

Use of bio-enriched yeast and stability of its vitamin D₂ in wheat dough baking

By

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Abstract

Vitamin D is one of the essential vitamins required by our body. It is vital for keeping our bone structure Stronger. For people living in countries where there is a shortage of sunlight vitamin D supplement is very crucial. For instance, in northern European countries (e.g., as in Finland) the oral intake of vitamin D either from dietary source or from supplementing tablets is very important. This study aimed to analyze the stability of vitamin D₂ in the bread baking process and investigates its impact on bread quality. Bio-enriched yeast which contains vitamin D₂ was added to the wheat dough for enrichment purpose, and natural fresh yeast was used for the fermentation process. To analyze the stability of vitamin D₂ in the baking process:

Different mixing intensities, variable cooking temperature-time combinations, and two different storage conditions were examined.

The results of the study have shown that changing the mixing conditions, the baking temperature/time combinations and the storage conditions did not affect the stability of the vitamin D₂. Quality wise low specific volume bread and evenly distributed crumb textures were obtained in all baking protocols.

Keywords: Vitamin D, Vitamin D fortification, fortified bread, vitamin D₂, vitamin D₃

Abbreviations

RDA, Recommended daily allowance; UVB, Ultraviolet B; 25(OH)₂5-hydroxyvitamin D; 1,25(OH)₂D₂, 1, 25-dihydroxyvitamin D₂; PTH, Parathyroid hormone; DBP, Vitamin D-binding proteins; VDR, Vitamin D receptors; cGMP, Cyclic guanosine monophosphate; 1-OHase, 1-alpha-hydroxylase; LC-MS/MS, Liquid chromatography tandem mass spectrometry; IG, Insulin like growth factors; GH, Growth hormone; PUFA, Poly unsaturated fatty acids; HTST, High temperature-short time; UHT, Ultra high treatment; FU, Farinograph units; BU, Brabender units; RH, Relative humidity; SV, Specific volume; SBP, Standard baking process; LEM, Low energy mixing; HEM, High energy mixing; LBT, Low baking temperature; HBT, High baking temperature; ANOVA, Analysis of variance; M_{wet}, Wet mass; M_{dry}, Dry mass; V_b, Bread volume; FFQ, food frequency questionnaire Standard measurement units; µg, micro gram; IUs, International Units; IU/g, International Units per gram; mg, Milligrams; mL, milliliters; nmol/L, nanomoles per liter; Kg, kilogram; g, gram; °F, Degree Fahrenheit; °C, Degree centigrade; Ø, Diameter; rpm, revolution per minute.

Introduction

Vitamin D is one of the essential vitamins required by the human body. It helps to have a stable health condition and stronger bone structures. It exists in two principal forms: namely as vitamin D₂ and vitamin D₃. Humans can obtain a sufficient amount of vitamin D from sunlight. However, people living in northern European countries they experience a shortage of sunlight in the winter season. In such countries between November and March, the oral intake of vitamin D is significant.¹

People can obtain natural vitamin D₃ from Fish and egg yolk whereas vitamin D₂ from Wild Mushrooms. Generally, Foods which naturally contain vitamin D are quite limited. Because of this vitamin D fortifications have been practiced in different countries.¹ For example, fortified commercial food products especially margarine and milk products widely became available in Finland market over the past few years. As a result, most people started to consume fortified commercial food products. This has shown improvement in the oral intakes of vitamin D in this country.²

However, the overall intake of vitamin D in most European countries is deficient. The weather condition of the region, personal food preferences and poor nutrition style are the main reasons for low vitamin D intake in this area. Besides, the limited choices of vitamin D containing foods are also a cause of low intake in most European countries.³

The recommendations of using sunscreen and avoiding excessive sun exposure have exacerbated the risk of vitamin D deficiency in different parts of the world. E.g., the idea of leading sun-safe life caused a rapid increase in vitamin D deficiency in Australia.⁴

Inadequate intake of vitamin D can be a cause for a deformed pelvis and complicating delivery of babies in young women. At the end of 1600s rickets was identified as a critical health problem for children in many countries. In the late 19th, 90% of children from modern cities of North America and European countries suffered from rickets.³

According to the International Osteoporosis Foundation (IOF) report, vitamin D deficiency associated with osteoporosis, hip fractures, skeletal and non-skeletal disquiets. Annually Osteoporosis causes more than 8.9 million bone fractures every 3 seconds.⁵

The recommended daily intake of vitamin D depends on individuals' initial vitamin D status, BMI, and their regular sunlight exposure. For people with proper sunlight exposure, a daily intake of <20µg of vitamin D/day is enough. However, obese people, people affected by osteoporosis, people having less access to sunlight and individuals with malabsorption problem should take a high amount of vitamin D (up to 50µg/day). Immigrants coming from none European countries and residing in Europe are mostly affected by vitamin D deficiency. Therefore, controlling their serum 25(OH)D level is very important.⁶

People at different age group require a specific amount of vitamin D per day to keep their serum 25(OH) D at the

optimum level. A 50nmol/l of serum 25(OH)D concentrations considered as an optimum. To keep serum 25(OH)D at the optimum level, 15µg/day of vitamin D is recommended for the age of <70. Whereas, 20µg/day of vitamin D is enough at the age of 71. According to Institute of Medicine (IOM), the safe upper limit of vitamin D intake is raised from 50 to 100µg/day for adults' and from 25 to 75µg/day for children.⁵

Nordic nutrition recommendation suggested different approaches for improving vitamin D status of the people. For instance, teaching the people about the importance of taking vitamin D daily and increasing commercially available fortified food products are some of the suggested approaches. The Nordic nutrition recommendations increased the daily vitamin D intake from 5µg /day to 7.5µg / day at the age between 2 to 60years.⁶ However, people's living in European countries and who do not consume fish or drink fortified milk suffer the consequence of low vitamin D intake.

Food fortification method has been practiced as a possible means of enriching foods with micronutrients for a long time. For example in 2010, Finland raised the concentration of vitamin D in fluid milk and margarine to 1µg/100 g and 20µg/100 g respectively. However, in Finland and other European countries, most of the commercially available fortified foods are enriched only with vitamin D₃. Generally, compared to vitamin D₃ the consideration given for vitamin D₂ is very less.

The first part of this study focuses on brief introductions of vitamin D and its metabolism process in the human body. Further, it reviews different factors which affect the stability of vitamin D in food products. The second part of this study focuses on a short introduction of the bread baking process and bread quality attributes. The experimental section of this study describes the steps followed to conduct this research especially the bread baking process and vitamin D stability test.

Literature review

Vitamin D

Vitamin D (calciferol) is a steroid hormone and exists as fat-soluble secosterols in few foods. However, with the help of UVB radiation, it can be photosynthesized in the skin of vertebrates.^{3,7}

Vitamin D exists as vitamin D₃ (cholecalciferol) and Vitamin D₂ (ergocalciferol). If people can get proper sunlight, their skin can synthesize sufficient amount of vitamin D₃. Vitamin D₂ can be synthesized by UVB irradiation of plant or yeasts.^{8,9} Irradiating the "ergosterol" a (5,7-diene phytosterol) in fungi and phytoplankton can also produce vitamin D₂.¹⁰ The UVB irradiation process first transforms ergosterol into pre-vitamin D₂. Then the thermal energy (≈37°C) converts the pre-vitamin D₂ to final vitamin D₂.

Ultraviolet conversion of ergocalciferol to vitamin D was started in the late 1920s and later this method is licensed for pharmaceutical companies to produce vitamin D₂ supplement called Viosterol.¹¹ Compared to vitamin D₃,

vitamin D₂ has a less hypercalcemic effect. For this reason, vitamin D₂ is considered for the potential therapeutic agent of cancer and other easily prorogating coetaneous disorders.¹²

According to different studies, both vitamin D₂ and vitamin D₃ have shown comparable efficiency in improving vitamin D status of the people. E.g., people who took 1250µg/g of vitamin D₂ twice per week for consecutive five weeks, their serum 25(OH)D level increased by 100%. Their bone hip and spine mineral densities are also well developed.⁴

Premature birth, skin pigmentation, inadequate sunlight exposure, obesity, improper vitamin D absorption, and age are some of the factors that intensify the vitamin D deficiency problem across the world. Severe vitamin D deficiency is a cause of rickets and osteomalacia in infants and children that leads to reduced bone mineralization. Besides, severe vitamin D deficiency can also cause improper intestinal calcium absorption which is a primary cause of reduced muscle strength and osteoporosis.⁷ Furthermore, individuals with vitamin D deficiency problem can also suffer from lower serum 1,25(OH)₂D concentration and low calcium level. These promote higher production of PTH that cause fast bone mass loss and increased bone resorption.⁸

Adequate intake of vitamin D either from dietary sources or supplementing tablets is essential to maintain a balanced level of serum 25(OH) D concentrations. For individuals living in a region where there is no sufficient sunlight, Vitamin D intake of 25µg/g per day is essential.

Vitamin D metabolism

Vitamin D₂ and vitamin D₃ have different metabolic pathways. The structural difference between them is the cause of dissimilarity in their respective metabolic pathway. Pre-vitamin D₂ and vitamin D₃ are biologically inactive. Therefore the metabolic reaction which takes place in the liver and kidney changes them to their active metabolites. However, both vitamin D₂ and vitamin D₃ yield similar final metabolites.

Vitamin D₂ has a methyl group on a C₂₄ and double bond at C₂₂-C₂₃ where the oxidative process takes place on the side chains. Even though the oxidative process of vitamin D₂ is different from vitamin D₃, the *in vivo* metabolic conversion of vitamin D₂ and vitamin D₃ takes place through similar hydroxylating enzymes. Later the hydroxylation process produces identical 24-/25- hydroxy final derivatives for both vitamin D types.¹²

If the source of the vitamin D is a dietary source, firstly it is fused into chylomicrons before it is absorbed in the lymphatic system. The vitamin D fusion in chylomicrons values 80% of vitamin D absorption in the small intestine.¹³ However, Vitamin D obtained from any sources pass through double hydroxylation process that converts it into final active metabolites.¹⁴

Vitamin D₂ metabolic pathway yields metabolically active hormone called 1,25-dihydroxy vitamin D₂ [1,25(OH)₂D₂]

J.¹⁵ The Liver metabolically converts vitamin D₂ to 25-hydroxyvitamin D[25(OH)D] whereas the kidney changes it to 1,25-dihydroxy vitamin D.⁴

The metabolic process starts with the transportation of pre-vitamin D to the liver by vitamin D- binding proteins (DBP). Once the delivery of pre-vitamin D takes place to the liver, enzyme- catalyzed hydroxylation process takes place on C-25, and this converts the inactive form of vitamin D to active 25-hydroxyvitamin D [25(OH)D] form. Microsomal cytochrome P450 enzyme CYP2R1 and/or mitochondrial cytochrome P450 CYP27A1 catalyze the hydroxylation reaction. In addition to these enzymes, various cytochrome P450 mixed function oxidase enzymes (CYP2C11, CYP3A4, CYP2D5, and CYP2J3) are also involved in the hydroxylation process. After the first hydroxylation process, 25(OH)D enters the circulation system. 25(OH)D has a shorter half-life for about two weeks, and the average serum 25(OH)D concentration between 25nmol/L to 200nmol/L is optimum to lead a healthy lifestyle.¹⁶

