Research Article

Natural and Synthetic Antioxidants Prevent the Degradation of Vitamin D_3 Fortification in Canola Oil during Baking and *in Vitro* Digestion

Sebastian Goebel
University of Applied Sciences Bremerhaven, 30 Ankerstraße, Bremerhaven, Germany

Sylvie Avallone
UMR QualiSud, University of Montpellier, CIRAD, Institut Agro, Université d'Avignon, Université de la Réunion, Montpellier, France

Pakkawat Detchewa and Patcharee Prasajak
Department of Agro-industry Technology and Management, King Mongkut's University of Technology North Bangkok, Prachinburi, Thailand

Wichien Sriwichai*
Department of Innovation and Product Development Technology, King Mongkut's University of Technology North Bangkok, Prachinburi, Thailand

* Corresponding author. E-mail: wichien.s@agro.kmutnb.ac.th DOI: 10.14416/j.asep.2021.01.005
Received: 14 October 2020; Revised: 23 November 2020; Accepted: 25 November 2020; Published online: 25 January 2021
© 2021 King Mongkut's University of Technology North Bangkok. All Rights Reserved.

Abstract
Vitamin D insufficiency is widespread in the northern and partly equatorial hemisphere countries. Fortification of vitamin D in commonly consumed vegetable oils can prevent rickets in children, osteoporosis and bone fractures in adults. Avoiding the loss of vitamin D_3 fortification in oils during cooking is beneficial for consumer's health. The aim of this work was to investigate the stability of cholecalciferol (vitamin D_3) fortification in canola oil during baking at 80 to 230°C for 10 to 40 min. The natural antioxidants (β-carotene and α-tocopherol) and the synthetic ones (butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ)) were used to prevent the degradation of vitamin D_3. The kinetic degradation of vitamin D_3, oxidative status of canola oil and the bioaccessibility in *in vitro* digestion were assessed. Vitamin D_3 was relatively stable at 80 and 130°C for 10 to 40 min. High temperatures of 180 and 230°C caused the highest loss of vitamin D_3 being up to 90%. Reaction rate of vitamin D_3 degradation ranged from 2.01 to 6.80×10^{-2} sec^{-1}. BHT and TBHQ had the highest antioxidant activity (> 50 %) to decrease the degradation of vitamin D_3 at 230°C. The oxidative status (peroxide value, malondialdehyde content) of canola oil was improved after incorporating antioxidant agents. The vitamin D_3 bioaccessibility was increased 1.5 fold after *in vitro* digestion. The consumption of 5 g brownie containing vitamin D_3 100 μg/L and antioxidant agents 180 mg/L in 1 mL of canola oil would cover the daily intake.

Keywords: Degradation, Cholecalciferol, α-tocopherol, β-carotene, BHT, TBHQ

Please cite this article as: S. Goebel, S. Avallone, P. Detchewa, P. Prasajak, and W. Sriwichai, "Natural and synthetic antioxidants prevent the degradation of vitamin D_3 fortification in canola oil during baking and *in vitro* digestion," *Applied Science and Engineering Progress*, vol. 14, no. 2, pp. 247–258, Apr.–Jun. 2021, doi: 10.14416/j.asep.2021.01.005.

1 Introduction

Vitamin D deficiency is widespread, especially in the northern hemisphere countries where the level of UVB exposure is limited. The prevalence of vitamin D deficiency (< 50 nmol/L) has been reported for 25% of the population in Canada, 22–36% in the USA, 45–52% in New Zealand, 47–65% in Korea, and 31% in Australia [1]. A lack of Vitamin D is also found in equatorial countries where people tend to stay indoors, cover their skin or using sunscreen. The deficiency of vitamin D is crucial for causing rickets in children and can precipitate and exacerbate osteopenia, osteoporosis and fractures in adults [2]–[6]. The recommended daily allowance (RDA) of vitamin D is 600–800 IU, which is equivalent to 10–15 µg/day [7].Consumption of food rich in vitamin D is sometimes insufficient to meet the vitamin D requirement. Fortification of commonly consumed vegetable oils in which vitamin D can be evenly distributed is an efficient way to improve the vitamin D status of population in a mass scale [8]. The bioavailability of the vitamin D_3 was improved when it was co-digested with vegetable oil [9]. Among the vegetable oils, canola oil is suitable for vitamin D_3 fortification since it contains omega-3 and omega-6 fatty acids being beneficial for health. Although instability of high unsaturated fatty acid in canola oil during heat cooking is well known. Drastic temperature of cooking provoked a degradation of vitamins and change the fatty acid profiles of oils caused by chemical degradation reactions [10], [11] Canola oil is produced, exported and consumed worldwide. However, the research on heat stability and bioaccessibility of micronutrient fortified in canola oil are needed to ensure a good nutritional status of consumers.

Baking is a common method of preparing food that uses dry heat in an oven. Different recipes of baked food using vegetables oils as an ingredient are used worldwide. Unfortunately, the loss of 13–31% of vitamin D_3 fortified in vegetable oils during different time and temperature of cooking methods was reported [12]. Heating in presence of air at 150°C showed the drastic destroy of almost the whole vitamin D_3 amount in the pure and dry form [13]. Recently, Zareie *et al.* [14] reported the instability and thermal degradation of vitamin D_3 in fortified canola oil and relationship between the increment of lipid oxidation products and the retention of vitamin D_3.

The addition of antioxidants to food could enhance the stability of vitamin D_3 during heating process. Natural antioxidant such as phenolic and vitamin E in olive oil were shown to delay the oxidative reaction during deep frying [15]. The natural antioxidants from Beijing grass extract showed a great potential to protect edible oil from lipid oxidation [16]. Synthetic antioxidants such as butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and the mixture of BHT and BHA have protective effects in stabilization of sunflower oils during storage and in frying process [17]. BHT and TBHQ is generally recognized as safe for fortification in food with a maximum of 0.02% of the fat or oil content [18]. However, incorporating the synthetic antioxidants is sometimes considered to be harmful for blood, cause circulatory problems and lung or liver tumours after long-term consumption for a health sensitive consumers [19], [20]. Using natural vitamins, such as vitamin A and E in an authorized amount; 700–900 µg and 15 mg, respectively can ensure more the security [21]. Although the addition of vitamin E for preventing lipid oxidation of vitamin D_3 fortified canola oil is useable, vitamin E may impose competitions for vitamin D absorption in intestine [22].

