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VALIDATION OF ANALYTICAL METHODS AND DETERMINATION OF ALPHA-LINOLENIC ACID (OMEGA 3) AND LINOLEIC ACID (OMEGA 6) IN SOME FORMULA MILK PRODUCT

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ABSTRACT

In certain conditions, formula milk, if necessary, can be used as a substitute for breast milk, which must have formulations comparable to breast milk. Alpha-linolenic acid (ALA) and linoleic acid (LA) are essential fatty acids that must be contained in formula milk based on The Regulation of Indonesian FDA, Number 1 of 2018. This study aims to obtain valid, simple, and reproducible ALA and LA analysis methods to guarantee the quality and safety of formula milk. Validation of ALA and LA analysis methods using AOCS Official Methods Ce 2b-11 modified in formula milk by gas chromatography using the capillary column DB *Fast FAME*. The analysis method meets the validation requirements. ALA had quantitation limit detection as low as 6.833 µg/mL, repeatability (RSD) less than 1.9 %, and recovery from 96.2 % to 103.9 %. LA had quantitation limit detection as low as 8.063 µg/mL, repeatability (RSD) less than 0.9 %, and recovery from 97.4 % to 99.2 %. This method was applied to determine the content of ALA and LA in 11 sample formula milk products. The analyzed contents of ALA between 50.850 ± 0.053 mg/100 kcal to 122.279 ± 0.113 mg/100 kcal and LA between 478.152 ± 0.267 mg/100 kcal to 956.606 ± 0.207 mg/100 kcal. This result showed that the samples contained ALA and LA by applicable requirements. However, by evaluating the label declaration, three samples contain ALA and four samples containing LA lower than label declaration.

KEYWORDS: Capillary column DB Fast FAME**,** alpha-linolenic acid, linoleic acid, bomb calorimeter, and formula milk.

INTRODUCTION

Breast milk is the golden standard for baby food because it contains essential nutrients and nutritional components[1] for optimal growth and development of infants,[2] which should be consumed by babies for two years. The average number of exclusively breastfed infants was 41 % worldwide,[3] while nationally, babies who received exclusive breastfeeding were 61.33 % .[4]

In certain conditions, formula milk, if necessary, can be used as a substitute for breast milk, which must have formulations comparable to breast milk. ALA is omega 3, and LA is omega 6 that plays a role in the growth and maturation of various organs in infants, especially the brain and eyes.[2] The determination of ALA and LA is essential to guarantee the quality and safety of formula milk on the market.

Analysis methods for the determination of fatty acids can be adopted for the determination of ALA and LA. In the Association of Official Agricultural Chemists (AOAC) Official Method 996.06, the procedure for analyzing fatty acids in food samples is carried out in several stages: hydrolysis, extraction, methylation measurement using gas chromatography.[5] The analysis phase is too long and not efficient enough to be applied. At present, a method of analyzing the determination of fatty acids is developed by direct transesterification.[6] Competition of the analysis of fatty acids using the direct transesterification method with the fat extraction method first gave results that were not significantly different.[7]

The American Oil Chemists' Society (AOCS) Official Method Ce 2b-11 has been used to analyze omega 3 in conventional foods and food supplements.[8] In this study, the method is used to analyze ALA and LA in formula milk. This method was chosen because, in this method, the sample is methylated directly without fat hydrolysis first, so it is simpler and faster.

Fatty acids are analyzed using BPX 70 capillary columns with LA retention times around 25 minutes.[9] Fatty acids are analyzed using DB-225ms capillary columns with LA retention times around 24 minutes, and ALA retention time is about 26 minutes and is almost the same as using the DB-23 capillary column.[10] Meanwhile, it takes approximately 19 minutes to elute LA and 22 minutes to elute ALA using Supelco-waxed capillary column 10.[8]

Gas chromatography is used with a flame ionization detector provides high reproducibility. The condition that the separation of fatty acids must be specific with a resolution meets the requirements because the characteristics of fatty acids are determined based on retention time. DB Fast FAME capillary column offers a short-term analysis of fatty acids with high resolution.[11]

The valid, reproductive, and straightforward ALA and LA analysis methods are obtained to guarantee the quality and safety of formula milk based on The Regulation of Indonesian FDA, Number 1 of 2018.[12] It is necessary to validate the determination analysis method ALA and LA using the American Oil Chemists' Society (AOCS) Official Methods Ce 2b-11 modified in milk formula by gas chromatography with capillary column DB Fast FAME.

