Determination of Docosahexaenoic Acid in Infant Formulas with Gas Chromatography

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ABSTRACT

Objective: Docosahexaenoic acid (DHA) is important for the development of infant's nervous and visual system because it is a major fatty acid in brain and retina phospholipids. However, the benefit of adding DHA in infant formulas is still controversial. The over intake of DHA should be considered because of its side effect. The aim of this study was to get a valid analysis method of DHA using gas chromatography (GC) to determine the concentration of DHA in infant formula.

Method: The milk fat was extracted in chloroform-methanol (1:2), continued with methylated in methanol-toluene (4:1) with acetyl chloride, and finally, injected to GC.

Result: The GC conditions were as follows: Injector temperature was 230°C, detector temperature was 250°C, oven temperature was programmed to increase from 130°C to 230°C by 2°C/min and held for 20 min, helium flow rate was 2.00 ml/min, and split ratio was 1:3. This method had passed the precision and recovery evaluation. The result of DHA determination in five infant formula samples was 27.49±0.62 mg/100 g, 31.14±0.43 mg/100 g, 11.63±0.38 mg/100 g, 19.34±0.58 mg/100 g, and 45.87±0.42 mg/100 g.

Conclusion: The method was valid and successfully applied to determine of DHA in infant formula.

Keywords: Docosahexaenoic acid, Gas chromatography, Infant formula.

INTRODUCTION

Quality of human being determined by the early growth and development. The right intake of nutrients is essential for the optimal development of genetic potency. The nutrients must be given correctly both the quality and the quantity [1,2]. Malnutrition in feeding will affect the quality of human being in the future. It is mainly related to the growth and development of vital organs, especially the brain that mostly grows fast during the last trimester of pregnancy and 1st months of life. This growing brain needs perfect nutrients intake [3].

Docosahexaenoic acid (DHA) is a major fatty acid in brain and retina phospholipids [4]. It has important functional membrane and cellular properties of neural tissue [5]. Human milk contains DHA, whereas infant formula, which made from cow milk, has no DHA. To fulfill the necessity of DHA as essential nutrient, DHA is added to infant formulas [3], but the benefit of DHA in infant formulas is still controversial [1].

Over intake of DHA can inhibit the formation of arachidonic acid and also compete with arachidonic acid for the cyclo-oxygenase, and thus reduce the formation of prostaglandin Histamine 2 and Histamine 3, thromboxane, and leukotriene that can inhibit inflammatory and immune response, which cause longer time of bleeding and decrease renin that has important role in renal regulatory [5].

The analysis method of DHA needed to avoid improper DHA concentration in infant formulas. The analysis method of DHA is not simple. First, the milk fat has to be separated from other components such as carbohydrate and protein. Then, before injected to GC, the fat has to be converted to methyl ester [6,7]. The extraction and esterification steps must be carried out carefully to avoid the losses of DHA, because of these complicated steps, the analysis method of DHA needs to be studied.

METHODS

Equipments
Gas chromatography (GC) (Shimadzu GC-17A), VB-wax capillary column (60 m ×0.32 mm ×0.25 µm) equipped with a flame-ionized detector, helium as carrier gas, Class GC solution data processor, and microsyringe 10 µl (SGE), oven, centrifuge (Kubota 6800 and 5100), analytic, borosilicate glass tubes with feflon-lined screw-caps (100 mm×13 mm), centrifugation tubes, vortex, micropipette (Socorex), and other glass-wares for quantitative analysis were commonly used.

Materials
DHA (Sigma, CAS no. 6217545), DHA oil (Tama Biochemical Co. Ltd. Lot 611151), methanol p.a. (Merck), chloroform p.a. (Merck), toluene p.a. (Merck), hexane p.a. (Merck), acetyl chloride for synthesis (Merck), sodium chloride p.a. (Merck), and potassium carbonate p.a. (Merck) were used.

Determination of GC analysis condition
About 25 mg of DHA standard was precisely weighed, then diluted with hexane until 10.0 ml, 300 µl of this 2500 µg/ml DHA standard solution were placed to teflon-lined caps reaction tube, dried under nitrogen stream, and was submitted to the esterification procedure described below.

