Mono-Diglyceride Fractions in Indonesian Infant Formula Products

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Abstract: Infant formula contains fat derived from a mixture of vegetable oils, which act as external source of fat. Oil or fat is an ester of fatty acid glycerol. This work aimed at quantifying the content of triglycerides (TAG), diglycerides (DAG) and monoglycerides (MAG) in Indonesian infant formula products. We observed infant formula in BPOM depository (year 2018), then stratified random sampling was applied to determine samples used. Fat content in all samples (50 products) was determined, as well as profile of acylglycerols. All 50 samples were then classified according to food category No. 13.1 (Standard of BPOM), resulting in 4 main groups: infant formula (FB, n = 11.21%), advanced formula (FL, n = 16.32%), growth formula (FP, n = 15.30%) and special formula (FK, n = 8.17%). As the results, some samples possessed a high content of MAG and DAG, in which they might be added as emulsifiers. In addition, correlation coefficient between DAG content and proportion of palm oil in samples was recorded at R^2 0.4 to 0.7, suggesting that higher level of palm oil would increase DAG content. PCA analysis clearly separated the distribution of DAG and TAG into 6 groups that exerted different characteristics of each group.

1 INTRODUCTION

Infant formula is included in the food category number 13.0, namely food products for special purposes, which mean that it needs a particular processing or formulation to preserve nutritions available for treatment of diseases or disorders (Republic of Indonesia Food and Drug Supervisory Agency, 2015). Based on the food category (Republic of Indonesia Food and Drug Supervisory Agency, 2015), Formula milk is divided into several types including infant formula, advanced formula and special medical formula.

Infant formula contains protein, carbohydrates, fats, vitamins and minerals. Generally, infant formula is made from cow's milk which is modified and fortified with other nutrients. An additional source of fat used in infant formula particularly includes a variety of vegetable oils, such as palm oil, coconut oil, soybean oil, sunflower seed oil and corn oil (Delplanque et al., 2015). However, detail information on food label related to this fat additive is often unclear. Oil or fat constitutes an ester of glycerol and fatty acids, composed of a mixture of most triglycerides (TAG) and a small number of other compounds, including diglycerides (DAG) and monoglycerides (MAG), free fatty acids, pigments, sterols, hydrocarbons, phospholipids, lipoproteins.

The physical, chemical and functional properties of oil or fat are determined by the profile of triglycerides and their fatty acid composition (Da Silva et al., 2010). MAG and DAG can be one of the factors that affect oil quality standards. MAG and DAG are minor components in oil that can be formed not only through lipase hydrolysis by TAG during the ripening, harvesting and transportation of fruit or seeds, but also through the pyrolysis of TAG at high temperatures, including conventional heating and deodorization (Shimizu et al., 2012). High level of MAG and DAG in vegetable oil represent a reduced quality. Regardless source of oils, distribution of MAG and TAG may differ, but commonly, the proportion of MAG is lower than that of DAG (Pacheco et al., 2014). In addition, the presence of DAG in infant formula could cause the formation of 3-MCPDE compounds (Hamelet et al., 2014). This compounds can cause a damage to the kidneys and testicles in experimental animals (Abraham et al., 2013; Liu et al., 2012).

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2 MATERIALS AND METHODS

Chemicals for analysis included standard monolaurin, a mixture of chloroform methanol (2:1) (Merck), heptane (Merck), acetone (Merck), NaCl (Merck) 0.88%, N-methyl-N-trimethylsilytrifluorocoacetamide, technical N_2 gas andtetrahydrofuran (Merck).

The main instrument used was Gas Chromatrography (Hawlett Packard) with DB-5HT column type (15 m × 320 nm), thickness = 0.1 μm and Flame Ionization Detector type detector.

