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Behaviour of galatolipids components in crude lipid extracts in *in-vitro* digestion emulsion model

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Abstract: Delays in lipid digestion may impart a prolonged feeling of satiety, thus rendering this research highly relevant to the current world-wide efforts to combat obesity. This study aimed to evaluate the efficacy of crude plant lipid extracts from spinach and pumpkin seed that are rich in DGDG to determine whether they can delay lipolysis when submitted to the in-vitro digestion emulsion model. The lipolysis of the O/W stabilised emulsions were followed using an in-vitro bulk model with two hours measurements in the stomach phase and a further two hours measurements in the duodenal phase. Prior to obtained processed O/W emulsion, four different vesicle solutions were prepared which later served as the continuous phase, namely LEC, LECDG, SPLIP and PULIP. The emulsion breakdown was followed using measurements of zeta potential and amounts of free fatty acids released using the pH stat method. The results of lipid digestibility, determined by the pH stat measurement, indicate that the LEC stabilised emulsion showed the highest rate of digestibility when compared against SPLIP and PULIP, which was lower respectively. Zeta potential results showed an increased in zeta value both in the stomach and in the duodenal phases. The results showed that SPLIP and PULIP are both efficient in delaying lipolysis as compared to LECDG; this indicate that DGDG in cruder extract shows an inhibition properties which resistant against digestion enzymes.

Key words: Qualitative TLC; Crude lipid extract; Glycolipid; In-vitro digestion

1. Introduction

Phytonutrients, as well as other bioactive components, are stored in lipids which are found in the chloroplast thylakoid membrane; this is the photosynthetic membrane of a green leaf. The thylakoid membrane of spinach, when added to food, has been reported to control fat regulation in in-vitro and in-vivo studies; specifically, it was shown to induce satiety in short-term studies in humans (Köhnke et al., 2009) as well as in long-term studies of rats and mice (Emek et al., 2010; Albertsson et al., 2007).

Spinach possesses a significant amount of galactolipid, in the form of digalactosyldiacylglycerol (DGDG). Galactolipids, mainly DGDG, can delay the rate of digestion; to illustrate, when a large portion of undigested lipids reach the ileum, they trigger the ileal break mechanism – this controls the way in which satiety is modulated (Chu et al., 2010; Chu et al., 2009). Based on the in-vitro assessment using purified DGDG, the suggested mechanism for delaying lipolysis is based on the fact that the large head groups, in the form of two galactose moieties with strong molecular interaction between the galactosyl residues, might sterically hindered the bile salts attack; this is the first step of lipid digestion (Chu et al., 2009). The work of Chu and colleagues (2009) specifically claiming that galactolipids, particularly DGDG, provide a steric hindrance that slows the duodenum enzyme's ability to absorb the emulsion lipid-water interfaces, and thus impedes the rate at which lipolysis occurs. Spinach thylakoid component is reported to significantly reduce body weight and improved glucose/lipid metabolism in obese individual (Erlanson-Albertsson & Albertsson, 2015).

Besides thylakoid spinach, pumpkin seed was chosen as another plant based material that has also been investigated as an alternative to delaying lipolysis; Pumpkin seed also containing a significant amount of DGDG (Nakea et al., 2000). Numerous studies have investigated pumpkin seed in food applications; however, as yet there is scarce information about in-vitro digestion of pumpkin seed.

To date, lipid in a crude extract which containing DGDG, is not known whether possesses delaying effect similar to purified DGDG. This study provides data on galactolipid behaviour when submitted in in-vitro digestion model.

2. Method

2.1. Extraction from spinach leaf

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The stem of the spinach leaves were cut off and weighed before being mixed with 0.3 M (M = molar) of the sucrose solution, at a ratio of 1:3 w/w, they were blended using a household blender for 30 seconds. Sucrose was used as osmotic agents, of which are commonly used in the isolation work of chloroplasts using buffer solutions (Honda et al., 1966). Once blended, the slurry was filtered using cheese cloth – the remaining supernatant was then poured into a 50 mL centrifuge tube and centrifuged at 3,500 g, 5°C, for 15 minutes. Finally, the supernatant was discarded and the pellet which remained was collected for further analysis.

