Title: Use of esterified palm acid oils with a different fatty acid positional distribution and acylglycerol composition in broiler chick diets

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Abstract

Esterified acid oils are obtained from the chemical esterification of acid oils with glycerol. Because esterified acid oils may have different physicochemical properties than have their respective native oil, a detailed characterization of these fats was performed. Subsequently, the effect of fatty acid (FA) positional distribution within acylglycerol molecules, and the effect of acylglycerol composition on FA apparent absorption, and their possible consequences on the subsequent metabolism, including the evolution of postprandial lipemia and growth performance, were assessed in young broiler chickens. For this purpose, 72 1-day-old female broiler chicks were randomly distributed into 18 cages. The three treatments used were the result of a basal diet supplemented with 6 wt% of native palm oil (N-TAG), esterified palm acid oil (E-TAG), and esterified palm acid oil high in mono- and diacylglycerols (E-MDAG). Esterified palm acid oils showed the same FA composition, but a different intramolecular structure than did N-TAG oil. Chemical esterification raised the fraction of palmitic acid at the *sn*-2 position from 9.63 mol% in N-TAG oil to 17.9 mol% in E-TAG oil. Furthermore, E-MDAG oil presented a high proportion of mono- and diacylglycerol molecules (23.1 and 51.2 wt%, respectively), with FA mainly located at the *sn*-1,3 positions, which resulted in a lower gross energy content and an increased solid fat index at the chicken's body temperature. However, the different acylglycerol structure of esterified palm acid oils did not improve fat absorption, and did not cause differences in lipid metabolism of young broiler chickens.

Key words

sn-2 position · Monoacylglycerol · Diacylglycerol · Fatty acid apparent absorption · Postprandial lipemia · Solid fat index

Abbreviations

DAG	Diacylglycerol(s)
DSC	Differential scanning calorimetry
FA	Fatty acid(s)
FFA	Free fatty acid(s)
MAG	Monoacylglycerol(s)
MIU	Moisture, impurities and unsaponifiable matter
MUFA	Monounsaturated fatty acid(s)
NMR	Nuclear magnetic resonance
PUFA	Polyunsaturated fatty acid(s)
SFA	Saturated fatty acid(s)
TAG	Triacylglycerol(s)

Introduction

Among the ingredients used in the formulation of animal diets, fats and oils are the most concentrated sources of energy, but also those with the most variable nutritive value [1]. Because of reasons of market availability and competitive price [2], native palm oil is a high-energy ingredient to be considered in animal nutrition. However, the high saturated fatty acid (SFA) content of this vegetable fat source, in particular of palmitic acid, compromises its absorption and, therefore, its metabolizable energy content, especially at early ages [3, 4].

The biological role of the chain length and the degree of unsaturation of fatty acids (FA) is well known [5]. However, evidence is accumulating that the

intramolecular structure of dietary fats is also of importance because it can affect their rates of digestion and absorption, and also its subsequent metabolism.

Both the number of FA bound to the glycerol molecules and the stereospecific position of FA within acylglycerol molecules play an important role in fat absorption. Tri- (TAG) and diacylglycerols (DAG) are too-large molecules and cannot be absorbed intact in the small intestine. Thus, before absorption, they must be broken down by pancreatic lipase to monoacylglycerols (MAG) and free fatty acids (FFA). Pancreatic lipase preferentially hydrolyzes the FA in the *sn*-1,3 positions of the acylglycerol molecules. Consequently, FA in the *sn*-2 position predominantly remain in this location and are directly absorbed as 2-MAG [6]. 2-MAG are easily absorbed regardless of their constituent FA, because their amphiphilic properties facilitate their incorporation into mixed micelles [7, 8]. However, the absorption of FFA varies greatly depending on their chemical structure. Mono- (MUFA) and polyunsaturated fatty acids (PUFA) are well absorbed, but long-chain SFA are poorly absorbed because they have high hydrophobicity, high melting points above body temperature, and a great ability to form insoluble soaps with divalent cations in the gut [9]. Therefore, stearic and palmitic acids are better absorbed if they are situated in the *sn*-2 position of the acylglycerol molecules than in the *sn*-1,3 positions [10–15].

On the one hand, native palm oil is mainly composed of TAG, and small proportions of DAG, MAG and FFA [16]. Nevertheless, higher contents of DAG and MAG can be achieved through chemical [17] or enzymatic [18] glycerolysis of fats or FA methyl esters, which could act as emulsifying agents, able to improve fat absorption. On the other hand, in native palm oil, SFA are predominantly located at the *sn*-1,3 positions of the acylglycerol molecules [19]. However, authors using randomized oils (obtained by chemical interesterification of native oils) have observed an equal

96 distribution of FA among all three positions of the glycerol molecule [3, 20–22], which
97 could benefit the absorption of SFA. Therefore, in this study the chemical esterification
98 of palm acid oil with glycerol (both, economically interesting by-products from oil
99 refining and biodiesel industries, respectively) has been proposed as a way to generate
100 esterified palm acid oils with a different intramolecular structure than native palm oil,
101 and thus increase its nutritive value.

102 To-date, and to the knowledge of the authors, there are no reports in the
103 literature using this kind of esterified palm acid oils. Because these technical fats may
104 have different physicochemical properties than their respective native oil, a detailed
105 characterization of the experimental fats was performed. Subsequently, the effect of FA
106 positional distribution within acylglycerol molecules, and the effect of acylglycerol
107 composition on FA apparent absorption, and their possible consequences on the
108 subsequent metabolism, including the evolution of postprandial lipemia and growth
109 performance, were assessed in young broiler chickens.

110

111 **Material and methods**

112

113 *Experimental fats*

114 Experimental fats were supplied by SILO S.p.a. (Florence, Italy). Esterified palm acid
115 oils were obtained by reacting palm acid oil with glycerol. According to the
116 stoichiometric proportion of acid oil and glycerol, the time, the temperature, and the
117 vacuum applied to the reaction, fats with the same FA profile, but with a different FA
118 positional distribution, and TAG, DAG and MAG proportions were obtained (Table 1).