2.1 Methods

2.1.1 Sample Identification and Sampling

The infant formula database was online-accessed in www.pom.go.id, finding 361 registered products. They were then categorized according to BPOM, in which formula milk belonged to Food Category No. 13 (Regulation No. 1/2015), known as food products for special nutritional needs. Specifically, it was included in No. 13.1. The formula product in this category is generally divided into 3 classes, namely infant formula (13.1.1), advanced formula (13.1.2), and infat formula for special medical use (13.1.3). The sample size was determined from Slovin method or the √N + 1 method as in Eq. 1.

$$n = \frac{[N]}{N\,(d)^2 + 1} \qquad \text{(Eq. 1)}$$

where N is total population; d is confidence level (10%); and n is number of sample.

In this work, stratified random sampling method was applied, enabling to divide the population into smaller groups. These groups were classified according to particular attributes or characteristics within population. Subsequently, proportional amount of sample was selected between groups. Samples were taken randomly and proportionally at each layer (category). The number of samples taken in this study amounted to 50 samples. The Microsoft Excel 2010 application was employed for random sampling from each category using the formula "= RANDBETWEEN (lower limit; upper limit)". Every selected registered formula milk brand was taken 2 batches (different production codes) as a test, and each test was analyzed 2 times. Products are purchased from markets in Bogor, West Java, Indonesia.

2.1.2 Fat Extraction

Extraction was carried out according to (Abraham et al., 2013). Sample (24 g) was macerated in 60 mL of chloroform and methanol solution (2:1) for 120 min while stirring using a magnetic stirrer. The maceration mixture was filtered using Whatman filter paper with the help of a vacuum pump, then the filtrate solution was added with 32 mL of 0.88% Cl and shaken to produce two layers. The lower layer (oil phase) was collected using filtration with a filter paper, then evaporated using rotary vacuum evaporator at 40°C to remove solvent residue. Afterwards, the extraction product was concentrated by blowing N_2 gas to eliminate the remaining solvent. The oil was stored in a dark bottle, tightly closed with parafilm coated and stored at 4°C until subsequent analysis. Fat content was calculated as in Eq. 2.

$$\text{Fat content (\%)} = \frac{W_t}{W_o} \times 100\% \qquad \text{(Eq. 2)}$$

where Wt is mass of extracted oil (g) and W0 is sample mass (g)

2.1.3 Determination of Acylglycerol Composition

Composition of acylglycerol was determined using Gas Chromatography (Hawlett Packard Series 6890) with Flame Ionization Detector, operated according to AOCS Official Method Cd 11b-91 2003 (Liu et al., 2012). The column used was DB-5HT (15 m × 320 nm) with thickness of 0.1 μm. The carrier gas used was helium, while the make up gas was N_2. Gas chromatography apparatus was equipped with split injection or injection column and FID, and run at following conditions: initial column temperature of 50°C increased to 180°C at rate of 15°C·min^{-1}, then subsequently increased to 230°C at rate of 7°C·min^{-1}, and increased again to 380°C, the temperature for detector and injector was set at 390°C with velocity of carrier gas 0.7 mL N^2 ·min^{-1}, while the air flow velocity was 450 mL·min^{-1} with injection volume of 1 μ.

Briefly, sample (0.0250-0.0255 g) was transferred in vial, then added with 10 μL of tetrahydrofuran and 50 μL of N-methyl-N-trimethylsilyl-trifluoroacetamide. After that, the tube was closed, mixed using vortex at 2400 rpm for 90 sec. The mixture was incubated in a dark room for 10 min, added with 2 mL of heptane, then mixed at 2000 rpm for 30 sec. The sample tube was covered with parafilm, then incubated at room temperature for approximately 30 min prior to injection at volume of 1 μL.

$$\text{DAG in product} = fat \times \frac{\text{Fat contain in product}}{100\ (g)} x100(g) \quad \text{(Eq. 3)}$$

$$\text{DAG + MAG (g/100mL)} = \text{DAG + MAG} \times \frac{g}{mL} \text{ (Serving size)} \quad \text{(Eq. 4)}$$

2.1.4 Data Analysis

The resulting data were statistically evaluated using Analysis of Variance (ANOVA) in SPSS software. Significance among means was verified using DMRT (Duncan Multiple Range Test) at $p<0.05$. Proportion of palm oil, diglycerides and triglycerides was determined by multivariate using Pricipal Component Analysis (PCA) in XLSTAT 2018 application.