The method used for lipid extraction was adapted from Bligh and Dyer (Folch et al., 1975). The fresh chloroplast pellet was added to a mixture of chloroform/methanol at a ratio of 2:1, it was then vortexed for 1 minute before 0.3 mL of a saline solution (0.9% NaCl) was added. NaCl was added to facilitate the partitioning of the lipids into the organic phase (Hinca et al., 1998). This mixture was then vortexed and left for 3 minutes before being centrifuged at 3,500 g, 5°C, for 15 minutes. The bottom layer, which contained the lipids, was transferred into a pre-weigh bottle and subsequently left in nitrogen dryer until it was dry. After which, the lipids were weighed. The total amount of lipid was calculated using Equation 1 below.

$$Lipid\ content\ (\%) = \frac{weight\ of\ lipid\ (g)}{weight\ of\ dried\ pellet\ (g)} \times 100\% \quad (1)$$

2.2. Extraction from pumpkin seed

Edible pumpkins were bought in October. They were cut using a sharp knife; the seeds were collected from the gourd and then washed with tap water before drying using a dehydrator at 37°C for 3 days. Then, the dried seeds were ground using a coffee grinder.

The method used for lipid extraction was similar to the method described for spinach lipid extract. Following the addition of the saline solution, the mixture was vortexed and transferred into a separator funnel and then left in a fume hood until a clear phase was observed on the bottom layer. The bottom layer, containing the lipids, was removed from the funnel into a pre-weigh bottle. Following this, the solvent was then evaporated under nitrogen gas until it was dried. The dried lipids were then weighed and the total amount of lipid was calculated using Equation 1.

2.3. Oil-in-water emulsions for in-vitro analysis of digestive behaviour

2.3.1. Purification of sunflower oil

Sunflower oil was purified using magnesium silicate particles at 4% w/w and stirring for 30 min at 600 rpm on a magnetic stirrer at room temperature. Silicate particles were then removed by centrifugation at ambient temperature and 2700 g during 15 min. Purification of sunflower oil removes surface active impurities and therefore allows for more precise assessment of the effect of added galactolipids and surfactants on fatty acid released.

2.3.2. Bulk vesicles preparation

The method referred to as hydration of a lipid cake was used to prepare the lipid vesicles because it is easy, simple and widely used (Lichtenthaler and Park, 1963; Hur et al., 2009) a bath sonicator was used to dissolve the dry lipid cake completely; this was followed by vigorous shaking for a recommended timeframe of an hour. Distilled water was used as the medium for hydration because the final dispersion of the hydrated vesicles would then be subsequently used as the continuous phase of the oil-in-water emulsions. The dispersion was then disrupted using a probe sonicator (Soniprep 150 Plus) at 14%, with 230V amplitude. The disruption was carried out repeatedly until the vesicles reached a size range of between 150 and 230 nm. The solutions obtained were then centrifuged at 20,000 g for 20 minutes to remove any titanium deposits, further filtrated were performed using a 0.4 µm syringe filter. The size of the vesicles was verified based on the particle size measurement method (Delsa-nano, Beckman Coulter, UK).

2.4. Processing

Oil-in-water (O/W) emulsions with a 15% w/w dispersed oil phase were prepared by adding 15 g of purified sunflower oil to 85 g of water containing lipid vesicles. These lipid vesicles represented the surfactant in the system; lecithin (LEC), mixture of lecithin with purified DGDG (LECDG), chloroplast spinach lipid extract (SPILP) and pumpkin seed lipid extract (PULIP). This was followed by processing using a high shear overhead mixer (Silverson L5M, Chesham, UK), at 8,000 rpm for 5 minutes. The emulsions were further processed through two further stages involving a high pressure homogeniser (Niro Soavi, Italy), running at 300 bar for 30 minutes in order to complete the emulsification process. This was later increased to 800 bar for 3 minutes, at which point the samples were collected. The emulsions were stored at 4°C and used within 24 hours.

