Characteristic on infants biscuit for complementary feeding through fortificant paste of natural folic acid as smart food

Agustine Susilowati*, Yati Maryati, and Aspiyanto
Research Centre for Chemistry, Indonesian Institute of Sciences,
452 Building, Kawasan PUSPIPTEK, Serpong - 15314, South Tangerang, BANTEN,
INDONESIA

*Email: agustine_1408@yahoo.co.id

Abstract. Infants biscuit as complementary feeding through mixture of fortificant paste from yellow nixtamalized corn (Zea mays indentata), soy (G. soya) tempeh and fermented spinach (Amaranthus sp.) has potential use as ‘smart food’ for the source of natural folic acid. This experiment aims to find out the best characteristic of biscuit on composition, the dominant folic acid monomer, volatile compounds, particle size and particle size distribution. This experiment was conducted by using 2 types of fortificant paste, in which fortificant pastes of A and B are mixture of yellow nixtamalized corn-soy tempeh – fermented spinach and mixture of yellow nixtamalized corn-soy tempeh – fermented broccoli at concentration of folic acid in fortificant subsequent 0, 200, 400, 600, and 800 µg/145 g of weight of biscuit base formula. The result of experiment activity showed that based on dissolved protein, optimization of biscuit formula by using fortificant pastes of A and B were achieved at concentration of fortificant paste of 800 µg/145 g of base formula, respectively, and yield both biscuits of A and B with compositions of folic acid of 191.152 and 112.48 µg/mL, dissolved protein of 2.400 and 2.291 mg/mL, total solids of 92.74 and 92.77%, total sugars of 276.94 and 318.79 mg/mL, reducing sugars of 50.73 and 50.56 mg/mL, respectively. Identification on folic acid monomer showed that biscuit A and biscuit B are dominated by 2 and 1 of folic acid monomer in which monomer with molecular weight (MW) of 442.65 and 442.34 Dalton (Da.), and 442.26 Da., meanwhile biscuit volatile compounds of A and B were dominated by compounds of vanillin (37.50 and 50.41%), furan (4.27 and 6.91%), and fatty acids, such as hexadecanoic acid (11.378 and 9.700%), octadecenoic acid (2.604 and 13.620%), linoleic acid (18.469 and 0.000%), pentanoic acid (2.976 and 2.820%) and oleic acid (0.00 and 11.98%) with particle size and biscuit particle distribution of 366.6 and 368.8 nm, and particle index of 0.795 and 0.790, respectively.

1. Introduction

A fermentation-based process on soy (G. soya) tempeh by Rhizopus oligosporus strain C1, and both spinach (Amaranthus sp.) and broccoli (Brassica oleracea L.) by Kombucha culture are performed to produce natural folic acid [1]. Nixtamalized corn is prepared by using horse dent corn (Zea mays var. indentata) and adding Ca(OH)2 to increase folic acid concentration as an effort in order to yield fortificant paste of natural folic acid [2]. Folic acid has an essential role as a precursor in synthesizing nucleotide to the remethylation of homocysteine on the period of cell division and cell growth, particularly to produce red blood cells and prevent anaemia [3,4], prevent Neural Tube Defects (NTDs), namely spina bifida and anencephaly on infants. Folic acid is a synthetic form of natural
folate consisting of a pteridine cycle, \textit{para}-aminobenzoate ($p$-ABA) moiety and L-glutamates molecules (C$_{19}$H$_{19}$N$_7$O$_6$, MW 441.4) \cite{5}, as shown in Figure 1.

\textbf{Figure 1.} Chemical structure of folic acid.

Folic acids are sensitive compounds on oxygen, light, and high temperature so that a secured and controlled process method is needed to achieve both fortificant and application product with minimum loss of folic acid. Mixture pasta of natural folic acid from a mixture of soy/mung beans tempeh/fermented spinach & broccoli/nixtamalized corn (1:1:1, w/w/w) is the best ratio in recovery of folic acid as fortificant of folic acid \cite{6,7}, meanwhile, the best formulation condition on supplement for pregnant women is obtained through emulsification 3\% of gelatin with rotation speed of 9500 rpm for 30 minutes \cite{7}. Application of fortificant paste of natural folic acid in the preparation of infants biscuit for complementary feeding has become priority as a functional food for supporting smartness aspect besides other important sources of nutrition for growth and development of infants aged 5 years. Infants biscuit is a product of dry bakery \cite{8} being produced through formulating and mixing processes for the whole material, dough, mould, and roast. Infant biscuit as complementary feeding for children aged more than 1 (one) year is purposed as food to adapt the digestive system of infants besides breastfeeding, to learn bitting so that its formulation is related to this function. The formulation in preparation of infants biscuit must be able to dilute directly like porridge being equal to 20 g per chip of biscuit \cite{8}. Formulation of infants biscuit by adding fortificant paste of natural folic acid is enabled to affect the composition of biscuit. Infants biscuit with particles size or small texture, specific aroma, and presence of folic acid monomer to part of the specific characteristic of infants biscuit and the whole compositions. Biscuit particle with nano-size enabling folic acid and all components are able to be adsorbed by infant digestive system.

