Food & Function

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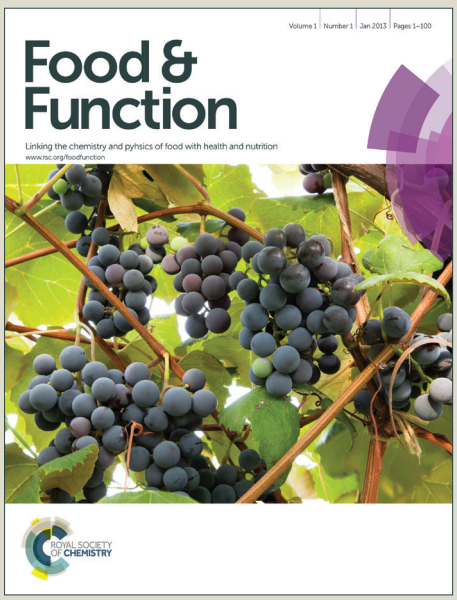

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A microfluidic device to study the digestion of trapped lipid droplets.

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ABSTRACT

At the junction of chemistry, physics and biology, digestion involves many processes. Studying the mechanisms in such a complex system is challenging because numerous interactions coexist. Even in an apparently simple system such as emulsion, many physicochemical characteristics affect lipid digestion. Moreover, these characteristics are difficult to control using conventional in vitro techniques. The goal of this work was to design a microfluidic device allowing the study of well-controlled individual oil droplets in gastrointestinal digestion conditions. Different parameters were investigated in order to validate the relevance of this device compared to conventional in vitro techniques using emulsions. Various triglycerides and digestion conditions were tested with droplets of the same initial diameter generated by a flow focusing device, then placed in individual traps of a microfluidic chamber for digestion with continuous digestive juice renewal. The results are in good agreement with those obtained with conventional in vitro techniques and open the way to screening of lipid digestion, in particular of lipophilic molecules bioaccessibility, a prerequisite for bioavailability studied in nutrition, pharmacology, and toxicology.

1. Introduction

Despite an in-depth knowledge of digestion physiology, the physicochemical aspects related to food composition and structure were poorly investigated. Efforts to include these aspects in the understanding of digestion are recent, mostly focused on lipids, starches and proteins.[1-3]

For lipids, emulsions are widely studied because they constitute a model dispersion that can include other nutrients while keeping good control of the structure. Still, their digestion involves the chemical aspect of hydrolysis (lipolysis when only lipid is studied) and the physical aspect of lipid solubilization in self-assembled structures (vesicles, micelles), the latter being also referred to as lipid bioaccessibility.[3] These aspects are difficult to study simultaneously, especially using conventional in vitro digestion techniques, usually not adapted to investigate interdependent mechanisms.[4] In previous articles, we reported alternatives to monitor digestion processes in situ using diffusion-based techniques on emulsion,[5] tensiometry and simulation techniques on a single droplet.[6-8] Drawbacks were the difficulty to monitor several lipids simultaneously in emulsion, and the large droplet size and non-spherical shape in tensiometry.

The latter issue was solved at the end of the 1990s by the advent of droplet microfluidics, allowing the generation and use of well-controlled individual droplets.[9] However, the application to biological systems is more recent and devices able to trap cells or droplets are quite new. In the context of enzyme kinetics, such devices were first developed to trap cells,[10,11] then adapted to trap water droplets.[12-15] The hydrolysis of a fluorescein galactoside by β-galactosidase[12,13] and the loss of water[13] in these droplets could then be monitored by using fluorescence and microscopy image analysis.

The goal of the present work was to develop a microfluidic device able to generate and trap individual oil droplets coated by β-lactoglobulin, and to study their digestion in terms of lipolysis and solubilization, with the longer term goal to include lipophilic micronutrients. The first challenge was to trap oil droplets in a microfluidic device for the first time, as only water droplets were trapped so far. The second challenge was to develop a microfluidic device allowing dynamic lipid digestion testing. Image analysis was used to monitor droplets of various triglycerides with the same initial diameter in several digestion conditions related to

the gastric and intestinal steps. The findings were compared to results previously obtained using conventional in vitro digestion techniques.

2. Materials and methods

2.a. Materials

Photoresist (SU8-2100) was from MicroChem Corp., provided by CTS (France). Poly(dimethylsiloxane) PDMS (RTV615) was from GE Silicones, provided by Eleco Produits. Propylene glycol methyl ether acetate PGMEA, Amano lipase A (534781, fungal lipase from Aspergillus Niger, 12-15 U/mg), pancreatic lipase (L3126, lipase from porcine pancreas type II, 100-400 U/mg), sodium glycodeoxycholate (G9910, a bile salt), tricaprylin (T9126), triolein (T7140), and highly refined olive oil (O1514) were provided by Sigma-Aldrich. The marine fish oil (EPAX 1050 TG) was from Polaris (France). β-Lactoglobulin was purified from whey protein isolate in our laboratory. In all preparations, Milli-Q water having an electrical resistivity of 18.2 MΩ.cm was used.

