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FORMULATION OF NATURAL HYDROCOLLOIDS AND VIRGIN COCONUT OIL AS A PLANT-BASED SALAD DRESSING

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ABSTRACT

Salad dressing is traditionally used as a seasoning to enhance consumers' appetite due to its creamy mouthfeel and special flavor. However, consumers are aware of the cholesterol level in egg yolk and the fat type applied in dressing products. This study aimed to produce low-fat and eggless salad dressing with virgin coconut oil (VCO). Hydrocolloids, including xanthan gum and modified starch, were used as independent variables by response surface methodology (RSM) to evaluate their impacts on the salad dressing's viscosity, stability, and firmness. The findings showed that optimum values for the hydrocolloids of xanthan gum and modified starch were 1.56% and 0.10%, respectively and the optimum experimental values were stability 0.33%, texture 1506.5 g, and viscosity 162.25 mpas. This optimized formulation's predicted and experimental data had no significant ($p>0.05$) differences that indicated this study's desired results. The proximate analysis of the optimized formulation was moisture content 47.91, ash 1.91, fiber 1.57, fat 21.97, protein 1.66, carbohydrate 24.98, and caloric values 296.29. The findings of this study were similar to the commercial products, which suggested a high potential for using optimum values for the hydrocolloids of xanthan gum and modified starch as an egg replacer and VCO in salad dressing to improve the quality and the biological functions of the product.

1.Introduction

Salad dressing is a kind of emulsion formulated with vegetable oil not less than 30% oil, egg yolk or emulsifier and stabilizer, flavoring agents, and acidifying ingredients (Ma et al., 2013a). The consumption of this product is growing as a preferable sauce worldwide due to its accepted flavor and sensory attributes resulting from the addition of starch or hydrocolloids, which gives the required consistency of that product (Ma and Boye, 2013b). Egg yolk is used in dressing products to improve the emulsion stability by decreasing interfacial tension and forming a layer that prevents the droplets from aggregation. However, its high content of high-density lipoproteins (HDL) and low-density lipoproteins (LDL) are associated with several cardiovascular diseases (Marventano et al., 2020). Besides, for vegans and vegetarians who wish to include plant-based fat in their diet for various reasons, a growing interest in developing egg-free food and low-in fat products has been generated according to the consumer's awareness. Vegan diets exclude all animal items, including meat, fish, dairy products, and eggs (Bradbury et al., 2017). The food industry has faced major challenges in producing varieties of salad dressing products that have reduced-calorie content, contain higher levels of plant-based ingredients, and are

high in beneficial ingredients that can be used. Several studies have recently focused on producing a new salad dressing or mayonnaise formulation to reduce the fat content and replace the egg yolk associated with heart diseases (Ma et al., 2016; Tekin and Karasu, 2020). This could be done by applying plant-based alternatives with acceptable characteristics to meet the consumers' desires. According to Chivero et al. (2016), emulsion-based mayonnaise stabilized by Xanthan gum instead of the egg yolk could be applied as an alternative hydrocolloid emulsifier to formulate mayonnaise. Hydrocolloid is one of the important ingredients to increase the stability and physicochemical properties of the salad dressing.

In addition, using the hydrocolloid improves the characteristics of the food due to its ability to alter the rheology of the food system. In addition, they usually act as stabilizers (stabilizing agents) of oil-in-water emulsions (Dickinson, 2009). Moreover, fat used in dressing products is usually hydrogenated fats associated with heart diseases. Ban has been announced against trans and hydrogenated fat in food products, which was recently effective in different countries (Chen, 2020). Therefore, replacing fats with functional oils that contain nutritional value with biological activities contributes to healthy diets required by consumers. Virgin coconut oil (VCO) is an appropriate fat substitute for the salad dressing formulation (Dayrit and Nguyen 2020). The VCO is frequently used to produce oil/water emulsions. The previous study showed the VCO application in the emulsion to be used as a new nutritional food supplement. The findings demonstrated that the stability of the emulsion, which was stored at 4°C and 25°C throughout the storage period, had no alter in the free fatty acid composition of the VCO (Khor et al., 2018). According to the recent review study by Mirzanajafi-Zanjani et al. (2019), several studies have attempted to replace the egg with plant-based emulsifiers to produce egg-free mayonnaise (Muhialdin et al., 2021). Optimizing the specific ratios of Xanthan gum and modified starch acts as hydrocolloid agents with a low-fat content of VCO as a fat alternative is challenging to produce healthy sauces.

The main objective of this work was to develop egg-free and low-fat mayonnaise with a high nutritional fat alternative. In detail, the following effects were studied: (1) using VCO as a fat substitute and preparing the oil in water emulsion, (2) replacing the egg yolk with plant-based hydrocolloids, and (3) optimizing the hydrocolloids (Xanthan gum and modified starch) used as egg replacers and evaluate the approximate analysis and sensory characteristics.

2. Materials and methods

2.1. Materials

Virgin Coconut Oil (VCO), salt, sugar, mustard powder, lemon juice, and acetic acid were purchased from a local bakery shop in Selangor, Malaysia. Xanthan gum, modified starch, and analytical-grade solvents were purchased from Sigma–Aldrich Co (St. Louis, MO, USA). Commercialized products were presented for comparison with the optimum formulation, namely Mayolite Salad Dressing Lady's Choice, Kewpie Half Salad Dressing, Mayonnaise Lady's Choice, and Kimball mayonnaise, and coded as C1, C2, C3, and C4, respectively.

2.2. Preparation of VCO salad dressing

The virgin coconut oil (VCO) salad dressing was prepared at different concentrations of hydrocolloids, xanthan gum and modified starch, according to the experimental design following Fonseca et al. (2009) method with a slight modification. Table 1 shows the formulations of salad dressing containing sugar 4%, mustard powder 7.5%, and salt 1.5% added to a bowl containing lemon juice 5.5% and acetic acid 4.5%, then mixed homogeneously using the mixer and homogenized for 10 min. Subsequently, hydrocolloids consisting of xanthan gum 1-1.9% and modified starch 0.1-1% were added to the solution as per the experimental design

(Table 2) and then stirred using a magnetic stirrer at room temperature for at least 6 h to ensure complete emulsification in order to prepare the aqueous phase. Finally, 75 ml of virgin coconut oil as the oil phase was added gradually into the aqueous phase and homogenized for 20 min. As a result, the aqueous and oil phases had a ratio of 75:25.

Table 1. Shows all the formulations with different percentages of hydrocolloid in VCO salad dressing

Ingredients	Percent (%)
Virgin coconut oil (VCO)	31.80
Coconut milk	45.81
Lemon juice	4.46
Acetic acid	4.46
Mustard powder	6.78
Salt	1.25
Sugar	3.44
Xanthan gum	1-1.9 *
Modified starch	0.1-1*
Total	100.0

*Amount varied according to the experimental design

2.3. Emulsion stability test

The stability of the dressing formulations was evaluated after 24 hours of storage, referring to the method performed by de Melo et al. (2016) with slight modification. The salad dressing formulations were centrifuged at (3500 × g) for 30 min at 25˚C. The emulsion stability was determined by comparing the oil percentages before and after centrifuging. The less oil that was calculated shows the highest stability.

2.4. Determination of texture

The texture determination was done according to Ng et al. (2014) with slight modification. It was performed by using the equipment of Texture Analyzer TA. XT2i (Stable Micro Systems Ltd, Surrey, UK). The 5 kg load cell was used for this penetration test to measure firmness and consistency. Using a probe (P/36R), aluminum radiused AACC with 10 mm penetration, 1 mm/sec pre-test speed, 1 mm/sec test speed, and 10 mm/sec, all the samples of VCO salad dressing were placed in a round plastic container at a depth of 30 mm. Then, this test was performed in triplicate.

2.5. Determination of rheological properties (Viscosity)

The method by Jung (2011), with slight modification, was used to determine the viscosity of VCO salad dressing using the equipment of a rheometer (Physical Rheolab, Anton Paar, Austria). First, the samples were added to the viscometer cup at about 10g. Next, each sample was recorded once the value shown on the viscometer became stable, it was started. Finally, the constant room temperature (25˚C) was used, and all samples were performed in triplicate.

2.6. Proximate analysis

The determination of moisture, protein, ash, fat, fiber contents, and caloric value of the low-fat and eggless VCO salad dressing samples were conducted as the official methods ascribed by the Association of Official Analytical Chemistry (AOAC 2006).

2.7. Determination of color

The determination of color was measured by using a Colorimeter (Ultra Scan Pro, Hunter Lab, USA). The emulsion of each sample was measured by referring to the value of the color system, L* = 97.10, a* = −0.07, b*=+1.97, shows the lightness meanwhile a* (+a is the red coordinate, −a is the green coordinate) and b* (+b is the yellow coordinate, and−b is the blue coordinate) represents the color coordinates. The color becomes more saturated as the a* and b* values rise. However, the value in this test will approximately approach zero for neutral colors such as black, grey, or white. All the samples were done in triplicate (Ng et al., 2014).

Table 2. Emulsion stability, texture, and viscosity of formulation salad dressing using face-centered Central Composite Design

Std Order	Run Order	PtType	Xanthan %	Modified Starch %	Stability (%)	Texture (g)	Viscosity (mpas)
12*	1(c)	0	1.45	0.55	0.81	149.197	1192.50
5	2	-1	1.00	0.55	1.40	139.325	1107.5
8	3	-1	1.45	1.00	2.46	122.820	1479.10
10*	4(c)	0	1.45	0.55	0.81	149.197	1192.50
9*	5(c)	0	1.45	0.55	0.81	153.260	1050.25
3	6	1	1.00	1.00	0.79	178.495	1624.85
1	7	1	1.00	0.10	0.05	123.800	1470.65
7	8	-1	1.45	0.10	0.04	178.200	1471.61
4	9	1	1.90	1.00	0.47	67.017	1452.05
13*	10(c)	0	1.45	0.55	0.30	153.260	1050.25
2	11	1	1.90	0.10	0.00	123.530	2682.89
6	12	-1	1.90	0.55	0.02	103.520	1992.60
11*	13(c)	0	1.45	0.55	0.30	153.260	1050.25

(c) center point.

2.8. Sensory evaluation

The sensory evaluation was done according to Liu et al. (2007) method with some modifications. The optimized formulation salad dressing was compared with 3 different commercial products named Mayolite Salad Dressing Lady's Choice (C1), Kewpie Half Salad Dressing (C2) and Mayonnaise Lady's Choice (C3). In this sensory evaluation, 30 untrained students were asked to perform a sensory test for appearance, color, odor, texture, flavor, and overall acceptability on a 5-point scale, presenting 1 as the least preferred and 5 as the most preferred. Three-digit random numbers were used to code the optimized samples and three commercial items. It was given to panelists on a tray in individual booths to eliminate prejudice. During the sensory evaluation, water was provided between samples as palate cleanses for each untrained panelist.

2.9. Statistical analysis

Minitab 17.0 (Minitab, Inc, State College Pennsylvania, USA) was used for the optimization study. This optimization research used a face-centered central composite design (CCD) with two independent variables: xanthan gum (1-190%) (x_1) and modified starch (0.1-1%) ($x2$). In addition, the emulsion stability (y^1), texture or (firmness) (y^2), and viscosity (y^3) were set as response variables at three coded levels (-1, 0, +1) and 5 replicates at the center point was programmed by the software, which a complete design consisted of 13 experimental runs were automatically generated for each salad dressing (Table 2). As a result, the effect of the two independent variables on the RSM was obtained.

The polynomial regression model equation was utilized, and the performance of the response surface was examined. The generalized response surface model is given below:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_{12} + \beta_{22} x_{22} + \beta_{12} x_1 x_2 \quad (1)$$

Where: y is the response calculated by the model; β_0 is a constant regression; and β_i, β_{ii} and β_{1j} are the linear, squared, and interaction coefficients, respectively. The $x_1$and x_2 is the coded independent variables. One-way analysis of variance (ANOVA) was used to find the differences using a method for all responses with a significant level of ($p < 0.05$) determined

using Turkey's test. All the data were reported in mean ± standard deviation with a significance letter.

3. Results and discussions

3.1. Response surface analysis

RSM was applied with the 23 CCD to determine the optimum hydrocolloids (xanthan and modified starch) for salad dressing formulation. The emulsion stability, viscosity, and texture (firmness) were set as the response for this model. Table 2 shows the experimental data of emulsion stability, viscosity, and texture from the optimization study. The model summary, as shown in Table 3 divided into linear, quadratic and interaction. From the data obtained, R2 from the viscosity response was essentially high, 0.9315, compared to the texture (firmness) and stability, 0.8962 and 0.3805, respectively. Therefore, the models explained viscosity and texture (firmness) well and reached more than 0.8. However, the response of emulsion stability had no desirable results, although the lack of fit was better than both ($p>0.05$).

Table 4 depicts the significance probability of the p-value and F-value from the main linear, quadratic, and interaction effects after the final reduced models. In order to obtain the best results, the p-value should be significant ($p<0.05$). In addition, the insignificant results ($p>0.05$) must be excluded to improve the model before the final reduced model is constructed. Based on the results obtained, there was only a linear effect on emulsion stability on modified starch. However, the model explained all the effects well regarding viscosity, as there were significant differences ($p<0.05$). Meanwhile, regarding the texture (firmness), there was only a quadratic square effect on modified starch that was insignificant ($p>0.05$) as compared to all the effects. In short, all the responses give the desired results except for the emulsion stability.

3.2. Responses to the optimization conditions

Based on the data obtained, all the responses were dependent on the percentages of hydrocolloids named xanthan gum and modified starch in this study. In terms of emulsion stability, the lowest percentages of oil expelled give the higher emulsion stability of the salad dressing. The higher percentages of the xanthan gum, 1.90%, with lower percentages of modified starch, 0.10%, give the higher emulsion stability 0.00% among all of the runs Table 2.

Table 3. Regression coefficient, R^2, adjusted R^2, probability values, and lack of fit for the final reduced models

Regression coefficient	Stability (Y_1)	Texture (Y_2)	Viscosity (Y_3)
Constant			
b_0	0.635	151.31	1122.4
Linear			
b_1	-	-24.59	320.8
b_2	0.604	-9.53	-178.2
Square			
b_1^2	-	-28.70	389.4
b_2^2	-	-	314.9
Interaction			
b_{12}	-	-27.80	-346.3
R^2	0.3805	0.8962	0.9315
R^2 (adj)	0.3242	0.8444	0.8825
Regression (p-value)	-	-	-
Lack of fit (F-value)	5.96	57.00	8.36
Lack of fit (p-value)	0.052	0.001	0.034

*b_1= xanthan gum, b_2= modified starch

Table 4. The significance probability (p-value & F-value) of regression coefficients in the final reduced models

Variables		Main linear effects		Quadratic effects		Interaction effects
		x_1	x_2	x_1	x_2	x_1x_2
Emulsion Stability (Y_1)	p-value	-	0.025	-	-	-
	F-value	-	6.76	-	-	-
Texture (Firmness)(Y_2)	p-value	0.001	0.087	0.003	-	0.002
	F-value	25.26	3.80	18.52	-	21.52
Viscosity (Y_3)	p-value	0.002	0.029	0.005	0.013	0.003
	F-value	24.48	7.55	16.60	10.85	19.01

* x_1= xanthan gum, x_2= modified starch

On the other hand, the combination of xanthan gum and modified starch with the percentages 1.45% and 1.00%, respectively, gives the highest value of 2.46%, which shows that this run had the lowest emulsion stability. Therefore, xanthan gum was more important in having strong emulsion stability than modified starch. Cabeza et al. (2002) revealed that emulsion stability could be increased by adding emulsifiers to reduce the interfacial tension or hydrocolloid to increase the viscosity. Therefore, it can reduce drop mobility. As supported by the previous study by Dolz et al. (2007), the synergistic of xanthan gum and modified starch promotes good stability, texture, and acceptability in low oil content in mayonnaise formulations. Moreover, the xanthan gum stabilizes the starch gel since its dispersion is not thixotropic, defined as the isothermal slow reversible conversion from gel to solution.

To choose the suitable viscosity range, this study refers to the commercial products in the market named C1, C2, C3, and C4, which are 1450-2200 mpas. From the results obtained, the combination of xanthan gum and modified starch at 1.45% and 1.00%, 1.00% and 1.00%, 1.00% and 0.10%, 1.45% and 0.10%, 1.90% and 1.00%, respectively give the data in the range of the commercialized products which were desirable. Hence, this result depicted that the percentages of xanthan gum were flexible in all the runs to get desired viscosity but must be synergistic with the modified starch. Furthermore, according to Dickie & Kokini (1983), a strong link was discovered between shear stress on the tongue and sensory thickness. Therefore, it was also proposed that the measured gap be utilized as an indicator of oral texture when creating a new salad dressing product.

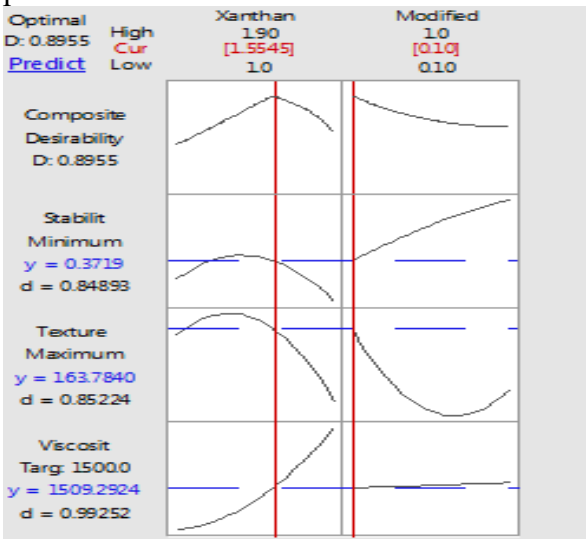

Figure 1. The optimization plot of the optimized formulation

Besides, the texture in terms of firmness was chosen for the response in this RSM. The maximum texture was chosen to be the desired firmness for the optimum condition. The highest firmness was the combination of xanthan gum (1.00%), and modified starch (1.00%) which was 178.495 g, while the lowest firmness was the combination of xanthan gum (1.90%) and modified starch (1.00%) was 67.017g. The combination of xanthan gum and modified starch of 1.45% and 0.10%, respectively, was the second highest which was 178.200 g, followed by xanthan gum (1.45%) and modified starch (0.55%) which was 153.260 g. The observation shows that the percentage of xanthan gum plays an important role compared to the modified starch. The percentages of xanthan gum are higher than modified starch to give the desired firmness of salad dressing. This was due to the addition of xanthan gum, which prevents the creaming phenomena by flocculating the emulsion droplets to create a weak particle network rather than showing its solution rheology on the dressing (Parker et al., 1995).

3.3. Optimization and validation condition

Figure 1 shows the optimization plot obtained from the RSM.

x1= xanthan gum, x2= modified starch, whereas y1= 0.3719%, y2= 163.784g, and y3= 1509.2924 mpas.

The optimization plot of the optimized formulation is shown in Figure 1. Based on the model analyzed, the factor of xanthan gum and modified starch that the model had optimized were 1.56% and 0.10%, respectively. Besides, the predicted data from the model predict the emulsion stability, texture (firmness), and viscosity as follows 0.37%, 1509.29 mpas and 163.78 g, respectively.

On the other hand, the emulsion stability was excluded due to the lowest R2, less than 0.8. The chosen parameter for the texture (firmness) was to get the maximum firmness of more than 160 g. Thus, this figure 1 explains that at the range 1.4-1.56% of xanthan gum and less than 0.2% of modified starch, or at the range 1.0-1.2 of xanthan gum and 0.8-1.0% of modified starch gives the desired firmness. Verbeken et al. (2006) reported that on cooling, modified starch forms thermally irreversible opaque gels, but xanthan gum exhibits high shear thinning and retains viscosity in the presence of electrolytes. Therefore, this combination of hydrocolloids can affect the firmness of the VCO salad dressing. This was supported by a previous study by Gibinski et al. (2006), which indicated that a thickening made up of starch and xanthan gum is a good choice for sauces with a shelf life of fewer than three months because it delivers consistent sensory and textural qualities. For the viscosity, the targeted parameter was 1500 mpas which was chosen based on the references from the commercial products. As reported by the previous study by Dolz et al. (2007), which looked at the effects of xanthan gum and locust bean gum on the flow and thixotropic behavior of food emulsions, including modified starch, found that the emulsion with the highest concentrations of gums had a greater viscosity than the modified starch reference emulsion.

Table 5. The validation of prediction and experimental optimization formulation salad dressing

	Emulsion Stability (%)	**Texture (Firmness) g**	**Viscosity (mpas)**
Prediction value	0.372[a]	163.78[a]	1509.29[a]
Experimental value	0.33 ± 0.119[a]	162.25 ± 1.129[a]	1506.5 ± 3.569[a]

Significance letter [a] shows insignificant differences between the standard and sample ($p>0.05$).

Table 5 shows the prediction and experimental value of the optimization formulation salad dressing to demonstrate the reduced models' accuracy and the validation of this RSM. Based on the results obtained for the experimental value, all the responses of emulsion stability, viscosity, and texture (firmness) showed an insignificant difference ($p>0.05$) as compared to the prediction values,

as shown in Table 5. Therefore, this validation proved the appropriate model for optimizing the xanthan gum and modified starch percentage to produce low-fat and egg-free salad dressing.

3.4. Proximate analysis of optimized formulation salad dressing

Table 6 shows the proximate analysis results of the optimized formulation salad dressing, including moisture and ash content, fiber, fat, protein, carbohydrate, and caloric values. Based on the results, the moisture content was highest among all the proximate analyses, 48.33%, compared to ash, crude fiber, crude fat, protein and carbohydrate, which were 1.96%, 1.76%, 21.02%, 1.66%, and 23.32%, respectively.

Table 6. Proximate composition of optimized formulation salad dressing.

	Moisture (%)	Ash content (%)	Fibre (%)	Fat (%)	Protein (%)	Carbohydrate (%)	Caloric values (kcal)
C1	47.91	1.91	1.57	21.97	1.66	24.98	296.29
C2	48.84	2.05	2.04	20.35	1.67	22.73	280.75
C3	48.24	1.93	1.67	20.75	1.65	22.24	282.31
C4	47.84	2.94	1.83	20.95	1.67	24.77	293.35
Total	48.33±0.47	1.96±0.08	1.76±0.25	21.02±0.84	1.66±0.01	23.32±1.46	286.45±8.56

*C1= Mayolite Salad Dressing Lady's Choice, C2= Kewpie Half Salad Dressing, C3= Mayonnaise Lady's Choice, C4= Kimball mayonnaise, VCO-salad dressing, Data are reported in mean ± standard deviation (n=3).

The higher moisture content was due to the high percentages of liquid content used in the formulation rather than solid content. Thus, these percentages increase the moisture content of this formulation. The second highest was the carbohydrate which depends on the composition of the others. The lower the composition, such as moisture, ash, fiber, fat, and protein, the increased carbohydrate content. Next, the fat was also relatively higher, 21.02%, due to the types of oil used. The ash, crude fiber, and protein were in the same range. According to the previous study by Babajide & Olatunde (2010), the proximate composition of salad dressing by using corn starch (Cs), cocoyam starch (Cy) with a different formulation of 100Cs, 75Cs: 25Cy, 50Cs:50cy, 25Cs:75Cy and 100Cy, respectively gives the results of moisture content, ash, fat, protein and carbohydrate at the range 48-49%, 0.59-0.79%, 27.04-27.99%, 2.63-3.28%, and 18.96-19.95%, respectively. These values were not too far from the optimized formulation of salad dressing from the present study. Besides, the different results obtained from the previous and present study can be due to the ingredients used in the formulations, such as types of oil, the addition of hydrocolloids, and others which contributed to the values of each composition in the products. This optimized formulation gives about 286.45 kcal for a 100 g serving caloric value. This caloric value is normal for salad dressing; usually, the consumer takes 20 g per serving, which will be 57.29 kcal.

3.5. Color Evaluation

The color of the low-fat and egg-less VCO salad dressing produced by the optimum percentages of xanthan gum and modified starch was evaluated. Besides, this formulation was compared with the commercialized products, which presented as C1, C2, C3, and C4. These color-system values of the formulated low-fat and egg-less VCO salad dressing and commercial products were expressed as lightness (L*), redness-greenness (a*), and yellowness-blueness (b*). Table 7 depicted that there was no significant difference ($p>0.05$) of L* for all the low-fat and egg-less VCO salad dressing and commercial products except for C1, which gives significant differences ($p<0.05$). It was the same goes for a*, which gave insignificant differences ($p>0.05$) for low-fat and egg-less VCO salad

dressing and commercial products, except for C1, which gave significant differences ($p<0.05$).

Table 7. The color of all formulations

Formulations	Color		
	L*	***a****	***b****
C1	92.89 ± 0.348^{a}	-2.73 ± 0.09^{b}	16.2 ± 0.121^{b}
C2	90.08 ± 0.639^{b}	-1.46 ± 0.215^{a}	29.26 ± 0.558^{a}
C3	89.55 ± 0.449^{b}	-1.64 ± 0.131^{a}	16.867 ± 1.438^{b}
C4	90.22 ± 0.973^{b}	-1.73 ± 0.09^{a}	21.2 ± 0.897^{b}
VCO-salad dressing	90.12 ± 0.125^{b}	-1.45 ± 0.02^{a}	19.88 ± 0.062^{b}

*C1= Mayolite Salad Dressing Lady's Choice, C2= Kewpie Half Salad Dressing, C3= Mayonnaise Lady's Choice, C4= Kimball mayonnaise, VCO-salad dressing, Data are reported in mean ± standard deviation (n=3). Significant letter a to b shows a significant difference ($p<0.05$) among all the formulations.

However, the b* gave an insignificant difference ($p>0.05$) of b* for low-fat and egg-less VCO salad dressing and commercial products except for C2, which gives significant differences ($p<0.05$). Thus, the results show that low-fat and egg-less VCO salad dressing was acceptable in the range with all the commercialized products.

The commercial dressing had a lighter appearance as the value of L* was increased. In addition, as Ying (2015) reported, the oil had beaten consistently and vigorously during emulsion preparation, eventually making the oil droplet smaller. As a result, the bigger the surface of oil contact with the liquid phase, the smaller the oil droplet. Hence, it increased the lightness due to more network interaction with the liquid phase.

3.6. Sensory evaluation

Figure 2 shows the sensory evaluation using the spider web plot of optimized formulation salad dressing, which was compared with three different commercial products named C1, C2, and C3. This sensory evaluation measured some attributes: appearance, color, aroma, texture, flavor, and overall acceptability.

The results showed no significant difference ($p>0.05$) between the optimized formulation salad dressing and C2, 3.7 ± 1.236 and 4.73 ± 0.583, respectively. Besides, in terms of color, there was no statistically significant difference ($p>0.05$) between the improved formulation and the C2 formulation, which gave 2.7 ± 1.178 and 3.57 ± 1.135, respectively.

This showed that the panelist accepted the appearance and color of this optimized formulation of VCO-salad dressing. In addition, the aroma of the optimized formulation was 2.7 ± 1.178, which gave no significant differences with C1, 3.57 ± 1.135.

Therefore, these results show that the optimized formulation was still acceptable even though there was a significant difference between C3 and C4, which were 3.4 ± 1.248 and 3.53 ± 1.106, respectively.

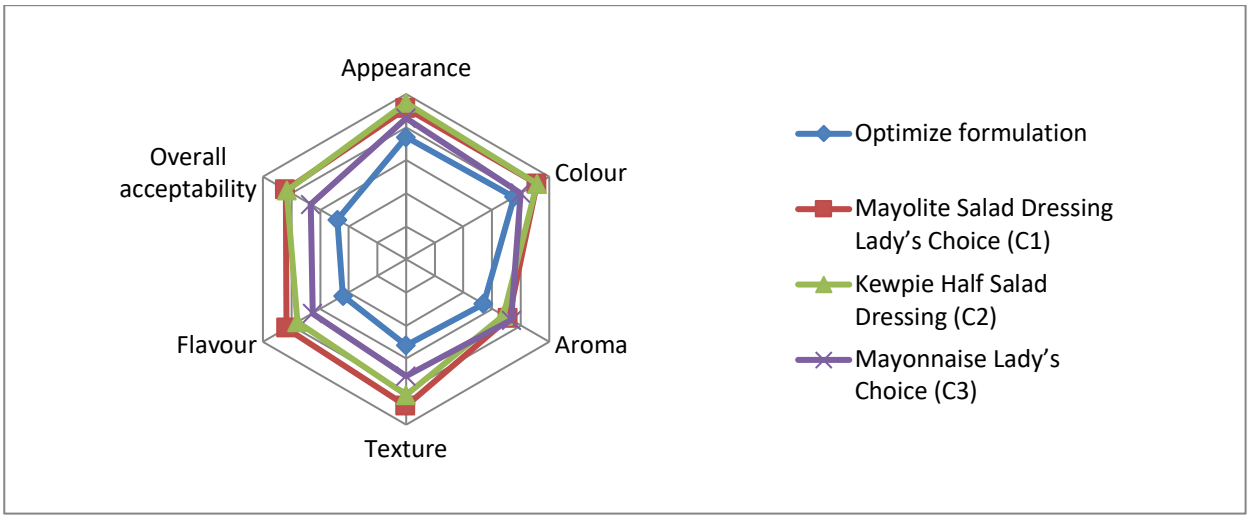

Figure 2. The sensory evaluation of optimized formulation salad dressing as compared with commercial products

Other than that, the optimized formulation salad dressing was 2.6 ± 1.102, which gave significant difference ($p<0.05$) with 3 different commercial products, C1, C2, and C3. This result shows that the consumer did not prefer the optimized formulation compared to the commercial products.

The same goes for the flavor, which gave the optimized formulation a significant difference ($p<0.05$) among C1, C2, and C3. This significant difference was maybe due to the presence of virgin coconut oil. According to Khor et al. (2018), because most customers detest the oily taste of pure VCO, creating a VCO-based emulsion product will indirectly enhance VCO use.

Therefore, the VCO-producing business will benefit from the transformation of VCO into a more pleasant and stable VCO-based emulsion product. On the other hand, some panelists do not like the flavor of the coconut as it can disturb the taste of salad dressing. In terms of overall acceptability, this obtained optimized formulation was significant ($p<0.05$) from all commercial products, C1, C2, and C3. This result shows that the panelist preferred this product, maybe due to the strong coconut presence.

4. Conclusions

The present study revealed that low-fat and egg-less virgin coconut oil salad dressing with optimum hydrocolloids of xanthan gum and modified starch showed desirable emulsion stability and physicochemical properties in viscosity, texture, and color. The optimum percentages of xanthan gum and modified starch were 1.56% and 0.10%, respectively. The validation of this optimized formulation was insignificant ($p>0.05$) between the predicted and experimental data, which indicated the desired results from this study. Besides, the physicochemical properties of the optimized formulation were compared with a few commercial products to choose the desired formulations based on these references. In addition, the proximate composition of the optimized formulation was reported. For the sensory evaluation, the optimized formulation had a significantly different score for texture, flavor, and overall acceptability ($p<0.05$) from the commercial products. However, the optimized formulation's appearance, color, and aroma are insignificant ($p>0.05$), with some commercial products still acceptable to the consumer. Hence, further study is required to improve the quality and evaluate the shelf life of the low-fat and egg-less VCO salad dressing.

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CHANGES IN PHYSICO-CHEMICAL PARAMETERS, BIOACTIVE COMPOUNDS AND SURVIVAL OF *Lactiplantibacillus plantarum* J12 IN FERMENTED CARROT JUICE

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ABSTRACT

Carrots are economically significant vegetables; however, their perishability necessitates their processing into a variety of products, especially juices. Therefore, this research evaluated the fermentation of carrot juice with the probiotic bacterium *Lactiplantibacillus plantarum* J12, as well as the modification of some features under storage. The tested strain showed good growth properties on carrot juice without any other supplementation. The viable cell counts of *L. plantarum* J12 was 4.38 10^6 CFU/ml at 0 h and reached 1.93 10^9 CFU/ml after 24 h of fermentation (increase of 10^3 CFU/ml). The fermentation had a partial effect on the physico-chemical parameters of the juice : a slight increase in the electrical conductivity, viscosity and turbidity of the lactofermented juice compared to the control was observed. Furthermore, lower sugar content with a good reduction in glucose was noticed. Phenolic and flavonoid compounds were higher in the control juice, but the antioxidant activity was better in the fermented juice. The sensory evaluation showed good acceptance with a better score attributed to the lactofermented juice. Cold storage for 21 days showed good strain survival, as well as pH and acidity stability. The results suggest that the probiotic *L. plantarum* J12 can be applied to produce functional juice for lactose-intolerant individuals and vegan population.

1.Introduction

The change in eating habits is the result of a rapid socio-cultural evolution; consumers are more concerned about their diet. Thanks to progress in the agri-food industry, food production has no longer the only purpose of satisfying basic needs, but of searching something more to add for health and to be able to compete with new products. Processed foods represent a growing part of the market, among these are functional foods. The latter, attract the attention of consumers because they are associated with the prevention or treatment of several illnesses comprising hypertension, diabetes, and cancer (Topolska et al., 2021).

Carrots and carrot juice are among those trending foods that have caught the attention of several researchers (Adiamo et al., 2018; Hu et al., 2019; Malik et al., 2019; Wuyts et al., 2018; Zhang et al., 2019). Due to its richness in carotenoids (mostly β-carotene), trace elements (iron, zinc, manganese, calcium, phosphorus and molybdenum), polyphenols (Martínez-Flores et al., 2015), and to its various biological activities (antioxidant, hypoglycemic activities, eyesight protection, anti-aging and immunity improvement) (Malik et al., 2019), carrot juice represents a very good solution to transform this perishable vegetable, which allows limiting major losses for farmers, and valorizing downgraded carrots which cannot be destined to the market because of their size (Rafiq et al., 2016).

Existing food probioticification is one of the strategies utilized to develop new functional foods. Several microorganisms, mostly lactic acid bacteria (LAB), may provide this probiotification (Zhang et al., 2019). The ability of these microorganisms to enhance the nutritional, hygienic, and sensory properties of fermented foods, as well as to give therapeutic benefits such as the reduction of type 2 diabetes, has received interest (Hu et al., 2019). Generally, LAB are carried by dairy products, but as there are many people who do not consume these products either for lack of interest or for health reasons such as lactose intolerance, carrot juice is therefore a good alternative to convey probiotic bacteria within this class of the population (Rafiq et al., 2016).

The interest of functional foods had to be scientifically proven; this is why we tried through this work to evaluate the combined effect of *L. plantarum* J12 and the carrot juice by physicochemical analyzes and testing the antioxidant activity of this probiotic juice, as well as carrying out a sensory evaluation to ensure its taste, which will allow us to formulate a functional juice.

2. Materials and methods

2.1. Materials

2.2.1. Bacterial strain and inoculum preparation

L. plantarum J12 was isolated from chicken's crop at the Laboratory of Biotechnology, Environment and Health, University of Jijel, Algeria. The strain was identified by the 16S rDNA technique. The gene sequence (1406 bp) was determined and deposited at the GenBank database under the accession number KJ690574.

To make the inoculum, a *L. plantarum* J12 glycerol stock culture tube was put to a 250 ml beaker with 100 ml MRS broth. Cultures were grown at 37 °C until their cell density attained 0.600, as measured spectrophotometrically (OD590), which corresponds to 9.00 Log CFU/ml on the MacFarland scale. After centrifugation at 5000 x g for 5 minutes, the cells were collected. For starting inoculation, the cell pellets were recovered, rinsed, and resuspended in peptoned water (0.1%).

2.2.2. Juice preparation

Local carrots (Daucus carota L) were acquired from a market (Jijel, Algeria). The carrots were properly rinsed with distilled water, scraped, and divided into about 3.5 cm by 0.5 cm by 0.5 mm in thickness pieces. In the next step, the fragments of carrots were blanched in distilled water with 3 g/l of citric acid for 3 minutes at 90 °C. Then, they were grinded (Zhao et al., 2014). Finally, a centrifugation at 4000 g for 5 min was performed to separate the juice from the pulp. The juice was filled in glass bottles and pasteurized at 70 °C for 2 min. The contents of the flask were split into two groups, one as a control and the other was inoculated with 5% v/v by the *L. plantarum* J12 culture (final concentration approximately 10^6 CFU/ml). After that, the fermentation process was carried out at 37 °C for 24 h (Tamminen et al., 2013). After fermentation, the flasks were kept at 4 °C for 03 weeks.

2.2. Methods

Various parameters were investigated to elucidate the effect of fermentation immediately after recuperation of flasks (physical parameters, content of bioactive compounds). To investigate the effect of conservation, samples were analyzed every 3 days during 3 weeks (bacterial counts, pH and acidity measurements). Sensory evaluation was performed 24 h after the end of fermentation.

2.2.1. Viable cells count

L. plantarum J12 was cultured on MRS agar to determine its viability (Difco Laboratories, Sparks, MD). Plates were then incubated 24 h hours at 37 °C (Malik et al., 2019).

2.2.2. pH and titratable acidity determination

After calibrating a pH meter (Model Hanna pH 211, USA), the pH of carrot juice samples was measured by immersing an electrode directly into 50 ml of juice at 20 °C (Zhang et al., 2016).

Titratable acidity (TA) was measured through titration with 0.1 N NaOH solution in the presence of phenolphthalein (Daneshi et al., 2013). TA was based on lactic acid percentage.

2.2.3. Total soluble solids assay, protein and ash determination

A refractometer was used to determine the total soluble solids (TSS) in Brix degree (Model Atago HSR-500, Japan) at room temperature (Jin et al., 2019). The Kjeldahl technique was used for proteins determination. The ash was measured after incineration of 1 g of the juice in the muffle furnace at 550 °C.

2.2.4. Measurement of electrical conductivity

The electrical conductivity of the juice was evaluated by immersing the electrode of the conductivity meter (Bioblock, Belgium) in a beaker containing 30 ml of sample; the reading was done directly on the display of the conductivity meter at 23 °C.

2.2.5. Turbidity

A portable Turbidimeter (Aqualytic SN 084591, Germany) was used to determine the turbidity of the juice. Results were expressed in nephelometric turbidity units (NTU).

2.2.6. Viscosity

The viscosity was measured with an AR 1000 rotary viscometer (Haake, Viscotester, Germany) equipped with a cone (angle 2°, diameter 40 mm) and a spindle (spindle R1, 20 x g). Its container was filled with 250 ml of sample and then the probe was introduced into the container by changing the scale of measurement until the exact viscosity value was obtained on the screen of the apparatus. The viscosity value was expressed in mPa/s.

2.2.7. Determination of sugar content

Five ml of carrot juice samples were homogenized and filtered through 0.45 µm cellulose acetate membrane (VWR, Mississauga, ON, Canada). Using an HPLC system (Knauer Co., Ltd, Germany), the filtrate was utilized to evaluate the concentration of sucrose, fructose, and glucose in carrot juice samples. The mobile phase comprised of water: acetonitrile (75:25, v/v) and a column of (4.6 / 250 mm) was utilized. External standards with sucrose, glucose and fructose HPLC grade were used for quantification, the findings were reported in terms of grams per liter of carrot juice (Zhang et al., 2016).

2.2.8. Extraction of phenolic compounds

One milliliter of carrot juice was used to extract phenolic compounds, with 10 ml of methanol in dark and agitation in an orbital shaker for 24 h (Infors AG CH-4103 Bottmingen, Switzerland). Supernatants were collected after centrifugation at 5000 x g for 5 min (Sigma, Germany) and stored at 4 °C (Martínez-Flores et al., 2015).

2.2.9. Total phenolic compounds (TPC)

200 µl of the extracted solution was combined with 4.0 ml of distilled water and 400 µl of Folin-Ciocalteau reagent with a Vortex (MS2 Mini shaker, VWR) and maintained at room temperature (25 °C ± 1 °C) for 10 min. Next, 1.25µl of 20% Na2CO3 solution was added, stirred, and maintained for 120 min in the dark. Using a spectrophotometer (Model Ultrospec 100 pro Shimadzu, Germany) at 760 nm, the absorbance was read. The findings were represented in µg of gallic acid equivalents per milliliter of sample (µg GAE /ml) (Adiamo et al., 2018).

2.2.10. Total flavonoids contents (TFC)

After adding 1 ml of each sample of extract to 1 ml of $AlCl_3$ solution and letting it sit for 10 min at room temperature, the OD was taken at 415 nm. The concentration of flavonoids was deduced from a calibration curve established with quercetin and expressed in equivalent µg of quercetin per ml of sample (Talbi et al., 2015).

2.2.11. Total Carotenoids

The total carotenoids were analyzed using the methodology given by Martnez-Flores et al (2015). Two ml of carrot juice and ten ml of chloroform/methanol (2:1, v/v) were stirred for 5 min. After shaking, the organic and aqueous phases were separated, and the latter was filtered

and recovered using filter paper. The operation was repeated four times with the aqueous phase using 5 ml chloroform/methanol. The extracts were then combined and diluted using chloroform/methanol to a maximum volume of 50 ml. Carotenoids were quantified at room temperature, at an absorbance of 450 nm, using a spectrophotometer (Model Specord 50 plus, Analytic jena, Germany). Standard solutions of β-carotene were used at different concentrations (2–10 µg/ml). Results were expressed as µg β-carotene equivalent per ml of carrot juice.

2.2.12. Antioxidant capacity

The antioxidant capacity was determined using the method published by Kourouma et al (2019). 2.5 mg of DPPH was dissolved in 100 ml of methanol to create a stock solution (0.0625 mmol/L) that was stored in the dark at room temperature. Then, 200 µl of carrot juice was combined for 30 min in the dark with 3 ml of DPPH solution. Using a spectrophotometer (Model Specord 50 plus, Analytic jena, Germany) and methanol as a blank, the absorbance was measured at 517 nm. According to the following equations, radical scavenging ability (RSA) was expressed in terms of the percentage of DPPH inhibition:

$$DPPH\ RSA\ (\%) = [(A_0 - \mathrm{As})/A_0] \times 100 \quad (1)$$

where A_0 was the absorbance of DPPH radical solution without sample and As was the absorbance of the sample.

2.2.13. Sensory evaluation

The sensory assessment was conducted using Zhang et al approach's (2016). An untrained panel of students (9 male, 9 female) aged between 19 and 28 years at the University of Jijel were volunteered to take part in this study. To be considered, panelists had to be non-smokers and not allergic to carrots. A randomized block was designed to determine carrot juice order. Each carrot juice was drunk by every volunteer independently of the others. A questionnaire was used to measure the preference of each carrot juice's taste, color, appearance, aroma and overall acceptability on a 9-point hedonic scale with a degree of liking where 1 = dislike extremely and 9 = like extremely. The subjects were given carrot juice samples in little plastic cups, and they were allowed to note their responses on the reverse of the questionnaire.

2.2.14. Statistical analysis

Tests were conducted in triplicate, and findings were presented as mean ± SD. Differences between samples were calculated by analysis of variance (ANOVA) using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was assigned statistical significance.

3. Results and discussions

3.1. Lactofermentation process

As displayed in Table 1, supplementation of *L. plantarum* J12 to carrot juice causes a little change in the juice's approximate composition. Comparable to the control sample (CJ), lactofermented juice (LJ) had a considerably decreased moisture content which went from 93.78% to92.86%. The opposite was noticed with the protein and ash content that have increased from 0.46% and 0.16% to 0.74% and 0.24% respectively. Furthermore, sugar contents were significantly lower than in CJ ($P < 0.05$). These modifications may be the result of the probiotic's action and the production of metabolites. Our findings are consistent with those of Rafiq et al (2016), who formulated a probiotic carrot juice with *L. acidophillus*, *L. plantarum*, *L. casei* and *Bifidum longum*. However, their results were slightly higher than ours.

The carrot juice was inoculated with *L. plantarum* J12, at an initial cell density of about 10^6 CFU/ml. *L. plantarum* J21 was found to be capable of growing well on pasteurized carrot juice without any nutrient supplementation. Zhang et al. (2019) has reported that *L. plantarum* WZ-01 strain could proliferate rapidly in native carrot juice.

Table 1. Effect of lactofermentation by *L. plantarum* J12 on physicochemical parameters, bioactive compounds and antioxidant activity of carrot juice.

		Control juice	Lactofermented juice
Moisture (%)		93.78 ± 1.24	92.86 ± 2.33
Protein (%)		0.46 ± 0.23	0.74 ± 0.17
Ash (%)		0.16 ± 0.4	0.27 ± 0.06
Sugars *(g/l)*	**Sucrose**	2.722 ± 0.13	2.570 ± 0.17
	Glucose	4.031 ± 0.15	1.599 ± 0.19
	Fructose	3.657 ± 0.16	3.359 ± 0.11
TSS ***(°Brix)***		7.8 ± 0.1	7.1 ± 0.2
Bioactive compounds	**TPC** *(µg GAE/ml)*	296.58 ± 4.05	211.47 ± 2.87
	TFC *(µgRE/ml)*	79.21 ± 3.24	57.43 ± 2.82
	Carotenoids *(µg/ml)*	1.05 ± 0.03	0.98 ± 0.08
	DPPH (%)	51.7 ± 5.78	62.6 ± 6.46
Physical parameters	**Viscosity** *(mPa/s)*	1.36 ± 0.09	1.46 ± 0.04
	Turbidity *(NTU)*	2655 ± 122	2990 ± 156
	Conductivity	0.436 ± 0.06	0.523 ± 0.08

The growth of *L. plantarum* requires the minerals Mg^{2+} and Mn^{2+}. A good quantity of carbohydrates, magnesium, phosphorus, calcium, iron, potassium, sulphur, manganese, and copper can be found in carrots (Rafiq et al., 2016). Therefore, carrot juice may serve as a substrate for probiotic bacteria like lactic acid bacteria (Malik et al., 2019). *L. plantarum* is an auxotrophic bacterium. it starts to use carbohydrates (mainly glucose) present in the juice, then it will attack proteins. moreover, it is able to convert some amino acids into others, such as the conversion of methionine into cysteine, which allows it to survive in the carrot juice (Wegkamp et al., 2010).

During 24 h of fermentation in carrot juice, the growth of *L. plantarum* J21 increased logarithmically from an initial population of 4.90×10^6 CFU/ ml to reach 1.93×10^9 CFU/ml (Figure 1). Our results were in agreement with those reported by Wuyts et al. (2018) when they checked microbial community dynamics using high-throughput sequencing of 16S rRNA gene of 38 household carrot juice fermentations, where the number reached 3.37×10^9 CFU/ml after 3 days of fermentation. Zhang et al. (2019) prepared a lactofermented carrot juice with *L. plantarum* WZ-01, and they obtained a cell density of about 5.3×10^8 CFU/ml in 48 h. Data given by Nguyen et al. (2019) showed that the cell count of *L. plantarum* 299V was significantly higher in comparison of that of the *L. acidophilus* La5 strain where both strains were used in the fermentation of pineapple juice. According to these authors, the origin of the strain plays an important role in its adaptation to the fermentation substrate. A strain isolated from a plant source grows better than one isolated from an animal source. Despite this, our strain has been adapted very well to the carrot juice.

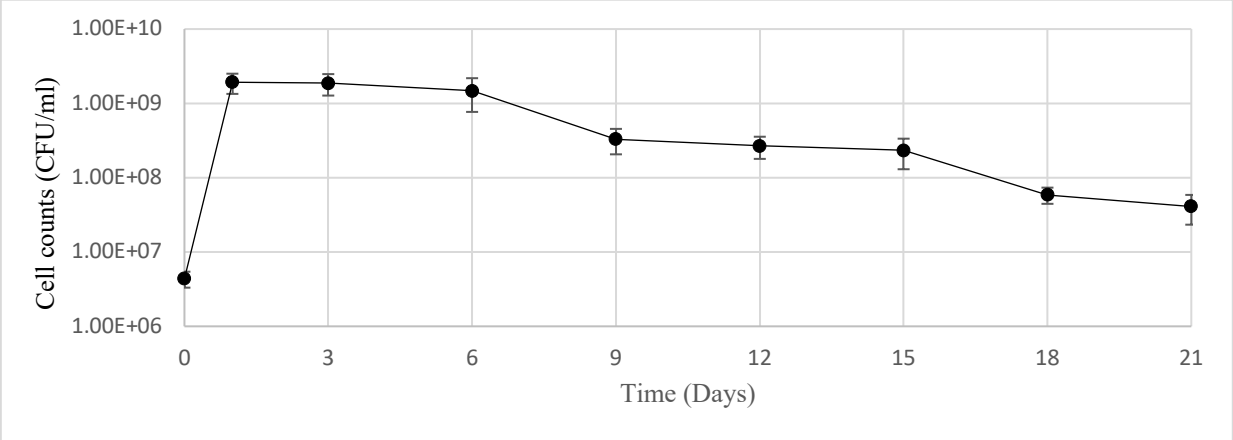

Figure 1. Fermentation process and survival of the strain *L. plantarum* J12 during storage at 4 °C

3.2. Effects on the physicochemical parameters of the juice

Physical parameters, bioactive compounds, and contents of some sugars after 24 h of fermentation are summarized in Table1**.** It had been reported that *Lactiplantibacillus* have high requirements of fermentable carbohydrates, vitamins, free amino acids and peptides for growth (Śliżewska and Chlebicz-Wójcik, 2020). Interestingly, our carrot juice was rich in fermentable sugars.

The pH of the produced carrot juice before fermentation was about 5.45; then after the fermentation process, the pH dropped to 4.10 at 24 h. The pH of the CJ, which is placed under the same conditions, remained almost unchanged, with a value of 5.41 (Figure 2). On the other hand, the acidity increased considerably during the fermentation and reached 0.69. Lactic acid is the main acid produced by *L. plantarum*, although other acids can be also produced because of hydrolysis and microbial activity (Jin et al., 2019). The concentration of these acids increases as the biomass increases which leads to a considerable drop in pH. Similar results for pH decrease were reported by Demir et al. (2006) when they studied the properties of carrot juice inoculated by *L. plantarum* at different concentrations. The increase of acidity was reported by Malik et al. (2019) in carrot juice substrate fermented with *L. plantarum* which increased from 0.15% to 0.88% during 72h.The main function of *L. plantarum* is the conversion of sugar to lactic acid, which explains the drop in pH and the sugar content during fermentation. Indeed, the concentration of sucrose, glucose and fructose at the CJ were 2.722, 4.031 and 3.657 g/l, then they passed to 2.570, 1.599 and 3.359 g/l, respectively at the end of the fermentation (Table 1). Some works reported that many LAB strains, especially *L. plantarum* are natively able to utilize simple sugars (Garcia et al., 2020; Wuyts et al., 2018). The order of utilization of sugars by *L. plantarum* J12 was glucose>sucrose>fructose. Nguyen et al. (2019) gave an order of fructose > glucose ≥ sucrose with pineapple juice fermented by *Lactiplantibacillus* and *Bifidobacterium* strains. After 24 h of fermentation, the TSS of LJ decreased from 7.8 to 7.1 °Bx (Table 1). This result agree with those of Do and Fan (2019).

Electrical conductivity is an important parameter for evaluating the quality of any juice; it gives an idea on the freshness of the product. It is low when the juice is fresh and increases when it loses its freshness. It also makes possible to detect changes in the structure of the treated product. The obtained results show that the conductivity of the LJ is 0.523 ± 0.08; it was a little higher than that of the CJ which was 0.436 ± 0.06, but this difference is not significant statistically ($P > 0.05$). These values are similar to those found by Rodrigo et al. (2003) who reported conductivities of 0.416 ± 0.11, 0.375 ± 0.01 and 0.435 ± 0.03 in three types of orange-carrot juice mixtures stored at different temperatures.

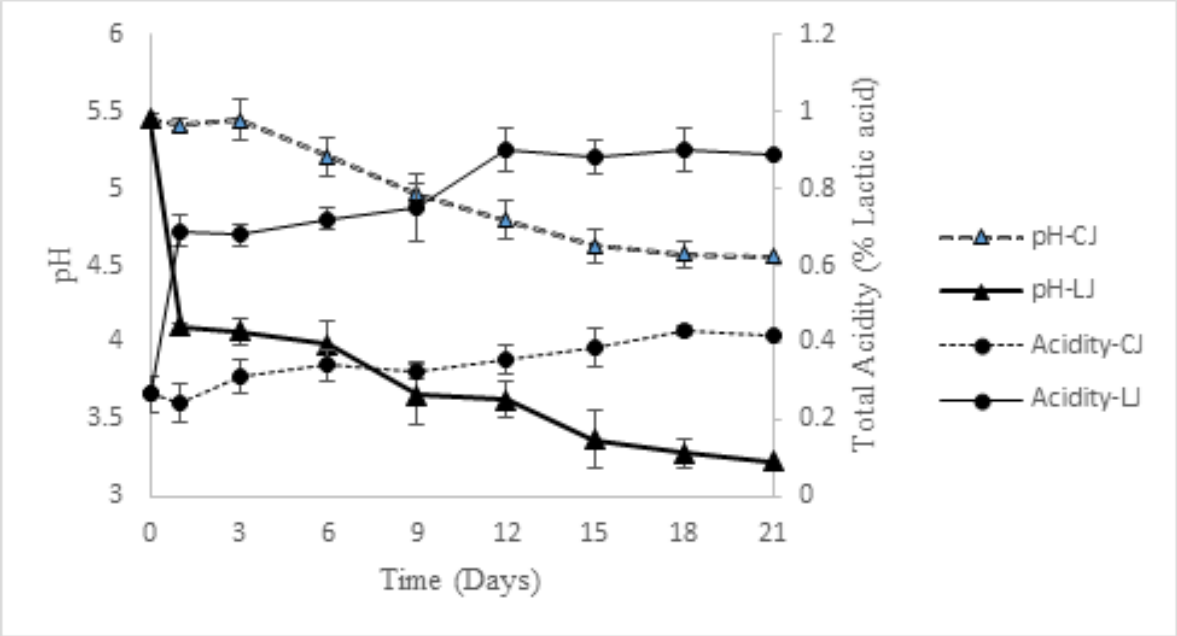

Figure 2. Variations in pH and acidity of CJ and LJ during storage at 4 °C

Electrical conductivity is influenced by the pH of the solution, the valence of the ions and the degree of ionization. This small improvement in electrical conductivity might be related to mineral components or vitamins released from carrot tissues throughout fermentation.

Viscosity represents the resistance to flow and movement of a liquid. Statistical analysis showed a non-significant difference ($P > 0.05$) between the values of juice samples. However, we notice that the viscosity of LJ (1.46 ± 0.04) is a little higher than that of CJ (1.36 ± 0.09). These results are lower than those found by Martínez-Flores et al. (2015). This partial rise in viscosity may be attributed to an increase in the solvation of pectin in cell wall membranes (Xiang et al., 2014).

Bottom sediment formation in carrot juice is a cloud stability problem, but it do not affect the visible loss of turbidity (Reiter et al., 2003). As shown in Table 1, the turbidity of the carrot juice was increased after fermentation, it changed from 2655 NTU to 2990 NTU, but statistically there is no significant difference ($P > 0.05$). This increase in turbidity could be explained by an increase in the number of particles related to degradation of substrates contained in the juice such as pectins, these particles are too small to affect the particle size distribution (Chen et al., 2019). Nevertheless, the turbidity values of our juices are lower than those given by other authors (Reiter et al., 2003; Yu and Rupasinghe, 2012).

3.3. Changes in bioactive components and antioxidant activity

Phenolic compounds are among the most important phytochemicals that can usually be found in foods of plant origin. As shown in Table1, CJ showed the highest TPC of 296 µg GAE/ml, which was lower than that reported by Zhang et al. (2016) in raw carrot juice. Perla et al. (2012) have reported that phenolic content decreased after pasteurisation because of thermal degradation. After fermentation, a decrease in the TPC value was noticed in LJ sample, which declined to 211 µg GAE/ml. Ciniviz and Yildiz (2020) have concluded that fermentation decreases total phenolics. TFC in LJ dropped from 79.2 to 57.4 µg/ml (Table 1), which is equivalent to a reduction yield of 27.52%. These results were also proven by Malik et al. (2019) who reported that TFC of carrot juice decreased during fermentation with *L. plantarum*, *L. acidophilus*, and *L. casei*. Furthermore, the decreased TPC and TFC did not affect the enhanced antioxidant activity of fermented carrot juice. Li et al. (2019) explained this finding by the fact that supplementation

with LAB leads to the production of certain metabolites with higher antioxidant activity during the fermentation process.

Carotenoids are the major natural pigments that give carrots their color characteristic and have too powerful biological functions. Since human can not produce carotenoids, they have to be provided to him mainly by fruits and vegetables or their juices. Carotenoids in LJ were not degraded by *L. plantarum* J12. The results were unchanged after fermentation. Zhang et al. (2019) obtained similar results after fermentation of carrot juice with *L. plantarum* WZ-01.

Carrots are rich in antioxidants; however, during the transformation of carrots into juice, a significant part of this antioxidant activity is destroyed by technological processes, so it must be recovered by other sources. Fermentation with probiotic and/or LAB remains an effective means because these bacteria produce various metabolites such as antioxidant exo-polysaccharides, superoxide dismutase and glutathione (Tang et al., 2017; Zhang et al., 2019). Table 1 showed the antioxidant capacity of carrot juice determined by DPPH assay. Scavenging activity of DPPH had been enhanced from 51.7% to 62.6% during the fermentation of carrot juice by *L. plantarum* J12. Various studies reported that *L. plantarum* enhance antioxidant activity in carrot juice (Zhang et al., 2019), pineapple juice (Nguyen et al., 2019), beet roof juice (Malik et al., 2019) and apple juice (Li et al., 2019).

3.4. Sensory evaluation

The sensory assessment of a juice has a significant impact on customer acceptability and preference. The sensory evaluation of both CJ and LJ was performed 24h after the end of the fermentation process. The sensory score (Figure 3) increased during fermentation, indicating that *L. plantarum* J12 could improve the sensory attributes of the carrot juice. The scores of overall acceptability, flavor and acidity increased from 6.42, 3.78 and 5.14 to 7.14, 4.92 and 6.35, respectively. There were no visible color or appearance variations amongst the samples. Several authors (Malik et al., 2019; Tamminen et al., 2013; Zhang et al., 2019) have showed that carrot juice fermentation by lactic acid bacteria improves its sensory qualities. In addition, fermentation reduces the sugar content and the produced acids give a refreshing taste.

3.5. Survival of *L. plantarum* during storage

The international standards describe that for health and functional point of view, the probiotic products should contain at least of 10^6 viable bacterial cells per gram of product at the moment of consumption (Daneshi et al., 2013). The storage of the carrot beverage was carried out with and without supplementation. Viable counts (log CFU/ml) of our strain in carrot juice during storage at 4 °C over 21 days are presented in Figure 1. The microbial population did not change significantly in this period. For all storage period, strains reached a viable cell number reduction of less than 2 log CFU/ml and remained greater than 7 log CFU/ml. The cell viability depends on various factors such as final acidity of the product, the used strain, the concentration of lactic acid and acetic acid, oxygen content and interaction between existing species.

During refrigerated storage (4 ºC), the pH of the CJ did not show significant change (Figure 2) in the first six days but it was decreased to 4.55 after 21 days. Moreover, there is a large drop in pH of the LJ after the fermentation process (from 5.42 to 4.09), then a gradual drop during refrigeration. At the same time, the acidity of the two juices increased slightly during storage, with the exception of the brutal increase noted in the LJ during fermentation. Nualkaekul and Charalampopoulos (2011) reported similar results in fruit juices lacto-fermented by *L. plantarum* and stored under refrigeration for 6 weeks.

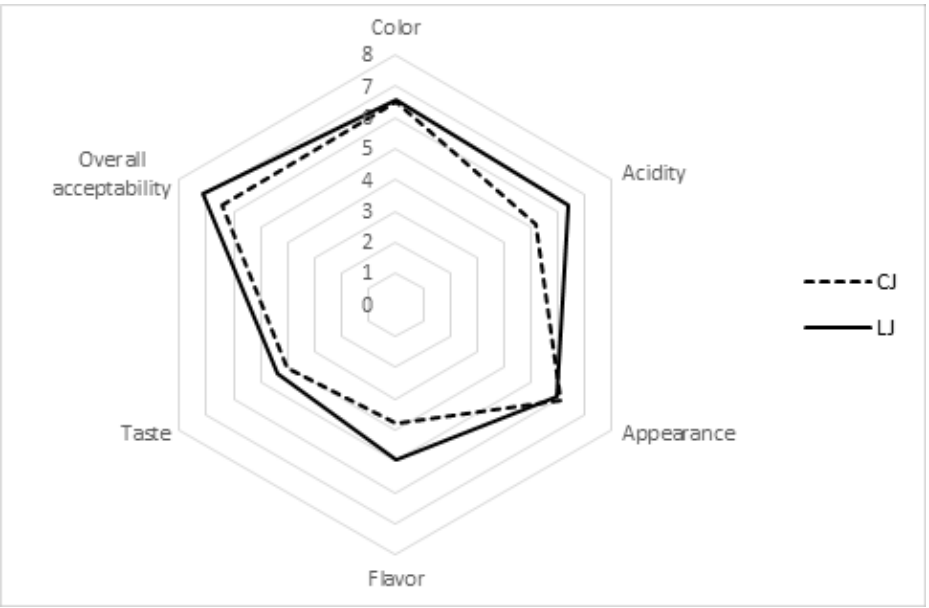

Figure 3. Sensory analysis profile of CJ and LJ

4. Conclusions

The present study confirms that the strain *L. plantarum* J12 proliferates well in carrot juice and can survive during storage at 4 ºC. Viability was satisfactory with less than a two log CFU/ml decline. Furthermore, it was noticed that TPC and TFC reduced in the fermented carrot juice. At the end of the fermentation, the antioxidant capacity had risen. The lacto-fermented juice had a better acceptability from the tasting panel. It may be established that *L. plantarum* J12 has a promising use in the fermentation of carrot juice., which can be a functional drink especially for people who do not consume dairy products. Further studies, *in vivo*, concerning the beneficial effects of this strain should be carried out.

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PHYTOCHEMICAL AND BIOLOGICAL PROFILES OF FENNEL FRUITS (*FOENICULUM VULGARE* MILL. VAR. *DULCE* MILL.)

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ABSTRACT

The aim of this article is to determine the phytochemical composition and biological activity of sweet fennel fruits, cultivated during two harvest years in Bulgaria (2020 and 2021). First, we researched the chemical composition of fennel fruits and determined their protein content (15.74% and 14.30%), crude fiber (32.88% and 31.50%), ash (6.49% and 7.99%), and mineral elements (58373.22 mg/kg and 102825.96 mg/kg), respectively for 2020 and 2021 harvest years. Then, the fruits were subjected to hydrodistillation and the essential oil was isolated (8.38% and 7.57%), respectively for 2020 and 2021 harvest years. At the end, the lipid fraction was extracted from the ground fruits (9.07% and 5.58%), respectively for 2020 and 2021 harvest years. Polar and unpolar fractions were isolated from the fruits. The results reveal that the fruits of the 2020 harvest have a higher content of protein, essential oil and lipid fraction. Some significant differences were also found in the amounts of macro elements, while the content of micro and toxic elements was comparable. Regarding the chemical composition, both essential oils were high in anethole and low in fenchone. The essential oils of the fennel fruits have antimicrobial activity, which is more pronounced against molds (*Aspergillus brasiliensis* and *Fusarium moliniforme*) and yeasts (*Candida albicans*). The main compounds of polar fraction were saccharides (mono- and di-), followed by sugar alcohols, organic acids, sugar acids, amino acids, and others. In both lipid fractions the main sitosterols are *β*-sitosterol and stigmasterol, and major tocopherol is *γ*-tocotrienol. Overall, the obtained results demonstrate that with its balanced composition of protein, crude fiber, essential oil, polar and lipid fraction, and mineral elements, the fennel fruits can be used as a supplement to the food of humans and animals.

1. Introduction

Fennel (*Foeniculum officinale* All. = *F. vulgare* Mill. = *F. capillaceum Gilib*) belongs to the Apiaceae family. The main species are dulce (var. *dulce* (Mill.) Thell.) and bitter (var. *vulgare* (Mill.) Thell). They differ both in the shape of the fruits and in chemical composition. The fruits of the fennel are mainly used in the traditional medicine and in the food industry (Anka *et al.*, 2020). The fruits are

processed in order to obtain essential oil and lipid fraction.

The main component of the sweet fennel essential oil is *trans*-anethole, and its content in essential oils from different countries ranges from 65 to 91% (most often 78-88%). The composition of the oil also contains fenchone (1.0-12.0%), limonene (up to 22.4%, most often 2-7%), methyl chavicol (2.3-7.3 and up to 67%, most often 2.3-4.0%) and many other components, the amount of which varies depending on the origin of the raw material (Conforti *et al*., 2006; Diaz-Maroto *et al*., 2006; Coşge *et al*., 2008; Renjie *et al*., 2010; He and Huang, 2011; Zheljazkov *et al*., 2013; Najdoska-Bogdanov *et al.,* 2015; Akhatou *et al*., 2016; Ali *et al*., 2016; Bahmani *et al*., 2016; Ahmad *et al*., 2018; Wodnicka *et al*., 2019). The essential oil is characterized by antimicrobial activity (Dadalioglu and Evrendilek, 2004; Damianova and Stoyanova, 2007; Mohsenzadeh, 2007; Kaur and Arora, 2010; Renjie *et al*., 2010; He and Huang, 2011; Jamwal *et al*., 2013; Ali *et al*., 2016; Balouiri *et al*., 2016; Wodnicka *et al*., 2019; Anka *et al*., 2020), antioxidant effect (Conforti *et al*., 2006; He and Huang, 2011; Jamwal *et al*., 2013; Zheljazkov *et al*., 2013; Anka *et al*., 2020) and other biological properties (He and Huang, 2011; Rather *et al.*, 2012; Jamwal *et al*., 2013; Dikova, 2014; Dikova *et al*., 2017; Anka *et al*., 2020). The pharmacological activities of the essential oil are attributed to its main compound – anethole (Baser and Buchbauer, 2010).

The main fatty acids in the lipid fraction are petroselinic acid (67.0-83.3%), oleic acid (12.0-16.4%), linoleic acid (6.50-8.97%), and palmitic acid (3.25-6.80%). Their amount varies depending on the origin of the raw material and the method of obtaining the lipid fraction (Coşge *et al*., 2008; Morales *et al*., 2012; Najdoska-Bogdanov *et al*., 2015; Ahmad *et al*., 2018).

The fennel fruits contain about 20% protein, phenolic compounds, flavonoids, minerals, vitamins, *etc*., which explains its biological properties and application (Jamwal *et al*., 2013; Anka *et al*., 2020).

In Bulgaria, the fennel fruits are processed only to obtain essential oil, and the remaining raw material after distillation is used as an additive to feed mixtures.

The studies on the composition of fruits and their biological activity are scarce. Therefore, the aim of the present work is to determine the phytochemical composition and biological activity of sweet fennel fruits cultivated during two harvest years – 2020 and 2021. This, in our opinion, will enrich the data for a more versatile application of the fennel fruits, as well as for the content of various biologically active compounds in them.

2. Materials and methods

2.1. Materials

2.1. Plant material

The fennel fruits (*Foeniculum vulgare* Mill. var. *dulce* Mill.), family Apiaceae, harvest 2020 and 2021, were used. They were provided by a company, producer of essential oil plants, located in North Eastern Bulgaria near the town of Razgrad (43°32'00" N and 26°32'30" E). The ripe greenish-yellow fennel fruits were air dried and stored in sacks in well-ventilated warehouses (20-25°C), with no sunlight, in cool, and dry conditions.

Before analysis, the fruits were grounded for 30 s to a size of 0.5 mm, using the electric blender Bosch MKM 6003, Stuttgart, Germany.

2.2. Methods

2.2.1. Chemical composition of the plants

Protein content, crude fiber, ash and moisture were determined using methods, described in AOAC. All results in the study are reported at dry weight.

2.2.2. Isolation of mineral elements

The mineral contents (trace elements, heavy metal and other elements) were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) after pressure digestion (BSS-EN 17053, BSS-EN 15763).

2.2.3. Metabolic profiling by fennel fruits

0.050 g from each sample (fruits) were mixed with 1.0 mL methanol/distilled water (75:25, v/v) mixture, followed by heating at 70°C for 30 min in a laboratory thermo mixer (Analytik Jena AG, Germany), and then cooled to 25ºC. Next, 500.0 mL chloroform and 200.0 mL distilled water were added, and then the resulted mixture was centrifuged at 13000 rpm for 5 min at 22°C. The lower phase was designed for the analysis of non-polar substances (essential oil, lipid fraction, *etc*. – fraction "A"), whereas the upper phase – for the polar constituents (amino and organic acids, carbohydrates, *etc*. – fraction "B"). The two phases obtained were vacuum-dried in a centrifugal vacuum concentrator (Labconco Centrivap, US) at 40ºC. 1.0 mL 2% H_2SO_4 in methanol was added to the dried residue of fraction "A", and the mixture was heated on Thermoshaker TS-100 for 1 h, at 96ºC and 300 rpm. The solution was left to cool and then extracted with *n*-hexane (3x500.0 mL). Combined organic layers were vacuum-dried in the centrifugal vacuum concentrator at 40ºC.

Prior to the gas chromatography-mass spectrometry (GC-MS) analysis, fractions "A" and "B" were derivatized using the procedures: 100.0 µL pyridine and 100.0 µL N, O-Bistrifluoroacetamide (Sigma Aldrich, St. Louis, MO, United States) were added to the dried residue (fraction "A"), then heated on Thermoshaker TS-100, Analytik Jena AG, Germany for 45 min, at 70ºC and 300 rpm, followed by injecting 1.0 µL from the solution into the GC-MS. 300.0 µL solution of methoxyamine hydrochloride (20.0 mg/mL in pyridine) was added to dried residue (fraction "B"), and the mixture was heated for 1 h at 70ºC and 300 rpm. After it was cooled, 100.0 µL BSTFA were added to the mixture then heated for 40 min, at 70ºC and 300 rpm, injecting 1.0 µL from the solution into the GC-MS system.

GC-MS analysis was carried out on Agilent 7890A gas chromatograph (Santa Clara, CA 95051, US) interfaced with an Agilent 5975 C mass selective detector (Santa Clara, CA 95051, US). Separations were performed using a DB-5ms silica-fused capillary column (30 m × 0.25 mm (i.d.)), coated with 0.25 µm film of poly (dimethylsiloxane) as a stationary phase. The carrier gas (helium) flow rate was maintained at 1.0 mL/min. The injector and the transfer line temperature were kept at 250°C. The oven temperature program used was 100°C for 2 min then 15°C/min to 180°C for 1 min then 5°C/min to 300°C for 10 min. The injection volume was 1 µL, and was carried out in a 1:20 split mode. The mass spectrometer was scanned from 50 to 550 m/z. All mass spectra were acquired in electron impact (EI) mode with 70 eV.

A mixture of aliphatic hydrocarbons (C_8-C_{40}) (Sigma) was injected into the system under the above mentioned temperature program, in order to calculate the retention index RI (as Kovàts index) for each compound. The compounds in the polar fraction were identified as trimethylsilyl (TMS) derivatives with the help of the NIST 08 database (NIST Mass Spectral Database, PC-Version 5.0-2005, National Institute of Standardization and Technology, Gaithersburg, MD, USA), and other plant-specific databases: the Golm Metabolome Database (http://csbdb.mpimp-golm. mpg.de/csbdb/gmd/home/gmd_sm.html accessed on 15 December 2021). In order to calculate the retention index RI of each compound, a mixture of aliphatic hydrocarbons (C_8-C_{40}) (Sigma-Aldrich, St. Louis, MO, USA) was injected into the system using the above mentioned temperature program. In order to identify the amino acids, a mixture of standard AA (Amino Acid Standard Solution Prod. No A 6407; Sigma-Aldrich, St. Louis, MO, USA) was used as well. The amount of identified metabolites was considered by the percentage peak area that appeared in the total ion chromatogram (TIC) in the GC-MS analysis.

2.2.4. Essential oil Isolation by hydrodistillation

The fruits were hydrodistilled for 4 hours in a British Pharmacopoeia laboratory glassware apparatus, modified by Balinova and Diakov, in 1974. The essential oil was dried over

anhydrous sodium sulfate and stored at 4°C in dark vials. After hydrodistillation, the fruits were air-dried (humidity 7.00±0.06%) for 10 days at 25°C.

Determination of chemical composition

The chemical composition of the essential oils was determined as described in subchapter 2.2.3.

Determination of antimicrobial activity

Antimicrobial activity of the studied essential oil was tested against the following test microorganisms: Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876; Gram-negative bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella abony* NTCC 6017 yeasts: *Saccharomyces cerevisiae* ATCC 2601, *Candida albicans* ATCC 10231 and molds *Aspergillus brasiliensis* ATCC 16404, *Fusarium moniliforme*. Test micro-organisms strains were provided by the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria.

Antimicrobial activity was determined by the diffusion method in agar wells. Growth media were Tryptic soy agar (Merck) for the tested bacteria and Sabouraud-Dextrose-Agar (Merck KGaA, Darmstadt, Germany) for yeasts and molds. Media were inoculated with 24-hour suspension of bacterial species and 48-hour suspension for yeasts and molds with turbidity – 0.5 McFarland standards.

The melted and cooled media at 50°C±2°C were inoculated with 1% of the prepared suspensions of the microorganisms to be tested. 20 mL of inoculated medium was poured into sterile Petri dishes (∅ = 90 mm). 50 µL of essential oil was added drop wise to each well, and then the Petri dishes were placed in thermostatic chambers and incubated at 37°C or 28°C for 24 and 48 h depending on the microbial seasoning.

The diameter of the inhibition zones was measured using digital caliper, and were interpreted as follows: microbial culture up to 15 mm is weakly sensitive; from 15 to 25 mm – sensitive and above 25 mm – very sensitive (Balouiri *et al*., 2016; Hussein *et al*., 2020).

2.2.5. Lipid fraction by extraction Isolation of lipid fraction

The lipid fraction was extracted from ground fruits with *n*-hexane using a Soxhlet extractor (ISO 659).

Fatty acid composition

The determination fatty acid composition of triacylglycerol's was done using GC method (ISO 12966-1). The triacylglycerols were pre-esterified with methanol in the presence of sulfuric acid in order to obtain fatty acid methyl esters (FAMEs) (ISO 12966-2). Determination of FAMEs was carried out on Agilent 8860 gas chromatograph equipped with a capillary DB-FastFAME column, (30 m x 0.25 mm x 0.25 µm (film thickness)) and a flame ionization detector (FID). The injector and detector temperatures were set at 270°C and 300°C.

The column temperature was from 70°C (1 min), at 6°C/min to 180°C (0 min), and at 5°C/min to 250°C, and the split ratio was 50:1. A standard Supelco, USA mixture (FAME mix 37 components, Supelco, USA) was used for the identification of FAMEs.

Determination of sterols

Unsaponifiables were determined according to the ISO 18609 standard. Sterols were isolated from the unsaponifiable matter by thin layer chromatography (TLC) (Ivanov *et al*., 1972) and their total content was determined spectrophotometrically at a 597 nm wavelength. Individual sterol composition was determined on HP 7890 gas chromatograph equipped with DB – 5 (25 m x 0.25 mm) capillary column and FID. Identification was established by comparing retention times with those of a standard mixture of sterols (Across Organics, New Jersey, USA) (ISO 12228-1).

Determination of tocopherols

Individual tocopherols were determined by Merck-Hitachi (Merck, Darmstadt, Germany)

high-performance liquid chromatography (HPLC). The column was Nucleosil Si 50-5 (250 mm x 4 mm). Fluorescence detection was used (excitation at 295 nm and emission at 330 nm). The mobile phase used was *n*-hexane: dioxane, 96:4 (v/v) and the flow rate were set at 1 mL/min. (ISO 9936).

Determination of total phospholipids

Total phospholipid content was determined spectrophotometrically at 700 nm after mineralization of the lipid fraction with a mixture of perchloric and sulphuric acid (1:1, v/v) (ISO 10540-1).

2.2.6. Statistics

All measurements in the study were performed in triplicate ($n = 3$). The results were presented as the mean value with the corresponding standard deviation (SD). ANOVA and Tukey multiple comparison test were used as statistical tools in the assessment of significant differences at $p < 0.05$.

3. Results and discussions

3.1. Chemical composition of fennel fruits

The chemical composition of the fruits is presented in Table 1. The data show that the fruits of the 2020 harvest have a higher content of protein, essential oil and lipid fraction, which can be explained by the influence of climatic conditions, for example at a higher temperature and drier air, the essential oil evaporates more easily from the plants (Baser and Buchbauer, 2010). For the region of the city of Razgrad, the average monthly temperature in August 2020 was 36.6°C, and the average precipitation is 15 mm, while in 2021 these values were 38.1°C and 10 mm, respectively (Annual Hydrometeorological Bulletin). The amount of essential oil in both samples is higher than reported in the literature, for example 2.5-5% (Kaur and Arora, 2010); 3% (He and Huang, 2011); 1.74% (Diao *et al*., 2014); 3.64-4.14% (Wodnicka *et al*., 2019); from 1.1 to 4.8% (Bahmani *et al*., 2016). The content of the lipid fraction in both samples was lower compared to literature data, for example 19.80% (Ahmad *et al*., 2018); from 12.2 to 22.8% (Ali *et al*., 2016); 20% (He and Huang, 2011); 20.90% (Gulfraz *et al*., 2005). Protein content also differed from literature data 9.5% (Kaur and Arora, 2010); 24.12% (Gulfraz *et al*., 2005). The amount of fiber determined differed as well from literature data: 18.5% (Kaur and Arora, 2010); 9.50% (Gulfraz *et al*., 2005). It was found that the ash value was lower than the data, reported in literature: 7.86% (Unal *et al*., 2013); 8.93-9.78% (Moser *et al*., 2014).