To our knowledge, few studies were conducted on the effect of natural antioxidants (*i.e.*, vitamin A and E) and some synthetic ones (*i.e.*, BHT and TBHQ) on the stability of vitamin D_3 fortified in canola oil during baking and digestion. Therefore, the purpose of this work was to study the stability of cholecalciferol (vitamin D_3) fortification in canola during baking at 80 to 230°C for 10 to 40 min. The antioxidant capacity of natural antioxidants (*i.e.*, β-carotene, α-tocopherol) and synthetic ones (*i.e.*, BHT and TBHQ) were used to prevent the degradation of vitamin D_3. The chemical indicators of lipid oxidative status of canola oil, which are the peroxide value and malondialdehyde content, were assessed. The kinetic degradation of vitamin D_3 through different antioxidants were compared. The bioaccessibility of vitamin D_3 in *in vitro* digestion of dark chocolate brownie replacing the butter by canola oil in which vitamin D_3 was fortified was evaluated.

2 Materials and Methods

2.1 *Materials and chemicals*

Canola oil was purchased from supermarket (Prachinburi,

Thailand). Solvents for high performance liquid chromatography (HPLC), standard (β-carotene, α-tocopherol, BHT and TBHQ) and enzymes (*i.e.*, porcine gastric mucin, pepsin from porcine gastric mucosa (800–2,500 U/mg), pancreatin from porcine pancreas, porcine lipase (100–400 U/mg) and porcine bile extract B8631)) were obtained from Sigma-Aldrich Chemie GmbH (Germany). Potassium iodide and starch from Ajax Finechem Pty Ltd (Australia), acetic acid from ORec SDN BHD (Malaysia), sodium thiosulfate from Loba Chemie Pvt.Ltd (India), toluene from Mallinckrodt Chemicals (USA), n-hexane from Lab-scan Asia Co. Ltd (Thailand), and Dimethyl sulfoxide from Finisher scientific (UK). The other reagents and solvents were from Sigma-Aldrich Chemie GmbH (Germany). The polytetrafluoroethylene (PTFE) membranes were received from Sartorius (Palaiseau, France).

2.2 *Vitamin D_3 and antioxidant reagents incorporation in canola oil*

Vitamin D_3 was carefully incorporated in five different batches of canola oil (100 mg/L) and gently homogenized for 10 min. The authorized amount of antioxidants agents were added in canola oil. Natural antioxidants: β-carotene and α-tocopherol (5 g/L) were fortified in different batches of canola oil. Synthetic antioxidants: BHT and TBHQ were incorporated in the same concentration (180 mg/L) in canola oil by the same way as the natural antioxidants. All samples were stored in the dark and kept away from oxygen until the analysis of vitamin D_3 degradation during baking started.

2.3 *Baking method and heating conditions*

Fortified canola oil incorporating antioxidants agents were baked in an oven (Binder FD 115, USA). The variation of temperature was controlled for ± 2°C. When the oven had to reach the desired temperature, approximately 20 mL of each batch of samples in triplicates were put in an uncovered aluminium cup and then subjected to an oven. A thermocouple was used to measure the temperature in the middle of the samples during baking.The duration of baking to evaluate the kinetic of vitamin D_3 degradation were 10, 20, 30, and 40 min for each temperature of baking, which were 80, 130, 180, and 230°C. After that samples were cooled on ice for the vitamin D_3 determination. The oxidative status of canola oil and antioxidant activity of antioxidant agents were then evaluated.

2.4 *Food preparation*

The bioaccessibility of vitamin D_3 was assessed by replacing butter with canola oil fortified in vitamin D_3 and incorporating four different types of antioxidants agents (β-carotene, α-tocopherol, BHT and TBHQ) in a dark chocolate brownie. Approximately 70 mL of fortified canola oil was mixed with an egg, 5 g cocoa powder, 175 g sugar and 70 g flour. Four distinguished formulas of brownie containing vitamin D_3 and different types of antioxidant agents were baked in an oven at 180°C for 20 min. The samples were cooled down and then subjected to *in vitro* digestion.

2.5 *In vitro digestion protocol*

In vitro digestion protocol was carried out according to the standardised INFOGEST protocol [23]. The solutions for oral (Simulated Saliva Fluid, SSF), gastric (Simulated Gastric Fluid, SGF), and small intestinal (Simulated Duodenal Fluid, SDF) phase were prepared before the *vitro* digestion. Brownie in triplicates of 5 g were weighted into an amber screw-capped bottle. After that, 5 mL SSF fluid containing mucin was added and the sample was then incubated at pH 7.0 ± 0.2 for 2 min at 37°C in a water bath under constant stirring at 100 rpm. In gastric phase, sample from oral phase was mixed to 10 mL of SGF containing porcine gastric pepsin (2000 U/mL in the final digestion mixture), pH adjusted to 3.0 ± 0.2, and the sample was incubated again for 2 h. In the final small intestinal step 20 mL SDF containing porcine pancreatin and bile salts (trypsin activity of 100 U/mL and a lipase activity of 2000 U/mL in the final mixture) was added to the sample. The pH was adjusted to 7.0 ± 0.2 and samples incubated for another 2 h. Digesta were centrifuged at 4000 × g for 30 min at 10°C (Hettich, Rotana A35R, Germany).

2.6 *Vitamin D_3 extraction*

The supernatant from digesta after *in vitro* digestion was collected with a needle fitted to a syringe and

filtered through a 0.2 μm cellulose acetate membrane to recover the micellar fraction. Vitamin D_3 was then extracted by adding 10 mL of ethanol/hexane (4 : 3, v/v). Samples were homogenised and then washed with 10 mL of distilled water. After 10 min in the dark, the organic phase was re-extracted and washed. The organic phase was dried under nitrogen. The residues were dissolved in 400 μL of acetone. The extracts were immediately filtered through a 0.2 μm PTFE membrane and analysed using HPLC. The content of bioaccessible vitamin D_3 corresponds to the amount of compounds transferred to the micellar phase at the end of *in vitro* digestion. The bioaccessibility in % corresponds to the amount of compound transferred to the micellar phase compared to the initial contents in the raw food.