1.0 µl of the aliquot of the upper toluene phase was injected into the chromatograph. The experiments to determine the GC analysis were done in isothermal condition and by temperature programmed. Elusion temperature on GC column was 120°C, 130°C, and 140°C and also a variation of flow rate at 1.35, 1.80, and 2.00 ml/min.
RESULTS AND DISCUSSION

GC analysis condition

The obtained GC analysis conditions were as follows: Injector temperature was 230°C, detector temperature was 250°C, oven temperature was programmed to increase from 130°C to 230°C by 2°C/min, and held for 20 min. Helium flow rate was 2.00 ml/min, and split ratio was 1:3. Among the other conditions, it had the highest value of theoretical plates or the lowest value of HETP [11]. The results clearly showed in Table 1 and Fig. 1.

Determination of DHA in DHA oil

From the triple experiments, the average DHA concentration in DHA oil was 22.76%. The measurement of DHA in DHA oil was plotted to the linear regression equation of the calibration curve of DHA standard. The equation of calibration curve of DHA standard was 

\[ y = -3349.6516 + 66.8654x \]

with a value of the coefficient of correlation was 0.9999. The obtained GC analysis condition was used to make calibration curve and calculate the limit of detection (LOD) and limit of quantification (LOQ)

Esterification of fat

The fat extract was put into Teflon-lined caps reaction tube and dissolved in 0.40 ml of toluene and 1.6 ml of methanol, then it was shaken well. 0.2 ml of acetyl chloride was added slowly over 1 min while the tube was shaken slowly. Tube was closed tightly and methanlysis was conducted at 100°C for 1 h. After tube had been cooled in water, 5 ml of 6% K$_2$CO$_3$ solution was added slowly to stop the reaction and neutralize the mixture. The tubes were shaken and centrifuged, and an aliquot of the toluene upper phase was injected into the chromatograph. The correlation was 0.9993. The result clearly is presented in Table 2 and 3.

Linearity test, calibration curve, and calculation of LOD and LOQ

The linear regression equation of calibration curve was 

\[ y = −3349.6516 + 66.8654x \]

with a value of the coefficient of correlation was 0.9999. The result clearly is presented in Table 2 and 3.
was 0.9999. With the obtained value of the coefficient of correlation, the calibration curve of DHA concluded linear. LOD was 74.33 µg/g and LOQ was 247.77 µg/g. These values were still below the smallest concentration in the calibration curve.

**Precision test**

In this research, precision test was conducted at low, medium, and high concentration of calibration curve. The concentrations were 689.95 µg/g, 2051.45 µg/g, and 4066.25 µg/g. The precision of esterification and chromatography analysis of DHA was good with the coefficient of variations of each concentration, respectively, was 1.73%, 1.46%, and 1.85%. Since the coefficient of variations was below 2%, this method concluded having a precise result. The result clearly is shown in Table 4.

**Recovery test**

Recovery test was conducted using absolute method, which calculated as the percentage of recovery of DHA was added to the blank milk. The amount of DHA that added to the blank milk was 0.025% from total weight of milk. This evaluation had a satisfactory result with the percentage of recovery of DHA was 96.40%. The recovery requirement which has concentration below 0.1% is 95.0–105.0%. With this result, it concluded that the analysis method used was accurate enough. The result clearly showed in Table 5.

**Result of DHA determination in some infant formulas**

After determining five different samples of infant formulas, three samples had DHA concentration significantly higher than the concentration written in the packaging label. DHA concentrations in five samples, respectively, were 27.49±0.62 mg/100 g, 31.14±0.43 mg/100 g, 11.83±0.38 mg/100 g, 19.34±0.58 mg/100 g, and 45.87±0.42 mg/100 g.

Sample A had DHA 8.36% less than the concentration written in packaging label, sample B had 55.68% more DHA, sample C had 51.70% more DHA, sample D had 136.44% more DHA, and sample E had 5.44% more DHA than the concentration written in packaging label. The result clearly showed in Table 6.

**CONCLUSION**

The optimum GC analysis conditions were as follows: Injector temperature was 230°C, detector temperature was 250°C, oven temperature was 60°C, and the temperature of injection was 100°C. The precision of esterification and chromatography analysis of DHA was good with the coefficient of variations of each concentration, respectively, was 1.73%, 1.46%, and 1.85%. Since the coefficient of variations was below 2%, this method concluded having a precise result. The result clearly is shown in Table 4.

