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Changes in secondary structure of gluten proteins due to emulsifiers

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HIGHLIGHTS

- Changes in the secondary structure of gluten proteins were analyzed.
- Emulsifiers additions were analyzed by Raman Spectroscopy.
- Protein folding was induced by 0.25% of Sodium Stearoyl Lactylate (SSL).
- High levels of SSL and Diacetyl Tartaric Acid Esters of Monoglycerides (DATEMs) led to more disordered protein structures.

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ABSTRACT

Changes in the secondary structure of gluten proteins due to emulsifiers were analyzed by Raman Spectroscopy. The protein folding induced by 0.25% SSL (Sodium Stearoyl Lactylate) (GS0.25, Gluten + 0.25% SSL) included an increase in α-helix conformation and a decrease in β-sheet, turns and random coil. The same behavior, although in a less degree, was observed for 0.5% gluten–DATEM (Diacetyl Tartaric Acid Esters of Monoglycerides) system. The low burial of Tryptophan residues to a more hydrophobic environment and the low percentage area of the C–H stretching band for GS0.25 (Gluten + 0.25% SSL), could be related to the increased in α-helix conformation. This behavior was also confirmed by changes in stretching vibrational modes of disulfide bridges (S–S) and the low exposure of Tyrosine residues. High levels of SSL (0.5% and 1.0%) and DATEM (1.0%) led to more disordered protein structures, with different gluten networks. SSL (1.0%) formed a more disordered and opened gluten matrix than DATEM, the last one being laminar and homogeneous.

1. Introduction

Improvements in wheat flours quality, and the requirement of maintaining good organoleptic and textural characteristics of bread, resulting in an adequate acceptance by consumers, promote the utilization of additives for improving breadmaking quality. Emulsifiers have been employed as anti-staling agents, dough modifiers, shortening sparing agents, and as improvers for the production of common and high-protein breads. Sodium Stearoyl Lactylate (SSL) and Diacetyl Tartaric Acid Esters of Monoglycerides (DATEMs) are common emulsifiers used in breadmaking (Fig. 1). Due to their high hydrophilic/lipophilic balance these additives produce strong doughs, in which the lipophilic tail of the molecule would be bound to hydrophobic sites of gluten proteins. SSL is a surfactant known to improve volume and softness in fresh and frozen dough products [1,2]. This additive promotes emulsifying and air incorporation into dough [3]. In breadmaking, SSL improves crumb and crust texture, diminishes water loss, and decreases starch recrystallization percentage in bread [4], thus extending the shelf life of the product [5,6]. In dough pieces frozen up to 8 weeks, SSL and DATEM incorporation produced dough with higher resistance to extension and bread with significantly higher loaf volume than bread without emulsifier [7].

DATEM are anionic oil-in-water emulsifiers that are used as dough strengtheners to improve bread quality [8]. They improve mixing tolerance, gas retention, and resistance of dough to collapse. In bread, these emulsifiers increase loaf volume [9] and generate a crumb with a resilient texture, fine grain, and good slicing properties [10]. DATEM may promote the aggregation of gluten proteins in dough by binding to the protein hydrophobic surface and, through hydrogen bonds, with glutamine [11]. These interactions generate a strong protein network, which in turn will produce bread with a better texture and increased volume [12]. Hydrophilic emulsifiers may also form lamellar liquid–crystalline phases in water, which associate with gliadins. The formation of

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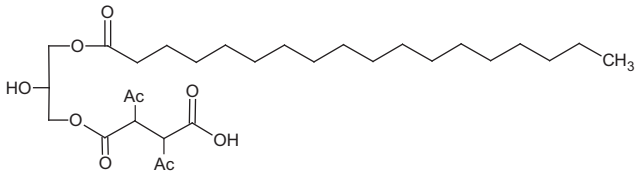
DATEM:



SSL:

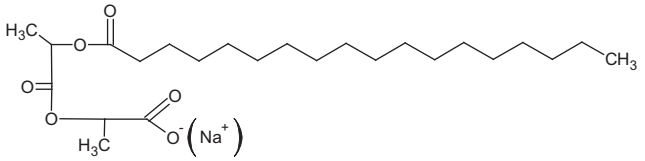


Fig. 1. Structures of the Sodium Stearoyl Lactylate (SSL) and Diacetyl Tartaric Acid Esters of Monoglycerides (DATEMs).

such structures allows the expansion of gas cells and contributes to dough elasticity, resulting in increased bread volume [9].

Spectroscopy techniques are adequate for studying secondary and tertiary protein structure. Modifications in protein structure due to several factors (temperature, pressure, pH) as well as the interaction of certain type of molecules like emulsifiers with proteins can be analyzed [13,14]. As FT-Raman spectra of polypeptides and proteins exhibit characteristic bands, they are utilized for monitoring structural changes in food proteins. Peptide bond has nine bands, being the most important Amide I and Amide III bands. In addition, changes due to protein treatment, the conformation around the disulfide bonds, the involvement of phenol groups and Tyrosine residues in hydrogen bonding, and the degree of exposure of aromatic residues, are all reflected in Raman bands. Analysis of Amide I and Amide III bands, as well as skeletal stretching modes related to the polypeptide backbone, can be used to estimate the secondary structure of proteins [15,16] studied changes in gluten structure due to chemical amidation of proteins and found that this treatment led to transitions from ordered to random coil structures.

