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Thermal Degradation Kinetics of Ascorbic Acid and Some B-Complex Vitamins in Black Mulberry (*Morus nigra*) Juice

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Abstract

In this paper, L-ascorbic acid, thiamine (vitamin B_1), riboflavin (vitamin B_2) and niacin (vitamin B_3) thermal degradations depending on different thermal treatment times (0, 5, 10, 15, 20, 25 and 30 min) and temperatures (70, 80, 90 and 95°C) were investigated and results were presented. Firstly, black mulberry juice was obtained from fresh black mulberry fruits and then thermal treatment was carried out. L-ascorbic acid, thiamine, riboflavin and niacin were analyzed by using HPLC method with 25 min separation time. L-ascorbic acid, thiamine, riboflavin and niacin thermal degradation followed first order kinetic model during thermal treatments. Particularly, increment in temperature from 80°C to 90°C significantly increased rate constants of compounds. Activation energy, which indicates temperature dependence of reaction, was calculated using Arrhenius equation. Activation energies were found to be 58.5 kJ mol^{-1}, 40.5 kJ mol^{-1}, 45.9 kJ mol^{-1} and 52.1 kJ mol^{-1} between 70°C and 95°C for ascorbic acid, thiamine, riboflavin and niacin, respectively. L-ascorbic acid, thiamine, riboflavin and niacin thermal degradation in black mulberry juice was investigated for the first time. Thus, this study will be beneficial for future studies.

Keywords: Ascorbic acid, B-complex vitamins, black mulberry, HPLC, kinetic

1. Introduction

The black mulberry (*Morus nigra*) is a member of Moraceae family and its native countries are India, China and Japan [1]. It also grows especially well in the north America and Africa [2].

Black mulberry is regarded as a source of bioactive compounds. The main importance of black mulberry is its high content of total phenolics, total flavonoids, and vitamin C [3] and nutritional values of protein, carbohydrates, fat, fiber, mineral matter (particularly calcium, iron, phosphorus) and water-soluble vitamins (thiamine, riboflavin, nicotinic acid and ascorbic acid) [4]. Black mulberries have been used as a medicine (expectorant, hypoglycemic, anthelmintic, laxative, emetic, odontalgic and warming agents) since prehistoric times [5]. In addition, black mulberry fruits have antioxidant, anti-inflammatory and antimicrobial properties with the contain of various phytochemicals including phenolics [6].

Because of its physiological, nutritional and technological properties, black mulberry fruits have great importance. The black mulberry has sweet and pleasant taste. It is a fruit that is difficult to be harvested, transported and marketed due to its soft texture and its short-term deterioration. For this reason, the fruit is mostly consumed as processed [7]. Turkey is an important country in term of traditional and indigenous food production techniques. Evaluation form of black mulberry consists of raw, dried or processed foods such as pekmez, juice, marmalades, liquors, pestils and komes [7, 8].

Vitamins are essential organic compounds for human and animal bodies in terms of body resistance, biochemical functions and growing. Water-soluble and fat-soluble vitamins are different subgroups of vitamins. Studies on determining the vitamin values of black mulberry juice are very limited. Fruit maturity, harvest time, storage conditions and duration, pre-consumption cooking etc. affect the vitamin content of fruit [9]. As known, vitamins can degrade during processing and storage of foods because of chemical reactions.

Quantitative analysis of vitamins has great importance for manufacturers due to their essential role in nutrition and their relative instability [10]. For separation of vitamins, HPLC is suggested method with solid phase extraction because of removing interfering components [11].

Determination of kinetic properties of quality parameters is important to maintain the highest level of quality in new food process designs [12]. Therefore, prediction of probable quality changes in foods can be provided by determining kinetic properties of quality parameter [13]. No study on HPLC determination of thiamine (TH), riboflavin (RB), niacin (NC) and degradation of L-ascorbic acid (LAA), TH, RB and NC in black mulberry juice (BMJ) are current in the literature. The aim of this study is a) to determine loss of LAA, TH, RB and NC during thermal treatments at different temperatures b) to define kinetic properties of thermal degradation of LAA, TH, RB and NC such as reaction order, rate constant of reaction, activation energy, half-life and Q_{10}.

