Encapsulation of betalain into w/o/w double emulsion and release during *in vitro* intestinal lipid digestion

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ABSTRACT

A water-in-oil-in-water (w/o/w) double emulsion was prepared with water extract of red beet as the inner water phase, rapeseed oil as the oil phase and polysaccharides solution as the outer water phase. Polyglycerol polyricinoleate and polar lipid fraction from oat were used as emulsifiers for primary water-in-oil (w/o) emulsion and secondary w/o/w emulsion, respectively. Their mean droplet sizes were approximately 0.34 µm and 5.5 µm, respectively. The double emulsion showed a high encapsulation efficiency of 89.1 % and had a pink coloration due to encapsulated betalain. The double emulsion was subjected to *in vitro* intestinal lipid digestion and the evolution of structures and release of betalain were monitored. During the first 2 hours of digestion, coalescence of the inner water phase droplets was observed, and the sizes of the double emulsion droplets increased quickly because of aggregation. This period also corresponded to release of betalain, reaching about 35 %. After 3 hours of digestion, no more release was measured, corresponding to no further increase in droplet sizes. In contrast, the encapsulation efficiency and droplet sizes were not affected after 3 hours in the same digestion conditions but without the bile salts and lipase, showing they were responsible for the release.

HIGHLIGHTS

- Betalain encapsulated in the inner water phase of a w/o/w double emulsion
- Effect of *in vitro* lipid digestion on emulsion structure and betalain release studied
- Aggregation of w/o/w droplets and coalescence of w/o droplets during *in vitro* digestion
- 35 % release of betalain during *in vitro* intestinal lipid digestion

KEYWORDS: betalain, double emulsion, encapsulation, *in vitro* intestinal digestion

ABBREVIATIONS

CMC	critical micelle concentration

D3,2	surface mean diameter (Sauter mean diameter)
D4,3	volume mean diameter (De Brouckere mean diameter)
DLS	dynamic light scattering
LD	laser diffraction
NaGDC	sodium glycodeoxycholate
o/w/o	oil-in-water-in-oil (emulsion)
o/w	oil-in-water (emulsion)
PGPR	polyglycerol polyricinoleate
SD	standard deviation
w/o	water-in-oil (emulsion)
w/o/w	water-in-oil-in-water (emulsion)

1. INTRODUCTION

In recent years, there have been reports of artificial colorants and preservatives having relation to hyperactivity in children (McCann et al., 2007; Nigg, Lewis, Edinger, & Falk, 2012). As consumers are becoming more and more aware of health issues, these findings have made natural pigments, such as carotenoids, anthocyanins and betalains, more favorable to be used as food colorants. However, their use as food colorants is hindered by their instability and solubility properties, which narrow the possible applications. Betalains are water-soluble yellow, red or violet natural pigments, which have antioxidative properties, but are sensitive to high temperature, basic or very acidic pH, light, air (oxygen), and high water activity (Cai, Sun, & Corke, 2003; Herbach, Stintzing, & Carle, 2006; Cai, Sun, & Corke, 1998). The stability of betalains, and also other natural pigments, could be improved with encapsulation technologies, for example spray-drying or emulsification (Gandia-Herrero, Jimenez-Atienzar, Cabanes, Garcia-Carmona, & Escribano, 2010; Rodriguez-Huezo, Pedroza-Islas, Prado-Barragan, Beristain, & Vernon-Carter, 2004).

Double (or multiple) emulsions can be described as being emulsions within emulsions. The first phase is dispersed into the second as small droplets, and this emulsion is again dispersed as droplets into a third phase. There are two major types of double emulsions: water-in-oil-in-water (w/o/w) emulsions, which have water droplets dispersed into oil droplets dispersed into a continuous water phase, and oil-in-water-in-oil (o/w/o) emulsions, which have oil droplets dispersed into water droplets dispersed into a continuous oil phase. Since most foods are constituted of an aqueous continuous phase, the w/o/w double emulsions have more potential for food applications. They offer the possibility to incorporate both lipophilic and hydrophilic compounds which are isolated from the surrounding aqueous environment. Besides food industry, the possibilities of double emulsions as encapsulation systems have been extensively studied for the drug and cosmetic industries. (Jiao & Burgess, 2008; Leal-Calderon, Schmitt, & Bibette, 2007)

In our previous research, we used a polar lipid fraction from oat (*Avena sativa*) to produce o/w emulsions, which were colored yellow with lutein (Kaimainen et al., 2012). In that study we showed a rapid creaming of these emulsions, but we have thereafter managed to significantly delay the creaming by adding small amounts of long chain polysaccharides to the emulsions (data not published). In the present research, the first step was to formulate a w/o/w double emulsion encapsulating hydrophilic betalain colorant and using an oat polar lipid emulsifier to produce natural and stable pink-colored emulsions, with the longer term goal to study the color stability during shelf life. As betalain is also a bioactive displaying antioxidative properties *in vivo*, the next step was to follow the evolution of both the betalain encapsulated and the structure of the double emulsion during *in vitro* intestinal digestion, in order to understand its release.