The influence of pedo-climatic conditions, as well as the care given to the cultivation of plants, could lead to the appearance of these differences (Conforti *et al*., 2006; Ehsanipour *et al.,* 2012; Yaldiz and Çamlica, 2019).

The polar compounds are presented in Table 2. From the data it can be seen that the identified components (% of the composition) in the 2020 harvest are 98.69%, and in the 2021 harvest their amount is 98.44%.

Table 1. Chemical composition of the fennel fruits

Compounds, %	Harvest 2020	Harvest 2021
Moisture	13.49±0.11 [a]	14.70±0.12 [b]
Proteins	15.74±0.14 [b]	14.30±0.13 [a]
Crude fiber	32.88±3.00 [b]	31.50±3.00 [a]
Ash	6.49±0.05 [a]	7.99±0.07 [b]
Essential oil	8.38±0.33 [b]	7.57±0.09 [a]
Lipid fraction	9.07±0.08 [b]	5.58±0.04 [a]

Results: mean value ± standard deviation ($n = 3$). Different letters in the same row indicate significant differences ($p < 0.05$).

Table 2. Polar metabolites, trimethylsilyl esters.

RT [2]	RI [3]	Compounds	Content, % of TIC [1]	
			Harvest 2020	Harvest 2021
Amino acids				
4.80	1088	Alanine 2TMS	0.14±0.11 [a]	0.12±0.10 [a]
5.81	1224	Valine 2TMS	0.21±0.02 [a]	0.17±0.01 [a]
6.17	1302	Proline 2TMS	0.12±0.11 [a]	0.10±0.01 [a]
7.71	1470	Aspartic acid 3TMS	0.18±0.01 [a]	0.15±0.01 [a]
Organic acids				
5.20	1196	Malonic acid 2TMS	2.20±2.00 [b]	1.76±1.50 [a]
6.32	1307	Succinic acid 2TMS	1.14±0.09 [b]	0.91±0.09 [a]
6.45	1341	Glyceric acid 2TMS	0.17±0.01 [a]	0.14±0.01 [a]
6.58	1345	Fumaric acid 2TMS	0.19±0.01 [a]	0.16±0.01 [a]
6.96	1397	Glutaric acid 2TMS	0.12±0.01 [a]	0.10±0.01 [a]
7.94	1485	Malic acid 3TMS	6.96±6.00 [b]	5.57±5.00 [a]
8.24	1498	Adipic acid 2TMS	0.80±0.07 [b]	0.64±0.06 [a]
9.71	1636	*o*-Methoxymandelic acid 2TMS	0.35±0.03 [b]	0.28±0.02 [a]
9.97	1673	*p*-Methoxymandelic acid 2TMS	0.51±0.04 [a]	0.41±0.04 [a]
10.70	1736	*(E)*-Aconitic acid 3TMS	5.29±5.00 [a]	5.41±5.00 [a]
11.38	1790	Shikimic acid 4TMS	2.86±2.00 [b]	1.25±1.00 [a]
12.16	1804	Citric acid 4TMS	2.26±2.00 [b]	1.78±1.12 [a]
Sugar alcohols				
5.89	1265	Glycerol 3TMS	13.03±1.10 [a]	10.65±0.90 [a]
8.12	1489	Threitol 4TMS	0.19±0.01 [a]	0.16±0.01 [a]
8.16	1493	Erythritol 4TMS	0.55±0.04 [a]	0.44±0.04 [a]
10.49	1690	Xylitol 5TMS	9.65±8.00 [b]	7.72±7.00 [a]
10.57	1703	Arabitol 5TMS	1.91±1.00 [b]	1.36±1.00 [a]
15.86	2144	Myo-Inositol	3.79±3.00 [a]	4.55±0.40 [b]
Sugar acids				
14.69	2050	Gluconic acid 5TMS	2.18±2.00 [a]	2.60±2.00 [b]
15.01	2072	Glucaric acid 6TMS	5.21±5.00 [a]	6.25±0.55 [b]
Saccharides (mono- and di-)				
12.76	1855	Fructose oxime 5TMS isomer	1.62±1.10 [a]	1.95±1.50 [b]
13.03	1864	Fructose oxime 5TMS isomer	0.67±0.05 [a]	0.80±0.07 [a]
13.30	1881	Glucose oxime 6TMS isomer	1.42±1.30 [a]	1.70±1.50 [b]
13.87	1901	Glucose oxime 6TMS isomer	3.96±3.00 [a]	4.71±4.00 [b]
19.11	2298	Glucose 6-phosphate 6TMS	9.24±9.00 [a]	11.0 ±1.00 [b]
25.13	2611	Sucrose 8TMS isomer	19.16±1.80 [a]	22.91±2.10 [b]
25.96	2656	Sucrose 8TMS isomer	1.50±1.10 [a]	1.80±1.70 [b]
Others				
8.90	1575	*n*-Dodecanol 1TMS	1.11±0.09 [b]	0.89±0.08 [a]

[1] – total ion current; [2]– retention time, min; [3]– retention (Kovat's) index; Results: mean value ± standard deviation (n = 3); Different letters in the same row indicate significant differences ($p < 0.05$).

The distribution of the polar metabolites by groups (expressed as percentage of the identified) is presented in Figure 1. The data show that the saccharides (mono- and di-) predominated in both samples, followed by sugar alcohols, organic acids, sugar acids, others, and amino acids.

From the group of organic acids, the amount of malic acid 3TMS and (E)-aconitic acid 3 TMS was the largest. It is known that aconitic acid has a role as a fundamental metabolite and shikimic acid is polyphenol precursors, as well as it participates in the genesis of aromatic amino-acids like: tyrosine, tryptophan, and phenylalanine (Bontpart *et al*., 2016).

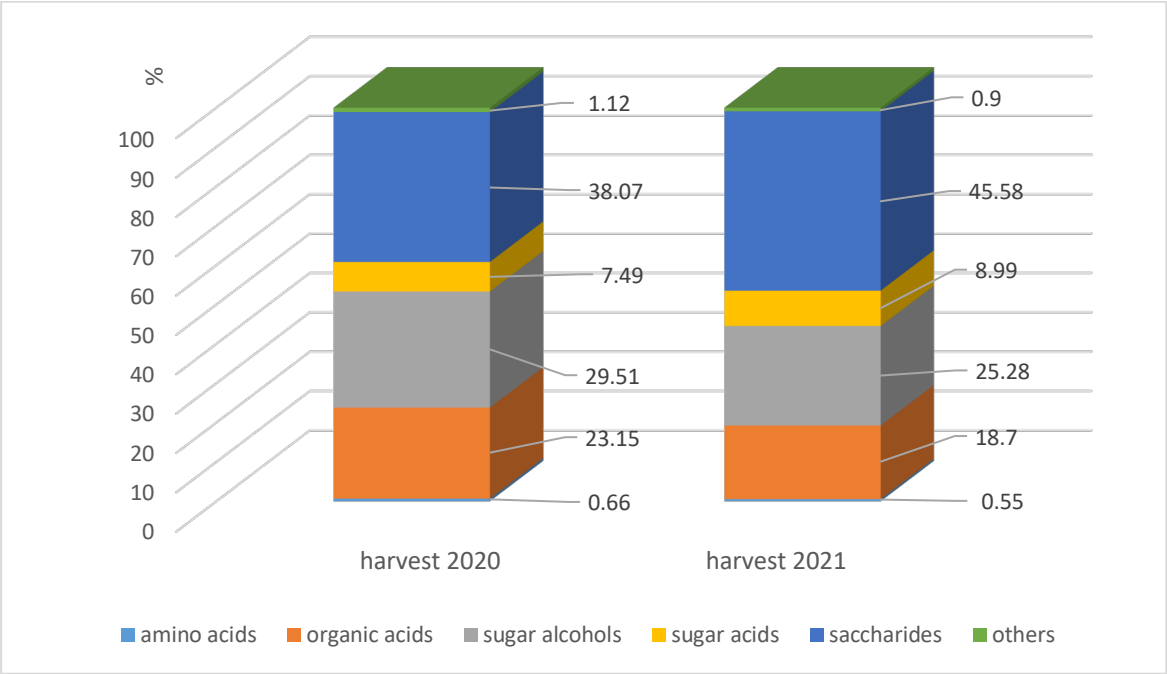


Figure 1. Distribution of compounds in polar fraction from fennel fruits.

The sugars that supply energy to the body are precursors for primary and secondary metabolites (Akhatou *et al*., 2016), represented by different group. The data show that in both samples fruits from the group of sugar alcohols predominate xylitol 5TMS and myo-inositol, which are important for health (Chhetri, 2019; Benahmed *et al*., 2020). Sugar acids glucaric acid 6TMS and gluconic acid 5TMS are also biologically important, as the content of the former is twice that of the latter.

From the group of carbohydrates, the content of sucrose is the highest.

The wide variety of polar metabolites defines the fennel fruit as a potential additive in human and animal food.

Mineral elements play a major role in metabolic processes and in the normal functioning of both human and animal bodies. Their main source is fruits and vegetables, and their consumption is a major factor against various diseases and functional disorders. Data in the literature show that the determination of mineral elements is also the subject of research on plant raw materials that are not used for direct consumption, but can be used as an additive in the food of humans and animals.

The mineral composition of fennel fruits is presented in Table 3. The data show significant differences in the amounts of macro elements, while the content of micro and toxic elements was comparable. Generally, the concentrations of the identified macro elements were lower in harvest 2020 compared with harvest 2021, and the greatest differences were with respect to potassium (about 2.5 times), sodium (about 2 times), and magnesium (3 times) contents. The comparative analysis shows that some of the macro elements do not differ from the data in the literature, for example potassium (0.57-40.60 mg/g), sodium (0.07-1.32 mg/g), magnesium (0.57-5.38 mg/g), calcium (1.00-16.78 mg/g), phosphorus (2.13-56.58 mg/g), and sulfur (0.35-52.12 mg/g) (Yaldiz and Gamlica, 2019). However, a lower phosphorus (9 367.80 mg/kg) and calcium (16 452.88 mg/kg) content was found in bitter fennel (Ozcan and Akbulut, 2007). A study by Ullah *et al*., 2012 reported very high values of calcium (70 mg/kg), magnesium (34 mg/kg), and sulfur (21.4 mg/kg). The observed differences in macro elements in our investigation and literature data were probably due to growth conditions, cultural applications

or genetic factors (Guil *et al*., 1998; Ozcan and Akgul, 2007).

Microelements, regardless of their small amounts, participate in various metabolic and regulatory processes in the body of humans and animals. Iron content is highest, followed by magnesium and zinc.

The trace toxic elements, lead, arsenic, cadmium, and mercury, were identified in both harvests.

Table 3. Minerals in fennel fruits

Minerals	Harvest 2020	Harvest 2021
Macro elements, mg/kg		
Potassium (K)	18 328.31±150.00 [a]	45 003.02±400.00 [c]
Calcium (Ca)	18 904.20±17.00 [a]	19 426.64±18.00 [b]
Phosphorus (P)	9 771.80±9.50 [a]	13 201.33±12.00 [h]
Sulfur (S)	5 984.93±5.20 [a]	7 909.11±7.20 [b]
Magnesium (Mg)	4 661.64±4.10 [a]	15 871.62±14.00 [b]
Sodium (Na)	523.14±0.50 [a]	1 213.22±11.00 [b]
Aluminum (Al)	33.37±0.02 [a]	43.33±0.03 [b]
Micro elements, mg/kg		
Iron (Fe)	65.66±0.05 [a]	70.54±0.06 [b]
Manganese (Mn)	51.67±0.04 [b]	40.31±0.03 [a]
Zinc (Zn)	32.29±0.02 [a]	30.23±0.02 [a]
Copper (Cu)	14.16±0.01 [a]	13.80±0.01 [a]
Nickel (Ni)	1.53±0.01 [a]	2.12±0.0 [b]
Cobalt (Co)	0.11±0.0 [a]	0.20±0.0 [a]
Selenium (Se)	0.14±0.0 [a]	0.14±0.0 [a]
Toxic elements, mg/kg		
Chromium (Cr)	0.27±0.0 [a]	0.35±0.0 [b]
Arsenic (As)	trace[1]	trace
Lead (Pb)	trace	trace
Cadmium (Cd)	trace	trace
Mercury (Hg)	trace	trace

[1] up to 0.01 mg/kg; Results: mean value ± standard deviation (n = 3); Different letters in the same row indicate significant differences ($p < 0.05$).

Data for micro- and toxic elements do not differ from data in the literature (Yaldiz and Gamlica, 2019).The established differences in the amount of mineral elements reflect the influence of environmental factors on their presence and accumulation during the vegetation of plants.

The obtained results show that with its balanced composition of mineral elements, the fruits of the fennel can be used as a supplement to the food of humans and animals.

3.2. Chemical composition and antimicrobial activity of essential oil

All the essential oils were easily mobile liquids, with a pale-yellow color and a characteristic odor of anethole.

The chemical composition of the essential oils is presented in the Table 4.

From the data it can be seen that the identified components (% of the composition) in the essential oils obtained by hydrodistillation are 98.45% (harvest 2020) and 98.79% (harvest 2021). In this variant the two essential oils have a similar composition. In the 2020 harvest, the main components (above 2%)

were: anethole (73.72%), fenchone (17.09%), and estragole (2.67%). The essential oil obtained from the fruits of the 2021 harvest had the following main components (over 2%): anethole (76.15%), fenchone (13.70%), *α*-pinene (2.27%), and estragole (2.05%). Comparison of chemical composition data shows that both samples were high in anethole and low in fenchone. The increased amount of anethole in the essential oil obtained from the fruits of the 2021 harvest and the lower amount of fenchone can be explained by the climatic conditions when the plant was grown. In terms of content of essential components, the essential oils correspond to the data from the literature for oils of sweet fennel. In the variant extraction/derivatization the identified components (% of the composition) in both essential oils are 98.92%. The essential oils do not differ in chemical composition. Their main components (above 2%) were: anethole (74.03-75.20%), fenchone (14.38-15.25%), estragole (2.15-2.80%), and *α*-pinene (1.88-2.39%).

The quantity of the main components in all investigated essential oils differ from the data in the literature: anethole (82%), limonene (6.55%), estragole (3.53%) (Renjie *et al*., 2010); anethole (46.2%), limonene (27.78%), fenchone (12.78%), estragole (6.34%), and *α*-pinene (3.26%) (Shahat *et al*., 2012); anethole (68.53%), estragole (10.42%), and limonene (6.24%) (Diao *et al*., 2014); *trans*-anethole (82.9-87.4%), estragole (4.47-5.87), and limonene (3.64-4.97%) (Najdoska-Bogdanov *et al*., 2015); anethole (1.2-88.4%), estragole (0.2-59.1%), fenchone (1.1-14.7%), and limonene (5.3-15.7%) (Bahmani *et al*., 2016); anethole (69.95%) and fenchone (18.14%) (Wodnicka *et al*. 2019).

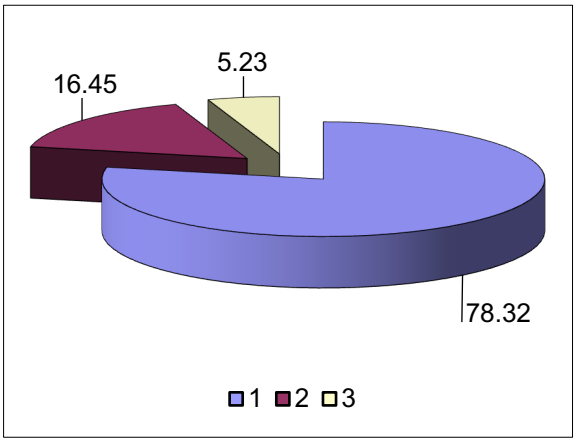
The distribution of the components by functional groups (expressed as percentage of the identified) is presented in Figure 2.

Table 4. Chemical composition of essential oils of fennel fruits (% of TIC[1])

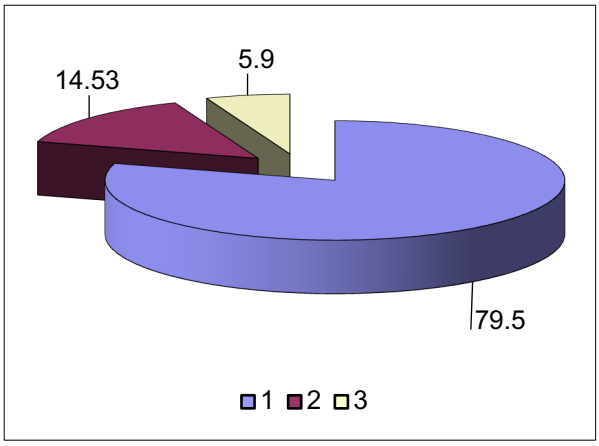
RT[2]	RI[3]	Compounds	By hydrodistillation		By extraction/ derivatization	
			Harvest 2020	Harvest 2021	Harvest 2020	Harvest 2021
Monoterpene hydrocarbons						
10.09	933	*α*-Pinene	1.79±0.16 [a]	2.27±0.21 [b]	1.88±0.17 [a]	2.39±2.22 [b]
10.59	947	Camphene	0.21±0.02 [a]	0.24±0.02 [a]	0.22±0.02 [a]	0.25±0.02 [a]
11.36	968	Sabinene	0.08±0.0 [a]	0.11±0.01 [a]	0.08±0.0 [a]	0.11±0.0 [a]
11.51	979	*β*-Pinene	0.10±0.0 [a]	0.13±0.01 [a]	0.10±0.0 [a]	0.14±0.01 [a]
11.94	988	Myrcene	0.49±0.04 [b]	0.36±0.03 [a]	0.51±0.05 [b]	0.38±0.03 [a]
12.46	1001	*α*-Phellandrene	0.25±0.02 [a]	0.32±0.03 [b]	0.27±0.02 [a]	0.34±0.03 [b]
13.11	1024	Limonene	1.64±0.15 [a]	1.73±0.16 [b]	1.72±0.15 [b]	1.82±0.06 [c]
13.44	1033	*(Z)-β*-Ocimene	0.07±0.0 [a]	0.08±0.0 [a]	0.07±0.0 [a]	0.09±0.0 [a]
14.04	1054	*γ*-Terpinene	0.43±0.04 [a]	0.52±0.04 [b]	0.45±0.03 [a]	0.54±0.04 [b]
14.93	1073	Terpinolene	0.09±0.0 [a]	0.07±0.0 [a]	- [4]	-
Oxygenated monoterpenes						
13.22	1027	Eucalyptol	0.10±0.0 [a]	0.13±0.01 [a]	0.11±0.01 [a]	0.14±0.01 [a]
14.52	1062	*(Z)*-Sabinene hydrate	0.11±0.01 [a]	0.08±0.0 [a]	0.12±0.01 [a]	0.09±0.0 [a]

15.36	1085	Fenchone	15.47±1.40 [c]	13.70±1.20 [a]	15.25±1.40 [c]	14.38±1.30 [b]
16.10	1114	endo-Fenchol	0.09±0.0 [a]	0.10±0.00 [a]	0.10±0.0 [a]	0.11±0.0 [a]
16.30	1139	*(Z)*-Pinene hydrate	0.08±0.0 [a]	0.06±0.0 [a]	0.09±0.0 [a]	0.07±0.0 [a]
16.84	1165	Camphor	0.34±0.03 [a]	0.28±0.02 [a]	0.35±0.03 [a]	0.29±0.02 [a]
Phenyl propanoids						
12.95	1020	*p*-Cymene	0.10±0.01 [a]	0.08±0.0 [a]	0.12±0.01 [a]	0.08±0.0 [a]
18.37	1195	Estragole	2.67±0.21 [b]	2.05±0.21 [a]	2.80±0.22 [b]	2.15±0.20 [a]
20.03	1237	*o*-Anisaldehyde	0.15±0.01 [a]	0.11±0.01 [a]	0.16±0.01 [a]	0.12±0.0 [a]
20.43	1248	*p*-Anisaldehyde	0.46±0.04 [b]	0.22±0.02 [a]	0.49±0.04 [b]	0.23±0.02 [a]
21.15	1290	Anethole	73.72±7.20 [a]	76.15±7.50 [b]	74.03±7.00 [a]	75.20±7.20 [b]

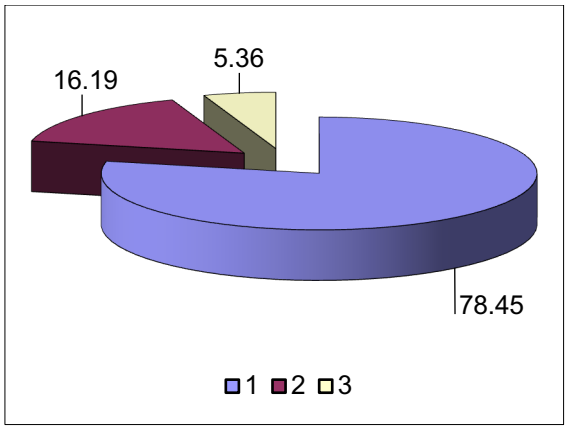
[1] – total ion current; [2] – retention time, min; [3]– retention (Kovat's) index; [4] – not identified; Results: mean value ± standard deviation ($n = 3$); Different letters in the same row indicate significant differences ($p < 0.05$).



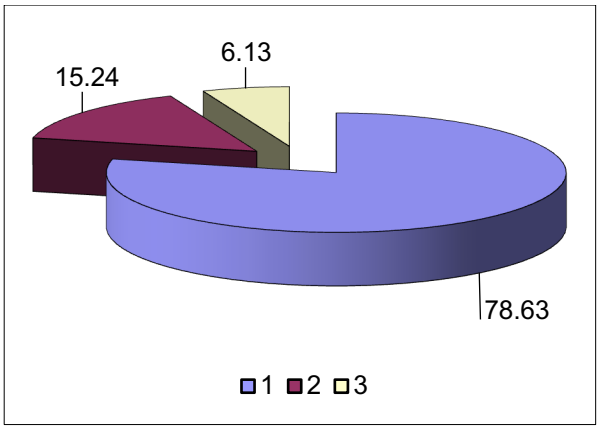
By hydrodistillation, Harvest 2021



By hydrodistillation, Harvest 2021



By extraction/derivation, Harvest 2020



By extraction/derivation, Harvest 2020

Figure 2. Distribution of compounds in essential oils from fennel fruits:
1 – phenyl propanoids; 2 – oxygenated monoterpenes; 3 – monoterpene hydrocarbons.

The data show that in variant hydrodistillation phenyl propanoids were the dominant group in both essential oils. The comparative analysis shows that the amount of phenylpropanoids and monoterpene hydrocarbons was higher in the 2021 harvest, and the monoterpene oxygen derivatives was higher in the 2020 harvest.

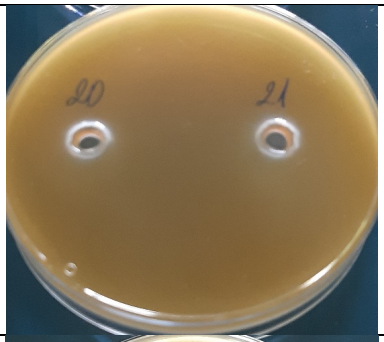
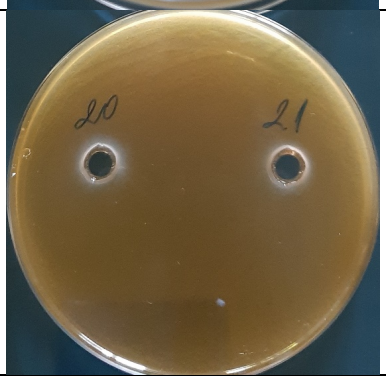
In variant extraction/derivatization the distribution of components in both essential oils by group of compounds does not differ: phenyl propanoids (from 78.32% to 79.50%), followed by oxygenated monoterpenes (from 14.53% to 16.45%), and monoterpene hydrocarbons (from 5.23% to 6.13%). These groups of compounds in all essential oils differ from the literature: monoterpene hydrocarbons (6.20%), oxygenated monoterpenes (11.40%), and phenyl propanoids (74.37%) (Ahmad *et al*., 2018).

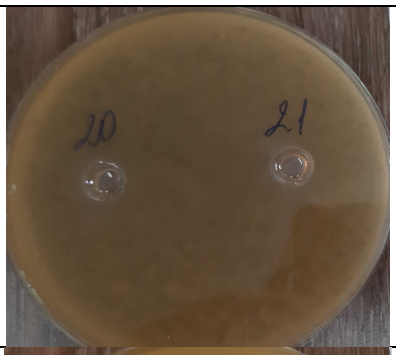
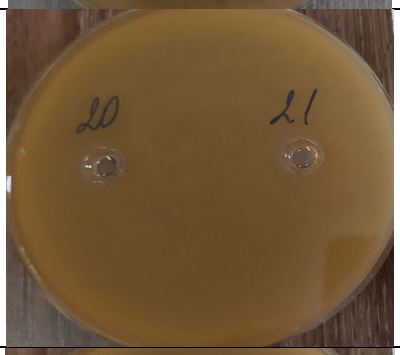
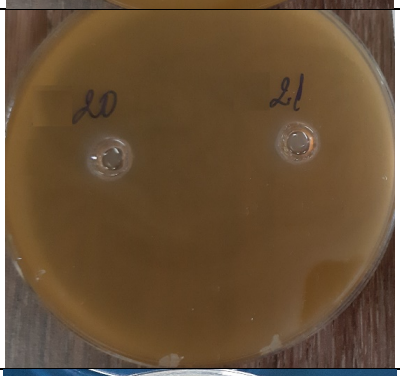
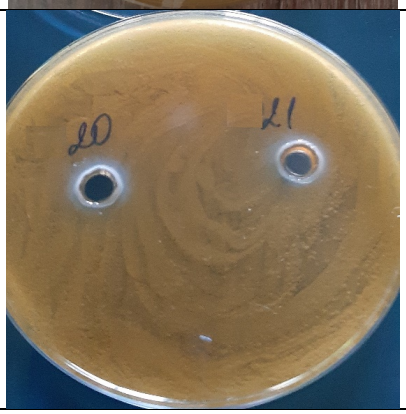
The differences in the aromatic compounds of the investigated essential oils and those from the literature can be both explained by the climatic conditions in different countries.

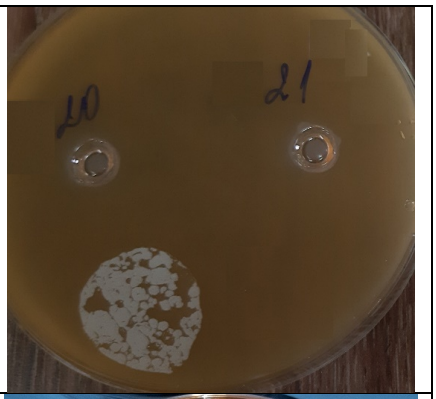
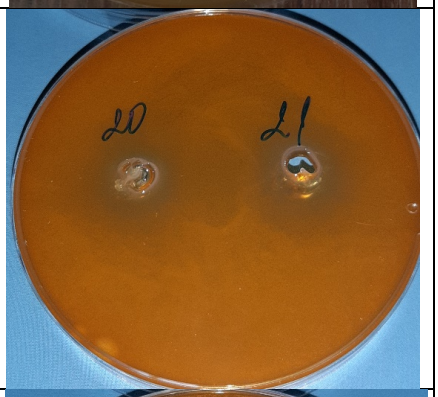
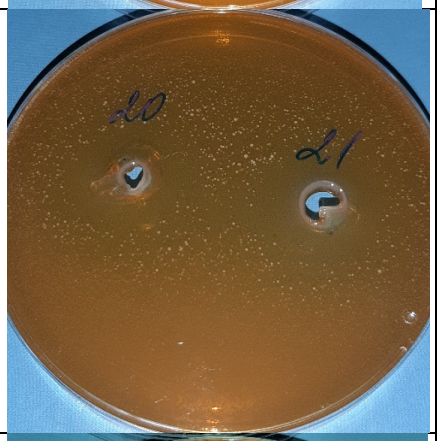
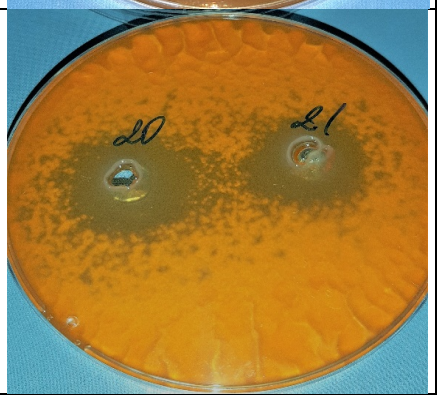
Anethole is characterized by various biological properties – antimicrobial, antioxidant, anti-inflammatory, *etc*. (Ghelardini *et al*., 2001; Freire *et al*., 2005; Tognolini *et al*., 2007; Huang *et al*., 2010; Ponte *et al*., 2012; Giustarini *et al*., 2014; Kim *et al*., 2017). However, anethole taken orally in large quantities has an effect on the central nervous system, which is why essential oils containing it have limited use in the food industry and cosmetics (Poon and Freeman, 2006; Aschenbeck and Hylwa, 2017; Horst *et al*., 2017; Sarkic and Stappen, 2018).

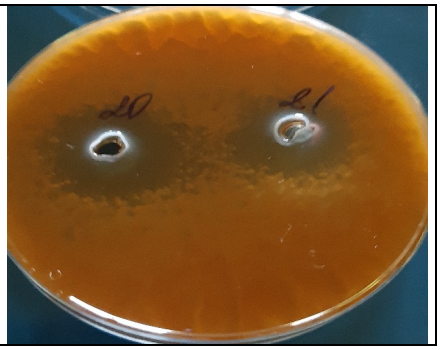
The results for the antimicrobial activity of the essential oils of the fennel fruits are presented in Table 5.

Table 5. Antimicrobial activity of essential oils from fennel fruits

Tested microorganisms	**Inhibition zone (mm)**		
	Harvest 2020	**Harvest 2021**	
Staphylococcus aureus ATCC 6538	8.0±0.0 [a]	8.0±0.0 [a]	
Staphylococcus epidermidis ATCC 12228	8.0±0.0 [a]	8.0±0.0 [a]	

Bacillus subtilis ATCC 6633	8.0±0.0 [a]	8.0±0.0 [a]	
Bacillus cereus ATCC 10876	8.0±0.0 [a]	8.0±0.0 [a]	
Escherichia coli ATCC 8739	8.0±0.0 [a]	8.0±0.0 [a]	
Pseudomonas aeruginosa ATCC 9027	16.3±0.3 [b]	14.9±0.4 [a]	

Salmonella abony NCTC 6017	8.0±0.0 [a]	8.0±0.0 [a]	
Candida albicans ATCC 10231	13.6±0.2 [a]	13.7±0.5 [a]	
Saccharomyces cerevisiae ATCC 9763	22.7±0.7 [a]	21.2±0.3 [a]	
Aspergillus brasiliensis ATCC 16404	19.0±0.5 [b]	17.8±0.6 [a]	

Fusarium moniliforme	17.4±0.4 [a]	18.3±0.2 [b]	

Results: mean value ± standard deviation (n = 3); Different letters in the same row indicate significant differences ($p < 0.05$).

It can be seen that they have an almost identical profile of antimicrobial activity – good against yeasts and molds and weak to none antibacterial activity.

The strongest activity of the oils was observed against the yeast *S. cerevisiae* and the molds *A. brasiliensis* and *F. moniliforme*. The yeast *C. albicans* is weakly sensitive to the tested essential oils. These results are also confirmed by the research of other authors (Javed *et al*., 2012; Atanasova-Pančevska *et al*., 2021).

Among the bacteria tested, only the Gram-negative bacterium *Pseudomonas aeruginosa* was sensitive to the oils. The remaining bacteria are not affected by the two oils.

Research by other authors confirmed the low antibacterial activity of the essential oil of the fennel against *S. aureus*, *L. monocytogenes, B. cereus,* and *Salmonella* sp. (Miguel *et al*., 2010) and against pathogenic and bacteria that cause spoilage of food products (Borotova *et al.*, 2021).

Other studies have found high inhibitory activity of the essential oil against the bacteria *A. baumannii*, *E. coli*, *P. aeruginosa*, *S epidermidis*, and *S. aureus* (Diao *et al*., 2014; Barrahi *et al*., 2020).

The established differences in the antimicrobial activity of the oils are due to differences in their chemical composition, due to the geographical origin of the fennel fruits, differences in the methods of collection, drying and preparation of the essential oil (Bozin *et al*., 2006).

3.3. The chemical composition of lipid fractions

The lipid fractions are a viscous mass with a green to dark green color and a specific odor.

In Table 6 it is presented the fatty acid composition of the lipid fraction.

In the variant of extracting the fruits after their distillation the sum of petroselinic + oleic acids predominated in both oils (84.1 and 79.5%, respectively), followed by saturated palmitic (6.5 and 9.5%), the unsaturated linoleic (4.0 and 4.2%) acid, and stearic acid (1.6 and 2.1%). The other fatty quantities are negligible (from 0.1 to 0.7%). The main fatty acid in both samples was C $_{18:1}$. In this case, it is a mixture of isomers that cannot be separated under the conditions of the analysis. In this variant a tendency to increase the content of palmitic acid and decrease of linoleic acid was observed. This was probably due to the possible transformation of these acids under the influence of temperature, resulting in oxidation of the methylene group near the carboxyl. Free radicals were produced, which in subsequent oxidative degradation are cleaved off, resulting in acids with a shorter chain (Gonstone, 1967).

In the variant extraction/derivatization very few fatty acids were identified. The main fatty acid was petroselinic acid (72.7-74.2%), followed by oleic acid (3.4-3.8%), and palmitic acid (2.95-3.2%).

The values of the identified fatty acids differ from the data in the literature: petroselinic acid (74.80%), linoleic acid (12.74%), and palmitic acid (5.34%) (Ahmad *et al*., 2018); linoleic acid (37.01%), linolenic

acid (35.54%), and palmitic acid (17.7%) (Morales *et al*., 2012); petroselinic acid (75.18%), linoleic acid (11.18%), oleic acid (6.15%), and palmitic acid (4.76%) (Cosge *et al*., 2008); ($C_{18:1}$) methyl esters (80.9-83.0%), linoleic acid (10.8-11.9%), and palmitic acid (4.27-5.15%) (Najdoska-Bogdanov *et al*., 2015). The established differences in the amount and fatty acid composition of the studied oils, and those from the literature can be explained both by the varietal characteristic of the fennel and by the climatic conditions of the respective years during which the studied fruits ripened.

The distribution of saturated and unsaturated fatty acids shows that in the extraction version after distillation, unsaturated fatty acids predominated in both oils (89.6 and 85.7%), and the monounsaturated ones were in a greater amount (85.3 and 81.1%) than the poly-unsaturated ones (4.3 and 4.6%). Saturated fatty acids were found to be 10.4% (2020 harvest) and 14.3% (2021 harvest) respectively.

Table 6. Fatty acid composition of lipid fraction from fennel fruits (variant extraction after distillation).

Fatty acids, %		**By extraction after distillation**		**By extraction/ derivatization**	
		Harvest 2020	**Harvest 2021**	**Harvest 2020**	**Harvest 2021**
Saturated fatty acids					
$C_{8:0}$	Caprylic	0.3±0.0[a]	0.3±0.1[a]	-	-
$C_{10:0}$	Capric	-[1]	0.1±0.0 [a]	-	-
$C_{12:0}$	Lauric	0.1±0.0 [a]	0.1±0.0 [a]	-	-
$C_{14:0}$	Myristic	0.3±0.1 [a]	0.4±0.0 [a]	-	-
$C_{15:0}$	Pentadecanoic	0.1±0.0 [a]	0.2±0.0 [a]	-	-
$C_{16:0}$	Palmitic	6.5±0.5 [b]	9.5±0.7 [c]	2.95±0.2 [a]	3.2±0.2 [a]
$C_{17:0}$	Margaric	0.3±0.0 [a]	0.2±0.0 [a]	-	-
$C_{18:0}$	Stearic	1.6±0.2 [b]	2.1±0.1 [c]	0.3±0.1 [a]	0.4±0.1 [a]
$C_{20:0}$	Arachidic	0.7±0.1 [b]	0.7±0.2 [b]	0.1±0.0 [a]	0.1±0.0 [a]
$C_{22:0}$	Behenic	0.5±0.1 [a]	0.7±0.2 [a]	-	-
Monounsaturated fatty acids					
$C_{14:1}$	Myristoleic	-	0.1±0.0 [a]	-	-
$C_{15:1}$	Pentadecenoic	0.2±0.0 [a]	0.2±0.0 [a]	-	-
$C_{16:1}$	Palmitoleic	0.2±0.0 [a]	0.3±0.1 [a]	-	-
$C_{17:1}$	Heptadecenoic	0.4±0.1 [a]	0.5±0.1 [a]	-	-
$C_{18:1}$ (n-9)	Oleic	-	-	3.8±0.2 [a]	3.4±0.2 [a]
$C_{18:1}$ (n-12)	Petroselinic	-	-	72.7±7.0 [a]	74.2±7.0 [a]
$C_{18:1}$	(Petroselinic + Oleic)	84.1±8.0 [b]	79.5±7.8 [b]	-	-
$C_{20:1}$	Gondoic	0.3±0.0 [a]	0.3±0.0 [a]	-	-
$C_{22:1}$	Erucic	0.1±0.0 [a]	0.2±0.0 [a]	-	-
Polyunsaturated fatty acids					
$C_{18:2}$ (n-6)	Linoleic	4.0±0.2 [b]	4.2±0.3 [b]	1.6±0.2 [a]	1.5±0.2 [a]
$C_{18:2}$	*trans*-Linoleic	0.1±0.0 [a]	0.1±0.0 [a]	-	-
$C_{18:3}$ (n-6)	γ-Linolenic	0.1±0.0 [a]	0.1±0.0 [a]	0.2±0.1 [a]	0.2±0.1 [a]
$C_{18:3}$ (n-3)	α-Linolenic	0.1±0.0 [a]	0.1±0.0 [a]	-	-
$C_{20:3}$ (n-3)	Eicosatrienoic	-	0.1±0.0 [a]	-	-

[1] – not identified; Results: mean value ± standard deviation (n = 3); Different letters in the same row indicate significant differences ($p < 0.05$).

In the variant extraction/derivatization the quantity of saturated fatty acids was (2 times) as low compared to the variant extraction after hydrodistillation. The amounts of the monounsaturated and polyunsaturated fatty acids were comparable. Petroselinic acid, which is the main fatty acid in the lipid fraction, is an important raw material for oleochemical processes and can be easily processed into lauric and adipinic acid. This acid also plays a significant role in the food, chemical, and cosmetics industries (Uitterhaegen *et al*., 2016).

In Bulgaria there is a tradition of growing the fennel fruits. Due to the high content of petroselinic acid, the fruits can be a raw material for its preparation, replacing the fruits of coriander.

Unsaponifiable matter in both lipid fractions is presented in Table 7 and their values were found to be comparable. They contain terpenic (sterols, tocopherols, tocotrienols, carotenoids, *etc.*) and aliphatic (fatty alcohols, saturated and unsaturated hydrocarbons) compounds (Fontanel, 2013). The data show that their content is higher than that in other lipid fractions from maize (2.8%), rape seed (2.0%), grape seed oil (2.0%), and sunflower (1.5%) (Codex Stan 210).

Total sterols in both lipid fractions were 1.17-1.34% and phospholipids were found to be 0.92 and 1.33%. The total amount of sterols in the oil is higher compared to that of other vegetable oils, for example, from sunflower, soybean, cotton, safrole, *etc*. (0.24-0.64 %) (Codex Stan 210). Since numerous studies demonstrate that phytosterols from the lipid fraction lower total and LDL cholesterol levels, therefore the sterols from the lipid fractions from fennel fruits could constitute a sanogenic ingredient in the prevention and treatment of hypercholesterolemia.

Tocopherols are a class of organic compounds that have vitamin E activity, contained in the lipid fraction of various oils - olive, sunflower, soybean, *etc*. Total tocopherols in the fractions were relatively low, but their content in the lipid fraction from the fruits harvest 2020 (197 mg/kg) was higher than in the fraction from fruits harvest 2021 (160 mg/kg). However, their content in both lipid fractions is lower than that of soy (600-3370 mg/kg), corn germ (330-3720 mg/kg) and rapeseed (430-2680 mg/kg) (Codex Stan 210). The biological role of phospholipids in the construction of cell membranes is known, as well as their antioxidant properties (Tlili *et al*., 2011; Küllenberg *et al.*, 2012). Their quantity is higher in the 2021 harvest. Sterol and tocopherol profiles of the examined lipid fractions are given in Table 7. The data show significant differences in individual sterols. The main ones in the 2020 harvest were *β*-sitosterol (61.1%) and stigmasterol (24.6%), and in the 2021 harvest – *β*-sitosterol (39.7%), stigmasterol (41.2%), and Δ^5-avenasterol (13.8%). In the content of *β*-sitosterol, the main sterol is comparable to that of other lipid fractions such as olive, soybean, and sunflower oil (Codex Stan 210). For tocopherols, the values of the individual components are comparable. The main tocopherol in both lipid fractions is *γ*-tocotrienol, followed by α-tocopherol. In composition, the lipid fraction differs from other commonly used, such as sunflower (where *α*-tocopherol predominated), soybean (*γ*- and *δ*-tocopherols were the main components) and corn oils (where *γ*-tocopherol predominated but there were also traces of *α*- and *δ*-tocopherols), and was rather similar to those of sesame oil (where *γ*-tocopherol was also the major one) (Codex Stan 210).

The biological components in the lipid fraction define the fennel fruit as a suitable supplement to the food of humans and animals.

Table 7. Biological component, sterol and tocopherols in lipid fraction from fennel fruits.

Compounds	Harvest 2020	Harvest 2021
Biologically components		
Unsaponifable matter, %	25.7±0.2 [a]	27.4±0.2 [a]
Sterols, %	1.17±0.12	1.34±0.08 [b]
Phospholipids, %	0.92±0.10 [a]	1.33±0.07 [b]
Tocopherols, mg/kg	197.0±14.0 [d]	160.0±8.0 [d]
Sterols		
Cholesterol	0.4±0.1 [a]	0.5±0.1 [a]
Brassicasterol	0.1±0.0 [a]	-[1]
Campesterol	2.3±0.1 [a]	4.1±0.1 [b]
Stigmasterol	24.6±0.3 [a]	41.2±0.2 [b]
Δ^7-Campesterol	2.1±0.2 [b]	0.7±0.1 [a]
β-Sitosterol	61.1±0.4 [b]	39.7±0.3 [a]
Δ^5-Avenasterol	9.4±0.2 [a]	13.8±0.2 [b]
Tocopherols		
α-Tocopherol	41.6±0.2 [b]	38.2±0.1 [a]
γ-Tocotrienol	58.4±0.2 [a]	61.8±0.4 [b]

[1]- not identified; Results: mean value ± standard deviation (n = 3); Different letters in the same row indicate significant differences ($p < 0.05$).

4. Conclusions

As the fennel fruits are widely used in the food industry, in this research we focus at determining the phytochemical composition and biological activity of sweet fennel fruits, cultivated during two harvest years 2020 and 2021 in North Eastern Bulgaria. The content of protein, crude fiber, essential oil, polar and lipid fractions, and mineral elements was determined in the fennel fruits. The polar metabolites are present in saccharides (mono- and di-), sugar alcohols, organic acids, sugar acids, others, and amino acids. Fennel fruits have high content of macro elements potassium, sodium, and magnesium, and low content of toxic elements. The main components of both essential oils are anethole and fenchone. The essential oils have antimicrobial activity, more pronounced against molds and yeasts. The main sitosterols in both lipid fractions are β-sitosterol and stigmasterol, and major tocopherol is γ-tocotrienol. Based on the results we achieved, we can conclude that because of its biological components in the lipid fraction and with its balanced composition of mineral elements, the fennel fruits can be used as a suitable supplement to the food of humans and animals.

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EFFECT OF JAVANESE GINSENG MUCILAGE AND ORANGE OIL CONCENTRATIONS ON PROPERTIES AND OXIDATIVE STABILITY OF BEVERAGE EMULSIONS

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ABSTRACT

The objective of this study was to investigate the effect of Javanese ginseng mucilage and orange oil concentrations on the properties (viscosity and droplet size) and oxidative stability (total oxidation value) of beverage emulsions. The mucilage was firstly extracted from the fronds of Javanese ginseng and determined to exhibit a lower IC_{50} (i.e. the concentration of sample required to scavenge 50% of free radicals) as compared to gum Arabic, reflecting its higher antioxidant capacity. Fourteen emulsions varying in mucilage (5 – 10%) and orange oil (6 – 10%) concentrations were further prepared based on a two-factor central composite design. The emulsions exhibited a thixotropic behaviour with the viscosity range of 3920 – 20050 mPa.s and the droplet size range of 3.14 – 16.49 µm. All emulsions were stable towards lipid oxidation with low total oxidation values (< 10). The data were subsequently fitted to the quadratic model using a response surface modelling to further elucidate the effects of both factors on each response. There was a significant ($p < 0.05$) synergistic interaction between mucilage and orange oil which positively increased the emulsion viscosity. However, significant ($p < 0.05$) quadratic effects of orange oil resulted in too much increase in the viscosity and thus undesirably led to the formation of large droplets and high total oxidation value. The results suggested that the mucilage and oil should be used around 8 – 9.5% and 6 – 8.5%, respectively in order to provide the emulsions with desired properties and oxidative stability.

1. Introduction

Javanese ginseng, botanically known as *Talinum paniculatum* (Jacq.) *Gaertn*. is a succulent subshrub from the family of Talinaceae. This edible ornamental plant is one of the perceptible medicinal plants, which is also commonly known as Fameflower, Som Java and Jewels-of-Opar. A leaf cross-sectional of this plant exhibits a single-layered epidermis covered with a thin cuticle and contains a large number of mucilaginous cells (Tolouei *et al.*, 2019). The abundance of mucilaginous cells in the leaves (and also in young stems) makes it possible of extracting a significant amount of a complex soluble polysaccharide called mucilage. The mucilage could be easily obtained via a hot water extraction, followed by a solvent (e.g. ethanol) precipitation. With extraction conditions that vary in water to fronds (young leaves and stems) ratio (0.5:1 – 12:1),

temperature (25 – 90˚C) and pH (3 – 11), the range of mucilage that could be obtained is 2.32 – 4.90% (Nor Hayati *et al*., 2019). The mucilage exhibits a significant surface-active property, a key functional property for application in emulsion-based food products. The surface-active property of Javanese ginseng mucilage is strongly associated with its significant protein content (up to 30.97%). Interestingly, the mucilage could be proposed as a functional food ingredient with health-promoting properties. Tolouei *et al*. (2019) demonstrated that the leaf extract (or mucilage) obtained by ethanol precipitation has significant diuretic and cardioprotective effects as well as non-toxicity. The extract also contains bioactive compounds such as chlorogenic acids, organic acids, and O-glycosylated flavones, which are thought to be responsible for its antioxidant and related medicinal properties (Lestario *et al*., 2009; Thanamool *et al*., 2013; Reis *et al*., 2015).

For decades, food hydrocolloids have become important ingredients for the food industry. Besides, it was made constant efforts from the scientists to search for new sources and varieties of food hydrocolloids to function as emulsifiers, stabilizers and texturizers mainly in emulsion-based food products. In addition to *T. paniculatum* mucilage, other examples of leaf mucilage that exhibit the said functionalities are the mucilage extracted from leaves (or young fronds) of *Asplenium australasicum* (Lai and Liang, 2012), *Pereskia aculeate* (Martin *et al.*, 2017; Lago *et al.*, 2021), *Perekia bleo* (Nurul Farhanah *et al*., 2019), *Basella alba* (Hung and Lai, 2019), and *Malva parviflora* (Munir *et al.*, 2021). However, very limited studies concern about their functionality performance in model or real food emulsion systems. To the best of our knowledge, there is one related study done by Lago *et al*. (2019) who have examined the performance of *P. aculeate* leaf mucilage in soybean oil-in-water nanoemulsions. The study highlighted that the mucilage concentration should be set at 1.0 to 1.5% with the oil concentration should be less than 5% to obtain the nanoemulsions with the desired properties and stability. In particular, no information is available regarding the performance of Javanese ginseng mucilage in any food system including beverage emulsions. Moreover, the effectiveness of the mucilage in playing a role as a natural antioxidant in such a complex system is yet to be investigated.

Indeed, this research focus is consistent with the recent trend of using food ingredients with high antioxidant activity in the food system (Paraiso *et al*., 2020; Souza *et al*., 2020a). Thus, the objective of the present study was to determine the effects of Javanese ginseng mucilage and orange oil concentrations on the properties and oxidative stability of beverage emulsions. The effects were elucidated in a meaningful way by using response surface modelling, which also allows one to empirically estimate the concentration limit of both ingredients in the formulation.

2. Materials and methods

2.1. Materials

Javanese ginseng fronds were collected from a controlled farm in Kelantan, Malaysia. To obtain the mucilage powder, the extraction was carried out according to our previous report (Nor Hayati *et al*., 2019) using the optimized extraction condition i.e. water to fronds ratio (8.4:1), temperature (90°C) and pH (8). Orange oil was purchased from Mee Soya Sdn. Bhd. Other ingredients for the emulsion formulation were purchased from SIM Company Sdn. Bhd.

2.2. Determination of antioxidant activity of mucilage

To better understand the mucilage's ability to inhibit lipid oxidation in beverage emulsions, the antioxidant activity of the mucilage was first analysed in terms of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity test according to the modified method of Kong *et al*. (2010). Briefly, 2.0 mL DPPH solution (2 mM ethanol) was added to 2.0 mL of mucilage in water at different concentration of 100 – 800 µg/ml. The mixture was shaken and left to stand for 30 min at room temperature in the dark. The absorbance was measured at 517 nm with a UV–Vis spectrophotometer (UV-160,

Shimadzu, Kyoto, Japan). The DPPH radical scavenging effect was calculated as follows:

$$Scavenging\ activity\ (\%) = \frac{(Ao - (A - Ab))}{Ao} x\ 100 \quad (1)$$

where Ao is the absorbance (A_{517}) of DPPH without sample, A is the A_{517} of sample and DPPH, and Ab is the A_{517} of sample without DPPH. Sample concentration providing 50% scavenging activity (IC_{50}) was determined from the plot of scavenging activity (%) against the sample concentration. Butylated hydroxytoluene (BHT) was used as a positive control and commercial gum Arabic was used for comparison. All assays were carried out for triplicate samples.

2.3. Preparation of beverage emulsions and experimental design

A response surface methodology was used to determine the effect of two most significant emulsion components namely Javanese ginseng mucilage and orange oil on viscosity, droplet size, and total oxidation (TOTOX) of beverage emulsions. Fourteen model beverage emulsions were prepared according to two factor central composite design (face-centred). The two factors involved were mucilage (5-10%) and orange oil (6-10%). Other ingredients namely carboxymethyl cellulose (CMC) (0.5%), sodium benzoate (0.08%), citric acid (2%) and distilled water (mark up to 100%) were constant. Hydrocolloid dispersion was firstly prepared by blending the mucilage with CMC at 80°C for 2 h and subsequently stored at room temperature (25±2 ºC) overnight. The dispersion was then added with distilled water, citric acid and sodium benzoate. The mixture was homogenized for 2 min at 6000 rpm. After that, orange oil was mixed to the mixture and then homogenized for 3 min at 10000 rpm. Homogenization was done using a high-speed homogenizer (Ultra Turrax T-25, IKA Instruments, IKA-Werke GmbH & Co., Germany). The emulsions were observed to be stable after homogenization with desirable pH of 3.0 ± 0.2 (Guzey & McClements, 2006).

2.4. Determination of emulsion viscosity

Determination of viscosity was carried out by using a viscometer (Brookfield DV-III Viscometer, Brookfield, Brookfield Engineering Laboratories, Inc., USA) equipped with a small sample adapter and Spindle No. 1 at 100 rpm were used. The viscosity was measured at room temperature for 12 min.

2.5. Determination of emulsion droplet size and microstructure

The droplet image for the emulsions was captured under a polarized light microscopy at room temperature. Droplet microstructures were visualized by Eclipse 80i Advanced Research microscope (Nikon, Nikon Instruments, Inc., USA) at 100x magnification after being equilibrated for 2 min. The average size of the predominant droplets in each emulsion were recorded. In addition, the smallest, medium and largest size of the droplets were also determined in order to estimate the emulsion polydispersity.

2.6. Determination of emulsion oxidative stability

Peroxide value (PV) and Anisidine value (AV) of the stored emulsions (30 days at 5°C in order to stimulate the normal storage condition) were determined by the standard iodometric AOAC Method 965.33 (AOAC, 1990) and based on Egan *et al*. (1981), respectively. The oxidative stability was expressed in a TOTOX value calculated as 2PV + AV.

2.7. Statistical analysis

A one-way ANOVA with Tukey's post-hoc test was applied to the DPPH radical-scavenging data. For other data, the statistical analysis was done based on a response surface regression analysis involving determination of estimated coefficients of model terms and ANOVA. Independent variables of mucilage (X_1) (5 – 10 %) and orange oil (X_2) (6 – 10 %) and their relationship with dependent variables (responses) (Y_i) was expressed by a multiple linear regression equation as follows:

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

where β_0 is a constant, β_1 and β_2 are regression coefficients for the linear effects, β_{11} and β_{22} are coefficients for the quadratic effects and β_{12} is coefficient for the interaction effects. The interaction effect between independent variables on the responses were also shown in the response contour plots. Model lack-of-fit and coefficient of determinations (R^2) were used to judge the model adequacy. All analyses were carried out by using a Minitab statistical software package (Release 14, Minitab Inc, USA). The level of confidence used was at α-0.05.

3. Results and discussions

3.1. Antioxidant activity of Javanese ginseng mucilage

In the DPPH assay, BHT served as a positive control since it is known as a synthetic antioxidant which typically exhibits higher antioxidant activity in most antioxidant analyses as compared to plant extract. The result for BHT will demonstrate that the procedure used in the present study was appropriate. In addition, commercial gum Arabic served as a sample for comparison. This is due to the fact that it is a plant extract that has been shown to have antioxidant activity, as well as some functional similarities (e.g. stabilizing/emulsifying effects on emulsion) with Javanese ginseng mucilage. As shown in Table 1, the DPPH radical-scavenging activities gradually increased as the sample concentration increased from 100 to 800 µg/ml. For each concentration tested, there were significant differences ($p < 0.05$) among the samples.

One promising finding was that Javanese ginseng mucilage had nearly twice the free radical scavenging activity as gum Arabic at all concentrations tested. The average 50% inhibitory concentration (IC_{50}) values of Javanese ginseng mucilage and gum Arabic were 536 and 1327 µg/ml, respectively, indicating that the mucilage has a higher antioxidant activity.

The IC_{50} of Javanese ginseng mucilage was lower than that of Hsian-tsao leaf mucilage (Lai *et al*. (2001) and partially purified mucilage from Japanese yam (860 µg/ml) (Hou *et al*., 2002). Moreover, the value was within the IC_{50} range (380 – 650 µg/ml) of Balangu (*Lallemantia royleana*) seed gums as reported by Sardarodiyan *et al*. (2019). Meanwhile, Lin *et al*. (2001) reported a comparable value of the IC_{50} (547 µg/ml) for *Dioscorea alata* L. cv. Tainong 2 (Taiwanese yam) mucilage.

Table 1. Scavenging activity of Javanese ginseng mucilage as compared to butylated hydroxytoluene (BHT) and gum Arabic

	Scavenging activity (%)[1]		
Concentration (µg/ml)	**BHT**	**Gum Arabic**	**Javanese ginseng mucilage**
100	42.24 ± 0.07[a]	9.35 ± 3.52[c]	18.06 ± 5.26[b]
200	61.87 ± 0.18[a]	12.98 ± 1.42[c]	25.88 ± 2.51[b]
400	84.16 ± 0.01[a]	20.24 ± 4.05[c]	41.60 ± 0.74[b]
800	98.12 ± 0.01[a]	32.32 ± 2.98[c]	68.96 ± 0.34[b]
Regression model ($R^2 > 0.98$)	Y = 0.1287x + 19.903	Y = 0.0769x + 8.8143	Y = 0.0329x + 6.346
IC_{50} (µg/ml)[2]	146	1327	536

[1] Means with standard deviation from three independent replications. Means with different superscripts within the same row are significantly different ($p < 0.05$)

[2]The concentration of sample required to scavenge 50% of free radicals (determined by the respective regression models).

In contrast, The IC_{50} of Javanese ginseng mucilage was substantially lower than that of the mucilage from *M. parviflora* leaves, with an IC_{50} of 154.27 µg/ml (Munir *et al*., 2021). According to some previous studies (Ragavee *et al*., 2018; Gemede *et al*., 2018; Souza *et al*., 2020b), the

antioxidant activity of the mucilage or gum could be well related with their phenolic acid and flavonoid compounds. As for Javanese ginseng mucilage, chlorogenic acids and O-glycosylated flavones (Tolouei *et al*., 2019) are examples of the said compounds possibly responsible for its antioxidant activity.

3.2. Fitting the experimental data of emulsions to the quadratic model

The experimental data obtained for the prepared emulsions in terms of viscosity, droplet size and TOTOX values (responses) are presented in Table 2 and the data were fitted to a quadratic model using response surface modelling. Table 3 depicts the regression model for each response addressing the significance of the coefficient of estimation for each linear, quadratic and interaction terms. All the fitted models were found to be significant ($p < 0.05$) and thus pertinent to indicate the relationship between the factors and the responses. The high values (> 0.8) of R^2 indicate that both mucilage and orange oil as factors in the model could explain well the variation observed in the responses. There were insignificant lack-of-fits ($p > 0.05$), suggesting that the models fitted well to the data and are able to accurately predict future responses. This study also found that removal of non-significant terms from the initial models fitted to the viscosity and TOTOX value did not significantly increase the R^2 and thus the initial models were unreduced.

In contrast, the removal of non-significant interaction term from the initial model fitted to the droplet size data seemed to be essential to improve the goodness of fit.

Table 2. A Central Composite Design with the experimental data of beverage emulsions

Run	Sample code	Factors (Actual value)		Responses		
		Mucilage (%)	Orange oil (%)	Viscosity (mPa.s)	Droplet size (µm)	Total oxidation (TOTOX)
1	7.5%M-8%O	7.5	8	11040	8.77	5.67
2	7.5%M-10%O	7.5	10	12560	9.68	9.37
3	7.5%M-8%O	7.5	8	9760	7.39	7.12
4	10%M-8%O	10	8	17440	7.22	3.86
5	5%M-8%O	5	8	4000	3.14	5.84
6	7.5%M-8%O	7.5	8	6960	12.39	4.50
7	7.5%M-6%O	7.5	6	8960	7.60	4.46
8	5%M-6%O	5	6	3920	6.20	6.73
9	7.5%M-8%O	7.5	8	7600	12.72	6.70
10	7.5%M-8%O	7.5	8	9520	8.80	4.81
11	10%M-10%O	10	10	20050	16.49	7.07
12	10%M-6%O	10	6	15120	10.36	2.94
13	7.5%M-8%O	7.5	8	9920	10.13	4.22
14	5%M-10%O	5	10	6800	8.13	8.39

Note: All beverage emulsions had the same composition of carboxymethyl cellulose (CMC) (0.5%), sodium benzoate (0.08%), and citric acid (2%) except that distilled water concentration was varied between 79.42 – 86.42%, following the composition of mucilage (5 – 10%) and orange oil (6 – 10%).

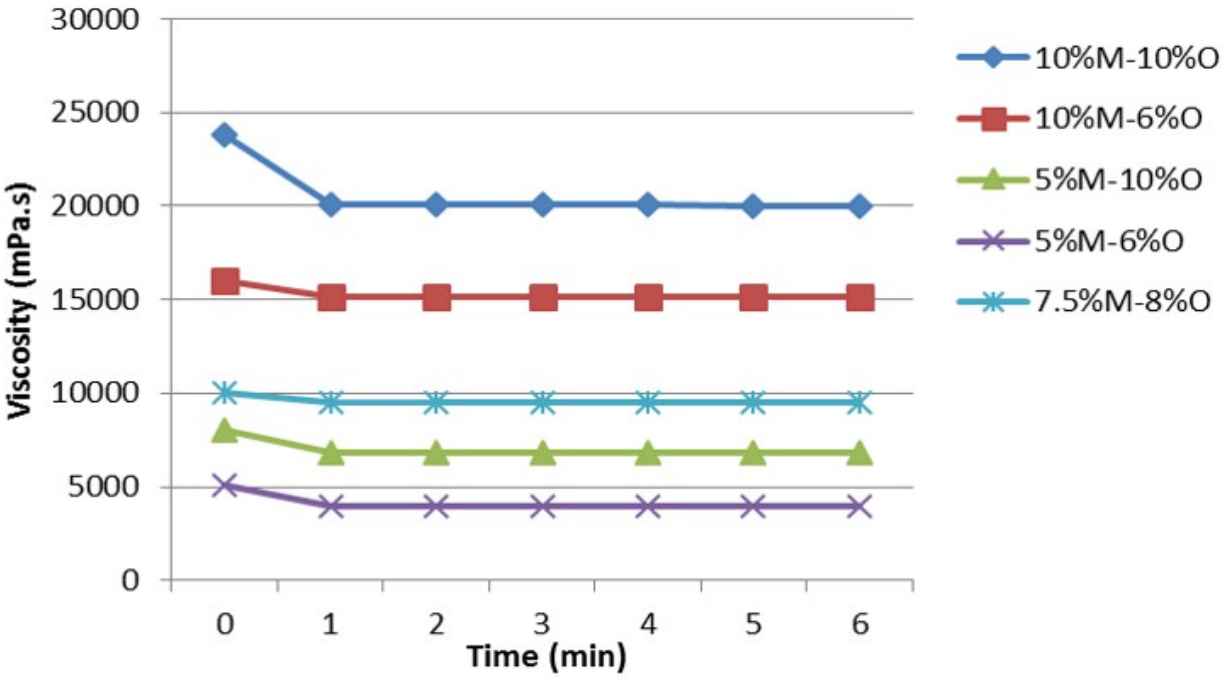


Figure 1. Viscosity profile of representative beverage emulsions varying in Javanese ginseng mucilage (M) and orange oil (O) concentrations. See Table 2 for the description of each sample code.

3.3. Effect of mucilage and orange oil concentrations on emulsion viscosity

The viscosity values of the emulsions were in the range of 3920–20050 mPa.s which were greatly influenced by the mucilage and oil concentrations (Table 2). The viscosity of all emulsions notably decreased within the first 2 min of measurement as shown by the representative emulsions in Figure 1, revealing a thixotropic behavior. This behavior is due to the fact that aggregated droplets (held by weak forces) were gradually disrupted and collapsed because of the shearing force, causing reduction of the viscosity over time (McClements, 2005).

According to the results, the combination of mucilage and orange oil at their highest concentrations (10%) could produce an emulsion with higher viscosity than other combinations. This is because a higher mucilage concentration contributes to a higher emulsion continuous phase viscosity. Similarly, increasing the oil concentration in the dispersed phase will result in more droplet-droplet interactions, making the emulsion more resistant to flow, as indicated by an increase in viscosity. According to Zeng and Lai (2014), increasing the volume ratio of oil has increased the emulsion viscosity and slowed the potential of phase separation in
the emulsions containing *A. australasicum* mucilage. However, in the present case, it was discovered that the emulsions with the lowest amounts of mucilage (5%) and oil (6%) displayed the lowest viscosities, which may encourage the phase separation during storage.

Changes in mucilage and oil concentrations caused abrupt changes in emulsion viscosity, implying that their respective effects may not be simply linear. According to the fitted regression model (Table 3), the quadratic (square) and interaction terms of mucilage and orange oil had significant ($p < 0.05$) increasing effects on viscosity (i.e. positive sign of the estimated regression coefficients). This is consistent with previous research, which found that increasing the concentration of beet pectin increased the viscosity of secondary lactoferrin-coated orange oil emulsions (Zhao *et al*., 2015). The quadratic effect of oil (X_2), on the other hand, appeared to be stronger than that of mucilage (X_1) as indicated by its higher coefficient (221). Moreover, it is worth noticing an interesting

finding on a synergistic interaction effect of mucilage and oil on the emulsion viscosity.

With a positive coefficient of estimation (104 of X_1X_2), the result revealed that when the mucilage and oil concentrations increased, there would be a sudden increase in the viscosity in a non-linear manner. The contour plot (Figure 2a) gives a better picture of the interaction effect on the emulsion viscosity when both mucilage and orange oil concentration increased. The interaction could be attributed to movement restriction of oil dispersed droplets due to increased viscosity caused by the increase in the mucilage concentration. At the same time, an increase in oil concentration would increase the packing fraction of the droplets which in turn would also restrict the droplet movement.

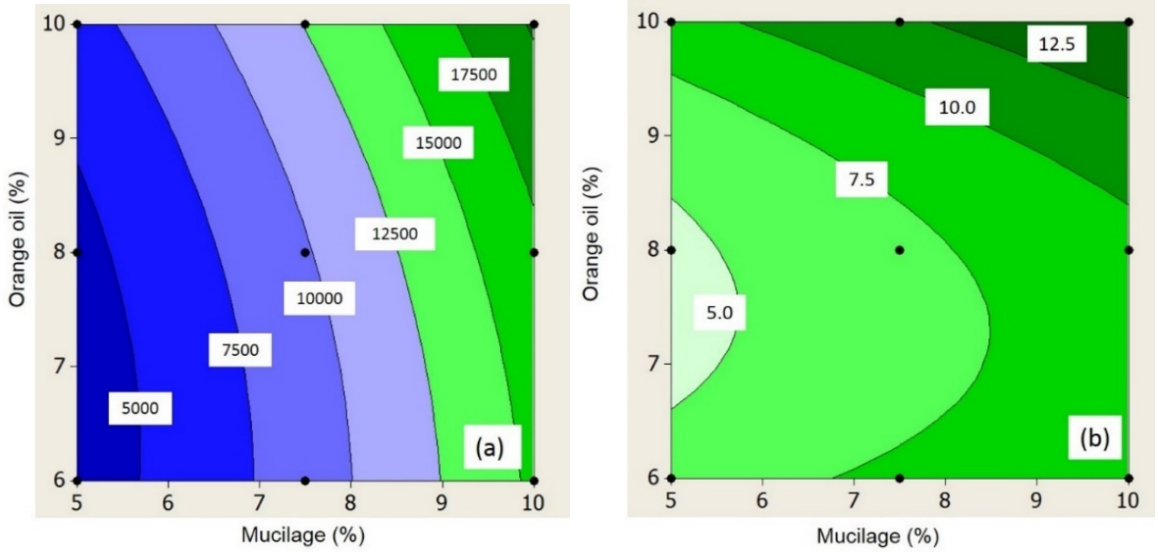
3.3. Effect of mucilage and orange oil concentrations on emulsion droplet size

Droplet size was measured using the average size of predominant droplets ranging from 3.14 to 16.49 µm (Table 2) among the emulsions due to the large difference in mucilage-oil concentration. Furthermore, the presence of droplets of various sizes in the representative micrographs (Figure 3) demonstrated the polydispersity characteristic of each emulsion.The emulsions demonstrated a typical droplet packing of emulsions with a low amount of oil (6 – 10%), with the flocculated droplets being scarcely visible, especially for the emulsion with the lowest amounts of mucilage-oil (Figure 3a), which had a narrower size range of 0.91 – 13.77 µm in comparison to others.

Table 3. Summary of ANOVA results for the fitted regression models

Responses	Fitted regression models	R^2	Lack-of-fit
Viscosity (mPa.s)	$11240 - 335X_1 - 3371X_2 + \mathbf{135X_1X_1^*} + \mathbf{221X_2X_2^*} + \mathbf{104X_1X_2^*}$	0.998	0.184
Droplet size (µm)	$48.5 + 0.1X_1 - \mathbf{12.4X_2^*} + \mathbf{0.8X_2X_2^*} - 0.1X_1X_2$	0.810	0.090
TOTOX value	$34.5 - 0.8X_1 - \mathbf{7.1X_2^*} - 0.1X_1X_1 + \mathbf{0.4X_2X_2^*} + 0.1X_1X_2$	0.928	0.400

X_1, Javanese ginseng mucilage; X_2, orange oil; TOTOX, total oxidation. *Significant at $p > 0.05$.



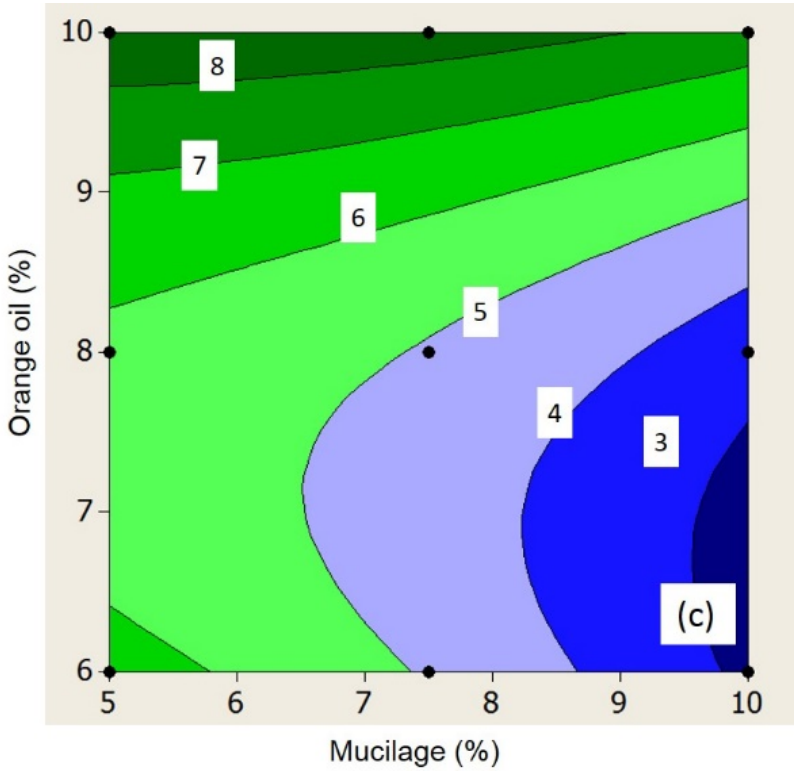
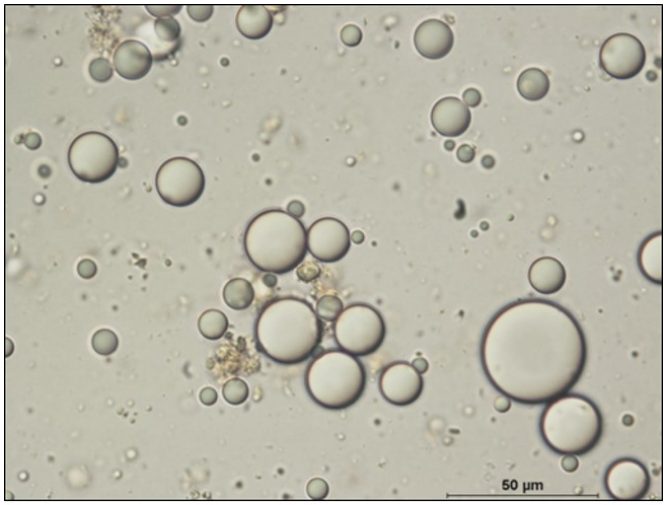
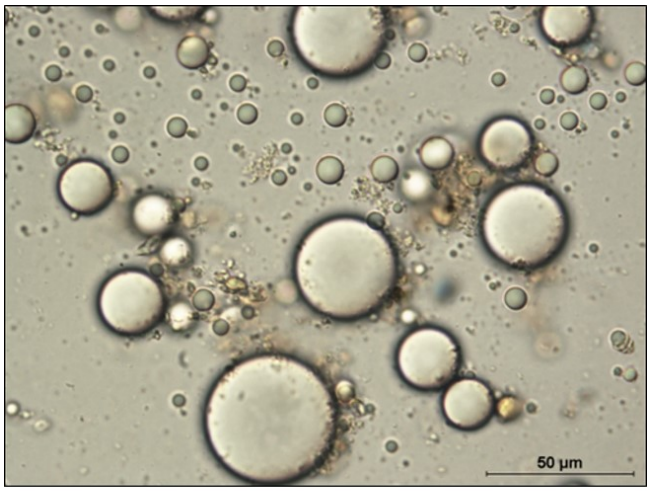


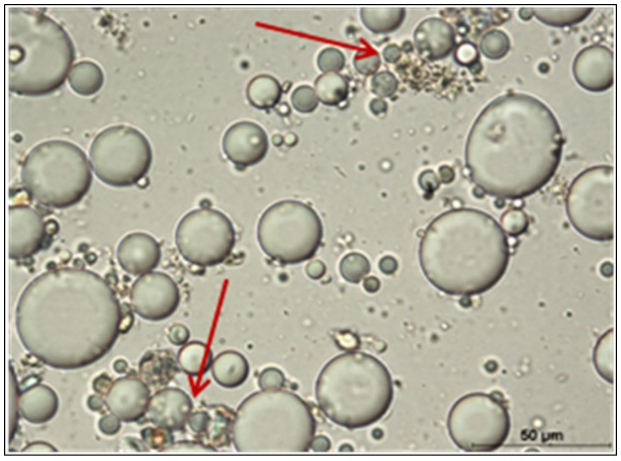
Figure 2. Contour plots showing the interaction effect of mucilage and oil on (a) Viscosity, mPa.s (measured at 0 min); (b) Droplet size, μm and (c) Total oxidation value of beverage emulsions.



(a) 5%M-6%O (0.91-13.77 μm)



(b) 10%M-10%O (0.87 – 28.87µm)



(c) 5%M-10%O (0.59-16.41 µm)

Figure 3. Droplet microstructure (100x magnification) of representative beverage emulsions varying in Javanese ginseng mucilage (M) and orange oil (O) concentrations. See Table 2 for the description of each sample code.

Nevertheless, a previous study found that the emulsion droplets became bigger, non-spherical and non-uniform as relative percentage of *A. australasicum* mucilage in the emulsion decreased (Zeng and Lai, 2014). In the present case, the use of maximum levels of mucilage-oil seemed to produce an emulsion with a wider size range (0.87 – 28.87 µm) or higher degree of polydispersity (Figure 3b). It is meaningful to observe that the minimum level of mucilage (5%) appeared to be fairly enough to cover the droplet surroundings at the maximum level of oil (10%) as the emulsion exhibited an intermediate droplet size range of 0.59 – 16.41 µm (Figure 3c). However, as seen in the micrograph, the emulsion flocculated due to an increase in the number of small droplets (as shown by arrows). As referred to the fitted model in Table 3, the linear increment of orange oil was predicted to significantly ($p < 0.05$) reduce the droplet size as indicated by the negative coefficient of estimation (–12.4 for X_2) whilst its quadratic increment would give the opposite effect (0.8 for X_2^2). In this study, the emulsification of oil into fine droplets was primarily caused by the presence of Javanese ginseng mucilage, which has a significant surface property (Nor Hayati *et al*., 2019), as no emulsifier was used in the formulation. The finding implies that the emulsification was only efficient when the

increase in orange oil was linear. However, if the increase was quadratic, it was no longer efficient because the droplets formed were quite large. For this reason, it is important to sustain the interaction term (X_1X_2) in the model despite its insignificant effect, to give insight into the mucilage-oil interaction. As illustrated by the contour plot in Figure 2b, increases in the mucilage concentration approximately from 7 to 10% did not seem to be efficient to produce predominant droplets with small sizes (< 7.5 μm) when the oil concentration increased > 8.5%. Remarkably, when the oil concentration was < 8.5%, the predominant droplets formed were not more than 10 μm in size. One possible explanation for this observation is the increased viscosity of the continuous phase resulting from the increase in the mucilage concentration could have restricted the energy input during homogenization and thus disturbed the mucilage's emulsifying activity. With > 8.5% oil concentration, this drawback seemed to be more prominent as the mucilage-oil interaction had substantially increased the emulsion viscosity while also requiring more oil to be emulsified.

3.5. Effect of mucilage and orange oil concentrations on emulsion oxidative stability

The oxidative stability of the emulsions was evaluated based on the TOTOX value, a comprehensive oxidation index considering hydroperoxides and aldehydes that formed during the primary and secondary stages of lipid oxidation, respectively. Based on Table 1, the TOTOX values were in the range of 2.94 – 9.37 after the emulsions have been stored at 5°C for 30 days. The values < 10 reveal that the prepared emulsions were considerably stable towards lipid oxidation (Rossell & Hamilton, 1986). The result reasonably showed that higher mucilage concentrations at 7.5 and 10% were more efficient to inhibit lipid oxidation in the emulsion system (lower TOTOX values) as opposed to 5%. This is due to the antioxidant activity of the Javanese ginseng mucilage, which is as supported by the DPPH result discussed earlier. Phenolic compounds such as chlorogenic acids may function as hydrogen donators, reducing the consumption of oxygen in emulsion system. Consequently, the amount of hydroperoxides that decomposed into the secondary oxidation products, could be successfully reduced. The lowest TOTOX value (2.94) can be achieved at the minimum orange oil concentration (6%) and maximum Javanese ginseng mucilage concentration (10%). The result is in agreement with a walnut oil-beverage emulsion (28 days of storage) containing 3% walnut oil, 10% gum Arabic and 0.12% xanthan gum giving the lowest TOTOX value (2.347) (Gharibzahedi *et al*., 2012).