The second enzyme assisted hydroxylation process takes place in kidney and enzyme 1-α-hydroxylase (CYP27B1) assists the hydroxylation process. The hydroxylation process takes place at carbon 1 of the A ring and yields bioactive metabolite called calcitriol or 1,25-dihydroxy vitamin D₃ [1,25(OH)₂D₃]. This bioactive metabolite (calcitriol) is responsible for the most biologic function of vitamin D.^{14,17}

Calcitriol (1α, 25(OH)₂D₃) starts its biological function in the kidney. 1α,25(OH)₂D has a shorter life for about 5 hours. With the help of DBP Calcitriol is transported to different favorable tissue destinations (vitamin D receptors) and act in genomic and nongenomic ways. The target tissue of acting includes bone, intestine and parathyroid glands. VDR intermediates the calcitriol gene expression process within few hours. In contrary, the nongenomic calcitriol activity takes place within a few seconds or minutes. Nongenomic calcitriol activity includes acceleration of phosphoinositide metabolism, rising intracellular calcium, and cyclic guanosine monophosphate (cGMP) concentration, promoting intestinal calcium and phosphate flow and stimulation of protein kinase- C activity.¹⁶

All 25-hydroxylated molecules bind to DBP (transcalciferin). Whereas none hydroxylated molecules formed at the first stage of vitamin D activation stored in the adipose tissue.^{15,18}

The healthy individual's serum of 25(OH)D₃ concentration lies between 95.4-159nmol/L. If a person serum 25(OH)D₃ concentration is <15.9nmol/L it is a sign of a severe vitamin D deficiency problem possibly reflected by osteomalacia or rickets.^{14,17}

CYP27B1 is 1-α-hydroxylase (1-OHase) enzyme and catalyzes the formation of the active form of vitamin D called 1,25-dihydroxy vitamin D [1,25(OH)₂D].¹⁹ Calcium, parathyroid hormone (PTH), calcitonin, growth hormone (GH) and insulin-like growth factors (IGF) positively control CYP27B1 activity. In contrary, phosphates, fibroblast growth factors (FGF)-23, klotho, and (1,25(OH)₂D₃) negatively control CYP27B1 activity. Low calcium level in our serum

fastens the release of PTH that promotes the high renal activity of CYP27B1 and production of (1, 25(OH)₂D₃).

However, if the calcium level in the serum is at the optimum amount it suppresses the CYP27B1 activity. Generally, 1, 25(OH)₂D₃ controls the serum calcium level in three principal ways. It minimizes the renal calcium discharge, enhances intestinal calcium assimilation or enhances maturity of osteoclasts which release calcium from bones.¹⁴

Bile is the primary organ responsible for Vitamin D excretion process. The vitamin D metabolic pathway produces water-soluble metabolites like calcitroic acid and excreted by the kidney through the urine systems (Figure 1).¹³

The diagram shows the coetaneous conversion of pro-vitamin D₃ (7-dehydrocholesterol) to pre-vitamin D₃ by UVB irradiation to form vitamin D₃ (cholecalciferol). Firstly, pre-vitamin D₃ is isomerized in the epidermal basal layer then attached to vitamin binding proteins (DBP) and transported to the liver. Vitamin D₃ is converted to 25-hydroxycholecalciferol (25(OH)D₃) by hydroxylases (25-OHase) enzyme. 25(OH)D₃ further hydroxylated in the kidney to yield active secosteroid called 1α, 25(OH)₂D₃ (calcitriol). This Calcitriol is the final metabolite and acts on different target tissues. PTH positively promotes the production of 1α 25(OH)₂D₃ whereas Ca²⁺, Pi, and 1α, 25(OH)₂D₃ negatively hinders the production of Calcitriol (Table 1).²⁰

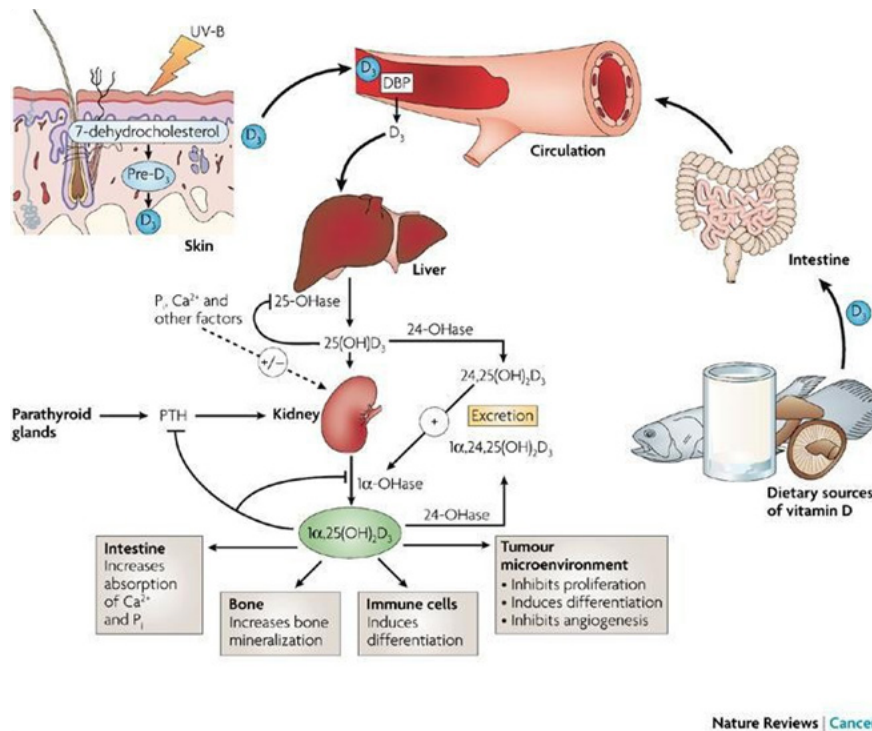


Figure 1 Vitamin D metabolism.

Table 1 The associations between osteomalacia, plasma phosphate and 1.25(OH)D₂D₃ in various clinical disorders²¹

State	Plasma phosphate	Plasma 1.25(OH)D ₂ D ₃	Osteoid mineralization
Vitamin D deficiency	Low	Low	Impaired
Hyperparathyroidism	High	Low	Normal
Renal Failure	Always High	Absent	Normal (in variable proportion of patients)
Vitamin D-resistant rickets	Low	Normal	Impaired
Vitamin D-dependent rickets, Type I	Low	Low	Impaired
Vitamin D-dependent rickets, Type II	Low	Normal	Impaired
Phosphate deprivation	Low	High	Impaired

Vitamin D has a different role in the physiological activity of the human body. It supplies calcium and phosphate to a gut, bone, and kidneys. It also facilitates the production of PTH and calcitonin. Vitamin D is inactive at a physiological level. Therefore it is primarily metabolized to an active form of 1,25(OH)₂D₃. Low level of 1,25(OH)₂D₃ promotes poor calcium absorption in the human body. Physiological doses of 1,25(OH)₂D₃ is given for individuals suffering from chronic renal failure and hyperparathyroidism to reinstate their health status. The concentration of 1,25(OH)₂D₃ within the plasma is 1000-fold less than 25(OH)₂D₃ and its physiological production rate ranges 0.2 to 1 μg per day.²¹

Metabolic pathway difference between vitamin D₂ and vitamin D₃: Vitamin D₂ and vitamin D₃ have slight structure

difference on their side chains. Because of this structural difference, they follow different metabolic pathway and produce different biologically active metabolites.¹¹ Presence of unsaturated carbon on C-22/C-23 and a methyl group on C-24 affect their respective hydroxylation process. However, both forms of the vitamin D pass through a 25-hydroxylation process.¹⁵

Following the 25-hydroxylation, 25(OH)D pass through the second hydroxylation process which takes place on C24 and yields 24,25(OH)₂D, i.e., the corresponding vitamin D₂ final metabolite. Likewise, 1,25(OH)₂D undergo a hydroxylation process on the same C24 and yields 1,24,25(OH)₃D₂. Both hydroxylation processes take place in kidneys (Figure 2).

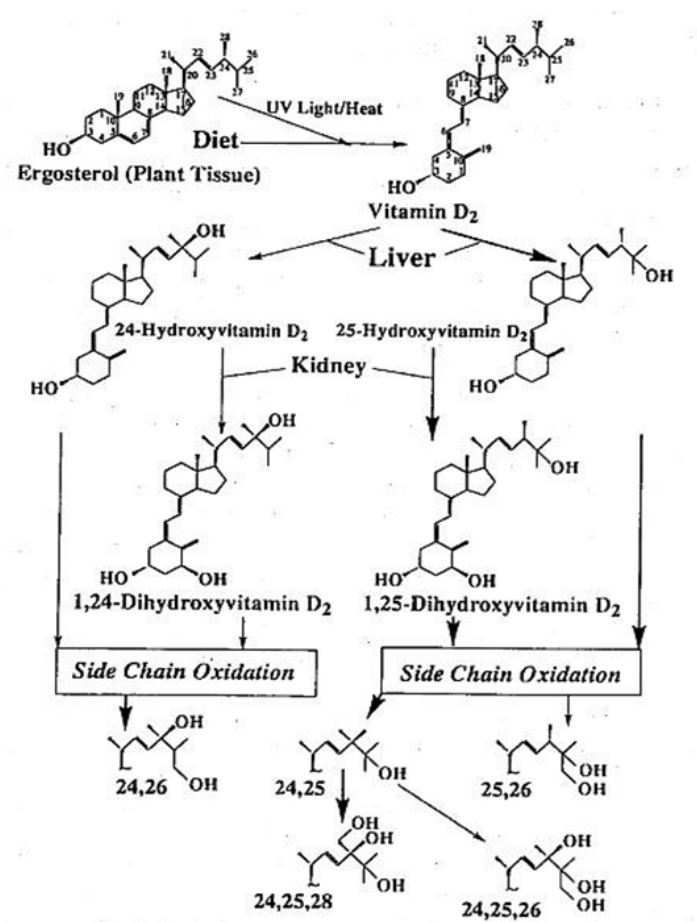


Figure 2 Vitamin D₂ metabolism pathway.¹⁵

Formation of 1,24,25(OH)₃D₂ deactivate vitamin D₂ molecule. In contrary, when the corresponding vitamin D₃ metabolite (1,24,25(OH)₃D₃) pass through further side-chain oxidation, it biologically deactivates vitamin D₃ molecules. Compared to 1,25(OH)₂D₃, 1,24, 25(OH)₃D has a higher binding ability to VDR (>40%). A higher binding ability to VDR promotes the greater biological activity of 1, 24, 25(OH)₃D.¹¹

The 24-hydroxylation process of vitamin D₂ metabolism takes place in the liver and yields 20–50% of 24(OH)D₂. Following the 24-hydroxylation process in the liver, the

kidney further transforms 24(OH)D₂ to 1,24(OH)₂D₂. The affinity of vitamin D metabolites to bind themselves to VDR determines their biological activity or function. Compared to 1,25(OH)₂D₃ and 1,24(OH)₂D₃, 1,24(OH)₂D₂ has less affinity to VDR. Generally, the bioefficacy of vitamin D₃ is better than vitamin D₂.¹¹