2. MATERIALS AND METHODS

2.1. Materials

The oat polar lipid fraction used as o/w emulsifier was extracted from oat flakes (*Avena sativa*) using a supercritical fluid process described by Aro et al. (Aro, Järvenpää, Könkö, Huopalahti, &

Hietaniemi, 2007). It consists mainly of different glycolipids (monogalactosyldiacylglycerol, digalactosyldiacylglycerol and steryl glucoside) and phospholipids (phosphatidyl choline). The polyglycerol polyricinoleate (PGPR) used as w/o emulsifier was a sample of PGPR 4175 received from Palsgaard (Juelsminde, Denmark). The betalain pigment was extracted with hot water (70 °C, 30 min) from red beets (*Beta vulgaris*) bought at a local grocery store (ratio of water:beet was 2:1). After extraction, the solid material was filtered out through a filter paper under vacuum. The extract was further concentrated to 60 % of original volume with a rotary evaporator at 50 °C, 7-8 kPa. The rapeseed oil used for the oil phase of double emulsion was bought at a local grocery store and used as such without any purification. For making the buffer solution, citric acid monohydrate was purchased from Carlo Erba Reagenti (Milano, Italy) and disodium hydrogen phosphate heptahydrate from Riedel-de Haën (Seelze, Germany), and both salts were of analytical grade. Guar gum (Meypro Rein guarin) was purchased from Meyhall Chemical AB (Kreuzlingen, Switzerland) and xanthan gum (Rhodigel® xanthane) from Rhodia (Lyon, France). For *in vitro* digestion, sodium glycodeoxycholate, NaGDC (G9910) and pancreatic lipase type II (L3126, activity 100-400 units/mg protein, using olive oil) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Water used was purified reverse osmosis water (Milli-Q Plus ultra-pure water system, Millipore, Molsheim, France).

2.2. Preparation of double emulsions

First the oil phase of the primary w_1/o emulsion was prepared by adding PGPR at 20 mg/g into rapeseed oil. Beet extract was added slowly with mixing at 10 000 rpm with a SilentCrusher M high-speed mixer (Heidolph, Schwabach, Germany) so that the amount of the inner water phase w_1 was 0.3 ml/g of the total w_1/o emulsion. After the whole amount was added, the emulsion was homogenized at 20 000 rpm for 5 min. This primary w_1/o emulsion was slowly added to the outer water phase w_2 with mixing at 13 000 rpm so that the amount of w_1/o emulsion was 0.03 ml/g of the total double emulsion, and after the whole amount was added, the double emulsion was

homogenized at 18 000 rpm for 5 min. The outer water phase w_2 was prepared as described by Kaimainen et al. for simple o/w emulsions (Kaimainen et al., 2012) with slight modifications. A pH of 5.8 instead of 2.6 was chosen because the polysaccharides used for stabilizing the emulsion are degraded at low pH values. Citrate-phosphate buffer with pH 5.8 was prepared by mixing 0.1 mol/L citric acid solution, 0.2 mol/L disodium hydrogen phosphate solution and water in proportion 197:303:500, respectively. This buffer was used to prepare three solutions containing either 10 mg/g oat polar lipid emulsifier (dissolved at 50 °C with magnetic stirring for one hour), 10 mg/g guar gum (dissolved at 80 °C with magnetic stirring for two hours), or 10 mg/g xanthan gum (dissolved at 80 °C with magnetic stirring for two hours). Different compositions for the outer water phase w_2 were formulated by mixing these four solutions in different proportions as preliminary tests. Particularly, a composition of w_2 phase consisting of 5 mg/g oat polar lipid emulsifier, 2 mg/g guar gum, 2 mg/g xanthan gum and 39 mg/g glucose (for adjusting the osmolarity of the solution) in the pH 5.8 citrate phosphate buffer was used in this study. The osmolarities of inner and outer water phases were measured with a Micro-Osmometer type 13 Autocal (Roebling, Berlin, Germany) and glucose was added to the outer water phase to balance the osmolarity of the inner and outer water phases.

2.3. Encapsulation efficiency

Encapsulation efficiency was measured by centrifuging a double emulsion sample at 3000 x g for 10 minutes and filtering the outer water phase through a 0.45 µm PTFE syringe filter. The absorbance at 530 nm of the filtered sample was measured with a Multiskan GO spectrophotometer (Thermo Scientific, Vantaa, Finland) against a blank sample made of filtered (0.45 µm PTFE syringe filter) outer water phase and the value was compared with a standard curve prepared by adding calculated amounts of beet extract to the filtered (0.45 µm PTFE syringe filter) outer water phase corresponding to 5, 10, 20, 40, 60 or 100 % release of the inner water phase, i.e. 95, 90, 80, 60, 40 or 0 % encapsulation efficiency. The double emulsion sample and all the standards were

prepared as duplicate samples, and each sample was measured twice for a total of four measurements per sample or standard. Duplicate measurements of the two duplicate standard sets were very close to each other; relative standard deviations were all less than 1 %, and for most solutions less than 0.1 %. The relative standard deviations calculated from all 4 measurements of each standard point were slightly higher, between 0.14 % and 7.41 %, so the major source of error came from the preparation of solutions and not from the actual measurement. Standard deviation for 90 % encapsulation efficiency standard point was 24 %, but this was due to an error in the preparation of one of the standard solutions. For this reason, that standard solution was excluded and the point of 90 % encapsulation efficiency was calculated only from one standard solution instead of two (and two measurements instead of four). The resulting standard curve had a squared correlation coefficient of 0.9986.