The TOTOX value for the emulsions was also successfully fitted with a quadratic model showing that both linear and quadratic terms of orange oil significantly ($p < 0.05$) affected the TOTOX value (Table 3) but in a dissimilar way. The negative coefficient (–7.1) of X_2 suggests that there will be a decrease in the TOTOX value with a linear increase in the orange oil concentration. This is thought to be due to sufficient mucilage coverage surrounding the droplet surfaces which could prevent diffusion of pro-oxidants from the oil-water interface into the oil droplets. Conversely, the value is predicted to be increased when the oil concentration increased in a quadratic manner (0.4 of X_2^2) possibly due to self-interaction of the oil droplets which could favor the oxidation process. Furthermore, it is worth mentioning that increasing the mucilage concentration could increase the oxidative stability of the emulsion even though its' linear and quadratic effects were not significant. This is expressed by the negative sign of the related coefficients (–0.8 of X_1 and –0.1 of X_1^2). This clearly reflects the ability of Javanese ginseng mucilage to inhibit the lipid oxidation in the emulsion system via its high antioxidant activity, as well as formation of the thick layer around the droplet surface. However, the mucilage-oil interaction somehow leveraged this effect as indicated by the positive coefficient (0.1) of X_1X_2. As referred to the contour plot of the TOTOX value (Figure 2c), the decreasing effect is seen towards a higher

concentration of the mucilage (7 – 10%) when the oil concentration was limited to < 8.5%, revealing the effectiveness of the mucilage antioxidant activity. Undesirably, the TOTOX value was substantially increased when the oil concentration increased over the limit, even with > 7% of mucilage present in the system. It is strongly believed that, the mucilage-oil interaction at this stage had led to undesirable increase in the emulsion viscosity (Figure 2a), giving rise to inefficiency of free radical scavenging activity due to restricted mobility of the antioxidant compounds (Nor Hayati *et al*., 2020). In addition, the increase in the droplet size (Figure 2b) as discussed before, is a sign of insufficient mucilage coverage to effectively protect the droplets from the free radical chain reaction.

3.6. Concentration limits of Javanese ginseng mucilage and orange oil in beverage emulsion

Based on the elucidated effects (linear, quadratic and interaction) of Javanese ginseng mucilage and orange oil on the viscosity, droplet size and TOTOX values of the emulsions, it is possible to estimate the concentration limit of both ingredients in the formulation. With the guide of the pattern of their respective contour plots, the desirable range for viscosity, droplet size and TOTOX values were set as 10000 – 20000 mPa.s, 5 – 10 µm and 3 – 5, respectively. The contour plots were then superimposed to give the feasible region of desirable viscosity, droplet size and TOTOX values as depicted in Figure 4. In a formulation with 6 – 8.5% orange oil, it is recommended to employ a concentration of Javanese ginseng mucilage of 8 – 9.5%. Out of these limits, the emulsions were expected to have undesirable high viscosity and large droplet size which will further reduce their oxidative stability.

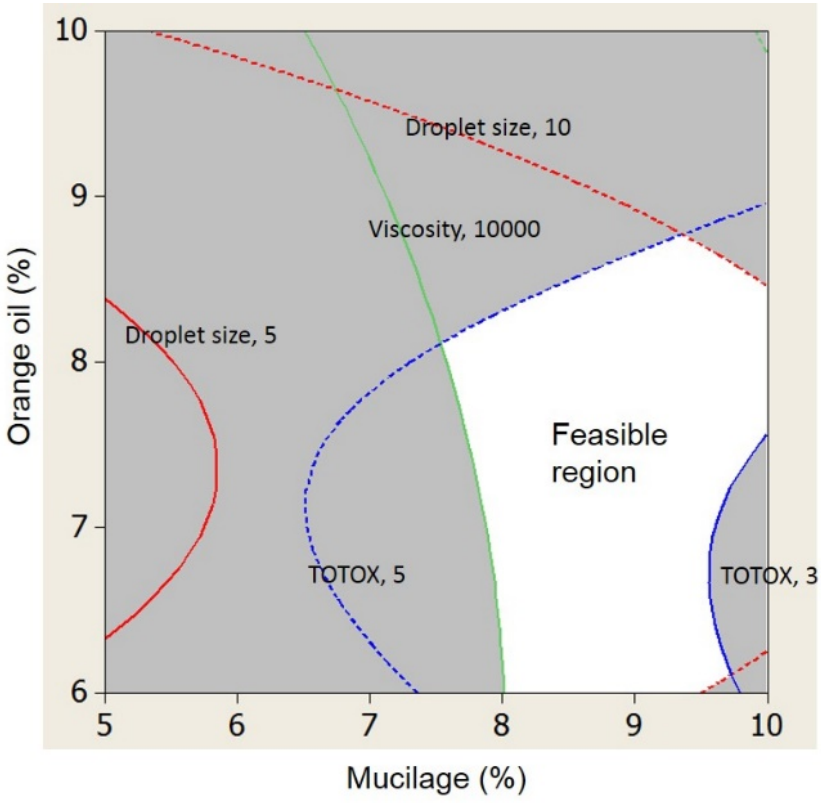


Figure 4. Superimposed contour plots of viscosity, droplet size and total oxidation value (responses) showing the feasible region adhered to concentration limit of the Javanese ginseng mucilage and orange oil.

4. Conclusions

The present findings led to a conclusion that, with certain considerations, it is highly possible to prepare a stable beverage emulsion by using Javanese ginseng mucilage as thickener and emulsifier as well as a natural antioxidant source. It was elaborated that the effects of Javanese ginseng mucilage and orange oil on the viscosity, droplet size and oxidative stability of the prepared emulsions were not simply linear. Increasing the oil concentration in a quadratic manner was predicted to result in too much increase in the emulsion viscosity. This undesirably would favor the formation of large droplet size and also intrude the antioxidant activity of the mucilage to inhibit lipid oxidation in the system. This study also revealed that understanding of interaction between the mucilage and oil is vital in order to formulate the emulsion with desirable properties and stability. Further work is certainly required to better understand a more complex interaction among the mucilage with other ingredients (e.g. protein, salt, sucrose, etc.) with the aim to extend utilization of the mucilage in other various emulsion-based food systems.

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COMPARATIVE ANALYSIS OF THE QUALITY INDICATORS OF ORANGE JUICE OBTAINED BY DIFFERENT EXTRACTION METHODS

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ABSTRACT

The samples used for the extraction of raw juice from oranges were prepared as follows: the first sample was pulp peeled and subjected to centrifugation for juice extraction, the second sample was made from oranges cut in half and squeezed by twisting, the third sample was made from whole oranges, chopped and juiced by centrifugation, and a sample was taken from commercially available orange juice. The raw juice was compared to the commercial juice that is processed and pasteurized. The impact of the type of extraction on the production yield and on the specific consumption was examined. The quality parameters such as soluble dry matter, relative density, pH, total acidity, SSC/TTA ratio, refractometric index, viscosity, color, turbidity were assessed and compared. The non-sensory and sensory analyses were used to compare the quality of the juices and the consumer preference. The values of the determined quality parameters were real and dependent on the juice extraction method. The values which were the closest to the reference were the ones resulted for the OJCW sample (taken from the whole fruit and de-juiced by centrifugation), and these values are 10.75ºBx, pH=3.8, TTA = 0.41g/100g expressed in citric acid, the refractive index is 1.3484.

The sensory analysis proved that the most favorable preferences were obtained for sample 278 (OJSQ - orange cut in half and squeezed by twisting). For the development of the technologies at an industrial level, the Squeeze Juicer for the, *Valencia* orange variety is recommended.

1. Introduction

The orange juice is one of the most consumed juices in the world because it is rich in nutrients and energy, in addition to its refreshing characteristic and antioxidant abilities. The health benefits of this juice are remarkable (Pontifex *et al*., 2021). The premium orange juice is a food product with a rising consumption trend in Europe and North America and is a lucrative business (Business-Wire, 2020). The history of juice extraction dates back to the 19th century. The methods of extracting juice from fruit has progressed enormously from the old boring method of squeezing to an automatic juicer used worldwide, making it an essential tool for citrus farmers. The juicing machines are generally classified into four types: centrifugal juicers, masticating juicers, triturating juicers and presses, which can be manually or electrically operated (Ugwu *et al*., 2020). Conventionally, various types of pressing machines have been used to extract juice from fruits and vegetables, and the juices have been used fresh or minimally processed (Mushtaq, 2018). The juice can be obtained using extraction machines that have progressed in recent years (Ugwu *et al.*, 2020). Further processing of the juice can

also influence its quality characteristics (Wang *et al.*, 2022; Gomes *et al*., 2022).

The classification of juice extractors can be done as follows: centrifugal juicer (net 2); masticating juicer (Cold Press Juicers) (net 2); triturating Juicer (net 2); press Juicer (citrus juicer) (net 3), manual citrus juice extractor like hand presser (net 1), hand Squeeze Juicer, Hand-held Juicer, dome shape juicer (net 4), cup hand- held citrus Juicer, an orange juice Extractor (Aye *et al.*, 2012) and electric power operated orage Juice Extractors ca masticating machiness, Juice pulping machine (Emelike *et al*., 2015), Juice extractor machine (Adewumi, 2005; Odewole *et al*., 2018), mini orange juice extractor (Olaniyan, 2010); monitorized Fruit Juice Extractor (Bamidele, 2011), a multi fruit juice extractor (Odewole *et al*., 2018), mechanized fruit juice extracting (Gbasouzor *et al*., 2014), fruit juice extractor machine (Boih, 2015), Modified fruit juice machine (Nwoke, 2017), motorized juice extractor machine (Omoregie *et al*., 2018), centrifuge beach juicer machine (net 5) and triturating and citrus orange extractor machine like automatic orange extractor (net 5). The high content of peel oil and the pasteurization process decrease the loss of pulp in the orange juice. The extraction and the degree of processing, the pasteurization and the oil content are major factors influencing the flavor of the orange juice (Baldwin *et al*., 2012). There are approximately 200 volatile components that form the aroma and taste of the oranges (Maarse, 1991; Baldwin,1993; Johnson *et al.*,1996) and they consist of esters, terpenes, aldehydes, hydrocarbons, ketones, alcohols (Plotto *et al.*, 2008; Plotto *et al*., 2004). When processing the raw juice, a large part of these substances are lost (Perez-Cacho *et al.*, 2008; Jordan *et al*., 2001). Many substances are found in the juice but also in the peel oil. The predominant ones are limonene, linalool, myrcene, α-pinene, and sabinene (Coleman *et al*., 1971; Pin *et al*., 1992; Verzera *et al*., 2004). In order to reach the consumer, the raw juice is subjected to processing, the floating and sinking pulp content is corrected, the flavoring components in the form of peel oil are added, then the juice is pasteurized or it can be subjected to different preservation methods.

The aim of this study was to compare the quality indicators of the orange juice of the same variety and lot, which was obtained from peeled pulp and subjected to juice extraction by centrifugation, oranges cut in half and squeezed by twisting, whole oranges, crushed and de-juiced by centrifugation and a sample of commercially available orange juice. The values of the quality indicators will be compared with those of the commercial sample. The influence was also studied on some technological parameters such as yield, losses and specific consumption.

2. Materials and methods

2.1. Materials

2.1.1. Samples collection

Commercial oranges of the "*Valencia*" variety were purchased. For the reference sample, OLIMPUS orange juice, 100% natural, cloudy juice, pasteurized, which was purchased from the refrigerated sector, was analyzed.

2.1.2. Orange juice preparation

The quantity of oranges was divided into three. For the preparation of juices, the juicer BOSCH MES 25 A0, centrifugal, juicer was used. Samples for analysis are: OJSQ – orange juice hand squeezer; OJM – Orange juice from market; OJCW-Orange juice from the whole fruit; OJCP-orange juice from pulp used centrifugal juicer. The codes for sensory analysis were: 456– OJCP; 278 – OJSQ; 369-OJM; 765-OJCW.

2.2 Methods

2.2.1. Technological studies

For calculating production and extraction yield the formula was used:

$\eta = (W_{juice} \times 100)/W_{orange,}$ (%) and for calculating specific consumption, the formula was used $c_{orange} = W_{orange}/W_{juice}$ (kg/kg), in wich: η is yield; W_{juice} is the weight of the juice; W_{orange} is the weight of the orange; c_{orange} is specific consumption.

2.2.2. Physical and chemical analyses

To determine the values of the quality indicators for the work options mentioned for orange juice, the methods used were:titratable acidity (TTA)(expressed as citric acid g/100g) (EN 12147:1998); relative density (EN 1131-1993-pycnometer, DMA-35 and areometer); pH-value (NMKL 179:2005, pH-meter, Orion Type 2-STAR, England); soluble solid content (SSC)(ºBrix), refractive index at 20ºC (AOAC 932.12, refractometer *Krüss,* Germany, connected to a bath room ultrathermostated *Brookfield*, with the outer circulation, Germany); kinematic viscosity (cSt)(ISO 3105:1994, Ubbelhode viscometer with 3 tube); turbidity (NTU)(Method 180.1,TB 100 Portable Turbidity Meter, China); vitamin C (iodometric method, Helmenstine, 2019), SSC/TA.

2.2.3. Determination of color characteristics

Color characteristics were measured using Chroma Meter-CR-400/410 (HunterLab, Japan)(Hsu *et al.*, 2003) and were measured L*CIE lightness coordinate, a*CIE red (+)/green(-) color atribute, b*CIE yellow (+)/blue(-) color attribute. They were calculated: C*–croma using the equation (Granato and Mason, 2010)

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

h*- hue angle, using the equation (Barreiro *et al.*,1997; Lopez *et al.*, 1997)

$$h* = \tan^{-1}\left(\frac{b*}{a*}\right) \quad (2)$$

YI – Yellowness index, using the equation: (Rhim *et al.*, 1989).

$$YI = \frac{142.86b*}{L*} \quad (3)$$

The samples were studied in 3 replicates.

2.2.4. Sensory analyses

The method of preferential organoleptic analysis was used which adopted for the characteristics: color, smell, taste, aroma, freshness, general impression, stages of appreciation of the hedonic scale with 9 levels (extremely pleasant, very pleasant, pleasant, slightly pleasant, indifferent, slightly unpleasant, semi-unpleasant, completely unpleasant, extremely unpleasant), in a first working version. For appreciation the intensity of some taste descriptors, at first impression were analyzed: herbal taste, bitter, sour, astringent, natural orange, boiled. Intensity testing it was done using the preference method with a 5-level hedonic scale: extremely, with the characteristic of, regardless, very little, it is not. They were used a group of trained panelists, students of the Faculty of Agricultural Sciences, Food Industry and Environmental Protection," Lucian *Blaga*" University, girls and boys aged between 19 and 38 years.

2.2.5. Non sensory analyses

The nonsensory analysis was carried out by filling in an online questionnaire containing general questions related to the consumption of orange juice and 22 people answered.

2.2.6. Statistical analysis

The samples ware study in triplicates. The mean value, deviation from the mean, squared probable error, mean squared error, mean squared error of the selection mean, confidence interval were calculated, tabular "t" was used for a 0,05 significance levels and two degrees of freedom and then the actual value of the quality indicator was calculate.

3.Results and discussions

3.1. Non sensory analysis

In order to justify the appropriateness of this study, an assessment was carried out by means of non-advised consumers using online questionnaires. The results are shown in figure 1. Thus, the feedback received from 22 consumers can be summarized as follows: the yellow color of the juices can attract the consumer in proportion of 48%; if it is premium orange juice, 44% of the interviewed would choose it; it is consumed occasionally say 100% of the orange juice consumers. 68%

of the consumers consider that the juice that is freshly squeezed is preferred (52%) and consumed mainly for health purposes. Taking into account the results shown in figure 1, the yellow drinks and the orange juice seem to be the most preferred by consumers, by the general public. The orange juice is consumed occasionally, fresh, for health purposes and mandatorily of premium quality.

3.2. Technological studies

The samples to be studied were prepared by following the steps shown in the technological diagram in figure 2. The peculiarity of obtaining the raw juice can be observed. It consists in the method of preparing the fruit for extraction. The physical process used was the extraction by centrifugation and cold pressing. The values of the important technological indicators are shown in table 1. The production yield is slightly affected and the extraction yield has the highest value when using the variant obtained by centrifugation from the peeled pulp (OJCP). The specific fruit consumption is also 3.23% lower in the OJCP sample compared to OJSQ and OJCW samples. This is a technological advantage. The best production and extraction yield (64%) was obtained in the OJCP sample and a specific consumption of 2.39 kg/kg. In the literature, the production yield for the orange juice is between 23 and 72%. Losses in this juice sector are known to be high due to the special morphology of the oranges.

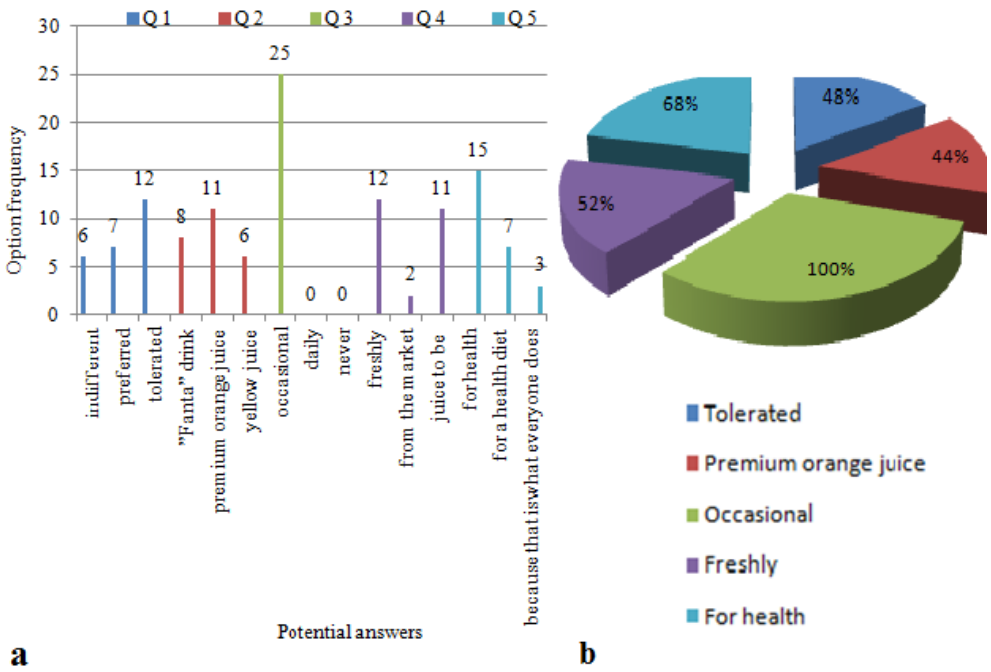

Figure 1. Results of the nonsensory analysis (a), about the consumption of orange juice and yellow juices in the Transylvanian region of Sibiu and the answers with the highest percentage (b).

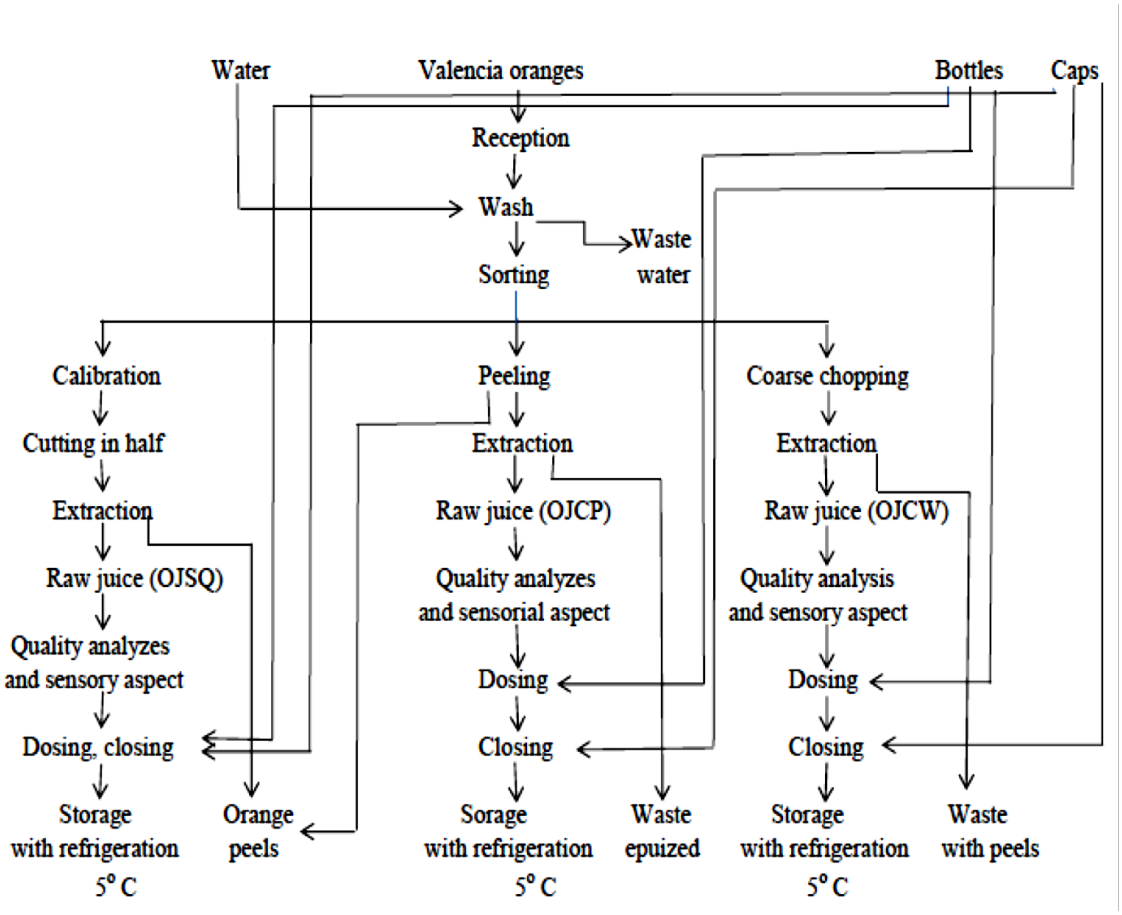

Figure 2. Overview of orange juice production using various modalities of processing before extraction and analysis of samples

Table 1. Basic technological indicators upon obtaining through various procedures, of orange juice. Result are means ±SD (n =3) for singnificant level P ≤ 0,05

Sample	Production yield, (%)	Extraction yield, (%)	Total losses, (%)	Specific consumption of oranges, (kg/ kg)
OJCW	41 ± 0,1	43 ± 0,09	63,49 ± 0,16	2,47 ± 0,14
OJSQ	40,45 ± 0,2	41 ± 0,18	59 ± 0,09	2,47 ± 0,89
OJCP	41,5 ± 0,01	64 ± 0,01	43,15 ± 0,215	2,39 ± 0,09

OJCW-orange juice centrifugal of whole fruit; OJSQ-orange juice hand squezer juicer; OJCP-orange juice centrifugal without peel

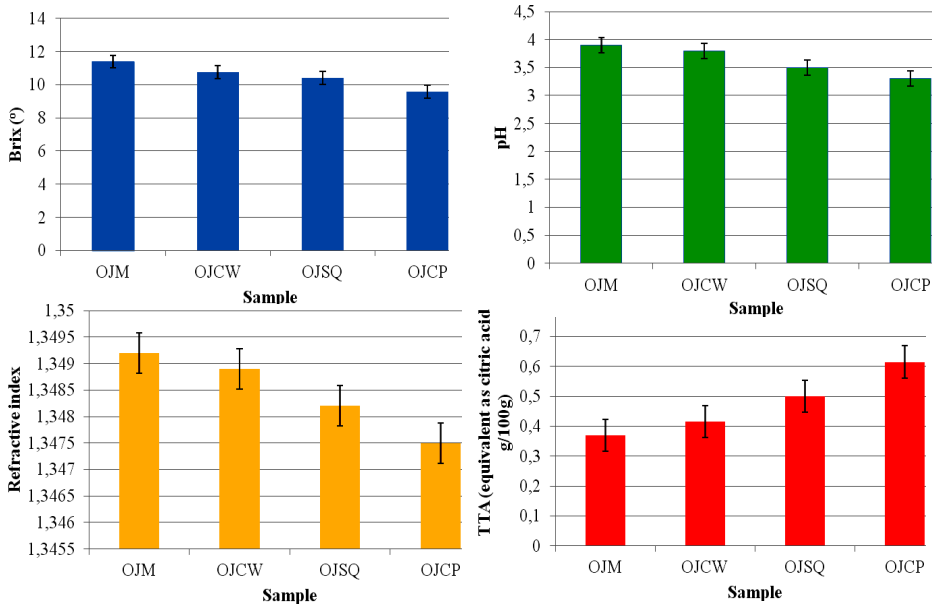

Figure 3. Variation of SSC, refractive index, pH and TTA in the studied orange juice samples: OJM – orange juice from market; OJCW- orange juice centrifugal of whole fruit; OJSQ- orange juice hand squezer juicer; OJCP- orange juice centrifugal without peel.
Result are means ±SD (n =3) for singnificant level P ≤ 0,05

3.3 Physicochemical indicators

3.3.1. Soluble solid content (SSC), pH, refractive index, total titratable acidity (TTA)

The soluble dry matter content in the orange juices indicates the sugar content and is the most important quality indicator. The variation of the values of the Brix, pH, refractive index, and titratable acidity are shown in figure 3. These quality indicators are the most important and are used in the classification of juices. They are also the ones that vary with any technological change. The SSC value ranges between 9.57 and 11.4° Bx, the pH decreases and the acidity increases compared to the commercial sample used as a reference sample. These values are comparable to those in the literature (IFU Analysis, 2005). There are also the determined or calculated values of the relative density that correspond to the determinations conducted in this study. In the literature, the orange juices subjected to different treatments show the value of SSC = 11.73-11.39 °Bx, pH=3.34-3.35 (Timmermans *et al.*, 2011, Wang *et al.*, 2022). The values of the SSC are also influenced by the presence of low molecular weight substances such as esters, aldehyde alcohols soluble in water (Perez-Cacho *et al.*, 2008). In the commercial juice (OJM), these flavor substances are dosed and the SSC is given by the sugars. In the raw juice, according to the graph, the SSC is decreasing, and this means that the quantity of sugars is decreasing. The acidity is increasing which means that the non-sugar is present in the juice. The sample of orange juice extracted from the whole crushed fruit in a centrifugal juice extractor (OJCW) was noted here.

3.3.2 Relative density, correlation indices

The relative density is an important quality indicator that is influenced by the temperature and the composition of the juice and that influences the values of the other quality indicators such as: the acidity, the viscosity, the color, and the pH. The standard (pycnometric and areometric) method is used for the determination, but also the method using DMA 35 is employed. Since the principles underlying these physical determinations are different, it is

necessary to make some correlations between the results. Some regression equations are obtained and the corresponding correlation indices are calculated. These variations are shown in figure 4. The correlation indices were calculated and R^2=1 was obtained, based on certain polynomial regression equations as follows:

$y_{OJSQ} = -0{,}0026x^2 + 0{,}0111x + 1{,}0322$

$y_{OJCW} = 0{,}0006x^2 – 0{,}0029x + 1{,}0433$

$y_{OJCP} = 0{,}0091x – 0{,}0414x + 1{,}0804$

$y_{OJM} = - 0{,}0003x^2 + 0{,}0006x + 1{,}0398$

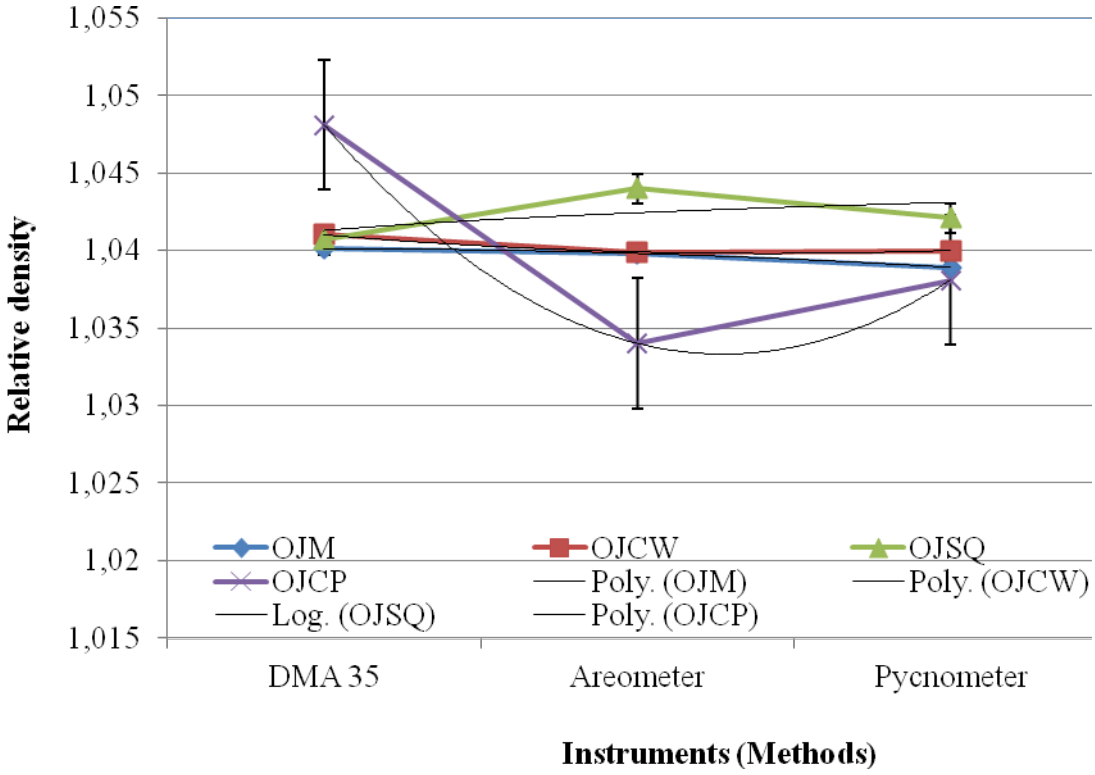

Figure 4. The variation of the relative density of orange juices and the correlation of the values that were obtained by measurement based on different physical principles. Result are means ±SD (n=3) for singnificant level $P \leq 0.05$.

Devices operating according to different principles were used on the same samples and for the same quality indicator, namely the relative density. The best correlation was obtained on the reference sample considered to be the commercially available juice. These values are consistent with the values stipulated in the literature that are proportional to the values for the SSC (IFU Analysis, 2005). The size of the particles in the juice influenced by the extraction mode led to different results. Therefore, either of these methods can be used to determine the relative density of the orange juice.

3.3.3. Viscosity, turbidity, vitamin C, SSC/TTA

The values of the determined quality indicators: viscosity, turbidity, vitamin C, SSC/TTA, are shown in table 2. The values of the kinematic viscosity are increased compared to the reference value. These values are directly proportional to the percentage of the floating pulp and turbidity.

The values closest to the reference were those from the OJCP sample, i.e. the juices obtained from the peeled pulp and extracted by centrifugation. The vitamin C content is low because the method used is not sensitive

enough. In the literature, the 2.6 dichlorophenolindophenol or HPLC method was used to determine the vitamin C content, which is 529 mg/l (Vermont *et al*., 2011). Thus, the orange juice has a vitamin C content of 40.91-33.33 mg/100g (Kujawińska *et al*., 2022). It seems that this content is influenced by the method of juice extraction and the storage time of the oranges after harvesting. The longer the juice was exposed to the oxygen in the air, the lower the vitamin C content. From this point of view, the OJCW sample (obtained from the whole fruit, with peel, by centrifugation) had the highest vitamin C content.

Table 2. Non-traditional quality characteristics of orange juice extracted by different methods. Result are means ±SD (n =3) for singnificant level P ≤ 0,05

Sample	Viscosity (cSt)	Floating pulp (%)	Turbidity (NTU)	Vitamin C mg/100g	ºBrix/TTA
OJCW	undetermined	87,00 ± 0,08	450 ± 3,7	53,71 ± 0,64	21,48
OJSQ	1,9628±0,054	18,24 ± 0,37	397,6 ± 0,74	43,02 ± 0,0025	20,8
OJCP	1,7459±0,0068	14,71 ± 0,03	382,7 ± 0,938	54,90 ± 0,469	15,36
OJM	1,573±0,0037	11,1 ± 0,009	384,5± 0,059	50,01 ± 0,292	30,8

OJM – orange juice from market; OJCW- orange juice centrifugal of whole fruit; OJSQ- orange juice hand squezer juicer; OJCP- orange juice centrifugal without peel. Result are means ±SD (n =3) for singnificant level P ≤ 0,05

3.3.4. Color characteristics

The values of the parameters characterizing the color can be found in table 3.

L* is the luminance and the closer it is to 100, the closer it is to the absolute luminance value. In the obtained juices for which it is considered that the reference is made to an assortment of juice existing on the market (OJM), the luminance value is different. The lowest value is observed on the sample OJCW, of 48.3 at a difference of 25% compared to the commercial sample (OJM). It is followed by the OJEP sample with 58.6. The OJSQ juice sample has the value of the L* closest to that of the commercial juice. This is because it is generally the most used method of extracting juice from oranges. An increased luminance value is a quality of the juice. It is influenced by the mechanical way of extracting the juice.

Table 3. CIE color parameters L*, a*, b* measured and C*, h*, YI* calculated
Result are means ± SD (n=3) for singnificant level P ≤ 0,05

Sample	L^*	a^*	b^*	C^*	h^*	YI
OJCW	48,3 ± 0,74	22,2 ± 0,84	28,23 ± 0,42	53,02	87,39	83,28
OJSQ	65,37±1,506	6,5 ± 0,24	31,33 ± 0,129	66,2	78,48	68,06
OJCP	58,6 ± 1,79	15,1±1,07	32,6 ± 1,2	60,39	65,11	79,36
OJM	64,75 ± 2,25	5,76 ± 0,27	37,5 ± 2,7	37,93	81,27	82,64

OJM – orange juice from market; OJCW- orange juice centrifugal of whole fruit; OJSQ- orange juice hand squezer juicer; OJCP- orange juice centrifugal without peel. Result are means ±SD (n =3) for singnificant level P ≤ 0,05

In the literature, the L* value ranges between 56.3 and 57.6 and is influenced by the treatments that are applied during the processing of the orange juice, such as: thermal treatments, high pressure, pulsed electric field processing (Vervoort *et al*, 2011). The addition of pulp, the pasteurization, the extraction and deacidification led to L* values between 53.95 (extraction) and 65.3 (deacidified juice) (Akyildiz *et al*., 2022; Wang *et al*., 2022). The non-frozen orange juice has L*= 61.43-73.41

and the thermally treated one 64.80-76.79 (Vervoort *et al*., 2011).

The values for the a* parameter are all positive, which means that it tends towards the reddish color. It is observed that compared to the OJM reference sample that was thermally treated, the value of a* increases. Thus, the highest value of 22.2 is obtained in the OJCW sample. In the literature, the values of a*=1.83-2.07 (for processed, untreated, treated at high pressure or pulse electric field processed orange juice) (Vervoort *et al.,* 2011). The method of extracting and processing can lead to a value of the a* parameter of 11-14.42. (Wang *et al*., 2022). The preservation treatment can increase the value of a* up to 13.94 and the thermal treatment can decrease it down to 3.41 (Vervoort *et al.,* 2011).

The values for the b* parameter are all positive, which means that it tends towards the yellow color. It is observed that compared to the OJM reference sample that was thermally treated, the value of b* decreases. Thus, the lowest value of 28.23 is obtained in the OJCW sample. Therefore, the extraction method can influence the color variation. In the literature, the value of the b* parameter is very high: 63.51 (Vervoort *et al*., 2011), 81.73 (Wang *et al*., 2022), 50.52 (Melendez-Martinez *et al.*, 2011), compared to the values obtained in this study.

The chroma (C*), considered the quantitative attribute of color fullness, is used to determine the degree of hue difference compared to a gray color of the same lightness. The higher the chromatic values, the higher the color intensity of the samples, perceived by humans. The values obtained in this study are increased compared to the pasteurized reference sample (OJM). This means that the consumer's eye perception of color intensity is high. The highest value was obtained for the unpasteurized OJSQ sample, namely 66.2. This is also confirmed by other studies. In the literature, values between 50.68 and 63.97 were obtained for the C* parameter (Melendez-Martinez *et al*., 2011).

The hue angle (h*), considered the qualitative attribute of color according to which colors were traditionally defined as: purplish-red, bluish-green, is used to define the difference of a certain color with reference to the gray color with the same lightness. This attribute is related to the differences in absorbance at different wavelengths, with a larger hue angle representing a smaller yellow property in the samples. An angle of 0° or 360° represents the red hue, while the angles of 90°, 180° and 270° represent hues of yellow, green and blue. It has been widely used in the assessment of the color parameters in green vegetables, fruits and meat (Barreiro *et al*., 1997; Lopez *et al*., 1997). In this study the values of h* are not high. They vary around 90^{o} which means that the yellow property of the studied orange juices increases. This is also confirmed by other studies in which values between 82.62 and 86.64 were obtained for the h* parameter (Melndez-Martıinez *et al*., 2011).

The L* luminance is high, positive and close to the maximum value (100), which means that the samples have an intense color which is perceived by the consumer, tending towards the yellow hue of the spectrum (yellow-orange). This aspect is influenced by the presence of the pigments in the fruit, and the method of juice extraction, being lower where there are large particles in the slurry, respectively in the OJCW sample, which is not to be desired.

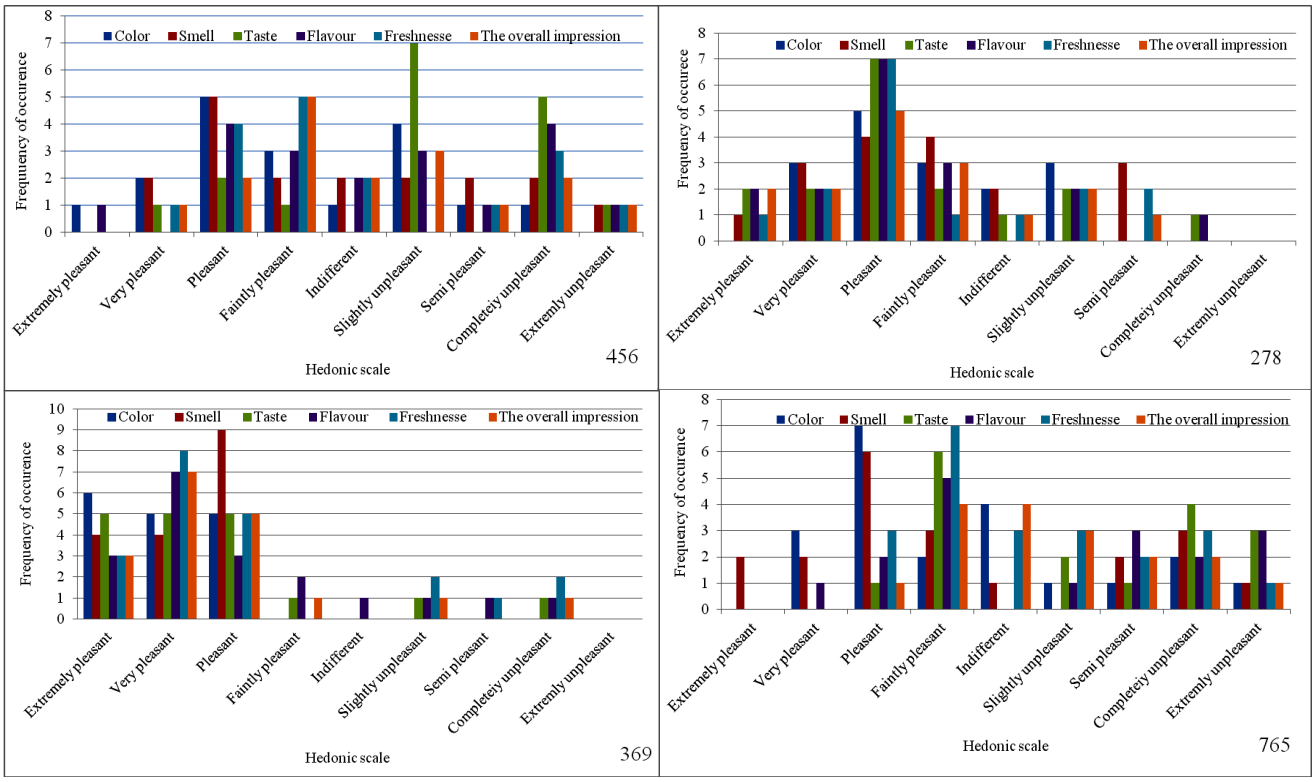

Figure 5. Sensory profiles, preferences (hedonic scale from 9 points), for all characteristics and general impression of the studied orange juices: 456 (OJCP); 278 (OJSQ); 369 (OJM); 765 (OJCW)

The values of the a* parameter, CIE red (+)/green(-), are positive, therefore tending towards red and increasing in the raw juices compared to the reference sample which is pasteurized.The values of the b* parameter, CIE yellow (+)/blue(-), are positive and therefore tending towards yellowness, and decreasing compared to the reference sample. The C* chroma, has large, positive values, higher than the reference sample, OJM, which means that the color intensity of the sample, as perceived by the human eye, is high. The hue angle h* has values around 90º, which means that the yellowish hue in the studied juices is increased. The orange-yellow pigments found in the juice vesicles of the pulp and the membranes adhering to the particles in the peel, the oil, cause the redness to diminish. These particles improve the light diffusion effect (Baldwin *et al.*, 2012).

3.3.5. Sensory Analysis

These aspects are regulated in the juice processing and preservation process and therefore it has higher values in this study. 22 male and female panelists aged between 19 and 38 were used. They were trained for 60 days, in 2-hour sessions, once a week. The types of juices similar to those studied here were tasted during training. They were also trained on the taste descriptors that were used in this study to be able to create the taste profile. The panelists have thorough knowledge of the biochemistry of juices, the technology of obtaining and preserving them and the quality control. They are generally consumers of this type of food. Using these graphic representations (figure 5) of the results, both the sensory characteristics and the intensity of the taste descriptors were highlighted. Thus, the method of extraction of the studied samples influenced the options of the panelists. For example, the overall impression decreased in the following order: 369 (very pleasant → pleasant), 278

(pleasant→mildly pleasant), 456 (mildly pleasant); 765 (mildy pleasant→indifferent). For sample 369, which is the commercially available juice, the most feedback was of very pleasant to pleasant. In the other studied samples, on average, the level of pleasant → mildly pleasant was chosen. The description of one of the most important sensory characteristics, namely the taste, the results of which are shown in figure 6, led to numerous conclusions. In sample 456, juice obtained by extraction only from the pulp, the descriptors of bitter, sour, astringent with no boiled or herbal flavor were noted. In sample 278, squeezed by twisting, the descriptor of natural taste of orange, bitter sour, no boiled or herbal taste was observed. Sample 369, which is a commercial sample, stood out for its natural orange taste as a basic characteristic, balanced, perfect, very close to what the consumer is used to.

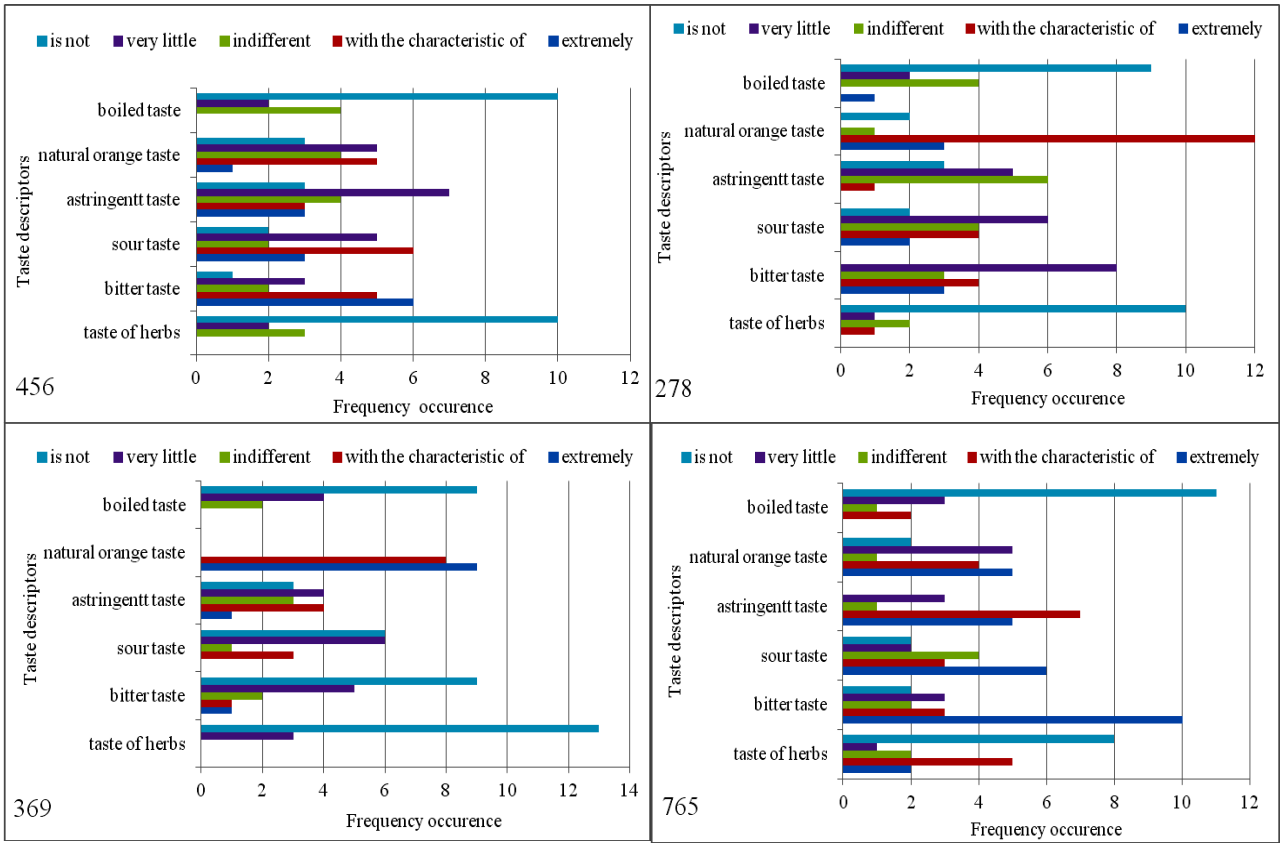

Figure 6. The taste profiles, preferential, with a hedonic scale with 5 levels of intensity of the specific descriptor, for the studied orange juices: 456(OJCP); 278(OJSQ); 369(OJM); 765(OJCW)

In sample 765, which is juice obtained from the whole split orange, the results are completely different from the others and from the commercial sample. The conclusion was reached that the juice was bitter, sour, astringent, but with a strong taste of oranges, without a boiled, herbal, unbalanced flavor. From a sensory point of view, the panelist clearly preferred the commercial sample (OJM). The sample closest to the reference sample was number 278 (OJSQ – which uses the squeezer juicer principle) and the descriptor of natural orange taste was noted in the taste profile. Since this work variant was distinguished from a sensory point of view, this is the variant that is also recommended in terms of technology and quality indicators. The soluble dry matter content of this working

variant (OJSQ) is within normal values, 10.4 ^{o}Bx, pH=3.5, SSC/TTA ratio = 20.8. This ratio value indicates a balanced taste that is very close to that of the OJCW sample that stood out in terms of quality indicators. The analysis of the taste profile has shown that the OJCW sample is not preferred by the consumers. The OJCW coloration indicates a tendency towards yellowness, yellow orange, the weakest compared to all the studied samples. The vitamin C content is lower, and the turbidity is high just like its viscosity due to the presence of the particles in the slurry.

4. Conclusions

The comparative evaluation of the techniques of juice extraction from a single variety of oranges has proven that there are small differences between the values of the determined and calculated quality indicators and large differences in the sensory analysis. The technological studies revealed a better extraction yield in the OJCP sample (raw juice from peeled pulp and extracted by centrifugation) (64%) and with a specific orange consumption of 2.39 kg/kg. The composition of the raw juices is different from the OJM reference sample (the commercial sample). The OJCW sample (juice from the whole fruit with peel, split and extracted by centrifugation) is thus distinguished due to its SSC, pH, and relative density. The values of these indicators are the closest to those of the reference sample, but in the sensory analysis, this juice received an overall negative vote. The recommended methods of evaluating the density of the raw orange juice are the pycnometric method, the areometric method and the one using the "U-tube" principle (DMA-35). The color change is not visible to the naked eye, but using the instrumental method it was discovered that the luminance tends to intensify, a* tends towards red and b* is positive and oriented towards yellowness. The color intensity increases in the ordinary raw juices obtained using the squeeze juicer and the centrifugal method. The preferred sensory profiles revealed that the most appreciated sample, immediately after the reference sample, was sample 278 (OJSQ– extracted by twisting). The preferred taste profiles have highlighted that the most balanced sample was number 278 (OJSQ-orange juice obtained by twisting) in which the descriptor of natural orange taste was noted more than in the reference sample. The squeeze juicer method of orange juice extraction is recommended as the best option to combine the two important aspects in the juice industry, namely the advantageous extraction yield and the premium juice quality.

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BUILDING AND SOLVING THE MATHEMATICAL MODEL OF TRANSIENT HEAT TRANSFER DURING THE PEANUT ROASTING PROCESS TO DETERMINE THE ROASTING PARAMETERS

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ABSTRACT

In this study, a mathematical model to describe the transient heat transfer of peanut roasting process was established to determine the relationship among the center and surface temperatures of peanuts, and the roasting time. The results were used to simulate the temperature gradient of peanuts over roasting time as well as to establish the technological mode for the peanut roasting process. Besides, the obtained results could be used to calculate and design a peanut roasting system. The results of the mathematical model established the suitable roasting conditions for peanuts as follows: the roasting temperature was 100°C and the roasting time was 25 minutes. At these conditions, the peanut product after roasting had bright yellow color, 100% of seed coats were peeled off with the ratio of broken grains was as low as 1.2%, and the sensory value of products was very good.

1. Introduction

Peanut (*Arachis hypogaea L.*), along with legume and Pea (*Pisum sativum L.*), is part of the legume family. It provides abundant protein in the diet and has great health benefits. Peanut is used to make many delicious dishes such as roasted with salt, dry roasted, boiled, or even eaten raw. In addition, peanut can be used to make butter, candy, ... Therefore, supplementing peanut in the diet was recommended to support good health (Smyth, et al., 1998; Smith, et al., 2014). Peanut has many benefits such as:

Improve fertility: Peanut contains high content folic acids, which are essential for women's fertility. The literature shows that women consuming a daily intake of 400 micrograms of folic acids before and during early pregnancy could reduce the baby's risk of neural tube defects after birth by 70% (Arzandeh and Jinap., 2011; Smith, et al., 2014).

Figure 1. Peanuts grown in the Southeast of Vietnam with pods removed

Support blood circulation: A quarter cup of peanuts (about 30 grams) can provide 35% of the required amount of manganese for the body. Manganese is a mineral that plays an important role in fat and carbohydrate metabolisms,

calcium absorption and blood sugar regulation (Smith, et al., 2014).

Reduce the risk of cognitive impairment: according to Martins et al ., (2017), high niacin foods like peanuts reduce the risk of Alzheimer's disease by at least 70%. A quarter cup of peanuts a day can provide efficient daily amount of niacin.

Protect against cancer: A form of phytosterol called beta-sitosterol (SIT) is found abundantly in some vegetable oils, such as peanut oil. Phytosterols not only protect against cardiovascular disease by interfering with cholesterol absorption but also protect the body against cancer by inhibiting the growth of tumors (Arzandeh and Jinap., 2011).

Reduce the risk of weight gain: Peanut can also help the body lose weight. According to Martins et al,. (2017), those who ate peanut at least twice a week were less likely to gain weight than those who almost never ate peanuts.

It can be seen that peanuts are rich in nutrients that are good for human health. However, due to the high nutrient and moisture contents, fresh peanuts are susceptible to mold development if stored incorrectly (M. Gürses, 2006). Therefore, the peanuts are prone to be contaminated with aflatoxin, a mycotoxin produced naturally after being molded. Aflatoxins are known to be extremely toxic and highly carcinogenic (Dzung N.T, et al**.,** 2021). Therefore, moldy peanuts should absolutely not to be eaten.

Currently, the food industry has used peanuts as raw materials to make several products for trade and export. Most of current processes have the peanuts roasted to remove the contaminated aflatoxins and microorganisms, to change the color and taste, and to improve the texture as well as the sensory value of the products. Therefore, there have been many studies on the peanut roasting such as those of Smyth, et al (1998), Arzandeh and Jinap (2011), M. Gürses (2006), Smith, et al (2014), Martins, et al,. (2017). These studies were mainly conducted by experiments under specific conditions to determine the appropriate roasting conditions. Nevertheless, the generalization of the peanut roasting process to apply in all conditions of production has not been concerned. Therefore, in this study, the building and solving a mathematical model of heat transfer for the peanut roasting process were conducted to determine the optimal roasting conditions. The obtained results could be used to apply in actual production to produce high quality roasted peanuts for trade and export.

2. Building and solving a mathematical model describing the roasting process

2.1. Assumptions

- *System analysis approach*

- Qualified peanuts were placed in a roasting pan with $T_e = 100^0C$ = const (equal to evaporation). During the roasting process, the peanuts must be stirred continuously so that the seeds were evenly cooked and not burned.
- Stirring time was about 30 minutes.
- After roasting, peanuts must meet the following standards: the seed were uniformly cooked and not burned, not broken, had pale yellow and delicious taste.
- 100 random peanut seeds (Figure 1) were observed to model the heat transfer problem. The results showed that seeds with cylindrical shape and spherical shape (15%) were the majority (75% and 15%, respectively), and the rest were in different shapes (10%). Because of the highest percentage of the cylindrical seeds, it was possible that the peanut was approximate to the cylindrical shape to build the heat transfer mathematical model.
- The ratio of length to diameter (*l/d*) of 100 cylindrical particles were measured, and the results are shown in Table 1.

Table 1. The ratio of the length (path of birth) to the diameter of the cylindrical peanut

l \ *d*	5	6	7
10	20%	11%	5%
12	5%	45%	2%
15	2%	2%	4%

Results in Table 1 show that peanuts with size of 6 x 12 mm were the majority (45%). Therefore, to calculate the heat transfer for the

roasting process, a peanut seed model can be considered as a cylinder with radius $2R = d = 6$ mm = 6.10^{-3} m and length $l = 2h = 12$ mm = 12.10^{-3}m.

- *Modeling the roasting process of peanut by an object model*

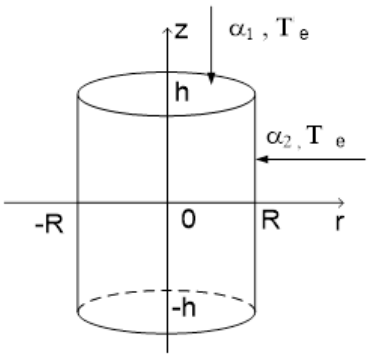

Figure 2. The object model of a peanut

- The object model was a solid cylinder with length $l = 2h =$ 12 mm, diameter $d = 2R =$ 6 mm, the coordinate axis was at the center of the object.

- *The thermophysical properties affecting the roasting of peanut*

- The peanut had the initial temperature evenly distributed and equal to the ambient temperature T_0= const ($\tau = 0$).

- At the beginning of the process ($\tau > 0$), the temperature of roasting medium was $T_e \neq T_0$.

- Heat emission coefficient of roasting environment on the two bottom surfaces was α_1, heat emission coefficient of the environment surrounding the cylinder was α_2. Due to the continuous roasting process, it can be inferred that $\alpha_1 = \alpha_2 = \alpha$ (Gebhart B., 1992).

- Peanuts had thermophysical properties as folows: specific heat capacity c_p (J/(kg.K)), density ρ (kg/m^3), thermal conductivity λ (W/(m.K)), thermal diffusivity a (m^2/s), the residual water content W (%), the critical residual water content W_c (%), and the oil content M (%). These parameters were determined in Table 2.

2.2. Building the mathematical model

Peanut had no internal heat source ($q_v = 0$). Based on the energy balance equation, the heat transfer model was established as follows (Luikov, 1975; Holman J., 1992):

$$\begin{cases} \dfrac{\partial T}{\partial \tau} = a\left(\dfrac{\partial^2 T}{\partial r^2} + \dfrac{1}{r}\dfrac{\partial T}{\partial r} + \dfrac{\partial^2 T}{\partial z^2}\right) \\ T = T(r, z, \tau);\ -R \le r \le R;\ -h \le z \le h \end{cases} \quad (1)$$

Initial-boundary value conditions:

- Initial conditions:

$$T(r, z, 0) = T_0 = \text{const} \quad (2)$$

- Peanut roasting temperature:

$$T_e = \text{const} \quad (3)$$

- Boundary conditions:

$$\frac{\partial T(R, z, \tau)}{\partial r} = -\frac{\alpha}{\lambda}\left[T(R, z, \tau) - T_e\right] \quad (4)$$

$$\frac{\partial T(r, h, \tau)}{\partial z} = -\frac{\alpha}{\lambda}\left[T(r, h, \tau) - T_e\right] \quad (5)$$

$$\frac{\partial T(0, z, \tau)}{\partial r} = \frac{\partial T(r, 0, \tau)}{\partial z} = 0 \quad (6)$$

2.3. Solving the mathematical model

Separation of variables (the Fourier method) were used to solve the system of equations (1) to (6), the root of a dimensionless temperature θ was found as follows:

$$\theta = \frac{T(r, z, \tau) - T_e}{T_o - T_e} = \sum_{n=1}^{\infty}\sum_{m=1}^{\infty} A_n A_m P_{nm} \exp\left[-\left(\mu_n^2 Fo_R + \mu_m^2 Fo_h\right)\right] \quad (7)$$

where:

$$P_{nm} = J_o\left(\mu_n \frac{r}{R}\right)\cos\left(\mu_m \frac{z}{h}\right) \quad (8)$$

$$A_n = \frac{2.J_1(\mu_n)}{\mu_n\left[J_o^2(\mu_n) + J_1^2(\mu_n)\right]} \quad (9)$$

$$A_m = \frac{2.\sin(\mu_m)}{\mu_m + \sin(\mu_m).\cos(\mu_m)} \quad (10)$$

Bi: Biot number

$$Bi_R = \frac{\alpha R}{\lambda} \quad (11)$$

$$Bi_h = \frac{\alpha h}{\lambda} \quad (12)$$

μ_n - roots of specific equation (13)

$$\frac{J_0(\mu_n)}{J_1(\mu_n)} = \frac{\mu_n}{Bi_R} \quad (13)$$

μ_m - roots of specific equation (14)

$$\cot an(\mu_m) = \frac{\mu_m}{Bi_h}$$

or $\mu_m \tan\mu_m = Bi_h$ (14)

Fo: Fourier number

$$Fo_R = \frac{a\tau}{R^2} \quad (15)$$

$$Fo_h = \frac{a\tau}{h^2} \quad (16)$$

Thermal diffusivity of the peanut:

$$a = \frac{\lambda}{c_p \rho}, \text{ m}^2/\text{s} \quad (17)$$

$J_0(\mu_n)$, $J_1(\mu_n)$: Bessel functions type 1 of the zero and first order**.**

$$J_0(x) = 1 - (\frac{1}{2}x)^2 + \frac{(\frac{1}{2}x)^4}{1^2.2^2} - \frac{(\frac{1}{2}x)^6}{1^2.2^2.3^2} + \frac{(\frac{1}{2}x)^8}{1^2.2^2.3^2.4^2} - ... \quad (18)$$

$$J_1(x) = -J_0'(x) = \frac{1}{2}x - \frac{(\frac{1}{2}x)^3}{1^2.2} + \frac{(\frac{1}{2}x)^5}{1^2.2^2.3} - \frac{(\frac{1}{2}x)^7}{1^2.2^2.3^2.4} + ... \quad (19)$$

Some equations to determine the thermophysical properties of peanuts affecting the roasting process are as follows (D.R. Heldman and Daryl B. Lund, 1992; Figura and Teixeira, 2007):

- The average critical residual water content of the peanut was $W_c = (8 \div 9)\%$, hence it should be able to choose $W_c = 8\%$.
- Peanut roasting temperature was T = 100 ^{0}C = 373.15 K
- Density of peanut:

$\rho = 1176 + 500 \times W_c$, kg.m^{-3} (20)

- Specific heat capacity of peanut was approximate according to the following equation:

$c_p = 1753.18 + 13.6 \times M$
$+ (0.95 + 0.019 \times M)(T - 291)$, J.kg^{-1}.K^{-1} (21)

Where: M is the average oil content in peanuts (M = 50%).

- Thermal conductivity of peanut was approximate according to the following equation:

$\lambda = 1.367 \times (0.007 \times W_c + 0.86) \times [0.42 \times 10^{-6} \times \rho \times (273.15 + T) + 0.0254]$, W.m^{-1}.K^{-1} (22)

- Heat emission coefficient of roasting environment α (short contact when roasting) was calculated by the following equation:

$$\alpha = \frac{2}{\sqrt{\pi}} \sqrt{\frac{\lambda c_p \rho}{\tau_{tx}}} \quad (23)$$

- Maximum contact time when roasting was calculated as follows:

$$\tau_{tx}^{max} = 381.5 \frac{\delta^2}{a} \quad (24)$$

3. Materials and methods

3.1. Materials

Peanuts grown in the Southeast region of Vietnam were used in this study. The peanuts had their pod removed (Figure 1), roughly shaped like cylinders with average diameter $d = 2R = 6.10^{-3}$ m and height $l = 2h = 12.10^{-3}$ m.

3.2. Apparatus

- In this study, the roasting process was conducted by using a rotary roster equipment GD20 from Grande, manufactured in 2020 (Figure 3).
- The GD20 roasting device automatically measures and controls the technological parameters such as roasting temperature and time, the rotation speed of the drum, hence hot air could evenly contact all surfaces of the seeds, leading to a uniform temperature and shorter roasting time.

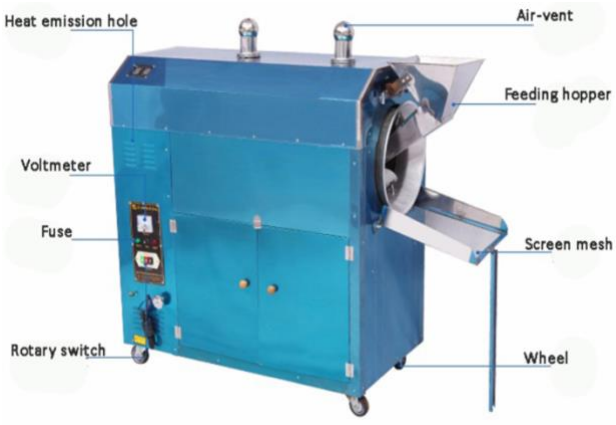

Figure 3. The peanut roaster

3.3. Methods

- In this study, the system analysis approach was used to build the mathematical model for the roasting process (Dzung et al., 2012a). In addition, modeling and optimization methods were used to build and solve the mathematical model for the peanut roasting process (Dzung et al., 2012b).

- Temperature of roasted materials were determined by two methods, experimental method and calculation method from the mathematical model.

- Mathematical tools and softwares such as Microsoft Excel 2020, Matlab 7.0 and Visual Basic 8.0 were used to build and solve the mathematical model (Dzung et al., 2012b).

4. Results and discussions

4.1. Determine the thermophysical properties

The thermophysical properties of peanuts were estimated by using equations (11), (12), (20) - (24). Results are summarized in Table 2.

Table 2. Thermophysical properties of peanuts

Parameter	Unit	Value
R	m	3×10^{-3}
h	m	6×10^{-3}
T_e	K	373.15
W_c	%	8.0
M	%	50
ρ	$kg.m^{-3}$	1216
ρ_v	$kg.m^{-3}$	854
λ	$W.m^{-1}.K^{-1}$	0.254
c_p	$J.kg^{-1}.K^{-1}$	1838.8
a	$m^2.s^{-1}$	11.36×10^{-8}
τ_{tx}^{max}	s	30224
τ_{tx}	s	1800
α	$W.m^{-2}.K^{-1}$	20.04
Bi_R	-	0.2367
Bi_h	-	0.4734

By the bisection algorithm programmed by the Visual Basic 8.0 software, roots of specific equations (13) and (14) were found. These roots (μ_m, μ_n) were then substituted into equation (7). The results showed that: series of numbers (7) converged quickly to 0 when n, m ≥ 2. That was:

$$\sum_{n=2}^{\infty}\sum_{m=2}^{\infty} A_n A_m P_{nm} \exp\left[-\left(\mu_n^2 Fo_R + \mu_m^2 Fo_h\right)\right] \to 0$$

Therefore, the roots of specific equation (13) and (14) were as follows: μ_n = 0.6506 (n = 1); μ_m = 0.6282 (m = 1).

4.2. Determine the peanut roasting temperature

- The roots of specific equations (13) and (14) (μ_n = 0.6506 (n = 1), μ_m = 0.6282 (m = 1)) and the thermophysical properties in Table 2 were substituted into equation (7). Visual Basic language programmed in Macro of Microsoft Excel 2020 was used to calculate the center temperature $T(0,0,\tau)_M$ and surface temperature $T(\mathrm{R},\mathrm{h},\tau)_M$ of peanut. The results are shown in Table 3 and Table 4.

- The experiments of the peanut roasting process were also carried out to determine the center temperature $T(0,0,\tau)_E$ and surface temperature $T(\mathrm{R},\mathrm{h},\tau)_E$ of the peanuts. The experimental results are also shown in Table 3 and Table 4.

Table 3. Variation of center temperature of peanuts according to roasting time

τ (minutes)	$T(0,0,\tau)_M$	$T(0,0,\tau)_E$	Er (%)
0.0	30.00	30	0.00
1.5	54.66	47.6	14.82
3.0	**75.03**	**65.2**	**15.07**
4.5	86.25	76.6	12.60
6.0	92.43	86.7	6.61
7.5	95.83	90.5	5.89
9.0	97.70	95.2	2.63
10.5	98.74	96.4	2.42
12.0	99.30	97.6	1.75
13.5	99.62	98.5	1.13
15.0	99.79	98.7	1.10
16.5	99.88	99.1	0.79
18.0	99.94	99.2	0.74
19.5	99.96	99.4	0.57
21.0	99.98	99.5	0.48
22.5	99.99	99.6	0.39
24.0	99.99	99.7	0.29
25.5	100.00	100	0.00
27.0	100.00	100	0.00
28.5	100.00	100	0.00
30.0	100.00	100	0.00

Table 4. Variation of surface temperature of peanuts according to roasting time

τ (minutes)	$T(\mathrm{R},\mathrm{h},\tau)_M$	$T(\mathrm{R},\mathrm{h},\tau)_E$	Er (%)
0.0	30.00	30	0.00
1.5	**67.44**	**58.2**	**15.87**
3.0	82.07	71.1	15.43
4.5	90.12	82.3	9.51
6.0	94.56	87.6	7.95
7.5	97.01	91.5	6.02
9.0	98.35	95.2	3.31
10.5	99.09	96.6	2.58
12.0	99.50	97.8	1.74
13.5	99.72	98.3	1.45
15.0	99.85	99.4	0.45
16.5	99.92	99.5	0.42
18.0	99.95	99.6	0.36
19.5	99.97	99.7	0.28
21.0	99.99	99.8	0.19
22.5	99.99	99.9	0.09
24.0	100.00	100	0.00
25.5	100.00	100	0.00
27.0	100.00	100	0.00
28.5	100.00	100	0.00
30.0	100.00	100	0.00

- From the data in Table 3, Matlab 8.0 software was used to simulate the variation in center temperature of peanuts according to roasting time. The results are shown in Figure 4.

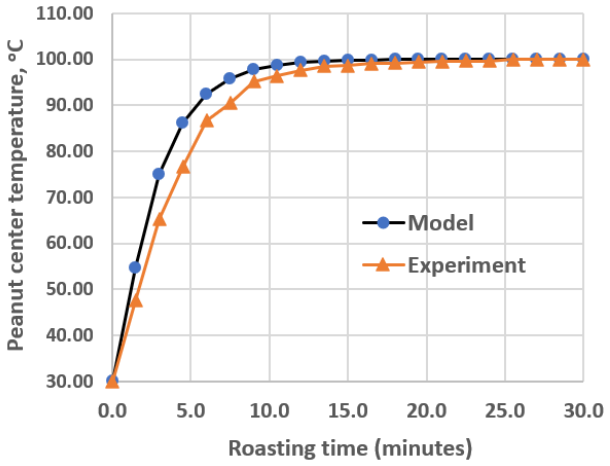

Figure 4. The relationship between center temperature of peanuts and roasting time

- From the data in Table 4, Matlab 8.0 software was used to simulate the varation in center temperature of peanuts according to roasting time. The results are shown in Figure 5.

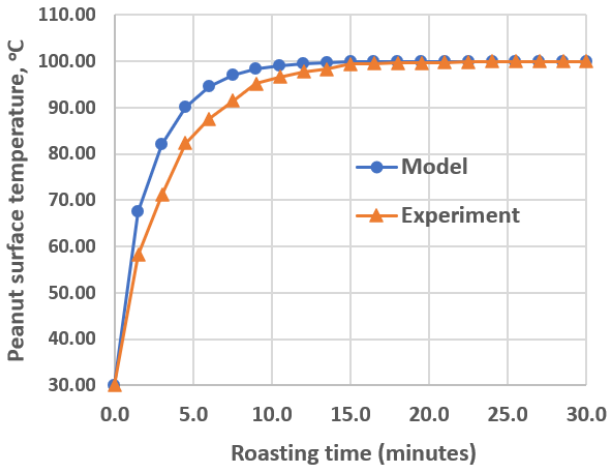

Figure 5. The relationship between surface temperature of peanuts and roasting time

- Evaluate the error of model: the term Er (%) was used to express the error between the calculated data and the experimental data. The error of the mathematical model was determined by the following expression:

$$Er = \frac{\left|T(r,z,\tau)_M - T(r,z,\tau)_E\right|}{T(r,z,\tau)_E}.100\% \quad (25)$$

- Table 3 shows that the maximum error between $T(0,0,\tau)_M$ - center temperature of peanuts calculated from the model (7), and $T(0,0,\tau)_E$ - temperature of peanuts determined by experiments was 15.07% when τ = 3.0 minutes (Figure 4). Similarly, the results in Table 4 also show that the error between $T(R,h,\tau)_M$ - surface temperature of peanuts calculated from the model (7), and $T(R,h,\tau)_E$ - surface temperature of peanuts determined by experiments was 15.87% when τ = 1.5 minutes (Figure 5). These errors could be attributed to the relatively cylindrical shape of peanuts. Besides, the thermophysical properties of peanuts were not constant but varied with roasting temperature. Moreover, instead of being a moderate unstable heat transfer, the first stage of roasting was a complicated transient heat transfer; the input energy was used to heat peanuts from the initial temperature to the roasting temperature to perform the roasting process according to the required method.

- It can be seen in Figure 4 and Figure 5 that as the roasting time increased, the errors decreased gradually. This is because the complicated transient heat transfer converted into the stable heat transfer because at this stage the thermophysical properties of peanuts had reached constant values and the moisture evaporation at the peanut surface was no longer similar to that at the initial roasting period.

- Results from Table 3, Table 4, Figure 4 and Figure 5 show that from roasting time τ = 1.5 mins to τ = 4.5 mins, the errors between the experimental data and the calculated data are relatively high. However, as analyzed above, the causes leading to the errors of the mathematical models were acceptable. Therefore, the mathematical models from (1) to (19) were suitable to describe the heat transfer of the roasting process of peanuts. These models could be used to calculate and determine the appropriate conditions for the roasting process, and be applied to the actual production.

4.3. Sensory evaluation of roasted peanuts

Raw peanuts were roasted at T_e = 100°C at three differents roasting times of 25 minutes; 27.5 minutes and 30 minutes to evaluate their color, peeling capacity of seed coats and ratio of broken of peanut seeds after roasting. The results are shown in Figures 6 and Table 5.

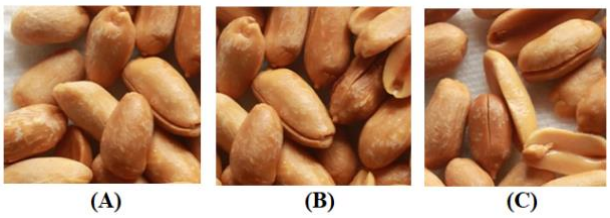

Figure 6. Roasted peanut seeds

(A)– Peanut seeds roased for 25 minutes; (B)- Peanut seeds roasted for 27.5 minutes; and (C)– Peanut seeds roasted for 30 minutes.

Results from Table 5 show that at roasting time of 25 minutes, the products had bright yellow colour with 100% of seed coats peeled off (Figure 6A) and the lowest ratio of broken seeds (only 1.2%). On the other hand, as being roasted for 27.5 minutes and 30 minutes, the products had darker color with 100% of seed coats peeled off (Figure 6B and 6C), but the ratio of broken seeds increased by 27.82% and 46.31%, respectively. This was because when the products were fully cooked, prolonging roasting time at 100 ^{o}C would cause the burning or decomposing of peanut chemical components, resulting in a significant decline in peanut quality.

Table 5. Characteristics of peanut after roasting

Roasting time, τ (minutes)	Color	Peeling capacity of seed coats, (%)	Ratio of broken seeds (%)
25.0	Bright yellow	100	1.20
27.5	Gray yellow	100	27.82
30.0	Dark yellow	100	46.31

Another roasting experiment was conducted at roasting temperatures of 105^{o}C for 20 minutes. Results show that the peanuts and seed coats had burnt color (Table 6). In contrast, roasting peanuts at lower 95^{o}C would lead to a longer roasting time of 35 minutes to remove the seed coats, but final products also had dark yellow color. Therefore, it was recommended to choose the roasting temperature of T_e = 100^{o}C.

Table 6. Color of roasted peanuts at different roasting conditions

Peanut roasting temperature, ^{o}C	Roasting time, τ (minutes)	Color roasting peanut (product)
95.0	35	No bright yellow
100.0	25	Bright yellow
105.0	20	Black

4.4. Determine the roasting conditions for peanuts

From the results of building and solving the mathematical models (1) to (19) in Table 3, Table 4, Figure 4, Figure 5 as well as experimental verification in Table 5, Table 6 and Figure 6, the roasting conditions for peanuts were established as following:

- Roasting temperature was 100^{o}C;
- Roasting time was 25 minutes.

After roasting at these conditions, the temperature of roasted peanuts calculated from the mathematical model was consistent with the experimental temperature. The roasted peanuts had good sensory quality with bright yellow color, 100% of seed coats were peeled off and the proportion of broken seeds was as low as 1.2%. Hence, these roasting conditions could be potentially applied in practice.

5. Conclusions

- Peanut roasting process was modeled in this study using mathematical models from (1) to (6). This was a model of transient heat transfer that accurately expressed the nature of the complex physical chemistry of peanut roasting process.
- Solving the mathematical models from (1) to (6) resulted in the roots of the mathematical model (7). Experimental verification showed that mathematical model (7) was completely suitable to describe the roasting process for peanuts. Therefore, model (7) could be used to calculate the roasting conditions as

well as to calculate, design and fabricate a roasting equipment.

- The roasting conditions for peanuts were also established in this study as follows: roasting time was 25 minutes; roasting temperature was 100°C. After roasting at these conditions, the seed coats were completely peeled off and the proportion of broken seeds was the lowest, only 1.2%. The roasted products had a bright yellow color, the sensory quality was dramatically improved (Fig *6 (A)*).

Nomenclature

$R = 0.003$ m : Radius of the peanut;
$h = 0.006$ m : Half height of the peanut;
$T_e = 100^oC$: Roasting temperature;
$W_c = 0.8 = 8\%$: The critical residual water content of the peanut;
$M = 0.5 = 50\%$: Oil content in the peanut;
$c_p = 1838.8$ J.kg^{-1}.K^{-1} : Specific heat of the peanut;
$\rho = 1216$ kg.m^{-3} : Density of the peanut;
$\rho_v = 854$ kg.m^{-3} : Density of the peanut block;
$\lambda = 0.254$ W.m^{-1}.K^{-1} : Thermal conductivity coefficient of the peanut;
$\alpha = 20.04$ W.m^{-2}.K^{-1} : Heat emission coefficient of roasting environment;
$a = 11.36\times10^{-8}$ m^2.s^{-1} : Thermal diffusivity of the peanut;
τ (s) roasting time;
$\tau_{tx} = 1800$s : Short contact time when roasting;
$\tau_{tx}^{max} = 30224$s : Maximum contact time when roasting;
Bi_R, Bi_h: Biot number of the peanut;
Fo_R, Fo_h: Fourier number of the peanut;

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POMEGRANATE SEED OIL: EXTRACTION, SHELF LIFE PREDICTION, AND MICROENCAPSULATION

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ABSTRACT

Interest in oils rich in polyunsaturated fatty acids has increased in recent years about their beneficial effects on human health. Pomegranate seed is an industrial by-product that has a large variety of nutritional value. Pomegranate seed oil (PSO) has an exceptional fatty acid profile. Lipid oxidation is one of the important parameters that limits its use in food products. Thus, the objectives of this study were to evaluate the effects of extraction methods (Soxhlet and stirring) and solvents (*n*-hexane, petroleum ether, diethyl ether, and ethanol) on oxidation parameters in the extraction of PSO, to predict the shelf life of PSO by Rancimat and to encapsulate PSO by spray drying to extend its shelf life. PSO yield varied between 1.88 and 14.32%. The lowest peroxide (2.88 meq O_2/kg), *p*-Anasidine (6.56 meq *p*-Anisidine/kg), and TOTOX (12.13) values were observed when PSO was extracted by stirring using n-hexane as solvent. The shelf lives of PSO at 0, 4, and 20 ºC using Rancimat were predicted as 11593, 8088, and 1916 h, respectively. The main fatty acid in PSO was punicic acid and derivatization had a significant effect on the determination of punicic acid by GC (sodium methoxide, 84.75% and potassium hydroxide, 81.79%). Inlet air temperature of 140 °C, oil: wall material ratio of 1:2 (w/w), and maltodextrin: gum Arabic ratio of 1:1 (w/w) were selected as favorable conditions in microencapsulation of PSO. The addition of antioxidants into feed solutions had no positive effects on oxidation during spray drying.