**Table 2: Result of DHA standard measurements for calibration curve**

| Concentration (µg/g) | Area (µV/s) |
|----------------------|-------------|
| 388.58               | 24495       |
| 776.85               | 50342       |
| 1552.50              | 99519       |
| 2326.94              | 146129      |
| 3100.19              | 203598      |
| 3872.23              | 259360      |

DHA: Docosahexaenoic acid

**Table 3: Result of DHA determination in DHA oil**

| Area (µV/s) | Concentration (µg/ml) | DHA concentration (%) | Average concentration (%) |
|-------------|-----------------------|-----------------------|--------------------------|
| 133947      | 2389.711              | 23.78                 | 22.76                    |
| 133956      | 2389.868              | 23.78                 |                           |
| 133573      | 2383.201              | 23.72                 |                           |

DHA: Docosahexaenoic acid

**Table 4: Result of precision test**

| Concentration (µg/g) | Area (µV/s) | Measured Concentration (µg/g) | Mean measured Concentration (µg/g) | Standard of deviation | Coefficient of variation (%) |
|----------------------|-------------|-------------------------------|-----------------------------------|-----------------------|-----------------------------|
| 689.95               | 49277       | 740.338                       | 721.164                           | 12.4506               | 1.73                        |
| 2051.45              | 47014       | 706.118                       | 1975.261                          | 28.7647               | 1.46                        |
|                      | 48238       | 724.627                       |                                     |                       |                             |
|                      | 47500       | 713.467                       |                                     |                       |                             |
|                      | 47603       | 715.010                       |                                     |                       |                             |
|                      | 48662       | 731.038                       |                                     |                       |                             |
|                      | 130731      | 1972.039                      |                                     |                       |                             |
| 4066.25              | 133947      | 2020.669                      |                                     |                       |                             |
|                      | 133956      | 2020.805                      |                                     |                       |                             |
|                      | 133573      | 1988.597                      |                                     |                       |                             |
|                      | 131826      | 1950.627                      |                                     |                       |                             |
|                      | 129315      | 2015.034                      |                                     |                       |                             |
|                      | 133573      | 4102.496                      |                                     |                       |                             |
|                      | 271621      | 4129.322                      |                                     |                       |                             |
|                      | 273395      | 4129.322                      |                                     |                       |                             |
|                      | 280094      | 4230.620                      |                                     |                       |                             |
|                      | 269217      | 4066.144                      |                                     |                       |                             |
|                      | 276658      | 4178.663                      |                                     |                       |                             |

**Table 5: Result of recovery test**

| Added DHA (µg) | Result of DHA determinations |
|---------------|-----------------------------|
|               | Measured (µg) | Recovery (%) | Mean Recovery (%) |
| 419.694       | 407.928       | 97.20        | 96.27             |
|               | 402.315       | 95.86        |                   |
|               | 401.834       | 95.74        |                   |
|               | 512.903       | 97.77        |                   |
| 524.618       | 499.957       | 95.30        | 96.05             |
|               | 498.857       | 95.09        |                   |
|               | 596.784       | 94.80        |                   |
| 629.542       | 616.623       | 97.95        | 96.88             |
|               | 616.342       | 97.90        |                   |

DHA: Docosahexaenoic acid
temperature was programmed to increase from 130°C to 230°C by 2°C/min and held for 20 min, helium flow rate was 2.00 ml/min, and split ratio was 1:3. The extraction, esterification, and chromatography method of DHA determination used in this research had passed the linearity, precision, and recovery evaluation so that this method can be applied to determine DHA in infant formula. DHA concentrations in five infant formula samples, respectively, were 27.49±0.62 mg/100 g, 31.14±0.43 mg/100 g, 11.83±0.38 mg/100 g, 19.34±0.58 mg/100 g, and 45.87±0.42 mg/100 g.

| Sample | Result of DHA determination |
|--------|-----------------------------|
|        | Concentration (mg/100 g) | Mean concentration (mg/100 g) | Concentration written in the packaging label (mg/100 g) |
| A      | 27.46                     | 27.46                        | 30.0                                      |
|        | 26.89                     |                               |                                           |
|        | 28.13                     |                               |                                           |
|        | 31.46                     |                               |                                           |
| B      | 30.64                     | 31.14                        | 20.0                                      |
|        | 31.31                     |                               |                                           |
|        | 11.96                     |                               |                                           |
| C      | 11.41                     | 11.83                        | 7.8                                       |
|        | 12.14                     |                               |                                           |
|        | 19.98                     |                               |                                           |
| D      | 19.21                     | 19.34                        | 8.2                                       |
|        | 18.83                     |                               |                                           |
|        | 45.69                     |                               |                                           |
| E      | 45.57                     | 45.87                        | 43.5                                      |
|        | 46.34                     |                               |                                           |

DHA: Docosahexaenoic acid

AUTHORS CONTRIBUTIONS
All the authors have contributed equally.

CONFLICT OF INTERESTS
Declared none.

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