2.b. Device fabrication

Microfluidic device was fabricated using PDMS soft lithography techniques.[16] The design of the microstructures was made using Adobe Illustrator software and printed out on transparencies by high resolution printing as photomask in UV lithography. Then a master with positive relief microstructures was produced by coating a silicon wafer with SU8-2100 photoresist using a SPIN150 spin-coater (SPS-Europe). This was prebaked for 5 min at 65°C then for 20 min at 90°C, and placed in a UV LED masker (UV-KUB 2, Kloé) for 40 s through the photomask. After postbaking for 5 min at 65°C then for 10 min at 95°C, microstructures 120 μm in height were developed using PGMEA. To fabricate the microfluidic device, a mixture of PDMS (90%) and crosslinker (10%) was poured on the master and in a Petri dish, and both parts were placed at low pressure (50 mbar) for degassing, then cured overnight at

70°C. The cured PDMS was cut and peeled off the master, then access holes were punched. Both parts were exposed to an oxygen plasma for 3 min using a gas mixer PlasmaFlo and a plasma cleaner PDC-002 (Harrick Plasma) to enable their assembly and make their surfaces hydrophilic. Once treated with the oxygen plasma and assembled in the Petri dish, the microfluidic device was ready for use.

These operations were done for two masters, one for a flow focusing device allowing the formation of droplets, the other one for a digestion chamber containing traps. The fabricated devices were connected using polytetrafluoroethylene (PTFE) tubing (11919445, Fisher Scientific) ended by stainless steel tubing (Coop 23G/15mm, Phymep). The whole microfluidic device and its dimensions, as measured by profilometry, are shown in fig. 1.

2.c. Droplet formation and trapping

The microfluidic device in a Petri dish was placed on a heating plate under an IX51 inverted microscope (Olympus), and observed with a 4x objective. Using PTFE tubing and a Luer lock needle LS22 (Phymep), each inlet of the flow focusing channel was connected to a disposable plastic syringe (Terumo) mounted on a syringe pump PHD 2000 (Harvard Apparatus). To make oil droplets coated by β-lactoglobulin, the oil (several types were tested: tricaprylin, triolein, olive oil, fish oil) was distributed through the central channel and the aqueous phase (7.5 mg/mL β-lactoglobulin in 10 mM NaH_2PO_4 adjusted to pH 7.5) through the two lateral channels. Flow rate for both syringes was initially set to 15 µL/min, then the oil flow rate was decreased to 2 µL/min, producing initial droplets about 300 µm in diameter. Then the aqueous phase flow rate was increased with small 15 µL/min steps in order to maintain flow equilibrium, up to a maximum of 300 µL/min. The final diameter of the droplets in the digestion chamber did not depend on the type of oil, with an overall value of 137 ± 17 µm.

Droplet traps were designed following Huebner et al.,[13] and positioned in the digestion chamber so that initial large droplets do not get stuck, passing through the chamber from one

side or between the traps. As shown in fig. 1, the traps had a droplet capacity of 150 μm, preventing larger or multiple droplets to get in. An exhaust channel of minimum width 10 μm was included, allowing flow through the traps to guide the droplets in.

2.d. Droplet digestion

Once all traps were filled with droplets, the oil flow was stopped and the aqueous phase flow rate was set to 50 μL/min. The temperature of the heating plate was then set to 70 °C, inducing a fluid temperature of 37 ± 1 °C at the outlet hole of the digestion chamber. Then, the flow focusing tubing was replaced with another tubing connected to the digestive juice syringe pump. This tubing was always pre-filled before replacement in order to prevent air from entering the digestion chamber. The digestion was then initiated by flowing the digestive juice through the digestion chamber at 50 μL/min. This flow rate was maintained throughout the digestion experiment.

Digestion consisted in either a gastric step followed by an intestinal step, or an intestinal step alone. When a gastric step was included, it was run for 55 min with a digestive juice of 0.5 mg/mL Amano lipase A in 100 mM KCl adjusted to pH 3.5. Then, replacing the tubing and the syringe pump, the intestinal step was run for 60-180 min with a digestive juice of 0.5 mg/mL pancreatic lipase and 10 mg/mL sodium glycodeoxycholate in 100 mM NaH_2PO_4 adjusted to pH 7.5. At 50 μL/min, the fluid in the digestion chamber was entirely renewed in less than 2 min.

2.e. Digestion analysis

Droplets were observed using a C4742-95 camera (Hamamatsu) integrated to the microscope and controlled by a software developed in-house. Images were recorded automatically with a periodicity of 5 min. The field of view included up to six trapped droplets.