2.4. Droplet size

Droplet sizes were measured by laser diffraction (LD) using a Mastersizer S equipped with a 2 mW He–Ne laser of 633 nm and a 300RF lens (Malvern Instruments Ltd., Worcestershire, UK). The detection limits were 0.05 and 900 µm. Calculations to determine the droplet size distribution were based on a o/w emulsion model with a refractive index n_0 of the aqueous phase of 1.33, and that of rapeseed oil of 1.457. The absorption was set to 0.001. Emulsions were diluted with distilled water in the dispersion unit to reach a droplet volume concentration near 0.03% for the circulation in the measurement cell. For each sample, triplicate measurements were done. For undiluted samples, droplet sizes and overall double emulsion appearance were also investigated on standard slides with a light microscope Axioskop 2 (Zeiss, Oberkochen, Germany) equipped with a Prosilica EC1350 CCD camera. Back-scattering measurements of the primary w/o emulsion droplet size distribution were obtained by dynamic light scattering (DLS) using a Zetasizer Nano ZS equipped with a 4 mW He–Ne laser of wavelength 633 nm (Malvern Instruments Ltd., Worcestershire, UK). The exact angle between the laser beam and the detector (avalanche photodiode) was 173°. The laser power

was automatically attenuated to collect an optimal scattered intensity. The measurement position was set to the maximum of 4.65 mm, which 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 dilution factor of the w/o emulsion in rapeseed oil was 100-fold. The optical properties were the same as previously mentioned for laser diffraction. A 30 s acquisition was generally enough to obtain a stable measurement.

2.5. *In vitro* lipid digestion

In vitro lipid digestion was performed as described by Marze et al. (Marze, Choimet, & Foucat, 2012), using only the intestinal step as the interest was to understand the role of lipase and bile salt during digestion. No protease was included because there was no protein in the double emulsion formulation. For the same reason, the gastric step was not studied, and also because gastric lipases are poorly available, with no commercial supplier worldwide. The digestion medium was a 130 mmol/L NaH_2PO_4 buffer (pH 7.5) with 20 mg/mL NaGDC (a bile salt) and 1 mg/mL pancreatic lipase used to mimic the intestinal step of the gastrointestinal tract. Equal volumes of freshly prepared double emulsion and digestion medium were mixed together and the digestion was performed at 37 °C under magnetic stirring. Samples were taken with a micropipette for measurements of droplet size (LD) and encapsulated betalain (spectrophotometry) at 0, 30, 60, 90, 120 and 180 minutes of digestion. Using the protocol described in the Encapsulation efficiency section 2.3, absorbance at 530 nm was measured in the centrifuged outer water phase. However, the background was constantly changing because the digestion modified the chemical and physical properties of the lipids. Thus the true betalain absorbance was deduced by subtracting the background measured on the centrifuged outer water phase of a double emulsion with 70 mg/g glucose instead of beet extract as the internal water phase and digested in the same conditions. The encapsulated betalain was calculated from this absorbance considering the linear relationship of the

standard curve. Microscopic investigation was also done at 0, 10, 20, 50, 80, 110 and 170 minutes of digestion. These investigations were done in duplicate.

3. RESULTS AND DISCUSSION

3.1. Preparation and visual inspection of double emulsions

The osmolarity of the beet extract after concentration was 387 mosm/L and the osmolarity of the outer water phase before glucose addition was 173 mosm/L. This difference would have caused very high osmotic pressure into the double emulsion droplets, and that is why we added 39 mg/g glucose into the outer water phase, after which its osmolarity was 388 mosm/L. After preparation the double emulsions had a milky appearance typical to emulsions, and a faint pink coloration, typical to dilute beet pigment solutions. Formation of a double emulsion was confirmed upon microscopic investigation, as primary w_1/o emulsion droplets were observed inside the o/w_2 emulsion droplets, as can be seen in figure 1. Freshly prepared double emulsions were also homogenous, but after storage at room temperature for a few days, a slight creaming was observed. The pink color seemed to concentrate on the cream layer, and this was confirmed with centrifugation at 3000 x g for 10 minutes, after which all color was found to be in the cream layer upon visual inspection. This indicated a good encapsulation efficiency of the beet extract even before additional quantitative measurements.

3.2. Encapsulation efficiency

The four measurements from double emulsions gave absorbance values of 0.0219, 0.0217, 0.0286 and 0.0284, which correspond to encapsulation efficiencies of 90.8 %, 90.9 %, 87.2 % and 87.4 %, respectively, with a mean encapsulation efficiency of 89.1 %. As with the standards, two measurements from the same sample had much smaller relative standard deviations than different samples; 0.65 % or 0.50 % vs. 15.39 %, calculated from the measured absorbance values. This is most likely due to pipetting imprecision during the preparation of double emulsions and standards,

as small volumes and viscous liquids were pipetted. Encapsulation efficiency of 89.1 % is quite high, but it is not unusual to reach encapsulation efficiencies over 95 %, or even up to 99 % with w/o/w double emulsions (Sapei, Naqvi, & Rousseau, 2012; Benichou, Aserin, & Garti, 2007; Mun, Choi, Rho, Kang, Park, & Kim, 2010; Hasegawa, Imaoka, Adachi, & Matsuno, 2001). However, lower encapsulation efficiencies of less than 20 % have also been reported and it is likely that encapsulation efficiency depends on the composition of the whole system and the properties of the encapsulated ingredient (O'Regan & Mulvihill, 2010; Fechner, Knoth, Scherze, & Muschiolik, 2007).

