DETERMINATION OF TOTAL FAT AND FATTY ACIDS COMPOSITION IN SELECTED INFANT FORMULA AND FOLLOW UP FORMULA AVAILABLE IN MALAYSIA

by

NUR ATIQAH BINTI NASARUDIN

Thesis submitted in partial fulfillment of the requirement for the degree of

Bachelor of Health Sciences (Nutrition)

JUNE 2016

Acknowledgment

I would like to express my deepest gratitude to my project supervisor, Dr Marina bt. Abdul Manaf for her kind guidance and patient in sharing her knowledge with me. Dr had assist and helped to ease many problems that I encountered during conducted this project and encouraged me to maintained a continuous enthusiasm for this project. Besides, I would like to thanks Prof. Wan Manan bin Wan Muda as course coordinator for his selfless help and valuable support in aiding me to make this project a success.

I would also like to give thanks to the staff at the Makmal Pemakanan namely Cik Fadzlina, Kak Azila and Kak Mira for their handy assistance and guidance during my practical and technical work in the lab. Besides, I would like to thanks my senior and friend, Kak Ain , Anisah, Faezah and Syikin for their valuable advice and friendly help during my laboratory works.

Special thanks go to Unit Pengurusan Makmal Sains (UPMS), School of Health Sciences, USM for their help and supply of material throughout my project study. I also want to thanks En Rosliza b. Haron, for his generous help in the basic laboratory instrument usage.

Lastly, I would like to express my greatest thanks to my parent for their continuous encouragement and support to me in pursuing my studies in Nutrition course here. This dissertation is dedicated to all of you.

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Abstrak

Rumusan bayi dan rumusan susulan adalah salah satu sumber pemakanan utama untuk bayi dan kanak-kanak. Dalam kajian ini, jumlah komposisi lemak dan asid lemak dalam rumusan bayi (n = 6) dan rumusan susulan (n = 6) telah dianalisis. Lemak telah diekstrak menggunakan kaedah Folch et al (1957), dan komposisi asid lemak (kumpulan dan individu) telah dianalisis menggunakan gas kromatografi. Komposisi utama lemak yang dapat dikesan dalam rumusan bayi adalah, asid lemak tepu (SFA) (1.442 ~ 20.715%), asid lemak monotaktepu (MUFA) (1.646 ~ 10.584%) dan asid lemak poli tak tepu (PUFA) (60.345 ~ 73.065%). Bagi rumusan susulan, purata jumlah kandungan SFA adalah 2.319 ~ 26.321%, manakala kandungan MUFA dan PUFA masing-masing adalah daripada 0.712 ~ 17.774% dan 44.258 ~ 91.406%. Di samping itu, kadar DHA dalam rumusan bayi dan rumusan susulan adalah masing –masing dari pada 3.487 ~ 6.084 dan 0.516 ~ 15.373%. Sebanyak 1.624 ~ 4.456% asid linoleik / asid linolenik dikesan dalam produk yang dianalisis (rumusan bayi dan rumusan susulan). Hanya sedikit asid lemak trans (2.626 ~ 10.144%) telah dikesan dalam sampel rumusan bayi dan rumusan susulan.

Abstract

Infant formula and follow up formula is one of the main nutritional sources for infants and children. In this study, the total fat and fatty acid compositions of infant formula (n=6) and follow up formula (n=6) were analyzed. Fats were extracted using the Folch et al (1957) method, and fatty acid compositions (group and individual) were analyzed by gas chromatography. In infant formulas, saturated fatty acid (SFA) (1.442~20.715%), monounsaturated fatty acid (MUFA) (1.646~10.584%) and polyunsaturated fatty acid (PUFA) (60.345~73.065%) were the major group of fatty acids detected. Follow up formula products contained 2.319~26.321% of total SFA content, whereas the content of MUFA and PUFA ranged from 0.712~17.774% and 44.258~91.406% respectively. In addition, the range of DHA in infant and follow up formula were 3.487~6.084% and 0.516~15.373% respectively. About 1.624~ 4.456% of linoleic acid/linolenic acid was detected in the analyzed products (infant and follow up formula). A small amount of trans fatty acids (2.626~10.144%) were also detected in both infant anf follow up formula.