MATERIALS AND METHODS

Materials

The internal standard (IS) glyceryl tridecanoate (C13:0), standard linolenic acid, and standard linoleic acid were purchased from Sigma Aldrich (purity ≥ 99%). 25% sodium methoxide solution in methanol (Sigma Aldrich, Germany), 20% boron trifluoride solution in methanol (Merck), sodium chloride (Merck, Germany), n-hexane (Merck, Germany) and double-distilled water. Formula milk samples (n = 11) consist of infant formula (n=6), advance formula (n=3) and growth formula (n=3). The sample was registered product at The Indonesian FDA, produced domestically (MD), and was sampling in 3 food distribution facilities in Padang, West Sumatra, Indonesia.

Methods

Sample preparation

The sample base was prepared on AOCS *Official Method* Ce 2b-11 adopted by Li and friends.[8] Into the test tube was added 1 mL of standard internal solution (2.5 mg/mL), and the existing solvent was evaporated by slowly flowing nitrogen gas. The sample test was weighed 100 mg and added to the test tube. Then, in the test tube, 2 mL of 2 % sodium methoxide solution in methanol was added and heated in a water bath at 80 ^{o}C for 10 minutes. Then, in the test tube, 2.5 mL of 20 % boron trifluoride solution in methanol was added, and the test tube returned to a water bath at 80 ^{o}C for 5 minutes. Then, into the test tube 5 mL of n-hexane was added, and the solution was heated in a water bath for 2 minutes at 80 ^{o}C. Next, a 3 mL saturated NaCl solution was added into the test tube, centrifuged at 2500 rpm for 5 minutes. The top layer is pipetted and put in a gas chromatography vial.

Changes in the condition of AOCS *Official Method* Ce 2b-11 adopted by Li and friends[8] were analyzed using 2 % sodium methoxide solution in methanol instead of 0,5 M NaOH in methanol.

Standard Solution preparation

The stock standard solution of ALA and LA was prepared by dissolving the weighted standard in n-hexane to get final concentration, respectively, 0.5 mg/mL and 5 mg/mL. The working standard solution was prepared in 6 levels with each standard level being prepared the same as the sample to obtain ALA concentration equivalent to 12.5; 25; 50; 100; 150; 200 µg/mL and LA concentrations equivalent to 125; 250; 500; 1000; 1500; 2000 µg/mL.

Gas chromatography analysis

The gas chromatography system used is the Shimadzu GC-2010 Plus, AOC-6000 injector, flame ionizer detector, and Agilent J&W DB Fast FAME capillary column (30 m x 0.25 mm, ID: 0.25 µm). Gas chromatography system conditions are using helium carrier gas at a pressure of 19 psi, injector temperature of 250 °C, split inlet mode at a ratio of 1:50, program temperature starts at 50°C, hold for 0.5 minutes. The temperature is raised to 194 °C with an increase of 30 °C/minute, hold for 3.5 minutes, the temperature is raised to 240 °C with an increase of 5 °C/minute hold for 1 minute.[11] Changes in the condition of Wu and Zu at the detector temperature used is 250 °C.

The system suitability tests are carried out to ensure adequate gas chromatography system conditions for the analysis of ALA and LA in formula milk. The system suitability test is done by injecting a mixture of standards ALA and LA seven times. The parameters measured include resolution, the number of plates, HETP, tailing factor, and area ratio.

Validation of the assay

The method was validation according to guidelines for the validation of chemical processes for the FDA FVM Program, 2nd Edition.[13] The calibration curves of ALA and LA were prepared from 6 levels of working standard. LOD and LOQ were determined using an external calibration curve method. The precision of the quantitative approach was checked through the repeatability (RSD) of 3 sample formula milk (infant formula, advance formula, and growth formula) in triplicate (n = 3). The accuracy of ALA and LA determination was evaluated in 3 levels spiked of standard solution into the blank matrix of formula milk.

Determination of ALA and LA in formula milk

Requirements for ALA and LA-based on The Regulation of Indonesian FDA, Number 1 of 2018 are calculated based on the heat contained in 100 mL of ready-to-use formula milk, for that analysis of the samples also carried out a review of the energy contained in the sample in triplicate (n=3) using the method bomb calorimeter (Parr, The 1341 Oxygen Bomb Calorimeter).