3.3. Droplet size

The droplet size analysis of double emulsion showed two populations of droplets, as can be seen in figure 2. The smaller droplet population accounted for 20 % of the total droplet volume, and was centered around a peak at 313 nm, and the larger droplet population accounted for 80 % of the total droplet volume and was centered around a peak at 6.6 µm (see control 0 min from table 1 for mean values). As the dispersed water phase w_1 accounts for 30 % of the total mass of the primary w_1/o emulsion, the smaller droplets population could correspond to the primary w_1/o emulsion droplets. Moreover, these primary emulsion droplets were also visible with light microscopy, so they can likely cause laser diffraction and thus can be detected in the droplet size measurement. We checked this assumption by measuring the droplet size distribution of the primary w_1/o emulsion by dynamic light scattering (also shown in figure 2) and indeed recovered a similar range and a peak at 342 nm. The size of emulsion droplets greatly affects the creaming rate of the emulsion so that larger droplets cream faster (Walstra, 2003). In our previous study, we observed significant creaming in an o/w emulsion with a mean droplet size of 1-2 µm (Kaimainen et al., 2012), so with a mean droplet size of 5.5 µm for the double emulsion, rapid creaming could be expected. However, based on visual investigation of the prepared double emulsions during a few days, only a slight creaming occurred. This slow creaming can be explained by two phenomena: 1) the viscosity of the

continuous phase was increased by the addition of small amounts of guar gum and xanthan gum (not present in the formulation of Kaimainen et al., 2012), 2) the density difference between the oil droplets and the continuous water phase was lowered by the presence of inner water droplets. So the first objective to formulate a double emulsion encapsulating betalain and stable to creaming for a few days (and anticipating the next results, also stable for at least 3 hours in intestinal digestion conditions without the bile salt and lipase) was fulfilled.

3.4. *In vitro* lipid digestion

Before the *in vitro* digestion experiment, the osmolarity of the digestion medium and 1:1 mixture of digestion medium and double emulsion outer water phase were measured. The osmolarities were 309 mosm/L and 341 mosm/L, respectively. The osmolarity of the digestion medium was not balanced with that of the double emulsion, because this was more representative of the actual digestion conditions. The droplet size distributions during *in vitro* intestinal lipid digestion are shown in figure 3. Multimodal droplet size distributions can be seen during the whole experiment, the smaller size population likely corresponding to the primary w_1/o emulsion droplets. Indeed, even during digestion, the position of this population was in good agreement with that for the w_1/o primary emulsion alone as can be seen when comparing figures 2 and 3. The surface mean diameter D3,2 was calculated, and it was indeed close to the diameter for the primary w_1/o emulsion alone measured by DLS, although a bit higher because the double emulsion distribution was multimodal so the larger size population influenced the calculation of the D3,2. The volume mean diameter D4,3 was also calculated, representing the double emulsion droplets, and it was in contrast insignificantly influenced by the smaller size population. The D3,2 and D4,3 with standard deviations from duplicate measurements for all digestion time points are shown in table 1. Both increased quickly as digestion progressed up to 120 min. This reflected a coalescence process of the inner droplets, as well as an aggregation process of the outer droplets, characterized by the appearance and growth of a population of large sizes from 30 min of digestion. At the end of the

digestion, from 120 min, the size of both types of droplets did not change anymore. Under microscope, the aggregation was detected very early and the coalescence was detected gradually and was clearly complete in some emulsion droplets after 110 min of digestion, as can be seen in figure 4.

The release of encapsulated betalain during *in vitro* intestinal lipid digestion is shown in figure 5. The release was fast during the first 120 min of digestion, but nevertheless slowed down as the digestion progressed. Then, the last point at 180 min showed no further release, and the final amount released was around 35 %. Such low release could be explained by the fact that although the inner droplets coalesced, the double emulsion structure was retained throughout the digestion, as seen under microscope, indicating a low release of the inner phase and hence of betalain. Thus, there seems to be a link between the structural evolution of the droplets and the betalain release during this digestion experiment. This link is confirmed quantitatively in figure 6, where relations are evidenced between both D4,3 and D3,2 and the encapsulated betalain during digestion. To go further, a control experiment in the same digestion conditions but without the bile salt and lipase was done. Only a very small amount of betalain was released (figure 5) and the droplet sizes were not changed significantly after 180 min (table 1). So the changes during intestinal lipid digestion were not due to environmental conditions (temperature, pH, osmolarity), but really to the digestion processes. From these results, we can suggest the following processes for the betalain release: 1) reduction of the lipid barrier through digestion by lipase and solubilization of the digestion products in bile salt micelles, inducing the inner droplets coalescence, 2) release of betalain facilitated by the thinning of the lipid barrier and the decrease of the inner droplets number, 3) simultaneous bridging flocculation by lipase, inducing the outer droplets aggregation that progressively inhibits digestion by lipase, thus stopping further release of betalain.

This scheme is in agreement with the literature for simple o/w emulsions. Several studies indeed reported an increase of the droplet size during the digestion, usually due to aggregation of the oil droplets (Mun, Decker, & McClements, 2007; Nik, Wright, & Corredig, 2011; Salvia-Trujillo,