Unlike Vitamin D₃, Vitamin D₂ is directly metabolized to 24(OH)D, and this is because Vitamin D₃ metabolites have higher affinities to hepatic 25-hydroxylase, DBP and VDR.¹¹

Sources of vitamin D & its bioavailability

Under sufficient sunlight exposure, human skin can provide 80 to 100% of vitamin D need. However, individuals living in areas where there is a shortage of sunlight they require a dietary source of vitamin D or vitamin D supplementing tablets.⁷ Enriched milk, varies dairy-based products, orange juices and in some fortified cereal products can provide Vitamin D. Vitamin D₃ is primarily obtained from sunlight. Fish contains 3 to 12.5 µg of vitamin D₃ per 3-oz of serving, and this accounts 50 to 200% of the daily recommended intake. Most foods other than fish and marine products contain a low amount of vitamin D₃. Unfortified whole milk and cheese can only provide 1% of the daily recommended intake, and this is insufficient for proper nutrition. Therefore fortified foods are extra food options for our daily vitamin D need.²²

Oily fishes and lanolin are some of the natural sources of vitamin D₂. UVB treated mushrooms and yeasts are a potential source of vitamin D₂. Both Vitamin D₂ and vitamin D₃ can also be provided as a supplementing tablet.²³

Studies have shown that different population groups cannot get enough vitamin D intakes. Environmental, cultural and physiological factors can be reasons for improper vitamin D intake, and this restricts the people not to fulfill their daily vitamin D need from sunlight (UVB). Therefore, other dietary sources of vitamin D are essential. To fulfill the vitamin D need of a people fortification method has been practiced in different countries. For example, in Canada, it is compulsory to fortify milk with 2.5 µg /250mL of vitamin D and margarine with 13.25µg of vitamin D/10g. In USA fortification of milk is not compulsory. However, breakfast cereals and fruit juices are fortified from 1-3.5µg of vitamin D/ serving.²⁴

Several bio-availability studies have been conducted to analyze the efficacy of consuming vitamin D fortified foods on balancing people's serum 25(OH)D concentration. For instance, a single serving of rye and wheat bread fortified with 10µg of vitamin D₃/100g of bread have increased the serum 25(OH)D concentration of the people without affecting their parathyroid hormone (PTH). However, increasing the amount of vitamin D₃ added in rye and wheat bread to 125µg of vitamin D₃/serving and lengthening the intervention time to 1year has increased the serum 25(OH)D concentrations and reduced their PTH.²⁴

In a study conducted on people, who consumed cheese fortified with 100µg cholecalciferol/ds for consecutive eight weeks, their serum 25(OH)D concentration has shown increment to nearly 75nmol/L.¹ According to a study conducted in the USA adults who consumed fortified orange juice more than one glass per day, their vitamin D status is well improved. Fruit juices fortified with 25 µg of vitamin D₃/236.6mL of juice given to the examinees, and they consumed for consecutive 12weeks. At the end of the intervention time, the examinees' serum 25(OH)D concentrations have increased by 150%.²⁴

In another study consumption of Wild mushroom has also shown an effect of increasing serum 25(OH)D

concentrations of examinees i.e. Equivalent of taking 10µg of vitamin D₂ supplements. However, bio-availability studies related to vitamin D₂ are insufficient to deeply understand its effect on balancing serum 25 (OH) D concentrations.²⁴

Vitamin D fortification and the required accuracy in the fortification process

People living in the subtropical region of our world obtain an inadequate amount of vitamin D. This is mainly related to the shortage of sunlight in the region. Therefore, people living in these areas require extra sources of vitamin D either from fortified foods or supplementing tablets.²⁵ Food fortification process has been practiced as a means of preventing micronutrient deficiency problems across the world. The effectiveness of the fortification method is mostly dependent on the skill and experience of the expertise involved in the fortification tasks.²⁶

Failing to deliver the required amount of micronutrients to the consumers and less stability of fortificants are the main challenges of food fortification process.²⁵ To complete the fortification process successfully it is highly recommended to identify the compatibility of the food vehicle and fortifying agents. Furthermore, Identifying the resistivity of fortificant toward degradation and reduction of losses in the fortification processes is also essential.²⁷ For instance, multiple fortificants within the same food vehicles may result in a high cost of production and reduced bioavailability.²⁶ Working with the proper dosage of fortifying ingredients is essential for two fundamental reasons. Firstly, the fortification process should sufficiently meet the recommended daily allowance (RDA) of micronutrients for the targeted consumers. Secondly, since a low amount of fortificant is used in the food enrichment process, it is decisive to use extraordinary equipment which can stream the right amount of fortificant in the food vehicles. E.g., the daily recommended intake of vitamin D in most European countries is 5µg/day for adults and 10µg between the age of 60 to 65years.²⁸ Therefore, to deliver the right amount of vitamin D through chosen fortified foods, the process should be done at a high level of accuracy.²⁵ Besides, controlling the required amount of micronutrients in fortified foods is also essential to keep the original organoleptic property of the food and for the stability of micronutrients. Recently, different types of equipment which have a capacity of protecting vitamin D degradation caused by UV light and by refrigeration system are available on the market. These types of equipment can maintain the colloidal stability of vitamin D in food matrixes.²⁷

Stability of vitamin D in fortified foods

Most studies conducted concerning vitamin D fortification process and identification of its stability in foods is carried out in milk and milk products. Studies conducted in other types of food are insufficient.¹ Even though different studies have conferred vitamin D is stable under different processing and storage conditions, heating the pure and dry form of vitamin D at 150°C in the presence of air has shown its total loss.²⁹⁻³¹ There is also a study which shows the susceptibility of vitamin D to oxidation and its poor

stability in extruded food storages.³²

Stability of vitamin D in milk and other milk products:

Stability of vitamin D in low-fat strawberry yogurt, in HTST treated-processed milk and UHT treated-processed chocolate milk was studied. Besides, the effect of increasing vitamin D concentration on the sensory property and its stability in different storage condition were studied in the above milk products. Within this study the content of vitamin D added was increased from 2.5µg/serving to 6.29µg/serving and a cold-water spreadable vitamin D₃ concentrate was used. The storage times were 21days for HTST-processed 2% fat milk, 60days for UHT processed 2% fat chocolate milk and 42days for Low-fat strawberry yogurt. Vitamin D measurement was carried out throughout the storage days. The sensory analysis was also conducted on day 14 for HTST-processed 2% fat milk, on day 40 for UHT for processed 2% fat chocolate milk and day 28 for Low-fat Strawberry yogurt. Increasing the amount of vitamin D added for enrichment purpose to 6.29µg/serving did not affect the sensory perception of the consumers, and there was no observed vitamin D loss in all milk products. The Vitamin D was also stable in all milk products after each storage days.²⁹

Stability of vitamin D₃ and its impact on the flavor of the food vehicle were examined in fortified Cheddar cheese. In this study 5 and 10µg/serving emulsion form of vitamin D₃ was added in cheese milk. The result of this experiment confirmed that vitamin D₃ had shown stability for nine months. Compared to the unfortified cheese the flavor and taste of fortified cheddar cheese were equivalently liked by consumers. The flavor of the cheese was also unchanged for 9months of storage.²⁵

Effects of using different forms of vitamin D in the fortification process were also studied in Cheddar cheese production. To examine this 10µg/L of vitamin D were added in three different forms, these are in commercial water-soluble emulsion form (Vitex D), homogenized crystalline liposoluble vitamin D in a portion of cream and as water-soluble vitamin D entrapped in multilamellar liposomes (Prolipo-Duo™). Comparing the vitamin D content in fortified cheese and control cheese, forms of vitamin D used in this study did not significantly alter the protein, fat, moisture and salt content of the cheese. However, the vitamin D was best recovered when it was entrained (61.5±5.4%) in liposomes unlike when it is in a portion of cream or Vitex D form. While 40.5±2.2% of vitamin D was recovered in a portion of cream, 42.7±1.7% of vitamin D was recovered in a Vitex D form. Vitamin D stability was not affected after 3-5months of cheese ripening. However, liposome-encapsulated vitamin D has shown poor stability in the after five months of ripening. Vitamin D recovery measurement in the cheese indicates that 39% of vitamin D added in liposome form, 59 % of vitamin D added in Vitex D form and 57% added in cream milk form were either lost in whey or destroyed in cheese making process. The acidification caused from lactic acid bacteria fermentation or oxygen might have caused instability of vitamin D in cheese making process.

The highest vitamin D recovery in an encapsulated liposome can be associated with the protection of vitamin D in the encapsulation process. In contrary, the lowest recovery of vitamin D was observed when vitamin D was added to milk in the homogenization method. The homogenization process might give less protection to vitamin D compared to Vitex D form.

Ripening of cheeses in the absence of air (in a vacuum packaging) and low-temperature storage in the absence of light best maintained the stability of vitamin D in the cheese products.³³ Pasteurizing cheddar cheese milk, which contains vitamin D₃ at 72°C for 6sec, did not affect the stability of vitamin D₃.¹ Likewise, vitamin D which naturally exists in some foods or fortified foods has shown resistance to pasteurization and sterilization temperature of 62.8°C/30min and 115.6°C/15min respectively.³⁴

Vitamin D₃ has shown resistance to heat in fortified Cheddar cheeses and low-fat cheese production. 91% and 55% of vitamin D₃ were recovered in fortified Cheddar and low-fat cheese production respectively. The remaining percentage of vitamin D₃ assumed to be entrapped in the whey protein. Processing cheeses at 232°C/5min and ripening it between 3-8°C for over one year did not affect the stability of vitamin D. Vitamin D₃ is also retained when fortified cheese was stored between 4-6°C and 21-29°C for more than nine months. It is also homogeneously dispersed all over the cheese pieces. Furthermore, the chemical property and flavor of the Cheddar cheese was not altered throughout the storage times.

