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► To cite this version:

Maëla Choimet, S. Marze. In vitro digestion of emulsions: mechanistic and experimental models. *Soft Matter*, 2012, 8 (42), pp.10982. 10.1039/C2SM26336J . hal-03615514

HAL Id: hal-03615514

<https://hal.inrae.fr/hal-03615514>

Submitted on 21 Mar 2022

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In Vitro Digestion of Emulsions: Mechanistic and Experimental Models

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ABSTRACT

Digestion is a complex combination of physical, chemical and biological processes. In order to investigate the impact of food structure on the digestion of lipids, we work on a controllable triglyceride-based system: emulsion. In this study, the emulsion was composed of a single triglyceride (tricaprylin or triolein), decanal as a model lipophilic micronutrient, and a single emulsifier (β -lactoglobulin or sodium oleate) at different concentrations. We investigated the effects of these parameters on an in vitro intestinal static digestion, which was monitored using classic physicochemical methods: fatty acid titration, lipids extraction/chromatography and sizing.

To interpret the results, we developed several mechanistic models based on mass transfer kinetics, which enable a direct comparison and identify the factors influencing the digestion. Those are the molar mass of the lipids, the initial interfacial area (droplet size) and dispersed volume fraction, the interfacial tension and dilatational viscoelasticity.

We also developed an experimental digestion model based on a single droplet using tensiometry. This technique was able to monitor the kinetics of lipolysis and micellar solubilization simultaneously.

All methods confirmed the result from our previous study that the type of triglyceride is the major parameter influencing the digestion. Moreover, the mechanistic and experimental models allowed to evidence that digestion was usually faster for β -lactoglobulin emulsions/droplets compared to sodium oleate ones. There was no clear effect of the emulsifier concentration.

1. Introduction

Evaluating the health, safety and nutritive aspects of foods is nowadays an important activity as more and more evidence shows that diet has impacts on chronic diseases.¹ At the same time, there are also proofs that the structural aspects of foods influence the bioavailability of some nutrients and micronutrients.² This means that the effective nutritive intake may differ significantly from the food content if e.g. a structure retaining a micronutrient is not broken down during digestion.

In this context, questions arise about the impact of structuration and formulation of foods on their nutritional values. To study this relation, most of the works presently focuses on the in vitro characterization of the digestion of model food systems, enabling to distinguish the roles and interactions of different structures and physicochemical processes.³ A widely used model food system is emulsion, a liquid-liquid dispersion. It is minimally composed of lipid droplets dispersed in water and stabilized by an emulsifier at the oil/water interface (surfactant, protein, lipid...). But carbohydrates, dietary fibers and micronutrients (vitamins, minerals...) may be added to tend towards a real food.

Even with a model emulsion, there are still many factors to account for to understand its digestion.³⁻⁵ In particular, the lipids and emulsifiers roles and interactions with some digestive molecules are not well-known. With similar emulsifiers, the conclusions of several studies are indeed contradictory.⁶⁻⁹

In this article, we study some emulsion formulations based on one pure triglyceride of short or long chain length and one emulsifier of protein or lipid nature at different concentrations. Our previous results based on newly developed diffusion methods only showed a clear effect of the triglyceride on the digestion.¹⁰ Using classical physicochemical methods, our goal is now to check for the roles of the other factors. Moreover, we develop mass transfer models that identify the mechanistic parameters and help the data interpretation. A single droplet model experiment is also developed to study the interfacial characteristics of digestion in order to discriminate between lipolysis and solubilization.

2. Materials and methods

2.a. Materials

Tricaprylin TC (T9126), triolein TO (T7140), sodium oleate NaO (O7501), decanal (W236209), sodium glycodeoxycholate NaGDC (G9910) and pancreatic lipase type II (L3126) were provided by Sigma-Aldrich France. β -lactoglobulin (β LG) was purified from whey protein isolate in our laboratory. In all preparations, Milli-Q water having an electrical resistivity of 18.2 $M\Omega.cm$ was used.

2.b. Emulsion preparation

Oil/water emulsions of dispersed oil volume fraction $\phi = 0.2$ were prepared the day of the in vitro digestion using a 7.5 $mg.mL^{-1}$ solution of either NaO or β LG (as emulsifier) in 10 mM NaH_2PO_4 buffer adjusted to pH 7.5 as the aqueous phase, and either tricaprylin or triolein (both containing 10 wt% decanal as a model micronutrient) as the oil phase. A total volume of 10 mL was placed in a 50 mL plastic vial, pre-emulsified for 2 min at 15000 rpm using a rotor-stator homogenizer (SilentCrusher M equipped with the 12F generator, from Heidolph Instruments, Germany). Immediately after, the pre-emulsion was sonicated using a Misonix Sonicator 4000 equipped with a microtip probe 419 (Qsonica, Connecticut, USA) for 2 min ,

alternating 15 s sonication and 15 s pause. A total of three cycles were performed with a 2 min pause between them to let the emulsion cool. The total energy brought to the emulsions was always about 1.8 kJ.

All solutions were made by magnetic stirring at ambient temperature for 30-60 min and adjusted to pH 7.5 the day of the in vitro digestion. Only the dissolution of NaO in water was stirred at 37 ± 0.5 °C overnight and the high resulting pH brought back to 7.5 with addition of μL amounts of 1 M HCl (see ¹¹).

2.c. Emulsion digestion

The day of the in vitro digestion, an intestinal solution of 20 mg.mL^{-1} NaGDC (bile salt) and 1 mg.mL^{-1} pancreatic lipase was made in 130 mM NaH_2PO_4 buffer and adjusted to pH 7.5. The variants we called “+” for excess were obtained by adding 12 mg.mL^{-1} of either NaO or βLG to this solution, corresponding to the NaO or βLG emulsion respectively. The role of these variants was to add excess emulsifier in the bulk, as the initial emulsions were formulated so that most emulsifier be adsorbed at the oil/water droplet interface.¹²

First 2 mL of emulsion was placed in a plastic vial and diluted in 130 mM NaH_2PO_4 buffer alone with a volume ratio 1:1 to mimic the gastric dilution, then in the intestinal solution with a volume ratio 1:1 to start the digestion (the progressive increase of the emulsion ionic strength was designed to avoid flocculation during dilution¹²). The plastic vial was closed and placed in a Thermocenter oven (SalvisLab, Switzerland) at 37 ± 0.5 °C on a magnetic stirrer Multipoint HP15 (Variomag, Germany) in the shake mode at 100 rpm. The final emulsions had a ionic strength of 100 mM, a pH of 7.5 and a dispersed volume fraction of 0.05 for a total volume of 8 mL. They contained 10 mg.mL^{-1} NaGDC and 0.5 mg.mL^{-1} pancreatic lipase and either 1.5 or 7.5 mg.mL^{-1} emulsifier, in the normal or in the excess (+) case respectively.

2.d. HPLC

1 *mL* emulsion samples were collected each hour during digestion and immediately acidified using a couple of HCl 1 *M* drops in a 15 *mL* centrifuge tube. Then a lipid extraction was performed. 10 *mL* of a hexane-isopropanol mixture (3:2 v:v) and 1 *mL* of NaCl 150 *mM* were added. The tube was vortexed at 3000 *rpm* for 2 *min* then centrifuged at 2000 *g* for 5 *min*. About 7.5 *mL* of supernatant was obtained, of which 7 *mL* was collected and placed in a glass flask of known mass for solvent evaporation under nitrogen. After complete evaporation, the flask was weighted to deduce the lipid mass. Then, 5 *mL* chloroform was added and the sample was placed in a freezer at -80 °C for subsequent analyses.

Samples were analyzed at 30±1 °C in a HPLC system UltiMate 3000 RSLC (Dionex, France) coupled to an evaporative light scattering detector Sedex 85LT (Sedere, Alfortville, France). The analytical column was packed with a silica normal phase. The eluent was first pure chloroform, progressively replaced by a mixture of methanol/28% ammonia in water/chloroform (92:7:1 v:v:v) in order to elute the polar lipids. Identification of the peaks according to the retention time and calibration plots relating the area under the peaks to different masses of the lipid classes were obtained in the same conditions using a lipid standard mono-, di-, & triglyceride mix (1787-1AMP) plus oleic acid (O1008) provided by Sigma-Aldrich or TC alone. For the TC emulsions indeed, the technique did not detect the digestion products, presumably because of their relatively high polarity. Likely for the same reason, decanal was also not detected.

