Effect of light scattering in monitoring milk coagulation process

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Introduction

Milk coagulation takes place due to the precipitation of casein. Casein is the major, but not the unique, nitrogen compound in milk. The word "casein" is an approximation of a much more complex protein structure which includes various sub-units of casein: α-s_1, α-s_2, β, $\gamma_{1,2,3}$, k-casein, each having its own structure and composition. The sub-units form what is called "micelle", and the sub-units are kept together by phosphorus and calcium bridges. All the sub-units are almost completely hydrophobic, except for the k-casein which contains a side chain of sugar, able to create bridges with water, sufficient to keep the micelle in suspension in the water matrix. The hydrolysis of the sugar portion from the k-casein removes the agent capable of keeping the micelle in suspension.

Rennet is a complex of hydrolytic enzymes able to break the sugar portion from the protein. This process takes some time to be completed, depending on various factors such as type of milk, enzyme concentration, pH, temperature, and ionic strength. During the hydrolytic process when the hydrophilic part is removed, the remaining hydrophobic structures attach themselves to each other, forming small aggregates. As the hydrolysis goes ahead, the aggregates get larger and at the same time less sugar is bound to the protein, consequently there is lower ability to keep the micelle in suspension (coupled to the increasing weight and size of the aggregates). When the aggregates are large enough, they precipitate, forming a network of casein micelles which entrap fat globules and some whey proteins. Precipitation takes place when around 80% of the sugar has been removed from the k-casein.[1] The formation of small aggregates is not visible, visual observation is only possible when large flocks are formed.

The near infrared (NIR) spectrum of milk at time 0 contains a scattering contribution. After rennet addition and the initiation of hydrolytic process, released sugar binds to water molecules, modifying the water absorption band, a little at the beginning and in a larger amount later (when more sugar has been removed). Meanwhile the aggregation of micelles starts, consequently an increase in scattering and decrease in percentage reflectance or increase in log 1/R is observed. When the hydrolysis of sugar reaches the point where micelle suspension is no longer possible, due to an insufficient number of hydrophilic bonds, curd flocks appear and there is a change in the milk physical state, i.e. transition from the liquid state to the gel state.

Milk macro-composition, expressed as percentage of total solids, total protein, total fat, lactose, and salts, does not change during coagulation, except for the variation due to rennet addition, causing a rearrangement in the sub-micelle structure but not a change in composition. Also the κ-casein, losing the sugar chain, does not change the amino-acid sequence. The sugar released, exhibits the same functional groups as when bound to the protein. The phenomenon is independent from fat presence or absence.

Since protein absorption bands are a-specific, due mainly to the peptide bond absorption, it's comprehensible that protein bands are not influenced by the particle size and do not change in intensity. However, significant modifications of a different nature occur. The progressive sugar release in the water matrix changes the ratio of free to bound water, modifying the water absorption band.

Extended multiplicative scatter correction (EMSC)[2] has been extensively used in modelling the scattering contribution to biological sample NIR spectra. The light scattering contribution to measured NIR absorption has been used in a previous study[3] to improve knowledge about actual contribution of water to the total NIR absorption of milk. Giangiacomo et al. [4] have identified the main events taking place during the early stage of milk coagulation by NIR spectroscopy.

Extensive debate on the interpretation of the spectra and on the effect of light scattering on the spectra, both in transmission and reflectance, can be found in the NIR Discussion Forum (http://www.impublications.com) initiated on June 08, 2010. This paper is a further addition to the debate, showing a procedure able to differentiate the contribution of absorption and scattering in the spectral properties of milk during coagulation.

Reference paper as:
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Materials and Methods

Samples

Several milk coagulations were carried out at 35 ± 1°C using raw and heated (60°C, 15 min), whole and defatted milk (which had 0.2% fat and was obtained by centrifugation at 3000 g for 15 min) that were produced at the Fodder and Dairy Productions Research Centre (Lodi, Italy) farm . Sodium azide was added to the milk samples as a preservative.