In another study, 25 to 30% of vitamin D₃ was lost when cheese milk is heated at 232°C for 5minutes.³⁵ There are studies which have shown susceptibility of Vitamin D₂ to temperature, oxygen, light, and moisture.³⁶ Stability of vitamin D₂ in milk processing, in different packaging types and various storage conditions, were investigated. Three different types of heat treatment techniques were compared to see their effect on vitamin D₂ stability in milk. Effect of sterilization at 121°C/15min at 15psi, Pasteurization at 63°C/30min and boiling temperature on the stability of vitamin D were studied. The results of the study have shown that there was no significant loss of vitamin D₂ in all heat treatment techniques. To investigate the effect of storage condition on the stability of the vitamin D₂ the milk samples were stored in plastic and glass bottles for consecutive seven days in refrigeration storage. There was no observed difference in vitamin D₂ retention between the two packing types. However, the polyethylene bag has shown minor loss of vitamin D₂ from 14.9µg to 13.7µg which can be related with vitamin D₂ sorption in polyethylene pouches.³⁷

To investigate the effect of light on vitamin D₂ stability fortified milk samples were stored in polyethylene pouch and glass packaging under three different light intensities (namely 1485, 2970 and 4455 lux). The results have shown that glass packaging was compelling enough to maintain the vitamin D₂ stability better than the polyethylene pouch.³⁷ Vitamin D₂ has lower bioactivity which makes it sensitive to

lose. In particular exposing the crystalline vitamin D₂ powder to temperatures, humidity, and different storage vessels can fasten its lose.¹¹

Vitamin D stability in none-milk products: Effect of household cooking methods on the stability of vitamin D₃ in fortified bread, egg, and margarine were studied. According to one the study, 39-45% of vitamin D₃ was retained when eggs and margarine heated in the oven for 40min. 60% loss of vitamin D after the oven cooking process indicates that vitamin D in eggs might be bind to protein or esters (the non-specific storage form of vitamin D) that couldn't protect the vitamin D from heat destruction. Unlike cooking method in the oven better retention of vitamin D (82-84%) in eggs and margarine is observed by frying method. Boiling eggs also has shown comparable retention of vitamin D₃ like the frying method (86-88%). Even though cooking methods play a central role in vitamin D loss, type of the food on which the cooking method applied also affect the vitamin D loss.³⁸

Bread is a standard diet across the world. For this reason, it has drawn attention for using it as a fortification vehicle. Stability of Vitamin D₃, its dispersion property in bread and its bioavailability from fortified wheat and rye bread were studied. To study these rye and wheat bread were fortified with 12mg/100g of Cholecalciferol (Vitamin D₃) and their stability after the baking process was analyzed. The stability test result has shown that 79 to 109% of cholecalciferol was recovered after the baking process and this confirmed its stability in the bread baking process. Besides, Cholecalciferol was evenly distributed throughout the dough matrix and in the final bread texture.³⁹

Compared to rye bread the wheat bread has shown better retention of both vitamin D₂ and vitamin D₃. 69 and 85% of vitamin D₃ were retained in rye and wheat bread respectively. Similarly, 73% and 89% of vitamin D₂ were retained in rye and wheat bread respectively. Unlike wheat bread, rye bread had shown poor vitamin D retention when it was baked at 200°C for 30min. The final temperature of rye bread was lower than the wheat bread. The authors of the study concluded that baking temperature was not the reason for lower retention of vitamin D in the rye bread instead they have speculated that the Lower pH in rye bread might be promoted the acidic isomerization of the vitamin D to isotachysterol and this resulted in a lower retention of vitamin D in the rye bread.^{38,40}

Vitamin D₃ stability in fortified orange juices was studied. The orange juice was fortified with 25µg of vitamin D₃/240mL and stored at 4°C for consecutive 30days. The study result showed that the vitamin D₃ was stable throughout the storage days.

Vitamins D₂ and D₃ also exist as white to yellowish water-insoluble crystalline powders. They exhibit reversible thermal isomerization that creates equilibrium fusion containing their equivalent pre-vitamins. Stability of vitamin D in fats and oils is similar to the stability of the fat itself. If vitamin D is not confined to the food matrix, it is vulnerable

to oxygen and light- induced destruction. Exposure of vitamin D containing thin films to heated air or diffusion of vitamin D containing alcoholic solution into the aqueous phase in the presence of oxygen promotes its destruction. Vitamin D is stable in alkaline condition. However, in the acidic environment or mild acidity, it isomerizes to 5.6-trans and isotachysterol, and this makes the vitamin D incapable of curing/preventing rickets (poor antirachitic activity).⁴¹

Different works of literature have shown lower bioactivity and reduced stability of vitamin D₂ crystalline powder. Exposing vitamin D₂ crystalline powder to unstable temperature, humidity or different storage container promotes its instability.¹¹ However, studies related with vitamin D₂ stability in foods and fortification process is quite limited.

Source of vitamin D₂ that was used in this research work was obtained from yeast *Saccharomyces cerevisiae*. The yeast is treated with UV light to photo-chemically convert the endogenous ergosterol component in the yeast to vitamin D₂ alternatively named as ergocalciferol.⁴²

Bread baking process

Bread recipes: Wheat flour constitutes 50 to 60% (formula weight) of bread recipes, and it takes the highest proportion of bread ingredients. Other ingredients such as leavening agents (yeast or different bacterial strains, chemical leavenings), dairy materials, egg, and starch are minor ingredients. Minor ingredients are usually added 5 to 10% (formula weight). Micro ingredients are added up to 5% (usually to nearly 0.1%) and function as oxidation enhancer and fortification ingredients. Micro ingredients also improve the baking activity and nutritional quality of baked products.⁴³

Salt is one of characterizing ingredient in bread baking process mainly added as a flavor enhancer and addition of salt (NaCl) between 1.5-2% maintains better bread volume.^{44,45} Sugar is used as a substrate in the bread dough fermentation process.⁴⁶ The inclusion of oil mixture and solid fat in bread formula plays an essential role in gas retention. Effect of adding palm oil/Palm Sterin based shortening in white bread recipes have shown bread quality improvements such as improvement of loaf volume, beautifying bread texture and formation of golden brown crust color.⁴⁷ Water will have an impact in overall dough rheological property and formation of a continuous viscoelastic gluten network.⁴⁸ The Farinograph (empirical mixing machine) is used to determine the water absorption property of wheat flour, the optimum time for proper dough development and measures the stability of dough.⁴⁹ The water absorption property of wheat flour is represented on the Farinograph by the volume of water added to the dough to reach 500 Brabender units during the mixing process.^{11,44}

The leavening agent yeast: In bread baking process the yeast (*Saccharomyces cerevisiae*) is used as a leavening agent. It promotes the generation of gas bubbles which is best for dough proofing and oven spring. Yeast is the solely leavening agent used in bread baking unlike other chemical

leavening agents used in many other bakery products. Yeast converts sugars formed from enzymatic hydrolysis of wheat starch or added sugars to carbon dioxide and alcohol. The alcohol which is created from enzymatic hydrolysis later evaporates during the oven baking processes. The most common types of yeast used in bread baking process are fresh compressed yeast, yeast cake or crumbled yeast, liquid cream yeast, or active dry yeast.⁵⁰

Besides functioning as a leavening agent yeast also functions as a nutritional ingredient. Nutritional yeasts are wealthy in containing significant amount nutrients such as the B vitamins, chromium, sixteen different amino acids, more than fourteen types of minerals, and seventeen different vitamins (except vitamins A, C, and E). Furthermore, yeast is also prosperous in phosphorus, protein and it is high energy food.⁵¹

UV irradiation of baker's yeast (*Saccharomyces cerevisiae*) at 254nm can produce vitamin D₂. The irradiation process transforms the ergosterol in the yeast to vitamin D₂ (ergocalciferol). The irradiation process cannot affect the natural gassing power of the yeast that makes it usable in

yeast-leavened bread, rolls, and pastry. The gene mutation caused by UV treatment process of the yeast is negligible that would not allow mutants to prevail in the final fortified products. The vitamin D enriched novel yeast can serve as a naturally available food option for vegan diets.⁵²

Overview of the bread baking process

Dough development process includes sequential steps of weighing the intended ingredients, mixing of the ingredients, fermentation process, dough dividing, molding, and panning of proved dough pieces for a final baking process (Figure 3). In the straight dough makeup process, ingredients are mixed, and the dough allowed to rest for a short time. Mixing unit operation is the first step of a baking process and at this stage desired level of the major-ingredients like wheat flour, yeast, salt and other micro-ingredients are mixed. In addition, the mixing process promotes incorporation of air bubbles and develops the gluten network. Development of gluten network further expands the bread structure and allows the incorporation of gases liberated from the fermentation process.⁵⁰

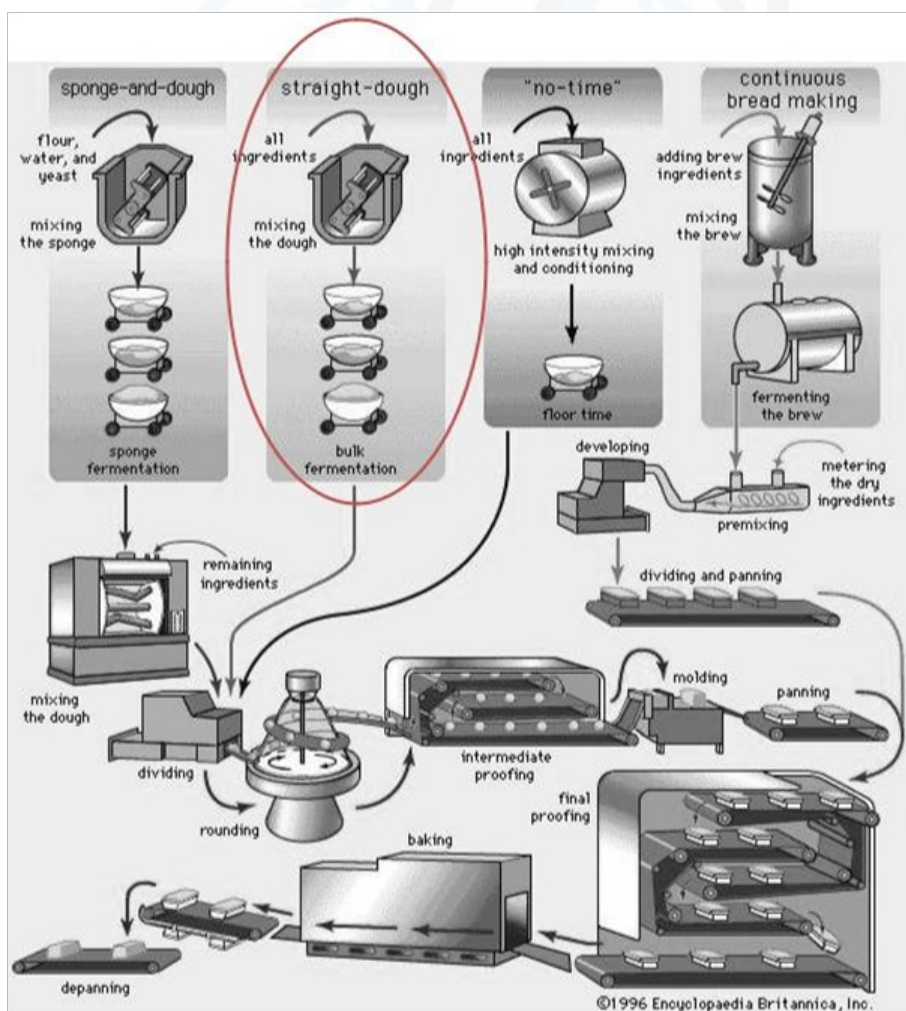


Figure 3 Summarized explanation of commercial bread Baking process.