2.5. Characterization

2.5.1. Visual observation

Emulsion instability is observed by placing the emulsion in a tube that is stored in quiescent conditions. When destabilisation occurs it is indicated by the separation of the two phases: firstly the top layer is opaque and/or turbid; while, secondly, the bottom layer is transparent (Herrera, 2012).

2.5.2. Microscopy

The microstructure of the freshly extracted emulsions was observed using a light microscope (Nikon Microscope Eclipse E400, Nikon Corporation, Japan). Specifically, a drop of the sample was placed on a glass slide and covered with a cover slip; a digital camera was attached to the microscope to capture images of the samples. The scales of the images were calibrated against a glass mounted graticule (1 mm, 0.01 mm division from Graticules Ltd, Tonbridge, Kent, UK).

2.5.3. Zeta potential measurement

Initially, for the zeta potential measurement, 90 µL of the emulsion was added to 20 mL of dH^2O, and stirred using a Pasteur pipette. This solution was then loaded into the chamber of a particle electrophoresis instrument (Particle Analyser, Beckman Coutler, Inc. USA), using the following settings: temperature 25°C; refractive index of dispersant = 1.330; viscosity of dispersant = 0.891 mPas; relative dielectric constant of dispersant = 79.0; electrode spacing = 50.0 mm. The zeta potential was then determined by measuring the direction and velocity of the droplets in an applied electrical field, from which the zeta potential was calculated using Beckman Coulter software. Each zeta potential data reported was an average of three readings that were taken per sample.

2.6. In-vitro analysis of lipid digestion

2.6.1. Emulsion digestion model

The in-vitro digestion model was modified from the works of Beysseriat et al. (2006), Mun et al. (2007) and McClements et al. (2009). This model focuses on mimicking digestion in the stomach and in the duodenum phase as the lipid itself is not digested in the mouth.

The samples prepared in these experiments were examined every hour, for a total of 4 hours of digestion. Light microscopy and zeta potentials were all used to characterise the emulsion digestion.

2.6.1.1. Gastric phase

The prepared emulsions (10 mL) were placed in amber bottles and added to 0.15 M NaCl. The mixtures were then adjusted to pH 2.5 with 1 M HCl, followed by the addition of pepsin and gastric lipase analogue to the system. The final concentrations of the mixtures were 146 units/mL pepsin and 84 units/mL of gastric lipase. A pH level of 2.5 was used because it is the range of a normal fasting human stomach, which ranges from pH 1 to 3 (Beysseriat et al., 2006; Reis et al., 2009; Sarkar et al., 2009b). The samples were then incubated, in an incubator, for 1 and 2 hours at 37°C, they were also stirred using a magnetic stirrer at 130 rpm.

2.6.1.2. Duodenal model

The gastric mixture was carried forward to the duodenal model. The pH of the samples was immediately adjusted to pH 5, by adding 0.9 N $NaHCO_3$. This addition mimicked the normal duodenal conditions (Sarkar, 2009b) whereby $NaHCO_3$ is secreted in preparation of the optimum condition for lipid digestion. Bile extracts were then added to the system. The samples were, at this stage, adjusted to pH 6.5 with 0.9 N $NaHCO_3$, followed by the addition of pancreatic lipase and colipase. The final concentrations of constituents were: 4.4 mg/mL bile extract; 54 units/mL pancreatic lipase and 3.2 µg/mL colipase. The duodenal digestion then continued for 2 hours at 37°C in the incubator, with stirring at 130 rpm.

During lipolysis, the free fatty acids (FFAs) were released from the lipids. In order for them to maintain a neutral pH condition, alkali needed to be added. The pH stat method had previously been used for *in-vitro* characterisation of lipid digestion under duodenal conditions (McClements & Li, 2010a); hence, for the purpose of this study, the pH stat method was used to measure the volume of alkali (NaOH) that must be added to the sample to maintain a neutral pH over time, as this would neutralise the FFAs produced. Specifically, 15 mL of emulsion was checked for the pH reading before being poured into a 20 mL beaker and warmed up at 37°C for 120 minutes. Then, the enzyme was added. Since this method was used to measure the rate of digested TAG, as complementary to the duodenal phase, the same concentration enzyme was used. The pH was kept at 7 using a 0.1 M NaOH solution and a tritator (702 SM Titrino, Metrohm, Runcorn, UK). The volume of NaOH that was added to the emulsion was recorded every 5 minutes; the measurements were carried out twice on two different samples. The percentages of FFAs released by the lipolysis was calculated from the number of moles of NaOH required to neutralized the FFA divided by the number of moles of FFA that could be produced from the triacylglycerols if they were all digested (assuming 2 FFA produced per 1 triacylglycerol molecule) (Eq. 2):