Each oil sample was analyzed in triplicate. Moisture (method 926.12 of the AOAC [23]), impurities (ISO 663:2007), and unsaponifiable matter (method 933.08 of the AOAC [23]) (MIU) content was determined as a quality control.

The acylglycerol composition of experimental fats was analyzed according to the ISO 18395:2005, in which TAG, DAG, MAG, and FFA are separated according to their molecular size. Briefly, a solution of approximately 10 mg of oil/mL of tetrahydrofurane was injected into an Agilent 1100 series HPLC chromatograph (Agilent Technologies; Santa Clara, CA, USA) equipped with a refractive index detector and two Styragel columns (Styragel HR 1 and Styragel HR 0.5) of 30 cm x 0.78 cm i.d., filled with a spherical styrene divinylbenzene copolymer of 5 μm particle size (Water Associates; Milford, MA, USA) connected in series. The mobile phase consisted of tetrahydrofurane. The acylglycerol molecules were quantified by internal normalization. Moreover, given the potential importance of different positional isomers of MAG and DAG molecules in the digestion and absorption processes, we also analyzed the experimental fats by high-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Thus, 2-MAG were distinguished from 1(3)-MAG, and 1(3),2-DAG from 1,3-DAG species by area integration of the individual resonances corresponding to the central CH at the *sn*-2 position in each type of compound. These species can be detected in the area covering 5.3-3.8 ppm, clearly differentiating the H2 protons belonging to 1(3),2-DAG (5.05 ppm), 1,3-DAG (4.03 ppm), 2-MAG (4.88 ppm) and 1(3)-MAG (3.89 ppm) derivatives (Figure 1). The degree of unsaturation and the chain length of FA do not influence the chemical shift values [24]. Briefly, oil samples (about 6 mg) were dissolved in deuterated chloroform and placed into a 5-mm-diameter NMR tubes. Conventional one-dimensional ^{1}H NMR spectra were collected under routine conditions on a Bruker 600 MHz spectrometer (Bruker; Billerica, MA,

144 USA), equipped with a triple-channel TXI probe. All experiments were recorded at 298
145 K, using a recycle delay of 3 s and 4 scans per sample. After Fourier transformation and
146 base-line correction, the areas of the selected H2 proton signals of the spectrum were
147 quantified by area integration.

148 The total FA composition of experimental fats was determined by gas
149 chromatography, according to the methylation method described by Guardiola et al.
150 [25]. Briefly, 50 mg of oil were methylated with sodium methoxide (0.5 N), followed
151 by boron trifluoride (20 wt% in methanol), and FA methyl esters were extracted with n-
152 hexane. Subsequently, FA methyl esters were analyzed using an Agilent 4890D gas
153 chromatograph (Agilent Technologies; Santa Clara, CA, USA), equipped with a flame
154 ionization detector and a polar capillary column (SP-2380, 60 m x 0.25 mm i.d., 0.2 μm
155 from Supelco; Bellefonte, PA, USA). Helium was used as the carrier gas. FA methyl
156 esters were identified by matching their retention times with those of their relative
157 standards (Supelco 37 component FAME Mix, Sigma-Aldrich Co.; St. Louis, MO,
158 USA) and quantified by internal normalization.

159 The FA composition at the *sn*-2 position of the acylglycerol molecules was
160 determined by the EU official method (Commission Regulation (EEC) No. 2568/91 –
161 Annex VII). Briefly, the original fat was hydrolyzed by pancreatic lipase (EC 3.1.1.3
162 from porcine pancreas Type II, Sigma-Aldrich Co.; St. Louis, MO, USA) to selectively
163 cleave the ester bonds at the *sn*-1,3 positions. 2-MAG were isolated by thin-layer
164 chromatography using silica gel plates (Merck; Darmstadt, Germany), impregnated in
165 boric acid (5 wt% in methanol). The 2-monoolein and 1-monoolein standards (Sigma-
166 Aldrich Co.; St. Louis, MO, USA) were spotted for identifying the 2-MAG zone spot.
167 The developing solvent was a mixture of chloroform/acetone (90:10, by vol). The zone
168 spot was visualized under UV light after being sprayed with 0.2 wt% of 2,7-

dichlorofluorescein in methanol. Then, 2-MAG were scraped, and the FA composition of 2-MAG was determined as described above. Finally, to assess the distribution of each FA within the three positions of the acylglycerol molecules, a modification of the formula suggested by Mattson [26] was used. These authors calculated the proportion of each FA that is located at the *sn*-2 position of the acylglycerol molecules (*sn*-2 %), applying the following formula:

(1) $$sn\text{-}2\ \% = (sn\text{-}2\ /\ \text{Total}) \times a \times 100,$$

where *sn*-2 is the FA composition at the *sn*-2 position (converted to mol%), Total is the total FA composition in the original fat (converted to mol%), and *a* is the ratio between the moles of FA located at the *sn*-2 position and the moles of total FA. Thus, in the original formula, *a* was equal to 0.33, since it was designed for native oils that are mainly constituted by TAG. In our study, however, experimental fats were a mixture of TAG, DAG, MAG, and FFA. For this reason, *a* was calculated from the acylglycerol composition of the fat, and the average molecular weight (according to the total FA composition of the fat) and the glycerol-to-FA ratio for each molecular species. These calculations were also used to obtain an estimation of the global glycerol-to-FA ratio of our experimental fats (Table 1).

Additionally, because changes in both FA positional distribution and acylglycerol composition may cause differences in the physical properties of the fat, the melting behavior of the experimental fats was studied by differential scanning calorimetry (DSC; Perkin-Elmer Diamond Calorimeter; Waltham, MA, USA). Briefly, oil samples (about 6 mg) were weighed into 50-µl aluminium pans, and covers were sealed into place. An empty pan was used as a reference. Samples were cooled and heated at 2 ºC·min^{-1} between -60 and 60 ºC. Thermograms were analyzed to obtain the total and partial melting enthalpies; assuming that the total melting enthalpy of a fat is

194 the total energy required to convert the substance from a solid state to a complete melt.
195 Then, the solid fat index was calculated for each 5ºC interval. The result of these
196 calculations is a graphic indication of the loss of solids due to the melting process
197 (Figure 2).