There is quite information about the improving effect of SSL and DATEM emulsifiers on bread quality, but few knowledge about structural changes of gluten proteins produced by these emulsifiers was reported. In a brief earlier communication [17] the analysis of the intensity of the Amide I band and some side chain bands of the gluten protein modified by the presence of the SSL emulsifier, was presented. In this paper, we present a more thorough investigation that includes conformational studies of proteins together with studies of the changes in the protein secondary structure including deconvolution and fitting process of the Amide I band to calculate the percentage contribution of the different types of conformations to the area of all the components. It complements the previously published information, including the analysis of the conformational changes produced for another common emulsifier used in breadmaking, DATEM and an exhaustive comparison of the changes the structure of the protein produced by both additives.

2. Materials and methods

2.1. Preparation of gluten samples

Wheat flour (000 type flour) for breadmaking (*Triticum aestivum* L.) was provided by an Argentinean mill (Molinos Campodónico Ltd., Argentina). Sodium Stearoyl Lactylate (SSL) and Diacetyl Tartaric Acid Esters of Monoglycerides (DATEMs) were obtained from DANISCO (Copenhagen, Denmark) both contain calcium carbonate as anti humectants.

Gluten samples were prepared from wheat flour (control sample) or a blend composed by flour and an emulsifier: SSL, DATEM or SSL + DATEM (1:1); at levels of 0.25%, 0.5% and 1.0% (flour basis). Emulsifier levels higher than 1.0% did not allow gluten formation. Samples were codified as: G for native gluten (without emulsifier), GS for gluten–SSL, GD for gluten–DATEM and GSD for gluten–SSL + DATEM. Levels of emulsifiers were written after the codifying letters. The numbers after the letters indicate the concentrations of emulsifiers utilized for dough preparation, before obtaining gluten throughout dough washing process. Wheat dough and gluten samples were prepared in the Glutomatic equipment [18]. Samples were prepared as described previously [17]. Gluten samples, prepared in duplicate, were freeze-dried, milled, and stored at 4 °C until analysis.

2.2. Secondary structure of gluten proteins

Parameters related to secondary and tertiary structure of gluten proteins were determined by FT-Raman Spectroscopy according to [17]. Raman spectra of dried gluten samples were collected on a Bruker IFS 113 FT-IR spectrophotometer provided with the NIR Raman attachment equipped with an Nd:YAG laser (1064 nm). Frequency calibration of the instrument was performed using the sulfur line at 217 cm^{-1}. Spectra were recorded at 25 °C with a laser power of 500 mW, and a spectral resolution of 6 cm^{-1}. Each spectrum was obtained after collecting and averaging 1000 scans in order to obtain spectra with high signal-to-noise ratios. FT-Raman spectra were plotted as intensity (arbitrary units) against Raman shift in wavenumber units (cm^{-1}). All spectra were vector normalized to the intensity of the phenylalanine band at 1004 cm^{-1}. In Amide I region, a straight baseline passing through the ordinates at 1700 and 1600 cm^{-1} was adjusted in order to calculate this band intensity. To calculate the secondary structure components, this region was truncated and fitted. The resulting fitted curve was analyzed taking into account the band assignment for the secondary structure previously reported in the literature [19–21]. The assigned structures were β-antiparallel: 1675–1695 cm^{-1}, turns: 1666–1673 cm^{-1}, α-helix: 1650–1658 cm^{-1}, random coil: 1637–1645 cm^{-1}, solvated helix: 1625–1637 cm^{-1}, β-sheet: 1613–1625 cm^{-1}. In order to calculate the percentage contribution of the different types of conformations to the area of all the components, bands assigned to a given conformation were summed and divided by the total Amide I area. The obtained number was taken as the proportion of the polypeptide chain in the corresponding conformation. The fitting procedure of the CH stretching band was performed by passing straight baseline through the ordinates at 3090 and 2800 cm^{-1}. Band assignment of the major vibrational motions of the side chains or the peptide backbone was based on comparison to Raman data reported in the literature [15–19]. In both cases the following procedure was applied: the frequencies, the number of peaks to be fitted, and the half-width of each peak to start a least square iterative curve-fitting procedures were those obtained from the second derivative of the original spectra. The areas of the bands were calculated by integration of the corresponding fitted band. The curve-fitting procedure was performed by stepwise iterative adjustment towards a minimum root mean-square error of the different parameters determining the shape and position of the Raman bands. It was carried out by assuming an initial mixed Lorentzian–Gaussian line-shape function, with full width band at half-height (FWHH) of 10–13 cm^{-1} and a maximum resolution factor. The intensity values obtained for the Tyrosine doublet were calculated relative to the local baseline of each peak

(830 and 850 cm^{-1}). Plotting, processing, normalizations and evaluation of spectra were carried out through Grams/32 (Galactic Industries Corporation, USA) software and OPUS software (Bruker Optics, Germany). All analyses were performed on three independent experiments, and the results were reported as averages of these replicates. Before spectra were analyzed the raw data were subjected to a quality test using Opus software. Spectral quality was ensured by taking into account the signal-to-noise ratios (rs1 and rs2). The signals were taken as peak heights of the band S1 and S2 at the 3000–2800 cm^{-1} and 1700–1600 cm^{-1} regions, respectively; and the noise was determined between 2500 and 2300 cm^{-1} as peak-to-peak. Signal-to-noise ratios were calculated by the quotient between the corresponding signals and noise values.