1.Introduction

The food industry is one of the sectors generating valuable wastes and by-products. Generally, food wastes are considered an environmental problem whereas some by-products and bioactive compounds derived from these materials can be ingredients or raw materials of products with high-added value (Puértolas & Barba, 2016).

Fresh pomegranate (*Punica granatum L.*) and pomegranate products such as jam, jelly, and fruit juice are commonly consumed worldwide. Pomegranate seed remains as an industrial by-product after the production of pomegranate juice whereas it is a potential source of functional lipids. Pomegranate seed oil (PSO) contains an exceptional conjugated fatty acid called punicic acid (9*Z*,11*E*,13*Z*-octadecatrienoic acid), which is a ω-5 long-chain polyunsaturated fatty acid that makes up approximately 65-80% of PSO (Lansky & Newman, 2007). Punicic acid is structurally very similar to conjugated linoleic acid and α-

linolenic acid which have been found to have various health benefits including cancer prevention (Grossmann, Mizuno, Schuster, & Cleary, 2010). Polyunsaturated fatty acids are prone to oxidation; however, conjugated counterparts are more susceptible to oxidative deterioration than the former ones. Oxidative degradation of lipids limits their use in food products due to the development of undesired flavor and odor (Çam, Erdoğan, Aslan, & Dinç, 2013; Loughrill et al., 2019). To date, both traditional and advanced extraction techniques have been used for the recovery of PSO (Abbasi, Rezaei, Emamdjomeh, & Mousavi, 2008; Đurđević et al., 2017; Eikani, Golmohammad, & Homami, 2012; Athanasia M. Goula, 2013; Liu, Xu, Hao, & Gao, 2009). Among these techniques, traditional extraction techniques such as Soxhlet and stirring extraction are still the most commonly used due to simple application processes and low investment costs. However, it is necessary to select suitable extraction techniques and solvents to obtain oil with desirable characteristics such as high yield and good oxidative stability. Besides, even if appropriate extraction conditions are selected, oils rich in conjugated fatty acids have a shorter shelf life compared to other unsaturated fatty acids because of the faster formation of unstable free radicals from conjugated fatty acids (Zhang & Chen, 1997). Thus, it is critical to examine their oxidative stability. Nowadays, different accelerated methods such as Differential Scanning Calorimetry (DSC), Thermal Gravimetric Analysis (TGA), and Rancimat are used to rapidly predict the shelf life of oils. DSC curves help to estimate the oxidation of oils exposing different isothermal temperatures under oxygen flow. A sharp exothermic curve as a function of heat provides information about an oxidation reaction. When the curve is seen, it could be interpreted that a rapid exothermic reaction occurs between oil and oxygen (Pardauil et al., 2011). TGA is also used to estimate the oxidation stability of oils by determining their mass gain or mass loss through oxygen uptake or thermal degradation (Tengku-Rozaina & Birch, 2015). Shelf-life prediction of oils by Rancimat is conducted by using different temperatures in presence of O_2 (Ghosh, Upadhyay, Mahato, & Mishra, 2019). High correlation between thermal analysis and Rancimat results was reported by previous work (Arain, Sherazi, Bhanger, Talpur, & Mahesar, 2009; Symoniuk, Ratusz, Ostrowska-Ligęza, & Krygier, 2017; Tengku-Rozaina & Birch, 2015). In the literature, there is no report regarding the shelf life prediction of PSO by any accelerated methods.

The microencapsulation process, on the other hand, is widely used to improve the oxidative stability of polyunsaturated fatty acids against lipid oxidation and to increase their shelf life. The efficiency, yield, and stability of microcapsules depend on the selection of suitable wall materials and operational conditions for the encapsulation process (Carneiro, Tonon, Grosso, & Hubinger, 2013; Tonon, Grosso, & Hubinger, 2011). PSO has been microencapsulated by using skim milk powder (Goula & Adamopoulos, 2012); maltodextrin, skim milk powder, and gum Arabic (Goula & Lazarides, 2015); starch derivates and whey protein concentrate mixes (Sahin-Nadeem & Afşin Özen, 2014); and capsule (modified corn starch) (Bustamante, Hinojosa, Robert, & Escalona, 2017). Nevertheless, there is no information on the evaluation of the oxidative stability of PSO by using synthetic or natural antioxidants during spray drying.

The objectives of this study were: (i) to evaluate effects of extraction methods and solvents on oxidation of PSO, (ii) to predict the shelf life of PSO under accelerated oxidation conditions, (iii) to determine effects of spray drying temperature and coating materials on microencapsulation of PSO (iv) to observe effects of natural and synthetic antioxidants on oxidation of PSO during spray drying.

2. Materials and methods

2.1. Materials

The seeds from mixed pomegranate cultivars as a by-product of the fruit juice industry were kindly provided by a local producer (Göknur, Niğde, Turkey). The seeds were dried in the dark at room temperature for 7

days and, then stored at 4 ºC until subsequent analyses. Pomegranate peel phenolics were extracted by a method described by Çam, İçyer, and Erdoğan (2014). The chemicals, standards, and wall materials were obtained from Sigma Aldrich Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany) unless otherwise stated.

2.2. Methods

2.2.1. PSO extraction

Before extraction, pomegranate seeds were immediately ground by a laboratory mill to increase the mass transfer. PSO from the seeds was extracted using two extraction methods (Soxhlet and stirring extractions) and four solvents (*n*-hexane, petroleum ether, diethyl ether, and ethanol).

In Soxhlet extraction, 50 g of the milled seeds were placed into a cellulose thimble which is then put into the main chamber of the Soxhlet extractor. The solvent (250 mL) was poured into the main chamber where it was heated under reflux at the boiling temperature of the solvent, and extraction was maintained for 120 min. After extraction, the solvent was removed at 40 ºC by a vacuum evaporator (Buchi, Flawil, Switzerland) and the oil was dried to constant weight in an oven at 105 ºC.

In stirring extraction, 50 g of milled pomegranate seeds were weighed into a glass beaker. After the addition of the solvent (250 mL), it was stirred at room temperature for 120 min. The mixture was centrifuged at 3,000 g for 5 min, the resulting supernatant was collected and then the solvent was evaporated at 40 ºC under vacuum (Buchi, Flawil, Switzerland). Subsequently, the oil was dried to constant weight in an oven at 105 ºC.

2.2.2. Lipid oxidation measurements

Peroxide value (PV), p-Anisidine value (*p*-AV), and total oxidation (TOTOX) value were determined to assess the oxidative stability of PSOs. PSO extraction was performed as described in the PSO extraction section except that the solvent was removed under nitrogen flow.

Peroxide value was determined by employing a method given in the literature (Wrolstad et al., 2004). Briefly, 5 g of PSO was dissolved in 30 mL of acetic acid-chloroform (3:2, v/v) solution. A 0.5 mL of saturated KI solution and 30 mL of distilled water were added to this mixture. This mixture was slowly titrated with $Na_2S_2O_3$ (0.01N) until the yellow color almost disappeared. After the addition of 0.5 mL of starch solution (1%), the titration was slowly maintained with Na_2S2O_3 until the violet color fully disappeared. The PV was calculated by following Eq. (1):

$$\mathrm{PV} = \frac{(\mathrm{S}-\mathrm{B})*\mathrm{N}*1000}{\mathrm{w}} \quad (1)$$

where S: $Na_2S_2O_3$ volume (Sample), B: $Na_2S_2O_3$ volume (Blank), N: normality of Na_2S2O_3, W: mass of sample.

p-AV was also measured spectrophotometrically according to AOCS Official Method Cd-18-90 (AOCS, 1998). PSO was dissolved in isooctane and then 1mL of *p*-Anisidine solution in acetic acid (0.25 g/mL) was added to this mixture. After 10 min, the absorbance was measured at 350 nm. The *p*-AV was calculated by following Eq. (2):

$$p-\mathrm{AV} = \frac{25*(1.2*\mathrm{A2}-\mathrm{A1})}{\mathrm{W}} \quad (2)$$

where A1: absorbance of fat solution, A2: absorbance of fat-anisidine solution, W: mass of sample.

PV and *p*-AV were used to measure TOTOX value (Sun-Waterhouse, Zhou, Miskelly, Wibisono, & Wadhwa, 2011). TOTOX value was calculated by following Eq. (3):

$$\text{TOTOX value} = (2 * \text{Peroxide value}) + p-\text{Anisidine value} \quad (3)$$

2.2.3. Oxidative stability and shelf-life prediction by Rancimat

To evaluate the thermal oxidation stability of PSO, the oil (5 g) was exposed to different temperatures (60, 70, 80, 90, 100, 110, 120 and 130 ºC) with an airflow rate of 20 L/h using Rancimat (743 model, Metrohm, Switzerland). The oxidative stability of PSO was expressed as an induction period (IP) in the respective temperature. The IP was the time required to

cause a sudden increase in conductivity. In addition, commercial sunflower and canola oils were used as the references at 110 and 120 ºC. Subsequently, the shelf life prediction of PSO was performed by plotting the temperatures (T, ºC) vs. IPs using Eq. (2). The plot was extrapolated to lower temperatures to predict the shelf life of PSO at 0, 4, and 20 ºC.

$$t = A * e^{(B*T)} \quad (4)$$

where A and B are the coefficients of the equation.

2.2.4. Fatty acid composition by GC-FID

Fatty acid methyl esters (FAMEs) of PSOs were prepared according to two base-catalyzed methods using sodium methoxide (Wrolstad et al., 2004) and methanolic potassium hydroxide (AOCS, 1997) to determine the effects of derivatization methods on the content of punicic acid. Subsequently, the fatty acid composition of PSO was determined by gas chromatography (GC-6890, Agilent, USA) equipped with a flame ionization detector (FID). FAMEs were separated in a capillary column (100 m x 0.25 mm, 0.2 µm film thickness, HP-88) (J&W Scientific, Folsom, Calif., USA). The chromatographic conditions were as follows: column temperature was programmed from 140 ºC (kept for 5 min.) to 240 ºC at 4 ºC/min (kept for 15 min); injector and detector temperatures, 260 and 280 ºC, respectively. The split ratio was 1:30. The flow rate of hydrogen as the carrier gas was 1 mL/min. FAMEs were identified by comparing their retention times with FAME standard mixture (Bellefonte, PA, USA).

2.2.5. Preparation of emulsions and microencapsulation by spray drying

The microencapsulation process was conducted according to McNamee, O'Riorda, and O'Sullivan (2001) with some modifications in the process parameters. Briefly, five different emulsions were prepared to evaluate the effects of three factors, including the inlet temperatures of 140, 160, and 180 ºC, oil: wall material ratios of 1:2 and 1:3, and MD: GA ratios of 1:1 and 2:1. In emulsification, MD was dissolved in 100 mL distilled water under continuous mixing at 24 000 rpm with an Ultra-Turrax homogenizer (IKA-T18 Basic, Staufen, Germany) for 10 min. PSO and GA were slowly added into the solution and the resulting emulsion was homogenized for a further 10 min. In all treatments, the emulsions had a total mass fraction of 30% and a volume of 200 mL.

After emulsification, the emulsions were spray-dried using a laboratory scale spray dryer (Buchi-B290, Flawil, Switzerland) with a chamber diameter of 16.5 cm and a chamber length of 60 cm. The airflow and feed rates were 600 mL/h and 8 mL/min, respectively. PSO microcapsules were collected with the help of a cyclone separator and then stored in a plastic container at 4 ºC until required for use.

2.2.6. Microencapsulation yield

The total amount of solid mass in feed emulsion and mass of microcapsules were used to calculate microencapsulation yield according to Eq.5 based on dry matter content:

$$\text{Microencapsulation yield (\%)} = \frac{\text{Mass of microcapsules (g)}}{\text{Total amount of solid mass (g)}} * 100 \quad (5)$$

2.2.7. Microencapsulation efficiency

Total oil content: An enzymatic digestion method was used to determine the total oil content of PSO microcapsules (Curtis, Berrigan, & Dauphinee, 2008). Briefly, 30 mg of porcine pancreatin and 250 mg of PSO were mixed. Ten mL of sodium phosphate buffer was added to the mixture. The mixture was vortexed and then shaken at 37 °C at 60 rpm for 1 h in a water bath. Ten mL of ethyl acetate was added after the mixture was cooled to room temperature. The mixture was centrifuged at 1000 rpm for 10 min. The top layer (1-2 mL) was removed and its weight was recorded. This layer was dried under continuous nitrogen flow at 40 °C for 1 h. The final weight of extracted oil was recorded.

Surface oil content: To determine surface oil content, 50 mL of isohexane and 5 g of PSO were mixed and shaken for 10 min at 225 rpm. The slurry was filtered and washed three times with 20 mL of isohexane. Isohexane was evaporated at 40 °C for 25 min. Then, the remaining oil was dried at 90 °C for 30 min to completely remove isohexane from the oil. The

final weight of the extracted oil was recorded (Anwar & Kunz, 2011).

Calculation of microencapsulation efficiency: The ratio between the amount of total oil and surface oil of microcapsules were used to determine microencapsulation efficiency according to Eq.6 based on dry matter content:

$$\text{Microencapsulation efficiency (\%)} = \left(1 - \frac{\text{Surface oil (g)}}{\text{Total oil (g)}}\right) * 100 \quad (6)$$

2.3. Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate the differences between extraction technique, solvents, and microencapsulation conditions. The significant difference at $p<0.05$ was tested using the Tukey HSD test in 22.0 SPSS (SPSS Inc., Chicago, USA).

3.Results and discussion

3.1. Oil yield

The effects of the extraction methods and solvents on oil yield are shown in Fig. 1. The extraction methods were carried out under similar conditions (50 g pomegranate seeds with 250 mL solvent and extraction time of 120 min) to investigate the effect of methods. The extraction of PSO by the Soxhlet method resulted in a higher oil yield compared to the stirring method regardless of extraction solvents. Oil yields were also influenced by extraction solvents but were not changed significantly ($p>0.05$) when used *n*-hexane, petroleum ether, and diethyl ether.

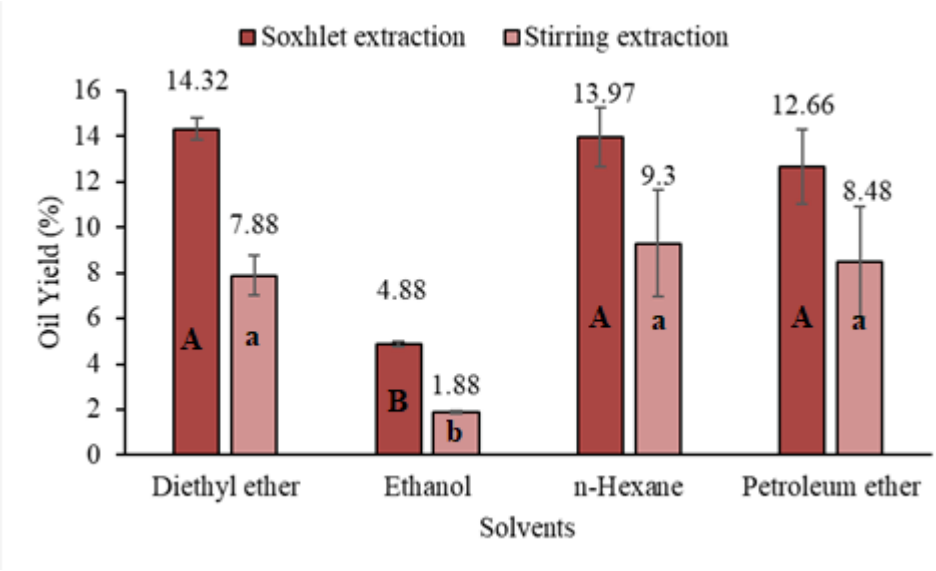

Figure 1. PSO yields for extraction techniques and solvents. (†[a-b] Means on bar different letters differ significantly ($p<0.05$)).

These solvents produced oil yields in the range of 12.66-14.32% for the Soxhlet method and 7.88-9.30% for the stirring method. A similar result (12.74%) was observed in the study of Khoddami, Man, and Roberts (2014) where PSO was extracted by sequential extraction using cold press and Soxhlet with petroleum ether. Besides, the reported results were partially closed to those investigated by Fadavi, Barzegar, and Hossein Azizi (2006) showing total lipid content (66.3-193 g/kg) in seeds of 25 varieties of pomegranates by Soxhlet with petroleum benzene. In a previous study, Abbasi, Rezaei, and Rashidi (2007) reported that PSO yields were 18.6% for Soxhlet with n-hexane (extraction time, 6 h) and 13.0% for stirring with n-hexane (extraction time, 4 h). The lower results in our study could be attributed to shorter extraction time (2 h) and varietal differences among pomegranate cultivars. In the present study, the lowest oil yields were observed when used ethanol as solvent in both

Soxhlet (4.88%) and stirring (1.88%) methods. In a recent study, the effects of certain solvents including hexane, petroleum ether, chloroform, chloroform:methanol and ethanol were examined in the extraction of PSO (Aruna, Manohar, & Singh, 2018). The authors reported that hexane was the best solvent giving the highest yield whereas ethanol was inefficient for PSO extraction.

3.2. Lipid oxidation and oxidative stability

The PSO obtained by ethanol was not included in the current stage of the present study since the PSO yield was found to be significantly lower than the other three solvents used in the previous stage.

Table 1. Oxidation parameters for techniques-solvents combinations of PSO

Extraction method	Solvent	PV (meq O2/kg)	*p*-AV (meq p-Anisidine/kg)	TOTOX value
Soxhlet	Diethyl ether	5.71±0.44^{b}	20.57±1.42^{b}	31.99±1.63^{c}
	n-Hexane	4.49±1.29^{b}	8.54±1.04^{a}	17.52±2.83^{b}
	Petroleum ether	11.16±0.59^{c}	8.41±1.38^{a}	30.72±1.21^{c}
Stirring	Diethyl ether	4.83±1.17^{b}	18.69±1.92^{b}	28.36±3.75^{c}
	n-Hexane	2.78±0.11^{a}	6.56±1.13^{a}	12.13±1.12^{a}
	Petroleum ether	5.09±0.31^{b}	5.45±2.03^{a}	15.63±2.01^{ab}

†$^{a-c}$ Means within each column with different letters differ significantly ($p<0.05$).

Table 2. Induction periods of PSO and commercial oils at different temperatures

Sample	Induction period (h)							
	60 °C	70 °C	80 °C	90 °C	100 °C	110 °C	120 °C	130 °C
PSO	54.02	18.54	7.82	3.02	1.15	0.58	0.44	0.31
Sunflower oil	-	-	-	-	-	6.73	4.05	-
Canola oil	-	-	-	-	-	10.10	5.90	-

Table 1 shows the oxidation parameters for methods and solvents. The effects of methods and solvents on primary and secondary products in lipid oxidation of PSO were assessed through the PV, *p*-AV, and TOTOX values. PVs and *p*-AVs ranged from 2.78 to 11.16 meq O_2/kg and 5.45 to 20.57 meq *p*-Anisidine/kg depending on the extraction methods and solvents, respectively. The lowest TOTOX value was obtained by stirring with *n*-hexane. Although this could be attributed to the room temperature being convenient for preventing oxidation of polyunsaturated fatty acids, this positive effect was not observed for the other solvents in the stirring method. The chemical composition of the extracted oil can be affected by solvents and methods used in the extraction (Uoonlue & Muangrat, 2018). The oxidative stability of the oil is directly related to the chemical composition of the oil such as the presence or absence of antioxidants in the oil of interest.

Oxidative stabilities of PSO determined by Rancimat at different temperatures are shown in Table 2. The IP values of PSO at 60, 70, 80, 90, 100, 110, 120 and 130 ºC were 54.02, 18.54, 7.82, 3.02, 1.15, 0.58, 0.44 and 0.31 h, respectively. It was also determined the IPs of commercial sunflower oil and canola oil at 110 and 120 ºC for comparison purposes. With high punicic acid content, PSO can be assumed as an unstable oil against thermal oxidation compared to commercial vegetable oils. The IP value of PSO at 110 ºC was shorter than those reported by Habibnia, Ghavami, Ansaripour, and Vosough (2012) (0.73-1.02 h), Basiri (2013) (3.03 h) and Melo et al. (2016) (0.72 h). However, the IP of PSO at 80 ºC was highly longer than those reported by Costa, Silva, and Torres (2019) (0.10-0.22 h) for commercial cold-pressed PSOs. These differences between our results and literature could be due to extraction methods, solvents, and pomegranate varieties as well as operational parameters of Rancimat (Farhoosh, 2007).

3.3. Shelf-life prediction

Shelf-life prediction of PSO was conducted using a linear relationship between the temperature in the range of 60-110 ºC and IP by the following equation:

$$IP = 11592.82 * e^{(-0.09*T)} \quad (7)$$

The correlation coefficient (R^2) of Eq. (5) was 0.9972. The shelf lives of PSO at 0, 4, and 20 ºC were estimated as 11593, 8088 and 1916 h, respectively (Fig. 2).

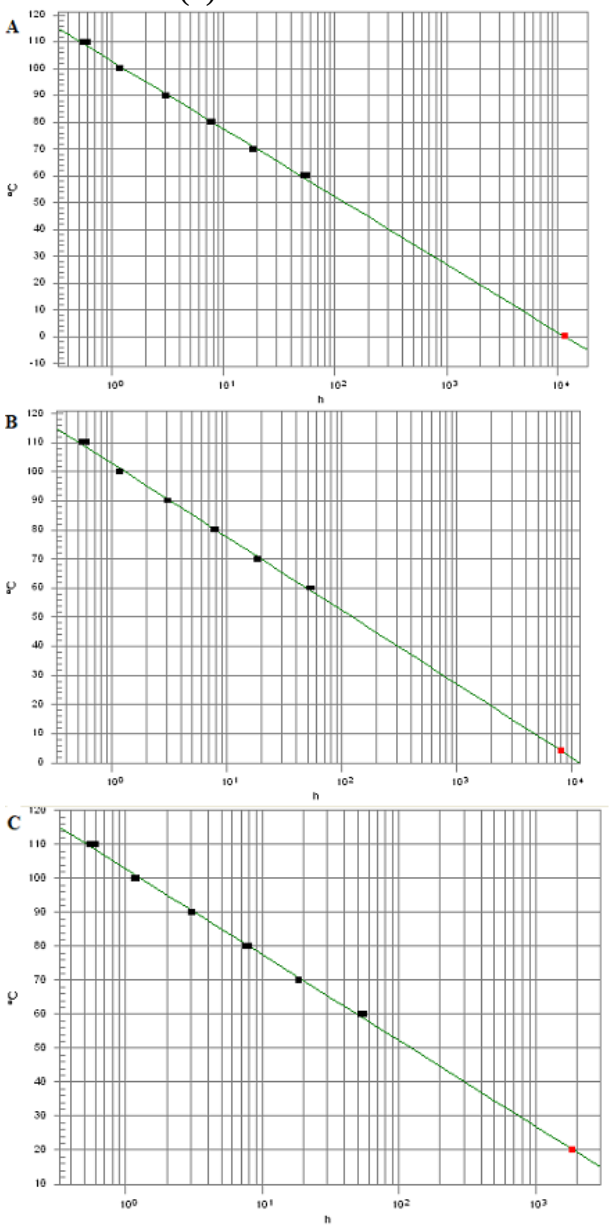

Figure 2. Shelf life prediction of PSO by Rancimat at 0 (A), 4 (B) and 20 (C) °C

There were no comparable shelf life results for PSO. However, shelf life at 20 ºC by the Rancimat method was reported between 4291 and 5687 h for soybean oil (Farhoosh, 2007) and 3789 h for sunflower oil (Upadhyay & Mishra, 2014). It was also reported that shelf lives of sunflower, sesame oils, and their blends at 25 ºC by Rancimat were between 2638 (109.94 days) and 5220 h (217.50 days) without antioxidant addition (Ghosh et al., 2019). This low shelf life at 20 ºC showed that PSO was very prone to oxidative deterioration compared to the above-mentioned studies. Shelf-life prediction by Rancimat can result in either overprediction or

underprediction depending on Rancimat parameters as shown in the study of Farhoosh (2007). Nevertheless, the results obtained by Rancimat are preferable to long-term storage studies because of its shorter analysis time.

3.4. Fatty acid composition

The effects of extraction type (Soxhlet or stirring) and transesterification method (potassium hydroxide or sodium methoxide) on the determination of the fatty acid composition of PSO were determined by ANOVA. The general fatty acid composition of PSOs did not change with extraction types and transesterification methods. Fourteen fatty acids were identified with GC-FID in all conditions. The results showed that the extraction type had no significant effects ($p=0.218$), however, the transesterification method had a significant effect ($p=0.001$) on the fatty acid composition of PSO, especially punicic acid. As can be seen in Table 3, when PSO was methylated with catalysts, the content of punicic acid for sodium methoxide and potassium hydroxide was 84.75 and 81.79%, respectively. Besides, the total punicic acid isomer content of PSO changed depending on the catalyst type used. Previous research evaluated the use of acid and base catalysts on the fatty acid profile of PSO (Sassano et al., 2009). The authors suggested the use of a base catalyst (sodium methoxide) in the determination of punicic acid in PSO. Melo et al. (2016) also showed a superior effect of a base catalyst in the determination of punicic acid in PSO compared to acid catalyst. According to our results, sodium methoxide was more effective than potassium hydroxide in the determination of the PSO fatty acid profile.

3.5. Microencapsulation

The effects of emulsion (MD: GA ratio, oil: wall material ratio) and spray drying parameter (Inlet air temperature) on ME and MY of PSO microcapsules are presented in Table 4.

Firstly, PSO microcapsules were produced using MD: GA ratios of 1:1 (T_1) and 2:1 (T_2). The results indicated that MD: GA ratio of 2:1 led to a decrease in ME and MY of PSO microcapsules. This can be associated with the lower emulsifying capacity of MD. It was reported that MD usage in the wall matrix decreased lipid oxidation by reducing oxygen permeability of the wall matrix for microencapsulated avocado oil because of hydrophilicity of MD and hydrophobicity of oxygen (Bae & Lee, 2008).

Secondly, oil: wall material ratio of feed emulsion was changed from 1:2 (T_2) to 1:3 (T_3). The increase in the proportion of the wall matrix, from 1:2 to 1:3, had no significant effects on ME and MY. Besides, this application decreases oil content in the total mass of microcapsules. Microcapsules having high oil content and ME are preferable for the enrichment of food products because a higher amount of oil (active material) can be incorporated into food products by using lower microcapsule powder. On the other hand, the use of less wall material is a cost-effective application.

Lastly, the effect of inlet air temperature on ME and MY of PSO microcapsules was tested. The inlet air temperatures of 140 and 180 ºC showed no significant effect on MY and ME of PSO microcapsules compared to 160 ºC. Tonon et al. (2011) reported that temperatures higher than 170 ºC promoted lipid oxidation during spray drying. Hence, PSO should not be spray-dried at high temperatures because of its polyunsaturated fatty acid content, especially punicic acid. Lavanya, Kathiravan, Moses, & Anandharamakrishnan (2019) showed that increasing outlet temperature led to an increase in PVs of microencapsulated chia and fish oils. In this study, outlet air temperature increased as a function of inlet temperature. High inlet air temperature can accelerate oxidation during spray drying, however, the microcapsules are exposed to outlet air temperature in the collection bottle during the total drying time. Thus, the selection of a lower outlet temperature is important to prevent further oxidation. The inlet air temperature of 140 ºC had no negative effects on MY and ME of PSO microcapsules, and the lowest outlet temperature was observed at this temperature. Hence, it was selected as optimal temperature for microencapsulation of PSO.

Table 3. Effect of extraction methods and catalysts on the fatty acid profile of PSO

Extraction method	**Soxhlet**		**Stirring**	
Catalyst type	**Sodium methoxide**	**Potassium hydroxide**	**Sodium methoxide**	**Potassium hydroxide**
Fatty acids	**Area (%)**	**Area (%)**	**Area (%)**	**Area (%)**
C14:0	0.02±0.01	0.41±0.32	0.02±0.01	0.54±0.07
C15:0	0.03±0.01	0.10±0.03	0.02±0.01	0.10±0.01
C16:0	2.42±0.02	3.74±1.03	2.42±0.03	4.02±0.26
C16:1	0.05±0.01	0.13±0.05	0.07±0.02	0.13±0.03
C17:0	0.07±0.01	0.12±0.07	0.06±0.02	0.11±0.00
C18:0	1.75±0.03	2.07±0.21	1.74±0.02	2.18±0.11
C18:1	4.79±0.08	5.51±0.47	4.73±0.02	6.23±0.95
C18:2	4.44±0.06	4.41±0.02	4.28±0.02	4.59±0.60
C20:0	0.44±0.01	0.41±0.05	0.45±0.02	0.43±0.00
C18:3 n-3	0.08±0.02	0.16±0.09	0.08±0.01	0.10±0.00
C20:1	0.67±0.04	0.67±0.01	0.64±0.04	0.64±0.02
C18:3 n-5	84.15±0.11^{a}	81.79±2.10^{b}	84.67±0.08^{a}	80.56±2.24^{b}
C18:3 n-5 isomer 1	0.80±0.05	0.29±0.13	0.59±0.04	0.29±0.19
C18:3 n-5 isomer 2	0.29±0.01	0.21±0.03	0.24±0.02	0.10±0.03

†$^{a, b}$ Means within each column with different letters differ significantly ($p<0.05$).

Table 4. Experimental conditions and responses for microencapsulation of PSO

Treatments	**MD (g)**	**GA (g)**	**Oil (g)**	**MD:GA (g/g)**	**Oil: wall material (g/g)**	**T_i (°C)**	**T_o (°C)**	**MY (%)**	**ME (%)**
T_1	20	20	20	1:1	1:2	160	90±2.0	29.2±8.8^{a}	73.4±2.5^{b}
T_2	26.7	13.3	20	2:1	1:2	160	90±1.0	27.4±0.9^{a}	69.4±1.9^{b}
T_3	22.5	22.5	15	1:1	1:3	160	94.5±2.5	31.9±2.9^{a}	89.8±2.2^{a}
T_4	20	20	20	1:1	1:2	180	105±3.0	31.1±0.6^{a}	77.8±1.8^{ab}
T_5	20	20	20	1:1	1:2	140	72±1.0	28.7±8.9^{a}	74.1±7.2^{ab}

† $^{a, b}$ Means within each column with different letters differ significantly ($p<0.05$). MD: Maltodextrin; GA: Gum Arabic; T_i: Inlet temperature; T_o: Outlet temperature; MY: Microencapsulation yield; ME: Microencapsulation efficiency.

3.6. Effect of antioxidants on microencapsulation

PSO microcapsules were further obtained at 140 °C with MD: GA ratio of 1:1 and oil: wall material ratio of 1:2 with the addition of synthetic and natural antioxidants to check the effects of antioxidants on PSO microcapsules. Butylated hydroxyanisole (BHA) and pomegranate peel phenolics were used in the same ratios (0.02%) in feed solutions. The control solution was prepared without any antioxidant. The proportion of BHA (0.02%) was selected according to the maximum permitted amount of this synthetic antioxidant in food products (Fortin, 2016). Although there is no limit for pomegranate peel phenolics, the same amount (0.02%) was used in the preparation of emulsions for a meaningful comparison. The addition of antioxidants into emulsions showed a statistically insignificant effect on lipid oxidation during the spray drying of PSO as shown in Table 5.

Table 5. Effect of natural and synthetic antioxidants on oxidative stability of PSO

PSO microcapsules	PV (meq O_2/kg)	*p*-AV (meq *p*-anisidine/kg)	TOTOX
Control	5.20±1.55[a]	8.45±1.03[a]	18.85±3.74[a]
PSO microcapsules with BHA	5.68±1.75[a]	7.04±1.06[a]	18.39±4.57[a]
PSO microcapsules with pomegranate peel phenolics	7.83±2.42[a]	5.44±1.50[a]	21.11±5.35[a]

†Means within each column with the same letters ([a]) are not significantly different (p>0.05).

The result was consistent with previous findings of Binsi et al. (2017). The authors reported that the addition of sage polyphenols to feed solution did not show a protective effect against oxidation during spray drying. However, the authors reported the protective effects of incorporated sage polyphenols during the post-storage of the microcapsules. A previous study was also reported that the addition of the mixture of 0.05% rosemary, 1% broccoli sprout, and 1% citrus extracts to feed solutions effectively reduced lipid oxidation during post-storage of microencapsulated seed oil (Ahn, Kim, & Kim, 2012). In another study, Yeşilsu and Özyurt (2019) evaluated the antioxidant effect of rosemary, thyme, and laurel extracts on lipid oxidation in fish oil microcapsules, and 1500 ppm rosemary extract addition was the most effective one. Although the oils rich in polyunsaturated fatty acids are known to be prone to thermal oxidation, they are exposed to inlet air temperature for only a few seconds during spray drying. Thus, it can be concluded that the use of antioxidants during spray drying had no or minute effects on the oxidation of microcapsules. The positive effects of antioxidants might be apparent during long time storage of microcapsules. Another possible explanation is the exposure of oil particles to oxidation before the formation of microcapsules (Binsi et al., 2017), specifically in the emulsification process.

4. Conclusions

Extraction methods and solvents had significant effects on the oxidation of PSO. Rancimat analysis showed that PSO was highly susceptible to oxidation compared to commercial oils. PSO was characterized by a high level of punicic acid, a polyunsaturated fatty acid. The derivatization procedure affected identifiable punicic acid content and the highest content of punicic acid was found in esterification with sodium methoxide. Although inlet air temperature, oil: wall ratio, and MD: GA ratio affected ME, they showed no significant effect on MY. The addition of synthetic and natural antioxidants to the feed solution did not prevent oxidation during spray drying. Further storage studies are necessary to attain better knowledge regarding the effect on the shelf life of PSO microcapsules of oxidation.

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MOROCCAN WALNUT ACCESSIONS RESEARCH USING MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR ANALYSES

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ABSTRACT

The application of different methods to assess genetic diversity is crucial for plant breeding and conservation programs. In this study, the morphological traits, the biochemical parameters and 11 ISSR molecular marker were used to investigate 11 Moroccan walnut accessions. The results of the morphological study revealed an important variation of the analyzed traits. The largest variation revealed in the position of the pad on the suture (CV=45.02%), whereas for leaflet margin no variation was found. The matrix of the Euclidean distance coefficient exhibited a large morphological variation between accessions, ranging from 3.1 to 12.1. In addition, the Mantel test showed no correlation between morphological and geographical distance of the accessions (r=0.159, p=0.84). Regarding the biochemical results, the parameters revealed a large variation. The coefficient of variation of energy value was the lowest (3.68%) while that of total flavonoids was the highest (62.02%). For the molecular analysis, the results indicated a high percentage of polymorphism (89%) as well as efficiency of the primers UBC836 and UBC841 in the research of the genetic diversity of this species. The principal component analysis divided the 66 trees into three groups independently of geographic origin and mountain range type. In addition, the altitude provided a weak effect on the structuring of the accessions (1.8%). Finally, the mantel test showed no significant correlation between the molecular and morphological markers (r= 0.591; P value= 0.998), molecular and biochemical markers (r=0.38; P=0.475), and morphological and biochemical markers (r=0.602, P=0.784). The results of this work can be useful for the conservation and improvement program of walnut in Morocco.

1.Introduction

The conservation and development of plant genetic resources are crucial for increasing agricultural productivity and sustainability (FAO, 1997). Genetic diversity is the fundamental basis for durability since it constitutes the raw material for the adaptation, evolution and survival of species and individuals, especially under modified environmental, social and pathological conditions (Hammer, 2003). The evaluation of genetic diversity has been greatly facilitated by the availability of a number of marker systems (Beyene *et al*., 2005). The morphological and biochemical markers are widely used to assess the genetic diversity, even if they are influenced by environmental factors (Zhang *et al*., 2011). In addition, DNA markers (RAPD, RFLP, AFLP, SSR, ISSR, SNP, ...) are also applied and were considered the efficient systems for genetic diversity analysis (Zhang *et al*., 2011). The choice of technic depends on the objective of the study, financial constraints, skills and available resources (Beyene *et al*., 2005).

Walnut (*Juglans regia* L.) belongs to the family *Juglandacea*e. Its natural origin is extended from the Carpathian Mountains of Eastern Europe to the Southern Caucasus, northern Turkey, Iran, Tian Shan province of western China, Himalayan states of India, Sikkim, and Bhutan (Angmo *et al*., 2013). In 2017, the global walnut production reached 3.829.626 tons for an area of 1.18 million hectares. The main nut-producing countries are China which is the first producer with 51.15% of the world production on an area of 487.007 ha. The United States of America occupies the second place with 15.18%, followed by Iran (9.27%) and Turkey (5.57%) (FAOSTAT, 2018). The walnut kernel consists of about 60% of lipids and represents a good source of macronutrients, micronutrients and other bioactive (Souci *et al*., 2008; Bolling *et al*., 2010; USDA, 2018; Yerlikaya *et al*., 2012). Indeed, previous studies revealed its richness in phosphorus, potassium, magnesium, iron, zinc, sodium, calcium and natural antioxidants such as polyphenols, folates, tannins (Li *et al*., 2006, Cosmulescu, *et al*., 2009; Tapia *et al*., 2013). As a result of this composition, the walnut has beneficial effects on human health, leading to an increasing demand in the market (Carvalho *et al*, 2010).

In this context, studying the genetic diversity and biochemical composition of the walnut provides useful information for genetic

resource management and the development of new high-yielding cultivars better adapted to drought conditions (Shamasbi *et al*., 2018). The objectives of this study are the evaluation the biochemical composition and genetic diversity of 11 walnut accessions as well as their structuration and relationships. Finally, the evaluation of the level of correlation between the morphological and molecular distance.

2. Material and methods

2.1. Plant Material

The plant material used in this study collected in September 2014 from 11 Moroccan accessions covering the main walnut-growing area. Table 1 and Figure 1 present the accessions studied with their geographical origins and ecological factors. In fact, developed leaves and healthy nuts were collected randomly from different sides and at different elevation of tree.

2.2. Morphological analysis

Based on the instructions provided by the IPGRI and UPOV descriptors (IPGRI, 1994; UPOV, 1999), a total of 31 leaf and fruit traits were considered (Table 2). The measurements were carried out on ten fresh leaves and 20 nuts after one month of harvest when the moisture content was less than 8%. (UPOV, 1999).

2.3. Biochemical analysis

The following chemical parameters are performed on walnut kernel from three trees of each accession:

The moisture was calculated on the basis of dry weight and fresh weight (Mikdat, 2010). The Ash was determined by incineration of 5 g of kernels at 600 °C for 240 minutes using a muffle furnace (AOAC, 1995). Total oil was extracted by Soxhlet apparatus using 5 g of ground kernels with N-hexane at 55-60 °C for 8 hours (AOCS, 1998). The protein content was obtained by Kjeldahl method (AOAC, 1995). The carbohydrates content and the energy value were estimated applying the following formulas: Carbohydrate content (%) = 100 % - (moisture (%) + protein (%) + oil (%) + ash (%) (Grosso *et al*., 2000) and Energy kcal = 4 x (protein g + carbohydrate g) + 9 x (lipid g) (Pereira *et al*., 2008) respectively. The crude fiber was determined with 5 g of ground samples which were digested in H_2SO_4 (1.25 %) for 45 min. Then the mixture was filtered and washed with hot distilled water before a second digestion in 100 ml of 1.25 % NaOH solution for 60 min. The resulting product was filtered and washed with hot deionized water, followed by over drying and measurement. The residue is incinerated in a furnace at 550 °C for 3 hours. Weight loss represents the amount of crude fiber. (Aryapak and Ziarati, 2014).

In order to determine the phenolic and flavonoids content as well as the scavenging activity of walnut, 5 g of ground kernel were macerated for 48 hours using 50 ml of 80 % methanol (Jacki *et al*., 2011). The Phenolic compounds was estimated according to Singleton and Rossi (1965) method. Briefly, 1 ml of extracts was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The absorbance measured at 725 nm after 90 minutes in the dark and room temperature. The results are expressed as mg of gallic acid equivalents per 100 g of DM. The flavonoids content quantified using a modified colorimetric method of Yang (2009). Briefly, 1:10 diluted extracts were mixed with distilled water and then with 0.07 ml of sodium nitrite solution (5%). Afterwards, 0.15 ml of aluminum chloride (10%) was added and allowed to react for another 6 minutes before the addition of 0.5 ml of one molar sodium hydroxide. Finally, distilled water was added to all samples in 1 ml portions. The absorbance was immediately measured at 510 nm. The results are expressed as mg rutin equivalents per 100g of DM

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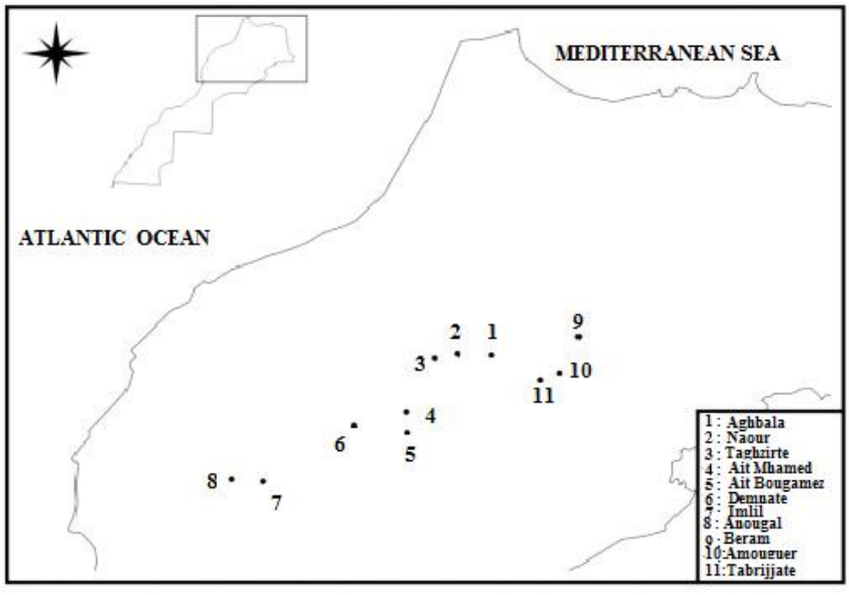

Figure 1. Geographical location of the 11 accessions studied

Table 1. Geographic and ecological parameters of walnut accessions studied

Accessions	Code	Geographic origin	Altitude (m)	Latitude N	Longitude W	Zone	Rainfall average (mm)
Aghbala	AGH	32 Km North east of Aghbala	1673	32°32’	5°39’	Middle Atlas	450
Naour	NAO	Central Naour	1300	32°29’	5°58’	Middle Atlas	600
Taghzirte	TAG	12 Km East of Tagzirte	650	32 26	6° 12’	Middle Atlas	700
Ait Bougamez	ABZ	Ait Bougamez Centre	1996	31°38’	6° 28’	High Atlas	580
Ait Mhamed	AMD	20 Km South east of Azilal	1728	31° 25’	2° 28’	High Atlas	450
Demnate	DEM	3 km South east of Demnate	932	31° 43’	6° 58’	High Atlas	350
Imlil	IML	17 km South of Asni	1763	31° 8’	7° 55’	High Atlas	459
Anougal	ANG	40 km South of Amzmiz	1569	31° 9’	8° 15’	High Atlas	681
Beram	BER	5 km South of Midelt	1521	32° 40’	4° 44’	High Atlas	210
Amouguer	AMG	40 km West of Rich	1569	32° 12’	5° 8’	High Atlas	250
Tabrijjate	TBR	70 km East of Imilchil	1831	32° 16’	4° 56’	High Atlas	319

Table 2. Morphological traits analyzed in Moroccan walnut accessions.

Leaf	Width of Pad on Suture
Leaf Length	Prominence of Pad on Suture
Leaf Width	Shape of Base Perpendicular to Suture
Number of Leaflet	Shape of Apex Perpendicular to Suture
Leaflet Shape	Prominence of Apical Tip
Leaflet Width	Structure of Surface of Shell
Leaflet Length	Shell Color
Leaflet Margin	Shell Strength
Leaf Color	Adherence of Two Halves of Shell
Rachis Color	Thickness of Shell
Nut	**Kernel**
Nut Shape in Longitudinal section through Suture	Difficulty of Removal of Kernel
The nut Shape in Longitudinal section Perpendicular to Suture	Kernel Weight
Nut Width	Kernel Percentage*
Nut Length	Kernel Fill
Nut Weight	Kernel Color
Nut: Position of Pad on Suture	Kernel Flavor

*Kernel percentage = Kernel weigh/nut weight ∗100

Regarding the scavenging activity of kernel extracts, using the free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH), it was monitored according to a method reported by Hatano *et al.* (1988). The amount of 0.3 ml of sample extracts were mixed with 2.7 ml of methanolic solution of DPPH (6.10^{-5}mol.l^{-1}). The mixture incubated in the dark for 30 min, before the absorbance measurement at 517 nm. The radical scavenging activity was calculated by the following formula.

$$\text{Scavenging (\%)} = \frac{Ab_{517\ control} - Ab_{517\ sample}}{Ab_{517\ control\ nm}} \times 100 \qquad (1)$$

Where:
$Ab_{517\ control}$: control absorbance.
$Ab_{517sample}$: control sample absorbance.

The results were expressed as µmol Trolox equivalent. g^{-1} DW.

2.4. Molecular analysis

For each accession, the young leaves belonging to six trees randomly selected, were collected. The extraction of DNA was carried out using the method described by Doyle and Doyle (1990). Then, a concentration of 10 ng/µl was prepared for PCR amplification. Indeed, 66 PCR amplification of the DNA were carried out using 11 ISSR primers, which are previously tested (Christopoulos *et al.*, 2010; Aiqing *et al.*, 2014; Shamasbi *et al.*, 2018) (Table 5). The final volume of the reaction was 12.5µl, contained: 15 ng of DNA template, 1x reaction buffer, 1 mM of $Mgcl_2$, 0.8 mM of dNTPs, 0.8 µM of each primer and 0.75 U of My Taq™ DNA polymerase. PCRs

were conducted in a Multigene gradient thermocycler (Labnet, NJ. USA). The DNA amplification is performed according to the following program: a pre-denaturation at 94 °C for 5 min, followed by 45 cycles of amplification. Each cycle includes a denaturation step at 94 °C for 45 s, a hybridization step for 45 s and an elongation step at 72 °C for 2 min. A final elongation at 72 °C for 7 min is programmed. The amplification products are separated by electrophoresis on 0.7 % agarose gel submerged in 0.5 x TBE buffer and then stained with 1 µg/µl of ethidium bromide. The DNAs were visualized under UV light using the Gel Doc system (EnduroTM GDS, Labnet). A 1 Kb DNA HyperLader™-Bioline was used for molecular weight estimation of PCR product.

2.5. Statistical analysis

Biochemical data. Average value, standard deviation, minima, maxima and coefficients of variation were determined for the studied parameters. The coefficient of variation (CV) was calculated using the following formula: CV (%) $=\frac{SD}{\bar{X}} \times 100$.

Morphological data. Average value, standard deviation and coefficients of variation were calculated for the analyzed traits. Moreover, the morphological divergence among the 11 accessions determined by the STATISTICA (Statistica StatSoft, 2011) using the UPGMA method. A Mantel test was used to research any correlation between the morphological and the geographic distance of the accession using Mx Comp of NTSys-pc software verion 2.02g.

Molecular data. The ISSR binary matrix obtained was used to carried out the principal component analysis (PCA) with the XLSTAT, (2014). The analysis of molecular variance (AMOVA) was applied to estimate the amount of difference between three altitude groups of accessions: Very low (Demnate and Taghzirte accessions), low (Naour and Anougal accessions) and moderate (Aghbala, Imlil, Ait Mhamed, Ait Bougamez, Amouguer and Beram accessions). Finally, the correlation between the Euclidean distance matrix based on morphology and the F_{ST} pairwise distance matrices obtained with ISSR markers were analyzed using the approach developed by Mantel (1967). The data analyses were performed using Mx Comp of NTSys-pc software version 2.02g.

3. Results and discussion

3.1. Variation of morphological traits

The description and coefficient of variation values related to leaf, nut and kernel trait were summarized in Table 3. The majority of examined traits showed an important value of coefficient of variation, indicting a high level of morphological variation. Indeed, the coefficient of variation varied from 45.02 % to 00.00%. In general, the large variation in leaf characters was registered in leaflets shape (CV=24.59 %), while the leaflets margins reveled with CV=00.00 % meaning no variation between accessions for this character. Concerning the leaf length and leaf width of Moroccan walnut tree, they have shown a CV of 12.29 % and 14.24 % respectively. Regarding the traits related to the nut, the highest variation was observed for the position of pad on suture, with a CV value of 45.02 %, followed by Shell Color (CV=40.50 %). However, the width, length and weight of nut are characterized by a moderate variation with a CV of 9.68 %, 10.28 %, and 26,27 % respectively. In addition, the kernel weight indicted an important variation with a CV value of 36.63 %, followed by the kernel color (CV=27.41 %), while the kernel percentage recorded a CV of 25.97%.

Morphological divergence of the 11 accessions was determined by calculating a morphological distance matrix (Table 4). The Euclidean distance coefficient matrix showed a large morphological divergence among accessions ranging from 3.1-12.1. Indeed, the Demnate and Amouguer accessions, separated by a geographical distance of 204 km, registered a coefficient of 12.1, which means that these accessions are the most divergent. Whereas, the Ait Mhamed and Anougal accessions were found to be less divergent (3.1), even though a geographic separation of 186 km. This finding is in agreement with the Mantel test, which indicated no correlation between morphological and geographical distance (r=0.159, p=0.84) for the accessions studied.

3.2. Variation of biochemical parameters

Among all biochemical parameters (Table 5), the maximum values of moisture, Ash, oil, protein, carbohydrates, energetic value, crude fiber, total phenols, total flavonoids and scavenging activity were 4 %, 2.7 %, 70.58 %, 22.75 %, 32.27 %, 734.06 Kcal, 7.52 %, 52 mg GAE 100 g^{-1}, 84.17 mg RE 100 g^{-1} and 87.3 %, while the minimum values were 0.2 %, 1.12 %, 53.72 %, 5.25 %, 641.9 Kcal, 3.32 %, 6.17 mg GAE 100 g^{-1}, 2.15 mg RE 100 g^{-1} and 72.23 %, respectively. Based on the values obtained, the oil has the abundant component. Concerning the coefficients of variation of the same parameters, they were 37.64 %, 17.31 %, 7.3 %, 25.59 %, 30.5 %, 3.68 %, 19.78 %, 56.02 %, 62.02 % and 5.48 %, respectively. The coefficient of variation of energetic value was the smallest (3.68 %) and the coefficient of variation of total flavonoids was the largest (62.02 %).

3.3. Molecular analysis

The electrophoretic gels of 66 trees using 11 ISSR primers, exhibited a high

polymorphism level in Moroccan walnut. The ISSR primers generated 135 bands, including 123 polymorphic and 12 monomorphic. These data have allowed to record a high percentage of polymorphism of 89% (Table 6). Although the results confirmed the ability of these primers to reveal the molecular polymorphism of Moroccan walnut accessions, no primers produced a specific band, thus no specific markers were revealed in the ISSR assay.

Table 3. Descriptive statistics of morphological characters analyzed

Character	Description	CV (%)
Leaf		
Leaf Length (cm)	41.47±5.10	12.29
Leaf Width (cm)	25.95±3.70	14.24
Leaflet Width (cm)	4.9±0.79	16.13
Leaflet Length(cm)	10.57±5.101.63	15.45
Leaflets color	Green	18.12
Leaflets shape	Broad elliptic	24.59
Leaflets margins	Entire	0
Rachis colour	Green	18.12
Number of Leaflet	8±1	12.3
Nut		
Nut Width (mm)	30.24±2.93	9.68
Nut Length (mm)	35.69±3.67	10.28
Nut Weight (g)	9.47±2.49	26.27
Thickness of Shell (mm)	1.6±0.42	26.33
Nut Shape in Longitudinal section through Suture	Broad elliptic	33.11
The nut Shape in Longitudinal section Perpendicular to Suture	Broad elliptic	30.43
Nut: Position of Pad on Suture	On upper 2/3 of nut	45.02
Width of Pad on Suture	Medium	26.35
Prominence of Pad on Suture	Medium	21.41
Shape of Base Perpendicular to Suture	Rounded	37.91
Shape of Apex Perpendicular to Suture	Truncate	28.36
Prominence of Apical Tip	Weak	27.87
Structure of Surface of Shell	Moderately grooved	27.67
Shell Color	Medium	40.5
Shell Strength	Intermediate	21.55
Adherence of Two Halves of Shell	Medium	23.01
Kernel		
Kernel Weight (g)	3.7±1.36	36.63
Kernel Percentage (%)	38.29±9.95	25.97
Difficulty of Removal of Kernel	Medium	26.56
Kernel Fill	Well	24.08
Kernel Color	Light amber	27.41
Kernel Flavor	Satisfactory	27.09

CV: Coefficient of variation

Table 4. Morphological distance among the accessions

	Tagzirte	**Tbrijjate**	**Amouguer**	**Anougal**	**Ait Mhamed**	**Imlil**	**Beram**	**Aghbala**	**Naour**	**Ait bougamez**	**Demnate**
Tagzirte	0										
Tbrijjate	6.2	0									
Amouguer	6.3	7.41	0								
Anougal	8.11	4.9	10.4	0							
Ait Mhamed	6.6	3.84	9.2	**3.1**	0						
Imlil	8.42	5.64	7.3	6.9	5.82	0					
Beram	5.89	3.73	6.4	5	4.28	5.4	0				
Aghbala	7.33	8.18	10.9	7.6	7.4	11.2	7.95	0			
Naour	5.59	5.51	7.2	7.3	5.52	7.7	5.53	6.2	0		
Ait bougamez	5.48	5.85	3.9	8.5	6.98	5.9	5.38	9.3	5.88	0	
Demnate	8.98	8.32	**12.1**	6.6	7.4	11.1	6.95	8	9.3	11.2	0

Table 5. Minima, maxima, average value, standard deviation and coefficients of variation of biochemical parameters

Parameters	Min	Max	Mean	SD	CV (%)
Moisture (%)	0.2	4	2.52	0.95	37.64
Ash (%)	1.12	2.7	2.08	0.36	17.31
Oil (%)	53.72	70.58	60.86	4.45	7.3
Protein (%)	5.25	22.75	15.59	3.99	25.59
Carbohydrates (%)	5.36	32.27	18.92	5.77	30.5
Energetic value (Kcal)	641.9	734.06	685.85	25.24	3.68
Crude fiber (%)	3.32	7.52	5.51	1.09	19.78
Total phenols (mg GAE 100 g^{-1})	6.17	52	25.79	14.58	56.02
Total flavonoids (mg RE 100 g^{-1})	2.15	84.17	37.18	23.08	62.09
DPPH (%)	72.23	87.3	79.73	4.37	5.48

Min: Minima, Max: Maxima, SD: Standard deviation, CV: coefficient of variation

Furthermore, the PCA was performed to determine the main primers contributing to the classification of walnut accessions. The first two components explained 15.78% of the observed variation (Table 7). The PC1, with an Eigen value of 9.76, contributed 8.07% of the total variability, while PC2, with an Eigen value of 9.08, accounted for 7.51% of the total variability. In PC1, primers USB811, USB834, USB836, USB841, and UBC889 with the eigenvalues of 24.55, 20.05, 18.79, 10.94, and 17.15, respectively, were the most efficient. Whereas the main primers contributing to PC2 were UBC818, UBC836, UBC841 and UBC889 with eigenvalues of 18.48, 29.85, 16.47 and 10.35, respectively. This study indicates that primers UBC836 and UBC841 may be the most suitable for investigating molecular variation in this species in future studies, while the USB855 with low contribution is the least informative. In addition, the PCA biplot divided the 66 trees into three groups. The first one formed by one accession (DEM2) from the Haut Atlas and the second group composed specifically by trees belonging to ANG, TBR, IML and BER from Haut Atlas Mountain as well as some trees from ABZ, AMG, DEM and AGH. The last group comprised by all trees of NAO and TAG belong to Middle Atlas Mountain, which were added to the trees from ABZ, AGH, AMG and DEM (Figure 2). According to the results obtained, the 66 trees are structured independently of their origin geographic and mountain type.

For hierarchical AMOVA, it was applied to examine the structuration of 66 trees according to altitude level (Table 8). The result showed a low percentage of genetic variation among the altitude groups (1.8 %). Therefore, altitude has no significant effect on the structuration of these accessions.

Table 6. Properties of 11 ISSR primers used in this study.

ISSR Loci	Sequence (5'-3')	Simple size	Number of bands amplified				Polymorphism (%)	Monomorphism (%)
			Total band	Polymorphic band	Monomorphic band	Unique loci		
UBC 807	(AG) 8T	66	9	9	0	0	100	0
UBC 810	(GA)8T	66	10	7	3	0	70	30
UBC 811	GA(AG)7C	66	10	10	0	0	100	0
UBC 814	(CT) 8A	66	10	8	2	0	80	20
UBC 818	(CA) 8 G	66	7	6	1	0	85	15
UBC 834	(AG) 8YT	66	15	14	1	0	93	7
UBC 836	(AG) 8YA	66	17	17	0	0	100	0
UBC 840	(GA) 8YT	66	14	12	2	0	85	15
UBC 841	(GA)8YC	66	19	19	0	0	100	0
UBC 855	(AC) 8YT	66	9	6	3	0	66	34
UBC 889	(AC) 7	66	15	15	0	0	100	0
Total		726	135	123	12	0		
Average			12,27	11,18	1,09	0	89	11

Note. Y = (C, T).

Table 7. Eigen-vectors on the two principal components

Primers	PC1	PC2
UBC807	2.73	5.64
UBC810	2.67	1.65
UBC 811	24.55	1.78
UBC 814	1.45	3.63
UBC 818	0.27	18.42
UBC 834	20.05	3.95
UBC 836	18.79	29.85
UBC 840	1.19	4.97
UBC 841	10.94	16.47
UBC 855	0.2	3.28
UBC 889	17.15	10.35
Eigen value	9.762	9.088
Variance (%)	8.068	7.51
Cumulative	8.068	15.578

Table 8. Analysis of molecular variance (AMOVA) for 11 walnut accessions

Source of variation	d.f	Sum squares	Variance component	Percentage of variation	*F Statistic*
Among altitude groups	2	34.083	0.11995 Va	1.89	FCT : 0.018
Among accessions Within groups	8	119.583	1.74132 Vb	27.37	FSC : 0.279***
Within accessions	55	247.500	4.50000 Vc	70.74	FST : 0.2926***
Total	65	401.167	6.36127		

Signification level (p<0,05), *** Very high significant.

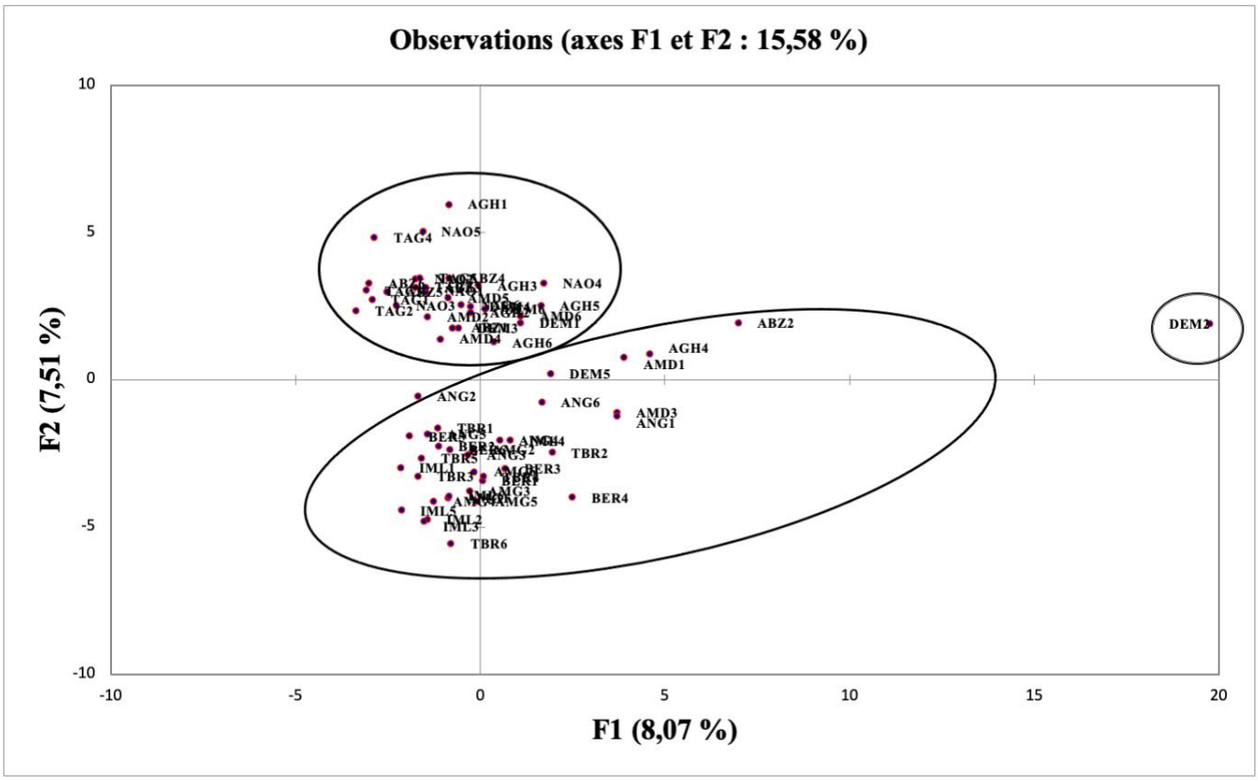

Figure 2. The plot of the 11 accessions examined on ISSR marker

3.4. Correlation between molecular and morphological analysis

The congruence between morphological and molecular markers is analyzed by the Mantel test. The result of correlation between morphological and molecular distance matrices, molecular and biochemical distance matrices, and morphological and biochemical distance matrices exhibited no correlation between these three markers (r= 0.591; P value= 0.998; r=0.38; P=0.475; r=0.602, P=0.784 respectively).

3.5. Discussion

The morphological results showed considerable variation specially for the position of pad on suture and shell color, which showed the highest coefficient of variation (45.02 and 40.50 % respectively), while the leaflets margin was Entire for all accessions (00.00 %). In

addition, the result obtained revealed a great variation of leaf length and width (12.29 and 14.24 % respectively). These values are higher than that registered by Ghanbari *et al.* (2018), in Iranian walnut (4.41 and 0.625 % respectively). Regarding the length, width and weight of the nut, they recorded a CV of 9.68, 10.28 and 26.27 % respectively. Similarly, Khadivi-Khub *et al.* (2015) published the coefficients of variation of nut length of 12.87%, nut width of 11.62% and nut weight of 20.49% in Iranian walnut. Other similar results were revealed in Romanian walnut by Cosmulescu, (2013) (9.53, 11.42 and 19.18% respectively). For the kernel, presenting the edible part, revealed with a CV of 36.63% for kernel weight, 25.97% for kernel percentage and 27.41% for kernel color. These values are higher of those reported by Khadivi-Khub *et al.* (2015) (25.76, 11.99 and 24.19% respectively) and by Cosmulescu, (2013) (22.34 and 12.19% respectively). These finding provides large combinations of traits in order to obtain a genotype with desired traits to satisfy the requests of breeders, farmers, consumers and industry. Demnate and Amouguer accessions, both belonging to the High Atlas and separated by 204 km, revealed the great morphological divergence while the weak divergence is found between Angual and Ait Mhamed of the High Atlas and distant by 186 km. This result suggests that the long distance between accessions leads to large differentiation. In contrast, the Mantel test, revealed no significant correlation between geographic and morphological distances (r=0.159, p=0.84), indicating that geographic distance is not the main factor in the morphological differentiation of walnut accessions. Indeed, the same climatic conditions have an equal effect on morphological traits (Khadivi-Khub *et al.* 2015). Nevertheless, a large geographic distance may limit gene flow (Sefc *et al.* 2000), as well as the small population effect, genetic drift and geographic isolation may be the main factors causing genetic differentiation among populations (Li *et al.* 2018). These random factors could be an important reason for the absence of correlation between geographic and genetic distances (Zhang *et al.*, 2015).

The results of the biochemical parameters of Moroccan walnut revealed large level of moisture (0.2-4 %), Ash (1.12-2.7 %), oil (53.72-70.58 %), protein (5.25-22.75 %), carbohydrates (5.25-32.27 %), energetic value (641.9-734.06 Kcal), crude fiber (3.32-7.52 %), total phenols (6.17-52 mg GAE 100 g^{-1}), total flavonoids (2.15 -84.17 mg RE 100 g^{-1}) and scavenging activity (72.23-87.3 %). These results are in agreement with the ranges recorded by Erdoğan *et al.* (2021) which are 53.75-71.43 % for total oil, 10.21-20.71 % for protein, 14.31-27.52 % for carbohydrates and 1.64-3.32% for Ash content. Regarding the total phenol content obtained in this study, it is comparable to that registered in the superior genotypes of Iranian walnuts (46.6 to 61.5 mg GAE g-1) (Sarikhani *et al.*, 2021). In fact, recent studies on walnut kernels showed different groups of monomeric and polyphenolic compounds with great antioxidant activity (Zhang *et al.*, 2009). High content of phenolic compounds and protein in walnut kernels had positive effect on human health (Labuckas *et al.*, 2008; Zhang *et al.*, 2009). According to the coefficient of variation ranging from 3.68 to 62.09 %, there are a great variation of biochemical parameters between the different accessions, providing a huge breeding selection potential.

The efficiency of a molecular marker technic is determined by the percentage of polymorphism demonstrated among the accessions under investigation. The results obtained in Moroccan walnut registered a polymorphism level of 89 %. Similarly, several studied reported a high polymorphism rate in the walnut tree (Malvolti *et al.*, 2010, 73.8 %; Christopoulos et al., 2010, 82.8 %; Aiqing *et al.*, 2014, 92.31 %). This finding is consistent with the common observation of high variation levels detected in long-lived, wind-pollinated tree species (Streiff *et al.*, 1998; Victory *et al.*, 2006). Effectively, the ISSR primers used in this work appears very useful, especially the UBC836 and UBC841 primers, which revealed the high eigenvalues in two first PC. This result can be confirmed by the values of PIC, MI and Rp (Kabiri *et al.*, 2019).

Concerning the classification of walnut trees into three groups, it was carried out undependably of the geographic origin and the mountain range with a weak effect of altitude (1.8%). These finding is in addition to the low genetic differentiation between regional (13.24%) and bioclimatic groups (1.31%) of 11 Moroccan walnut accessions (Kabiri *et al.*, 2019). These low rates of differentiation indicated limited adaptation of Moroccan walnut accessions to the local environment. Similarly, Farrokhi Toolir and Mozaffari, (2020) reported that ten genotypes of wild walnuts originating from Iran, are grouped independently of the geographical distances between them. Whereas, Geethanjali *et al.* (2017) indicated a clustering of genotypes into two main groups corresponding to the geographic origins.

The result of correlation analysis between morphological and molecular matrix was not significant (r= 0.591; P value= 0.998). The same result was observed in walnut trees grown in Iran (Ebrahimi *et al.*, 2011), as well as in an olive cultivars from Campania (Corrado *et al.*, 2009). Moreover, the comparison of ISSR and morpho-agronomic traits similarity matrices was not significant in the study of genetic variation among cumin accessions from Iran, Syria and Afghanistan (Rostami-Ahmadvandi

et al., 2013). There are two reasons why there is little or no correspondence between molecular and morphological variation. On the hand, molecular markers generally cover a large genome, including both coding and non-coding regions. On the other hand, non-specific molecular markers (such as ISSR) are generally used to measure genetic diversity and are not subject to artificial selection (Semagn, 2002).

4. Conclusions

Walnut with its various benefices, occupies a great place in nutritional history and habits of Moroccan people. These studies have shown a biochemical richness as well as the efficiency of morphological and molecular markers in the study of genetic diversity of walnut. Moreover, the Mantel test showed no significate correlation between the morphological and molecular markers. These finding would permit the selection of a high-performance genotype with the desired characteristics to improve yield and quality of Moroccan walnut.

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OPTIMIZATION OF SPRAY DRYING FOR COCONUT MILK POWDER USING RESPONSE SURFACE METHODOLOGY AND INVESTIGATION OF THE POWDER PROPERTIES

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ABSTRACT

The main objective of this work was to optimize the spray drying of coconut milk using response surface methodology (RSM). Maltodextrin was added to the feed coconut milk as the carrier agent in the laboratory scale experiments and the same were designed using Box-Behnken design approach. The effect of the chosen input variables on the responses namely product yield, moisture content, hygroscopicity, and angle of repose of the powders were evaluated using 3D surface plots. Among the chosen input variables of the process, the temperature of the inlet air in the spray drying chamber was found to significantly affect the characteristics of the powder. The flowability properties of the synthesized powders were experimentally obtained and the values were found to be satisfactory. The functional and morphological properties, and color of the powder produced at the optimal condition were compared to those of a commercially available coconut milk powder. For the synthesized powder of the optimal condition, an enhancement of 30°C for the glass transition temperature was seen over to that of the commercial powder.

1. Introduction

The storage of aqueous extract of coconut endosperm (commonly known as coconut milk) in its native liquid form is not suitable due to rancidity caused by microbial activity (Seow and Gwee 1997; Hettiarachchi et al. 2019). Coconut milk is an oil-in-water emulsion and very unstable due to insufficient quality and quantity of the protein mass and a high percentage of fat. These factors culminate to the separation of milk into two separate layers (creamy layer and dense aqueous serum) upon storage (Tangsuphoom and Coupland 2008; Maidannyk et al. 2020). Also, coconut milk is highly susceptible to spoilage (undesirable odor and taste) due to microbial activity, chemical deterioration due to lipid autoxidation, lipolysis, and oxidation of unsaturated fatty acids. Considering the importance of coconut milk in the food industry due to its nutritional importance, anti-cancerous and anti-inflammation properties, several methods were proposed to increase the shelf-life of coconut milk. For short-term storage, nevertheless, heat treatment alone is effective. Sterilization is an option for long-term storage but the physical instabilities within the milk due to long-term standing cannot be avoided (Ho et al. 2019).

In the past, spray drying or dehydration of coconut milk to powder form was shown as a commercially viable option for long-term storage with no major loss of the nutrients (Zafisah et al. 2018; Fournaise et al. 2020). Although some inevitable loss of favoring volatile components cannot be overruled in the drying process, almost all of the original milk attributes can be achieved under normal

conditions when the milk is prepared from the powder.

Spray drying is considered as a better option for the drying thermo-sensible liquids due to shorter exposer times of the liquids to higher temperatures in the drying chamber with almost no thermal degradation of the milk constituents (Sosnik and Seremeta 2015; Voronin et al. 2021). The carrier agents added to the feed milk can effectively encapsulate the hydrophobic and hydrophilic active compounds and increase the shelf-life of the milk powder (Chávez-Servín et al. 2015).

In the spray drying of coconut milk, higher viscosity of the milk due to the presence of fat globules can result in the improper spraying and the nozzle clogging in the drying chamber. To avoid these undesirable features, carrier agents are added to the feed milk for the encapsulation of the fat content and to prevent the formation of lumps and avoid powder particles sticking to the wall of the drying chamber. Maltodextrin, lactose, whey protein, and Gum arabic are generally used as carrier agents for this purpose (Carlos et al. 2018). Further, addition of microencapsulating agents to the feed milk can increase the product yield up to 60% by weight (Simuang et al. 2004; Manikantan et al. 2015). Emulsifiers and stabilizers are also added to the precursor milk for the stability in avoiding the formation of larger oil droplets (Hassan 1985). As the desirable physio-chemical properties for the powder (stability, solubility, flow properties, particle size and shape, density, and compatibility, etc.) can be achieved in the spray drying process (Kim et al. 1996), the production of coconut milk powder using spray drying was highly recommended (Negizj and Lagergren 1995; Langrish and Fletcher 2001; Millqvist-Fureby 2003; Krishnan et al. 2005; Piatkowski and Zbicinski 2007).

Another important parameter of the powder is its glass transition temperature (Adhikari et al. 2005). It is defined as the temperature at which amorphous glassy powder changes to rubbery type (Le Meste et al. 2002). If the glass transition temperature of the powder is higher than the maximum storage temperature, then lumping of the powder particles can be prevented upon storage. Commonly, the sticky nature of the particles can be seen at 10 to 20°C above the glass transition temperature. Hence, higher glass transition temperatures are desired for longer shelf-life. The flow properties of the powder are also equally important in the design of handling machines (Prescott and Barnum 2000). The other important functional properties are morphology and density. The hydration properties (wettability, sink ability, dispersibility and solubility) of the powder must be desirable in the successful preparation of milk from the powder (Schober and Fitzpatrick 2005; Hammes et al. 2015).

For several engineering applications, response surface methodology (RSM) has been successfully proven as an effective option for the optimization of input parameters (Nwabueze 2010). RSM is a collection of several mathematical and statistical techniques and can be utilized to design new formulations in the development of useful products (Islam Shishir et al. 2016). The Central Composite Design (CCD) and Box-Behnken Design (BBD) are the most famous approaches and these methods are generally chosen based on the application of interest and operability. RSM was used to optimize the experimental parameters for the spray drying of high-value oyster byproducts and the development and utilization of aquatic shellfish byproducts (Chen et al. 2018). To optimize the spray drying of pink guava extract, RSM was used with central composite face-centered design of experiments (Islam Shishir et al, 2016).

The four salient parameters of the spray drying of coconut milk are feed flow rate, inlet temperature of the air, atomizer speed and the concentration of the carrier agent in the feed. At higher feed flow rates, the rate of evaporation drastically reduces. The formation of a rigid impermeable layer on the droplets hinders the uniform drying at higher temperatures inside the drying chamber. Due to high-fat content of the coconut milk, it is necessary to add carrier agents to ease the spray drying process.

The main objective of the proposed work was to find the optimal operating parametric conditions for the production of coconut milk powder of improved quality. Three input parameters namely inlet temperature, concentration of carrier agent and atomizer speed were selected for this purpose. Box-Behnken Design (BBD) was employed for the design of lab-scale experiments. Using RSM, the effect of the three input parameters were correlated with the product yield and three important properties of the powders namely angle of repose, hygroscopicity, and moisture content. The flow properties of the powders were experimentally calculated and presented. The morphology, angle of friction and glass transition temperature of the milk powder produced at the optimal conditions were compared to those of a commercial coconut milk powder sample.

2. Materials and methods

2.1. Materials

Coconuts of 8-9 months of age were collected and de-husked. The endosperms were made brown testa free and the white coconut endosperm flesh was thoroughly washed and grated. The grated coconut flesh was kept in a measuring jar of 1-liter capacity and the container (top open to atmosphere) was partially immersed in a hot water bath (50 °C) for 15 minutes with no physical contact of the hot water and coconut flesh. Thereby, the smoothened flesh was cooled to room temperature and mixed with distilled water (1:1 by mass). The mixture was taken on a cheesecloth, and the cloth was then wrapped followed by mechanically squeezing for the milk. The coconut milk was pasteurized at 72 °C for 1 min and cooled to room temperature. In the current work, a carbohydrate component namely Maltodextrin (DE=20) was employed as the carrier agent.

2.2. Experimental procedure

Spray drying experiments were performed in a spray mate JISL type spray dryer. The dryer can operate up to 250°C of the inlet air. In Figure 1, a schematic diagram and the working model of the spray dryer in the co-current operation mode was shown with the locations of the temperature measurements. Precisely, the hot air from the heater, through an air distributor, comes into contact with the sprayed coconut milk in the drying chamber. The inlet pressure of air was kept at 2 atm throughout the experiments.

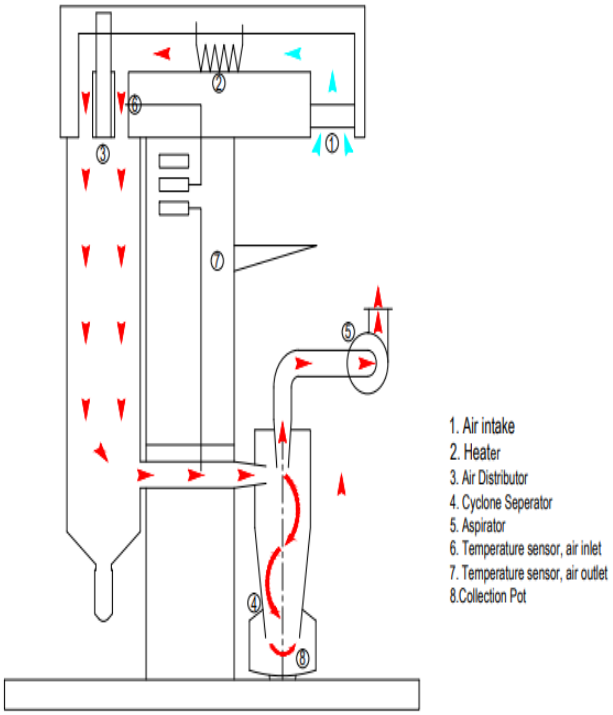


Figure 1. Schematic diagram of the airflow during spray drying

2.3. Characterization

The hygroscopicity of the milk powder was determined as follows. A sample of milk powder was placed in a desiccator for one week under a saturation solution of Na_2SO_4. The hygroscopic moisture was expressed as g of moisture per 100 g dry solids. The hygroscopicity was calculated as given below.

$$Hygroscopicity = \frac{(W_f - W_i)\times 100}{W_i \times \left(100 - \frac{(W_f - W_i)}{100}\right)} \quad (1)$$

where W_f and W_i are the final and initial weights of the powder. The moisture content of dried powder was determined gravimetrically by oven drying at 102°C for 2 hours (standard AOAC

1990). In general, lower values of hygroscopicity and moisture content are desired for the food powders.