Image analysis was conducted manually by measuring the change of diameter (in pixel) of each droplet as a function of digestion time, then converting the pixel size to μm using a

microscope ruler for calibration. The diameter of each droplet as a function of digestion time was normalized by its initial diameter. The final result was expressed as the average of these normalized diameters for all droplets. The variability was expressed as the amplitude of the individual values: $(Max - Min) / 2$. Two repetitions were conducted, presented separately in the figures in order to visualize the variability between droplets during the same digestion and the variability between two different digestions.

3. Results and discussion

3.a. Microfluidic device

Following the design principles of Huebner et al.[13] for water droplets trapping, optimizations had to be made to adapt to oil droplets trapping. First, the generation of oil droplets required hydrophilic surfaces which were obtained by the oxygen plasma treatment, that substitutes the methyl group in Si-CH_3 by the more polar hydroxyl group to form silanols Si-OH. The persistency of hydrophilicity was tested by generating oil droplets for two days using the same device. Within the day of the microfluidic device fabrication, oil droplets of reproducible size were obtained whereas on the next day, poor size reproducibility or even water droplets were obtained. Thus, the microfluidic device was always fabricated in the morning and used the same day.

Moreover, to produce small oil droplets, a high flow rate was needed for the aqueous phase, 150-fold higher than the oil flow rate (cf. 2.c.). When the outlet of the flow focusing part was connected to the inlet of the digestion chamber, the flow equilibrium was perturbed, interrupting the droplets flow. The digestion chamber was thus connected from the beginning of the droplets generation. So, the positioning of the traps had to let the initial large droplets pass through, hence the spaces let in one side of the digestion chamber and between the traps. Flow equilibration was also improved by adding a winding channel following the flow

focusing junction. During generation, no droplets coalescence was observed in the microfluidic device. At the end of the droplets generation, most traps were occupied by droplets. Occasionally, a few free droplets remained in the digestion chamber, but usually flowed out during digestion.

Design variations of the droplet trap of Huebner et al.[13] were used by the same team for different applications,[14,15] yet always for water droplets. This device was used differently in this work, showing for the first time that oil droplets can be trapped in a PDMS microfluidic device with hydrophilic surfaces obtained by an oxygen plasma treatment. Moreover, this is the first time such a device is used to study the gastrointestinal digestion of oil droplets, of which the results are presented below.

3.b. Droplet digestion

Images of an intestinal digestion of olive oil droplets is presented in fig. 2. First, practical aspects are visible. One is the accumulation of a precipitate in front of the traps, which likely comes from the crude pancreatic lipase extract used, as its absence resulted in no precipitate. The second is the impossibility to analyze some of the droplets, sometimes hidden by the pancreatic lipase precipitate, or rarely sticking to the trap surface, likely because of a local hydrophilicity defect. Most of the time, five droplets could be analyzed throughout the digestion experiment.

Then, concerning the lipid digestion, it proceeded as anticipated, inducing a decrease of the droplet size. Indeed, it is known that triglyceride lipolysis by pancreatic lipase produces fatty acid and monoglyceride, which are soluble in bile salt micelles, thus transferring from the droplet into the aqueous phase.[3] As we used sodium glycodeoxycholate (a bile salt) at a concentration of 10-20 x CMC (critical micelle concentration)[17,18] in the continuously renewing intestinal digestive juice, micelles were always present to solubilize digestion products.

Fig. 3 shows the individual decrease of the diameter of the five droplets during the intestinal digestion presented in fig. 2. Although the initial diameters differed, the trends are very similar. Indeed, once normalized by their initial diameters, the initial variability is suppressed, and only a small digestion variability remains. This also shows that the precipitate did not influence the digestion, as all droplets behaved similarly although some accumulated more precipitate upstream (fig. 2). In the following sections, the effects of various digestion parameters will thus be evidenced using normalized diameters. However, an alternative normalization based on the droplet volume (which is equivalent to the diameter normalization raised to the power 3) is interesting because it gives an indication of the fraction of mass solubilized into the aqueous phase, neglecting the decrease of the oil phase density during digestion.[5] The scale of this volume normalization is indicated in the right-hand ordinate of fig. 3. It shows in this case that more than 99% of the initial olive oil mass was digested in each droplet.

