Journal Pre-proofs

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PII: S0308-8146(20)30044-3
DOI: https://doi.org/10.1016/j.foodchem.2020.126197
Reference: FOCH 126197

To appear in: *Food Chemistry*

Received Date: 29 August 2019
Revised Date: 13 December 2019
Accepted Date: 9 January 2020

Please cite this article as: Olajide, T.M., Liu, T., Liu, H., Weng, X., Antioxidant properties of two novel lipophilic derivatives of hydroxytyrosol, *Food Chemistry* (2020), doi: https://doi.org/10.1016/j.foodchem.2020.126197

Antioxidant properties of two novel lipophilic derivatives of hydroxytyrosol

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ABSTRACT

Two novel lipophilic derivatives of the natural olive oil phenol, hydroxytyrosol (HT), were synthesized using 3,4-dihydroxyphenylacetic acid as starting material. Their antioxidant activities and kinetics compared to HT and TBHQ were assessed by Rancimat, Schaal Oven, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and deep-frying methods. All experiments, including kinetic data analysis based on the Arrhenius equation, utilized in assessing antioxidant activity except the DPPH assay revealed that the new lipophilic HT derivatives exhibited much stronger antioxidant activity than hydroxytyrosol. *Tert*-butylhydroquinone exhibited stronger antioxidant activity in bulk oil at 65 °C than the new HT derivatives, but showed much lower activity at higher temperatures (>110 °C). This demonstrates that the introduction of bulky alkyl moiety to the *ortho*-diphenolic structure of HT increased its antioxidant activity. It can be concluded that the new lipophilic HT derivatives satisfy industrial demands for bioactive compounds with strong antioxidant potential at high temperatures.

Keywords: Antioxidant activity; Hydroxytyrosol derivatives; Kinetic parameters; Lipophilicity; Rancimat test

Chemical compounds studied in this article:

Hydroxytyrosol (PubChem CID: 82755); 3,4-Dihydroxyphenylacetic acid (PubChem CID: 547); 2, 2-Diphenyl-1-picrylhydrazyl radical (PubChem CID: 15911)

1. Introduction

Food quality deterioration and off-flavor generation generally decrease nutritional value and shelf-life of food. This condition in fat and oil is caused by lipid oxidation—a sophisticated process involving three major steps: initiation, propagation and termination (Alamed, Chaiyasit, McClements, & Decker, 2009). Different methods have been developed to protect against lipid oxidation effects in foods; however, the addition of antioxidants remains the most effective method.

Antioxidants have become a vital group of food additives mainly due to their unique properties of elongating shelf-lives of food products without any negative effect on their sensory and/or nutritional qualities. The phenol types such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or *tert*-butylhydroquinone (TBHQ) have been utilized extensively for food preservation due to their good antioxidant capacity, but have been questioned due to their probable side effects for human health and also for their weak potency at high temperature food processing like deep frying (Olajide, Pasdar, & Weng, 2018).

The general aversion of consumers in the use of synthetic antioxidants has given rise to a recent trend in which phenolic antioxidants are synthesized structurally based on efficient natural antioxidants. Hence, the synthesis of novel antioxidants with better antioxidant potential and less toxicity which could better preserve food or prevent certain diseases, is very desirable (de Pinedo, Penalver, & Morales, 2007).

Hydroxytyrosol (HT) is the main simple phenol that gives olive oil its wide range of health benefits such as inhibition of inflammations, microbial activities, neurological damage, tumor growth, metabolic syndrome, cardiovascular diseases, as well as maintaining the oxidative stability of oil (Bañares, Martin, Reglero, & Torres, 2019; de Pinedo et al., 2007; Pereira-Caro et al., 2009; Trujillo et al., 2006). HT was recently approved as a novel food additive (Dominique et al., 2017). It has been of high interests to food, cosmetic and/or pharmaceutical industries (Sun, Zhou, & Shahidi, 2018), however its use is still very limited possibly due to its hydrophilic nature, which hinders its application under lipophilic conditions, as well as its isolation and purification from aqueous solutions (Mateos et al., 2015; Tofani, Balducci, Gasperi, Incerpi, & Gambacorta, 2010). Another drawback, which is of particular focus in this article, is the ineffectiveness of HT or its previously reported derivatives under high temperature food processing (Dominique et al., 2017).

Several authors have synthesized and reported the antioxidant activities of various derivatives of HT such as hydroxytyrosol acetate (Gordon, Paiva-Martins, & Almeida, 2001), alkyl hydroxytyrosyl ethers (de Pinedo et al., 2007; Pereira-Caro et al., 2009), alkyl nitrohydroxytyrosyl ether (Gallardo et al., 2016), isochromans (Mateos et al., 2015) and those with variable lengths of saturated and unsaturation fatty acid side chains (Mateos et al., 2008; Sun et al., 2018; Tofani et al., 2010). However, while some of these derivatives are yet to be tested in bulk oils, the few that have been tested only showed little or no antioxidant capacity over HT. More importantly, as of yet, there has been no report of any HT derivative that has good antioxidant activity at high temperature despite its numerous bioactivities. In terms of modifying phenolic compounds for better lipid solubility and antioxidant capacity at high temperature, the influence of *tert*-butyl group linked to the phenyl ring is greater than long-chain

alkanes (Huang, Jiang, & Liao, 2014; Shi, Liao, Olajide, Liu, Jiang, & Weng, 2017; Silva et al., 2000). Nevertheless, the analysis of kinetic parameters is useful for predicting the oxidative stability and temperature dependence of fats and oils in the presence and absence of antioxidants during heat processing, storage, and distribution (Hashemi et al., 2016). Also, kinetic data of edible oils oxidation from animal sources under Rancimat conditions with ease of use and appropriate reproducibility are scarce.

Therefore, taking into account the bioactivity attributed to hydroxytyrosol and the practical application of a lipophilic derivative that can withstand high temperature food processing; in this work, two novel butylated hydroxytyrosol derivatives, 3-(tert-butyl)-4,5-dihydroxyphenylacetic acid methyl ester (BuMT) & 3-(tert-butyl)-5-(2-hydroxyethyl)benzene-1,2-diol (BuHT), were synthesized and their antioxidant activities were assessed by Rancimat, Schaal Oven, DPPH and deep frying tests. The kinetics of BuMT and BuHT as efficient antioxidants provided by Arrhenius parameters, activation enthalpy (ΔH^{++}), entropy (ΔS^{++}), temperature coefficient (t_{coeff}) and Q_{10} in lard was also carried out.

2. Materials and methods

2.1. Materials

HT, 3,4-dihydroxyphenylacetic acid (DOPAC), thionyl chloride ($SOCl_2$), lithium aluminum hydride ($LiAlH_4$), and *p*-anisidine were purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). DPPH, TBHQ, tertiary butanol, anhydrous methanol (MeOH), anhydrous tetrahydrofuran (THF), ethanol, diethyl ether, glacial acetic acid, isooctane, dichloromethane (DCM), phosphoric acid (H_3PO_4), hydrochloric acid (HCl), sodium hydroxide (NaOH), petroleum ether (PE), ethyl acetate (EA), phenolphthalein indicator, potassium hydroxide

(KOH), sodium thiosulphate and starch were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

Lard was carefully rendered in the laboratory and stored below −18 °C for subsequent use. Potatoes were purchased from the local market. All solvents and reagents used in this experiment were of analytical grade and were used without further purification.

