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Potentials of African Nutmeg (*Monodora myristica*) as a Flavourant in Cookie Production

Kazeem K. Olatoye[a*], Omololu O. Fapojuwo[a], Joshu A. Olorunshola[a], and Julianah O. Ayorinde[a]

[a] Department of Food Science and Technology, College of Agriculture, Kwara State University, Malete, P.M.B 1530, Ilorin, Kwara State, Nigeria
[*]Corresponding author
luckykaykay@yahoo.com

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Abstract

African nutmeg, a possible local substitute for a commercial food flavourant, remains largely underutilized in Nigeria. Its application potential in cookie production was investigated in this study. African nutmeg (*Monodora myristica*) seed flour (ANM) was produced using a standard method. The flour was substituted for vanilla flavour (VFL) in ratio of 0, 1, 2, 3, and 3.5 g and functional properties of the flour blends (water absorption capacity (WAC), oil absorption capacity (OAC), and bulk density) were determined, using standard methods. Cookies were developed and characterized chemically, physically (colour) and organoleptically using the AOAC method, a colourimeter and sensory panellists respectively. Data were analysed using ANOVA at $p<0.05$. Replacement of vanilla with African nutmeg had no significant effect on bulk density (0.62 g cm^{-3}-0.68 g cm^{-3}), but significantly affected WAC (133-142 %) and OAC (147-156 %) of flour blends. Crude protein (9.44-15.49 %), crude fat (3.17-6.50 %), total ash (2-2.73 %) and crude fibre (0.12-0.23 %) contents of the cookie increased, whilst metabolizable energy (385.33-367 kcal) decreased. There were reductions in pH (6.83-6.53) and TSS (5.80-1.20). Brown index of the snack increased with addition of nutmeg. Antioxidant indicators (flavonoids, DPPH and phenol) varied among cookie samples. Antinutrients, saponin, tannin and oxalate, were within tolerable limits. All cookie samples were judged acceptable by the panellists, with SLZ being the most acceptable. An acceptable and nutritious snack was produced at 100 % replacement. *M. myristica* seed could serve as substitute for vanilla in the production of cookies and other related snacks.

Keywords: *Monodora myristica*; Snack; Flavourant; Food additive; Substitution

1 Introduction

Snacks are identified as foods eaten at times other than meals and could be mindfully or unmindfully consumed. They include a variety of products and can take different forms, including potato chips and cereal-based snacks (FAO, 2011; Sajilata & S. Singhal, 2005; Sumargo, 2016). Globally, the impact of snacks is felt daily mostly in big cities, possibly due to convenience driven lifestyles, job demands and dietary habits (Kruger, 2012; Olatoye & I. Lawal, 2016). Many people earn their livelihood through snack production and marketing (Norfezah, 2013). Quite often, some snacks are perceived to be sugary and their excessive consumption has been attributed to incidence of obesity, diabetes and coeliac diseases (Ajieroh, 2010). These might be connected with the composition of their ingredients.
Among other ingredients, flavourings remain

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indispensable with application of commercial flavourings in snack production being common, as this is meant to improve the taste and entice consumers. Ironically, some of these flavourings are synthetic and unhealthy. However, producing healthy snacks from locally available materials is the current trend and innovative development in the snack food industry. Consequently, it is necessary to explore natural flavourings and spices that may be considered as possible alternatives to synthetic ones (Enwereuzoh et al., 2015). One example of such possible alternatives is M. myristica, commonly known as African nutmeg. It is a perennial edible plant, belonging to the Ananacea family. It is a berry with many seeds grown in the evergreen forests of West Africa (Burubai, Akor, Igoni, & Puyate, 2007), with almost every part of the tree possessing both economic and medicinal usefulness. Its pod is used as a seasoning spice in Southern and Eastern Nigeria (Aladesanmi, 2007; Essien, Izunwane, Aremu, & Eka, 1994). The seeds, which are embedded in the white sweet-smelling pulp of the fruit are considered most economically important (Stephen, O., Oboh, & Eseosa, 2014). The seed powder is used as spices to prepare pepper soup and also used as stimulant to relieve constipation. Currently, the tendency to use the oil extracted from *M. myristica* and *Tetrapleura tetraptera* to flavour popcorn has proven to justify the use of the spices as flavourings with good acceptability and no adverse effect (Enwereuzoh et al., 2015). There have been few studies on the use of African nutmeg incorporated into snacks as it is mostly used in seasoning soups and salads. Largely, Nigerian food industries are still relying on the importation of flavourings at the expense of possible local substitutes (Enwereuzoh et al., 2015). Therefore, the objective of this research was to investigate the potential of African nutmeg as a flavouring agent in the production of cookies.

2 Materials and Methods

2.1 Sources of Materials

The African nutmeg was obtained from Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan, Nigeria. The wheat flour, sugar, salt, egg, and vanilla were bought from modern market, Ilorin, Nigeria. The chemicals and reagents used were of analytical grade A and were obtained from Bumlab Nigeria Limited, Ring Road, Ibadan, Nigeria.

2.2 Production of African nutmeg powder

The seeds of *M. myristica* were shelled manually with the use of mortar and pestle and then milled into powder using an electric blender, sieved with a 250 μm mesh and bottled in a sterile plastic container prior to use.

2.3 Cookie production

The method described by Eneche (1999) was used to prepare cookies with slight modifications. A fluffy mixture of butter and sugar was obtained by manually mixing 125 g of baking butter with 120 g of sugar for 5 min. Another mixture of flour (250 g), salt (2.5 g) and baking powder (10 g) was prepared separately and combined with the butter-sugar mixture to get a dough. A measured amount of water (20 ml) was added gradually and the dough mixed continuously until a good textured, firm dough was obtained. The dough was kneaded on a clean flat surface for 4 min and transferred to a cutting table where a shape maker was pressed over the dough to give the desired shapes. Dough pieces were then transferred into liquid-fat-greased baking trays and baked at 150 ^{o}C for 20 min, cooled and packaged for analysis.

2.4 African nutmeg-vanilla flavour formulation for cookie

African nutmeg (ANM) was substituted for vanilla flavour (VFL) in different proportions of 0, 1, 2, 3, and 3.5 g i.e. (0, 30, 60, 90 and 100 %) respectively, to give five (5) samples (Table 1), according to the modified method described by Adeboye, Babajide, Shittu, Omemu, and Oluwatola (2013).

Table 1: Substitution of African nutmeg for vanilla flavour in cookie production

Samples	African nutmeg flour (g)	Vanilla flavour (g)
KMO	0.00 (0 %)	3.5 (100 %)
JLK	1.00 (30 %)	2.50 (70 %)
VRU	2.00 (60 %)	1.50 (40 %)
PQS	3.00 (90 %)	0.50 (10 %)
SLZ	3.50 (100 %)	0.00 (0 %)

2.5 Determination of functional properties of flour blends

Water absorption capacity (WAC) and oil absorption capacity (OAC) were determined using the method described by Sosulski (1962). For the WAC, 1 g of the sample was added into 15 ml of distilled water in a pre-weighed centrifuge tube, while for the OAC, oil with a known density was added to the sample. The tube with its contents was agitated for 2min and centrifuged at 4000 rpm for 20 min on a SorvallGLC-1 centrifuge (Model 06470, USA). The clear supernatant was discarded and the centrifuge tube was weighed with the sediment. The amount of water or oil bound by the sample was determined by difference and expressed as the weight of water bound by 100 g dry of flour. Bulk density was determined using the method of AOAC (2000). The flour sample (7 g) was weighed into a 50 ml graduated measuring cylinder. The cylinder was tapped gently against the palm of the hand until a constant volume was obtained, and the bulk density (BD) calculated as shown in equation 1.

$$\text{BD} = \frac{\text{weight of sample}}{\text{volume of sample after tapping}} \quad (1)$$

2.6 Determinations of proximate composition and energy values of cookie samples

Proximate composition, including crude protein, fat, moisture content, crude fibre, ash and carbohydrate of the cookie samples were carried out using standard methods described by AOAC (1995). The energy value of the cookie was calculated from percentages of major nutrients in kilojoules per 100 g and the values were converted to kcal by dividing them by the conversion factor (4.184) (Maclean et al., 2003) as shown in equation 2.

$$\text{EV(kcal)} = \frac{\text{Carb} \times 17 + \text{Prot} \times 17 + \text{Fat} \times 37}{4.184} \quad (2)$$

Where EV = Energy value and Carb, Prot and Fat are the composition in carbohydrates, proteins and fats

2.7 Determination of physicochemical properties of cookie samples

A Fisher pH meter (Model 210, Fisher Scientific) was used to determine the pH and an Abbe refractometer was used to determine the total soluble solids content (Brix) at 20 ^{o}C. The physical colour of the cookies was quantitatively determined with the aid of a hand-held Colour Tec PCM/PSMTM1 colour meter as described by Babajide and Odulate (2015) using a white tile as reference and recording the brightness (L*), redness (a*) and yellowness (b*) values, Hunter Lab values.

2.8 Determination of Antioxidant profile of formulated cookie

Phenol

Total phenol was determined by the Folin-Ciocalteu spectrophotometric method (AOAC, 1990). Extraction of total phenol was done by dissolving 200 mg of the sample in 10ml methanol (>99 % w/w). At room temperature, the mixture was shaken well for 30 min and centrifuged

at 500 rpm for about 15 min. The extract from each sample (1ml) was treated with an equal volume of Folin-Ciocalteu reagent together with addition of 2 % Na_2CO_3 solution. Standard phenol solution was prepared and diluted to the desired concentration. The standard solution (1ml) was treated with Folin-Ciocalteu reagent and Na_2CO_3 solution. A spectrophotometer was used to measure the absorbance of the resulting blue coloration at 560 nm. Measurement was made with a reagent blank at zero. The phenol content was calculated using the formula below:

$$\%phenol = \frac{100}{W} \times \frac{Au}{As} \times C \times \frac{Vt}{Va} \quad (3)$$

Where W = weight of sample, Au = absorbance of test sample, As = absorbance of standard phenol sample, C = concentration of standard phenol sample, Vt = total extract volume and Va = volume ofextract analysed.

Flavonoids

Flavonoid content was determined using the modified method of Harborne (1973). The cookie sample (5 g) was refluxed for 40 min and allowed to cool before being filtered. The filtrate was treated with an equal volume of ethyl acetate and then transferred to a separation funnel. The flavonoid extract (contained in the ethyl acetate portion) was collected using a filter paper. The weight was obtained after drying in the oven and cooling in a dessicator. The weight was expressed as percentage of weight of sample analyzed and was calculated as shown below:

$$\%flavonoid = \frac{W_2 - W_3}{W_1 of sample} \times 100 \quad (4)$$

Where W_2 and W_3 represent flavonoid extract in ethyl acetate portion before and after drying respectively. W_1 is the initial weight of sample.

DPPH (2, 2-dipheny-1-pycryl hydrazine)

The DPPH-radical scavenging activity of the cookies sample was determined using a modified method of Blois (1958) as reported by Cakir et al. (2003). Different concentrations (0 to 350 μg ml^{-1}) of sample extract were pipetted into clean, dry test tubes in triplicate and the volumes adjusted to 1 ml with 10 mM acetate buffer, pH 4.5. This was followed by the addition of 2 ml of 0.2 mM DPPH solution in methanol. The reaction mixture was mixed thoroughly by inversion and then incubated in the dark for 30 min. The absorbance was read at 517 nm against the blank that contained 1 ml of 10 mM acetate buffer, pH 4.5 and 2 ml of 0.2 mM DPPH solution in methanol. For ascorbic acid (1 mg mL^{-1}) and rutin (1 mg mL^{-1}) standard, the procedure described above was followed. Scavenging activity was evaluated in percentage, using the expression:

$$\% \text{ of scavenging activity} = \frac{Ac - Au}{Ac} \times 100 \quad (5)$$

Where Au = absorbance of test sample, Ac = absorbance of the control

2.9 Determination of anti nutrient contents of cookie samples

Tannin

Tannin content of the sample was determined by Folin Denis colorimetric method (Kirk, Sawyer, et al., 1991). 5 g of the cookie was weighed and thoroughly dissolved in 10 ml distilled water. The solution was shaken well for 30 min at room temperature and filtered to obtain the extract. Standard tannic solution was prepared and a 2 ml portion mixed with equal volume of distilled water in a separate 50 ml volumetric flask, to serve as standard and reagent blank respectively. Then 2 ml of each of the sample extracts were put into labeled flasks. Contents of each flask were mixed with 35 ml of distilled water and 1ml of the Folin Denis reagent was added to each, followed by addition of 2.5 ml of saturated Na_2CO_3 solution. Thereafter, each flask was diluted to the 50 ml mark with water and incubated for 90 min at room temperature. Absorbance was read using a spectrophotometer at 760 nm with the reagent blank at zero. The tannin content was calculated as shown below:

$$\%tannin = \frac{100}{W} \times \frac{Au}{As} \times C \times \frac{V_t}{V_a} \quad (6)$$

Where W = weight of sample, Au = absorbance of test sample, As = absorbance of the standard tannin solution, C = concentration of the standard tannin solution, V_t = total volume of extract, V_a = volume of extract analyzed.

Oxalate

The determination of oxalate content was carried out according to Day and Underwood (1986). One gram of each sample was put into separate plastic bottles followed by the addition of 75ml of 0.1N H_2SO_4. The content was mixed properly and allowed to extract for 1 h with constant agitation using a mechanical shaker. This was then filtered and 25 ml of the filtrate was titrated with 0.1ml $KMnO_4$ while hot (80-90 ^oC) until a purple colour was observed at the end point. The titre value (volume of $KMnO_4$ used at the end point) was then multiplied by 0.9004 to get the result expressed as mg g^{-1}.

Saponin

Modified method of Fenwick and Oakenfull (1981) was used to determine saponin content of the cookies. A reflux condenser containing pure acetone was used to extract saponin for 2 h. Exhaustive re-extraction over a heating mantle with methanol in the Soxhlet apparatus carried out for 2 h. Methanol was allowed to evaporate and the extract was weighed. Saponin content was calculated as a percentage of the sample.

2.10 Sensory evaluation:

Cookie samples were evaluated for sensory attributes; appearance, taste, crispness, flavor, hardness and acceptability using thirty panellists on a hedonic scale (9-point) where 1 represented dislike extremely and 9 extremely like (Iwe, 2002).

2.11 Statistical analysis

All analyses were carried out in triplicate and the results were subjected to statistical analysis using Statistical Package for Social Sciences (SPSS version 21). Separation of means was carried out ($P \leq 0.05$) using the Duncan multiple range test.

3 Results and discussion

3.1 Functional properties of flour blends

Replacement of vanilla with African nutmeg had no significant effect on bulk density except for sample JLK and ranged from 0.62 g cm^{-3} to 0.68 g cm^{-3}. However, it significantly affected WAC (133 - 142 %) and OAC (147 - 156 %) of flour blends (Table 2). Water absorption capacity is an important functional property in the development of ready-to-eat foods, since a high WAC may assure product cohesiveness (Houssou & Ayernor, 2002). The WAC decreased as the amount of *M. myristica* increased. The oil absorption capacity (OAC) is a critical assessment of flavour retention and increases the palatability of foods (Kinsella & Melachouris, 1976). The addition of *M. myristica* decreased the OAC capacity of the flour blend. This could be as a result of the oil content of *M. myristica* (22.71-29.1 %), reported by Ekeanyanwu, Ogu, and Perpetua (2010) and Stephen et al. (2014).

3.2 Proximate composition of formulated cookie

The crude protein (9.44-15.49 %), crude fat (3.17-6.50 %), total ash (1.22-2.73 %) and crude fibre (0.12-0.23 %) contents of the cookies increased (Table 3), while metabolizable energy (385.33-367 kcal) decreased (Table 4) with addition of African nutmeg. In this study, the protein content was close to the value reported by Enwereuzoh et al. (2015) in similar study. Increased protein can contribute significantly to the recommended daily intake of proteins for adults (34-56 g day^{-1}) and children (13-19 g day^{-1}) (Food and Nutrition Board, 2002). Protein is an essential nutrient in the human diet as it helps to repair worn-out tissues (Baah, 2009). Fat content of the cookie was however lower (12.96–15.21 %) than documented by Giwa and Abiodun (2010) for a biscuit produced from composite flour of wheat and quality protein maize. It is important to note

Table 2: Functional properties of flour blends

Samples	Bulk density (g/cm^3)	WAC (%)	OAC (%)
KMO	$0.66^{a}\pm0.01$	$142^{a}\pm0.02$	$156^{a}\pm0.01$
JLK	$0.62^{b}\pm0.02$	$141^{ab}\pm0.02$	$155^{a}\pm0.01$
VRU	$0.67^{a}\pm0.02$	$139^{b}\pm0.04$	$152^{b}\pm0.02$
PQS	$0.68^{a}\pm0.01$	$135^{c}\pm0.01$	$151^{b}\pm0.01$
SLZ	$0.68^{a}\pm0.02$	$133^{d}\pm0.01$	$147^{c}\pm0.01$

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were significantly different ($P < 0.05$)

Table 3: Proximate composition of baked cookie (%)

Sample	Moisture	C. Protein	Crude fat	Crude ash	Crude fibre	Carbohydrate
KMO	$6.20^{d}\pm0.26$	$9.44^{d}\pm0.62$	$3.17^{d}\pm0.07$	$1.22^{c}\pm0.07$	$0.17^{c}\pm0.01$	$79.80^{a}\pm1.02$
JLK	$8.33^{c}\pm0.29$	$12.67^{c}\pm0.67$	$4.27^{c}\pm0.03$	$1.70^{b}\pm0.10$	$0.12^{d}\pm0.01$	$72.91^{b}\pm1.00$
VRU	$8.91^{b}\pm0.16$	$12.96^{bc}\pm0.80$	$4.45^{b}\pm0.09$	$1.74^{b}\pm0.10$	$0.17^{c}\pm0.00$	$71.77^{b}\pm1.14$
PQS	$9.20^{b}\pm0.96$	$14.44^{ab}c\pm0.95$	$4.49^{b}\pm0.01$	$1.82^{b}\pm0.07$	$0.19^{b}\pm0.01$	$69.85^{c}\pm0.89$
SLZ	$13.41^{a}\pm0.19$	$15.49^{a}\pm1.10$	$6.50^{a}\pm0.10$	$2.73^{a}\pm0.20$	$0.23^{a}\pm0.01$	$61.64^{d}\pm1.05$

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were significantly different ($P < 0.05$)

that the amount of fat in a food product plays a major role in its shelf life. High fat content could be undesirable in ready-to-eat snacks as it can promote rancidity, leading to development of unpleasant sensory properties (Ihekoronye & Ngoddy, 1985). Hence, the low fat was an added advantage with respect to the keeping quality of this cookie. Ash content (1.34-2.58 %) in this study was in agreement with reported by Eke, Achinewhu, and Sanni (2008) and was an indication of adequate mineral status of the cookie (Baah, 2009). Fibre is regarded as essential nutrient in human diet as it absorbs water and provides roughage for the bowels, assisting intestinal transit (Alaise & Linden, 1999). The crude fibre content was low, which is however helpful to the digestive process (Alaise & Linden, 1999). Substitution of African nutmeg for vanilla resulted in reduced carbohydrate (Table 3) and energy contents of the cookie (Table 4). This may help in the prevention of over-weight and obesity. Accurate information on energy value of foods is paramount, when it comes to the challenges of normal nutrition, under nutrition and obesity (Merrill & Watt, 1973).

3.3 Physicochemical properties and colour characteristics of formulated cookie

Substitution of African nutmeg for vanilla brought about significant ($p \leq 0.05$) reductions in pH (6.83-6.50) and TSS (5.80-1.20) (Table 5), with a concomitant increase in brown index (Table 6) of the cookie samples. The pH decreased with increased level of African nutmeg, with sample SLZ (100 %) exhibiting lowest pH value, hence highest acidity. Reduction of pH is an indication of improved shelf stability for this snack as most spoilage micro-organisms grow best at pH 6.8-7.2, close to neutral (Zahra & Safaa, 2015). Total soluble solid (TSS) followed similar trend. TSS is an indication of the amount of total soluble sugar, in particular sucrose contents of cookie samples. The control (KMO) had the highest value and SLZ (100 % African nutmeg) the least. This is a pointer to the suitability of cookie sam-

Table 4: Physicochemical properties of baked cookie

Samples	pH	TSS(Brix)
KMO	6.83^{a}±0.12	5.80
JLK	6.67^{ab}±0.06	5.00
VRU	6.63^{ab}±0.06	2.80
PQS	6.53^{b}±0.20	1.40
SLZ	6.50^{b}±0.00	1.20

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were significantly different ($P < 0.05$)

Table 5: Energy content of the different samples of cookie

Samples	Energy values (kcal)
KMO	390.62^{a}±0.12
JLK	385.48^{b}±0.11
VRU	383.63^{b}±0.01
PQS	382.18^{b}±0.00
SLZ	370.87^{c}±0.01

ple SLZ in control of dietary sugar intake, especially for overweight, obese and diabetic individuals. Most spices have been associated with a bitter principle which is believed to be capable of reducing blood sugar concentrations (Uhegbu, Iweala, & Kanu, 2011). The lightness, redness and yellowness as well as the brown index of the cookie were significantly affected by substitution of *M. myristica*. Brown index in particular increased with addition of African nutmeg (Figure 1). This might be attributed to the initial colour of African nutmeg and probable processing effect particularly, the baking temperature (Okafor & Ugwu, 2014). Colour is an essential parameter in judging quality of snack foods. It reflects the suitable of raw material used for preparation and provides information about the formulation and quality of the product (Abu-Salem & Abou-Arab, 2011).

3.4 Antioxidant profile of cookie samples

Replacement of vanilla flavour with *M. myristica* significantly ($p \leq 0.05$) influenced antioxidant characteristics among cookie samples, except flavonoids (Table 7). Sample SLZ (100 % African nutmeg) possessed highest values of total phenol and DPPH (2, 2-dipheny-2-pycryl hydrazine) contents. Similar results were documented by Stephen et al. (2014). Phenolic compounds have been reported to inhibit the activities of digestive and hydrolytic enzymes such as amylase, trypsin, chymotrypsin and lipase (Shetty, 1997). They also possess anti-carcinogenic, anti-viral, anti-microbial, anti-inflammatory, hypotensive and anti-oxidant activities (Shetty, 1997). The value 0.08 % obtained was slightly lower than values (0.18 %) reported by Uhegbu et al. (2011) and (0.15 %) Ndulaka, Ekaiko, Ogbonna, and Asiegbu (2016), in a similar study involving the incorporation of some spices into snacks. Flavonoids are potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer and anti- ulcer activity (Agbaire, 2011). It might, in addition, offer protection against the different levels of carcinogenesis.

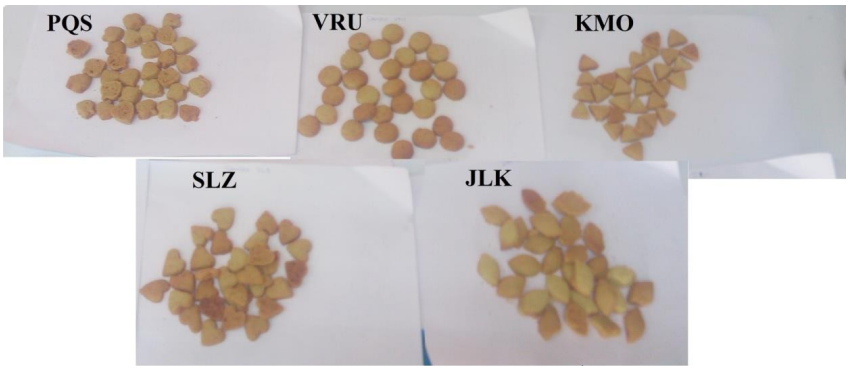

Figure 1: Cookie samples with different levels of African nutmeg as flavouring

Table 6: Colour characteristics of baked cookie samples

Samples	L*	a*	b*	BI
KMO	$48.38^{a}\pm0.29$	$7.42^{a}\pm0.03$	$21.61^{a}\pm0.05$	$51.57^{d}\pm0.07$
JLK	$48.43^{a}\pm0.07$	$3.83^{d}\pm0.02$	$19.62^{b}\pm0.01$	$51.62^{d}\pm0.03$
VRU	$47.18^{b}\pm0.04$	$3.78^{e}\pm0.06$	$19.20^{c}\pm0.03$	$52.82^{c}\pm0.04$
PQS	$46.43^{c}\pm0.03$	$5.48^{c}\pm0.06$	$18.84^{d}\pm0.01$	$53.57^{b}\pm0.03$
SLZ	$42.79^{d}\pm0.02$	$7.29^{b}\pm0.03$	$18.55^{e}\pm0.01$	$57.21^{a}\pm0.2$

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were significantly different ($P < 0.05$)

Table 7: Antioxidants profile of formulated cookie (%)

Samples	Flavonoid	Phenol	DPPH
KMO	$0.08^{a}\pm0.004$	$0.14^{e}\pm0.003$	$1.13^{c}\pm3.9$
JLK	$0.08^{a}\pm0.01$	$0.14^{d}\pm0.005$	$3.12^{b}\pm8.8$
VRU	$0.08^{a}\pm0.007$	$0.17^{c}\pm0.002$	$3.48^{b}\pm3.8$
PQS	$0.08^{a}\pm0.002$	$0.18^{b}\pm0.003$	$3.75^{b}\pm4.5$
SLZ	$0.08^{a}\pm0.008$	$0.24^{a}\pm0.004$	$5.22^{a}\pm1.7$

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were significantly different ($P < 0.05$)

Table 8: Anti-nutritional profile of cookie (%)

Samples	Saponin	Tannin	Oxalate
KMO	$0.45^{b}\pm0.12$	$0.16^{c}\pm0.02$	$0.11^{b}\pm0.02$
JLK	$0.53^{ab}\pm0.05$	$0.17^{bc}\pm0.006$	$0.11^{b}\pm0.02$
VRU	$0.77^{ab}\pm0.24$	$0.17^{bc}\pm0.008$	$0.13^{b}\pm0.05$
PQS	$0.77^{ab}\pm0.2$	$0.19^{b}\pm0.002$	$0.15^{b}\pm0.01$
SLZ	$0.82^{a}\pm0.21$	$0.21^{a}\pm0.008$	$0.20^{a}\pm0.00$

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were significantly different ($P < 0.05$)

Table 9: Sensory evaluation scores for cookies

Samples	Taste	Colour	Crispness	Flavour	Hardness	Overall acceptability
KMO	$7.23^{a}\pm1.61$	$7.77^{ab}\pm1.43$	$7.17^{a}\pm1.58$	$7.37^{a}\pm1.27$	$7.57^{a}\pm1.16$	$7.63^{a}\pm1.3$
JLK	$7.67^{a}\pm1.21$	$8.33^{a}\pm0.80$	$7.60^{a}\pm1.07$	$7.23^{ab}\pm1.38$	$7.83^{a}\pm1.34$	7.87^{a}a±1.07
VRU	$6.97^{a}\pm1.45$	$7.10^{b}\pm1.75$	$7.00^{a}\pm1.62$	$6.57^{b}\pm1.59$	$7.30^{a}\pm1.58$	$7.47^{a}\pm1.25$
PQS	$7.53^{a}\pm1.07$	$7.70^{ab}\pm1.02$	$7.33^{a}\pm1.09$	$7.10^{ab}\pm1.4$	$7.67^{a}\pm1.09$	$8.00^{a}\pm0.91$
SLZ	$7.37^{a}\pm1.10$	$7.30^{b}\pm1.34$	$7.50^{a}\pm0.97$	$7.40^{a}\pm1.04$	$7.43^{a}\pm1.25$	$7.80^{a}\pm0.76$

Mean ± standard deviation of triplicate readings, mean values followed by different superscripts within columns were were significantly different ($P < 0.05$)

3.5 Anti nutritional properties of cookie samples

The anti-nutrient content increased significantly ($p < 0.05$): saponin (0.45-0.82 %), tannin (0.16-0.21 %) and oxalate (0.11-0.20 %) with substitution of *M. myristica* for vanilla (Table 8). African nutmeg was earlier reported to be high in phytochemicals (Ekeanyanwu et al., 2010; Stephen et al., 2014). However, the levels of these phytochemicals in this product were within the ranges earlier considered safe in humans (Ugwu & Oranye, 2006). Health benefits of some of these phytochemicals are documented (Ugwu & Oranye, 2006). These include reductions of pathogenesis of cancer development and damage to intestinal tract (Makkar & Becker, 1996; Ugwu & Oranye, 2006). The low levels of tannin content in the cookie samples corresponded to the values reported by Uhegbu et al. (2011) and (Ndulaka et al., 2016).

3.6 Sensory attributes of formulated cookie

There was no significant difference ($p < 0.05$) between the mean sensory scores for most attributes of cookie samples and control, except in colour and flavour (Table 9). Cookie samples increased in brownness with increased levels of *M. myristica*, which may be due to the colour of the nutmeg powder. Similar trend was observed for flavour, which is the main criteria that makes the product to be liked or disliked (Abu-Salem & Abou-Arab, 2011). The assessment of flavour was done by the combination of taste and smell. Sample SLZ (100 % African nutmeg) was adjudged the most acceptable in terms of flavour by the panelists. The overall acceptability of the samples was based on their (panelist) individual performance on evaluation. Iwe (2002) considered a product with overall acceptability score of 7.0 as being accepted by consumer. It was observed that all samples were generally accepted by panellists, but SLZ (sample with 3.5 g of *M.*

myristica and 0 g of vanilla flavour) being the most acceptable.

4 Conclusions

Cookies of good nutritional standard and acceptable sensory qualities were produced from replacement of vanilla flavour with African nutmeg in the formulation. Functional properties of the flour blends were not affected markedly as a result of *M. myristica* seed flour substitution for vanilla flavour. Increased addition of *M. myristica* significantly influenced the proximate composition, physicochemical properties, phytochemical content and antioxidative potential of the cookies at the 95 % confidence level. The cookies compared very well with the control in virtually all the sensory attributes with sample SLZ (100 % replacement) being the most acceptable to the panellists. *M. myristica* seed could serve as a flavourant in the production of cookies and similar snacks and the replacement of vanilla flavour with African nutmeg is potentially possible. Studies on microbial characteristics and storage stability of such products are important and therefore recommended.

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Effects of "Starch:Water" Ratio on Gelatinization of *pinhão* Starch from Nine Germplasm Collections, Measured by Differential Scanning Calorimetry

Camila D. Bet[a], Rossane C. B. Godoy[b], Layse P. Cordoba[a], Ivo M. Demiate[a], Luiz G. Lacerda[a], and Egon Schnitzler[a*]

[a] State University of Ponta Grossa, Av. Carlos Cavalcanti, 4748, 84030-900, Ponta Grossa, PR, Brazil
[b] Embrapa Forestry–Brazilian Agricultural Research Corporation - Estrada da Ribeira, Km 111 - P.O. Box 319, 83411-000 - Colombo, PR, Brazil
[*]Corresponding author
egons@uepg.br
Tel: +55 042 3220-3093

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Abstract

Native starch was extracted from nine germplasm collections of *Araucaria angustifolia* seeds in aqueous medium and they were characterized by Pasting Properties (RVA), X-ray Powder Diffractometry (XRD) and Scanning Electron Microscopy (SEM). The gelatinization process of each sample was evaluated at different ratios of starch:water by Differential Scanning Calorimetry (DSC). A slight displacement in the gelatinization curves was observed for the *pinhão* starches prepared with different amounts of water. With an increase in water content, most of the samples presented a decrease in the peak, the conclusion temperatures, and the range of gelatinization temperatures, while the enthalpy did not follow a standard behavior. A displacement or a narrowing of the gelatinization temperature range occurred with increasing water content. *Pinhão* starch showed pasting temperature in the range of 60-67 ^{o}C and there were differences in the pasting properties and degree of relative crystallinity between the analyzed samples. The C-type diffraction pattern was found for all the samples and the morphology of starch granules was similar, with oval and round shapes. Therefore, different characteristics were found among starches from nine germplasm collections, encouraging the protection of the biological diversity of selected species, aiming at future applications.

Keywords: *pinhão* starch; Gelatinization; Pasting properties; Germplasm; Thermal analysis

1 Introduction

Starch, among the carbohydrates, is one of the most abundant renewable sources, widely used for industrial purposes due to its physicochemical properties and low cost. The main economic sources of starches are plants, in which this biopolymer occurs in various sites (cereal grains, seeds, roots and tubers, stems, etc.) as tiny white granules. They may present an oval, spherical, round, polygonal or lenticular shape, with a diameter range of <1 up to 100 μm. Starch granules are composed mainly of two glucose polymers called amylose (with a linear form consisting of up to 3000 glucose units; interconnected primarily by α-1,4 glycosidic linkages) and amylopectin (a large branched polymer with α-1,4 linkages that serve as a backbone and α-1,6 bridges at the branch points) (Bicudo et al., 2009; de Conto, Vicente Plata-Oviedo, Steel, & Chang, 2011; Pinto, Moomand, et al.,

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2015; Pinto et al., 2012; Thys, Aires, Marczak, & Noreña, 2013).

"*Pinhão*" is the name of the seeds from "*Paraná pine*", a tree that belongs to the *Araucariaceae* family (*Araucaria angustifolia syn. Araucaria brasiliensis*). This tree grows in forests of Brazil (South Region), Paraguay, Argentina, and Chile; however, it is threatened with extinction due to excessive extraction of its wood. The *pinhão* seeds are consumed by humans (mainly during the winter) after cooking in water and later peeling and also used as flour in regional cuisine. The main component of *pinhão* seeds is starch (68-72%, dry basis), but protein (around 3%), lipid (around 1%), soluble sugars (around 2.4%), as well as dietary fibers, phenolic compounds and minerals such as copper and magnesium are also found (Cordenunsi et al., 2004; Daudt, Kuelkamp-Guerreiro, Cladera-Olivera, Silveira Thys, & Ferreira Marczak, 2014; Ribeiro et al., 2014).

Germplasm collections are sources of genetic variability for plant breeding. These techniques are available and necessary with the aim to protect the biological diversity of selected species, mainly those that are at risk for extinction. Thus, EMBRAPA Forests created a germplasm bank with 224 different types of conifer Araucaria, in the city of Colombo, PR, Brazil (Bello-Perez et al., 2006; Oliveira Gomes da Costa et al., 2013; Villalobos, Ferreira, & Mora, 1991).

Gelatinization is a process that occurs when the starch-water slurry is heated, and the starch granules swell. The energy required for molecular order disruption of starch granules differs according to their botanical origin. With the DSC technique, this molecular disorder is often observed as an endothermic phenomenon (Kohyama, Matsuki, Yasui, & Sasaki, 2004; Malucelli et al., 2015). As starch is used in the food industry to confer some functional properties, this investigation was carried out to characterize the starches from nine different sources of *pinhão* seeds and evaluate the gelatinization process at different ratios of starch:water.

2 Materials and Methods

2.1 Materials

The genetic resources of the *pinhão* seeds were collected in the germplasm bank (Embrapa Forestry-Colombo-PR-Brazil) and all the seeds were collected in June (2016), at the same maturation stage.

2.2 Starch extraction

The starch extraction was carried out according to Bello-Perez et al. (2006) with the following modifications proposed by Oliveira Gomes da Costa et al. (2013): the main coat (hard) of seeds was removed as well as the second coat (a thin layer) adhered to the surface. Isolated seeds were milled, and an equal mass of distilled water was added. The suspension was mechanically stirred for 10 min, sieved (200 mesh or 0.075 mm) and centrifuged (5000 rpm for 10 min). Obtained starch was carefully dried in an oven with forced air circulation at 40 ^{o}C for 24 h. Finally, the purified starch was kept in a desiccator over anhydrous calcium chloride up to constant mass.

2.3 Differential scanning calorimetry (DSC)

The DSC curves were obtained using a DSC-Q200 model (TA-Instr. Co., USA). Initially the instrument was calibrated with 99.99% standard indium, m.p. = 156.6^{o}C, ΔH = 28.56 J g^{-1}. Each sample was prepared, in triplicate, as follows: 2.0 mg of starch sample and 8.0 μL of distilled water were added to an aluminum crucible (sample with starch:water ratio 1:4). Afterward, the crucible was sealed and held at rest for 60 min (samples A1 to A9) to perform DSC analysis. The same procedure was adopted for other samples with starch:water ratios of 1:5 (samples B1 to B9) and 1:6 (samples C1 to C9). The conditions of the instrument for each analysis were: heating rate of 10 ^{o}C min^{-1} from 30 ^{o}C to 100 ^{o}C under air flow of 50 mL min^{-1} and an empty aluminum crucible was used as reference. The temperature onset (T_o), peak temperature (T_p), endset or conclusion temperature (T_c), as well as

gelatinization enthalpy (ΔH_{gel}) were calculated (Malucelli et al., 2015).

2.4 Pasting properties (RVA)

The pasting profiles of the studied starches were obtained in triplicate according to the literature (Hornung, de Oliveira, Lazzarotto, da Silveira Lazzarotto, & Schnitzler, 2016) using the Rapid Visco Analyser (RVA-4, Newport Sci., Australia). Sample moisture was previously measured to prepare a suspension containing 8 % starch (dry basis), with the addition of water to make up the total mass of 28 g in the RVA canister.

The suspensions were subjected to a programmed heating and cooling cycle (STD-2, Thermocline for Windows), where they were continuously stirred at 160 rpm. The temperature was maintained at 50 ^{o}C for 2 min, then heated to 95 ^{o}C at a rate of 6 ^{o}C min^{-1} and held at this temperature for 5 min. Finally, the mass was cooled to 50 ^{o}C at a rate of 6 ^{o}C min^{-1} and maintained for 2 min.

2.5 X-ray diffractograms (DRX)

Each sample was analyzed in triplicate on a glass support and exposed to CuKα radiation (wavelength 1.5418 $\AA$), subjected to 40 kV and current of 20 mA in the X-ray diffractometer (Ultima 4, Rigaku Co., Japan). The scattered radiation was detected in the angular range of 5-50^{o} (2Θ) with scanning speed of 8 min^{-1} and a step of 0.06^{o}. The relative crystallinity was calculated in agreement with the method previously described (Zhang, Xie, Zhao, Liu, & Gao, 2009).

2.6 Morphological analysis (SEM)

Scanning electron microscopy (SEM) was carried out with the instrument VEGA3 (TESCAN, Czech Rep.). The parameters of analysis were: 20 μm in the reading scale, with a voltage of 20 kV in the electron beam, tungsten filament and retro mirrored electron detector. The instrument is based on electrons passing through the previously prepared sample. Initially, the sample was attached to a carbon tape and prepared by a metallization process with gold and palladium plasma. The mean diameter of the granules was calculated with the aid of AZtec software (Bet, Cordoba, Ribeiro, & Schnitzler, 2016).

2.7 Statistical analysis

After assuming normality of the data and verifying the homoscedasticity of the variances ($p > 0.05$) by Levene's test, differences between the means were verified by analysis of variance (ANOVA) and compared by Duncan's test ($p<0.05$). The software programs used were ACTION^{TM} and SASM-Agri^{TM} 8.2.

3 Results and discussion

Heating of starch in the presence of water leads to an irreversible transformation known as gelatinization, which is characteristic of the botanical origin and molecular composition of each starch. This phenomenon is an endothermic process that results in the disruption of molecular order (double helical and crystalline structures) within the starch granules with the creation of a molecular dispersion called a "paste" or "gel" (Klein et al., 2013; Kohyama et al., 2004). Among the different analytical methods, differential scanning calorimetry (DSC) has been preferred for measuring starch gelatinization (Wani et al., 2012). Figure 1 shows the DSC curves and it is possible to observe a slight displacement in the thermal event for the samples containing different proportions of water.

Differences between the *pinhão* starches from nine germplasm accessions in the DSC results can be visualized in Table 1. It was observed that, by comparing all the samples, the highest onset and peak temperature values were exhibited by samples 1, 5 and 7 (T_o between 60.3-62.5^{o}C; Tp between 66.2-67.5^{o}C). Lower values of gelatinization temperature (T_o between 51.0-53.7^{o}C; T_p between 58.1-60.6^{o}C) were observed for samples 4 and 9; and T_p around 61.4-62.4^{o}C for samples 2 and 3.