297 Qian, Martín-Belloso, & McClements, 2013), but the extent varied, for example as a function of the
298 type and amount of the emulsifier used (Mun, Decker, & McClements, 2007; Nik, Wright, &
299 Corredig, 2011; Yao et al., 2013) or the initial droplet size of the emulsion (Salvia-Trujillo, Qian,
300 Martín-Belloso, & McClements, 2013; Troncoso, Aguilera, & McClements, 2012). Moreover, Mun
301 et al. found that o/w emulsion with lecithin (phospholipids) as emulsifier was significantly more
302 resistant to digestion by lipase than o/w emulsion with sodium caseinate or whey protein isolate as
303 emulsifier, whereas emulsions with non-ionic surfactants as emulsifiers where even more resistant
304 than those with lecithin (Mun, Decker, & McClements, 2007). In another study, it was shown that if
305 the amount of low-molecular weight surfactant emulsifier is much larger than the critical micelle
306 concentration (CMC), the excess surfactant molecules compete with bile salts and lipase at the oil-
307 water interface, thus inhibiting the digestion of lipids (Yao et al., 2013). As our $w_1/o/w_2$ double
308 emulsion was stabilized by an emulsifier based on phospholipids and glycolipids, at a concentration
309 above its CMC (based on a preliminary study, data not shown) and had quite large outer droplets
310 aggregating during digestion, these results from the literature refine our explanation for the low
311 release of betalain, not only the aggregation of the outer droplets inhibiting the lipase activity, but
312 also the high concentration of phospholipids.
313 More specifically for w/o/w double emulsions, only one study investigated the structure of a similar
314 system during digestion (Shima, Tanaka, Kimura, Adachi, & Matsuno, 2004), yet using a marker as
315 the hydrophilic bioactive in a double emulsion with a much higher primary w/o emulsion
316 concentration. These authors showed that small outer droplets released more marker than large ones
317 because the latter were not hydrolyzed by lipase. For small outer droplets, the release and
318 hydrolysis started first and fast, then after a lag time the outer droplets coalesced, limiting further
319 release. Our results are in agreement with these findings, as the extensive aggregation of the outer
320 droplets from 120 min likely limits the release (figures 5 and 6). One other study investigated the
321 structure of double emulsions containing anthocyanin in digestion conditions, yet only qualitatively
322 (Frank, Walz, Gräf, Greiner, Köhler, & Schuchmann, 2012). These authors showed that there was

no release of the inner water phase (and indirectly of the anthocyanin) when no intestinal enzymes were present, all other conditions being the same. When intestinal enzymes were present, observations after 5 hours showed that this release was complete, as the double emulsion became a simple w/o emulsion. This was explained by the coalescence of the inner w_1/o droplets between themselves and at the outer o/w_2 interface. Our results evidenced the first coalescence mechanism but not the second, as the double emulsion structure was retained throughout the digestion.

4. CONCLUSIONS

The present study shows that betalain pigments can be encapsulated in w_1/o/w_2 double emulsions with high encapsulation efficiency and emulsion stability. Indeed, the stability of a control double emulsion for 180 min was high and thus the release of betalain was very low, but long times storage needs to be investigated further. Whether the technique can improve the chemical stability of the pigment in food applications is evaluated in on-going experiments, where pigment shelf-life is tested within food applications. The *in vitro* intestinal lipid digestion of the w/o/w double emulsion showed that the structural changes of the inner and outer droplets influenced the bioactive release, induced by coalescence of the inner droplets and reduced by aggregation of the outer droplets. Based on these findings, it seems that oil in w/o/w emulsion can act as a barrier to protect hydrophilic bioactives, of which a gradual release can be obtained during oil digestion. As confirmed here, the release is controlled by the destabilization mechanisms of the structures of the double emulsion. These mechanisms are similar than in simple emulsions (creaming, aggregation, coalescence, available surface area), so the same parameters are expected to play a role in double emulsions (presence of polysaccharide, emulsifier type and concentration, oil type and concentration, sizes). However, these mechanisms are even more interdependent during the digestion of double emulsions, so this needs to be studied further, as well as the role of the gastric step before the intestinal step.

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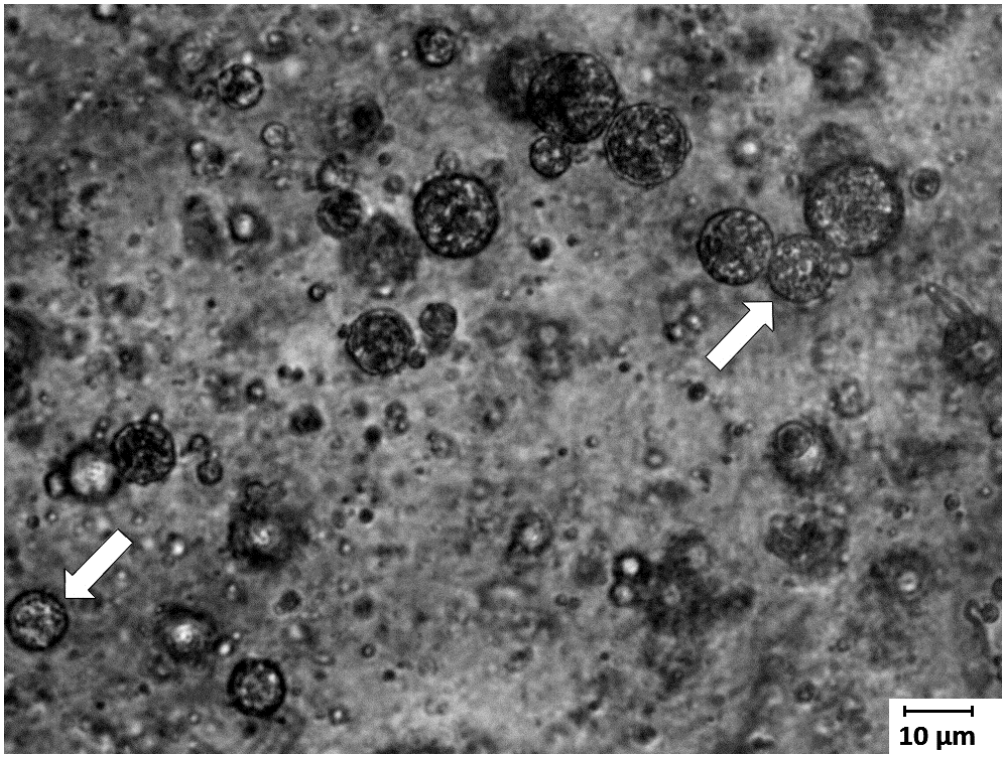
TABLES