2. Materials and Methods

2.1. Material

The fresh black mulberry fruits were provided from Gümüssu Food Co., a well-established local factory in Gümüshane province in Turkey. Nearly 300 kilograms of fresh black mulberries taken from the factory were transported to the Department of Food Engineering at Pamukkale University, Denizli, Turkey by refrigerated vehicles and processed for BMJ.

2.2. Production of BMJ

The black mulberry fruit is first washed in water in a clean container and removed dirt, leaves and foreign materials. After grounding by a fruit grinder (Model KMS6000, Vestel), extraction of raw black mulberry juice was done in a cloth bag by using a hydraulic press (Bucher-Guyer AG, Niederweningen, Switzerland). Black mulberry juice, obtained after pressing of the mulberries were filtered through a filter (25 µm pore size) and the filtered juice was then added into pyrex tubes (a three-necked round bottom flask, 75 x 10 mm ID) for the thermal processing and kept at 4 °C till the thermal treatment.

2.3. Thermal Treatment

Temperatures were selected as 70, 80, 90 and 95 °C for the thermal treatment. 25 ml of BMJ samples added into pyrex tubes and heated by placing in thermostatic water bath (Model 3047, Kottermann, Hänigsen/Germany). After the temperature of samples reached the desired temperature, the application time is started. In all heating treatment, reaching the desired temperature took less than 10 minutes. To prevent evaporation, the tubes were tightly capped and placed in a thermostatic water bath. Juice samples were removed from the water bath at regular time intervals (0, 5, 10, 15, 20, 25 and 30 min) and then were rapidly cooled in an ice water. LAA, TH, RB and NC were determined by sampling with test tubes filled with BMJ from water bath at 5 minutes intervals. Sampling was carried out in triplicate. All experiments and calculating the reaction rate constants of each temperature were performed in triplicate.

2.4. Selection of Temperatures and Heating Periods

The lowest temperature is 70°C for the industrial production of BMJ and concentrates. However, bottled BMJ are kept in boiling water for 20-30 minutes in traditional production. Therefore, temperature of bottled BMJ can reach to 95°C.

2.5. Analysis of Water-Soluble Vitamins

2.5.1. Equipment

The HPLC apparatus (Shimadzu Corporation, Kyoto, Japan) used in the study consisted of a PDA detector (Model SPD-M10 AVP, Shimadzu), a pump (Model LC-10AT-VP, Shimadzu), a column oven (Model CTO-10ASVP, Shimadzu) and a degasser (Model DGU 14A, Shimadzu). The peak areas were analyzed by using Shimadzu Software Program. The column for separation was reversed-phase C18 (15 cmx4.6 mm ID, 5µm particle size, Cat. No: 504955) and provided from SUPELCO (Bellefonte, PA, USA). Also, injection volume to HPLC was 20 µL of the samples and performed by using a micro syringe.

2.5.2. Reagents

Doubly distilled and deionized water was used in the experiments. Methanol (HPLC grade) and potassium dihydrogen phosphate (extra pure) was purchased from Merck (Darmstadt, Germany). LAA, TH, RB and NC analytical standards were provided from Sigma (Sigma-Aldrich Chemie GmbH, Deisenhofen-Germany) and stock standards were prepared in mobile phase. Linear calibration curves (r^2=0.999) involving five point were prepared by plotting concentration (g ml^{-1}) versus peak area (mAU) for each compound. Following then sonication, stock standards were transferred to amber glass to prevent light damage and stored under refrigeration.