Other values of T_p from gelatinization of *pinhão* starch reported in the literature were: 66.65 (Pinto et al., 2012), 62.44 (Ribeiro et al., 2014), 55.55 (Daudt et al., 2014), 63.4 (Bello-Perez et

Table 1: DSC, XRD and SEM results of nine different species of *pinhão* starch, where A corresponds to the samples prepared in a ratio of 1:4 (starch:water); B in a ratio of 1:5 and C in a ratio of 1:6 for DSC analysis

Samples		DSC gelatinization					XRD Degree of relative crystallinity/%	SEM da/μm
		T_o/^{o}C	T_p/^{o}C	T_c/^{o}C	ΔT/^{o}C	ΔH_{gel}/J g^{-1}		
	A	61.5 ± 0.2^{aB}	67.2 ± 0.1^{aA}	74.1 ± 0.1^{aC}	12.7 ± 0.3^{bGHIJ}	7.6 ± 0.3^{cHI}		
1	B	60.6 ± 0.1^{bC}	66.2 ± 0.1^{bD}	73.3 ± 0.2^{bD}	12.7 ± 0.1^{bGHIJ}	8.2 ± 0.2^{bFG}	22.1 ± 0.8^{a}b	12.9 ± 0.8^{d}
	C	60.3 ± 0.1^{bC}	66.3 ± 0.1^{bCD}	73.5 ± 0.2^{bD}	13.2 ± 0.1^{aFG}	11.3 ± 0.2^{aB}		
	A	57.3 ± 0.2^{bF}	61.4 ± 0.2^{aGH}	68.5 ± 0.2^{aFG}	11.2 ± 0.2^{aM}	4.7 ± 0.2^{bL}		
2	B	57.4 ± 0.2^{abEF}	61.5 ± 0.3^{aG}	66.7 ± 0.2^{bI}	9.2 ± 0.1^{bN}	5.8 ± 0.2^{aK}	15.4 ± 0.6^{e}	16.1 ± 0.8^{a}
	C	57.7 ± 0.2^{aE}	61.6 ± 0.4^{aG}	66.8 ± 0.3^{bI}	9.1 ± 0.3^{bE}	5.9 ± 0.1^{aK}		
	A	55.7 ± 0.3^{cH}	62.4 ± 0.2^{aF}	69.5 ± 0.1^{aE}	13.8 ± 0.2^{aE}	12.5 ± 0.4^{aA}		
3	B	56.1 ± 0.1^{bH}	61.7 ± 0.2^{bG}	69.7 ± 0.1^{aE}	13.7 ± 0.1^{aEF}	9.7 ± 0.2^{bD}	18.2 ± 0.7^{d}	14.6 ± 0.8^{b}
	C	56.5 ± 0.1^{aG}	61.4 ± 0.2^{bGH}	68.6 ± 0.3^{bFG}	12.1 ± 0.3^{bJK}	7.3 ± 0.1^{cI}		
	A	52.9 ± 0.2^{bL}	58.1 ± 0.1^{bL}	65.1 ± 0.1^{bJ}	12.2 ± 0.1^{abIJK}	7.9 ± 0.2^{bGH}		
4	B	52.7 ± 0.1^{bL}	58.3 ± 0.2^{bL}	64.5 ± 0.4^{cK}	11.8 ± 0.3^{bKL}	7.9 ± 0.3^{bGH}	12.8 ± 0.9^{b}	17.3 ± 0.6^{d}
	C	53.7 ± 0.3^{aK}	59.0 ± 0.3^{aK}	66.5 ± 0.3^{aI}	12.8 ± 0.5^{aGHI}	9.2 ± 0.1^{aE}		
	A	60.6 ± 0.2^{aC}	67.2 ± 0.1^{aA}	75.7 ± 0.3^{aA}	15.1 ± 0.5^{aC}	8.3 ± 0.1^{aF}		
5	B	60.3 ± 0.1^{aC}	66.8 ± 0.2^{bB}	75.3 ± 0.5^{aA}	15.0 ± 0.5^{aC}	8.6 ± 0.2^{aF}	21.3 ± 0.6^{bc}	12.5 ± 1.0^{d}
	C	59.6 ± 0.3^{bD}	66.2 ± 0.2^{cCD}	73.3 ± 0.3^{bD}	13.6 ± 0.6^{bEF}	7.7 ± 0.2^{bHI}		
	A	54.2 ± 0.1^{aIJ}	61.0 ± 0.4^{aHI}	68.9 ± 0.3^{aF}	14.7 ± 0.2^{aCD}	9.3 ± 0.2^{aDE}		
6	B	54.1 ± 0.5^{aJ}	60.5 ± 0.5^{aJ}	68.3 ± 0.7^{abG}	14.1 ± 0.3^{aDE}	9.2 ± 0.2^{aE}	20.5 ± 0.8^{c}	12.8 ± 0.9^{d}
	C	54.6 ± 0.4^{aI}	60.3 ± 0.3^{aJ}	67.7 ± 0.3^{bH}	13.1 ± 0.5^{bFGH}	8.2 ± 0.1^{bFG}		
	A	62.5 ± 0.2^{aA}	67.5 ± 0.4^{aA}	75.2 ± 0.2^{aA}	12.7 ± 0.2^{aGHI}	10.3 ± 0.3^{aC}		
7	B	61.6 ± 0.2^{bB}	66.6 ± 0.3^{bBC}	73.4 ± 0.4^{bD}	11.8 ± 0.3^{bKL}	8.6 ± 0.3^{bF}	23.3 ± 0.8^{a}	14.7 ± 0.8^{b}
	C	62.3 ± 0.3^{aA}	66.5 ± 0.2^{bBCD}	73.8 ± 0.2^{bCD}	11.4 ± 0.4^{bLM}	6.6 ± 0.2^{cJ}		
	A	54.3 ± 0.1^{bIJ}	61.5 ± 0.2^{cG}	68.5 ± 0.3^{cFG}	14.2 ± 0.4^{cDE}	10.4 ± 0.3^{bC}		
8	B	57.1 ± 0.1^{aF}	66.1 ± 0.1^{aD}	75.7 ± 0.3^{aA}	18.6 ± 0.2^{bB}	8.5 ± 0.3^{cF}	21.3 ± 0.8^{bc}	14.9 ± 0.7^{b}
	C	54.4 ± 0.4^{bIJ}	64.5 ± 0.2^{bE}	74.7 ± 0.1^{bB}	20.3 ± 0.2^{aA}	12.6 ± 0.2^{aA}		
	A	53.7 ± 0.2^{aK}	60.6 ± 0.2^{aIJ}	68.5 ± 0.1^{aFG}	14.8 ± 0.2^{aC}	7.7 ± 0.1^{cHI}		
9	B	52.1 ± 0.2^{bM}	58.3 ± 0.3^{bL}	64.6 ± 0.2^{bK}	12.5 ± 0.1^{bHIJ}	10.4 ± 0.1^{aC}	17.4 ± 0.5^{d}	13.9 ± 0.8^{c}
	C	51.0 ± 0.2^{cN}	56.8 ± 0.1^{cM}	63.8 ± 0.3^{cL}	12.8 ± 0.5^{bGHI}	9.6 ± 0.2^{bD}		

Values expressed as mean ± standard deviation. Values followed by the same lowercase letter in the same column did not differ from each other by the Duncan's Test ($p < 0.05$), comparing different concentrations of water from the same sample. Values followed by the same capital letter in the same column did not differ from each other by the Duncan's test ($p < 0.05$), comparing different samples. T_o "onset" or initial temperature, T_p peak temperature, T_c "endset" or conclusion temperature, ΔH_{gel} gelatinization enthalpy. The degree of relative crystallinity was calculated as a percentage, peaks are determined at 2Θ. da, average diameter.

Table 2: VA results of *pinhão* starch from nine different germplasm collections (1-9)

Samples	Pasting temperature/^{o}C	Viscosity peak/cP	Peak time/sec	Setback/cP	Breakdown/cP	Final viscosity/cP
1	64.8 ± 0.5^{b}	3087.0 ± 85.1^{a}	335.4 ± 5.0^{f}	1309.5 ± 40.0^{bc}	1798.0 ± 42.5^{a}	2606.8 ± 70.2^{a}
2	60.8 ± 0.6^{e}	1933.8 ± 45.0^{g}	445.7 ± 7.8^{a}	1345.4 ± 31.7^{ab}	813.2 ± 45.5^{f}	2467.7 ± 69.6^{b}
3	62.4 ± 0.5^{d}	2960.3 ± 62.6^{b}	363.7 ± 6.5^{c}	1360.8 ± 65.8^{ab}	1673.6 ± 60.7^{b}	2664.5 ± 40.6^{a}
4	60.2 ± 0.4^{e}	2703.5 ± 55.1^{cd}	319.6 ± 5.5^{g}	1190.1 ± 70.0^{de}	1493.1 ± 40.4^{c}	2386.4 ± 44.2^{bc}
5	66.3 ± 0.4^{a}	2743.8 ± 66.8^{c}	357.6 ± 2.1^{cd}	1257.8 ± 40.2^{cd}	1437.5 ± 60.1^{cd}	2591.5 ± 34.9^{a}
6	63.6 ± 0.4^{c}	2350.9 ± 50.0^{f}	350.3 ± 6.5^{de}	1148.7 ± 45.4^{e}	1050.3 ± 50.5^{e}	2445.0 ± 42.3^{b}
7	67.0 ± 0.3^{a}	2714.8 ± 41.0^{cd}	367.0 ± 4.6^{c}	1383.5 ± 30.6^{ab}	1406.8 ± 40.4^{cd}	2694.8 ± 75.0^{a}
8	64.3 ± 0.5^{b}	2556.8 ± 50.3^{e}	378.3 ± 3.7^{b}	1171.0 ± 33.7^{e}	1385.5 ± 73.5^{d}	2339.9 ± 60.1^{c}
9	60.0 ± 0.2^{e}	2634.2 ± 35.5^{de}	348.0 ± 56.0^{e}	1433.8 ± 56.0^{a}	1416.2 ± 68.7^{cd}	2654.8 ± 49.2^{a}

Values followed by the same lowercase letter in the same column did not differ from each other by the Duncan's Test ($p < 0.05$)

cP "centipoises", sec "seconds".

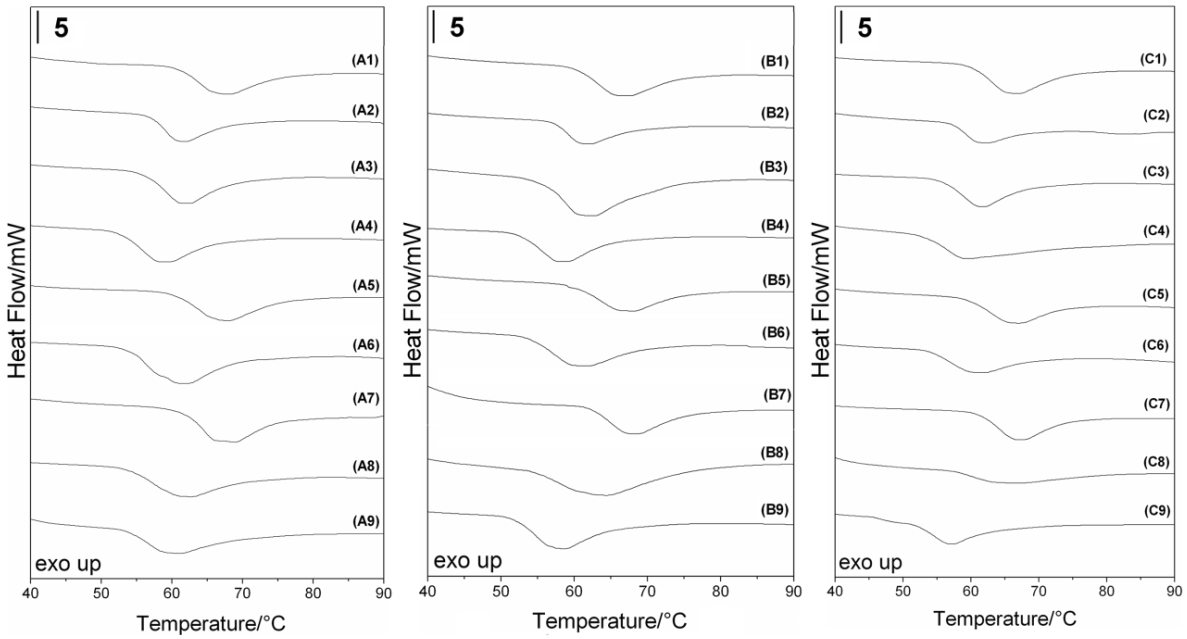

Figure 1: DSC gelatinization curves of different species of *pinhão* starch, where A1 to A9 correspond to the samples prepared in a ratio of 1:4 (starch:water); B1 to B9 in a ratio of 1:5 and C1 to C9 in a ratio of of 1:6

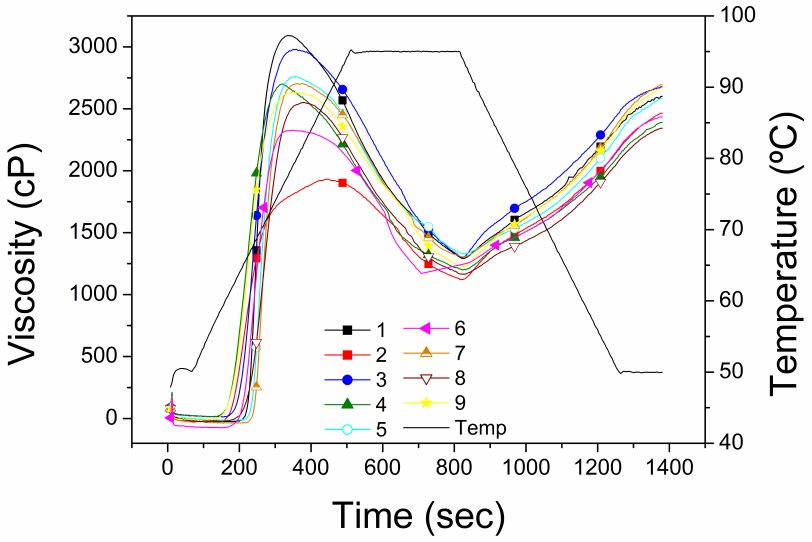

Figure 2: RVA curves of *pinhão* starch from nine different germplasm collections (1-9)

al., 2006), 60.15 (Pinto, Vanier, Deon, et al., 2015) and 66.69^{o}C (Klein et al., 2013), suggesting that these samples were acquired in respective local commerce (from different cities) in the southern region of Brazil.

Zortéa-Guidolin et al. (2017) found values of T_p between 58.3-64.5^{o}C for *pinhão* starches from seven accessions of the same germplasm bank used in the present study.

Similar results for the transition temperatures were reported by Oliveira Gomes da Costa et al. (2013) for *pinhão* starch from four germplasm banks in Embrapa (Brazil), with a 1:4 ratio (starch:water). However, the gelatinization enthalpy was higher (24.23, 20.23, 16.87 and 10.40 J g^{-1}) than that found in this study, where samples 3A (1:4 starch:water ratio) and 8C (1:6 starch:water ratio) had the highest values (12.5 and 12.6 J g^{-1}, respectively, yet with no significant difference by Duncan's Test, $p<0.05$) and sample 2A had the lowest value (4.7 J g^{-1}). Zortéa-Guidolin et al. (2017) also reported higher values of ΔH_{gel}, between 12.5 to 14.5 J g^{-1}.

Considering the starch:water ratios (1:4, 1:5 and 1:6), slight differences in the peak temperatures (T_p) were observed. For most of the samples (1, 3, 5, 7 and 9), the increase in the water content led to decreased T_p. In addition, a decrease in the transition temperature range was observed for samples 2, 3, 5, 6, 7 and 9, and an increase for samples 1, 4 and 8. This was also observed for flour and starch of chestnuts in different water contents (Torres, Moreira, Chenlo, & Morel, 2013).

It was suggested that this occurred due to the plasticising action of water over starch crystals, which assists in the conduction of energy, favoring a greater mobility of chains, and therefore, a lower temperature is necessary for this irreversible transition (Schirmer, Zeller, Krause, Jekle, & Becker, 2014). The water content did not significantly affect ($p<0.05$) the onset and the peak temperatures for samples 2 and 6. Only samples 4 and 8B showed an increase in T_p value. The increase in the water content promoted a decrease in the conclusion temperature, suggesting a displacement or a narrowing of the gelatinization range.

The gelatinization enthalpy (ΔH_{gel}) showed differences between each sample. Samples 1, 2 and 4 showed an increase in ΔH_{gel} proportional to the starch:water ratio increase, as was observed for flour and starch of chestnut (Torres et al., 2013). Samples 3, 5, 6 and 7 showed a decrease and the others did not follow this behavior.

Some differences in DSC values for peak temperature (T_p) and gelatinization enthalpy (ΔH_{gel}) of native *pinhão* starches among different authors can be attributed to differences among the samples (samples from market or selected from germplasm banks), different starch extraction processes (aqueous medium or chemical), instrument conditions (heating rate, starch:water ratio) and architecture of the crystalline region. In addition, the initial moisture as well as the way the water diffuses between the chains may be a determinant in the gelatinization of the starch (Hoover, 2001).

The pasting properties of each analyzed *pinhão* starch were performed and the RVA curves are shown in Figure 2.

Differences can be noted among the samples, and for better visualization the results are presented in Table 2. *pinhão* starch showed lower pasting temperature values, different from other unmodified starches such as corn (Malucelli et al., 2015), cassava (Hornung et al., 2016; Hornung, Granza, de Oliveira, Lazzarotto, & Schnitzler, 2015), common vetch (Bet et al., 2016), avocado (Lacerda et al., 2015), European chestnut (Lopes et al., 2016), carioca bean (Granza et al., 2015) and potato (Leivas et al., 2013).

In this investigation, samples 7 and 5 showed the highest values of pasting temperature (67.0 and 66.3 ^{o}C, respectively, without significant difference by Duncan's Test, $p<0.05$) and samples 2, 4 and 9 showed the lowest (60.8; 60.2 and 60.0 ^{o}C, respectively, without significant difference by Duncan's Test, $p<0.05$). These data corroborate that observed in T_o, determined by DSC, although the values by this analysis were lower, as observed by Park, Lbanez, Zhong, and Shoemaker (2007). DSC is a highly sensitive method, allowing the detection of temperatures at the beginning of the gelatinization process as a thermal response. On the other hand, the pasting temperature is related to the minimum cooking temperature of the starch, when the viscosity increases due to the maximum volume of swollen granules, which will already be disinte-

grated due to the shear used in this analysis. Thus, these temperatures can be correlated, and a lower value is expected from the higher sensitivity analysis (DSC) (Noisuwan, Bronlund, Wilkinson, & Hemar, 2008).
The highest viscosity of starch slurry was found for sample 1 (3091.0 cP) and the lowest for sample 2 (1931.5 cP), which also had the lowest enthalpy (DSC). The final viscosity of *pinhão* starch, which indicates the ability to form a viscous paste, was highest for samples 1, 3, 5, 7 and 9 (without significant differences by Duncan's Test, $p<0.05$) and lowest for samples 2, 4 and 8. Zortéa-Guidolin et al. (2017) also observed high final viscosity from seven accessions of the same germplasm bank, but the values of the bonding properties were different from the present study.
The relative crystallinity of the studied *pinhão* starches was quantitatively calculated based on the relationship between the peaks and the total area of diffraction patterns, and values are shown in Table 1. The lowest value was obtained for sample 2, which also showed the lowest enthalpy value of gelatinization. The highest relative crystallinity was exhibited by samples 1 and 7 (without significant differences by Duncan's Test, $p<0.05$). According to Hoover (2001), enthalpy may be correlated to the quantity and order of the double helices of amsylopectin, mainly to the distribution of short chains, that is to say, the molecular architecture of the starch. In another study with *pinhão* starch from four germplasm collections, authors found values between 25.43-28.43% (Oliveira Gomes da Costa et al., 2013). In a recent study (Zortéa-Guidolin et al., 2017), authors described values of relative crystallinity of *pinhão* starch from seven germplasm collections and their results were between 26.37-30.46%.
The X-ray diffractograms of native *pinhão* starch showed peaks at 15^o, 17^o and 23^o (2Θ), and another small peak at approximately 5.7^o, typical of C-type structures, and as also observed by other authors (Daudt et al., 2014; Pinto et al., 2012; Zortéa-Guidolin et al., 2017).
Regarding SEM analysis, the morphology of all samples presented oval and round shapes as previously reported by Pinto, Moomand, et al. (2015) and Bello-Perez et al. (2006) and hemispherical or truncated ellipsoid shapes with smooth surfaces (Zortéa-Guidolin et al., 2017).
In this investigation, the average diameter of the samples is shown in Table 1, with values between 12.8-16.1 μm. In other studies, the average diameter of starches from *pinhão* was 22.9 μm Pinto, Moomand, et al. (2015) and 13.98 μm (Ribeiro et al., 2014). Other values of average diameter of *pinhão* starches from germplasm accessions reported in the literature were 12.41 to 16.21 μm (Oliveira Gomes da Costa et al., 2013) and 12 μm (Zortéa-Guidolin et al., 2017).

4 Conclusions

The proposed study shows differences in gelatinization processes of *pinhão* starch from nine germplasm collections. When a starch suspension was prepared in a small amount of water, higher peak and conclusion temperatures and gelatinization temperature ranges were observed, since water is a limiting factor for gelatinization of the starch. It was not possible to relate the enthalpy to the water content.
New studies may be carried out, such as the determination of amylose/amylopectin ratio, which directly influences starch properties and may be different for species from the same botanical source. Differences in the pasting properties were identified between the samples. The X-ray diffraction pattern indicated that the starch was C-type, and an oval shape was observed for the starch granules. Since germplasm is related to genetic resource protection, further characteristics must be evaluated for its preservation and other rational uses.

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Effect of Osmotic Drying on Physicochemical Properties of Pansies *(Viola × Wittrockiana)*

Luana Fernandes[a, b, c], Susana Casal[b], Agostinho Magalhães[a], Paula Baptista[a], José A. Pereira[a], Jorge A. Saraiva[c], and Elsa Ramalhosa[a*]

[a] Centro de Investigação de Montanha (CIMO)/School of Agriculture, Polytechnic Institute of Bragança, Portugal
[b] LAQV@REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto University, Portugal
[c] QOPNA & LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Portugal
[*]Corresponding author
elsaramalhosa@ipb.pt
Tel: +351-273303308
Fax: +351-273325405

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Abstract

The objective of this work was to study the effect of osmotic drying, using different hypertonic solutions (sucrose and sodium chloride), on physicochemical characteristics of pansies (*Viola × wittrockiana*). The same treatments were applied to lettuce to compare the behavior of flowers with other vegetables. Pansies' superhydrophobic surface structure, called papillae, increased the resistance to exchanges with hypertonic solutions. No weight loss was observed after most treatments (sucrose: between 2.2 and 6.8 %; NaCl: between -23.0 % and 1.5 %), a_w maintained high values (> 0,94) and monomeric anthocyanins were preserved (fresh 0.10 and 0.19 mg Cy-3glu/g fresh matter for 20%/1 h in NaCl and 60%/1 h in sucrose). When applying more drastic conditions, as sodium chloride for more than 1 hour, undesirable textural and color changes were observed. For lettuce, all treatments caused osmotic dehydration, weight loss (ranged between -9.3 to -30.3 % for 80%/1 h in sucrose and 15%/1 h in NaCl) and a reduction in a_w (< 0,97) and carotenoids, with sodium chloride causing more damage in visual appearance than sucrose. Therefore, immersion in osmotic solutions can be applied to lettuce but the desired effect was not achieved for pansies due to the morphological structure of the flowers' epidermis.

Keywords: *Viola×wittrockiana*; Lettuce; Osmotic dehydration; Carotenoids; Monomeric anthocyanins

1 Introduction

Garden pansies (*Viola × wittrockiana*) result from extensive hybridizing and selection involving the species of *Viola tricolor, Viola lutea, Viola altaica* and others (Lim, 2014). Pansies are edible flowers, added to salads or used to garnish desserts (frosted cakes, sorbets and iced drinks). They can also be crystallized and eaten as a sweet delicacy. Furthermore, these flowers are rich in health-promoting compounds, such as anthocyanins, carotenoids, flavonoids, potassium and phosphorus (Gamsjaeger, Baranska, Schulz, Heiselmayer, & Musso, 2011; Rop, Mlcek, Jurikova, Neugebauerova, & Vabkova, 2012; Vukics, Kery, & Guttman, 2008), contributing for a healthy diet, while providing protection against cancer and cardiovascular diseases, among oth-

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ers (Lu, Li, & Yin, 2016). However, edible flowers have a limited shelf-life, with petal abscission, discoloration, wilting, dehydration and tissue browning occurring soon after harvest. The most common ways to preserve edible flowers include refrigeration, drying or canning in sugar, and preservation in distillates. However, these processes can have a negative impact on their nutritional and sensory quality and appearance. In response to recent culinary trends, demanding an increased availability and shelf life of edible flowers, it is essential to find new technologies or pretreatments which are able to extend edible flowers' shelf life with minimal impact on their sensory and textural properties.

Immersion in osmotic solutions is a common procedure used to dehydrate foods, and it can be regarded as a way to decrease the water activity and maintain the appearance of pansies. However, few studies on the effect of osmotic dehydration have been conducted on edible flowers. This technology has already been applied to broccoli (Xin, Zhang, & Adhikari, 2013) and cauliflower (Jayaraman, Das Gupta, & Rao, 1990; Vijayanand, Chand, & Eipeson, 1995), edible flowers less known by consumers, using sweet (sucrose or trehalose) and/or salty (ex: sodium chloride) approaches, but no studies on osmotic drying were found for pansies.

The main objective of the present work was to investigate the effect of osmotic dehydration in sucrose (60 and 80%, w/v) and sodium chloride (15%, 20%, 25%, w/v) solutions, at room temperature, on the quality of pansies (*Viola* × *wittrockiana*). Visual appearance, weight loss, a_w and color were evaluated before and after treatments. Simultaneously, the contents of carotenoids and monomeric anthocyanins were determined in the treated flowers. Lettuce, a leafy vegetable with a homogeneous color distribution, was used as a control, being subjected to the same treatments and evaluations.

2 Materials and Methods

2.1 Samples

Fresh white/violet pansies (*Viola* × *wittrockiana*) were collected at the greenhouse of the School of Agriculture, Polytechnic Institute of Bragança (Bragança, Portugal) and transported immediately to the laboratory under refrigeration. The production mode was organic. Fresh lettuce was bought at a local market in Bragança city that is located in the Northeast of Portugal.

2.2 Preparation of osmotic solutions

The osmotic agents, sucrose and sodium chloride, were of commercial grade, purchased from a local market. The different concentrations of 60 and 80% (w/v) of sucrose and 15%, 20% and 25% of sodium chloride were prepared by dissolving the required amounts of sucrose or sodium chloride in distilled water.

2.3 Osmotic dehydration

Pansies or lettuce leaves were placed into the vessels containing the different sucrose/sodium chloride solutions, remaining totally submerged. The ratio of raw material to osmotic solution was 1:4. At each sampling time (15 min, 30 min, 1 h and 2 h for sodium chloride / 1 h and 2 h for sucrose), the flowers and lettuce leaves were taken out, gently dried with adsorbent paper and weighed. These conditions were selected after several preliminary tests, where higher times and mechanical agitation were shown to induce degradation. Each treatment/time was performed in triplicate, on both pansies and lettuce.

A portion of each sample, including fresh ones, was immediately analyzed for weight, a_w, color, and microscopy, while the remainder was preserved by freeze-drying for analysis of bioactive compounds, as detailed in Section 2.4.

2.4 Physicochemical characterization of samples

Color, water activity (a_w) and weight loss

The color of pansies (white and violet parts) and lettuce were evaluated in three samples of each treatment/time, with a colorimeter Minolta CR-400 (Osaka, Japan), using CIE*Lab* mode. L^*,

a^* and b^* coordinates were measured, where L^* varies between 0 (completely black) and 100 (completely white), a^* -100 (green) to +100 (red), and b^* from -100 (blue) to +100 (yellow). Furthermore, the Chroma (C^*) and Hue Angle (h^*) values were determined. In order to analyse the color variations, the total color difference (ΔE^*) was also calculated, according to equation 1:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta b^*)^2 + (\Delta a^*)^2} \quad (1)$$

where Δ was the difference between the parameters' values after the immersion in osmotic solutions and before it (fresh sample).
Water activity (a_w) was determined in a portable water activity meter (Novasina, LabSwift-a_w, Lachen, Switzerland). This parameter was evaluated on three samples before treatment (control) and after immersion in the osmotic solutions. Weight measured in a digital balance (Kern ACJ/ACS, Balingen, Germany), is the weight variation expressed in relative percentage of initial weight.

Freeze-dried

Flowers and lettuce, treated and untreated, were frozen and lyophilized (Scanvac, Coolsafe, Lynge, Denmark) for 2 days. They were ground to a homogenous powder and then stored for protection from light and moisture until extraction.

Carotenoids

The carotenoids content was determined according to the method used by Aquino-Bolaños, Urrutia-Hernandez, Lopez Del Castillo-Lozano, Chavez-Servia, and Verdalet-Guzman (2013). One gram of frozen-dried powder of the osmotic dehydrated and fresh pansies and lettuce was extracted twice with 20 ml acetone:hexane solution (1:1, v/v). Both extracts were put into a separation funnel, where 200 ml of distilled water was added to eliminate acetone. The acetone-free phase was mixed with 5 g anhydrous sodium sulphate to eliminate any residual water. The remaining solution was filtered and completed to 100 ml with hexane. Carotenoids content was determined by reading the absorbance at 450 nm and comparing the result to a β-carotene calibration curve (0.22–8.8 μg/ml). Results were expressed in μg β-carotene/g fresh matter.

Monomeric anthocyanins

Extraction was based on the method described by Li et al. (2014) with slight modifications. Dried powdered sample (0.5 g) was extracted with 20 ml of methanol, at 37 ^{o}C, for 30 min under agitation (IKA, RCT Model B, Staufen, Germany) at a frequency of 900 rpm. The methanol extracts were filtered and concentrated in a rotary evaporator (Stuart RE3022C, Staffordshire, United Kingdom), frozen and lyophilized for 2 days. The extract obtained was re-dissolved with methanol to a concentration of 50 mg extract/ml, covered with aluminium foil and preserved under freezing conditions until further analysis.
The total monomeric anthocyanin contents of fresh pansies and lettuce, as well as of the samples immerged in the osmotic solutions, were estimated by the pH differential method, following the methodologies used by Bchir et al. (2012) and (Rajasekar, Akoh, Martino, & MacLean, 2012). The method consisted of using two buffer systems: potassium chloride buffer at pH 1.0 (0.025 M) and sodium acetate at pH 4.5 (0.4 M). Extracts' portions were diluted on both buffers, and allowed to stand for 30 min at room temperature. Subsequently, the absorbance readings were made on a UV-Visible spectrophotometer (Thermo, Genesys 10 UV, Waltham, USA) at the wavelengths of 510 and 700 nm, and the absorbance difference (A) determined by equation 2:

$$A = (A_{510nm} - A_{700nm})_{pH1.0} - (A_{510nm} - A_{700nm})_{pH4.5} \quad (2)$$

The monomeric anthocyanin pigment concentration was expressed as cyanidin-3-glucoside and was determined by equation 3:

$$MAP = A \times MW \times DF \times \frac{1000}{\varepsilon} \quad (3)$$

where MAP= Monomeric anthocyanin pigment (mg Cy 3-glu/l) MW= molecular weight (449.2), DF = dilution factor and ε = Molar absorptivity (26,900). All measurements were performed in triplicate. The results were expressed in mg Cy 3-glu/g fresh matter.

2.5 Microscopic analysis of pansies petals

Thin sections of petals were cut into random transversal and longitudinal sections, mounted in glycerin (previously dehydrated in an upward series of ethanol) and observed on a light microscope equipped with a digital camera (Leica DFC49012, Heerbrugg, Switzerland). The structure of the petals' epidermis was analyzed at different points.

2.6 Statistical analysis

The SPSS Statistical software, v.18.0 (SPSS Inc., Chicago, IL), was used for the statistical treatment of the data. The normality of the data was verified by the Shapiro-Wilk test. Analysis of variance (ANOVA) or ANOVA Welch was carried out to determine if there were significant differences ($p < 0.05$) between samples, depending on the existence or not of homogeneity of variances. Additionally, if significant differences were detected between treatments, a *post hoc* analysis was performed, namely: the Tukey's honestly significant difference test, if variances in the different groups were identical, or Games-Howell test if they were not. The homogeneity of the variances was tested by Levene's test.

3 Results and discussion

3.1 Visual appearance and color

Figure 1 shows the visual appearance of fresh pansies and lettuce before and after treatments with different concentrations and immersion times in osmotic solutions (sucrose and sodium chloride). Flowers and lettuce subjected to the four treatments with sucrose (1h or 2h at 60 and 80%) and lower concentration of sodium chloride (15% for 15 or 30 min) showed similar appearance to fresh samples. However, when higher concentrations of sodium chloride (20 and 25%) or longer immersion times (1 and 2 h) were applied, structural damage was observed, as well as color changes. Furthermore, when pansies were removed from these higher concentrated osmotic salt solutions they shrank, making them more fragile. Lettuce leaves also became more brittle and showed a darkening in color.

The total color difference, ΔE^*, which is a combination of L^*, a^* and b^* values, is a colorimetric parameter used to characterize the variation of colors in food during different treatments. For pansies and lettuce, higher values of ΔE^* and standard deviations were observed in samples treated with sodium chloride (Fig. 2 and 3, respectively). Taking into account that a ΔE^* of up to 3 CIELab units indicates color differences appreciable to the human eye (Trivellini et al., 2014), both samples showed color differences detectable by consumers after treatments. Regarding pansies, a distinct behavior was observed in the two colored parts studied, white and violet. Generally, the white part showed lower values of ΔE^* than the violet one, without significant differences between treatments in the white part, with the exception of the treatment 15% NaCl for 1h. On the contrary, the violet part was more color sensitive, with the more significant alterations observed with the following treatments: 80% sucrose/2h, 15% NaCl/15 min, 15% NaCl/30 min, and 15% NaCl/2h. Lettuce, probably due to its increased color homogeneity, showed constant values of ΔE^*, without significant differences detected between treatments, except for 60% sucrose/2h.

3.2 Weight and a_w variation

Immersion time and sucrose/sodium chloride concentration effects on weight and a_w variation in pansies and lettuce are also given in Fig. 2 and 3, respectively.

Pansies' weight (Fig. 2) showed significant increases in both osmotic solutions among treatments. It could be expected that, after immersion treatment, pansies should lose weight due to diffusion of water, even though accompanied by simultaneous counter diffusion of solutes from the osmotic solution into the tissue. Furthermore, it is desired to have a high water loss for preservation purposes and a low solids gain, which will lead to high weight variations. These exchanges of solute and water probably did not happen because pansies' petals have a structure named papillae (conical epidermal cells) in the

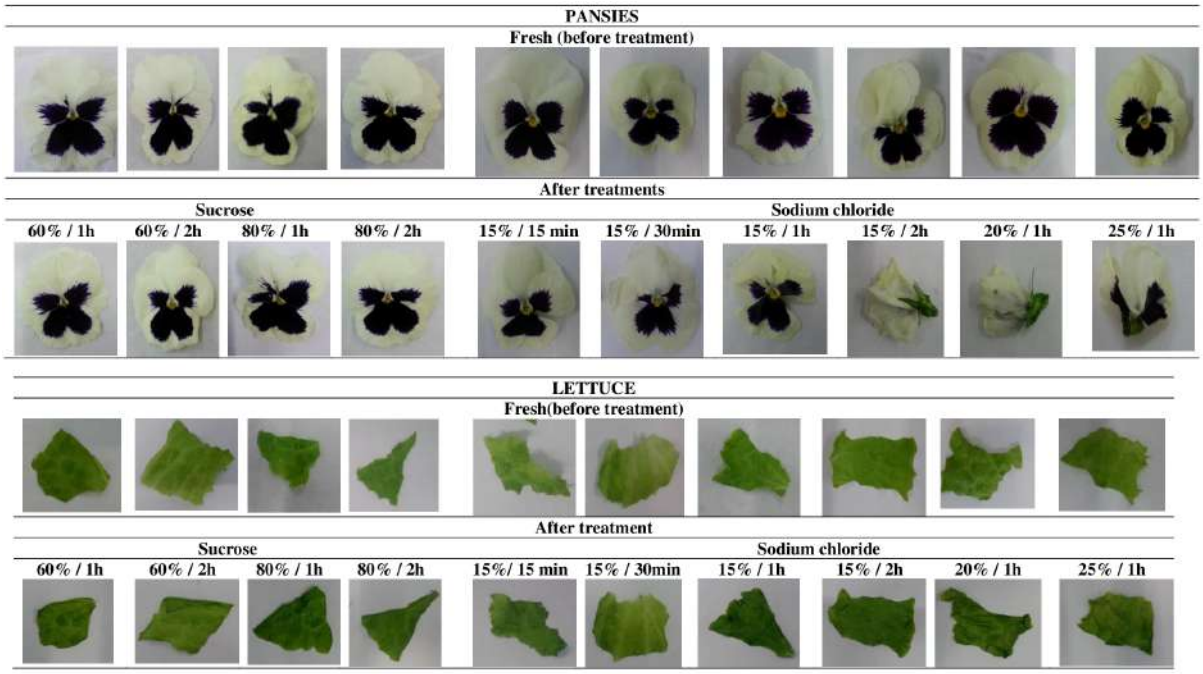

Figure 1: Visual appearance of pansies (A) and lettuce (B) before and after immersion in osmotic solutions with sucrose and sodium chloride

epidermis on both sides of all the petals (spurred, lateral and upper) (Weryszko-Chmielewska & Sulborska, 2012) and this structure has been reported to be superhydrophobic (Schulte, Droste, Koch, & Barthlott, 2011). In the present work, we observed pansies' petals by microscopy (Fig. 4). The papillae were very visible, explaining the obtained results. In some situations, the increased weight observed in pansies could be due to some sugar or salt dried on the outside of the flowers. However, when salt was used in different concentrations (15, 20, 25%) during 1 hour, pansies lost weight because probably some cell rupture occurred, contributing to the bad visual appearance (Fig. 1). Regarding lettuce, all treatments induced weight loss, giving rise to effective osmotic dehydration. Comparing different concentrations of sucrose (60% and 80%) at 1 h of immersion, 80% of sucrose was less effective than at 60%. This is in line with Ponting, Watters, Forrey, Jackson, and Stanley (1966), who stated that above 60% sugar concentration, an additional increase in sugar concentration did not promote further water loss in vegetables. However, a decreased weight can be observed in lettuce by increasing the immersion time (1 and 2 hours) for both sucrose concentrations. Regarding salt treatments, lettuce showed a higher decrease in weight with higher times of immersion (more than 30 min). However, it was observed that the effect was not as evident as when the concentrations of the osmotic solution increased. Based on these observations, it seems that pansies' structure does not allow the exchange of solutes with osmotic solutions, and therefore is not adequate for osmotic drying.