3 RESULTS

3.1 Sample Identification and Sampling

Among 361 infant formula products listed in BPOM, we observed that advanced formula became the most abundant product, i.e. 223 items (62%), while infant formula and formula for special medical use was 77 (21%) and 61 (17%) items, respectively. The category "advanced formula" is divided into two categories, i.e. formula for ages 6-12 months (advanced formula) and formula for 1-3 years (growth formula). Previously, have been also reported categories of formula products by the age: 0-6 months, 6-12 months, and 1-3 years (Liu et al., 2012). In this study, we investigated 50 samples of milk formula.

3.2 Fat Content

Content of fat showed a noticeable difference between samples, in which the highest one was attributed to infant formula (FB10), reaching up to 25.86%, then advanced formula (FL9), i.e. 23.61%, growth formula (FP4), i.e. 20.10%, and then special formula (FK3), i.e. 25.64% (Table 1).

Table 1: Fat content of formula milk samples.

Categories	Code	Weight of sample [g]	Weight of sample extract [g]	Fat content [%db]
Infant Formula	FB1	24.03	4.24	18.01
	FB2	24.02	5.26	22.10
	FB3	24.02	5.08	21.56
	FB4	24.12	4.52	19.09
	FB5	24.06	5.11	21.61
	FB6	24.11	5.26	22.16
	FB7	24.03	5.56	23.54
	FB8	48.02	4.91	10.40
	FB9	24.02	5.52	23.44
	FB10	24.05	6.13	25.86
	FB11	24.00	5.36	22.73
Advanced formula	FL1	24.04	4.40	18.60
	FL2	24.00	4.74	20.12
	FL3	24.03	5.19	21.77
	FL4	24.09	4.34	18.18
	FL5	24.02	5.40	22.95
	FL6	24.04	4.14	17.61
	FL7	42.06	2.79	6.82
	FL8	24.05	3.97	16.94
	FL9	24.05	5.58	23.61
	FL10	24.10	3.59	15.20
	FL11	24.01	5.26	22.35
	FL12	24.02	4.22	17.99
	FL13	24.01	4.48	18.84
	FL14	42.05	3.20	7.76
	FL15	24.01	4.44	18.77
	FL16	24.02	4.41	18.75
Growth Formula	FP1	42.03	2.15	5.24
	FP2	45.04	2.32	5.26
	FP3	24.01	4.11	17.33
	FP4	24.18	4.82	20.10
	FP5	24.03	3.19	13.58
	FP6	24.03	3.72	15.80
	FP7	24.03	2.99	12.72
	FP8	45.78	3.41	7.62
	FP9	24.02	3.38	14.38
	FP10	30.01	1.56	5.33
	FP11	24.06	2.85	12.10
	FP12	45.02	2.91	6.58
	FP13	30.02	3.99	13.57
	FP14	30.03	5.62	19.09
	FP15	30.59	3.68	12.25
Special Formula	FK1	42.06	5.29	12.9
	FK2	45.02	2.79	6.30
	FK3	24.00	6.06	25.64
	FK4	30.35	2.29	7.68
	FK5	42.03	1.70	4.11
	FK6	42.06	2.45	5.96
	FK7	48.47	2.10	4.44
	FK8	42.02	9.38	22.68

3.3 Composition of Acylglycerol Fraction

The results demonstrated that MAG was only found in FB11, reaching up to 0.13% (Table 2). This compound is intentionally incorporated by manufacture as it occurs on the label. Furthermore, DAG ranged from 0.3 to 1.8%, with an average of 1.67%. meanwhile, TAG was found at range of 83-97%, with an average of 92.32%.