The strength of mixing intensity required for optimum dough development depends on different factors such as on mixing speed, mixer arrangements, and wheat flour properties. In the dough mixing process it is essential to control temperature rise caused by the mixing intensity. High temperature caused from the mixing process can weaken the dough structure and forms sticky dough complex. Therefore, maintaining a dough temperature between 30 to 32°C is the very crucial requirement for desired chemical and enzymatic reaction in the dough.⁵⁰ Maintaining the dough mixing intensity above critical mixing speed and controlling the energy input are essential for optimum dough development.⁵³ However, the relationship between optimum dough development, dough aeration, and dough rheology is not much known.⁵⁴

Following the mixing process, dough resting takes place. At dough resting stage rheological modifications and improvements of dough pieces takes place.^{45,50} After necessary rheological modifications on dough resting stage, the dough is divided into pieces and rounded before it goes to the intermediate proving stage. Dough dividing and first molding stages are the consecutive stages that give the dough its desired weight and regular shape. The intermediate proving which lasts 4 -10minutes helps the dough to relax before the final molding process. Following the intermediate proving stage, a Low pressure is applied to the dough, and this gives final shape for the dough. The dough having the desired shape is forwarded to the final proving stage. Final proving takes place between 27°C to 40°C and a relative humidity of 85% for 41 to 60 minutes. The final proving fastens the yeast activity and dough pieces expansion.^{50,55}

Oven baking is the final stage of the bread making process before bread cooling and storage.⁴⁵ Closely monitoring the temperature and time combination is very important during the oven baking process.⁵⁰ For most bread types the baking temperature and time combinations ranges from 200 to 240°C for 10 to 30min respectively.⁵⁶ Oven spring, coagulation of protein, gelatinization of starches, crust development and crust browning are some of the central phenomenon happening in the oven baking process.⁵⁰

Major bread quality indicators

Loaf volume is one of the leading bread quality indicators. Loaf volume tells the level of gases produced during the fermentation process and the amount of gas retained after the baking process. Loaf volume can also indicate the quality of the flour protein and the effectiveness of the baking process in developing well-structured gluten network.⁵⁰ The specific volumes of bread tell the efficacy of dough fermentation process and plasticity of gluten network caused from prolonged fermentation time. Prolonged fermentation time causes poor gluten plasticity which results in a low loaf volume. Besides, the specific volume of bread also tells the intensity of dough kneading process. Extensive dough kneading process results in poor loaves volume.⁵⁷ Oven spring is another quality symbol

which indicates bread height rise in oven baking process. It compares the difference in height between the standard proving stage and after the oven baking process. Monitoring the optimum level of oven spring is very important. Less control on oven spring stage cause easy disassembling of crust structure from the top surface of the bread.⁵⁰

The amount of damaged starch in the flour can affect a yeast activity and crust color formation. If the amount of damaged starch in the flour is meager, the α -Amylase enzyme will have a small access to starches to liberate enough sugars for the yeast activity. If the amount of sugar liberated is lower, it can be an indicator that a negligible amount of sugars is left for Millard reaction that is the sole source for crust color formation. However, if there is a high level of a damaged starch or extra sugar added as milk powder or other sugar recipes, it can promote high α -Amylase activity which drives excessive crust color formation.⁵⁰

Gas bubbles formed from the mixing process have a significant role in forming right bread texture. If the gas bubbles are not kept intact during the subsequent baking processes, it is possible that the crumb color can be a gray color and the texture can be firmer.⁵⁰ Hollow structure formation on the crumb surface of bread is also another quality indicator. It tells the effectiveness of the dough handling process to keep intact the whole dough structure. It also shows how much the dough is weak concerning added recipes and processing conditions.⁵⁰

Experimental researches

Aims

Previous studies which were related to vitamin D have been only focusing on vitamin D₃ especially on its bio-availability and its stability.

The studies related to vitamin D₂ are quite limited. The correlation between unit operations of food processing and the stability of vitamin D₂ in foods is not well known. Moreover, there are studies which consider vitamin D₂ as an inappropriate nutrient for supplementation and food fortification process.¹¹

Therefore, this research aimed the following fundamental objectives:

- i. Investigate the stability of vitamin D₂ in white wheat bread and analyze loss of vitamin D₂ associated with the fortification process.
- ii. It studies the effect of bread baking unit operations on the stability of vitamin D₂. With the hypothesis of vitamin D₂ susceptibility to oxygen and temperature, the effect of mixing and baking temperature will be examined.
- iii. It studies vitamin D₂ stability in the bread under different storage conditions.
- iv. It studies the effect of vitamin D₂ containing bio-enriched yeast on bread quality.

Materials and methods

Ingredients used for the experiment: The main ingredient wheat flour was obtained from Myllyn Paras Oy (wheat milling company in Finland). The flour extraction rate, moisture content, ash content and protein content of the flour were 77%, 11.55%, 0.65% and 12.5% respectively. The bread formula designed for this experiment was based on flour weight,⁵⁸ and the final targeted dough mass was 2.5kg. The leavening agent used for the baking process was a yeast cream obtained from LALLEMAND Inc. The relative percentage and the amount of each recipe used listed on the Table 2. The rest of ingredients used for the baking process were obtained from commercially available products.

$$\text{amount of total flour} = \frac{\text{intended dough weight}}{\text{total percentages/total of flour percent}(100)}$$

The amount of recipes shown above (Table 2) was calculated based on the formula given below⁵⁸

Table 2 Recipes of the experimental bread

List of Recipes	Per flour weight (%)	Weight (g)
Wheat flour	100%	1470
Water	59.33%	872.2
Yeast cream	4.80%	42.35
Salt	29.40%	29.4
Sugar	1.20%	17.64
Rape seed oil	5%	73.5
Vitamin D ₂ yeast	0.05%	1.3645
Total	199.73	2505.14

Fortification ingredient: Novel yeast obtained from LALLEMAND Inc used as a fortification agent. The novel yeast is UVB treated to enrich it with Vitamin D₂. Even though this yeast has a gassing power, it was not used as the primary baker’s yeast. Instead, it is used only for enrichment purpose. The yeast is enriched with 432.5µg of vitamin D/g of yeast. Doing the necessary calculation 1.3645g of the novel yeast was added to the targeted dough mass of 2.5kg. The study aimed to provide 25µg of vitamin D₂/90g of bread. The weighed amount of yeast was dissolved in lukewarm water and mixed with the dough.

Preliminary experiments

Before starting the bread baking process the wheat flour moisture content, water absorption and the mixing behavior during dough formation were analyzed. Detail of the preliminary experiments described as the follows:

Moisture content determination: Determining the moisture content in the wheat flour is essential to understand the shelf life instability of bread caused by enzymatic and microbial activity. Besides, understanding the moisture content in the flour is helpful for controlling the dough consistency during the mixing process.

Oven drying method was used to determine the moisture content in the wheat flour.⁵⁹ The method compares the moisture content difference before and after the oven

drying. Firstly, two aluminum crucibles with an opened lid were placed in an oven for 1h at 130°C. After 1hour of oven drying the crucibles were kept in desiccators for 60minutes. Following this, the empty crucibles were weighed with closed lids. 5g of wheat flour sample was placed in tarred crucibles, and a combined weight of sample and crucibles was registered. Then the samples were dried at 130°C letting the lids stay open for 6hours. Closing back the lids the crucibles were kept in desiccators for 60minutes for cooling. After sufficient cooling, the dried crucibles were weighed, and the values were used for calculating the moisture content. The following formula was used to calculate the moisture content. The formula indicated below was adopted from⁶⁰ and customized for this research purpose.

$$\{Moisture\ content(\%)\} = \frac{M_{wet} - M_{dry}}{M_{wet}} \times 100$$

Where: M_{wet} is the weight of the crucible which contains wheat flour sample before drying; M_{dry} is the weight of crucible which contains wheat flour sample after drying.

After analyzing three sample replications, the moisture content in the wheat flour was measured as 11.5%.

Water absorption and mixing behavior determination:

The Farinograph was used for determining the quality and mixing behavior of the wheat flour. Understanding the quality and mixing behavior of the flour helps to get consistent dough characteristics. This method is applied based on method 54-21 of AACCC⁶¹ and was performed on 300g of flour at 14% moisture base. The Farinograph determines the water absorption behavior of the wheat flour and mixing behavior of the dough. It also measures dough development time (min), dough stability (min) and degree of dough softness. The mixing behavior of the dough was recorded throughout the mixing process. Firstly, a calculated amount of wheat flour (291.7g) was added to the mixing bowl, and then water from the burette was continuously added to the wheat flour until the dough consistency reached 500FU. The value of 500 FU alternatively called Brabender units (BU) shows the arrival of the average dough firmness. To increase the accuracy, three sample replications were taken. The results of the Farinograph were customized to a high energy mixing machine which is later used for the whole baking process.

The bread baking process

Using formula (1) indicated above the required amounts of ingredients were calculated and measured according to the Table 1. The baking protocols followed in this research are shown in Figure 4. The amount of water added to the dough corresponds to the Farinograph test result. The targeted dough mass was 2.5kg and a high energy mixing device, Diosna SP160 spiral mixer was used. All measured ingredients were poured into this machine. The first baking protocol was a standard baking process. It had mixing time for 2+4minutes. The second baking protocol was a short mixing time (2+2minutes), and the third baking protocol was a long mixing time (2+6minutes) respectively.

At the end of each protocol, dough samples were taken for vitamin D₂ analysis. Following the mixing process, the dough rested for 18 minutes. Then the dough was sheeted,

molded by hand and cut into 300g pieces. Each dough pieces was placed in a baking pan having a dimension of (15cmX6.5cmX9cm) and proved for 55minutes at 35°C and

RH of 75%. All baking protocols had a similar proving setup (35°C and 75% of RH for 55minutes). Finally, optimally proved dough pieces were baked in the electric oven.

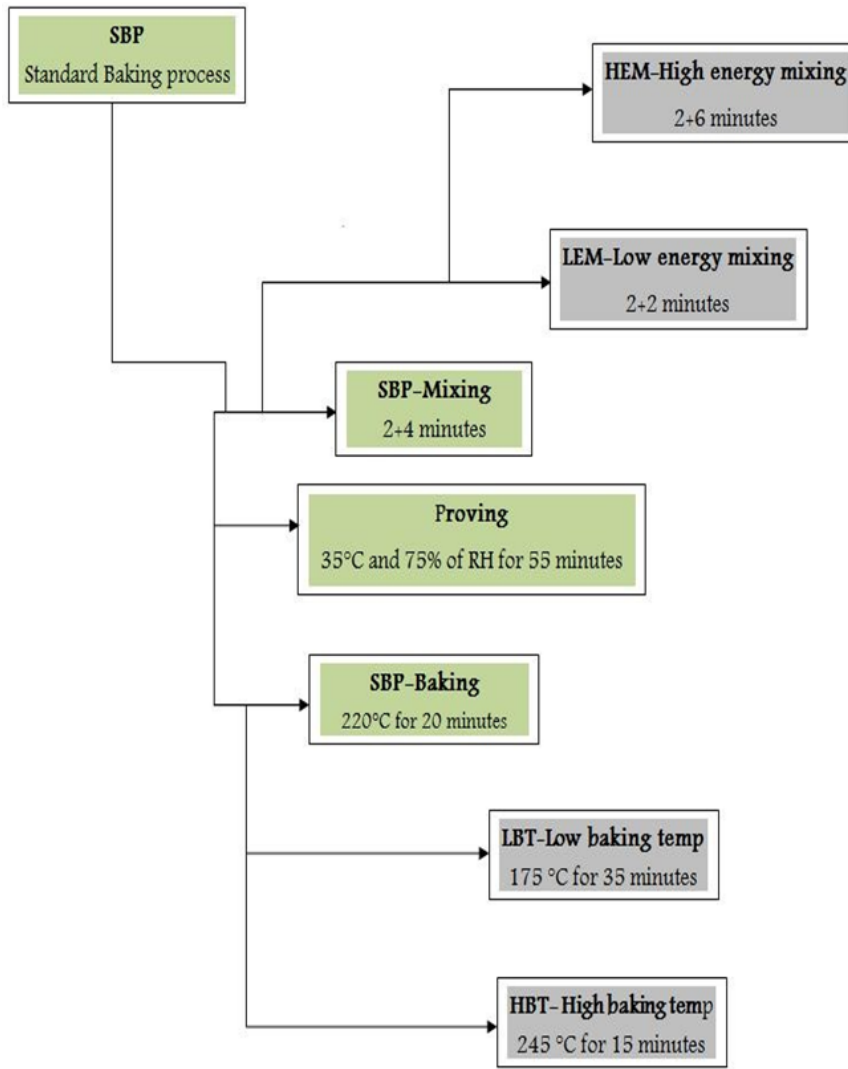


Figure 4 The parameters of the experimental protocols.