$$\%\text{FFA} = 100 \times \left(\frac{V_{NaOH} \times m_{NaOH} \times M_{Lipid}}{w_{Lipid} \times 2}\right) \qquad (2)$$

Here V_{NaOH} is the volume of sodium hydroxide required to neutralize the FFA produced (in mL), m_{NaOH} is the molarity of the sodium hydroxide solution used (in M), w_{Lipid} is the total weight of oil initially present in the reaction vessel (15 g) and $M_{Lipid}$is the molecular weight of the oil, 876.16 g/mol.

3.2. Properties of the emulsions prepared for in-vitro analysis of lipid digestion

3.2.1. General appearance of O/W emulsion

The four different stabilised emulsions are shown in Fig. 1 and were stable for at least 2 days. After emulsification, all of the prepared emulsions were kept at 4°C, and used within 24 hours. Obviously, the emulsions prepared are different in colours. SPLIP shows greenish coloured following by PULIP which is light green in coloured. This appearance possibly due to the chlorophyll components in SPLIP and PULIP; which suggested that the colour of emulsion can vary depending on the surfactant used and characteristic of the oil and water. On the other hand, LEC and LECDG showed a common colour of an emulsion; which is opaque.

The SPLIP and PULIP stabilised emulsions showed instability phenomenon faster than the LEC and LECDG. Water and oil possess different densities and due to gravity they tend to separate. By adding surfactants, the tendency to separate is minimised and the ability to adsorb on the droplets surface is increased; thus encourage the water and oil molecules to stick together. Each of the surfactants used possess different properties; consequently their ability to absorb, on the droplets surfaces, also varies. In fact, the stability of an emulsion is influenced by the type of surfactant used (McClements, 2005) since the emulsions stabilised with LEC and LECDG were more resistant to the instability phenomenon, it is possible to infer that lecithin helps to increase the absorbability of the droplets molecules. Thus increasing the chances by which both water and oil molecules will hold together to prevent separation. It is suggested that the water molecules are transferred from being aqueous solutions into the oil phase through hydration of the adsorbed lecithin (Shchipunov and Schmiedel, 1996); hence, a gel-like layer is formed which aids stability of the emulsion. In contrast, although phospholipids are also contained in both the SPLIP and PULIP stabilised emulsions, it is not presumed that the gel-like layer is formed due to the complex properties of SPLIP and PULIP, despite the fact that this may contribute to the stability of the emulsion for a short time.

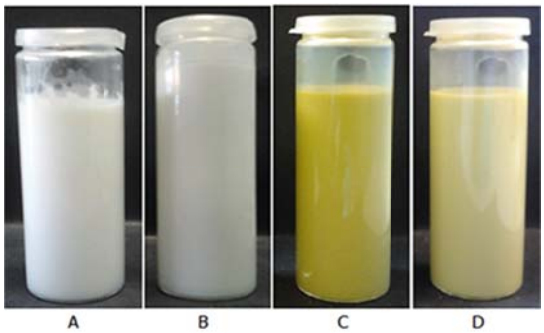

Fig. 1: Visual observation of stabilised emulsion prepared with: A) LEC B) LECDG C) SPLIP and D) PULIP

3.2.2. Microstructure

The effect of in-vitro lipid digestion on the microscopic appearance of the oil-in-water emulsions is shown in Fig. 2. It is noteworthy that remarkable microstructure changes occurred following the in-vitro lipid digestion processing for all of the oil-in-water emulsions that were stabilised with the four different types of surfactant.

