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CONTENT

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VALORIZATION OF CAROB SEEDS AS A FUNCTIONAL FOOD

Samira Lagha-Benamrouche[1,2]✉, Khaled Boudjema[1,3], Rezgui Walid[3,4], Djeziri Mourad[3,4] and Djamila Hezil[1]

[1]Department of Biology, Faculty of Sciences, M'Hamed Bougara University, Boumerdès 035000, Algeria
[2] Research Laboratory Soft Technology, Valorization, Physicochemistry of Biological Material and Biodiversity, Faculty of Sciences, UMBB University, Boumerdès 35000, Algeria
[3]Research Laboratory in Food Technology, Faculty of technology, University M'hamed Bougara of Boumerdès 35000, Algeria
[4] Center for scientific and technical research in Physico-chemical analysis (CRAPC) BP 384 Bouismail, Tipaza. Algeria.
✉*s.lagha@univ-boumerdes.dz*

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ABSTRACT

Our study aims to promote carob seeds as a functional food. For this; the nutritional value, the compounds with beneficial physiological effects and functional properties (fibers and gums), as well as the antioxidant potential were determined. Analysis of the chemical composition of carob seeds reveals their high protein, ash and fat content. However, the total sugars content was estimated to be moderate. The quantification of the compounds with a beneficial physiological effect shows that the seeds are rich in crude dietary fibers (8.39%). Regarding the gums, the yields are evaluated at 39.44% for the crude gums and at 4.026% for the purified gums. The phytochemical assays reveal a richness of the seeds in total polyphenols, in total flavonoids with a moderate content of flavonols and hydrolyzable tannins. The antioxidant potential was studied using two methods: reduction of the free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and the iron reduction method. According to the results, the carob seeds have a discreet antioxidant potential compared to the standards tested (gallic acid and quercetin).

1. Introduction

The carob tree (*Ceratonia siliqua* L.) is a fruit and forest tree, native to arid and semi-arid areas of the Mediterranean and the Arabian Peninsula. It is considered one of the best performing trees since all its parts (leaves, flowers, fruits, wood, bark and roots) are useful and have values in several areas (Benmahioul *et al.*, 2011).

The carob tree is used not only in animal feed but also in medicine and human food. Carob flour is used in the pharmaceutical industry, mainly against pulmonary tuberculosis, bronchial diseases and gastrointestinal disorders. The fibers contained in carob powder act as a natural laxative in case of constipation and tannins fight against diarrhea. These would retain the water present in the stool and act as a binder. Flour is also used above all in the food industry as an ingredient for the preparation of certain products, such as cakes, candies, ice creams, sauces and mayonnaise and as a cocoa substitute for the manufacture of chocolate (Serairi-Béji *et al.*, 2000).

Carob seeds are also a very good source of soluble and insoluble fiber, useful for regulating transit. They are rich in minerals (calcium, iron,

magnesium, phosphorus), vitamins (B3, A and E) and phenolic compounds. Another essential product is obtained from locust bean, which is gum, which is extracted from the albumen of the seeds. The latter is used in the food industry as a thickener known under the standardized code E 410, it replaces pectin and gelatin (Benmahioul *et al.*, 2011).

In Algeria, the carob tree remains much neglected and has not yet had the place, it deserves in reforestation programs, despite the socio-economic repercussions that this plant can have at the national and especially regional level and this despite the enthusiasm and interest shown in it for several decades by manufacturers. Few studies have been carried out so far on locust bean seeds, in particular on their nutritional and antioxidant values. The topics covered particularly concern the extraction and identification of gums, the analysis of the chemical composition, the determination of the energy value and the antimicrobial potential of the fruit pulp. Our interest in this product follows on from these observations and the present work consists in filling the lack of information on the nutritional, antioxidant and functional properties of carob beans. Our work aims to enhance the value of the carob tree through the valorization of its seeds.

2. Materials and methods

2.1. Collection and preparation of samples

The plant material, consisting of ripe carob tree pods, was collected in a forest in Bordj-Menail (region located in the Wilaya of Boumerdes, northern Algeria). The harvest was done randomly from several trees in August 2019. According to identification tests, this is the variety Belhessenat and Bouzegza widely responded to in the area. In the laboratory, we separated the pulps from the seeds. These were grinded and stored in dark glass bottles for subsequent analyzes.

2.2. Analysis methods

2.2.1. Determination of physicochemical parameters

Some physicochemical parameters were analyzed (moisture content and solids content (AOAC, 1995), pH and titratable acidity (AOAC, 2000)). The seed rate (SR %) is expressed as a percentage of mass, is given by the following formula:

$$SR\ \% = (m_2 / m_1)\ x100. \quad (1)$$

With: SR %: Seed rate in g per 100 g of whole fruit; m_1: the mass of whole fruits (in g) and m_2: the mass of seeds (in g).

2.2.2. Determination of biochemical composition

The total sugar content was determined according to the method of Dubois et al. (1956). In the presence of sulfuric acid and hot, carbohydrates are dehydrated to furfural compounds which combine easily with phenol and give a pinkish-salmon color. The reduction of Fehling's liquor by sugars makes it possible to determine the reducing sugars, the hydrolysis of the defecated solution in acidic and hot medium allowed us to determine the total sugars (reducing sugars + hydrolysable sugars) and to deduce indirectly the rate of non-reducing sugars (total sugars-reducing sugars) (Chidan-Kumar et al., 2014). The protein content is determined by the Kjeldhal method using the conversion factor of 6.25 (AOAC, 1995). The extraction of the fat was carried out by organic solvent (Hexane) with a Soxhlet type apparatus, according to the method of ISO 659 (1998). The ash content was determined by the destruction of any organic matter under the effect of the high temperature (550°C for 3 to 5 hours) to obtain a white or greyish-white powder (AOAC, 2002). The determination of mineral elements such as Na, Ca, Zn, Fe, Mn, Cd, Cu and Mg was carried out using atomic absorption spectrophotometry (AFNOR, 1986). The fiber content was determined according to method detailed by Henneberg and Stohmann (1860). It consists of treating the sample to be analyzed successively with sulfuric acid and potash. Acid / base

hydrolysis (hot) solubilizes almost all of the cellular content with the exception of dietary fiber and mineral salts. The extraction of the gums was carried out by acid decorticating followed by washing and soaking in water according to the protocol described by Dakia *et al.* (2007). Purification is carried out by precipitation in two solvents: ethanol and isopropanol.

The yield was determined by the following formula:

Yield of crude gum = (mass of crude gum / mass of seeds) X 100. (2)

Yield of purified gums = (mass of purified gum / mass of seeds) X 100 (3)

The extraction of phenolic compounds was performed according to Lagha-Benamrouche et Madani (2013). Total polyphenols are quantified according to Meyers *et al.* (2003), the condensed tannins were determined by the vanillin method described by Ba *et al.* (2010). The flavonoids were determined according to Bahorun *et al.* (1996) by direct dosing with aluminum chloride.

2.2.3. Determination of the overall energy value

The overall energy value is the energy released by the combustion of proteins, fats and carbohydrates contained in the diet, taking into account the digestibility of each of these macromolecules and their Atwater coefficients. The Atwater coefficients are defined as the metabolizable energy in kcal per 1g of nutrient. For carbohydrates and proteins, this coefficient is equal to 4 kcal, or 17 kJ and for lipids, it corresponds to 9 kcal or 38 kJ (AFNOR, 1987). The overall energy value is calculated from the relationship below.

$$E = (9 \times L) + (4 \times C) + (4 \times P) \quad (4)$$

With, E: global energy value in kcal, L: total lipid content in g per 100 g of sample, C: total carbohydrate content in g per 100 g of sample, P: total protein content in g per 100g of sample and 9, 4 and 4: the Atwater coefficients of lipids, carbohydrates and proteins.

2.2.4. Antioxidant activity

In this study the antioxidant activity was evaluated using both methods; the scavenging activity of the free radical 1, 1-diphenyl-1-2-picrylhydrazyl(DPPH) (Brand-Williams *et al.*, 1995) and the reducing power (Oyaizu, 1986). The presence of reducing agents in the extracts induces a reduction of ferric ions (Fe^{+3}) into ferrous ions (Fe^{+2}). This reduction is measured by the intensity of the resulting blue-green color. An increase in absorbance indicates high reducing power. In the presence of RLs scavengers, diphenyl picryl-hydrazyl (DPPH) having a violet color is reduced to a yellow compound, diphenyl picryl-hydrazine, the color intensity of which is inversely proportional to the capacity of the antioxidants present in the medium to donate protons

2.3. Statistical analysis

The statistical analysis of the results was carried out using the STATISTICA 5.5 software and the degree of significance is taken at the probability $p \leq 0.05$. We performed a one-way analysis of variance followed by a Tukey's test. All data represent the mean of the three tests ± standard deviation.

3.Results and discussions

3.1. Physicochemical parameters

The physicochemical characteristics of the analyzed carob seeds are illustrated in the Table 1.

Table 1. Physicochemical characteristics of carob seeds

pH	Acidity (g CAE/L)	Brix (%)	Moisture (%)	Seed rate (% of fruit)
6.453 ± 0.025	2.689 ± 0.665	19 ± 1	1.856 ± 0.016	8.79 ± 0.57

EAC : Equivalent Acide citrique

The pH of the seeds is estimated at 6.4. Our result is slightly higher than that found by Yousif and Alghzawi (2000). These report pH values of around 5.96 and 4.81 for unroasted and roasted carob flour, respectively. This difference in results can be explained by the nature of the part of the plant studied (pod or seed) and by the technological process applied (effect of the roasting process).

The titratable acidity tells us about the total acid concentration. From the results of Table 1, it can be seen that the acidity of the seeds is estimated at 2.69 g EAC/L. Our result is slightly higher than that found by Meziou-Chebouti *et al.* (2015) for carob pulp (2.1 g EAC/L).

The Brix generally tells us about the sugar content but the other components of soluble solids can however influence this rate if their proportion increases. The Brix rate of carob seeds is estimated at 19%. This rate is lower than those found by Gaouar (2011) (28.40% to 30.80%). The soluble solids content of carob seeds is much lower compared to the edible part (the pulp). Gaouar (2011) reports Brix levels in the order of 88.68% to 90.40% for the pulp against 28.40% to 30.80% for the seed.

The results illustrated in Table 1 show overall that the moisture of the seeds is estimated at 1.86%. The moisture contents of the seeds are compared to those of the pulp; it is observed that the seeds are less humid. Özcan *et al.* (2007), Youssef et al. (2013) and Loullis *et al.* (2018) report contents of 6%, 5.3% and 6 to 11% for the carob pulp, respectively.

The seed rate is estimated at 8.79% of the fruit. According to Yousif and Alghzawi (2000), the seeds represent 10 to 20% of the weight of the pod. This rate depends on the number of seeds contained in the seed which is estimated between 15 to 20 seeds (Sidina *et al.*, 2009).

3.2. Chemical composition

The chemical composition of the carob seeds studied is illustrated in the Table 2. From the obtained results, the protein content is estimated at 30.04%. Our result is more superior to those reported by Meziou-Chebouti *et al.* (2015) and Loullis *et al*. (2018) for carob pulp. These report protein contents in the range of 7% and 2 to 7.6%, respectively. From these results, it can be seen that the carob seeds are richer in protein than the pulp. The unequal proportion in pulp and seed depends on the biological activity of the two parts of the plant (Linden and Lorient, 1994).

Table 2. Chemical composition of carob seeds

Compounds	Contents
Proteins (g BSA E/100 g)	30.04±0.03
Total sugars (g G E /100g)	27.36±0.01
Reducing sugars (g/100 g)	06.36±0.03
Non-reducing sugars (g/100 g)	15.09±0.06
Fat (g/100 g)	9.74±0.06
Ash (g/100 g)	5.66±0.08
Total fibers (%)	8.39 ±0.77
Yield of crude gums (%)	39.44±0.11
Yield of purified gums (%)	4.03±0.00
Global energy value (Kcal / 100g)	317.21

BSA E: Bovine Serum Albumine Equivalent. GE: Glucose Equivalent.

Carob is a fruit rich in simple sugars which gives it its very sweet flavor and high energy value and which makes it a feed for cattle. According to the results of Table 2, the level of total sugars in carob seeds is estimated at 27.36% with 6.36% for reducing sugars and 15.09% for non-reducing sugars. By comparing our result with that found by Meziou-Chebouti

et al. (2015), we see that the carob pulp is richer in sugar compared to the seed (27.36% against 50.9%).

From the results of Table 2, it can be seen that the fat content of the seeds is estimated at 9.73%. This value is higher than those reported by Özcan *et al.* (2007) and Loullis *et al.* (2018) (0.4 to 1.3% and 0.2%, respectively). Multiple parameters influence the fat content such as particle size, humidity, the nature of the solvent and the extraction method used (Gaouar, 2011).

The ash content is estimated at 5.66%. This content is higher than that found by Bezzala (2005) (4%). This difference found can be explained by the cultivar, the nature of the soil, climatic and irrigation conditions and the edaphic characteristics of soils (Bezzala, 2005). The ash content of the seeds exceeds that of the pulp. El Batal *et al.* (2016) and Bezzala (2005) report contents between 2.4 to 3.9% and 2.1 to 2.4% for carob pulp. These findings were also confirmed by Gaouar (2011). The latter found that the mineral content of the seed is greater than that of the pulp (4% against 2.67%). The unequal proportion in pulp and seed depends on the biological activity of the two parts of the plant (Linden and Lorient, 1994).

Atomic absorption spectroscopy has allowed the determination of some minerals such as Ca, Zn, Na, Mg, Fe, Cd, Cu and Mn (Table 3).

Table 3. Mineral composition of the carob seeds studied.

Mineral	Contents (mg/g)
Manganese	0.055±0.003
Iron	0.089±0.003
Cadmium	Nd
Calcium	10.411±0.253
Copper	Nd
Magnesium	3.114±0.015
Sodium	2.215±0.024
Zinc	0.071 ± 0.0006

Nd: Not determined

From the results in the Table 3, the various minerals found in the carob seeds and which are in the dominant quantity are calcium at 10.41 mg/g, followed by Magnesium at 3.11 mg/g and sodium at 2.215 mg/g. Zinc, iron and manganese are found in trace form. By comparing our results to those found by Hafize *et al.* (2020), we see that our sample is richer in calcium (10.41 mg/g against 8.3 mg/g) and in magnesium (3.114 mg/g against 0.89 mg/g) but poor in zinc (0.071 mg/g against 0.12 mg/g) and manganese (0.055 mg/g against 0.19 mg/g). In comparison with the data obtained by for the pulp, it can be seen that the latter is richer in minerals than the seed. According to Gubbuk *et al.* (2010), Afoakwa *et al.* (2013), Khlifa *et al.* (2013) and Torres-Moreno *et al.* (2015), the mineral composition of the pulp is as follows: Ca (285.4 - 480 mg/g), Mg (54-170 mg/g), Zn (0.4-2.7 mg/g), Fe (1.8-5.1 mg/g) and Mn (0.2-2.7 mg/g).

Based on our results (Table 2), it is observed that the crude fiber content of the analyzed carob seeds is 8.39%. Our result exceeds twice that of Gaouar (2011). The latter reports a rate of 4% for carob seeds of Algerian origin. By comparing the raw fiber contents recorded for the analyzed samples with the bibliographic data, it can be seen that the seeds provide as much fiber as the pulp (8.01% according to Albanell *et al.* (1991) for varieties from Spain, 10.99% according to Yousif and Alghzawi (2000) for varieties from Jordan, 10 % according to Gaouar (2011) and 11% for Moroccan varieties according to Salih and Jilal (2020)).

The yields of crude and purified gums are estimated at 39.44% and 4.026%, respectively. The percentage of purification is estimated at 10.29%. According to Lopez Da Silva *et al.* (1990), locust bean gum constitutes one third of the total weight of the seed. It is mainly

composed of galactomannans (around 93%), protein (about 4-5%), lipids (1%) and minerals (1%). Purification removes cellulose, lignin and lipids, as well as considerably decrease the amounts of minerals and proteins. We compare our results to those found by Lopez da Silva *et al*. (1990) and Dakia *et al.* (2008) we find that our raw gum yield far exceeds that of Lopez Da Silva *et al.* (1990) (20%) and it is included in the range given by Dakia *et al.* (2008) (37% -61%). The yield has a direct relationship with the composition of the endosperm and the method used for extraction. The results of the Table 2, show that the overall energy value of carob seeds is estimated at 317,212 kcal/100g. This value is lower than that found by Kamal *et al.* (2013) (346.95 kcal/100g) for carob seed powder. This difference in results can be explained by the variability of the nutrient quantification methods, the degree of ripening of the fruit, the sweetness of the fruit and humidity of the sample, etc. (Dakia *et al*. 2007).

3.3. Antioxidant activity

The results of Table 4 show that the total polyphenol contents of the carob seeds analyzed are estimated at 283.68 mg GAE/g. The results also show that the content of flavonoids is four to five times higher than that of hydrolyzable tannins (12.65 mg QE/g against 2.79 mg TAE/g) and that the class of flavonols represents a quarter of total flavonoids.

Table 4. Levels of phenolic compounds in carob seeds

Total phenol (mg GAE/g)	Flavonoids (mg QE /g)	Flavonols (mg QE /g)	Hydrolyzable tannins (mg TAE/g)
283.68±6.89	12.65±1.01	3.23±0.10	2.79±0.45

EAG: Gallique acid Equivalent, QE: Qercetin Equivalent, TAE: Tannic acid Equivalent.

The results of the phytochemical assays obtained for our sample of carob seeds are far superior to those found by Mekhoukhe *et al.* (2018) and Ben Ayache *et al.* (2020). The latter report much lower contents of total polyphenols and flavonoids (12.24 mg/g, 1.33 mg/g, 9 mg/g and 1.76 mg/g, 0.30 mg/g, 6 mg/g, against 283.68 mg/g and 12.65 mg/g, respectively). While a minimal difference in results is seen with Mekhoukhe *et al*. (2018) for the flavonol contents in favor of our sample (3.23 mg/g and 2.97 mg/g, respectively). Our recorded result for hydrolyzable tannins far exceeds those of Avallone *et al.* (1997) and Gaouar (2011). The latter report contents of 0.95 mg/g and 0.04mg/g, respectively. However, the content of hydrolyzable tannins obtained for our analyzed sample (2.79 mg/g) was found to be low compared to that reported by Ben Ayache *et al.* (2020) (6 mg/g) and intermediate between those recorded for total and condensed tannins (4.29 mg/g and 0.53 mg/g, respectively) (Mekhoukhe *et al.,* 2018). This difference in results can be explained according to Lee *et al.* (2003), by several factors such as: the climate, the environment (the geographical area, drought, the nature of the soil, aggressions and diseases, .etc.), the genetic factor, the cultivar, the harvest period, the stage of development as well as conservation methods, extraction and quantification of these substances. According to Ydjedd *et al.* (2017), the solubility of phenolic compounds depends on their degree of polymerization, the solvent used and their interaction with other components of the cell matrix as well as the formation of insoluble complexes.

The antioxidant potential of the seeds was estimated using two methods: the potassium ferricyanide reduction method and the scavenging of the stable radical DPPH (Table 5).

Table 5. Reducing power and antioxidant activity against the DPPH radical of the aqueous organic extract of carob seeds and standards (quercetin and gallic acid)

Reducing power (Absorbances at 700nm)		Scavenger activity against the DPPH radical (%)	
Carob seeds (1 mg/mL)	0.12 ± 0.02^{c}	Carob seeds(1 mg/mL)	38.69 ± 2.66^{c}
Gallic acid (20 µg/mL)	0.29 ± 0.07^{b}	Gallic acid (40 µg/mL)	92.00 ± 0.55^{a}
Quercetin (20 µg/mL)	0.45 ± 0.17^{a}	Quercetin (40 µg/mL)	64.00 ± 0.27^{b}

DPPH: 1.1-diphenyl 1-2-picrylhydrazyl. The carrying values of the different letters for each analyzed parameter present significant differences (P≤0.05). The results are listed in descending order: a> b> c.

Analysis of the reducing power of the aqueous organic extract of carob seeds at a concentration of 1 mg/mL leads to an absorbance of 0.120 (Table 5). This absorbance is significantly low ($p\leq0.05$) compared to those of gallic acid and quercetin tested at 20 µg/mL. As can be seen from the results, it is quercetin which has significantly ($p\leq0.05$) the highest absorbance and therefore the most pronounced reducing power followed by gallic acid.

As indicated in Table 5, the anti-free radical activity for our studied extract (at a concentration of 1mg/mL) is estimated at 38.69%. The latter is significantly very lower ($p\leq0.05$) than those of the standards tested (gallic acid (92%) and quercetin (64%)).

4. Conclusions

Our study aims to promote carob seeds as a functional food source by determining their nutritional and energy value, extraction and purification of gums, determining the level of crude dietary fibers and studying their antioxidant potential.

Physicochemical analyzes show that the carob seeds have an estimated pH of 6.453 with a total acidity of 2.689 g EAC/L. An average moisture content of between 1.872% and 1.840% with a soluble solids content of around 19 ° Bx and an estimated seed rate of 8.79% of the fruit. Analysis of the chemical composition of the seeds indicates their high protein content (30.04 g BSA E/100 g) and fat content (7.74 g/100 g). However, the total sugars content was estimated to be moderate (27.36 g GE/100 g). Carob seeds are also found to be rich in minerals. The mineralogical analysis carried out on the ashes of the seed reveals a predominance of calcium with a content of 10.41 mg/g, followed by magnesium (3.11 mg/g) and sodium (2.215 mg/g). Manganese (0.055 mg/g), zinc (0.071 mg/g) as well as iron (0.089 mg/g) are found in trace form. According to the data, the carob seeds are very energetic; its overall energy value has been estimated at 317.21 kcal/100g. The gum yields are evaluated at 39.44% for the crude gums and at 4.026% for the purified gums. The percentage of purification is estimated at 10.29%. The results of the crude fiber assay show that the seeds are rich in these compounds with beneficial physiological effects. The crude fiber content was evaluated at 8.39%. The phytochemical assays reveal that the carob seeds analyzed are rich in total polyphenols (283.68 mg GAE/g), in total flavonoids (12.65 mg QE/g) with a moderate content of flavonols (3.23 mg QE/g) and hydrolyzable tannins (2.79 mg TAE/g).

The antioxidant potential was studied using two methods: reduction of the free radical1.1-diphenyl-2-picryl-hydrazyl (DPPH) and the iron reduction method. According to the results, the carob seeds have a discreet antioxidant potential compared to the standards tested (gallic acid and quercetin).

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TECHNOLOGICAL FEATURES OF GOAT'S AND COW'S HARD CHEESE PRODUCTION USING BIOLOGICAL PROCESSING OF MILK

Svitlana Kolesnikova[1], Tsvitana Korol[1]✉, Yaroslava Zhukova[1], Alla Bovkun[1], Serhiy Petryshchenko[2], Oksana Vialets[1]

[1]Institute of Post-Diploma Training of the National University of Food Technologies, Kyiv, Ukraine
[2]The Institute of Food Resources, Kyiv, Ukraine
✉tsvetana.korol@ukr.net

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ABSTRACT

The article presents a study of biological processing of milk of cows, goats and the mixture of cow's and goat's milk (50:50) by applying the cultures of *L. acidophilus* during the production of cheeses with a low temperature of the second heating. This approach accelerates the technological process by 3-4 times and guarantees a long-term storage of products without deteriorating their quality.

The reduction of the time of rennet curd formation, whey removal, kneading, hot self-pressing, salting and ripening were specific features of the production process.

The study showed the dynamics of fermenting microbial flora's quantity as well as the content of benzoic and sorbic acids, which guarantee the long-term storage of cheeses.

We have studied the influence of our technology on physicochemical and rheological parameters of cheeses in relation to the type of milk.

1. Introduction

The demand for wider selection of cheeses is growing, thereby giving rise to measures aimed at changing and stabilizing biochemical and technological parameters of raw milk; triggering the development of special fermenting agents, various physicochemical and biological methods of raw material processing, and so on.

In order to improve the technological and microbiological properties of raw milk, additional measures are used: bacteriological purification by cream settling and bacterial separation, introduction of nitrates, lysozyme, inhibitory yeasts, nisin, exogenous enzymes and peroxide-catalase treatment.

The quality of milk is important because there is a risk of contamination by spontaneous microbial flora during storage of raw chilled milk (Richard & Auclair, 1989). The use of low-quality raw milk in cheese-making is possible only if, in addition to traditional pasteurization, supplemental methods of neutralization of foreign microflora are introduced, particularly the additional heat treatment of milk and its ripening. These methods help to effectively destroy foreign microflora of raw materials without adversely affecting the technological properties (Shulga, 2003).

Ripening of milk reduces the duration of technological stages, as it intensifies biochemical processes, which increase the content of total, soluble and non-protein nitrogen. One of the promising areas of milk ripening could be a method of introducing microorganisms, enzymes lactase (β-galactosidase) or protease. The combinations of drugs including *Lactococcus lactis* ssp. *lactis, L. lactis* ssp. *cremoris, L. lactis* subsp. *lactis*

biovar. *diacetylactis* are used during the ripening of milk (Richard & Auclair, 1989; Davidson et al., 2015). Samples with the strains of *Lactobacillus plantarum*, *L. lactis* ssp. *lactis*, which have an antagonistic effect on pathogens of butyric fermentation in cheese are also applied as preventive measures during the production of cheeses with a high temperature of the second heating (Kuznetsov & Shiler, 2003; Hassan et al., 2021).

To increase the term of storage flavoring and preserving agents of chemical origin are being used more and more in food industry, particularly in cheese production (Beltyukova & Liventsova, 2013).

At the same time healthy diet is a world tendency which encourages refusing chemical treatment of milk replacing it with biological treatment during cheese-making (Beltyukova & Liventsova, 2013; Davidson et al., 2015).

European Union regulates the usage of food additives with the Regulation No.1333/2008 from Dec 16, 2008, emphasizing that it is important to inform consumers about food additives in food.

In cheese-making it is preferably to exclude the chemical additives like nitrates (e.g. E251, E252). Potassium nitrate (E252) in cheeses could reduce to potassium nitrite (E249) with a participation of xanthinoxidase or nitrate reductase of milk (Munksgaard & Werner, 1987).

As a result the accumulated nitrites inhibit unwanted and dangerous microbiological processes, particularly a growth of *Clostridium* bacteria, and prevent a raw cheese detachment, but also may have a negative influence on the fermenting flora (Park et al., 2016; Hassan et al., 2021).

The preservatives in the form of potassium nitrate or sodium nitrate with a standard of up to 20 g/100 kg of milk are allowed in EU countries, except France, Greece, Italy and Switzerland (Park et al., 2016).

In addition to nitrates, lysozyme is widely used in cheese-making as it prevents the development of butyric acid bacteria by breaking the bonds in the molecules of cell walls. This is a fundamental difference between the mechanisms of action of lysozyme and nitrates (Davidson et al., 2015).

There are acceptable prices for the other permitted preservatives for surface treatment of cheeses: sorbic acid (E200) and its salts (maximum level in the product is 1 mg/kg); benzoic acid and its salts; dehydroacetic acid, at the maximum level of 5 mg/kg. They could be of chemical rather than natural origin. These additives are effective in inhibiting the growth of yeast, mold, and cause an inhibitory effect on a wide range of bacteria (Tfouni & Toledo, 2002; Mroueh et al., 2008).

Benzoic acid is widely used in the food industry due to its low cost, colorlessness, lack of taste and relatively low toxicity (Beltyukova & Liventsova, 2013; Del Olmo et al., 2017; Bartáková et al., 2021). However, it is not allowed as an additive to dairy products in most countries, including Ukraine (Order of the Ministry of Health No. 222 of 23.07.96 On Approval of "Sanitary Rules and Norms for the Use of Food Additives").

A number of scientists (Kurisaki et al., 1973; Sieber et al., 1995; Tfouni & Toledo, 2002; Urbienė & Leskauskaitė, 2006; Esfandiari et al., 2013; Horníčková et al., 2014; Gucer et al., 2016; Han et al. 2016; Yerlikaya et al., 2021) investigated the formation of benzoic acid in the production of dairy products and cheese.

It is known, that benzoic acid is formed by various biochemical pathways: a) the formation of benzoic acid from hippuric acid in cheese production; b) through the hydrolysis of phenylalanine as an alternative pathway in cheeses (Sieber et al., 1995; Urbienė & Leskauskaitė, 2006; Horníčková et al., 2014); c) autooxidation of benzaldehyde produced by some strains of lactic acid bacteria and yeast (Mroueh et al., 2008; Gucer et al., 2016). Therefore, benzoic acid can be considered as one of the natural components of milk and dairy products.

The content of benzoic acid in fermented dairy products and cheeses depends on the applied lactic acid cultures, the origin of raw milk, duration and temperature of the fermentation process. It is known that strains of

Lactococcus lactis ssp. *lactis, L. lactis* ssp. *cremoris, L. lactis* ssp. *lactis* biovar. *diacetylactis, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Streptococcus thermophilus* produce benzoic acid in milk (Garmiene et al., 2010; Han et al. 2016; Bartáková et al., 2021).

The concentration of benzoic acid in dairy products can range from 2-5 mg/kg to 50 mg/kg (Urbienė & Leskauskaitė, 2006; Esfandiari et al., 2013), and in hard cheeses - from 1.6 to 90 mg/kg (Kurisaki et al., 1973; Horníčková et al., 2014).

Because numerous studies have shown the presence (Iammarino et al., 2011) of benzoic acid at the level of 20.5÷28.7 mg/kg in hard, semi-hard, soft, semi-soft, pickled and grated cheeses in which there were no chemical preservatives, it was proposed to introduce the maximum allowable level of benzoic acid - 40.0 mg/kg.

According to the data of Gucer et al. (2016), in Turkish cheeses (White Pickled, Kashar, Tulum), yogurts, ayran, butter, the content of benzoic acid varied in the amount of 2.3-160; 6.4–83; 0.6–12.8 and 0.0–7.3 mg/kg, respectively. All dairy products were made from the milk of cows, goats, sheep or mixtures thereof.

Milk usually contains a small amount of benzoates, but there are exceptions. There is evidence that this is due to contamination by foreign microflora, the use of veterinary drugs and feeds (soybean broth and especially flavorings of fruit pulp containing benzoic acid) (Sieber et al., 1995; Urbienė & Leskauskaitė, 2006).

The properties of sorbic acid are very similar to the properties of benzoic acid, but its content in dairy products is much lower. Sorbic acid (E200) and its salts - sorbates (E201-209) are also considered GRAS additives. In Ukraine, it is used in the production of condensed milk in the amount of 2000 mg/kg, and also during the manufacture of maturing cheeses and whizzed cheeses in the amount of 1000 mg/kg (Ministry of Health of Ukraine, 1996, No. 222). It has low toxicity because, it is rapidly metabolized by pathways similar to free fatty acids. There are data on the intolerance of sorbic acid in humans (Mroueh, et al., 2008; Park et al., 2016).

Dehydroacetic acid (E265) and its sodium salt (E266) are used for surface treatment of cheeses and cheese rind. These additives are effective against the development of mold, fungi and yeast. They can be added to animal feed (hay). E265 is active at pH values higher than benzoic acid. Although it is excreted in the urine, this process is very slow, therefore because of a constant consumption it is accumulated in the body. The daily consumption level should not exceed 3 mg/kg of body weight. In the EU (Official Journal of the European Union 354, 2008), the use of E265 and E266 is prohibited, in USA they are used for canning fruit (21CFR172.130, 2020), but in Ukraine they are allowed in food production (Ministry of Health of Ukraine, 1996, No. 222).

To avoid the use of chemicals in cheese manufacture, Ukraine has developed a method of biological treatment of milk with L. acidophilus of non-mucous race during the production of soft, semi-hard and hard cheeses (Fedin et al., 1985; Kolesnikova et al., 1991, Kolesnikova & Gening, 1994; Kolesnikova, 2000). At the same time, there is a trend of increasing the range of cheeses by using different raw materials: milk of cows, goats, buffaloes, sheep, deers, yaks and other animals.

The popularity of dairy products from goat's milk is due to their hypoallergenic properties, as well as due to the specific fatty acid and protein composition, which is good for people having gastric disorders, anemia, epilepsy, asthma, atherosclerosis and more.

Relevance: The development of technology for the production of cheeses capable of long-term storage, in which the content of chemical preservatives, dyes and other artificial substances is minimized or completely absent, is an urgent issue, especially taking into account the growing demand for this category of food.

Moreover, the population of goats and sheep in Ukraine has been increasing in recent years, which in turn highlights the need to create mini-enterprises and introduce technologies for the

production of various types of cheese from goat's and sheep's milk.

The purpose of our study were: to study the effect of biological treatment of milk using cultures of *L. acidophilus,* to show how it changes physicochemical and microbiological parameters of hard cheeses made from cow's milk, goat's milk and their mixture (50:50) during ripening and storage.

2. Materials and methods

The research was conducted in the Laboratory of the Institute of Post-Diploma Training of NUFT and the Department of Analytical Research and Food Quality of the Institute of Food Resources of the National Academy of Agrarian Sciences of Ukraine, Kyiv, Ukraine.

2.1. Materials

2.1.1. Samples

The objects of research were cheeses made from milk of cows and goats, obtained from the farm "Ostrivske" located in Bila Tserkva district of Kyiv region. The research was carried out during the indoor period (June – September) in 2017-2018.

The samples of milk were labeled as cow's milk - K-1, a mixture of milk from cows and goats - K-2, goat's milk - K-3.

During the production of cheeses, two starters' preparations produced by DDP IPR NAAS, Ukraine, were used: 1) dry lyophilized starter culture of *Lactobacillus acidophilus*; 2) the preparation "Iprovit-Active". The culture of *L. acidophilus* contained not less than $1.0 \cdot 10^9$ CFU/g (Colony-Forming Units per gram). The Iprovit-Active contained the cultures of *Lactococcus lactis ssp. lactic, Lactococcus lactis ssp. cremoris, L. lactis ssp. lactis biovar. diacetylactis, Lactobacillus casei* ($2.0 \cdot 10^{10}$ CFU/g).