To investigate the effect of baking temperature and time combination on the stability of vitamin D₂ three different baking conditions were designed as shown in Figure 4.

Specific volume of bread: The specific volume of a loaf of bread is determined based on AACC method,⁶² and it is calculated by dividing the volume (cc) of bread by its weight (g).⁶³ The process involves placing of loaves in a container of a specific volume, and rapeseeds were run until the container was full. The loaf volume is equal with the volume of seeds displaced. Finally using the following formula (5) the specific volume of loaves of bread (SVB) was calculated.

$$SVB = \frac{\text{Loaf volume(cc)}}{\text{Loaf weight(g)}}$$

Image-based crumb texture analysis

Crumb textures of loaves were analyzed by capturing their crumb image. At the end of the oven baking process and one hour of cooling a 300g of loaves were sliced into six parts. Pictures of the sliced bread were taken by Sony Cyber-shot digital camera which had 14.1 megapixels of resolution capacity. Images of the slices were analyzed using Image J application software.⁶⁴ The principle of the images analysis is it compares the contrast difference between the pore structures and other parts of the crumb surface. Firstly, the images taken were changed to grayscale, and the pixel sizes were calibrated to a known length of measurements. After resizing of the rectangular pictures of the slices, the distribution of the porous structure, evaluation of the radial pore size and analysis of porosity distribution all over the crumb surface were done by the Image J software.

Analytical method of vitamin D₂ content determination in dough and bread samples

Vitamin D₂ content in the dough and bread samples were measured to see how much vitamin D₂ was lost during the mixing and the oven baking process. Besides, the stability of the vitamin D₂ in two different storage conditions was analyzed. As part of the ingredient, novel yeast which contains 0.278µg of vitamin D₂/g was added before starting the mixing process. The vitamin D₂ concentration after the proving stage was checked to see the effect of the yeast fermentation on vitamin D₂ production and to investigate this standard baking protocol (SBP) was followed. The whole bread baking process was designed to have three different mixing and baking temperature arrangements. Samples were taken from all baking protocols. The amount of vitamin D₂ retained in controlled atmospheric storage of (23.4°C and 35% RH), and frozen storage at (-18°C) was also measured. The slices of bread stored for 15days in both storage types. The samples were analyzed based on the analytical method of vitamin D₂ determination at the National Food Institute of Denmark as described below. The measured values were statistically interpreted using JMP® Pro 11.0.0 software.

Method for vitamin D₂ (ergosterol) determination: The analytical method of vitamin D₂ determination earlier has been followed for determination of vitamin D in meat⁶⁵ and vitamin D₂ in the mushrooms.⁶⁶

Vitamin D₂ determination was run by couple HPLC technique (Waters, Milford, MA, USA). Semi- preparative HPLC was equipped with 600 controllers and pump, a refrigerated 717Plus Autosampler and a 2487 absorbance identifier. The system was equipped with a silica column (Luna, Si 60, 3mm, 150×4.6mm, Phenomenex, Torrance, CA, USA). The system for the analytical HPLC was equipped with a 515 HPLC pump, a 717Plus Autosampler, a 2996 Photodiode Array Detector (DAD) and a 2487 Absorbance Detector in combination with two VYDAC201TP54 (5µm, 250×4.6 ID, 5µm, 250×4.6ID, Phenomenex, Torrance, CA, USA). Waters Empower 3 (Waters, Milford, MA, USA) was used for interpretation and processing of chromatograms and data.

Before starting vitamin D₂ measurement, the bread and dough samples were homogenized in a food processor (Tecator 1094, Homogenizer, Foss Tecator, Höganäs, Sweden) for 1 min. Following this 10g of homogenized bread and dough samples were taken for analysis and vitamin D₃ was added as an internal standard. Firstly the samples were extracted by alkaline saponification and liquid-liquid extraction by petroleum ether: diethyl ether (50:50) followed by cleaning-up by solid silica phase. The eluent for the preparative HPLC system was 2-propanol: methyltert-butyl-ether: cyclohexan: n- heptan (0.7:2:48.65:48.65). The flow rate used was 1.2 ml min. The retention time for vitamin D₂ was approximately 9min; sample collection was done at 0.7minutes of a gap before and after vitamin D₂. The retention time for the vitamin D₂ was approximately 14min, which is a baseline for separation. To separate the vitamin

D₂ analytical system was run with acetonitrile: methanol (80:20) as mobile phase and Detection was performed by a photodiode array detector (220-320nm) and quantification at 265 nm. Retention time was 20.3 and 22.8 for vitamin D₂ and vitamin D₃ respectively.

Results

Dough mixing behavior and water absorption property

The water absorption behavior of the wheat flour was evaluated from the Farinograph curve. The result obtained has shown that the wheat flour had 59.5% of water absorption. As indicated on Figure 5 the flour had optimum dough development time of 2.4minutes and stability for 6.13 minutes.

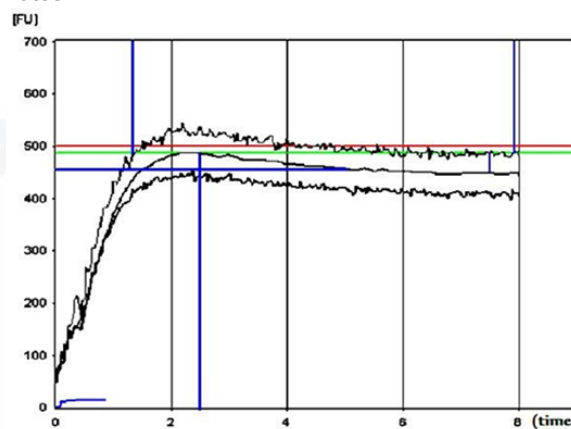


Figure 5 The Farinograph curve which shows the dough mixing behavior.

The above curve is interpreted as the following: as the mixing process progresses the curve is slightly falling and the curve has a broad bandwidth. These suggested that the dough was stiff enough to withstand the high stress applied from the mechanical mixer.

Bread quality

Specific volume of bread: The mean values of specific volume measurements are shown in Table 3. The mean weight and density of bread with standard deviations are also included in the table. One way ANOVA method is used to interpret the statistical values. Based on the interpretation there were no significant difference in bread weight, density and specific volume between all baking protocols ($P > 0, 05$) (Figure 6).



Figure 6 Image of loaf obtained from the standard baking protocol.

Image-based analysis of crumb texture and porosity distribution: Following the adjustment of the threshold of the pictures the average pore size and percentage of pored distribution on the surface of the slices are indicated

on Figure 7 and in Table 4. The median of pore diameter measurements and their distribution on sampled slices are indicated on the histograms (Figure 8) (Figure 9).

Table 3 The following data represent the mean values of weight, volume, density and specific volume (cm³ g⁻¹) of three sample loaves (n=3)±SD from each baking protocols

Bread samples taken form	Weight(g)	Volume(cm ³)	Density(ρ)	Specific volume
Standard-baking process (SBP)	270±4.36	655±25.24	0.41±0.05	2.43±0.07
2+2 min mixing process (LEM)	273±5.29	654±30.19	0.42±0.05	2.39±0.08
2+6 min mixing process (HEM)	268.67±1.15	654.5±22.29	0.41±0.05	2.44±0.08
Low-baking temperature (LBT)	270±10.83	656.7±32.66	0.41±0.10	2.43±0.16
High-baking temperature (HBT)	279.3± 1.53	658±11.14	0.42±0.03	2.36±0.04

Values are the mean±SD. There were no statistically significant differences between all banking protocols (P>0.05).

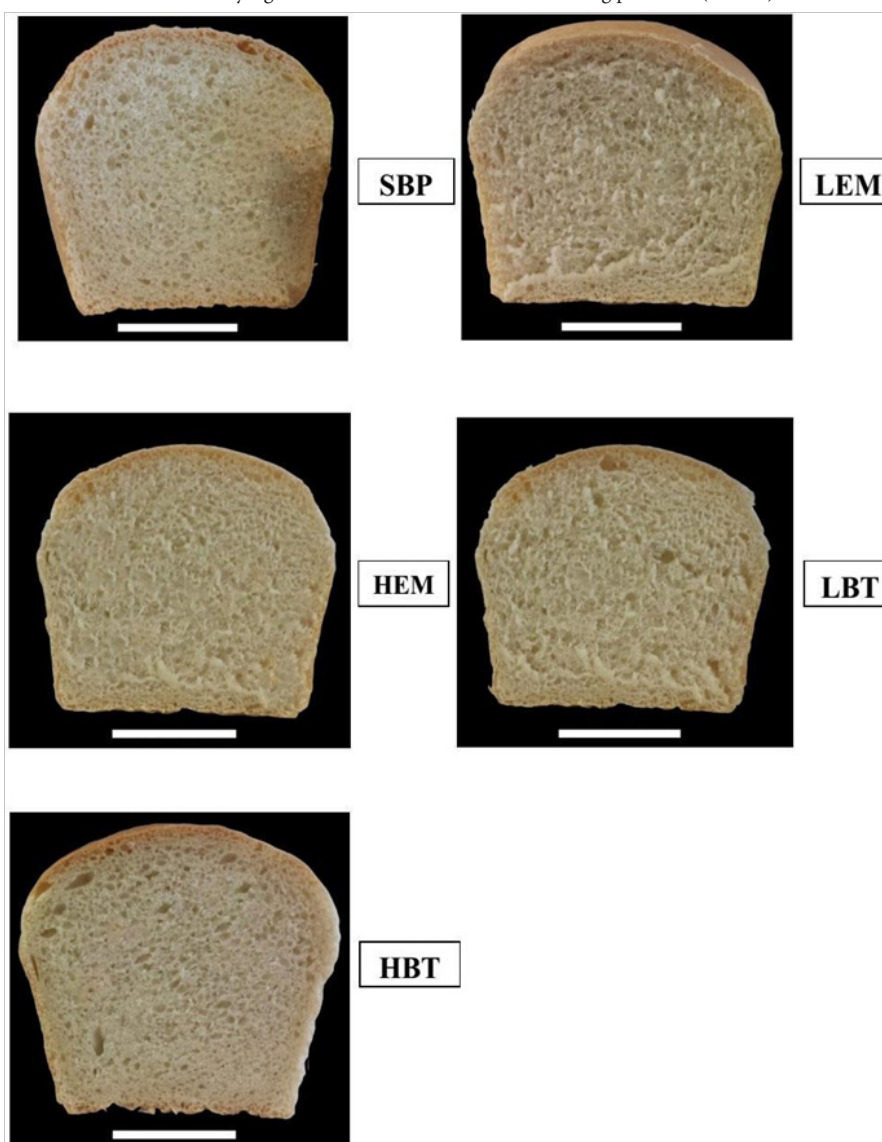


Figure 7 Scanned images of bread from all baking arrangements. These images are used for the Image J analysis. The line beneath the bread represents 2,54cm. Details of the analysis indicated in Table 4. SBP, standard-baking process; LEM, Low-energy mixing; HEM, High-energy mixing; LBT, Low-baking temperature; HBT, High-baking temperature.