198 Finally, combustion energies of the experimental fats were measured by an
199 adiabatic bomb calorimeter (IKA-Kalorimeter system C4000; Staufen, Germany).

200

201 *Animals and diets*

202 The trial was performed at the animal experimental facilities of the *Servei de Granges i*
203 *Camps Experimentals* (Universitat Autònoma de Barcelona; Bellaterra, Barcelona,
204 Spain). The experimental procedure received the prior approval from the Animal
205 Protocol Review Committee of the same institution. All animal housing and husbandry
206 conformed to the European Union Guidelines (EU86/609/EEC).

207 A total of 72 1-day-old female broiler chickens of the Ross 308 strain were
208 obtained from a commercial hatchery (Pondex SAU; Juneda, Lleida, Spain), where
209 birds with extreme weights were discarded. On arrival, chicks were wing-banded,
210 weighed (initial body weight, 46.6 ± 0.03 g) and randomly assigned to one of the three
211 dietary treatments, with four chicks per cage and six cages per treatment. Birds were
212 housed in wire-floor cages with excreta collection trays. Throughout the study, feed and
213 water were supplied *ad libitum*, and animals were raised under controlled conditions of
214 light and temperature, as recommended by the breeder.

215 The birds received a starter feed (in mash form) until Day 14. The wheat- and
216 soybean-meal-based diet was formulated to meet or exceed FEDNA requirements [27]
217 and to minimize basal fat levels. The three dietary treatments were the result of
218 including 6 wt% of one of the following experimental fats to the basal diet: native palm

219 oil (N-TAG), esterified palm acid oil (E-TAG), or esterified palm acid oil high in MAG
220 and DAG (E-MDAG). The composition of experimental diets is presented in Table 2.
221 The manufacturing of the experimental diets was carried out at the experimental station
222 of *IRTA Mas de Bover* (Constantí, Tarragona, Spain).

223 Analytical determinations of feeds were performed according to the methods of
224 the AOAC [23]: Dry matter (method 934.01), ash (method 942.05), crude protein
225 (method 968.06), crude fat (method 2003.05), and crude fiber (method 962.09). Gross
226 energy was determined as described previously, and FA content was analyzed following
227 the method of Sukhija and Palmquist [28], which consists of a direct transesterification
228 in which lipid extraction and FA methylation are achieved in only one step. Briefly,
229 samples (about 100 mg) were incubated with methanolic chloride, and a known amount
230 of nonadecanoic acid (C19:0, Sigma-Aldrich Chemical Co.; St. Louis, MO, USA) was
231 added as an internal standard. Then, the FA methyl esters were extracted with toluene
232 and submitted to gas chromatography [Agilent 6890 gas chromatograph, equipped with
233 a flame ionization detector, and a polar capillary column (DB23, 60 m x 0.32 mm i.d.,
234 0.25 μm) from Agilent Technologies; Santa Clara, CA, USA]. Helium was used as the
235 carrier gas. Peak areas were integrated and converted to concentration by comparison
236 with the internal standard-peak area, as follows:

237 (2) $FA = (\text{Area FA}/\text{Area C19}) \cdot [(\mu g\ C19/(\text{response coefficient} \cdot \text{mg sample weight})]$.

238 The macronutrient and the FA composition of the experimental diets are
239 presented in Table 3.

240

241 *Controls and sampling*

242 Feed consumption and weight gain were measured weekly to calculate average daily
243 feed intake, average daily gain and feed conversion ratio throughout the experiment.

From Day 7 to 10, a balance study was carried out using the total-excreta-collection method, according to the European reference method [29]. The last day of the balance, feed consumption was measured and total excreta was collected, weighed and homogenized, and a representative sample was frozen at -20 ºC. Contaminants such as feed, feathers, down, and scales were removed. Then, the excreta samples were freeze-dried, ground and kept at 5 ºC until further analysis.

Excreta samples were analyzed by the same methods as those described for feeds, to determine the apparent absorption of organic matter, fat (with previous acid hydrolysis, following method 954.02 of the AOAC [23]), fatty acids, and to determine the apparent metabolizable energy of the diets. The apparent absorption of the nutrients (X) was calculated as follows:

(3) % apparent absorption of X = [(X ingested – X excreted) / X ingested] x 100.

The postprandial lipemia kinetic study was carried out on the last day of the experiment. After 14 days feeding, chickens were made to fast for 5 hours, and then re-fed *ad libitum* for 20 min. Blood samples were drawn by jugular venopuncture at 0, 40, 80, 120, 160 and 200 min following consumption of the experimental diets (one extraction per bird; $n = 4$). Samples were immediately centrifuged at 2,000 *g* for 10 min and serum was stored at -20 ºC until analysis. Serum TAG concentrations were measured in each sample using a clinical chemistry autoanalyzer (Olympus AU400; Hamburg, Germany) and an enzymatic reagent (glycerol phosphate oxidase, Beckman Coulter; Galway, Ireland).

Statistical analysis

Normality of the data and variance homogeneity were verified. The effect of diet on FA apparent absorption and growth performance were statistically analyzed by one-way

ANOVA with diet as a main factor. Differences between treatment means were tested using Tukey's correction for multiple comparisons. The cage served as the experimental unit, so there were 6 units per diet.

The effect of diet on postprandial lipemia was statistically analyzed by two-way ANOVA. The model included time of extraction and diet as main factors and the two-way interaction. Data were plotted as variations in concentration over the fasting value (taking the fasting value as zero) to normalize the variations of initial values. It was not possible to analyze these data as repeated-measures because each blood extraction was performed in a different animal, due to the limited volume of blood that can be drawn from young chicks. The animal served as the experimental unit, so that there were 4 units per each time-point.