Twenty millilitres of liquid rennet (containing 22% of bovine pepsin, 134 IMCU.mL^{-1}, Chr. Hansen, Corsico, Italy) solution (0.8% in distilled water) was added to 500 mL of milk. The clotting time was measured by visual observation of the formation of the first flocks.

Near infrared spectroscopy

Milk clotting processes were monitored using a FT-NIR spectrometer (MPA Bruker Optics, Milan, Italy) in reflectance mode, over the range 1000–2500 nm (10000–4000cm^{-1}, resolution 16 cm^{-1}), using a fibre probe. For each coagulation process, 64 spectra were recorded every 70 s; a total of 55 spectra were collected.

Data Analysis

Spectral data were processed using MatLab version 7.1 (The MathWorks, Turin, Italy) and PLS Toolbox, version 4.0.2 (Eigenvector Research Inc. Wenalchee, WA, USA). The scattering contribution was calculated using the EMSC algorithm (EMSC Toolbox version 1.2 Eigenvector Research Inc. Wenalchee, WA, USA) with the first spectrum as the reference and applying a 4th order polynomial filter.[3] The signal that was filtered out was also considered.

Results and Discussion

Reflectance spectra of milk and defatted milk before and after EMSC preprocessing are shown in Figure 1. The main events taking place in the early stage of coagulation measured on the mean spectrum both in whole and defatted milk are reported in Figure 2.