Also, enzyme rennet (Semenko LLC, Ukraine) and calcium chloride were used in cheese production.

2.2. Methods

2.2.1. Preparation of starter cultures L. acidophilus and Iprovit-Active

According to the proposed technology, the experimental cheeses were produced using starter's preparations, which were applied in the activated state.

To activate 3 g of dry culture, *L. acidophilus* was added to 100 cm^3 of sterilized skim milk heated to a temperature of (38±1) °C.

The mixture was thermostated at (38±1) °C for (6.0±0.5) hours until the curd formation. The number of viable *L. acidophilus* cells in the starter culture was $5.0 \cdot 10^8$ CFU/cm^3.

Separately, up to 100 cm^3 of sterilized milk, and 5 g of dry preparation Iprovit-Active was added and kept at a temperature of (35 ± 1) °C for 9 hours until the curd formation. The number of viable Iprovit-Active cells in the activated state was equal to $(4.5\text{-}6.0) \cdot 10^9$ CFU/cm^3.

The cultures were then cooled to room temperature and stored at (5±1) ℃ before use for making hard cheese as described below.

2.2.2. Cheese making

Experimental cheeses (n = 5) were produced by the method of Kolesnikova, (2000). Cheeses were made from different types of milk. The samples were labeled as cheese No.1 (control) - from cow's milk; cheese No.2 - from a mixture of milk of cows and goats (1:1); cheese No.3 - from goat's milk.

The method involved pasteurization of milk (65°C for 30 minutes), cooling to coagulation temperature (32-34°C), introduction of a culture of *L. acidophilus* (1.5-2.5)%, calcium chloride, the preparation Iprovit-Active (1.5-2.5)% and rennet. After the formation of the rennet curd, the following steps were performed: cutting, setting the curd grain, draining the whey, the second heating at a temperature of 38-42°C, self-pressing at two temperatures: at room temperature and then heating the cheese head surface with hot water (65-75)° C, salting and ripening at (12±1) °C for 15 days (Figure 1). (Kolesnikova, 2000). The obtained finished cheese heads were coated with latex and stored for 2 months at a temperature of (5±1)°C.

2.2.3 Microbiological tests

Sampling and preparation of samples for microbiological studies were performed in accordance with the Ukrainian standard DSTU 7357:2013. The number of bacteria was determined by seeding dilutions in agar culture medium: lactic acid bacteria according to the standards GOST 10444.11-89 and DSTU 7999:2015; bacteria of the genus L. acidophilus on - MRS medium with 2% glucose or Rogoza (Rogosa et al., 1951), bacteria of the Escherichia coli group (coliforms) according to DSTU IDF 73A:2003; yeast and mold - DSTU 8447:2015; spore aerobic rod bacteria - DSTU 5093:2008.

Figure 1. The diagram of cheese production based on various raw milk in accordance with the experimental technology.

2.2.4. Physico-chemical analysis

The titratable acidity was determined according to the standard GOST 3624-92; active acidity - according to DSTU 8550:2015; mass fraction of fat according to DSTU ISO 1735:2005; mass fraction of dry matter - according to DSTU ISO 5534:2005; mass fraction of total protein and nitrogen-containing compounds by Kjeldahl method on the digester and distiller Fisher Bioblock Scientific according to DSTU ISO 8968-2:2005, DSTU ISO 8968-4:2005; DSTU ISO 17997-1/IDF 29-1:2009; DSTU 5038:2008.

The rheological index of shear force was performed on a SANS test machine series CMT2503 (Shenzhen SANS Testing Co. Ltd.).

2.2.5. Analysis of benzoic and sorbic acids

Sorbic and benzoic acids were detected using high-performance liquid chromatography - HPLC LC-20 (SHIMADZU Corp, Japan) with reversed phase and diode array detector.

The following work was performed to remove fat and proteins from the samples. A portion of the sample weighing 3 g was dispersed in 10 cm^3 of distilled water and then quantitatively transferred to a 100 cm^3 capacity measuring flask with two portions of 5 cm^3 of water. After that 25 cm^3 of 0.4% sodium hydroxide solution was added to the resulting solution. Then the resulting mixture was placed in an ultrasonic bath heated during 15 minutes at the temperatures of 70° C. Thereafter it was cooled to room temperature, and 0.5 normal sulfuric acid solution was added to reach pH (8.0±0.1). Then, to precipitate proteins and fats, 2 cm^3 of 10.6% potassium hexacyanoferrate (II) solution and 2 cm^3 of 21.6% zinc acetate solution were added to the reaction mixture. The flask was then shaken for 10 minutes and left alone for 15 minutes. After shaking, the flask was adjusted to the mark with methanol, stirred and allowed to stand for another 15 minutes. The supernatant was filtered through a membrane filter and used for the study.

Sample preparation was performed according to the standard ISO 9231:2008 (IDF139:2008). A Shim-Pack Velox C18 chromatographic column (5 μm, 4.6 × 150 mm) (SHIMADZU Corp, Japan) was used to separate the acids. The mobile phase consisted of 10 volume parts of methanol (A) and 90 parts of phosphate buffer pH 6.7 (B). Isocratic elution was performed at room temperature for 10 minutes at a flow rate of 0.8 cm^3/min Target components were detected at a wavelength of 227 nm and 250 nm. Sorbic and benzoic acids (Sigma-Aldrich, USA) were used as standards, eluting after 1, 2, 3 and 7 minutes, respectively.

Stock solutions of benzoic acids were prepared in distilled water (1000 mg/dm^3). Working standard solutions in the concentration range from 15 to 500 mg/dm^3 were obtained by diluting the starting material. The linearity of the procedure was determined by introducing a standard solution with a concentration from 3 to 500 mg/dm^3. The method of additives was used to determine the accuracy.

Similar procedures were performed for working standard solutions of sorbic acid with a concentration of from 0.02 mg/dm^3 to 2 mg/dm^3.

2.2.6. Statistical analysis

Analyzing the results of the main indicators of the three variants of experimental cheeses (n=5) was performed using Microsoft Excel 2010. Statistical analysis of the data was carried out by the methods of variation statistics; we used Statistica 6.0 software packages (StatSoft, Inc., 2001; www.statsoft.com).

3. Results and discussions

Cheeses made by traditional technologies have been in steady demand for decades, and in some cases even a century. Today there is a tendency to supplement the traditional range of cheeses with new varieties that are created taking into account the basic laws of technological processes of cheese making, which are fully consistent with modern conditions. In recent years, products from small cheese factories from farms and cooperatives have become popular.

The experimental technology of cheese-making presented in this article can be used at small cheese-making businesses as it provides hygienic reliability of finished products and the ability of cheeses to be stored.

3.1. Technological parameters of biological treatment of raw milk

The rennet coagulation ability of milk depends not only on the differences between the raw materials, but also on the processing of milk on the eve of the cheese-making process. The decisive effect of the acidity (pH) of milk on the rate of coagulation is still underestimated in the preparation of cheese at home and on mini-farms.

The whole milk obtained from the farm was pasteurized (Figure 1). As is known, the mode of pasteurization is chosen depending on the bacterial contamination of raw milk and the desired properties of the cheese curd.

The optimal mode of pasteurization of milk is heating to a temperature of 70-72 °C for 20-25 seconds. For the production of some types of cheese it is possible to use a higher temperature, it depends on the technology of production of a particular type of cheese. However, this type of pasteurization involves the appropriate equipment, that is advisable to apply for large volumes of products.

According to the experimental technology we applied a long-term pasteurization temperature (63-65 °C for 30 minutes) with constant stirring, which can be used on small farms. Then the milk mixture was quickly cooled to a temperature of (33-35) °C. The importance of following this regimen is to preserve the structure of milk protein and organoleptic properties of the finished product.

After adding a solution of calcium chloride at the rate of 20 g per 100 kg of milk to the milk mixture, activated acidophilic culture was added (Figure 1). Thus, the process of biological treatment of the milk mixture with constant stirring took place.

To determine the method and dose of *L. acidophilus* (1%, 2%, 3%) for biological treatment of milk with this culture, a number of previous model experiments were performed with different types of raw milk.

It was shown that when using 1% of the sample of *L. acidophilus* and holding for 30 minutes, there was a too slow decrease in acidity ($\Delta pN = 0.18 \div 0.22$), which resulted in longer subsequent period of rennet coagulation of milk mixture – up to 30-40 min.

When 2% acidophilic culture was added in milk with a processing time of up to 30 minutes, we observed an intensive increase in active acidity ($\Delta pH = 0{,}30 \div 0{,}42$), which allowed to obtain a rennet curd during 20 min. The produced cheese had a pleasant milk flavor.

When 3% acidophilic culture was added to milk with a processing time of up to 30 minutes, we observed an intense increase in active acidity ($\Delta pN = 0.58 \div 0.72$), which accelerated the rennet curd to 15 minutes. The result was a sour milk cheese with a sour flavour.

Therefore, the process of biological processing of milk should be carried out with 1.5-2% acidophilic culture for (30±2) minutes in the manufacture of this type of cheeses. The use of a culture of *L. acidophilus*, which does not form carbon dioxide during fermentation, and promotes the hydrolysis of a significant amount of lactose (70%) immediately after the grain is removed together with the whey, prevents swelling of the future product.

It is known that when using dry lyophilized cultures of direct application in cheese-making, it is necessary to increase the period of time from the moment of adding the starter to the moment of application of the coagulating enzyme (Kuznetsov & Shiler, 2003; Dağdemir et al., 2003; Shingareva et al., 2007).

According to the proposed technology, the experimental cheeses were produced using two fermenting cultures *L. acidophilus* and Iprovit-Active, which were applied in the activated state. The activation of fermenting cultures was carried out to avoid the prolonged lag phase of the cultures in milk raw materials.

The number of introduced cells of the fermenting microflora correlated with the data of active and titrated acidity. Figure 2 shows that the duration of the latent phase of cultures of Iprovit-Active and *L. acidophilus* was about 2 hours, after which the phase of active growth began. The obtained data coincide with Shingareva et al., 2007.

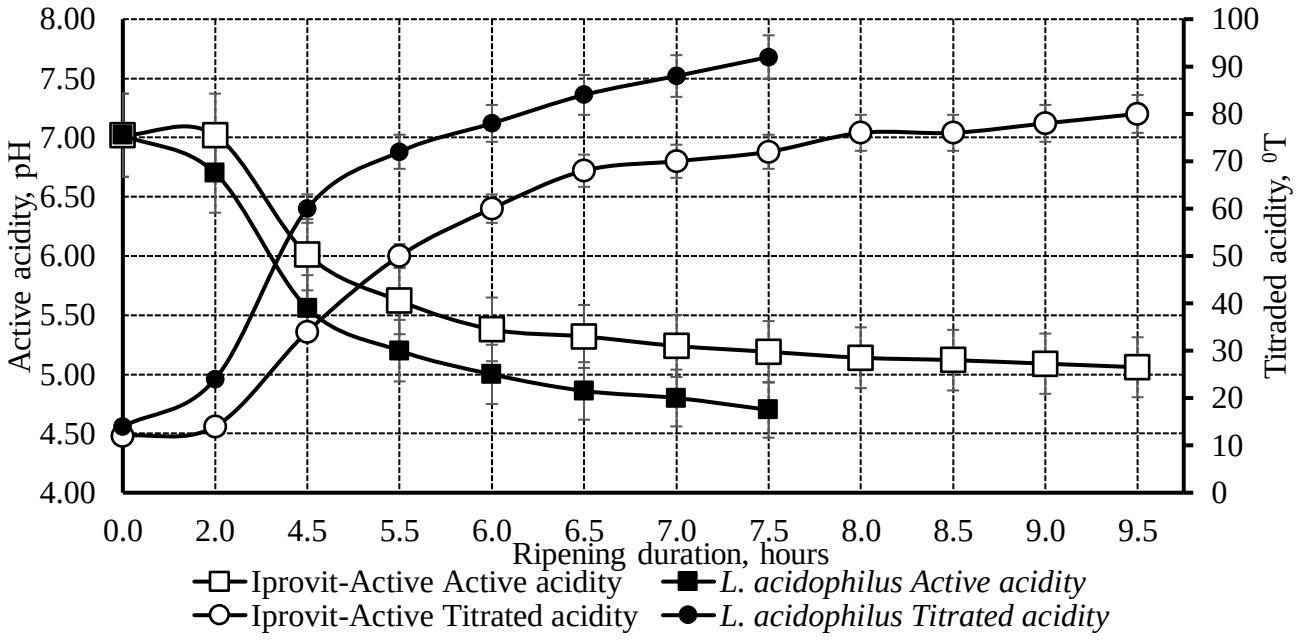

Figure 2. Active and titrated acidity during the activation of cultures.

3.2. Physico-chemical and biochemical parameters of hard cheeses during ripening and storage

After biological treatment of raw milk (the stage of rennet coagulation of the milk mixture) we applied the activated culture "Iprovit-Active" in the amount of (2.0 ± 0.5)% of the total amount of milk mixture and an aqueous solution of rennet enzyme in the amount of 1.9-2.0 g/100 kg (see Fig. 1).

The dependence of clotting time for milk coagulation and the formation of rennet curd on the applied raw material was established (Table 1).

Table 1. Technological data of curd grain and cheese mass characteristics

The name of data	Raw material		
	Cow's milk (K-1)	Mix of cow's milk and goat's milk (K-2)	Goat's milk (K-3)
Time of rennet curd formation, min.*)	23.25±1.95	20.11±1.50	18.82±1.63
Duration of curd grain stirring before the second heating, min.	7.5±2.5	7.5±2.5	7.5±2.5
Temperature of the second heating, °C	40±1	40±1	40±1
Active acidity of the cheese mass, units. pH**):			
- before self-pressing	6.24±0.03	6.20±0.02	6.17±0.02
- after self-pressing	5.85±0.02	5.80±0.02	5.73±0.03
Mass fraction of moisture in cheese mass, %**):			
- before self-pressing	57.1±0.5	58.3±0.4	60.1±0.6
- after self-pressing	47.8±0.2	48.5±0.20	49.0±0.3

*) Mean ± standard deviation (S_r) of five repetitions.

**) Mean ± standard deviation (S_R) of five repetitions.

It is known, that the ability and duration of milk to rennet coagulation largely depends on the composition of the casein complex, because it is known that there is a close relationship between the duration of coagulation and the content of β-casein, while the density of the gel

is more related to α_s-caseins. It has been shown, that the content of β-casein fraction in goat's milk can be 30% higher than the similar fraction in cow's milk, which is reflected in the duration of rennet coagulation, lower density and higher moisture retention capacity (Brule & Lenoir 1989; Beux et al., 2017).

However, under the research conditions, in samples based on goat's milk there was a tendency to reduce the duration of curd formation by 3-5 minutes (by 7-24 % comparing to the mixture of cow's milk and goat's or milk the cow's milk) ($P \leq 95.0\%$). This can be explained by pre-treatment of raw milk with *L. acidophilus*, as this culture developed more intensively in goat's milk, which allowed to increase the low titrated acidity of goat's milk 15-16°T to the characteristic of cow's milk 19-21°T.

The increase in the rate of curd formation at the time of decreasing pH of raw milk was observed to a certain extent (6.17-6.24) pH units (Table 1), because 6.2 pH units is optimal for the action of rennet enzyme. And a further decrease in acidity resulted in the acceleration of milk coagulation not due to the activation of rennet enzyme by hydrogen ions, but due to casein coagulation with acid (Beux et al., 2017).

So, the duration of coagulation of all milk mixtures with the use of both fermentation cultures ranged from 18.82±1.63 to 23.25±1.95 minutes, which differs from the technological regulations of cheeses produced by traditional technology for 15-30 minutes. The previous laboratory production of cheeses have shown, that a longer formation of rennet curd is impractical, because the lengthening of the process to 40 minutes was characterized by the formation of uneven grains and the curd began to crumble into fragments, which led to turbidity of the whey and an increase in protein in it, which reduced the yield of cheese.

As a result of organoleptic evaluation of the experimental samples after rennet coagulation of the milk base, the formation of curds with qualitative structural and mechanical parameters was recorded. In particular, the curds in all samples were dense, homogeneous, tender, they gave a split with sharp edges without the formation of protein flakes with a shiny surface, without the formation of cheese dust. And the taste of curds was pure fermented-milk. Goat's milk samples lacked the specific taste and odor of goat fat. The resulting curds were ready for further processing.

Particular attention was paid to the stage of curd grain formation. From the formed curds, which were cut by special cutting devices into uniform small cubes 2-4 mm in size, a curd grain was formed within 6±1 min. (Table 2).

Table 2. Technological data of separate operations during cheese manufacture

Technological operations*)	**Traditional technology**	**Experimental technology**
Rennet curd formation, min.	35±5	25±5
Grain formation, min.	17.5±2.5	6±1
Draining whey after the grain formation, min.	10±1	10±1
Grain stirring before second heating, min.	25±5	7.5±2.5
Draining whey removal before second heating, min.	10±1	Nil
Second heating at temperature 40±1°C, min.	25±5	10±1
Mixing after second heating, min.	30±10	10±1
Curd grain formation by stacking, min.	20±1	20±1
Self-pressing, min.	90±30	120±10
Pressing, min.	210±30	Nil
Duration of production until we get the pressed cheer of cheese, totally hours	7.78±1.47	3.48±0.25
Salting duration, hours	10.0±2.0	10.0±2.0
Ripening duration, days	55±5	17,5±2.5

*) Mean ± standard deviation (S_r).

The cutting was performed at a time when the curd was still highly mineralized and not very compacted to avoid the appearance of individual particles of the curd (cheese dust). Further syneresis proceeded for another 10±1 min. at rest, resulting in the formation of a rind on the curd grains (Figure 3).

Figure 3. Formed curd grain (size 2-4 mm) after rennet clotting of milk base.

After grain precipitation, the whey was removed in one step in the amount of (65±5) %. The whey released during the cutting of the curd was transparent, having a light green color and a sufficient level of acidity (increase in titrated acidity was 2 °T), turbidity of the whey was not visually observed.

According to traditional technology, the process of removing whey takes place in two stages: the first portion (30% by weight of the mixture) - after setting the grain, and the second (25-30) % - after mixing to the second heating (see Table 2). The reason to apply this approach is because of the slow rate of acidity due to the low dose of fermentation of mesophilic microorganisms (Kuznetsov & Shiler, 2003).

According to the experimental technology, the acidity quickly acquires maximum values, so the removal of whey is carried out in one step (Kolesnikova, 2000).

According to the experimental technology under the conditions of biological treatment of *L. acidophilus*, the curd grain with the rest of the whey was intensively mixed until the lumps disappeared, and then the second heating of the curd grain was carried out for (10±1) minutes applying hot water (70±5) °C in the amount of 15% of total weight of the milk mixture with increasing temperature to (40±1)°C in whey. Then partial salting in the grain was performed - a solution of sodium chloride (21±1) % (0.2% of the amount of the processing mixture) was added to the milk mixture and the curd grain was kneaded for 10 min and sent to perforated molds. Partial salting in grain shortens the salting process in brine.

The higher temperature regime provided uniform drying of curd grain in all samples. After self-pressing we obtained the required moisture content and the level of active acidity: 46.5÷49.2 % and 5.65÷5.78 pH units, respectively.

It was found that the level of active acidity could depend on the water content in the cheese mass: the cheese mass with a higher mass fraction of moisture had lower pH values (see Table 1). In particular, the acidity of the cheese mass in the sample made from goat's milk (K-3) was by (1.2-2.0) % lower, and the mass fraction of moisture was by (1.0-2.5) % higher compared to the samples from cow's milk (K-1) and the mixture (K-2).

According to this technology, there was one process of hot self-pressing, which accelerated the processes of self-pressing and pressing by 2.2-2.7 times comparing to the traditional technology (see Table 2). This process took place by heating the surface of the wheel of cheese with hot water (70±5) °C accompanied with rolling over the cheese perforated molds every 20 minutes for (120±10) minutes After that we cooled the wheel of cheese with cold water to room temperature and sent for salting.

Earlier in model laboratory experiments it was found that the duration of self-compression less than 100 minutes was insufficient, because the mass fraction of moisture in fresh and mature cheese can increase to 60% and over 45%, respectively, which is not typical for hard cheeses. And self-pressing over 130 minutes is impractical because (120±10) minutes is sufficient to close the surface of the wheel of cheese and achieve a mass fraction of moisture in the cheese after self-pressing and mature -

(48±2) % and (45±2) %, respectively, which corresponds to the technology of pressing hard cheeses.

The process of cheese ripening was carried out at a temperature of (12±1) °C and relative humidity of (80÷85) % for 15 days and storage at a temperature of (5±1) °C for 60 days.

It should be noted that there were no differences in the technological mode between different variants of experimental cheeses produced from different raw materials at the stages of self-pressing, salting and ripening.

Thus, biological processing of milk using *L. acidophilus* allowed reducing almost twice the duration of curd grain production, its formation in cheese wheels. Because *L. acidophilus* activated the fermenting lactic acid bacteria of the Iprovit-Active, the process of syneresis was active with the production of small grains from a delicate rennet curd. The grain settled quickly, which made it possible to remove (65±5) % of whey after the grain formation.

Starter culture of *L. acidophilus*, due to its probiotic properties is suitable for use in cheese production. Cultures of *L. acidophilus* produce natural antibiotics - acidophilus, lactocidin, acidolin and lactobacillin which are capable of inhibiting streptococci of serological groups A, B, C and H, and also staphylococci, *Proteus*, *Shigella*, *Salmonella*, *Mycobacteria* and moulds. The titer of antagonist activity toward *E. coli* i *Cl. butyricum* is greater than 1 (Ahmed et al., 2010, Chichik & Irkitova, 2013).

This approach is well established in the production of soft, semi-hard and hard cheeses in order to prevent their early and late swelling. In addition, in the manufacture of hard cheeses, this technology is effective, and particularly is cost-effective, as it accelerates by 2-3 times the entire process – from the curd grain formation to cheese ripening while maintaining the quality of cheese during long-term storage (Fedin et al, 1985; Kolesnikova et al, 1991, Kolesnikova & Gening, 1994; Kolesnikova, 2000).

During the ripening and storage of experimental cheeses, a change in their physicochemical parameters was recorded (Table 3).

Table 3. Physico-chemical and rheological parameters of experimental cheeses *)

Stages of the technological process	Cheese	Active acidity, pH units	Mass fraction of moisture, %	Mass fraction of fat in dry matter,%	Total nitrogen content,% of dry matter content	Cutting force, кН/м^2
Finished cheese (VII – cheese ripening 15 days)	No.1	5.50±0.03	45.50±0.17	48.57±0.15	7.21±0.20	27.34±1.05
	No.2	5.44±0.04	45.80±0.14	48.84±0.21	7.36±0.18	25.25±0.95
	No.3	5.39±0.03	46.20±0.15	49.00±0.20	7.43±0.16	23.41±1.20
30 days storage	No.1	5.37±0.03	42.80±0.20	48.90±0.18	7.17±0.18	36.50±0.85
	No.2	5.34±0.04	43.20±0.11	48.93±0.21	7.25±0.19	33.54±0.91
	No.3	5.22±0.04	43.90±0.13	49.01±0.17	7.46±0.20	31.42±0.96
60 days storage	No.1	5.30±0.02	42.10±0.18	49.14±0.20	7.14±0.18	49.15±0.70
	No.2	5.26±0.03	42.50±0.17	49.06±0.19	7.21±0.17	42.80±0.85
	No.3	5.16±0.02	43.00±0.17	49.20±0.18	7.44±0.20	40.63±0.83

Note*): the mass of the cheese wheels was 140±5 g.

**) Values are displayed as the mean ± standard deviation (S_R) of the five replications ($P < 0.05$).

Hard cheeses were characterized by a mass fraction of fat in the dry matter of not less than 40.0%, a mass fraction of moisture of not more than 47.0% according to the standard DSTU 6003:2008.

On the 15th day, organoleptic analysis of three types of cheese was performed. They had a pure fermented-milk taste and aroma with the features intrinsic to each type of cheese; dense, plastic consistency; appearance - the rind is thin,

dense, clean, had an imprint of perforation. In section the cheeses were characterized by a uniform pattern, consisting of holes (eyes) of regular shape with a size of 2 to 4 mm, the dough color was uniform throughout the mass: Cheese No.1 - bright yellow, Cheese No.2 - slightly yellow; Cheese No.3 - white.

According to the results of rheological studies obtained on a universal mechanical test machine "SANS" of SMT series, the density of the studied products significantly depended on the type of raw materials from which the cheese was produced (Table 3).

The process of cheese ripening takes place under the influence of living microorganisms, which gradually die, but the ripening process itself continues. It is caused by bacterial endoenzymes secreted from bacterial cells. This process can occur more intensively, in the case of increased moisture in the cheese mass (Blaya et al., 2018).

In the studied cheeses, the mass fraction of moisture decreased during ripening, and it resulted in the concentration of components.

The change in acid formation was observed after pressing in all cheeses in the range from 5.73 ÷ 5.85 pH units up to 5.50 ÷ 5.39 pH units after 15 days of ripening. The level of active acidity continued to decrease by 9.4-10.0 % in all cheeses until the 60th day compared with the cheeses after self-pressing.

The cheese No.3 made from goat's milk had a higher moisture content compared to other samples, so it resulted in a more intense change in the active acidity of the cheese mass compared to the rest of the samples (Table 3).

Finished cheeses lost moisture in the range of 6.9-7.5 % after two months of storage.

The cheeses had a good storage capacity, which was in line with the requirements for hard cheeses. The finished test products had a clean flat surface, pronounced, slightly acidic, sharp clean taste and smell, delicate, plastic, homogeneous consistency that corresponded to the highest quality products.

The main biochemical processes, due to which the specific characteristics of cheese are formed, are the reactions of splitting of protein and fat components of curd mass. As a result of the action of proteolytic enzyme systems of the microflora, caseins are broken down to form large peptide fragments, which in turn are hydrolyzed to low molecular weight peptides and free amino acids.

It is known that the process of formation of soluble nitrogen fractions and a pool of free amino acids depends on the proteolytic activity of bacteria that are part of the fermentation culture (McSweeney, 2004; Gudkov, 2004; Blaya et al., 2018).

One of the most important indicators in the study of cheeses is the amount of soluble nitrogen-containing compounds, as their level largely characterizes the degree of ripening and organoleptic properties of the finished product (Figure 4).

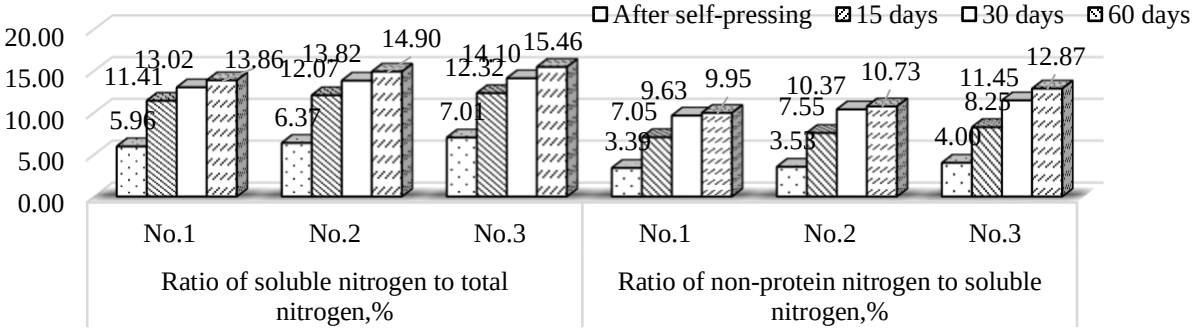

Figure 4. Dynamics of changes in the content of nitrogen-containing compounds during cheese ripening and storage *)

No.1 - cheese made from cow's milk; No.2 - cheese from a mixture of cow's milk and goat's milk (1: 1); No.3 - goat's milk cheese; 15 days - duration of cheese ripening; 30 days, 60 days - the duration of storage of cheese. Note*): the mass of the cheese wheels was 140±5 g.

Analysis of the dynamics of accumulation of nitrogen-containing compounds in cheeses made from milk of cows, goats and their mixture showed differences in the intensity of proteolytic processes already in fresh cheese and increased during storage. Soluble nitrogen-containing compounds accumulated most actively at the beginning of cheese ripening.

The ripening index of cheeses, which some authors (Zhukova et al., 2006; Andronoiu et al., 2015) prefer to calculate as the ratio of soluble nitrogen to total nitrogen, showed that for experimental cheeses No.1, No.2, No.3 on the 15th day of ripening it was 11.41%, 12.07%, 12.32%, respectively (Figure 4).

The highest percentage of soluble nitrogen after pressing was characteristic of cheese No.3 from goat's milk, and the lowest - for cheese No.1 from cow's milk. A similar pattern was observed in the composition of soluble non-protein nitrogen (Figure 4). The amount of non-protein nitrogen in the finished cheeses No.1-No.3 increased after pressing by 3.7-4.0 times, respectively.

The ratio of non-protein to soluble nitrogen fractions is considered to be interesting as well. This ratio for cheeses No. 1-3 was 15 days - 7.05; 7.55; 8.25, respectively.

This Figure indicates a high degree of cheese ripening. The highest concentration of this nitrogen fraction was in goat's milk cheese, and the lowest - from cow's milk.

Cheeses made from a mixture of raw milk had average values between cheese from the milk of cows and goats. The obtained indicators confirm that all experimental cheeses are quite mature on the 15th day, with a pure fermented-milk cheese taste.

During 2 months of storage, it was found that the accumulation of nitrogen-containing compounds varied depending on the raw material. The amount of soluble nitrogen increased by 1.27-1.33 times, non-protein nitrogen rose by 1.78-2.04 times. It should be noted that the ratio of non-protein to soluble nitrogen indicates a more significant accumulation of non-protein nitrogen in cheeses from goat's milk compared to cow's milk for the same period of time. In this case, the active accumulation occurred after self-compression until the 15th day, as well as from the 30th to the 60th day, which is associated with the activity of bacterial enzyme systems.

3.3. Microbial assessment

The content of fermenting and spontaneous microflora during the production and storage of cheeses was studied.

It was found that after the introduction of activated cultures of *L. acidophilus* and cultures of the culture Iprovit-Active, the number of lactic acid microflora was about 10^6 CFU/g in variants of raw milk obtained from milk of cows, goats and their mixture (50:50) (Figure 5).

The development of lactic acid bacteria was positively influenced by maintaining the temperature (33±1) °C during the coagulation of the milk mixture and before the second heating (Figure 1).

Analysis of the dynamics of fermentation microflora during cheese making showed that from the stage I to the stage III (introduction of fermentation starters into the milk mixture and self-pressing of cheese mass) more intensive development was observed in *L. acidophilus* cultures. While after the stage IV (cheese mass after salting), the fastest growth was noticed among Iprovit-Active lactic acid bacteria. The increase in the number of cultures of *L. acidophilus* between the stages I and II occurred 20.4-48.9 times, and the culture Iprovit-Active only 2.6-4.2 times. Thus, the intensive development of *L. acidophilus* cultures can be explained by the introduced stage of the second heating of curd grain to 40°C (see Figure 1).

At the stage III (Figure 5) in the cheese mass we observed an increase in the number of *L. acidophilus* cultures by 42.7-102.3 times and the microflora of the culture Iprovit-Active by 7.4-12.0 times. The results of microscopy of the samples showed that at this stage the number of lactobacillus *(L. acidophilus* and *L. casei*) in different fields of view was 15-20% more than the coccus microflora of the culture Iprovit-Active (*Lactococcus lactis ssp. lactic,*

Lactococcus lactis ssp. cremoris, L. lactis ssp. lactis biovar. diacetylactis).

Depending on the type of raw material used, the results of the dynamics of lactic acid microflora development showed significant variation.

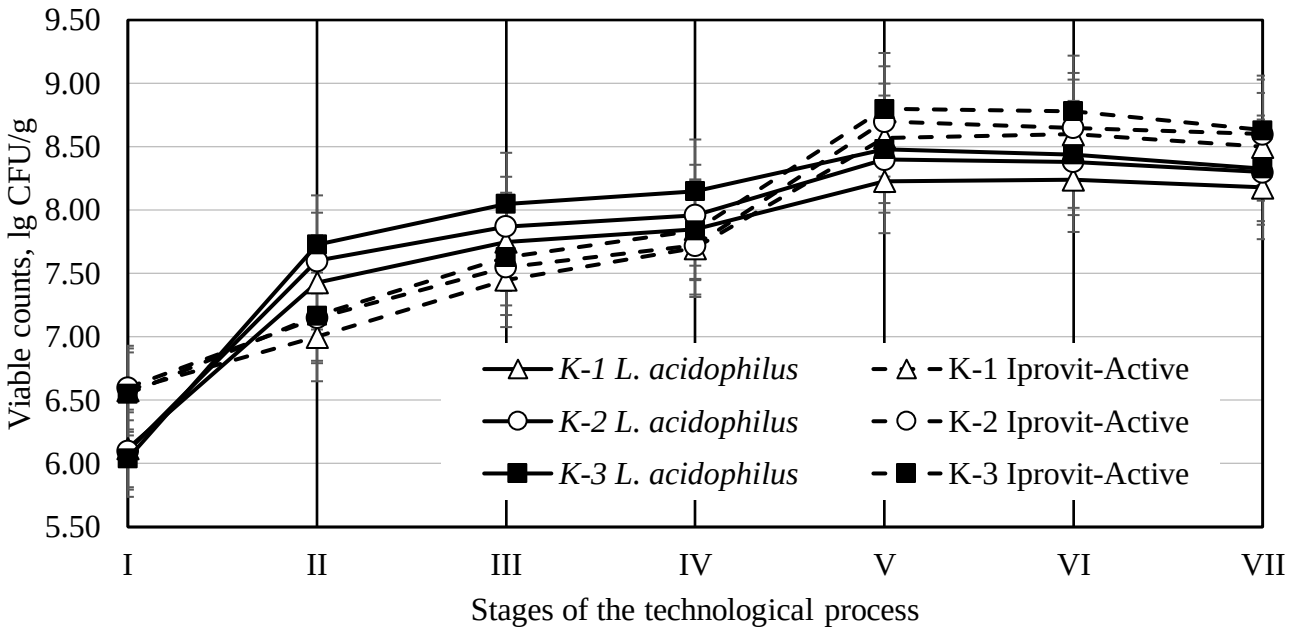

Figure 5. The change of lactic acid microflora during cheese production with the use of fermenting cultures

I - introduction cultures into the milk mixture; II - cheese mass after molding; III - cheese mass after self-pressing; IV - cheese mass after salting; V - 3 days' cheese ripening; VI - 7 days' cheese ripening, VII - 15 days' cheese ripening. Cow's milk - K-1, a mixture of milk from cows and goats - K-2, goat's milk - K-3.