2.7 *Vitamin D_3 and α-tocopherol analysis*

The method of the quantification of vitamin D_3 and α-tocopherol was adapted from a previous research [24]. Approximately, 100 μL of fortified canola oil with vitamin D_3 and antioxidant agents or concentrated samples after *in vitro* digestion were dissolved in 300 μL of acetone and filtrated on a 0.2 μm PTFE Minisart SRP4 membrane (Sartorius). Then 20 μL of the solution were used for the high performance liquid chromatography (HPLC, Knauer, Germany) equipped with a UV-visible photodiode array detector (Azura, Germany). The column was a polymeric C_{18} (4.6 mm i.d × 250 mm, 5 μm particle size, Vertex Plus Column). The mobile phase comprised two mixtures of solvents, the first mixture was methanol and Milli-Q water and the second one was methanol, methyl tert-butyl ether and Milli-Q water. The flow rate of analysis was 1 mL/min. The applied gradient was transformed from 100 to 0% at a temperature of 25°C and a period of 31 min. The chromatograms were analyzed at the wavelength of maximum vitamin D_3 and α-tocopherol absorption being 265 nm and 292 nm respectively. External calibration was used with the range of 0.5 to 100 mg/L. The initial α-tocopherol content in canola oil was subtracted from the amount of α-tocopherol added in sample as antioxidant agent.

2.8 *β-carotene analysis*

The amount of β-carotene in fortified canola oil was determined by spectrophotometry method [25]. Briefly, 50 mg of each oil samples were weighted and then dissolved in 5 mL cyclohexane. The solution was transferred into a quartz cuvette and the maximum absorbance wavelength at 445 nm was measured against cyclohexane. The β-carotene content of the sample was calculated and expressed in mg/L oil according to the Equation (1), below.

$$\beta\text{-carotene (mg/L)} = \frac{V \times 383 \times (A_s - A_b)}{100 \times W} \quad (1)$$

Where, V = volume used for analysis, 383 = extinction coefficient for β-carotene, A_s = absorbance of the sample, A_b = absorbance of the blank and W = weight of the oil sample.

2.9 *BHT and TBHQ analysis*

The method for BHT and TBHQ quantification was adapted from Espinosa-Mansilla *et al*. [26]. Firstly 0.5 mL of canola oil fortified with BHT or TBHQ in triplicate was transferred into a 10 mL test tube and filled up with DMSO. The control solution was made by using pure canola adding the DMSO. The absorption spectra of BHT and TBHQ on UV-visible spectrophotometer at 279.5 nm for BHT and 297.5 nm for TBHQ were measured respectively. The concentration of BHT and TBHQ was then calculated by extrapolation to the standard curve.

2.10 *Peroxide value determination*

The peroxide value was determined according to the standard protocol [27]. Briefly, 0.2 g of sample was dissolved in 2 mL of acetic acid followed by the addition of 0.2 mL freshly prepared saturated potassium iodide solution. The solution was gently mixed, incubated for 10 min in the dark and then diluted with 20 mL distilled water. Finally the mixture was slowly titrated with 0.01 N sodium thiosulfate ($Na_2S_2O_3$) in the presence of a starch solution (1%, 80 μL) until the dark blue color disappeared. The peroxide value was expressed in mmol of peroxide (or active oxygen) per kg of oil (meq O_2/kg). The peroxide value was calculated by the Equation (2), below.

$$PV(meq\ O_2/kg) = \left[\frac{(V_s - V_b) \times N}{W}\right] \times 1000 \quad (2)$$

Where V_s is the volume of $Na_2S_2O_3$ used in the canola oil sample (mL), V_b is the volume of $Na_2S_2O_3$ used in the blank sample (mL), N is the normality of $Na_2S_2O_3$ used for titration (meq/mL) and W is the weight of the canola oil (g)

2.11 *Malondialdehyde (MDA) determination*

Fortified canola oils (~1 mL) were mixed with 2 mL of distilled water, vortexed for 10 s and stirred for 5 min [28]. The samples were vortexed for 5 min and left for 10 min until phase separation. The upper phase was discarded and 750 µL of the aqueous extract were mixed with 250 µL of TBA (1% in NaOH 50 mM) and 750 µL of H3PO4 (440 mM). The solution was mixed then boiled for 60 min. The calibration curve was obtained with TEP which is naturally cleaved in MDA when dissolved in aqueous medium. A standard curve was made (0–10 µm) and the optical density was detected at 533 nm.

2.12 *Antioxidant activity*

The antioxidant activity of antioxidant agents in canola oil was estimated by using DPPH assay in triplicate measurements [29]. A freshly toluenic solution 10^{-4} mol/L of DPPH radicals was prepared for each experiments. The sample was transferred to a 10 mL volumetric flask for obtaining a concentration of 20 mg/L. After that 1 mL of prepared DPPH solution was added to the volumetric flask and was filled up with toluene. The mixture was well shaken and kept in the dark for 15 minutes. The control solution was prepared by mixing 1 mL of DPPH solution with toluene in a 10 mL volumetric flask. The absorption of the samples was measured at a wavelength of 520 nm by using a UV spectrophotometer. The inhibition percentage was calculated by the Equation (3) below.

$$\% \text{ Inhibition} = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

Where the A_c is the volume of control solution and whereas A_s is the one of sample.

2.13 *Kinetic of vitamin D_3 degradation parameters*

The degradation of vitamin D_3 during baking was based on the assumption follows first-order kinetics as given in the Equation (4). The rate of vitamin D_3 degradation was determined by the Arrhenius Equation (5) below.

$$\frac{C}{C_0} = exp^{(-kt)} \quad (4)$$

$$k = k_0\, e^{(Ea/RT)} \quad (5)$$

Where C is the vitamin D_3 concentration evaluated in time interval (t) and C_0 is the initial amount of vitamin D_3 before baking. k is the reaction rate constant (sec^{-1}), k_0 is the pre-exponential constant (sec^{-1}), Ea is the activation energy (kJ/mol), R is the universal gas constant (kJ/mol/K) and T is the absolute temperature (K).

2.14 *Statistical analysis*

All the experiments were realized in triplicate for each fortified canola oil batch. The data was assessed by analysis of variance (one-way ANOVA) using SPSS statistical software (Virginia, USA). Significance was accepted at probability $p < 0.05$ using the Duncan test. Normality of distribution and equality of variance was verified by using the Kolmogorow-Smirnov test.

3 Results and Discussion

3.1 *Initial state of canola oil fortification in vitamin D_3 and incorporating antioxidant agents*

Canola oil was selected for vitamin D_3 fortification because it is widespread used among vegetable oils in household cooking. Commonly, the level of temperature of cooking leads to the degradation of fatty acid and results in physical and chemical quality change of oils. In this work, natural and synthetic antioxidants were added in the fortified canola oil to prevent the degradation of the fortified vitamin D_3. The initial states of oil samples which were the peroxide value, MDA content and antioxidant activity of no heat canola oil were shown in Table 1.

