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Drying characteristics and some quality parameters of whole jujube (*Zizyphus jujuba* Mill.) during hot air drying

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PAPER

Abstract

Drying kinetics, water-soluble vitamins, total phenolic content (TPC), antioxidant capacity (AC) of the jujube fruits dried at 50, 60, and 70°C, and degradation kinetics of the quality parameters were investigated. The models fitted to drying were determined as Page at 50 and 70°C, Parabolic at 60°C. Increment in the drying temperature increased the drying rate and decreased the drying time. Water-soluble vitamins, TPC, and AC were significantly reduced by the drying process. Degradation of water-soluble vitamins increased with the drying temperature, although TPC and AC were not significantly affected by temperature. Thermal degradations of quality parameters were fitted to first-order kinetic.

Keywords: antioxidant capacity; degradation kinetics; drying kinetics; total phenol content; water-soluble vitamins

Introduction

Jujube fruit (*Zizyphus jujuba* Mill.), belonging to the *Rhamnaceae* family, is a drupe, which has a round-elliptic shape, apple-like taste, and is rich in various bioactive compounds and nutrients such as vitamin C, polysaccharides, minerals (especially potassium), and phenolic compounds (Chen *et al.*, 2015; Gao *et al.*, 2011; Wojdylo *et al.*, 2016). The jujube fruit has higher vitamin C content than the fruits that are known as sources of vitamin C such as kiwi, strawberry, and lemon (Frenich *et al.*, 2005; Wu *et al.*, 2012). In addition, the jujube fruit is a good source for thiamine, riboflavin, niacin, and pyridoxine as B complex vitamins (Gao *et al.*, 2013). B complex vitamins have an important role as coenzymes for enzymatic reactions in different biological systems (Calderón-Ospina and Nava-Mesa, 2020). Moreover, the jujube fruit has been considered as a good source of phenolic compounds compared to common fruits, which are widely known for being a source of phenolic compound such as berries (Gao *et al.*, 2011). In traditional Chinese medicine, the jujube fruits have been used as a crude drug for analeptic, palliative, and antibechic purposes for thousands of years (Li *et al.*, 2007; Rostami and Gharibzahedi, 2017). The jujube fruits are also used for pharmaceutical benefits such as antioxidant, anticancer, anti-inflammatory, antiepileptic, hepatoprotective, and neuroprotective effects (Choi *et al.*, 2012; Ji *et al.*, 2017). The pharmaceutical benefits of the jujube fruit have been associated with chemical ingredients, which mainly consist of vitamin C, phenolics, polysaccharides, triterpenic acids, and nucleosides (Ji *et al.*, 2017).

The jujube fruits have been consumed as fresh, dried, tea, alcoholic beverages, pickle, jam, compote, or candy (Elmas *et al.*, 2019; Wang *et al.*, 2016; Wojdylo *et al.*, 2016). Although the jujube fruits are generally consumed as fresh, the postharvest shelf-life of the jujube fruit is very short. Thus, the commercial value of the jujube fruits is less (Wang *et al.*, 2016; Zozio *et al.*, 2014). Chemical reactions, microbiological activity, and physical alterations in the pre- or post-harvest period of many plant-based foods mostly require high water content (Tepe and Tepe, 2020). Therefore, some preservation methods such as drying can be suggested to extend their shelf-life.

Drying, which is also called dehydration, is one of the oldest methods of food preservation, used since ancient times. The beneficial properties of drying can be ordered as reducing the water activity to microbiological safety zone and transport costs, providing easier process and extending shelf life (Elmas *et al*., 2019). Selection of drying methods has great importance for the preservation of quality parameters such as the content of phenolic compounds, vitamins, and antioxidant capacity (AC). Sun drying and hot air drying are traditional methods for jujube drying (Wang *et al*., 2016). The main mechanism of hot air drying is mass and heat transfer and phase transition (Tepe and Tepe, 2020). Hot air drying provides some advantages such as being free from the climate effects, reducing the drying cycle, and hygienic conditions in comparison to sun drying (Elmas *et al*., 2019). However, long drying time, loss of nutritional and bioactive value, and changes in sensory properties are disadvantages of hot air drying (Elmas *et al*., 2019; Onwude *et al*., 2017; Wang *et al*., 2019). In addition to the hot air drying method, microwave, vacuum, and freeze drying methods have been regarded as alternative drying methods. Condurso *et al*. (2019) notified that decrement in drying time and better product quality could be provided by microwave drying. In addition, vacuum drying is useful for drying of easily oxidizing foods. Besides, freeze drying has also many advantages such as preserving food quality, especially in heat-sensitive foods. These drying methods can be used alone or combined with each other such as microwave–hot air or vacuum–microwave drying. The combined drying methods may be useful for increasing the efficiency (energy efficiency, environment friendly, product quality) of these drying methods (Sun *et al*., 2019). To select the most appropriate drying method, mathematical modeling is needed along with the determination of product quality, energy efficiency, etc. In this context, thin-layer drying has been used for the determination of the drying kinetics of fruits and vegetables. The most appropriate drying conditions can be selected by using thin-layer drying technology, a kind of mathematical modeling. Thus, a drying process can be designed and optimized (Onwude *et al*., 2016).

There are some recent researches on the drying of jujube fruits in the literature. Elmas *et al*. (2019) have reported that moisture content, water activity, and the total phenolic content (TPC) decreased as the drying temperature increased in hot air drying. In addition, Anjum *et al*. (2020) have investigated the effects of drying methods (sun and hot air drying) on several physical properties, AC, content of vitamin C, and TPC. In that study, it was notified that the highest AC and TPC were observed in jujube fruits dried at 70°C, while the highest vitamin C content was obtained at 50°C (Anjum *et al*., 2020). The effects of different drying methods (convective, vacuum-microwave, convective pre-drying, and vacuum–microwave combination) on phenolic compounds, AC, and the color of jujube fruits were investigated by Wojdylo *et al*. (2019). Convective drying carried out at 50°C has been reported to be the best method in terms of polyphenol content, AC, and color parameters.

In this study, it was aimed to: (i) determine the drying characteristics of whole jujube fruits at different air temperatures (50, 60, 70°C); (ii) investigate the effect of drying on vitamin C and B complex content, TPC, and AC of fresh and dried whole jujube fruits; and (iii) determine thermal degradation kinetics of these bioactive compounds during the drying process.

Materials and Methods

Sample preparation

Fresh jujube fruits (*Zizyphus jujuba* Mill.) were provided from a local producer in Denizli, a province in Turkey. The fresh jujube fruits were carefully selected in terms of the same ripening stage (fully mature) and the same size. Before the analysis, fresh jujube fruits were washed to remove foreign materials. The fresh jujube fruits were stored at 4°C in a refrigerator. Determination of the initial moisture content of the samples was carried out in a drying oven at 105°C till any changes in the sample weight. The initial moisture content of whole jujube fruits was 65.26% ± 0.6.

Drying experiment

A cabinet dryer (Yücebaş Makine Ltd. Inc., Izmir, Turkey) was used for the drying experiments. For the condition stabilization, the cabinet dryer was turned on approximately 30 min before drying. Samples (500 g) were weighted on a drying tray and placed in a cabinet dryer for each drying experiment. Drying temperatures were selected as 50, 60, and 70°C, similar to other researches on the drying of jujube fruits (Fang *et al*., 2009a; Motevali *et al*., 2012; Wojdylo *et al*., 2016, 2019). Besides, air velocity and relative humidity were 2 m s^{-1} and 20%, respectively. In practical application, the moisture content of the dried jujube fruits needs to be below 25% on wet basis (WB) (Fang *et al*., 2009a). Therefore, the drying experiments were continued until the moisture content of the samples achieved to 21% on WB, similar to the results of the studies by Fang *et al*. (2009a) and Yi *et al.* (2012). All of drying experiments were performed in triplicate.

Drying characteristics of whole jujube fruits

To design the best drying conditions, thin-layer drying models are very important. The thin-layer mathematical models selected in the current study are listed in Table 1. Significant information about drying temperature and time can be provided with these models (Demiray *et al.*, 2017).

Moisture ratio (MR) of whole jujube fruits was calculated using Eq. (1):

$$\mathrm{MR} = \frac{M_t - M_e}{M_i - M_e} \tag{1}$$

M_i: initial moisture content of the samples (g water g^{-1} dry matter);

M_t: moisture content at any point of time (g water g^{-1} dry matter);

M_e: equilibrium moisture content (g water g^{-1} dry matter).

M_e can be ignored because of its insignificant value in comparison to M_i and M_t (Fang *et al.*, 2009a).

Drying rate (DR) was determined using Eq. (2):

$$\mathrm{DR} = \frac{\mathrm{M}_{t+\Delta t} - \mathrm{M}_t}{\Delta t} \tag{2}$$

$M_{t+\Delta t}$: moisture content at time difference;

Δt: difference of time between two measuring points.

The relation between the predicted and the experimental data of whole jujube fruits dried at different drying temperatures is explained with determination coefficient (R^2), root-mean square error (RMSE), and reduced chi-square (χ^2). RMSE is a statistical parameter, which expresses the deviation between the predicted and the experimental values. The best equation predicting experimental data is determined according to the lower values of χ^2 and RMSE, and the higher value of R^2. The RMSE (Eq. 3) and chi-square (χ^2) (Eq. 4) values were calculated as follows:

Table 1. Thin-layer mathematical models.

Model name	Model	References
Logaritmic	aexp(-kt) + c	Demiray *et al.* (2017)
Lewis	exp(-kt)	Demiray *et al.* (2017)
Henderson and Pabis	aexp(-kt)	Tepe and Tepe (2020)
Page	exp(-kt^n)	Demiray *et al.* (2017)
Parabolic	a + bt + ct^2	Bi *et al.* (2015)
Wang and Sing	1 + at + bt^2	Tepe and Tepe (2020)

$$\mathrm{RMSE} = \left[\frac{1}{N}\sum_{i=0}^{N}(MR_{pre,i} - MR_{exp,i})^2\right]^{\frac{1}{2}} \tag{3}$$

$$\chi 2 = \frac{\sum_{i=0}^{N}(MR_{pre,i} - MR_{exp,i})^2}{N - n} \tag{4}$$

$MR_{pre,i}$: predicted MR;

$MR_{exp,i}$: experimental MR;

N: number of observation data;

n: constants of thin layer drying models.

Thin-layer modeling and statistical parameters were calculated using the MATLAB software (R2015a, version 8.5) non-linear curve fitting toolbox with the trust-region algorithm.

Determination of effective moisture diffusivity and activation energy in hot-air drying

Fick's diffusion equation has been accepted to describe the drying characteristics of biomaterials. Crank (1975) has suggested a solution to this equation, which can be used for spherical products. Eq. (5) has been recommended for spherical products by assuming constant effective diffusivity and no shrinkage (Doymaz, 2006).

$$MR = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-n^2\pi^2 D_{eff} t}{r^2}\right) \tag{5}$$

D_{eff}: effective moisture diffusivity ($m^2 s^{-1}$);

r: arithmetical average of radius of samples at measured intervals (m).

Eq. (5) can be simplified for the first term of the series (Saravacos and Raouzeos, 1986). The new equation is written as given below, Eq (6):

$$ln(MR) = \ln\left(\frac{6}{\pi^2}\right) - \left(\frac{\pi^2 D_{eff}}{r^2}\, t\right) \tag{6}$$

The plot gives a straight line with a slope as follows, Eq. (7):

$$\text{Slope} = -\frac{\pi^2}{r^2} D_{eff} \tag{7}$$

Arrhenius equation in hot air drying process was used for the calculation of activation energy (Fang *et al.*, 2009a):

$$D_{eff} = D_0 \exp\left(\frac{-E_a}{RT}\right) \tag{8}$$

R: universal gas constant (8.314 J mol^{-1} K^{-1} or 1.987 cal mol^{-1} K^{-1});

T: absolute temperature (K);

E_a: activation energy (kJ mol^{-1} or kcal mol^{-1});

D_0: pre-exponential constant (m^2 s^{-1}).

After regulation of the natural logarithm of Eq. (8), Eq. (9) can be written as given below:

$$lnD_{eff} = lnD_0 - \frac{E_a}{RT} \tag{9}$$

Natural logarithm of effective moisture diffusivity versus T^{-1} gives a straight line with a slope, which represents activation energy.

Analysis of water-soluble vitamins

An extraction method, recommended by Dönmez (2015), was used for water-soluble vitamins. In order to determine the water-soluble vitamins, 5 g of each sample was weighted. After homogenization with distilled water (1:9, w:v), the homogenate was centrifuged at 2355 × g for 10 min (Nüve NF 800R). The supernatant obtained from centrifugation was filtrated using a 0.45 μm filter to be injected into the high performance liquid chromatography (HPLC).

A micro syringe was used for injecting 20 μL of the last filtrate into the HPLC column. Mobile phase consisted of 0.1 M HPLC grade KH_2PO_4 at pH 7. An HPLC device (SHIMADZU), column oven at 25°C (SHIMADZU CTO-20A), Column ACE C18 (7.8 × 300 mm), pump (SHIMADZU LC-20AD), degasser (SHIMADZU DGU-20A3), photo-diode array (PDA) detector (SPD-M20A) at 254, 261, 324, 234 nm for ascorbic acid, niacin, pyridoxine, and thiamin, respectively were used for analysis. The mobile phase was isocratic with 0.7 mL min^{-1} flow rate. For riboflavin analysis, the column is Macherey-Nagel NH_2 (4.6 × 250 mm), column oven temperature is 40°C, and the wave length is 266 nm. The same mobile phase was isocratic with 1 mL min^{-1} flow rate.

The content of water-soluble vitamins was calculated using an equation obtained from a calibration curve consisting of different concentrations of stock solutions (5, 10, 25, 50, 75, and 100 ppm) with a high R^2 (0.9999). Results were given as mg 100 g^{-1} in dry weight (DW) for vitamin C and μg 100 g^{-1} for niacin, pyridoxine, thiamine, and riboflavin. Each analysis was performed in triplicate.

Analyzes of TPC and AC

Analyses of TPC and AC were performed with methanolic extraction with a slight modification, as suggested by Choi *et al.* (2012). Jujube fruit samples (5 g) and 45 mL of 90% methanol were homogenized using a laboratory-type blender. The homogenate was centrifuged at 2355 × g for 10 min. After centrifugation, the supernatants were collected and filtrated using a filter paper.

TPC analysis was performed according to Singleton and Rossi (1965) with a slight modification. Folin-ciocalteu solution (1500 μL) (10% v/v) was added into 300 μL of the extract, and the mixture was kept in a dark place for 5 min. After adding 1200 μL of aqueous 7.5% Na_2CO_3 into the mixture, the mixture was incubated at room temperature in a dark place for 2 h. At the end of the incubation, the absorbance of samples was measured at 760 nm using a spectrophotometer (T80, PG Ins. UK.). Each analysis was carried out in triplicate, and TPC was expressed as mg gallic acid equivalent (GAE) 100 g^{-1} in DW.

The AC analysis was carried out using a method suggested by Thaipong *et al.* (2006) with slight modification. Extracts (150 μL) and DPPH methanolic solution (2850 μL), whose absorbance is 1.1 at 515 nm, were mixed. After incubation for 60 min at room temperature in a dark place, the absorbance of samples was measured at 515 nm. Each sample was analyzed in triplicate, and AC was expressed as mmol trolox equivalent (mmol TE) g^{-1} in DW.

Color measurement

Reflectance color value of the whole jujube fruit skin was measured using Hunter Lab Color Miniscan XE (45/0-L, USA). The samples were placed on a white background, and the measurement was done by covering a transparent glass. ΔE was calculated with the equations below (Horuz *et al.*, 2017):

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \tag{10}$$

ΔE: Total color differences;

L: Lightness (0 = black, 100 = white) value at the end of the drying process;

L_0: Initial lightness value of the fresh jujube fruits;

a: Redness (a+ = red, a- = green) value at the end of the drying process;

a_0: Initial redness value of the fresh jujube fruits;

b: Yellowness (b+ = yellow, b- = blue) value at the end of the drying process;

b_0: Initial yellowness value of the fresh jujube fruits.

Calculation of kinetic parameters

Labuza and Riboh (1982) and Kadakal *et al.* (2017) have suggested Eq. (11) as the general equation to describe the reaction rate of compounds degrading or forming:

$$\frac{dC}{dt} = k\left[C\right]^m \tag{11}$$

For the zero-order kinetic model, Eq. (12) can be written as below:

$$C = C - kt \tag{12}$$

When Eq. (1) is integrated, if m equals one, Eq. (3) is written as follows:

$$\ln C = \ln C_0 - kt \tag{13}$$

ln C: natural logarithm of the residual vitamin C, B complex vitamins, TPC, and AC;

ln C_0: initial content of vitamin C, B complex vitamins, TPC, and AC;

k: rate constant (h^{-1});

t: time.

Temperature dependence of vitamin C, B complex vitamins, TPC, and AC can be calculated with Eq. (14) (Kadakal *et al.*, 2017; Labuza and Riboh, 1982):

$$k = k_0 \; x \; e^{-\frac{E_a}{RT}} \tag{14}$$

When the Eq. (14) is regulated, Eq. (15) is written as follows:

$$lnk = \left(-\frac{E_a}{R}\right)x\left(\frac{1}{T}\right) + lnk_0 \tag{15}$$

k_0: frequency factor (h^{-1});

R: universal gas constant (8.314×10^{-3} kJ mol^{-1} K^{-1} and 1.987×10^{-3} kcal mol^{-1} K^{-1});

T: absolute temperature (K);

E_a: activation energy (kcal mol^{-1} or kJ mol^{-1}).

Quotient indicator (Q_{10}) expresses the temperature-dependence of reaction rate and is calculated with Eq. (16) (Kadakal *et al.*, 2017):

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)} \tag{16}$$

Half-life time, a time required for half of the concentration, for each temperature is calculated with Eq. (17) for the first-order kinetic (Kadakal *et al.*, 2017);

$$t_{1/2} = -\ln(0.5)xk^{-1} = 0.693xk^{-1} \tag{17}$$

D represents the time that it takes for the compound or quality criterion to lose 90% of its quality and is calculated for first-order kinetics as written below (Eq. 18):

$$D = 2.303xk^{-1} \tag{18}$$

Statistical analysis

All of the data were statistically analyzed using the SPSS software (ver. 22 SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate differences between treatments, with a significance level of $p = 0.05$. The differences between groups were determined using the Duncan test.

Results and Discussion

Drying characteristics of whole jujube fruits during hot air drying

MR and DR of whole jujube fruits during hot air drying are shown in Figure 1. As understood from Figure 1, drying temperature has statistically affected DR and the drying time of whole jujube fruits. It was clearly observed that DR increased with the increment in drying temperature. Accordingly, the drying time was reduced and found to be 48, 30, and 18 h for 50, 60, and 70°C, respectively. Likewise, Yi *et al.* (2012) reported that drying times of whole jujube fruits at 45, 55, and 65°C for constant air velocity (2 m s^{-1}) were about 45, 25, and 20 h, respectively. It could be explained with increasing of the heat transfer coefficient by increment in drying temperature. Generally, two periods as constant rate and falling rate are the main

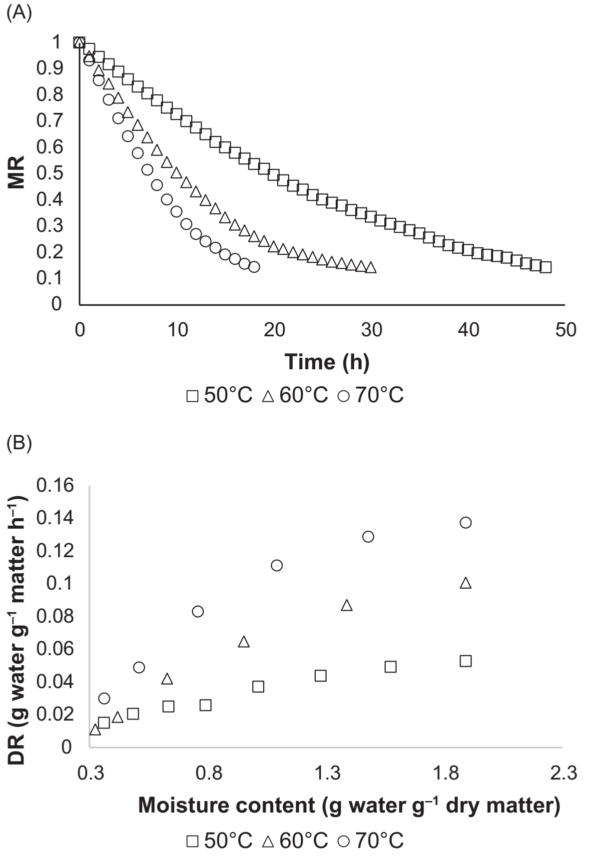

Figure 1. Moisture ratio and drying rate of whole jujube fruits during hot air drying.

constituents of the drying process of agricultural products such as fruits and vegetables. In the current study, the falling rate period was observed. This statement was found to in sync with other studies, in which the jujube fruits were dried with hot air, by Fang *et al.* (2009a), Yi *et al.* (2012), Baomeng *et al.* (2014), and Chen *et al.* (2015).

MR of whole jujube fruits during hot air drying was used to be fitted mathematical models that are listed in Table 1. Statistical parameters to describe the most suitable model are presented in Table 2. Demiray *et al.* (2017) reported that the lower RMSE and χ^2 and the higher R^2 are required for goodness of the fit. As seen from Table 2, the parabolic model was the best model for predicting the experimental MR of whole jujube fruits for 60°C, while experimental MRs of jujube dried at 50 and 70°C were described with Page model with the lowest RMSE and χ^2 and the highest R^2 values.

Effective moisture diffusivity and activation energy of whole jujube fruits during hot air drying

D_{eff} and E_a values of whole jujube fruits are presented in Table 3. D_{eff} values of whole jujube fruits were calculated in the range of 6.43×10^{-11} and 1.80×10^{-10} m² s⁻¹. In comparison to the other drying temperatures, the highest value of D_{eff} was obtained from the drying process

Table 2. Models, constants, and statistical parameters of thin-layer drying curves.

Models	Temperature	Model constants			χ^2	RMSE	R^2
Lewis	50°C	k = 0.03638			0.00043784	0.020710	0.9936
	60°C	k = 0.06996			0.000355573	0.018550	0.9953
	70°C	k = 0.10140			0.000841207	0.028230	0.9899
Page	50°C	k = 0.02349	n = 1.135		2.00099E–05	0.004381	0.9997
	60°C	k = 0.05734	n = 1.074		0.00022413	0.014480	0.9972
	70°C	k = 0.06731	n = 1.182		5.10587E–05	0.006759	0.9995
Henderson and Pabis	50°C	k = 0.03820	a = 1.041		0.000225899	0.014720	0.9968
	60°C	k = 0.07249	a = 1.032		0.00024309	0.015080	0.9970
	70°C	k = 0.10720	a = 1.049		0.000524835	0.021670	0.9944
Logaritmic	50°C	k = 0.05256	a = 0.9309	c = 0.1427	0.001495566	0.037470	0.9794
	60°C	k = 0.10390	a = 0.9326	c = 0.1428	0.001457264	0.036280	0.9825
	70°C	k = 0.14750	a = 0.9333	c = 0.1439	0.002635481	0.047110	0.9733
Wang and Singh	50°C	a = –0.03016	b = 0.0002595		2.90427E–05	0.005278	0.9995
	60°C	a = –0.05971	b = 0.0010510		4.66771E–05	0.006608	0.9994
	70°C	a = –0.08348	b = 0.0019720		0.000105507	0.009716	0.9988
Parabolic	50°C	a = 1.003	b = –0.03039	c = –0.0002634	2.92706E–05	0.005242	0.9996
	60°C	a = 1.008	b = –0.06080	c = 0.0010800	3.94992E–05	0.005973	0.9995
	70°C	a = 1.017	b = –0.08723	c = 0.0021410	6.1355E–05	0.007188	0.9994

RMSE, root-mean square error.

Table 3. D_{eff} and E_a values of whole jujube fruits.

Temperature	D_{eff} ($m^2 s^{-1}$)	E_a (kJ mol^{-1})	E_a (kcal mol^{-1})
50°C	6.43×10^{-11}	47.41	11.33
60°C	1.11×10^{-10}		
70°C	1.80×10^{-10}		

performed at 70°C. It is a fact that drying temperature is one of the most important factors affecting the D_{eff} value. Increment in D_{eff} value means more easy evaporation of moisture content of the sample and consequently an increment in DR. In addition, a proportional relationship between D_{eff} and DR was reported by Demiray *et al.* (2017). Elmas *et al.* (2019) and Fang *et al.* (2009a) notified D_{eff} values of jujube fruits ranging from 1.27×10^{-9} to 3.55×10^{-9} and 5×10^{-11} to 2×10^{-10} $m^2 s^{-1}$, respectively. When compared to these studies, the result of the current study was less than that reported by Elmas *et al.* (2019) and very similar to the findings notified by Fang *et al.* (2009a). This difference might be because of drying conditions, equipment, and the shape of dried fruits (sliced or whole). Arrhenius relation between D_{eff} and T^{-1} is presented in Figure 2. E_a value of whole jujube fruits was found to be 47.11 kJ mol^{-1} and 11.33 kcal mol^{-1}. Various E_a values were reported for drying of the jujube fruit. Fang *et al.* (2009a) have reported that the E_a value of the jujube fruit was 54.51 kJ mol^{-1}. On the contrary, Elmas *et al.* (2019) have found the E_a value of sliced jujube fruit to be 28.183 kJ mol^{-1}. Besides, Motevali *et al.* (2012) have notified that the E_a value of the jujube fruit ranged from 34.97 to 74.20 kJ mol^{-1}.

Color properties of whole jujube fruits during hot air drying

Color properties of fresh and dried whole jujube fruits are shown in Table 4. When compared to the initial L, a, and b values of whole jujube fruits, these parameters were significantly decreased depending on the drying process ($p < 0.05$). The lowest L, a, and b values were obtained at 50°C. It could be because of the longer time of the drying process at 50°C. ΔE represents differences between the colors of the samples (Horuz *et al.*, 2017). ΔE values of dried whole jujube fruits depending on drying temperature and time ranged from 16.84 to 20.17.

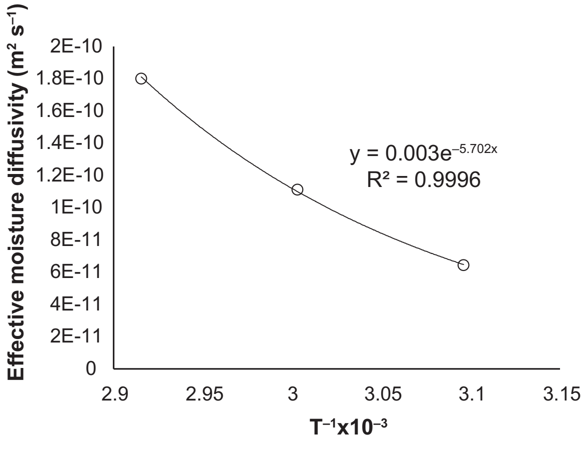

Figure 2. Arrhenius type relation between D_{eff} and T^{-1}.

Table 4. The color properties of whole jujube fruits.

	L	a	b	ΔE
Fresh	21.92 ± 0.01^a	15.95 ± 0.12^a	9.91 ± 0.12^a	0.00
50°C	10.31 ± 0.05^b	2.04 ± 0.04^b	1.04 ± 0.02^b	20.17
60°C	11.09 ± 0.03^c	2.23 ± 0.02^c	1.34 ± 0.07^c	19.47
70°C	12.25 ± 0.04^d	4.57 ± 0.02^d	2.12 ± 0.08^d	16.84

*The different letters in the same column are significantly different ($p < 0.05$).

The effect of the drying process on the water-soluble vitamins, TPC, and AC

The effect of the drying process on the water-soluble vitamins of whole jujube fruits is given in Table 5. Water-soluble vitamins were significantly affected by the drying process. Vitamin C content of the jujube fruit differs depending on some factors such as geographical conditions and cultivars. In the current study, vitamin C content of fresh whole jujube fruits was determined as 78.90 ± 0.96 mg 100 g^{-1} DW. Vitamin C content of the whole jujube fruit was considerably reduced by the drying process, mainly the drying temperature ($p < 0.05$). Fang *et al.* (2009b) have similarly reported decrement in vitamin C content of the whole jujube fruit during hot air drying. Reduction in vitamin C content of sliced jujube fruit during hot air drying was also notified by Chen *et al.* (2015). Vitamin C is a heat-sensitive compound and, thus, might be degraded by a heating process such as drying (Chin *et al.*, 2015). In addition, vitamin C oxidation can occur more rapidly at higher temperatures (Orikasa *et al.*, 2014; Santos and Silva, 2008). The highest loss of vitamin C content of whole jujube fruits was 81.91% at 70°C, while the lowest loss was 55.51% at 50°C. Loss of vitamin C content of whole jujube fruits increased with the increment in drying temperature. This result was similar to Chen *et al.* (2015) but contrary to Fang *et al.* (2009b). In other fruits, vitamin C has been reported to be more degraded with the increasing air temperature (Chin *et al.*, 2015; Kaya *et al.*, 2010; Orikasa *et al.*, 2014; Vega-Galvez *et al.*, 2009).

Initial content of thiamine (B1), riboflavin (B2), niacin (B3), and pyridoxine (B6) in fresh whole jujube fruits were determined as 27.33 ± 1.52, 41.00 ± 1.00, 883.33 ±

Table 5. The effect of the drying process on water-soluble vitamins of whole jujube fruits.

	Vitamin C	% Loss	Thiamine (B1)	% Loss	Riboflavin (B2)	% Loss	Niacin (B3)	% Loss	Pyridoxine (B6)	% Loss
Fresh	78.90 ± 0.96^{a}	0	27.33 ± 1.52^{a}	0	41.00 ± 1.00^{a}	0	883.33 ± 15.27^{a}	0	80.33 ± 2.08^{b}	0
50°C	35.10 ± 0.36^{b}	55.51	19.93 ± 0.21^{b}	27.07	19.73 ± 0.15^{b}	51.88	761.67 ± 4.04^{b}	13.77	ND*	100
60°C	20.93 ± 0.64^{c}	73.47	18.63 ± 0.15^{b}	31.83	18.13 ± 0.15^{c}	55.78	689.33 ± 4.16^{c}	21.96	ND	100
70°C	14.27 ± 0.55^{d}	81.91	16.47 ± 0.21^{c}	39.74	15.43 ± 0.15^{d}	62.37	631.00 ± 2.00^{d}	28.57	ND	100

*ND, not detected; Vitamin C was expressed as mg 100 g^{-1}; DW, B complex vitamins were expressed as μg 100 g^{-1} DW. The different letters in the same column are significantly different ($p < 0.05$).

15.27, and 80.33 ± 2.08 μg 100 g^{-1} DW, respectively. Yaşa (2016) had reported thiamine (B1), riboflavin (B2), niacin (B3), and pyridoxine (B6) content of jujube cultivated in Denizli, a province of Turkey, to be 0.018, 0.036, 0.82, and 0.076 mg 100 g^{-1}, respectively. Results of the current study were in good agreement with those reported by Yaşa (2016). In addition, Li *et al.* (2007) have reported thiamine and riboflavin content of five Chinese jujube cultivars in the range of 0.04–0.09 mg 100 g^{-1} and 0.05–0.09 mg 100 g^{-1}, respectively. These values were also similar to those reported by Gao *et al.* (2013), Pareek (2013), Yaşa (2016), and Li *et al.* (2007). Drying temperature has a great impact on B complex vitamins. As seen from Table 5, B complex vitamins of whole jujube fruits remarkably decreased at the end of the drying process ($p < 0.05$). The highest losses in thiamin (B1), riboflavin (B2), and niacin (B3) content occurred at 70°C as 39.74, 62.37, and 28.57% ($p < 0.05$), respectively, whereas the drying process at 50°C resulted in the lowest losses with the percentages of 27.07, 51.88, and 13.77 ($p < 0.05$), respectively. On the other hand, pyridoxine was the highest affected compound among B complex vitamins. No pyridoxine content of whole jujube fruits was determined at the end of the drying process ($p < 0.05$). It could be under the limit of detection. It was similarly notified by Yaşa (2016) that thiamin (B1), riboflavin (B2), and niacin (B3) content of whole jujube fruits decreased during drying. In addition, no pyridoxine content was reported at the end of the drying process by Yaşa (2016).

The effect of the drying process on TPC and AC of whole jujube fruits are presented in Table 6. As seen in Table 6, TPC and AC of fresh whole jujube fruits were found to be 1911.4 ± 47.32 mg GAE 100 g^{-1} DW and 0.214 ± 0.001 mmol TE g^{-1} DW. TPC and AC of whole jujube fruits significantly decreased with hot air drying ($p < 0.05$). The loss percentages of TPC in whole jujube fruits dried at 50, 60, and 70°C were calculated as 78.10, 76.26, and 74.68, respectively. In the current study, the increment in the drying temperature has no significant effect on the reduction in TPC ($p > 0.05$). Likewise, Vega-Galvez *et al.* (2009) noted no significant change in the TPC of red pepper during hot air drying. Similarly, TPC of sour cherries was also notified to have decreased during drying; however, no there were no significant differences between the drying temperatures (Horuz *et al.*, 2017). On the contrary, Yaşa (2016) has reported that the TPC of whole jujube fruits was reduced by hot air drying and the loss of TPC increased with an increment in the drying temperature. Likewise, Elmas *et al.* (2019) notified more decrement in the TPC of sliced jujube fruits based on an increment in the drying temperature. Furthermore, AC of whole jujube fruits dried at 50, 60, and 70°C decreased with the percentage of 61.22, 60.75, and 59.35, respectively. No significant difference was found between the drying temperatures ($p > 0.05$). Long drying times might decrease AC of foods (Garau *et al.*, 2007). On the contrary, Wojdylo *et al.* (2016) have notified that AC of three different jujube cultivars decreased with hot air drying, and an increment in the drying temperature increased the reduction of AC in these cultivars. A decrement in AC of hot-air-dried red pepper was reported by Vega-Galvez *et al.* (2009). However, no significant differences between drying temperatures were notified in the same study.

Kinetic parameters of vitamin C

To the best of our knowledge, degradation of vitamin C in whole dried jujube fruits was investigated for the first time. Thermal degradation of vitamin C in whole dried jujube fruits is shown in Figure 3. As seen in Figure 3, thermal

Table 6. The effect of the drying process on TPC and AC of whole jujube fruits.

	TPC	% Loss	AC	% Loss
Fresh	1911.40 ± 47.32^{a}	0	0.214 ± 0.001^{a}	0
50°C	418.59 ± 12.18^{b}	78.10	0.083 ± 0.001^{b}	61.22
60°C	453.71 ± 4.61^{b}	76.26	0.084 ± 0.001^{b}	60.75
70°C	484.03 ± 6.21^{b}	74.68	0.087 ± 0.001^{b}	59.35

*TPC was expressed as mg GAE 100 g^{-1} DW; AC was expressed as mmol TE g^{-1} DW. The different letters in the same column are significantly different ($p < 0.05$).
TPC, total phenolic content; AC, antioxidant capacity.

degradation of vitamin C in whole dried jujube fruits followed the first-order kinetic model. Thermal degradation of vitamin C is reported to frequently fit to the first-order reaction model in different dried foods by Demiray *et al.* (2013), Kadakal *et al.* (2017), Orikasa *et al.* (2014), and Kurozawa *et al.* (2014). Kinetic parameters of vitamin C are listed in Table 7. The rate constant of vitamin C thermal degradation increased depending on an increment in the drying temperature. Accordingly, the values of $t_{1/2}$ and D decreased. Likewise, Demiray *et al.* (2013) and Akdaş and Başlar (2015) have reported an increment in the degradation rate constant of vitamin C in tomato and mandarin, respectively, as drying temperatures were raised. In addition, the values of $t_{1/2}$ decreased with an increase in the degradation rate constant. Kadakal *et al.* (2017) have notified an increment in degradation rate constant and decrement in the values of $t_{1/2}$ and D of vitamin C thermal degradation in rosehip nectar during thermal treatment. Also, in another study, vitamin C degradation in hot-air-dried kiwi fruits showed an increment with an increase in the drying temperature (Orikasa *et al.*, 2014). Kurozawa *et al.* (2014) have indicated a rate constant of vitamin C thermal degradation in papaya with an increment in temperature during the drying process. The result of the current study is in good agreement with other reports.

Activation energy reflects the reaction's temperature sensitivity. Higher E_a indicates higher sensitivity to temperature changes. Besides, higher E_a means higher stability to thermal degradation (Bell, 2020; Kadakal *et al.*, 2017). Arrhenius equation, which was used for the calculation of the E_a of vitamin C thermal degradation, is given in Figure 4. The E_a of vitamin C thermal degradation in whole dried jujube fruits was calculated as 80.87 kJ mol^{-1}. This value was higher than 46.248 and 46.99 kJ mol^{-1} by Akdaş and Başlar (2015) and Demiray *et al.* (2013), respectively, meaning that vitamin C was more stable to thermal degradation, and its thermal degradation reaction was more sensitive to temperature changes in whole dried jujube fruits. Q_{10} value, a criterion reflecting the effect of raising temperature by 10°C on the rate of reaction, is also used as an indicator of the reaction's temperature sensitivity. Higher Q_{10} values denote greater temperature sensitivity (Bell, 2020; Kadakal *et al.*, 2017). In the current study, the value of Q_{10} from 50 to 60°C was found to be slightly higher than from 60 to 70°C. This means that the thermal degradation of vitamin C was more affected by an increment of temperature from 50 to 60°C than from 60°C to 70°C. Kadakal *et al.* (2017) and Demiray *et al.* (2013) have similarly reported that the Q_{10} value of vitamin C thermal

Table 7. First-order kinetic parameters of water-soluble vitamins, TPC, and AC of whole dried jujube fruits.

Compound	Temperature	k (h^{-1})	$t_{1/2}$ (h)	D (h)	R^2	E_a (kcal mol^{-1})	E_a (kJ mol^{-1})	Q_{10} (50–60°C)	Q_{10} (60–70°C)
Vitamin C	50°C	0.0170	40.76	135.47	0.9865	19.33	80.87	2.62	2.21
	60°C	0.0445	15.57	51.75	0.9894				
	70°C	0.0983	7.05	23.43	0.9890				
Thiamine	50°C	0.0066	105.00	348.94	0.9798	16.23	67.90	1.89	2.31
	60°C	0.0125	55.44	184.24	0.9776				
	70°C	0.0289	23.98	79.69	0.9869				
Riboflavin	50°C	0.0162	42.78	142.16	0.9810	13.23	55.37	1.70	1.96
	60°C	0.0275	25.20	83.75	0.9817				
	70°C	0.054	12.83	42.65	0.9918				
Niacin	50°C	0.0034	203.82	677.35	0.9733	18.83	78.78	2.47	2.24
	60°C	0.0084	82.50	274.17	0.9856				
	70°C	0.0188	36.86	122.50	0.9922				
Pyridoxine	50°C	0.0505	13.72	45.60	0.9898	15.63	65.41	1.75	1.72
	60°C	0.0886	7.82	25.99	0.9886				
	70°C	0.1524	4.55	15.11	0.9999				
TPC	50°C	0.0332	20.87	69.37	0.9815	9.33	39.02	1.51	1.54
	60°C	0.0503	13.77	45.78	0.9648				
	70°C	0.0775	8.94	29.72	0.9796				
AC	50°C	0.0194	35.72	118.71	0.9619	10.68	44.68	1.62	1.63
	60°C	0.0314	22.07	73.34	0.9683				
	70°C	0.0512	13.54	44.98	0.9684				

TPC, total phenolic content; AC, antioxidant capacity.

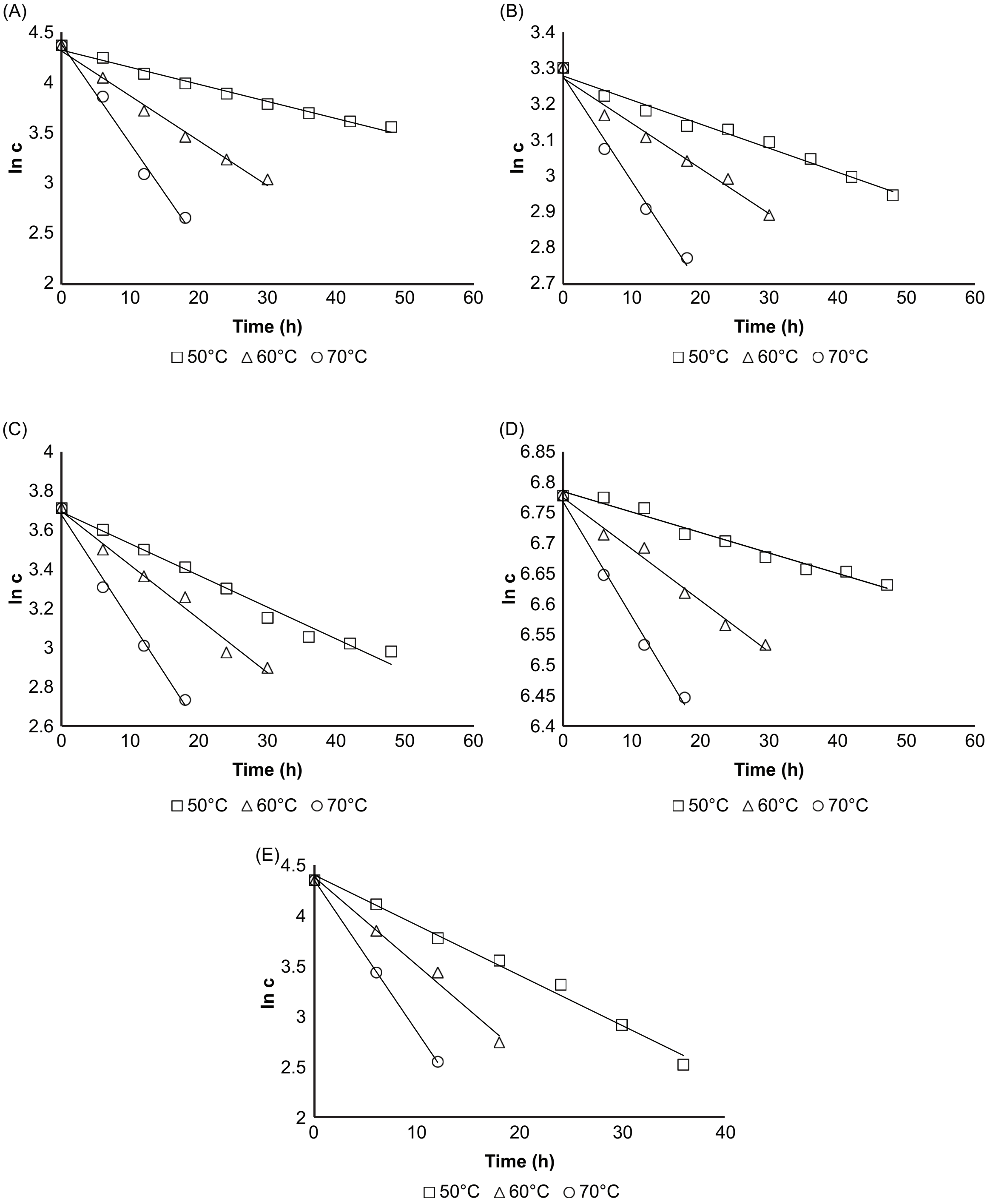

Figure 3. First-order plots of (A) vitamin C, (B) thiamine, (C) riboflavin, (D) niacin and (E) pyridoxine during drying of the whole jujube fruits

degradation decreased with an increment in the process temperature.

Kinetic parameters of B complex vitamins

To the best of our knowledge, no data on B complex vitamin degradations in jujube fruits during hot air-drying process have been published as yet. Thermal degradation kinetics of B complex vitamins in jujube fruits during hot air-drying process was investigated for the first time in this study. Thermal degradation of B complex vitamins is given in Figure 3. However, no pyridoxine content was determined at the end of the process. Therefore, kinetic modeling of pyridoxine thermal degradation was conducted until the last point where pyridoxine was

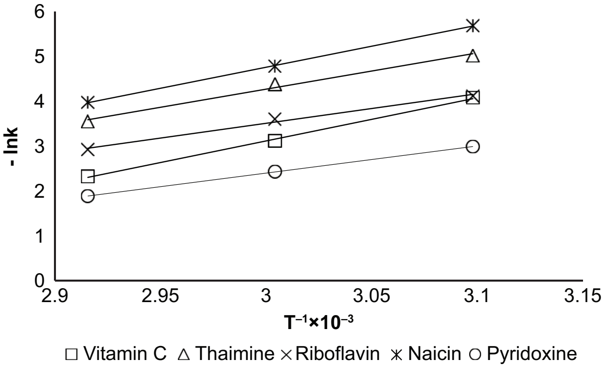

Figure 4. Arrhenius plots of water-soluble vitamins of whole dried jujube fruits.

detected. Thermal degradation of B complex vitamins followed the first-order kinetic model. Likewise, Kadakal *et al.* (2017) have reported that thermal degradation of thiamine and riboflavin in rosehip nectar fitted to the first-order kinetic model during thermal treatment for 30 min. Thermal degradation of niacin in cooked potato for 60 min in the temperature range of 50–120°C was notified to follow the first-order kinetic model by Nisha *et al.* (2009). Nisha *et al.* (2005) and Rekha *et al.* (2004) have reported a first-order kinetic of thermal degradation of riboflavin and thiamin in cooked spinach and red gram splits at a temperature range of 50–120°C for 60 min, respectively. Kinetic parameters of B complex vitamins' thermal degradation are presented in Table 7. In the current study, degradation rate constants of B complex vitamins increased with the increment in drying temperature. Accordingly, degradation rapidly occurred at higher temperatures. In consequence, the values of $t_{1/2}$ and D of B complex vitamins decreased. In the current study, degradation rate constants of thiamine, riboflavin, niacin, and pyridoxine ranged from 0.0066 to 0.0289 h^{-1}, 0.0162 to 0.054 h^{-1}, 0.0034 to 0.0188 h^{-1}, and 0.0505 to 0.1524 h^{-1}, respectively. Kadakal *et al.* (2017), Nisha *et al.* (2009), Nisha *et al.* (2005), and Rekha *et al.* (2004) have also reported an increment in degradation rate constant of B complex vitamins in different foods with an increase in process temperatures.

Figure 4 shows Arrhenius equation of B complex vitamins' thermal degradation. The E_as of thiamine, riboflavin, niacin, and pyridoxine were found to be 67.90, 55.37, 78.78, and 65.41 kJ mol^{-1}, respectively. Sensitivity of a reaction to temperature change can be explained with the E_a as stated before. Accordingly, niacin was more sensitive to temperature changes but shows the highest stability due to the highest E_a value in comparison to other B complex vitamins in jujube fruits. Nisha *et al.* (2005) have found the E_a of riboflavin thermal degradation in cooked spinach to be 21.72 kJ mol^{-1}. Kadakal *et al.* (2017) have also reported that the E_as of thiamine and riboflavin thermal degradation in rosehip were 36.38 and 37.15 kJ mol^{-1}, respectively. Nisha *et al.* (2009) have notified the E_a of niacin in cooked potato cubes to be 16.70 kJ mol^{-1}. The E_as of thiamine, riboflavin, niacin, and pyridoxine of jujube fruits were found to be higher than those reports. This means that thiamine, riboflavin, niacin, and pyridoxine in jujube fruits were more sensitive to temperature changes; however, they were more stable to thermal degradation when compared to those reports. On the other hand, niacin was the highest affected compound by 10°C temperature increment from 50 to 60°C due to the highest Q_{10} value when compared to Q_{10} values.

Kinetic parameters of TPC and AC

The current study presented the first data on TPC thermal degradation in whole dried jujube fruits. TPC thermal degradation is shown in Figure 5. It was found that TPC thermal degradation followed the first-order kinetic model. Akdaş and Başlar (2015) have similarly reported a first-order reaction of TPC of mandarin during the oven drying process. Sarpong *et al.* (2018) have notified that TPC thermal degradation in banana samples during convective drying was described using the first-order kinetic model. The result of the current study was in good agreement with reports by Akdaş and Başlar (2015) and Sarpong *et al.* (2018). Kinetic parameters of TPC thermal degradation are listed in Table 7. Rate constant of TPC thermal degradation showed an increment as drying temperatures increased. It was obvious that this increment caused a decrement in the values of $t_{1/2}$ and D. Rate constant of TPC thermal degradation ranged from 0.0332 to 0.0775 h^{-1}. Akdaş and Başlar (2015), Sarpong *et al.* (2018), and Kadakal and Duman (2018) have reported an increment in TPC thermal degradation in different foods with an increase in temperature. TPC thermal degradation was slightly affected by 10°C temperature increment. E_a was calculated using the Arrhenius equation as given in Figure 6. The E_a of TPC thermal degradation was calculated as 39.02 kJ mol^{-1}. Sarpong *et al.* (2018) have reported the E_a of convective dried banana at 60, 70, and 80°C as 14.29 kJ mol^{-1}. Likewise, The E_as of TPC thermal degradation in Starking Delicious, Golden Delicious, and Granny Smith apple cultivars dried at 65, 70, and 75°C were notified as 27.52, 29.84, and 32.48 kJ mol^{-1}, respectively (Ertekin Filiz and Seydim, 2018). Akdaş and Başlar (2015) have reported the E_a as 55.037 kJ mol^{-1} for oven-dried mandarin at 55, 65, and 75°C. The result of the current study was higher than those reported by Sarpong *et al.* (2018) and Ertekin Filiz and Seydim (2018); however, it was lower than those reported by Akdaş and Başlar (2015). On the other hand, thermal degradation of TPC was not affected by the 10°C temperature increment.

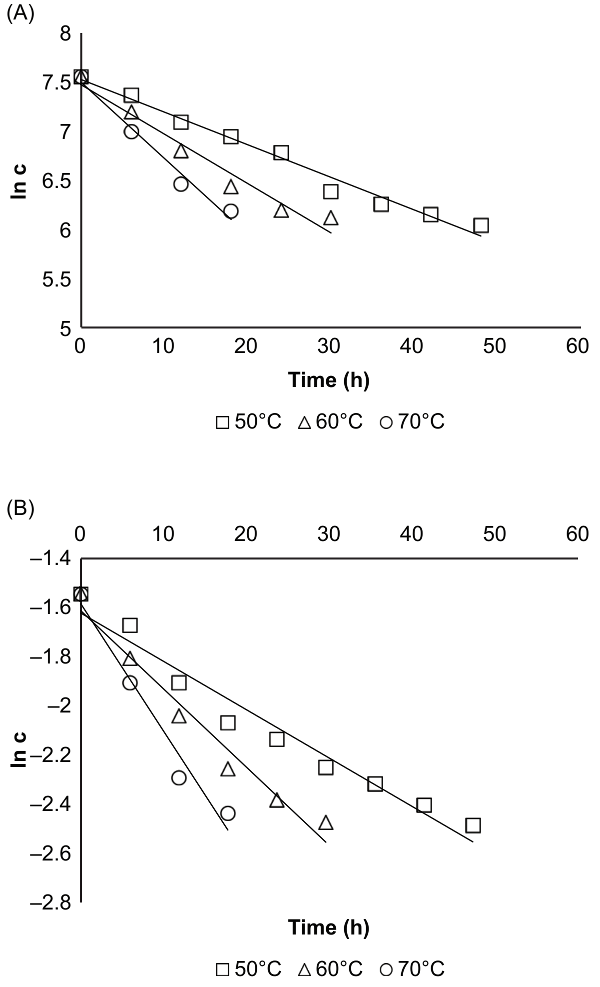

Figure 5. First-order kinetics of total phenolic content (A) and antioxidant capacity (B) of whole dried jujube fruits.