The distribution of MAG, DAG and TAG in advanced formula samples is presented in Table 3. The results exhibited that FL15 and Fl16 became two samples that contained MAG, i.e. 0.35% and 0.26%, respectively. In fact, both products confirmed presence of MAG, as written on the label. Furthermore, DAG in advanced formula samples ranged from 0.4 to 4.3%, with an average of 1.17%. The DAG content is greater than 4%, while the 3-MCPD ester level is generally greater than 5 ppm. The TAG content in the sample of the advanced formula category occured between 78-100%, with an average of 94.09%.

Table 4 presents the content of MAG, DAG, and TAG in growth formula category. Our data revealed that two samples (FP13 and FP15) were evidenced to contain MAG at 0.16% and 0.81%, respectively. Additionally, producers of both samples did not provide information on the label related to addition of MAG. DAG was found at range of 0.1-1.8%, with an average of 0.69%. The highest DAG content was detected in FP5, no information was given on the label. Afterwards, TAG ranged from 86 to 100%, with an average of 94.3%. In terms of special formula category, one sample was evidenced to contain MAG, i.e. FK7 (3.78%). However, manufacture has declared the addition of was found at 91-100%, with an average of 96.27%. We also detected percentage of TAG reaching up to 100%, found in FK3, FK6 and FK8. This presumably represents administration of Medium Chain Triglyceride (MCT) in the sample (AOCS Official Method Cd 11b-91, 2003). MAG since it occurred on the label. For DAG, it ranged from 0.1 to 0.4%, with an average of 0.34%, while proportion of TAG.

Table 2 : The content of MAG, DAG, TAG and label composition in samples of infant formula categories.

Category	Sample	MAG		DAG		DAG+MAG (g/100mL ready to eat product)	TAG % in fat/oil	Label composition	
		% in oil	% in product	% in oil	% in product			MAG	MCT
Infant Formula	FB1	-	-	5.57	1.00	0.15	94.43	-	-
	FB2	-	-	5.52	1.22	0.18	94.48	-	-
	FB3	-	-	2.70	0.58	0.08	97.30	-	-
	FB4	-	-	5.70	1.09	0.16	94.30	-	-
	FB5	-	-	7.76	1.68	0.25	92.24	-	-
	FB6	-	-	13.71	3.04	0.45	86.29	-	-
	FB7	-	-	12.33	2.90	0.42	87.67	-	-
	FB8	-	-	3.69	0.38	0.06	96.31	-	-
	FB9	-	-	3.73	0.87	0.13	96.27	-	-
	FB10	-	-	6.85	1.77	0.25	93.15	-	-
	FB11	0.55	0.13	16.95	3.85	0.57	83.05	√	-

Table 3: The content of MAG, DAG, TAG and label composition in samples of advanced formula categories.

Categories	Sample	MAG		DAG		DAG+MAG (g/100mL ready to eat product)	TAG %in fat/ oil	Label composition	
		% in oil	% in product	% in oil	% in product			MAG	MCT
Advanced Formula	FL1	-	-	5.74	1.07	0.15	94.26	-	-
	FL2	-	-	21.74	4.37	0.62	78.26	-	-
	FL3	-	-	6.08	1.32	0.22	93.92	-	-
	FL4	-	-	2.73	0.50	0.08	97.27	-	-
	FL5	-	-	2.96	0.68	0.11	97.04	-	-
	FL6	-	-	-	-	-	100	-	-
	FL7	-	-	6.17	0.42	0.06	93.83	-	-
	FL8	-	-	-	-	-	100	-	-
	FL9	-	-	3.27	0.77	0.12	96.73	-	-
	FL10	-	-	4.90	0.74	0.11	95.10	-	-
	FL11	-	-	6.11	1.37	0.22	93.89	-	-
	FL12	-	-	13.99	2.52	0.38	86.01	-	-
	FL13	-	-	2.81	0.53	0.09	97.19	-	-
	FL14	-	-	5.70	0.44	0.14	94.30	-	-
	FL15	1.84	0.35	3.12	0.59	0.13	95.05	√	-
	FL16	1.40	0.26	6.00	1.13	0.20	92.60	√	-

Table 4: The content of MAG, DAG, TAG and label composition in samples of growth formula categories.

