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OPINION PAPER

COLOR CHANGE KINETICS AND TOTAL CAROTENOID CONTENT OF PUMPKIN AS AFFECTED BY DRYING TEMPERATURE

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ABSTRACT

The color changes kinetics of pumpkin slices during convective hot air drying was investigated at drying temperatures of 50, 60, 70 and 80°C. The hunter lab L* a* and b* color coordinates were used as assessment indicators. The total color change ΔE, Chroma value, hue angle and brownness index (BI) of the pumpkin slices where also determined. To determine the most suitable kinetics model for the prediction of the color changes of pumpkin, the zero-order, first-order, and fractional conversion models were fitted to the experimental data, using linear regression analysis. The activation energy of the color change parameters (L*, a*, b* and ΔE) was estimated and found to be 41.59, 16.287 63.856 and 73.390 kJ/mol respectively. The fresh pumpkin samples contained a mean total carotenoid content of 25 μ-g/g, while the total carotenoid content of samples dried at 50, 60, 70 and 80°C were 146, 56.4, 37.9 and 102.5 μ-g/g, respectively. Further, the results of ANOVA showed there was significant difference between the total carotenoid content of the fresh pumpkin samples and those dried by convective hot air dryer at 5% ($p<0.05$) significant level.

Keywords: thin layer drying, color kinetics, color measurement, carotenoid, pumpkin

1. INTRODUCTION

Pumpkins are very good sources of carotenoid which are used either in small or large quantities for industrial utilization such as the production of food products, supplements, pharmaceutical, health care and cosmetic products (SEO *et al*., 2005; LEE and LIM, 2011; DURANTE *et al*., 2014). However, carotenoid content in pumpkins are highly degradable and must be preserved properly in order to increase shelf life and enhance their availability. Drying is one of the oldest methods of agricultural and food preservation (ALONGE and ONWUDE, 2013). It is also an operation unit in food processing that can result in longer shelf life, high retention of nutritional properties and a reduction in the bulk of products, thereby reducing the storage space required. Convective hot air drying is the most common method of industrial drying. This drying process has been reported to be largely influenced by factors such as drying temperature, material thickness, drying air velocity and relative humidity (KROKIDA *et al*., 2003; MISHA *et al*., 2013). These factors could adversely affect important product properties, such as moisture content, texture, color and total carotenoid content. The color properties and the total carotenoid content have been reported to be the most affected properties during drying of highly perishable fruits as a result of high processing temperature and longer drying time (KOCA *et al*., 2007; DEMIRHAN and ÖZBEK, 2009).

During drying, color changes occur due to the degradation of carotenoid and non-enzymatic reactions. Carotenoids constitute a large group of over 650 structures, which are responsible for most red, orange and yellow coloring in fruits. They are organic pigments and bioactive compounds synthesized from plants, fruits, vegetables and other photosynthetic plants (CRUPI *et al*., 2012). They are richly colored molecules and serve as a major source of vitamin A and its precursors, which include: α-carotene, β-carotene, lycopene and lutein (SEO *et al*., 2005; DURANTE *et al*., 2014). According to JOHNSON (2002), carotenoids are thought to be very beneficial due to their role as antioxidants. However, they have other health benefits such as anti-cancer, enhancement of human response and reduction in the risk of degenerative cardiovascular diseases (MOLDOVAN and RABA, 2010; DJUIKWO *et al*., 2011; NORSHAZILA *et al*., 2012). Carotenoids undergo degradation during drying due to factors such as a long processing time, high processing temperature and seasonal variations (AKANBI and OLUDEMI 2004; NOR, 2013). Degradation of carotenoids not only affects the attractive color of foods but also their nutritive value and flavor (PESEK and WARTHESEN, 1990). Thus, engineering processes that will optimize carotenoid retention become very essential.

Most often the optical (color) parameters used in the classification of fruits and vegetables are the linear color parameters, RGB (red, green and blue) and the nonlinear color parameters of HSI (hue, saturation and intensity) and L*a*b* (lightness, redness and yellowness) (HASHIM *et al*., 2012). RGB is difficult to measure due to the non-uniform scaling (CHENG *et al*., 2001). Thus, it is necessary to overcome this problem by modelling the color change kinetics using the L*a*b* color measurement parameters.

Regardless of the drying method, the optical properties and total carotenoid content (TCC) of most fruits decreases with longer drying time and higher drying temperature (NAWIRSKA, *et al*., 2009). Reducing drying time and temperature, and the time lag between material preparation and processing improve color quality and TCC retention significantly (RODRIGUEZ-AMAYA, 2003). High processing temperature and short time drying have also been reported to be a good alternative (RODRIGUEZ-AMAYA 2002, 2003). Modelling this processes can help in selecting the appropriate or optimum drying conditions, so that the drying process of pumpkin can be optimized as a means to maintain the product's optical properties, reducing carotenoid losses and improving product quality. Thus, color kinetic modelling approach such as the reaction order, rate

constant and activation energy are indispensable in predicting the quality of foods with regards to color changes due to the degradation of carotenoid. Its application is therefore essential for a wide range of technologies, including online monitoring of the drying process of fruits and vegetables (NADIAN *et al*., 2015).
Few studies on the color kinetics of food materials have been reported in the scientific literature. GAMLI (2011) studied the color changes of tomato puree and the kinetic modelling of the color changes of some fruits, such as kiwifruits (MOHAMMADI *et al*., 2008), apple, banana and carrots (KROKIDA *et al*., 2007) have also been investigated. More so, studies on the effect of process storage conditions and packaging on the color properties and carotenoid compositions of some fruits have been reported (BECHOFF *et al*., 2011; GUINÉ *et al*., 2011; BECHOFF *et al*., 2015). However, no study has been reported on the color change kinetics and total carotenoid content of pumpkin during convective drying process, with particular emphasis on the selection of a suitable kinetic model in predicting the color stability and optimum drying conditions. Therefore, the objective of this study is to investigate the color change kinetics and the enhancement of the optical properties of pumpkin slices, under different drying conditions during the forced air conventional drying.

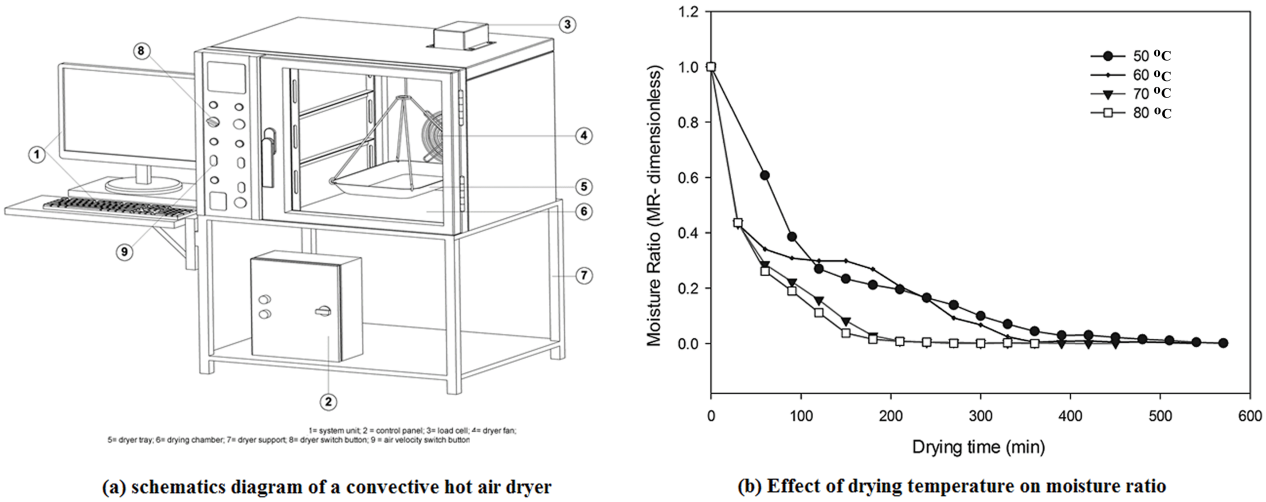
2. MATERIALS AND METHODS

2.1. Materials

Freshly harvested pumpkin fruits of the *Cucurbita moschata* variety were purchased locally in Malaysia. The samples were stored at 11°C before the drying experiments, in order to slow down respiration, physiological and chemical changes. The homogenous samples were sorted visually for color (maturity) and size with no evidence of physical damage. Before each drying experiment, the selected samples were hand peeled, washed in running water and the pulp was sliced into 5 mm thickness using the Nemco slicer (55200AN, USA). A total of 40 samples with three replications each were used to perform the experiments.

2.2. Drying experiments

Pumpkin samples were dried using an automated convective hot air dryer (Fig. 1a) designed and developed by the Department of Biological and Agricultural Engineering, Faculty of Engineering, Universiti Putra, Malaysia. The dryer used was a NFC-3D Series Electric Convection Dryer at a voltage of 380/220 V, a power of 4.5 kW and a frequency of 50 Hz. Details of the dryer can be found in the work of ONWUDE *et al*. (2016). During the drying experiments, the actual velocity was measured from the center of the drying chamber using a vane anemometer (Extech, 451104) with an accuracy of ± 0.030 m/s.
The drying was conducted at temperatures of 50, 60, 70 and 80°C, respectively. The relative humidity ranged from 40 to 50% throughout the experiments and the drying air velocity was constant at 1.2 m/s. During each drying experiments, the sample weight was recorded after every 1 hour periodically. Each drying experiment was allowed to run until there was no further change in the masses of two consecutive measurements. A total of 12 experimental runs were carried out with three replications and the average values were used to estimate the loss in moisture during drying, expressed in the form of dimensionless moisture ratio. The initial moisture content analysis was performed using the oven dry method at 103 ± 2°C (ASAE, 2005). Triplicate samples were analyzed and the average moisture content was found to be 5.63 dry basis (d.b.).



(a) schematics diagram of a convective hot air dryer **(b) Effect of drying temperature on moisture ratio**

Figure 1. Schematic diagram of the convective hot air oven and effect of temperature on moisture ration of pumpkin.

The experimental data obtained for different temperatures was further expressed in the form of the moisture ratio (MR) versus time as given in the equation (2) below:

$$MR=\frac{M_t-M_e}{M_{o-M_e}} \quad (1)$$

M_t is the moisture content at any time t, Mo is the initial moisture content of the pumpkin sample and M_e is the equilibrium moisture content.

2.3. Color measurement

Each pumpkin sample was measured before and during drying at 1-hour time intervals until equilibrium moisture content was obtained using a Chroma Meter colorimeter (CR-10, Konica Minolta Sensing Americas, Inc.). The colorimeter was calibrated against a standard black and white plate in order to accurately determine the color parameters of the Hunter L* a* b* values. For each of the samples and replicates, at least four measurements were performed at different sample positions and the average value was estimated. The measurements were represented by the L* a* b* color parameters which were used to describe the kinetics of the color changes of the convective hot air drying of the pumpkin. The L* represents the degree of brightness or lightness with a range from 0 (black) to 100 (white). The a* shows the degree of redness with color values ranging from – 60 (green) to + 60 (red), and the b* shows the degree of yellowness when positive, and blueness when negative with a range from − 60 (blue) to + 60 (yellow).

2.4. Estimation of color change kinetics

Being difficult to develop an adequate kinetic model for color change during drying, there is a need to combine more than one kinetic model.

Generally, the kinetics of the color change of most foods have been estimated by the zero, first order kinetic and fractional conversion models given as follows (XIAO *et al*., 2014; NIAMNUY *et al*., 2008; DADALı *et al*., 2007; KROKIDA *et al*., 2007):

$$\frac{dC}{dt} = -k(C)^n \quad (2)$$

where k is the rate constant, C is the value of the three color parameters varying with time (t), n is the order of the reaction and $\frac{dC}{dt}$ is the rate of change of C with time (HASHIM *et al*., 2012).
For zero-order reactions, the three color parameters C, are not dependent on the rate of reaction as shown below:

$$-\frac{dC}{dt} = k \quad (3)$$

Integrating Eq. 3 results in:

$$C = C_0 - kt \quad (4)$$

Eq. 4 is known as the zero-order reaction rate model. In terms of color kinetics, the solution of first order reaction rate (Eq. 2), which is also dependent on the color parameter, can be expressed as:
Integrating Eq. 2 gives:

$$C = C_0 e^{-kt} \quad (5)$$

Now, the first-order kinetic model (Eq. 5) can further be represented by fractional conversion, taking into account the final color value. Thus, according to XIAO *et al*. (2014), the color parameter can be expressed as a fractional ratio as shown below:

$$\frac{C - C_f}{C_0 - C_f} = e^{-kt} \quad (6)$$

Where C_0 is the sample color value at time zero and C_f is the final color value of the sample.
Furthermore, the rate constant is temperature dependent and follows an Arrhenius relationship (LAU *et al*., 2000). Thus, the relationship between the rate constant of the total color change, the L*, a* and b* color parameters of the pumpkin and the drying temperatures were determined as follows:

$$K = K_0 \exp\left[-\frac{E_a}{R}\left(\frac{1}{T+273.15} - \frac{1}{T_{ref}+273.15}\right)\right] \quad (7)$$

E_a is the activation energy in kJ/mol, R is the universal gas constant (R = 8.31451 x 10^{-3} kJmol−1 k−1), K_0 is the pre-exponential factor, T_{ref} is the reference temperature, which is the median of the drying temperatures (65°C) and T is the air temperature expressed in °C.

The Total color change (ΔE or TCD) of the pumpkin samples was calculated using following equation:

$$\Delta E = [(L_0^* - L^*)^2 + (a_0^* - a^*)^2 + (b_0^* - b^*)^2]^{1/2} \quad (8)$$

The polar coordinate Chroma, which is an indication of how dull/vivid the product is (ranging from 0 to 60), was estimated from the a* and b* Cartesian co-ordinates as follows:

$$\text{Chroma} = \left(a^{*2} + b^{*2}\right)^{1/2} \quad (9)$$

Another measure is the degree to which the appearance color of fruits and vegetables can be said to be similar to or different from the primary colors of red, green, blue, and yellow. An angle of 0° or 360° represents red hue, while angles of ≤ 90° indicates yellow, angle of ≤180° and ≤270° signifies green and blue hues, respectively. This parameter has been widely used in the determination of the color parameters in fruits and vegetables (PATHARE *et al*., 2012) and is expressed as:

$$\text{Hue Angle} = \tan^{-1}(b^*/a^*) \quad (10)$$

where L^* is the degree of lightness, L_0^* is the initial value of L^*, a^* is the degree of redness, a_0^* is the initial value of a^* while b^* signifies the degree of yellowness and b_0^* is the initial value of b^*.

The Browning index, which is defined as brown color purity (MOHAMMADI *et al*., 2008; DADALI *et al*., 2007) is associated with the browning of fruits during drying. This was estimated as:

$$BI = \frac{[10(x-0.31) * 10^3]}{17} \quad (11)$$

where

$$x = \frac{(a+1.75L)}{(5.645L+a-3.012b)} \quad (12)$$

The order of reaction for the color parameters during convective hot air drying of the pumpkin was determined by using linear regression analysis on Eq. 4, Eq. 5 and Eq. 6. In each case, the best fit was selected and the kinetic rate constant for each processes was determined.

2.5. Extraction and separation

A method described by RAVELO-PEREZ *et al*. (2008) and RODRIGUEZ (2001) with some modifications was employed to extract the carotenoid content from pumpkin samples. Approximately 5 g of fresh and dry samples (for each drying temperature) were ground and weighed on a digital weigh balance (A and D GF-10k, USA) and transferred into a beaker. 20 mL of 0.05% (w/v) BHT in hexane, 20 mL of acetone and 10 mL of ethanol in the ratio of 2:2:1 (v/v/v) were added, stirred and filtered. The process was continued with the residue until it became colorless in order to obtain optimum extract. The crude extract was evaporated in a rotary evaporator (Eyela OSB – 2100, Japan) attached to a vacuum pump at a temperature of 35°C, using glass pearl for optimization of the recovery in the

re-dissolving process (RODRIGUEZ-AMAYA, 2001; NORSHAZILA *et al*., 2012). The process was performed under subdued light and analyzed within one day in order to minimize degradation of carotenoids.

2.6. Spectrophotometry analyses

The total amount of carotenoid was determined using a UV-spectrophotometer (1800 series, Shimadzu, Japan). Preliminary analysis of lutein and β-, α- carotenes in pumpkin samples tested resulted in absorbance peak values within the range of 431-475 nm (visible region spectrum), which is in agreement with the absorbance peak values reported by SCOTT (2001). However, the absorbance values used in determining the total carotenoid content were those read at 450 nm (absorbance peak for pumpkin's lutein and β-, α-carotene in hexane and ethanol solvent). All analyses were performed in triplicate.
The total carotenoid content (TCC) was calculated from modified Lambert-Beer law using the formula reported by DE CARVALHO *et al*. (2012) as follows:

$$\text{Total Carotenoid content } (\mu g/g) = \frac{A\ x\ V(ml)\ x\ 10^4}{A_{1cm}^{1\%}\ x\ w(g)} \qquad (13)$$

where A = absorbance; V = total extract volume; w = sample weight; $A_{1cm}^{1\%}$= 2500 (it represents the carotenoid extinction coefficient for hexane on pumpkin samples where majority of the carotenes are dominant) (RODRIGUEZ-AMAYA, 2001; SCOTT, 2001).

2.7. Statistical analysis

Experimental data for the different color parameters were fitted to the zero, first order and fractional conversion kinetic models and processed using SIGMA plot 12.0 software (Systat Software Inc., California, USA). The coefficient of determination (R^2) and the sum of square error (SSE), were used to evaluate the goodness of fit of the best drying conditions for the color parameters. For the total carotenoid content, all data were recorded as the mean ± standard deviation of triplicate determination. One way analysis of variance (ANOVA) was also used to compare means of TCC and absorbance of pumpkin samples at different drying temperatures (5% significant level). Post-hoc Tukey's tests were used as the indicators for means comparison. The Kolmogorov–Smirnov test was used to access the normality of the data.

3. RESULTS AND DISCUSSIONS

3.1. Color changes

The effect of drying temperatures and drying time on the moisture content of pumpkin (*Cucurbita moschata*) is shown in Fig. 1b. An increase in temperature resulted in a decrease in the drying time and a corresponding decrease in the moisture content of pumpkin, which is expressed in the form of a dimensionless moisture ratio. In particular, a safe moisture ratio under 0.05 was reached after 480 min at drying temperature of 50°C, 420 min at temperature of 60°C and 70°C and 300 min at drying temperature of 80°C. Consequently, a decrease in the drying time of about 38% was observed as the temperature increased from 50°C to 80°C.

Figure 2 shows that temperature and time have a significant effect on the color properties of pumpkin during convective hot air drying. The L*, a*, b* and total color change (ΔE) values obtained during the drying experiments are presented in Fig. 2a-d. The L* value of the pumpkin slices decreased with an increase in drying time. Also, the L* value of pumpkin dried at a higher temperature (80°C) declined rapidly with an increase in the drying time. Furthermore, it can be seen that the L* value decreased from with increasing drying temperature. This decrease in the L* value is in line with the decrease in the moisture content at higher temperatures (BAL *et al*., 2011; DEMIRHAN and ÖZBEK, 2011; DEMIRHAN and ÖZBEK, 2009; AVILA *et al*., 1999). In addition, towards the end of the drying process the change rate of L* became slower similarly to the rate of moisture loss from pumpkin (Fig. 1b) (HASHIM *et al*., 2014; DOYMAZ, 2007). Thus, it can be seen that the moisture loss is also a factor that affect the changes in the brightness of pumpkin slices during drying.

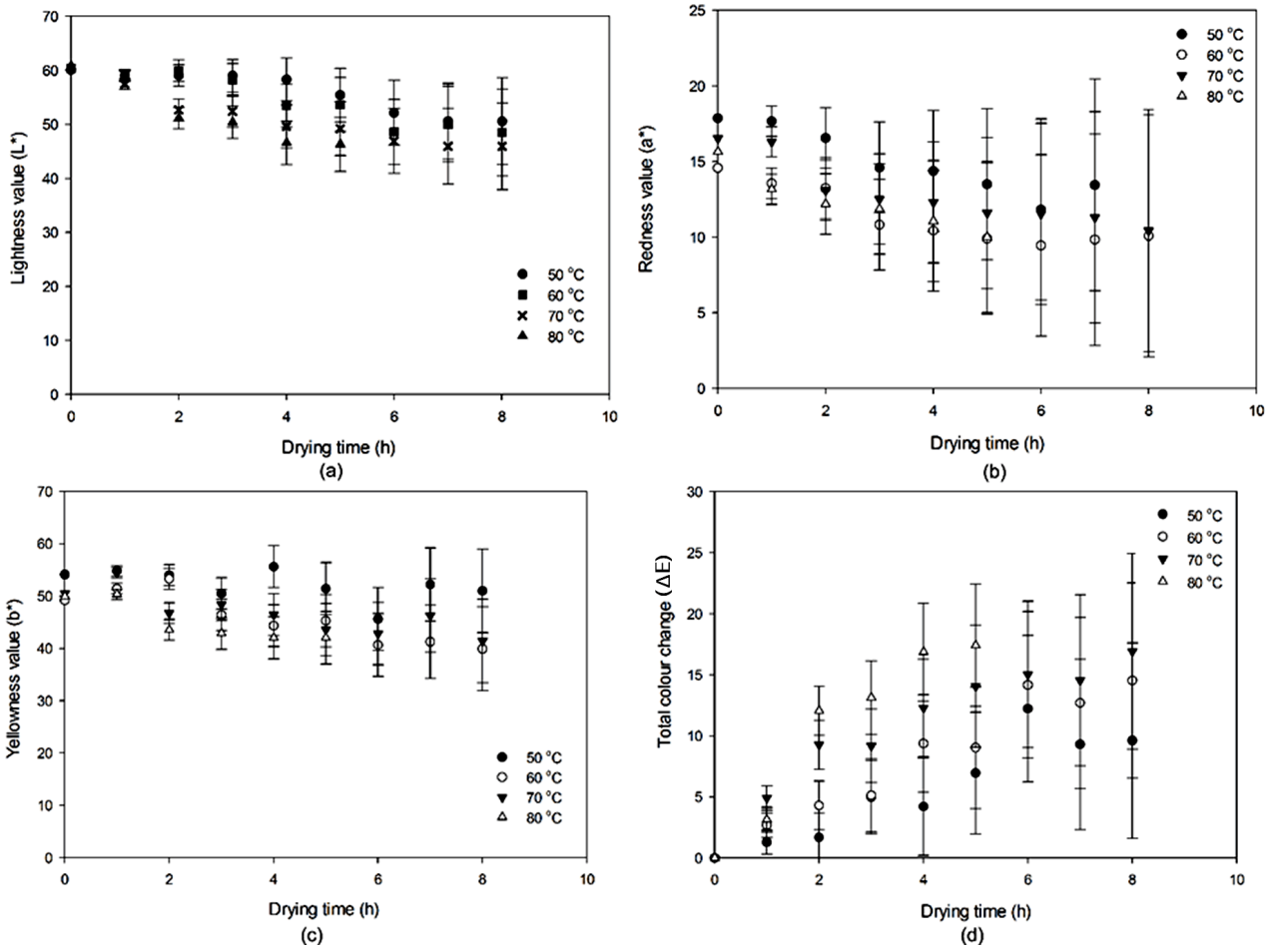


Figure 2. Pumpkin color degradation (L* a* b* and ΔE parameters) during convective hot air drying (bars represent standard error of the mean).

The variation of the redness values during drying is shown in Fig. 2b. From the figure, a decrease in the redness value with increase in drying time can be observed. Also, the final a* value decreased with an increase in the drying temperature. However, the standard deviation of the a* values of pumpkin increases as the drying time increases at

temperatures (50, 60, and 70°C). This could be due to the prolong exposure to heat. Thus, there is no justification in prolonging drying of pumpkin, as this would adversely affect the a* value of pumpkin. However, at higher drying temperatures (70 to 80°C), there was a decrease in the a* value. This behavior can be attributed to the heat and mass transfer from within the pumpkin slices to the surface, which is faster at higher temperatures during drying. Furthermore, the decrease of the a* value during drying could be due to the autoxidation of carotenoids which gradually caused the redness color of the pumpkin to deteriorate at much higher temperatures and longer drying times. For this study, there was no significant difference in the a* value of pumpkin slices under all drying conditions. Similar results, on the reduction of a* during drying have been reported by VEGA-GÁLVEZ *et al*. (2008) concerning the convective dehydration of red bell pepper and KOCA *et al*. (2007) on the dehydrated carrots.

Fig. 2c shows a decrease in the b* value with an increase in both temperature and time. The final reduction in the b* value varied from 51.00 at 50°C to 42.02 at 80°C. The loss in b* value indicates that the yellowness of pumpkin under convective hot air drying decreased with a corresponding increase in the drying temperature and time. According to PRAKASH *et al*. (2004), the high rate of β-carotene loss during the solar cabinet drying of carrot was caused by longer drying times and exposure to light, which leads to light induced oxidation of the β-carotene. In addition, reduction in β-carotene during drying at high temperatures has been attributed to the oxidation of its highly unsaturated chemical structure (OLIVEIRA *et al*., 2015). Thus, the reduction in the b* value of pumpkin slices in a convective hot air dryer was due to the oxidation of the dominant carotenes (β-carotene and lutein). Furthermore, the partial formation of brown pigments (quinones and melanins) could also be responsible for the reduction of b* at higher temperatures. Overall, the total color change (ΔE) of the pumpkin slices increased significantly with increase in the drying temperature and time during convective hot air drying, with a value ranging from 10.6 to 17.46 at a temperature range of 50°C to 80°C as shown in Fig. 2d.

Generally, slight differences in the color parameters were observed at time 0. This was as a result of samples variability used during the drying experiments. The decrease in L*, a* and b* values and the corresponding increase in ΔE values during the convective hot air drying of pumpkin could be attributed to the changes that occurs as a result of the oxidation of β-carotene, when drying at higher temperature and longer time. However, at temperature of 80°C, there was gradual stability in the reduction rate, due to shorter drying time.

Chroma, hue angle and browning index are also very important color parameters. These parameters were estimated from the experimental data by using Eq. (9) to Eq. (12) and the results are shown in Fig. 3a-c. The Chroma value decreased with a corresponding increase in temperature and drying time (Fig. 3a). The final Chroma value varied from 52.74 at 50°C to 43.19 at 80°C. The high Chroma value signifies that the dominant pumpkin colors of yellow and red are pure and intense. Conversely, the Hue angle increased with an increase in drying time (Fig. 3b). However, as the drying temperature was raised from 50 to 60°C, the Hue angle increased rapidly and began to stabilize at 77° after 4 to 5 hours. A further increase in the drying temperature showed a slight increase in the Hue angle and stabilized at 75° before further decrease as the drying continued. Generally, the results indicated a good hue angle as the hue angle for the pumpkin at different drying conditions ranged between 71° to 78° (< 90° for the yellow-orange-red HSB/HSL color). Fig. 3c shows the effect of temperature on the browning of pumpkin under convective hot air drying. The BI which was estimated from Eq. (11) and Eq. (12) and fitted to the experimental data, increased slightly with an increase in drying time. Similar results have been reported during the drying of coconut slices (YUN *et al*., 2015). Generally, in order to

minimize the oxidation of carotenoid during drying, it is not recommended to dry at a temperature above 80°C.

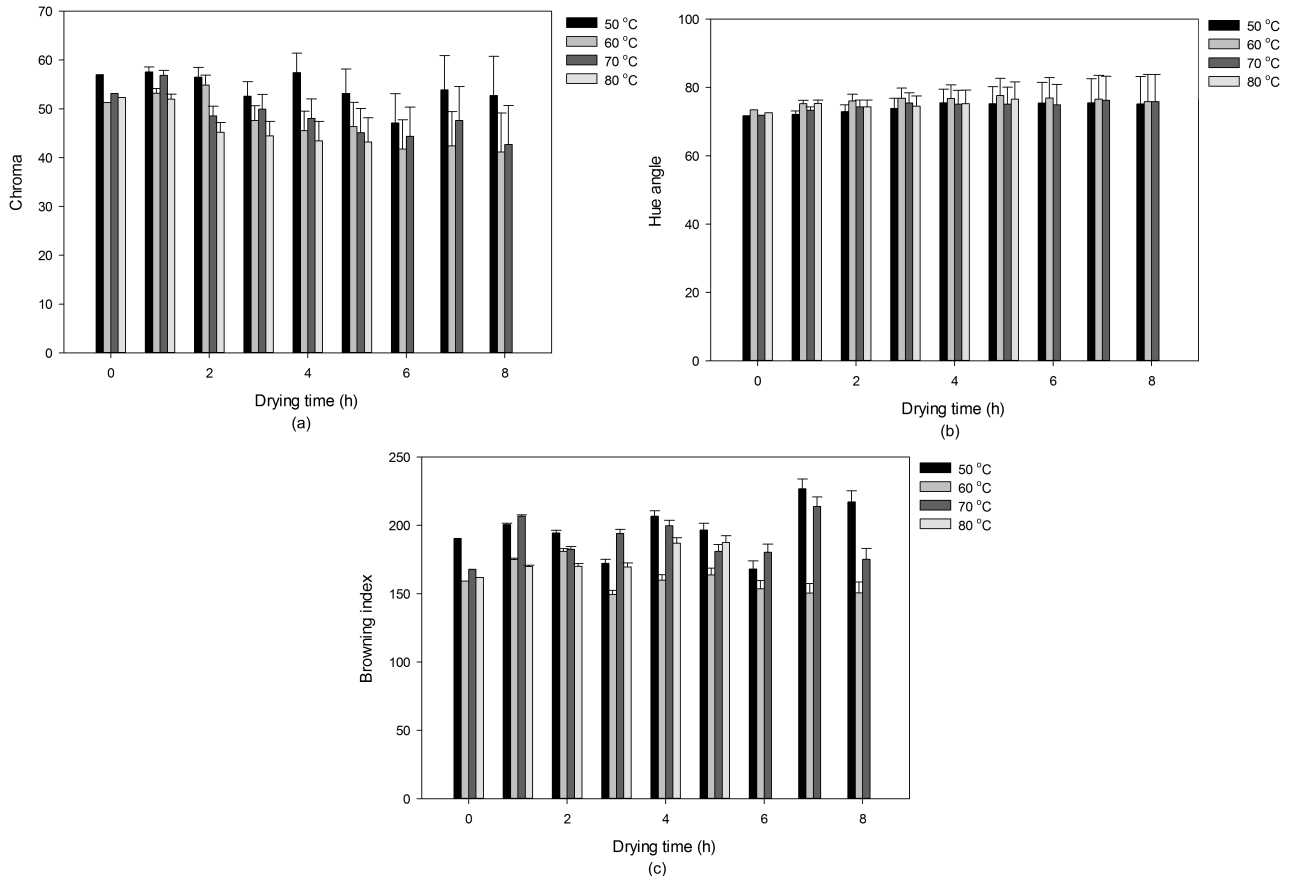


Figure 3. Effect of drying temperature on the (a) Chroma, (b) hue angle, and (c) browning index of pumpkin slices (bars represent standard error of the mean).

3.2. Kinetic modelling

Kinetic modelling of the color changes of pumpkin slices during convective hot air drying was investigated using the zero-order, first-order and fractional conversion (Eq. 4 to Eq. 8) kinetic models respectively. These models were fitted to the experimental data of the color parameters, and the most suitable kinetic constant and model were selected based on the highest coefficient of determination (R^2) and least sum of square error (SSE) of linear regression analysis.

Tables 1 and 2 show the results of the kinetic modelling of the color change of pumpkin slices. From the results, the most suitable model for the color parameters (L*, a*, b*, ΔE, Chroma and Hue angle) was generally the fractional conversion model, even though the first order model gave the best correlation at a temperature of 80°C for a* and b*, respectively. The zero order kinetic model was not found to be suitable for the prediction of pumpkin color changes during convective hot air drying, this model gave the lowest R^2 statistical values for all but one of the color parameters (BI at 80°C) and temperature conditions.

Table 1. Estimated regression results of zero-order, first-order and fractional conversion models for L*, a*, b*, and ΔE at various drying temperatures.

		Zero-order model			First-order model			Fractional conversion model		
Temp. (°C)	Parameter	K	R^2	SSE	K	R^2	SSE	K	R^2	SSE
50	L*	0.954	0.876	1.133	0.0166	0.864	1.181	0.0013	0.971	0.0731
	a*	0.826	0.871	0.781	0.0536	0.883	0.741	0.0662	0.909	0.160
	b*	0.524	0.288	2.561	0.0100	0.288	2.560	7.1E-9	0.237	4.934
	ΔE	-1.404	0.8517	1.6294	0.1476	0.571	7.2762	0.0320	0.8777	0.1645
60	L*	1.3322	0.8843	1.4392	0.0237	0.8803	1.4642	0.0138	0.9460	0.1028
	a*	0.7592	0.7492	0.9625	0.0645	0.8265	0.8005	0.0891	0.9581	0.0935
	b*	1.0707	0.6884	2.6741	0.0232	0.6762	2.7261	0.0044	0.8156	0.2365
	ΔE	-1.958	0.9435	1.2458	0.1979	0.6157	9.9669	0.0960	0.9457	0.0898
70	L*	1.9717	0.7752	2.1610	0.0374	0.8234	1.9155	0.3076	0.9826	0.0500
	a*	0.8648	0.7951	0.9805	0.0645	0.8588	0.8140	0.2392	0.9368	0.0953
	b*	1.0316	0.6669	2.3349	0.0218	0.6657	2.3393	0.0533	0.6973	0.2608
	ΔE	-2.440	0.7818	2.5498	0.3820	0.3148	12.5844	0.3337	0.9765	0.0529
80	L*	3.3011	0.9123	1.8214	0.0617	0.9295	1.6334	0.2249	0.9678	0.0852
	a*	1.2229	0.8445	0.7673	0.0954	0.8989	0.6187	0.5793	0.5068	0.2709
	b*	1.8982	0.7853	1.8159	0.0416	0.7996	1.7547	1.216e-5	0.7828	0.01367
	ΔE	-4.023	0.9015	2.2730	0.4266	0.4919	13.5336	0.2717	0.9763	0.0716

The fractional conversion kinetic rate constant for L* increased from 0.0013 to 0.3076 min^{-1}, a* increased from 0.0662 to 0.2392 min^{-1}, b* increased from 0.0100 to 0.0533 min^{-1} and the total color change, ΔE, increased from 0.032 to 0.3337 min^{-1} as the drying temperature was raised from 50 to 70°C. This implies that the rate of color reduction as a result of carotenoid degradation was faster with an increase in the drying temperature due to the high energy transferred to the inside of the food material. However, the value of the kinetic rate constant reduced with a further rise in the drying temperature to 80°C. This may be attributed to a shorter drying time experienced at temperatures above 70°C. This shorter drying time results to lesser exposure of the pumpkin to light and heat, thus reducing the rate of carotenoid degradation at temperatures between 70°C to 80°C. Therefore, the fractional conversion model can be used to predict the color change kinetics of pumpkin during convective hot air drying.

Similarly, several authors from the literature have reported that the fractional conversion and first-order kinetic models can adequately predict the change of different color parameters of some agricultural food products during drying (XIAO *et al.*, 2014; HOSSEINPOUR *et al.*, 2013; AVILA *et al.*, 1999).

Table 2. Estimated regression results of zero-order, first-order and fractional conversion models for Chroma, Hue angle and Browning index at various drying temperatures.

		Zero-order model			First-order model			Fractional conversion model		
Temp (°C)	Parameter	K	R^2	SSE	K	R^2	SSE	K	R^2	SSE
50	Chroma	0.7130	0.3866	2.6415	0.01310	0.3882	2.6379	0.02790	0.4133	0.6528
	Hue Angle	-0.5858	0.7838	0.7248	8.6736E-019	0.2817	3.04560	0.05170	0.9699	0.08260
	BI	-1.9244	0.1278	17.825	1.7347E-018	0.1315	20.302	7.9442E-009	0.3510	0.6142
60	Chroma	1.2303	0.7404	2.5771	0.02580	0.7288	2.6340	0.007200	0.8483	0.2073
	Hue Angle	-0.5473	0.2117	1.3450	1.4229E-018	0.3288	3.07380	1.3734	0.4021	0.4335
	BI	0.5342	0.04690	10.971	0.003400	0.04630	10.975	51.414	0.8938	3.1117
70	Chroma	1.2335	0.7153	2.3657	0.02510	0.7167	2.3602	0.07080	0.7441	0.2290
	Hue Angle	-0.6247	0.5037	0.9581	1.5658E-018	0.4876	3.2966	0.5198	0.9125	0.1080
	BI	-3.9046	0.4954	18.824	3.2747E-018	0.1361	27.260	51.414	0.8938	3.1117
80	Chroma	2.1608	0.8220	1.7858	0.04550	0.8397	1.6948	0.03140	0.9816	0.07050
	Hue Angle	-0.7779	0.5157	0.9285	3.7663E-019	0.2240	2.7419	0.7182	0.7046	0.2019
	BI	-4.9892	0.8287	4.3260	1.6660E-018	0.6778	17.103	0.04430	0.7951	0.2076

In addition, Fig. 4 shows the relationship between the experimental color data and predicted color data, based on the fractional conversion model for L* color parameter. The values fitted close to a straight line with $R^2 > 0.9$. This validates the application of the fractional conversion model in predicting the color change kinetics of pumpkin during convective hot air drying.

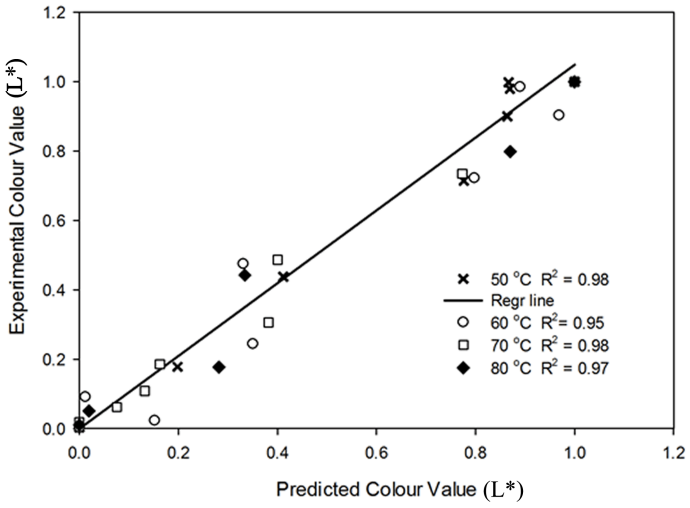


Figure 4. Validation of the fractional conversion model for the prediction of color change kinetics.

3.3. Estimation of activation energy

The activation energy of the color change can be estimated from a plot of the kinetic constant against the drying temperatures as shown in Fig. 5.

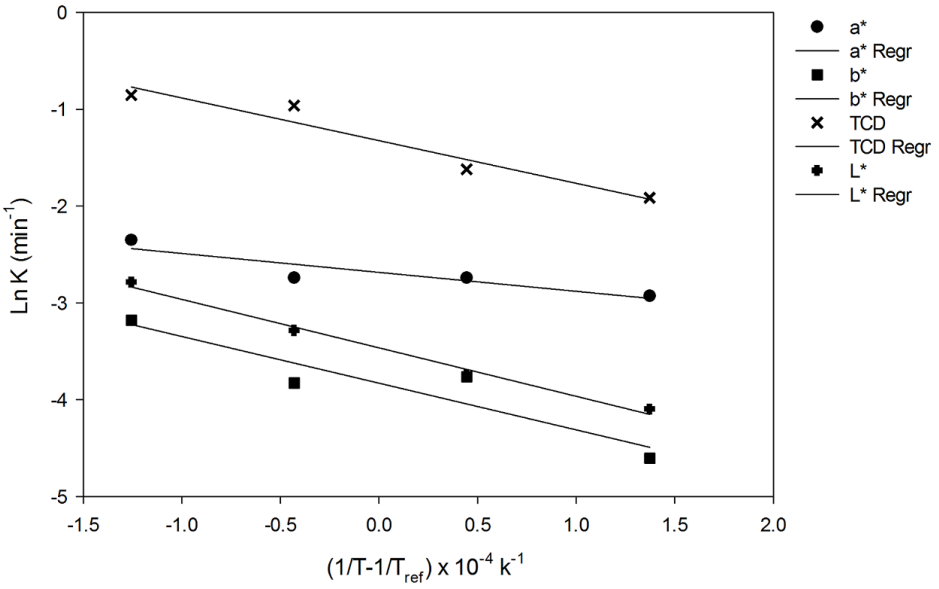


Figure 5. Estimation of activation energy.

Table 3 shows the linear models of the kinetic rate constant and temperature for different color parameters. The activation energy for the L*, a* and b* was estimated by linear regression using Eq. 7 and was found to be 41.59, 16.287 and 63.856 kJ/mol respectively. Higher values of L*, b* and ΔE indicates that higher energy is required in order to cause a significant change in the brightness and yellowness of pumpkin during convective drying, and therefore monitoring the color change during drying becomes more indispensable. Accordingly, a lower minimum energy is required to reduce the redness of pumpkin during the drying process due to its lowest activation energy. Overall, the minimum energy required to cause a significant total color change (ΔE) during the drying of pumpkin is 73.390 kJ/mol. In addition, the results of the activation energies for L∗, a∗, and b∗ color change kinetics were lower than those of peach puree (AVILA *et al*., 1999), the L* , a* were lower than those of celery leaves (DEMıRHAN and ÖZBEK, 2011) and the a*, and b* were lower than those of American ginseng (XIAO *et al*., 2014). Similarly, the activation energies for the L* a∗ b∗ and ΔE color change kinetics were higher than those of Okra (DADALI *et al*., 2007) and carrot (KOCA *et al*., 2007). Thus, the variation in the activation energies of the color parameters of pumpkin as compared to other fruits and vegetables may be due to the variations and compositions of different food products, including the method and drying process conditions.

Table 3. Activation energy for the color degradation of pumpkin for different major color kinetics parameter.

Color kinetics parameter	Linear modelled equation	R^2	E_a(kJ/mol)
L*	$Lnk = -3.4618 - 5002.1\left(\frac{1}{T_{abs}}\right)$	0.9898	41.592
a*	$Lnk = -2.6831 - 1958.9\left(\frac{1}{T_{abs}}\right)$	0.8335	16.287
b*	$Lnk = -4.01073 - 7680.1\left(\frac{1}{T_{abs}}\right)$	0.8687	63.856
ΔE	$Lnk = -2.01781 - 8826.7\left(\frac{1}{T_{abs}}\right)$	0.9411	73.390

3.4. Effect of drying conditions on total carotenoid content

From Table 4, the total carotenoid content (TCC) and absorbance values of pumpkin (*Cucurbita moschata*) can be observed. The results showed a mean total carotenoid content of 146 μg/g for samples dried at 50°C, 56.4 μ-g/g for samples dried at 60°C, 37.9 μ-g/g for samples dried at 70°C and 102.5 μ-g/g for samples dried at 80°C. These values are lower than those reported by de CARVALHO *et al*. (2012) in landrace pumpkin, with values ranging from 234.21 to 404.98 μ-g/g (wet matter). The lower TCC values may be attributed to the specie of pumpkin used in this study and also nature of materials used during extraction (dried pumpkin in this study and fresh pumpkin in the study of De CARVALHO *et al*. (2012). On the other hand, the TCC values of this present study are similar to those reported by ŞLEAGUN *et al*. (2007), with values ranging from 110.62 to 40.41 μ-g/g (dry matter). This similarity can be attributed to nature of material before extraction, and the drying method (thin layer hot air drying) used in both cases. Further, the TCC of the pumpkin (*Cucurbita moschata*) in this study are within the acceptable range of 20.3 μ-g/g to 158.56 μ-g/g for most pumpkin varieties (AZIZAH *et al*., 2009; SAHABI *et al*., 2012).

Further, the results show that convective hot air drying gives a significant effect on the TCC of pumpkin. From Table 4, it can be seen that there is significant statistical difference between the TCC of pumpkin samples at 5% ($p<0.05$) significant level and 95% confidence interval.

Table 4. Total carotenoid content and absorbance values of Pumpkin (*Cucurbita moschata*) as affected by drying conditions.

Samples	Total carotenoid content (μg/g x 10^3)
50°C	0.146^{a}±0.083
60°C	0.0564^{b}±0.055
70°C	0.0379^{b}±0.018
80°C	0.1025^{a}±0.064

Means ± Standard deviation of samples.
[a,b] Variations in the letters of samples in a column indicate significant difference at 5% ($p\leq0.05$) using Tukey's test.

As shown in Fig. 6, the TCC is highest in samples dried at 50°C. TCC values decreased when the drying temperature is 70°C. A further rise in the temperature to 80°C resulted in a higher TCC value. This high TCC value is due to the shorter drying time required to achieve a desired moisture content with a decrease in the drying time of about 38% as the temperature was raised from 50°-80°C as stated above. More so, the result showing that carotenoid contents are lower for samples which had been dried at temperatures between 50° and 70°C can be further explained by the fact that a longer drying time is needed at these lower drying temperatures. The increase in drying time at lower temperatures increases the exposure of pumpkin samples to light and heat. This further increases the rate of oxidation and carotenoid degradation. Similar results on different fruits have been reported by previous researchers (STAHL, 1992; PRAKASH *et al*., 2004; ALAM *et al*., 2013; MARIA *et al*., 2014). Therefore, from the results, it can be said that convective hot air drying at appropriate drying temperature can reduce the loss of carotenoid in pumpkin.

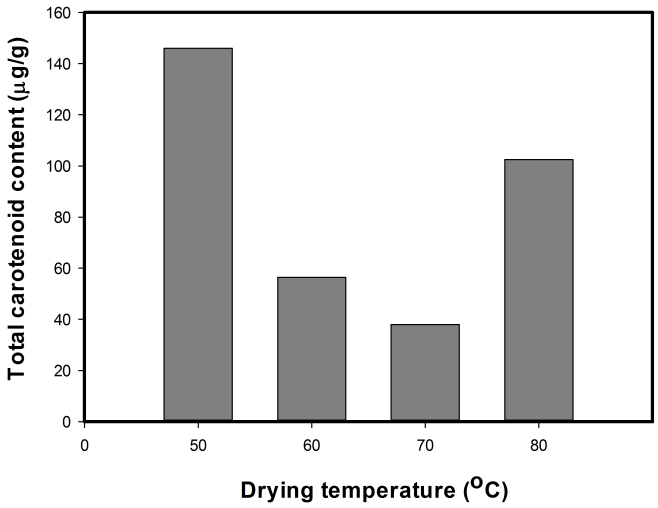


Figure 6. Effect of drying temperature on TCC.

4. CONCLUSIONS

This study showed that convective hot air drying affects the moisture content, color parameters and total carotenoid of pumpkin (*Cucurbita moschata*). Drying at a higher temperature resulted in a shorter drying time. The color parameters and total carotenoid content were significantly affected by drying temperatures and longer drying time. The final values of L *, a*, b*, total color change ΔE, Chroma, hue angle and browning index were also influenced by the drying temperature and time. The results of the total color change and the browning index further showed that drying at higher temperatures above 80°C will result in the darkening and significant pumpkin color change as compared to the fresh slices.

In order to make a better estimate of the color change kinetic parameters, a fractional conversion kinetic model was selected as the most appropriate model for predicting the color change kinetic of pumpkin. Also, the first order model sufficiently fitted the experimental data for certain drying conditions. Thus, can be used to predict the color

changes of pumpkin during drying. In addition, the values of the activation energy were 41.59 kJ/mol for L*, 16.287 kJ/mol for a*, 63.856 kJ/mol for b*, and 73.390 kJ/mol for ΔE, respectively. From these values, it can be seen that a significant amount of energy is required to cause the change in the color of pumpkin during drying.

From the results of this study, it can be concluded that convective hot air drying can be used to enhance the shelf life of pumpkin, maintain the optical properties while enhancing the availability of carotenoid in pumpkin (*Cucurbita moschata*). Thus, in practice a drying temperature between 50°C to 80°C could be used in the convective drying and extraction of carotenoid from pumpkin, considering the combined effects on moisture content, optical parameters and total carotenoid content. However, for the optimization of the drying process, a drying time of less than 300 minutes at a drying temperature between 70 to 80°C can be used. This research can be useful in evaluating the color change kinetics models as a reliable tool for predicting, controlling and optimizing the effect of drying conditions on the color and carotenoid degradation of pumpkin. Finally, it is suggested that different kinetic models could be used for different drying levels in future research.

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PAPER

CHEMICAL COMPOSITION AND THERMAL BEHAVIOUR OF TROPICAL FAT FRACTIONS FROM SOLVENT-ASSISTED PROCESS: A REVIEW

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ABSTRACT

Fractional crystallization is a process applied to plant and animal fats to obtain fat products with new functional properties and nutritional values. Fractionation is generally performed through either a dry or a solvent-assisted process. As solvents can wash off the liquid molecules able to entrap the solid component inside, the solvent-crystallization seems to be more efficient than other fractionation procedures. In recent times, fractional crystallization has been investigated for avocado (Persea Americana) butter, engkabang (Shorea macrophylla) fat, palm oil-moringa oil blend, and mee fat (Madhuca longifolia) to prepare solid (stearin) and liquid (olein) fractions. This review sums up the main and recent published studies on chemical and thermal (upon differential scanning calorimetry) properties of fat fractions obtained using solvent-assisted process. It has been argued that the ease of a fat fractionation mainly depends on the thermal characteristics of its triacylglycerol molecules. Fats with low-and high-melting thermal transitions in wide separation would yield fat components with remarkable changes in physico-chemical characteristics, fatty acid and triacylglycerol compositions as well as thermal profiles. The activities involving the use of fat components, during food formulation, may significantly benefit from a further learning of the fats behavior.

Keywords: differential scanning calorimetry, fats thermal behaviour, solvent-assisted fractionation, tropical fat fractions

1. INTRODUCTION

Modification of lipids plays an important role in the oil and fat processing industry. It has become the integral part of the industry as it helps to widen the product range of the native fats (COSSIGNANI *et al*., 2008). The main processes available for the lipid modification are: interesterification, fractional crystallization and hydrogenation (O'BRIEN, 2004). Fractional crystallization is the ultimate technique to alter composition and physical properties of fats for a number of reasons. Firstly, preparing a hard component out of native fats via fractional crystallization is more economical than partial hydrogenation. Secondly, fractional crystallization is advantageous over partial hydrogenation because it does not promote trans fatty acids in the finished products (TIMMS, 2005) that are well known to present unfavorable effects on human body (Onacik-Gür *et al*., 2015). Thirdly, fractional crystallization would contribute to the isolating of fat components of various physical and functional properties through as the so-called "multi-step fractionation" process (DEFFENSE, 1993; TAYLOR, 1976). By overall, the component lipid products isolated are likely to new ingredients suitable for applications where the native fat performs poorly.

Fractionation is generally accomplished by either a dry or a solvent-assisted process (TAYLOR *et al*., 2000; O'BRIEN, 2004, TIMMS, 2005). The principle of the dry fractionation is based on cooling the oil under controlled conditions slowly and with no solvents (O'BRIEN, 2004). The solvent-assisted fractionation follows a similar principle though, the difference lying in the use of the solvent to ease the crystallization. In the solvent-assisted process the melted native fat was used to mix with a solvent medium and was left to crystallize at a lower temperature. The precipitated fat was filtered off in order to give a high-melting fraction (HMF) while the mother-liquor was evaporated to produce a liquid called low-melting fraction (LMF) (DEFFENSE, 1993; KELLENS et. al, 2007; O'BRIEN, 2004; TAYLOR, 1976; TIMMS, 2005). In recent times, solvent-assisted fractionation has been investigated for a series of tropical fats that include palm oil (MARIKKAR and GHAZALI, 2011), coconut oil (MARIKKAR *et al*., 2013), cocoa butter (YANTY *et al*., 2013), engkabang fat (YANTY *et al*., 2013a), avocado butter (YANTY *et al*., 2013b), mee fat (MARIKKAR *et al*., 2010), and lard (YANTY *et al*., 2011). The current review report has investigated the method of solvent-assisted crystallization, which was effective and sharp while outlining that the yield recoveries of liquid fractions were usually better than those otherwise obtained. Moreover, it was of great concern for getting specialty products such as cocoa butter equivalent, lauric cocoa butter replacers, non-lauric cocoa butter replacers, confectionary products, medium chain triglycerides and high stability liquid oil (O'BRIEN, 2004). As the above mentioned fats and oils are generally known for possessing triacylglycerol (TAG) molecules of different kinds with different melting points, they are found to give lipid components of various kinds through fractionation. In most of these cases, it has been customary to use thermal analysis to cross-check the process and the outcome (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010). Among the various thermal analysis methodologies, differential scanning calorimetry (DSC) has stood out as an important tool for assessing the thermal behavior of oils and fats (VECCHIO *et al*., 2009; CERRETANI *et al*., 2012; TAN and NEHDI, 2015). Its use in fat modification studies was shown to give a great deal of information about the fractionation efficiency as well as the fractions thermal characteristics.

The aim of this review is to present an overview of the papers recently published on the chemical composition and thermal properties of the main fractions obtained from fractional crystallization of tropical fats, as the knowledge of these aspects are fundamental for improving the use in food formulation. Properties of avocado (Persea

Americana) butter (AB), engkabang (*Shorea macrophylla*) fat (EF), palm oil-moringa oil blend (PO-MO) and mee fat (*Madhuca longifolia*) (MF) are discussed.

2. MATERALS AND METHODS

2.1. Fractionation

The procedures for obtaining high melting and low-melting components from all the four fats were roughly similar, but with slight variations. Briefly, a portion of the melted sample of individual fat was mixed with acetone in 1:2 (w/v) ratio. The solution was boiled at 60°C until it became uniformly dissolved, and then it was cooled and left at 5±1°C for 2 hr to crystallize. The precipitated fat was filtered off to give a high melting fat fraction called stearin. After removing the precipitate, the mother-liquor was evaporated under reduced-pressure to yield a liquid fraction called olein (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010).

2.2. Analytical methodology

Cloud point (CP), slip melting point (SMP) and iodine value (IV) of the fat samples were determined according to AOCS method Cc.6.25, AOCS method Cc.3.25, and AOCS method Cd Id-92, respectively (AOCS, 1999). The TAG compositional analyses were carried out on a Waters Model 510 liquid chromatography equipped with a differential refractometer Model 410 as a detector (Waters Associates, Milford, MA) using Merck Lichrosphere RP-18 column (5 μm) (12.5 cm × 4 mm i.d.; Merck, Darmstadt, Germany). The mobile phase was a mixture of acetone:acetonitrile (63.5:36.5 v/v) and the flow rate was 1.5 mL/min (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013;YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010). Fatty acid methyl esters analyses were performed on a gas chromatograph (Agillent Technologies, Singapore) fitted with a FID detector using the polar capillary column RTX-5 (0.32 mm internal diameter, 30 m length and 0.25 μm film thickness; Restex Corp., Bellefonte, PA) (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010). Thermal analyses of the samples were carried out on a Mettler Toledo differential scanning calorimeter (DSC 823 Model) equipped with a thermal analysis data station (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 and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010). In all cases, the temperatures of onset (T_{on}), offset (T_{end}) and at the peak maximum of the melting thermal transitions, recorded during the DSC experiments, were determined using instrument's thermal analysis software.

2.3. Statistical analysis

In all analyses, three replicates were used and the results were expressed as mean value ± standard deviation. Data were statistically analyzed by one-way analysis of variance

(ANOVA), by using Tukey's test of MINITAB™ Statistical Software (MINITAB® Release 14.12.0, New York, USA) at 0.05 probability level.

3. RESULTS AND DISCUSSIONS

3.1. Physico-chemical parameters and potential uses of fractions

3.1.1 High-melting fractions

The physical nature of lipids is particularly relevant when it comes to their functional properties and applications (EL-KALYUOBI *et al*., 2011). It is hence important to assess the basic physico-chemical parameters such as CP, SMP and IV of fractions. According to Table 1, EF had the highest SMP (37.3°C) value followed by MF (35.5°C), PO-MO (30.5°C) and AB (30°C). As explained in several previous studies (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010), this variation was primarily due to the differences in the melting points of constituent TAG molecules of these fats. An inverse relationship between the SMP values of these fats to their degree of unsaturation was observed ($r = -0.9967$, $p < 0.05$). If SMP value of either the native fat or its fractionated components is below the physiological temperature of the human body (37.0ºC), it would make the fat suitable for direct use in food. Because of a lower state of hardness the direct use of the native fat in a formulation was often not found and all this implied a lack of the satisfactory quality attributes. In chocolate products, for instance, the hardness of the fat ingredient plays an important role to keep its texture (EL-KALYUOBI *et al*., 2011). When compared to normal milk fat, the HMF of milk fat was more compatible with cocoa butter (DEFFENSE, 1993). This links to HMFs generally reporting/showing higher proportions of the saturated TAG components when compared to their respective native samples. According to several previous studies (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010), the high-melting components obtained from fractional crystallization improved in terms of hardness due to higher SMP values [AS (42.5°C), MS (46.5°C), PS (55.5°C) and ES (38.5°C)] (Table 1) as well as elevated solid fat content profiles. NARINE and HUMPHREY (2004) explained that elevated solid fat contents usually helped the high-melting fractions to form stronger fat crystal networks.

Among the HMFs mentioned here, the highest increase of SMP value was noted for PS (25.0 units), followed by AS (12.5 units), MS (11.0 units) and ES (1.3 units). The HMF isolated from AB was AS, which was found to possess higher SMP (42.5 ºC) and lower IV (42.8) indicating that it was more or less comparable to those of HMF isolated from PO-MO blend (MARIKKAR and GHAZALI, 2011; MISKANDAR and NOR AINI, 2010). As similar to palm stearin in characteristics, AS might be suitable for fat-based products such as spreads and chocolates. Apart from this, it might also be useful as a fat ingredient in sticks and other solid formulations. According to Table 1, the SMP of MF was 35.5ºC, which was barely similar to those of either cocoa butter (ANON A, 2000) or Borneo Illipe butter (MARIKKAR and YANTY, 2012).

Table 1. Basic physico-chemical characteristics of avocado butter, engkabang fat, mee fat, palm oil-moringa blend, and their fractions[1].

Sample	Iodine value (g I_2/ 100 g)	Slip melting point (°C)	Ref.
AB	84.3±0.1	30.0±0.7	[Yanty *et al.*, 2013b]
AS	42.8±0.1	42.5±0.7	[Yanty *et al.*, 2013b]
AO	103.7±0.2	-	[Yanty *et al.*, 2013b]
MF	61.1±0.4	35.5±0.5	[Marikkar *et al.*, 2010]
MS	47.1±0.1	46.5±0.7	[Marikkar *et al.*, 2010]
MO	64.4±0.5	-	[Marikkar *et al.*, 2010]
PO-MO	54.0±0.5	30.5±0.1	[Marikkar and Ghazali, 2011]
PS	22.5±0.3	55.5±0.1	[Marikkar and Ghazali, 2011]
PO	59.0±1.3	-	[Marikkar and Ghazali, 2011]
EF	30.5±0.7	37.3±0.5	[Yanty *et al.*, 2013a]
ES	28.1±0.6	38.5±0.4	[Yanty *et al.*, 2013a]
EO	44.8±0.6	25.5±0.7	[Yanty *et al.*, 2013a]

[1]Abbreviations: AB, avocado butter; AS, avocado stearin; AO, avocado olein; EF, engkabang fat; ES, engkabang stearin; EO, engkabang olein; PO-MO, palm oil-Moringa oil blend; PS, palm stearin; PO, palm olein; MF, mee fat; MS, mee fat stearin; MO, mee fat olein

According to some reports (MARIKKAR *et al*., 2010, MARIKKAR and YANTY, 2012), MF can become a halal alternative fat for lard since these two had similar SMP values and their solid content profiles tallied in most temperatures. In a later study, MARIKKAR and YANTY (2011) compared the thermal properties of MS to lard stearin and suggested that MS might have applications such as alternative for lard stearin. Although the SMP value of MS was higher than the physiological temperature, its value was within the range of the palm stearins commercially available (ANON B, 2006). Hence, it might be useful as a raw-material for preparing fat blends simulating commercial shortenings. Similarly, the SMP values of the HMF isolated from the other two fats, namely PO-MO and EF, were also found to be higher than those of their respective native samples. Although the SMP value of ES exceeded the physiological temperature, its value was within the range of the commercially available palm stearin that may be useful as a raw-material in the preparation of either hard margarine or commercial shortenings (ANON B, 2006).

3.1.2 Low-melting fractions

LMFs were softer material when compared to their respective native samples – as the SMP and IV values of Table 1 outline accordingly. In several previous reports (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010), the IV values of the LMFs were higher than their native samples while a situation vice versa was observed for SMP values of LMFs. Among the IV of LMFs, the highest increment was noticed for AO (19.4 units), followed by EO (14.2 units), PO (5.0 units), and MO (3.3 units). In PO-MO, the highest IV was assessed for the LMF obtained under dry-fractionation condition at 18°C and the next lower value was for the LMF obtained under solvent-fractionation condition at 10°C (MARIKKAR and GHAZALI, 2011). However, IV of the LMFs isolated from PO-MO was found to be slightly higher

than palm olein obtained under dry fractionation of palm oil (ANON B, 2006). This could be probably due to the influence of *Moringa* oil factor, which is used to contribute more oleic acid into LMF. When compared to the IV of any other LMFs shown in Table 1, AO revealed a higher IV (103.7). That said, there is a possibility that AO fully maintains its liquid status at low-temperature ranges after longer period of storage. However, AO may be likely to undergo auto-oxidation, as observed in some other liquid oils (RAMLI *et al*., 2008). AO could be pragmatically useful in lotions since it could give a lighter skin-feel and better skin penetration.
The assessment of SMP of LMFs may be difficult seeing that some of them could remain entirely liquid at lower temperatures. It is, therefore, customary to use CP as it is the temperature at which dissolved solids are no longer completely soluble, precipitating as a second phase giving the fluid a cloudy appearance (AOCS, 1999). The CP value of MO was 10.5°C, which was within the range found in most of the commercially available palm olein samples (ANON B, 2006). However, some of the liquid oleins extracted from high-oleic palm oil were found to have CP values as low as 6°C (RAMLI *et al*., 2008). If its intended use was cooking medium in temperate climatic regions, MO may have some resistance to clouding effect. The LMF of EF was quite different from the other three LMF discussed so far. Although it remains solid at room temperature, its SMP and IV were significantly different from those of the native sample. Hence, this fraction (EO) may not be comparable to the properties of palm olein or any other oleins isolated from any other fatty material straightaway (ANON B, 2006). Alternatively, EO could be used in fat blend preparation suitable for manufacturing soft margarine (MISKANDAR and NOR AINI, 2010).

