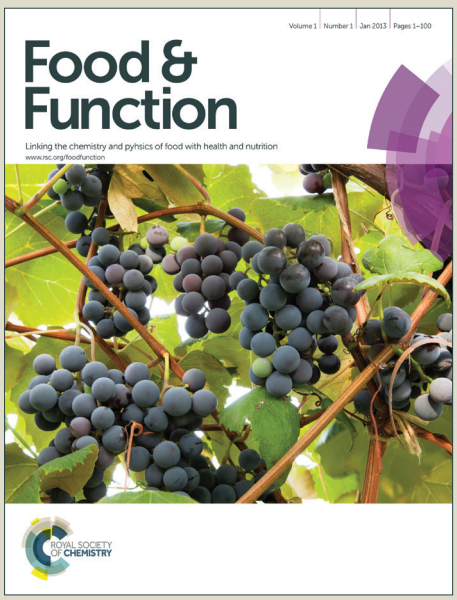
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

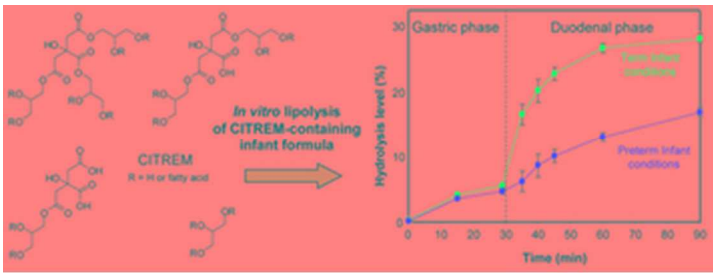
Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction



29x11mm (300 x 300 DPI)

In vitro digestion of citric acid esters of mono- and diglycerides (CITREM) and CITREM-containing infant formula/emulsions

Sawsan Amara[a], Amaury Patin[b], Francesca Giuffrida[b], Tim J Wooster[b], Sagar K. Thakkar[b], Anaïs Bénarouche[a], Isabelle Poncin[a], Sylvie Robert[a], Vanessa Point[a], Sacha Molinari[a], Hélène Gaussier[a], Sadia Diomande[a], Frédéric Destaillats[b], Cristina Cruz-Hernandez[b*] and Frédéric Carrière[a*]

[a] CNRS, Aix Marseille Université, Enzymologie Interfaciale et Physiologie de la Lipolyse UMR7282, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

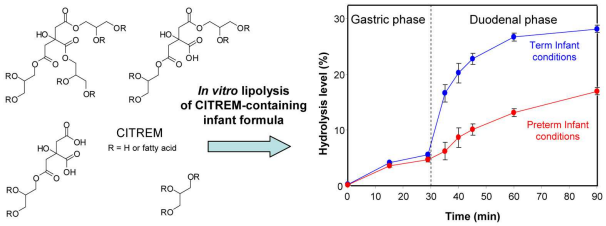
[b] Nestlé Research Center, Vers-Chez-Les-Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland

[*] Correspondence to:

Frédéric Carrière: carriere@imm.cnrs.fr – Tel +33 4 91 16 41 34 – Fax +33 4 91 71 58 57

Cristina Cruz-Hernandez: cristina.cruz-hernandez@rdls.nestle.com

Table of Content entry



The gastrointestinal lipolysis of CITREM, an emulsifier used in infant formula, is investigated for the first time using various digestive lipases and a two-step *in vitro* digestion model.

Abstract

CITREM is an emulsifier used in the food industry and contains citric acid esters of mono- and diglycerides (GCFE). It is generally recognized as safe but no publication on its digestibility under gastrointestinal conditions and impact on fat digestion was available. It was shown here that fatty acids are released from CITREM by gastric lipase, pancreatic lipase, pancreatic-lipase-related protein 2 and carboxylester hydrolase. A two-step *in vitro* digestion model mimicking lipolysis in the stomach and upper small intestine of term and preterm infants was then used to evaluate the digestibility of CITREM alone, CITREM-containing infant formula and fat emulsions, and isolated GCFE fraction. Overall, it was shown that fat digestion is not significantly changed by the presence of CITREM, and fatty acids contained in CITREM compounds are released to a large extent by lipases. Nevertheless, undigestible water-soluble compounds containing glycerol and citric acid units were identified, indicating that the ester bond between citric acid and glycerol is not fully hydrolyzed throughout the proposed digestion.

Introduction

Citric acid esters of mono- and diglycerides (also known as citroglycerides or CITREM) are commonly used as emulsifiers and additives (E472c) in the food industry [1]. CITREM is found for instance in infant formulae based on crystalline amino acids or protein hydrolysates or both. Combined with the antimicrobial substance lauric arginate, CITREM is used in many food preparations such as beverages, seasonings, ice cream coatings and frying margarine. It is also intended to replace the common emulsifiers used for flow control in the confectionery of chocolate and chocolate compounds such as soybean lecithin and polyglycerol polyricinoleate.

CITREM is in fact a mixture of various glycerides obtained by reaction of citric acid or its anhydride with acylglycerols (edible oils, mixtures of diacylglycerides (DAG) and monoacylglycerides (MAG), distillated MAG) in the presence of an acid catalyst, e.g., acetic acid [2, 3]. During the reaction citric acid typically reacts with more than one glycerol backbone and the composition of CITREM varies according to the reaction time, temperature and ratio between the raw materials. The composition of CITREM can be very complex but the main components are usually: 1,2(2,3)-DAG, MAG and citric acid esters of MAG and DAG. In these latter compounds, one to three glyceride moieties can be covalently linked to citric acid and their glycerol backbones can be esterified with 0 to 6 fatty acids as shown in Scheme 1.

CITREM is considered as a food substance of very low toxicity and its levels in foods are not considered to represent a hazard to human health [4]. Moreover, acceptable daily intake (ADI) is not specified by expert committees and no Food Chemical Codex specifications exists for CITREM [4]. However, it is described as “generally recognized as safe” (GRAS; [5]) for direct addition to foods (reference number 977093-28-9 by FDA’s Center for Food Safety and Applied Nutrition). In 1967, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) noted in its evaluation of citric acid esters of monoacylglycerides that they are

completely hydrolyzed in the gastrointestinal (GI) tract into components which are normal constituents of the diet (free fatty acids, glycerol, citric acid; Scheme 1) [4]. This assumption was based on *in vitro* digestion experiments performed with pancreatic lipase and liver esterase by a contract research organization (Huntington Research Centre, 1966; Unpublished report submitted to JECFA by Emulsion A/B) but no article related to this work or similar works was ever published in scientific journals. So far, there is no report on CITREM digestion by the various lipases found in the human GI tract; gastric lipase (HGL), that initiates lipolysis of dietary fats in the stomach [6], and at least three important pancreatic enzymes acting in the small intestine, pancreatic lipase (HPL), pancreatic lipase-related protein 2 (HPLRP2) and pancreatic carboxyl ester hydrolase (HCEH) also known as bile salt-stimulated lipase (BSSL) [7].

The objectives of this study were first to test *in vitro* lipolysis of CITREM by individual gastrointestinal lipases to identify the enzymes potentially involved in CITREM digestion *in vivo*. Second, the lipolysis of CITREM by a combination of enzymes was tested under *in vitro* conditions mimicking the physiological conditions found in the human GI tract, both in the stomach and the upper small intestine, in order to quantify the extent of CITREM hydrolysis. Since CITREM is currently used in the composition of infant formula due to its ability to stabilize such complex food matrixes, both preterm and term infant conditions were tested. Third, the lipolysis of a CITREM-containing infant formula was tested under the same *in vitro* conditions to assess the effects of CITREM on fat digestion. This part of the study was completed by testing the lipolysis of various fat emulsions containing the same amount of fat as in infant formula, with and without CITREM.

Materials and methods

Reagents and chemicals

Commercially available citric acid esters of mono- and diacylglycerides (Grinsted® CITREM LR 10 extra) was provided by DaniscoA/S (Brabrand, Denmark). Triolein, diolein, monoolein, oleic acid, $CaCl_2$, sodium taurodeoxycholate (NaTDC), and anhydrous $MgSO_4$ were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). Whey protein isolate (WPI), containing 95.8% protein – Bipro, was purchased from Davisco Foods International, US. Sodium methoxide (30% in methanol) was obtained from Merck (Germany) and the standard FAME mixture from Nu-Chek-Prep (Elysian, MN). All the solvents were purchased from SDS (Peypin, France), Sigma-Aldrich (St Quentin-Fallavier, France) and Merck (Darmstadt, Germany) and were of HPLC grade. Thin-layer chromatography (TLC) glass plates (10 × 20 cm) coated with silica gel 60 were from Merck. All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Infant formula and fat emulsions