Categories	Sample	MAG		DAG		DAG+MAG (g/100mL ready to eat product)	TAG % in fat/ oil	Label composition	
		% in oil	% in product	% in oil	% in product			MAG	MCT
Growth Formula	FP1	-	-	5.93	0.31	0.06	94.07	-	-
	FP2	-	-	3.11	0.16	0.03	96.89	-	-
	FP3	-	-	3.56	0.62	0.08	96.44	-	-
	FP4	-	-	-	-	-	100	-	-
	FP5	-	-	13.40	1.82	0.36	86.60	-	-
	FP6	-	-	9.14	1.44	0.24	90.86	-	-
	FP7	-	-	6.00	0.76	0.15	94.00	-	-
	FP8	-	-	5.65	0.43	0.07	94.35	-	-
	FP9	-	-	6.42	0.92	0.16	93.58	-	-
	FP10	-	-	3.56	0.19	0.03	96.44	-	-
	FP11	-	-	2.88	0.35	0.06	97.12	-	-
	FP12	-	-	3.15	0.21	0.03	96.85	-	-
	FP13	1.17	0.16	2.90	0.39	0.10	95.93	√	-
	FP14	-	-	8.43	1.61	0.24	91.57	-	-
	FP15	6.59	0.81	3.61	0.44	0.23	89.80	-	-

3.4 Coefficient Correlation between Levels of Palm Oil and Diglyceride

The coefficient correlation between the proportion of palm oil and DAG content in samples of infant formula was depicted in Figure 1A. The test results showed a linear curve with the equation $y = 0.4547x - 2.2717$ with $R^2 = 0.4789$, suggesting that proportion of palm oil positively correlates with level of DAG. In case of advanced formula, the linear curve was arranged with the equation $y = 0.0459x + 0.3131$, and $R^2 = 0.4017$ (Figure 1B). Similarly, higher proportion of palm oil also resulted in a higher level of DAG. For growth formula category, the equation was $y = 0.1176x - 0.1426$, with $R^2 = 0.7774$ (Figure 1C), indicating that content of DAG increases as more palm oil is added.

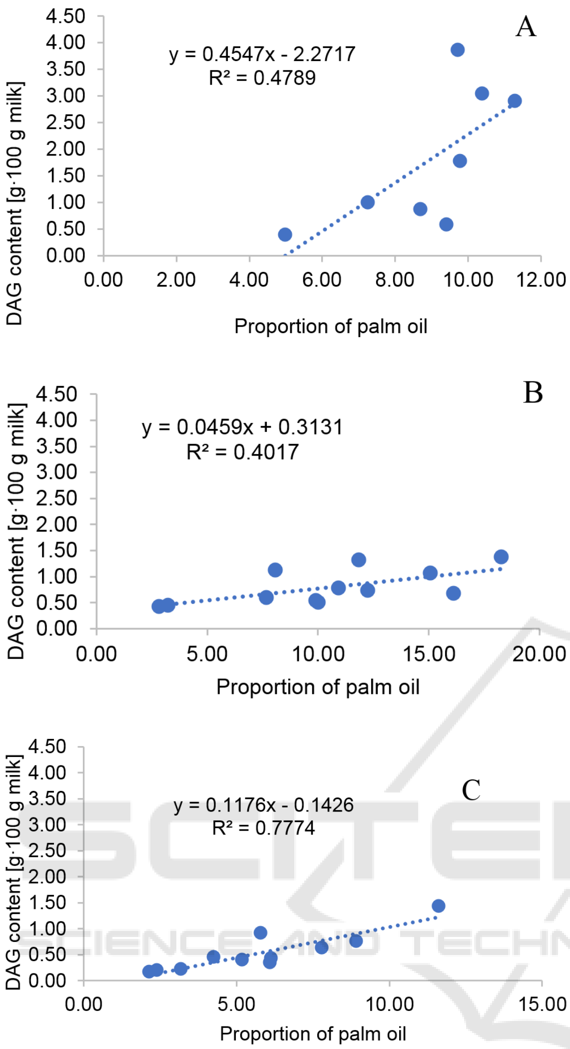

Figure 1: Plot of palm oil and DAG content in three groups of samples: infant (A), advanced (B) and growth (C) formula.