Chapter 1: Introduction

1.1 Background of study

Infant formula has been consumed during the critical growth period and the only source of nutrition for non-breastfed infant (Pehrsson, Patterson, & Khan, 2014). Infant formula can be the main source of energy and nutrition requirement for infant at first 12 months of life (Qawasmi, Landeros-Weisenberger, Leckman, & Bloch, 2012). The uses of infant formula definitely can be advantageous for the proper growth and development of children but they are rated as second best to natural breast milk (Olu-Owolabi, Fakayode, Adebowale, & Onianwa, 2007). As for growing up/follow up formula, it is design for toddler with ages range from 1 to 3 years old. Basically nutrition requirement between infant and toddler is a bit difference because infant only needs breast milk or formula to meet all their nutritional need while toddlers need additional calories from fat to ensure proper growth and development (Stettler, Bhatia, Parish, & Stallings, 2011).

According to Céline Yockney and Venise Comfort (2013), the usage of formula is different according to ages. For example, infants under 6 month old use infant formula either as an additional to breast milk or as full replacement for breast milk. The same concept is use for infant ages from 6 to 12 months old but most of the parent will provide the formula product as a component of foods as they start to introduce solid foods into their child's diet. As for toddler ages from 12 to 24 months old, the follow up formula is used as supplementary nutrition. As the child get older, their eating habit become wide and need sufficient volume of solid foods, therefore follow up formula tend to be used as a beverage and nutrition source (Céline Yockney and Venise Comfort, 2013).

All formula for infant and toddler must be safe and suitable to meet requirement and to promote growth and development of infant because it is basically used as main source of nutrition and as the liquid element in progressively diversified diet after introduction of complementary feeding (Panel, 2014). Nutrient and substance added in the formula should only in amount that can be serve as nutritional and can provided benefit to the infant. Any additional amount higher than those serving for benefit may become a burden to the infant's metabolism or other physiological function (Panel, 2014).

Therefore, minimum and maximum content of nutrient and other substance in infant formula and follow-up formula should be based on generally accepted scientific evidence which establish the nutrient requirement of all infant and toddler. Panel (2014) stated that the intake level of micronutrient is considered adequate for the majority of infant in the first six months of life was derived from mean nutrition intake from breast milk. The expected compositional requirement for follow up formula was less stronger than infant formula because it is not use as main source of energy and the other food can contribute to nutrient and energy intake in various amount to the toddler. U.S. Food and Drug Administration (FDA) are responsible to evaluate the content of composition in infant formula (Satchithanandam, Fritsche, & Rader, 2002).

The composition of fat in infant and follow up formula is influence by the need of energy and essential fatty acid for growth. Fat can help in absorption of fat soluble vitamin (Panel, 2014). About 50-55% of the total calories are provided by lipid and it is also the main energy source in infant formula (Satchithanandam et al., 2002). Nowadays, the amount of added essential fatty acid which is linoleic acid in infant formula seems to be adequate for healthy growth of infant. In recent finding, they stated that the long chain polyunsaturated fatty acid (LCPUFA) particularly, docosahexaenoic acid (DHA) and arachidonic acid (AA) are also playing a crucial part in growth and development of infant (Satchithanandam, Fritsche, & Rader, 2001). Recommendation for optimal levels of DHA and AA supplementation in infant formula has been published by international expert group. Based on world wide range of DHA and AA concentration in breast milk, general recommendation for DHA and AA in the infant formula should be between 0.2% to 0.4% and 0.35% to 0.7% respectively (Abad-Jorge, 2008).

There are numerous of studies which demonstrate the impact of DHA and AA intake from formula that been supplemented with DHA and AA (Abad-Jorge, 2008). According to the research, infant taking unsupplemented formula only had approximately 4% of DHA in their red blood cell, whereas infant taking formula with higher level of DHA had the higher level of red blood cell DHA (10% to 12%). Other than that, DHA and AA also have impact on visual acuity and cognitive development outcome (Abad-Jorge, 2008). Panel (2014) state that, the reason for adding DHA in infant formula is (1) DHA is essential structural component of the nervous tissue and retina which involve in normal brain and visual development, (2) in the first two years of live, brain has to accumulate large amount of DHA in order to develop, (3) even though DHA can be synthesized in the body by ALA , the intake of DHA from diet generally can increase red blood cell DHA as same as intake from breast milk.

There is no evidence stating that one company's milk is better that other company therefore, most of the parent does not need to focus or stick on one brand. Many commercialized formula available in Malaysia is have different level of protein, lipid and micronutrient which may affect tolerance of the child (UNICEF, 2010). Therefore it is significant to determine the level of fatty acid including saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and *trans* fatty acid present in difference brand of infant formula and follow up formula (OVEYSI et al., 2006).

