Alternative Use of Modified Cassava Flour (Mocaf) as Carrier of Natural Folic Acid in Infant Biscuits for Complementary Feeding

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Abstract. Modified cassava flour (mocaf) is an alternative food ingredient used in preparation of biscuits as a binder and a carrier of folic acid fortificant. This work aimed to evaluate the mocaf addition with natural folic acid fortificants in two infant biscuit formulations. Mocaf was added at concentration of 0, 0.3, 0.6, 1.2, and 2.4% (w/w, control formula of infant biscuit) with folic acid fortificant A (mixture of nixtamalized corn, soy tempeh and fermented broccoli) and B (mixture of nixtamalized corn, mung beans tempeh and fermented broccoli). The optimum condition was achieved at mocaf concentration of 0.3% and resulted in infant biscuits A and B with folic acid content of 212.23 µg/mL and 438.74 µg/mL, dissolved protein 27.41 mg/mL and 28.88 mg/mL, total sugars of 358.20 mg/mL and 322.72 mg/mL, reducing sugars of 35.10 mg/mL and 39.78 mg/mL, total solids of 94.82% and 92.43%, respectively. The formulations of biscuits A and B increased folic acid content by 80.05% and 199.55% compared to the counterpart without mocaf addition. The predominant folic acid monomers had molecular weight of 442 m/z. Volatile compounds were identified as acetic acid, furan, and fatty acids. Biscuits A and B displayed average particle size of 69.07 μm and 69.78 μm with hardness of 8.44 N and 16.94 N, and lightness of 35.68 and 36.86, respectively.

1. Introduction
Infant biscuits with folic acid fortificant have potential for complementary feeding (CF). Infant biscuits for CF are made to adapt the digestive system of infants. These biscuits are meant to complement breast milk or infant formula, so thus infants can learn to hold and bite their foods. Infant biscuits are designed for easy dilution into porridge. These biscuits are usually weighed 20 g per piece [1].

Folic acid is necessary for proper growth, particularly during pregnancy, so thus the baby can produce adequate red blood cells and prevent Neural Tube Defects (NTDs) [2], whilst the expecting mother can avoid anemia [3]. Folic acid (L-glutamic acid, pteroyl-L-glutamic acid), also known as Vitamin B9, consists of three parts: pteridine heterocyclic, para-aminobenzoate (p-ABA) and L-glutamic acid [4]. Folic acid is sensitive to oxidative degradation, which is triggered by light, high temperature, and mechanical treatment [5].

Mocaf, added in the formulation of infant biscuit to increase crispiness, can also act as a carrier or a binder of folic acid. Mocaf is produced through fermentation of thin cassava slices by lactic acid bacteria (LAB), followed by drying and grinding. Mocaf addition is a potential gelling agent since it
increases viscosity and imparts gelation, with good hydration power [6]. The potential role of mocaf as a binder and a carrier of bioactives in biscuits has not been explored yet. Mocaf is expected to be a good binder for folic acid due to smaller size of starch granule.

The objective of this experiment study was to determine the optimum mocaf concentration as a binder and a carrier of folic acid in infant biscuits, as observed through analysis of folic acid monomer. Physicochemical properties of the resultant biscuits were also analysed.

2. Materials and Methods

2.1. Materials and equipments
Materials used in this experimental work were mocaf (a gift from Research Center for Biotechnology, LIPI), dried yellow corn (Zea mays var. indentata) from a local corn plantation (Tangerang Selatan), soy beans, mung beans, broccoli, sucrose salt, whole milk powder, vanilla extract, unsalted butter, baking powder, wheat flour, and corn starch were purchased from local market. Rhizopus oligosporus strain C1 and Kombucha culture were the collection of Research Center for Chemistry, LIPI.

Main equipments implemented in this experimental activity were analytical balance (Fujitsu, Japan), autoclave (CHENG YI, LS–50 L, China), homogenizer (Ultra-Turrax, Ika Labortechnik, T50, Jane & Kunkel, Germany), blender (local, National), container, incubator (local), electrical oven, and cabinet dryer (local). 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 (Horiba Laser Scattering Particle Size Distribution Analyzer LA-960), GC-MS (Shimadzu, Japan) and Texture Analysis (TMS-Pro, Stable Micro System, USA).