The development of *L. acidophilus* cultures in goat's milk cheese was 139% and 101% more intense comparing to cow's milk and the mixture of two types of milk, respectively. The amount of lactic acid microflora of Iprovit-Active increased less actively than the culture of *L. acidophilus*. The maximum increase in Iprovit-Active lactic acid microflora was also revealed in goat's milk cheese, which was higher by 42-62% compared to other variants.

Keeping cheeses in salt brine (20% NaCl) for 10.0±2.0 h, at temperature (8±2) °C caused inhibition of growth of lactic acid microflora (Figure 5). In all the studied samples from the III to the IV stage of the technological process, no increase in the number of microflora was observed. It is significant that the survival rate of the microflora was the lowest (about 72%) during salting on the surface of the cheese mass in direct contact with the brine. However, already at a distance of (2±1) cm from the surface layer of cheese mass, this indicator increased significantly (up to 95%), and in the central part of the cheese mass the number of lactic acid microorganisms was up 12-15%.

At the stage IV after salting in all samples, the microflora of the culture Iprovit-Active overtook the development of *L. acidophilus* culture (7.99 lg CFU/g) and their average content in the cheese mass was about 7.75 lg CFU/g.

The maximum amount of microflora in all cheeses was observed on the 3rd day of ripening. During this period, the number of cultures of *L. acidophilus* reached the level $(1.7\text{-}3.0)\cdot10^8$ CFU/g and the microflora of the culture Iprovit-Active - $(3.7\text{-}6.2)\cdot10^8$ CFU/g.

At the V-VII stages of cheese ripening, from the 3rd to the 15th day (see Figure 5), a slowdown in the development of lactic acid microflora and a slight decrease in the number

of Iprovit-Active cultures and *L. acidophilus* by 1,2-1, 5 times and 1.1-1.4 times, respectively.

Thus, on the 15th day of ripening, the fermentation microflora increased in all types of cheeses, in particular Iprovit-Active cultures by 83.2-117.5 times, and *L. acidophilus* by 114.8-194.9 times in comparison with the initial content.

On the 15th day of ripening all cheeses made from milk of different origin had microbiological parameters that met the requirements of current regulations.

Therefore, the intensity of development of lactic acid microflora depended on the raw materials and fermenting cultures and could be different by much. It should be taken into account when developing technological modes of cheese production.

During 2 months of storage at a temperature of (5±1) °C (Figure 6) the gradual extinction of the fermenting microflora in all experimental cheeses was recorded.

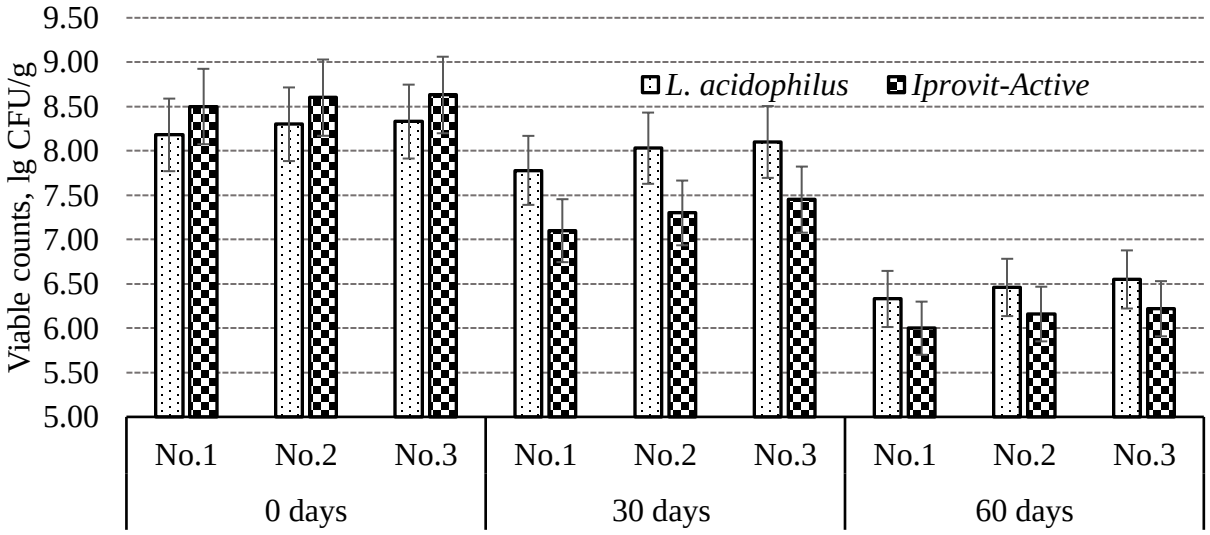

Figure 6. Dynamics of lactic acid microflora content during cheese storage.
No.1 - cheese made from cow's milk; No.2 - cheese from a mixture of cow's milk and goat's milk (1: 1); No.3 - goat's milk cheese.

The number of lactobacilli of the culture Iprovit-Active decreased more intensely by 4.0-4.4 times than the culture of *L. acidophilus* (Figure 6).

On the 60th day of storage in goat's milk cheese there was a minimal decrease in the number of both Iprovit-Active microflora and *L. acidophilus* culture. Thus, the slowdown was 18.7% and 5.8 % compared to cows' milk cheeses, and 14.9 % and 12.6 % compared to cheeses made from the mixture, respectively.

As a result, of this process, higher levels of benzoic and sorbic acids were observed in goat's milk cheeses.

In addition to studying the dynamics of fermenting microflora accumulation in cheeses, the analysis of the accompanying microflora was carried out, namely: coliform bacteria, spore-forming microorganisms, yeast and moulds after the self-pressing of cheese mass (the stage III), on the 15th day of ripening (the stage VII), as well as for 2 months of storage at a temperature of (5 ± 1) ° C (Figure 7).

During the production of cheeses from the III to the VII stage of the technological process, the content of foreign microflora decreased. This was especially true of spore-forming bacteria, the number of which in cheeses decreased by 3.1-3.3 times, which is explained by the use of cultures of *L. acidophilus* for biological processing of raw materials.

During storage at a temperature of (5±1) °C, a very slow development of foreign microflora was observed (Figure 7). The increase in spore-forming microorganisms occurred in 2.67-3.00 times, and yeast and mold - in 2.09-2.41 times compared with the content at the beginning of storage. This range of increase did not affect the organoleptic properties of cheeses and met the requirements of current regulations for these products according to DSTU 6003:2008.

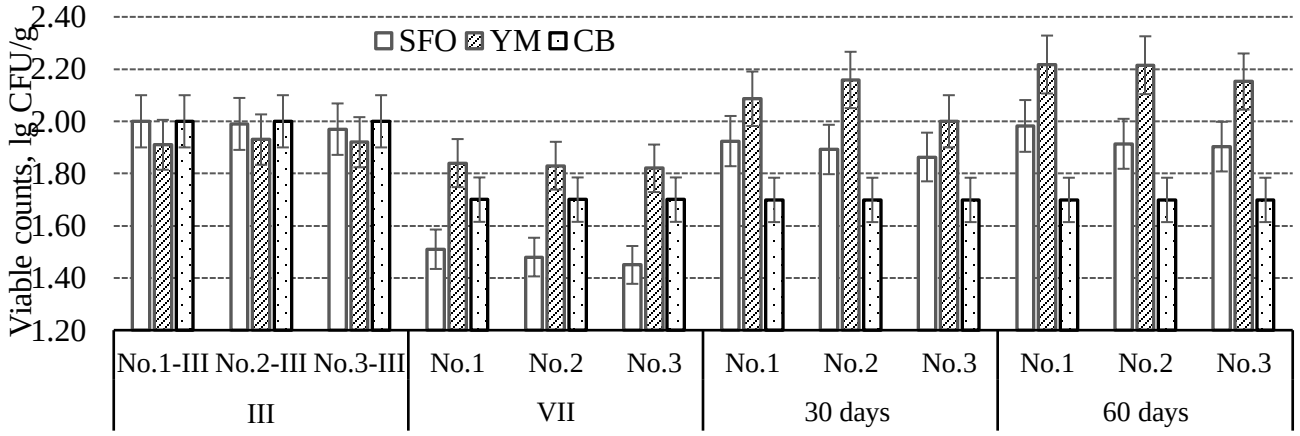

Figure 7. Changes of foreign microflora during ripening and storage of cheeses made from milk of different origin*)

III – cheese mass after self-pressing; VII –the 15th day of cheese ripening; No.1-III - cheese mass from cow's milk; No.2-III - cheese mass from a mixture of cow's milk and goat's milk; No.3-III - cheese mass from goat's milk; No.1 – cheese made from cow's milk; No.2 - cheese from a mixture of cow's milk and goat's milk (1: 1); No.3 - cheese from goat's milk, SFO – spore-forming organism; YM – yeast and moulds; CB – Coliforming bacteria.

*) Values are displayed as the mean ± standard deviation (S_R) of five replications. The average value in one row, followed by different indices, differed significantly ($P < 0.05$).

Thus, experimental cheeses can be stored for 60 days at a temperature of (5±1) °C while maintaining their quality, which exceeds the period specified in DSTU 6003:2008 for cheeses of this group for 15 days.

3.4. Investigation of benzoic and sorbic acids content during ripening and storage of cheeses

It has been found that some organic acids, in particular benzoic and sorbic, which are natural preservatives, may be present in milk and dairy products.

Benzoic acid is formed as a result of the activity of lactic acid microflora, and its content depends on the duration of fermentation, types of starter cultures, temperature and raw milk.

It was shown that during the fermentation of milk by starter cultures of S. *thermophilus, L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris, L. lactis* ssp. *diacetylactis, Leuconostoc mesenteroides* ssp. *cremoris, Lactobacillus paracasei* the content of benzoic acid was 0.0÷12.5 mg/kg. At the same time the highest level of benzoic acid was observed in skim milk fermented with R707 (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*) (Han et al., 2016).

According to Urbienė & Leskauskaitė (2006), the content of benzoic acid in milk can range from 2 to 5 mg/kg, and after fermentation of milk by starter cultures (La-5, ABT-2 and YC-180) - can be 14-23 mg/kg, and the content of sorbic acid - 0.06-0.09 mg/kg. The maximum formation of organic acids was detected at 3–6 h of fermentation of raw milk, ie during the log-phase, and depended on the type of lactic acid bacteria. The highest concentration of organic acids was found in milk for 7 hours of

cultivation by starter culture of *Lactobacillus acidophilus* La-5.

Bartáková et al., (2021) determined the range of benzoic acid in yogurts fermented with cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, in the amount (13.38±3.56); (21.31±5.66); (43.26±5.11) mg/kg depending on the raw material used - cow's, goat's or sheep's milk.

A study of cheeses (Cream, Curd, Cottage, Hard) found that the level of benzoic acid can reach 5.1-90 mg/kg (Gucer et al., 2016; Horníčková et al., 2014), and sorbic acid in Kashar and Fresh cheeses - 21.3÷511.3 mg/kg (Özdemir et al., 2020).

There are also data on a positive correlation of benzoic acid content with the number of lactic acid bacteria (LAB) in Feta cheese at (r = 0.827) (Heshmati et al., 2017), while there is no correlation for cream cheese. The concentration of benzoic acid was 41.80 mg/kg and 8.52 mg/kg in feta cheese and cream cheese, respectively.

Because the levels of benzoic and sorbic acids are controlled for food safety, in particular their chemical or natural origin, the study of the content of these compounds in fermented dairy products is of particular importance (Gucer et al., 2016; Özdemir et al., 2020; Bartáková et al., 2021; Yerlikaya et al., 2021).

Determination of the content of benzoic and sorbic acids in the process of making hard cheeses showed some differences depending on the applied raw milk. In the milk mixture No.3-I at the first stage of the technological process the content of benzoic acid was almost 4 times higher than in the milk mixture No.1-I. The content of sorbic acid was detected in trace amounts.

During the third stage, in the curd mass after self-pressing the increase in benzoic acid content occurred 3.7 times in cheeses No.1-III and 2.6 times in cheeses No.3-III compared to the first stage of the technological process; during the seventh stage, the cheese on the 15th day of ripening, the content of benzoic acid increased by 4.0 and 2.4 times, respectively, compared with the third stage (Table 4).

Table 4. Benzoic and sorbic acids content during cheese production (mg/kg)

The stage of the technological process	**Samples**	**Acid content, mg/kg**	
		benzoic	**sorbic**
I – adding fermenting culture in milk mixture	No.1-I	1.02±0.12	-
	No.2-I	3.13±0.48	-
	No.3-I	4.05±0.34	-
III – curd mass after self-pressing	No.1-III	3.79±0.62	0.060±0.004
	No.2-III	8.65±0.79	0.071±0.003
	No.3-III	10.55±1.24	0.092±0.002
VII – cheese, 15 days' ripening	No.1	14.80±1.76	0.068±0.003
	No.2	20.64±1.95	0.097±0.003
	No.3	25.44±1.83	0.123±0.003

Note. "-"- not found. Values are displayed as the mean ± standard deviation (S_R) of the five replications ($P<0.05$).

During storage, the accumulation of benzoic acid slowed down in all sample variants.

Compared with the finished cheese (at the seventh stage of manufacture), the content of this compound for 30 days of storage increased by 1.1-1.23 times and almost did not change during the next 30 days (Table 5).

The content of sorbic acid was much lower than benzoic acid in all samples of experimental cheeses at all stages of manufacture and storage. At the same time during storage of the first 30 days its content increased in comparison with finished cheeses. At the end of the next 30 days, a slight variation in the data was recorded, which

can be explained by the slowdown in the development of the fermenting microflora.

Table 5. The content of benzoic and sorbic acids in cheese during 2-months storage (mg/kg)

Storage period, days	Cheese samples	Acid content, mg/kg	
		benzoic	sorbic
30	No.1	18.37±0.45	0.067±0.004
	No.2	23.21±0.78	0.095±0.004
	No.3	28.20±0.52	0.120±0.004
60	No.1	18.41±0.53	0.062±0.003
	No.2	25.08±0.77	0.092±0.003
	No.3	29.77±0.60	0.118±0.002

Note. Values are displayed as the mean ± standard deviation (S_R) of the five replications ($P < 0.05$).

Thus, it can be assumed that organic acids are the main culture to prevent spoilage of cheeses during long periods of storage.

4. Conclusions

We have worked out the method of hard cheese production at a low temperature of the second heating using biological processing of raw milk, a shelf life was 60 days.

A feature of this approach is the step of introducing *L. acidophilus* cultures and keeping them in pasteurized milk for 30 minutes before adding the fermentation culture.

Comparing to the traditional technology, this approach has reduced the duration of rennet curd formation by about 10-15 minutes, grain formation by 10 minutes, and reduced protein and fat losses.

Also, the proposed approach involves the use of hot self-pressing, which reduces the stages of self-pressing and pressing by traditional technology by 180±50 minutes, i.e. by 2-3 times. The surface of the cheese wheels was treated with hot water (70±5) °C every 20 minutes and then there was a compaction and formation of a rind on the product's surface.

Our method of cheese making is valuable also because of the stages of grain mixing and whey draining. According to the proposed technology, the removal of whey occurs in one step in the amount of (65 ± 5) %, in contrast to the traditional technology which involves two stages.

Treatment of raw milk with cultures of *L. acidophilus* allowed to increase the shelf life of the finished product to 60 days without deterioration of organoleptic properties by preventing the development of spontaneous microflora.

It should be noted that the addition of two starter preparations, which included *L. acidophilus, L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris, L. lactis* ssp. *lactis biovar. diacetylactis, Lactobacillus casei,* could also promote the accumulation of benzoic and sorbic acids in the experimental cheeses. At 60 days of storage, the content of benzoic acid was 18-30 mg/kg of cheese, which avoids additional measures for the treatment of finished cheeses with artificial preservatives.

It has been shown that this technology can be applied to cheeses made from various dairy raw materials - cow's, goat's or sheep's milk. However, the stage of biological treatment of raw milk with *L. acidophilus* culture should remain a mandatory stage. It was found that under these production conditions, the maximum content of benzoic acid was observed in cheeses made from goat's milk or a mixture. The peculiarity of this technology is the possibility of its implementation on mini-farms for small batches of products.

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PHYTOCHEMICAL SCREENING, GC–MS AND FTIR ANALYSIS OF BIOACTIVE COMPOUNDS PRESENT IN VEGETABLES AND FRUITS

Niaz Ali Malghani[1]✉, Sarfaraz Ahmed Mahesar[1], Jameel Ahmed Baig[1], Farah Naz Talpur[1], Samina Sohu[2], Syed Tufail Hussain Sherazi[1]

[1]National Centre of Excellence in Analytical Chemistry, University of Sindh, Jamshoro-76080, Pakistan
[2]Dr. M. A. Kazi Institute of Chemistry, University of Sindh, Jamshoro-76080, Pakistan
✉niazalimalghani12@gmail.com

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ABSTRACT

Vegetables and fruits are among the most regularly consumed foods because of their physiological effects. This study aimed to check the potential phytochemical substances in the vegetables and fruits of Larkana, Sindh, Pakistan, using qualitative and quantitative analysis. The vegetables and fruits extract screening analysis showed important phytochemicals such as phenols, proteins, quinones, alkaloids, flavonoids, tannins, terpenoids, and carbohydrates. The GC-MS identified different phytochemical compounds. The major components present in vegetables and fruits were Benzoic acid (7.73%), Lupeol (13.44%), 1-Eicosene (11.49%), N-Tetratetracontane (8.41%), 2-Pentadecanone, 6,10,14-trimethyl (9.33 %), Hexadecanoic acid methyl ester (12.89%), Nonadecane (12.19%), 3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)- (9.14%), Ethyl benzoate (14.43%) and 5-Hydroxymethylfurfural (15.06%). FTIR spectroscopy was also used to identify typical functional groups in freeze-dried materials. Data revealed the strong absorption around 3600-3200 cm^{-1} due to the O-H stretching vibrations and C-H stretching vibration at 3000-2800 cm^{-1}. The representing C=O and C-O stretching vibrations appeared at 1700-1750 cm^{-1} and 1200-1000 cm^{-1}. The C-N stretching vibration was observed at 1300-1200 cm^{-1}.

1. Introduction

Phytochemicals, commonly known as phytonutrients, are non-nutritive plant compounds with disease-preventing or protective properties. Most food, especially fruits and vegetables, contain these complex compounds. Fruits and vegetables have shown several beneficial features, mostly in terms of preserving excellent health and nutritional potential (Aman, Schieber, & Carle, 2005). Vegetables and Fruits contain high concentrations of dietary fibre, vitamins, minerals, electrolytes, antioxidants, and phytochemicals to consider these for nutritional recommendations (Slavin & Lloyd, 2012). Large numbers of phytochemicals have been known in vegetables and fruits. They have been divided into several classes based on biological function and their chemical structure like Phenols, proteins, quinones, alkaloids, flavonoids, tannins, and terpenoids are examples of these phytochemical compounds (Shahidi, McDonald, Chandrasekara, & Zhong, 2008). These substances are produced by live organisms or secondary metabolism. Secondary metabolites are a taxonomic and chemically varied group of molecules with a mysterious purpose. They are active in human therapy, veterinary medicine, agriculture, scientific research, and a variety of other fields (Goud, Suryam, & Charya, 2009). Vitamins and minerals have long been thought to play an important role in disease prevention; however, current research suggests that phytochemicals may contribute more to vitamins or other nutrients. Phytochemicals have been linked to

preventing chronic illnesses such as heart disease, cancer, diabetes, osteoporosis, and eyesight problems. Many forms of cancer, including stomach cancer, lungs cancer, breast cancer, and colon prostate cancers, are adversely connected to fruit and vegetable consumption (Kris-Etherton et al., 2002; Temple & Gladwin, 2003). Fruits and vegetables include phytochemicals that have a preventive impact against certain illnesses. Phytochemicals can also help limit the spread of cancer by reducing the multiplication of cancer cells (Hirsch et al., 2000; Schneider et al., 2000). As a result, there is a need to assess the potential of local vegetables in providing essential nutrients and phytochemicals, which will aid in selecting appropriate green leafy vegetables by food processors, nutritionists, dieticians, and consumers. Biochemicals are typically referred to as secondary metabolites in this environment, and these biochemicals are valuable for the traditional medical system these can be identified by using Gas chromatography-mass spectrometry (GC-MS) technique (Prasain, Wang, & Barnes, 2004). GC-MS has established itself as a fundamental technical platform for analyzing secondary metabolites profiling in vegetables and fruits in recent years (Ganesan & Raja, 2021; Kell et al., 2005; Robertson, 2005).

2. Materials and Methods

2.1. Chemicals and Reagents

The chemicals and reagents used in this study were obtained from Merck (Darmstadt, Germany) for the analysis of phytochemicals such as methanol, ferric chloride, sodium hydroxide, copper sulphate, ammonia solution, sulphuric acid, Wagner's reagent, chloroform, Benedict's reagent and distilled water.

2.2. Sample collection

For the present study, three leafy vegetables and two fruits samples were collected from different areas of Larkana, Sindh, Pakistan, such as Spinach (Spinacia oleracea), Coriander leaves (Coriandrum sativum), Fenugreek leaves (Trigonella Foenum-graefum), Jujuba (Ziziphus Jujube) and Guava (Psidium Guajava). In the lab, samples of vegetables and fruits were placed through a three-step washing process that included agitating and rinsing with distilled water first, then three successive washings with ultra-pure water. The freeze-drying process was used to dry the clean vegetable and fruit samples (Shah, Rasapalli, Mello, Singh, & Cai, 2012). In an Agate mortar, the freeze-dried fruit and vegetable samples were powdered and sieved through a nylon sieve with a mesh size of 7 mm.

2.3. Preparation of vegetables and fruits

The collected vegetables and fruits samples were dried and crushed to powder form. Each vegetable and fruit sample was steeped in 50 mL of water, ethanol, and hexane individually. For 48 h, the whole combination was incubated at 4°C. After the incubation time, the mixture was filtered and centrifuged at 4°C for 10 min at 10,000 rpm. In a rotary evaporator (IKA-RV 10 Control), the extracts were concentrated to dryness and kept at 4°C until further analysis.

2.4. Phytochemical analysis tests

2.4.1. Test for Phenols or Ferric chloride test

For analysis of phenols content in vegetables and fruits, the procedure was used as reported earlier (Jigna & Sumitra, 2008). In the test tube, 2 mL of each sample extract was added, followed by 4 mL of distilled water and a few drops of aqueous ferric chloride solution. The presence of phenols is indicated by the production of a blue or green color.

2.4.2. Detection of proteins or Biuret test

The method described by (Jigna & Sumitra, 2008) was used to determine the proteins in vegetables and fruits. 2 mL of each extract were taken into the test tube and reacted with 5% NaOH and 1% (w/v) $CuSO_4$ formed violet colored complex indicates that protein is present.

2.4.3. Detection of quinones

A procedure reported earlier by (Raja & Ravindranadh, 2014) was used to detect quinones in vegetables and fruits. In short, 2 mL of each extract were put into the test tube and reacted with dilute NaOH, which formed the red or blue-green color, indicating quinones' presence.

2.4.4. Alkaloids test or (Wagner's reagent)

Wagner's reagent was used to analyze alkaloids test as reported by (Jigna & Sumitra, 2008). In brief, 2 mL of each extract were put into the test tube and added 2 mL of Wagner's reagent (2 g of KI and 1.27 g of I2 in 100 mL of (H_2O). After mixing the solution, it left for a few min to appear reddish-brown color, indicating alkaloids presence.

2.4.5. Flavonoids test

For confirmation of flavonoids in vegetables and fruits, 2 mL of each extract were put into the test tube, added 5 mL of a dilute solution of ammonia and concentrated H_2SO_4. The yellow color formation confirms the presence of flavonoids, as described by (Jigna & Sumitra, 2008).

2.4.6 Tannins test or Braymer's test

For analysis of tannins, 2 mL of each extract of vegetables and fruits were taken into a test tube and reacted with 0.1% $FeCl_3$ solution. The blue color confirms the presence of tannins, as described by (Jigna & Sumitra, 2008).

2.4.7. Terpenoids

The method applied for the analysis of terpenoids in vegetables and fruits was reported by (Jigna & Sumitra, 2008). In the test tube, 2 mL of each extract of vegetables and fruits were taken, added 4 mL of chloroform and 5 mL concentrated (H_2SO_4). After mixing, the presence of reddish-brown color in the solution confirms the presence of terpenoids.

2.4.8. Carbohydrates (Benedict's test)

The method reported by (Jigna & Sumitra, 2008) was used to analyze carbohydrates in vegetables and fruits. Briefly, 2 mL of each extract were taken into a test tube and mixed with Benedict's reagent. The solution was heated gently in a water bath for 5 min and observed the formation of orange-red precipitate that indicates the presence of carbohydrates.

2.5. Gas chromatography-mass spectrometry (GC-MS) analysis

The chemical compositions of ethanol extract were investigated through GC-MS. The GC-MS 6890 N from Agilent Technology was interfaced to a Mass Spectrometer equipped with an HP-5MS capillary column (30mx 0.25mm x 0.25µm film thickness). The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–550 m/z. The GC-MS was controlled using Agilent Chem Station 6890 Scale Mode software. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min. 1 µL each sample was inserted as a split mode ratio of 10:1. The starting oven temperature was 110°C (isothermal for 2 min) and increased to 200°C with an increase of 10°C /min, then 5°C/min to 280°C and stayed for 10 min. The injector temperature was selected at 250°C, for detector temperature at 270°C. All analysis was performed in triplicate and the relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

2.6. Fourier transform infrared (FTIR*)*

For qualitative analysis, FTIR was used to identify the typical groups (functional) in the dried vegetable and fruits powders. The spectra of vegetable and fruits samples were collected using Nicolet iS10 (Thermo) spectrometer equipped with a DTGS detector and controlled with OMINIC software (version 9). Other parameters such as resolution 4 cm^{-1}, scan 16, range 4000-650 $cm^{-1,}$ and diamond SB-ATR accessory was used. Before each analysis, the crystal was cleaned with a solvent, and a background spectrum was collected (Sandosh, Peter, & Raj, 2013; Talukdar, Choudhury, Chakraborty, & Dutta, 2010).

3. Results and Discussion

3.1. Analysis of phytochemicals

The phytochemicals are found in vegetables possess various therapeutic applications (Okwu, 2001). Phenols are widely distributed in vegetables. They contribute color, flavor, and astringency to vegetables. These phenols are considered secondary metabolites of plant metabolism, which contributes to physiological or ecological functions (Oyeyinka & Afolayan, 2020). Isoprenoids are another name for terpenoids. Many vegetables contain these abundant and architecturally varied natural compounds. They are a diverse category of

natural compounds that may be found in all types of living organisms.

Alkaloids in the vegetables reveal their beneficial importance. Alkaloids in the diet may help in the healing of wounds, ulcers, haemorrhoids, and burns. These alkaloids have antiviral and cytotoxic properties, as well as a variety of other physiological effects (Ding et al., 2017). Multiple viruses have been shown to be inhibited by flavonoid compounds. These are water-soluble antioxidants and free radical scavengers that protect cells from oxidative damage and have potent anticancer properties (Duraipandiyan, Ayyanar, & Ignacimuthu, 2006). Dietary flavonoids provide several health benefits, including antioxidant and antiproliferative activities that may protect the human body from a variety of illnesses (Duraipandiyan et al., 2006). Phytochemical analysis of leafy vegetables and fruit extracts gave information which type of phytochemical compounds is present in vegetables and fruits. For qualitative analysis of all vegetables and fruits extract, a total of eight phytochemicals were analysed, such as phenols, proteins, quinones, alkaloids, flavonoids, tannins, terpenoids, carbohydrates. The results of the qualitative analysis of vegetables and fruits are shown in Table 1, where (+) represents the phytochemical is present and (-) indicates the absence of phytochemicals.

Table 1. Screening analysis of vegetables and fruits

Compounds	Spinach	Coriander Leaves	Fenugreek leaves	Jujube	Guava
Phenols	+	+	+	+	+
Proteins	+	+	+	+	+
Quinones	+	+	+	+	-
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	-
Tannins	+	+	-	-	+
Terpenoids	+	+	-	-	+
Carbohydrates	+	+	+	+	+

Proteins and carbohydrates were present in all the vegetables and fruits. Spinach and coriander leaves among the analyzed samples were the rich sources of phytochemicals and showed presence of all eight phytochemicals (Aborisade, Olagoke, & Abdulhakeem Dapo, 2018) also studied for spinach leaves is rich source of flavonoids, phenol, and tannins, whereas other phytochemicals were absent. (Al-Marzoqi, Hameed, & Idan, 2015) was reported Alkaloids, Flavonoids were absence in coriander leaves Phenols Tannins, Carbohydrate and Proteins were presence. Tannins and phenols were absent in fenugreek leaves, whereas all the studied phytochemicals were present. The tannins, terpenoids were absent in jujube, whereas phenols, proteins, quinones, alkaloids, flavonoids, terpenoids, and carbohydrates were present. In a study (Roughani & Miri, 2019) reported a similar trend of phytochemicals for jujube where terpenoids and tannins were absent, on the other hand, phenol, flavonoids, and alkaloids were present in the extract. In guava extract, except quinines and flavonoids, all other phytochemicals such as phenols, proteins, alkaloids, tannins, terpenoids, and carbohydrates were present.

3.2. Characterization of GC-MS analysis

In general, GC-MS is utilised to investigate compounds found in vegetables and fruits directly. In recent years, GC-MS studies for the analysis of vegetables and fruit have become more common since this technique has proven to be a useful tool for the examination of phytochemicals (Marston, 2007; Sridharan, Vaidyanathan, Venkatesh, & Nayagam, 2011). The molecular formula and peak area are used to confirm the phytochemical compounds' identity. The name of phytochemical compounds and concentration (%) is given in Table 2, which is important for regulating several significant functions like lipid levels, blood pressure, immune response, and inflammatory response to injuries. The GC-MS analysis in the vegetables and fruits described the presence of different phytochemical compounds that could contribute to the antioxidant and therapeutic properties of vegetables and fruits.

Table. 2 GC-MS analysis of spinach leaves

S.no	Compounds	Area (%)
1	3,4-dimethyl-1H-Pyrazole	2.03
2	Thymol	0.12
3	N-Methyl propionic acid amide	1.05
4	(S)-(+)-Glutamic acid	3.06
5	3-Methyl-3-pyrazolin-5-one	5.12
6	Glycine betaine	4.05
7	Propanoic acid, 2-(hydroxyimino)-, methyl ester	1.11
8	Thiole	6.50
9	Benzeneacetaldehyde	0.91
10	3-Hydroxypyrrolidine	1.19
11	Phenol, 2-methoxy-4-vinyl-	7.13
12	Benzoic acid	7.73
13	Ephedrin	5.62
14	Pyrollidine, 2,5-bis(imino)-	3.27
15	N-methyl piperazine	3.16
16	2-Pyrrolidinone	5.36
17	2,5-Diethylphenol	2.23
18	D-Valine	1.53
19	Monoethylaminoethanol	0.20
20	Thiophene	2.09
21	Succinic acid, diethyl ester	4.76
22	1-Eicosanol	1.94
23	Pyrrolidin-1-acetic acid	3.73
24	Palmitic acid ethyl ester	2.39
25	1-Methyl-5-D1-1,2,4-Triazole	1.33
26	L-Proline, 5-oxo-	2.21
27	Phthalic acid, dipropyl ester	5.22
28	Lauric acid	4.22

3.2.1. GC-MS analysis of spinach leaves

The GC-MS analysis shows different phytochemical compounds in the methanol extract of spinach leaves, as shown in Table 2. These compounds include 3,4-dimethyl-1H-Pyrazole (2.03%), Thymol (0.12%), N-Methyl propionic acid amide (1.05%), (S)-(+)-Glutamic acid (3.06%), 3-Methyl-3-pyrazolin-5-one (5.12%), Glycine betaine (4.05%), Propanoic acid,2-(hydroxyimino)-, methyl ester (1.11%),

Thiole (6.50%), Benzeneacetaldehyde (0.91%), 3-Hydroxypyrrolidine (1.19%), Phenol, 2-methoxy-4-vinyl- (7.13%), Benzoic acid (7.73%), Ephedrin (5.62%), Pyrollidine, 2,5-bis(imino) (3.27%), N-methyl piperazine (3.16%), 2-Pyrrolidinone (5.36%), 2,5-Diethylphenol (2.23%), D-Valine (1.53%), Monoethylaminoethanol (0.20%), Thiophene (2.09%), Succinic acid diethyl ester, (4.76%), 1-Eicosanol (1.94%), Pyrrolidin-1-acetic acid (3.73%), Palmitic acid ethyl ester (1.33%), L-Proline, 5-oxo- (2.21%), Phthalic acid dipropyl ester (5.22%), Lauric acid (4.22%). These phytochemical compounds indicate that spinach leaves can reduce the risk of different diseases.