Principle of Particle Size Analyzer (PSA) use in analyzing particle size distribution by means of Coulter SZ 100 (Horiba Nano Partica) is laser radiation (\textit{\lambda} 750 nm) through spatial filter and projector lens to form beam collecting and through cell samples, in which dispersed particles in liquid are scattering beam being hit in certain characteristic related to particles size. Particles size distribution is calculated by means of Fraunhofer or Mie method from measuring 126 detectors placed with angle 35° from optic axis \cite{9,10}. Identification of folic acid monomer through Liquid Chromatography coupled with Mass Spectrometry (LC-MS) is conducted to find out the dominant monomer based on specific relative intensity on each monomer indicating the characteristic of biscuit. LC-MS will separate a mixture of molecular according to the difference in migration speed and molecule distribution in stationary phase (adsorbent) and mobile phase (eluent), meanwhile, mass spectrometry (MS) will ionize analyte based on principle electrospray ionisation (ESI) to the gas phase (fine aerosol) \cite{11}. Whereas, the aroma of biscuit caused by its emergence of volatile compounds originating from fortificant and interaction with the raw material of biscuit in the roasting period. Using Gas Chromatography-Mass Spectrometry (GC-MS) will separate different biscuit compounds based on volatile level by flowing mobile phase (gas) and carries stationary phase (biscuit extract) in column and compounds spectrum is further collected out from spectrometer mass column \cite{12}.

The goal of this experiment activity is find out influence on type and concentration of the best fortificant powder in formulating preparation of infants biscuit as complementary feeding for children aged more than 6 months – less than 1 year on the whole components composition, particularly the
best folic acid and its characteristic on folic acid monomer, type of volatile compounds, particle size and particle size distribution of biscuit.

2. Materials and Methods

2.1. Materials and Equipment

Materials used in this experimental work were perforated plastic, distilled water, soybeans, spinach and broccoli purchased from a domestic market, dry yellow corn from kind of horse dent procured from a local corn plantation center (South Tangerang), sucrose (local), inoculum of Rhizopus oligosporus strain C1, Kombucha culture (Research Center for Chemistry – LIPI), wheat flour (local, Bogasari), corn starch (Honig, Netherland), vanilla, salt, powder milk, unsalted butter, baking powder (local). Whereas, chemicals used in these process and analysis were Ca(OH)$_2$ (E.Merck), standard folic acid (Aldrich), hydrochloric acid (E.Merck), sodium nitrite (E.Merck), and methanol. All the chemicals used were of reagent grade procured domestically and used without further purification. Main equipment utilized in this experimental work were balance (Fujitsu, Japan), autoclave (CHENG Y1, LS – 50 L, China), homogenizer (Ultra-Turrax, Ika Labortechnik, T50, Jane & Kunkel, Germany), a series of system of nixtamalization process in laboratory scale, blender (local, National), container, a series of microbiology process equipments, system of laminar flow chamber (local), incubator (local), and sieve of 80 and 100 mesh (Retsch, Germany). Main instruments for analysis were UV-vis Spectrophotometer (Model RF-550, Shimadzu, Japan), Liquid Chromatography-tandem Mass Spectrometry (LC-MS) (Mariner Biospectrometry) with LC (Hitachi L 6200), Particle Size Analyzer (PSA) (Horiba Nano Partica-Backman) and Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu, Japan).