NMR spectra were recorded with a Bruker Avance 600 MHz spectrometer (USA) and data are reported as chemical shift δ (ppm) values referenced to the solvent used. Thin-layer chromatography (TLC) was conducted on 0.25 mm pre-coated silica gel plates with detection under UV light at 254 nm Induction periods (IP) of the antioxidants were measured by Rancimat 743 (Metrohm, Herisau, Switzerland) and UV-2450 spectrophotometer (Shimadzu Corp, Kyoto, Japan) for UV spectroscopy.

2.2. General synthesis of compounds

The synthesis of compounds **(3 & 4)** was done according to a previous method (Zhang, Xiao, Chen, & Lian, 2010) with some improvement (Fig. 1).

DOPAC methyl ester (**2**): 30 mmol of DOPAC (5 g) was dissolved in anhydrous methanol (60 mL) under stirring at 0°C in the presence of 40 mmol of $SOCl_2$ (3 ml) as acid catalyst. The mixture was stirred at room temperature for 9 h. After solvent was evaporated under low pressure, desired compound **2** (94% yield) was isolated as colorless oil by silica gel column chromatography using petroleum ether/ ethyl acetate (3:1, v/v) as eluent. Analytical data were as reported in Zhang et al. (2010).

3-(tert-butyl)-4,5-dihydroxyphenylacetic acid methyl ester (**3**): 20 mmol of DOPAC methyl ester (3 g) was added to a mixture of 200 mmol tertiary butanol (20 ml) and 98% phosphoric acid (7 ml) under nitrogen condition. The mixture was heated under reflux for 8 h, and then

evaporated to dryness under low pressure to remove residual solvent. After solvent removal, residue was washed with warm distilled water (50 ml) followed by ethyl acetate (30 ml) and the organic layers were evaporated to dryness under vacuum. The product mixture was applied to silica gel column chromatography using dichloromethane/methanol (20:0.1, v/v) as eluent to afford **3** (**BuMT**) (71% yield) as white crystals after recrystallizing from methanol: mp 99-100 °C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.70 (d, *J* = 1.9 Hz, 1H), 6.68 (d, *J* = 2.0 Hz, 1H), 3.71 (s, 3H), 3.51 (s, 2H), 1.39 (s, 9H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 173.5, 143.0, 142.7, 136.4, 124.0, 120.1, 113.6, 52.3, 40.8, 34.6, 29.5; HRMS (ESI): Calcd for $C_{13}H_{19}O_4^+$ $(M+H)^+$ m/z 239.12779, found 239.12756. The purity of BuMT was determined by HPLC as 99.4%.

3-(tert-butyl)-5-(2-hydroxyethyl)benzene-1,2-diol (**4)**: 10 mmol of BuMT (1.3 g) was added to a stirred solution of 20 mmol lithium aluminum hydride (0.6 g) in anhydrous tetrahydrofuran (80 ml) at 0 °C under nitrogen condition. The mixture was stirred at room temperature for 9 h, and then a 0.5 M aqueous hydrochloric acid solution (35 ml) was added to the mixture at 0 °C. After 30 minutes, the acidic mixture was washed with ethyl acetate (30 ml × 3), and the organic layers were combined and evaporated to dryness under low pressure. The residue was subjected to column chromatography using petroleum ether/ethyl acetate (2:1, v/v) as eluent to furnish **4** (**BuHT**) (91% yield) as white crystals after recrystallizing from methanol: mp 109-110 °C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.68 (d, *J* = 2.0 Hz, 1H), 6.60 (d, *J* = 2.0 Hz, 1H), 3.84 (t, *J* = 6.3 Hz, 1H), 2.74 (t, *J* = 6.3 Hz, 1H), 1.40 (s, 9H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 143.1, 142.1, 136.7, 128.7, 119.5, 113.6, 63.9, 38.6, 34.6, 29.5; HRMS (ESI): Calcd for $C_{12}H_{19}O_3^+$ $(M+H)^+$ m/z 211.13287, found 211.13269. The purity of BuHT was determined by HPLC as 98.9%.

2.3. Rancimat test

The antioxidant activities of TBHQ, HT, BuMT and BuHT in lard were determined according to Olajide et al. (2018) on a Rancimat 743 apparatus (Metrohm, Herisau, Switzerland). Lard samples of 3 ± 0.05 g containing different concentrations of antioxidants (0.01, 0.02, and 0.04%) were subjected to accelerated oxidation at temperatures between 100 and 140 °C under an air flow rate fixed at 20 L/h. Each test was carried out in duplicate and results are expressed as induction period (IP) of lard samples with antioxidants in hours corresponding to the stability of lard without antioxidant evaluated. The protection factors (Pf) of each antioxidant was determine according to Bañares et al. (2019):

$$Pf = IP_w/IP_{wo}$$

where IP_w and IP_{wo} represent the IP values with and without antioxidant, respectively. Pf values < 1 indicate a pro-oxidant effect of antioxidant, whereas when equal to 1 means no antioxidant effect and values of Pf > 1 are related to an antioxidant effect.

2.3.1. Kinetic parameters analysis

Temperature coefficients (t_{coeff}, $°C^{-1}$) were determined from the slopes of the lines obtained by regressing log (OSI) vs. temperature (T, °C):

$$log\ IP = a(T) + b \quad (1)$$

where $a = t_{coeff}$ and b represent slope and intercept, respectively.

Q_{10} number, which is defined as the increase in rate of oxidation reaction for every 10 °C rise in temperature, was calculated from slopes of the lines obtained above using this equation:

$$Q_{10} = 10^{-10a} \quad (2)$$

Activation energies (*Ea*, kJ/mol) and pre-exponential or frequency factors (*A*, h^{-1}) were evaluated from the slopes and intercepts respectively, of the lines obtained by regressing ln *k* vs. $1/T$ (K^{-1}) according to the Arrhenius equation:

$$\ln k = \ln A - (Ea/RT) \quad (3)$$

where *k* represents the reaction rate constant or reciprocal induction period (h^{-1}), *T* is the temperature in Kelvin, and R is the universal gas constant (8.3143 J/mol K)

Activation enthalpies (ΔH^{++}) and entropies (ΔS^{++}) were determined from the slope and intercept of the lines obtained by linear regression of ln (k/T) vs. $1/T$ according to the equation derived from the activated complex theory:

$$\ln (k/T) = \ln (k_B/h) + (\Delta S^{++}/R) - (\Delta H^{++}/RT) \quad (4)$$

where k_B represents the Boltzmann constant ($1.3806586 \times 10^{-23}$ J/K) and *h* is the Planck's constant ($6.62607556 \times 10^{-34}$ Js).