2.3. Free sulfhydryl groups of gluten proteins

Free sulfhydryl groups of gluten samples were determined using the method developed by Beveridge and coworkers [22]. Dried gluten samples were suspended (1 mg/mL) in 0.086 mol/L Tris–HCl – 0.09 mol/L glycine – 4 mmol/L EDTA – 8 mol/L urea – pH 8 buffer. Dispersions were stirred at 25 °C during 10 min at 500 rpm in a thermomixer and then centrifuged at 13,600*g* (10 min, 25 °C) (Eppendorf, Hamburg, Germany). Supernatant was incubated with Ellman's reagent (4 mg DTNB/mL methanol). Absorbance at 412 nm was measured in a Heλios β Thermo Spectronic spectrophotometer (Cambridge, UK). The molar extinction coefficient of NTB (13,600 L mol^{-1} cm^{-1}) was used. Assays were performed in duplicate. Protein concentration of extracts was determined by the Bradford method [23].

2.4. Microstructure of gluten samples

The microstructure of gluten samples (G, GS1 and GD1) was analyzed by scanning electron microscopy (SEM). Gluten samples were immersed in 2.5% glutaraldehyde and then washed with 0.5 mol/L phosphate buffer before starting the dehydration procedure. Samples were dehydrated in a graded acetone series: 25%, 50%, and 75% and three times with 100%. In order to preserve gluten structure during the electron incidence, samples were totally dried using CO_2 fluid at its critical point. Dehydrated samples were coated with gold particles in a sputter coater (Pelco, Redding, California, USA). Images were taken in a JOEL JSM 35 CF scanning electron microscope (Tokyo, Japan) with a 5 kV acceleration voltage.

2.5. Statistical analysis

Results were subjected to a one-way analysis of variance according to the general linear model procedure with least-square means effects. A multiple range test was applied to determine which means were significantly different ($p < 0.05$) according to Fisher's Least Significant Differences (LSDs). Statistical analysis was carried out using Statgraphics Plus 5.1 (Statpoint Technologies, Inc., Virginia, USA).

3. Results and discussion

3.1. Secondary structure of gluten proteins

Conformational changes produced on gluten due to protein interaction with SSL, DATEM and SSL + DATEM were analyzed by FT-Raman spectroscopy. Values of normalized band intensity of Amide I are shown in Fig. 2. According to previous studies [17], in the gluten–SSL systems (GS) the intensity of the Amide I band increased with the incorporation of SSL to gluten with a maximum

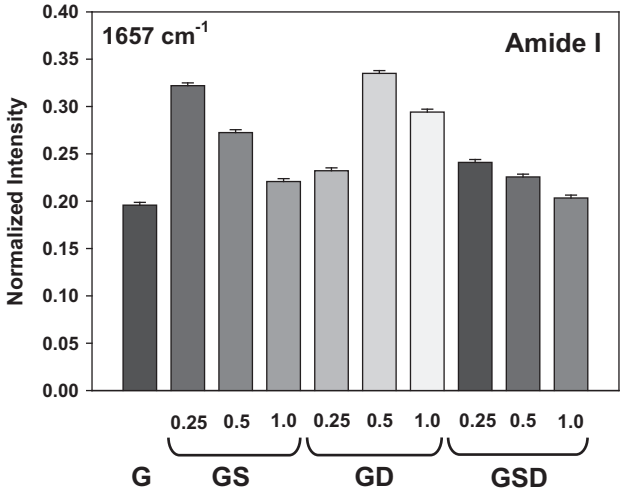


Fig. 2. Normalized intensity of Amide I band (c.a. 1657 cm^{-1}). Native gluten (G), gluten–SSL (GS), gluten–DATEM (GD), gluten–SSL + DATEM (GSD). Emulsifier concentrations: 0.0, 0.25, 0.5, 1.0%. Data belong to native gluten and SSL–gluten samples, was obtained from Ref. [17]. Normalized intensity values correspond to the average three independent experiments. Vertical bars represent confidence intervals at $p < 0.05$.

corresponding to 0.25%. This increment of intensity in Amide I band was related to an increased protein folding [19]. As in GS0.25 sample, the 0.5% gluten–DATEM (GD) system showed the most ordered gluten structure (Fig. 2). The increase in Amide I band intensity of gluten–SSL + DATEM samples (GSD) was not so pronounced as in systems containing only one additive (Fig. 2). In GSD samples would be occur a steric hindrance due to the different chemical structure of both emulsifiers. This difference could minimize the interaction between this additives and gluten proteins, leading to the formation of a more disordered network.

In order to obtain a deeper insight into gluten modifications, a quantitative analysis of the corresponding secondary structure in the Amide I region was performed [19]. The percentages of the different secondary structure conformations, obtained by curve fitting of the Amide I band, are shown in Table 1. Fig. 3 shows the fitting of Amide I band corresponding to the native gluten sample (Fig. 3 A) and the systems that presented the most significant changes in gluten matrix (0.25% SSL and 0.5% DATEM) (Fig. 3B and C). The main band, located at 1657 cm^{-1}, belongs to Amide I. As can be seen, emulsifiers increased the α-helix content of gluten proteins. The addition of 0.25% of SSL (GS0.25) enhanced the α-helix structure to the detriment of turns and β-sheets structures [17]. The quantitative analysis performed in this work corroborated that SSL increased protein order throughout the increment of α-helix structure (68.53%) comparing to native gluten (40.09%) (Table 1). Results obtained by calculations of the intensity of the Amide I band for SSL [17] were confirmed with data from the percentage contributions of each component of this band. Samples with a higher concentration of SSL (0.5% and 1.0%) showed a lower proportion of α-helix conformation (Table 1), suggesting that proteins had a more disordered structure than GS0.25. Compared with SSL, DATEM also enhanced protein ordering with an increase in α-helix conformation and a decrease in turn contents (Table 1), mainly at 0.5 and 1% levels. A decrease in β-sheet and an increase in β-antiparallel Amide I components of the secondary protein structure were also detected for GD gluten samples (Table 1). The percent area of the 1630 cm^{-1} band (solvated helix) was associated to short-segment chains interconnecting α-helix segment [24]. This percent area showed a slight increase, being consistent with the increment of α-helix proportion and protein folding, resulting in a more ordered protein structure.