RESULT AND DISCUSSION

The standard mixture used in the system suitability test is first esterified into its methyl ester form. Acid analysis using gas chromatography results in poor separation due to polarity so that the derivatization step must be taken before the sample is analyzed using a gas chromatography system.[14] Derivatization of fatty acids is also known as methylation to form a methyl ester, which has a more stable polarity.

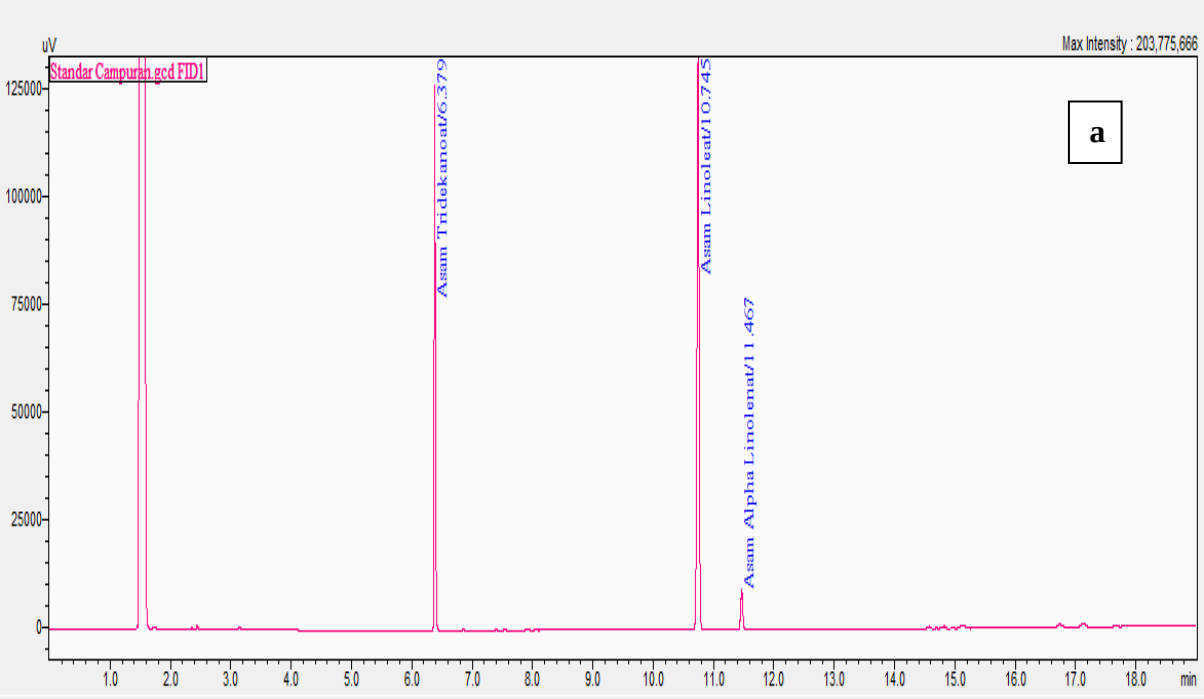
The primary purpose chromatography system is separate the analyte in the sample through a column where the separation between the tops of the analyte one with another is called resolution.[15] The average resolution between the IS peaks with LA peak was 87.425, while the ordinary resolution between LA peak and ALA peak was 11.391. The optimal resolution has a value of ≥ 1.5.[16]

The number of plates depicts the efficiency of the analyte separation tested.[15] The average of the number of plates for LA was 479844 and ALA was 506154. The average HETP value for LA was 0.313, and ALA was 0.297. Optimal HETP values range from 0.1-1.[15] The average tailing factor value for LA was 0.934, and ALA was 1.040. Symmetrical chromatograms have a tailing factor value of 1 while tailing factor values > 1 indicate an asymmetric chromatogram.[16] In this study, the calculation of concentration uses an area ratio between LA and ALA with IS. So that the system suitability test calculated is the RSD from the area ratio. The RSD of the area ratio of LA to IS is 0.477 %, and RSD of the area ratio of ALA to IS is 0.636 %.

The measurement of the resolution value, the number of plates, HETP, tailing factor, and area ratio in the system suitability test shows that the condition of the gas chromatography system used is adequate for the analysis methods of ALA and LA. In this study, the AOCS Official Method Ce 2b-11 method was modified in standard and sample preparation. To ensure that

the process accurate and reliable for analyzing linolenic acid and alpha-linoleic acid present in formula milk, method validation is then performed. Validation parameters include specificity, linearity, sensitivity, precision, and accuracy.