2.2. Experimental Design
Mocaf was added to the control formula of infant biscuits at concentration of 0, 0.3, 0.6, 1.2, and 2.4% (w/w). The biscuits contained either fortificant A or B which was equivalent to 1,000 µg of folic. Fortificant A was a mixture of nixtamalized yellow corn, soy tempeh, and fermented broccoli. Fortificant B consisted of the same ingredients except the use of mung beans instead of soy tempeh.

The infant biscuits were prepared according to Indonesian National Standard (SNI) [20] for infant foods. The requirement of folic acid is 20% from the whole vitamin requirement according to recommended daily intake (RDA) or approximately 400 µg. The weight of one piece of biscuit ranged from 20 to 25 gr, with equivalent energy of 20 kcal. The biscuits were predicted to contain folic acid of 100–50 µg per piece of biscuit.

Analyses were performed on both forticants and resultant infant biscuits included total solid (gravimetric method), dissolved protein (spectrophotometry) [7], total sugar (phenol sulphate method), reducing sugars (Somogyi Nelson method) [8], and folic acid (spectrophotometry) contents [9]. Identification on folic acid monomer was conducted using LCMS (Mariner Biospectrometry) tandem with LC (Hitachi L 6200) [10]. Other analyses included particle size distribution (Horiba LS-PSA LA-960) [11], volatile compounds (Shimadzu, Japan) [12], hardness (TMS Pro Texture Analyser), and colour measurement (Konika Minolta CR-410) [13].

2.3. Procedure
Folic acid forticants were prepared by mixing either soy bean (fortificant A) or mung bean (fortificant B), fermented broccoli and nixtamalized yellow corn at mass ratio of 1:1:1, followed by homogenation, drying, size reduction, and sieving through 60-80 mesh until fine powder of fortificant A and B were obtained [14].

The biscuits were prepared from wheat flour (33.62%), corn starch (11.2%), sucrose (15.17%), vanilla (0.79%), salt (0.79%), whole milk powder (9.86%), unsalted butter (1.86%), baking powder (0.86%), and water (11.73%) (w/w, control formula). Fortificant was added at concentration
equivalent to 1,000 µg of folic acid per 100 g of control formula. Mocaf was added at concentrations of 0, 0.3, 0.6, 1.2, and 2.4 % (w/w, control formula).

Butter, sucrose, and milk powder was beaten using an electrical mixer until fluffy. Fortificants A or B, salt, vanilla, wheat flour, corn starch, baking soda, and water were added and gently mixed with spatula. The dough was then moulded and baked in oven at 150 °C for 15–20 minutes. The biscuits were subsequently cooled and packed.

3. Results and Discussions

3.1. Characteristics of materials
Fortificant B (130.54 µg/mL) had higher content of folic acid than fortificant A (101.89 µg/mL) (Table 1). Fortificant B also showed higher total sugars, dissolved protein, and total solids. It, however, contained lower reducing sugar than fortificant A. Higher folic acid content in fortificant A was possibly due to the use of soy tempeh which contained 503.60 µg/mL of folic acid. While fortificant B used mung beans tempeh that contained lower folic acid (381.5 µg/mL) [15].

During the fermentation of either soy or mung beans by *Rhizopus oligosporus* strain C1, the protein degraded to amino acids, particularly glutamic acid, which was a component for folic acid formation. The results of protease activity on the soy or mung bean substrates was expressed as dissolved protein [14]. Meanwhile, broccoli was fermented by Kombucha culture using sucrose as source of carbon in order to produce folic acid through synthesis of de novo [16, 17]. The remaining reducing sugars achieved from this synthese was soluble in biomass as a source of energy and contributed to the taste of the product.