2.e. Dynamic light scattering

Back-scattering intensity autocorrelation function (IACF) was obtained at 37±0.5 °C using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 4 *mW* He-Ne laser of $\lambda = 633$ *nm*. The exact angle θ between the laser beam and the detector (avalanche photodiode) is 173°. The laser power is automatically attenuated to collect an optimal scattered intensity. The measurement position was set to the maximum of 4.65 *mm*,

that is 3.65 mm inside the sample as we used disposable 12 mm square polystyrene cuvettes with 1 mm thick walls (Brand, Wertheim, Germany). The refractive index n_0 of the aqueous phase was 1.335 and those of TC and TO were 1.445 and 1.465 respectively. The absorption was set to 0.001. A 30 s acquisition was generally enough to obtain a stable IACF.

30 μL of the final emulsion was diluted in 1.5 mL of 100 mM NaH_2PO_4 buffer, of which 1 mL was placed in a capillary cell (DTS1060) at 37 ± 0.5 °C so that the digestion directly occurred in the measurement cell. The IACF was recorded automatically every 2 min to continuously monitor the effect of digestion.

2.f. pH-stat

To measure the fatty acids release using a pH-stat setup, separate digestions were performed with no buffer in the emulsion nor in the digestion media, keeping all other conditions the same. The digestion was followed using 0.02 M NaOH to titrate the fatty acids production by maintaining a pH of 7.5, which is close to that of the intestinal artificial medium and gives a high pancreatic lipase activity.¹³ The result was calculated as the percentage of fatty acids molecules released deduced from the volume of NaOH added, considering that 1 triglyceride TG produces 2 fatty acids FA (see ⁸). This volume was recorded every 3 s (1200 points / hour). Note that when NaH_2PO_4 was included, this volume was systematically lower because it buffered a part of the fatty acids produced.

2.g. Experimental digestion model: drop tensiometry

The Tracker drop tensiometer (I.T.Concept-Teclis, Longessaigne, France) was used to perform model digestions on a single rising oil droplet at the end of a vertical curved needle. The droplet characteristics are controlled by a motor acting on the syringe according to droplet shape analysis feedbacks. The needle was placed in an optical glass cuvette of 8 mL (Hellma GmbH, Müllheim, Germany) filled with the desired solution. Just before setting up,

the triglyceride oil was purified in a Sep-Pak Silica cartridge (WAT051900, Waters SAS, France).

The interfacial tension between a triglyceride oil and a 10 *mM* NaH₂PO₄ solution (without β LG or with β LG at the same concentration as for emulsion) at constant interfacial area was allowed to reach equilibrium at 25 °C. Then, half the volume of the aqueous phase was removed and replaced by the same volume of 130 *mM* NaH₂PO₄ buffer using a micropipette. This had a small instantaneous effect on the interfacial tension. Temperature was set to 37 °C and the interfacial tension was allowed to reach equilibrium. Then, half the volume of the aqueous phase was removed and replaced by the same volume of intestinal medium (same concentrations as for emulsion) to start digestion. Before and at the end of the digestion step, the interfacial area was oscillated at 0.02 *Hz* with an amplitude of $\pm 5\%$ in order to measure the interfacial dilatational viscoelasticity.

For NaO, the initial solution was turbid so we could not measure the interfacial tension before digestion. We then directly performed the measurements using the final mixture which was clear. We checked the validity of such a protocol by comparing it to the step-by-step one using β LG.

At least 2, usually 3 independent emulsions, digestions and associated measurements were done on different days in order to check the overall repeatability of the study. The results are presented as averages and standard deviations, except if stated otherwise.

3. Mechanistic models

In this section, we develop several approaches to model the kinetics of triglycerides in a monodisperse emulsion. The quantities we want to express as a function of time *t* are the

solubilized mass fraction of digestion products $w_s(t)$ and the droplet radius $r(t)$, underlined in the text.

3.a. Zeroth order kinetics

Following Li and McClements,⁸ we write a zeroth order equation depending on the interfacial area of the dispersed droplets A_d :

$$\frac{dw_s}{dt} = \frac{1}{m_0} \frac{dm_s}{dt} = \frac{k.A_d.M_s}{m_0} \quad (1)$$

where m_s is the solubilized mass of digestion products, m_0 is the initial mass of dispersed triglycerides, M_s is the molar mass of the solubilized digestion products, k is an effective mass transfer coefficient and $A_d = N4\pi.r^2$ with N the number of droplets.

Substituting mass for volume V then volume for radius in (1) by noticing that

$$\frac{dV_d}{dr} = N4\pi.r^2 = A_d, \text{ and reversing the mass flux, we get:}$$

$$\frac{dr}{dt} = \frac{-k.M_s}{\rho_d} \quad (2)$$

where ρ_d is the dispersed phase density, of which we neglect the temporal variation.

Equation (2) is easily solved to find:

$$r(t) = r_0 - \frac{k.M_s}{\rho_d} t \quad (3)$$

It is more difficult to solve equation (1) for w_s , as we have to explicit A_d because it is a

function of time. It can be calculated that $\frac{A_d}{V_T} = \frac{3\phi}{r}$, where V_T is the total emulsion volume

and ϕ is the dispersed droplet volume fraction, which is also a function of time. Expressing

$$A_d \text{ in the initial conditions we find } \frac{N4\pi.r_0^2}{V_T} = \frac{3\phi}{r_0}, \text{ thus:}$$

$$N = \frac{3\phi_d V_T}{4\pi r_0^3} = \frac{3\phi_d V_T}{4\pi r^3} \quad (4)$$

supposing the number of droplets N is constant because the emulsion is monodisperse. In the initial conditions, we also have $m_0 = \phi_d \rho_d V_T$.

Expressing A_d , N and m_0 in (1) and replacing r using (3) gives:

$$\frac{dw_s}{dt} = \frac{3k.M_s r^2}{\rho_d r_0^3} = \frac{3k.M_s}{\rho_d r_0^3} \left(r_0 - \frac{k.M_s}{\rho_d} t \right)^2 \quad (5)$$

which can finally be solved to give:

$$w_s(t) = 1 - \left(1 - \frac{k.M_s}{\rho_d r_0} t \right)^3 \quad (6)$$

3.b. First order kinetics

Following McClements and Dungan,¹⁴ a first order equation is obtained:

$$\frac{dw_s}{dt} = \frac{1}{m_0} \frac{dm_s}{dt} = \frac{k.A_d}{V_T} \frac{(m_\infty - m_s)}{m_0} \quad (7)$$

where m_∞ is the maximum mass of digestion products that can be solubilized.

Using the same method as for equation (1), we get an expression for r from equation (7):

$$\frac{dr}{dt} = \frac{-k}{V_T} \frac{(m_\infty - m_s)}{\rho_d} \quad (8)$$

3.b.1. Initial model

Supposing the whole dispersed phase can solubilized (because the emulsion is very diluted

and the enzymes and bile salts are in excess), it can be calculated that $\phi = \frac{1}{V_T} \frac{(m_\infty - m_s)}{\rho_d}$.

Using the latter and (4), we obtain:

$$\frac{dr}{dt} = -k.\phi \left(\frac{r}{r_0} \right)^3 \quad (9)$$

Integrating (9) gives:

$$r(t) = \left(\frac{r_0^3}{r_0 + 2k \cdot \phi \cdot t} \right)^{1/2} \quad (10)$$

Similarly as in equation (1), expressing A and N in (7) and replacing r using (10) gives:

$$\frac{dw_s}{dt} = \frac{3k \cdot \phi \cdot r^2}{r_0^3} (w_\infty - w_s) = \frac{3k \cdot \phi}{r_0^3} \left(\frac{r_0^3}{r_0 + 2k \cdot \phi \cdot t} \right) (w_\infty - w_s) = \frac{3k \cdot \phi}{r_0 + 2k \cdot \phi \cdot t} (w_\infty - w_s) \quad (11)$$

Integrating (11) gives:

$$w_s(t) = w_\infty - w_\infty \left(\frac{r_0}{r_0 + 2k \cdot \phi \cdot t} \right)^3 \quad (12)$$

supposing there are no solubilized products at $t = 0$.

3.b.2. Model integrating Kelvin equation

In this model, we take into account the factors influencing the solubility using the Kelvin equation.¹⁵

$$m_s = m_\infty \exp \left(\frac{2\gamma \cdot M_s}{\rho_d \cdot N_A \cdot k_B \cdot T_K \cdot r} \right) \approx m_\infty \left(1 + \frac{2\gamma \cdot M_s}{\rho_d \cdot N_A \cdot k_B \cdot T_K \cdot r} \right) \quad (13)$$

If no solubilizes re-enter the droplets, using (13) in (8) gives:

$$\frac{dr}{dt} = \frac{-k}{r} \frac{2\gamma \cdot M_s \cdot m_\infty}{V_T \cdot \rho_d^2 \cdot N_A \cdot k_B \cdot T_K} = -k \frac{\gamma}{r} C \quad (14)$$

where γ is the interfacial tension and $C = \frac{2M_s \cdot m_\infty}{V_T \cdot \rho_d^2 \cdot N_A \cdot k_B \cdot T_K} = \frac{2M_s \cdot \phi_\infty}{\rho_d \cdot N_A \cdot k_B \cdot T_K}$, with N_A the

Avogadro constant, k_B the Boltzmann constant and T_K the temperature in Kelvin.