3.2 Fatty acid compositions of fat fractions

3.2.1 Fatty acid composition of high-melting fractions

The fatty acid distributions of AB, PO-MO, MF, EF, and their fractions have been presented in multiple reports (Table 2). AB, MF, and PO-MO consisted of higher proportions of unsaturated fatty acids (USFA) than saturated fatty acids (SFA), while EF had a higher proportion of SFA than USFA. In AB (YANTY *et al*., 2013b), oleic (43.7%) was the major fatty acid followed by palmitic acid (30.4%). PO-MO was also found to have oleic (45.5%) as the main fatty acid followed by palmitic acid (38.1%). However, MF and EF had somewhat different fatty acid compositions when compared to the other two fats. In MF, oleic (44.0%) was the major fatty acid followed by stearic acid (22.0%) while in EF, stearic (47.8%) was the major fatty acid followed by oleic acid (32.5%). These differences would definitely have some influence on the crystallization behavior of these fats under the same circumstance/given the same situation/in the same situation.
The HMFs of all fats showed increments in SFA contents due to the migration of the palmitic and steric acids into the solid phase during crystallization. In AS, the percentage of palmitic acid was increase to 59.6% while there was a decrease in the oleic (29.3%) and linoleic acids (5.6%) contents (Table 2). In PS, the increase in the proportion of palmitic acid (68.3%) caused a drop in the proportions of oleic (20.6%) and linoleic acids (3.4%) (MARIKKAR and GHAZALI, 2011). A similar trend was also noted in the fractionation of MF, where SFA content of MS increased tremendously (54.3%) with a concurrent decrease in USFA content (44.1%) (MARIKKAR *et al*., 2010). However, somewhat different situation was observed in the fractional crystallization of EF where ES reported only very little change in SFA and USFA contents. For instance, the changes in stearic and oleic acid contents were merely 1-2%, which was quite a smaller when compared to the fractional crystallization behaviors of AB, PO-MO, and MF as seen earlier.

Table 2. Fatty acid compositions (%) of avocado butter, engkabang fat, mee fat, palm oil-moringa blend, and their fractions[1].

Sample	Fatty acid (%)										Ref.
	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3	C 20:0	SFA	USFA	
AB	-	30.4±0.1	5.2±0.0	1.3±0.0	43.7±0.0	17.5±0.1	-	-	31.7	68.3	[Yanty *et al.*, 2013b]
AS	-	59.6±0.1	2.2±0.0	2.9±0.0	29.1±0.1	5.6±0.0	-	-	62.5	37.5	[Yanty *et al.*, 2013b]
AO	-	26.2±0.0	6.1±0.0	1.1±0.0	60.5±0.1	5.9±0.0	-	-	27.3	72.7	[Yanty *et al.*, 2013b]
MF	-	20.9±1.5	-	22.1±0.9	44.0±1.1	7.9±0.8	-	-	43.4	51.6	[Marikkar *et al.*, 2010]
MS	-	25.3±1.3	-	29.0±0.8	38.4±1.6	5.7±0.6	-	-	54.3	44.1	[Marikkar *et al.*, 2010]
MO	-	19.3±1.2	-	16.2±0.7	53.1±0.9	9.6±0.9	-	-	35.5	62.7	[Marikkar *et al.*, 2010]
PO-MO	0.9±0.0	38.1±0.2	0.4±0.0	4.6±0.1	45.5±0.1	8.2±0.0	1.0±0.1	0.5±0.0	45.0	55.1	[Marikkar and Ghazali, 2011]
PS	1.3±0.1	68.3±0.7	-	5.4±0.1	20.6±0.8	3.5±0.2	0.7±0.1	0.4±0.0	75.4	24.6	[Marikkar and Ghazali, 2011]
PO	0.9±0.1	35.2±0.3	0.5±0.0	4.4±0.1	48.3±0.2	8.7±0.0	1.0±0.1	1.0±0.1	41.5	58.5	[Marikkar and Ghazali, 2011]
EF	-	16.6±0.4	-	47.8±0.1	32.5±0.1	1.0±0.0	-	2.1±0.0	66.5	33.5	[Yanty *et al.*, 2013a]
ES	-	16.9±0.2	-	49.1±0.0	30.9±0.0	1.0±0.0	-	2.0±0.1	68.1	31.9	[Yanty *et al.*, 2013a]
EO	-	21.5±0.1	-	24.9±0.0	44.3±0.0	8.3±0.0	-	1.1±0.0	47.5	52.6	[Yanty *et al.*, 2013a]

[1]Abbreviations: AB, avocado butter; AS, avocado stearin; AO, avocado olein; EF, engkabang fat; ES, engkabang stearin; EO, engkabang olein; PO-MO, palm oil-Moringa oil blend; PS, palm stearin; PO, palm olein; MF, mee fat; MS, mee fat stearin; MO, mee fat olein; SFA, saturated fatty acids; USFA, unsaturated fatty acids.

3.2.2 Fatty acid composition of low-melting fractions

The LMFs of all fats experienced increments in the proportions of USFA with the concurrent decreases of SFA contents (Table 2). When TAG molecules with more saturated fatty acids have undergone crystallization easily, the liquid phase would naturally become enriched in unsaturated fatty acids (MARIKKAR and GHAZALI, 2011; YANTY *et al*., 2013a; MARIKKAR *et al*., 2010). In AO, oleic acid content was increased from 43.7 to 60.5% while palmitic acid content was decreased from 30.4 to 26.2%. Similar trends of fatty acid changes resulted in LMFs of MF and PO-MO. As PO was the LMF isolated from PO-MO, the oleic acid content increased from 45.6 to 48.3% with the concurrent decrease of palmitic acid content from 38.1 to 35.2%. In MO, oleic acid content increased from 44.0 to 53.1% while there were drops in the proportions of palmitic acid (20.9 to 19.3%) and stearic acid (22.0 to 16.2%). In this case, the decline in stearic acid content was more marked than the decrease of the palmitic acid content. In EO, oleic acid content increased from 32.5 to 44.3% while stearic acid content decreased from 47.8 to 24.9%. In this particular case, however, the palmitic acid content was found to increase from 16.6 to 21.5%. This was considered as an anomalous behavior when compared to the general trend observed in other fats.

3.3 TAG Compositions of fat fractions

3.3.1 TAG composition of high-melting fractions

In multiple reports (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010), the comparative TAG distributional profiles of AB, EF, PO-MO, MF and their fractions have been presented. TAG composition of fat fractions is an important determining factor of their chemical properties. Among the native fats, AB, MF, and PO-MO consisted of higher proportions of di-unsaturated TAG molecules while EF had a higher proportion of mono-unsaturated TAG molecules (Table 3). When these fats were subjected to fractional crystallization, the majority of the tri-saturated and di-saturated TAG molecules tended to migrate into the solid phase, leaving mono-saturated and tri-unsaturated TAG molecules in the liquid phase. In AS, for instance, the tri-saturated and di-saturated TAG molecules such as PPP (38.3%) and PPO (30.1%) increased with concurrent decreases in OOL (1.1%), POL (1.6%), and OOO (5.3%). The proportion of PPO in AS was almost equivalent to that of palm mid fraction as reported previously (ANON B, 2006). When MF was fractionated, a similar phenomenon was noticed in TAG composition. In MS, the proportions of di-saturated TAG molecules (MARIKKAR *et al*., 2010) increased from 42.2 to 73.2% with concurrent reductions in mono-saturated (18.2%) and tri-unsaturated (4.9%) TAG molecules. TAG molecular species such as POP, POS, and SOS experienced increments while PLO, OOO, OOP, and OOS experienced decreases (MARIKKAR *et al*., 2010). The proportion of SPO in MS was almost equivalent to that of cocoa butter as reported previously (SIMONEAU *et al*, 1999; ANON A, 2000). In the fractionation of PO-MO, migration of PPP into the solid phase was considerable. Similar observations were also made by other researchers in the dry fractionation of palm oil, as reported previously (MISKANDAR and NOR AINI, 2010; RAMLI *et al*., 2008). As a result, tri-saturated TAG molecules of PS was increased from 7.1 to 50.4% with concurrent reductions in mono-saturated (9.0%) and tri-unsaturated TAG molecules (2.2%) (Table 3).

Table 3. Triacylglycerol (TAG) compositions (%) of avocado butter, engkabang fat, mee fat, palm oil-moringa blend, and their olein and stearin fractions[1].

TAG	AB	AS	AO	MF	MS	MO	PO-MO	PS	PO	EF	ES	EO
LLLn	1.9±0.0	-	2.7±0.1	-	-	-	-	-	-	-	-	-
LLL	0.9±0.1	0.5±0.0	1.2±0.1	-	-	-	-	-	-	-	-	-
OLL	3.2±0.0	0.5±0.0	6.0±0.1	-	-	-	-	-	-	-	-	-
OOA	-	-	-	-	-	-	-	-	-	-	-	1.7±0.1
OOL	9.0±0.0	1.1±0.0	17.0±0.2	3.0±0.3	1.5±0.0	2.6±0.1	1.6±0.1	0.3±0.1	2.0±0.0	-	-	0.4±0.0
OOO	11.4±0.0	5.3±0.0	22.4±0.4	9.9±0.2	3.5±0.0	12.6±0.1	10.0±0.2	1.9±0.2	9.9±0.5	0.1±0.0	0.4±0.0	3.3±0.1
OOSt	0.5±0.0	0.6±0.1	1.1±0.1	17.8±0.0	7.4±0.1	24.1±0.0	4.6±0.6	0.5±0.1	4.1±0.2	1.4±0.0	1.3±0.1	29.7±0.2
OPO	22.8±0.0	12.3±0.0	8.8±0.2	22.9±0.9	9.0±0.0	28.6±0.1	24.1±1.4	6.1±0.0	26.1±0.1	0.7±0.0	0.4±0.0	16.0±0.4
MMM	-	-	-	-	-	-	0.4±0.0	-	0.2±0.0	-	-	-
MPL	-	-	-	-	-	-	0.2±0.1	-	0.2±0.0	-	-	-
PLL	4.2±0.0	0.2±0.0	5.6±0.1	-	-	-	1.5±0.0	0.3±0.1	2.1±0.3	-	-	0.3±0.0
POL	19.3±0.1	1.6±0.0	13.2±0.2	4.3±0.4	1.8±0.0	2.6±0.2	8.9±0.4	2.1±0.1	10.8±0.1	-	-	1.7±0.2
POSt	-	-	-	-	-	-	-	3.4±0.2	5.2±0.1	35.6±0.1	38.2±0.1	12.3±0.2
PPL	4.0±0.1	1.7±0.1	0.2±0.0	1.2±0.0	0.4±0.0	Tr.	6.9±1.4	6.1±0.1	10.1±0.0	0.2±0.0	0.1±0.0	3.1±0.1
PPO	12.4±0.1	30.1±0.4	0.2±0.0	11.9±0.7	14.0±0.1	12.1±0.0	29.8±1.8	28.9±0.1	28.71±0.1	7.3±0.0	7.3±0.0	16.5±0.3
PPP	2.9±0.1	38.3±0.5	0.6±0.0	Tr.	0.2±0.1	Tr.	6.0±1.2	42.1±0.2	0.4±0.0	-	-	-
PPSt	0.1±0.0	4.0±0.1	-	Tr.	1.8±0.0	Tr.	0.7±0.2	8.3±0.1	-	0.3±0.0	0.2±0.0	1.0±0.1
StOA	-	-	-	-	-	-	-	-	-	3.8±0.1	3.7±0.2	-
StPO	0.6±0.0	1.7±0.0	-	19.3±0.4	35.1±0.0	14.1±0.0	5.4±0.1	-	-	-	-	-
StStO	-	-	-	9.7±0.6	23.8±0.0	3.5±0.2	-	-	-	50.0±0.1	47.8±0.3	2.7±0.3
StStSt	-	-	-	-	-	-	-	-	-	0.3±0.0	0.3±0.0	-
Others	6.8±0.0	2.3±0.9	21.0±1.6	-	1.9±0.1	-	-	-	-	0.4±0.0	0.2±0.0	11.4
SSS	3.5	42.3	0.6	-	2.0	-	7.1	50.4	0.6	0.6	0.6	1.0
USS	17.0	33.5	0.4	42.2	73.2	30.2	42.3	38.4	42.2	96.8	97.1	34.6
UUS	46.8	14.7	28.7	45.0	18.2	55.3	39.0	9.0	43.01	2.1	1.7	49.4
UUU	26.4	7.4	49.3	12.9	4.9	15.2	11.6	2.2	11.94	0.1	0.4	3.7
Ref.	[Yanty *et al.*, 2013b]	[Yanty *et al.*, 2013b]	[Yanty *et al.*, 2013b]	[Marikkar *et al.*, 2010]	[Marikkar *et al.*, 2010]	[Marikkar *et al.*, 2010]	[Marikkar and Ghazali, 2011]	[Marikkar and Ghazali, 2011]	[Marikkar and Ghazali, 2011]	[Yanty *et al.*, 2013a]	[Yanty *et al.*, 2013a]	[Yanty *et al.*, 2013a]

[1]Abbreviations: O, oleic; P, palmitic; L, linoleic; Ln, linolenic; St, stearic; Tr., trace; UUU, triunsaturated; UUS, diunsaturated; USS, disaturated; SSS, triunsaturated. For other abbreviations see Table 1.

In contrast to the three native fats discussed so far, a different situation was noticed in the fractionation of EF (YANTY *et al*., 2013a). This could be due to the fact that EF was a fat dominated by stearic acid enriched TAG molecules (NESARETNAM and MOHD ALI, 1992) while others were dominated by palmitic and oleic enriched TAG molecules. This fact was further confirmed in a separate study carried out by NUR ILLYIN and co-workers (2013) who used EF as one of the raw materials to formulate a fat substitute for lard. Although there was an increase in the proportion of di-saturated TAG molecules of ES with decreasing proportions of mono-saturated TAG molecules, the changes were not remarkable. Interestingly, StOSt and POSt together accounted for 86% of all TAG molecules of ES.

3.3.2 TAG composition of low-melting fractions

TAG composition of LMFs isolated from AB, PO-MO, MF and EF are presented in Table 3. LMFs of these fats were generally found to possess higher proportions of di-unsaturated and tri-unsaturated TAG molecules. For instance, AO was found to possess higher percentages of OOO (22.4%) and OOL (17.0%) with respect to the native AB sample. The increased proportions of di-unsaturated and tri-unsaturated TAGs in AO were compatible to the increased amounts of oleic and linoleic acids of AO, as noticed in the fatty acid distribution (Table 2). MO also had remarkably higher proportions of di-unsaturated and tri-unsaturated TAG molecules. In this case, TAG molecular species such as OOO, OOP and OOS experienced increments while TAG species such as POS and SOS underwent decreases. PO (MARIKKAR and GHAZALI, 2011) consisted of di-unsaturated (43.0%), di-saturated (42.2%) and tri-unsaturated (11.9%) TAG molecules. This was somewhat similar to the findings reported previously for dry fractionation of palm oil (MISKANDAR and NOR AINI, 2010, RAMLI *et al*., 2008). In EO, the TAG molecular species namely SOO and POO experienced increments while TAG species such as POS and SOS have undergone decreases. The relative increase in the proportions of di-unsaturated and tri-unsaturated TAG molecules would have led to the increase of oleic acid content as noticed earlier (Table 2). By overall, the changing nature of TAG composition in the LMF caused an increase in the iodine value of AO (103.7), MO (64.4), PO (59.0), and EO (44.8) (Table 1) (MARIKKAR and GHAZALI, 2011; YANTY *et al*., 2013a; MARIKKAR *et al*., 2010).

3.4. Thermal behaviors of fat fractions

3.4.1 Thermal behavior of high-melting fractions

The DSC thermal profiles of AB, PO-MO, MF, and EF have been documented in multiple reports (MARIKKAR and GHAZALI, 2011; MARIKKAR *et al*., 2013; YANTY *et al*., 2013a; YANTY *et al*., 2013b; MARIKKAR *et al*., 2010). Past research (MARIKKAR *et al*., 2013; RAMLI *et al*., 2008) has shown that DSC thermal profiles have greatly contributed to the understanding of the fractional crystallization behavior of individual fats. Particularly, the distribution of thermal transitions in these fats provided some important basis for predictions of the outcome of fractionation of individual fat (CHE MAN *et al*, 1999). This was also confirmed in several studies conducted on dry fractionation of palm oil (RAMLI *et al*., 2008; ZALIHA *et al*., 2004). According to Figs. 1-4, native samples of AB, PO-MO, and MF displayed cooling curves with a low-melting thermal transition below 0°C and a high-melting thermal transition above 0°C. Likewise, the melting curves of these three fats were also found to have two well-separated endothermic transitions, which represented the low- and high-melting regions. This phenomenon was well-evidenced in the thermal studies of palm oil reported by other investigators (CHE MAN *et al*, 1999; YANTY *et al*.,

2012; SHUKAT *et al*., 2012; SAMI *et al*., 2012). As EF had a somewhat different composition in terms of fatty acid (Table 2) and TAG (Table 3) distributions, its cooling curve was different from those of AB, PO-MO and MF. It had a strong single major exothermic thermal transition at 16.40°C with minor thermal transitions at 8.6°C and -9.2°C. According to previous reports (YANTY *et al*., 2013a; NUR ILLYIN *et al*., 2013), co-crystallization of TAG molecular species within a narrow temperature region would have caused EF to display a single major thermal transition.

The thermal behaviors of HMFs were presented in Figs. 1-4. Depending on the fatty acid and TAG compositions, the nature of cooling and heating curves displayed by individual HMF was found to vary. This was in accordance with the findings reported previously for whole lipids in other studies (MARIKKAR *et al*., 2011; LONG *et al*., 2005). As shown in Fig. 1A (Curve 3), the thermal curve of AS had a strong high-melting thermal transition with duplet at 40.5ºC [peak C_1] and 32.5ºC [peak C_2].

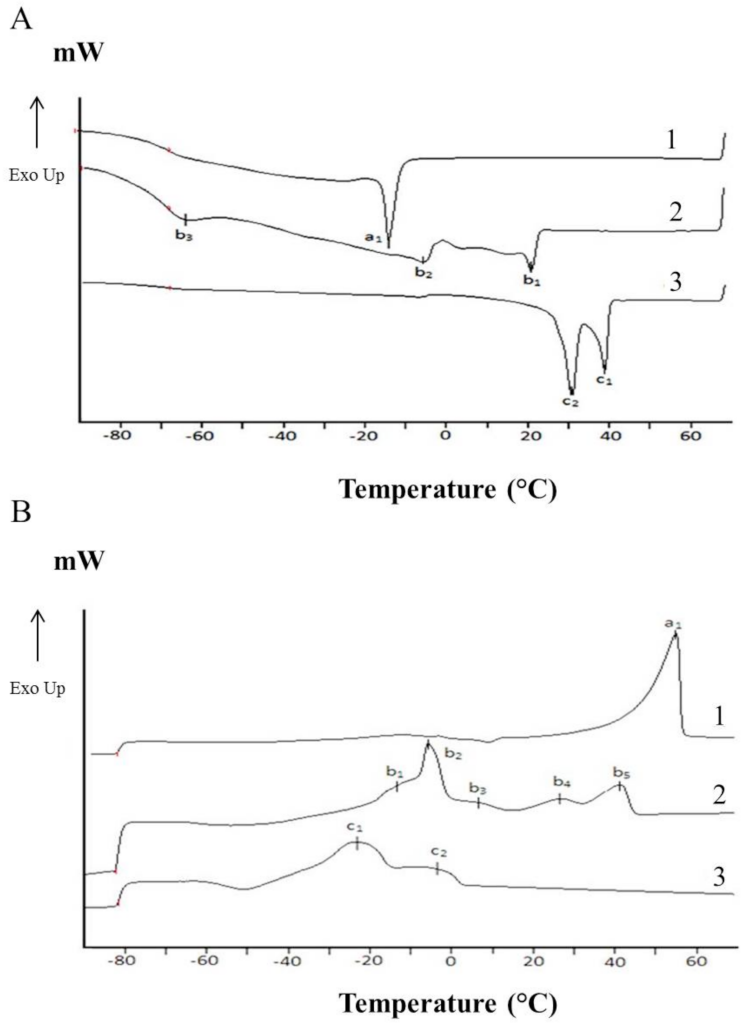


Figure 1. (A) DSC cooling curves of avocado fat (curve-2), avocado olein (curve-1), and avocado stearin (curve-3) and (B) DSC heating curves of avocado fat (curve-2), avocado stearin (curve-1), and avocado olein (curve-3).

This curve did not show any low-melting transition below 10.0ºC, mainly due to the fact that it had lost most of the di-and tri-unsaturated TAG molecules during fractional crystallization. This was commonly noticed in several previous studies related to the dry fractionation of palm oil (MISKANDAR and NOR AINI, 2010, CHE MAN *et al.*, 1999). With respect to the cooling curve of native sample, T_{on} and the peak maximum of thermal transitions shifted toward the high-temperature region (Table 4). These changes were positively correlated ($r = +0.996$, $p < 0.0001$) with increasing proportions of disaturated and trisaturated TAG molecular species of AS (Table 3) as well as SFA of AS with respect to that of the native sample (Table 2).

According to O'BRIEN (2004), these groups of TAG molecules were generally found to have higher melting temperatures. The fractionation behavior of MF (Fig. 2) was slightly different from those of AB and PO-MO since it had more stearic acid esterified to TAG molecular species (Table 3). The cooling curve representing MS (as shown in Fig. 2A, curve 3) was distinguishable from that of the native sample as the high-melting thermal transition (peak c_1) of the curve became more dominant with the gradual reduction of the low-melting transition (peak c_3). As seen before with HMFs of AB and PO-MO, the positions of thermal transitions of MS in the cooling curve also shifted towards higher temperatures (Tables 4 and 5), owing to the decline of TAG with high degree of unsaturation. In fact, the changing DSC thermodynamic parameters were well-correlated with the changing proportion of di-unsaturated and tri-unsaturated TAG groups in the fractions (Table 3).

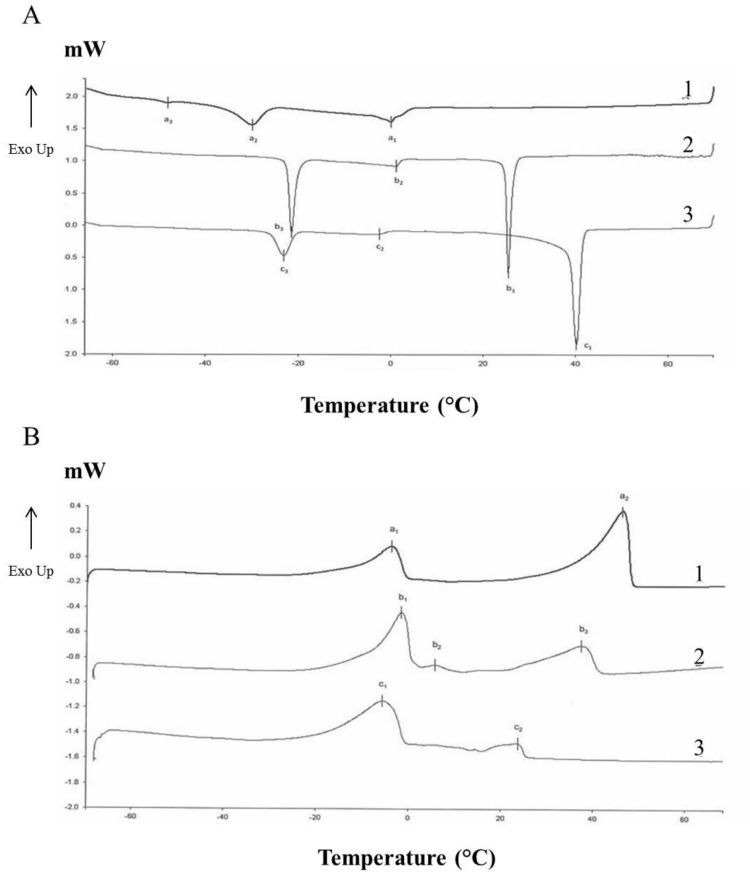


Figure 2. (A) DSC cooling curve of Mee fat (curve-2), Mee olein (curve-1) and Mee stearin (curve-3) and (B) DSC heating curves of Mee fat (curve-2), Mee stearin (curve-1) and Mee olein (curve-3).

The melting curve representing MS, as shown in Fig. 2B (curve 1), was distinguishable from that of the native sample as its high-melting thermal transition has become more dominant than the low-melting transition. It had its major transition at 46.3ºC (a_2) with a minor transition at -3.7ºC (a_3).

Table 4. Thermal parameters associated with the cooling curves of avocado butter, mee fat, engkabang fat, palm oil-moringa oil blend, and their olein and stearin fractions[1].

Sample	T_{On} (°C)	Tran1 (°C)	Tran2 (°C)	Tran3 (°C)	Ref.
AB	23.9±0.2	22.2±0.0	-2.7±0.1	-64.4±0.2	[Yanty *et al.*, 2013b]
AS	41.3±0.0	40.5±0.1	32.5±0.1	-	[Yanty *et al.*, 2013b]
AO	2.5±0.2	-12.9±0.1	-	-	[Yanty *et al.*, 2013b]
MF	29.6±0.0	25.4±0.0	1.2±0.2	-21.4±0.0	[Marikkar *et al.*, 2010]
MS	42.4±0.1	40.2±0.1	-2.2±0.0	-23.2±0.0	[Marikkar *et al.*, 2010]
MO	5.2±0.2	0.0±0.0	-30.0±0.1	-47.3±0.3	[Marikkar *et al.*, 2010]
PO-MO	20.4±0.2	18.8±0.1	0.7±0.0	-	[Marikkar and Ghazali, 2011]
PS	44.4±0.1	42.8±0.3	-	-	[Marikkar and Ghazali, 2011]
PO	2.8±0.1	0.5±0.0	-	-	[Marikkar and Ghazali, 2011]
EF	17.6±0.1	16.4±0.2	8.6±0.1	-9.2±0.1	[Yanty *et al.*, 2013a]
ES	19.0±0.0	17.2±0.0	8.9±0.1	-7.8±0.1	[Yanty *et al.*, 2013a]
EO	16.8±0.0	17.0±0.1	7.2±0.1	-	[Yanty *et al.*, 2013a]

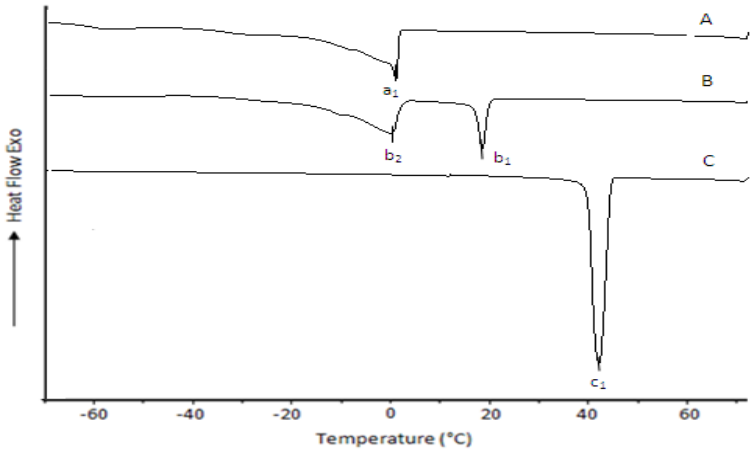
[1]Abbreviation: T_{On}, Onset of crystallization; Trans, thermal transition. For other abbreviations see Table 1.

Table 5. Thermal parameters associated with the melting curves of avocado butter, mee fat, engkabang fat, palm oil-moringa oil blend, and their olein and stearin fractions[1].

Sample	Tran1 (°C)	Tran2 (°C)	Tran3 (°C)	Tran4 (°C)	Tran5 (°C)	T_{End} (°C)	Ref.
AB	-12.2±0.0	-5.5±0.0	7.6±0.1	26.6±0.1	41.3±0.4	44.7±0.1	[Yanty *et al.*, 2013b]
AS	55.2±0.2	-	-	-	-	57.1±0.0	[Yanty *et al.*, 2013b]
AO	-24.0±0.3	-2.1±0.3	-	-	-	3.4±0.0	[Yanty *et al.*, 2013b]
MF	-1.5±0.1	5.6±0.1	37.7±0.1	-	-	38.9±0.1	[Marikkar *et al.*, 2010]
MS	-3.7±0.2	46.3±0.2	-	-	-	49.8±0.4	[Marikkar *et al.*, 2010]
MO	-5.4±0.1	23.8±0.1	-	-	-	26.2±0.2	[Marikkar *et al.*, 2010]
PO-MO	1.3±0.1	5.2±0.1	21.5±0.0	42.1±0.1	-	39.3±0.2	[Marikkar and Ghazali, 2011]
PS	51.4±0.0	63.3±0.2	-	-	-	66.4±0.1	[Marikkar and Ghazali, 2011]
PO	6.3±0.2	10.1±0.3	-	-	-	15.3±0.0	[Marikkar and Ghazali, 2011]
EF	17.0±0.0	20.3±0.1	26.6±0.2	33.7±0.0	-	35.3±0.1	[Yanty *et al.*, 2013a]
ES	-	-	27.1±0.1	-	-	37.5±0.0	[Yanty *et al.*, 2013a]
EO	-10.5±0.1	5.3±0.0	19.2±0.1	-	-	28.1±0.1	[Yanty *et al.*, 2013a]

[1]Abbreviation: Trans, thermal transition; T_{End}, end of melting temperature. For other abbreviations see Table 1

The fractional crystallization behavior of PO-MO was expected to be similar to that of AB since both of these were found to possess TAG molecular species predominantly esterified by oleic and palmitic acids. The cooling curve of PS, as shown in Fig. 3A (curve 3), was found to possess a high-melting thermal transition only at 29.25°C (peak c_1) with no strong low-melting transition appearing in the low-temperature region.



(Y)

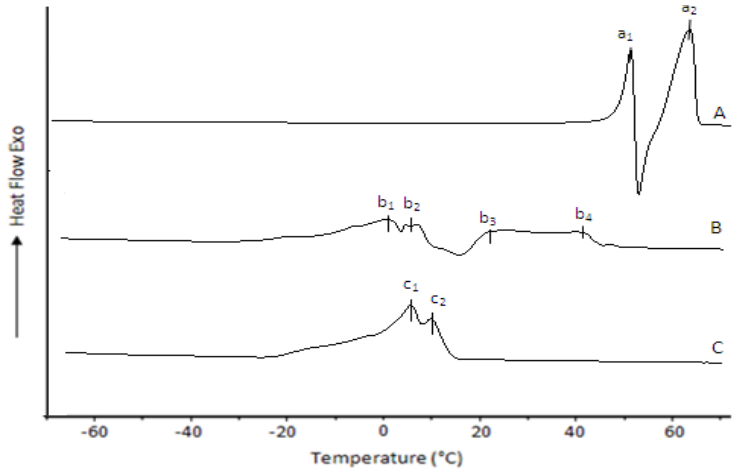


Figure 3. (X) DSC cooling curves of palm olein (curve-A), palm oil (curve-B), and palm stearin (curve-C) and (Y) DSC heating curves of palm stearin (curve-A), palm oil (curve-B), and palm olein (curve-C).

As reported by other researchers (MISKANDAR and NOR AINI, 2010; CHE MAN *et al.*, 1999; ZALIHA *et al.*, 2004) about the PS cooling curve, this phenomenon was frequently attributed to the occlusion of small amount of liquid oil within the crystal matrix during the crystallization process. This difference was primarily due to the high-fractionation efficiency of solvent-assisted processes when compared to dry fractionation technique. As mentioned previously, the use of solvent in fractionation helped to wash away the small amount of liquid oil occluded within the crystal matrix (MARIKKAR and GHAZALI, 2011). As the overall cooling profile of PS shown in Fig. 3A (curve 3) was slightly different with respect to that of native palm stearin, some differences could be expected in the onset

of crystallization temperatures as well as the position of the thermal transitions. These changes were mainly due to differences in the conditions adopted during commercial fractionation (MISKANDAR and NOR AINI, 2010, CHE MAN *et al*., 1999; ZALIHA *et al*., 2004). The DSC thermodynamic parameters were positively correlated ($r = 0.986$, $p < 0.001$) to the increasing proportions of di-saturated and tri-saturated TAG molecular species in PS (Table 3). This was also positively correlated to the increasing proportion of the SFA of PS with respect to that of the native sample (Table 2).

The fractionation behavior of EF was completely different from those of AB, PO-MO and MF. As discussed before, this anomalous behavior was mainly due to the nature of the composition of EF resulting from the combination of TAG molecules esterified with more stearic fatty acid (Tables 2 and 3). The cooling curve of ES as shown in Fig. 4A (curve 3) displayed a major sharp transition at 17.15°C (peak c_1) with two minor thermal transitions at 5.7°C (peak c_2) and -5.2°C (peak c_3) (Table 3) while its melting curve displayed the major transition at 27.05°C (peak a_1, Fig. 4B, curve 1) (Table 4). In the melting curve, the end set temperature of melting of ES was slightly higher than that of the native sample. By overall, ES showed significant changes in the values of T_{on}, T_{end} as well as rest of the DSC parameters of both cooling and heating curves.

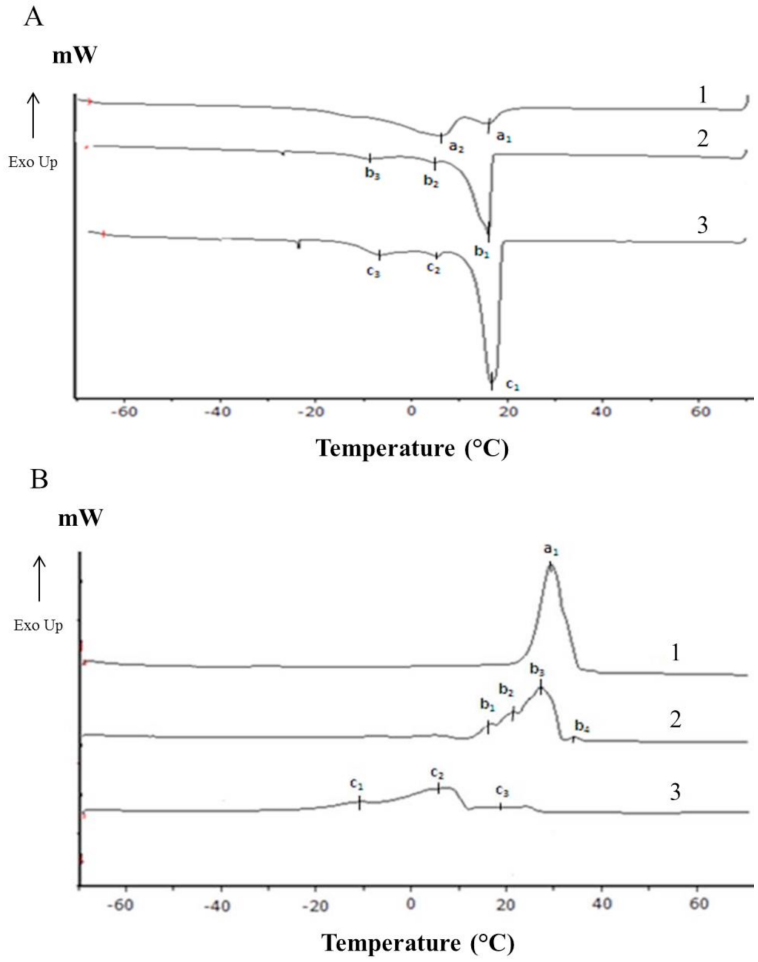


Figure 4. (A) DSC cooling curves of engkabang olein (curve-1), engkabang fat (curve-2), and engkabang stearin (curve-3) and (B) DSC heating curves of engkabang stearin (curve-1), engkabang fat (curve-2), and engkabang olein (curve-3).

3.4.2 Thermal behavior of low-melting fractions

Thermal behaviors of LMFs were quite different when compared to their respective native fats. According to several previous reports, this has been true even for dry fractionation of palm oil and milk fat (TIMMS, 2005; DEFFENCE, 1993; RAMLI *et al*., 2008; CHE MAN *et al*., 1999; ZALIHA *et al*., 2004; LONG *et al*., 2005). Owing to migration of highly saturated TAG molecules into the solid phase, they were largely composed of TAG molecules esterified with more unsaturated fatty acids (Tables 2 and 3). These TAG molecules would make LMFs to have low solid fat and display low-melting points (DE MAN, 1999). Since they contain lesser amounts of high-melting TAG groups, their cooling and melting curves tend to differ from those of their native fats by not having any major thermal transition in the high-temperature region of the DSC cooling curve (Figs. 1-4). This phenomenon was evidently seen in liquid palm olein isolated from dry fractionation of palm oil (RAMLI *et al*., 2008; ZALIHA *et al*., 2004; MARIKKAR *et al*., 2011) and palm oils enriched with diacylglycerols (LONG *et al*., 2005). The cooling curve of AO [Fig. 1 (A); Curve 1] isolated from AB was found to display thermal transitions in the lower-temperature region with peak maximum at -12.9ºC (peak a_1). Hardly, any significant thermal transition was noticed in the temperature region above 2.5ºC. Likewise, the melting curve of AO shown in (Fig. 1 B) also displayed a broad thermal transition in the lower-temperature region with peak maximum at -24.0ºC (peak c_1) and -2.10ºC (peak c_2), but hardly any significant thermal transition in the temperature region above 2.5ºC. With respect to the native fat, the peak maxima of thermal transitions in both cooling and melting curves were also found to shift toward the low-temperature region (Tables 4 and 5). This changing pattern of DSC thermodynamic parameters was also positively correlated to the increasing proportion of the USFA and di-and tri-unsaturated TAG molecules of AO (Tables 2 and 3) ($r = 0.974$, $p < 0.001$). The shifting of thermodynamic transitions of low-melting fractions is a common phenomenon even noted in commercial dry fractionation of crude palm oil (CHE MAN *et al*., 1999; ZALIHA *et al*., 2004).

The cooling and melting curves of the LMF of MF are depicted in Fig. 2. As shown in Fig. 2 (A), the DSC cooling curve of MO had its major exothermic transitions at 0ºC (peak a_1) and -30.0ºC (peak a_2) and its onset of crystallization was at 5.2ºC, which was slightly higher than that of AO. This could be attributed to the difference in SFA (%) contents of MO and AO (Table 2). Past studies already showed that onset temperatures of crystallization of LMF of palm oil from different suppliers were found to vary with the changes in fatty acid (MARIKKAR *et al*., 2011). According to Fig. 2 (B), the DSC melting curve of MO had its major thermal transition in the lower-temperature region at -5.4ºC (peak c_1) while a minute high-melting transition appeared at 23.8ºC (peak c_1) (Table 4). This was an indication that MO has lost majority of the high-melting TAG molecules during fractionation. According to Table 3, there was 12% drop in the proportion of di-saturated TAG molecules after fractionation. TAN and NEHDI (2015) earlier discussed the co-relationship between DSC thermodynamic parameters and chemical composition of plant oils, and the same co-relationship was also found to hold true in the case of fractions isolated from tropical fats selected in this study.

The LMF isolated from PO-MO also exhibited a similar trend as seen before in AO and MO. According to Fig. 3(A), PO did not show the sharp high-melting transition possessed by the native sample. The onset temperature of crystallization of PO was 2.8ºC (Table 4) and there was hardly any exothermic thermal transition in the temperature region above this point. By overall, this characteristic feature was comparably similar to those of palm olein samples reported previously by other researchers (CHE MAN *et al*., 1999; MARIKKAR *et al*., 2011). According to Fig. 3(B), the melting curve of PO displayed dual melting thermal transitions at 6.3ºC (peak c_1) and 10.1ºC (peak c_2) and the end of melting

was at 15.3ºC. These values were slightly different from those of palm olein reported previously by ZALIHA *et al* (2004) and CHE MAN *et al* (1999). This deviation could be attributed to the influence of fractionation conditions adopted in the two different fractionation approaches. Particularly, the starting material used in the former was palm oil while the starting material used in the later was PO-MO blend.
A totally different result could be observed with respect to the LMF of EF. As shown in Fig. 4A, EO did not show any significant low-melting transition in the negative temperature region, but instead it displayed a broad thermal transition with a doublet having peak maxima at 17.0°C (peak a_1) and 7.2°C (peak a_2). This was quite an anomalous behavior when compared to the thermal behavior noticed in the cooling curves of AO, PO, and MO. The native sample of EF itself did not display any strong low-melting transition in the low-temperature region of the DSC cooling curve (YANTY *et al*., 2013a). As mentioned before EF was a fat containing TAG molecules, esterified mostly with stearic acid (Table 3). These were the group of TAG molecules reappearing in the TAG composition of EO. Apart from the changing pattern of the thermal transitions, there were significant changes in the onset temperature of crystallization as well as the other DSC parameters. As already noted with the LMFs of AB, PO-MO, MF etc., the onset temperature of crystallization of EO was also found to decrease with respect to the native sample (Table 3). As shown in Fig. 4B (curve 3), the melting curve of EO displayed the major thermal transition at 5.3ºC (peak c_2) with a shoulder peak at - 10.5ºC (peak c_1) and a minor transition at 19.2ºC (peak c_3). Similar to the cooling curve pattern seen before, the melting behavior pattern was completely different from that of the native sample (curve 2). In addition, EO had its end of melting at 28.1ºC, which was quite lower when compared to that of the native sample (Table 5).

4. CONCLUDING REMARKS

The current study compared the fractional crystallization behavior differences of AB, PO-MO, MF, and EF using the analytical data obtained from GLC, HPLC and DSC techniques. Since the thermal characteristics of oils and fats are generally related to their TAG composition, the outstanding differences in the thermal profiles of the LMF and HMF fractions were well-related to the changing TAG composition and to the degree of the fatty acids unsaturation. The HMF fractions of all fats experienced increases in the percentage of saturated fatty acids, while increases in the proportions of unsaturated fatty acids resulted in the LMF fractions of all fats. The iodine value differences among them clearly showed that the HMF and LMF fractions underwent changes in their fatty acid and TAG compositions. Due to the fatty acid and TAG compositional differences, the fractions isolated through crystallization are new products with differing functional properties. The HMF fractions of these fats tended to emerge from the high-melting TAG molecular species of native fats. The reason lies in the fact that these TAG molecular species are usually represented by relatively more saturated TAG molecules, which would undergo solidification rapidly. While the LMF fractions obtained can be generally used as cooking and frying oils, the HMF fractions may be considered as a raw ingredient for preparing sticks, hard margarine, commercial shortening and other solid formulations because of their hard nature and higher SMP values. The fractionation at times yielded exotic specialty fat products, such as palm mid-fraction, that could be used as cocoa butter substitute. In some instances, fractionation helped to come out with confectionary fats and oils with higher oxidative stability. In conclusion, it should be noted that the fractional crystallization approach is a mere physical process with a low environmental impact, therefore it provides several advantages over other methods of fat derivative formation.

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PAPER

SIMULATING INTERNATIONAL SHIPMENTS OF VEGETABLE OILS: FOCUS ON QUALITY CHANGES

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ABSTRACT

This investigation evaluated the quality changes of commercial vegetable oils after different simulated shipments. In particular, the oils were placed in containers with or without thermal insulation and subjected to two simulated shipments, from Italy to Los Angeles and to Quebec. The temperature profiles were monitored to simulate the real shipments conditions in laboratory through properly developed climate chambers. Different quality parameters were evaluated before and after the simulations, showing a high degree of oxidation for samples shipped to Los Angeles in standard containers. In this study, the thermal insulation container was effective in protecting samples from potential oxidative damage during simulated shipping.

Keywords: edible vegetable oils, food quality, oxidation, simulated shipment; thermal insulation

1. INTRODUCTION

Vegetable oils such as sunflower, palm kernel, and soybean oils are extensively used for cooking purposes. These types of fatty food products are more susceptible to oxidation than animal fat because of their content of unsaturated fatty acids (PARKER *et al.*, 2003). In 2014, about 168 million tons of vegetable oil was produced worldwide (USDA, 2014). Among vegetable oils, Italy is considered as the dominant supplier of olive oils to Canada and USA, and about 72 % and 60 % of olive oil imported in 2014 to Canada and USA, respectively, was from Italy. Furthermore, in 2014, Italy exported around 230,000 metric tons of virgin olive oil (IOC, 2014b). During transportation by sea, the desired temperature for most edible oils is ambient temperature (CAC, 2013b). Considering that solidification and crystallization of the product occurs at 3-4°C (PISCOPO and POIANA, 2012), edible oils may suffer from deterioration in quality, which involve hydrolytic and oxidative modifications promoted by several factors, such as temperature and humidity in the stages of pumping and tank filling, in addition to the effect of light exposure for samples transported in clear bottles (BTM, 2013). Raw edible oils, even after soft refining, as well as virgin olive oils, contain a range of minor compounds such as chlorophylls, tocopherols, carotenoids, and phenolic compounds that function as natural antioxidants by enhancing the stability of the oil during storage (KRISTOTT, 2000). The monounsaturated/polyunsaturated fatty acid ratio, as well as the presence of phenolic compounds, make virgin olive oil more stable towards heat induced oxidation (BENDINI *et al.*, 2004). Moreover, the hydrolysis of acylglycerols, catalyzed mainly by an increase in temperature during storage, as well as the presence of moisture, oxygen, or light (FRANKEL, 1991), plays an important role in development of off-flavors, thus making edible oils unpalatable and shortening their shelf-life (KRISTOTT, 2000). High temperatures increase the rate of oxidation, while very low freezing temperatures may also change the availability of some micro components, such as phenolic compounds, water distribution around crystals, and the physical characteristics of olive oil (BENDINI *et al.*, 2007). Several studies have been carried out on the simulated transportation of foodstuffs. For example, an interesting report done by BURGER (1985) studied the effect of bulk storage and transportation on the quality of palm oil, and found that during the 25 days of an actual journey at temperatures ranging between 37-55°C, there was a slight increase in free acidity, while peroxide values were doubled at the final stage of the voyage. The effect of different thermal conditions registered in the food supply chain during transportation of edible oils was recently studied by our group (VALLI *et al.*, 2013). In that study, we investigated the effect of simulated shipment on the quality of different types of edible oils from Italy to Taiwan, starting from the stage of truck loading and ending at the truck delivery phase. It was found that vegetable oils underwent a loss of quality and deterioration after the journey, especially in terms of primary and secondary oxidation products. The simulation runs were conducted using ad-hoc closed-loop controlled chambers (MANZINI and ACCORSI, 2013), in order to measure and control the effects of transportation on the quality of edible oil. Moreover, we have also compared the performance of these containers (ACCORSI *et al.*, 2014; MANZINI *et al.*, 2014).

In the present study, changes in the quality of three kinds of vegetable oils (extra virgin olive oil, rice oil, and grape seed oil) after two simulated shipments were investigated. The first journey was characterized by high temperatures during 37 days of shipment from Italy to Los Angeles (USA), and the latter by lower temperatures during 30 days of shipment from Italy to Quebec (Canada). Such temperatures were monitored using a thermal data logger during actual shipping and then reproduced in the laboratory. In particular, both the shipping profiles experienced by the bottles were tracked within a standard container (SC), i.e., a general-purpose one or dry container, and a thermal liner

containers (TLC), i.e., a basic dry containers equipped with a thermal liner that can partially or completely insulate cargo from climate stresses. This study evaluated the ability of the thermal insulated container to protect the quality of the oils in both shipments. With this aim, quality parameters such as free acidity, oxidation indexes (peroxide value, thiobarbituric acid content, and oxidative stability index) as well as sensory analysis and other physicochemical parameters (water amount, turbidity, and CIElab color indexes) were evaluated before and after the simulated shipments.

2. MATERIALS AND METHODS

2.1. Samples

The two simulated shipments were carried out using three different kinds of commercial vegetable oils: extra virgin olive oil (EVOO), grape seed oil (GSO), and rice oil (RO). In particular, two bottles (1 liter each) of oil were subjected to the simulated shipments. The two bottles of each oil for each destination (Quebec, coded as "Q" or Los Angeles, coded as "LA") contained edible oil coming from the same production line batch.
For both bottles, the primary packaging is a glass bottle. The cork is made of aluminum with a PET pourer. The bottles are contained within a secondary package made by corrugated carton (i.e., wrap package). The shipping profiles experienced by the two sets of bottles are tracked respectively within a standard container (SC), i.e., a general-purpose 40-foot equivalent units (FEU) container or dry container, and thermal liner containers (TLC), i.e., a basic dry containers equipped with a thermal liner that can partially or completely insulate cargo from climate stresses. A more detailed definition of these two containers is given in ACCORSI *et al.* (2014), while for the primary (i.e., bottle) and secondary packaging (i.e., carton) (ACCORSI *et al.*, 2015).

2.2. Simulation process

The temperature profiles were reproduced using closed-loop climate-controlled chambers placed in standard or thermally insulated containers (Fig. 1). The two container solutions have been previously described in a paper by the same research group (MANZINI *et al.*, 2014). The simulation chambers reproduced temperature cycles to fit the monitored temperatures registered during actual shipments. The temperature inside the chambers covers the possible range of -20°C to 65°C. The integrated cooling system consists of an evaporator utilizing 21 g of R600a iso-butane as a refrigerant. A closed-loop algorithm, developed with LabView National Instrument software, controls the actuators so that the chamber temperature reaches a defined set point. The first international simulated shipment (coded as "Q") from Italy to Quebec started on January 30 from the port of origin (Livorno) and ended on March 1 at the port of final destination (Quebec); the temperature profile of this shipment is illustrated in Fig. 2. The second international shipment (coded as "LA") from Italy to Los Angeles started on June 26 from the port of origin (Livorno) and ended on August 2 at the port of final destination; the temperature profile of this shipment is shown in Fig. 3.

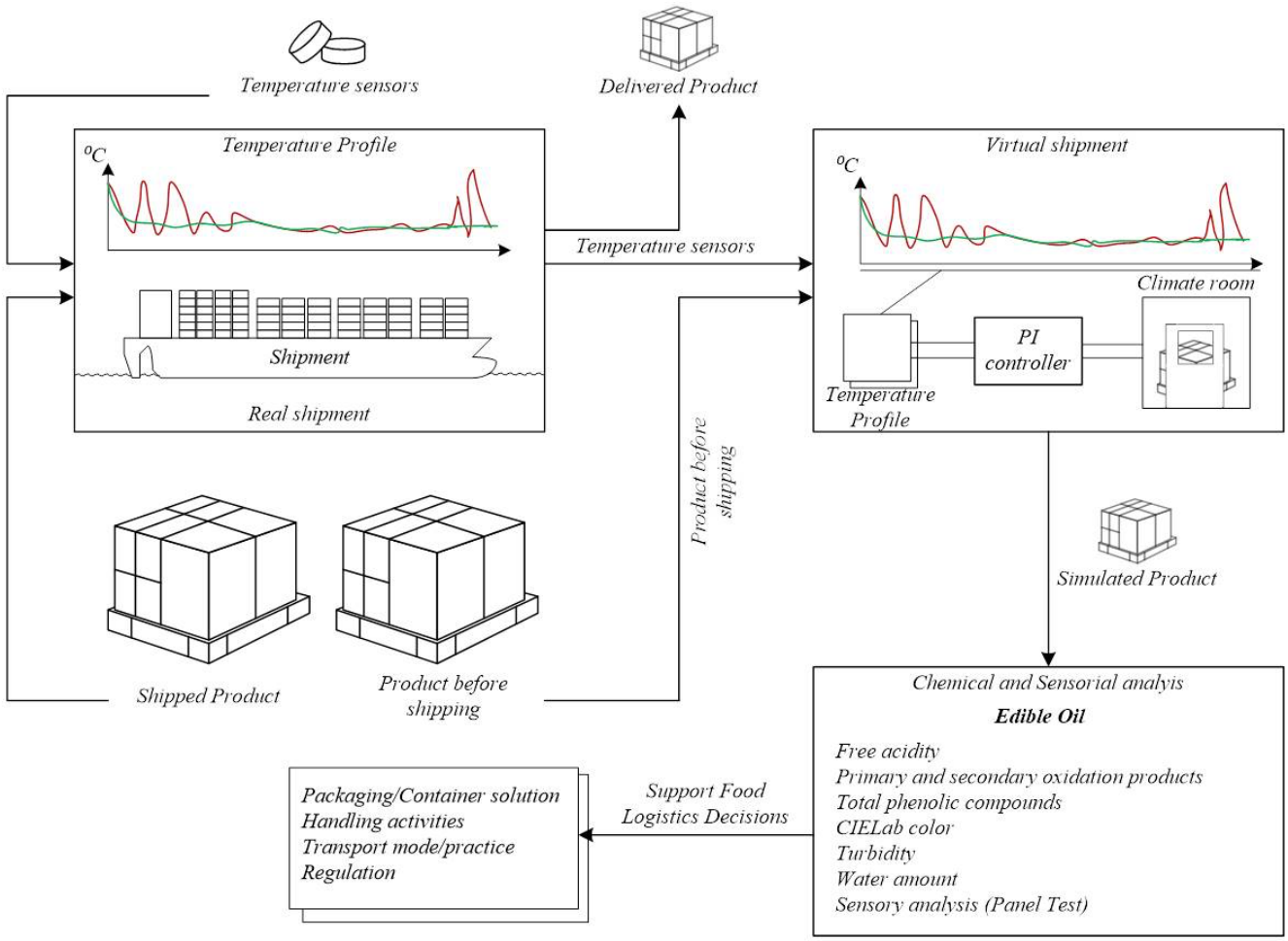

Figure 1. Closed-loop protocol system for simulation of shipping.

2.3. Chemical, physical and sensory analyses

2.3.1 Free acidity and thiobarbituric acid reactant substance content (TBARs)

Free acidity (FA) expressed as g oleic acid 100 g^{-1} oil and peroxide value (PV) expressed as milliequivalent O_2 kg^{-1} oil were determined for EVOO according to the official methods described in EEC. Reg. 2568/91 and successive amendments (EEC Reg. 1348/2013). For the two other edible oils, free acidity values (AV) were obtained by the Codex Alimentarius official method (CAC 2013a), and expressed in mg KOH g^{-1} oil. Thiobarbituric acid reactant substance content (TBARs) was determined in triplicates according to the AOCS Official Method Cd 19-90 (AOCS, 2006) and expressed as TBA value (milligram of malonaldehyde equivalent per kilogram of oil). Oil sample (50-200 mg) was weighted into 25 ml volumetric flask and dissolved with a small portion of 1-butanol. The solution volume was then filled by using 1-butanol. A portion (5 ml) of the dissolved sample was transferred into a screw-capped test tube. The reagent solution (200 mg of 2-thiobarbituric acid dissolved in 100 ml of 1-butanol) was added, and the mixture was thoroughly mixed. The tubes were then placed in a water bath at 95°C for 2 h. After cooling at room temperature, absorbance was determined at 530 nm by using 1 ml glass

cuvettes with a UV-vis 1800 spectrophotometer (Shimadzu Co., Kyoto, Japan). The reagent blank was prepared simultaneous to sample preparation.
TBA value was obtained using the following equation:

$$TBA = 50 \times (\text{abs of the sample - abs of the blank}) / \text{weight of the sample (mg)}$$

2.3.2. Spectrophotometric determination of total phenolic content (TP)

Phenolic compounds were extracted according to the method of PIRISI *et al*. (2000). Absorbance was determined at 750 nm by using a UV-vis 6705 spectrophotometer (Jenway, United Kingdom) through the method reported by Singleton and Rossi (1965). Briefly, each sample (2 g) was dissolved in 1 ml of *n*-hexane and extracted three times with 2 ml of methanol–water solution (60:40 v/v). In each extraction, the mixture was shaken with a vortex mixer for 1 min and then centrifuged for 5 min at 3,000 rpm. The aqueous phase was collected and transferred into another test tube after each centrifugation cycle. *n*-hexane (2 ml) was added to the collected phenolic extract, mixed on the vortex, and then centrifuged for 5 min at 3,000 rpm. After the *n*-hexane phase was removed, the extract was evaporated using a rotary evaporator at 35°C. The residue was dissolved with 5 ml of methanol-water solution (50:50 v/v). Absorption was determined with a spectrophotometer, and a standard calibration curve was prepared using different concentrations of gallic acid. The results were calculated and expressed as milligram of gallic acid per kilogram of oil.

2.3.3 Evaluation of the colour (CIELab)

CIELab color for EVOO samples was determined (GOMEZ-CARAVACA *et al.*, 2007), using a Hunterlab (Reston, VA, USA) colorflex instrument and expressed as L*, a*, b* chromatic coordinates. Turbidity (TD) of samples was determined using a Ratio turbidimeter model 18900 (Hack, Colorado, USA) and expressed as nephelometric turbidity units (NTU).

2.3.4 Determination of the water content

Water amount was determined at 103°C through air drying technique (ISO 662:1988). Oil sample (10 g) was weighed in an empty aluminum moisture dish (approximately 50 mm in diameter and 30 mm height, with a flat bottom). The samples were heated for 1 h in a drying oven at 103±2°C, and the dish was cooled in the desiccator and weighed. The sample was reheated for another 0.5 h, cooled, and then weighed again. The half-hour reheating, cooling, and weighing cycle may be repeated until the difference between the final successive weights was lower than 2 mg. The water amount was calculated with the following equation: weight of sample − weight of dried sample / weight of sample.

2.3.5 Sensory analysis

Sensory analysis of EVOO samples was performed according to the procedure outlined in EEC Reg. 640/2008 by a fully trained panel of 8 expert and trained tasters of the Department of Agricultural and Food Sciences of the University of Bologna.

2.3.6 Statistical analysis

All analyses were run in triplicate and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed using XLSTAT 7.5.2 software (Addinsoft, NY, USA) at a 95% confidence level (Fisher LSD, $p < 0.05$) to evaluate significant differences between means.

3. RESULTS AND DISCUSSION

3.1 Effect of simulated shipment on hydrolytic degradation

Free acidity is considered as an important parameter to determine the hydrolysis of triacylglycerol in olive oil. Moreover, acidity values are considered as a basic criterion to classify the different categories of olive oil. The results in Table 1 show that FA increased slightly during shipments to both destinations. In addition, there was a slight increasing trend in FA for EVOO LA shipped in a standard container compared with that before shipping, which was influenced by the increase in temperature during the simulated journey (PARADISO *et al.*, 2010). However, none of the shipped EVOO samples reached the limit of 0.8% accepted for the extra virgin olive oil category (EEC Reg. 1348/2013).

Table 1. FA, free acidity (g oleic acid 100 g^{-1} oil); PV, peroxide values (meq O_2 kg^{-1} oil); TBARS, thiobarbituric acid reactive substances value (mg of malonaldehyde equivalent kg^{-1} oil); TP, total phenols (mg gallic acid kg^{-1} oil) tested before simulation and after simulation of shipping in insulated and standard containers for evoo samples to the two final destinations (EVOO Q, Quebec and EVOO LA, Los Angeles).
Values (mean ± standard deviation) with different superscript capital letters in each column and for each sample were significantly different between the simulated shipping conditions ($p < 0.05$; Fisher's test).

Sample	Experimental condition	FA (g oleic acid 100 g^{-1})	PV (meq O_2 kg^{-1})	TBARs (mg of malonaldehyde equivalent kg^{-1})	TP (mg gallic acid kg^{-1})
EVOO Q	Before shipping	0.52^{B}±0.04	11.7^{C} ±0.7	0.013^{B} ± 0.001	353^{B} ± 25
	Insulated container	0.59^{A}±0.01	13.1^{B} ±0.3	0.012^{B} ± 0.001	372^{B} ± 38
	Standard container	0.60^{A}±0.01	17.0^{A} ±0.8	0.016^{A} ± 0.001	478^{A} ± 30
EVOO LA	Before shipping	0.45^{B}±0.01	8.8^{C} ± 0.2	0.015^{C} ± 0.001	259^{A} ± 2
	Insulated container	0.45^{B}±0.01	9.2^{B} ± 0.1	0.028^{B} ± 0.001	257^{A} ± 8
	Standard container	0.48^{A}±0.01	10.4^{A} ± 0.1	0.040^{A} ± 0.001	222^{B} ± 3

Acid value results (Table 2) of GSO stored in the standard container for both simulated shipments were significantly higher in comparison with the thermally insulated samples and that before shipping. Considering the RO samples shipped to Quebec which, before starting the simulation, had an AV higher than the accepted limit of 0.6% for edible oils (CAC 2013a), the AV registered for the sample stored in the standard container was significantly higher than both the respective values for samples with and without thermal insulation.

Table 2. AV, acid values (mg KOH g^{-1}); PV, peroxide values (meq O_2 kg^{-1} oil); TBARS, thiobarbituric acid reactive substance values (mg of malonaldehyde equivalent kg^{-1} oil) of vegetable oil samples [grape seed oil (GSO) and rice oil (RO)] tested before and after simulation of shipping in insulated or standard containers to the two final destinations (coded as "Q" to Quebec and as "LA" to Los Angeles). Values (mean ± standard deviation) with different superscript capital letters in each column and for each sample were significantly different between the simulated shipping conditions ($p < 0.05$; Fisher's test).