In the specificity, the test used solvents, standard solutions, samples, spiked samples, blank matrix, and empty spiked matrix to determine the ability of the analytical method to distinguish analyzed analytes (ALA and LA) from other components in the sample. The standard analysis of IS, LA, and ALA had consecutive retention times at minutes 6.378, 10.735, and 11.561, which showed different retention times. Profiles of standard and sample chromatograms can be seen in Figures 1a and 1b. The resolution of IS, LA, and ALA meets the criteria. It shows the analysis method and specific chromatographic system conditions for the analysis method of LA and ALA.



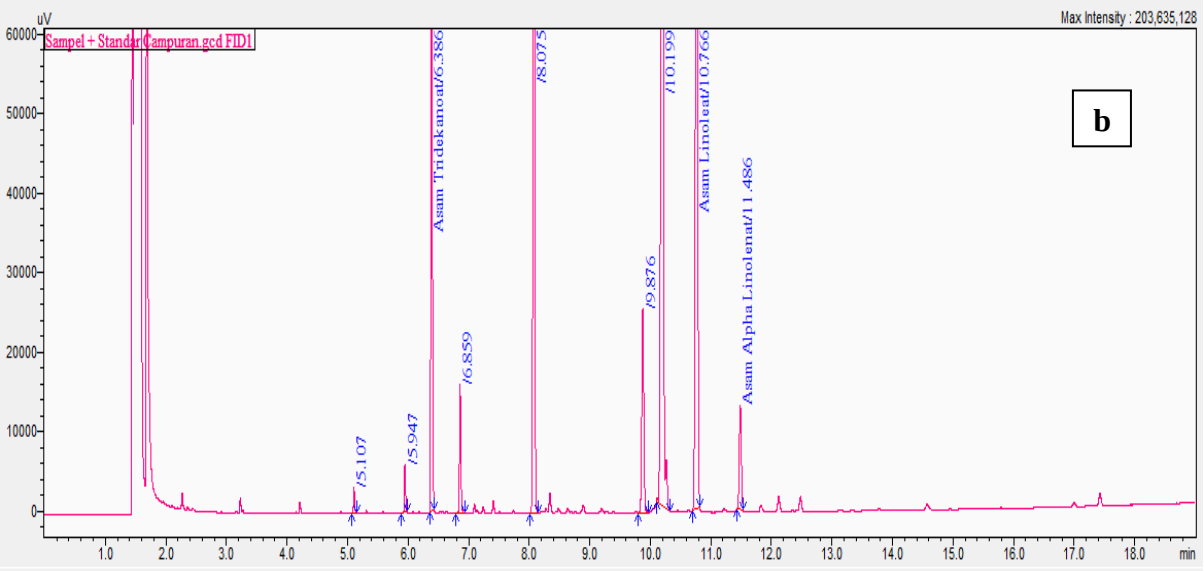


Figure 1. (a) Chromatogram of standard ALA and LA, (b) Chromatogram of sample milk formula

Linearity is the ability of method to obtain test results that are directly proportional to the concentration of the analyte in the sample [16]. The range of ALA and LA levels are regulated in such a way that they can meet The Regulation of Indonesian FDA, Number 1 the Year 2018. In this study, the range of ALA was 12.969 - 207.504 µg/mL, and the range of LA on the calibration curve was 125.730 - 2011, 680µg/mL. The ratio between ALA to LA is 1: 10. This analysis method uses internal standards. Internal standards can minimize errors that occur during sample preparation, inaccuracies originating from equipment, and systematic errors from instruments (including the volume of samples injected).[15] The internal standard used is tridecanoic acid, which is also known as glyceryl tridecanoate. The internal standard used must have a chemical structure similar to the analyte tested, elute at different times, not contained in the sample.[15]

Linearity test results from the relationship of ALA concentration with the ratio of the ALA area to the IS area are shown in the calibration curve of Figure 2a with the line equation y = 0.001635 x - 0.010884, r^2 = 0.997. Linearity test results from the relationship of LA concentration with the LA area ratio to the IS area are shown in the calibration curve of Figure 2b with the line equation y = 0.002021 x - 0.090106 r^2 = 0.998. ALA has a detection limit of 2.050µg/mL and a quantitation limit of 6.833µg/mL. LA has detection limit 2,419µg/mL and quantitation limit 8.063 µg/mL.