Integrating (14) gives:

$$r(t) = \left(r_0^2 - 2k \cdot \gamma \cdot C \cdot t \right)^{1/2} \quad (15)$$

Using (15) in equation (11), we obtain:

$$\frac{dw_s}{dt} = \frac{3k \cdot \phi}{r_0^3} \left(r_0^2 - 2k \cdot \gamma \cdot C \cdot t \right) (w_\infty - w_s) \quad (16)$$

Integrating (16) gives:

$$\underline{w_s(t) = w_{\infty} - w_{\infty} \exp\left(-3\phi\left(k.t / \eta - k^2.t^2.\gamma.C / \eta^3\right)\right)} \quad (17)$$

3.b.3. Models integrating interfacial dilatational viscoelasticity

Following Kloeck et al.¹⁶ and Dickinson et al.,¹⁷ we can further decompose the interfacial tension as:

$$\gamma = \gamma_{eq} + 2\varepsilon \ln\left(\frac{r}{\eta}\right) + \frac{2\kappa}{r} \frac{dr}{dt} \quad (18)$$

where γ_{eq} is the equilibrium interfacial tension, ε is the interfacial dilatational elasticity and κ is the interfacial dilatational viscosity.

Using (18) in equation (14), we obtain the general case:

$$\frac{dr}{dt} = \frac{-k.C}{r} \left(\gamma_{eq} + 2\varepsilon \ln\left(\frac{r}{\eta}\right) + \frac{2\kappa}{r} \frac{dr}{dt} \right) \quad (19)$$

Analytical integration of (19) is possible but results in a form which is hardly usable for fitting.

We rather treat two cases separately, the purely viscous and purely elastic behaviors.

3.b.3.1 Purely viscous

In this case, $\varepsilon = 0$ in equation (19), which is integrated to give:

$$\underline{t(r) = \frac{r_0^2 - r^2}{2k.\gamma_{eq}.C} - \frac{2\kappa \ln(r / \eta)}{\gamma_{eq}}} \quad (20)$$

3.b.3.2 Purely elastic

In this case, $\kappa = 0$ in equation (19). We make the approximation $\ln(r / \eta) \approx (r / \eta) - 1$ to be able to integrate it. This gives:

$$\underline{t(r) = \frac{2\varepsilon(1 - r / \eta) + (\gamma_{eq} - 2\varepsilon) \ln[1 - (2\varepsilon / \gamma_{eq})(1 - r / \eta)]}{k(2\varepsilon / \eta)^2 C}} \quad (21)$$

3.b.3.3 Comments

Both (20) and (21) are presented as an expression of time because the radius can not be expressed analytically. For this reason, we did not obtain any equation describing $w_s(t)$ for the models integrating interfacial rheology.

4. Results

4.a. HPLC assay

Figure 1 shows the mass percentage of each lipid class during digestion for the TO emulsions. From these results, it appears that the TO digestion is already quite advanced after one hour as more than 40 wt% of TG disappeared and more than 30 wt% MG+FA were produced. Their evolutions are then fast for 4 hours whereas after they only evolve marginally. The percentage of DG is more or less constant. Given the deviations, there is no clear effect of the type and concentration of emulsifier. Converting the mass percent into mol percent, it was checked that MG and FA are produced with a molar ratio close to 1:2 throughout the digestion.

Figure 2 shows the disappearance of the TG during digestion for the TC emulsions. Most of the TG was digested within the first hour. Then its evolution is fast for 4 hours whereas after it only evolves marginally, as for TO emulsions. After 6 hours of digestion, the residual TC percentages tend towards 1-2 wt%, much smaller than the TO ones around 15 wt%. From 2 hours on, a significant effect of the excess emulsifier is seen, the emulsions containing more emulsifier being digested more quickly.

4.b. pH-stat titration

Figures 3 and 4 show the release of fatty acid during digestion, calculated in mol%, what is equivalent to wt% because it is relative to the total amount of the fatty acid. Comparing TC and TO emulsions, the latter are clearly digested more slowly and to a lower extent. Contrary to the HPLC results, the pH-stat titration evidences an effect of the emulsifier, which is the same for TC and TO emulsions. The FA release kinetics indeed follows the order $\beta\text{LG}^+ >$

β LG > NaO > NaO+. For TC emulsions, the FA release was not monitored above 80 % except for β LG+ which reaches 90 % after about 2 hours then slowly tends towards 90-95 %.

4.c. DLS sizing

Figures 5 and 6 show the DLS volume-based mean droplet diameter versus the digestion time. Given the deviations, the freshly made emulsions have a similar mean droplet diameter except TC-NaO(+) for which it is significantly smaller. Comparing the size evolution, the significant effects are those of the triglyceride and of the emulsifier type but no effect is seen with an excess of emulsifier. After 6-7 hours of digestion, the emulsions reach a similar diameter only depending on the triglyceride, of $40 \pm 2 \text{ nm}$ for TC and $54 \pm 4 \text{ nm}$ for TO.

4.d. Mechanistic models

All models developed in this article were tested to fit the experimental results of FA release and mean droplet diameter. The model of Li and McClements⁸ for the FA release (eq. 11 in their article) was included for comparison. Figures 3-6 show the best fits for these models together with the fitting parameters values, essentially the mass transfer coefficients k .

As seen from the curves shape and the coefficient of determination values, the best results for the mean droplet diameter are obtained for first order kinetics including the interfacial dilatational viscoelasticity, especially viscosity. Only the TC- β LG cases are not well fitted by this model, unable to reproduce the sudden slowing of the droplet decrease around 5400 s.

For the FA release, the best results are obtained for the zeroth order kinetics of Li and McClements⁸ and our initial first order kinetics. Similarly the HPLC assay of the digestion end products (MG+FA) was fitted as the average of all TO emulsions. Again, the same two models give the best results, as shown in figure 7.

Note that all mass fraction models were fitted imposing a 100 % FA release as a long time limit. This enables a direct comparison between the fitted k values, and does not change the ranking of the models compared to a free limit fitting. Such free limit fitting gives coefficient

of determination values above 0.9 for all models (results not shown), demonstrating the overall adequacy of mass transfer models.

4.e. Drop tensiometry

Interfacial tension curves as a function of time were obtained for single droplets. Representative curves for TO are presented in figure 8. We are interested in the digestion part. It always shows two characteristics: an instantaneous decrease followed by a gradual decrease. This was also seen in another study,¹⁸ in which a mouth and a gastric steps preceded the intestinal step. However, because we compare different emulsifiers or even no emulsifier at all, the interfacial tensions before digestion are usually different. In order to compare the systems quantitatively, we introduce the interfacial tension variation $\Delta\gamma$. To plot the interfacial tension variation (difference), the origin of time is set at the beginning of the digestion step (a few seconds after the addition of the medium) and the interfacial tension is lowered by the value at the end of the previous step (a few seconds before the addition of the medium).

Figure 9 shows $\Delta\gamma$ as a function of the digestion time for all formulations plus a control one for which pancreatic lipase was not included. Without lipase (bile salt alone), the instantaneous interfacial tension decrease is the same than with lipase, showing that bile salt adsorption is responsible for this instantaneous decrease. The interfacial tension then re-increases and stabilizes, in contrast with the usual gradual decrease in the presence of lipase, which can thus be attributed to MG and FA production.¹⁹ The overall variation of the interfacial tension is always larger for TO compared to TC and there is no clear effect of the emulsifier type.

To further characterize the interface, we use the Henry isotherm equation²⁰ to calculate the interfacial concentration Γ during digestion:

$$\Gamma = \frac{\Pi}{2N_A k_B T_K} M_s \quad (22)$$

where Π is an interfacial pressure (i.e. an interfacial tension difference) and M_s is used to convert mol into mass. As the Henry isotherm only characterizes one interfacial molecule, Π does not equal $\Delta\gamma$, but is taken as the interfacial tension difference starting just after the bile salt adsorption, for instance from the arrow in figure 9. Considering the gradual decrease is due to MG and FA production, the M_s we use is an average for these two products. Figure 10 shows the interfacial concentration of MG+FA as a function of digestion time. This is another way to quantify the effect of the formulation, Γ being larger for TO compared to TC but similar for all emulsifier types (including no emulsifier).