1.2 Rationale

In Europe, the composition of commercial infant formula is regulated by the EU Regulation 609/2013 under Recommendation of an ESPGHAN Coordinated International Expert Group (Koletzko et al., 2005). The composition of human milk always has been view as a main reference in the development of infant formula as it is been used as a substitute of breast milk. However, the great variability in the composition of nutrient in human milk and the significant difference in the bioavailability and metabolic effect of same nutrient in human milk and milk formula, had led the ESPGHAN to declare that "infant formula should only contain component in amount that can serve as nutritional purpose or can provide another benefit". This is because other unnecessary nutrient or excessive amount of some nutrient can also give impact in physiological function of the new born (Zunin, Boggia, Turrini, & Leardi, 2015).

As far as lipid fraction is concerned, ESPGHAN also had recommended that the fat content in infant formula must be between 4.4 and 6.0g/kcal, together with a α-linoleic acid content between 0.3 and 1.2 g/kcal (Zunin et al., 2015). Linolenic acid is important for the synthesis of docosahexaenoic acid but due to the limited information regarding the efficiency of these metabolic pathways, ESPGHAN had recommend the linoleic acid can only be in minimum content (50mg/100kcal). As for the content of lauric acid and myristic acid, EU regulation had accepted higher level which is 20% of the total fat and for unsaturated trans fatty acid and erucic acid both of them must be <3 % and < 1% of total fat respectively due to their negative effect on baby's health (Zunin et al., 2015). Therefore, all infant formula and follow up formula must not only be adherence with the label but also need to meet recommendation level (Pehrsson et al., 2014).

Many questions had arisen about the intake of long chain polyunsaturated fatty acid (LCPUFA) in healthy term infant and these issues have received a lot of attention and become focus of much research over the past decade (Uauy, Hoffman, Mena, Llanos, & Birch, 2003). Essential LCPUFA which is docosahexaenoic (DHA) and arachidonic acid (AA) are important in early central nervous system development. According to Jacobson et al. (2008), the only n-3 fatty acid found in significant amount in brain is DHA while AA is most crucial component of n-6 fatty acid. Both DHA and AA have high concentration in the gray matter of cerebral cortex specifically on the membrane of neuronal synapses (Ghys, Bakker, Hornstra, & Van den Hout, 2002). DHA also present in high concentration at outer segment membrane of the retina which make it critically important for vision and learning whereas AA is a key for normal growth as it is a precursor of the series 2 eicosanoid that make a labor (Innis, 2003).

Supplemented formulas with both DHA and AA have been shown to be safe for term and preterm infant (Caplan, 2007). But it is well advised that the amount of DHA in breast milk had to be a guideline to estimate the amount of DHA that should be added in infant formula for 2 to 6 months old infant (Panel, 2014). Only small amount of DHA needed to achieve optimal growth and development of infant and children. It is approximately 20 to 50 mg per day of DHA for 0 to 6 month olds. This amount is estimated from DHA content and daily breast milk volume which is 7 to 8 mg/dL and 500 to 750 ml. As for child ages range from 6 to 24 months, 100 mg per day should be adequate.

Most of the formula company nowadays had supplemented the formula with DHA and AA. Nowadays, there are several brands and types of infant formula with different company names. However, there is no such evidence that can ensure that their formula can provide better nutrition for infant (UNICEF, 2010). Many of commercially available formula differs from each other in term of processing method, sources of nutrient and level of protein, lipid and micronutrient . Most of the parents want to have the best source of nutrition for their child so milk's manufacturer had to compete with each other to produce the best formula as closely resemble the composition of breast milk (UNICEF, 2010).

The significant of this study is to provide analytical data on both infant formula and follow up formula available in Malaysia and thus to improve the accuracy of the estimated fatty acid content in this important food. This data can be used by researcher and nutritional public policy makers to get more accurate assessment of the fatty acid level intake of very young children (Pamela et al, 2014). Moreover, this study also come up with the information on how well the label claims follow the actual fatty acid content and whether these milk product comply with recommendation of specific fatty acid concentration (Pamela et al, 2014).

Thus, it is very crucial to identify and compare the fatty acid content in different brands of infant formula and follow up formula with the standard essential composition provided. On the other hand, comparison of fatty acid content also important to determine which formula follows the nutritional requirement of infant and toddler. It is very important to analyze each element in each types/brand of infant formula and follow up formula as the results may vary significantly from each other. Other than that, there is a lack of information or analysis of nutritional composition on infant formula and follow up formula available in Malaysia.

Therefore, this study can help to identify the formula that contain highest level of fatty acid and the best nutritional value among difference brand of infant formula and follow up formula available in Malaysia. This can help parents to choose the best formula suitable for their children in order to have normal growth and development. Other than that, we also can informed the parent and give them a guidance to choose the best infant formula and follow up formula for their children. In this study, fatty acid contents of the different brands of infant and follow up formula will be analyzed.