2.5.3. Sample Preparation (Solid Phase Extraction=SPE)

Due to chromatographic interference of many components of black mulberry with LAA, TH, RB and NC, a SPE cartridges (Sep-Pak C18, 500 mg) were used to remove components which cause interfering. 20 g of deionized water added into 5 g of BMJ. After homogenization, centrifugation of homogenate was

carried out at 9x103 rpm for 10 min (Model 2-16, Sigma Bioblock Scientific). A method suggested by Cho, Ko, and Cheong [11] was used for SPE extraction of LAA, TH, RB and NC with slight modifications. To activate stationary phase, 10 ml of pure water at 4.2 pH and 10 ml of methanol were mixed and added into SPE as flushing liquid. After the activation of stationary phase, the black mulberry juice (10 mL) was loaded. 0.005 M HCl solution was used for pH adjustment of acidified water. The elution of samples was performed with 5 ml of acidified water and 10 ml of methanol with 1 ml min of flow rate. Following that, eluents were collected and evaporated to dryness. The mobile phase was used to dissolve the residue and the residue was filtered through a 0.45 µm microfilter (Schleicher & Schuell, Darmstadt-Germany). 20 µl of filtered sample was injected to HPLC.

2.6. Methods

Detection wavelengths of LAA, TH, RB and NC were selected as 265, 234, 266 and 261 nm for the monitoring of column elution, respectively. After degassing by sonication, mobile phase was filtered through 0.45 µm filter. The mobile phase consisted of methanol (90:10) and 0.1 mol L^{-1} potassium dihydrogen phosphate (pH: 7) and 0.7 ml min^{-1} was the flow rate. 20 µl of the samples was injected into the system and the column temperature was 25 °C (room temperature). Concentrations of the LAA, TH, RB and NC were calculated using integrated areas of the corresponding standards and the samples.

2.7. Recovery of Water-Soluble Vitamins

Determining the recovery was carried out adding three additional levels of standard LAA, TH, RB and NC into the BMJ samples containing known amounts of LAA, TH, RB and NC. Therefore, two determinations were performed with two replicates for each addition level.

2.8. Calculation of Kinetic Parameters

General equation for degradations of food components can be written as given below [14]:

$$-\frac{d[C]}{dt} = k[C]^m \quad \textbf{(2.1)}$$

$[C]$: concentration of the compound
m: reaction order
k: rate constant of reaction.

Thermal degradation of LAA, TH, RB and NC in BMJ followed first-order kinetic model. First order kinetic model (Eq. 2.2) is described by integrating of Eq. (2.1).

$$\ln\left(\frac{[C]_t}{[C]_0}\right) = -kt \quad \textbf{(2.2)}$$

C_0: the initial LAA, TH, RB and NC
C_t: the residual LAA, TH, RB and NC
k: rate constant of reaction (min^{-1})
t: time (min)

Arrhenius equation (Eq. 2.3) was used to describe temperature dependence of LAA, TH, RB and NC.

$$k = k_0 \times e^{-E_a/RT} \quad \textbf{(2.3)}$$

k_0: factor of frequency (min^{-1}),
E_a: activation energy (kJ mol^{-1})
T: absolute temperature (K),
R: universal gas constant (8,314 x 10^{-3} kJ $mol^{-1}K^{-1}$).

Quotient indicator (Q_{10}) expresses temperature-dependence of reaction rate and calculated with Eq. (2.4):

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{\frac{10}{T_2-T_1}}_{0} \quad \textbf{(2.4)}$$

T: temperature,
k_1: rate constants of LAA, TH, RB and NC degradation at temperatures T_1
k_2: rate constants of LAA, TH, RB and NC degradation at temperatures T_2

The time required for the initial concentration to be halved is calculated by the following equation 2.5, given below:

$$t_{1/2} = 0.693/k \quad \textbf{(2.5)}$$

Where k is the reaction rate constant (per min).

2.9. Further Determinations

Total solid (%), water soluble solid (Bx), pH and total acidity (dry citric acid) was measured by using the Method of Association of Official Analytical Chemists [15]. the Lane-Eynon method was used for the determining the amount of total sugar in the BMJ [16].

2.10. Statistical Analysis

All data were statistically evaluated by using "Statistical Analysis Systems" of SAS® software [17]. Statistical significance differences were considered at the level of $p < 0.05$

3. Results and Discussion

Studies on the LAA content of black mulberry and its products have been frequently published but limited studies on TH, RB, NC content and their changes during processing are current in the literature. Degradation kinetics of LAA, TH, RB and NC with HPLC determination in BMJ were investigated for the first time. Thus, contribution of this study is critical for the literature.

