EUROPEAN SYNCHROTRON RADIATION FACILITY

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Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office using the **Electronic Report Submission Application:**

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The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

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All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

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- 1st March for experiments carried out up until June of the previous year;
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Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

	Experiment title: Interaction of tannins with caseins in milk	Experiment number: SC2165
Beamline: ID02	**Date of experiment**: from: 19/07/07 to: 20/07/07	**Date of report**: 29/08/07
Shifts: 3	**Local contact(s)**: A. Shukla, T. Narayanan	*Received at ESRF:*

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Report:

Aims

The aim of this test project was to investigate the morphological changes of casein micelles caused by adding tannins to milk. This experiment was motivated by our recent results [1] on pure beta-casein in aqueous mixture with small (monomer, dimer, trimer and pentamer) and with large (28-mer) tannins. Briefly, the results of this previous study was that in presence of small tannins beta-casein-tannin micelles undergo significant compatization by increasing the aggregation number and keeping the size constant, while large tannins caused aggregation of micelles into large clusters.
In the present experiment we explored if similar effects persist in the case of complex casein micelles of the real milk.

Materials

The procyanidin molecules were extracted from apples by Sylvain Guyot and Alain Baron from the URC group (INRA, Le Rheu). The polymerization degree (DP) of resulting oligomers was measured by standard thiolysis, followed by HPLC.[2-4] In addition to the apple tannins, we used also the total polyphenols of a red wine (VT hereafter), extracted and purified by the Montpellier SPO ENSAM/INRA group (Aude Vernhet and her colleagues). We also used the catechin (monomer unit of procyanidins, ab. Cat) and epigallocatechin-gallate (alias EGCG).
We used the skim milk from commerce.

Experiments

For our SAXS experiments we used the ID02 beamline. Acquisition time was between 0.2 and 1s in series of 3 or 4 runs. We checked for irradiation; some attention was necessary with tannins alone in solution where irradiation cased some declination of the signal. All experiments were thermostated at 25°C. Samples with

milk and tannin were prepared by mixing 10% (v/v) of milk and adjusting the concentration of tannins in the remaining 90%v/v by mixing tannin stock solutions and water.

Results

Tannins in water: conformations and molecular mass

*Conformation*s of polymeric tannins are rather compact as seen from the exponent d_F: its values increase with molecular mass of the tannins from 2.2 for VT tannin to 2.9 for DP150 tannin. The fact that all values are greater than 2 reveals the tannin's tendencies to hide as much as possible of its hydrophobic surfaces from water. This "folding" is more pronounced at higher DP where the effects of persistence length are less important.

Molecular masses: Ratios of intensities extrapolated to q=0 indicate the weight average molecular masses compared to the mass of catechin. These ratios are (cat:EGCG:VT:MM:DP28:DP150) 1 : 2.50 : 10.4 : 25.6 : 76.8 : 560.