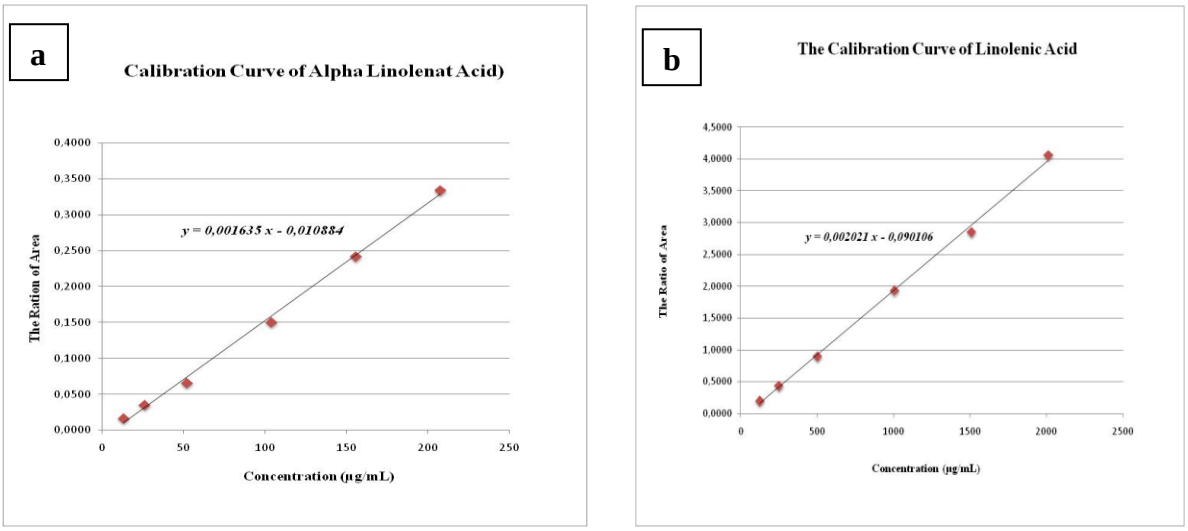


Figure 2. (a) Calibration Curve of Alpha-Linolenic Acid, (b) Calibration Curve of Linoleic Acid.

Recoveries of ALA 96.2 – 103.9 % with a range of analysis 36.313 – 145.253µg/mL. Besides, recoveries of LA 97.4 – 99.2 % with a variety of study 352.044 – 1408.176 µg/mL. Precision was performed on repeatability (RSD) of ALA less than 1.3 % and LA less than 0.9 %. The validation of the analysis method of ALA and LA in formula milk that refers to the modified AOCS Official Method Ce 2b-11 met the criteria for the validated parameters. The recapitulation of the results of the validation method of ALA and LA in formula milk can be seen in Table 1.

Tabel 1. The recapitulation of the results of the validation method of ALA and LA.

Analytes	Regression Equation	r^2	LOD	LOQ	RSD of Repeatibility	Range	Recovery
Alpha Linolenat Acid	y = 0,001635 x – 0,010884	0,997	2,050 µg/mL	6,833 µg/mL	1,3%	36,313 - 145,253 µg/mL	96.16 - 103.88 %
Linoleat Acid	y = 0,002021 x – 0,090106	0,998	2,419 µg/mL	8,063 µg/mL	0,9%	352,044 - 1408,176 µg/mL	97.42 - 99.16 %

ALA and LA play a role in the growth and maturation of various organs in infants, especially the brain and eyes.[2] Determining ALA and LA is essential to guarantee the quality and safety of formula milk on the market.

Based on The Regulation of Indonesian FDA, Number 1 of 2018, formula milk is processed food for special diets of infants and children consisting of infant formula, advanced formula,

and growth formula. Requirements for the content of ALA in infant formula, advanced formula, and growth formula are the same, which is a minimum of 50 mg/100 kcal. Requirements for LA content in infant formula, advanced formula and growth formula; 300 - 1400 mg/100 kcal, 300 - 1200 mg/100 kcal, and 300 - 1200 mg/100 kcal. The ratio requirements of ALA and LA 1: 5-15.