2.2. Experimental Design

The experimental work was conducted by preparing nixtamalized corn, both fermented spinach and broccoli, and soybean tempeh. Further, mixing and homogenizing materials mentioned above was done in order to yield fortificant paste. Fortificant paste A is a combination amongst nixtamalized yellow corn, soybean tempeh and fermented spinach, whereas fortificant paste B is a combination amongst nixtamalized yellow corn, soybean tempeh and fermented broccoli. Formulation of biscuit was prepared by adding fortificant paste being equivalent to folic acids concentration of 0, 100, 200, 400, 600, and 800 µg folic acid/145g of the base formula of biscuit, mixing/doughing, moulding, and roasting in the oven. Analyses were performed at initial material of fortificant and products of biscuit on total solids (Gravimetric method according to AOAC) [13], dissolved protein [14], total sugars (Phenol-Sulphate method), reducing sugars (Somogyi Nelson method) [15] and folic acid (LC-MS) [16]. Identification of folic acid, volatile compounds, and particles size distribution was performed by means of LC-MS [16], GC-MS [12], and Particle Size Analyser, respectively [9]. Experiment data processing was done through descriptive manner with average of triplicate of analysis from a duplicate of process. Procedure

2.2.1. The fermentation process of soybeans (tempeh)

A number of soybeans were sorted, washed, blanched for 30 - 45 minutes, cooled, and steeped at pH 5 overnight. Next day, processed beans were hulled, washed, allowed, inoculated by tempeh inoculum of Rhizopus oligosporus strain-C1 at concentration 0.2 % (w/w) uniformly, packaged in perforated plastic, and incubated at room temperature (28 – 30 °C) for 24 – 36 hours.

2.2.2. The fermentation process of broccoli and spinach

Spinach and broccoli were blanched at 80 °C for 15 and 5 minutes, respectively, and pulverized at the ratio of vegetables and water of 1: 4 to produce vegetable suspension as the substrate in order to get folic acid. The vegetable suspension was then poured by appropriate vegetable inoculum with types of vegetables of 15% (v/w, vegetable suspension) and sucrose 10% (w/w, vegetable suspension), and stored in a closed container with
cheesecloth in a dark room and room temperature for 6 and 3 days, respectively. All activities were conducted aseptically. Biomass produced was fermented vegetable suspension [2].

2.2.3. Nixtamalization process. A number of yellow corn and white corn of horse dent was subsequently washed and steeped in water at the ratio of corn and water of 1: 4. On both steeped corn was then added Ca(OH)2 of 20% (w/w, dissolved corn), cooked at 90 °C for 60 minutes, cooled, rinsed to remove cooking water and excess lime, allowed, grounded and sieved through 80 mesh to produce nixtamalized corn powder composed of different kinds of particles.

2.2.4. Mixing, homogenizing, drying, size reduction and sieving. The preparation process of mixture of tempah paste and fermented vegetables was conducted by mixing them at the ratio of tempah paste and fermented vegetable of 1: 1 [2], homogenized at 8000 rpm for 30 minutes until it is generated a mixture of tempah and fermented vegetable. Nixtamalized corn was added into the mixture of tempah paste and fermented vegetable at the ratio of the mixture of tempah paste and nixtamalized corn of 1: 1, homogenized at 8000 rpm for 30 minutes to produce a fortificant paste.

2.2.5. Preparing infant biscuit. Preparation of infant biscuit was carried by adding fortificant paste A and B was equivalent to as folic acid with concentrations which of 0, 200, 400, 600, and 800 µg folic acid/100 g base formula of biscuit (145 g), respectively. Composition on base formula of biscuit consisted of wheat flour, corn starch, smooth sucrose, vanilla, salt, powder milk, unsalted butter, baking powder and water of 33.62, 11.20, 15.17, 0.79, 0.79, 9.86, 1.86, 0.96, and 11.70% (w/w, base formula). Preparation process of infant biscuit was by agitating unsalted butter, smooth sucrose, milk, and water up to swell, adding fortificant paste, salt, and vanilla followed by adding wheat flour, corn starch and baking soda, moulding, roasting baking in oven at approximately 150 °C by means of electrical power for 15 – 20 minutes, cooling, and packaging.

2.3. Identifying and analyzing folic acid by means of LC-MS. Suitable samples in biscuit use fortificant paste A and B, and standard folic acid. The oligomer was analyzed by means of an LC-MS using Mariner Biospectrometry. LC system was integrated with Q-TOP MS through Electrospray Ionization (ESI) system, in which scan mode was performed in a range of m/z 100 – 1200 at 140 °C. LC was carried out on a 15 mm x 2 mm C18 (RP 18) Supelco column and particles size of 5 μm. Solvent types were a mixture of 80 parts of methanol and 20 parts of water with flow rate of 0.1 mL/minute and injection volume of 5 uL [16]. In order to know the folic acid concentration, the curve of standard folic acid is made in various concentrations followed by identifying through LC-MS. Recovery of abundance data at each peak is entered to the curve of standard concentration (ug/mL) vs abundance/area so that it is achieved equation of linear regression of standard. Data abundance of sample is plotted in standard folic acid in order to know its concentration.