To go further in the analysis of this single droplet digestion, we use the droplet injected or withdrawn volume, which is a measured quantity. In the digestion context, the injection is a measure of the volume transferred from the droplet into the micellar aqueous phase at constant interfacial area, thus at constant droplet volume because its shape is only slightly changed. Figure 11 shows the volume injection/withdrawal normalized by the interfacial area. Positive values mean that oil was injected from the syringe into the droplet whereas negative values mean that oil was withdrawn from the droplet into the syringe. Starting at 0 at the beginning of the digestion, we can see there is mostly injection. This means that digestion products transferred into the micellar aqueous phase, replaced by TG from the syringe. As expected for TC without lipase, there were no digestion products so there was no transfer. The volume withdrawal is the one needed to keep the interfacial area constant as the droplet slightly changes shape. This is also why a slight volume withdrawal is seen at the beginning of digestion for the TO droplets.

To fully quantify the transfer of digestion products into the micellar aqueous phase (release), we re-plot these data as the mass of micellar MG+FA normalized by the interfacial area,

considering that this mass is equivalent to the mass of injected TG. To enable the comparison between TC and TO, we shift the TO results up to cancel the droplet shape effect at the beginning of the digestion. The curves are shown in figure 12. The trends are qualitatively similar as in figure 11, showing that the release out of TC droplets is faster than that out of TO droplets. Moreover, there is an effect of the emulsifier, which is the same for TC and TO droplets. The release kinetics indeed follows the order $\beta\text{LG} > \text{NaO} > \text{No Emulsifier}$.

Finally, table 1 gives the values of the measured interfacial dilatational moduli before the digestion and at its end.

5. Discussion

All the techniques in this study were used to follow the kinetics of digestion. Nevertheless, some experimental conditions vary from one to another. So we start by comparing the results qualitatively. First, the dominant parameter is the type of TG, and all methods agree on faster kinetics for TC compared to TO emulsions, confirming our results using other techniques¹⁰ or real fish oils,¹⁸ as well as the results of Li and McClements.⁸ The role of the emulsifier is less clear, however both pH-stat and single droplet experiment show that βLG induces a faster digestion compared to NaO. This result is similar to that of Mun et al.⁶ finding faster digestion using whey protein isolate WPI (similar to βLG) compared to Tween (similar to NaO). This is also in agreement with Reis et al.⁷ finding a much faster digestion using βLG compared to 2-monopalmitin (similar to NaO). Nevertheless, two other articles report a minor effect of these emulsifiers on the digestion kinetics.^{8,9} Concerning the excess of emulsifier in the bulk, our results show only a small effect except for the pH-stat measurements, for which $\beta\text{LG}+ > \beta\text{LG}$ and $\text{NaO} > \text{NaO}+$.

Now using the models, we compare the mass transfer coefficients obtained from the mean droplet diameter and the pH-stat measurements. For the TC emulsions, those are correlated,

confirming that β LG emulsions are more quickly digested than NaO ones, and also that β LG+ > β LG and NaO > NaO+. For TO emulsions, the mass transfer coefficients are only correlated for the emulsifier concentration, confirming that NaO > NaO+ and β LG+ > β LG. For the type of emulsifier, the mean droplet diameter measurements show much less effect. Concerning the mass transfer coefficient values, they are statistically in the range 3-70 $nm.s^{-1}$ for the mean droplet diameter measurements and 0.008-0.2 $nm.s^{-1}$ for the pH-stat measurements when first order kinetics models are used. Such a difference is not surprising as the emulsions are much more diluted for the mean droplet diameter measurements ($\phi = 0.001$), what induces larger mass transfer coefficients as already found.⁸ In good agreement with our results, Ariyaprakai and Dungan^{21,22} reported mass transfer coefficients in the range 1-100 $nm.s^{-1}$ for different emulsified alkanes solubilized in Tween micelles at comparable low ϕ . They found the mass transfer coefficient to increase as the alkane aqueous solubility increased. As already pointed out in other articles,^{10,18} this applies to our results and many others.^{8,23,24} For the zeroth order kinetics models, the mass transfer coefficients are statistically in the range 4-80 $nmol.m^{-2}.s^{-1}$, only slightly depending on the type of measurement. This is because the mass transfer coefficient is constructed to characterize the transfer per unit interfacial area, which is the only parameter that changes between the two measurements because of the dilution. So the coefficients are now normalized and thus show a better agreement, even though they still slightly depend on the interfacial area.⁸ Compared to those found by Li and McClements,⁸ they are 10 to 100 times smaller. All other parameters being comparable, the main factor is likely the absence of $CaCl_2$ in our digestion medium, which is known to accelerate the digestion, indeed much faster in their conditions. Concerning the models including the interfacial dilatational viscoelasticity, which usually give very good results, we now comment the second fitted parameter. For the elastic behavior, the normalized interfacial dilatational elasticity is close to 0.5, which is within the ones measured using tensiometry before and at

the end of the digestion. For the viscous behavior, the normalized interfacial dilatational viscosity is in the range 2600-6100 s, whereas the measured ones lie in the range 1-10 s. It was found that a value of 0.5 for the normalized interfacial dilatational elasticity is already enough to slow down a solubilization, whereas a high value for the normalized interfacial dilatational viscosity is needed.^{16,25,26} This does not seem to be the case in our experiments, except if such a viscosity is due to a slow process. Concerning the predictions of the fitted parameters effects, all models are in agreement with that of Li and McClements,⁸ predicting an increase of the mass transfer coefficient k with two factors: a decreasing molar mass of the lipids and an increasing initial droplet size. Contrary to the zeroth order kinetics models, the first order ones include the initial dispersed volume fraction (and can also include the interfacial tension and dilatational viscoelasticity), of which the decreases are predicted to result in an increasing k . These predictions are in good agreement with the experimental data trends, although some are more difficult to interpret due to multiple parameters variation (for instance comparing TC-NaO(+) and TO-NaO(+) for which the molar mass, the initial droplet size and the interfacial properties are different). The predicted influence of the initial droplet size is in apparent contradiction with most literature reporting a slower digestion with bigger droplets.^{3,4} In fact, one has to keep in mind that the initial droplet size never varies alone because it has dependant variables, which are related as $r_0 = \sqrt{A_{i0} / 4\pi N_0}$, and also to the dispersed volume fraction (see eq. 4). For example, in emulsion production (including bile emulsification), the dispersed volume fraction is fixed and the other parameters usually vary altogether as droplets are split, resulting in the decrease of r and in the increase of A_d and N . In the example of a dispersed phase solubilization, N is fixed and all the other parameters decrease. A more correct way to express the size influence is then that the mass transfer coefficient k increases with decreasing initial number of droplets and increasing initial

interfacial area, in fact in agreement with the size (sometimes explicitly the interfacial area) influence reported in the literature.^{3,4,9}

To finish, we focus on the single droplet digestion experiment to interpret our results according to the interfacial behavior. It was seen that such an experiment allows the simultaneous measurements of the digestion products adsorption at the oil/water interface and solubilization into the micellar aqueous phase. Comparing figures 10 and 12, it appears that the limiting process is the solubilization one, as it is linear with time, indicating that enough digestion products are available for solubilization. Moreover, it is delayed and slower for the TO droplets, whereas the interfacial concentration increases faster and higher for these droplets, resulting in the saturation of the interface with digestion products. This is in agreement with results indicating a much higher solubilization capacity (more than 10 fold) in bile salts of 1-monocaprylin compared to 1-monoolein,²³ or oleic acid,²⁴ whereas the pancreatic lipase activity on TC is about 2 fold that on TO.^{27,28}

The interfacial concentrations of 0.34 ± 0.02 and $0.16 \pm 0.02 \text{ mg.m}^{-2}$ for TO and TC droplets only represent the digestion products concentrations, neglecting lipase and bile salt. An estimation of the total interfacial concentration can be obtained from the experiments without emulsifier, considering that bile salt is responsible for the instantaneous interfacial tension decrease (figure 9). Using (22) with the molar mass of NaGDC, the interfacial concentration of bile salt is calculated to be $1.47 \pm 0.03 \text{ mg.m}^{-2}$. The total interfacial concentrations are then of 1.81 and 1.63 mg.m^{-2} , which is an usual order of magnitude.^{5,29,30} The digestion products thus account for about 19 wt% and 10 wt% of the interface for TO and TC droplets.