Low water activity reduces the growth of microorganisms and decreases biochemical reactions, which is important for the preservation of food. Regarding the water activity (a_w) values in pansies, the highest values were obtained in fresh samples. A decrease in a_w values was observed after all treatments, except with 15% NaCl for 30 min. However, when using sucrose no significant differences were detected between treatments. With NaCl, higher contact times (ex: 1

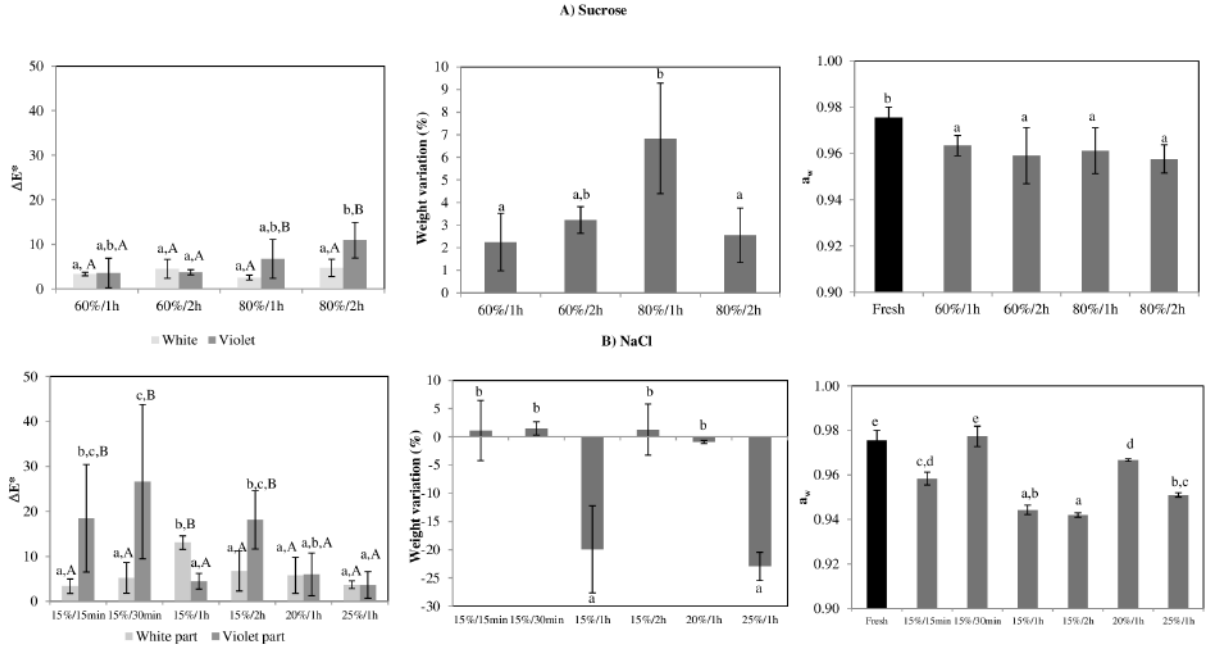

Figure 2: a_w, weight (%) and ΔE^* variation before and after pansies treatments with sucrose (A) and sodium chloride (B) solutions (lower letters compare treatments while caps compare different parts of pansies (white and violet). Values with the same letter are not statistically different (p>0.05))

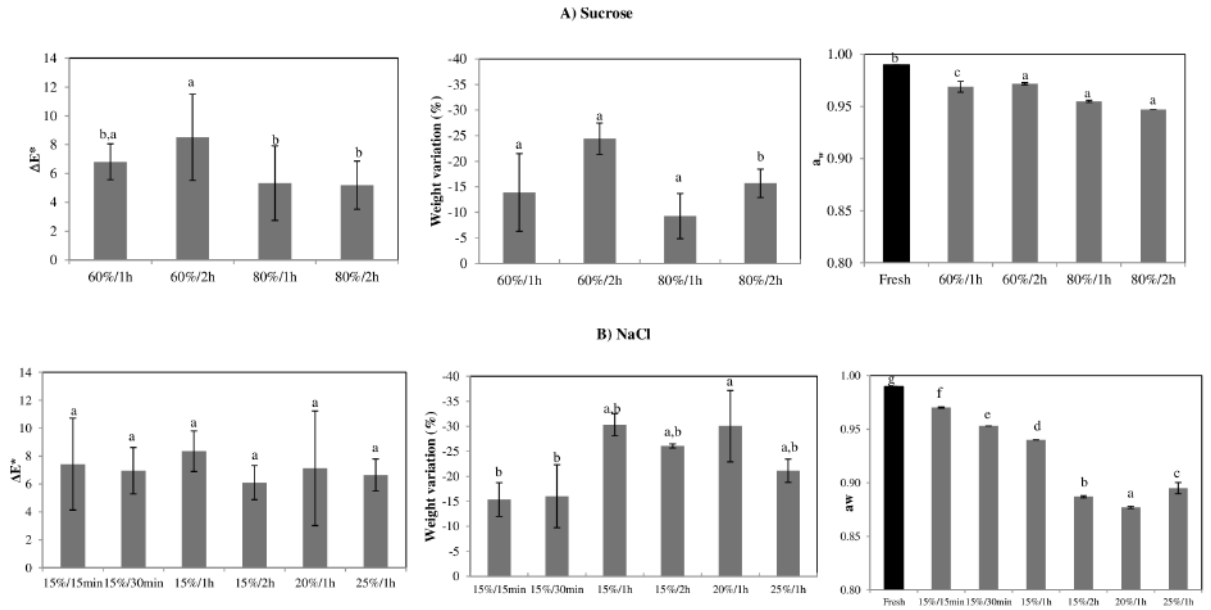

Figure 3: a_w, weight variation and ΔE^* contents before and after lettuce treated with sucrose (A) and sodium chloride (B) solutions

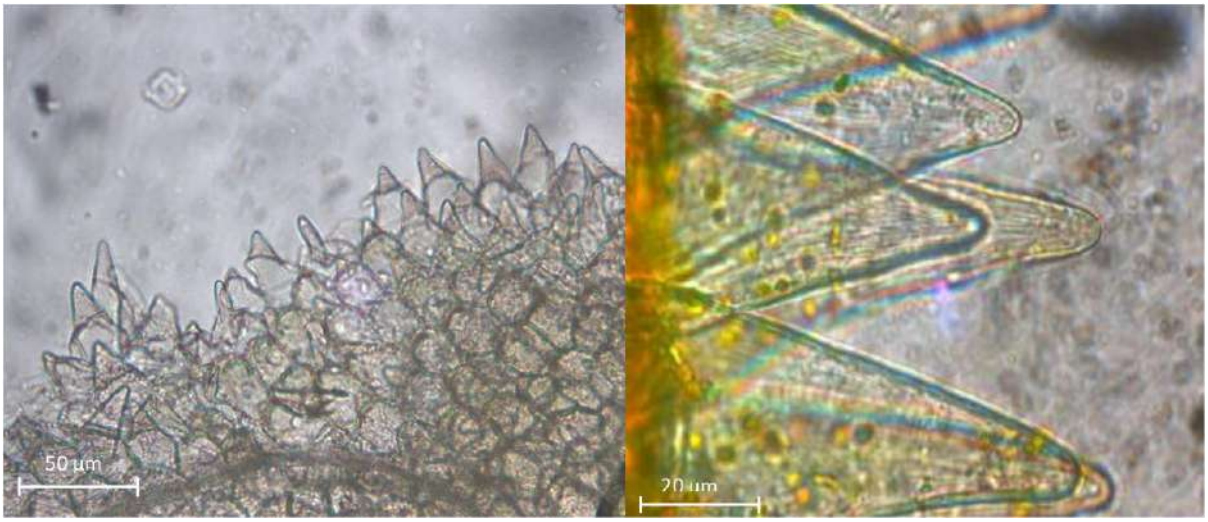

Figure 4: Conical papillae on the surface of *Viola×wittrociana*

and 2 h at 15%, w/v) or higher salt concentrations (20 and 25%; w/v) caused an effective decrease in the a_w values. As expected for lettuce subjected to different osmotic solutions, a_w values also decreased compared to the fresh sample. In general, increased immersion times and concentrations induced a decrease in a_w, with a linear decreasing effect. Furthermore, this decrease was more pronounced when using sodium chloride as an osmotic solution.

3.3 Carotenoids

Fig. 5 presents the carotenoids content of pansies (A) and lettuce (B) before and after immersion in osmotic solution. The highest concentrations were observed in the fresh sample for pansies and lettuce (75.7 and 72.8 μg β-carotene /g fresh matter, respectively). In general, a decrease of carotenoids content was observed with the osmotic treatments. This decrease might be due to partial leaching of these pigments as the osmotic stress increased, due to the breakage of the cell structures (Tadesse, Abera, & Worku, 2015), or the susceptibility of carotenoids to oxidation, isomerisation and other chemical changes during processing because of their extensive conjugated double-bond systems (Shi & Le Maguer, 2000). Samples immersed in sucrose showed lower contents of carotenoids than in sodium chloride. The effect of different concentrations of osmotic solutions and immersion time on carotenoids content of lettuce and pansies were significant ($p < 0.05$). For pansies, the best conditions to retain the highest amount of carotenoids was 15% sodium chloride with 30 min of immersion, and for lettuce was 15% of sodium chloride for 15 min. For pansies, when using sucrose, no significant differences were obtained between treatments.

3.4 Monomeric anthocyanins

The total monomeric anthocyanins content in fresh pansies and those immersed in osmotic solutions are shown in Fig. 6A (sucrose) and 6B (NaCl). Lettuce anthocyanins, if present, were below the detection limit.

Anthocyanins are the main compounds responsible for the wide range of colors in pansies. Some authors report that the anthocyanins present in the *Viola* species are formed from malvidin, peonidin and petunidin anthocyanidins (Gamsjaeger et al., 2011; Skowyra, Calvo, Gallego, & Azman, 2014; Zhang et al., 2012). From all the treatments, only immersion in 60% sucrose during 1h induced a significant increase of to-

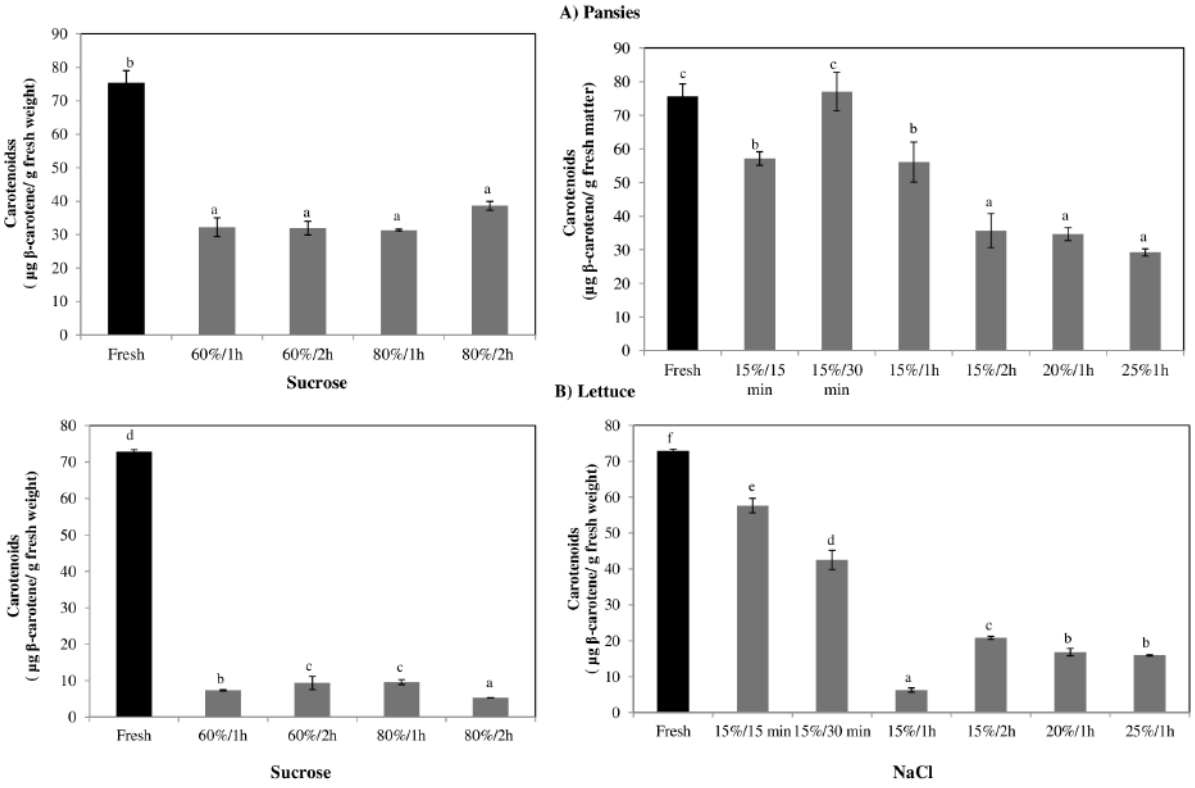

Figure 5: Carotenoids content (μg β-carotene/g fresh matter) before and after pansies (A) lettuce (B) treatments with sucrose solutions and sodium chloride

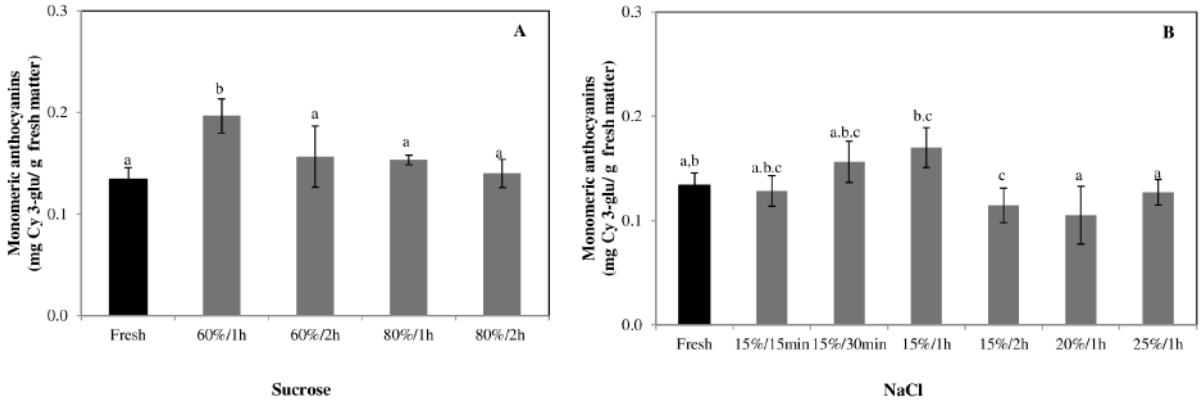

Figure 6: Monomeric anthocyanins content (mg Cy 3-G/g fresh matter) before and after pansies treatments with sucrose and sodium chloride solutions

tal monomeric anthocyanins compared with fresh samples. A direct interpretation of this result is not as expected because anthocyanins are soluble in water and they exist in epidermal and subepidermal cells, dissolve in vacuoles or accumulate in vesicles called anthocyanoplasts (Karami, Yousefi, & Emam-Djomeh, 2013). Therefore, they could leak into the osmotic medium through the cuticle and skin ruptures (Karami et al., 2013). Within the other sucrose treatments, no significant differences were observed between assays, and no indications of degradation or leaching of these compounds existed. Furthermore, the sugar concentrations applied in the present work were not sufficient to increase the pH of the solution, which may raise the percentage of anthocyanins in the colourless carbinol base form that is very unstable, making the pigment more susceptible to degradation by oxygen (Karami et al., 2013; Stojanovic & Silva, 2007).
For NaCl, only the treatment with 15% and 2h of immersion caused a decrease of the monomeric anthocyanins content when compared to the fresh state. This result might be due to the increase in the contact time of pansies in the sodium chloride solution leading to an increase in osmotic pressure and enhanced water loss. High water loss resulted in a higher loss of anthocyanins because they are water-soluble pigments.

4 Conclusions

In summary, pansies present a superhydrophobic structure that increases the resistance to osmotic dehydration with both sucrose and sodium chloride solutions. In general, when pansies were submitted to osmotic solutions, an almost general weight gain, a_w maintenance and high monomeric anthocyanins contents were observed. However, high concentrations of sodium chloride induced damage in pansies' structure and color alterations. Compared to pansies, all treatments applied to lettuce caused effective osmotic dehydration, with weight loss and reduced a_w, as expected when performing the osmotic dehydration of vegetables. So, immersion in osmotic solutions cannot be applied to pansies due to their morphological structure, namely the presence of papillae (conical epidermal cells) that are extremely hydrophobic.

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Detection of Long Storage and Sunflower Adulteration of Olive Oils Using an Ultra-violet (UV) Spectroscopy Method

M. Al-Bachir[a*] and Y. Othman[a]

[a] RadiationTechnologyDep. AtomicEnergyCommission of Syria, P.O.Box: 6091, Damascus, Syria
[*]Corresponding author
ascientific@aec.org.sy
Tel: 009-63-11-2132580

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Abstract

The purpose of the study was to evaluate the effects of Syrian Kaissy olive oil (SKOO) adulteration and long storage periods on the basic quality indices namely, peroxide value (PV), acid value (AV), and absorbance of UV (K_{232}, K_{270}, ΔK and R (K_{232}/K_{270}) values).
This study revealed that the quality indexes, including AV, PV, K_{232}, K_{270} and ΔK values, of all SKOO specimens increased with increasing storage time. It was revealed that the K_{232}, K_{270} and ΔK values of SKOO specimens stored for 1 and 2 years remained under the limits, established by International Olive Council (IOC), of 2.5, 0.22 and 0.01 units, respectively. While in some cases, the K_{232}, K_{270} and ΔK values exceeded their respective limits after 8, 9 and 10 years of storage. The index of R (K_{232}/K_{270}) decreased with time of storage.

Keywords: Adulteration; Olive oil; Oxidation; Storage period; Syria; UV absorbance

1 Introduction

Olive oil has been regarded as "liquid gold" from antiquity. Among the several types of edible oils, extra virgin olive oil (EVOO) is considered as the best organoleptically, which is obtained only by mechanical or physical methods, without involving any thermal alteration or treatment. It is an important oil that has a high nutrition value (Gonzalez, Aparicio-Ruiz, & Aparicio, 2008). Olive oil contains unsaturated fats in the form of poly unsaturated fatty acids and mono unsaturated fatty acids which are good for health (Al-Bachir & Sahloul, 2017; Al-Bachir & Koudsi, 2016).
Due to its higher price, virgin olive oil is often adulterated with cheaper vegetable oils (Nikolova, Eftimov, Antova, & Brabant, 2013). There is a need to develop reliable analytical methods to ensure compliance of olive oil quality with labeling, and to determine the genuineness of the product by detection of eventual defects during adulteration, processing and storage (Abbadi et al., 2014).
In the literature, different methods used in the detection of olive oil adulteration are reported (Frankel, 2010). They include the iodine value, saponification value, oxidation value as well as refractive index, density, viscosity, volatile compounds, and olive oil antioxidants measurements (Amereih, Barghouthi, & Marowan, 2014; Fernandes, Umebara, Lenzi, & Teixeira Silva, 2013). To determine the type of adulterant, chemical methods such as gas chromatography or high performance liquid chromatography are used (Inthiram, Mirhosseini, Tan, Mohamad, & Lai, 2015). Ultraviolet (UV) spectroscopy can provide information on the degree of oxidation of the olive oil (Nikolova, Eftimov, Perifanova, & Brabant, 2012). More specifically, the quality of olive oil is

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analyzed based on the absorption band between 200 and 300 nm, which can be used to evaluate the presence of refined oils in virgin olive oil (Aued-Pimentel, Alves da Silva, Takemoto, & Cano, 2013).
Current European Union regulation (European Communities, 2013) and the International Olive Council International Olive Council (2015) require olive oils to be graded based on sensory assessment and three fundamental chemical parameters: free acidity, peroxide value and UV absorbance. By comparing oils' scores with threshold values, these are categorised as extra virgin olive oil (EVOO), virgin olive oil (VOO) and other low-quality ordinary olive oil (OOO).
This study was conducted to determine if storage period could induce measurement changes in the Ultra-violet UV spectrophotometric absorbance values at 232 nm and 270 nm (K232, K270, ΔK and R (K232/K270) values) of Syrian Kaissy olive oil (SKOO). Samples were stored at room temperature conditions and the results obtained under different storage periods (1, 2, 8, 9 and 10 years) were compared.

2 Materials and Methods

2.1 Chemical and reagents

Olive oil, from olives harvested in the 2007, 2008, 2009, 2015 and 2016 seasons, was obtained from olive fruits of Kaissy cultivar grown in orchards located at the Deer Al Hajar research station, southeast Damascus, Syria (33^o 21´ N, 36^o 28´ E) at 617 m above sea level, under conventional agriculture practices. The oil was extracted at the shortest time possible using mechanical and physical processes (Blatchly, Delen, & O'Hara, 2014). Olive fruits were crushed with a hammer crusher and slowly mixed for about 30 min at 27 ^{o}C. Then, the mixed paste was centrifuged at 3000 rpm for 3 min without addition of water to extract the oil. Finally, the oils were decanted and immediately transferred into dark glass bottles and stored at room temperature. Olive oils were extracted and stored at our lab. Chemical and physical analyses were performed on SKOO samples in 2017 (after 1, 2, 8, 9 and 10 years of storage).

Adulteration was carried out in triplicate in 2017 in our lab by mixing olive oil samples produced in 2016 with sunflower oil (SO), purchased from a local supermarket in 2017, at different blending ratios (0:100, 2:98. 4:96, 6:98, 8:92, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0 (v/v)). Physical analyses were performed on SKOO, SO and mixed oil samples in 2017 (after 1 year of storage).

2.2 Chemical and physical analysis of oils

Acid value (AV, oleic acid %) and peroxide value (PV, meq O_2 kg^{-1}) were determined according to standard methods (Association of Official Analytical Chemists, 2010). Absorbance of UV was used to detect abnormal oxidation compounds in olive, sunflower and mixed oil samples according to norms established by IOC (Ait Taleb, Boutoial, Kzaiber, & Oussama, 2016). K_{232} and K_{270} were calculated from absorption at 232 and 270 nm, respectively. ΔK was calculated from 266, 270 and 274 nm absorptions using the following equation:

$$\Delta K = K_{270} - (K_{266} - K_{274}) \cdot \frac{1}{2} \qquad (1)$$

The R value was calculated as K_{232} divided by K_{270}. A T70 UV-Visible spectrophotometer (PG Instruments Limited, England) was used with a 1% solution of oil in cyclohexane and a path length of 1 cm. 0.25 g of oil was dissolved in 25 mL of cyclohexane and the absorbance of the solution was then measured at the specified wavelengths with reference to pure solvent. Specific extinction is calculated from the spectrophotometer reading (UVWin5.0 program) using a quartz cell with 1 cm optical path.

2.3 Statistical analysis

The five storage periods and 15 levels of adulterations were distributed in a completely randomized design, with three replicates. Data were subjected to an analysis of variance test (ANOVA) using the SUPERANOVA computer package (Abacus Concepts Inc., Berkeley, CA,

USA; 1998). The P value of less than 0.05 was considered for statistical significance.

3 Results and Discussions

3.1 Basic quality parameters of investigated virgin olive oils

Acid value (AV): Table 1 shows the averaged values obtained for AV indices of SKOO and their respective standard deviations during the whole storage time (1, 2, 8, 9 and 10 years). It is worth mentioning that during storage at room temperature, the AV of stored SKOO increased with increasing time of storage. Data presented in Table 1 shows that the AV of stored SKOO for one year (2.71%), 2 years (2.95%), and 8 years (2.17%) were below the maximum levels. The AV of SKOO stored for 9 years (4.16%) and 10 years (4.39%) exceeded the maximum levels established by the international regulations, which are the maximum levels established by the IOC for an oil to be considered as an ordinary virgin olive oil. According to the standard of the International Olive Council International Olive Council (2015), there should be a maximum of 1.0, 2.0 and 3.3% acidity in extra virgin olive oil (EVOO), virgin olive oil (VOO) and ordinary olive oils (OOO), respectively. The free fatty acids, measured as acidity (% oleic acid (C18:1)), is a very important quality and classification index for olive oils. It is a measurement of the hydrolytic breakdown of fatty acid chains from triglycerides into diglycerides and monoglycerides, liberating free fatty acids (Tanoitu, Elamrani, Serghini-Caid, & Tahani, 2011).
Peroxide value (PV): According to the IOC standard, the maximum allowable PV for EVOO, VOO and OOO is 20 meq O_2 kg^{-1} oil (International Olive Council, 2015). It is worth mentioning that PV in SKOO samples stored for 1 year were less than the value of 20 meq O_2 kg^{-1} (16.68 meq O_2 kg^{-1}), while the PV in SKOO samples stored for 2 and 8 years were around the value of 20 meq O_2 kg^{-1} (20.78 and 20.74 meq O_2 kg^{-1}, respectively) and the PV in SKOO samples stored for 9 and 10 years exceeded the value of 20 meq O_2 kg^{-1} (26.16 and 27.58 meq O_2 kg^{-1}, respectively) (Table 1). During storage, an increase in PV was observed as a consequence of the action of both diluted and headspace oxygen in the bottle that induce a rapid deterioration. Then the PV was progressively reduced because of the degradation into secondary products. This could be explained as the evolution of autoxidation which depends on the light, temperature and vacuum activity (Vekiari, Papadopoullou, & Kiritsakis, 2007). These results are in line with those reported by Papuc, Nicorescu, and Durdun (2009) where PV is taken as a measure of primary oxidation compounds produced in the oxidation of oil samples. Oxidation of oil generates a multitude of compounds, such as acids with short chain molecules formed by the oxidation of aldehydes. The quality of olive oil reduced during storage due to oxidation that leads to rancidity (Vacca, Del Caro, Poiana, & Piga, 2006). Variation during olive oil storage and transportation leads to lipid oxidation, which is due to natural or climatic conditions (Silva, Rodrigues Anjos, Cavalcanti, & dos Santos Celeghini, 2015). Similar results have been reported for extra-virgin olive oil which was stored for 6 years, where free acidity exceeded the limit recommended for EVOO by IOC, whereas, the stability against oxidation persisted for a longer period of time due to the high concentration of oleuropein derivatives (Baiano, Terracone, Viggiani, & Alessandro Del Nobile, 2014).
Lipids containing polyunsaturated fatty acids and their esters are exposed to autoxidation by molecular oxygen. Autoxidation of lipids has been recognized as a major deterioration process affecting the quality properties of foods, which limits shelf-life (Ait Taleb et al., 2016). The importance of virgin olive oil is related to its high levels of monounsaturated fatty acids (mainly oleic acid (C18:1)), and the low levels of polyunsaturated fatty acids (linoleic acid (C18:2) and linolenic acid (C18:3)) (Al-Bachir & Sahloul, 2017; Al-Bachir & Koudsi, 2016).

3.2 Influence of storage period on UV spectrophotometric absorbance of SKOO

The mean ± standard deviation values of K_{232}, K_{270}, ΔK and R (K_{232}/K_{270}) of SKOO samples

Table 1: Effect of production year on acid value and peroxide value of Syrian Kaissy olive oil

Storage period (years)	Production year	Acid value (oleic acid %)	Peroxide value (meq O_2kg^{-1} oil)
1	2016	2.71±0.01	16.68±1.30
2	2015	2.95±0.09	20.78±0.54
8	2009	2.17±0.29	20.74±2.15
9	2008	4.16±0.87	26.16±1.01
10	2007	4.39±2.64	27.58±7.05
P-Value		**0.0070**	**0.0004**

Table 2: Specific extinction coefficient UV at 232, 266, 268, 270 and 274 nm of Syrian Kaissy olive oil

Storage period (years)	Production year	K_{232}	K_{270}	ΔK	R (K_{232}/K_{270})
1	2016	2.161±0.002	0.174±0.001	0.002±0.000	12.41±0.062
2	2015	2.128±0.001	0.147±0.000	0.002±0.000	14.48±0.010
8	2009	2.290±0.280	0.352±0.120	0.010±0.000	6.51±1.610
9	2008	2.367±0.030	0.469±0.004	0.020±0.000	5.05±0.036
10	2007	2.574±0.030	0.597±0.150	0.030±0.000	4.31±0.060
P-Value		*	*	*	*

*: Significant at $p<0.05$

tested and their variation with time of storage are given in Table 2. The maximum values for K_{232}, K_{270} and ΔK are, respectively, 2.5, 0.22 and 0.01 for extra virgin olive oils, 2.6, 0.25 and 0.01 for virgin olive oils and 2.6, 0.30 and 0.01 for ordinary virgin olive oils (International Olive Council, 2015). After 1 and 2 years of storage, SKOO samples presented K_{232}, K_{270} and ΔK values less than the maximum limits established for the extra virgin category. However, a sample of SKOO presented a K_{232} value of 2.574 after 10 years of storage, K_{270} value of 0.352 after 8 years of storage and ΔK value of 0.02 after 9 years of storage, and these values are higher than the maximum limit established for the EVOO category. Finally, the R value (K_{232}/K_{270}) of SKOO decreased with time of storage (Table 2). R values were 12.41, 14.48, 6.51, 5.05 and 4.31 for SKOO samples stored for 1, 2, 8, 9 and 10 years respectively. Similar results for the effect of storage time on the quality parameters of olive oil have been reported by several authors. Abbadi et al. (2014) reported that the extinction coefficients, K_{232} and K_{270}, of olive oil stored at elevated temperature increased continuously and significantly with extending time of storage. In recent years, several authors have reported results for different olive oil varieties under storage conditions lasting 12-18 months. Low temperatures preserve the properties of a fresh olive oil better than room temperatures (Brkić Bubola, Koprivnjak, Sladonja, & Belobrajić, 2014; Mulinacci et al., 2013). The extinction coefficients, K_{232} and K_{270}, of virgin olive oil depended on the year of production (2001, 2002 and 2003) (Ninfali et al., 2008). Determination of the absorption coefficients in the ultra-violet region (232 nm and 270 nm) reflects the stage of oxidation for olive oil during storage (Kiritsakis, Kanavouras, & Kiritsakis, 2002). The K_{232} value reportedly is correlated with peroxide value, not only at time zero but also during storage. Higher readings of K_{232} in these samples may indicate improper storage of the oils (Ogutcu, Mendes, & Yilmaz, 2008).

K_{232}, K_{270} and ΔK parameters indicated a substantial increase of primary oxidation, measured by the peroxide value (formation of hydroperoxides) and K_{232} (formation of conjugated dienes), and of secondary oxidation, as shown by K_{270} and ΔK (formation on conjugated trienes) starting from the seventh year of storage (Baiano et al., 2014). Under room temperature storage, the increase in oxidation, confirmed by the increase in extinction coefficients, occurred in both due to the increase in the number of compounds resulting from peroxide degradation (Vekiari et al., 2007). Silva et al. (2015) reported that K_{232} and K_{270} values varied in relation to storage time.
Because of the significant variation of K_{232} and K_{270} values during olive oil storage, in response to oil oxidation, these parameters may be of major quality control importance for stored extra virgin olive oil in terms of determining the time at which they will lose their extra category (Abbadi et al., 2014).

3.3 Influence of storage period and adulteration ratios on UV spectrophotometric absorbance of SKOO

Figure 1 shows the changes in the K_{232} specific extinction coefficient at different storage times and adulteration ratios. It was found that both storage time and adulteration ratio significantly affected the K_{232} specific extinction coefficient ($P<0.05$). The initial K_{232} specific extinction coefficient value was less than 2.5, the maximum value established by the national and international regulations for EVOO (International Olive Council, 2015). An increase in storage time and adulteration ratio increased the K_{232} specific extinction coefficient significantly ($P<0.05$), and reached 2.574 after 10 years of storage for SKOO and 4.902 in a mixture of SKOO and SO at a ratio of 9:1 (SO:SKOO). This indicates that adulteration of SKOO with SO has a greater effect on the formation of conjugated dienes in SKOO than the long storage time.
The changes in K_{270} specific extinction coefficient at different storage time and adulteration ratio are illustrated in Figure 2. The results of analysis of variance (ANOVA) indicate that both the storage time and adulteration ratio significantly affected the K_{270} specific extinction coefficient ($P<0.05$). The initial K_{270} specific extinction coefficient value was less than 0.22, the maximum value established by the national and international regulations for EVOO (International Olive Council, 2015). An increase in storage time and adulteration ratio increased the K_{270} specific extinction coefficient significantly ($P<0.05$), and reached 0.597 after 10 years of storage for SKOO and 2.761 in a mixture of SKOO and SO at a ratio of 9:1 (SO:SKOO). This indicates that adulteration of SKOO with SO has a greater effect on formation of conjugated trienes in SKOO than the long storage time.
Figure 3 shows the changes in ΔK value of SKOO at different storage time and adulteration ratio. It was found that both the storage time and adulteration ratio significantly affected the ΔK value ($P<0.05$). The initial ΔK value was less than the 0.01, the maximum value established by the national and international regulations for EVOO (International Olive Council, 2015). An increase in storage time and adulteration ratio increased the ΔK value significantly ($P<0.05$), and reached 0.03 after 10 years of storage for SKOO and 0.37 in a mixture of SKOO and SO at a ratio of 9:1 (SO:SKOO). This indicates that adulteration of SKOO with SO has a greater effect on the ΔK value in SKOO than the long storage time.
The changes in R value (K_{232}/K_{270}) at different storage time and adulteration ratio are illustrated in Figure 4. The results of analysis of variance (ANOVA) indicate that both storage time and adulteration ratio significantly affected the R value ($P<0.05$). The initial R value of SKOO was around 12.42. An increase in storage time and adulteration ratio decreased the R value significantly ($P<0.05$), and reached 4.31 after 10 years of storage for SKOO and 1.78 in a mixture of SKOO and SO at a ratio of 9:1 (SO:SKOO). This indicates that adulteration of SKOO with SO has a greater effect on R value in SKOO than the long storage time.
UV spectrophotometric measurements are widely used in both olive oil authentication and quality assessments (Escudero, Ramos, La Rubia García, & Pacheco, 2016). European Commission Regulation No 2568/91 defines

specific extinction coefficients at 232 nm and 270 nm as the parameters related to the oxidation state of olive oil (Jenisová, Braniša, Jomová, & Porubská, 2014). Absorbance at specific wavelengths in UV is a more delicate indicator of oxidation, and is related to the presence of a conjugate diene and triene system. Ultraviolet absorbance at 232 and 270 nm, respectively are conventionally indicated by K_{232} (an indication of conjugated polyunsaturated fatty acids in olive oil), and K_{270} (an indication of carbonylic compounds: aldehydes and ketones) (Amereih et al., 2014; Noorali, Barzegar, & Sahari, 2014). When linoleic acid is oxidized to form hydroperoxides, a shift of the double bonds occurs producing a conjugated diene that can be measured by UV absorbance at 232 nm. The increase of absorptivity at 232 nm was progressive for all oils, especially for oils rich in linoleic acid (sunflower, maize germs and soy oil) (Papuc et al., 2009). The oxidation stability of oil is a function of the fatty acid composition, and decreases with higher contents of linoleic and linolenic acids (Souza, De Almeida, Batista, De, & Rios, 2011). However, SKOO contains more oleic acid and less linoleic and linolenic acids than other vegetable and seed oils. Oleic acid is a monounsaturated fatty acid, whereas linoleic and linolenic acids are polyunsaturated fatty acids. The main fatty acids in SKOO are: palmitic acid (C16:0) (13.11-15.89%); stearic (C18:0) (2.19-3.83%); oleic acid (C18:1) (68.94%), (68.15-72.64%); linoleic acid (C18:2) (7.65-12.51%); Linolenic acid (C18:3) (0.41-1.09%.) (Al-Bachir & Sahloul, 2017; Al-Bachir & Koudsi, 2016).

4 Conclusion

This study evaluated the changes in oxidative stability of SKOO during long storage time and adulteration ratios with SO. The results of analysis of variance (ANOVA) indicated that both storage time and adulteration ratio significantly affected the K_{232}, K_{270}, ΔK and R (K_{232}/K_{270}) values ($P<0.05$). The adulteration of SKOO with sunflower oil had a greater effect on K_{232}, K_{270}, ΔK and R (K_{232}/K_{270}) values in SKOO than a long storage time. The

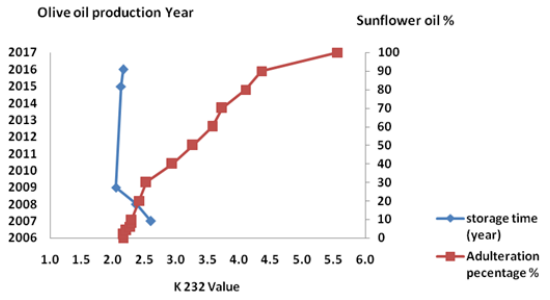

Figure 1: Effect of production year and adulteration percentage on the increase in K_{232} value of Syrian Kaissy olive oil

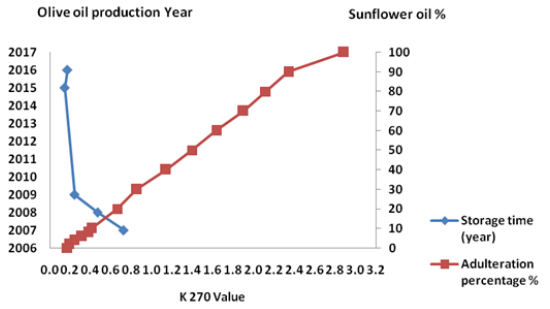

Figure 2: Effect of production year and adulteration percentage on the increase in K_{270} value of Syrian Kaissy olive oil

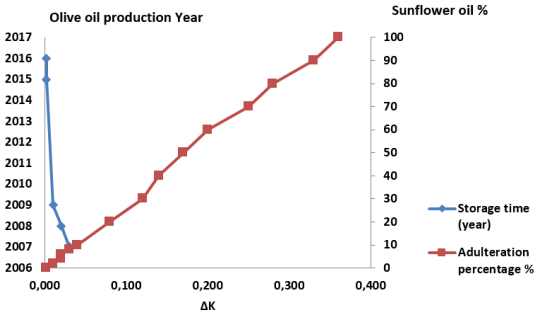

Figure 3: Effect of production year and adulteration percentage on the increase in delta-K value of Syrian Kaissy olive oil

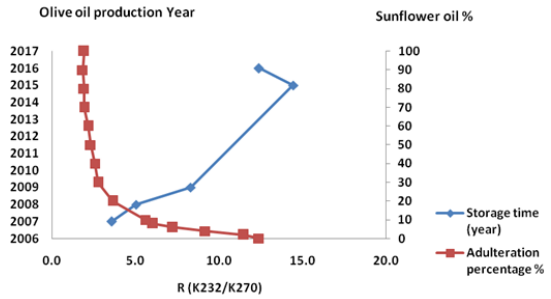

Figure 4: Effect of production year and adulteration percentage on the increase in R value (K_{232}/K_{270}) of Syrian Kaissy olive oil

K_{232} specific extinction coefficient of SKOO exceeded the maximum allowable limit of national and international regulations after 10 years of storage (2.574) and above an adulterant ratio of 1:2 (SO:SKOO)(2.526). The K_{270} specific extinction coefficient of SKOO exceeded the maximum allowable limit after 8 years of storage (0.352) and above an adulterant ratio of 1:24 (SO:SKOO)(0.24). The ΔK value of SKOO exceeded the maximum allowable limit after 9 years of storage (0.02) and above an adulterant ratio of 1:24 (SO:SKOO)(0.02).

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Antidiabetic Activity of Herbal Green Tea Extract from White Mangrove (*Avicennia marina*) Leaves towards Blood Glucose Level of Diabetic Wistar Rats (*Rattus novergicus*)

Hardoko[a, b*], BintiNafi'ah[a], Bambang B. Sasmito[a], and YuniwatyHalim[b]

[a] Faculty of Fisheries and Marine Sciences, Universitas Brawijaya Jalan Veteran No. 1 Malang 65113, Indonesia
[b] Food Technology Department, Universitas Pelita Harapan Jalan M.H. Thamrin Boulevard, Lippo Karawaci, Tangerang 15811, Indonesia
[*]Corresponding author
hardoko@ub.ac.id
Tel: +62-0341-553512
Fax: +62-0341-557837

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Abstract

Green tea *Camellia sinensis* contains polyphenol that has antidiabetic activity. Mangrove leaves also contain polyphenol which potentially gives these leave antidiabetic activity. The aim of this research was to determine the ability of herbal green tea extract from white mangrove (*Avicennia marina*) leaves to decrease blood glucose level of diabetic Wistar rats (*Rattus novergicus*). The method used was experimental and involved giving a herbal green tea extract from white mangrove leaves with concentration of 100, 200 and 300 mg/200g BW/day, and positive control, i.e. glybenclamide (0.09 mg/200 g BW/day), to diabetic rats injected with Streptozotocin (STZ) and Nicotinamide (NA). The rats were observed on day 0, 5, 10 and 15. The results showed that the herbal green tea extract from white mangrove leaves decreased the blood glucose level of diabetic rats. The effective extract dose that decreased the blood glucose level of diabetic rats was 300 mg/200 g BW, which is comparable to the effect produced by glybenclamide (antidiabetic medicine). This dose could decrease the blood glucose level of diabetic rats to reach a normal blood glucose level after 20 days.