The angle of repose is an indicator of the flowability of the powder. Smaller values of the angle of repose indicate better flowability of the powders and this parameter is a crucial one in the design of processing and packaging equipment. The angle of repose (Θ) of the powder was determined as the inverse of the tangent of the ratio of height (*h*) to the radius (*r*) of the cone of powder formed when 100 g of powder is poured through a funnel.

$$\Theta = tan^{-1}\left(h/r\right) \quad (2)$$

The yield of coconut milk powder (in percentage) is expressed as

$$Yield, \text{wt}\% = \frac{W_2}{W_1} \times 100 \quad (3)$$

where W_2 = weight of coconut milk powder; W_1 = weight of coconut milk plus carrier agent. The moisture contents of the powder samples were evaluated with moisture Analyzer AND MX – 50 with a tolerance limit of 0.01 %.

The insolubility index is an indicator of the solubility of the powder in water while reconstitution. IDF Standard 129A method summarizes the evaluation of insolubility of powders. To calculate insolubility index, 10 g of dry powder is added to 100 mL of water at 50°C with continuous mixing for 5 min. The suspension was stirred with a spatula and 50 mL was filled to a graduated 50 mL centrifuge tube and the tube was centrifuged at 5070 rpm. The same procedure was repeated after siphoning off the sediment-free liquid. The sediment in the bottom was dried at 70°C overnight until it approaches a constant weight. The insolubility index is expressed as the weight of the sediment after drying (mg). Lower insolubility index values are desired for the powders.

Dispersibility is the ability of the powder to separate and form individual particles upon mixing. The dispersibility of the powder is calculated as,

$$\%\ Dispersibility = \frac{(10+a)\,TS}{a\left(\frac{100-b}{100}\right)} \quad (4)$$

where a = Powder in g, b = percentage of moisture content, TS =Total solids in the coconut milk.

The powder characteristic to quickly form a stable suspension can be depicted as the solubility time. It is expressed as the time taken for the uniform suspension formation after mixing. Bulk density (ρ_b) is a measure of the mass of powder occupying a unit volume that include the inter particle voids of the powder. Tapped density (g/cm^3) of powder is defined as the ratio of the mass of the powder to the volume occupied by the powder after it has been tapped for a defined period. The tapped density (ρ_t) of the powder can be calculated as $\rho_t = M/V_f$, where M and V_f are mass of powder in grams and tapped volume in milliliter or cm^3, respectively.

Using ρ_b and ρ_t values, the flowability characteristics of the powder such as Compressibility index (*Carr's Index*) and *Hausner ratio* are determined as given below.

$$Carr's\ Index = \frac{\rho_t - \rho_b}{\rho_t} \times 100 \quad (5)$$

$$Hausner\ ratio = \frac{\rho_t}{\rho_b} \quad (6)$$

The thermal characterization of the milk powders was performed using differential scanning calorimetry (DSC). The DSC Thermogram can be used to estimate the glass transition temperature of the powder. The morphology of the powders was evaluated in the scanning electron microscopy (SEM) imaging. It is very easy to visualize caking, bridge formation among the powder particles using this technique.

2.4. Box-Behnken Design of experiments

The spray drying experiments were systematically designed and the responses were statistically interpreted for the optimization of chosen independent variables. The three selected input variables namely inlet air temperature (*A*), atomizer speed (*B*), the

concentration of the carrier agent (*C*) were considered as independent variables. Feed flow rates of the milk and inlet air were kept at their maximum allowable values. Three different coded levels (Low (-1), Medium (0), High (+1)) were selected for the Box-Behnken Design of experiments (DOE) approach. For each response, a quadratic polynomials correlating the independent variables was obtained. The experimental design matrix of the Box-Behnken design is depicted in Table 1. It shows the range of the values of independent variables and response variables of the performed experiments.

The constraints for the optimization were maximum product yield, minimum values of hygroscopicity and moisture content, and the angle of repose should be between 10° and 35°. The predicted and adjusted R^2 values, and coefficient of variation (*CV*) were evaluated to understand the lack of the model fit for the data. terms concerning the independent variables were generated ($p < 0.01$). The experimental data fitted for a response R_j and independent variables *A*, *B*, and *C* is given below.

The analysis of variance (ANOVA) against each response was generated and all the interaction

$$R_j = \beta_{0j} + \beta_{1j}A + \beta_{2j}B + \beta_{3j}C + \beta_{12j}AB + \beta_{13j}AC + \beta_{23j}BC + \beta_{11j}A^2 + \beta_{22j}B^2 + \beta_{33j}C^2 \quad (7)$$

For j = 1 to 4 where β_{0j} is a constant; β_{1j}, β_{2j}, and β_{3j} are the coefficients of linear regression; β_{12j}, β_{13j}, and β_{23j} are the coefficient of interaction & β_{11j}, β_{22j} and β_{33j} are the coefficients of quadratic regression.

3. Results and discussions

Several preliminary experiments were performed to identify the range of selected independent operating parameters. The suitable temperature range for the inlet air was found to be 160°-190°C. Watery product or insufficient drying condition was observed when the temperature of inlet air was kept below 160°C. insufficient drying was very much bothersome with the nozzle clogging in the spray drying chamber. At lower temperatures of the inlet air, stronger outer crust may not form on the powder particles. Above 190°C, the product color was dark due to overheating. Powders with desirable properties were produced when the maltodextrin (DE=20) concentration in the feed milk is in the range of 5% - 15% (w/w). Fig. 2a shows the sample containing coconut milk and MD (DE=20) before spray Drying. Fig 2b shows the dripping of milk in the spray drier column when the air inlet temperature is < 160°C.

Table 2 shows the experimental range and level of variables as per the Box-Behnken Design. The experiments were performed at different factor values, called levels. Each experiment involves the combinations of the levels of factors. In BBD, only three levels required to run an experiment and the three levels of independent variables are chosen were also depicted in Table 1.

Figure 2. a) Coconut Milk b) dripping of coconut milk for inlet air temperature <160°C

The experimental results were utilized to derive the polynomial equations. The optimal values of independent variables for the given constraints were obtained with the Response Surface Methodology (RSM) in Design Expert® software.

Table 1. Design matrix of the Box-Behnken design obtained from RSM

Std	Run	*A*	*B*	*C*	R_1	R_2	R_3	R_4
		°C	w/w	rpm	g	deg	g/100g	%
16	1	0.00	0.00	0.00	5.1	32.005	32.01	6.8
3	2	-1.00	1.00	0.00	5.11	29.56	27.01	8.5
14	3	0.00	0.00	0.00	5.1	32.005	32.01	6.8
2	4	1.00	-1.00	0.00	8.21	25.1	37.1	6.8
1	5	-1.00	-1.00	0.00	8.1	29.61	28.6	8.2
9	6	0.00	-1.00	-1.00	5.6	31.27	32.87	6.75
13	7	0.00	0.00	0.00	5.1	32.005	32.01	6.8
5	8	-1.00	0.00	-1.00	5.1	29.4	27.1	6.3
7	9	-1.00	0.00	1.00	8.5	31.27	27.45	7.9
4	10	1.00	1.00	0.00	5.1	25.3	38.01	5.1
11	11	0.00	-1.00	1.00	8.15	31.27	27.4	7.56
8	12	1.00	0.00	1.00	5.1	25.1	37.1	5.32
15	13	0.00	0.00	0.00	4.98	32.005	32.01	6.8
12	14	0.00	1.00	1.00	5.1	31.55	31.9	7.25
10	15	0.00	1.00	-1.00	5.01	32.56	32.65	7.12
6	16	1.00	0.00	-1.00	4.25	25.73	38.05	5.62
17	17	0.00	0.00	0.00	5.1	32.005	32.01	6.8
A: Temperature; *B*: Concentration; *C*: Atomizer speed R_1: Yield %, R_2: Angle of repose, R_3: Hygroscopicity, R_4: Moisture content								

Table 2. The Experimental range and level of variables chosen for the Box-Behnken design

Variable, unit Symbol	Low (-1)	Center (0)	High (+1)
Inlet temperature, °C	160	170	180
Maltodextrin concentration, w/w	5	10	15
Atomizer speed, rpm	1400	1700	2000

3.1. Product yield

The product yield results are plotted in Figure 3. As shown in Fig 3(a), for a fixed concentration of Maltodextrin, the yield decreased with the increase of inlet air temperature. For a fixed temperature, the effect of maltodextrin concentration on the yield was almost absent. Atomizer speed and inlet air temperature are having a significant impact on yield (Fig 3(b)). At the highest values of inlet temperature and atomizer speed (190°C and 2000 rpm respectively), the maximum yield was seen. Similarly, maximum yield was obtained at the highest values of maltodextrin concentration and atomizer speed as depicted in Fig 3(c). The heat transfer rate increases and becomes effective with respect to the increase in the atomizer speed. Overall, the product yield was mainly quite sensitive with respect to inlet air temperature and atomizer speed. The polynomial equation in terms of coded factors for the product yield is given below.

$$R_1 = 5.10 - 1.65\,A + 0.041\,B + 0.04\,C - 0.078\,AB - 0.095\,AC + 0.1\,BC + 1.48\,A^2 - 0.051\,B^2 + 0.061\,C^2$$

ANOVA results of the fitted polynomials of the product yield are shown in Table 3. The model is significant as the Model F value is 468.84. There is only a 0.01% chance that a "Model F-value" of this large could occur due to noise. Since the values of "Prob > F" are less than 0.05, it indicates the significance of model terms. Hence, in the predicted polynomial, the

terms encompassing *A*, *B*, *C* and *AB* are significant. A value of 0.9735 for the "Pred R-Squared" is in reasonable agreement with the "Adj R-Squared" of 0.9962. The well-dispersed nature of the data with better reproducibility and repeatability was seen as the *CV* (Coefficient of variation) less than 10 %.

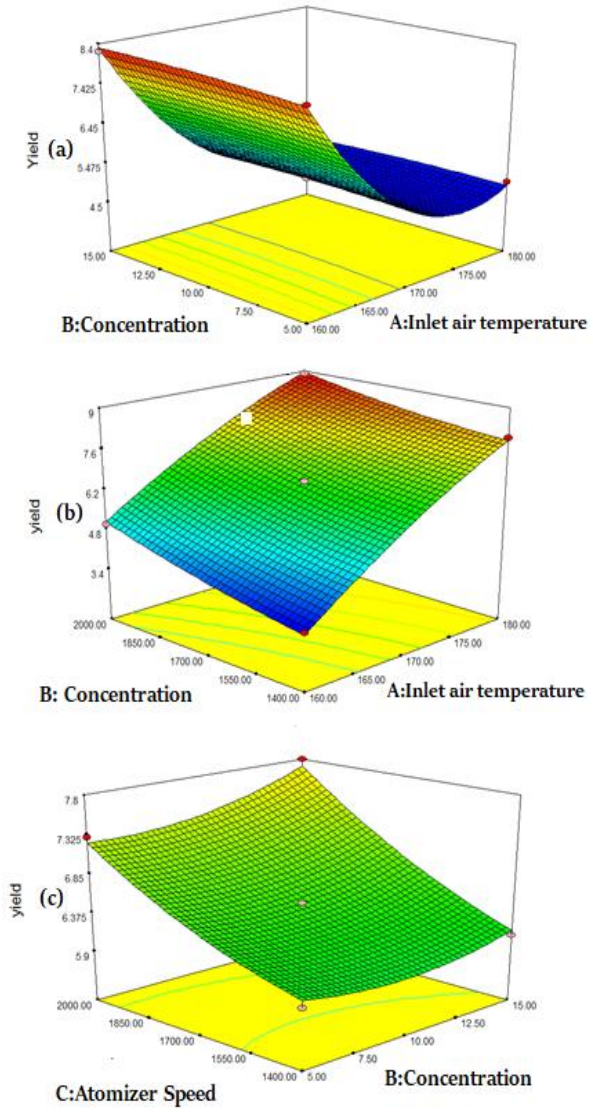


Figure 3. Response surface plots: effect of independent variables on the product yield

The probability value of 0.01 signifies the statistical stability of the responses and their agreement with a lack of fit value. The greater adequate precision value suggests that the respective model equations are valid for the experimental data. Figure 4 shows the experimental and predicted values of yield suggesting a close agreement between the predicted and actual values of the yield.

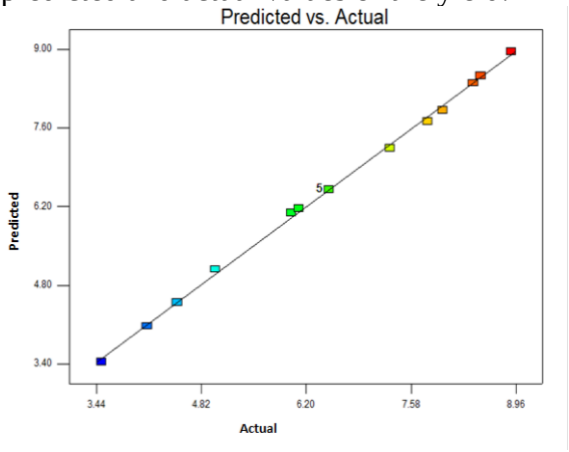


Figure 4. Experimental and predicted yield of powder

3.2. Angle of repose (Ɵ)

If the angle of repose value is less than 40°, the flow behavior of the powder is classified as an easily flowable type. In Figure 5, the variation of the angle of repose concerning the three independent variables is represented. The values of angle of repose were less than 40° in all the experiments. With respect to the inlet air temperature, the angle of repose exhibits a maximum value of 32.4 ℃. The effect of atomizer speed and concentration of the carrier agent was quite insignificant as the variation in the value of Ɵ is very small as shown in Fig 4(a). The inlet air temperature has a significant effect on the angle repose in comparison to that of the other two input variables as shown in Fig 4(b) and 4(c). Maximum values of angle repose were observed approximately in the range of 165^{o}-170^{o}C and ANOVA results are satisfactory (data not shown). The angle of repose (Y_2) is written as

$$R_2 = 32.0 - 2.07\,A + 0.17\,B + 0.27\,C + 0.062\,AB - 0.20\,AC + 0.35\,BC - 4.26\,A^2 - 0.35\,B^2 - 0.19\,C^2$$

Table 3. Analysis of variance (ANOVA) for the fitted polynomial model of yield

Source	Sum of squares	df	Mean Square	F value	P-Value Prob > F
Model	31.30	9	3.48	468.84	<0.0001 significant
A-Inlet air temperature	21.81	1	21.81	2940.61	< 0.0001
B-Concentration	0.014	1	0.014	1.84	0.2176
C-Atomizer speed	0.013	1	0.013	1.73	0.2304
AB	0.024	1	0.024	3.24	0.1149
AC	0.036	1	0.036	4.87	0.0632
BC	0.044	1	0.044	5.95	0.0449
A^2	9.27	1	9.27	1249.62	< 0.0001
B^2	0.011	1	0.011	1.49	0.2616
C^2	0.016	1	0.016	2.13	0.1879
Residual	0.052	7	7.418×10^{-3}		
Lack of Fit	0.052	3	0.017		
Pure Error	0.000	4	0.000		
Cor Total	31.35	16			
Std. Dev	0.086	R^2	0.9983		
Mean	5.80	Adj R^2	0.9962		
C.V. %	1.48	Pred R^2	0.9735		
PRESS	0.83	Adeq Precision	54.291		

3. Hygroscopicity

Hygroscopicity is an important parameter for the long time storage and shelf life. The hygroscopicity values of food powders should generally be lesser. With a careful observation of Fig 6(a-c), one can see that the inlet air temperature had a great significance on the hygroscopicity of powder as the hygroscopicity increased with the increase of inlet air temperature of the spray drying. The increased removal of mositure quantity from the powder with the increase of inlet air temperature could be a reason for this trend. The hygroscopicity increased from 27% to 39 % for the temperature increase from 160°C to 180°C (Fig 6(a)). The effect of the other two independent variables was quite insignificant. The hygroscopicity is related to the three independent variables as given below

$$R_3 = 32.14 - 5.48\,A - 0.29\,B - 0.16\,C - 0.025\,AB + 0.23\,AC + 0.047BC + 0.88A^2 + 0.4B^2 - 0.24\,C^2$$

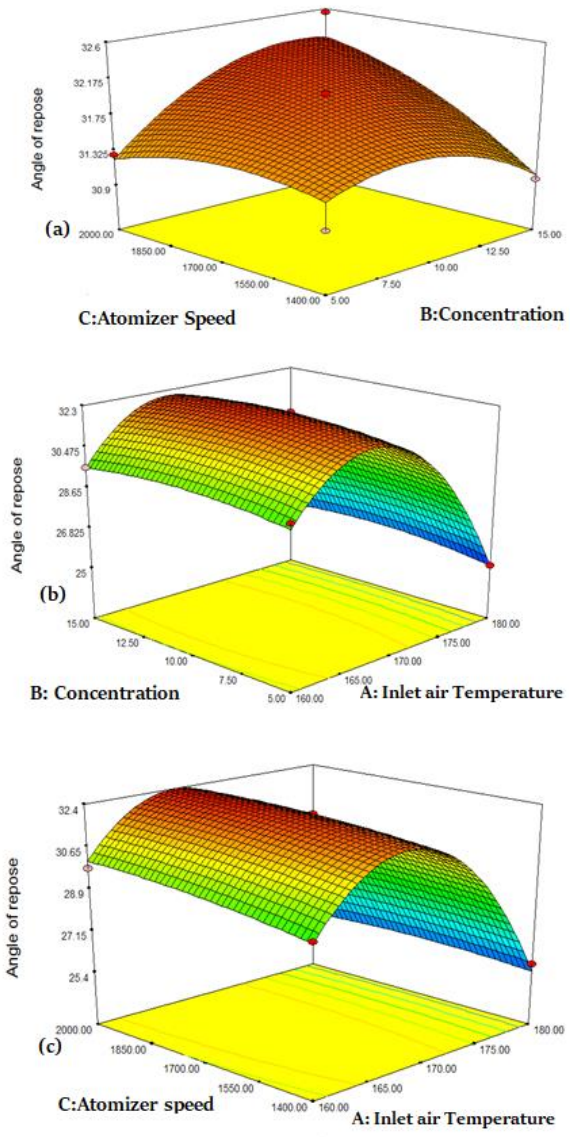


Figure 5. Response surface plots: effect of independent variables on the angle of repose of powder

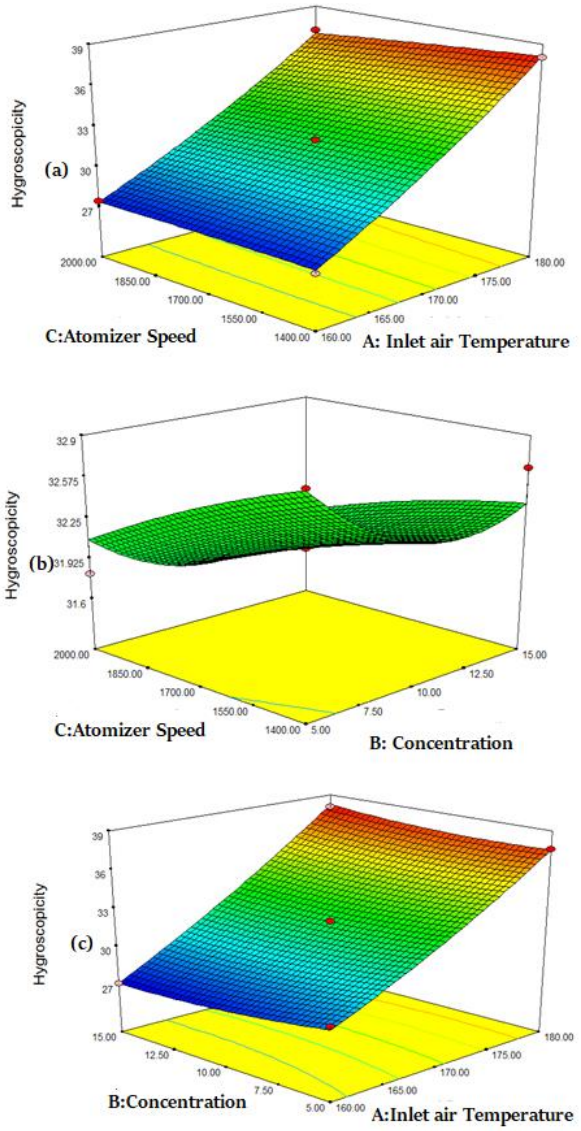


Figure 6. Response surface plots: effect of independent variables on Hygroscopicity of spray-dried coconut milk powder.

3.4. Moisture content

The moisture content decreased with an increase in inlet air temperature (Fig 7(a) and Fig 7(b)). It is obvious since higher inlet air temperatures cause increased moisture removal from the powder. The effect of the other two input parameters was again not much pronounced as evident in Fig 7(c).

The equation for moisture content is obtained as given below.

$$R_4 = 6.80 - 2.42A + 0.028\,B + 0.029\,C - 0.99\,AB + 0.13\,AC + 0.068\,BC + 0.83\,A^2 - 0.29\,B^2 + 0.50\,C^2$$

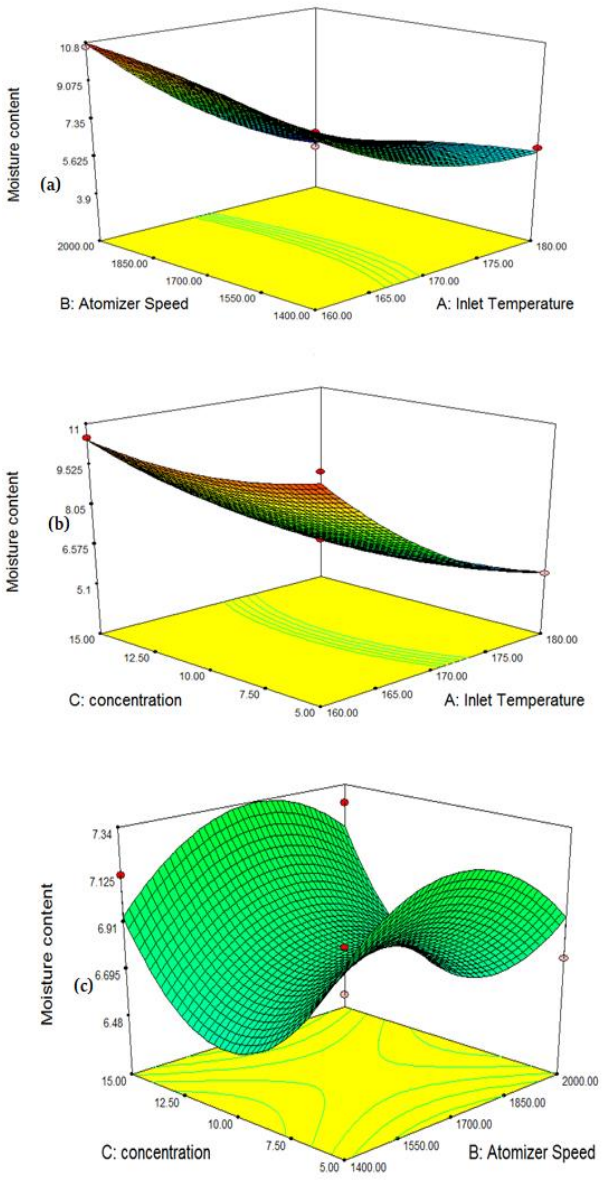


Figure 7. Response surface plots: effect of input variables on moisture content of spray-dried coconut milk powder.

3.5. Optimization

The overall desirability of the responses concerning the independent variables wass 0.94. The optimized values for the three independent variables are 160°C (inlet air temperature), 9.71w/w (maltodextrin concentration), 1400 rpm (atomizer speed). For these input values, the product yield, moisture content, angle response, and hygroscopicity are 8.14 g/200 mL of coconut milk, 8.35 wt%, 29.17°, and 27.01 respectively.

3.6. Flow roperties of the powder

In Table 4, the flow properties of the milk powders of the spray drying experiments were tabulated. It can be seen that an increase in the inlet air temperature increases the insolubility index values. Also, the higher inlet air temperatures result in the denaturing and can adversely effect the quality. The dispersibility values of the coconut milk powders were in the acceptable range of 80.55 to 96.2%. The obtained compressibility Index (*Carr's Index*) values were in the range of 0.5-3.6. The Hausner ratio values range from 1.19 to 1.22 The results show that the powders exhibits good flowability.

The physical and flow properties of the optimized powder, is compared to those of a commercial coconut milk powder sample. All the experiments were done in triplicates and the average values were tabulated in Table 5. The optimized powder exhibits desirable values of the dispersibility, flowability, and hygroscopicity. The values of the other parameters (moisture content, angle of repose, bulk density, tapped density) were comparable to those of the commercial powder.

3.7. Angle of internal friction

The flowability of the powders depends on many factors which include physical properties of the powder and the quality of the equipment used for handling, storing, and processing. Here, to calculate the angle of internal friction of the two powder samples (prepared and commercial), the direct shear method was used. This Method evaluates the shear force required to overcome the cohesive strength at different vertical loads. The shear stress (S) at the failure was plotted against the normal stress (σ) and the curve can be fitted to a linear equation as

$$S = C + \sigma \tan \Theta \quad (7)$$

where S: shear strength; σ: normal stress; C: cohesion; Θ: angle of shearing resistance.

Table 4. Flowability properties of the powder samples

S. No	Inlet Temperature (°C)	Concentration (w/w)	Atomizer Speed (RPM)	Bulk (g/cm^3)	Tapped Density (g/cm^3)	Hausner Ratio	Compressibility Index	Solubility index (mg)
1*	180	10	1400	0.214	0.219	1.023	2.283	14.25
2	180	10	2000	0.208	0.215	1.033	3.256	16.85
3	170	10	1700	0.317	0.417	1.460	23.98	10.01
4	170	10	1700	0.339	0.4	1.18	15.25	10.15
5	160	15	1700	0.3077	0.363	1.18	15.23	9.41
6	170	10	1700	0.384	0.465	1.211	17.42	10.12
7	160	5	1700	0.238	0.293	1.231	18.77	9.34
8	170	5	1400	0.333	0.377	1.132	11.67	10.23
9	180	5	1700	0.268	0.272	1.015	1.47	16.52
10	170	15	2000	0.379	0.413	1.09	8.232	10.03
11	170	5	2000	0.377	0.416	1.10	9.375	10.27
12	170	10	1700	0.384	0.408	1.06	5.88	10.16
13	160	10	2000	0.488	0.625	1.28	21.92	9.21
14	170	15	1400	0.476	0.625	1.31	23.84	10.28
15*	160	10	1400	0.212	0.236	1.11	10.17	9.18
16	180	15	1700	0.202	0.215	1.065	6.046	15.32
17	170	10	1700	0.235	0.289	1.23	18.685	10.15
* Dispersibility tests were conducted for the powders of these test runs (For (1) 80.79% and (15) 92.36%								

Table 5a. The properties of commercial coconut milk powder and the powder synthesized at optimal conditions

Bulk density (g/cm^3)	Tapped Density (g/cm^3)	Hausner Ratio	Compressibility Index	Flowability Index	Solubility (sec)	Dispersibility (%)
0.215 (0.236)	0.302 (0.263)	1.40 (1.14)	28.80 (10.04)	0.216 (0.181)	298 (272)	94.3 (87.5)

Moisture content (%)	The angle of repose (Θ)	Hygroscopicity(g/100g)
9.56 (8.35)	30.2 (29.17)	31.2 (27.01)

The variation of shear stress with respect to normal stress values of the two samples were shown in Fig 8 (a) and 8(b). The obtained Θ values for both samples were close to 30° and it can be interpreted that the samples exhibit good flowability.

3.8 Color analysis

Color is a sensory attribute that can be evaluated as the measure of quality and consumer acceptability. Color analysis of both the optimized and commercial powder were evaluated values are represented by *L**, *a** and *b** using KONICA MINOLTA spectrophotometer.

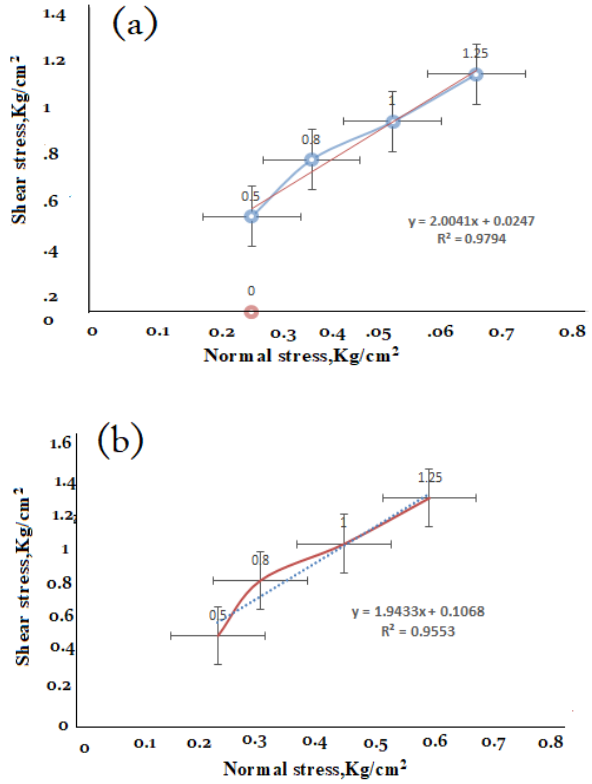


Figure 8. Plots showing the angle of friction for (a) optimized and (b) commercial powder**.**

Table 6 shows that the values of the both optimized and commercial powder are very much similar color attributes. The *L** values indicated that the colors of both powders were more aligned towards brightness. The negative and positive values of *a** and *b** imply lighter green and yellow shades of the powders respectively. The overall appearance of both powders was white and similar.

Table 6. The color analysis of the powders

Item	*L**	*a**	*b**
Optimized powder	70.66	-0.27	3.5
Commercial powder	70.89	-0.22	3.61

3.9. Differential Scanning Calorimetry (DSC)

Using differential scanning calorimetry (DSC), a plot of the temperature of powder sample versus heat flow (mW) difference between the sample and the reference was plotted. In the plot curve, a number of phase changes (glass transition, cooling crystallization, melting etc.) for the sample can be witnessed. After the glass transition, there would be the relaxation of enthalpy in the DSC curve. The DSC curves of two powder samples (commercial and optimized) are shown in Figure 9.

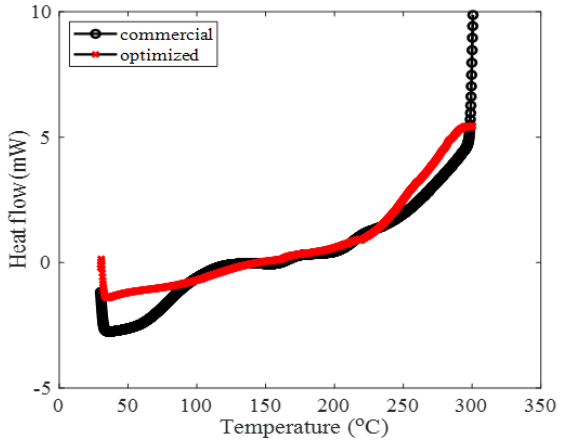


Figure 9. DSC Thermograms of commercial and optimized powder

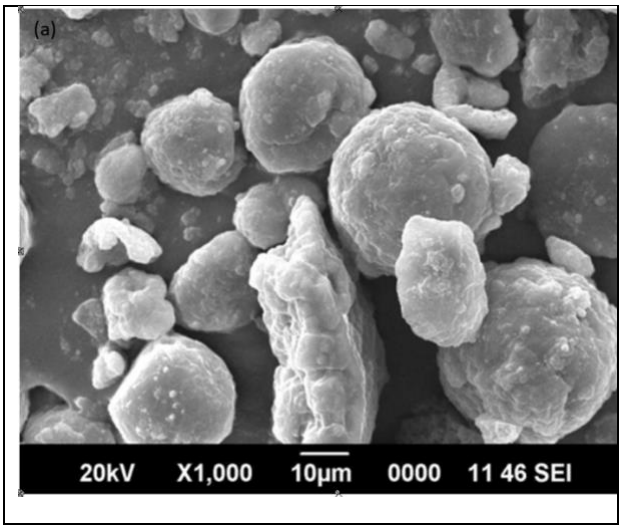
Glass transition temperatures of commercial and optimized powders were estimated to be around 120.3°C and 152°C respectively. The higher glass transition temperature of the optimized powder ensures its adaptability at the elevated temperatures with no loss of quality upon storage.

3.10. Scanning Electron Microscopy (SEM)

SEM images of the commercial coconut milk powder and the milk powder prepared at the optimum conditions are shown in Figure 9. The commercial and synthesized powders were imaged after 30 days of storage to study the structural stability of the powders. Figure 9 (a) shows that the particles of synthesized powder at the optimum conditions were globular with desirable structural stability. On the other hand, the commercial powder exhibited undesirable distorted structures with water bridges formed among the non-globular and irregular particles of the powder.

4. Conclusions

The operation of spray drying of coconut milk to milk powder was optimized using response surface methodology (RSM). Box-Behnken design of experiments approach was used for this purpose. The effect of input variables on the responses were analyzed using the 3D surface plots between the inputs and responses. In the analysis, it was found that inlet air temperature in the spray drying chamber has an significant effect on the physical properties of the powder. The solubility, flowability and dispersibility values of the powders synthesized were in the acceptable range.



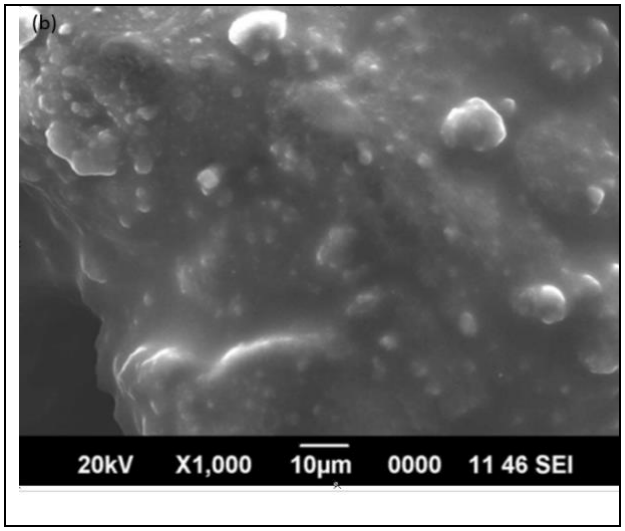


Figure 9. SEM images of (a) powder synthesized at optimum conditions and (b) commercial powder

The optimized values of the input parameters were obtained as follows: inlet temperature 160 ℃, Maltodextrin concentration 9.71 w/w, atomizer speed 1400 rpm. Correspondingly, for the powder synthesized at the optimal conditions, the values of the four responses were as follows: 8.14 g/200 ml of milk (yield), 8.35 wt% (moisture content), 29.17° (angle of repose), and 27.01 (hygroscopicity). The properties of the powder prepared at the optimal conditions were compared with those of a commercial powder sample and found to be in god agreement. Moreover, the optimized powder exhibited superior structural stability.

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DETERMINATION OF CONCENTRATIONS ^{2}H AND ^{17}O BY NMR SPECTROSCOPY METHOD IN DRINKING WATER, FRUIT AND VEGETABLES FRESH JUICES WITH INTRODUCTION OF $EU(CF_3SO_3)_3$ TO THE REFERENCE SAMPLE

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ABSTRACT

This research presents results of approbation of method for quantitative determination of ^{2}H by NMR spectroscopy using lanthanide shift reagent introduced to the probe as an external NMR standard regarding to which the measurement is taken. Within the process of the conducted studies it was confirmed that the most suitable chemical compound to create a reference sample was europium (III) trifluoromethanesulfonate. It was defined that the highest content of deuterium was determined in Argentine pear juice (144.6 ± 2.3 ppm), the least concentration of deuterium was found in potato juices of Russian and Egyptian origin (≈100 ppm), as well as tomatoes from Morocco. The developed method is suitable for analysis of isotope ratios in juices.

1.Introduction

The main reasons that cause changes in isotopic composition of the elements, besides radioactive transformations of atoms, are various physicochemical and biological processes (Bila, *et al*. 2017; Bowen, 2011), where the main function is implemented by kinetic and thermodynamic isotope effects, which in general lead to fluctuations in isotopic composition of the same chemical compounds in different regions of our planet (Moody, *et al.* 2012; Belkoum and Houha, 2017). That's why study of isotope composition of environmental substances in natural and synthetic materials can be used for determination of geographical origin of food and drinks (Ekaykin, *et al.* 2016; Sartori, *et al.* 2015).

For determination of isotopic composition of substances various instrumental methods are used: mass spectroscopy (Reynard, *et al*. 2016; Kuo, *et al.* 2012), IR-spectroscopy (Xiong, *et al.* 2013), nuclear magnetic resonance (NMR), including methodological approach SNIF-NMR (Jamin, *et al*. 2007; Sharifi, *et al.* 2017).

These studies are of high significance and topicality, it is explained by demand of wide range of scientific disciplines for study of isotopic composition of organogenic elements, which composition varies in biological objects of various types (Lehn, *et al*. 2015). Wherein fractionation of isotopes in nature is more expressed in those elements, which take more active part in circulation of substances in the inorganic and organic world: oxygen, hydrogen, carbon, nitrogen, sulfur (Schmidt, *et al*. 2015; Symes, *et al*. 2017).

The growing significance is conferred to study of isotope composition of water

(Ehleringer, *et al*. 2016; Chenaker, *et al.* 2017; Basov, *et al.* 2020), which influences to practically all biochemical and biophysical processes in organism (Svidlov, *et al.* 2021; Somlyai, *et al*. 2020). In particular water with artificially changed content of deuterium can significantly influence to metabolic processes in living systems (Avila, *et al.* 2012; Yaglova, *et al.* 2020, 2021; Fatemi, *et al*. 2020; Dzhimak, *et al.* 2014; Zlatska, *et al*. 2020, Zhang, *et al*. 2020).

It is known that quantitative NMR spectroscopy is one of the main precision analyze methods of deuterium concentration in liquids (Ma, *et al*. 2012), which, due to simplicity of sample preparation procedures and analytical process (Hosseini, *et al*. 2016), can be used as an express method for study of isotopic ($^2H/^1H$) composition of liquids (Basov, *et al*. 2019a).

In connection with the foregoing the purpose of this research is to develop a new method for quantitative determination of oxygen isotopes ^{17}O and ^2H content in liquid media with help of NMR spectroscopy using a lanthanide shift reagent introduced to the probe as an external NMR standard, in relation to which the measurement is taken.

2. Materials and methods

In this work there was used a device - NMR Fourier spectrometer 400 MHz (FT NMR SYSTEM model JNM-ECA 400) with a sensor 40TH5AT / FG2 of the following technical characteristics: induction of constant magnetic field - 9.389766 T; frequency range - from 10 to 400 MHz with increment of 0.01 Hz; the resonance frequency of ^2H nuclei is 61.37 MHz, the resonance frequency of ^{17}O nuclei is 54.22 MHz (tuned by the device); 1H sensitivity: ≥ 220 (for 0.1% solution of ethylbenzene in deuterochloroform); drift of constant magnetic field: less than 4 Hz/h; the width of ^1H NMR signal at half-height: not more than 0.45 Hz (for 0.1% solution of ethylbenzene in deuterochloroform).

For the studied sample the calibrated main NMR-ampoule with a diameter of 4.97 ± 0.013 mm and a length of 178 mm was used. The substance studied for its isotopic composition was placed into the ampoule. Also there was a calibrated internal NMR-ampoule (coaxial external standard) with length of end capillary 32 mm. and capacity of 40 mcl, which was inserted into the main NMR-ampoule and contained a reference substance with the same molecular structure as the substance being determined but with known isotopic composition (Fig. 1).

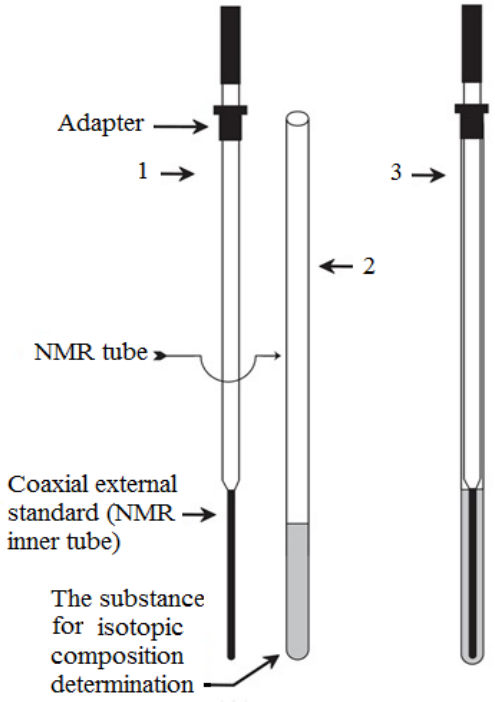

Figure 1. Schematic image of the NMR-ampoule (2) with the substance studied for isotopic composition; coaxial external standard (1) with solution $(CF_3SO_3)_3Eu$ in water with known concentration of ^2H and ^{17}O; the system of the standard inserted into the ampoule (3)

There was used a number of calibration samples of pure water with impurity content of no more than 0.01% by mass and with known content of isotope deuterium and oxygen ^{17}O in accordance with the international standard introduced by the IAEA (VSMOW and SLAP). For the method of determination of ratio $^2H/^1H$ and $^{17}O/^{16}O$ the sample of water containing $(CF_3SO_3)_3Eu$ was placed into the internal NMR-ampoule, wherein the content of deuterium or ^{17}O water in this solution must correspond to the

studied range of deuterium concentrations in the object being measured.

All experiments with the series of samples, including calibration samples, were carried out under identical conditions of NMR measurements and under the same settings of device. For measurements on ^2H nuclei we have chosen the optimum values: amplification 60; displacement 5 ppm; sweep 10 ppm; time of observation of free induction decay 6 s.; scans range 256; relaxation delay $10 \cdot T_1$ (^2H_2O) ≥ 7 s.; temperature inside the resonator is 25 ° C. For measurements on 17O nuclei we selected the optimal parameters: amplification 90; displacement (-10) ppm; sweep 80 ppm; time of observation of free induction decay 60 ms.; scans range 10000; relaxation delay $10 \cdot T_1$ ($H_2{}^{17}O$) ≥ 0.1 s; temperature inside the resonator is 25 ° C.

The important requirement is that the spectrum must be photographed without rotation of the sample in the spectrometer, otherwise, as we have found, there may be additional errors in the determination of the isotope ratios.

For determination of optimal concentration of lanthanide shift reagent (LSR) in the reference sample containing water, we studied the concentration dependence of europium salt from induced paramagnetic shift of the deuterium nuclei included into molecules of water composition. There was prepared a series of aqueous solutions containing 0.080 mol/l, 0.040 mol/l, 0.020 mol/l, 0.010 mol/l and 0.005 mol/l of shift reagent. Whereas for determination of concentration of oxygen isotope 17O there was prepared a series of aqueous solutions containing 0.200 mol/l, 0.100 mol/l, 0.050 mol/l, 0.025 mol/l of the shift reagent.

3.Results and discussions

3.1. Justification of lanthanide shift reagent choice

The quantitative NMR experiment is based on imaging of spectrum on the nucleus of the investigated isotope under absence of magnetic saturation, wherein the measurements are taken in relation to a special probe with certain parameters and known content of the corresponding isotopes. At this time the main task and complexity in implementation of methods for isotopes determination by quantitative NMR methods lies in development of optimal specific probes with suitable magnetic-relaxation characteristics. In this research a solution to the problem of magnetic-relaxation delay by using lanthanide shift reagents was found, which solution drastically shortens the time of experiment in comparison with the previous methods of NMR quantitative measurements. In this regard the internal NMR-ampoule contained a dissolved lanthanide shift agent (europium (III) trifluoromethanesulfonate, Sigma Aldrich, anhydrous and water-soluble) due to which reagent the NMR signal from the substance in the internal ampoule was displaced in relation to the substance being determined from the main ampoule, which allowed integration of individual spectrum signals relatively to each other (Fig. 2).

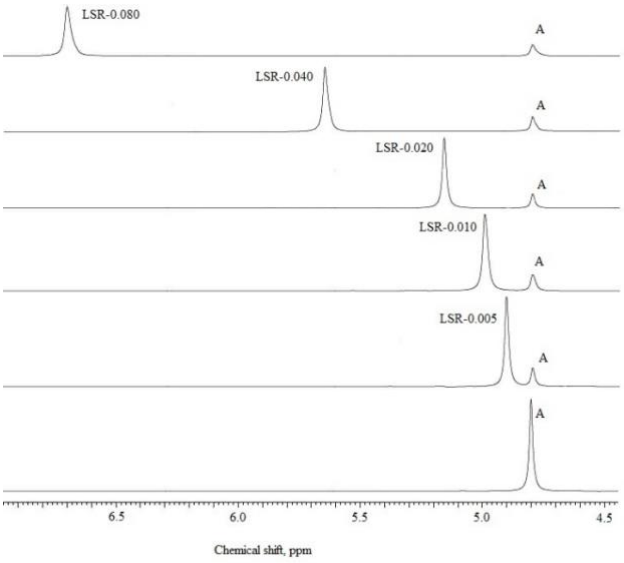

Figure 2. NMR spectra of solutions containing lanthanide shift reagent (LSR), LSR-0.005 - solution containing Eu $(CF_3SO_3)_3$ in concentration of 0.005 mol/l; LSR-0.010 - a solution containing Eu $(CF_3SO_3)_3$ in concentration of 0.010 mol/l; LSR-0.020 is a solution containing Eu $(CF_3SO_3)_3$ in

concentration of 0.020 mol/l; LSR-0.040 - solution containing Eu $(CF_3SO_3)_3$ in concentration of 0.040 mol/l; LSR-0.080 is a solution containing Eu $(CF_3SO_3)_3$ in concentration of 0.080 mol/l.

The europium (III) compound was chosen from all lanthanides as the lanthanide shift reagent due to the fact that while sufficient shift effect of Eu^{3+} the influence for the time of spin-lattice and spin-spin nuclear relaxation is small in relation to other rare-earth element compounds. This is explained by the fact that for Eu^{3+} the main electronic state is diamagnetic ($^7F_0$ term), and paramagnetism arises only due to the excited $^7F_1$ state. Because of this the spin-lattice and spin-spin electron relaxation times T_{1e} and T_{2e} are extremely short and are about from 10^{-12} to 10^{-13} s. And if one takes, for example, the gadolinium ions Gd^{3+} and Eu^{2+}, the broadening lines of NMR have the order of electronic relaxation values 10^{-8}-10^{-9} sec, thus representing good NMR relaxants since its ground state is $^8S_{7/2}$. For this term the value of the total angular momentum is determined only by the spin, since the orbital momentum is equal to zero, and, consequently, under such conditions they do not interact with the electric field, i.e. the Stark effect does not appear, so the relaxation time of electron spin Gd^{3+} and Eu^{2+} will be much larger than for Eu^{3+}.

3.2. Determination of optimal concentration of Eu^{3+} for estimation of content of ^2H and ^{17}O in liquid media

We conducted a series of NMR experiments to determine the spin-lattice and spin-spin nuclear relaxation times for ^2H and ^{17}O nuclei in presence of Eu^{3+} in a wide range of concentrations (from 0.001 to 0.200 mol/l of Eu^{3+}) by method "inversion-recovery" (for T_1) and CPMG (for T_2). The obtained results confirmed the theoretical justification of choice of europium (III) as a LSR for purposes of this research, as the changes of time of nuclear relaxation depended insignificantly from increase of Eu^{3+} concentration. For ^2H nuclei without introduction of Eu^{3+} the T_1 time was 0.43 ± 0.02 s., while introduction of 0.080 mol/l of Eu^{3+} changed this time to 0.36 ± 0.02 s. Accordingly T_2 without Eu^{3+} was 0.35 ± 0.05 s., and with addition of 0.080 mol/l of Eu^{3+} this time changed to 0.23 ± 0.05 s. Similarly, for ^{17}O nuclei without addition of Eu^{3+} the T_1 time was 0.007 ± 0.001 s., and with addition of 0.200 mol/l of Eu^{3+} this time changed to 0.005 ± 0.001 s. Moreover, the ^{17}O nucleus, having a high electric quadrupole moment, relaxes under quadrupole mechanism, consequently $T_1 = T_2$, which was observed by us in the experiment. Accordingly, T_2 without Eu^{3+} was equal to 0.007 ± 0.001 s., and with addition of 0.200 mol / l of Eu^{3+} it changed to 0.005 ± 0.001 s.

The results of the obtained spectra of deuterium in the prepared solutions show an appreciable displacement of the NMR signal in dependence of concentration of Eu^{3+} ions in the semi-heavy water (Fig. 2). This is caused by presence of strong pseudo-contact (dipole-dipole) interaction of Eu^{3+} ions with deuterium atoms. In NMR spectra on deuterium nuclei we observe two spectra spaced along the scale of chemical displacements of NMR signals – from deuterium of water with europium (III) trifluoromethanesulfonate dissolved there, located in the inner insert into the ampoule, and from the deuterium of studied water in the ampule itself.

By changing the percentage of deuterium water content, from the natural content of about 0.015% to 98% of deuterium contained in heavy water, we showed that the paramagnetic shift is the same for the whole range of deuterium concentrations and depends only on concentration of Eu^{3+} ions. On the basis of quantitative experiments to determine the isotope ratio of ^2H / ^1H in water (Fig. 2), water solutions and in biological fluids, it was found that the most optimal value of Eu^{3+} concentration in a reference water sample was 0.045 ± 0.005 mol/l.

We also studied the concentration dependence of Eu^{3+} ions from induced paramagnetic shift of ^{17}O oxygen nuclei included into water molecules composition. To find the optimal concentration of europium (III) trifluoromethanesulfonate in a reference sample

containing water, a series of water solutions containing 0.200 mol/l, 0.100 mol/l, 0.050 mol/l, 0.025 mol/l of europium (III) trifluoromethanesulfonate was prepared. From the obtained calibration dependence it is obvious that there is a significant displacement of NMR signal of ^{17}O of water containing Eu^{3+} ions to strong field, which is characterized by a change of overall magnetic susceptibility of solution due to the presence of contact interaction of Eu^{3+} ions with ^{17}O. After analyzing the obtained data we chose the optimal concentration of Eu^{3+} ions for purposes of this study at the level of 0.120 mol/l (Fig.3).

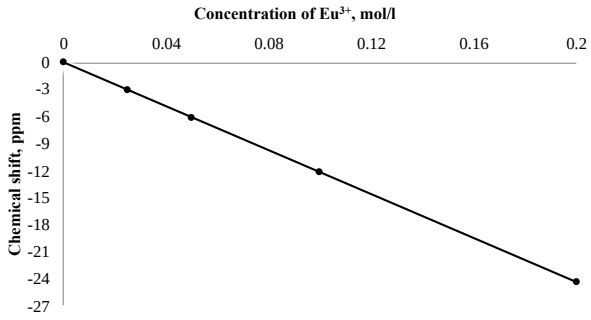

Figure 3. Dependence of paramagnetic chemical displacement of ^{17}O water nuclei from concentration of Eu^{3+} ions in solution

Table 1. Deuterium content in various water samples

Water	Deuterium concentration, ppm	Water	Deuterium concentration, ppm	Water	Deuterium concentration, ppm
Tap water	150.2±0.9	Essentuki 4	151.9±1.6	Arkhyz	147.4±0.9 #
Distilled water	150.4±0.8	Dzhermuk	152.1±1.1	Vittel	157.0±2.1 #
Bidistilled water	150.1±1.3	Piligrym	155.2±1.5 #	Evian	155.6±1.6 #
Goryachiy Klyuch, borehole 934	153.0±0.8 #	Narzan	144.0±1.6 #	Aquamin erale	149.5±1.8
Serebryany istochnik	153.4±0.7 #	Mercury	145.8±2.0 #	Miniliya	159.8±1.4 #
Goryachiy Klyuch Aroma-yug	152.0±1.4	Kubay	167.3±1.9 #	Bonaqua	150.7±1.2

Note. # - Reliability of differences ($p<0.05$) in comparison with values of tap water.

3.3. Study of 2H concentration in fresh juices, bottled and natural waters

Among the bottled water samples a noticeably lower content of deuterium in comparison with the SMOW standard was observed in the following: the least content was found in "Narzan" water, whose deuterium values were below
the SMOW standard by 7.6% and in comparison with tap water at 4.1%, which indicates the advisability of its use in food ration to reduce content of hydrogen heavy isotopes in patient's body (Table 1). The applicability of this issue is caused by the fact that one of the important tasks in the concept of healthy nutrition of the population is the formation of regional programs of healthy food, among other thing taking into account the distribution of heavy isotopes in food, especially in regions with an unfavorable ecological environment (Ehleringer, *et al.* 2016), that will allow reducing impact of adverse factors to the human body. In the recent researches number of authors, while studying of deuterium depleted water (DDW) influence for isotope composition of blood and tissues plasma, it was noted that during use of this water in all organs of body there is decrease of deuterium concentration expressed in various levels (Dzhimak, *et al.* 2018; Kozin, *et al.* 2021). The most significant changes in D level were peculiar for kidneys, which after 2 weeks of experiment showed 10.8% lower deuterium content than the one of the liver and 14.2% less than the D values of the heart. An even more

significant decrease in concentration of D content was observed in blood plasma, which change was accompanied by a change in the direction of isotope D / H gradient ("plasma>>tissues" to "plasma<<tissues") (Basov, *et al*. 2019b). The above-described changes are caused by low rate of exchange of deuterium to protium in tissues: in carbon-hydrogen bonds (R_3C-D) in composition of organic substrates that have no atoms with an unshared electron pair, i.e. incapable, in contrast to hydroxyl (–O-H), sulfhydryl (–S-H), primary ($–NH_2$) and secondary (=N-H) amino groups, to form complexes with hydrogen bonds capable to rapid exchange with D atoms while obtaining predominantly protium with water in the process of the food consumption. The observed changes of isotope composition in tissues cause nonspecific changes in metabolic and functional activity of immune defense systems, which is apparently related to the sub-stress effect of the isotope D/H gradient, including the energy exchange of the cell (Dzhimak, *et al*. 2014).

In addition, the obtained results allow not only comparative assessment of the D / H isotopic load on the population while forming the food nutrition, but also will allow tracking the movement of people in different regions, which can be used in forensic studies. Thus, in a study conducted in two regions of the United States of America: East Greenbush (New York) и Fairbanks (Alaska), it was shown, that the content of deuterium in the hair and urine correlates with its content in the food ration (O'Brien, *et al*. 2007). And when a person moves between regions, the concentration of deuterium in his/her urine changes in accordance with its concentration in the food intake. The authors suggested using similar studies to track geographical movements of a person. Similar approaches are also applicable in biology to track geographical location and migration of various fauna representatives (Reynard, *et al*. 2016; Schmidt, *et al*. 2015). Therefore, the developed method for measurement isotope ratios by the NMR method using a probe containing a lanthanide shift reagent can be used in studies of environment and for environment condition monitoring, including monitoring of isotopic composition of liquids of natural and synthetic origin, as well as for determination of blood plasma isotopic composition.

Unlike the variability of deuterium concentrations in bottled waters, the concentration of ^{17}O there did not differ significantly from its concentration in tap water, in which deuterium concentration was 371.9±1.4 ppm.

Taking into account the above-stated, we conducted investigations of the isotope D/H composition of freshly squeezed juices, the data are presented in Table 2.

Table 2. Concentration of deuterium in fruit and vegetables freshly squeezed juices from various geographical regions

Fresh juice, manufacturer country	**Deuterium concentration, ppm**	**Fresh juice, manufacturer country**	**Deuterium concentration, ppm**
from tangerine, Egypt	126.2±1.5	from tomato, Morocco	112.0±0.8
from tangerine, Pakistan	127.9±1.0	from tomato, Turkey	122.4±0.9
from tangerine, Spain	132.7±1.8	from tomato, Spain	125.2±1.3
from orange, Egypt	128.5±1.7	from tomato, Russia	120.7±0.8
from orange, Turkey (1)	121.3±1.2	from cabbage, Poland	100.6±2.2
from orange, Turkey (2)	122.1±1.1	from cabbage, Russia	113.1±2.4
from pomegranate, Israel	129.7±1.3	from cabbage, the Netherlands	114.8±1.7
from pomegranate, Turkey	122.4±0.8	from potato, Egypt	99.6±2.4
from pomegranate, Azerbaijan	120.2±0.9	from potato, Israel	102.3±1.9
from apple, USA	118.3±0.6	from potato, Russia	105.4±2.6
from apple, Spain	131.5±1.1	from pear, Argentina	144.6±2.3
from apple, Italy	123.7±1.5	from pear, South Africa	118.1±1.6

from apple, Poland	127.6±1.2	from pear, USA	122.3±0.9
from apple, Russian	128.3±0.7	from pear, Russia	126.6±1.2

To obtain water samples the freshly squeezed juices were centrifuged at speed 3000 rpm for 15 minutes. It was found that the highest content of deuterium was in juice from a pear brought from Argentina (144.6 ± 2.3 ppm), while the lowest concentration of deuterium was found in the juice from Egyptian potato (99.8 ± 2.4 ppm). In general, the reliably lower deuterium content was detected in freshly squeezed potato and cabbage juices grown in different countries, in comparison with other fruit and vegetable fresh juices. The least range of differences in the isotope D/H composition was found in freshly squeezed juices (tomato, pomegranate and oranges) from Turkish producers (deuterium concentration there ranged from 121.3 to 122.4 ppm), which evidences similar climatic conditions and technologies for growing plant products in this country.

Among the tomato fresh juices the least deuterium values were found in the juice squeezed from tomatoes grown in Morocco (112.0 ± 0.8 ppm), which was 10.5% lower than the value in tomato juice grown in Spain, 8.4% lower than in juice from tomatoes grown in Turkey, 7.2% lower than in juice of tomatoes grown in Russia. All this allows us to recommend tomato juice from Morocco in order to correct isotope violations associated with accumulation of heavy stable hydrogen isotopes in ecologically unfavorable regions.

In addition, the obtained data, especially about fruit and vegetable juices from Turkish producers, allow us to recommend isotope D/H studies as alternative confirmation of its geographical origin. The sufficiently significant variations in content of heavy non-radioactive hydrogen isotopes were noted in juices from vegetables and fruits grown in Egypt (concentration of D in juices varied from 99.6 to 128.5 ppm), Poland (concentration of D in juices varied from 100.6 to 127.6 ppm) and Russia (concentration of D in juices varied from 105.4 to 128.3 ppm). The latter factor may be caused by the peculiarities of agrarian production, including the features of water sources used to irrigate plant crops in these regions. Therefore, to confirm this suggestion the isotopic D/H composition in water samples from various regions of southern Russia was investigated (Table 3).

Table 3. Deuterium content in water samples, taken from surface and underground water sources in the southern region of the Russian Federation

Subject of investigation	**Water from borehole 1**	**Water from borehole 2**	**Glacial water**	**Water from artesian well**
Deuterium concentration, ppm	151.8± 2.3	153.2± 1.6	132.7± 2.0	149.1± 1.4

It was found that depending on the water source the ratio of light and heavy non-radioactive hydrogen isotopes can significantly differ in various geographic regions, especially significant differences between glacial water and water pumped from boreholes (ΔD accounted from 16.4 to 20.5 ppm). That's why the change of deuterium content in plant products will also depend on the water supply source (surface or underground), used for irrigation of the plant cultures. Certain influence on fluctuation in deuterium concentration in plants can be caused by climatic factors, primarily atmospheric precipitations, which ensure formation of specific hydrological mode in each territory. It is necessary to note that much lower content of deuterium is found in freshly squeezed juices from potatoes and cabbage in comparison with other fruit and vegetable fresh juices.

At the same time the concentration of ^{17}O did not significantly change in waters obtained from surface and underground sources in the southern regions of the Russian Federation and varied within the range from 371.6 to 373.8 ppm, which indicates lower variability of

concentrations of heavy oxygen isotopes in comparison with deuterium concentrations, and first of all allows us to recommend determination of the D/H ratio in analysis of isotopic composition of products from different geographical origin.

Taking into account the data on capability of reduced deuterium concentrations in blood and tissues of internal organs to change the functional activity of the nonspecific immune defense system, the consumption of products and water with lower content of heavy non-radioactive hydrogen isotopes may increase the nonspecific resistance of the organism. In connection with this the determination of isotope D/H composition of phytonutrients by this developed method using the lanthanide shift reagent $Eu(CF_3SO_3)_3$ is the additional criterion for defining of quality of the manufactured food products of plant origin.

4. Conclusions

As a result of the implemented research, there was developed a method for measuring the content of ^2H and ^{17}O isotopes in liquid medium using quantitative nuclear magnetic resonance. Among the considered paramagnetic metal ions the three-charged cation of europium (Eu^{3+}) proved to be the most suitable for relaxation and shifting characteristics for the purposes of this research. The most suitable chemical compound containing Eu^{3+} for its use in capacity of shift reagent for water solutions for study of isotopic composition was europium (III) trifluoromethanesulfonate. According to the results of research of the dependence of chemical shifts of ^2H and ^{17}O nuclei from concentration of europium (III) trifluoromethanesulfonate, the specific values of paramagnetic chemical shift were determined, which allowed to establish the necessary concentration of Eu^{3+} ions for external standards (reference samples) to determine the isotopic composition of substances. The concentration of Eu^{3+} ions in determination of $^{17}O/^{16}O$ ratio was 0.120 mol/l, and the ratio $^2H/^1H$ was 0.045 mol/l. It is shown that use of shift reagents makes it possible to increase the informational content of the NMR spectra. The developed methods for measurement of isotope ratios by NMR method using probes containing shift reagent can be used to solve environmental problems and to monitor the environment state, including for control of the isotopic composition of liquids of natural and synthetic origin.

With the help of developed method the concentration of deuterium was measured in fresh fruit and vegetables juices obtained from range of vegetables and fruits of various geographical origin. It was found that the highest content of deuterium was in juice of the Argentine pear (144.6 ppm), the least concentration of deuterium was found in the potato juices of Russian and Egyptian origin (≈100 ppm). Among the tomato fresh juice the least deuterium values were found in the juice obtained from tomatoes grown in Morocco (112.0 ppm). In general, the significantly lower deuterium content was detected in freshly squeezed juices of potato and cabbage grown in different countries, in comparison with other fruit and vegetable fresh juices. The concentration of ^{17}O did not change significantly in waters obtained from surface and underground sources in the southern regions of the Russian Federation and varied within the range from 371.6 to 373.8 ppm, which indicates lower variability of heavy oxygen isotopes concentrations in comparison with deuterium concentrations in the same natural water sources.

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NUTRACEUTICALS COMPOUNDS EXTRACTION OPTIMIZATION FROM OPEN AIR AND SWELL-DRIED BANANA PEEL POWDERS

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ABSTRACT

The aim of this study was to optimize the operating conditions of two drying processes on banana peels: open air and Instant Controlled Pressure Drop technique (DIC) assisted Swell-Drying at 40°C in order to obtain high quality final powders. The optimization of three extraction conditions including extraction temperature (40-100°C), extraction time (10 – 60 min) and particle size (60 – 363 µm) from open air banana peel powder was investigated). Additionally, three DIC texturing conditions were improved. DIC involves maintaining banana peels at a high temperature for 20 to 220s, high steam pressure (p=0.3 to 0.6 MPa) and varying the Number of cycles from 1 to 7. Modelling of some nutraceutical compounds (Total Flavonoids Content and carotenoids) from open air and Swell-Dried banana peels powders by applying experiments design using Response Surface Methodology and Desirability Function. The antioxidant activity was also investigated by the determination of the % of DPPH inhibition.

The optimal conditions derived from the multi-Responses-Desirability Function were as follows: 60.47°C; 10min; and particle size Φ= 348.648 µm yielding a TFC=5.13 (mg QE/g d.b), TCC=0.48 (mg /g d.b) and % of DPPH inhibition=73.05%, with an optimal desirability coefficient d=0.7 (open air process). The following optimized DIC operating parameters with maximum desirability coefficient d=1, t=24.46s P=0.59MPa and number of cycles N=6.38 yielding a TFC=4.07 (mg QE/g d.b), a TCC= 1.37 (mg /g d.b) and a % of DPPH inhibition=75.97 %. Banana peel could be a good source of bioactive substances, which could be further used as a natural antioxidant.

1.Introduction

The production of banana fruit, a tropical perishable fruit, amounts to 119 million tonnes per year (FAO, 2022), with around 36 million tonnes of peel as a waste product. Banana peel contains a variety of bioactive compounds, including flavonoids, tannins, carotenoids, vitamins, and other elements with anti-inflammatory and antioxidant properties (Havsteen, 1983; Toh et *al*., 2016; Pereira et

al., 2017). According to (Iman and Akter, 2011), the eucocyanidin flavonoid is gaining popularity as medication for stomach ulcers.

Banana peel contains mucilages, which are naturally occurring polysaccharides that are chemically linked to proteins and minerals (Gemede et *al*., 2015). These substances have a range of medicinal effects including anticancer, antioxidant, antimicrobial, hypoglycemic, and antiulcer properties (Dantas et *al*., 2021).

Proteins and other compounds have the ability to form phenolic insoluble complexes. Dopamine is the only phenolic amino acid found in banana peels according to Happy (Emaga et *al*., 2007).

In terms of applications, the banana's peel has long been used in traditional medicine to cure many health disorders such as burns, anemia, and diarrhoea (Vu et *al*., 2018).

In Algerian traditional medicine, dried banana peel infusion is used to cure gastric disorders.

Furthermore, a water extract of *Musa acuminata* peel dried in the open air could be deemed an efficient treatment for reducing gastrointestinal inflammation in rats after 30 days. This work was conducted at Pasteur Institute in Algeria and is currently being published.

The impact of pre-treatment on the availability of phenolic chemicals recovered from banana peels have been reported in a number of studies. These pre-treatments include, among others, blanching (Hernandez-Carranza, 2016), and freezing (ISO, 2000).

To our knowledge, no scientific research has been conducted to investigate the impact of the two drying processes -open air drying and Swell-Drying using DIC- on the availability of nutraceutical compounds of banana peel and determine the parameters under which it can be used.

The current research was carried out in order to analyse the biochemical composition of banana peels and to optimize the extraction conditions of some nutraceutical compounds (flavonoids and carotenoids) and to evaluate their antioxidant activity by determination of the % of DPPH inhibition from open air and DIC Swell-dried banana peels powders by applying experiments design using Response Surface Methodology (RSM) and Desirability Function (DF).

2. Materials and methods

2.1. Plant material

Banana fruits of the *Musa acuminate* species were acquired at a French market in La Rochelle (France). Table 1 summarizes some physicochemical properties of fresh Musa acuminate peels.

Table 1. Some physicochemical composition of fresh *Musa acuminate* peels (n=3)

Parameters	Average contents
pH at 22°C	5.45±0.07
Humidity level (%)	7.76±0.18
Ash rate (%)	14.18±4.14
Organic matter (%)	85.82±4.14
Titratable acidity (g of citric acid /100g of sample)	0.09±0.01
Total sugars (g/l)	17.11±0.02
Reducing sugars (g/l)	0.36±0.01
Sucrose (g/l)	15.91±1.64

2.2. Methods

2.2.1. Drying processes of banana peels

Two drying processes were used in this study: open air under dark condition and Instant Controlled Pressure Drop process DIC assisted Swell-Drying at 40°C. This work was conducted at the Unit Laboratory of Materials, Processes & Environment (UR-MPE), M'Hamed Bougara University of Boumerdes (Algeria) with the collaboration of the laboratory of Engineering Science for

Environment (LaSIE) UMRER7356 CNRS, La Rochelle University (France).

All banana fruits were washed under tap water to remove impurities, then the peels were manually recovered and cut into small pieces before being dried in the open air under dark condition and textured with DIC using equipment from ABCAR-DIC Process (La Rochelle, France) followed by Swell-Drying at 40°C until the final weight was constant.

The DIC treatment involves four steps, which are as follows: 1): a primary vacuum is created; 2): high saturated steam pressure is injected and maintained for a short time (10 to 30 s); 3): pressure is abruptly dropped toward a vacuum within few milliseconds, and finally released to atmospheric pressure.

The dried peels were crushed in an electric grinder and sieved using an ORTO ALRESA sieve between 60 and 363 µm. The final powders were stored in hermetic glass bottle at room temperature until their analysis

2.2.2. Experimental design

Using a banana peel that had been dried in the open air and DIC assisted Swell-Drying, the extraction of nutraceutical compounds (Total of flavonoids and carotenoids) and antioxidant activity were improved as revealed by the response optimizer function of Statgraphics Centurion 18 software. The independent variables used in the RSM design of a banana peel that had been dried in the open air are listed in Table 2. Three DIC operating conditions optimization of banana peel: treatment time (X_1) from 20 to 220s**,** steam pressure P (X_2) from 0.3 to 0.6 MPa, and the number of cycles (X_3) adjusted from 1 to 7 were also investigated (Table 3).

The response for nutraceutical components (TFC and TCC) and the percentage of DPPH inhibition of water extract from banana peels dried in open air and DIC-assisted Swell-Drying were assessed using a Central Composite Design (CCD) with four central points (Tables 4, 5).

A second-order polynomial of the following form was employed to express the responses using multiple regressions:

$$Y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_i X_i^2 + \sum_{i=1}^{3}\sum_{j=1}^{3} a_{ij} X_i X_j \quad (1)$$

Where Y is the predicted responses used as dependent variables, $X_i (i = 1, 2\ and\ 3)$ are the independent variables, and a_0 and $a_{ij} (i = 1, 2, 3;\ j = i, \dots, 3)$ are the model coefficient parameters.

All water extracts were prepared from open air and DIC-assisted Swell- Dried banana peels powders at 5%.

Only the textured banana peels powders underwent a one-night maceration in distilled water at 4°C.

All measurements were made in triplicate, and the results were reported as mean standard deviation. Except for the pivot points matching the experiments run N° 4, 7, 10, 13, 16 and 19 in Table 5, nothing else was repeated.

Table 2. Central Composite Design independent variables and their levels (Case of open air)

Variables	Coded level				
	-1.68	-1	0	1	1.68
$\mathbf{X_1}$	40	52	70	88	100
$\mathbf{X_2}$	60	122	212	302	363
$\mathbf{X_3}$	10	20	35	50	60

X_1= Extraction Temperature T (°C); X_2= Particle size (µm); X_3= Extraction time t (min)

Table 3. Central Composite Design independent variables and their levels (case of DIC)

Variables	Coded level				
	-1.68	-1	0	1	1.68
$\mathbf{X_1}$	20	60,54	120	179,46	220
$\mathbf{X_2}$	0.3	0,36	0.45	0,54	0.6
$\mathbf{X_3}$	1	2,22	4	5,78	7

X_1 = Treatment time t (s); X_2 = Steam Pressure P (MPa); X_3 = Number of cycles (-)

Table 4. Central composite design and observed response for nutraceutical compounds and the % of DPPH inhibition of banana peel dried in open air

	Coded variable levels			Uncoded variable levels			Responses		
Run	X_1	X_2	X_3	X_1	X_2	X_3	TFC (mg QE/g d.b)	TCC (mg/g d.b)	% of DDPH inhibition (Water extract)
1(C)*	0	0	0	70	212	35	2.57±0.03	0.398±0.005	75.14±0.417
2	-1	-1	-1	52	122	20	4.00±0.12	0.632±0.010	67.23±0.11
3	+1	-1	-1	88	122	20	3.5±0.27	0.446±0.031	72.05±0.298
4	-1	+1	-1	52	302	20	3.69±0.08	0.530±0.005	63.23±0.468
5	+1	+1	-1	88	302	20	3.55±0.02	0.365±0.011	76.35±0.192
6	-1	-1	+1	52	122	50	3.80±0.10	0.524±0.018	64.7±0.358
7	+1	-1	+1	88	122	50	3.38±0.08	0.400±0.019	70.45±0.298
8	-1	+1	+1	52	302	50	3.16±0.08	0.422±0.005	69.11±0.063
9	+1	+1	+1	88	302	50	2.93±0.19	0.302±0.057	67.05±0.298
10(C)*	0	0	0	70	212	35	2.36±0.01	0.414±0.017	64.7±0.358
11(C)*	0	0	0	70	212	35	2.52±0.02	0.428±0.013	72.23±0.11
12	-1.68	0	0	40	212	35	2.81±0.10	0.482±0.006	62.57±0.415
13	+1.68	0	0	100	212	35	2.01±0.02	0.430±0.039	61.6±0.032
14	0	-1.68	0	70	60	35	3.33±0.16	0.299±0.021	67.35±0.192
15	0	+1.68	0	70	363	35	5.13±0.32	0.356±0.007	75.58±0.405
16	0	0	-1.68	70	212	10	2.76±0.11	0.487±0.037	68.23±0.468
17	0	0	+1.68	70	212	60	2.06±0.01	0.377±0.044	81.03±0.308
18(C)*	0	0	0	70	212	35	2.61±0.03	0.474±0.041	64.56±0.421

X_1: Extraction Temperature T (°C); X_2: Particle size Ps (µm); X_3: Extraction Time t (min); TFC: Total Flavonoids Content (mg QE/g d.b); TCC: Total Carotenoids Content (mg/g d.b); % of DPPH inhibition (water extract); (C)*: Central point

Table 5. Central composite design and observed response for nutraceutical compounds and % of DPPH inhibition from Swell-dried textured banana peels by DIC

	Coded variable levels			Uncoded variable levels			Responses		
Run	X_1	X_2	X_3	X_1	X_2	X_3	TFC (mg QE/g d.b)	TCC (mg/g d.b)	% of DDPH inhibition (Water extract)
1(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
2	+1.68	0	0	220.00	0.45	4.00	2.3 ± 0.045	0.78± 0.007	71.34± 6.219
3	0	0	+1.68	120.00	0.45	7.00	1.79 ± 0.105	0.59± 0.004	68.20± 0.117
4(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
5	0	+1.68	0	120.00	0.60	4.00	1.11 ± 0.17	0.8± 0.007	67.46± 0.462
6	0	-1.68	0	120.00	0.30	4.00	1.67 ± 0.032	1.23± 0.063	66.41± 0.491
7(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
8	+1	-1	+1	179.46	0.36	5.78	1.74 ± 0.126	0.46± 0.008	62.23± 0.498
9	-1	-1	-1	60.54	0.36	2.22	1.26 ± 0.084	0.42± 0.008	62.38± 0.534
10(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
11	+1	+1	-1	179.46	0.54	2.22	1.19 ± 0.21	0.99± 0.01	66.56± 0.471
12	-1	+1	+1	60.54	0.54	5.78	2.01 ± 0.155	1.06± 0.004	70.29± 0.222
13(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
14	-1	+1	-1	60.54	0.54	2.22	2.32 ± 0.182	1.19± 0.005	69.55± 0.448
15	-1	-1	+1	60.54	0.36	5.78	2.07 ± 0.195	0.42± 0.004	74.77± 0.249

16(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
17	-1.68	0	0	20.00	0.45	4.00	3.09 ± 0.371	0.44± 0.004	67.76± 0.249
18	+1	+1	+1	179.46	0.54	5.78	2.04 ± 0.351	0.54± 0.006	68.65± 0.241
19(C)*	0	0	0	120.00	0.45	4.00	1.42 ± 0.071	0.43± 0.004	66.11± 0.685
20	+1	-1	-1	179.46	0.36	2.22	3.65 ± 0.138	0.49± 0.003	69.10± 0.132
21	0	0	-1.68	120.00	0.45	1.00	3 ± 0.184	0.48± 0.003	69.25± 0.209
22(C)*	0	0	0	120.00	0.45	4.00	2.89 ± 0.032	0.64± 0.003	70.14± 1.053

X_1: Treatment time t (s); X_2: Steam pressure P (MPa); X_3: Number of cycles (−); TFC: Total Flavonoids Content (mg QE/g d.b); TCC: Total Carotenoids Content (mg/g d.b); % of DPPH inhibition (water extract); (C)*: Central point

2.2.3. Physicochemical analysis

- Hexane extraction was used to extract the oil from banana peels. The fatty acid profile (Table 6) was determined using CHROMPACK CP 9002 gas phase chromatography. The methyl esters were produced by esterification using the ISO 5509 technique (ISO, 2000).
- An Atomizer (VARIAN AA 240, Australia) was used to determine the mineral composition (Table 7) of banana peels. This measurement is based on dissolving 1g of ashes in 5mL of HCL acid (0.5N) (Adrian, et *al.* 1995).
- The mucilage was extracted from banana peel powder according to the method described by Dick et *al.* (2019). 30 g of banana peel powder were mixed with 100mL of distilled water and heated for 54 to 96 minutes at 50°C. The mucilage was lyophilized after being precipitated with 35mL of ethanol.
- A colorometric method was used to determine the Total Flavonoids Content (TFC) (Bahorun et *al.* 1996). 1mL of the water extract was mixed with 1mL of a 2% AlCl3 solution. After a 10 minutes incubation period, the absorbance was measured using a spectrophotometer at 430nm. The TFC in different banana peel water extracts was estimated using a regression equation that used Quercetin as a standard and was estimated in milligrams of Quercetin Equivalent per gram dry basis (mg QE/g d.b).
- The Total Carotenoids Contents TCC was extracted using the Sass-Kiss et *al.* (2005) method. 20 mL of a solvent mixture of hexane-acetone-ethanol (2V: 1V: 1V) were added to 0.5g of banana peel powder. After 30 minutes of agitation, the upper phase was recovered. For a second extraction, 10 mL of hexane were added. The absorbance was measured using a spectrophotometer at 450nm. Carotenoid concentrations were estimated by referring to the regression equation with carotene as a standard and were expressed in milligrams per gram dry basis (mg /g d.b).
- The DPPH free radical scavenging method was employed to assess the antioxidant activity of aqueous extracts of banana peels dried in the open air and DIC-assisted Swell–drying (Kroyer, and Hegedus, 2001). A volume of 0.1mL of extract was added to 3.9mL of DPPH (60mM). After 30 minutes of incubation in the dark, the absorbance was measured at 517 nm. The percentage of DPPH radical inhibition is expressed as follows:

$$\%\ \text{of DPPH radical inhibition} = \frac{(\text{OD517 of Reference} - \text{OD517 of water extract})}{\text{OD517 of Reference}} \times 100 \quad (2)$$

Table 6. Fatty acids (%) of *Musa acuminate* peel powder

Fatty Acids	Names	Content (%)
C12 :0	Lauric A.	1.32
C16 :0	Palmitic A.	28.32
C16 :1ω7	Palmitoleic A.	1.53
C18 :0	Stearic A.	5.54
C18 : 1ω9	Oleic A.	3.93
C18 : 2ω6	Linoleic A.	19.35
C18 : 3ω3	Linolenic A.	22.13
C22 :0	Behenic A.	3.13
C14 : 0	Myristic A.	1.50
C24: 0	Lignoceric	2.81

Table 7. Mineral composition of *Musa acuminate* peel powder

Minerals	Content (g/Kg d.b)
Ca	30.254
Mg	25.836
Na	54.701
K	24.178
Fe	0.441
Cu	0.019
Zn	0.018
Cr	-
Cd	-
Pb	-

2.2.4. Statistical analysis

Using Statgraphics Centurion 18 software, the Response Surface Methodology (RSM) was utilized to define the relationship between independent variables and to deduce the optimum extraction conditions of bioactive substances from banana peel powders. A quadratic model to predict the response (TFC, TCC and percentage of the DPPH radical inhibition) was determined from all the experiments, and the regression coefficients for the linear, quadratic and interaction factor were calculated and statistically examined using variance analysis (ANOVA). The multi-response optimization was defined using the desirability approach (Response Optimizer function in Statistica). The desirability function is a multi-criteria approach for assessing the overall desirability that considers each response's desirable value or the best accepted.