Table 1
Raman determination of secondary structure percentages of native gluten (G), SSL–gluten (GS) and DATEM–gluten samples (GD). Emulsifier concentrations: 0.0%, 0.25%, 0.5%, 1.0%.

	β-Antiparallel	Turns	α-Helix	Random coil	Solvated helix	β-Sheet
G	2.59	23.48	40.09	7.24	9.10	17.42
GS0.25	8.65	6.82	68.53	nd	9.42	6.56
GS0.5	16.72	9.34	55.97	nd	3.83	14.35
GS1	15.79	9.23	53.76	nd	5.43	15.69
GD0.25	21.19	18.10	42.99	nd	10.56	7.13
GD0.5	11.37	11.65	54.98	nd	11.99	10.0
GD1	10.37	11.64	53.13	nd	11.86	13.0

nd: Not detected.
Frequency ranges. β-Antiparallel: 1675–1695 cm^{-1}, turns: 1666–1673 cm^{-1}, α-helix: 1650–1658 cm^{-1}, random coil: 1637–1645 cm^{-1}, solvated helix: 1625–1637 cm^{-1}, β-sheet: 1613–1625 cm^{-1}.

It was not possible to perform a good fit of the Raman spectra for the gluten–SSL + DATEM (GSD) systems. However, in all spectra with higher α-helix contribution, the band attributable to the skeletal C–C vibrations of this conformation (c.a. 933 cm^{-1}) increased in intensity (data not shown).

The Tyrosine (Tyr) ring vibrations are located at 850 and 830 cm^{-1}, and the ratio between the intensities of these bands ($I_{850/830}$) is related to the presence of "exposed" and "buried" Tyr residues, so that $I_{850/830}$ increases with the number of exposed Tyrosine residues [19]. It has been also stated that the doublet arises from Fermi resonance and that $I_{850/830}$ is a good indicator of the nature of hydrogen bonding and of the ionization state of the phenolic hydroxyl group.

According to the results outlined previously [17], a low content of SSL (0.25%) produced a decrease in the $I_{850/830}$ ratio (Fig. 4), indicating the burial of Tyr groups and confirming the protein folding observed with secondary structure results. On the other hand, the 1.0% SSL concentration produced an increase of the $I_{850/830}$ ratio, in comparison to native gluten (G) (Fig. 4). The higher value of intensity ratio (>1.5) observed in GS1 sample (Fig. 4) was well correlated with the $-O^-$ form of the phenol group of Tyrosine [21]. This unusually high intensity ratio may be caused by SSL–protein interactions that allowed Tyr residue to act as a positive charge acceptor, favoring charge repulsion between protein molecules.

All gluten–DATEM samples showed lower $I_{850/830}$ values than native gluten (Fig. 4). No significant differences were found between DATEM levels, indicating a protein folding with no exposed Tyr groups in opposition that occurred with SSL addition. Gluten prepared with SSL + DATEM blends (GSD) presented a behavior similar to that of GS samples. In addition, GSD1 showed the highest $I_{850/830}$ ratio (Fig. 4), suggesting that DATEM interaction would potentiate the Tyr exposition produced by SSL on gluten protein.

Modifications in the characteristic 760 cm^{-1} Tryptophan (Trp) band were also related to conformational changes in proteins. The intensity of the Trp bands has been related to the hydrophobicity of the microenvironment of the indole ring [25], so that high values indicate that Tryptophan is buried. In general, the addition of the selected emulsifiers caused an increase in the intensity of this band (Fig. 5), suggesting that Trp residues changed from a middle-exposed (gluten sample) to a buried (emulsifier–gluten sample) hydrophobic microenvironment, being GD0.5 the sample with the higher intensity value (Fig. 5).

Some side chains bands also provide information about the local side chain conformations or environments. In particular, a direct analysis of the stretching vibrational bands in the 450–550 cm^{-1} region (disulfide S–S region) provides information concerning to disulfide bridges. Three vibrational modes have been associated to the disulfide bridge: the gauche–gauche–gauche (g–g–g), the gauche–gauche–trans (g–g–t) and the trans–gauche–trans (t–g–t) conformations [19]. Table 2 shows the frequencies belonging to stretching vibrational bands of disulfide bridges. In the gluten sample (G) the bands associated to these vibrational modes were located at 534 (t–g–t) and 503 cm^{-1} (g–g–g). The g–g–g band was the most intense one (data not shown) that corresponds to the more stable conformation. As reported recently [17], the addition of 0.25% of SSL (GS0.25) caused the appearance of three bands, the first one (533 cm^{-1}) assigned to the t–g–t conformation and the others (514 and 497 cm^{-1}) corresponding to g–g–g conformation. As has been suggested, bands associated to g–g–g conformation would be related to two types of S–S configuration: intrachain (lower frequency value, longer S–S bond) and interchain (higher frequency value, shorter S–S bond) disulfide bonds [26] (Table 2). Concentration of SSL of 0.5% (GS0.5) also produced the splitting of the band associated to g–g–g conformation. When SSL content was higher than 0.25% (GS0.5 and GS1), new bands appeared in the g–g–t region, suggesting changes in the local disulfide bonding conformation (Table 2). The appearance of these bands and the splitting of the band corresponding to g–g–g conformation suggest a major protein folding phenomenon. Compared with SSL samples, in all gluten–DATEM samples (GD) the three conformations were present, including the split bands in the g–g–g region. For the gluten–SSL + DATEM samples (GSD) the g–g–g and t–g–t conformations were present. In GSD1 (0.5% of each emulsifier) a band related to the g–g–t conformation was also observed (Table 2). The presence of SSL in the blend enhances protein order.