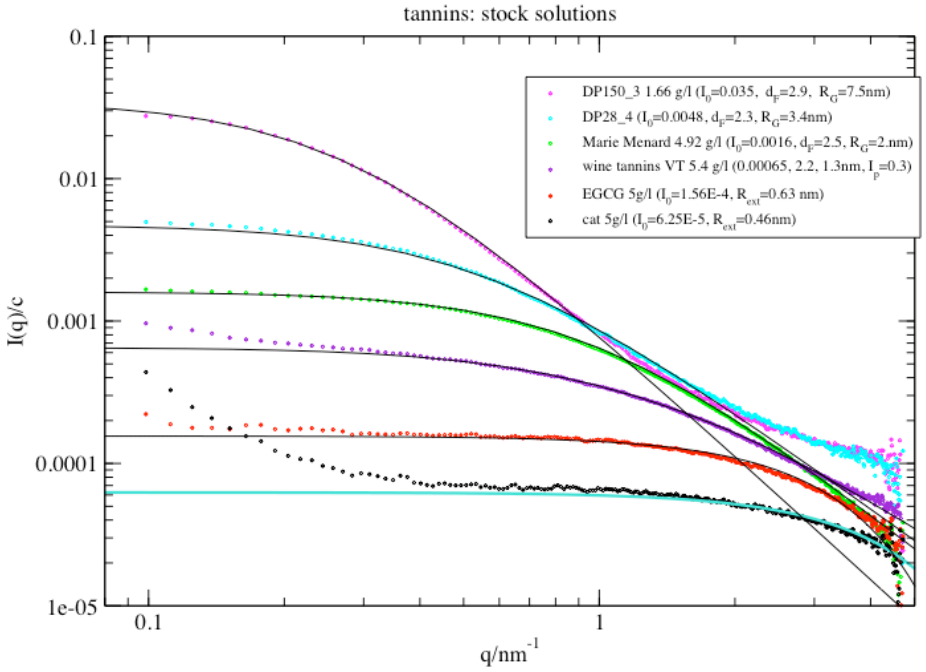

Figure 1 SAXS spectra of the tannin polymers used in this study.

Milk with tannins

Experiments with milk and tannins were performed on skim milk and four different tannins. After visual observation we concluded that casein+small tannin micelles remain stable at macroscopic length scales: no precipitation was observed with EGCG and catechin. However, phase separation (gelification) was observed visually on samples with long tannins DP28 and VT.

On the intermediate length scales, the general feature, common to all tannins used, *including catechin*, is that tannins compactify the casein micelles without changing their size. This means that the number of molecules per micelle increases with increasing tannin concentration. Which molecules are that, tannin, or both casein and tannin, we don't know yet. However, a number of details about the tanning effect on casein micelles can be extracted from the spectra.

The EGCG tannin is the most efficient enhancer of the micelles mass. As it can be seen on the figure, the gyration radius of the super-micelle and of the sub-micelle remains constant over the whole range of the tannin concentrations, while the intensity at q=0, revealing the aggregation number was increased by a factor of 3.7. The first compactness exponent 3.5 did not change, but the second, corresponding presumably to the sub-micelles increased from 3.2 without tannin to 3.8 at maximal tannin concentration used (4.5 g/l). This indicates that the super-micelles are compatized at the level of their sub-micelles. One question remains open: it's what is the fraction of tannins in micelles. If this fraction is high, the enhancement of the scattering intensity at q=0 can be due to simply bound tannins in micelles, and not to presumed supplementary caseins brought into micelles by tannins. In order to answer this question, we have to do more experiments on a set of protein concentrations, keeping constant tannin/protein stoichiometry. Than, the results can be compared to the calculated profiles used the extended shell model [1].

Another interesting effect is the evolution of the 0.7/nm structure upon adding tannins. We still don't know what are the mechanisms involved in this effect.