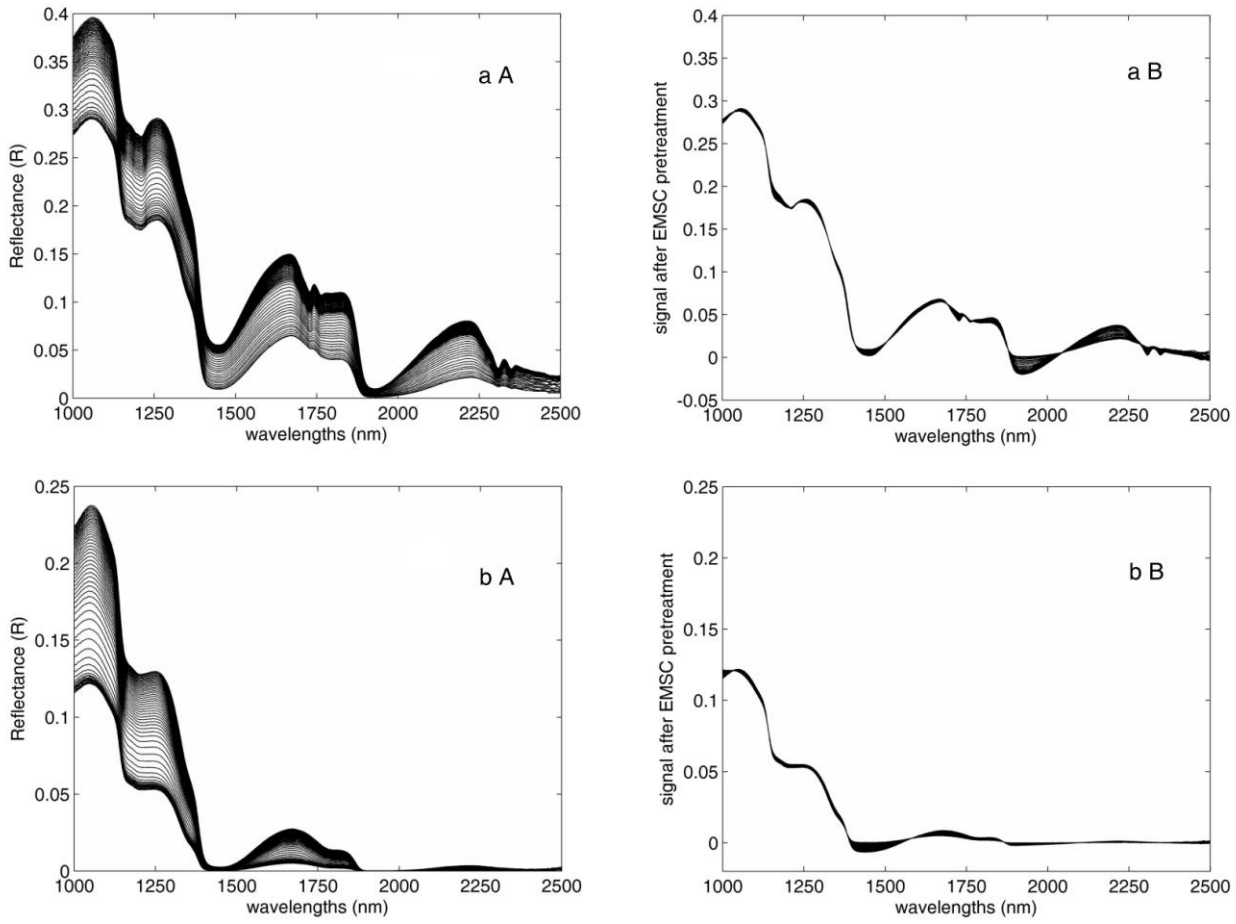

Figure 1. Reflectance spectra of whole (a) and defatted milk (b) before (A) and after (B) EMSC pre-treatment.

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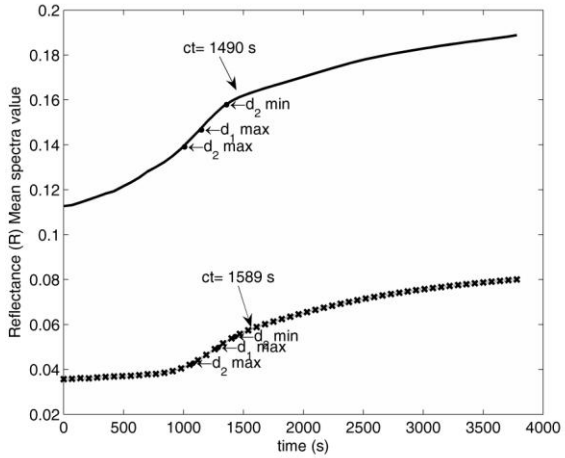

Figure 2. Coagulation curves for whole (full line) and defatted (dashed line) computed from the mean NIR spectra (ct= visual clotting time; d1max= maximum reaction rate; d2max = maximum acceleration d2min= maximum deceleration)

It was observed that the visual clotting time (formation of flocks) occurred earlier in whole milk compared to defatted milk. This was a consequence of the larger size and weight of the coagulum, due to the entrapment of fat globules in the casein network, leading to faster precipitation. Also, the maximum reaction rate (d_1) and the maximum acceleration ($d_2$max) and maximum deceleration ($d_2$min) appear delayed in whole milk. It is worth noting that the maximum of deceleration corresponds to the gelation time, i.e. the point where coagulation is almost complete and which appears, by spectroscopic measurements, before the visual evaluation of flocks formation, confirming the good NIR prediction power for monitoring milk coagulation.[5] At this point the coagulum sets and there is a shift from sol to sol/gel and finally to gel.

By computing the scattering contribution to the spectra using EMSC and plotting these residuals for each time point the presence of an isosbestic point at 1150 nm, and a maximum at 1336 nm was observed for skim milk (Figure 3). It was also observed that there was a group of spectra which did not meet the isosbestic point (Figure 3a). This group of spectra were collected before the clotting time and allowed the determination of critical points before coagulation. The wavelength 1150 nm was associated with a water band, as also suggested by Norris during his personal communication at the IDRC 2010 in Chambersburg (PA, US). Analysis of this wavelength across the time series indicated that after coagulation the milk system had a new equilibrium between water and organic constituents and no further significant increases in scattering occurred at this wavelength (Figure 3b).