Relevant percentages of Raman shifts of amino acids related to bands assigned to proteins in the CH-stretching region are shown in Table 3. The CH-stretching vibrational region (2800–3100 cm^{-1}) is generally related to hydrophobic groups. The main changes were associated to the bands related to the Tyrosine moiety (2934–2945 cm^{-1}) and also to other amino acids, mainly those containing OH groups; such changes were specially observed in GS samples. Previous results indicated that for GS0.25, the band located at 2935 cm^{-1} split into two components placed at 2939 and 2950 cm^{-1} [17]. Moreover, gluten samples with higher SSL content (GS0.5 and GS1) presented the same behavior. In addition, the total percent area (2935 cm^{-1} band) of GS0.25 sample was the lowest of the three GS samples and this can be related to a more folded protein structure. These results follow the same trend than $I_{850/830}$ values observed in Fig. 4 [15]. In gluten–DATEM systems only the GD1 sample presented a split in the 2935 cm^{-1} band, with an almost equal contribution of percent shift (9.8 and 8.1) (Table 3). There was no band splitting in gluten–SSL + DATEM samples (GSD) and the main percent contribution to area was for 0.25% SSL + 0.25% gluten–DATEM sample (GSD0.5). The main protein band near 2940 cm^{-1} (2934–2945 cm^{-1} region) was tentatively assigned to the aromatic and aliphatic amino acids, some charged amino acids and also Proline, Threonine, and Histidine. The presence of two contributions in this region could be attributed to some type of interaction between amino acids and the emulsifiers.

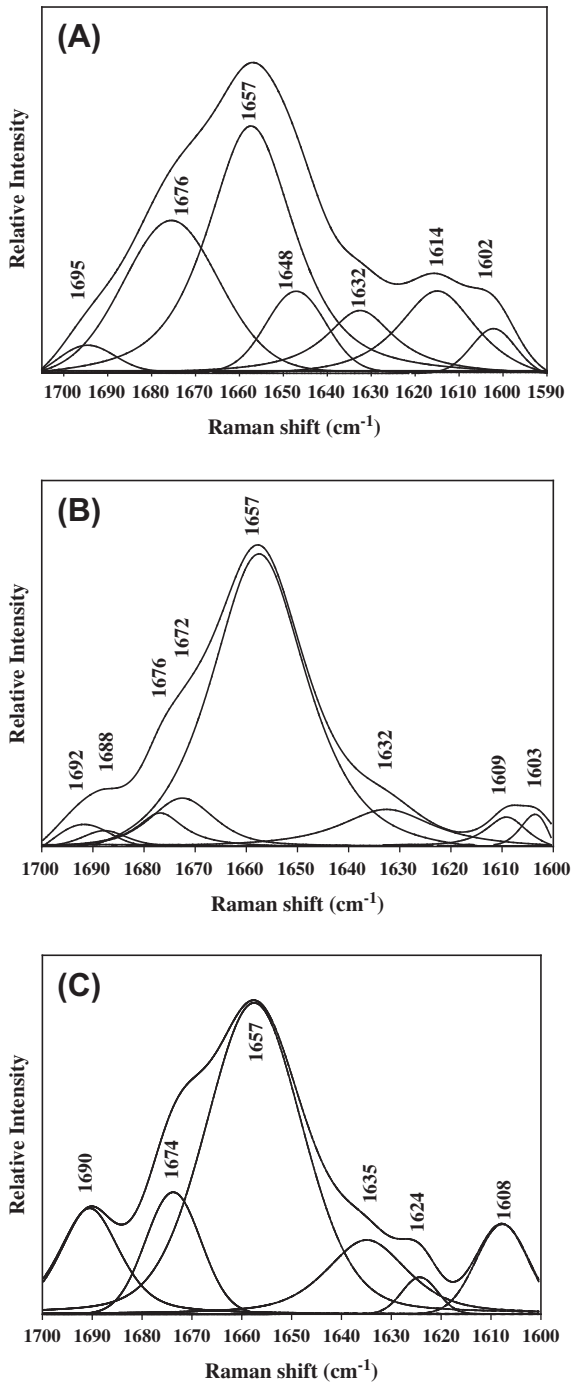


Fig. 3. (A) Amide I band of native gluten (G), (B) Amide I band of 0.25% gluten–SSL system (GS), (C) Amide I band of 0.5% gluten–DATEM system (GD). The secondary structure determination by curve-fitting procedure is indicated.