1.3 Research Objective

General objective

1. To determine the fatty acid profile in infant formula and follow up formula.

Specific objective

1. To compare the fatty acid profile in selected brands of infant formula available in Malaysia.
2. To compare the fatty acid profile in selected brands of follow up formula available in Malaysia.

1.4 Hypothesis

Null hypothesis 1

There is no significant difference in fatty acid content among different brands of infant formula available in Malaysia

Hypothesis 1

There is significant difference in fatty acid contents among different brands of infant formula available in Malaysia

Null hypothesis 2

There is no significant difference in fatty acid contents among different brands of follow up formula available in Malaysia

Hypothesis 2

There is significant difference in fatty acid contents among different brands of follow up formula available in Malaysia

Chapter 2: Literature Review

2.1 Infant formula and follow up formula

Infant formula is use for infant under 6 month ages as either an additional to breast milk or as full replacement (primary) of breast milk (Céline Yockney and Venise Comfort, 2013). During 6 to 12 month old, parent will either provided infant formula or follow up formula as supplementary source of energy because at these ages infant had started weaning. As for children ages 12 to 24 months old, follow up formula or toddler milk can act as supplementary nutrition and as beverage. This is because as the child goes older, their food intake become wider and they can get sufficient volume of solid food. Children ages range from 24 to 36 months old had similar concept with child in 12 to 24 months old but the volume and frequency had decrease (Céline Yockney and Venise Comfort, 2013).

The main difference between infant formula and follow up formula is nutritional composition which been optimizing by manufacturer based on ages and stage. Infant formula contain full range of nutrient require by babies up to 12 months old which means it can be used as complete source of nutrient for infant (Céline Yockney and Venise Comfort, 2013). As for follow up formula and toddler milk, it contain age formulated nutrient that suit infant ages between 6 to 12 months and it also provide some specific nutrient such as additional iron and protein to help growth and development of children. Parent nowadays had understood that difference stage of ages need difference nutritional content to meet the varying of dietary need for children to grow. Therefore, they are more concern to ensure that their child consuming the correct nutrient need for their ages (Céline Yockney and Venise Comfort, 2013).

Infant formula and follow up formula also can be differentiating by types and brand. There are many types of formula available such as soy-based, cow-based or goat-based. Manufacturer also identifies a range of type including types for allergies or intolerance and reflux (Céline Yockney and Venise Comfort, 2013). Different brand available can provide parent a choice to choose which one of the formula that suits their children requirement. In Malaysia, the formula brands available are Nurture, S26, NAN, Neocate, Mamia, Novalac, Dutch Lady, DUMEX, Nestie and Mead Johnson.

Age	Purpose	Usage option
0to 6 months	Primary	Exclusive formula feeding
		Mixed milk feeding or addition to breast milk.
6 to 12 months	Primary	Only milk based beverage consumed in additional to small amount of complementary foods.
		Mixed milk feeding (include breast milk) in additional to small amount of complementary food
	Supplementary (may or may not include breast milk)	As a component of food
		As a comforter (in the evening)
		As a snack (between meals)
12 months +	Supplementary	As a milk based beverage
		As a component of food
		As a comforter (in the evening)
		As a snack (between meals)

Table 2.1: Usage of Infant Formula and Follow up Formula by Age

Note. Form "Understanding Caregivers' Perception and Use of Follow Up Formula and Toddler Milks in New Zealand and Australia", by Céline Yockney and Venise Comfort. (2013). New Zealand.

2.2 Standard fatty acid content in infant formula and follow up formula

Triacylglycerol, phosphatidycholine, and cholesterol can be classified as dietary fat or lipid. Together with protein, carbohydrate, and alcohol, fat are the main source of energy for human body (Authority, 2010). Fatty acid also involves in structural component of cell membrane, precursor for bioactive molecules, regulator of enzyme activities and regulation of gene expression. Classification of fatty acid is based on their number of double bond. Saturated fatty acid had no double bond while monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) have one and two or more double bond respectively. These double bond have two types which either cis or trans configuration. Unsaturated fatty acid in diet mostly have cis configuration but trans configuration can also present as either trans MUFA or trans PUFA. Cholesterol also types of fat that play important role in many metabolic process but it does not provide energy (Authority, 2010).

Dietary recommendation for total fat intake is separated for saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid and Trans fatty acid (Authority, 2010). As for polyunsaturated fatty acid, it frequently divided into n-6 polyunsaturated fatty acid, n-3 polyunsaturated fatty acid and n-3 long chain polyunsaturated fatty acid. In breast fed infant, fat intake is high during breastfeeding period but it can gradually decrease in after 6 months old as the infant started to have complementary feeding (Authority, 2010).