The maximum of the scattering reflectance was registered at 1336 nm (Figure 3a), and was associated with a rearrangement in milk protein structures during coagulation without the formation of new chemical species. This theory is in accordance with the WAMACS table[6] where some interconnections between water and protein groups are identified at this wavelength. Considering that the events under investigation are due to rearrangements in protein constituents and not to new chemical species, it can be inferred that the aggregation of these new structures changes the size of the micelles, inducing an increase in scattering properties of the medium. This event occurs when micelle aggregation starts and the size of these aggregates interferes with the incident radiation reaching a dimension of about 1 μm. This evidence was highlighted only using skimmed milk, where no fat contribution could overlap the response of other milk components, potentially involved in the same process. The significant contribution of protein scattering to total scattering is evident. The percent contribution in reflectance due to scattering between the isosbestic point and the maximum value observed is about 53% (Figure 3c).

When plotting reflectance spectra against time at 1440 nm (water absorption band), it is possible to detect several changes in both absorption and scattering, as reported in Figure 4a, b, c. It is shown that at this wavelength there is a limited contribution to total reflectance and that the scattering curve vs. time (Figure 4b) register the same behaviour as the coagulation spectrum (Figure 4a) with scattering increasing with time. The reflectance curve showed an opposite trend (Figure 4c), indicating a decreasing, but still noticeable, reflectance due to water rearrangement around the protein network.

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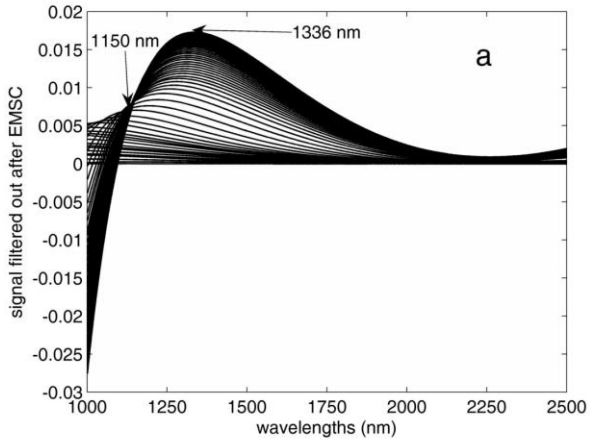