Keywords: Diabetic; Extract; Blood glucose; Herbal mangrove green tea

1 Introduction

Diabetes mellitus is an endocrine condition that is caused by the decrease of insulin effectivity, i.e. a hormone that plays a role in the carbohydrate metabolism. Insufficient insulin secretion causes blood glucose level to exceed the normal acceptable physiological limit. Chronic high blood glucose levels causes nerve, blood vessel and coronary artery damage. In addition, this condition increases coronary disease risk, stroke, kidney failure and other disease complications (Fadilah, 2017).

Diabetic treatment generally involves dietary management and the use of synthetic chemical drugs or natural traditional medicine. The treatment using chemical drugs is considered less safe because of its unwanted side effects. Treatment using traditional medicine is considered safer because it has less side effects; therefore many diabetic patients are interested in it. One of the natural ingredients that can be used as a natural medicine is tea from *C. sinensis* leaves. Green tea is used to decrease obesity (Purwanto, Darmawati, Purwaningsih, & Ners, 2014; Rahmanisa & Wulandari, 2016; Sari, 2015), dia-

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Nomenclature

STZ	Streptozotocin	BW	Body Weight
NA	Nicotinamide	GOD/PAP/POD	Enzymatic colorimetric method

betes (Bait, 2010; Cheng, Shen, & Wu, 2009; Efendi, Damayanthi, Kustiyah, & Kusumorini, 2010; Holidah, Yasmin, & Christianty, 2018; Ilma, 2016; Rohdiana, Firmansyah, Setiawati, & Yunita, 2012; Wibowo, Kusmana, Suryani, Hartati, & Oktadiyani, 2009; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012); black tea to treat diabetes (Bait, 2010; Deswati & Nur Maryam, 2017; Holidah et al., 2018; Rosalia, Indrasari, Tangsilan, Jayadi, & Danu, 2017); white tea to treat diabetes (Trinoviani, Kholisoh, Ar-Rifa, & Rustamsyah, 2016) and kombucha green tea is used as a hepato protective agent. The role of tea in health is related to the presence of beneficial compounds such as polyphenol, theophylline, flavonoids, tannins, Vitamin C and E, catechins and some minerals (Majid & Nurkholis, 2010).

Considering the reported health benefits of tea people started brewing tea from leaves other than *C. sinensis* leaves, which is known as herbal tea. Some examples are, herbal tea from stevia leaves to treat diabetes (Trinoviani et al., 2016), mulberry leaves tea (Bait, 2010; Efendi et al., 2010), guava leaves herbal tea for diabetes (Cheng et al., 2009), stevia leaves tea for antioxidant (Ariviani & Ishartani, 2009), avocado leaves' tea for antioxidant, soursop leaves' black tea for uric acid treatment (Hardoko, Puspitasari, & Amalia, 2015), soursop leaves' extract to treat diabetes (Moniaga, 2014), soursop leaves' green tea to treat diabetes (Hardoko, 2015), fragrant pandan (*Pandanus amaryllifolius* Linn) leaves' water extract to treat diabetes (Prameswari & Widjanarko, 2014). The roles of plants or products from plants as natural medicines are related to their bioactive compounds, which vary with the type of the plants.

Mangrove plant also has potential to be a natural medicine because it contains compounds, such as tannins, polyphenols, flavonoids, etc that in other plants and have been known as bioactive compounds. Other than their antioxidant capacity, compounds from the polyphenol group also play a role in improving glucose metabolism (Scalbert, Manach, Morand, Rémésy, & Jimenez, 2005). Phenolic compounds have the ability to bind protein; therefore, the activity of α-glucosidase activity can be inhibited (Rachmania, Supandi, & Cristina, 2016). It has also the ability to trigger glucose metabolism by increasing tissue sensitivity towards insulin to prevent glucose accumulation in the blood (Prameswari & Widjanarko, 2014). Of the many species of mangrove, the one that is dominant and widely available is white mangrove (*A. marina*). This mangrove contains high levels of alkaloids, saponin and glycosides (Wibowo et al., 2009). Based on this, white mangrove has high potential for development as a component of functional foods. To ease the consumption of white mangrove, its leaves can be processed into herbal tea and promoted as a health beverage especially in areas such as Indonesia and other geographical areas where tea is widely consumed. The brew of white mangrove leaves has brown colour, similar to tea, is easy to produce and should be acceptable alternative to tea drinkers. A previous research on white mangrove (*A. marina*) leaves characterized the flavonoid content that was used as an antioxidant agent (Handayani & Nurjanah, 2013). The aim of this research was therefore to determine the ability of the green tea extract from white mangrove leaves

to reduce the blood glucose level of diabetic Wistar rats (*R. norvegicus*).

2 Materials and Methods

2.1 Materials

Herbal green tea extract from white mangrove (*A. marina*) leaves used, was obtained from '*UKM Tani Mangrove*' at Rungkut Village, Wonorejo Subdistrict, Surabaya, Indonesia. 96% ethanol (Merck, Germany) was used for extraction process, filter paper (Whatmann no.1) and label paper (brand: Tom&Jerry).

Rat feeds were obtained from Centre for Food and Nutrition Research Laboratory (Gajah Mada University, Yogyakarta, Indonesia), these included corn flour, casein, sucrose, soybean oil, fiber, mineral mix AIN-93-MX/100 g of feed (1 g of NaCl; 15 g of $MgSO_4.7H_2O$; 25 g of $NaH_2PO_4.2H_2O$; 32 g of KH_2PO_4; 20 g of $Ca(H_2PO_4).2H_2$; 2.5 g of Fecitrate (Kawano, Egashira, & Sanada, 2007), vitamin mix AIN-93-VX /100 g of feed (Thiamin 6 mg; Riboflavin 10 mg; Piridoxine 4 mg; Cyanocobalomine 0.01 mg; Vitamin C 500 mg; Niacin 40 mg; Capantothenate 10 mg; Inositol 200 mg; Biotin 0.6 mg; Folic acid 1.5 mg; R-amino benzoic acid 5 mg; Vitamin K3 5 mg; Vitamin A 4000 IU; Vitamin D3 4000 IU (Takeuchi, 1988)), L-Cystine, Choline bitartrate and Tert butylhydroquinone. Antidiabetic drug used as a positive control was glibenclamide 5 mg (PT Kalbe Farma, Indonesia).

Streptozotocin (STZ) and nicotinamide (NA) (Nacalai Tesque, Kyoto) were used to condition rats with diabetes mellitus. Materials used for phytochemical analyses of herbal green tea extract were Magnesium, 2N HCl, concentrated H_2SO_4, 1% $FeCl_3$, sodium acetate anhydrous, chloroform, $HgCl_2$, KI (Merck, Germany), aquadest (Hydrobatt) and label paper. Material used for blood glucose analysis was Glucose Kit GOD-PAP (Diasys, Germany).

3-month old male wistar rats (*R. novergicus*) with body weight of about 200 gram and their maintenance equipment, obtained from Centre for Food and Nutrition Research Laboratory (Gajah Mada University, Yogyakarta, Indonesia) were used for the experiments.

Equipment used for herbal green tea preparation included scissors, stove, balance (Kabuto 5000), basin, tray, steamer and baking pan.

Equipment used for herbal tea from mangrove leaves extraction were analytical balance (Mettler Toledo AB204-S), blender (Philips, Indonesia), 1000 ml beaker glass (Pyrex), 250 ml Erlenmeyer (Pyrex), 100 ml measuring cylinder (Pyrex), spatula, funnel, spoon and rotary vacuum evaporator (Buchi R124).

Rats' blood was collected in Eppendorf tubes and haematocrit tubes andplaced on trays. Blood glucose level analysis required the use of a centrifuge (Sigma 3-18K), dropping pipettes, vortex mixer(VM-2000), reaction tubes (Schott Duran) and rack, micropipette (Accumax Pro 10-100μL), spectrophotometer and micro cuvettes (Spectroquant Pharo 300).

2.2 Research Methods

Ethical clearance permission letter number 509-KEP-UB was 'obtained from the Animal Care and Use Committee, Universitas Brawijaya, Malang, Indonesia. The experimental methods involved adminstering a dose of extract of green tea from white mangrove (*A. marina*) leaves extract to diabetic rats, with negative control (rats that were fed with standard feeds) and positive control (diabetic rats that were given glybenclamide at a level of of 0.09 mg/ 200 g BW/day).The dosed rats were sampled at different times. The doses of green tea from white mangrove (*A. marina*) leaves extract were administered orally at the following concentrations per unit body weight: 100 mg/200 g BW, 200 mg/ 200 g BW and 300 mg/200 g BW. The rats were fed for 15 days according to the treatments indicated and samples were taken for blood glucose determinations on day 0, 5, 10 and 15. The observed parameters were blood glucose level, body weight, the amount of feed consumption and feces weight. The experimental design used in this research was a randomized factorial design, followed by Duncan test.

Preparation of Herbal Green Tea Extract from White Mangrove Leaves (*A. marina*)

Herbal green tea from white mangrove (*A. marina*) was prepared from picked young mangrove (*A. marina*) that were cleaned and processed using steam, chopping and drying to obtain dry herbal green tea. Extraction was carried out using a maceration method with 96% ethanol as a solvent with a ratio of 1:4 (w/v). This mixture was then put in a shaker for 6 hours and left at room temperature for 24 hours (12 hours dark and 12 hours light). The mixture was then filtered and the solvent was evaporated from the filtrate using a rotary evaporator at a temperature of 45 ^{o}C until all the solvent has been evaporated. A thick extract was obtained that was ready for use in the experiment.

Preparation of Diabetic Rats and Feeding of Herbal Green Tea Extract from Mangrove Leaves

Male Wistar Rats (2.5-3 month old) were adapted for 7 days by putting each rat individually in a cage with sufficient light, ventilation, at room temperature under *ad libitum* standard feed and drink conditions, and weighed at the end of the adaptation phase. The rats were then induced with STZ (45 mg/200 g BW) and NA (110 mg/200 g BW) by intraperitoneal injection. After the diabetic condition was achieved, the rats were given several treatments: green tea extract from mangrove leaves of 100 mg/200 g BW/day, 200 mg/200 g BW/ day and 300 mg/200 g BW/day using gavage needles, positive control (induced with glybenclamide with a dose of 0.09 mg/200 g BW/day) and negative control (rats without any treatment). The experiment lasted for 15 days. On day 0, 5, 10 and 15, the rats' blood was collected from the orbital sinus and analysed for its blood glucose level.

Qualitative Phytochemical Analyses

Phytochemical analyses were based on methods used by Dia, Nurjanah, and Jacoeb (2015). *Tannin analysis*: 0.5 g of sample was put into a reaction tube to which was added 10 ml of boiling aquadest, and then filtered. 3 drops of 1% $FeCl_3$, were added to the filtrate obtained. The presence of tannins was indicated by formation of brownish green colour or blackish blue colour.
Alkaloid analysis: 1 ml of sample was put into a reaction tube to which 3 drops of Meyer reagent were added. These two solutions were mixed and diluted to 100 ml). A positive result was indicated by formation of yellowish white precipitate.
Flavonoid analysis: To 0.05 g of sample were added the following: 0.1 mg of Magnesium powder, 0.4 ml of amyl alcohol (mixture of 37% hydrochloric acid and 95% ethanol with the same volume) and 4 ml of ethanol. The mixture was then shaken. The presence of flavonoid was indicated by formation of a red, yellow or orange colour in the amyl alcohol layer.
Steroid and terpenoid analysis: 0.5 g of sample was put into a reaction tube and to it added 10 ml of boiling aquadest, the resulting mixture was then then filtered. The filtrate obtained was evaporated to dryness, and followed by the addition of the following reagents: 2 ml of acetic acid glacial and 3 ml of concentrated H2SO4 to form layer. Formation of a blue-green colour indicated the presence of steroids, whereas the formation of reddish-purple indicates the presence of terpenoids.
Saponin analysis: 0.5 g of extract was diluted with 10 ml of boiling aquadest, let cool and shaken manually for 30 second and changes that occur were observed. 2 N HCl was dropped into the tube and changes that occur were also observed. If there is a solid foam formed (last for at least 30 seconds), then it indicates the presence of saponin.

Blood Glucose Analysis

Pre-prandial blood glucose was measured on day 0, 5, 10 and 15. The method used in this research was glucose the GOD-PAP method. This method involves the oxidation of glucose by gluco-oxidase (GOD) into gluconic acid and H_2O_2. H_2O_2 was then reacted with phenol and 4-aminoantipyrine to form chinonimine, which has red colour, and H_2O. This reaction was catalysed by peroxidase enzyme (POD). The chinonimine formed was equivalent to glucose, which

means the colour measured from chinonimone was equivalent to the glucose level.

3 Results and Discussion

3.1 Characteristics of Mangrove Green Tea Extract

Mangrove green tea extract had a yield of 37.81%, a moisture content of 6.46% and an ash content of 10.5%. Yield was influenced by the initial moisture content and expected final moisture content (Hutami & Harijono, 2014). Moreover, the moisture content of a product was influenced by the heating process and the initial moisture content (Pratama, Rostini, & Liviawaty, 2014), whilst the ash content was influenced by the mineral content of the raw materials (Lestari & Tjahjani, 2015).
Phytochemical analysis results showed that the mangrove (*A. marina*) green tea extract contained tannins, alkaloids, flavonoids and saponins. These compounds form part of the polyphenol group of compounds (Hardoko et al., 2015), which have antidiabetic and anti-uric acid activity (Hardoko, 2015).

3.2 Preparation of Diabetic Rats

Rats were declared diabetic when the increase in the blood glucose reached levels considered to demonstrate hyperglycaemia, i.e. increase of blood glucose level above the normal limit. The blood glucose level before and after induction with STZ and NA can be observed on Table 1.
From Table 1, it can be seen that on day 0, before inducing using STZ, all rats had a normal blood glucose level, i.e. about 54-58 mg/dL. Normal blood glucose level of rats is about 50-135 mg/dL (Johnson-Delaney, 1996). On day 3 after induced using STZ, all rats had an increase in blood glucose level to reach *diabetes mellitus* condition, except for control (-) rats. Rats are considered to be diabetic when the pre-prandial blood glucose level is above 126 mg/dL (Setiawan, 2012).

3.3 The Effect of Herbal Mangrove Green Tea Extract on Blood Sugar Level of Diabetes Rats

Anova results showed that treatment of duration and dose of mangrove green tea extract, and the interaction of both treatments, gave a significant effect on blood glucose level of diabetic rats ($p<0.05$). The post hoc test result using Duncan method can be seen on Figure 1.
From Figure 1, it can be inferred that the longer the feeding and the higher the dose of herbal mangrove green tea extract, the greater the decrease of blood glucose level on diabetic rats. This result may be due to the higher dose of extract that contains a higher concentration of bioactive compounds, which may have a greater effect on blood glucose level, compared to the lower dose of extract. It was presumed that tannins, flavonoids, alkaloids and saponins in herbal mangrove (*A. marina*) green tea extract play a role in reducing the blood glucose level on diabetic rats. Tannins consist of condensed tannins and hydrolysable tannins. Proanthocyanidins is the other name given to condensed tannins (Frutos, Hervás, Giráldez, & Mantecón, 2004). There are several mechanisms of the effect of tannins ion the lowering of blood glucose levels, i.e. tannins decrease the blood sugar levels by inhibiting glucose absorption in intestines and tannins can induce the regeneration of pancreatic beta cells which cause adipose cells, to increase insulin activity (Kumari & Jain, 2012). Moreover, an alkaloid extract has been proven to have the ability to increase the regeneration of damaged pancreatic beta cells (Arjadi & Susatyo, 2010). The increase of insulin secretion is caused by a sympathetic nerve stimulation effect (sympathomimetic) by alkaloids. Saponins as an antidiabetic agent were described in previous research, in which after histopathologic examination of the pancreas, saponins could regenerate pancreas, so that the amount of pancreatic beta cells and islets of langerhans would increase, causing an increase in insulin secretion. The increase in insulin secretion could then decrease the blood glucose level (Firdous, Koneri, Sarvaraidu, Harish, & Shubhapriya, 2009).

Table 1: Blood glucose level of rats induced using STZ and NA

Treatment	Blood Glucose Level (mg/dL)	
	Before inducing using STZ (day 0)	After inducing using STZ (day 3)
Negative control	54.68±1.08	55.01 ± 1.08
Positive control	56.47± 1.30	221.91±1.32
100 mg/200g BW/day	58.39 ±1.10	214.13±2.76
200 mg/200g BW/day	57.43 ±2.55	210.95±5.21
300 mg/200g BW/day	57.79 ±0.75	215.08±4.06

Table 2: Percentage of blood glucose level changes by herbal mangrove green tea extract

Day	Blood glucose level changes				
	Negative control	Positive control	100mg/200gBW	200mg/200gBW	300mg/200gBW
5	+1.31%	-14.02%	-22.23%	-22.23%	-27.71%
10	+1.44%	-38.34%	-34.84%	-38.34%	-46.35%
15	+2.16%	-55.39%	-37.74%	-42.37%	-50.81%

Notes: (+) = increase; (-) = decrease

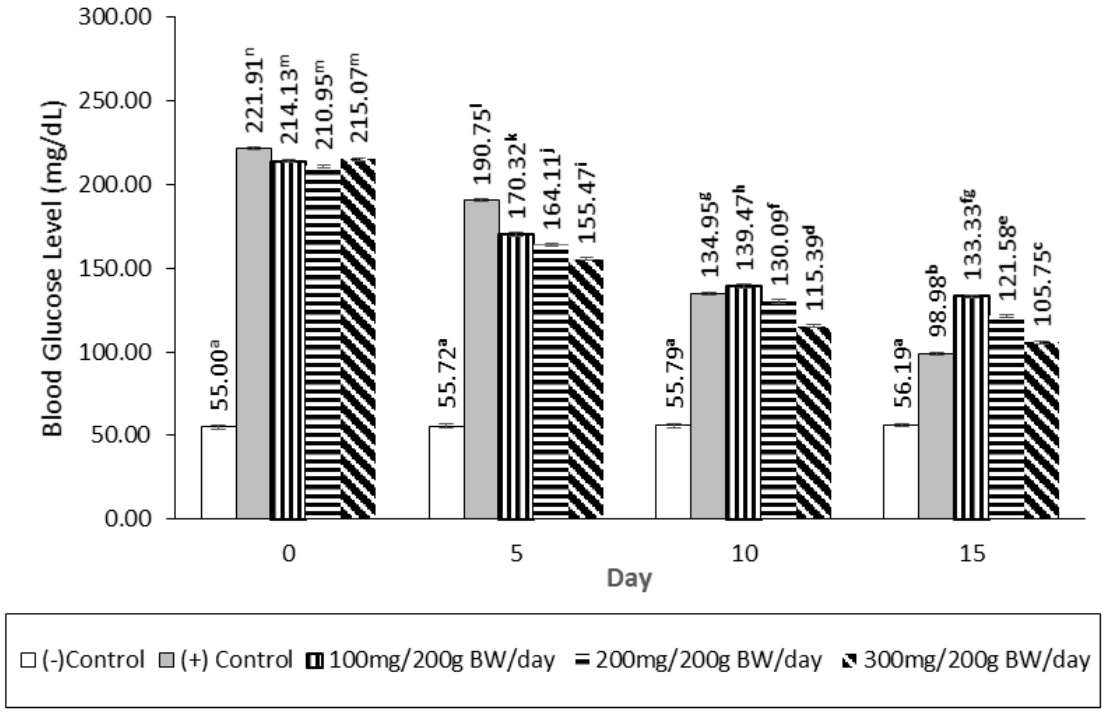

Figure 1: Histogram of blood glucose level of rats for 15 days. Notes: different superscript notation shows significant difference ($p<0.05$)

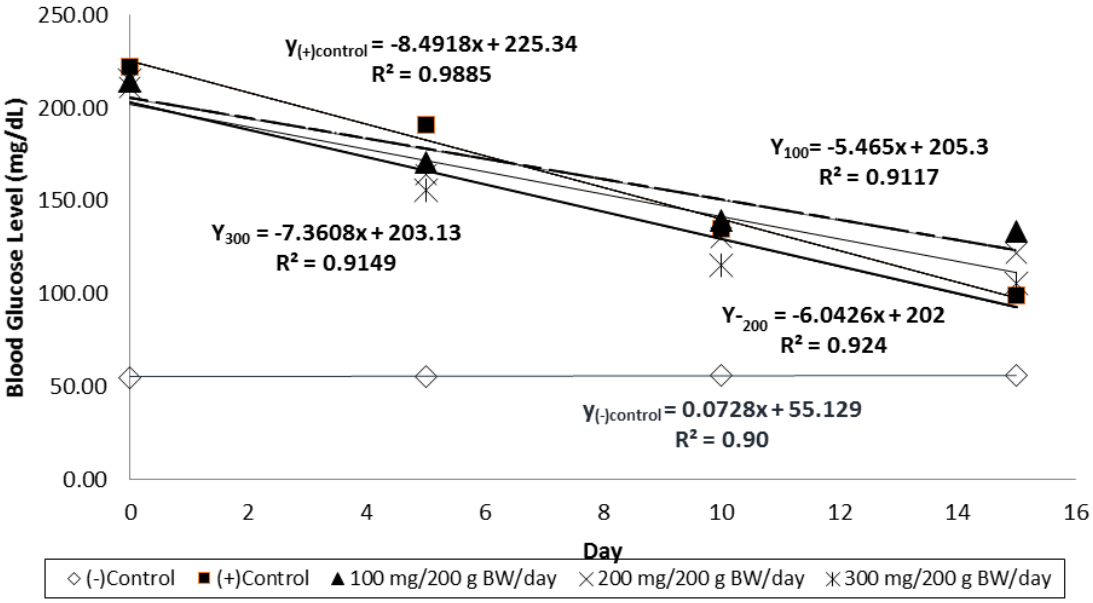

Figure 2: Linear regression of of rats' decreased blood glucose level

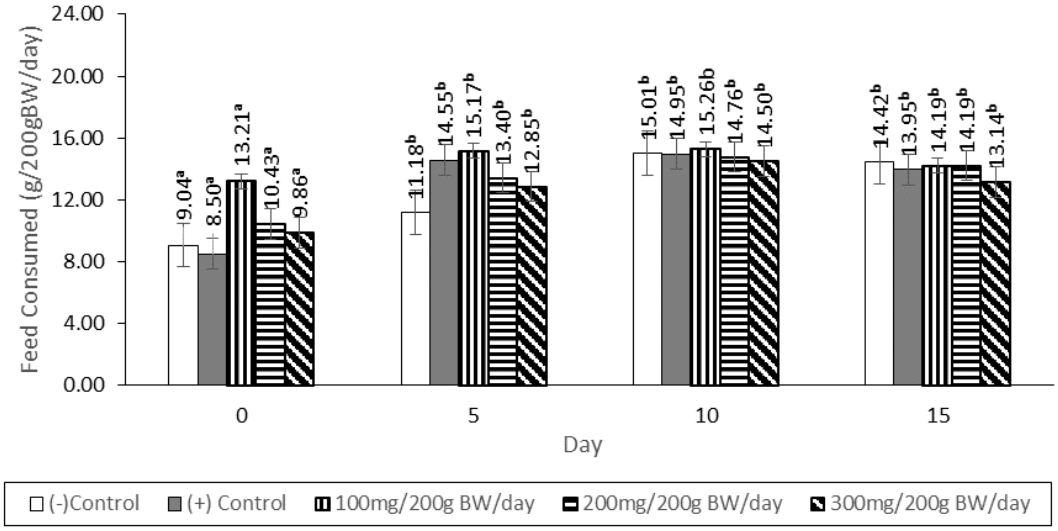

Figure 3: Histogram of the amount of feed consumed by rats during experiment. Notes: different superscript notation shows significant difference ($p<0.05$)

To determine the effect of herbal white mangrove green tea extract on the blood glucose level, the calculation of the percentage of lowering of blood glucose is presented on Table 2. The higher the dose, the more effective the blood glucose levels decrease in diabetic rats. It is expected that because the bioactive compounds, which play a role in decreasing the blood glucose level, are different in each dose given, and there is also a difference in each rat's body response towards the dose given. The best dose of herbal mangrove green tea extract to decrease blood glucose level was 300 mg/200 g BW/day, i.e. about -50.81%.
To predict on which day the herbal mangrove green tea extract feeding could decrease the diabetic blood glucose level to the normal blood glucose level, a linear regression that shows the correlation of blood glucose level and the dose of extract given was constructed (Figure 2). The intersection of the regression equation of negative control and extract dose shows the day on which the blood glucose level of diabetic rats decrease to normal. The day on which blood glucose level of diabetic rats decreases to normal can also be calculated by changing the Y-axis value with blood glucose level of control negative rats, i.e. 55.675 ± 1.30 mg/dL.
The results based on the regression equation shows that the blood glucose level of positive control rats are normal at day 20, rats treated with an extract dose of 100 mg/200 g BW/day are normal on day 28, rats treated with an extract dose of 200 mg/200 g BW/day are normal on day 24 and rats treated with an extract dose of 300 mg/200 g BW/day are normal on day 20. Thus, the dose of herbal mangrove green tea extract that is comparable to glybenclamide (0.09 mg/200g BW/day) was 300 mg/200g BW/day.

3.4 Amount of Feed Consumed by Rats

The amount of feed consumed by rats was determined by counting the difference between the amount of feed given and the remaining feed, which was not consumed by the rats. Anova statistical result shows that the duration gave a significant effect on the amount of feed consumed ($p<0.05$), whereas the extract dose and the interaction of both did not give a significant effect on the amount of feed consumed ($p>0.05$). Post hoc test result using Duncan method can be seen in Figure 3.
The amount of feed consumed could influence blood glucose level of rats (Wahyu, 2004). However, in this research, the amount of feed consumed on the same day of observation was not significantly different (Figure 3). This implies that the decrease in blood glucose level of rats was only affected by the herbal mangrove green tea extract given. The other factors that could influence the amount of feed consumed are palatability, appetite, environmental conditions and in case of illness (Wahyu, 2004).

4 Conclusions

Supplementation of herbal white mangrove (*A. marina*) green tea extract for 15 days could decrease the blood glucose level of diabetic Wistar rats (*R. novergicus*).
The effective dose of herbal white mangrove (*A. marina*) green tea extract in decreasing the blood glucose level on diabetic Wistar rats (*R.novergicus*) is 300 mg/200g BW/day, which is comparable to glybenclamide.

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Consumer Awareness of the Use of Food Labels in Lagos State, Nigeria

Samuel T. Danilola[a*], Olubunmi A. Omotesho[a], and Jubril Animashaun[a]

[a] Department of Agricultural Economics and Farm Management, University of Ilorin, Kwara state, Nigeria
[*]Corresponding author
samueldanilola@yahoo.com
Tel: +2348068569911

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Abstract

The increase in relative level of education and the growing middle-class income earners in Nigeria have made packaged food items attractive to consumers. These foods provide handy, nutritious and convenient food for consumers; however, they also come with public health concerns. From a policy perspective, food labelling can gauge consumers' response to the safety conditions of packaged food. However, understanding consumers' awareness and the use of food label information has attracted little research attention in many developing countries in sub-Saharan Africa. In this study, we investigated consumers' awareness of the use of food safety labels in Lagos state, Nigeria. We used primary data collected using pretested structured questionnaire from 220 food shoppers who bought pre-packaged foods. A two-stage sampling technique was used to select the respondents. Consumers' awareness and use of food safety information was revealed using descriptive statistics; Ordinal Regression Analysis was used to examine the socioeconomic factors determining the frequency of reading food safety labels. We found evidence of the influence of socioeconomic characteristics on how consumers read food labels. The study therefore recommends that there should be continuous awareness campaigns on the importance of food labels in ensuring safety and food producers should make sure their food labels are legible.

Keywords: Food label; Food safety; Nigeria; Ordinal Regression; Pre-packaged food

1 Introduction

Globalization, the demand for healthy food and the significant progress in food science and technology have resulted in an increase in the consumption of imported pre-packaged foods and local food producers following internationally acceptable standards. In addition, because the change in food and consumption lifestyle often comes with public health concerns, regulators often emphasize the use of food labelling to address the health concerns that may arise from the changing food lifestyle. Food labelling enables local consumers to make informed decisions on food choices. In many countries, food product labelling is a policy tool used by regulators to gauge the response of consumers to health information and is used by food companies for product differentiation strategy (Kaur, Nirmal Kaur, & Neha Qumar, 2016). Unlike in many industrialized countries, there have been very few studies in Nigeria on consumers' awareness of food labelling.

According to a report by the Office of Agricultural Affairs of the USDA/Foreign Agricultural Service in Lagos, Nigeria (David, 2009), the National Agency for Food and Drug Administration and Control (NAFDAC) has been empowered by law - Government of Nigeria Act No 19 of 1993 (as amended) and the Food and Related

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Products (Registration) Act No. 20 of 1999 - to bring up standards for food production as well as laws against food commodities that are deleterious to human health. NAFDAC regulations require that food labels be detailed and exact, and not fraudulent or misleading:

- Listed below are the outline of the minimum labelling requirements of NAFDAC:
  a) A product's brand name must be in bold letters.
  b) Name and detailed "location" address of the manufacturer showing country of origin must be provided on the product label.
  c) The batch number, date of manufacture and best before/expiry date should be written.
  d) Net content, specifying essential ingredients in standard weight for solids and standard volume for liquids should be written.
  e) Ingredients must be listed by their public names in order of their prominence by weight.
  f) Food additives and colours must be written, if present, on the label. Spices, flavours and colours may be listed as such, without naming the specific material, but any artificial colour or flavour should be identified as such.
  g) NAFDAC's registration number must be a part of the information on the product label. Labelling should be in English. If it is in another language, an English transcription has to be provided on the label or package insert (where applicable).
- Stick-on labels meeting NAFDAC requirements are permitted provided they don't remove easily.
- Foreign labels must be affixed prior to the product arrival at the Nigerian port of entry.
- Foreign labels must be applied prior to export.
- Sample-size products or institutional packed products have no special labelling requirements.
- For production and expiry dates, the day is written before the month.
- NAFDAC regulation stipulates that all food products should carry best-before dates and/or shelf life on their packaging. The regulation states that the expiry date should be "at least half the shelf life as at time of inspection." This implies at the time of inspection (by NAFDAC after clearing Customs), that the period from the inspection date until the expiration date should be equal to or greater than half of the total shelf life of the product (date of production until expiry):
- NAFDAC does not grant exceptions to labelling requirements

With respect to Nutritional Labelling, the specific requirements include:

- NAFDAC accepts the standard U.S. nutritional fact panel.
- Every nutritional claim on a product's label must be warranted. Nutritional labelling is obligatory for any pre-packaged food item for which the manufacturer makes a nutrition or dietary claim.
- Foods for special dietary uses with claims of disease prevention, treatment, mitigation, cure or diagnosis must comply with NAFDAC's guidelines for registration of drugs and be registered as medicinal products or *nutriceuticals*. Labels must contain directions for safe usage.
- Additional nutritional labelling information is voluntary.

In addition, the regulations guiding the use of food additives are also indicated in the Government of Nigeria Act 19 of 1993. For example, NAFDAC requires the fortification of wheat and maize flour, vegetable oil and sugar with Vitamin A, while salt must be iodized. NAFDAC applies the food additive standards of the Codex Alimentarius Commission, EU and FDA in its assessment of food safety:

- No person may manufacture, import, advertise, sell or present any food item or beverage containing a non- nutritive sweetener for human consumption unless the product is "specified for special dietary usage."
- Non-nutritive sweeteners, including saccharin and cyclamates, may be used in low-calorie, dietary foods/beverages but are not permitted in any food or beverage to be consumed by infants or children.
- Potassium bromate as a bread improver is not permitted. Other several bread improvers are now available.

As at the time of this study, Nigeria had no laws regulating products of biotechnology. However, a draft biosafety bill has been developed and is before the National Assembly for consideration. The draft bill advocates compulsory labelling of food items containing products of biotechnology to protect consumers' right to know. When the bill is passed, NAFDAC will be the enforcing agency.

The extant literature has focused on the importance of reading food labels due to its role as a major source of information on food to consumers (Grunert & Wills, 2007) Other literature has identified how food labels inform consumers' purchasing behaviour (Henson & Caswell, 1999; Naspetti & Zanoli, 2009; Ortega, Wu, Wang, & Widmar, 2011; Rijswijk & Frewer, 2008; Smith & Riethmuller, 2000) in developed countries. Together, these studies provide evidence that gaps still exist in research about how consumers respond to threats of unsafe foods in the global food market and if it influences their use of food labels during purchases in developing countries like Nigeria.

There is evidence of the effect of some socioeconomic characteristics on the use of food labels around the world. For example, the studies by Basarir (2012), Du Plessis and Rousseau (2003), Ali and Kapoor (2009), Wyma et al. (2012), Talagala and Kalukottege (2015) and Ezeh and Ezeh (2014) highlight the importance of education, gender, and income in promoting the use of food labels. However, there is no empirical study on the factors influencing the frequency of using food labels in Nigeria. For example, Falola (2014) studied the willingness to pay for food labels, and Ezeh and Ezeh (2014) only studied the attitude of students of higher education institutions towards food label information. These leave some gaps in knowledge that this study was designed to fill.

Therefore, in this study we investigated the level of awareness and use of food safety labels; identified the socioeconomic and psychological factors influencing the awareness, and use of food safety information on labels, and the constraints to the use of food label information before food purchase.

2 Materials and Methods

2.1 Study Area

This study was conducted in Lagos state, southwest Nigeria. Lagos is geographically positioned on longitude $2^o42'$–$4^o20'$ East and latitude $6^o22'$–$6^o42'$ North. It is surrounded on the West by the Republic of Benin, the North and East by Ogun State and on the South, the Atlantic Ocean (Lawal & Aliu, 2012).

Lagos, according to World population review website, is the largest city in Africa and Nigeria Population Commission estimates its population to be about 21 million people (World Population Review, 2016) and it produces less than 10% of the food that is consumed in the city (Mhango, 2013). Being the commercial city of Nigeria, it is home to virtually all ethnic groups in Nigeria and it has the smallest land area, 356,861 ha, of all the states in Nigeria, of which 75,755 ha is marshland and 169,613 ha is designated for agriculture (John, Wakilu, & Olateju, 2013). Lagos state presented the perfect choice for the study as it has the highest literacy level (96.30%) in Nigeria; and remains the focal point of Nigeria's economic and social development owing to its sea and airports (Nigeria Data Portal, 2016). In addition, it is the biggest spender on most non-food commodities like rent, fuel and lighting, household goods and transport, which reflects the high purchasing power of its populace (Mhango, 2013). It was therefore expected that Lagos state would have the highest level of consumers who are aware of, and use, food safety

information in Nigeria.

2.2 Source of Data

Primary data was obtained via the use of pre-tested structured questionnaire from buyers of pre-packaged food in Lagos state. Using a confidence level of 95% and a confidence interval of 6.5, 220 food shoppers were found to be representative (from a population of 21 million people) for the study. Then, a two-stage sampling technique was used to select the sample of food shoppers. The first involved the selection of two shopping (Shoprite) malls from a list of five shopping (Shoprite) malls across Lagos state as they allowed their facilities to be used for data collection. Shoprite malls were used in the study because anecdotal evidence revealed they are the most widely accepted shopping malls in Lagos based on quality and prices of products sold. The second stage was the systematic selection of every fifth food shopper who purchases pre-packaged products until a population of 110 respondents were interviewed in each mall; this made a total of 220 food shoppers from each of the malls.

2.3 Data Collection

Data was collected from respondents using a structured questionnaire in an interview schedule. The questionnaire was split into three different sections:

- the first part aimed at assessing the socio-economic characteristics of the consumer;
- the second, assessed the awareness and use of the different types of information on the food labels and which information is most utilised. It also obtained information on constraint to the use of food safety information; and
- the third part measured the factors that motivate consumers to read food safety information.

2.4 Data Analysis

Descriptive statistics were used to present the socio-economic characteristics of the respondents and the constraints to the use of food label information before food purchase. Cluster analysis was used to elicit the level of awareness and use of food safety labels, then presented using percentages and frequencies. Ordinal Regression Analysis was used to determine the factors influencing the frequency of reading food safety information contained food labels.

Descriptive statistics

The perception of a consumer's level of awareness and use of food safety labels was measured on a Likert scale. A scale ranging from 'never (1)' to 'always (5)' was used to measure the knowledge of those information cues printed on the food label. The mean of the responses from each respondent on each information cue was then obtained as a measure of the consumer's knowledge of the food safety information. Another scale ranging from 'Always (3)' to 'Never (1)' was used to elicit the level of use of those information cues. Then, the mean of the responses from each respondent on each information cue was obtained as a measure of that consumer's use of the food safety information. The information cues printed on food labels in Nigeria, according to NAFDAC, include: Product name, Brand name, Date of manufacture, Expire/best before date, Net quantity, Country of Origin, Ingredient list, Food additives, Name/address of manufacturer, Direction for use/storage, Warning statement, Health/nutrition claims, Information about allergens, Nutrition panel, and Trade mark.
In addition, a scale ranging from 'strongly agree (5)' to 'strongly disagree (1)' was used to measure the constraints to the use of food safety information contained in labels. This measure was obtained after calculating the mean of the responses by the respondents to the questions.

Two-step Cluster Analysis

A two-step cluster analysis was used to identify clusters of respondents based on their knowledge

and use of the food safety information printed on the food labels during their purchase. The cluster analysis grouped the respondents into two based on their level of awareness and use of food safety information as done in the study by Talagala and Kalukottege (2015).

Ordinal Regression Analysis

Ordinal regression model is a type of regression model best suited for predicting the effects of independent variables on an ordinal dependent variable. It can be performed with the use of a generalized linear model (GLM) that fits both a coefficient vector and a set of thresholds to a dataset (McCullagh, 1980; Winship & Mare, 1984). This model has also been used in other studies involving ordinal dependent variables (Lall, Campbell, J Walters, & Morgan, 2002; Reddy & Alemayehu, 2015). This was therefore used to obtain the determinants of frequency of reading food safety information. This model was selected due to the level of measurement of the dependent variable - frequency of reading food safety information, which was ordinal.

The respondents were asked, 'A variety of information is provided on food labels, how often you read them?' The responses were categorised into never, rarely, sometimes, and often. It is assumed that the alternative responses (y) were related to a continuous, latent variable y* that signified a respondent's frequency of reading food safety information. The answers to each statement were taken as the dependent variable in the ordinal regression model.

The model specification is given in equation 1

$$\begin{aligned} y_i^* =& \beta_0 + \beta_1 X_{1i} + \beta_1 X_2 + \beta_3 X_3 + \\ & \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + \beta_8 X_8 + \\ & \beta_9 X_9 + \beta_{10} X_{10} + \beta_{11} X_{11} + \beta_{12} X_{12} + \varepsilon_i \end{aligned} \tag{1}$$

Where 0 if y* is never; y = 1 if y* is rarely; 2 if y* is sometimes; 4 if y* is always.

β_0 = Constant; β_i = Regression coefficients of the ith independent variable; X_{1i} = Marital status (i could be Single – 1, Married, Divorce, Cohabiting partners or Widowed); X_2 = Sex (1= Male; 0 = female); X_3 = Age (years); X_4 = Occupation/Sector (nominal); X_5 = Perceived severity of food safety issues (continuous); X_6 = perceived vulnerability to food safety issues (continuous); X_7 = response efficacy (continuous); X_8 = self-efficacy (continuous); X_9 = Frequency of shopping (categorical variable); X_{10} = Awareness of food safety information (continuous); X_{11i} = Educational status of respondent (i = No formal education, primary education, secondary education or tertiary education); X_{12} = Use of food safety information (continuous); ε_i

2.5 Measurement of Variables

The variables – perceived severity of food safety issues, perceived vulnerability to food safety issues, response efficacy, and self-efficacy were the measure of the psychosocial factors that could influence the frequency of reading food labels. These are constructs from the Protection Motivation Theory postulated by Rogers (1975). The theory has been used to explain consumer intention to consume foods in several health related studies (Floyd, Prentice-dunn, & Rogers, 2000). Three of the constructs (Perceived severity of Food Safety Issues, Perceived Vulnerability to Food Safety Issues, and Response Efficacy) were measured using consumers' responses to three items on a scale of 1 (strongly disagree) to 5 (strongly agree), while Self-efficacy was measured using their responses to two items on a scale of 1 (strongly disagree) to 5 (strongly agree).