Sources of fat in infant and follow up formula are generally from cow's milk, goat's milk and different types of vegetable oils. Cow's milk usually has palmitic acid and medium chain fatty acid (MCFA). Due to hydrogenation of PUFA by rumen bacteria, cow's milk has relatively high in trans fatty acid and low in PUFAs content (Authority, 2010). As for goat' milk, the fat content is similar to other ruminant species which percentage of unsaturated fatty acid does not have much different with cow's milk. Many difference sources of vegetable oil can be used in production of infant formula which most of them have high content of PUFAs and lower content of SFA. Long chain polyunsaturated fatty acid (LCPUFA) is not present in cow's milk or vegetable oil; therefore other sources that can supply LCPUFA are fish oil, DHA rich algal oil and egg phosphatidylcholone from egg yolk (Authority, 2010).

The use of myristic and lauric acid in infant and follow up formula had become major concern due to their cholesterol increasing effect in adults (Authority, 2010). However, most dominant saturated fatty acid (SFA) in breast milk which is palmitic acid also can increase the cholesterol level. Moreover, composition of plasma cholesterol is higher in breast milk than in formula fed infant, therefore it is no support evidence that can prove this fatty acid has any long term adverse health effects (Authority, 2010).

Compulsory composition	Infant formula				Follow up formula			
	g/100kcal		FA%		g/100kcal		FA%	
	Min	max	min	max	min	max	min	max
Total fat	4.40	6.00			4.00	6.00		
Trans- fatty acid				3.0				3.0

Lauric acid + myristic acid				20.0				20.0
Erucic acid				1.0				1.0
LA (18:2, n-6)	0.30	1.20			0.30	1.20		
ALA (18:3, n-3)	0.05	0.24			0.05	0.24		
Voluntary addition								
Total n-3 LCPUFAs				1.0				1.0
Total n-6 LCPUFAs				2.0				2.0
ARA (20:4, n-6)				1.0				1.0
DHA (22:6, n-3)	Shall not exceed total n-6 LCPUFA				Shall not exceed total n-6 LCPUFA			
EPA (20:5, n-3)	Shall not exceed DHA				Shall not exceed DHA			
Phospholipids	2g/L				2g/L			

Table 2.2: Current composition requirement of infant formula and follow up formula with respect to total fat, fatty acid and PLs

Note: From Europe Food Safety Authority (EFSA) NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the Essential Composition Of Infant And Follow-On Formulae. EFSA Journal.2014, pg 3

2.3 Role of fatty acid in infant and children nutrition

In the past decade, the curiosity about lipid nutrition has focused on the benefit of essential lipids in central nervous system development, and the role of specific fatty acid and cholesterol in lipoprotein metabolism (Joint, 2009). Lipids are structural component of all tissue and essential in manufacturing of membrane of cell and cell organelles. Long chain polyunsaturated fatty acid (LCPUFA) is particularly abundant in brain, retina and other neural tissue. Other than that, dietary lipid can affect cholesterol metabolism at an early age and this will later associated with cardiovascular mobility and mortality in life (Joint, 2009).

Fat and fatty acid has impact on the development of nutritional related chronic disease (NRCD) throughout the lifespan. According to Uauy, Mize, and Castillo-Duran (2000), fat particularly EFA and LCPUFA, also has effect on neural development and function. There are strong evidence indicate that specific fatty acid will maintain their effect by modifying the physical properties of membrane, including membrane-related transport systems, ion channel, enzymatic activity, receptor function and various signal transduction pathways.

The quality of dietary lipid provided in early life has received much attention because lipid is a major determinant of growth, infant development and long term health. Fat has capability to exhibit slow gastric emptying and intestinal motility which can prolong satiety. This is particularly important for infant and children due to their small stomach size. Therefore, the selection of fat and fatty acid sources during the first years of life is considered to be critically important (Uauy et al., 2000).

Most of infant formula nowadays used fat based on a mixture of vegetable oils and it became much less complex composition compared to human milk fat (Delplanque, Gibson, Koletzko, Lapillonne, & Strandvik, 2015). Sources of fat in infant formula are varying according to the brand and manufacturer. Most common sources of fat used is included single cell oil, fractioned lipid, repeated esterified structure lipid, egg phospholipid and fish oil. Hence, fatty acid composition in each infant formula is varies based on the lipid sources used by manufacturer. This is because difference sources of lipid has difference specific feature. As example, palm oil has high content of palmitic acid but there is no content of short or medium chain fatty acid and this fatty acid highly present in coconut oil. Therefore, uses of difference lipid source can reflex the content and proportions of short and medium chain fatty acid such as lauric acid, myristic acid, palmitic acid and oleic acid (Delplanque et al., 2015).