Overall, all techniques agree on faster kinetics for TC compared to TO, as we already found using other experiments.^{10,18} Most of the techniques indicate faster kinetics for β LG compared to NaO, and no effect of the emulsifier excess. These emulsifier effects would perhaps be more important in other concentrations ranges, as found by Reis et al.⁷ Some identical

quantities can display very different values, as seen for the FA release obtained using a HPLC assay or a pH-stat titration. The latter indeed always gives lower values, as was also seen by Helbig et al.³¹ The accumulation of digestion products at the interface might explain a part of the discrepancy (10-20 %). Another reason might be the presence of vesicles,¹⁰ inside which a significant part of the protons is located.

6. Conclusion

In this study, we compared the *in vitro* digestion of emulsions with different formulations using various techniques. Mechanistic models were derived to quantitatively interpret the data. An experimental model based on the digestion of a single droplet was designed.

All techniques showed that the type of triglyceride is the dominant parameter explaining the emulsion digestion, and the models revealed that the molar mass and interfacial properties were responsible for this effect. The use of models was also helpful to discriminate the roles of various factors and compare the data from different experiments and also from the literature. The best results were obtained using first order kinetics including interfacial dilatational viscoelasticity.

Going further with these models now requires the inclusion of structural elements such as the formation of vesicles and micelles.

Using the single droplet experiment, a comparable digestion products adsorption, but a delayed and slower solubilization into the bile micelles were found for TO compared to TC. Many techniques showed that β LG induces faster digestion kinetics than NaO. No clear effect of the emulsifier excess was observed.

Our results also raised questions concerning the pH-stat method which could be very sensitive to the emulsifier used and the structures formed during the digestion. We indeed confirmed that it systematically gives values lower than those using chromatography.

Large-scale radiation techniques are currently explored to investigate the fast structural changes during digestion.

Acknowledgments

We thank Michèle Viau and Hernan Kenmogne-Domguia for their help with HPLC.

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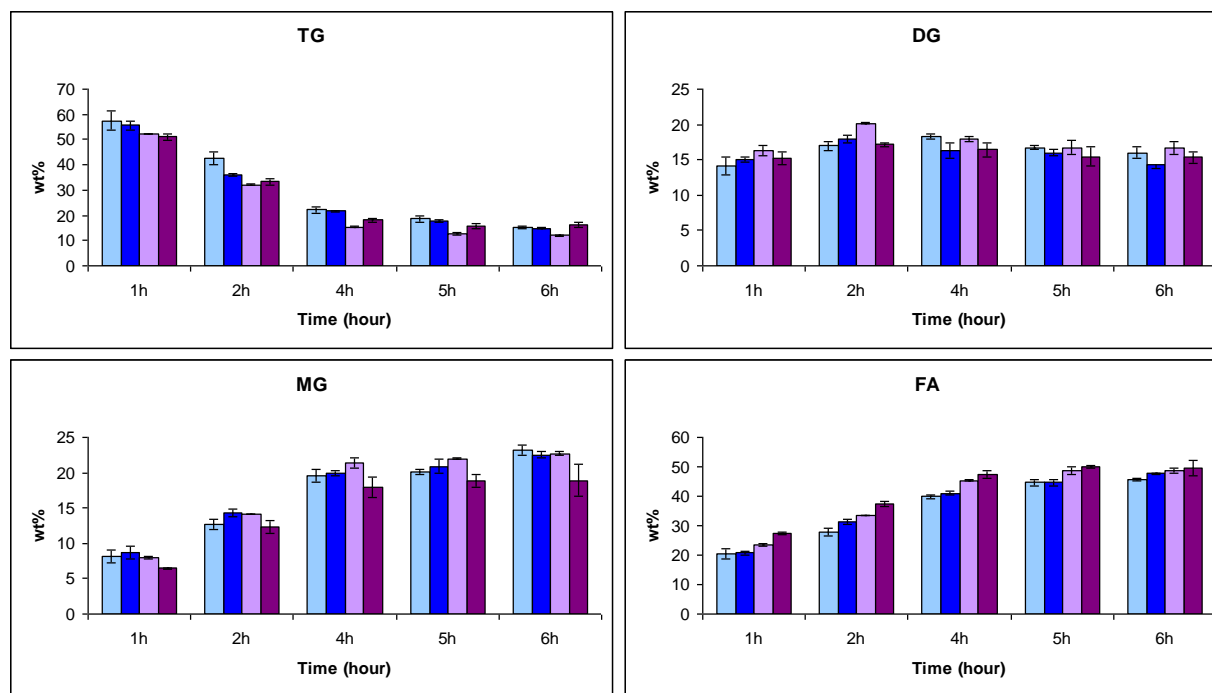


Figure 1: Mass percentage of extracted lipid classes as a function of digestion time for TO emulsions, stabilized by β LG, β LG+, NaO or NaO+, from left to right for each time.

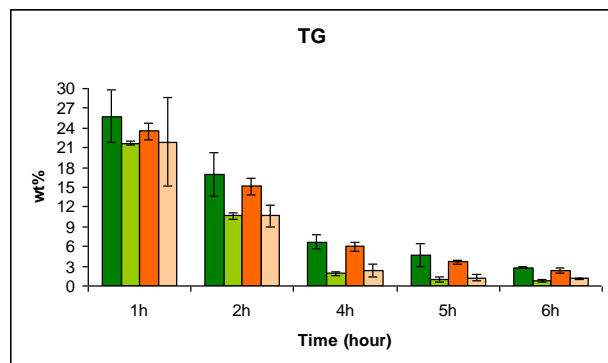


Figure 2: Mass percentage of triglyceride as a function of digestion time for TC emulsions, stabilized by β LG, β LG+, NaO or NaO+, from left to right for each time.

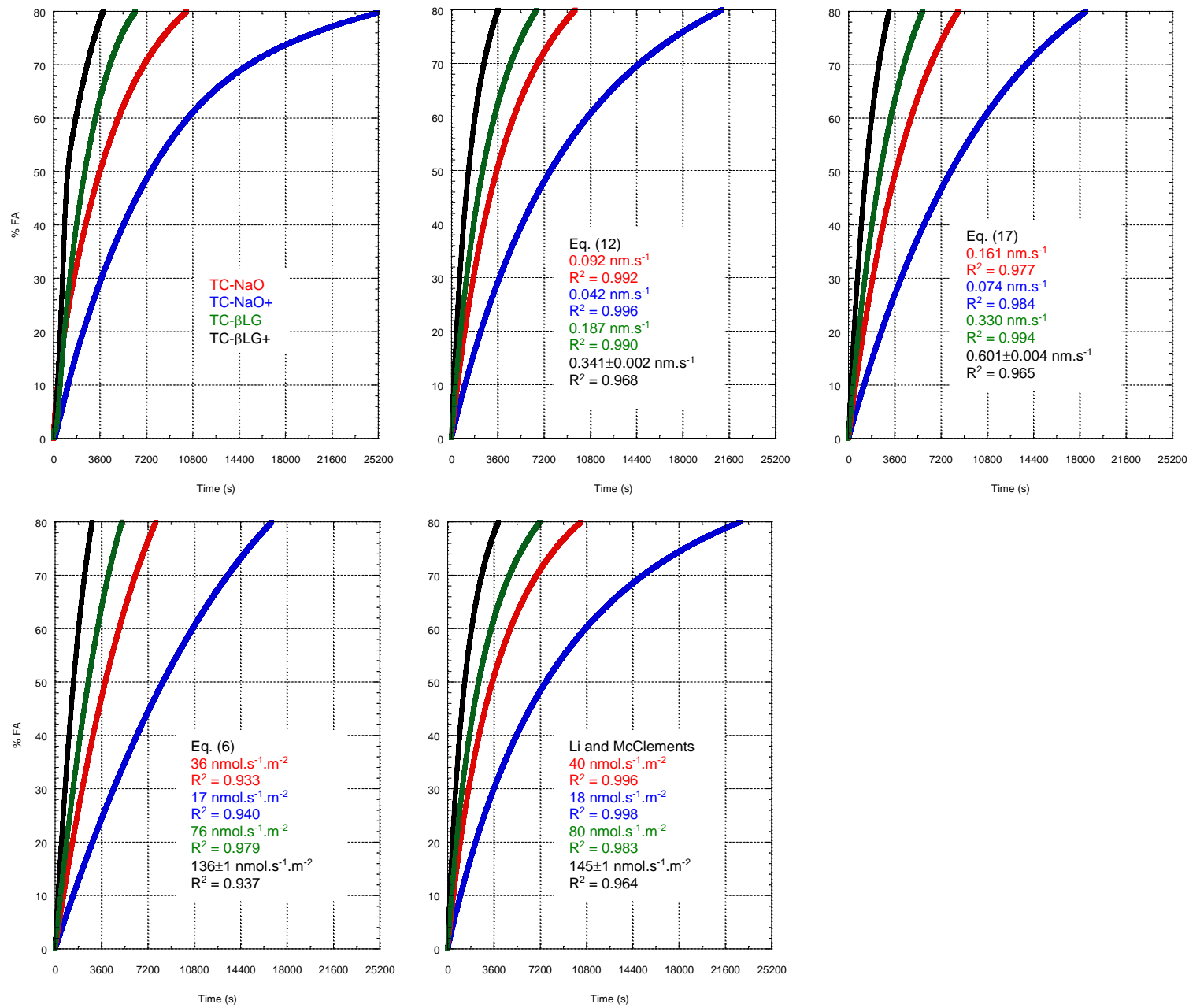


Figure 3: Top left: Mol% of fatty acids released during digestion, obtained by averaging the data of two digestions of TC emulsions, stabilized by β LG+, β LG, NaO or NaO+, from top to bottom at 7200 s. For clarity, the deviation of about ± 3 mol% is not represented. From left to right (up then down): Models with indication of the fitted mass transfer coefficient and the coefficient of determination.