Chemical components have a wavelength giving maximum absorbance. Therefore, LAA, TH, RB and NC were sensitively determined by setting detector at specific wavelength that give maximum absorbance. As

seen from Figure 1, LAA, TH, RB and NC were separated well by isocratic elution within 25 min. Unknown peaks in the chromatogram were detected but no interfering with LAA, TH, RB and NC.

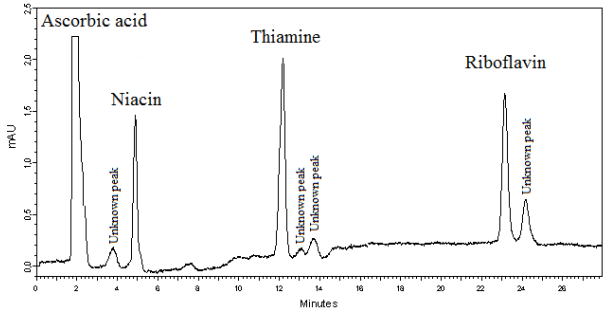

Figure 1. Chromatographically separated LAA, TH, RB and NC by isocratic elution.

3.1. Analytical Characteristic of the HPLC Method

3.1.1. Linearity, Detection Limit, Recovery and Precision

Detection limit, linearity of standard curve, recovery and precision of proposed method for determination of LAA, TH, RB and NC in BMJ is shown in Table 1. The *"R^2"* values of LAA, TH, RB and NC were determined as 0.9992, 0.9996, 0.9989 and 0.9990, respectively. Coefficients of determinations (r^2) were determined above 99.74% for LAA, TH, RB and NC. The detection limits of LAA, TH, RB and NC were calculated with "S/N" (signal/noise) of 3 [18] and found to be 0.1, 0.01, 0.01 and 0.01 mg/l, respectively. Standard addition procedure was used for recovery experiments to determine the reliability of the method. In this context, recovery experiments were performed by adding standard solution of LAA, TH, RB and NC, whose contents were 50, 0.1, 0.2 and 0.5 mg/L, respectively, to the samples. Recovery values of LAA, TH, RB and NC in BMJ were calculated as 96.6±0.5%, 97.2±0.5%, 93.9±0.7% and 103.2±1.0%, respectively. The precision of the method was determined under the same conditions (apparatus and reagents) with triplicate experiments. Calculation of precision was done by applying intra- and inter-day tests and the results were given as relative standard deviation (*RSD*, %). Low *RSD* values indicate good precision for HPLC. *RSD* values of LAA, TH, RB and NC were found to be as 0.6%, 2.8%, 3.6% and 2.1%, respectively.

Table 1. Linearity of standard curve, detection limit, recovery and precision of the proposed method for determination of AA, TH, RB and NC in BMJ.

Vitamin	Linear range (mg/L)	*R*	r^2	Detection limit (mg/L)	Initial content (mg/L)	Content after addition (mg/L)[a]	Recovery (%)	Precision
							Mean SD[b]	R.S.D. (%)
L Ascorbic acid	5.0-250.0	0.9992	99.67	0.1	34.50	82.8±0.34	96.6±0.5	0.6
Thiamine	1.0-50.0	0.9996	99.93	0.01	0.124	0.221±0.016	97.2±0.5	2.8
Riboflavin	1.0-40.0	0.9989	99.83	0.01	0.212	0.399±0.010	93.9±0.7	3.6
Niacin	1.0-20.0	0.9990	99.84	0.01	0.524	1.04±0.010	103.2±1.0	2.1

a50 mg for ascorbic acid, 0.1 mg for thiamine, 0.2 mg for riboflavin and 0.5 mg for niacin bMean ± standard deviation