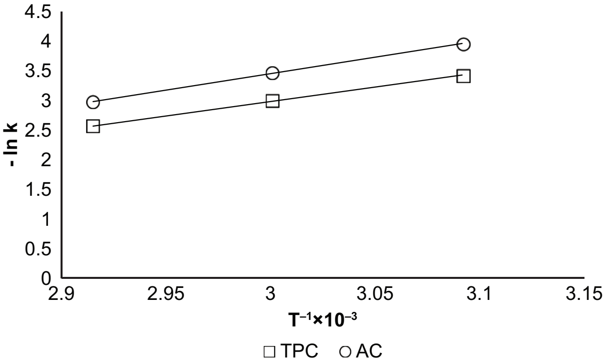

Figure 6. Arrhenius plots of total phenolic content and antioxidant capacity of whole dried jujube fruits.

AC thermal degradation could be described using the first-order kinetic model (Başlar *et al.*, 2014; Oancea *et al.*, 2017; Sarpong *et al.*, 2018). In contrast to this, Orikasa *et al.* (2014) and Ertekin Filiz and Seydim (2018) have reported that zero-order kinetic model may also be used to describe AC thermal degradation. To the best of our knowledge, AC thermal degradation in whole dried jujube fruits was investigated for the first time. AC thermal degradation is given in Figure 5. In the current study, AC thermal degradation followed the first-order kinetic model. Oancea *et al.* (2017), Başlar *et al.* (2014), and Sarpong *et al.* (2018) have reported the first-order kinetic of AC in sour cherry extracts, oven-dried pomegranate, and convective dried banana during the thermal process. Kinetic parameters of AC thermal degradation are given in Table 7. Rate constants of AC thermal degradation increased with an increment in the drying temperature as expected. Rate constant of AC thermal degradation was reported to increase with process temperature by Oancea *et al.* (2017), Başlar *et al.* (2014), and Sarpong *et al.* (2018). Accordingly, the values of $t_{1/2}$ and D were reduced. On the other hand, a 10°C temperature increment had no significant effect on AC thermal degradation. Figure 6 presents the Arrhenius equation of AC thermal degradation that was used for the calculation of the E_a. The E_a of AC thermal degradation was 44.68 kJ mol^{-1}.

Conclusions

In the current study, drying characteristics and some quality parameters of the health-promising fruit, jujube (*Zizyphus jujuba* Mill.), in Turkey were investigated under different drying conditions.

1. DR of jujube was highly influenced by the drying temperature. The longest drying time was found to be 48 h at 50°C, and the shortest was 18 h at 70°C.

2. The best predicting models of experimental MR were determined as Parabolic model for 60°C and Page model for 50 and 70°C.

3. Effective moisture diffusivity showed an increment with an increase in the drying temperature. The most effective moisture diffusivity was obtained at 70°C.

4. When compared to dried jujube fruits regardless of the drying temperature, water-soluble vitamins, TPC, and AC of fresh jujube fruits were determined to be higher. While the highest loss of water-soluble vitamin occurred at 70°C because of a more rapid enzymatic and nonenzymatic degradation, TPC and AC were not significantly affected by the drying temperature.

5. Vitamin C and B complexes, TPC, and AC thermal degradation were fitted to the first-order kinetic model.

6. Vitamin C and niacin were very susceptible to temperature change. On the contrary, TPC and AC were the lowest sensitive compounds.

7. Vitamin C and B complexes were strongly temperature dependent, while TPC and AC were not significantly affected by increment in temperature.

In further studies, the effect of different drying methods, such as microwave, vacuum, combinations of vacuum–microwave, microwave–hot air, should be investigated for dehydration of jujube fruits. Thus, the most suitable conditions and methods may be optimized by observing the loss of nutritional compounds during the process.

Acknowledgements

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Drying characteristics and degradation kinetics in some parameters of goji berry (*Lycium Barbarum* L.) fruit during hot air drying

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PAPER

Abstract

Drying kinetics, color properties, water-soluble vitamins, antioxidant capacity, total phenolic content, and thermal degradation kinetics of bioactive compounds in goji berries were investigated. Drying experiments were conducted at 50°C, 60°C, and 70°C. Page model was determined as the best model to predict experimental moisture ratio for all temperatures. Increment in drying temperature increased effective moisture diffusivity and drying rate values. Vitamins C and B_6, antioxidant activity and total phenolic content were significantly reduced by drying. Thermal degradation of vitamins C and B_6, antioxidant capacity and total phenolic content were found to fit the first-order kinetic model.

Keywords: antioxidant capacity, degradation kinetics, drying kinetics, goji berry, total phenolic content, water-soluble vitamins

Introduction

Goji berry (*Lycium barbarum* L.), which belongs to *Solanaceous* family, is grown in China, Tibet, and some regions of Asia. Goji berry is primarily found in East Asia and grown particularly in Japan, South Korea, and South China (Gao *et al*., 2017). *Lycium barbarum* L. is a deciduous woody shrub, mostly thorny, that grows 1–4 m in height (Griffiths and Huxley, 1992). Fruits are two-chambered, mostly orange-red in color, juicy, and fleshy (Chen *et al.*, 2018). Ripe goji berries are 3–10 mm in diameter, 6–20-mm long, and have oblong or ellipsoid shape (Gao *et al*., 2017).

Goji berry contains a high amount of anthocyanin (Cui *et al*., 2011), carotenoids (lycopene, zeaxanthin dipalmitate, beta-carotene, zeaxanthin, and lutein), vitamins (tocopherol, glucopyranosyl ascorbic acid, and ascorbic acid), betaine, fatty acids, and peptidoglycans (Islam *et al*., 2017). Fatty acids commonly found in goji berry are linoleic, oleic, and palmitic acids (Cossignani *et al*., 2018). The fruit also includes organic acids such as malic, citric, shikimic, and fumaric acids (Mikulic-Petkovsek *et al.*, 2012), monosaccharides, which are mannose, rhamnose, galactose, xylose, arabinose, and glucose, 18 amino acids, and galacturonic acid (Amagase and Farnsworth, 2011). Owing to bioactive substances and healthy functions, goji berries are popular in the western world (Bertoldi *et al*., 2019).

In traditional Chinese herbal medicine, goji berry has been used as a supplement for more than 2000 years (Burke *et al*., 2005). It is used to protect the liver, kidneys, and eyes, strengthen the eyesight (Shan *et al.*, 2011), and reduce serum lipids and blood glucose levels. Also, Goji berry has other health-promoting effects such as anti-radiation, immune-enhancing, anti-fatigue, and anti-aging effects, stimulating hematopoiesis, and treating male infertility (Luo *et al*., 2004; Tian *et al*., 2013).

Drying is a protection technique that is widely applied to fresh products. Dehydration prolongs stability of fruits and vegetables by decreasing water content and minimizing physicochemical changes and microbial growth (Tepe and Tepe, 2020). Besides, the drying process protects valuable foods under effective conditions, prolongs shelf life, and reduces storage, transportation, and packaging costs because of decrement in the volume and weight of food products (Önal *et al.*, 2019). One of the most important steps in the food processing industries is the dehydration process.

Sun drying is a preferred method because it is economical and does not require investment, but it is a disadvantageous method due to microbial reliability and loss in quality such as color and aroma (Göztok and Içier, 2017). Convective drying is one of the most used drying methods to protect agricultural products compared to other drying methods because it is simple and low costing. On the contrary, this method causes changes in sensory properties and nutritional values (Orikasa *et al.*, 2014). The convective drying can be used as an alternative method instead of conventional (sun) drying. It has more advantages than conventional drying in terms of preventing microbial contamination, component protection, and involving lower drying time (Lewicki, 2006). Adiletta *et al.* (2015) and Fratianni *et al.*, (2018) studied goji berry drying, but no data are available in literature about drying characteristics, degradation kinetics of water-soluble vitamins, antioxidant capacity (AC), and total phenolic content (TPC) of dried goji berry fruit.

Absence of data on drying characteristics and degradation of some bioactive compounds can be regarded as a gap in the drying process of goji berry fruit. In this context, determining drying characteristics and degradation of some bioactive compounds can be useful for the designing of drying process. Thus, the aims of this study are to: determine the drying characteristics of goji berry fruit at 50°C, 60°C, and 70°C, examine the influence of drying process on contents of vitamins C and B_6, antioxidant capacity and total phenolic content of goji berries, and calculate the degradation kinetics of these bioactive compounds.

Materials and Methods

Materials and sample preparation

Goji berry fruits, of NQ1 variety, were obtained from Redlife in the Çivril district of Denizli province of Turkey. Geographical location of Denizli is between 28°30’–29°30’ east meridians and 37°12’–38°12’ north parallels and is located in the Aegean region of Turkey. The fruits were carefully collected in July 2019 from 10 randomly selected plants. Fresh fruits were washed to remove any foreign material and kept at –18°C before analysis.

Method

Drying process of samples

Goji berry samples were dried in a tray drying cabinet (Yücebaş Makine Tic. Ltd. Şti., İzmir, Turkey). Dryer comprised an electronic proportional controller (EUC442 model, ENDA, Turkey), an electric heater, and a centrifugal fan to provide airflow. The dryer’s internal size was 70 cm × 55 cm × 100 cm, the range of workable temperature was 40–120°C, and the range of workable relative humidity was 20%–95%. Three different drying air temperatures were used in the experiments: 50°C, 60°C, and 70°C. The cabinet was heated for 1 h before the start of drying process to reach a constant temperature; and 200 g of samples were uniformly placed on the drying tray. The drying process was performed up to the targeted dry matter content at a relative humidity of 20% and air velocity of 2 m/s. The drying experiments were performed in triplicate and weighed at certain time intervals with a 0.001 g precision digital scale.

Drying characteristics of goji berry fruit

Empirical models are more useful because theoretical drying models are complicated and the former models offer a direct relationship between drying time and moisture content (Moradi *et al.*, 2020). Thin-layer drying models have great significance in designing the best drying conditions.

Moisture ratio (MR) must be calculated by $M_{i,}$ M_t, and M_e values to choose the best model.

$$MR = \frac{M_t - M_e}{M_i - M_e} \qquad \text{(Eq. 1)}$$

where

M_i: initial moisture content of samples (g water/g dry matter);

M_t: moisture content at any time (g water/g dry matter);

M_e: equilibrium moisture content (g water/g dry matter).

However, if the equilibrium moisture content (M_e) is very low than M_t and M_i, it can be neglected and Equation 2 is used (Yousefi *et al.*, 2013):

$$MR = \frac{M_t}{M_i} \qquad \text{(Eq. 2)}$$

Drying rate (DR) was determined using Equation 3:

$$DR = \frac{M_{t+\Delta t} - M_t}{\Delta t} \qquad \text{(Eq. 3)}$$

where

$M_{t+\Delta t}$: moisture content at time difference;

Δ_t: time difference between two measuring points.

The relation between predicted and experimental data of goji berry fruits dried at different drying temperatures is explained with root mean square error (RMSE), reduced chi-square (χ^2), and determination coefficient (R^2). RMSE is a statistical parameter that expresses deviation between experimental and predicted values. The best equation predicting experimental data is determined accordingly with lower RMSE and χ^2 and higher R^2 values. The chi-square (Equation 4) and RMSE (Equation 5) values were calculated as follows:

$$\chi^2 = \frac{\sum_{i=0}^{N}\left(MR_{pre,i} - MR_{\exp,i}\right)^2}{N-n} \qquad \text{(Eq. 4)}$$

$$RMSE = \left[\frac{1}{N}\sum_{i=0}^{N}\left(MR_{pre,i} - MR_{\exp,i}\right)^2\right]^{1/2} \qquad \text{(Eq. 5)}$$

where

$MR_{pre,i}$: predicted MR;

$MR_{exp,i}$: experimental MR;

N: number of observation data;

n: constants of thin layer drying models.

MATLAB software was used to calculate thin-layer modeling and for statistical analyses.

Determination of effective moisture diffusivity and activation energy in hot air drying

Fick's diffusion equation described the drying characteristics of biomaterials. Crank (1975) suggested a solution to this equation to be used for spherical products. Equation 6 is recommended for spherical products, provided that there is no shrinkage and constant effective diffusivity (Doymaz, 2006):

$$MR = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-n^2\pi^2 D_{eff}}{r^2}\right) \qquad \text{(Eq. 6)}$$

where

D_{eff}: effective moisture diffusivity (m^2/s)'

r: arithmetical average of radius of samples at measured intervals (m).

Equation 6 can be reduced (Saravacos and Raouzeos, 1986) and a new equation is provided below:

$$\ln(MR) = \ln\left(\frac{6}{\pi^2}\right) - \left(\frac{\pi^2 D_{eff}}{r^2}t\right) \qquad \text{(Eq. 7)}$$

Equation 8 shows a straight line with a slope provided in the plot:

$$Slope = -\frac{\pi^2}{r^2}D_{eff} \qquad \text{(Eq. 8)}$$

The Arrhenius equation of hot air-drying process was used for calculation of activation energy (Fang *et al.*, 2009):

$$D_{eff} = D_0 \exp\left(\frac{-E_a}{RT}\right) \qquad \text{(Eq. 9)}$$

where

R: universal gas constant [8.314 J/mol (K) or 1.987 cal/mol (K)];

T: absolute temperature (K);

E_a: activation energy (kJ/mol or kcal/mol);

D_0: pre-exponential constant (m^2/s).

After regulation of natural logarithm in Equation 8, Equation 9 can be written as follows:

$$\ln D_{eff} = \ln D_0 - \frac{E_a}{RT} \qquad \text{(Eq. 10)}$$

Natural logarithm of effective moisture diffusivity versus 1/T gives a straight line with a slope that represents activation energy.

Analysis of water-soluble vitamins

An extraction method proposed by Donmez (2015) was used for analysis of water-soluble vitamins. To determine water-soluble vitamins, a sample of 5 g of goji berries was taken, and after homogenization with distilled water (1:9, w:v), the homogenate was centrifuged at 4500 rpm for 10 min (Core NF 800R). The supernatant obtained from centrifugation was filtered using a 0.45-μm filter to be injected into a high-pressure liquid chromatography (HPLC) column.

Using a micro syringe, 20 μL of filtrate was injected into the HPLC column. Mobile phase consisted of 0.1 M HPLC grade KH_2PO_4 at pH 7.

The HPLC device (Shimadzu), in which analysis of water-soluble vitamins was performed, consisted of column oven (Shimadzu CTO-20A, Japan), pump (Shimadzu LC-20AD, Japan), degasser (Shimadzu DGU-20A3, Japan), photodiode array (PDA) detector, and HPLC software in a computer. The column used in the analysis was ACE C18 column (7.8 × 300 mm), column temperature was 25°C, and the flow rate of mobile phase was 0.8 mL/min (isocratic). Wavelengths used in analysis were 254 nm, 261 nm, 324 nm, and 234 nm for ascorbic acid, niacin, pyridoxine, and thiamine, respectively. Analysis was performed in triplicate.

A calibration curve of different concentrations of stock solutions (5, 10, 25, 50, 75, and 100 ppm) with high R^2 (0.9999) was obtained. The content of water-soluble vitamins was calculated by the equation obtained from the calibration curve.

Analysis of antioxidant capacity and total phenolic content

The AC and TPC analysis was carried out using the methanolic extraction method proposed by Otağ (2015). A sample of 5 g of goji berries was mixed with 45 mL of 90% methanol and homogenized using a laboratory blender. The homogenate was centrifuged at a speed of 4500 rpm for 10 min. After centrifugation, supernatants were collected and filtered using a filter paper.

The TPC analysis was performed according to Singleton and Rossi (1965) with modifications. In this analysis, 1500 μL Folin–Ciocalteu solution (10% v/v) was added into 300 μL of extract and the mixture was kept in a dark place for 3 min. Then 1200 μL aqueous 7.5% Na_2CO_3 was added into the mixture. The final mixture was incubated for 2 h at room temperature in a dark place. After incubation, the absorbance measurement of the samples was carried out at a wavelength of 760 nm using spectrophotometer (T80, PG Ins., UK.). Analysis was carried out in triplicate, and TPC was expressed as mg/100 g gallic acid equivalent (GAE) dry weight (DW).

The AC analysis was carried out using the method suggested by Thaipong *et al.* (2006) with modifications. Here, 150 μL of extracts and 2850 μL of DPPH methanolic solution (absorbance value: 1.1 at a wavelength of 515 nm) were mixed. Absorbance of samples was measured at a wavelength of 515 nm using spectrometer after 60 min incubation in a dark place at room temperature. Each sample was analyzed in triplicate and AC was expressed as mmol Trolox equivalent (TE)/g DW.

Color measurement

Reflectance color value of goji berry skin was measured by using Hunter Lab Color Miniscan XE (45/0-L, USA). The samples were placed on a white background and the measurement was performed by covering with a transparent glass. The highest color difference (ΔE) was calculated using Equation 11 (Horuz *et al.*, 2017):

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \qquad \text{(Eq. 11)}$$

Calculation of kinetic parameters

The following equation (Equation 12) was used as a general equation to describe the reaction rate of the compounds that are degraded or formed by Labuza (1984):

$$\frac{dc}{dt} = k[C]^m \qquad \text{(Eq. 12)}$$

For the zero-order kinetic model, equation can be written as follows:

$$C = C_0 - Kt \qquad \text{(Eq. 13)}$$

If Equation 13 is integrated and m = 1, then Equation 14 is written as follows:

$$\ln C = \ln C_0 - kt \qquad \text{(Eq. 14)}$$

where
ln C: natural logarithm of the residual vitamins C and B complex, TPC, and AC;
ln C_0: initial content of vitamins C and B complex, TPC, and AC]
k: rate constant (1/h);
t: time.

Temperature dependence of vitamins C and B complex, TPC, and AC can be calculated using Equation 15 (Labuza and Riboh, 1982):

$$k = k_0 x e^{-\frac{E_a}{RT}} \qquad \text{(Eq. 15)}$$

When Equation 15 is regulated, Equation 16 is written as follows:

$$\ln k = \left(-\frac{E_a}{R}\right) x \left(\frac{1}{\tau}\right) + \ln k_0 \qquad \text{(Eq. 16)}$$

where
k_0: frequency factor (1/h);
R: universal gas constant [8.314×10^{-3} kJ/mol (K) and 1.987×10^{-3} kcal/mol (K)];
T: absolute temperature (K);
E_a: activation energy (kcal/mol or kJ/mol).

Quotient indicator (Q_{10}) expresses temperature-dependence of reaction rate and is calculated using Equation 17 (Labuza and Schimidl, 1985):

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)} \qquad \text{(Eq. 17)}$$

Half-life time, time required for half of concentration, for each temperature is calculated using Equation 18 for first-order kinetics (Labuza, 1984):

$$t_{1/2} = -\ln(0.5) x \frac{1}{k} = 0.693 x \frac{1}{k} \qquad \text{(Eq. 18)}$$

Time taken by the compound, or quality criterion, to lose 90% of its quality is expressed as D and is calculated for first-order kinetics as follows (Equation 19):

$$D = 2.303 x \frac{1}{k} \qquad \text{(Eq. 19)}$$

Statistical analysis

SPSS 22.0 software (IBM Corporation, Armonk, NY) was used for statistical analysis and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to evaluate differences between treatments with the significance level P = 0.05. Differences between groups were determined using the Duncan test.

Results and Discussion

Drying characteristics of goji berry fruits during hot air drying

The drying rate and moisture ratio values of goji berries during hot air drying are presented in Figure 1. Drying time and drying rate of goji berry fruits were significantly affected by drying temperature, and it is clearly seen that drying rate increases with the increment in drying temperature.

Drying time decreased depending on the increment in temperature, so drying time was found to be 24 h at 50°C, 19 h at 60°C, and 9 h at 70°C. Adiletta *et al.* (2015) determined drying time as 21 h at 60°C for hot air-drying treatment of goji berries. Fratianni *et al.* (2018) dried goji berry fruits in a convective dryer and the drying process was finished in 45 h at 50°C, 21 h at 60°C, and 12 h at 70°C, and the velocity of air was 2.1 m/s. It could be that the increment in drying rate with increase in temperature might be due to the increase in heat transfer coefficient. The results of this study were similar to the results of the studies examined in literature.

Mathematical models used in modeling the drying process, constants, and the statistical data of mathematical models are listed in Table 1. Demiray *et al.* (2017) reported that the lower RMSE and χ^2 and the higher R^2 values are required for determining the best model. As seen in Table 1, the Page (1949) model is the best model predicting experimental moisture ratio of goji berry fruits for all drying temperatures (50°C, 60°C, and 70°C), with the lowest RMSE and χ^2 and the highest R^2 values.

Effective moisture diffusivity and activation energy of goji berry fruits during hot air drying

The effective moisture diffusivity (D_{eff}) and activation energy (E_a) values of goji berry fruits are presented in Table 2, and the D_{eff} values were calculated in the range of 2.98×10^{-8}–1.04×10^{-8} m^2/s. Effective moisture diffusivity is a useful indicator of dehydration effectiveness (Chen *et al.*, 2016). When compared with other drying temperatures, the highest D_{eff} value was determined in the drying process conducted at 70°C. Increase in the D_{eff} value means the moisture content in goji berry samples is evaporated more easily. As understood from Equation 9 mentioned above, it is a known fact that the drying temperature is an important factor affecting the D_{eff} value.

No mention of D_{eff} value during the drying of goji berry fruits with hot air was found in literature. Senadeera *et al.* (2014) found D_{eff} values in the range of 1.32×10^{-6}–1.34×10^{-6} m^2/h because of the drying process executed on different types of grapes at 50°C, 0.5 m/s air velocity, and 20% moisture content. Chen *et al.* (2016) carried out hot air drying of wine grapes, grown in Canada, between 25°C and 80°C and determined the D_{eff} value at 25°C and 80°C as 0.05×10^{-10} m^2/s and 0.49×10^{-10} m^2/s, respectively, at MR = 0.2. They observed that the D_{eff} values increased 10 times with increase in temperature from 25°C to 80°C. Dong *et al.* (2013) studied the drying process of grapes at 30°C, 35°C, 40°C, and 45°C and found that the D_{eff} value was higher at the highest temperature. In other words, the D_{eff} value increases with increase in drying temperature, and the data examined in literature support our study.

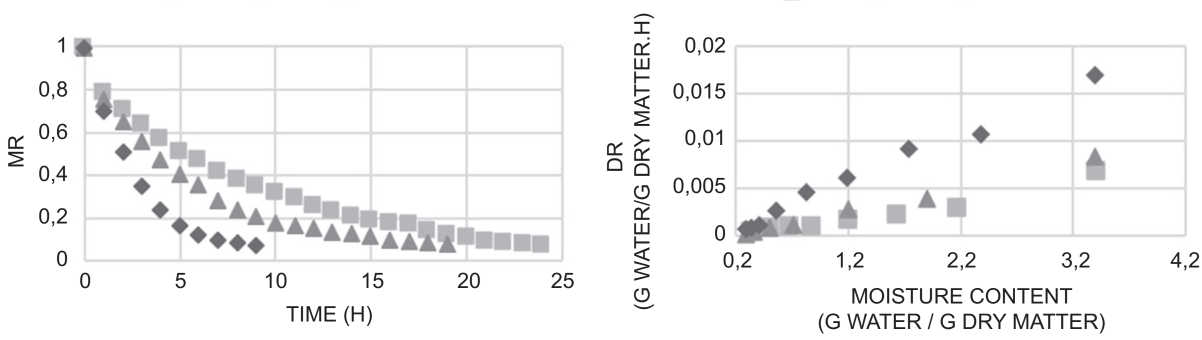

Figure 1. Moisture ratio and drying rate of goji berries during hot air drying.

Table 1. Thin-layer mathematical models, model constants, and statistical parameters of thin-layer drying curves.

Model Names and References	Model	Temperature	Model Constants			χ^2	RMSE	R^2
Lewis / Lewis (1921)	exp(-kt)	50°C	k = 0.1186			0.001344007	0.03592	0.9793
		60°C	k = 0.1737			0.001277711	0.03484	0.9819
		70°C	k = 0.3450			0.000251669	0.01505	0.9977
Page / Page (1949)	$\exp(-kt^n)$	50°C	k = 0.1812	n = 0.8161		0.000264861	0.01561	0.9962
		60°C	k = 0.2508	n = 0.8107		0.000165378	0.01220	0.9979
		70°C	k = 0.3574	n = 0.9716		0.000290322	0.01524	0.9979
Henderson and Pabis / Henderson and Pabis (1961)	aexp(-kt)	50°C	k = 0.1075	a = 0.9150		0.000576501	0.02303	0.9918
		60°C	k = 0.1607	a = 0.9316		0.000866761	0.02793	0.9890
		70°C	k = 0.3438	a = 0.9964		0.000316013	0.01590	0.9977
Logaritmic / Doymaz (2011)	aexp(-kt) + c	50°C	k = 0.1341	a = 0.8742	c = 0.07044	0.000666050	0.02421	0.9910
		60°C	k = 0.2094	a = 0.8951	c = 0.07613	0.000296302	0.01587	0.9964
		70°C	k = 0.4267	a = 0.9425	c = 0.07490	0.000811922	0.02384	0.9948
Wang and Singh / Wang and Singh (1978)	$1 + at + bt^2$	50°C	a = -0.09269	b = 0.002374		0.003999604	0.06066	0.9433
		60°C	a = -0.13020	b = 0.004536		0.004329174	0.06242	0.9450
		70°C	a = -0.26180	b = 0.018240		0.001641672	0.03624	0.9880
Parabolic / Bi et al. (2015)	$a + bt + ct^2$	50°C	a = 0.8701	b = -0.07149	c = 0.001653	0.001539746	0.03681	0.9800
		60°C	a = 0.8800	b = -0.10560	c = 0.003485	0.002166198	0.04291	0.9754
		70°C	a = 0.9556	b = -0.24320	c = 0.016610	0.001492261	0.03232	0.9917

RMSE, root mean square error.

Table 2. Effective moisture diffusivity and activation energy of goji berry fruit.

Temperature	D_{eff} (m^2/s)	E_a (kJ/mol)	E_a (kcal/mol)
50°C	1.04×10^{-8}	48.37	11.56
60°C	1.31×10^{-8}		
70°C	2.98×10^{-8}		

The Arrhenius-type relation between D_{eff} and 1/T is presented in Figure 2. The E_a values of goji berry fruits were found to be 48.37 kJ/mol and 11.56 kcal/mol. In literature, no activation energy data for hot air drying of goji berry fruits were found. When compared with similar berry fruits dried with hot air, Vega-Galvez *et al.* (2009) found E_a = 48.34 kJ/mol in blueberries. In another study done on hot air drying, Abdulla (2012) found E_a = 51.31 kJ/mol for golden fruits. López *et al.* (2010) and Shi *et al.* (2008) reported the E_a values of blueberry as 57.85 kJ/mol and 61.2 kJ/mol, respectively. Although the values found in some studies are similar to the values found in our study, others were higher. Differences between the results of the current study and other studies, in which other fruits were used, may be due to different factors such as different fruit structures, temperature, airflow rate, and relative humidity.

Effect of drying process on water-soluble vitamins, total phenolic content, and antioxidant capacity

Effects of drying on water-soluble vitamins of goji berries are provided in Table 3. Carr and Frei (1999) indicated that vitamin C easily scavenges nitrogen species and reactive oxygen and thereby may prevent oxidative damage to nontrivial biological macromolecules such as proteins, lipids, and DNA. It is extremely important to maintain vitamin C during the drying process or to carry out this process with minimal loss; however, vitamin C is significantly affected by the drying process. In this study, value of vitamin C in fresh goji berries was found to be 112.75 ± 2.23 mg/100 g DW. Donno *et al.* (2015) found the concentration of vitamin C in goji berries to be 42 mg/100 g FW (fresh weight). The United States Department of Agriculture (USDA) has found the amount of vitamin C in dried goji fruit to be 48.4 mg/100 g (Koçyiğit and Sanlier, 2017). When compared with literature, it could be said that goji berries grown in Turkey are rich in vitamin C. There are statistical losses in values of vitamin C at all drying temperatures ($P < 0.05$). Vitamin C values at 50°C, 60°C and 70°C were determined as 39.45 ± 2.21, 26.48 ± 1.16 and 21.87 ± 0.971 mg/100 g DW, respectively. Since vitamin C has low stability against heat treatments, it is established as

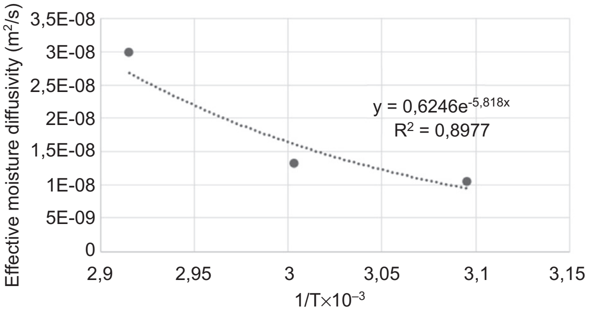

Figure 2. The Arrhenius-type relation between effective moisture diffusivity and 1/T.

a quality index in foods during the processing (DiScala and Crapiste, 2008). Besides kinetic parameters, vitamin C may be a significant quality parameter in goji berries' drying process. López *et al.* (2010) reported that there were significant loss in vitamin C values of blueberries at all drying temperatures, and the highest loss was 92% at 80°C. In a detailed review on ascorbic acid, Santos and Silva (2008) stated that a significant loss in ascorbic acid was seen due to hot air drying of fruits and vegetables. They even stated that no vitamin C was left in some drying processes applied on tomatoes over 100°C. Other studies (Araya-Farias *et al.*, 2011; Kadakal *et al.*, 2017) have demonstrated that the hot air-drying process significantly reduces the amount of vitamin C.

In our study, the amount of vitamin B complex was analyzed in fresh goji berry fruits and the kinetic data were obtained during the drying process. The amount of pyridoxine (B_6) in fresh goji berries was determined as 2.19 ± 0.046 mg/100 g DW but thiamine, riboflavin, and niacin were not detected. Right after hot air-drying process at different temperatures, amount of pyridoxine was determined as 0.937 ± 0.055, 0.681 ± 0.061, and 0.49 ± 0.034 mg/100 g DW at 50°C, 60°C, and 70°C, respectively. The highest loss appears to be in the drying process at 70°C.

Ryley and Kajda (1994) stated that loss in the values of water-soluble vitamins was observed with the effect of heat treatment in various foods. In consequence of drying at different temperatures, an important decrease in the amount of pyridoxine was observed in goji berries. Decrease in the amount of water-soluble vitamin B_6 increases with the increment in drying temperature.

Effects of hot air drying on total phenolic content and antioxidant capacity of goji fruits are presented in Table 3. The TPC and AC values of fresh goji berries were found as 1838.43 ± 37.47 mg/100 g DW and 0.077 ± 0.002 mmol TE/g DW, respectively. Islam *et al.* (2017) determined the TPC value of red goji berry fruits as 217–448 mg GAE/100 g. Ban *et al.* (2015) determined the TPC value of fresh goji berries in the range of 449.92–450.48 mg GAE/kg FW. Zhang *et al.* (2016) determined the TPC values of goji berry fruits in the range of 5840–7340 mg GAE/100 g FW. Pedro *et al.* (2018) investigated TPC by extraction of goji berry fruits in different concentrations of methanol and found it in the range of 1052.53–1736.36 mg GAE/100 g. The TPC of goji berry fruits because of drying processes at 50°C, 60°C, and 70°C was determined to be 491.00, 450.17, and 404.45 mg GAE/100 g DW, respectively. Islam *et al.* (2017) and Zhang *et al.* (2016) determined the AC value of red goji berry fruits as 16.07–17.47 mg µmol TE/g and 77.41–85.46 µMTE/g FW, respectively. Pedro *et al.* (2018) found the AC values of goji berry in the range of 0.94–1.51 mmol TE/100 g. Mikulic-Petkovsek *et al.* (2014) stated that a significant difference in the content of fruits is seen when grown at different locations. The compositional difference seen in the same varieties of fruits and vegetables is influenced by numerous factors such as environmental conditions of the region where the product is grown, especially soil quality, cultivation technique and cultural measures, maturity level, transportation and storage, and so on (Gökkür and Çelik, 2016). The reason why our results are different from those found in literature may be due to the reasons explained above.

Table 3. Effect of drying process on vitamins C and B_6, total phenolic content, and antioxidant capacity of goji berries.

	Vitamin C*	Loss percentage (%)	Pyridoxine (B_6)*	Loss percentage (%)	TPC**	Loss percentage (%)	AC**	Loss percentage (%)
Fresh	112.75 ± 2.23^{a}	0	2.19 ± 0.046^{a}	0	1838.43 ± 37.47^{a}	0	0.077 ± 0.002^{a}	0
50°C	39.45 ± 2.21^{b}	65.03	0.937 ± 0.055^{b}	56.56	491.00 ± 7.96^{b}	73.29	0.017 ± 0.001^{b}	77.92
60°C	26.48 ± 1.16^{c}	76.84	0.681 ± 0.061^{c}	69.04	450.17 ± 8.26^{b}	75.51	0.014 ± 0.001^{bc}	81.82
70°C	21.87 ± 0.971^{d}	80.5	0.492 ± 0.034^{c}	77.48	404.45 ± 6.89^{c}	78	0.011 ± 0.001^{c}	85.71

*Vitamins C and B_6 was expressed as mg/100 g DW.

**TPC was expressed as mg GAE/100 g DW, AC was expressed mmol TE/g DW.

***Different letters in the same column are significantly different values ($P < 0.05$).

TPC, total phenolic content; GAE, gallic acid equivalent; TE, trolox equivalent; DW, dry weight.

Color properties of goji berry fruit during hot air drying

Color properties of fresh and dried goji berries were presented in Table 4. When compared with initial L*, a*, and b* values of goji fruits, values were significantly decreased due to drying process ($P < 0.05$) and the lowest L*, a*, and b* values were obtained at 70°C. ΔE indicates differences between colors of samples (Horuz *et al.*, 2017). The ΔE value of dried goji fruits depends on drying conditions and ranges from 10.87 to 13.91. The highest ΔE was obtained from the goji berry fruits dried at 70°C.

Table 4. Color properties of goji berry fruits.

	L*	a*	b*	ΔE
Fresh	25.97 ± 0.12^{a}	25.16 ± 0.13^{a}	17.30 ± 0.05^{a}	
50°C	23.11 ± 0.09^{b}	16.45 ± 0.07^{b}	11.03 ± 0.11^{b}	10.87
60°C	22.79 ± 0.05^{c}	14.41 ± 0.09^{c}	10.67 ± 0.07^{c}	13.01
70°C	21.99 ± 0.06^{d}	14.67 ± 0.08^{d}	9.62 ± 0.05^{d}	13.91

*Different letters in the same column are significantly different values ($P < 0.05$).

Kinetic parameters of vitamins C and B6

To the best of our knowledge, vitamin C degradation in the hot air-drying process of goji berries was investigated for the first time in this study. Thermal degradation of vitamin C in goji berries is shown in Figure 3; its content in fully dried goji berries is found to fit the first-order kinetic model. It is stated by Gamboa-Santos *et al.* (2014), Hiwilepo-van Hal *et al.* (2012), Kadakal *et al.* (2017), and Wang *et al.* (2017) that the thermal degradation of vitamin C fits the first-order kinetic model in different dried foods.

Air-drying may have a negative effect on the physical properties of products and cause degradation of aromatic compounds and nutrients (Araya-Farias *et al.*, 2011). In other words, losses are observed in the compounds found in all foods, especially vitamin C, by heat treatment. Dağhan *et al.* (2018) studied the hot air drying of Isot at different temperatures and found that there was significant loss in vitamin C. They found the highest loss at 75°C and stated that vitamin C is highly sensitive to changes in temperature. Marfil *et al.* (2008) performed tomato hot air drying at different temperatures and reported that the loss of vitamin C in tomatoes increased with increase in drying temperature. Kinetic parameters of vitamin C in goji berries are presented in Table 5. Vitamin C degradation rate constants of goji fruits at 50°C, 60°C, and 70°C were found to be 0.047, 0.075, and 0.182 1/h, respectively. It is clearly observed that the rate constant increased but

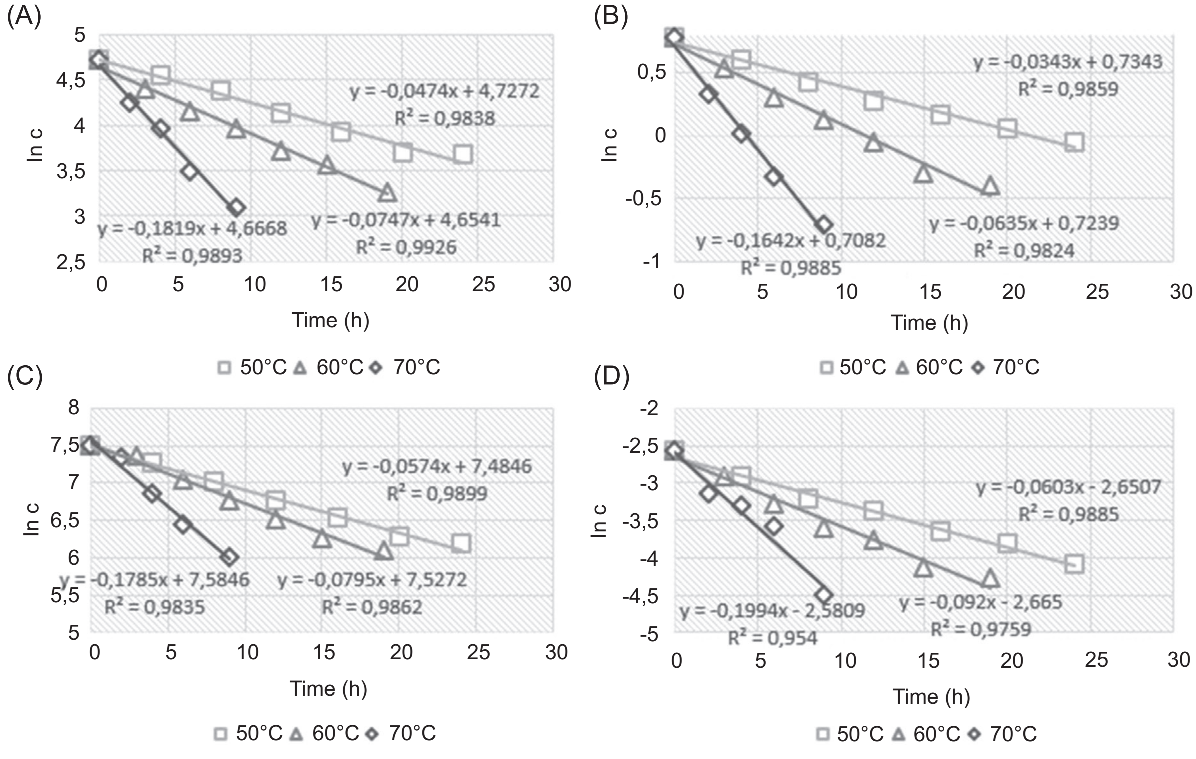

Figure 3. First-order kinetics of (A) vitamin C, (B) pyridoxine, (C) total phenolic content (TPC), and (D) antioxidant capacity (AC) of goji berries.

Table 5. First-order kinetic parameters of vitamins C and B_6, total phenolic content, and antioxidant capacity of dried goji berries.

Compound	Temperature	*k* (1/h)	$t_{1/2}$ (h)	*D* (h)	R^2	E_a (kcal/mol)	E_a (kJ/mol)	Q_{10} (50–60°C)	Q_{10} (60–70°C)
Vitamin C	50°C	0.047	14.62	48.59	0.984	14.75	61.72	1.58	2.44
	60°C	0.075	9.28	30.83	0.993				
	70°C	0.182	3.81	12.66	0.989				
Pyridoxine (vitamin B_6)	50°C	0.034	20.2	67.14	0.986	17.19	71.94	1.85	2.59
	60°C	0.064	10.91	36.27	0.982				
	70°C	0.164	4.22	14.03	0.989				
TPC	50°C	0.057	12.07	40.12	0.990	12.43	52.01	1.39	2.25
	60°C	0.080	8.72	28.97	0.986				
	70°C	0.179	3.88	12.90	0.984				
AC	50°C	0.060	11.49	38.19	0.989	13.12	54.90	1.53	2.17
	60°C	0.092	7.53	25.03	0.976				
	70°C	0.199	3.48	11.55	0.954				

TPC, total phenolic content; AC, antioxidant capacity.

$t_{1/2}$ and *D* values of vitamin C decreased due to the increment in temperature. Similarly, Demiray *et al.* (2013) stated that the *k* value increased with the increment in drying temperature. They also stated that the $t_{1/2}$ value decreased with the increase of drying temperature in drying of tomatoes. Kadakal *et al.* (2017) stated that the degradation rate constant of vitamin C was increased due to the thermal increase in rosehip nectar while the $t_{1/2}$ and *D* values were decreased. Our results are compatible with literature. Also, the Q_{10} value from 60°C to 70°C was found to be higher than from 50°C to 60°C. With this data obtained in our study, it is understood that the thermal degradation of vitamin C is more sensitive to the increment of temperature from 60°C to 70°C. The Q_{10} value of vitamin C thermal degradation increased with the decrement in drying temperature (Demiray *et al.*, 2013; Kadakal *et al.*, 2017). Kadakal *et al.* (2017) stated that high activation energy of reaction indicates that the reaction sensitivity of temperature is very high. The Arrhenius equation of vitamin C thermal degradation is given in Figure 4.

To the best of our knowledge, the degradation of vitamin B_6 in the hot air-drying process in goji berries has been investigated for the first time. Thermal degradation of vitamin B_6 is shown in Figure 3, and the kinetic parameters of vitamin B_6 thermal degradation are presented in Table 5. The thermal degradation of vitamin B_6 content in fully dried goji berries is found to fit the first-order kinetic model. Vitamin B_6 degradation rate constants of goji berries at 50°C, 60°C, and 70°C have been found to be 0.034, 0.064, and 0.164 1/h, respectively. Rate constant increased, but the $t_{1/2}$ and *D* values of vitamin B_6 decreased due to the increment in temperature. Kadakal *et al.* (2017) stated that the degradation rate constant in vitamin B complex increased due to thermal increase in rosehip nectar. Also, the Q_{10} value from 60°C to 70°C was found to be higher than that from 50°C to 60°C. When Q_{10} values vitamins C and B_6 were compared, it was understood that vitamin B_6 is more sensitive to increase in temperature. The E_a value of vitamin B_6 was found to be 71.94 kJ/mol. When the E_a values of vitamins C and B_6 were compared, the E_a value of vitamin B_6 was higher than that of vitamin C, which means that vitamin B_6 is more stable than vitamin C. At the same time, vitamin B_6 is more sensitivity to changes in temperature than vitamin C.

Kinetic parameters of total phenolic content and antioxidant capacity

There are no data about the kinetic parameters of TPC in dried goji berries. The TPC thermal degradation is shown in Figure 3, and the kinetic parameters of TPC thermal degradation are listed in Table 5. The TPC thermal degradation rate constant increased and values of $t_{1/2}$ and *D* decreased with the increment in drying temperature. Thermal degradation of TPC content in fully dried goji berries was found to fit the first-order kinetic model. The rate constant of TPC thermal degradation in goji fruits ranged from 0.057 to 0.179 1/h. TPC thermal degradation increases depending on the increment of temperature (Kadakal and Duman, 2018; Sarpong *et al.*, 2018). López *et al.* (2010) reported that the TPC value decreased with increase in the temperature of drying air. The activation

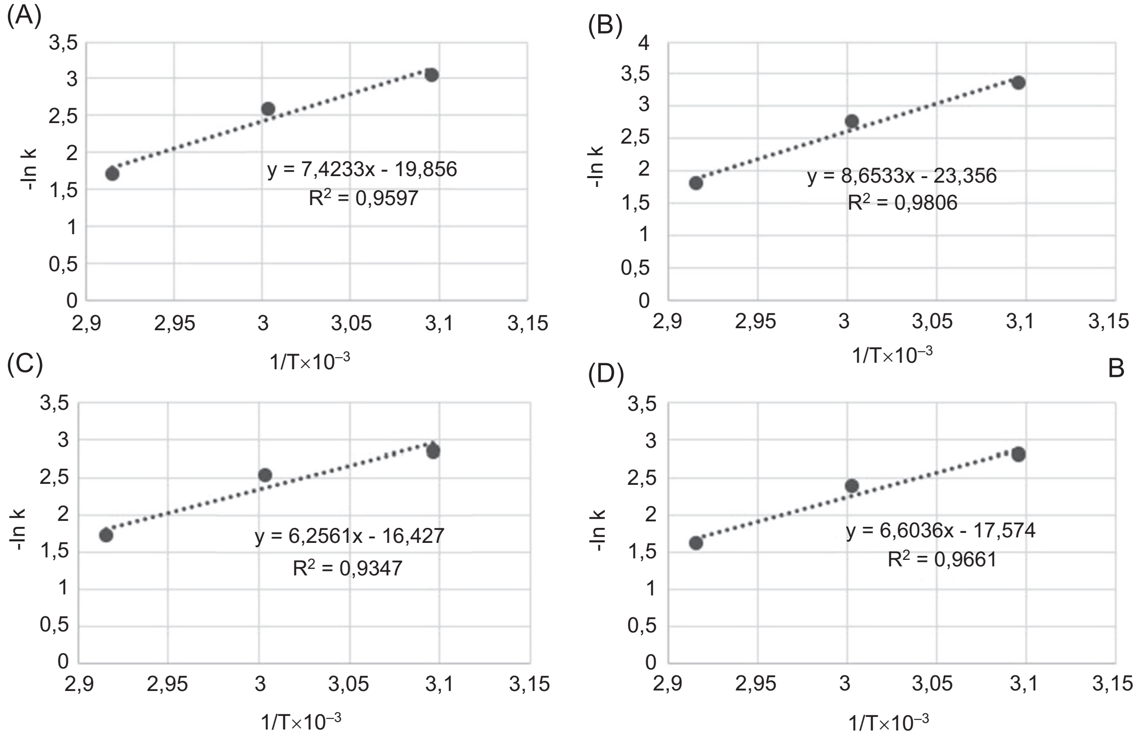

Figure 4. Arrhenius plots of dried goji berries: (A) vitamin C, (B) pyridoxine, (C) total phenolic content (TPC), and (D) antioxidant capacity (AC).

energy was calculated using the Arrhenius equation presented in Figure 4 and found to be 52.01 kJ/mol. The Q_{10} value from 60°C to 70°C was found to be higher than that from 50°C to 60°C. Thus, the thermal degradation of TPC is more sensitive to the increment of temperature from 60°C to 70°C.

To the best of our knowledge, AC thermal degradation in dried goji fruits was studied for the first time in the current study. The AC thermal degradation is shown in Figure 3, and the kinetic parameters of AC thermal degradation are given in Table 5. In the current study, thermal degradation of AC in fully dried goji berries was found to fit the first-order kinetic model. Oancea *et al.* (2017) used the first-order kinetic model on AC thermal degradation in sour cherry extract. Owing to temperature increment, the rate constant increased but $t_{1/2}$ and D values of AC decreased. Oancea *et al.* (2017) and Sarpong *et al.* (2018) reported that the rate constant of AC increased with increase in temperature in sour cherry extract and banana slices, respectively. The Arrhenius equation of AC thermal degradation is presented in Figure 4 and E_a was found to be 54.90 kJ/mol. The Q_{10} values from 50°C to 60°C and from 60°C to 70°C were found as 1.53 and 2.17, respectively. In this context, the increment in Q_{10} value from 60°C to 70°C indicates that the thermal degradation of AC is more sensitive than that in the range of 50–60°C.

Conclusions

In this study, for the first time, drying characteristics and thermal degradation of some ingredients in goji berry (*Lycium barbarum* L.) grown in Turkey were investigated under different drying conditions. Page model was determined to be the best model to predict experimental moisture ratio at all drying temperatures (50°C, 60°C, and 70°C). Drying temperature affects the drying speed and drying time. Drying time ranged from 9 to 24 h at 50–70°C. With increase in drying temperature, effective moisture diffusivity increased and the highest effective moisture diffusivity was determined at 70°C. The drying process showed losses in vitamins C and B_6, TPC, and AC, and the highest loss was observed at 70°C. The highest percentage loss was found in AC. The thermal degradation of Vitamins C and B_6, TPC, and AC is found to fit the first-order kinetic model, and the drying rate values of all these in goji berries increased by drying temperature increment. Vitamins C and B_6 were very susceptible to temperature increment, but TPC and AC were the lowest sensitive compounds in dried goji berries. The highest color difference (ΔE) was obtained in the goji berries dried at 70°C. The shortest drying time was observed in the goji berries dried at 70°C, and the drying process at 50°C provided the highest retention of bioactive compounds in goji berries. According to the data obtained and evaluated, the optimal drying temperature for goji berries is 50°C in hot air drying.

As additional studies, research should be conducted on obtaining dried goji berries with different and combined drying method, which could be a more efficient drying process with less component loss. Also, the content differences in goji berries grown at different locations should be investigated.

Acknowledgments

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Does iron-fortified chewing gum influence the biochemical profile of school-going children (6–10 yrs.)?

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PAPER

Abstract

Iron deficiency has become a common nutritional problem of developing countries, especially in children. This study approached to tackle the issue of iron deficiency by inexpensive fortified food such as chewing gums, which is commonly consumed by children. In this study, iron-fortified chewing gums were prepared by adding ferrous sulfate ($FeSO_4$) and sodium iron EDTA (NaFeEDTA) 30 mg/100 g. An efficacy trial was conducted to determine the impact of iron-fortified chewing gums on the blood profile and iron status of school-going children (n = 300). Results showed maximum increase in blood profile and iron status that is, serum ferritin (10.43%), hemoglobin (3.22%), hematocrit (3.42%), red blood cells (3.05%), mean cell volume (1.55%), mean cell hemoglobin (5.43%), total white blood cells count (9.09%), and platelets count (4.40%) as compared with control whereas decrease in mean cell hemoglobin concentration (1.90%) and neutrophils (3.33%) was also observed. The study concluded that $FeSO_4$ and NaFeEDTA (1:1) fortification of chewing gums is an appropriate approach for mitigating iron deficiency among the target population.