In general, the LEC stabilised emulsion showed larger droplets than the LECDG, this was followed by the SPLIP and PULIP stabilised emulsions over the 4 hour *in-vitro* digestion period. The larger droplets observed suggest that flocculation may occur in the system; which promotes coalescence. In the LEC stabilised emulsion, coalescence was observed at 1 and 2 hour intervals during the stomach phases, it was also more pronounced after 2 hours in the duodenal phases. Coalescence in the duodenum has been reported and ascribed to the formation of free fatty acids (FFAs) and monoacylglycerols (MAGs) at the droplets surfaces during lipolysis (McClements and Li, 2010b).

Similarly, coalescence was also observed in the LECDG, SPLIP and PULIP stabilised emulsions at 1 and 2 hours in the stomach phases. In addition, the microscopy images also revealed that flocculation was observed for the LEC, LECDG and PULIP stabilised emulsions systems at 1 and 2 hours in the duodenal phase. Conversely, the SPLIP stabilised emulsion showed only a small amount of flocculation when compared to the other three emulsions. It is presumed that in the SPLIP stabilised emulsion, repulsive forces were occurring between the droplets that were sufficient enough to keep them apart.

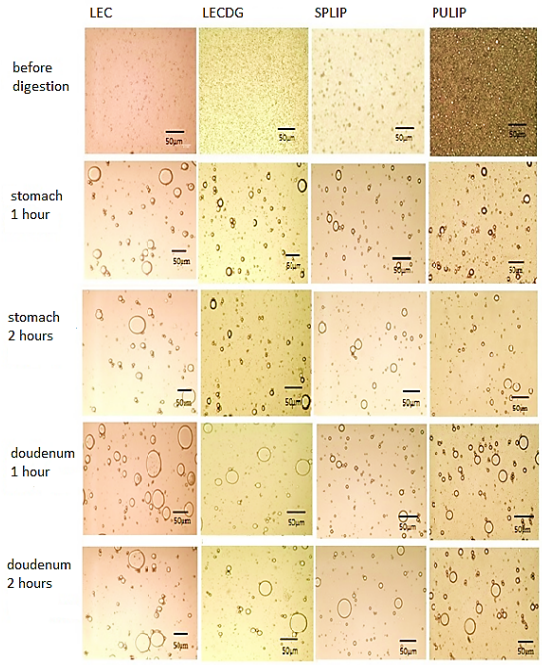

Fig. 2: Optical microscopy images of o/w emulsions before and during in-vitro digestion

3.2.3. Zeta potential data

The effects of digestion enzymes, in the stomach and in the duodenum, on the droplet charges of oil-in-water emulsions stabilised using LEC, LECDG, SPLIP and PULIP were investigated. In general, all of

the emulsions systems exhibited increasing droplet charges after they were subjected to 1 and 2 hours in the stomach phases and again in the duodenal phases, as shown in Fig. 3 Increases in the droplets charge values suggest that the emulsions are digested which presumably the increased charges caused by the accumulation of digestion by-products; these results were supported by microscopy images (Fig. 2).

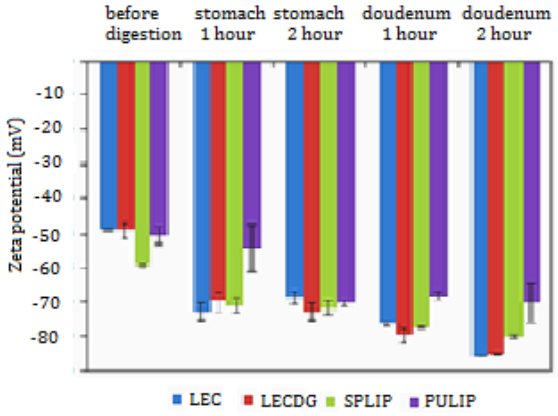

Fig. 3: The zeta potential (mV) of the droplet was measured before and during the in-vitro digestion process to examine the interactions of bile salts and lipase, using four different types of surfactant

For this discussion, the numerical values have also been presented in Table 1.