Nixtamalised yellow corn not only gave a better binding of folic acid, but also acted as a source of carbohydrate and imparted texture [18]. Folic acid is sensitive on mechanical treatment, light and temperature [5], and processed foods might experience reduction of folic acid. Therefore, folic acid fortification will help to supply adequate folic acid in the final products. The control infant biscuit already showed folic acid content of 109.09 µg/mL, which obtained from the baking ingredients i.e., wheat flour, corn starch, sugars, powder milk, and unsalted butter.

| Materials          | Folic acid (µg/mL) | Total solids (%) | Dissolved protein (mg/mL) | Reducing sugars (mg/mL) | Total sugars (mg/mL) |
|--------------------|--------------------|------------------|---------------------------|------------------------|----------------------|
| Fortificant A*     | 101.89             | 92.91            | 5.80                      | 28.00                  | 78.28                |
| Fortificant B**    | 130.54             | 93.07            | 6.00                      | 19.14                  | 89.65                |
| Control biscuit    | 109.09             | 69.17            | 12.30                     | 30.48                  | 143.96               |
| Mocaf              | 22.26              | 96.13            | 1.17                      | 5.50                   | 96.13                |

* A, mixture of nixtamalized yellow corn, soy tempeh and fermented broccoli
** B, mixture of nixtamalized yellow corn and mung beans tempeh and fermented broccoli

![Figure 1](image_url)
3.2. Effect of formulation on infant biscuits

3.2.1. Folic acid (µg/mL) and dissolved protein (mg/mL) contents. The addition of mocaf to the formulation of either biscuit A or B, in general, increased the folic acid content. The increase of folic acid content, however, was not linearly correlated with the increase of mocaf addition. Biscuit B displayed higher content of folic acid compared to biscuit A across percentages of mocaf additions (Figure 1a). This was due to higher concentration of folic acid in fortificant B (130.54 µg/mL) than that of fortificant A (101.89 µg/mL). Besides, the interaction of mocaf and other ingredients in each biscuit might be different. Mocaf in biscuit B was possibly better in binding folic acid and thus the folic acid could handle the baking process better. Therefore, more folic acid remained in biscuit B after the exposure to baking temperature (150 ºC for 20 minutes).

Mocaf addition led to increase of available carbohydrate for Maillard reaction. At higher mocaf concentration, the Maillard reaction [19] between sugars (carbohydrate) and protein was accelerated, and thus more melanoidin was produced. This might also contribute to a drop of final folic acid content in the biscuit at higher mocaf concentrations. The highest folic acid content was achieved at mocaf addition of 0.3% for both biscuits, which resulted in final folic acid content of 212.23 µg/g and 438.74 µg/g for biscuit A and B, respectively. Compared to the control biscuits (biscuits with folic acid fortification at 1,000 µg/g and without mocaf addition), the mocaf addition was able to increase folic acid content in biscuit by 80.05% and 199.55%.

![Graph showing folic acid and dissolved protein contents](a)

![Graph showing dissolved protein contents](b)

Figure 2. Relationship between type of fortificant and mocaf concentration on recovery (a) folic acid and (b) dissolved protein from infant biscuit as source of folic acid.

Increasing mocaf concentration in biscuits also resulted in fluctuvative values of dissolved protein. Dissolved protein is the whole proteins and its derivate, particularly amino acids and folic acid structured by glutamic acid, pteridine heterocyclic and para-aminobenzoate (p-ABA) [4]. The highest values of dissolved protein in biscuits A and B were 28.52 mg/mL and 34.27 mg/mL respectively, which were both reached at mocaf concentration of 1.2%. Compared to the control positive biscuits, the mocaf addition at 1.2% increased dissolved protein by 8.77% and 55.63%. The initial dissolved protein concentration in fortificants A and B (5.8 and 6.0 mg/mL, respectively) might contributed to the different dissolve protein contents of the final biscuits. In addition, the rate of Maillard reaction and protein denaturation in each biscuit was possibly different due to different fortificant ingredients although the mocaf addition was the same. However, both biscuits experienced a decrease of dissolved protein content at mocaf concentration of 2.4 mg/mL. The Maillard reaction at this mocaf concentration was possibly too fast and resulted in more melanoidin compounds that were undetected by the Lowry method [7].
3.2.2. Total sugars (mg/mL), reducing sugars (mg/mL) and total solids (%). Mocaf addition, in general, increased the total sugars of biscuit A. The increase of total sugars in biscuit B, however, did not follow a smooth pattern (Figure 2a). The highest total sugars of biscuits A (408.99 mg/mL) and B (429.97 mg/mL) were achieved at mocaf concentration of 2.4 and 0.6 % (w/w), respectively. Total sugars of biscuit A was most of the time higher than that of biscuit B across percentage of mocaf addition, except at 0.6% mocaf addition. The initial fortificant analysis showed that fortificant A had a lower (78.28 mg/mL) total sugars compared to fortificant B (89.65 mg/mL). This might explain the reason beyond a lower total sugar of biscuit A at 0.6% mocaf addition. More available sugars in the formulation of biscuit B implied more intensive Maillard reaction took place in biscuit B compared to that of biscuit A. Therefore, less sugars remained in biscuit B for most of mocaf concentrations as revealed by the phenol sulphate method [8].