Keywords: complete blood count (CBC), daily value (DV), ferrous sulfate ($FeSO_4$), iron deficiency anemia (IDA), sodium iron EDTA (NaFeEDTA)

Introduction

Iron (Fe) deficiency anemia (IDA) is known as a predominant nutritional deficiency worldwide. It has depicted substantial economic losses and health problems to distress a massive segment of global population (Petry *et al.*, 2016). IDA causes maternal hemorrhage, reduced productivity, decreased school performance, and innumerable mortalities in vulnerable population. Nearly 700 to 800 million people worldwide are affected by the IDA, which severely distresses 60–70 million people in developing countries. Approximately 65% of pregnant women in South Asia suffer from IDA (Siddiqui *et al.*, 2007).

Numerous reports have revealed that 65–78% of children aged <5 years are suffering from IDA; their hemoglobin (Hb) levels were found below 11 g/dL (Akhtar *et al.*, 2013; Menon and Yoon, 2015). According to another report, the occurrence of anemia was estimated as 82.90%, 85%, 83%, and 78% among children, adolescent girls, pregnant, and lactating women, respectively (Akhtar *et al.*, 2013).

Survey of semi-urban areas of Abbottabad and Peshawar exhibited the occurrence of iron deficiency among children aged <2 years as 68% and 69%, respectively (Idris and Anis-ur-Rehman, 2005). Similarly, in urban slums of Karachi, occurrence of IDA has been depicted as up to 61% (Hb < 11 g/dL) and low hematocrit (PCV) (63.80 % in children aged 6–60 months). The prevalence of anemia among rural-based pre-school children of Karachi has shown highly significant results (Molla and Khurshid, 1992). Likewise, according to another survey conducted in Pakistan, approximately 69% children aged <2 years, 40–50% of pre-school and primary school children, 39% of adolescents, 30% of adult females, and 54% of young girls were reported to be affected by IDA (Khor, 2005). In Pakistan, IDA has also been demonstrated as a severe health problem in pre-school children, followed by pregnant and nonpregnant women (World Health Organization [WHO], 2011).

The WHO has recommended four basic strategies to control and prevent IDA, which include increased iron intake, control of infection, supplementation, and food fortification (Prentice *et al.*, 2017). A lot of iron fortification success stories have been reported in literature, that is, countries of the Middle East, Mongolia, Kyrgyz Republic, Kazakhstan, Tajikistan, Azerbaijan, North Africa, and Uzbekistan have successfully implemented iron fortification in wheat and corn flour (Mclean *et al.,* 2008). In fortification of food, either voluntarily or mandatorily, in order to achieve optimum results and to avoid side effects, concentration of iron fortificants should be carefully monitored. Additionally, extensive research has to be practiced to preserve flavor, color, and appearance of food (Kuong *et al.*, 2016). Predominant constraints for the success of any fortification depend upon the types and appropriate concentration of fortificants, fortified food, and viability of plans. Isotopic studies indicated that iron absorption from NaFeEDTA was possibly two to three times higher than the other form of iron used as a fortificant (Hurrell, 2002). Huo *et al.* (2002) investigated that NaFeEDTA-fortified soy sauce intervention within concentrations of 5 and 20 mg Fe/day significantly improved the iron status in children (aged 11–17 years). Davidsson *et al.* (2002) evaluated bioavailability of iron in foods based on corn tortillas and black bean paste fortified with ferrous sulfate ($FeSO_4$), ferrous fumarate, and Na-FeEDTA, and concluded that NaFeEDTA retains the higher geometric mean bioavailability (9.0%) than $FeSO_4$ (5.5%). Bouhouch *et al.* (2016) investigated and concluded that the influence of wheat flour biscuits fortified with iron and EDTA resulted in improved iron status significantly, but failed to show any positive influence on cognitive scores. In another study, maize flour, fortified with iron such as NaFeEDTA at in high and low concentrations (56 and 28 mg/kg) along with electrolyte iron (56 mg/kg) was fed to school children (aged 3–8 years) in Kenya. Its findings revealed that only high concentration of NaFeEDTA improved the iron status in children (Andang'o *et al.*, 2007). Arcanjo *et al.*(2010) used $FeSO_4$-fortified drinking water with different concentrations of $FeSO_4$ (5, 7.5, and 10 mg/L) for a period of four months, and suggested that 7.5 mg/L of $FeSO_4$-fortified drinking water enhanced the hemoglobin level along with significant reduction in anemia. Moretti *et al.* (2006) advocated the influence of extruded rice fortified with ferric pyrophosphate in Indian school children and showed that IDA reduced from 15 to 30% within the group using iron-fortified meals whereas the control group indicated nonsignificant difference ($P > 0.05$). Indeed, bioavailability of iron relies on the composition of meals, and presence of enhancers and inhibitors during iron absorption (Davidsson *et al.*, 2002). Besides selecting suitable vehicles during fortification, processing of food is also crucial. Traditionally, in many countries, cereal products are used as vehicles for fortification. Other vehicles include fish sauce, sugar, common salt, and cookies (Sari *et al.*, 2001). However, chewing gums are selected because they are more preferred by children in every economic segment of population. Currently, $FeSO_4$ and NaFeEDTA are being used to fortify food products. Presently, stability, acceptability, and bioavailability of iron compounds in the final product are critical issues and need to be explored to a greater extent.

The objective of the present study was to prepare the cheaper fortified chewing gums and to elucidate changes in proximate composition, minerals, and texture; its impact on the blood profile and iron status of school-going children was also explored.

Materials and Methods

Materials

Ingredients required for the preparation of chewing gums were procured from the local market. All reagents (analytical) were procured from Merck (Merck KG_a A, Darmstadt, Germany) and Sigma-Aldrich (Sigma Aldrich, USA). All other chemicals and reagents used were of analytical grade.

Preparation of iron-fortified chewing gums

Water (10 kg) and glucose (30 kg) were mixed, followed by the addition of 50-kg sucrose and 24 kg gums. The mixture was heated at 154°C followed by the removal of vacuum to get a better color, texture, and flavor. Furthermore, vacuum application decreased mixing to wrapping time. Other ingredients such as color (0.9 g), flavor (4.5 mL), black salt (2.5 g), and citric acid (2.5 g) were added per kilogram of the above mixture and mixed uniformly. Successively, after the approval of ethical committee, $FeSO_4$ (30 mg) and NaFeEDTA (100 g) were added in 1:1 ratio. After cooling, the mixture was rolled into a sheet and passed through roller press to get chewing gums of uniform size (5 g). Wrapping was done through a central seal machine to remove air and to extend shelf life of the product.

Proximate composition

Chewing gum samples were analyzed on a dry weight basis for crude protein (Method No. 46-30), crude fat (Method No. 30-25), crude fiber (Method No. 32-10), ash (Method No. 08-01), and nitrogen-free extract (NFE) according to their respective procedures. Minerals' analysis was done using the procedure described by Azeem *et al.* (2019).

Textural analysis

Hardness of samples was measured using a texture analyzer (TA-XT2, Plus, Stable Microsystems, Surrey, UK) interfaced with a computer. To compare the hardness of chewing gums, 2-mm cylinder probe (P/2) with a 5-kg load was used. For data analysis, Texture Expert program, version 4.0.9.0, was used.

Energy value

Energy value of chewing gums was determined by taking 0.5 g of sample using Oxygen Bomb Calorimeter (C2000 Basic, IKA®-Werke GMBH & Co., KG, Staufen, Germany) as described by Krishna and Ranjhan (1981).

Sensory acceptability

Sensory acceptability was assessed using a 9-point hedonic scale system, commencing from 1 = dislike extremely up to 9 = like extremely, for sensory attributes like color, flavor, mouthfeel, stickiness, texture, hardness, and overall acceptability by following the instructions of (Meilgaard *et al.*, 1999).

Efficacy trial

Healthy school-going children (boys) aged 6–10 years, with a hemoglobin concentration of <11 g/dL, from low- to moderate-income families were selected for efficacy study after the approval of the ethical committee of Allied Hospital, affiliated to the Department of Medicine (No. 09-0234), Punjab Medical College, Pakistan. An efficacy trial was carried out from 10 March 2016 to 15 April 2016 in two middle schools of district Jhang, Pakistan. Parents and children were invited in a seminar to introduce them the purpose, procedure, potential risks, and benefits of the study, and parents' written consent was taken. Complete enrollment and randomization procedure was explained. Parents of 318 children showed willingness to get enrolled in the study. An acceptability trial was conducted prior to efficacy studies to familiarize the children with sensory properties of the proposed iron-fortified chewing gums. Children were examined by a physician to determine whether they are healthy enough to be included in the study. Eighteen children were excluded, who were found diseased, infected, stunted, and malnourished. Finally, 300 healthy children, free from any chronic and acute disease, were stratified by computer-generated random numbers into four groups (T_1–T_4) with 75 children in each group. Fortified chewing gums (5 g) were given during the recess time to each selected child for 45 days continuously. In case of absence of child from school, missed chewing gums were compensated by provision of one extra chewing gum on the following day with advice to consume at midnight. The consumption and distribution of chewing gums were monitored by the authors and teachers. The blood samples were collected at baseline and after 45 days of intervention by a well-trained phlebotomist in the presence of a medical doctor through venipuncture procedure. Each group consumed chewing gums according to the following pattern.

T1: Group of children served with chewing gums without fortificants (control group); **T2**: Group of children served with chewing gums fortified with $FeSO_4$ (100%); **T3**: Group of children served with chewing gums fortified with $FeSO_4$ (50%) and NaFeEDTA (50%), and **T4**: Group of children served with chewing gums fortified with NaFeEDTA (100%). T_1 group was a placebo group. During the period of intervention, parents were advised to remain consistent with their children's dietary habits, constraining them from using any supplements. During pretrial, students complaining diarrhea were managed by decreasing the concentration of iron to the required level (30 mg/100 g). Since placebo group was considered as a control group, parents knew that their children were receiving chewing gums but were not aware whether chewing gums possessed iron or not.

Complete blood count (CBC)

Blood samples (5 mL) of each child were collected at the baseline and after 45 days of intervention through venipuncture procedure by a well-trained phlebotomist. Each 5-mL blood sample was divided into two equal parts and transferred into an erythrocyte sedimentation rate (ESR) tube and a gel tube (anticoagulant-free tube) to collect serum. Immediately after sample collection, CBC was carried out in ESR tubes separately using a CBC analyzer (Sy-Bh192, Sunny Medical Equipment Limited., Guangzhou, China), which analyzed the following parameters: hemoglobin, hematocrit, red blood cells (RBCs), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), total white blood cells count (TWBCC), neutrophils, platelets count (PLT), and erythrocyte sedimentation rate. Samples collected in gel tubes were centrifuged to isolate serum that was placed in refrigerator at −20°C for further tests.

Serum ferritin and iron

Serum ferritin (SF) concentrations were measured according to the method described by Sari *et al.* (2001) within 1 h of blood collection by using the enzyme immunoassay method (IMx System; Abbott, Abbott Park, IL). Iron concentration was performed according to the method described by Schoorl *et al.* (2012) using semi-automatic clinical chemistry analyzer (YSTE 168, Guangzhou Yueshen Medical Equipment Co. Ltd., Guangzhou, China).

Statistical analysis

All samples were analyzed in triplicate, and the results were presented as mean ± standard deviation. The statistical analyses were performed using the one-way analysis of variance (ANOVA) to evaluate differences between treatments followed by the Tukey test used to check differences between mean values with SPSS version 17.0 (SPSS Inc., USA). $P < 0.05$ was considered statistically significant.

Results and Discussion

Proximate and mineral composition

Compositional analysis of the iron-fortified chewing gums revealed significant differences for ash- and nitrogen-free extract but nonsignificant results were found for moisture, crude protein, and crude fat (Table 1). The highest ash content was found in T_3 (2.60 ± 0.09%), followed by T_4 (2.56 ± 0.05%), while the lowest ash content was found in T_2 (2.30 ± 0.05%). The overall ash content of iron-fortified chewing gums was in the range of 2.30 ± 0.05–2.60 ± 0.09% whereas the ash content of non-fortified (control) chewing gums was 1.30±0.05%. This increase in ash content was possibly due to the addition of fortificants. It was found in another study that the ash content of flour was increased with the fortification of iron from 1.63 to 1.76% (Akhtar *et al.*, 2005). The highest nitrogen content was found in T_2 (95.55 ± 0.70%), followed by T_4 (95.42 ± 0.60%) whereas the lowest nitrogen content was found in T_3 (95.12 ± 0.10%). Overall, the nitrogen content of iron-fortified chewing gums was in the range of 95.12±0.1–95.55±0.70%, while the nitrogen content of non-fortified (control) chewing gums was 96.64±0.90%. Besides, mean values of moisture, protein, fiber, and fat were found in the range of 1.3±0.26–1.4±0.17%, 0.33±0.12–0.60±0.22%, 0.01±0.05–0.03±0.04%, and 0.49±0.16–0.53±0.17%, respectively, whereas mean values of moisture, protein, fiber, and fat of non-fortified chewing gums were 1.7±0.17, 0.27±0.17, 0.2±0.005, and 0.60±0.21%, respectively. A similar pattern of the contents was found in another study, where tomato-based candy was prepared using 40% sugar solution. The results found similarity with the findings of Manjula and Suneetha (2014), who investigated that crude fiber in a hard candy prepared from pumpkin juice ranged from 1.20 to 2.24%.

Mean values for the iron content of iron-fortified chewing gums revealed significant results (Table 1). The highest iron content (334.03 mg/kg) was found in T_4, followed by T_2 (333.36 mg/kg) whereas the minimum iron content was found in T_2 (330.43 mg/kg). Current results showed similarities with the findings of Manjula and Suneetha (2014).

Texture analysis

Mean values for the hardness of iron-fortified chewing gums were nonsignificant among treatments ($P > 0.05$). Overall hardness content of iron-fortified chewing gums remained in the range of 36.27–39.59 (N) (Table 1). The texture of chewing gums was the same due to the addition of the same ingredient except for iron salt, which showed that addition of salt does not affect the texture. Textural values of chewing gums were comparable with that of tomato candy prepared from 40% sugar solution (Kamruzzaman *et al.*, 2014).

Energy value

Mean values of energy of iron-fortified chewing gums revealed a significant difference among treatments ($P < 0.05$) (Table 1). Mean values of iron-fortified chewing gums revealed that the highest energy value was found in T_3 (326.0 kcal/100 g), followed by T_2 (325.50 kcal/100 g). The lowest energy was found in T_4 (324.63 kcal/100 g). The overall energy of iron-fortified chewing gums was in the range of 324.63–326.0 kcal/100 g. T_1 had less energy value, probably because of missing iron salt whereas other treatments have differences because of salt characteristics.

Sensory evaluation

Sensory evaluation of iron-fortified chewing gums revealed significant difference in flavor, mouthfeel,

Table 1. Mean values for the effect of treatments on physiochemical characteristics of iron-fortified chewing gums.

Treatment	Moisture (%)	Crude protein (%)	Crude fiber (%)	Crude fat (%)	Ash (%)	NFE (%)	Energy (kcal/100 g)	Fe (mg/kg)	Texture (N)
T_1	1.7 ± 0.17	0.267 ± 0.17	0.02 ± 0.05	0.60 ± 0.21	1.30 ± 0.05^d	96.64 ± 0.9^a	322.14 ± 3.26^d	$0.01 + 0.25^d$	38.40 ± 0.81
T_2	1.3 ± 0.26	0.43 ± 0.17	0.01 ± 0.05	0.53 ± 0.17	2.30 ± 0.05^c	95.55 ± 0.7^b	325.50 ± 1.35^b	$330.43 + 0.24^c$	39.59 ± 0.12
T_3	1.3 ± 0.12	0.60 ± 0.22	0.02 ± 0.02	0.50 ± 0.12	2.60 ± 0.09^a	95.12 ± 0.1^d	326.0 ± 2.73^a	$333.36 + 0.17^b$	37.91 ± 0.68
T_4	1.4 ± 0.17	0.33 ± 0.12	0.03 ± 0.04	0.49 ± 0.16	2.56 ± 0.05^b	95.42 ± 0.6^c	324.63 ± 4.44^c	$334.03 + 0.13^a$	36.27 ± 0.45

Different letters in the same column indicate significant differences ($P < 0.05$).
T_1: 0% $FeSO_4$ and 0% NaFeEDTA; T_2: 100% $FeSO_4$; T_3: 50% $FeSO_4$ and 50% NaFeEDTA; T_4: 100% NaFeEDTA.

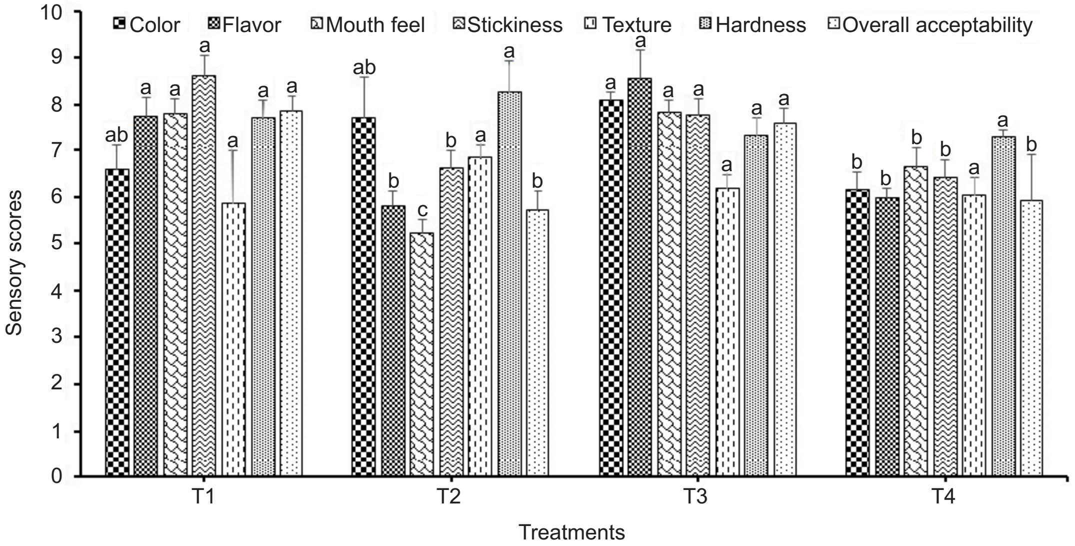

Figure 1. Sensory profile of $FeSO_4$- and NaFeEDTA-fortified chewing gums. Different letters on the same respective parameter bar indicate significant differences between mean values ($P < 0.05$).

stickiness, and overall acceptability but nonsignificant difference for color, texture, and hardness (Figure 1). The best flavor was found in T_3 (8.54 ± 0.62), followed by T_4 (5.99 ± 0.19) whereas the lowest acceptable flavor was observed in T_2 (5.81 ± 0.31). The flavor of non-fortified (control) chewing gums was 7.74 ± 0.40. T_3 (7.84 ± 0.26) secured the highest scores for mouthfeel, followed by T_4 (6.63±0.41) whereas the lowest scores for the stated trait was found in T_2 (5.23 ± 0.29). The score for mouthfeel of non-fortified (control) chewing gums was 7.79 ± 0.33. The highest stickiness was found in T_3 (7.76 ± 0.37), trailed by T_2 (6.61 ± 0.38); however, the lowest stickiness was found in T_4 (6.40 ± 0.39). The stickiness of non-fortified (control) chewing gums was 8.62 ± 0.42. The highest overall acceptability was found for T_3 (7.61 ± 0.30), followed by T_4 (5.91 ± 0.98), while the lowest overall acceptability was shown by T_2 (5.72 ± 0.41). However, the score of overall acceptability of non-fortified (control) chewing gums was 7.87 ± 0.30. Mean values for color and hardness were found in the range of 6.59 ± 0.55–8.10 ± 0.16 and 7.31 ± 0.39–8.27 ± 0.42, respectively, while mean values for color and hardness of non-fortified chewing gums were 6.59 ± 0.55 and 7.71 ± 0.39, respectively. The highest sensory scores for texture was found in T_2 (6.86 ± 0.28), followed by T_3 (6.19 ± 0.25), while the lowest value was found in T_4 (6.03 ± 0.39). The score for texture of non-fortified (control) chewing gums was 5.87 ± 1.12. Indeed, the overall acceptability of food products was affected by fortificant types (Chadare *et al.*, 2019; Davidsson *et al.*, 2005). Current outcomes were consistent with the findings of Durrani *et al.* (2011), who elucidated the color scores of honey-based candy as 7.56–9.23. The flavor's sensory scores reported in the previous study on the quality evaluation of aonla (amla) candy prepared from steep preserved fruits ranged from 6–8%, which were in agreement with the recent findings. The mouthfeel scores in the present study ranged from 5–6%, which showed similarities to the scores reported by Bhattacherjee *et al.* (2013). The maximum score was given to T_3 because of the combination of $FeSO_4$ and NaFeEDTA, as NaFeEDTA has the capability to mask the off-flavor produced by $FeSO_4$. The lowest score was given to T_2 because of bitter aftertaste imparted by $FeSO_4$.

Efficacy study

Hemoglobin

Hemoglobin level was found significantly increased among the children fed with fortified chewing gums (Figure 2). Maximum increase in hemoglobin (3.22%) was observed in children consuming chewing gums fortified with 100% NaFeEDTA, which ranged from 11.05 to 11.80 g/dL (Table 2) whereas an increase in control group was 0.96% during the study trial of 45 days. Minimum increase (2.31%) was noted in children fed with 100% $FeSO_4$-fortified chewing gums. Overall, a significant improvement in the hemoglobin level of subjects was observed during the study period. It is concluded from the present exploration that the consumption of iron-fortified chewing gums is helpful to increase blood hemoglobin levels in school-going children. This increase was due to iron fortification, which is the basic component of hemoglobin, so an increase in iron in diet is directly related to the serum hemoglobin level (Longfils *et al.*, 2008).

Hematocrit

Hematocrit percentage in school-going children revealed a significant increase after the consumption of iron-fortified chewing gums (Figure 2). This fortification induced improvement in hematocrit by up to 3.42% in children consuming chewing gums fortified with 100% NaFeEDTA that range from 35.92 to 38.12% as compared to the control group (0.98%). The least increase (1.03%) was found in the diet group fed with 100% $FeSO_4$-fortified chewing gums. It is concluded that iron-fortified chewing gums are helpful to increase hematocrit percentage in the blood. The results of hematocrit are related to the findings of hemoglobin. In another study, fortification was done using the micro-capsulated iron pyrophosphate (500 mL/d) providing 18 mg of iron in fruit juice and the influence was determined in menstruated women for eight weeks. Finding indicated that after the intervention, hematocrit increased by 3% as compared to control (Blanco-Rojo *et al.*, 2011). Similarly, in another study

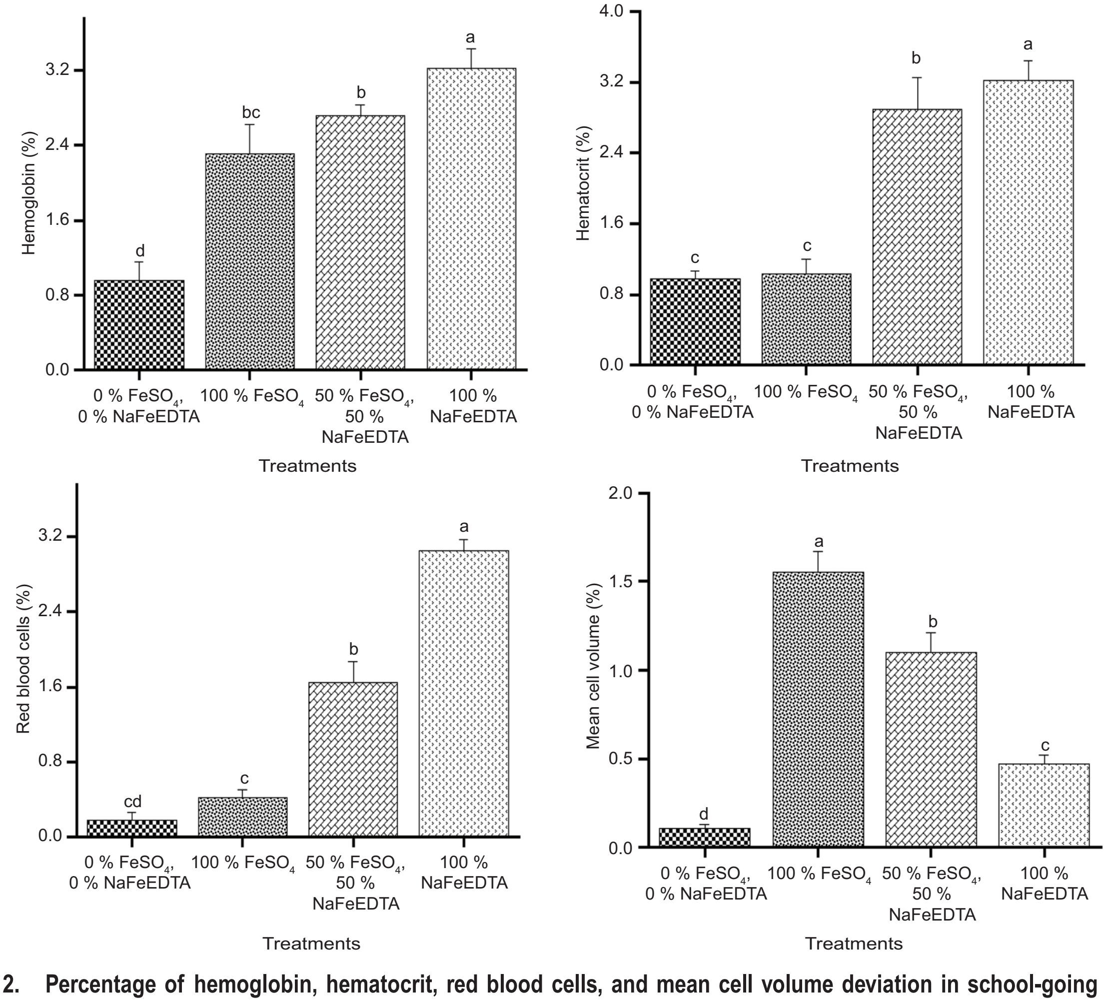

Figure 2. Percentage of hemoglobin, hematocrit, red blood cells, and mean cell volume deviation in school-going children influenced by the consumption of $FeSO_4$- and NaFeEDTA-fortified chewing gums.

hematocrit increased by 2% as compared to control after consumption of iron-fortified food (Patil *et al.*, 2013).

Red blood cells

Changes in red blood cell count in children who consumed iron-fortified chewing gums are shown in (Figure 2). This fortification induced the highest red blood cells (3.05%) in children consuming chewing gums fortified with 100% NaFeEDTA, which increased from 4.12 to 4.38 M/UI, followed by the control group (0.18%). The lowest increase in red blood cells (0.42%) was found in the group fed with 100% $FeSO_4$-fortified chewing gums. This increase in the levels of red blood cells is related to improvement in the levels of hemoglobin. In another study, the fortification was done using the micro-capsulated iron pyrophosphate (500 mL/d) by providing 18 mg of iron in fruit juice to menstruated women for four weeks. This resulted in increase of red blood cells by up to 2% as compared to the control group (Blanco-Rojo *et al.*, 2011).

Mean cell volume

Deviation in percentage values of MCV in school-going children is described in Figure 2. This fortification induced the highest MCV (1.55%) in children consuming chewing gums fortified with 100% NaFeEDTA that improved from 82.95 to 85.91 femtoliters (fL), followed by the control group (0.11%). The lowest increase in MCV (0.47%) was found in the diet group fed with 100% $FeSO_4$-fortified chewing gums. It is obvious from the present results that recent fortificants are effective in increasing the MCV. The results of MCV are directly related to hemoglobin because hemoglobin is an iron-based protein in red blood cells. In another study done in India, tolerability and cost of three iron fortificants, such as ferrous fumarate (100 mg), ferrous biglycinate (100 mg), and carbonyl iron (100 mg), among pregnant women were determined for 60 days; MCV concentration increased by 0.60% as compared to the control group (Patil *et al.*, 2013).

Mean Cell Hemoglobin

Percentage values of MCH in school-going children by the consumption of iron-fortified chewing gums are presented in Figure 3. This fortification induced the highest MCH (5.43%) in children consuming chewing gums fortified with 100% NaFeEDTA that range from 23.47 to 26.17 picograms (pg), followed by the control group (0.98%) (Table 2). The lowest increase in MCH (0.98%) was found in the group fed with 100% $FeSO_4$-fortified chewing gums. The findings of MCH are related to the findings of hemoglobin.

Total White Blood Cells Count

Percentage changes in TWBCC by the consumption of iron-fortified chewing gums by school-going children are shown in Figure 3. This fortification induced the highest TWBCC (7.98%) in children consuming chewing gums fortified with 50% $FeSO_4$ and 50% NaFeEDTA, followed by the control group (3.35%). The lowest increase in TWBCC (6.90%) was found in the diet group fed with 100% NaFeEDTA, followed by (6.90%) chewing gums fortified with 100% $FeSO_4$. It is obvious from the present findings that the consumption of iron-fortified chewing gums may be helpful in increasing TWBCC.

Platelet Count

Percentage changes in platelet count by consumption of iron-fortified chewing gums by school-going children are presented in Figure 3. This fortification induced the highest platelet count (4.40%) in children consuming chewing gums fortified with 100% NaFeEDTA that increased from 136.75 to 89.50 thousands per cubic milliliter (K/uL), followed by the control group (0.59%) (Table 2). The lowest increase in platelet count (0.61%) was found in the diet group fed with 100% $FeSO_4$ followed by the diet group fed with 50% $FeSO_4$ and 50% NaFeEDTA (2.3%). The results showed that fortificants were effective in increasing the platelet count in normal individuals. Furthermore, increase in platelet count is mainly done in the bone marrow and affected by alcohol, drugs, hepatitis, medication, and anemia. Current results are consistent with the finding of Kulnigg-Dabsch *et al.* (2012).

Serum Ferritin

The results of increase in serum ferritin in school-going children after consuming iron-fortified chewing gums are presented in Figure 3. This fortification induced the highest increase in serum ferritin (7.43%) in children consuming chewing gums fortified with 50% NaFeEDTA and 50% $FeSO_4$, followed by the control group (2.08%) (Table 2). The lowest increase in serum ferritin (6.52%) was found in the group fed with 100% NaFeEDTA-fortified chewing gums, followed by 100% $FeSO_4$-fortified chewing gums (5.3%). In another study, impact of different iron fortificants, such as NaFeEDTA (60 mg/kg) and $FeSO_4$ (20 mg/kg), in wheat were observed in anemic school children and concluded that concentration of serum ferritin increased significantly by 3% as compared to the control group (Huang *et al.*, 2009).

Iron

Percentage of iron consumption changes with the consumption of iron-fortified chewing gums in normal school-going children as shown in Figure 4. This fortification induced the highest percentage of iron (29.82%) in children consuming chewing gums fortified with 100% NaFeEDTA, followed by the control group (16.14%). The minimum increase in iron (23.47%) was found in the diet group fed with 50% NaFeEDTA- and 50% FeSO4-fortified chewing gums. It is clear from the result that iron-fortified chewing gums may be supportive in children fed

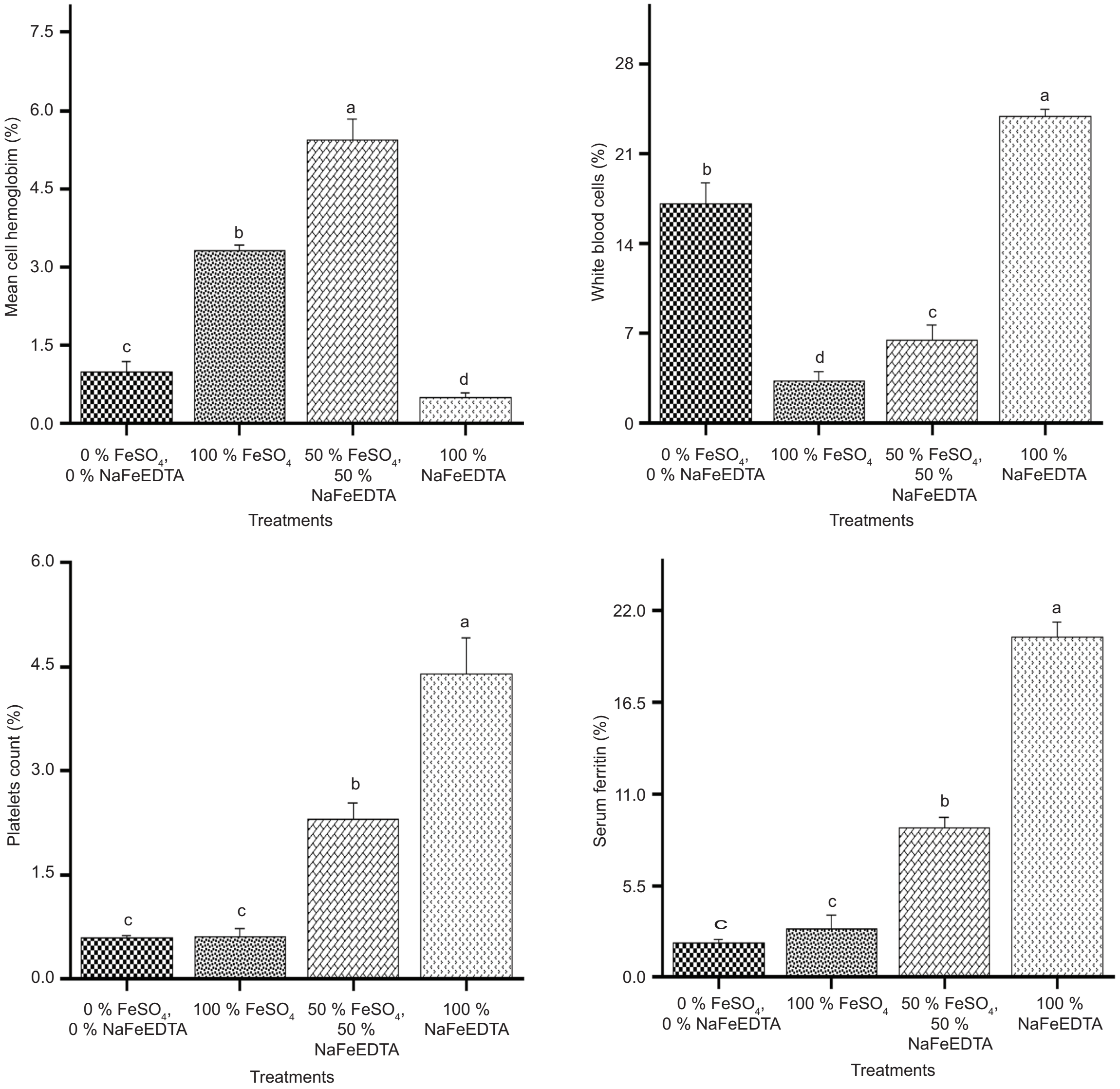

Figure 3. Percentage mean cell hemoglobin, total white blood cell count, platelets count, and serum ferritin deviation in school-going children affected by the consumption of $FeSO_4$- and NaFeEDTA-fortified chewing gums.

for enhancing iron consumption. The current findings were consistent with the findings of Ma *et al.* (2016).

Conclusion

Synthesized evidence revealed that consumption of iron-fortified chewing gums were found effective to improve hemoglobin, serum ferritin and iron status in school-going children aged of 6–10 years, and this is an inexpensive way to combat iron deficiency in low- to middle-income population. Chewing gums fortified with $FeSO_4$ and NaFeEDTA (1:1) obtained highest sensory scores. Proximate composition and texture showed nonsignificant differences ($P > 0.05$), except ash, which might be due to the involvement of different iron salts. Conclusively, the findings of the current study infer that chewing gums fortified with $FeSO_4$ and NaFeEDTA (1:1) possess significant potential to ensure nutritional security. The cost of chewing gums was calculated as PKR 2 per child per day. Diarrhea, widespread malabsorption, and enteropathy may be a barrier to this approach for the acquisition of maximum results. Further research in the future is needed to explain the mechanism underlying these outcomes along with rigorous randomized trials needed to resolve clinical and safety effectiveness.

Acknowledgments

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Table 2. Mean values of blood biochemical profile of school-going children.

Treatments	T_1		T_2		T_3		T_4	
Days	**0**	**45**	**0**	**45**	**0**	**45**	**0**	**45**
Hb (g/dL)	11.25 ± 0.32^{b}	11.47 ± 0.37^{a}	13.70 ± 0.26^{b}	14.35 ± 1.74^{a}	11.65 ± 0.26^{b}	12.30 ± 0.22^{a}	11.05 ± 0.50^{b}	11.80 ± 0.35^{a}
PCV (%)	34.32 ± 1.55^{b}	36.07 ± 0.86^{a}	42.85 ± 2.04^{b}	43.75 ± 0.17^{a}	34.37 ± 0.10^{b}	36.42 ± 1.44^{a}	35.72 ± 0.74^{a}	35.92 ± 0.74^{a}
RBC (M/uL)	5.27 ± 0.44^{a}	5.29 ± 0.53^{a}	4.70 ± 0.15^{a}	4.66 ± 0.08^{a}	4.17 ± 0.15^{b}	4.31 ± 0.13^{a}	4.12 ± 0.35^{b}	4.38 ± 0.43^{a}
MCV (fL)	90.40 ± 1.0^{a}	90.20 ± 0.80^{b}	83.20 ± 0.60^{b}	84.0 ± 0.30^{a}	71.50 ± 0.90^{b}	73.10 ± 0.10^{a}	82.95 ± 0.50^{b}	85.91 ± 0.40^{a}
MCH (pg)	26.87 ± 0.88^{b}	27.15 ± 0.16^{a}	29.75 ± 0.78^{a}	29.17 ± 0.45^{b}	27.15 ± 0.49^{b}	29.02 ± 0.63^{a}	23.47 ± 1.74^{b}	26.17 ± 0.10^{a}
TWBC (K/uL)	7.91 ± 0.21^{b}	8.46 ± 0.31^{a}	9.37 ± 0.70^{b}	10.70 ± 0.11^{a}	10.67 ± 0.80^{b}	12.52 ± 0.87^{a}	7.62 ± 0.70^{b}	8.75 ± 0.24^{a}
PLT (K/uL)	280.17 ± 1.80^{a}	276.83 ± 1.0^{b}	286.10 ± 1.10^{a}	285.75 ± 0.90^{b}	260.75 ± 0.71^{a}	249.0 ± 0.40^{b}	136.75 ± 0.79^{b}	189.50 ± 0.90^{a}
SF (ng/mL)	21.30 ± 0.15^{b}	22.57 ± 0.76^{a}	25.45 ± 0.90^{b}	28.35 ± 1.02^{a}	24.40 ± 1.83^{b}	28.32 ± 0.73^{a}	28.95 ± 0.34^{b}	32.97 ± 1.46^{a}
ESR (mm/h)	26.0 ± 15.03^{b}	28.17 ± 22.04^{a}	9.15 ± 4.12^{b}	10.57 ± 4.87^{a}	12.5 ± 4.15^{b}	20.0 ± 6.04^{a}	26.25 ± 3.39^{b}	51.42 ± 7.41^{a}
Fe (ug/dL)	88.8 ± 44.94^{b}	123.00 ± 23.54^{a}	60.85 ± 19.24^{b}	119.35 ± 30.98^{a}	51.75 ± 17.71^{b}	83.50 ± 20.33^{a}	53.92 ± 16.94^{b}	99.75 ± 8.25^{a}

Different letters in treatment (consecutive two rows) indicate significant differences ($P < 0.05$).
Hb: hemoglobin, PCV: hematocrit, RBC: red blood cells, MCV: mean cell volume, MCH: mean cell hemoglobin, TWBCC: total white blood cells count, PLT: platelet count, MCHC: mean cell hemoglobin concentration, SF: serum ferritin, ESR: erythrocyte sedimentation rate, Fe: iron.
T_1 = 0% $FeSO_4$ and 0% NaFeEDTA, T_2 = 100% $FeSO_4$; T_3 = 50% $FeSO_4$ and 50% NaFeEDTA; T_4 = 100% NaFeEDTA.

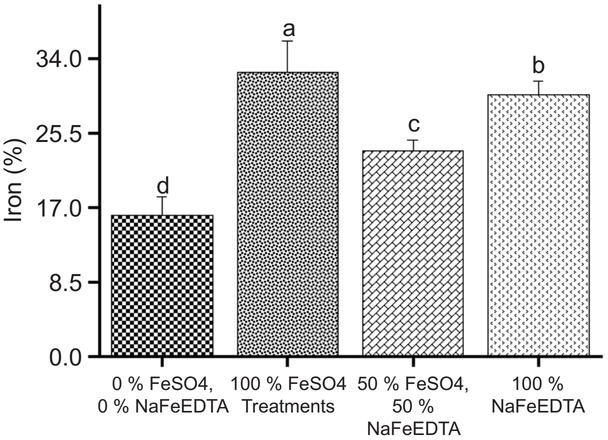

Figure 4. Percentage of iron deviation in school-going children affected by the consumption of $FeSO_4$- and NaFeEDTA-fortified chewing gums.

Conflict of Interest

The authors declared no conflict of interest.

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The antimicrobial activity of two phenolic acids against foodborne *Escherichia coli* and *Listeria monocytogenes* and their effectiveness in a meat system

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PAPER

Abstract

Ready-to-eat meats are susceptible to pathogenic contamination during their production, distribution, and sale. This study evaluated the antimicrobial effects of two phenolic acids (caffeic and ferulic acids) against foodborne pathogens in cold-cut meat at low-temperature conditions. The individual and combined antibacterial activities of caffeic and ferulic acids against *Escherichia coli* O157:H7 ATCC 43888 and *Listeria monocytogenes* ATCC 7644 were determined by diffusion disk assay in broth media and cold-cut meat. Broth media and meat samples already inoculated with *E. coli* and *L. monocytogenes* were treated with caffeic acid, ferulic acid, and their combination at the concentrations of 150 ppm and 200 ppm and stored at 4°C. Microbial growths were monitored at 0, 24, 48, and 72 h. Caffeic acid at 200 ppm exhibited a zone of inhibition of 12.33 mm on *E. coli*, and ferulic acid revealed a zone of inhibition of 11.00 mm on *L. monocytogenes*. The combination of caffeic-ferulic acid at a concentration of 200 ppm was most effective against *E. coli*, demonstrating a synergistic effect over 72 h at 4°C in both broth media and meat. For meat samples, the combination of caffeic acid and ferulic acid exhibited a log reduction of 3.63 CFU/g at 150 ppm and 2.51 CFU/g at 200 ppm against *E. coli* O157:H7 at the end of cold storage. Caffeic acid alone exhibited an overall log reduction of 2.48 CFU/g at 150 ppm and 2.75 CFU/g at 200 ppm against *L. monocytogenes*. These results indicate the ability of caffeic and ferulic acids, individually and in combination, to reduce pathogenic contamination and improve safety of cold-cut meats.

Keywords: antibacterial activity; caffeic acid; ferulic acid; cold-cut meat; *E. coli*; *L. monocytogenes*

Introduction

Cold-cut meat products such as ham are one of the most commonly consumed ready-to-eat products. They are often in sliced forms and used as fillings for sandwiches and similar foods. They are manufactured from raw pork and/or beef and characterized by physical and biochemical changes that occur during curing (Marušić *et al.*, 2014). In most modern cold-cut meats, nitrites (either sodium nitrite or potassium nitrite) are employed during curing to prevent bacterial growth and survival and enhance safety and storage stability. However, owing to the toxicity of nitrites, some country regulations specify maximum allowable contents in the final product (Campion *et al.*, 2017). This indulges public health concern because, under certain conditions, nitrites in meat can react with degradation products of amino acids, forming nitrosamines, which are known carcinogens

(Shpazier *et al.*, 2018). Furthermore, foodborne pathogenic bacteria may persist and replicate in cold-cut meats due to their low effective doses, antimicrobial resistance, and capability of adapting to stress conditions without observable sensorial changes in the product (Rybarczyk *et al.*, 2017).

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacterium that thrives in water, soil, and foods (Chen *et al.*, 2017). It is the causative organism for listeriosis and has the potential to contaminate different forms of foods, including low-moisture foods, at different stages within the food chain. It has been implicated in many foodborne disease outbreaks, and some of the most common vehicles involved in these outbreaks include ready-to-eat meals, unpasteurized milk, dairy products, meat, fruits, and vegetables (Pernin *et al.*, 2019). Ingestion of foods contaminated with *L. monocytogenes* could result in different symptoms from mild gastroenteritis to severe central nervous system infections. High-risk individuals prone to *L. monocytogenes* infection include pregnant women and immunocompromised people (Hunjak *et al.*, 2019). *Escherichia coli* (*E. coli*) is also a Gram-negative, rod-shaped, ubiquitous bacterium, which also lives in the gut of humans and suppresses the growth of harmful bacteria (Saxena *et al.*, 2015). One of its pathogenic strains, *E. coli* O157:H7, is listed by the United States Centers for Disease Control (CDC) as a pathogen that has attracted increasing attention due to the number of victims who had to be hospitalized following its ingestion (*De Souza et al.*, 2018). The CDC estimated that in the US, there were 10,000 illnesses, thousands of hospitalizations, and hundreds of deaths attributed to the *E. coli* O157:H7 yearly. Most *E. coli* O157:H7 outbreaks are associated with fecal-oral transmission due to poor hygiene and food handling practices. Sources of human infection include poorly cooked meat, apple juice, water, and milk products such as yogurt and unpasteurized milk. These bacteria have been adjudged the most threatening foodborne pathogens because of their severe debilitating effects on humans upon ingestion (Buchanan *et al.*, 2017).

Although various measures have been implemented in the past to control and reduce the prevalence of *L. monocytogenes* and *E. coli* O157:H7, reports of foodborne disease outbreaks as a result of their infection subsists. Chemical preservatives are capable of inhibiting or inactivating bacterial growth in foods. However, the use of natural preservatives is currently encouraged due to increasing bacterial resistance to chemical preservatives, food safety regulations, and increased awareness of the adverse effects of chemicals (Pernin *et al.*, 2019). Phenolic compounds have attracted much interest due to their antioxidant potential and antimicrobial properties. Phenolic compounds are secondary metabolites produced by plants and can be classified into three different groups based on their chemical structures. These classes include non-flavonoids, flavonoids, and tannins in their simple or complex forms (Pernin *et al.*, 2018). In plant-based foods, phenolic compounds are responsible for the color of red fruits, juices, and wines, substrates involved in enzymatic browning and are also considered to contribute to the health benefits associated with dietary consumption of fruits and vegetables (Cheynier, 2012).

In particular, caffeic and ferulic acids possess aromatic rings with a hydroxyl group (-OH) on the fifth position of their rings while their difference is in the presence of methoxy group ($-H_3CO$) for ferulic acid and hydroxyl group (-OH) at the sixth position of aromatic rings (Sanchez-Maldonado *et al.*, 2011). They are well distributed in plants, and they possess antioxidant and prooxidant properties (Nystrom *et al.*, 2005) substantiated by their functions in the reduction of auto-oxidation by acting as radical scavengers, inhibitors of lipid peroxidation, and low-density lipoproteins (Maurya & Devasagayam, 2010). Their antioxidant and prooxidant abilities in foods have been adduced to factors such as concentration, chemical structure, and the nature of substrates or foods (Maurya & Devasagayam, 2010).

Previous studies have validated the antimicrobial properties of phenolic compounds against bacteria, including pathogenic species. These findings justified that their use could be an alternative means of inhibiting or inactivating the growth of *L. monocytogenes* and *E. coli* (Vaquero *et al.*, 2007). When combined, phenolic acids could reflect greater antimicrobial efficacy against foodborne pathogens, compared to individual phenolic acids. Therefore, this study was conducted to investigate the individual and combined antimicrobial effects of caffeic and ferulic acids on the survival of *L. monocytogenes* ATCC 7644 and *E. coli* O157:H7 ATCC 43888 in cold-cut meat products under low-temperature storage conditions.

Materials and Methods

Preparation of inoculum

The bacterial strains *L. monocytogenes* ATCC 7644 and *E. coli* O157:H7 ATCC were obtained from the microbial culture collection of the Department of Biotechnology and Food Technology, Durban University of Technology. Stock cultures of *L. monocytogenes* were grown aerobically for 24 h at 30°C in brain heart infusion (BHI) broth. For *E. coli*, stock cultures were grown at 37°C for 24 h in tryptic soy broth (TSB). A loopful of the stock culture of each microorganism was transferred to 10 mL of BHI and TSB, as applicable, and incubated at 37°C for

24 h. Turbidity of cultures was adjusted to match that of a 0.5 McFarland standard to achieve an approximate 10^8 CFU/mL in each case.

Preparation of phenolic acid extracts

Caffeic acid and ferulic acid (>98% purity) were purchased from Sigma-Aldrich (Johannesburg, South Africa). To elucidate the antimicrobial properties, these phenolic compounds were dissolved in 99.8% ethanol (Merck, Johannesburg, South Africa). However, for broth and meat experiments, the phenolic acids were dissolved in water at 1% w/v (Zhang *et al.*, 2016). The solutions were filtered through 0.22-μm membrane filters in each case and stored at 4°C in sterilized glass containers until needed.

Antibacterial activity determination

For agar disk diffusion assay, 0.1 mL of each bacterial culture suspension was transferred into Petri dishes containing Mueller Hinton agar (Merck, Johannesburg, South Africa) and were uniformly spread onto the surface with the help of a sterile plate spreader. Fifty microliters of phenolic acids solutions, at different concentrations (150 and 200 ppm), were pipetted on 6 mm sterile filter paper disks (Whatman No. 1) and air-dried in a laminar flow chamber. Distilled water was used as the negative control, while Ciprofloxacin (Merck, Johannesburg, South Africa) was used as the positive control for its effectiveness against both Gram-positive and Gram-negative bacteria. These dried disks were then transferred into inoculated plates and incubated at 30°C for 24 h for *L.monocytogenes* and 37°C for *E. coli*. Antibacterial activity for each phenolic acid and their combination was determined by measuring the inhibition zone around the disk following a 24-hour incubation period. Each experiment was replicated at least three times.

Broth study

In each case, 1 mL bacterial suspension containing $1x10^8$ CFU/mL *E. coli* and *L. monocytogenes* were transferred into either BHI broth for *L. monocytogenes* or TSB broth for *E. coli* O157:H7. Thereafter, 50 μL of individual solutions of caffeic and ferulic acid of 150 ppm and 200 ppm concentration as well as a combination (1:1) of caffeic and ferulic acid solutions (150 ppm:75 ppm of each and 200 ppm:100 ppm of each) were added to inoculated broths. An inoculum containing a bacterial suspension of $1x10^8$ CFU/mL of each bacterium that was not treated by the phenolic acids served as the control samples. The samples were then stored at 4°C for 72 h. Microbial survival in each broth treatment was determined at 12, 24, 48, and 72 h by pour-plating six serial dilutions on appropriate growth agar. Plates were incubated at 37°C for 24 h before enumeration. Each experiment was repeated at least thrice.