3.2.2. GC-MS analysis of coriander leaves

The GC-MS analysis shows different phytochemical compounds in the methanol extract of coriander leaves (Table 3). The most phytochemical compounds present in the extract are n-Tetracosane (2.71%), Glycerin (2.99%), Benzenesulfonyl chloride (5.11%), N-Tetratetracontane (8.41%), 2-Methoxy-4-vinylphenol (6.22%), Tetradecanoic acid (2.66%), L(+)-isoleucine (3.85%), Cyclohexadecane (1.97%), 2-Isopropyl-5-methyl 1heptanol (2.51%), Cyclohexadecane (1.88%), Octadecane (1.72%), 9,12-Octadecadienoic acid (Z, Z)-methyl ester (3.29%), 1-Mercapto-4-methylbicyclo[2.2.2]octane (3.48%), Hexadecanoic acid (4.19%), Hexadecanoic acid (4.19), 1-Eicosene (13.44%), Squalene (6.31%).

Table 3. GC-MS analysis of coriander leaves

S. no	Compounds	Area (%)
1	n-Tetracosane	2.71
2	Glycerin	2.99
3	Benzenesulfonyl chloride	5.11
4	N-Tetratetracontane	8.41
5	2- Methoxy-4-vinylphenol	6.22
6	Tetradecanoic acid	2.66
7	L(+)-isoleucine	3.85
8	Cyclohexadecane	1.97
9	2-Isopropyl-5-methyl-1-heptanol	2.51
10	Cyclohexadecane	1.88
11	Octadecane	1.72
12	9,12-Octadecadienoic acid (Z, Z)-methyl ester	3.29
13	1-Mercapto-4-methylbicyclo[2.2.2]octane	3.48
14	Hexadecanoic acid	4.19
15	1-Eicosene	11.49
16	Lupeol	13.44
17	Squalene	6.31

3.2.3. GC-MS analysis of fenugreek leaves

The compounds identified by GC-MS in alcoholic extracts of fenugreek leaves are described in (Table 4) as Stigmasterol (2.23%), Hexadecane (4.19%), Diazoprogesterone (3.88%), L-Isoleucine (2.43%), Z-2-Dodecenol (1.81%),2-Pentadecanone, 6,10,14-trimethyl (9.33%), Methyl linolenate-Linolenic acid, methyl ester(5.15%), Hexadecanoic acid, methyl ester (12.89%), Nonadecane (12.19%), Hexadecanoic acid, ethyl ester, (6.31%), 10,13-Octadecadienoic acid, methyl Ester (4.17%), Phytol (8.23%), Glutamic acid (6.72%), Hexadecanoic acid, butyl ester (7.44%), Octadecane (1.11%), Campesterol (1.69%), Isophytol (3.42%),n-Hexadecanoic acid (2.18%), 3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)- (9.14%).

3.2.4. GC-MS analysis of Jujube

GC-MS identified the compound in alcoholic extracts of Jujuba are described in Table 5, these include stigmasterol (1.57%),

formic acid (1.33%), molinate (3.18%), 5-Hydroxymethylfurfural (15.06%), Oleic acid (1.09%), Maltol (1.33%), 2-Propyloctanoic acid (5.89%), Beta-D-Glucopyranoside, methyl (5.97%), Pentanoic acid nonyl ester (1.17%), Thymine (4.09%), D-Allose (4.63%), Polygalitol (6.73%), 3,4-Altrosan (3.91%), Glucose (5.77%), Erucic acid (1.09%), 1,5-Anhydroglucitrol (7.89%), Tetradecanoic acid (2.59%).

3.2.5. GC-MS analysis of Guava

The compounds identified by GC-MS in alcoholic extracts of guava are described in Table 6, such as Pentadecanoic acid, (2.14%), Ethyl butanoate (3.28%), Butyl-2-methylbutyrate (4.69%), 9, 12-octadienoic acid (4.22%), Oleic acid, (6.51%), (+)-Aromadendrene (1.53%), Decyl fluoride (1.79%), Phytol, acetate (1.63%), β-Caryophyllene (3.29%), Arachic acid (3.23%), Acetoin (3.34%), (S)-(−)-Limonene (1.91%), Palmitic acid (1.11%), Nonanal (1.16%), (Z)-β-Farnesene (2.66%), cis-3-Hexenyl butanoate (1.01%), Eicosanoic acid (8.81%), α-Terpineol (3.37%), Methyl 3-propionamidobenzoate (2.13%), cis-3-Hexenyl isobutanoate (7.16%), Ethyl octanoate (6.23%), (−)-α-Copaene (5.29%), Ethyl butanoate (1.18%), (E, Z)-α-Farnesene(2.19%), γ-bisabolene (2.45%).

Table 4. GC-MS analysis of fenugreek leaves

S.no	Compounds	Area (%)
1	Stigmasterol	2.23
2	Hexadecane	4.19
3	Diazoprogesterone	3.88
4	L-Isoleucine	2.43
5	Z-2-Dodecenol	1.81
8	2-Pentadecanone, 6,10,14-trimethyl	9.33
9	Methyl linolenate-Linolenic acid, methyl ester	5.15
10	Hexadecanoic acid, methyl ester	12.89
11	Nonadecane	12.19
12	Hexadecanoic acid, ethyl ester	6.31
13	10,13-Octadecadienoic acid, methyl Ester	4.17
14	Phytol	8.23
15	Glutamic acid	6.72
16	Hexadecanoic acid, butyl ester	7.44
17	Octadecane	1.11
18	Campesterol	1.69
29	Isophytol	3.42
20	n-Hexadecanoic acid	2.18
21	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	9.14

Table 5. GC-MS analysis of Jujube

S.no	Compounds	Area (%)
1	Stigmasterol	1.57
2	Formic acid	1.33
3	Molinate	3.18
3	5-Hydroxymethylfurfural	15.06
5	Oleic acid	1.09
6	Maltol	1.33
7	2-Propyloctanoic acid	5.89
8	Beta-D-Glucopyranoside, methyl	5.97

9	Pentanoic acid, nonyl ester	1.17
11	Thymine	4.09
12	D-Allose	4.63
13	Polygalitol	6.73
14	3,4-Altrosan	3.91
15	Glucose	5.77
16	Erucic acid	1.09
17	1,5-Anhydroglucitrol	7.89
19	Tetradecanoic acid	2.59

Table 6. GC-MS analysis of Guava

S. no	Compounds	Area (%)
1	Pentadecanoic acid	2.14
2	Ethyl butanoate	3.28
3	Butyl-2-methylbutyrate	4.69
4	9, 12-octadienoic acid	4.22
5	Oleic acid	6.51
6	(+)-Aromadendrene	1.53
7	Decyl fluoride	1.79
8	Phytol, acetate	1.63
9	β-Caryophyllene	3.29
10	Arachic acid	3.23
11	Acetoin	3.34
12	(S)-(−)-Limonene	1.91
13	Palmitic acid	1.11
14	Nonanal	1.16
15	(Z)-β-Farnesene	2.66
16	Ethyl benzoate	14.43
17	cis-3-Hexenyl butanoate	1.01
18	Eicosanoic acid	8.81
19	α-Terpineol	3.37
20	Methyl 3-propionamidobenzoate	2.13
21	cis-3-Hexenyl isobutanoate	7.16
22	Ethyl octanoate	6.23
23	(−)-α-Copaene	5.29
24	Ethyl butanoate	1.18
25	(E,Z)-α-Farnesene	2.19
26	γ-bisabolene	2.45

3.3. Fourier Transform Infrared Spectrophotometer (FTIR) analysis

The qualitative characteristics of organic substances in freeze-dried vegetables and fruits were identified using FTIR spectroscopy. Chemical components of bioactive molecules are represented by many indicator bands associated with various functional groupings. The fingerprint area has distinct bands in the FT-IR spectrum. The infrared spectrum identifies the major components and shows a spectral variation to find differences among the vegetables and fruits. The typical functional groups observed in the vegetables and fruits are shown in Table 7

Table 7. FTIR peak assignment of vegetables and fruits.

Functional group	Spinach	Coriander Leaves	Fenugreek leaves	Jujube	Guava
O-H stretching vibration presence of alcohols, phenols	3362	-	-	-	-
	3276	3276	3275	3280	3273
C-H stretching vibration presence of alkenes	2959	2920	2918	2930	2920
	2917	-	-	-	-
	2850	2851	2849	2855	2851
C=O s stretching vibration presence of alcohols, carboxylic acids, esters, ethers	-	1733	1732	1724	-
-C=C- stretching vibration presence of alkenes	1635	1601	1616	1634	1626
C-C stretching vibration presence of aromatics	-	-	-	1416	-
N-O stretching nitro compound	1539	1558	1558	-	1557
	-	1540	1539	-	-
O-H bending	1371	-	1334	1338	1378
	1338	1394	1398	-	1320
	1321	-	-	-	-
C-N stretching	1241	1241	1261	1239	1241
stretching of C-O group	1099	-	-	1147	1155
	-	-	-	1051	-
	1046	1014	1027	1027	1028
	1030	-	-	-	-
C=C bending	-	-	893	918	-
	827	-	-	815	-
C-H bending	775	-	-	-	-

3.3.1. FTIR analysis of Spinach

In the spinach**,** a very strong absorption band in the region 3600-3200 cm^{-1} was observed, representing O-H stretching vibrations that may be due to the presence of alcohols. Absorption bands at 2959 cm^{-1}, 2917 cm^{-1}, 2850 cm^{-1} represent the presence of alkanes' C-H stretching vibration. The C=C stretching vibration of alkenes showed an absorption band at 1635 cm^{-1}. The band at 1539 cm^{-1} represents the N-O stretching due to the nitro compound. The absorption bands at 1371cm^{-1}, 1338 cm^{-1}, and 1321 cm^{-1} appeared due to the bending of O-H. The C-N stretching vibration shows an absorption band at 1241 cm^{-1}. The absorption bands at 1099 cm^{-1}, 1046 cm^{-1}, and 1030 cm^{-1} appeared due to the stretching of the C-O group. Two absorption bands appeared at 827 cm^{-1} and 775 $cm^{-1,}$ representing C=C bending and C-H bending.

3.3.2. FTIR analysis of Coriander leaves

In the coriander leaves, a very strong absorption band in the region 3200 cm^{-1} was observed, representing O-H stretching vibrations that may be due to the presence of alcohols. Absorption bands at 2920 cm^{-1}, 2851 cm^{-1} represent the presence of alkanes' C-H stretching vibration. The C=O stretching vibration presence may be due to alcohols showed an absorption band at 1733 cm^{-1}. Absorption bands at 1601 cm^{-1} C=C stretching vibration presence of alkenes. The absorption band at 1558 cm^{-1},1540 cm^{-1} shows N-O stretching due to nitro compound. The absorption bands at 1394 cm^{-1} appeared due to the bending of O-H. The C-N stretching vibration shows an absorption band at 1241 cm^{-1}. The absorption bands at 1014 cm^{-1} appeared due to the stretching of the C-O group.

3.3.3. FTIR analysis of Fenugreek leaves

In the Fenugreek leaves**,** a very strong absorption band in the region 3275 cm^{-1} was observed, representing O-H stretching vibrations that may be due to the presence of alcohols. Absorption bands at 2918 cm^{-1}, 2849 cm^{-1} represent the presence of alkanes C-H stretching vibration. The C=O stretching vibration that may be due to esters, alcohols,

ethers, carboxylic acids showed an absorption band at 1732 cm^{-1}. The C=C stretching vibration of alkenes showed an absorption band at 1616 cm^{-1}. The band at 1558 cm^{-1} and 1539 cm^{-1} represents the N-O stretching due to the nitro compound. The absorption bands at 1334 cm^{-1} and 1398 cm^{-1} appeared due to O-H bending. The C-N stretching vibration shows an absorption band at 1261 cm^{-1}. The absorption bands at 1027 cm^{-1} appeared due to the stretching of the C-O group. The absorption bands appeared at 893 cm^{-1}, representing C=C bending.

3.3.4. FTIR analysis of Jujuba

In the Jujuba**,** a very strong absorption band in the region 3280 cm^{-1} was observed, representing O-H stretching vibrations that may be due to the presence of alcohols. Absorption bands at 2930 cm^{-1},2855 cm^{-1} represent the presence of alkanes C-H stretching vibration. The C=O stretching vibration that may be due to esters, alcohols, ethers, carboxylic acids showed an absorption band at 1724 cm^{-1}. The C=C stretching vibration of alkenes showed an absorption band at 1634 cm^{-1}. The band at 1416 cm^{-1} represents the C-C stretching vibration presence due to the aromatics compound. The absorption bands at 1338 cm^{-1} appeared due to the bending of O-H. The C-N stretching vibration shows an absorption band at 1239 cm^{-1}. The absorption bands at 1147 cm^{-1}, 1051 cm^{-1}, and 1027 cm^{-1} appeared due to the stretching of the C-O group. Two absorption bands appeared at 918 cm^{-1} and 815 cm^{-1}, representing C=C bending

3.3.5. FTIR analysis of Guava

In the guava**,** a very strong absorption band in the region 3273 cm^{-1} was observed, representing O-H stretching vibrations that may be due to the presence of alcohols. Absorption bands at 2920 cm^{-1}, 2851 cm^{-1} represent the presence of alkanes C-H stretching vibration. The C=C stretching vibration of alkenes showed an absorption band at 1626 cm^{-1}. The band at 1557 cm^{-1} represents the N-O stretching due to the nitro compound. The absorption bands at 1378 cm^{-1} and 1320 cm^{-1} appeared due to O-H bending. The C-N stretching vibration shows an absorption band at 1241 cm^{-1}. The absorption bands at 1155 cm^{-1} and 1028 cm^{-1} appeared due to the stretching of the C-O group.

4. Conclusions

Natural antioxidants are increasingly being used in place of synthetic antioxidants. Vegetables and fruits are common dietary sources that contain many nutritional, functional, antioxidant, and other medicinal characteristics. The results of phytochemical screening, GC-MS, and FTIR indicated the presence of different phytochemical compounds with different compositions in freeze-dried vegetables and fruits. Hence, this study recommends that vegetables and fruits are essential for increasing potential health and safe food since they contain beneficial bioactive substances.

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QUALITY CHARACTERISTIC ANALYSIS OF BADUY PALM SUGAR

Dwining Putri Elfriede[1]✉, Fransisca[1], Rike Tri Kumala Dewi[1], Ni Nengah Ari Widiastuti[1]

[1]Department of Food Business Technology, School of Applied Science, Technology, Engineering, and Mathematics, Universitas Prasetiya Mulya, Tangerang, Indonesia

✉*dwiningputrie@gmail.com*

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ABSTRACT

The palm sugar center of Banten Province, Indonesia, is in Lebak Regency with a production contribution of approximately 70%. Baduy's palm sugar has the potential to be the best palm sugar in Banten. The quality of palm sugar is based on chemical and organoleptic properties such as water insoluble material, water content, ash content, reducing sugar and saccharose sugar as well as sensory tests. The distribution of variance and deviation of its standard was analyzed by statistical means, such as, histogram, control chart and Pareto chart. The results of this study are expected to increase the confidence of the domestic and foreign people to consume it so that it becomes one of the food tourism destinations in Banten. This study aims to determine: the characteristics of product quality, the diversity and the deviation of chemical characteristic compared to Indonesian National Standard (abbreviated SNI) Palm Sugar.

The average value of Baduy's palm sugar chemical characteristic, namely water content, ash content, water insoluble content, reducing sugar content and saccharose sugar content, respectively were 8.3749 %bb, 1.6773%bb, 0.5946%bb, 0,5625 %bb and 85.78%bb. The percentage of discrepancy from the chemical characteristics analysis of Baduy palm sugar with SNI, namely the sugar content as saccharose reached 63%, ash content reached 15% and water content reached 12%. While the results of reducing sugar content and water insoluble content analysis were in accordance with SNI. Based on the Pareto diagram, the results of the chemical characteristic analysis of Baduy palm sugar that most do not comply with SNI were the sugar content as saccharose. The average saccharose sugar content reached 85.78% bb, did not meet the SNI at 77% bb. In the sensory analysis, Baduy palm sugar had characteristics that were close to ideal compared to other commercial palm sugar.

1.Introduction

Sugar commodity in Indonesia is second commodity under rice commodity in terms of demand (Kurniasari et al. 2015). There are various types of sugar in Indonesia based on the ingredients, namely cane sugar, palm sugar and coconut sugar. The need for sugar in Indonesia will continue to increase along with the population and income increase. The International Sugar Organization states that Indonesia's sugar consumption will grow 4% per year to meet the needs of the national population of 240 million people. The limited knowledge and low level of education of sugar craftsmen have caused less attention to process sanitation from tapping to product packaging. This results the unbalance demand for sugar based on the total sugar offered by domestic production

Palm sugar is one of sugar types in Indonesia which is made from palm sap by boiling the

sweet sap which is harvested from the male flower stalks of Arenga pinnata. Palm sugar has a peculiarity compared to other sugar's types, such as it is more soluble, the condition is dry and clean and has a distinctive aroma. Based on the chemical composition, palm sugar contains higher sucrose of 84.81% than coconut sugar 71.89% and palm sugar 76.86%, so palm sugar is able to provide higher energy. Based on the nutritional content, palm sugar contains higher protein and phosphorus and lower fat than coconut sugar and palm sugar. This shows that palm sugar is better for consumption and more beneficial for body health compared to other sugar's types.

Banten is one of the palm sugar production centers in Indonesia which has palm plants with a large area of 3,040 ha. Sugar palm production in Banten experienced an increasing trend from 2016 - 2018 with an average of 2,691 tons/year (BPS, 2019). The palm sugar center in Banten Province is in Lebak Regency. Palm sugar production in Lebak has experienced unstable growth over the past 3 years, namely 3,527 tons (2016), 2,945 tons (2017) and 3,827 tons (2018). This is due to the presence of plants that have decreased productivity, unproductive plants and seasonal changes.

Lebak Regency contributes 70% palm sugar in Banten. Baduy (Lebak Regency's original tribe) has the potential to produce the best palm sugar in Banten. The advantages of Baduy palm sugar come from organic plants that grow in the hills or mountains so that they are not exposed to chemical fertilizers that can harm health. Baduy consists of two groups, namely the inner Baduy and the outer Baduy. This sugar production is only allowed for the outer Baduy people in Lebak, South Banten. In accordance with applicable customary rules, the Inner Baduy people is only allowed to drink the basic ingredients before turning it into sugar, namely palm wine.

Quality grading for sugar craftsmen is generally only based on sensory properties, namely color and texture. According to BSN (2021), the quality of palm sugar is based on chemical and organoleptic properties such as water insoluble material, water content, ash content, reducing sugar and saccharose sugar as well as sensory tests. One of the problems currently faced by Baduy palm sugar craftsmen is the high diversity and deviation of the chemical characteristic of the the palm sugar produced. This causes the low competitiveness of palm sugar products in the market. The distribution of diversity and deviation of Baduy palm sugar can be analyzed by statistical quality control techniques. Statistical quality control techniques that can be used are the histogram method, control chart and Pareto diagram (Haryanti and Mustaufik, 2020).

Although Baduy palm sugar has been widely sold freely, however, no research has been conducted to determine the quality of this product. This characteristic test is needed to determine the suitability of Baduy palm sugar with Indonesian national standards and can provide recommendations in improving the quality of Baduy palm sugar based on SNI of Palm Sugar.

2. Materials and methods

This research was carried out in three stages of primary data, namely: the first stage is analysis of sample quality characteristics including water insoluble material, water content, ash content, reducing sugar, and saccharose sugar as well as sensory testing; the second stage is data analysis of variance and deviation of the chemical characteristic. Secondary data collection was also carried out to support primary data by conducting field observations and interviews with Baduy palm sugar craftsmen.

Sampling was done by purposive random sampling method with a directed system, which was selected about 20% of the 25 palm sugar craftsmen randomly scattered in Kanekes Village. Each sample of palm sugar from 5 different places was taken as much as 1 kg. Sampling was carried out twice at each place in the same time.

2.1. Analysis of Quality Characteristics

2.1.1. Water Content Analysis (BSN[a], 1992)

The sample was weighed 2 g in a closed weighing glass which weight is known. The samples were dried in an oven at 105oC for 3 hours. The sample was cooled and weighed to a constant weight.

2.1.2. Ash Content Analysis (BSN[a], 1992)

The sample was weighed 3 g into a porcelain dish which the weight is known. Samples were ashed in a kiln at a maximum temperature of 550oC until complete ashing. The sample was cooled and weighed to a constant weight.

2.1.3.Analysis of Insoluble Parts in Water (BSN, 2021)

The sample was weighed 20 g, put into a glass, added 200 ml of hot water, stirred until dissolved. In hot conditions, the water-insoluble part was poured into filter paper which had been dried and weighed. The filter paper was dried in an oven at 105^{o}C for 2 hours, the sample was weighed to a constant weight.

2.1.4. Reducing Sugar Analysis

The main principle is that the sugar will reduce ions Cu^{2+} to ions Cu^{+} when heated (Wirajana et al. 2016). A total of 0.5 mL of the enzyme was mixed with 0.05 g of palm sugar sample. 2 mL buffer fosfat pH 6 0,1 M was added to the solution. The solution mixture was incubated at 70°C for 15 minutes. 0.5 mL of 10 mg/mL main glucose solution and 2.0 mL of DNS reagent were added to the solution. Incubate at 90 °C for 15 minutes. The mixed solution was added with 1.0 mL of 40% potassium sodium tartrate and allowed to stand for 20 minutes. The solution mixture was centrifuged at 5000 rpm for 1 minute. The absorbance of the supernatant was measured at a wavelength of 540 nm. The calculation of reducing sugar content formed at an incubation time of 15 minutes was performed using a UV-Vis spectrophotometer.

2.1.5. Analysis of Sugar as Saccharose (BSN[b], 1992)

50 ml of the reducing sugar filter was pipetted into a 100 ml volumetric flask. 25 ml of 25% HCl was added, a thermometer was installed and hydrolysis was carried out on a water bath. When the temperature reaches 68-70 ^{o}C, the temperature is maintained for 10 minutes. 30% NaOH was added until neutral (pink color) with phenolphthalein indicator. 10 ml of the solution was pipetted and put into a 500 ml Erlenmeyer. 15 ml of distilled water, 25 ml of Luff's solution and a few grains of boiling stone are added to the solution. The solution was heated for 10 minutes then removed and cooled in a bath filled with ice. 10 ml of 20% KI solution and 25 ml of 25% H_2SO_4 solution were added to the solution. Titar with 0.1 N tio solution with 0.5% starch solution as an indicator. The blank determination solution was carried out with 25 ml of water and 25 ml of Luff's solution.

Table 1. List of Sensory Attributes

Appearance	Odor	Texture	Taste
light brown color	palm odor	sticky texture	honey taste
dark brown color	acid odor	light texture	palm sap taste
cloudy appearance	sweet odor	thick mouthfeel	
clear appearance	bitter odor	lumpy mouthfeel	
foamy sugar	metalic odor	liquid texture	
clean sugar	honey odor	sandy texture	
	caramel odor		
	palm sap odor		

2.2.6. Sensory Analysis

Sensory analysis was carried out to compare the sample with commercial palm sugar and also get the ideal product and its characteristics. Sensory testing used the Check-All-That-Apply (CATA) method with 50 untrained panelists who are palm sugar consumers (Giacalone, 2013). Twenty-two sensory attributes tested can be seen in Table 1. The data obtained in the sensory were then analyzed using the XL STAT 2020.5.1 software (Addinsoft, New York, USA, 2021). Additionally, Principal Component Analysis (PCA) was conducted to describe the relationship between products and attributes.

2.2.Data Analysis of Diversity and Deviation of Chemical Characteristic

Data analysis used statistical quality control techniques. Analysis of the chemical characteristics (water content, ash content, insoluble parts in water, reducing sugar and sugar as saccharose) of Baduy palm sugar were carried out using histogram and control chart methods. Analysis of characteristic deviation used Pareto diagram.

3. Results and discussions

3.1. Analysis of Quality Characteristics

3.1.1. Water Content Analysis

Based on the analysis result of water content of the Baduy palm sugar samples, the average result was 8.3749 %b/b. This value is still within the specification limit of SNI Palm Sugar, which the maximum set 10% b/b. The data total that meets the specifications which can be seen in Table 2.

Table 2. Result of Water Content of Baduy Palm Sugar

Sample Number	SNI Standard (%b/b)	Result (%b/b) Test 1	Test 2	Average	Standard Deviation	Description
G1	Maximum 10	10,1273	10,1085	10,1179	0,0094	Abnormal
G2		6,8826	6,8962	6,8894	0,0068	Normal
G3		9,8306	9,8586	9,8446	0,0140	Normal
G4		8,2117	8,1596	8,1857	0,0261	Normal
G5		8,6731	8,6055	8,6393	0,0338	Normal

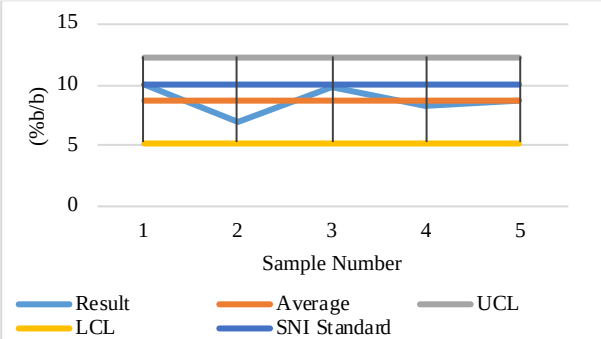

Figure 1. Diversity Water Content

The water content in palm sugar can vary depending on the production process, storage temperature, and conditions when storing the product (Susi, 2013). When compared to sugar from other regions, it tends to be high. The water content of palm sugar in Tasikmalaya is about 5%, while palm sugar in Banyumas is around 6%. Baduy palm sugar is produced at high temperatures, before being packaged, the sugar does not undergo a cooling process for a long time so that steam condensation occurs after the packaging process.

Diversity Data Analysis

Based on the water content analysis, there are two measurement data that are above the SNI Palm Sugar limit, namely G1 and G3 sample. This indicates that it needs the improvement process to improve the quality of Baduy palm sugar, referring to the average water content of 8.3749, because this number still tends to be high compared to palm sugar from other regions.

The standard deviation with the histogram diagram shows a value of 1.3097, where this number indicates the diversity of the data which tends to be high. This can occur due to production method differences or the absence of standards that are used simultaneously the results of the water content obtained tend to vary.

In Figure 1, it can be seen that the scattered data tends to approach the average test results. This means that the distribution of the data tends to be normal. In the control chart, the water content has a Cp value of 1.27 so that the process capability can be good but still needs to be increased.

Improving the quality of this water content can be done by controlling the temperature consistently in the cooking of palm sap. In addition, packaging can also be done after the sugar conditions have dried or cooled. Baduy palm sugar packaging can also be developed using water-resistant materials.

3.1.2. Ash Content

The average ash content of Baduy palm sugar is 1.6773% b/b, still below the specification limit contained in SNI Palm Sugar, which is a maximum of 2% b/b. The percentage of sugar that does not meet the standard is 40% which can be seen in Table 3. The ash content can determine whether the food processing process is good, the higher the ash content, the possibility of process improvement is needed to reduce the amount of inorganic substances in food (Lukito and Guyarto, 2017). The ash content of Baduy palm sugar samples tends to be high when compared to other palm sugar such as Tasikmalaya palm sugar with an ash content of 0.56% and Banyumas palm sugar with an ash content of 0.4% (Susi, 2013)

Table 3. Result of Ash Content of Baduy Palm Sugar

Sample Number	SNI Standard (%b/b)	Result (%b/b) Test 1	Test 2	Average	Standard Deviation	Description
G1	Maximum 2,0	2,0682	2,0625	2,0654	0,0029	Abnormal
G2		1,3196	1,3231	1,3214	0,0017	Normal
G3		1,2394	1,2482	1,2438	0,0044	Normal
G4		1,6333	1,6131	1,6232	0,0101	Normal
G5		2,1375	2,1333	2,1354	0,0021	Abnormal

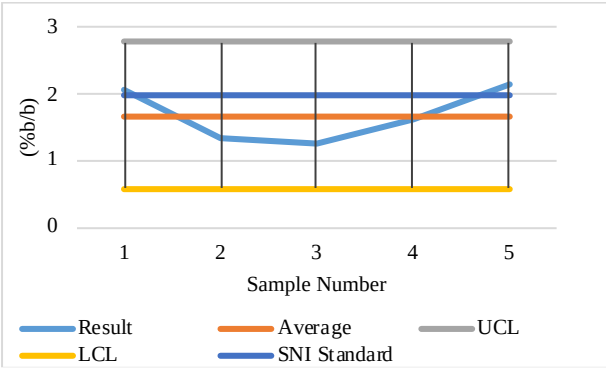

Figure 2. Diversity Ash Content

Diversity Data Analysis

Based on the test results presented in Table 3, there are four test data that pass the SNI specification limit, namely G1 and G5 sample. The standard deviation with the histogram diagram shows a value of 0.4131, where this number indicates the diversity of the data which tends to be low. This high ash content can be caused by production preparation conditions, production conditions, cooking temperatures that are too high (Fadilla, 2021). Temperature control is very necessary to reduce the ash content in Baduy palm sugar. The packaging process is not clean so that there are still organic and non-organic minerals in palm sugar products.

In Figure 2, it can be seen that the scattered data tends to be close to the average. The Cp value of the ash content control chart is 1.24 so this process has high capability, but process control still needs to be done. Ash content is closely related to the mineral content of the product. Poor filtering process can cause high ash content in foodstuffs. The ash content shows how much metal, sand or mineral impurities are in the food. High ash content will be able to affect the taste of a food ingredient.

3.1.3. Water Insoluble Content

The average analysis result is 0.59%, which still below the specification limit for Palm Sugar SNI, where the maximum is 1.0% b/b. The percentage of content that meets the standard is 100% can be seen in Table 4. The palm sugar production process has the possibility of contamination of water insoluble materials into the product. In the extraction process, natural materials can enter the palm sap and pass through the filtering process because the production location is close to the trees. In addition, the processing equipment can be insoluble in water. Although the test results show conformity, the presence of this content can be controlled so that the quality of Baduy palm sugar will increase.

Diversity Data Analysis

All samples are within the limits of the SNI specification with a standard deviation of 0.110 which indicates that the diversity of test results tends to be small. This happens because the materials used have the same characteristics. However, there are still four data that have deviations close to the limit, namely G4 and G5 samples. This parameter can affect other components such as sugar content and product organoleptic characteristics. Improving the quality of Baduy palm sugar can be controlled through a hygienic production process to reduce the potential for contamination of insoluble materials that can enter the product.

In Figure 3, it can be seen that the data is spread close to the average. The Cp value of the water insoluble content is 6 so that the process capability is very good and the quality control is good.

Table 4. Result of Water Insoluble Content of Baduy Palm Sugar

Sample Number	SNI Standard (%b/b)	Result (%b/b)		Average	Standard Deviation	Description
		Test 1	Test 2			
G1	Maximum 1,0	0,5315	0,5309	0,5312	0,0003	Normal
G2		0,4899	0,4894	0,4897	0,0003	Normal
G3		0,5214	0,5238	0,5226	0,0012	Normal
G4		0,7029	0,7068	0,7049	0,0020	Normal
G5		0,7249	0,7244	0,7247	0,0002	Normal

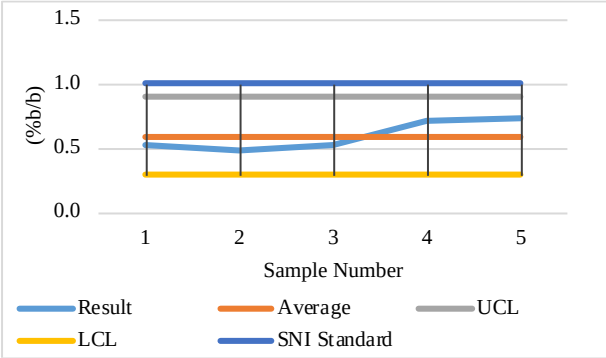

Figure 3. Diversity Water Insoluble Content

3.1.4. Reducing Sugar Content

The content of reducing sugar has a close relationship with the water content. The higher the reducing sugar content, it is easier for food to absorb water from its environment. Table 5 shows that the average reducing sugar is 0.5625%. This figure is still below the SNI limit, which is a maximum of 10% b/b. The percentage of content that meets the standards is 100%. This happens because the average palm sap only contains 0.5-1% reducing sugar.

The tapping process occurs in 3-4 days, it is possible that a fermentation process occurs which causes a decrease in reducing sugar levels. The reducing sugar content in Baduy palm sugar can occur due to changes in the pH of the palm sap or during cooking. A decrease of pH can increase the inversion reaction or change non-invert sugar (saccharose) to invert sugar (reduction). The speed of the inversion reaction is strongly influenced by the cooking temperature, cooking time, and the pH of the solution.