3. Results and Discussion

3.1. Characteristic of materials
Soy tempeh contributes the highest dissolved protein (0.81 mg/mL) as compared to both fermented spinach (0.71 mg/mL) and fermented broccoli (0.30 mg/mL), nixtamalized yellow corn (0.27 mg/mL) in formulation of fortificant. Tempeh is produced from soybeans fermented by protease enzyme activities of Rizosphus oligosporus strain C1 causing the degradation of protein of soy to form amino acids, particularly glutamic acid as components of formation of folic acid calculated as dissolved protein [1]. As a source of carbohydrate, nixtamalized yellow corn yields the highest total sugars (316.14 mg/mL) as compared to other materials. On these materials have been performed modification of corn nixtamalization process in order to increase composition, particularly its folic acid concentration [2]. Nixtamalized yellow corn generates also the highest total solids (54.46%). Amongst other materials will have role in the whole compositions and fortificant texture. Fermentation of
spinach and broccoli by Kombucha culture yields the highest reducing sugars of 21.82 and 18.27 mg/mL, meanwhile reducing sugars concentration in soybean tempeh and nixtamalized corn are 7.13 and 0.75 mg/mL. This fermentation uses sucrose as a source of carbon to produce folic acid through synthesis *de novo* [17] by Kombucha culture as microbe degrading the components of vegetable, particularly polyphenol. Remainder reducing sugars from this synthesis are dissolved in biomass and are the source of energy affecting the *taste* of application product. The whole composition of materials is shown in Table 1.

**Table 1.** Characteristic of materials in preparation of infant biscuit as complementary feeding using fortificant paste of natural folic acid.

| Kind of material          | Components          | Dissolved protein (mg/mL) | Total solids (%) | Total sugars (mg/mL) | Reducing sugars (mg/mL) |
|---------------------------|---------------------|----------------------------|------------------|----------------------|-------------------------|
| Nixtamalized yellow corn  |                     | 0.27                       | 54.46            | 316.14               | 0.75                    |
| Soy bean tempeh           |                     | 0.81                       | 40.02            | 60.00                | 7.13                    |
| Fermented spinach         |                     | 0.71                       | 7.66             | 45.92                | 21.82                   |
| Fermented broccoli        |                     | 0.30                       | 8.09             | 40.64                | 18.27                   |
| Fortificant paste A*       |                     | 2.42                       | 24.88            | 274.47               | 54.57                   |
| Fortificant paste B*       |                     | 2.24                       | 31.57            | 237.73               | 36.82                   |
| Basic formula of biscuit**|                     | 1.23                       | 69.17            | 143.96               | 30.48                   |

Legend : *A, Mixture of nixtamalized yellow corn, soybean tempeh & fermented spinach; B, the mixture of nixtamalized yellow corn, soybean tempeh & fermented broccoli; **total 145 g.

Fortificant processes are achieved through a series of homogenization at rotation speed at 4000 - 6000 for approximately 30 minutes between soy tempeh and fermented spinach/broccoli at the ratio of 1: 1 and is added nixtamalized yellow corn (1 part). This formulation increases the composition of fortificant paste compared with each material prior to formulation. Fortificant paste A is a mixture of yellow nixtamalized corn-soy tempeh – fermented spinach and is deep suspension, smoother granular and darker in colour compared with fortificant paste B which is mixture of yellow nixtamalized corn-soy tempeh – fermented broccoli. Composition on dissolved protein (2.42 mg/mL), total sugars (274.47 mg/mL) and reducing sugars (54.57 mg/mL), however total solids is lower (24.88%) compared with fortificant paste B. Composition of base formula of biscuit demonstrates dissolved protein of 1.23 mg/mL, total sugars of 143.96 mg/mL, reducing sugars of 30.48 mg/mL, and total solids of 69.17% affecting on formulation of biscuit. Preparation of infant biscuit is performed based on Recommended Dietary Allowances (RDA) on folic acid according to standard SNI [8] for infant food. It had been known that need of folic acid is 20% from all need of vitamins according to RDA or approximately 400 µg per chip (w/w, biscuit) being equivalent to 20 – 25 gram or 20 Kkal. In other words, fortificant paste contains 100 – 150 µg/g per serve (biscuit chip). Figures 2a, 2b, 2c, 2d, 2e, and 2f show nixtamalized yellow corn, fermented spinach, fermented broccoli, soy tempeh, fortificant paste A (mixture of soy tempeh & fermented spinach), and fortificant paste B (mixture of soy tempeh & fermented broccoli), respectively.