3.c. Effect of the oil type

Fig. 4 compares the intestinal digestion of droplets made of four types of oil, showing that this parameter significantly affected the digestion kinetics. The rate of the decrease of the droplet size followed the order tricaprylin > triolein ≈ olive oil > fish oil. As the main fatty acid in olive oil is oleic acid, this is not surprising that triolein and olive oil digestion kinetics are close. Nevertheless, olive oil digestion is a bit slower, reflecting the mixed constitution of its long chains triglycerides (mainly oleic, linoleic and palmitic acids), worse substrates for lipase compared to pure triolein.[8] For tricaprylin, a medium chain triglyceride, the faster rate was always observed, even though the digestion was usually incomplete because droplets flowed through the exhaust channel of the traps prematurely, due to the low viscosity of the oil phase. The digestion of fish oil, composed of long and very long chains triglycerides,[6]

obeyed especially slow kinetics, with at best 40% diameter decrease after 180 min (in terms of mass, it represents more than 70%).

These results are in agreement with our bulk measurements monitoring emulsion digestion with the same oils, and support our previous conclusions that the type of oil is a key parameter affecting emulsion digestion.[5-8] This was explained in terms of a) lipase specificity on triglycerides, the longer their fatty acids, the lower the lipase activity, and b) digestion products solubilization in bile salts, the longer the fatty acids, the lower the solubilization ratio.[8] The latter article includes references from other research teams showing the same trend in various systems. A recent article, not discussed in ref. 8, shows the same trend for fatty acids absorption in vivo.[19]

In the present experiments, bile salt was always in large excess relative to the mass of digestion products to solubilize. This excess was more than 300-fold even at the higher rates of digestion obtained with tricaprylin droplets. Thus, the results should mainly relate to the lipase specificity on triglycerides, which should be the limiting process. This was confirmed by calculating the maximal fatty acid release rate per droplet surface area,[20] in the linear region of the droplet volume normalization curves. These rates were of 26±2, 57±8, 23±2, and 4.7±0.7 μmol s^{-1} m^{-2} for triolein, tricaprylin, olive oil, and fish oil respectively. Relative to the triolein fatty acid release rate, that of tricaprylin was thus 2.2-fold, that of olive oil 0.9-fold, and that of fish oil 0.18-fold. This is indeed in very good agreement with the relative lipolysis rates found for emulsions in the literature data.[8] Concerning the absolute values, they are about one order of magnitude higher compared to those obtained for similar compositions using emulsions.[20] This could be explained by several facts, mainly that our droplets are highly diluted in an aqueous phase rich in lipase and bile salt, and that our droplets are isolated so no flocculation or coalescence, which would reduce the surface area available for digestion,[1] can occur.

As it is known that the initial droplet diameter also influences the digestion kinetics,[3] we reported the mean value for each experiment in fig. 4. For two repetitions, the mean value only differed significantly for olive oil, but the kinetics was unaffected, so the difference was not large enough to induce an effect. The effect of the oil type was also significant whatever the diameter mean values were, so there was no effect of the minor variations of the droplet diameter in these experiments. Moreover, compared to bulk emulsion droplets, these microfluidic droplets have a diameter more than two orders of magnitude larger, yet the relative lipolysis rates are identical. To study digestion mechanisms, individual droplets is thus a good model extrapolating bulk emulsion droplets.

3.d. Effect of the intestinal juice composition

To emphasize the role of digestive juice constituents, fig. 5 compares the digestion kinetics in presence or absence of pancreatic lipase or bile salt (sodium glycodeoxycholate). As expected, the control experiment in absence of pancreatic lipase resulted in a constant droplet diameter, because tricaprylin was not lipolyzed, thus bile salt micelles had no digestion products to solubilize, not being able to solubilize tricaprylin. In absence of bile salt, pancreatic lipase lipolyzed tricaprylin into caprylic acid and monocaprylin, which have a solubility in water of about 1.2 mg/mL. Thus these digestion products were able to solubilize into the aqueous phase, although at a slower rate than in presence of bile salt. This is because bile salts enhance both pancreatic lipase activity and digestion products solubilization into the aqueous phase.[3] Again, in these experiments the mean value of the initial droplet diameter did not play a significant role.

3.e. Effect of the gastric step

Figure 6.A shows the results of digestions with a gastric step preceding the intestinal step. During the gastric step, the decrease of the droplet size was only seen with tricaprylin, yet the variability is high, showing this was not an efficient process. This is not surprising as the

fungal lipase used has a very low activity compared to the pancreatic lipase, especially towards long chains triglycerides. In terms of mass, it could nevertheless release 10-20% of the initial mass of tricaprylin, but none of the initial mass of fish oil. During the intestinal step following the gastric step, the decrease of the droplet size was repeatable and again did not depend on the mean value of the initial droplet diameter.