The reducing sugars of both biscuits, in general, increased with increased mocaf concentrations. The highest amounts of reducing sugars for biscuits A and B was 42.67 mg/mL and 49.94 mg/mL, respectively, at mocaf concentration of 2.4% (Figure 2b). Compared to the control biscuits, the mocaf addition increased the reducing sugars in biscuit A and B up to 111.87% and 66.46%, respectively.

The increasing percentages of mocaf addition in both biscuits did not give a clear pattern. Particularly at mocaf concentration of 1.2% which gave a lower total solid content for both biscuits compared to the control biscuits. Interaction between ingredients during baking process might lead to reduction of total solids due to water evaporation and shrinkage of materials. The highest total solids of biscuits A and B were 94.82% and 94.13%, respectively. These values were achieved at different mocaf concentrations. At 0.3%, mocaf displayed its best interaction with fortificant A and other ingredients. This left unbound water which later evaporated during baking. For biscuit B, the highest total solid content was achieved at 2.45. This indicated that the interaction between mocaf, fortificant, and other ingredient in each biscuit was unique.
3.2.3. Characteristic of Volatile Compounds for Infant Biscuit. Volatile analysis was performed on biscuits A and B which were added with 0.3% (w/w) since they displayed the best folic acid recovery. Chromatogram of biscuit A (Figure 3a) shows 25 peaks which were dominated (46.1%) by acetic acid and ethyl/methyl ester acetic acid. Other peaks represented furan and furan derivatives (18.64%) e.g., furfural, ethylhydroxylamine, furanmethanol, furanone, hydroxymethylfurfural and rhamnose. Fatty acids (11.93%) consisted of undecanoic acid, tridecanoic acid, pentadecanoic acid, hexadecanoic acid, 9-octadecenoic acid, methyl stearate, and butanoic acid. Other methyl ester (9.68%) were mercaptoacetone, cyclopentanol, 2-propanamine, benzaldehyde, n-hexane and oixane.

Chromatogram of biscuit B (Figure 3b) displays 33 peaks that were dominated (37.12%) by acetic acid and ethyl/methyl ester acetic acid. Furan and its derivatives (29.77%) consisted of furfural, 2-furan methanol, 4H-pyran-4-one, (3H)-furanone, and 5-hydroxymethylfurfural. Fatty acids (7.8%) comprised of methyl ester, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, and 10-octadecenoic acid. Alcohol and its derivates (15.35%) were dihydroxyacetone, 2-Cyclopenten-1-one, mercaptoacetone, 2-propen-1-ol, 2-butanol, etraethoxyethylene, benzaldehyde, cyclopropyl carbinol, and trihydroxy-2-butoxy.

3.2.4. Identification of folic acid monomer. Folic acid has MW of 441 Da. The LCMS analysis of folic acid standard showed the retention time (RT) was 3.27 min (Figure 4a) and MW of 441.37 (Figure 4b). The chromatogram of biscuit A had RT of 1.44 min (Figure 4c). The spectra (Figure 4d) demonstrated six folic acid monomers with MW of 441.12–441.83 Da, dominated by folic acid monomer with MW of 441.83 Da. The LCMS analysis of biscuit B showed a RT of 2.12 min (Figure...
with eight folic acid monomers with MW 440.90–441.98 Da, dominated by three folic acid monomers with MW of 441.09, 441.44, and 441.59 Da (Figure 4f).