2.4. DPPH test

The DPPH radical scavenging activity of TBHQ, HT, BuMT and BuHT was determined according to previous method reported by Jiang, Weng, Huang, Hou, and Liao (2014) with slight modification. 0.5 ml of each antioxidant in methanol was mixed with 3 ml methanolic solution of DPPH (0.1 mM). The final concentrations of antioxidants in the reaction mixtures were 1.5, 3, 6, 12, 24, and 48 μM. The resulting mixtures were shaken vigorously and left to react in a dark chamber for 30 min, and then the decreasing absorbance of DPPH was read at 517 nm against blank on a UV-2450 spectrophotometer (Shimadzu Corp, Kyoto, Japan). Methanol was used as blank solution and 2.5 ml DPPH solution in 0.5 ml methanol served as the control. The radical scavenging activity of the antioxidants was expressed as EC_{50}, which is the effective

concentration required to obtain a 50% antioxidant capacity of a compound (Chen, Bertin, & Froldi, 2013). DPPH radical scavenging activity was calculated using the equation below:

Scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

2.5. Deep frying test

The lipid matrix used in this experiment was obtained from soybean oil by purification through a silica gel column according to the method described by Lampi, Kataja, Kamal-Eldin and Piironen (1999) with some modification. The purification process ensured the complete removal of endogenous pro-oxidants and antioxidants from the oil. Two replications of 30 h deep frying trials were conducted. Each oil sample (500 g) containing 0.02% (w/w) of antioxidants were heated to 180 ± 0.5 °C and kept at this temperature for 15 h on two consecutive days. Fresh potato slices (50 g) were fried in the oils every hour for 8 min. Oil samples were collected and stored below −18 °C after every frying until the conjugated dienes (CD) and acid values (AV) of all the samples were evaluated according to the IUPAC method (Paquot, 1979). Oils were not replenished during the frying trial.

2.6. Schaal Oven test

Soybean oil samples (50 g each) with and without antioxidant were placed in 100 ml capacity conical flasks and heated in an oven set at 65 ± 0.5 °C. The peroxide value (PV) and *p*-anisidine value (p-AV) in the samples were measured in duplicate at intervals of 0, 3, 6, 9, 12, and 15 d. The induction period of the samples was dependent on the oil reaching a peroxide value of 80 mEq O_2/kg oil (Nissiotis, & Tasioula-Margari, 2002) and an anisidine value of 10 (Maszewska et al., 2018). After PV and p-AV were determined, total oxidation (TOTOX) values were calculated according to the equation: TOTOX = 2PV + p-AV.

2.6.1. Peroxide value (PV)

The PV of oil samples was measured based on the method described in AOCS Method Cd8b-90 with some modification.

2.6.2 p-Anisidine value (p-AV)

p-Anisidine value (p-AV) was determined according to AOCS Official Method (1995). This method involves the spectrophotometric determination of products formed in the reaction between *p*-anisidine and aldehydes in the oil. Each oil sample (1 g) was dissolved in 25 ml isooctane and the absorbance of this test solution was read at 350 nm. Then 5ml of the above mixture was mixed with 1 ml *p*-anisidine (0.25% in glacial acetic acid, w/v) and absorbance was measured at 350 nm after 10 min. Reference solution was prepared by adding 1 ml of *p*-anisidine reagent to 5 ml isooctane. The p-AV was calculated according to the equation:

$$\text{p-AV} = 25(1.2A_s - A_b)/m$$

Where A_s is the absorbance of test solution after reaction with *p*-anisidine reagent; A_b is the absorbance of test solution only, and m is the mass in grams of oil samples.

2.7. Statistical analysis

All tests were performed in duplicate. Data are presented as mean ± standard deviation (SD). Significance differences between means were examined by analysis of variance (ANOVA) using OriginPro version 9.1 followed by Duncan's multiple range test ($P < 0.05$).

3. Results and discussion

3.1. Preparation, purification and characterization of BuMT and BuHT

The new lipophilic HT derivatives were obtained as illustrated in Fig. 1. BuMT was prepared by a two-step reaction involving the esterification of DOPAC followed by alkylation of

the *ortho*-diphenolic ring. BuHT was subsequently obtained by a one-step reduction process from BuMT using $LiAlH_4$ as reducing agent. Both compounds were synthesized in good yields at a purity of 99.4% (BuHT) and 98.9% (BuMT), as determined by HPLC. Their identity was confirmed by NMR spectroscopy and HRMS (ESI). The Rf values of HT, BuMT and BuHT were 0.28, 0.87 and 0.50, respectively (petroleum ether/ethyl acetate, 1:1). In addition, the theoretical partition coefficient (Log P_{theor}) values of HT, BuMT and BuHT were calculated as 0.96, 2.72 and 2.66, respectively, using ChemBioDraw Ultra Software. Rf values were coincidental with Log P_{theor} values, showing the following order: HT < BuHT < BuMT. This means that in terms of lipophilicity, HT had the highest polarity amongst all the evaluated compounds, showing a Log *P* value which is in agreement with previously published data (Gallardo et al., 2016; Mateos et al., 2015). BuMT and BuHT both showed less hydrophilic attributes due to the presence of higher carbon skeleton density in the form of bulky hydrophobic alkyl group bonded to their phenyl moiety. The methyl ester group attached to the aliphatic side chain of BuMT resulted in decreased polarity compared to BuHT. The UV spectra of the compounds were quite similar exhibiting two broad absorption bands in the range of 200–380 nm. BuMT and BuHT both had strong absorptions at 222/ 224 nm, and weak absorptions at 283/ 282 nm respectively, in the UV spectra. However, when KOH was added to the solutions, absorptions at 222 and 224 nm were strengthened and there were red shifts to 223 and 225 nm, respectively. Meanwhile, the weak absorption of BuMT had a red shift to 295 nm and BuHT to 293 nm. This phenomenon confirmed that the compounds were both phenolics. In the ^1H NMR spectrum of BuMT, phenolic protons were not observed. Two duplets around 6.70 and 6.68 ppm were assigned to the aromatic protons, and methyl of the ester moiety was observed at 3.71 ppm as a sharp singlet. The methylene protons were observed at 3.51 ppm while a proton singlet at

1.39 ppm was observed for tertiary butyl group attached to the aromatic ring. In the ^{13}C NMR, the most downfield signal 173.5 ppm was assigned to the carbonyl carbon of the ester group. Six aromatic carbon peaks were observed from 143.1 to 113.6 ppm. Methylene and methyl group carbon signals were assigned to 52.3 and 40.8 ppm, respectively, and tertiary butyl group carbon signals to 34.6 and 29.5 ppm. Similarly, phenolic protons were not observed in the ^{1}H NMR spectrum of BuHT. Two duplets around 6.68 and 6.60 ppm were assigned to the aromatic protons. Two methylene protons were observed at 3.84 and 2.74 ppm, while a proton singlet at 1.40 ppm was observed for tertiary butyl group attached to the aromatic ring. In the ^{13}C NMR, six aromatic carbon peaks were observed from 143.1 to 113.6 ppm. The methylene group carbon bearing OH and the one attached to the aromatic ring exhibited signals at 63.9 and 38.6 ppm, respectively, and the tertiary butyl group carbon signals were assigned to 34.6 and 29.5 ppm. In the HRMS (ESI) spectrum of BuMT, a protonated molecular ion peak was found at m/z 239.12756 for $[M+H]^+$, whereas for BuHT, a protonated specie at m/z 211.13269 was observed, which was assigned to $C_{12}H_{19}O_3^+$ $(M+H)^+$. All spectra data fully characterizes BuMT and BuHT, and as far as is known, neither has been previously described in any literature.