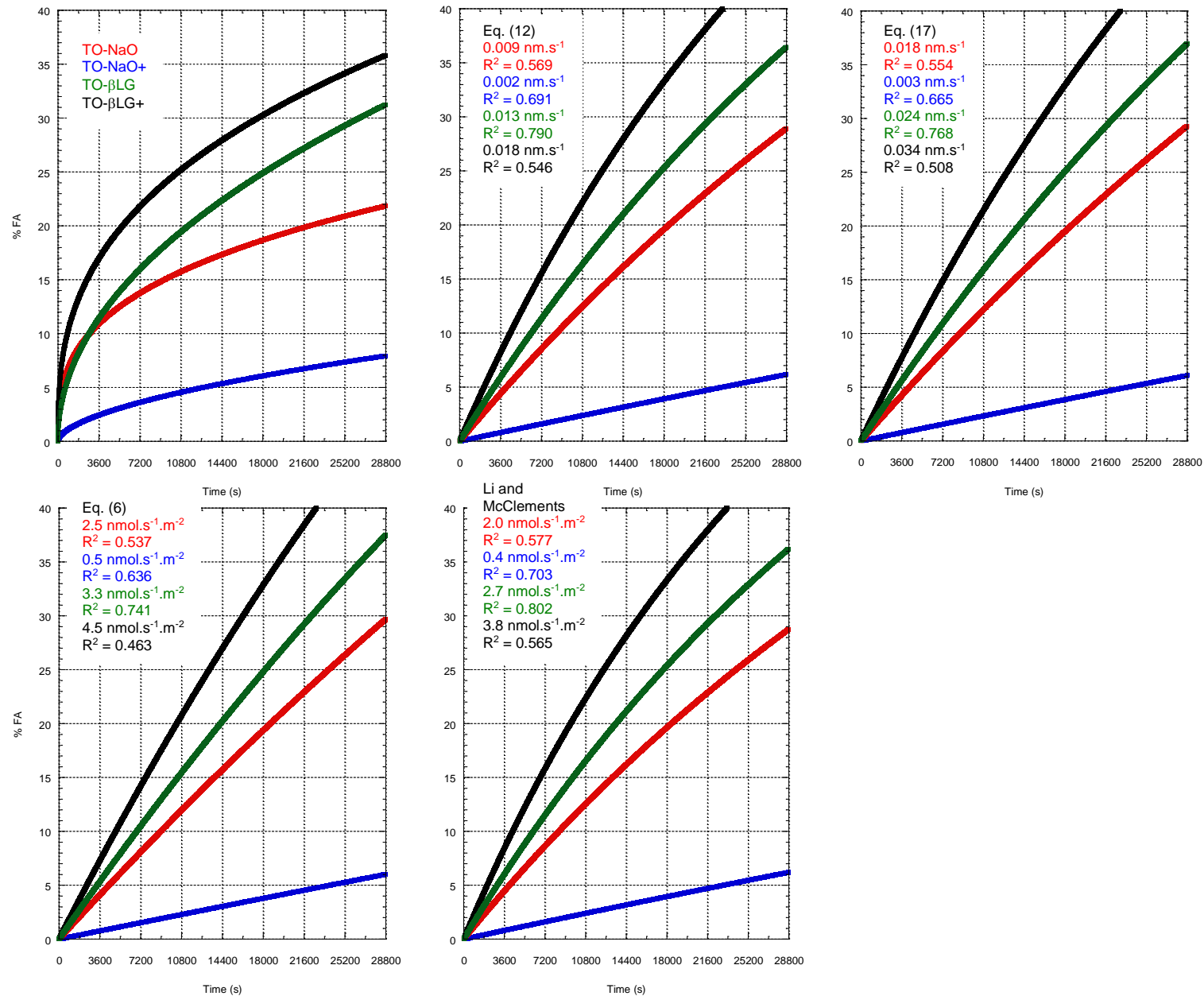


Figure 4: Top left: Same as figure 3 for TO emulsions, stabilized by β LG+, β LG, NaO or NaO+, from top to bottom at 3600 s. For clarity, the deviation of about ± 2 mol% is not represented. From left to right (up then down): Same as figure 3.

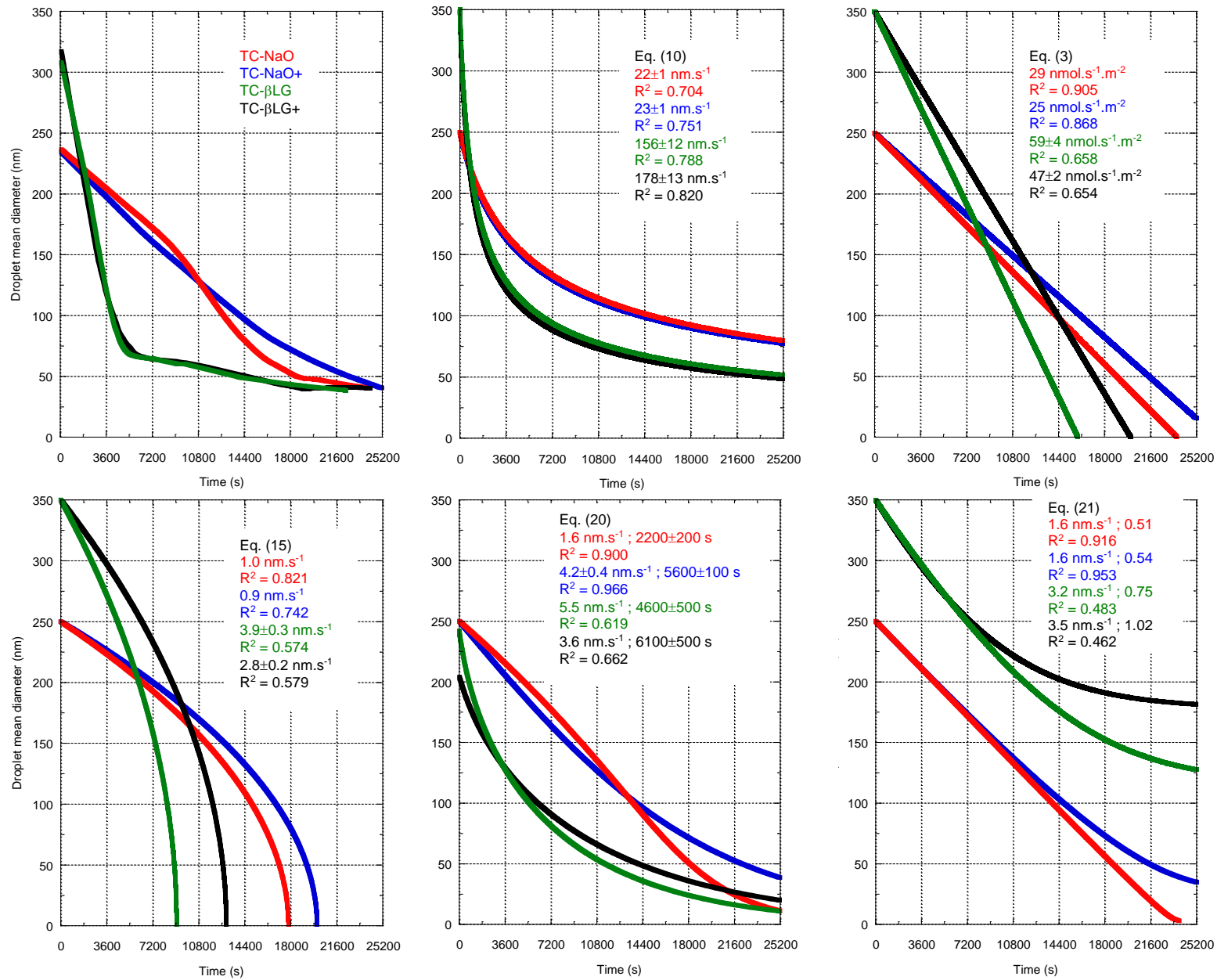


Figure 5: Top left: Experimental DLS volume-based mean droplet diameter variation during digestion, obtained by averaging the data of two digestions of TC emulsions, stabilized by NaO, NaO+, βLG+ or βLG, from top to bottom at 7200 s. For clarity, the deviation is not represented. During the first hour, it is about ± 50 nm. Then, it is about the line thickness.