Results in tables are reported as means, differences were considered significant at $P < 0.05$, and trends were discussed at $P < 0.10$. All procedures were carried out using the SAS statistical package (version 9.2, SAS Institute Inc.; Cary, NC, USA).

Results

Characterization of experimental fats

The chemical analysis of the experimental fats is presented in Table 1. Experimental fats had similar and low levels of MIU. Regarding total FA composition, experimental fats also showed a similar FA profile. This indicated that the esterification of palm acid oil with glycerol did not substantially modify the FA composition of the fat. Therefore, the FA composition of esterified acid oils agreed with that reported for native palm oil, being more than 80 wt% of the total FA composed of palmitic (45.0 ± 1.01 wt%) and oleic (38.0 ± 0.94 wt%) acids.

294 The natural preference for a specific FA positional distribution was evident in N-
295 TAG oil, since FA were not randomly distributed over the three positions of the
296 glycerol molecule. As expected, the FA at the *sn*-2 position of N-TAG oil contained less
297 palmitic acid than did those at the *sn*-1,3 positions of the glycerol molecule. On the
298 contrary, although chemical esterification did not result in a complete random
299 distribution of FA, this process raised the fraction of palmitic acid at the *sn*-2 position
300 from 9.63 mol% in N-TAG oil to 17.9 mol% in E-TAG oil, and 14.5 mol% in E-MDAG
301 oil.

302 Another important difference among experimental fats was their acylglycerol
303 composition. E-TAG oil presented the highest TAG content (86.2 wt%), followed by N-
304 TAG oil (66.6 wt%), and E-MDAG oil (25.6 wt%). In contrast, E-MDAG oil presented
305 the highest DAG content (51.2 wt%), followed by N-TAG oil (19.9 wt%), and E-TAG
306 oil (12.1 wt%). E-MDAG oil also presented an important amount of MAG (23.1 wt%),
307 and N-TAG oil of FFA (11.4 wt%). Because of the relevance in the digestion process,
308 by using ^{1}H NMR we found that most FA from MAG and DAG molecules of our
309 experimental fats were located at the *sn*-1,3 positions. Thus, in E-MDAG oil, the
310 isomeric ratio between 1(3)-MAG:2-MAG was 94:6 mol/mol, and that between 1,3-
311 DAG:1(3),2-DAG was 68:32 mol/mol.

312 The different acylglycerol composition observed in experimental fats was
313 closely related to its glycerol-to-fatty-acid ratio and, in turn, to their subsequent gross
314 energy content. Given that the average heat of combustion of palm FA (9,455 kcal/kg)
315 is more than twice that of glycerol (4,346 kcal/kg), an increase in the glycerol-to-fatty-
316 acid ratio has a negative impact on the gross energy content. N-TAG and E-TAG oils,
317 due to their high TAG content, showed nearly the same glycerol-to-fatty-acid ratio,
318 close to 0.33 mol/mol, (N-TAG oil: 0.34 and E-TAG oil: 0.35 mol/mol), and nearly

identical gross energy content (N-TAG oil: 9,307 and E-TAG oil: 9,298 kcal/kg). Nevertheless, the glycerol-to-fatty-acid ratio of E-MDAG oil increased up to 0.56 mol/mol, because of its high MAG and DAG content, and this difference represented a decrease of about 4 % on its gross energy content (8,947 kcal/kg).

The melting thermograms (Figure 2a), determined by DSC, allowed us to calculate the total and partial melting enthalpies. Although experimental fats showed different and highly complex melting patterns, a similar amount of energy was needed to bring them from the solid to the liquid state (N-TAG oil: 106, E-TAG oil: 99, and E-MDAG: 104 J/g). The melting profile of these oils (Figure 2b), obtained by calculations of the above enthalpies, showed that N-TAG and E-TAG oils had nearly the same melting profile and the same melting range, although N-TAG oil started to melt and finished melting earlier than did E-TAG oil (N-TAG oil: - 25 to 35 ºC and E-TAG oil: -20 to 40 ºC). However, E-MDAG oil started to melt earlier and finished melting later, expanding its melting range (-50 to 50 ºC). Nevertheless, the most important physical property of fats in lipid nutrition is their solid fat index at the chicken's body temperature (41.5 ºC). While N-TAG and E-TAG oils were totally or almost completely liquid at 41.5 ºC (N-TAG oil: 0 wt% and E-TAG oil: 1 wt%), E-MDAG oil still had a 16 wt% of solid fat index at this temperature.

Digestibility balance

The effects of dietary treatments on the apparent absorption coefficients are presented in Table 4. As expected, unsaturated FA were better absorbed than were SFA, and stearic acid was less absorbed than was palmitic acid. However, no statistically significant differences were observed for fat and FA apparent absorption ($P > 0.05$), despite the great numerical differences observed among treatments (about a 15 % difference was

344 detected between N-TAG and E-MDAG treatments for total FA apparent absorption).
345 The great variability observed among cages within the same dietary treatment may have
346 prevented researchers from seeing statistically significant differences.

347 In this sense, neither statistical differences among treatments were observed for
348 apparent metabolizable energy, which was consistent with the organic matter absorption
349 results ($P > 0.05$). The lower gross energy found for E-MDAG oil was too small to be
350 reflected in the feed, since fat only accounted for 17% of the total-feed gross energy.

351

352 *Postprandial lipemia*

353 The fasting levels of serum TAG (0 min) did not differ significantly between treatments
354 (N-TAG: 35.0 ± 3.19 mg/dL, E-TAG: 39.5 ± 2.02 mg/dL and E-MDAG: 41.75 ± 3.22
355 mg/dL; $P = 0.287$). Changes in serum TAG concentration after feeding the experimental
356 diets are represented in Figure 3. Statistical analysis showed that serum TAG
357 concentrations differed significantly ($P < 0.001$) throughout the postprandial period.
358 However, there were no significant differences ($P = 0.124$) in the postprandial
359 responses among treatments.

