Gamma \) which was 37% higher at pH 3.0 (2.01 mg/m²) than at pH 7.0 (1.47 mg/m²). No difference was observed between the two NaCl concentrations. In emulsions made with granules, \( \Gamma \) was much larger than in emulsions prepared with plasma and it varied in much larger proportions: the lowest value of \( \Gamma \) was observed at pH 7.0 and 0.55 M NaCl (2.77 mg/m²) and a concentration more than three times as important was determined at pH 3.0 and 0.55 M NaCl. The values of \( \Gamma \) in emulsions made with granules at pH 3.0 were much higher than those usually recorded in the case of adsorption of soluble proteins; they resulted from the adsorption of aggregates as it was previously established by Aluko and Mine.

Table 1

<table>
<thead>
<tr>
<th>Molecular weight (kDa)</th>
<th>Fraction</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>175</td>
<td>Plasma</td>
<td>apo-LDL</td>
</tr>
<tr>
<td>137</td>
<td>Plasma</td>
<td>apo-LDL</td>
</tr>
<tr>
<td>105</td>
<td>Granules</td>
<td>apo-HDL</td>
</tr>
<tr>
<td>83</td>
<td>Plasma</td>
<td>( \alpha )-livetin</td>
</tr>
<tr>
<td>79</td>
<td>Granules</td>
<td>apo-HDL</td>
</tr>
<tr>
<td>60–70</td>
<td>Plasma</td>
<td>( \gamma )-Livetin and apo-LDL</td>
</tr>
<tr>
<td>53</td>
<td>Granules</td>
<td>apo-HDL</td>
</tr>
<tr>
<td>46</td>
<td>Granules</td>
<td>Phosvitin</td>
</tr>
<tr>
<td>38–40</td>
<td>Plasma</td>
<td>( \beta )-Livetin</td>
</tr>
<tr>
<td>32</td>
<td>Granules</td>
<td>apo-HDL</td>
</tr>
<tr>
<td>15</td>
<td>Plasma</td>
<td>apo-LDL</td>
</tr>
</tbody>
</table>

Fig. 7. SDS-PAGE profiles of soluble (s), unadsorbed (u) and adsorbed (a) proteins in emulsions prepared with egg yolk at different pH and NaCl concentrations: Sd, molecular weight standards; Y, native egg yolk; P, plasma; G, granules.
was confirmed by the more intensively revealed granular proteins at this pH confirmed that granules aggregates were displaced from the interface by soluble proteins. Putting this result together with the poor adsorption of granules proteins at pH 3.0. At this pH, we observed that the 15 kDa polypeptide, constitutive of apo-HDL, appeared in the profile of the soluble proteins at pH 3.0. Therefore, at pH 7.0, which allows sufficient granules protein solubilisation, plasma and granules seemed to contribute roughly to the same proportion in the interfacial film. Referring to the relative contribution of plasma and granules to yolk proteins (52 and 48%, respectively), granules tended to be slightly over-represented in the interfacial film.

4. Discussion
4.1. Emulsifying properties of yolk matched those of plasma

In emulsions prepared with egg yolk, we found that the oil droplet size was not affected by either pH or NaCl concentration of the aqueous phase, whereas the stability to creaming was. The comparison between yolk and fractions pointed out similarities between yolk and plasma, whereas granules exhibited distinct properties. Our results confirmed previous comparative studies (Anton & Gandemer, 1997; Dyer-Hurdon & Nnanna, 1993; Le Denmat, Anton & Gandemer, 1999). This brings evidence of the driving contribution of plasma constituents to yolk emulsifying properties.

In acidic conditions, plasma exhibited much better emulsifying properties than granules. At pH 3.0, indeed, emulsions made with granules exhibited larger oil droplet size and very low stability to creaming. As explained earlier, the native aggregated structure of the granules, which is maintained under these conditions, was responsible for their poor emulsifying properties. Thus, in acidic conditions yolk took up the emulsifying properties of its most surface active fraction. At neutral pH, emulsions made with granules exhibited the same droplet size as those made with plasma. Concurrently, they showed better stability to creaming, which confirmed previously reported results (Anton & Gandemer, 1997; Le Denmat et al., 1999). At this pH, the native aggregated structure of the granules was disrupted; the disruption was partial with low (0.15 M) and complete with high (0.55 M) NaCl concentration. Thus, soluble granules constituents isolated from egg yolk, and especially HDL, appear to be efficient emulsion stabilisers. But the stability of emulsions prepared with yolk still corresponded to that of plasma in these conditions. As a result, when soluble granules constituents compete with plasma constituents in plain egg yolk at pH 7.0, they still fail to impose their emulsion stabilisation properties to yolk emulsions.