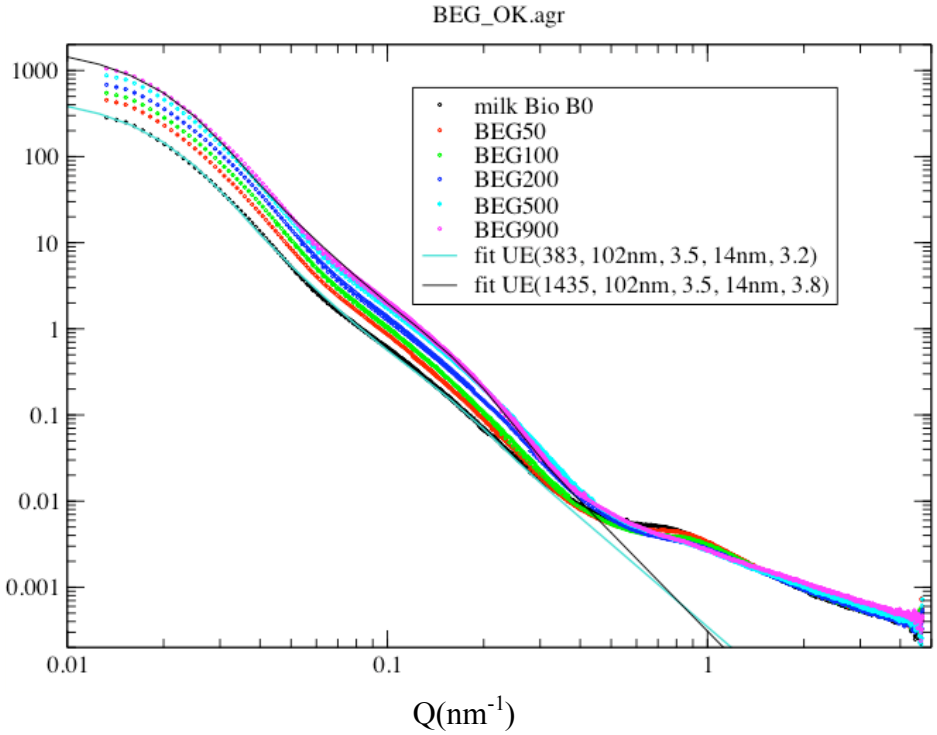

Figure 2: SAXS spectra of skim milk with EGCG tannin. Intensity at small q increases with tannin concentration.

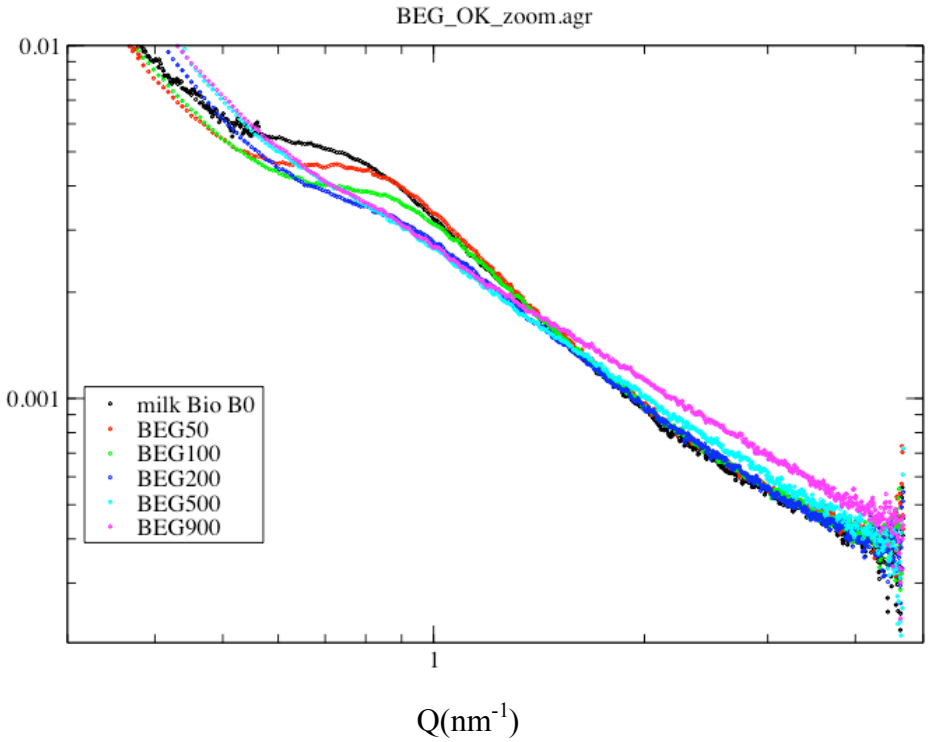

Figure 3 : The feature at q=0.7/nm is affected by tannins. Correlations are first enhanced and than washed out as the tannin content is increased.

The extrapolation of the scattering intensity to q=0 we reveals the micelle mass enhancement by all tannins used (see figure 4). The size of the micelles is independent of the tannin concentration: the gyration radius of the micelles is 100 nm, within less than 5% error. Increasing micelle mass with constant size implies that the casein micelles became more compact as we increase tannin concentration. Furthermore, the profiles on the figure 4 have a characteristic shape, which indicates that at about 4g/l of tannin the complexation with proteins starts to saturate. This saturation is probably determined by the number of available prolines in caseins.