3.2. DPPH test

The DPPH radical scavenging assay is a simple, rapid and easily reproducible method used for evaluating the efficiency of antioxidants as radical scavengers against stable DPPH free radicals. Scavenging activities of the evaluated antioxidants are shown in Fig. 2. Results are expressed as EC_{50}, which is the effective concentration required to decrease the initial DPPH radical concentration by 50% (Chen et al., 2013; Pereira-Caro et al., 2009). Among all the tested compounds, HT (EC_{50}= 10.10 μM) showed the highest scavenging capacity, which was more

than double that of TBHQ (EC_{50}= 20.43 μM), BuHT (EC_{50}= 23.59 μM) and BuMT (EC_{50}= 25.31 μM). This finding coincides with earlier reports by Sun et al. (2018), where HT had highest scavenging activity as the most polar antioxidant. This phenomenon is explained by the polar paradox and interface theories in which the polarity of antioxidants is proportional to their DPPH radical scavenging activity. It is known that the presence of two or more hydroxyl groups on the phenyl ring can increase radical-scavenging activity by allowing more hydrogen atoms of the phenolic hydroxyl groups be donated to stabilize the free radicals (Shahidi, & Wanasundara, 1992). Herein, however, the presence of bulky *tert*-butyl group in the BuMT and BuHT chemical structures decreased their radical scavenging abilities compared with HT because of steric hindrance effects, which inhibited the ease of DPPH binding. Some researchers have also observed this phenomenon in other phenolic antioxidants and their derivatives. For instance, Shi et al. (2017) reported a lower free radical scavenging activity for butylated caffeic acid (BCA) than caffeic acid itself. In a separate study by Huang et al. (2014), a methylbenzenediol derivative, 3-(tert-butyl)-5-methylbenzene-1,2-diol (TBHPC), having a bulky alkyl group linked to the *o*-position of one hydroxyl also showed lower scavenging activity compared to its mother compound, 4-methylcatechol (HPC) which is similar to HT in structure. In summary, the hydrogen-donating ability, molecular structure, and subsequent stabilization of phenoxyl radical influence the antioxidant activity of the phenolic compounds (Silva et al., 2000). Thus, the steric hindrance induced by the bulkiness of the alkyl groups varied the antioxidant capacity of the HT derivatives in contrast with HT (Roleira et al., 2010).

3.3. Antioxidant activity in oil – Rancimat and deep-frying tests

The efficacy of BuMT and BuHT as antioxidants in comparison with HT and TBHQ was evaluated using the Rancimat test at temperatures in the range 100–140 °C, and concentrations at

0.01, 0.02 and 0.04% (w/w) in air saturation conditions. The results were expressed as induction period (IP) corresponding to the oxidative stability of lard, protection factor (Pf) of antioxidants and Arrhenius kinetic parameters from which the effect of temperature on the rates of oxidation was evaluated as shown in Table 1. According to Wang et al. (2000), a higher Pf value means stronger antioxidant activity: Pf < 1 shows pro-oxidant activity; Pf = 1 shows no antioxidant activity; 2 > Pf > 1 shows weak antioxidant activity; 3 > Pf > 2 shows potent antioxidant activity and Pf > 3 shows the compound has a strong antioxidant activity. In this study, concentrations up to 400 ppm were investigated to accurately evaluate the variability of antioxidant protection at a broader range of doses. Nevertheless, there is a 215 mg/kg safety limit established for HT (Dominique et al., 2017) and 200 mg/kg for synthetic antioxidants like TBHQ in vegetable oils (Saad et al., 2007).

Pf values greater than 1 were observed for all evaluated antioxidants at 0.02% (w/w) in the temperature range studied (Table 1a) which indicate a protective effect against accelerated oxidation. The highest Pf was observed for BuMT, followed by BuHT, then the slightly less stable, HT and finally, TBHQ. Averagely, both BuMT and BuHT showed an induction period (IP) 51 h higher than HT and about 126 h more than TBHQ. This demonstrates that they are much stable than both HT and the common commercial phenolic antioxidant, TBHQ. Similarly, the newly synthesized lipophilic HT derivatives, BuMT (0.01%, Pf = 10.58; 0.04%, Pf = 32.05) and BuHT (0.01%, Pf = 12.66; 0.04%, Pf = 27.94), showed substantially higher antioxidant activity than HT (0.01%, Pf = 10.13; 0.04%, Pf = 17.75) or TBHQ (0.01%, Pf = 3.98; 0.04%, Pf = 7.63) in the lipid matrix at 120 °C (Table 1b). As expected, IP and Pf values increased as concentration of the antioxidants increased. Summarily, the antioxidant activity of the phenolic antioxidants as a function of temperature and concentration decreased as follows: BuMT ≈

BuHT > HT >> TBHQ > Control. HT has been reported to display the strongest antioxidant activity in olive oil, having higher induction period in Rancimat test than caffeic acid or gallic acid (Ranalli, Lucera, & Contento, 2003). Nevertheless, in this experiment, the stronger antioxidant activity of BuMT and BuHT compared to HT and TBHQ was partly due to higher molecular weight, which reduced their partial volatilization. A similar finding has been reported (Huang et al., 2014; Jiang et al., 2014; Olajide et al., 2018 & Shi et al., 2017). It has been established that the catechol group is one of the main contributors to the antioxidant capacity of phenolic compounds, enhancing their redox potential, radical scavenging ability and metal-chelating capacities (Pereira-Caro et al., 2009). Additionally, alkylating *ortho*-diphenolic structures with electron donating substituents like bulky *tert*-butyl group at the 2, 4 and 6-positions can further increase their antioxidant activity (Kajiyama, & Ohkatsu, 2001; Weng, & Huang, 2014; Zhang, Wu, & Weng, 2004). Hence, the Rancimat results demonstrate that alkylation of HT at the *o*-position preserved the *ortho*-diphenolic group, and positively influenced antioxidant potentials of the new HT derivatives.