Table 5. Result of Reducing Sugar Content of Baduy Palm Sugar

Sample Number	SNI Standard (%b/b)	Result (%b/b)		Average	Standard Deviation	Description
		Test 1	Test 2			
G1	Maximum 10,0	0,5700	0,5252	0,5476	0,0224	Normal
G2		0,5670	0,5753	0,5712	0,0042	Normal
G3		0,6206	0,6484	0,6345	0,0139	Normal
G4		0,5106	0,5182	0,5144	0,0038	Normal
G5		0,5262	0,5639	0,5451	0,0189	Normal

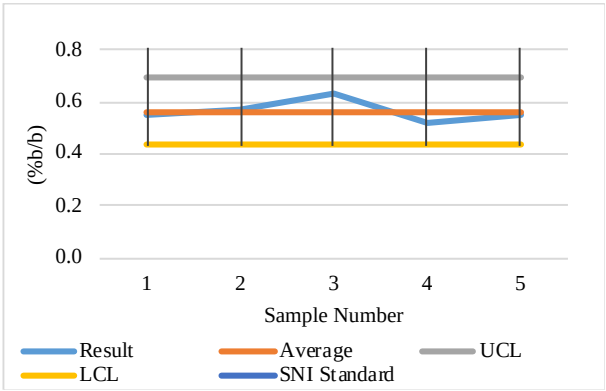

Figure 4. Diversity Reducing Sugar Content

Diversity Data Analysis

All palm sugar samples meet the standards that have been set. Even the test results of reducing sugar are very low compared to the SNI specifications. The reducing sugar parameter does not need special attention in improving the quality of Baduy palm sugar. The standard deviation shows a value of 0.0450 which indicates that the tested data has low diversity. The results of reducing sugar content are strongly influenced by the pH of the sap. The data above has little diversity because the raw material for Baduy palm sugar comes from palm sap which both grow in the Baduy area.

The distribution of data on the control chart in Figure 4 is close to the average value. All samples contain reducing sugars that are not much different and the testing process has been carried out properly. This is in line with the Cp value of 1.5, so that the process capability can be said to be very good and it is in good statistical control quality.

3.1.5. Saccharose Sugar Content

The content of saccharose is closely related to the content of reducing sugars. The lower the saccharose content, the higher the reducing sugar content. Based on Table 6, it can be concluded that all Baduy palm sugar samples do not meet the SNI standard, which the maximum is 77%. The average value of the saccharose sugar analysis was 85.78%. This is in line with the low reducing sugar content, which is below 1%.

Saccharose levels in foodstuffs can affect the texture. The higher the saccharose sugar content, the harder the texture will be. The decrease in the saccharose sugar content can be done by increasing the cooking time (Lay and Heliyanto, 2011). However, this can change the color into darker so that a preservative such as sodium metabisulfite is needed in accordance with SNI limits.

Table 6. Result of Saccharose Sugar Content of Baduy Palm Sugar

Sample Number	SNI Standard (%b/b)	Result (%b/b) Test 1	Test 2	Average	Standard Deviation	Description
G1	Maximum 77	86	86	86,0000	0,0000	Abnormal
G2		85,3	85,3	85,3000	0,0000	Abnormal
G3		84,2	84,2	84,2000	0,0000	Abnormal
G4		86,9	86,9	86,9000	0,0000	Abnormal
G5		86,5	86,5	86,5000	0,0000	Abnormal

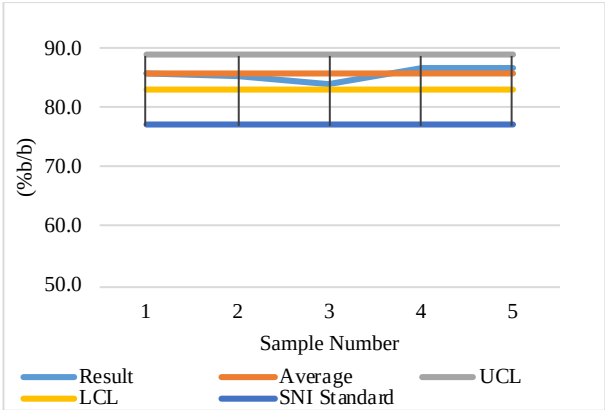

Figure 5. Diversity Saccharose Sugar Content

Diversity Data Analysis

All samples did not meet the specifications or standards set by SNI. The distribution of data does not match the normal distribution and shows varying frequencies. The standard deviation shows a value of 1.0663, this means that the diversity of the data tends to be high. The amount of sugar content as saccharose is influenced by the waiting time for the hardening process, cooking time and reducing sugar content. The amount of sugar content as saccharose is influenced by the waiting time for the hardening process, cooking time and reducing sugar content.

The uniformity of product quality can be controlled by making a Standard Operating Procedure (SOP) for palm sugar production so that the craftsmen have a reference and do not change any time. Saccharose sugar affects the density of sugar. The higher the saccharose sugar content, the denser and tougher the product will be. This causes the standard setting of 77%, so that sugar is easily crushed when it will be processed as an additive.

The Cp value of the control chart in Figure 5 is 5.7, so the process capability is very good. Generally, palm sugar can contain 80-90% total sugar, in other words the total sugar content in Baduy palm sugar can be said to be normal (Christina et al. 2017). However, to increase saccharose sugar, producers must reduce the amount of product impurities.

Table 7. Sensory Analysis Sugar Profile

Sugar Name	Sample Code	Origin of Sugar
Probolinggo palm sugar	256	Probolinggo
Javara palm sugar	741	Bekasi
Cap badak palm sugar	397	Tangerang
Sample G2	163	Outer Baduy
Sample G4	574	Outer Baduy

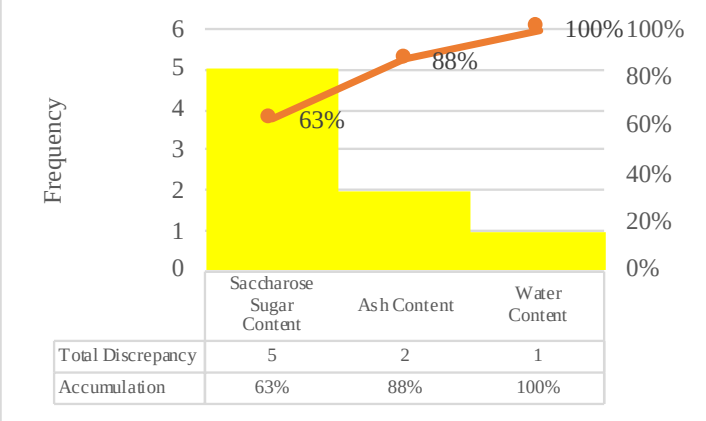

Figure 6. Pareto Diagram Chemical Characteristics of Baduy Palm Sugar

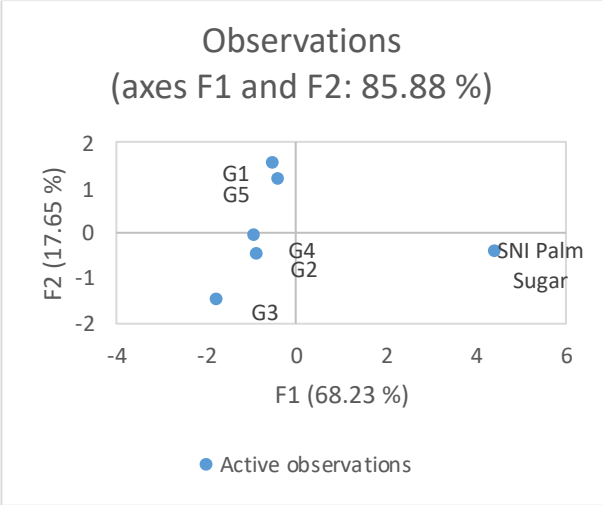

Figure 7. Principle Component Analysis of Samples Based on Chemical Characteristics

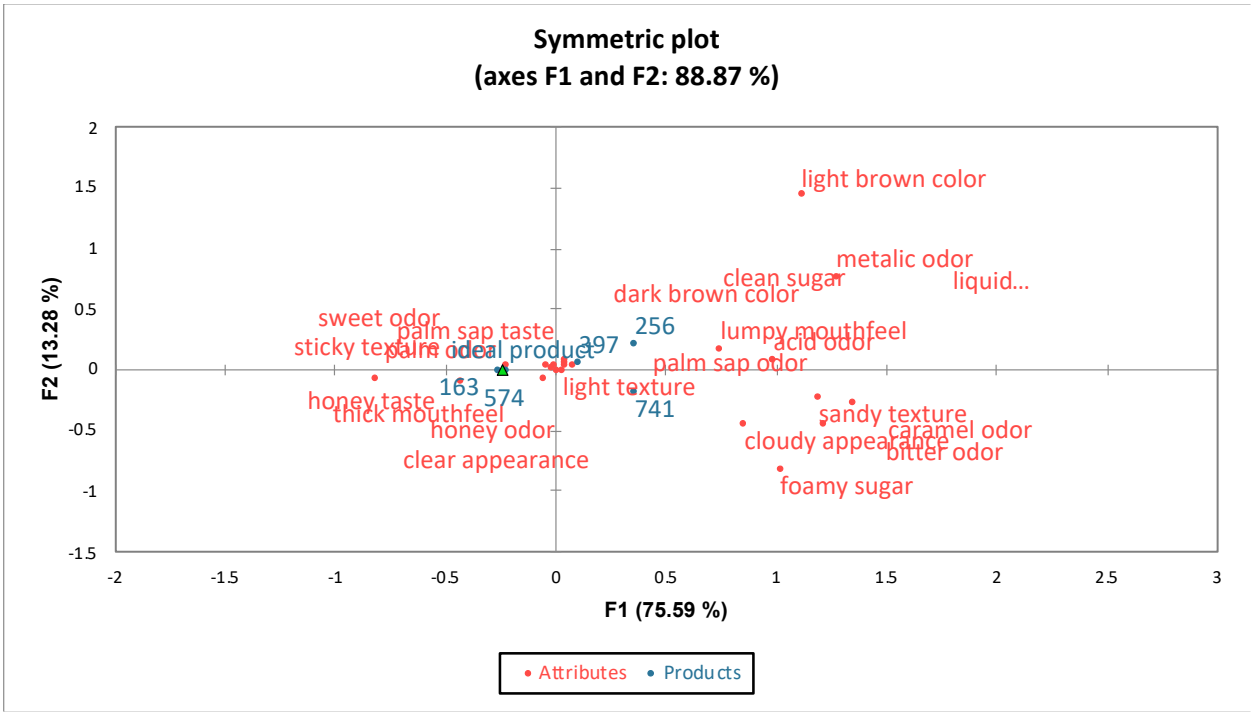

Figure 8. Symetric Plot Sample

3.2. Deviation Data Analysis

The total discrepancy in the Pareto diagram of Figure 6 shows a value of 63%, where the highest to smallest deviations are sugar content as saccharose, ash content and moisture content. Meanwhile, other parameters are free from deviations or meet the specifications set by SNI Palm Sugar. This indicates that the three parameters must be improved immediately in order to improve the quality of Baduy palm sugar.

3.3.Sensory Analysis

PCA is conducted to determine the sample that meet with SNI (BSN, 2021). The Biplot in Figure 7 shows the relationship between samples and chemical characteristics compared to Standard. The selection of sample codes G2

and G4 were caused by these samples had the best results of chemical analysis compared to other samples. The sugar sample used in sensory analysis can be seen in Table 7.

In Figure 8, based on consumer, the ideal product has the attributes of dark brown color, clear appearance, clean sugar, palm odor, sweet odor, sticky texture, honey odor, palm sap odor, honey taste and palm sap taste. It can be seen that palm sugar with sample codes 574 and 163 is in the same quadrant as the ideal product. The two samples are Baduy palm sugar samples which have the attributes of sweet aroma, palm sap taste, palm aroma, honey taste, sticky texture, and the appearance of clear sugar.

Meanwhile, sugar with code 397 which is considered as Badak sugar and code 256 which is considered as Probolinggo sugar is in quadrant two. Both of these sugars have the attributes of clean sugar, light texture, light brown color, palm sap aroma, and metallic aroma. While the code 741 which is Javara sugar is in quadrant 3 has the attributes of foamy sugar, light texture, sandy texture, bitter aroma, and caramel aroma. In this plot, it can be concluded that the panelists considered that Baduy palm sugar was close to the ideal palm sugar characters.

4. Conclusions

The average value of the chemical characteristics of Baduy palm sugar, namely water content, ash content, water insoluble content, reducing sugar content and saccharose sugar content respectively were 8.3749 %bb, 1.6773%bb, 0.5946%bb, 0,5625 %bb and 85.78%bb. The percentage of discrepancy in the analysis of the chemical characteristics of Baduy palm sugar with SNI 3743-2021, namely the sugar content as saccharose reaches 63%, ash content reaches 15% and water content reaches 12%. Baduy palm sugar, namely G2 and G4 are sugars that are most similar to ideal palm sugar compared to other commercial palm sugar. It means Baduy palm sugar has good potential to be traded in a wider market and can compete with other commercial palm sugar**.**

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DETERMINATION OF PROTEIN VALUE AND WATER ABSORPTION IN CHICKPEA (*CICER AIIETINUM L.*) SEEDS DURING GERMINATION

Aynur Ay Tezcan[1], Sukru Karatas[2]✉, Indrani Kalkan[3]

[1] *Department of Nutrition and Dietetics, Istanbul Aydin University, Kucukcekmece, 34295 Istanbul, Turkey;*
[2] *Department of Nutrition and Dietetics, Istanbul Arel University, Cevizlibagi, 34200 Istanbul, Turkey;*
[3] *Department of Nutrition and Dietetics, Istanbul Medipol University, Kavacik, 34810 Istanbul, Turkey.*
✉*karatassukru@gmail.com*

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ABSTRACT

Protein and water absorption values were investigated in Cagatay and Gokhoyuk varieties of chickpea (*Cicer arietinum*) seeds after germination. The crude protein content were determined as 29.14% by dry weight in Cagatay and 26.13% in Gokhoyuk varieties respectively. Following imbibition, the amount of water absorption increased up to approximately 50% to 60% in both varieties. Crude protein content increased by 10.4% and 14.2% and carbohydrate content increased by 0.45% and 0.41% respectively for Cagatay and Gokhoyuk varieties of chickpea seeds. In addition, total viability of microorganisms, yeasts and molds were also determined after germination. Inactivation of microorganisms (including yeast and molds) to acceptable safe limits were noted, after soaking in hot water at 95^0 C for five minutes.

1.Introduction

Chickpea (*Cicer arietinum* L.) is one of the major source of plant dietary protein and particularly important for vegetarian segments of the world population. It is also used as a protein supplement in Europe and Australia and has been suggested to have significant effect on dietary quality, satiety and bowel health (Glemente and Olias, 2017; Murty *et al.*, 2010). Chickpea is an ancient crop and has been grown and consumed in tropical, sub-tropical and temperate regions for centuries. Chickpea is valued for its nutritive properties, economic source of protein and potentially health-beneficial bio-active compounds (Sofi *et al*., 2020).

Chickpea is used exclusively as a food item in many countries (Gupta *et al.*, 2017; Jukanti *et al.*, 2012) and its traditional uses include boiling, roasting, canning or processing into humus, salad, soup and stew. It is also known for its use in cosmetic and herbal medicine (Mahjour *et al.*, 2018; Ahmadi *et al.*, 2020). Apart from bioactive compounds, chickpea also contains high quantities of insoluble and soluble fibers which are associated with lowering of blood cholesterol levels, thereby lessening the risk of heart disease, stroke, type 2 diabetes and regular bowel movements (Hall *et al.*, 2016; Jukanti *et a*l., 2012).

Several studies have been conducted regarding amino acid content (Khan *et al*.,1995; Sanjeewa *et al*., 2010; Gupta *et al.*, 2018*)* chemical composition (Viveros *et al.*, 2001; El-Adawy 2002; Suhasini and Malleshi 2003), carbohydrate content (Hawkins and Johnson., 2005) and mineral content (Nestares *et al.*, 1999) in non-germinated chickpea seeds.

The aim of this research was to investigate the water absorption and changes in crude protein and carbohydrate contents in chickpeas

during germination at 5° C, total viability of microorganisms in the samples and extent of their inactivation following hot water treatment at 95^0 C for five minutes.

2. Materials and methods

2.1.Chickpea samples used in the study

Gokhoyuk (Vd.1.104) and Cagatay (Vd.1.101) varieties of chickpea seeds (*Cicer arietinum* L.) harvested in October 2020, were obtained from Blacksea Agricultural Research Institute of Turkey based in Samsun. The samples were obtained in dry form (vaccum packed) in polyethylene bags. Nutrient composition of the chickpea varieties are shown in Table 1. The collected samples were stored in refrigerator for physical and chemical analysis.

Table 1. Nutrient composition of Cagatay and Gokhoyuk chick pea varieties

Genotype	Protein (%)	Ash (%)	Ca (ppm)*	Cu (ppm)	Fe (ppm)	K (ppm)	Mg (ppm)	Mn (ppm)	P (ppm)	Zn (ppm)
Vd.1.101 (Cagatay)	21.82	2.96	1434.50	11.68	35.48	12536.71	1712.69	27.77	3172.46	46.82
Vd.1.104 (Gokhoyuk)	24.05	2.80	1719.43	10.36	41.17	12751.74	1743.84	27.30	3502.05	39.49

2.2. Preparation of the samples for germination procedure

150 g of samples were weighed from each chickpea sample and placed in separate conical flasks. 600 ml of double distilled water was added to each and the flasks and covered with stretch film. The flasks were stored in the refrigerator at 5°C for 6 days. On the 7^{th} day, the flasks were taken out, 50 g of moist samples were weighed out from each variety of chickpeas and placed in covered petri dishes and stored in the dark at 21°C temperature for 48 hours for germination (Ferreira et al., 2019). Duplicate samples were prepared, from each chickpea variety, for germination. During germination, the samples were sprayed with distilled water twice in a day. Protein contents were determined on 1, 2, 3, 4, 5 and 6^{th} day of soaking in water (Kajihausa *et al.*, 2014).

2.3. Determination of crude protein

The protein content, of chickpea samples, was measured by the Dumas Method, an official AOAC method (993.13) using a LECO FP 828 nitrogen analyzer (Jung *et al.*, 2003).

The Jones conversion factor of 6.25 was used to convert the nitrogen (N) content as percentage (%) of protein in the sample N % x 6, 25 = protein % (Chang and Zhang., 2017; Jones., 1931)

The amount of crude protein in the samples was calculated as follows:

Protein content on a wet weight basis / dry solids (Chang and Zhang., 2017)

Table 2 shows the crude protein contents of the two varieties of chickpeas.

Table 2. Effects of soaking and germintation on crude protein content of chickpea samples

Chickpea Variety	Before Germination	After Germination	After Germination (water soluble ash)	After Germination (water insoluble ash)
Cagatay Chickpea Vd.1.101	3.14	2.87	77.81	22.19
GokhoyukChickpea VD.1.104	3.13	2.72	81.02	18.97

2.4. Determination of moisture content

The moisture content, of the chickpea samples, was determined from the weight loss, following the evaporation process (Pearson., 1973). Figure 1 indicates the water absorption by the chickpea samples on 1, 2, 3, 4, 5 and 6th day of soaking in water.

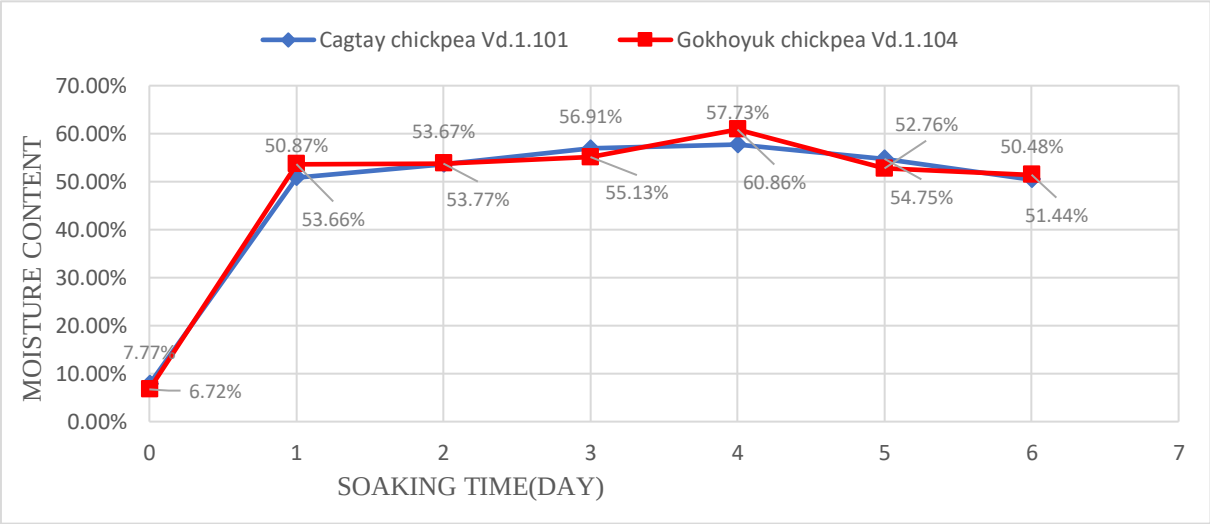

Figure 1. Changes in moisture content in chickpea during soaking

2.5. Determination of the ash content

The ash content, of chickpea samples, was determined by the standart AOAC method 923.03. which is based on the principle of calculating the amount of inorganic matter remaining as a a residue after the burning of organic materials (FSSAI,2016).

The formulation for calculating the ash content is as follows:

Total ash on dry basis (% by weight) = $\frac{W_2-W}{W_1-W} x100$

Where,

w_2- weight of the dish with ash (in g)

w- weight of the empty dish (in g)

w_1- weight of the dish with the dry sample (in g)

2.7. Determination of alkalinity of water-soluble ash in chickpeas after germination

The alkalinity of water-soluble ash, in germinated chickpea samples, was determined using the AOAC method 900.02. 10 ml of HCl was added to the water-soluble ash sample. It was titrated with 0.1N NaOH until a color change occurred using methyl orange indicator.

2.6.Determination of water-soluble and ınsoluble ash in chickpeas after germination

After germination process, water-soluble (organic) and insoluble (inorganic) ash amount in chickpeas was determined according to AOAC method 900.02. The chickpea samples were incinerated in the ash furnace. After combustion or complete acid-facilitated oxidation of organic matter, the organic ash was dissolved in boiling water and filtered to obtain the insoluble fraction (Harris and Marshall., 2017).

The water soluble and insoluble ash contents of chickpea samples before and after germination are given in Table 3.

The NaOH volume required for titration was used to determine the alkalinity of water soluble ash in the sample (Harris and Marshall., 2017).

2.8. Determination of total bacteria, yeast and mold

Fresh germinated (sprouted) and blanched (vaccum packed in a vacuum device, kept in a 95°C water bath for 5 minutes and cooled)

chickpea samples from both varieties were used for analyses. 10 g of each sample (fresh and blanched) was blended and homogenized (Stomacher AES Smasher) with 90 ml sterile peptone solution (0.1%) and serially diluted using sterile peptone water. Plate Count Agar (PCA) was used for total plate count after incubation at 35°C for 24 hours (Livingstone *et al.,* 1992). Yeast Extract Glucose Chloramphenical (YGC) agar was used for yeast and mold count after incubation at 25°C for 5 days (AOAC 997.02., 2000).

3. Results and discussion

3.1.Effect of germination on chemical composition of chickpea

Germination may be characterized as a complicated bio-process consisting of three separate stages, depending on the seed's microstructure and water absorption capacity. The first stage is the imbibition stage, characterized by rapid water uptake. In the second stage, water intake decreases; however, with the sprouting of roots out of the seed coat in the third stage, water intake increases again (Ohanenye et al., 2020). Germination, may be also identified as an effective and inexpensive method for improving the nutritional quality of grains and legumes (Khattak et al., 2007).

Following 48 hours of germination, protein content of chick pea was found to increase substantially (from 18.48% to 24.46%). The close result was observed for Gamma Aminobutyric Acid (GABA) to increase from 6.42 mg/100 g to 24.576 g/100g (Ferreira et al., 2019).

Germination process carried out under optimal conditions increased antioxidant activity and total phenolic compounds content of desi chickpea seeds significantly by (5707-14,361mmol TE /100g sample) and (97 to 201 mg GAE /100g sample) respectively. In addition protein content was also found to increase by 16.4% as compared to un-germinated samples ($p < 0.05$). Germination process was suggested to be an effective alternative method for increasing the nutritive value of chickpeas (Domínguez-Arispuro et al., 2018).

In this study, the crude protein content of non-germinated Cagatay chickpea variety was found to be 25%, ash content 3.14% and moisture content 7.77%. For the Gokhoyuk chickpea variety, crude protein was 23.41%, ash 3.13% and moisture content 6.72%. Similar results were reported in a study conducted by Alajaji et al., (2006) on non-germinated chickpea having crude protein content of 23.64%, ash 3.72% and moisture content of 10.35% (Alajaji and El-Adawy., 2006).

In addtion to that, samples from two chick pea varieties (Cagatay and Gokhoyuk) were kept soaked in water for 6 days followed by a germination period of 2 days (48 hours). Significant increases in moisture and protein content was noted in both varieties. The highest crude protein content (28.83%) was recorded on the 5th day of soaking for the Cagatay chickpea variety. On the other hand, the highest crude protein content (30.53%) was detected on the fourth day for Gokhoyuk chickpea samples as given in Table 2 and Figure 1. As likewise, after 2 days (48 hours) of germination, an increase of 14.20% in the amount of crude protein for the Cagatay chickpea variety and an increase of 10.40% for the Gokhoyuk chickpea variety were observed.

Similar results were reported by Ferreira et al., (2019); In their study, the amount of protein content of the chickpea sample initially was 18.4% but increased to 24.6% after 48 hours of germination (Ferreira et al., 2019). In another study conducted by Xu et al., (2019), the crude protein content of chickpea samples increased by 3.39g /100g, after 6 days of germination (Xu et al., 2019).

The increased amount of crude protein during germination, was explained by the synthesis of enzymes by the seed during germination and the compositional change resulting from the degradation of other constituents (Xu et al., 2019). In a study conducted by Dipnaik and Bathere., (2017), the protein content of chickpeas increased from 32% to 48% and a significant increase in alanine transaminase activity was observed after 12 hours of soaking and germination, compared to

raw chickpea samples (Dipnaik and Bathere., 2017).

The highest moisture rate was found to be 57.73% for Cagatay and 60.86% for Gokhoyuk chickpea varieties on the fourth day. As of the fifth day, a decrease in the moisture content was observed for both chickpea varieties as given in Figure 1. At the end of 48 hours of germination, moisture content of Cagatay chickpea type was determined as 56.60% and for Gokhoyuk chickpea it was determined as 52.36%.

Similar results were observed in the study conducted by Kajihausa et al., (2014), moisture and protein content of flour obtained from sprouted sesame seeds increased with soaking and sprouting. The seeds were immersed in water for 16 hours and germinated for a period of 36 hours. Moisture content increased from 3.97% to 4.99% and protein content increased from 26.09% to 47.64% in the 10th hour in the soaked samples. It was found that protein content increased from 26.09% to 45.64% (at 8th hour) and 48.70% (at 12th hour) holding time. However, with increasing the soaking time further, protein content decreased to 48.27% (14th hour) and 47.81% (at 16 hour) holding time, respectively (Kajihausa et al., 2014).

In a study conducted by Fouad and Rehab., (2015) on lentils, germination was found to increase moisture content (from 25.42% to 39.25%), crude protein content (7.33% to 12.60%), and ash content (2.77 to 3.35%). On the other hand a decrease in total carbohydrate content (from 41.69% to 48.70%) and fat content (2.2g/100g to 0.90g/100g) was noted. It was stated that the increase in the amount of moisture was related to the increase in the number of hydrated cells, as the germination period extended. On the other hand, the decrease in the amount of fat it was associated with the increased lipolytic enzyme activity during germination. Finally, the authors reported that the decrease in the amount of ash was associated with the increase in phytase enzyme activity. Fat and total carbohydrate ratios that decreased during germination were attributed to the possibility of being used as an energy source during the germination phase (Fouad and Rehab., 2015).

In this study, ash content in non-germinated chickpeas was determined as 3.14% and 3.13% for Cagatay and Gokhoyuk varieties, respectively. The germination process resulted in a reduction in the ash content of both chickpea varieties this may be due to the amount of carbohydrate increased during germination (Table 3).

Table 3. Ash content of chickpea before and after germination (%)

Chickpea Varieties	Crude Protein Content (%) During Soaking							Crude Protein Content (%) After Germination (2 days)
Days	0	1	2	3	4	5	6	
Cagatay Vd.1.101	25.0	22.79	25.14	25.52	28.74	28.83	23.92	29.14
Gokhoyuk Vd.1.104	23.41	24.06	23.40	26.52	30.53	25.08	25.43	26.13

Similar results were found by Ferreira et al., (2019) in their study with chickpeas. After 48 hours of germination, it was determined that the amount of ash, which was 3.3% in non-germinated form, decreased to 3.0% at the end of germination (Ferreira et al., 2019b).

In a study conducted by Xu et al., (2019) a small increase in ash content (3.13g/100g to 3.26g/100g) was observed on chickpea flour obtained after 6 days of germination (Xu et al., 2019). In another study performed by the same authors, an increase in the amount of ash content

(4.72g/100g to 5.07g/100g) was observed in protein isolates obtained from germinated chickpeas (Xu et al., 2020). The results were not consistent with our research this may be related with varieties of chickpeas.

On the other hand, Cornejo et al., (2015) reported a slight decrease in the amount of ash content (2.85g/100g to 2.35g/100g) in brown rice, at the end of the 48-hour germination process (Cornejo et al., 2015).

For those living in tropical and subtropical regions, legumes are included in diets as an indispensable protein source, as animal foods are consumed in limited quantities. In addition, chickpeas are considered a good source of carbohydrates, minerals and vitamins for vegans and vegetarians who meet most of their protein needs from legumes. In another study conduced by Oghbaei and Prakash., (2020) chickpeas were examined for nutritional quality after germination in distilled water supplemented with iron and zinc. A control sample was maintained consisting of chickpeas germinated in plain water. Ash content of control sample was 2.71g/100g whereas ash content of chickpeas germinated in water supplemented with 100 mg and 200 mg Fe was 3.11 g/100g and 4.11g/100g, respectively. Similarly, a significant increase in ash content (3.63g/100g) was also observed in chickpeas germinated in water supplemented with 100mg zinc (Oghbaei and Prakash., 2020).

3.2. Microbial load of germinated chickpea and effect of blanching

Following the Escherichia coli epidemic outbreaks in Germany and France in the spring and summer of 2011, the European Food Safety Authority (EFSA) assessed the public health risks of Escherichia coli producing Shiga toxin and other pathogenic bacteria that could contaminate sprouted seeds that could pose microbial food safety concerns, and therefore recommended that the general EU food safety hygiene guidelines be followed throughout the food chain, up to the final product (EFSA., 2011).

Bergspica et al. (2020) investigated Shiga toxin producing Esherichia coli (STEC), Salmonella spp. and Listeria spp. in 45 samples of microgreens, sprouted and un-sprouted seeds from retail markets. Listeria monocytogenes was not detected in any of the samples tested. In addition, Listeria innocua was detected in two (4.4%) of the samples. Three (6.7%) dried sprouted samples were found positive for STEC virulence genes. Salmonella spp. were detected in one sunflower seed sample (2.2%). According to the results of the study, it was reported that microgreens and seeds were generally safe, but since E.coli virulence genes were identified in 3 samples of dried sprouts, there could be some concerns regarding consumption of dried sprouts (Bergspica et al., 2020).

In this study, microflora changes that occurred during germination were examined and colonies were counted for yeast, mold and total bacteria which are important for health and were responsible for food spoilage. Microbiological analyses indicated that there were no increase in the numbers of yeast and molds during germination, however number of total bacterial colonies were too high to be counted by naked eyes. Total bacterial (plate) count were found to decrease to safe levels ($<10^3$cfu/g) after blanching.

4.Conclusions

It may be concluded that consumption of germinated chickpeas is of significant importance from health perspectives due to its increased protein content, as compared to its non-germinated form. On the other hand, boiled or cooked forms of chickpeas must be preferred instead of non-cooked ones in terms of microbial contamination.

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IMPACTS OF SOAKING TIME AND STEAMING TIME ON PROXIMATE, VITRO-STARCH DIGESTIBILITY AND AMYLOSE CONTENT OF SHORT, MEDIUM AND LONG RICE GRAIN TYPE

M.S. Sanusi[1]✉, J.B. Hussein[2]

[1]*Department of Food Engineering, Faculty of Engineering and Technology, University of Ilorin, Ilorin, Nigeria*
[2]*Department of Food Science and Technology, Modibbo Adama University, Yola, Adamawa State, Nigeria.*
✉*sanusi.ms@unilorin.edu.ng*

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ABSTRACT

This study aimed to evaluate the impacts of soaking time, steaming time and rice grain type (FARO 15 (short rice grain), FARO 60 (medium rice grain) and FARO 62 (long rice grain) on the proximate composition, in-vitro starch digestibility (rapidly digestible starch (RDS), slowly digestible starch (SDS), resistance starch (RS) and glycemic index (GI)), amylose and amylopectin contents of rice using Taguchi design. A Pareto chart was used to identify the most significant process factor. The highest crude protein content (12.14%), ash content (1.06%) and carbohydrate content (83.85%), amylose (25.06%) and GI (67.24%) were obtained in long grain rice while the highest RS (22.46%) and amylopectin (81%) were obtained in medium grain rice. The type of rice grain had the most significant impact on moisture, carbohydrate, ash, RS and GI. In contrast, the interaction of rice grain type and soaking time had the most significant influence on crude protein, fibre and SDS. The interaction of rice grain type and steaming time influences amylose and amylopectin contents. This study provides valuable information for rice processors on the nutritional and starch digestibility of three different classes of rice varieties and their optimum processing conditions.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most consumed staple foods globally, which contributes to the nutrients intake in the diet of humankind (Sanusi *et al.*, 2017). Rice consumers often consume short grain, medium grain or long grain rice as one of the primary sources of carbohydrates and a staple food for almost half of the world's population. The nutrient quality of rice is usually affected by post-harvest handlings, such as parboiling, which involves soaking, steaming and drying (Ayamdoo *et al.*, 2015). Parboiling is a simultaneous hydration and heat treatment executed on paddy rice to improve rice quality. The parboiling process is a crucial hydrothermal process that significantly enhances rice milling quality, cooking characteristics, and nutrient retention (Ejebe *et al.*, 2015; Sanusi and Akinoso, 2020). Parboiling results in alteration of rice, which causes the vitamins and minerals to be transferred from the germ and aleurone into the starchy rice endosperm. These transfigurations, however, cause a reduction in whiteness and give de-husked rice a more pellucid appearance during polishing (Ejebe *et al.*, 2015). The parboiling process includes soaking paddy rice in hot water, steaming to ensure starch gelatinization, and drying before de-husking and milling operations (Onmankhong *et al.*, 2020; Sanusi and Akinoso, 2022). Relevant information on starch digestibility, glycemic index, resistance starch, amylose and amylopectin of rice are important factors influencing proper nutrition and

prevention of diseases. According to Nakayama *et al.* (2017), rice with high amylopectin content and glycemic content could increase the risk of diabetes because of the high rate of digestible starch that leads to a rise in blood glucose level within the short term. Resistance starch is also directly proportional to the amylose content (Khatun *et al.*, 2019). This means an increase in amylose content and resistance starch contents resulted in a lower glycemic index. Zafar (2018) stated that resistance starch reduces the postprandial blood glucose level due to its dietary fibre in the gastrointestinal. Several studies have been reported on the influence of parboiling conditions on rice milling quality and nutritional composition. Ayamdoo *et al.* (2015) said that parboiling causes changes in the physical and chemical composition of Jasmine 85 and NERICA 14.