**Figure 2.** (a) Nixtamalized corn, (b) fermented spinach, (c) fermented broccoli, (d) soy tempeh, (e) fortificant paste A (mixture of soy tempeh & fermented spinach) and (f) fortificant paste B (mixture of soy tempeh & fermented broccoli).
3.2. Effect of formulation and baking on the composition of biscuit

3.2.1. Dissolved protein (mg/mL) and total solid (%). Preparation process of infant biscuit is conducted through mixing unsalted butter, sugars, salt up to white and thick by means of mixer followed by adding wheat flour, corn starch, baking powder (NaHCO₃), fortificant paste of folic acid, vanilla flavor and water, doughing, moulding, and roasting at 150 °C for 15 – 20 minutes. The whole processes produce biscuit with appearance (texture, color, aroma) and specific composition in each treatment. Increasing folic acid in fortificant paste increases dissolved protein of biscuit on using both types of fortificant. Biscuit with fortificant paste A (biscuit A) produces higher dissolved protein compared with biscuit with fortificant paste B (biscuit B), as shown in Figure 3a. Optimization of dissolved protein is achieved by biscuit A (2.4 mg/mL) and biscuit B (2.29 mg/mL) at folic acid concentration of 800 µg/145 g of base formula of biscuit increasing dissolved protein of 95.12 and 86.18% compared with biscuit with base formula without fortificant (1.23 mg/mL). This increase of dissolved protein is caused by the contribution of both fortificant paste A and B of 2.42 and 2.24 mg/mL in the base formula of biscuit despite it takes place denaturation during roasting. Process of adding fortificant paste with higher concentration produces total solids of biscuit fluctuating, as indicated in Figure 3b. Total solids in biscuit A will increase and reach optimum by adding 200 µg of folic acid (94.96%), whereas biscuit B achieves optimum condition by adding 600 µg of folic acid resulting total solids 93.24% followed by dropping to the highest concentration of folic acid (800 µg). In this condition, both biscuits A and B raises total solids of 37.28 and 34.80% compared with biscuit using base formula without fortificant (69.17%). This increasing total solid is not only caused by the contribution of fortificant on base formula of biscuit, but also by baking as a consequence of water mass evaporation. Adding fortificant becoming more and more high will generate biscuit with optimum total solids up to a limit, in which with higher concentration of fortificant will take place a lysis or dissociating components due to caramelization to form brown pigments of melanoidin or volatile compounds so that it is not detected as solids according to Gravimetric method [13].

3.2.2. Total sugars (mg/mL) and reducing sugars (mg/mL). Both biscuits A and B have the optimum concentration of total sugars by adding fortificant paste at folic acid concentration equal to 800 µg/145 g of base formula of biscuit of 276.94 and 318.79 mg/mL, as shown in Figure 4a. In this condition, adding both fortificant paste A and B increase total sugars of biscuit of 92.37 and 121.44% (1.2-folds), respectively compared with biscuit using base formula without fortificant (143.96 mg/mL). Meanwhile, reducing sugars at both biscuits A and B with the optimum condition is yielded by adding fortificant paste at folic acid concentration equal to 800 µg/145 g of base formula of biscuit of 50.73 and 50.56 mg/mL, as displayed in Figure 4b. In this condition, adding fortificant paste raises reducing sugars on biscuits A and B of 66.44 and 65.88% compared with reducing sugars of biscuit using base formula without fortificant (30.48 mg/mL). This increasing reducing sugar is caused by the contribution of fortificant on base formula of biscuit despite caramelization occur in roasting by
forming melanoidin as a consequence of Maillard reaction [18] being undetected according to Phenol-Sulphate method and Nelson Somogyi method [15].

![Figure 4](image_url)

**Figure 4.** Relationship between the kind of fortificant pte and folic acid concentration on the recovery of (a) total sugars and (b) reducing sugars in infant biscuit as complementary feeding.

### 3.3. Optimum process condition of biscuit formulation

From evaluating the best composition in preparation of infant biscuit as complementary feeding is based on the highest dissolved protein as an important indication to get the best folic acid. The optimum process condition is achieved at concentrations of fortificant pastes A and B is equal to folic acid of 800 µg/145 g of base formula of infant biscuit. In this condition is yielded infant biscuit A and B with the composition of dissolved protein of 2.40 and 2.29 mg/mL, total solids of 92.74 and 92.77%, total sugars of 276.94 and 318.79 mg/mL, and reducing sugars of 50.73 and 50.56 mg/mL, as demonstrated in Figure 5.