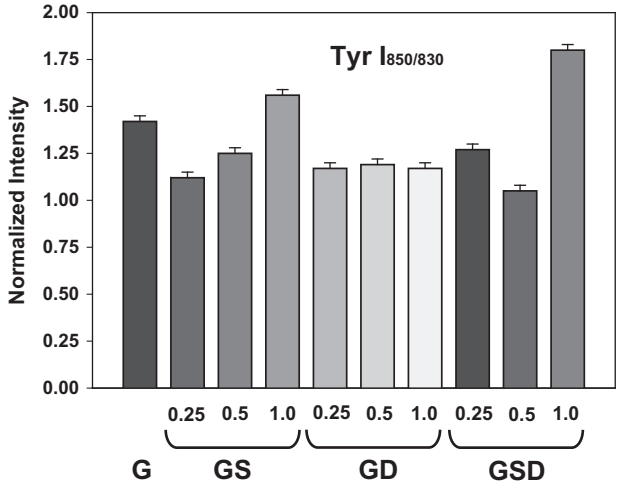


Fig. 4. Intensity ratios $I_{850/1830}$ of Tyrosine bands of different gluten samples. Native gluten (G), gluten–SSL (GS), gluten–DATEM (GD), gluten–SSL + DATEM (GSD). Emulsifier concentrations: 0.0%, 0.25%, 0.5%, 1.0%. Data belong to native gluten and gluten–SSL samples were obtained from Ref. [17]. Normalized intensity values correspond to the average three independent experiments. Vertical bars represent confidence intervals at $p < 0.05$.

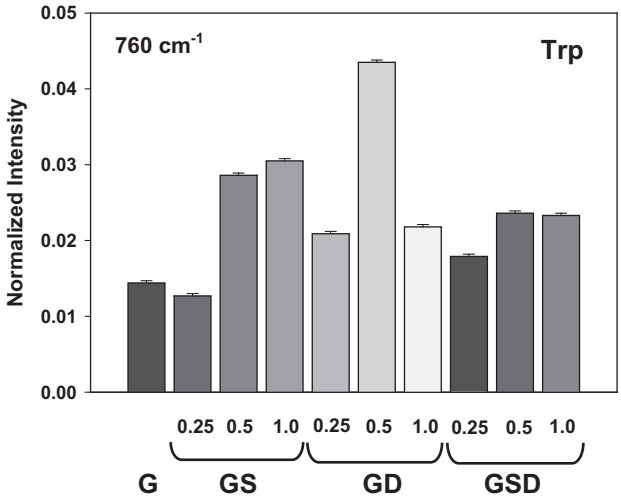


Fig. 5. Normalized Intensity of Tryptophan band (c.a. 760 cm^{-1}). Native gluten (G), gluten–SSL (GS), gluten–DATEM (GD), gluten–SSL + DATEM (GSD). Emulsifier concentrations: 0.0%, 0.25%, 0.5%, 1.0%. Data belong to native gluten and gluten–SSL samples were obtained from Ref. [17]. Normalized intensity values correspond to the average three independent experiments. Vertical bars represent confidence intervals at $p < 0.05$.

The decrease in doublet intensity associated to Tyr in GD samples was independent of emulsifier concentration (Fig. 4). This behavior, together with the absence of splitting of bands in the 2934–2945 cm^{-1} region observed in GD0.25 and GD0.5 (Table 3), would be related to the fact that the amino acids that contribute to these bands are not participating in protein–DATEM interaction.

Noticeable differences were also observed in the bands in the range of 2889–2897 cm^{-1}, which predominantly showed aliphatic amino acid groups involvement. For SSL–gluten samples there was a tendency to maintain the splitting, inverting its percent contribution as compared to G. Only one contribution appeared for the other samples (GD and GSD), being the exception GD0.5 (the GD with higher α-helix content) which did not present contribution to this region. This behavior would be related to the way in which charged amino acids contribute to protein structure changes.

SSL is an ionic molecule formed by the ester union between stearic acid and sodium lactilate, whereas DATEM has a glycerol backbone with a stearic acid attached to C_1 and a diacetil tartaric acid to C_3. Nevertheless, SSL is considered a less hydrophobic emulsifier than DATEM, deduced from their hydrophilic/lipophilic balance (HLB). Armero and Collar [2] reported HLB values of 21 and 9.2 for SSL and DATEM, respectively. Armero and Collar [2], Indrani and Rao [27] and Köler [28] proposed HLB value for DATEM of 9.2. The polar hydrophilic chain (sodium lactilate) of SSL interacts with gluten proteins through ionic bonds, producing gluten agglomeration and the strengthening of dough structure during baking [29].

Table 2
Raman S–S stretching vibrations in the 470–550 cm^{-1} region. Native gluten (G), SSL–gluten (GS), DATEM–gluten (GD) and SSL + DATEM–gluten (GSD). Emulsifier concentrations: 0.0%, 0.25%, 0.5%, 1.0%.

	t–g–t (535–545 cm^{-1})	g–g–t (515–525 cm^{-1})	g–g–g (470–515 cm^{-1})
G*	534		503
GS0.25*	533		514, 497
GS0.5	543	526	515, 501
GS1	540	527, 520	513
GD0.25	540	526	508, 498
GD0.5	534	525	514, 500
GD1	542	523	514, 500
GSD0.25	541		513, 500
GSD0.5	537		513, 500
GSD1	538	522	509, 501

* Data belong to G and GS0.25 was obtained from Ref. [17].

Table 3
Relevant percentages for Raman shifts of amino acids components related to the bands associated with proteins in the CH-stretching region. Native gluten (G), SSL–gluten (GS), DATEM–gluten (GD) and SSL + DATEM–gluten (GSD). Emulsifier concentrations: 0.0%, 0.25%, 0.5%, 1.0%.