A prototype of an Infant formula containing 3.3 % w/w fat and CITREM at 0.21 g/100 mL was provided by Nestlé Health Science (Konolfingen Product Technology Centre (Konolfingen, Switzerland). Various emulsions containing the same fat blend as the infant formula were also prepared for testing CITREM effects on lipolysis. First, CITREM was dissolved at 6.25 % w/w in the fat blend and this mixture was added directly in the reaction vessel used for the *in vitro* digestion experiments. In this case, the emulsion was formed by mechanical stirring within the reaction vessel. Second, a 10 % w/w fat emulsion stabilized by 0.625 % w/w CITREM was prepared by dispersing the oil phase into the emulsifier solution using a Silverson LMR5A lab Mixer with a 1mm circular mesh at 10,000 rpm for 2 minutes

and then transferred into the reaction vessel. Third, a 10 % w/w fat emulsion was prepared using 2 % w/w whey protein isolate instead of CITREM. This was aimed as a control for the latter identification of CITREM-specific digestion products. The particle size of each emulsion was measured with laser light scattering using a Mastersizer 2000 (Malvern, Worcestershire, United Kingdom). The emulsion sample was dispersed in recirculating water in the Hydro SM measuring cell until an obscuration rate of ~14% was obtained. Information about emulsion particle size was then obtained via a best fit between light scattering (Mie) theory and the measured light scattering pattern. A differential refractive index of 1.1045 (1.469 for oil/1.33 for water) and the absorption of 0.001 were used as the optical properties of the emulsion. The mean emulsion droplet size is quoted as the volume-weighted mean diameter D4,3 ($= \Sigma n_i d_i^4 / \Sigma n_i d_i^3$). The measurement was carried out in duplicate on two emulsion samples prepared separately. The particle size distributions of the two 10 % w/w fat emulsions (mean diameter D4,3 of 9.2 ± 0.1 µm) were matched to each other to remove the artefact of initial droplet size distribution from the digestion behaviour. The emulsions were chosen to be of a size that was relatively similar to the fat droplets of the rehydrated formula. The emulsions contained sodium azide as an antimicrobial agent. They were kept at < 4 °C and were used before their estimated shelf half-life (2-3 weeks) was reached. The fatty acid composition (% w/w) of the fat blend used for making the infant formula and model emulsions is given in Table 1. It was enriched in medium chain fatty acids (around 25 % w/w of total fatty acids).

Lipases

Recombinant dog gastric lipase (rDGL) was chosen because this enzyme has similar properties to the human enzyme HGL and it was used in previous *in vitro* digestion studies involving a gastric phase of lipolysis [8-11]. rDGL displays activities on acylglycerols with

various acyl chain length, PEG esters and vinyl esters. It does not display activity on phospholipids, cholesterol esters and p-nitrophenol esters. rDGL was produced in transgenic maize by Meristem Therapeutics (Clermont-Ferrand, France) and was purified according to Roussel et al. [12]. The powder contains pure lipase (>98% proteins) mixed with lactose and salts (50 % w/w). The specific activity of the powder is 150±25 U/mg using tributyrin as substrate and the standard assay conditions of DGL[13]. For lipolysis experiments, rDGL stock solutions at 1 and 0.1 mg rDGL/mL were prepared in 10 mM MES, 150 mM NaCl, pH 6.0.

Recombinant human pancreatic lipase (rHPL) and pancreatic lipase-related protein 2 (rHPLRP2) were produced in the yeast *Pichia pastoris* and purified according to Belle et al. [14] and Eydoux et al. [15], respectively. Purified rHPL and rHPLRP2 have specific activities of 8,000 and 900 U/mg, respectively, using tributyrin as substrate and their respective standard assay conditions[15, 16]. For lipolysis experiments, rHPL and rHPLRP2 solutions at 1 mg/mL were prepared in 10 mM MES, 150 mM NaCl, pH 6.0. Experiments with rHPL were performed in the presence of colipase at a 2 to 1 molar excess. Porcine colipase was purified according to Maylié et al [17].

Native porcine pancreatic lipase (PPL) and human pancreatic carboxyl ester hydrolase (HCEH) were purified to homogeneity from porcine pancreas and human pancreatic juice, respectively, according to methods previously described [18-20]. Purified PPL and HCEH have specific activities of 8,000 and 300 U/mg, respectively, using tributyrin as substrate and their respective standard assay conditions[16].

Porcine pancreatic extract (PPE or pancreatin) was chosen as a mixture of pancreatic enzymes for mimicking human pancreatic juice (HPJ). The selected PPE batch contained 5.8 mg PPL /100 mg and its specific activity was 464 U/mg using tributyrin as substrate and pancreatic lipase standard assay conditions[16].

Assay of individual enzyme activity on CITREM by the pHstat technique

The substrate (45 to 500 mg Grinsted CITREM LR 10 extra, Danisco) was first dispersed in 15.0 mL of an assay solution (150 mM NaCl; 0.5 or 4 mM NaTDC; 1.4 mM $CaCl_2$; 1 mM Tris-HCl) previously found to be adapted to the activity measurements of all lipases tested in this study [8]. The CITREM dispersion was formed by mechanical stirring (450 rpm) in the temperature-controlled (37 °C) reaction vessel of a pH-stat apparatus (718 STAT Titrino, Metrohm, Switzerland). After adding the lipase sample, the release of free fatty acids (FFA) from CITREM was measured by titration at a constant pH (2 to 8) with 0.1 M NaOH. When testing rHPL and PPL, a 2-fold molar excess of porcine colipase was added in the reaction vessel before the lipase. For the pH values at which FFAs were only partly ionized, total FFA were estimated from back-titration experiments. The CITREM dispersion in the assay solution was incubated first with the lipase for 3 min. The pH end-point value was then shifted to pH 9.0 using the pH-stat device and the volume of NaOH required to reach this pH value was measured using the automated burette. Control experiments without any enzyme were performed to determine the amounts of NaOH required to reach pH 9.0 in the absence of FFAs released by the lipase. Subtraction of these amounts of NaOH to the total amounts of NaOH delivered in presence of the lipase allowed to determining the amounts of FFAs released, and therefore, lipase activity. Lipase activities on CITREM were expressed in international units: 1 U corresponds to 1 µmol of FFAs released per minute. Specific activities were expressed as U per mg of pure enzyme (rDGL, rHPL, PPL, rHPLRP2 or HCEH) and per mg of powder when PPE was used as the source of lipolytic enzymes.

In vitro lipolysis of CITREM and infant formula under conditions prevailing in term infants

A two-step static *in vitro* digestion model including a gastric phase and a duodenal phase of lipolysis was used. CITREM (48 mg or 144 mg) or infant formula powder (2.45 g containing 31.5 mg CITREM) was dispersed in 16 mL of an assay solution containing 150 mM NaCl, 1.4 mM $CaCl_2$, 10 mM MES. The corresponding CITREM initial concentrations were 0.3 g/100 mL, 0.9 g/100 mL and 0.21 g/100 mL, respectively. This mixture was mechanically stirred in a temperature-controlled reaction vessel at 37 °C and the pH was adjusted to 5.5. At t=0 min, 3 mL of a freshly prepared rDGL solution at 100 µg/mL was added to the reaction vessel to obtain a final concentration of 17 µg/ml of rDGL and the pH was kept constant at 5.5 during 30 min, via an automated titration of FFAs with 0.1 M NaOH using a pH-stat device. At t=30 min, 11 mL of PPE/bile salts solution was added to the mixture and the pH shifted to 6.25 and then kept constant for 60 min. The PPE/bile salts solution was prepared using 117.8 mg PPE at 5.8% w /w PPL (i.e., 6.8 mg PPL) and 56.2 mg NaTDC. After adding the pancreatic enzyme solution to the reaction vessel, the final pancreatic lipase concentration was 250 µg/mL, the final bile salt concentration was 4 mM and the gastric phase was diluted 1.7-fold. One-mL samples for TLC analysis of lipolysis products were collected at various times (0, 15, 29, 35, 40, 45, 60 and 90 min), immediately acidified and used for the extraction of residual CITREM and its lipolysis products.

Similar experiments were also performed with the various emulsions prepared with the fat blend used in the composition of infant formula. In each case, the initial reaction mixture contained 500 mg fat in 16 mL of assay solution.

In vitro lipolysis of CITREM and infant formula under conditions prevailing in premature infants

The same two-step static *in vitro* digestion model was used for testing preterm infant conditions, except that both gastric and duodenal pH values were adjusted to 6.0. The volume of PPE/bile salt solution added to the mixture was reduced 10-fold compared to term infant conditions. The final concentrations of pancreatic lipase and bile salts were 25 µg/mL and 0.4 mM, respectively. The gastric phase was diluted 1.04-fold. The pancreatic lipase concentration used in these experiments was based on pancreatic lipase levels in duodenal aspirates of two premature infants born at 35 weeks and fed with 35 mL Premilumel formula (unpublished data from F. Carriere).

Lipid extraction and analysis by thin-layer chromatography

Lipid extraction was performed immediately after sampling by mixing vigorously each 1-mL sample with 200 µL 0.1 N HCl and 5 mL chloroform-methanol (2:1 v/v) in a 15-mL glass tube with a screw-cap. After phase separation, the lower organic phase was collected using a Pasteur pipette, transferred to a 15-mL test tube, and dried over anhydrous magnesium sulfate ($MgSO_4$). After total precipitation of $MgSO_4$, the clear organic phase was filtered and transferred into a 2-mL vial with a screw-cap and the vial was kept at -20 °C until the analyses were performed.

Quantitative analysis of CITREM and infant formula lipolysis products was performed by thin-layer chromatography (TLC), staining and densitometry. One to 50 µL of lipid extracts or lipid standards (triolein, diolein, monoolein and oleic acid) at known concentrations were spotted automatically as 5-mm bands onto thin-layer silica plates using a Linomat IV apparatus (Camag, Muttenz, Switzerland) equipped with a 100-µL Hamilton Syringe. The elution of the lipids was then performed using either heptane/ether/formic acid

(55/45/1 v/v/v) for the single migration of neutral lipids or chloroform/methanol/H_2O (65/25/4 v/v/v) followed by heptane/ether/formic acid (55/45/1 v/v/v) for the double migration of polar and neutral lipids. Following chromatography, the plates were dried at room temperature for 10 min and then immediately sprayed with a cupric acetate-orthophosphoric acid solution prepared by mixing a saturated aqueous solution of cupric acetate with 85% phosphoric acid in a 1-to-1 volume ratio. The plates were then placed in an oven to ensure heating at 110 °C for 10 min. Densitometry analysis of the stained lipids on the TLC plates was carried out using a Camag TLC Scanner II (Camag, Muttenz, Switzerland). Lipid bands were scanned at 500 nm with a 0.5 × 7-mm slit, at a speed of 2.5 cm/min. The densitometric signals of all tracks (2-3 scan repeats per track) were integrated using a D2000+ Chromato-Integrator (Merck). Quantities of lipids on the TLC plates were estimated from the calibration curves established with pure lipid standards.