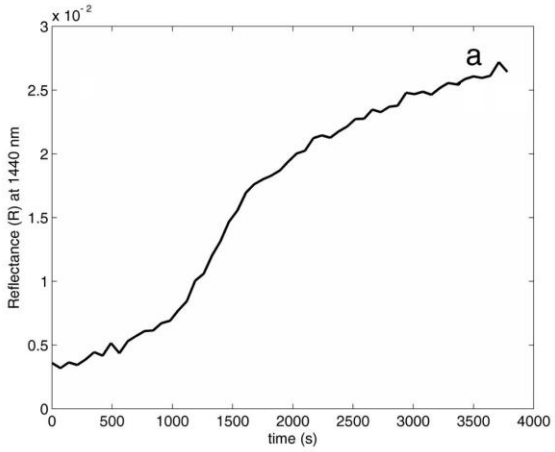

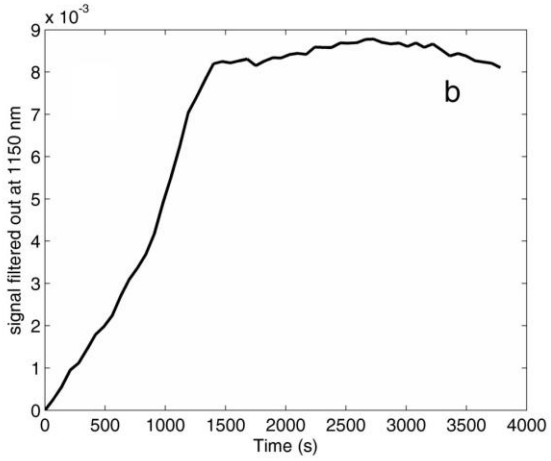

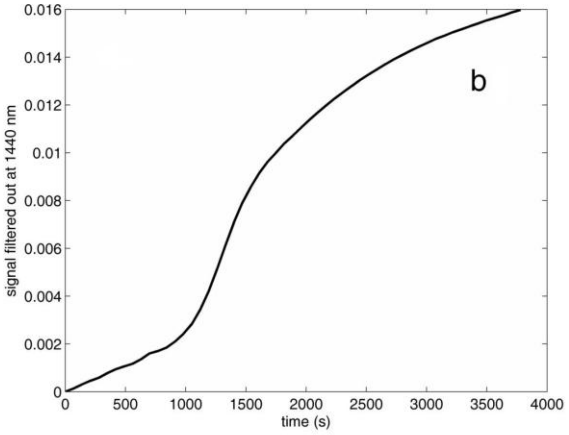

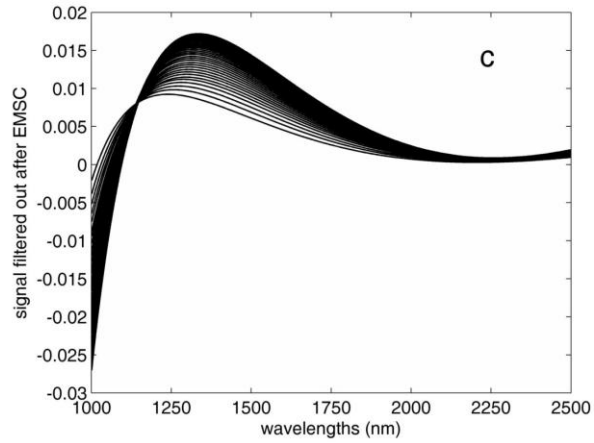

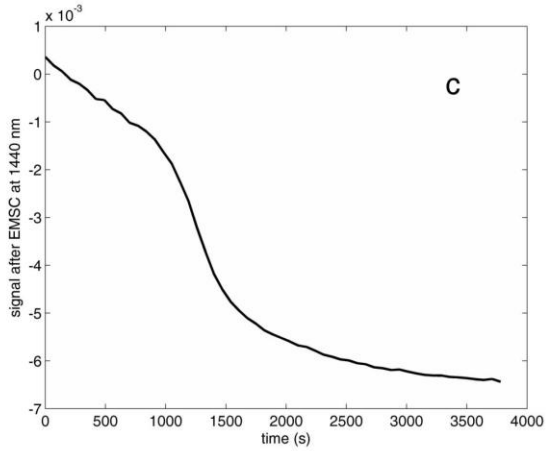

Figure 3. Defatted milk. **a)** Filtered out signals against time; **b)** Coagulation trend at the isosbestic point; **c)** Scattering contribution after the isosbestic point.

Figure 4. Defatted milk. **a)** Coagulation trend at 1440 nm; **b)** Scattering trend at 1440 nm; **c)** EMSC pre-processed signal at 1440 nm against time

By applying the same procedure to whole milk, a significant contribution to total reflectance can be observed at 1440 nm (5a) with the scattering (5b) representing almost entirely the change in total reflection. The signal after EMSC pre-treatment showed an opposite trend to skimmed milk, but with lower intensity, indicating a very low effect of absorption on total reflectance. The effect of scattering in the wavelength domain is represented in Figure 5b giving a reason for the differences observed in Figure 1 in the same domain.