Buggenhout et al. (2013) reported that parboiling could significantly influence rice's milling and physicochemical properties. Ebuehi and Oyewole (2007) examined the influence of parboiling on the physical properties of raw ofada and aroso rice varieties, while Min et al. (2014) examined the effects of soaking and cooking on antioxidant compounds from red, purple and brown genotypes. Paiva *et al.* (2016) reported the effect of polishing and parboiling on the nutritional properties of red and black rice varieties. Sivakamasundari *et al.* (2020) reported the effect of parboiling on the amylose and amylopectin Karaya cultivar and waxy cultivar. Sanusi and Akinoso (2021) studied the impacts of parboiling variables on the energy consumption and quality of brown rice. However, literature is sparse on the impacts of soaking time, steaming time and rice grain type on the proximate composition, in-vitro starch digestibility, amylose and amylopectin contents of rice. The study of the influence of soaking temperature and steaming time during parboiling of different rice varieties on the proximate composition, in-vitro starch digestibility, amylose and amylopectin contents of could provide an avenue to understand the impact processing variables and conditions on the nutritional contents of the rice varieties. It could as well be useful for commercialization purposes based on rice grain type optimum conditions for better nutrient attributes. The rice varieties under consideration in this study are short rice grain, medium rice grain and long rice grain. The selection of rice varieties with superior nutritional content is critical for consumers and producers. This study is expected to provide valuable information to rice producers and consumers on the impact of soaking time, steaming time and rice grain type on the proximate composition, in-vitro starch, amylose and amylopectin contents. Therefore, the objective of this study was to evaluate the impact of soaking time (4 h, 5 h and 6 h), steaming time (30 min, 35 min and 40 min), and rice grain type (FARO 15, FARO 60 and FARO 62 on the proximate composition, in-vitro starch digestibility (Readily digestible starch (RDS), slowly digestible starch (SDS) and resistance starch (RS) and glycemic index (GI), amylose and amylopectin contents of rice.

2. Materials and Methods

2.1. Materials

This study used three varieties of paddy rice; FARO 15 (short rice grain), FARO 60 (medium rice grain) and FARO 62 (long rice grain), which were purchased grain quality laboratory NCRI, Nigeria.

2.2. Experimental design

Taguchi experimental design was used to interact soaking time, steaming time and rice grain type on proximate composition, in-vitro starch digestibility, amylose and amylopectin contents of rice, as shown in Table 1. The experimental design consisted of three independent variables; soaking time (4, 5 and 6 h) and steaming time (30, 35 and 40 min) and rice grain type (FARO 15, FARO 60 and FARO 62). The dependent variables were proximate composition (moisture content, crude protein, crude fat, crude fibre, ash and carbohydrate), in-vitro-starch (Readily digestible starch (RDS), slowly digestible starch (SDS) and resistance starch (RS)), glycemic index (GI), amylose and amylopectin contents of the rice.

Table 1. Taguchi experimental design for the impact of rice type, steaming time and soaking time

Process Factors	Unit	Level 1	Level 2	Level 3
Rice grain type		1	2	3
Soaking Time	h	4	5	6
Steaming Time	min	30	35	40

where; 1 is the FARO 15; 2 is the FARO 60 and 3 is the FARO 62

2.3. Paddy rice processing

The paddy rice of FARO 15, FARO 60 and FARO 62 were cleaned to remove foreign materials using rice cleaner. Rice grain type of 6 kg each was used and steeped in a dual-powered rice parboiler, where water was heated to 80°C. FARO 15 was first introduced and soaked for 4 h, 5 h and 6 h, respectively. After achieving 30% paddy rice moisture content, the paddy rice was steamed at 100°C for 30 min, 35 min and 40 min, respectively. The same procedure was repeated for FARO 60 and FARO 62 based on the Taguchi experimental design. The steamed rice paddies of FARO 15, FARO 60 and FARO 62 were dried to 14% moisture content (wb) using an oscillatory rice dryer. To obtain milled rice, the dried paddy rice of each type was milled using a rice miller (Model MLNJ-15-13, India). The resulting milled rice for each rice grain type under different processing conditions was analyzed for their proximate composition, in-vitro-starch, amylose and amylopectin contents. Pareto chart was used to determine the significant processing parameters (soaking time, steaming time and rice grain type. In the pareto chart a reference line is drawn on the chart to indicate the P=0.05 threshold for statistically significant effect.

2.4. Proximate composition

The proximate composition in terms of the moisture content, crude protein, crude fat, ash and crude fibre were analyzed using AOAC (2016), while the carbohydrate content was determined using the difference method.

2.5. Amylose and amylopectin content

The amylose content of the rice samples was determined by using the method of Al-Rabadi et al. (2009) while amylopectin was determined using equation 1

$$Amylopectin = 100 - (amylose) \quad (1)$$

2.6. In-vitro starch digestibility

In vitro starch digestibility was determined using a modified Englyst method. 100 mg of dried sample was suspended in 2 mL 0.1M sodium acetate buffer (pH 5.2) and incubated at 37°C for 5 min before the addition of 100 µL of diluted α-amylase and 100 µL of diluted amyloglucosidase. Samples were incubated in a shaking water bath at 37°C for 120 mins. 50 µL aliquots were pipetted into 2 mL micro-centrifuge tubes containing 400 µL of cold ethanol after 20, 60 and 120 min of digestion. The glucose released at each interval was determined using the glucose oxidase / peroxidase method and was converted to the percentage of starch hydrolyzed by multiplying by 0.9. Starch digested at 20 min is defined as rapidly digestible starch (RDS), starch digested between 20 and 120 min is defined as slowly digestible starch (SDS), and starch not digested after 120 min incubation is defined as resistance starch (RS).

2.7. Glycemic index

The glycemic index was calculated from the ratio of the increment area under the curve of the glucose response curve of test food sample containing 50 g of available carbohydrate and the same amount of reference food and expressed as a percentage.

2.8. Statistical analysis

All analysis was carried out in triplicate and the experimental data were reported as mean$\pm$ standard deviation (SD). The data were subjected to analysis of variance (oneway ANOVA). The significant difference was determined by Duncan's (significance difference) test $p \leq 0.05$ and Pareto chart was

used to determine the most significant factor at $p \leq 0.05$.

3. Results and Discussions

3.1. Proximate Composition

Table 2 shows the effect of rice grain type, soaking time and steaming time on the proximate composition. The moisture content ranged between 5.06 and 13.75% crude protein (8.02 and 12.14%), crude fat (0.85 and 1.27%), crude fibre (0.44 and 0.61%), ash content (0.51 and 1.06%) and carbohydrate content (75.02 and 83.85%), respectively. The highest moisture content (13.75%) was observed in FARO 15 while the lowest moisture content (5.06%) was observed in FARO 62. The difference in the moisture content could be traced to the rate at which the rice grain type imbibes water and become moist. In addition, 40 min steaming time used in FARO 62 could also reduce the moisture content because, during long steaming, excess moisture content evaporates to vapour due to reduction from saturation point to a normal level. Thus, the moist starch would be transformed into a gelatinous substance. This finding agreed with Ayamdoo *et al.* (2015) that an increase in steaming duration decreases the moisture content of Nerica-14 and Jasmine-85.

Table 2. Effect of rice grain type, soaking time and steaming time on proximate content (%)

Rice variety	Soaking time (mins)	Steaming time (mins)	Moisture	Crude protein	Crude fat	Crude fibre	Ash	CHO
FARO 15	4	30	13.06±0.01b	8.04±0.00h	1.22±0.00e	0.46±0.01f	0.51±0.00e	76.71±0.00h
FARO 15	5	35	13.75±0.01a	9.16±0.00f	0.95±0.00h	0.52±0.00e	0.60±0.01d	75.02±0.02i
FARO 15	6	40	8.15±0.00c	9.08±0.00g	1.11±0.00a	0.57±0.00d	0.63±0.00c	80.45±0.01e
FARO 60	4	40	6.45±0.00d	10.48±0.00d	0.85±0.00g	0.61±0.00a	1.03±0.01b	80.57±0.02d
FARO 60	5	30	5.86±0.01f	12.07±0.01b	1.30±0.00c	0.52±0.00e	1.02±0.00b	79.23±0.02e
FARO 60	6	35	6.14±0.01e	10.26±0.00f	1.02±0.00f	0.58±0.00c	1.05±0.00a	80.94±0.00e
FARO 62	4	35	5.25±0.00e	8.02±0.01i	1.21±0.00b	0.60±0.00b	1.06±0.00a	83.85±0.01a
FARO 62	5	40	5.06±0.01g	10.52±0.00c	1.27±0.00d	0.43±0.00g	1.06±0.00a	81.65±0.02b
FARO 62	6	30	6.14±0.01eh	12.14±0.01a	1.02±0.01f	0.52±0.00e	1.03±0.00b	79.14±0.02g

Means in a column, within processing condition, not followed by a common letter are significantly different at $P < 0.05$

The Pareto chart in Figure 1 (a) showed that rice grain type, quadratic effect of rice grain type and interaction of rice grain type and steaming time were the three most significant factors influencing the moisture content. The highest crude protein (12.14%) was observed in FARO 62 while the lowest crude protein content (8.02%) was observed in FARO 62 but under different conditions. FARO 60 was observed to have high crude protein content than FARO 15 regardless of the processing conditions. This could be due to the inherent difference in the rice varieties' protein content and the processing conditions. Kale *et al.* (2015) reported that the protein content of Basmatic rice differs based on soaking duration. Also, Ayamdoo *et al.* (2015) observed differences in the protein content of different rice varieties. The Pareto chart in Figure 1 (b) showed that quadratic effect of rice grain type and interaction between rice grain type and soaking time had the most significant influence on crude protein content. The highest crude fat (1.30%) was observed in FARO 60 while the lowest (0.85%) was also observed in FARO 60 at 4 h soaking time and 40 min steaming time. The low crude fat content could be attributed to the long steaming duration, which causes the oil in the rice embryo to diffuse and dissolve. The Pareto chart in Figure 1 (c) shows that the quadratic effect of steaming time and interaction of rice grain type and soaking time had the most significant influence on crude fat content. Patindol *et al.* (2008) reported an increase in the crude fat content as the parboiling conditions increased, while Ayamdoo *et al.* (2015) reported a difference in the crude fat

content of Nerica 14 and Jesmine 85 due to steaming time. The highest crude fibre (0.61%) was recorded in FARO 60. The lowest crude fibre (0.44%) was observed in FARO 62. The Pareto chart in Figure 1 (d) showed that the interaction of rice grain type and soaking time and quadratic effect of rice grain type had the most significant influence on crude fibre content. The low amount of crude fibre observed in this study and the difference in the crude fibre of the rice varieties could be attributed to the low heat generation during the soaking and steaming process, thus do not significantly degrade the fibre within the rice varieties.

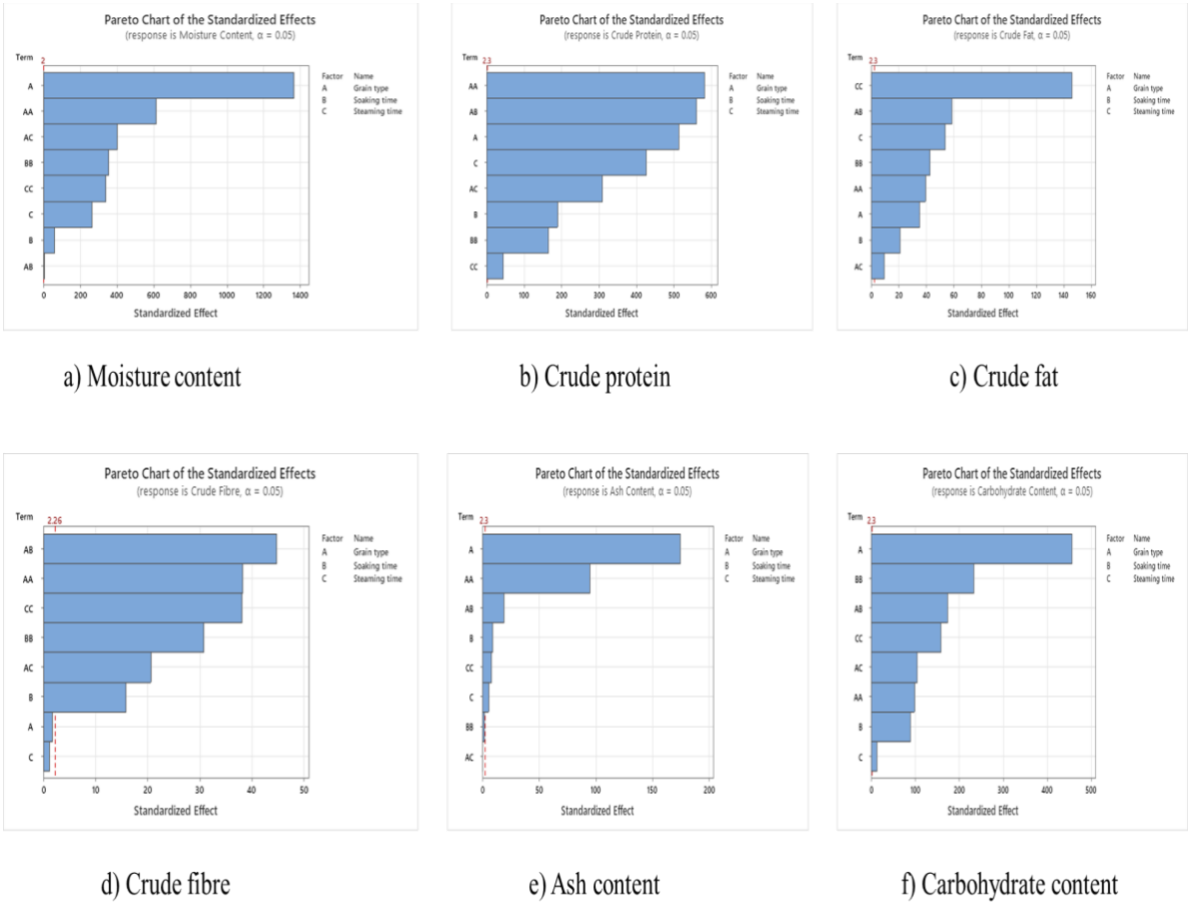


Figure 1. Impact of soaking time, steaming time and rice grain type on proximate composition of rice

In addition, the genetic makeup of the rice varieties may also influence it. This result corroborates with the findings of Kale *et al.* (2015) that crude fibre is low in brown rice and polished rice. The highest ash content (1.06%) was observed in FARO 60 and 62 while the lowest ash content (0.51%) was observed in FARO 15. The Pareto chart in Figure 1 (e) showed that rice grain type and quadratic effect of rice grain type influenced the ash content. The result agrees with Ayamdoo *et al.* (2015) that the rice grain type has significantly affected ash content. Therefore, it can be deduced that the ash content of the medium (FARO 60) and long rice (FARO 62) is more than the short rice (FARO 15). The highest carbohydrate content (83.85%) was observed in FARO 62 while the lowest (75.02%) was observed in FARO 15. The difference in the carbohydrate content could be attributed to the rice grain type and the soaking condition. During soaking, exudation of endosperm starch occurs due to low amylose content that might be present in the variety, causing severe grain deformation that reduces the carbohydrate content. Islam *et al.* (2004) reported similar findings that rice grain type and

soaking process influence carbohydrate content. The Pareto chart in Figure 1(f) showed that rice grain type and quadratic effect of soaking time significantly influenced the carbohydrate content.

3.2. Vitro-Starch Digestibility

The digestibility of starch is associated with the proportion of starch that is absorbed in the small intestine, and based on this absorption rate, starch is classified into Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS) and Resistant Starch (RS) (Muttagi and Ravindra, 2021). The RDS are rapidly converted into glucose by the body while SDS slowly breaks down. Conversely, the portion of starch or starch products that resist digestion as they pass through the small intestine is referred to as resistance starch (Tsuiki *et al.,* 2016). That is, the body cannot easily digest them and could pass through the digestive system untouched, similar to dietary fiber. The effect of rice grain type, soaking time and steaming time on vitro starch digestibility of rice is shown in Table 3.

Table 3. Effect of rice grain type, soaking time and steaming time on in-vitro starch, amylose and amylopectin contents

Rice Variety	Soaking Time (h)	Steaming Time (mins)	RDS %	SDS %	RS	Glycemic index	Amylose Content %	Amylopectin Content %
FARO 15	4	30	74.85±0.03a	16.92±0.03g	8.23±0.06e	66.24±0.00b	23.36±0.00c	76.64±0.00f
FARO 15	5	35	69.40±0.01d	22.46±0.01a	8.14±0.01e	64.22±0.00c	20.06±0.00f	79.94±0.00c
FARO 15	6	40	71.83±0.00h	21.14±0.00c	7.03±0.00f	62.18±0.00d	24.03±0.00b	75.97±0.00g
FARO 60	4	40	72.92±0.00i	16.90±0.00g	10.18±0.01a	58.66±0..00f	23.05±0.01d	76.95±0.00e
FARO 60	5	30	72.06±0.00e	17.88±0.01f	10.06±0.01a	60.26±0.00e	18.25±0.00h	81.00±0.00a
FARO 60	6	35	69.56±0.00c	20.44±0.00e	10.00±0.00a	58.75±0.70f	20.19±0.00e	79.81±0.00d
FARO 62	4	35	71.59±0.00g	19.73±0.00d	8.67±0.00d	50.41±0.23g	19.35±0.00g	80.65±0.00b
FARO 62	5	40	72.56±0.00f	18.14±0.24e	9.29±0.24c	67.24±0.02a	24.06±0.01b	75.94±0.01g
FARO 62	6	30	72.13±0.00b	18.11±0.00e	9.76±0.00b	52.16±0.00g	25.06±0.07a	78.06±2.40h

Means in a column, within processing condition, not followed by a common letter are significantly different at $P < 0.05$

The RDS, SDS, RS and glycemic index content ranged between 69.40 and 74.85%, 16.92 and 22.46%, 7.03 and 10.18% and 50.41 and 67.24%, respectively. The highest RDS content (74.85%) was observed in FARO 15 while the lowest RDS (69.40%) also was observed in FARO 15 but at different processing conditions. Polesi *et al.* (2017) reported 32.30 to 35.30% of RDS for raw rice and 74.00 to 80.40% for cooked rice of the same variety. However, a relatively lower range of 48.22 to 61.31% of RDS was reported by Muttagi and Ravindra (2021) for cooked traditional rice varieties. The Pareto chart in Figure 2 (a) shows that soaking time and the interaction of rice grain type and soaking time had the most influence on the RDS. The highest SDS (22.46%) was observed in FARO 15 while the lowest SDS (16.90%) was observed in FARO 60. A range of 41.10 to 44.00% of SDS for raw rice and 0.70 to 1.60% for cooked rice of the same variety was reported by Polesi *et al.* (2017). However, a relatively lower range of 4.87 to 10.02% of SDS was reported by Muttagi and Ravindra (2021) for cooked traditional rice varieties. As expected, the three varieties showed higher levels of RDS and lower levels of SDS due to starch gelatinization (Polesi *et al.*, 2017). The Pareto chart in Figure 2 (b) shows that the interaction of rice grain type and soaking time had the most significant influence on SDS. Considering the health benefits of SDS, the result shows that the interaction of rice grain type and soaking time could improve the rice acceptability from the point of view of starch digestibility. The highest RS (10.18%) was observed in FARO 60 while the lowest RS (7.03%) was observed in FARO 15. No

significant difference ($p>0.05$) was observed within the same rice variety; however, significant difference ($p<0.05$) was observed among the rice variety. This implies that irrespective of the processing conditions, rice varieties significantly affect the RS of rice. Polesi *et al.* (2017) reported that the starch structures are responsible for RS in rice samples and the rate of hydrolysis by gelatinising the starch makes it more easily available for enzymatic attack.

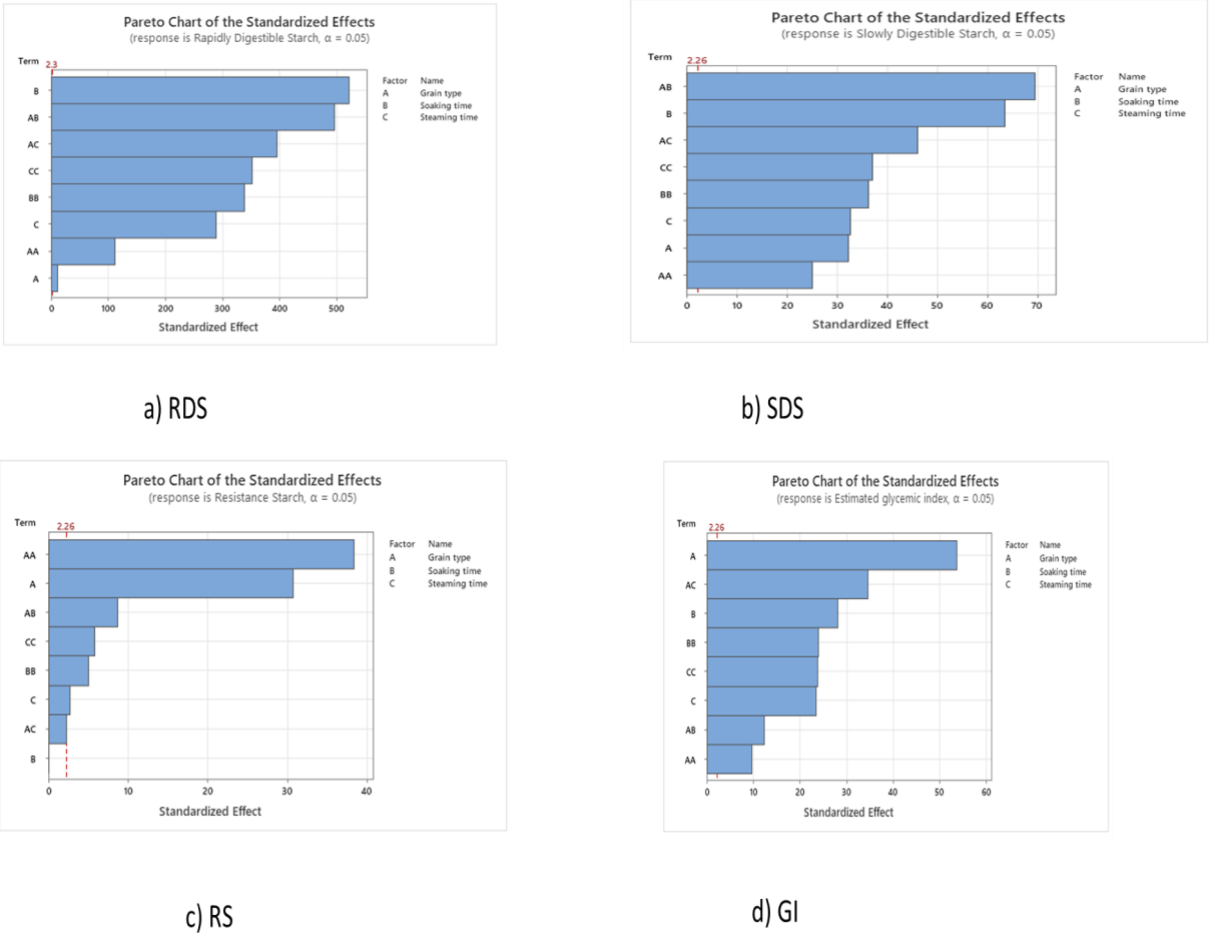


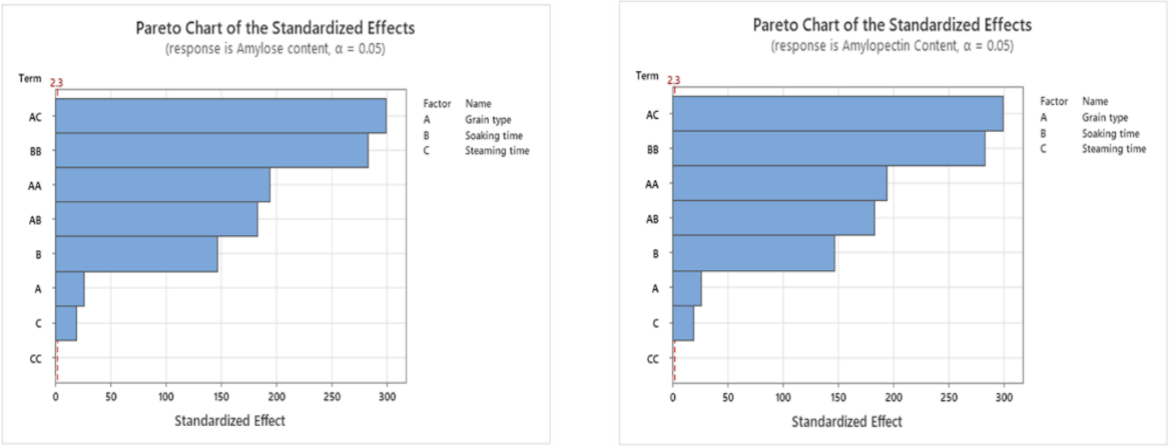
Figure 2. Impact of soaking time, steaming time and rice grain type on in vitro starch digestibility

The Pareto chart in Figure 2 (c) shows that the quadratic effect of rice grain type had the most significant influence on RS. The kinetic component of starch digestion is mainly associated with the so-called glycaemic index (GI), which strongly influences postprandial metabolism. The GI quantifies the postprandial blood glucose response to starchy foods. It is a tool for their characterisation and classification in terms of the physiological response they elicit (Miao *et al.*, 2015; Bello-Perez *et al.*, 2020). The highest GI (67.24%) was observed in FARO 62 while the lowest (50.41%) was also observed in FARO 62 at different processing conditions. The Pareto chart in Figure 2 (d) shows that rice grain type and the interaction of rice grain type and steaming time had the most significant influence on the GI. Tsuiki *et al.* (2016) reported that the difference in structure affects the digested rate of starch. Starch with high amylose content and resistant starch have a lower GI value. GI affects the chemical structure of the starch, amylose and amylopectin, and the level of GI in the body affects the health of the human being. Low GI foods have consistently improved the blood lipid concentration and prevented further diabetic complications (Upadhyaya *et al.*, 2016). Thus, rice grain type

with high amylose content and RS could result in a lower GI, which is essential to produce healthier foods and reduce the risk of diabetes and cardiovascular diseases.

3.3. Amylose and Amylopectin content

Starch is a natural polymer, or polysaccharide, meaning that it is a long chain comprising one type of molecule. This molecule is glucose which occurs in two forms: amylose and amylopectin. Amylose is a linear or straight-line polymer, whereas amylopectin forms a branched-chain (Bello-Perez *et al.*, 2020). Table 3 shows the effect of rice variety, soaking time, and steaming time on rice's amylose and amylopectin content. The amylose content ranged from 18.25 to 25.06, and amylopectin content ranged from 75.94% to 81.00%. The highest amylose content (25.06) was observed in FARO 62 at 6 h soaking time, 30 min steaming time, while the lowest amylose (18.25%) was observed in FARO 60 at 5 h soaking time, 30 min steaming time. The difference in values could be attributed to the interaction of rice grain type type and steaming time, which has the highest significance on the amylose content of rice, as shown in Figure 3(a).



a) amylose content b) Amylopectin content

Figure 3. Impact of soaking time, steaming time and rice grain variety on amylose and amylopectin contents

Muttagi and Ravindra (2021) also reported that rice grain type had a significant influence on the amylose content, affecting the starch digestibility. They also indicated that other factors such as physicochemical properties, granule size, and degree of crystallinity might also substantially affect starch digestibility. The highest amylopectin value (81.00%) was observed in FARO 60 at 5 h soaking time, 30 min steaming time), while the lowest (75.94%) was observed in FARO 62 at 5 h soaking time, 40 min steaming time). The difference in values could be attributed to the interaction of rice grain type and steaming time (AC) which has the highest significance on the amylopectin content of rice, as shown in Figure 3(b). The range of amylose and amylopectin shows that they are normal starches according to Bello-Perez *et al.* (2020).

4. Conclusions

The impacts of soaking time, steaming time and rice grain type on the proximate composition, in-vitro starch digestibility (RDS, SDS, RD and GI), amylose and amylopectin contents of rice were investigated. The results demonstrated significate effects of processing conditions evaluated on the test samples. The type of rice grain had the most significant impact on moisture, carbohydrate, ash, RS and GI. Conversely, the interaction of rice grain type and soaking time had the most crucial influence on

crude protein, fibre and SDS. The soaking time and the interaction of rice grain type with soaking time had the most impact on the RDS contents. On the other hand, the interaction of rice grain type with steaming time alone influences the amylose and amylopectin contents. These results will help the Nutritionists select the best processing combinations to provide a more suitable source of starch for chronic disease patients. Also, the food processors could plan adequate diets, especially for school children and elderly ones. Other processing combinations such as cooking methods, soaking temperatures, cooking water uptake, etc can be investigated for further study.

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CHARACTERISTICS OF NATIVE CHICKEN BREAST MEAT SOAKING IN JUICE OF PINEAPPLE HUMP AND CHAYOTE FRUIT

Wardah[1], Feni Avinda[2] and Tatang Sopandi[2]✉

[1] Study Program of Agroindustry, Vocational Faculty, University of 17 Agustus 1945, Jl. Semolowaru 45, Surabaya 60118, East Java, Indonesia

[2] Study Program of Biology, Faculty of Science and Technology, University of PGRI Adi Buana, Jl. Dukuh Menanggal XII No.17. Surabaya 60234. East Java. Indonesia.

✉tatang_sopandi@yahoo.co.id

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ABSTRACT

Kampong or native chicken has an important role in providing meat for the people of Indonesia. Special treatment is needed to increase the tenderness of native chicken meat which has a tough of texture. This study aims to evaluation the physical, chemical, and sensory characteristics of native chicken meat soaked in a combination of pineapple hump juice and chayote juice. The study was conducted experimentally using a completely randomized design with a factorial pattern. The first factor was soaking pineapple hump juice which consisted of 4 concentrations and second factor was 4 the concentration of chayote juice. The results showed that the soaking of native chicken meat in pineapple and chayote hump juice significantly ($P<0.05$) decreased the total collagen content, water holding capacity, increased tenderness, cooking loss, and favorite sensory characteristics of color, aroma and taste of native chicken meat. Chayote can be used as a meat tenderizer, either independently or in combination with other tenderizers. Based on the tenderness data and the preferred sensory characteristics of color, aroma and taste, the combination of soaking native chicken breast meat in 7.5% pineapple hump and 5.0% chayote is the most optimal.

1.Introduction

Native or kampong chicken has an important role in the provision of meat in Indonesia and is claimed to have low cholesterol content with a savory meat taste. However, special treatment is needed before cooking because native chicken meat has a tough texture. Texture, especially tenderness, is an attribute of meat quality and is an important selection criterion used by consumers when buying meat (Fanatico *et al*., 2007).

Tenderization of native chicken meat can be done by adding protease enzymes that can hydrolyze protein peptide bonds into simpler compounds such as dipeptides and amino acids (Naidu, 2011). The use of protease enzymes from plants for the purpose of tenderizing meat is still limited to the use of papain from papaya (Mamboya and Amri, 2012) and bromelain from pineapple (Ketnawa *et al*., 2011). Protease enzymes derived from plants such as papain, bromelain and ficin are widely used as meat tenderizers and are the most important enzymes in several food industries which account for 60% of the total market share (Gaur *et al*., 2010). Papain and bromelain are the two main protease enzymes in the food industry which take up 8% of the market demand (Adulyatham and Owusu-Apenten, 2005).

The use of papaya to tenderize meat can produce a bitter taste (Gerelt *et al*., 2000) or lead to the undesirable appearance of mushy flesh

(Ashie *et al*., 2002). The use of bromelain enzymes from pineapple extract can reduce the texture of the meat to become too tender, due to the broad substrate specificity, and cause an unpleasant taste (Manohar *et al*., 2016). Diversification of meat tenderizers is needed to provide more options for the food industry.