Estimation of CITREM and infant formula/TAG emulsions lipolysis levels

CITREM lipolysis levels were estimated from the quantification of FFA by TLC-densitometry and their expression as percent of the total fatty acids esterified in CITREM compounds (Table 1). Infant formula and TAG emulsion lipolysis levels were estimated from the quantification of residual TAG, FFA, DAG and MAG by TLC-densitometry. Mass amounts were converted into moles using mean molecular masses of 784.43 g/mol for TAG, 248.8 g/mol for FFA, 553.65 g/mol for DAG and 322.87 g/mol for MAG. These molecular masses were estimated from the fatty acid composition of the fat blend used for making infant formula and fat emulsions (Table 1). Lipolysis levels were expressed as FFA% *versus* total fatty acids esterified in CITREM compounds or fat present in infant formula and fat emulsions.

Fast Gas Chromatography (GC)

Fatty acid composition of CITREM and fat blend samples was determined by derivatization of lipids into fatty acid methyl esters (FAME) as described in Destaillats et al. [21] using acidic conditions. Analyses of total FAMEs were performed on a 7890 Agilent gas chromatograph (Agilent Technologies, Palo-Alto, CA), equipped with a fused-silica BPX-70 capillary column (10 m, 0.1 mm i.d., 0.2 µm film thickness; SGE, Melbourne, Au), hydrogen as a carrier gas. Split injector and flame-ionization detector were operating at 250°C, volume of the oven was about 5400 cm^3. Oven temperature programming was 50°C isothermal for 0.2 min, increased to 180°C at 120°C/min, isothermal for 1 min at this temperature then increased to 220°C at 20°C/min and then to 250°C at 50°C/min.

NMR spectroscopy

NMR spectroscopy was used for the identification of CITREM compounds and for monitoring their hydrolysis. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 spectrometer equipped with a broadband multinuclear z-gradient probe head, at room temperature (25 °C). For ^{1}H-NMR spectra, the proton chemical shifts (δH) are given in ppm (parts per million) relative to internal tetramethylsilane (Me_4Si) standard, with the following residual solvent resonances used for chemical shift calibration: $CDCl_3$ at δH 7.26 ppm. Deuterated solvent ^{1}H and ^{13}C resonances used for chemical shift calibration were taken from reference.

Liquid chromatography coupled with mass spectrometry (LC-MS)

Water soluble fractions of digested CITREM were separated by LC using an Aquity HSS T3 column (1.8 µm; 2.1×100 mm i.d.; Waters). The chromatography system consisted of Infinity1290 modules (Agilent Technologies, Basel, Switzerland) coupled to high scan speed

with high resolution (>20,000 FWHM) and high mass accuracy (2 ppm) mass spectrometer (SCIEX 5600 TripleTOF High Resolution Hybrid Mass Spectrometer, Ontario, Canada).

All analyses were performed at 40°C. Solvent A was composed of 0.1% acetic acid in water and solvent B of 0.1% acetic acid in acetonitrile. Gradient conditions were as follows; time= 0 min 99% solvent A, time=1.0 min 99% solvent A, time=16 min 70% solvent A, time=17 min 0% solvent A, time=25 min 0% solvent A, time=26 min 99% solvent A, time=27 99% solvent A. Flow rate was 0.4 mL/min. The mass spectrometer was equipped with electrospray ionization ion source (ESI). The ESI mass spectra were recorded in the negative ion mode under the following conditions; ion spray voltage (IS) -2500 V, temperature of the source 400 ºC, declustering potential (DP) -60 V and collision energy (CE) -10V. The *m/z* transitions of soluble products potentially generated from CITREM hydrolysis (Scheme 2; *m/z*: 92.047 – 192.027 – 266.064 – 340.101 – 414.137 – 440.080 – 514.117) were monitored. Data were collected and processed using Analyst software 5.1. Peak view and Formula finder (Applied Biosystems, Sciex, Ontario, Canada).

Partial purification of glycerol citrate fatty acid esters (GCFE) from CITREM

The aim of this purification procedure was to isolate citrate-containing compounds from the complex mixture of compounds present in CITREM, in order to further test the activity of digestive lipases on these compounds. CITREM (504 mg) was fractionated by column chromatography (SiO_2) on puriflash system using petroleum benzine and ether (+1% HCO_2H) as eluent from 60% to 100%. Fractions 1 to 23 (260 mg; 51.6 % w/w) mostly contained TAG and DAG but no citrate signals in ^{1}H-NMR. Fractions 24 to 29 (70 mg; 13.9 % w/w) mostly contained 1- and 2-MAG but no citrate signals in ^{1}H-NMR. Then the column was eluted with 100% MeOH to remove the remaining compounds off the column. After concentration of all the remaining fractions (from Fraction 30 to the end + MeOH elution), citrate protons were observed in ^{1}H-NMR and a second separation was undertaken by column chromatography

(SiO_2) on puriflash system using chloroform/methanol 5% to 50%. Fractions 4 to 10 contained a viscous oil and citrate protons were observed in ^{1}H-NMR. They were pooled (sample E1: 90 mg; 17.8 % w/w) and mainly contained citrate esters since most citric-acid-free glycerides had been removed in the previous fractions (1 to 29) of the first chromatography. Citrate protons were also observed by ^{1}H-NMR in fractions 11 to the end and these fractions were pooled separately (sample E2; 87 mg, 17.3 % w/w). Some very polar compounds like glycerol alone or glycerol citrate (without any FA) might be present in this sample. Due to the complexity of CITREM composition, the E1 and E2 GCFE fractions were obtained as mixtures of compounds, but they were nevertheless devoid of citric-acid-free glycerides (DAG, MAG and TAG).

Results

CITREM chemical structure and composition

The commercial CITREM used in this study was made from the reaction of citric acid with refined high oleic sunflower oil (Danisco) and oleic acid represents 79.1 % of total fatty acids (Table 1). The back-titration experiments performed for measuring lipase activities on CITREM allowed to estimate an average pKa of 6.3 for the FFA released from CITREM. This pKa value is consistent with the fatty acid composition of CITREM (Table 1).

TLC analysis showed that 1,3-DAG, 1,2(2,3)-DAG, MAG and GCFE, as well as traces of TAG and FFA were present in CITREM (Figure 1). These various compounds were separated by flash-chromatography and their respective proportions were found to be 51.6 % w/w, 13.9 % w/w and 34.5 % w/w for DAG (+TAG traces), MAG and GCFE. These results were in good accordance with the CITREM composition provided by the manufacturer (64% of glycerides and 36% of GCFE).

^{1}H and ^{13}C NMR analysis were performed on complete CITREM, enzymatically-digested CITREM and CITREM fractions obtained by flash chromatography and preparative TLC. They confirmed that CITREM is composed of a complex mixture of numerous combinations between glycerol, citrate and fatty acids. Despite this complexity, the various residues of CITREM (glycerol, citrate and fatty acid moieties) could be clearly identified by ^{1}H NMR (Figure 2). By comparison with ^{1}H NMR spectra of pure MAG, 1,2-DAG, 1,3-DAG, TAG and GCFE fractions isolated from CITREM (data not shown), it was possible to identify and quantify them in CITREM. NMR could then be used as an analytical tool for studying the hydrolysis of CITREM. The citrate moiety signals were found to be a key element for investigating the cleavage of the ester bond between citric acid and glycerol.

In vitro lipolysis of CITREM by individual gastrointestinal lipases

Preliminary lipolysis experiments with various gastrointestinal lipases and amounts of CITREM (45, 135, 250 and 500 mg in a total reaction volume of 15 mL) were performed to establish the best kinetics and measure optimum enzyme activities using the pH-stat technique. All lipase activities were found to be close to their maximum using 135 mg CITREM per 15 mL (0.9 g/100 mL). This substrate concentration corresponds to the maximum CITREM concentration currently found in commercial infant formula and was therefore chosen for testing lipase activities at various pH values and in the presence of bile salts. NaTDC was chosen as a model bile salt commonly used in lipase studies and two NaTDC concentrations were tested to simulate normal bile secretion and bile salt concentration in the intestinal contents (4 mM NaTDC, >CMC) and preterm conditions in which bile secretion is not fully established (0.5 mM NaTDC, <CMC) [22].

All the purified lipases tested and PPE were found to be active on CITREM (Figure 3), with pH optima ranging from 5-6 for gastric lipase (rDGL; Figure 3A) to 8 for porcine

pancreatic extracts (PPE; Figure 3F). The concentration of bile salts had a clear effect on the levels of activity of rDGL, rHPL, PPL and rHPLRP2 (Figures 3A to 3D), with optimum activities shifted to lower pH values when NaTDC concentration was increased from 0.5 mM to 4 mM. These effects were weak in the case of HCEH and PPE (Figures 3E and 3F).

rHPLRP2 was found to be the most active lipase with a specific activity of 252.7 ± 16.8 U/mg at pH 6, in the presence of 4 mM NaTDC (Table 2). Recombinant human (rHPL) and native porcine (PPL) pancreatic lipases displayed similar levels of activities, but slightly distinct pH-dependent profiles. It is worth noticing that PPE shows an optimum activity at pH 8 whatever the NaTDC concentration, whereas purified PPL shows an optimum activity at pH 6 with 0.5 NaTDC and at pH 5 with 4 mM NaTDC. These findings indicate that PPL is probably not the only enzyme acting on CITREM present in PPE. Porcine CEH is also known to be present in PPE and its activity on CITREM could explain the optimum at pH 8 since the homologous human enzyme (HCEH) shows an optimum activity at pH 8 in the presence of 0.5 mM NaTDC. PPE displays a much lower specific activity compared to purified lipases but the batch used in this study only contains 5.8 % w/w PPL.