The levels of LA listed on the sample packaging label are 435.7 - 716.3 mg/100 kcal. ALA levels listed on the sample packaging label vary from 52.2 - 89 mg/100 kcal. The ratio of ALA to LA energy test results on 11 samples varied from 4.501 ± 0.027 kcal/gram to 5.266 ± 0.092 kcal/gram with RSD lower than 1.8 %. Furthermore, energy is calculated in 100 mL of ready-to-use formula milk based on the serving size printed on the packaging label of each formula milk—the power in samples formula milk 64.366 – 86.196 kcal/100 mL (Tabel 2, Figure 3).

Tabel 2. Analysis of energy in milk formula.

Sample Milk Formula	Energy		
	Label Declaration (kcal/100 mL)	Analysis Result (kcal/100 mL)	Requirement (kcal/100 mL)
Infant Formula 1	70,0	71,1	60 - 70
Infant Formula 2	67,0	64,4	60 - 70
Infant Formula 3	66,0	65,9	60 - 70
Infant Formula 4	-	70,5	60 - 70
Infant Formula 5	74,0	76,8	60 - 70
Advance Formula 1	80,0	85,3	65 - 80
Advance Formula 2	70,0	71,0	65 - 80
Advance Formula 3	70,0	72,3	65 - 80
Growth Formula 1	70,0	72,0	60 - 85
Growth Formula 2	81,8	97,4	60 - 85
Growth Formula 3	80,0	86,2	60 - 85

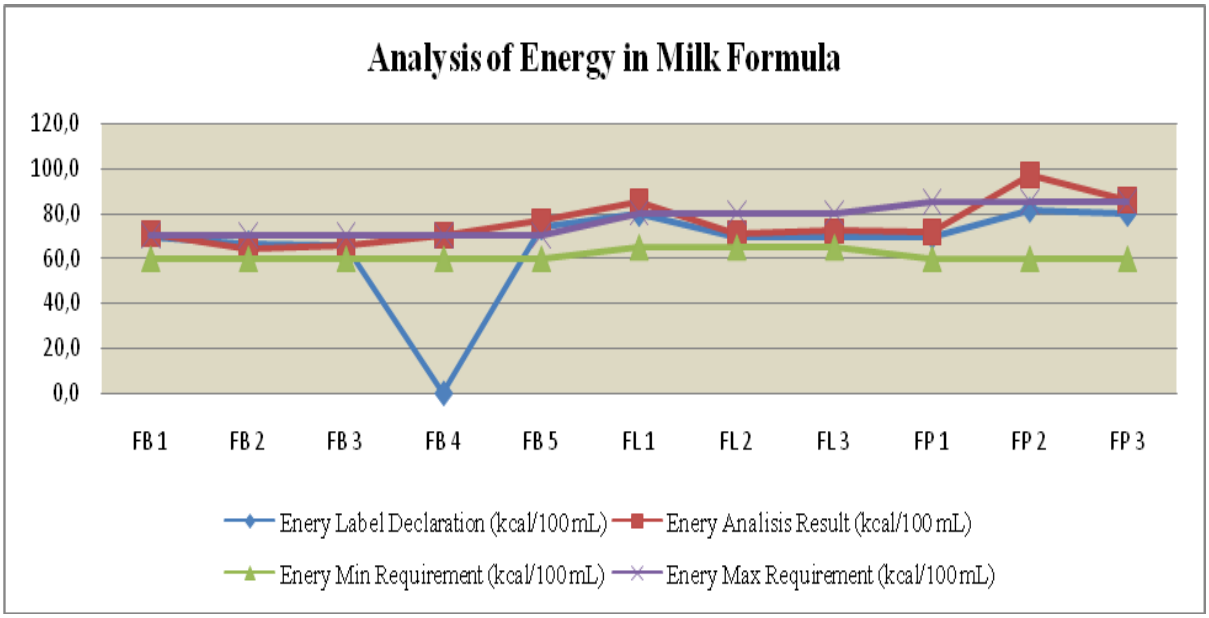


Figure 3. Analysis of energy in milk formula.

ALA levels found in formula milk samples ranged from 50.850 – 122.279 mg/100 kcal. All samples meet ALA content requirements based on The Regulation of Indonesian FDA, Number 1 of 2018. However, if we evaluate the packaging label's claims, there are three samples of formula milk containing ALA lower than the levels claim (Table 3 and Figure 4).

Tabel 3. Analysis of alpha-linolenic acid in milk formula.