From left to right (up then down): Models with indication of the fitted mass transfer coefficient and the coefficient of determination. For the last two, the other fitted quantity indicated is κ / γ_{eq} or $\varepsilon / \gamma_{eq}$ respectively.

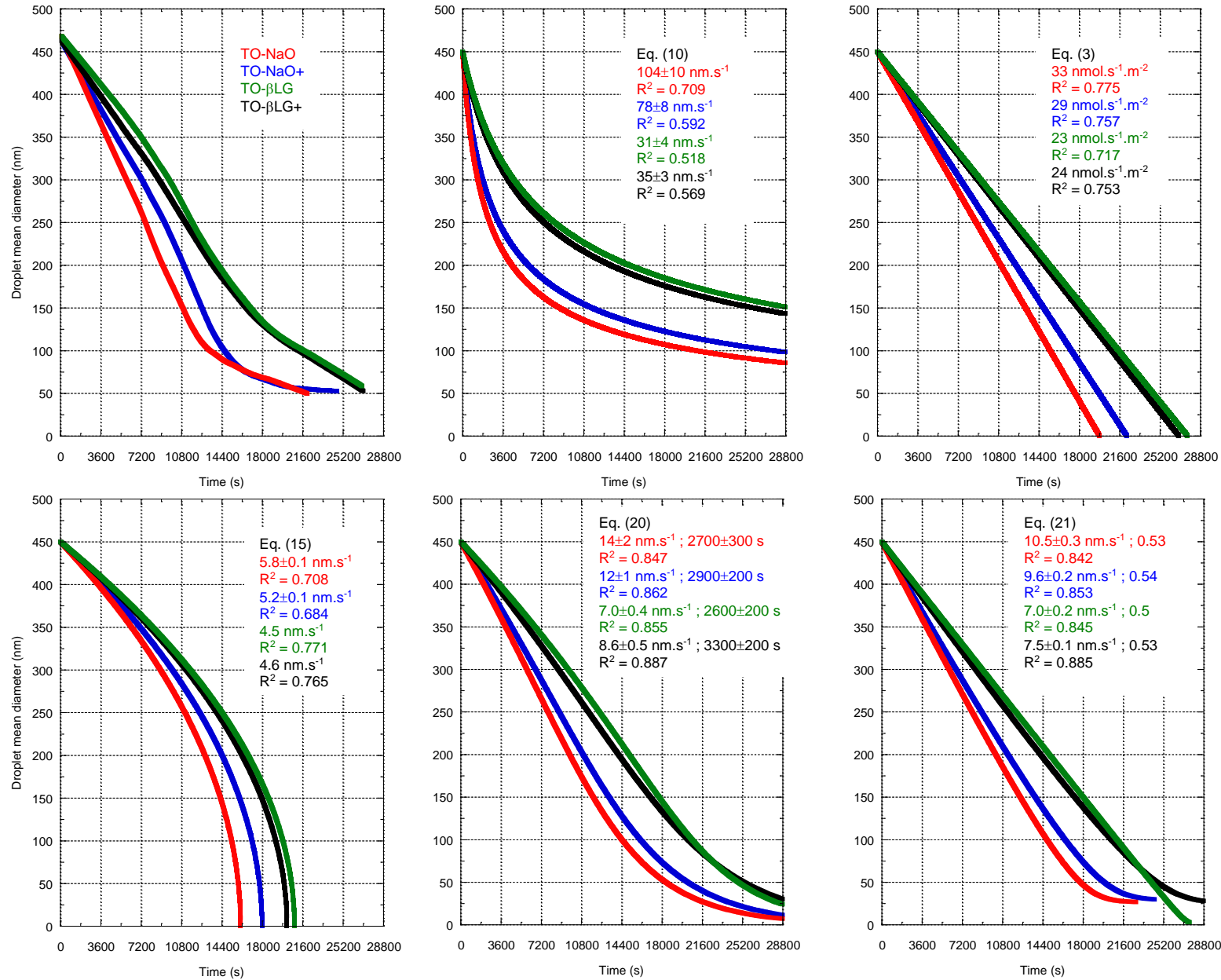


Figure 6: Top left: Same as figure 5 for TO emulsions, stabilized by β LG, β LG+, NaO+ or NaO, from top to bottom at 7200 s. For clarity, the deviation is not represented. During the first hour, it is about $\pm 100 \text{ nm}$. Then, it is about the line thickness. From left to right (up then down): Same as figure 5.

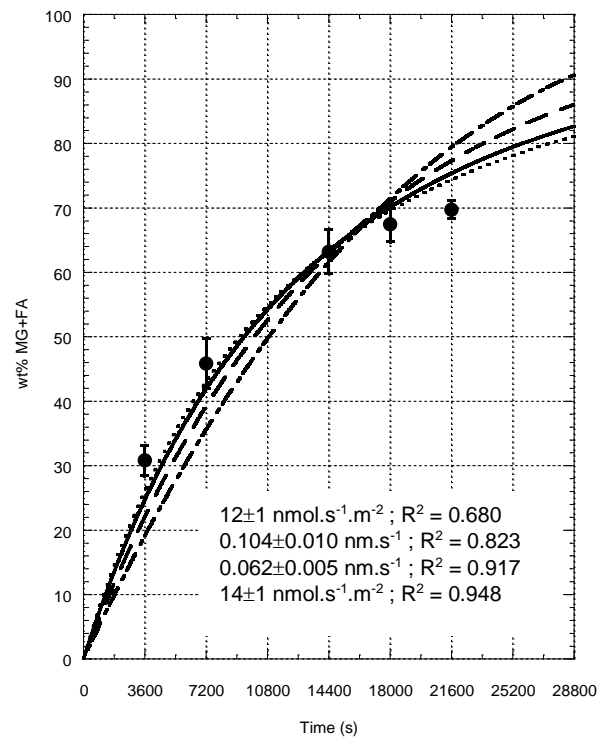


Figure 7: Average mass percentage of MG+FA as a function of digestion time for all TO emulsions, fitted by equation (6), (17), (12) or Li and McClements, from top to bottom at 28800 s, respectively. The fitted mass transfer coefficient and the coefficient of determination are indicated in the same order.

	γ_{eq} before digestion	ε before digestion	$\kappa \cdot \omega$ before digestion	γ_{eq} after digestion	ε after digestion	$\kappa \cdot \omega$ after digestion
TC	24.0 ± 0.6	7.6 ± 3.1	<i>3.4 ± 3.2</i>	2.7 ± 0.2	0.26 ± 0.03	<i>0.03 ± 0.02</i>
TO	23.7 ± 0.6	13 ± 2.6	<i>3.1 ± 2.9</i>	1 ± 0.1	0.09 ± 0.04	0.15 ± 0.02
TC- β LG	10.4 ± 1.3	13.6 ± 2.0	4.8 ± 1.7	2.8 ± 0.1	0.42 ± 0.19	<i>0.14 ± 0.13</i>
TO- β LG	8.7 ± 0.4	14.7 ± 0.6	3.9 ± 0.5	1 ± 0.1	0.19 ± 0.07	0.17 ± 0.03
TC-NaO				2.3 ± 0.1	0.17 ± 0.06	0.13 ± 0.06
TO-NaO				1 ± 0.1	0.17 ± 0.05	0.08 ± 0.04
TC no lipase	24.0 ± 0.6	7.6 ± 3.1	<i>3.4 ± 3.2</i>	6.9 ± 0.2	0.51 ± 0.20	<i>0.19 ± 0.18</i>

Table 1: Interfacial dilatational viscoelastic moduli and interfacial tension measured before and after digestion. The unit is $mN.m^{-1}$. Values in italics are statistically unreliable. The angular frequency is calculated as $\omega = 2\pi f \approx 0.125 \text{ rad.s}^{-1}$.