To evaluate the effect of the gastric step on the following intestinal step, we renormalized the droplet diameter from the start of the intestinal step and compared it to the previous results of figure 4 with intestinal step alone. The results are shown in figs. 6.B and 6.C, indicating no effect of the gastric step in the conditions tested. This may be explained by the low activity of the fungal lipase, which did not pre-lipolyzed the droplets enough during the gastric step. Thus the bile salt did not have a significant amount of digestion products to solubilize at the very beginning of the intestinal step. Higher concentrations of this fungal lipase could be tested with tricaprylin to reach a release of about 30% of the initial oil mass, which is the average amount produced during the gastric step in vivo.[21] Concerning long chains triglycerides, their insoluble digestion products are known to inhibit lipolysis,[22] so very little release during gastric step was expected in these cases, as confirmed using fish oil.

3.f. Comparison to other in vitro digestion techniques

For food digestion in general, in vitro techniques were reviewed recently,[4] so the discussion here focuses on lipid digestion. The main issue of the widespread static technique is its stoichiometric nature, that is the use of fixed quantities of lipases, bile salts, and triglycerides, in other words of fixed digestive molecules/substrates ratios. For instance, bile salts are often in limiting quantity relative to the expected quantity of digestion products, because neither bile salts are renewed nor digestion products are removed (whereas they are by absorption in vivo). This situation can be partially corrected using dynamic techniques where the quantities are adjusted during digestion. However, this still obeys predefined stoichiometry as the

digestion products are usually not removed. In the most sophisticated versions of dynamic techniques, dialysis membrane or intestinal cell culture can be used to remove digestion products, but even in these situations bile salts may also be removed. Another drawback of all these techniques is the volumes needed for digestion, in the range 10-100 mL for static and reaching 1000 mL or more for dynamic techniques. When testing purified molecules, the resulting cost may be high.

When compared to static in vitro techniques for emulsions, the microfluidic device developed here solves the problem of stoichiometry, as the droplets constituting the substrate are trapped, so that the lipase and bile salt can be continuously renewed and the digestion products continuously removed around the droplets, up to a complete solubilization of the digestion products. This is an important feature, because, although the relative digestion rates obtained using emulsions are in quantitative agreement with our values, a complete solubilization of digested emulsions is seldom reached, especially with long chains triglycerides requiring a higher concentration of bile salts.[6] Another advantage is the control of the fluid mechanics, as the shear rate acting on each droplet is directly proportional to the fluid flow rate. The digestion chamber geometry was thus designed so that the flow rate induces a shear rate in the range found in the literature,[23,24] of 0.23 s^{-1} for the present 50 µL/min flow rate. The effect of the shear rate could in principle be tested by varying the flow rate. However, this would affect in turn the quantity of enzymes brought by the digestive juice, which would thus need to be less concentrated. And changing the geometry would affect the trapping of the droplets. This interdependency of some design parameters is a limitation of this microfluidic device. Another limitation is the impossibility to reproduce certain bulk effects. Only translucent aqueous phases can be used, so dilution might be necessary for a transposition to opaque food matrices. Moreover, as droplets are isolated, the

effect of flocculation and coalescence can not be studied, although those processes influence the digestion rate.[1]

Finally, as our technique is based on microfluidics, the volumes needed are low, of less than 1 mL for the oils and less than 10 mL for the digestive juices. The cost of the microfluidic device preparation being low as well, the overall cost is much lower than that of conventional in vitro techniques. These features make it a promising screening tool in the context of ingested molecules, in nutrition, pharmacology, and toxicology studies.

4. Conclusion

This work showed that a microfluidic device could be used to miniaturize an in vitro digestion setup, with equivalent results and several advantages over conventional techniques. The initial model systems based on droplets studied here can be incremented to include micronutrients (especially lipophilic ones), different proteins and polysaccharides, and their associated enzymes. In preliminary tests, the digestion of lipophilic vitamins inside oil droplets was already successfully monitored using confocal microscopy with spectral imaging. The effects of these constituents will be investigated to understand the interplay between triglyceride droplet digestion and lipophilic micronutrient solubilization (bioaccessibility), in parallel to coarse-grained simulations on the same subject. The effects of the initial droplet size, the shear rate, and of the type of emulsifier (protein, lipid) can also be studied, especially the latter in comparison to droplet tensiometry techniques. To optimize these fundamental studies, improvements of the setup will be made with regard to a) the surface treatment to increase the hydrophilicity persistency, b) the dimensioning of the microfluidic device to obtain different initial droplet sizes, c) the number of droplets analyzed, d) the automation of image analysis. Using this microfluidic device as a screening tool to test the bioaccessibility of purified

lipophilic molecules is also a promising perspective in the fields of nutrition, pharmacology, and toxicology.