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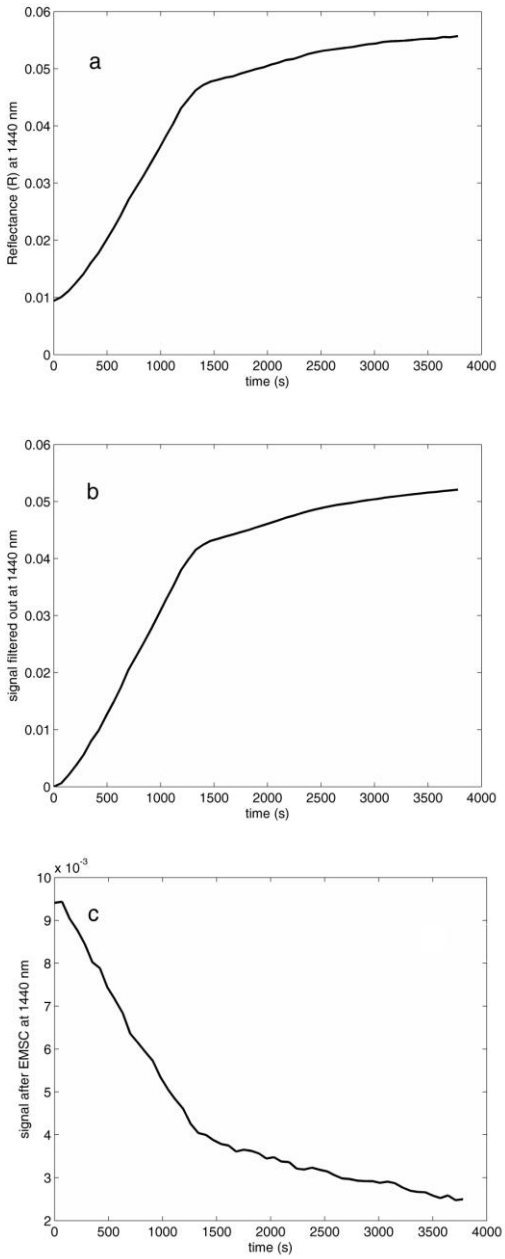

Figure 5. Whole milk. **a)** Coagulation trend at 1440 nm; **b)** Scattering trend at 1440 nm; **c)** EMSC pre-processed signal at 1440 nm against time.

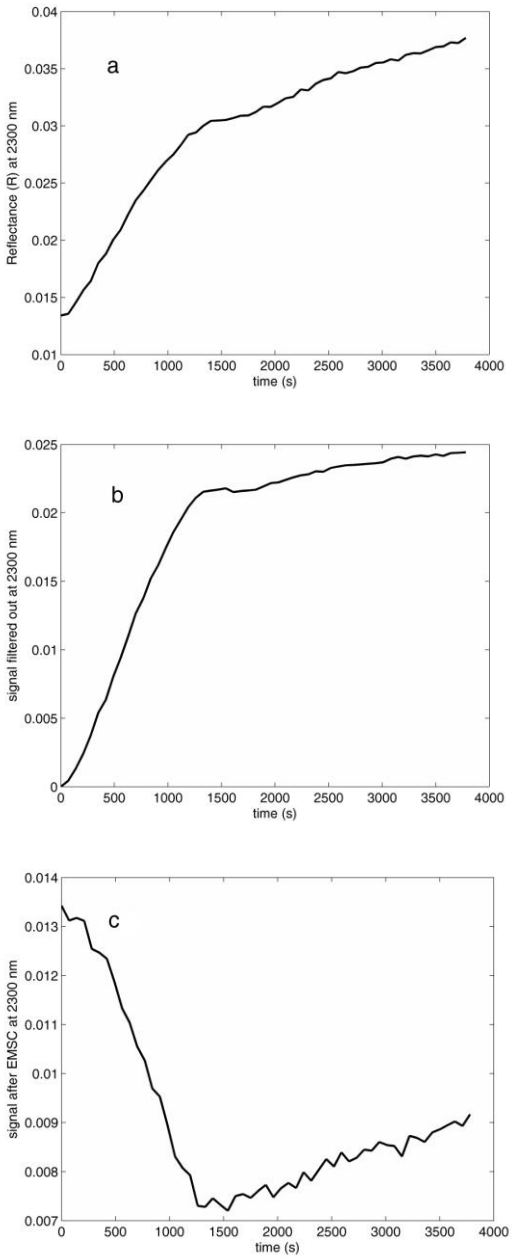

Figure 6. Whole milk. **a)** Coagulation trend at 2300 nm; **b)** Scattering trend at 2300 nm; **c)** EMSC pre-processed signal at 2300 nm against time.