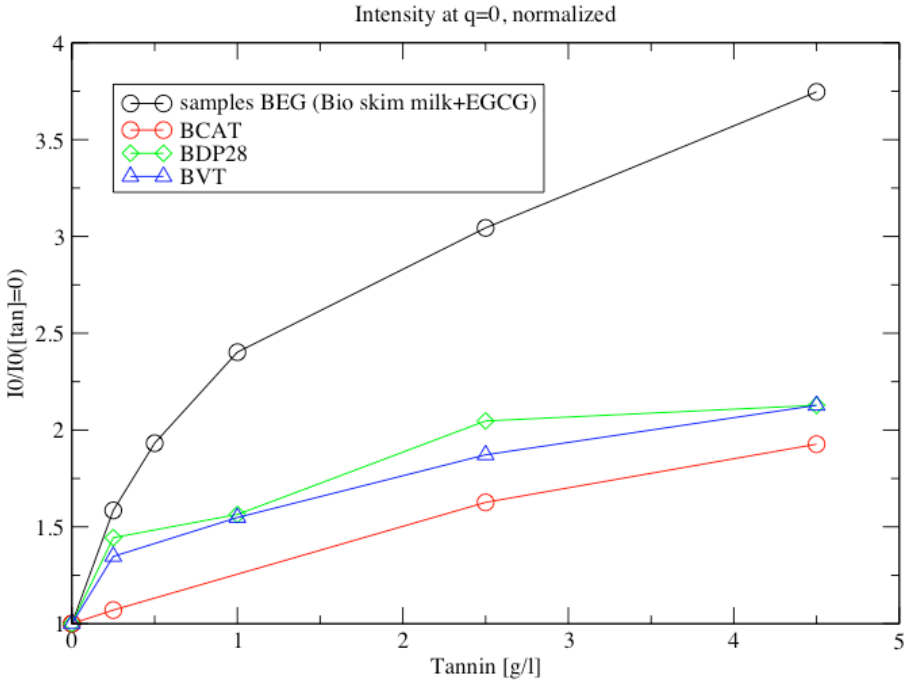

Figure 4 The scattering intensity extrapolated to Q=0 as function of tannin concetntration for milk with EGCG tannin (BEG), catechin (BCAT), apple 28-mer tannin (BDP28) and wine tannin (VT).

Interestingly, different tannin fractions affect in different way each of three characteristic hierarchical levels of casein structure. The EGCG tannin (see spectra BEG) affects the internal structure of both 100nm- and 14nm-objects, while DP28 tannin is less efficient at global level (100nm), but enhances strongly the compactness.

Conclusion and perspectives

Upon adding tannins the total mass of the casein micelles and compactness of the sub-micelles increase, but the size and the existence of the hierarchical structure of the casein super-micelles remains non affected by presence of tannins. This result is consistent with our findings on pure beta-casein with different tannin polymers.[1]

Another interesting observation is the effect of tannins on the q=0.7/nm feature in the milk spectrum. As far, we can only speculate about the tannin-induced loss of this well defined quasi-order within the submicelles. The effect is visible with all tannins.

Our project is to continue to explore the effects of tannins on both milk and on beta-casein. In particular, we want to know in details how the structure of the micelles depends on both tannin and protein concentrations and on temperature. The interactions and mechanisms involved in protein-tannin micellization will be further explored by stop-flow and T-jump experiments. The resulting kinetics will be compared with the results of our theoretical model. [1]

References

[1] D. Zanchi, T. Narayanan, D. Hagenmuller, B. Cabane, A. Baron, S. Guyot and S. Bouhallab, Interaction of beta-casein with tannins, to be submitted to PRL; D. Hagenmuller and D. Zanchi, to be published.

[2] A. Baron, S. Guyot and D. Zanchi, Solvent induced conformations of tannin macromolecules, paper in preparation.

[3] D. Zanchi, A. Vernhet, C. Poncet-Legrand, D. Cartalade, C. Tribet, R. Schweins, B. Cabane, Colloidal Dispersions of Tannins in Water–Ethanol Solutions, Langmuir (2007), in press.

[4] T. Hatano and R. W. Hemingway, J. Chem. Soc., Perkin Trans. **2,** 1035 (1997).

[5] N. J. Baxter, T. H. Lilley, E. Haslam, and M. P. Williamson, Biochemistry **36**, 5566 (1997).