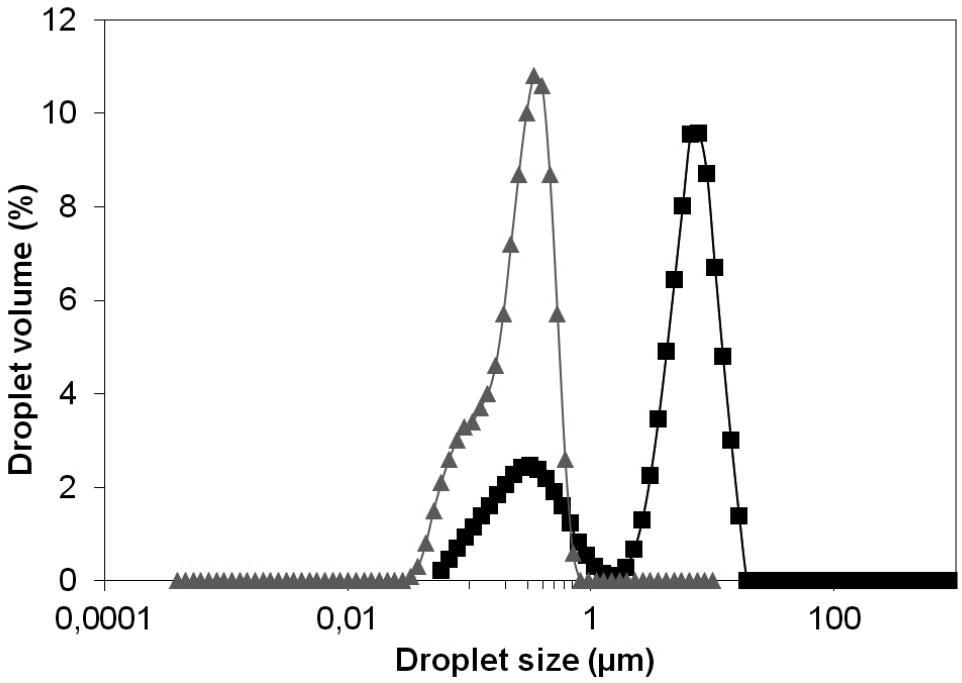
Table 1. Droplet size calculations during the *in vitro* lipid digestion showing calculated surface means (D3,2) and volume means (D4,3) with standard deviations (SD) of duplicate experiments. The control experiments are in the same conditions but without the bile salt and lipase.

Digestion time	D3,2(µm)	SD(µm)	D4,3(µm)	SD(µm)
Control 0 min	0.81	0.15	5.49	0.42
Control 180 min	0.75	0.07	5.34	0.67
0 min	1.04	0.09	6.28	0.25
30 min	1.66	0.13	11.95	1.75
60 min	2.02	0.37	26.74	1.60
90 min	2.55	0.18	35.30	0.62
120 min	2.98	0.27	38.82	6.58
180 min	2.74	0.39	43.63	3.40

FIGURES AND FIGURE CAPTIONS



433 Figure 1 Microscopic image of the double emulsion. Small inner water phase w_1/o droplets can be
434 seen inside the larger oil droplets confirming the structure of a double emulsion. White arrows point
435 examples of $w_1/o/w_2$ droplets.



436

437 Figure 2 Droplet size measurements of the $w_1/o/w_2$ double emulsion and the primary w_1/o emulsion.
438 *Black square* $w_1/o/w_2$ double emulsion measured by laser diffraction (LD), *grey triangle* primary
439 w_1/o emulsion measured by dynamic light scattering (DLS). The LD measurement shows a bimodal
440 distribution, corresponding to the primary w_1/o emulsion droplets (smaller peak) and the double
441 emulsion droplets (larger peak).