The kinetic parameters obtained from Rancimat test are listed in Table 1c. Activation energy (*Ea*), which is the minimal energy of molecules required to initiate oxidation reaction was employed to evaluate the dependence of the rate of lipid oxidation on temperature. *Ea* of the various oil sample oxidation ranged from 66.16 to 86.32 kJ/mol. Hashemi et al. (2016) reported the activation energies of blended oils spiked with antioxidants obtained in a higher temperature range 120–140 °C to be between 68.34 and 82.47 kJ/mol. The activation energy of HT, BuMT and BuHT oil samples were lower compared to that of TBHQ. A greater *Ea* value implies that a smaller temperature change is needed to increase the rate of oxidation Bañares et al. (2019). In other words, oils containing BuMT and BuHT were more

resistant to oxidative degradation (*Ea* = 84.72 and 86.30 kJ/mol, respectively) at higher temperature than TBHQ (*Ea* = 86.32 kJ/mol). Interestingly, the *Ea* value of control showed an opposite trend to the result expected, but is however comparable to the study by Bañares et al. (2019), where the kinetic parameters of *Echium* oil including *Ea* (74.1 kJ/mol) in the temperature range 50-110 °C were lower compared to antioxidant (rosemary extract) spiked oil samples, *Ea* (78.7 kJ/mol). The oxidation pathway at different temperatures may vary due to saturated and unsaturated (MUFA + PUFA) fatty acid ratio, reactivity of metal ions, presence of antioxidants and prooxidants and also because the solubility of oxygen in oil reduces by 25% for every 10 °C decrease in temperature (Bañares et al., 2019; Farhoosh & Hoseini-Yazdi, 2014). The pre-exponential or frequency factor (*A*) for various oil sample oxidation ranged from $5.2x10^8$ to $4.2x10^{10}$. It is the number of collisions that produce chemical change; hence, a higher *A* value means higher probability of successful collisions leading to oxidation. In general, frequency factor (*A*) reduces in the presence of antioxidants. The TBHQ sample had higher frequency factor than the new antioxidants. This increase of frequency factor in the presence of TBHQ may be ascribed to its instability at higher temperature leading to reduced hydrogen donating ability and consequently the loss of rotational freedom in the transition state.

The ΔH^{++} and ΔS^{++} values were computed based on activated complex theory from linear regression parameters summarized in Table 1c. ΔH^{++} and ΔS^{++} values ranged from 62.89 kJ/mol and -88.71 J mol^{-1} K^{-1}, to 84.06 kJ/mol and -52.02 J mol^{-1} K^{-1}, respectively, for the oxidation of oil samples. The positive sign of ΔH^{++} depicts an endothermic nature of activated complex formation. In this work, the enthalpy and entropy of TBHQ sample were higher and lower, respectively, than that of BuMT and BuHT, therefore the concentration of the activated complex in oil containing the new antioxidants was lower than oil with TBHQ. The greater negative ΔS^{++}

value of oil depicts a more ordered activated complex than the reactants, and hence a lower probability of faster lipid oxidation reaction (Hashemi et al., 2016).

The temperature coefficients (t_{coeff}) and Q_{10} numbers computed from log (IP) *vs*. *T* (°C) linear representation are shown in Table 1c. The t_{coeff} values ranged from -2.2×10^{-2} to -2.9×10^{-2} °C^{-1} and Q_{10} values were between 1.66 and 1.96 for all the samples. The t_{coeff} and Q_{10} values presented for control in this study are comparable to that reported in Farhoosh and Hoseini-Yazdi (2014) for lard at -2.55×10^{-2} °C^{-1} and 1.8, respectively. A higher *Q*10 number implies that a smaller change in temperature is needed to increase the rate of lipid oxidation. Antioxidants can decrease the dependence of the rate of lipid oxidation on temperature (Bañares et al., 2019). Herein, the temperature dependence of lipid oxidation rate in the presence of BuHT and TBHQ were similar, but lower than that of BuMT. This means that BuMT was fairly active than BuHT but more active than TBHQ at high temperatures as evidenced by the IP and Pf values in Table 1a. Finally, the reaction rate constant (*k*), which is the reciprocal of induction period was also employed to evaluate the protective effect of the antioxidants at temperatures between 100 and 140 °C. The higher the oxidation reaction rate constant, the lower the antioxidant protection. Hence, the *k* values for the oil samples relative to antioxidant activity of the antioxidants as a function of temperature decreased as follows: BuMT ≈ BuHT > HT >> TBHQ > Control.

Deep frying is a common process where food is wholly immersed in hot oil to produce crispy food with better texture and taste. Conjugated diene (CD) and acid values (AV) are useful quality control parameters used for determining the activity of antioxidants in the deep-frying test. The results of CD measured at 233 nm are expressed in Fig. 3a. A gradual increase was observed in CD contents for BuHT and BuMT compared to a faster rate for HT, TBHQ and control relative to the frying time. Initial CD value was 0.53%, which at the end of 30 h deep

frying reached 12.10, 8.88, 7.03, 3.83 and 3.34% for control, TBHQ, HT, BuHT and BuMT groups, respectively ($p < 0.05$). The continuous formation of CD may be related to the presence of high contents of polyunsaturated fatty acids in soybean oil (Marinova et al., 2012). The increase in CD is proportional to the uptake of oxygen and a greater level of CD means lower oxidative stability of the oils (Bushra, Farooq, & Roman, 2007). According to Fig. 3b, the acid value (AV) of BuHT and BuMT spiked oil samples at the end of frying trials increased slowly to about 2.32 and 2.24 mg KOH/g, respectively, in comparison with HT, TBHQ, and control groups, that increased to 3.44, 3.71 and 4.17 mg KOH/g of oil, respectively. This shows that the new HT derivatives were able to suppress lipid oxidation better than HT or TBHQ leading to lower acid values, which is an attribute of good quality oil. Thus, the CD and AV changes in oil samples indicate that the antioxidant activities of compounds during frying decreased as follows: BuMT ≥ BuHT > HT > TBHQ > control. This finding is in agreement with published report by Shi et al. (2017), where the superior antioxidant activity of butylated caffeic acid (BCA) as with BuHT and BuMT under Rancimat and deep-frying tests was due to the presence of *tert*-butyl group at the *ortho*-position to their diphenolic structure. In addition, the presence of a catechol moiety and an alkyl side-chain with primary alcohol linked to phenyl ring are structural factors that can increase antioxidant activity (de Pinedo et al., 2007). These phenomena provided strong steric hindrance influence that helped stabilized the radical resonance of the antioxidant structure in capturing more peroxyl radicals, and contributed to an increased molecular mass that enhanced less volatilization than HT and TBHQ.

Contrarily, the tendency found in the Rancimat and deep-frying tests for the new phenolic antioxidants derived from HT under study was different to the one found in the DPPH test results. This finding, which was largely due to the bulkiness of the DPPH radical as it can bind

easily with phenoxyl radicals with less steric hindrance like HT (Huang et al., 2014), is in agreement with similar studies by others (Jiang et al., 2014; Olajide et al., 2018; Shi et al., 2017; Weng & Huang, 2014).

3.4. Antioxidant activity in oil –Schaal Oven test

PV is one of the most commonly used tests for the determination of oxidative rancidity in oils and fats. The antioxidant potential of antioxidants at 200 ppm in soybean oil evaluated in this work is also evident from the measurement of primary oxidation products by the peroxide value (PV) as a function of time (in days) in an oven at 65 ± 0.5 °C. Oil samples were oxidized until a peroxide value of 80 mEq /kg was reached. As shown in Fig. 4a, the PV of fresh oil was 0.53 mEq O_2/Kg oil, indicating that the oil used was in good condition for oxidative stability determination and meets the requirements for edible oils specified by Codex Alimentarius (1999) (PV <15 mEq O_2/Kg). Except for control, the oxidation of oils containing TBHQ, HT, BuHT and BuMT proceeded at a slower rate initially, but gradually increased dynamically with storage time. Oil sample without antioxidant (control) reached a maximum PV of 79.2 ±0.85 mEq/kg after 6 days of storage, whereas for HT and BuMT spiked samples it required 11 and 15 days to reach a PV value of 80 mEq/kg, respectively. Interestingly, both TBHQ and BuHT suitably sustained the oxidative stability of oil samples with PV values of 7.7 ± 0.13 and 27.33 ± 0.18 mEq/kg, respectively, after 15 days of storage.