Since the pH-stat assay of lipase activity is based on the titration by NaOH of released acids and CITREM contains both fatty acid and citric acid esters, it was checked that free fatty acids (FFA) were generated in the pH-stat vessel by extraction of the reaction medium and TLC analysis. All lipases were found to release FFA as illustrated in the case of rHPLRP2 in Figure 1 (Panel A, lane 2). A simultaneous decrease in DAG, the main compounds present in CITREM was also clearly observed.

In vitro lipolysis of CITREM under simulated physiological conditions

The lipolysis of CITREM was tested under *in vitro* conditions mimicking the physiological conditions found in the GI tract of both adults fed with a liquid test meal [6, 23],

also considered as those found in term infants, and preterm infants fed with a formula [24]. Experiments were performed according to a two-step static *in vitro* digestion model in which CITREM was first incubated for 30 min with a gastric lipase (rDGL) solution reproducing the conditions (lipase concentration, pH) found in the stomach at half-gastric emptying of a test meal for term conditions [25] or an infant formula for preterm conditions [24]. In a second step, a mixture of pancreatic enzymes and bile salts was added to simulate the dilution of gastric contents in the upper small intestine at half-gastric emptying time and the mixture was incubated for 60 more minutes [25]. Experiments therefore lasted for a total duration of 90 minutes. The durations of 30 min for the gastric phase and 60 min for the small intestine phase were selected from previous studies in which good *in vitro-in vivo* correlations were obtained between *in vitro* lipolysis of test meals and their gastrointestinal lipolysis in the stomach and the duodenum for the whole digestion period[23, 25]. CITREM was tested at two initial concentrations (C_{min}= 0.3 g/100 mL and C_{max}= 0.9 g/100 mL) corresponding to the minimum and maximum concentrations found in commercial infant formula.

The FFA released from CITREM were measured by TLC-densitometry after lipid extraction and they were expressed as a percentage of the total fatty acids esterified in CITREM compounds, which corresponds to the hydrolysis level. As shown in Figure 4A, the overall hydrolysis level recorded at 90 min was maximum with term infant conditions and was found to increase with CITREM concentration (19.9% to 24.3%). Whatever this concentration, a clear jump in the release of fatty acids was observed when pancreatic enzymes were added at 30 min. This jump was not observed with preterm infant conditions. In this case, the highest hydrolysis level (20.4%) was recorded at 90 min with the lowest CITREM concentration. Gastric lipolysis by rDGL (0-29 min) was found to be slightly higher with preterm infant conditions, probably because the pH was higher (6.0 *vs.* 5.5 for term infant conditions) and rDGL shows its highest specific activity on CITREM at pH 6 in the

presence of a low bile salt concentration (Figure 3A). Overall, hydrolysis levels at 90 min ranged from 14.6% to 24.3% and therefore, only one fourth of CITREM fatty acids could be released under these conditions mimicking gastrointestinal lipolysis in the stomach and the upper part (duodenum) of the small intestine.

In vitro lipolysis of CITREM-containing infant formula under simulated physiological conditions

As described previously with pure CITREM, the lipolysis of CITREM-containing (0.21 g/100 mL) infant formula was tested under *in vitro* conditions mimicking the physiological conditions found in the GI tract of both term and preterm infants.

As shown in Figure 4B, the hydrolysis levels (FFA% *vs.* total fatty acids) obtained during the gastric phase of infant formula lipolysis (0-29 min) were similar for term (5.64±0.39 %) and preterm (4.71±0.39 %) infant conditions. The FFA generated during that period mainly resulted from the hydrolysis of TAG (Figure 5). When pancreatic enzymes were added at 30 min, a clear jump in the release of fatty acids, 1,2-DAG and MAG was observed for term infant conditions while TAG decreased at a higher rate (Figure 5A). Only a slight increase in the rate of FFA release was observed with preterm infant conditions (Figure 5B), as expected from the reduced amounts of pancreatic enzymes. It is worth noticing that in both cases, the levels of 1,3-DAG initially present in the infant formula did not change significantly during the lipolysis experiment. These 1,3-DAG are normally never formed by digestive lipases and the present results suggest that they are not hydrolyzed either under these experimental conditions. At the end of the duodenal phase of infant formula lipolysis (t=90 min), the hydrolysis level reached 28.11 ± 0.68 % when term infant conditions were tested, whereas it was only 16.97 ± 0.68 % with preterm infant conditions (Figure 4B). Under the best conditions, a little bit more than one fourth of infant formula fatty acids were

therefore released under these conditions mimicking gastrointestinal lipolysis in the stomach and the upper part of the small intestine. A similar hydrolysis level was observed with CITREM alone (Figure 4A) but it was not possible to distinguish the lipolysis products resulting from CITREM hydrolysis from those resulting from fat hydrolysis in the experiments performed with the CITREM-containing infant formula. Hydrolysis levels were therefore estimated from the overall measurements of residual TAG, DAG, MAG and FFA.

In vitro lipolysis of CITREM-stabilized fat emulsions under simulated physiological conditions

Since general infant formulas are complex food matrixes composed of proteins (whole, hydrolyzed or amino acids), carbohydrates, vitamins and minerals, etc. in addition to fat and CITREM, the effects of CITREM on fat digestion alone were tested with CITREM-stabilized emulsions prepared with the same fat blend used to prepare the infant formula. Fat alone and a fat emulsion stabilized by proteins were used as controls. Using term infant conditions, hydrolysis levels similar to those obtained with the infant formula (Figure 4B) were obtained with the various fat emulsions (Figure 4C). The hydrolysis levels obtained during the gastric phase of lipolysis (0-29 min) were; 3.42±0.39 %, 3.48±0.39 %, 5.28±0.39 % and 3.47±0.39 % with Fat alone, Fat-CITREM 6.25%, pre-emulsified Fat 10%-CITREM 0.625% and pre-emulsified Fat 10%-Protein 2%. The preparation of fat emulsions, including infant formula, had therefore only a weak effect on gastric hydrolysis levels, except when fat was pre-emulsified with CITREM before being mixed with gastric lipase solution. This is in good agreement with the biochemical properties of gastric lipase that is less sensitive than pancreatic lipase to interfacial properties. Some slight differences were then observed during the duodenal phase of lipolysis (Figure 4C). The rate of lipolysis during the 30-60 min period was lower with Fat alone compared to Fat-CITREM 6.25%, Fat 10%-CITREM 0.625%, Fat

10%-Protein 2% (Figure 4C) and infant formula under term conditions (Figure 4B). This probably reflects the fact that pancreatic lipase activity is dependent on the state of fat emulsification [26]. At the end of the duodenal phase of lipolysis (t=90 min), the hydrolysis levels reached 29.39±0.68 %, 29.18±0.68 % and 29.14±0.68 % with Fat alone, Fat-CITREM 6.25%, and Fat 10%-Protein 2%. These levels were identical to the hydrolysis level recorded with infant formula using term conditions. A higher hydrolysis level (34.88±0.68 %) was obtained with Fat 10%-CITREM 0.625%, probably because this Fat-CITREM mixture was pre-emulsified (10% fat in water) before performing the lipolysis experiments. As already mentioned for the experiments performed with the infant formula, it was not possible to distinguish the lipolysis products resulting from CITREM hydrolysis from those resulting from fat hydrolysis.

Enzymatic hydrolysis of the glycerol citrate fatty acid esters (GCFE) fractions of CITREM

In order to test the lipolysis of GCFE independently from the presence of acylglycerols (mainly DAG and MAG), CITREM was fractionated by flash chromatography. Two GCFE fractions giving citrate proton signal in NMR (samples E1 and E2) and containing no “free glycerides” were obtained (Figure 1, panel B). The relative composition of E1 and E2 in terms of citric acid, oleic acid and glycerol units were estimated from ^{1}H NMR analysis (comparison of the area under the curve of specific NMR signals). Citric acid/glycerol/oleic acid molar ratio were found to be 1/3.5/2.7 for E1 and 1/2.6/1.6 for E2. This composition was further used to estimate the mean molecular masses of E1 (1174.367 g/mol) and E2 (800.602 g/mol), assuming that all citric and fatty acids were esterified and bound to the glycerol backbone. These values were further used for the estimation of lipolysis levels of E1 and E2.

E1 and E2 samples (30 mg each) were submitted to the enzymatic digestion using the two-step *in vitro* digestion model (Figure 4D) and the results obtained were compared with

those obtained for the hydrolysis of the whole CITREM (Figure 4A). At the end of the duodenal phase of lipolysis (t=90 min), the amounts of FFA released were 9.21 ± 0.96 mg for E1 and 9.84 ± 0.05 mg for E2. In both cases, about one third of GCFE mass could therefore be converted in FFA (w/w). Mass amounts of FFA were converted into moles and hydrolysis levels of 47.3 ± 4.9 % for E1 and 58.1 ± 0.3 % for E2 were estimated (Figure 4D). These levels were higher than those measured with the whole CITREM (24.3% at maximum; Figure 4A) under similar conditions, indicating that the lipolysis rate of GCFE depends on other components of CITREM such as DAG and MAG. Nevertheless, these results show that the fatty acids esterified in GCFE can be released to a largest extent by digestive lipases.