3.5 PCA Analysis

As depicted in Table 5, there is a significant difference between content of DAG, TAG content and proportion of palm oil for each groups. Group A showed the lowest proportion of palm oil compared to other groups; on the contrary, group B had the highest level of DAG among groups. Meanwhile, the highest proportion of palm oil was detected in group C. The discrepancy between group D and E occured in the gradient of group E closing to TAG gradient, which means that TAG in group E is higher than in group D. Furthermore, gradient of group D is also close to DAG gradient, suggesting that DAG in group D is higher than group E. Group F has the highest TAG content, i.e. 100%. This links to the use of medium chain triglycerides (MCT), while also contains lower amount of palm oil compared to other groups.

Table 5: Distribution of diglycerides and triglycerides.

Groups	n	DAG	TAG	Palm Oil Proportion
A	3	9.86 ± 3.08^{d}	90.14 ± 3.08^{b}	0.00 ± 0.00^{a}
B	7	13.81 ± 4.51^{e}	86.19 ± 4.51^{a}	46.57 ± 11.24^{c}
C	6	5.60 ± 0.40^{c}	94.40 ± 0.40^{c}	71.00 ± 1.10^{d}
D	12	5.77 ± 1.00^{c}	93.25 ± 1.28^{c}	42.17 ± 8.12^{c}
E	16	3.16 ± 0.33^{b}	96.66 ± 0.58^{d}	45.31 ± 6.64^{c}
F	6	0.00 ± 0.00^{a}	100.00 ± 0.00^{e}	28.67 ± 22.46^{b}

4 DISCUSSION

MAG is a common emulsifiers applied in milk-based recombination products such as infant formula. In short, presence of MAG in sample is associated with its functionality as emulsifiying agent. Maximum threshold of mono-diglycerides as emulsifier in infant formula is 0.4g·100mL^{-1} Sun et al., 2016). Our data revealed that content of these chemicals in 27.27% of samples was evidenced to be much higher than standard, i.e. FB6, FB7 and FB11. The product does not include an emulsifier in its composition. The high content of MAG and DAG that exceeds the limit possibly results from vegetable oil which does not fit the requirements. The existence of MAG and DAG depends on the process, storage and shelf life of the oil Risma et al., 2019) or the condition of raw materials that did not meet standard. Besides, they are added intentionally by manufacturers to give emulsifying properties, but not mentioned in list of composition.

The high content of DAG needs to receive serious concern related to its potentiality as precursor for the formation of 3 MCPD esters (CODEX Alimentarius, 2017). MAG and DAG in samples studied are used as emulsifiers. Compared to standard of Codex, we found 16 samples (6.25%) did fit the criteria (> 0.4g·100mL^{-1}), meanwhile FL2 showed no compliance with regulations because the product did not include the addition of emulsifiers. Based on regulation issued by Codex Sun et al., 2016), we concluded that all samples of growth formula categories met the standard regarding addition of MAG and DAG. Compared to regulation of Codex Sun et al., 2016), level of MAG and DAG present in all samples of special formula is accordance with the standard.

5 CONCLUSION

Categorization of 50 samples based on food category No. 13.1 resulted in 4 major classes: infant formula (n = 11.21%), advanced formula (n = 16.32%), growth formula (n = 15.30 %) and formula for special medical purposes (n = 8.17%). The experiment successfully detected presence of MAG and DAG in samples, which might be linked to intentional addition by manufacture considering their function as emulsifiers. Besides, the results found 27.27% of infant formula samples and 6.25% of advanced formulas containing MAG and DAG that exceed the maximum threshold of Codex. This presumably relates to hydrolysis of vegetable oils used in the samples, and may be intentionally added as emulsifiers despite not mentioned on label. PCA analysis successfully mapped proportion of palm oil, MAG, DAG and TAG into 6 groups, having distinctive feature for each group.

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