The *p*-anisidine value (p-AV) is a good method for evaluating secondary oxidation products formed during the oxidative degradation of oil. For a good quality oil or fat, p-AV lower than 10 is expected (Tadesse, Ret, & Beyero, 2017). According to Fig. 4b, all samples had a p-AV less than 10 after 15 days of storage at 65 ± 0.5 °C except for the control and HT groups. As with PV above, TBHQ-spiked sample had the lowest p-AV level compared to other

antioxidants, gradually reaching a maximum of 2.24±0.04 from an initial value of 1.61±0.01, whereas BuHT and BuMT smoothly increased to 4.71±0.23 and 7.62±0.11, respectively, after 15 days of storage.

TOTOX represents overall oxidative deterioration since it considers both peroxides and aldehydes produced during oxidation. The lesser the TOTOX value, the better the oil quality. As shown in Fig. 4c, similar pattern in peroxide and *p*-anisidine values were observed for TOTOX values of the samples with storage time. Therefore, the antioxidant activities of compounds studied under the Schaal Oven test decreased as follows: TBHQ > BuHT > BuMT > HT > control. The difference in efficacy of antioxidants may be accounted for on the basis of their chemical structures. That is, the rate of propagation of oxidation reactions in the lipid matrix was reduced by the antioxidants ability to stabilize phenoxy radicals (Ying et al., 2010). BuMT and BuHT both provided a much stronger protective effect than TBHQ at higher temperature experiments (> 110 °C) such as Rancimat and deep frying, however at lower temperatures (< 65 °C) their protective effect seems to diminish. In addition, the superior antioxidant activity demonstrated by TBHQ in comparison with HT and its new lipophilic derivatives, especially BuMT, at lower temperature in this work is also evident in the studies done by Zhang et al. (2004). This observation was as a result of the two *para*-hydroxyl groups on TBHQ which can release hydrogen atoms to active peroxyl radicals in edible oils to interrupt the oxidative chain reaction (Nissiotis & Tasioula-Margari, 2002).

4. Conclusion

In this work, a thorough study of the antioxidant activity of two novel lipophilic derivatives of HT, a natural antioxidant predominantly found in olive oil, has been conducted using the Arrhenius kinetic parameters, Rancimat, Schaal Oven, DPPH and deep-frying tests. The results

demonstrated that BuMT and BuHT are good antioxidants in bulk oils at high temperatures, but their potency tends to slightly decrease at lower temperatures (< 65 °C). Although, they exhibited a weak DPPH radical scavenging activity compared to HT and TBHQ, clearly the butylated *ortho*-diphenolic structure, as well as the alkyl side-chain bearing a primary alcohol (for BuHT) and a methyl ester (for BuMT) highly influenced their antioxidant capacity. The polar paradox is not applicable to the antioxidant capacity found for these new phenolic antioxidants, except from a very general perspective. Nevertheless, these new lipophilic HT derivatives satisfy the requirements for new antioxidants with strong antioxidant potential at high temperatures, and therefore maybe used as functional antioxidant ingredients in food, cosmetics and pharmaceuticals after proper evaluation of their safe consumption, which will be further studied.

Acknowledgement

The authors are thankful to Ms. Yanhong Song and Dr. Faiz-Ur Rahman of the Center for Supramolecular Materials and Catalysis, Shanghai University, for helping with the NMR and HRMS (ESI) analyses.

Conflict of Interest

The authors declare no conflict of interest in this experiment.

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FIGURE CAPTIONS

Fig. 1. Synthetic route leading to 3-(tert-butyl)-4,5-dihydroxyphenylacetic acid methyl ester (BuMT, **3**) and 3-(tert-butyl)-5-(2-hydroxyethyl)benzene-1,2-diol (BuHT, **4**).

Fig. 2. DPPH radical scavenging activity of lipophilic hydroxytyrosol derivatives (BuMT & BuHT), HT, and TBHQ**.** Values are expressed as means ± SD (n=2).

Fig. 3. Changes in conjugated diene (a) and acid values (b) of soybean oil spiked with and without antioxidant during deep frying. Values are expressed as mean ± SD (n=2).

Fig. 4. Changes in peroxide value (a), *p*-anisidine value (b) and Totox (c) of soybean oil spiked with and without antioxidant during oven test. Values are expressed as mean ± SD (n=2).