Sample	Experimental conditions	AV (mg KOH g^{-1})	PV (meq O_2 kg^{-1})	TBARs (mg of malonaldehyde equivalent kg^{-1})
GSO Q	Before shipping	$0.27^{C} \pm 0.00$	$4.2^{B} \pm 0.1$	$0.018^{A} \pm 0.001$
	Insulated container	$0.36^{B} \pm 0.03$	$6.3^{A} \pm 0.9$	$0.020^{A} \pm 0.003$
	Standard container	$0.43^{A} \pm 0.00$	$6.2^{A} \pm 0.1$	$0.017^{A} \pm 0.002$
RO Q	Before shipping	$0.74^{C} \pm 0.01$	$4.4^{B} \pm 0.2$	$0.017^{B} \pm 0.001$
	Insulated container	$0.86^{B} \pm 0.03$	$4.8^{A} \pm 0.1$	$0.016^{B} \pm 0.002$
	Standard container	$0.98^{A} \pm 0.08$	$4.1^{B} \pm 0.1$	$0.022^{A} \pm 0.003$
GSO LA	Before shipping	$0.24^{B} \pm 0.04$	$1.6^{B} \pm 0.0$	$0.018^{C} \pm 0.001$
	Insulated container	$0.24^{B} \pm 0.03$	$3.3^{A} \pm 0.5$	$0.020^{B} \pm 0.001$
	Standard container	$0.35^{A} \pm 0.02$	$3.0^{A} \pm 0.2$	$0.043^{A} \pm 0.001$
RO LA	Before shipping	$0.46^{A} \pm 0.01$	$3.3^{B} \pm 0.3$	$0.014^{B} \pm 0.001$
	Insulated container	$0.45^{A} \pm 0.03$	$3.5^{B} \pm 0.4$	$0.020^{A} \pm 0.001$
	Standard container	$0.51^{A} \pm 0.03$	$4.9^{A} \pm 0.4$	$0.020^{A} \pm 0.001$

The results for the RO sample to Quebec revealed a drastic effect of temperature variation, and in particular for low quality edible oils. In fact, as recorded during the simulation in a standard container to Quebec, the temperature decreased to -10°C (Fig. 2). Such low temperatures probably facilitate hydrolytic processes due to water droplets in the liquid phase that surrounds the lipid crystals (KRISTOTT 2000). In the case of RO in the simulated shipment to Los Angeles, on the other hand, the change in AV after simulation in both the standard and thermally insulated containers was not significant; in this case, the samples experienced a slight temperature fluctuation during 13 days of simulated shipment before reaching the final destination.

3.2. Influence of simulated shipment on oxidation stability

In order to estimate the effect of shipment on EVOO and other vegetable oils, oxidation quality was tracked by evaluating i) PVs, which indicate the increase in primary oxidation products, such as hydroperoxides, and ii) TBAR values, which detect the formation of malondialdehyde from fatty chains with three or more double bonds (FRANKEL, 1991), and indicate the trend in secondary oxidation products in edible oil. As seen in Table 1, the PV was significantly higher in the EVOO sample for which the simulated shipment was conducted in a standard container compared to that shipped in a thermally insulated container for both destinations. TBARs values were also significantly higher when a standard container was used to transport EVOO samples compared with those subjected to simulation in a thermally insulated container for both destinations. These results suggest that thermally insulated containers have a beneficial effect, compared with a standard container, in terms of protecting EVOO samples against oxidative stress. Moreover, starting from similar values for both samples before shipping, higher TBARs

values were reported for EVOO sent to Los Angeles compared with the sample sent to Quebec; this may be related to the higher temperature stress applied in the Los Angeles simulation (Figs. 2 and 3).

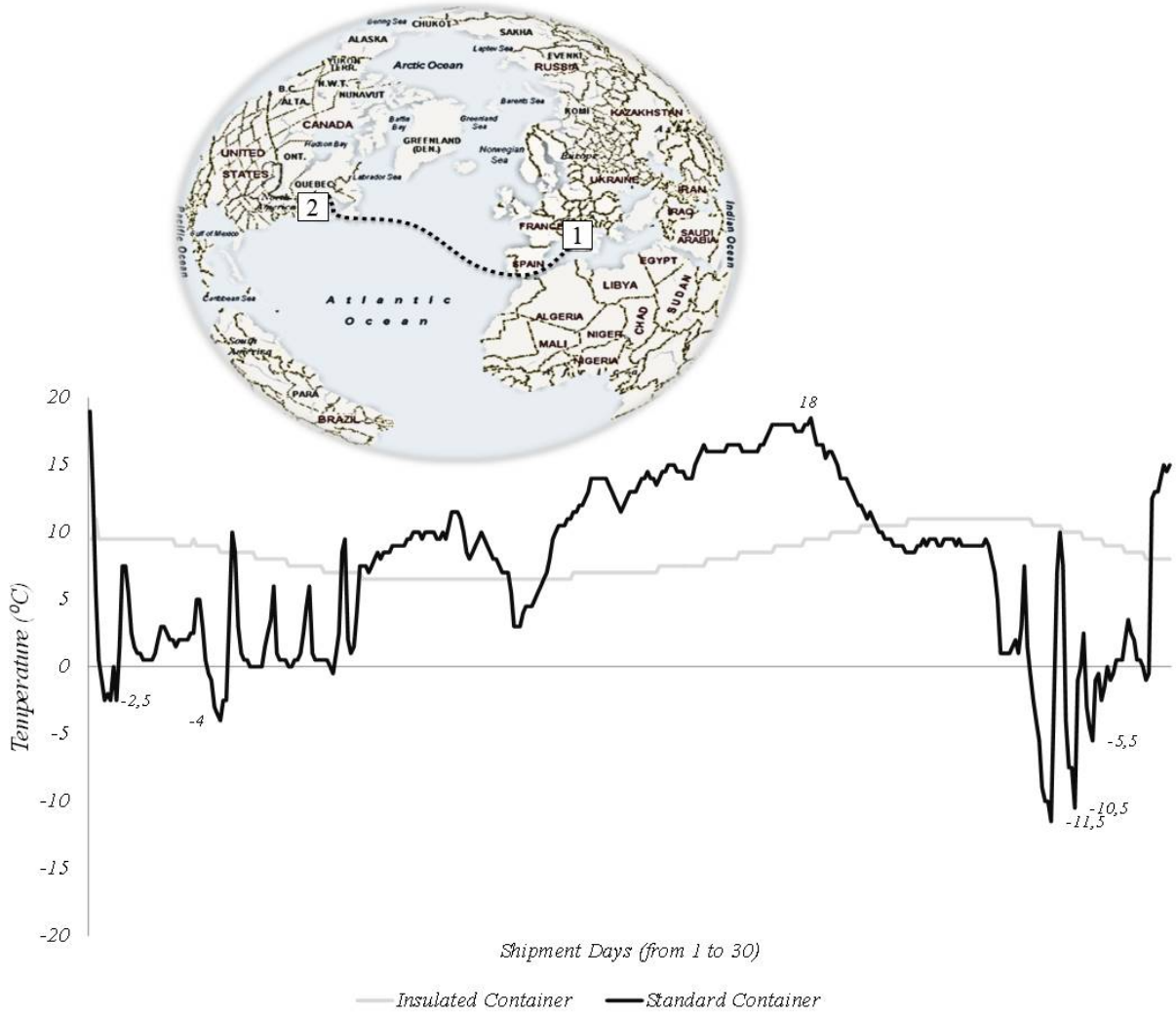

Figure 2. Temperature profile monitored using data loggers for the Quebec simulation (in the world map, 1: Livorno port; 2: Quebec port). a: inside standard container; duration: 30 days; highest temperature: 19°C; lowest temperature: -11.5°C. b. inside thermal insulated container; duration: 30 days; highest temperature: 11°C; lowest temperature: 6.5°C.

Regarding the other vegetable oils, the PVs (Table 2) had higher values after simulation compared with those before shipping, for both destinations, except for RO shipped in a standard container to Quebec. Considering RO to Los Angeles, a higher increase was observed in PVs in a standard container compared with thermally insulated samples, which indicate more advanced formation of peroxides in the standard container. On the other hand, the lower PV values seen in RO to Quebec in a standard container compared with samples shipped in an insulated container reveals possible additional transformation of peroxides to secondary oxidation products, which was also confirmed by the increase in TBAR observed in the same sample (Table 2). The higher impact on oxidative status on all

edible oils by the Los Angeles simulation is also demonstrated by considering the changes in total phenols in EVOO (Table 1): these minor components, in addition to their nutritional role, act as antioxidants in EVOO (BENDINI *et al.*, 2007). Before simulation, EVOO samples contained about 353 and 259 mg gallic acid kg^{-1} oil, respectively, for samples sent to Quebec and Los Angeles (Table 1); after shipping, these values tended to decrease in standard container for the samples sent to Los Angeles. This reduction was more pronounced for samples stored in the standard container than after the non-thermally insulated journey due to the effect of higher temperature stress (Fig. 3). The anomalous increase in total phenolic content registered for the EVOO shipped to Quebec after simulation in standard container could be attributed to the higher extractability of phenolic molecules after a crystallization and subsequent thawing out caused by the low temperatures reached during the simulation (Fig. 2). On the other hand, for the sample EVOO LA shipped at higher temperature, this effect was not observed.

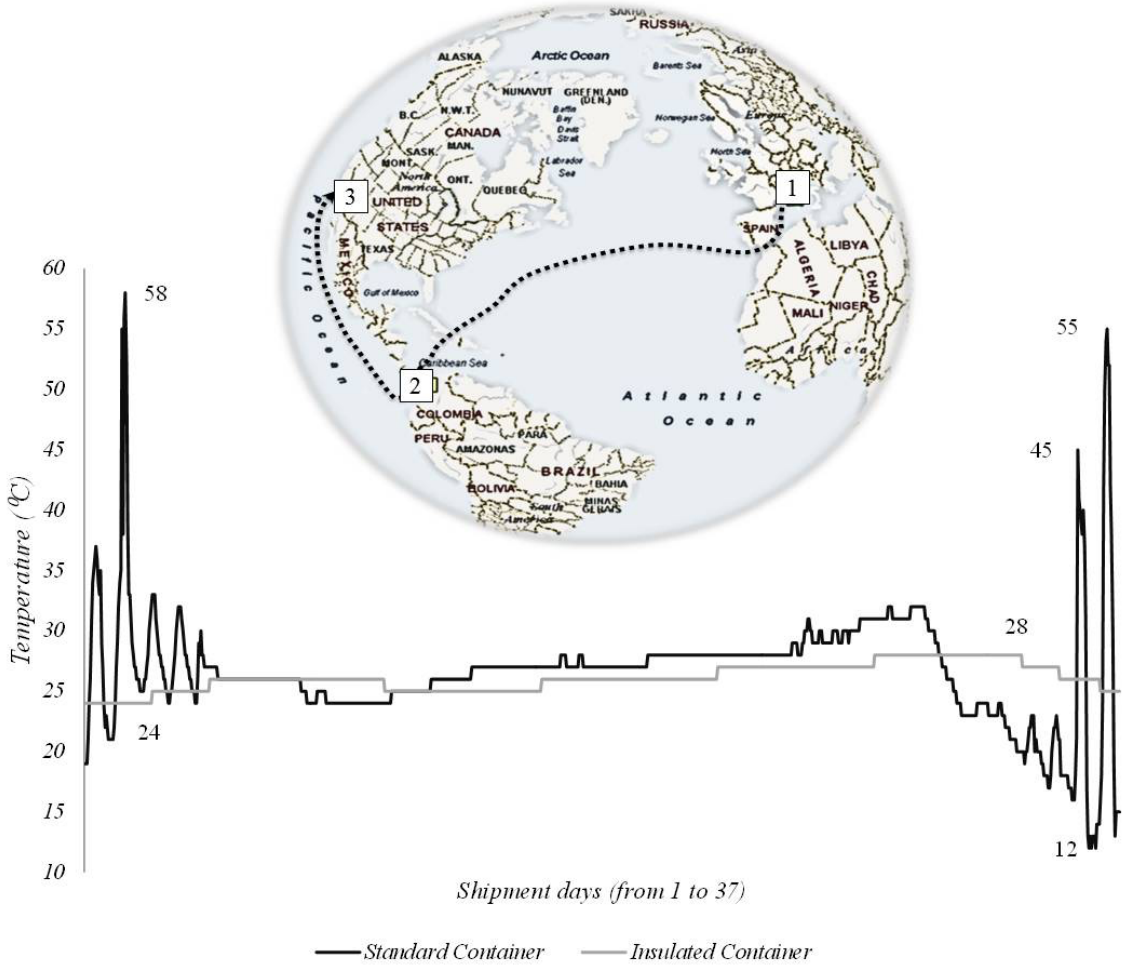

Figure 3. Temperature profile monitored using data loggers for the Los Angeles simulation (in the world map: 1, Genoa port; 2, panama canal ; 3, Los Angeles port). duration: 37 days, highest temperature: 58°C, lowest temperature: 11.5°C.

3.3. Influence of simulated shipment on physical and sensory properties

Color changes in EVOO reflect the visual color appearance that is considered to be an important factor in consumer satisfaction (MOYANO *et al.*, 2010). The color of olive oils, in general, is principally affected by two classes of minor compounds, namely chlorophylls and carotenoids. The degradation of these compounds is due to different conditions of stress, such as temperature and light, which may alter color in addition to clarity and transmittance (SIKORSKA, *et al.*, 2007). Color indexes were expressed as chromatic coordinates: L* corresponds to brightness and positive b* to yellowish color, while negative a* corresponds to light green color (MINGUEZ-MOSQUERA *et al.*, 1991). As seen in Table 3, there were significant changes in the brightness (L*) and b* indices for EVOO samples sent to Quebec after simulation in the standard and insulated containers (more bright and more yellowish). However, a reduction in L* values (meaning less bright oils) was seen in both shipping conditions for the simulated shipment to Los Angeles. A reduction was also observed for b* values (less yellow toward light blue) of samples shipped to Los Angeles, corresponding to the degradation of yellow chromophores (pigments), that function as natural antioxidants, such as carotenoids and pheophytins (PSOMIADOU and TSIMIDOU 2002), since oxidation is promoted by the increased temperature (MORELLO *et al.*, 2004) during the simulation to Los Angeles (Fig. 3). As previously reported, degradation of natural pigments such as carotenoids occurs at around 40°C (THAKKAR *et al.*, 2009). Moreover, an increase in a* values (partial loss of green color toward redness) was recorded for samples sent to Los Angeles: such a partial loss of green color, in general, may correspond to partial degradation of chlorophylls, which are partially converted into other gray/brown compounds, and specifically to pyropheophytin a which is formed from pheophytin a due to degradation triggered by inadequate temperatures during the storage of oil (APARICIO-RUIZ *et al.*, 2014). Consequently, the increased degradation of chlorophyll and carotenoid pigments is likely related to the increased temperature (up to 58°C) in the final stages of the Los Angeles simulation (Fig. 3).
In addition, variations in water amount and turbidity were not significant (Table 3) in either simulation. Sensory analysis, realized according to the EU Reg. 640/2008, is an essential technique for the assessment of the quality of EVOO. The sensory evaluation (results not shown) indicated that no sensory defects developed after simulated shipment to Quebec or Los Angeles, and all samples remained within the "extra virgin" category in both thermally insulated and standard containers.

Table 3. Color coordinates (l*, a*, b*); TD, turbidity (NTU); WA, water amount (mg kg^{-1} oil) before and after simulated shipping in an insulated and standard container for EVOO samples to the two final destinations (EVOO Q, Quebec and EVOO LA, Los Angeles).
Values (mean ± standard deviation) with different superscript capital letters in each column and for each sample were significantly different between the simulated shipping conditions ($p < 0.05$; Fisher's test).

Samples	Experimental conditions	L*	a*	b*	TD (NTU)	WA (mg kg^{-1} oil)
EVOO Q	Before shipping	$54^{B} \pm 0.1$	$4.9^{A} \pm 0.0$	$80^{B} \pm 0$	$11.7^{A} \pm 0.2$	$719^{A} \pm 98$
	Insulated container	$55^{A} \pm 0.1$	$4.8^{B} \pm 0.0$	$84^{A} \pm 0$	$11.3^{A} \pm 0.1$	$621^{A} \pm 6$
	Standard container	$55^{A} \pm 0.1$	$4.6^{C} \pm 0.0$	$84^{A} \pm 0$	$11.5^{A} \pm 0.2$	$708^{A} \pm 92$
EVOO LA	Before shipping	$63^{A} \pm 0.0$	$4.3^{B} \pm 0.1$	$89^{A} \pm 0$	$11.6^{A} \pm 0.2$	$650^{A} \pm 30$
	Insulated container	$50^{B} \pm 1.4$	$5.8^{A} \pm 0.2$	$71^{C} \pm 1$	$11.5^{A} \pm 0.2$	$607^{A} \pm 64$
	Standard container	$52^{B} \pm 1.5$	$5.5^{A} \pm 0.2$	$79^{B} \pm 2$	$11.4^{A} \pm 0.1$	$562^{A} \pm 72$

4. CONCLUSIONS

It is important to point out that this study is related to two specific simulations, and thus the results cannot be generalized to all shipments of vegetable oils to Los Angeles or Quebec. From parallel study of two simulated shipments to different destinations with different thermal conditions, it was found that thermal isolation is associated with significant benefits in terms of avoiding an increase in degradative reactions for edible oils, and especially on oxidative status. Considering the different parameters evaluated, the quality of the edible oils subjected to the simulation to Quebec was higher than those shipped to Los Angeles, which was due to the different thermal profiles of the two journeys. The aim of future studies is the adoption of a proposed ex-post simulation analysis on edible oils having different ages, shipped in different periods of the year and to different destinations, in agreement with specific logistic decisions (storage, material handling, transportation modes, etc.) and packaging solutions including primary, secondary, tertiary packaging, and containment equipment.

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CHARACTERIZATION OF CHITINASE ISOFORMS FROM GRAPE JUICE

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ABSTRACT

Grape chitinases are recognized as being mainly responsible for protein haze formation in white wines. *Vitis vinifera* L. cv. Manzoni Bianco grape juice proteins were fractionated using anion exchange and hydrophobic interaction chromatographies. According to SDS-PAGE and zymography, six protein bands with chitinolytic activity were subjected to mass spectrometry (MALDI-TOF/TOF MS), which assigned all the bands to *Vitis vinifera* class IV chitinases. These grape chitinase isoforms showing different electrophoretic and chromatographic behaviours are likely to be also distinct in their functionality in wine. This could be relevant to understand the involvement of single chitinase components in wine hazing and to develop specific winemaking techniques for their removal from wine.

Keywords: chitinase, electrophoresis, glycol chitin, grape juice, isoform, mass spectrometry

1. INTRODUCTION

The problem of protein haze formation in white wines is still unresolved, despite wine hazing being a serious quality defect because consumers perceive hazy wines as faulty products. Protein haze is caused by the presence of relatively low concentrations (from 15 to 700 mg/L) of Pathogenesis-Related (PR) proteins, namely Thaumatin-Like Proteins (TLPs) and chitinases (FERREIRA *et al.*, 2001; WATERS *et al.*, 2005; VINCENZI *et al.*, 2005; VAN SLUYTER *et al.*, 2015).
Chitinases are the most active protein components in causing wine turbidity (FALCONER *et al.*, 2010; MARANGON *et al.*, 2011). These proteins derive from grapes, are present in different isoforms (MARANGON *et al.*, 2011; GAZZOLA *et al.*, 2012), are tolerant to low pH in juice and wine and are resistant to proteolytic enzymes, as most of the PR proteins (FERREIRA *et al.*, 2001; WATERS *et al.*, 2005; VAN SLUYTER *et al.*, 2015).
The first step of the mechanism leading to haze formation in wines should involve protein denaturation (VAN SLUYTER *et al.*, 2015). Grape chitinases can denature within minutes at temperatures >40°C, compared to weeks for TLPs under the same conditions, with a predicted half-lives of only 14 hours at a realistic temperature of 35°C (FALCONER *et al.*, 2010). Moreover, these grape enzymes seem to maintain their activity in wine at least for some months after alcoholic fermentation (MANTEAU *et al.*, 2003) and the consequences of this activity on wine quality are unknown. Chitinases have antifungal properties resulting from their activity toward chitin, a major structural component of many fungal cell walls (GRAHAM and STICKLEN, 1994). However, a chitinase purified from *Vitis vinifera* L. cv. Manzoni Bianco grape juice, although showing both endo- and exo-chitinase activities, was not able to inhibit wine yeast growth (VINCENZI *et al.*, 2014).
Chitinases have been successfully purified by others (MARANGON *et al.*, 2009; VAN SLUYTER *et al.*, 2009; DUFRECHOU *et al.*, 2013), despite their low concentration and strong interaction with endogenous polyphenols and other non-protein compounds (FERREIRA *et al.*, 2001; GAZZOLA *et al.*, 2012). In spite the interest of these type of wine components, in-depth knowledge surrounding them is still incomplete. Therefore it is important to develop robust systems for a better characterisation of these components and in particular to establish the role of each chitinase isoform found in grape in wine haze formation and development. In this paper, the purification, the electrophoretic characterisation and the Mass Spectrometry identification of some chitinase isoforms from Manzoni Bianco grape juice is described.

2. MATERIALS AND METHODS

2.1. Protein extraction from grape juice

According to VINCENZI *et al.* (2014) with minor modifications, fifteen kg of *Vitis vinifera* L. cv. Manzoni Bianco berries were manually crushed and treated with 7.5 g/kg polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, St. Louis, MO), 0.15 g/kg ascorbic acid (Baker, Deventer, Holland) and 0.375 g/kg potassium metabisulfite (Carlo Erba, Milano, Italy). The grape juice (10 L) was treated overnight at 4°C with 3 g/L of pectolytic enzymes (Pectazina DC, Dal Cin SpA, Milano, Italy), and centrifuged (5000 g, 20 min, 4°C). The free run juice was dialysed (3500 Da cut-off) against distilled water, concentrated by ultrafiltration (3000 Da cut-off) and freeze-dried.

2.2. Protein separation by chromatography

A two-step chromatographic separation was performed using an ÄKTA purifier FPLC (GE-Healthcare, Uppsala, Sweden) equipped with an UV detector. Data were processed by the Unicorn 5.11 software (GE-Healthcare). Each solution used and samples to load were previously filtered with 0.20 μm cellulose acetate filters (Millipore, Vimodrone, Italy).
The first chromatographic step was Anion Exchange Chromatography (AEC). ≈ 50 mg of freeze-dried extract were dissolved in 20 mM Tris-HCl pH 9.0 (buffer A) and loaded onto a Tricorn MonoQ 5/50 column (GE-Healthcare) equilibrated with buffer A at a flow rate of 1 mL/min. Bound proteins were eluted at 1 mL/min with a gradient of buffer B (20 mM Tris-HCl, 1 M NaCl, pH 9.0) as follows: 0 to 14% B in 70 min and 14 to 100% B in 3 min (VINCENZI *et al.*, 2011 with minor modifications). AEC fractions were pooled on the basis of 280 nm elution profiles and analysed by SDS-PAGE after being concentrated and dialysed against water (Vivaspin 50, 3000 Da cutoff, Sartorius, Göttingen, Germany).
The second purification step was performed by Hydrophobic Interaction Chromatography (HIC) according to VINCENZI *et al.* (2014). The pooled and selected AEC fractions were fractionated at 0.5 mL/min on a HIC BioSuite Phenyl 10 μm HIC 7.5 x 7.5 mm column (Waters, Milford, MA) with a 60 min linear gradient to 100% buffer B (20 mM tartaric acid pH 3.5) in buffer A (20 mM tartaric acid pH 3.5 containig 1.25 M ammonium sulfate).

2.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analyses were performed according to LAEMMLI (1970) in a Mini-Protean III apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Samples were prepared by precipitating proteins from 5 to 50 μL (depending on protein concentration on samples) of pooled chromatographic fractions by the KDS method (VINCENZI *et al.*, 2005; GAZZOLA *et al.*, 2015). Precipitated proteins were solubilized in 20 μL of 0.5 M Tris-HCl buffer, pH 6.8, containing 15% (w/v) glycerol and 1.5% (w/v) SDS (Bio-Rad Laboratories) and heated at 100°C for 5 minutes before loading. In order to detect the presence of disulphide linked protein aggregates, SDS-PAGE was performed also in reducing conditions. This was done by adding 4% (v/v) 2-mercaptoethanol to the loading buffer.
Electrophoresis was carried out at 25 mA constant current until the tracking dye Bromophenol Blue ran off the gel. The molecular weight standard proteins were the Broad Range Molecular Weight Markers (Bio-Rad Laboratories). 1.5 mm thick gels were prepared with T = 12% (SDS-PAGE in Fig. 5) or 14% (acrylamide-N, N' metilenbisacrylamide 29:1; Sigma-Aldrich) and stained with Colloidal Coomassie Brilliant Blue G-250 (Sigma-Aldrich) or with the PAS (Periodic Acid-Schiff) method for glycocompounds detection (SEGREST and JACKSON, 1972).

2.4. Chitinolytic activity detection on SDS-PAGE gels

Chitinolytic activity detection was assayed according to VINCENZI and CURIONI (2005). Samples were prepared with the same reagents used for SDS-PAGE and loaded into a gel (T = 14%) containing glycol-chitin (0.01% or 0.05% w/v). After protein separation, the gels were incubated overnight at room temperature in a 50 mM sodium acetate buffer pH 5.5 containing 1% (w/v) Triton X-100 (Sigma-Aldrich). Afterwards, gels were incubated for 20 minutes in 0.5 M Tris-HCl buffer pH 8.9, containing 0.01 % (w/v) Calcofluor White MR2 (Sigma-Aldrich), followed by a wash in distilled water (for at least 1 h). Protein bands with chitinolytic activity were digitized with an EDAS290 image capturing system (Kodak, Rochester, NY).

2.5. Protein identification by MALDI-TOF/TOF MS

The selected bands were excised from SDS-PAGE gels, dehydrated with acetonitrile for 10 min and dried in Speed Vac concentrator. Disulphide bridges were reduced with 10 mM dithiotreitol (1 h, 56°C, in the dark) and cysteines were alkylated with 55 mM iodoacetamide (1 h, room temperature, in the dark). Gel bands were repeatedly washed with 50 mM NH_4HCO_3 and acetonitrile, and dried under vacuum. In gel protein digestion was performed using sequencing grade modified trypsin (Promega, Madison, WI). 10 μL of trypsin (12.5 ng/μL in 50 mM NH_4HCO_3) were added to each band, and digestion was carried out at 37°C overnight. Peptides were extracted with 50 μL of 50% acetonitrile and 1% formic acid (3 times), dried under vacuum and dissolved in 10 μL of 0.1% formic acid. The digested sample was mixed with an equal volume of matrix solution (a-cyano-4-hydroxycinnamic acid, 5 mg/mL in 70% acetonitrile, 0.1% trifluoroacetic acid) and 1 μL was spotted on a 384-well AB OptiTOF MALDI stainless steel target plate (SHEVCHENKO *et al.*, 2006). Samples were analysed using a MALDI-TOF/TOF 4800 Analyzer (Applied Biosystems, Toronto, Canada) with 4000 Series Explorer v3.5.3 software. MS data were acquired automatically over a mass range of 900–3500 Da in the positive-ion reflector mode. In the MS spectrum, the 10 most abundant MS peaks were selected for MS/MS.
MS/MS data were searched using the Mascot search engine (Matrix Science, London, UK) against the MSDB database (3239079 sequences; 1079594700 residues; Taxonomy: Viridiplantae, 247880 sequences). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 50 ppm for the precursor ion and 0.3 Da for the fragment ions and carbamidomethylcysteine as fixed modification.

3. RESULTS AND DISCUSSIONS

3.1. SDS-PAGE analysis of the grape juice proteins

Vitis vinifera L. cv. Manzoni Bianco (Riesling Renano x Pinot Bianco) was used for the grape protein extraction, as this variety shows a high protein content and gives wines generally requiring fining treatments with significant amounts of bentonite for protein stabilization (VINCENZI *et al.*, 2011).
From the free run juice, 2.6 g of grape juice macromolecular powder (crude extract, CE) was obtained. An aliquot of CE was analyzed by SDS-PAGE, showing main protein bands in the region between 20 and 30 kDa (Fig. 1a).
These bands have been previously identified as grape Pathogenesis-Related (PR) proteins including Thaumatin-Like Proteins (TLPs) and chitinases (WATERS *et al.*, 1996; MONTEIRO *et al.*, 2007; VAN SLUYTER *et al.*, 2015). High molecular weight protein bands were also evident in the 45-80 kDa range. The protein with relative molecular mass (*Mr*) of 65 kDa is likely to be the grape vacuolar invertase which is known to be one of the most abundant proteins in grape juice and wine, reaching 14% of Chardonnay wine proteins (DAMBROUCK *et al.*, 2005). The minor bands with *Mr* ranging from 45 to 60 kDa have also been identified by proteomic analysis in a Semillon grape juice as (*Vitis vinifera*) "unnamed protein product" and class IV chitinase (MARANGON *et al.*, 2009). Finally, the band of 12 kDa is likely to correspond to the Lipid Transfer Protein (LTP), whose presence has already been reported in grapes where was indicated as one of the major allergens (PASTORELLO *et al.*, 2003). Staining the gel for sugar residues confirmed the presence of glycocompounds, probably polysaccharides, which barely entered the gel (Fig. 1b) (VINCENZI *et al.*, 2012).

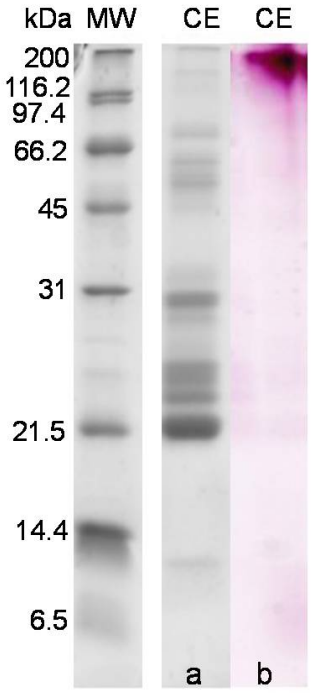

Figure 1 Non-reducing SDS-PAGE of the grape berries crude extract (CE), stained for proteins (a) and glycocompounds (b). Molecular weight standard proteins are on the left.

3.2. Protein fractionation and characterization

Starting from the CE, the grape juice macromolecules (>3.5 kDa) were initially fractionated by Anion Exchange Chromatography (AEC). Since the grape proteins have very similar MWs and different pI (MONTEIRO *et al.*, 2001), this chromatographic technique proved to be very effective at this stage. AEC has already been applied previously to those compounds (WATERS *et al.*, 1992; DORRESTEIN *et al.*, 1995; PASTORELLO *et al.*, 2003), allowing to obtain a good resolution of protein peaks.
A representative AEC chromatogram for grape juice macromolecules (≈ 50 mg) is shown in Fig. 2.
The material not retained by the column (Flow through) was very little or at least had a low UV absorption. Almost all peaks were eluted at relatively low NaCl concentrations (0.08-0.12 M), while a last peak was obtained with a high NaCl concentration (1 M), indicating that at pH 9 the eluting fractions have different charge properties.
Six separated fractions were collected (Fig. 2) and analysed by SDS-PAGE in non-reducing conditions (Fig. 3).
As expected, the Flow through did not contain any proteins. The first two peaks (1a and 1b) both displayed a band at ≈ 20 kDa. Fraction 1b contained also some minor bands around 25 kDa. All these bands probably correspond to TLPs according to literature data (WATERS *et al.*, 1996; MARANGON *et al.*, 2011; MARANGON *et al.*, 2014). Moreover, peak 1b showed a 40 kDa band which could correspond to a β-glucanase (ESTERUELAS *et al.*, 2009; SAUVAGE *et al.*, 2010). Peak 2 contained only one band that showed a *Mr* similar to that of TLPs (WATERS *et al.*, 1996; MARANGON *et al.*, 2011; MARANGON *et al.*, 2014). Peaks 3a and 3b showed several bands with *Mr*s similar to that of TLPs and a protein of ≈ 66 kDa, probably corresponding to invertase (MARCHAL *et al.*, 1996).

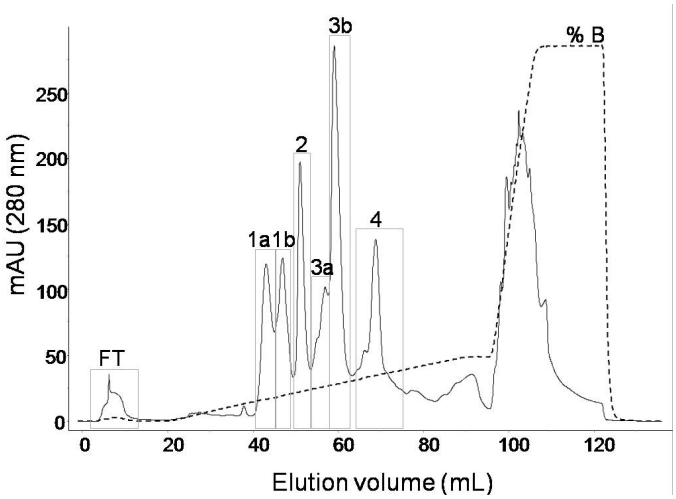

Figure 2. Anion exchange chromatogram for Manzoni Bianco crude extract (50 mg). Collected fractions are indicated by numbered boxes. The dotted line indicates the salt gradient.

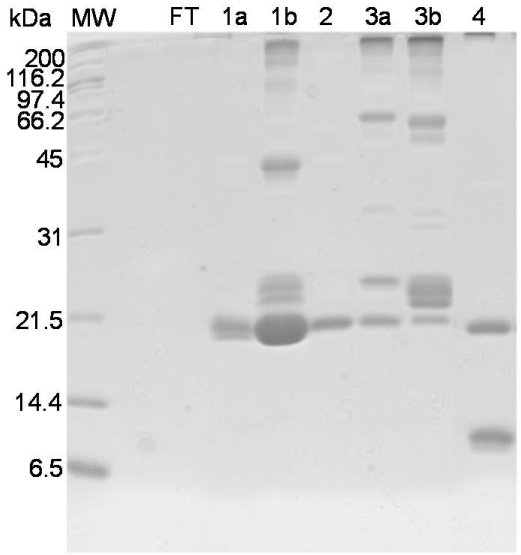

Figure 3. Non-reducing SDS-PAGE of the fractions collected from Anion Exchange Chromatography. Molecular weight standard proteins are on the left.

The presence of faint bands with MWs of ≈ 31 and ≈ 32 kDa is noteworthy, which could correspond to grape chitinases (WATERS *et al.*, 1996; MARANGON *et al.*, 2009). Peak 3b also contained a ≈ 52 kDa band, whose identity was investigated in this work. Finally, peak 4 showed another band with the same mobility of TLPs and a low MW band that could correspond to the grape LTP (PASTORELLO *et al.*, 2003) (Fig. 3). According to this chromatographic behaviour it is clear that different protein isoforms assigned to both TLPs and chitinases on the basis of their SDS-PAGE mobility show different charge properties at pH 9 being eluted from the AEC column at different NaCl concentrations (from ≈ 0.08 to ≈ 0.12 M). This is obviously related to a heterogeneity of the grape juice proteins at the amino acid level (MONTEIRO *et al.*, 2001) which is likely to affect their net

charge also at the low pH of the wine. Since charge is one of the main factors involved in protein functionality in terms of colloidal behavior (VINCENZI *et al.*, 2011), it is likely that different forms of the same protein detected by SDS-PAGE in the different AEC fractions play specific roles in the phenomena leading to haze formation in wine. Indeed the pH-dependent variation of protein charges has been indicated as one of the factors that strongly affect protein aggregation in wine (DUFRECHOU *et al.*, 2012). The chitinase-containing peaks (3a and 3b in figures 2 and 3), from 15 chromatographic separations of 50 mg of protein each, all giving the same results (not shown), were combined and freeze dried. Since this sample (from now on named "peak 3") was contaminated by other proteins (Fig. 3, lanes 3a and 3b), a further purification step involving Hydrophobic Interaction Chromatography (HIC) was used, resulting in the separation of protein fractions differing in surface hydrophobicity (VAN SLUYTER *et al.*, 2009). The protein peak 3 from AEC gave six peaks after HIC (Fig. 4).
SDS-PAGE analysis of the proteins of HIC peaks under reducing and non-reducing conditions, showed several protein bands, differing in both *Mr* and staining intensity (Fig. 5).
Also in this case, proteins with the same SDS-PAGE mobility were detected in more than one peak, indicating differences in surface hydrophobicity of components showing very similar charges (those of fractions 3a and 3b of the AEC) and apparent molecular weight (by SDS-PAGE). Hydrophobicity is also an important property affecting the interaction of a protein with other components (SIEBERT *et al.*, 1996). Therefore the propensity to form haze in wine can be different for wine protein isoforms with different hydrophobicity, as demonstrated, for example, by studying the reactivity of wine protein fractions differing for this parameter with tannins (MARANGON *et al.*, 2010).

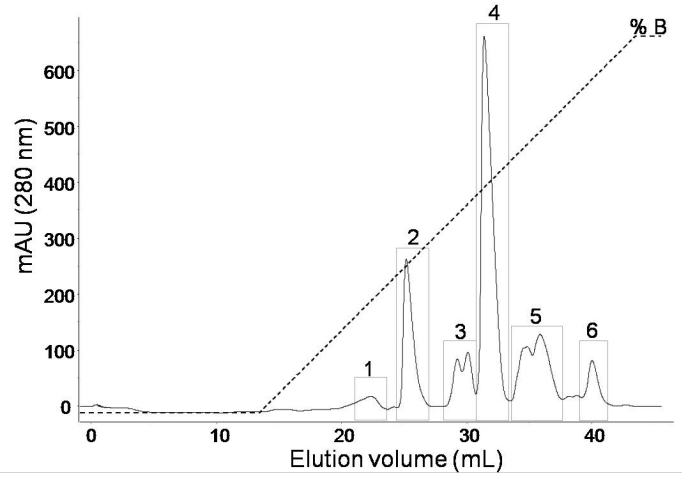

Figure 4. Hydrophobic interaction chromatogram of AEC fraction 3 (pooled fractions 3a and 3b). Collected fractions are indicated by numbered boxes. The dotted line indicates the linear gradient.

The bands of HIC fractions 3 and 4 were found to be almost pure when analysed by SDS-PAGE in reducing conditions (Fig. 5a). However, when the same samples were analysed in non-reducing conditions a variation of the electrophoretic patterns was noted.
In addition to a shift of the main bands to a slightly higher apparent molecular weight, a minor low mobility band of ≈ 52 kDa was detected when the gel was run under non-reducing conditions (fig. 5b). Similar bands of 50-52 kDa found by analysing the proteins present in the natural wine haze, and not directly in the grape juice as done here, where

identified by NanoLC-MS/MS as *Vitis vinifera* class IV chitinase (MARANGON *et al.*, 2011). However, in that study it was not clear the origin of these high MW chitinase bands, which could be artefacts produced during the extraction procedure (MARANGON *et al.*, 2011), but also the result of the protein aggregation leading to haze. In contrast, the 52 kDa bands here detected, which were obtained directly from the grape juice, are clearly due to the presence of disulphide-linked proteins, being present only when the fractions were not treated with a reducing agent (fig. 5b).

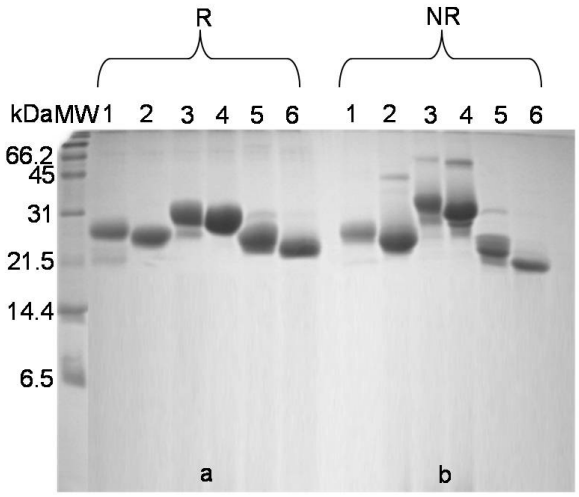

Figure 5. Reducing (a) and non-reducing (b) SDS-PAGE of the fractions (1 to 6) from hydrophobic interaction chromatography. Molecular weight standard proteins are on the left.

When tested for chitinolytic activity on gel (VINCENZI and CURIONI, 2005), peaks 3 and 4 confirmed to contain active chitinases corresponding to the bands with the MW expected for grape chitinases (CHI 1, CHI 2, CHI 3 and CHI not delayed) (in the range 30-32 kDa) but also to that of the disulphide linked components of ≈ 52 kDa (CHI dimer I and II) (Fig. 6).

In contrast the other chromatographic fraction did not show bands with well marked chitinolytic activity (not shown).

Confirming what was previously reported (VINCENZI and CURIONI, 2005), the presence of glycol chitin in the non-reducing SDS-PAGE gel caused a *Mr* decrease of the chitinase bands CHI 1, CHI 2, CHI 3 and this shift was proportional to the quantity of glycol chitin incorporated (Fig. 6). This result indicates that grape chitinases interact with the substrate during the electrophoretic migration if not reduced, likely due to the presence of the chitin-binding domain typical of the type IV chitinases (COLLINGE *et al.*, 1993). However, one minor chitinase isoform (CHI not delayed) did not show the same behaviour (Fig. 6), suggesting that the chitin-binding domain involved in the interaction with chitin was lacking in this component.

It is also interesting to note that the ≈ 52 kDa band was retarded in these conditions, showing the same behaviour of the main bands with higher *Mr*. This result and the disappearance of the ≈ 52 kDa band in reducing conditions suggest that this protein could be a dimer of chitinases linked by S-S bonds. As a matter of fact, all these bands, including that at ≈ 52 kDa, showed chitinolytic activity after staining the gels for its detection (Fig. 6).

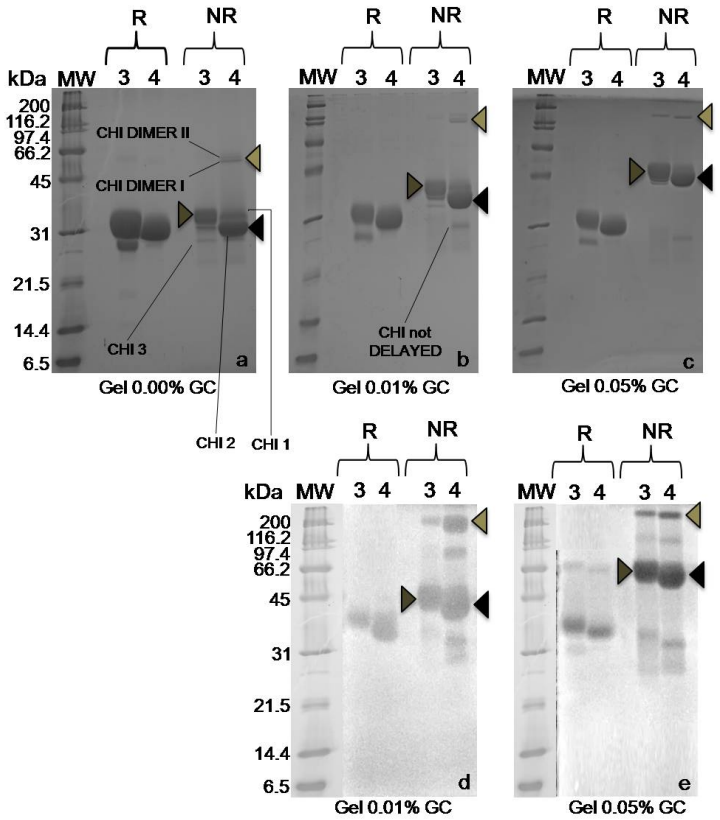

Figure 6. SDS-PAGE analysis of HIC fractions 3 and 4 under reducing (R) and non-reducing (NR) conditions. Gels contained 0 (a), 0.01 (b, d), and 0.05 (c, e) % glycol chitin (GC). Panels a-c: staining for proteins. Panel d-e: staining for chitinolytic activity. The arrowheads indicate bands retarded in the presence of glycol chitin. The bands selected for MALDI-TOF/TOF MS analysis are indicated in panels a and b. Molecular weight standard proteins are on the left.

3.3. MS protein identification

The six bands showing chitinolytic activity (CHI 1, 2 and 3, CHI dimers I and II, and CHI not delayed, Fig. 6) were excised from the SDS-PAGE gels and analysed by MALDI-TOF/TOF MS.

According to database searching using Mascot, all bands were found to belong to *Vitis vinifera* class IV chitinases, including that named "CHI not delayed". As mentioned above, these chitinases are characterised by the presence of a chitin binding domain of the hevein type in the N-terminal region (COLLINGE *et al.*, 1993), and this would be the reason for being retarded in glycol chitin containing gel (VINCENZI and CURIONI, 2005). Therefore, since the electrophoretic migration of the minor "CHI not delayed" band was unaffected by the presence of glycol chitin in the gel, it is likely that this grape chitinase has in common with the typical class IV chitinases only one part of its structure, but not the chitin binding domain.

In most of the cases the analysed proteins were assigned to two isoforms of class IV chitinases (accessions>gi|2306811|gb|AAB65776.1| and >gi|33329392|gb|AAQ10093.1|). Only for the band named 'CHI DIMER II' there was only one sequence matched (>gi|2306811|gb|AAB65776.1|), and three in the case of 'CHI 3' (Table 1).

Table 1. Selected proteins identified by MALDI-TOF/TOF MS.

Sample	Protein identification name	NCBI accession number	Sequence coverage (%)	Number of peptides matched
CHI 1	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	17%	5
	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	17%	5
CHI 2	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	20%	6
	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	20%	6
CHI 3	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	15%	5
	class IV endochitinase [*Vitis vinifera*]	>gil2306813lgblAAB65777l	15%	5
	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	15%	5
CHI not DELAYED	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	20%	6
	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	20%	6
CHI dimer I	class IV chitinase [*Vitis vinifera*]	>gil33329392lgblAAQ10093.1l	17%	5
	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	17%	5
CHI dimer II	class IV endochitinase [*Vitis vinifera*]	>gil2306811lgblAAB65776.1l	18%	5

Two reasonable hypotheses can be given to explain why bands with different electrophoretic and chromatographic behaviour are recognised as chitinases corresponding to the same isoforms: i) the MS data do not provide complete coverage of any sequence and therefore a precise identification of the proteins is not possible. In addition, because of the partial lack of available grape protein sequences, there is a chance that the selected peptides do not exactly match corresponding database entries; ii) the proteins could be modified forms of the same original chitinase isoforms, affecting only the chromatographic and electrophoretic behaviour but not the catalytic activity or the capacity to bind chitin. Indeed, it has been shown that some partial modification of the chitinases could occur during juice preparation (WATERS *et al.*, 1998; MANTEAU *et al.*, 2003; DAHIYA *et al.*, 2006). Overall, the results of the MS analysis are the same of those reported for the proteins found in natural wine haze (MARANGON *et al.* 2011), confirming that grape protein components related to type IV chitinases are actually those mainly involved in haze formation in wines.

4. CONCLUSIONS

Grape chitinases are considered as one of the main protein components involved in protein haze formation in wines (FALCONER *et al.*, 2010; MARANGON *et al.*, 2011). Here we have confirmed that these proteins are present in the grape juice as different isoforms, which, although sharing common amino acid sequences related to type IV chitinases, can be distinguished on the basis of their electrophoretic and chromatographic behaviours. Moreover, chitinases are present in the grape juice also in the form of S-S-linked dimers, and also in a form apparently lacking the chitin-binding domain. Since all these characteristics can be related to differences in the functional properties of the single components, it is likely that the different chitinase isoforms found in grape juice have different impacts on their hazing potential in wines, as it has been demonstrated for TLPs (GAZZOLA *et al.*, 2012; MARANGON *et al.*, 2014). In particular, differences in charge, hydrophobicity and also molecular weight can affect the interactions of the single chitinase components when they are present in a complex colloidal system as wine, leading to different tendencies to form haze. This can be an important point to be clarified, not only to better understand the mechanisms of wine hazing, but also for practical pourposes. For example, the identification of the most unstable protein components will help to develop protein instability tests much more specific than those currently in use, thus allowing the winemaker to be more precise in applying the wine stabilisation treatments. Moreover, also these treatments can be improved by a deep knowledge of the molecular characteristics and functionality of the single wine protein components, which will allow to design stabilisation treatments tailored to specifically remove the desired proteins and not the others. For example, the discovery that wine chitinases are able to bind chitin was the rational basis for the application of chitin as a specific adsorbent to remove these unstable proteins from wine (VINCENZI *et al.*, 2005). In conclusion, the biochemical and molecular characterisation of the different protein components of grape, as done here, can be of great help to develop "precision" winemaking techniques aimed to improve wine quality.

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PAPER

ASSESSING OIL OXIDATIVE STABILITY IN TARALLINI BY OXITEST®

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ABSTRACT

The shelf life of the typical Italian bakery snack "tarallini" depends on the recipe and on the cooking and storage conditions. In this work, the lipid oxidative stability of tarallini was measured using an OXITEST® instrument, an accelerated oxidation test. The OXITEST® methodology was optimised taking into account sample amount and the sample particle size.

Homemade tarallini prepared using sunflower oil, extra virgin olive oil and a blend of extra virgin olive oil and lard were cooked for two different cooking times. The results showed a good ability of OXITEST® to discriminate between lipid unsaturation and cooking time, providing information on the lipid shelf life of complex food matrices, such as tarallini.

Keywords: accelerated oxidation, bakery products, OXITEST®, shelf life, Tarallini

1. INTRODUCTION

Lipid oxidation is one of the main deteriorating reactions in food chemistry; food quality is deeply affected by lipid oxidation (CALLIGARIS *et al*., 2008). In particular, it damages lipids, especially essential fatty acids (CHOE and MIN, 2006), in fat-rich foods, such as bakery products, biscuits and snacks. Moreover, lipid oxidation is a promoter of off-flavours, producing the worst sensorial properties, reducing nutritional value and increasing the production of potentially toxic compounds (VERLEYEN *et al*., 2005). Thus, bakery product formulation must consider the characteristics of the used lipids, the matrix effect that influences their contact with oxygen and the technological treatment (cooking) that the products undergo.

Due to influence of food composition on shelf life, formulation can play an important role in food quality, even in terms of lipid oxidation. In fact, the interaction of lipid oxidation products with sugar, proteins and Maillard reaction products greatly affect the development of lipid rancidity in complex food (FRENKEL, 1984).

Moreover, different other factors (temperature, light, oxygen partial pressure, etc.) could affect and enhance lipid oxidation during storage. In particular, among them, partial oxygen pressure affect oxidation rate (KACYN *et al*. 1983): higher is the headspace partial oxygen pressure and higher is the amount of oxygen dissolved in food. Consequently, the oxygen available for lipid oxidation is increased and, in the dark, it will be enough to reach a value of peroxide value of 10mEqO_2/kg of fat (PRZYBYLSKI and ESKIN, 1988).

In addition, fatty acid composition, in particular fatty acid unsaturation degree, storage time and pro- and antioxidant compounds significantly affect the auto-oxidation rate (CALLIGARIS *et al*., 2008).

Briefly, when the oxidation occurs, fatty acids are converted at first into hydroperoxide, can then decompose to form volatile molecules, hydroxylated, keto- or epoxy- compounds or react with other oxidised fatty acids to form dimers or polymers.

Many different methods have been developed to assess these oxidation compounds in various food ingredients and products. GC analyses of volatiles compounds, free fatty acid or mono and diglicerides, HPLC evaluation of oxidised fatty acid or spectrophotometric determination of peroxide value, conjugated diene and trienes are the main traditional methods applied to asses lipid oxidation in foods (VERARDO *et al*., 2010; VERARDO *et al*., 2011)

However, the lipid oxidation rate is usually slow at room temperature and the rancidity threshold, which is strictly related to the consumer rejection of foods, could take months.

Moreover, oxidation products analyses are often time consuming and for the food industry it is very important to check as quickly as possible the food stability (MÀRQUEZ-RUIS *et al*., 2003).

Thus, excessively time-consuming shelf-life tests are useless for industry needs, and it is essential to apply methods that give quick answers (GÓMEZ-ALONSO *et al*., 2004).

An interesting way to reduce the time of analysis of lipid oxidation compounds is using accelerated oxidation tests, allowing foods lipid stability assessment in a significantly shorter time than under real storage conditions (PRZYBYLSKI and ESKIN, 1988).

Usually, in this type of test, one or more parameters (temperature, oxygen pressure, light, etc.) (WAN *et al*., 2000) that can increase the lipid oxidation rate are modulated, but the temperature is the most critical factor affecting the oxidation rate; thus, it is the most commonly considered (RAGNARSSON and LABUZA, 1977; LABUZA and SCHMIDT, 1985; WATERMAN and ADAMI, 2005).

Several tests were developed to evaluate accelerated lipid oxidation. In particular, most of them like Rancimat or Oxidative Stability Instrument (OSI) tests are suitable only for oils

or fat extracted from foods, but are not applicable to the whole food. In this way, however, the effect of food matrix on lipid oxidation onset cannot be considered.
A newer instrument, the OXITEST® reactor, has been used to asses oils lipid oxidation in several studies (AMATO *et al*., 2015; CAVAZZA *et al*., 2015; CLAUS *et al*., 2015; CLAUS *et al*., 2015 b; MORA *et al*., 2009; Mora *et al*., 2011) but it is particularly fitted to investigate the oxidation sensitivity also in solid foods (VERARDO *et al*., 2013; KWON *et al*. 2015), such as bakery products, with limited preparation and without any fat extraction (VELP SCIENTIFICA, 2006; MARUYAMA *et al*., 2014; FERREIRA SILVA *et al*., 2015).
The OXITEST® reactor subjects the sample to an oxidative stress environment at high temperature and high oxygen pressure; the drop in oxygen pressure inside the oxidation chambers is monitored according to ability of the food to oxidise and is expressed as the induction period (IP) which is theoretically defined as the time required to obtain a continuous oxidation cycle in the oxidation process; it is measured as the time required for a sudden and rapid change in the oxidation rate (FRENKEL, 1998).
Considering the importance of the rapid determination of lipid oxidation in foods as a marker of quality during shelf life, the aim of this work was to evaluate the performance of OXITEST® as a new screening instrument to assess lipid stability directly on bakery products, tarallini, a typical Italian snack. In particular, the sample amount, particle size, and formulations were setted in order to enhance the discriminating power of the technique.
Traditionally, tarallini are a typical southern Italian salted snack formulated with wheat flour, oil, water, salt and white wine. The oil used during its preparation plays the main role in the oxidative stability and thus shelf life of this snack; homemade tarallini is generally prepared using extra virgin olive oil (EVOO), but the industrial one could be formulated with sunflower oil (cheaper than EVOO) or with a blend of EVOO and LARD, more expensive but with longer shelf life (CAPONIO *et al*., 2009). The longer shelf life is mainly due lower susceptibility of fat blend to lipid oxidation, related to the fatty acids composition of fats and the presence of natural antioxidants in EVOO.
In addition, cooking time is critical for tarallini quality. In particular, cooking time should be optimised for a golden colour without negatively altering the crunchiness and the texture of the product.
In fact, a cooking time or a cooking temperature too high could led to a product extremely dry and with a darker colour compared to the typical one. Moreover, a longer cooking enhance the heat stress promoting lipid oxidation onset and reducing the shelf life of the snack.

2. MATERIALS AND METHODS

2.1. Solvents and reagents

All of the solvents were purchased from VWR International (Radnor, Pennsylvania, USA). The reagents were provided by Sigma Aldrich (St. Louis, MO, USA).

2.2. Samples

The analysed samples were Italian typical salted snacks ("tarallini") prepared following three different recipes (reported in Table 1):
Recipe I: Wheat flour, water, salt and sunflower oil.
Recipe II: Sunflower oil was replaced with extra virgin olive oil (EVOO).
Recipe III: A blend of EVOO and lard (4:1 w/w) was used as a fat source.

Table 1: Recipes of formulated tarallini.

Ingredients	SO tarallini	EVO Tarallini	EVO/LA Tarallini
Wheat flour (g)	1000	1000	1000
Water (mL)	350	350	350
Salt (g)	20	20	20
Oil (g)			
* Sunflower oil	200	0	0
* Extravirgin olive oil	0	200	160
* Lard	0	0	40

In all of the recipes, the dough was kneaded for 15 minutes, manually formed (~4 cm diameter, 0.7 cm thickness) and then oven cooked at 220°C for different times: 8 and 11 minutes to evaluate the ability of the instrument to detect minimal differences in thermal-induced oxidation of the products. The cooking times were chosen based on the typical commercial golden colour of the products (8 minutes), and giving more heat stress, compatibly with the snack's crunchiness (11 minutes).
The samples that were used as control to optimise the OXITEST® analytical conditions were commercial tarallini snacks purchased from a local supermarket in Cesena (Italy). The commercial products that were used for the analytical optimisation conditions were formulated with wheat flour, water, salt and a mixture of EVOO and lard.
The oxidation tests evaluation with OXITEST® was carried immediately after sample production.

2.3. Fatty acid analysis of fats used for the formulation of tarallini snacks

The fatty acid methyl esters (FAMEs) of oils used for the lab scale tarallini productions were identified after alkaline treatment, as described by CHRISTIE (1989), on a GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector (FID) according to the method of VERARDO *et al*. (2013a). Peaks were identified by comparing peak retention times with GLC-463 from Nu-Check (Elysian, MN, USA) and FAME 189-19 standard mixtures from Sigma Aldrich Chemicals (St. Louis, MO, USA).
The fatty acids were expressed as weight percentages of total FAME.

2.4. OXITEST® analysis optimisation

The method of analysis was optimised using commercial samples. The OXITEST® reactor (Velp Scientifica, Usmate, Milan, Italy) was fitted with two separate oxidation chambers at 90°C with an oxygen pressure of 6 bar, as indicated by VELP SCIENTIFICA (2006).
At first, the ground commercial sample, milled mechanically using a water-cooled mill (IKA-WERKE M20 mill, speed 20000 rpm, maximum particle size 6-7 mm) (TFG), was analysed in different amounts (10, 20 and 30 g) to determine the best compromises between quantity, time of analysis and repeatability. Each sample was analysed three times and monitored twice during each test (in chambers A and B). The results, expressed as IPs, were obtained using the two-tangent method.
After establishing the sample amount, an OXITEST® analysis was performed on commercial samples with different particle sizes: whole product (WT) and after mechanical (TFG) (using IKA-WERKE M20 mill) and manual (TCG) milling. Briefly, 10 g

of tarallini was oxidised as whole (WT) after milling five times for 30 seconds using a water-cooled mill (TFG) and after grinding manually in a mortar (TCG) to obtain bigger particles.
Inter-days tests were performed to evaluate the instrument repeatability with different types of samples. Thus, each type of sample was monitored twice by the OXITEST® reactor over five days. *Intra*-day tests were not allowed by the experiment duration (greater than ten hours).

2.5. OXITEST® analytical conditions

All of the analyses were carried out under the same conditions of temperature (90°C) and oxygen pressure (6 bar). After optimisation of analytical conditions, the amount of handmade tarallini that was analysed was 10 g. The samples were analysed immediately after production.

2.6. Statistical analysis

One-way (Tukey's honest significant difference multiple comparison) and multifactorial analyses of variance (ANOVA), were carried out to establish significant differences. All of the statistical tests were evaluated using Statistica 8.0 software (StatSoft, Tulsa, OK, USA), and p values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSIONS

3.1. Chemical composition of SO, EVOO and EVOO/LARD

The analysis of the oils used as raw materials showed a high amount of unsaturated fatty acids, as expected. In EVOO, the most abundant fatty acid was oleic acid (C18:1), 70.0%, followed by palmitic acid (C16:0, 13.7%) and linoleic acid (C18:2 9.2%).
Linoleic (C18:2, 59.0%), oleic (C18:1, 28.9%) and palmitic (C16:0, 6.4%) acids were also the main fatty acids in sunflower oil.
The fatty acid composition of the EVOO/LARD (4:1 w/w) blend closely reflected the composition of EVOO, with some changes in the percent distribution of each fatty acid. Oleic acid (C18:1) accounted for 63.4%, palmitic acid (C16:0) 15.4%, and linoleic (C18:2) and stearic (C18:0) acids 9.7% and 4.3%, respectively, of the total fatty acids. In small quantities, this blend also contained saturated medium chain fatty acids, such as lauric acid (C12:0, 0.02%) and myristic acid (C14:0, 0.28%), typical of lard, as shown in Table 2.
The oxidative status of the fats used during formulation was assessed by an OXITEST® reactor, at the same condition applied for tarallini analyses (90°C, 6 bar oxygen pressure) but weighting 5 g of products. The results showed that EVOO was characterised by a similar and not significantly different oxidative stability compared to EVOO/LARD blend (1371 vs 1408 minutes, respectively) but by a greater oxidative stability than the one of sunflower oil (699 minutes), confirming the results reported by COMANDINI *et al*. (2009).

3.2. Optimisation of the analytical parameters of the OXITEST® reactor

Preliminary tests performed on commercial tarallini were aimed to to optimise the conditions of analysis of bakery products by OXITEST®; in particular they were aimed to determine the amount of sample and particle size that allow the best repeatability. Thus, different quantities loaded into the reactor chambers were tested. Moreover, once decide

the best amount to load, trying to reach the most homogenous and repeatable contact with oxygen, the samples were analysed as whole (WT) or ground mechanically with a mill (TFG) or manually with a mortar (TCG).

Table 2: Fatty acid composition (%) of fats used in tarallini formulation.

	SO	EVOO	EVOO/LA
C12:0	0.00±0.00	0.00±0.00	0.02±0.00
C14:0	0.07±0.00	0.00±0.00	0.28±0.02
C16:0	6.41±0.01	13.70±0.09	15.38±0.01
C16:1t	0.02±0.00	0.13±0.04	0.16±0.01
C16:1c	0.11±0.00	1.13±0.03	1.30±0.00
C17:0	0.06±0.00	0.13±0.00	0.20±0.01
C17:1c	0.03±0.00	0.21±0.01	0.22±0.01
C18:0	3.25±0.02	2.03±0.02	4.32±0.07
C18:1	28.90±0.22	70.01±0.05	63.36±0.21
C18:2 tt	0.23±0.05	0.00±0.00	0.00±0.00
C18:2 n6	58.97±0.24	9.22±0.09	9.73±0.00
C18:3n6	0.06±0.01	0.07±0.01	0.10±0.03
C18:3n3	0.09±0.02	0.57±0.01	0.58±0.01
C20:0	0.24±0.02	0.39±0.01	0.37±0.00
C20:1	0.15±0.04	0.30±0.02	0.42±0.02
C20:2	0.00±0.00	0.00±0.00	0.13±0.01
C20:3n6	0.10±0.06	0.37±0.14	0.90±0.13
C22:0	0.71±0.01	0.14±0.00	0.10±0.00
C20:5+C22:1	0.00±0.00	0.06±0.03	0.11±0.02
C22:2	0.06±0.00	0.52±0.02	0.44±0.02
C22:3+C22:4	0.00±0.00	0.16±0.06	0.39±0.09
C24:0	0.33±0.03	0.39±0.15	0.69±0.02
C24:1	0.10±0.00	0.15±0.01	0.33±0.08
C22:5	0.14±0.01	0.33±0.02	0.46±0.05

Oxidising 30, 20 and 10 g of TFG, the IP values were 757, 873 and 788 minutes, respectively, and there were no significant differences in term of IPs between the three amounts tested (Table 3). Thus, in order to save the sample to use, 10 g was chose as the amount to use in the following trials. Thirty grams of sample was the maximum quantity per plate in a single oxidation chamber; a higher amount, in fact, needed to be placed on more than one, causing a not-reliable comparison between trials with smaller amounts. Lower amounts were not chosen because quantities below 10 g did not allow the total covering of the steel plate in the oxidation chamber, leading to a possible irregular oxygen distribution on the sample and showing a non-linearity of response.
After choosing the more repeatable amount of sample and testing the instrument repeatability, the instrument capacity of discrimination for different sample particles size was tested.