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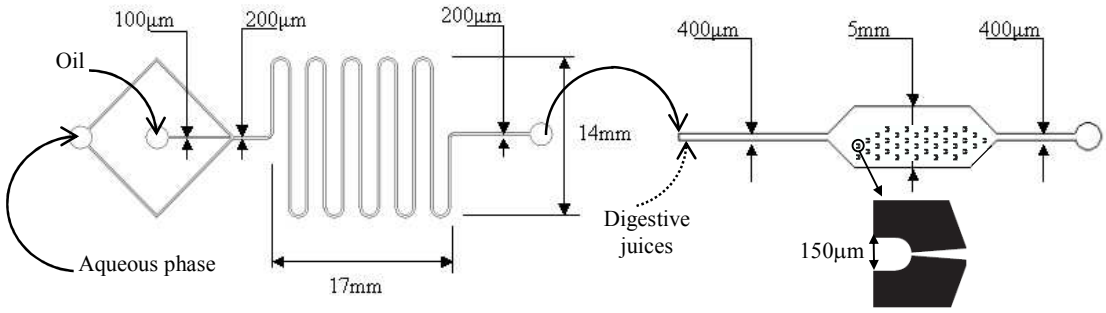


Figure 1: Design of the whole microfluidic device, the left part representing the flow focusing device, the right part representing the digestion chamber, showing the positioning of the traps, and a single trap in a zoomed view.

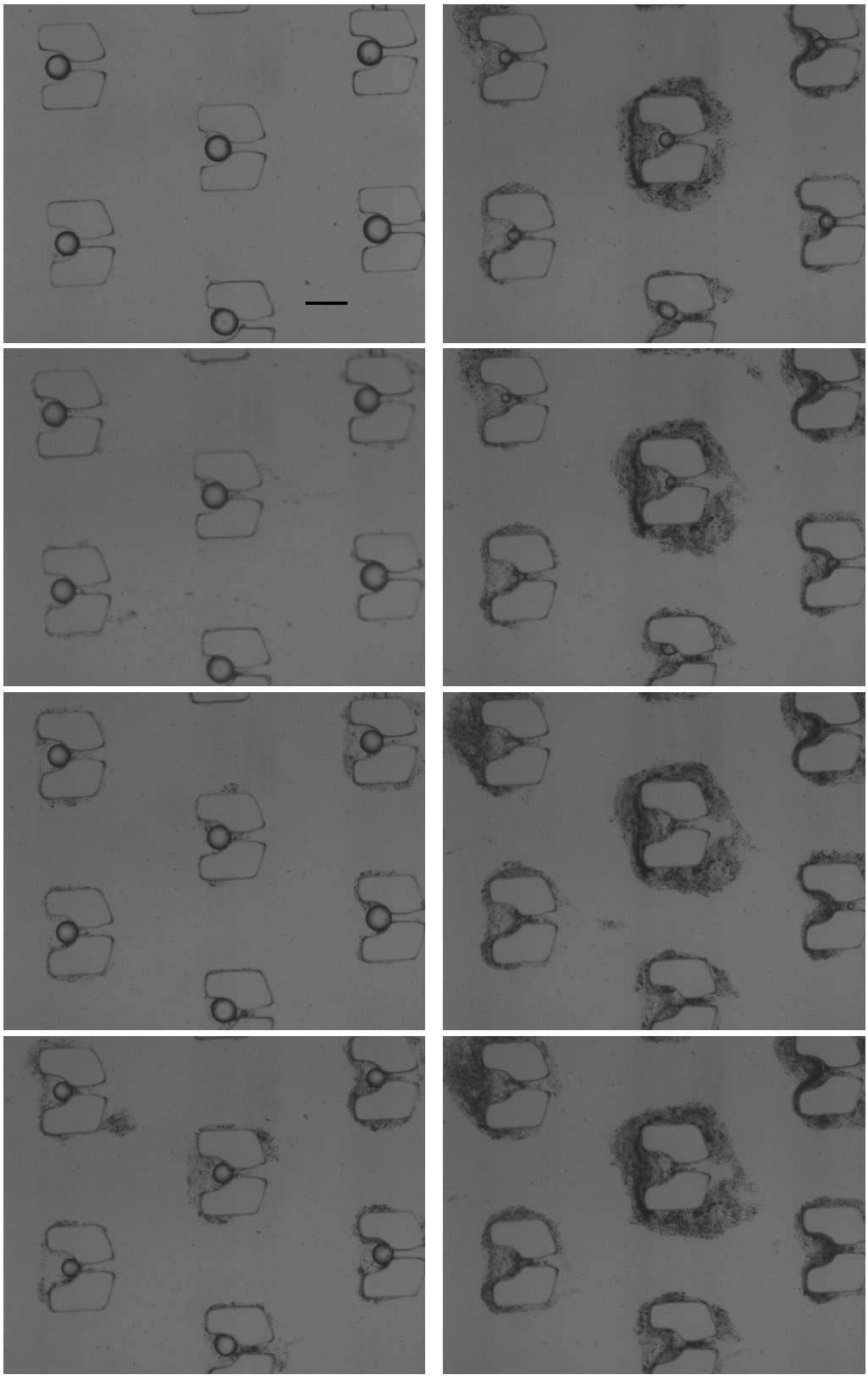


Figure 2: Images showing the intestinal digestion of olive oil droplets coated by β-lactoglobulin at digestion times 0, 15, 30, 45, 60, 75, 90, 105 min, from top to bottom, left then right. Scale bar represents 200 μm.