<table>
<thead>
<tr>
<th>pH 7.0: 0.15 M NaCl</th>
<th>Adsorbed</th>
<th>Adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native egg yolk</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>pH 3.0: 0.15 M NaCl</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>pH 7.0: 0.55 M NaCl</td>
<td>53</td>
<td>51</td>
</tr>
</tbody>
</table>

(1998) and Anton et al. (2000). Emulsions prepared with plain egg yolk exhibited values of $I'$ in the range of those observed with plasma. As for plasma, $I'$ tended to increase at pH 3.0 but the increase was reduced to 11% and was not statistically significant ($p > 0.05$).

We used SDS-PAGE to compare the peptide profiles in the interfacial films and in the continuous phases of the emulsions made with yolk (Fig. 7). The bands were identified from their molecular weight, from the comparison with the profiles of reference samples of yolk, plasma and granules and by comparison with previous studies (Anton & Gandemer, 1999; Ito et al., 1983; Mine, 1998b). We distinguished 15 major bands in plain egg yolk that have been identified as described in Table 1. Densitometric analysis of the bands enabled us to estimate the relative proportion of plasma and granules proteins in the peptide profiles of adsorbed and unadsorbed protein material from emulsion prepared with egg yolk (Table 2).

We found that variations of pH brought about alterations in peptide profiles whereas the influence of NaCl concentration was negligible. At pH 3.0 and whatever the NaCl concentration, we found that plasma proteins represented a smaller proportion in the unadsorbed peptide profiles (25% on average) compared to their contribution in native egg yolk (52%). Concurrently, plasma proteins were over-represented in the adsorbed peptide profiles at this pH (62% on average). These results clearly indicated a preferential adsorption of plasma proteins at pH 3.0. At this pH, we observed that the 15 kDa polypeptide, constitutive of apo-LDL was the major peptide at the interface: it accounted for 33% of the peptides in the interfacial film at pH 3.0 and 0.15 M NaCl and 41% at pH 3.0 and 0.55 M NaCl. These percentages represented 2.1 and 2.6 times, respectively, the proportion in native egg yolk. Granules proteins did not appear in the profile of the soluble proteins at pH 3.0. Putting this result together with the poor adsorption of granules proteins at this pH confirmed that granules aggregates were displaced from the interface by soluble proteins.

At pH 7.0, granular proteins became more soluble as it was confirmed by the more intensively revealed granular peptides in the profiles of soluble proteins. Concurrently, the proportion of granules peptides among adsorbed peptides rose sharply. It reached 53% at pH 7.0 and 0.15 M NaCl and 51% at pH 7.0 and 0.55 M NaCl. At pH 7.0 the 105 kDa peptide coming from apo-HDL became the major peptide in the interfacial film. It made 26% of the adsorbed peptides whatever the NaCl concentration; this represented 1.2 times its proportion in native egg yolk. Therefore, at pH 7.0, which allows sufficient granules protein solubilisation, plasma and granules seemed to contribute roughly to the same proportion in the interfacial film. Referring to the relative contribution of plasma and granules to yolk proteins (52 and 48%, respectively), granules tended to be slightly over-represented in the interfacial film.
4.2. The interfacial film, and especially adsorbed apo-LDL, had a major contribution to the measured stability to creaming in emulsions made with yolk

Since there was no significant difference of droplet size in emulsions made with yolk whatever the pH or NaCl concentration, droplet size could not explain the differences in the stability to creaming that we measured. Moreover, the emulsions were prepared from low-viscosity Newtonian solutions so that the viscosity of the aqueous phase could not limit creaming. We assessed flocculation in emulsions made with yolk by measuring the droplet size without dilution in SDS (data not shown). We found that the emulsions which creamed more (pH 7.0) were those which were significantly flocculated. Flocculation is likely to enhance creaming velocity in static conditions of storage. However, in our measurement conditions which involved centrifugation, we do not think that the differences in stability to creaming could be due to this effect on creaming velocity. Indeed, the oil droplets were forced to cream in any case due to centrifugation. The final volume of the creamed phase was more likely a consequence of the ability of the droplets to oppose packing. The less tight the droplets were packed, the higher the volume of the creamed phase and consequently the higher the stability to creaming that we measured. Thus, the interfacial film around oil droplets is of major importance, insofar as it regulates colloidal interactions between droplets.