Sample Milk Formula	Alpha Linolenat Acid		
	Label Declaration (mg/100 kcal)	Analysis Result (mg/100 kcal)	Requirement (mg/100 kcal)
Infant Formula 1	70,0	89,7	Minimal 50
Infant Formula 2	60,0	95,9	Minimal 50
Infant Formula 3	89,0	113,6	Minimal 50
Infant Formula 4	81,0	80,9	Minimal 50
Infant Formula 5	54,0	64,9	Minimal 50
Advance Formula 1	73,1	84,7	Minimal 50
Advance Formula 2	56,4	53,6	Minimal 50
Advance Formula 3	62,1	66,2	Minimal 50
Growth Formula 1	64,3	92,0	Minimal 50
Growth Formula 2	52,2	50,9	Minimal 50
Growth Formula 3	65,6	122,3	Minimal 50

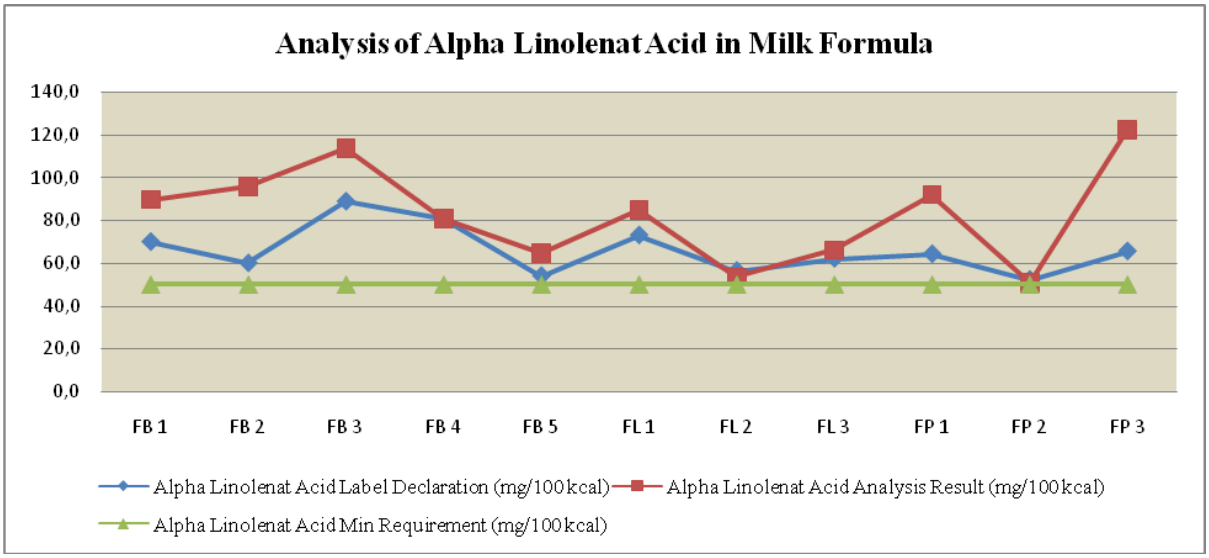


Figure 4. Analysis of alpha-linolenic acid in milk formula.

LA levels found in formula milk samples ranged from 478.152 – 956.606 mg/100 kcal. All samples meet the LA content requirements based on The Regulation of Indonesian FDA, Number 1 of 2018. However, if we evaluate the claims of the packaging label, there are four samples of formula milk containing LA lower than the levels claimed on the packaging label. Data results of the analysis of LA formula milk samples can be seen in Table 4 and Figure 5.

Tabel 4. Analysis of linoleic acid in milk formula.

Sample Milk Formula	Linoleat Acid		
	Label Declaration (mg/100 kcal)	Analysis Result (mg/100 kcal)	Min Requirement (mg/100 kcal)
Infant Formula 1	665,0	847,9	300 - 1400
Infant Formula 2	661,0	884,2	300 - 1400
Infant Formula 3	642,0	680,4	300 - 1400
Infant Formula 4	610,0	513,4	300 - 1400
Infant Formula 5	667,0	758,0	300 - 1400
Advance Formula 1	716,3	744,2	300 - 1200
Advance Formula 2	652,9	590,7	300 - 1200
Advance Formula 3	642,9	604,8	300 - 1200
Growth Formula 1	435,7	478,1	300 - 1200
Growth Formula 2	650,0	578,8	300 - 1200
Growth Formula 3	656,3	956,6	300 - 1200