Amino acid bands	G	GS0.25	GS0.5	GS1	GD0.25	GD0.5	GD1	GSD0.25	GSD0.5	GSD1
2874–2879	10.6	4.9	9.3	18.7	6.5	22.8	3.7	10.4	2.6	11.1
His, Leu, Lys, Ile, Val			7.4		5.9				7.8	
2889–2897	1.4	4.5	8.9	4.3	6.8		10.1	13.7	5.5	1.6
Thr, Pro	12.8	7.3		3.8						
2934–2945	37.0	2.8	15.3	8.7	18	25.8	9.8	20.4	29.7	14.2
Met, Trp, Arg, Cys, Leu, Lys, Ile, Phe, Tyr, Glu, Thr, Val, Pro, His, Asp, Asn		17.5	11.7	23.6			8.1			

Although DATEM is also a polar molecule (diacetil tartaric acid residue) it works cooperatively with gluten proteins and flour lipids at the air/water interface, improving gas-holding ability of dough. These interactions may be established mainly through hydrophobic bonds mediated by the hydrophobic part of the molecule [30].

Spectroscopic results suggest that for SSL–gluten systems interactions involve mainly amino acids with OH or charged residues (Table 3). Band splitting related to g–g–g conformation would be related to the formation of disulfide bonds (inter and intrachain) that would participate in gluten structure stabilization.

For gluten–DATEM systems, except for GD1 sample, amino acid residues producing signal in the 2934–2945 cm^{-1} region would act jointly, without undergoing differentiation. This behavior suggests that they are not directly involved in protein structure stabilization. On the other hand, in these systems disulfide bonds belong to both g–g–g and g–g–t conformations.

Differences between emulsifiers are probably related to the ability of DATEM to interact via hydrophobic and/or hydrogen bonds in contrast to the ionic interactions that SSL is capable to establish with protein molecules. Therefore, DATEM–protein interaction would be mainly through hydrophobic bonds.

Considering all the Raman results, several points have to be taken into account:

For the gluten–SSL system, the increment of the Amide I band was correlated with the percentage increase of the α-helix component in comparison with native gluten. In particular, this behavior is more pronounced for 0.25% of SSL on gluten matrix (Table 1) and it is accompanied by a lost of the percentage area contribution of the band related to turns and β-sheets structures and the disappearance of the percentage contribution to random coil structures. This behavior is consistent with protein folding and the tendency to a more stable structure. In addition, the modifications observed in the bands arising from protein tertiary structure also support this assumption: environment of Tyrosine side chain showed that, for samples with 0.25% and 0.5% of SSL content, the $I_{850/830}$ ratio decreased, indicating the burial of Tyr groups. Only for the sample with 1% of SSL content, an increase in the ($I_{850/830}$) ratio is presented which can be related to the formation of Tyr positive charge acceptor. The changes in disulfide bridges conformations also can be associated to the new disulfide bonding formation during the folding process of the protein: appearance of the band belonging to g–g–t conformations for GS0.5 and GS1 and the splitting of the band associated to g–g–g conformation for GS0.25.

For gluten–DATEM system, it was observed that DATEM addition in all its proportions also improved protein ordering with the following changes in the percentage area contribution: an increase of α-helix conformation, a decrease of turn contents and β-sheet constituents and an increase in β-antiparallel component (Table 1), principally at 0.5% and 1% levels. In the same way, the diminution of the $I_{850/830}$ values, the increment of the Tryptophan band intensity and the presence in all gluten–DATEM samples of the three typical S–S bands suggest new interactions inside the protein probably leading to a folding structure.

In the gluten–SSL + DATEM samples the increase in the Amide I band intensity was not so pronounced in comparison with the others systems. In all the tested proportions of emulsifiers, the band intensity of the Trp increases while the $I_{850/830}$ ratio decreases for the lowest emulsifier proportions being the highest value only when the content of the SSL–DATEM is 1% in percentage. In comparison with the other mixtures (gluten–SSL and gluten–DATEM), there is a less tendency to the formation of the g–g–t conformation for the disulfide bridges. The cause of these alterations probably arises of some kind of steric effect due to the simultaneously presence of the two emulsifiers in the gluten matrix.

Table 4
Protein solubility (S) and content of free sulfhydryl groups (SH_F) of native gluten (G) and 1.0% of SSL (GS1), DATEM (GD1) and SSL + DATEM (GSD1) samples.

	G	GS1	GD1	GSD1
S (mg/mL)	1.96a	2.16b	2.13b	2.35c
SH_F (μmol/g protein)	4.06a	3.56b	2.86c	3.45b

Values followed by the same letter in the same row are not significantly different ($p < 0.05$).