Table 3: IP Values of different amounts of tarallini.

Samples	IP values (minutes)
TFG 30 g	757±38[a]
TFG 20 g	873±48[a]
TFG 10 g	788±37[a]

TFG: Tarallini fine ground, ground mechanically; **TCG**: Tarallini coarse ground, ground by mortar; **WT**, Whole tarallini). Different letters indicate significantly differences between values ($p < 0.05$).

As reported in Table 4, statistically significantly differences in the IPs were observed between the two different types of milled products (TCG and TFG) and between WT and the TCG. These differences could be due to the initial heating effects that, related to the milling process, increase the susceptibility to oxidation in TFG with a decrease in the IP value compared to TGC. Moreover, for ground products, as smaller was the particles size (TFG respect to TCG) as higher was the susceptibility of the product to lipid oxidation, as reported by TAN *et al*. (2002)
As concerning WT, the IP value was not significantly different to the one of TFG but significantly different from TGC. However should be noticed that WT IP showed a high standard deviation suggesting that OXITEST® were less repeatable when used on whole product compared to the ground one; this can be probably connected to the lower sample homogeneity (e.g slightly different shape and thickness) of the whole product, that could affect interaction between oxygen an oil fraction.

Table 4: IP values of whole tarallini and tarallini ground differently.

Samples	IP values (minutes)
TFG 10 g	788±37[a]
TCG 10 g	902±54[b]
WT 10 g	788±168[a]

(TFG: Tarallini fine ground, ground mechanically; **TCG**: Tarallini coarse ground, ground by mortar; **WT**, Whole tarallini). Different letters indicate significantly differences between values ($p < 0.05$).

The results showed that the larger the particles (TCG compared to the same quantity of TFG saqmple) and thus the smaller the surface area-to-volume ratio exposed to oxygen, the higher the IP.
However, the above hypothesis is confirmed only by ground products because the results obtained by the oxidation of the whole sample are very changeable, probably due to the shape of tarallini, which could lead to a different exposure to oxygen and different oxidation rates among trial tests. The whole product showed an induction period very close to that milled by grinder (TFG) but was characterised by a higher variation between replicates.
The results of *inter*-day experiment showed a lower CV (4.1%) for TFG compared to TCF (7.5%) and WT (23.2%). These results suggest the product ground mechanically (TFG) as the one to choose for OXITEST® analysis; thus, handmade tarallini was ground by a water-cooled mill before analysis (TFG).

3.3. Evaluation of the oxidative stability of handmade tarallini

To assess the effect of food composition ("matrix effect") on lipid rancidity, handmade tarallini formulated with three different fat sources were oxidised by the OXITEST® reactor, testing the reliability of this instrument for the screening of the oxidative stability of baked snacks.
Comparing the IP values of tarallini formulated with different recipes, it can be noticed that the most oxidable products were sunflower oil tarallini snacks, showing the lowest IPs (274 and 599 minutes). A low IP means high oxidation susceptibility.
Increasing lipid saturation (EVOO and EVOO/LARD as fatty sources), the IPs increased, reaching values more than double (1254 and 1150 minutes for EVOO snacks and 1192 and 1100 minutes for EVOO/LA tarallini) compared to those of sunflower snacks. This trend may be associated with the fatty acid composition of the fat used: the higher degree of unsaturation of sunflower oil reduces the oxidative stability of the produced snacks (Table 5).

Table 5: Induction period (IPs) of handmade tarallini snacks cooked for 8 and 11 minutes and reduction of IPs by increasing cooking time.

Samples	8 minutes cooking	11 minutes cooking	Reduction of IPs
EVOO	1254 [a]	1150 [a,b]	8.3%
EVOO/LA	1192 [a,b]	1100 [b]	7.7%
SO	599 [c]	274 [d]	54.3%

Different letters mean significantly differences between values ($p < 0.05$)

To better explain these results, the unsaturation degree was expressed as the "unsaturation point" and calculated as the sum of the main fatty acids weighted by the number of double bounds of the fatty acid.
Sunflower oil had the highest unsaturation point (147), followed by those of EVOO (88.5) and the EVOO/LARD blend (82.8). Although the EVOO/LARD blend had a lower unsaturation point compared to that of EVOO, it showed greater susceptibility to oxidation. This is probably due to the blend's lower content of natural antioxidants, typical of EVOO, which protects the fat against oxidation.
In addition to lipid unsaturation degree, cooking time also played a significant role in lipid oxidation onset in tarallini snacks as reported in Tables 5 and 6.
Cooking for a longer time decreases the IPs of the samples analysed due to an increase in the lipid oxidation rate. The effect of cooking time was emphasised in the products characterised by the higher unsaturation degree (SO tarallini), with a significant reduction of IP values (274 vs 599 minutes).
Even if the IP values decreased with 11 minutes of cooking, significant differences were not observed in tarallini made with EVOO or EVOO/LA when cooking time was changed. Anyway, the highest IP value was shown by tarallini made of EVOO and cooked for 8 minutes (IP=1254 minutes). This result may indicate that the phenolic compounds naturally present in EVOO have a strong antioxidant activity that can help to prevent lipid oxidation. The IP values of EVOO tarallini were higher, even if not significant, also

compared to that of a more saturated lipid (EVOO/LA tarallini), confirming the report of HRNCIRIK and FRITSCHE (2005) in different EVOO samples.

Table 6: Multifactorial ANOVA (univariate results).

Source of Variation	Probability
Oil	***
Baking time	***
Oil x Baking time	*

$p < 0.05$; *** $p < 0.001$

Thus, the cooking heating effect caused a significant increase in the oxidation onset only in snacks formulated with highly oxidable oils, such as sunflower oil. In this case, comparing snacks formulated with the same fat but cooked for different times, it can be seen that even only 3 minutes of cooking changes the oxidation stability of the products, suggesting that heat effect plays a dominant role in the high unsaturated lipid oxidation onset.
The CV (all values below 5%, except for tarallini made with EVOO and cooked for 11 minutes, where the CV was 9%) for handmade snacks confirms the good repeatability of the OXITEST® reactor even when fats with different susceptibilities to oxidation were used for formulations.

4. CONCLUSIONS

Tarallini ground in different ways and with different particle sizes showed different behaviour during the accelerated oxidation process due to the different surface/volume ratio and, consequently, different contact with oxygen. These results highlight the necessity to perform an analysis on ground foods, especially when their shape is not homogeneous.
The OXITEST® instrument also showed good performance for the discrimination of different fat used in snacks formulations. Moreover, baking tarallini for different cooking times led to an ~50% reduction of IPs, highlighting the power of the OXITEST® to discriminate between products under different thermal stresses.
Thus, OXITEST®, different from other accelerated shelf life tests, is suitable for solid, liquid and doughy foods (VELP SCIENTIFICA, 2006).
The possibility of screening lipid stability to oxidation with good repeatability of results in complex foods, such as bakery products, avoiding lipid extractions and with the minimal preparation of the sample, suggest the OXITEST® instrument as a good option to save time in the preliminary evaluation of lipid oxidation. Moreover, this instrument considers the complexity of formulated foods giving more reliable results due to interactions between compounds that can be considered.
Nevertheless, more investigations are needed to study this accelerated oxidation test with other foodstuffs.

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ABBREVIATIONS

IP, Induction Period; OXITEST, Oxidation Test; EVOO, Extra virgin olive oil; SO, Sunflower oil; LA, Lard; TFG, Tarallini fine ground; TCG, Tarallini coarse ground; WT, Whole tarallini.

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PAPER

EFFECT OF THE SOIL NATURE ON SELECTED CHEMICO-PHYSICAL AND THERMAL PARAMETERS OF EXTRA VIRGIN OLIVE OILS FROM *CV* CHEMLALI

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ABSTRACT

The current study assessed the effect of different soils (clay, EVOOsC, stony, EVOOsS, brown, EVOOsB, limestone and gypsum, EVOOsLG) on the chemico-physical (free acidity, peroxide value, oxidative stability, fatty acids, pigments, colour, viscosity, heat capacity) and thermal (upon DSC) quality of four 'Chemlali' extra virgin olive oils. EVOOsC showed the lowest peroxide value, the highest amount of monounsaturated fatty acids and the narrowest crystallization range. EVOOsLG had the highest content of saturated and polyunsaturated fatty acids as well as the lowest stability to oxidation and melting enthalpy. EVOOsS and EVOOsB revealed the highest viscosity, while the heat capacity measurement didn't show any difference among the oils. EVOOsB exhibited the highest b^* colour parameter in relation to the highest carotenoids content. These preliminary findings have showed that the nature of the soil has an effect on the final quality of EVOO, as also revealed by the PCA analysis.

Keywords: composition, DSC, extra virgin olive oil, physical properties, soil

1. INTRODUCTION

EVOO is a traditional product of the Mediterranean countries. Among these the olive culture represents one of the main economic and agricultural strategic sectors in Tunisia. About 60 million olive trees are distributed and spread on 1.6 million hectares extended from the northern to the southern regions of the country. Tunisia contributes to more than 4% of the world olive oil production the 'Chemlali' being the most abundant olive variety (ISSAOUI *et al*., 2010). The chemical characterization of 'Chemlali' olive oils, as influenced by different either natural or human factors, was the object of several scientific studies (BEN HASSINE *et al*., 2014; HANNACHI *et al*., 2007; BEN HASSINE *et al*., 2015). Significant variability on fatty acids (FA) composition other than minor components such as polyphenols and/or pigments and oxidative stability was found.

Closely related to chemical composition, but less debated in literature, is the physical and thermal characterization of the oils, which could be considered of great interest for consumers and industries. The use of differential scanning calorimetry (DSC) has been mainly suggested as an alternative interesting approach for the characterization of the oils from different vegetable sources (TAN and CHE MAN, 2002) or for the evaluation of different oxidative stability (CHIAVARO *et al*., 2009; CHIAVARO *et al*., 2011; CHIAVARO *et al*., 2012) by means of the phase transition behaviour. Recently, DSC has also been applied for the characterization of Tunisian olive oils on the basis of the cultivar–environment interaction showing good correlations between thermal and chemical properties(KOTTI *et al*., 2009).

DSC can be also used for the determination of the heat capacity (Cp). SANTOS *et al*. (2005) reported an increase in the Cp of vegetable oils, measured by DSC, as a function of the fatty acids saturation. The measurement of viscosity along with the heat capacity provides useful information to optimize the equipment design (settling and storage tanks, centrifuges, pumps, etc.) thanks to the determination of their behaviour during different technological processes. The correlation between viscosity and chemical composition of vegetable oils was slightly mentioned only when, after prolonged heating, an increasing of the viscosity was observed in relation with the increased saturation of the oil constituents and the generation of different polymer compound classes (KALOGIANNI *et al*., 2011).

The colour is a basic criterion affecting the consumer preference although the European Union regulations do not require its measurement for an assessment of the virgin olive oil quality. Olive fruits contain two main classes of pigments that are transferred to the virgin olive oil during the extraction process: the green chlorophylls and the yellow and orange carotenoids. They can be used as indicator of the olives genetic make-up other than on the habitat where the olive trees are grown(CERRETANI *et al*., 2008).

It is well-known that the unique nutritional and organoleptic properties of the olive oil are closely related to olives genotype other than agronomic, environmental and technological factors (GARCÍA-GONZÁLEZ *et al*., 2010). Among these factors, the effect of the soil nature on the chemical composition of the oils is barely debated and often just regarded to as a combined effect with other factors (BEDBABIS *et al*., 2015; PAPADIA *et al*., 2011; ROMERO *et al*., 2015). Moreover, to the authors' best knowledge, no study on the impact of the soil nature on physical and thermal properties of olive oils has been reported in literature so far. This literature gap has led us to perform a complete characterization, in terms of composition (fatty acids, quality parameters, main pigments), oxidative stability, physical (viscosity, colour, heat capacity) and thermal properties, of four monovarietal 'Chemlali' EVOOs, differing only in the nature of the cultivation soils being cultivated in the same area under the same agricultural conditions and harvested in the same season at the same ripening index.

2. MATERIALS AND METHODS

2.1. Oil samples preparations

EVOO samples were obtained from fruits of the main Tunisian olive cultivar, 'Chemlali', which were picked by hand at the same stage of maturity, according to the IOOC (2004) classification, from three trees, during the crop season 2012/ 2013 (October), placed in 4 locations with different soil nature: clay (EVOOsC), stony (EVOOsS), brown (EVOOsB), limestone and gypsum (EVOOsLG), located in Sousse center of Tunisia (35°.49′ N, 10°.30′ E). Olive trees were subjected to identical fertilisation regime and to all common olive cultivation practices. The same laboratory mill was used to prepare the olive oil samples. Only healthy fruits without any kind of infection or physical damage were processed. After harvesting, fresh olives (1.5-2.0 kg) were washed and defoliated, smashed with a hammer crusher and then paste mixed at 25°C for 30 min, centrifuged without addition of warm water (oil produced from each extraction was 200-250 mL/kg) and then transferred into dark glass bottles and stored in the dark at 4°C until analysis. Three samples of each oil were analysed and triplicate analyses were carried out for each sample.

2.2. Chemical analysis

Free acidity, expressed in oleic acid (18:1) percentage, and peroxide value (POV), given as milli-equivalents of active oxygen per kilogram of oil ($meqO_2/kg$) were determined according to the analytical methods described in the European Union Commission Regulations (EEC/2568/91; EEC/1429/92).
Oxidative stability (OSI) was evaluated by the Rancimat method (GUTIÉRREZ ROSALES *et al*., 1989). Stability was expressed as the oxidation induction time (h), measured with the Rancimat 743 apparatus (Metrohm, Herisau Switzerland), using an oil sample of 3.6 g. The oil temperature was 101.6°C and the air flow was 10 L/h.
Chlorophyll and carotenoid contents were determined colorimetrically, as previously described (MINGUEZ-MOSQUERA *et al*., 1991). Briefly, the method consists in quantitatively assessing the chlorophyll fraction by measuring the absorbance of the olive oils at 670 nm and the carotenoid fraction at 470 nm through the use of appropriate molar absorption coefficients. The results were expressed in g/kg.
The fatty acids (FA) were converted to fatty acid methyl esters before analysis by shaking a solution of 0.2 g oil and 3 mL of hexane with 0.4 mL of 2-N methanolic potassium hydroxide, and analyzed using a Hewlett-Packard (HP 4890D; Hewlett-Packar Company, Wilmington, DE) chromatograph equipped with a capillary column (Supelcowax: 30 m × 0.53 mm; 0.25 mm), a split/splitless injector and a flame ionization detection (FID) detector. The carrier gas was nitrogen at a flow rate of 1 mL/min. The temperatures of the injector, the detector and the oven were held at 220, 250 and 210°C, respectively. The injection volume was 1 μL. The results are expressed as relative area percentage of total FAs.

2.3. Thermal analysis

EVOO samples (8-10 mg) were weighed into aluminium pans, covers were sealed into place and the whole analyzed with a DSC Q100 (TA Instruments, New Castle, DE). Indium (melting temperature 156.6°C, ΔH_f= 28.45 J/g) and *n*-dodecane (melting temperature -9.65°C, ΔH_f= 216.73 J/g) were used to calibrate the instrument and an empty pan was used as reference. Oil samples were equilibrated at 30°C for 8 min and then cooled at -80°C at the rate of 2°C/min, equilibrated at -80°C for 8 min and then heated

from -80 to 30°C at 2°C/min. Dry nitrogen was purged in the DSC cell at 50 cm^3/min. DSC curves were analysed with Universal Analysis Software (Version 3.9A, TA Instruments) to obtain enthalpy change for transition (ΔH, J/g), onset temperature of transition (Ton,°C), offset temperature of transition (Toff,°C) and peak temperature at the maximum (Tp) for the two main events of cooling and heating transitions (Tp1 and Tp2,°C). Range of transition was calculated as the temperature difference between Ton and Toff. Heat flow (W/g) of the main peak (p1) of both cooling and heating was also calculated.
Heat capacity (Cp, J/g°C) measurements were taken with a DSC Q100 (TA Instruments, New Castle, DE). Each determination was performed in triplicate according to the combination of two methods (ASTM E1269-05; HEIDENREICH *et al*., 2007). According to the method (Minguez-Mosquera *et al*., 1991) three individual DSC experiments referred to as: baseline, reference, and sample were performed using the same procedure: equilibration at 5°C; isothermal for 15 min; ramp at 5°C/min up to 300°C; isothermal for 20 min. For determination of the heat capacity calibration constant E(T), the heat capacity of the sapphire ($c_{p,s}$), the mass of the sapphire (m_s) and the temperature-dependent heat flow of sapphire (Q_s) and of the empty pan (Q_E) were considered in Eq.1.

$$E(T) = \frac{c_{p,s}\dot{q}m_s}{60[\dot{Q}_S(T) - \dot{Q}_E(T)]} \qquad \text{Eq. 1}$$

The constant E(T) was then used to calculate the heat capacity of the sample by using the appropriate heat flow and mass (Q_c and m_c, respectively).

$$c_p = \frac{60E(T)[\dot{Q}_c(T) - \dot{Q}_E(T)]}{\dot{q}m_c} \qquad \text{Eq. 2}$$

2.4. Viscosity measurements

Measurements were made by means of a concentric cylinder Brookfield® DV-I Prime rotational viscosimeter (Brookfield, Middleboro, Massachusetts, USA). Data capture was obtained connecting the viscosimeter to a computer, monitoring rotation per minutes (RPM) and the apparent viscosity (Centipoise) at 1s intervals. Control of temperature was obtained connecting the jacket of the measuring cell to a water bath whose temperature was checked at 25°C±0.5. Two cylindrical spindles were used for the rheological analyses. The ULA spindle (Brookfield, Middleboro, MA, USA) (viscosity range from 0.06 to 2000 mPa s) and the SC4-18/13R spindle (Brookfield, Middleboro, MA, USA)(viscosity range from 0.3 to 9998 mPa s). Rheological behaviour was described in terms of viscosity (mPa s) at various shear rates (dv/dy). Since the apparatus measures the viscosity as a function of spindle RPM, shear rate values (1/s) were obtained multiplying RPM by a specific constant for every spindle (e.g.: 1.224 for Ultra Low Adapter, and 1.32 for the spindle SC4-18 of the Small Sample Adapter). The viscosity (μ) value was obtained from the Newton's law

$$\sigma = \mu\dot{\gamma} \qquad \text{Eq. 3}$$

where σ is shear stress (mPa), $\dot{\gamma}$ is the shear rate (1/s) and μ is viscosity (mPa s).

2.5. Instrumental colour

The software ImageJ, v.1.38x, fitted with the plugin Color Inspector 3D v. 2.3, was used to assess the oil colour applying the CIELAB colorimetric system. Each time 20 ml of samples

were put into a glass Petri dish. The images of each Petri dish were acquired with a scanner (Hewlett Packard, Palo Alto, CA, USA) at 600 dots per inch (dpi). The colour brightness coordinate L^* measures the whiteness value of a colour and ranges from black at 0 to white at 100. The chromaticity coordinate a^* measures red when positive and green when negative, and chromaticity coordinate b^* measures yellow when positive and blue when negative (MOYANO *et al*., 2008).

2.6. Statistical analysis

Means and standard deviations were calculated with SPSS (version 22.0 SPSS Inc., Chicago, IL, USA) statistical software. SPSS was used to perform one-way analysis of variance (ANOVA) and Tukey's honest significant difference test (HSD) at a 95% confidence level ($p<0.05$) to identify differences among samples. Pearson correlation coefficients were calculated among the variables at a 95% and 99% confidence levels ($p<0.05$ and $p<0.01$). Principal component analysis (PCA) was also performed by means of Statistica software (version 8.0, Stat-Soft, Tulsa, OK, USA). PCA has been used as descriptive statistical technique plotting the selected vectors (independent variables) versus all cases (samples) with the aim to find relationships among the variables, able to describe differences among the four cases.

3. RESULTS AND DISCUSSION

3.1. Chemical analysis

3.1.1 Quality parameters, oxidative stability and main pigment content

Chemical quality parameters and oxidative stability of the four samples are shown in Table 1.

Table 1. Chemical quality parameters of the EVOO samples.

	EVOOsC	EVOOsS	EVOOsB	EVOOsLG
Free acidity (%)	0.70 ± 1.00^{a}	0.75 ± 0.05^{a}	0.55 ± 0.15^{a}	0.70 ± 0.01^{a}
POV ($meqO_2$/Kg)	2.5 ± 0.50^{c}	4.5 ± 0.51^{b}	4.5 ± 0.50^{b}	10.5 ± 0.49^{a}
OSI (h)	15.58 ± 0.41^{b}	14.84 ± 0.15^{c}	18.01 ± 0.16^{a}	3.34 ± 0.27^{d}
Chlorophylls (mg/kg)	3.48 ± 0.09^{b}	3.01 ± 0.22^{b}	4.32 ± 0.10^{a}	4.68 ± 0.44^{a}
Carotenoids (mg/kg)	1.27 ± 0.00^{b}	1.20 ± 0.11^{b}	1.83 ± 0.02^{a}	1.60 ± 0.12^{a}

Data are expressed as mean±standard deviation of three determinations ($n = 3$, $p<0.05$). Different letters in the same row are statistically different ($p<0.05$). EVOOsC, extra virgin olive oil from soil clay, EVOOsS extra virgin olive oil from soil stony, EVOOsB, extra virgin olive oil from soil brown, EVOOsLG extra virgin olive oil from soil limestone and gypsum.

The free acidity value (5.5-7.5 g/kg) was lower than the legal limit for the commercial category of extra virgin olive oil in all the samples (ECC/61/2011) and it does not seem to be affected by the nature of soil. Similarly other authors didn't find any difference in the free acidity on 'Chemlali' olive oils obtained from olives cultivated on soils irrigated with

different quality waters (BEDBABIS *et al*., 2015) or from different geographical origins (BEN HASSINE *et al*., 2014). The peroxide value (POV), on the other hand, showed (Table 1) significant differences among the four 'Chemlali' EVOO according to the nature of the soil. Among all the samples, EVOOsC showed the lowest POV value (2.5 meqO_2/kg), while EVOOsLG had the highest one (10.5 meqO_2/kg) (Table 1); however they were all below the legal limit of 20 meqO_2/kg of oil for extra virgin olive oil (EEC/2568/91). Contradictory results were previously reported in literature according to this quality parameter in EVOO. BEDABIS *et al*. (2015) didn't find any difference among 'Chemlali' EVOOs obtained from olives irrigated with different types of water. Other authors (ISSAOUI *et al*., 2010; BEN HASSINE *et al*., 2014) found differences comparing 'Chemlali' olive oils belonging to different geographical sites in Tunisia. The oxidative stability (OSI) of the 'Chemlali' samples turned out to be influenced by pedologic conditions as well significant differences among the four oils (Table 1) have been observed and EVOOsB being the most stable (18 h). In accordance with the highest POV values EVOOsLG reported the lowest oxidative stability (3h). In general, the oxidative status and the stability to the oxidation of an olive oil depend on the oil composition, both in terms of lipids profile and micro-components (BEN MANSOUR *et al*., 2015). The oil composition is demonstrated to be itself affected by several environmental factors (ISSAOUI *et al*., 2010; BEN HASSINE *et al*., 2014; HANNACHI *et al*., 2007), including the soil nature (PAPADIA *et al*., 2011).
As shown in Table 1, the oils pigments content seems to be influenced by the nature of the soils, showing significant differences among the samples. The chlorophyll content was in the range 3.0-4.7 mg/kg with EVOOsLG and EVOOsS as the richest and the poorest samples, respectively. The same trend was observed for the carotenoids, being however in the concentration range of 1.2-1.8 mg/kg (Table 1). Several authors reported different pigments concentration in 'Chemlali' olive oils as affected by the growing area in Tunisia (ISSAOUI *et al*., 2010; BEN HASSINE *et al*., 2014; BEN MANSOUR *et al*., 2015) being however in the same order of magnitude of this study. BEDBABIS *et al*. (2015) reported different pigments contents in 'Chemlali' EVOOs obtained from olives irrigated with different types of water. These authors outlined that the pigments content of the olive oils is influenced by the olives ripening, being the latter itself influenced by the salinity of the soil.

3.1.2 Fatty acid composition

The fatty acids (FA) composition of the four 'Chemlali' EVOO is shown in Table 2.
Oleic, linoleic and palmitic acids were the main FA present in the samples being oleic the main abundant compound. The FA distribution was within the range expected for high quality olive oils ECC/61/2011. The FA composition found in this study traced the ones found for extravirgin 'Chemlali' oils from other authors (ISSAOUI *et al*., 2010; BEN HASSINE *et al*., 2015; BEN MANSOUR *et al*., 2015), with differences attributable to the environmental factors. As summarized in Table 2, EVOOsLG exhibited the lowest C18:1 content (471.6 g/kg) and the highest amount of C16:0 and C18:2 (206.6 and 256.4 g/kg, respectively). On the contrary, EVOOsC showed significantly highest content of C18:1(610.5 g/kg) and lowest of C16:0 and C18:2 (180.6, 158 g/kg, respectively). Stearic acid content did not show a significant difference among samples, showing concentration around 20 g/kg. EVOOsS and EVOOsB exhibited a very similar composition and intermediate between the ones of the other two oils. While EVOOsLG exhibited the highest amount of SFA and PUFA other than the lowest amount of MUFA, EVOOsC had the highest content of this fatty acid category, among all. In particular, MUFA correlated positively with OSI ($p \leq 0.01$, $R=0.738$) and negatively with POV ($p \leq 0.01$, $R= -0.922$),

inversely PUFA correlated positively with POV ($p \leq 0.01$, $R=0.948$) and negatively with OSI ($p \leq 0.01$, $R= -0.780$). Similarly, BEN HASSINE *et al*. (2015) associated the oxidative stability of an olive oil to the value of the oleic/ linoleic acids ratio. Interestingly, PAPADIA *et al*., (2011) found significant correlations between the amount of oleic and linoleic acids in mono-varietal extra virgin olive oils and the concentration of B, Cr, Mn and Zn in the cultivation soil.

Table 2. Main fatty acid composition (%) of the EVOO samples.

	EVOOsC	EVOOsS	EVOOsB	EVOOsLG
C16:0	18.06±0.52^{c}	20.40±0.30^{ab}	19.77±0.02^{b}	21.07±0.43^{a}
C16:1	2.27±0.07^{c}	3.13±0.11^{ab}	2.88±0.01^{b}	3.40±0.16^{a}
C18:0	1.99±0.06^{a}	2.00±0.07^{a}	1.99±0.02^{a}	1.91±0.05^{a}
C18:1	61.05±0.53^{a}	53.66±0.30^{b}	53.65±0.08^{b}	47.16±0.75^{c}
C18:2	15.80±0.05^{d}	20.14±0.07^{c}	20.66±0.08^{b}	25.64±0.20^{a}
C18:3	0.82±0.02^{a}	0.67±0.02^{b}	0.85±0.03^{a}	0.82±0.01^{a}
SFA	20.05±0.52^{c}	22.40±0.24^{ab}	21.95±0.02^{b}	22.98±0.38^{a}
MUFA	63.32±0.46^{a}	56.79±0.19^{b}	56.53±0.08^{b}	50.57±0.59^{c}
PUFA	16.63±0.07^{d}	20.80±0.08^{c}	21.51±0.11^{b}	26.45±0.21^{a}

Data are expressed as mean ±standard deviation of three determinations ($n = 3$, $p<0.05$). EVOOsC, extra virgin olive oil from soil clay, EVOOsS extra virgin olive oil from soil stony, EVOOsB, extra virgin olive oil from soil brown, EVOOsLG extra virgin olive oil from soil limestone and gypsum. FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturatedfatty acids. (C16:0) palmitic, (C16:1) palmitoleic, (C18:0) stearic, (C18:1) oleic, (C18:2) linoleic, (C18:3) linolenic.

3.2. Thermal analysis

3.2.1 Cooling curves

The DSC cooling curves of the four 'Chemlali' EVOO (Fig. 1a) were similar to that previously reported for extra virgin olive oils (CHIAVARO *et al*., 2010) and those of 'Chemlali' samples in particular (KOTTI *et al*., 2009).
The DSC cooling curves of the olive oils are well known to be simply influenced by the chemical composition of the oils (TAN and CHE MAN, 2000). All the cooling profiles (Fig. 1a) showed two well distinguishable exothermic events. In all the samples, the major event (Peak 1 of Fig. 1a), at lower temperature (~ -45°C), showed a symmetrical line shape, indicating a highly cooperative and more ordered transition due to the homogeneity of the crystallizing molecules; the minor exothermic transition (Peak 2 of Fig. 1a), (~ -10°C) showed an asymmetrical line shape, suggesting a more heterogeneous crystallization process (TAN and CHE MAN, 2000). The major exothermic event was previously attributed to the crystallization of TAG rich in oleic acid, while the other was attributed to the crystallization of the more saturated TAG (TAN and CHE MAN, 2000; TAN and CHE MAN, 2002; CHIAVARO *et al*., 2010). Besides, a less defined exothermic event was also quite evident appearing as a shoulder of the major peak.

The thermal parameters extrapolated from the cooling thermograms (Table 3) exhibited differences among the four studied EVOOs, probably attributable to the nature of the soil on which they were grown.

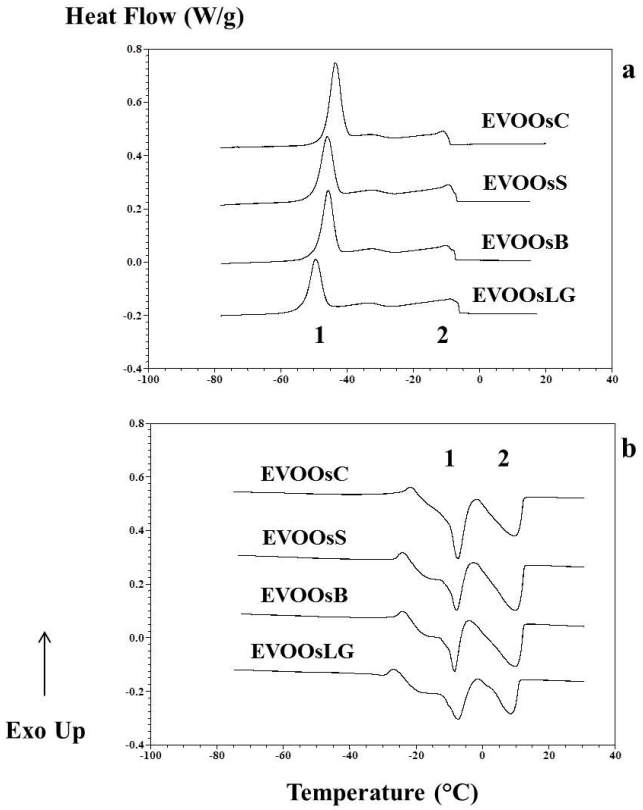

Figure 1. Cooling a) and heating b) curves of the EVOO samples. The main transitions are indicated with number 1 and 2. EVOOsC, extra virgin olive oil from soil clay; EVOOsS extra virgin olive oil from soil stony; EVOOsB, extra virgin olive oil from soil brown; EVOOsLG, extra virgin olive oil from soil limestone and gypsum.

Very different values were found for EVOOsC and EVOOsLG, while similar parameters were registered for EVOOsB and EVOOsS, intermediate from the other two. For EVOOsC, the major exothermal peak (Fig. 1a-Peak 1) resulted as the tallest among all the samples, as visible from the significant highest heat flow (Table 3). This observation could be related to the highest content of MUFA (CHIAVARO *et al*., 2010), in particular oleic acid, of this olive oil (Table 2). Moreover, it is reported that olive oils rich in oleic acid and MUFA, crystallized at lower temperature than samples rich in palmitic acid and SFA (CHIAVARO *et al*., 2010), as confirmed from our results (Tables 2-3). The minor exothermal event (Fig. 1a-peak 2) of EVOOsLG was peaked at higher temperatures (Table 3) than the other oils. This may be related to the significantly higher degree of lipid saturation (SFA content) of EVOOsLG (Table 2), as already reported for olive oil (CHIAVARO *et al*., 2010; KOTTI *et al*., 2009). EVOOsLG and EVOOsC exhibited respectively the broadest and the narrowest range of transition (Table3). Narrow range of transition has been associated to high oleic virgin olive oil (JIMENEZ MARQUEZ *et al*., 2003). About this, a high statistical correlation was found between the oleic acid content and the range of crystallization ($p \leq 0.01$; $R = -0.964$). Moreover high statistical correlations were also found between the range of crystallization and oxidative status of the oils by means of peroxide values ($p \leq 0.01$; $R = -0.912$) and oxidative stability ($p \leq 0.01$; $R = 0.797$). VITTADINI *et al*. (2003) reported that the

broadening of the crystallization phenomena is one of the changes related to the formation of lipid oxidation products that could form mixed and disordered triglyceride crystals. These crystals require lower energy to crystallize, and retard the occurrence of phase transition. The crystallization enthalpy on the other hand was not able to discriminate among the four EVOO according to the nature of the soil (Tab.3), as already observed for different 'Chemlali' EVOO from different geographical origin (KOTTI *et al*., 2009).

Table 3. DSC data obtained from the cooling and heating thermograms of the different samples.

	Enthalpy (J/g)	T_{on} (C°)	T_{off} (C°)	Range (C°)	Heat Flow (W/g)	T_{p1} (°C)	T_{p2} (°C)
Cooling							
EVOOsC	73.46±0.34 a	-8.83±0.32 c	-52.95±0.67 a	44.12±0.83 c	0.37±0.00 a	-43.84±0.45 a	-11.36±0.07 c
EVOOsS	74.72±2.38 a	-6.79±0.14 b	-56.79±0.58 b	50.00±0.67 b	0.32±0.02 bc	-46.38±0.45 b	-9.60±0.51 b
EVOOsB	77.52±1.81 a	-7.21±0.00 b	-55.70±0.29 b	48.49±0.29 b	0.36±0.02 ab	-45.51±0.34 b	-10.12±0.16 b
EVOOsLG	75.30±4.30 a	-5.88±0.08 a	-60.77±1.00 c	54.89±1.00 a	0.28±0.01 c	-49.67±0.29 c	-8.79±0.09 a
Heating							
EVOOsC	83.78±1.56 a	-24.75±0.22 a	13.31±0.08 a	38.06±0.17 c	0.30±0.01 c	-7.11±0.63 a	9.75±0.07 a
EVOOsS	76.20±3.82 ab	-27.31±0.22 b	13.07±0.16 a	40.38±0.25 b	0.25±0.01 ab	-7.32±0.79 ab	9.81±0.09 a
EVOOsB	79.54±2.08 a	-27.26±0.14 b	12.74±0.08 b	40.00±0.08 b	0.28±0.01 bc	-8.49±0.14 b	9.90±0.08 a
EVOOsLG	70.76±4.23 c	-30.25±0.14 c	11.79±0.16 c	42.04±0.21 a	0.24±0.01 a	-7.11±0.25 a	8.52±0.08 b

Data are expressed as mean±standard deviation of three determinations (n = 3, $p<0.05$). Same letters within each column do not significantly differ. Range(C°): Temperature difference between T_{on} and T_{off}; Heat Flow (W/g): value measured on the peak 1. EVOOsC, extra virgin olive oil from soil clay, EVOOsS extra virgin olive oil from soil stony, EVOOsB, extra virgin olive oil from soil brown, EVOOsLG extra virgin olive oil from soil limestone and gypsum.

3.2.2 Heating curves

The heating curves of the four EVOO (Fig. 1b) showed shapes similar to those reported in literature for 'Chemlali' samples (KOTTI *et al*., 2009). The thermal properties during heating were previously found to be largely influenced by difference in macro-components (FA) (CHIAVARO *et al*., 2009), despite those curves appeared to be more complex than cooling ones, due to the polymorphism of oils and fats (TAN and CHE MAN, 2000). Multiple exo-endothermic events were observed for all the studied samples (Fig. 1b). A first exothermic peak, visible around -25°C could be attributed to the crystallization of the minor components that did not solidify under the cooling regime used in this study and/or to a solid–solid polymorphic transformation resulting from the rearrangement of a portion of the crystals formed during cooling (BARBA *et al*., 2013). The following increase of temperature caused two major endothermic events: the melting of the unsaturated components (PUFA, MUFA) (Fig. 1b-peak1), at lower temperatures (~ -7.5°C), followed by that of the SFA ones (Fig. 1b-peak2), at higher temperatures (~ 9.5°C), as previously reported (JIMENEZ MARQUEZ *et al*., 2013). Peak 1 exhibited a quite different profile between the samples (Fig. 1b). It appeared more symmetric for EVOOsC,

probably in relation to the highest content of oleic acid and thus, to a more cooperative transition. The presence of a small endothermic event, as a shoulder of the major endothermic peak, was also observed in all the samples (~ -20°C), and with a lower intensity for EVOosC. It is generically attributed to the melting of lowest stables polymorphic forms of TAG (e.g. α) (BARBA *et al*., 2013).
As shown in Table 3, the thermal parameters of heating thermograms showed significant difference among the studied 'Chemlali' EVOO, according to the different nature of the soils. Heating enthalpy values were significantly different among samples; EVOOsC exhibited the highest value and EVOOsLG showed the lowest one. Positive correlations between heating enthalpy and oleic acid were found ($p \leq 0.01$; $R = 0.829$) in this study, as also previously observed on EVOO (ILYASOGLU *et al*., 2011). EVOOsC exhibited the highest and EVOOsLG the lowest T_{on} of heating. EVOOsLG moreover had a significant lowest T_{off} than the other oils, in relation to the high instauration of its FA profile, as previously suggested (KOTTI *et al*., 2009). Finally, EVOOsB and EVOOsS presented similar values for mostly of the heating thermal properties, probably in relation with the similarity shown in their chemical composition.

3.2.3 Heat capacity

The variation of the heat capacity as a function of temperature, in the range 26.85 – 296.85°C, is reported in Fig. 2 for all the samples.

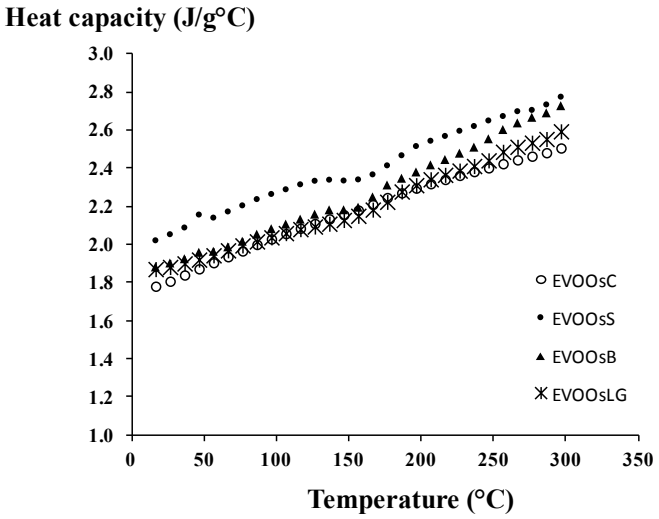

Figure 2. Heat capacity as a function of temperature (16.85 – 296.85°C) for the four EVOO samples. EVOOsC, extra virgin olive oil from soil clay; EVOOsS extra virgin olive oil from soil stony; EVOOsB, extra virgin olive oil from soil brown; EVOOsLG, extra virgin olive oil from soil limestone and gypsum.

The values obtained at 50°C were in accordance with those reported in literature for olive oil and calculated with the same method (1.99 ± 0.02)(HEIDENREICH *et al*., 2007). A linear increase of the heat capacity was observed with temperature, showing coefficient of linearity (R^2) of 0.9943, 0.9876, 0.9894 and 0.9905 for EVOOsC, EVOOsS, EVOOsB and EVOOsLG respectively.
The values obtained, by means of interpolation, at room temperature (25°C) are summarized in Table 4 ranging from 2.1 to 1.8 (J/gC°) for EVOOsS and EVOOsC

respectively, without any significant differences. Thus, the measurement of the heat capacity seems to not discriminate among the EVOO according to the nature of the soil.

3.3. Physical analysis

3.3.1 Viscosity

Edible oils are almost all Newtonian liquids (KALOGIANNI *et al*., 2011). The linear relationship of shear stress to shear rate found in this study (data not shown) indicates that all the vegetable oil samples in the tested shear rate conditions exhibited a Newtonian behaviour. The viscosity value at room temperature (25°C) was therefore obtained from the fitting of the slope of experimental shear stress-shear rate data to the Newton's law of viscosity equation (Eq. 1)(FASINA *et al*., 2008) and reported in Table 4.

Table 4. Physical quality parameters of EVOO samples.

	EVOOsC	EVOOsS	EVOOsB	EVOOsLG
Viscosity (mPa s)	59.21±0.12^{b}	61.58±0.24^{a}	61.79±0.19^{a}	59.24±0.12^{b}
Heat capacity (J/gC°)	1.81±0.07^{a}	2.06±0.18^{a}	1.89±0.07^{a}	1.88±0.09^{a}
Colour				
*L**	57.67±1.15^{a}	57.67±0.58^{a}	56.00±0.00^{a}	57.67±0.58 ^{a}
*a**	-3.67±0.58^{a}	-5.00±0.00^{b}	-5.33±0.61^{b}	-3.33±0.59^{a}
*b**	14.33±1.53^{c}	21.00±0.00^{b}	41.0±2.65^{a}	14.0±1.73^{c}

Data are expressed as mean standard deviation of three determinations. Different letters in the same row are statistically different ($p<0.05$). EVOOsC, extra virgin olive oil from soil clay, EVOOsS extra virgin olive oil from soil stony, EVOOsB, extra virgin olive oil from soil brown, EVOOsLG extra virgin olive oil from soil limestone and gypsum.

The values of viscosity found in this study are in accordance to that reported from other authors (GILA *et al*., 2015; BONNET *et al*., 2011) on virgin olive oils at room temperature. EVOOsB and EVOOsS showed values of viscosity (61.6 and 61.8 mPas respectively) significantly higher than that of EVOOsC and EVOOsLG (59.2 mPas for both). Some authors reported slight but significant correlation between viscosity and olive oil composition (GILA *et al*. 2015). GILA *et al*. (2015) observed an increase of viscosity increasing the oleic acid content of virgin olive oils from different varieties. BONNET *et al*. (2011) affirmed however that the intravarietal variance of the fatty acid and TAG compositions is much smaller than the intervarietal variance, therefore viscosity differences should not be visible. Thus, the differences found in this study may be ascribable to other factors influencing the final composition, such as the oxidative status, as effect of the soil nature. In support of this hypothesis, a correlation was found in this study between viscosity measurement and the oxidative stability of the oils ($p\leq0.05$ $R=0.617$). It is reported that lipid oxidation products may weak and/or hinder intermolecular bonding between TAG molecules leading to the increase of the oil viscosity (VITTADINI *et al*., 2003).

3.3.2 Instrumental colour

The colour of the oils showed significant differences related to the nature of the soil, except for *L** (Table 4), which was found to be not statistically different among the samples, even if the lowest value was registered for the EVOOsB, the sample richest in carotenoids. In general, *L** is reported to increase with the reduction of the pigment content in the oils, as pigments would capture part of the light, instead of transmitting it (CERRETANI *et al*., 2008). The values of *a**, ranging from -3.3 to -5.3, described a green colour for all the samples. It resulted significantly higher for EVOOsC and EVOOsLG than for the other two oils. The values of *b**, representing the yellow colour, ranged from 41 to 14. EVOOsB resulted the most yellow among all the samples in relation with the highest content of carotenoids, as already reported (MOYANO *et al*., 2008). Two Pearson's correlations were also found between the chlorophylls/carotenoids ratio and the chromatic coordinates *a** ($p<0.01$, $R=0.841$) and *b** ($p<0.01$, $R= -0.828$), confirming the function of the pigments on the color of the studied olive oils, as affected by the soil nature.

3.4. PCA analysis

Based on previous works (CAPONIO *et al*., 2013; CHIAVARO *et al*., 2013), in which PCA analysis was successfully performed to discriminate among olive oils according to different refined steps or oxidative status by means of the correlation among thermal and chemical properties, it was again proposed in this study to tentatively discriminate among the four EVOO according to the nature of the soils. Twenty one variables were selected after factor extraction using as selection criteria loading values higher than 0.7, with PC1 and PC2 being the first two principal components that explained about 85% of the total variance. In Fig. 3a, the projection of the variables on the factor plane is reported.
All the DSC thermal properties, except the cooling enthalpy, were represented on the plane and better described by the PC1. Most of the DSC thermal properties showed positive factor loadings on PC1, being instead negative only for onset temperature of cooling (T_{on} cooling) and peak 2 cooling temperature at the maximum (T_{p2} cooling). As far as the chemical properties are concerned, positive factor loadings on PC1 were observed for MUFA and OSI, being instead negative for SFA, PUFA and POV. Few variables were represented on PC2, having this component a low contribution on the total results, as revealed from the high distance of the vectors from the boarders if compared to the ones on PC1. Positive factor loading on PC2 were showed by viscosity, carotenoids and colour parameter *b**, being instead negatives for *L**, *a** and peak 1 heating temperature at the maximum (T_{p1} heating). The heat capacity was not able to discriminate among the four EVOOs, thus it was excluded from the factor analysis.
The score plot obtained for the samples was shown in Fig. 3b. PC1 clearly divided EVOOsLG and EVOOsC in different clusters, having the first negative scores and the second positive ones. EVOOsS showed an intermediate behavior among all the samples, being instead EVOOsB better described by PC2 with positive scores. By comparing the score with loading plots it is evident how EVOOsLG and EVOOsC were well discriminate by the thermal parameters, being themselves influenced by the fatty acids composition and the oxidative stability. In particular, EVOOsC, the EVOO richest in MUFA, resulted well discriminated by the thermal properties of the cooling and heating major peak, confirming previous correlation found among this peaks with this class of fatty acids (CHIAVARO *et al*., 2007).

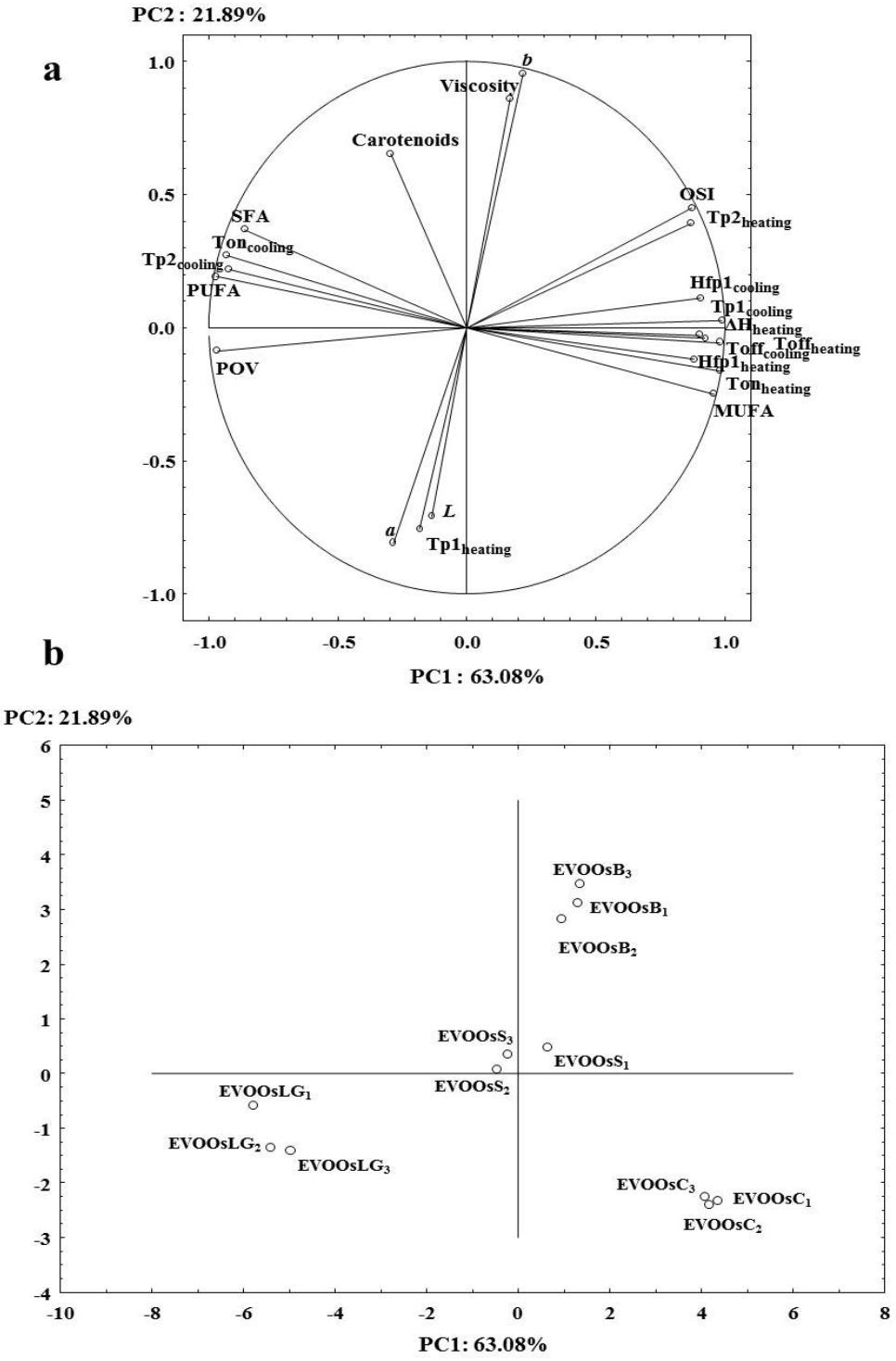

Figure 3. Principal Component Analysis (PCA) results obtained for the two principal components, showing a) the projection of the cases on the factor plane, and b) the projection of the variables on the factor plane. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; OSI, oxidative stability; POV, peroxide value; *T*on, onset temperature of transition; *T*off, offset temperature of transition; Hfp, peak heat flow at the maximum; *T*p, peak temperature at the maximum; Δ*H*, enthalpy change of transition; EVOOsC, extra virgin olive oil from soil clay; EVOOsS extra virgin olive oil from soil stony; EVOOsB, extra virgin olive oil from soil brown; EVOOsLG extra virgin olive oil from soil limestone and gypsum.

EVOOsLG on the other hand, with its high content of SFA and PUFA and the related POV value, was well described by the cooling parameters of the thermal event peaking at higher temperatures: T_{p2} and T_{on} of cooling, having also an inverse relation with the heating enthalpy. EVOOsS and EVOOsB had an intermediate composition in comparison to the other two oils, thus they showed intermediate values on PC1. EVOOsB was better discriminated on PC2 particularly from the viscosity value and from the b^* colour parameter, being the latter related to the carotenoid content, as observed in this study and already reported in literature (MOYANO *et al*., 2008).

4. CONCLUSIONS

The current study has investigated for the first time how the soil may impact on different quality parameters of four 'Chemlali' extra virgin olive oils considering physical and thermal variables other than the traditional chemical parameters. The PCA analysis helped in identifying the variables able to discriminate among the four studied EVOOs. EVOOsC and EVOOsLG resulted better discriminated by their chemical composition and thus by the related thermal properties. In particular, EVOOsC resulted as the sample with the highest MUFA content, exhibiting the highest heat flow of the first cooling peak and the narrowest cooling range in relation with lowest POV. EVOOsLG was the richest in SFA and PUFA showing the lowest heating enthalpy and the highest cooling and heating ranges together with the lowest OSI value. EVOOsB was distinguished on the base of its colour, being more yellow than the others, as revealed from the b^* parameter in relation to the highest carotenoid content, and having also the highest OSI value. EVOOsS showed intermediate properties to the other samples.
These first encouraging results have revealed the effective influence of the soil on chemico-physical and thermal properties of 'Chemlali' EVOOs which need further confirmation through the analysis of a larger set of samples to sort out the best pedologic conditions for the olive cultivation. Their relation with the soil composition should be also considered in the future.

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PAPER

PHENOLIC COMPOUNDS , CAROTENOIDS AND ANTIOXIDANT ACTIVITY IN FIVE TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) CULTIVARS

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ABSTRACT

In this study we examined the antioxidants content (polyphenols and carotenoids) and the total hydrophilic (HAA) and lipophilic (LAA) antioxidant activity of red (cv. Shiren and Red Pear), yellow (cv. Yellow Pear-shaped), pale yellow (cv. Snowball) and black (cv. Black Trifele) ripe tomato fruits. For each studied cultivars, the HAA was higher than the LAA. The correlation between antioxidants (polyphenols and carotenoids) and TEAC values (HAA and LAA) was also estimated and the only significative correlation was obtained between rutin and TEAC-HAA. Statistical analysis shows significative differences in antioxidants content (especially for lycopene and β-carotene) between the analyzed tomato cultivars.

Keywords: ABTS, antioxidants, β-carotene, HAA, LLA, lycopene, rutin, TEAC, tomato

1. INTRODUCTION

The nutraceutical quality of tomato fruits is related to their antioxidant and antitumoral properties. The habitual consumption of tomatoes has been associated with decreased risk of chronic degenerative diseases including certain types of cancer and heart diseases (LAVELLI *et al*., 2000; GIOVANNUCCI, 1999, 2002; RAO and AGARWAL, 1998). Epidemiological findings confirmed that the beneficial health effects are due to the presence of bioactive molecules such as carotenoids, particularly lycopene, and polyphenolic compounds, particularly flavonoids. Lycopene has been considered the most efficient for quenching singlet oxygen (DI MASCIO *et al*., 1989). Its protective role in human health has been well described in numerous papers which showed that the dietary intake of lycopene is associated with a decreased risk of prostate cancer and degenerative diseases (GIOVANNUCCI, 1999, 2002; RAO and AGARWAL, 1998, 1999). In human diet, tomatoes and tomato products are the predominant sources of lycopene. The lycopene contents and the qualitative and quantitative composition of tomato fruits depend on cultivar, ripening stage, climatic conditions, etc (ABUSHITA *et al*., 2000; ZANFINI *et al*., 2007). Many authors have focalized their research on the variations in the carotenoid profile in relation to different cultivars (ABUSHITA et al., 1997, 2000; LEONARDI *et al*., 2000; ZANFINI *et al*., 2007). Similar studies focused their attention on the polyphenolic compounds such as flavonoids and hydroxycinnamic acids. The phenolic fraction of tomato fruits contains quercetin, naringenin, rutin and chlorogenic acid as the main compounds (CLIFFORD, 1999; HERTOG *et al*., 1992; JUSTESEN *et al*., 1998). Their quantitative changes in relation to cultivar, season and country of origin were also investigated and the ability of these compounds as scavengers of peroxyl radicals has been well described (HALLIWELL, 1999; STEWART *et al*., 2000).
The antioxidant capacity of tomato fruits has been widely investigated and a clear influence of the genetic factors (type of cultivar) has been found. The tomato cultivars characterized by a high carotenoid contents showed the highest antioxidant activity and a strong correlation between the antioxidant power and lycopene content was also found (RAFFO *et al*., 2002, 2006; ZANFINI *et al*., 2010).
Today, especially in the Mediterranean area, black, white or yellow tomato fruits are commonly present in local markets and used as fresh products for salads or for culinary preparations. The aim of the present study was to examine the polyphenolic fraction, the lycopene and β-carotene contents and the hydrophylic and lipophilic antioxidant activities of red, yellow, pale yellow and black tomato fruits. This work is justified by the fact that the color of fruits and vegetables can be an index of the antioxidant potential and may be assist in the selection of food consumption (CHÁVEZ-MENDOZA *et al*., 2015). We analyzed five different cultivars: two red cultivars, the cv. Read Pear which is characterized by a typical pear shaped fruit and the more commercial cv. Shiren which is a conventional cherry type tomato; the cv. Yellow Pear-shaped with yellow pear shaped fruits; the cv. Snowball with pale yellow fruits and the black cultivar cv. Black Trifele.

2. MATERIALS AND METHODS

2.1. Reagents and standards

All solvents used were of HPLC grade from BHD (Poole, England). The β-carotene, lycopene, vanillin, kaempferol-3-O-glucoside, naringin, naringenin, and the acids gallic, protocatechuic, vanillic, chlorogenic (4-CQA), caffeic, benzoic and cinnamic were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany); lutein, quercetin

and rutin standard from ICN Biochemical Inc. (Ohio, USA); Folin-Ciocalteau reagent, magnesium oxide and silica gel 60 (0.063-0.200 mm) from Merck (Darmstadt, Germany); β-apo-8'-carotenal and ABTS from Fluka Chemie (Buchs, Switzwrland); Trolox from Hoffman La Roche Aldrich Chem. Co. (Saint Louis, MO, USA).

2.2. Plant material and tomato sampling

Different tomato cultivars were investigated: *Lycopersicon esculentum* Mill. cv. Red Pear, cv. Snowball, cv. Black Trifele and cv. Yellow pear-shaped. Seeds were from Graines Baumaux - Mirecourt - France and from Baker Creek Heirloom Seeds Co., Mansfield (MO), USA. The commercial cherry type cv. Shiren was also analyzed.
Seeds were allowed to germinate under glass in February-March 2014, then plants were transplanted and cultivated in open air nearby Siena (Southern Tuscany, Italy, 43°15'11.8"N 11°36'11.5"E) under the climatic conditions typical of the Mediterranean area.
Figure 1 shows for illustration purpose the colors of various tomato cultivar and below are listed their morphological characteristics.

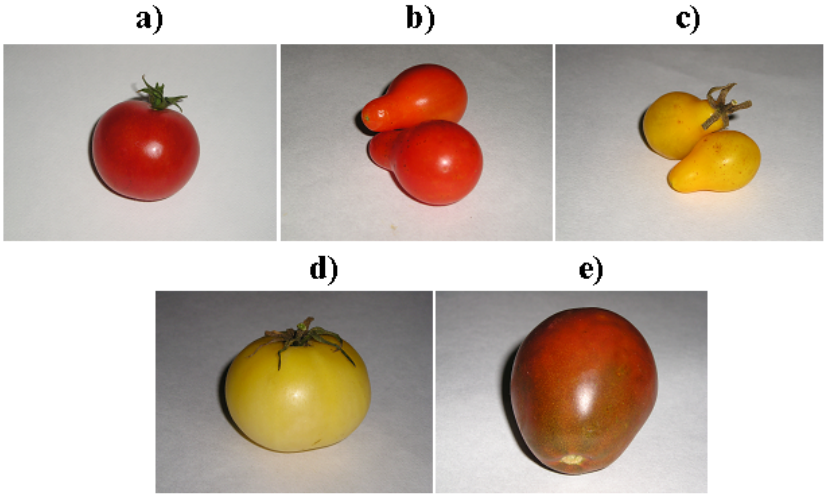

Figure 1. Sample of various tomatoes fruits used in the analysis: (a) cv. Shiren; (b) cv. Red Pear; (c) cv. Yellow Pear-shaped; (d) cv. Snowball and (e) cv. Black Trifele.

Yellow Pear-shaped (or Yellow Pear): plants are indeterminate and hardy. They produce bright yellow, pear-shaped cherry tomatoes with a sweet, mild flavor. They ripe in 75-80 days and are 25-50 mm long. Red Pear (or Red Pear-shaped) is almost analogous, with bright red fruits. Snowball (or White Beauty) is a cultivar introduced in North America in the mid-1800s. Plants are indeterminated and yield very heavy crops of 140 up to 220-340 g, 65-75 mm, yellow-white, somewhat flattened round tomatoes with very mild sweet flavors. They ripe in 80-85 days. Skin and flesh are pale yellow to parchment or creamy white, with a pink blush on the blossom end. Black Trifele (or Japanese Black Trifele) is, despite the name, a cultivar of Russian origin. Pear-shaped fruit has green-streaked shoulders, deepening to a burnished mahogany and finally to a darkened, nearly black base. They ripe in 80-85 days, reach 65-75 mm long and wide, 170 g weight. Shiren is a more recently obtained cultivar. Plants are indeterminate, compact and particularly suitable for full-sun or greenhouse cultivation. The plant is very resistant to many viruses and produces a very elegant "Fishbone" cluster with long shelf life fruits. Fruits are

cherry-like globose in shape, with a diameter of 35 mm and a weight of 10-20 g; they ripe in 55-68 days.
Full ripe (4.5-5.5°Brix) and healthy fruits harvested during July and August 2014 were analyzed for antioxidant content as well as for hydrophilic (HAA) and lipophilic antioxidant activity (LAA). For the analysis ten tomatoes were sampled from four different representative plants and combined into one sample which is analysed as reported below. The ripeness was determined both visually and by determination of ° Brix after chopped and centrifuged an aliquot of the various tomato samples with Exacta Optech GmbH (Munchen, Germany) refractometer.

2.3. LC–UV-MS analysis of hydrophilic extracts

LC-MS system consisted of an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA) including a vacuum solvent degassing unit, a binary high-pressure gradient pump, an UV detector and a 1100 MSD model VL benchtop mass spectrometer with API-ES interface. Nitrogen was used as nebulizer gas and drying gas (350°C). The nebulizer gas, the drying gas, the capillary voltage, and the vaporizer temperature were set at 40 psi, 9 L/min, 3000 V and 350°C, respectively and fragmentor 70 eV. The LC-ESI-MS determination was performed by operating the MSD in both positive and negative ion mode. Mass spectra were acquired over the scan range m/z 50-1500 using a step size of 0.1 μ. The chromatographic separation was performed using a Pursuit C18 (3 μm, 5x2.0 mm) (Varian) column. The sample was injected (20 μl) after filtration. The separation was performed by using linear gradient elution for 60 min with a mobile phase of 0.2% (v/v) formic acid in water and acetonitrile (from 90:10 to 30:70 v/v in 60 min) at the flow rate of 1.5 ml/min. After the chromatographic separation an aliquot of the eluent (400 μl/min) was directed to MSD for spectra analysis. The MS analysis was used for the identification of the main compounds present in the polyphenolic fraction. Their identification was obtained for comparison with the retention time and with the fragmentation pattern of the relative standards. The UV-Vis analysis was used for quantitative purposes. The quantitative analysis was performed for the main identified compounds using calibration curves obtained by injecting the standard solutions at various concentrations. All analyses were run in triplicates and results were expressed with the standard deviation of the means.