![Figure 5](image_url)

**Figure 5.** Infant biscuit using (a) fortificant paste A and (b) fortificant paste B.

### 3.4. Identification of folic acid monomer and analysis of folic acid in biscuit

Identification of standard folic acid and biscuit examples are subsequently indicated in Figures 6a, 6b, 6c, 6d, 6e, and 6f. Identification of folic acid monomer in standard folic acid is achieved 1 (one) peak (T 2.6) with retention time range of 0 – 10 minutes and relative intensity of 100%, in which at mass spectra m/z 439 – 448 from T 2.6 shows domination of monomer with MWs of 442.66, 442.85 and 442.99 Da. and relative intensities of 100, 58.48%, and 45.46%, as indicated in Figures 6a and 6b.
Figure 6. (a) Chromatogram of standard folic acid, (b) mass spectra of standard folic acid, (c) chromatogram and (d) mass spectra of biscuit A, (e) chromatogram and (f) mass spectra of biscuit B.

It had been known that folic acid has a molecule weight (MW) of 441 Da. By means of LC-MS method had been known that a compound indicated the difference in MW, in which its possibility is as M+, M+ Na+, 2M++ or 2M+, Na+. This matter is caused by its presence of ionization as a consequence of the sensitivity of LC-MS instrument related to eluent used. Operation condition of LC-MS is injection volume of 5 μL, flow rate of 0.2 mL/minutes, methanol/water (8 : 2, v/v) as eluent, and C-18 column (15 mm x 2 mm i.d.) [16]. Identification of folic acid monomer on biscuit A and B is conducted by adding fortificant paste with folic acid concentration equal to 800 µg/145 g of base formula. Figures 6c and 6d show chromatogram at biscuit A with 3 peaks dominated by T2.1 for retention time 0 – 10 minutes and relative intensity of 100%. Mass spectra T2.1 give 8 monomers of folic acid dominated by monomer with MW of 442.34 Da., m/z 441.80 – 443.10, and relative intensity of 100%. Figures 6e and 6f give chromatogram at biscuit B with 1 (one) peak dominated by T2.1, retention time 0 – 10 minutes and relative intensity of 100%. Mass spectra T2.1 yield 9 monomers of folic acid dominated by monomer with MW of 442.26 Da., m/z 441.69 – 443.17 and relative intensity of 100%. The concentration of folic acid in biscuit A and B is 127.23 and 117.38 µg/mL.

3.5. Identification of volatile compounds in biscuit

Identification of volatile compounds was done on biscuit A and B from by adding fortificant with the concentration of 800 µg/145 g of base formula. Biscuit A shows chromatogram with 8 peaks dominated by volatile compounds of Vanillin (C₈H₈O₃), as shown in Figure 7a. Vanillin is at the formulation of infant biscuit (1.15 g/145 g of base formula or 0.79% of formula weight). The baking process does not reduce the effect of vanillin aroma of 37.50% from the whole emerged volatile compounds. Other volatile compounds are furan derivative, furan methanol (4.27%) and fatty acids (hexadecanoic acid, 11.378%, octadecenoic acid, 2.604%, linoleic acid, 18.469%, pentanoic acid, 2.976%, and ester methyl 1-octadecenyl, 3.246%). Biscuit B shows chromatogram with 10 peaks dominated by volatile compound of vanillin (46.05%), furan compounds (2-Furanmethanol, 3.25%, Furanmethanamine, 3.66%, and benzamide, 1.99%), fatty acids (pentanoic, 2.82%, hexanoic, 9.70%, octadecanoic, 13.62%, oleic acid, 11.98% and butyric acid, 2.45%), as showed in Figure 7b. The difference in the type of volatile compounds for both biscuits is detected much more ester (linoleic acid ethyl ester) in biscuit A so that it has more specific aroma compared to biscuit B despite it has higher vanillin. These compounds are obtained as fortified and biscuit formulas which are possible to change their structure by heat processing during roasting.
3.6. Distribution of particle size