Evaluation of enzymatic hydrolysis of CITREM by NMR and LC-MS

NMR analysis were first performed using the organic extracts obtained from CITREM and CITREM-containing formula/emulsions in the course of the *in vitro* digestion experiments reported in Figure 4. The peaks corresponding to the citrate moiety of GCFE in the ^{1}H-NMR spectra (Figure 2C) were well identified in all samples at time 0 and were further used as a reference. These specific signals should not remain after complete hydrolysis of CITREM compounds. After 90 min of *in vitro* digestion, a decrease of these NMR signals was observed in all organic extracts. From NMR signal integration, it was estimated that 20 to 30% of citrate still remained after digestion of CITREM alone. Moreover, NMR was found to be sensitive enough, although not perfectly quantitative, detect the citrate moiety and follow the hydrolysis of CITREM in complex mixtures such as the infant formula or the emulsions prepared with the same fat blend. It was thus shown that CITREM was not fully hydrolyzed during the *in vitro* digestion experiments after incubation for 90 min with gastric lipase and pancreatic enzymes.

Since it was suspected that partial hydrolysis could result from the inhibition of lipases by the glycerides present in CITREM, the enzymatic hydrolysis of purified GCFE fractions (experiments reported in Figure 4D) was also analyzed by ^{1}H-NMR. This time, the citrate signals were not present anymore in the organic extract obtained at 90 min and only some glycerides and FFA could be detected by NMR (data not shown). These results confirmed the negative effect of glycerides on the rate of GCFE hydrolysis by lipases and the fact that GCFE could be largely converted into water-soluble compounds after removal of fatty acids.

The enzymatic decomposition of GCFE was further investigated. Although the NMR signal for citrate disappeared from the organic extracts, water-soluble compounds combining glycerol and citric acid could be still present in the digestion samples. The production of some water soluble compounds resulting from complete or incomplete digestion of CITREM was anticipated and they could be detected using LC-MS technique. Actually, several polar compounds containing glycerol and citric acid (Scheme 2; compounds **1** to **7**) were identified by LC-TOF in the aqueous phase obtained after the digestive process. It was thus shown that digestive lipases can release the fatty acids of GCFE as FFA or glycerides (DAG, MAG) but the resulting glycerol citric acid esters (GCE) are not further hydrolyzed by these enzymes into glycerol and citric acid as initially expected [4].

Discussion

The lipolysis of CITREM by individual gastrointestinal lipases was tested *in vitro* to identify the enzymes potentially involved in CITREM digestion *in vivo*. Using the pH-stat technique and titration of the free fatty acids released upon lipolysis, it was shown that CITREM is hydrolysed by gastric lipase at pH values ranging from 2 to 7, suggesting that CITREM digestion can already start in the stomach. Gastric lipase was found to be active on CITREM in the absence (Figure 4A) and presence (Figure 3A) of bile salts, indicating that this enzyme could also contribute to the CITREM digestion in the small intestine. All pancreatic enzymes were found to be active on CITREM, including recombinant human pancreatic lipase (+ colipase), human pancreatic lipase-related protein 2 (rHPLRP2) and human pancreatic carboxyl ester hydrolase (CEH), the same enzyme as bile salt-stimulated lipase (BSSL) present in human milk. Porcine enzymes commonly used for *in vitro* digestion studies were also tested and it was found that both purified porcine pancreatic lipase and total pancreatic extracts (e.g., pancreatin) were active on CITREM like human enzymes. Pancreatin could therefore be used as a substitute for human pancreatic juice during *in vitro* digestion experiments. Pancreatic enzymes displayed optimum activities at pH values ranging from 5 to 8, with and without micellar concentrations of bile salts and could thus be active on CITREM under the conditions found in the small intestine in both term and preterm infants. No evidence was established at this stage that lipases could hydrolyze the ester bond of glycerol citrate, whereas the release of free fatty acids was confirmed by TLC analysis.

CITREM digestion was then tested *in vitro* using a two-step static digestion model including a gastric phase with gastric lipase (30 min) and an intestinal phase with pancreatin (60 min), under conditions mimicking the physiological conditions found in the stomach and duodenum of term infants (high pancreatic enzyme and bile salt concentrations) and preterm infants (low pancreatic enzyme and bile salt concentrations). CITREM concentrations in the

assay were based on the minimum and maximum concentrations found in commercial infant formula (0.3-0.9 g/100 mL). The overall hydrolysis level (%FFA *versus* total CITREM fatty acids) was found to be maximum with term infant conditions but did not exceed 24.3% after 90 min of incubation (gastric + duodenal phases). Therefore, only one fourth of CITREM fatty acids could be released under conditions simulating gastrointestinal lipolysis in the upper part of the GI tract.

The two-step *in vitro* digestion model was further used to evaluate the digestibility of an infant formula containing CITREM. With the term infant conditions, it was found that the fat from infant formula was hydrolyzed at the same rate as various emulsions prepared with the same fat blend and containing only TAG emulsified with either CITREM or protein. Therefore, fat digestion was not significantly changed by the presence of CITREM. It was not possible from TLC analysis to discriminate the fatty acids released from CITREM and formula TAG, but assuming that the main part came from TAG lipolysis, the maximum hydrolysis level (%FFA *versus* total fatty acids) of infant formula reached 28.1 % with term infant conditions, whereas it was only 16.9 % with preterm infant conditions. Again, a little bit more than one fourth of infant formula fatty acids was released under the best conditions. The validity of the two-step *in vitro* model for testing the digestion of CITREM and infant formula was questioned at that point, although similar hydrolysis levels were previously obtained during the *in vitro* digestion of casein-stabilized emulsions of rapeseed oil and olein, using the same two-step digestion model [27]. The maximum hydrolysis levels obtained in these studies are however consistent with some data obtained in the course of *in vivo* studies. Indeed, similar hydrolysis levels were recorded at the Angle of Treitz in healthy volunteers fed with a solid-liquid test meal (27.7±6.8 % [23]; 28.1±13.5 % [28]). The two-step digestion model used here is therefore suitable for simulating lipid digestion in the upper part of the GI tract (stomach and duodenum). It is not well adapted however for reproducing the entire

digestion process and the completion of lipolysis that occurs further down in the small intestine and depends on the removal of lipolysis products by intestinal absorption. Additional experiments are therefore required for investigating the complete digestion of CITREM and infant formula.

One way to proceed was to study the hydrolysis of the GCFE fraction (34% w/w of CITREM) in the absence of the acylglycerol fraction (mainly DAG and MAG; 66% w/w), thus mimicking an acceleration of the overall lipolysis reaction and intestinal absorption. In this case, the hydrolysis levels (47.3 to 58.1 %) obtained using the two-step *in vitro* model were 2-fold higher than those recorded with complete CITREM, thus indicating that GCFE hydrolysis can be potentially increased depending on the mixture in which they are present (i.e., presence or not of DAG and MAG). GCFE hydrolysis was monitored by NMR after GCFE could be identified thanks to the presence of specific citrate moiety signals in the NMR spectra. A complete disappearance of these citrate signals could be observed in the lipid extracts obtained from GCFE digestion. Nevertheless, it appeared that CITREM was not fully hydrolyzed since undigested GCE were detected in the water phase. Several water soluble compounds resulting from incomplete digestion of CITREM could be identified using LC-MS (Scheme 2), including mono-, di- and triglycerol citrate, as well as other GCE containing additional glycerol or citric acid units, but no citric acid or glycerol were detected.

Conclusion

It was shown that the fatty acids contained in CITREM compounds can be released to a large extent by the action of digestive lipases on the ester bonds linking fatty acid and glycerol units. NMR data support the hypothesis that fatty acids are released from GCFE in the form of free fatty acids or alternatively as acylglycerols (MAG and DAG). Considering the identification of deacylated GCEs in the water phase, the proposed model indicates that ester bonds between citric acid and glycerol units are not totally hydrolyzed during the digestion process as initially expected. The complete the understanding of CITREM digestibility, the degree of hydrolysis of GCEs during human digestion remains as a topic to be studied.

Acknowledgements

We are grateful to Kaouthar Dridi (EIPL, CNRS UMR7282, Marseille) for her technical assistance during the assays of CITREM lipolysis by individual lipases.

The authors would also like to thank Dr. Claudia Roessle (Nestlé Research Center, Lausanne), Dr Flemming vang Sparso and his team (Danisco, Denmark) for the many discussions and analysis of the composition and production of CITREM.

This work was carried out with the financial support of the Nestlé company (Nestec Ltd., Avenue Nestlé 55, CH-1800 Vevey, Switzerland) and several authors (A. Patin, F. Giuffrida, T.J. Wooster, S.K. Thakkar, F. Destaillats, C. Cruz-Hernandez) of this article are currently employed by Nestlé Research Center in Lausanne, Switzerland.

The EIPL laboratory (UMR7282 CNRS-Aix Marseille Université) is a member of the LISA Carnot Institute (Lipids for health and industry).