2.4. Lycopene, β-carotene and lutein analysis

Carotenoids and xanthophylls were extracted using a procedure previously published with small variations (SETIAWAN et al., 2001). A sample of 5 g of homogenized fresh tomatoes was extracted using 10 ml THF in presence of 0.01% butylated hydroxytoluene (BHT) and internal standard (β-apo-8'-carotenal). This extraction was performed twice. The organic fractions were collected and evaporated to dryness under nitrogen. The residue was dissolved in chloroform, appropriately diluted with the mobile phase mixture (methanol: acetonitrile: dichloromethane 50:48:2), then filtered (0.45 mm Minisart SRP 4 filter, Sartorius, Germany) and analyzed by using HPLC. LC 410 Series Perkin-Elmer apparatus (Norwalk, Connecticut, USA) equipped with a UV/VIS LC295 Perkin-Elmer detector set at 290 nm and TotalChrom ver.6.3.1 software were used (Perkin Elmer). Chromatographic separation was done using a reversed-phase LiChrospher 100 RP 18 column (5 μm, 125 x 4.6 mm) (Merck). Elution was carried out using a mixture of methanol: acetonitrile: dichloromethane (50:48:2) at a flow rate of 1.0 ml/min. Quantitative analysis of lutein, lycopene and β-carotene was based on the internal standard method. Three replications were carried out to examine each sample.

2.5. Lipophilic and hydrophilic extracts and their antioxidant activities determination

Total antioxidant activity was measured both, for hydrophilic and lipophilic extracts of the various tomato cultivars. In brief, 5 g of fresh tomato sample was extracted with 10 ml of CH_2Cl_2 and then centrifuged at 1620 g for 10 min. The extraction was performed twice and the supernatant fractions were collected and evaporated to dryness under nitrogen. The dried residue was dissolved in 3 ml of CH_2Cl_2 and analyzed for the determination of lipophilic antioxidant activity (LAA). The pellet was extracted with 10 ml of 60% methanol in Milli-Q water. The samples were then sonicated with Sonorex RK103H apparatus (Bandelin electronic, Berlin, Germany) for 10 min and centrifuged at 1620 g for 10 min. The supernatant was then transferred to a new tube and used for determination of hydrophilic antioxidant activity (HAA) and polyphenol content.
The antioxidant activity was measured using ABTS radical cation ($ABTS^{\cdot}$) decolorization assay (RE et al., 1999). In brief, 1 ml of the ABTS solution was added to different volumes of the lipophilic or hydrophilic extract (20, 40 or 60 μl) and diluted at a final volume of 2 ml using ethanol. The solution was vortexed for 10 s and the decolourization produced by the presence of antioxidants was measured at 751 nm (UV/Visible Lambda 2 spectrophotometer, Perkin-Elmer, Norwalk, Connecticut, USA), 10 min after initial mixing. The TEAC assay is a standard method used for antioxidant activity assessment of vegetables with numerous advantages such as reproducibility, simplicity and a good estimate of the antioxidant activity of pure compounds and complex matrices (THAIPONG et al., 2006). Trolox was used to prepare the standard curve and the activity was reported as equivalent millimolar Trolox related to fresh weight (mM TEAC/100 g FW). The LAA and HAA were measured in triplicates for each extract.

2.6. Statistical evaluation of data

All analyses were run in triplicates and results were expressed with the standard deviation of the means. A one-way analysis of variance (ANOVA) was performed to test the significance of the observed differences (using Stata 9.0, StataCorp LP). Data were analyzed considering the tomato cultivar as experimental factor and when the observed differences were significant ($P \leq 0.05$) the mean values were then compared by Bonferroni's multiple comparison test.

3. RESULTS AND DISCUSSION

3.1. Polyphenolic fraction analysis

3.1.1 Identification of the main polyphenolic compounds by LC-UV-MS

In the present study 13 different polyphenolic compounds were detected and identified in the extracted samples. The identified compounds belonging to five groups of phenolic compounds (benzoic acids, hydroxycinnamic acids, phenolic aldehydes, flavonols and flavanones) are listed in Table 1. Identification of the chromatographic peaks was made by comparison of mass spectra with those provided by commercial standards and by comparing their retention times in correlation to MS fragmentation patterns.

Table 1. List of polyphenolic compounds identified in tomato samples.

Compound	Retention time (min)	$[M+H]^+$	$[M+H]^-$	Identification
		(*m/z*)		
1	5.1	---	169	Gallic acid
2	9.5	155	153	Protocatechuic acid
3	19.7	169	167	Vanillic acid
4	20.4	355	353	Chlorogenic acid
5	21.3	181	179	Caffeic acid
6	23.4	153	151	Vanillin
7	28.4	123	121	Benzoic acid
8	30.1	611	609	Rutin
9	32.6	287	285	Kaempferol-3-O-glucoside
10	33.1	581	579	Naringin
11	41.1	149	147	Cinnamic acid
12	44.7	303	301	Quercetin
13	47.1	273	271	Naringenin

Under the experimental conditions used, the most intensive signals detected for the main compounds was the pseudomolecular ion ($[M+H]^+$ or $[M-H]^-$).
Some benzoic acid derivates or phenolic acids were identified. Gallic acid comes out at Rt 5.1 min, giving the characteristic molecular ion at 169 *m/z*. The acids p-hydroxybenzoic acid, vanillic acid, protocatechuic acid were also identified by their retention times, their pseudomolecular ion ($[M+H]^+$ or $[M-H]^-$) and mass fragmentations in comparison with standard solutions. A comparison with the MS spectra described in literature was also carried out (GUASH-JANÉ *et al*., 2004; TIAN *et al*., 2005).
Hydroxycinnamic acids were also identified. The presence of chlorogenic acid and caffeic acid was detected in all the analyzed samples. The phenolic aldehyde vanillin was also detected. Flavonols were mainly characterized by quercetin (303 *m/z*) and rutin (611 *m/z*). The presence of kaempferol-3-O-glucoside (449 *m/z*) was also detected. Flavanones were mainly represented by naringenin (273 *m/z*).
Comparison of total ion content (TIC) and UV (289 nm) chromatograms obtained from the LC-UV-MS analysis of the various cultivars revealed similar profiles (same compounds were observed even if in different quantity). Additionally, we found that the profiles obtained from the analysis of the various cultivars were essentially identical with some qualitative differences detected for minor components (unknown compounds).

3.1.2 Quantitative analysis of the main identified compounds

The main polyphenolic components identified were quantified as previously reported and the most abundant polyphenols detected in tomato cultivars are reported in Table 2. The most abundant were chlorogenic acid, rutin and quercetin. The determination of phenolic compounds showed that Snowball and Black Trifele cultivars contained the lowest concentration of antioxidant compounds, while the cultivar that possessed the highest concentration, especially of rutin (40.18 μg/g of FW), was Shiren. These observations are perfectly in agreement with HAA values showed below.
Quantitative determination of phenolic compounds showed that contents were in agreement with the few analytical data reported on red, purple or yellow cultivars

(MARTÍNEZ-VALVERDE *et al*., 2002; VALLVERDÚ-QUERALT *et al*., 2011; GARCÍA-VALVERDE *et al*., 2013; CHOI *et al*., 2014; RAIOLA *et al*. 2016).

Table 2. Mean content (μg/g of FW)±SD of the main polyphenolic compounds in the analyzed tomato cultivars.

	Tomato cultivar				
	Red Pear	**Snowball**	**Black Trifele**	**Yellow Pear**	**Shiren**
Gallic acid	1.03±0.06^{c}	1.09±0.08^{c}	0.04±0.01^{b}	nd ^{a}	0.62±0.02^{d}
Chlorogenic acid	12.53±1.09^{b}	15.08±1.03^{b}	9.26±0.83^{a}	17.54±1.26^{b}	8.86±0.06^{a}
Caffeic acid	0.69±0.02^{c}	0.47±0.05^{b}	0.01±0.01^{a}	0.03±0.01^{a}	1.32±0.09^{d}
Rutin	14.46±1.11^{c}	9.96±0.95^{a}	12.40±0.92^{b}	26.32±1.86^{c}	40.18±2.45^{d}
Kaempferol-3-O-glucoside	0.13±0.01^{a}	0.10±0.02^{a}	0.13±0.01^{a}	0.54±0.02^{c}	0.20±0.01^{b}
Naringin	0.12±0.01^{b}	0.06±0.01^{a}	0.23±0.01^{c}	0.23±0.01^{c}	0.50±0.02^{d}
Quercetin	23.91±1.16^{d}	16.92±1.23^{c}	8.01±0.75^{b}	15.34±1.14^{c}	0.15±0.01^{a}
Naringenin	3.63±0.27^{b}	0.54±0.04^{a}	10.14±1.01^{c}	5.36±0.43^{b}	10.21±0.92^{c}

Different letters in the rows represent statistically significant differences (P>0.05).
nd = not detected.

3.2. Lutein, Lycopene and β-carotene analysis

We carried out a quantitative analysis of lutein, lycopene and β-carotene in the different investigated tomato cultivars. All samples exhibited a similar chromatographic profile, showing that the two carotenoids lycopene and β-carotene were the main component of the lipophilic extracts for the cultivars with red fruits. The compounds were identified by comparing the retention time with those obtained from the reference standard injections.
The quantitative data were obtained using the internal standard method and are presented in Table 3. Lycopene content ranged from 1.02 μg/g (recorded for Yellow pear tomatoes) to 184.42 μg/g of fresh weight (recorded for Shiren tomatoes), while β-carotene ranged from 2.60 μg/g (recorded for Yellow pear tomatoes) to 64.81 μg/g (recorded for Shiren tomatoes). Additionally, the quantitative data showed that the Yellow pear cultivar had a β-carotene content more abundant than the lycopene content. The two investigated carotenoids were not detected in the Snow ball cultivar in which only lutein was detected. Lutein was also very abundant in Black Trifele cultivar.

Table 3. Principal carotenoids (lycopene and β-carotene) and xantophylls (lutein) content of various tomatoes cultivars.

Tomato cultivar	mg/g of fresh fruit		
	lutein	**lycopene**	**β-carotene**
Red Pear	0.18±0.02^{b}	42.18±2.31^{e}	12.4±0.12^{d}
Snowball	0.08±0.01^{a}	nd^{a}	nd^{a}
Black Trifele	1.12±0.04^{d}	60.9±1.92^{c}	8.42±1.66^{c}
Yellow Pear	0.20±0.02^{b}	1.02±0.28^{b}	2.60±0.84^{b}
Shiren	0.21±0.02^{bc}	184.42±2.61^{d}	64.81±0.37^{e}

Different letters in the columns represent statistically significant differences (P>0.05).
nd = not detected.

From the results of table 3 emerged, that there is a significant difference in the ability of tomatoes to synthesize carotenoids in relation to cultivar. The cultivar with the highest content of carotenoids was Shiren, on the contrary, the Yellow pear cultivar had low ability in carotenoids production.
Characterization of carotenoid content of yellow tomatoes and the differences with the red ones dates back to the 50s (JENKINS and MACKINNEY, 1955) and many authors have determined significant influence of cultivars on the content of tomato carotenoids (ABUSHITA *et al*., 2000; BINOY *et al*., 2004; CHOI *et al*. 2014; ILAHY *et al*., 2011; RAIOLA *et al*. 2016; ZANFINI *et al*. 2007). Our results are in accord with data reported by these authors and demonstrate that lutein, lycopene and β-carotene content is significantly related with the cultivar and the color of the tomatoes as earlier reported by other authors (LI *et al*., 2013).

3.3. Antioxidant activity

The antioxidant activity of tomato lipophilic (LAA) and hydrophilic (HAA) extracts was also performed using the Trolox equivalent antioxidant capacity (TEAC) assay. For each studied cultivar, the HAA was higher than the LAA and the Shiren type tomatoes, characterized by a high carotenoid and total phenolic contents, showed the highest antioxidant activity (Table 4).

Table 4. TEAC values (μM Trolox/100 g FW) measured for each investingated cultivar.

Tomato Cultivar	HAA - TEAC	LAA- TEAC
Red pear	502.8±52.3^{a}	75.4±2.0^{c}
Snow ball	472.8±48.7^{a}	47.7±1.3^{a}
Black Trifele	499.2±60.3^{a}	121.7±0.9^{d}
Yellow pear	643.5±54.1^{ab}	58.3±1.0^{b}
Shiren	706.0±19.3^{b}	131.0±2.1^{e}

Different letters in the columns represent statistically significant differences (P>0.05).

Statistical analysis shows significative differences in antioxidants activity only for LAA between the analyzed tomato cultivars.
The correlation between the content of the various antioxidant (independent variable) and the TEAC value (dependent variable) was also estimated. The only significative correlation was obtained between rutin and TEAC HAA ($r^2 = 0.982$, $p<0.05$).

4. CONCLUSIONS

This study has confirmed the important role played by cultivar in determining the antioxidant potential of fresh raw tomatoes. Results show that phenolic compounds but especially carotenoids are responsible for the differences among tomatoes in accord with the cultivar. In effect the values of HAA and LAA reflect the contribution of different antioxidants to the total antioxidant activity of the various tomato cultivars.

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PAPER

SHELF LIFE VALIDATION BY MONITORING FOOD ON THE MARKET: THE CASE STUDY OF SLICED WHITE BREAD

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ABSTRACT

The aim of this work was to develop a protocol to validate the shelf life obtained in laboratory based on the product performance on the market. Packed sliced white bread was chosen as target product. Bread shelf life was assessed by measuring the changes in firmness during storage at 25°C and by determining the firmness of bread samples directly purchased from the market with different storage life. Firmness data were modelled by the restricted Avrami equation. Results suggest that shelf life estimates obtained in laboratory should be validated by checking the evolution of product quality on the market.

Keywords: laboratory storage, market conditions, shelf life validation, white bread

1. INTRODUCTION

Shelf life can be defined as a finite length of time after production, during which the food product retains a required level of quality - the so-called acceptability limit - under well-defined storage conditions (NICOLI, 2012a). Shelf life is an important feature of all foods, including raw materials, ingredients and semi-manufactured products. Every packed food has its own shelf life and all the subjects involved in the food chain should be aware of it. Shelf life assessment of foods is a vital process for food companies not only to comply with the legal obligations, but above all to maintain their brand reputation on the market avoiding product recalls. Indeed, the challenge of every food business operator is to ensure that the product characteristics fulfil the requirements of consumers whenever they buy and eat the food.

The adoption of a systematic approach is always necessary to get reliable shelf life data. Shelf life assessment is generally performed under laboratory conditions by developing experimental storage trials, as extensively described by many authors (FU and LABUZA, 1993; ROBERTSON, 2009; GALIC *et al*., 2009; NICOLI, 2012b). The preliminary steps of any shelf life assessment imply the identification of the most critical chemical, physical or biological event leading to the product quality depletion. A suitable quality decay indicator should be then selected and the relevant acceptability limit defined. The next step is the evaluation of the changes of the quality indicator as a function of storage time under laboratory conditions, either mimicking the most probable conditions experienced by the product on the market (real-time shelf-life testing) or able to accelerate deteriorative reactions (accelerated shelf life testing - ASLT) (LABUZA and SCHMIDL, 1995; MIZRAHI, 2000; ROBERTSON, 2009; NICOLI, 2012a). Finally, kinetic data are modelled to estimate reaction rate. The latter is used to compute the shelf life, which is the time needed to reach the acceptability limit (LABUZA and SCHMIDL, 1985; VAN BOEKEL, 1996; CALLIGARIS *et al*., 2012).

Shelf life assessment under laboratory conditions is applied in different stages of the product life-cycle (FU and LABUZA, 1993; NICOLI, 2012b). It cannot be disregarded during the development of new products to assign a suitable food dating. Moreover, it is employed for the on-going shelf life validation of consolidated products. The drawback is that the shelf life values, commonly obtained in laboratory, hold only when the product, in its real life, experiences the same environmental conditions suffered during the shelf life experiments. Obviously laboratory shelf life does not take into account any changes in environmental conditions (e.g. temperature, light) during distribution and storage. In addition, laboratory shelf life tests should be run on a number of different product batches to take into account production variability (CALLIGARIS and MANZOCCO, 2012). It is a matter of fact that the number of batches adopted should be increased proportionally to the lack of production standardization.

Although the discrepancy between laboratory and market shelf life is recognized as a critical issue, there is a lack of information about the possible error deriving from attributing to a product a shelf life based only on laboratory tests. In the light of the continuous improvement process required to increase food industry competitiveness, it could be very profitable to verify if the laboratory shelf life estimates really account for the shelf life of the product on the market shelves.

To our knowledge this study is a first attempt to develop a useful protocol to validate the shelf life obtained in laboratory based on the product performances on the market. To this purpose, packed sliced white bread was chosen as target ambient stable product since it represents a widespread bakery good. The shelf life attributed by the producers to this food category is about 2-3 months. As well studied, sliced bread quality depletion during storage is mainly affected by the crumb firmness increase (HE and HOSENEY, 1990; GIL *et*

al., 1999; RONDA *et al*., 2011). The latter was thus chosen as critical indicator to estimate the product shelf life. The research was divided in two parts. In the first one, bread shelf life was assessed by means of a typical laboratory shelf life test by measuring the changes in firmness of sliced bread during storage at 25°C. Firmness data were then modelled by the restricted Avrami equation and used to estimate shelf life based on reasonable acceptability limits. The shelf life laboratory test was replicated four times by using bread from different production batches. In the second part of the experimental activity, a high number of bread samples, each belonging to different production batches and having different storage life, were directly purchased from the shelves of stores with different size. Also in this case, bread firmness was evaluated and used to estimate shelf life following the same modelling procedure applied in the laboratory shelf life tests. Discrepancies between laboratory and market shelf life estimates were then discussed.

2. MATERIALS AND METHODS

2.1. Laboratory shelf life test

Freshly made commercial sliced white bread was kindly provided by an Italian factory. According to the producer indications, the shelf life of bread was 75 days, bread slices were superficially treated with ethanol and packed in conventional atmosphere. The original polypropylene bags of bread slices contained 285 g of product corresponding to 16 bread slices having dimensions 9x9x0.9 cm. Twenty-five original packages, all from the same batch, were stored just after production at 25°C in a SMD34 thermostated cell (Dexion, Brescello, Italy). At increasing storage time up to 72 days, two sample packages were removed from the thermostated cell and bread slices were analyzed for firmness. The storage test was replicated four times using different bread batches produced between January and June 2013. The overall number of samples considered in the laboratory shelf life test was equal to 100.

2.2. Market shelf life test

Sliced white bread samples analogous to those considered in the laboratory shelf life test (same producer, production plant, number and dimensions of slices) were purchased on the market by visiting 35 different stores located in the north-eastern part of Italy in the period from November 2012 to April 2013. Selected stores were grocery stores serving a limited number of persons in a small area (small stores); supermarkets with a larger number of customers than traditional groceries (medium stores) and hypermarkets of well-known retailer brands (large stores). The ratio among the number of the small, medium and large stores was 1:1:1. Each sample (two bread packages) was from a different production batch as declared on the label. The storage life after production was calculated from the production date declared by the producer on the product bar code.

2.3. Analytical determinations

2.3.1 Firmness

Firmness of bread crumb was measured by a puncture test using an Instron 4301 (Instron LTD., High Wycombe, UK). The instrumental settings and operations were accomplished using the software Automated Materials Testing System (version 5, Series IX, Instron LTD., High Wycombe, UK). Four slices were taken from each bread package. The central

part of the slice (4.5x4.5x0.9 cm) was sampled by manual cut with a sharp knife. A uniaxial compression test was performed at ambient conditions (20±2°C, ambient humidity). Samples were penetrated using a cylindrical probe of 12.7 mm diameter mounted on a 100 N compression head. Crosshead speed was set at 5 cm/min. Force-distance curves were obtained and firmness was taken as the force (N) required to compress the bread crumb by 0.3 cm. Five measurements were performed in different places of each bread slice.

2.3.2 Moisture content

Moisture content was determined by AOAC gravimetric method n. 925.09-1925 (AOAC, 1980) by drying the samples in a vacuum oven (1.32 kPa) at 75°C until a constant weight.

2.4. Data analysis

The results reported in this work are the average of at least twenty firmness determinations for each bread sample. All determinations are expressed as the mean ± standard error (SE).
The changes in bread firmness were analyzed by the Avrami equation (AVRAMI, 1939; 1940; COLWELL *et al*. 1969):

$$\theta_t = \frac{F_{\max} - F_t}{F_{\max} - F_0} = e^{-kt^n} \qquad (1)$$

where θ_t is the non-firmed fraction of bread at time t, F_{max} is the maximum firmness, F_0 is bread firmness at time 0, F_t is bread firmness at time t, n is the Avrami exponent and k is the rate constant having units depending on the value of n (days^{-n}). F_0 was assumed to be the lowest firmness value observed in just prepared bread (0.49 N), while F_{max} was taken as the highest firmness value (3.05 N) observed in bread stored for the longest time (72 days). The restricted Avrami equation considering n=1.0 was also applied.
The Avrami equation was fitted using non-linear regression. Parameter estimates relevant to bread from different batches were compared using t test with Bonferroni adjustment for multiple testing. Shelf life estimates and related confidence intervals were first computed on the log scale for better normal approximation and delta method was used to compute standard errors. Statistical analysis was performed by using R v. 3.0.2 (The RStudio Foundation for Open Access Statistics).

3. RESULTS AND DISCUSSION

3.1. Laboratory shelf life test

The first step in facing a shelf life assessment test is the definition of the critical indicator to be used to monitor product quality decay during storage. In this work, the evolution of bread quality was monitored by assessing the changes in crumb firmness. This parameter was chosen in accordance with literature data, indicating that crumb firmness is well related to the product rejection by consumers (GAMBARO *et al*., 2004; GIMENEZ *et al*., 2007; GIL *et al*., 1999; RONDA *et al*., 2011).

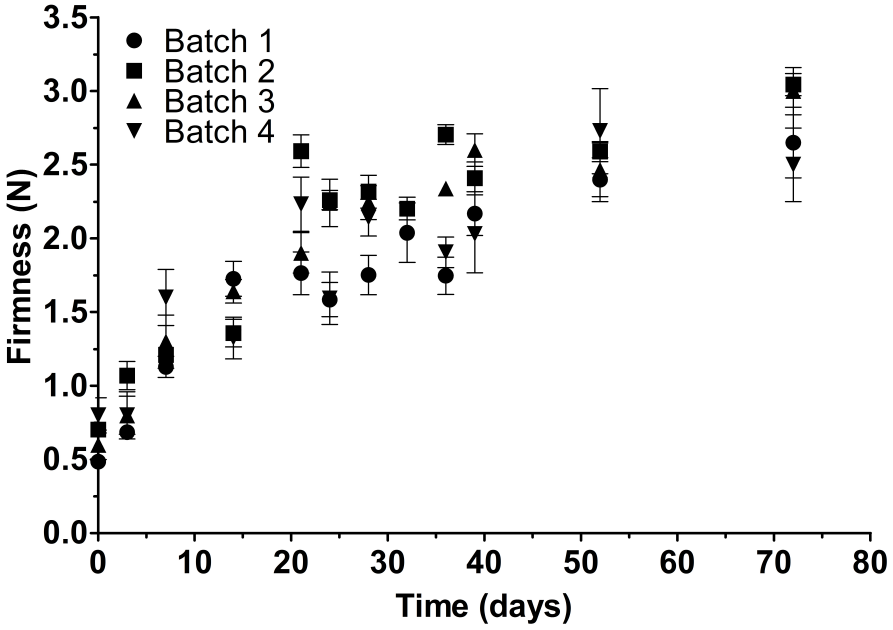

Figure 1: Firmness of sliced bread from different batches stored in the dark at 25°C under laboratory conditions.

Figure 1 shows the firmness increase of the crumb of bread from four different batches during storage at 25°C under laboratory conditions. Just produced samples showed different mean values of firmness accounting for the product batch variability. Although the increase in firmness of the different bread batches proceeded according to similar trends, samples from batches 1 and 4 were generally characterized by lower firmness values than those of batches 2 and 3.

The increase in bread crumb firmness during storage is known to be a complex phenomenon in which multiple mechanisms operate, mainly involving starch retrogradation of gelatinized starch and moisture redistribution (KULP and PONTE, 1981; ATWELL *et al*., 1988; ZOBEL and KULP, 1996; GIL *et al*., 1999; GALIC *et al*., 2009; RONDA *et al*., 2011). Since no changes in moisture were recorded during bread storage at 25°C confirming the moisture barrier properties of the packaging material (data not shown), crumb firming can be mainly attributed to starch recrystallization phenomena. This reversible aggregation involves the progressive re-association of the non-linear amylopectin fraction to form a molecular structure with increased order, which can be assimilated to a crystalline one (ZOBEL and KULP, 1996). Based on these considerations, starch recrystallization in bread can be considered a nucleation controlled phenomena and the changes in firmness can be analyzed by the Avrami equation (COLWELL *et al*., 1969) (equation 1). Firmness data reported in Figure 1 were thus used to predict the value of θ (Fig. 2).

In order to fit the Avrami model to the data, non-linear regression was performed to estimate the model parameters k and n. Table 1 shows that the Avrami exponent n, although close to 1.0 for all batches, actually assumed different values for each batch. According to the Avrami model, the exponent n should be an integer that indicates the crystal growth mechanism. In particular, values close to 1.0 indicate that crystallization

occurs by instantaneous nucleation, with nuclei appearing all at once early on the process, and proceeds with formation of rod-like crystals.

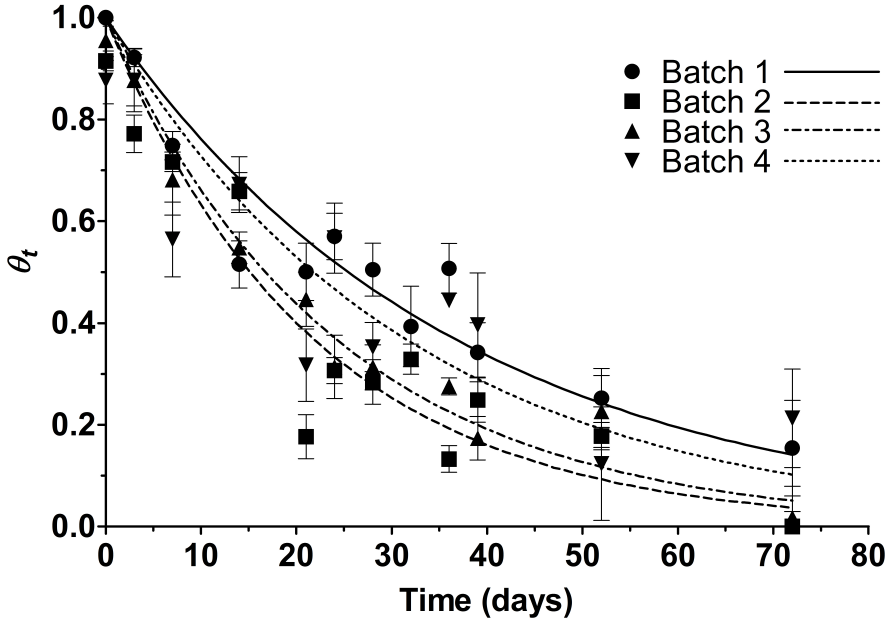

Figure 2: θ_t values (non-firmed fraction) of sliced bread from different batches stored in the dark at 25°C under laboratory conditions. Points: experimental data; lines: predicted values under the restricted Avrami model.

For this reason, the restricted Avrami equation, with n set equal to 1, was fitted to θ_t (Table 1). Reaction rates obtained in this case have the same unit ($days^{-1}$) and can thus be compared. Statistical analysis actually showed that the evolution of sliced bread firmness was significantly affected by the bread batch ($p < 0.05$). It is evident that the production of the bread samples considered in this study is not completely standardized and that samples with higher or lower firming tendency are produced.

Table 1: Parameter estimates of Avrami and restricted Avrami models for sliced bread from different batches stored in the dark at 25°C under laboratory conditions.

	Avrami model		Restricted Avrami model	
	n	k ($days^{-n}$)	n	k ($days^{-1}$)
Batch 1	0.770±0.109	0.060±0.022	1.0	0.027±0.002^{b}
Batch 2	0.869±0.167	0.069±0.038	1.0	0.046±0.005^{a}
Batch 3	0.871±0.083	0.063±0.017	1.0	0.041±0.002^{a}
Batch 4	0.651±0.168	0.102±0.058	1.0	0.032±0.004^{ab}

^{ab}: Estimates indicated by the same letter are not significantly different ($p > 0.05$) with the t test with Bonferroni adjustment.

The definition of shelf life implies that there should be a quality level discriminating products that are still acceptable for consumption from those no longer acceptable (MANZOCCO, 2012). To estimate the shelf life of sliced bread based on its crumb firmness changes, it is thus necessary to define the acceptability limit. The latter corresponds to the maximum bread firmness the company can tolerate at the end of shelf life. In particular, a company can choose a certain level of θ_t as possible criterion to estimate the limit firmness. For instance, if θ_t at shelf life is chosen to be equal to 0.20, the end of bread shelf life is assumed to be reached when 80% of starch crystallization is achieved. In our samples, this condition would be reached when bread firmness is equal to 2.53 N, as calculated from θ_t defined in equation (1). More restrictive choices can be done by high quality standard companies which may decide that the product is out of shelf life when θ_t is equal to 0.50 (50% starch crystallization), corresponding to a firmness equal to 1.77 N in our samples. Reversely, companies addressing consumers less sensitive to quality aspects could accept a θ_t value equal to 0.10, associated with a 90% starch crystallization. Our bread samples reached this level of staling at a firmness of 2.79 N. Based on these considerations, a reasonable simulation of company choices was performed by considering values of firmness equal to 1.77, 2.53 and 2.79 N as acceptability limits to estimate sliced bread shelf life. Table 2 shows shelf life estimates of sliced bread from different batches in correspondence of these acceptability limits.

Table 2: Shelf life estimates in correspondence of different θ_t values criteria and acceptability limits (firmness) for sliced bread from different batches stored in the dark at 25°C under laboratory conditions. 95% lower and upper confidence limits (LCL and UCL) of shelf life are also reported.

Batch	θ_t	Firmness (N)	Shelf life (days)		
			LCL	Estimate	UCL
1	0.10	2.79	73	85	101
	0.20	2.53	51	59	70
	0.50	1.77	22	25	30
2	0.10	2.79	41	50	65
	0.20	2.53	29	35	45
	0.50	1.77	12	15	19
3	0.10	2.79	50	56	64
	0.20	2.53	35	39	44
	0.50	1.77	15	17	19
4	0.10	2.79	56	72	104
	0.20	2.53	39	51	73
	0.50	1.77	17	22	31

It can be noted that the shelf life estimate was strictly dependent on the choice of the acceptability limit. This result is in agreement with data reported by GUERRA *et al*. (2008), showing that the acceptability limit easily affects the final shelf life by more than 20%. As expected based on the different reaction rates (Table 1), the shelf life of sliced bread resulted significantly affected by the batch. In particular, for a given acceptability limit, the shelf life of sliced bread from batches 2 and 3 resulted comparable and almost half than that of batch 1. Even if batch 4 shelf life estimates were similar to those of batch 1, they were characterized by large confidence levels suggesting the existence of a high variability

within this batch. In other words, each bread batch may show specific firmness variability, begetting shelf life values with considerably different confidence interval. The latter dramatically increase the uncertainty of shelf life.
It is noteworthy that the producer attributes this sliced bread a commercial shelf life equal to 75 days. It can be thus inferred that the company highly tolerated bread firming at shelf life. The latter actually corresponded to an acceptability limit approaching 0.10 θ_i, equal to 2.79 N bread firmness. By selecting such an acceptability limit, the shelf life assessment tests carried out in the laboratory would indicate a shelf life, computed as the average value of the shelf life estimates of the four batches (Table 2), equal to 66±16 days. It is evident that this shelf life estimate intensely suffered by the variability of the firmness kinetics of the four bread batches considered in the experiment. Under these conditions, the application of the conventional laboratory procedure to estimate shelf life would lead to a product dating strictly dependent on the bread batch considered in the experimental test.

3.2. Market shelf life test

To validate whether the laboratory shelf life estimates really account for the shelf life of the product available on the market, bread samples analogous to those considered in the laboratory shelf life test were purchased in local stores having different size, sale volume, logistics and distribution platforms. Collected bread samples not only had different storage time but also had suffered storage conditions that varied depending on the logistics and distribution modalities of the selected store. Bread samples were then analyzed for firmness (Table 3).
It can be noted that, in some cases, samples having the same storage time but taken from different stores exerted considerably different firmness. For instance, samples stored for 43 days presented firmness values between 2.81 and 1.76 N.
Firmness data were then used to compute the evolution of θ_i value (Fig. 3) and modelled according to the restricted Avrami equation (equation 1 with n = 1.0). The relevant rate constant resulted equal to 0.024±0.002. Statistical analysis showed that the rate was not significantly different from that observed for bread slices from batches 1 and 4 stored under laboratory conditions ($p > 0.05$), but significantly lower than those observed for batches 2 and 3.
Table 4 reports the shelf life values of sliced bread taken from the market assuming as acceptability limits firmness values analogous to those selected for shelf life estimate of bread stored under laboratory conditions. Shelf life values similar to those observed for bread slices from batch 1 and 4 (Table 2). The 95% confidence limits appeared similarly wide to those of batch 1, indicating that the shelf life test performed in the lab on samples from batch 1 provided a reasonable estimation of the shelf life of the product stored on the market. By contrast, confidence limits of market shelf life were largely lower than those of batch 4, indicating that the use of this batch to estimate the shelf life of the product on the market would provide an uncertain shelf life value. Finally, performing the shelf life test on samples from batch 2 and 3 would imply an almost 50% underestimation of the actual shelf life of the product on the market.
The discrepancy observed between laboratory and market shelf life could be attributed to a high batch variability as well as changes of environmental conditions during storage and distribution. Results suggest that shelf life estimates obtained in the laboratory should be validated by checking the evolution of product quality on the market. Although time consuming and cost effective, this process could become highly sustainable when integrated in the analysis of historical data concerning the "real" performance of the product on the shelves, including consumer complaints and recall data.

Table 3: Firmness of bread samples collected in different stores and belonging to different production batches as a function of storage time.

Batch	Storage time (days)	Firmness (N)
1	7	0.68±0.03
2	13	1.02±0.09
3	13	1.02±0.16
4	18	1.43±0.13
5	19	1.46±0.16
6	20	1.45±0.23
7	20	1.90±0.03
8	24	1.58±0.10
9	24	1.73±0.02
10	24	2.43±0.20
11	24	2.62±0.09
12	27	0.98±0.10
13	31	1.71±0.14
14	31	1.77±0.13
15	34	1.39±0.04
16	34	2.00±0.09
17	34	2.11±0.09
18	35	1.62±0.27
19	38	1.77±0.17
20	38	2.01±0.20
21	43	1.76±0.14
22	43	1.93±0.15
23	43	2.58±0.10
24	43	2.81±0.18
25	51	1.92±0.09
26	51	2.18±0.15
27	56	2.34±0.18
28	56	2.62±0.02
29	61	2.40±0.22
30	61	2.59±0.15
31	69	2.47±0.11
32	69	3.03±0.06
33	75	2.23±0.19
34	75	2.34±0.10
35	75	2.38±0.23

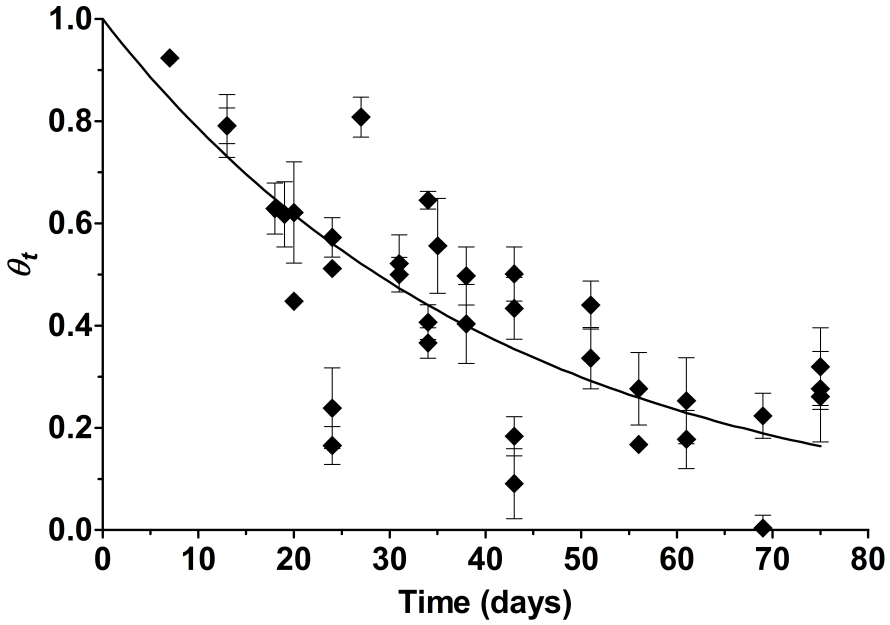

Figure 3: θ_t values (non-firmed fraction) of sliced bread taken on the market. Points: experimental data; lines: predicted values under the restricted Avrami model.

Table 4: Shelf life estimates in correspondence of different θ_t values criteria and acceptability limits (firmness) for sliced bread taken from the market. 95% lower and upper confidence limits (LCL and UCL) of shelf life are also reported.

θ_t	Firmness (N)	Shelf life (days)		
		LCL	Estimate	UCL
0.10	2.79	83	95	112
0.20	2.53	58	67	79
0.50	1.77	25	29	34

4. CONCLUSIONS

The methodology here proposed could represent a powerful tool to validate the reliability of laboratory shelf life dating. Moreover, its application would allow the continuous improvement of shelf life accuracy by progressively adding new data coming from further product batches. On the other hand, the availability of market shelf life data could be exploited to easily identify batch outliers, obtaining indications about possible issues deriving from variability of raw material, formulation or processing conditions.
Although data were relevant to the study case of sliced white bread and additional studies should be accomplished to fully validate the proposed approach, results suggest that caution should be paid to shelf life estimation carried out only under laboratory conditions. It is a matter of fact that further complications should be expected in products stored under chilled and frozen conditions, for which slight temperature changes could dramatically modify the kinetics of quality depletion as well as the prevalent event affecting shelf life.

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PAPER

ARABINOXYLANS AND β-GLUCANS ASSESSMENT IN CEREALS

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ABSTRACT

Arabinoxylans (AX) and β-glucans are the major source of soluble dietary fibre in cereals and have a significant role as functional/bioactive ingredients implicated in lowering plasma cholesterol, postprandial blood glucose and improving lipid metabolism. In this work, the variation in the content and solubility of AX and β-glucans in different cereal species and varieties were studied. Different methods (phloroglucinol, orcinol-HCl, HPAEC-PAD) for AX analysis were tested. The results confirmed the variability in contents of both polymers (AX and β-glucans) in cereal species and varieties as well as providing additional information useful for their characterization.

Keywords: Arabinoxylans, barley, β-glucans, emmer, spelt, wheat

1. INTRODUCTION

The most common source of dietary fibre are the outer layers and the endosperm cell walls of cereal grains (wheat, barley, oat, rye etc.). The non-starch polysaccharides (NSP) found in mature cereal grain include the arabinoxylans (AX), which make up the pentosan portion of the insoluble fibre fraction and the β-glucans which are major components of starchy endosperm and aleurone cell walls.

AX have been identified in a variety of tissues of major cereals: wheat, rye, barley, oats, rice, sorghum (FINCHER and STONE, 1986). Although these polysaccharides are minor components of the whole caryopsis, they still account for a substantial fraction of the cell walls and thus constitute a major portion of the dietary fibre of wheat flours (SKENDI *et al*., 2011). Wheat varieties differ in AX amount and properties (MASLEN *et al*., 2007) such as average molecular weight and distribution, branching pattern, extractability with water (FINNIE *et al*., 2006) and interaction with other cell wall components such as lignin or cellulose (REVANAPPA *et al*., 2007).

AX consist mainly of a xylan chain with *β*-1,4-linked D-xylopyranosyl residues (Xyl) to which mostly single *α*-L-arabinofuranose units (Ara) are linked at the O-2 and/or O-3 positions of the xylose units as side residues (IZYDORCZYK and BILIADERIS, 1995). In wheat AX, approximately 66% of the xylose residues, that form the backbone chain, are unsubstituted Xyl (SAULNIER *et al*., 2007b). Moreover, some Ara units carry ferulic acid residues esterified to O-5 of Ara linked to O-3 of the xylose residues (SOSULSKI *et al*., 1982). Extractable AX and unextractable AX structures are similar, showing only slightly differences in molecular weight and in the Ara/Xyl ratio (IZYDORCZYK and BILIADERIS, 1995).

AX represent the major polysaccharides in the aleurone fraction (65%) of the wheat caryopsis, and the arabinose to xylose ratio decreases from the pericarp to the endosperm; furthermore AX from the aleurone layer as well as AX from starchy endosperm have a lesser degree of branching than acidic AX from pericarp/testa, which may improve their solubility and their digestibility (BROUNS *et al*., 2012; SAULNIER *et al*., 2007a).

β-glucans are linear polymers of high molecular weight consisting of D-glucose molecules linked by β-(1-4) and β-(1-3) linkages; the presence of β-linkages (1-3) gives to the molecule an irregular shape that makes the β-glucans flexible and partially soluble in water (PAPAGEORGIOU *et al*., 2005; SHELAT *et al*., 2011).

From a nutritional point of view, as major non-starch polysaccharides of various cereals and main constituents of cell walls of wheat, rye, barley and oat, β-glucans and soluble AX have nutritional benefits in humans (WARD *et al*., 2008). The positive effects of AX are related to the ability to reduce postprandial blood glucose levels (GARCIA *et al*., 2007; LU *et al*., 2000), lowering levels of potentially toxic ammonia in the colon and the ability to reduce levels of triglycerides in the blood (GARCIA *et al*., 2006). Over the last two decades β-glucans have been considered as bioactive ingredients due to their capacity in lowering plasma cholesterol, improving lipid metabolism, and reducing the glycaemic index (MÄKELÄINEN *et al*., 2007; WOOD, 2007). These positive effects increased the popularity and consumption of cereal-based foods as well as of many other foods fortified with cell wall-enriched grain fractions, β-glucan concentrates and isolates (LAZARIDOU and BILIADERIS, 2007).

Furthermore, the Commission Regulation (EU) No 432/2012 of 16 May 2012 (Official Journal of the European Union 25-05-2012) included arabinoxilans and β-glucans in the list of authorized health claims.

In this study, the content of AX and β-glucans in several cereal wholemeal flours was determined using different techniques, whose efficacy was compared.

2. MATERIALS AND METHODS

2.1. Samples

- Five barley varieties, Acquarelle, Braemar, Kelibia, Naturel, USA 2 (waxy variety), were provided by Agroalimentare Sud S.p.A. (Melfi, Potenza, Italy).
- Five spelt varieties, Ebners Rotkorn, Hercule, Oberkulmer, Redoute, Triventina, were provided by Agenzia Regionale per lo Sviluppo Agricolo, Rurale e della Pesca (ARSARP, Campobasso, Italy).
- Five emmer varieties, Molise, Angelo, Garfagnana, Molise Colli, Guardiaregia, were provided by ARSARP (Campobasso, Italy).
- Two durum wheat varieties, Cappelli, Saragolla, were provided by ARSARP (Campobasso, Italy).
- Three soft wheat varieties, Roscetta, Solina 1, Bianchetta, were provided by ARSARP (Campobasso, Italy).

Samples were milled in a refrigerated laboratory mill IKA A 10 Labortechnic (Tanke & Kunkel, Gmbh & Co., Staufe, Germany) and stored in aliquots of 100 g at 4°C in a closed containers until analysis.
Data reported for all parameters are the average values of three different aliquots of each sample. All results are expressed as % dry weight (d.w.). Moisture content was determined according to ICC method 109/1 (ICC, 1995).

2.2. Reagents

NaOH 50% (p/v) was purchased from Baker (Mallinckrodt Baker B.V., Deventer, Holland), high-purity laboratory water was produced by means of a MilliQ-Plus apparatus (Millipore S.p.A., Milano, Italy); glucose, xylose, arabinose, fructose, standards were from Sigma Chemical Co. (St. Louis, MO, USA); all other chemicals and reagents of HPLC grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. AX analysis by phloroglucinol method

Total AX analysis was performed as reported by DOUGLAS (1981). Flour (5.0 mg) was added to 2 mL of water followed by 10 mL of a solution of glacial acetic acid, hydrochloric acid and phloroglucinol in a stoppered tube. The tube was placed in a boiling water bath for 25 min and the absorbance of the resulting solution measured at 552 nm and 510 nm.

2.4. AX analysis by orcinol-HCl method

Water soluble AX and total AX analysis by orcinol-HCl method was performed as reported by HASHIMOTO *et al*. (1987).
Water soluble AX: 100 mg of flour sample were shaken in water at 30°C for 2 h and centrifuged. Aliquots of the supernatant were hydrolyzed with 4N HCl at 100°C. The AX content was estimated, after treatment in boiling water bath with $FeCl_3$ and orcinol and by reading the absorbance at 670 nm.
Total AX: flour (10 mg) was weighed into a glass tube, where 2 mL of 2N HCl was added, and the mixture was hydrolyzed at 100°C for 150 min. After cooling, neutralization was carried out by adding 2N sodium carbonate and fermentable sugars were removed by means of fresh compressed yeast. The mixture was then centrifuged and an aliquot of the supernatant was treated with $FeCl_3$ and orcinol in boiling water bath; the absorbance was read at 670 nm.

2.5. AX analysis by HPAEC-PAD

Determination of arabinoxylans was performed as reported by MESSIA *et al*. (2016). Briefly, for water soluble AX: 100 mg of a flour sample were shaken in 10 mL of water at 30°C for 2 hours and centrifuged. Aliquots (1 mL) of the supernatant were hydrolyzed with 1 mL of 4N HCl for 2 hours. For total AX, flour sample (10 mg) was weighed into a glass tube, 2 mL of 2N HCl were added, and the mixture was hydrolyzed at 100°C for 150 min. After cooling, neutralization was carried out by the addition of 2N sodium carbonate. Diluted samples were injected in a chromatographic system equipped with a Rheodyne injector (Cotati, CA, USA) with a 25 μL loop. The chromatographic separation was carried out with a Carbopac PA1 (250x2 mm) (Dionex Corporation, Sunnyvale, CA, USA) analytical column. The chromatographic run (22 min) and the quantitative determination were conducted with a 0.25 mL/min flow rate, using a mobile phase of water and 200 mM sodium hydroxide (90%-10%). The control of the instrument, the data collection and the total quantification were carried out by the chromatographic software Chromeleon (Dionex). An HPAEC-PAD Dionex system (Dionex Corporation, Sunnyvale, CA, USA) composed of a gradient pump (mod GP50) with an on-line degaser and electrochemical detector (model ED40) was used. The flow-through electrochemical cell (Dionex) consisted of a 1 mm diameter Gold Working Electrode, a pH reference electrode, and a titanium body of the cell as the counter electrode. The optimized time-potential waveform used was: 0.1 V at 0-0.40 s, -2.00 V at 0.41-0.42 s, 0.60 V at 0.43 s, -0.10 V at 0.44-0.50 s.
Total and soluble AX were quantified on the basis of the Ara and Xyl content in the hydrolyzed sample: ([Ara] + [Xyl] x D x 0.88), where: D = dilution factor; 0.88 =adjustment for free sugar to anhydrous sugar.

2.6. β-glucans analysis

Total β-glucans were determined using the K-BGLU assay kit (Megazyme International Ltd., Ireland). Insoluble fractions were determined after extraction of soluble β-glucans with water for 2 h at 38°C (ÅMAN and GRAHAM, 1987). Soluble β-glucans were calculated as the difference between the total and insoluble components.

2.7. Statistical analysis

All analyses were carried out in triplicate. Results were expressed as means±Standard Deviation (SD).
A one-way analysis of variance (ANOVA) was performed, considering species, variety or analytical method as factor. When significant differences ($p < 0.05$) were detected, Fisher's least significant difference (LSD) was computed. All the statistical tests were performed using the software IBM SPSS statistics 23.

3. RESULTS

3.1. AX analysis and quantification

A comparison between three different analytical methods (HPAEC-PAD and colorimetric methods) was carried out in order to devise a practical system for screening of different varieties of grains such as barley, wheat, spelt and emmer (Table 1).

Table 1. Total and soluble AX content in different cereal wholemeals determined by three different methods (g/100g d.w.±SD).

Sample	Phloroglucinol	Orcinol-HCl		HPAEC-PAD				
				Total		Soluble		Soluble AX/ Total AX
	Total AX	Total AX	Soluble AX	Total AX	A/X	Soluble AX	A/X	
Barley								
Acquarelle	4.92±0.05^{b}	6.42±0.06^{d}	0.09±0.03^{a}	6.18±0.03^{d}	0.85	0.08±0.02^{a}	0.65	0.013
Braemar	4.30±0.04^{d}	5.94±0.05^{e}	0.06±0.06^{a}	5.21±0.07^{e}	0.75	0.07±0.03^{a}	0.60	0.013
Kelibia	5.08±0.06^{c}	7.30±0.08^{b}	0.08±0.04^{a}	6.87±0.05^{b}	0.73	0.10±0.03^{a}	0.66	0.015
Naturel	4.85±0.03^{b}	7.01±0.03^{c}	0.08±0.06^{a}	6.76±0.04^{c}	0.72	0.09±0.04^{a}	0.62	0.013
USA 2	6.17±0.08^{a}	9.75±0.04^{a}	0.11±0.05^{a}	8.96±0.03^{a}	0.47	0.12±0.05^{a}	0.71	0.013
mean	**5.06^{b}$_{\alpha}$**	**7.28^{a}$_{\beta}$**	**0.08^{A}$_{\beta}$**	**6.80^{a}$_{\beta}$**	**0.70**	**0.09^{A}$_{\alpha}$**	**0.65**	**0.013**
Wheat								
Cappelli*	5.10±0.04^{c}	8.78±0.05^{a}	0.09±0.02^{a}	8.21±0.04^{a}	0.78	0.10±0.03^{a}	1.25	0.012
Saragolla*	5.18±0.03^{bc}	8.17±0.04^{d}	0.09±0.03^{a}	7.90±0.02^{b}	0.80	0.10±0.06^{a}	0.80	0.013
Roscetta**	5.49±0.05^{a}	8.76±0.05^{a}	0.11±0.03^{a}	8.16±0.10^{a}	0.66	0.12±0.03^{a}	0.71	0.015
Solina 1**	5.15±0.04^{bc}	8.54±0.07^{b}	0.10±0.04^{a}	7.97±0.04^{b}	0.71	0.12±0.04^{a}	0.71	0.015
Bianchetta**	5.23±0.06^{b}	8.32±0.04^{c}	0.11±0.03^{a}	7.98±0.03^{b}	0.74	0.13±0.05^{a}	0.71	0.016
mean	**5.23^{c}$_{\alpha}$**	**8.51^{a}$_{\alpha}$**	**0.10^{A}$_{\alpha}$**	**8.04^{b}$_{\alpha}$**	**0.74**	**0.11^{A}$_{\alpha}$**	**0.84**	**0.014**
Spelt								
Ebners Rotkorn	4.18±0.05^{b}	5.57±0.04^{b}	0.14±0.04^{a}	5.41±0.05^{a}	0.66	0.11±0.02^{a}	0.77	0.020
Hercule	4.29±0.06^{a}	5.97±0.05^{a}	0.14±0.03^{a}	5.34±0.07^{a}	0.68	0.12±0.02^{a}	0.85	0.022
Oberkulmer	3.99±0.04^{d}	5.18±0.06^{c}	0.12±0.03^{a}	4.75±0.05^{c}	0.64	0.12±0.04^{a}	0.95	0.025
Redoutè	3.89±0.07^{c}	5.13±0.05^{c}	0.14±0.03^{a}	4.60±0.06^{d}	0.61	0.10±0.02^{a}	0.80	0.022
Triventina	4.20±0.04^{b}	5.63±0.10^{b}	0.11±0.02^{a}	5.03±0.03^{b}	0.65	0.11±0.03^{a}	0.78	0.022
mean	**4.11^{c}$_{\beta}$**	**5.50^{a}$_{\gamma}$**	**0.13^{A}$_{\alpha}$**	**5.03^{b}$_{\gamma}$**	**0.65**	**0.11^{A}$_{\alpha}$**	**0.83**	**0.022**
Emmer								
Molise	3.65±0.04^{c}	6.48±0.04^{b}	0.23±0.04^{a}	5.89±0.04^{b}	0.67	0.18±0.05^{a}	1.04	0.031
Angelo	3.76±0.03^{b}	5.26±0.06^{c}	0.11±0.05^{b}	5.03±0.05^{d}	0.70	0.10±0.06^{b}	1.16	0.020
Garfagnana	4.12±0.09^{a}	5.63±0.03^{d}	0.11±0.03^{b}	5.29±0.03^{c}	0.62	0.11±0.05^{b}	1.00	0.021
Molise Colli	4.06±0.05^{a}	5.31±0.05^{c}	0.09±0.05^{b}	4.90±0.08^{e}	0.58	0.07±0.04^{b}	1.10	0.014
Guardiaregia	3.60±0.06^{c}	6.76±0.07^{a}	0.12±0.04^{b}	6.32±0.04^{a}	0.69	0.08±0.05^{b}	1.40	0.013
mean	**3.84^{c}$_{\gamma}$**	**5.89^{a}$_{\gamma}$**	**0.13^{A}$_{\alpha}$**	**5.49^{b}$_{\gamma}$**	**0.65**	**0.11^{A}$_{\alpha}$**	**1.14**	**0.020**

*durum wheat; **soft wheat

Different superscript letters (total AX=lower case, soluble AX=upper case) between species means within a row indicate statistically significant differences at $P< 0.05$.

Different superscript letters between means (species=Greek font, variety=italic font) within a column indicate statistically significant differences at $P< 0.05$.

The systematic quantification of AX in cereal wholemeal flours revealed substantial differences between the colorimetric procedures. Although phloroglucinol and orcinol-HCl procedures are both based on colorimetric assessments, for the measurement of total AX, the phloroglucinol method provided values that were significantly lower than those of the orcinol-HCl method. This underestimation of total AX can be attributed to the type of extraction performed for the phloroglucinol assay, that leads to a relevant release of hexoses from starch hydrolysis, which greatly exceeds pentoses, influencing their

evaluation. In the orcinol-HCl method, the high glucose concentrations are removed by the action of the yeast *Saccharomyces cerevisiae*, thus providing a better assessment of the total AX; additionally the orcinol-HCl method allows to evaluate not only the total AX but also the soluble AX, that cannot be quantified with the phloroglucinol method.
A significant advantage of the HPAEC-PAD method is to provide more detailed information, as it offers the possibility to assess the content of individual sugars Ara and Xyl, which are constituents of the AX chain, and it allows to calculate the ratio Ara/Xyl (A/X), an important factor related to the behavior of the flour during technological processes. HPAEC-PAD assessments also give a more accurate estimation of the sugars present in the chain of AX without suffering problems caused by glucose interference, thus providing a more accurate and precise quantitative analysis. Finally, the HPAEC-PAD can be applied to matrices with high contents of AX (bran) as well as to refined flour (low AX content).
To sum up, the assessment made using the phloroglucinol is not reliable, whereas the values of total and soluble AX found in varieties of barley, wheat, spelt and emmer by using of either the orcinol-HCl or HPAEC-PAD methods are similar and comparable to the values of AX reported in the literature (BERGER and DUCROO, 2005, HENRY, 1987) for these cereals.
GEBRUERS *et al*. (2008) have published data about the content of AX in refined flours and in bran of wheat, spelt and einkorn showing a comparable value of total AX in the different types of flour examined. Instead, the results obtained in this trial show a much lower content of total AX in spelt an emmer (≈ 5.26%) compared to barley and wheat (6.80% and 8.04% respectively). This is in spite of the fact that spelt and emmer are phylogenetically close to wheat.
The data obtained also show a generally close relationship between AX content and cereal varieties. Among barleys, USA2, a waxy (i.e. having a low amylose content) variety has the highest content of β-glucans (9.5%) (Table 2) and of total AX (6.2%). The content of AX in barley also depends on genetic and environmental factors (IZYDORCZYK and DEXTER, 2008) but appears to be less variable than that of β-glucans.
The HPAEC-PAD method allows to compute the ratio A/X which indicates the degree of branching in the polymer chain, thus permitting to deduce information about the AX structure of different species and varieties. A high A/X ratio corresponds to a higher proportion of mono-substituted xylosyl residues and a lower proportion of unsubstituted xylosyl residues. The degree of substitution of xylan backbone is relevant for predicting the cereal behavior when subjected to different technological processes. Emmer varieties showed an A/X ratio of soluble AX (1.14), much higher than the ratios of barley (0.65), wheat (0.84) and also spelt (0.83).
From a technological point of view, the quantification and the assessment of variations in the overall AX content is relevant because AX is generally considered to have a significant effect on wheat functionality and also to affect suitability of flours for certain applications (GEBRUERS *et al*., 2008). Cereal varieties rich in AX, have a strong potential for the production of healthy or even health promoting food products that contain not only a high overall dietary fiber content but also increased levels of soluble dietary fiber as well as prebiotic oligosaccharides which are produced by the in situ action of xylanases (GEBRUERS *et al*., 2008).

3.2. β-glucans analysis and quantification

Total, insoluble and soluble β-glucans were quantified in different cereals (barley, wheat, spelt, emmer) flours (Table 2). Results showed a different β-glucans distribution in tested species and between waxy and non waxy barley varieties.

Table 2. β-glucans content, AX+ β-glucans and AX/β-glucans in different cereal wholemeals (g/100g d.w.±SD).

Sample	β-glucans				AX+β-glucans	AX/β-glucans
	Total	Insoluble	Soluble	Soluble/ Insoluble		
Barley						
Acquarelle	4.43±0.13^{c}	1.50±0.01^{e}	2.94	1.97	10.61	1.40
Braemar	3.89±0.11^{e}	1.92±0.07^{b}	1.97	1.03	9.07	1.34
Kelibia	4.17±0.07^{d}	1.61±0.02^{d}	2.57	1.60	11.04	1.65
Naturel	4.67±0.11^{b}	1.81±0.04^{c}	2.86	1.58	11.43	1.45
USA 2	9.49±0.09^{a}	3.42±0.08^{a}	6.08	1.78	18.45	0.94
mean	**5.33^{A}**	**2.05^{A}**	**3.28**	**1.59**	**12.12**	**1.36**
Wheat						
Cappelli *	0.52±0.03^{c}	0.41±0.03^{b}	0.11	0.27	8.73	15.79
Saragolla *	0.42±0.02^{d}	0.22±0.01^{c}	0.20	0.93	8.32	18.81
Roscetta **	0.60±0.05^{b}	0.42±0.06^{b}	0.19	0.45	8.76	13.60
Solina 1 **	0.53±0.03^{c}	0.37±0.03^{b}	0.16	0.42	8.50	15.00
Bianchetta **	0.79±0.01^{a}	0.60±0.03^{a}	0.19	0.31	8.77	10.10
mean	**0.57^{B}**	**0.40^{B}**	**0.17**	**0.47**	**8.62**	**14.66**
Spelt						
Ebners Rotkorn	0.68±0.02^{ab}	0.44± 0.05^{ab}	0.24	0.55	6.09	7.96
Hercule	0.63±0.05^{b}	0.42± 0.04^{ab}	0.21	0.50	5.97	8.48
Oberkulmer	0.70±0.02^{a}	0.49± 0.03^{a}	0.21	0.43	5.45	6.78
Redoutè	0.65±0.03^{ab}	0.47± 0.03^{a}	0.18	0.38	5.25	7.08
Triventina	0.57±0.02^{c}	0.40±0.03^{b}	0.17	0.43	5.60	8.82
mean	**0.65^{B}**	**0.44^{B}**	**0.20**	**0.46**	**5.67**	**7.82**
Emmer						
Molise	0.48±0.03^{b}	0.37±0.01^{a}	0.12	0.32	6.37	12.27
Angelo	0.41±0.02^{c}	0.29±0.05^{b}	0.12	0.42	5.44	12.27
Garfagnana	0.37±0.01^{c}	0.28±0.03^{b}	0.09	0.30	5.66	14.30
Molise Colli	0.53±0.02^{a}	0.35±0.03^{ac}	0.18	0.51	5.43	9.24
Guardiaregia	0.48±0.03^{b}	0.30 ±0.02^{bc}	0.18	0.61	6.8	13.17
mean	**0.45^{B}**	**0.32^{B}**	**0.14**	**0.43**	**5.94**	**12.25**

*durum wheat; **soft wheat.
Different superscript letters between means (species=upper case, variety=lower case) within a column indicate statistically significant differences at $P< 0.05$.

In barley, the variable β-glucans content can be influenced by genotype, culture practices and environmental growing conditions (NEWMAN and McGUIRE,1985; Newman and

Newman, 2008). The presence of waxy genes can influence polysaccharides biosynthesis and their composition in the kernel. In the "normal" barley genotype, the starch is composed of about 25% of amylose and 75% amylopectin while in the waxy genotypes the starch is almost exclusively composed of amylopectin (95-100%). The reduced level of starch is usually accompanied with an increased content of β-glucans in the cell walls of the starchy endosperm (ANDERSSON *et al*., 2008). In fact, according to many research reports (ABDEL-AAL *et al*., 2005; WOOD *et al*., 2003) a waxy barley variety (USA2) showed a β-glucans content which was twofold that of non waxy varieties.