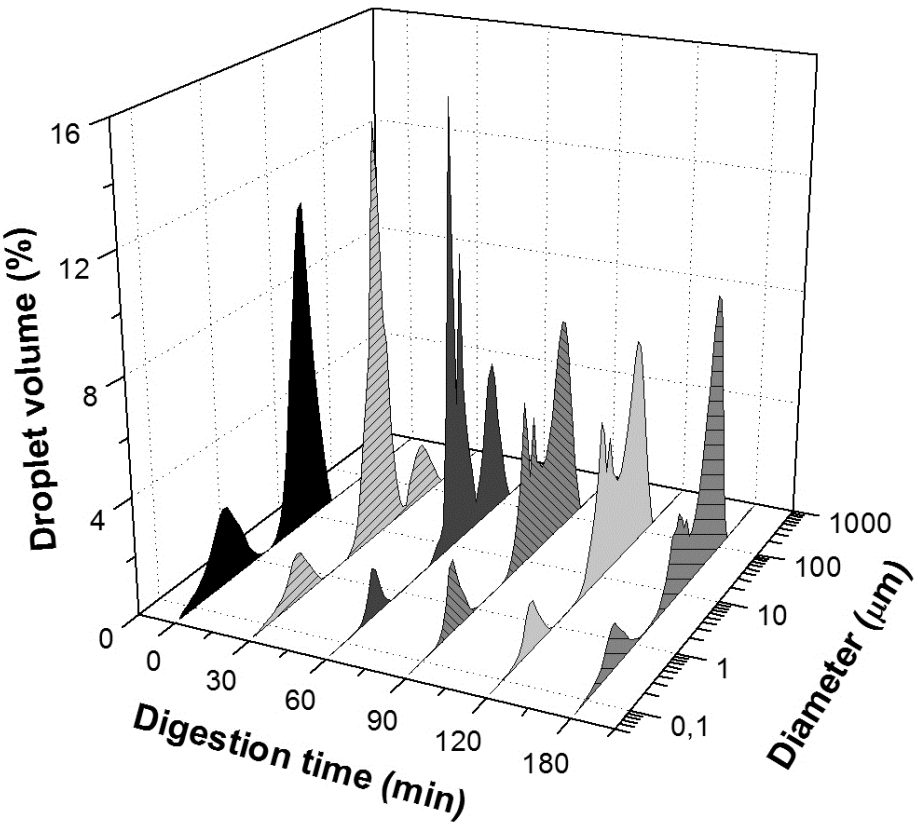


Figure 3 Droplet size distributions of the double emulsion during *in vitro* intestinal lipid digestion as a function of digestion time measured by laser diffraction. Time 0 is just after the mixing of the digestion fluid and the double emulsion. A population of larger droplets forms as digestion progresses due to aggregation of $w_1/o/w_2$ droplets.

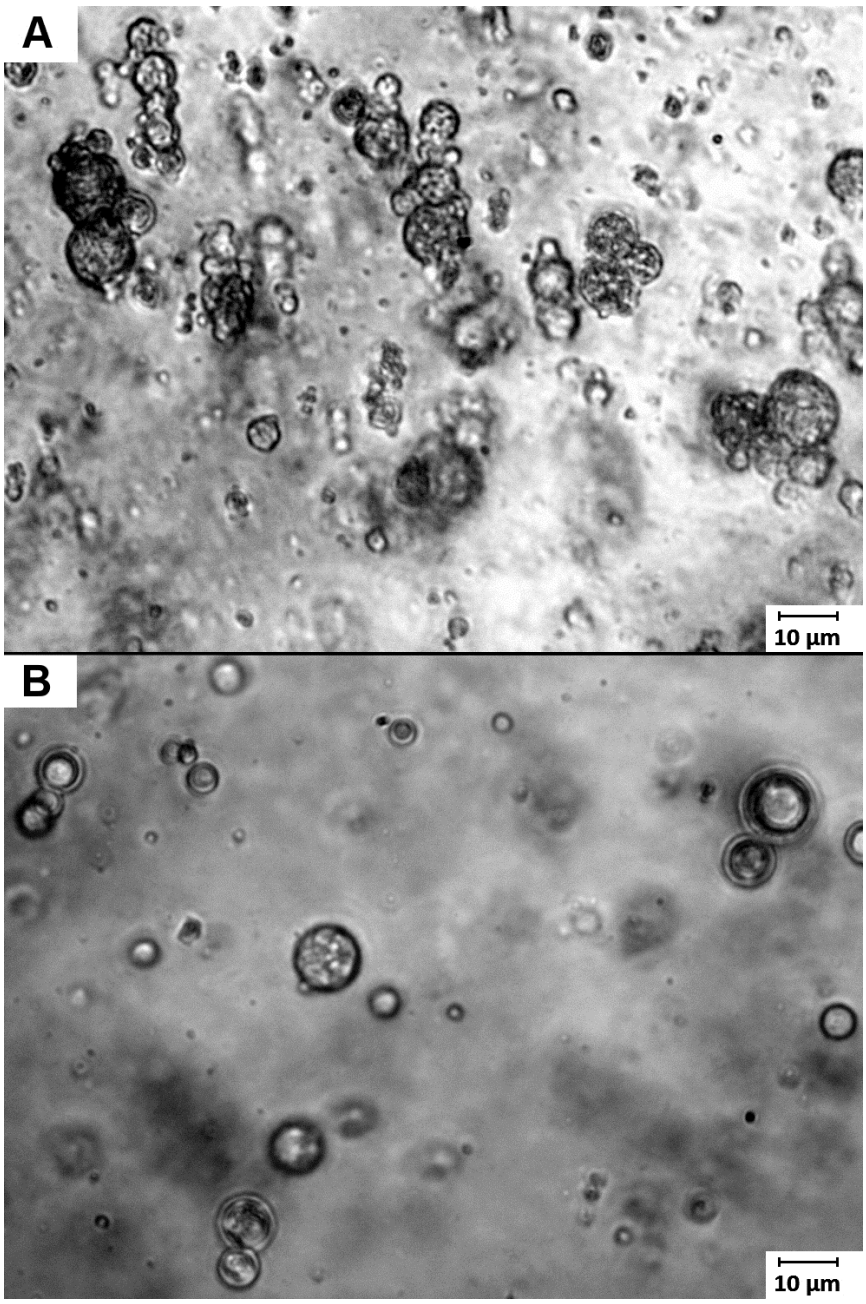
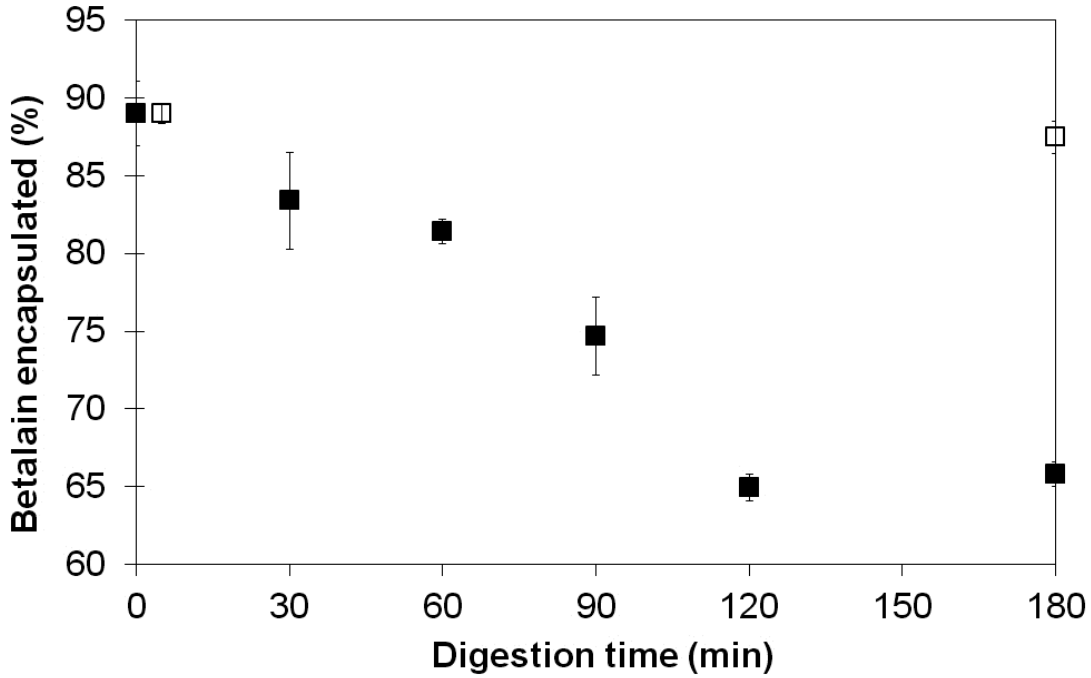
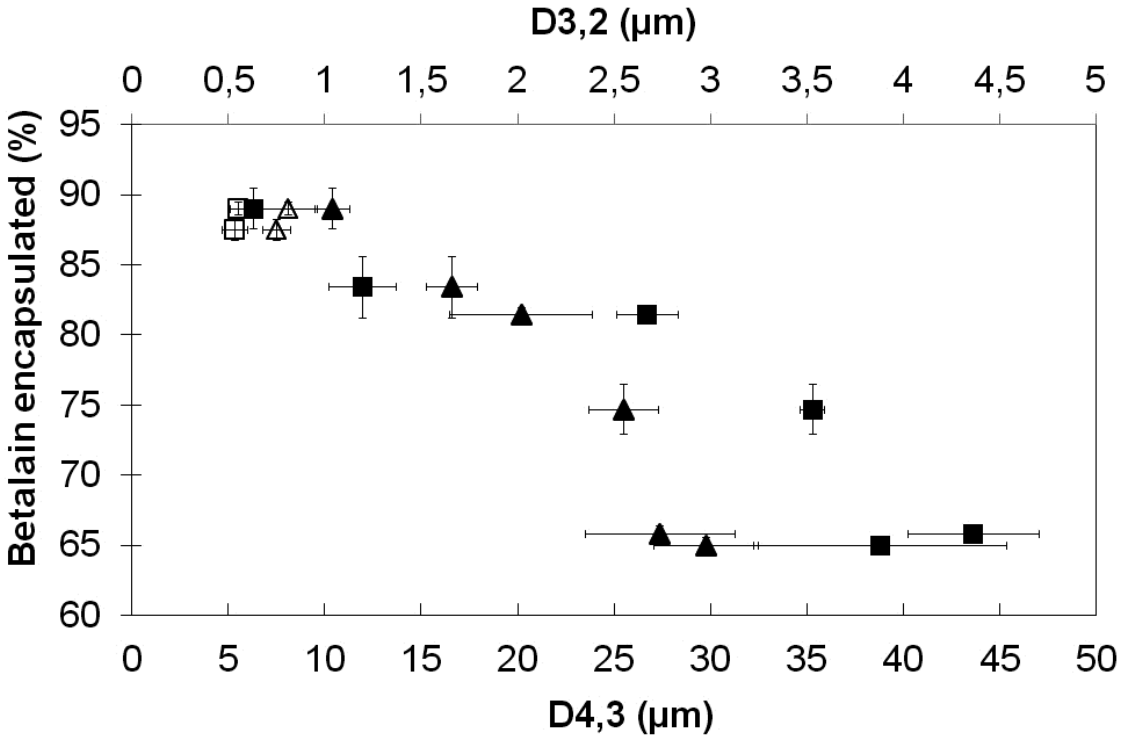