Chayote (*Sechium edule*) is an annual vegetable plant containing several bioactive compounds, polyphenols component, vitamins and carotenoids (Vieira *et al*., 2019). Chayote production in Indonesia in 2019 reached 407,962 tons (Badan Pusat Statistik Indonesia, 2019). Most of the chayote is used for vegetable food, but has not been used as a meat tenderizer. Chayote can be used as a source of meat tenderizing protease enzymes that are easy to obtain and safe for consumption (Okfrianti, 2011). The crude chayote extract is reported to contain protease enzymes (Ratnayani and Kusumaningrum, 2015). Documentation and scientific publications on the use of chayote as a meat tenderizer, either independently or in combination with other tenderizers, are still limited. This study aims to evaluate the chemical, physical and sensory characteristics of native chicken meat breast soaked in pineapple hump juice and chayote juice.

2. Materials and methods

This study was also conducted using a completely randomized design with a factorial pattern consisting of 4 levels of pineapple hump juice concentration (0.0, 2.5, 5.0 and 7.5%) and 4 levels of chayote juice concentration (0.0, 2.5, 5.0, 7.5%). Each treatment of meat soaking was repeated 20 times.

2.1. Preparation of pineapple hump juice and chayote juice

A total of 65 kg of ripe pineapple (*Ananas comosus* queen variety) from local supermarket was peeled, cleaned and the hump part was taken. A total of 49.5 kg of pineapple hump were divided into 3 parts of 8, 17.5 and 24 kg, respectively, then each portion was divided into 12. Meanwhile, 50 kg of chayote (*Sechium edule*) obtained from a local supermarket were peeled, cleaned, washed and as much as 49.5 kg was divided into 3 parts of 8, 17.5 and 24 kg, then each portion was divided by 12. Pineapple hump, chayote and each mixture of pineapple hump and chayote, put in a glass beaker (1.5 L) containing 1 L of water, crushed with a blender for 2 minutes, squeezed and filtered using a flannel cloth. The combination of soaking treatment with pineapple weevil juice and chayote juice consists of;

P_0C_0 = soaking in distilled water (0% pineapple hump juice and 0% chayote juice)
$P_0C_{2.5}$ = soaking in pineapple hump juice 0% and chayote juice 2.5%
$P_0C_{5.0}$ = soaking in pineapple hump juice 0% and chayote juice 5.0%
$P_0C_{7.5}$ = soaking in pineapple hump juice 0% and chayote juice 7.5%
$P_{2.5}C_0$ = soaking in pineapple hump juice 2.5% and chayote juice 0%
$P_{2.5}C_{2.5}$ = soaking in pineapple hump juice 2.5% and chayote juice 2.5%
$P_{2.5}C_{5.0}$ = soaking in pineapple hump juice 2.5% and chayote juice 5.0%
$P_{2.5}C_{7.5}$ = soaking in pineapple hump juice 2.5% and chayote juice 7.5%
$P_{5.0}C_0$ = soaking in pineapple hump juice 5.0% and chayote juice 0%
$P_{5.0}C_{2.5}$ = soaking in pineapple hump juice 5.0% and chayote juice 2.5%
$P_{5.0}C_{5.0}$ = soaking in pineapple hump juice 5.0% and chayote juice 5.0%
$P_{5.0}C_{7.5}$ = soaking in pineapple hump juice 5.0% and chayote juice 7.5%
$P_{7.5}C_0$ = soaking in pineapple hump juice 7.5% and chayote juice 0%
$P_{7.5}C_{2.5}$ = soaking in pineapple hump juice 7.5% and chayote juice 2.5%
$P_{7.5}C_{5.0}$ = soaking in pineapple hump juice 7.5% and chayote juice 5.0%
$P_{7.5}C_{7.5}$ = soaking in pineapple hump juice 7.5% and chayote juice 7.5%

2.2. Meat soaking

A total 320 carcasses from 8-month-old native chicken with an average weight of 650 g per carcass obtained from a local chicken slaughter house in Surabaya, East Java, Indonesia. Each carcass was taken at random and each was soaked in the juice of pineapple

and chayote and the mixture for 60 min at a temperature of 25ºC. After soaking, analysis of the total collagen content, tenderness, pH, water holding capacity, cooking loss and sensory characteristics of color, aroma, and taste of native chicken meat breast were carried out.

2.3. Determination of total collagen

Determination of the total collagen of native chicken meat was soaked in various combinations of pineapple hump juice and chayote juice using the method described by Fang *et al*. (1999) and Moon (2018). Briefly, 10 g of thigh meat from each treatment were crushed, put into a 50 mL centrifuge tube containing 24 mL of Ringer's solution and then stirred. The mixture was then incubated in a water bath at a temperature of 77℃ for 65 min with a stirring interval of 15 min. After incubation, the mixture was centrifuged for 10 min at 4000 g. The supernatant was taken and collected, while the precipitate was mixed with 8 mL of Ringer's solution and centrifuged again for 10 min. The precipitate was rinsed, the supernatants from the two centrifugation stages were combined. The supernatant and precipitate of all samples were hydrolyzed separately in 30 mL 6 N sulphuric acid (H_2SO_4) at 110℃ for 16 h. The hydrolyzate was diluted to 250 mL with Ringer's solution. The absorbance of the hydrolyzate was then measured at 560 nm and recorded. Standard curves were made with concentrations of 0.0, 0.1, 0.2, 0.3 0.4, 0.5, 0.6, 0.8, 0.9 and 1% using hydroxyproline which had been neutralized with a solution of 4.37 mL of 1 M sodium hydroxide (NaOH). The total collagen content was calculated from the sum of the hydroxyproline concentrations in the precipitate and supernatant using a conversion factor of 7.25 (Goll *et al*., 1963) and expressed as mg/g wet weight.

2.4. Determination of tenderness

Meat tenderness was measured using a penetrometer according to Hinnergardt and Tuomy (1970) method. Briefly, as much as 10 g thigh meat with a size of 2.5x2.5 cm from each soaking treatment were placed on the bottom of the penetrometer. The pointer needle was set in contact with the surface of the meat sample and the needle scale shows zero. The base of the needle is loaded with 50 g, the penetrometer lever was pressed for 10 s, released and the penetration scale of the penetrometer needle is read as mm 10/s/50 g.

2.5. Determination of meat acidity

Determination of the acidity (pH) of meat was done using a pH meter. A total of 10 g thigh meat from each immersion treatment was added with 10 mL of distilled water, stirred until homogeneous, then the pH of the homogenate was measured with a pH meter that was calibrated at pH 4.0 and 7.0 in phosphate buffer solution.

2.6. Determination of water holding capacity

Determination of water holding capacity (WHC) is determined by the Hamm method (Chaurasiya *et al*., 2015) with modification. Briefly, a 10 g thigh meat from each soaking treatment was finely chopped and put into a 100 mL centrifuge tube containing 50 mL of distilled water. The tube was centrifuged at 3000 rpm for 20 min. After centrifugation, the volume of the supernatant was measured and the water holding capacity of the meat was calculated using the formula WHC (%) = (volume before centrifuge - volume of supernatant)/volume before centrifuge) x 100

2.7. Determination of cooking loss

Determination of cooking loss of meat is done using the method according to the AOAC International 950.46 method (AOAC, 2005). Briefly, as much as 100 g thigh meat from each immersion treatment was put in plastic, tightly closed, then boiled in a water bath with a temperature of 60°C for 60 min. The liquid part on the surface of the meat was absorbed using tissue paper and the boiled part of the meat was weighed. Cooking loss was calculated as the percentage of the raw weight lost, based on the weights of all steaks before and after cooking. The mass changes were expressed as a percentage of the initial mass (w/w, wet basis).

2.8. Enumeration of total aerobic plate count

Aseptically, 25 g of chicken thigh meat samples with skin from each immersion and replication treatment were put in a sterile stomacher bag containing 225 mL of a sterile 0.1% peptone solution and homogenized in a stomacher for 2 min at room temperature. The mixture was transferred to a test tube and followed by serial dilutions to 10^{-6} dilutions. From each dilution, 0.1 mL was taken with a sterile micropipette, dripped and spread with a glass hokey stick on nutrient agar media. The agar medium was then incubated at 28°C for 48 h in an inverted position. Bacterial colonies were counted using a colony counter.

2.9.Determination of sensory characteristics

The sensory characteristics of color, aroma, and taste of breast meat breast from each soaking treatment were observed by 20 trained panelists with a taste threshold of 0.5% sugar content in tea water, not color blind and not smoking. A total of 320 breast meat from each soaking treatment was steamed at a temperature of 60°C for 60 min. Panelists were asked to evaluate the color, aroma and taste of the meat using a linkert scale, namely 1 very poor, 2 for poor, 3 for neutral, 4 for good, and 5 for very good.

2.10. Data analysis

All observational data were analyzed using analysis according to a completely randomized design with a two-way factorial pattern at a significance level of 0.05. Prior to the analysis of variance, the sensory characteristics of color, aroma and taste were first transformed into log+0.5 numbers and data on the number of bacteria were transformed into log numbers 10. Further tests to see the location of the differences between treatments were carried out using the Tukey test at a significance level of 0.05 if there were significant differences between treatments in the analysis of variance. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 22 software.

3.Results and discussions

3.1.Total collagen

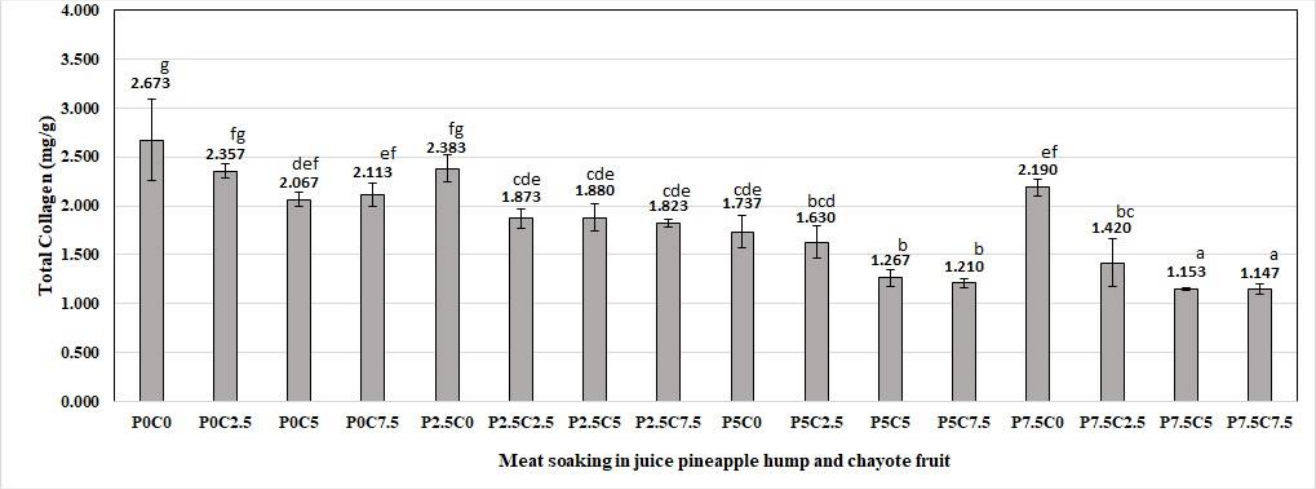

Figure 1. Total collagen of native chicken meat breast soaked in various combinations of pineapple hump juice (P) and chayote juice (C). The mean values which are not the same are significantly different ($p<0.05$).

The results of this study (Fig. 1) indicated that soaking in pineapple hump juice at concentrations of 5.0 and 7.5% (P_5C_0, and $P_{7.5}C_0$), chayote juice at concentrations of 5.0 and 7.5% (P_0C_5 and $P_0C_{7.5}$) and the combination of the mixtures were significant ($p<0.05$) decreased the total collagen content of native chicken meat breast. However, in the independent treatment, the concentration of pineapple hump was 2.5% ($P_{2.5}C_0$) and the

concentration of chayote 2.5% ($P_0C_{2.5}$) have no significant effect ($p>0.05$) on the total collagen content of meat. The results of this study also showed that the lowest total collagen content of native chicken meat breast was found in the combination treatment of soaking 7.5% pineapple weevil juice and 5.0% chayote juice and 7.5% pineapple hump juice and 7.5% chayote juice ($P_{7.5}C_{5.0}$ and $P_{7.5}C_{7.5}$).

The decrease in total collagen of native chicken meat breast soaked in pineapple hump juice and chayote juice is thought to be due to the activity of the protease enzymes contained in both. Several investigators have reported the content of the protease enzyme bromelain in pineapple hump (Wuryanti, 2004; Monahar *et al*., 2016; Gul *et al*., 2021). While Ratnayani and Kusumaningrum (2015) reported that chayote contains protease enzymes. Protease enzymes found in fruits degrade myofibrillar proteins and collagen that can soften meat (Santos *et al*., 2020). Rawdkuen *et al*. (2013) reported that proteases derived from plants can hydrolyze collagen and elastin in meat.

3.2. Tenderness of meat

The results of this study (Fig. 2) show that the independent treatment of P_5C_0 and $P_{7.5}C_0$, P_0C_5 and $P_0C_{7.5}$ as well as the combination of the mixture significantly ($p<0.05$) increased the tenderness of native chicken meat breast. However, in the independent treatment $P_{2.5}C_0$ and $P_0C_{2.5}$ have no significant effect ($p> 0.05$) on the tenderness of meat. The results of this study also showed that the highest tenderness of native chicken meat was found in the combination treatment of $P_{7.5}C_5$ and $P_{7.5}C_{7.5}$.

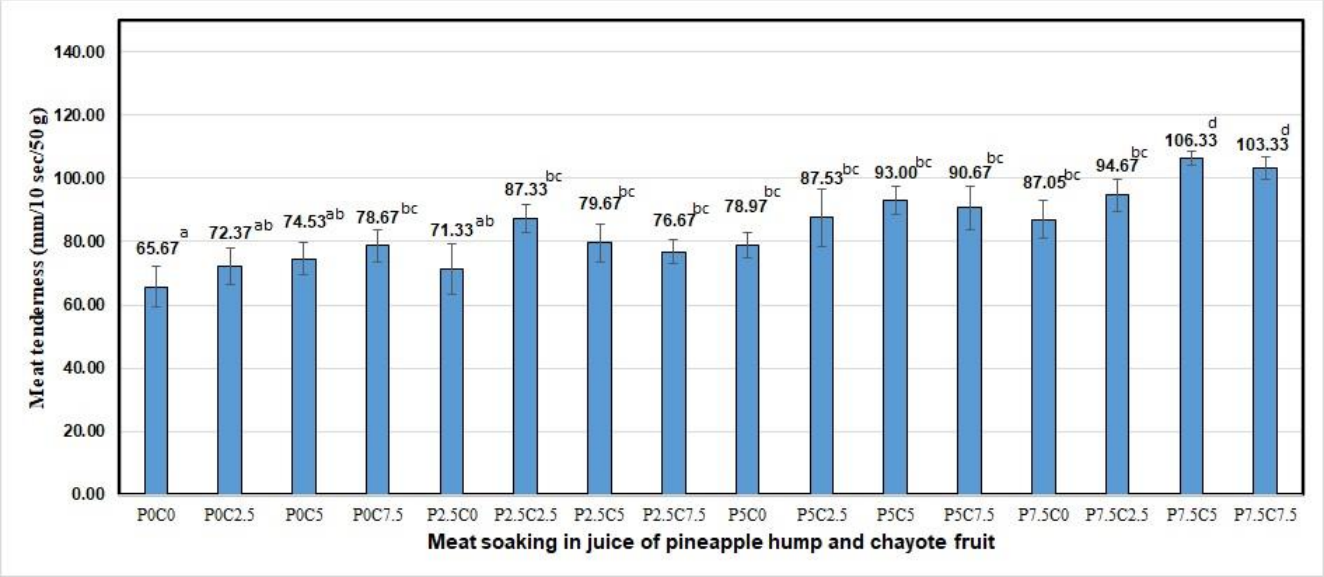

Figure 2. Tenderness of native chicken meat breast soaked in various combinations of pineapple hump juice (P) and chayote juice (C). The notated mean values did not differ significantly ($p<0.05$).

The increase in tenderness of native chicken breast soaked in pineapple hump juice and chayote juice is thought to be due to the activity of the bromelain and protease enzymes contained in each fruit. Several investigators have documented the effects of protease enzymes on meat tenderness. Protease enzymes can cause the breakdown of the fibrous structure of the muscle and the fragmentation of collagen cross-links or split into smaller segments (Endalk *et al*., 2020). During the protease enzyme treatment process, there was hydrolysis of muscle fiber protein, binding weaves and changes occurred such as thinning and destruction of the sarcolemma, dissolution of the nucleus of muscle fibers and connective tissue and the release of muscle fiber attachments to produce soft tissue. Nadzirah *et al*. (2016) and Gerelt *et al*. (2000) reported that proteolytic enzymes increase the rate of myofibril

fragmentation in meat and break down intramuscular connective tissue structures. Santos *et al*. (2020) suggested that bromelain affects the structure of actin and myosin filaments in meat myofibrillar proteins. Jahidin and Monica (2018) reported that soaking young pineapple extract can improve the texture and increase the tenderness of buffalo meat. Widiastuti *et al*. (2017) reported that antemortem injection of protease enzymes hydrolyzes proteins at cross-linkages between collagen and increases the tenderness of rejected laying hens.

3.3. Meat acidity

The results of this study (Fig. 3) showed that the treatment of soaking in pineapple hump $P_{5.0}C_0$ and $P_{7.5}C_0$ significantly ($p<0.05$) reduced the pH of native chicken meat breast. Soaking in chayote juice $P_0C_{2.5}$, $P_0C_{5.0}$ and $P_0C_{7.5}$ had no significant effect ($p>0.05$) on the pH of native chicken meat breast. However, the combination of soaking in a mixture of pineapple hump juice and chayote juice significantly ($p<0.05$) reduced the pH of meat.

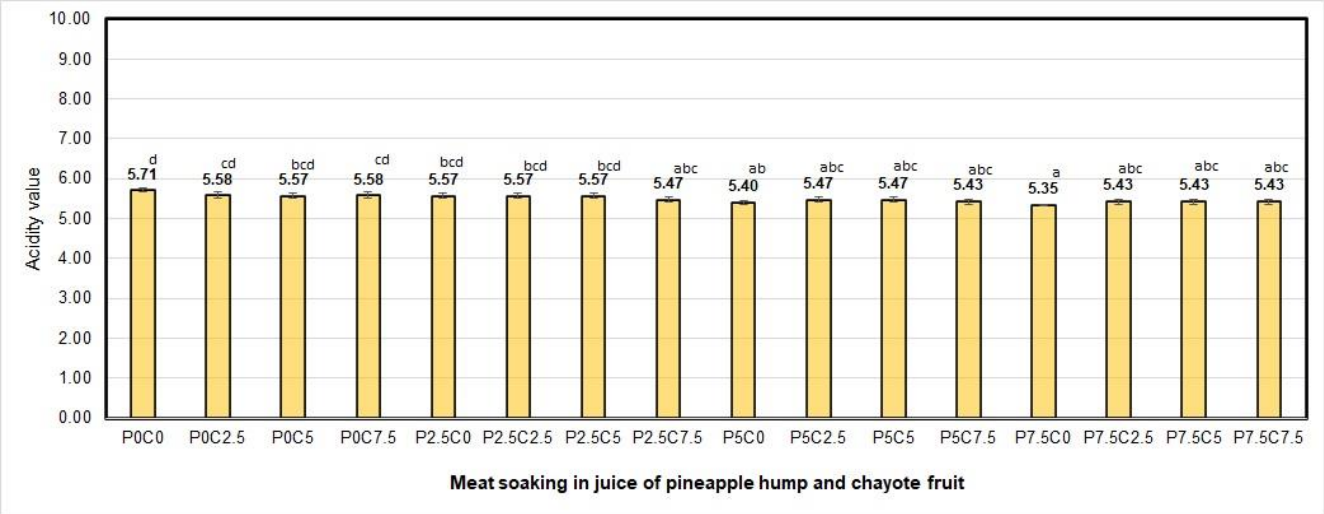

Figure 3. Acidity (pH) of native chicken meat breast soaked in various combinations of pineapple hump juice (P) and chayote (C). The mean values which are not the same are significantly different ($p<0.05$).

The decrease in the pH of native chicken meat breast soaked in pineapple hump juice is thought to be due to the organic acid components contained in the fruit. Pineapple was reported to contain organic acids such as citric acid, malic acid, succinic acid, and acetic acid. Meanwhile, chayote juice only contains ascorbic acid (Sangma *et al*., 2019). Ketnawa *et al*. (2011) reported that hydrolysis of bromelain in meat muscle can cause amino acid cleavage and lower pH.

3.4. Water holding capacity

The results of this study (Fig. 4) showed that the treatment of soaking in pineapple hump juice $P_{5.0}C_0$ and $P_{7.5}C_0$ and chayote juice $P_0C_{5.0}$ and $P_0C_{7.5}$ significantly ($p<0.05$) reduced the WHC of native chicken meat breast. The decrease in WHC of native chicken meat breast soaked in pineapple hump juice, chayote juice and their combination is thought to be the effect of protein hydrolysis activity of protease enzymes that break down myofibril and collagen proteins causing the volume of meat muscle fibers to expand and water holding capacity to decrease. Huff-Lonergan and Lonergan (2005) suggested that most of the water in the muscle is trapped in the cell structure, including the intra and extra-myofibrillar spaces, changes in the intracellular structure of the cell can affect the ability of muscle cells to retain water.

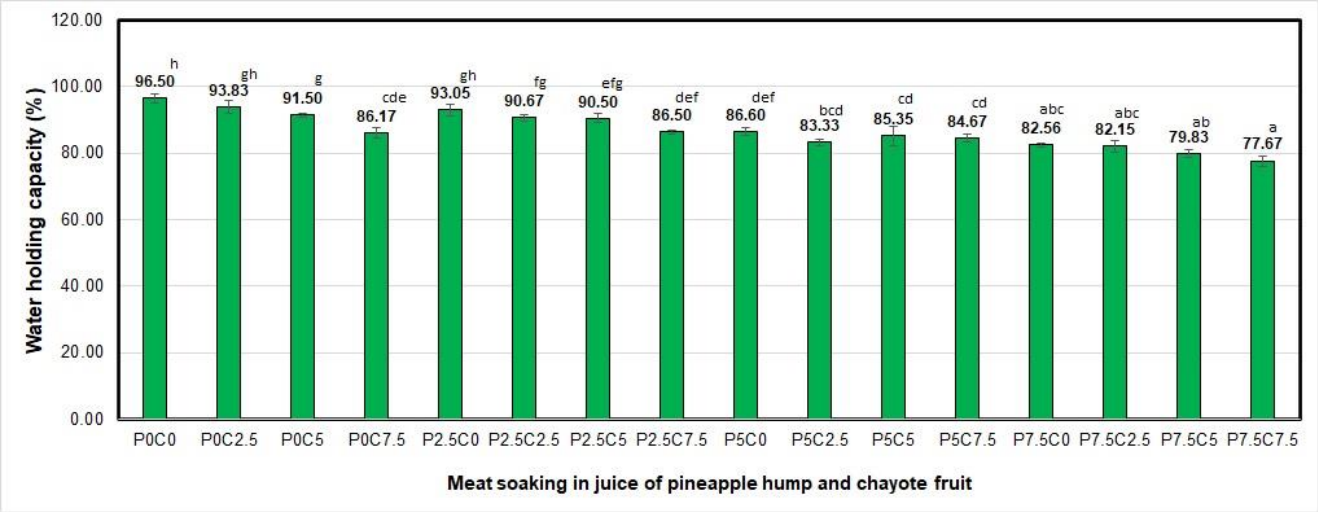

Figure 4. Water holding capacity of native chicken meat soaked in various combinations of pineapple hump juice (P) and chayote juice (C). The mean values which are not the same are significantly different ($p<0.05$).

The effect of using plant-derived meat tenderizing enzymes on WHC differs between meat origin and type, origin of tenderizing enzyme, concentration and method of tenderization. Naveena *et al*. (2004) reported that the use of ginger homogenate for 48 h significantly increased the WHC of buffalo meat. Abdeldaiem *et al*. (2014) reported that the use of fresh ginger extract at concentrations of 15, 30 and 45% increased the WHC of camel meat. Gokoglu *et al*. (2017) reported that the use of bromelain and papain solutions as softeners did not significantly increase the WHC of squid muscles. Our study showed that soaking in pineapple hump juice and chayote juice reduced the total collagen of native chicken meat breast. Ha *et al*. (2012) suggested that the hydrolysis and breakdown of meat connective tissue varies between plant extracts, properties and concentrations. Woinue *et al*. (2001) suggested that the bromelain enzyme can denature myofibrillar proteins that play a role in air retention. Ketnawa and Rawdkuen (2011) suggested that myofibrils and the movement of water from the myofilaments space to the extracellular space due to enzymatic action can reduce WHC.

3.5. Cooking loss

Cooking loss is an important indicator of meat quality because it is related to the amount of water lost and water-soluble nutrients due to the influence of cooking. The results of this study (Fig. 5) indicated that the immersion in pineapple hump juice $P_{5.0}C_0$ and $P_{7.5}C_0$ was significant ($p<0.05$) and the immersion in chayote juice $P_0C_{5.0}$ and $P_0C_{7.5}$ was significant ($p<0.05$) respectively, increase the cooking loss of native chicken meat. This study also showed that the combination of soaking in pineapple hump juice and chayote juice significantly ($p<0.05$) increased the cooking loss of native chicken meat.

The increase in cooking loss of native chicken meat breast soaked in pineapple hump juice, chayote juice and their combination is thought to be due to the activity of protease enzymes that break down the muscle connective tissue of the meat. Several investigators have reported the effect of using enzymes and plant extracts on the cooking loss of meat. Nowak (2011) reported that a solution of pineapple extract was able to break down peptide bonds into soluble amino acids and increase the shrinkage of chicken meat. Abdeldaiem *et al*. (2014) reported that the use of fresh ginger extract at concentrations of 15, 30 and 45% increased the cooking loss of camel meat. Zhang *et al*. (2017) reported that the use of actinidin and papain enzymes as tenderizers caused high muscle cooking loss in rabbits due to the

breakdown of more extensive muscle tissue at higher temperatures.

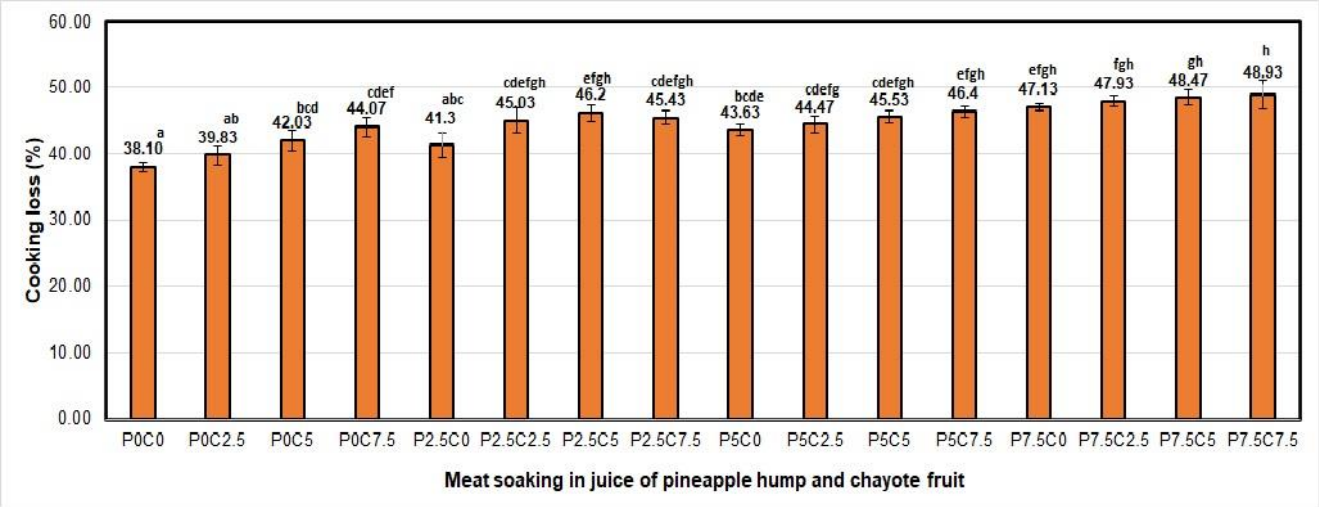

Figure 5. Cooking loss of native chicken meat soaked in various combinations of pineapple hump juice (P) and chayote juice (C). The mean values which are not the same are significantly different ($p<0.05$).

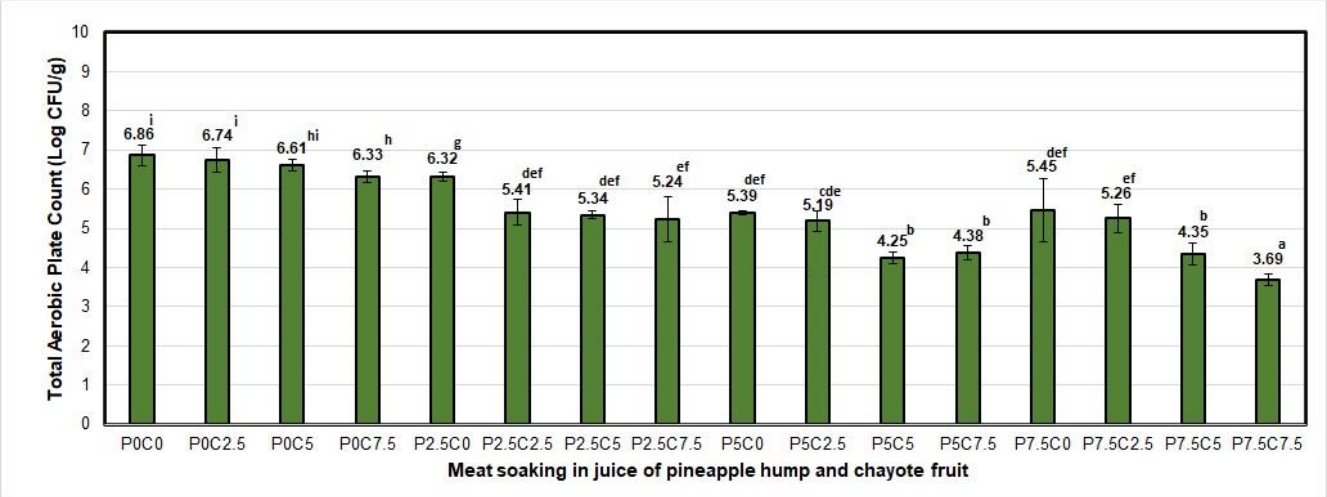

Figure 6. Total aerobic plate count of native chicken meat soaked in various combinations of pineapple hump juice (P) and chayote juice (C). The mean values which are not the same are significantly different ($p<0.05$).

3.6. Total aerobic plate count

Assessment of sanitation quality, sensory acceptability and conformity to good manufacturing practices in general can use aerobic plate count (Kim *et al*., 2018). The results of this study (Fig. 6) indicate that soaking chicken meat in chayote juice independently or in a mixture with pineapple hump juice significantly ($p<0,05$) reduce APC and the lowest APC was found in meat soaked in the treatment combination $P_{7.5}C_{7.5}$. The decrease in APC in chicken meat soaked in pineapple hump juice and chayote, either independently or in combination, was thought to be due to the antibacterial activity of the components contained in pineapple hump and chayote. Several investigators have reported that pineapple hump extract can inhibit bacterial

growth. Pineapple hump was confirmed to contain alkaloids, flavonoids, and saponins (Soulissa *et al*., 2021; Liliany *et al*., 2018). Meanwhile, Sibi *et al*. (2013) reported that the alkaloids, flavonoids, saponins and terpenoids contained in the chloroform and methanol extracts of chayote fruit could inhibit the growth of *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri* bacteria.

3.7.Sensory characteristics

Table 1. Sensory characteristics of color, aroma and taste of native chicken meat breast soaked in various combinations of pineapple hump juice (P) and chayote juice (C). The mean value given not the same in the direction of the column is significantly different ($p<0.05$).

Soaking treatment	Panelist assessment		
	Color	Aroma	Taste
P_0C_0	3.75 ± 0.46^{ab}	3.13 ± 0.35^{a}	2.50 ± 0.53^{a}
$P_0C_{2.5}$	3.75 ± 0.46^{ab}	3.13 ± 0.35^{a}	2.75 ± 0.71^{ab}
P_0C_5	3.63 ± 0.52^{ab}	3.25 ± 0.46^{ab}	3.25 ± 0.46^{c}
$P_0C_{7.5}$	3.13 ± 0.35^{a}	3.25 ± 0.46^{ab}	3.38 ± 0.52^{c}
$P_{2.5}C_0$	3.63 ± 0.52^{ab}	3.38 ± 0.52^{abc}	3.88 ± 0.35^{cd}
$P_{2.5}C_{2.5}$	3.50 ± 0.53^{ab}	3.50 ± 0.53^{bc}	4.03 ± 0.11^{d}
$P_{2.5}C_5$	3.50 ± 0.53^{ab}	3.63 ± 0.52^{bc}	4.04 ± 0.12^{d}
$P_{2.5}C_{7.5}$	3.63 ± 0.52^{ab}	3.75 ± 0.46^{bc}	4.00 ± 0.00^{d}
P_5C_0	4.25 ± 0.46^{bc}	4.03 ± 0.11^{bc}	4.00 ± 0.00^{d}
$P_5C_{2.5}$	3.63 ± 0.52^{ab}	4.13 ± 0.35^{c}	4.13 ± 0.35^{d}
P_5C_5	3.75 ± 0.46^{ab}	4.25 ± 0.46^{c}	4.13 ± 0.35^{d}
$P_5C_{7.5}$	3.88 ± 0.35^{abc}	4.25 ± 0.46^{c}	4.25 ± 0.46^{de}
$P_{7.5}C_0$	4.25 ± 0.52^{bc}	4.50 ± 0.53^{cd}	4.50 ± 0.53^{e}
$P_{7.5}C_{2.5}$	4.13 ± 0.42^{bc}	4.25 ± 0.46^{c}	4.25 ± 0.46^{de}
$P_{7.5}C_5$	4.63 ± 0.35^{c}	4.75 ± 0.46^{d}	4.50 ± 0.53^{e}
$P_{7.5}C_{7.5}$	4.45 ± 0.46^{c}	4.65 ± 0.48^{d}	4.88 ± 0.35^{e}

The results of this study (Table 1) showed that soaking in pineapple hump juice and a mixture of pineapple hump juice with chayote juice significantly ($p<0.05$) increased the preference for color, aroma and taste of native chicken breast meat. Soaking in chayote juice independently have no significant ($p>0.05$) effect on color and aroma, but significantly ($P<0.05$) increased the taste of native chicken meat breast at concentrations of 5.0% ($P_0C_{5.0}$) and 7.5% ($P_0C_{7.5}$). In this study, the color, aroma and taste of native chicken meat soaked in a combination of pineapple hump juice and chayote juice $P_{7.5}C_{5.0}$ and $P_{7.5}C_{7.5}$ were the most preferred. The increase in preference for the color of native chicken meat breast soaked in pineapple hump juice and chayote juice is thought to be due to the activity of protease enzymes and the components contained in pineapple hump and chayote.