The vitamin D_3 amount was the same for all initial oil samples (100 mg/L). However, the PV and MDA content were significantly decreased when antioxidants agents were added to the oil. The lowest PV and MDA content were in the oil samples with added THBQ and BHT, 1.1 ± 0.2 meq O_2/kg and

10.2 ± 0.8 mg/kg respectively, being in the same range as the previous study [30]. In the contrast, antioxidant activity was overall 7 fold higher compared to the control oil (*i.e*., no antioxidants added). The antioxidant agents increased the antioxidant activity by inhibiting the free radicals being responsible for the vitamin D_3 degradation.

Table 1: Peroxide values, malondialdehyde content and antioxidant activity of fortified canola oils (no heat)

Fortified Canola Oils	PV (meq O_2/kg)	MDA (mg/kg)	Antioxidant Activity (%)
Control	3.3 ± 0.9^{a}	$14.1 \pm 2.2^{a,b}$	14.0 ± 0.028^{d}
α-tocopherol	3.5 ± 0.6^{a}	13.1 ± 1.2^{b}	99.3 ± 0.003^{a}
β-carotene	1.2 ± 0.3^{c}	14.3 ± 0.6^{a}	95.6 ± 0.007^{b}
BHT	2.3 ± 0.2^{b}	10.2 ± 0.8^{c}	97.3 ± 0.004^{b}
TBHQ	1.1 ± 0.2^{c}	10.9 ± 0.7^{c}	93.7 ± 0.003^{c}

Notes: Data are presented as the mean ± standard deviation. Values with distinct lowercase superscript letters in the same column are significantly different ($p < 0.05$) for $n = 3$.

3.2 *Degradation of vitamin D_3 during baking*

The degradation of vitamin D_3 was monitored in canola oil at 80, 130, 180, and 230°C as a function of baking time recorded at 10, 20, 30, and 40 min. The total baking period was 40 min for all baking temperatures. The dimensionless vitamin D_3 concentrations in canola oil with or without antioxidant agents incorporation are presented in Figure 1. For all baking temperatures, the dimensionless concentration of vitamin D_3 decreased as a function of the baking time. In all treatments, vitamin D_3 was found to decrease markedly and quickly in the first 10 minutes of baking. The vitamin D_3 was 50% less degraded at 80 and 130°C compared to 180 and 230°C after 40 min of baking.

In contrast, the drastic temperature of 230°C for 10 min caused the highest dimensionless vitamin D_3 concentration drop, being up to 90%. The results were in a good accordance to the study on the vitamin D_3 retention in sunflower oil in an oven with low temperature and a long heating time and with high temperature and short heating time. The retention of vitamin D_3 was 85% at low temperature while the high temperature process caused a lower vitamin D_3 retention of 76% [31]. A lower heating temperature combined with longer heating time, instead of a high temperature combined with a short heating time had been founded out to cause a less loss of vitamin D_3 [12].

Anyway, in this study, all antioxidant agents could inhibit the degradation of vitamin D_3 compare to the control sample (no antioxidant adding). The corresponding degradation rates (r) of vitamin D_3 were used to compare the effect of antioxidants agents against degradation reaction, in Table 2.

Table 2: Degradation rate (r) of vitamin D_3 in canola oil with or without antioxidants adding at 80, 130, 180 and 230°C

Fortified Canola Oils	Degradation Rate : $r \times 10^{-2}$ (s^{-1})			
	80°C	130°C	180°C	230°C
Control	$2.7 \pm 0.5^{b,c}$	$3.0 \pm 0.9^{a,b}$	5.4 ± 0.3^{b}	$7.5 \pm 0.2^{a,b}$
α-tocopherol	$3.4 \pm 0.6^{a,b}$	2.7 ± 0.3^{b}	5.4 ± 0.1^{b}	$7.5 \pm 0.3^{a,b}$
β-carotene	2.2 ± 0.2^{c}	$3.0 \pm 0.7^{a,b}$	$5.5 \pm 0.2^{a,b}$	7.2 ± 0.1^{b}
BHT	2.3 ± 0.7^{c}	1.9 ± 0.2^{c}	4.7 ± 0.1^{c}	7.2 ± 0.2^{b}
TBHQ	2.0 ± 0.5^{d}	2.7 ± 0.4^{b}	4.0 ± 0.5^{d}	6.7 ± 0.5^{c}

Notes: r : Degradation rate. Data are presented as the mean ± standard deviation. Values with distinct lowercase superscript letters in the same column are significantly different ($p < 0.05$) for $n = 3$.

The degradation rates of the vitamin D_3 in canola oil with and without antioxidants adding increased as time and temperature increased. The highest value was reached at 230°C with a 2.7 fold higher value than at 80°C. Canola oil with added α-tocopherol had the degradation rate value in the same range for each treatment with and without antioxidant adding. In contrast, oil with β-carotene incorporation delayed the degradation reaction, thus has the lower degradation rate. Natural antioxidants (*i.e*., α-tocopherol and β-carotene) may self-degraded at high temperature of baking at 180 and 230°C and could not protect the degradation of vitamin D_3. However, the synthetic antioxidants (*i.e*., BHT and TBHQ) were both retarded greatly the degradation reaction of vitamin D_3. These antioxidants were more stable than the natural antioxidants. Their degradation rates were 0.5 to 1.4 fold lower at the low, moderate and high temperature of baking compare to sample with no antioxidants adding.

Generally, the chemical reactions require some activation energy to go forward. The activation energy (Ea) represents the minimum amount of energy required to activate a chemical degradation reaction. In the present study, the Ea of vitamin D_3 degradation was calculated when the temperatures of baking changed from 80 to 230°C. The Ea of vitamin D_3 degradation of canola oil without antioxidant adding and incorporated α-tocopherol, β-carotene, BHT and TBHQ were