360 Following consumption of experimental diets, the rates of lipid transport sharply
361 increased. The incremental change in serum TAG concentration rose to a postprandial
362 level of about 39.6 ± 3.44 mg/dL at 80 min after feeding. Then, serum TAG
363 concentrations rapidly returned to baseline levels, with no differences among
364 treatments. Thus, it can be stated that both rates of absorption and clearance of serum
365 TAG were not affected by the different dietary acylglycerol structure of our
366 experimental fats.

367

368 *Growth performance*

369 The effect of dietary fat source on growth-performance traits is reported in Table 5.
370 Differences were only found for the average daily feed intake. The increased MAG and
371 DAG content of E-MDAG treatment caused a statistically (P = 0.014) or a nearly
372 statistically (P = 0.096) significant decrease in feed intake, when compared with E-TAG
373 and N-TAG treatments, respectively. Weight gain, feed conversion, and final body-
374 weight were not statistically affected by the diet (P > 0.05), although figures were also
375 numerically lower for average daily gain and final body-weight in the E-MDAG group.
376
377 **Discussion**
378
379 *Fatty acid positional distribution within acylglycerol molecules*
380 Our hypothesis was that the chemical esterification of palm acid oil with glycerol would
381 increase SFA content at the *sn*-2 position, and therefore its absorbability, in particular
382 that of palmitic acid. Although E-TAG oil showed a higher percentage of palmitic acid
383 at the *sn*-2 position than did N-TAG oil, no improvements in the apparent absorption of
384 this FA or other individual FA were observed in this study. On the contrary, a decrease
385 of about 6 % was detected for the total FA apparent absorption of E-TAG when
386 compared with N-TAG. Similarly, Smink et al. [3] also found that randomization of
387 palm native oil did not significantly affect fat and FA absorption in young broiler
388 chickens. In contrast, other studies carried out with human newborn infants [22], rats
389 [20, 30, 31] and broiler chickens [32, 33] reported that palmitic acid present at the *sn*-2
390 position of dietary TAG was more readily absorbed than was palmitic acid at the *sn*-1,3
391 positions. While most of these studies used fats with a high palmitic acid content at the
392 *sn*-2 position (from 33 to 84 mol%), in our study E-TAG oil only had a 17.9 mol% of
393 the total palmitic acid content located at the *sn*-2 position.

On the other hand, the melting point is also considered to have an important influence on fat absorption, because fats that are crystalline solids at body temperature form micelles less readily, and the rate of micelle formation is a critical step in determining the rate of lipolysis [34]. As established by Lida et al. [35] and reviewed by Small [9], native palm oil mainly consists of di-saturated (mainly POP with a melting point of 35.2 ºC) and di-unsaturated (POO with a melting point of 18.2 ºC) TAG, where P is palmitic acid and O is oleic acid. Although, in this study, the chemical esterification process did not reach a complete randomization of FA within acylglycerol molecules, this was probably enough to lead to changes in TAG composition. Thus, the slightly higher solid fat index observed in E-TAG oil was probably due to the increased tri-saturated (mainly PPP with a melting point of 66.4 ºC) TAG content, as well as a simultaneous decrease in the di-saturated and di-unsaturated species mentioned above, as reported by Berry and Sanders [34].

Once in the blood, SFA in the *sn*-2 position may delay the TAG clearance. The increased proportion of high melting MAG may alter the physical properties of the surface layer of remnant particles, impeding further hydrolysis, and slowing removal by the liver [36, 37]. However, in our experiment, E-TAG animals did not show a more prolonged postprandial lipemia response than did N-TAG animals, just the contrary. Several authors [38–41] did not find significant differences in plasma lipids of adult men and women after consuming TAG with different FA positional distribution. Because the extent of postprandial lipemia is determined by both rates of absorption and clearance of dietary TAG, the reasons for not finding differences in the peak TAG concentration or the time to maximal TAG concentration following consumption of dietary treatments suggests that fats studied were digested and cleared from the blood at the same rates, or that the small difference observed in the FA positional distribution of

our experimental fats, added to the high individual variations, prevented us from seeing any statistical effect on the postprandial lipemia response.

Regarding growth performance traits, the lack of differences found between N-TAG and E-TAG groups are in good agreement with the findings observed by Lin and Chiang [32] and Smink et al. [3], who also fed broiler chickens with native and randomized palm oils.

Acylglycerol composition

For the second objective, the hypothesis tested was that the presence of MAG and DAG molecules, due to their amphiphilic properties, would act as emulsifying agents, able to enhance fat digestion and absorption. In the study of Garrett and Young [8], the efficacy of free oleic acid in enhancing palmitic acid absorption in broiler chickens was compared with that of the MAG of oleic acid (monoolein). Monoolein, due to its higher micellar solubility, was superior to oleic acid in promoting the absorption of palmitic acid at several different ratios. Nevertheless, in our study, although the increased glycerol-to-fatty-acid ratio of E-MDAG oil resulted in a greater amount of MAG and DAG molecules, no improvements in the apparent absorption of fat and individual FA were observed in comparison with TAG-rich diets. On the contrary, a decrease of about 9 % was detected for the total FA apparent absorption of E-MDAG when compared with E-TAG. Taguchi et al. [42] reported that the fecal excretion of FA after feeding rats with DAG was almost the same as that with TAG, suggesting that the intestinal absorption of DAG was comparable to that of TAG, although they used fat sources rich in oleic and linoleic acids.