Infant biscuit is generated through a series of formulation process covering mixing formula of biscuit and fortificant paste, agitating dough, moulding, and baking at 150 °C for 15 – 20 minutes. Particle size distribution on biscuit is affected by the type of components and processes being done by each material. Main raw materials used in this product are wheat flour (33.62%), corn starch (11.21%), sugars (15.17%), and other materials (flavor, salt, milk, butter, baking powder) of 28.27% and water. Using fortificant pastes A and B at a concentration of 800 µg/145 g of base formula or equal to 2.47 and 1.44 g of fortificant paste so that the largest contribution in biscuit is wheat flour and corn starch. This matter affects the possibility of particle size and particle size distribution of biscuit, moulding, and baking. Using mixer is part of size reduction and emulsification, whereas baking causes its occurrence of mass water evaporation as a consequence of smaller particle size. Table 2 shows particle size distribution both types of biscuit, in which biscuit A results in smaller particle size (366.6 nm) with higher Particle Index (PI) (0.795) compared with biscuit B (368.8 nm) and dispersed particles (Particle Index) of 0.790. The difference in this type of biscuit is not only caused by the difference in the type of fortificant, but only by processes used. Fortificant paste A has lower total solids (24.88%) compared with fortificant paste B (31.57%) so that it has smaller particle size of biscuit. This matter affects the possibility of adsorbing infant digestive system. Total solids are the accumulation of the whole components both dissolved and undissolved according to Gravimetric method [13]. In this analysis of particle size distribution according to the DLS method, particle size becoming more and smaller will be small in PI. In other words, dispersed particles are more uniform and homogenous, in which PI is smaller than 1 showed that particles size distribution is more uniform, whereas PI is higher than 1 showed that particles size distribution is not uniform [8]. Both type of biscuits has PI smaller than 1 so that they display homogenous distribution of particles size.

| Kind of paste fortificant | Distribution of nano-folate particles (nm) |
|-------------------------|------------------------------------------|
|                         | Z-Average (nm)* | PI**        |
| Biscuit A               | 366.6          | 0.795       |
| Biscuit B               | 368.8          | 0.790       |

Legend :*Diameter of nano particles and **dispersed particles (Particle Index).

This difference seems in Figure 8a, in which particle size distribution of infant biscuit with fortificant paste A result particles with diameter size (Ø) of 100 – 400 nm (< 1000 nm) at frequency of dispersed particles of 17.5%, however, the whole diameter sizes is yielded particles with Ø of 100 – 1000 nm at frequency of dispersed particles of 35%. Meanwhile, infant biscuit with fortificant paste B result particles with Ø of 100 – 600 nm (< 1000 nm) at frequency of dispersed particles of 17.5% and particles with Ø of 100 – 1000 nm at frequency of dispersed particles of 35%, as shown in Figure 8b.
4. Conclusion

Fortificant paste on the mixture of soy/mung beans tempeh - fermented spinach/broccoli -nixtamalized corn has potential use as source of natural folic acid in the preparation of infants biscuit for complementary feeding. Fortification on various concentrations increases dissolved protein, total sugars, reducing sugars, meanwhile, total solids fluctuates on both types of biscuit. Optimization on fortificant paste in both biscuit A and B is achieved at concentration of folic acid of 800 µg/145 g of base formula yielding biscuits with composition of folic acid of 127.23 and 117.38 µg/mL, dissolved protein of 2.40 and 2.29 mg/mL, total solids of 92.74 and 92.77%, total sugars of 276.94 and 318.79 mg/mL, and reducing sugars of 50.73 and 50.56 mg/mL, respectively. In this condition, using both fortificant paste A and B increase dissolved protein on biscuit of 95.12 and 86.19%, total solids of 34.07 and 34.11%, total sugars of 92.37 and 121.44 % (1.2-folds), reducing sugars of 66.44 and 65.88% compared with biscuit for base formula without fortificant from each component, respectively. Biscuit A and B is dominated by folic acid monomer with MW of 442.34 and 442.26 Da., dominant volatile compounds are vanillin flavour (37.50 and 50.41%), furan (4.27 and 6.91%), fatty acids, such as hexadecanoic acid (11.378 and 9.70%), octadecenoic acid (2.604 and 13.62%), linoleic acid (18.469 and 0%), pentanoic acid (2.976 and 2.82%), and oleic acid (0 and 11.98%), respectively. Particles size distribution in biscuit A and biscuit B displays particles size of 366.6 and 368.8 nm with particles index of 0.795 and 0.790, respectively.

Acknowledgement

The authors wish to thank the Kemenristekdikti throughout Program Insentif Riset Sistem Inovasi Nasional (INSINAS) Fiscal Year 2019 supporting this research in Program INSINAS Riset Pratama Individu on Research Field for developing functional food–based local natural resources.