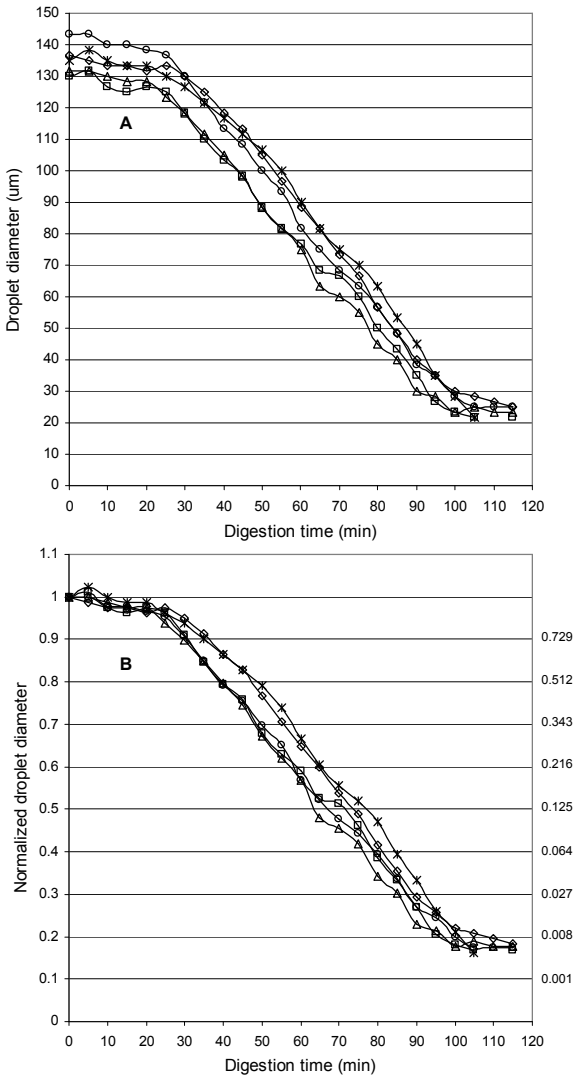


Figure 3: Evolution of droplet diameter (A) and normalized droplet diameter (B) for the digestion showed in figure 2. Right ordinate in (B) shows the normalized droplet volume scale.

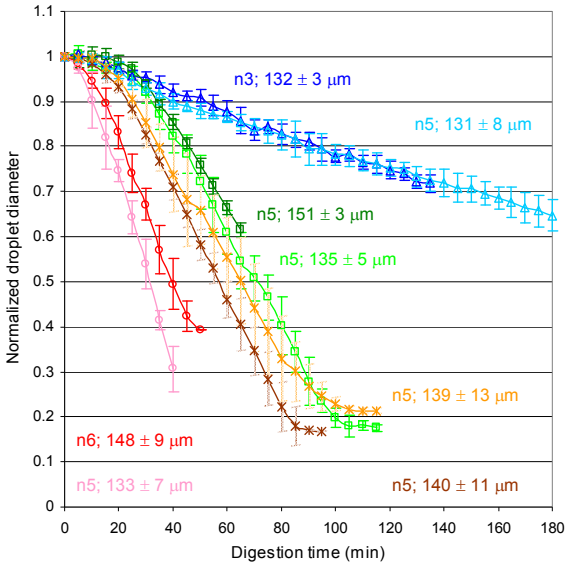


Figure 4: Evolution of the normalized droplet diameter for intestinal digestions of different oils, from top to bottom at 50 min: fish oil (triangle), olive oil (square), triolein (star, light error bar), tricaprylin (circle). Two separate digestions were done for each oil. The number of droplets n, the average initial diameter and its standard deviation are indicated for each digestion near the data points.