References

1. J. B. Lauridsen, *J. Am. Oil Chemists' Soc.*, 1976, **53**, 400-407.
2. G. L. Hasenhuettl, in *Food Emulsifiers and Their Applications.*, ed. G. L. Hasenhuettl and R. W. Hartel, Springer, New York, 2008, ch. 2, pp. 11-37.
3. *United States Pat.*, US4071544 A, 1978.
4. FAO, *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilisers, flour-treatment agents, acids and bases.*, Food and Agricultural Organization of the United Nations, Geneva, 1967.
5. FDA, *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food ("Redbook II").* US. Food and Drug Administration, Center for Food Safety and Applied Nutrition., Washington, DC, 1993.
6. F. Carrière, J. A. Barrowman, R. Verger and R. Laugier, *Gastroenterology*, 1993, **105**, 876-888.
7. J. C. Bakala N'Goma, S. Amara, K. Dridi, V. Jannin and F. Carrière, *Therapeutic Delivery*, 2012, **3**, 105-124.
8. S. Fernandez, V. Jannin, J. D. Rodier, N. Ritter, B. Mahler and F. Carriere, *Biochim Biophys Acta*, 2007, **1771**, 633-640.
9. S. Fernandez, J. D. Rodier, N. Ritter, B. Mahler, F. Demarne, F. Carriere and V. Jannin, *Biochim Biophys Acta*, 2008, **1781**, 367-375.
10. S. Fernandez, S. Chevrier, N. Ritter, B. Mahler, F. Demarne, F. Carrière and V. Jannin, *Pharm. Res.*, 2009, **26**, 1901-1910.
11. S. Fernandez, V. Jannin, S. Chevrier, Y. Chavant, F. Demarne and F. Carriere, *Pharm Res*, 2013, **30**, 3077-3087.
12. A. Roussel, N. Miled, L. Berti-Dupuis, M. Riviere, S. Spinelli, P. Berna, V. Gruber, R. Verger and C. Cambillau, *J Biol Chem*, 2002, **277**, 2266-2274.

13. F. Carrière, H. Moreau, V. Raphel, R. Laugier, C. Bénicourt, J.-L. Junien and R. Verger, *Eur. J. Biochem.*, 1991, **202**, 75-83.

14. V. Belle, A. Fournel, M. Woudstra, S. Ranaldi, F. Prieri, V. Thome, J. Currault, R. Verger, B. Guigliarelli and F. Carriere, *Biochemistry*, 2007, **46**, 2205-2214.

15. C. Eydoux, J. De Caro, F. Ferrato, P. Boullanger, D. Lafont, R. Laugier, F. Carriere and A. De Caro, *J Lipid Res*, 2007, **48**, 1539-1549.

16. C. Erlanson and B. Borgström, *Scand. J. Gastroenterol.*, 1970, **5**, 293-295.

17. M. F. Maylié, M. Charles, M. Astier and P. Desnuelle, *Biochem. Biophys. Res. Comm.*, 1973, **52**, 291-297.

18. E. Mas, N. Abouakil, S. Roudani, F. Miralles, O. Guy-Crotte, C. Figarella, M. J. Escribano and D. Lombardo, *Biochem. J.*, 1993, **289**, 609-615.

19. E. Mas, N. Abouakil, S. Roudani, J. L. Franc, J. Montreuil and D. Lombardo, *Eur. J. Biochem.*, 1993, **216**, 807-812.

20. R. Verger, G. H. de Haas, L. Sarda and Desnuelle, *Biochim Biophys Acta*, 1969, **188**, 272-282.

21. F. Destaillats and C. Cruz-Hernandez, *J Chromatogr A*, 2007, **1169**, 175-178.

22. D. N. Challacombe, S. Edkins and G. A. Brown, *Arch Dis Child*, 1975, **50**, 837-843.

23. F. Carrière, C. Renou, S. Ransac, V. Lopez, J. De Caro, F. Ferrato, A. De Caro, A. Fleury, P. Sanwald-Ducray, H. Lengsfeld, C. Beglinger, P. Hadvary, R. Verger and R. Laugier, *Am. J. Physiol. Gastrointest. Liver. Physiol.*, 2001, **281**, G16-28.

24. C. Roman, F. Carriere, P. Villeneuve, M. Pina, V. Millet, U. Simeoni and J. Sarles, *Pediatr Res*, 2007, **61**, 83-88.

25. F. Carrière, C. Renou, V. Lopez, J. De Caro, F. Ferrato, H. Lengsfeld, A. De Caro, R. Laugier and R. Verger, *Gastroenterology*, 2000, **119**, 949-960.

26. G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta*, 1965, **105**, 121-136.

27. C. Vors, P. Capolino, C. Guerin, E. Meugnier, S. Pesenti, M. A. Chauvin, J. Monteil, N. Peretti, M. Cansell, F. Carriere and M. C. Michalski, *Food Funct.*, 2012, **3**, 537-546.

28. F. Carrière, P. Grandval, C. Renou, A. Palomba, F. Prieri, J. Giallo, F. Henniges, S. Sander-Struckmeier and R. Laugier, *Clin Gastroenterol Hepatol*, 2005, **3**, 28-38.

Figure legends

Scheme 1: *CITREM chemical composition and expected products from enzymatic hydrolysis*

Scheme 2: *Water-soluble compounds expected from CITREM hydrolysis*

Fig. 1: *TLC analysis of CITREM.* Panel A: separation of neutral lipids using heptane/ether/formic acid (55/45/1 v/v/v). Lanes 1 and 2 correspond to CITREM and CITREM hydrolyzed by rHPLRP2, respectively. Panel B: separation of polar and neutral lipids using a double elution with chloroform/methanol/H2O (65/25/4 v/v/v) first and heptane/ether/formic acid (55/45/1 v/v/v) second. Lanes 3, 4 and 5 correspond to CITREM, GCFE fractions E1 and E2, respectively. Triolein (TO), oleic acid (OA), 1,2-diolein (1,2-DO), 1,3-diolein (1,3-DO) and monoolein (MO) were used as reference standards (RS). TAG, triacylglycerides; FFA, free fatty acids; 1,3-DAG, 1,3-diacylglycerides; 1,2-DAG, 1,2-diglycerides; MAG, monoacylglycerides; GCFE, Glycerol Citrate Fatty acid Esters.

Fig. 2: *^{1}H-NMR spectra of CITREM and glycerol citrate fatty acid esters (GCFE).* (A) complete CITREM ^{1}H-NMR spectrum showing the characteristic chemical shifts of FA, glycerol and citrate protons. (B) close-up view of glycerol protons chemical shifts with the identification of glyceride molecular species (TAG, DAG, MAG). (C) ^{1}H-NMR spectrum of the GCFE E1 fraction obtained from CITREM showing characteristic chemical shifts of citrate protons.

Fig. 3: *Variations with pH in the specific activities (U/mg) of individual lipases on CITREM.* Values are mean ± SD (n=3) and were obtained using the pH-stat technique with back-titration to pH 9 of FFA released at the various pH tested.

Fig. 4: *Lipolysis levels of CITREM alone (A), infant formula containing CITREM (B), various fat emulsions including those stabilized by CITREM (C) and GCFE fractions of CITREM in a two-step in vitro digestion model.* In panel A, data are FFA% and are expressed as the percentage of total fatty acids esterified in CITREM compounds. In panels B and C, data are FFA% and are expressed as the percentage of total fatty acids esterified in the TAG initially present in infant formula and fat emulsions. In panel D, data are FFA% and are expressed as the percentage of total fatty acids esterified in GCFE fractions (E1 and E2). Both term and preterm infant conditions were tested with CITREM alone (panel A) and infant formula containing CITREM (panel B). Fat emulsions (panel C) were prepared with the same fat blend used for making the infant formula (panel B). All values are mean ± SD obtained from triplicates

Fig. 5: *Variations with time in the individual lipolysis products generated during the in vitro lipolysis of infant formula using the two-step digestion model. Both term (A) and preterm (B) conditions were tested.* Individual lipolysis product levels are expressed in µmoles present in the reaction mixture. TAG, residual triacylglycerides; FFA, free fatty acids; 1,3-DAG, 1,3-diacylglycerides; 1,2-DAG, 1,2-diacylglycerides; MAG, monoacylglycerides. Values are mean ± SD obtained from triplicates.

Table 1: *Fatty acid composition (% w/w) of CITREM and fat blend used for making model emulsions and infant formula.*

Fatty acid	Relative % (w/w) of total fatty acids	
	CITREM[a]	Fat blend[b]
8:0		13.72
10:0		11.54
11:0		0.03
12:0	0.06	0.28
14:0	0.13	0.23
15:0	nd	0.03
16:0	5.77	7.43
16:1	0.08	0.09
17:0	0.04	0.05
17:1	0.04	nd
18:0	3.74	2.11
18:1 t	0.16	0.06
18:1 n-9	79.11	40.83
18:1 n-7	0.69	0.99
18:2 tt	0.12	0.00
18:2 ct	0.09	nd
18:2 n-6	8.48	18.79
18:3 n-6	0.04	nd
18:3 n-3	0.29	2.02
20:0	0.30	0.27
20:1 n-9	nd	0.39
20:2 n-6	nd	nd
20:3 n-6	nd	0.03
20:4 n-6	nd	0.24
22:0	0.69	0.40
22:1 n-9	nd	0.06
24:0	0.16	0.15
24:1	nd	0.03
22:6 n-3	nd	0.23
Total	100.00	100.00