3.2. Characteristics of BMJ Samples Used

The characteristics of the BMJ used in the study are as follows: 16.15 % total solid, 15.15 Bx soluble solid, 3.4 pH value, 0.35 % total acidity, 11.50 % total sugar, 34.50 mg/l LAA, 0.124 mg/l TH, 0.212 mg/l RB and 0.524 mg/l NC. Soluble solid, total acidity and pH values of BMJ were reported between 11.55-19.04°Brix, 1.37-2.24 g/100ml in citric acid equivalent and 3.63-4.18, respectively [19]. Snapyan et al. [20] studied the physiochemical properties of BMJ and reported that it contained ascorbic acid (15.4 mg/100g), thiamine (0.49 µg/ml) and niacin (19.05 µg/ml). Okatan [21] reported the highest value of vitamin C as 31.34 mg/100g in mulberry genotypes collected from natural resources of Uşak province. In addition, 15.37 mg/100g LAA, 0.040 mg/100g RB and 1.60 mg/100g NC content (fresh weight basis) in black mulberry (*Morus nigra*) fruits [22] and 30 g/100g LAA, 0.04 mg/100 g TH and 0.08 mg/100 g RB content in mulberry fruits were reported [23].

3.3. Kinetic Parameters of LAA, TH, RB and NC Thermal Degradation in BMJ

Thermal stability of LAA, TH, RB and NC in BMJ were studied at 70, 80, 90 and 95 °C. The initial LAA, TH, RB and NC contents determined by HPLC were 34.50 mg/l, 0.124 mg/l, 0.212 mg/l and 0.524 mg/l, respectively and their concentrations highly decreased during the 30 min heating periods of 95 °C, with the reductions of 38.70%, 51.61%, 46.70% and 41.41%, respectively. On the other hand, 30 min heating periods of 70 °C in BMJ resulted with the reductions of 12.61%, 22.60%, 20.30% and 16.22% for LAA, TH, RB and NC, respectively.

Degradations of LAA, TH, RB and NC in BMJ during thermal treatment were presented in Figure 2. As understood from Figure 2, first-order kinetic model was found to be the most suitable kinetics model to describe degradations of these compounds. Based on the increment in temperature and time, degradations of LAA, TH, RB and NC increased. Determination coefficients of LAA, TH, RB and NC degradations at each constant temperature based on time were found to be over 0.98 for first-order kinetic model. This indicates a linear relation between natural logarithm of LAA, TH, RB and NC concentrations and time. Degradations of quality parameters may follow zero or first-order reactions, but differences may be insignificant between zero and first-order reactions [14]. On the other hand, no studies on degradation kinetics of LAA, TH, RB and NC in BMJ are current in the literature. The results of this study were compared with degradations of these compounds in other foods. Likewise, it was reported by Karhan, Aksu, Tetik, and Turhan [24] that ascorbic acid degradation in rosehip pulp was fitted to first-order kinetic model at different temperatures. Moreover, Rekha, Singhal, and Pandit [25] reported that degradation of thiamine in red gram splits (*Cajanus cajan L.*) follows first-order kinetic model at different pH values (4.5, 5.5 and 6.5) and temperature ranging from 50 to 120°C. Additionally, Mulley, Stumbo and Hunting [26] notified that degradations of vitamins in foods and model systems under different conditions are fitted to first-order kinetic model.