2.3.1. Saturated fat

Function of saturated fatty acid is not only in providing energy but it also has structural and metabolic function. It can be synthesized in human either from nonfat sources or by β-oxidation from unsaturated fatty acid (Brenna, 2002). Saturated fatty acid can be range according to the size which is from 6 to 24 carbons, but the most common saturated fatty acid used in infant diet is 12, 14, 16, and 18 carbon chain length.

Medium chain length fatty acid (MCFA) range from C6-C10 can be highly absorbed into the hepatic portal vein and been transport to the liver in order to be oxidized for energy (Koletzko, Poindexter, & Uauy, 2014). Additional of MCFA in preterm infant diet may be useful because it has potential to facilitate the fat absorption but there is no evidence on benefit of MCFA in energy balance and growth of the infant (Lapillonne, Groh-Wargo, Gonzalez, & Uauy, 2013). However, additional of dietary medium chain triglycerides had shown to have benefit in children with severe fat malabsorption such as intestine failure due to the short bowel syndrome or severe cholestatic liver disease (Goulet et al., 2013).

Palmitic acid in palm oil based formula is mainly located at the sn-1 or sn-3 position and it has potential to impair absorption of calcium and fat that can cause in insoluble calcium soap which has negatively influence in early bone accretion (de Queiroz Leite et al., 2013). The sn-2 position of palmitate can be duplicated in infant formula by adding repeat esterified β-palmitate and these will cause stool consistency and the absorption of palmitic acid and calcium similar to those seen in breast fed infant (Carnielli et al., 1995). Palmitate level can reach 16% to 20% in formula with dairy fat based but the physiological and health consequence of these composition are still unknown and would require further investigation (Delplanque et al., 2015).

2.3.2. Monounsaturated fatty acid

Second most common fatty acid in both breast milk and infant formula is monounsaturated fatty acid. The main monounsaturated fatty acid (MUFA) are oleic acid (C18:1n-9) and palmitoleic acid (C16:1n-7). Although both of this fatty acid is largely present, their potential functionalities have not been explored in infant and the nutritional relevance is still unknown. Moreover, possible impact of monounsaturated fatty acid on the immune system and other functional outcomes in infant is still questionable and need to be explored further (Delplanque et al., 2015).

Even though the level of monounsaturated fatty acid in both breast milk and infant formula is low, the role of 24-carbon nervonic acid (C24:1n-9) in the body is important for myelination and may play a role in brain growth and development (Delplanque et al., 2015). Nervonic acid is the main long chain fatty acid in sphingomyelin and will dramatically accretion around the time of delivery (Martínez & Mougan, 1998). Unfortunately, nervonic acid also be endogenously synthesized in newborn and the role of the dietary supply of this fatty acid are remain speculative (Delplanque et al., 2015).

2.3.3. Polyunsaturated fatty acid

<u>**Linoleic acid (LA) and α- linolenic acid (ALA)**</u>

Both linoleic acid and α- linolenic acid are essential fatty acid that play an important role in metabolic process such as lowering plasma cholesterol. The ratio difference of LA and ALA is a key for the endogenous synthesis of the respective LC-PUFA. This is because these 2 precursor fatty acid will compete each other to denaturizes and elongases in PUFA conversion pathway (Figure 1) (Jensen et al., 1997). The current guideline regarding the level of LA and ALA in infant formulae is aim to avoid an extremely high LA: ALA ratio because it may reduce ALA conversion to n-3 LCPUFA (Koletzko et al., 2012). According to Innis (2007), extraordinary high level of LA in formulae may cause unexpected effect as some of their oxygenated metabolites have proinflammatory function.

2.4 Benefit of infant formula and follow up formula supplemented with DHA and AA

The question about benefit of dietary supply of docosahexaenoic acid (DHA) and arachidonic acid (AA) had been debated for many years (Hoffman, Boettcher, & Diersen-Schade, 2009). DHA and AA are naturally present in breast milk and these fatty acids are needed for rapid synthesis of cell membrane specifically neural cell. Therefore, it is important to provide sufficient supply of DHA and AA during infancy as both of this fatty acid is rapidly continues to accumulate in brain gray matter during first 2 years of life. As infant is rapidly growing, there is a high demand for these complex lipids to form vital cell membrane structure (Hoffman et al., 2009).