Meat study

Cold-cut meat samples were bought from a trusted retail outlet and sliced into 5 g portions. For sterilization, the samples were dipped into 95% ethanol solution and left to dry in a laminar flow chamber. Afterward, 0.1 mL of each bacterial suspension was spot-inoculated onto the meat samples and spread out onto the surface of the meat using sterile spreader. Inoculated meat samples were then dried for 1 h at room temperature. Thereafter, samples were dipped in phenolic acid solutions containing a concentration of 150 ppm and 200 ppm of each phenolic acid and their combination (1:1) for 60 s. Broth inoculum containing a bacterial suspension of $1x10^8$ CFU/mL for each strain was used as the control. The samples were stored at 4°C for a 72-hour period in sterilized stomacher bags containing phosphate buffer solution (PBS). Microbial survival was also evaluated at 12, 24, 48, and 72 h. Plates were incubated at 37°C for 24 h prior to enumeration. Each experiment was repeated at least three times.

Statistical analysis

All experiments were performed in triplicate. Experimental data were analyzed by ANOVA (Analysis of Variance) ($P<0.05$). The mean values of the experimental growth data were compared using Duncan's multiple range test.

Results and Discussion

Antibacterial activity of phenolic acids using disk diffusion assay

The effects of caffeic acid, ferulic acid, and their combination on the growth of *L. monocytogenes* and *E. coli* O157:H7 are shown in Table 1. They demonstrated various degrees of inhibition against the two pathogenic strains. Ferulic acid was found to be most effective against the *E. coli* O157:H7 strain at 150 ppm concentration, with a zone of inhibition of 10 mm, compared to caffeic acid and their combination at the same concentration. There was no difference ($P<0.05$) in the inhibitory actions of caffeic acid and the combination of caffeic and ferulic acids at this same concentration (150 ppm). With an increase in the ferulic acid concentration to 200 ppm, the zone of inhibition was found to increase further. For *L. monocytogenes*, caffeic acid was the most effective antibacterial agent at the two concentrations tested, giving zones of inhibition of 7.66 and 11 mm at 150 and 200 ppm, respectively. The structure-function relationships between the antimicrobials and the respective microorganisms could justify the contrasting activities of the phenolic acids against Gram-positive and Gram-negative bacteria (Sánchez-Maldonado *et al.,* 2011). Distilled water did not inhibit the growth of organisms as no inhibition zones was detected during screening. Ciprofloxacin, used as the positive control, demonstrated greater inhibitory potential for *L. monocytogenes,* possibly due to differences in the cell wall structures of Gram-positive and Gram-negative bacteria. The combination of caffeic acid and ferulic acid exhibited a joint effect at both concentrations, suggesting an affinity relationship between the two antimicrobials. However, they tend to possess a greater antimicrobial activity individually than in combination.

Inhibitory effect of phenolic acids against *E. coli* O157:H7 and *L. monocytogenes* in broth

The growth response of *E. coli* O157:H7 and *L. monocytogenes* in broth media treated with phenolic acids at 150 or 200 ppm and stored at 4°C for a 72 h period is presented in Table 2. The high levels of *L. monocytogenes* and *E. coli* inocula in broth and meat samples in this study are necessary to clearly understand the efficiency of the different concentrations of phenolic acids and the extent of their activity in highly contaminated food materials. Inoculum levels excess of 10^7–10^9 have been used by previous authors (De Souza *et al.,* 2018; Kwon *et al.,* 2019; Rodriguez-Vaquero *et al.,* 2007). In the control medium, the number of viable cells of *E. coli* O157:H7 increased from 7.70×10^8 to 8.01×10^8 CFU/mL at the end of the incubation period (72 h). For *L. monocytogenes*, the number of viable cells in the control medium increased from 7.87×10^8 after 12 h to 8.21×10^8 at the end of the incubation period. At a concentration of 150 ppm, ferulic acid was the most effective antimicrobial agent against *E. coli* O157:H7, showing a reduction of 2.98 log CFU/mL, while caffeic acid was the most effective against *L. monocytogenes* with a log reduction of 2.17 log CFU/mL following the incubation period. The combination of caffeic acid and ferulic acid in a 1:1 ratio demonstrated a synergistic effect whereby the viability of microorganisms diminished by 2.42 and 2.14 log cycles, respectively. At a concentration of 200 ppm, the order of effectiveness remained the same for both Gram-positive and Gram-negative strains; however, a higher inhibition rate was observed (Table 2) – a reduction of 3.49 log CFU/mL against *E. coli* O157:H7 by ferulic acid and 2.35 log CFU/mL against *L. monocytogenes* by caffeic acid. The synergistic effect of the combination of caffeic acid and ferulic acid enhanced the reduction of viability of microorganisms by 2.42 and 2.25 log CFU/mL (at 150 ppm) as well as 3.14 and 2.62 log CFU/mL for *E. coli* and *L. monocytogenes*, respectively. In a previous study, the combination of caffeic acid and gallic acid, at 100 ppm and 200 ppm solution produced 1 log CFU/mL and 2 log CFU/mL reduction of *L. monocytogenes*, respectively, in meat (Rodriguez-Vaquero *et al.,* 2011). In broth media, phenolic acids were less effective against *L. monocytogenes.* A possible explanation could be that the microorganism can proliferate under refrigeration conditions, allowing it to be more adaptable to the stress imposed by phenolic acids.

Inhibitory effect of phenolic acids against *E. coli* O157:H7 and *L. monocytogenes* in cold-cut meats

Table 3 illustrates the growth response of *E. coli* O157:H7 and *L. monocytogenes* in cold-cut meats treated with phenolic acids and stored at 4°C for over 72 h. The number of viable cells in the control samples increased from 7.49×10^8 to 7.75×10^8 CFU/mL for *E. coli* O157:H7 and

Table 1. Antibacterial activity of phenolic compounds against *E. coli* O157:H7 and *L. monocytogenes* determined by disk diffusion assay.

Treatments	Concentration	Zone of inhibition (mm) Organism	
		E. coli O157:H7 (ATCC 43888)	*L. monocytogenes* (ATCC 7644)
Caffeic acid	150 ppm	7.00 ± 1.73^b	7.66 ± 2.51^b
	200 ppm	9.33 ± 1.15^a	11.00 ± 3.00^a
Ferulic acid	150 ppm	10.00 ± 2.00^b	6.00 ± 1.72^b
	200 ppm	12.33 ± 2.51^a	8.33 ± 2.52^a
Combination (1:1)	150 ppm	7.00 ± 1.73^b	6.67 ± 1.15^b
	200 ppm	8.67 ± 1.16^a	7.66 ± 2.51^a
Control	Positive	26.67 ± 2.88^a	36.67 ± 2.89^a
	Negative	ND	ND

Superscripts indicate significant differences (P < 0.05) across the columns only for each phenolic acid and concentration.

Table 2 The effect of phenolic compounds (at 150 ppm and 200 ppm) against *E. coli* O157:H7 and *L. monocytogenes* at 4°C for over 72 h in a broth medium.

Phenolic acids (150 ppm)	**Population (log CFU/mL) over storage period (h) of**			
	12	**24**	**48**	**72**
***E. coli* O157:H7 (ATCC 43888)**				
Control	7.70 ± 0.04^{a}	7.80 ± 0.04^{a}	7.92 ± 0.06^{a}	8.01 ± 0.04^{a}
Caffeic acid	6.08 ± 0.07^{b}	5.73 ± 0.05^{b}	5.71 ± 0.05^{b}	5.63 ± 0.06^{b}
Ferulic acid	5.89 ± 0.03^{c}	5.57 ± 0.03^{b}	5.55 ± 0.05^{b}	5.03 ± 0.05^{b}
Combination (1:1)	6.01 ± 0.03^{b}	5.70 ± 0.13^{b}	5.61 ± 0.03^{b}	5.59 ± 0.07^{b}
***L. monocytogenes* (ATCC 7644)**				
Control	7.87 ± 0.03^{a}	8.08 ± 0.05^{a}	8.17 ± 0.04^{a}	8.21 ± 0.03^{a}
Caffeic acid	6.24 ± 0.02^{b}	6.13 ± 0.02^{b}	6.08 ± 0.03^{b}	6.04 ± 0.05^{b}
Ferulic acid	6.36 ± 0.03^{b}	6.18 ± 0.02^{b}	6.14 ± 0.03^{b}	6.11 ± 0.03^{b}
Combination (1:1)	6.33 ± 0.03^{b}	6.18 ± 0.03^{b}	6.12 ± 0.02^{b}	6.07 ± 0.04^{b}
Phenolic acids (200 ppm) ***E. coli* O157:H7 (ATCC 43888)**				
Control	7.70 ± 0.04^{a}	7.80 ± 0.04^{a}	7.92 ± 0.06^{a}	8.01 ± 0.04^{a}
Caffeic acid	6.30 ± 0.02^{b}	6.27 ± 0.02^{b}	5.34 ± 0.01^{b}	5.29 ± 0.02^{b}
Ferulic acid	6.29 ± 0.03^{b}	6.21 ± 0.02^{b}	4.85 ± 0.04^{c}	4.52 ± 0.06^{c}
Combination (1:1)	6.33 ± 0.05^{b}	6.26 ± 0.03^{b}	4.97 ± 0.04^{c}	4.87 ± 0.04^{c}
***L. monocytogenes* (ATCC 7644)**				
Control	7.87 ± 0.03^{a}	8.08 ± 0.05^{a}	8.17 ± 0.04^{a}	8.21 ± 0.03^{a}
Caffeic acid	6.19 ± 0.02^{b}	6.01 ± 0.03^{b}	5.92 ± 0.06^{b}	5.86 ± 0.08^{b}
Ferulic acid	6.24 ± 0.03^{b}	6.09 ± 0.03^{b}	6.03 ± 0.05^{b}	6.01 ± 0.02^{b}
Combination (1:1)	6.22 ± 0.02^{b}	6.05 ± 0.03^{b}	6.01 ± 0.04^{b}	5.96 ± 0.03^{b}

Superscripts indicate significant differences (P<0.05) across the columns only for each phenolic acid used.

7.51x10^{8} to 7.96x10^{8} CFU/mL for *L. monocytogenes*. At a concentration of 150 ppm, the combination of caffeic acid and ferulic acid was found to be most effective against *E. coli* O157:H7 in comparison to caffeic acid, and ferulic acid was applied individually, with a log reduction of 3.38 CFU/mL at 72 h. As observed in broth media, caffeic acid revealed the greatest effects against *L. monocytogenes* in cold-cut meats with a log reduction of 2.44 CFU/mL at the maximum incubation period. At a concentration of 200 ppm, a similar pattern of effectiveness was documented for both Gram-positive and Gram-negative strains, with a higher inhibition rate (Table 3). The survival rate was lower for both strains, indicating that the synergistic effect of the combination of caffeic acid and ferulic acid was more effective in the meat compared to the broth. This could be attributed to the presence of macromolecules (proteins, lipids) and micromolecules (vitamins) in the food matrix (meat), which provided enhanced affinity for phenolic compounds. Arima *et al.* (2002) reported that the combinations of quercetin and quercitrin, quercetin and morin, and quercetin and rutin portrayed synergistic effects resulting in improved efficacy than individual flavonoids against *Bacillus cereus* and *Salmonella enteritidis*.

The inhibitory effect of phenolic compound mixtures was greater at 4°C incubation temperature than at 20°C for meat (Rodriguez-Vaquero *et al.*, 2011) and fish (Rodríguez-Vaquero *et al.*, 2013), reducing the viability of *L. monocytogenes* at two concentrations (100 and 200 mg/L). This is because their mode of action depends on their migration into bacterial membranes, which reduce fluidity at lower temperatures (Ultee *et al.*, 2000). Beuchat *et al.* (1994) substantiated the improved antibacterial effect of phenolic compounds at low storage conditions. The shelf lives of foods could be further preserved by hurdle technology, such as in the case of combining refrigeration temperatures between 0°C and 4°C with modified atmosphere packaging to preserve foods (Leistner & Gorris, 1995).

Conclusions

Caffeic acid, ferulic acid, and their combination have potentials for the effective reduction or inhibition of foodborne pathogens; thus, providing a good alternative to chemical additives. Zones of inhibition of *E. coli* and *L. monocytogenes* were widened with an increase in the

Table 3 **The effect of phenolic compounds (at 150 ppm) against *E. coli* O157:H7 and *L. monocytogenes* at 4°C for over 72 h in meat.**

Phenolic acids (150 ppm)	Population (log CFU/g) over storage period (h) of			
	12	24	48	72
***E. coli* O157:H7 (ATCC 43888)**				
Control	7.49 ± 0.06^{a}	7.70 ± 0.05^{a}	7.76 ± 0.06^{a}	7.75 ± 0.11^{a}
Caffeic acid	5.67 ± 0.04^{b}	5.51 ± 0.04^{b}	5.22 ± 0.05^{b}	4.98 ± 0.05^{b}
Ferulic acid	5.49 ± 0.02^{c}	5.31 ± 0.04^{c}	5.06 ± 0.08^{b}	4.51 ± 0.11^{b}
Combination (1:1)	5.42 ± 0.03^{c}	5.21 ± 0.04^{c}	4.91 ± 0.02^{b}	4.37 ± 0.04^{c}
***L. monocytogenes* (ATCC 7644)**				
Control	7.51 ± 0.05^{a}	7.71 ± 0.05^{a}	7.87 ± 0.04^{a}	7.96 ± 0.02^{a}
Caffeic acid	6.29 ± 0.02^{c}	6.09 ± 0.03^{b}	5.76 ± 0.03^{b}	5.52 ± 0.04^{b}
Ferulic acid	6.52 ± 0.02^{b}	6.39 ± 0.02^{b}	6.12 ± 0.03^{b}	5.97 ± 0.06^{b}
Combination (1:1)	6.41 ± 0.03^{c}	6.27 ± 0.08^{b}	5.99 ± 0.03^{b}	5.68 ± 0.04^{b}
Phenolic acids (200 ppm) ***E. coli* O157:H7 (ATCC 43888)**				
Control	7.49 ± 0.06^{a}	7.70 ± 0.05^{a}	7.76 ± 0.06^{a}	7.75 ± 0.11^{a}
Caffeic acid	5.34 ± 0.08^{b}	5.21 ± 0.05^{b}	4.94 ± 0.07^{b}	4.67 ± 0.08^{b}
Ferulic acid	5.24 ± 0.05^{b}	5.10 ± 0.02^{b}	4.76 ± 0.08^{b}	4.23 ± 0.04^{c}
Combination (1:1)	5.19 ± 0.02^{b}	5.07 ± 0.06^{b}	4.66 ± 0.08^{b}	4.09 ± 0.02^{c}
***L. monocytogenes* (ATCC 7644)**				
Control	7.51 ± 0.05^{a}	7.71 ± 0.05^{a}	7.87 ± 0.04^{a}	7.96 ± 0.02^{a}
Caffeic acid	6.02 ± 0.04^{b}	5.63 ± 0.02^{b}	5.52 ± 0.05^{b}	5.25 ± 0.02^{c}
Ferulic acid	6.19 ± 0.02^{b}	5.77 ± 0.04^{b}	5.74 ± 0.04^{b}	5.63 ± 0.04^{b}
Combination (1:1)	6.12 ± 0.03^{b}	5.77 ± 0.04^{b}	5.68 ± 0.03^{b}	5.49 ± 0.02^{c}

Superscripts indicate significant differences ($P<0.05$) across the columns only for each phenolic acid used.

concentration of phenolic acids, with ferulic acid having the greatest inhibition effect on *E. coli* at 200 ppm. For the two concentrations tested, phenolic acids were more efficacious against *E. coli* in broth and meat as the storage hours increased, compared to *L. monocytogenes.* However, the greater effects were obtained at 200 ppm for the two phenolic acids and their combination for both microorganisms. These results show the effectiveness of phenolic acids tested, at the stipulated concentrations, against the pathogenic organisms investigated and, as such, may allow the formulation of new antimicrobial products for potential use as food preservatives. Further studies involving sensory evaluation may be necessary to determine consumer acceptability of caffeic and ferulic acid-treated meats.

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Chemical characterization of ‘Pecorino Di Farindola’ cheese during ripening

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PAPER

Abstract

This study evaluated the nutritional and sensorial characteristics of Pecorino di Farindola cheese at different commercial ripening stages. Moreover, in order to assess effectively the peculiar features of this product, the evolution of proteolysis and lipolysis, together with that of free amino acids (FAAs), was studied throughout ripening. A marked proteolysis of Pecorino di Farindola was found. At the end of ripening, FAAs with the highest content were glutamic acid, valine, leucine and lysine. Long-ripened cheeses had a light spicy feature that distinguishes them from other Italian Pecorino cheeses.

Keywords: free amino acids, lipolysis, Pecorino di Farindola, pig rennet

Introduction

The term ‘Pecorino’ refers to a cheese obtained from ewe milk, in most cases of protected origin. In Italy, however, several cheeses, even not of protected origin, have typical features and are prepared in limited geographical areas (Coda *et al.*, 2006). Pecorino cheeses are often produced traditionally in central and south Italy and are characterised by different ripening stages (Di Cagno and Gobbetti, 2011; Schirone *et al.*, 2011), and the use of different milk, rennet and technology of production (Gobbetti, 2016). ‘Pecorino di Farindola’ is a traditional food product (Prodotto Agroalimentare Tradizionale – PAT, published in the Official Gazette of the Italian Republic on 20th February 2020, General series n. 42 , Ordinary supplement n. 9) and has a limited production in the eastern part of the Gran Sasso area, Abruzzo, Italy. This is an original Pecorino cheese, since it is made from pig rennet that gives the cheese particular flavours and taste. The milk comes from sheep of Pagliarola Appenninica breed, which are bred in the wild, with a limited milk production. The cheese can be found at a short ripening time (3 months), demonstrating a soft texture and a yellow crust, or at a long ripening time (over 12 months), having a harder texture, a more intense and spicy flavour and a darker crust (Schirone *et al.*, 2011). The cheese-making process is reported by Schirone *et al.* (2011). It starts from raw milk without the addition of natural cultures or selected starters, added with porcine rennet. The microbiota, derived mostly from mesophilic lactobacilli coming from the raw milk and the cheese-making environment, plays an important role during ripening, contributing to the development of typical aromas of this cheese (Aquilanti *et al.*, 2007; De Angelis *et al.*, 2001; Tofalo *et al.*, 2015). The chemical and microbiological features of 10 Pecorino di Farindola cheeses coming from different dairies of the Consortium, at 90 days of ripening, are reported by Schirone *et al.* (2011). Information about the chemical and microbiological characteristics of Pecorino di Farindola, together with that of proteolytic and lipolytic phenomena during its ripening process is still limited (Di Giacomo *et al.*, 2013; Suzzi *et al.*, 2014; Tofalo *et al.*, 2015). The aim of this work is to have a deeper insight into the chemical and nutritional characterization of the Pecorino di Farindola cheese and to evaluate its proteolysis and lipolysis at different ripening stages.

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Materials and Methods

Sample collection and preparation

Three batches of Pecorino di Farindola cheeses were analysed. The three batches were different according to ripening times: three samples were ripened for 3 months (3R), three for 5 months (5R) and three for 12 months (12R). Samples came from a dairy in the area of Pecorino of Farindola, which includes nine towns located within the provinces of Pescara and Teramo (reported in the 'Production Disciplinary of Pecorino di Farindola'- https://www.pecorinodifarindola.it/disciplinare/). All cheeses were produced following the cheese-making phases stated in the production disciplinary. The 3- and 5-month cheeses were produced during spring and summer, respectively, while the 12-month ripened samples in the autumn season. Cheeses were grounded and carefully mixed. Three bulk samples were prepared by combining the samples of each ripening month and stored at –20°C until analysis.

Chemical–physical analysis

For each ripening stage, each sample was analysed in triplicate. Cheese samples were analysed, following the international methods of Association of Official Analytical Chemists (AOAC, 2000), for fat (method: 933.05), protein (method: 920.123), moisture (method: 948.12) and ash (method: 935.42). Proteolysis was assessed by determining the content of water-soluble nitrogen (SN) and non-protein nitrogen (NPN), as done in Niro *et al.* (2014). The amount of SN and NPN, expressed as a percentage of total nitrogen (TN) (SN/TN% and NPN/TN%) indicates the extent of proteolysis. The nitrogen content was determined by the Kjeldahl method (AOAC, 2000; method: 920.123). Free amino acids (FAAs) were analysed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK), with a Li-cation-exchange column (20 × 0.46 cm). A mixture of basic, acid and neutral amino acid (AA) of a known concentration (Sigma Chemical Co., St. Louis, MO) was used as standards. The FAA extraction procedure is reported in Niro *et al.* (2017a). Lipolysis was expressed as Acid Degree Value (ADV) (Deeth and Fitz-Gerald, 1976).

Sensorial analysis

Each sample was evaluated for three times. Samples were evaluated by a panel comprising 10 trained components. Sensory evaluation was conducted according to the Etana method described by Bozzetti *et al.* (2004) modified by Chiavari *et al.* (2006). The evaluated attributes were flavour and aroma: odour intensity, aroma intensity, hardness, solubility, sweet, salty, bitter, spicy and acidic. The definition of the descriptive attributes is reported in Niro *et al.* (2014). Samples were served at room temperature. The intensity of each attribute was rated on an increasing scale from 1 to 10 (from absence to maximum).

Statistical analysis

An ANOVA was applied to the data. Least significant differences were obtained using the least significant difference test ($P < 0.05$).

Results and Discussion

The chemical composition of different Pecorino cheeses (g/100 g fresh weight), at different ripening stages, is reported in Table 1. The moisture value ranged from 29.6% in 3R samples to 28.5% in 5R and 23.2% in 12R samples. Significant differences ($P < 0.05$) were found in proteins and fats, depending on different raw milk, which, in 3R and 5R samples, came from grazing sheep, while in 12R samples from sheep fed with forage. Different ash values could depend on the variability of salting process. The cheese composition is in accordance with the values reported in the 'Production Disciplinary' and the ranging values are from literature for Pecorino di Farindola (Bellocci *et al.,* 2018). Schirone *et al.* (2011) and Tofalo *et al.* (2015) have reported lower fat values in several Pecorino di Farindola cheeses. Different Pecorino cheeses were found to have similar or higher fat contents (Coda *et al.*, 2006; Di Cagno *et al.*, 2007).

The proteolysis and lipolysis indices are shown in Table 2. The value of SN/TN% increased significantly during ripening, ranging from 32.5% at 3 ripening months (3R)

Table 1. Proximal composition of Pecorino di Farindola cheeses at different ripening times (g/100-g fresh weight) (mean ± S.D).

Ripening time	Moisture	Proteins	Fats	Ash
3 months	29.6 ± 0.04^{a}	25.7 ± 0.29^{a}	39.0 ± 0.09^{a}	4.1 ± 0.03^{a}
5 months	28.5 ± 0.03^{b}	27.7 ± 0.16^{b}	36.3 ± 0.12^{b}	5.4 ± 0.02^{b}
12 months	23.2 ± 0.08^{c}	31.6 ± 0.27^{c}	40.1 ± 0.27^{c}	5.0 ± 0.00^{c}

Different letters within the same column indicate a significant difference ($P < 0.05$).

Table 2. Proteolysis and lipolysis indices of Pecorino di Farindola cheeses at different ripening times (mean ± SD).

Ripening time	Proteolysis indices		Lipolysis index
	SN/TN%	NPN/TN%	ADV (meq KOH/100-g fat)
3 months	32.5 ± 0.75^{a}	12.1 ± 0.61^{a}	2.9 ± 0.34^{a}
5 months	33.7 ± 1.91^{a}	13.8 ± 1.68^{b}	4.0 ± 0.23^{b}
12 months	37.6 ± 0.12^{b}	19.6 ± 0.24^{c}	7.0 ± 0.05^{c}

Different letters within the same column indicate a significant difference ($P < 0.05$).

to 37.6% at 12 ripening months (12R). This value is an indicator of hydrolysis of casein caused by the action of rennet and milk proteases present at the beginning of the ripening process. The SN is very variable for composition, including high-, medium-, low-molecular weight peptides and AAs. Moreover, a significant part of SN is produced during curd acidification and, consequently, it is partly lost into the water or brine (Alichanidis and Polychroniadou, 2008). The breaking off of casein and high- and medium-molecular mass peptides by microorganisms, endogenous enzymes and rennet into low-molecular mass peptides and AAs, which are soluble in 12% trichloroacetic acid (TCA), is expressed by the NPN/TN% (Corradini, 1995). This value increased significantly from 12.1% in 3R samples to 19.6 % in 12R samples. These results demonstrated a marked proteolysis of Pecorino di Farindola cheese, similar of that of common Italian Pecorino cheeses (Di Cagno and Gobetti, 2011). The principal proteolytic agents in the curd are coagulant, microbial proteinases and peptidases and indigenous milk proteinase (plasmin) (Di Cagno and Gobetti, 2011). The proteinases and peptidases of rennet are among some of the proteolytic enzymes acting during the cheese-making process and the ripening phase (Fox and Stepaniak, 1993). Tofalo *et al.* (2015) found faster casein breaking off in Pecorino di Farindola cheeses, attributed to higher proteolytic activity of the enzymes of pig rennet. On the contrary, a lower proteolytic activity of pig rennet than that of calf rennet was found by Di Giacomo *et al.* (2013) in Pecorino di Farindola cheese samples.

Table 3. Free amino acid (FAA) content of Pecorino di Farindola cheeses at different ripening times (mg/100-g fresh weight).

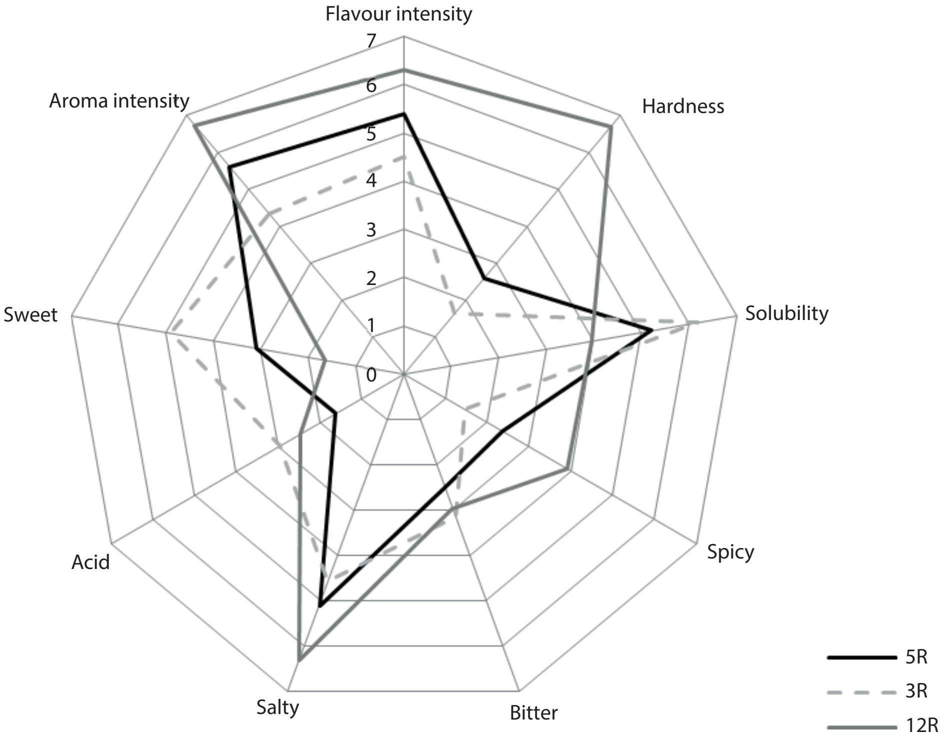
FAA	3 months*	5 months	12 months
Aspartic acid	10.74^{a}	32.20^{b}	45.26^{c}
Threonine	3.24^{a}	10.06^{b}	17.49^{c}
Serine	0.00^{a}	5.68^{b}	11.01^{c}
Asparagine	10.15^{a}	13.27^{b}	54.20^{c}
Glutamic acid	15.36^{a}	99.14^{b}	146.21^{c}
Glutamine	4.56^{a}	4.01^{a}	9.64^{b}
Glycine	4.34^{a}	6.45^{a}	20.89^{b}
Alanine	11.35^{a}	14.13^{b}	43.82^{c}
Valine	30.71^{a}	42.08^{b}	88.03^{c}
Methionine	7.95^{a}	14.62^{b}	33.68^{c}
Isoleucine	12.03^{a}	25.50^{b}	60.10
Leucine	42.97^{a}	57.66^{b}	115.54^{c}
Tyrosine	0.00^{a}	2.27^{b}	12.41^{c}
Phenylalanine	26.75^{a}	37.07^{b}	63.56^{c}
γ-Aminobutyric acid	21.55^{a}	30.02^{b}	56.98^{c}
Ornithine	14.33^{a}	15.08^{a}	10.64^{a}
Lysine	20.34^{a}	38.92^{b}	115.87^{c}
Histidine	0.00^{a}	9.13^{b}	31.15^{c}
Total	236.37^{a}	457.28^{b}	936.48^{c}

*Different letters within the same raw indicate a significant difference ($P < 0.05$).

A measure of lipolysis is represented by the determination of ADV. This value is a measure of the content of free fatty acids (FFA) dissolved in a certain amount of fat by lipases that can be correlated to the sensorial quality of finished products (Deeth and Fitz-Gerald, 1976; Mcsweeney and Sousa, 2000). The 3-, 5- and 12-month ripened cheeses demonstrated an ADV value of 2.9, 4.0 and 7.0 meq KOH/100-g fat, respectively (Table 2). Similar values were reported for ewe's cheeses (Georgala *et al.*, 2005), while higher indices were found in mixed cow/ewe Caciocavallo cheeses by Niro *et al.* (2014).

The evolution of FAAs of cheeses during ripening is shown in Table 3. The concentration of total free amino acids (TFAA) increased significantly ($P < 0.05$) to 936.48 mg/100 g at 12 months of ripening. At different ripening stages, the average TFAA content is in accordance with literature data for other Italian Pecorino cheeses (Coda *et al.*, 2006). No proline and arginine were found in all analysed samples. The latter evidence could be due to its consumption by bacteria; in fact, many species of LAB are able to convert arginine to citrulline and ornithine (Diana *et al.*, 2014; Niro *et al.*, 2017a). During ripening, the content of single FAAs increased, with the exception of ornithine. As also reported by Di Giacomo *et al.* (2013), at 12-month ripening, the highest FAAs found were glutamic acid (Glu), valine (Val), leucine (Leu) and lysine (Lys) in Pecorino di Farindola cheese at 6-month ripening and in long-ripened Pecorino cheese (Coda *et al.,* 2006; Mangia *et al.,* 2008). Glutamic acid is one of the most abundant amino acids in milk protein and, as almost free, in mature cheeses (Redruello *et al.,* 2020) and is strictly correlated with the 'umami' taste (McSweeney and Sousa, 2000).

Leu, phenylalanine (Phe) and Lys e Val can be found in other long-ripened cheeses such as Caciocavallo cheeses (Corsetti *et al.,* 2001; Niro *et al.,* 2017b; Succi *et al.,* 2016), Idiazabal (Barcina *et al.,* 1995), Picante (Freitas *et al.,* 1998), Serra da Estrela (Tavaria *et al.,* 2003), Teleme (Pappa and Sotirakoglou, 2008), goat cheeses (Poveda *et al.,* 2016) and Italian hard cheeses (Niro *et al.,* 2017b). Leu, with the other branched chain AA, isoleucine (Ile) and Val, the aromatic AA, Phe and tyrosine (Tyr), and metionine (Met) are the main precursors of key aroma compounds (Yvon and Rijnen, 2001). Some authors have attributed the bitter flavour in cheese to the concentration of arginine (Arg) (Barcina *et al.,* 1995; Pappa and Sotirakoglou, 2008). In all samples, ornithine (Orn) and γ-aminobutyric acid (GABA) were found; they do not originate from casein but are the products of microbial metabolism and are established functional AAs (Diana *et al.,* 2014; Tofalo *et al.*, 2019). As for GABA, its anti-hypertensive and anti-diabetic properties and its



	3R	5R	12R
Flavour intensity*	a	b	c
Hardness	a	b	c
Solubility	a	a	b
Spicy	a	b	c
Bitter	a	a	a
Salty	a	a	b
Acid	a	a	a
Sweet	a	b	c
Aroma intensity	a	b	c

*Different letters within the same row indicate a significant difference (P < 0.05)

Figure 1. Sensorial profile of Pecorino di Farindola cheeses at different ripening times. 3R: three-month ripening; 5R: five-month ripening; 12R: 12-month ripening.

ability to reduce stress and anxiety are widely recognised (Redruello *et al.,* 2020; Tofalo *et al.,* 2019). GABA is synthesised by glutamate decarboxylase through the decarboxylation of L-glutamate; milk origin, milk treatment, proteolytic activity, fat content, texture, ripening time and climate are reported to be the key factors governing its accumulation (Redruello *et al.,* 2020). Tofalo *et al.* (2019), in Pecorino di Farindola cheeses made from pig rennet, reported similar amounts of GABA than those found in this study, higher than the values of different commercial cheeses.

Regarding sensorial analysis, all samples were characterised by low acid and bitter attributes and a high salty score (Figure 1). As reported by Suzzi *et al.* (2014), cheeses made from pig rennet demonstrated the lowest elasticity, bitter taste and fruity and hay flavour intensities, compared with the cheeses made from calf and kid rennet. These data were also confirmed by Di Giacomo *et al.* (2013). Low-ripened cheeses had a higher solubility, and tasted sweeter, less hard and less spicy than the corresponding long-ripened cheeses ($P < 0.05$). Flavour and aroma intensity increased during ripening, reaching the highest score in Pecorino at 12-month ripening ($P < 0.05$). The 12-month ripened cheeses had a light spicy feature that distinguishes them from other Italian Pecorino cheeses, probably because of the use of pig rennet instead of the commonly used lamb rennet. As reported by different authors (Di Giacomo *et al.,* 2013; Kindstedt *et al.,* 2004), cheeses with a more evident lipolysis differ for strong flavours. The effects of FAAs on taste and flavour are reported by McSweeney and Sousa (2000) and Yvon and Rijnen (2001). In raw milk cheeses, similar to Pecorino di Farindola, the native microbiota may have played an important role and contributed to the distinct sensorial characteristics (McSweeney and Sousa, 2000; Niro *et al.,* 2014).

Conclusions

Different Pecorino di Farindola cheeses were characterised by certain variability because of different composition of the used raw milk, the low standardization of the cheese making process and different salting and ripening conditions. The use of raw ewe's milk and pig rennet contributed to the peculiar features of lipolysis and proteolysis and the sensorial attributes of the Pecorino of Farindola cheese. Sensorial analysis confirmed Pecorino of Farindola as sweet, lightly spicy and never bitter cheese, even when the ripening is extended a distinctive feature that is appreciated by the consumer.

Data emerging from this work could add new knowledge to the investigations of this cheese, giving an effective characterisation of the final nutritional and sensorial quality of the product. An additional investigation could be conducted to have a deeper insight into the fatty acid evolution and the influence of lipolysis and proteolysis on the profiles of volatiles during ripening.

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Sensory properties of iodine-biofortified potatoes

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PAPER

Abstract

The present study assessed the sensory impact of potatoes biofortification with iodine and the stability of iodine during six months of storage. Four biofortified cultivars (*Cupido*, *Marabel*, *Orchestra* and *Universa*) and their controls (non-biofortified) were evaluated. Descriptive analysis was applied with a panel to describe the sensory properties, and triangle tests were applied with consumers to evaluate perceivable differences between controls and respective biofortified samples at the end of shelf life. Iodine content was quantified on raw potatoes for three periods of storage. Descriptive analysis showed some differences between controls and iodine-biofortified samples, especially in texture (hardness). However, consumers did not significantly discriminate fortified from unfortified samples. Iodine was stable during storage in all varieties. *Orchestra* cultivar showed the highest iodine content, while *Universa* the lowest.

Keywords: biofortification, descriptive analysis, essential micronutrient, functional food, potato, triangle test

Introduction

Iodine is an essential micronutrient consumed with diet, and is necessary for the biosynthesis of thyroid hormones which regulate metabolic functions (Trumpff *et al.*, 2013). The daily recommended iodine intake ranges from 90 to 250 μg, depending on several factors, among others, age and physiological status (e.g. childhood, adultness, pregnancy/lactation) (Zimmermann *et al.*, 2012). Inadequate iodine intake is currently one of the main micronutrient deficiencies worldwide, leading to a spectrum of clinical and social issues called 'Iodine deficiency disorders' (Gonzali *et al.*, 2017)' and iodine deficiencies still represent a severe problem in certain geographic areas (Khattak *et al.*, 2017). Iodine biofortification in vegetables is a promising strategy to increase iodine intake and overcome iodine deficiencies in human diet. However, the main critical issues in biofortification of iodine vegetables rely on the stability of the mineral during storage and the potential sensory modifications, which can compromise acceptability of fortified food.

There are different strategies to address increase in iodine such as mineral supplementation, food fortification and biofortification of crops (Comandini *et al.*, 2013). Biofortification is applied to increase the bioavailable nutrient content of the edible portion of crop plants (Hotz, 2013). Biofortification of plant foods can be obtained by the following three ways: agronomic biofortification, conventional plant breeding of selected cultivars, and genetic engineering (Carvalho and Vasconcelos, 2013). The aim of the agronomical approach is to increase micronutrients through mineral fertilisers and/or through the improvement of soil mineral elements' mobilisation (Saltzman *et al.*, 2013). Recently, many authors have developed and tested specific agronomical practices to increase iodine content in different vegetables and crops, such as potato and tomato (Caffagni *et al.*, 2011; Zanirato

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and Mayerle, 2009); barley and wheat (Caffagni *et al.*, 2011); spinach and lettuce (Smoleń *et al.*, 2014; Weng *et al.*, 2013); cabbage, coriander, cucumber, eggplant, long cowpea and hot pepper (Weng *et al.*, 2013); carrot and onion (Zanirato and Mayerle, 2009).

As a general rule, food fortification should not alter the stability, colour or flavour of the vehicle food (Dwyer *et al.*, 2015). Iodine salts may theoretically be involved in colour or oxidative reactions, since iodide is a strong reducing agent and iodate is a strong oxidizing agent (Winger *et al.*, 2008). Since the modification of the sensory properties of food can negatively affect consumers' hedonic responses, it is necessary to maintain an adequate level of acceptance when dealing with fortified foods. In fact, in spite of certain consumers that compromise on taste when consuming healthy foods (Verbeke, 2006), often functional foods are characterised by critical sensory properties which are more disliked by consumers compared to conventional foods (Dal Bello *et al.*, 2017; Torri *et al.*, 2016).

The effect of iodine on food sensory quality has been studied by many authors (Greis *et al.*, 2018; West and Koning, 1995). On biofortified vegetables, particularly considering potatoes, studies were performed regarding the process of biofortification (Caffagni *et al.*, 2011; Zanirato and Mayerle, 2009), the stability of iodine during cooking (Caffagni *et al.*, 2012; Comandini *et al.*, 2013) and the bioavailability of iodine (Tonacchera *et al.*, 2013).

However, to the present authors' best knowledge, no study has explored the stability of iodine during storage in biofortified potatoes and its effect on sensorial properties.

Therefore, the aim of the present study was two-fold: (1) to evaluate the effects of iodine biofortification on sensory properties by describing the sensory properties of controls and biofortified samples (descriptive analysis [DA]) and by verifying whether biofortified cultivars were discriminated from non-fortified controls (triangle test); and (2) to evaluate the chemical stability of iodine in raw biofortified potatoes during storage (iodine analysis). Since prior results showed that iodine is stable during domestic cooking, including baking (Comandini *et al.*, 2013), the analysis of iodine was done directly on raw potatoes to establish the following: (1) the starting natural quantity of iodine in each cultivar; (2) the effect of iodine biofortification on each cultivar immediately after harvesting (storage time at which potatoes are extremely fresh) and (3) stability at the end of six months (storage time corresponding to end of shelf life, the moment at which the potatoes are the least fresh). Sensory analyses were conducted at the end of shelf life (i.e. six months), which was potentially the most critical moment, since the product was not fresh.

Materials and Methods

Growing and processing of potato cultivars

Potatoes (*Solanum tuberosum*) are grown in a limited zone of Emilia-Romagna region in Italy, and these were kindly donated by Pizzoli S.p.A. (Budrio, Italy). Four international cultivars were chosen for the biofortification process: *Cupido*, *Marabel*, *Orchestra* and *Universa*. These cultivars were chosen because of their maximum usage to produce fresh potatoes for global commercial level. For each cultivar, control and biofortified samples were obtained from the same field of growth to minimise any variability factor other than biofortification process. One batch was analysed for each cultivar. In text, letter 'C' is used to identify controls, while letter 'B' is used for biofortified samples. In all, eight samples were evaluated (*Cupido_C*, *Marabel_C*, *Orchestra_C*, *Universa_C*, *Cupido_B*, *Marabel_B*, *Orchestra_B* and *Universa_B*). Iodized potatoes were obtained by means of a patented agronomic procedure (Zanirato and Mayerle, 2009) through foliar fertilisation realised during the growing season. The harvest was done three weeks after the iodine treatment. Potatoes were stored under conventional industrial storage conditions: in a plastic box put in a warehouse designed for potato storage in absence of light, and in ventilated and conditioned atmosphere at 8°C and 80% of relative humidity during all storage period. No sprout inhibitors were used during storage. A period of six months was chosen as a shelf life, since it is a reasonable/representative storage time for commercial potatoes in controlled conditions before they are packaged and distributed immediately.

Sensory evaluation

All sensory tests were performed after six months of storage. Participants freely joined the sensory activities and written informed consent was obtained from all participants before inclusion in the tests. The study was in conformity with the Declaration of Helsinki. Prior to sensory evaluations, assessors received both verbal and written instructions regarding the evaluation procedures.

Preparation of samples

Whole unpeeled potatoes were washed in tap water and cooked for 80 min in a steam oven (Chef top, Unox S.p.A., Padova) at 100°C and 100% of relative humidity. After cooking, whole potatoes were cooled at a room temperature of 20°C for 2 h. Approximately 30 min prior to sensory evaluations, potatoes were cut in cubes with side of 15 mm and put on white plastic plates codified with a random three-digit code. Each container was closed with a plastic lid. Water was provided as palate cleanser at the start and between successive samples. The same preparation procedure was used in both sensory tests.

Descriptive analysis

Sensory profiles of eight potato samples were determined by the sensory panel of ASTRA laboratory (Imola, Italy). Test room was designed and managed following the ISO 8589:2007 (ISO 2007). The panel comprised eight trained judges (60% females, aged 27–45 years). The sensory panel was selected, trained and continuously monitored following the ISO 8586-1:2012 (ISO 2012). The panel evaluated the eight potato samples with an internally adapted procedure to establish a sensory profile developed following the ISO 13299:2016 (ISO 2016). The sensory profile approach is referred to in the text as descriptive analysis. A list of six attributes (two for tastes, one for flavour, and three for texture) was finally selected by the panel. Attributes, each of which was defined by a specific definition, were evaluated on nine-point scale (Table 1). For the purpose of tasting attribute, assessors were required to taste the sample, swallow and evaluate attributes for taste and flavour. Then, panellists were required to cut the sample with a knife to re-taste it and to evaluate texture properties. Samples were presented to panellists monadically and served in single-use, white plastic containers codified with a random three-digit code. A 1-min break was enforced between samples, when panellists rinsed their mouth with water. The descriptive analysis was conducted in two replicates. Each descriptive evaluation session lasted for approximately 1.5 h.

Triangle tests

The triangle test approach was chosen to find any difference between samples without specifying the sensory characteristic(s) that differ. Triangle tests were performed following ISO 4120:2004 (ISO 2004). Four triangle tests were performed in total (12 samples). The evaluation session included two subsets, each comprising two triads. Each triad had three codified samples, two of which were identical, and one was the odd sample. Assessors must identify the odd sample. Within each triad, the comparison was between the biofortified sample and the correspondent control. Samples were served according to a randomised design (ABB, BAA, AAB, BBA, ABA and BAB). A group of 46 subjects were involved (68% females, aged 24–50 years). Instructions required the assessors to taste the samples according to the provided order (from left to right), and to select the sample that they perceived as different from the other two within each triad. Assessors were asked to provide an answer even if they were not sure. Re-tasting was permitted. No time limitation was imposed on assessors. The presentation order of the triads was randomized across subjects. All evaluations were conducted between 12:00 noon and 2:00 pm.

Chemical analysis

Reagents

High purity grade solvents were used for iodine extraction. Tetramethylammonium hydroxide solution (TMAH, 1 M) was bought from Sigma Aldrich (St. Louis, MO), and hydrogen peroxide solution (H_2O_2 30% m/m) was from Carlo Erba (Arese, MI, Italy). Ion exchange water (18 M Ω) was obtained from Millipore Direct Q5 system (Millipore Co., Bedford, MA).

Iodine analysis

Iodine analysis was performed on controls and treated potatoes at three different periods: immediately after harvest (T0), after three months (T3) and after six months of

Table 1. Sensory attributes and definitions used by the trained panel in descriptive analysis to describe iodine-biofortified potatoes and controls.

Sensory modality	attribute	Definition	Scale
Taste	Sweet	The perception of sweet taste on the tongue	1 = very low; 5 = moderate; 9 = very intense
	Salty	The perception of salty taste on the tongue	1 = very low; 5 = moderate; 9 = very intense
Flavour	Typical potato flavour	The presence of an overall flavour typical for cooked potato perceived after swallowing	1 = very low; 5 = moderate; 9 = very intense
Texture	Hardness	The resistance of flesh to knife cutting and mastication (to the force impressed by teeth at the first bite)	1 = soft; 5 = neither soft nor hard; 9 = hard
	Moistness	The presence of liquid in the cut surface and perception of suiciness in the mouth during mastication	1 = dry; 5 = neither dry nor wet; 9 = wet
	Granulation	The presence of granules with a certain dimension, perceived during mastication	1 = coarse; 5 = neither coarse nor fine; 9 = fine

storage (T6). T3 and T6 were measured only for a group of biofortified samples. A representative quantity of fresh potatoes was homogenised. About 0.5 g of sample was weighed directly in pyrex test tubes. Iodine was extracted by adding 6 mL of TMAH solution (0.25 M) and 2 mL of H_2O_2 (30%) followed by microwave mineralisation (Mars Express 5, CEM srl, Cologno al Serio, Italy). The extract was diluted with ultrapure water, centrifuged and filtered following the procedure previously developed (Comandini *et al.*, 2013). Iodine content was determined with an inductively coupled plasma mass spectrometer (ICP-MS) (Agilent, Palo Alto, CA) using the following parameters: RF power 1550 W, and argon flow rates of 1.05 L/min and 0.2 L/min, respectively, for carrier gas and make-up gas. Instrument calibration was performed by employing iodine standards of up to a concentration of 100 mg/L in diluted TMAH solutions. Iodine analysis was done in triplicate.

Statistical analysis

The effect of samples on the perceived intensity of descriptors from panel was estimated using two-way mixed Analysis of Variance (ANOVA) models (random factor: judge; fixed factor: sample; model without interactions) separately conducted on each variable (sensory descriptor) considering eight products. All ANOVA models were followed by Tukey HSD test ($P < 0.05$). A Principal Component Analysis (PCA) was conducted on the mean intensity ratings of significant attributes obtained from a two-way mixed ANOVA, with the option of bootstrap hulls, which permitted to visualise the confidence areas of each sample. *T*-tests ($p<0.05$) were used to estimate Differences between the mean values obtained by the panel for both control samples and means obtained by the relative biofortified samples. For the triangle test, the following parameters were defined for 46 assessors: an α-risk of 0.10, a β-risk of 0.30 and a p_d of 0.20. The mean value of the three replicates was used for iodine analysis. Values of iodine are expressed in the text as mean and standard error of the mean for each storage time. A one-way ANOVA was conducted to estimate the effect of cultivar on the initial iodine content after harvesting among control samples (fixed factor: cultivar). The effect of iodine treatment within each cultivar was estimated after harvesting by four *t*-tests ($P < 0.05$), each comparing the content of iodine in the biofortified sample versus the related control sample. To assess the combined effect of the cultivar and the storage time on the final iodine content, a two-way fixed ANOVA was conducted (fixed factors: cultivar, and time of storage; model with interactions) considering 12 samples (four biofortified cultivars × three storage periods). Analyses were conducted with XLStat 2019.1.1 (Addinsoft, Boston, USA; package sensory).

Results and Discussion

Sensory properties

Sensory profiles

The sensory effects of addition of iodine to food have been described in previous papers (Greis *et al.*, 2018; West and Koning, 1995). However, to the best of present authors' knowledge, this is the first study reporting on the systematic description (done with a trained panel) of the sensory profiles obtained from biofortified potatoes baked in an oven.

The two-way mixed ANOVA models indicated a significant effect of the sample ($P < 0.05$) for all attributes except for salty (F = 2.04, $P = 0.06$). Mean intensity values obtained from replicates of significant attributes were submitted for PCA. Two principal components were extracted, which accounted for 82.5% of the total data variance, with PC 1 accounting for 58.1% and PC 2 for 24.4% variance (Figure 1A).

The first component (PC 1) was positively associated with two textural attributes: moistness and granularity, and sweetness, and negatively associated with the typical potato flavour. Granulation, moistness and sweetness were highly correlated to each other. The second component (PC 2) was positively associated with hardness, and negatively associated with granularity. The two *Universa* samples (*Universa_B* and *Universa_C*) were highly and positively associated with PC 1, thereby showing a positive correlation with attributes having a positive loading on this component. Instead, *Marabel* (*Marabel_B* and *Marabel_C*), *Cupido* (*Cupido_C* and *Cupido_B*) and *Orchestra_B* samples had a negative correlation on PC 1, thereby associated with a high intensity of typical potato flavour. However, *Cupido_B*, *Cupido_C*, *Marabel_C*, *Orchestra_C* and *Universa_C* had a negative score on PC 2. The bootstrap hulls plot showed samples with their confidence areas (Figure 1B). Some overlapping was observed in terms of perceptive properties, especially between *Marabel_C* and *Cupido_B* and between *Cupido_C* and *Marabel_B*, suggesting perceptive similarities between these two cultivars. Instead, *Universa_B* was clearly the most diverse sample which did not show perceptive overlaps with other samples.