We found that maximum stability to creaming in emulsions made with yolk was achieved when the interfacial concentrations of both emulsifier types, proteins and phospholipids, were maximum. On a first approach, this suggested that the contributions of proteins and phospholipids could not be distinguished. However, assuming that plasma constituted the governing emulsifying fraction of yolk, observing it separately brings about a simplified view of the effective functional constituents of yolk, deprived of the ineffective contaminating granules constituents. With plasma, creaming variations were consistent with interfacial protein concentrations variations since they both fluctuated with pH. On the contrary, interfacial phospholipid concentrations varied with NaCl concentration exclusively and did not correlate, therefore, with emulsion creaming. Maximum stability against creaming was achieved when the interfacial protein concentration was highest. These observations ascertained the driving contribution of plasma proteins to the control of the creaming rate in emulsions prepared with plasma. Therefore we assume that they still play a major role in emulsions prepared with plain yolk. The high phospholipid concentration at the interface in yolk emulsions at acidic pH may result from co-adsorption of proteins and phospholipids within undisrupted granules.

The SDS-PAGE profiles of adsorbed proteins confirmed that plasma proteins were present at the interface in important proportion at any pH and NaCl concentration. At pH 3.0, they adsorbed preferentially over granules proteins so that their governing influence is easily conceivable. However at pH 7.0, granules proteins adsorbed efficiently and got even slightly quantitatively dominant at the interface.

This confirms the observation of Mine (1998b) and it may be regarded as an apparent contradiction that plasma constituents impose their properties whereas granular proteins, namely apo-HDL predominated at the interface at pH 7.0. Considerations about the structure of proteins at the interface may provide an explanation. Depending on their flexible or globular structure, proteins can spread at the interface or, conversely, keep a compact structure which does not allow coverage of an extended interface area (Hunt & Dalgleish, 1994). As a result, in mixed protein films covering oil droplets, a flexible protein which does not predominate quantitatively can all the same cover a majority of the interface area and therefore control the colloidal interactions.

This hypothesis may provide promising investigation perspectives since, even though yolk lipoproteins have not been precisely characterised yet, apo-LDL are thought to adopt flexible structure at the interface as was argued by Shenton (1979) whereas apo-HDL are considered to be globular-like proteins (Cook & Martin, 1969).

4.3. Steric repulsion must be the dominant colloidal interaction controlling droplet aggregation

Adsorbed plasma proteins, namely apo-LDL, take part in interaction forces between droplets; they especially provide repulsive forces which can be electrostatic or steric in nature (McClements, 1999). We stated that at pH 3.0 and 0.55 M NaCl and at pH 7.0 whatever the NaCl concentration, the interfaces did not support charges important enough to produce electrostatic repulsion. Therefore, in these conditions, the only repulsive contribution to the interaction potential between oil droplets is steric repulsion. This is generally the case in food emulsions stabilised by biopolymers, namely proteins, as has been stated by Bergenstahl and Claesson (1997). For a given polymer, the strength of steric repulsion that it imposes when it adsorbs at the interface depends, partially, on the amount of polymer adsorbed (Hunter, 1986; McClements, 1999). Therefore the more pronounced steric repulsions at pH 3.0 were consistent with the higher interfacial protein concentration. At pH 3.0 and 0.15 M NaCl, an additional electrostatic repulsion occurred. This could explain the slightly higher stability to creaming compared to pH 3.0 and 0.55 M NaCl.

5. Conclusions

In this study, we have shown evidence of the major contribution of plasma proteins to yolk emulsifying properties. We presumed that adsorbed apo-LDL, especially a dominant peptide of 15 kDa, control the colloidal
interactions between oil droplets and regulate packing of the creamed oil droplets. Steric repulsion is the main repulsive interaction. However, further investigations of the structure of the interface are required to clarify this issue. This emphasises the importance of developing purification of yolk proteins to allow precise characterisation of their interfacial properties.

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