Figure 1

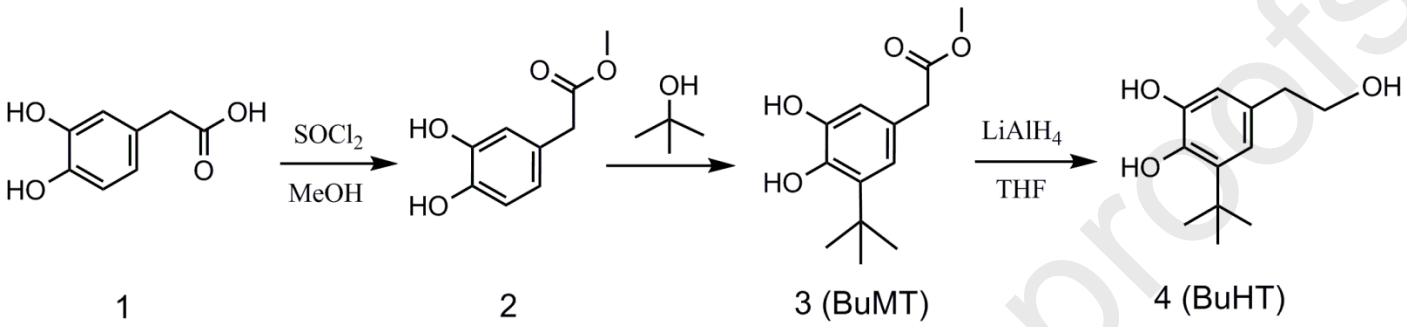

Figure 2

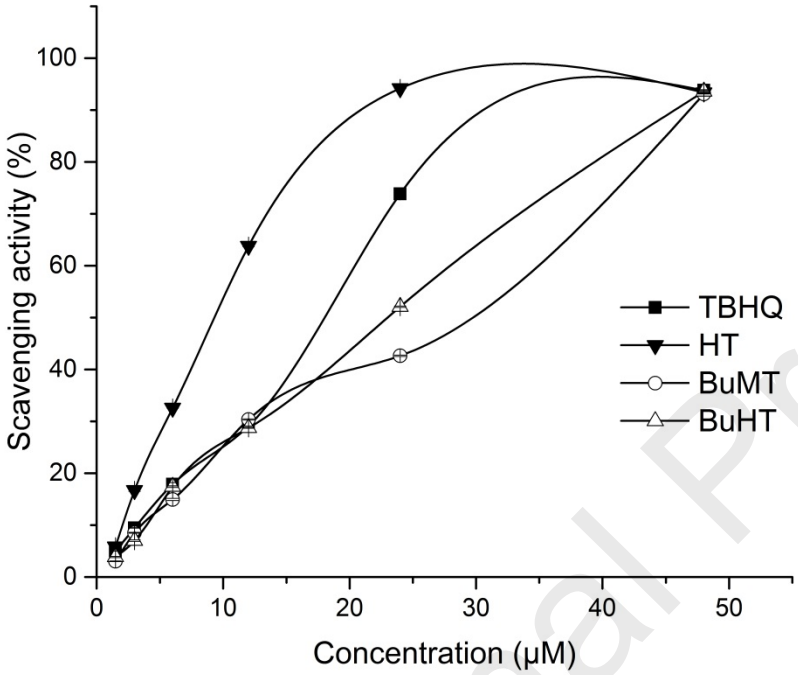

Figure 3

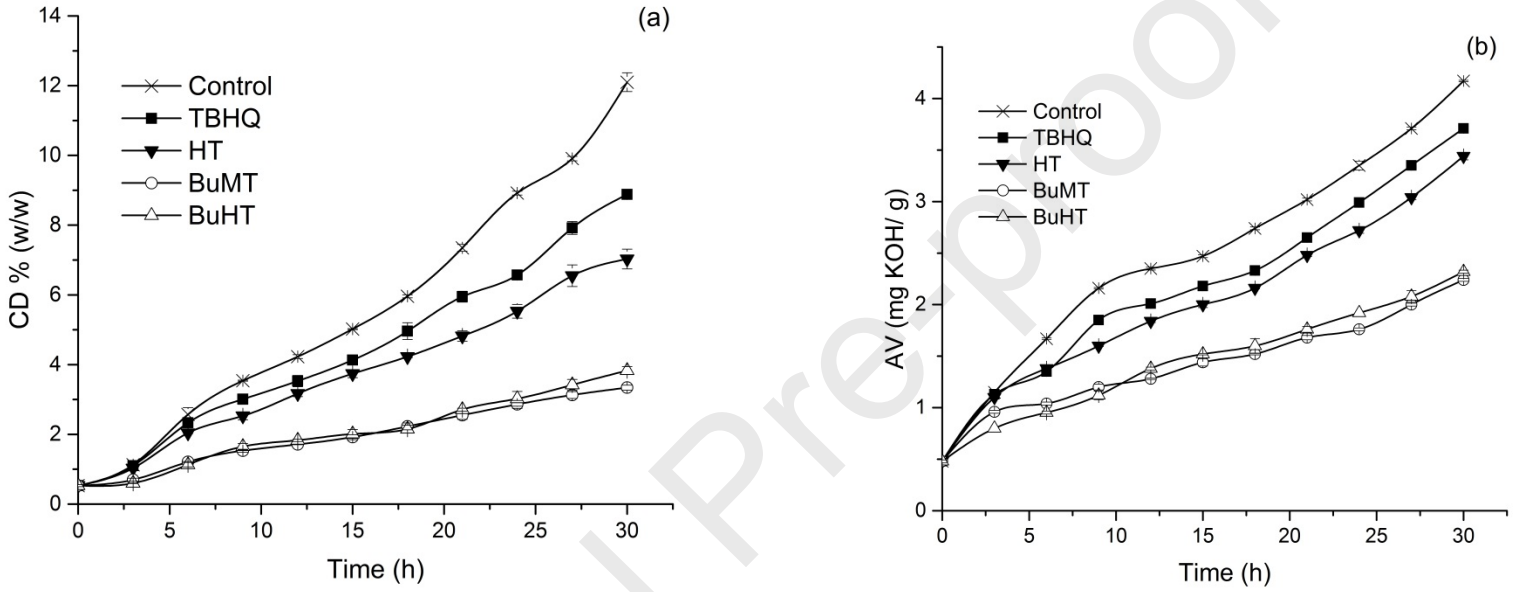

Figure 4

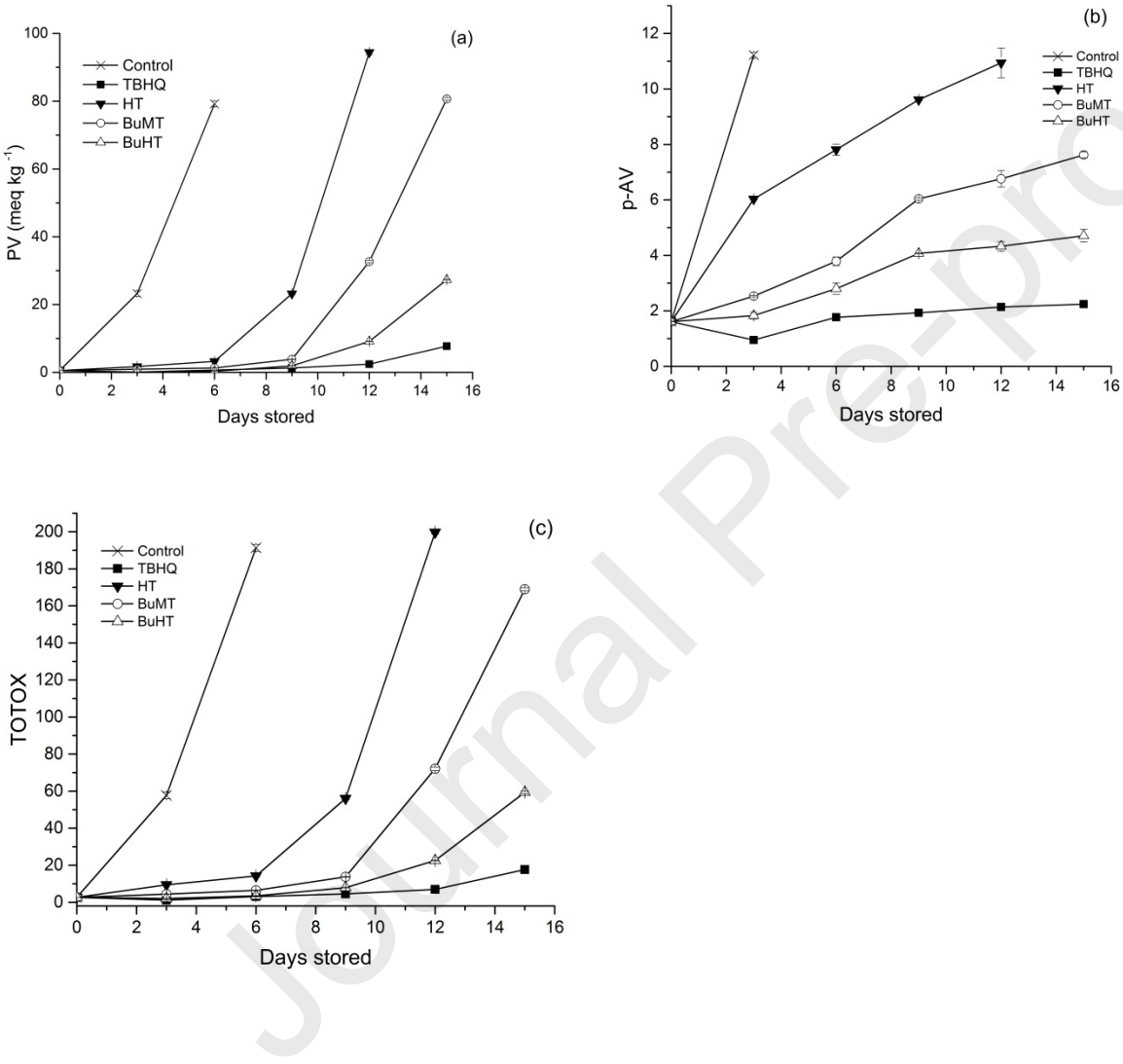

Table 1

Induction periods (IP), protection factors (Pf), activation enthalpy (ΔH^{++}), entropy (ΔS^{++}), temperature coefficient (t_{coeff}), Q_{10} and Arrhenius parameters obtained for lard spiked with different concentrations of antioxidants in the temperature range 100-140 °C under Rancimat test.

(a)	Antioxidant presence at 0.02% (w/w)							
Temperature (°C)	IP (h)				Pf			
	TBHQ	HT	BuMT	BuHT	TBHQ	HT	BuMT	BuHT
100	32.58 ± 0.04	72.98 ± 0.64	101.34 ± 0.63	102.99 ± 0.45	8.35	18.70	25.98	26.41
110	11.79 ± 0.08	29.68 ± 3.35	40.62 ± 0.38	41.67 ± 0.04	6.24	15.70	21.49	22.05
120	6.85 ± 0.01	18.65 ± 0.28	23.79 ± 0.82	23.23 ± 0.17	5.98	16.22	20.69	20.20
130	3.69 ± 0.26	8.58 ± 0.55	12.74 ± 0.80	12.13 ± 0.04	5.23	12.08	17.94	17.20
140	2.02 ± 0.06	4.83 ± 0.09	6.66 ± 0.04	6.61 ± 0.02	4.12	9.86	13.59	13.49

(b)	Antioxidant activity at 120 °C							
Concentration (%)	IP (h)				Pf			
	TBHQ	HT	BuMT	BuHT	TBHQ	HT	BuMT	BuHT
0.01	7.04 ± 0.13	17.93 ± 0.32	18.74 ± 0.05	22.40 ± 0.71	3.98	10.13	10.58	12.66
0.02	7.84 ± 0.02	18.65 ± 0.28	23.79 ± 0.82	23.23 ± 0.17	6.83	16.22	20.69	20.20
0.04	13.51 ± 0.77	31.43 ± 0.80	56.72 ± 1.58	49.45 ± 1.92	7.63	17.75	32.05	27.94

(c)	Kinetic Parameters at 0.02% (w/w)						
Oil samples	$\ln k = A(1/T) + B$						
	A	B	R^2	A (h^{-1})	Ea (kJ mol^{-1})	ΔH^{++} (kJ mol^{-1})	ΔS^{++} (J mol^{-1} K^{-1})

Control	-7957.15 ± 54.7	20.06 ± 0.11	0.992	$5.2x10^8$	66.16	62.89	-88.71
TBHQ	-10382.5 ± 10.6	24.48 ± 0.03	0.990	$4.2x10^{10}$	86.32	84.06	-52.02
HT	-10286.5 ± 23.3	23.34 ± 0.10	0.993	$1.4x10^{10}$	85.52	82.26	-61.44
BuMT	-10190.5 ± 112	22.78 ± 0.31	0.995	$7.8x10^9$	84.72	81.46	-66.14
BuHT	-10380.5 ± 2.12	23.27 ± 0.01	0.996	$1.3x10^{10}$	86.30	83.04	-62.02

log IP = a(T) + b

	a = t_{coeff} (°C^{-1})	b	R^2	Q_{10}
Control	-0.022 ± 0.0001	2.78 ± 0.03	0.995	1.66
TBHQ	-0.029 ± 0.0003	4.36 ± 0.00	0.990	1.96