Perceived severity of Food Safety Issues (X_5)

The respondents were asked to state their perception about the following statements: 'food safety incidents occur frequently in Nigeria'; 'unsafe food is widely spread in Nigeria food system'; and 'people's health is at risk due to unhealthy food'. The mean of the responses from each respondent was then used as the measure of the respondent's perceived severity of food safety issues.

Perceived Vulnerability to Food Safety Issues (X_6)

The respondents were asked to state their perception about the following statements: 'my health is at risk due to unhealthy food'; 'it is quite I possible I will ever get unsafe food'; and 'my health risk is highly related to food safety'. The mean of the responses from each respondent was then used as the measure of the respondent's perceived vulnerability to food safety issues.

Response Efficacy (X_7)

The respondent's perceptions were obtained from their responses to the following statements: 'reading these food safety labels helps prevent choosing unsafe food'; 'these food safety labels provide me with useful information on purchasing safe food'; and 'reading these food safety labels relieves my concern about food safety'. The mean of the responses from each respondent was then used as the measure of the respondent's response efficacy to food safety issues.

Self-efficacy (X_8)

The respondents were asked to state their perception - on a scale of 1 (strongly disagree) to 5 (strongly agree) - about the following statements: 'it is easy for me to read these food labels before purchasing food'; and 'I feel confident in my capability to study these food labels before purchasing food'. The mean of the responses from each respondent was then used as the measure of the respondent's self-efficacy to food safety issues.

The Frequency of Shopping (X_9)

This was measured as a categorical variable. The respondents were asked to respond how frequently they went on food shopping: whether daily, at least twice a week, fortnightly, or monthly.

Awareness of Food Safety Information (X_{10})

Consumers' awareness was obtained from their responses to their level of awareness about the 14 information cues available on most food labels in Nigeria as stated earlier on a 5-point Likert scale.

Use of Food Safety Information (X_{12})

This was also measured on a 3-point Likert scale using the same set of information cues used for awareness of food safety information.

3 Results and discussion

3.1 Socio-economic characteristics of Respondents

A total of 220 respondents took part in this study as shown in Table 1 . The study revealed that males made up a larger proportion (58.2%) of the shoppers of pre-packaged food that were interviewed.

The age distribution of the respondents was within the youth bracket with the largest proportion (61.6%) being 19 to 29 years old. The food shoppers were made up of 82.3% single persons and only about 20.5% of the food shoppers had a child or children.

The study also revealed that about 99% of the respondents had at least a secondary education with 53.5% either presently in a tertiary institution or had completed tertiary education. This was reflective of the high literacy rate in Lagos state. It also revealed that 51.6% of the respondents were technical and professional workers and about 34.4% unemployed.

On the frequency of food shopping, the largest proportion of the respondents (29.6%) claimed to go food shopping at least twice a week.

3.2 Awareness and Use of Food Safety Information

The 2-step cluster analysis of the awareness and use of food safety information identified two broad categories within which each respondent fell. The clusters as shown in table 2 were high level of awareness and high use of food safety information and high level of awareness and low use of food safety information.

The study revealed that 61.8% had high level of awareness and high use of the information

Table 1: Socio-economic Characteristics of Respondents

Variable	Frequency	Percent
Sex of consumer		
Female	92	41.6
Male	128	57.9
Age distribution of consumer		
<= 18	19	8.6
19 – 29	130	58.8
30 – 40	54	24.4
41 – 51	7	3.2
52+	1	0.5
Marital status of consumer		
Single	181	81.9
Married	39	17.6
Is the consumer a parent?		
No	175	79.2
Yes	45	20.3
Educational status of consumer		
Primary education	2	0.9
Secondary education	30	13.6
Tertiary education	117	52.9
Postgraduate education	70	31.7
Occupation of consumer		
Unemployed	76	34.4
Technical and Professional Workers	113	51.1
Sales workers	2	0.9
Transport workers	6	2.7
Agricultural workers	1	0.5
Artisans and Craftsmen	18	8.1
Administrative and managerial workers	3	1.4
Frequency of food shopping		
Monthly	61	27.6
Fortnightly	56	25.3
At least twice a week	64	29.0
Daily	36	16.3

Source: Field Survey, 2017

Table 2: Cluster analysis for the awareness and use of food safety information

Clusters	High-high	High-low
Size	61.8% (136)	38.2% (84)

Source: Field Survey, 2017

cues on food labels, whereas 38.2% fell in the second category of high level of awareness but low use of the information cues on food labels. This confirmed the finding of similar studies (Affram & Darkwa, 2015; Basarir, 2012; Van der Merwe, Bosman, & Ellis, 2014) where consumers reported high level of use of food labels before making food purchases.

The high level of awareness was reflective of the literacy rate of the respondents, while the low level of use in some quarters could have been due to the low level of trust on food labels to provide information on food safety.

3.3 Factors Influencing the Frequency of Reading Food Safety Information on Food Labels

The frequency of reading food label is indicated in Table 3 : of the 220 respondents (15 missing items) only 3.9% never read food labels. Many of the consumers read food labels; hence producers/processors should ensure the labels are clear enough for easy comprehension.

To check the reliability of the constructs of Protection Motivation Theory, the Cronbach alpha test was conducted on the different constructs (Table 4).

The ordinal regression model was utilised to obtain the determinants of the frequency of reading food safety information by respondents before food purchases in the study area. Since the frequency of reading food labels is an ordinal variable, the ordinal regression model was the most appropriate model to be used. The dependent variable of the model was frequency of reading food label and the ranked alternatives were never, rarely, sometimes and often. The result of the model is given in Table 5 . Only the variables important to the model were presented in the table.

It can be deduced from the results that sex, secondary education, tertiary education, unemployment, technical and professional workers and transport workers were all significant. In addition, the response efficacy and awareness of food label information of consumers both positively influenced the frequency of reading food safety information on food labels.

By taking the exponent of the pooled estimate of each variable i.e. $\exp^{\beta}$, the common odds ratio of each variable differing by one unit was obtained. Therefore, male consumers were 0.561 times less likely than females to be reading food safety information in food labels frequently. This was very closely related to the findings of Ezeh and Ezeh (2014) and Falola (2014), where it was reported that females read food labels more than males.

Consumers with secondary education were 0.247 times less likely to read food labels frequently, and those with tertiary education 0.44 times less likely to read food labels frequently, than consumers with postgraduate education. This was in consonance with the studies that showed the frequency/likelihood of reading food labels increases with increases educational attainment (Basarir, 2012; Falola, 2014). The unemployed consumers were 1.46 x 10^{-9} times less likely, technical and professional workers 2.615 x 10^{-9} times less likely and transport workers 4.34 x 10^{-9} less likely, when compared with artisans and craftsmen, to read food safety information frequently.

The odds of reading food labels frequency by consumers would likely increase by 1.826 for every unit increase in the response efficacy of consumers to food safety threat. This is expected because as the consumers see the reading of food labels as one of several coping strategies against food safety threats, it would most probably result in increased frequency of reading food safety information.

The odds of reading food safety information was also expected to increase, but by a factor of 1.504, for a unit increase in the awareness of food safety information. This is plausible as the awareness of food safety information makes consumers realise the importance of knowing what they eat, and this culminates in the habit of frequency reading food labels.

Table 3: The frequency of reading food labels

Frequency of reading food labels	**Frequency**	**Percentage**
Never	8	3.9%
Rarely	28	13.6%
Sometimes	84	40.8%
Often	86	41.7%
Valid	206	100.0%

Source: Field Survey, 2017

Table 4: Reliability Test for the Different Variables

S/N	**Variable**	**Cronbach's Alpha**	**N of items**
1	Awareness of food safety information	1.00	14
2	Use of food safety information	1.00	14
3	Perceived severity of food safety threats	0.56	3
4	Perceived vulnerability to food threats	0.63	3
5	Perceived response efficacy	0.73	3
6	Perceived self-efficacy	0.72	2

Source: Field Survey, 2017

Table 5: Ordinal logistic regression analysis for the determinants of frequency of reading food labels

Variable[1]	**Coefficient**	**Std. Error**	**Wald**
Sex	-.579*	.339	2.918
Age	.031	.036	.714
Marital status	-.528	.655	.648
Secondary education	-1.405**	.521	7.284
Tertiary education	-.817**	.367	4.961
Unemployed	-20.347***	.593	1177.465
Technical and professional workers	-19.762***	.548	1300.238
Transport workers	-19.255***	1.015	360.145
Admin and managerial workers	-.639	7365.302	.000
Perceived Severity	.234	.258	.820
Perceived Vulnerability	-.251	.169	2.198
Self-Efficacy	.051	.205	.062
Response Efficacy	.602**	.195	9.573
Awareness of Food Label Information	.408*	.232	3.095
Use of Food Label Information	.170	.322	.277

Log Likelihood function: 393.591; Chi squared: 71.095. *: significant at 0.10 level; **: significant at 0.05 level; ***: significant at 0.01 level.

[1] The reference variables for secondary and tertiary education are postgraduate education, while for employment status is the artisan and craftsmen

Source: Field Survey, 2017

Table 6: Constraints to the Reading of Food Safety Labels

Constraints	Mean score	Rank
Buying familiar foods	3.54	1^{st}
Smallness/illegible prints	3.36	2^{nd}
Lack of time	3.30	3^{rd}
Positioning of food labels	3.15	4^{th}
Interpreting food label information	3.14	5^{th}
Health issues	3.10	6^{th}
Importance	2.75	

Source: Field Survey, 2017

3.4 Constraints to Consumers' Awareness and Use of Food Safety Information

Consumers are faced with some challenges to the use of food safety information in food labels. Table 6 shows the constraints faced by the respondents.

The respondents perceived buying of familiar foods as the major constraints to the reading of food safety information on food labels. This is due to the belief that they have checked out the food label in the past and don't expect significant change in the product information in the current purchase, although they check for the expiry/best before date in some products. In addition, the respondents also identified smallness/illegibility of most food label prints and lack of time to go through them as constraints to the reading of food labels. As a result, producers of pre-packaged food are advised to ensure the print is clear enough for the consumers to read without stress and that these labels should contain concise information for them to read in little time. On the flip side consumers did not believe food labels are unimportant as seen by the mean score (2.75) being below the passing score (3.00).

4 Conclusions

This study examined consumers' awareness and use of food safety information among food shoppers in Lagos state. Primary data were collected by means of interview schedule through the use of a structured questionnaire. Data were analysed using descriptive statistics, cluster analysis, and ordinal regression model. A large percentage of the shoppers of pre-packaged were aware of food safety information on food labels. However, a cluster analysis of the respondents showed that only about 61.8% of consumers accorded high importance to the awareness and use of food safety information.

Analysis from the ordinal regression model showed that female, more educated consumers, and those consumers who are artisans and craftsmen were more likely to read food labels frequently. It also showed that the frequency of reading food labels could be increased among the consumers with increased response efficacy to food safety threat and awareness of food safety information. The habit of buying familiar food is a constraint to reading food labels for the average food shopper. Based on the findings of this research, it can be concluded that a large percentage of the food shoppers are aware of food safety information present on food labels but only few use them to inform purchases.

The following recommendations would be made to policy makers and other relevant stakeholders based on the findings of the research:

Educational and awareness programmes encouraging food consumers to pay closer attention to the use of food labels beyond just the product name and expiry date as food labels. This would help maximise the use of the contents of food labels especially the health and nutrition claims as consumers also check labels for health reasons.

Stakeholders on food labelling should address the challenges faced by consumers in the utilisation of food labels especially on the illegibility

of prints. Although buying familiar food seems not like an issue but legibility of print and time constraints are big issues in the reading and use of food labels.

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Optimization Conditions of UV-C Radiation Combined with Ultrasound-Assisted Extraction of Cherry Tomato (*Lycopersicon esculentum*) Lycopene Extract

Alexandre R. Lima[a, b], Nathana L. Cristofoli[a, b], Josamaique G. Veneral[a], Alcilene R. M. Fritz[a], and Margarida C. Vieira[b, c*]

[a] Department of Chemical Engineering and Food Engineering (EQA), Federal University of Santa Catarina, SC Cidade Universitária, Trindade, 88040970, Florianópolis (SC), Brazil
[b] Department of Food Engineering, Institute of Engineering, University of Algarve, Faro, Portugal
[c] MeditBio-Centre for Mediterranean Bioresources and Food, Faculty of Sciences and Technology, University of Algarve, Faro, Portugal
*Corresponding author
mvieira@ualg.pt
Tel: +351 289 800 100

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Abstract

The aim of this work was to study the effect of UV-C radiation on ultrasound assisted extraction (UAE) of cherry tomato bioactive compounds. Cherry tomatoes were exposed to two UV-C radiation doses (0.5 and 1.0 J cm^{-2}) and stored at 20 ± 0.5 °C for 7 days. Next, they were lyophilized, and the bioactive compounds were extracted by UAE at 20 KHz. To evaluate the effectiveness of the extraction process of the bioactive compounds, a CCRD (central composite rotational design) was used together with RSM (response surface methodology), for extraction times from 4 to 12 minutes and concentrations (g of lyophilized product / L of ethanol) of 1:10, 1:20 and 1:30. The extracts obtained from the irradiated tomatoes presented 5.8 times more lycopene content than the controls and higher antioxidant activity was obtained for 4 and 8 min, in the concentrations 1:10 and 1:20 (m v^{-1}). Through numerical model optimization, optimal extraction conditions were obtained. The results demonstrated that by previously irradiating tomatoes with UV-C light, the UAE yielded considerably higher amounts of lycopene and other bioactives.

Keywords: UV-C radiation; Cherry tomato, Ultrasound extraction; Lycopene

1 Introduction

Tomatoes are at the top of the list of fruits with high antioxidant power due to the presence of compounds like lycopene, flavonoids, phenolics and vitamins responsible for their antioxidant capacity (Artes, Gomez, Aguayo, Escalona, & Artes-Hernandez, 2009). Tomato's major carotenoid is lycopene which is responsible for the red color on ripe tomatoes and tomato products (Honda et al., 2017). In addition, consumption of lycopene is often associated with the prevention of some diseases, such as prostate cancer (Di Mascio, Kaiser, & Sies, 1989). Shi and Le Maguer (2000), showed that tomato, because of its innumerable evidence on cancer risk reduction, has assumed its functional food status.
UV-C radiation and ultrasounds are often applied to food to replace thermal treatments (or decrease their severity) providing products with better physical and chemical attributes. UV-C

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light application has benefical impacts in several quality parameters of fresh fruits and vegetables. UV-C light reduces maturation rate, thus improves firmness, increases pigmentation levels and shelf life (Artes et al., 2009). UV- C light is used to reduce microbial load on fruits and vegetables and others food products (Karg et al., 2008; Stevens et al., 1999). Several authors reported that after UV-C irradiation of fruits and vegetables the concentration of antioxidant compounds increased (Liu, Hu, Zhao, & Song, 2012; Majeed et al., 2016).

As well as UV-C light, ultrasound is a good technology for food preservation. High intensity ultrasound assisted extraction (UAE) is an important process that is used to extract bioactive compounds from fruits and vegetables (Riera et al., 2004). UAE does not have operational toxicity and uses low amount of organic solvents (Feng, Luo, Tao, & Chen, 2015). In addition, its low operating temperatures and high power probes that generate ultrasound are versatile and diversified for application in food industry (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). Ultrasound can stimulate diverse processes of extraction because of propagation of high-level pressure waves producing cavitation phenomenon that causes disturbance in plants' cell walls. Throught violent implosions, cavitation promotes an acceleration process of diffusion and increase of mass transfer through the surface fragmentation of the solid matrix (Chemat et al., 2017). Therefore, there is increasing erosion and surface fragmentation followed by increased of heat and mass transfer, resulting in an easier release of bioactive compounds which in turn increases the extracted yield quickly and efficiently (Chemat, Zill-e-Huma, & Khan, 2011; Vilkhu, Mawson, Simons, & Bates, 2008).

The main objective of the present study was to investigate the effect of UV-C radiation on the ultrasound-assisted extraction of bioactive compounds from cherry tomato.

2 Materials and Methods

2.1 Cherry tomato sample

Tomatoes were selected from those which had uniform size (diameter 20-30 mm), red color, about 5.5 Brix of total soluble solids, and physical integrity. Samples were washed under running water and sanitized in a 200 ppm chlorine solution for 15 minutes. After that, the fruits were separated into three groups: UV1-C and UV2-C light treatments, plus one control group (C).

2.2 UV-C light treatments and storage

UV-C light treatments were carried out with ten UV germicidal lamps (2G11-OSRAM, Munich, Germany) that emitted light at 254 nm.

Tomatoes were exposed to radiation doses of 0.5 J cm^{-2} UV1 and 1.0 J cm^{-2}. A portable radiometer (UV-Integrator - Model UV int 150+ Integration Technology Ltd., United Kingdom) was used to measure the radiation doses. The irradiated intensity was 1.5 W m^{-2} for the UV1 group and 3 W m^{-2} for the UV2 group. The irradiated cherry tomatoes were rotated 180^o horizontally and exposed to the same dose for 60 min/side on the surface. The three groups of samples (UV1-treated, UV2-treated, and control (C)) were distributed and stored in rigid plastic trays (18.0 x 12.5 x 8.0 cm) and stored at $20^oC \pm 0.5^oC$ for 7 days. Then, tomatoes without seeds were lyophilized (Freeze dryer - model L101- Liotop, São Paulo, Brazil). Tomato powder was obtained from dried samples ground in a knife grinder.

2.3 Ultrasound-assisted extraction of the lycopene from cherry powder tomatoes

An ultrasonic tip sonicator (QR500 - ECO SONICS, São Paulo, Brazil) was immersed in a beaker (200 mL) containing 5 g of sample and 50, 100 and 150 mL of ethanol as extraction solvent having concentrations of 1:10, 1:20 and 1:30 in m V^{-1}, respectively.

Power was set at 400 W (RMS), with an ultrasonic frequency of 20 kHz. The temperature was controlled and maintained at 20^{o}C using an ice bath. The extraction times selected were 4, 8 and 12 minutes. After the extraction, the extracts were filtered under vacuum, and residual solvent was removed in a vacuum rotary evaporator (Model 802-FISATOM Ltda., São Paulo, Brazil). The extracts were collected in amber glass and stored at -18^{o}C until further analyzes were performed.

2.4 Experimental design and statistical analysis

Response surface methodology (RSM) using a central composite rotational design (CCRD) and with a reduced cubic model (Design-Expert trial version 11) was employed to determine the best levels of the three independent variables (X_{UV}, UV-C radiation dose, X_{ET}, extraction time, X_{SC}, extraction solvent concentration). Three levels (-1.0, 0, +1.0) were used to evaluate the optimum combinations regarding four responses (lycopene content (LC), total phenolic compounds (TPC), trolox equivalent antioxidant capacity (TEAC) and (DPPH) radical scavenging activity).

The analysis of variance (ANOVA) and Tukey test were used to determine the statistical significance between lycopene content, antioxidant activity, and TPC values with a 95% confidence level.

UV-C radiation varied between 0.5 and 1.0 J cm^{-2} and the control (no-radiation), the ratio of extraction solvent for the solid matter ranged from 50 to 150 mL, extraction time from 4 to 12 min. All the ranges for the parameters were selected based on preliminary experimental work and literature. The range of independent variables and their levels were depicted in Table 1.

The variation of LC, TPC, TEAC and DPPH radical scavenging activity related to the three variables X_{UVC}, X_{ET} and X_{SC} were evaluated using a polynomial second degree model given by the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

Where Y is the predicted response, β_0 is the fixed response at central point, β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction coefficients, respectively. X_i and X_j are the levels of the independent variables.

2.5 Lycopene content (LC)

Lycopene content of the cherry tomatoes extracts (C, UV1 and UV2) was quantified by spectrophotometry according to methodology proposed by Fish, Perkins-Veazie, and Collins (2002) with modifications. The absorbance was measured at 503 nm with a spectrophotometer (Hitachi UV/Vis U-2000 - Hitachi Ltd., Tokyo, Japan). The lycopene content of samples was estimated using $LC = (A_{503}\ x\ 31.2)/g_{extract}$ (where 31.2 g cm^{-1} is the extinction coefficient for lycopene in hexane) and expressed as mg g^{-1} (Fish et al., 2002).

Total phenolic compounds (TPC)

The total phenolic content was quantifield using the Folin-Ciocalteu method described by Singleton, Orthofer, and Lamuela-Raventos (1999). The absorbance was measured at 765 nm in spectrophometer. The TPC was calculated using a standard curve prepared previously with gallic acid as a standard compound. The assays were performed in triplicates and the average results were expressed as milligrams of gallic acid equivalent (GAE) per gram extract (mg GAE g^{-1}) ± standard deviation.

2.6 Antioxidant activity (AA)

Trolox equivalent antioxidant capacity assay (TEAC)

TEAC assay was employed to measure AA capacity based on the procedure described by Re et al. (1999). The absorbance was measured at 734 nm in spectrophotometer. Results were expressed as TEAC values (μg Trolox g^{-1}). In order to find TEAC values, a separate concentration response curve for standard Trolox solutions was prepared and results were presented by average ± standard deviation from triplicate assays.

Table 1: Independent variables and their respective levels

Independent variable	Symbol	Level		
		-1	0	1
UV-C radiation (J/cm^2)	X_{UVC}	0*	0.5	1.0
Extraction time (min)	X_{ET}	4	8	12
Solvent concentration (mL)	X_{SC}	50	100	150

Free radical scavenging activity (DPPH)

The free radical scavenging of cherry tomato extracts was evaluated using the (DPPH) method according to the procedure reported by Mensor et al. (2001). The absorbance values were measured in spectrophotometer at 517 nm and converted into percentage of antioxidant activity (AA%). This activity was also presented as the effective concentration at 50% (EC50), which is the solution concentration required to give 50% decrease in the absorbance of the test solution compared to a blank solution and expressed in $\mu g_{extract}$ mL^{-1}. The EC_{50} values were calculated by linear regression from the curves of the AA% obtained for all extract concentrations. The AA% and EC_{50} for all extracts were obtained by taking the average of triplicate assays.

3 Results and Discussions

3.1 Fit of models and obtained responses

The results for LC, TPC, TEAC, DPPH radical scavenging activity analysis are presented in Table 2.

Experimental variables, UV-C radiation dose, extraction time, and solid/liquid ratio (solvent concentration) were the determinants of the combination of UV-C radiation and UAE technologies.

The results of ANOVA showed that the models were significant with good determination coefficients (R^2) for lycopene content, TPC, TEAC and DPPH radical scavenging activity, implying that the correlation between response and independent variables was satisfactory (p> 0.05). In addition, the F-values and p-values were obtained for the reduced cubic model (Table 3). Thus, values of R^2 closer to 1.0 mean higher accuracy of the model.

The high values for predicted and adjusted determination coefficients also illustrate the model suitability in relation to the experimental data (Badwaik, Prasad, & C. Deka, 2012). Adequance Precision (Table 3) measures the signal to noise ratio and a ratio greater than 4 is desirable. By observing the values (Table 3), all are higher than the desired minimum value and it is possible to state that the proposed models can be used to navigate the design space.

3.2 Checking assumptions with residual plots

Through the use of residual plots, it is possible to investigate the normality, constant variation and linearity hypothesis of the simple linear regression model. If these assumptions are true, then the observed residual using the equation $e_i = y_i - \hat{y}_1$ (where, e_i: observed sample residue; y_i: y-value observed; $\hat{y}_1$: y-value predicted) should behave in a similar fashion.

Therefore, it is possible to observe the error terms of distribution normality, constant variance along X and the values independence of X, through the analysis of the graphs residual vs run (Fig. 1D, 2D, 3D and 4D).

In the interval between -3.6 and 3.6, it can be observed that for any of the dependent variables, the residual plots contradict the linearity assumption, behaving in a nonlinear way with a random distribution throughout the space of X axis. These residual plots prove that the assump-

Table 2: Values of response variables (LC, TPC, TEAC and DPPH) obtained at treatment conditions used in experimental design by RSM

UV-C treatments	UAE t (min)	UAE C (m:v)	LC (mg g^{-1})	TPC (mg GAE g^{-1})	TEAC (μM TEAC g^{-1})	DPPH EC50 (μg mL^{-1})
C (control)	4	1:10	24.4 ± 0.8	15.8 ± 2.2	137.5 ± 0.4	565.5 ± 1.3
	4	1:20	32.4 ± 0.8	12.7 ± 1.6	107.8 ± 0.6	689.4 ± 3
	4	1:30	31.9 ± 1	17.1 ± 1.7	97.5 ± 1.8	679.9 ± 1.4
	8	1:10	27.7 ± 1.1	9.8 ± 0.5	241.3 ± 1	903.5 ± 4
	8	1:20	25.2 ± 0.6	36.1 ± 2.2	128.1 ± 2.5	632.3 ± 7.2
	8	1:30	17.4 ± 0.6	14.1 ± 0.9	105.7 ± 0.1	688.3 ± 2.4
	12	1:10	21.4 ± 0.9	15.4 ± 0.5	107.14 ± 0.1	668.7 ± 2.5
	12	1:20	14.6 ± 0.9	16.6 ± 0.9	106.2 ± 0.2	515.1 ± 2.7
	12	1:30	19.9 ± 0.7	9.4 ±0.9	144.6 ± 0.3	424.8 ± 2.3
UV1 (0.5 J/cm^2)	4	1:10	147.5±0.3	41.5 ± 1.1	258.1 ± 2.4	471.6 ± 4.7
	4	1:20	132.5 ± 0.7	22.9 ± 1.4	194.5 ± 0.5	712.5 ± 2.8
	4	1:30	146.2 ± 0.6	23.6 ± 1.6	147.2 ± 3.2	401.5 ± 1.6
	8	1:10	143.4 ± 0.6	16.5 ± 1.8	137.9 ± 0.5	530.8 ± 2.6
	8	1:20	168.2 ± 0.7	24 ± 1.8	214.8 ± 1.1	351.4 ± 4.4
	8	1:30	147.1 ± 0.8	19.5 ± 0.8	132.5 ± 0.4	392.8 ± 2.5
	12	1:10	146 ± 0.9	17.3 ± 1.3	135.3 ± 0.6	312.0 ± 9
	12	1:20	162.7 ± 0.4	18.1 ± 1.5	198.4 ± 1	513.7 ± 1.8
	12	1:30	155.4 ± 0.6	19.4 ± 1	279.7 ± 1.3	336.3 ± 2.2
UV2 (1.0 J/cm^2)	4	1:10	139.4 ± 0.9	22.7 ± 0.4	268.7 ± 0.7	511 ± 2.3
	4	1:20	135.4 ± 0.4	14.7 ± 0.9	100.6 ± 0.2	655 ± 4
	4	1:30	144.3 ± 0.6	15.9 ± 0.7	169.9 ± 0.7	645.9 ± 3.3
	8	1:10	130.5 ± 0.8	44.4 ± 1.7	241.9 ± 0.4	383.1 ± 2.8
	8	1:20	139.9 ± 0.6	20.3 ± 1.4	352.2 ± 2.2	596.9 ± 5.1
	8	1:30	143.6 ± 0.2	18.7 ± 0.8	171.4 ± 0.8	745.5 ± 4
	12	1:10	139.6 ± 0.1	34.4 ± 2	218.7 ± 1.3	550.7 ± 1.1
	12	1:20	138.1 ± 0.6	31.9 ± 2.4	94.3 ± 0.2	586.9 ± 5.3
	12	1:30	139.1 ± 0.4	15.6 ± 1	94.7 ± 0.4	668.0 ± 1.3
*BHT				465.5 ± 3.1	1039 ± 2.2	72.2 ± 3.5

Table 3: Fit Statistics for coefficient regression (ANOVA)

Response variables	R^2	R^2 Adjusted	R^2 Predicted	Adeq. Precision	F-value	p-value
LC	0.9926	0.9906	0.9883	54.525	449,97	<0,0001
TPC	0.8796	0.8423	0.7963	16.752	23,63	<0,0001
TEAC	0.8661	0.8276	0.7783	17.433	49,83	<0,0001
DPPH	0.9432	0.9243	0.9020	23.004	22,46	<0,0001

tions of our model be real and do not present nothing abnormality.

3.3 Effects of UV-C light and UAE treatments on the lycopene content

Equation (2) describes the relationship between the significant independent variables and the lycopene content response. For optimization purposes, the second-order polynomial empirical model is widely accepted (Myers, Montgomery, & Anderson-Cook, 2016). The factors were coded, and the the p-values were less than 0.0001.

$$LC = +105.49 - 30.777X_{sc} + 0.52X_{UVC}X_{sc} \quad (2)$$

By performing the ANOVA analysis, the cubic reduction model is significant for LC, with an F 499.47 and $p < 0.0001$. The regression coefficient of LC ($R^2 = 0.9926$) showing an optimal fit of the model to the experimental values. In addition, the R^2 predicted ($R^2 = 0.9883$) agrees with the R^2 coefficient ($R^2 = 0.9906$) (Table 3). The extracts from the UV1 and UV2 groups yielded mean lycopene content 5.8 times higher than the extracts from the control (C), as represented in Table 2 and Fig. 1.

This result corroborates the results obtained by Pataro, Sinik, Capitoli, Donsi, and Ferrari (2015) which obtained 5.23 times more LC in the irradiated tomatoes compared to the control, using a dose of 2000 mJ cm^{-2}, higher than that used and 60 min irradiation time, (similar to the present study).

The increase in lycopene content in tomato as a function of UV-C light treatment was already observed by other researchers. Segovia-Bravo, Guignon, Bermejo-Prada, Sanz, and Otero (2012) reported an increase of 1.2 to 1.8 times in the LC in tomatoes stored at room temperature for 8 days at doses ranging from 0.1 to 1.2 J cm^{-2}. Likewise, Liu, Zabaras, Bennett, Aguas, and Woonton (2009) observed an increase in the lycopene content, but from irradiated green tomatoes with a dose of 1.37 J m^{-2}, in a storage cycle that lasted 21 days, this increase was only significant from the 15^{th} day on, when compared to untreated tomatoes. Although, in the present work the process conditions and origin of the raw material are different from those used in the literature, the same trend in the results is observed.

The control samples (C) differed significantly among the samples under the extraction conditions studied. The highest content was 32.4 and 31.9 mg g^{-1} after the 4 min extraction, at the concentrations of 1:20 and 1:30 m v^{-1} solvent, respectively (Table 2).

3.4 Effects of UV-C light and UAE treatments on TPC values

The phenolic content is related to stress conditions that plants undergo while being processed and radiation, mainly UV light, is one of the factors that can influence changes in the amount of this compound in certain vegetable species (Reay & Lancaster, 2001). Another factor is the temperature control of UAE, taking into account that low extraction temperatures lead to higher yields of secondary metabolites such as as phenolics compounds (Ma, Chen, Liu, & Ye, 2009). Mathematical model (Eq. 3) correlates total phenolic content (TPC) with process variables in terms of coded factors excluding non-significant terms, with the ratio represented by ANOVA with $R^2 = 0.8796$ (Table 3), where the p-value was less than 0.0001:

$$\begin{aligned} TPC = {} & +19.62 - 3.59X_{ET} - 4.79X_{SC} \\ & - 5.54X_{UVC}X_{SC} + 4.85X_{ET}X_{SC} \\ & + 3.03X_{UVC}X_{ET}X_{SC} + 7.01X_{UVC}^2X_{SC} \end{aligned} \quad (3)$$

TPC content of the extracts ranged from 9.4 to 44.4 mg EAG g^{-1}, values well below the 465.5 mg EAG g^{-1} obtained for the synthetic antioxidant BHT, which differed significantly from all extracts ($P < 0.05$) (Table 2).

Among the samples from tomatoes treated with UV-C radiation there was no significant difference that induced the increase of the TPC content, even when compared to the control (C) extracts. This result shows that UV-C radiation, in the doses used, did not induce large accumulation of phenolic compounds in the samples.

Among the extraction conditions evaluated, the

best results obtained were for conditions with lower ethanol concentration (1:10) combined with extraction times of 4 and 8 minutes, as shown (Fig. 2). The choice of ethanol as the solvent, was based on the results of previous studies, in which ethanol was the one that presented the best results among many solvents used to extract phenolic compounds from vegetable matrices, which is associated with its polar characteristic (Biscaia & Ferreira, 2009). Also, the intermiscibility theory states that phenolic compounds are easily dissolved from plant cells when there is similarity between phenolic and solvent polarities (Majeed et al., 2016).

The results obtained for TPC emphasize that the use of lower solvent concentrations and reduced and moderate extraction times, lead to more favorable results for phenolic compounds. This behavior partially corroborates results obtained by Feng et al. (2015), who observed increased phenolic extraction with increasing solvent concentration at the beginning of extraction but noted the inverse behavior when the rate of phenolic extraction reached a certain value, considering that at this moment the polarity of the solvent was inverted, and the extraction started to decrease.

In general, the exposure time is the main factor may have contributed to different results in relation to those found in the literature, but the tomato variety, harvesting and storage conditions, equipment type, arrangement of the fruits during the irradiation and the maturation differences are considerable factors. The time of 60 minutes may not have been enough for the UV-C light to induce a stress response of the fruits that provoked the activation of the phenolic biosynthesis pathway, leading to the accumulation of phenolic compounds, as well as the low UV-C radiation doses used.

3.5 Response surface analysis of TEAC and effects of UV-C radiation and UAE

The second-order polynomial model correlating TEAC and the three variables in this study was obtained in Eq. 4, excluding non-significant terms, below:

$$\begin{aligned} TEAC = {} & +148.81 - 27.70X_{ET} + 29.1X_{SC} \\ & + 23.33X_{UVC}X_{ET} + 21.24X_{ET}X_{SC} \\ & + 30.21X_{UVC}X_{ET}X_{SC} + 6.87X_{UVC}^2X_{SC} \\ & - 61.32X_{ET}^2X_{SC} \end{aligned} \quad (4)$$

The statistical significance of the model was checked by the F-test where the model F-value of 22.46 implied that the model to be significant ($p < 0.0001$). The regression coefficient ($R^2 = 0.8661$) of the experimental model also indicated the model suggested to be a good fit for combining the UV-C and UAE technologies in the antioxidant compounds extraction and showed an acceptable relation with the predicted value ($R^2 = 0.7783$) (Table 3).

UV-C treatment ($p < 0.0001$) and solvent concentration ($p < 0.0001$) had a significant effect on the antioxidant activity, as well as its interactive effects. The extraction time ($p = 0.0317$) as well as its interactive terms had no significant effect on antioxidant activity by the TEAC method.

From the observation of results (Table 2), none of the UV-C treatments combined with the UAE yielded better results better results than the obtained for the synthetic compound BHT (1039 μM TEAC g^{-1}). However, the treatment with UV-C positively influenced the extracts of the UV1 and UV2 groups, not being significantly different from each other, but with a significant difference in relation to the control (C) samples (Fig. 3).

Low radiation doses can promote a significant increase in antioxidant activity in postharvest tomatoes, agreeing with Ribeiro, Canada, and Alvarenga (2012), as low doses of UV-C radiation (<1000 mJ cm^{-2}) promote the formation and increase the bioactive compounds with antioxidant functions, increasing the nutritional value of the treated products.

Samples extracted at times (4 and 8 min) and concentrations (1:10 and 1:20 m V^{-1}), in the 3 groups, presented AA significantly higher than the other extracts. These results characterize a favorable behavior of the extraction conditions in relation to the antioxidant activity.