Comparing the data from different species (Table 2), it is evident that there is a significant difference in the average content of total β-glucans among barley and wheat, spelt and emmer (5.33% d.w. for barley, 0.57% d.w for wheat, 0.65% d.w. for spelt and 0.45% d.w. for emmer), confirming the data reported in the literature by other authors (SKENDI *et al*., 2003).

With regard to soluble β-glucans content, varieties of wheat, spelt and emmer are characterized by very low levels of soluble β-glucans (average: 0.17%, 0.20% and 0.14% d.w., respectively), probably related to a higher ratio of cellotriose/cellotetraose, which generally amounts to 4.6 and 3.3 respectively in wheat and barley. Statistically, although the distribution of cellotriose and cellotetraose units linked by β (1-3) is random (WOOD *et al*., 2003), the probability of a repetition of ordered cellotriose units is greater in wheat than in barley and oats (CUI *et al*., 2000). Since the structure is more ordered and more inter chain associations are favoured, the water solubility of β-glucans derived from wheat is lower than that of other cereals (LAZARIDOU and BILLIADERIS, 2007).

The soluble β-glucans content affects the soluble/insoluble ratio, which is greater in barley (1.59) than in wheat, spelt and emmer (0.47, 0.46 and 0.43 respectively). Data on total content of AX + β-glucans and their ratio (AX/β-glucans) (Table 2) showed significant differences between the analyzed wholemeals. The high content of AX + β-glucans in barley corresponded to a low AX/β-glucans ratio, which is further reduced in the waxy barley variety USA 2 (0.94). While β-glucans are mainly present in barley, the AX are distributed in all the analyzed species and varieties. The relevant presence of two polymers in barley makes this cereal an excellent ingredient for the preparation of products with an increased content of total and soluble dietary fiber, capable of enhancing both the physiological effects and health benefits (VERARDO *et al*., 2011a; VERARDO *et al*., 2011b; VITAGLIONE *et al*., 2010).

Moreover, β-glucans and AX are the chief structural constituents of cell wall in various tissues of the barley grain. In the starchy endosperm of mature barley grain, the matrix-phase AX and β-glucans may represent up to 85% of total cell wall polysaccharides. The endosperm cell walls are mainly made up of β-glucans and contains a smaller amount of AX, while aleurone cell walls are composed primarily of AX (67-71%), with smaller amounts of β-glucans (26%). Various studies have been carried out as to the possibility of producing barley flour enriched in β-glucans using air classification and dry milling methods to produce an enriched flour that can be used for producing different cereal products, such as bread, muffins, and pasta (MARCONI *et al*., 2000).

4. CONCLUSIONS

The present study revealed a wide variability in the content of AX and β-glucans in wholemeal of different cereal species and varieties.

Barley varieties showed a higher β-glucans and AX content compared to other cereal species/varieties. The assessment of the A/X ratio for AX and soluble/insoluble β-glucans ratio, which directly affect the behavior of cereal flours during transformation, are useful for deciding the end use of grains and evaluating the nutritional quality of the analysed grains.
The HPAEC-PAD method proved to be advantageous compared to colorimetric methods. It allows to compute the ratio A/X which indicates the degree of branching in the polymer chain, thus permitting to deduce information about the AX structure of different species and varieties.
The quantification and the assessment of variations in the overall AX content is relevant because AX is generally considered to have a significant effect on cereal flours functionality and also to affect suitability of flours for certain applications.
Cereal varieties rich in AX and β-glucan, have a strong potential for the production of healthy or even health promoting food products that contain not only a high overall dietary fiber but also increased levels of soluble dietary fiber, that should meet the health claims listed in the Commission Regulation (EU) No 432/2012.

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SENSORY PROPERTIES AND PHYSICO-CHEMICAL CHANGES IN THE *BICEPS FEMORIS* MUSCLE DURING PROCESSING OF DRY-CURED HAM FROM CELTA PIGS. EFFECTS OF CROSS-BREEDING WITH DUROC AND LANDRACE PIGS

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ABSTRACT

We investigated how cross-breeding Celta pigs with Landrace and Duroc pigs affected the physico-chemical properties and sensory characteristics of the *biceps femoris* muscle during the manufacturing of dry-cured ham. The intramuscular fat (IMF) content was significantly (P<0.001) affected by cross-breed: the IMF content of hams from the Duroc x Celta crosses (13.92%) was higher than that of hams from pure-bred Celta pigs (8.03%), and the IMF content of hams from the Landrace x Celta crosses was intermediate (12.27%). Instrumental colour parameters were also slightly affected by cross-breed: hams from cross-bred pigs were yellower (CIE b*-value) and lighter (CIE L*-value) than hams from the pure-bred Celta pigs. At the end of the process, shear force did not differ significantly (P>0.05) between groups, although the values were lowest in hams from Duroc x Celta crosses. In sensory analysis, panellists described hams from cross-bred pigs as of softer texture (P<0.01) and juicier (P<0.01) than hams from the pure-bred Celta pigs.

Keywords: *biceps femoris* muscle, crossbreeding, dry-cured ham, physico-chemical characteristics, sensory properties

1. INTRODUCTION

The Celta breed was the typical breed of pig raised on farms in Galicia (NW Spain) until the middle of the 20th century, when it underwent a strong decrease in numbers until near disappearance (< 200 head) due to the introduction of improved breeds and crosses (GÓMEZ and LORENZO, 2013). However, in recent years native pig breeds have become highly appreciated for their rusticity and also for the quality of their meat products. Dry-cured meat products have an important added value for the pig industry, and production of Celta pigs is mainly focused on obtaining raw meat to manufacture products such as dry-cured ham (BERMÚDEZ *et al.*, 2012), dry-cured "lacón" (LORENZO *et al.*, 2014) and sausages (GÓMEZ and LORENZO, 2013).

Dry-cured meat products represent a large proportion of the meat products on the European market, especially in the Mediterranean countries, and dry-cured ham is recognised as a high quality product of increasing economic importance (JIMÉNEZ-COLMENERO *et al.*, 2010). However, the quality of the end product is closely linked to the characteristics of the raw material, especially those related to the degree of marbling and the fatty acid composition, which in turn depend on factors such as breed, slaughter age and finishing feed (BERMÚDEZ *et al.*, 2012; GARCÍA-GONZÁLEZ *et al.*, 2008). In this regard, FRANCI *et al.* (2007) concluded that breed had a marked effect on the physico-chemical and sensory properties of Tuscan dry-cured ham.

Current pig breeding schemes in Europe are based on a backcross or on a three-four cross. In Spain, the most common cross is one that uses different breeds such as Landrace, Large White, Pietrain and Belgian Landrace. During the last few years, the Duroc breed has been introduced by national breeders to supply dry-cured meat producers who prefer hams with higher levels of intramuscular fat (GOU *et al.*, 1995). One of the options most often used to improve the productive parameters of the Celta pig is to cross this breed with the Duroc or Landrace breed (FRANCO *et al.*, 2014). FRANCO *et al.* (2014) concluded that cross-breeding with Landrace and Duroc lines affected the carcass characteristics and meat quality. Therefore, the Landrace and Duroc genotypes may affect the physico-chemical and sensorial properties of Celta dry-cured ham. The aim of this study was to evaluate how cross-breeding Celta pigs with Landrace and Duroc pigs affects the physico-chemical and sensorial properties of the *bicep femoris* muscle throughout the manufacturing of dry-cured ham.

2. MATERIALS AND METHODS

2.1. Animals and management

A total of 52 pigs (26 entire females [EF] and 26 castrated males [CM]) were divided into three groups according to genotype: 16 pure-bred Celta pigs (C), 20 Landrace x Celta (C × L) cross-bred pigs, and 16 Duroc x Celta (C × D) cross-bred pigs. The pigs in each group were balanced for gender (half males/half females), they were born at around the same time and the live weight at birth of all pigs was similar. All of the Celta pigs were registered in the record of births in the farm stud book. Animals were reared all together in an outdoor system by *Porco Celta* (a pig farming co-operative operating in the province of Lugo, Galicia, NW Spain). The pigs were fed ad libitum a commercial diet (17% protein, 2.4% fat and 3250 kcal/kg metabolic energy) and were provided free access to water. As the pigs were reared in a natural environment, part of the diet was obtained from natural vegetation. Campsite style huts and trees in the area provided shade. The pigs were reared following the recommendations of the legislation for pig welfare and protection

(COUNCIL DIRECTIVE 2008/120/EC, 2009). The pigs were slaughtered when they reached live weights of 167.30±11.65, 168.90±14.81 and 165.43±7.54 kg (P>0.05) for the C, C×L and C×D groups respectively; these weights were achieved at an age of around 12 months for C group, and at around 10 months for the C×L and C×D groups. The animals were transported to the abattoir the day before slaughter. The pigs from different groups were not mixed at any time, and various measures were taken to minimize stress. Pigs were slaughtered in an accredited abattoir (*Matadero Municipal de Sarria*, Lugo, Spain) and were stunned using carbon dioxide, according to the specifications outlined in the Spanish legislation.

2.2. Samples

Ham pieces were obtained after refrigerating the carcasses for 24 h at 4 °C. A total of 90 ham pieces (30 randomly chosen from each group) were used in the study. Raw pieces were dry salted with an excess of coarse salt (approximately 0.5 kg/kg of ham). A pile was formed by alternating layers of ham pieces and salt. The pieces were thus totally covered with salt. Ham pieces were salted for 11 days in a salting room at 2-5 °C and 90-95% relative humidity. After the salting stage, the pieces were removed from the pile and then brushed, washed and transferred to a post-salting room where they were held for 120 days at 3-6 °C and around 85-90% relative humidity. After the post-salting stage, the pieces were ripened for 115 days in a room where the temperature was gradually increased to 30 ºC and the relative humidity was gradually decreased to 40% for adequate drying of the thighs. The hams were then left to mature for a further 11 months ("bodega" step) in a chamber at 12-24 ºC and 70-80% relative humidity.
Samples were taken from the fresh pieces, and at the following times: end of the salting stage, end of the post-salting period, end of the drying-ripening stage and after 165 and 330 days of the "bodega" step. At each stage, a total of five ham pieces from each group were randomly collected and analyzed. Hams were transported to the laboratory under refrigerated conditions (< 4 °C) for analysis. Once in the laboratory, the entire pieces were skinned, boned, and the *biceps femoris* (BF) muscles were obtained. The muscle samples were vacuum packed and stored at -30 ºC for no more than four weeks until analysis.

2.3. Determination of pH, water activity and colour parameters

The pH of the samples was measured using a digital pH-meter (Thermo Orion 710 A+, Cambridgeshire, UK) with a penetrating probe. Water activity was assessed using a water activity meter Fast-lab (GBX, Romans-sur-Isère Cédex, France), previously calibrated with sodium chloride and potassium sulphate solutions. Colour measurements were made with a CM-600d colorimeter (Minolta Chroma Meter Measuring Head, Osaka, Japan). Briefly, at each stage of the process the muscle from each ham piece was cut and, after a blooming period of 30 min, the colour of the slices was measured three times. The CIELAB colour space lightness (L*), redness (a*) and yellowness (b*) were determined. Before each series of measurements, the instrument was calibrated with a white ceramic tile.

2.4. Determination of the chemical composition

Moisture, fat, ash and protein (Kjeldahl N x 6.25) were quantified according to the respective ISO recommended standards - 1442:1997 (ISO, 1997), 1443:1973 (ISO, 1973), 936:1998 (ISO, 1998) and 937:1978 (ISO, 1978). Total chlorides were quantified according to the Charpentier-Volhard method (ISO standard 1841-1:1996) (ISO, 1996).

2.5. Warner-Bratzler (WB) test

A Texture Analyzer TA-XT2 (Stable Micro Systems Ltd., Godalming, Surrey, UK) was used to perform Warner-Bratzler (WB) test. The samples for WB shear test were obtained by cutting pieces of approximately 1 x 1 x 2.5 cm (height x width x length). The pieces were cut across the fibers with a WB shear blade with a triangular slot cutting edge (1 mm thickness) at a crosshead speed of 3.33 mm/s. Maximum shear force, shear firmness and total work required to cut the samples were determined.

2.6. Assessment of lipid oxidation

Lipid stability was evaluated using the method proposed by VYNCKE (1975) with the modifications described by LORENZO and CARBALLO (2016). Briefly, a meat sample (2 g) was dispersed in 5% trichloroacetic acid (10 mL) and processed in an Ultra-Turrax homogenizer (Ika T25 basic, Staufen, Germany) for 2 min. The homogenate was maintained at -10 °C for 10 min and centrifuged at 2360 x g for 10 min. The supernatant was filtered through a Whatman No. 1 filter paper. The filtrate (5 mL) was reacted with a 0.02 M TBA solution (5 mL) and incubated in a water bath at 96 °C for 40 min. The absorbance was measured at 532 nm. Thiobarbituric acid reactive substance (TBARs) values were derived from a standard curve for the quantification of malondialdehyde (MDA), constructed using known concentrations of 1,1-3,3 tetraetoxipropane. The TBARs values were expressed as mg MDA/kg sample.

2.7. Sensory analysis

At the end of the manufacturing process (after 330 days of the bodega step), samples from the *biceps femoris* muscle of the five pieces of each pig group were analysed by sensory evaluation. The sensory evaluation was conducted by eight panellists from the Meat Technology Centre of Galicia. Therefore, forty qualifications were obtained for each attribute in each pig group. The panellists received training (for four months) in the attributes and the scale to be used according to the method proposed by the ISO regulations (ISO, 2012). The panellists carried out the sensory evaluations in individual cubicles, according to the ISO regulations (ISO, 2007). The panellists were given water at the beginning of the session and between tasting samples to clean the palate and remove residual flavours. The samples were individually labelled with random three-digit numbers. Ten sensory traits of the muscle, grouped according to appearance (lightness, colour of lean meat, fat yellowness and marbling), odour (intensity, rancidity and cured), taste (saltiness), and texture of the lean meat (hardness and juiciness) were assessed according to the methodology proposed by the ISO regulations (ISO, 1991; ISO, 1994; ISO, 2006). The intensity of each attribute was expressed on a structured scale ranging from 0 (very low) to 9 (very high). The normality of data was checked using the Shapiro-Wilk's normality test.

2.8. Statistical analysis

Analysis of variance (ANOVA) was applied to all variables considered in the study by using IBM SPSS Statistics 19.0 software (IBM Corporation, Somers, NY, USA). The least squares means (LSM) were separated using Duncan's t-test. A significance level of $P<0.05$ was used in all LSM tests. Cross-breed and ripening time were included as fixed effects in the model, to study physico-chemical and sensorial properties of the BF muscle.

The model used was expressed as follows:

$$Yij = \mu + Ci + Rj + \varepsilon ij$$

where Yij represents the observed values of the dependent variables, μ is the overall mean, Ci is the effect of cross-breed, Rj is the effect of ripening time, and εij is the residual random error associated with the observation. Pearson´s linear correlation coefficients were determined for the different variables with the above-mentioned statistical software package.

3. RESULTS AND DISCUSSION

3.1. Influence of cross-breed on physico-chemical changes during the manufacturing of dry-cured ham

The effects of cross-breed on the pH, water activity and chemical composition (moisture, intramuscular fat, protein, ash and chlorides) of BF muscle throughout the manufacturing process of dry-cured ham from Celta pig are summarized in Table 1. The mean pH (at 24 h) of samples from Duroc x Celta cross-bred pigs was significantly higher ($P<0.01$) than the mean pH of the samples from the other pigs. This is consistent with findings reported by ALONSO *et al.* (2009), who noted a relatively high pH of meat from Duroc crosses, and could be related to a lower glycogen content or to a higher buffer capacity in the Duroc x Celta pig muscle. Significant differences ($P<0.001$) in pH values were observed during the manufacturing of dry-cured ham. An increase in the final pH relative to the pH of raw pieces (from 5.59, 5.82 and 5.69 to 6.00, 5.92 and 5.90 for respectively C, C×D and C×L) was observed. The final pH values were similar to those reported by other authors for different types of ham, such as Iberian (MARTÍN *et al.*, 1998) and Serrano ham (GOU *et al.*, 1995), but lower than those reported for Teruel P.D.O. dry-cured hams matured for 20 months (CILLA *et al.*, 2005). The increase in pH values throughout the manufacturing process may be related to the release of low-weight nitrogen molecules and ammonia ascribed to endogenous and exogenous proteolytic enzyme activities (VIRGILI *et al.*, 2007).
The average moisture content in raw pieces was similar across groups, although the lowest values corresponded to samples from the Celta x Landrace cross-bred pigs (73.69%, 73.48% and 73.16% for respectively C, C×D and C×L). The water content decreased during the post-salting stage (around 4-5%) owing to the osmotic effect produced by the salt covering the entire surface of the hams. At subsequent stages (drying-ripening and "bodega") the decrease is due to dehydration process. The water loss during the drying-ripening and "bodega" stages of hams from pure-bred Celta pigs was significantly ($P<0.05$) higher than in hams from the cross-bred pigs (around 5-6%, Table 1). In terms of water loss, the dehydration was more intense during the "bodega" stage due to the duration of this stage and the environmental conditions (higher T and lower RH) in the storage chamber. The moisture content was significantly ($P<0.001$) higher in hams from the cross-bred pigs than in those from pure-bred Celta pigs (52.36%, 58.30%, and 58.93% for respectively C, C×D and C×L). This outcome is not consistent with findings reported by CARRAPISO and GARCÍA (2005), who did not observe any significant effect ($P>0.05$) of cross-breed on the moisture content of Iberian ham. Moisture contents were positively correlated with instrumental colour attributes of CIE L*-values ($r = 0.722$, $P<0.01$), CIE a*-values ($r = 0.633$, $P<0.01$) and CIE b*-values ($r = 0.555$, $P<0.01$) and negatively correlated with pH values ($r = -0.666$, $P<0.01$). Similarly, water activity decreased gradually throughout the manufacturing process (Table 1).

The decrease in water activity can be attributed to salt diffusion and the intense dehydration that the pieces undergo during the drying-ripening stage. The a_w values were positively correlated with moisture content (r = 0.918, $P<0.01$) and negatively correlated with salt content (r = -0.882, $P<0.01$). In addition, a_w was also correlated with shear force (WB test) (r = -0.285, $P<0.05$).

The IMF content of fresh pieces from the Duroc x Celta cross-bred pigs (13.92%) was significantly higher ($P<0.001$) than in hams from pure-bred Celta pigs (8.03%), while the levels were intermediate in hams from the Landrace x Celta cross-bred pigs (12.27%). This finding is consistent with those reported by ALONSO *et al.* (2009) and LATORRE *et al.* (2003), who observed a higher IMF content in meat from Duroc cross-bred pigs, as Duroc pigs are generally fatter than the other breeds. However, other authors (CARRAPISO and GARCÍA, 2005; TEJEDA *et al.*, 2002) observed that the Duroc line did not modify the IMF content in crosses with the Iberian pig. Finally, FRANCI *et al.* (2007) found that the IMF content was higher in hams from Cinta Senese pigs than in hams from Large White pigs, and the IMF contents of hams from the crosses between these breeds were intermediate. Data on IMF contents are of particular interest because of the influence of this parameter on essential quality traits. IMF has a clear effect on quality of meat as it reduces the shear force during chewing, making separation of muscle fibres easier, thus improving the sensations of juiciness and tenderness (LAWRIE, 1998). The contribution of IMF to juiciness is particularly important in dry-cured ham because of the strong dehydration of the product during the ripening process (VENTANAS *et al.*, 2005). Juiciness has been indicated to be the main trait influencing the overall quality of Iberian dry-cured ham (RUIZ *et al.*, 2002) and IMF has been closely associated with this parameter (RUIZ CARRASCAL *et al.*, 2000). The significantly higher IMF contents in the BF muscle of hams from Duroc x Celta and Landrace x Celta cross-bred pigs than in those from pure-bred Celta pigs may affect the acceptability of the final product.

Total chloride content (expressed as g/100 g of dry matter) increased significantly ($P<0.001$) during the salting and post-salting stages as a result of diffusion of salt throughout the whole pieces. During the next steps, the concentration of total chlorides in BF muscle continued to increase until the end of the process (Table 1). In this type of muscle (internal muscle), the differences between groups may be due to intrinsic heterogeneity, because the connective tissue, skin, fat, bones, etc. act as barriers to NaCl diffusion. The BF muscle, which is covered by subcutaneous fat and also contains intermuscular fat, is one of the muscles with the lowest concentration of sodium chloride during the first stages of the process; the concentration depends on thickness of subcutaneous fat. The final mean contents (15.87%, 15.14% and 16.59% of dry matter for respectively C, C×D and C×L) were within the range of values (13-20% of dry matter) reported by other authors (BUSCAILHON *et al.*, 1994; GOU *et al.*, 1995) for dry-cured hams. However, these NaCl contents were higher than those reported by some authors for Iberian ham (MARISCAL *et al.*, 2004; MARTIN *et al.*, 1998), Serrano ham (MARISCAL *et al.*, 2004), and Italian ham (MØLLER *et al.*, 2003) (9.29-11.4% of dry matter).

The colour of dry-cured ham is one of the most important characteristics of appearance (RUIZ *et al.*, 2002) and it is assumed to influence the consumer's choice of sliced ham in the supermarket. The influence of ripening time and cross-breed on colorimetric parameters is shown in Table 2. The luminosity (CIE L*-value) values decreased throughout the whole process. Lightness is related to the thin aqueous layer on the muscle surface, and the CIE L*-value depends on the movement of moisture (dehydration) towards the surface.

Table 1. Effect of cross-breeding on the changes of pH, water activity and proximate composition of *bicep femoris* muscle during the manufacture of dry-cured Celta ham. Results expressed as means±standard deviation of values from five samples in each group and sampling point.

	Fresh piece	After salting	After post-salting	After drying-ripening	"Bodega" stage		SEM	Significance time
					First point	Second point		
pH								
C	5.59±0.06^{a,1}	5.63±0.06^{a,1}	5.76±0.03^{b}	5.90±0.05^{c}	5.86±0.05^{c,1}	6.00±0.05^{d,2}	0.03	***
C×D	5.82±0.09^{a,2}	5.77±0.05^{a,2}	5.85±0.06^{a,b}	5.98±0.10^{c,d}	6.04±0.04^{d,2}	5.92±0.028^{b,c,1}	0.02	***
C×L	5.69±0.09^{a,1}	5.59±0.11^{a,1}	5.80±0.09^{b}	5.98±0.07^{c}	5.98±0.05^{c,2}	5.90±0.02^{b,c,1}	0.03	***
Sig. genotype	**	**	n.s.	n.s.	***	***		
a_w								
C	0.98±0.00^{e,2}	0.96±0.00^{d,2}	0.96±0.01^{d}	0.94±0.01^{c}	0.90±0.01^{b,1}	0.87±0.02^{a,1}	0.01	***
C×D	0.96±0.00^{d,1}	0.99±0.01^{e,3}	0.94±0.01^{c}	0.95±0.01^{c}	0.92±0.01^{b,2}	0.90±0.01^{a,2}	0.01	***
C×L	0.99±0.00^{d,3}	0.95±0.01^{c,1}	0.96±0.01^{c}	0.95±0.01^{c}	0.92±0.01^{b,2}	0.90±0.01^{a,2}	0.01	***
Sig. genotype	***	***	n.s.	n.s.	***	*		
Moisture (%)								
C	73.69±0.81^{e}	72.46±0.46^{e,2}	68.05±0.59^{d}	65.36±0.93^{c}	56.56±1.60^{b,1}	52.36±3.09^{a,1}	1.47	***
C×D	73.48±0.58^{f}	71.29±0.80^{e,1}	67.82±0.46^{d}	65.67±1.36^{c}	61.01±1.17^{b,2}	58.30±1.01^{a,2}	1.00	***
C×L	73.16±0.58^{f}	70.63±0.61^{e,1}	67.29±1.34^{d}	65.34±0.99^{c}	61.97±1.13^{b,2}	58.93±1.19^{a,2}	0.92	***
Sig. genotype	n.s.	**	n.s.	n.s.	***	***		
IMF (% dry matter)								
C	8.03±0.24^{a,b,1}	7.74±0.09^{a,1}	8.28±0.53^{b,1}	8.02±0.34^{a,b,1}	8.03±0.28^{a,b,1}	7.83±0.42^{a,b,1}	0.07	n.s.
C×D	13.92±1.29^{b,c,d,3}	15.21±2.68^{d,2}	14.86±1.97^{c,d,3}	12.78±0.40^{a,b,c,3}	11.07±0.96^{a,2}	12.43±1.45^{a,b,2}	0.38	**
C×L	12.27±1.05^{b,2}	13.94±0.94^{c,2}	10.17±0.69^{a,2}	11.39±0.50^{b,2}	10.20±0.37^{a,2}	11.95±0.37^{b,2}	0.27	***
Sig. genotype	***	***	***	***	***	***		
Protein (% dry matter)								
C	85.04±1.15^{e,2}	83.40±1.16^{d,3}	75.86±1.36^{c}	73.32±0.92^{b,2}	71.00±1.13^{a,2}	71.49±1.34^{a,2}	1.09	***
C×D	80.93±2.35^{e,1}	76.70±1.31^{d,1}	71.33±2.15^{c,1,2}	68.44±1.96^{b,1}	69.02±1.01^{b,1}	65.11±0.69^{a,1}	1.03	***
C×L	81.12±0.81^{f,1}	78.78±1.33^{e,2}	74.99±0.79^{d,2}	71.73±0.72^{c,2}	70.32±1.00^{b,1,2}	66.59±1.37^{a,1}	0.94	***
Sig. genotype	**	***	***	***	*	***		
Ash (% dry matter)								
C	4.59±0.18^{a,1,2}	8.28±0.71^{b,2}	14.86±0.95^{c,3}	16.60±1.26^{d}	19.80±1.06^{e,2}	19.68±1.11^{e}	1.09	***
C×D	4.33±0.14^{a,1}	6.09±0.76^{b,1}	11.49±1.17^{c,1}	15.98±0.86^{d}	17.11±1.17^{d,1}	18.32±0.85^{e}	1.02	***
C×L	4.85±0.31^{a,2}	5.92±0.69^{b,1}	12.82±0.65^{c,2}	15.95±0.47^{d}	18.22±0.96^{e,1}	19.80±1.29^{f}	1.08	***
Sig. genotype	*	***	***	n.s.	**	n.s.		

Chlorides (% dry matter)								
C	0.51±0.19^{a}	3.62±1.10^{b}	11.74±1.87^{c,2}	13.02±1.79^{c}	16.15±1.14^{d,2}	15.87±1.81^{d}	1.16	***
C×D	0.34±0.41^{a}	3.24±0.74^{b}	8.18±1.06^{c,1}	13.09±1.14^{d}	12.20±2.04^{d,1}	15.14±0.80^{e}	1.02	***
C×L	0.69±0.20^{a}	2.85±0.51^{b}	9.57±0.60^{c,1}	14.35±0.56^{d}	14.45±1.18^{d,2}	16.59±1.52^{e}	1.13	***
Sig. genotype	n.s.	n.s.	**	n.s.	**	n.s.		

$^{a-f}$Means in the same row (corresponding to the same genotype and parameter) not followed by a common letter are significantly different (P<0.05; Duncan test) (differences among sampling points). $^{1-3}$Means in the same column and parameter not followed by a common number are significantly different (P<0.05; Duncan test) (differences among genotypes). Significance: n.s.: not significant; * (P<0.05); ** (P<0.01); *** (P<0.001). SEM is the standard error of the mean.

Table 2. Effect of cross-breeding on the changes of colour parameters of *bicep femoris* muscle during the manufacture of dry-cured Celta ham. Results expressed as means±standard deviation of values from five samples in each group and sampling point.

	Fresh piece	After salting	After post-salting	After drying-ripening	"Bodega" stage		SEM	Sig. time
					First point	Second point		
Lightness (L*)								
C	48.90±2.39^{d}	47.84±2.28^{d,1}	40.35±1.45^{b,1}	43.06±1.19^{c,1}	39.73±1.46^{a,b,1}	37.12±2.51^{a,1}	0.89	***
C×D	50.59±1.72^{c}	50.17±2.44^{c,2}	45.58±2.00^{a,b,2}	47.53±1.67^{b,2}	46.14±1.14^{b,2}	43.65±0.96^{a,2}	0.64	***
C×L	47.97±1.57^{d}	47.03±2.46^{d,1}	41.22±1.25^{a,1}	45.03±1.74^{c,d,1}	44.23±2.26^{b,c,2}	41.87±1.27^{a,b,2}	0.60	***
Sig. genotype	n.s.	*	***	**	***	***		
Redness (a*)								
C	18.14±0.27^{c,2}	18.16±1.98^{c,2}	13.48±1.17^{b}	13.05±0.86^{a,b,1,2}	13.82±1.63^{b,2}	11.52±0.98^{a}	0.52	***
C×D	13.43±1.68^{b,c,1}	13.97±0.27^{c,1}	13.32±1.13^{b,c}	12.01±0.80^{a,1}	12.30±0.87^{a,b,1,2}	11.02±0.68^{a}	0.31	***
C×L	16.69±1.89^{b,2}	16.44±2.17^{b,1,2}	13.53±0.97^{a}	13.59±1.45^{a,2}	11.60±1.14^{a,1}	11.18±1.31^{a}	0.49	***
Sig. genotype	***	*	n.s.	n.s.	*	n.s.		
Yellowness (b*)								
C	13.13±1.17^{b,2}	13.00±1.66^{b}	7.55±0.91^{a,1}	7.65±0.79^{a,1}	7.15±0.97^{a,1}	8.63±0.80^{a,1}	0.52	***
C×D	11.35±1.40^{b,1}	12.24±0.79^{c}	12.24±1.54^{b,c,3}	9.71±1.14^{a,2}	11.13±0.64^{a,b,2}	11.04±0.81^{a,b,2}	0.27	***
C×L	12.96±0.99^{c,1,2}	13.30±0.62^{d}	9.83±0.67^{a,b,2}	8.94±0.51^{a,2}	10.39±0.87^{b,2}	10.09±0.67^{b,2}	0.37	***
Sig. genotype	n.s.	n.s.	***	**	***	**		

$^{a-d}$Means in the same row (corresponding to the same genotype and parameter) not followed by a common letter are significantly different (P<0.05; Duncan test) (differences among sampling points). $^{1-3}$Means in the same column and parameter not followed by a common number are significantly different (P<0.05; Duncan test) (differences among genotypes). Significance: n.s.: not significant; * (P<0.05); ** (P<0.01); *** (P<0.001). SEM is the standard error of the mean.

The decrease in CIE L*-values may be related to decrease in moisture content (r = 0.722, $P<0.01$). To this regard, SANABRIA *et al.* (2004) observed that moisture loss increased the concentrations of pigments (e.g. myoglobin) and led to a reduction in CIE L*-values. On the other hand, CIE L*-values also decreased as salt concentration increased (r = -0.741, $P<0.01$). A similar trend in CIE L*-values was reported by MARUŠIĆ *et al.* (2011) and PÉREZ-PALACIOS *et al.* (2011) for dry-cured ham. However, CILLA *et al.* (2005) did not report any significant changes in the colour parameters, except for the BF muscle redness index in dry-cured Teruel P.D.O hams matured for different lengths of time (between 12 and 26 months). Relative to CIE a*-values, a decrease in redness was observed until the end of the process; this may be due to the decrease in moisture content (r = 0.633, $P<0.01$) and an increase in salt content (r = -0.644, $P<0.01$). The final values were within the range of those described by SANABRIA *et al.* (2004) in Iberian ham, and very similar to those reported by MARUŠIĆ *et al.* (2011) for Istrian ham and by CILLA *et al.* (2005) for Teruel ham. Finally, the yellowness values decreased as the processing time increased, and the decrease was more pronounced during the post-salting stage (Table 2). The decrease in CIE b*-values may also be due to a decrease in moisture content (r = 0.555, $P<0.01$) and an increase in salt content (r = -0.696, $P<0.01$). A similar pattern was reported by SANABRIA *et al.* (2004) for Iberian ham. The final values of this parameter were also similar to those reported by other authors for different varieties of ham (CILLA *et al.*, 2005; MARUŠIĆ *et al.*, 2011; PÉREZ-PALACIOS *et al.*, 2011). The instrumental colour parameters were also slightly affected by cross-breed (Table 2), as hams from the cross-bred pigs were yellower (CIE b* value) and lighter (CIE L* value) than those from pure-bred Celta pigs. Thus, the higher lightness (CIE L*) values in dry-cured hams from crosses between Celta pigs and both Duroc and Landrace pigs may be related to the higher IMF content. This is consistent with the findings of RAMÍREZ and CAVA (2008), who reported a positive correlation between CIE L*-value and IMF content in Iberian ham. In the present study, CIE L*-value was positively correlated with the IMF content (r = 0.535, $P<0.01$).

The changes in TBARs values during the manufacturing of dry-cured ham are shown in Fig. 1. The TBARs values increased significantly ($P<0.001$) during the salting and post-salting periods, reaching the highest values after the drying-ripening period in hams from C and C×L groups, and after the post-salting stage in hams from the Celta x Duroc cross-bred pigs. The increase in malondialdehyde contents during the post-salting and drying-ripening stages may be related to the pro-oxidant action of metallic ions present as impurities in the salt used in the curing process. The TBARs values were positively correlated with NaCl content (r = 0.542, $P<0.01$). From these maximum values, a significant decrease ($P<0.001$) was observed until the end of the process, reaching final average values of 1.33, 1.07 and 1.00 mg MDA/kg of muscle for C, C×D and C×L respectively. The decrease in TBARs values was associated with the advanced reactions of secondary lipid oxidation products with protein residues, especially for conditions of low water activity to yield oxidatively modified proteins (KIKUGAWA *et al.*, 1991). Increased TBARs values in dry-cured ham during the first stages of production followed by a decrease toward the final stages have previously been reported for Iberian ham (ANDRÉS *et al.*, 2004) and Parma ham (KOUTINA *et al.*, 2012). At the end of the process, there were no significant differences ($P>0.05$) between groups, although the fat of hams from pure-bred Celta pigs tended to be more oxidized. This finding seems contravene previous observations, since lipid oxidation is usually positively related to fat content (JO *et al.*, 1999). However, the highest fat unsaturation of Celta pig compared to most of the other pig breeds (FRANCO *et al.*, 2006) could be responsible for the highest oxidation of fat in hams from pure Celta breed.

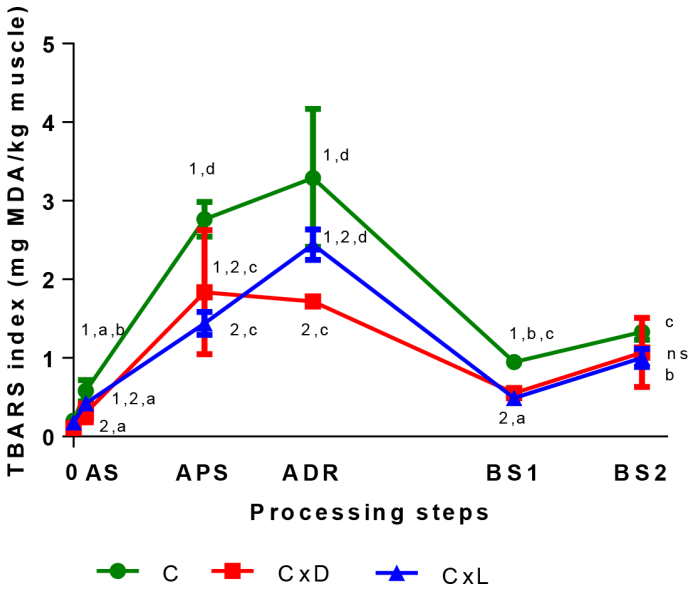

Figure 1. Effect of cross-breed on the changes in TBARs index of *bicep femoris* muscle during the manufacture of dry-cured Celta ham. Plotted values are means and standard deviations from five samples in each group and sampling point. AS = After salting, APS = After post-salting, ADR = After drying-ripening, BS1 = After bodega stage 1, BS2 = After bodega stage 2. [a-d] Means in the same genotype not followed by a common letter are significantly different ($P<0.05$) (differences among processing steps). [1-2] Means in the same processing step not followed by a common number are significantly different ($P<0.05$) (differences among genotypes).

The final mean value (1.13±0.17 mg MDA/ kg muscle) was two times higher than values obtained in Istrian ham (MARUŠIĆ *et al.*, 2011), Iberian ham (ANDRÉS *et al.*, 2004) and Teruel P.D.O. ham (CILLA *et al.*, 2006).

The changes in maximum shear force during the manufacturing of dry-cured hams are shown in Fig. 2. Shear force increased significantly ($P<0.001$) during the process. The final mean value of shear force (3.58 kg/cm^2) was lower than those obtained by SORIANO PÉREZ (2001) and FRANCI *et al.* (2007), who reported values of 6.84 kg/cm^2 and 20.9 kg/cm^2 respectively. A similar trend was observed by SERRA *et al.* (2005), who suggested a negative non-linear relationship between hardness and water content. In the present study, moisture content was negatively correlated with shear force (r = -0.541, $P<0.01$). In addition, texture has previously been linked to IMF content (RUIZ-RAMÍREZ *et al.*, 2005); this is consistent with our observations as IMF content was negatively correlated with shear force (r = -0.756, $P<0.01$). MONIN *et al.* (1997) also included other variable in the dry-cured ham process, such as level of proteolysis, because they observed that changes in hardness depended on both water content and protein state. These authors found that muscles initially became harder during the early processing stages, because of the decrease in protein solubility and water content, and they then became softer in texture as proteolysis occurred. At the end of process, shear force did not differ significantly ($P>0.05$) between groups, although the lowest values were found in hams from Landrace x Celta cross-bred pigs (see Fig. 2). This finding is consistent with those reported by FRANCI *et al.* (2007) who observed that shear force values were higher in pure-bred Cinta Senese pigs than in Cinta Senese x Large White cross-bred pigs.

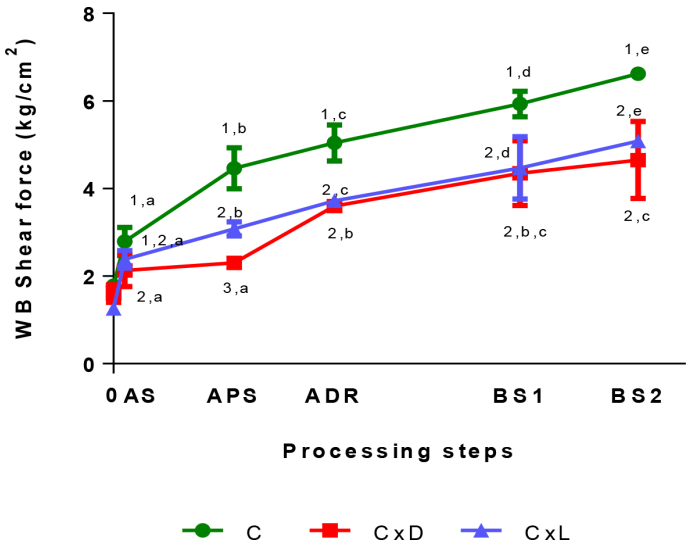

Figure 2. Effect of cross-breed on the changes in maximum shear force of *bicep femoris* muscle during the manufacture of dry-cured Celta ham. Plotted values are means and standard deviations from five samples in each group and sampling point. AS = After salting, APS = After post-salting, ADR = After drying-ripening, BS1 = After bodega stage 1, BS2 = After bodega stage 2. [a-e] Means in the same genotype not followed by a common letter are significantly different ($P<0.05$) (differences among processing steps). [1-3] Means in the same processing step not followed by a common number are significantly different ($P<0.05$) (differences among genotypes).

3.2. Influence of cross-breed on sensory characteristics

Ten descriptors were evaluated in the sensory analysis of the dry-cured ham (Fig. 3). Within appearance, lightness and lean redness scores were similar for the dry-cured hams from all three groups of pigs considered, although the lightness values were highest for samples from cross-bred pigs, while the redness of the lean meat was highest in samples from pure-bred Celta pigs. Instrumental and sensory results of lean meat colour indicate that discrimination between Celta and crosses with Landrace and Duroc pigs is difficult. This confirms the results obtained in other comparisons between local breeds and cross-breeds (Iberian compared with Iberian×Duroc) by CARRAPISO *et al.* (2003), who concluded that instrumental colour measurement of the lean portion of dry-cured ham is not particularly useful for assessing differences perceived by panellists. However, marbling scores differed significantly between groups and were highest in ham samples from cross-bred pigs (1.5, 5.6 and 5.4, $P<0.001$ for C, C×D and C×L respectively). These outcomes are consistent with data reported for instrumental colour determination and IMF and moisture contents. Thus, the CIE L* values were positively correlated with IMF content (r = 0.751, $P<0.01$), marbling (r = 0.516, $P<0.01$) and moisture content (r = 0.617, $P<0.01$). These findings are consistent with those reported by CARRAPISO and GARCÍA (2008) and RAMÍREZ and CAVA (2008), who also obtained a significant correlation between the CIE L* value and marbling and suggested that the colour of Iberian ham is more strongly influenced by fat distribution than by the chemical IMF content of the muscle.

The odour (intensity, rancidity and cured) traits were not significantly ($P>0.05$) affected by cross-breed, although odour intensity was highest in samples from pure-bred Celta pigs (7.8, 7.3, 7.2, $P>0.05$ for C, C×D and C×L respectively). Similarly, the IMF content was negatively correlated with odour intensity (r = -0.543; $P<0.01$) as reported by RAMÍREZ

and CAVA (2008), who observed a close relationship between IMF/marbling, aroma and odour intensity. On the other hand, salty taste was not significantly (P>0.05) affected by cross-breed, although slightly higher values were obtained in ham samples from the crosses (5.3, 5.7 and 5.5 for C, C×D and C×L respectively).

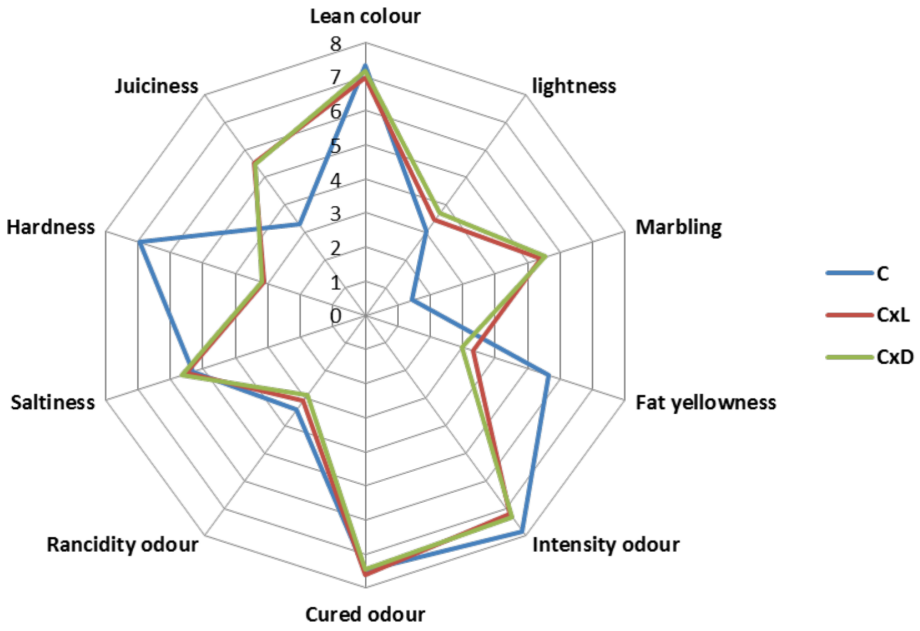

Figure 3. Effect of cross-breed on the sensory characteristics of dry-cured Celta ham at the end of the manufacturing process. Plotted value for each attribute and pig group is the mean of forty evaluations.

Finally, panellists observed significant between-group differences in texture traits that were consistent with those detected by instrumental methods (see Fig. 2). Hardness scores were correlated with WB shear force (r = 0.574, P<0.01). Panellists described hams from the cross-bred pigs as softer in texture (P<0.01) and juicier (P<0.01) than hams from pure-bred Celta pigs (Figure 3). These results are consistent with those reported by RAMÍREZ and CAVA (2008), who also observed a positive correlation between hardness scores and TPA hardness and WBSF.

Differences in sensory textural attributes can be caused by several factors: (i) the final pH of fresh meat, (ii) the IMF content and (iii) the moisture content (RAMÍREZ and CAVA, 2008). Increasing the IMF content reduces the force required to chew the meat, by easing separation of muscle fibres, and causes an enhanced perception of meat tenderness (ESSÉN-GUSTAVSON *et al.*, 1994). Thus, better sensory texture parameters have been reported in Iberian hams with higher IMF content, with significant positive correlations between marbling and juiciness (RAMÍREZ and CAVA, 2008). In the present study, juiciness scores were correlated with IMF content (r = 0.529, P<0.01), marbling (r = 0.585; P<0.01) and moisture content (r = 0.527, P<0.01).

4. CONCLUSIONS

The physico-chemical and sensory properties of dry-cured ham from Celta pig can be improved by crossing this breed with others. Hams from Celta x Duroc and Celta x Landrace cross-bred pigs had more intramuscular fat than hams from pure-bred Celta

pigs. The results of the WB test also suggest an effect of cross-breed, as hams from Celta x Duroc and Celta x Landrace cross-bred pigs were of softer texture than hams from pure-bred Celta pigs. Sensorial analysis demonstrated that hams from Celta pure breed were harder and less juicy than those from crosses. Although the two crosses improve the quality of hams, overall considering the texture and sensory traits it seems that the crossing with the Duroc genotype has the better improvement results.

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INFLUENCE OF FAT CONTENT ON QUALITY OF COW'S MILK

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ABSTRACT

The aim of the study was to verify whether changes in the percentage of fat in highly selected cows produce variations in the physical structure of the fat and changes in milk composition. Individual milk was sampled from 50 cows. Fat was evaluated in each individual in order to create two groups of animals with lower and higher percentage. The group with higher fat content showed a significantly larger diameter of the fat globules, less C14:0 and more C16:1. In conclusion the diameter variations observed result in few changes in milk fatty acid composition, thus maintaining a consistent nutritional quality.

Keywords: cow, fatty acids, milk fat globules, milk quality

1. INTRODUCTION

The nutraceutical composition of foods consumed daily is becoming increasingly important. Particular focus has been on consumers belonging to all age groups especially with regard their food lipid intake, since lipids have been implicated in several diseases such as obesity, insulin resistance and atherosclerosis (OLOFSSON *et al*., 2009). For these reasons, the number of studies on the physical and chemical structure of fat in several edible products of animal origin have increased (LE *et al*., 2014, MARTINI *et al*., 2016)
Cow's milk is consumed on a daily basis from childhood, thus it is important to know and monitor the milk lipid changes that occur naturally, or are induced by different farming techniques. It is thus necessary for consumers to drink throughout their life a guaranteed product from a nutritional and nutraceutical point of view. For the dairy industry it is important to assess how changes in the morphometry of the milk fat globules (MFGs) lead to changes in yields, ripening and the nutritional quality of cheeses (MARTINI *et al*., 2016).
Milk lipids are spherical structures and research has shown that the differences related to the diameter and number of MFGs are of interest with regard to the chemical-physical and nutritional properties of milk and lipid digestion and absorption (MARTINI *et al*., 2013).
The secretion of milk fat globule is not yet completely understood. Furthermore it is known that their size and distribution vary as a function of factors such as species, breed, parity, stage of lactation and amount of fat secreted (MARTINI *et al*., 2016). Some authors (WIKING *et al*., 2004 and CAROLL *et al*., 2006) have found positive relationships between daily fat yield and the average diameter of the MFGs. These findings are probably due to the fact that part of the membrane of the mammary epithelial cell is sacrificed to envelop the globule, then larger MFGs may be secreted to reduce the amount of membrane lost by the cell (ARGOV *et al*., 2008). Some authors have hypothesized also that relationships between fat secretion and MFG size are due to the effects on the mammary metabolism of the energy balance and the availability of nutrients (MARTINI *et al*., 2013). Moreover, the effects of energy balance on the metabolism of the mammary gland are not yet fully known (GERMAN, 2011).
The purpose of this study is to check whether small changes in fat secretion in highly selected cows, which have been reared in the same conditions, would result in variations in the physical structure of the fat and in changes in the milk composition.

2. MATERIALS AND METHODS

2.1. Animals and sampling

Milk from the morning milking of 50 pluriparous Friesian cows (151 ± 25 days in milk) was sampled. The cows were reared intensively in a single dairy farm in Tuscany (central Italy). All the cows were fed with the same diet consisting in a mixed ration formulated according to NRC (2001) requirements for dairy cattle and made up of corn silage, corn meal, alfalfa hay, hay ryegrass, soybean meal 44% PG, integral cotton seed, cane molasses, and commercial complementary feed. All individual milk samples were taken during the same day by in-line milk meters (De Laval) and were refrigerated at 4°C before being taken to the laboratory for analysis.

2.2. Milk analysis

All the fresh samples were analysed in duplicate for total solids (TS), fat, protein, casein content according to AOAC methods (2004). Fat extraction was performed using hexane and ethanol, according to Rose-Gottlieb's method modified by SECCHIARI *et al*. (2003).

Methyl esters of fatty acids (FAME) were obtained after transesterification with sodium methoxide according to Christie (1982). The composition of the milk fatty acids was determined by gas chromatography using a Perkin Elmer Auto System (Perkin Elmer, Norwolk, CT, USA) equipped with a flame ionization detector and a capillary column (30 m × 0.25 mm; film thickness 0.25 mm; FactorFour Varian, Middelburg, the Netherlands). The helium carrier gas flow rate was 1 mL·min^{-1}. The oven temperature program was as follows: level 1, 50°C held for 2 min, level 2, 50 to 180°C at 2°C·min^{-1} then held for 20 min, level 3, 180 to 200°C at 1°C·min^{-1} then held for 15 min, and finally level 4, 200 to 220°C at 1°C·min^{-1} then held for 30 min. The injector and detector temperatures were set at 270 and 300°C, respectively. Individual FAs were identified by comparing their retention times with those of the following authenticated standards: FIM_FAME mix (Restek Corporation, 110 Benner Circle, Bellefonte, PA 16823) and methyl 9(Z), 11(E)-octadecadienoate (Matreya LLC International Dealers & Representatives-Superchrom S.r.l., Via C. Menotti 11, Milan, 20129)
In addition, nonadecanoic acid methyl ester (C19:0 Restek Corporation, 110 Benner Circle, Bellefonte, PA 16823) was used as internal standard to calculate the recovery of the fatty acid methyl esters. The desaturase index was calculated for: cis-9 14:1/14:0, cis-9 16:1/16:0, cis-9 18:1/18:0, following KELSEY *et al*. (2003).

2.3. Morphometric analysis of milk fat globules

A direct method (MARTINI *et al*., 2013) was used to determine the diameter (μm) and the number of fat globules per mL of milk in each fresh sample by a fluorescence microscope (Leica Ortomat Microsystem, Milan, Italy) equipped with a camera (TiEsseLab, Milan, Italy) and TS view 2.0 image software.

2.4. Statistical analysis

The frequency distribution of the total counted and measured MFGs was evaluated and globules were grouped into three size-categories: small globules (SG) with a < 2μm diameter, medium-sized globules (MG) with a diameter from 2 to 5 μm, and large globules (LG) with a > 5 μm diameter.
The variability in the fat secreted from each of the 50 cows was evaluated in order to create two groups of animals (groups A and B) secreting lower fat percentages (less that 32 g kg^{-1}, made up of 16 individuals) and higher (more than 37 g kg^{-1}, made up of 12 individuals) respectively, compared to the average value (34 g kg^{-1}). Individuals whose fat percentage was between 32 and 37 g kg^{-1} were excluded.
The results were analyzed by ANOVA using JMP software (2002)considering the group (A or B) as the fixed factor. Least significance means were compared by *t*-test. Significant differences were considered at $P< 0.05$.

3. RESULTS

Milk yield, chemical and physical characteristics are reported in Table 1.
Milk yield in the two groups of animals was similar. The higher fat content of group B corresponded to a significantly larger average diameter of globules and to increases in the percentages of the large globules. In group B, 22% of the total number of globules was made up of large globules, whereas in group A, the percentage of large globules was only 12%. This confirms the hypothesis of other authors (MARTINI *et al*., 2016) who have linked the positive relationship between the fat secreted and the average diameter of the

MFGs with a balance of the mammary gland cell. In fact, as the secretion of fat is an apocrine mechanism, it involves losses in the membrane, which is sacrificed to envelop the globules. Larger MFGs may therefore be secreted to reduce the amount of membrane lost by the cell per unit volume of fat.

Table 1. Milk yield, quality and characteristics of the fat globules in the milk from two groups of cows.

	Group A (fat<32 g kg^{-1}) (n‡=16)	Group B (fat >37 g kg^{-1}) (n‡=12)
Average daily milk yield (kg^{-1}) †	29.64±1.65	28.58±1.92
Fat (g kg^{-1})	27.9±0.50**	40.5±0.60**
Proteins (g kg^{-1})	30.50±1.00	31.70±1.1
Casein (g $kg^{-1)}$	24.34±0.996	25.08±1.068
Casein/fat ratio	0.90±0.039**	0.62±0.045**
Protein/fat ratio	1.13±0.045**	0.81±0.052**
Dry matter (g kg^{-1})	134.4±5.30	133.9±6.20
Ash (g kg^{-1})	6.9±0.10	6.9±0.10
Number of milk fat globules (n/mL)	$11.82X10^{9}$±9.53	$8.48X10^{9}$±1.10
Milk fat diameter (μm)	2.90±0.18*	3.50±0.21*
SG § (%)	38.15±3.35	30.45±3.89
MG ¶ (%)	49.96±3.44	47.54±4.00
LG ††(%)	11.89±3.13*	21.98±3.64*

* P<0.05; ** P <0.01
†Calculated as kg of milk produced until the day of sampling /days in milk at the day of sampling.
‡ n= number of cows;
§SG=Small globules with a diameter <2 μm.;
¶ MG=Medium globules with a diameter between 2 and 5 μm;
††LG=Large globules with a diameter >5 μm.

In addition, as previously observed in sheep (MARTINI *et al*., 2012),subjects with a smaller average diameter (group A) showed a higher number of globules/ml of milk (+39%).
From the point of view of the milk quality, total nitrogen did not differ significantly in the two groups of cows, however lower casein/fat and protein/fat ratios were observed in group B. This may be a limiting factor, especially for cheese-making since protein/fat ratio is a critical characteristics for the cheese yield and for the recoveries of fat and water in the cheese (GUINEE *et al*., 2007). Moreover, some authors have suggested that there is a link between milk protein synthesis and fat (HEID and KEENAN, 2005). CEBO *et al*. (2012) have also reported that genetic polymorphism at the *α*s1-casein (*CSN1S1*) affects both the structure and composition of the MFGs.
On the other hand, the milk of group A showed a lower average diameter of MFGs (P <0.05). It has been observed that milk with smaller sized globules increases cheese yield in Emmental production (MICHALSKI *et al*., 2004), and that in sheep a greater diameter of the MFG leads to a worsening of the rheological parameters, due to negative relations with the content of casein and the casein:fat ratio (MARTINI *et al*., 2016).
Studies on the variability in the average MFG diameter in Friesian cows have shown contrasting results in terms of the relations between diameter and the milk yield and quality. Some authors have reported positive correlations between the diameter and the amount of fat (BRIARD-BION *et al*., 2008), whereas others have not observed any relationship (LOGAN *et al*., 2014).

In addition, the diameter of MFGs has been reported to affect milk fatty acid composition (LOPEZ *et al.,* 2011).
In our study, the changes in the fatty acid profile (Table 2) were limited and concern an increase ($P < 0.05$) in myristic acid in group A (+11% g kg^{-1} of the total fatty acids), and an increase ($P < 0.05$) in palmitoleic acid in group B (+19% g kg^{-1} of the total fatty acids).
This study highlights that, although statistically significant, small changes (0.6 microns) in the diameter of the milk fat globules in homogeneous animals only have a slight effect on the milk fatty acid profile.

Table 2. Fatty acid composition (g kg-1of total fatty acids), classes of milk fatty acids (%) and desaturase indexes in milk from two groups of cows.

	Group A (fat <32.00 g kg^{-1}) (n†=16) g kg^{-1} of the total fatty acids	Group A (fat <32.00 g kg^{-1}) (n†=16) g kg^{-1} of milk	Group B (fat>37.00 g kg^{-1} (n†=12) g kg^{-1} of the total fatty acids	Group B (fat>37.00 g kg^{-1} (n†=12) g kg^{-1} of milk
C4:0	25.88±1.04	0.72±0.029	25.74±0.86	1.04±0.035
C6:0	20.49±0.71	0.57±0.020	19.43±0.85	0.79±0.034
C8:0	12.98±0.48	0.36±0.013	12.2±0.67	0.49±0.027
C10:0	31.07±1.30	0.87±0.036	28.34±0.16	1.15±0.006
C11:0	2.88±1.44	0.08±0.040	2.75±0.17	0.11±0.007
C12:0	36.62±1.45	1.02±0.040	33.34±1.76	1.35±0.071
C13:0	1.36±0.06	0.04±0.002	1.32±0.07	0.05±0.003
C14:0	125.31±3.65*	3.50±0.101*	113.25 ±4.41*	4.59±0.179*
C14:1	13.67±0.40	0.38±0.010	13.19±0.49	0.53±0.020
C15:0	13.59±0.42	0.38±0.012	13.28±0. 51	0.54±0.021
C15:1	3.06±0.15	0.09±0.004	3.26±0.18	0.13±0.007
C16:0	346.77±7.75	9.67±0.216	344.19±9.36	13.94±0.379
C16:1	13.13±0.68*	0.37±0.019*	15.58±0.83*	0.63±0.034*
C17:0	7.81±0.26	0.22±0.007	8.00±0.31	0.32±0.013
C17:1	2.88±0.23	0.08±0.006	3.17±0.28	0.13±0.011
C18:0	89.55±3.65	2.50±0.102	91.84±4.40	3.72±0.178
C18:1 *trans-9*	5.52±0. 27	0.15±0.007	5.52±0.33	0.22±0.013
C18:1 *trans-11*	6.90±0.28	0.19±0.008	7.05±0.34	0.29±0.014
C18:1 cis-9	192.89±9.21	5.38±0.257	203.38±11.12	8.24±0.450
C18:2 *trans*-9,12	2.31±0. 08	0.06±0.002	2.19±0.10	0.09±0.004
C18:2 cis-9,12	35.64±1.90	0.99±0.053	33.53±2.29	1.36±0.093
C18:3n3	3.52±0.15	0.10±0.004	3.60±0.18	0.15±0.007
C18:3 n6	13.06 ±0.11	0.36±0.003	12.03±0.13	0.49±0.005
C20:0	1.17±0.05	0.03±0.001	1.23±0.01	0.05±0.001
CLA *cis*-9, *trans*11	5.34±0.11	0.15±0.003	5.17±0.13	0.21±0.005
C20:1	0.57±0.05	0.02±0.001	0.56±0.06	0.02±0.002
C21:0	0.77±0.01	0.02±0.001	0.86±0.01	0.03±0.001
C20:2	0.22±0.03	0.006±0.001	0.17±0.03	0.01±0.001
C20:3n6	1.17±0. 06	0.03±0.002	1.17±0.07	0.05±0.003
C20:4	0.11±0.04	0.003±0.001	0.14±0.05	0.01±0.002
C20:3 n3	0.25±0.03	0.007±0.001	0.21±0.03	0.01±0.001
C22:0	0.73±0.06	0.02±0.002	0.70±0.07	0.03±0.003
C22:1	1.61±0.11	0.04±0.003	1.42±0.13	0.06±0.005

C20:5	0.24±0.03	0.007±0.001	0.22±0.04	0.01±0.002
C23:0	0.34±0.02	0.01±0.001	0.33±0.03	0.01±0.001
C22:2	0.34±0.03	0.01±0.001	0.42±0.04	0.02±0.002
C24:0	0.43±0.03	0.01±0.001	0.44±0.04	0.02±0.002
C24:1	0.19±0.06	0.005±0.002	0.30±0.06	0.01±0.002
C22:5	0.77±0.04	0.02±0.001	0.74±0.05	0.03±0.002
C22:6	0.94±0.21	0.03±0.006	0.55±0.25	0.02±0.010
	Group A (fat <32.00 g kg^{-1}) (n†=16) % of the total fatty acids	**Group A (fat <32.00 g kg^{-1}) (n†=16) g kg^{-1} of milk**	**Group B (fat>37.00 g kg^{-1} (n†=12) % of the total fatty acids**	**Group B (fat>37.00 g kg-1 (n†=12) g kg-1 of milk**
Short chain FA‡ (≤C10)	9.04±0.28	2.52±0.078	8.57±0.34	3.47±0.014
Medium chain FA (≥C11≤C17)	55.66±1.14	15.53±0.032	55.13±1.37	22.33±0.055
Long chain FA (≥C18)	35.29±1.28	9.85±0.036	36.30±1.54	14.70±0.062
Saturated FA	70.73±1.11	19.73±0.031	69.72±1.34	28.24±0.054
Monounsaturated FA	24.04±1.07	6.71±0.030	25.34±1.21	10.26±0.049
Polyunsaturated FA	5.18±0.22	1.45±0.006	4.89±0.49	1.98±0.020
	Group A (fat <32.00 g kg^{-1}) (n†=16)		**Group B (fat>37.00 g kg^{-1} (n†=12)**	
Unsaturated /Saturated FA ratio§	0.42±0.03		0.44±0.03	
n3/n6 ratio¶	0.06±0.005		0.07±0.006	
Desaturase C14 index††	0.10±0.003		0.11±0.004	
Desaturase C16 index††	0.04±0.002		0.04±0.003	
Desaturase C18 index††	0.32±0.01		0.31±0.01	

* P<0.05

†n= number of cows; ‡ FA: Fatty Acids; § Unsaturated /Saturated FA ratio: :∑unsaturated fatty acids/:∑saturated fatty acids; ¶n3/n6 ratio:∑n3 fatty acids /∑n6 fatty acids; †† Desaturase C14 index: [C14:1]/[C14:1+C14:0].; Desaturase C16 index: [C16:1]/[C16:1+C16:0]; Desaturase C18 index: [C18:1c9]/[C18:1cis 9+C18:0]

4. CONCLUSIONS

In high-selected dairy breeds significant variations in fat secreted influence the morphometry of the fat globules, casein:fat and protein:fat ratios. However, the range of variation observed in the diameter does not lead to significant changes in the fatty acid composition. Thus maintaining a consistent nutritional quality of bovine dairy milk.