Qualitative comparison between chromatograms revealed that biscuit B had more folic acid monomers than biscuit A. This was most likely due to differences of fortificants and the further interactions between fortificants and other biscuit ingredients during baking.

Figure 6. Chromatograms and spectra of standard folic acid (a and b), biscuit A (c and d), and biscuit B (e and f). The selected biscuits A and B were prepared with 0.3% (w/w) mocaf addition.
3.2.5. Particle size and particle size distribution for infant biscuits. The particles analysis of biscuit A showed the D10, D50, and D90 was 8.32, 25.81, and 195.34 µm, respectively (Figure 5a) with mean diameter size of 69.07 µm. Meanwhile the D10, D50, and D90 of biscuit B was 8.33, 26.37, and 196.38 µm, with mean diameter size of 69.78 µm. In general, biscuit B demonstrated larger particle size than biscuit A. This matter is possibility caused by difference in fortificant and interaction between formula and baking process, which resulted in different particle agglomeration.

![Figure 5a](image)

![Figure 5b](image)

**Figure 5.** Particle size distribution of biscuit A (a) and biscuit B (b), both were prepared with 0.3% (w/w) mocaf addition.

3.2.6. Identification of crispiness and sharpeness of biscuit. Both hardness and lightness analysis were performed on biscuits with 0.3% (w/w) mocaf addition. Biscuit B showed higher hardness (16.94 N) compared to biscuit A (8.44 N) eventhough total solids of biscuit A (94.82%) was higher compared with total solids of biscuit B (92.88%). The hardness of biscuit B which was two fold that of biscuit B was possibly due to the nature of fortificant B. Fortificant B was more sticky than fortificant A. Thus, the dough of biscuit B was harder.

![Figure 6](image)

**Figure 6.** Level of hardness from infant biscuit of A and infant biscuit of B at mocaf concentration 0.3% (w/w, control formula of biscuit).

The colour analysis of both biscuits are presented as Table 2. Biscuit B showed lower ∆E (14.95±0.32) than that of biscuit A (25.31±0.61). Value of ∆E demonstrated how different the colour of material from standard white tile. Both biscuits were very much different from standard white tile, as diplayed by their ∆E that exceeded 5.

| Type of biscuit | L* ± a* ± b* | ∆E ± |
|----------------|--------------|------|
| Biscuit A      | 65.45 ± 1.09 | 11.51 ± 0.26 | 35.68 ± 0.68 | 25.31 ± 0.61 |
| Biscuit B      | 81.18 ± 2.52 | 5.88 ± 0.59  | 36.86 ± 0.55 | 14.95 ± 0.32 |

**Table 2.** The three stimulus of colour parameters of biscuits A and B and their corresponding ∆E against standard white tile.
The bigger $\Delta E$ value of biscuit A indicated a faster Maillard reaction that resulted in melanoidin and other advanced glycation end products [19]. This was also reflected by green-red coordinate ($a$) in Table 2; the $a$ value of biscuit A was higher than that of biscuit B. Although biscuit B had higher dissolved protein and reducing sugars than the counterpart biscuit A, the total sugars of biscuit B (322.72 mg/mL) was lower compared to biscuit A (358.20 mg/mL). This possibly lead to a lower rate of Maillard reaction in biscuit B, and thus generated a higher lightness.

4. Conclusions

Mocaf has potential use as folic acid binder which also impart texture in infant biscuits. The optimum mocaf addition that resulted in the best folic acid recovery was achieved at 0.3% (w/w) for both biscuits A and B. These biscuits were prepared to result in 1,000 µg/g folic acid with different fortificants, namely fortificant A and B. The formulations produced infant biscuits A and B with folic acid of 212.23 and 438.74 µg/mL, dissolved proteins of 27.41 and 28.88 mg/mL, total sugars of 358.20 and 322.72 mg/mL, reducing sugars of 35.10 and 39.78 mg/mL, and total solids of 94.82 and 92.43%, respectively. Compared to control biscuits (biscuits with 1,000 µg/g folic acid without mocaf addition), the 0.3% mocaf addition was able to recover folic acid in biscuits A and B as much as 80.05% and 199.55%, respectively.

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