Initially, when the emulsions were exposed to 1 hour of digestion in the stomach, there were increases in the zeta potential values as shown in Table 1. The incremental changes in the charge of the droplet values, for before digestion and after 1 hour of digestion in the stomach phase, were significant ($p < 0.05$) using the t-test analysis, this was with the exception of the PULIP stabilised emulsion. A possible explanation is that there could be less absorption of gastric lipase on the droplets surfaces, this would result in fewer droplets being digested and less exposure of the negative phospholipid on the droplets which would ultimately attribute to lower charges associated with these droplets.

On the other hand, after 2 hours in the stomach phase, there were no significant ($p > 0.05$) changes in droplet charge values for the LEC, LECDG or SPLIP stabilised emulsions, with the exception of the PULIP stabilised emulsion which showed a significant increase ($p < 0.05$) in droplet charge. This can possibly be explained in terms of longer exposure in the stomach phases which allows the stomach enzymes to have sufficient time to absorb on the droplets surfaces, as a consequence the droplets surface will be digested somewhat to expose phospholipids on the droplets surfaces. Free exposure to the negatively charged phospholipid will result in increases to the negative charge of the droplets observed.

Table 1: Zeta-potential (mV) value of the droplets which were measured before and during the *in-vitro* digestion process to examine the interactions of bile salts and lipase, using four different types of surfactant

Emulsion systems	Zeta-potential (mV)				
	Before digestion	Stomach 1 hour	Stomach 2 hours	Duodenum 1 hour	Duodenum 2 hours
LEC	-49.1 ± 0.1	-72.7 ± 2.3^{a}	-68.7 ± 2.4	-76.1 ± 0.6^{b}	-85.3 ± 0.1^{b}
LECDG	-49.1 ± 2.4	-69.7 ± 3^{a}	-72.7 ± 2.4	-79.5 ± 2.2^{b}	-84.7 ± 0.2^{b}
SPLIP	-59.2 ± 0.1	-71.0 ± 1.9^{a}	-71.7 ± 1.9	-77.0 ± 0.3^{b}	-80.0 ± 0.6^{bc}
PULIP	-50 ± 3	-54.0 ± 7.1	-70.3 ± 0.9^{b}	-69.2 ± 1.2^{bd}	-70 ± 6^{bd}

During the 2 hours in the duodenal phase, the zeta potential of the emulsion droplets increased as shown in Table 1. The mean droplet charges during 1 hour and 2 hours in the duodenal phases for the PULIP stabilised emulsion were fairly static (-69.2 ± 1.2 mV to -70.1 ± 5.5 mV); thus, suggesting that no further digestion occurred on the droplets surface over the time scale investigated.

To summarise, the main physical changes observed for all of the emulsions indicated an increase in zeta potential when they were subjected to the duodenum phases, this was in comparison to the stomach phases. This is indicative that more emulsion breakdown occurs in the duodenal phases – this is also supported by the microscopy image results. The emulsion instability phenomenon was investigated from the least stable to the more stable when they were subjected to both the stomach phases and the duodenal phases. Their instabilities were recorded and they are now listed from least to most stable: LEC stabilised emulsion, LECDG stabilised emulsion, SPLIP stabilised emulsion and PULIP stabilised emulsion.

3.2.4. Free fatty acid analysis

The results for the in-vitro digestion emulsion method were utilised to determine the amount of free fatty acids (FFAs) during lipolysis of the droplets in the duodenal phases. Fig. 2 shows the results of the amount of NaOH (mL) used to neutralise the FFAs from the emulsion systems. During the 2 hours of lipolysis, the amount of NaOH (mL) used to neutralise the FFAs released from the emulsion systems were observed in terms of the higher amount to the least amount, as follows: LEC stabilised emulsion, LECDG stabilised emulsion, SPLIP stabilised emulsion, PULIP stabilised emulsion. The percentages of FFAs released were: 89%, 68%, 52% and 40% for the stabilised emulsions of LEC, LECDG, SPLIP and PULIP. The rate of lipolysis was recorded in decreasing levels for the stabilised emulsions of LEC, LECDG, followed by SPLIP and then, finally, PULIP. Initially, up to 20 minutes, the volume of NaOH (mL) added in the system increased quickly before a fairly constant value was reached for all of the emulsion systems; thus suggesting that the FFAs were rapidly released.