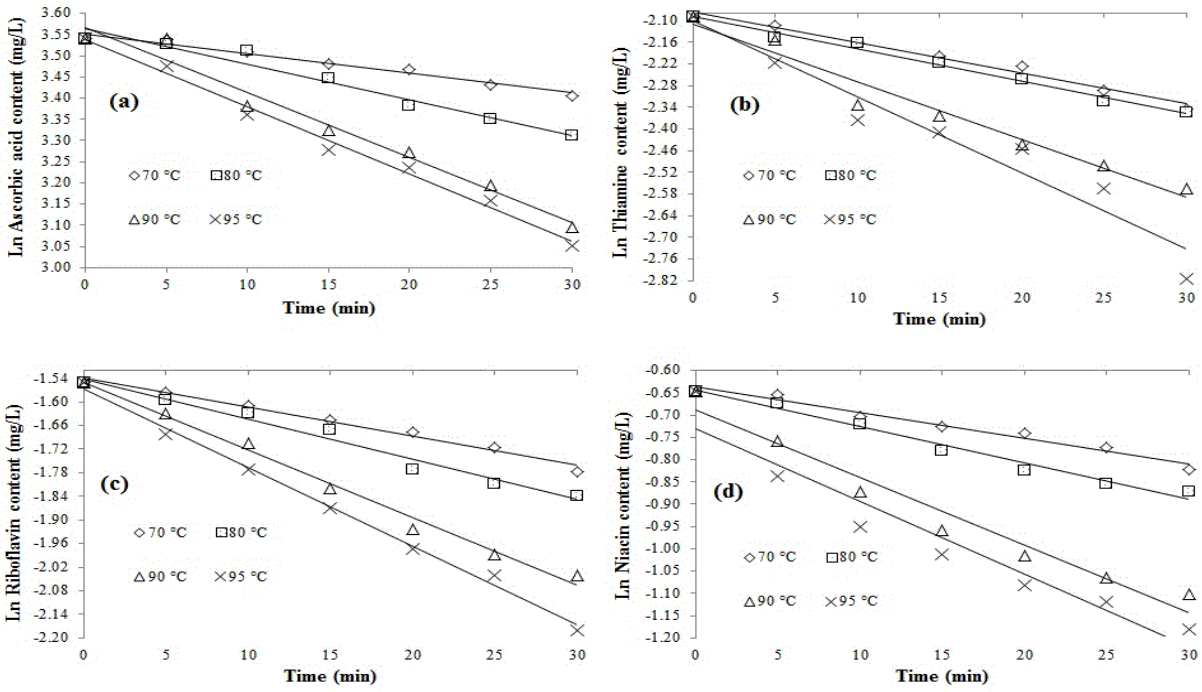

Figure 2. First order degradation kinetics of **(a)** LAA, **(b)** TH, **(c)** RB and **(d)** NC during thermal treatment.

Kinetic parameters of thermal degradation of LAA, TH, RB and NC in BMJ were listed in Table 2. The rate constants of LAA, TH, RB and NC increased depending on the increment in temperature. This result indicates that LAA, TH, RB and NC degrade as temperature-dependent. The lowest degradation rate constant was obtained from LAA, followed NC, RB and TH. This shows that LAA is less thermal sensitive in comparison to NC, RB and TH. Also, temperatures rising from 70 to 80 and 80 to 90°C were found to be more effective on the rate constant of LAA than from 90 to 95°C. On the contrary, while a slight increasing in rate constants of TH, RB and NC at 70-80°C and 90-95°C temperature range was observed, the highest increment in rate constants of TH, RB and NC was determined at 80-90°C temperature range. The *k* values of LAA, TH, RB and NC varied between $4x10^{-3}$-$15x10^{-3}$, $8x10^{-3}$-$21x10^{-3}$, $7x10^{-3}$-$20x10^{-3}$ and $5x10^{-3}$-$16x10^{-3}$ over the temperature range of 70-95 °C, respectively (Table 2). In conclusion, it was observed that thermal treatment and time have great effect on degradation of LAA, TH, RB and NC. Increment in temperature and time caused a rapid degradation in LAA, TH, RB and NC. Combs [27] notified that stability of LAA is higher at acidic medias when compared to alkaline or neutral medias. Several studies on thermal stability of RB are current in various foods and model systems. However, there are very limited data on thermostability of TH, RB and NC in fruit juice and no studies are current in BMJ.

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As shown in Table 2, the half-life values ($t_{1/2}$) for LAA, TH, RB and NC decreased with the increment of temperature. It was observed that higher temperatures provide faster degradation of LAA, TH, RB and NC. Thermal stability of LAA was higher in comparison to TH, RB and NC due to having the highest $t_{1/2}$ values among the other components at the same temperatures.

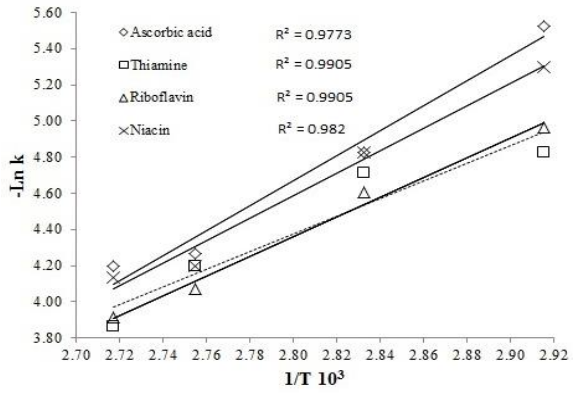

Figure 3. Arrhenius plots for degradation of LAA, TH, RB and NC in BMJ during heating.