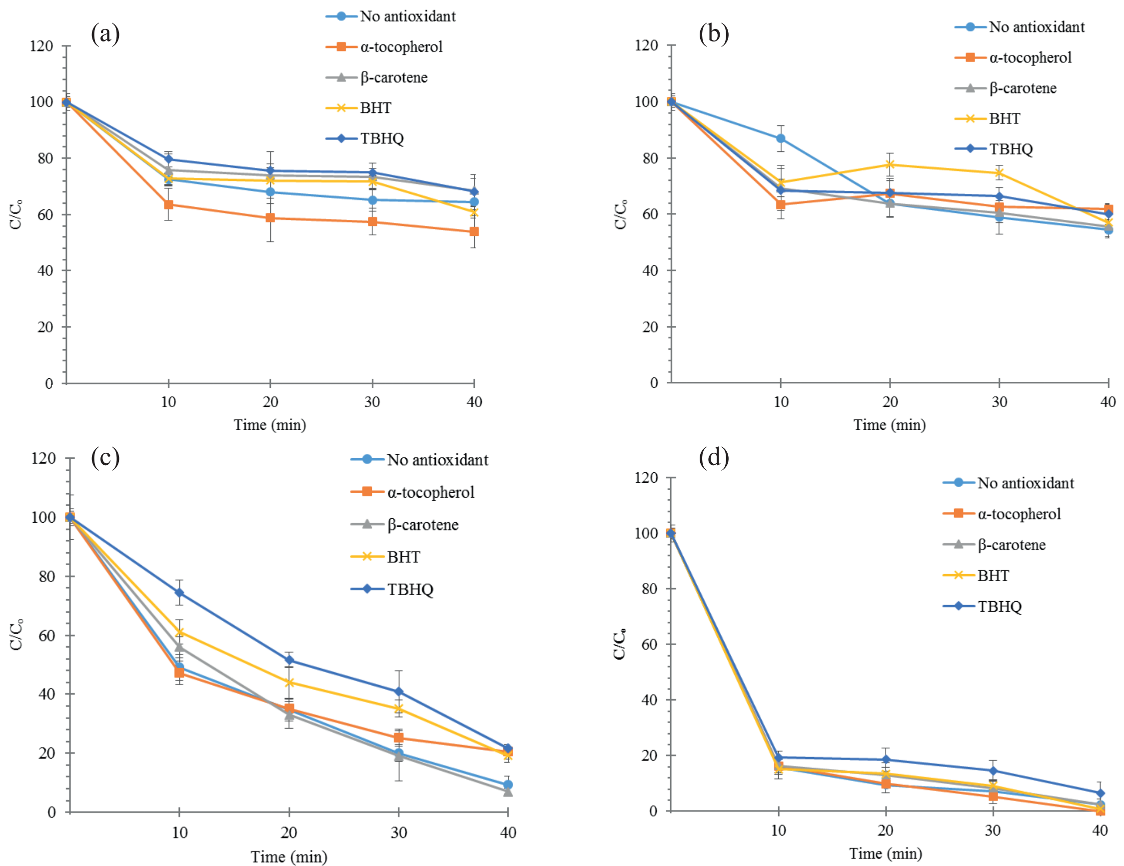

Figure 1: Evolution of the dimensionless concentrations (C/C_0) of vitamin D_3 at baking temperature at (a) 80, (b) 130, (c) 180, and (d) 230°C for n = 3. Error bars represent the standard deviation.

10.2, 7.7, 11.9, 11.2, and 11.9 kJ/mol, respectively. Obviously, the oils with the highest *Ea* value had the lowest degradation rates; consequently vitamin D_3 was more retained. The results were in a good accordance with the previous kinetic study of β-carotene and lutein degradation in oils during heat treatment [30].

3.3 *Antioxidant content during baking*

Different concentrations of antioxidants were added in canola oil according to the Recommended Dietary Allowances (RDAs). A concentration of 5 g/L for α-tocopherol and β-carotene as well as 180 mg/L BHT and TBHQ were used in this study. The amount of antioxidants were examined during four coupled times and temperatures being 80°C for 40 min, 130°C for 30 min, 180°C for 20 min and 230°C for 10 min, which can be seen in Figure 2(a) and (b). The significant loss of α-tocopherol and β-carotene content (1.9–5 fold) were observed at 80°C for 40 min compared to the initial concentration Figure 2(a).

The α-tocopherol was stable when the temperature increased. It was greatly retained at high temperature and short time of baking. However, the rate of vitamin D_3 degradation with added α-tocopherol was high, which means it may not be suitable to add α-tocopherol for delaying the vitamin D_3 degradation due to the heating condition in this study. In contrast, the other antioxidant contents were decreased progressively as temperature increased. The β-carotene and BHT were almost completely destroyed at 230°C for 10 min. Nonetheless, the TBHQ content was apparently the best retaining antioxidant after the highest temperature and lowest time of baking (230°C for 10 min). Literately, the TBHQ had been shown to be more effective in vegetable oils than BHA and BHT due to its diphenolic

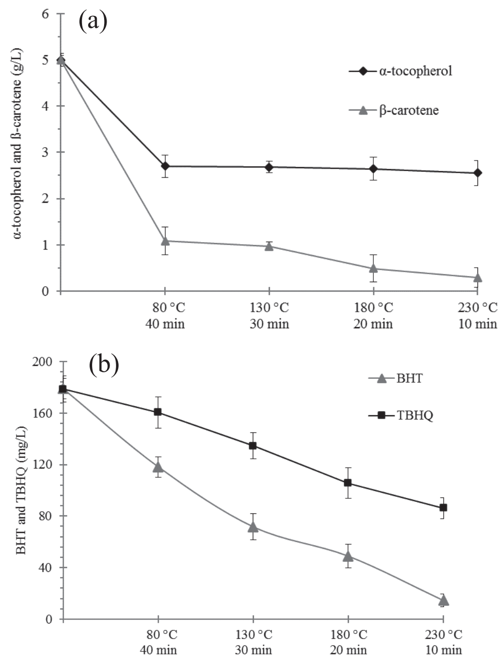

Figure 2: Antioxidant contents at different coupled temperatures and times: 80°C for 40 min, 130°C for 30 min, 180°C for 20 min and 230°C for 10 min. (a) α-tocopherol and β-carotene, (b) BHT and TBHQ for n = 3. Error bars represent the standard deviation.

chemical structure. It is stable to heat treatments and recognized as the most effective antioxidant in preventing the oxidation of frying oils [32]. Despite of the different antioxidant activities, the content of antioxidants confirmed that the more antioxidant was retained the better the vitamin D_3 was preserved and the rate of degradation were inversely low.

3.4 *Antioxidants activity and oxidation status of canola oil*

A previous study had demonstrated an inverse relationship between lipid oxidation products and vitamin D_3 retention at high heating temperatures [14]. Therefore, the antioxidant activity and oxidative status of canola oil were monitored during baking in order to find out whether the degradation of vitamin D_3 was influenced by the latter factors. The antioxidant activity of antioxidant agents and the primary and secondary oxidation products (*i.e.*, PV and MDA content) of canola oil were evaluated and illustrated in Figure 3.