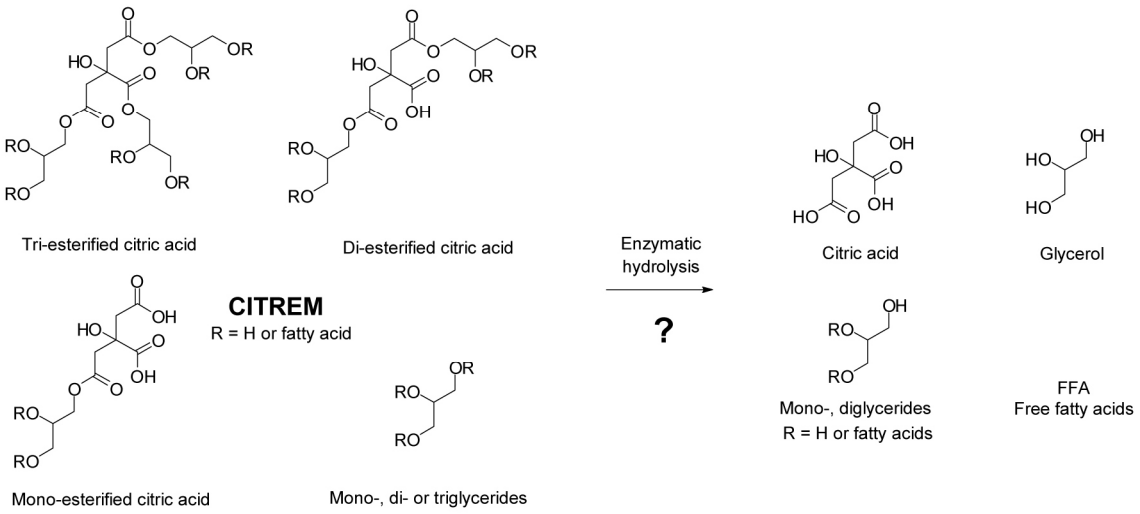
[a]CITREM contains 66.73 g of fatty acids per 100 g. [b]Fat blend contains 86.73 g of fatty acids per 100 g of oil. Values are means obtained from triplicates.

Table 2: *Maximum specific activities (U/mg) of individual lipases on CITREM.*

Enzyme	Bile salt concentration (mM)	Maximum specific activity (U/mg) [a]	Optimum pH
rDGL	0.5	50.9 ± 26.1	6
	4	73.8 ± 15.7	5
rHPL+colipase	0.5	120.1 ± 2.8	6
	4	76.2 ± 24.6	5
PPL+colipase	0.5	141.8 ± 10.7	6
	4	112.6 ± 13.9	5
rHPLRP2	0.5	162.0 ± 26.2	6
	4	252.7 ± 16.8	6
HCEH	0.5	24.0 ± 3.0	8
	4	30.7 ± 6.0	6
PPE	0.5	5.8 ± 0.3	8
	4	5.7 ± 0.6	8

[a]Values are mean±SD (n=3)