The Surface Responses were generated using the polynomial equations regressions coefficients. These were utilized to analyse the variables under investigation as a function and to present the most favourable acceptance.

To highlight the interaction effects of independent variables of each drying process, one variable remained constant at its centre level while the other two variables changed within the experimental range.

3.Results and discussions

3.1. Physicochemical analysis

Musa acuminata peel dried in the open air studied here contains oil with a yield of 10±0.5%. This oil is rich in unsaturated fatty acids such as palmitic acid (28.32%), linolenic acid (22.13%), linoleic acid (19.35%) and oleic acid (3.39%) (Table 6). These fatty acids are beneficial to people's health and are thought to

help in the prevention of cancer, atherosclerosis and obesity (Kaleem, 2013).

High levels of macro-metals (30.02; 5.47; 2.583 g/Kg d.b) for Ca, Na and Mg respectively and low levels of trace metals (Fe, K) were found in *Musa acuminata* peel (Table 7). Heavy metals like Cr and Cd are not present.

Banana peel, which is rich in minerals, could be used as a powder to correct mineral deficiencies.

The size of the particle determines how much mucilage can be produced from banana peels powder 21.25±12.37% (for a particle size Φ=363µm); 17.25±3.181% (Φ=208µm); 14±5.656% (Φ=69µm).

The amount of mucilage is higher than that of flaxseed, which Mazza and Biliaderis, (1989), and Benahmed Djilali et *al*. (2022) reported to yield, respectively, 7.08 and 9.4%.

Additional factors, such as the temperature and extraction time, have also an impact on the mucilage content (Mazza, and Biliaderis, 1989).

The mucilage of banana peels was verified using the FT-IR method (Fig.1). A strong band is visible at 3438 cm-1 (-OH stretching) and 2935 cm-1 (-CH stretching) is observed (Sawut et *al*. 2014).

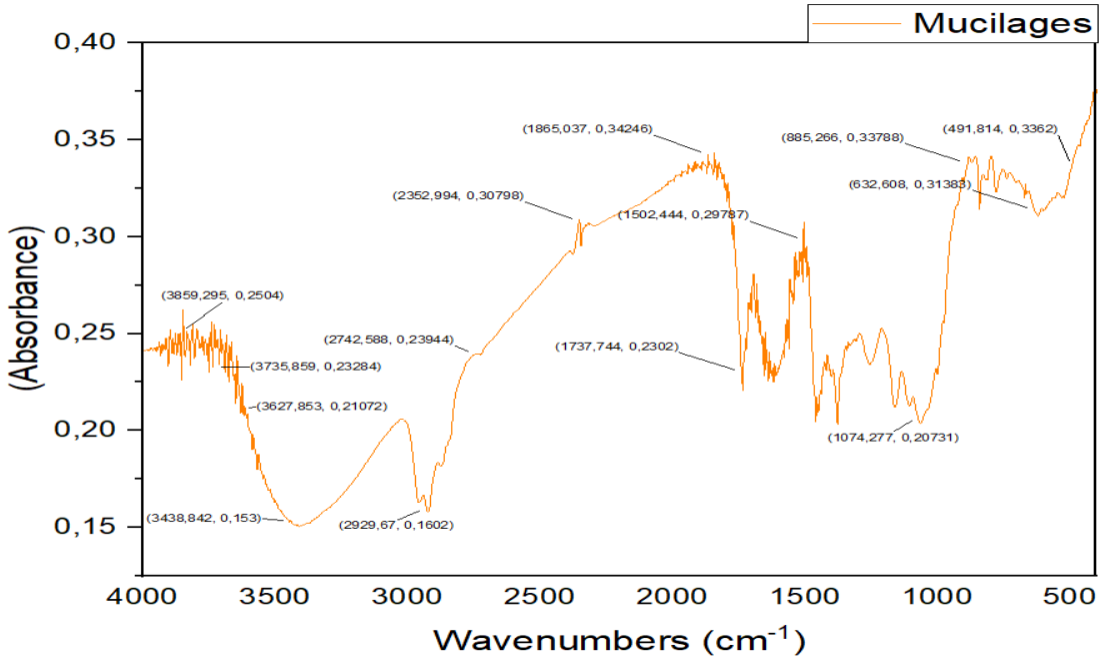


Figure 1. FT-IR spectrum of banana peel mucilage

The vibrational peaks at 1215 and 1074 cm-1 (CO, ether) and (C-C of pyrenoids), respectively, indicate the presence of polysaccharides (mucilage) in banana peel (Pereira et *al*. 2017).

The peaks at 1750 and 1733 cm-1 are caused by stretching vibrations of the C=O group (Kpodo et *al*. 2017).

The carboxyl group COOH of Galacturonic Acid (GalA) is responsible for the peaks between 1650 and 1733 cm-1 (Nejatzadeh-Barandozi, and Enferadi, 2012 ; Kpodo et *al*. 2017).

In the food industry, this polysaccharide can be used for stabilizing beverages and adding texture to dairy desserts, among other uses (Qin et *al*. 2005).

3.2. Fitting the models

3.2.1. Case of open air drying

The following empirical models for the Total Flavonoids Content, Total Carotenoids Content and % of the DPPH inhibition from

open air banana peels powder and their regression (Table 8).

Where X_1: Extraction Temperature T (°C); X_2: Particle size Ps (µm); X_3: Extraction Time t (min).

$$\text{Total Flavonoids Content}\left(\text{mg}\frac{\text{QE}}{\text{g}}\text{d.b}\right) \quad (3)$$

$$= 8.661 - 0.047X1 - 0.036X2 - 0.016X3 + 0.0002X1^2 + 0.00004X1X2$$
$$- 0.000005X1X3 + 0.00009X2^2 - 0.00008X2X3 + 0.0003X3^2$$

$$\text{Total Carotenoids Content(mg QE/g d.b)} \quad (4)$$

$$= 1.136 - 0.014X1 + 0.001X2 - 0.009X3 + 0.00006X1^2$$
$$+ 0.000002X1X2 + 0.00005X1X3 - 0.000003X2^2 - 0.000002X2X3$$
$$+ 0.00005X3^2$$

$$\%\ \text{of DPPH radical inhibition (water extract)} \quad (5)$$
$$= 22.172 + 1.358X1 - 0.041X2 - 0.131X3 - 0.007X1^2$$
$$+ 0.000038X1X2 - 0.0066X1X3 + 0.00011X2^2 + 0.000066X2X3$$
$$+ 0.0092X3^2$$

The fitted models for TFC and TCC (Eq.2 and Eq.3) using the open-air drying process were satisfactory, with significant regressions responsible for residual values and satisfactory determination coefficients R^2 (76 and 68%), which represents acceptable equation fitting. However, the percentage of DPPH inhibition, presents a low value of R^2 (54%) (Table 8).

Statistical analysis of the experimental design shows that the Total Flavonoids Content extract from open air banana peels powder is only affected by DIC operating parameters (particle size); the quadratic second-order effect was significant (P< .05) as observed in Fig.2.

Fig. 3 and Eq. 3 allow noting the extraction temperature T has an impact on the quantity of Total Carotenoids extracted from open air banana peels powder (a negative linear first-order effect was significant (P< .05)). When the extraction temperature decreases the TCC increases.

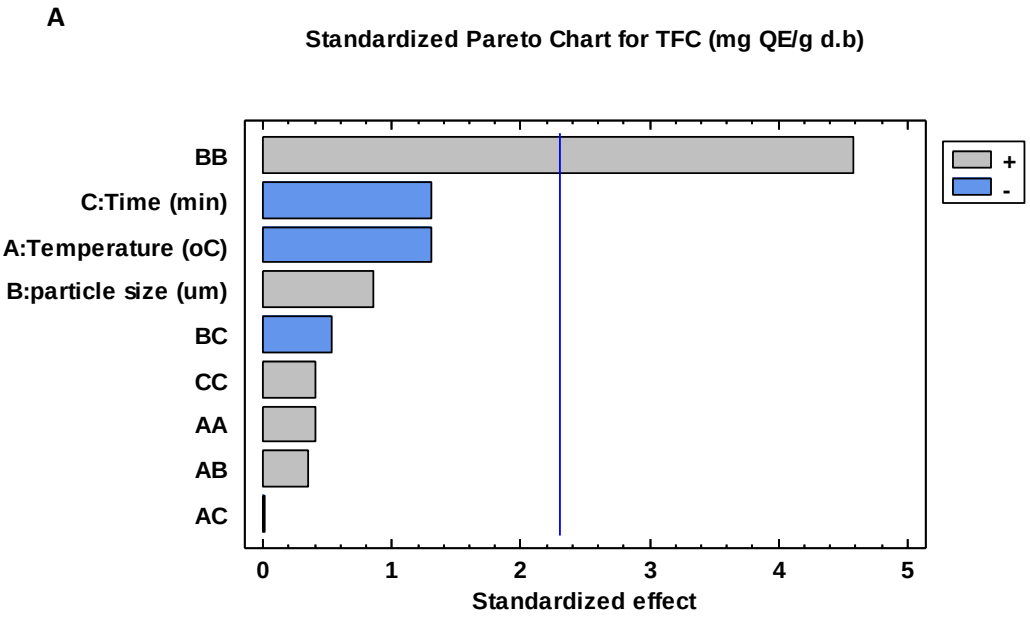
Here too, statistical analysis showed that the percentage of DPPH inhibition was not affected by any of the studied parameters (Temperature, Particle size and time), and the other terms from eq.4 are not statistically significant (P>05) (Fig.4).

The absence of interaction effects for TFC, TCC, and the percentage of DPPH inhibition suggests that there is no synergistic interaction between the components being studied.

Table 8 Regression coefficients of the second-order polynomial and their significance (open air)

	TFC		TCC		% of DPPH inhibition (Water extract)	
Regression coefficients	Values	*P*-value	Values	*P*-value	Values	*P*-value
a_0	8,66179		1,13642		22,1717	
a_1	-0,0472928	0,2291	-0,0139118	0,0247	1,35838	0,3235
a_2	-0,0356211	0,4181	0,00101351	0,2785	-0,0407254	0,4486
a_3	-0,0162992	0,2269	-0,00940816	0,0736	-0,130885	0,4893

a_{11}	0,000197904	0,6917	6,41098E-05	0,3089	-0,0075248	0,1363
a_{22}	8,6661E-05	0,0018	-3,13178E-06	0,2143	0,00011356	0,543
a_{33}	0,000284981	0,6917	0,0000539181	0,5434	0,00923629	0,1957
a_{12}	4,24383E-05	0,7315	1,92901E-06	0,8984	3,78086E-05	0,9741
a_{23}	-7,68519E-05	0,6063	-1,57407E-06	0,9308	6,57407E-05	0,9624
a_{13}	-4,62963E-06	0,995	4,9537E-05	0,5883	-0,00659722	0,358
R^2	76,43 percent		68,97 percent		54,62 percent	



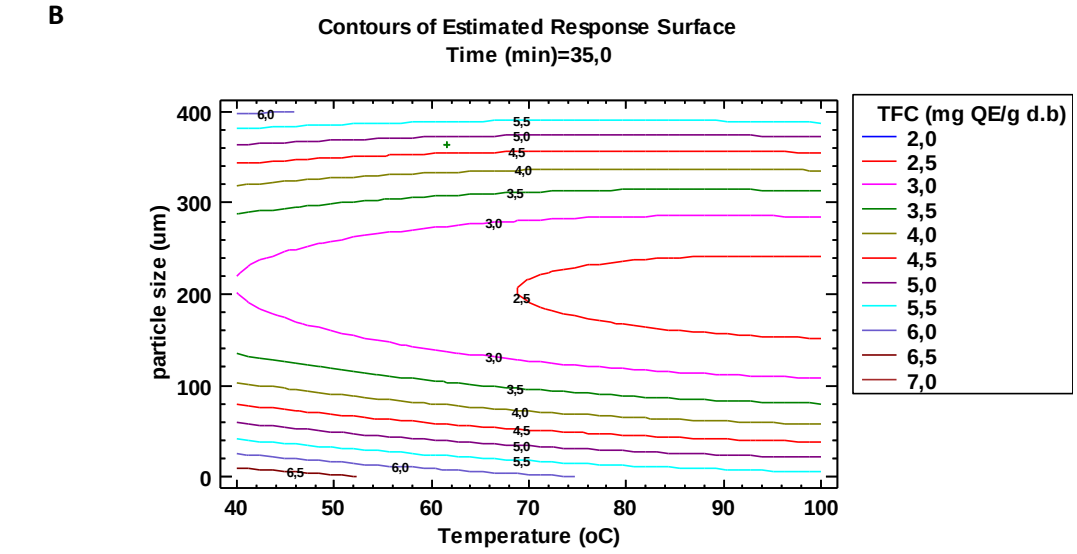


Figure 2. Effect of open-air extraction parameters (temperature (oC), particle size (µm) and extraction time (min)) on the Total Flavonoids Content: (a) Pareto Chart; (b) Response surface

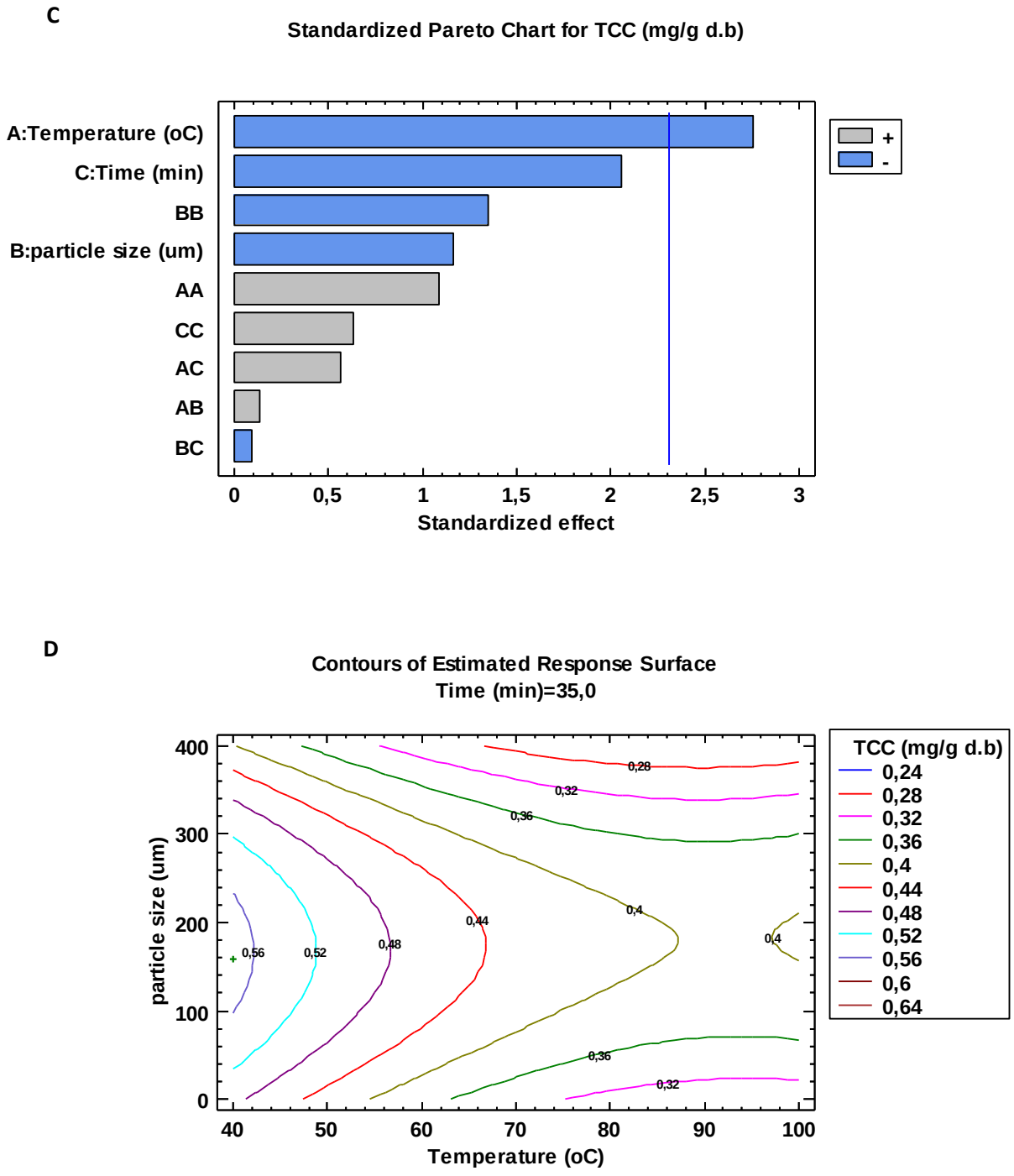


Figure 3. Effect of open-air extraction parameters (temperature T(oC), particle size Ps (µm) and extraction time t(min)) on the Total Carotenoids Content: (c) Pareto Chart; (d) Response surface

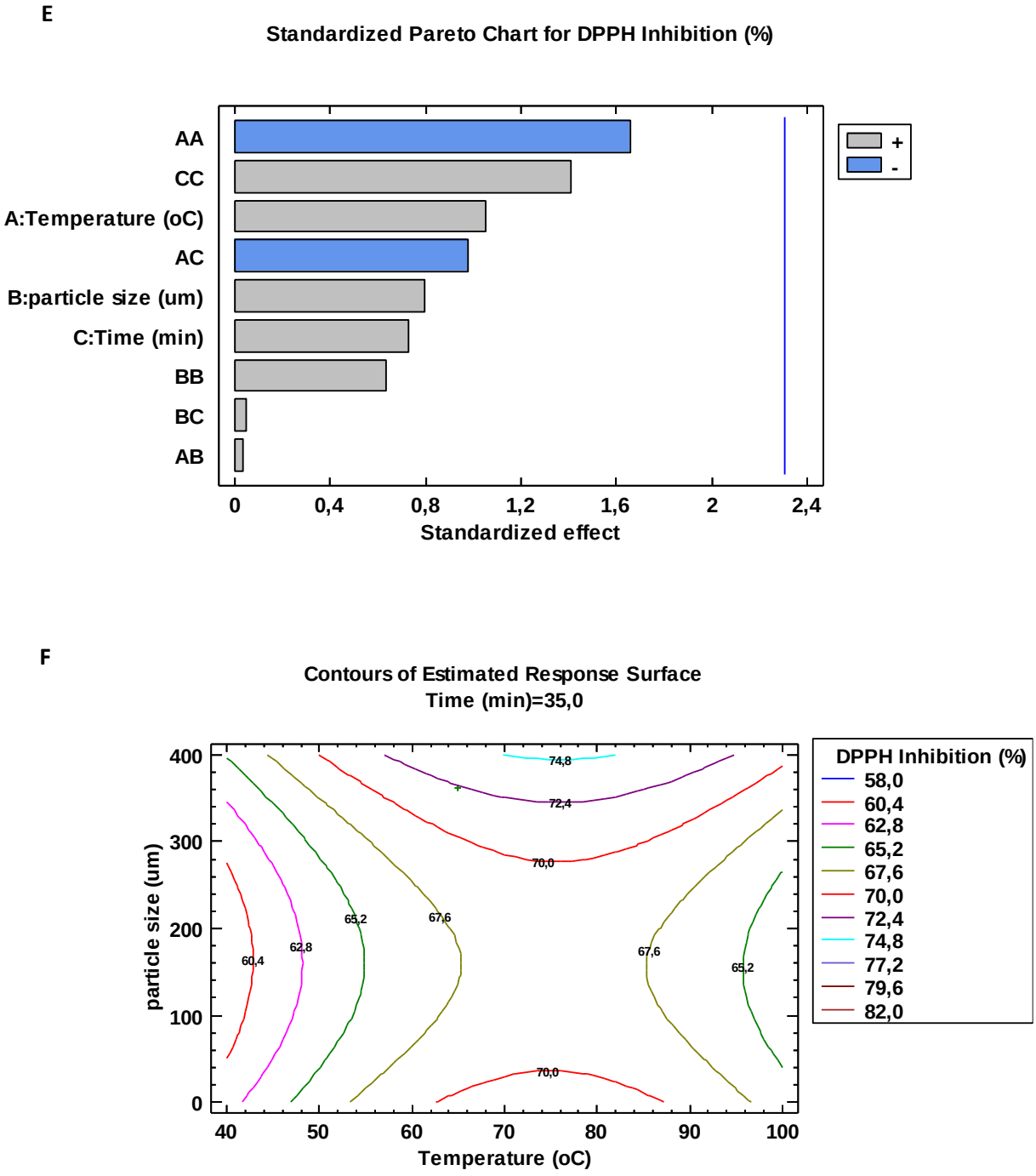


Figure 4. Effect of open-air extraction parameters (temperature T(oC), particle size Ps (µm) and extraction time t(min)) on the Total Carotenoids Content: (e) Pareto Chart; (f) Response surface

3.2.2. Case DIC assisted Swell-drying

The following responses form of a second-order polynomial for TFC, TCC and % of the DPPH inhibition from Swell-Dried banana peels powders and their regression (Table 9).

Where X_1: Treatment time t (s);
X_2: Steam pressure P (MPa);
X_3: Number of cycles (−).

$$\text{Total Flavonoids Content}\left(\text{mg}\frac{\text{QE}}{\text{g}}\text{d.b}\right) \quad (6)$$
$$= 0{,}258343 + 0{,}0161853X1 + 14{,}0994X2 - 1{,}08437X3 + 0{,}0000997754X1^2 - 0{,}0738125X1X2 - 0{,}00184242X1X3 - 13{,}4117X2^2 + 1{,}27965X2X3 + 0{,}0778587X3^2$$

$$\text{Total Carotenoids Content}\left(\text{mg}\frac{\text{QE}}{\text{g}}\text{d.b}\right) \quad (7)$$
$$= 3{,}0694 + 0{,}00720274X1 - 15{,}9562X2 + 0{,}175842X3 + 0{,}0000130335X1^2 - 0{,}0193875X1X2 - 0{,}000413364X1X3 + 23{,}3667X2^2 - 0{,}429151X2X3 + 0{,}00617456X3^2$$

$$\%\text{ inhibition of DPPH radical of water extract} \quad (8)$$
$$= 57{,}4982 + 0{,}00424404X1 + 0{,}00424404X2 + 2{,}16904X3 + 0{,}000260012X1^2 + 0{,}0277965X1X2 - 0{,}0211525X1X3 - 0{,}650717X2^2 - 2{,}09894X2X3 + 0{,}198078X3^2$$

The DIC technique was used to successfully fit the TFC and TCC (Eq.5 and Eq.6). The resulting models were suitable with acceptable determination coefficients R^2, with similar values (~ 56%), indicating satisfactory equation fitting and appropriate adjustment models, except for the percentage of DPPH inhibition, which had a low value of R^2 (42.55%) (Table 9).

Table 9 Regression coefficients of the second-order polynomial and their significance (DIC)

	TFC		TCC		% of DPPH inhibition (Water extract)	
Regression coefficients	Values	*P*-value	Values	*P*-value	Values	*P*-value
a_0	0,258343		3,0694		57,4982	
a_1	0,0161853	0,874	0,00720274	0,9658	0,00424404	0,6759
a_2	14,0994	0,3739	-15,9562	0,1718	12,4278	0,4358
a_3	-1,08437	0,2764	0,175842	0,6346	2,16904	0,5362
a_{11}	9,97754E-05	0,0436	1,3034E-05	0,4568	0,00026001	0,2202
a_{22}	-13,4117	0,5007	23,3667	0,0082	-0,650717	0,9942
a_{33}	0,0778587	0,1408	0,00617456	0,7497	0,198078	0,3946
a_{12}	-0,0738125	0,0947	-0,0193875	0,2372	0,0277965	0,883
a_{23}	1,27965	0,365	-0,429151	0,4258	-2,09894	0,7398
a_{13}	-0,00184242	0,388	-0,00041336	0,6094	-0,0211525	0,0429
R^2	55,84 percent		56,07 percent		42,55 percent	

According to the study of the variance, Fig. 5 and Eq.5 allow noting that DIC treatment time t is only significant for the increase of Total Flavonoids Content (a positive quadratic effect was significant (P< .05)).

Statistical analysis of the experimental design showed the steam pressure P to be the only significant DIC operating condition affecting the increase of Total Carotenoids recovered from DIC swell-dried banana peels powder (a positive quadratic second-order effect was significant (P< .05)) (Fig.6).

Statistical analysis of the experimental design allowed obtaining Pareto Chart and Response surface (Fig.7), as well as the empirical model (Eq.7), which show that treatment time t and number of cycles are the main significant operating parameters. (a negative linear effect was significant (P< .05)). When the treatment time t and the Number of

cycles increase, the percentage of DPPH inhibition decreases.

3.3.Optimization of extraction conditions

3.3.1. Case of open air

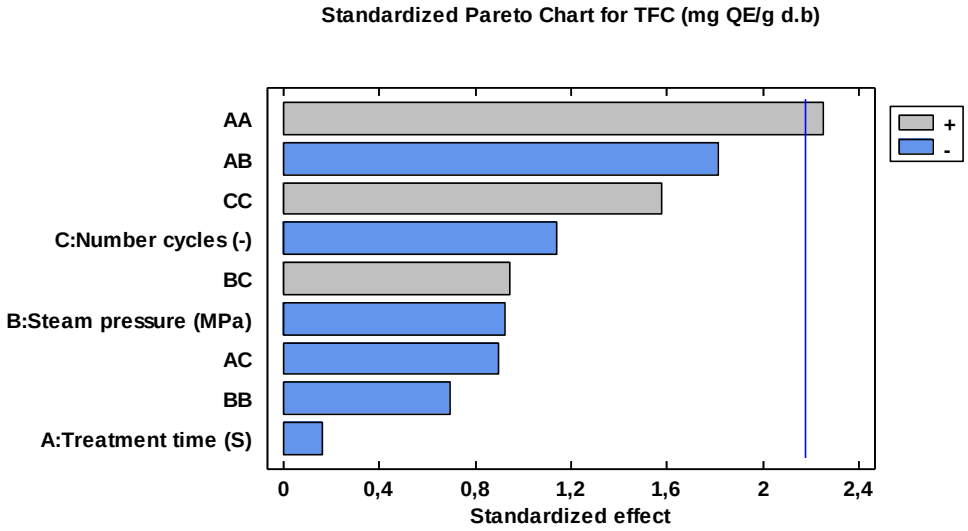
The optimal values for the variables used in the open air drying of banana peels, with maximum desirability were found to be as follows: temperature, 60.47°C; extraction time, 10min; and particle size, 348.648µm. The predicted values are satisfactory because the optimal desirability d=0.7.

The values for the responses at the optimized point were as follows (Fig.8):

Total Flavonoids Content= 5.13 (mg QE/g d.b) with a desirability d=1;

Total Carotenoids Content = 0.48 (mg /g d.b) with a desirability d=0.54;

% of DPPH inhibition=73.05% with a desirability d=0.63.



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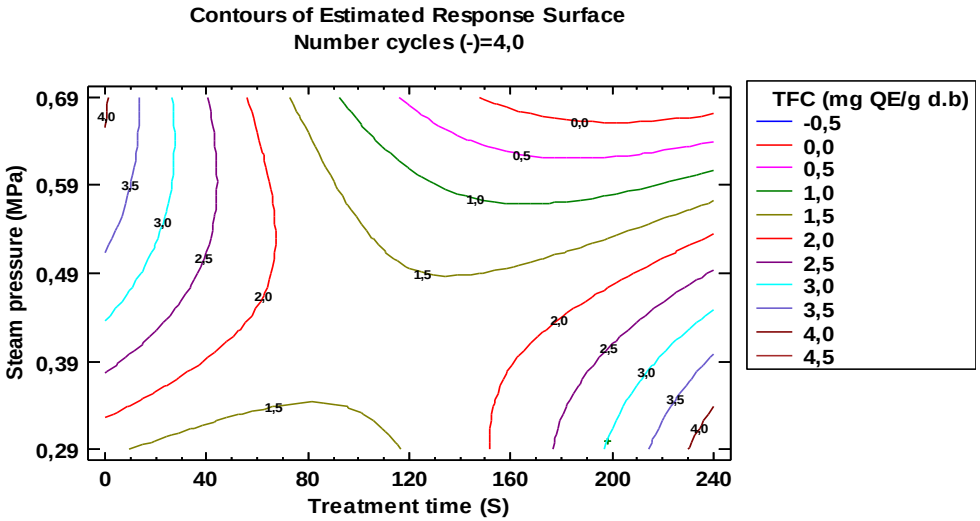

Figure 5. Effect of Swell-Drying with DIC parameters (Treatment time t(s), steam Pressure P (Mpa) and Number of Cycles)) on the Total Flavonoids Content: (g) Pareto Chart; (h) Response surface

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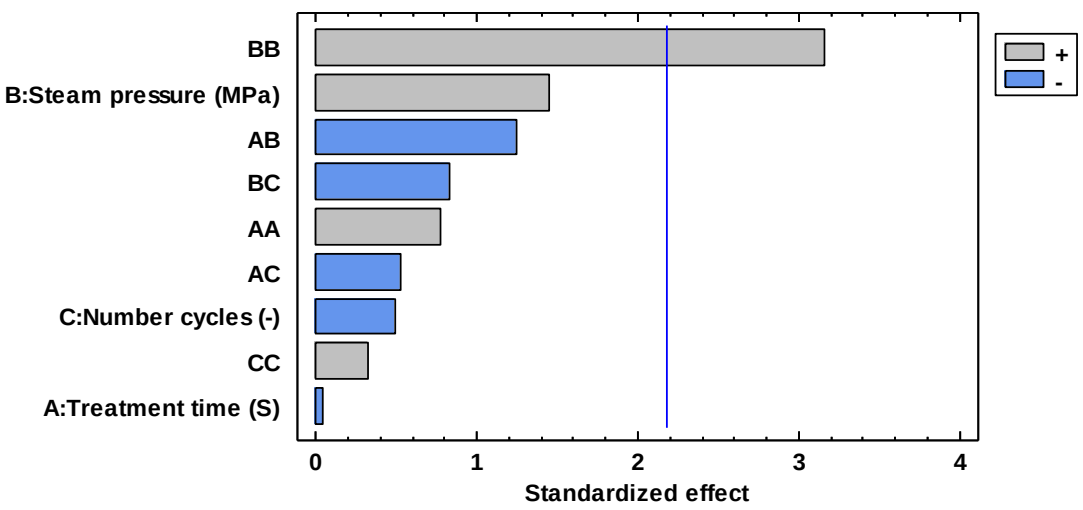

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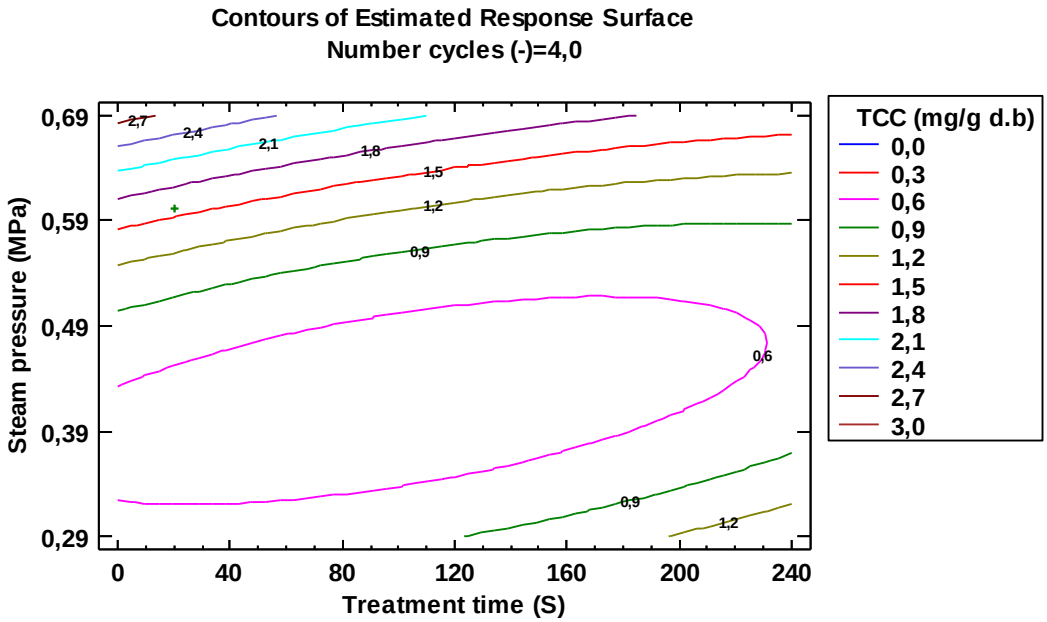

Figure 6. Effect Swell-Drying with DIC parameters (Treatment time t(s), steam Pressure P(Mpa) and Number of Cycles)) on the Total Carotenoids Content: (i) Pareto Chart; (k) Response surface

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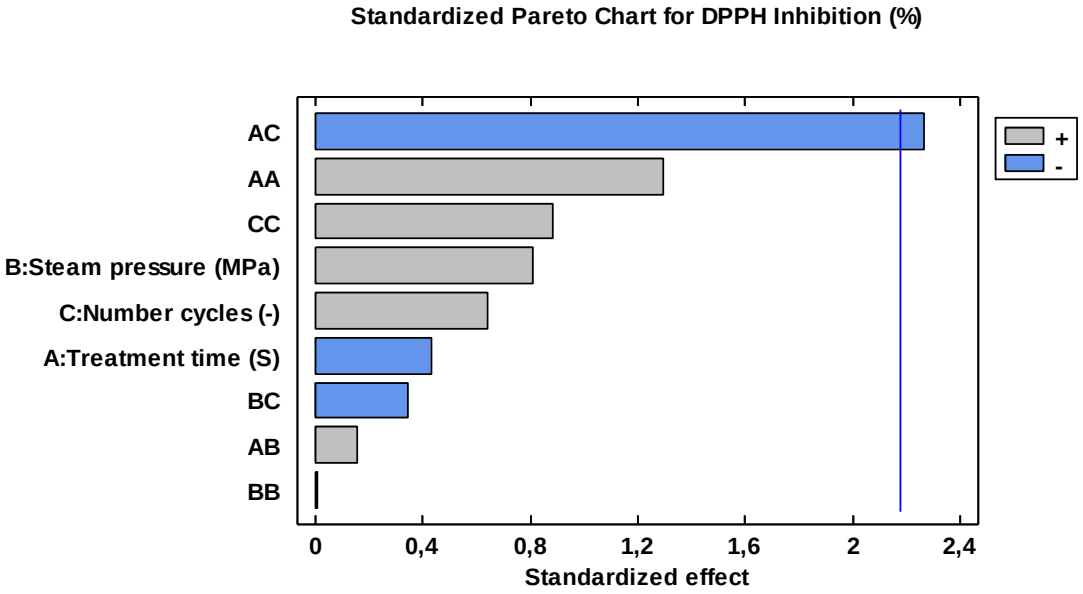

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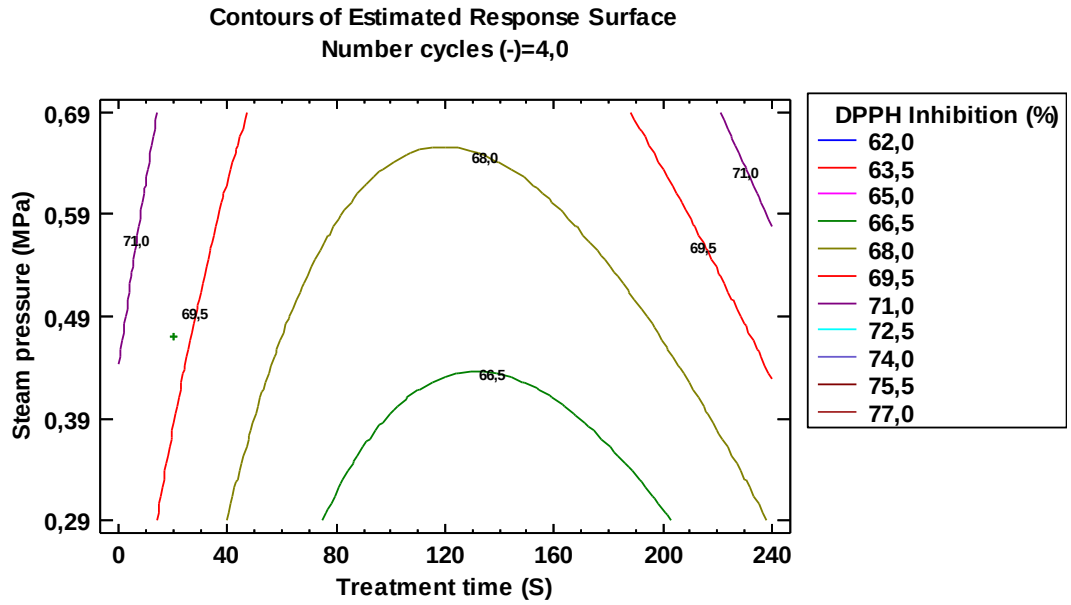

Figure 7. Effect Swell-Drying with DIC parameters (Treatment time t(s), steam Pressure P(Mpa) and Number of Cycles) on the % of the DPPH inhibition: (l) Pareto Chart; (m) Response surface

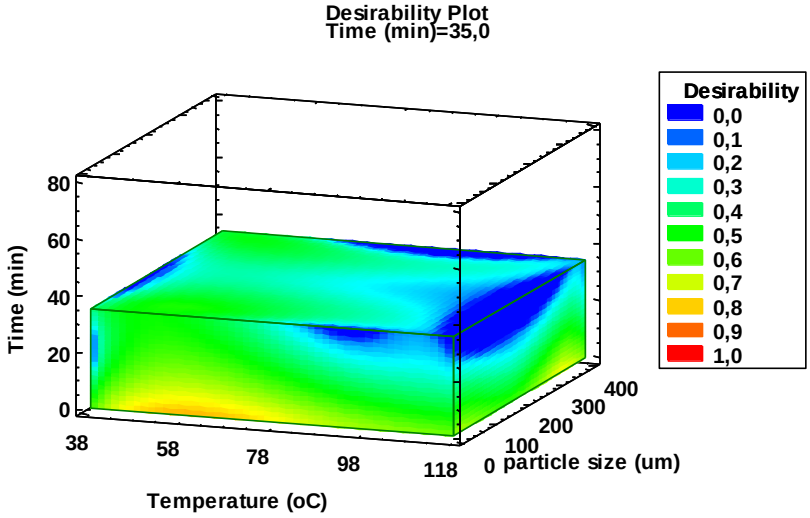

Figure 8. Response surface of the effect of open-air extraction parameters (temperature (oC), particle size (µm) and extraction time (min)) on desirability

3.3.2. Case of Swell- drying by DIC

The optimized DIC operating parameters with maximum desirability were found to be as follows: treatment time, 24.46 s; steam pressure, 0.59MPa; number of cycles, 6.38.

Fig.9 allowed obtaining the following response with the highest optimized desirability (d=1):

TFC=4.07 (mg QE/g d.b),
TCC= 1.37 (mg /g d.b)
DPPH inhibition=75.97 %

Hence, the predicted values are perfect (d=1).

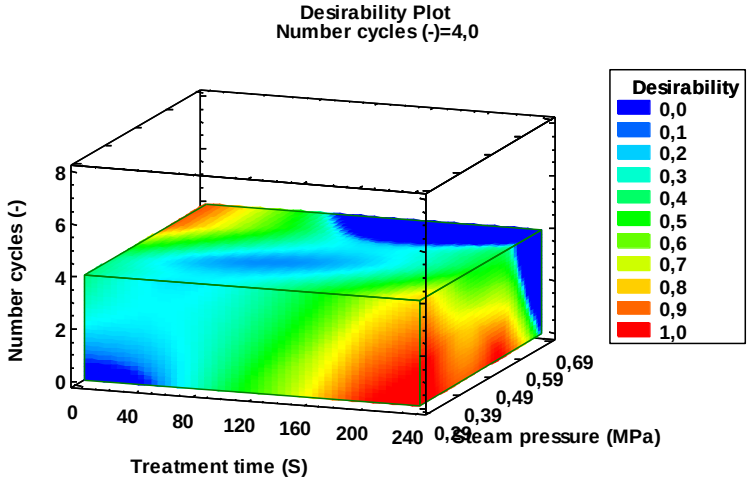

Figure 9. Effect of DIC-assisted Swell-Drying parameters (Treatment time t (s), steam Pressure P (Mpa) and Number of Cycles Nc)) on the Desirability

3.4. Discussions

The Response optimizer Function was used to optimize the operating conditions of two drying processes (open air and DIC assisted Swell Drying) in order obtain a maximum of nutraceutical components (flavonoids and carotenoids) from banana peels.

In this study, various quantitative findings on the biochemical properties were investigated. This concerns minerals, fatty acids, mucilage, flavonoids, and carotenoids.

The optimized extraction parameters for open-air dried banana peel were found at 60.47°C, extraction time (10min) and particle size (348.648µm). Additionally, the thermal treatment time (24.46s), saturated steam pressure (0.59MPa) and cycles Number 6.38 were the optimized operating DIC texturing parameters that were found. Under the selected optimum conditions, the suitability of the models to predict the responses of the Total Flavonoids Content and Total Carotenoids Content and % of DPPH inhibition was evaluated.

The richness of banana peels in nutraceuticals compounds (flavonoids and carotenoids) was linked to the drying processes and the operating conditions. The highest optimized concentration of Total Flavonoids Content (5.13 mg QE/g d.b) was observed from open-air dried banana peels and correlated positively with the particle size.

However, the TFC released from DIC Swell-dried banana peels was correlated positively with the heat treatment time with DIC at the optimum value (4.07 mg QE/g d.b).

In plant tissues, phenolic compounds appear as glucosides and have a lot of hydroxyl groups. This produces a large variety of these molecules with various properties, including solubility (Muzolf-Panek, and Gliszczynska-Swiglo, 2022).

Using water as an extraction solvent, in order to hydrate the particle, which intensifies mass transfer by diffusion (Ghitesci et *al*. 2015).

Our findings are comparable to those found by Someya et *al*. (2002), who showed that banana peels had a high level of total phenolic content (907mg/100g). On the other hand, banana peels heat-dried by microwave irradiation have a higher phenolic component level than freeze-dried banana peels (Vu et *al*. 2018).

Conversely, increasing temperature resulted in a loss of sensitive compounds and decreased the antioxidant activity. According to Passo Tsamo et *al*. (2015), boiling banana peels decreased the amount of flavonoids while increased the ferulic acid.

According to earlier studies, the use of other extraction procedures was responsible for the loss of some phenolic compounds (Gonzalez-Montelongo, 2010; Toh et *al*. 2016; Hernandez-Carranza et *al*. 2016).

The variation of phenolic compounds depends on the variety, cultivation condition and the maturity of fruit.

Additionally, it can be said that the heat treatment with DIC is an effective process for releasing bound phenolic compounds from their phenolic acids. This has attracted a lot of interest due to their antioxidant qualities.

When using DIC-texturing, biological composition is preserved thanks to the expanded granule powder with a high level of interaction (porosity and capillarity) (Benahmed Djilali et *al*., 2016; Benseddik et *al*., 2022).

The level of carotenoids was maximised at 1.72 mg /g d.b by using Swell-drying with DIC.

Our findings are comparable to those of Nguyen et *al*. (2014), who showed that carrots subjected to Swell-Drying assisted by DIC, which maximised porosity for the steam pressure (P=0.5MPa) and thermal holding time (~ 36 s). Furthermore, the optimal effectiveness of an extract cannot result from one active compound but rather form the combined synergistic action of various constituents.

The effects of temperature, extraction time, and methanol concentration on the TFC from *Nigella sativa* seeds were examined in the study by Muzolf-Pan and Gliszczynska-Swiglo (2022). TFC increased with an increase in temperature and extraction time up to T =

67oC, t = 208 min, and methanol concentration = 50%, allowing the maximum predicted value of TFC =7.68 mg QE/g.

4. Conclusions

Some operational parameters of the two drying processes (open air and Swell-Drying with DIC were optimized to maximise the availability of Total Flavonoids and carotenoids using single- and multi-response desirability functions.

Texturing banana peel by DIC promotes high levels of carotenoids and maximised the antioxidant activity

Banana peel water extract could be a good source of bioactive substances, which could be further used as a natural antioxidant in various industrial settings.

However, for the sake of this study it would be interesting to apply the optimum operational parameters resulting from the open air to the second drying process.

Future study will focus on identifying the phenolic components of the obtained powders, optimizing additional parameters, and using those components to elaborate medicinal forms.

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6. Abbreviations

db Dry basis (g)
DIC Instant Controlled Pressure-Drop
DPPH 2,2-diphenyl-1-picrylhydrazyl
SD Swell-Drying
SD Swell-Drying
TFC Total Flavonoids Content
TCC Carotenoids Content

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ENHANCEMENT OF PHYSICAL PROPERTIES OF GELATIN-BASED FILM BY BOVINE SERUM ALBUMIN

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ABSTRACT

Biopolymer-based films are created from plant or animal materials such as carbohydrates and proteins. In this study, a biopolymer-based film from gelatin and Bovine Serum Albumin (BSA) was created to form a film through cross-linking interaction of both biopolymers. The synthesized biopolymer film was characterized using Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), and Fourier Transform Infrared (FTIR) as morphological evaluation. Besides, the encapsulation efficiency of BSA-gelatin blend film was also evaluated. The coagulation process was performed using acetone carried out at 35 ºC for 4 hours under stirring. The formed particles were separated from the solution using high centrifugation around 12,000 rpm for 30 min and then kept at -80 ºC.

Based on the FTIR spectra showed that BSA and gelatin have the same functional group such as hydroxyl (OH) and Amina I, II, and III which appear at 3278 cm^{-1}, 1633 cm^{-1}, 1542 cm^{-1}, and 1241 cm^{-1}, respectively. After linkage reaction, the gelatin-BSA interaction created more strength interaction that caused no vibration functional groups seen. It also increased glass transition temperature where the resulted melting point of gelatin-based and 70% BSA/gelatin-based films are 152.2 and 260 °C, respectively. The encapsulated BSA in the blend film reached above 90% in the BSA/gelatin ratio of 70%.

1.Introduction

In the last decade, nanotechnology is a fascinating field that was interested some researchers. The expansion of nanotechnology eventually is considered in this stage because it possesses various advantages and is favorable in some industry fields. Hence, some products were designed via nanotechnology, including nanofibers (Jeong and Park, 2014; Panzalvota et. al., 2014) and nanocomposites (Umamaheswari et. al., 2015; Premlatha and Kothai, 2015; Satapathy et. al, 2017).

One of the raw materials commonly used and interested by the researcher is gelatin. Gelatin has been exploited and developed via nanotechnology to be converted to nanomaterial products, named; gelatin nanoparticles (GNPs). The application of gelatin is mostly used in several products beneficial to drugs delivery system (Foox and Liberman, 2015), as well as delivery of protein including Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) (Kaintura et al., 2015), and as food

packaging potential (Hanani et al., 2014), and hard and soft capsule (Prasad, 2017).

The application of gelatin as a macromolecule transport, including protein delivery models, produces many advantages because it can protect from protein degradation and control the release time (Thakur et al, 2013). Besides, the application of gelatin nanoparticles also increases the bioavailability of the drug (Azimi et al, 2014). Gelatin applications are also a proficient carrier for large or small molecules (Jahanshahi and Babaei, 2008).

The gelatin application as a protein delivery system has been carried out and reported by several studies. Azimi et al. (2014) reported that gelatin from bovine skin is used as BSA delivery using a two-step desolvation method. The encapsulated BSA into gelatin nanoparticles using the water/oil (w/o) emulsion method has an average particle diameter is 840 nm (Li et al, 1998). The study from Azimi et al. (2014) showed that encapsulated BSA into gelatin nanoparticles using a two-step desolvation method resulted in a particle size around 200-300 nm. This study is not applying gelatin nanoparticles as a delivery system of BSA but it is focused on the evaluation of the biopolymer gelatin-based and BSA/gelatin-based film that is able to support the application of food packaging or hard and soft capsule. A previous study by Panzavolta et al., (2014) created gelatin-based film with a simple method.

2. Materials and methods

2.1. Synthesis of Blend Film from BSA and Gelatin

The synthesis of BSA-gelatin-based film follows the research from Sailaja and Amareshwar (2012) with slight modification. Gelatin nanoparticles were synthesized using acetone as a desolvating agent via a one-step desolvation method. 1 g of hydrolyzed gelatin was dissolved with 10 mL aquades and heated at 35^0C for 5 min. The pH of the gelatin solution was adjusted around pH range 2.5-3 using HCl 0,1 N and then heated at 35^0C for 5 min. After that, 25 mL of the acetone solution was added to the gelatin solution dropwise for 10 min. In the last step, this solution was added with 500 uL glutaraldehyde 8% and stirred for 4 hours until raising the particles. To obtain the gelatin nanoparticles from the solution was separated using centrifugation at 1000 rpm for 30 min. Afterward, the gelatin nanoparticles were dried to get the solid form. The encapsulation process of BSA level into gelatin nanoparticles is performed using a similar method with gelation nanoparticles producing. BSA/gelatin ratio used is between 0-100%.

2.2. The Entrapment Calculation of Bovine Serum Albumin (%) into Blend Film

The entrapped procedure of BSA into gelatin nanoparticles was determined using a spectrophotometer at λ = 278 nm (Azimi et al, 2014). The unloading of BSA was used to calculate the encapsulation efficiency (EE, %) using the equation:
(Abdala et al., 2015)

EE (%) = [(total BSA added – amount of free BSA)/total BSA added] x 100. (1)

2.3. Characterization of gelatin nanoparticles

The characterization of the gelatin nanoparticle products was analyzed using Scanning Electron Microscopy (SEM) (Phenom Pro X) to detect the morphology of the nanoparticles and Differential Scanning Colorimetry (DSC) (1-STARe, Mettler Toledo, Columbus, OH) to characterize the melting point of gelatin nanoparticle resulted. The Fourier Transform Infrared (FTIR) (Perkin Elmer) was used to detect the functional group vibrations of nanoparticle products.

3.Results and discussions

3.1. Identification of Interaction between BSA and Gelatin using FTIR

The gelatin-based film was synthesized using the one-step desolvation method because it is a more simple and convenient method. This method is able to encapsulate biopolymer is approximately 99% (Wang et al., 2018) and can produce stable gelatin nanoparticles in ranging small particle sizes of around 200-300 nm

(Mohanty and Bohidar, 2003; Azimi et. al, 2014). BSA/gelatin-based film is a new material because the previous research was focused on the application of gelatin as a BSA delivery system. Both gelatin and BSA are composed of different protein monomers. The amino acid levels in gelatin are glycine of 33%; Proline of 21%; Hydroxyproline of 10%, and X (other amino acid monomers) of around ~36% (Djabourov, et al., 1988; Yasmin et al., 2017). While the amino acids of BSA are Lysine, Threonine, Valine, Leucine, Isoleucine, Tryptophan, Histidine, Methionine, Methionine + Cysteine, Tyrosine, Phenylalanine, Phenylalanine + Tyrosine, Aspartic acid, Serine, Glutamic acid, Proline, Glycine, Alanine, and Arginine (Prata and Sgarbieri, 2008).

To evaluate the chemical interaction between gelatin and BSA in blend film can be seen in Figure 1. Figure 1 described that BSA possesses the vibration fields in 3278 cm^{-1} as a hydroxyl (OH) stretching or NH_2 stretching vibration (Amide A) (Qiu et al., 2019). The vibration of C-H alkane arises of around 2972 cm^{-1} and the supported other wavenumbers at 1538 cm^{-1} and 1454 cm^{-1} as CH_3 and CH_2 vibration, respectively (Figure 2). Besides, the wavenumber at 2972 cm^{-1} also illustrates the vibration asymmetric of Amide B as free $–NH^{3+}$ from lysine or terminated N (Yang et al., 2019). While the functional group vibration at 1633 cm^{-1}, 1542 cm^{-1}, and 1241 cm^{-1} indicate molecule vibrations from amide groups such as amide I, amide II, and amide III (Kaintura et al., 2015; Yang et al., 2019).

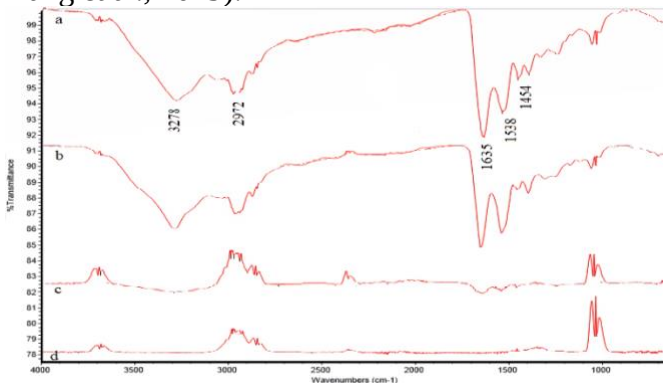

Figure 1. FTIR Spectrum from; a. BSA ; b. Hydrolyzed Gelatin; c. Gelatin-based film; d. 70% BSA/gelatin-based film

The blended gelatin-BSA film and gelatin film have changed the vibrations of functional groups. Previous research from Kaintura et al., (2015) reported that the indication of protein encapsulated into gelatin nanoparticles would change their vibrations and decrease FTIR intensity. However, the gelatin nanoparticles involve intermolecular interaction through hydrogen bonds. It caused the vibration of functional groups in the gelatin structure to be unable to vibrate freely. Therefore, the intensity of the FTIR spectrum of gelatin nanoparticles has decreased significantly compared with gelatin structure (Figure 1). It is also seen in the blended gelatin-BSA as an indication that BSA functional group also interacts with gelatin functional groups forming a rigid molecule.

3.2. Melting point of BSA/Gelatin-based Film

The rigidity of BSA/gelatin-based film had influenced the melting point of the material. DSC analysis also reveals differences in melting point transposition between gelatin nanoparticles and BSA encapsulated into gelatin nanoparticles. Both particles have been observed that the melting point of the gelatin-based film is an exothermic peak of about 152.20°C, while BSA encapsulated into the gelatin-based film is able to increase the melting points which is an exothermic peak of around 201.43°C (Figure 2).

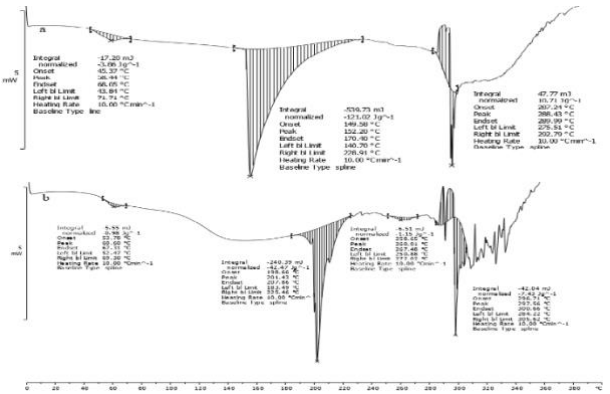

Figure 2. The Result of Differential Scanning Colorimetry (DSC) of nanoparticle; a. Gelatin-based film; b. 70% BSA/gelatin-based film

Based on this result, the interaction of BSA with gelatin is able to build a rigid structure, it can occur through the inclusion of complex interaction between BSA and gelatin causing its melting point to increase significantly. Yasmin et al., (2017) explained that mechanical properties of gelatin nanoparticles covering thermal properties and water swelling ability were significantly affected by the interaction gelatin formation, especially in complex biopolymers. Previous research reported several characteristics of the material applied for edible plasticizer film excipients (Table 1).

Table 1 explained that the development of blended polymers can improve their physical properties and quality. Occasionally, they can be fabricated as phenolic-polymeric hybrids in nutraceutical and drugs delivery systems, edible films, hydrogels, and nanoparticles (Liu et al., 2019). One of the features evaluated for the polymeric-based film is the T_g value (Liang et al., 2019). The T_g value indicates generally to amorphous structure or semicrystalline materials formed (Hoque et al., 2011; Acevedo et al., 2014), while the T_m value is related to the dissociation of hydrogen bond and polymers chains cleavage occur (Cai et al., 2019). The result of this study showed that the gelatin-BSA composite can increase the T_g value indicating both polymers interact strongly. The intermolecular reaction of both polymers will increase of T_g value (Hosseini et al., 2013), indicating that the moisture content in the matrix decreased (Avecedo et al., 2014). This phenomenon will affect other physical and mechanical properties of the materials. The rigidity of this film was also seen in SEM analysis (Figure 3).

Table 1. The development of blended polymeric as edible film material

Polymeric Agent 1	Polymeric Agent 2	Physical Properties	Applications	References
Chitosan	Gelatin	$T_{g\ (gelatin)}$: 50.6°C, T_m: 69.6 °C $T_{g\ (gelatin\text{-}chitosan)}$: 51.1°C, T_m: 75.0°C	Biofilm	Acevedo et al., 2014
Chitosan	Gelatin	$T_{g\ (gelatin\text{-}chitosan)}$: 56.18°C	Edible film	Cai et al., 2019
Chitosan	Fish gelatin	$T_{g\ (gelatin)}$: 29.8°C $T_{g\ (chitosan)}$: 56.1°C $T_{g\ (gelatin\text{-}chitosan)}$: 55.9°C	Edible film	Hosseini, et al., 2013
Chitosan	Cellulose	$T_{g\ (chitosan)}$: 91.03°C $T_{g\ (chitosan\text{-}cellulose)}$: 91.33°C	Biofilm	Liang et al., 2019
Chitosan	HPMC	$T_{g\ (chitosan)}$: 114.06°C $T_{g\ (HPMC)}$: 164.56°C $T_{g\ (chitosan\text{-}HPMC)}$: 196.23°C	Edible film	Rotta et al., 2011
Chitosan	Methyl Cellulose	$T_{m\ (chitosan)}$: 118°C $T_{m\ (methyl\ cellulose)}$: 115°C $T_{m\ (chitosan\text{-}methyl\ cellulose)}$: 110°C	Edible film	Rachtanapun and Wongchaiya, 2012
Gelatin	-	$T_{g\ (gelatin)}$: 60 °C, T_m: 214,02°C	Biofilm	Perkasa et al., 2013
Gelatin	MPI	$T_{g\ (gelatin)}$: 33.70°C $T_{g\ (MPI)}$: 22.83°C $T_{g\ (gelatin\text{-}MPI)}$: 30.08°C	Edible film	Hoque et al., 2011
Gelatin	PVA	$T_{g\ (gelatin\text{-}PVA)}$: 43-53°C, T_m: 98.5-138.9°C	Edible film	Sobral et al., 2011
Gelatin	BSA	$T_{g\ (gelatin)}$: 58.44 °C, T_m: 152.20°C $T_{g\ (gelatin\text{-}BSA)}$: 60.60°C, T_m: 201.43°C	Potential for edible film and hard capsule	This work

PVA: Poly Vinyl Alcohol; HPMC: hydroxypropylmethylcellulose; MPI: mungbean protein isolate
Tg: glass transition temperature, Tm: melting temperature

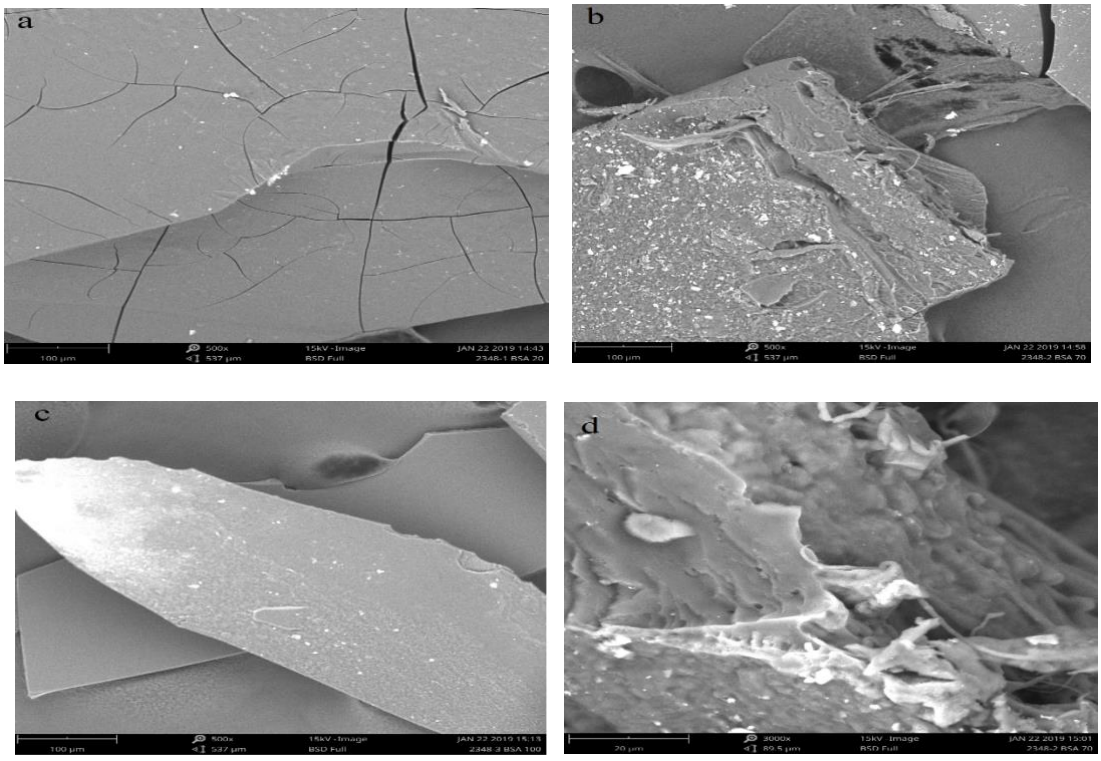

Figure 3. Scanning Electron Microscopy of Different of BSA Concentration loaded into Gelatin-based film; a. 20 % (w/w) BSA; b. 70% (w/w) BSA; c. 100% (w/w) BSA (all scale pictures were seen at 100 µm) and, d. 70% BSA (scale picture was seen at 20 µm)

3.3. Morphology analysis of gelatin-based film using SEM

Based on figure 3. the effect of BSA concentration affected the different morphology of the produced gelatin-based film. The use of 20% BSA loaded into gelatin-based film generate particle properties that have easily cracked. Whereas the 70% BSA and 100% BSA can create a rigid material and a smooth material, respectively. Even the 70% BSA in gelatin-based film produces strong fibers (figure 3b and 3d). This fiber is an aggregate of spherical shape from gelatin nanoparticles because of the temperature conditions in the gelatin-based film processing. Besides, the suggestion of this study is to use a 70% BSA/gelatin ratio in the production of BSA/gelatin-based film because gelatin-based film can encapsulate the optimum of BSA concentration in 50-70% BSA/gelatin ratio up to more than 90% (Figure 4). The encapsulation efficiency (EE) resulted was similar to previous research from Kaintura et al., (2015) reported that the encapsulation efficiency of Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) into gelatin nanoparticles which is 90% and 80%, respectively. Meanwhile, Azimi et al., (2014) also explained that the two-step desolvation method can produce a particle size range of gelatin nanoparticles of 200-300 nm with entrapment efficiency of BSA reached 87.4%. Based on this result, these physical properties are supporting the use of modified materials as edible plasticizer film excipients or hard capsule materials. However, the blended gelatin-BSA is also applied as a soft capsule or an edible plastic because BSA creates smooth material in a high BSA/gelatin ratio.

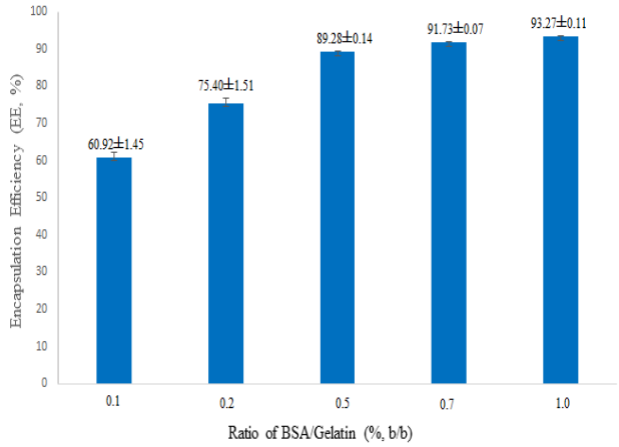

Figure 4. Encapsulation Effeciency (EE) of BSA Loaded into Gelatin-based film

4. Conclusions

The encapsulated BSA into gelatin nanoparticles affects several properties of the material including vibration of functional groups, melting point, and physical properties. The BSA/gelatin ratio of 70% produced a strong fiber that is agglomerating with other fiber become a solid material and the encapsulation efficiency reached above 90%. Based on this research, the effect of the encapsulated BSA into gelatin nanoparticles can improve material properties, including rigid characteristics and melting point. Therefore, it may be applied as edible plasticizer film excipients.

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IMPACTS OF PRETREATMENTS AND DRYING TECHNIQUES ON THE PHYSIOCHEMICAL, MICROBIAL, AND SENSORY PROPERTIES OF WHITE YAM (*Dioscorea Rotundata*) FLOUR

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ABSTRACT

The impacts of pretreatments and drying techniques on the physicochemical, microbial, and sensory properties of white yam are examined in this study. Fresh tubers were cleaned, pretreated, uniformly sliced, dried, and turned into flour. Physicochemical, microbial, and sensory attributes of the flours were assessed using accepted techniques. The findings demonstrated that, with the exception of the gelatinization temperature, all physicochemical parameters under investigation were significantly ($p<0.05$) influenced by the pretreatments and drying techniques. The moisture ranged from 3.00 to 11.50%, the bulk density was 0.67 to 0.71 g/mL, the water absorption capacity was 1.80 to 2.47 g/g, the swelling capacity was 27.30 to 70.00%, the gelatinization temperature was 74.00 to 80.00°C, and the least gelation capacity was 8.00 to 14.00%. Compared to solar and oven drying, the total viable bacterial count in open sun dried yam flour was significantly higher ($p<0.05$). *Alternaria* was isolated with using open sun and solar drying, *Aspergillus niger* and *Aspergillus flavus* were isolated using open sun drying, *Candida albicans* was isolated with using open sun and oven drying, *Rhizopus sp*. was isolated using all drying techniques taken into account. The *amala* produced from yam flour pretreated with blanching and 0.1 percent sodium metabisulphite along with oven and solar drying was preferred by the panellists to untreated and open sun-dried ones, according to the sensory analysis of the *amala*. The extensive variation in the physicochemical characteristics of the flour samples could be used as a database to enhance the processing of yam flour.

1. Introduction

The term "yam" refers to a number of plant species in the family Dioscoreaceae that produce edible tubers that are a staple crop in West Africa (Asiedu, 1992). It is a multi-species crop that was primarily developed in Africa and Asia before being introduced to other regions of the world (Hahn, 1995). Perennial herbaceous vines known as yams are grown for their starchy tubers throughout many temperate and subtropical areas. There are up to 600 species of yam tubers, some of which are crucial for the economy. *Dioscorea rotundata* (white yam), *Dioscorea cayenensis* (yellow yam), *Dioscorea alata* (water yam), *Dioscorea bulbifera* (aerial yam), and *Dioscorea esculenta* (Chinese yam) are among them. *Dioscorea rotundata* (white yam) and *Dioscorea alata* (water yam) are the two species that are most prevalent in Nigeria. *Dioscorea dumetorum*, *Dioscorea opposita*, *Dioscorea japonica*, *Dioscorea hispida*, and *Dioscorea transversa* are additional yam species with minor economic importance in some tropical areas (Asiedu *et al.*, 1997).

Yams can be eaten in a variety of ways, including boiled, pounded, fried, or baked. It is

frequently dried and ground into flour for a variety of products, including *amala* and other products made from composite flour. Like other tubers, yams have a number of limitations, including high production costs and post-harvest losses. Yam falls under the category of semi-perishable foods because of its relative high moisture content and susceptibility to physiological deterioration over time after harvest (Jimoh and Olatidoye, 2009). Thus, to make it available all year long, processing it into less perishable products like yam flour through a drying process is required (Ogunlakin *et al.*, 2012; Abiodun and Akinoso, 2014). The oldest method of food preservation is food drying, which is used to preserve agricultural produce to make it available all year long, lower post-harvest losses, and achieve food security (Hussein and Filli, 2018; Hussein *et al.*, 2021).

Dried yam (*gbodo*) and its flour (*elubo*) are traditional transformation methods for processing and storing yam. This yam flour is the primary ingredient in making *kokonte* in Ghana, and *amala* is eaten mainly by ethnic Yorubas of the southwestern part of Nigeria when reconstituted (Abiodun and Akinoso, 2014). Numerous people in the yam zone of West Africa depend on these products as their primary source of carbohydrates. They are renowned for feeding both adults and children (Mestres *et al.*, 2004). The appearance and taste are permanent features of *amala*. However, drying causes deterioration of both the eating quality and the nutritional value of *amala*. The colour is thus a significant attribute of *amala* and predominantly affects its acceptability by consumers. The changes in the shade are thought to be linked to browning caused by the polyphenol content of tubers and bad drying conditions (Chilaka *et al.*, 2002).

Open sun, oven, and solar drying are some of the most widely used drying techniques. However, the physicochemical characteristics of the dehydrated products are significantly impacted by these drying techniques (Ogunlakin *et al.*, 2012). Another issue that lowers the acceptability of the finished products is inadequate processing combination and microbial contamination as a result of poor processing handling. Food products are typically pretreated with hot water blanching and sulphating before drying to stop oxidative browning. According to Babajide *et al.* (2006a), blanching can be used to inhibit enzymes that might cause quality degradation and enhance the finished product. As a result, the objective of this study was to assess how pretreatments and drying techniques affected the physicochemical, microbial, and sensory properties of white yam flour.

2. Materials and methods

2.1. Materials

The Yam Market (Kasuwan Guari) at Yola Bye-Pass, Adamawa State, Nigeria, was where we bought the white yam tubers (*Dioscorea rotundata*). In order to conduct scientific identification, the samples were delivered to the Department of Crops and Horticulture. The analytical-grade chemicals and reagents that were used were obtained from the food processing laboratory of the Department of Food Science and Technology at the Modibbo Adama University, Yola, Adamawa State, Nigeria.

2.2. Methods

2.2.1. Experimental design and sample preparation

The experimental design was carried out using a completely randomised design (CRD). A total of 1 yam variety (white yam), 3 drying techniques (open sun, oven, and solar drying), 4 pretreatments (0.1% metabisulphite, blanching, blanching and soaking, and untreated), and 1 x 3 x 4 = 12 samples were used in the production of the yam flour. Three times of this were done, yielding a total of 12 x 3 = 36 samples. The yam tubers (16 tubers, each weighing about 1.5 kg) were sorted, then washed in clean tap water to remove any soil that had adhered, and finally divided into 4 portions (4 tubers each).

2.2.2. Pretreatment of the yam tubers

The first portion of the yam tubers was hand-peeled and sliced with stainless steel knife into smaller sizes of 0.5 cm thickness inside water to prevent enzymatic browning. The sliced yam

was treated with 0.1% sodium metabisulphite ($Na_2S_2O_5$). Nine and a half grams (9.5 g) of sodium metabisulphite was dissolved in 5 litres of water, and the sliced yams were submerged in it for 10 minutes, drained and then set for drying. The second portion of the yam tubers was first blanched at 100^{o}C for 1 minute, after which it was immediately hand-peeled and sliced with stainless steel knife into smaller sizes of 0.5 cm thickness. It was then further blanched at 100^{o}C for 30 seconds, drained and set for drying. The third portion of the yam tubers was blanched at 75^{o}C for 5 minutes and left to soak in water overnight (12 hours) before being drained and set for drying. The yam tubers' residual portion was hand-peeled and sliced with stainless steel knife into smaller sizes of 0.5 cm thickness inside water to prevent enzymatic browning. The sliced yam was not treated with anything and served as control samples.

2.3. Drying Processes

2.3.1. Open sun drying technique

On four different wire meshes made in Nigeria, 1 kg of each pretreated yam slice and 1 kg of the control were spread out in a single layer and sun dried until equilibrium moisture content was reached. The drying period lasted for four (4) days from 8:00 am to 5:00 pm every day, with an average atmospheric temperature of about 37 to 42°C.

2.3.2. Oven drying technique

Another portion of the same pretreated samples and control were placed in a convective hot air oven (Gallenkamp plus II Oven-OPL150.TSI.B, UK) and dried to a constant weight at 50^{o}C for 36 hours.

2.3.3. Solar drying technique

The last portion of the same pretreated samples and control were dried in a constructed solar dryer by Hussein *et al.* (2017) using the principles of the greenhouse effect for 3 days to achieved equilibrium moisture content. Daily from 8:00 am to 5:00 pm, the drying process took place at an atmospheric temperature range of 37 to 42 °C. The dried yam chips were cleaned to remove dust and other impurities before being reduced in size as much as possible and pounded in a clean mortar and pestle to improve milling. To prevent mixing, each sample was carefully removed, and the mortar and pestle was thoroughly cleaned afterward. The yam pieces were ground into fine flour using a commercial mill before being allowed to pass through a sieve with a 1 mm mesh size. In order to prevent evaporation until they were used for further analysis, the acceptable yam flour samples were stored airtight at room temperature in polythene nylon.

2.4. Determination of the Physicochemical Properties of Yam Flour

The AOAC (2016) guidelines were used to determine the moisture content of the yam flour. Hussein *et al.* (2016)'s methodology was used to calculate the bulk density and water absorption index. Onwuka (2005)'s method was used to determine swelling capacity, and Msheliza *et al.* (2018a)'s was used to determine gelatinization temperature and least gelation capacity.

2.5. Microbial Analysis

According to Jideani and Jideani (2006), the materials were autoclaved at a pressure of 1 kg/cm^2 (121°C) for 15 minutes to sterilise them. Petri dishes, pipettes, glass rods, measuring cylinders, beakers, and conical flasks were among the glassware that underwent detergent soaking, washing, and rinsing. They were sterilised by dry-heating them at 170°C for 60 minutes while inverted, wrapped in aluminium foil paper. Using cotton wool dipped in 70% ethanol, the working area was also cleaned and disinfected. Plate Count Agar and Sabouraud Dextrose Agar were the two media, and they were prepared in accordance with the manufacturer's instructions. Prior to use, they were sterilised for 15 minutes at 1 kg/cm^2 (121°C) and submerged in a water bath set at 100°C to prevent gelling. Additionally, a Bunsen burner was used to sterilise the wire loop, "heat fix" smears, and other tools. For culturing and sub-culturing, inoculation techniques such as streaking and pour plating were used. Secondary observations were made after preliminary tests and observations.