HT	-0.029 ± 0.0000	4.72 ± 0.01	0.991	1.95
BuMT	-0.029 ± 0.0003	4.83 ± 0.03	0.992	1.95
BuHT	-0.029 ± 0.0002	4.89 ± 0.00	0.993	1.96

Temperature (°C)	k ± SD (× 10^{-2}) [h^{-1}]				
	Control	TBHQ	HT	BuMT	BuHT
100	25.70 ± 0.89	3.10 ± 0.00	1.40 ± 0.01	0.99 ± 0.00	0.97 ± 0.00
110	53.10 ± 1.49	8.50 ± 0.06	3.40 ± 0.37	2.50 ± 0.02	2.40 ± 0.00
120	87.40 ± 2.70	14.60 ± 0.02	5.40 ± 0.08	4.20 ± 0.15	4.30 ± 0.03
130	141.90 ± 4.30	27.10 ± 0.05	11.70 ± 0.75	7.90 ± 0.49	8.20 ± 0.02
140	206.20 ± 3.00	49.50 ± 0.35	20.70 ± 0.39	15.00 ± 0.08	15.10 ± 0.05

*3.00 ± 0.05 g lard was used; IP of lard: 3.90 ± 0.13, 1.89 ± 0.05, 1.15 ± 0.04, 0.71 ± 0.02 and 0.49 ± 0.01 h at 100, 110, 120, 130 and 140 °C, respectively; air flow rate was 20 L/h. IP values are expressed as mean ± standard deviation (n=2). Pf = IP of lard with antioxidant/ IP of control.

Term	**Definition**
Xinchu Weng & Tosin M. Olajide	Conceptualization

Term	Definition
Tosin M. Olajide, Xinchu Weng, Tao Liu & Haian Liu	Methodology
Tosin M. Olajide	Software
Tosin M. Olajide & Xinchu Weng	Validation
Tosin M. Olajide, Xinchu Weng, Tao Liu & Haian Liu	Formal analysis
Tosin M. Olajide, Xinchu Weng	Investigation
Tosin M. Olajide, Xinchu Weng & Tao Liu	Resources
Tosin M. Olajide & Xinchu Weng	Data Curation
Tosin M. Olajide & Xinchu Weng	Writing - Original Draft
Tosin M. Olajide & Xinchu Weng	Writing - Review & Editing
Tosin M. Olajide, Xinchu Weng, Tao Liu & Haian Liu	Visualization
Tosin M. Olajide & Xinchu Weng	Supervision
Tosin M. Olajide & Xinchu Weng	Project administration
Xinchu Weng	Funding acquisition

Highlights

- Butylated hydroxytyrosol (HT) derivatives are excellent antioxidants in lipophilic media.
- HT derivatives showed stronger antioxidant potentials than HT and TBHQ at high temperatures (>110 °C).
- Antioxidant activities of the lipophilic HT derivatives were stronger due to the introduction of bulky alkyl group to the *ortho*-diphenolic structure of HT.

- Scavenging activity against DPPH radical decreased in the presence of bulky alkyl moiety.
- Arrhenius kinetic parameters were evaluated.