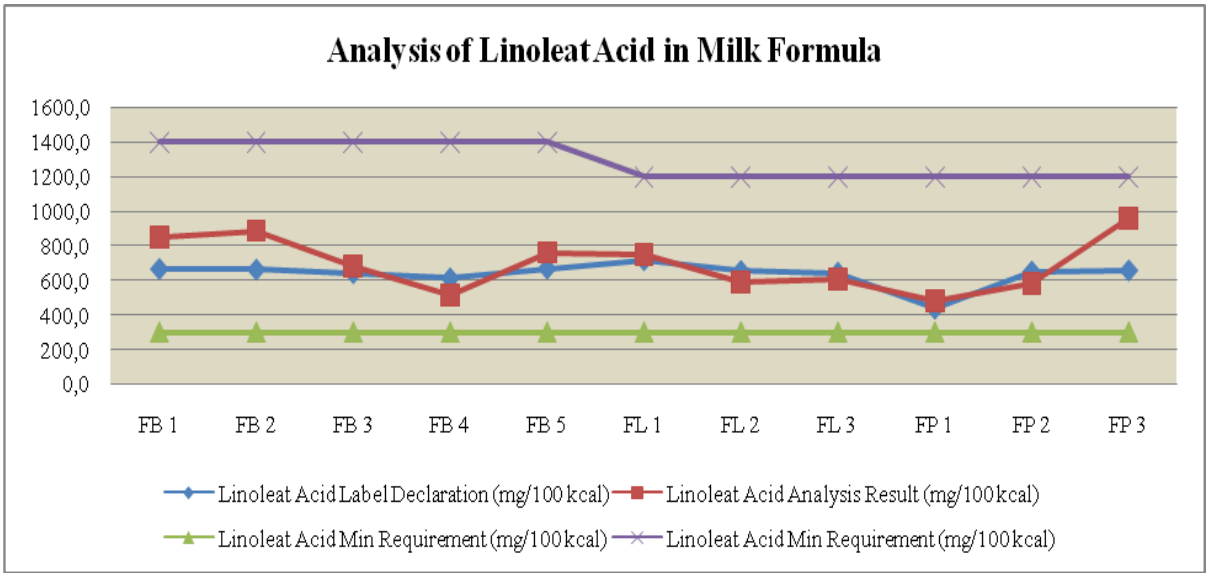


Figure 5. Analysis of linoleic acid in milk formula.

The ratio of ALA to LA ranged from 1: 5.197 to 1: 11.688. All samples meet the requirements of the ratio of ALA to LA-based on The Regulation of Indonesian FDA, Number 1 of 2018. However, if we evaluate the claim of the packaging label, there are four samples of formula milk whose ratio of ALA to LA is lower than at the levels claimed on the packaging label. Data from the calculation of the ratio of linoleic acid to alpha-linolenic acid can be seen in Table 5.

Tabel 5. The ratio of linoleic acid: alpha-linolenic acid in milk formula.

Sample Milk Formula	Ratio Linoleat Acid : Alpha Linolenat Acid		
	Label Declaration	Analysis Result	Requirement
Infant Formula 1	9,5 : 1	12,1 : 1	15 : 1
Infant Formula 2	11,0 : 1	14,7 : 1	15 : 1
Infant Formula 3	7,2 : 1	7,6 : 1	15 : 1
Infant Formula 4	7,5 : 1	6,3 : 1	15 : 1
Infant Formula 5	12,4 : 1	14,0 : 1	15 : 1
Advance Formula 1	9,8 : 1	10,2 : 1	15 : 1
Advance Formula 2	11,6 : 1	10,5 : 1	15 : 1
Advance Formula 3	10,3 : 1	9,7 : 1	15 : 1
Growth Formula 1	6,8 : 1	7,4 : 1	15 : 1
Growth Formula 2	12,5 : 1	11,1 : 1	15 : 1
Growth Formula 3	10 : 1	14,6 : 1	15 : 1

CONCLUSIONS

The development analysis methods in determining ALA and LA in formula milk by adopted AOCS Official Methods Ce 2b-11 modified using gas chromatography with capillary columns DB *Fast FAME* has been successfully validated and meets the validation requirements. Determination ALA and LA results showed that the samples contained ALA

and LA by applicable requirements. Still, by evaluating the label declaration, three samples contain ALA and four samples containing LA lower than label declaration.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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