The reason why MAG and DAG molecules did not enhance micellar solubilization may be due to their increased melting point. Each acylglycerol molecule

has specific melting and crystallization characteristics. It is well known that tri-saturated TAG (PPP with a melting point of 66.4 ℃), due to their high melting temperatures, are almost not digested [9]. However, di-saturated DAG have even higher melting points (74.9 and 70.1 ℃ for 1,3-PP and 1(3),2-PP, respectively), and saturated MAG are still crystalline above body temperature (1(3)-P and 2-P have melting points of 70.5 and 68.5 ℃, respectively). Thus, the presence of high amounts of saturated MAG and DAG may promote the formation of crystalline structures on the surface of fat globules, preventing the hydrolytic action of pancreatic lipase. In addition, the melting points of saturated 1,3-DAG and 1(3)-MAG, major isomers in our experimental fats, are higher than are their respective 1,2-DAG and 2-MAG isomers, which may have contributed to further increase the solid fat index of E-MDAG oil.

Otherwise, Kondo et al. [43] and Murata et al. [44] reported that the main end-products of lipase action on 1,3-DAG and 1(3)-MAG were free glycerol and FFA. It is not surprising, therefore, that the increased MAG and DAG content of E-MDAG, rather than benefiting, may have impaired the absorption of fats.

There is still one more possible hypothesis. Because it has been suggested that the end products of MAG and DAG digestion are less readily re-synthesized to TAG [43, 44], this may slow the FA absorption rate from the intestinal lumen to the enterocytes. The re-synthesis of TAG in the intestinal mucosal cells can be done *via* two pathways: the 2-MAG pathway and the glycerol-3-phosphate pathway. Under normal conditions, these pathways account for 80 and 20 % of the mucosal TAG re-synthesis, respectively, since the final digestion products of TAG are 2-MAG and FFA [45]. As mentioned above, the main end-products of 1,3-DAG and 1(3)-MAG are glycerol and FFA. On the one hand, free glycerol would be readily absorbed and transported into the blood circulation or phosphorylated to *sn*-glycerol-3-phosphate and utilized for the

469 synthesis of TAG. On the other hand, FFA may be less readily re-synthesized to TAG
470 because such synthesis may proceed *via* the glycerol-3-phosphate pathway, which is
471 less active than the 2-MAG pathway.

472 This suggested slower TAG re-synthesis may have caused changes in lipid
473 metabolism. Compared with consumption of TAG, several studies have reported that
474 consumption of DAG produces lower postprandial [44, 46–48] and fasting serum TAG
475 concentrations [49, 50] in humans, rats and pigs. Another important aspect to take into
476 account in the magnitude of postprandial lipemia is the higher FA β-oxidation activity
477 observed in rodents fed DAG. According to Murase et al. [51], dietary DAG was found
478 to up-regulate genes involved in FA transport, β-oxidation and thermogenesis in the
479 small intestine, and Murata et al. [52] observed an increased activity of enzymes
480 involved in the β-oxidation pathway in the liver.

481 However, there are several reasons related to our experimental design that can
482 explain why we did not observe changes on postprandial lipemia response among
483 treatments. Most studies assessed postprandial lipemia after only one single meal;
484 however, in our study, the animals had been fed with experimental fats since the first
485 day of life. The shift from the 2-MAG pathway to the glycerol-3-phosphate pathway
486 requires an up-regulation of the enzymes involved in this pathway. Thus, a permanent
487 diet rich in MAG and DAG may have changed the relative expression of genes involved
488 in both pathways, increasing the rate of TAG re-synthesis through the glycerol-3-
489 phosphate pathway. On the other hand, while previous studies used TAG and DAG oils
490 with oleic and linoleic acids as the major FA, our experimental fats were rich in palmitic
491 and oleic acids. Other limitations of our study may have been the small amount of fat
492 consumed by the birds and the inability to perform serial blood extractions from each
493 animal.

494 Concerning growth performance, the significantly lower average daily feed
495 intake observed in the E-MDAG group could be in good agreement with the reported
496 lesser feeling of hunger and appetite observed in women fed a DAG oil-rich diet [53].
497 Authors suggested that this could be related to the higher β-hydroxybutyrate
498 concentrations found in blood, as a result of the increased fat oxidation suggested
499 above, which are inversely associated with appetite and food intake [54]. However,
500 other studies feeding animals with TAG and DAG oil-rich diets did not find differences
501 in either feed intake or weight gain [42, 52].

502 Taken together, it is concluded that the chemical esterification of palm acid oil
503 with glycerol can be used to obtain fats with the same saturation degree and isomeric
504 state of FA, but with a different intramolecular structure and melting profile. However,
505 the differences observed for the FA positional distribution within acylglycerol
506 molecules and acylglycerol composition in esterified palm acid oils did not improve fat
507 absorption and did not cause differences in lipid metabolism of young broiler chickens.
508

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516

517 **Conflict of interest**

518 All authors declare no conflict of interest.

519

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Table 1. Chemical analyses of the experimental fats[a].

Item		N-TAG oil	E-TAG oil	E-MDAG oil
Moisture, wt%		0.03	0.01	0.07
Impurities, wt%		<0.50	<0.50	<0.50
Unsaponifiable matter, wt%		1.44	1.03	1.38
Fatty acid composition and distribution, wt%				
C16:0	Total	43.5	44.5	46.9
	sn-2 %[b]	9.63	17.9	14.5
C18:0	Total	4.60	4.51	4.76
	sn-2 %[b]	11.6	18.4	19.5
C18:1 n-9	Total	38.6	39.3	36.1
	sn-2 %[b]	38.5	41.7	20.1
C18:2 n-6	Total	10.1	9.19	8.40
	sn-2 %[b]	42.8	49.3	19.9
Minor fatty acids		3.25	2.56	3.79
SFA	Total	49.5	50.2	53.9
	sn-2 %[b]	10.2	18.3	14.6
MUFA	Total	39.8	40.5	37.6
	sn-2 %[b]	37.9	41.3	20.1
PUFA	Total	10.7	9.33	8.58
	sn-2 %[b]	42.4	49.3	19.9
Acylglycerol composition, wt%				
TAG		66.6	86.2	25.6
DAG	Total	19.9	12.1	51.2
	1(3),2-DAG %[c]	16.7	13.6	31.5
MAG	Total	2.08	0.40	23.1
	2-MAG %[d]	12.5	50.0	6.45
FFA		11.4	1.32	0.00
Glycerol:fatty acid ratio[e], mol/mol		0.34	0.35	0.56
Gross Energy, kcal/kg		9,307	9,298	8,947

[a] Native palm oil (N-TAG oil), esterified palm acid oil (E-TAG oil), and esterified palm acid oil high in MAG and DAG (E-MDAG oil).