Formula fed infant who consume formula without supplementation of DHA had to rely on endogenous synthesis from essential fatty acid precursor to meet their long chain polyunsaturated fatty acid (LCPUFA) requirement (Caplan, 2007). However endogenous synthesis is limited and varies widely among individual and can approaching zero for some infant (Hoffman et al., 2009). Several studies had demonstrated that concentration of DHA in infant blood is flexible to the amount of DHA supply in diet. Other than that, DHA also have impact on visual development. According to Birch et al. (2007), infant formula supplemented with DHA and AA can support visual acuity and IQ maturation similar to that breast fed infant.

As a result for growing evidence of benefit of DHA and AA supplemented on infant development, several expert group have publish level of recommendation additional of DHA and AA to infant formula (Hoffman et al., 2009). The recommendation must base on level of DHA and AA found in breast milk.

Organization	LCPUFA (% of total fatty acid)	
	DHA	AA
British Nutrition Foundation	~0.4	~0.4
FAO/WHO Expert Panel	~0.35	~0.7
Expert Panel Convened By ISSFAL	~0.35	~0.5
Child Health Foundation, Germany	≥0.2	≥0.35
American Dietetic Association (ADA) And Dietitians of Canada	≥0.2	≥0.2
World Assoc. of Perinatal Med./Early Nutrition Academic/ Child Health Foundation	0.2-0.5	≥ 0.2

Table 2.3: Expert Positions for DHA and AA Level in Term Infant Formula

Note: From Toward optimizing vision and cognition in term infants by dietary docosahexaenoic and arachidonic acid supplementation: a review of randomized controlled trials. By Hoffman, et al., (2009). *Prostaglandins, Leukotrienes and Essential Fatty Acids, 81*(2), p.156

2.4.1 Impact on blood DHA level and neurodevelopmental outcome

Many studies reveal a dose response relationship between infant dietary intake of DHA (either from breast milk or formula) and level of DHA found in the red blood cell. Infant fed with unsupplemented formula had only 4% of DHA in their red blood cell (Auestad et al., 2001). On the other hand, infant fed with breast milk and formula with higher DHA level (0.29% and 0.36%) had shown the highest level of DHA in red blood cell. Therefore, level of DHA in red blood cell is directly proportional to the level of DHA in breast milk or formula.

The potential effect of DHA on neurodevelopment of infant and children had been studies. Assessment of neurodevelopment of children is a bit complex because of the wide range of developmental domain therefore difference assessment had existed within each domain and has wide variation in performance measure and psychometric characteristic (Panel, 2014). The effect of additional DHA and AA in infant formula have been investigated using performance on the mental development index (MDI) and psychomotor development index (PDI) of Bayley Scales of Infant Development (BSID) on term infant at difference age. The result from these studies stated that there are no significant differences between intervention group which consume infant formula with DHA and AA and control group in MDI score for infant at three months ages, six months and at one year of ages. However, one study report demonstrates that at 18 month of ages there are significant difference in MDI score (Panel, 2014).

2.4.2 Impact on Visual Acuity and cognitive outcome

High concentration of DHA in photo receptor cell membrane of the retina had become a key to the role of DHA in visual development. Since both retina and brain tissue derived from neuroectoderm, measuring functional outcomes in the visual system will provide a readily accessible index to neurodevelopmental milestones. Most of the studies investigate infant visual acuity by using visual evoked potential (VEP) which are electrophysiological responses generated by brain in response to specific visual stimulation (Hoffman et al., 2009).

Various levels of DHA and AA in formula had appeared to give impact on visual acuity during first two year of life. Birch et al. (2007) had investigated visual acuity in term infant during a four months feeding study and he stated that infant fed with supplemented formula has similar visual acuity with breast fed infant. Moreover, at the ages of 18 months the VEP value was significantly higher in supplemented formula group as compare with unsupplemented group.

The benefit of DHA and AA in cognitive development also has been proven in numerous of study during infancy and preschooler (Caplan, 2007). The gold standard for assessing infant mental and psychomotor development is by using Bayley Scales of Infant Development. This scale can generate (1) Mental development Index (MDI) which can measure perception, cognitive, language and social and sensorimotor skill while (2) Psychomotor development index (PDI) can measure gross and fine motor skill.

According to the study, term infant who fed with DHA and AA supplemented formula have significantly higher in MDI score as well as PDI score at the ages of 18 months compare to unsupplemented group. As for children ages 4 years old, DHA supplementation at appropriate level can result in improvement of developmental score (Caplan, 2007).