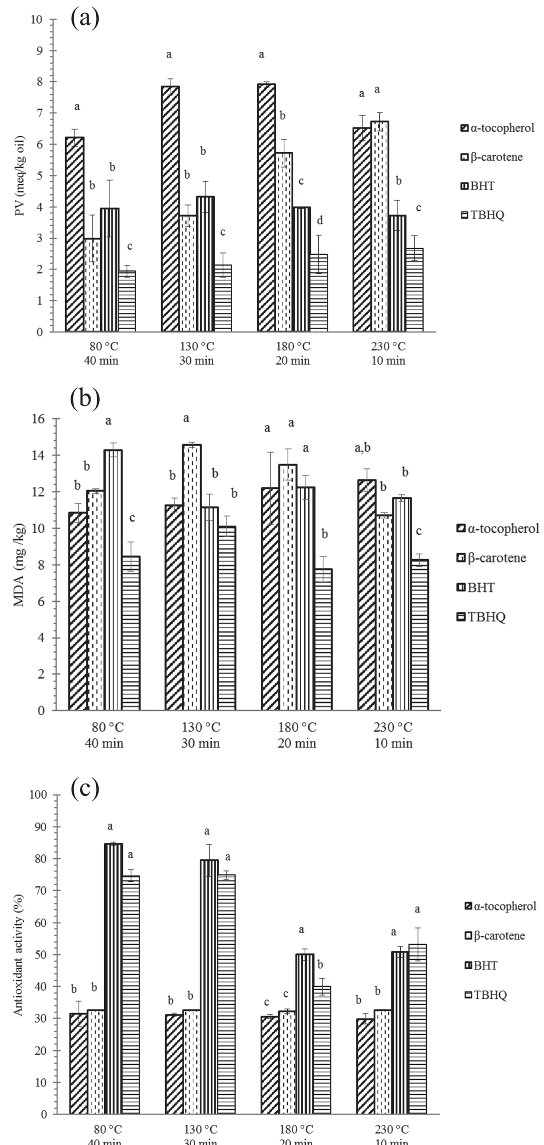

Figure 3: Peroxide value (a), malondialdehyde content (b) of canola oil and antioxidant activity (c) for n = 3. Different letters above each bars indicate significant difference ($p < 0.05$). Error bars represent the standard deviation.

The PV of baked oils increased as temperature increased for all treatments which varied from 2.0 to 7.9 meq O_2/kg oil. It was 2 to 8 fold higher than the initial ones. Canola oil fortified with α-tocopherol had the highest PV with 0.5 to 2 fold higher than the other

samples. Contrariwise, all canola oils incorporated in TBHQ had the lowest PV and their PV was quite stable for all treatments (PV < 4.5 meq O_2/ kg oil). However, the PV was still not perceived as rancid since the value was less than 20 meq O_2/kg, which can be considered as fresh oil.

The MDA content of samples was lower compared to the no heated ones. The antioxidants may inhibit the lipid peroxidation of polyunsaturated fatty acids of canola oil, thus decrease the secondary product of lipid peroxidation during baking. The MDA content ranged from 7.9 to 14.5 mg/kg and the lowest one was in canola oil incorporated in TBHQ. In this research, the samples with added BHT and TBHQ showed the highest antioxidant activity for all treatments, up to 85%. The antioxidant activity decreased as temperature increased because antioxidant contents were lost; hence the vitamin D_3 was more degraded. It had been demonstrated in the previous researches that α-tocopherol is more effective at high oxygen pressure than β-carotene. Moreover, the chain breaking antioxidant α-tocopherol is able to prohibit lipid peroxidation by scavenging peroxyl radicals faster than polyunsaturated fatty acid [33]. A higher dosage of vitamin A can cause instead of the antioxidant effect, oxidative stress and additionally an excess intake of vitamin A can cause vitamin D deficiency since it interferes with absorption of vitamin D [34].

In addition, oil samples lost their color and intensity when the temperature increased. However a low amount of TBHQ (180 mg/L) seems to be more effective in term of antioxidant activity against the vitamin D_3 degradation compared to the natural antioxidants (5 g/L). TBHQ was recognized as the most effective antioxidant against oxidation in polyunsaturated vegetal oils and often fortified in soybean oil. It was the most active antioxidant at 45°C in soybean oil and was more stable against heat treatment compared to ascorbic acid, ascorbyl palmitate, BHA, BHT and propyl gallate (PG) [35].

3.5 *Bioaccessibility of vitamin D_3*

The total vitamin D_3 content, the bioaccessible content and bioaccessibility were determined before and after the *in vitro* digestion of 5 g brownie with the four added antioxidants in canola oil as presented in Figure 4(a) and (b).

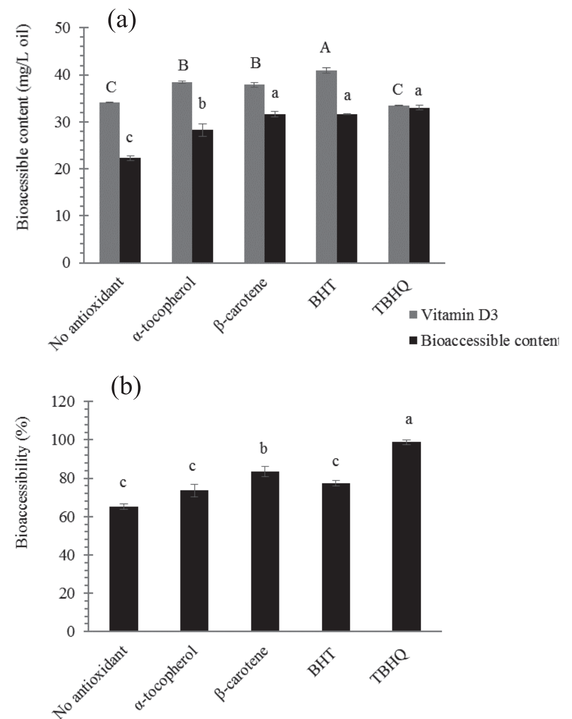

Figure 4: The contents in bioaccessible of vitamin D_3 (mg/L oil) (a) and its relative bioaccessibility (%) (b). Different big and small letters above each bars indicate, respectively, significant difference ($p < 0.05$) of vitamin D_3 content and their bioaccessible content of each antioxidants for $n = 3$. Error bars represent the standard deviation.