[b] The proportion of a particular FA that is located at the acylglycerol *sn*-2 position (*sn*-2 %) was calculated as follows: *sn*-2 % = (*sn*-2 / Total) x *a* x 100, where *sn*-2 is the FA composition at the *sn*-2 position, Total is the total FA composition in the original fat, and *a* is the ratio between the moles of FA located at the *sn*-2 position and the moles of total FA. *a* was 0.24, 0.30, and 0.17 for N-TAG, E-TAG, and E-MDAG oils, respectively.

[c] The proportion of 1(3),2-DAG relative to total DAG.

[d] The proportion of 2-MAG relative to total MAG.

[e] Estimated calculation based on the values of the acylglycerol composition.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols; FFA: free fatty acids

Table 2. Ingredient composition of the experimental diets.

Ingredients, wt%	
Wheat	51.4
Soybean meal 48 %	38.6
Experimental fats[a]	6.00
Dicalcium phosphate	1.69
Calcium carbonate	1.30
Sodium chloride	0.40
Vitamin and mineral pre-mix[b]	0.30
DL-Methionine	0.23
L-Lysine	0.07
Enzyme supplement[c]	0.05
Antioxidant[d]	0.02

[a] Native palm oil (N-TAG), esterified palm acid oil (E-TAG), or esterified palm acid oil high in mono- and diacylglycerols (E-MDAG).

[b] Provides per kg of feed: vitamin A (E-672) 13,500 IU; vitamin D_3 (E-671) 4,800 IU; vitamin E (alpha-tocopherol) 45 mg; vitamin B_1 3 mg; vitamin B_2 9 mg; vitamin B_6 4.5 mg; vitamin B_{12} 16.5 µg; vitamin K_3 3 mg; calcium pantothenate 16.5 mg; nicotinic acid 51 mg; folic acid 1.8 mg; biotin 30 µg; Fe (E-1) (from $FeSO_4 \cdot 7H_2O$) 54 mg; I (E-2) (from $Ca(I_2O_3)_2$) 1.2 mg; Co (E-3) (from $2CoCO_3 \cdot 3Co(OH)_2 \cdot H_2O$) 0.6 mg; Cu (E-4) (from $CuSO_4 \cdot 5H_2O$) 12 mg; Mn (E-5) (from MnO) 90 mg; Zn (E-6) (from ZnO) 66 mg; Se (E-8) (from Na_2SeO_3) 0.18 mg; Mo (E-7) ($(NH_4)_6Mo_7O_{24}$) 1.2 mg.

[c] Provides per kg of feed: β-glucanase 350 IU; xylanase 1125 IU.

[d] Etoxiquin 66%.

Table 3. Analyzed macronutrient content and fatty acid composition of the experimental diets[a].

Item	N-TAG	E-TAG	E-MDAG
Macronutrient content, wt%			
Dry matter	90.4	90.4	90.4
Crude protein	24.3	24.0	23.5
Crude fat	7.50	7.50	7.61
Crude fiber	2.72	2.88	2.70
Ash	7.06	7.05	6.84
Gross energy, kcal/kg	4,244	4,243	4,222
Fatty acid composition, wt%			
C16:0	36.9	37.1	37.1
C18:0	4.41	4.36	4.73
C18:1 n-9	32.6	33.5	31.1
C18:2 n-6	22.3	21.6	22.6
C18:3 n-3	1.91	1.77	1.96
Minor fatty acids	1.92	1.72	2.56
SFA	42.4	42.3	43.4
MUFA	33.5	34.4	32.0
PUFA	24.2	23.3	24.5

[a] Diets with 6 wt% of native palm oil (N-TAG), esterified palm acid oil (E-TAG) or esterified palm acid oil high in mono- and diacylglycerols (E-MDAG).
All samples were analyzed at least in duplicate.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

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Table 4. Apparent absorption coefficients (%) according to different fat sources in diet.

Item	Dietary treatments[a]			RMSE[b]	*P*-values
	N-TAG	E-TAG	E-MDAG		
AME, Kcal/Kg	2,976	2,888	2,883	119.0	0.341
Organic matter	69.8	68.3	69.0	2.01	0.630
Crude fat	52.5	43.7	39.2	13.48	0.252
Total fatty acids	53.8	47.9	38.9	12.61	0.156
SFA	44.9	39.0	31.5	11.89	0.183
MUFA	61.5	54.9	43.8	13.97	0.119
PUFA	58.6	53.6	45.8	12.55	0.234
C16:0	46.6	40.8	32.7	11.64	0.151
C18:0	31.9	25.3	17.1	13.08	0.181
C18:1 n-9	62.2	55.7	45.3	13.61	0.128
C18:2 n-6	58.3	53.2	45.2	12.64	0.224

[a] Diets with 6 wt% of native palm oil (N-TAG), esterified palm acid oil (E-TAG), or esterified palm acid oil high in mono- and diacyglycerols (E-MDAG).

[b] RMSE: Root Mean Square Error of six observations per treatment (the experimental unit is the cage).

AME: apparent metabolizable energy; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table 5. Growth performance of broiler chickens according to different fat sources in diet.

Item	Dietary treatments[a]				
	N-TAG	E-TAG	E-MDAG	RMSE[b]	*P*-values
ADFI, g/bird per day	32.9[ab]	33.8[a]	30.8[b]	1.59	0.016
ADG, g/bird per day	24.1	23.8	22.1	1.79	0.142
FCR, g/g	1.37	1.42	1.40	0.064	0.419
BW at 13 days, g	359	357	337	25.4	0.262

[a] Diets with 6 wt% of native palm oil (N-TAG), esterified palm acid oil (E-TAG), or esterified palm acid oil high in mono- and diacylglycerols (E-MDAG).