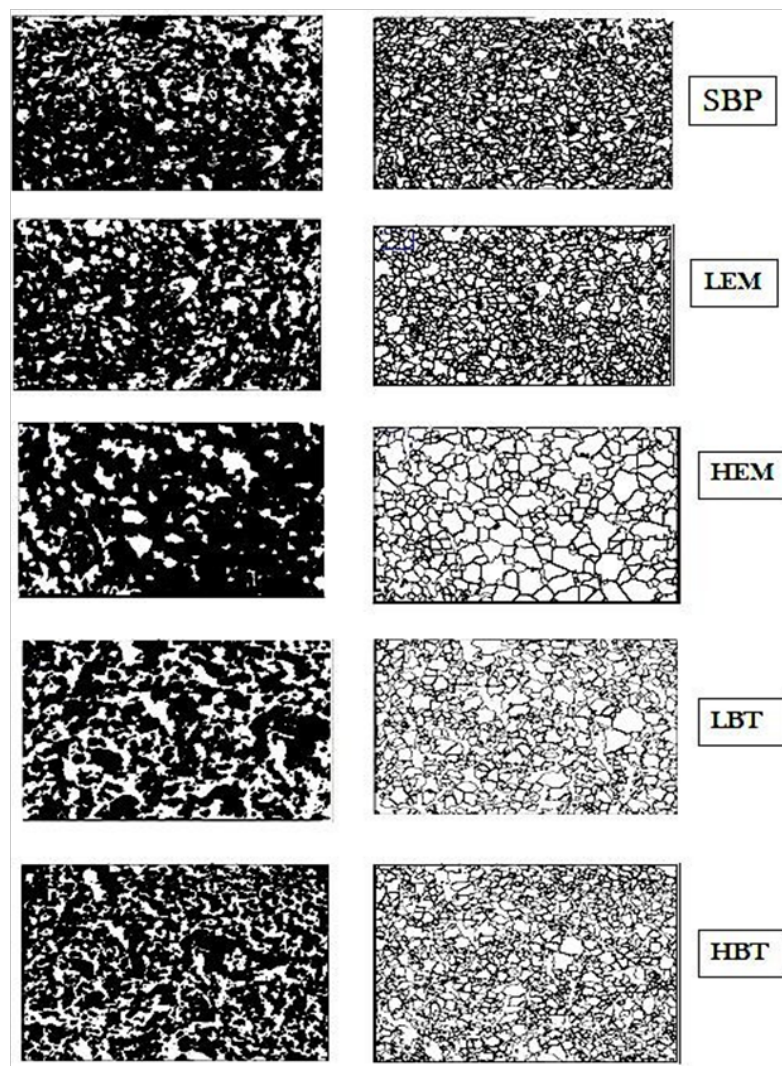


Figure 8 The figure shows how the Image J software applies contrast difference in the scanned image to analyze the corner of pores and determines the regions representing voids before measuring their areas. SBP, standard-baking process; LEM, Low-energy mixing; HEM, High-energy mixing; LBT, Low-baking temperature; HBT, High-baking temperature.

The average pore size measurements are tabulated in Table 4. These measurements are restricted to rectangular bread samples with an approximate dimension of 70mm width and height 90mm.

The one way ANOVA statistical analysis results have shown that there was a significant difference in the mean pore size distributions between the baking protocols ($P < 0.0001^{***}$).

Performing Tukey-Kramer mean comparison technique the mean porosity differences between the slices of high-energy mixing (HEM), LBT (low-baking temperature) and HBT (high-baking temperature) protocols were insignificant. However, the porosity of the slices from low-energy mixing (LEM) and standard-baking process (SBP) was significantly different from the rest of the baking arrangements. Furthermore, there was no significant difference in the average pore size of slices from LEM and SBP. The highest and the lowest

pore sizes were observed from the HEM and SBP protocol respectively. These results are shown in Figure 10 and Table 4. The difference of porosity distributions over the surface of the slices is insignificant between all protocols ($P > 0.0001$).

Based on one way ANOVA analysis the mean values of pore distribution in all baking protocols were compared. Hence, the highest porosity distribution was recorded from LEM protocol which was 175.96%. Even though the porosity distribution differences between the protocols were insignificant, the lowest distribution up to 165.57% was observed from HEM protocol. A comparable number of pores which are the size of 0-20 μ m were seen in SBP and LBT baking process. However, the slices from HEM process had the lowest number of small pores with a diameter of 0-20 μ m.

Table 4 Measurements of the average pores size and their distributions on the slices of bread in each baking protocols. Values are presented as a mean±standard error of the mean (n=3)

	Average pore size (xc) (µm)	Pore distributions (%)
Standard-baking process (220°C for 20 minutes)	29.09±4.09 ^a	174.97±5.03 ^a
Low-energy mixing (2+2 minutes)	32.87±3.13 ^a	175.96±1.18 ^a
High-energy mixing (2+6)	60.75±2.55 ^b	165.57±11.83 ^a
Low-baking temperature (175°C, 35 minutes)	58.35±0.35 ^b	167.93±1.73 ^a
High-baking temperature (245°C, 15minutes)	58.51±4.91 ^b	169.53±5.53 ^a

^b Mean values within the columns and with different superscripts are significantly different from each other (p<0.0001***).

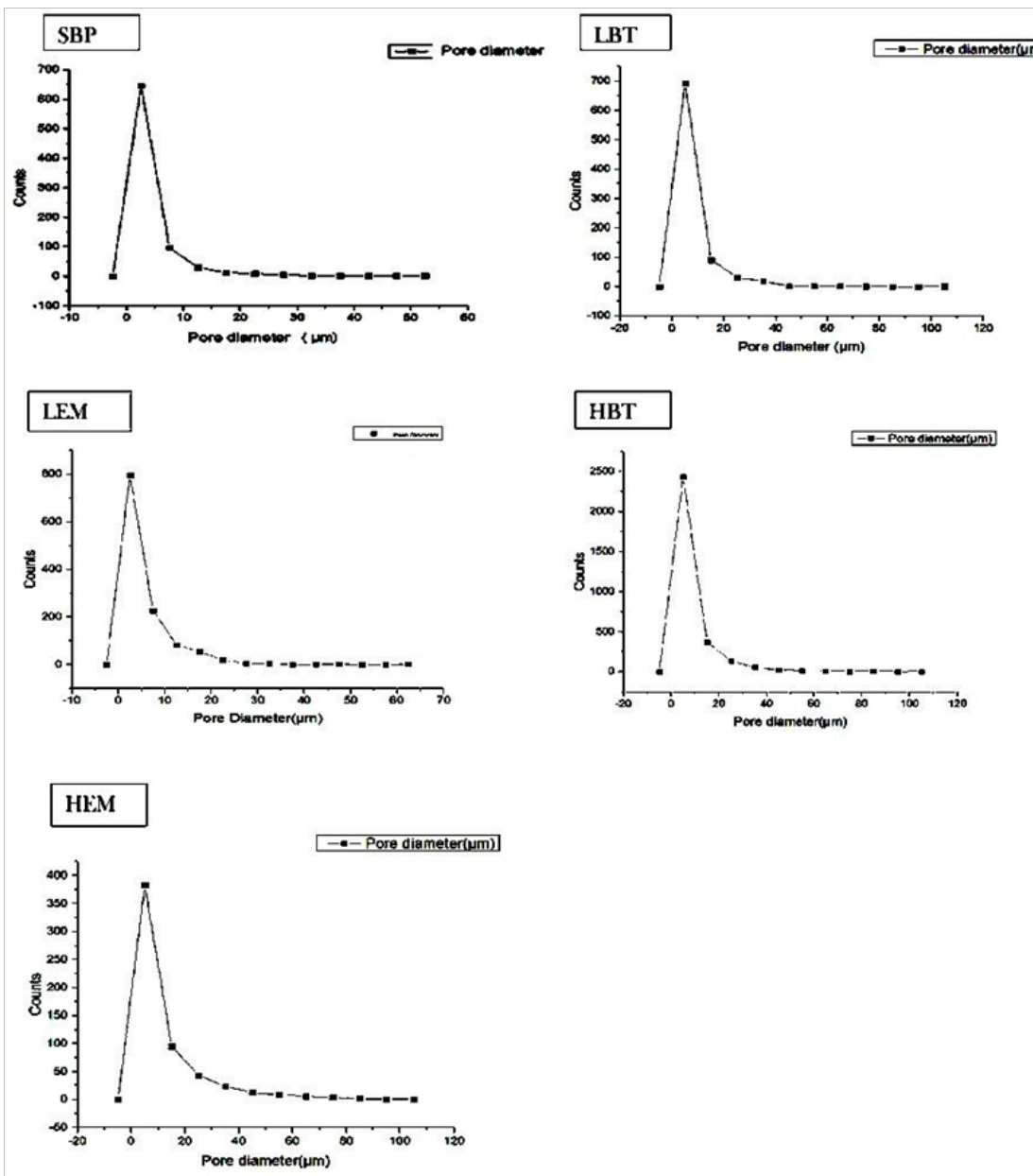


Figure 9 The porosity distributions on the slices of baking protocols.

Vitamin D₂ content in dough and bread samples

According to the results obtained from the vitamin D₂ stability measurements in bread and dough samples, the variations of the mixing time and baking temperature-time combinations did not affect the stability of vitamin D₂. Besides, the difference in vitamin D₂ retention between the mixing protocols was insignificant ($P>0.05$). A negligible difference in vitamin D₂ retention also observed between the different baking temperature-time combinations ($P>0.05$). The mean value of vitamin D₂ concentrations after each mixing and baking protocols are shown on Figure 10.

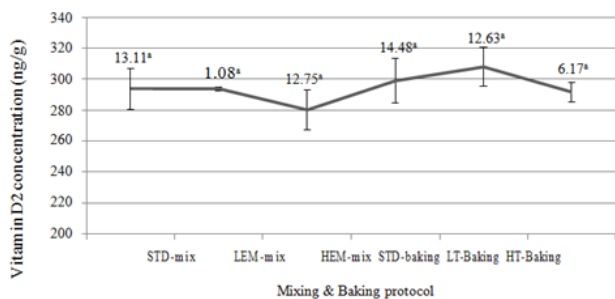


Figure 10 Vitamin D₂ concentration after each mixing and baking protocols. Superscripts with a letter 'a' show the standard deviations.