Figure 4 Microscopic images of the double emulsion during *in vitro* intestinal lipid digestion showing the aggregation of the $w_1/o/w_2$ droplets after 20 min (A) and the complete coalescence of some inner w_1/o emulsion droplets after 110 min (B).



451

452 Figure 5 Release of betalain from the inner water phase of the double emulsion expressed as the
453 relative amount of betalain still encapsulated as a function of digestion time with standard
454 deviations of duplicate experiments. *Black square* intestinal lipid digestion experiment, *empty*
455 *square* control experiment in the same digestion conditions but without the bile salt and lipase (the
456 point at 0 min is arbitrarily shifted to avoid overlapping data points). Betalain is slowly released
457 during digestion up to 120 minutes, whereas no release is seen in the control experiment.



458

459 Figure 6 Betalain encapsulated represented as a function of the double emulsion volume mean
460 diameter D4,3 and surface mean diameter D3,2 during the *in vitro* intestinal lipid digestion with
461 standard deviations of duplicate experiments. *Black square* D4,3 and *black triangle* D3,2 for the
462 digestion experiment, *empty square* D4,3 and *empty triangle* D3,2 for the control experiment in the
463 same digestion conditions but without the bile salt and lipase. The release of betalain corresponds to
464 an increase in droplet sizes as digestion progresses.

465