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PAPER

A COMPARATIVE STUDY OF THE PHYSICOCHEMICAL PROPERTIES AND EMULSION STABILITY OF COCONUT MILK AT DIFFERENT MATURITY STAGES

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ABSTRACT

Based on chemical analysis, mature coconut (MC) milk had the highest moisture content ($p<0.05$), followed by immature coconut (IMC) and overlay mature coconut (OMC) milk, respectively. OMC milk had the highest lipid content while IMC milk showed the lowest lipid content ($p<0.05$). The lowest protein and carbohydrate contents were found in MC milk ($p<0.05$). Cocosin with MW of 55 kDa was observed as the major protein in all coconut milks; however, the band intensity slightly decreased with increasing maturity stages. Increase in oil droplet size was observed with increasing maturity stages. Therefore, maturity stages have an influence on the chemical compositions, properties and emulsion stability of coconut milk.

Keywords: coconut milk, droplet size, emulsion stability, physicochemical properties, protein pattern

1. INTRODUCTION

Coconuts (*Cocos nucifera)* are extensively used in many traditional foods of the Asian and Pacific regions (ONSAARD *et al.*, 2005). Coconut milk is commonly used in several cuisines such as curries and desserts (TANSAKUL and CHAISAWANG, 2006). It contains high amounts of medium chain saturated fatty acids (MCFAs), especially lauric acid (RAGHAVENDRA and RAGHAVARAO, 2010). Lauric acid is converted into a very valuable compound known as monolaurin, which has antiviral and antibacterial properties. The consumption of coconut milk may help to protect the body from infections (DEBMANDAL and MANDAL, 2011).

Coconut milk is a milky white oil-in-water emulsion extracted from grated coconut meat with or without the addition of water. The emulsion in coconut milk is naturally stabilised by coconut proteins (globulins and albumins), as well as phospholipids (RAGHAVENDRA and RAGHAVARAO, 2011). The major protein (~65%) in coconut endosperm is an 11S globulin known as cocosin with a molecular weight (MW) of 55 kDa (GARCIA *et al.*, 2005), and is believed to play a significant role in stabilising the coconut milk emulsion (TANGSUPHOOM and COUPLAND, 2008). Generally, both intrinsic factors (e.g. protein compositions, etc.) and environmental conditions (e.g. pH, temperatures, etc.) can affect the stability of the coconut milk emulsion (RAGHAVENDRA and RAGHAVARAO, 2010).

On the other hand, the instability of the coconut milk emulsion is required for the production of virgin coconut oil (VCO). In recent years, coconut milk is immensely used for the extraction of VCO. Moreover, VCO has gained much popularity in the scientific community due to the presence of MCFAs, its high degree of saturation and good stability. It can be obtained by breaking the emulsion of coconut milk using different extraction methods (RAGHAVENDRA and RAGHAVARAO, 2010). Thus, to maximise the yield of VCO, coconut milk emulsion must be destabilised to a high degree, so that oil can be released and separated effectively

The quality and stability of coconut milk emulsion could be governed by intrinsic factors, especially at different maturity stages. However, no information exists regarding the influence of maturity stages on the characteristics and emulsion stability of coconut milk. A better understanding of the physicochemical properties and emulsion stability of coconut milk at different maturity stages could be beneficial in the manufacturing of VCO with prime quality and high yield. Therefore, this comparative study was carried out to evaluate the physicochemical properties and emulsion stability of milk obtained from coconut at three different maturity stages.

2. MATERIALS AND METHODS

2.1. Chemicals

Sodium hydroxide, boric acid and Nile blue A were purchased from Sigma (St. Louis. MO, USA). Sodium dodecyl sulphate and isooctane were obtained from Merck (Darmstadt, Germany). Methanol, ethanol, acetic acid, chloroform, petroleum ether, hydrochloric acid, sulphuric acid, n-hexane and cyclohexane were procured from Lab-Scan (Bangkok, Thailand). Chemicals for electrophoresis were obtained from Biorad (Richmond, VA, USA) and protein molecular weight marker was procured from GE healthcare (Buckinghamshire, UK).

2.2. Preparation of coconut meat and coconut milk

Coconuts at three different maturity stages including immature coconut (IMC) (9-10 months old from pollination), mature coconut (MC) (11-12 months old from pollination) and overlay mature coconut (OMC) (14-15 months old from pollination) were purchased from a plantation site in Yaring District, Pattani Province, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. Coconuts were subjected to deshelling, paring and removal of water. Coconut kernel was collected manually and grated using a rotary wedge cutter machine. To prepare coconut milk, the grated coconut meat was pressed using a hydraulic press machine (Model stainless steel hydraulic press A2, Sakaya, Bangkok, Thailand) with a maximum pressure of 10.35 MPa for 2 min. Thereafter, coconut milk was collected and analysed.

2.3. Proximate analysis of coconut meat and coconut milk

Coconut meat and coconut milk at three different maturity stages were analysed for moisture, ash, lipid and protein contents according to the method of AOAC (AOAC, 2000). The protein content was calculated using 6.25 (as the factor) and the carbohydrate content was calculated as the difference from the sum total of the aforementioned proximate analysis components. The values were expressed as g/100 g (wet weight basis).

2.4. Colour determination

Coconut milk colour was measured using a colourimeter (HunterLab, Model colourFlex, VA, USA). The colour was reported as L^*, a^*, b^* values, indicating lightness, redness/greenness and yellowness/blueness, respectively. Total difference in colour (ΔE^*) and the difference in chroma (ΔC^*) were also calculated using the following equations:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding colour parameter of the sample and the white standard ($L^* = 93.55$, $a^* = 0.84$, $b^* = 0.37$).

$$\Delta C^* = C^*_{sample} - C^*_{Standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

2.5. pH measurement

A digital pH meter (Eutech, pH700 Thermo Scientific, USA) was used to measure the pH values of coconut milk.

2.6. SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

The protein patterns of coconut milk were determined by SDS-PAGE, according to the method of LAEMMLI (1970), using 4% staking gel and 12% separating gel. The coconut milk samples (10 mL) were homogenised with 10 mL of 50 g/L SDS at a speed of 12,000 rpm for 1 min. The homogenate was heated at 95°C for 1 h, followed by centrifugation at 7000 × g for 10 min at 25°C using a centrifuge (Beckman coulter, Allegra™ centrifuge, CA,

USA). The protein concentration of the supernatant was determined by the Biuret method (ROBINSON and HOGDEN, 1940), using bovine serum albumin (BSA) as a standard. The prepared samples were mixed with sample buffer containing 2% SDS, 10% glycerol and 0.05% bromophenol blue in 0.5 M Tris-HCl, and the resulting solution had a pH of 6.8. Under reducing condition, β-mercaptoethanol was added to the sample buffer in order to obtain a final concentration of 5% and the mixtures were heated at 95°C for 3 min prior to loading. The prepared mixtures (20 μg protein) were loaded onto the gel. Electrophoresis was performed using a vertical gel electrophoresis unit (Mini-protein II; Bio-Rad Laboratories, Richmond, VA, USA) at a constant voltage of 20 mA/gel. After electrophoresis, the gels were stained with 0.5 g/L Coomassie Brilliant Blue R-250 in 500 mL/L methanol and 75 mL/L acetic acid for 30 min. Finally, they were destained with a mixture of 500 mL/L methanol and 75 mL/L acetic acid for 30 min and destained again with a mixture of 50 mL/L methanol and 75 mL/L acetic acid for 1 h. The relative mobility (R_f) of proteins was calculated and their molecular weight was estimated from the plot between R_f and log (MW) of standards.

2.7. Microstructure determination of oil droplets

2.7.1 Confocal laser scanning microscopy (CLSM)

The microstructures of coconut milk samples were examined with a confocal laser scanning microscope (CLSM) (Model FV300; Olympus, Tokyo, Japan.). The samples were dissolved in Nile blue A solution (1:10) and manually stirred until uniformity was obtained. Fifty microlitres of sample solutions were smeared on the microscope slide. The CLMS was operated in the fluorescence mode at excitation and emission wavelengths of 533 and 630 nm, respectively; using a Helium Neon Red laser (HeNe-R) for lipid analysis. A magnification of 400x was used.

2.7.2 Phase contrast microscopy

Oil droplets in coconut milk were observed under a phase contrast microscope (Model IX50; Olympus, Tokyo, Japan) equipped with camera. Samples were placed on a glass slide, covered with cover slip and observed at 400x magnification.

2.7.3 Determination of particle size

The particle size distribution of coconut milk emulsion was determined using a laser particle size analyser (LPSA) (Model LS 230, Beckman Coulter®, Fullerton, CA, USA) as per the method of CASTELLANI *et al.* (2006). Prior to analysis, the sample (5 mL) was diluted with 1 mL sodium dodecyl sulphate (SDS) in order to dissociate flocculated droplets. The surface-weighted mean particle diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured.

2.7.4 Determination of coalescence and flocculation

Coconut milk samples were diluted with distilled water in the presence and absence of SDS. The coalescence index (C_i) and flocculation factor (F_f) were calculated using the following equations (INTARASIRISAWAT *et al.*, 2014):

$$F_f = \frac{d_{43}\text{-SDS}}{d_{43}+\text{SDS}}$$

$$C_i = \frac{(d_{43}+SDS,t - d_{43}+SDS,in)}{d_{43}+SDS,in} \times 100$$

where d_{43}+SDS and d_{43}-SDS are the volume-weighted mean particle diameter of the emulsion droplets in the presence and absence of SDS, respectively; d_{43}+SDS, in and d_{43}+SDS, t are the volume-weighted mean particle diameter of the emulsion droplets in the presence of SDS at time 0 and the designated storage time (24 h), respectively. Determination was conducted at room temperature (28-30°C).

2.7.5 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For paired comparison, T-test was used (STEEL and TORRIE, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Proximate compositions of coconut meat and milk

The proximate compositions of coconut meat and milk at three different maturity stages are shown in Table 1. MC meat had the highest moisture content (61.07 g/100 g), followed by IMC (53.94 g/100 g) and OMC (39.50g/100 g), respectively. A similar trend was observed in coconut milk, in which MC had the highest moisture content (61.55 g/100 g), followed by IMC (55.36 g/100 g) and OMC (36.59 g/100 g), respectively. The high moisture content of MC meat and milk was more likely due to the absorption of coconut water inside the endosperm, since the beginning of germination. Water uptake is an essential step towards germination (BEWLEY and BLACK, 1994). On the other hand, low moisture content was observed in OMC meat and milk. The result suggested that the absorbed water in the endosperm was utilised during embryo development (BEWLEY and BLACK, 1994). It was found that there was a general increase of lipid content in coconut meat and milk with increasing maturity. OMC meat and milk were found to have the highest lipid content ($p<0.05$). The lipid content of coconut increased with maturity stage due to the accumulation of lipids in the endosperm (LÓPEZ-VILLALOBOS *et al.*, 2001). Lower protein content was observed in both MC meat and milk as compared with those of IMC and OMC ($p<0.05$). Cocosin is a reserve protein found in coconut endosperm and serves as nitrogen source during germination (BALASUNDARESAN *et al.*, 2002). The result suggested that proteins could be degraded and utilised at the beginning of germination. Since water was utilised during the germination of OMC, the proportion of proteins was slightly increased. The ash content of both meat and milk decreased with maturity. The ash contents are indices of the mineral content (OBASI *et al.*, 2012). It has been reported that coconut water contains sugars, vitamins, minerals, amino acids and phytohormones(YONG *et al.*, 2009). The decrease in ash content in OMC meat and milk suggested that minerals are more likely used up during the germination process. A significantly lower carbohydrate content was observed in MC meat and milk ($p<0.05$). The obtained results are in accordance with that of JEGANATHAN (1970) who found that coconut milk at the mature stage, had a carbohydrate content of 5.5%. WHITE *et al*. (1989)

found that coconut milk at the mature stage is composed predominantly of galactose and arabinose with a small amount of mannose and glucose. BALASUBRAMANIAM (1976) reported that galactomannans and cellulose are present in the kernel of maturing and matured coconuts, whereas mannans are almost absent from very immature kernel and increased with maturation. Endosperm rich nutrients appear to function as a food reservoir for embryo development (BALASUNDARESAN *et al.*, 2002). Thus, reserved materials, particularly carbohydrates, were degraded and utilised during maturity. The decrease in carbohydrate plausibly led to the increased proportion of lipid in MC, as compared with the IMC sample. The results revealed that different maturity stages had marked impact on the chemical composition of coconut meat and milk.

Table 1. Proximate composition of coconut meat and milk at three different stages of maturity.

Content (g/100 g)	Coconut Meat			Coconut Milk		
	IMC	MC	OMC	IMC	MC	OMC
Moisture	53.94±0.60b	61.07±1.02a	39.50±0.82c	55.36±0.15b	61.55±0.13a	36.59±1.05c
Lipid	18.59±0.89b	20.86±0.95b	32.45±0.35a	17.28±1.46c	30.34±0.96b	44.20±0.85a
Protein	4.79±0.16a	3.95±0.09b	4.45±0.56a	3.35±0.29a	2.90±0.06b	3.34±0.49a
Ash	1.15±0.02a	1.14±0.04a	1.04±0.03b	1.03±0.05a	1.00±0.04a	0.80±0.03b
Carbohydrate	21.53±0.98a	13.05±0.95b	22.34±0.85a	22.98±1.21a	4.21±0.93c	15.07±1.63b

IMC: Immature Coconut, MC: Mature Coconut and OMC: Overlay Mature Coconut.
Values are presented as Mean±SD (n=3).
Different lowercase letters in the same row, within the same commodity, indicate significant difference ($p<0.05$).

3.2. Colour of coconut milk

L^*, a^* and b^* values of coconut milks at three different stages of maturity are shown in Table 2. The coconut milks were milky white in colour as evidenced by high L^*-value (lightness). In general, coconut milk is an oil-in-water emulsion, where oil droplets are dispersed in the water phase. Light scattering of oil droplets is mostly associated with the white colour of coconut milk. All samples had low a^* and b^*- values, suggesting that deterioration did not occur in all samples. It was observed that ΔE^* and ΔC^* decreased with increasing maturity stages, where IMC had the highest values, followed by MC and OMC samples, respectively. The highest L^*- value was found in the MC sample ($p<0.05$). The turbidity, cloudiness, or opaque appearance of emulsion is dependent on light scattering which is mediated by the dispersed oil droplets (MCCLEMENTS, 2002).

Table 2. Colour and pH of coconut milk at three different stages of maturity.

Samples	L^*	a^*	b^*	ΔE^*	ΔC^*	pH
IMC	92.90±0.24a	0.10±0.05c	5.09±0.06a	4.83±0.05a	4.09±0.06a	7.00±0.01a
MC	94.86±1.83c	-0.28±0.04b	4.48±0.12a	4.48±0.13b	3.84±0.12b	6.39±0.02b
OMC	93.09±0.18b	-0.35±0.03a	4.22±0.08b	3.88±0.08c	3.23±0.08c	5.58±0.13c

IMC: Immature Coconut, MC: Mature Coconut and OMC: Overlay Mature Coconut.
Values are presented as Mean±SD (n=3).
Different lowercase letters in the same column indicate a significant difference ($p<0.05$).

Lightness is not only determined by lipids or oils, but also by the size of oil droplets, which is another prime factor governing the colour, particularly the lightness of coconut milk. Some differences in indigenous pigments present in coconut milk of different maturity stages were also presumed.

3.3. pH of coconut milk

The pHs of freshly prepared coconut milks at three different stages of maturity are shown in Table 2. IMC milk was found to have a pH of 7.0 while MC milk was slightly acidic in pH (pH 6.39). The lowest pH (5.58) was obtained in OMC milk ($p<0.05$). Reserved food materials such as proteins, carbohydrates and lipids provide nourishment to growing embryo (SAMSON *et al*., 1971). The breakdown of these stored food materials by some enzymes, possibly occurred for embryo development. Acidic metabolites or degradation products such as acidic amino acids may contribute to the lowered pH. The pH of 5.58 found in OMC milk is close to the isoelectric point of coconut proteins (pI = 4-5) (SAMSON *et al.*, 1971; MONERA and DEL ROSARIO, 1982; Kwon *et al.*, 1996). Therefore, pH may affect the emulsifying properties of proteins in coconut milk, especially those stabilising oil droplets in the aqueous phase.

3.4. Electrophoretic patterns of coconut milk proteins

The protein patterns of coconut milks at different stages of maturity under reducing and non-reducing conditions are shown in Fig. 1. Under non-reducing condition, there were six protein bands with MW of 55, 46, 33, 25, 18 and 16 kDa. The major protein in coconut endosperm is 11S globulin, which is referred to as cocosin, with MW of 55 kDa (GARCIA *et al.*, 2005). A hexamer (55 kDa) consists of acidic (32-34 kDa) and basic (22-24 kDa) subunits, which are linked by a disulphide bridge (GARCIA *et al.*, 2005; TANGSUPHOOM and COUPLAND, 2008). Some proteins present in the aqueous phase of coconut milk could act as emulsifier to stabilise fat globules (PEAMPRASART and CHIEWCHAN, 2006). Cocosin plays a prominent role in regulating the stability of coconut milk (TANGSUPHOOM and COUPLAND, 2008). In the present study, smaller bands of proteins with MW higher than 55 kDa were found. Bands with higher intensity were found in IMC as compared with others. In the OMC sample, protein bands with MW of 70 and 46 kDa almost disappeared, indicating the degradation of proteins during germination. Under the reducing condition, several major protein bands with MW of 55, 33, 31, 25, 21, 20, 18 and 16 kDa were observed, which are in agreement with previous reports of GARCIA *et al.* (2005) and DEMASON and SEKHAR (1990). Under the reducing condition, cocosin dissociated into acidic and basic polypeptides with the coincidental appearance of proteins with MW of 32 and 22 kDa. Other proteins with MW greater than 55 kDa were not found. The occurrence of protein with MW of 20 kDa was also observed under reducing conditions. Protein with MW of 36 kDa was found only in the MC sample. In general, the OMC sample showed the lower band intensity of most proteins as compared with others. The result confirmed that the protein composition of coconut milk changed with maturity stages.

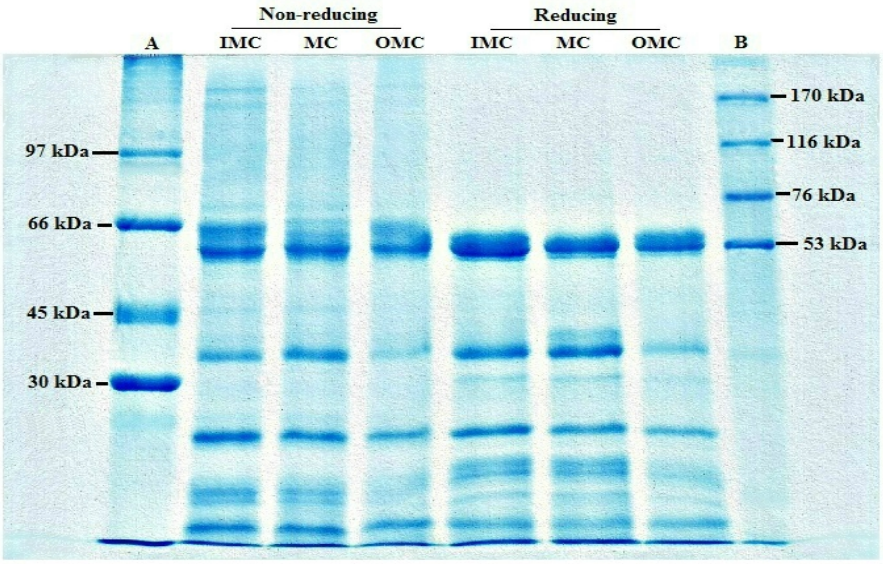

Figure 1. SDS-PAGE patterns of coconut milk proteins with three different maturity stages. A: low molecular weight standards; B: high molecular weight standards; IMC: Immature Coconut, MC: Mature Coconut and OMC: Overlay Mature Coconut.

3.5. Microstructure of oil droplets in coconut milk

The microscopic structures of coconut milk emulsions at three different stages of maturity were visualised by confocal laser scanning microscopy (CLSM) and phase contrast microscopy (Fig. 2). In the same coconut milk sample, similar results were observed when both CLSM and phase contrast microscopies were used. CLSM generally provides higher clarity and better resolution images of the emulsion microstructure than convention optical microscopy. The observation of the microstructure of the emulsion was facilitated using a fluorescence dye such as Nile blue A, in order to label the lipid. However, phase contrast microscopy provides excellent contrast, and a halo is formed even around a small oil droplet. For IMC (Fig. 2a), smaller oil droplets with uniform shape and size were distributed uniformly in the aqueous phase. An emulsion with the same size of oil droplets is referred to as a monodisperse emulsion, whereas that containing a range of droplet sizes is referred to as a polydisperse emulsion (MCCLEMENTS, 2004). Coconut milk is an oil-in-water emulsion naturally stabilised by coconut proteins (BIROSEL *et al.*, 1963). In the present study, IMC had higher protein/lipid ratio as compared with the MC sample (Table 1). High protein content can lead to efficient localisation of protein films at the oil-water interphase. Thus, this could increase the emulsion stability of coconut milk. Moreover, proteins could stabilise the coconut milk emulsion by lowering the interfacial tension between two phases, in which oil droplets are dispersed uniformly throughout the water phase. However, polydisperse emulsion was observed in MC and OMC (Figs. 2b and c) with a wide range of oil droplet sizes. Large sizes of oil droplets were abundantly observed in the OMC. In OMC, coconut milk contained a high amount of lipid. Thus, the present proteins may not be sufficient to stabilise the emulsion. The low pH of OMC milk could be another factor enhancing the destabilisation of emulsion, by lowering the repulsion of protein film surrounding the oil droplets. In general, the emulsion was less stable as evidenced by the larger droplets with non-uniform distribution. The results clearly indicated that maturity stages affected oil droplet size.

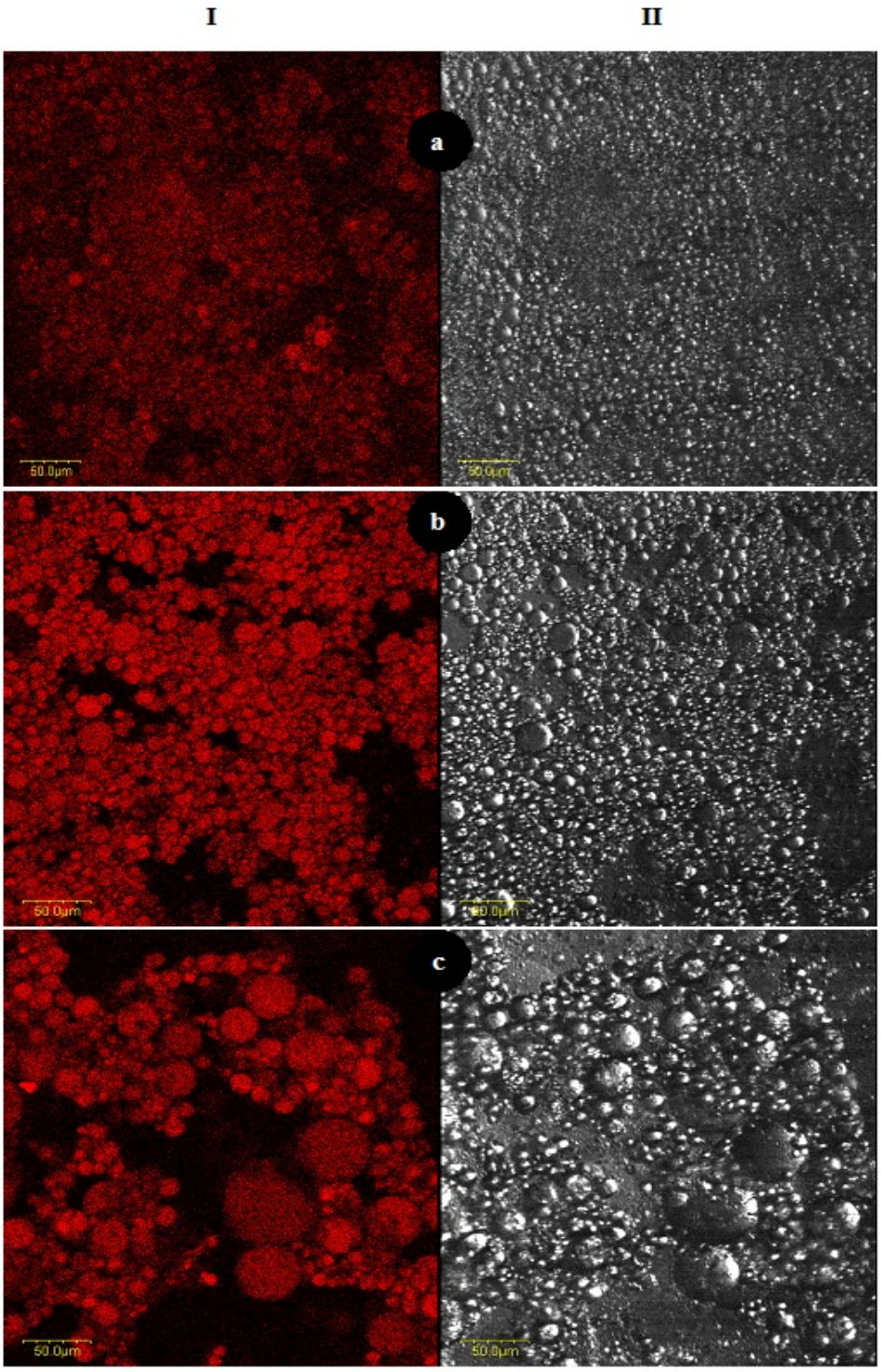

Figure 2. Confocal laser scanning micrographs (I) and phase contrast microscopy (II) of coconut milk at three different stages of maturity: (a) IMC: Immature Coconut; (b) MC: Mature Coconut; (c) OMC: Overlay Mature Coconut. Magnification: 400×. Scale bar = 50 μm.

3.6. Particle size distribution

Particle size distributions expressed as d_{32} and d_{43} of coconut milk emulsions at three different stages of maturity are shown in Table 3. The d_{32} increased from 3.38 μm (IMC) to 5.48 μm (OMC), while d_{43} increased from 5.29 μm (IMC) to 13.38 μm (OMC) with increasing maturity stages. Coconut milk from OMC contained the largest oil droplets (d_{43} and d_{32}), followed by those from MC and IMC. The d_{32} is related to the average surface area of droplet exposed to the continuous phase per unit volume of emulsion. The smaller d_{32} indicates higher specific surface area (INTARASIRISAWAT *et al.*, 2014). The d_{43} is the sum of the volume ratio of droplets in each size class multiplied by the mid-point diameter of the size class. The d_{43} can be used as the index of coalescence and flocculation (HEBISHY *et al.*, 2013). The proteins in coconut milk are known to function as emulsifier, which stabilises the oil droplets in coconut milk (DIONISIO, 1963; MONERA and DEL ROSARIO, 1982). The largest size of oil droplets in OMC can be attributed to the pH of the OMC milk, close to pI. As a result, there was a decrease in repulsion between protein films surrounding the oil droplets, thereby facilitating the coalescence. When the repulsive forces dominate, the droplets tend to remain as individual entities (MCCLEMENTS, 2004) and form a stable emulsion. After 24 h of storage, both d_{32} and d_{43} increased (Table 3), indicating the coalescence of oil droplets. Among all samples, a slight increase in d_{32} and d_{43} were observed in the OMC sample. The result suggested that the collapse of emulsion in the OMC milk was pronounced at the initial time and less coalescence occurred after 24 h. Conversely, the emulsion collapsed continuously in the MC and IMC samples.

Table 3. Droplet size and stability of coconut milk at three different stages of maturity.

Samples	Storage Time (h)	d_{32} (μm)	d_{43} (μm)	F_f	C_i
IMC	0	3.38±0.10C	5.29±0.20C	1.18±0.03A	-
	24	4.16±0.40b	8.10±0.10c	0.77±0.04b	53.22±0.59a
MC	0	5.07±0.25B	12.31±0.15B	0.90±0.90A	-
	24	5.55±0.60a	14.22±0.21a	0.78±0.03b	15.52±0.89b
OMC	0	5.48±0.13A	13.38±0.03A	1.26±0.04A	-
	24	5.66±0.26a	13.44±0.01b	1.26±0.01a	0.45±0.50c

F_f: Flocculation factor, C_i: Coalescence index.
IMC: Immature Coconut, MC: Mature Coconut and OMC: Overlay Mature Coconut.
Values are presented as Mean±SD (n=3).
Different uppercase letters in the same column at the initial storage time (0 h) indicate significant difference ($p<0.05$). Different lowercase letters in the same column after the designated storage time (24 h) indicate significant difference ($p<0.05$).

3.7. Coalescence and flocculation

The coalescence index (C_i) and flocculation factor (F_f) of the coconut milk emulsions were investigated to determine the instability of the emulsion as shown in Table 3. Emulsions are thermodynamically unstable due to the unfavourable contact between oil and water (FREDRICK *et al.*, 2010) and their physical structures are likely to change over time by various mechanisms including coalescence and flocculation. In IMC, higher *Ci* was observed after 24 h, as compared with the MC and OMC samples ($p<0.05$). On the other

hand, F_f decreased, suggesting that the individual oil droplets assembled to form larger oil droplets as evidenced by the increase in droplet size. The increase in d_{43} also reconfirmed the assembly of individual droplets into larger flocs (INTARASIRISAWAT *et al.*, 2014). The formation of larger oil droplets indicates poor emulsion stability (FREDRICK *et al.*, 2010). The interactions between oil droplets depend on the quality and quantity of proteins (DAMODARAN, 2005). The proteins in IMC were plausibly not effective in stabilising the coconut milk emulsion, especially after the extended storage. However, the lowest rate of C_i was observed for OMC. This coincided with the lowest rate of changes in d_{32} and d_{43} of OMC. For OMC, initial pH close to pI might not favour the solubility of proteins and as such, it was presumed to have poor emulsifying property. Additionally, the partial crystallization of lipid within the oil droplets could be another factor that favours the destabilisation of emulsion by coalescence (ROUSSEAU, 2000). Nevertheless, the emulsion initially found was slightly altered upon storage time. The results suggested that the oil droplet size and emulsion stability of coconut milk depended on the maturity stages.

4. CONCLUSIONS

Coconut milk and meat at three different maturity stages had varying proximate compositions. Cocosin with MW of 55kDa was predominantly observed in coconut milks, regardless of the maturity stage. Polydisperse emulsion was observed in coconut milk at the mature and overlay mature stages, whilst the monodisperse counterpart was found in coconut milk of the immature stage. The stability of coconut milk emulsion depends on intrinsic factors, mainly pH and protein content. Thus, the maturity stages had influence on the physicochemical properties and emulsion stability of coconut milk. The present study has provided a better understanding of the impact of maturity stage on the characteristics and emulsion stability of coconut milk, used as the starting material for VCO production. OMC was more appropriate for the production of VCO with higher yield as compared with other stages.

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PAPER

PURIFICATION AND IDENTIFICATION OF ANTIOXIDANT PEPTIDES FROM GELATIN HYDROLYSATES OF UNICORN LEATHERJACKET SKIN

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ABSTRACT

Antioxidant peptides from a gelatin hydrolysate of unicorn leatherjacket skin prepared using a partially purified glycyl endopeptidase were purified using Sephadex G-25 gel filtration, DEAE-cellulose anion-exchange and reverse phase high-performance liquid chromatography. The fractions with the highest ABTS radical scavenging activity were analyzed using UPLC–ESI-MS/MS to identify the peptide sequences therein. Four of the identified peptides, Glu-Pro-Gly-Pro-Val-Gly (555.27 Da), Leu-Pro-Gly-Pro-Ala-Gly (511.29 Da), Leu-Asp-Gly-Pro-Val-Gly (557.30 Da) and Glu-Gly-Pro-Leu-Gly (472.24 Da), were subsequently synthesized. Glu-Gly-Pro-Leu-Gly exhibited the highest antioxidant activity (4.95 μmol TE/g solid). Therefore, peptides from unicorn leatherjacket skin gelatin hydrolysate could be further employed as functional food ingredient.

Keywords: gelatin hydrolysate, unicorn leatherjacket, antioxidant activity, identification, UPLC, mass spectrometry

1. INTRODUCTION

There is an increased interest in the isolation of natural antioxidants from different sources as the use of synthetic antioxidants is restricted due to the potential risks to human health (HRAŠ *et al*., 2000). Antioxidant peptides have been found in several foodstuffs such as milk (PHELAN *et al*., 2009), cereals (MALAGUTI *et al*., 2014) and algae (CORNISH and GARBARY, 2010). Fish protein hydrolysates, especially gelatin hydrolysates, have been shown to have the ability to scavenge free radicals (MENDIS *et al*., 2005), inhibit lecithin liposome peroxidation (KARNJANAPRATUM and BENJAKUL, 2015a; 2015b) and reduce the oxidation of Fe^{3+} to Fe^{2+} (ALEMAN *et al*., 2011). The antioxidant activity of protein hydrolysates is mainly governed by the parent protein sequence, the specificity of enzymes used and the conditions used in the hydrolysate preparation (PEÑA-RAMOS and XIONG, 2011). The amino acid sequences of several antioxidant peptides have been identified (SUETSUNA *et al*., 2000; SAIGA *et al*., 2003; FAN *et al*., 2012), demonstrating an association between bioactivity and the amino acid sequence (SHAHIDI and ZHONG, 2008). Gelatin peptides have an abundance of glycine (Gly), proline (Pro) and hydroxyproline (Hyp), which may contribute to their enhanced bioactivity in comparison with peptides isolated from other sources. Pro residues have a scavenging effect on radicals and the percentage of hydroxylation has been correlated with antioxidant activity (ALEMAN *et al*., 2011).
Antioxidant gelatin hydrolysates from unicorn leatherjacket skin have been produced using different methods. The autolysis-assisted process mediated by indigenous protease in combination with thermal or enzymatic hydrolysis was implemented to prepare antioxidant gelatin hydrolysates from unicorn leatherjacket skin (KARNJANAPRATUM *et al*., 2015b). Glycyl endopeptidase (GE), isolated from papaya latex, was shown to yield gelatin hydrolysates from unicorn leatherjacket skin with higher antioxidant activity, compared to the crude extract from papaya latex (KARNJANAPRATUM *et al*., 2015a). Gelatin hydrolysates from unicorn leatherjacket skin prepared using GE with autolysis-assisted process also demonstrated antioxidant properties in *in vitro* cellular model systems (KARNJANAPRATUM *et al*., 2015). Nevertheless, no information on the structure and sequence of potential antioxidant peptides from skin gelatin hydrolysates of unicorn leatherjacket exists. The aims of this study were to purify and identify the amino acid sequences of antioxidant peptides from gelatin hydrolysate of unicorn leatherjacket skin prepared using glycyl endopeptidase.

2. MATERIALS AND METHODS

2.1. Chemical

2,2′-Azinobis (3-thylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and other reagents were obtained from Sigma Chemical Co. (Dublin, Ireland). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Fluka Chemie (Buchs, Switzerland). All solvents were high performance liquid chromatography (HPLC) grade and were procured from Merck (Darmstadt, Germany).

2.2. Preparation of gelatin hydrolysate from unicorn leatherjacket skin

2.2.1 Preparation of fish skins

The skins of unicorn leatherjacket (*Aluterus monoceros*) were obtained from a dock in Songkhla, Thailand. The skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2). Skins were subjected to alkaline pretreatment to remove non-collagenous proteins as per the method of KAEWRUANG *et al*. (2013). The autolysis of pretreated skin was conducted following the method of KARNJANAPRATUM and BENJAKUL (2015a). The resulting autolyzed skin was used as a substrate for preparation of the gelatin hydrolysate.

2.2.2 Preparation of partially purified glycyl endopeptidase from papaya (*Carica papaya*) latex

The glycyl endopeptidase (GE) was fractionated from the latex using the method of KARNJANAPRATUM and BENJAKUL (2014). An aqueous two phase system (ATPS) with 10% PEG 6000 and 10% ammonium sulphate $(NH_4)_2SO_4$ was used for fractionation of GE. The obtained GE was stored at -40°C until use.

2.2.3 Production of gelatin hydrolysates

The antioxidant gelatin hydrolysate from skin of unicorn leatherjacket was prepared as described by KARNJANAPRATUM and BENJAKUL (2015a). Autolyzed skin solution (3%, w/v) was hydrolysed using GE (8%, w/w based on solid matter) at 40°C for 60 min. The resulting gelatin hydrolysate (GH) was lyophilized using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark) and was then stored at -20°C until use for analysis.

2.3. Purification of antioxidant peptides

From a previous study, it was found that GH demonstrated strong ABTS radical scavenging activity in comparison with ferrous chelating activity and ferric reducing antioxidant power (KARNJANAPRATUM *et al*., 2015a). ABTS radical scavenging activity was therefore selected to monitor antioxidative activity of purified peptide in each step.

2.3.1 Size exclusion chromatography

GH (2 mL, 80 mg/mL) was subjected to gel filtration (2.5 x 50 cm column) using Sephadex G-25 (Product number 17-0032-01, GE Healthcare Bio-Science AB, Uppsala, Sweden) as described by KARNJANAPRATUM and BENJAKUL (2015a). The fractions of 3 mL were collected. The absorbance of the eluent was recorded at 220 and 280 nm. All fractions were measured for ABTS radical scavenging activity.

2.3.2 Anion-exchange chromatography

The fractions exhibiting the highest antioxidant activity obtained from size exclusion chromatography were pooled, lyophilized and further subjected to anion-exchange chromatography (DEAE-cellulose, Whatman, England) column (1.0×50 cm) coupled with a fraction collector (Model 2128, Bio-RAD Laboratories Ltd.). The elution was carried out using a constant flow rate (0.5 mL/min) with a linear gradient of NaCl (0-0.4 M). The

absorbance at 220 and 280 nm was monitored and 3 mL fractions were collected. ABTS radical scavenging activity of all fractions was measured and the fractions exhibiting high antioxidant activity were pooled (Peak B-1, B-2, B-3), desalted into DI water using size exclusion chromatography (Sephadex G-25, 2.5×50 cm column) and subsequently lyophilized.

2.3.3 High performance liquid chromatography (HPLC)

The fraction with the highest ABTS radical scavenging activity obtained from anion-exchange chromatography was further separated using reversed phase- (RP-HPLC) on a 201TP C18 (4.6×250 mm) column (Grace Davision Discovery Science, Epping, Australia). The HPLC system consisted of a spectra system P2000 pump (Thermo Electron corporation, Wisconsin, United State), sample injector (Spectra system AS3000, Thermo Electron corporation) and a detector (Spectra system UV6000LP, Thermo Electron corporation). The column was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A), and a linear gradient was developed using solvent A and solvent B (acetonitrile containing 0.1% (v/v) TFA). To separate the peptides, elution was performed with the following conditions: 0.0-10.0 min, 5% B; 10.0-60.0 min, 5.0-30.0% B; 60.0-70.0 min, 100% B; 70.0-80.0 min, 100% A, at a flow rate of 1.0 mL/min. The fractions were manually collected, based on the peaks of A_{220} and A_{280}. Each fraction was evaporated to dryness in a miVac centrifugal vacuum Quattro concentrator (Genevac Ltd., Ipswich, UK) and ABTS radical scavenging activity of each fraction was then determined. Total activity/A_{220} of each fraction was then calculated.

2.4. ABTS radical scavenging activity

The ABTS radical scavenging activity of gelatin hydrolysates was determined as described by BINSAN *et al*. (2008). The activity was expressed as μmol Trolox equivalent (TE)/g sample.

2.5. Peptide identification using ultra performance liquid chromatography (UPLC)-electrospray ionization (ESI) mass spectrometry (MS) and tandem MS (MS/MS)

Samples were separated on an ACQUITY UPLC (Waters, Milford, MA, USA) and analyzed on an Impact HD™ mass spectrometer (Bruker Daltonics, Bremen, Germany). Mobile phase A was 0.1% formic acid (FA) in MS-grade H_2O and mobile phase B was 0.1% FA in 80% MS-grade acetonitrile. Peptides were separated on an Acquity UPLC BEH 300 C18 RP column (1.7 μm×50 mm, Waters). A flow rate of 0.25 mL/min with isocratic elution was used for 5 min at 100% mobile phase A, followed by gradient elution to 80% mobile phase B from 5 to 25 min.
MS/MS analysis was performed using two different methods: (i) a broad range method targeting peptides having a wide range of molecular masses and (ii) a short peptide method specifically targeting peptides having low molecular masses, as developed by O'KEEFFE and FITZGERALD (2015), was used. The Impact HD™ (Bruker Daltonics) was calibrated using ESI low molecular mass tune mix (Agilent Technologies, Cork, Ireland) for the broad range method while sodium formate (10 mM NaOH, 0.2% formic acid in isopropanol) was used as calibrant for the short peptide method. Mass spectra were acquired in positive ion mode and scans were performed for Auto MS/MS between 100 and 2500 *m/z* for the broad range method and between 50 and 600 *m/z* for the short peptide method. MS/MS conditions were as follows: capillary voltage: 4500 V; collision gas:

nitrogen; nebulizer pressure 1.8 bar; dry heater temperature: 220 ºC and dry gas flow: 8 L/min. Specific broad range method conditions were: collision energy 7.0 eV; commission cell radio frequency (RF): 1500 Vpp and transfer time: 100 μs. Specific short peptide MS/MS conditions were: collision energy 5.0 eV; collision cell RF was stepped between 200 and 350 Vpp (50% of the time each) and transfer time was stepped between 36.1 and 51.1 μs (50% of the time each).
All MS/MS spectra were searched against the SwissProt database, limited to phylum Chordata using PEAKS Studio 7.5 (Bioinformatics Solutions Inc., Waterloo, Canada) and MASCOT (version 2.3, Matrix Science, London, UK). Further peptide identification was carried out by *de novo* sequencing using PEAKS Studio 7.5, Data Analysis (version 4.0, Bruker Daltonics) and Biotools (version 3.2, Bruker Daltonics) software.

2.6. Peptide synthesis

The identified peptides with the typical collagen sequence (Gly-Pro-X motif) and with homology of peptide to collagen proteins ≥75% as well as ≥65% average local confidence (ALC) were selected, in which the presence of Gly at C-terminal, attributed to glycyl endopeptidase cleavage, would be the major criteria. The selected peptides were synthesized by DgPeptides Co., Ltd (Hangzhou, Zhejiang, China). The purity of the synthesized peptides was greater than 95% as determined by HPLC. All peptides were assayed for ABTS radical scavenging activity as described previously and results were expressed as μmol Trolox equivalent (TE)/g peptide.

2.7. Statistical analysis

Experiments were carried out in triplicate. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (STEEL and TORRIE, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Purification of antioxidant peptides

Previous work (KARNJANAPRATUM and BENJAKUL, 2015a), found that ABTS radical scavenging activity had the highest activity in comparison with other antioxidant assays used. Additionally, this assay has been shown to measure the antioxidant activity of both hydrophilic and hydrophobic antioxidants (AGHDAM *et al*., 2011). Therefore, the ABTS assay is suitable for measuring the antioxidant activity of gelatin peptides, which contain both hydrophobic and hydrophilic amino acids in the peptides sequence, as the hydrophilic–hydrophobic property of the peptide is a critical factor affecting its antioxidant activity (MENDIS *et al*., 2005). Thus, the ABTS radical scavenging activity was carried out for screening and selecting the most antioxidant fraction from gelatin hydrolysate.
Initial isolation of the peptides with the highest antioxidant activity from GH was carried out using Sephadex G-25 size exclusion chromatography. Two fractions (A and B) were collected and ABTS radical scavenging activity of each was determined (Fig. 1). Fraction B, which contained smaller lower molecular mass peptides showed the higher antioxidant activity (82.46 μmol/g solid), compared to GH (65.49 μmol TE/g solid) and fraction A

(9.31 μmol TE/g solid) (P<0.05). In general, lower molecular mass peptides possess higher antioxidant activities (SUN *et al*., 2013; INTARASIRISAWAT *et al*., 2013; FAN *et al*., 2012).

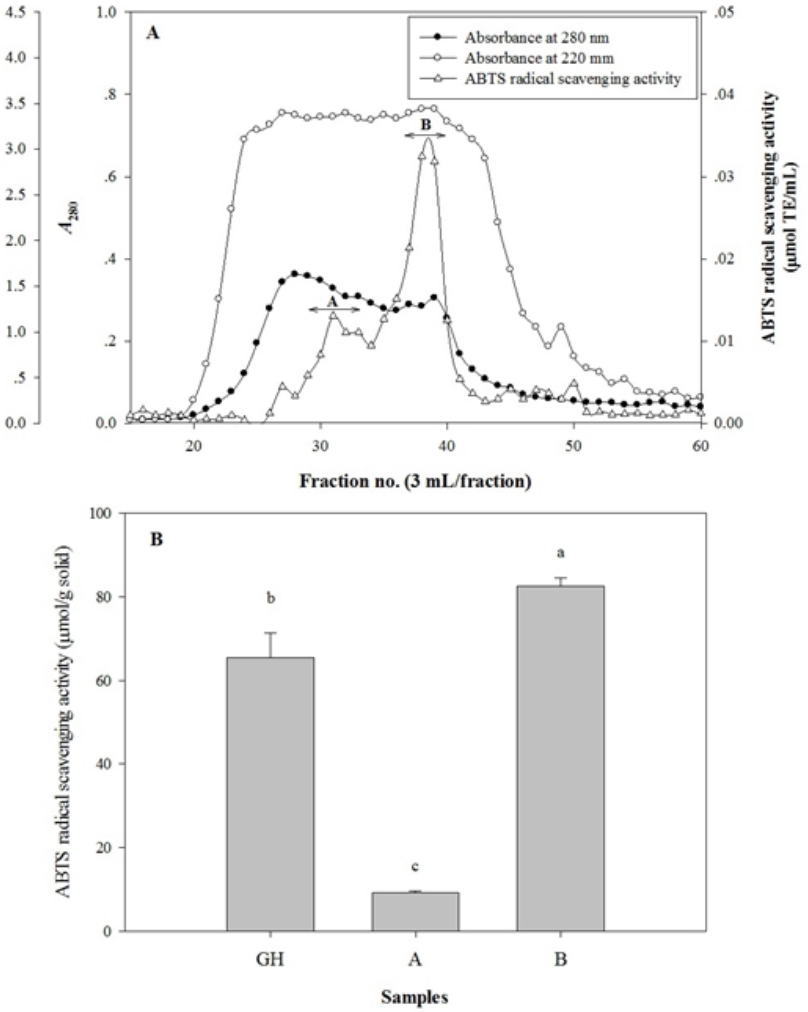

Figure 1. Elution profile of gelatin hydrolysate (GH) from unicorn leatherjacket skin on a Sephadex G-25 column (A) and ABTS radical scavenging activity of GH and its fractions (B). Values represent mean ± SD. Different letters on the bars indicate significant differences ($p < 0.05$) between values.

Fraction B obtained from Sephadex G-25 size exclusion chromatography was further separated by DEAE-cellulose column. Three fractions (B-1, B-2 and B-3) with ABTS radical scavenging activity were obtained from anion exchange chromatography (Fig. 2). The results suggested that most of the peptides in fraction B were negatively charged and those with lower negative charge were dominant (fraction B-1). The peptides with higher negative charge (B-2 and B-3) were eluted with increasing concentrations of NaCl. Fraction B-2 showed the highest ABTS radical scavenging activity (P<0.05) (Fig. 2B). A similar result was reported for antioxidant peptides from tilapia skin gelatin hydrolysates, in which highly charged peptides showed the highest antioxidant activity (ZHANGE *et al*., 2012). Peptide charge can vary depending on hydrolysis conditions such as the type of enzyme and substrate used. When peptides from a skipjack roe hydrolysate were separated by cation exchange, fractions with lower charges had stronger antioxidant

properties (INTARASIRISAWAT *et al*., 2013). In order to remove salt from fraction B-2, a Sephadex G-25 column was used. The desalted fraction was subsequently lyophilized.

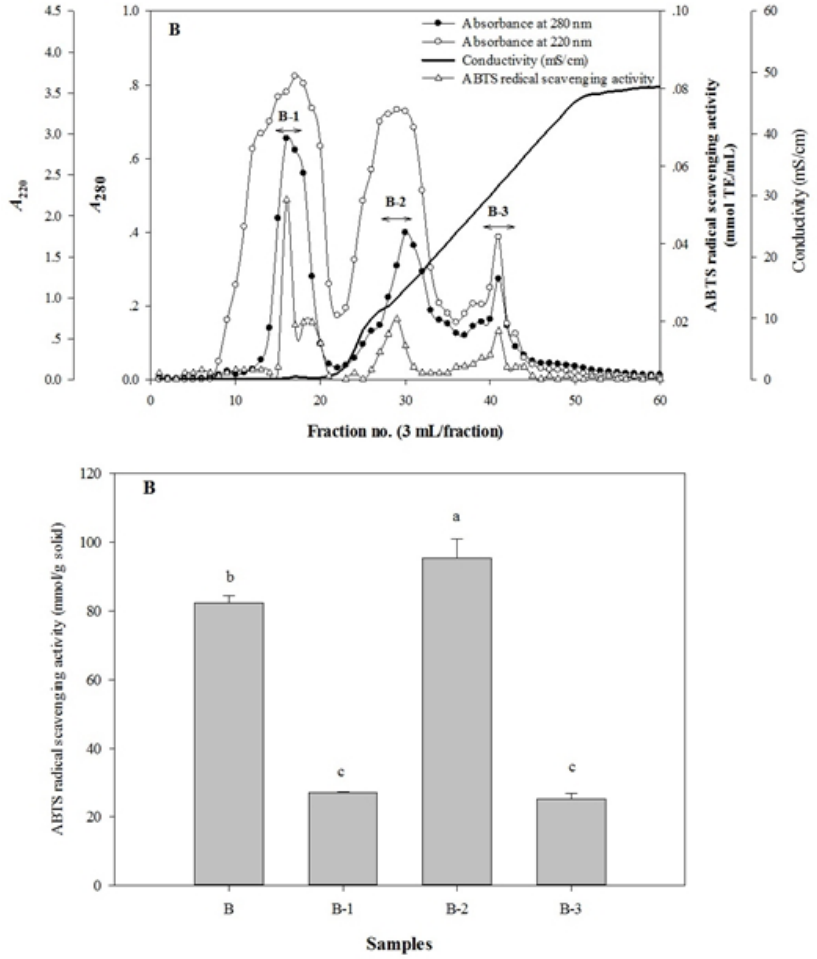

Figure 2. Elution profile of fraction B on a DEAE-cellulose column (A) and ABTS radical scavenging activity of fractions (B). Values represent mean ± SD. Different letters on the bars indicate significant differences ($p < 0.05$) between values.

Fraction B-2 was further fractionated by RP-HPLC on a C-18 column using a linear gradient of acetonitrile containing 0.1% TFA. As shown in Fig. 3, eighteen fractions were obtained from the RP-HPLC column and their ABTS radical scavenging activities were evaluated. The highest antioxidant activity was observed in fraction B-2/4 (0.72 total activity/A_{220}), followed by fraction B-2/8 (0.64 total activity/A_{220}). The fractions with higher hydrophobicity were eluted with increasing acetonitrile concentration. It was reported that the more hydrophobic peptides fractionated from enzymatic hydrolysates of tilapia frame protein using semi-preparative C18 RP-HPLC exhibited the highest antioxidant activity (FAN *et al*., 2012). In contrast, the more hydrophilic peptides obtained from a gelatin hydrolysate of tilapia skin using RP-HPLC column had the stronger antioxidant activity (ZHANG *et al*., 2012). In the present study, fraction B-2/4 and B-2/8, which demonstrated the highest antioxidant, activity were selected for peptide sequence identification using UPLC-MS/MS.

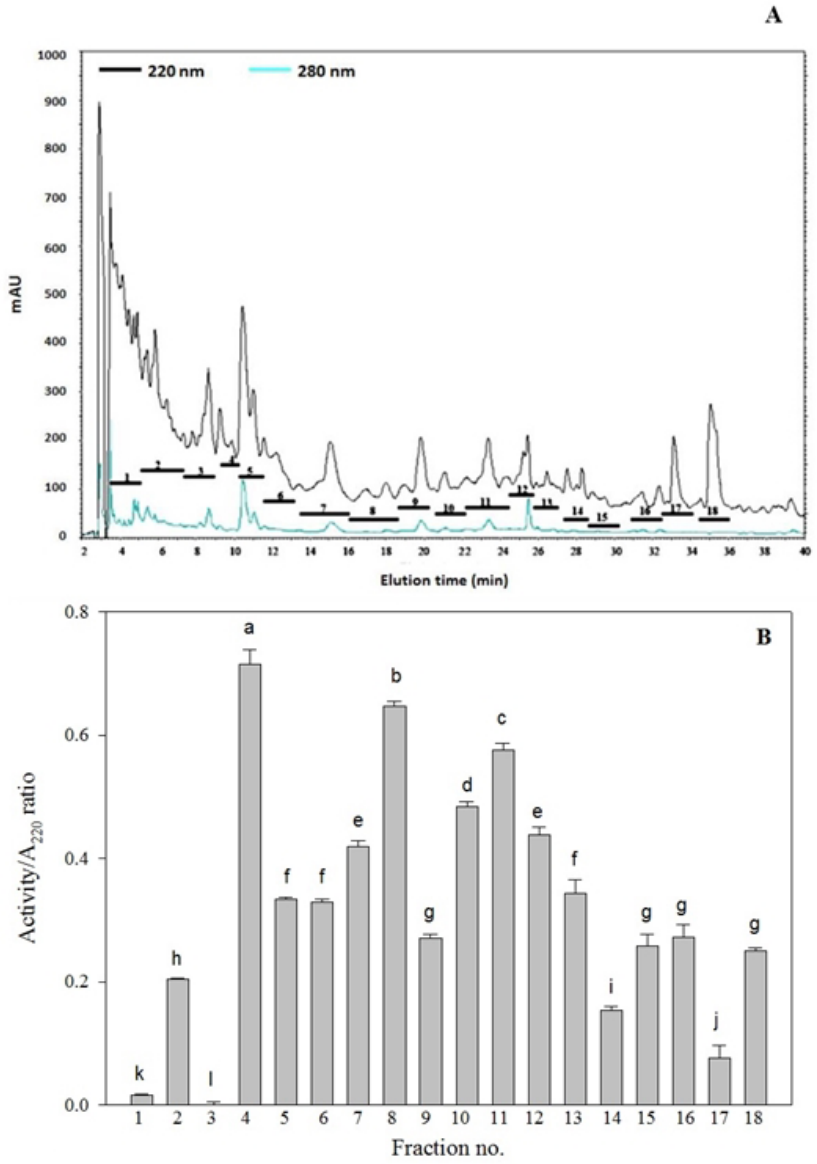

Figure 3. Elution profile of fraction B-2 on RP-HPLC (A) and ABTS radical scavenging activity/A_{220} ratio of fractions (B). Absorbance at 220 nm (-). Absorbance at 280 nm (-). Values represent mean ± SD. Different letters on the bars indicate significant differences ($p < 0.05$) between values.

3.2 Identification of antioxidant peptides

The identification of peptide sequences within selected fractions obtained from RP-HPLC (fraction B-2/4 and B-2/8) were determined by UPLC-MS/MS. Thirty peptides were identified with molecular weight range from 279.17 to 555.28 Da in fraction B-2/4. In fraction B-2/8, twenty six antioxidant peptides observed had MW ranging from 366.20 to 897.37 Da. Two peptides were selected from each fraction followed the criteria as mentioned above (Table 1). Glu-Pro-Gly-Pro-Val-Gly (555.27 Da) and Leu-Pro-Gly-Pro-Ala-Gly (511.29 Da) were identified from fraction B-2/4 (Fig. 4). Another two peptides, Leu-Asp-Gly-Pro-Val-Gly (557.30 Da) and Glu-Gly-Pro-Leu-Gly (472.24 Da), were

identified from fraction B-2/8 (Fig. 5). In this study, each identified peptide possessed a specifically arranged amino acid sequence typical of collagen and gelatin, where glycine strictly represents every third amino acid residue (LI *et al*., 2007). Moreover, the molecular weight of the peptides was similar to the antioxidant peptides isolated from tilapia gelatin hydrolysate (317.33-645.21 Da) (ZHANG *et al*., 2012; SUN *et al*., 2013). In addition, the selected peptides contained Gly at the C-terminal, indicating that the glycyl endopeptidase activity used for preparing the gelatin hydrolysates specifically cleaves peptide bonds with Gly at P_1 position (KARNJANAPRATUM *et al*., 2014).

Table 1. ABTS radical scavenging activity of synthesized peptides

Peptide sequence	Molecular weight (Da)	ABTS radical scavenging activity (μmol TE/g solid)
Glu-Pro-Gly-Pro-Val-Gly	555.28	1.25±0.36^b
Leu-Pro-Gly-Pro-Ala-Gly	511.29	1.22±0.10^b
Leu-Asp-Gly-Pro-Val-Gly	557.30	1.36±0.12^b
Glu-Gly-Pro-Leu-Gly	472.24	4.95±0.65^a

Values represent the mean ± SD for three separated experiment. Different letters in the same column indicate significant differences ($p < 0.05$) between values in the same column.

3.3. Antioxidant activity of synthesized peptides

Selected peptides (Glu-Pro-Gly-Pro-Val-Gly, Leu-Pro-Gly-Pro-Ala-Gly, Leu-Asp-Gly-Pro-Val-Gly and Glu-Gly-Pro-Leu-Gly) were synthesized for further study. All four synthetic peptides had antioxidant activity (Table 1) with Glu-Gly-Pro-Leu-Gly having the highest ABTS radical scavenging activity ($P<0.05$). The remaining three synthetic peptides all demonstrated similar antioxidant activity ($P>0.05$). Therefore, the amino acid sequence played an important role in antioxidant activity. Peptides rich in hydrophobic amino acids (Gly, Pro), which contribute substantially to free radical scavenging, were previously found in Pacific cod skin gelatin hydrolysate (NGO *et al*., 2011). The side chain of Gly consists of a single hydrogen atom and may confer high flexibility on the peptide bond. The pyrrolidine ring of Pro tends to interrupt the secondary structure of the peptide, thus imposing conformational constraints (RAJAPAKSE *et al*., 2005; ALEMAN *et al*., 2011). Glu and Leu acted as direct radical scavengers in a peptide purified from fish skin gelatin (ZHANG *et al*., 2009; MENDIS *et al*., 2005). It was noted that GH herein showed a higher ABTS radical scavenging activity (65.49 μmol TE/g sample) (Fig. 1B), compared with those of the synthesized peptides (1.22-4.95 μmol TE/g sample). This suggests that several peptides and/or free amino acids identified within the antioxidant fractions may exert a synergistic effect on radical scavenging activity.

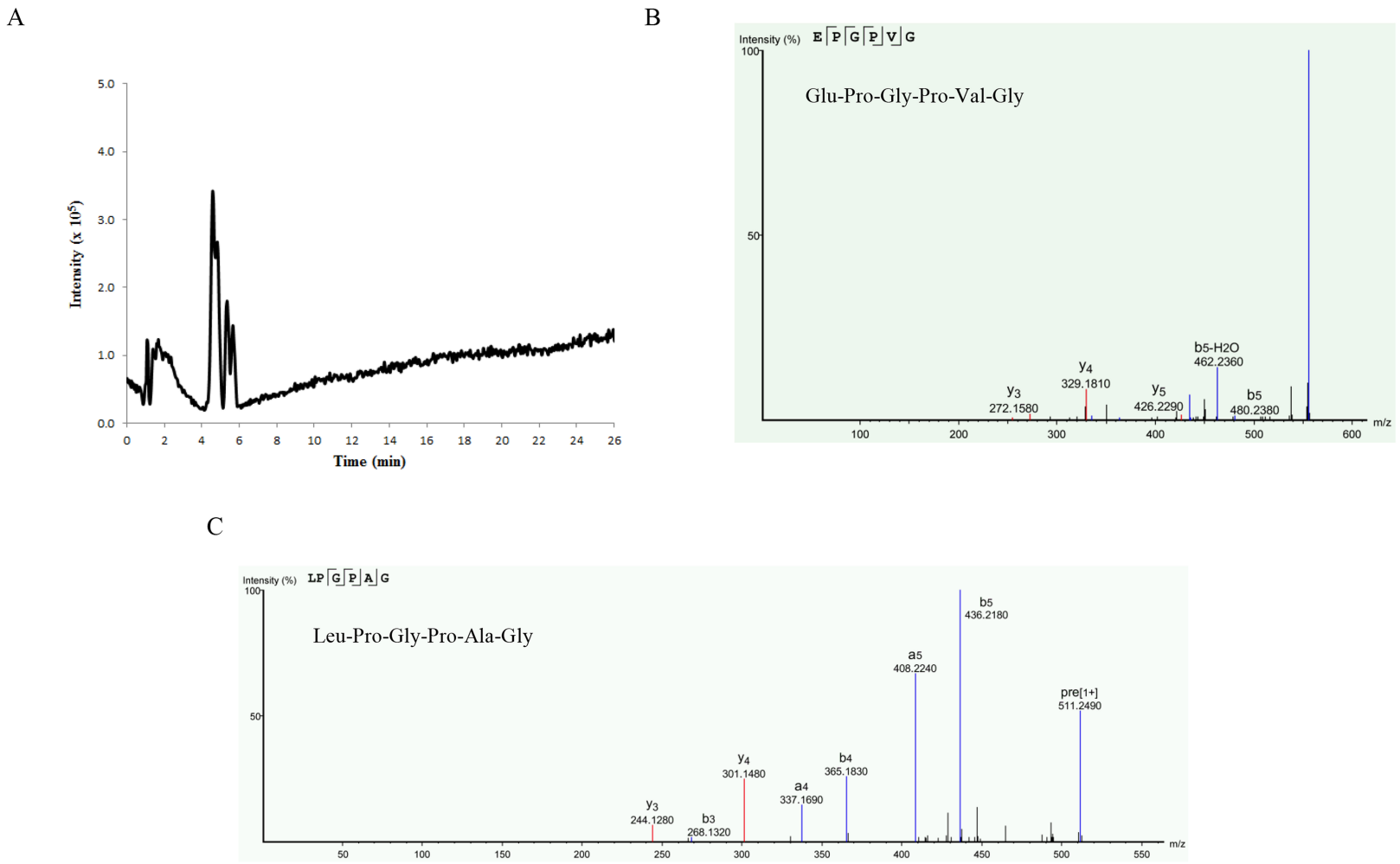

Figure 4. Base peak chromatogram (BPC) of B-2/4 fraction separated by Acquity UPLC BEH C18 column (A) and representative MS/MS spectra of peptides Glu-Pro-Gly-Pro-Val-Gly (B) and Leu-Pro-Gly-Pro-Ala-Gly (C). The x-axis shows the *m/z* of the precursor (pre) and fragment ions while the y-axis shows the relative intensity. The deduced sequence can be seen on the top left.