2.5.1. Determination of total bacterial count

To create a 10^{-1} dilution, 1 g of each sample of yam flour was added to a bottle with 9 ml of sterile distilled water and thoroughly mixed. That is, using a sterile pipette, 1 ml of the stock solution was aseptically transferred from bottle one to bottle two, bottle two to bottle three, and bottle three to bottle four, all of which contained sterile distilled water. The sample was then spread on the agar medium's surface using a sterile glass spreader. The medium's pH was modified before sterilisation. The inoculated plates were inverted and incubated at 37°C for 18–24 hours to promote bacterial growth. Utilizing a Leica Quebec dark field colony counter (Model: 3325), colonies were then counted. Using the equation in Equation 1, the counts were converted into colony-forming units per millimetre. Other samples went through the same process again.

$$Colony\ forming\ unit\ per\ gram\ \left(\frac{Cfu}{ml}\right) = \frac{NOC}{VTP} \times dilution\ blank\ factor \quad (1)$$

Where: NOC = No of colonies
VTP = Volume transferred to plate

2.5.2. Determination of the presence of moulds and yeasts

Twelve (12) plates were prepared by dissolving 16.30 g of sabouraud dextrose agar (SDA) powder into 250 mL of distilled water. Both heating and mixing were used to make it dissolve. After autoclaving the mixture for 15 minutes at 121°C, it was cooled to 47°C to sterilise it. The agar was mixed very well, and 20 ml was poured into each Petri dish. A sterilized glass spreader was then used to spread the sample on the surface of the agar media. The SDA on the Petri dishes were allowed to solidify. The sample was inoculated at 37ºC on each Petri dish using a sterilized mountain needle. Fungal growths were observed after 3 days. The procedure was repeated for other samples (Jideani and Jideani, 2006).

2.5.3. Isolation and identification of bacterial isolates

Bacterial identification tests were conducted to differentiate and identify the various bacterial species associated with the prepared yam flour samples. The collected samples were plated onto blood, nutrient, and MacConkey agars, and then incubated for 48 hours at 37°C. Isolated colonies were further subjected to purification and subculture after the incubation period. The isolates' pure colonies were chosen using nutrient agar with 5% sheep blood. Gram stain, catalase, and oxidase tests were used to identify each isolate preliminary. If the isolates were thought to be E. coli, selective media like Eosin methylene blue agar (EMB) were then used (Bhetwal *et al.*, 2017).

2.6. Sensory Evaluation

Sensory evaluation was carried out on both the primary product (yam flour) and the secondary product (*Amala*). For each sample, 50 g of yam flour was combined with 150 ml of boiling water to create a paste known as *amala*. To prevent crowding and to allow for impartial judgement, the containers containing the samples were coded and kept far apart. A 50-member panel of judges who were familiar with the products received the samples for sensory evaluation. The panellists were chosen based on the fundamental criteria for a panellist, including availability for the entire assessment period, interest, willingness to serve, good health (not having a cold), and no allergies or sensitivities to the emulated product, as described by (Msheliza *et al.*, 2018b). Seven-point hedonic scale (7-Like extremely, 6-Like very much, 5-Like moderately, 4-Neither like nor dislike, 3-Dislike moderately, 2-Dislike very much, and 1-Dislike extremely) was used by the fifty semi-trained panellists to score the sensory attributes (Iwe, 2010). The sensory attributes evaluated for the yam flour were appearance (colour), aroma and overall acceptability. At the same time, *amala's* appearance (colour), texture, flavour and overall acceptability were evaluated.

2.7. Statistical Analysis

The results of each experiment were run in triplicate, and they were presented as means ± standard error. The graphs were plotted using the OriginPro 2021 programme, and the Duncan's Multiple Range Test was used to determine the significance of the means. The confidence level was set at $p > 0.05$.

3. Results and discussions

3.1. Physicochemical properties of Yam Flour Samples

Table 1 displays the findings of the physicochemical characteristics of yam flour samples. The moisture content ranged from 3.00 to 11.50%. The sample that had been pretreated with sodium metabisulphite had the lowest moisture content. In contrast, the samples that were dried after being soaked in water for a night had the highest values. This demonstrates that the samples' high water absorption during soaking is what caused their higher moisture content. The low moisture content in those samples of pretreated sodium metabisulphite, on the other hand, is a reflection of the significant impact of the sulphating pretreatment on the drying kinetics. According to Orikasa *et al.* (2018), physical damage to the sample causes sodium metabisulphite to inhibit the hardness surface, destroy cell membrane stability, and change the resistance to internal moisture diffusion. All of the values found in this study, however, were within the permitted range of not more than 10% for long-term storage of flour (Polycarp *et al.*, 2012).

Bulk densities varied between 0.63 and 0.71 g/mL. These values are comparable to the range of 0.64 to 0.76 g/cm^3 reported by Udensi *et al.* (2008) for various water yam flour varieties. The outcome revealed no distinction between the pretreatments used for each drying technique ($p > 0.05$). However, the solar drying technique had the highest bulk density, followed by the oven and open sun drying techniques. Because of its high bulk density, yam flour has the potential to be used as a thickener in the food industry to give yoghurt and other foods more body and mouthfeel (Adepeju *et al.*, 2011). Additionally, Hussein *et al.* (2016) noted that the bulk density of the flour has an impact on its packaging situation; the greater the bulk density, the more convenient the packaging will be. Therefore, it is preferable to have a high bulk density, which provides a more significant packaging advantage by allowing for the packing of a larger volume with a smaller amount of material (Adepeju *et al.*, 2011).

The range of the water absorption capacity (WAC) was 1.80 to 2.47 g/g. Sample with pretreatment of sodium metabisulphite has the highest value. For the three drying techniques taken into consideration, the control sample had the lowest values. According to the results, there was no discernible difference between the pretreatments used for each drying technique; however, oven-dried samples had the highest WAC, followed by solar and open sun drying techniques. The outcome also demonstrated that higher bulk density was caused by lower water absorption capacity. This implies that more shrunk products will have a higher bulk density and be more capable of absorbing moisture. This will be significant when cooking because it is predicted that the mixture's yield will increase (Hussein *et al.*, 2016). Water absorption capacity is a crucial factor in the development of ready-to-eat foods; a high WAC may ensure the cohesiveness of the flour product (Housson and Ayenor, 2002), while a low WAC product ensures the flour's easy digestibility (Bolarinwa *et al.*, 2015).

The ability of flour to swell when combined with water is indicated by the swelling index, which is a crucial functional characteristic. Additionally, it denotes the presence of amylase, which affects how much amylose and amylopectin are present in the yam flour (Oke *et al.*, 2013). The yam flour's swelling index ranged from 27.30 to 70.00 %. The results were significantly different ($p<0.05$); the sodium metabisulphite pretreated sample had the lowest swelling index while the untreated sample had a higher value. The outcome also revealed that oven-dried yam flour came in second place to sun-dried yam flour in terms of swelling index. This suggests that different pretreatments and

drying techniques have an impact on the swelling index, which could account for the variation in the swelling capacity of yam flour. However, the relatively higher swelling capacity found in this study suggests that the starch granules in yam flour likely contain a high amount of amylose. Oke *et al.* (2013) reported a similar finding for water yam flour.

Table 1. Physicochemical properties of the yam flour produced

Drying Techniques	PRT	Moisture Content (%)	Bulk Density (g/ml)	Water Absorption Capacity (g/g)	Swelling Capacity (%)	Gelatinization Temperature (ºC)	Least Gelation Capacity (%)
Sun dried	**MTB**	6.00 ± 0.12^{de}	0.69 ± 0.03^{ab}	2.10 ± 0.01^{bc}	45.00 ± 5.00^{d}	80.00 ± 5.00^{a}	12.00 ± 2.00^{b}
	BLC	7.50 ± 0.50^{cd}	0.68 ± 0.01^{bc}	2.30 ± 0.30^{ab}	60.00 ± 10.00^{b}	80.00 ± 5.00^{a}	10.00 ± 2.00^{c}
	SON	9.50 ± 0.50^{b}	0.66 ± 0.01^{cd}	2.30 ± 0.10^{ab}	60.00 ± 10.00^{b}	76.00 ± 4.00^{a}	12.00 ± 1.00^{b}
	CTR	8.00 ± 1.00^{bc}	0.68 ± 0.02^{bc}	2.40 ± 0.20^{a}	70.00 ± 10.00^{a}	80.00 ± 0.01^{a}	8.00 ± 1.00^{d}
Oven dried	**MTB**	4.50 ± 0.50^{ef}	0.68 ± 0.03^{bc}	2.20 ± 0.10^{abc}	40.00 ± 10.00^{e}	80.00 ± 5.00^{a}	14.00 ± 4.00^{a}
	BLC	5.00 ± 0.01^{e}	0.64 ± 0.03^{cd}	2.20 ± 0.01^{abc}	47.40 ± 2.60^{cd}	78.00 ± 3.00^{a}	12.00 ± 2.00^{b}
	SON	8.00 ± 2.00^{bc}	0.64 ± 0.02^{cd}	2.20 ± 0.20^{abc}	50.00 ± 5.00^{c}	78.00 ± 7.21^{a}	12.00 ± 4.00^{b}
	CTR	5.50 ± 0.50^{e}	0.63 ± 0.01^{d}	2.47 ± 0.12^{a}	70.00 ± 10.00^{a}	74.00 ± 4.00^{a}	8.00 ± 2.00^{d}
Solar dried	**MTB**	3.00 ± 1.00^{f}	0.69 ± 0.01^{ab}	2.30 ± 0.01^{ab}	27.30 ± 1.70^{g}	80.00 ± 0.01^{a}	10.00 ± 2.00^{c}
	BLC	6.00 ± 1.00^{de}	0.71 ± 0.02^{a}	2.00 ± 0.01^{cd}	35.00 ± 5.00^{f}	80.00 ± 5.00^{a}	14.00 ± 2.00^{a}
	SON	11.50 ± 0.50^{a}	0.69 ± 0.02^{ab}	2.10 ± 0.10^{bc}	27.30 ± 2.70^{g}	76.00 ± 2.00^{a}	12.00 ± 2.00^{b}
	CTR	8.00 ± 2.00^{bc}	0.71 ± 0.01^{a}	1.80 ± 0.20^{d}	40.00 ± 5.00^{e}	80.00 ± 5.00^{a}	8.00 ± 1.00^{d}

Mean in the same column bearing different superscript are significantly different at ($p<0.05$) for drying technique.
Where; PTR = Pretreatment, MTB = Metabisulphite, BLC = Blanching, SON = Blanching and Soak overnight, CTR = Control

The yam flour gelation temperature ranged from 74.00 to 80.00°C. The differences between the values were not statistically significant ($p>0.05$). This suggests that the pretreatments and drying procedures used had no significant impact on the yam flour's gelatinization temperature. The flour's least gelation capacity ranged from 8 to 14%. For the three drying techniques that were taken into consideration, a weak gel of 8% was noted for the untreated samples. The treated samples showed gel ranging from a strong gel at 10% to a very strong gel at 14%. This suggests that for untreated samples as opposed to treated ones, a lower flour concentration would be needed to form a gel. All of the flours' least gelation concentrations were relatively lower than the values (30–50% w/v) reported by Udensi *et al.* (2008)) for various D. alata varieties. It was, however, a little higher than the figures for cocoyam flour (6.00–8.00%) and yam flour (2.00–5.00 w/v) reported by Ogunlakin *et al.* (2012) and Wahab *et al.* (2016), respectively. The relative ratios of various constituents, such as proteins, carbohydrates, and lipids, may be the cause of the variation in this yam flour property that has been observed.

3.2. Effect of Pretreatment and Drying Method on the Microbial Composition of Yam Flour

As shown in Figure 1, the results of the microbial analysis revealed that the samples that were left untreated and dried using the open sun technique had the highest microbial loads (496 x 10^3 cfu/ml), while the samples that were pretreated with sodium metabisulphite and oven-dried had the lowest loads (140 x 10^3 cfu/ml). This demonstrated that untreated samples and samples dried in the sun had a higher overall viable count than other drying techniques. This supports the finding from Djeri *et al.* (2010) that sun-dried chips had a higher germ count than oven-dried chips. This demonstrates unequivocally how the pretreatment process and the state of the drying environment affect the microbial load of yam samples. Since the yam sample is sliced fresh until it is completely dried, airborne germs and

their spores are constantly in contact with the chips during the sun-drying process at room temperature. The environment is sealed off and free from airborne bacteria during solar drying. In contrast, the higher air temperature created during oven drying prevents microbial growth. The high microbial load of untreated samples in comparison to treated samples in all drying techniques demonstrated that bacterial growth may be caused by contaminants but does not manifest after pretreatments.

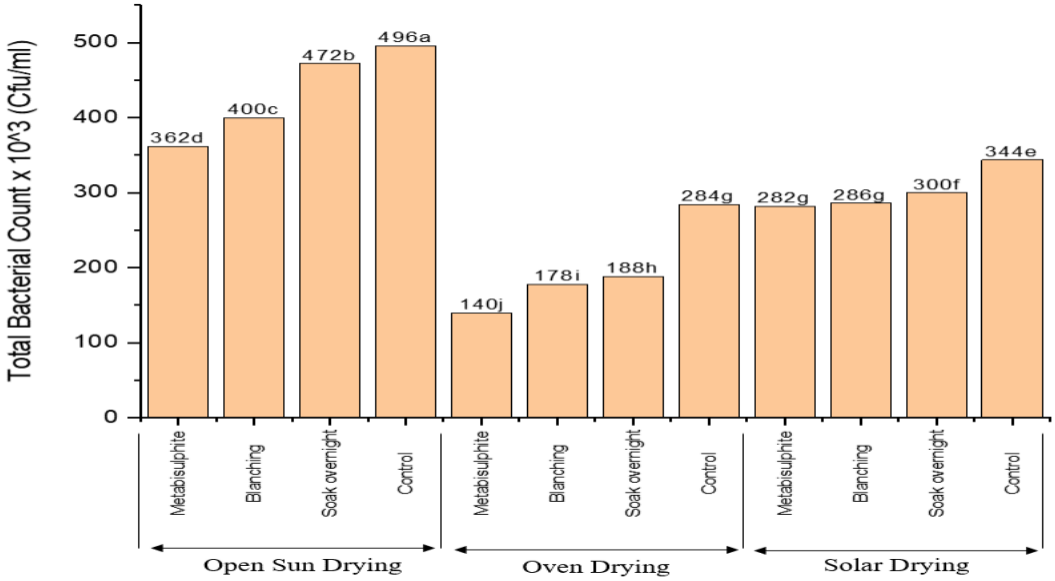

Figure 1. Total bacteria count of dried yam flour

Table 2. Morphological Characteristics of Fungi Isolates

Growth on Sabouraud Dextrose Agar (SDA)	**Microscopy**	**Likely Organism**
Black appearance on culture.	Conidial/heads are dark brown to black.	*Aspergillus niger*
Yellow green appearance on culture.	Spreading yellow green colonies, rough walled stipes, mature vesicle bearing phialides over their entire surface and conspicuously echinulate conidia.	*Aspergillus flavus*
White colonies (dense cotton) that turn yellowish brown with sporulation	Mass of non-septate hyphae bearing sporangia on sporangiospore Rhizoids present	*Rhizopus sp.*
White to cream coloured smooth, glabrous and yeast like appearance	Spherical to sub spherical budding yeast-like cell or blastoconidia	*Candida albicans*
Cottony colonies grey to olive brown on the surface with short aerial hyphae	Dark Septate hyphae Zigzag appearance, conidia are brown, ovoid with an elongated beak-like apical cell	*Alternaria*

3.3. Morphology Characteristics of Fungi Isolates

The test results for moulds and yeast in dried yam flour samples that were pretreated differently and dried using three different drying techniques are shown in Table 2. The outcome demonstrates that fungi had grown on every sample of yam flour. According to growth patterns on the media and examination, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus species*, *Candida albicans*, and *Alternaria* are among the most likely fungi isolates. The results of this study confirmed what had previously been reported for dried yam flour sold in South-West Nigeria by Bankole and Adebanjo (2003), Bankole and Mabekoje (2004), and Babajide *et al.* (2006b). Mold isolates including *Aspergillus flavus*, *Aspergillus niger*, *Penicillium spp*., and *Rhizopus spp*. were also highlighted in the study by Djeri *et al.* (2010). According to Somorin *et al.* (2011), both white yam flour and water yam flour contained *Fusarium oxysporum*,

Aspergillus niger, and *Rhizopus nigricans*. However, *Penicillium citrium*, *Penicillium oxalicum*, *Aspergillus fumigatus*, and *Aspergillus flavus* were also isolated from white yam flour.

Table 3 displays the percentage of each isolated fungus found in the samples of yam flour. *Aspergillus flavus* and *Aspergillus niger* are the least common, with one isolate each representing 4.2%, according to the table, which shows that *Rhizopus species* is the most common organism with nine (9) representing 75% of the isolates out of the twelve samples analysed. There are also six (6) isolates of *Alternaria* and seven (7) isolates of *Candida albicans*. Microorganisms may infect yam at any stage of its growth, from the seedling stage to postharvest (Amusa *et al.*, 2003). According to Okigbo and Ikediugwu (2000), yams are susceptible to a number of diseases; some fungi have been linked to the deterioration of yam tubers during storage. Fungal pathogens that enter wounds in the tubers brought on by insects, nematodes, and improper handling before, during, and after harvest and infect the inner tissue are to blame for this.

Yam flour might also have microbial contamination from the drying environment. According to Djeri *et al.* (2010), improper drying is the root of the mould and yeast contamination. These microorganisms produce the toxins that cause food poisoning infections and are to blame for the decline in the nutritional value and flavour of food. According to a study by Somorin *et al.* (2011), some milling equipment may introduce fungi known to produce mycotoxins into the yam flour. Due to some unhygienic practises used during the milling process, they reported that milling yam chips into flour in the equipment available at markets increased the microbiological contamination of the yam chips by 10^1 to $>10^2$ folds. This has implications for the microbial quality and safety of the yam flour meal consumed.

3.4. Sensory Attributes of Yam Flour Samples (*Elubo*) and Thick Yam Dough (*Amala*)

Figure 2 displays the sensory characteristics of yam flour (Elubo) as reported by the 50 panellists. The ratings had a 95 percent probability level of statistical significance. This suggests that the test samples had a lot of variation. Based on three sensory qualities (appearance, aroma, and overall acceptability) evaluated, oven-dried yam flour samples were rated as the best drying technique, followed by solar and open sun drying. In all drying techniques taken into consideration, the samples pretreated with sodium metabisulphite and blanching received higher ratings (6.90), while the untreated samples received lower ratings (4.10).

Table 3. Percentage occurrence of each fungi isolate obtained from the yam flour samples

Fungal isolate	**Open sun drying**				**Oven drying**				**Solar drying**				**No of isolate**	**Occurrence (%)**
	MTB	BLC	SON	CTR	MTB	BLC	SON	CTR	MTB	BLC	SON	CTR		
Aspergillus niger	-	+	-	-	-	-	-	-	-	-	-	-	1	4.2
Aspergillus flavus	-	+	-	-	-	-	-	-	-	-	-	-	1	4.2
Rhizopus sp.	+	-	+	+	-	-	+	+	+	+	+	+	9	37.5
Candida albicans	+	+	-	+	+	+	+	+	-	-	-	-	7	29.1
Alternaria	+	+	-	-	-	-	-	-	+	+	+	+	6	25
Total													24	100

Where; MTB = Metabisulphite, BLC = Blanching, SON = Blanching and Soak overnight, CTR = Control, + = Present and - = Absent

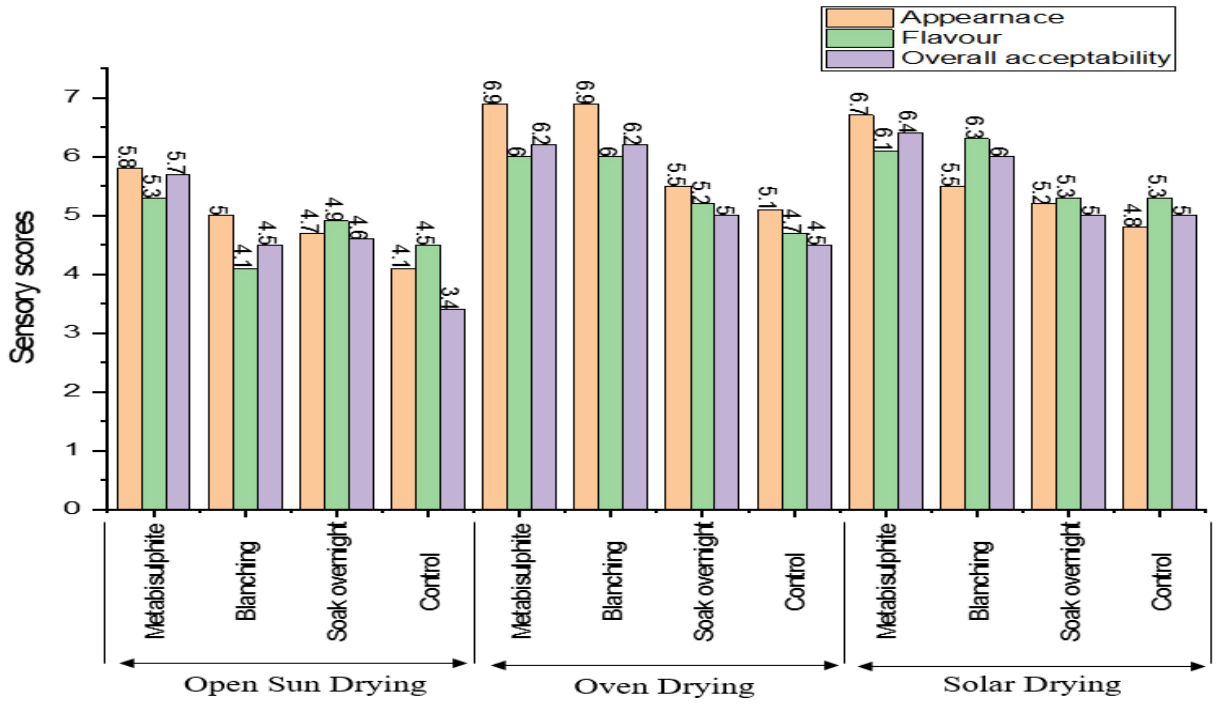

Figure 2. Sensory attributes of yam flour samples (*Elubo*)

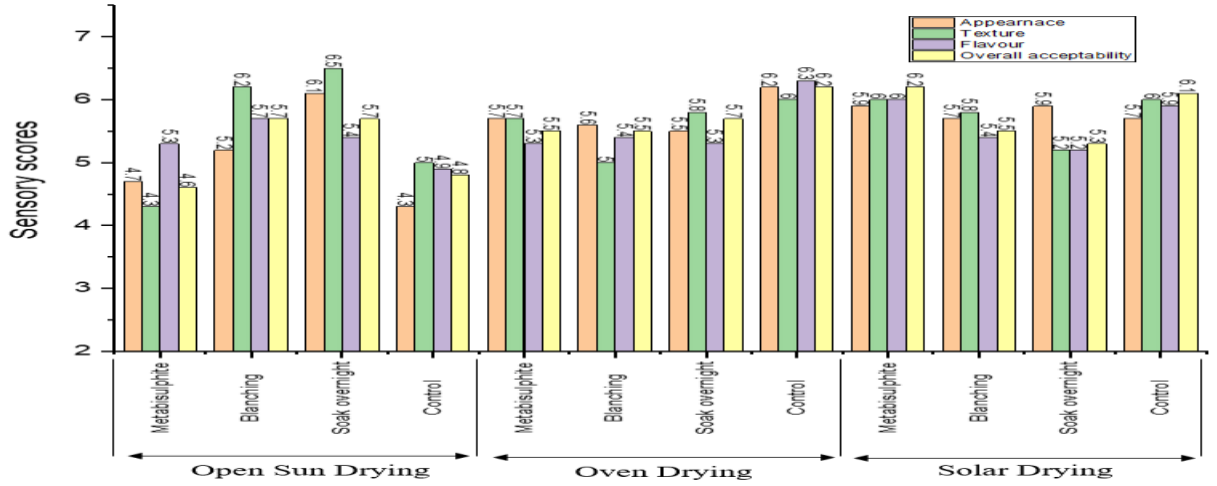

Figure 3. Sensory attributes of *amala* (Yam flour dough meal)

This might be because the pretreated samples were dried out more quickly than the untreated ones. Additionally, it might be because of the dust and other environmental toxins to which sun-dried yam flour was exposed while drying in the open sun. According to Somorin *et al.* (2011), the process of exhuming raw yam from the soil, peeling and cutting it into chips, and then sun-drying it on mats or broom-swept cement floors may expose the dried yam chips to dirt and other environmental contamination, lowering their acceptability.

Figure 3 displays the sensory assessment of yam flour-based *amala*. The flour and *amala* made from the flour sample that was blanched and oven-dried received the highest ratings for appearance (6.20 and 6.30). The sample that was left untreated but dried in the sun received the lowest rating (4.30 and 4.90). The samples that

had been pretreated by soaking overnight and being sun-dried received the highest texture ratings (6.50), while the samples that had not been pretreated and been sun-dried received the lowest ratings (4.30). The samples that were pretreated with blanching, 0.1 percent sodium metabisulphite, and oven and solar dryer drying were equally rated highest for overall acceptability (6.2). The sample that wasn't pretreated and dried in the sun, on the other hand, received the lowest rating (4.6). Overall, the evaluation revealed that solar and oven-dried yam flour was preferred to open-sun-dried yam flour for making *amala*. The panellists gave the pretreated samples a higher rating than the untreated ones, but they both agreed that the *amala* made from oven- and solar-dried flour was acceptable. The untreated and dried in the open in the sun received low ratings, neither like nor dislike. The untreated and dried outdoors in the sun received a low rating, or neither like nor dislike. The negative impact of the open sun drying technique may be responsible for the poor reception of these *alama* samples. Abiodun and Akinoso (2014) noted a similar finding for yam flour in stiff dough *amala*.

4. Conclusions

The findings of this study demonstrated that the physicochemical, microbial, and sensory characteristics of white yam flour were significantly influenced by the pretreatments and drying techniques. The temperature at which gelatinization occurs was unaffected by the pretreatments or drying techniques employed. In comparison to solar and open sun drying techniques, oven drying produces the best results because it shortens drying times and minimises microbial contamination. The *amala* produced from yam flour pretreated with blanching and 0.1 percent sodium metabisulphite along with oven and solar drying was preferred by the panellists to untreated and open sun-dried ones, according to a sensory evaluation of the *amala*. The extensive variation in the physicochemical characteristics of the flour samples could be used as a database to enhance the processing of yam flour.

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STABILIZATION OF OXIDATIVE PROCESSES IN COOKED SAUSAGES BY OPTIMIZATION OF INCORPORATED BIOLOGICALLY ACTIVE SUBSTANCES

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ABSTRACT

Nowadays, many substances of plant origin are used for their antioxidant properties. The aim of the study was to stabilize oxidative processes in cooked sausages by optimization of the incorporated biologically active substances (BAS). For this purpose, a full factorial design with three factors at two levels was used. Sodium L-ascorbate (x1); dihydroquercetin from *Larix sibirica* Ledeb (x2) and lyophilized (30% w/v) ethanol extract from dry distilled rose (*Rosa damascena* Mill) petals (DDRPE) (x3) were used as BAS. The antioxidant activity (DPPH and FRAP assays), the stabilizing effect on the oxidative processes in the lipid and protein fraction, and the color stability of the cross-cut surface were investigated. The optimization was performed according to the target functions as follows: Minimum TBA values and protein carbonyls as well as maximum color lightness (L*) and redness (a*). Cooked sausages with optimized BAS composition were characterized by: TBA value = 0.80 mg MDA / kg; PC = 0.118 nmol DNPH / mg protein; L* = 56.69 and a* = 18.73. The mathematically defined combined optimum according to the four target functions was determined at: 0.10 g x1/ kg; 0.09 g x2/ kg and 0.10 g x3/ kg.

1.Introduction

The ongoing oxidative changes in cooked meat products lead to a decrease in nutritional value (Dominguez *et al.,* 2019) and an increased risk to human health (García-Lomillo *et al.,* 2017; Estévez, 2021). The malondialdehyde (MDA) and protein carbonyls (PC) as end products of the lipid and protein oxidation are used as indicators for the quality of cooked meat products (Estévez *et al.,* 2005).

The substances capable of electron donation or antioxidants are those who inhibit the oxidative processes. Their origin can be either synthetic or natural and all of them comes with their pros and cons (Aminzare *et al.,* 2019). Synthetic antioxidants such as butylated hydroxytoluene (BHT) were used for years to improve the food quality and extend the shelf life (Hashemi Gahruie *et al.,* 2017), but there are evidences linking them to an increased health risks (Carocho *et al.,* 2015). Recently, the so-called "clean label" products gained interest (Oswell *et al.,* 2018; Estévez, 2021). In this regard, more and more extracts of natural origin (Aminzare *et al.,* 2019; Rather *et al.,* 2016) have established themselves as possible additives in the production of meat products. Found in fruits and herbs, bioactive substances such as polyphenols, carotenoids, and tocopherols with strong antioxidant properties (Oswell *et al.,* 2018) can slow down the development of lipid oxidation (García-Lomillo *et al.,* 2017). Ascorbic acid and its salts like sodium L-

ascorbate are widely used in meat processing and under the regulation of the EC (Cenci-Goga *et al.,* 2020). Dihydroquercetin isolated from Siberian larch (*Larix sibirica* Ledeb) has been used to suppress oxidative processes in meat products (Ivanov *et al.,* 2009; Balev *et al.,* 2017). Kobyalko *et al.,* (2009) suggest the combination of dihydroquercetin and L-ascorbic acid as a possible option. Vlahova-Vangelova *et al.,* (2014) suggest the use dry distilled rose (*Rosa damascena* Mill.) petals extract in combination with dihydroquercetin. Therefore, the quality of cooked sausages can be improved due to the rich content of biologically active substances (BAS) with well-defined antioxidant and antimicrobial properties (Baydar and Baydar, 2013; Dragoev *et al.,* 2021).

Our hypothesis is that the triple optimized BAS blend (Sodium L-ascorbate - x1, Dihydroquercetin - x2 and Dry distilled rose petals extract - x3) can increase the oxidative stability of cooked sausages. Therefore, the aim of the study is to inhibit lipid and protein oxidation expressed by the TBA value and the formation of protein carbonyls (PC) as well as to limit the discoloration (L*, a*) of the cross-cut surface of the model system of cooked sausages by optimized incorporation of BAS.

2. Materials and methods

2.1. Materials

The chilled deboned beef shoulder and pork bacon were delivered from the slaughterhouse of Unitemp Ltd., Voyvodinovo village, Bulgaria (48 h *post mortem*). The experiment was carried out in the Department of Meat and Fish Technology at the University of Food Technologies, Plovdiv, Bulgaria. The beef and pork (1:1 w/w), chilled to -1°C are ground in a mincer with mesh diameter of 3 mm and then divided into 9 portions. Each part of minced meat is mixed with the appropriate pre-prepared amounts of BAS (Table 1) and mixed for 10 min using a mixer. Mixed filling masses are separately stuffed in polyamide coatings with diameter of 50 mm. An industrial steam boiler (ALLROUND – SYSTEM "RONDAIR", Rauch and Wärmetechnik GmbH&Co.KG, West Germany) was used for the cooking process. The cooking is considered as finished upon reaching 72°C in the diametrical center. The cooked sausages are water cooled for 20 min and stored at 0+4°C for 7 days.

Table 1. Full factorial design of the experiment

Design points	Coded values of added antioxidant compounds			Sodium L-ascorbate, g/kg	Dihydroquercetin isolate, g/kg	Lyophilized DDRPE, g/kg
	x1	x2	x3	x1	x2	x3
1	-	-	-	0.00	0.00	0.00
2	+	-	-	0.10	0.00	0.00
3	-	+	-	0.00	0.10	0.00
4	+	+	-	0.10	0.10	0.00
5	-	-	+	0.00	0.00	0.10
6	+	-	+	0.10	0.00	0.10
7	-	+	+	0.00	0.10	0.10
8	+	+	+	0.10	0.10	0.10
9	C	C	C	0.05	0.05	0.05

Triple BAS blend: sodium L-ascorbate (x1) which is commonly used, antioxidant with defined synergistic properties (Staykov *et al.,* 2016) is food grade and bough form certified

local distributor; Dihydroquercetin isolate of *Larix sibirica* Ledeb (x2) was bought from Flavit Ltd. (Pushtino, Russia) (dihydroquercetin (96%), dihydrokaempferol (3%) and naringenin (approx. 1%) (Ivanov *et al.,* 2009); the lyophilized (30% w/v) ethanol extract of dry distilled rose *Rosa damascena* Mill petals (x3) was prepared in the Department of Food Preservation and Refrigeration Technology at the University of Food Technologies. The dried distilled rose petals are a by-product of the rose oil industry, containing more than 30 polyphenolic compounds with antioxidant properties (Dragoev *et al.,* 2021).

All other reagents and standards are an analytical grade. Trichloroacetic acid, 2-Thiobarbituric acid, Malondialdehyde, 2,4-Dinitrophenylhydrazine, Hydrochloric acid (37%, puriss.), 2,4,6-Tris(2-pyridyl)-s-triazine, Trolox, 2,2-Diphenyl-1-picrylhydrazyl are purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Guanidine Hydrochloride from Fisher Scientific (New Jersey, USA). The Iron (III) chloride is from Reidel-de Haen (Germany). Sodium Chloride, Ethanol (99%), Ethyl acetate, Methyl alcohol, Sodium acetate and Acetic acid (99%) are from Fillab Ltd. (Plovdiv, Bulgaria).

2.2. Methods

A homogenized mean sample was prepared according the recommendations of Esbensen & Wagner, (2014) and immediately tested.

2.2.1. Antioxidant activity analysis

For the methanol extraction, mixture of 10 g homogenized sample and 100 cm^3 methyl alcohol (99.9%) were left for 12 hours in a refrigerator (4 + 8° C). The solution was filtered through folded filter paper (Filtrax, Grade 391). The resulting extract is stored in a refrigerator.

Radical scavenging activity was determined by DPPH analysis, based on the method of Brand-Williams, Cuvelier and Berset (1995), with modifications by Dinkova et. al (2014). 250 μL extract directly mixed in a UV-macro cuvette with 2250 μL of methanolic DPPH solution (2,2-Diphenyl-1-picrylhydrazyl) (6×10^{-5} M). The absorbance is measured at 515 nm after 15 min in dark. The results are presented as μmol TE (Trolox equivalent)/100g sample.

The iron reducing potential was measured by FRAP test according to Benzie and Strain (1996) with some modifications by Dinkova et al. (2014). The FRAP reagent was prepared by mixing 2.5 mL of a solution of TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (10 mmol / L) in hydrochloric acid (40 mmol / L), 2,5 mL aqueous $FeCl_3$ solution (20 mmol / L) and 25 mL acetate buffer (0.3 mol / L, pH 3.6). 250 μL extract mixed with 2250 μL of FRAP reagent in a UV-macro cuvette. After 4 minutes in dark at room temperature the absorbance is measured at 593 nm. The results are presented as μmol TE (Trolox equivalent)/100g sample.

2.3. Color characteristics

A Konica Minolta colorimeter CR-410 (Konica Minolta Holding, New Jersey, USA) was used to assess the lightness (L*), redness (a*) and yellowness (b*) on the cross-cut surface of the cooked sausages (Hunt *et al.,* 2012).

2.4. Determination of lipid and protein oxidation's products

The 2-thiobarbituric acid (TBA) test was determined by the method proposed by Botsoglou *et al.,* (1994). Ten grams homogenized sample are mixed with 50 cm^3 0.9% NaCl and 50 cm^3 10% trichloroacetic acid, stirred, left for 10 min and filtrated. A 4 cm^3 extract is mixed with fresh 1% solution of 2-Thiobarbituric acid and heated at 70°C for 30 min. Absorbance is measured at 532 nm using a dual-beam UV-VIS spectrophotometer Camspec, model M 550 (Camspec Ltd., Sawston, UK). The results are presented as mg MDA/kg sample.

Protein oxidation products, expressed by the concentration of carbonyl groups, were determined by the method of Mercier et al., (2004). Briefly, two parallel 0.5 cm^3 aliquot homogenate for each sample were mixed with 10% trichloroacetic acid and centrifuged at 5500 rpm for 10 min. To the first one a 1 cm^3 of 2 N HCl is added and to the second a same volume of 0.2% (w/v) 2,4-dinitrophenylhydrazine

(DNPH) in 2 N HCl. After 1h incubation at room temperature and occasional shaking, bout sample were precipitated with 10% trichloroacetic acid and centrifuged at 5500 rpm for 10 min. The precipitate is washed twice ethanol: ethyl acetate (1:1v/v), and the left proteins are dissolved in 2 cm^3 6M guanidine HCl. Last centrifuging is done at 5500 rpm for 10 min to eliminate insoluble impurities. A dual-beam UV-VIS spectrophotometer Camspec, model M 550 (Camspec Ltd., Sawston, UK) was used to measure the absorbance at 280 nm for HCl controls and at 370 nm for the DNPH treated samples. The results are presented as nmol DNPH/ mg protein.

2.6. Data analysis

For the antioxidant activity analysis, a two-way ANOVA was used. The BAS mixture and time of storage were used as the two factors of the statistical analyze at $\alpha = 0.05$.

A full factorial design was determined by three factors (x1, x2 and x3) at two levels (0.00 and 0.10g/kg) following the methodology suggested by Severino et al., (2012) (Table 1). The optimization was performed according to the target functions as follows: minimum values of the lipid oxidation by-products (TBA value) and protein oxidation products (PC) as well as maximum values of lightness (L*) and redness (a*) of the color on the cross-cut surface. Target functions are assessed after 7 days of refrigerated storage at 0 - 4°C.

3.Results and discussions

3.1. Antioxidant activity of the cooked sausages

The free radical scavenging activity of the cooked sausages evaluated by the DPPH• radical showed that the triple BAS blend in concentrations of 0.10 g/kg led to a 95.6% ($p<0.05$) increased radical scavenging activity compared to control (Table 2).

Table 2. Antioxidant activity expressed by DPPH and FRAP assay

Design points	DPPH•, µmol TE/100 g	DPPH•, µmol TE/100 g	FRAP, µmol TE/100 g	FRAP, µmol TE/100 g
	1st day	7th day	1st day	7th day
1	$16.33^{a,x}\pm0.61$	$12.67^{a,y}\pm0.56$	$151.73^{a,x}\pm0.88$	$141.89^{b,y}\pm0.54$
2	$18.67^{b,x}\pm0.61$	$14.67^{b,y}\pm0.37$	$161.40^{b,x}\pm1.21$	$135.96^{a,y}\pm0.63$
3	$80.50^{c,x}\pm0.94$	$60.00^{c,y}\pm1.94$	$266.42^{d,x}\pm1.48$	$230.76^{d,y}\pm1.61$
4	$216.33^{f,x}\pm1.35$	$206.67^{g,y}\pm0.64$	$420.47^{g,x}\pm0.85$	$390.95^{h,y}\pm1.40$
5	$200.33^{e,x}\pm1.78$	$170.00^{e,y}\pm2.04$	$201.40^{c,x}\pm1.22$	$182.20^{c,y}\pm0.84$
6	$135.67^{d,x}\pm1.15$	$119.67^{d,y}\pm1.43$	$283.89^{e,x}\pm0.56$	$274.08^{e,y}\pm0.90$
7	$239.33^{h,x}\pm1.99$	$180.33^{f,y}\pm1.46$	$445.41^{h,x}\pm1.37$	$387.43^{g,y}\pm0.89$
8	$369.00^{i,x}\pm1.19$	$355.00^{h,y}\pm0.49$	$561.07^{i,x}\pm1.68$	$539.63^{i,y}\pm1.29$
9	$227.67^{g,x}\pm0.98$	$168.33^{e,y}\pm0.72$	$340.19^{f,x}\pm1.15$	$297.10^{f,y}\pm0.65$

*Results are presented as Means±SEM

a,b,c,d,e,f,g,h,i indexes indicating significant differences ($p<0.05$) between Means by columns

x,y indexes indicating significant differences ($p<0.05$) between Means by columns for one each parameter separately

The results from the ferric reduction activity potential (FRAP) assay showed similar to the DPPH• values, correlation between the combinations of the BAS. Both at 1-st and 7-th day of the storage (0+4°C), highest ($p<0.05$) FRAP values were measured in design point 8 (DP 8) (Table 2). A well-defined trend in both DPPH• and FRAP values was observed.

Lowest DPPH•/FRAP values – control < Single BAS< Combination of two BAS < Triple BAS blend - Highest DPPH•/FRAP values.

This trend confirmed our hypothesis that the combination of two or three BAS can increased the overall antioxidant activity and by that to increase the oxidative stability of the cooked sausages.

A decrease in the DPPH• and FRAP values on the seventh day of the experiment was evaluated. This could be explained by the ongoing oxidative processes, which leads to the consumption of substances with antioxidant properties.

3.2. Oxidative stability of the cooked sausages

Four response surfaces (Fig. 1) and four second-order polynomial equations (equation 1-4) were generated showing the correlation between the values of the target functions and either combination or concentrations of added BAS.

$$TBA = 0.925 + 0.853 * x1 + 0.483 * x2 + 0.173 * x3 - 6.225 * x1^2 - 12.500 * x1 * x2 + 3.500 * x1 * x3 - 3.225 * x2^2 - 6.500 * x2 * x3 - 4.225 * x3^2, mg\ MDA/kg \quad (1)$$

$$PC = 0.145 + 0.170 * x1 + 1.160 * x2 - 1.590 * x3 + 3.803 * x1^2 - 1.000 * x1 * x2 - 12.000 * x1 * x3 - 6.197 * x2^2 - 5.000 * x2 * x3 + 19.803 * x3^2, nmol\ DNPH\ /\ mg\ protein \quad (2)$$

$$L^* = 56.074 - 9.379 * x1 + 13.991 * x2 - 5.019 * x3 + 17.690 * x1^2 + 44.000 * x1 * x2 + 133.000 * x1 * x3 - 78.310 * x2^2 - 76.000 * x2 * x3 + 21.690 * x3^2 \quad (3)$$

$$a^* = 19.427 + 4.166 * x1 + 0.506 * x2 - 35.484 * x3 - 41.859 * x1^2 + 7.500 * x1 * x2 + 70.500 * x1 * x3 - 39.859 * x2^2 + 66.500 * x2 * x3 + 176.141 * x3^2 \quad (4)$$

Each of three design points 2, 3 and 5 showed an inhibitory effect on MDA formation (Fig. 2 a), Equation 1). However, the combination of the triple BAS blend (DP 8) was evaluated with the lowest ($p<0.05$) accumulation of MDA (Fig. 1 a). At the 7-th day of the cold storage (0+4°C), cooked sausages prepared with the triple BAS blend in concentration of 0.10 g/kg (DP 8) are characterized by the least amount of MDA (0.76±0.04 mg MDA/kg, $p<0.05$), which is 26.2% lower than the control (DP 1) – 1.03±0.04 mg MDA/kg ($p<0.05$). The formation of secondary products of lipid oxidation well correlates with the established antioxidant activity (DPPH and FRAP values) of the cooked sausages (Table 2).

The results for protein oxidation were similar to the obtained for lipid oxidation. Significant reduction on the formation of protein carbonyls was found at concentrations 3×0.05 g/kg of the triple BAS blend (DP 9 – 0.11±0.01 nmol DNPH/mg protein, $p<0.05$) (Fig. 1 b). The most pronounced was in DP 8 at 3×0.10 g/kg of the triple BAS blend (Fig. 2 b), Equation 2). The protein oxidation in the DP 8 was 33.3% (0.10±0.02 nmol DNPH/mg protein, $p<0.05$) lower than the control (0.15±0.01 nmol DNPH/mg protein, $p<0.05$). One possible reason for those results are the highest DDPH and FRAP values in DP 8 and DP 9.

Near to control (56.01±0.03) was L* value in sausages prepared with triple BAS blend (DP 9 – 56.30±0.05). The lightness of the color in DP 8 (56.75±0.03) after 7 days of cold storage (0+4°C) is comparable and a little bit higher (by 1.30%, $p\leq0.05$) to the control. In the design points prepared with single use of BAS or combination of two is observed a darkening of the color (lower L* values) of cross cut surface (Fig. 1 c) and 2 c), Equation 3).

The results evaluated in the a* values show opposite to L* values trend (Fig 1 d). The triple BAS blend in concentrations of 0.10 g/kg (DP 8) showed a slight decrease in a* values compared

to the control (Fig. 2 d), Equation 4). At the end of storage period the control was characterized by the highest redness of the color (19.36±0.07) which was 3.25% higher ($p \leq 0.05$) than the evaluated in DP 8 (18.73±0.07).

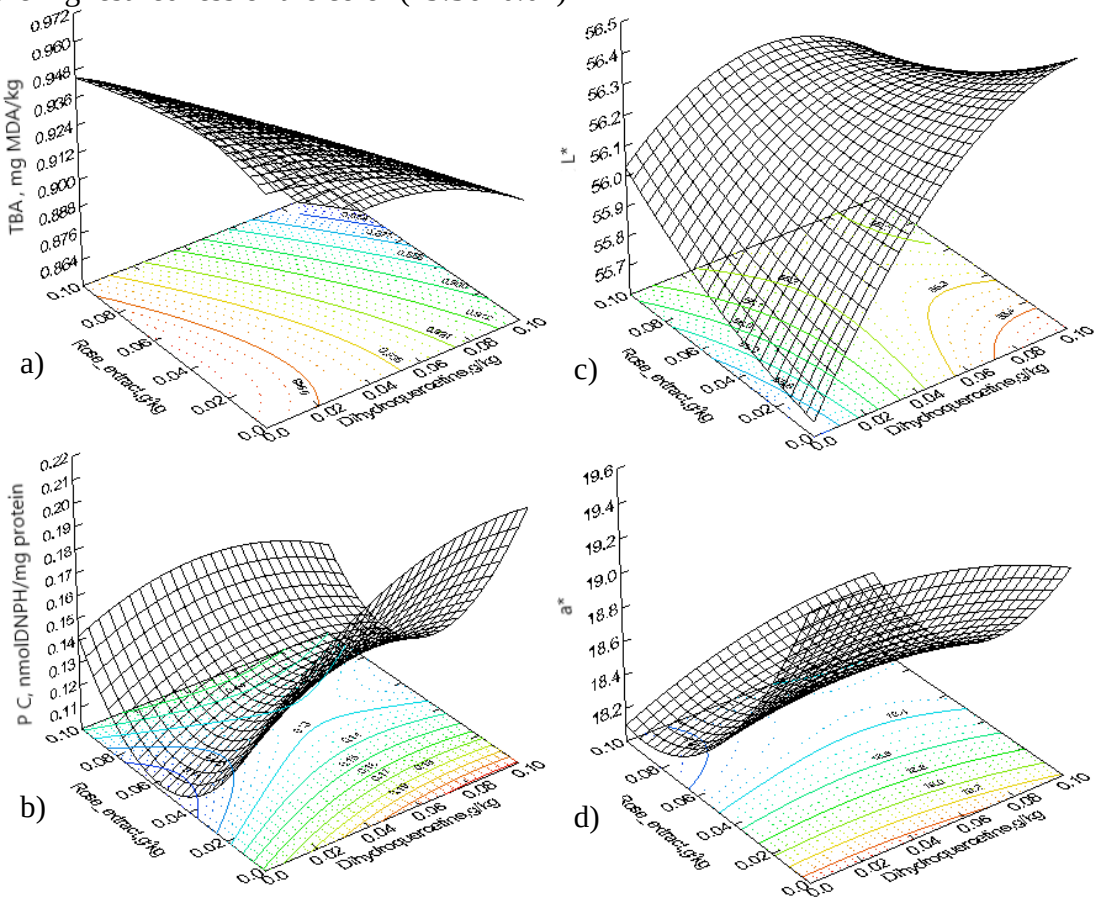

Figure 1. Response surfaces of the four target functions: a) TBA value = f(x2, x3), x1= const; b) PC = f(x2, x3), x1= const; L*= f(x2, x3), x1= const; a*= f(x2, x3), x1= const

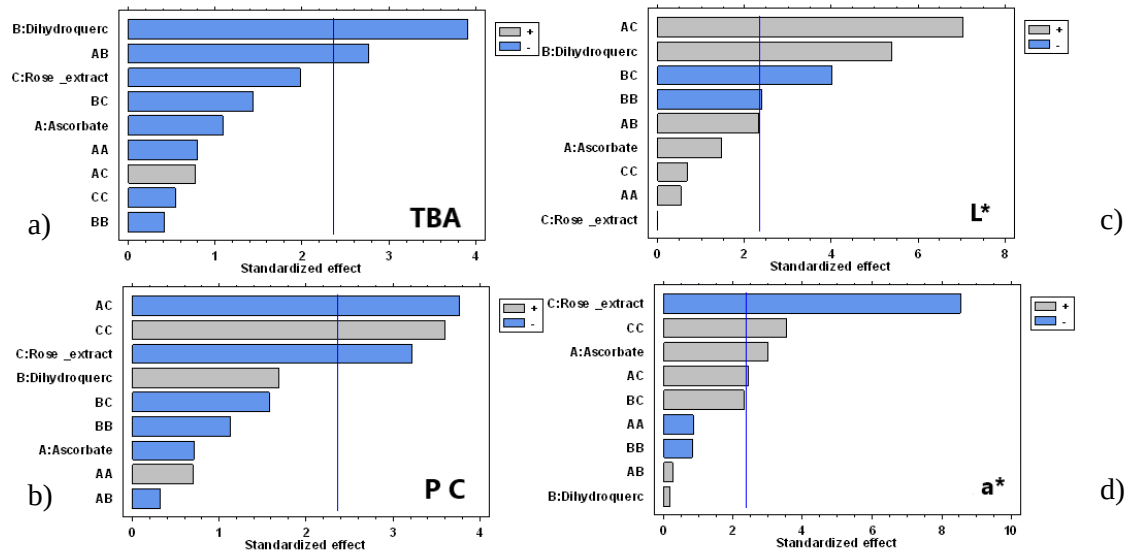

Figure 2. Pareto chart of standardized effects on the target functions of the experiment

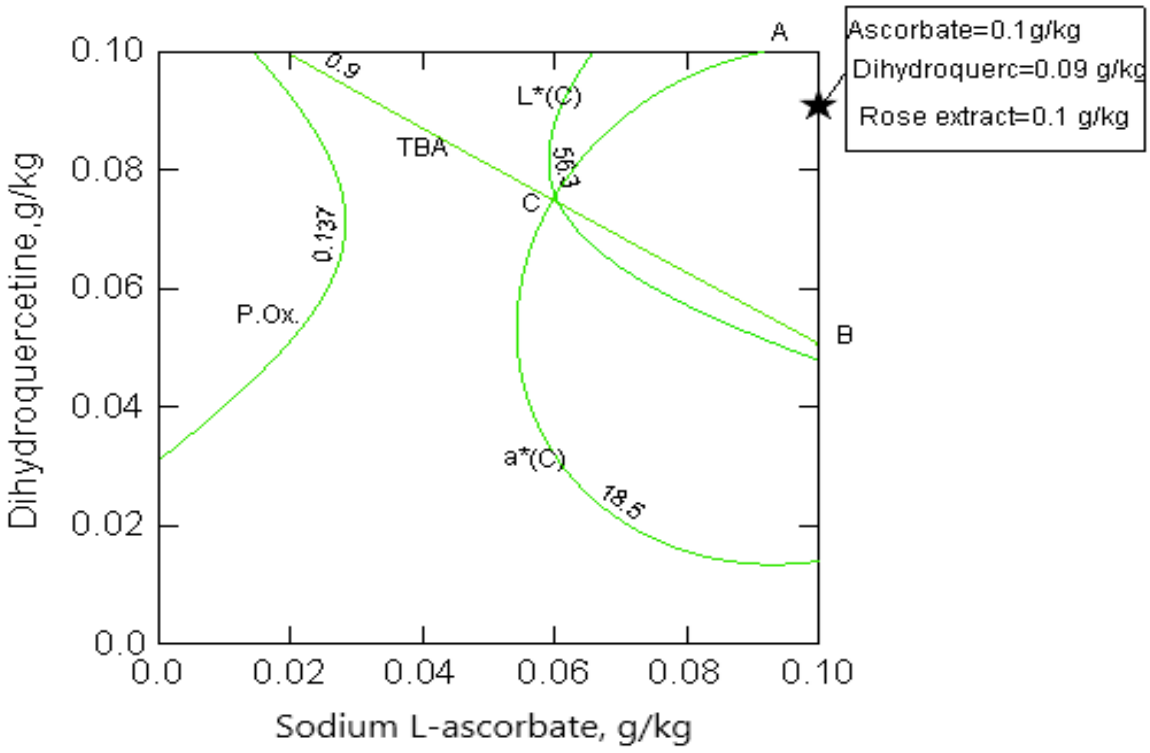

Figure 3. Graphical optimization of the added biologically active substances

The mathematically defined combined optimum (Fig. 3) according to the four target functions was determined at: 0.10 g sodium L-ascorbate (x1)/kg, 0.09 g dihydroquercetin (x2)/kg and 0.10 g DDRPE (x3)/kg.

The potential synergistic effect between the three used BAS may be the reason for the studied results. The use of triple BAS blend resulted in lower levels of MDA (inhibition of lipid oxidation), protein carbonyls (suppression of protein degradation) as well as prevention of the discoloration (inhibition of color pigments' oxidation) of the cooked sausages. In combination with a strong antioxidant (dihydroquercetin) the sodium L-ascorbate act as a synergist. Such synergism in three-component mixture of antioxidants (10 g/L dihydroquercetin, 5 g/L rosemary extract and 1 g/L L-ascorbic acid) was reported by Staykov et al., (2016). Minimal changes in color characteristics were found in DP 8 (Table 1). This suggests that antioxidant properties of used dihydroquercetin and DDREP could suspend the oxidation of the meat pigments (Ivanov et al., 2009; Vlahova-Vangelova et al., 2014). Kaempferol and quercetin glycosides found in DDREP (Baydar and Baydar, 2013; Dragoev et al., 2021) demonstrate free radical scavenging activity and ferric ion reducing antioxidant power (Table 2). The synergistic effects between dihydroquercetin and ascorbic acid, observed by Kobyalko et al., (2009) correspond to the inhibition of lipid and protein oxidative processes found by us. The protein oxidation could be inhibited when DDRPE was added to a matrix of cooked functional sausages with reduced nitrite content (Balev et al., 2019). Vlahova-Vangelova *et al.*, (2020) also reported the possibility of reducing the addition of nitrites in cooked sausages produced with the addition of dihydroquercetin or pork obtained from pigs fed with feed to which dry distilled rose petals had been added.

4. Conclusions

The increase of the antioxidant activity, the inhibition of the formation and accumulation of malondialdehyde, the decreased amounts of

protein carbonyls, and preservation of the cross-cut surface's lightness may be a consequence of synergism between the three biologically active substances.

The combination of sodium L-ascorbate, dihydroquercetin and lyophilized dried distilled rose petals extract in concentrations of 0.10 g/kg each, can be used as antioxidant mixture to inhibit both lipid and protein oxidation and to suspend the discoloration of the cross-cut surface of cooked sausages.

The optimized triple BAS blend can potentially be used in processing of cooked sausages with reduced nitrite addition or extended shelf life.

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DRINKING WATER MICROBIOLOGY: DESIRED AND UNDESIRED MICROBIOTA, LEGISLATION, OUTBREAKS AND ANALYSIS

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ABSTRACT

Water being a fundamental part of all living things is unique in nature, ubiquitous and needed every day for consumption so as to facilitate the metabolic process of the body, processing of food items and other domestic purposes. The taste, color and odor of water can be affected when contaminants in the form of pesticides, animal or human fecal materials is released into drinking water, making it unsafe and unfit for human consumption. According to World Health Organization (WHO), quality water going into distribution system should be devoid of coliform bacteria in 100ml of water. 95% water sample must contain no trace of *E.coli* in 100ml of the water sample throughout the year. No water sample should possess more than 10 coliforms organism in 100ml and no coliform organism should be seen in at least 100ml of at least two (2) consecutive samples. The *Escherichia coli* and coliform bacteria is used as indicators to prove if the water sample is safe and fit for consumption. Drinking water can convey some diseases which are threats to public health safety and they include cholera, typhoid fever, gastroenteritis diseases etc. Conventional and current methods employed in the assessment of drinking water are to prove the safety and quality of the water, but some limitations have been observed during the assessment. Finally, with proper regulatory and legislative measures in place to govern drinking water, the desired target of drinking water devoid of *E.coli* and coliforms as pollutants can be achieved.

1. Introduction

Potable water is a unique, ubiquitous liquid that is a fundamental part of all living things and needed as a fuel for sustaining the human life every day. Due to its unique nature and qualities water has continually been an incomplete research work for scientist. According to the National Research Council (NRC) water being a typical nutrient for life has been recommended for a daily intake of approximately 1mb/kcal of energy expended. Being a daily consumable substance, an adequate, portable and safe drinking water should be accessible due to its significant health effect. Therefore, constant improvement should be applied for quality drinking water purpose (WHO, 2008). Drinking water has been found to act as a body temperature regulator and also helps in the metabolic process of the body (Staci, 2005). Exclusively, water has not only been used for drinking purposes, but also suitable for washing, irrigation, preparation and production of food items, both domestic and industrial purposes. The need for water ranges based on different factors which include human body and animal metabolism, diet, change in climate and different choices of clothing. Water, being a good solvent, can readily take up impurities and continues to interest scientists for further research, owing to the uniqueness of its physical,

chemical, and biological properties in comparison to a set of standards. The wide use of water for the development of humans, animals, plants and industries has created a significant scientific approach to understanding its properties (Naveen, 2007).

Contaminants are chemicals or materials that, when discharged into drinking water, render it dangerous to consume. The flavor, color, and turbidity of the water can help identify some of them. Drinking water should be colorless, tasteless, and odorless, according to WHO (WHO, 1996) physical characteristics. According to other scientific literature, a high pH value leads humans to have an unpleasant taste, and a low pH value promotes corrosion (Chan *et al.*, 2007). Chemical pollution is one of the most common complaints regarding drinking water in rural areas. This is prevalent in areas where agrochemical usage results in a high level of pesticide deposit in the environment, which may migrate into nitrate from fertilizer use, or when water is pumped via led pipes, which can result in a high amount of lead (pb) in drinking water. Furthermore, exposure to increased levels of fluoride causes mottling of teeth and may lead to skeletal fluorosis and crippling. The biological properties of drinking water suggest that drinking water should be devoid of pathogenic or disease causing organism. Any bacteria found in drinking water that is indicative of fecal pollution is harmful and such water is unsafe for consumption.

2.Accepted Level of Microbiological Load in Potable Water

The usage of water for drinking has been linked to a variety of health advantages, including appetite stabilization, improved metabolism, increased energy levels, and lowering of blood pressure. Furthermore, water helps to stablize the body's homeostatic environment, control internal body temperature, and maintain bodily fluid balance (Uzma et al., 2015).

Microorganisms' inherent participation in water is responsible for a range of waterborne infections, as well as nutrient recycling in both marine and fresh water (Willey et al., 2008). The microbiological load of drinking water varies from nation to country, but the World Health Organization has established two reference criteria (WHO, 2011). They distinguished between a piped water supply system (which is defined as a communal public system by the EPA) and a single or small community supply. They also made a distinction between water that had left the treatment plant and water that had entered the distribution system.

The World Health Organization (WHO) recommended a standard for treated and disinfected water supplies, as well as a requirement that water entering the distribution system include no coliform bacteria in 100 mL of water. The standards for disinfected water are as follows: "(1) Throughout any year, 95 percent of samples should be devoid of *E. coli* in 100 ml; (2) No water sample should contain more than 10 coliform organisms per 100 ml; and, (3) Coliform organisms should not be seen in 100 ml of at least two consecutive samples." (9)The number of coliforms measured in non-piped systems should not exceed 10/100 ml.

Table 1. Guideline of microbial quality for drinking water (WHO, 1997)

Organism	Microbial critical limits
All water directly intended for drinking *E. coli* or thermotolerant coliform bacteria	No detection in any 100ml sample
Treated water entering the distribution system. *E.coli* or thermotolerant coliform bacteria	No detection in any 100ml sample
Treated water in the distribution system *E.coli* or thermotolerant coliform bacteria	No detection in any 100ml sample

2.1.Classification and Sources of Pathogens Involved in Drinking Water (Coliform bacteria, *E.coli* and other Pathogens)

Microorganisms in consumed water have formerly been used to signify that the water is unpleasant to drink. Indicator microbes may be followed by pathogens, but they do not cause sickness in and of themselves; their presence in drinking water, however, is a sign of pollution. This pollution can arise as a result of feces contamination from humans or other animals. Two important indicator microorganisms typically discovered in drinking water are *Escherichia coli* and coliform bacteria.

Some of the emerging drinking water pathogens include the *Microsporidia* include bacteria like *Mycobacterium avium intracellulare*, *Helicobacter pylori*, *Tsukamurella*, and *Cystoisospora* belli, as well as viruses including adenoviruses, parvoviruses, coronaviruses (SARS), and polyomaviruses (Woolhouse, 2006). Extensive investigation into these species has revealed that the majority of them have some level of chlorine resistance. Microsporidia, *Enterocytozoon bienusi*, *Encephalitozoon hellem*, and *E. intestinales* are all good examples. *M. avium* and certain viruses have been shown to contaminate several disinfectants used in drinking water, as well as being inactivated by UV radiation and heat pathogens (Nwachcuku and Gerba, 2004). *Salmonella* sp., *Shigella* sp., *Vibrio cholerae*, *Leptospira* sp., *Yersinia enterocolitica*, *Francisella tularensis*, *tularemia*; *Escherichia coli* (particular enteropathogenic strains); and *Pseudomonas aeruginosa* are some of the bacteria that have been discovered as drinking water pathogens.

Pathogens, on the other hand, are not easy to differentiate and recognize than indicator organisms, necessitating the use of specialized media and techniques. Other than pathogens, indicator organisms are commonly employed to grade water quality because they are easily identified using basic laboratory procedures, are more accurate, and take less time. When comparing infections with indicator organisms, diseases are more frequently detected in small quantities than indicator organisms, making them less likely to be separated.

The genus *Escherichia* is well defined and outstanding from different Enterobacteriaceae mixed-acid fermenters in general due to their capacity to ferment sugar, motility, production of indole from tryptophan, loss of urease, incapability to utilize citrate as the only carbon supply, and inhibition of growth by potassium cyanide. Despite this, the "coliform group" is not adequately explained. The "coliform group," according to the American Public Health Association's Standard Methods, is made up of all gram-negative, "aerobic and facultative anaerobic, non-spore-forming, rod-shaped bacteria that can digest lactose with gas production in 48 hours at 35 °C." *E.coli* is a member of this group, which also contains *Klebsiella pneumoniae* and *Enterobacter aerogenes*.

Coliform community is utilized as a fecal contamination indicator in drinking water; it's important to remember that coliform activity in water is quicker than diseases like *Salmonellae* and *Shigellae*. They are easily repressed by an increase in the number of various species, especially in untreated groundwater or when there is no free residual chlorine (Allen and Geldreich, 1975).

3.Diseases Associated With Drinking Unsafe Water/Examples of Outbreaks

Water has been seen as a vehicle for disease transmission because wastewater, which is a primary carrier of *E.coli*, coliforms, and other pathogens discharged into freshwaters and coastal seawaters, pollutes drinking water with human and animal feces, creating a significant health risk (Fenwick, 2006).

People suffering from severe microbial diarrhea in impoverished nations face significant public health issues, which may be caused by poor sanitation, a lack of hygienic facilities, or a lack of financial resources. This is seen in a large number of young children from Asian, African, and poor nations throughout the world (Seas *et al*., 2000). Microbial waterborne infections have an impact on advanced nations

across the world, according to the findings. According to scientific evidence, over 560,000 individuals in the United States suffer from severe waterborne diseases each year, while approximately 7.1 million people are infected with minor to slight infections, ensuing in an estimated 12, 000 fatalities each year (Medema *et al*., 2003).

Table 2. Major diseases transmitted via contaminated water and their causative agents (João, 2010).

Disease	Causative agent
Cholera	*Vibrio cholera, serovarieties* O1 and O139
Gastroenteritis caused by *vibrios*	*Vibrio parahaemolyticus*
Typhoid fever and other acute salmonellosis	*Salmonella enterica* subsp. *enterica* serovar *Paratyphi* *Salmonella enterica* subsp. *enterica* serovar *Typhi* *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*
Bacillary dysentery or shigellosis	*Shigella dysenteriae* *Shigella flexneri* *Shigella boydii* *Shigella sonnei*
Severe diarrhea and gastroenterititis	*Escherichia coli*, particularly serotypes such as O148, O157 and O124

Cholera, salmonellosis, and shigellosis are some of the most common gastrointestinal disorders caused by bacteria spread by water. Contaminated water that has been polluted with human and animal feces is the major vehicles that convey these infections. As a result, drinking water can get contaminated with *E.coli*, coliforms, and other harmful bacteria, posing a serious health problem. The colony of harmful bacteria in potable water on the other hand, is unusual and unstable, with a low number and inconsistent isolation and culture of these germs. As a result, microbiological testing of drinking water entails more than just looking for dangerous microorganisms. Healthy drinking water therefore, requires the absence of any harmful germs (George and Servais, 2002).

Some of the diseases associated with drinking contaminated water and their causative agents are displayed in Table 2.

3.1.Cholera

*Vibrio*s are Gram-negative rods that have one polar flagellum and a tiny, curved appearance. They are facultative anaerobes, meaning that they have both fermentative and respiratory metabolism. Extensive research has shown that *Vibrio cholerae* is therefore, the most significant of the vibrio species. *Vibrios* are mostly aquatic bacteria, and their circulation is heavily influenced by salt content and water temperature. *Vibrios* are typically found in marine and estuarine habitats, where they live in the open or on the surfaces of marine animals' intestinal walls. Their cells can grow at 40 °C with a pH of 9–10, and sodium chloride is usually present to help them grow (Farmer and Hickam-Brenner, 2003).

The toxin produced by *Vibrio cholerae* is an exotoxin that acts directly on its target cells. The toxin binds to a particular receptor (ganglioside Gl) on the cell membrane of intestinal cells, causing the enzyme adenylate cyclase to be activated. This causes the breakdown of internal ATP to continue indefinitely, resulting in the generation of cAMP and inorganic phosphate. Diarrhea is caused by an increase in the inner concentration of cAMP, which induces an outflow of water, sodium, potassium, chloride, and carbonate ions out of the cells of the mucous membrane.

Cholera has a development period of 1–3 days. Acute and very severe diarrhea characterizes the condition, which can reach a

rate of at least a liter per hour. Thirst for water, muscle cramps, and overall weakness are some of the signs of cholera, as are oliguria, hypovolemia, hemoconcentration, accompanied by anuria, lower potassium levels in the blood, patients feeling dull, and lastly, blood distribution quits and dehydration with cyanosis (Farmer and Hickam-Brenner, 2003).

Since the 19th century, cholera has been a well-known illness. Seven major pandemics were identified in the nineteenth and twentieth centuries. The first six pandemics all began in Asia, spread over Europe, and eventually reached South America. The seventh pandemic, which is still ongoing, began in Asia's Celebes Islands in 1961. The illness first spread through Asia in the 1960s, then to the Middle East and Africa in the 1970s, and finally to South America in 1991. (Sack *et al.*, 2004).

3.2.Salmonellosis

The genus *Salmonella* is a Gram-negative motile straight rod, belonging to the family of *Enterobacteriaceae*. Its Cells are catalase-positive and oxidase-negative, and they release gas from D-glucose using citrate as a primary carbon supply. *Salmonellae* contains different endotoxins viz antigens O, H and Vi (Popoff and Le Minor, 2005).

Infections due by *Salmonellae* can be divided into two types of salmonellosis which include (1) typhoid and paratyphoid fever and (2) the gastroenteritis. Infective doses enough to cause clinical symptoms is less than 1,000 cells. Newborns and toddlers infected with salmonellosis display certain signs involving a deadly typhoid-like illness with septicemia to a lowered or asymptomatic infection. This transmission usually occurs in pediatric wards via the hands of staff handling the children (Popoff, and Le Minor, 2005).

Salmonella is found mostly in the guts of humans and animals, but it is also gotten from environmental samples because it is expelled by humans, pets, farm animals, and wild animals (Le Minor, 2003). Communal sewage, farm pollution, and storm water runoff are the major sources of pathogenic bacteria and coliforms in drinking water (Arvanitidou *et al.*, 2005).

Between 1994 and 2004, Tunisia was hit by major epidemics of salmonellosis, including outbreaks in 1997, 1999, 2002, and 2004. In 1997, serovar Mbandaka produced a salmonellosis outbreak, and in 1999, three *salmonella* outbreaks were reported from hospitals across the country. Mbandaka, Livingstone, and Typhi Vi+ serotypes were used to distinguish each epidemic. *S. enterica* subsp. *enterica* serovar *S. enterica* serovar S. *enterica* serovar S. *enterica* serovar *S. enterica* serovar The Livingstone infection was detected in a separate ward of the same hospital that had previously reported an epidemic caused by serovar Typhi Vi+ in 1999. In Tunisia, the Livingstone serovar rose to first place in human infection in the same year. Furthermore, in 2004, a second major epidemic of serovar Typhi Vi+ occurred, with the pathogen's source being a fermented juice obtained locally from palm trees (Ben *et al.*, 2007).

3.3.Shigellosis or Bacillary Dysentery

Shigella are Gram-negative, non-spore producing, non-motile straight rod-like bacteria belonging to the *Enterobacteriaceae* family. Its cells are capable of fermenting carbohydrates without producing gas. Salicin, adonitol, and myo-inositol, on the other hand, are not fermented. Shigella cells do not create H_2S because they do not utilise citrate, malonate, or acetate as their sole source of carbon. Also lysine is not decarboxylated. They are catalase-positive and oxidase-negative, and members of the genus have a diverse antigenic pattern, with classification based on somatic O-antigens (Strockbine and Maurelli, 2005).

The incubation period for bacillary dysentery is 1–4 days, during which time early indications such as fever, anorexia, lethargy, and malaise appear. Carriers have a constant stream of tiny bloody feces (sometimes excessively purulent) and stomach discomfort. Diarrhea advances to dysentery, bloody mucus, and pus in stools that reduces in volume (no more than 30

mL of fluid per kg per day) around 12-36 hours later (Germani and Sansonetti, 2003).

Blood, inflaming components, and mucus flow into the intestinal lumen due to epithelial breakdown and eventual ulceration of the intestinal mucosa. The colon's ability to absorb water is hampered, and the amount of stool produced is determined by ileocecal flow. Therefore, the patient's feces will be constant, sparse, and dysenteric (Todar, 2009). *Shigella* is mostly located around the intestines of humans and other primates (Tetteh and Beuchat, 2003). It is most commonly transmitted by feces-contaminated drinking water or food, or through direct contact with an infected individual. *Shigellosis* may survive in water for up to six months at ambient temperature, making it ideal for transmission through water.

4.Conventional and Current Techniques of Assessing Water Potability

To retain records of water quality impairments, meet with regulatory criteria to assure continual safe drinking water, monitor sources of drinking water pollution, and eventually decrease and investigate illness outbreaks caused by drinking water, drinking water potability must be assessed.

4.1.Conventional Method

Many scientists used cultural methodologies for measuring the standard of drinking water in the previous century to preserve public health. Because of its ease of use, broth culture was quickly adopted for the isolation of specific groups of microorganisms, and it became the standard approach for isolating and identifying *Escherichia coli* and other coliform bacteria. To test microorganisms in drinking water, the MacConkey broth was introduced utilizing the Most Probable Number (MPN) (Anon, 1934). Percy and Grace Frankland used Koch's solid gelatin media technique to detect the total bacteria present in drinking water sources and slow sand filters in 1880, according to scholarly literature (Bulloch, 1979). Although these approaches were effective for detecting coliforms and *Escherichia coli* in drinking water, the procedure was found to be time demanding, taking around 48 hours to complete, and lacking in specificity.

4.2.Current Methods of Assessing Water Potability

Table 3 has an abstract for existing methodologies for investigating the microbiological profile and microbial activity in drinking water. Several approaches have been utilized to determine microbial density and structure (including certain opportunistic pathogens), as well as microbiological functions, in order to reliably monitor and describe the drinking water microbiology.

Table 3. An abstract for current methods used to investigate microbiological profile and microbial activities in drinking water (Ya Zhang and Wen-Tso, 2019)

Microbial density	**Community structure and composition**	**Microbial activities**
Cultivation methods (HPC, selective and differential media)	Community fingerprint (DGGE, T-RFLP, PCR-ALH, SSCP)	Adenosine triphosphate assay
Cell counting (microscopic counts, FCM)	16S Rrna gene amplicon analysis (clone library and sanger sequencing, NGS)	Enzymatic activity tests
Molecular methods (qPCR, viable qPCR, ddPCR)	16S rRNA gene hybridization (DNA microarray) Spatial distribution (FISH, SEM)	Assimilable organic carbon assay

According to scientific studies, no one approach can offer all of the necessary information about microorganisms and their activity in potable water. To increase the perspective of microorganisms in the drinking water utilized for study, the present strategy combines many ways. Furthermore, all of the approaches have been shown to have known biases during analysis, therefore caution should be used throughout monitoring (Sartory and Walkins, 1999). (1) Measuring microbial density, (2) evaluating microbial composition, and (3) quantifying microbial activity are some of the recent approaches used to analyze the standard of water.

4.2.1.Measurement of Microbial Density

The cultivation of bacteria is still commonly used today to determine the density of microorganisms in drinking water. HPC and bacterial indicators (total coliforms and *E. coli*) have been utilized as pollution signals for determining water quality and as a standard for density measurement (Bartram *et al.*, 2004). Isolation and precise identification of disease-causing bacteria are major problems in water evaluation, hence selective and differential media approaches are employed to culture distinct pathogenic bacteria in drinking water.

Cell counting is another approach for measuring microbial density that entails counting the cells in drinking water samples using microscopy or fluorescent dyes and then placing them under an epifluorescence microscope or flow cytometry (FCM) as a total cell count to be measured. The ability to distinguish temporal bacterial dynamics requires a high frequency of fully automated online FCM (Besmer *et al.*, 2016). Flow cytometry is currently limited to structures without residual disinfectants in drinking water microbial density (DSs). Due to low cell population and blockage of bacterium-like particles, membrane filtering to pretreat water with residual disinfectants is required to concentrate bacteria at a suitable density (Van Nevel *et al.*, 2017).

4.2.2.Measuring Microbial Composition

The first step in grasping the problems of microbial presence in drinking water structures is to characterize microbial populations. There are two types of PCR amplification procedures that can be used. The first category of PCR-based approaches is known as "Community Fingerprint." It examines the amplified 16rRNA genes and produces a sample-based network structure description, which is usually represented by a nucleic acid fragment banding fashion resolved through gel electrophoresis. In detail, these network fingerprinting methodologies permits researchers to quickly evaluate microbial heterogeneity inside a microbial environment, as well as compare and contrast the microbial communities formed by different ecosystems (Liu and Stahl, 2007).

The second molecular approach is to extract 16S rRNA cistron progression from the retrieved grouped genomic deoxyribonucleic acid and create a dataset. This was originally accomplished by creating a clone library of 16S rRNA cistron chain. Nowadays, next-generation sequencing (NGS) has been used to determine the amount of 16S rRNA cistron sequences in microbial samples. Each method describes the microbiological framework based on the number of distinct 16S rRNA sequences and hence the population of each 16S rRNA sequence. The 16S rRNA sequences can also be compared to all 16S rRNA arrangement in a publicly available database. This enables researchers to determine the evolution association of individual 16S rRNA sequences and confirm whether the sequences are unique or similar with known species, demonstrating sequence similarity. (Zhang and WenTso, 2019).

Finally, evaluating the spatial structure of microbial population in-situ may be used to estimate microbial composition. The most often utilized techniques to expose the spatial structure and organization of microbial ecology are fluorescent in-situ hybridization (FISH) and scanning electron microscopy (SEM) (Fischer *et al.*, 2005).

4.2.3.Measuring Microbial Activities Technology

The composition and activity of microbial communities present throughout treatment, storage in tanks, and distribution to consumers influence the standard of drinking water (Gray, 2008). Based on several scientific studies, the bacterial population present in drinking water ranges from 1,000 to 100,000 cells per millimeter (Proctor and Hammes, 2015). As a result, some opportunistic pathogenic bacteria represent a public health risk, induce corrosion in drinking water pipes, and eventually generate metabolites that alter the taste, odor, and color of drinking water (Srinivasan and Sorial, 2011). The requirement to measure the composition of microorganisms and their activity in drinking water is of highest relevance to the public and regulatory agencies in order to avoid the repercussions of eating infections associated with unclean water. Some of the currently available methods for measuring microbial activity include the use of an ATP assay, protein activity assays, and assimilable organic carbon (AOC) testing (Lautenschlager *et al.*, 2014). The total quantity of ATP determined using bioluminescence test is used to describe all active bacteria physiologically (Stutz *et al.*, 1986). Protein activity assays measure the increase in visible radiation intensities or absorbance as a function of time due to the breakdown of substrates by specific enzymatic activities such as polysaccharide degrading enzymes (a- and b-glucosidase, cellobiohydrolase, xylosidase, chitinase) (Lautenschlager *et al.*, 2014). The highest level of growth of two (2) microorganism isolates (*Pseudomonas fluorescens* P-17 and *Spirillum* sp. strain NOX) in a drinking water sample is measured by determining the portion of dissolved organic carbon that can readily support microbial growth and is estimated by determining the highest rate of growth of two (2) microorganism isolates (*Pseudomonas fluorescens* P-17 and *Spirillum* sp (Vanderkooij et al., 1982). However, due to time constraints and labor intensiveness, microbial activity assessment has not been routinely used in water microbiome investigations.

5.Limitations of Assessing Water Potability

The cultivation of microbes, which is known to be time-consuming, low sensitivity, and low in recovery of microorganisms during culture, are all limitations that impact the evaluation of drinking water (Hammes *et al.*, 2008). Furthermore, despite their widespread usage in HPC and *E. coli* testing, culture-based enumeration approaches fail to capture disinfectant-injured or genetically engineered bacteria with injected antibiotic resistance genes (ARGs) (Li *et al.*, 2017). These pathogens are often located in the drinking water habitat, but they are often missed since they can reactivate under the correct test situation and present a health risk to consumers. As a result, most current research is dependent on molecular equipment to get deeper into the microbiome evaluation of drinking water.