The highest total vitamin D_3 content of 40.9 mg/L oil was found in BHT after baking at 180°C for 20 min. The bioaccessible content of vitamin D_3 and its bioaccessibility ranged from 22.3 to 33.0 mg/L oil and 65.2 to 98.7% respectively. The brownie with incorporated TBHQ in oil had the highest bioaccessibility with 98 %. The bioaccessibility of vitamin D_3 in four formulas were 5–10 fold higher than the other lipid soluble vitamins such as provitamin A and vitamin K [36], [37]. The chemical structure and polarity of vitamins may play an important role to incorporation into the micelles during *in vitro* digestion. In case food is co-digested with antioxidant agents, the degradation of micronutrients may be protected during digestion condition (pH and digestive enzymes) [22].

As mentioned previously the RDA for vitamin D is 10–15 μg/day, for vitamin A 900 μg for men and 700 μg

for woman and for vitamin E 15 mg for men and women. According to the results, the consumption of 5 g brownie containing approximately 1 mL of canola oil fortified with vitamin D_3 (100 mg/L), in which the most powerful antioxidant TBHQ (180 mg/L) is incorporated, followed by baking at 180°C for 20 min, would cover the daily intake of vitamin D. However the bioavailability vary depending on the health status of each consumer to meet their vitamin D requirement. Therefore, the bioavailability and bioefficiency of vitamin D in oily food with added antioxidant agents have to be further carried on to ensure vitamin D content in the human body.

4 Conclusions

This research demonstrated the effect of four antioxidant agents on the heat stability of vitamin D_3 fortified in canola oil during baking method. TBHQ (180 mg/L) oil was demonstrated to be the most effective to retard the vitamin D_3 in canola oil degradation. TBHQ was also identified to increase the bioaccessibility of vitamin D_3 compared to the other antioxidant agents. Using an appropriate antioxidant agents allows to delay the chemical change and may extend the shelf life of vegetable oils. The carefulness of food additives has to be considered by the food industries in term of using the antioxidant agents as oil stabilizer. They have to pay attention, importantly on the amount suitable for food regulation to be safe for the consumers. The consumption of canola oil fortification in vitamin D_3 in which an appropriate amount of antioxidant were added could avoid the toxic effects after long-term consumption and solve the problem of vitamin D deficiency. As a matter of fact, this research can help to estimate the amount of vitamin D_3 available for gut absorption. The result could help many countries to achieve the goal of decreasing the number of population suffering the vitamin D deficiency by using food fortification method.

Acknowledgments

This work was supported by a grant from the faculty of Agro-industry, King Mongkut's University of Technology North Bangkok. The authors report no conflicts of interest. The authors are responsible for the content and writing of the paper.

References

[1] R. M. Daly, C. Gagnon, Z. X. Lu, D. J. Magliano, D. W. Dunstan, K. A. Sikaris, P. Z. Zimmet, P. R. Ebeling, and J. E. Shaw, "Prevalence of vitamin D deficiency and its determinants in Australian adults aged 25 years and older: A national, population-based study," *Clinical Endocrinology*, vol. 77, no. 1, pp. 26–35, Jul. 2012.

[2] M. F. Holick, T. C. Chen, Z. Lu, and E. Sauter, "Vitamin D and skin physiology: A D-lightful story," *Journal of Bone and Mineral Research*, vol. 22, no. S2, Dec. 2007.

[3] A. Kruavit, L. Chailurkit, A. Thakkinstian, C. Sriphrapradang, and R. Rajatanavin, "Prevalence of vitamin D insufficiency and low bone mineral density in elderly Thai nursing home residents," *BMC Geriatrics*, vol. 12, no. 1, p. 49, Sep. 2012.

[4] M. G. J. Balvers, E. M. Brouwer-Brolsma, S. Endenburg, L. C. P. G. M. de Groot, F. J. Kok, and J. K. Gunnewiek, "Recommended intakes of vitamin D to optimise health, associated circulating 25-hydroxyvitamin D concentrations, and dosing regimens to treat deficiency: Workshop report and overview of current literature," *Journal of Nutritional Science*, vol. 4, pp. 1–8, Jan. 2015.

[5] C. M. O'Neill, A. Kazantzidis, M. J. Ryan, N. Barber, C. T. Sempos, R. A. Durazo-Arvizu, R. Jorde, G. Grimnes, G. Eiriksdottir, V. Gudnason, M. F. Cotch, M. Kiely, A. R. Webb, and K. D. Cashman, "Seasonal changes in vitamin D-Effective UVB availability in europe and associations with population serum 25-hydroxyvitamin D," *Nutrients*, vol. 8, no. 9, p. 533, Aug. 2016.

[6] D. Parekh, J. M. Patel, A. Scott, S. Lax, R. C. A. Dancer, V. D'Souza, H. Greenwood, W. D. Fraser, F. Gao, E. Sapey, G. D. Perkins, and D. R. Thickett, "Vitamin D deficiency in human and murine sepsis," *Critical Care Medicine*, vol. 45, no. 2, pp. 282–289, Feb. 2017.

[7] Institute of Medicine, *Dietary Reference Intakes for Calcium and Vitamin D*. Washington, D.C: National Academies Press, 2011.

[8] P. B. Ottaway, "19 - Stability of vitamins during food processing and storage," in *Chemical Deterioration and Physical Instability of Food and Beverages*. Cambridge, England: Woodhead Publishing, 2010, pp. 539–560.

[9] B. Nikooyeh, A. Zargaraan, A. Kalayi, N. Shariatzadeh, M. Zahedirad, A. Jamali, M. Khazraie, B. Hollis, and T. R. Neyestani, "Vitamin D-fortified cooking oil is an effective way to improve vitamin D status: An institutional efficacy trial," *European Journal of Nutrition*, vol. 59, pp. 2547–2555, Oct. 2019.

[10] Y. Cui, P. Hao, B. Liu, and X. Meng, "Effect of traditional Chinese cooking methods on fatty acid profiles of vegetable oils," *Food Chemistry*, vol. 233, pp. 77–84, Oct. 2017.

[11] M. Maszewska, A. Florowska, E. Dłużewska, M. Wroniak, K. Marciniak-Lukasiak, and A. Żbikowska, "Oxidative stability of selected edible oils," *Molecules*, vol. 23, no. 7, Jul. 2018.

[12] Z. Saghafi, B. Nikooyeh, A. Jamali, M. Mehdizadeh, and A. Zargaraan, "Influence of time and temperature on stability of added vitamin D_3 during cooking procedure of fortified vegetable oils," *Nutrition and Food Sciences Research*, vol. 5, no. 4, pp. 43–48, Oct. 2018.