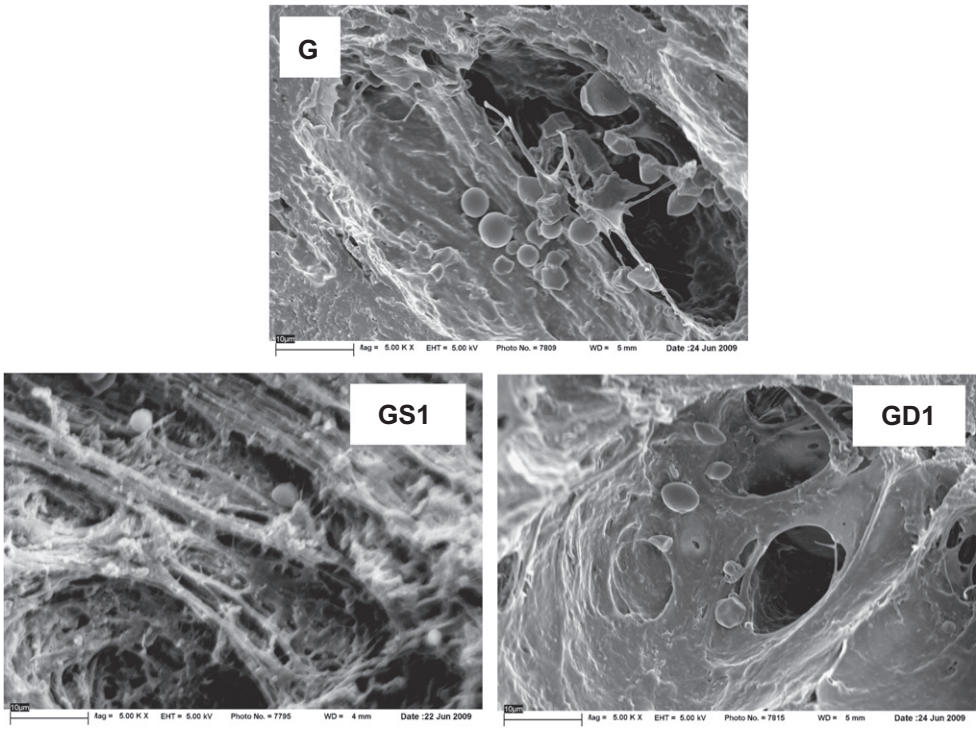


Fig. 6. Scanning electron microscopy of gluten prepared with emulsifier (1.0%). Native gluten (G), gluten–SSL (GS1), gluten–DATEM (GD1). Magnification: 5000×.

3.2. Gluten network

The free sulfhydryl content (SH_F) of soluble proteins may proportionate additional information about the SH/S–S interchange reaction in gluten samples. The highest value of SH_F was obtained for native gluten, G (Table 4). Free sulfhydryls of gluten samples prepared with emulsifier (1.0%) were significantly decreased as compared to G. Results suggest a greater formation of disulphide bonds in the presence of emulsifiers. In addition, in comparison with G sample, a high amount of protein was extracted from emulsifier–gluten samples (Table 4). Therefore, changes in secondary gluten proteins structure, due to the presence of emulsifiers, influenced the type of gluten network formed (Fig. 6). The homogeneous network observed for native gluten (G) was modified in the presence of SSL so that GS1 presented a more fibrous structure. DATEM (GD1) incorporation caused the formation of a laminar gluten network (Fig. 6). The major formation of disulfide bonds (lower SH_L content) and the structural changes in gluten proteins observed by FT-RAMAN assay, caused modifications in gluten matrix. On gluten matrix surface some hydrophobic amino acids generate hydrophobic regions. At low concentrations (0.25 and 0.5%) the lipophilic tail of the SSL probably binds to these hydrophobic regions, incorporating the negatively charge of its polar head to gluten matrix. This phenomenon would promote interactions between the polar head of the emulsifier and the positively charged amino acids of gluten proteins, leading to a more folded structure. The addition of 1% of SSL (GS1) probably produced both simultaneous phenomenon's, the unfolding of gluten proteins due to the repulsion generated by an excessive negative charge incorporated to the matrix, and the aggregation of proteins mediated by the hydrophobic interactions as a result of an excess of emulsifier. On the other hand, low concentrations of DATEM (GD0.25) favor the formation of hydrophobic bonds. In addition, interactions between the hydrophilic head of DATEM and charged amino acids of gluten proteins would be less effective than SSL, because it is a less hydrophilic emulsifier [2,27]. Therefore, the low proportion of ionic interactions established with DATEM lead to a gluten network of low degree of protein folding. Higher quantities of DATEM, 1% (GD1), due to the greater amount of hydrophobic bonds, cause a major interaction with gluten proteins but, comparing to SSL (GS1), with less unfolding as a consequence of a low charge repulsion. Thus, the observed differences would be attributed to the different hydrophilic/lypophilic balance (HLB). SSL presents a HLB value of 21 [2,28], due to the polar chain lactylate, which allows SSL to interact through ionic bonds with positive charged amino acid residues of gluten proteins. In contrast, due to its lower HLB (HLB = 9.2) [2,27] DATEM is a molecule that mainly interacts with the hydrophobic domains of gluten proteins.

4. Conclusions

The incorporation of SSL and DATEM emulsifiers caused different changes in gluten protein structure. SSL at 0.5% and mainly at 0.25% level produced a protein folding with an increase in α-helix conformation and a decrease in β-sheet, turns and random coil. Protein folding was confirmed by changes in vibrational modes of stretching of disulfide bonds, by the low exposition of Tyrosine residues, and also by the great burial of Tryptophan residues from a hydrophobic microenvironment. The lowest percent values of C–H stretching band (2934–2945 cm^{-1}) intensity detected in the presence of 0.25% of SSL would be related to the lower mobility of Tyrosine, suggesting a certain protein structure order, correlating with the higher content of α-helix previously detected.

The highest emulsifier concentration of SSL (1.0%) produced a higher degree of structural disorder than the 0.25% level, as evidenced by a more opened and labile structure. Differences in gluten structure produced by SSL or DATEM could be attributed to the distinct chemical structures of these emulsifier molecules, which condition their hydrophilic/lipophilic balance and therefore the type of interaction that they establish with gluten proteins. These differences will directly influence structural, rheological

and thermal properties of dough and consequently will improve the bread quality.

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