[b] RMSE: Root Mean Square Error of six observations per treatment (the experimental unit is the cage).

Values within the same row with no common superscript are significantly different, $P < 0.05$.

ADFI: average daily feed intake; ADG: average daily gain; FCR: feed conversion ratio; BW: body weight.

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669 **Figure 1.** Expanded area of the ^{1}H NMR spectrum of a fat sample, with the ^{1}H chemical
670 shift assignment of the glycerol signals corresponding to the differently substituted
671 glycerol units.
672
673 **Figure 2.** Melting thermogram (a) and melting profile (b) of native palm oil (N-TAG),
674 esterified palm acid oil (E-TAG) and esterified palm acid oil high in mono- and
675 diacylglycerols (E-MDAG), determined by differential scanning calorimetry. The
676 vertical line on Figure 2b shows the solid fat index at chicken's body temperature (41.5
677 ºC).
678
679 **Figure 3.** Postprandial changes (Δ) of triacylglyceride (TAG) concentrations in serum
680 of broiler chicks, following consumption of native palm oil (N-TAG), esterified palm
681 acid oil (E-TAG) or esterified palm acid oil high in mono- and diacylglycerols (E-
682 MDAG) supplemented diets. Values are expressed as means ± SE, n = 4 for each time-
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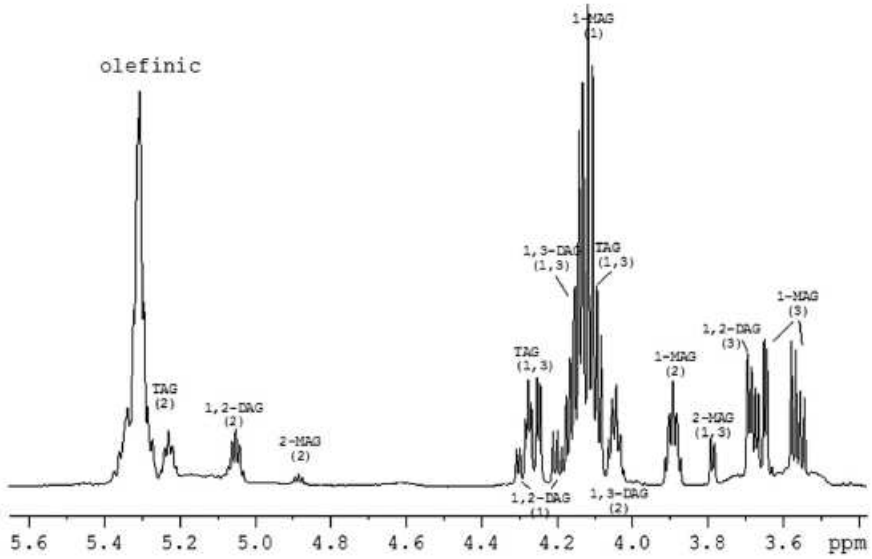

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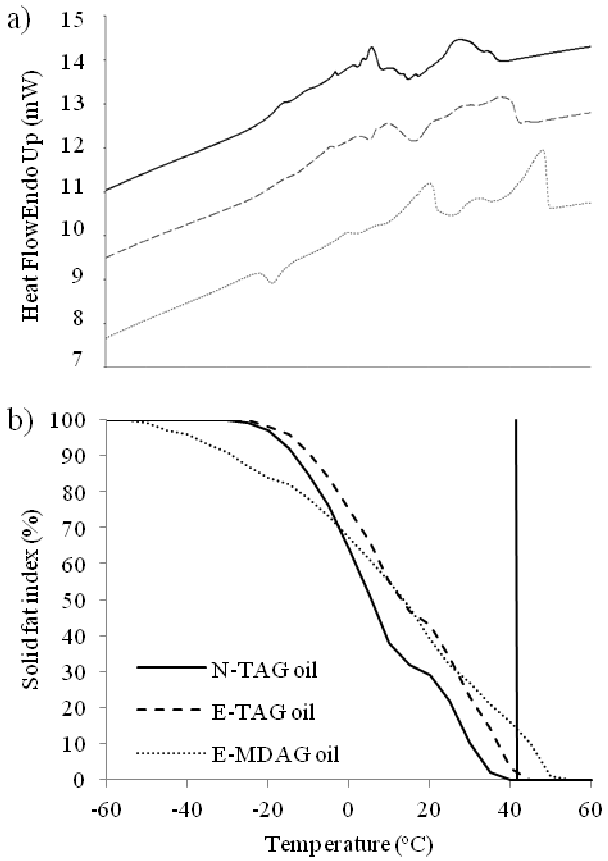

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705 Figure 2

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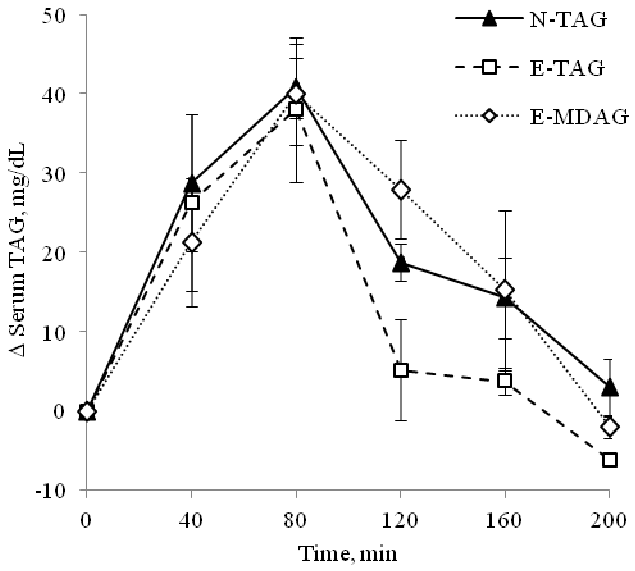

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719 Figure 3