[13] P. S. Chen, A. R. Terepka, K. Lane, and A. Marsh, "Studies of the stability and extractability of vitamin D," *Analytical Biochemistry*, vol. 10, no. 3, pp. 421–434, Mar. 1965.

[14] M. Zareie, A. Abbasi, and S. Faghih, "Thermal stability and kinetic study on thermal degradation of vitamin D_3 in fortified canola oil," *Journal of Food Science*, vol. 84, no. 9, pp. 2475–2481, Sep. 2019.

[15] S. Casal, R. Malheiro, A. Sendas, B. P. P. Oliveira, and J. A. Pereira, "Olive oil stability under deep-frying conditions," *Food and Chemical Toxicology*, vol. 48, no. 10, pp. 2972–2979, Oct. 2010.

[16] K. Kittipongpittaya, W. Rungjang, and S. Klomlab, "Investigation of using beijing grass extract as a natural antioxidant in edible oil," *King Mongkut's University of Technology North Bangkok International Journal of Applied Science and Technology*, vol. 9, no. 1, pp. 45–52, 2016.

[17] H. Akhtar, I. Tariq, S. Mahmood, S. Hamid, and R. Khanum, "Effect of antioxidants on stability, nutritional values of refined sunflower oil during accelerated storage and thermal oxidation in frying," *Bangladesh Journal of Scientific and Industrial Research*, vol. 47, no. 2, pp. 223–230, Jul. 2012.

[18] Office of the Federal Register, *Code of Federal Regulations: 1985–1999*. Washington DC: Office of the Federal Register, 1985.

[19] C. P. Jayalakshmi and J. D. Sharma, "Effect of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) on rat erythrocytes," *Environmental Research*, vol. 41, no. 1, pp. 235–238, Oct. 1986.

[20] R. Kahl and H. Kappus, "Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin E," *Zeitschrift für Lebensmittel Untersuchung und Forschung*, vol. 196, no. 4, pp. 329–338, Apr. 1993.

[21] World Health Organization and Food and Agriculture Organization of the United Nations, *Vitamin and Mineral Requirements in Human Nutrition*. 2nd ed., Geneva, Switzerland: World Health Organization, 2004.

[22] V. K. Maurya and M. Aggarwal, "Factors influencing the absorption of vitamin D in GIT: An overview," *Journal of Food Science and Technology*, vol. 54, no. 12, pp. 3753–3765, Nov. 2017.

[23] M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. L. Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Ménard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies, and A. Brodkorb, "A standardised static *in vitro* digestion method suitable for food – An international consensus," *Food and Function*, vol. 5, no. 6, pp. 1113–1124, May 2014.

[24] C. Renaud, J. Berger, A. Laillou, and S. Avallone, "Quantification of vitamin A in fortified rapeseed, groundnut and soya oils using a simple portable device: comparison to high performance liquid chromatography," *International Journal for Vitamin and Nutrition Research*, vol. 83, no. 2, pp. 122–128, 2013.

[25] *Methods of Analysis of Fats and Fatty Oils: Other Methods. Determination of Carotene in Vegetable Oils*, BS 684-2.20:1977, 1976.

[26] A. Espinosa-Mansilla, F. Salinas, M. D. Olmo, and I. de O. Payá, "Determination of synthetic food Antioxidants in multicomponent mixtures

using UV-visible spectrophotometry and partial least-squares calibration," *Applied Spectroscopy*, vol. 50, no. 4, pp. 449–453, Aug. 1996.

[27] D. Hornero-Méndez, A. Pérez-Gálvez, and M. I. Mínguez-Mosquera, "A rapid spectrophotometric method for the determination of peroxide value in food lipids with high carotenoid content," *Journal of the American Oil Chemists' Society*, vol. 78, no. 11, pp. 1151–1155, Nov. 2001.

[28] A. Papastergiadis, E. Mubiru, H. V. Langenhove, and B. D. Meulenaer, "Malondialdehyde measurement in oxidized foods: Evaluation of the Spectrophotometric Thiobarbituric Acid Reactive Substances (TBARS) test in various foods," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 38, pp. 9589–9594, Sep. 2012.

[29] M. F. Ramadan, L. W. Kroh, and J.-T. Mörsel, "Radical scavenging activity of black cumin (*Nigella sativa* L.), Coriander (*Coriandrum sativum* L.), and Niger (*Guizotia abyssinica* Cass.) crude seed oils and oil fractions," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 24, pp. 6961–6969, Nov. 2003.

[30] N. Achir, V. A. Randrianatoandro, P. Bohuon, A. Laffargue, and S. Avallone, "Kinetic study of β-carotene and lutein degradation in oils during heat treatment," *European Journal of Lipid Science and Technology*, vol. 112, no. 3, pp. 349–361, 2010.

[31] P. Ložnjak and J. Jakobsen, "Stability of vitamin D_3 and vitamin D_2 in oil, fish and mushrooms after household cooking," *Food Chemistry*, vol. 254, pp. 144–149, Jul. 2018.

[32] F. Shahidi and Y. Zhong, "Antioxidants: Regulatory status," in *Bailey's Industrial Oil and Fat Products*. Georgia: American Cancer Society, 2005.

[33] G. W. Burton and K. U. Ingold, "β-Carotene: An unusual type of lipid antioxidant," *Science*, vol. 224, no. 4649, pp. 569–573, 1984.

[34] C. E. Schnorr, M. da Silva Morrone, A. Simões-Pires, R. F. da Rocha, G. A. Behr, and J. C. F. Moreira, "Vitamin A supplementation in rats under pregnancy and nursing induces behavioral changes and oxidative stress upon striatum and hippocampus of dams and their offspring," *Brain Research*, vol. 1369, pp. 60–73, Jan. 2011.

[35] E. N. Frankel, "Chapter 9 - Antioxidants," in *Lipid Oxidation*. 2rd ed., Cambridge, England: Woodhead Publishing, 2012, pp. 209–258.

[36] D. Hornero-Méndez and M. I. Mínguez-Mosquera, "Bioaccessibility of carotenes from carrots: Effect of cooking and addition of oil," *Innovative Food Science and Emerging Technologies*, vol. 8, no. 3, pp. 407–412, Sep. 2007.

[37] F. Sun, T. C. C. Jaspers, P. M. V. Hasselt, W. E. Hennink, and C. F. V. Nostrum, "A mixed micelle formulation for oral delivery of vitamin K," *Pharmaceutical Research*, vol. 33, no. 9, pp. 2168–2179, Sep. 2016.