5.1.Regulatory and Legislative Measures of Drinking Water

The availability of sufficient regulation, standards, and norms is perfect for effective programs to control drinking-water high quality. One of the tasks of the basic rules is to define the characteristics, authority, and obligations of the water-supply and monitoring businesses. Standards and rules should outline the standard of water to be provided to consumers, as well as the methods to be utilized in choosing and creating water assets, treatment processes, distribution structures, and strategies for approving water systems in terms of water quality. The method of the regulation in a particular country will be determined by national constitution, and other factors (WHO, 1997).

As a result, water-quality criteria should be carefully considered so that acceptance by the public health or environmental health regulatory bodies complies with health-protection regulations. Typically, such law provides for the continuation and modification of drinking-water safety rules and recommendations, as well as

regulations for the enhancement of drinking-water sources, as well as the production, continuous regulation, and dissemination of safe drinking water. It also establishes the legal capabilities and importance of the water-delivery agency, stating unequivocally that, as a corporation that sells and/or delivers water to consumers; this company has a legal obligation to provide safe and healthy water that aligns with legally established water-quality standards (WHO, 1997).

The new EU Directive on Drinking Water, which came into effect in 1998, lays forth rules for the importance of drinking water, water sold in bottles or cans, and water used in food production. Mandatory and non-mandatory parameter values are suggested by the Directive. Mandatory standards for mains water, which comprise twenty-eight microbiological and chemical characteristics, are vital for human health and the environment, and must be satisfied on specific dates. For monitoring reasons, non-mandatory indicator values are necessary, in addition to microbiological, chemical, and physical characteristics. Any violation of an indicator value must be watched, but corrective action is best conducted when there is a public health danger (Council Directive, 1998).

When water fulfills the requisite microbiological load and defined requirements established by regulatory agencies for quality water and healthy living, it is considered to be wholesome. Microbiological, chemical, and physical recommendations all have standard concentrations or values. In addition to the Directive's provisions, national legislation has a few additional norms and obligations. All water that is covered by the laws should be free of microorganisms. Microbiological parameter concentrations and values are based on an already established indicator species such as coliform bacteria, *E. coli*, *Enterococci*, *Clostridium perfringens*, and colony counts. In order to achieve quality, water must be free of any microorganism (aside from a parameter) or parasite that might pose a health risk to humans at a specific level (Council Directive, 1998).

Because this strategy captures the hazard from the water sources to the consumer's tap, the WHO suggest water safety plans as the sole option for constantly making sure of the protection of drinking water supply (Bartram *et al.*, 2009). Approach to the water safety plan is entirely anchored on the hazard analysis and critical control point system, which is often utilized in the food sector to manage food quality. Under appropriate law, the Department of the Environment, Food and Rural Affairs regulates the quality of bottle water and packaging containers in the United Kingdom (Statutory Instrument, 1999). The EU Directives (Council Directive, 1996) on the misuse and advertising of natural mineral waters are implemented by these regulations, which also integrate the legislation on other types of bottled water. All water backed by these laws must be bacteriologically sound, as determined by indicator organisms once again.

6.Conclusions

Microbial load evaluation in drinking water should be a standard worldwide since potable water has become a major global concern in the twenty-first century. Extensive microbiological research should be conducted for novel techniques of monitoring drinking water, with the goal of using biosensors to overcome the limits of time-consuming analysis and poor sensitivity. Apart from monitoring *E.coli* and coliform bacteria, which are considered as important indicators of drinking water pollution, more study should be focused on monitoring other pathogens in drinking water in order to prevent disease transmission through drinking water. Finally, the government and drinking water regulatory organizations should verify that enterprises that produce potable water follow stringent guidelines for creating safe and natural mineral water.

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MICROORGANISMS RESPONSIBLE FOR DETERIORATION OF FOOD PRODUCTS: REVIEW

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ABSTRACT

Every year, tons of food is thrown away because of changes in odor, taste, texture or color. Food spoilage has a very important effect at this point. Microorganisms have a significant position in food spoilage. The type of microorganisms seen varies depending on factors such as the amount of water, acidity, carbohydrate, protein or fat ratios of foods, packaging type, and oxygen levels. In this section, basic organisms that cause food spoilage (bacteria, mold, yeast) and microorganisms that are effective in specific food groups (meat products, poultry products, dairy products, seafood products, egg products, cereal products, fruits and vegetables) are covered. Approaches to reduce spoilage organisms are as important as identifying spoilage organisms. Therefore, recommendations for controlling and preventing spoilage organisms are also included in this section.

1.Introduction

People need food to grow, develop, protect and maintain health, and meet their daily macro and micronutrient requirements. At this point, some problems may be experienced in reaching healthy and delicious foods. Food spoilage is one of these problems (Hammond *et al.*, 2015). Food spoilage is a metabolic process that adversely affects food consumption due to changes in sensory properties. Deteriorated foods can be consumed safely, in other words, since there are no pathogens or toxins, consuming these foods may not cause any disease. On the other hand, food spoilage causes the rejection of nutrients by changes in texture, odor, taste, or appearance. It is thought that this situation is created especially by microorganisms. So, the food is rejected by the animals and the microorganisms keep the food source for themselves (Burkepile *et al.*, 2006).

Among the main causes of food spoilage are physical changes that occur in situations such as transportation and storage. Animals such as insects or rodents also cause food spoilage. Exposure to chemicals and autolytic enzymes are also important risk factors for food spoilage. In addition to all these, microorganisms that facilitate the effect of all these factors on the food can also affect the quality and texture of the food. Certain species from different organisms found in food cause this spoilage. At this point, the physical qualities of the food have an important effect. These are water content, pH, temperature, gaseous conditions, texture, and nutrients (Modi, 2009).

Considering the water content, the probability of spoilage of food rises as the number of water increases. Foods with a higher water content are susceptible to spoilage by a wide variety of organisms. Therefore, the drying process is used to prevent food from spoiling (Kayacan Çakmakoğlu *et al.*, 2020). When the water content of different foods is examined, it has been observed that foods such as skim milk,

strawberries, watermelon, and spinach have a water content of 90% and above. Foods such as fruit juices, yogurt, oranges, and carrots contain 80-89% water. Foods such as banana, avocado, baked potato, cottage cheese have a water content near 70-79%. Foods such as legumes, pasta, chicken breast, salmon, ice cream contain 60-69% water. Foods such as minced meatballs and sausage contain 50-59% water. Foods such as bread, bagels, cakes, and biscuits contain 20-39% water. Butter and margarine have 10-19% water content, while oils and sugar have 0% water content (Baker *et al.*, 2014).

When the pH is evaluated; the pH value of food limits the microorganisms that can grow. Foods can be spoiled by the growth of a wide variety of organisms, the effect of which is to change the flavor, texture and appearance of the affected item. At this point, the acidity of food with an acidic pH can be changed and microorganisms that can grow in acidic pH can be prevented. Thus, food spoilage can be prevented. The spoilage of canned foods also depends on the pH all contain after heat treatment (Ratzke and Gore, 2018). While more fungal growth in acidic foods, more bacterial growth occurs in fruits and vegetables with pH value above 4.5 (Rawat, 2015).

When the temperature is evaluated; especially molds and yeasts grow best at room temperature. When the tissue is evaluated; fluid and solid-liquid mixed foods spoil quickly. Solid foods begin to degrade from their outer surfaces. When the food is evaluated; foods rich in protein are more attacked by proteolytic organisms. Foods rich in carbohydrates are more prone to attack by fermentative organisms. Fat-rich foods tend to be attacked by lipolytic organisms. In the gaseous conditions; the oxygen tension and oxidation-reduction state of the food directly affect the microorganisms that cause food spoilage. The degradation by aerobic organisms occurs on the surfaces of foods (Modi, 2009).

Food losses from farm to fork have environmental and economic impacts. According to the Food Waste Index 2021 report, 17% of the food produced is wasted before it reaches the consumer. In this case, it is thought that at least 40% of the food produced is wasted. In numerical terms, it is thought that 2.5 billion tons of food are lost or wasted annually. 44% of these foods are fish and seafood, 26% fruits and vegetables, 15% roots, tubers and oil crops, 14% cereals and pulses, 12% meat and animal products and 6% consists of other foods (WWF-UK, 2021). At this point, the importance of food losses in fighting hunger and improving food security comes to the fore, especially in poor countries around the world. It is affected by factors such as food production conditions, transportation, distribution conditions and infrastructure. These preventable food losses directly or indirectly influence the financial situation of both farmers and consumers negatively. Food spoilage and loss also have environmental effects. When a certain percentage of the product is lost, the fertilizer, water and energy used to grow that crop are also wasted at the same rate (Rawat, 2015). While the water footprint spent for the production of 1 kilogram of red meat is more than 15,000 liters on average, the water used for the production of 1 kg of eggs is 3,265 liters, and the water footprint for 1 kg of wheat is 1,827 liters (Mekonnen and Gerbens-Leenes, 2020).

2. Food Spoilage Microorganisms

Spoilage of food; it is defined as the loss of qualitative characteristics in terms of color, taste, texture, smell, or shape in foods. At this point, various microorganisms cause chemical reactions that cause disturbing sensory changes in foods. These microorganisms use food as a source of carbon and energy. Microorganisms that cause food spoilage are bacteria, yeasts, and molds (Gram *et al.*, 2002).

2.1. Yeast

Yeasts are a subset of a large group of organisms, including molds and fungi. These are generally adapted to living in liquid environments. Yeasts can reproduce with or without oxygen. It also plays an important role in the formation of bread and alcoholic beverages through fermentation. It is known that

food spoilage caused by yeast does not directly lead to infections or toxic product formation in humans. But the simultaneous growth of other microorganisms can cause these problems (Barnett *et al.*, 2000).

2.1.1. Zygosaccharomyces and related genera

Zygosaccharomyces and its species *Lachancea*, *Torulaspora* and *Zygotorulaspora* generally affect high sugar and high salt foods (Barnett *et al.*, 2000). Yeasts also affect wine and cause significant economic losses as these produce negative organoleptic properties in wine (Kuchen *et al.*, 2021). Because these species can grow slowly, it can take months for food to deteriorate. The most obvious problem seen in spoiled products is the accumulation of carbon dioxide in the package of the product. This species can also cause food staining, foul odor and taste (Grinbaum *et al.*, 1994).

2.1.2. Saccharomyces and related genera

Saccharomyces cerevisiae and *Saccharomyces bayanus* are involved in the fermentation of alcoholic beverages (Parapouli *et al.*, 2020).

S. cerevisiae and *Saccharomyces exiguus* have a position in the fermentation of cereal products (Rose and Harrison, 1993). *Saccharomyces* species generally have positive effects on food. But some species produce high amounts of hydrogen sulfide and acetic acid. In this case, the raw material is wasted. Regrowth of *S. cerevisiae* or *S. Bayanus* in beer or wine causes turbidity and off-flavor (Oda and Ouchi, 2000).

2.1.3 Candida and related genera

Candida is an asexual, imperfect and anamorphic yeast genus. Candida is the nomenclatural type genus of the family *Candidaceae*. Candida species make up about a quarter of all known yeasts. *Candida* is a type of yeast that also causes infections in humans (Blackburn, 2006). It is known that *Candida* species have effects especially on milk and dairy products, fruits and vegetables. Nearly 24 different candida species were affected by the deterioration of milk and dairy products (Tomičić *et al.*, 2017).

2.1.4. Dekkera/Brettanomyces spp:

These mainly cause an undesirable and repellent taste in wines. *Dekkera/Brettanomyces* also play a role in the spoilage of dairy products and fermented foods. Judging by its effect, it produces volatile phenolic compounds that cause bad taste (Couto *et al.*, 2005).

2.2. Molds

Molds are filamentous fungi that are most visible to the naked eye and do not form fruit bodies. Molds are involved in the recycling of dead plant and animal remains in nature. These are well adapted to growth on and through solid substrates. These need oxygen for their metabolic processes to take place. Most molds can grow in both acidic and basic environments. In addition, molds can grow in dry foods in very low levels of water activity. Their spores are resistant to harsh environmental conditions. These are generally sensitive to heat treatment, with some exceptions (Carlile *et al.*, 2001).

2.2.1. Zygomycetes

Zygomycetes reproduce in soil and dead plant material. These are also a good source of fertilizer and generally scavenge simple carbon sources rapidly. Many *Zygomycetes* are associated with living organisms as parasites or mycorrhizal fungi. These can infect many insect species and microscopic creatures as parasites (Martin *et al.*, 2004). *Zygomycetes* are most associated with food spoilage; *Absidia*, *Mucor* and *Rhizopus* genera. Ecologically, these reproduce in soil, plant residues and manure. *Rhizopus* and *Absidia* species are found in cereals, fruits and vegetables, and meat and dairy products (Hesseltine, 1991).

2.2.2. Penicillium and related genera

Fungi belonging to the *Penicillium* genus are among the most common microorganisms. It is hardly to find an area that does not contain penicillium spores. Interestingly, *Penicillium* species showed that these are substrate-specific. Some *Penicillium* species do not cause spoilage because their growth is limited. However, *Penicillium* species are a major cause of food spoilage. It can cause food spoilage in fruits, vegetables and grains (Blackburn, 2006).

Penicillium spp. also causes deterioration in foods such as butter, margarine, cheese (Garnier *et al.*, 2017).

2.2.3. *Aspergillus* and related teleomorphs

The genus *Aspergillus*, which includes approximately 350 species, is very important for public health (Bennett, 2010). *Aspergillus* spores can also survive in extremely harsh environmental conditions where usual mold growth would not occur (Pagano *et al.*, 2010). The genus *Aspergillus* is an important genus. The reason for this is that while it causes spoilage in foods, it also produces mycotoxins. This genus is quite common in products such as cereals, nuts, and spices. *Aspergilli* can generally thrive even at higher temperatures or lower water activity than *Penicillia*. *Aspergillus* species are more prominent in the tropics, while *Penicillium* species are more common in temperate regions (Hocking, 2006).

2.3. Bacteria

2.3.1. *Pseudomonas* and related genera

Pseudomonas, *Shewanella*, and *Xanthomonas* are among the bacteria that have the widest effect on food spoilage (Tarlak and Pérez-Rodríguez, 2021). These can be found in many environments such as soil and water. *Pseudomonads* have a very important place in aerobic degradation and biological degradation. Therefore, its effects on the environment are undeniable. *Pseudomonas fluorescens*, *Pseudomonas lundensis*, *Pseudomonas viridiflava* and *Pseudomonas fragi* are species that are particularly associated with food spoilage. When foods are spoiled, these cause a visibly watery and sticky structure and an unfavorable odor (Andreani ve Fasolato, 2017).

2.3.2. Lactic acid bacteria

Lactic acid bacteria are closely related to food fermentation and food spoilage. These bacteria are especially encountered in environments with vacuuming or modified atmosphere, low pH and low temperature, low oxygen content. It is the main microbial group that emerges under vacuum or modified atmosphere conditions (Kalschne *et al.*, 2015). Some lactic acid bacteria have a strong tolerance to salt and sugar. In a study, the most resistant species to salt was *Lactobacillus delbrueckii* subsp. bulgaricus was identified (Kwun *et al.*, 2020). Lactic acid bacteria also include *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* species that are useful in the production of fermented foods such as yogurt and pickles. In undesirable formations due to lactic acid bacteria, problems such as greening in the color of meat, mold formation in cheese, swelling in pickles appear. Lactic acid bacteria can produce exopolysaccharides that cause degradation (Audenaert *et al.*, 2010).

2.3.3. Spore-forming bacteria

Many of the spore-forming bacteria are important causes of food spoilage. These bacteria, which have more than 200 species, have a role in the deterioration of heat-treated foods because these can survive at high temperatures. Because spores can survive at high temperatures**.** These bacteria, which have resistance to environmental factors, wait in a dormant state when there is no suitable opportunity. When suitable conditions occur again, these continue their normal metabolism. These Gram-positive bacteria can be strict anaerobes or facultative (growth with or without oxygen) (Ande *et al.*, 2017). In foods kept at high temperatures, these bacteria produce species-specific hydrogen sulfide, hydrogen or carbon dioxide. In particular, these are a significant risk factor for the deterioration of milk and dairy products (Gopal *et al.*, 2015).

2.3.4. Enterobacteriaceae

Enterobacteriaceae are gram-negative, facultative anaerobic bacteria. Many organisms such as *Salmonella*, *E. coli*, *Shigella*, *Yersinia* are included in this group. These bacteria are common in soil, plant surfaces and the digestive systems of animals**.** *Enterobacteriaceae* is associated with pneumonia, especially in elderly individuals. In addition to their negative health effects, some members of this family are responsible for significant economic losses in some sectors of the food industry (Jenkins *et al.*, 2017).

Some microorganisms are common in many types of spoiled food, while others are less common. More than one species can often be identified in a single spoiled foodstuff. However, it may be a dominant species that is primarily responsible for the production of compounds that cause a difference in odor or taste. At this point, the basic indicator is the substance that becomes usable or depleted in the spoiled food. Thus, the microorganism population inside can increase or decrease (Gram *et al.*, 2002). In this review, food spoilage in many foods and food groups (meat products, poultry products, dairy products, seafood products, egg products, cereal products, fruits and vegetables) will be evaluated under headings

3. Spoilage Organisms in Meat Products

Degradation in meat products is one of the significant issues. Searching for the words (spoilage) and (meat) on PUBMED revealed that 1682 different articles have been published since 1947. While only certain microorganisms were identified in the beginning, hundreds of different species have been recognized today (Zhao *et al.*, 2015). The deterioration of meat products causes significant economic losses every year (Teixeira *et al.*, 2020).

Normally the muscle tissue of healthy animals is sterile, but a large number of microorganisms are seen after slaughter. Moreover, processes such as transportation and storage are also associated with the growth of microorganisms that cause degradation. The determination of these microorganisms and their growth conditions is important for the solution of the problem. Deterioration gene markers in microorganisms that cause spoilage are also being investigated (Mohareb *et al.*, 2015). Meat spoilage includes changes in the composition and sensory quality of meat. These changes are divided as enzymatic and non-enzymatic. Microorganisms are responsible for the deterioration of meat using macronutrients and other nutrients in meat. As a result, different metabolites are produced (Wang *et al.*, 2017).

Microorganisms in the degradation of red meat are molds, yeasts and bacteria. Bacteria are especially involved in the deterioration of raw meat. Molds and yeasts are more prominent in baked foods which have low moisture. However, degradation by molds and yeasts appears to be rare (Zhao *et al.*, 2015). The spoilage bacteria in red meat include Gram-negative Pseudomonas, *Acinetobacter*, *Psychrobacter*, *Aeromonas*, *Shewanella putrefaciens*, *Enterobacteriaceae*; Gram-positive lactic acid bacteria and *Brochothrix thermosphacta* (Ercolini *et al.*, 2011). There are also other bacteria responsible for spoilage. *Achromobacter* is a gram-negative bacteria. *Achromobacter* species can grow even at low temperatures, especially in pork sausage**.** Moraxella is particularly found in animal mucus. Although it has a low percentage of meat spoilage, it can be seen in vacuum-packed pork products (Li, 2006). As a gram-negative bacterium, *Psychrobacter* can grow at low temperatures. Clostridium can be seen especially in vacuum-packed products (Huang *et al.*, 2018).

Packaging conditions also have a direct effect on the deterioration of red meat. It has the positive outcome of removing dioxygen in packaging to delay the occurrence of spoilage. In the presence of dioxygen, the carbon dioxide ratio in gas mixtures directly affects the deterioration (Luong *et al.*, 2020). Temperature is another basic factor that is associated with degradation. Microorganisms need the optimal temperature to grow. For bacteria, this temperature range is 4–60 °C. As a result, meat products should be kept below 4 °C to prevent spoilage (Ercolini *et al.*, 2011). In PCR-denaturing gradient gel electrophoresis analysis to assess bacterial contamination sources, the bacterial diversity of cattle and sheep slaughter lines was significantly different. The reason for this is thought to be due to the hand slaughter of sheep. Salmonella enterica was observed most frequently in sheep with 30% and beef with 28%. It has been observed that the risk of salmonella contamination is significantly

reduced by washing carcasses (Fletcher *et al.*, 2018).

3.1. Control and Prevention of Spoilage Microorganism in Meat Products

Physical (low temperature, high temperature and high pressure), chemical and microbiological methods can be used for the preservation of meat products. First, when looking at the physical methods; chilling is the reduction of the carcass temperature to the appropriate temperature for storage without the formation of ice crystals. Rapid carcass cooling has a reducing effect on microbial growth. Freezing is one of the most effective methods. Since both temperature and water activity decrease, it is effective in preserving meat without spoiling. Many microorganisms are destroyed by exposure to high temperatures (Tarlak, 2021). Another method used to preserve meat is drying. In drying, which is one of the old traditional methods, water is removed. In this way, microbial growth is prevented. Exposing meat to radiation is one of the most effective protection methods. The applied radiation can break down and destroy the DNA of microorganisms. In this way, it allows dealing with microorganisms. Packaging meat products is another effective method of protecting against spoilage effects (Triki *et al.*, 2018).

Looking at chemical methods; in ancient times, when refrigerators were not available, the way of curing meat was often used. Today, curing is done by adding substances such as sodium, potassium chloride, sodium nitrite to raw meat (Teixeiria *et al.*, 2019). Health concerns, especially with the addition of nitrites and nitrates, are still a major concern (Sebranek ve Bacus, 2007). At this point, the search for natural products appeared. Recently, essential oils as a natural product have attracted attention with their antimicrobial potential. Moreover, these essential oils have benefits for human health. Essential oils have antioxidant effects. It is thought that more data are needed to use essential oils as preservatives in meats (Chivandi *et al.*, 2016).

Finally, looking at the microbiological methods; The fermentation of meat, as a traditional method, has a protective effect against microorganisms. However, it gives a different flavor, color and texture to the product. Lactic acid is responsible for the antimicrobial properties of fermented meats. This formation occurs from the conversion of glycogen reserves in the carcass tissues and the sugar added during fermentation (Di Gioia *et al.*, 2016).

4. Spoilage Organisms in Poultry Products

Microorganisms have an essential place as the main factor causing deterioration (Tarlak and Khosravi-Darani, 2021). Microorganisms are seen in the feathers, skin, feet, and feeding ways of poultry. Various processes performed on these creatures generally reduce microbial contamination. But cross-contamination from the environment can be seen. In addition, processes such as transportation and storage may result in the formation and degradation of microorganisms (Wang *et al.*, 2017).

Campylobacter spp. and *Salmonella* spp. has a high risk in poultry products. Other main microorganisms responsible for degradation in poultry are pseudomonads and other Gram-negative bacteria. Apart from these, Acinetobacter is another organism responsible for spoilage in poultry carcasses (Barnea and Thornley, 1966). Yeast formation in poultry carcasses has also been described. *Candida*, *Cryptococcus*, *Debaryomyces* and *Yarrowiawere* species were identified in carcasses (Zhang *et al.*, 2012). Chicken liver is one of the perishable products. *Pseudomonas* spp. found to have an effect mainly. *Salmonella* managed to survive at 0 °C in chicken liver (Dourou *et al.*, 2021).

4.1. Control and Prevention of Spoilage Microorganism in Poultry Products

High-temperature exposure is one of the most effective methods for the control of microorganisms in poultry. In addition, hot water, chlorine, organic acid, or different chemicals have positive effects on the control and prevention of microorganisms (Wang *et al.*,

2017). Trisodium phosphate has been shown to reduce spoilage-causing bacteria. Moreover, it takes place without affecting the sensory quality of the products (Hinton and Ingram, 2005).

5. Spoilage Microorganisms in Seafood Products

Seafood is one of the foods that have the risk of perishable quickly. The spoilage of seafood is closely related to the composition of the microbiota. Factors such as the aquaculture environment and storage temperature affect this microbiota (Zhuang *et al.*, 2020). In addition to these, storage conditions, packaging atmosphere and preservatives also affect the microbiota in seafood (Sørensen *et al.*, 2020).

During the storage of seafood, carbon and nitrogen-derived compounds are metabolized. This situation is closely related to the formation of microorganisms. Structural proteins are hydrolyzed to peptides and amino acids. At this point, microbial proteases are involved (Zhuang *et al.*, 2019). The peptides produced are transferred to bacterial cells. The resulting amino acids result in the production of amines such as ammonia in the cytostome. Among the amino acids found in seafood, those containing sulfur, branched chains and aromatic amino acids are effective in microbial deterioration and malodor formation (Biji *et al.*, 2016).

Bacteria such as *Photobacterium*, Psychrobacter *Pseudomonas* are abundant in the intestines of fish (Shehata *et al.*, 2020). Gram-negative bacteria are more common in seafood grown in temperate waters. Gram-positive bacteria predominate in seafood grown in tropical environments (Françoise, 2010). *Vibrio* and *Pseudoalteromonas* are important microorganisms responsible for degradation in shellfish (Madigan *et al.*, 2014). In the following stages, the dominant microorganism changes. The main reason is that shellfish have a high carbohydrate content (Fernandez-Piquer *et al.*, 2012). In dried seafood, *Aspergillus niger*, *Cladosporium cladosporioides* and *Penicillium citrinum* are responsible for degradation (Park *et al.*, 2014).

5.1. Control and Prevention of Spoilage Microorganism in Seafood

Products Packaging method in seafood is one of the essential factor in preventing microorganisms. At this point, modified atmosphere packaging was found to be more successful than vacuum packaging in seafood (Aberoumand and Baesi, 2020). It has been observed that the CO_2 content in packaging has positive effects on preventing bacterial growth. Traditional preservatives such as salt and chemical additives can be used to store seafood (Olatunde *et al.*, 2020). Recently, due to health concerns, phytochemicals have been used as preservatives instead of chemicals (Ribeiro-Santos *et al.*, 2017). As in meat products, essential oils come to the forefront as a protector against microbial degradation in seafood. Freezing, smoking, high pressure, dehydrating, ozone and ionizing radiation are also helpful preservatives which are used in seafood products (Wang *et al.*, 2017).

6. Spoilage Microorganisms in Dairy Products

Milk and dairy products are nutritious foods with the proteins, essential amino acids, oils, minerals and vitamins these contain. However, this content is also suitable for the growth of different heterogeneous microorganisms (Fusco *et al.*, 2020). Psychrotrophic microorganisms, *pseudomonads*, gram-negative and rod-shaped bacteria are the predominant microorganisms seen in raw milk. Pasteurization has an extensive effect on food safety. However, *Pseudomonas* and Acinetobacter spp. microorganisms such as can produce proteases and lipases even after pasteurization. Global warming is also one of the important risk factors at this point. Because of that, the risk of mycotoxin increases in milk and dairy products (Fusco *et al.*, 2020).

It is known that fungi also alter the deterioration of dairy products. In particular, these microorganisms are responsible for the formation of undesirable odor, color and taste. Coliforms, heterofermentative lactic acid bacteria, yeasts and spore-forming bacteria can cause gas formation in cheese. Methods such as

the type of microorganisms that deteriorate, production, processing, packaging, storage, transportation have an impact. When the microorganisms in different milk and dairy products are examined; There are especially *Psychrotrophs*, spore formers in pasteurized milk. Spore-forming bacteria and osmophilic fungi are dominant in concentrated dairy products. Butter *Psychrotrophs*, cottage cheese *Psychrotroph*s, coliforms, yeasts, molds are dominant. Yeasts predominate in yogurt, fungi and coliforms in other fermented milk products. While fungi and spore-forming bacteria are common in cream cheese and processed cheese, Psychrotrophs, coliforms, fungi, and lactic acid bacteria are dominant in fresh cheese (Ledenbach and Marshall, 2009).

6.1. Control and Prevention of Spoilage Microorganism in Dairy Products

Heating milk to high temperatures kills most pathogenic bacteria and spoilage bacteria. The filling equipment of milk and dairy products must be sterile (Ledenbach and Marshall, 2009). Exposing the milk to high hydrostatic pressure, in particular, ensures the destruction of many bacteria. Spores are more resistant at this point (McClements *et al.*, 2001). Pasteurization eliminates the risk of most psychrotrophic microbes, coliforms, *leukonostoks* and many *lactobacilli*. Pasteurized milk has a limited shelf life due to the formation of psychrotrophic contaminants. Lactic acid bacteria are effective here and cause the formation of a sour taste (Erkmen and Bozoglu, 2016).

Freezing inhibits microbial growth and enzyme activity. Therefore, the microbial degradation in frozen desserts is thought to be due to previous contamination. Pasteurization of milk destroys most of the acid-forming bacteria. Heat-resistant bacteria can survive. During the ultra high temperature processing (UHT) procedure, contamination of spore-forming bacteria can be seen (Ledenbach and Marshall, 2009).

7. Spoilage Organisms in Egg Products

Eggs are a powerful food both economically and nutritionally. Eggs contain essential oils, protein, vitamins and minerals. Egg; It is the lowest cost animal food for protein, vitamin B12, vitamin A, iron, choline, riboflavin. It is the second-lowest cost animal food for zinc and calcium. The egg has an average of 76.1% water. Looking at the macronutrients; It contains 12.6% protein, 9.5% fat and 0.7% carbohydrates (Réhault-Godbert *et al.*, 2019). When looking at the microorganisms in the egg, Gram-positive bacteria such as *Staphylococcus*, *Streptococcus*, *Aerococcus* and Micrococcus are seen in the egg shell. Apart from this, bacteria such as Salmonella and Escherichia draw attention. Gram-positive bacteria predominate on the eggshell surface. Gram-negative bacteria are responsible for degradation in egg content. When eggs and egg-containing foods are spoiled, a black or green color change is usually observed with the formation of a foul odor (De Reu *et al.*, 2006).

Salmonella is responsible for more than half of all microbial outbreaks. It is also the pathogen associated with an increased risk of death and the most frequently reported pathogen responsible for 81% of deaths. Potential risk in a *Salmonella* outbreak was found to be egg-containing foods and equipment contaminated with eggs (Gurtler *et al.*, 2015).

Bacillus cereus has been found in raw and pasteurized eggs and bakery products made from them. This microorganism draws attention with its ability to form spores and survive at low-temperature treatments (Reis *et al.*, 2014). *Campylobacter*, as another microorganism, is transmitted mainly through poultry. It is seen in unpasteurized eggs and their products (Sato and Sashihara, 2010). 17.4% of raw egg products were found to contain Listeria (Rivoal *et al.*, 2010). As a result of the analysis of 1125 eggs, Staphylococci were found in 45.6% of them (Stepień-Pyśniak *et al.*, 2009).

7.1. Control and Prevention of Spoilage Microorganism in Egg Products

Heat treatment has an important position in the destruction of microorganisms in egg products. It is adequate to be exposed to heat between 65-68 °C for 5-6 minutes. In egg white, shorter time and lower temperature are sufficient. Methods such as adding sugar or salt and drying are also effective in preventing deterioration. Again, the realization of the right storage conditions has positive effects in preventing and controlling the deterioration of eggs and egg-containing products (Techer *et al.*, 2014).

8. Spoilage Organisms in Cereal Products

Grains are often contaminated with microorganisms during collection, transportation and storage**.** Microorganisms can be encountered in many situations and conditions. Moreover, many microorganisms can adversely affect health. When looking at cereal products, food poisoning is very rare. At this point, the underlying factor is thought to be the high temperature applied during cooking and the low amount of water associated with it (Cook and Johnson, 2009). Although food poisoning seems rare, the taste of cereal products changes after degradation by microorganisms and may become unusable. Molds can cause defective odor in grain products. In addition, mycotoxins may form. This may cause adverse health effects (Gupta and Srivastava, 2014). Humidity, temperature and oxygen are the most effective factors in the degradation of grains and the growth of microorganisms. The dominant bacteria occurring in cereals belong to the families of *Pseudomonadaceae*, *Lactobacillaceae*, *Micrococcaceae* and *Bacillacea*e. Molds are predominantly *Alternaria*, *Helminthosporium*, *Fusarium*, and *Cladosporium*.

8.1. Control and Prevention of Spoilage Microorganism in Cereal Products

Different methods such as ozone, radiation, antimicrobial agents are used to reduce microorganisms in cereals. Recently, the hazard analysis system of critical control points has been implemented to ensure food safety (Hulebak and Schlosser, 2002***).*** Grinding in cereal products can reduce microbial formation. However, molds and some microorganisms may remain inside. Especially whole wheat flour and foods made from this flour are at higher risk in terms of microorganisms, since their bran is not separated. As a newer method, superheated steam pasteurization is one of the prominent microorganism control methods in grain products (Wang *et al.*, 2017).

Yeast is more resistant to disinfectants and preservatives. For this reason, quaternary ammonium can be preferred for surface cleaning. Packaging grain products quickly after cooking is one of the methods that can prevent mold contamination. This will prevent different microorganisms that may come from the environment. Packaging in a sterile atmosphere after cooking may be preferred (Saranraj, 2012).

Antimicrobial preservatives can be used, especially for bacteria, to ensure food safety. Examples of these are sorbic acid, calcium propionate and potassium sorbate. These are used especially in the bread production stage. Vinegar and malic acid can also be used to prevent acid degradation. Because these products will lower the pH. Spices with antimicrobial properties such as cinnamon and black pepper can also be added. In addition, starch can be added to the grain product to reduce the amount of water (Jay, 2012).

9. Spoilage Organisms in Fruits and Vegetables

Fruits and vegetables are basic sources of fiber, vitamins and minerals. Because of its high-water content, pathogens can easily develop in fruits and vegetables (Blackburn, 2006). Fruits and vegetables are very abundant in nutrients. In addition, vegetables have a pH value close to neutral. The natural acidic structure of fruits protects food from many microorganisms, especially bacteria. Fungi are responsible for the degradation of both fruits and vegetables. Fungi from microorganisms generate extracellular pectinases and

hemicellulases for degradation (Miedes and Lorences, 2004).

Storage and transportation conditions are critical in the prevention of microorganisms. *Penicillium expansum* and *Botrytis cinerea* must be removed from the fruit before storage. If it is not removed, it may cause deterioration in other fruits. These appear, especially in pectin-rich fruits (van Kan, 2006). *Erwinia carotovora* subsp. *carotovora* is responsible for the degradation of fruits and vegetables (Lund, 1982).

Viruses, bacteria and fungi have different roles in degradation. Fungi, in particular, are responsible for mycotoxin growth (Marin *et al.*, 2013). Decay is mostly caused by fungi and bacteria. Rot and discoloration of citrus fruits are associated with *Penicillium digitatum* and *Penicillium italicum* molds (Caccioni *et al.*, 1998). *Colletotrichum musae* is a microorganism that is particularly effective in the blackening of bananas (Zakaria *et al.*, 2009). Food decay can often be seen in foods such as pears and apples. *Botryosphaeria obtusa* and *Physalospora cydoniae* are dominant in these fruits. In grapes, *Erysiphe necator* is responsible for degradation. Differences in color changes seen in fruits vary according to the type of microorganism (Wang *et al.*, 2017). In a study, it was seen that the most common fungi in fruits and vegetables were Penicillium and then Rhizopus (Saleh and Al-Thani, 2019).

9.1. Control and Prevention of Spoilage Microorganism in Fruit and Vegetables

It is tried to protect freshly cut fruits and vegetables by methods such as hot water, hot steam and hot sterilization. However, these heat treatments can cause deterioration of the quality of the product. Technologies that do not include heat treatment include physical and chemical processes. As physical processes, there are methods such as high pressure, ultraviolet radiation and ultrasound. As chemical processes, different liquid and gaseous forms (ozone and chlorine dioxide) are used to provide sanitation (Wang *et al.*, 2017). It has been observed that the application of ultrasound in fruits and vegetables reduces the microbial load and can prevent color change in the products (Roknul Azam *et al.*, 2020). Disinfectants are also used to destroy pathogens on the surfaces of fruits. Especially sodium hypochlorite, chlorine dioxide, ozone and chlorine dioxide are frequently preferred (Fukuzaki, 2006). As a natural alternative to chemicals, essential oils also have strong antimicrobial activity. Modified atmosphere packaging processes have emerged to prevent microbial growth by actively adding antimicrobial agents to packages (Gong *et al.*, 2016; Tarlak *et al.*, 2020).

Finally, ethylene has a significant effect on the deterioration of fruits and vegetables. Although studies on the subject are limited, it is thought that the use of ethylene scavengers in food packaging may be propitious (Wei *et al.*, 2021). Coating fruits and vegetables with aloe vera gel have a reducing effect on ethylene biosynthesis. Aloe vera coating can also prevent or delay processes such as softening, discoloration, rot (Hasan *et al.*, 2021).

10. Conclusions

In this review, the microorganisms responsible for deterioration of food products were comprehensively compared, and food preservation methods to minimize food spoilage were discussed in detail.

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ASSESSMENT OF FOOD ADDITIVES IMPACT ON ACRYLAMIDE FORMATION IN POPCORN SUPPLIED IN TEHRAN, IRAN: A RISK ASSESSMENT STUDY

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ABSTRACT

Acrylamide (AA) is a probable toxic substance that forms naturally, especially in carbohydrate-rich foods. Adding flavorings to foods and food additives makes people new food habits and customer friendly. This study investigated the AA concentration in 34 commercial popcorn samples with different additives. Also, health risk assessment was carried out using the Simulation of Monte Carlo methods. The highest and lowest mean AA concentration was detected in salty popcorn and cheese popcorn, respectively. The differences in acrylamide amounts in popcorn samples are due to differences in the food additives used for each popcorn type production. Hazard quotient (HQ) was determined for three age groups (<18, 18-60, and >60 years old). For all three age groups, mean HQ was lower than 1, but the probabilistic approach has shown that the HQ 90% was higher than 1 for both age groups <18 and 18-60 years old and revealed that AA might have potential health problems. Cancer risk for all three age groups indicates a potential concern for consumers. Therefore, controlling and monitoring AA demand in the different flavors and brands is essential, especially for the children group.

1. Introduction

The contamination of food products with pollutants such as heavy metals and mycotoxins raised notable concerns during last decade (Bangar, Sharma, Bhardwaj, & Phimolsiripol, 2022; Bounar, Boukaka, & Leghouchi, 2020; Gao et al., 2022; Heshmati, Mehri, Karami-Momtaz, & Khaneghah, 2020; L. Hu et al.,

2022; Khaneghah et al., 2023; Luo et al., 2022; Pires et al., 2022; Rezaei et al., 2020). However, the issue of contaminants in food products also is associated with other toxic compounds like acrylamide (Atabati et al., 2020; Hwang & Kwon, 2022; Mousavi Khaneghah, Fakhri, Nematollahi, Seilani, & Vasseghian, 2020; Nematollahi et al., 2020a; Nematollahi, Meybodi, & Khaneghah, 2021a; P. E. Shahrbabki et al., 2018; Zokaei et al., 2020). One of the neurotoxin compounds is Acrylamide (AA) (Costa, Freita, Mendes, Roviero, & Mutton, 2018; Hwang & Kwon, 2022). This compound is classified as a "probable human carcinogen" and as well as listed with IARC (international agency for cancer research) as a carcinogenic compound through experimental studies (Bušová, Bencko, Kromerová, Nadjo, & Babjaková, 2020; Deribew & Woldegiorgis, 2021; Hamid, Yaqub, Ahmed, & Aziz, 2017; Mucci & Wilson, 2008). Since the 1950s, it has been utilized in the chemical industry to manufacture polyacrylamide polymers and enhance polymers' adhesive bonding. Notably, incomplete polymerization processes may result in the residual monomer of AA in products; also, the carcinogenic effect happened at the maximum concentration limit of 5 mg/kg (Khan, Alothman, Naushad, Alomary, & Alfadul, 2018; Medeiros Vinci, Mestdagh, & De Meulenaer, 2012). Rather than being a food contaminant, the primary way to create AA in foods is through a sequence of Maillard reactions involving an amino acid, mainly asparagine, and a reducing sugar such as glucose or fructose during cooking. Such conditions, including temperature, can affect the formation of AA, which can occur in some foodstuffs during high-temperature cooking operations at temperatures between 120°C and 180° C (Mottram, Wedzicha, & Dodson, 2002; Nematollahi, Meybodi, & Khaneghah, 2021b). As a result, AA is formed during thermal processing such as (Mohsen et al., 2020)roasting, frying, and baking. Thus, the European Union (EU), in 2007-2009, suggested that member states conduct annual monitoring of AA levels in specific foods including drinking water, potato crips, fresh potato and dough, popcorn, type of coffee for control and monitoring the permissible limit of AA according to µg/kg (Authority, 2012; Koszucka, Nowak, Nowak, & Motyl, 2020; Ma et al., 2016; Pacetti et al., 2015; Park, 2021; Wenzl & Anklam, 2007). For example, according to the mean body weight of people in several counties such as Italy, Germany, and the Netherlands, the recommended average daily intake of AA was 0.45, 0.32, and 0.32 µg/kg bw per day, respectively (Koszucka et al., 2020).

The AA presence in a range of heat-treated foods is relatively low, and the risk of carcinogenicity is considerable (Peivasteh-Roudsari et al., 2022). Increasing snacking and fast-food consumption lead to a high AA intake. In addition, in several studies, the presence of AA in popcorn and corn seeds was measured (Baskar & Aiswarya, 2018; Bocharova, Reshta, & Bocharova, 2017; Khan et al., 2018). Since the different types of popcorn with different flavours are among the most consumed foodstuffs among adults and especially children in Iranian people (Shaviklo, Dehkordi, & Zangeneh, 2015). Making popcorn invariably entails processing at high temperatures, which builds enough pressure within the kernels to cause them to burst (Bocharova et al., 2017; Byrd & Perona, 2005).

Although remarkable contention exists as to exposure levels associated with AA's carcinogenicity in humans, data on the presence of AA in foods needs to be analysed. For the importance of AA monitoring amounts in the different types of foods, new technologies have developed and increased AA exposure through the intake of roasted foods, seeds, and popcorns in recent years. In recent years, numerous published methods for determining the quantity of AA monomer have been developed, including distinct extraction methods in different food matrixes. Firstly, the presence of AA was determined in a variety of heat-treated meals using the isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach (Byrd & Perona, 2005; F. Hu et al., 2017; Kamankesh, Nematollahi, Mohammadi, & Ferdowsi, 2021; Sun, Fang, & Xia, 2012). Also, other techniques such as

HPLC, CE-MS (Capillary Electrophoresis-Mass Spectrometry), NACE (Non-Aqueous Capillary), HPLC-MS (High-Performance Liquid Chromatography-Mass Spectrometry), GC-MS (Gas Chromatography-Mass Spectrometry), Solid-Phase Micro Extraction-Gas Chromatography (SPME-GC), MSPD (Matrix Solid-Phase Dispersion), ELISA methods (Enzyme-Linked Immunosorbent Assay), and Micro Emulsion Electro Kinetic Chromatography (MEEKC) were as standard methods to AA and other contaminant detection (Arvanitoyannis & Dionisopoulou, 2014; De Souza, Khaneghah, & Oliveira, 2021; Maqbool et al., 2021; Tang, Qu, Cao, Wang, & Lou, 2020). However, many food samples must be examined and analysed to determine AA. Although in this case, it is vital to develop a reliable and low-cost analytical approach for determining AA (Mollakhalili-Meybodi, Khorshidian, Nematollahi, & Arab, 2021).

For the first time, AA concentrations in popcorn, a product consumed widely by children and adults in Iran, were found in this study.

2.2. Instruments

A Hewlett Packard (HP) 1090 M type HPLC system (UV-vis) with a quaternary pump and vacuum degasser was used to quantify AA. Chromatographic separations were carried out on a 25cm×4, 6 Mm Zorb ax ODS (5m) column. The blank sample was analyzed for confirmation using the same device linked to a UV diode array detector (UV-DAD). The flow rate of the mobile phase (acidic water) was adjusted to 0.5 mL/min during routine analysis of food samples. At 202 nm, the absorbance of AA was determined.

Acrylamide spiked Chromatogram in popcorn (with HPLC-UVvis chromatogram), the analyses were performed by a sensitive HPLC equipped with a UV-vis detection system. There were several clean-ups and removing interference steps in the used procedure and an enrichment phase that we dried the extracted sample and reconstituted it with an appropriate solvent that was finally injected into the HPLC. Some other reports revealed obtaining adequate LOQ to quantirandomly from several places in Tehran with different production dates to extract AA. fy such low AA levels in foodstuffs by HPLC-UV detection (H. Wang, Feng, Guo, Shuang, & Choi, 2013).

Electrophoresis), LC-MS, Pressurized Fluid Extraction (PFE

Thus, the primary objective of this study is to identify the probability of AA production in popcorn with different food additives in the different brands, with HPLC (UV-DAD), and to estimate the health risk of carcinogenic and non-carcinogenic disease was also determined by Monte Carlo Simulation (MCS) (Heshmati, Khorshidi, & Khaneghah, 2021; Heshmati et al., 2020; Jafari et al., 2021; Makkaew, Chaloeijitkul, & Vattanasit, 2022; Tang et al., 2020).

2. Materials and Methods

2.1. Chemicals and solutions

Sigma provided Acrylamide (>99 percent) (Deisenhofer, Germany). Methanol, acetonitrile, acetic acid, and acetone were of analytical grade, while Merck supplied potassium hexacyanoferrate (Carrez I) and zinc sulfate (Carrez II) (Darmstadt, Germany). The AA content was determined using distilled, double deionized, and 0.20 μm filtered water.

2.3. Sampling

Five popular Iranian popcorn products with four flavours were collected (6-8 each) and alphabetically nominated (A, B, C, D, and E). All popcorn samples were collected.

2.4. Sample Preparation

The AA content was achieved using Shahrbabki Peah, B. et al. (2018) and Oroian, M.S., (2015) methods with some modifications (Oroian, Amariei, & Gutt, 2015a; P. E. a. H. Shahrbabki, B. and Shoeibi, S. and Elmi, M. and Yousefzadeh, A. and Conti, G.O. and Ferrante, M. and Amirahmadi, M. and Fakhri, Y. and Mousavi Khaneghah, A. (2018) Probabilistic non-carcinogenic and carcinogenic risk assessments (Monte Carlo simulation method) of the measured acrylamide content in Tah-dig using QuEChERS extraction and UHPLC-MS/MS. Food and Chemical Toxicology, 118. pp. 361-370). At first, samples were smashed (less than 1 mm), and 1 gram of each sample was put into a 50 mL centrifuge tube. Samples were

mixed well with 100 µL acetamide as internal standard (Kim, Hwang, & Lee, 2011), and mixed with appropriate AA solutions at different concentrations to assess the percentage of relative recovery. Each sample was shaken enough and aqueous phase-separated in another falcon tube from solid samples. It is critical to eliminate solid remnants from the supernatant thoroughly, and samples were centrifuged again if necessary. Then 5 mL deionized water, 5 mL acetonitrile, and 2.5 mL hexane were applied to remove protein and a fat portion from samples. The sample tube was shaken for 30 minutes using a shaker.

Then samples were centrifuged at 3000 rpm (30 min) at 250 rpm. Centrifugation separates the sample tubes into three layers: the top layer (which includes proteins), the middle layer (which contains acrylamide), and the bottom layer (which contains acrylamide) (sample precipitate). The upper-fat layer was removed, and middle layer containing AA was transferred into a clean test tube. This will separate the water-soluble co-extractive components from the pure aqueous phase.

The separated phase was treated with 1 mL Carrez I and II solutions to precipitate the remained protein and soluble carbohydrates (Norouzi, Kamankesh, Mohammadi, & Attaran, 2018). After centrifuging the sample for 30 minutes at 250 rpm, the supernatant was transferred to a conical flask and evaporated until dry in an evaporator. The residue was dissolved in (acidic water concentration of 4 mL with pH=3) for 2 minutes using a vortex. Thus, following shaking (250 rpm) and centrifuging for 20 minutes at 3000 rpm, the AA residue was wholly dissolved in the water phase, while the leftover lipids and lipid-soluble co-extractive chemicals were separated into the test tube wall. Filtration of the fluid was performed using a 0.2 µm syringe filter. Twenty microliters of the finished solution were loaded into a high-performance liquid chromatography column for quantification (Ghalebi, Hamidi, & Nemati, 2019; Sarion, Dabija, & Codină, 2020).

2.5. Statistical analysis

SPSS software version 22 was used to conduct statistical analyses. The independent T-test and one-way analysis of variance (ANOVA) were used to compare the mean concentration of AA in different brands with different flavours. Additionally, to determine the probabilistic health risk, MCS was performed using Crystal Ball software (version 11.1.1.1, Oracle, Inc, USA) with a total of 10,000 trials.

2.6. Monte Carlo simulation for Health risk assessment

The current study assessed the risk of carcinogenic and non-carcinogenic effects of acrylamide in various brands and flavours of popcorn (Eisenbrand, 2020; Sanaei et al., 2021) (Eq 1-3).

$$EDI = \frac{C \times IR}{BW} \quad (1)$$

Where EDI represents the estimated daily intake of AA (mg/kg body weight. Day), C represents the concentration of AA in investigated samples (mg. kg1), IR represents the popcorn intake rate for each age group (kg/day), and BW represents the reference body weight (kg) (Morales-Moo et al., 2020).

Hazard quotients (HQ) were determined by Eq (2) to assay the potential non-carcinogenic health risk of popcorn consumption containing AA.

$$HQ = \frac{EDI}{RfD} \quad (2)$$

HQ is the target hazard quotients (Almendares Calderon, Garcia Mena, & Roman Miranda, 2020; Hamid, Wasim, Azfar, Amjad, & Nazir, 2020; Qasemi et al., 2022; Yao, Lin, Yan, Huang, & Chen, 2021), whereas RfD denotes the reference dosage of acrylamide (2 ng/kg/day)) (Nematollahi et al., 2020b). When the HQ>1 indicates a potential risk for consumers, and when the HQ<1, popcorn consumption is the safe level for non-carcinogenic risk. This study evaluated lifetime carcinogenic risk (LTCR) using Eq (3).

$$LTCR = EDI \times CSF \quad (3)$$

CSF is the cancer slop factor for acrylamide that is 0.5 mg/kg of the day (Eslamizad et al., 2019; Nematollahi et al., 2020b). The parameters used to determine the health risk of acrylamide in industrial popcorn are shown in Table 1.

Table 1. Distributions of parameters for different age groups used in risk assessment model with Monte Carlo simulation.

Parameters	Unit	Description	Distribution	age groups (year)			Ref
				<18	> 18 <60	>60> 18 <60	> 18 <60> 18 <60>
EDI	mg/kg/day	daily intake through ingestion	-	Will be assessment with Eq. (1)			(Morales-Moo et al., 2020)
HQ	-	Target Hazard Quotient Index	-	Will be assessment with Eq. (2)			(Nematollahi et al., 2020)
C	mg/kg	Acrylamide Concentration	Log normal	3.45 ± 3.52			-
IR	g/day	ingestion rate	Log normal	28 ± 5	23 ± 41	5 ± 3	(Morales-Moo et al., 2020)
BW	Kilogram	Mean of Body weight	Log normal	52 ± 13	66 ± 12	67 ± 10	(Morales-Moo et al., 2020)
RfD	µg/kg/day	Oral reference dose	Fixed value	2			(Nematollahi et al., 2020)

3. Results and discussion

3.1. The concentration of Acrylamide in the different brands with different flavours

The concentration of acrylamide in several brands with varying tastes. The presence of acrylamide in a variety of popular Iranian popcorn products was determined in this investigation. Microwave popcorn manufactured in Iranian facilities consists of a microwave popping bag, kernel popcorn, fat, and, in most cases, salt, vinegar, cheese, ketchup. Popcorn was made by combining kernel popcorn, salt, and melted fat into a paste and dropping the paste into the selected chamber of the popcorn bag prior to the final closing. Recently, microwave popcorn products have grown in popularity, and as a result, their acrylamide concentration must be determined.

The results of identifying acrylamide concentrations in popcorn products are presented in Table 2. AA level in popcorn contains food additives is variable between 0.23±0.32 mg/kg to 8.9±1.9 mg/kg. There is no difference between Brand A, B, C, D, and E ($p>0.05$).

Table 2. Acrylamide concentrations in industrial popcorns with various flavorings agent in different brands (mg/kg).

Flavoring's agent	Brand A	Brand B	Brand C	Brand D	Brand E
Salty popcorn	1.8±1.4	1.5±0.04	-	4.7±6.4	8.9±1.9
Vinegar popcorn	-	1.8±0.13	0.96±0.62	-	5.6±7.9
Cheese popcorn	2.5±0.2	1.4±0.09	0.86±0.01	0.23±0.32	4.8±5.7
Ketchup popcorn	2.2±1.6	1.4±0.24	1. 04±0.36	5.5±6.7	8.1±1.8

The formation of AA was dependent on several parameters, including raw ingredients, extrusion circumstances, and the presence of CO_2 (Masatcioglu, Gokmen, Ng, & Koksel, 2014). The monosaccharides found in maize endosperms, such as glucose, fructose, galactose, ribose, and mannose, are the Maillard reactions' initial stage (Inglett, and, & Press.).

The addition, food additives affected AA levels in packaged popcorn. Some additives are considered safe or beneficial to consumers, especially popcorn with low AA levels. For example, in the recent study in Iran, AA concentration was evaluated in the snack samples, that AA levels were generated faster in potato-based samples than in corn-based samples. Thus, compared to the other two variables, the composition of potato-based samples had the most significant influence (Kamankesh et al., 2021). Numerous research has been conducted on the amount of AA found in various food products worldwide. Abramson et al. found that AA level in popcorn was 500 µg/kg (Svensson et al., 2003), and Das et al. detected 400 µg/kg AA levels in popcorn that meet data in this survey (Das & Srivastav, 2012). In the study by Sanchez-oter et al., in 2017, the highest amount of AA was 5.80 mg/kg for potato chips, so the AA concentration differs according to the type of food and cooking condition (Sanchez-oter et al., 2017). In other words, the level of sugars in the different foods, especially in potato, cereal, and coffee products, depended on various factors and parameters, including the type of food, compounds of these food, heating and cooking process, and condition of storage of this food. Also, adding some additives during cooking reduces AA formation (Pedreschi, Mariotti, & Granby, 2014).

Among 5 additives into popcorn samples, the mean concentration of AA according to different brands of popcorn shows in Figure 1, that highest concentration of AA was in the E> D> A> B> C. As indicated by the data, the quantity of AA in popcorn products is higher in Brand E (8.91.9 for salty tastes has the greatest concentration) than in others, which may be related to differences in manufacturing methods. Popcorn in Brand E is heated to 200–220 °C for 2 minutes, whereas samples A, B, C, and D are heated to 180–200 °C for less than 2 minutes. Compared to the other samples, Sample C with vinegar flavour contains low AA. However, extreme caution must be exercised when picking food additives with kernel popcorn. The influence of various food additives used in popcorn production on the development of AA is still being investigated. In the case of recipes, considerable cost savings can be gained by substituting the chemical raising agent ammonium bicarbonate for the comparable sodium salt in items such as gingerbread (Lineback, Coughlin, & Stadler, 2011). However, since the focus on AA was globally pointed out about health risks (around the year 2002), there has been a gradual reduction of AA in snacks (Al-Jawaldeh et al., 2021; Başaran, 2020; Soares, 2015).

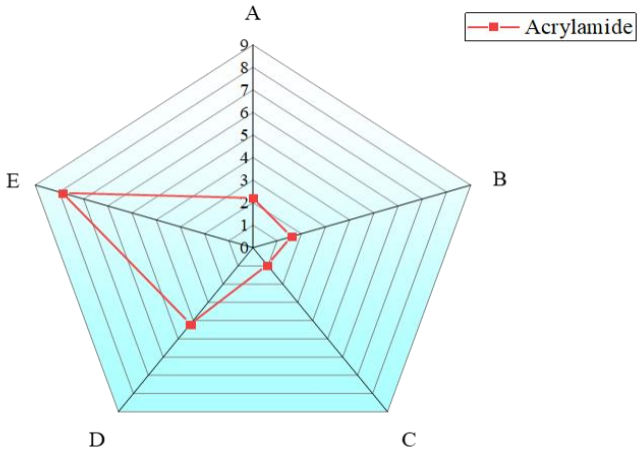


Figure 1. AA mean concentration in the popcorn according to type of brand

Because AA in food raises cancer risk for consumers of all ages, mitigating techniques are used to keep AA levels in food as low as possible. Mitigating AA in popcorn products without lowering dietary AA intake (Bušová et al., 2020; Maan et al., 2020). Mean concentration of AA according to different flavours of popcorn shows in Figure 2 that the highest concentration of AA was in the salty> ketchup> vinegar> cheese flavours.

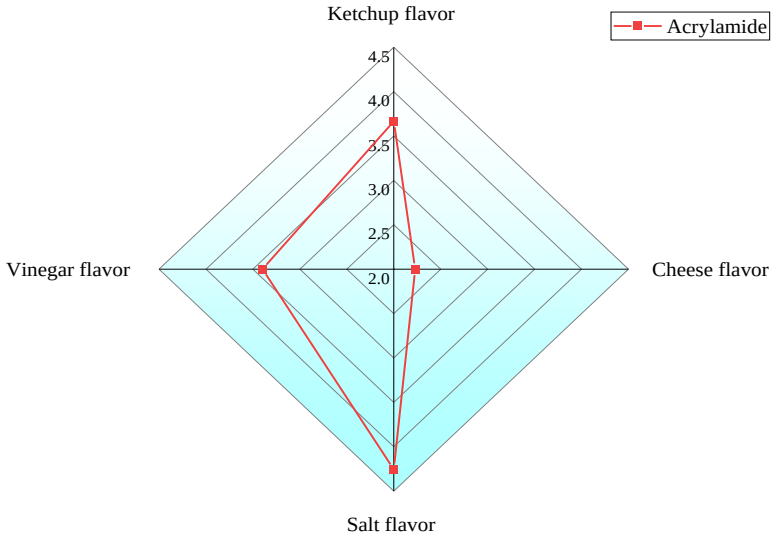


Figure 2. Mean concentration of AA in the popcorn according to different flavours.

A study by Eslamizad et al. (Eslamizad et al., 2019), shows the mean concentration of AA in the different bread with the different conditions of cooking. Their average AA concentration in the semi-industry trial sangak bread and traditional sangak in Shiraz were 48.5 and 43.4 ng/g, respectively. Also, the amount of AA according to the location and eating habits of people from different countries are different as well as age, sex, income, and exposure to passive smoking (Alpözen, Güven, Özdestan, & Üren, 2015; Alyousef, Wang, Al-Hajj, & Koko, 2016; Choi, Ko, Kang, Hwang, & Lee, 2019; Khan et al., 2018; Norouzi et al., 2018; Sadeghi et al., 2016).

Comparing this study, the type of food and amount of surges compound are the factors that affected the AA concentration rather than our study. Another study measured AA in different food. Their results show that the AA mean concentration in the corn-based extruded snack was about 257 ng/g, which was significantly lower than potato crips (1162 ng/g) and significantly higher than Roasted peanuts (21 ng/g). Also, AA concentration in the corn-based extruded snack was lower than AA levels in the salty flavour of popcorn in brand E (Esposito, Nardone, Fasano, Triassi, & Cirillo, 2017). The method of popcorn cooking is a critical component in reducing the production of AA. Thus, the existence of D-allose in microwaved popcorn was established. AA has not discovered infractions of popcorn treated for 2, 5, or 8 minutes in a microwave oven. The absence of asparagine, a critical component of AA synthesis, in the pericarp of maize kernels was the primary factor affecting the results obtained (Bocharova et al., 2017). AA concentration was measured in several foods such as corn breakfast cereal (498 µg/kg) and microwave popcorn (607 µg/kg) in the México

3.2. Health risk assessment

3.2.1. Non-carcinogenic risk

The rank order of EDI (mg/kg/day) for age groups were lower than 18 years (1.86E-3)> age group 18-60 years (1.2E-3) > age group higher than 60 years old (2.57E-4), respectively.

According to the results shown in Figure 3, for all of the samples in the all-age groups (age

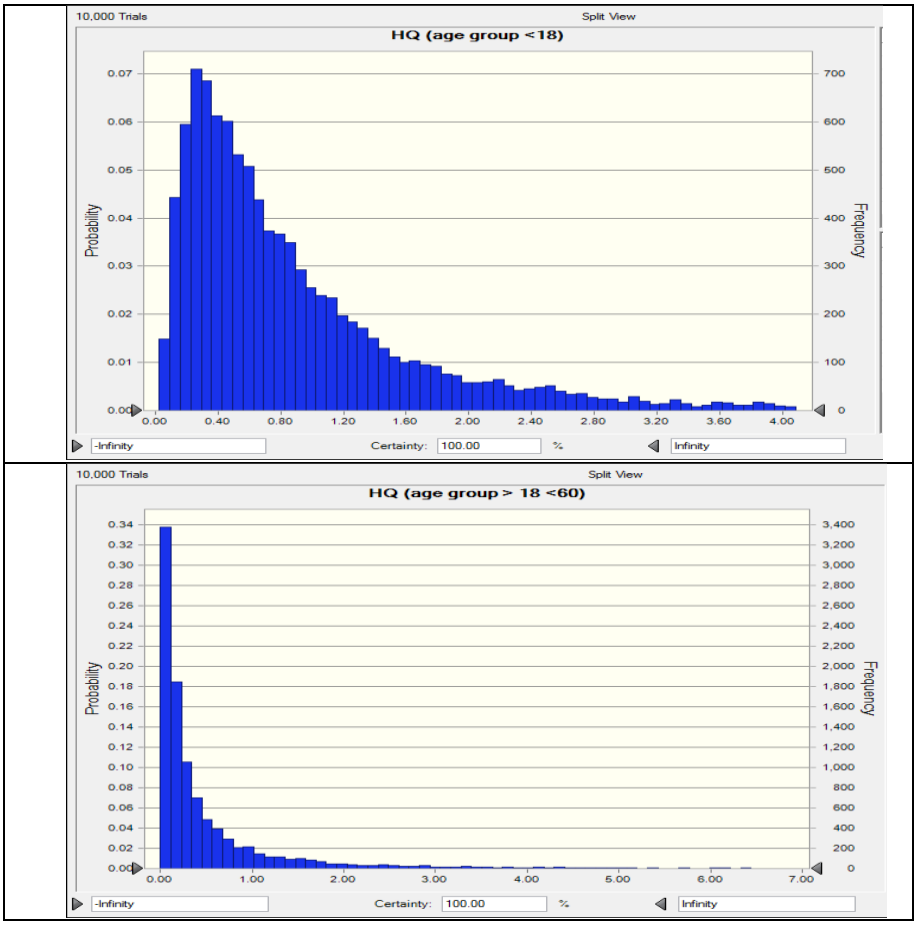
<18, age >18<60, and age > 60 years old), the mean value of HQ was lower than 1, which indicate there was no concern about non-carcinogenic risk due to acrylamide consumption through industrial popcorn with different flavors and brands.

Factors such as the condition of cooking, including traditional and industrial processes, are the foremost important parameters in the formation of AA. Also, the time of heating and temperature of cooking and type of food is vital to reduce the HQ due to AA intake (Eslamizad et al., 2019); similar to these studies for all age groups, the mean value of HQ was less than 1. It is notable that in this study, while the mean value of HQ was lower than 1, the results of MCS in Table 3 indicated that the percentile 90% HQ values for age groups <18 and 18- 60 years old was higher than 1, which indicated potential health risk for these population (Naeem et al., 2022; SHARIF & NAZIR, 2018).

The only noncarcinogenic and non-genotoxic effect of AA in humans is neurotoxicity (oral intake from food). However, in animal experiments, AA causes testicular injury and detrimental effects on fertility. In vivo, AA is genotoxic to somatic and germ cells and can cause heritable gene and chromosome damage (Park, 2021; B. Wang, Guerrette, Whittaker, & Ator, 2020; WHO, 2002)

Table 3. Hazard quotient values of AA exposure levels for different age groups.

	Age group	**50%**	**90%**	**Mean**
Probabilistic approach	< 18	0.65	2.10	0.98
	> 18 < 60	0.21	1.43	0.66
	> 60	0.08	0.30	0.13



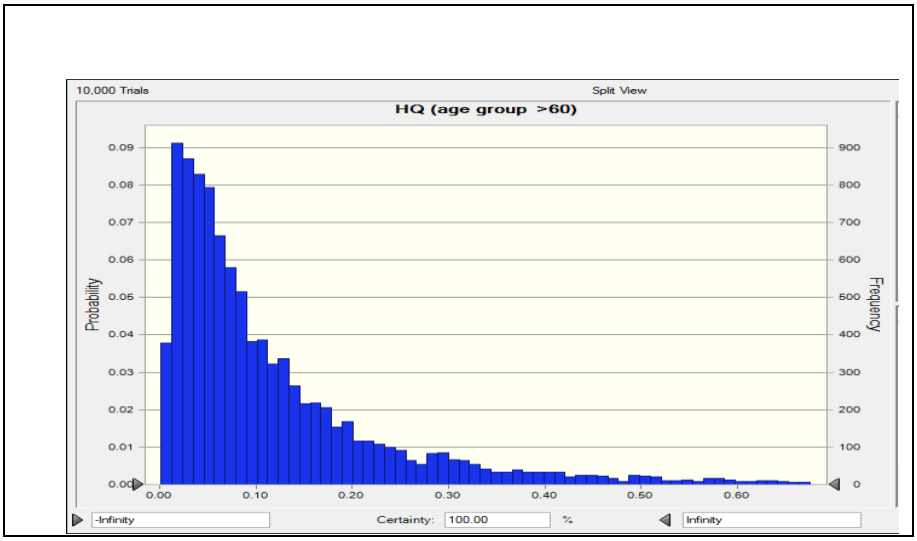
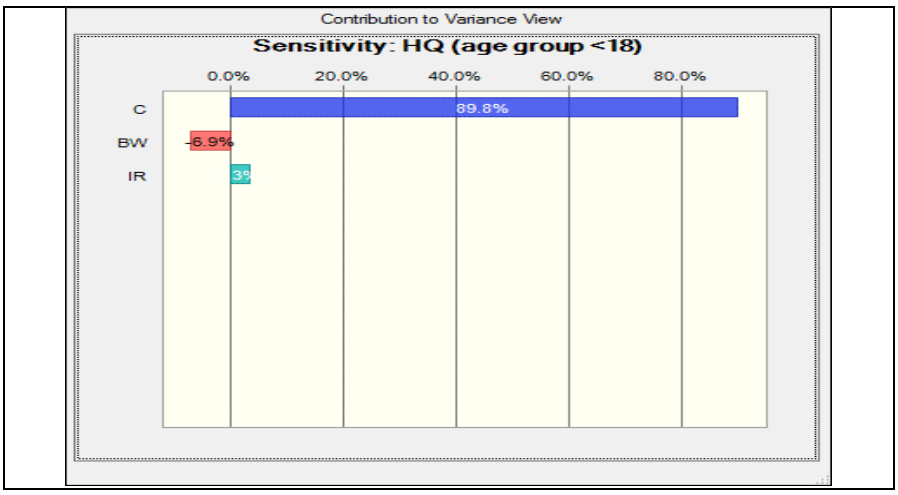


Figure 3. Hazard Quotient Index (HQ) of Acrylamide for three age groups (<18, > 18 <60, and >60 year) (The result of Crystal Ball, HQ defined as a Forecast and the assumptions were IR, BW, and C, calculated by 10000 trials number).

Figure 4 depicts a sensitivity analysis of noncarcinogenic risk for three age groups. Sensitivity analysis was used to discover the most influential risk factors for noncarcinogenic illnesses. The results for the age groups tested are as follows: among those aged 18 to 60 years, the most influential parameter is AA concentration in the examined samples. The volume of popcorn consumed was the most influential factor in the age range > 18 - 60 years old. However, the content of acrylamide played a significant influence as well.



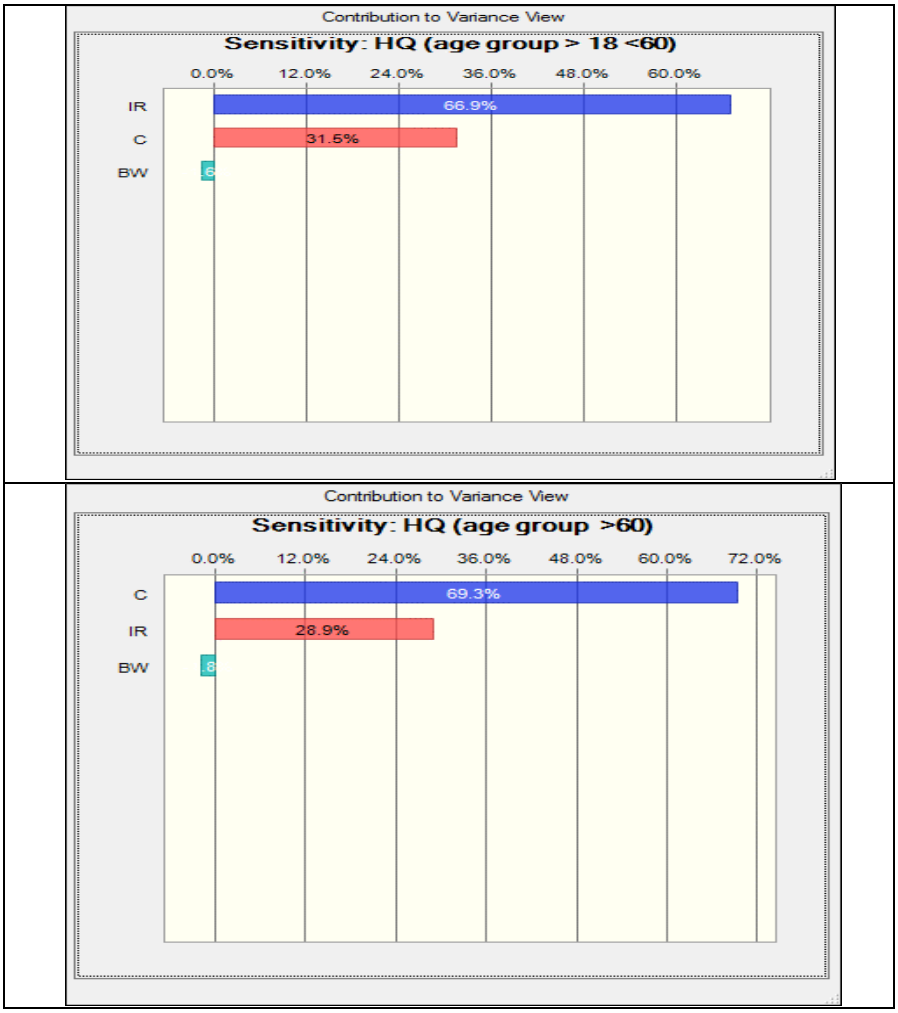


Figure 4. Sensitivity analysis of noncarcinogenic heath risk for three age groups (<18, > 18 <60, and >60 year) (The result of Crystal Ball software with 10000 trails, HQ defined as a Forecast and the assumptions were IR, BW and C).

3.2.2. Carcinogenic risk

According to the results shown in Figure 5, for all of the samples in the all-age groups (age <18, age >18<60, and age > 60 years old), there is the cancer risk that indicates there was concern about carcinogenic risk due to acrylamide consumption through industrial popcorn with different flavours and brands. In this study, the rank order of LTCR for age group<18, >18<60, and >60 years were 9.29E-4, 6.01E-4, and 1.29E-4, respectively. According to the USEPA (2009), where the LTCR was higher than 10-4, there is the potential concern about people's health and acceptable safe level that is between 10^{-4}-10^{-6} (Fathabad et al., 2020). So, according to the LTCR of popcorn consumption for all-age groups, AA concentration in this study has a positional risk for carcinogenic diseases. According to another study, AA has a carcinogenic risk for animals and human health, but another metabolite of AA, such as GA (glycinamide), is a hazardous and toxic effect rather than AA. So, type of food, cooking condition, time-heating, brands of food, and additives are the critical variables that affect AA concentration (Mollakhalili-Meybodi et al., 2021; Xu et al., 2014). Leilani et al. show that the AA level under cooking conditions and industrial or traditional methods and in the different nuggets (with chicken, meat, and shrimp) are different. Also, LTCR was safe for all samples (Seilani et al., 2021). In another study carried out by Eslamizad et al. on the

wheat flour of bread, similar to this study, all age group was at elevated carcinogenic risk (Eslamizad et al., 2019). While, in contrast to our study results, the result of a study in the Romanian population shows that there was no cancer health risk for consumers (Oroian, Amariei, & Gutt, 2015b).

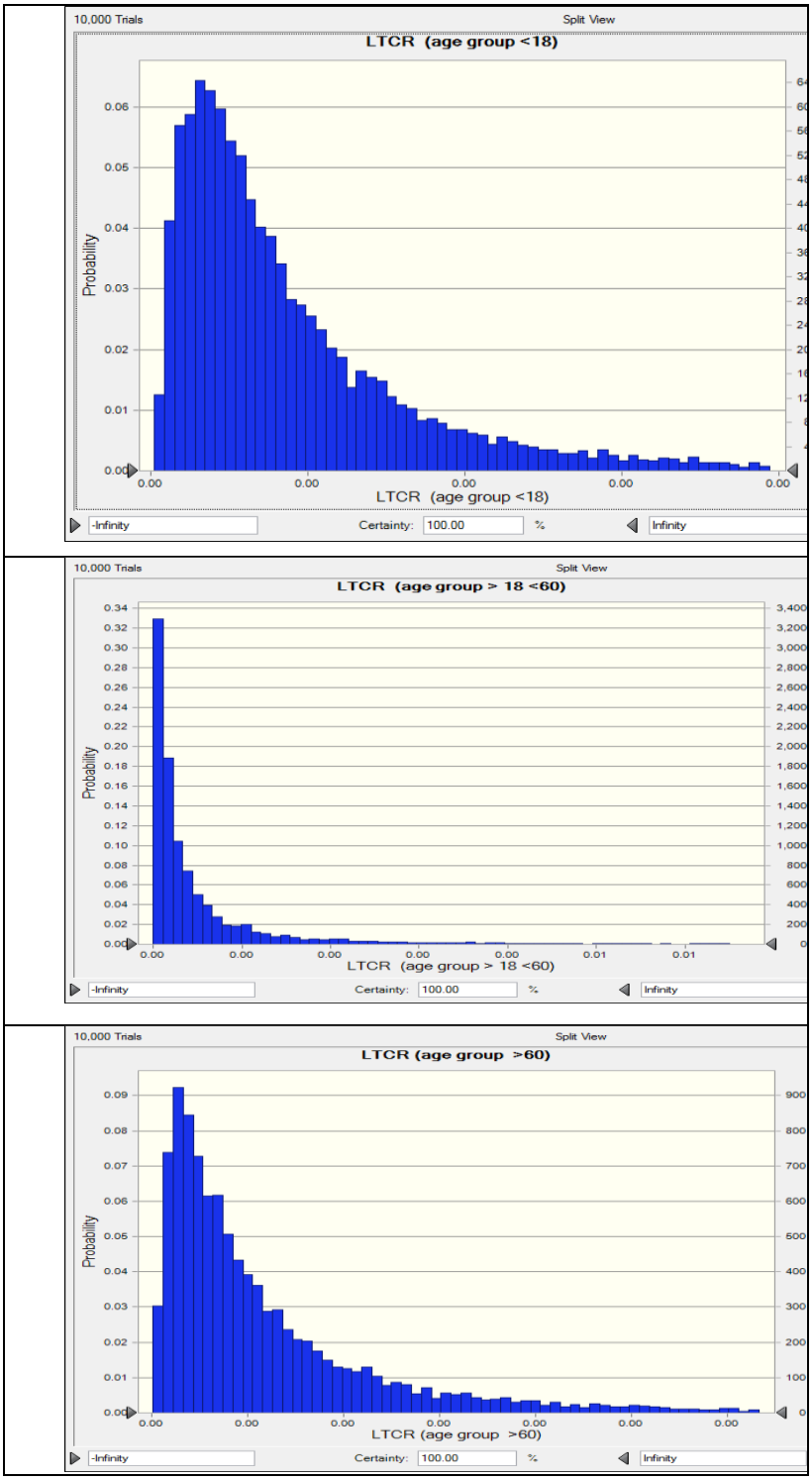


Figure 5. LTCR for different age groups that consumption the industrial popcorn.

Another study examined the dietary AA intake of Chileans for the first time and conducted risk evaluations using the margin of exposure (MOE) approach. MOEs for individuals aged 12 to 65 years were less than 10-4. According to the EFSA standards, there is a risk to public health. It is noteworthy that potatoes and bread provided around 77 percent of dietary acrylamide exposure in Chile, and children (12-17 years old) have the most significant dietary acrylamide consumption of all age groups (Barrios-Rodríguez et al., 2021), whose finding was consistent with the current study.

4. Conclusions

This study detected AA concentration in popcorn products in the different brands with 5 flavours kind. The AA concentration in popcorn was calculated using HPLC (UV-DAD). Results show that the AA concentration in some popcorn brands that are produced in the domestic factories is high, so it needs to control and monitor AA mitigation in such popcorn products, which are used extensively by Iranian people, especially children aged 6-12 years old, till not to impose a health risk to humans. Also, health risk assessment in the three age groups showed that in all samples, the mean HQ value for AA intake was lower than 1, but it is notable, while the mean value of HQ was lower than 1, for part of populations age groups ranged from less than 18 years old and age groups higher than 18 and less than 60 years old, the HQ values was higher than 1, which indicated probable health risk for these population. Also, LTCR results for all consumers indicated the potential cancer risk. Therefore, control and monitoring of the different popcorn materials (oil, additives, and heat (°C) and production processes are essential.

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