The sensory profiles of the four cultivars comparing the control samples with the related biofortified samples at T6 are shown in Figure 2. Observed modifications in the sensory properties may have been induced by the iodine treatment in field, which was done in the last period of growth of tubers (about 2 weeks before harvest). In general, a few differences were found between controls and biofortified samples, and the sensory modifications observed from the *t*-test were small, similar to a recent

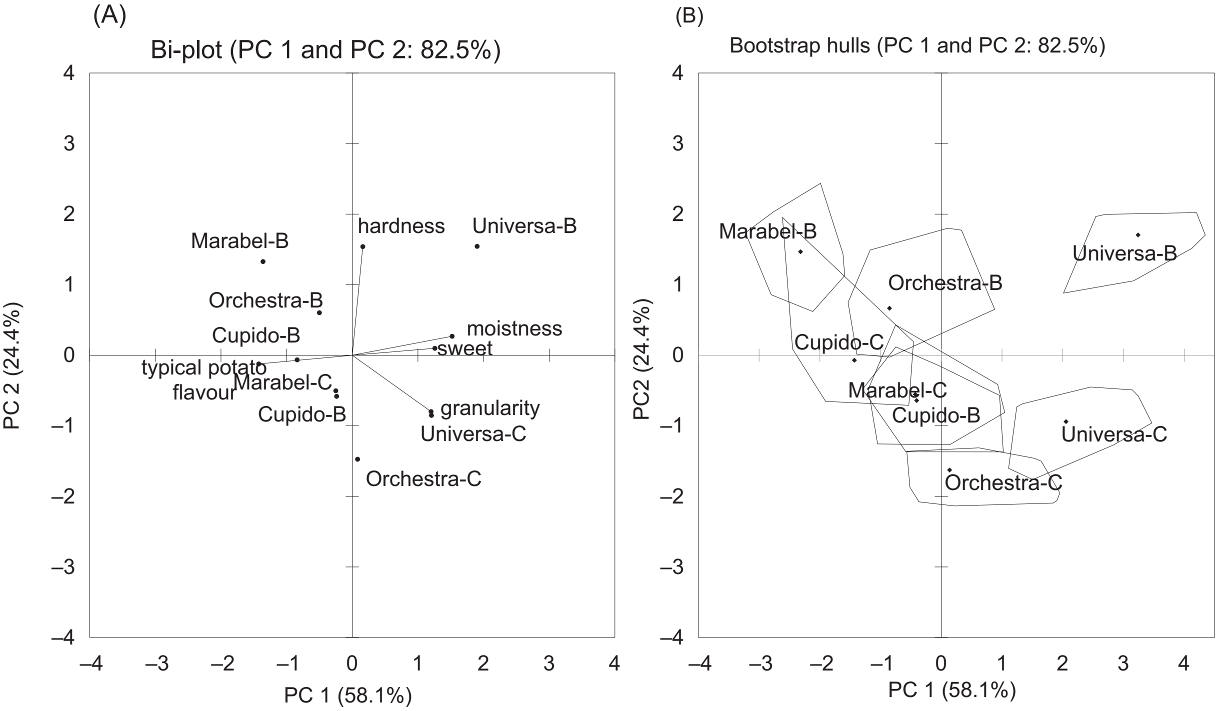

Figure 1. Bi-plot (1A) and bootstrap hulls plot (1B) from principal component analysis (PCA) of significant attributes used in descriptive analysis to describe eight samples of potatoes, including four controls (*Cupido_C*; *Marabel_C*; *Orchestra_C*; and *Universa_C*) and four biofortified samples (*Cupido_B*; *Marabel_B*; *Orchestra_B*; and *Universa_B*).

study which assessed the sensory modifications induced by addition of iodine in wheat bread, sausages and pickled cucumbers (Greis *et al.*, 2018).

Cupido was the only cultivar that did not show any significant differences in the comparison of profiles (Figure 2A).

The typical potato flavour and sweetness were not affected by the treatment of iodine in any variety. The intensity of descriptor 'typical potato flavour' was in agreement with a previous study, showing that the addiction of salt containing 400-mg iodine from potassium iodide or iodate did not affect the flavour of boiled potatoes and boiled rice (West and Koning, 1995). Moreover, iodine content of up to 100 mg/kg did not affect the sensory attributes of pickle (Greis *et al.*, 2018).

On the contrary, in the current study, the biofortification seems to influence the texture, with a general increase in the perceived hardness, which significantly increased in three out of four biofortified samples (*Marabel*, *Orchestra* and *Universa*).

The two other textural attributes (moistness and granularity) varied depending on the cultivar.

In *Marabel*, the biofortified sample (*Marabel_B*) showed a significant decrease ($P = 0.03$) in granularity and a significant increase in hardness ($P = 0.02$) (Figure 2B). In biofortified *Orchestra*, the hardness significantly increased ($P \leq 0.01$), while the perceived saltiness decreased ($P = 0.03$) (Figure 2C). In biofortified *Universa* (Figure 2D), both hardness ($P \leq 0.001$) and moistness ($P \leq 0.01$) increased significantly.

In general, the texture of potatoes is determined by several mutually dependent factors (Jarén *et al.*, 2016). The consistent increase in hardness found in the current study in biofortified samples may have been linked to the modification induced by the iodine within the starch structure. In fact, it is known that iodine may be bounded within the helical V-amylose component (Yu *et al.,* 1996), and this modification in the structure can translate into a microscopical increase in the perception of hardness. A few existing studies on the effect of iodine on texture seems inconsistent across different food products. Greis and colleagues (2018) found the largest deviation from the reference for tenderness for sausages but no difference in hardness for iodized and non-iodized pickles. Meat samples (sausages) containing a high quantity of iodine were tenderer (Greis *et al.*, 2018), probably because of the variation in their water-holding capacity (WHC). Iodate appeared to soften vegetable pickles in brine during storage (12 days) (Amr and Jabay, 2004), which goes opposite to our results. Taken together, these results suggest that a few sensory modifications induced by the iodine biofortification strongly depend on the type of food matrix used.

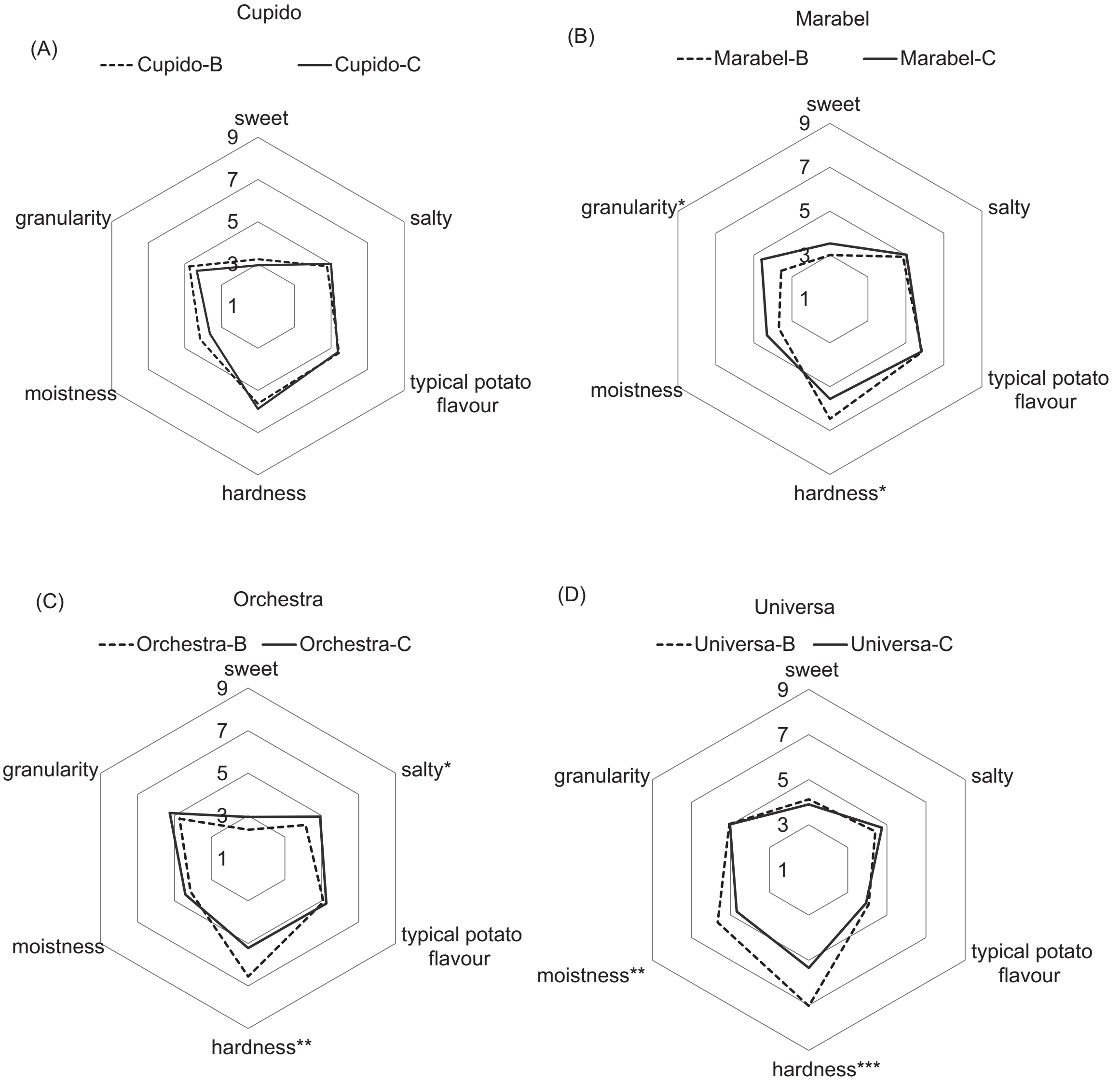

Figure 2. Sensory profiles of the control samples (continuous lines) and biofortified samples (dashed lines) for the cultivars: (A) *Cupido*, (B) *Marabel*, (C) *Orchestra* and (D) *Universa*. Asterisks indicate significant different mean values from *t*-tests for the considered attribute (P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001).**

Overall sensory difference

Results of triangle tests for the four cultivars are shown in Table 2.

No significantly perceivable differences were found between any control sample and its respective biofortified sample at T6, thus indicating that the biofortification was not perceived in the population of subjects. The fact that no significant differences were found from triangle tests between treated samples and control samples suggested that differences found from descriptive analysis were minor, and therefore it was unlike that naive consumers may have noticed them. The two sensory techniques gave some slightly different outputs because descriptive analysis was conducted by trained assessors, while triangle tests with naive consumers. Apart from the different expertise of the two groups, the approach was fully different. Triangle test is an overall difference test (Meilgaard *et al.*, 2007b), while descriptive analysis is an approach that requires an analytical evaluation of each descriptor (Meilgaard *et al.*, 2007a).

Iodine content and stability

Table 3 shows the iodine content after harvest (T0) in controls, and that in biofortified cultivars at three storage periods (T0, T3 and T6). The amount of iodine present in control samples (not treated) was derived from the environment elements (soil, water and air)

Table 2. Results of triangle tests conducted at T6 to estimate significant differences between biofortified samples and their respective controls. Each row shows the results of each cultivar from comparison between the biofortified samples and the relative control samples.

Cultivar	Assessors (N)	Critical number of correct responses	Correct responses given (n)	p_d	*P*-value
Cupido	44	20	18	0.11	0.18
Marabel	44	20	19	0.15	0.11
Orchestra	47	21	20	0.14	0.12
Universa	46	20	17	0.05	0.35

Note: p_d is the maximum proportion of assessors being able to detect a difference between products.

Table 3. Iodine content (µg/100 g) in controls ('C') and relative iodine-biofortified samples ('B') at three different periods of storage (after harvesting = T0, after three months = T3 and after six months of storage = T6).

Cultivar	Samples	Storage time		
		T0*	T3	T6
Cupido	*Cupido_C*	2.2 ± 0.3		
	Cupido_B	40.1 ± 3.9^{a,b,c,d*}	36.4 ± 3.9^{a,b,c,d}	37.0 ± 3.9^{a,b,c,d}
Marabel	*Marabel_C*	3.2 ± 0.3		
	Marabel_B	32.1 ± 3.9^{b,c,d,e*}	24.5 ± 3.9^{c,d,e}	22.9 ± 3.9^{c,d,e}
Orchesta	*Orchestra_C*	6.7 ± 0.3		
	Orchestra_B	52.4 ± 3.9^{a*}	42.4 ± 3.9^{a,b,c}	48.7 ± 3.9^{a,b}
Universa	*Universa_C*	2.8 ± 0.3		
	Universa_B	23.7 ± 3.9^{c,d,e*}	20.9 ± 3.9^{d,e}	15.7 ± 3.9^{e}

Values are mean ± standard error of three measurements.
*Mean values of biofortified samples significantly differ from mean values of respective control samples at T0 from *t*-test ($P \leq 0.01$).
Different letters in mean values across the 12 biofortified samples indicate significantly different iodine contents from two-way ANOVA followed by Tuckey's HSD test ($P < 0.05$).

(Osterc *et al.*, 2011). At T0, *Orchestra_C* had the significantly highest iodine content (6.7 ± 0.3 µg/100 g) compared to the other control samples, suggesting that this cultivar is naturally richer in iodine.

In general, standard errors associated with the iodine analysis were high. This was because the sample preparative phase of this method (extraction) suffers from an intrinsic variability of sub-samples (replicates) that can greatly vary despite a proper homogenisation of the matrix (e.g. for the water content of single tuber, for different accumulations of micro- and macro-components in tuber etc.), thus resulting in high variations in standard errors.

As expected, *t*-test confirmed that the iodine content significantly ($P \leq 0.01$) and strongly increased in all biofortified samples, independent of the initial iodine content (Table 3). This suggests that the agronomic treatment was effective in fresh potatoes, confirming results of other authors (Caffagni *et al.*, 2011, 2012; Zanirato and Mayerle, 2009).

The four cultivars showed different accumulation efficiencies in agreement with a previous study, which observed that the accumulation efficiency varied not only across potatoes (Caffagni *et al.*, 2012) but also for different amounts of iodine provided (Caffagni *et al.*, 2011). The increased ratio (the ratio of amount of iodine in biofortified and control samples) was approximately 18 (for *Cupido*), 10 (for *Marabel*) and 8 times more (for *Orchestra* and *Universa*). Therefore, *Cupido* showed the highest increased ratio. These differences in accumulation efficiency across varieties could be derived from different responses of cultivars to iodine treatment. Since the iodine behaviour in a soil–plant system is very complex due to the high number of factors involved (Fuge and Johnson, 2015), different varieties of plants absorb iodine differently.

The two-way ANOVA showed a significant strong effect of the biofortified cultivar (F = 23.352, $P < 0.001$) on the final iodine content but no effect of the storage time (F = 3.088, $P = 0.064$) nor of the interaction of cultivar and storage time (cultivar × storage time; F = 0.471, $P = 0.823$) (data not shown). The lack of effect of storage time indicated that the content of iodine is substantially stabile during the considered time, in agreement with a previous study (Caffagni *et al.*, 2012), which noted no significant effect of storage time on iodine content in potato tubers. The lack of effect of interaction (cultivar × storage time) indicated similar trends in iodine contents over time across the considered cultivars.

Instead, the strong effect of cultivar indicated a difference in the total iodine amount across the biofortified cultivars, as shown in Table 3. After six months of storage, among the biofortified samples, *Orchestra_B* had significantly the highest amount of iodine (48.7 ± 3.9 μg/100 g), not significantly different from *Cupido_B* (37.0 ± 3.9 μg/100 g), followed by *Marabel_B* (22.9 ± 3.9 μg/100 g) and *Universa_B* (15.7 ± 3.9 μg/100 g), which did not differ significantly from each other. *Orchestra* cultivar seemed to be the most suited variety for iodine biofortification, while *Universa* cultivar is the least suited because of its lowest accumulation efficiency (according to the iodine increased ratio after treatment) and the lowest final content of iodine after six months.

Conclusions

On the one hand, the data obtained from the present work confirmed the effectiveness of the agronomical practice of iodine biofortification in potatoes. Iodine content was stable for six months under the conventional storage conditions in all cultivars. On the other hand, this study extended the knowledge by providing a systematic description of the sensory effects of iodine-biofortified potatoes, considering four different potato cultivars (*Cupido*, *Marabel*, *Orchestra* and *Universa*).

The descriptive analysis highlighted a few differences between biofortified and control samples, mainly in texture (hardness), probably because of modifications in the starch structure of potatoes induced by iodine. However, differences were small and were not detected by consumers in triangle tests. Therefore, iodine biofortification seems to be a suitable and promising technique for potatoes, since it does not compromise the sensory properties and it allows to develop functional vegetables (that is products with improved nutritional characteristics), which could be successful by consumers. A further development of the study could be the exploration of consumers' perception related to liking of biofortified samples, targeting specific consumers (e.g. consumers with specific nutritional needs).

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Author Contributions

All authors contributed to the study's conception and design. M. Piochi undertook the analyses and wrote the paper. All authors critically contributed and commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

The research was conducted on human volunteers, and it was in conformity with the Declaration of Helsinki. Written informed consent was obtained from all participants before inclusion in the sensory tests.

Conflict of Interest

The authors declare that they have no conflict of interest. Vegetables were supplied free of charge with no obligation between researchers and suppliers.

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The addition of *Capsicum baccatum* to Calabrian monovarietal extra virgin olive oils leads to flavoured olive oils with enhanced oxidative stability

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ORIGINAL ARTICLE

Abstract

This study aimed to evaluate the influence of *Capsicum baccatum* L. Aji Angelo and Bishop crown cultivars to the quality parameters of flavoured olive oils (FOOs) obtained by the addition of both fresh and dried pepper powders (1%) to Dolce di Rossano and Roggianella monovarietal extra virgin olive oils (EVOOs). First, pepper extracts were investigated for their total phenolic, flavonoid, carotenoid content as well as phenolic acids, fatty acid profile, and vitamin C and E content. In order to evaluate the impact of both fresh and dried peppers on the oxidative stability of FOOs, the Rancimat test was applied. 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene bleaching (B-CB) and ferric reducing antioxidant power (FRAP) assays were used to investigate the antioxidant potential. Bishop crown dried extracts showed the highest phenolic, carotenoid and vitamin content, whereas Aji Angelo had the highest amount of capsaicinoids. Among EVOOs, Roggianella EVOO showed the highest antioxidant activity as well as the highest induction time (39.6 h). Remarkably, FOO obtained by the addition of Bishop crown dried pepper extract to Roggianella EVOO showed a higher induction time (44.9 h) with respect to the corresponding EVOO.

Keywords: antioxidant activity; *Capsicum*; chemical profile; monovarietal extra virgin olive oil; oxidative stability

Introduction

According to the definition of the European Union Commission (EC, 2003; EEC, 1991), an extra virgin olive oil (EVOO) must be extracted "only from olives with superior quality, cannot undergo any treatment other than washing the fruits, and decanting, centrifuging and filtering the extracted olive oil. It excludes oils obtained from seeds by chemical or mechanical methods or the use of solvent extraction or re-esterification methods, and those mixed with oils from other sources." Thus, the addition of herbs, spices or other fruits to an EVOO generates a product that cannot be called "extra virgin olive oil," but can be defined as flavoured olive oil (FOO) (Baiano *et al.*, 2010). These FOOs can be characterised by improved nutritional values, enriched sensory characteristics and increased shelf-life. Recently, several FOOs have been introduced into the market (Issaoui *et al.*, 2016).

Capsicum genus comprises 30 species, however, only five (*C. baccatum, C. annuum, C. frutescens, C. chinense* and *C. pubescens*) are those mainly cultivated (Tripodi and Kumar, 2019). *Capsicum* phytochemicals include phenolics, carotenoids, capsaicinoids and other metabolites (Wahyuni *et al.*, 2013). Several of these compounds are well-known as antioxidants accounting for the traditional use of peppers as food preserving agents from ancient times. In the last decades, our research group investigated the chemical composition and bioactivity of many food plants including different *Capsicum* species collected in Calabria (Southern Italy) (Fazio *et al.*, 2018; Loizzo *et al.*, 2015, 2017; Menichini *et al.*, 2009; Tundis *et al.*, 2013).

This study aimed to evaluate the effect of *Capsicum baccatum* Aji Angelo and Bishop crown cultivars on the quality parameters of FOOs obtained by the addition of pepper powder to Dolce di Rossano and Roggianella monovarietal EVOOs. For this purpose: (i) total phenolic, flavonoid and carotenoid contents, as well as vitamins C and E were assessed in both EVOOs, pepper extracts and FOOs; (ii) fatty acids, sterols, phenolics and capsaicinoids were also quantified; (iii) the protective effect of pepper on FOO oxidative stability; and (iv) the antioxidant potential was investigated by different *in vitro* methods.

Materials and Methods

Chemicals and reagents

All reagents were purchased from Sigma-Aldrich S.p.a. (Milano, Italy), whereas analytical-grade solvents were obtained from VWR International s.r.l. (Milan, Italy). The following standards were used: FAME Mixes-Analytical Standards (CRM47885); Cholesterol (PubChem CID: 5997); 24-Methylene-Cholesterol (PubChem CID: 92113); Campesterol (PubChem CID: 173183); Campestanol (PubChem CID: 119394); Stigmasterol (PubChem CID: 5280794); Δ^7-Campesterol (PubChem CID: 5283646); Clerosterol (PubChem CID: 5283638); Clerosterol (PubChem CID: 5283638); β-Sitosterol (PubChem CID: 222284); Sitostanol (PubChem CID: 6743); Δ^5-Avenasterol (PubChem CID: 5281326); $\Delta^{5,24}$-Stigmastadienol (PubChem CID: 286499); Δ^7-Stigmastenol (PubChem CID: 3080632); Δ^7-Avenasterol (PubChem CID: 12795736); Erythrodiol (PubChem CID: 101761); Uvaol (PubChem CID: 92802); 4-Hydroxybenzoic acid (PubChem CID: 135); *p*-Coumaric acid (PubChem CID: 637542); *o*-Coumaric acid (PubChem CID: 637540); Ferulic acid (PubChem CID: 445858); Hydroxytyrosol (PubChem CID: 82755); Tyrosol (PubChem CID: 10393); (+) Pinoresinol (PubChem CID: 73399); 3,4-DHPEA-EDA (PubChem CID: 18684078); *p*-HPEA-EDA (PubChem CID: 11652416); Hydroxytyrosol acetate (PubChem CID: 155240); Apigenin (PubChem CID: 5280443); Luteolin (PubChem CID: 5280445); Chlorogenic acid (PubChem CID: 1794427); Quercetin (PubChem CID: 5280343); Beta-carotene (PubChem CID: 5280489); Vitamin C (PubChem CID: 54670067); Vitamin E (PubChem CID: 14985); Capsaicin (PubChem CID: 1548943); Dihydrocapsaicin (PubChem CID: 107982).

Materials, drying process and enrichment procedure

Dolce di Rossano and Roggianella monovarietal EVOOs were supplied in November 2019 by Frantoio Meringolo, Corigliano Calabro (Cosenza, Italy). All samples have accomplished the UNI10939, 2001 certification. EVOOs were stored in green glass bottles without headspace before analysis. Fruits of *C. baccatum* Aji Angelo and Bishop crown cultivars were obtained from Miceli s.r.l. farm (Scalea, Cosenza, Italy) that provides its authentication. Fruits were picked up at the maturity stage, defined by a visual colour change and size measurement. Before analyses, fruits were examined for integrity and absence of insect contamination, devoid of peduncles and seeds and cut into small pieces. To obtain dried peppers, fruits were sun-dried at 35°C for 2 weeks. Both fresh and dried peppers were grounded and the powders (50 mg) were added to 5 g of EVOOs and stirred to obtain FOOs that were left for 30 days in the infusion. After that, FOOs were filtered to remove the powder and analysed. Samples were stored at −20°C until analysis.

EVOOs and FOOs

Quality parameters

Extra virgin olive oil quality parameters (free acidity, peroxide index, UV light absorption sterol and fatty acid profiles) were analysed according to the EC Regulation methods (EU, 2016). To compare oxidative stability of FOOs and EVOOs, Rancimat equipment (Metrohm, Basel, Switzerland) at 98°C and with airflow of 10–12 L/h was used, according to a known protocol (Firestone, 1993).

EVOO phenolic extract and high-performance liquid chromatography (HPLC) analysis

The EVOO phenolic extract was obtained by using a previously described procedure (Montedoro *et al.*, 1992). The obtained extract was dissolved in 1 mL of methanol/water (1:1, *v*/*v*) and after filtration injected into a high-performance liquid chromatography (HPLC) instrument, Knauer instrument (ASI – Advanced Scientific Instruments, Berlin, Germany), coupled with UV-VIS detector of Waters Company (model Waters 486 Tunable), as previously described (Sicari *et al.*, 2010). The mobile phase was constituted by water/acetic acid (98:2, *v*/*v*) (A) and methanol/acetonitrile (1:1, *v*/*v*) (B), with a flow rate of 1 mL/min. Data from three independent experiments were acquired with Clarity software (Chromatography Station for Windows) and expressed as mean ± SD.

Total phenolic, flavonoid, carotenoid and chlorophylls contents

Total phenolic content (TPC) was determined as previously described by Gao *et al.* (2000). The absorbance was read at 765 nm. For the total flavonoid content (TFC), the method reported by Yoo *et al.* (2008) was applied. Absorbance was read at 510 nm. Total carotenoid content (TCC) and chlorophyll content were determined following a previously reported procedure (Mínguez-Mosquera *et al.*, 1900). According to this method, the index K670 assesses the total chlorophylls and their derivatives, whereas the index K470 assesses TCC.

Capsicum baccatum

Extraction procedure

Both *C. baccatum* Bishop crown and Aji Angelo cultivars (250 g) were subjected to maceration in ethanol (500 mL) for three times. Extracts were stored until analysis at −20°C in amber bottles.

Total phenolic, flavonoid and carotenoid contents

Total phenolic content, TFC and TCC were determined as described for EVOO samples in the previous section. TPC was expressed as mg chlorogenic acid (CA) equivalents/100 g dry weight (DW). TFC was expressed as mg quercetin equivalents (QE)/100 g DW. TCC was expressed as mg β-carotene (βC) equivalents/100 g DW (Menichini *et al.*, 2009).

Capsaicin and dihydrocapsaicin content

Capsaicin and dihydrocapsaicin contents were determined according to a previously reported method (Menichini *et al.*, 2009), by using a GC17A gas chromatograph (GC) (Shimadzu, Milan, Italy) equipped with a Flame Ionization Detector (FID). Analyses were performed in isothermal conditions at 210°C. The capsaicinoids content was done and data are expressed as mean ± SD in μg/g DW.

Vitamin C and E content

The content of vitamin C in pepper samples was determined according to a previously reported method (Klein and Perry, 1982). For determination of vitamin E content gas chromatography-mass spectrometry (GC-MS) analyses were performed (Loizzo *et al.*, 2015). Vitamin content was expressed as mg/100 g DW.

Antioxidant activity of EVOOs and pepper extracts

Radical scavenging activity by ABTS and DPPH tests

Both 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) tests were applied to examine the radical scavenging activity of *Capsicum* extracts and EVOO phenolic fraction using the procedure previously described by Leporini *et al.* (2018). In both cases, ascorbic acid was used as a positive control.

Evaluation of protection of lipid peroxidation

In the β-carotene bleaching (B-CB) test, a mixture of linoleic acid (LA), β-carotene and Tween 20 was prepared and the resulting emulsion was mixed with samples (Loizzo *et al.*, 2019). The absorbance was read after 30 min of incubation at 470 nm. Propyl gallate was used as a positive control.

Ferric reducing activity power (FRAP) assay

Both pepper extract and EVOO phenolic fraction (at a concentration of 2.5 mg/mL) were tested. Also, the ability of samples to protect the iron from redox reaction is evaluated (Loizzo *et al.*, 2019). Butylated hydroxytoluene (BHT) was used as control.

Calculation of relative antioxidant capacity index (RACI)

The relative antioxidant capacity index (RACI) was used to establish antioxidant rank of samples (Sun and Tanumihardjo, 2007).

Statistical analysis

Data were obtained from three different experiments (n = 3) and expressed as means ± standard deviation (SD). Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to calculate the concentration that yielded a 50% inhibition (IC_{50}) value as a result of the concentration-response curve. *Pearson's* correlation coefficient (*r*) was calculated using Microsoft Excel 2010 software. ANOVA followed by Dunnett's test ($\alpha = 0.05$) was applied to evaluate the differences between data and positive control result in biological assays, however, Tukey's test was used to determine any significant difference among all treatments at different levels $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

Principal component analysis (PCA) is the most common form of factor analysis and is categorised as a multivariate statistical technique. It is used to analyse interrelationships among a large number of variables. For the evaluation of the results of the chemical (TFC, TPC, TCC, DPPH, ABTS, β-carotene-bleaching, FRAP and induction time), analyses of Roggianella and Dolce di Rossano EVOOs and FOOs, PCA was applied.

Results and Discussion

Dolce di Rossano and Roggianella EVOO quality parameters

Based on the results reported in Table 1 and according to EC regulation, both Dolce di Rossano and Roggianella EVOOs could be classified as "extra virgin" (EC, 2003; EEC, 1991).

Table 1. Chemical and qualitative parameters of monovarietal Dolce di Rossano and Roggianella EVOOs produced in Calabria (Italy) during 2018–2019 season.

Quality parameter	EVOO		Significance
	Dolce di Rossano	Roggianella	
Free acidity (%)	0.79 ± 0.02^a	0.73 ± 0.01^b	**
Peroxide value (meqO$_2$/kg)	15 ± 1^a	13 ± 1^b	**
K232	1.9 ± 0.9^a	1.8 ± 0.8^b	**
K270	0.15 ± 0.02^a	0.15 ± 0.02^a	n.s.
ΔK	0.001 ± 0.00^a	0.001 ± 0.00^a	n.s.
TPC (ppm)	73 ± 2^b	537 ± 6^a	**
TFC (ppm)	18 ± 1^a	15 ± 1^b	**
TCC (ppm)	3 ± 2^a	2 ± 3^b	n.s.
Chlorophyll (ppm)	4 ± 2^a	4 ± 3^a	n.s.

TPC: Total Phenolic Content; TFC: Total Flavonoid Content; TCC: Total Carotenoid Content.
** Significance at $P < 0.01$, n.s. not significant.
Results followed by different letters in the same row are significantly different by Tukey's multiple range test.

Our values agreed with those reported by Lavelli and co-workers for EVOO Pendolino, Leccino, Moraiolo and Taggiasca cultivars (Lavelli and Bondesan, 2005).

A significant difference ($P < 0.01$) was observed between the phytochemicals content (TPC, TFC and TCC) of two investigated oils. In particular, Roggianella EVOO showed a TPC value that was seven-time higher than that found for Dolce di Rossano (Table 1). The TPC value is in line with those found by Sicari *et al.* (2017) for Roggianella EVOO from Reggio Calabria whereas lower values were recorded for Calabrian Ottobratica and Carolea EVOO, respectively (Piscopo *et al.*, 2016). A statistically significant difference was also observed in TFC with values of 18 and 15 ppm for Dolce di Rossano and Roggianella EVOOs, respectively.

The pigment content of EVOOs is an important quality parameter since consumers directly evaluate it based on their colour (Gargouri *et al.*, 2013). Moreover, their content is strictly related to EVOO stability. As reported in Table 1, TCC and chlorophyll content were not statistically different for Dolce di Rossano and Roggianella EVOOs. This TCC content is similar to that reported by Borrello and Domenici (2019) for Tuscan EVOOs from Frantoio, Leccino, Moraiolo and Pendolino cultivars. Previously, Tuberoso *et al.* (2016) found chlorophylls values in the range from 6.5 to 10.5 ppm, and from 20.9 to 47.6 ppm with respect to TCC for Sardinian EVOOs Semidana and Tonda di Cagliari, respectively.

EVOO chemical profile

As expected, oleic acid (OA, ω-9) was the main monounsaturated fatty acid (MUFA) whereas palmitic acid (PA) was the major saturated fatty acid (SFA) in both EVOOs (Table 2). A statistical difference was observed in OA/ LA (ω-6) ratio ($P < 0.01$), as Roggianella EVOO showed the highest OA/LA ratio, an indicator of the EVOO stability (Alvarruiz *et al.*, 2003). Our data are in line with data found for Roggianella EVOO from Reggio Calabria province (Sicari *et al.*, 2010). More recently, several Italian monovarietal EVOOs including Leccino, Frantoio, Dolce Agogia and Moraiolo were investigated by Blasi *et al.* (2019). The percentages of OA and PA were quite similar to those found in our samples. Values from 71.84 to 73.20% and from 16.09 to 19.30% were recorded for OA and PA, respectively, in Sari Hasebi and Halhali green EVOOs from Turkey (Yorulmaz and Konuskan, 2017).

In general, the sterolic composition of the two investigated EVOOs was significantly different ($P < 0.01$). As expected, β-sitosterol represented the most abundant phytosterol (Table 3). Two-time higher content of Δ^5-avenasterol was found in Roggianella EVOO with respect to Dolce di Rossano. A statistically significant difference in the campesterol/stigmasterol index was observed between investigated EVOOs. Both EVOOs showed values of campesterol lower than 4%, the maximum limit established by European regulations and by the IOC (EU, 2011; IOC, 2009). Our data were in agreement with those reported for Turkish Sari Hasebi and Halhali green EVOOs, that were found in the range from 80.72 to 87.81% of β-sitosterol, respectively, followed by Δ^5-avenasterol (3.34–5.29% for Halhali and Sari Hasebi, respectively) (Yorulmaz and Konuskan, 2017). The total sterol content was 1698 and 1971 mg/kg for Dolce di Rossano and Roggianella, respectively, i.e. much higher than 1000 mg/kg indicated by the European regulations and by the IOC as a minimum sterol content for an EVOO (EU, 2011; IOC, 2009).

Table 2. Fatty acid composition of monovarietal Dolce di Rossano and Roggianella EVOOs produced in Calabria (Italy) during 2018–2019 season.

Fatty acid	EVOO		Significance
	Dolce di Rossano	Roggianella	
Myristic acid (C14:0)	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	n.s.
Palmitic acid (PA, C16:0)	14 ± 2^{a}	13 ± 2^{b}	**
Palmitoleic acid (C16:1)	1.3 ± 0.1^{a}	0.9 ± 0.1^{b}	**
Margaric acid (C17:0)	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	n.s.
Heptadecenoic acid (C17:1)	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}	n.s.
Stearic acid (SA, C18:0)	1.4 ± 0.2^{b}	1.5 ± 0.1^{a}	**
Oleic acid (OA, C18:1)	72 ± 4^{b}	79 ± 6^{a}	**
Linoleic acid (LA, C18:2)	8.4 ± 0.9^{a}	6.6 ± 0.8^{b}	**
α-Linolenic acid (ALA, C18:3)	0.40 ± 0.02^{a}	0.40 ± 0.02^{a}	n.s.
Arachidic acid (C20:0)	0.90 ± 0.03^{a}	0.80 ± 0.03^{b}	*
Gadoleic acid (C20:1)	0.20 ± 0.01^{b}	0.30 ± 0.01^{a}	**
Behenic acid (C22:0)	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	n.s.
OA/LA	8.6^{b}	12.0^{a}	**
$\sum$SFA	16.43^{a}	15.43^{b}	**
$\sum$MUFA	73.54^{b}	80.60^{a}	**
$\sum$PUFA	8.8^{a}	7.0^{b}	**
MUFA/PUFA	8.36^{b}	11.51^{a}	**

SFA: saturated fatty acid; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids.
**Significance at $P < 0.01$, * Significance at $P < 0.05$, n.s. not significant.
Results followed by different letters in the same row are significantly different by Tukey's multiple range test.

Table 3. Sterol composition of Dolce di Rossano and Roggianella EVOOs produced in Calabria (Italy) during 2018–2019 season

Sterol	EVOO		Significance
	Dolce di Rossano	Roggianella	
Cholesterol (%)	0.12 ± 0.01^{a}	0.07 ± 0.02^{b}	**
24-Methylene-Cholesterol (%)	0.07 ± 0.01^{b}	0.29 ± 0.01^{a}	**
Campesterol (%)	2.23 ± 0.03^{b}	2.67 ± 0.05^{a}	**
Campestanol (%)	0.16 ± 0.01^{a}	0.11 ± 0.01^{b}	**
Stigmasterol (%)	0.80 ± 0.02^{b}	1.81 ± 0.04^{a}	**
Δ^{7}-Campesterol (%)	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}	n.s.
Clerosterol (%)	0.87 ± 0.01^{b}	0.98 ± 0.02^{a}	**
β-Sitosterol (%)	88.21 ± 0.08^{a}	81.71 ± 0.06^{b}	**
Sitostanol (%)	0.98 ± 0.03^{a}	0.63 ± 0.02^{b}	**
Δ^{5}-Avenasterol (%)	5.01 ± 0.05^{b}	10.3 ± 0.8^{a}	**
$\Delta^{5,24}$-Stigmastadienol (%)	0.62 ± 0.04^{a}	0.51 ± 0.01^{b}	**
Δ^{7}-Stigmastenol (%)	0.37 ± 0.01^{a}	0.39 ± 0.05^{a}	n.s.
Δ^{7}-Avenasterol (%)	0.51 ± 0.01^{a}	0.43 ± 0.01^{b}	**
Apparent β-Sitosterol (%)	95.68^{a}	94.16^{b}	**
Campesterol/Stigmasterol	2.79^{a}	1.48^{b}	**
β-Sitosterol/Δ^{5}-Avenasterol	17.61^{a}	7.89^{b}	**
Total sterols (mg/kg)	1698^{b}	1971^{a}	
Erythrodiol (%)	1.52 ± 0.02^{a}	0.92 ± 0.02^{b}	**
Uvaol (%)	0.79 ± 0.01^{a}	0.16 ± 0.01^{b}	**

All the sterols as well as erythrodiol and uvaol are expressed as percentage of the total sterol content.
** Significance at $P < 0.01$, * Significance at $P < 0.05$, n.s. not significant.
Results followed by different letters in the same row are significantly different by Tukey's multiple range test.

Table 4. Phenolic composition of monovarietal Dolce di Rossano and Roggianella EVOOs produced in Calabria (Italy) during 2018–2019 season.

Compound	Amount in EVOO (ppm)		Significance
	Dolce di Rossano	Roggianella	
4-Hydroxybenzoic acid	0.21 ± 0.01^{b}	0.6 ± 0.9^{a}	**
p-Coumaric acid	0.8 ± 0.2^{a}	0.20 ± 0.02^{b}	**
o-Coumaric acid	0.15 ± 0.01^{b}	0.24 ± 0.02^{a}	**
Ferulic acid	0.27 ± 0.02^{a}	0.14 ± 0.01^{b}	**
Hydroxytyrosol	3.12 ± 0.03^{a}	2.9 ± 0.8^{b}	**
Tyrosol	4.04 ± 0.04^{b}	6.2 ± 0.5^{a}	**
(+) Pinoresinol	8 ± 2^{b}	22 ± 2^{a}	**
(+) 1-Acetoxypinoresinol	14 ± 4^{b}	52 ± 7^{a}	**
3,4-DHPEA-EDA	17 ± 2^{b}	48 ± 5^{a}	**
p-HPEA-EDA	5.1 ± 0.9^{b}	18 ± 1^{a}	**
Hydroxytyrosol acetate	9 ± 1^{b}	21 ± 2^{a}	**
3,4-DHPEA-EA	3.6 ± 0.3^{b}	8.26 ± 0.09^{a}	**
p-HPEA-EA	0.87 ± 0.08^{b}	1.15 ± 0.01^{a}	**
Apigenin	0.00 ± 0.00^{b}	0.79 ± 0.01^{a}	**
Luteolin	0.48 ± 0.02^{b}	1.12 ± 0.08^{a}	**
∑ Identified phenolics	66.64^{b}	178.52^{a}	**

3,4-DHPEA-EDA: Oleacein, *p*-HPEA-EDA: oleocanthal, 3,4-DHPEA-EA: Oleuropein aglycone, *p*-HPEA-EA: Ligstroside aglycone.
** Significance at $P < 0.01$.
Results followed by different letters in the same row are significantly different by Tukey's multiple range test.

The phenolic composition of both Dolce di Rossano and Roggianella EVOOs is reported in Table 4. In general, the total amount of identified phenolic compounds in the two EVOOs was significantly different ($P < 0.01$). As expected, secoiridoids and lignans were the most abundant compounds. Roggianella EVOO resulted to be richer in phenolic compounds than Dolce di Rossano. 1-Acetoxypinoresinol followed by 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA) were the two most abundant compounds in both EVOOs. The amounts of these compounds in Roggianella were 3.7- and 2.8-times higher than those in Dolce di Rossano. Roggianella EVOO was also characterised by a significant content of pinoresinol and hydroxytyrosol acetate. Dolce di Rossano EVOO variety showed a slightly higher tyrosol content than Roggianella (Table 4). Roggianella EVOO showed a two-times higher content of luteolin than Dolce di Rossano variety. Among phenolic acids, 4-hydroxybenzoic acid was the main abundant acid in Roggianella oil whereas *p*-coumaric acid was the most abundant in Dolce di Rossano EVOO.

Our data were in line with those previously reported for EVOOs from Roggianella cultivars (Giuffrè and Louadj, 2013). Mean values of 48.54, 38.02 and 16.24 µg/g have been found for 3,4-DHPEA-EDA, 1-acetoxypinoresinol and hydroxytyrosol acetate, respectively. Values in the range 21.11–76.80 µg/g were found for 3,4-DHPEA-EDA in Grignano and Leccino EVOOs (Baiano *et al.*, 2009). The lower content of phenolic compounds has been found in Sardinian EVOOs where 3,4-DHPEA-EDA ranged from 1.0 to 11.6 µg/g in Tonda di Cagliari and Semidana, respectively (Tuberoso *et al.*, 2016). It is interesting to emphasise that 1-acetoxypinoresinol, one of the main phenolics identified in our Calabria EVOO, was not revealed in Tonda di Cagliari and found in a very low quantity in Tonda di Villacidro, Semidana and Bosana oils. However, Bosana contained a high amount of tyrosol (22.5 ppm). A perusal analysis of literature evidenced a great variability in EVOO phenolic profile due to several factors including geographical origin, genetic factors and environmental conditions. This is particularly true for hydroxytyrosol that was found in a high amount in Colozzese EVOO (16.1 ppm), whereas its amount ranged from 0.2 to 8.8 ppm in Spina and Oliva Grossa samples (Negro *et al.*, 2019). With respect to apigenin and luteolin, a higher amount of both phenols were found by Giuffrè *et al.* (2010) in Roggianella EVOO from Reggio Calabria during the season 2006.

Phytochemical content in C. baccatum cultivars

Fresh and dried *C. baccatum* Bishop crown and Aji Angelo fruits were subjected to extraction by maceration. The highest extraction yields were obtained with dried fruits (Table 5).

Table 5. Investigated *Capsicum baccatum* cultivars.

Capsicum baccatum		Colour	Length (cm)	Width (cm)	Extraction yield (%)
Bishop crown	Fresh	Red	5–7	6–8	6.06
	Dried				29.92
Aji Angelo	Fresh	Red	5–7	6–8	6.06
	Dried				27.90

Table 6. Total phenolic, flavonoid, carotenoid, capsaicin and dihydrocapsaicin, vitamin C and vitamin E content in *Capsicum baccatum* cultivars.

Content	Bishop crown		Aji Angelo		Significance
	Fresh	Dried	Fresh	Dried	
TPC[1]	6.80 ± 0.02^{c}	67 ± 4^{a}	7.4 ± 0.4^{c}	63 ± 2^{b}	**
TFC[2]	1.40 ± 0.03^{c}	10.90 ± 0.02^{b}	1.10 ± 0.01^{c}	11.9 ± 0.2^{a}	**
TCC[3]	2036 ± 8^{d}	4785 ± 9^{a}	2763 ± 7^{c}	3388 ± 8^{b}	**
Vitamin C[4]	3.90 ± 0.22^{b}	5.2 ± 0.3^{a}	3.40 ± 0.09^{c}	4.0 ± 0.1^{b}	**
Vitamin E[4]	5.1 ± 0.5^{b}	5.3 ± 0.2^{b}	5.2 ± 0.4^{b}	5.5 ± 0.9^{a}	**
Capsaicin[5]	44 ± 3^{c}	125 ± 7^{b}	19 ± 5^{d}	175 ± 9^{a}	**
Dihydrocapsaicin[5]	24 ± 4^{c}	58 ± 6^{b}	15 ± 1^{d}	156 ± 7^{a}	**

[1] TPC: Total Phenolic Content, expressed as mg of chlorogenic acid equivalents/100 g dried weight (DW), [2] TFC: Total Flavonoid Content, expressed as mg of quercetin equivalents/100 g DW, [3] TCC: Total Carotenoid Content, expressed as mg of β-carotene equivalents/100 g DW. [4] Expressed as mg/g DW, [5] Expressed as µg/g DW. Results followed by different letters in the same row are significantly different ($P < 0.01$, **) by Tukey's multiple range test.

Dried peppers contained the highest amounts of all analysed phytochemicals. In particular, Bishop crown showed the highest TPC and TCC values whereas Aji Angelo had the highest TFC (Table 6). The same trend was also observed in the extracts obtained from fresh samples.

As reported in Table 6, dried pepper extracts were rich in both capsaicin and dihydrocapsaicin capsaicinoids with values ranging from 58 to 125 µg/g DW for Bishop crown and Aji Angelo dried pepper extract, respectively. Bishop crown dried pepper showed the highest vitamin C content while vitamin E was particularly abundant in Aji Angelo dried pepper extract.

Antioxidant activity

Despite it has been previously reported that antioxidant ability determined by DPPH and ABTS *in vitro* assays can significantly differ due to different mechanisms of inactivation of the radicals (Antolovich *et al.*, 2002; Plastina *et al.*, 2018), we observed similar trends for the investigated samples. Promising radical scavenging activity was found for Roggianella EVOO phenolic extracts in both applied assays (Table 7). These values are better than that reported for different Calabrian monovarietal EVOOs by Sicari (2017) for Sinopolese and Roggianella and Leporini and co-workers (2018) for Frantoio EVOO (average value of 117.2 and 131.9 µg/mL for ABTS and DPPH, respectively). Previously, Negro and co-workers found IC_{50} values in the range 91–160 g/oil for DPPH radical scavenging activity for Oliva Grossa and Spina, respectively (Negro *et al.*, 2019). The radical scavenging potential of EVOOs phenolic fraction was positively correlated to TFC and TCC with *r* values of 1.0.

Among investigated peppers, Bishop crown resulted to be the most active with IC_{50} values of 148 and 167 µg/mL for DPPH and ABTS tests, respectively. No significant differences between Bishop crown and Aji Angelo dried pepper extracts were evidenced in the protection from lipid peroxidation. Promising ferric reducing ability was observed for both Dolce di Rossano and Roggianella EVOO, respectively. Previously, Loizzo *et al.* (2017) investigated the antioxidant activity of *C. annuum* and *C. chinense* fresh and processed peppers and found the highest DPPH radical scavenging potential with Effix fresh pepper sample (IC_{50} value of 3.9 µg/mL). A high radical scavenging potential against ABTS has been previously observed for dried *C. annuum* cv Pellegrino and Idealino samples with IC_{50} values of 45.2 and 45.7 µg/mL, respectively (Loizzo *et al.*, 2017). Great variability in antioxidant potential was observed with oleoresin obtained from different varieties of fresh peppers including

Table 7. Antioxidant activities of Dolce di Rossano and Roggianella EVOOs and *C. baccatum* extracts.

Sample	ABTS[1]	DPPH[1]	β-carotene bleaching[1]	FRAP (μM Fe(II)/g)	RACI values
EVOO phenolic extracts					
Dolce di Rossano	48 ± 3****	54 ± 2****	205 ± 9****	79 ± 3	71
Roggianella	36 ± 1****	30 ± 1****	127 ± 5****	57 ± 3*	–71
C. baccatum extracts					
Bishop crown					
Fresh	170 ± 2****	162 ± 3****	33 ± 3****	15 ± 1****	0.04
Dried	167 ± 3****	148 ± 3****	21 ± 1***	14 ± 2****	–0.47
Aji Angelo					
Fresh	453 ± 4****	186 ± 2****	77 ± 3****	9.1 ± 0.4****	0.86
Dried	256 ± 4****	157 ± 3****	20 ± 1***	9.2 ± 0.8****	–0.43
Positive controls					
Ascorbic acid	1.71 ± 0.03	5.0 ± 0.8			
Propyl gallate			1.00 ± 0.01		
BHT				63 ± 4	

[1] IC_{50} value (μg/mL). Data are expressed as means ± S.D. (n= 3). DPPH Radical Scavenging Activity Assay, Antioxidant Capacity Determined by Radical Cation ($ABTS^{+}$), β-carotene bleaching test, Ferric Reducing Antioxidant Power (FRAP). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****$P < 0.0001$, ***$P < 0.001$, compared with the positive controls.

C. annuum and *C. chinense*. The RACI approach was applied to estimate the rank of antioxidant activity for EVOOs and pepper samples. Roggianella EVOO resulted as the most active one whereas Bishop crown dried pepper extract exhibited the highest antioxidant potential among investigated pepper extracts (Table 7).