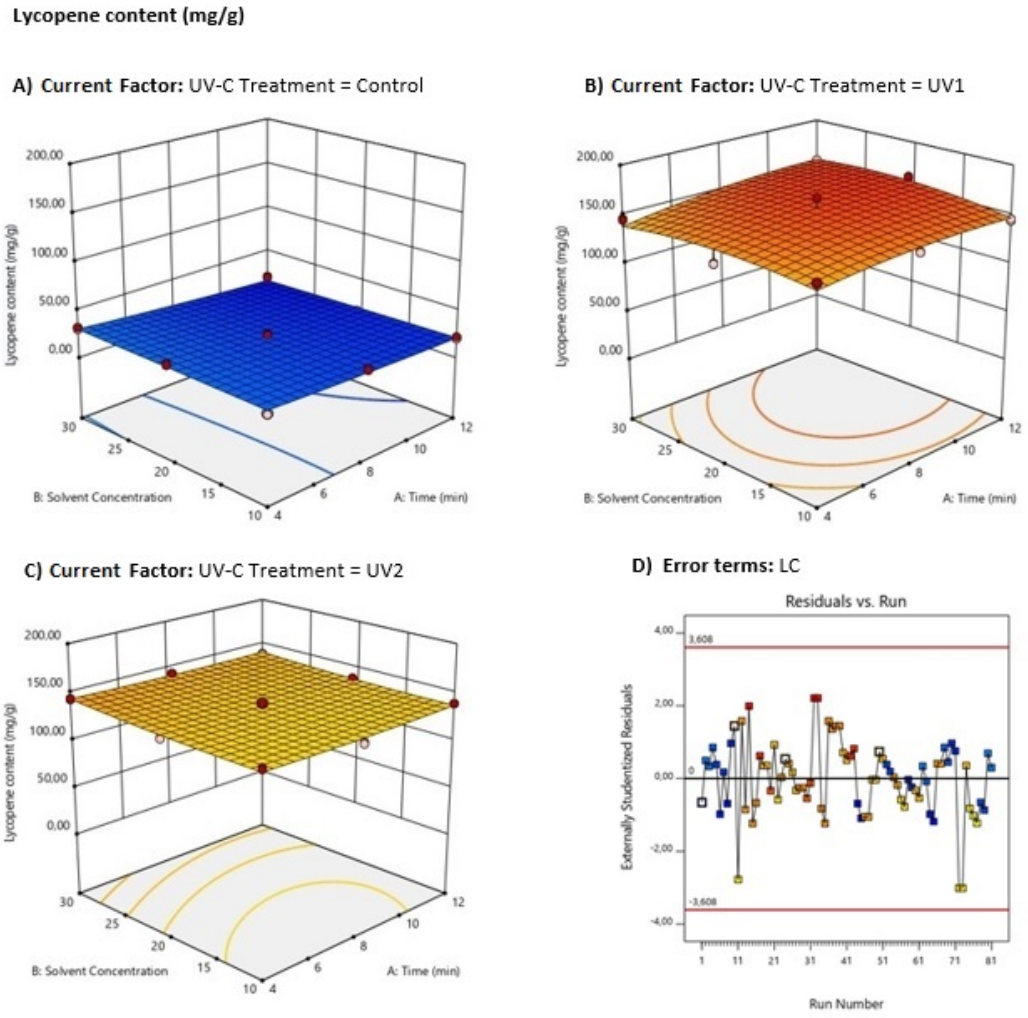

Figure 1: Response surface plots (3D) of LC extraction produced by the polynomial model (eq. 2) (A) UAE; (B) UV-C1 + UAE; (C) UV-C 2 + UAE; and (D) Analysis of error terms

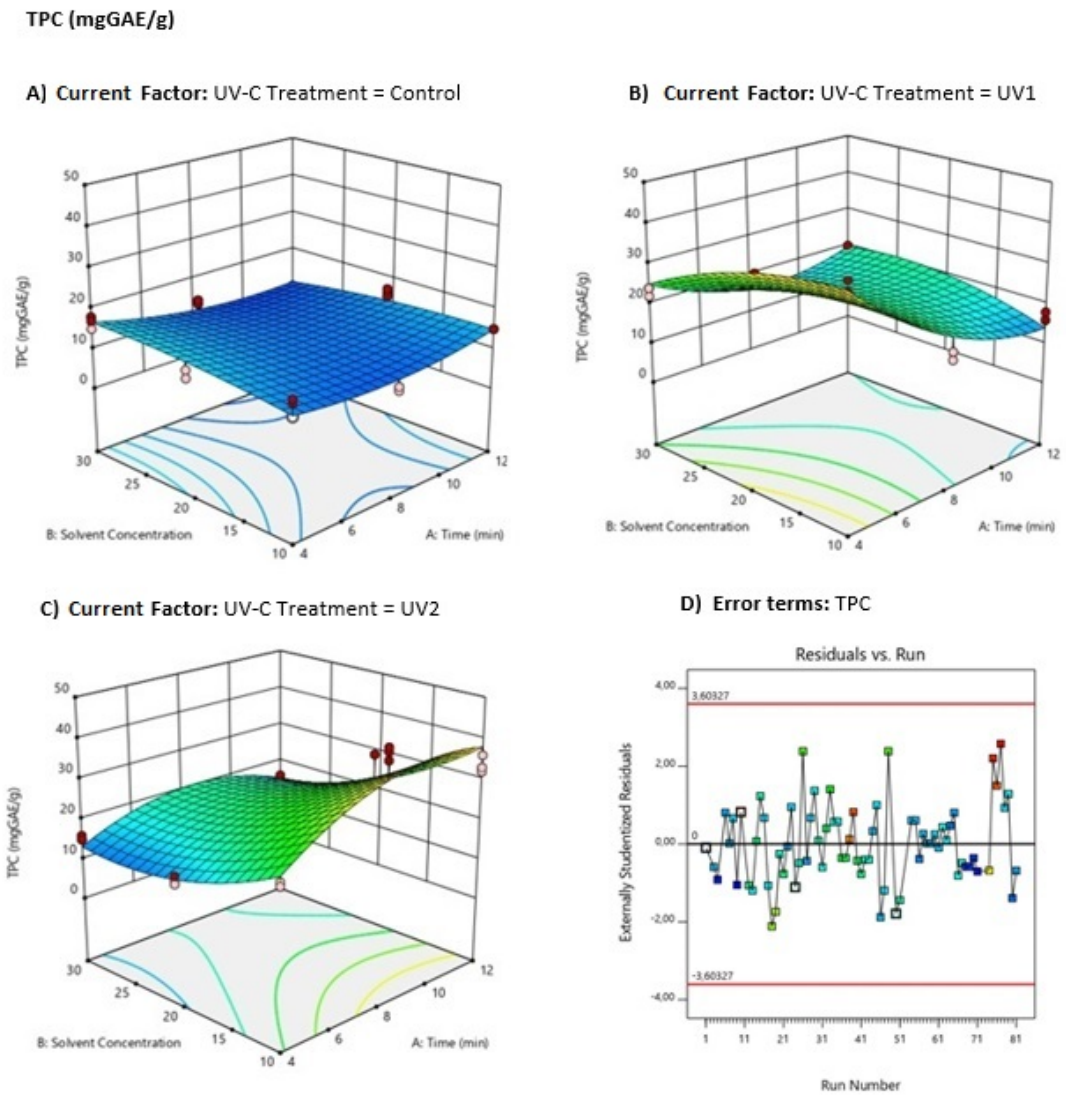

Figure 2: Response surface plots (3D) of TPC analysis as a function of significant interaction between factors; (A) UV-C treatment and time; (B) UV treatment and solvent concentration; (C) time and solvent concentration; (D) Analysis of error terms

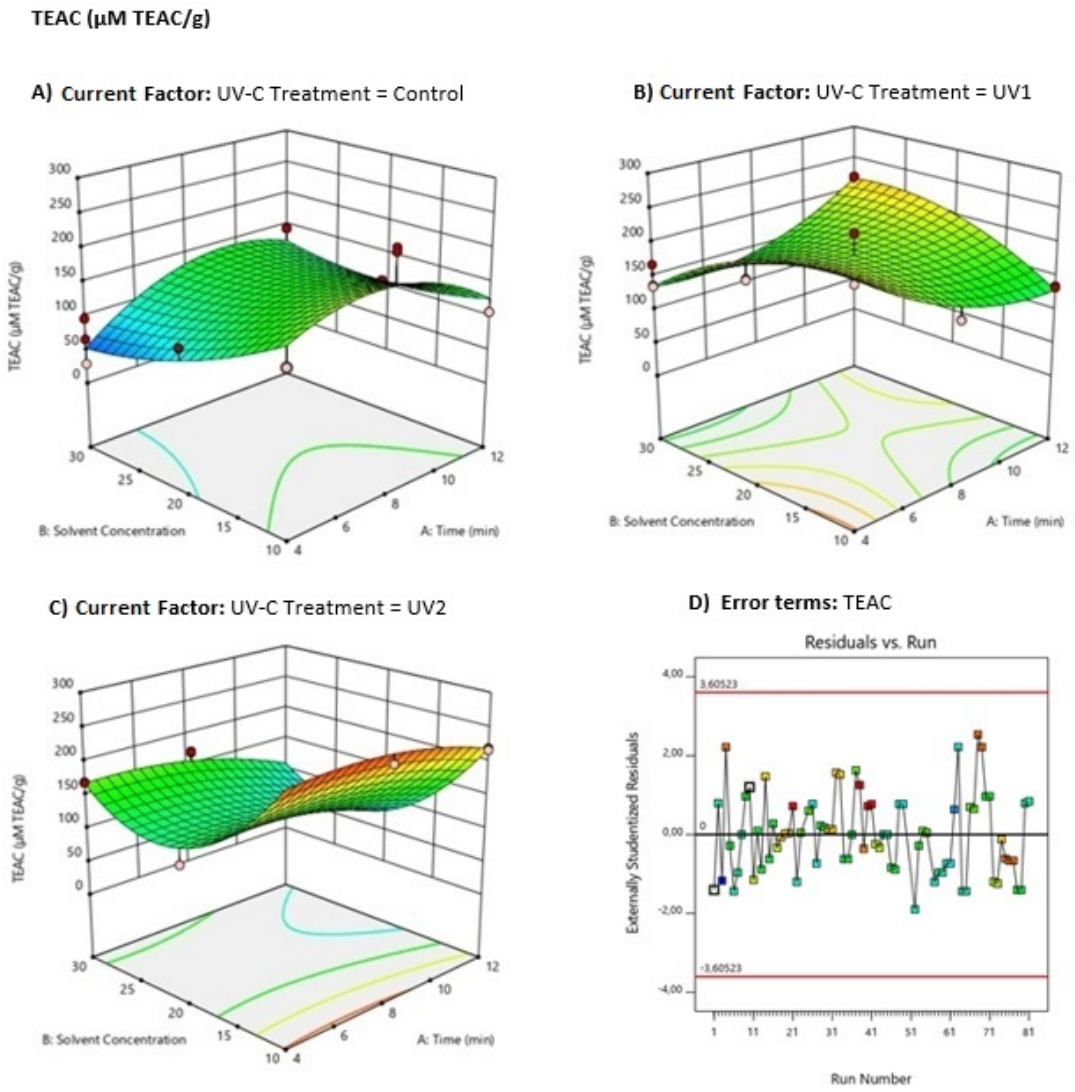

Figure 3: Response surface plots (3D) of antioxidant activity (TEAC analysis) as a function of significant interaction between factors; (A) UV-C treatment and time; (B) UV treatment and solvent concentration; (C) time and solvent concentration; (D) Analysis of error terms

3.6 Effects of UV-C radiation and UAE on AA by DPPH method and RSM

The AA results of the cherry tomato extracts obtained by the DPPH radical capture method are shown in (Table 2) and compared to the result found for the synthetic antioxidant BHT of 72.2 μg mL^{-1}.

The mathematical model Eq. 5 demonstrates the relationship among the DPPH scavenging activity and significant independent variables. For the model fitted, the sample variation of 94.32% for the DPPH radical scavenging activity was related to the independent variables, and only 5.68% of the total variation could not be explained by the model (Table 3). p-value was less than 0.0001% and the model was valid.

Following Eq. 5 represents the coded extraction parameters of the DPPH optimization analysis:

$$\begin{aligned} DPPH = &+663.08 - 42.46X_{UVC} + 107.75X_{SC} \\ &- 23.83X_{UVC}X_{ET} + 15.72X_{UVC}X_{SC} \\ &- 69.04X_{ET}X_{SC} - 103.83X_{ET}^2 - 18.09 \\ &- X_{UVE}X_{EX}X_{SC} - 129.75X_{UVC}^2X_{SC} \\ &- 44.58X_{ET}^2X_{SC} \end{aligned} \quad (5)$$

UV-C radiation was effective in providing an increase in antioxidant activity in the extracted samples. The best DPPH value was obtained in the irradiated samples that were extracted at the moderate and higher times (8 and 12 min.), respectively. The ANOVA identified that the solvent concentration (SC) of UAE was not significant (p-value <0.0001%) for TEAC of the samples (Fig. 4).

The treatments with UV-C of the UV1 group (593 mJ cm^{-2} dose, intensity of 1.5 W m^{-2}), presented higher AA, with a significant difference (P<0.05) for the other treatments. The results were above 250 μg mL^{-1}, a limit value for materials with high antioxidant potential (de Campos, Leimann, Pedrosa, & Ferreira, 2008). In general, the low antioxidant capacity found can be attributed mainly to the decrease in the phenolic compounds content (Fig. 2).

The data currently found in literature on the effects of low-dose UV-C treatment on the antioxidant potential are not only scarce, but also present contradictory conclusions. However, some studies corroborate the results presented in this research. Liu et al. (2012), Pataro et al. (2015), Segovia-Bravo et al. (2012) observed positive effects of UV-C treatment at low doses on distinct tomatoes varieties and maturation stages after storage period. There was no significant effect of UV-C radiation on AA in tomatoes (Jagadeesh et al., 2011) and mushrooms (Guan, Fan, & Yan, 2012). These studies are in line with the results obtained in the present study for UV2 treatments (doses of 992 mJ cm^{-2}) (Table 2).

The significantly (P<0.05) better results obtained for AA were 312.0 and 336.3 μg mL-1, after 12-min extraction with solvent concentrations of 1:10 and 1:30 m V-1, which were not the best conditions for TPC (Table 2). This indicates that the antioxidant activity of the extracts tested was not directly related to phenolic content. Therefore, it can be concluded that AA depends not only on the presence of the phenolic compounds, but also on other compounds with high antioxidant activity that may have had their bioavailability reduced or impaired due to factors inherent to the experimental conditions.

3.7 Optimization of the experimental model

An optimization process was carried out to determine the best conditions for the combination process, which yield the maximum LC extraction, maximum TPC and antioxidant activity (lower TEAC and maximum DPPH). The results are shown in Fig. 5.

The optimal conditions selected were a combination of UV2 treatment with UAE during 8.5 min with a minimum solvent concentration (1:10). Under these conditions the predicted values for all the parameters evaluated are presented in Table 2. Lycopene (135.28 mg g^{-1}), TPC (38.17 mg GAE g^{-1}), antioxidant activity (252.6 μM TEAC g^{-1}) and DPPH radical scavenging activity (425.8 μg g^{-1}) were within a 95% mean confidence interval of the experimental values coinciding with two experimental points used in the model, as summarized above, suggesting a good correlation between observed and predicted val-

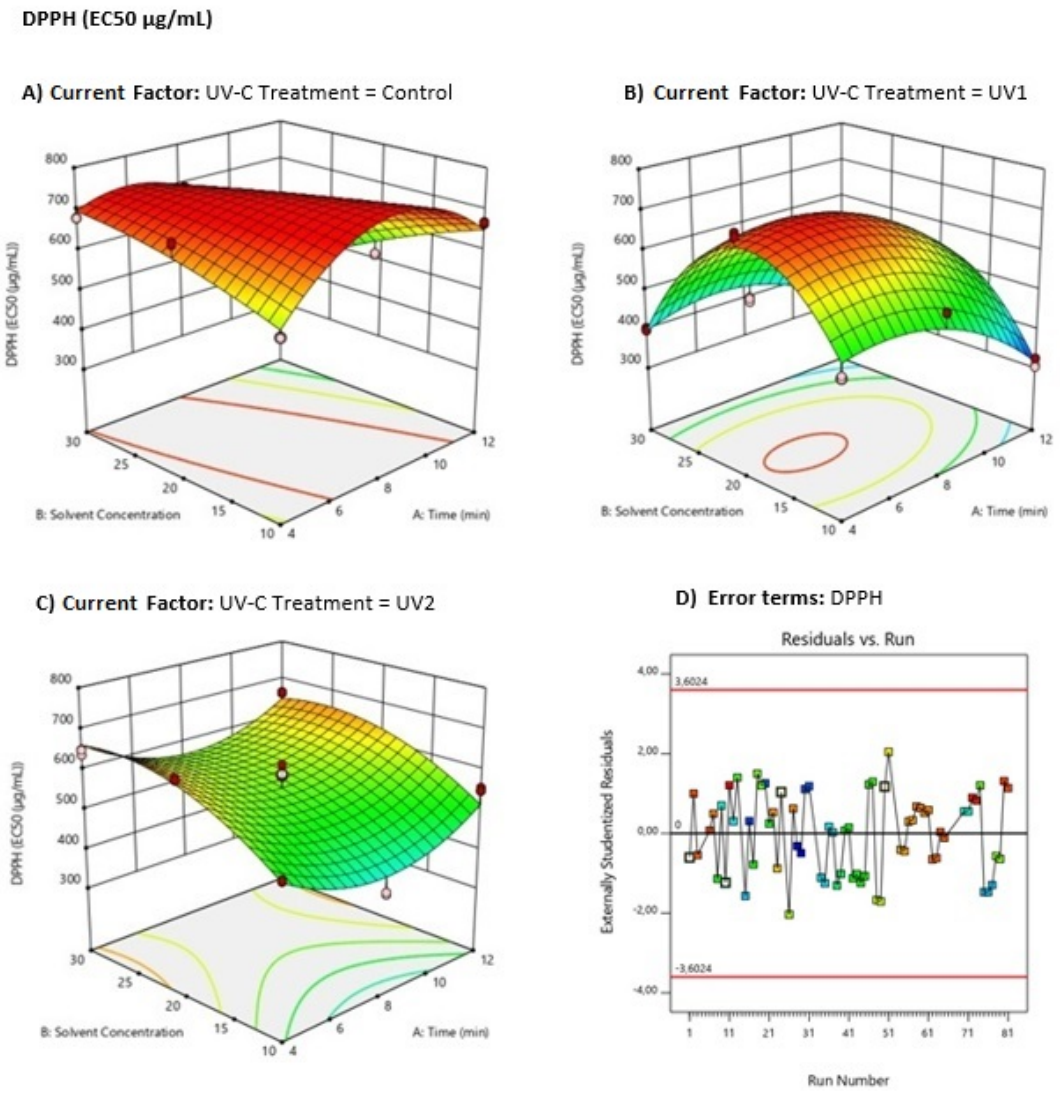

Figure 4: Response surface plots (3D) of total antioxidant activity (DPPH analysis) as a function of significant interaction between factors; (A) UV-C treatment and time; (B) UV treatment and solvent concentration; (C) time and solvent concentration; (D) Analysis of error terms

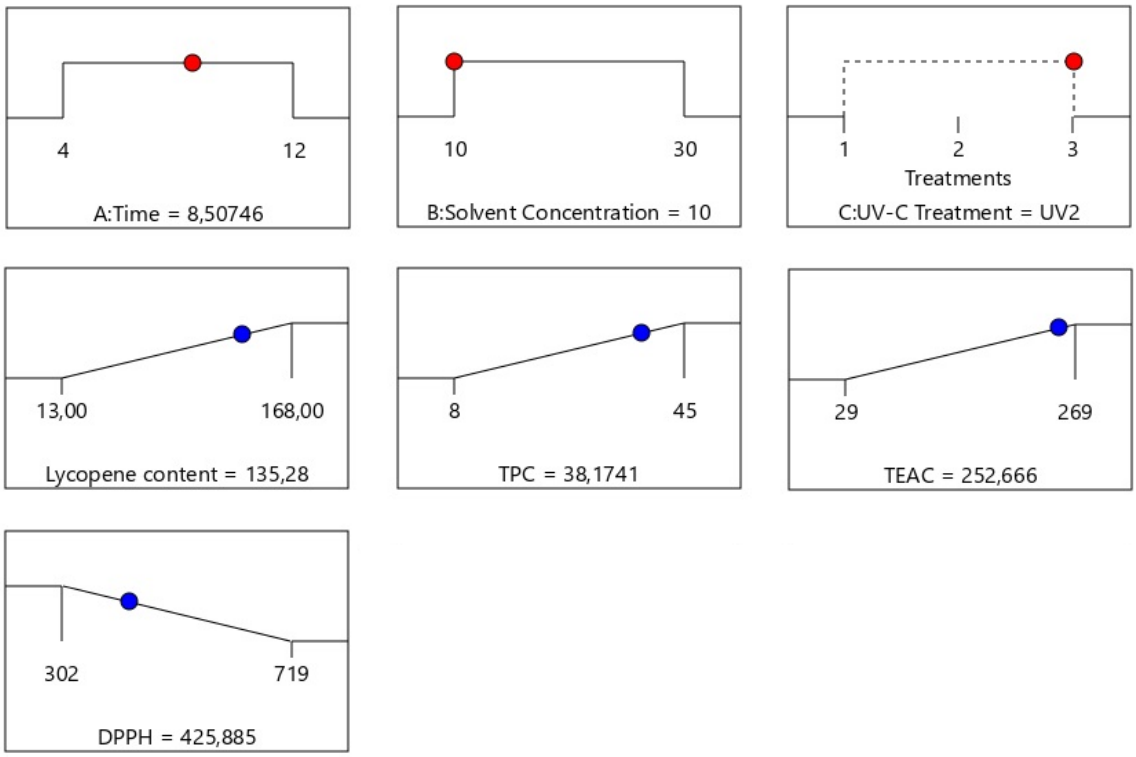

Figure 5: Graphical representation of process optimization

ues.

Based on optimized results it is possible to evaluate the UAE efficiency by observing the low solvent consumption associated with the low extraction time and the easy reproducibility of this technique, demonstrating its high process industrialization potential (Chemat et al., 2017).

In addition to these factors, the good results obtained using ethanol (compounds with antioxidant activity) show that the technology adapts to green solvents, and therefore contributing to environmental impact reduction (Sicaire et al., 2016). These responses are in agreement with Cristofoli, Lima, Vieira, Andrade, and Ferreira (2018) that also obtained consistent results througth UAE with ethanol for compounds with antioxidant activity in another food matrix when compared to other technologies.

4 Conclusion

This first attempt to investigate and optimize the combination of UV-C radiation and UAE technologies to obtain cherry tomato extracts (*Lycopersicon esculentum* Mill) with high antioxidant bioavailability was effective.

The results obtained suggest strongly that the combination of the UV-C radiation treatment with the UAE process was effective not only in promoting oxidative stress in the raw fruit contributing to the increase of lycopene and the antioxidant content availability in these fruits, but also to help the release of these bioactive compounds from tomato matrix. This study may therefore serve as guidance to industrial extraction processes at environmental temperature and short-time periods by using emerging technologies such as UV-C and ultrasounds promoting the increase of value-added products with a high bioactive compounds index.

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Application of Response Surface Methodology to Optimize the Drying Conditions of Black Tea using a Superheated Steam Dryer

Rumaisa Nordin[a, b], Norazatul H. M. Rozalli[a*], and Tajul A. Yang[a]

[a] Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

[b] Department of Chemical Engineering Technology (Food Technology), Faculty of Engineering Technology, University Malaysia Perlis (UNIMAP), UNICITI Alam Campus, 02100 Sg. Chucuh, Padang Besar, Perlis, Malaysia

*Corresponding author

norazatulhanim@usm.my

Tel: +604 653 6361

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Abstract

Drying conditions of black tea using superheated steam (SHS) were optimized based on a central composite design (CCD) of response surface methodology (RSM). The effects of independent variables (temperature and time) towards the total phenolic content (TPC), scavenging free radical ability, ferric-reducing antioxidant power assay and sensory attributes (infusion colour, aroma, flavour and astringency) were analysed using regression polynomial equations. Analysis of variance (ANOVA) showed that a quadratic model fitted the experimental design well, with a p-value less than 0.05 and a highest coefficient of 0.9474. The optimum drying conditions were achieved at 166.7^{o}C and 21.2 minutes where the experimental values were in accordance with predicted values, with percentage errors (PE) less than 10%. Temperature (140^{o}C – 180^{o}C) was concluded to have the most significant effect and showed a positive direction in most responses. This effect was definitely able to support the application of a SHS dryer for the quality production (antioxidant and sensory properties) of black tea.

Keywords: Drying; Black tea; Superheated steam; Response surface methodology; Antioxidant; Sensory properties

1 Introduction

Tea is consumed and well-liked worldwide due to its pleasant flavour and health benefits. Black tea accounts for 80% of tea consumed in the world (Tea Fact Sheet, 2016). Special attention had been paid to processing technologies because they directly influence the quality of tea produced (Botheju, Amarathunge, & Abeysinghe, 2011). The drying process is especially responsible for finalising the flavour of tea and removing undesired moisture to give a longer shelf life (Kavish, S. Botheju, & De Silva, 2016).

Drying is an energy demanding process that consumes around 10-15% of national energy in most developing countries (Mujumdar & Devahastin, 2000). The same authors mentioned most conventional dryers use hot air that consume a large amount of energy and cause inefficiency during drying. Furthermore, traditional hot air dryers, where firewood (wooden logs) are the main source of energy, are used, especially in Kenya, Sri Lanka and Malaysia, for tea drying (Wickramasinghe & Munasinghe, 2014). This causes

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adverse environmental impacts in terms of deforestation and air pollution (the release of carbon dioxide after burning).

SHS has been widely proposed by researchers as an effective solution to these problems in food drying. SHS is produced when saturated steam is further heated under constant pressure. SHS drying permits the recovery of the latent heat through condensation of exhaust steam, while in hot air drying this retrieval of heat in the released humid air is hard to achieve (Romdhana, Bonazzi, & Decloux, 2015). SHS drying of food offers many advantages such as high energy efficiency (due to a higher drying rate than hot air), low net energy consumption, elimination or reduction of odour emission, oxygen-free environment (no fire and explosion hazards), lower nutrient loss and less impact on quality (colour, texture, shrinkage and rehydration) (Mujumdar, 2006). Several researchers have applied SHS drying to foodstuffs such as potatoes (Tang & Cenkowski, 2000), cocoa beans (Zzaman, Bhat, & Yang, 2014) and noodles (Markowski, Cenkowski, Hatcher, Dexter, & Edwards, 2003). Previous work, done on drying mate leaves, claimed that a higher concentration of phenol content was achieved by applying SHS drying as compared with conventional hot air drying. Recently, Zzaman et al. (2014) also observed that a minimal loss of antioxidant properties and nutrients can be achieved using a SHS dryer when heat treating cocoa beans.

The effects of drying temperature and time may be vital for optimization of the processing methods used to obtain a cup of black tea with good flavour (Kaack & P. Christensen, 2008). RSM is a powerful tool that enables optimal drying conditions to be generated with fewer experimental runs (Chakraborty, Bera, Mukhopadhyay, & Bhattacharya, 2011). This statistical technique can also evaluate the relationship between independent and dependent variables, and describe the effects of all combined variables (Erbay & Icier, 2009). Even with much work done, using RSM for optimization of food processes, there is a lack of literature covering the effects of drying conditions on black tea product quality using a SHS dryer. Therefore, the aim of this research is to optimize the performance of a SHS dryer for the quality production of black tea. The quality attributes of concern are phenolic content, antioxidant ability and sensory qualities (liquid colour, aroma, flavour and astringency taste). Additionally, there are limited comprehensive studies dealing with the correlation between chemical composition, antioxidant activity and sensory attributes. Herein, this study focused on providing a better understanding of the relationship between these variables, with the aim of ultimately improving product quality in the development and production of black tea.

2 Materials and Methods

2.1 Drying procedure

Freshly fermented tea leaves (60%, moisture content), collected from the BOH Sg Palas plantation, Cameron Highland, Malaysia, were dried using a superheated steam dryer (Healsio, AX-1500 V, SHARP, Osaka, Japan). The dryer consisted of a 900 W steam heater and a backward-curved blade centrifugal fan, and had a 16 cc/min steam generation power (Zzaman et al., 2014). Tea leaves were thinly spread as a single layer on a steel broil pan (359 × 256 × 339 mm). Steam generated by the boiler was continuously heated until it reached the superheated state. Once steam reached the superheated state, the SHS dryer was fully operated on pure steam. The heat was then transferred onto the product by the centrifugal fan to dry and evaporate the water. The temperature and time ranges were between 140-180^{o}C and 20-25 minutes respectively. These ranges were chosen based on preliminary results to produce tea with a moisture level of 3-4%. After drying, the samples were left to cool in a desiccator at room temperature before being vacuum packed into fully metalized bags, for storage prior to analysis.

2.2 Optimization of Superheated Steam Drying

RSM was used to investigate the effects of two independent variables, namely temperature (^{o}C) and time (minutes), on seven dependent variables which are TPC, scavenging free radical, reducing power assay, and sensory attributes of tea

Table 1: Face centered CCD settings in the original and coded form of the independent variables (X_1, X_2) for black tea drying using a SHS dryer

Runs	Temperature (^oC)		Time (min)	
	Coded Level (x_1)	Actual Level (X_1)	Coded Level (x_1)	Actual Level (X_2)
1	1	180	1	25
*2	0	160	0	22.5
*3	0	160	0	22.5
4	-1	140	1	25
5	-1	140	-1	20
6	0	160	1	25
7	-1	140	0	22.5
8	1	180	0	22.5
9	0	160	-1	20
*10	0	160	0	22.5
11	1	180	-1	20
*12	0	160	0	22.5
*13	0	160	0	22.5

infusion colour, aroma, flavour and mouth astringency. The two factors (X_1 and X_2) and three levels (-1, 0 and 1) of a face centred CCD were chosen for all the variables within the desired ranges. A total of 13 experimental runs was obtained, including five centre points in the design performed for estimation of pure error. Table 1 shows the experimental design generated using Design Expert Version 6.0.10 (Stat-Ease Inc., Minneapolis, USA). Experiments were conducted in a random order and repeated twice for the 13 runs to minimize error due to extraneous factors.

Statistical analyses were performed to evaluate an analysis of variance (ANOVA) of the data obtained. Criteria for all statistical significance followed the rule of having a 5% significant level ($p \leq 0.05$). Model adequacy was determined with a coefficient of determination (R^2) value above 80% and an insignificant lack of fit ($p > 0.05$) as the best indicators for model fitting with experimental data (Myers, Anderson-Cook, & Montgomery, 2016).

2.3 Validation of model

Optimum conditions to dry tea leaves using SHS were provided by RSM based on the maximum value of TPC, antioxidant activity and target scores for sensory attributes. Predicted values of all the responses were also derived from the optimum model. Under these conditions, a validation experiment was carried out to verify the adequacy of the model equation. Experimental values obtained were compared with predicted values by calculating their percentage error (PE). The PE should be less than 10% to indicate a good fit.

$$PE(\%) = \frac{m_{ev} - m_{pv}}{m_{ev}} \times 100 \quad (1)$$

where m_{ev} is the experimental value and m_{pv} is the predicted value.

2.4 Determination of total phenolic content (TPC)

1 g of fine particles of dried black tea were extracted in 100 ml of boiling water, with continuous swirling for 5 minutes. The Folin–Ciocalteu procedure used by Chan, Lim, and Chew (2007)

was followed, with slight modification. Tea samples (300 µl) were transferred into test tubes to be mixed with 1.5 ml of Folin–Ciocalteu's reagent (diluted 10 times) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were then left to incubate for 30 minutes at room temperature. After incubation, the absorbance reading was measured at 765 nm against a blank solution. TPC was expressed as Gallic acid equivalents (GAE) in mg GAE/g material.

2.5 DPPH free-radical scavenging assay

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay of black tea extraction was carried out in accordance with the procedure reported by Omwamba and Hu (2010), with slight modifications. Different dilutions of the extract were mixed with 2 ml of DPPH (5.9 mg/100 ml methanol) and then incubated for 30 minutes. The absorbance reading was then taken at 516 nm against a control sample. The free radical scavenging ability of black tea extracts were calculated as follows:

$$Scavenging\ ability\ (\%) = \left[1 - \left(\frac{A_{516}\ of\ sample}{A_{516}\ of\ control}\right)\right] \times 100 \quad (2)$$

2.6 Ferric reducing power assay

The ferric-reducing antioxidant power (FRAP) of black tea infusion was determined according to an adapted method of Benzie and Szeto (1999). FRAP reagent was prepared by adding together 100 ml of acetate buffer (0.3 M, pH 3.4), 10 ml of 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ) solution in 40 Mm HCl and 10 ml of iron (III) chloride ($FeCl_3 \cdot 6H_2O$). 4.5 ml of FRAP reagent was then mixed with different dilutions of the extracts. The mixture was later incubated at 37^{o}C for 4 minutes before the absorbance reading was measured at 593 nm. A standard curve of ferrous sulphate was prepared at different concentrations.

2.7 Sensory evaluation

The protocol for sensory evaluation in this study was approved by the Human Research Ethics Committee of University Sains Malaysia (USM). Sensory evaluation of the investigated black tea was performed by quantitative descriptive analysis according to ISO 3130 (1980). It was conducted by professional tea tasters, aged between 30-40 years old, with over 10 years' experience in tea tasting. The analysis took place at BOH Company, Banting, Malaysia. The sensory attributes are tea infusion colour, aroma, flavour and mouth astringency. During the quantitative assessment, the assessors evaluated the intensity of the specified attributes on a numeral ordinal 15-unit scale with the end values labelled as weak and strong. The tea samples were served in randomized order. The average scores of sensory attributes, based on the scores given by three panellists, were presented as evaluation results.

3 Results and Discussions

3.1 Model fitting

A second order polynomial equation was employed on the experimental data to obtain multiple regression analysis for each response as follows:

$$\Upsilon = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (3)$$

Where Υ is a response variables (Υ_1 = TPC; Υ_2 = DPPH scavenging ability; Υ_3 = FRAP metal chelating activity; Υ_4 = infusion colour; Υ_5 = aroma; Υ_6 = flavour and Υ_7 = mouthfeel (astringency) and β_0 is a constant coefficient of the model. Other coefficients are β_1 and β_2 (linear), β_{11} and β_{22} (quadratic) and β_{12} (interaction regression). X represents the coded independent variables where X_1 is temperature and X_2 is time. The experimental data of various responses during drying of tea leaves are presented in Table 2. The ANOVA results (Table 3) indicate that the quadratic model is the best fit model based on its statistical significance in comparison with the linear and two-factor interaction (2FI) models for all responses. Figures 1 and 2 (a-g) show the interaction effects of drying conditions (time and

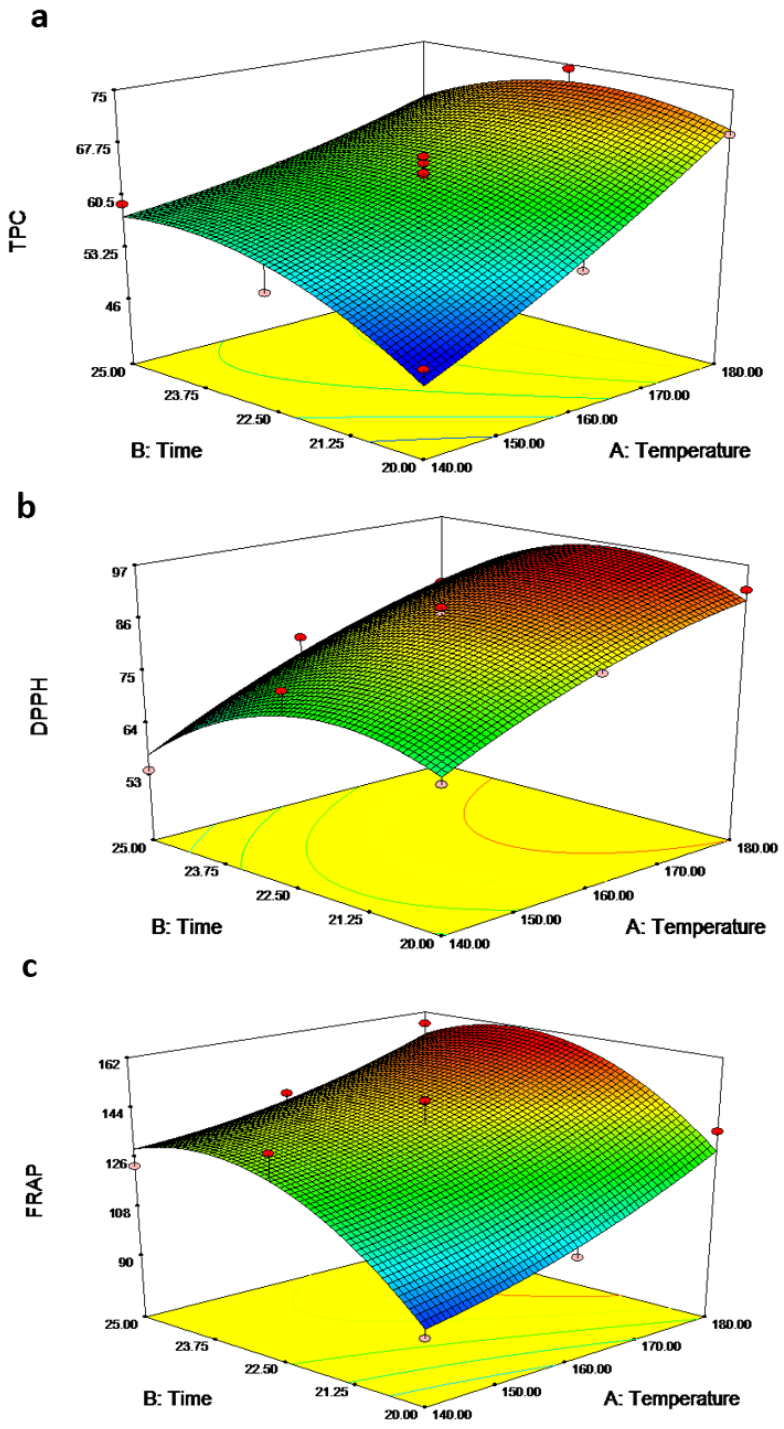

Figure 1: Response surface plots showing effects of drying temperature and time on the a) TPC, b) DPPH scavenging ability and c) FRAP

temperature) towards each response in a contour plot. The final equations for coded factors are as follows:

$$\begin{aligned}
\Upsilon_1 &= 62.36 + 8.16X_1 + 3.62X_2 \\
\Upsilon_2 &= 87.39 + 11.21X_1 - 5.05X_2 \\
\Upsilon_3 &= 140.33 + 14.91X_1 + 15.23X_2 \\
\Upsilon_4 &= 9.93 + 2.61X_1 + 1.03X_2 \\
\Upsilon_5 &= 6.52 - 0.79X_2 \\
\Upsilon_6 &= 7.83 + 2.17X_1 \\
\Upsilon_7 &= 8.92 + 2.29X_1
\end{aligned} \tag{4}$$

3.2 Effect of drying conditions on TPC

A major proportion of black tea comprises total phenolic compounds which are mostly responsible for the health effects and sensory characteristics of black tea (Luczaj & Skrzydlewska, 2005). In the present study, the initial value of TPC before drying was 42.15 mg/g (dry basis) and the TPC values of dried black tea using a SHS dryer at different temperatures and times were in the range 805.0 to 1096.0 mg/g (dry basis). Undried tea leaves have the lowest TPC, primarily due to the presence of enzymes that might degrade the antioxidants (Roshanak, Rahimmalek, & Goli, 2016). Dried tea leaves (3-4% moisture ratio) retain high phenolic compounds as result of decreased water content that deactivates enzymes that might degrade phenolic compounds (Rabeta & Lai, 2013). It is also interesting to point out that the TPC value in black tea from this study is 10% higher than those reported in the literature for conventional hot air drying (Chan et al., 2007). Loss of TPC in hot air and microwave drying may be due to the heat generated that allows oxidative condensation to take place within the system , thereby degrading polyphenols or even converting them into non-antioxidant forms (Lim & Murtijaya, 2007).

In the present investigation, the variable X_1 (temperature) was found to be highly significant with a p-value of 0.0009 followed by X_2 (time) with $p<0.05$. However, the other models (X_{12}, X_{1^2} and X_{2^2}) were not significant for the prediction of TPC in black tea as a result of drying (Table 3). The three dimensional (3D) surface plot (Figure 1-a) clearly shows an increase in TPC from 483.0 mg/g to 743.7 mg/g when increasing drying temperature from 140-180^{o}C. At all drying temperatures, the TPC only increased during the first 22.5 minutes and then slowly decreased after that drying time to 657.6 mg/ml. This suggests that high drying temperature accelerates the release of phenolic compounds, by breaking down the cell walls of the tea leaves, making them largely accessible by the extraction method (Tomaino, Cimino, Zimbalatti, Venuti, & De Pasquale, 2005). Indeed, TPC increased even at the highest temperature which indicates SHS can operate in the absence of oxygen thereby retaining the antioxidants, vitamins and other essential nutrients (Cenkowski, Pronyk, Zmidzinska, & Muir, 2007). However, a prolonged exposure of heat can result in loss of TPC. A similar trend was observed in a study carried out by Temple, Temple, van Boxtel, and Clifford (2001) that proved rapid drying is vital to prevent the loss of TPC.

3.3 Effects of drying conditions on DPPH

Black tea mostly consists of phenolic compounds that are capable of scavenging free radicals as effectively as green tea. In the present study, the ANOVA results of DPPH verify that drying temperature and time are statistically significant ($p<0.0005$) for antioxidant activity in black tea dried using SHS (Table 3). Figure 1-b depicts the 3D representation of the response surface that shows the influence of the independent variables on DPPH radical scavenging activity. SHS drying temperature significantly increased the antioxidant activities of black tea (53% - 93%), whereas a longer duration of drying caused the activities to decrease. Among the conditions, drying temperature and time of 180^{o}C and 22.5 minutes respectively provided the highest percentage of DPPH radical scavenging activity, which coincides with the TPC assay. These findings indicate that antioxidant ability is highly related to TPC as these compounds can act as free radical scavengers during oxidation reactions (Youssef & Mokhtar, 2014).