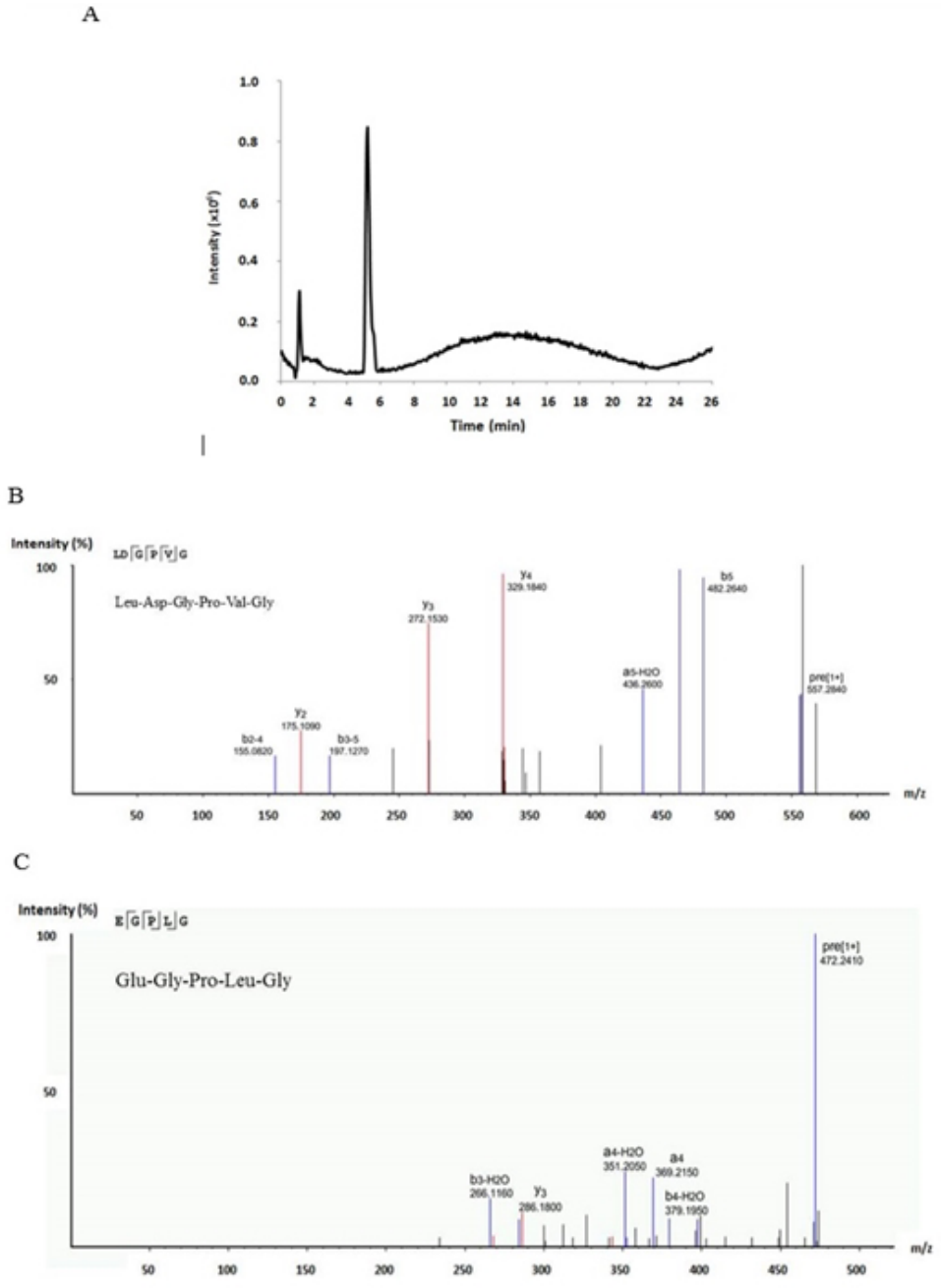

Figure 5. Base peak chromatogram (BPC) of B-2/8 fraction separated by Acquity UPLC BEH C18 column (A) and representative MS/MS spectra of peptides Leu-Asp-Gly-Pro-Val-Gly (B) and Glu-Gly-Pro-Leu-Gly (C). The x-axis shows the *m*/*z* of the precursor (pre) and fragment ions while the y-axis shows the relative intensity. The deduced sequence can be seen on the top left.

A similar result was reported for a patin (*Pangasius sutchi*) protein hydrolysate prepared using Alcalase and papain, in which the purified peptide obtained had a lower antioxidant activity, in comparison with a crude patin hydrolysate (NAJAFIAN *et al*., 2013). Therefore, the combination of peptides present in protein hydrolysates could produce higher bioactivity than the isolated peptides (ALUKO, 2015).

4. CONCLUSIONS

GH from unicorn leatherjacket skin prepared using glycyl endopeptidase contained peptides possessing free radical scavenging activity. Four antioxidant peptides were identified as Glu-Pro-Gly-Pro-Val-Gly (555.27 Da), Leu-Pro-Gly-Pro-Ala-Gly (511.29 Da), Leu-Asp-Gly-Pro-Val-Gly (557.30 Da) and Glu-Gly-Pro-Leu-Gly (472.24 Da). GH and its peptides could be potential candidates for functional food ingredients or nutraceuticals which protect against free radical generation and related diseases.

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PAPER

STUDY ON THE UTILIZATION OF PANEER WHEY AS FUNCTIONAL INGREDIENT FOR PAPAYA JAM

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ABSTRACT

The objective of the present study was to elaborate papaya jam by substituting 5% and 10% papaya pulp with same amount of paneer whey. Physico-chemical, sensory, microbial and texture analysis were performed on 0, 30th and 60th day of storage. Upon whey substitution in jams, protein content and texture of final products were significantly improved. Slight but not significant increase of acidity and decrease of pH were observed in all the formulations and during shelf life. Reducing sugar content in jam increased from16.20 to 37.49 and that of non-reducing sugar decreased from 45.18 to 26.23% during the storage period of 60 days. Absence of microbial growth was observed in all the jam formulations throughout the storage period. Taken together, whey can be efficiently substituted in papaya jam to improve nutritive value and texture of the product.

Keywords: jam, papaya pulp, protein, storage, substitution, texture, whey

1. INTRODUCTION

Jam is the most popular and shelf stable product made from fruits at household and commercial level. Fruit jams are good source of energy but poor source of protein (NAEEM *et al* 2015). Ingredients and fruit pulps used for preparing jams are commonly poor in protein content and ultimately results in less protein level in jam (NAEEM *et al* 2015). Commercially available jams are poor in protein content as reported by several workers i.e. apple jam - 0.04%, mango jam – 0.15%, jackfruit jam – 0.19%, papaya jam – 0.26%, blueberry jam – 0.31%, strawberry jam – 0.41%, grape jam – 0.27% and apricot jam – 0.43% pineapple jam – 0.46% and orange marmalade jam - 0.79% (AHMMED *et al*., 2015, TEANGPOOK and PAOSANTONG 2013, EKE-EJIOFOR and OWUNO, 2013 NAEEM *et al*., 2015). So there is a need to improve the protein content in jam to make it still a better nutritional food.

Whey is a valuable by-product obtained during manufacture of cheese, channa, casein paneer and shrikand as watery portion of milk after coagulation and removal of curd. Whey contains about 50% of milk solids together with 100% of lactose and 20% of protein. Lactose makes up about 75% of total whey solids (SISO, 1996). Whey represents about 80 to 90% of the volume of milk from which it is obtained (KHAMRUI and RAJORHIA, 1998). Whey protein is a complete, high quality source of protein with a rich amino acid profile. Whey has high protein efficiency ratio (3.6), biological value (104) and net protein utilization (95) is next only to egg protein in terms of nutritive value (RENNER, 1990). About 3 million tonnes of whey is produced annually in India containing about 2 lakh tonnes of valuable milk nutrients (NAIK *et al*., 2009). About 40% of total global production of whey is disposed as raw whey (REDDY *et al*., 1987) causing serious environmental pollution. The disposal of whey is problematic as the Biological Oxygen demand (BOD) of whey is 38,000 to 46,000 ppm (due to its high organic content) as compared to 200 ppm permissible limit for domestic sewage (MISHRA, 2008). Whey has to be treated appropriately to obtain commercial products (GUPTE and NAIR, 2010) or preheated before its discharge in inland water or rivers as per Environmental Protection Act (1986). So it is important to find alternative uses of whey to reduce the economic and environmental impact.

Whey is used in food industry for its high nutritional value, excellent functional properties and for reducing the cost of production of food products. Whey preparations are used in meat and meat products, reduced fat products, yoghurt, ice cream, cheese, bakery products, confectionary and pastry products, infant formula, whey beverage and for encapsulation of sensitive foods and edible coating of foods. The functional properties of whey proteins, mainly used in the production of food products are solubility, gelling, emulsifying, and water binding properties, antioxidant activity, flavour improvement and fat mimetics (Królczyk *et al*., 2016).

Papaya is considered as power house of nutrients as it is a rich source of carotenoids, Vitamin C, Vitamin E, niacin, riboflavin, Vitamin K, carbohydrate, folate, pantothenic acid and dietary fibre. It is also rich in Fe, Na, K, Ca, Mg, P, Cu, Zn and Mn (ARAVIND *et al*., 2013). Papaya is popularly used as dessert or processed into jam, puree or wine (MATSUURA *et al*., 2004).

In the present study, different percentages of papaya pulp were substituted with paneer whey in formulations for papaya jam. Upon substitution, its effects on functional and nutritional properties of jam were studied along with sensory and microbial analysis during storage period of two months.

2. MATERIALS AND METHODS

Plain condensed whey obtained from paneer (Dairy Plant, College of Veterinary and Animal Science, Thrissur) was utilized for study. Papaya used in the experiment was obtained from the local market. Pectin, Sugar (sucrose), citric acid (food grades) was purchased from local market.

2.1 Preparation of papaya jam

Three type's papaya jams were prepared by substituting papaya pulp with paneer whey. Control Jam group (T_0) served as control without whey substitution in papaya pulp (100 g papaya pulp + 0 g whey). Substitution of papaya pulp with condensed whey both for T_1 and T_2 are 5%(95 g papaya pulp + 5 g whey) and 10%(90 g papaya pulp + 10 g whey) respectively. Composition of prepared papaya jam was given in Table 1 and method of preparation was given in Fig. 1.

For substitution in jam, plain condensed whey (semi- solid form) was allowed for slow hydration for a period of 30 min by mixing with water at 60°C at the ratio of 1:2. Hydration was performed for optimal performance of whey protein during heat processing (ZHANG and ZHONG, 2010). The normal range pH range of jam was 2.5 to 3.5 (BROOMFIELD, 1996) and this pH range of jam helps in stability of whey protein.β-lactoglobulin in whey is heat stable at pH 3 (BOYE *et al*., 1996). Heat stability of whey protein also further improved by presence of sucrose in jam (KULMYRZAEV *et al*., 2000). According to DURANTI *et al*. (1989) heating to 85°C is critical for whey protein denaturation. So concentrated whey was substituted in the jam just 1 or 2 minutes before reaching end point of jam preparation (105.5ºC) to avoid denaturation. Prepared jams were stored in sterilized glass jars at room temperature (30ºC).

Table 1. Formulation of jam.

Composition	Control Jam (T_0)	T_1	T_2
Papaya Pulp (g)	100	95	90
Whey (g)	0	5	10
Sucrose (g)	75	75	75
Pectin (g)	1	1	1
Citric Acid (g)	0.6	0.6	0.6

2.2. Physical-chemical analysis

Physical-chemical analyses were performed in jam samples on 0^{th}, 30^{th} and 60^{th} days of storage and results were expressed as mean±standard error of mean. Following analysis were performed according to regulations and protocols described by Association of Official Analytical Chemists (2000): pH using a digital pH meter, total soluble solids (TSS,°Brix) by using an Abbe refractometer, water activity (Aw) using Aw Sprint - Novasina TH-500®, crude protein using micro Kjeldahl method,% acidity by titration with NaOH (0.1 M), ash by muffle furnace and moisture by drying in kiln. Estimation of fat in condensed whey was performed as per protocol described by AOAC (2000). Sugars in jams (total sugars, reducing sugar and non-reducing sugar) were determined as per Lane and Eynon method (1923).

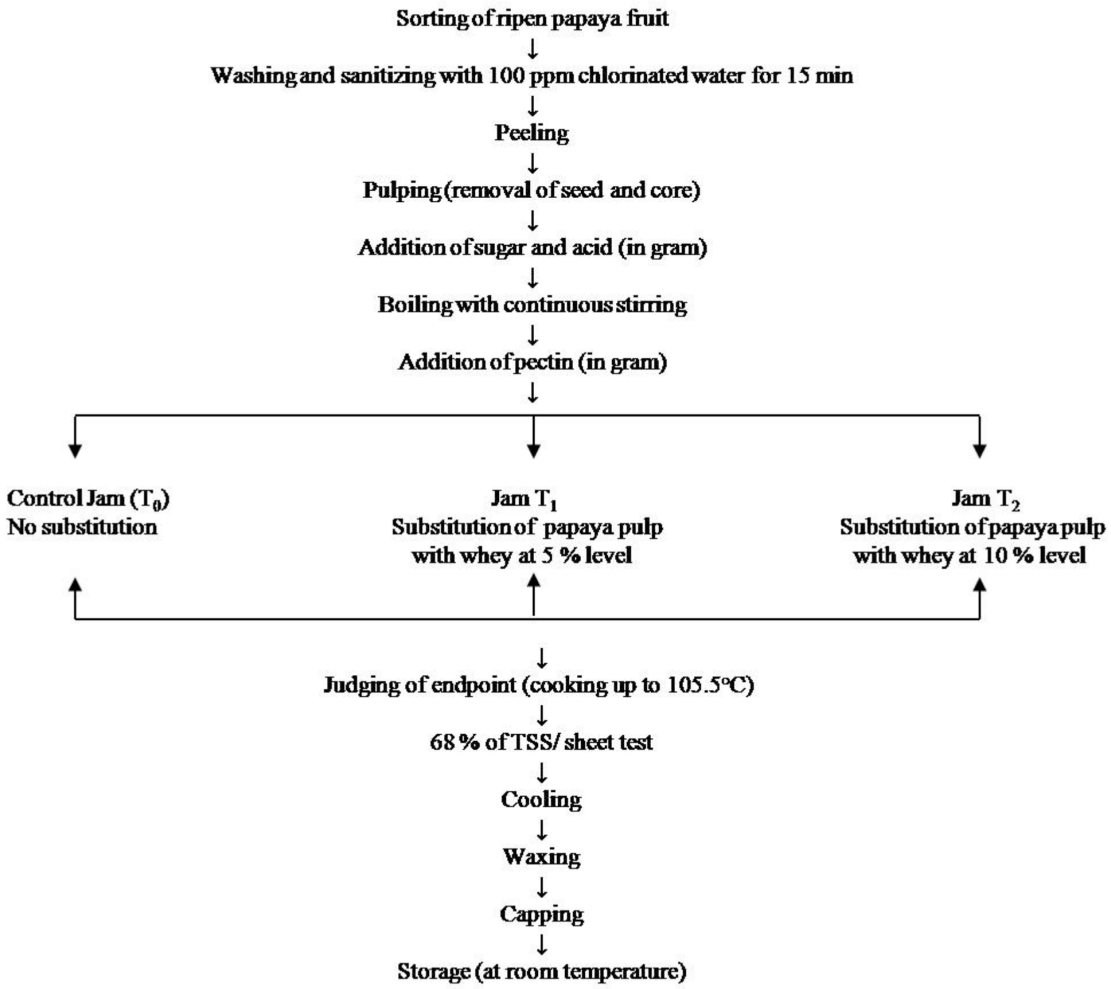

Figure 1. Flow chart for jam preparation.

2.3. Microbial analysis

Standard total plate count, spore count, coliform count, yeast and mould count of jams were performed as per RANGANNA (1986) on 0th, 30th and 60th day of storage period for T_0, T_1 and T_2 jams.

2.4. Sensory evaluation

Jam for organoleptic evaluation was prepared aseptically in clean transparent disposable closed containers and served fresh on test day in a perfectly homogeneous way, i.e. identical conditions of preparation, conservation and presentation. Sixteen trained members of panel were selected from the university community among postgraduate students for evaluating sensory characteristics (color and appearance, taste, texture and overall acceptability) of the samples using a 9-point hedonic scale as per RANGANNA (2008). During product test, panel members were allowed to clean their mouth at intervals with water. The sensory evaluation of jams was performed on 0th, 30th and 60th days of storage.

2.5. Texture analysis

Texture analysis was performed directly in jar containing jam at the ambient temperature with a Texture analyzer TA.XTplus® (Stable Micro System, United Kingdom), using back extrusion procedure. It was used to measure the force – time curve for a two cycle compression. A cylindrical probe (1 inch) was used to compress the samples. On the basis of the preliminary work, instrument working parameters were determined with test mode compression, pretest speed at 1.0 mm/s, test speed at 1.0 mm/s, post-test speed at 10.0 mm/s, distance 10.0 mm, trigger force at 10.0 g and data acquisition rate at 200 pp (YOUNIS *et al*., 2015). Data were analyzed using Texture expert Version 1.22® Software (Stable Micro System, United Kingdom) to measure hardness, consistency, cohesiveness and index of viscosity in the samples. All measurements were done in triplicates of jam samples.

2.6. Statistical analysis

ANOVA with Tukey's t-test, at the 5% level, was applied to data to establish significance of difference among the samples. Statistical analyses were performed using the statistical analysis package STATISTICA 7.0®.
The experiment was carried out with 6 replications

3. RESULTS AND DISCUSSIONS

The composition of plain condensed whey obtained from paneer is presented in Table 2. The papaya pulp had pH of 4.46, total soluble solid 10±0.56% and acidity 2.05±0.05% (Table 3). Similar findings were reported by ZAMAN *et al*. (2006) where pH of papaya fruit pulp ranged from 4.2 to 4.5, total soluble solids varied from 9.0 to 13.0%, the acidity (as citric acid) ranged between 2.00 to 2.30%, total sugar ranged from 6.96 to 10.5%, reducing sugar ranged from 3.42 to 6.92% and non-reducing sugar ranged from 3.17 to 3.58%. ARAVIND *et al*. (2013) reported that papaya pulp contain 0.61% crude protein. SARAN and CHOUDHARY (2013) reported that ash content of papaya pulp range from 0.31 to 0.66% and moisture from 85.9 to 92.6.

Table 2. Composition of plain condensed whey.

	Total solids%	Acidity%	Ash%	Fat%	Crude Protein%
Plain condensed	58.96±0.69	0.48±0.05	6.54±0.21	0.68±0.71	7.35±1.61

SEM – Standard error of mean

3.1. Physical-chemical analysis of jam

The physical chemical properties of jam are presented in Table 4. In this experiment, acidity values of all jams ranged from 0.55% to 0.59% during the storage period of two months). MAMEDE *et al*., 2013 reported that acidity of jam ranges between 0.5% and 0.8% of citric acid and jam with acidity above 1% shows syneresis as higher acidity value causes exudation of liquid from jam. Substitution with whey at 5% (T_1) and 10% (T_2) level did not affect significantly the acidity of jam; even if a slight but not significant rise in the acidity of all jams was observe during the storage period. TEANGPOOK and PAOSANTONG

(2013) reported that acidity of low sucrose lime juice papaya jam increased from 0.63 to 0.70% during the storage period of 6 months. Similarly SHAKIR *et al*. (2008) reported that there was increase in acidity from 0.6 to 0.78% in apple and pear mixed jam during the storage period of 3 months. Increase in acidity might be ascribed to rise in concentration of weakly ionized acids and their salts during storage. Further, rise in acidity might also be due to formation of acids by degradation of polysaccharides and oxidation of reducing sugars or by breakdown of pectic substances and uronic acid (HUSSAIN *et al*., 2008).

Table 3. Chemical Composition of fresh papaya pulp.

	pH	Total soluble solids %	Acidity %	Total sugar %	Reducing sugar %	Non-reducing sugar %	Crude Protein%	Ash %	Moisture %
Papaya pulp (Mean± SEM)	4.46±0.02	10±0.56	2.05±0.05	7.26±0.1	3.92±0.6	3.34±0.01	0.65±0.25	0.45±0.06	88.16±0.23

SEM – Standard error of mean

In jam, total soluble solids or °Brix is a measure of all soluble solids from natural fruit components, added sugar, acid, pectin and other ingredients. According to Food Safety and Standards Regulations (2011) total soluble solids of jam should be not less than 65%. For optimum gel formation in jam with good texture and sensory acceptance, the total soluble solids should range between 65 and 68% (MACRAE *et al*., 1993; DAMIANI *et al*., 2008). In current experiment, total soluble solids of all jams were > 65.0% without any significant differences among them. Substitution of papaya pulp with whey in jam at 5% (T_1) and 10% (T_2) level not caused any changes in total soluble solids level (Table 4). During storage period, all jams showed slight but not significant rise in TSS. TSS of T_1 increased from 66.25 to 67.06, T_2 from 67.52 to 68.01 and Jam C from 68.56 to 69.18. Similarly EHSAN *et al*. (2002 and2003) reported rise in TSS of watermelon lemon jam from 68.6 to 68.9 and grape fruit apple marmalade from 70.0 to 70.8 after 60 days of storage. SHAKIR *et al*. (2008) reported that there was increase in total soluble solids of apple and pear mixed fruit jam from 68.5 to 70.6 during the storage period of 90 days. Increase in TSS during storage might be due to acid hydrolysis of polysaccharides especially gums and pectin (LUH and WOODROOF, 1975).

Setting quality of jam can be improved by adequate pH maintenance. pH and titratable acidity are indicators for quantity of organic acids and their salts contained in a fruit. In our experiment pH value of all jams were ranged between 3.08and 3.36. This pH range was close to the optimal pH suggested by RAUCH (1965), which ranges from 2.5 (hard jam) to 3.45 (soft jam). Similarly, BROOMFIELD (1996) also reported that the pH range of 2.5 to 3.5 in jam was suggested for stable pectin-acid- sugar gel structure. The formation of gel structure in jam depends on the concentration of hydrogen ions and not that of the acidity. TEANGPOOK and PAOSANTONG (2013) reported that low sucrose lime juice papaya jam had pH of 3.22. There was slight decrease in pH of T_0 from 3.31 to 3.09, $T_1$from 3.36 to 3.15 andT_2 from3.27 to 3.08 during storage period of two month without any significant difference). KHAN *et al*. (2012) reported that pH value of the strawberry jam decreased from 3.20 to 2.91 during the storage period of 60 days.

Table 4. Physico-chemical analysis of jam.

		Control Jam (T_0)	T_1	T_2
Acidity	0th day	0.55±0.017	0.58±0.03	0.58±0.02
	30th day	0.57±0.04	0.58±1.25	0.59±1.28
	60th day	0.57±0.05	0.59±0.56	0.59±1.58
Total soluble solids (^{0}brix)	0th day	66.25±0.029	67.52±0.028	68.56±0.025
	30th day	66.78±0.09	67.91±0.18	68.92±0.08
	60th day	67.06±0.24	68.01±0.87	69.18±0.14
pH	0th day	3.31±0.12	3.36±0.25	3.27±0.71
	30th day	3.25±0.53	3.21 ±0.98	3.15±0.59
	60th day	3.09±0.78	3.15±0.65	3.08±0.27
Ash (%)	0th day	0.323±0.002	0.323±0.002	0.323±0.007
	30th day	0.323±0.006	0.324±0.006	0.324±0.006
	60th day	0.324±0.009	0.324±0.005	0.324±0.005
Moisture	0th day	26.24±0.21^{Aa}	28.74±0.21^{Ba}	30.32±0.25^{Ca}
	30th day	25.22±0.36^{Ab}	27.42±0.78^{Bb}	29.60±0.39^{Cb}
	60th day	24.36±0.54^{Ac}	26.19±0.87^{Bc}	28.91±0.17^{Cc}
Crude protein	0th day	0.92±0.028^{A}	3.15± 0.028^{B}	4.23±0.03^{C}
	30th day	0.85±0.58^{A}	3.14±0.13^{B}	4.20±0.04^{C}
	60th day	0.84±0.24^{A}	3.12±0.54^{B}	4.20±0.03^{C}
Reducing sugars	0th day	16.20 ±0.06^{Aa}	19.54 ±0.02^{Ba}	22.17 ±0.01^{Ca}
	30th day	18.47±0.03^{Ab}	25.63±0.01^{Bb}	29.35 ±0.05^{Cb}
	60th day	24.30 ±0.18^{Ac}	32.44 ±0.01^{Bc}	37.49 ±0.02^{Cc}
Non reducing sugar	0th day	45.18±0.01^{Ac}	43.16 ±0.01^{Bc}	40.28 ±0.02^{Cc}
	30th day	43.19±0.12^{Ab}	37.24 ±0.01^{Bb}	32.50 ±0.02^{Cb}
	60th day	38.54 ±0.29^{Aa}	31.55 ±0.08^{Ba}	26.23 ±0.03^{Ca}
Water Activity	0th day	0.80±0.001^{C}	0.78±0.002^{B}	0.75±0.003^{A}
	30th day	0.80±0.001^{C}	0.78±0.002^{B}	0.75±0.003^{A}
	60th day	0.80±0.001^{C}	0.78±0.002^{B}	0.75±0.003^{A}
Microbial Analysis	0th day	Nil	Nil	Nil
	30th day	Nil	Nil	Nil
	60th day	Nil	Nil	Nil

SEM – Standard Error of mean.

^{ABC} means on the same line without a common letter are significantly different at $P < 0.05$. (all samples at the same time).

^{abc} means on the same column without a common letter are significantly different at $P < 0.05$. (single sample during storage period).

jam significantly ($P < 0.05$) decreased with 5% whey substitution in T_1 (0.78) and with of 10% whey substitution in T_2 (0.75) substitution when compared to that of control jam T_0(0.80) TEANGPOOK and PAOSANTONG (2013) reported that low sucrose lime juice papaya jam had water activity of0.9. SANTOS *et al*. (2013) showed that water activity of gabiroba jams prepared with sucrose and sucralose were 0.78 and 0.80 respectively. In this study there was no change in water activity of all jams during the storage period of 60 days.

The decrease in pH of jam during storage may be attributed to formation of free acids by degradation of polysaccharides, oxidation of reducing sugar, ascorbic acid degradation and hydrolysis of pectin (HUSSAIN and SHAKIR, 2010).

There was no significant rise ($P>0.05$) in ash content of both T_1 and T_2due to whey substitution). Ash content of all jams ranged from 0.323% to 0.324% without any significant changes during storage. TEANGPOOK and PAOSANTONG (2013) reported low sucrose lime juice papaya jam had ash content of 0.51%. AHMED *et al*. (2011) reported ash content of sapota jam as 0.42%. VIDHYA and NARAIN (2011) reported that there was no change in ash content of wood apple jam during storage period of 90 days.

Statistical analyses revealed that addition of whey significantly ($P<0.05$) increased the moisture content of T_1 (28.74%) and T_2 (30.32%) when compared to that of control jam T_0 (26.24%). This may be due to higher water retention property of protein present in whey (VIDIGAL *et al*., 2012). T_2jam has significantly higher ($P<0.05$ moisture content than that of T_1. EKE-EJIOFOR and OWUNO (2013) reported that moisture content of jack fruit jam and pineapple jam in their experiments were 24.60% and 23.29% respectively. MAMEDE *et al*. (2013) observed that the moisture content of dietetic jam prepared from umbucaja fruit ranged from 26.7 to 31.98%.The moisture content of jam produced from dehydrated fruits (Tamarind Guava and Kumquarts) ranges normally between 28.6 – 30.1% (WINUS,2011). It is important to consider that the moisture content is directly related to the conservation of the product during storage. Reduction in moisture content of jam may decrease gel strength and thereby causes firmer jam with poor spreading ability (FASOGBON, 2013). In this experiment, there was significant reduction ($P>0.05$) in moisture content of all jams T_0 (from 26.24 to 24.36%), T_1 (from 28.74 to 26.19%), and T_2 (from 30.32 to 28.91%), during storage period of 60 days. Similarly, HUSSAIN and SHAKIR (2010) observed a decreasing trend in the moisture content of apricot and apple jam (from 16.08 to 4.3%) during the storage period of 60 days. ANJUM *et al.* (2000) observed decreased in% moisture from 79% to 77% after 60 days of storage in dried apricot diet jam. The loss of moisture in jam that is stored in sterilized glass container in room temperature is due to the exchange of moisture between the outside and inside of the glass by desorption, trapping the free water during gel formation in jam and due to Maillard reaction, which occurs at high temperatures (even at 25°C) in high-sugar products with low pH values by using the freely available water in jam (DAMIANI *et al* 2012).

Protein content of most fruit jam is very low due to the low protein content of most of fruit pulp and none of the ingredients used in jam preparation are an abundant source of protein (NAEEM *et al* 2015) In this experiment, T_2 contain significantly ($P < 0.05$) highest crude protein content (4.23%) due to 10% whey substitution. T_1 with 5% whey substitution contain crude protein content (3.15%) significantly ($P < 0.05$) higher than control T_0(0.92%). TEANGPOOK and PAOSANTONG (2013) reported that low sucrose lime juice papaya jam had protein content of only 0.26%. EKE-EJIOFOR and OWUNO (2013) prepared jackfruit jam using pineapple jam as control and reported that protein content ranged from 0.19% - 1.12% with pineapple jam had lowest and jackfruit jam had highest of the products. SALVADOR *et al*. (2012) reported that proteins were present in small amounts in yacon jams as yacon pulp had low protein content. CARVALHO *et al*. (2013) estimated that protein content of diet strawberry jam ranged between 1.31 and 1.35%. GEBHARDT and THOMAS (2002) reported that protein content present in one tablespoon (20 gram) of jam was in traces. CHAUDHARY and VERMA (2011) analysed the physicochemical properties of the processed fruits and vegetable products and found that protein content of jam was 0.48% which is least when compared to the protein content of other processed products (tomato sauce – 0.82%, pickle – 1.98 and orange juice – 1.97%). No significant change was observed in the value of protein content of all the jam samples during the storage period of two months.

Sugars that have high tendency to crystallize like pure dextrose (glucose) were not used in this study, in contrary refined sucrose is known to be a good sugar for addition to jam because of low tendency to recrystallization was used. Sucrose is partly inverted to glucose and fructose in the manufacturing process when the pH of the product is low. This fact was important because as it reduces tendency of sugar to form crystals (CANCELA *et al*., 2005). Statistical analyses revealed that substitution of whey significantly ($P<0.05$) increased the reducing sugar content in T_1 (19.54%) and T_2 (22.17%) when compared to control jam T_0 (16.20%). This increase in reducing sugar content in jam is due to the presence of lactose in whey and lactose itself is a reducing sugar (JENNESS AND PATTON, 1959). Presence of lactose may lead to formation of Maillard reaction products, which are known to have antioxidant properties in food systems (DE WIT 2001). The rise in reducing sugar by whey substitution has correspondingly significant decrease ($P > 0.05$) in non-reducing sugar content of jam T_1(43.16%) and T_2 (40.28%) when compared to T_0(45.18%). During storage period, there was significant ($P < 0.05$) rise in reducing sugar and decline in non-reducing sugar content of all jams. The results of reduction in non-reducing sugars in jams were in accordance with RIAZ *et al*. (1999) who reported reduction in non-reducing sugars from 44.64 to 32.35% in strawberry jam during storage period of 3 months. EHSAN *et al*. (2003) observed drop in non-reducing sugars of grape fruit apple marmalade during storage period of 60 days. Rise in reducing sugar may be due to inversion of non-reducing (sucrose) to reducing sugar (glucose + fructose) because of acid and high temperature during storage (MUHAMMAD *et al*., 2008). RIAZ *et al*. (1999) observed increasing trend in reducing sugars of strawberry jam during 3 months storage period. ANJUM *et al*. (2000) while working on apricot diet jam also observed similar increase in reducing sugars during storage period of 60 days. EHSAN *et al*. (2003) reported increasing trend in reducing sugars of grape fruit apple marmalade where reducing sugars increased from 16.55% to 31.36% after 60 days of storage.

Water activity (Aw) determines the lower limit of available water for microbial growth. Water activity for jams must be less than 0.95 in order to prevent the growth of pathogenic bacteria. In general, minimum Aw for most moulds were 0.8, yeasts - 0.85 and osmophillic yeasts- 0.6-0.7 (RAY and BHUNIA, 2014). Most yeasts, molds and bacteria do not survive at low water activities, which were directly correlated with a long shelf life (BEUCHAT, 1981) In this experiment water activity of papaya jam significantly ($P < 0.05$) decreased with 5% whey substitution in T_1 (0.78) and with of 10% whey substitution in T_2 (0.75) substitution when compared to that of control jam T_0 (0.80) TEANGPOOK and PAOSANTONG (2013) reported that low sucrose lime juice papaya jam had water activity of0.9. SANTOS *et al*. (2013) showed that water activity of gabiroba jams prepared with sucrose and sucralose were 0.78 and 0.80 respectively. In this study there was no change in water activity of all jams during the storage period of 60 days.

3.2. Microbial analysis

Microbial analysis of jam during 0^{th}, 30^{th} and 60^{th} day of storage revealed no bacterial or fungal growth in all three types of jam (T_0, T_1 and T_2). The presence of total soluble solids about 66 to 70% protects jam from microbial deterioration (TAUFIK and KARIM, 1992). In this experiment the total soluble solids of all jam ranges between 66.25 to 69.18%, which resulted in the absence of microbial growth during the storage period of two months. Jam was also prepared with hygienic measures during handling and storage to make it microbiologically safe.

The microbial results of this study were in agreement with findings of many authors like PARSI ROS (1976) who stated that papaya jam stored for 180 days at 29.4°C were found to be microbiologically safe. JOY and RANI (2013) reported that microbial analysis showed

no growth of any kind of bacteria or fungi in the plates of Aloe Vera strawberry jam, papaya jam and pineapple jam till the 60th day of storage. KERDSUPA and NAKNEANA (2013) observed no bacteria, fungi and yeast growth in low-sugar mango jam over 6 weeks of storage.

3.3. Texture analysis

Textural properties of papaya jam were significantly (P<0.05) increased by whey substitution (Table 5). Water retention capacity and gelling properties of whey protein increased texture of jam by avoiding syneresis. ANTUNES *et al*. (2003) also reported that β- lactoglobulin in the whey act as a main gelling agent due to presence of free sulfhydryl group. Firmness of T_2jam (283.83 N) was significantly higher (P<0.05) than T_1 jam (209.92 N) and control jam T_0(147.83 N). Firmness of T_1 jam was significantly higher (P<0.05) than control jam T_0.VIDIGAL *et al*. (2012) reported that addition of whey protein concentrate improved firmness but not adhesiveness and cohesiveness of ice cream. Adhesiveness of T_2jam (-1662.8 NS) was significantly higher (P<0.05) than T_1 jam (-1543.3NS) and control jam T_0 (-755.83 NS). Adhesiveness of T_1 jam was significantly higher (P<0.05) than control jam T_0. HERRERO and REQUENA (2006) showed that addition of whey protein concentrate enhanced textural characteristics of yoghurt prepared from goat milk by increasing its firmness, hardness and adhesiveness. Viscosity of T_2jam (10276.0 cP) was significantly higher (P<0.05) than T_1 jam (8739.20 cP) and control jam T_0 (5072.8 cP). Viscosity of T_1 jam was significantly higher (P<0.05) than control jam T_0. Increased viscosity is important in fruit jams as it gives better mouth feel, a greater sense of fruitiness and sweetness in final product (JAVANMARD and ENDAN, 2010).

Table 5. Texture analysis of jam.

Parameters		Control Jam (T_0)	T_1Mean±SEM	T_2Mean±SEM
Firmness (N)	0th day	147.83 ±1.51 [a]	209.92 ±0.75 [b]	283.83 ±0.87 [c]
	30th day	153.3 ±0.49 [a]	210.60 ±0.91 [b]	285.50±1.33 [c]
	60th day	155.5 ±0.56 [a]	221.1 ±0.47 [b]	291.5 ±0.67 [c]
Consistency (NS)	0th day	1672.2 ±6.01 [a]	2021.8±3.96 [b]	2549.0±4.42 [c]
	30th day	1679.0 ±2.85 [a]	2033.16 ±2.41 [b]	2561.16 ±2.77 [c]
	60th day	1683.0 ±4.4 [a]	2048.66 ±2.02 [b]	2579.33 ±2.11 [c]
Adhesiveness (NS)	0th day	-755.83±1.30 [a]	-1543.3±2.72 [b]	-1662.8±6.18 [c]
	30th day	-764.66±0.88 [a]	-1558.66±2.62 [b]	-1671.33±1.23 [c]
	60th day	-782.16±1.85 [a]	-1575.16±1.94 [b]	-1684.33±1.54 [c]
Viscosity (cP)	0th day	5072.8±7.74 [a]	8739.20±9.60 [b]	10276.0±1.30 [c]
	30th day	5119.33±3.45 [a]	8763.33±2.24 [b]	10313.66±1.87 [c]
	60th day	5152.16±2.12 [a]	8787.16±1.68 [b]	10344.16±1.74 [c]
Cohesiveness (N)	0th day	264.66±1.14 [a]	371.83±1.10 [b]	486.33±4.88 [c]
	30th day	272.00±0.96 [a]	381.50±0.71 [b]	504.83±1.13 [c]
	60th day	282.33±0.84 [a]	392.33±1.25 [b]	519.33±1.11 [c]

SEM – Standard Error of mean.

[abc] means on the same line without a common letter are significantly different at P < 0.05. (all samples at the same time).

LI and GUO (2006) showed that incorporation of polymerized whey protein (prepared by thermal denaturation method) in yoghurt significantly improved viscosity. Consistency of T_2jam (2549.0 NS) was significantly higher ($P<0.05$) than T_1 jam (2021.8 NS) and control jam T_0 (1672.2 NS). Consistency of T_1 jam was significantly higher ($P<0.05$) than control jam T_0.Whey proteins were added to yoghurts to increase total solid content of milk in order to provide better consistency, texture and creaminess to the product (Martinez *et al*., 2002). Cohesiveness of T_2 jam (486.33 N) was significantly higher ($P<0.05$) than T_1 jam (371.83 N) and control jam T_0(264.66 N). Cohesiveness of T_1 jam was significantly higher ($P<0.05$) than control jam T_0.RAJU *et al*. (2007) substituted refined wheat flour with whey protein up to 30% in biscuit manufacture and observed increase in cohesiveness of biscuit dough which reduced the fracture stress of biscuit. Addition of whey protein increased hardness, adhesiveness, gumminess and chewiness of frankfurters prepared with 5% and 12% fat (HUGHES *et al*., 1998).

The results of textural parameters of jam from both sensory evaluation by sensory panel and instrument (texture analyzer) were in accordance to each other. Jam with 10% whey substitution (T_2) showed significantly higher value than other jams (T_0 and T_1) in textural parameters both through textural analyzer and sensory panel.

3.4. Organoleptic evaluation

Sensory scores of jam samples (Table 6) was performed based on 9 point hedonic scale to evaluate colour and appearance, taste, flavour, texture and overall acceptability with 9 = like extremely, 5= neither like nor dislike and 1= dislike extremely. Appearance and texture revealed that there was no crystallization of sugars in all jams. Presence of off flavor was not noticed in jam samples.

Table 6. Sensory Eevaluation of jam.

Parameters		Control Jam(T_0)	T_1	T_2
Color and appearance	0^{th} day	8.5 ± 0.03^{A}	7.12 ± 0.21^{B}	7.75 ± 0.11^{AB}
	30^{th} day	8.4 ± 0.16^{A}	7.11 ± 0.08^{B}	7.64 ± 0.17^{AB}
	60^{th} day	8.3 ± 0.03^{A}	7.08 ± 0.27^{B}	7.52 ± 0.11^{AB}
Taste	0^{th} day	8.3 ± 0.08^{A}	7.37 ± 0.03^{B}	7.37 ± 0.15^{B}
	30^{th} day	8.1 ± 0.07^{A}	7.14 ± 0.24^{B}	7.22 ± 0.19^{B}
	60^{th} day	8.0 ± 0.23^{A}	7.18 ± 0.31^{B}	7.18 ± 0.43^{B}
Flavor	0^{th} day	8.93 ± 0.05^{B}	7.06 ± 0.26^{A}	7.93 ± 0.38^{AB}
	30^{th} day	8.89 ± 0.17^{B}	7.18 ± 0.27^{A}	7.85 ± 0.19^{AB}
	60^{th} day	8.74 ± 0.05^{B}	7.29 ± 0.07^{A}	7.71 ± 0.22^{AB}
Texture	0^{th} day	7.32 ± 0.06^{A}	8.06 ± 0.28^{AB}	8.75 ± 0.03^{B}
	30^{th} day	7.44 ± 0.08^{A}	8.08 ± 0.34^{AB}	8.55 ± 0.11^{B}
	60^{th} day	7.62 ± 0.51^{A}	8.18 ± 0.44^{AB}	8.60 ± 0.18^{B}
Overall Acceptability	0^{th} day	8.37 ± 0.08^{A}	7.87 ± 0.01^{B}	7.75 ± 0.09^{B}
	30^{th} day	8.25 ± 0.31^{A}	7.78 ± 0.13^{B}	7.65 ± 0.21^{B}
	60^{th} day	8.19 ± 0.07^{A}	7.71 ± 0.04^{B}	7.64 ± 0.06^{B}

SEM – Standard Error of mean

^{ABC} means on the same line without a common letter are significantly different at $P < 0.05$ (all samples at the same time).

The flavour, colour and appearance of control jam were significantly higher ($P < 0.05$) from that of Jam T_1. The taste and overall acceptability of control jam was significantly higher ($P < 0.05$) from that of Jam T_1 and Jam T_2. Texture of Jam T_2 was significantly higher ($P < 0.05$) than that of control jam. All the parameters of sensory evaluation were within liked limit (sensory score between 7.18 and 8.75) for the jam when substituted with whey both at 5% (T_1) and 10% (T_2). Thus sensory property of papaya jam is not affected by the substitution of whey up to 10% of papaya pulp.

4. CONCLUSIONS

Papaya jam with 10% whey substitution can be effectively assigned as best formulation as it has better textural property which is necessary for jam product, with normal range of pH, acidity, total solids and ash, with better water activity, reducing sugar and significant amount of protein content. The crude protein level in control jam (T_0), T_1, and T_2 jam are 0.92%, 3.15% and 4.23% respectively.
Further, it was substantially proved that whey can be efficiently substituted in jam with advantage of improving its nutritive, physico chemical and textural properties without any detrimental effect on sensory properties and also avoiding much denaturation of whey protein. Usually, fruit pulps incorporated in jam had less protein level, so whey can be used as economic protein source to improve its nutritive value. This experiment also paves a way for effective utilization of whey, which is produced abundantly as by product in dairy industry.

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ULTRASOUND-ASSISTED EXTRACTION OF LYCOPENE AND β-CAROTENE FROM TOMATO-PROCESSING WASTES

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ABSTRACT

Ultrasound-assisted extraction (UAE) of lycopene and β-carotene from tomato-processing wastes were investigated. Hexane:acetone:ethanol (2:1:1 v/v/v) including 0.05% (w/v) butylated hydroxyl toluene (BHT) was used as a solvent, with 1:35 w/v solid liquid ratio at 15±5°C. Ultrasonic power (50, 65, 90W) was applied in UAE for 1-30 min. Conventional organic solvent extraction (COSE) was applied under the same solvent and temperature conditions for 10-40 min.
UAE was more effective and required a shorter time than COSE. Maximum lycopene and β-carotene yields were obtained using 90W ultrasonic power for 30 and 15 min, respectively.

Keywords: lycopene, β-carotene, ultrasound assisted extraction, tomato processing wastes

1. INTRODUCTION

The tomato is popular because of its healthy composition and consumption both in raw and processed forms all over the world. The essential role of carotenoids, being a major dietary source of vitamin A, is having remarkable effects on the immune response and intercellular communication (TAYLOR *et al*., 2000; SU *et al*., 2002; SUN *et al*., 2010). Studies show that antioxidant-rich diet reduces or prevents risks of epithelial and prostate cancers, cardiovascular diseases and cataracts (TONUCCI *et al*., 1995; ARAB and STECK, 2000; STAHL and SIES, 2005; CAPANOGLU *et al*., 2008; LIANFU and ZELONG, 2008).
Lycopene and β-carotene are used in industry for their wide color range between yellow and red (SABIO *et al*., 2003; ALDA *et al*., 2009). However, for their great solubility in oil and fat, both of them are used as a natural colorant in the food, cosmetics, and pharmaceutical industries. β-carotene is used as pro-vitamin A, animal feed, an additive in cosmetics, multivitamin constituent and anti-oxidant (BEN-AMOTZ and FISHLER, 1998). The basic carotenoid in tomato is lycopene, and it is known for its high anti-oxidant capacity. Depending on protective properties of lycopene against cancer and oxidants, lycopene is one of the important components used in pharmaceuticals and cosmetic formulations (KUMCUOGLU *et al*., 2014; DOLATABADI *et al*., 2016).
Various amounts and type of wastes at various stages appear during tomato processing. Dry pomace consists of 44% seed and 56% pulp and skin (KAUR *et al*., 2008; LAVELLI and TORRESANI, 2011; DOLATABADI *et al*., 2016). A major part of waste is composed of seed and skin, which contain five times more lycopene than tomato pulp (SHI *et al*., 1999; TAYLOR *et al*., 2000). Since the skin is the major source of lycopene, it must be separated from other parts for better extraction (GEORGE *et al*., 2004; KAUR *et al*., 2008). Total lycopene content in tomato pulp and tomato skin varies between 90 to 190 mg/kg and about 120 mg/kg in fresh weight, respectively, while β-carotene is found to be about 3 mg/kg (BAYSAL *et al*., 2000; ALDA *et al*., 2009; POOJARY and PASSAMONTI, 2015; KUMCUOGLU *et al*., 2014). Besides having high antioxidant activity, lycopene degrades and isomerizes under light irradiation and high temperature treatments (CHEN *et al*., 2009). To isolate all-*trans*-lycopene and avoid isomerization, suitable and fairly controlled conditions should be satisfied. Extraction of lycopene is applied by classic solvent extraction (COSE) method traditionally (LIANFU and ZELONG, 2008). But in traditional extraction methods, long process time and high amounts of solvent are needed. Decreasing the solvent consumption, shortening the extraction time, increasing the extraction yield, and enhancing the quality of extracts can be reached by new methods such as ultrasound-assisted (UAE), microwave-assisted, or supercritical extraction (WANG and WELLER, 2006; FANTIN *et al*., 2007; WANG *et al*., 2008).
Sound waves produced by an ultrasonic probe at frequencies greater than human hearing cause a mechanical impact, allowing greater penetration of solvent into the plant body, known as the "sponge effect." Another effect of ultrasonic power is producing high-energy cavitation bubbles containing solvent vapor. These bubbles implode near cell walls causing very high local temperatures, pressure increase and cell wall destruction, which eases mass transfer from cell to solvent and enhances micro-streaming. The combination of these effects intensifies solvent penetration and satisfies sufficient mixing for extracting high amounts of active components. Besides easing extraction, ultrasound may also produce free radicals within the cavitation phenomenon such as highly reactive hydroxyl radicals in water-contained solutions (TOMA *et al*., 2001; VINATORU, 2001; MASON and LORIMER, 2002; WANG *et al*., 2008; JERMAN *et al*., 2010; SUTKAR *et al*., 2010).
The aim of this study was to evaluate the effects of time and ultrasonic intensity in UAE of lycopene and β-carotene from tomato-processing wastes and to compare the efficacy between UAE and COSE.

2. MATERIALS AND METHODS

2.1. Material

Tomato wastes used in this study were supplied from a tomato-paste-manufacturing pilot plant (Ege University, Agricultural Faculty Izmir, Turkey, 2012). The raw material was dried, from 75% to 4.90±2.50 % moisture content, in a vacuum drier at 40°C for 24 h. Before extraction process, dried raw material containing 48.80±4.70% skins and % 51.20±3.10 seeds was grounded in a laboratory scale hammer mill (Armfield Hammer Mill, England) and then subjected to sieving. The average particle size of the powder was determined by screen analysis. Samples with 286±24 μm average size were used in the extraction process. Then, the samples were packed under vacuum and stored at-40°C until the extraction process.

2.2. Methods

2.2.1 Conventional Organic Solvent Extraction (COSE)

Extraction of lycopene and β-carotene was carried out according to the method of Sadler *et al*. (1990) and modified as described by PERKINS-VEAZIE *et al*. (2001). The mixture of solvents hexane: methanol: acetone (2:1:1 v/v), containing 0.05% (w/v) BHT (butylated hydroxyl toluene), were used to extract carotenoids from the sample. A 1.14 g dried sample was placed into a 50 mL flask, and a 40 mL solvent mixture was added to satisfy 1:35 (w/v) ratio. Then, these flasks were agitated continuously in a shaking water bath. Extractions were applied at 15±5°C temperature for 10, 20, 30 and 40 minutes. After the extraction process 15 ml of cold distilled water was added after the extraction process and then suspension was centrifuged by 1000 rpm at 5°C. The solution was then allowed to stand for 5 min for separation of polar and non-polar layers. Non-polar phase was used for lycopene and β-carotene determination. Filtered non-polar phases were first dried under nitrogen flow and then kept at -40°C in amber bottles as described in previous study (KUMCUOGLU *et al*., 2014) for 6 months before subjected to HLPC analyses.

2.2.2 Ultrasound Assisted Extraction (UAE) Method

A high-intensity ultrasonic probe with maximal input power of 400 W and operating frequency of 24 kHz (Model UP400S, Dr. Hielscher, Germany) equipped with a H14 sonotrode (Dr. Hielscher, Germany) was used for the extraction experiments. Solvent composition and liquid-solid ratio were the same as applied in COSE. Ultrasonic probe was immersed 7 cm into the solution from the top of the 150 ml flask. Ultrasonic treatments were performed for 1, 2, 5, 10, 15, 20 and 30 min. In this study, ultrasonic power levels and corresponding ultrasonic intensities were 50, 65, 90 W and 32.50, 42.25, 52 W/cm^2 respectively. During UAE experiments, the temperature of samples was kept at 15±5°C by using indirect cold water circulation system. For lycopene and β-carotene determination, the same procedure was applied as the one implemented in COSE.

2.2.3 Lycopene and β-carotene Determination

Solvent-free extracts were dissolved in HPLC-grade hexane and then analyzed by high-performance liquid chromatography (HPLC 1200 Agilent Technologies/USA) using a

diode array detector (DAD) at 475 nm by applying BARBA *et al*. (2006) procedure. All extracts were filtered through a 0.22 μm filter membrane before injection to the HPLC column. Extracts were diluted with HPLC grade hexane to fit the concentrations to the calibration curves. The calibration curves were determined by injecting 20 μl samples of 2.5, 5.0, 7.5, 10 and 20 mg/kg of pure β-carotene (95% synthetic, HPLC grade-Sigma Chemical Co.) and samples of 20, 30, 50, 80 and 100 mg/kg of pure lycopene (90-95% from tomato, Sigma Chemical Co.). Correlation coefficients of calibration curves were 0.9975 and 0.9979, respectively.
C_{18} column (10 μm, 3x300 cm Waters/USA) was used for the separation. The column temperature was 30°C. Methanol: acetonitrile (90:10 w/w) was used as a mobile phase at a flow rate of 0.9 ml/min, while 20 μl hexane phase was injected for each sample. At the beginning of analyses of each extract, the column was washed with mobile phase in order to remove pigments other than lycopene and β-carotene, which are not soluble in this polar solvent. Identification of carotenoids in the extracts was done by comparing their retention time with the retention time of their standards. Results were calculated as mg/kg of dry weight.

2.2.4 Statistical analysis

All measurements were carried out in triplicate. The results were expressed as the mean value ± the standard deviation. Data were subjected to statistical analysis using SPSS 16.0 (SPSS Inc. Chicago, IL, USA), and analysis of variance (ANOVA) was applied using generalized linear model to determine the single and multiple effects of the parameters on the conditions by comparing the mean values. All significant differences were reported at $p<0.05$. Duncan's multiple range test (MRT) was used in post hoc analysis for multiple comparisons.

3. RESULTS AND DISCUSSIONS

3.1 Conventional Organic Solvent Extraction (COSE)

Effect of extraction time for lycopene and β-carotene in COSE is given in Fig 1. It has been observed that the amount of extracted lycopene and β-carotene increased with time at the beginning of the extraction period. Statistical evaluation of the results showed that time was an important factor in extraction of lycopene and β-carotene *($p<0.05$).There is an increase in yield for* 10 min and 20 min extraction periods *($p<0.05$)* while longer extraction periods such as 30 min and 40 min yields were similar *($p>0.05$)* for both lycopene and β-carotene yields. This can be explained by driving force decrease in osmotic balance; as the diffusion of carotenoids from material to the solution in COSE takes place slowly so that the osmotic pressure between the inside and the outside of the cell easily reached equilibrium (SUN *et al*., 2011; KUMCUOGLU *et al*., 2014).

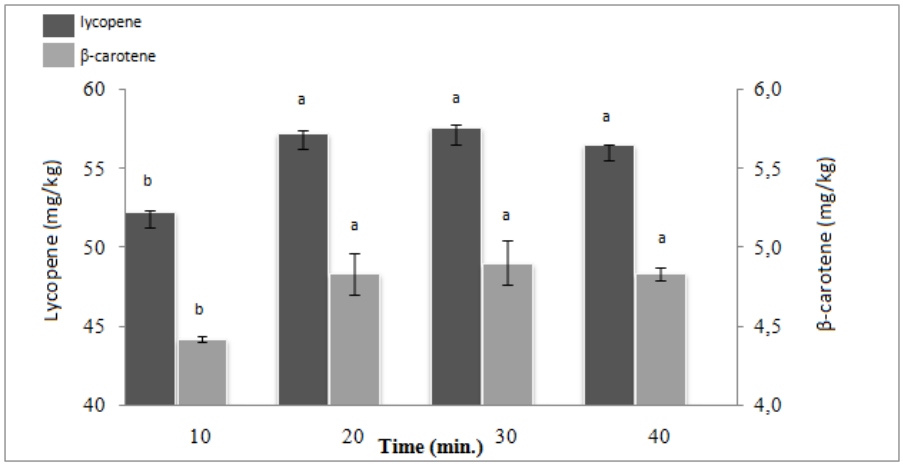

Figure 1: Effect of Extraction Time for Lycopene and β-carotene in COSE

In this study, the amount of extracted lycopene and β-carotene in dried samples were between 52.21- 57.19 mg/kg dry weight and between 4.42-4.90 mg/kg, respectively. In previous studies, similar results were found; extracted lycopene values of fresh tomatoes were reported between 8.5 - 136 mg/kg (LUGASI *et al*., 2003; KARAKAYA, 2007; KUMCUOGLU *et al*., 2014). The lycopene values in tomato skin were determined between 25.5 - 141 mg/kg, and extracted β-carotene values in fresh tomatoes varied between 0.5-9.5 mg/kg, depending on genetic, agronomic, climatic factors and processing conditions (RAO *et al*., 1998; RAO and AGARWAL, 1999; BAYSAL *et al*., 2000; TOOR and SAVAGE, 2005; BRAVO *et al*., 2012).

3.2 Ultrasound Assisted Extraction (UAE)

The results of the general linear model indicates that extraction time, ultrasonic power and their composite effects are significant for both lycopene and β-carotene yields as given in Table 1, and the applied model seems significant to describe extraction for each carotenoid *(p<0.05)*.

Table 1: ANOVA of response for ultrasound-assisted extraction experiments for lycopene and β-carotene

Lycopene				β-carotene			
Source	**SS**	**DF**	**P**	**Source**	**SS**	**DF**	**P**
Corrected Model	14296.275[a]	20	<0.0001	Corrected Model	49.510[b]	20	<0.0001
Intercept	179.031.254	1	<0.0001	Intercept	1447.072	1	<0.0001
power	6.725.223	2	<0.0001	power	12.436	2	<0.0001
time	6.828.752	6	<0.0001	time	32.242	6	<0.0001
power * time	742.299	12	<0.0001	power * time	4.832	12	<0.0001
Error	282.484	42		Error	1.359	42	
Total	193610.013	63		Total	1497.941	63	
Corrected Total	14578.759	62		Corrected Total	50.869	62	
a. R Squared = .981 (Adjusted R Squared = .971)				b. R Squared = .973 (Adjusted R Squared = .961)			

SS, sum of squares; DF,degrees of freedom, significant effects, α=0.05.

Table 2: Identification and Chromatographic Data for Lycopene and β-carotene after UAE.

Component	**Power (W)**	**Extraction time (min)**						
		1	**2**	**5**	**10**	**15**	**20**	**30**
Lycopene	50	28.44^{cE}±1.68	31.72^{cD}±0.69	37.39^{cC}±3.48	43.00^{cB}±2.20	44.20^{cAB}±0.03	47.03^{cA}±0.28	48.05^{cA}±0.65
	65	35.79^{bE}±1.43	39.23^{bD}±1.37	57.49^{bC}±5.75	60.02^{bB}±3.73	65.34^{bAB}±0.08	62.93^{bA}±0.24	63.00^{bA}±0.56
	90	35.89^{aE}±2.52	56.34^{aD}±0.42	67.34^{aC}±3.38	70.07^{aB}±2.20	73.73^{aAB}±0.04	75.90^{aA}±0.07	76.87^{aA}±0.33
carotene	50	3.46^{cE}±0.16	3.56^{cD}±0.10	4.05^{cC}±0.41	4.15^{cB}±0.13	4.72^{cA}±0.05	5.00^{cA}±0.02	5.24^{cA}±0.07
	65	3.07^{bE}±0.10	3.57^{bD}±0.06	4.71^{bC}±0.26	4.99^{bB}±0.08	5.28^{bA}±0.03	5.52^{bA}±0.01	5.66^{bA}±0.06
	90	3.92^{aE}±0.08	5.00^{aD}±0.05	5.30^{aC}±0.29	6.01^{aB}±0.20	6.12^{aA}±0.07	5.92^{aA}±0.01	5.41^{aA}±0.03

Data are means ± standard deviation. For every compound, different apices (capital letters) in a row indicate significant difference with respect to extraction time; different apices in a column (small letters) indicate significant differences with respect to ultrasonic power.
$p<0.05$, Duncan's multiple range test.

It was observed that the extraction yield increased exponentially in a few minutes (2 min), later increased gradually (10 min) and then became constant during extraction (Table 2). The initial sharp increase in the rate of extraction was due to the large β-carotene and lycopene concentration gradient between the extracting solvent and the cell. Later, the concentration gradient decreased as the extraction became difficult thanks to the interior location of cells. Similar results were observed for extraction of all-*trans*-lycopene from red grapefruit and lycopene from tomato-processing wastes (KUMCUOGLU *et al*., 2014; XU and PAN, 2013). In previous studies, similar results were observed for ultrasonic extraction of all-*trans*-lycopene, and time was found to be the most important factor affecting extraction yield. It is reported that most of all-*trans*-lycopene could be extracted during the 1/3 of total extraction period (30 min), and then lycopene degradation and isomerization led to the reduction of lycopene amount due to the side effect of sonication called ultrasonic degradation (WANG and WELLER, 2006; JERMAN *et al*., 2010; SUN *et al*., 2010; XU and PAN, 2013; KUMCUOGLU *et al*., 2014).

As the power increased from 50W to 65W, lycopene value increased significantly *($p<0.05$)*, while the increase in β-carotene value between 50W and 65W was not significant *($p>0.05$)*. But application of 90W had a significant effect on both lycopene and β-carotene contents *($p<0.05$)*.

Cavitation and thermal effects play an important role in UAE. With an increase in power, more energy was getting transferred for cavitation, and this resulted in the increase in lycopene and β-carotene yield. At low ultrasonic intensities, thermal effect can be ignored because the heat produced by ultrasound may be completely diffused. As the ultrasonic intensity is further increased, the cavitation effect becomes less important compared to thermal effects during extraction of sensitive products such as carotenoids (SORIA and VILLAMIEL, 2010; SUN *et al*., 2011; EH and TEOH, 2012). It was reported that during sonication the extreme physical conditions of temperature and pressure caused carotenoid isomerization (CHEN *et al*., 2009). Besides enhancing extraction efficiency, high ultrasonic power could cause thermal degradation to thermally sensitive components such as β-carotene (LIANFU and ZELONG, 2008; ADEKUNTE *et al*., 2010).

As a result, lycopene content increased with time from 15 min to 30 min at all ranges of ultrasonic power; β-carotene content started to decrease at 90W. This can be explained by sensitivity differences of lycopene and β-carotene in thermal effects. In previous studies, it was shown that lycopene was relatively resistant to thermal degradation compared to other carotenoids such as α-tocopherol and β-carotene (TAYLOR *et al*., 2000).

4. CONCLUSIONS

In this study, various parameters affecting the COSE and UAE of lycopene and β-carotene were investigated. Maximum lycopene and β-carotene yields were obtained in UAE at 90W for 30 min and 90W for 15 min extraction time, respectively. UAE extraction yields were significantly higher *($p<0.05$)* than COSE for both lycopene and β-carotene yields except when ultrasonic power of 50W was applied. Extracted values of β-carotene obtained from 50W treatments at 15±5°C after 5 min extraction were similar to the values from COSE at 20°C after 20 min extraction because the effect of heat was still a predominant factor for extraction efficiency of β-carotene at this ultrasonic intensity.

The results indicated that UAE was more effective and requires shorter time than COSE even at lower temperatures. The ultrasound was beneficial for extracting compounds from tomato waste while shortening extraction time and being able to extract heat-sensitive compounds by increasing mass transfer at a lower temperature.

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