Considering the coagulation curve at 2300 nm (Figure 6a), where there is a strong absorption of fat groups, it was observed that around this wavelength there was a low contribution to the total reflectance coagulation curve reported in Figure 2. It was also observed that the maximum reflectance value, reached at the end of coagulation process, represents the sum of the two signals related to scattering (Figure 6b = about 65 %) and absorption (Figure 6c = about 35 %) of "–CH_n" groups.

The processing of scattering curves as residuals after the application of EMSC to the whole milk dataset (Figure 7a) allowed the identification of a new isosbestic point, around 1260 nm, and a maximum around 1530 nm. In particular it can be noted that the maximum of reflectance takes place at the wavelengths where there is the absorption of-RNH stretching first overtone and –CH stretching first overtone. Analysing the scattering curves, the maximum of reflectance at 1530 nm indicated the presence of a double contribution due to the increase in particle size as consequence of the continuation of the milk coagulation process. The latter involves both the protein network and the fat globules entrapped in it and leads to an increase in scattering properties of the medium. This confirms the combined role of protein and fat. Considering the scattering variations at 1260 nm (isosbestic point) (Figure 7b) against time, it can be noticed that after 1400 s

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the milk system looks for a new equilibrium between water and organic constituents and no further significant increases in scattering at this wavelength takes place.

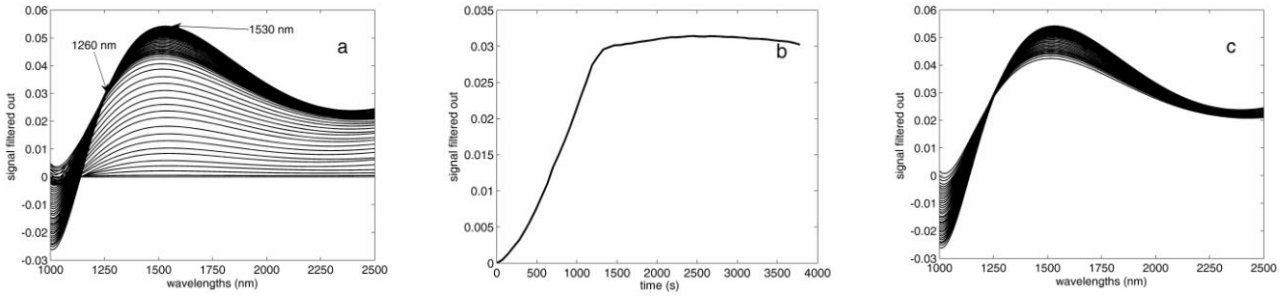

Figure 7. Whole milk. **a)** Filtered out signals against time; **b)** Coagulation trend at the isosbestic point; **c)** Scattering contribution after the isosbestic point.

It has to be considered that the scattering measured after this point in coagulated milk was due to the network of casein micelles with entrapped fat occurring in a percent contribution in reflectance of about 38% (Figure 7c). Conversely, the scattering in skimmed milk coagulum was due to the casein micelles network only, which justified the lower absolute value and indicated that the final modifications in scattering properties during the coagulum setting were characterised by a narrow range of variability in absence of fat.

Conclusion

This work has clarified that the major events taking place during the physical change in milk leading to gel formation are independent of fat presence. The contribution of the casein network to the scattering of milk during the coagulation process has been highlighted. The water rearrangement around proteins with progressive association of micelles has also been observed. This information further explains the physical milk modifications during coagulation as measured by NIR spectroscopy.

The evaluation of scattering properties of casein aggregation after the isosbestic point, i.e. during the phase of gel formation, could also be used for monitoring the protein network under various conditions, where other parameters involved in coagulation play a role such as temperature, pH, rennet strength, rennet amount.

Acknowledgements

It has been possible to present this work at NIR 2011 thanks to the financial contribution of ICNIRS supporting the participation of Roberto Giangiacomo by Emeritus Award.

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