Protective effect of pepper extracts against oil oxidation

Rancimat apparatus was used to investigate and compare the stability of EVOOs and the corresponding FOOs (Figure 1). In this method, the oxidation is induced by the passage of constant airflow through the oils that is kept under constant temperature. The volatile products of the reaction are collected in deionised water and are measured through the electric conductivity. During the development of the reaction, due to an increase of the conductivity, a curve is drawn from which the induction period is inferred. Stability was expressed as oxidation induction time (expressed in hours). Roggianella EVOO showed a greater induction time than Dolce di Rossano sample (39.6 versus 19.2 h, respectively). This is probably due to the higher amounts of TPC and identified phenolic compounds found in Roggianella EVOO. In line with our data, the following trend for resistance to oxidation has been previously observed for Calabrian EVOOs: Roggianella > Ottobratica > Sinopolese (Sicari, 2017) and Carolea > Ottobratica > Sinopolese > Grossa di Gerace (Piscopo *et al.*, 2016). Remarkably, all FOOs enriched with pepper extracts had a prolonged induction time with respect to the corresponding EVOO. Among them, FOOs obtained by the addition of Bishop crown dried extract to Roggianella EVOO showed the highest induction time of 44.9 h. This possibly depends on the high amount of bioactive phytochemicals, with the only exception of vitamin E and dihydrocapsaicin, found in Bishop crown dried extract. Moreover, by considering the protection factor quotient (PF), Bishop crown fresh pepper-FOO had a PF of 5.7 with respect to Dolce di Rossano EVOO followed by FOO obtained by the addition of the dried extract from the same pepper in Roggianella EVOO (PF = 5.3). Lower values were observed for FOOs enriched with Aji Angelo extracts with PF values ranging from 1.05 to 3.03. It has been previously reported that FOOs obtained by the addition of *C. frutescens* extract did not show any protective effect against the oxidation process (Gouveia *et al.*, 2006). This was probably due to the low content of capsaicinoids. By contrary, a protective effect towards oxidation was observed when dried *C. annuum* pepper was added to EVOO by infusion (10–20% w/w) up to 30 days (Caporaso *et al.*, 2013). The effect of the addition of spices or their extract/essential oils on oil oxidative stability gained attention by researchers working in the food area. The infusion of dried garlic, oregano and rosemary and hot pepper to Dauno monovarietal EVOO led to FOOs in which the formation of primary oxidation products was reduced without any modification on acidity parameter and secondary oxidation compounds (Gambacorta *et al.*, 2007). More recently, Ayadi

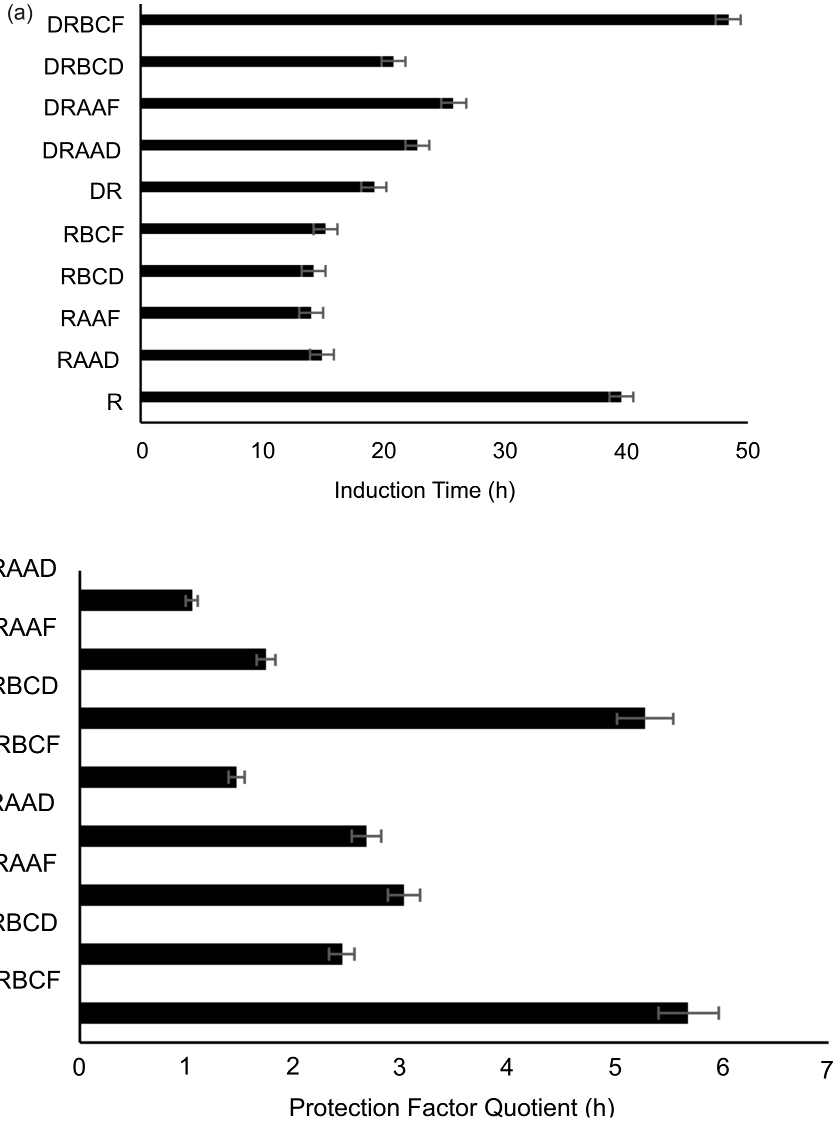

Figure 1. **(a) Induction time and (b) Protection Factor quotient of Dolce di Rossano (DR) and Roggianella (R) EVOOs and corresponding FOOs enriched with *C. baccatum* cv Bishop crown (BC) and Aji Angelo (AA) fresh (F) and dried pepper (D) extracts.**

et al. (2009) investigated the quality parameters of FOOs obtained by the infusion of 5% (*w*/*w*) of Mediterranean spices to Chemlali EVOO. Although the addition of spices has led to a slight increase in free acidity, an improvement of their thermal resistance and stability with this hierarchy: rosemary > thyme > lemon > basil ≥ EVOO control was also observed. This could be caused by the transfer of antioxidant compounds from spices to oil during the infusion. More recently, Ammar *et al.* (2017) evaluated the effect on quality parameters including oxidative stability of the addition of *Opuntia ficus-indica* flower extracts to "Chemlali" EVOO that results in FOO1 and FOO2 (5 and 15% (*w*/*w*), respectively). FOO1 was richer in phenolics by 3.4% than FOO2. In disagreement with our data, this addition did not improve oxidative stability. Also, the induction time obtained by the Rancimat method showed values of 2.73 and 2.42 h for FOO1 and FOO2, respectively, in comparison to the control EVOO (2.83 h). The authors speculated that this may be due to a diffusion of undesirable compounds during the flower maceration of olive oil. These compounds can take part in reactions that negatively interfere with oxidative stability (Ammar *et al.*, 2017).

The unfavourable effect of EVOO aromatisation on oxidative stability was also observed by Sena-Moreno and co-workers who proposed a new EVOO aromatisation method. In this study, liquid–liquid extractions of saffron aqueous extract in "Arbequina" EVOO was done. The sample obtained from the first liquid–liquid extraction was SO1, whereas SO2, SO3 and SO4 were those obtained from the second, third and fourth extraction, respectively. SO1 showed the highest safranal concentration. It is worthy of mention that in the first seven months, FOOs were characterised by a reduction of quality parameters including oxidative stability. After that FOOs parameters were still comparable to those of EVOO (Sena-Moreno *et al.*, 2018). The impact of enrichment with phenolic compounds from the olive cake and dried thyme on the quality parameters phenolic composition, oxidative stability and antioxidant activity of "Arbequina" EVOO was investigated by Rubió *et al.* (2012). Flavonoids from thyme were characterised by a higher transference ratio (average 89.7%) with respect to secoiridoids from an olive cake (average ratio of 35.3). In each case, all resulted FOOs were more resistant to oxidation with values of induction time of ~8 and ~20 h for control and enriched oil, respectively.

Principal component analysis

The projections of the observations on the first two principal component axes are shown in Figure 2. The accessions are distributed on the factor plane. These two coordinates represent 87.77% of the total variance (PC1 explained 56.17% of total variation, while PC2 explained 31.59% of total variation). The first component (PC1) was positively correlated with TPC, TFC, FRAP, β-carotene bleaching, RACI and ITDR. The second component (PC2) was positively correlated with TPC, ITDR and ITR. Figure 2 shows the space of oils and hot pepper samples (fresh and dried), samples and the bioactive attributes associated with FOOs. The PCA model showed that FOOs were characterised by a higher induction time than the corresponding EVOOs.

Conclusions

Flavoured olive oils gained attention by consumers not only as a new dressing for meat, fish or salad but also for the role of spice phytochemicals on human health. In this context, the protective effect of *C. baccatum* peppers cultivars on the oxidative stability of FOOs obtained by the addition of pepper to two Calabrian EVOOs was evaluated. Roggianella EVOO, characterised by a high phytochemicals content showed a longer induction time. The addition of *C. baccatum* Bishop crown to Roggianella EVOO led to a FOO with an increased induction time with respect to the corresponding EVOO. Promising results were also obtained with FOO resulted by the addition of Bishop crown fresh pepper to Dolce di Rossano. Therefore, the addition of *C. baccatum* Bishop crown peppers was able to enhance the oxidative stability of oils, regardless of the quality of the starting EVOO.

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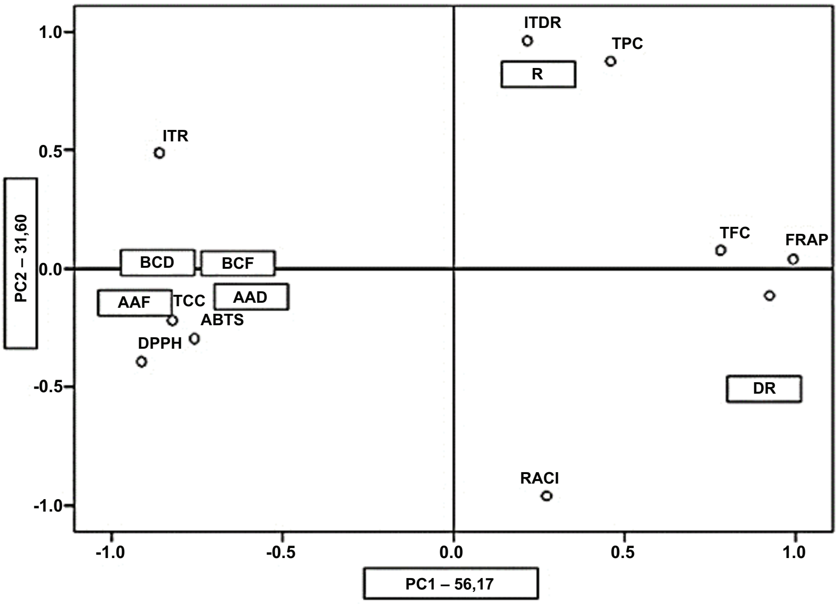

Figure 2. **Principal component analysis plot based on bioactivity attributes of extra virgin olive oils (EVOOs) and corresponding flavoured olive oils (FOOs) enriched with C. baccatum pepper extracts. DR: Dolce from Rossano; BCF: Bishop crown Fresh; BCD: Bishop crown dried; AAF: Aji Angelo Fresh; AAD: Aji Angelo dried; R: Roggianella.**

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Garlic greening: Pigments' biosynthesis and control strategies

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REVIEW

Abstract

Greening is a major problem for garlic's quality. This phenomenon leads to discoloration of the product and is directly related to the alliinase-catalyzed conversion of isoalliin into 1-propenyl-containing thiosulfates. Garlic crushing, refrigeration, and storage in normal atmosphere, as well as in the presence of monocarboxylic acids, are established the main factors that promote its greening. In last decades, the study of biochemical pathway of this phenomenon has allowed to effectively understand the main steps and key enzymes involved, and to identify optimum conditions for chemical and enzymatic reactions leading to discoloration. These findings have, in some cases, determined the development of new tools for the control of garlic greening on large scale. After providing an updated description of the biochemistry of green pigments produced in garlic, this review reports an overview on the strategies for controlling discoloration of garlic at industrial level.

Keywords: control strategies, discoloration, garlic, greening, isoalliin, *S*-alk(en)ylcysteine sulfoxides

Introduction

Garlic (*Allium sativum*) is a world-spread plant being used for centuries as a food for its health benefits (Banerjee *et al.*, 2003). Its health benefits have been exploited to treat diseases starting from ancient Sumerians, Egyptians, Greeks, and Romans up to the modern times (Zang *et al.*, 2013). Garlic cultivation was probably started in central Asia for more than 10,000 years ago (Block, 2010). Nowadays also, garlic cultivation is primarily concentrated in the same region, with China, India, and Bangladesh accounting for 86% of the world's production (FAOSTAT, 2016).

Garlic is a fundamental ingredient of many food preparations. Indeed, thanks to its antimicrobial properties and distinctive taste, it is used as a food preservative and/or seasoning in many typical recipes, and has become a key component of the Mediterranean cuisine (Lanzotti, 2006). Essentially, owing to its high content of sulfur aroma compounds (Speranza and Morelli, 2012), garlic is widely used as a taste and flavor enhancer of recipes all over the world. In these preparations, fresh garlic cloves are usually crushed immediately before use, a process that is essential to release the sulfur-containing compounds responsible for its typical flavor (Block, 1992). Indeed, crushing allows enzyme alliinase, which is originally found in cell vacuole, to get in contact with *S*-alk(en)ylcysteine sulfoxides, such as isoalliin and alliin, and convert them into 1-propenyl-containing thiosulfinates and allicin, respectively (Kubec and Velíšek, 2007). This latter compound is in turn the precursor for many flavoring molecules such as diallyl disulfide and diallyl sulfide (Yoshimoto *et al.*, 2019). In addition, the isoalliin degradation pathway leads to the synthesis of 1-propenyl-containing thiosulfinates, which are recognized as "color developers," i.e., compounds responsible for garlic greening (Kubec and Velíšek, 2007; Kubec *et al.*, 2004). This phenomenon of discoloration, generally desirable for many Asiatic preparations (i.e., Laba garlic;

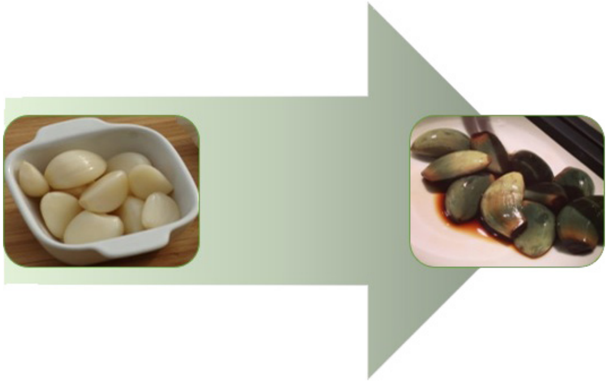

Figure 1. Greening in Laba garlic (retrieved from commons.wikimedia.org).

Figure 1), is undesirable in western countries, where any variation from the white color negatively affects garlic's marketability (Tao *et al.*, 2016).

Formation of pigment cannot be inhibited drastically (i.e., by interventions at the genetic level) as it shares the same biosynthetic pathway of bioactive and flavor compounds. Therefore, the phenomenon of greening needs to be controlled by preventive practices during garlic storage and/or processing (Zang *et al.*, 2013).

Many papers, reporting on the mechanisms of production of allicin (and related compounds), have primarily focused on their flavoring and health properties. In contrast, fewer and less recent works have dealt with the problem of garlic discoloration by analyzing the main greening controlling strategies employed at industrial level.

In this review, an updated biochemical description of the biosynthetic pathway leading to the production of pigments in garlic is reported. Subsequently, the available and possible new technologies to control garlic greening are described, and discussed critically.

Pigments' Formation

The starting point of pigments' biosynthesis is the conversion of isoalliin and other *S*-alk(en)ylcysteine sulfoxides into 1-propenyl-containing thiosulfinates (Kubec and Velíšek, 2007; Kubec *et al.*, 2004). This biochemical process gained more and more interest starting from the middle of the 20th century when *S*-alk(en)ylcysteine sulfoxides, including alliin, isoalliin, and methiin, as well as their conversion into allicin by the enzyme alliinase, were first described (Stoll and Seebeck, 1948, 1949a, 1949b, 1951).

A schematic overview on the main biochemical steps leading to the pigment's biosynthesis in *Allium sativum* plants is described in Figure 2 (Imai *et al.*, 2006b; Zang *et al.*, 2013). These different steps are discussed in detail in the following sections.

Biosynthesis of *S*-alk(en)ylcysteine sulfoxides

S-alk(en)ylcysteine sulfoxides are present in all plants belonging to the genus *Allium*, especially alliin (*S*-allylcysteine sulfoxide) and isoalliin (*S*-1-propenylcysteine sulfoxide) (Martins *et al.*, 2016). These compounds comprise analk(en)yl group, probably originating from valine catabolism, and a cysteine moiety derived from glutathione. The currently proposed alliin and isoalliin biosynthesis pathway (Figure 3 [Yoshimoto et al., 2019]), recently reviewed by Yoshimoto *et al.* (2019), includes: (i) the conjugation of glutathione to methacrylic acid or methacrylic-CoA (both derived from valine catabolism) to form *S*-(2-carboxypropyl) glutathione (Granroth, 1970; Lancaster and Shaw, 1989; Suzuki *et al.*, 1962); (ii) the formation of γ-glutamyl-*S*-1-propenylcysteine and γ-glutamyl-*S*-allylcysteine (precursors of isoalliin and alliin, respectively) after removal of glycyl from *S*-(2-carboxypropyl) glutathione and its conversion into the *S*-1-propenyl and/or *S*-2-propenyl group by oxidative decarboxylation; (iii) the sulfide formation after removal of the γ-glutamyl group catalyzed by γ-glutamyl transpeptidases enzyme (GGT); and (iv) the *S*-oxygenation of intermediate sulfides into alliin and isoalliin catalyzed by a flavin-containing *S*-oxygenase.

The reactions described at above-mentioned (i) and (ii) are probably catalyzed by still unknown enzymes. Conversely, the roles of GGT and flavin-containing *S*-oxygenase were assessed at molecular level (Cho *et al.*, 2012b; Yoshimoto *et al.*, 2015), and recently a consensus has been reached on this point (Yoshimoto *et al.*, 2019).

Even though it is known that *S*-alk(en)ylcysteine sulfoxides are accumulated in cytosol, the subcellular localization of several of the biosynthetic reactions described above has not been completely clarified (Yoshimoto *et al.*, 2019). Indeed, many of the above-mentioned enzymes and intermediates are probably located in different cell compartments of *Allium* plants. In garlic, the flavin-containing *S*-oxygenase is located in the cytosol (Yoshimoto *et al.*, 2015), and in onion (*Allium cepa*), which presents a similar *S*-alk(en)ylcysteine sulfoxides biosynthetic pathway and cell structure, γ-glutamyl peptides have been detected also in the cytosol, while glutathione was found in both cytosol and chloroplasts (Lancaster and Collin, 1981; Lancaster and Shaw, 1989). Conversely, GGT enzyme and methacrylic-CoA have never been precisely localized in *Allium* spp., although in plant cells they are usually found in vacuoles and mitochondria, respectively (Binder, 2010; Grzam *et al.*, 2007).

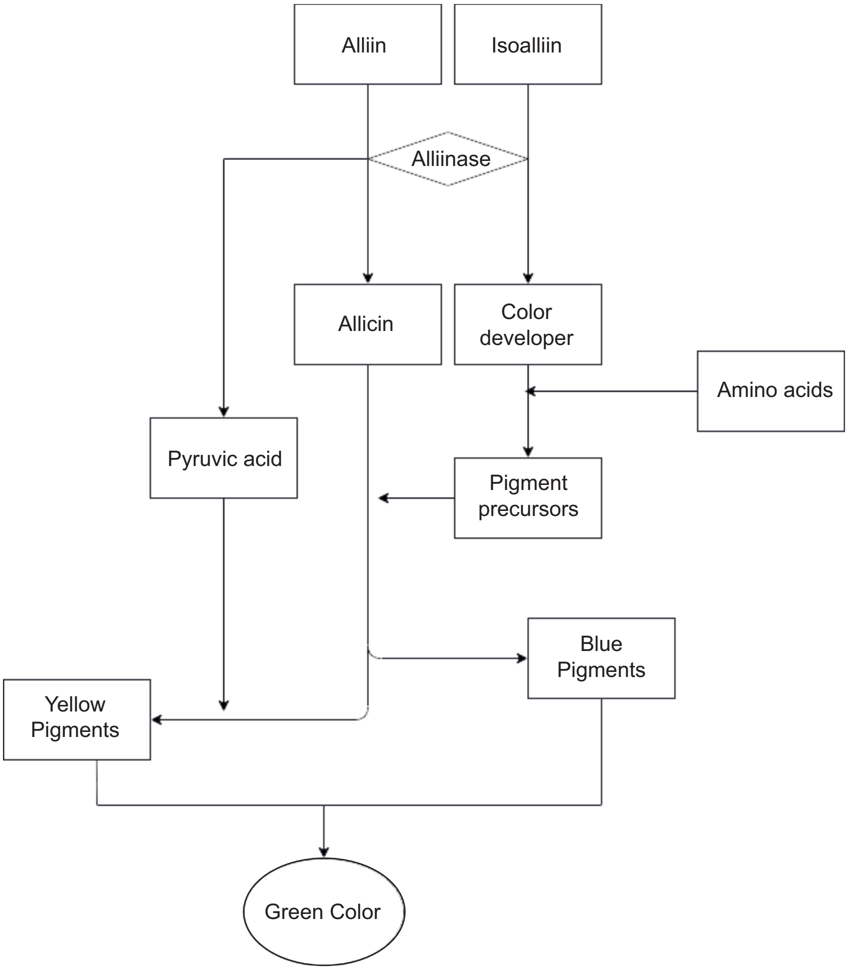

Figure 2. Main steps involved in pigments' biosynthesis pathway in garlic (*Allium sativum*) plants.

Allium plants synthesize *S*-alk(en)ylcysteine sulfoxides in a manner to accumulate nitrogen and sulfur as well as a defense mechanism against insects and pathogens (Aghajanzadeh *et al*., 2019; Yoshimoto *et al*., 2019).

Biosynthesis of thiosulfinates

In *Allium* plants, thiosulfinates are produced from the hydrolysis of *S*-alk(en)ylcysteine sulfoxides, which are catalyzed by the enzyme alliinase, a pyridoxal 5′-phosphate (PLP)-dependent C–S lyase (EC 4.4.1.4). Since alliinase is located in the vacuole and the *S*-alk(en)ylcysteine sulfoxides are stored in the cytosol, this reaction takes place only after crushing, cutting, maceration, or other practices leading to cell disruption (Lancaster and Collin, 1981; Li *et al*., 2015). Upon contact, alliinase removes a proton attached to the chiral carbon of the *S*-alk(en)ylcysteine sulfoxide, cleaving the C–S bond to yield sulfenic acid, pyruvic acid, and ammonia (Shimon *et al*., 2007). Sulfenic acids are then converted into thiosulfinates by spontaneous self-condensation (Yoshimoto *et al*., 2019). In onion, this step could be partially hindered by the activity of the enzyme lachrymatory factor synthase (LFS), which converts isoalliin into propanethial *S*-oxide, the "lachrymatory factor," which is the compound involved in lachrymation during slicing of onions (Imai *et al*., 2002; Silvaroli *et al*., 2017).

In garlic, 1-propenyl-containing thiosulfinates derived from isoalliin are called "color developers." A minor but significant role is played in the greening of garlic by the alliin-derived thiosulfinate allicin (Figure 4 [Kubec *et al*., 2017]). Allicin is established as the main thiosulfinate in garlic, where it is the principal responsible for the development of typical flavors and health-benefit properties (Borlinghaus *et al*., 2014; Kopec *et al*., 2013; Martins *et al*., 2016; Rahman, 2007).

However, for the greening phenomenon, the main role is played by isoalliin-derived 1-propenyl-containing thiosulfinates ("color developers"), as they constitute the basic structure for the formation of pigment precursors (Figure 2; Kubec *et al*., 2004, 2017; Zang *et al*., 2013).

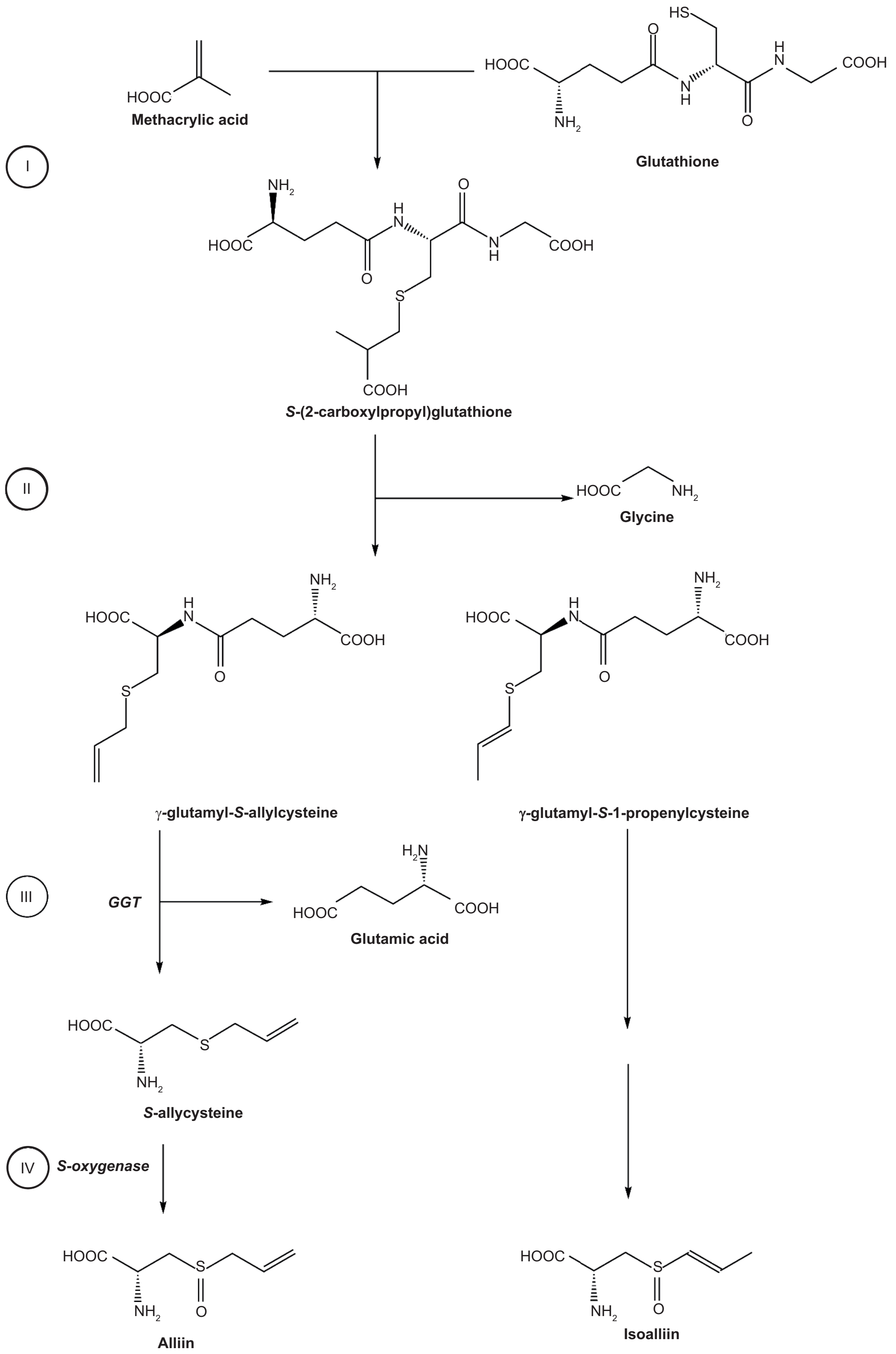

Figure 3. Biosynthetic pathways for alliin and isoalliin formation.

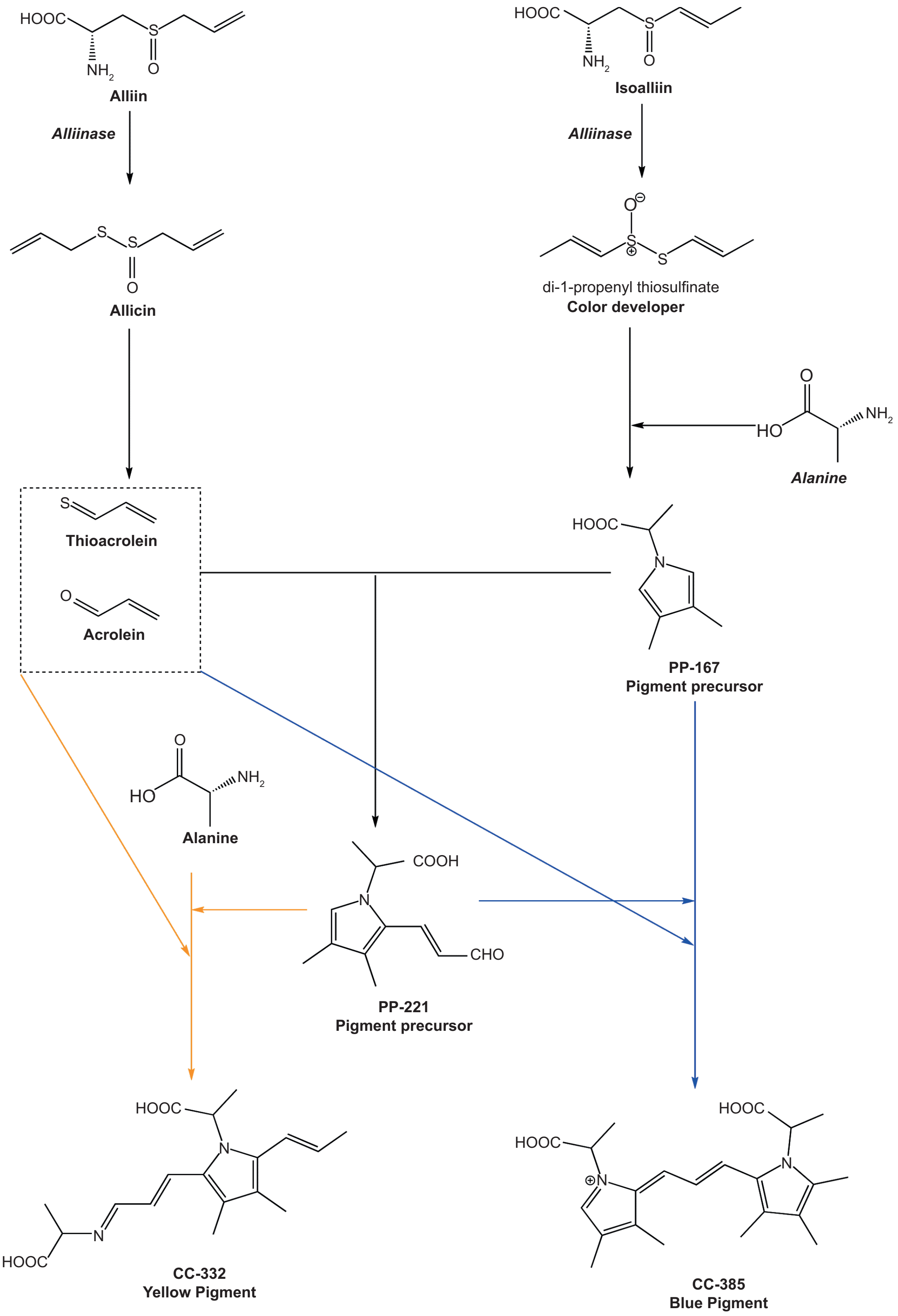

Figure 4. Formation of yellow (CC-332) and blue (CC-385) pigments from alliin and isoalliin. Other yellow and blue pigments can be formed, not shown in the figure.

Formation of pigments

Thiosulfinates are highly reactive, and thus they easily undergo nonenzymatic reactions yielding numerous sulfur-containing compounds responsible for sensorial attributes and bioactivities of *Allium* plants (Yoshimoto *et al*., 2019). Examples are acyclic sulfides, dithiines, and thiolanes that demonstrate bioactive properties similar to those of allicin (Block *et al*., 2018; Kubec *et al*., 2018; Martins *et al*., 2016; Štefanova *et al*., 2019).

1-Propenyl-containing thiosulfinates ("color developers") are converted into *N*-substituted 3,4-dimethylpyrroles, also called pigment precursors (PPs), which can further react with different (thio)carbonyl compounds generating colored compounds that are responsible for the pigmentation of many *Allium* bulbs, including garlic (*Allium sativum*), onion (*A. cepa*), and leek (*A. porrum*). Among these species, the biosynthetic pathway of these pyrroles is very similar even if it leads to different pigmentations, typically green for garlic and pink/red for onion and leek (Comparini *et al*., 2018; Kubec *et al*., 2015, 2017). On the other hand, the red pigment formed upon crushing of another *Allium* species, giant onion (*A. giganteum*), is not derived from isoalliin but from a different amino acid precursor, S-(2-pyrrolyl)cysteine sulfoxide (Kučerová *et al*., 2011).

In general, almost all abundant free amino acids present in onion, leek, and garlic, including alanine, glutamine, glutamic acid, asparagine, aspartic acid, arginine, and lysine, are largely involved in the production of pigments (Cho *et al*., 2009; Kubec and Velíšek, 2007; Lee *et al*., 2013). However, it has been established that different pigments and color intensities are related to different amino acids involved in formation of pyrroles. In 2010, Lee and colleagues detected several pink–red pigments, originated from onion juice added with 21 free amino acids, indicating that onion discoloration could be due to these different pigments. Lee *et al*. (2010) also demonstrated that some amino acids could be the precursors of more than one pigment, while others, such as histidine, serine, and cysteine, are not involved in the biosynthesis of pigments. In another study with hydrophobic amino acids, it was hypothesized that the intensity of garlic greening could be related to the dimension of the amino acid R group, namely, the smaller the size of the R group, the larger the color intensity (Wang *et al*., 2008b). Nevertheless, this hypothesis was not confirmed for hydrophilicamino acids as, in a following study, glutamic acid yielded a more intense green color compared to asparagine, which has a bigger R-group (Hu *et al*., 2010).

The typical discoloration that occurs in garlic cloves is represented by the development of a green pigmentation (Figure 1). This color is not due to the production of green pigments but rather arises from a mixture of yellow and blue pigments, originating following different pathways (Wang *et al*., 2008b). Even if a full consensus has not been reached on the single step, proposed mechanisms suggest that both alliin and isoalliin should be present for the onset of green coloration (Imai *et al*., 2006a; Kubec *et al*., 2017; Wang *et al*., 2008; Zang *et al*., 2013). According to Wang and colleagues (2008), yellow pigments result from the interaction between precursors derived from isoalliin and pyruvic acid originated from alliinase activity (Figure 2). In the same study, precursors derived from different amino acids (PP-AA) give rise to yellow pigments absorbing visible light in the range of 418–460 nm, following at 440 nm this order of intensity: PP-Ala > PP-Val > PP-Gly > PP-Ile > PP-Leu > PP-Phe. Conversely, the blue pigments contributing to green color were attributed to the interaction between different precursors and allicin. Even if several precursors developed a blue dye, only PP-Gly, PP-Ala, and PP-Val could determine a significant blue pigmentation in garlic (absorption in the range 590–610 nm). However, owing to their instability, blue dyes could in part be degraded into their yellow analogues (Wang *et al*., 2008; Zang *et al*., 2013), although it is unlikely that this hypothesized phenomenon is sufficient to prevent garlic discoloration.

Recently, a slightly different mechanism for the synthesis of both yellow and blue pigments was proposed by Kubec and colleagues (2017). In their study, two precursors and six color compounds (CC) were identified after a reaction between garlic extract and the enzyme alliinase. The proposed pathway for the formation of these color compounds (CC-332 and CC-385) should not involve pyruvic acid and exclude the possibility of mutual interconversion of blue- and yellow-colored species (Figure 4).

The control of garlic discoloration

With the exception of "Laba" preparation, in which garlic greening is desired (Tao *et al*., 2016), this phenomenon strongly affects the consumers' perception, reducing garlic marketability (Zang *et al*., 2013). Different strategies have been adopted by farmers, sellers, and retailers to control greening, including management of environmental conditions during storage and usage of additives added directly on garlic cloves (Madhu *et al*., 2019).

Discoloration control during fresh garlic storage

Discoloration in garlic is affected by several environmental variables that should be controlled during its storage and processing (Zang *et al*., 2013). Among these variables, acting on temperature offers the possibility of controlling the activity of GGT enzyme involved in the biosynthesis of greening precursors *S*-alk(en)ylcysteine sulfoxides. Indeed, the inhibition of this enzyme would lead to an early interruption of the pigments' biosynthesis pathway. To prevent germination, garlic is usually stored at a temperature of around –3°C for up to 9 months (Volk *et al.*, 2004). Under these conditions the GGT enzyme (also involved in germination) is almost inactive, resulting in low alliin and isoalliin accumulation (Lancaster and Shaw, 1991). After sale, storage of garlic at refrigeration temperatures (0–12°C) should be avoided, because the enzyme establish its maximum activity at 4°C, thus resulting in a high production rate of *S*-alk(en)ylcysteine sulfoxides. It was demonstrated that, upon a long-term refrigerated storage, garlic cloves developed, after crushing, much greener dye compared to those stored for a shorter time at the same conditions. On the contrary, it was observed that storing garlic at 35°C drastically reduced the activity of GGT, resulting in the decrease of greening and improvement in product's shelf-life (Li *et al.*, 2008). Despite the fact that temperature of 35°C appears to be optimum for alliinase activity (Mishra *et al.*, 2001), the concentration of its substrate (*S*-alk(en)ylcysteine sulfoxides) is reduced due to the thermal inhibition of GGT. In addition, the temperature of 35°C favors the spontaneous conversion of isoalliin (one of the major *S*-alk(en)ylcysteine sulfoxides) into cycloalliin, which is not involved in the greening phenomenon as it is not a substrate of alliinase. This mechanism could be exploited also in the case of refrigerated storage. Indeed, if cold storage conditions (below 0°C) are not available, cloves can be stored at refrigerated conditions and then subjected to warm storage (35–40°C for 2 weeks) to allow the conversion of isoalliin to cycloalliin and produce garlic bulbs without noticeable changes in color (Yamazaki *et al.*, 2012).

Another important factor that could be controlled during storage is the concentration of oxygen and carbon dioxide present in the atmosphere. Indeed, the storage in controlled atmosphere, normally applied to prevent spoilage, also aids in controlling garlic greening. Encouraging results in terms of final quality, sprout growth, spoilage delay, and discoloration of garlic bulbs have been reported after keeping garlic cloves for up to 6.5 months at 0–1°C in a controlled atmosphere containing 0.5% O_2, 5–15% CO_2, and 60–70% relative humidity, compared to normal atmosphere and modified atmosphere, in which only O_2 or CO_2 concentration was adjusted (0.5% O_2/normal CO_2 and normal O_2/10% CO_2) (Cantwell *et al.*, 2003). The positive effect of low O_2/high CO_2 conditions combined with cold storage was also demonstrated for onion, in which decrease in discoloration was attributed to a slowdown of alliinase activity (Uddin and MacTavish, 2003).

However, it is important to consider that the control of both temperature and atmosphere composition during storage does not appear to be resolutive; indeed, even if the activity of enzymes is inhibited and the production of *S*-alk(en)ylcysteine sulfoxides is limited, all conditions favorable for developing greening phenomenon could be restored at the end of the storage and/or during transportation and retail.

Discoloration control during fresh garlic processing

In Mediterranean countries, garlic is primarily consumed fresh, while in Asia and other regions it is generally processed to obtain products such as paste, powder, puree, and macerate cloves (Li *et al.*, 2015). Owing to crushing, these processes could increase the risk of greening of these products. Nevertheless, the addition of additives and/or the adoption of specific techniques, which are normally not available for fresh garlic, during the production process can offer some possibilities to control discoloration.

Control by acidification, heat treatments, and drying

The level of acidity (pH) affects both enzymatic and nonenzymatic reactions leading to pigment formation in garlic. Nonenzymatic reactions are promoted in strong acidic conditions (pH 2–3), but they still take place at pH 5–6, which makes these difficult to control by acting on acidity (Zang *et al.*, 2013). Conversely, enzymatic reactions, in general, and alliinase activity, in particular, are inhibited at pH < 3. Since the normal pH of garlic is in the range 4.5–5.5, acidification can represent a relevant tool to control greening of some garlic-based products (Bai *et al.*, 2006). The pH drop could be achieved by adding acidifying agents, such as organic acids, during maceration or immediately after cutting or crushing, resulting in the inhibition of alliinase activity. However, it is important to consider that monocarboxylic acids such as acetic acid are great promoters of garlic greening, since they facilitate tonoplast permeability, allowing alliinase to come in contact with its substrate *S*-alk(en)ylcysteine sulfoxides (Zang *et al.*, 2013). As observed previously, once imported into the cell, the dissociated form of acetic acid is able to damage tonoplast and other cellular structures (Bai *et al.*, 2006). Indeed, for some processed garlic products (such as "Laba"), maceration of cloves with vinegar promotes the desired greening in spite of low pH (between 2 and 3) of vinegar (Li *et al.*, 2015). This is due to the slow acidification of the inner parts of intact cloves during maceration that allows the acetic acid to permeate tonoplast before alliinase inhibition. Therefore, control of greening by organic acid additions should involve only non-monocarboxylic acids such as ascorbic acid, malic acid, and citric acid, which do not affect tonoplast's integrity, possibly because plants develop specific systems to manage these acids stored naturally in vacuole (Bai *et al.*, 2006). Indeed, when tested on garlic, ascorbic acid, malic acid, and citric acid reported a substantial inhibition of green color development (Bai *et al.*, 2006; Kim *et al.*, 1999).

As many other enzymes, GGT and alliinase can be inactivated by high temperature treatments. In fact, in garlic processing the inactivation of both the enzymes can be achieved by blanching. This treatment involves heating at 70–100°C for 1–30 min with hot water or microwaves and is applied to processed vegetables to achieve enzymatic inactivation and reduce microbial contamination. Blanching conditions are poorly tolerated by both GGT and alliinase, whose activity rapidly drops after 10 min at 70°C (Huang *et al.*, 2019; Yin *et al.*, 2009), whereas complete inactivation was reported after 30 min at 70°C for GGT and after 15 min at 90°C for alliinase (Mochizuki *et al.*, 1988; Rejano *et al.*, 1997). However, heat treatments also cause a decrease in the contents of allicin and other antioxidant molecules, leading to impoverishment in the bioactive properties of garlic. A recent study calculated the kinetics of GGT and alliinase inactivation along with that of decrease in concentration and antioxidant activity of allicin (Huang *et al.*, 2019). Following a holistic approach, these authors proposed a blanching treatment of 4 min at 90°C, which seemed to be a good compromise to obtain an effective enzyme inactivation and limited losses of allicin and other valuable compounds. Nevertheless, it should be established that blanching is effective only if performed before the accumulation of thiosulfinates formed by the catalytic action of alliinase because the following nonenzymatic reactions leading to production of pigments have established to be strongly promoted by high temperatures (Imai *et al.*, 2006b).

While the techniques to control greening based on pH modification and heat treatments negatively reduce the accumulation of allicin, drying could represent a compromise between the control of greening and the accumulation of bioactive compounds. It is well known that the drying process can limit many enzymatic and nonenzymatic reactions responsible of food spoilage and degradation by decreasing the activity of water (Baysal *et al.*, 2003; Fante and Noreña, 2015). In the case of garlic, recent studies have investigated the possibility to preserve its original color while maintaining a good content of allicin by drying fresh garlic immediately after cutting. All these studies established that the involvement of allicin (which was formed soon upon cutting) in pigment formation could be dramatically reduced by a drop in activity of water (Baysal *et al.*, 2003; Duan *et al.*, 2015; Fante and Noreña, 2015; İlter *et al.*, 2018; Utama-Ang *et al.*, 2018). Conventional air drying of garlic was also compared with more sophisticated techniques such as freeze-drying and microwave treatment. The last two methods reported effective results in terms of color maintenance by reducing the effect of the Maillard reaction, which is promoted at high temperatures/for long durations typical of conventional drying. In particular, microwave drying of garlic appeared to be suitable for industrial applications as it may offer the possibility of reducing treatment time and energy costs (Baysal *et al.*, 2003; İlter *et al.*, 2018).

Control by addition of additives

As mentioned above, isoalliin is involved in the discoloration process of garlic and other *Allium* plants. In onion, its corresponding sulfenic acid (1-propenesulfenic acid) can be converted into di-1-propenyl thiosulfinate (a pigment precursor) by a nonenzymatic reaction. In *Allium* species, this step could be partially inhibited by the action of the enzyme lachrymatory factor synthase (LFS). Indeed, LFS catalyzes the conversion of 1-propenylsulfenic acid into propanthial*S*-oxide (lachrymatory factor). Therefore, LFS is able to remove isoalliin from the pathway of formation of pigments, thus inhibiting discoloration (Cho *et al.*, 2012a; Zang *et al.*, 2013). By considering this discovery and that isoalliin is a key compound for garlic greening (Lukes, 1986), some authors hypothesized that the addition of LFS in garlic (where it is not naturally present) could prevent the appearance of green pigmentation. With the aim to suggest the application of LFS as a natural additive, in these studies dried onion was used as a source of LFS instead of adding the purified enzyme. It was reported that 5–15 g of onion powder per kilogram of fresh garlic prevented greening by decreasing the content of isoalliin-derived thiosulfinate (Cho *et al.*, 2012a; Lee *et al.*, 2012). Compared to others, this approach has the advantage of preserving the antioxidant activity of allicin because it allows this compound to remain available in garlic.

Other compounds have been applied to protect garlic from both greening and browning (Zang *et al.*, 2013). Addition of cysteine, ascorbic acid, sodium metabisulfite, and trisodium phosphate resulted in a significant drop of green pigmentation in ground garlic, and sodium metabisulfite was the most effective of all these compounds. In addition, it was demonstrated that binary, ternary, and quaternary mixtures of these additives, if containing sodium metabisulfite, were more effective than individual compounds in preventing both greening and browning (Kim *et al.*, 1999).

Control by other technologies

The need to control greening without affecting sensory properties of garlic has led to the application of other more advanced technologies. γ-Irradiation is a widespread technology that is used to process plant foods (for review, see Farkas, 2006). First attempts were made to evaluate the impact of application of both γ-irradiation and high hydrostatic pressure on development of green color during storage of garlic (Ceci *et al.*, 1992; Hong and Kim, 2001). Thanks to the slowing of certain metabolic processes and inhibition of enzymes such as polyphenol oxidase, both technologies were effective in preventing germination, senescence, and browning (Farkas, 2006; Hong and Kim, 2001; madhu *et al.*, 2019), but no impact on the development of greening and its related enzymes was recorded. In addition, high-pressure treatments probably damaged garlic tissues, thus promoting the contact between alliinase and its substrates *S*-alk(en)-ylcysteine sulfoxides (Tao *et al.*, 2016).

More recent studies have evaluated the impact of light of different spectra on developing greening during storage of garlic (Comparini *et al.*, 2018; He *et al.*, 2019). A significant delay in the process of greening was reported after the application of green, blue, and yellow lights, and a combination of them, during storage. Even if not resolutive, light-control could represent an additional tool to be integrated in strategies aimed to prevent greening. Indeed, the application of light has a negligible impact on garlic integrity, thus making this technique accessible and easily acceptable.

Conclusions

Greening represents a major technological problem significantly affecting garlic marketability. In spite of some missing information, great advancements have been made during the last decade in understanding the biosynthetic pathways leading to the formation of pigments in *Allium* plants. Some of these findings have improved the available strategies aimed at controlling garlic greening. Innovative approaches that would deserve more attention and research are those based on the use of natural additives, as in the case of dried onion addition. However, new strategies to be developed to prevent greening of garlic should in any case also consider the preservation of the compounds known to impact both consumer health and flavor of garlic.

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Optimization of stir-baked technology for Flos Sophorae Immaturus tea according to quadratic regression rotation-orthogonal design method and quality evaluation

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PAPER

Abstract

This study aimed to optimize the stir-baked technology for Flos Sophorae Immaturus tea (FSIT) and evaluate the quality of FSIT. The optimum stir-baked conditions were found to be as follows: amount, 3.9 kg; rotation speed, 400 r/min; and time required to reach the temperature of 120°C, 5 min and maintained for 3.9 min after adding 15 mL of 1% stevioside. The machine-made FSIT soup was clear and golden in color, with charred taste, no bitterness, no peculiar smell, and improved sensory quality under the above-mentioned conditions. Heavy metal contents and microorganisms did not exceed the national standards.

Keywords: Flos Sophorae Immaturus, quadratic regression rotation-orthogonal design, quality, stir-baked

Introduction

Flos Sophorae Immaturus (FSI), which originated from the flower buds of *Sophora japonica L.*, is a medicinal food homology crude drug planted throughout China. China, which has rich resources, is the origin of Flos Sophorae Immaturus. It is bitter, slightly cold, nontoxic, and distributed in the large intestine and liver channel. It is effective in cooling blood and stopping bleeding, clearing the liver, and reducing fire. Flos Sophorae Immaturus contains large amounts of flavonoids and glycosides, such as rutin, narcissoside, quercetin, isorhamnetin, kaempferol, genistein, total polysaccharides, saponin, tannin, sterol, and vitamin A (Liu *et al.*, 2016; Xie *et al.*, 2014; Krishna, *et al.*, 2012), and has good curative effect on myocardial circulation. Moreover, the drug clears body heat, detoxifies, lowers blood fat and blood pressure levels, softens the blood vessels, reduces inflammation, tones the kidney, prevents arteriosclerosis, and has beauty-holding and antiaging properties (Chua, 2013; He *et al.*, 2016). Flos Sophorae Immaturus has an extremely high nutritional value and contains 19 kinds of amino acids, including essential amino acids required for the human body. The protein content in Flos Sophorae Immaturus is as high as 19.03%, which is 2.2-fold that of common health food silver almond (Wang and Wang, 2009). With emphasis on healthcare, the research and development of functional foods containing Flos Sophorae Immaturus has become extensive, especially the development of Flos Sophorae Immaturus tea (FSIT), which has become a focus of research by the Flos Sophorae Immaturus processing industry.

The stir-baked method for Flos Sophorae Immaturus was first recorded in the Song dynasty, and the Chinese Pharmacopoeia (edition 2015) has also recorded stir-baked Flos Sophorae Immaturus. In Chinese medicine, stir-baked Flos Sophorae Immaturus can reduce the side effects of bitter cold, and is used in patients with spleen deficiency. The stir-baked substitute tea prepared from Flos Sophorae Immaturus has evident curative effects on diabetes, hypertension, vascular sclerosis, constipation, various hemorrhoids, pharyngeal dryness, sore throat, red eyes and heat, and heart irritability (Li *et al*., 2017). At present, domestic research on FSIT is primarily focused on handmade or simple mechanism and the development of new compound FSIT (Jiang and Chen, 2008), although scientific reports on the key technologies of the stir-baked process are relatively few. The evaluation method of FSIT is primarily limited to sensory evaluation, and the FSIT production cannot be evaluated through modern methods of measurement.

In the present study, the shape, soup color, aroma, and taste of FSIT are used as sensory indicators. Water extract content, total polysaccharides, total flavonoids, and rutin, narcisin, quercetin, and isorhamnetin contents are used as chemical indicators. The weight distribution was determined through projection pursuit clustering (PPC) with objective assignment, and comprehensive vector distance (*Ci*), used as an evaluation index, was obtained by analyzing the sensory and chemical indices through the Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) method. On the basis of single factor, the quadratic regression rotation-orthogonal combination design was applied for the optimization of the process conditions for stir-baked FSIT, development of an FSIT variant with burnt flavor, and without bitterness and peculiar smell, and evaluation of sensory, physical, and chemical indices of production. This design can provide reference for the development and utilization of Flos Sophorae Immaturus and quality control of FSIT.

Materials and Methods

Materials and reagent

The Flos Sophorae Immaturus sample was collected in 2018 at the Flos Sophorae Immaturus base of Maojiawan, Changping Township, Wanzhou District, Chongqing City. The sample was identified as a dry flower bud of *S. japonica L.* by a researcher (Longyun Li) of Chongqing Academy of Chinese Materia Medica. Rutin reference substance (batch number: MUST−16070511, purity 99%), quercetin reference substance (batch number: MUST−16072203, purity 98%), and isorhamnetin reference substance (batch number: MUST−16092001; purity of 98%) were purchased from Chengdu Mansite Biotechnology Co. Ltd. Narcissoside reference substance (batch number: TAQT−LRLA; purity of 93.1%) was purchased from National Institutes for Food and Drug Control. As a standard solution, the lead (Pb) and cadmium (Cd) standard solutions were purchased from American Sigma Company. Acetonitrile, methanol, and acetic acid of chromatographic grade were purchased from TEDIA Company (USA). All other solvents and chemical reagents were of analytical grade or effective.