Based on the SBP protocol, the change in vitamin D₂ concentration between the initial amounts (before the mixing process) and the final baking stage was analyzed. Overall, the result has shown an increment of vitamin D₂ concentration by 7.03% (Figure 11).

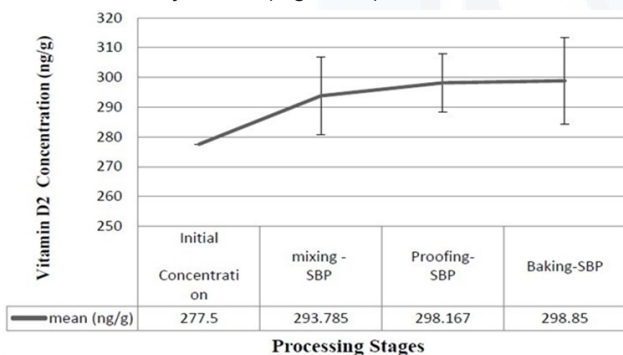


Figure 11 Vitamin D₂ concentration change in the SBP protocol.

Figure 12 shows the amount of vitamin D₂ retained in the controlled atmospheric storage (at 23.4°C and 35% RH) and freezer storage of -18°C. According to the analysis, there was no significant difference in vitamin D₂ retention between the storage protocols ($P>0.05$).

Discussions

Bread quality and vitamin D₂ stability

Bread quality and impact of the baking procedure on quality attributes: Different studies have shown the correlation between the specific volume of bread, dough mixing intensity and the amount of dough improver added in the mixing process. According to various studies, the mixing process has shown significant influence on the

specific volume of wheat bread. A high energy mixing intensity improves the stretching of the gluten matrix and incorporation of air bubbles, and these enhance the specific-volume of bread. However, studies which investigate the effect of the mixing process on the specific volume of bread only focus its impact in the presence of oxidative dough improvers. In the current study effect of mixing process was studied without the addition of dough improvers. The results of this study have shown that the designed mixing intensity and time length didn't affect the specific volume of bread. Besides, the impact of the baking temperature-time arrangements on the specific-volume of the loaves was insignificant ($P>0.05$).

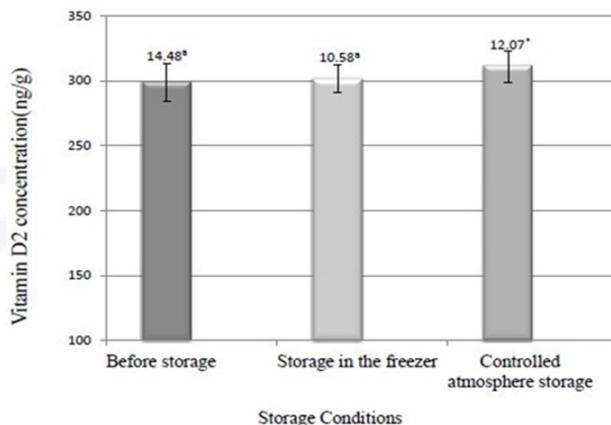


Figure 12 The amount of vitamin D₂ retained after 10 days of storage. Superscripts with symbol 'a' show the standard deviations.

Studies which show the impact of increasing the temperature-time length on the specific volume of bread baked from 100% of wheat flour are limited.^{56,67,68} Therefore, it was challenging to compare the current study result with other research works. e.g., the specific-volume of bread fortified with coriander was increased when the baking temperature-time was increased.⁵⁶ However, in the current study, we have observed that there was no significant difference of specific volume between the baking protocols. The specific volume of quality wheat bread is between 4,5-5ml/g and compared to this a low specific volume of bread was obtained in this study. The possible assumption for this is the technical failures of the proving cabinet that we encountered during this study, and this might influence the yeast activity.

The mixing process regulates the level of air inclusion within the dough matrix, and this affects the number of gas cell distributions on the crumb surface of the slices. The subsequent bread making unit operations further promotes enlargement of the gas cells formed in the initial phase of the mixing process.⁶⁹

The final bread texture is entirely dependent on the expansion of gas cells created in the initial phase of the mixing process. The number, size and even distributions of gas cells are dependent on the mixing procedure, the intensity of the mixing energy and the atmospheric condition of the mixer headspace.⁷⁰ Within the current study, a significant difference of crumb pore diameter was

observed between the designed protocols ($P < 0.0001^{***}$). The observed differences in crumb porosity associated with the mixing intensity and length of timing which caused the difference in the amount of air incorporated in the dough matrix.⁷¹⁻⁷⁶

The crumb structures of the slices obtained from SBP and LEM-Mixing protocols have shown the smallest pore diameters (or fine crumb textures), and this was significantly different from the other baking protocols. In contrary, the highest pore diameter was recorded from HEM protocol which was substantially different from SBP-mixing and LEM protocols and this result associated with the amount of air inclusion in HEM condition. The majority of the atmospheric air which was in the headspace of the mixing device might be included in the dough mass.⁷⁷⁻⁷⁹

As far as the oven baking is concerned the pore diameter of the slices obtained from SBP (220°C for 20minutes) was small compared to the pore diameter of LBT (175°C, 35minutes) and HBT (245°C, 15minutes). As it was shown in Table 4, the pore diameter of the slices from SBP is significantly different from the pore diameter of the slices from LBT and HBT processes ($P < 0.0001^{***}$).

Effect of mixing and baking parameters on the stability of vitamin D₂

Changing the mixing intensity from low energy- short time to high energy-long time mixing didn't affect the stability of vitamin D₂ in dough and bread samples. Most of the researches conducted concerning vitamin D₂ stability were mostly focused on the effect of heat treatment and storage the conditions and vitamin D₂ is described as vulnerable to temperature, oxygen, light, and moisture.³⁶ However, the result obtained from the current study implies that vitamin D₂ which obtained from bio-enriched yeast was resistant to dough aeration caused by different mixing intensities. As per the hypothesis of the present study the level of dough aeration didn't affect the vitamin D₂ stability. Furthermore, the difference in vitamin D₂ concentration of dough samples in each designed mixing conditions was insignificant (Figure 10). The vitamin D₂ which is used in this study was sourced from bio-enriched yeast, and it was stable under different temperature-time combinations. The vitamin D₂ concentration difference between the baking protocols was also insignificant (Figure 10). This result was consistent with previous studies which have shown a resistivity of vitamin D₃ to lose caused by heat treatments of pasteurization and sterilization of milk products.³⁴ In another study, vitamin D₃ was affected when the cheese milk was heated at 232°C for 5minutes.³⁵

The amount of vitamin D₂ retained after the baking process of wheat bread was analyzed.³⁸ This analysis is done based on one temperature set up of 200°C for 30min, and the result has shown vitamin D₂ is stable under this baking temperature. In the current research work, the vitamin D₂ in bread samples had also shown resistivity toward losing when the baking temperature-time combination varied from LBT (175°C, 35minutes) to HBT (245°C, 15minutes).⁸⁰⁻⁸²

The change in vitamin D₂ concentration in the dough and bread samples of SBP is shown in Figure 11. In the SBP protocol, the vitamin D₂ concentration has demonstrated an increment of 7.03%. We assume that this increase can be associated with a further production of vitamin D₂ when the yeast is exposed to light sources during the ingredient measurement and mixing operations. Since the baking process was not adequately protected from light exposure and it is possible that the yeast can keep producing vitamin D₂ in the mentioned unit operations.

Stability of vitamin D₂ under different storage conditions

Different studies have shown the stability of vitamin D in varies storage conditions.^{25,29,33,36} However, most of the studies focused on the stability of vitamin D in milk and milk products storage. e.g., the effect of plastic and glass bottles packaging on the stability of vitamin D₂ in milk samples was compared. According to this study the milk samples were stored under refrigeration temperature for consecutive seven days. The result shows that the vitamin D₂ in the milk samples was stable in both packaging types.³⁶ In the current study the stability of vitamin D₂ in bread and dough samples of the SBP protocol was studied in controlled atmospheric storage (at 23.4°C and 35% RH) and freeze storage of -18°C. The vitamin D₂ was also stable in both storage protocols. Besides, there was no significant difference in vitamin D₂ retention between the storage protocols.

Conclusion

This study aimed to investigate the stability of vitamin D₂ in the baking process and assessed its impact on bread quality. The source of vitamin D₂ used in this baking is sourced from UV treated bio-enriched yeast. The stability of vitamin D₂ was examined based on different mixing and baking protocols. The assessment is conducted to determine the amount of vitamin D₂ retained after each unit operations. The vitamin D₂ content within the dough and bread samples were measured based on the analytical method which runs by a couple of HPLC technique. Image J application software was used to analyze the texture of the experimental loaves of bread. Finally, the results obtained from the study were statistically analyzed using JMP® Pro 11.0.0 software.

In this study, we have got a low specific volume of bread which is different from the specific- volume of quality wheat bread. We also noticed that the difference of specific-volume between the baking protocols was insignificant. From this, we can deduce that variations of baking temperature-time combinations did not affect the loaves volume. However, we assume that the technical problem we encountered in our proving cabinet might slow down the yeast's activity. Therefore, we suggest that closely monitoring the efficiency of proving cabinet is essential.

Overall, the SBP (2+4min mixing and 220°C/20min baking) and LEM-baking process (2+2min mixing and 175°C/35min baking) have yielded bread with evenly distributed porosity and fine porous structure. From these result, we concluded that the SBP and LEM protocols could be the best protocols

for baking bread fortified with vitamin D₂ sourced from the bio-enriched yeast.

The result of vitamin D₂ retention measurement has shown that there was no observed loss of vitamin D₂ caused by the mixing and baking protocols. From this, we conclude that the vitamin D₂ was stable under different mixing intensity and baking temperature-time arrangements. Besides, the controlled atmospheric storage (at 23.4°C and 35% RH) and freeze storage of -18°C fully retained the vitamin D₂ content in the dough and bread samples after ten storage days. Therefore, bread fortified with vitamin D₂ sourced from bio-enriched yeast can be safely stored in both storage conditions. In the SBP protocol, the vitamin D₂ concentration was increased by 7.03%. In future studies to clearly understand how the vitamin D₂ increment has happened, we suggest that closely monitoring the proving cabinet efficiency will be significant.

The analytical method of vitamin D₂ measurement is time taking and expensive. Therefore, this research work is limited to a small number of samples. However, the conclusion given based on this research work can be satisfying ground for future experimental setups. We also recommend that it is essential to study the stability of vitamin D₂ obtained from UVB-treated yeast in different bread packaging types and different baked products. In conclusion, depending on the scope of this study the vitamin D₂ obtained from UV treated yeast was stable throughout the bread baking process, and its presence in bread fortification process did not affect the bread quality attributes.

Preference

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Conflicts of interest

Author declares that there is none of the conflicts.

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