Scheme 1



Scheme 2

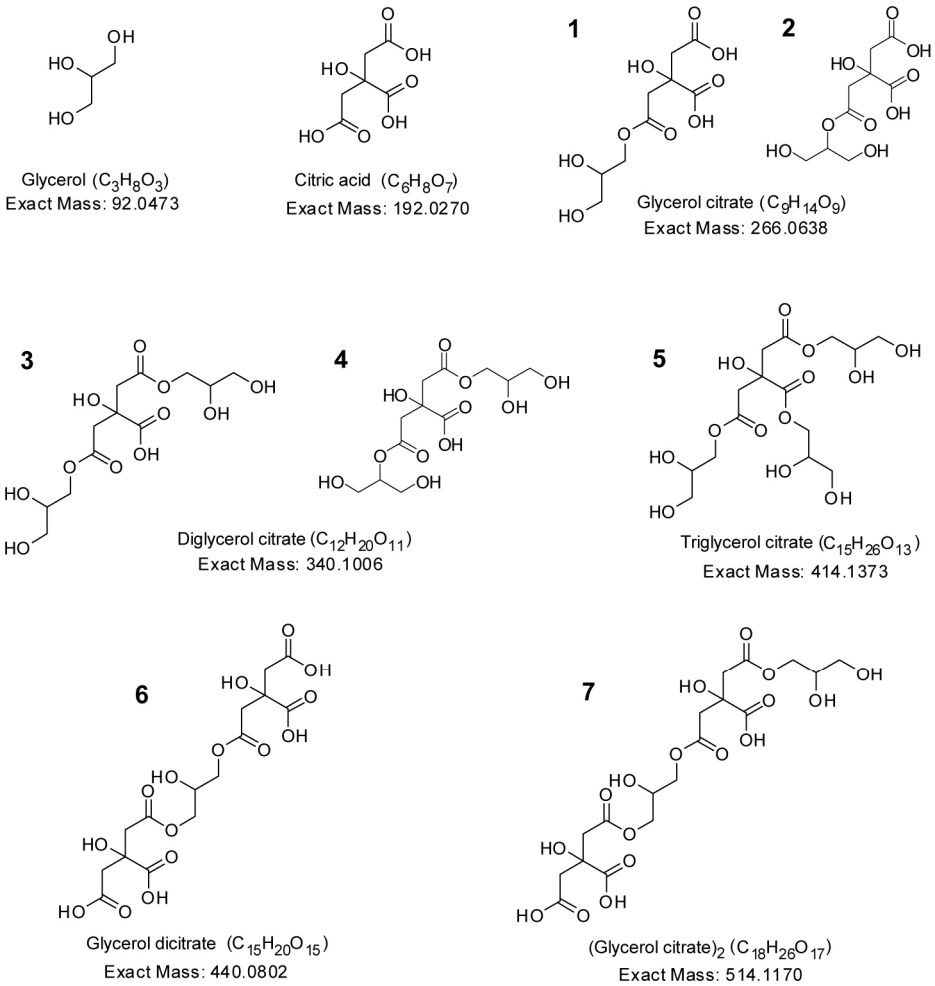


Figure 1

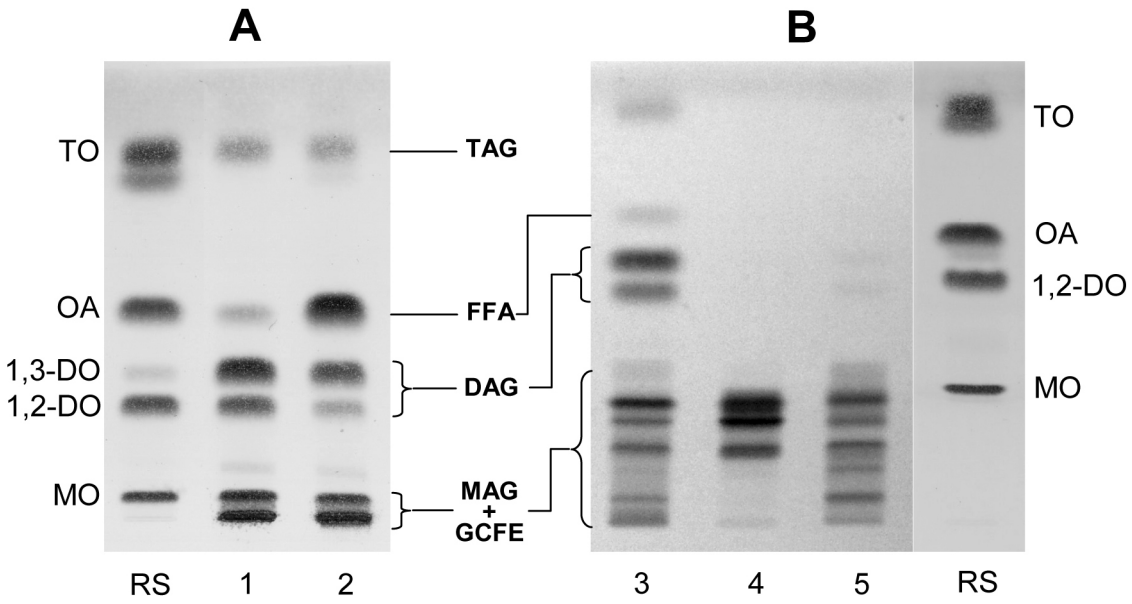


Figure 2

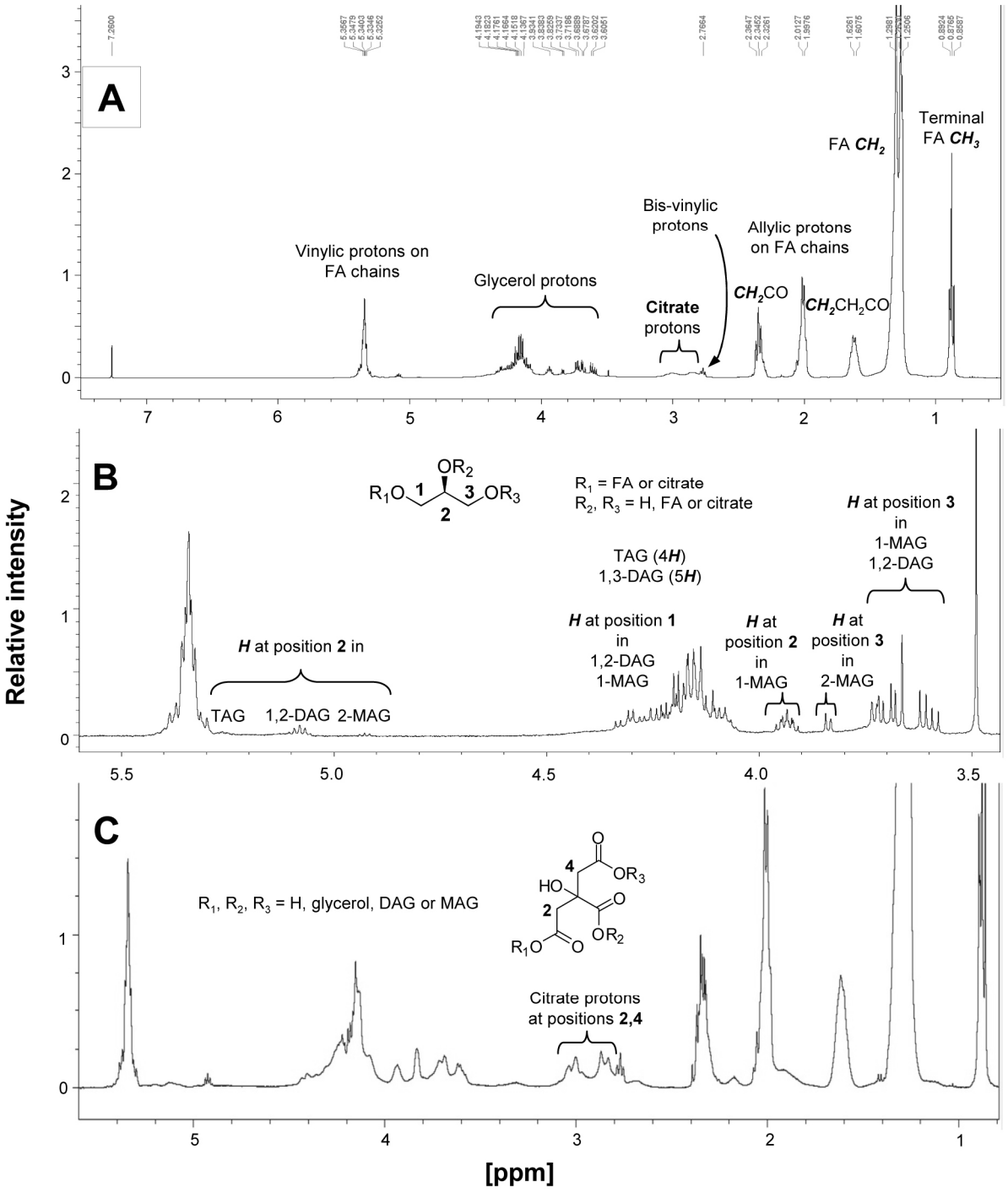


Figure 3

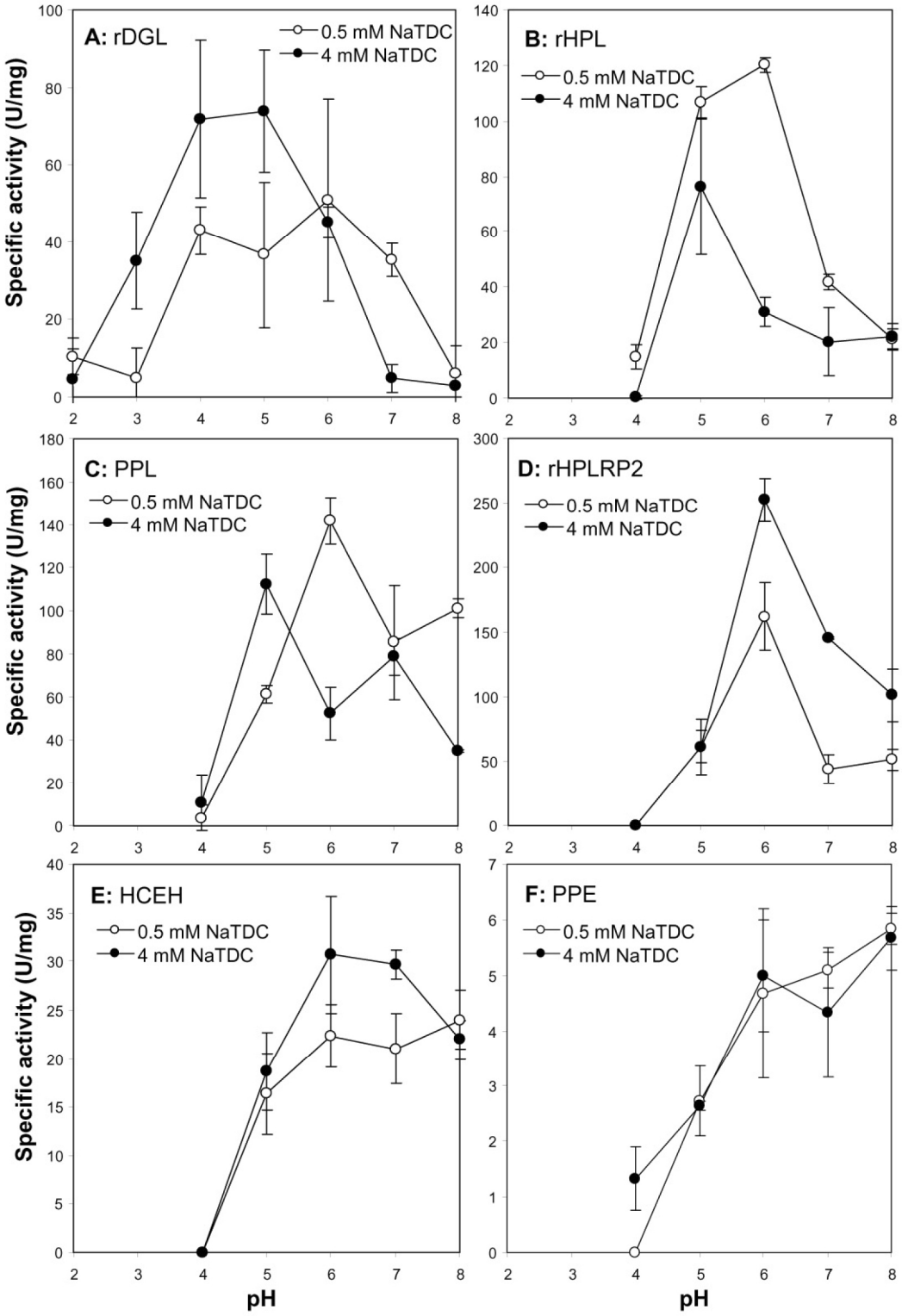


Figure 4

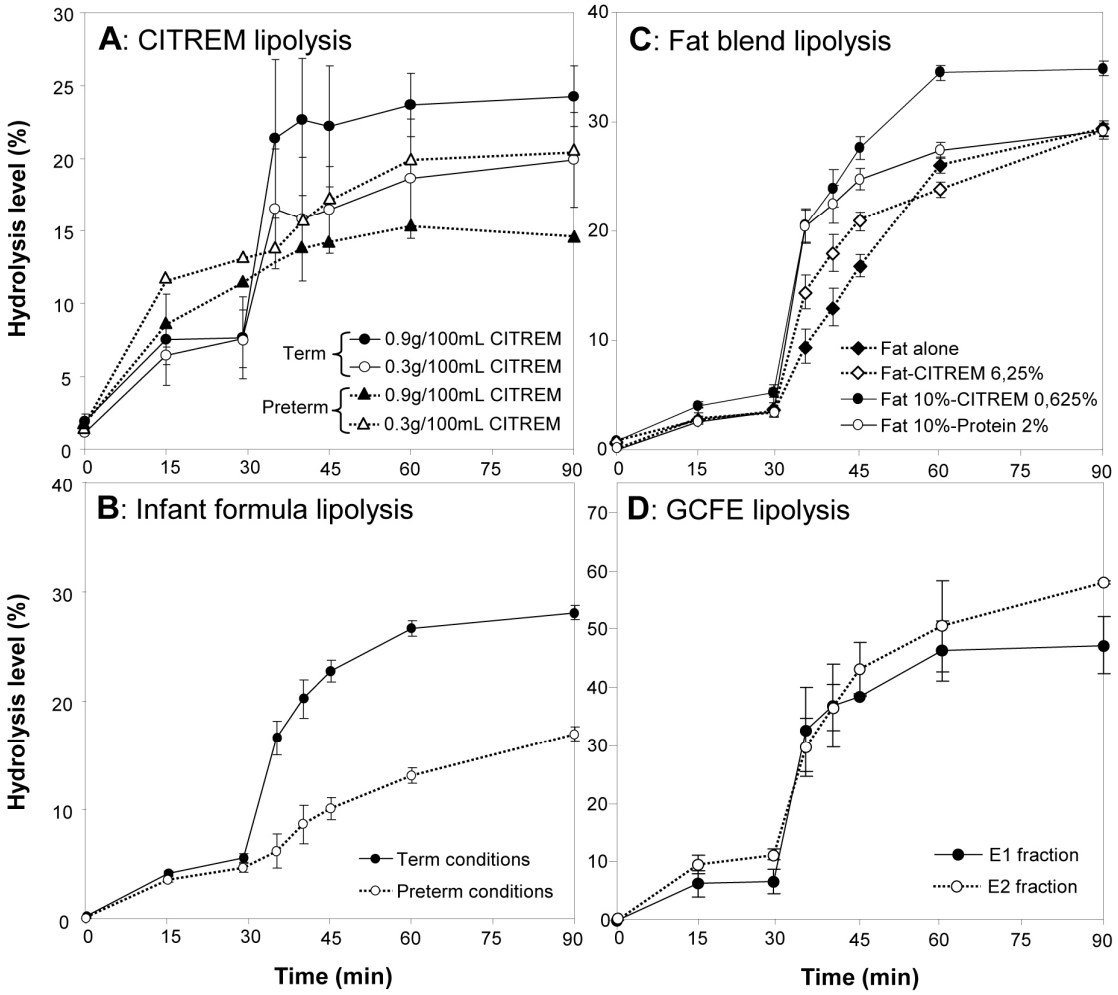


Figure 5