Table 2: Experimental values of TPC (mg/g), DPPH (%), FRAP (mg/g) and sensory attributes of dried tea leaves using a SHS dryer

Run	TPC (mg/g)	DPPH (%)	FRAP (mg/g)	Liquor colour	Aroma	Flavour	Mouthfeel
1	804.95	68.23	150.06	5.50	8.25	7.00	8.00
2	873.10	78.32	232.02	6.50	7.00	7.00	7.75
3	990.76	53.85	205.36	7.00	6.25	7.75	8.00
4	922.61	81.80	171.79	7.25	7.25	8.50	9.50
5	1063.20	84.02	244.76	10.50	6.67	8.25	9.00
6	1085.15	88.51	217.98	10.00	7.00	9.25	10.00
7	1100.66	86.52	245.18	9.50	6.83	7.25	9.75
8	1058.91	88.42	218.75	8.50	6.50	5.75	6.50
9	996.70	86.84	245.00	10.50	5.50	6.50	8.75
10	1009.08	75.73	232.68	10.25	6.00	11.50	9.75
11	1149.67	91.94	226.37	9.50	7.50	9.25	11.75
12	1239.44	93.64	247.74	14.00	7.00	12.50	12.75
13	1096.04	82.09	262.44	11.17	6.00	13.00	13.00

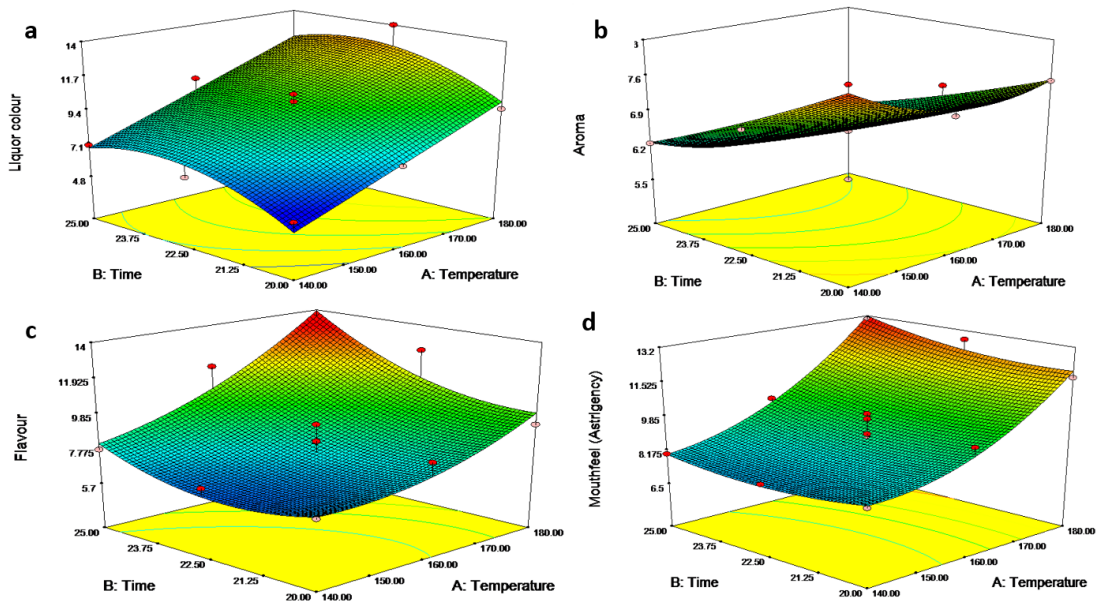

Figure 2: Response surface plots for the effects of drying temperature and time on sensory attributes of dried black tea extracts: a) liquor colour, b) aroma, c) flavour and d) mouthfeel (astringency)

Table 3: Summary for quadratic, linear and interaction model analysis, lack of fit and R^2 for measured responses

Source	Response variables								
	TPC			DPPH			FRAP		
	Quadratic	Linear	2FI	Quadratic	Linear	2FI	Quadratic	Linear	2FI
R^2	0.8403	0.8184	0.8326	0.9474	0.6491	0.6528	0.8511	0.6255	0.6332
Prob. >F	0.0103	0.0002	0.0008	0.0014	0.0053	0.0187	0.0426	0.0074	0.0237
Lack of fit	0.1013	0.2021	0.1830	0.0578	0.0043	0.0032	0.3449	0.1622	0.1310

Source	Response variables											
	Liquor colour			Aroma			Flavour			Mouthfeel (astringency)		
	Quadratic	Linear	2FI	Quadratic	Linear	2FI	Quadratic	Linear	2FI	Quadratic	Linear	2FI
R^2	0.8727	0.7584	0.7585	0.7501	0.6270	0.6370	0.7570	0.5872	0.6224	0.8157	0.7000	0.7086
Prob. >F	0.0049	0.0008	0.0039	0.0438	0.0072	0.0227	0.0401	0.0120	0.0268	0.0166	0.0024	0.0088
Lack of fit	0.2041	0.1597	0.1243	0.9111	0.8140	0.7608	0.3755	0.3338	0.3120	0.9404	0.7740	0.7175

3.4 Effects of drying conditions on FRAP

Antioxidant properties are also associated with the ability of phenolic compounds to form complexes with metal ions. In the present study, reducing capabilities of black tea extracts, undergoing different drying conditions, were in the range 150.1 to 262.4 mg/g (dry basis). From statistical measurements shown in Table 4, it was concluded that both temperature and time significantly affect the metal chelating ability of dried black tea ($p<0.01$). The contour plot (Figure 1-c) also showed that the reducing power of black tea increased as temperature increased. This trend was also observed with the DPPH scavenging ability assay, where the highest reducing power was attained at 180^oC and 22.5 minutes, while a prolonged drying time reduced both activities. Reducing capability at the highest temperature could be explained due to the fact that novel antioxidant compounds may be formed from Maillard reaction products (Manzocco, Anese, & Nicoli, 1998). In addition, an oxygen free environment in SHS also promotes the preservation of antioxidant compounds and reduces any oxidation reaction taking place.
In absolute terms, the three major parameters of TPC, free radical scavenging ability and FRAP are all interconnected between one another. They are highly affected by the drying conditions used with positive direction.

3.5 Effects of drying conditions on sensory attributes

The taste quality of black tea largely depends on its liquid colour, brightness, appearance, aroma, flavour and mouthfeel (astringency) (Tea Manufacturing, 2016). Primarily, liquid colour is an important factor not only in assessing the visual aspect of tea infusion but also for its relationship with antioxidant content (Ratti, 2001). Results clearly show that drying temperature and time largely affect the infusion colour of black tea extract. Specifically shown in Figure 2-a, the intensity of infusion colour of black tea increased with drying temperature and time. This result corroborates the work done by Teshome, Debela, and Garedew (2014) where the higher temperature and increase in drying time of tea leaves led to the loss of brightness and the tea infusion became darker and dull. This also hinted that the Maillard reaction (browning) occurred which may result in antioxidant ability during drying of black tea (Lou et al., 2015).
In the case of black tea aroma, only the single interaction of drying time can affect aroma scores with $p<0.05$ (Table 4). Figure 2-b clearly shows the effect of drying time on the aroma of tea infusions. At longer drying time, the aroma of tea

Table 4: ANOVA evaluation of quadratic terms for response variables of TPC, DPPH, FRAP and sensory properties

Source	Response variables								
	TPC			DPPH			FRAP		
	Sum of squares	F value	p-value	Sum of squares	F value	p-value	Sum of squares	F value	p-value
Model	491.31	7.37	0.0103	1324.36	25.21	0.0002	3707.89	8.00	0.0082
A	399.84	29.98	0.0009	754.32	71.81	<0.0001	1333.98	14.40	0.0068
B	78.65	5.90	0.0455	153.05	14.57	0.0066	1391.02	15.01	0.0061
AB	8.30	0.62		5.15	0.49		33.27	0.36	
A^2	$4.82x10^{-3}$	$3.623x10^{-4}$		20.78	1.98		50.54	0.55	
B^2	3.75	0.28		273.82	26.07	0.0041	926.10	10.00	0.0159
Residual	93.35			73.53			342.32		
CV (%)	5.91%			3.98%			7.19%		

Source	Response variables											
	Liquor colour			Aroma			Flavour			Mouthfeel		
	Sum of quares	F value	p-value	Sum of squares	F value	p-value	Sum of squares	F value	p-value	Sum of squares	F value	p-value
Model	54.37	9.60	0.0049	4.70	4.20	0.0438	48.40	4.36	0.0401	37.15	6.20	0.0166
A	40.91	36.11	0.0005	0.17	0.75		28.17	12.69	0.0092	31.51	26.27	0.0014
B	6.34	5.60	0.0499	3.76	16.82	0.0046	9.37	4.22		0.37	0.31	
AB	$6.944x10^{-3}$	$6.13x10^{-3}$		0.0063	0.28		2.25	1.01		0.39	0.33	
A^2	$3.649x10^{-4}$	$3.22x10^{-4}$		0.53	2.39		2.01	0.91		2.88	2.40	
B2	6.12	5.40		0.012	0.052		3.36	1.52		0.43	0.36	
Residual	7.93			1.57			15.54			8.40		
CV (%)	11.51%			7.01%			17.06%			11.44		

infusion scores dropped from 8.5 to 5.5. This behavior can be explained due to the evaporation of volatile compounds responsible for tea aroma (Tuan, Thinh, & Tu, 2016). Besides that, mishandling tea when all samples were prepared in one go for tasting could cause loss of aroma and inconsistent scores. Therefore, two or three sessions of tea tasting are suggested to be conducted to avoid the loss of aroma.

Both flavour and mouthfeel are only affected by temperature. Figure 2-c and 2-d show similarity between these two quality attributes as temperature increased, whereby the strength of flavour and mouthfeel also increased. The highest scores for both characteristics were achieved at 180^oC after 25 minutes of drying using a SHS dryer. In terms of flavour formation in black tea infusion, it is highly dependent on the content of essential oils in tea leaves that are strongly affected by temperature (Ho, Zheng, & Li, 2015). In contrast, the astringent taste in black tea was reported to be closely related to the theaflavins and thearubigins components (Scharbert, Jezussek, & Hofmann, 2004). Nevertheless, the key to good quality of black tea extracts is the balanced taste in terms of strength of flavour and the astringent and bitter taste (Scharbert et al., 2004). Thus, having a strong flavour can lead to a high bitter taste which results in loss of a balanced flavour in black tea (Wang et al., 2014).

3.6 Optimization and validation

A simultaneous multiple response optimization was employed to generate the optimum process conditions for SHS dying of black tea. Specific criteria of having maximum value of TPC and antioxidant ability, and a set target score of 7.5 for all sensory characteristics were assigned (Table 5). The target score for all sensory characteristics was set following the reference standard that was commercialized and accepted by con-

Table 5: Comparison of experimental and predicted values for all responses of dried tea leaves using a SHS dryer, with PE calculated

Name	Predicted value	Actual value	Relative difference	Percentage Error (%)
TPC (mg/g)	1142.20	1100.60	1.04	3.71
DPPH (%)	89.90	88.51	1.02	1.56
FRAP (mg/g)	233.23	217.90	1.07	6.80
Liquor colour	9.50	10.00	0.95	5.13
Aroma	6.80	6.50	1.05	4.51
Flavour	8.90	8.20	1.09	8.19
Astringency	9.95	9.75	1.02	2.03

sumers.

The optimal experimental conditions for the drying of black tea using SHS was found to be at 166.7^{o}C and 21.2 minutes with a desirability of 71.8%. Predicted values of each response were estimated to be 644.4 mg/g for TPC, 90.6% scavenging free radical ability, 133.3 mg/g for its reducing power and scores of 9.8, 7, 8.3 and 9.7 for tea infusion colour, aroma, and flavour and astringency levels respectively. Verification of the model was conducted. The percentage error calculated was not more than 10% of experimental data difference from predicted value thus confirming the suitability of the optimal conditions produced from the RSM.

4 Conclusion

RSM is a powerful tool that was successfully used in the present study to perform an optimization of drying conditions for black tea. The contour plot clearly visualized the interaction between all variables for the response of quality attributes in black tea drying. The optimal experimental conditions for the drying of black tea using SHS were found to be 166.7^{o}C and 21.2 minutes. Drying temperature is the most significant factor that affects TPC, antioxidant ability and sensory scores. The present results highlighted the drying approach of SHS is effective in preserving the antioxidant compounds and other quality aspects in black tea even at elevated temperature. Consequently, this study can help tea industries to consider this drying technique to retain quality aspects of their production, especially for black tea.

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Non-fermented Synbiotic Drink Based on Lactic Cheese Whey Which Incorporates *Lactobacillus rhamnosus* GG and *Lactobacillus paracasei*

Sima Taheri[a], Morteza Khomeiri[a*], Mehran Aalami[a], and Ali Moayedi[a]

[a] Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[*]Corresponding author
khomeiri@gau.ac.ir
Tel: +98 9111777143

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Abstract

The use of acid whey in food formulations is one way to reduce the environmental problems associated with its disposal. In the present study, a new formulation of a drinking dessert was prepared using Lactic cheese whey, milk, xanthan gum at 4 levels (0, 0.1, 0.2 and 0.3%), resistant corn starch at 4 levels (0, 0.5, 1 and 1.5%), cocoa powder and sugar. Samples containing starch and gum had higher viscosity and were completely stable, with no syneresis and sedimentation after a month of storage at 4 °C. Samples containing 0.3% xanthan gum and 1% corn starch were considered as the desired drink based on sensory analysis. Study of the optimal flow behavior indicated that the drinking dessert is a non-Newtonian pseudoplastic fluid, and the Herschel-Bulkily model was the best model to describe the flow behavior. The pH of the synbiotic dessert containing *L.* GG was almost constant after 7 days of storage at 4 °C, while the pH of samples containing *L. paracasei* decreased by 0.7. The population of both probiotic bacteria decreased during storage time at 4 °C. The rate of decrease was higher for *L. paracasei* than *L.* GG. However, both contained $>10^6$ CFU mL^{-1}, which is necessary for the health benefits of probiotic bacteria.

Keywords: Lactic cheese whey (LCW); Drinking dessert; Synbiotic; *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei*

1 Introduction

Whey is a by-product of cheese making in the dairy industry, which is obtained after the removal of casein from milk (Smithers, 2008). The amount of organic nutrients in whey is remarkable, and is responsible for its high Biological oxygen demand (BOD) and Chemical oxygen demand (COD). Owing to this fact, disposal of whey is expensive (Abdolmaleki, Mazaheri Assadi, & Jahadi, 2010; Smithers, 2008). Lactic cheese (LC) is widely consumed in Iran, and it is made by coagulation of milk using high acidity yogurt, usually more than 120 °D, and/or organic acids such as citric, lactic or acetic acids. A large amount of lactic cheese whey (LCW) is produced as a by-product of LC production. Drying of LCW to produce a powder is problematic due to its undesirable taste and acidity. Thus, a considerable amount of LCW is directly used in food formulation (Zadow, 1992).

There is an increasing use of probiotic bacteria and prebiotic carbohydrates in fermented and non-fermented dairy foods. Milk and whey are used to make functional foods, which due to their high nutritional value are good choices for human consumption and as carriers for probiotic bac-

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teria. For example, sweet and acid whey were studied to make a beverage in a fermented or non-fermented form (Abdolmaleki et al., 2010; Djurić, Carić, Milanović, Tekić, & Panić, 2004). The sour taste of fermented products is unpleasant for some people, so the production of non-fermented probiotic products would broaden the range of people interested in consuming probiotic products. One of the few studies carried out on non-fermented probiotic beverages is the production of an orange probiotic beverage using *Lactobacillus acidophilus* (Khamirian, Jooyandeh, Hesari, & Barzegar, 2016).

A dairy drinking dessert is a new type of beverage that, in addition to near neutral pH, usually has a high viscosity, opaque appearance and complete stability over the shelf-life (Beecher, Drake, Luck, & Foegeding, 2008). Addition of acid whey, at pH values below the natural pH of the milk (6.4-6.6), and starch may cause syneresis or sedimentation in the final product. Because texture, oral sensitivity and stability are three important factors affecting the consumer's opinion about a drinking dessert, selection of an effective hydrocolloid in an appropriate amount can play an important role in consumer's acceptance.

In this study, lactic cheese whey was used, a by-product of the cheese industry, to make a synbiotic drinking dessert which incorporated *L. rhamnosus* GG and *L. paracasei*, that had a complete stability and desirable sensory properties. Different physicochemical and microbial tests were also performed to determine the properties of this novel product.

2 Materials and Methods

2.1 Probiotic bacterial strain

Lactobacillus rhamnosus GG (ATCC53103) and *Lactobacillus paracasei* (L. casei 431) were obtained from Chr. Hansen (Denmark). 10 g Direct Vat Set (DVS) culture was added to 100mL 1.5% sterilized milk as a stock culture, and was frozen in liquid nitrogen and stored at -80 ^{o}C.

2.2 Determination of physicochemical properties of LCW

Physicochemical properties of LCW were analyzed using the following methods: Density using a lactodensimeter (Quevenne, Germany) (AOAC 925.225); pH by a digital pH meter (Knick model 766, Germany); acidity by a titration method with 0.1N NaOH in the presence of phenolphthalein as an indicator (AOAC 947.05); soluble solids using a digital refractometer (CETI, Belgium) (AOAC 923.12); dry matter by air drying in an oven (Nuve, Model FN120, Turkey) (AOAC 990.20); ash by incinerating in an electric furnace at 550 ^{o}C (Nabertherm, Germany) (AOAC 945.46); protein by the Kjeldahl method (Behr, Germany) using a factor of 6.38 (AOAC 991.23); and fat by the Gerber method (AOAC 2000.18). The lactose content of whey was determined by an HPLC system (Knauer, Germany), equipped with an analytical column from Eurokat (300 × 8 mm, 10 µm) and a refractive index detector. Sample preparation was performed as described by Chavez-Servin, Castellote, and Lopez-Sabater (2004). A standard curve was also prepared by HPLC analysis of different concentrations of pure lactose.

2.3 Preparation of dairy drinking dessert

In the formulation of this new dairy product, lactic cheese whey, milk, xanthan gum, resistant corn starch, sugar and cacao powder were used. The pre-tests were performed to obtain the proper proportion of milk and whey as the main components of the product. According to the primary tests, the best ratio for milk and whey in the mix was 1:1. Whey with Xanthan gum (Puratox, Belgium) at 4 levels (0, 0.1, 0.2 and 0.3%), resistant corn starch (Ingredion, USA) at 4 levels (0, 0.1, 0.2 and 0.3%), 5% sugar and 0.3% cocoa powder were mixed using a magnetic stirrer (Labtron, Iran). This mixture was sterilized at 110 ^{o}C for 10min. Then sterilized milk was added and the dessert mix was homogenized by a homogenizer (Heidolph, model D-91126, Germany) (10000 rpm for 2 minutes) which had been

Table 1: Formulation of 16 different treatments to prepare the drinking desserts

Treatments[1]	Xanthan gum (%)	Resistant corn starch (%)
1	0	0
2	0	0.5
3	0	1
4	0	1.5
5	0.1	0
6	0.1	0.5
7	0.1	1
8	0.1	1.5
9	0.2	0
10	0.2	0.5
11	0.2	1
12	0.2	1.5
13	0.3	0
14	0.3	0.5
15	0.3	1
16	0.3	1.5

[1]In all treatments, whey and milk were used in 1:1 ratio, cacao powder (0.3%) and sugar (5%)

sterilized using 70% alcohol. In this study, 16 treatments were used for preparation and testing of the desserts (Table 1).

2.4 Evaluation of storage stability

Syneresis and sedimentation were analyzed using a volumetric cylinder (Laurent & Boulenguer, 2003). 12 mL of each sample was poured into a volumetric cylinder and after one-week storage at 4 ^{o}C, the volume of sediment at the bottom of the tube and the volume of water in the upper part of the sample were measured in milliliters. Results were calculated using the following equations:

$$syneresis(\%) = \frac{Vs \times 100}{Vt} \quad (1)$$

$$sedimentation(\%) = \frac{Vp \times 100}{Vt} \quad (2)$$

where Vs is the supernatant volume, V_P is the sedimentation volume and V_t is the total volume of the sample in the tube.

2.5 Determination of viscosity

The viscosity of drinking desserts was measured by a viscometer (RV-DV II Brookfield, USA), equipped with a thermal circulator and using spindle NO. S00. All samples were subjected to a different shear rate and finally, depending on the texture of the sample, 15 rpm was selected. Therefore, 16 mL of the sample was poured into a special cylinder and the viscosity was read at 25 ^{o}C.

2.6 Rheological properties of optimal drinking dessert

Flow curves were obtained by increasing the speed of shearing from 15 rpm to 120 rpm. In order to determine the optimum flow behavior of the optimum sample of drinking dessert, and obtain the rheological parameters, three time-independent models were used: power-law ($\tau = k\gamma^n$), Herschel-Bulkley ($k\gamma^n + \tau$) and Bingham ($\tau=\mu_p\gamma + \tau_0$) (Bhattacharya & Bhattacharya, 1994).
In these models, k is the consistency coefficient

(Pa.sn), n is the flow behaviour index, γ is the shear rate, τ_0 is the yield stress (Pa) and μ_p is the Bingham plastic viscosity (Pa.s).

2.7 Sensory analysis

Samples that were completely stable during refrigerated storage were selected for sensory analysis. Samples were served in cups coded with three random alphabets. 15 trained panelists were asked to rate consistency, taste and mouthfeel on the basis of a 5-point hedonic scale (5- really good, 4- good, 3- normal, 2- bad, 1- really bad). The analysis was carried out in two stages. In the first stage, panelists were served samples in three groups of four (during three consecutive days), and for each group, the sample that had the highest score was selected for the second stage. Finally, among the three dessert samples, the sample with the highest score was selected as the desired dessert. It should be noted that due to the importance of mouthfeel for drinking desserts, the score of this parameter was weighted as 2 (Janhøj, Frøst, & Ipsen, 2008).

2.8 Preparation of synbiotic drinking dessert

After selection of the optimal formulation based on stability and sensory analysis, *L. rhamnosus* GG and *L. paracasei* culture were inoculated to the beverage individually at 4 ^{o}C. Beverage samples were stored in the refrigerator for a week and were analysed for pH, titratable acidity and probiotic viable counts just after production, and 1, 2, 4 and 7 days after storage.

2.9 Determination of pH and titratable acidity

pH was measured using a digital pH meter by direct immersion of the pH meter electrode in the sample.

Titratable acidity was assessed according to the method of Purwandari, Shah, and Vasiljevic (2007). 10mL of the sample was mixed with 10mL CO_2 free distilled water and the temperature was adjusted to 22 ^{o}C. The pH meter electrode was placed in the sample and the pH was adjusted to 8.3 with 0.1N NaOH. The following equation was used to calculate the results and expressed as ^{o}D.

$$A = V \times 10 \tag{3}$$

A = acidity percentage, V = volume of sodium hydroxide consumed in milliliters

2.10 Enumeration of probiotics viable counts

The viability of *L. rhamnosus* GG and *L. paracasei* was evaluated by selective enumeration according to the method of Tharmaraj and Shah (2003). 10 mL of sample was diluted in 90 mL of saline solution (0.85 g NaCl 100mL^{-1} distilled water) for the first dilution, and keep diluting with a 1:10 ratio until the appropriate dilution is reached. 1mL of appropriate dilutions were pour plated in MRS-Vancomycine agar and incubated under an aerobic condition at 37 ^{o}C for 72 hours and then two consecutive dilutions were counted. The following equation used to calculate the results (Cappuccino & Welsh, 2018)

$$N = \frac{\sum Ci}{V(n1 + 0.1n2)d} \tag{4}$$

where C is the sum of colonies on all plates counted, v is the volume applied to each plate, n_1 is the number of plates counted at the first dilution, n_2 is the number of plates counted at the second dilution and d is the dilution factor from which the first count was obtained.

2.11 Microbial quality control

Samples were analyzed for enumeration of coliforms, molds and yeasts immediately after production and 1, 2, 4 and 7 days after storage. The test for coliforms was performed according to the Iranian National Standard No. 11166, using Violet Red Bile Agar medium (Merck, Germany), and molds and yeasts according to the Iranian National Standard No. 10154, using Yeast Extract Glucose Chloramphenicol medium (Liofilchem, Italy).

2.12 Microbial quality control

Experiments were replicated three times following a completely randomized design using a factorial arrangement. All data were analyzed using the one-way ANOVA procedure, followed by Duncan multiple comparison tests, using SPSS version 23 (SPSS, USA). Probabilities of $P < 0.05$ were considered significant.

3 Results and Discussions

3.1 Physicochemical properties of LCW

The physicochemical properties of LCW used in this study were as follows: pH (5.7±0.8), acidity (17.5±0.44 ^{o}D), density (1.0238±0), dry matter (6.11±0.25%), fat (0.35±0.02%), protein (0.94±0.17%), lactose (4±0.01%) (Fig. 1), salts (0.46±0.02%) and ash (0.62±0.01%). Khamirian et al. (2016) found similar values for whey characteristics except for pH (6.63) and protein content (0.46) which were higher than those found in the present study. Differences in the process of cheese production can influence the whey characteristics. The difference in protein content could be due to the high efficiency of ultrafiltration in feta cheese production. The pH reported by Djurić et al. (2004) for acidic whey was 3.63, which could have been related to differences in type (lactic acid, acetic acid or citric acid) and amount of acid used in cheese production. Therefore, the composition of the main constituents of whey varies depending on the type of cheese and the type of milk used (Alsaed et al., 2013).

3.2 Effect of xanthan gum and resistant starch on syneresis and sedimentation

Addition of acid whey to a drinking dessert mix makes the milk protein network unstable and a clear layer of serum is formed in the beverage over time. On the other hand, the opinion of consumers is that the drinking dessert should be completely stable. Therefore, in the preparation of drinking desserts, hydrocolloids need to be added. Table 2 shows the values obtained for syneresis and sedimentation of drinking desserts containing different levels of xanthan gum and resistant starch. In the control sample (without starch and xanthan gum), a significant syneresis (33.33%) was observed, which can be due to denaturation of casein micelles at a pH below milk pH (de Kruif, 1998). Other samples containing xanthan gum did not have syneresis after 30 days storage at 4 ^{o}C. This is due to the binding of hydrocolloids to the casein micelles, which do not allow casein micelles to aggregate (Syrbe, Bauer, & Klostermeyer, 1998). However, there was no significant difference between the mean percentage (%) syneresis and sedimentation in all samples containing different amounts of gum ($P > 0.05$). This indicates that xanthan gum has a significant effect at the 0.1% level. In samples containing only starch, sedimentation was observed, which naturally could be due to starch sedimentation. Samples containing both starch and xanthan gum conferred a great stability, with no syneresis and sedimentation after 30 days of storage at 4 ^{o}C (Table 2), which demonstrates that a mix of resistant starch and gum is more effective in the production of a single-phase beverage. Many researchers have investigated the effect of different hydrocolloids on the stability of beverages containing whey (Janhøj et al., 2008; Laurent & Boulenguer, 2003; Mohammadi, Abbasi, & Hamidi, 2011). Paraskevopoulou et al. (2003) evaluated the effect of three polysaccharides (pectin, xanthan gum and guar gum) on the stability of whey-milk kefir. They showed that Xanthan gum was the most effective stabilizer at 0.2% level. Generally, polysaccharides contribute to the formation of a stable colloid system by increasing the viscosity of the aqueous phase and preventing particle movement (Parker, Gunning, Ng, & Robins, 1995).

3.3 Effect of xanthan gum and starch on the viscosity of drinking dessert

The viscosity of dairy desserts is primarily affected by the type and concentration of thickening agents, especially polysaccharide hydrocolloids such as gums and starch. Figure. 2 shows the viscosity analysis of the desserts. There

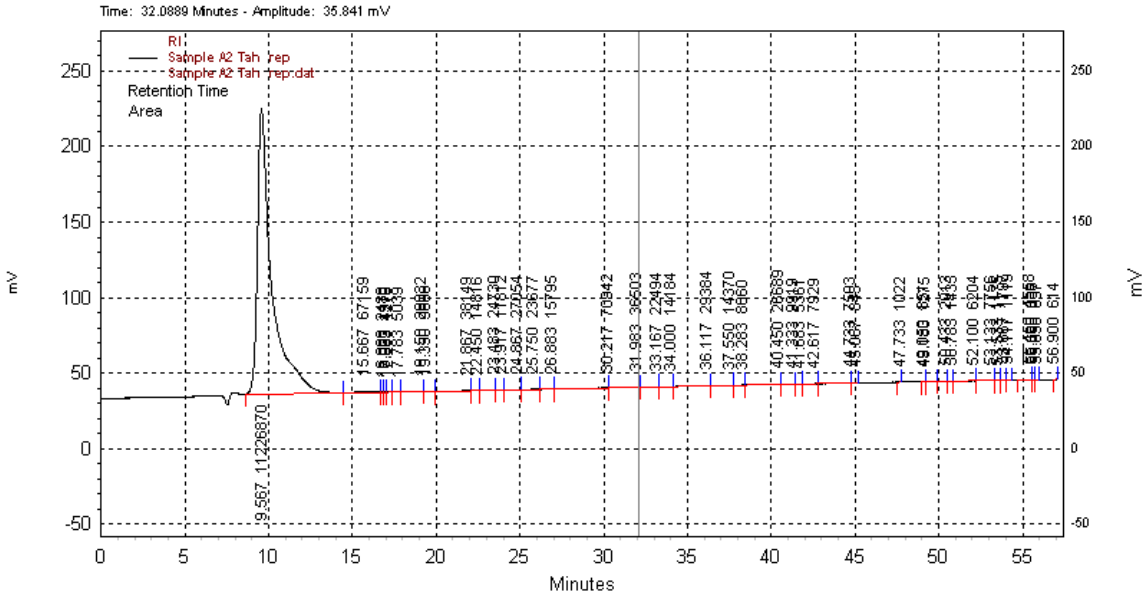

Figure 1: Chromatogram of whey lactose analysis by the HPLC–RI method. Peaks: 1, Lactose

Table 2: Effect of different amounts of gum and starch on sedimentation, syneresis and viscosity

Treatment	Sedimentation (%)	Syneresis (%)
1	0 ± 0^{c}	33.33 ± 4.8^{a}
2	61.11 ± 7.11^{a}	0 ± 0^{b}
3	58.32 ± 5.3^{b}	0 ± 0^{b}
4	50 ± 0^{b}	0 ± 0^{b}
5-16	0 ± 0^{c}	0 ± 0^{b}

$^{a-c}$ Data with different superscripts are significantly different ($p<0.05$) according to Duncan's comparison test.
The results are expressed as mean values ± standard error (n=3)

Table 3: Overall scores for different samples in sensory analysis. Stage 1

Sample no.	Overall score (out 20)	Sample no.	Overall score (out 20)	Sample no.	Overall score (out 20)
5	8.81 ± 0.41^{b}	9	13.25 ± 0.84^{a}	13	12.87 ± 1.03^{a}
6	9.68 ± 0.59^{b}	10	13.5 ± 0.85^{a}	14	14.37 ± 0.74^{a}
7	10.87 ± 0.9^{ab}	11	14.31 ± 0.89^{a}	15	14.43 ± 0.88^{a}
8	12.43 ± 0.87^{a}	12	11.06 ± 0.79^{a}	16	14.43 ± 1.08^{a}

$^{a-b}$ Mean values with different superscripts are significantly different ($p<0.05$) according to Duncan's comparison test
The results are expressed as mean values ± standard error (n = 3)

Table 4: Rheological parameters obtained using power law models for the best drinking dessert

Model	n	RMSE	SSE	R^2	K	τ_0	μ
Power law	0.298	0.395	2.34	0.998	10.11	-	-
Hershel-Bulkley	0.23	0.396	2.2	0.998	10.11	3.68	-
Bingham	-	9.146	1255	0.953	-	-119.2	4.41

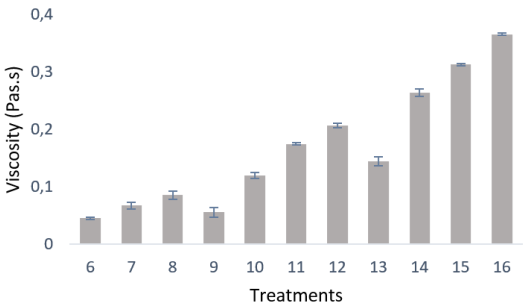

Figure 2: Effect of different percentages of Xanthan gum and resistant corn starch on viscosity of drinking dessert. Results for viscosity of samples 1 to 5 have not been reported, because they had a torque less than 10.

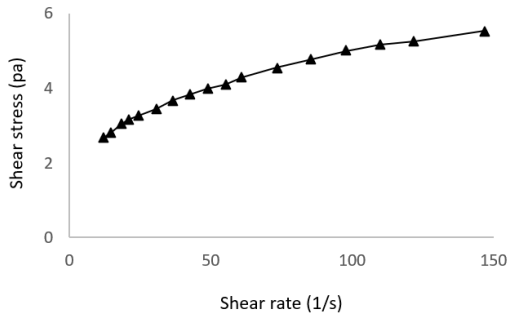

Figure 3: Flow curve of shear stress versus shear rate for the best drinking dessert

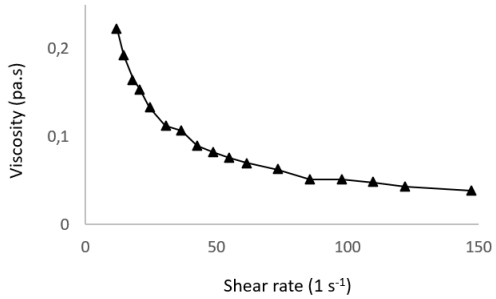

Figure 4: Apparent viscosity versus shear rate for the optimum drinking dessert

was a significant difference between the viscosity of samples for different treatments. With constant gum content and increasing starch content, the viscosity increased and has a maximum at 0.3% xanthan gum and 1.5% resistant starch. However, gum and starch in combination had a greater impact on the viscosity than their individual usage (Fig. 2), as a result of hydrocolloids in the continuous phase and starch in the dispersed phase. Sikora, Kowalski, and Tomasik (2008) studied the effect of starch and xanthan gum on viscosity and found that gum and starch individually form two separate phases, but when combined, they form one phase that increases viscosity. Wei, Wang, and Wu (2001) also obtained the same results when using gums and corn starch.

3.4 Sensory analysis

There were no significant differences between the samples in each group ($P > 0.05$), except for group number 3. The dessert containing 0.1% gum and 1.5% starch had the highest overall

acceptability in the first group. In the second group, due to the increase in gum concentration and the desired consistency, the desirable samples were those containing 0.2% gum and 1% starch. In the third group, samples containing 0.3% gum, 1.5% starch and 0.3% gum and 1% starch had the same score, however, the dessert formulation containing 1% starch and 0.3% gum was selected because of lower costs of preparation as a result of less starch. Thus, samples containing 0.1% gum and 1.5% starch, 0.2% gum and 1.5% starch, 0.3% gum and 1% starch were selected for the second stage of sensory analysis (Table 3).

In second stage, samples number 8, 11 and 15 gained overall scores of 13.37±3.9, 15.6±3.2 and 16.93±3.3 respectively. When comparing the three desserts with respect to overall acceptability, there was no significant difference. Nevertheless, the dessert containing 0.3% gum and 0.1% starch had the highest score and finally was considered as a best one to prepare the synbiotic dairy dessert.

3.5 Rheological properties of optimal drinking dessert

The study of viscosity and flow behavior of fluids is necessary for the design and engineering of equipment and systems, such as pumps, mixers and tubes (Augusto, Cristianini, & Ibarz, 2012). The rheological properties of dairy desserts mainly depend on the amount of milk fat, the type and concentration of starch and hydrocolloids and their interactions (Torres, Tárrega, & Costell, 2010). Figures 3 and 4 respectively show shear stress (δ) versus shear rate (γ) and apparent viscosity (η) versus shear rate for the optimal drinking dessert containing 0.3% xanthan gum and 1% resistant corn starch. With respect to shear stress, the increase in shear rate was not linear. By increasing the shear rate, the apparent viscosity of the sample decreased and shear stress increased, indicating that the dairy dessert had a shear-thinning behaviour. At the lower shear rate, due to the irregular molecular arrangement, the viscosity is high, while with increasing shear rate, the number of molecules that orient to one side is greater and hence the viscosity decreases (Mahdian, Mehraban, Karazhian, & Vaghei, 2014). Such behaviour has already been reported in dairy desserts (Bayarri, Chuliá, & Costell, 2010; González-Tomás, Bayarri, Coll-Marqués, & Costell, 2009). Many research studies have focused on the effect of hydrocolloids to increase the shear-thinning behaviour of foods (Panaras, Moatsou, Yanniotis, & Mandala, 2011). Because of the increasing viscosity of the serum phase, a condensed polysaccharide network is formed, which is very susceptible to shear rate (Panaras et al., 2011). Xanthan gum is used in dairy products as a semi-solid compound with a gel network which is very similar to a thick three-dimensional gel network. The gel formed by xanthan gum flows freely; therefore, it is extremely shear thinning (Sworn, 2009). The relatively low viscosity, at high shear rate, makes the food containing xanthan gum easy to mix, pour and swallow. Some features, such as inducing high viscosity at low levels and lack of gel formation, make xanthan gum a convenient viscosity control compound as well as a thickening agent, stabilizer and emulsifier in dairy products (Kang & Pettit, 1993).

According to the flow behaviour index (n<1), the drinking dessert is a non-Newtonian fluid. Although many food fluids have Newtonian behaviour, some liquids and semi-solids have non-Newtonian behaviour. The pseudoplastic behaviour found in this research, has also been reported for frozen soy yogurt containing beta-glucan and modified starch (Rezaei, Khomeiri, Kashaninejad, Aalami, & Mazaheri-Tehrani, 2017), frozen yogurt containing inulin (Rezaei, Khomeiri, Aalami, & Kashaninejad, 2014), ice cream containing xanthan gum (Toker, Dogan, Canıyılmaz, Ersöz, & Kaya, 2013) and dairy dessert based on starch containing inulin (Torres et al., 2010).

Among the models used to determine the flow behaviour of the best drinking dessert, both the power law and Herschel-Bulkley models could be identified as the best because of the high similarity between R^2 and RMSE (Table 4).

According to the Herschel-Balkley model, the consistency coefficient (K) was 10.11. This is higher than K value reported for frozen soy yogurt containing 1% resistant starch (Rezaei et al., 2017) (K=0.985) and lower than dairy dessert

starch based (2.5%) enriched with 7.5% inulin (K=11.81) (Torres et al., 2010) and frozen yogurt containing 1% inulin (K=15.45) (Rezaei et al., 2014). The consistency of our best drinking dessert was favourable to allow easy drinking with a straw and provide a proper mouthfeel, with acceptable thickness.

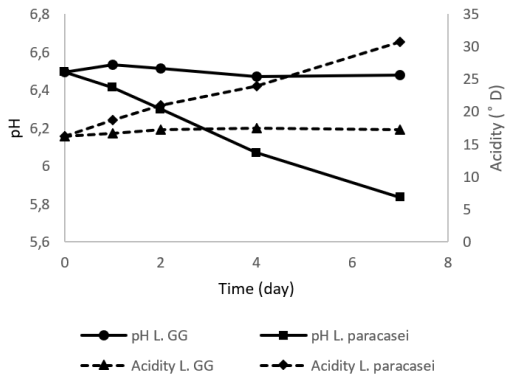

Figure 5: Changes in pH and acidity for both types of synbiotic drinking dessert at 4 ^{o}C

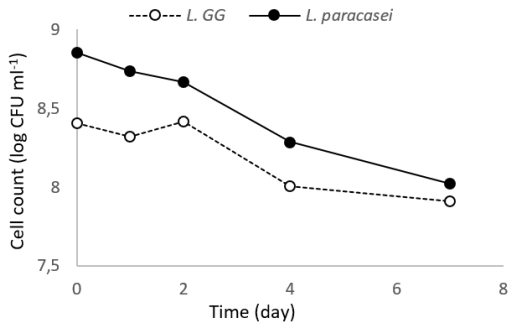

Figure 6: Changes in viable cell counts of probiotic bacteria at 4 ^{o}C

3.6 Changes in pH and acidity at refrigerator temperature

As shown in Figure 5, the pH of the refrigerated dessert containing *L. rhamnosus* GG was constant ($P > 0.05$) as *L. rhamnosus* GG is not capable of fermenting lactose and casein because of alterations in the nature of anti-terminator (*lac T*) and 6-phospho-β-galactosidase (*lac G*) genes (Kankainen et al., 2009). Therefore, the presence of simple sugars, such as glucose and fructose, and proteins, such as whey protein and amino acids as nitrogen sources, are essential for growth and survival of this bacterium. Karlton-Senaye and Ibrahim (2013) also reported that *L. rhamnosus* GG slowly reduces the pH of a dairy dessert during a month under refrigerated storage.

The pH of the synbiotic drinking dessert containing *L. paracasei* decreased and its differences were significant after a week. As no yeasts and molds and coliforms were detected during one-week storage, decreasing the pH could have been due to probiotic bacteria activity. Probiotic bacteria have enzymatic activity of β-galactosidase, β-glucosidase and α-glucosidase, which are inactive in most probiotic strains at refrigerator temperature but some strains could have β-galactosidase activity even at refrigerator temperature (Lipovová, Spiwok, Mala, Králová, & Russell, 2002). Lipovová et al. (2002) studied the activity of the β-galactosidase enzyme in some lactic acid bacteria, and the results showed that some *L. paracasei* strains at refrigerator temperature are able to ferment lactose, albeit slowly.