Instruments and equipment

Instruments and equipment included are as follows: Agilent 1263 HPLC (Agilent Company, USA), UV-2600 ultraviolet spectrophotometer (Shimadzu Corporation, Japan), Milli-Q Integral 5 pure water meter (Millipore Company, USA), BSA 124-S electronic scales (Sartorius Company, Germany), KQ-250 DB numerical control ultrasonic cleaning instrument (Kunshan Ultrasonic Instrument Co. Ltd., China), XMTB digital display electric thermostatic water bath (Shanghai Yuejin Medical Instrument Co. Ltd., China), G2X-9240 MBE electricity heat drum wind drying oven (Shanghai Boxun Industrial Co. Ltd., China), ZDHW temperature regulating electric heating sleeve (Beijing Zhongxing Weiye Instrument Co. Ltd., China), and CY-900 drum-type medicine stir-fry machine (Kanghua Pharmaceutical Machinery Co. Ltd., China).

FSIT preparation

High-quality Flos Sophorae Immaturus with full granules and large grain size was selected, cleaned, placed in a self-made sterilizing device (patent application number: 201920071851.3), piled up, steamed at a thickness range of 4–6 cm, and cooked for 15–20 min after boiling. Then it was taken out and dried for use. According to the stir-baked temperature, rotation speed, and time designed for the experiment, an appropriate amount of Flos Sophorae Immaturus was placed in a drum stir-baked machine for frying, and an appropriate amount of 1% stevia glycoside was added to it for flavor mixing. At the end of frying, after cooling for 1 h with a blower, the sample was placed for two times in a double-layer vibrating screening machine through 90-mesh screen. Finally, the sample was placed in the color selection machine for removing burnt paste and black Flos Sophorae Immaturus, and the finished product of FSIT was obtained.

Single factor design of stir-baked process for FSIT

Stir-baked amount

The fixed stir-baked temperature was 120°C, and the motor synchronous speed was 400 r/min. At 120°C, the selected stir-baked amounts were 2, 3, 4, 5, and 6 kg. We analyzed water extract, total polysaccharides, total flavonoids, and rutin, narcisin, quercetin, and isorhamnetin contents, and performed sensory evaluation for FSIT for determining preferred stir-baked amount.

Stir-baked temperature

The fixed stir-baked amount was 4 kg, the motor synchronous speed was 400 r/min, the selected stir-baked temperatures were 80, 100, 120, and 140°C. The sample was stir baked for 4 min after the target temperature was reached. Water extract, total polysaccharides, total flavonoids, and rutin, narcisin, quercetin, and isorhamnetin contents were analyzed, and sensory evaluation was performed for FSIT for determining the preferred stir-baked temperature.

Stir-baked rotation speed

The fixed stir-baked amount was 4 kg, and the stir-baked temperature was 120°C. The stir-baked time needed to reach the target temperature was 4 min, and the selected motor synchronous rotation speeds were 200, 300, 400, 500, and 600 r/min. We analyzed the water extract, total polysaccharides, total flavonoids, and rutin, narcisin, quercetin, and isorhamnetin contents, and performed sensory evaluation for FSIT to determine the preferred stir-baked rotation speed.

Stir-baked time

The fixed stir-baked amount was 4 kg, the stir-baked temperature was 120°C, and the motor synchronous rotated speed was 400 r/min. After stir-baking for 5 min and reaching 120°C, the stir-baked timings were 1, 2, 3, 4, 5, and 6 min for the test. We analyzed the water extract, total polysaccharides, total flavonoids, and rutin, narcisin, quercetin, and isorhamnetin contents, and performed sensory evaluation for FSIT to determine the preferred stir-baked time.

Secondary orthogonal rotating combination design of stir-baked process for FSIT

According to the results of the single factor test, four factors for stir-baking that is, amount, temperature, rotation speed, and time, were designed, thereby applying the quadratic regression rotation-orthogonal combination design with four factors and five levels to optimize the FSIT stir-baked process (Ye and Liu, 2017), see Table 1.

Table 1. Coding list of levels of various factors.

Levels code	Factors			
	Amount (kg) X_1	Temperature (°C) X_2	Time (min) X_3	Rotate speed (r/min) X_4
+2	6	140	6	600
+1	5	130	5	500
0	4	120	4	400
-1	3	110	3	300
-2	2	100	2	200

Deployment of flavor for FSIT

According to the best process parameters of the test, different volumes of 1% stevioside were sprayed with regard to time, and stir-baking was continued for a certain period. Ten professional tasters were invited to score different samples according to the sensory evaluation criteria mentioned in Table 2, and the average of the results was taken as the final sensory evaluation.

Determination of physical and chemical indicators for FSIT

Determination of moisture: Refer to the direct drying method in GB 5009.3-2016, 'Determination of moisture in foods'.

Determination of ash: Refer to the first method in GB 5009.4-2016, 'Determination of ash in foods'.

Determination of aflatoxin B1 content: Refer to the second method in GB/T 5009.22-2016, 'Determination of aflatoxin B and G in foods'.

Determination of lead, arsenic and cadmium content: Refer to the inductively coupled plasma-mass spectrometric method in GB/T 35876, Inspection of grain and oils—Determination of sodium, magnesium, kalium, calcium, chromium, manganese, iron, copper, zinc, arsenic, selenium, cadmium, and lead in cereals and derived products'.

Determination of water extract, total polysaccharides and total flavonoids: The sample processing and detection methods were tested according to the relevant methods furnished in the 2015 edition of the Pharmacopoeia of the People's Republic of China (National Pharmacopoeia Commission, 2015).

Determination of rutin, narcisin, quercetin, and isorhamnetin: Refer to the method introduced in a previous report (Tan *et al.*, 2018).

Determination of microbial indicators for FSIT

The total number of bacterial colonies and coliforms of salmonella and Staphylococcus aureus of S. japonica were determined with GB 4789-2016. Shigella assay was performed with GB 4789-2012.

Sensory evaluation of FSIT

According to GB/T 23776-2018, 'Methodology for sensory evaluation of tea', the method of password evaluation was adopted for sensory evaluation and score. The review procedure is as follows:

Sampling → comment on the shape → weighing 3 g →
brewing 200 mL of boiling water → turn over the
soup → look at the soup color → sniffing aroma → taste.

The product quality consisted of four factors, namely, shape, aroma, soup color, and taste, and each factor was evaluated at three levels, that is, poor, medium, and good. The scores were presented in percentage; the scores for shape, aroma, soup color, and taste were 25%, 30%, 10%, and 35%, respectively. Ten professional tasters were invited to evaluate taste according to the scoring criteria, and the average was taken as the final sensory score. The sensory score criteria are demonstrated in Table 2.

Weight determination by the projection pursuit clustering method

Pursuit Clustering is a new statistical method for processing and analyzing high-dimensional data. The basic idea is to project high-dimensional data onto low-dimensional subspace and determine the optimal projection direction reflecting data structure or characteristics in solving the comprehensive evaluation of high-dimensional problems (Tang, 2010). According to the size of the projection direction, the weight coefficient of each evaluation index can be determined. In this study, the weight of the quality index of FSIT was calculated by the PPC method in DPS software.

Application of technique for order preference by similarity to ideal solution (TOPSIS) method

TOPSIS, as proposed by Hwang and Yoon in 1981 (Hwang and Yoon, 1981), is a sorting method based on the proximity of finite evaluation objectives to ideal objectives. TOPSIS is an effective method that is used commonly in multi-objective decision-making analysis, whose basic idea is to explore optimal and worst solutions (represented by the optimal vector D_i^+ and worst vector D_i^-, respectively) in the finite solutions on the basis of normalized original matrix. Then the distance of each evaluation objective to the optimal and worst solutions is calculated. Thus, the relative proximity between the evaluation objective and optimal solution (represented by the C_i) as the basis of optimal or worst solution evaluation was obtained (Wang *et al.*, 2019; Tang, 2010). The specific method is as follows: *n* evaluation objectives and *m* evaluation indices are set, and the original data could be written as matrix $X = (X_{ij})_{n\times m}$. High- (the larger the better) and low-quality indices (the smaller the better), that is, $Z_{ij} = \frac{X_{ij}}{\sqrt{\sum_{i-1}^{n} X_{ij}^2}}$ and $Z_{ij} = \frac{X_{ij}}{\sqrt{\sum_{i=1}^{n} (1/X_{ij})^2}}$, respectively, were normalized. The normalized matrix was $Z = (Z_{ij})_{n\times m}$, with the optimal and worst vectors comprising maximum and minimum values in each column labeled as $Z^+ = (Z_{max1}, Z_{max2} \ldots Z_{max})$ and $Z^- = (Z_{min1}, Z_{min2} \ldots Z_{min})$, respectively. The distances between the evaluation objective *i* and the optimal and worst solutions were $D_i^+ = \sqrt{\sum_{j=1}^{m} (Z_{max\,j} - Z_{ij})^2}$ and $D_i^+ = \sqrt{\sum_{j=1}^{m} (Z_{max\,j} - Z_{ij})^2}$, respectively. The relative proximity between the evaluation objective *i* and optimal solution is as follows: $C_i = D_i^- / (D_i^+ + D_i^-)$.

Table 2. Sensory scoring standards for FSIT.

Factor	Grade	Index
Shape (25%)	Good (18–25 points)	With charred taste, even and coking yellow in color, full grain, texture solid, crisp
	Middle (9–17 points)	Light fragrance, uneven color, brownish yellow, individual grain broken
	Poor (0–8 points)	Light yellow or brownish brown, with obvious burnt smell and severe grain breakage
Soup Color (10%)	Good (8–10 points)	Clear and golden, no turbidity, no scattered particles
	Middle (4–7 points)	Brownish yellow, no turbidity, no scattered particles
	Poor (0–3 points)	Light green or tan, turbid, with scattered particles
Aroma (30%)	Good (21–30 points)	Strong taste with charred smell
	Middle (11–20 points)	Light fragrance, no charred smell
	Poor (0–10 points)	With charred smell
Taste (35%)	Good (26–35 points)	No astringency
	Middle (13–25 points)	With astringency
	Poor (0–12 points)	With paste taste and strong astringency

Index weight was introduced into the TOPSIS of DPS; the weight coefficient of the chemical indices was calculated using PPC; and the quality indices of the stir-baked FSIT were evaluated comprehensively by combining with TOPSIS. The larger the value of C_i, the better the effect of experimental method.

Results

Single-factor test on stir-baked process of FSIT

The factors influencing the stir-baked process of FSIT primarily included stir-baked amount, temperature, time, and speed. The assignment of water extract, total polysaccharides, total flavonoids, and rutin, narcissoside, quercetin, and isorhamnetin contents, as well as the sensory score in the preparation of FSIT, were evaluated by the objective PPC method and analyzed through the TOPSIS method using DPS software. The results are demonstrated in Table 3. Water extract, total polysaccharides, total flavonoids, and rutin, narcissoside, quercetin, and isorhamnetin contents, as well as the sensory scores, were used as evaluation indicators. According to the ranking of C_i, the FSIT sample with the best quality was obtained after the stir-baked amount was 4 kg, stir-baked temperature was 120°C, stir-baked rotation speed was 400 r/min, and stir-baked time was 4 min at a temperature of 120°C or above. Therefore, the following tests selected the stir-baked amount of 4 kg, the stir-baked temperature of 120°C, the stir-baked speed of 400 r/min, and the stir-baked time of 4 min at zero level.

Optimization of stir-baked process of FSIT by quadratic regression rotation-orthogonal combination design

According to single-factor experiments, the quadratic regression rotation-orthogonal combination design with four factors and five levels was used to optimize the stir-baked process of FSIT. A total of 36 combinations at five levels for each factor were present, and the experimental design and results are demonstrated in Table 4.

According to the test results (Table 4), the statistical analysis software DPS 17.10 was used to obtain the quadratic regression model of C_i for four experimental factors, such as stir-baked amount, temperature, time, and rotation speed, as follows:

$$Y = -19.80272 + 0.64512X_1 + 0.25508X_2 + 0.98855X_3 + 0.00966X_4 - 0.11938X_1^2 - 0.00106X_2^2 - 0.1095\ 2X_3^2 - 0.00001X_4^2 + 0.00189X_1X_2 - 0.00309X_1X_3 + 0.00021X_1X_4 - 0.00071X_2X_3 - 0.00001X_2X_4 - 0.00004X_3X_4$$

ANOVA was performed with F_1 = mean square loss/mean square error and F_2 = mean square regression/mean square residual. As demonstrated in Table 5, $F_{Lf} = F_1 = 0.3916 < F_{0.1(10,11)} = 2.25$ and the *P*-value was higher than 0.05 (0.9245), thereby suggesting that the lack of fit was not significant, that is, the regression equation fitted all the test points well, and no other unknown factors affected the results. $F_{regression} = F_2 = 11.2178 > F_{0.01\ (14,21)} = 3.03$ and $P = 0 < 0.\ 01$, thereby indicating that the regression equation was extremely significant, that is, the quadratic regression model was suitable. Among them, the influences of stir-baked temperature (X_2) and all quadratic terms on C_i were extremely significant at $a = 0.01$, stir-baked time (X_3) and stir-baked speed (X_4) were significant at a = 0.05, and the stir-baked volume (X_1) was significant at a = 0.1. However, all interaction terms were not significant. Therefore, at the significant level of $a = 0.1$, the regression equation was simplified by eliminating the nonsignificant terms as follows:

$$Y = -19.80272 + 0.64512X_1 + 0.25508X_2 + 0.98855X_3 + 0.00966X_4 - 0.11937X_1^2 - 0.00106X_2^2 - 0.1095\ 2X_3^2 - 0.00001X_4^2$$

The *P*-value influenced by the main effect of each factor can reflect the importance of each factor to the test index. The smaller the *P*-value, the greater the influence of the factor on test result, that is, the greater will be the importance (Sun *et al.*, 2016). As demonstrated in Table 5, the primary and secondary influences of the four factors on C_i followed the following order: stir-baked temperature (X_2) > stir-baked time (X_3) > stir-baked rotation speed (X_4) > stir-baked amount (X_1). The stir-baked temperature had a significant influence on C_i, followed by the stir-baked time, and the effect of stir-baked rotation speed was relatively small, but all reached a significant level ($P < 0.05$), and the stir-baked amount had no significant effect on experimental results ($P > 0.05$). The relationship between various factors and C_i is demonstrated in Figure 1. As demonstrated in Figure 1, the relationship between stir-baked amount (X_1) and C_i was nearly linear, thereby indicating that the stir-baked amount had minimal influence on C_i. The three other factors (i.e., X_2, X_3, and X_4) also had the same trend with increase in temperature, time, and rotation speed. C_i established an upward trend, among which the changed trend of temperature was the most evident one, thereby indicating that temperature had the most significant influence on C_i.

The frequency analysis method was used to find the good stir-baked conditions, and the frequency analysis results are demonstrated in Table 6. As demonstrated in Table 6, in the 95% Confidence Interval (CI), the average of C_i was more than 0.50. The optimized stir-baked scheme is as follows: stir-baked amount = 3.9 kg, stir-baked temperature = 121°C, stir-baked time after reaching the target temperature = 3.9

Table 3. **Results of single-factor test.**

Factor	Parameter	Content (%)							Sensory-score	C_i	Rank
		Water extract	Total polysaccharides	Total flavonoids	Rutin	Narcissoside	Quercetin	Isorhamnetin			
Amount (kg)	2	32.30	1.18	31.41	25.99	0.79	0.49	0.34	56	0.04	5
	3	31.63	1.89	32.70	26.75	0.88	0.69	0.35	70	0.74	2
	4	35.39	1.89	32.89	27.57	0.93	0.69	0.35	83	0.99	1
	5	32.47	1.74	32.97	27.54	0.91	0.49	0.34	79	0.57	3
	6	30.85	1.88	32.53	26.44	0.81	0.50	0.33	72	0.51	4
Temperature (°C)	80	30.98	1.66	30.24	26.10	0.86	0.64	0.33	49	0.35	4
	100	31.63	1.89	32.70	26.75	0.88	0.69	0.35	70	0.79	3
	120	39.92	2.03	33.12	27.28	0.88	0.69	0.36	75	0.90	1
	140	35.08	2.14	34.48	26.67	0.85	0.69	0.34	65	0.79	2
	160	33.72	1.89	30.92	25.58	0.81	0.63	0.33	32	0.15	5
Rotate speed (r/min)	200	29.31	1.68	26.08	27.42	0.81	0.49	0.28	60	0.04	5
	300	31.63	1.89	32.70	26.75	0.88	0.69	0.35	70	0.67	4
	400	38.21	1.78	32.44	26.17	0.87	0.62	0.38	78	0.91	1
	500	38.70	1.50	35.06	26.16	0.88	0.61	0.35	78	0.73	2
	600	38.15	1.24	34.44	25.47	0.85	0.59	0.34	79	0.68	3
Time (min)	1	31.73	2.12	32.00	26.01	0.88	0.58	0.30	37	0.30	6
	2	33.99	1.63	32.18	26.51	0.89	0.59	0.32	50	0.31	5
	3	31.63	1.89	32.70	26.75	0.88	0.69	0.35	70	0.72	3
	4	39.62	1.90	34.48	27.40	0.92	0.63	0.38	78	0.84	1
	5	34.37	1.94	34.32	25.62	0.87	0.60	0.35	73	0.75	2
	6	33.84	1.66	32.74	26.24	0.79	0.47	0.33	69	0.55	4

Note: D_i^+ is the optimal vector distance; D_i^- is the distance of the worst vector; C_i is the relative proximity of optimal value.

Table 4. Experimental design and results of quadric regression orthogonal rotation combination with four factors and five levels.

Test number	X_1	X_2	X_3	X_4	Content determination (%)							Sensory score	C_i
					Water extract	Total polysaccharides	Total flavonoids	Rutin	Narcissoside	Quercetin	Isorhamnetin		
1	1	1	1	1	33.80	1.67	38.68	26.88	1.03	0.56	0.33	82	0.28
2	1	1	1	–1	34.55	1.34	38.43	31.16	1.00	0.53	0.29	81	0.20
3	1	1	–1	1	47.02	1.26	34.81	29.83	0.98	0.54	0.32	79	0.27
4	1	1	–1	–1	38.20	1.58	33.31	28.4	0.96	0.55	0.33	75	0.23
5	1	–1	1	1	37.00	1.31	33.75	27.61	0.94	0.47	0.30	72	0.16
6	1	–1	1	–1	35.74	1.45	33.21	26.79	0.97	0.47	0.32	80	0.19
7	1	–1	–1	1	35.58	1.73	30.67	27.34	0.95	0.5	0.31	78	0.24
8	1	–1	–1	–1	35.85	1.25	34.42	28.08	0.96	0.44	0.28	77	0.15
9	–1	1	1	1	39.16	1.42	37.15	27.38	0.96	0.43	0.28	81	0.21
10	–1	1	1	–1	34.99	1.81	38.18	30.53	1.07	0.42	0.27	69	0.30
11	–1	1	–1	1	35.53	1.43	34.71	30.72	1.10	0.44	0.28	80	0.23
12	–1	1	–1	–1	36.83	1.87	40.86	30.88	1.10	0.47	0.29	81	0.35
13	–1	–1	1	1	34.52	1.8	38.24	31.01	1.08	0.45	0.28	76	0.30
14	–1	–1	1	–1	33.25	1.74	37.4	30.56	1.10	0.48	0.30	75	0.30
15	–1	–1	–1	1	28.10	1.69	38.04	29.82	1.12	0.47	0.30	70	0.28
16	–1	–1	–1	–1	30.34	1.58	38.41	31.77	1.10	0.45	0.29	79	0.26
17	–2	0	0	0	28.96	1.31	39.13	31.54	1.07	0.46	0.29	81	0.21
18	2	0	0	0	31.51	1.97	41.88	31.85	1.09	0.43	0.27	78	0.35
19	0	–2	0	0	34.27	1.99	40.44	30.33	1.08	0.44	0.28	70	0.35
20	0	2	0	0	34.40	1.81	41.01	31.97	1.09	0.42	0.27	71	0.31
21	0	0	–2	0	31.70	1.67	41.88	32.09	1.15	0.40	0.26	78	0.31
22	0	0	2	0	34.30	1.86	40.47	28.49	1.06	0.53	0.31	75	0.33
23	0	0	0	–2	37.22	1.9	37.33	27.12	0.83	0.68	0.35	73	0.30
24	0	0	0	2	38.86	1.84	34.88	26.44	0.83	0.73	0.37	78	0.31
25	0	0	0	0	38.62	1.91	39.94	29.18	1.284	0.73	0.37	83	0.49
26	0	0	0	0	37.38	1.96	38.17	30.52	1.18	0.64	0.40	88	0.44

(continues)

Table 4. Continued

Test number	X_1	X_2	X_3	X_4	Content determination (%)							Sensory score	C_i
					Water extract	Total polysaccharides	Total flavonoids	Rutin	Narcissoside	Quercetin	Isorhamnetin		
27	0	0	0	0	39.93	1.83	44.62	30.79	1.94	0.58	0.37	85	0.73
28	0	0	0	0	39.56	1.91	42.9	35.67	1.97	0.58	0.37	89	0.74
29	0	0	0	0	38.80	1.97	39.21	30.44	1.97	0.58	0.37	80	0.73
30	0	0	0	0	37.20	1.925	43.38	29.34	1.97	0.59	0.37	85	0.73
31	0	0	0	0	38.58	2.15	44.84	31.3	1.96	0.62	0.39	81	0.77
32	0	0	0	0	38.68	1.89	44.19	33.17	1.77	0.94	0.49	82	0.79
33	0	0	0	0	37.62	1.96	41.98	34.22	1.74	0.99	0.52	83	0.78
34	0	0	0	0	38.33	1.96	39.97	30.08	1.74	0.93	0.47	81	0.77
35	0	0	0	0	39.13	2.06	43.69	31.01	1.85	0.74	0.44	80	0.80
36	0	0	0	0	37.85	2.16	42.04	30.03	1.78	0.93	0.49	80	0.81

Table 5. ANOVA table of regression model.

Sources of variation	Sum of squares	Degrees of freedom	Average square	Standard regression coefficient	*F*-values	*P*-values
X_1	9.9885	1	9.9885	2.3281	1.7559	0.0937
X_2	156.1570	1	156.1570	9.2051	5.4658	0
X_3	23.4535	1	23.4535	3.5674	2.6906	0.0137
X_4	22.4100	1	22.4100	3.4871	2.6301	0.0156
X_1^2	22.3462	1	22.3462	–3.4822	6.6377	0
X_2^2	156.1020	1	156.1020	–9.2035	5.9017	0
X_3^2	18.8064	1	18.8064	–3.1945	6.0894	0
X_4^2	19.8862	1	19.8862	–3.2849	6.2617	0
X_1X_2	1.3810	1	1.3810	0.8657	0.7441	0.4650
X_1X_3	0.0075	1	0.0075	–0.0637	0.1214	0.9045
X_1X_4	0.3599	1	0.3599	0.4419	0.8424	0.4091
X_2X_3	0.1944	1	0.1944	–0.3248	0.2791	0.7829
X_2X_4	0.4848	1	0.4850	–0.5129	0.4408	0.6638
X_3X_4	0.0133	1	0.0133	–0.0851	0.1622	0.8727
Regression		14	0.1161		$F_2 = 11.2178$	0
Surplus		21	0.0103			
Loss of quasi		10	0.0057		$F_1 = 0.3916$	0.9245
Error		11	0.0146			
Sum		35				

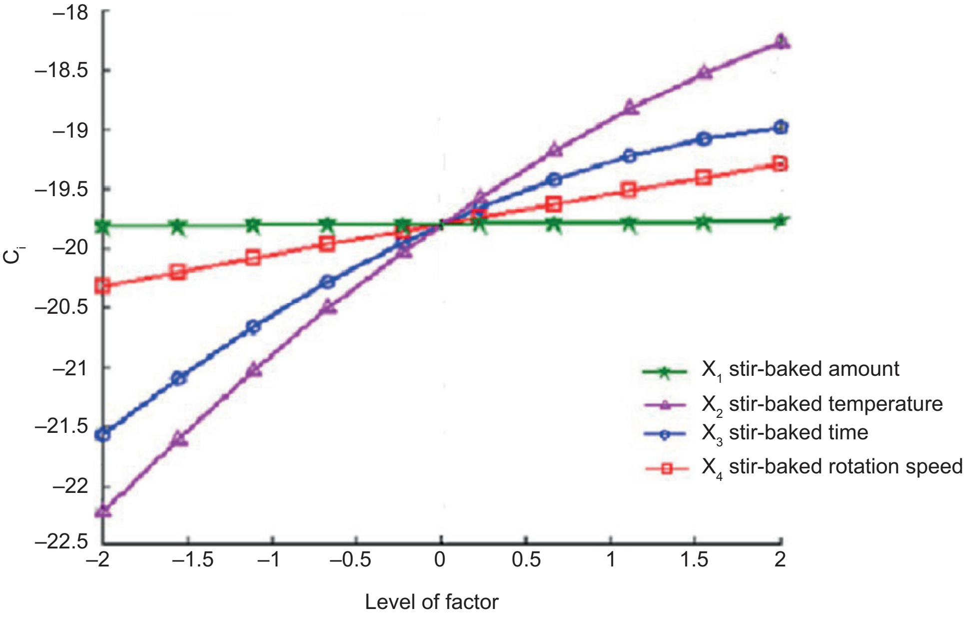

Figure 1. Influence of various factors on comprehensive vector distance (Ci).

min, and stir-baked rotation speed = 393 r/min. However, considering the practical operability of the equipment and production, the optimization scheme was appropriately modified. The modification scheme is as follows: stir-baked amount = 3.9 kg, stir-baked temperature w= 120°C, stir-baked time after reaching the target temperature = 3.9 min, and stir-baked rotation speed = 400 r/min.

Study on taste blending of FSIT

In Section 2.2.4, we obtained the best stir-baked process parameters, that is, the stir-baked amount was 3.9 kg, the stir-baked rotation speed was 400 r/min, and the stir-baked time after reaching 120°C was 5 min; 1% stevia glycoside of different volumes was added, and the stir-baked

Table 6. Value frequency distribution of stir-baked amount (X_1), temperature (X_2), time (X_3), and rotation speed (X_4).

Level	X_1 (kg)		X_2 (°C)		X_3 (min)		X_4 (r/min)	
	Number	Frequency	Number	Frequency	Number	Frequency	Number	Frequency
–2	0	0	0	0	0	0	0	0
–1	3	0.2	3	0.2	2	0.13	3	0.2
0	10	0.67	8	0.53	12	0.8	10	0.67
1	2	0.13	4	0.27	1	0.067	2	0.13
2	0	0	0	0	0	0	0	0
95% Confidence Interval (CI)	–0.3569~0.2236		–0.2774~0.4107		–0.2905~0.1571		–0.3569~0.2236	
Stir-baked conditions	3.63~4.22		117.22~124.11		3.71~4.16		364.31~422.36	
Average	3.9		121		3.9		393	

was conducted for 3.9 min. According to the results of scoring, the product obtained by adding 15 mL of 1% stevia glycoside tasted good. Therefore, the result of the taste blending scheme of FSIT is as follows: 15 mL of 1% stevia glycoside was added per 3.9 kg of Flos Sophorae Immaturus.

Study on quality of FSIT

The sensory evaluation of FSIT was conducted according to the requirements of sensory indices of the National Industry Standard of Substitute Tea. We observed the appearance of the obtained tea: the color was burnt yellow, the grain was full, the texture was firm and crisp, and the smell had a strong coke flavor. Under natural light, the soup of FSIT was clear and golden in color; had no turbidity; had no scattered particles; had no astringency; and had charred taste through watching, smelling, and tasting.

The physicochemical and microbial indicators of FSIT were determined according to the requirements of the Chinese Pharmacopoeia and the National Industry Standard on Physical and Chemical Indices of Substitute Tea and microbiological indicators on food microbial national standards. The results are demonstrated in Table 7; all the indicators complied with the relevant provisions of the industry standards, and the contents of all kinds of microorganisms did not exceed the values specified in the National Standards.

Discussion

In this experiment, the sensory and chemical indices of the multicomponent content were used to: evaluate the stir-baked process of the new mechanism of FSIT comprehensively; optimize the best process; avoid the limitation and inaccuracy caused by the evaluation of single index; make the stir-baked process of FSIT reasonable; and perfect the quality evaluation system. For the selection of the content evaluation indices, water extract was the general term of soluble substances that could be dissolved in hot water in the tea soup and a comprehensive index that represented the overall level of flavor components in the tea soup (Liu *et al*., 2014). Flavonoids could not only enhance the capillary and has anti-inflammatory, antispasmodic, antiulcer, hypolipidemic, and other pharmacological effects but could also cool the blood to stop bleeding, clear the liver, and purge fire and heat. Flavonoids are one of the substances with evident biological activity and physiological functioning in FSIT (Liu *et al*., 2018). Although the total content of polysaccharides in Flos Sophorae Immaturus is not high but has anti-inflammatory and antiviral effects, maintains vascular resistance, is rouge, and inhibits aldose reductase. The total content of polysaccharides is also one of the basic substances involved in life activities (Zhou and Xia 2011).

Table 7. Physical and chemical indices of FSIT.

Project	Index	Measured value
Total flavonoids (%)	>20	36.87 ± 0.38
Rutin (%)	>15	29.49 ± 0.72
Moisture (%)	≤13	2.43 ± 0.25
Total ash (%)	≤12	5.23 ± 0.25
Aflatoxin B_1 ($\mu g \cdot kg^{-1}$)	≤5	3.20 ± 0.10
Lead ($mg \cdot kg^{-1}$)	≤5	0.8 ± 0.10
Total arsenic ($mg \cdot kg^{-1}$)	≤0.5	0.13 ± 0.06
Cadmium ($mg \cdot kg^{-1}$)	≤0.5	0.26 ± 0.06
Total number of colonies ($cfu \cdot g^{-1}$)	≤30,000	17,000
Coliform ($MPN \cdot g^{-1}$)	≤30	15
Staphylococcus aureus	Not detected	Not detected
Salmonella	Not detected	Not detected
Shigella	Not detected	Not detected

The single-factor experiment selects PPC weight analysis combined with TOPSIS as a comprehensive evaluation method for initial screening and stir-baked conditions. Given the difference in the contribution of sensory and chemical indices in evaluating the quality of FSIT in the stir-baked process, the direct use of subjective valuation method to determine the weight coefficient is biased. The PPC method in DPS software is a relatively objective weight assignment method that avoids artificial interference factors of expert grading and eliminates grading steps. Therefore, single-factor experiment is more scientific, reasonable, and advantageous than the subjective weight assignment method. The TOPSIS method could provide effective solutions for the optimization of different evaluation indices and comprehensive evaluation of target groups (Ning *et al.*, 2018) and simplify the statistical analysis of multi-index variable data. The TOPSIS method could be used as an auxiliary analysis method to compare different cooking conditions of Flos Sophorae Immaturus in improving work efficiency and accuracy.

Orthogonal and uniform experiments are generally used to optimize the extraction or stir-baked process. Although these methods avoid a large number of repetitive experiments, they also have defects, such as poor precision and limited scope of application. In this experiment, four factors, including stir-baked amount, temperature, time, rotation speed, and five levels of each factor, were investigated in the stir-baked process of FSIT. The quadratic regression rotation-orthogonal combination design is the best choice. This design is a kind of experimental design method with orthogonal, regression, uniform, and high saturation. This design belongs to an advanced experimental design technique that not only overcomes the faults that the quadratic regression forecast variance in the regression orthogonal design depends on sites in the factor space position but also retains the advantages of the regression orthogonal design experiment (Ye and Liu, 2017) and provide scientific basis for industrialization production of FSIT.

Water extract and volatile components are the material basis of tea flavor. In this report, the descriptions of chemical characteristics of different tea soups prepared from the same fresh Flos Sophorae Immaturus with different stir-frying techniques are based on the relative content of each component. The fresh raw materials of tea and the biochemical composition of tea soup have their own unique index profiles. The multi-component chemical pattern recognition method of tea soup contributes to the better quality control of tea soup. The next step would be to study the overall change of the chemical profile of tea soups because of different fresh raw materials and the processing techniques. In addition, since there are various biochemical components in tea soups, it remains to be explored how to establish a stoichiometry scheme that correlates quality of the tea soup flavor with its main components (including sensory threshold).

Conclusions

The combined PPC-TOPSIS method with the quadratic orthogonal rotation combination design method was used to investigate the four factors, namely, stir-baked amount, temperature, time, and rotation speed, of the machine-made FSIT. The contents of seven components and sensory scores were taken as inspection indices to study the key technology of stir-baked FSIT, and the optimum stir-baked conditions are as follows: stir-baked amount: 3.9 kg, stir-baked rotation speed: 400 r/min, and time needed to reach the temperature of 120°C: 5 min and maintained for 3.9 min after adding 15 mL of 1% stevioside. The technology had high stability and simple operation, and the results established that the machine-made FSIT soup was clear and golden in color, with charred taste, no bitterness, no peculiar smell, and improved sensory quality under the above-mentioned conditions. The sensory, that is, physical and chemical indicators, and microbial indicators agreed with relevant national standards, and the technical standards for the production of stir-baked FSIT could be formulated accordingly. This research provides additional insight into the types of substitute teas and technical support for enterprises to develop the stir-baked technology of FSIT for industrial application, which has practical guiding significance.

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Influence of sugar concentration and sugar type on the polyphenol content and antioxidant activity in spiced syrup preparation

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PAPER

Abstract

Besides their culinary roles, spices in the eastern traditional medical practices serve as a medicinal diet therapy. The polyphenol-rich content of these spices contributes to their antioxidant properties, which convey the therapeutic elements. Traditionally, these phytonutrients are orally delivered via herbal decoction, including sugar-syrup-based decoctions. However, the impact of the addition of sugar into polyphenol content and their antioxidant activities are still insufficiently researched. Therefore, this study aimed to evaluate the influence of sugar concentration and its types (refined and unrefined) on the polyphenol content and their antioxidant activities. The results of the principal component analysis (PCA) did not exhibit any specific trend on either sugar concentration or its type. As indicative evidence, reducing sugar by less than 25% in such products can be considered for lower-calorie beverage development as a means for healthier diet choice.

Keywords: decoction syrup; low-sugar; polyphenol–sugar interaction; sugar-sweetened

Introduction

Spices are traditionally served as culinary condiments and habitually used in Ayurveda, Persian *Tebb-e Sonnati*, Traditional Chinese Medicine (TCM), and Unani (Graeco-Arabic) medicinal preparations. Phytochemicals in spices can provide a great deal of medicinal therapy in preventing or treating common ailments. For instance, a mixture of cinnamon, clove, ginger, allspice, nutmeg, and star anise is used in the sugar-sweetened beverage for diet therapy in alleviating metabolic syndrome (Block, 2015). Their phytotherapeutic benefits are administered mostly in crude forms, either orally as infusions (herbal teas), tinctures (alcoholic extracts), decoctions (boiled extracts), and syrups (extracts of herbs made with syrup or honey), or topically applied as poultices, balms, and essential oils (Ahmad *et al.*, 2016; Ramalingum and Mahomoodally, 2014). Among the common liquid forms, *Sharbat* or *Sérbét*, which has the Arabic root word *shariba* or “to drink,” is made from either decoction, infusion of herbal remedies, fruit juice, or flower petal mixtures. There are various types of *Sharbat* preparation, depending on the type of the substrate to be extracted. Commonly, dry substrates, like most culinary spices, are boiled until one-third of the water is left and allowed to cool before being filtered. The filtered decoction is usually sugar sweetened with two to three parts and further boiled to obtain a syrupy consistency.

Addition of sugar to the phytochemical extraction step is relatively similar to the fruit osmotic dehydration technique, whereby sugar syrup is used as an osmotic agent (Pereira *et al.*, 2014) to produce an aromatic and flavorful herbal syrup or extract (Geng and Zhou, 2010). The range of osmotic agent additions were from 2 to 50% of the herbal substrate, but the study did not indicate as to when the sugar should be added. This syrup extraction was traditionally meant to improve the flavor and aroma of the herbal extract but was not intended to enhance the extractability of antioxidant compounds or specifically the polyphenols. Previously, it was known that a hot aqueous extract of several spices (non-sugar addition) had a high antioxidant activity that was probably due to the phenolic and flavonoid compounds (Kim *et al.*, 2011). Later, a study revealed that the addition of sugar before or during processing might play a significant role in enhancing the polyphenol extraction, and simultaneously the antioxidant activities instead of improving only the gastronomic qualities (Loncaric *et al.*, 2014). However, studies that evaluate the impact of sugar addition on polyphenols' content and their antioxidant activities are still limited to ensure this positive enhancement. The latest findings showed that there were only slight polyphenol losses during food processing (Szymanowska *et al.*, 2017; Zeng *et al.*, 2017) to unapprehensive changes in antioxidant capacities (Baroni *et al.*, 2018) upon the introduction of sugar, which was probably due to some degree of protective mechanism. A similar mechanism was observed in chokeberry juice that was added with polysaccharide as the clarifying agent (Lachowicz *et al.*, 2018). However, the polyphenolic content became less as soon as the natural sedimentation appeared, resulting in polyphenol binding to polysaccharides.

A list of sugar-loaded syrups showed promising therapeutic potentials as novel sources of antioxidant polyphenols. Among the examples are the traditional prickly pear (*Opuntia ficus indica*) and the pink Tecoma (*Tabebuia impetiginosa*) inner-bark syrups were advocated for their anti-tumor potential (Dhaouadi *et al.*, 2013; Pires *et al.*, 2015), and the English plantain (*Plantago lanceolate*) leaf syrup was recommended for combating common cold (Mansoor *et al.*, 2017). A polyphenol-rich syrup can potentially be a novel functional ingredient like the bog bilberry (*Vaccinium uliginosum*) syrup (Malvidin-3-O-glucoside, petunidin-3-O-glucoside, and delphinium-3-O-glucoside), which is the chief flavonoid glucoside that is used to enrich wine for antioxidant capacities (Liu *et al.*, 2015). Carbohydrates can enhance the bioactivity and the bioavailability of polyphenol compounds through increased fermentation by microbiota in the large intestine, especially the oligosaccharides (Zhang *et al.*, 2014). However, the primary concern is monosaccharide and disaccharide, which are readily absorbed and digested in the small intestine and may cause an alarming glycemic response in patients with metabolic syndrome.

Nevertheless, the negative health impact of commonly consumed sugary beverages on the risk factors for metabolic syndrome cannot be validated with adequate clinical evidence (Angelopoulos *et al.*, 2016; Della Corte *et al.*, 2018) to confirm that the added sugar products are the chief culprits for the metabolic imbalance. While it is health-wise beneficial to consume added sugars beyond the average level, the reduction in sugar intake accompanied by restricted calorie intake may be more beneficial (Rippe and Angelopoulos, 2016). Therefore, this preliminary study was to evaluate the effect of sugar concentration and the sugar type by examining their influence on polyphenol contents and their antioxidant activities.

Material and Method

Four culinary spices comprising Chinese cinnamon (*Cinnamomum cassia*), dried ginger (*Zingiber officinale*), green cardamom (*Elettaria cardamomum*), sweet fennel (*Foeniculum vulgare*), and six types of sugar, namely, refined white sugar (RWS), filtered sugarcane brown sugars (FBS), unfiltered sugarcane brown sugar (NBS), organic molasses (OMO), Javanese coconut sugar (JCS), and Malaccan coconut sugar (MCS) were acquired from Jaya Grocer store, Bangi Gateway.

Spiced syrup preparation

Dirt and debris were removed from the spices by cleaning under running tap water, and the excess water was drained before they were air-dried. A mixture of spices, comprising 10% (w/v) cinnamon and 5% (w/v) other spices, was added to boiling sugar syrup (110°C) before it was simmered for 240 min at 80°C. The spice mixture was directly used without pounding into smaller sizes based on a traditional technique to avoid a strong flavor. The spiced syrup was prepared (Figure 1) according to a traditional recipe, whereby sugars were added to filtered water at ratios of 1:2 kg/L, 1:3 kg/L, 1:4 kg/L, and 1:6 kg/L to produce syrups at the corresponding concentrations (Table 1). The range of sugar additions was according to traditional practices in preparing a sugar-based decoction. However, the most common practice is to mix one part of sugar with four parts of water (25%, w/v). The non-spiced 25% sugar syrups (all selected sugar types) were boiled and simmered in the same way as the spiced syrup was prepared and were considered as the positive control (control, CTRL [sugar type]), and the spice decoction without sugar syrup (0% sugar) was considered as the negative control. The spiced syrup was then filtered with a clean muslin cloth before being bottled and

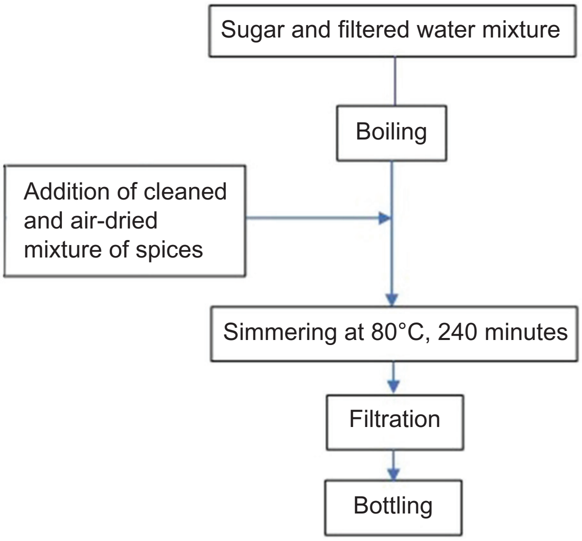

Figure 1. **Flow chart of spiced syrup processing**

Table 1. **Sugar ratio to sugar percentage conversion.**

Sugar to water ratio (kg/L)	Sugar used (g/1000 mL)	Sugar percentage, % (w/v)
1:2	500.0	50.0
1:3	333.3	33.3
1:4	250.0	25.0
1:6	166.7	16.7

stored at room temperature until use. Each spiced syrup was prepared in triplicates. The uncertainty due to spice sampling heterogeneity was kept below 13%, as recommended by Bodnar *et al.* (2013).

Determination of the total phenolic content

The Folin–Ciocalteu (FC) method (Singleton *et al.*, 1999) was used to quantify the phenolic content with slight modification on the reactant volumes for 96-well microplate suitability. A hundred microliters of 10% FC reagent were mixed with 20 µL of blank (deionized water)/standard/filtered syrup (diluted 50-fold with deionized water). After 5 min, 80 µL of 7.5% sodium carbonate was added and incubated at 37°C for 120 min. This spectrophotometric measurement was carried out at $\lambda = 765$ nm (Spectrostar Nano, BMG Labtech, Germany). The quantification of TPC by using the calibration curve of gallic acid was calculated by MARS Data Analysis Software Version 2.01. The concentration for the calibration curve ranged from 250 to 4000 ppm. The results were expressed as micrograms of gallic acid equivalents (GAE)/mL of syrup.

Antioxidant activity determination

Multiple spectrometric techniques were employed to evaluate the spice polyphenol antioxidant activities by three different mechanisms: organic radical scavenging, ferric reducing capacity, and peroxyl radical scavenging.

It was essential to evaluate the antioxidative compounds by using various techniques due to the unique identity of each polyphenol with different action mechanisms against oxidative substances. With these multiple antioxidant determinations, an almost complete profile of antioxidants can be attained.

The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities were determined according to Kodama *et al.* (2010). The 0.5 mM DPPH was pre-adjusted with methanol (1.00 ± 0.1 absorbance unit) before being mixed with diluted syrup (20-fold with distilled water) at a ratio of 9:1. After 30 min, the absorbance of mixtures was measured at 515 nm, and the DPPH radical scavenging activities were calculated as mM Trolox equivalent (TE)/mL of syrup.

Ferric reducing antioxidant power assay

A ferric reducing antioxidant power (FRAP) assay of spiced syrup was based on Musa *et al.* (2011), which required the FRAP reagent to be freshly prepared by sequentially mixing one part of 20 mM $FeCl_3 \cdot 6H_2O$ with one part of 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and thoroughly mixing with 10 parts of 300 mM acetate buffer, at pH 3.6. A freshly prepared FRAP reagent at a 1950 µL was mixed with 50 µL diluted syrup before a 30-min incubation at room temperature. The absorbance changes against the blank (distilled water) were measured at 595 nm. The Trolox calibration curve (3.125 mM to 100 mM) was used to equivalently estimate the FRAP reducing capacities, with results expressed as mM Trolox equivalents (TE)/mL of syrup.

Oxygen radical absorbance capacity assay

For the oxygen radical absorbance capacity (ORAC) value determinations, 150 µL of 10 nM fluorescein solution was mixed with 25 µL diluted syrup in a 96-well black microplate, as described by Payne *et al.* (2013). After incubation for 30 min at 37°C, the fluorescence degradation measurements were executed at the emission and excitation of 520 nm and 485 nm, respectively, by using a microplate reader (FLUORstar Omega, BMG Labtech, Germany). Automatic injection of 240 mM AAPH (25 µL) at the fourth cycle was made before measurement resumption, and the calculated results were expressed as µM Trolox equivalents (TE)/mL of syrup.

Statistical analysis

Data collected as means of triplicate readings were subjected to analysis of covariance (ANCOVA), correlation analysis by Pearson's correlation coefficient, and data analysis by principal component analysis (PCA) by using XLSTAT Premium 2018.1.4913 (Addinsoft Inc., New York, NY, USA). The ANCOVA was applied to evaluate whether TPCs were varied with sugar concentrations (quantitative) and types (qualitative), and verify a sensible linear model. The exploratory statistical tool, PCA, was applied to observe any typical or divergent features or degree of similarities among a set of variables.

Results and Discussions

Effect of sugar concentration and sugar type on the TPC of spiced syrups

Figure 2 shows that all CTRL sugars had a TPC that ranged from 500 to 1000 GAE mg/mL, except for RWS, which was the least with < 500 GAE mg/mL. Java coconut sugar, JCS had the highest TPC among the selected sugar types of approximately 1000 GAE mg/mL. Most brown or unrefined sugars contained a significant amount of polyphenol ($P < 0.05$), compared to refined sugar (Kongkaew *et al.*, 2014; Choong *et al.*, 2016). Brown sugar from sugarcane and coconut are commonly used in traditional medicine preparation as a sweetener because it is believed to possess therapeutic benefits. However, the 0% sugar decoction had 2-fold to 5-fold higher TPC than each CTRL sugar, and showed that sugar syrup of any type could contribute to a smaller portion of TPC value in the spiced syrup.

Overall, the higher the sugar concentration, the higher was the polyphenol content, except for OMO, which showed an opposite trend. This contradictory effect on OMO might be attributed to the OMO polyphenol antagonistic effect, reflecting the reduction of content. Meanwhile, in the RWS syrup, the TPC showed a unique bell-shaped trend, whereby intermediate sugar concentrations of 25 and 33.3% were exhibited among the highest contents (> 2500 GAE mg/mL). At higher sugar concentrations, the TPC high and low values were possibly due to the osmotic pressure, which allowed for desorption or prevention of spice polyphenols into the sugar syrup. It was postulated that the TPC decrement in the spiced syrup as the sugar concentration decreased was due to spice polyphenol degradation in the low solute matrix. However, the enhancement of the polyphenolic content in pumpkin by using concentrated flowering quince juice was attributed to the penetration of the polyphenol-rich osmotic solution into the pumpkin, which provided some stability (Lech *et al.*, 2018). The inconsistent variance among TPC in a syrup of different sugar concentrations and types might occur due to the interaction of Folin–Ciocalteu reagents with any reducing or readily oxidizable substances of nonphenolic compounds, causing an aberration of the total phenolic content (Essawet *et al.*, 2015; Ho *et al.*, 2017; Ramachandran and Nagarajan, 2014). It was postulated that different profiles of monosaccharide and disaccharide could play some interaction with the spice-polyphenols via a covalent or non-covalent bond, and thus, reflecting some variation in free polyphenol content.

The ANCOVA analysis showed that 63% of TPC variability (Table 2) was explained by sugar concentrations and types. However, the latter variability and interaction between sugar concentrations and types did not

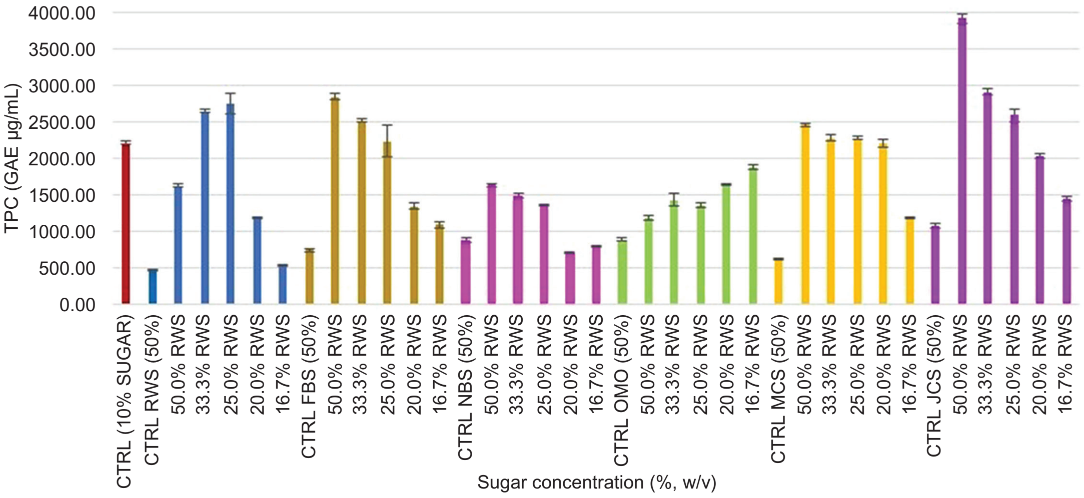

Figure 2. Total phenolic content (TPC) of spiced syrups prepared from different type of sugars and concentrations.

Table 2. Summary of ANCOVA for all variables.

		TPC	DPPH SC	FRAP RC	ORAC V
R^2		0.627	0.403	0.273	0.439
F		13.098	14.186	2.019	5.681
Pr > F		< 0.0001	< 0.0001	0.067	0.000
Sugar concentration (%)	F		0.730	10.740	
	Pr > F		0.401	0.003	
Sugar type	F	1.814	2.726		1.106
	Pr > F	0.150	0.018		0.395
Sugar concentration (%) * Sugar type	F	2.234		1.226	1.262
	Pr > F	0.084		0.321	0.320

ANCOVA, analysis of covariance; TPC, total phenolic content; ORAC, oxygen radical absorbance capacity; DPPH, 2, 2-Diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power.

significantly explain the TPC variability. Furthermore, the equation of the TPC linear model is proposed,

$$\begin{aligned}\text{TPC} = {} & 733.60 \times \text{Type of Sugar-C-FBS} + 1070.85 \\ & \times \text{Type of Sugar-C-JCS} + 602.73 \\ & \times \text{Type of Sugar-C-MCS} + 872.03 \\ & \times \text{Type of Sugar-C-NBS} + 880.13 \end{aligned} \tag{1}$$

Since the type of sugar did not carry significant information, the proposed model (Equation 1) was rejected. Based on Type 3 sum of squares, the interaction variable (type of sugar × sugar concentration) was the most influential contributor to the insignificant variation. Therefore, RWS can still be utilized in the production of the spiced syrup without a considerable loss in the TPC. One can avoid brown sugar in the mass production of the spiced syrup as it was uneconomically feasible.