References

[1] Susilowati, A., Aspiyanto, A. and Maryati, Y., 2018, December. Characteristic of folic acid monomer and distribution of both fermented broccoli (Brassica oleracea L.) and spinach (Amaranthus sp.) for forticant of natural nano folate. In AIP Conference Proceedings (Vol. 2049, No. 1, p. 030006). AIP Publishing.

[2] Susilowati, A., Lotulung, P.D. and Maryati, Y., 2018. Modification process in nixtamalization of folic acid-rich dent corn (Zea mays identata) and its identification as smart food fortificant. In MATEC Web of Conferences (Vol. 154, p. 01017). EDP Sciences.

[3] Bailey, L. B. 2017. Folate in Health and Disease, 2nd. Edition, June 13, Boca Raton, CRC Press.

[4] McKenzie, S. B. 1999. Megaloblastic and Nonmegaloblastic Macrocytic Anemias. In Textbook of Hematology, 2nd edition, Williams and Wilkins, Baltimore, p.179 – 199.
[5] Quinlivan, E. P., Roje, S., Basset, G., Shachar-Hill, Y., Gregory, J. F. and Hanson, A. D. 2003. The Folate precursor ρ-aminobenzoate is reversibly converted to its glucose ester in the plant cytosol, J. Biol. Chem., June 6, 278 (23), p.20731 - 20737. PMID : 12668665. DOI : 10.1074/jbc.M302894200.

[6] Susilowati, A., Maryati, Y., Lotulung, P. D. N., Aspiyanto. 2018. Formulasi Nikstamal Jagung, Tempe, dan Sayuran Terfermentasi dalam Perolehan Pasta Fortifikasi sebagai Sumber Asam Folat Alami, Jurnal Applikasi Teknologi Pangan (), Vol. 7, No.2, 2018. Indonesian Food Technologists https://doi.org/10.17728/jatp.2517. p-ISSN 2089-7693, e-ISSN 24605921.

[7] Susilowati, A., Lotulung, P.D. and Maryati, Y., 2018. Proses emulsifikasi dan pengeringan pasta campurun sumber asam folat alami dan identifikasi sanya dalam perolehan serbuk suplemen untuk wanita hamil. Jurnal Sains dan Kesehatan, 1(9), pp.484-496.

[8] SNI. 2007. Makanan Pendamping Air Susu Ibu (MP-ASI). Surat Keputusan Menteri Kesehatan Republik Indonesia No. 224/Menkes/SK II/2007. Jakarta.

[9] Backman. 2018. Laser diffraction measurement for non sperical particles. Backman Coulter Life Sciences, © Backman Coulter Inc., USA.

[10] Dass, Chreh. 2007. Dasar-dasar Spektrometri Massa Kontemporer. John Wiley & Sons, Inc., 151 – 194. doi : 10.1002/9780470118498.ch5. ISBN 9780470118498. Accessed at 20 April 2017.

[11] Onggo, D. 1998. General Principles in Electrospray Mass Spectrometry : A New Technique in Mass Spectral Analysis. Journal of Mass Spectrum, Vol. 3, No. 2, pp.115 – 131. Wikipedia. Accessed at 3 February 2019.

[12] Hussain, S. Z. and Maqbool, K. 2014. GC-MS : Principle, Technique and its application in Food Science, INT J CURR SCI, 13, p.116 - 126.

[13] A.O.A.C. 2016. Official Methods of Analysis. Association of Official Analytical Chemists International, 20th Edition, Washington D. C.

[14] Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951, 193, p.265 – 276. PMID 14907713.

[15] Hatanaka, C. and Kobara, Y. 1980. Determination of Glucose by a Modification of Somogyi-Nelson Method. Journal of Agricultural and Biological Chemistry, Volume 44, Issue 12, p.2943 – 2949. https://doi.org/10.1080/00021369.1980.10864408.

[16] Eichhorn, P. and Knepper, T. P. 2001. Electrospray ionization mass spectrometric studies on the amphoteric surfactant cocamidopropylbetaine, Journal of Mass Spectrum, June, 36 (6) : 677 – 684. DOI : 10.1002/jms.170.

[17] De Crécy-Lagard V., El Yacoubi B., de la Garza R. D., Noiriel A., and Hanson A. D. 2007. Comparative genomics of bacterial and plant folate synthesis and salvage : predictions and validations, BMC Genomics, July 23, 8, p.245. PMID : 17645794, PMCID : PMC1971073. DOI : 10.1186/1471-2164-8-245

[18] Manley, C. H. 1995. Process Flavours and Precursor Systems, in Savory Flavors (ed. T. W. Nagodawithana), Esteekay Associates, Wisconsin.