As can be seen in Fig. 4, a gradual increase in both LEC and LECDG stabilised emulsions occurred during the 2 hours of lipolysis. However, the

stabilised emulsion of LECDG showed a lower release rate of FFAs. Thus, the presence of DGDG in the emulsion systems actually decreased the rate of lipolysis. As the amount of FFAs released was higher in the LEC stabilised emulsion, this is indicative that the LEC stabilised emulsion underwent a higher degree of lipid digestion than the LECDG stabilised emulsion. As shown in previous report, the presence of DGDG at the interface decreases the rate of lipolysis in the duodenal conditions (Chu et al., 2009).

Interestingly, it became apparent that after just 20 minutes in the in-vitro digestion, the LECDG, SPLIP and PULIP stabilised emulsions showed different rates of lipolysis. Instead of a gradual increase as observed in the LECDG stabilised emulsion, both SPLIP and PULIP showed a different curve pattern; this could be as a result of the complex properties which are contained in both the SPLIP and PULIP extracts. The type of

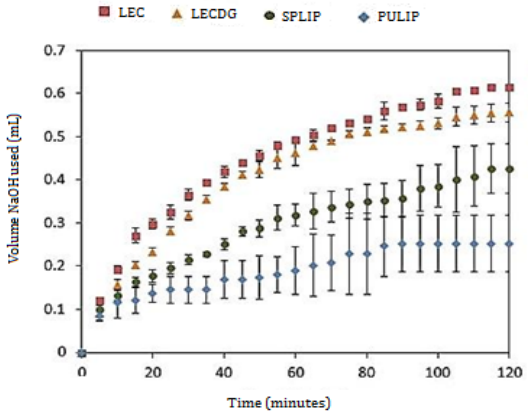

Fig. 4: Volume of NaOH added to the four different emulsion systems; the pH level was kept at 7, and the experiment was carried out at 37°C

surfactant used could impact on the way in which lipase interacts and binds to the droplets surfaces and, thus, inhibits lipolysis (Mun et al., 2007; Hur et al., 2009). It was expected that the concentration of DGDG would be approximated the same for all of the emulsion systems; nonetheless, the instability phenomenon observed could possibly be due to the efficiency of the DGDG extract when it was subjected to 2 hours in the duodenal phase. The results suggest that when DGDG is in a cruder form for both the SPLIP and PULIP stabilised emulsions then it is more efficient in inhibiting lipolysis, when compared to the DGDG (in a form of 98% purity) that was contained in the LECDG stabilised emulsion.

Within this experiment, on the time scale investigated, the PULIP stabilised emulsion underwent a slower rate of lipid digestion. It can, therefore, be suggested that the PULIP stabilised emulsion is more efficient in delaying lipolysis. This is possibly due to promising amount of TGDG components which is reported abundantly exist in pumpkin seed (Nakea et al., 2000); as such were helping to inhibit lipase molecules from accessing the droplets surfaces. It is hypothesis that for instance a bigger head group than DGDG is present which limits the lipolysis.

The rate of lipolysis in the PULIP stabilised emulsion levelled off approximately 90 minutes after it was first subjected to duodenal digestion. This could be due to the rate of lipolysis which was relatively slow and there was no more presence of FFAs in the emulsion.

Finding showed that the rate of lipolysis is highly affected by the type of surfactant used. The percentage of FFAs released were lower in the SPLIP stabilised emulsion, with the least being recorded from the PULIP stabilised emulsion when compared with the LECDG stabilised emulsion. To illustrate, only marginal lipolysis was observed in the PULIP stabilised emulsion, after 2 hours digestion in the duodenal phase, thus indicating that the PULIP stabilised emulsion was the most stable against the duodenum enzyme.

4. Conclusions

Results in the *in-vitro* digestion emulsion model suggests that SPLIP and PULIP are also effective and even better in hindering the access of lipase molecules, even in presence of bile salts in the system than the LECDG stabilised emulsion. To conclude, the crude extract behaves differently from pure extract; glycolipid as a whole fraction in a crude form is also efficient in delaying lipolysis than isolate DGDG.

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