Antioxidant capacities (DPPH scavenging, FRAP reducing activities, and ORAC values) of spiced syrups

From the summary of variable analysis (Table 2), 44% of the ORAC value variability was explained by the two explanatory variables (sugar concentration and type). The variability of the ORAC values in the spiced syrup was similar to TPC variability, which was not significantly explained by the type of sugar and the interaction variables of sugar concentration and sugar type. It may explain that the TPC content may have a corresponding influence on the ORAC values. All the spiced sugar syrups showed decrement trends in the ORAC values, excluding three negligible samples that showed increments (Figure 3). Meanwhile, the DPPH scavenging and the FRAP reducing capacities had 40 and 27% variabilities, respectively, explained. The sugar concentration did not give significant variation information in DPPH scavenging capacity ($P > 0.05$). However, the type of sugar played an influential role in explaining the variation in the capacity based on Type 3 sum of squares. It contradicted the FRAP reducing capacity, whereby the sugar concentration significantly influenced its variability.

The DPPH scavenging capacity of the spiced syrup showed decreasing trends in the RWS (0.6 to 72%) but the opposite trend in the MCS-based spiced syrup (31–53%), as compared to the control zero-sugar spiced decoction (Figure 4). The rest of the four-brown sugar-based decoctions exhibited mixed trends in the scavenging capacities. All FRAP reducing capacities showed reductions as negatively influenced by sugar, excluding two samples from the different types of sugar (Figure 5).

Visualization of the correlations between variables by using PCA

For distinguishing the spiced syrup-based TPC and antioxidant activities, the PCA was employed on the Pearson correlation matrix. As a classification/discrimination method, PCA permits a simplified data representation over a complex data matrix of the spiced syrup with different sugar concentrations and types of sugar use. The first two principal components (PC), PC1 and PC2, explained a total of 78.4% variance (Figure 6). Both PCs were almost equally explained, whereby the initial PC, explained up to 41.7% and the latter up to 36.7%. PC1 has a strong positive correlation with the TPC and all antioxidant variables, whereby the ORAC values have the highest correlation with the TPC ($P < 0.514$) (Table 3). The DPPH scavenging and FRAP reducing capacities have the least correlation with the other two variables and are negatively correlated with each other ($P < -0.465$).

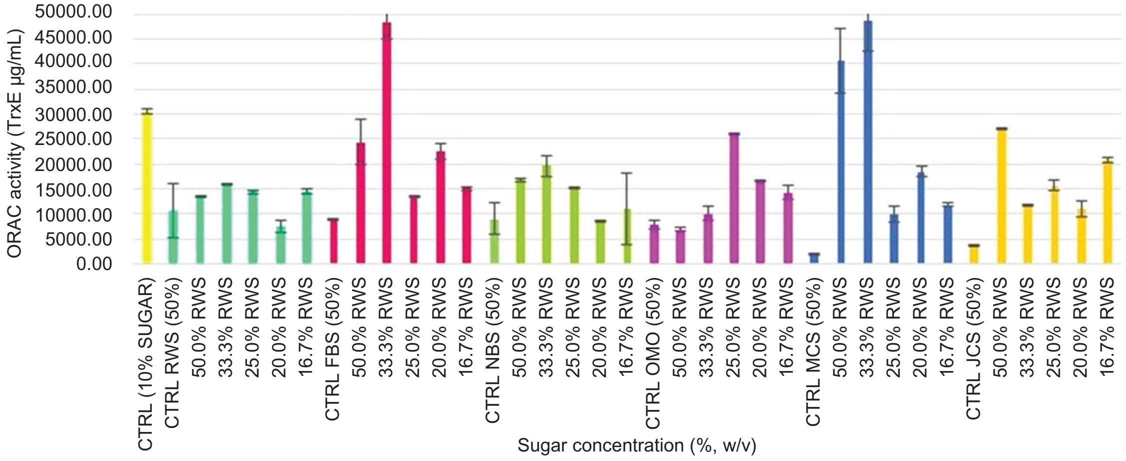

Figure 3. **ORAC values of spiced syrups prepared from different type of sugar and concentration.**

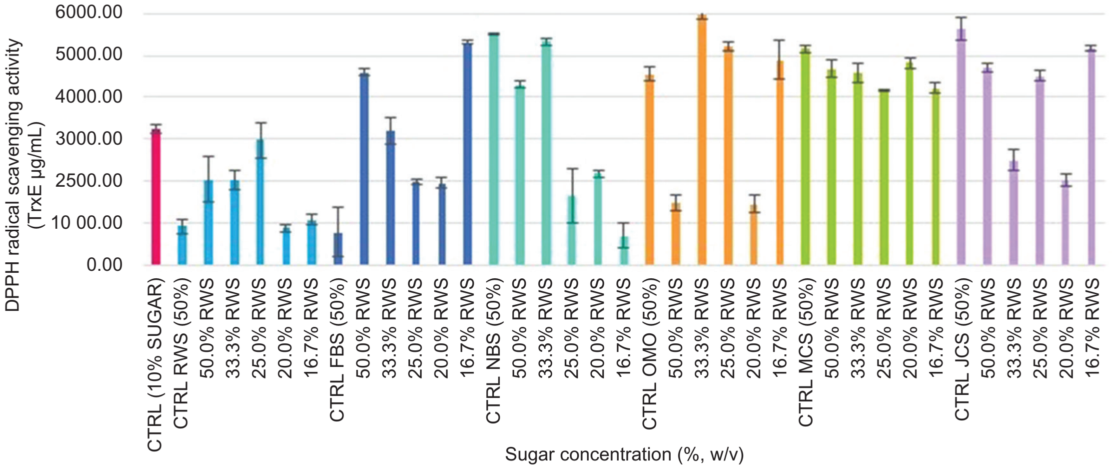

Figure 4. **DPPH radical scavenging activities of spiced syrups prepared from different type of sugar and concentration.**

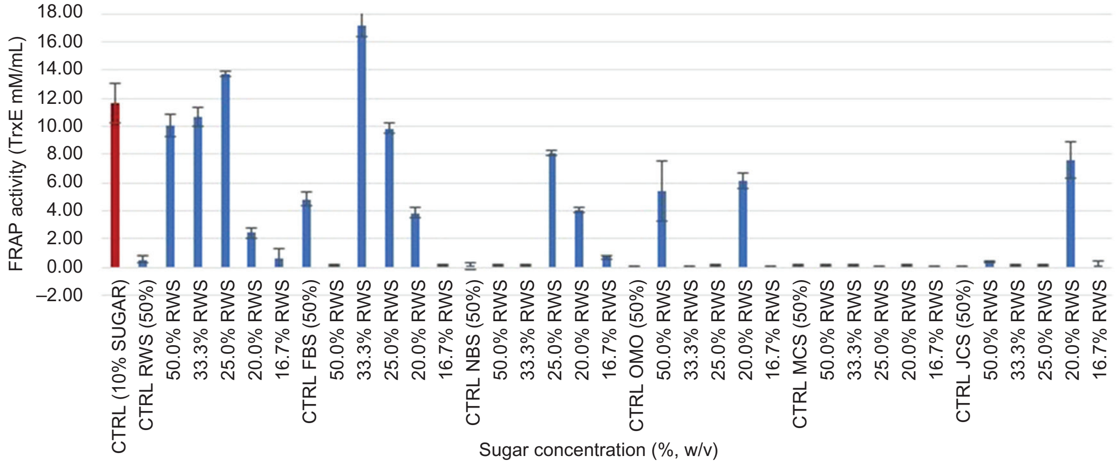

Figure 5. **FRAP values of spiced syrups prepared from different type of sugar and concentrations.**

From the biplot (Figure 6), there was no specific classification based on either concentration of sugar or the type of sugar exhibited on both the PCs. However, two clusters can be observed in the positive and negative loadings of PC2 without specific classification in each cluster. The PCA analysis results showed that either sugar concentration or sugar type significantly influences the TPC and their antioxidant activities.

Only hard ingredients, either from bark, seed, or heartwood, are traditionally subjected to simmering. In contrast, the soft or leafy ingredients are infused in boiled water to extract bioactive compounds. The duration of the former may take hours as compared to the latter, which only takes a few minutes. The decoction process started after the sugar syrup was boiled and the spices were added and terminated after 4 h of simmering. The spices were introduced into the boiled sugar syrup to obtain the glassy clear syrup in culinary practice. The point at which the sugar was added did not significantly impact taste (Madhavamenon and Maliakel, 2015). However, from the study observation, the addition of sugar toward the end of the decoction process only retained the liquid turbidity, which was visually less appealing.

Table 3. Correlation matrix (Pearson [n – 1]).

Variables	TPC	DPPH SC	FRAP RC	ORAC V
TPC	**1**	0.201	0.255	0.514
DPPH SC	0.201	**1**	−0.465	0.220
FRAP RC	0.255	−0.465	**1**	0.156
ORAC V	0.514	0.220	0.156	**1**

Values in bold are different from 0 with a significance level alpha = 0. ANCOVA, analysis of covariance; TPC, total phenolic content; ORAC, oxygen radical absorbance capacity; DPPH, 2, 2-Diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power.

Previous studies showed that mild thermal exposure, such as in the fortification of sugar syrup with functional fruit-based ingredients, retained its high polyphenol content (Tarko *et al.*, 2015). Those that were traditionally prepared fruit-based beverages also showed more pronounced antioxidant capacities than the commercially processed juice (Marjanović *et al.*, 2015). A traditional Calabrian fig syrup made from fresh figs' (*Ficus carica*) aqueous slurry, prolong boiling until the syrup concentrate has a high polyphenol content (3.92 mg of GAE per gram of syrup) with desirable sensorial qualities (Puoci *et al.*, 2011). It seemed to be not much affected by the lengthy thermal treatment. Therefore, the effect of the thermal treatment on the polyphenol content of the spiced syrup was not carried out. The decrement trend in radical scavenging capacities

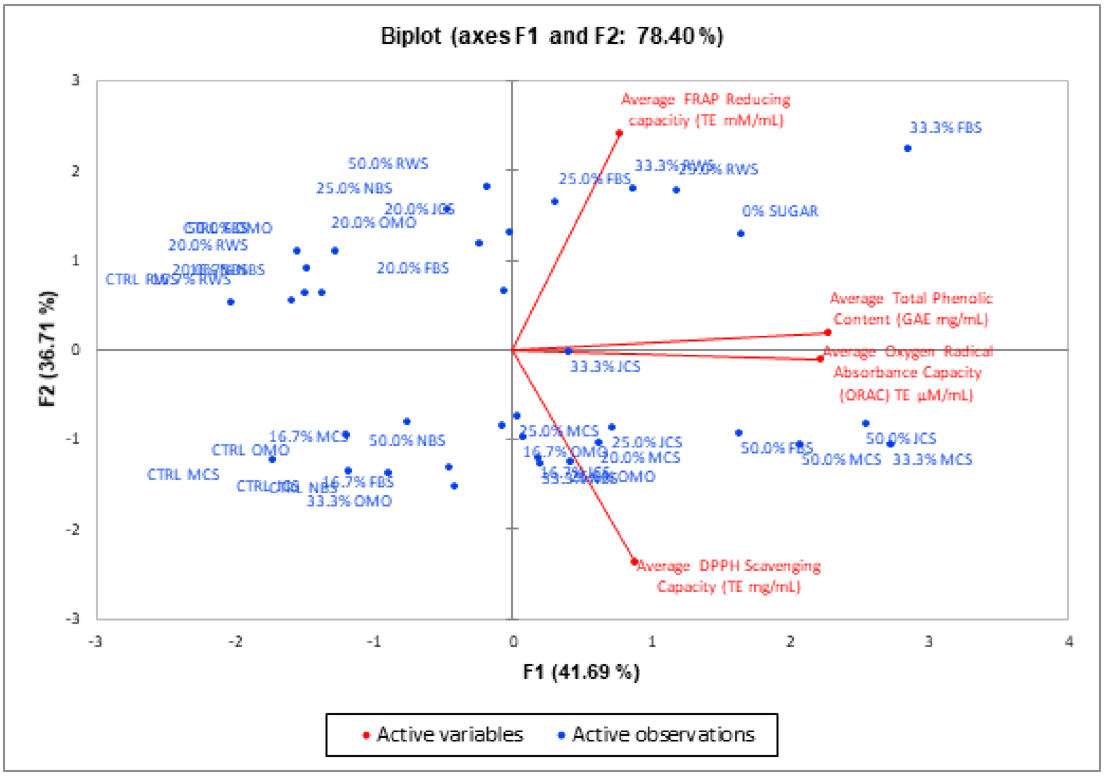

Figure 6. PCA biplots of spiced syrups made from different type of sugar and concentrations versus TPC and antioxidant parameters.

might be attributed to the glycosidic bond formation through the condensation reaction between a hydroxyl group of sucrose molecule and the hydroxyl group of phenolic compounds to form glycoside compounds. However, the increments were probably the result of phenolic reduced-form compounds due to the oxidation of phenolic compounds and sucrose molecules (Shalaby *et al.*, 2016). In addition, Jlassi *et al.* (2016) suggested that the dose-dependent manners of the polyphenol bioactivities may vary due to the polyphenol functional site reaction with ionized sucrose. Besides, the polyphenol profile may influence disaccharide action and give variability in antioxidant activities (Pengseng *et al.*, 2011).

Sugar-sweetened beverage consumption still gives a stir to the national health issues. Simultaneously, polyphenol-rich food intakes are linked with a positive impact on consumers' health. However, sugar addition to polyphenol-rich food products, especially beverages, can change the health benefits. Some suggested that the presence of sugars generally increases the bioavailability of polyphenols, but product formulation may influence the sugar impact to a certain degree (Ackar *et al.*, 2013). This study observed a specific range of sugar concentration and sugar type that accounts for a certain extent of the TPC. These two variables may introduce different profiles of disaccharides, which in turn react to a certain extent. It was not only the type of disaccharide that was reported to have an essential impact on phenolics but also their chemical isomers since the chemical behavior can be different in complex food matrices (Zlatić *et al.*, 2017). For example, maltose and trehalose are isomers of sucrose. The latter isomer has a more hydrophilic site than the former, leading to a higher interaction with hydrophilic polyphenols. In the study spice syrup, hydroxycinnamic acids derived from cinnamon, which was hydrophilic (Teixeira *et al.*, 2013), probably had more sugar interaction. In addition to the saccharide structure-related factors, evidence showed that the polyphenol interaction with saccharides was influenced by the polyphenol molecular weight (monomeric or polymeric) (Amoako and Awika, 2016). It was found that polymeric polyphenol (molecular weight, MW > 1000) polysaccharides via hydrogen bonding and hydrophobic interactions formed nondigestible complexes. This positive interaction can reduce calorie density and may help modulate glucose metabolism. Therefore, future research on the consequences of polyphenol–sugar interaction should uncover a positive impact on health.

In the food matrix, polyphenols can have either a covalent or noncovalent association with polysaccharides. A combination of hydrophobic interaction and hydrogen bond (between the phenolic hydroxyl group and the polysaccharide oxygen atom) can result in a noncovalent association. Simultaneously, the covalent association involved the oxidation of phenolic compounds (Le Bourvellec and Renard, 2012), which probably occurred in the spiced syrups and highly corresponded to the low antioxidant activities. It showed that both types of association have a determinant effect on the quality level of polyphenol-rich beverages. Sugars engage in the stability of polyphenols in the form of conjugated sugar or glycoside via a glycosidic bond (excluding the subclass of catechins) to one or more hydroxyl groups (Martin, 2009). Therefore, the addition of sugar may inhibit the autoxidation of polyphenols and contribute to high antioxidant activities in some spiced syrups. However, the profound antioxidant activities were not solely contributed by inhibition of autoxidation. It may partially be due to the advantage of additive or synergistic effect of different phenolics, flavonoids, reducing sugars, and vitamin C profiles like in the must of dried grapes (Peinado *et al.*, 2010), whereby an antagonist effect may be found in the spiced syrup for the reduced antioxidant activities. The glucoside forms (sugar conjugated) are likely to offer more therapeutic benefits than the aglycones (nonsugar conjugated polyphenol), as they are more bioaccessible, bioavailable, and stable under in vivo conditions (Fernandes *et al.*, 2017). In addition, the sugar moiety of glycoside can enhance the bioavailability, but dimerization weakens the ability. However, glycosides are usually weaker in vitro antioxidants than aglycones (Shashank and Pandey, 2013); this explains why some spiced syrups have lower antioxidant activities than the zero-sugar spiced decoction.

Conclusions

Various sugar concentrations and different types of sugar used in the extraction of polyphenols to produce the spiced syrup may influence the TPC and antioxidant activities to a particular extent with mixed variabilities, as observed from the results of the ANCOVA and the PCA. The weaving trends may uniquely contribute to the different profile components of sugar and water-soluble phytochemicals from the spice mixtures. These biochemical complexities of molecular weight and structure-related reaction produced a different level of antioxidant activities. From the compiled results of the ANCOVA and the PCA, it can be suggested that a sugar concentration of less than 25% is adequate to prepare the spiced syrup with many antioxidant activities, even while using the RWS. Moreover, further studies are required to better conclude the sugar–polyphenol interaction as in vivo.

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Analytical approaches for discriminating native lard from other animal fats

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PAPER

Abstract

Establishing the distinguishable characteristics of lard from other common animal fats might be helpful for authentication initiatives in foods and feeds. In this study, fatty acid and triacylglycerol compositions, thermal and spectroscopic characteristics of native lard (NL), respectively, were compared with those of beef tallow (BT), mutton tallow (MT), and chicken fat (CF) by using gas liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and differential scanning calorimetry (DSC). GLC analysis showed that the comparison of the overall fatty acid data might not be suitable for the discrimination of different animal fats, but the use of the principal component analysis and the percent palmitic acid enrichment factor [PAEF (%)] calculations were useful. HPLC analysis showed that NL displayed a TAG profile, which was quite different from those of either BT or MT, but appeared to be closely similar to that of CF. Results of DSC thermal analysis showed that both melting and crystallization curves of NL were remarkably different from those of other animal fats.

Keywords: animal fats; fatty acid composition; food authentication; thermal analysis; triacylglycerol composition

Introduction

Meat has been the main source of protein for humankind from time immemorial. Beef, pork, chicken, and mutton have been the preferred sources of meat for mankind. According to the worldwide meat intake assessment, pork is the most widely consumed meat accounting for over 36%, followed by poultry (35%) and beef (22%) (FAO, 2012). The consumption patterns of different animal species across the world might vary according to religious beliefs and cultural preferences. For instance, consumption of pork is not desirable for some segments of the society due to their religious restrictions. Either deliberate or accidental mixing of pork with other meat species is a great concern for adherents of Islam and Orthodox Judaism. According to some reports, mixing of pork with other commonly consumed meat types is reported to have occurred due to fraudulent reasons (Saeed *et al.*, 1989). Hence, a great deal of effort has been made to develop analytical methodologies to detect fraudulent practices related to the presence of pork in food systems. Researchers in the past frequently employed deoxyribonucleic-acid-based methodologies to differentiate between different meat species in meat products (Aida *et al.*, 2005). Lipid-based detection methods, on the other hand, are advantageous since lipid component in food matrices is minimally affected by the processing conditions (Che Man *et al.*, 2005; Marikkar *et al.*, 2005; Sawaya *et al.*, 1990b). As considerable amount of literature has been available on the detection of animal fats in food systems (Che Man *et al.*, 2011; Marikkar *et al.*, 2005; Nurjuliana *et al.*, 2011; Nina Naqiyah *et al.*, 2017; Rohman and Che Man, 2010; Rohman *et al.*, 2011), it would be highly beneficial to discuss the distinguishable characteristics of native lard (NL) from other common animal fats. Hence, the purpose of this article is to explore the literature and experimental studies on NL and other animal fats in terms of compositional and physico-chemical characteristics using different analytical techniques, such as gas liquid chromatography (GLC), high performance liquid chromatography (HPLC), and differential scanning calorimetry (DSC).

Materials and Methods

Sampling and reagents

Samples of animal fats, namely, NL, beef tallow (BT), mutton tallow (MT), and chicken fat (CF) were extracted by rendering adipose tissues of animals collected in triplicate from three slaughter houses located in different locations of Sri Serdang, Malaysia. Thirty-six samples were collected and analyzed in this study. All chemicals used in this experiment were of either analytical or HPLC grade.

Fat extraction protocol

The rendered fat was squeezed out using double-folded muslin cloth. The removal of impurities was carried out by filtering the melted animal fats through cotton wool. A small proportion of anhydrous sodium sulfate was added to remove residual moisture, and the samples were then filtered through Whatman No. 2 filter papers (Marikkar *et al*., 2001).

Analytical methodologies

Preparation of fatty acid methyl esters

The determination of fatty acid composition required a derivatization step with methyl ester for all the animal fats using sodium methoxide. Preparation of fatty acid methyl esters of animal fat was done according to the Association of Official Agricultural Chemists (AOAC) method 969.33. A 50-mg portion of animal fat was weighed into a 20-mL test tube (with screw cap). After adding 2 mL of 2N sodium hydroxide in methanol, the sample tube was closed and heated at 80°C for 1 h. After allowing the tube to cool down for a few minutes, 2 mL of 25% borontrifluoride solution in methanol was added. The tube was closed and heated again for 1 h at 80°C. Subsequently, 5 mL portions of water and hexane were added into this. The contents of the tube were shaken well and allowed to undergo phase separation. The clear supernatant of the solution was transferred into a 2-mL auto-sampler vial (AOAC, 2007).

Determination of fatty acid composition

The top hexane layer of the FAME solution was injected on an Agilent 6890N gas chromatograph (Agillent Technologies, Singapore) equipped with a polar capillary column RTX-5 (0.32 mm internal diameter, 30 m length, and 0.25 mm film thickness; Restex Corp., Bellefonte, PA) and a Flame Ionization Detector (FID). Split injection was conducted with a split ratio of 58:1 using nitrogen as a carrier gas at a flow-rate of 1.00 mL/min. The temperature of the column was 50°C (for 1 min), and was programmed to increase to 200°C at 8°C/min. The temperatures of the injector and detector were maintained at 200°C (Nina Naqiyah *et al*., 2013).

HPLC analysis of TAG composition

The separation of triacylglyceride (TAG) components of animal fats was performed on a Waters Model 510 liquid chromatograph equipped with a Merck Lichrosphere RP-18 column (5 μm) (12.5 cm × 4 mm i.d.; Merck, Darmstadt, Germany) and a differential refractometer Model 410 as a detector (Waters Associates, Milford, MA). Each fat sample was loaded in the sample vials after dissolving in chloroform and filtering through a Whtaman no. 40 filter paper. The isocratic mobile phase was a mixture of acetone:acetonitrile (63.5:36.5 v/v) and the flow rate was set at 1.5 mL/min. Each sample was chromatographed three times, and the data were reported as peak area percentages. The identification of the TAG peak of the samples was done in accordance with the TAG profiles of animal fats reported previously by Marikkar *et al.* (2002).

Thermal analysis by DSC

Thermal analyses of all animal fats were carried out on a Mettler Toledo differential scanning calorimeter (DSC 823 Model, Columbus, Ohio, USA). Data elaboration was performed with a thermal analysis software (STARe software, Version 9.0x, Schwerzenbach, Switzerland). Nitrogen (99.99% purity) was used as the purge gas at a rate of ~20 mL/min. Approximately 4–8 mg of molten sample was placed in a standard DSC aluminum pan and then hermetically sealed. An empty, hermetically sealed DSC aluminum pan was used as the control. The samples were held at 70°C isotherm for 1 min to eliminate the thermal history of the samples, then cooled at 5°C/min to −70°C and held for 1 min. The sample was then brought from −70°C to 70°C at the same rate (Marikkar *et al*., 2001; Nina Naqiyah *et al*., 2017).

Statistical analysis

All the analyses were performed in triplicate and the results were expressed as mean value ± standard deviation. Data were statistically analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test using MINITAB™ Statistical Software (MINITAB®

Release 14.12.0, New York, USA) at α = 0.05. Principal component analysis (PCA), which is a multivariate statistical method that entails data reconstruction and reduction, was carried out using Unscrambler 9.7 (Camo, USA) software.

Results and Discussions

Fatty acid distribution

Fatty acid composition is one of the important attributes that determine the nature of most natural lipids. In this study, the fatty acid composition of four common animal fats is compared as shown in Table 1. In NL and other animal fats, oleic acid ($C_{18:1}$) has been the most dominant fatty acid. Palmitic acid ($C_{16:0}$), linoleic acid ($C_{18:2}$), and myristic acid ($C_{14:0}$) are other fatty acid types that occur in appreciable amounts in NL. Other than these, occurrence of fatty acids such as $C_{14:1}$ and $C_{15:0}$ was rarely detected in NL, but they were detected in minute amounts in other animal fats. According to Haas (2005), the prevalent fatty acids of animal depot lipids are either 16 or 18 carbons in their chain length and are either fully saturated or contain one or two double bonds. Investigations by Marikkar and Yanty (2014) showed that NL was generally composed of higher amounts of unsaturated fatty acids (50.2–74.72%) than saturated fatty acids (33.76–46.08%). The relative variations of individual fatty acids in NL has been mainly attributed to differences in sex and the breed types, climatic conditions due to the diet, and the system of animal rearing (Enser, 1995; Muriel *et al.*, 2002). According to Che Man *et al.* (2005), experimental diets composed by soybean or canola oil could cause an increase in the proportions of unsaturated fatty acids in swine fat. Among samples extracted from the different body parts of the animals, the fatty acid composition of NL can also slightly vary. However, in the majority of the cases, oleic acid was always found to be the most predominant fatty acid of NL (Enser, 1995). Similar to NL, CF was also found to possess high oleic acid (43.94%), followed by palmitic acid (25.39%) (Table 1). These values were closely comparable to those reported previously by Lee and Foglia (2000a). In contrast to both NL and CF, the distribution of fatty acids in BT and MT would be remarkably different. Particularly, the stearic acid content of BT and MT might differ from that of NL, which is low in saturated fatty acids. Hence, this feature was thought to be helpful to distinguish NL from BT/MT. Even though the presence of high stearic acid content is a common characteristic feature of both BT and MT, it may be varied slightly due to the influence of various other factor factors, namely, breed, sex, and nutritional conditions (Grompone, 1989; Grompone and Moyna, 1983; Holia and Press, 1987; Yilmaz and Karakaya, 2009).

The degree of unsaturation could also be considered a parameter for the discrimination of NL from other common animal fats. As shown in Table 1, unsaturated/saturated (US/S) fatty acid ratio was found to be useful in discriminating NL from BT and MT, but not NL and CF. This finding was largely in conformity with the results previously reported by Rashood *et al.* (1996). If either overall fatty acids (FA) distribution or degree of unsaturation was considered singularly, differentiating NL from CF might not be feasible. In such situations, applying multivariate data analysis techniques such as PCA on the overall fatty acid data would be beneficial. In essence, PCA helps to reduce the number of observed variables into a smaller number of variables that accounts for most of the variance in a particular dataset. An outcome of PCA would possibly group samples with dissimilar characteristics into different groups. As shown in Table 1, fatty acids such as lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) were present in all four types of animal fats. Henceforth, they can be taken as independent variables to develop a PCA model.

The score plot shown in Figure 1 represents the projection of samples defined by the principal component 1 (PC1, which explains 86% of the dataset variance) and the principal component 2 (PC2 which explains 9% of the dataset variance). According to the score plot, a good group separation was achieved with the PCA model, in such a way that NL samples were located in the lower left quadrant, CF in the upper left quadrant, MT in the upper right quadrant, and BT in the lower right quadrant. According to the loading plot shown in Figure 2, stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) were the most discriminating parameters that influence separation along the PC 1 axis, while palmitic acid (C16:0) and palmitoleic acid (C16:1) were the most discriminating parameter along the PC 2 axis.

Determination of *sn*-2 positional distribution of fatty acids within the glycerol chain using the pancreatic lipase hydrolysis method was also adopted as an alternative option to find effective discrimination between NL and other animal fats. This procedure is laborious, as it involves isolation of triacylglycerol using column chromatography followed by preparation of 2-MAG by pancreatic lipolysis. Calculation of the fatty acid enrichment factor (FAEF) corresponding to individual fatty acid could be possible based on the FA distribution of 2-MAG. As shown in Figure 3, FAEF corresponding to the palmitic acid was quite high for NL (283%) when compared to that of any other animal fat [BT (69%); MT (70%); CF (84%)]. This was because more than 74% of the fatty acids present in 2-MAG of NL were saturated fatty acids, of which about 68% was palmitic acid (Marikkar

Table 1. Fatty acid compositions (%, peak area) of native lard, chicken fat, beef fat, and mutton fat.[1]

Fatty acids	Native lard	Chicken fat	Beef fat	Mutton fat
C10:0	0.08^{b} ± 0.01	—	—	0.29^{a} ± 0.06
C12:0	0.19^{a} ± 0.11	0.64^{a} ± 0.93	0.11^{a} ± 0.05	0.46^{a} ± 0.06
C14:0	2.28^{b} ± 1.10	1.62^{b} ± 0.65	6.15^{a} ± 0.31	6.40^{a} ± 0.49
C15:0	0.05^{c} ± 0.04	—	0.46^{b} ± 0.24	0.76^{a} ± 0.02
C16:0	24.64^{b} ± 1.90	25.39^{b} ± 1.01	31.07^{a} ± 0.78	27.38^{ab} ± 1.22
C16:1	1.07^{c} ± 0.46	5.32^{a} ± 0.48	2.56^{b} ± 0.07	0.52^{c} ± 0.18
C17:0	0.25^{b} ± 0.23	–	0.82^{b} ± 0.62	1.85^{a} ± 0.13
C18:0	11.53^{c} ± 1.67	4.84^{d} ± 0.18	16.53^{b} ± 1.25	30.90^{a} ± 0.50
C18:1	42.62^{a} ± 0.74	43.94^{a} ± 1.77	35.70^{b} ± 1.71	29.82^{c} ± 1.04
C18:2	17.29^{a} ± 3.11	18.26^{a} ± 1.64	6.59^{b} ± 0.61	1.61^{b} ± 0.06
∑SFA	39.02	32.48	55.15	68.05
∑USFA	60.98	67.52	44.85	31.95
US/S	1.56	2.08	0.81	0.47

[1] Each fatty acid value in the table represents the mean ± standard deviation of three replicates. Means within each row with different superscripts are significantly ($P < 0.05$) different.

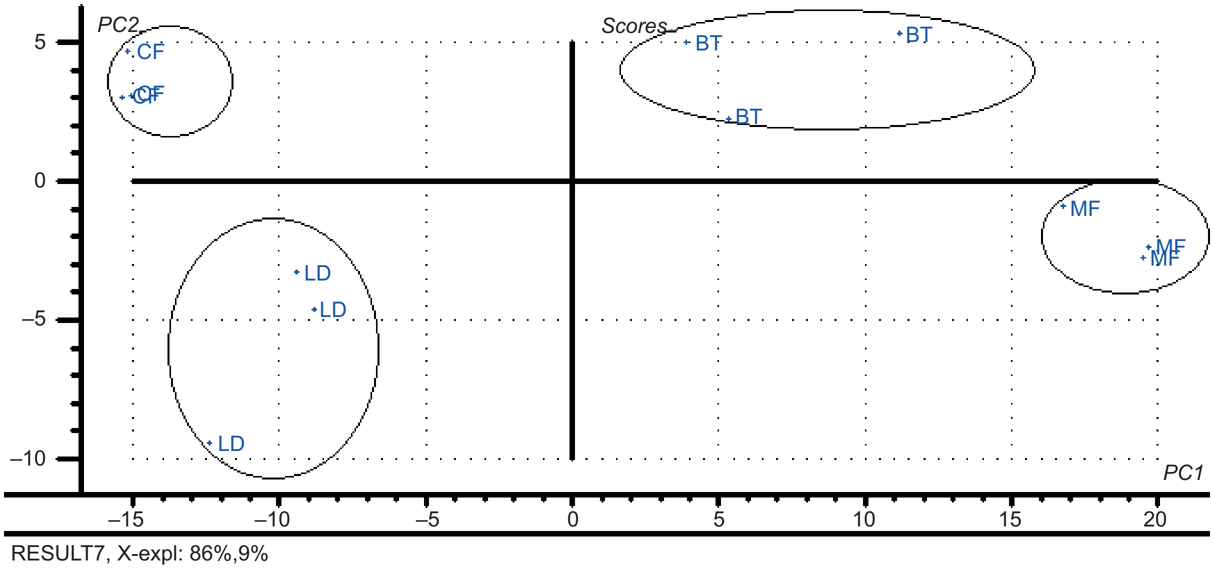

Figure 1. Score plot of PCA based on fatty acid composition for the discrimination of animal fats. Abbreviations: LD, lard; CF, chicken fat; BT, beef tallow; MT, mutton tallow.

et al., 2005; Rashood *et al.*, 1996). This characteristic feature could be effectively used to differentiate NL from other animal fats.

TAG distributional pattern

Comparative TAG distributional ratios of different triacylglycerol classes of animal fats are presented in Table 2. The TAG molecules present in most natural oils and fats could be classified into four subgroupings: trisaturates (SSS), monounsaturates (SUS/SSU), diunsaturates (SUU), and triunsaturates (UUU) by considering their degree of unsaturation. According to Table 2, there was hardly any significant ($P > 0.05$) difference among animal fats with respect to the amount of triunsaturates (UUU), but the amount of trisaturates (SSS) was remarkably higher for both BT and MT than those for either NL or CF. This was due to the fact that the major TAG molecular species of NL were PLL, OOL, LPO, OPO, PPO, and SPO, of which SPO, LPO, and OPO had become the most dominant TAG molecular species (Marikkar *et al.*, 2002; Yanty *et al.*, 2011).

On the other hand, the most commonly found TAG molecular species of BT and MT were SSP, SSS POO,

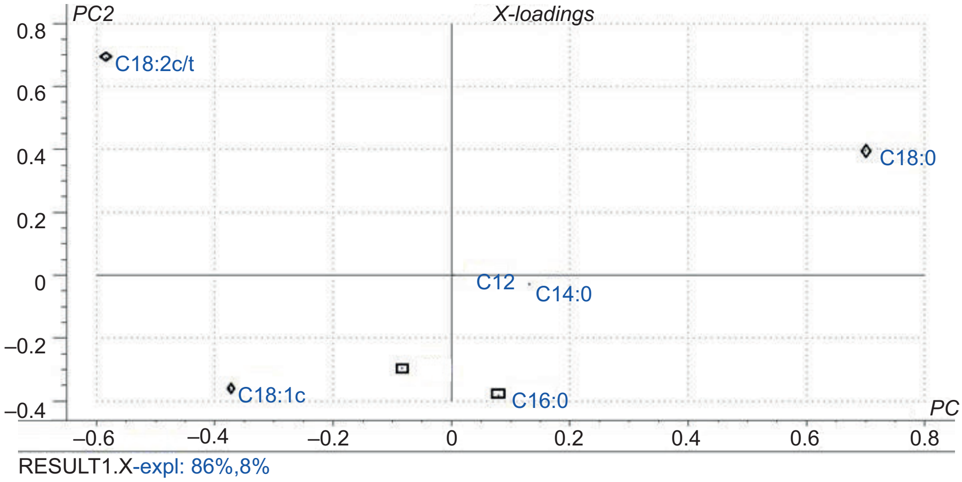

Figure 2. **Loading plot of PCA of the animal fats based on the fatty acid composition. (PC1: ◊, PC2: ☐).**

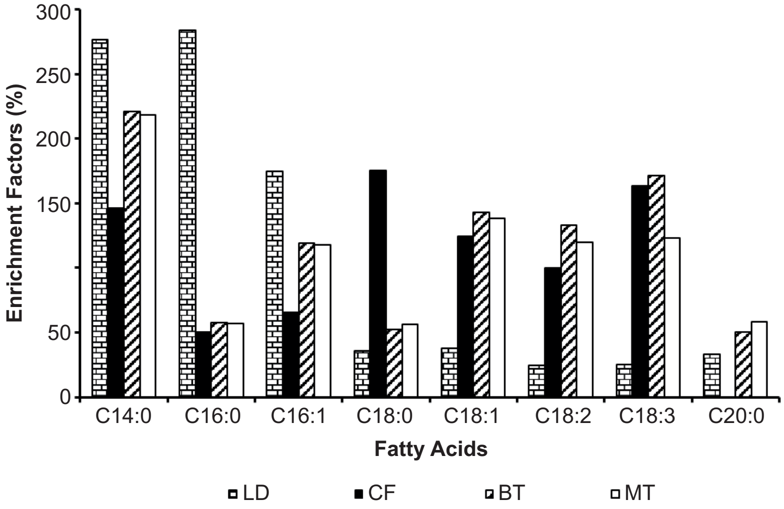

Figure 3. Comparative fatty acid enrichment factors (%) of lard and other common animal fats. Abbreviations: LD, native lard; BT, beef tallow; MT, mutton tallow; CF, chicken fat.

Table 2. **Comparison of trisaturated (SSS), monounsaturated (SSU), diunsaturated (SUU), and triunsaturated (UUU) triacylglycerol distribution among different animal fats.**[1]

Parameter	Native lard	Chicken fat	Beef fat	Mutton fat
% Trisaturated	36.15^{c} ± 0.30	33.99^{d} ± 0.44	63.07^{a} ± 0.65	55.11^{b} ± 0.58
% Monounsaturated	44.86^{b} ± 0.26	51.52^{a} ± 0.72	34.62^{d} ± 0.35	41.86^{c} ± 0.51
% Diunsaturated	18.22^{a} ± 0.28	13.76^{b} ± 0.55	1.84^{c} ± 0.33	2.11^{c} ± 0.45
% Triunsaturated	0.69^{a} ± 0.10	0.75^{a} ± 0.14	0.51^{a} ± 0.07	0.95^{a} ± 0.17
US/S ratio	1.76:1	1.94:1	1:1.71	1:1.23

[1] Each value in the table represents the mean of three replicates. Means within each row with different superscripts are significantly ($P < 0.05$) different.

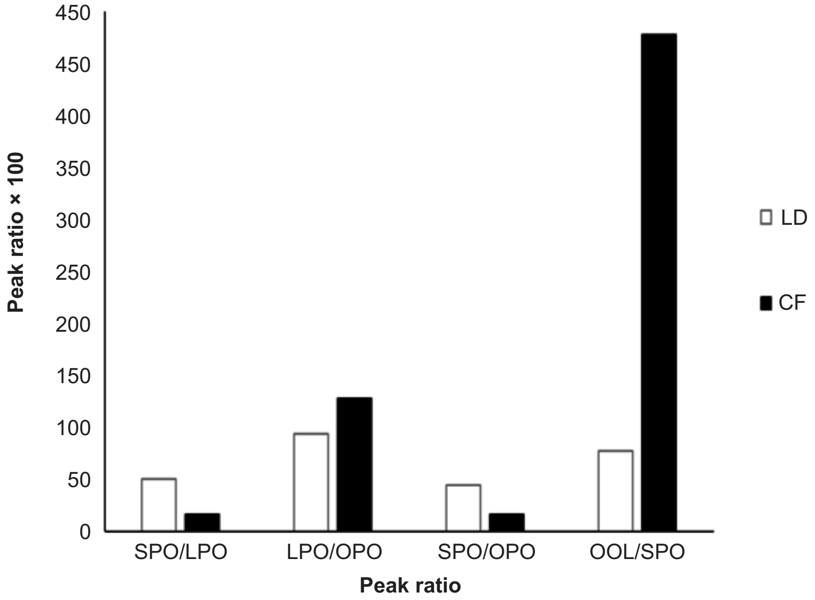

Figure 4. Selected TAG peak ratio of lard and chicken fat. Abbreviations: LD, native lard; CF, chicken fat.

PPO, PPP, SOO, PSO, SPP, SSO, and OOO (Marikkar *et al.*, 2002). Some early investigations of the 1960s suggested that NL possessed 38% of SSU, 41% of USU, and very low percentages of SUS (1%) and UUS (7%) (Chacko and Perkin, 1965). According to the literature, UUU and SUU were the most abundant TAG species present in CF (Lee and Foglia, 2000b). Among these, LOO, OOO, OPO, and PSO were found to be predominant in CF (Marikkar *et al.*, 2002). Some major differences in the TAG distribution were found between NL and BT/MT, and those differences can make them easily distinguishable. Hence, the US/S ratio for NL (1.76:1) was remarkably different from those of either MT (1:1.23) or BT (1:1.71). On the contrary, the differences in the US/S ratio between NL (1.76:1) and CF (1.94:1) were minor, which could make their discrimination more difficult. In order to discriminate between NL and CF, TAG peak ratios such as SPO/LPO, LPO/OPO, SPO/OPO, and OOL/SPO can be calculated. According to Figure 4, NL and CF displayed only slight differences among ratios of SPO/LPO, LPO/OPO, and SPO/OPO, but showed a strong difference with respect to the OOL/SPO ratio. In this case, CF was found to show higher OOL/SPO ratio than that of NL.

DSC thermal profiles

The overlay presented in Figure 5 shows DSC cooling curves of NL (Curve A), BT (Curve B), MT (Curve C), and CF (Curve D). DSC cooling curves are recognized worldwide to provide thermal characteristics of plant oils (Tan and Che Man, 2000), animal body fats (Yilmaz and Karakaya, 2009), lard (Yanty *et al*, 2011), milk fat (Campos *et al.*, 2002), etc. According to the cooling curve of NL (Figure 5; Curve A), there were two well-separated and defined sharp exothermic thermal transitions, indicating two steps of crystallization; transition above 0°C was defined as the high-melting group (HMG) while the transition below 0 °C was identified as the low-melting group (LMG). These thermal features are largely in conformity with the results mentioned in previous reports (Marikkar *et al.*, 2001; Rashood *et al.*, 1996; Yanty *et al.*, 2011). However, a few differences observed in the onset temperatures and heat flux profiles could be possible due to either sample to sample variation or variation in the DSC scanning rate. In an early report, Campos *et al.* (2002) demonstrated the effect of different cooling rates on the DSC curve of NL. As illustrated in Figure 5, the cooling profiles of other animal fats were more complex heat flux curves involving multiple thermal transitions as compared to that of NL. Particularly, peak broadening is a characteristic feature seen in Curves B, C, and D, indicating a wider range of melting points of the constituent TAG molecules. Both BT and MT showed sharp thermal transitions at 29.8 and 26.5°C, and broad crystallization transitions at 5.9 and 4.9°C, respectively. The cooling profile of BT was comparable to that of buffalo body fat reported previously by Lambelet and Ghanguli (1983). The occurrence of broader melting peaks in the low-temperature range in BT and MT could be due to the difference in the nature of TAG distributional pattern in these animal fats as compared to that of NL. The cooling profile of CF represented by curve D, displayed a single sharp peak at 8.9°C and a broad peak at –1.9°C. Overall, the thermal events recorded by CF in this study were roughly similar to the one reported previously by Lee and Foglia (2000b). As these features were quite dissimilar from those of NL, the thermal profile obtained

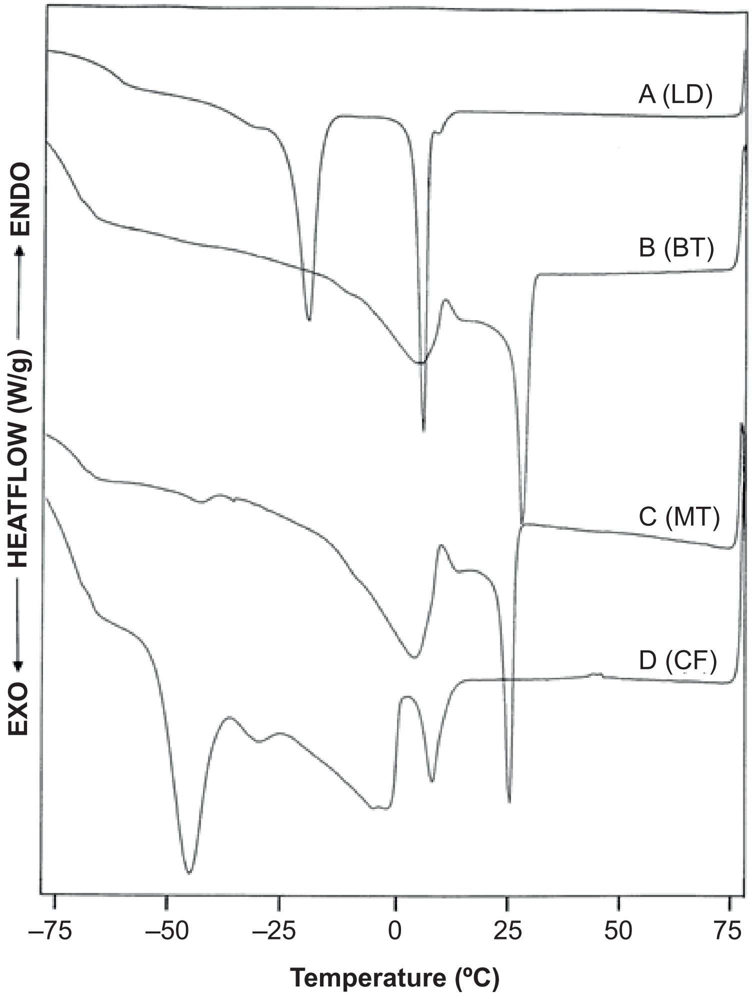

Figure 5. DSC Cooling thermograms of lard and other common animal fats. Abbreviations: LD, native lard; BT, beef tallow; MT, mutton tallow; CF, chicken fat.

from this study could provide a basis for differentiating CF from NL.

A comparison between the heating profiles of NL and other animal fats is shown in Figure 6. As previously seen with cooling curves, the heating curves of NL also had two well-separated endothermic transitions, identified as the low- and high-melting regions (Curve A). The low-melting region consisted of a major sharp peak at 1.10°C with a shoulder-peak at -17.2°C, while the high-melting transition was represented by a sharp peak with the maxima at 28.25°C. In the melting profile of CF (Curve B), there was substantial overlap between the low- and high-melting transitions. The low-melting region consists of a major peak at 1.85°C with two shoulder peaks at -8.0 and -23.0°C, respectively, while the high-melting region is represented by a broad peak resulting from the overlap of two adjacent peaks appearing at 17.2 and 35.6°C, respectively. In the case of BT and MT, their melting curves were comparably similar to each other, but they were remarkably different from those of NL and CF. In the melting curve of BT, the major peak was located at 12.1°C, with two smaller shoulder peaks at 6.5 and –15.0°C, respectively, representing the low-melting region, while the peak appearing at 47.5°C with a shoulder at 34.1°C represented the high-melting region (Figure 6). This curve was comparably similar to the DSC melting profile of BT reported previously by Rodriguez *et al* (2001) and Aktas and Kaya (2001). The melting profile of MT (Figure 6, Curve D) also showed four steps of melting transitions. The low-melting region of MT is represented by a major peak at 11.4°C with two smaller shoulder peaks at 4.05 and -15.00°C, respectively, while the HMG fraction had a major peak at 45.10°C with a shoulder peak at 32.65°C.

Conclusions

A comparison between lard and other common animal fats has been made on the basis of fatty acid, TAG composition, and DSC thermal profiles. The different nature

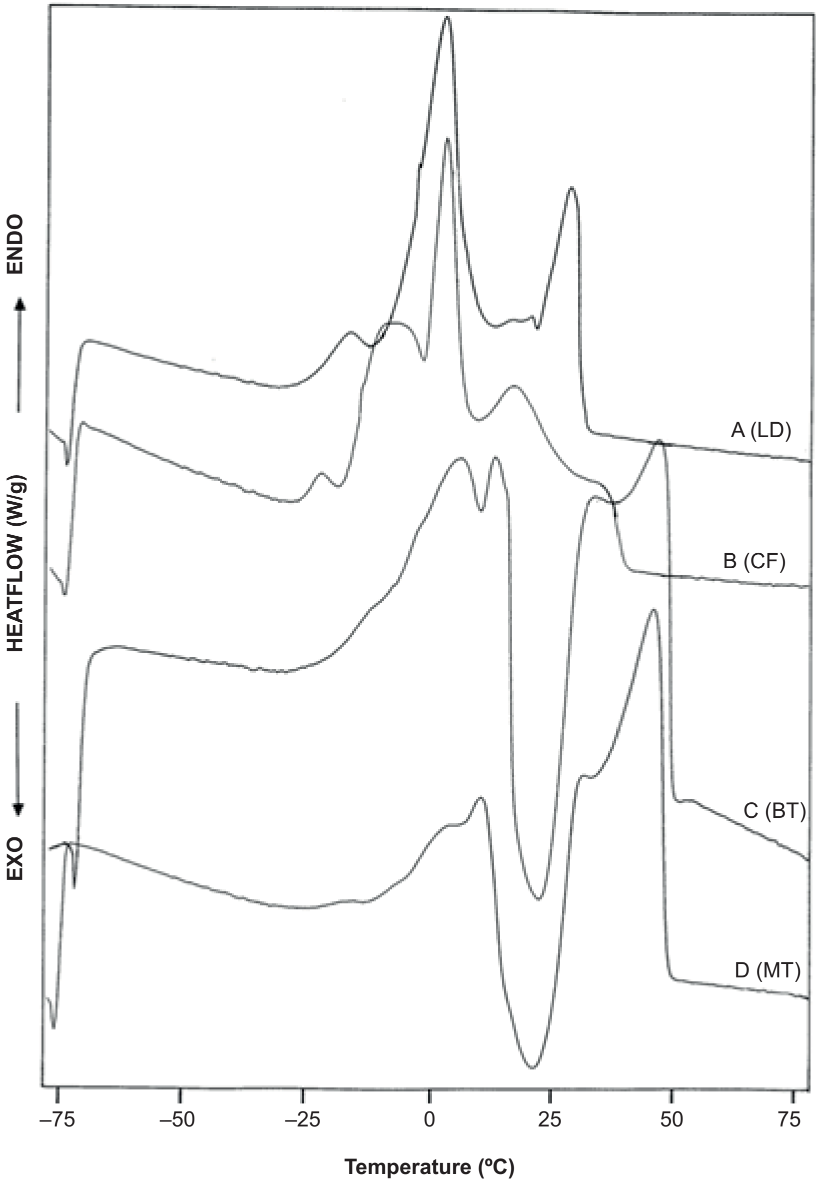

Figure 6. DSC heating thermograms of lard and other common animal fats. Abbreviations: LD, native lard; BT, beef tallow; MT, mutton tallow; CF, chicken fat.

of esterification of different FA in TAG molecules is the primary factor that makes animal fats to differ in their chemical and physical characteristics. Fatty acid compositional analysis showed that PAEF of lard was much higher than those of other common animal fats. Major differences between NL and BT/MT were also found on the basis of TAG composition. The degree of unsaturation of the TAG species in animal fats had a strong influence on the TAG separation in the reverse-phase silica column and could help differentiate different animal fats. Lard displayed a DSC cooling curve with two distinct exothermic sharp peaks at 4.9 and -16.9°C, respectively, while cooling curves of other animal fats were more complex, involving multiple thermal transitions, resulting in broader peaks.

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