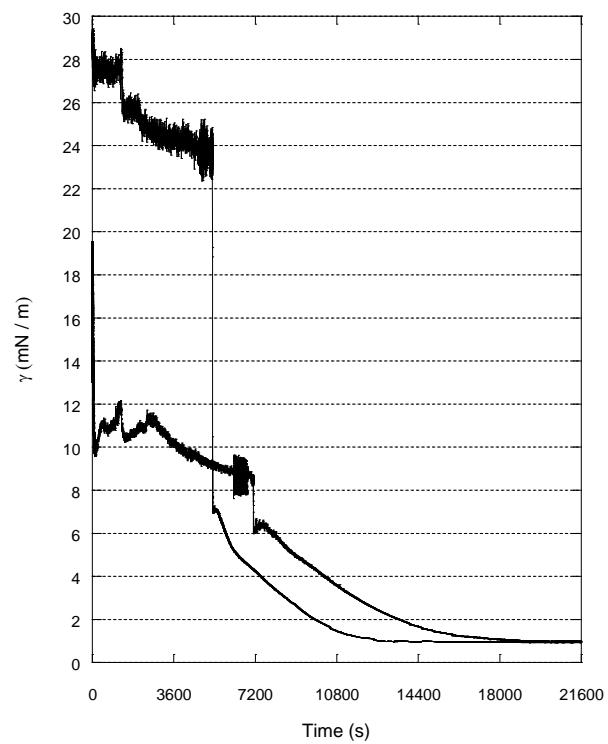


Figure 8: Representative measurements of the interfacial tension as a function of time for TO (top) and TO- β LG (bottom). Notice the small effects of the addition of 100 mM NaH_2PO_4 buffer and of the change of temperature from 25 °C to 37 °C. Notice the instantaneous decrease at the start of the digestion.

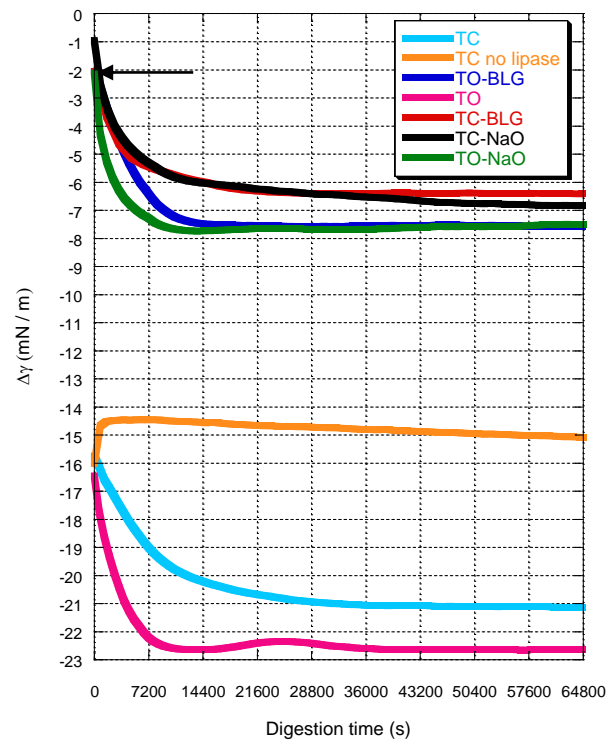


Figure 9: Representative measurements of the interfacial tension variation as a function of digestion time. The deviations are within the line thickness. The arrow shows the magnitude of the instantaneous interfacial tension decrease for TO- β LG, TO-NaO and TC- β LG. See colors for legend.

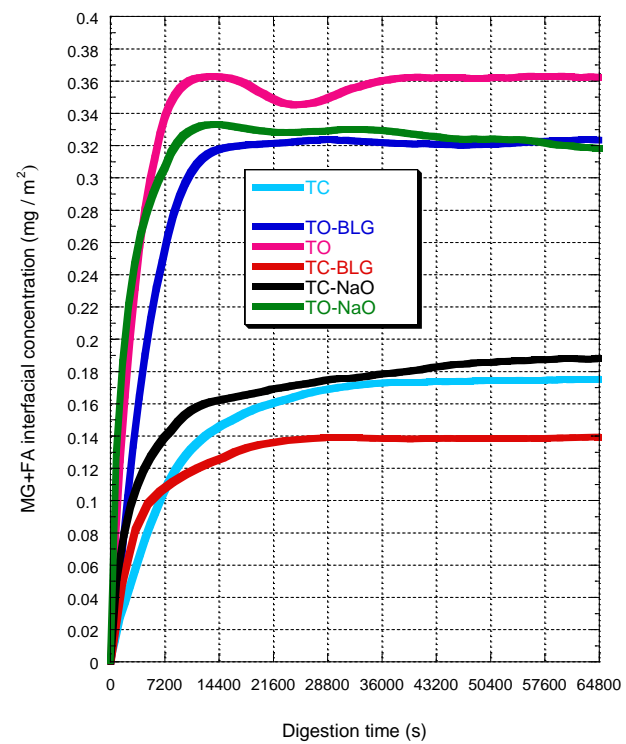


Figure 10: Calculated interfacial concentration of the digestion products MG+FA as a function of digestion time from the data of figure 9. See colors for legend.

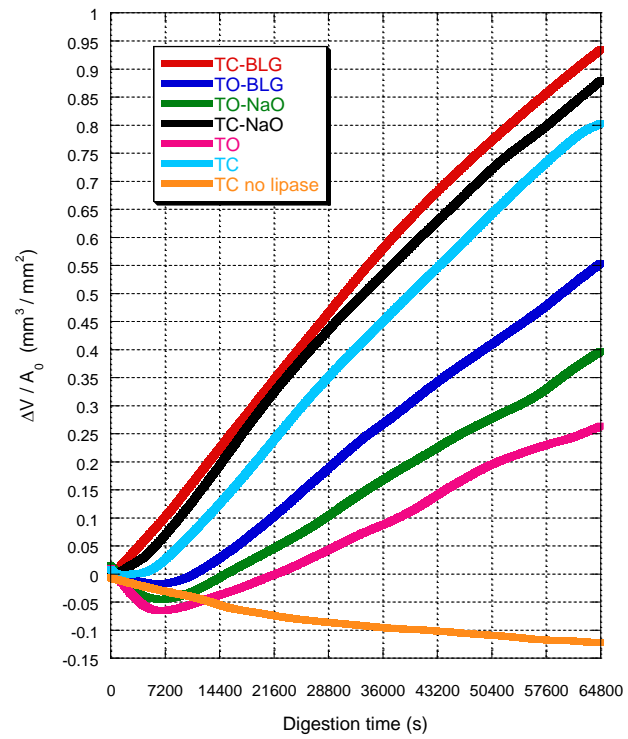


Figure 11: Representative measurements of the volume injection/withdrawal normalized by the droplet interfacial area as a function of digestion time (same experiments as figure 9). The deviations are within the line thickness. See colors for legend.

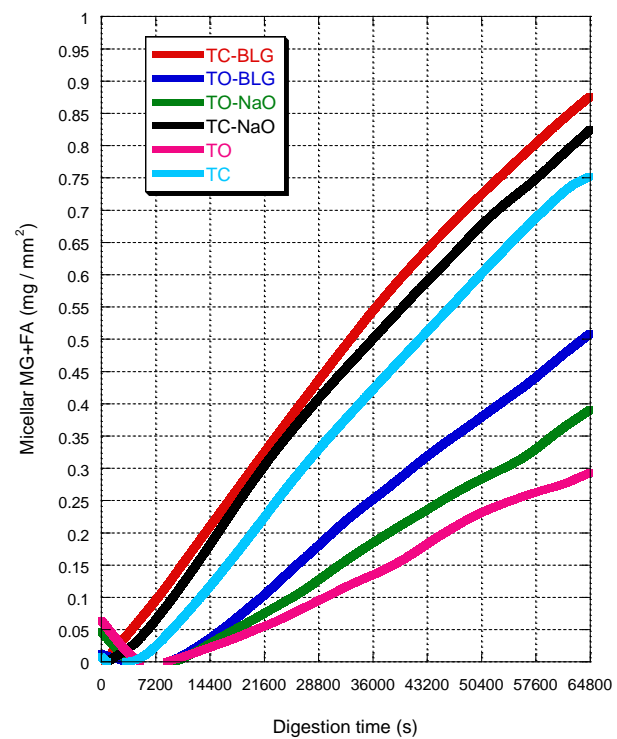


Figure 12: Calculated mass of MG+FA transferred into the micellar aqueous phase normalized by the droplet interfacial area as a function of digestion time from the data of figure 11. See colors for legend.