As shown in Figure 5, the acidity of the samples containing *L. paracasei* increased and decreased the pH. But the acidity of the sample containing *L. rhamnosus* GG was relatively constant and significant changes were not observed ($P > 0.05$). Similar results were obtained by Mani-López, Palou, and López-Malo (2014). They reported that the acidity of yogurt containing probiotic bacteria *L. casei, L. reuteri* and *L. acidophilus* increased at 4 ^{o}C temperature with respect to decreasing pH.

3.7 Cell count of probiotic bacteria

The population of probiotic strains were analyzed for 7 days of storage at 4 ^{o}C. In the whole 7 days, because of increasing acidity, the population of *L. rhamnosus* GG decreased 0.5 log CFU mL^{-1} compared to the initial time of inoculation

and was significant. The population of *L. paracasei* decreased significantly in all 7 days up to 7.89 log CFU mL^{-1}. (Fig. 6) The lower counts of *L. paracasei* than *L. rhamnosus* GG. could be due to the intense decrease in pH.
The viable counts of both probiotic bacteria remained above 10^6 CFU mL^{-1} during the whole refrigerated storage period which is necessary for the health benefits (Shah, 2007). The results of the present study suggest that it is possible to produce a non-fermented synbiotic drinking dessert, incorporating *L. rhamnosus* GG, which is not able to reduce the pH at refrigerated temperature and is not sour.
Sarvari, Mortazavian, and Fazei (2014) studied the population of *L. animalis* and *L. acidophilus* at three different pH values (4.5, 4.7 and 4.9) in a mixture of probiotic yogurt. They reported a significant decrease in the population of the probiotic bacteria at lower pH as observed in this study. Vinderola, Bailo, and Reinheimer (2000) also examined the viability of two probiotic bacteria, *B. bifidum* BBI and *L. acidophilus* LAI in Argentinian yogurt during cold storage. Their results showed that the decrease in *B. bifidum* population was not significant at pH 6.5, 5.5, and 4.5, while the population of *L. acidophilus* significantly decreased at these pH values.

4 Conclusion

In this research, we tried to introduce a new formulation of a dairy drinking dessert as a non-fermented probiotic product using LCW. The results showed that the addition of both xanthan gum and corn starch have a positive effect on the viscosity of the samples and their complete stability during storage at refrigerated temperature. The drinking dessert is a non-Newtonian pseudoplastic fluid, where the Herschel-Bulkley model is the best to describe the behaviour of this food fluid. To produce a non-fermented probiotic product with a sweet taste, it is important to select a probiotic bacteria with the lowest possible activity at refrigerated temperature. *L. rhamnosus* GG was considered as a suitable species to produce a dairy dessert of non-fermented form due to inactivity at refrigerated temperature.

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Development and Incorporation of Nanoemulsions in Food

Antonio de J. Cenobio-Galindo[a], Rafael G. Campos-Montiel[a*], Rubén Jiménez-Alvarado[a], Isaac Almaraz-Buendía[a], Gabriela Medina-Pérez[b], and Fabián Fernández-Luqueño[c]

[a] Instituto de Ciencias Agropecuarias, Universidad Autónoma del Estado de Hidalgo. Av. Rancho Universitario s/n Km. 1. Tulancingo, Hidalgo, C.P. 43600, México
[b] DCTS, Cinvestav-Zacatengo, Ciudad de México, CP 07360, México
[c] Sustainability of Natural Resources and Energy Program, Cinvestav-Saltillo, Coahuila, México
[*]Corresponding author
ragcamposm@gmail.com
Tel: +(52)7717172000 ext. 2422

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Abstract

Currently, nanoencapsulation of bioactive compounds is promising, and is one of the methods that has been proven very effective. The development of food-grade nanoemulsions is in a state of constant innovation due to the interesting features that this method of encapsulation has, such as small droplet size, kinetic stability and appearance. With this technology, it is possible to control some food properties, such as texture, taste and stability. In this article, we present a review of the most commonly used methods in the creation of nanoemulsions, the recent developments of these dispersions, the relevant applications of nanoemulsions in food matrices, the most commonly used food-grade materials and the functionality of nanoemulsions, which are designed primarily to encapsulate compounds with biological activity. Nanoemulsions have been shown to be effective in preventing degradation and improving the bioavailability of bioactive compounds, such as oil-soluble vitamins, antimicrobials, flavours and antioxidants. At the end of this article, facts of interest about acceptance issues and nanotechnology regulatory policies in the food industry are presented.

Keywords: Shelf life; Antimicrobial; Essential oils; Encapsulation

1 Introduction

The word "nano" is used in all areas of science and technologythat deal with the characterization, manufacture and operation of structures, devices or materials with sizes on the nanometre scale (Duncan, 2011; Pradhan et al., 2015). All foods developed and consumed since ancient times co contain natural nanomaterials because plant and animal products contain multiple nanostructures; and based on their size, foods can be classified within these categories (Magnuson, Jonaitis, & Card, 2011).

The food industry has made important advances with the application of nanotechnologies, such as nanoliposomes, nanoemulsions, nanofibres, and nanocapsules, to obtain fresh and better-tasting foods (Raj, Ragavi, Rubila, Tirouthchelvamae, & Ranganathan, 2013). Nanoemulsions are colloidal systems of particular interest because they can be made from food-grade ingredients and use processes in the food industry, such as blending, thermal treatment and homogenization (Rao & McClements, 2011). The food industry uses these science and technology principles of emulsions to create and transform a wide variety of

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foods and beverages (McClements, 2010).
One of the main advantages of using nanoemulsions is that the food manufacturer can improve some properties, such as appearance, texture and/or taste, through careful selection of the ingredients and the processes used to make them (McClements, 2015). Another advantage of nanoemulsion use is that emulsions can have better stability against the coalescence of droplets (McClements & Rao, 2011). Additionally, droplets are transparent when dispersed, so they are suitable for addition to food without modifying the appearance characteristics (Mason, Wilking, Meleson, Chang, & Graves, 2006); the appearance of a colloidal dispersion tends to become translucent or transparent when the particle radius falls below approximately 100 nm. These dispersions can increase the bioavailability of certain nanoemulsified compounds (Acosta, 2009). Recent research on nanoemulsions has focused on their manufacture, characterization and use as release systems (McClements, 2012). Therefore, the present work aims to review the application trends of nanoemulsions in food, the materials used, and the risks and regulations to consider in the use of nanotechnology.

2 Nanoemulsions

According to McClements (2012), a nanoemulsion is a thermodynamically unstable colloidal dispersion consisting of two immiscible phases (nanoemulsions have better stability in particle aggregation and gravitation separation due to their small droplet sizes compared to that of macroemulsions). One of the phases contains dispersed particles and small droplets in the other (normally r<100 nm); this resultant dispersion has a translucent appearance. These nanoemulsions may be emulsions of oil-in-water (O/W) or water-in-oil (W/O).
The proportions of water and oil in a nanoemulsion must be carefully designedas a large variety of components are often incorporated in the aqueous phase (such as proteins and polysaccharides, among others) (McClements & Rao, 2011). Polysaccharides (pectin, gums, and others) are used to stabilize these systems because of their high viscosity (Choi, Kim, Cho, Hwang, & Kim, 2011). In the creation of nanoemulsions, several oils (such as essential oils), acylglycerols and free fatty acids are used (Shah, Bhalodia, & Shelat, 2010). Lipophilic active ingredients, such as vitamin E, carotenoids, and curcumin, are also used. The addition of these active components totally changes the physicochemical properties and activity of the nanoemulsions (Jin et al., 2016; Shakeel et al., 2010). Commercially, nanoemulsions have been designed to encapsulate components with a lipophilic nature because these components may not be well dispersed in aqueous media. However, it is possible to manufacture W/O-type nanoemulsions with rheological behaviour such as colour, appearance, texture and stability (Jafari, Assadpoor, He, & Bhandari, 2008) that produce desirable characteristics in food applications. Emulsions are systems that are not in equilibrium, and they tend to separate into their constituent phases (Gutierrez et al., 2008). For a nanoemulsion, the free energy of the emulsion is greater than the free energy of the separate phases (oil and water), which means that a nanoemulsion is thermodynamically unstable. A nanoemulsion can be made kinetically stable (metastable) by ensuring that there is a sufficiently large energy barrier between the two states. The height of the energy barrier is mainly determined by physicochemical interactions that prevent the droplets from coming into close proximity with each other, such as repulsive hydrodynamic and colloidal (e.g., steric and electrostatic) interactions operating between droplets (McClements, 2012). Thermodynamic instability occurs through certain mechanisms, such as sedimentation, coalescence, flocculation and Ostwald ripening (Gharibzahedi, Mousavi, Hamedi, & Ghasemlou, 2012). According to Solans, Izquierdo, Nolla, Azemar, and Garcia-Celma (2005), nanoemulsions can possess high kinetic stability and persist for many years. The stability depends on the preparation method, which affects properties, such as the droplet size. The characteristics of a nanoemulsion will be good if the preparation method uses a high shear or high energy accumulation in the system (Gutierrez et al., 2008). These dispersions exhibit better stability against separation than macroemulsions because of their relatively small droplet size and the effects of Brownian mo-

tion, which dominates gravitational forces (McClements, 2015). In addition, food-grade nanoemulsions are designed to increase the bioactivity of some compounds that are poorly absorbed (Qian & McClements, 2011).
One of the most frequent instabilities in nanoemulsions is Ostwald ripening, or molecular diffusion (Gupta, Eral, Hatton, & Doyle, 2016; Tadros, Izquierdo, Esquena, & Solans, 2004). This phenomenon occurs when a large droplet increases its size, incorporating a smaller droplet by molecular diffusion (Ahmed, Li, McClements, & Xiao, 2012). According to Tadros et al. (2004), the high energy required for the formation of nanoemulsions can be understood from the pressure difference between the inside and the outside of a large drop that will divide into smaller drops; the drop must be strongly deformed, which can be shown when a spherical drop deforms into an ellipsoid. The force necessary to deform the drop is greater when the drop is smaller since the energy is generally transmitted by the surrounding liquid through agitation; therefore, more energy is needed to produce smaller drops. The dispersed phase can be controlled through osmotic pressure. In dilute volume fractions, the droplets are spherical, while in high fractions, the surfaces of the droplets are strongly repelled by the surfaces of the neighbouring droplets. This can cause the droplets to deform and become non-spherical. An appropriate surfactant can inhibit the droplets from recombining through interfacial coalescence, making the nanoemulsions stable (Graves, Meleson, Wilking, Lin, & Mason, 2005). This is due to the difference in the ratio of the curvature of the drops. In addition, this process can be observed in terms of a reduction in the free energy of the system via the destruction of the interfacial zone (Taylor, 1998). With a properly selected emulsifier, Ostwald ripening can be effectively controlled (Kabalnov & Shchukin, 1992).

2.1 Methods used to form nanoemulsions

Understanding the formation of nanoemulsions is essential to create a small droplet size; these dispersions are representatively developed in a two-step procedure in which a macroemulsion is developed and then transformed into nanodroplets (Gupta et al., 2016). Nanoemulsions are created using a series of specific methods, and they can be grouped based on the energy input, i.e., low energy and high energy. Optimal droplets are achieved by these methods, and methods are based on the materials designed for droplet preparation (Jin et al., 2016).

Low-energy methods

In these methods, a nanoemulsion is obtained when there is a phase inversion in the system due to variations in the conditions or concentrations of the emulsion and a state of low interfacial tension is reached (Gupta et al., 2016). Spontaneous emulsification, phase inversion composition and phase inversion temperature methods are some of the commonly used low-energy methods (Jin et al., 2016).

Method of spontaneous emulsification

In this method, a nanoemulsion is obtained when organic and aqueous phases are mixed (Anton & Vandamme, 2009; Miller, 1988). The elaborate procedure can be performed in different ways: the composition between the phases can be modified; the ambient conditions can be altered (temperature or pH); or the process conditions can also be altered (e.g., addition order). For example, a hydrophobic oil, a hydrophilic emulsifier, and an organic solvent could be incorporated into water (Anton & Vandamme, 2009). In addition, water can be incorporated into an organic phase containing an emulsifier and oil (Sonneville-Aubrun et al., 2009). Fine droplets are obtained by altering the composition of the phases and the process characteristics. There are currently no reports in which polysaccharides or proteins have been employed as emulsifiers in this process, which may be due to physicochemical limitations (McClements & Rao, 2011). Surh, Decker, and McClements (2017) produced nanoemulsions by means of spontaneous emulsification when they encapsulated lutein and obtained nanoemulsions that were stable against the aggregation of drops when the nanoemulsions were stored at room temperature for up to 1 month;

however, some colour fading occurred due to the degradation of lutein.

Phase Inversion Composition (PIC)

The PIC method optimizes the curvature of emulsifiers by changing the configuration at a certain temperature. The phase change is driven by the Gibbs free energy of the system and results in a spontaneous inversion of the emulsifiers. The PIC method is used to obtain an O/W emulsion by aqueous phase dilution in W/O emulsions (Jin et al., 2016; Roger & Cabane, 2012; Sonneville-Aubrun et al., 2009). Another process used to prepare nanoemulsions by this method is to alter the electrical charge (modifying the pH) of the emulsion (McClements & Rao, 2011). Pagan, Berdejo, Espina, Garcia-Gonzalo, and Pagan (2018) prepared nanoemulsions incorporating citral, using the PIC method to evaluate their antimicrobial activity; nanoemulsions were prepared from an oil phase mixture by slowly adding an aqueous phase during gentle magnetic stirring and then increasing the amounts of water added to the system with continuous agitation. The amount of water added to a W/O emulsion was progressively increased until a phase inversion occurred, and an oil-in-water emulsion was formed. The results of the study showed that the citral nanoemulsion was more effective than the free-form citral. At the same time, researchers applied a heat treatment and observed that there was an antimicrobial synergistic effect.

Phase Inversion Temperature (PIT)

The PIT method is based on modifications in the relative solubility of non-ionic emulsifiers with respect to certain temperature changes (Gutierrez et al., 2008). Nanoemulsions are formed using the PIT method via modifications in the structure of non-ionic emulsifiers when the temperature in the system is varied. At low temperatures, the head group of a non-ionic emulsifier is highly moistened and is more soluble in the aqueous phase. Conversely, as the temperature increases, the head group is dehydrated, and the solubility of the emulsifier in this phase decreases (Anton, Gayet, Benoit, & Saulnier, 2007; McClements & Rao, 2011). Chuesiang, Siripatrawan, Sanguandeekul, McLandsborough, and McClements (2018) formulated nanoemulsions of cinnamon oil by heating a mixture of cinnamon oil, a non-ionic surfactant and water above the PIT of the system and then quickly quenched this mixture with continuous agitation, which led to the spontaneous generation of small drops of oil. They formed nanoemulsions with a 101 nm drop diameter and reported that their nanoemulsions were stable for at least 31 days when stored at 4^oC or 25^oC.

High-energy methods

High-energy methods are made using various types of mechanical equipment, such as ultrasonic homogenizers, high-pressure homogenizers, or microfluidizers, that generate enough levels of intensive energy to rupture oil and water, producing small droplets. The energy inputs regulate the properties of the dispersions and prevent droplet rupture and coalescence (Jin et al., 2016).

High-pressure homogenization

High-pressure homogenization subjects an emulsion to high pressures and then passes it through a controlled valve. High pressure forms droplets due to breaking, and the droplets have a small size. The homogenization parameters, such as the pressure or temperature, modify the final characteristics of the nanoemulsions (Jin et al., 2016; Lee & Norton, 2013). Galvão, Vicente, and Sobral (2018) designed nanoemulsions with pepper extracts using a high-speed homogenizer followed by a high-pressure homogenizer. Nanoemulsions were evaluated based on their environmental (centrifugal and thermal) and storage stabilities, and it was found that the nanoemulsions were stable during centrifugation and under most thermal stresses when stored at 4 ^{o}C and room temperature for more than 120 days.

Microfluidizer homogenization

Microfluidization is very similar to the high-pressure homogenization method, and the terms are used interchangeably. The equipment has a narrow orifice through which the emulsions are pumped. The droplets undergo high shear to

form nanoemulsions. Similar to high-pressure homogenization, microfluidization requires multiple recirculation so that the droplets will reach a desired final size (Gothsch et al., 2011; Gupta et al., 2016). Raviadaran, Chandran, Shin, and Manickam (2018) optimized a palm oil-based nanoemulsion to encapsulate curcumin using a microfluidizer and considered the microfluidizer pressure, the number of cycles and the concentration of surfactant (Tween 80) to obtain droplet sizes from 200 nm to 300 nm.

Ultrasonication

Ultrasound technology relies on mechanical waves at a frequency above the threshold of the human ear (> 16 kHz). With this method, it is possible to obtain fine emulsions, which transform into smaller, more stable emulsions than those produced by other conventional techniques (Silva, Rosa, & Meireles, 2015). It is one of the most effective methods for the preparation of nanoemulsions for research purposes in the food industry (Moghimi, Aliahmadi, McClements, & Rafati, 2016; Sugumar, Ghosh, Mukherjee, & Chandrasekaran, 2016). The dispersions produced by ultrasound are more stable, and the small droplets show an excellent particle size distribution (Silva et al., 2015). Because ultrasonication uses high-intensity waves to generate intense disturbing forces within mixtures of oil and water, it promotes a decrease in the droplet size (Ghosh, Mukherjee, & Chandrasekaran, 2014; Moghimi et al., 2016; Tan et al., 2016). The use of ultrasound in the formation of nanoemulsions has shown promising results because there are no extra requirements for emulsion manufacturing (Nejadmansouri, Hosseini, Niakosari, Yousefi, & Golmakani, 2016). Shao et al. (2018) prepared a new eugenol nanoemulsion using ultrasonication as an emulsification technique, and the droplets obtained had a regular spherical shape with a size of 80-100 nm. In addition, the nanoemulsions showed high storage and thermal stabilities and presented excellent antioxidant capacity and antimicrobial activity, among others.

Materials used in the development of nanoemulsions

Nanoemulsion applications in food are becoming popular in the food industry to encapsulate components with potential biological activity, such as some fatty acids, liposoluble flavours, vitamins and others (Rao & McClements, 2011). Emulsifiers help in the production of emulsions by adsorbing at the water-oil interface during homogenization to decrease the interfacial tension (Yang, Leser, Sher, & McClements, 2013). The emulsifier must have certain characteristics: (1) it must rapidly reduce the interfacial tension at the newly formed water-oil interface; (2) it must be strongly bound to the interface once adsorbed; and (3) it must protect the droplets against destabilization. In many food process situations, the adsorption kinetics and interface stabilization processes are complicated because the emulsifiers have polydispersed sizes and are very different in their chemical compositions (Dickinson, 2009). The stability of electrostatically and sterically stabilized dispersions can be controlled by the charge of the electrical double layer and the thickness of the droplet surface layer formed by a non-ionic emulsifier (Capek, 2004). Emulsifiers and surfactants can be classified in various ways. One of the most common classifications is based on their application, i.e., emulsifiers, foaming agents, wetting agents and dispersants, among others. They can also be classified according to some physical characteristics, such as their affinity for water/oil or stability in hostile environments, and this is the classification most commonly used. According to the production source, surfactants can be classified as synthetic or natural. Moreover, it is possible to group them into four classes of primary surfactants: anionic, nonionic, cationic and amphoteric (Myers, 2005).
The substitution of synthetic emulsifiers with natural emulsifiers is a growing tendency since many synthetic emulsifiers are not permitted for use in many countries or can only be used at low levels due to legislative, monetary, or sensorial problems (Rao & McClements, 2011). Because of this situation, naturally occurring emulsifiers are a better choice for the development of nanoemulsions. Natural emulsifiers include proteins, polysaccharides and gums. Proteins act as

emulsifiers because of their amphiphilic characteristics, which confer the ability to adsorb at the oil-water interface and decrease interfacial tension. Polysaccharides, on the other hand, provide stability to emulsions mainly by increasing the viscosity of the continuous phase (Silva et al., 2015).

Silva, Cerqueira, and Vicente (2012) listed the emulsifiers that have been used in the development of nanoemulsions. Non-ionic emulsifiers, such as polyethylene glycol castor oil, polyoxyethylene-660-12-hydroxystearate, polyoxyethylene 4-lauryl ether, sucrose fatty acid ester (L1695), Span 20, Span 80, Tween 20, Tween 80, and Tween 40, have been used. Some anionic emulsifiers, such as Alkanol-XC and sodium dodecyl sulfate, have been used. The amphiphilic emulsifiers that have been used include some proteins, such as gelatin, whey protein isolate (WPI), whey protein concentrate (WPC), and whey hydrolysed protein (WPH). Modified starch and pectin have been used as anionic emulsifiers.

2.2 Use of nanoemulsions in foods

Currently, nanoemulsions have been designed to contribute to food safety via antimicrobial activity and the improvement of the availability of bioactive compounds due to the controlled release of the emulsion (Ranjan, Dasgupta, Ramalingam, & Kumar, 2017) There are bioactive compounds that are difficult to incorporate into foods. These are non-polar compounds with high melting points and low solubilities in water. The use of nanoemulsions is an excellent method used to incorporate these bioactive compounds into foods (Angel Robles-Garcia et al., 2016; Lu, Kelly, & Miao, 2016; Oehlke et al., 2014). Bioactive compounds, such as polyphenols, come mainly from plants and fruits (Hernandez-Fuentes et al., 2015; Valdes et al., 2015). Polyphenols have antioxidant, antimicrobial and anti-inflammatory properties (Perumalla & Hettiarachchy, 2011). Emulsion-based polyphenol encapsulation systems have been developed for obtaining food with novel properties (Pimentel-Gonzalez et al., 2015). Consumers seek to find natural conservators in foods to replace synthetic conservators (Seow, Yeo, Chung, & Yuk, 2014). Essential oils are natural conservators with characteristics such as high reactivity and hydrophobicity that make them difficult to add to industrialized foods. Nanoemulsions are a way to add essential oils and keep the organoleptic properties of the foods in which they are incorporated.

The nanoemulsion development with applications in the food area and on the edible coating of food is shown in Table 1. These dispersions are generally O/W and have mainly been designed to increase the stability of the encapsulated compounds, as well as to evaluate the behaviour of the nanoemulsions after exposure to gastric simulations. The work developed by Sari et al. (2015) incorporated curcumin in the oil drops of medium chain triglycerides using whey protein concentrate-70 and Tween-80 as emulsifiers to observe the in vitro release kinetics and found that the nanoemulsion was relatively resistant to digestion with pepsin, but with pancreatin, the compounds were released. The authors concluded that nanoencapsulation of highly unstable lipophilic compounds is an effective means to increase certain properties, such as bioavailability. Silva et al. (2018), who also incorporated curcumin in a multilayer nanoemulsion using layers of alginate and chitosan, performed another study that observed bioavailability. These authors simulated in vitro digestion conditions and found that this type of nanoemulsion improved control over the speed and degree of digestibility when compared to the level of control observed with uncoated nanoemulsions. These results showed in vitro digestion is an alternative method that can be used with functional foods to achieve an increase in satiety by delaying the digestion of lipids. Some other researchers developed nanoemulsions to observe their stability for incorporation into a food matrix, as is the case in the work performed by Golfomitsou, Mitsou, Xenakis, and Papadimitriou (2018), where a nanoemulsion was used as a vehicle for the enrichment of milk emulsions with vitamin D3 in. The stability was examined for several weeks, and it was found that the encapsulation of vitamin D3 was directly related to the size of the nanoemulsion, indicating that the encapsulation of this type of biocomposite was viable.

Table 1: Development and characterization of food grade nanoemulsions

Active compound	Area of application	Objective	Reference
β- Carotene	Development in the food area	Characterization	Yin, Chu, Kobayashi, and Nakajima (2009)
Phenolic compounds of grape marc	Development in the food area	Characterization and stability	Sessa et al. (2013)
Thymol	Packaging/edible coatings	Characterization and antioxidant activity	Ramos, Jimenez, Peltzer, and Garrigos (2014)
D-Limonene	Development in the food area	Characterization and stability	Zahi, Wan, Liang, and Yuan (2014)
Nigella sativa essential oil enriched with nanogold particles	Development in the food area	Bioactivity	Guler et al. (2014)
Black seed oil (*Nigella sativa*) and wheat germ oil	Development in the food area	Biological impact	Gumus et al. (2015)
Curcumin	Development in the food area	Bioavailability	Sari et al. (2015)
Oregano, thyme, lemongrass and mandarin oils	Development in the food area	Characterization and stability	Ines Guerra-Rosas, Morales-Castro, Araceli Ochoa-Martinez, Salvia-Trujillo, and Martin-Belloso (2016)
Carvacrol y cinnamaldelyde	Packaging/edible coatings	Characterization	Otoni, Avena-Bustillos, Olsen, Bilbao-Sainz, and McHugh (2016)
Tea polyphenols	Packaging/edible coatings	Characterization and stability	Liu et al. (2017)
Curcumin	Development in the food area	Characterization and bioaccessibility	Silva et al. (2018)
Vitamin D	Development in the food area	Characterization and stability	Golfomitsou, Mitsou, Xenakis, and Papadimitriou (2018)

Table 2: Antimicrobial effects used in the development of nanoemulsions in the food industry

Active compound	Effect against	Reference
Thyme essential oil	*Zygosaccharomyces bailli, Saccharomyces cerevisiae, Brettanomyces bruxellensis, and Brettanomyces naardenensis.*	Ziani, Chang, McLandsborough, and McClements (2011)
Lemon essential oil	*Escherichia coli, Listeria monocytogenes and Salmonella Typhimurium, Pseudomonas aeruginosa and Bacillus cereus*	Buranasuksombat, Kwon, Turner, and Bhandari (2011)
Thyme essential oil	*Zygosaccharomyces bailii*	Chang, McLandsborough, and McClements (2012)
Eucalyptus essential oil	*Bacillus cereus, Staphylococcus aureus and Escherichia coli*	Sugumar et al. (2013)
Basil oil	*Escherichia coli*	Ghosh, Mukherjee, and Chandrasekaran (2013)
Cinnamaldehyde	*Escherichia coli, Salmonella enterica, Listeria monocytogenes and Staphylococcus aureus*	Otoni, Pontes, Medeiros, and Soares (2014)
D-Limonene	*Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Saccharomyces cerevisiae*	Zhang, Vriesekoop, Yuan, and Liang (2014)
Thymol, carvacrol, linalool and eugenol	*Yeast and mold counting, coliforms and Salmonella sp.*	Khaled, Ramadan, and Ashoush (2014)
Salvia officinalis	*Escherichia coli, Shigella dysentery and Salmonella typhi*	Moghimi, Aliahmadi, McClements, and Rafati (2016)
Thymol and eugenol	*Listeria monocytogenes and Escherichia coli*	Ma, Davidson, and Zhong (2016)
Clove essential oil	*Listeria monocytogenes, Staphylococcus aureus and Escherichia coli*	Majeed et al. (2016)
Carvacrol	*Escherichia coli and Listeria innocua,*	Tastan, Ferrari, Baysal, and Donsi (2016)
Clove and cinnamon essential oils	*Escherichia coli, Bacillus subtilis, Salmonella typhimurium and Staphylococcus aureus*	Zhang, Zhang, Fang, and Liu (2017)
Geraniol and carvacrol	*Bacillus cereus and Escherichia coli*	Syed and Sarkar (2018)
Betel essential oil	*Staphylococcus aureus, Bacillus cereus, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa*	Roy and Guha (2018)
Citral essential oil	*Staphylococcus aureus, Escherichia coli, Pseudomonas Aeruginosa, Enterococcus faecalis, Salmonella typhimurium and Listeria monocytogenes*	Lu et al. (2018)

Table 2 shows the antimicrobial effect of nanoemulsions with bioactive compounds, which mainly contain essential oils or their derivatives. For example, the work performed by Buranasuksombat, Kwon, Turner, and Bhandari (2011), in which the authors developed a nanoemulsion containing lemon myrtle oil and compared it to a nanoemulsion of soybean oil that supposedly did not exhibit antimicrobial activity, confirmed antimicrobial action against *Salmonella typhimurium, Escherichia coli* 0157: H7, *Pseudomonas aeruginosa, Bacillus cereus* and *Listeria monocytogenes* by all emulsions. It was found that all of the emulsions with essential oils had the same level of antimicrobial effects against the five (5) bacteria, while all soybean oil emulsions had no antimicrobial effect. This antimicrobial activity was attributed to the encapsulated compounds. Another example of the antimicrobial use of nanoemulsions is in the work developed by Moghimi et al. (2016), where they encapsulated *Salvia officinalis* extract in a nanoemulsion using non-ionic surfactants (Tween 80, Span 80) and sonication. The antimicrobial activity of the nanoemulsions against foodborne bacteria (*E. coli, Shigella dysentery* and *S. Typhimurium*) was compared with that of the pure essential oil, and it was found that the antimicrobial effect of the nanoemulsion was four times greater than that of the oil. In addition, the results showed extensive damage of the bacterial cell membrane after treatment with the nanoemulsion. Zhang, Zhang, Fang, and Liu (2017) prepared nanoemulsions to encapsulate clove and cinnamon essential oils using Tween 80 and ethanol as a surfactant and cosurfactant, respectively. These nanoemulsions showed high antimicrobial activities against four tested microorganisms (*E. coli, Bacillus subtilis, S. typhimurium* and *Staphylococcus aureus*) when the nanoemulsions were at low concentrations, which shows that these compounds potentiate their effect when they are mixed and encapsulated in a stable system. The antimicrobial activity of emulsions based on geraniol and carvacrol against Bacillus cereus and Escherichia coli was evaluated, evaluated by Syed and Sarkar (2018) using ultrasonication, and it was demonstrated that this nanoemulsion prolonged the antibacterial efficacy of the combined emulsion of essential oils against both pathogens. The essential oils contained in a nanoemulsion can pass through the cell membrane, affecting its functionality and providing a high antimicrobial effect (Donsi & Ferrari, 2016).

At present, several studies have been carried out in which nanoemulsions serve to grant specific characteristics to complex food matrices. The high antimicrobial activity of nanoemulsions is the primordial effect that is sought when incorporating a nanoemulsion into food or coating food with a nanoemulsion, but the stability presenting when food is added should also be analysed. Table 3 shows several recent additions of nanoemulsions in foods. Several nanoemulsions have been tested in a few vegetal matrices to evaluate the effect after their application, e.g., in zucchini. Donsi, Cuomo, Marchese, and Ferrari (2014) observed the effect of the incorporation of a nanoemulsion based on carvacrol on the infusion rate. The results showed that emulsions of nanometric drop size smaller than the characteristic size exhibited a significantly improved effective diffusivity, which promoted a more effective antimicrobial action from carvacrol. Zambrano-Zaragoza et al. (2014) developed a nanoemulsion containing α-tocopherol and applied it to freshly cut apples to evaluate their enzymatic behaviour and texture changes with respect to time. Results showed that the activity of the pectin methylesterase in the coated apples was lower in the submicron-size samples, helping to maintain the firmness of the apples coated with the nanoemulsion; the authors mention that the particle size of the emulsion drops is a determining parameter in controlling the texture and browning index in foods. In grapes, Oh et al. (2017) investigated the effects of the droplet size of a nanoemulsion containing lemongrass oil. This was carried out to determine the effectiveness of the emulsion coating in improving microbiological safety. Their results showed higher initial inhibition of *Salmonella typhimurium*; greater inhibition of the growth of total mesophilic aerobes, yeasts and molds; higher colour retention; and higher antioxidant activity, among others.

The effect of nanoemulsions has also been determined in food of animal origins, such as chicken pâté (Moraes-Lovison et al., 2017) in which several quantities of oregano were incorporated into a nanoemulsion using the phase inversion tem-

perature method to obtain stable nanoemulsions. The authors found that the incorporation of nanoemulsions in chicken meat did not modify the physicochemical characteristics of the meat product. Therefore, the results obtained indicated that nanoemulsions are suitable for their incorporation into food formulations to prevent and control microbial growth and extend food's useful life. In trout fillets, the use of nanoemulsions has been reported to extend their useful life. Shadman, Hosseini, Langroudi, and Shabani (2017) incorporated different concentrations of *Zataria multiflora Boiss* into nanoemulsions based on sunflower oil, incorporated the emulsion into fillets and evaluated the physicochemical and sensorial properties during storage. The results revealed that the nanoemulsion achieved a reduction in lipid oxidation, a characteristic attributed to the high contents of carvacrol and thymol. The nanoemulsion also contributed to increasing the acceptability of taste, odour and texture. Noori, Zeynali, and Almasi (2018) developed a sodium caseinate-based coating that contained a nanoemulsion that encapsulated ginger essential oil and applied the coating to chicken breast fillets to observe their behaviour. The coatings caused a decrease in the count of total aerobic psychrophiles and maintained the colour, achieving a better acceptance of the coated fillets and becoming an alternative to improve the shelf life of raw chicken meat.

Nanoemulsions have been applied in bakery products to observe their behaviour with respect to time. Otoni, de Moura, et al. (2014) produced nanoemulsions with the essential oils clove and oregano and incorporated them into methylcellulose films, improving the mechanical properties of the films. Slices of bread were coated with these films, and it was found that both oils managed to reduce mold and yeast counts. The authors mention that because of the particle size, bioavailability is improved, and thus, less preservative content can be used to obtain the same antimicrobial efficacy. It is evident that nanoemulsions help maintain some properties with respect to the shelf life of the product to which they were applied. Donsi and Ferrari (2016) classified four major macro-areas for the applications of nanoemulsions containing essential oils: a) Direct incorporation in liquid food, b) disinfection of the surface of the food with an antimicrobial dispersion, c) immersion into porous food matrices, and d) coverage with a biopolymer layer containing the nanoemulsion.

There are some cases of nanoemulsion use by world-class companies, such as Nestlé and Unilever. Nestlé has developed W/O nanoemulsions to make microwave defrosting easier for some foods by the incorporation of polysorbates and other compounds. Unilever produce a reduced-fat ice cream with the application of nanoemulsions (Silva et al., 2012). The German company AQUANOVA in its product line NovaSOL® has used nanoemulsions in the food industry and developed a varied product line focused on the protection and shelf life of various products, such as beverages and supplements, with essential oils, vitamins, and pigments, among others (AquaNova, 2016). The products based on nanoemulsions are very attractive because they are translucent in appearance and can increase the bioavailability of the bioactive component they possess. Preferably, nanotechnology should be simple to use and inexpensive, and it should have easily perceived benefits that may be considered as a real alternative to established techniques. Therefore, the food industry should use nanoemulsions from legally acceptable and ecologically and economically feasible ingredients (Ranjan et al., 2017).

3 Risks and regulations of the utilization of nanotechnology in food

There are some reports from the media about the possible adverse health effects of nanotechnology, and this has resulted in increased negative perceptions about this field and the food industry. Although there are risks associated with some nanomaterials used in foods, they should not be considered as potential risks as natural nanomaterials have been consumed since the Neolithic era (Bouwmeester et al., 2009; Rogers, 2016; Szakal et al., 2014).

In general, each country should have an organization that is responsible for regulating and verifying the risks associated with nanotechnology. The legislation available for the European Union

Table 3: Applications of nanoemulsion in food

Active compound	Applied in:	Objective	Reference
Carvacrol	Zucchini	Antimicrobial effect and mass transfer	Donsi, Cuomo, Marchese, and Ferrari (2014)
Lemongrass essential oil	Apples	Characterization, stability and antimicrobial effect	Jo et al. (2014)
α- Tocopherol	Apples	Shelf life	Zambrano-Zaragoza et al. (2014)
Lemongrass essential oil	Apples	Characterization, stability and antimicrobial effect	Salvia-Trujillo, Alejandra Rojas-Graue, Soliva-Fortuny, and Martin-Belloso (2015)
Oregano essential oil	Lettuce	Antimicrobial effect	Bhargava, Conti, da Rocha, and Zhang (2015)
Essential oils of carvacrol, bergamot, lemon and mandarin	Broccoli	Antimicrobial effect	Severino et al. (2014)
Carvacrol, mandarin, bergamot and lemon oils	Green beans	Antimicrobial effect	Severino et al. (2015)
Lemongrass essential oil	Grapes	Characterization, stability and antimicrobial effect	Kim, Oh, Lee, Bin Song, and Min (2014)
Lemongrass essential oil	Grapes	Antimicrobial effect	Oh et al. (2017)
Trans-cinnamaldehyde	Melon juice	Antimicrobial effect	Jo et al. (2015)
Curcumin	Milk	Characterization and antioxidant activity	Joung et al. (2016)
Thymol	Milk and cantaloupe juice	Antimicrobial effect	Xue, Davidson, and Zhong (2017)
Oregano essential oil	Chicken pate	Characterization and antimicrobial effect	Moraes-Lovison et al. (2017)
Oregano essential oil	Reduced fat cheese	Antimicrobial effect and shelf life	Artiga, Fani, and Martin-Belloso (2017)
Essential oil of Zataria multiflora Boiss	Trout fillet	Shelf life	Shadman, Hosseini, Langroudi, and Shabani (2017)
Essential oils of rose, laurel, thyme and sage	Trout fillet	Characterization and antimicrobial effect	Ozogul et al. (2017)
Clove and oregano essential oils	Bread	Antimicrobial effect	Otoni, de Moura, et al. (2014)
Curcumin	Chicken fillets	Characterization and shelf life	Abdou, Galhoum, and Mohamed (2018)
Quercetin	Chicken pate	Shelf life	de Carli, Moraes-Lovison, and Pinho (2018)
Ginger essential oil	Chicken fillets	Shelf life	Noori, Zeynali, and Almasi (2018)
Carotenoids	Carrot puree	Bioaccessibility	Salvia-Trujillo et al. (2019)

can be consulted online at http://EUR-LEX.europa.eu/. In the United States, food additives are always subject to authorization by the Food and Drug Administration (FDA). The FDA has published some papers that approach the problems of nanotechnology, for example: "Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology" (U.S. Food and Drug Administration, 2014). In Canada, nanotechnology issues are reviewed by the Canadian Food Inspection Agency (CFIA); in Switzerland, by the Swiss Federal Office of Public Health (FOPH); in Japan, by the Ministry of Health, Labour and Welfare; and in China, by the Ministry of Health. Most countries continue to rely on existing legislation about nanomaterials, although a significant challenge exists between nanotechnology legislation and industrial application (Amenta et al., 2015). Improvement in the regulatory system is crucial to avoid consumer disinformation in relation to applied nanotechnology in food. Although the relevant legislation is still being developed, further action can be taken to obtain consumer acceptance in foods containing nanotechnology-based materials. This includes ensuring the use of food-grade ingredients to produce nanosystems (Singh, 2016). It is inevitable that human exposure to nanomaterials will increase in various ways, whether intentional or not. Nanomaterials, which serve as food additives (including nanoemulsions), come into direct contact with humans, resulting in higher levels of exposure depending on their addition to food. Thus, it is important to consider the risks of nanoemulsions in biochemical (solubility, metabolism/excretion) and toxicity profiles (Amenta et al., 2015; Landsiedel et al., 2012). For the evaluation of nanotoxicity, several criteria can be considered, such as exposure assessment, nanoparticle toxicology, biological fate, transport, persistence and transformation of nanoparticles, among others (Chau, Wu, & Yen, 2007; Dreher, 2004). Therefore, the use of nanoemulsions with the incorporation of bioactive compounds in food should significantly attract the attention of the public and governmental sectors (Jovanovic, 2015).

4 Conclusion

The use of nanoemulsions, mainly as effective encapsulation systems, has received great interest from the food industry due to their advantages, including small droplet size, transparency and high stability. The food industry seeks to implement these nanosystems of encapsulation for the addition of compounds with biological activity, e.g., functional lipophilic substances.

Nanoemulsions have been used mainly in the development of some foods and beverages to enrich the bioavailability of certain compounds and to contribute to the stability of the foods that contain them. An interesting challenge involves the development of nanoemulsions that are compatible with the product in which they will be incorporated; therefore, there must be adequate knowledge about the formulation and functionality of the product. In addition, the application of nanoemulsions in food systems presents challenges, e.g., reducing production costs.

Consumer acceptance should be achieved based on research showing that nanoemulsions do not cause toxicity and that they are not toxic and do not accumulate in the human body. In addition, effective legislation for nanotechnology in food safety must be established. The use of nanotechnology in the food sector is a field of study in development that could improve food systems that provide functionality and improve aspects related to health.

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