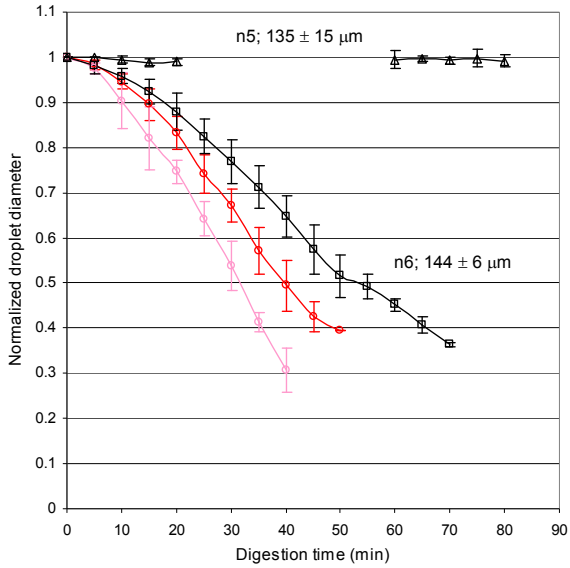
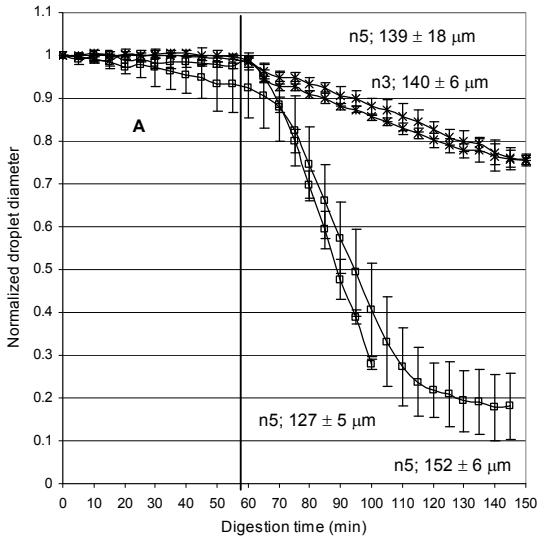
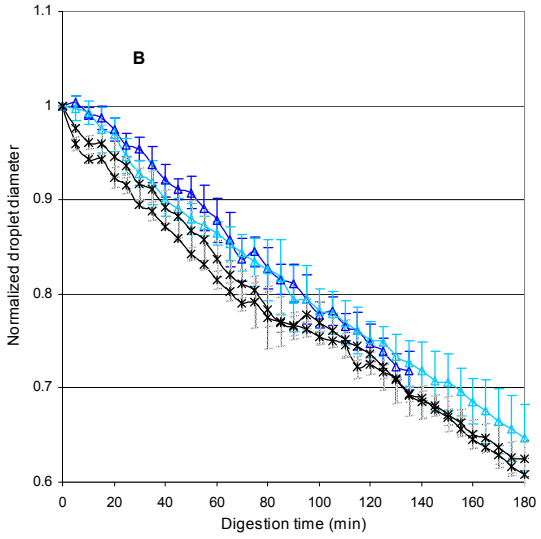


Figure 5: Evolution of the normalized droplet diameter for intestinal digestions of tricaprylin, using different intestinal juices: including pancreatic lipase and sodium glycodeoxycholate (circle, same as figure 4), without pancreatic lipase (triangle), without sodium glycodeoxycholate (square).





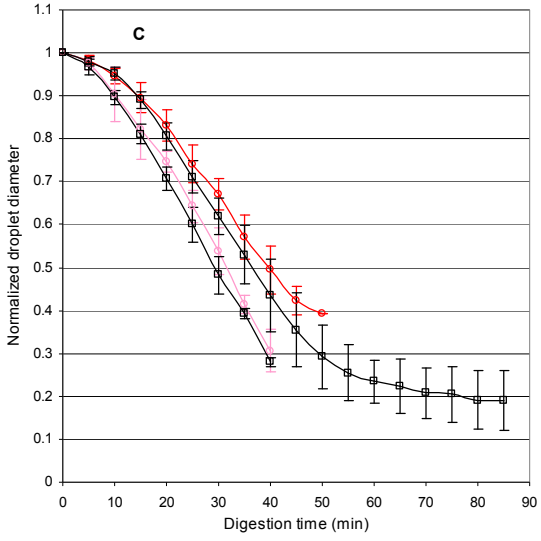


Figure 6: A) Evolution of the normalized droplet diameter during gastric step followed by intestinal step for fish oil (star) and tricaprylin (square), the vertical line showing the gastric/intestinal transition. B) Evolution of the droplet diameter renormalized from the start of the intestinal step for fish oil after the gastric step (star, light error bar) compared to fish oil from figure 4 (no preceding gastric step). C) Same as (B) for tricaprylin (square).

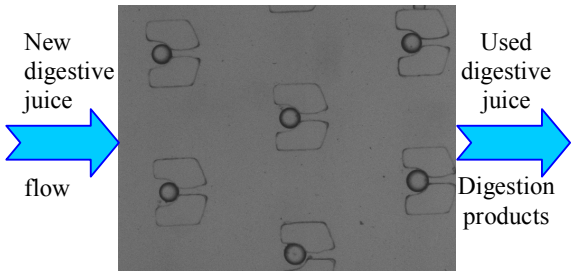


Table of contents entry: Digestion of 140 µm lipid droplets generated and trapped in a microfluidic device, with continuous digestive juice renewal.