Figure 3 shows Arrhenius type relation of LAA, TH, RB and NC during thermal treatment. Arrhenius equation explains relation between degradation rate constants and temperature. If the natural logarithms of the degradation rate constants are plotted vs absolute temperature, a linear curve is obtained. Ea can be calculated by means of the slope of the linear curve. It was found that activation energies (E_a) of LAA, TH, RB and NC degradation in BMJ differ by temperature (70-95°C). The highest activation energies were obtained from LAA, NC, RB and TH, respectively.

When compared to NC, TH and RB, thermal stability of LAA is higher. Sensitivity of a reaction to temperature change can be explained with activation energy. Higher activation energy indicates higher sensitivity. Therefore, LAA, TH, RB and NC degraded more at 95°C than 70°C. The activation energy of LAA was calculated as 58.5 kJ mol^{-1}. When comparing to other reports, activation energies of heated rosehip (70-95°C), raspberry (60-90°C) and citrus juice were reported to be 47.5 kJ mol^{-1}, 15.7 kJ $mol^{-1}$and 35.9 kJ mol^{-1}, respectively [24, 28, 29]. The activation energy of LAA was found to be higher than those reports. On the contrary, the activation energy of LAA was lower than 115.5 kJ $mol^{-1}$for orange juice [30] and 81.67 kJ mol^{-1} for pomegranate juice [31].

Table 2. Kinetic parameters of LAA, TH, RB and NC in BMJ.

Vitamin	T (°C)	k 10^3 (min^{-1})	$t_{1/2}$ (min)	Q_{10}			E_a (kJ mol^{-1})
				70-80 °C	80-90 °C	90-95 °C	
Ascorbic acid	70	4	173.3	2.00	1.75	1.15	58.5
	80	8	86.6				
	90	14	49.5				
	95	15	46.2				
Thiamine	70	8	86.6	1.13	1.67	1.96	40.5
	80	9	77.0				
	90	15	46.2				
	95	21	33.0				
Riboflavin	70	7	99.0	1.43	1.70	1.38	45.9
	80	10	69.3				
	90	17	40.8				
	95	20	34.7				
Niacin	70	5	138.6	1.60	1.88	1.14	52.1
	80	8	86.6				
	90	15	46.2				
	95	16	43.3				

Q_{10} values of vitamin degradation in BMJ were presented in Table 2. Q_{10} values were found to be different for each 10°C temperature increment. The temperature ranges of 70 to 80 ºC, 90 to 95 ºC, 80 to 90 ºC and 80 to 90 ºC were observed for the highest Q_{10} values of LAA, TH, RB and NC, respectively. As understood from this result, these temperature ranges significantly affected the degradations of these compounds. For example, when compared to other temperature ranges, Q_{10} values of LAA and NC were lower at the range of 90-95°C and very close. This indicates that degradation of LAA and NC was slightly affected by the temperature change in this range. The results could not be compared with literature since no data on this subject are current. However, comparison between Q_{10} values of LAA, TH, RB and NC in BMJ and other fruits was made. Karhan, Aksu, Tetik and Turhan [23] reported that Q_{10} value of LAA in the rosehip was 1.21 during thermal treatment under anaerobic conditions.

4. Conclusion

Degradation of examined compounds fit the first-order kinetics. Degradation rate of LAA, TH, RB and NC increased with the increment of heating time and the temperature. The best temperature of long-term heating of black mulberry juice was found as 70°C for less loss of LAA, TH, RB and NC. Results of this study may be benefit for the BMJ producing industry for prevent the loss of examined for water-soluble vitamins which are important quality parameters. Therefore, designing a thermal treatment with a high retention of water-soluble vitamins may be easier when the degradation kinetics of LAA, TH, RB and NC is known.

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Author's Contributions

Cemre Sernikli: Performed the experiments, wrote the manuscript.
Çetin Kadakal: Performed the model, design of the experiments, statistical analysis, wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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