Chapter 3: Methodology

3.1 Reagents and Standard

The entire reagent used for sample preparation was analytical-reagent grade. Standard grades of lauric, palmitic, stearic and linoleic acid were used from stock available in analytical laboratory of School of Health Science, Universiti Sains Malaysia. The 37- component mixture was available as a 100-mg neat mixture, containing C4-C24 FAMEs (2%-4% relative concentration). Standard solution was diluted in 10 ml hexane before used. Final concentration of each FAMEs was 0.2-0.4 mg/mL. Chloroform, methanol, hexane, and sodium methoxide, were obtained from Unit Pengurusan Makmal Sains (UPMS). The apparatus used in this study were supplied by nutrition lab and UPMS. All chemical reagents and laboratory equipment, used in this research project are listed in table 3.1 to 3.2.

Table 3.1: List of chemical and reagent

Chemical reagent
Methanol
Chloroform
Sodium chloride
n-hexane
Sodium methoxide
Saturated sodium chloride
Sodium sulphate

Table 3.2: List of laboratory equipment used

Equipment
Gas chromatography FID (GC Agilent 7890 A)
Centrifuge (Universal 32R)
Rotatory extraction

3.2 Samples

Different brands of infant formula and follow up formula were randomly selected and purchased from supermarket and pharmacy available in Kubang Kerian, Kelantan. The samples were labeled as sample 1, 2, 3, 4, 5, and 6 therefore there were 12 samples because 6 sample of difference brand of infant formula and 6 sample of difference brand of follow up formula. All the formula was labeled as products that suitable for infant and toddler.

Inclusive Criteria:

1) Sample will be purchased in supermarket and pharmacy in Kelantan
2) Selection for follow up formula only for 1-3 years old
3) Only normal/ standard formula been used
4) only original flavor will be used

Exclusive Criteria:

1) follow up formula above 3 year old
2) specific purpose formula
3) formula with vanilla, chocolate or honey flavor

3.3 Fat extraction from the samples

Folch extraction method is chosen because it is a standard method and well established to determine total lipids (Folch, Lees, & Sloane-Stanley, 1957). Folch extraction is gravimetric quantification using 1-step solvent extraction with mixture of water and chloroform:methanol (2:1) followed by washing with 0.9% NaCL. About 3-4 g of sample was weighed and poured into 250ml of conical flask. Then, 40ml of methanol was added and shaken to homogenize the sample. Then the solution was poured in 250ml separating funnel. After that, 80ml of chloroform was added to wash off the conical flask and then poured in the separating funnel. The conical flask was been rinse once again with 40 ml 0.9 % of sodium chloride solution and then added in separating funnel. The separating funnel was closed and been left overnight to allow separating of solution. After been left overnight, 2 layer would be formed which upper layer consist of methanol that dissolve in water and lower layer consist of chloroform and lipid. Chloroform layer was transferred to pre- weighed round bottom flask. The chloroform was evaporated by using rotary evaporator.

Calculation for total fat content:

Weight of conical flask = a

Weight of conical flask with sample =b

Weight of round bottom flask = c

Weight of round bottom flask with lipid extract= d

Weight of sample = (b-a)

Weight of lipid extract = (d-c)

% lipid = (d-c)/(b-a) X 100

3.4 Fatty acid analysis: Using gas chromatography

3.4.1 Preparation of FAMES

Fatty acid methyl esters were prepared according to AOCS (1996) method. Firstly, 0.5 g of oil was dissolved in 25 ml hexane. Then 5 ml of the mixture was transferred into a test tube and mixed with 250µl of 0.5M sodium methoxide in methanol. The mixture was then been shaken vigorously in a vortex mixer for 60 second. After that, 5 milliliter of saturated sodium chloride solution was added and the mixture was once again shaken vigorously for 15 seconds. After 10 minutes, 3 ml of hexane layer containing FAME (the top layer) was transferred into a vial followed by additional of small amount of anhydrous sodium sulfate. Finally, approximately 1 ml of sample was injected manually into the gas chromatography. The percentage of each component was calculated by dividing the individual peak area with total peak area.

3.4.2 GC-FID determination of FAMEs

Fatty acid methyl esters (FAMEs) were analyzes by GC-FID and the model of GC-FID used was GC Agilent 7890 A. The FAMEs profile was determined by spilt injection (10:1) on a fused silica Agilent J&W GC column (30m X 250µm X 0.25 µm film thickness). The flushing solution used was hexane. The initial temperature of 50°C was maintained for 1 minute, raised to 180°C at rate of 7 °C/min, and kept at 180°C for 10 minutes. Later it was increase to final temperature at 230 °C at the rate of 1.8 °C/min and kept for 4 minutes. The injector temperature was 260°C at 0.5 min.