Botinestean *et al*. (2018) reported that meat tenderized with protease enzymes increased consumer acceptance, although it could decrease yields. Dewanto *et al*. (2017) suggested that the bromelain enzyme enters the meat causing the myoglobin bonds to break down and change the color of the meat. Zulfahmi *et al*. (2013) reported that immersion in pineapple peel extract caused a change in the color of rejected duck meat from white to yellowish. Ismanto

(2017) reported that soaking in pineapple extract increased the aroma of rejected parent stock chicken meat because the pineapple fruit aroma was quite strong. Zhao *et al*. (2020) reported that treated of proteinase papain, bromelain and flavour enzyme causes the changes in volatile compounds and odors of beef longissimus dorsi. Okfrianti *et al*. (2011) reported that the addition of protease enzymes from pineapple extract caused the flesh to be quite sweet, typical of pineapple, because pineapple contains high glucose.

4. Conclusions

This study concluded that soaking native chicken meat breast in pineapple hump juice and chayote juice for 60 minutes at 25°C independently and/or in combination could increase tenderness and cooking loss, reduce total collagen content and water binding capacity, but did not change pH. The use of pineapple hump juice and its combination with chayote juice as a meat tenderizer can increase consumer preferences. Referring to the tenderness data and sensory characteristics, we concluded that the combination of 7.5% pineapple hump juice and 5.0% chayote juice was the most optimum concentration. Chayote has the potential to be used as a meat tenderizer, either independently or in combination with other tenderizers. Identification and characteristics of protease enzymes in chayote need to be investigated further.

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EFFECT OF COCONUT OIL ENRICHED CASSAVA STARCH BASED EDIBLE COATINGS ON QUALITY OF MINIMALLY STRAWBERRIES (*FRAGARIA ANANASSA*)

Rafaela Oliveira da Silva[1], Ianca Dalila Arguelho[1], Sandriane Pizato[2]✉, Rosalinda Arévalo Pinedo[1], William Renzo Cortez-Vega[1]

[1]Federal University of Grande Dourados – UFGD, Laboratory of Bioengineering, Rod. Dourados km 12, Itahum, Zip code – 79804970, Dourados, MS – Brazil
[2]Federal University of Amazonas – UFAM, Faculty of Agricultural Sciences, Rodrigo Otávio Avenue, Japiim, Zip code – 69077000, Manaus, AM – Brazil
✉sandrianepizato@yahoo.com.br

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ABSTRACT

The purpose of this work was to evaluate the effects of applying cassava starch coating with the addition of different concentrations of coconut oil in minimally processed strawberries. The strawberries were selected and sanitized, had their leaves and peduncles removed, and were submerged in the coatings (40ºC) for three minutes. Four treatments were obtained: T1 - control (strawberries without coating); T2 - coconut oil (1.0%) + cassava starch (3.0%); T3 - coconut oil (1.5%) + cassava starch (3.0%); T4 - coconut oil (2.0%) + cassava starch (3.0%). After receiving the coatings, the strawberries were placed in PET (polyethylene terephthalate) containers and stored at 5±1°C for 12 days. Physical, chemical, sensory analysis and microbiological evaluations were performed. T2 and T3 samples were more efficient in reducing mass loss (14.59% and 14.52%). They were also effective in maintaining texture and color for longer, as they influenced sensory and microbiological analysis, increasing shelf life and slowing growth, especially of molds and yeasts. The study may help small-scale establishments to increase the shelf life of minimally processed strawberries. The use of small concentrations of coconut oil prolonged the quality of strawberries during refrigerated storage.

1.Introduction

Strawberry (*Fragaria ananassa*) is a non-climacteric fruit containing a great variety of bioactive compounds including phenolic constituents, anthocyanins, vitamins and minerals (Giampieri *et al*., 2015; Pizato *et al.*, 2022). However, postharvest handling and storage of fresh strawberries is difficult mostly due to their high susceptibility to mechanical injury, water loss, microbial decay, physiologic al deterioration and high respiration rate (Liu *et al*., 2018).

Studies show the possibility of developing packaging or coatings using natural and biodegradable macromolecules, such as proteins, lipids and polysaccharides (Alves-Silva *et al*., 2022). There are numerous studies that reported the beneficial effects of edible coating on the minimally processed fruit and vegetables such pineapple (Pizato *et al*., 2019; Padrón-Mederos *et al*., 2020), papaya (Holsbach *et al*., 2019), broccoli (Pizato *et al*., 2020), guavas (Arroyo *et al*., 2020), strawberry (Martínez-González *et al*., 2020; Pizato *et al*., 2022).

Starch is one of the most studied biodegradable polymers for obtaining films and coatings, and present low cost (Borges *et al*.,

2015). It presents possibilities of chemical and physical modification for application in use as coatings (Holsbach *et al*., 2019). Starch is a biopolymer composed of several units of amylose and amylopectin, being classified as a polysaccharide (Oliveira *et al*., 2020).

Coconut oil is a natural food product rich in lauric acid. There is evidence that a part of this acid converts endogenously to monolaurin that is known to possess a broad spectrum of antiviral, antibacterial and antifungal activities (Liberman *et al*., 2006). Coconut oil coating closed the opening of stomata and lenticels thereby, reducing the transpiration and respiration rate and also reduce microbial activity (Nasrin *et al*., 2020).

Thus, the objective of the present work was to evaluate the effect of an edible coating based on cassava starch with the addition of coconut oil to increase the shelf life of minimally processed strawberries.

2. Materials and methods

2.1. Material

Fresh strawberries (*Fragaria ananassa*), cassava starch and coconut oil from the local market in Dourados-MS, Brazil, were used. The fruits were selected and classified according to their skin color, without physiological defects. The strawberries were transported to the bioengineering laboratory of the Federal University of Grande Dourados in plastic boxes, free of solar rays and at a temperature of approximately 10-12ºC. They were then stored at 5±1°C until processing.

2.2. Preparation of strawberries

The leaves and stalk of the strawberry were removed with the help of a stainless-steel knife, then they were sanitized in an organic chlorine solution at 2 $g.L^{-1}$, for 5 minutes, the water was drained for 2 to 3 minutes on sieves.

2.3. Preparation and application of coatings

The solutions were prepared according to the methodology of Chevalier *et al.* (2016), by slow homogenization of cassava starch (3%) in distilled water, until complete dissolution, followed by heating to 70ºC, for 20 minutes, and cooling to 40ºC. After this step, the coconut oil (Origin: Brazil; white colour at solid form but colourless above 30ºC with melting point in 25ºC and smoking point in 177ºC) was added according to the concentration established for each treatment.

The strawberries were totally submerged in the different coverings for three minutes and then drained with the help of sieves. Four treatments were obtained: T1 - control (strawberries without coating); T2 - coconut oil (1.0%) + cassava starch (3.0%); T3 - coconut oil (1.5%) + cassava starch (3.0%); T4 - coconut oil (2.0%) + cassava starch (3.0%). After applying the treatments, the strawberries were stored in a quantity of nearly 50 grams per package in PET (Polyethylene terephthalate), with lid (SANPACK) and with external measurements of 15x10x4 cm, and these packages were stored under refrigeration at 5±1°C for a period of 12 days.

2.4. Physical, chemical and microbiological analysis

The physical, chemical and microbiological analyses was performed in triplicate, on the day of processing, being considered as day 0 and after 1, 3, 5, 7, 9 and 12 days of storage.

2.4.1. Loss mass

The strawberries were stored at a cooling temperature of 5±1°C and UR of 60±2% and weighed in an analytical balance on days 0, 1, 3, 5, 7, 9 and 12 of storage. The loss of mass was obtained through the difference in the initial and final mass of the strawberry multiplied by 100 at each analysis. The results were expressed as a percentage of mass loss.

2.4.2. pH analysis

The analysis was performed according to the method described by AOAC (2000).

2.4.3. Total soluble solids content (ºBrix)

The total soluble solids content was determined using an Abbé bench refractometer, and the results were expressed in ºBrix (AOAC, 2000).

2.4.4. Titratable acidity

The titratable acidity was determined by titrating 10 mL of the sample (previously homogenized with 100 mL of distilled water) and phenolphthalein using 0.1 $mol.L^{-1}$ NaOH.

The results were expressed as a percentage of citric acid (AOAC, 2000).

2.4.5. Color measurement

The color measurements were made using a previously calibrated Konica Minolta colorimeter (Model CR-400/CR-410), using the CIE L*a*b* (Comission Internationale de L'Eclairage) system. The results were expressed as L*, a* and b* (Minolta, 1994).

2.4.6. Firmness measurement

The determination of the firmness of the strawberries was obtained by cutting and shearing the samples in a uniaxial way, with the aid of the texturometer (TA-TX Plus). The tests were performed with the aid of a 12 mm diameter cylindrical probe. The samples were centered on the slit of the blade, which was sheared at a speed of 2 mm s^{-1} uniaxially. The distance traveled was pre-fixed in 35mm and the complete shearing of the sample occurred. The shearing force was expressed in Newton (N) (AMSA, 2015).

2.4.7. Microbiological analysis

The microbiological tests performed were for molds and yeasts, psychrotrophic, *Salmonella* ssp, and *Escherichia coli*, following the methods described in APHA (2001).

2.4.8. Sensory evaluation

The sensory evaluation followed the methodology described by Chevalier *et al*. (2018), using 12 trained judges. For each treatment, attributes of texture, color, aroma and overall evaluation were evaluated only through sight, smell, and touch (under no hypothesis it was proven) during 12 days of storage at 5 ± 1°C, using a scale that ranged from 5 to 3, where 5 meant a sample of very good quality (fresh, aromatic, and without darkening); 4 meant regular (not very fresh, less pronounced aroma, and moderate darkening); 3 the sample of very bad quality (without freshness and aroma and with a high degree of darkening and presence of mold).

2.5. Statistical analysis

The analyses were realized in triplicate and the results expressed by the average. The results obtained were statistically evaluated through the Analysis of Variance (ANOVA) and Tukey's test at a 5% significance level, with the help of the STATISTICA® 7.0 program.

3.Results and discussions

3.1. Loss mass

Table 1 shows the values of loss of mass evaluated for strawberries over a period of 12 days stored at a temperature of 5±1ºC.

We can observe in Table 1 that T2 and T3 treatments had lower mass loss. The T4 treatment presented the highest mass loss (27.58%), values that were above those found by Martínez-González *et al*. (2020), who obtained values between 9.7% - 13.2% after eight days of storage, when they evaluated the effect of coatings with chitosan, chitosan nanoparticles and propolis on the behavior of ripening and antioxidant capacity of strawberries. As a non-climacteric fruit, strawberries have high water loss due to their considerable respiratory rate, in addition to being susceptible to mechanical damage and disease. All these factors lead the fruit to a rapid deterioration (Cunha Júnior *et al*., 2012). Mass loss increased significantly ($p>0.05$) during the entire storage period for all treatments. These results agree with those observed by Virgen-Ortiz *et al*. (2020), who evaluated the application of pectic oligosaccharides in different concentrations in the post-harvest quality of strawberries, where they found a 22.9% mass loss at the end of the storage period for untreated strawberries, while for strawberries coated with 9 g L^{-1} of pectic oligosaccharides the mass loss was 17.8%.

Table 1. Mass loss (%) of strawberries coated with cassava starch and different concentrations of coconut oil at 5±1ºC, for 12 days

Days	Treatments			
	T1	**T2**	**T3**	**T4**
0	0^{gA}	0^{gA}	0^{gA}	0^{gA}
1	0.61 ± 0.12^{fB}	1.22 ± 0.23^{fA}	1.20 ± 0.61^{fAB}	1.36 ± 0.37^{fA}
3	2.10 ± 0.16^{eB}	2.04 ± 0.44^{eB}	2.01 ± 0.08^{eB}	5.77 ± 0.71^{eA}
5	5.59 ± 0.21^{dB}	4.78 ± 0.09^{dC}	4.17 ± 0.17^{dD}	10.64 ± 0.22^{dA}
7	11.80 ± 0.43^{cB}	8.65 ± 0.31^{cC}	7.98 ± 0.32^{cC}	14.81 ± 0.58^{cA}
9	16.53 ± 0.11^{bA}	13.58 ± 0.11^{bB}	12.12 ± 0.09^{bC}	16.43 ± 0.04^{bA}
12	23.38 ± 0.94^{aB}	14.59 ± 0.59^{aC}	14.52 ± 0.06^{aC}	27.58 ± 0.49^{aA}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

Mass loss over 10% is enough to compromise the strawberry's appearance, causing wrinkled and dull epidermis (Hernandéz-Muñoz *et al*., 2006). Until the seventh day, T2 and T3 presented values below 10%, while T4 treatment presented values above 10% from the 5th day of storage. These results are close to those found by Muley and Singhal (2020), who used chitosan conjugate coating and whey protein isolate in strawberries stored at 5±1ºC and 20 ±1ºC.

3.2. pH analysis

Table 2 presents the evaluated pH data for strawberries during a 12 days storage period.

The pH values observed on day 12 decreased in relation to day 0 of storage for all the evaluated treatments, and the T3 and T4 treatments presented the highest final values, statistically differing from the other treatments. This higher pH value verified along the storage days for the samples T3 and T4 was already expected, since these samples were the ones that received the highest concentration of coconut oil. According to Muley and Singhal (2020), non-volatile organic acids, especially citric acid formed during the storage of strawberries, contribute and regulate pH. These organic acids are consumed in various metabolic pathways associated with the ripening process of strawberries (Lan *et al*., 2019). In this study, pH decreases during storage, and Yan *et al*. (2019) report that this decrease is combined with storage conditions, such as temperature, relative humidity and light, storage life, and physiological conditions of the fruits.

Studies by Bose *et al*. (2019) observed a slight increase in pH values with the passing of the days of storage when they worked with strawberries coated with alginate. Similar effect was found in the pH values by Virgen-Ortiz *et al*. (2020), working with the application of pectic oligosaccharides in the concentration of 9 g/L in the post-harvest quality of strawberries, where they observed a variation from 3.49 to 3.77 in 14 days of storage. These works demonstrated that a greater consumption of organic acids and sugars occurred during the respiratory metabolism of the strawberry. These results disagree with the present study, because a decrease of the pH values was observed with the passing of the storage days.

Table 2. pH values of strawberries coated with different proportions of coconut oil and cassava starch at 5±1ºC for 12 days

Days	Treatments			
	T1	**T2**	**T3**	**T4**
0	3.83 ± 0.04^{aA}	3.83 ± 0.04^{aA}	3.83 ± 0.04^{aA}	3.83 ± 0.04^{aA}
1	3.83 ± 0.02^{aA}	3.51 ± 0.01^{bC}	3.74 ± 0.03^{bB}	3.72 ± 0.02^{bB}
3	3.29 ± 0.02^{dC}	3.31 ± 0.01^{dC}	3.44 ± 0.05^{cB}	3.65 ± 0.01^{cA}
5	3.27 ± 0.03^{dA}	3.09 ± 0.00^{eD}	3.17 ± 0.00^{dB}	3.13 ± 0.00^{fC}
7	3.46 ± 0.04^{bcA}	3.29 ± 0.02^{dB}	3.45 ± 0.02^{cA}	3.27 ± 0.02^{eB}
9	3.54 ± 0.04^{bC}	3.54 ± 0.01^{bC}	3.73 ± 0.01^{bB}	3.84 ± 0.00^{aA}
12	3.46 ± 0.01^{cB}	3.45 ± 0.00^{cB}	3.50 ± 0.01^{cA}	3.50 ± 0.00^{dA}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

3.3. Total soluble solids content (ºBrix)

Table 3 presents the values of the total content of soluble solids found for strawberries over a period of 12 days stored at 5±1ºC.

Table 3. Values of total soluble solids (°Brix) of strawberries coated with different proportions of coconut oil and cassava starch at 5±1ºC, for 12 days

Days	Treatments			
	T1	**T2**	**T3**	**T4**
0	7.00 ± 0.00^{aA}	7.00 ± 0.00^{aA}	7.00 ± 0.00^{aA}	7.00 ± 0.00^{aA}
1	6.17 ± 0.24^{bA}	6.00 ± 0.00^{cA}	5.17 ± 0.24^{bcB}	6.00 ± 0.00^{bA}
3	5.50 ± 0.41^{cB}	7.00 ± 0.00^{aA}	5.00 ± 0.01^{cB}	4.00 ± 0.00^{eC}
5	5.00 ± 0.00^{dB}	5.50 ± 0.03^{dA}	5.00 ± 0.00^{cB}	4.00 ± 0.00^{eC}
7	5.50 ± 0.16^{cB}	6.37 ± 0.17^{bA}	5.40 ± 0.14^{bB}	6.07 ± 0.05^{bA}
9	5.33 ± 0.24^{cAB}	5.30 ± 0.14^{dAB}	5.50 ± 0.24^{bA}	5.00 ± 0.02^{dB}
12	5.13 ± 0.03^{cC}	5.08 ± 0.01^{eC}	5.77 ± 0.21^{bA}	5.20 ± 0.00^{cB}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

There was a decrease in the values of soluble solids with the passing of the storage days for all the evaluated treatments: the T3 treatment presented the smallest decrease (17.57%) and the T1 and T2 treatments presented the largest reductions, 26.71 and 27.41%, respectively. According to Pelayo *et al.* (2003), the reduction of the total soluble solids occurs because of the hydrolysis of sucrose, which uses the respective sugars as a substrate for respiration.

Petriccione *et al.* (2015) found different results in strawberries covered with chitosan (1% and 2%) and non-covered, where the content of soluble solids increased gradually over nine days of cold storage. A study by Virgen-Ortiz *et al.* (2020) also observed an

increase in the content of soluble solids in all treatments evaluated when they worked with the application of pectic oligosaccharides on strawberries. A possible explanation for this phenomenon is the solubilization of polyuronides and hemicelluloses present in the cell wall and also the loss of water due to the perspiration of the fruit (Nguyen *et al*., 2020).

3.4. Titratable acidity

Table 4 presents the results of titratable acidity found for strawberries during a period of 12 days stored at refrigerated temperature.

The results obtained of titratable acidity expressed in percentage (%) of citric acid show that there was a decrease in titratable acidity between day 0 and day 12 for treatments T1 (from 8.22 to 7.26), T2 (8.22-5.87) and T4 (8.22-6.31). According to Vargas *et al*. (2006), the enzymatic activity during storage is one reason why the decrease of fruit acids occurs. Taste and flavor are results of the presence of sugars and organic acids in the fruits, and many organic acids are secondary metabolites formed by the citric acid cycle that is used during breathing (Moshari-Nasirkandi *et al*., 2020). With this, it was perceived that the T1, T2 and T4 treatments went through a more accentuated respiratory process than the T3 treatment, because they obtained higher acidity losses in 12 days of storage.

Petriccione *et al*. (2015) found values close to the present study when working with strawberry chitosan coatings. These authors demonstrated that the acidity decreased significantly during the storage of strawberries at 2 ºC, with lower values in uncoated fruits compared with chitosan coated fruits. This work found final values of titratable acidity in treatments with less and more coconut oil (T2 and T4, respectively).

Table 4. Titratable acidity (citric acid %) of strawberries coated with different proportions of coconut oil and cassava starch at 5±1ºC, for 12 days

Days	Treatments			
	T1	**T2**	**T3**	**T4**
0	8.22 ± 0.33^{bA}	8.22 ± 0.33^{abA}	8.22 ± 0.33^{bcA}	8.22 ± 0.33^{bA}
1	4.59 ± 0.10^{dD}	6.11 ± 0.09^{dC}	6.88 ± 0.00^{dB}	7.45 ± 0.14^{cA}
3	7.26 ± 0.05^{cB}	8.41 ± 0.05^{aA}	8.60 ± 0.14^{abA}	7.45 ± 0.08^{cB}
5	9.36 ± 0.17^{aA}	7.93 ± 0.06^{bcC}	7.26 ± 0.05^{dD}	8.79 ± 0.05^{aB}
7	9.36 ± 0.19^{aA}	8.60 ± 0.08^{aB}	8.03 ± 0.08^{cC}	7.07 ± 0.21^{cD}
9	8.22 ± 0.12^{bA}	7.64 ± 0.12^{cB}	5.92 ± 0.12^{eC}	5.16 ± 0.14^{eD}
12	7.26 ± 0.19^{cB}	5.87 ± 0.09^{dD}	8.78 ± 0.09^{aA}	6.31 ± 0.29^{dC}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

3.5. Color measurement

The results found for the color parameters L* (Brightness), a* (Chroma a*) and b* (Chroma b*) are shown in Table 5.

Through Table 5 it is possible to observe that the parameters were affected by the presence of 3% cassava starch coating and by the different concentrations of coconut oil studied, since significant differences ($p\geq0.05$) were observed between the samples with the passing of the storage days.

The parameter L* (luminosity) is an indicator of darkening of the fruit, expressing the change in color during the period of senescence, where the fruit tends to be darker and redder throughout storage (Borges *et al*., 2015). The use of the cover on strawberries did not show significant changes in the initial coordinates (day 0) of color of the fruit. With the passing of the storage days, a decrease of L* values were observed for all the evaluated treatments, tending to a darker coloration, and

the T4 and T1 treatments were the ones that presented the smallest decrease of this parameter (20.72% and 19.77%, respectively), and the T3 treatment presented the smallest luminosity variation during the evaluated period (10.45%). The final values (days 12) of the highest brightness parameter of the treatments that the coverings (T2, T3
and T4) received occur by the cassava starch cover, together with different proportions of coconut oil that gave the strawberry a greater brightness.

Table 5. Color obtained from strawberries coated with cassava starch and different proportions of coconut oil at 5±1ºC for 12 days

Analyzed Parameters	Days	Treatments			
		T1	T2	T3	T4
L*	0	34.14 ± 0.76^{aB}	37.42 ± 0.87^{aA}	38.16 ± 0.22^{aA}	38.64 ± 0.82^{aA}
	1	33.94 ± 1.12^{aB}	37.30 ± 0.58^{aA}	38.06 ± 0.19^{abA}	37.64 ± 0.36^{abA}
	3	32.49 ± 0.33^{abB}	36.19 ± 1.12^{abA}	37.24 ± 0.52^{abcA}	36.01 ± 0.75^{bcA}
	5	31.50 ± 1.23^{bcB}	36.05 ± 0.09^{abA}	36.79 ± 0.71^{bcA}	34.57 ± 1.01^{cdA}
	7	30.31 ± 0.45^{cdD}	35.11 ± 0.47^{bcB}	36.12 ± 0.09^{cdA}	33.14 ± 0.26^{deC}
	9	29.72 ± 0.61^{cdC}	34.30 ± 0.08^{cdAB}	35.22 ± 0.16^{deA}	32.07 ± 0.06^{efB}
	12	28.18 ± 0.24^{dC}	32.15 ± 0.36^{dAB}	34.17 ± 0.88^{dA}	30.63 ± 0.34^{fB}
Chroma a*	0	29.48 ± 0.18^{aA}	31.24 ± 0.09^{aA}	31.89 ± 0.14^{aA}	32.13 ± 0.11^{aA}
	1	29.11 ± 0.43^{aC}	31.16 ± 0.15^{aB}	31.80 ± 0.09^{abA}	31.02 ± 0.07^{bB}
	3	27.70 ± 0.65^{bC}	30.73 ± 0.08^{aAB}	31.54 ± 0.07^{bA}	30.48 ± 0.09^{cB}
	5	26.11 ± 0.58^{cC}	29.61 ± 0.13^{aB}	30.98 ± 0.11^{cA}	29.31 ± 0.14^{dB}
	7	27.34 ± 0.53^{bC}	29.14 ± 0.05^{aB}	30.01 ± 0.03^{dA}	28.55 ± 0.17^{eB}
	9	26.03 ± 0.12^{cD}	28.03 ± 0.09^{aB}	29.79 ± 0.12^{dA}	27.01 ± 0.03^{fC}
	12	23.68 ± 0.18^{dD}	27.41 ± 0.27^{aB}	28.88 ± 0.05^{eA}	26.41 ± 0.07^{gC}
Chroma b*	0	25.03 ± 0.59^{abB}	26.63 ± 0.02^{aA}	26.78 ± 0.12^{bA}	27.22 ± 0.05^{aA}
	1	25.03 ± 0.21^{abC}	26.24 ± 0.14^{abB}	26.71 ± 0.09^{bA}	27.04 ± 0.19^{aA}
	3	24.36 ± 0.76^{abB}	25.86 ± 0.03^{abA}	26.25 ± 0.05^{cA}	25.07 ± 0.05^{bAB}
	5	26.04 ± 0.64^{aA}	26.21 ± 0.31^{abA}	27.08 ± 0.13^{aA}	26.79 ± 0.41^{aA}
	7	25.12 ± 0.30^{abC}	25.85 ± 0.30^{abB}	26.81 ± 0.09^{abA}	25.35 ± 0.07^{bBC}
	9	24.22 ± 0.06^{bB}	25.71 ± 0.02^{bA}	25.91 ± 0.14^{dA}	25.01 ± 0.22^{bAB}
	12	22.10 ± 0.17^{cC}	25.56 ± 0.09^{bA}	25.64 ± 0.04^{dA}	24.84 ± 0.09^{bB}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

The trend in the decrease of L* values found in this study were similar to those found by Virgen-Ortiz *et al*. (2020), when they worked with coverings in strawberries based on pectic oligosaccharides and also Garrido-Bigotes *et al*. (2018). These authors observed that the strawberries slowly darken during storage due to the induction of anthocyanin production. Furthermore, Perdones *et al*. (2012) found a decrease in luminosity values in uncoated strawberries coated with chitosan and essential

oil of lemon, and those with the coating showed lower losses of luminosity at the end of storage.

The Chroma a* varies from negative to green and positive to red. Chroma a* is a measure of redness and is highly correlated with the anthocyanin concentration in strawberry (Virgen-Ortiz *et al*., 2020).

A decrease of a* values was observed with the passing of the storage days for all the evaluated treatments (Table 5). The control treatment (T1) presented the greatest decrease (19.67%), and the T3 treatment had the smallest decrease (9.43%) in 12 days of storage. Hernández-Muñoz *et al*. (2008), report that the decrease of Chromas indicates a change to less vivid colors. A study by Virgen-Ortiz *et al*. (2020) disagrees with the present study, because when they worked with coverings of pectic oligosaccharides in the concentration of 5 and 9 g/L, they observed an increase in the values of Chroma a* with the passing of storage days.

Regarding Chroma b*, it can be observed that a small decrease in values occurred when compared between day 0 and day 12 of storage, and the control treatment (T1) showed the largest decrease (11.70%), while treatment T2 showed the smallest decrease in parameter b* (4.01%), followed by treatment T3 (4.25%). However, T2 and T3 treatments showed no significant difference between them ($p \leq 0.05$) at the end of the days of storage.

3.6. Firmness measurement

Changes in the texture of the controlled and treated strawberries during storage at 5±1ºC are shown in Table 6.

It can be seen in Table 6 that the firmness has decreased with the passing of the storage days for all treatments. The control treatment was the one that presented a greater decrease in texture at the end of 12 days (49.33%), while the T2 and T3 treatments presented the smallest loss, 24.72% and 22.07%, respectively, not differing statistically between them at the end of the evaluated period. During fruit ripening, the loss of firmness is mainly associated with the rupture of the medium lamella and the modification of the composition and structure of the polymers existing in the primary cell wall (Luo, 2006), respiration, water loss, and damage to structural tissues caused by molds (Chu *et al*., 2020). The process of disassembling the cell wall involves the depolymerization of the xyloglucan-cellulose matrix and the solubilization of pectins, which contribute to the softening of the fruits (Posé *et al*., 2019). As a result, it can be seen that the use of covers helped to slow down the loss of firmness and were efficient in keeping the cell wall of the fruit stiffer for longer.

Table 6. Firmness (N) of strawberries coated with cassava starch and different proportions of coconut oil at 5±1ºC, for 12 days

Days	Treatments			
	T1	**T2**	**T3**	**T4**
0	4.50 ± 0.11^{aA}	4.53 ± 0.11^{aA}	4.53 ± 0.11^{aA}	4.51 ± 0.11^{aA}
1	3.63 ± 0.09^{bB}	4.35 ± 0.06^{aA}	4.42 ± 0.23^{aA}	4.15 ± 0.05^{bA}
3	3.11 ± 0.16^{cB}	4.19 ± 0.14^{abA}	4.21 ± 0.09^{abA}	3.85 ± 0.17^{cAB}
5	3.01 ± 0.10^{cC}	4.02 ± 0.07^{bA}	4.09 ± 0.11^{bA}	3.59 ± 0.09^{cB}
7	2.78 ± 0.02^{dC}	3.82 ± 0.05^{cA}	3.94 ± 0.02^{bA}	3.37 ± 0.03^{dB}
9	2.57 ± 0.04^{eC}	3.70 ± 0.03^{cA}	3.81 ± 0.03^{cA}	3.13 ± 0.04^{eB}
12	2.28 ± 0.12^{fC}	3.41 ± 0.07^{dA}	3.53 ± 0.32^{cA}	3.02 ± 0.06^{eB}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

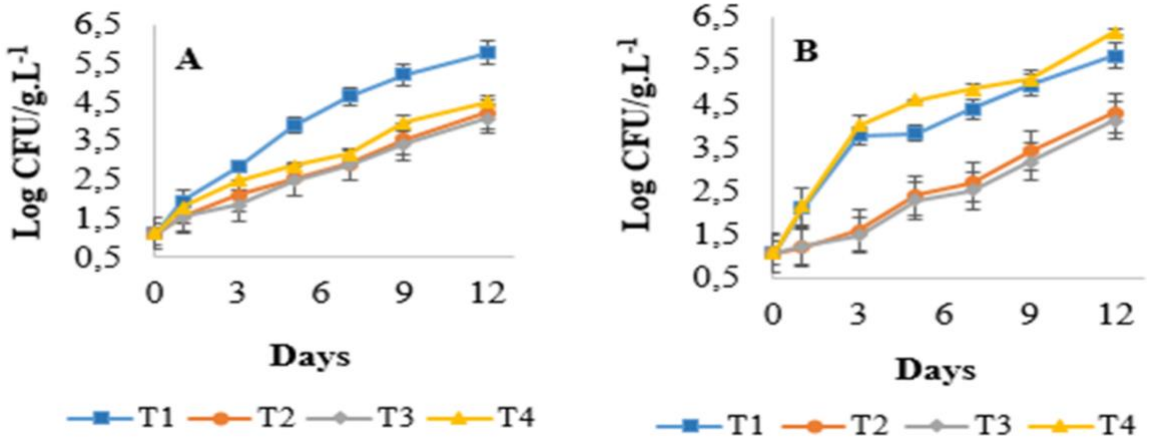
Some works found in the literature are in accordance with those observed in this study. Petriccione *et al*. (2015) demonstrated the efficiency of coatings to maintain the texture of strawberries for longer, when they worked with 2% chitosan in the coatings. When Martínez-González *et al*. (2020) studied the application of chitosan, along with nanoparticles of chitosan and propolis, in concentrations of 10% and 20%, they observed that at the end of eight days of storage the strawberries showed a greater firmness when compared to the control sample. These results are also in line with Restrepo and Aristizábal (2010), which reported higher firmness in strawberries covered with mucilaginous Aloe Vera gel and carnauba wax compared to the control sample. Ventura-Aguilar *et al*. (2018), in turn, evaluated the effect of chitosan and cinnamon essential oil applied to strawberries at 5 and 20ºC and observed a 33% higher firmness retention in the fruit treated with coatings compared to the control sample. Chitosan-based coatings plus oleic acid significantly reduced the rate of respiration and slowed the loss of strawberry texture (Vargas *et al*., 2006).

3.7. Microbiological analysis

No *Salmonella* ssp. and *Escherichia coli* were detected in the minimally processed strawberry samples. The minimally processed products must be similar to the fresh product, but with microbiological quality guaranteed by the reduction of pathogenic and deteriorating microorganisms. RDC Nº. 12 of 2001 (Brasil, 2001) establishes that for fresh fruit "*in natura*", prepared (peeled or selected or fractionated), sanitized, chilled, or frozen for direct consumption, the *Salmonella* ssp. bacteria must be absent in 25 grams of sample and that the maximum values of thermotolerant coliforms must be $5x10^2$ CFU.g^{-1}. Therefore, the present work is within these important specifications by legislation. Chevalier *et al*. (2018), when they studied the application of coatings obtained from protein isolate of tilapia (*Oreochromis niloticus*) in minimally processed melons did not observe the presence of *Salmonella* ssp. and *Escherichia coli*.

Figure 1 (A and B) shows the values of psychrotrophic and mold and yeast found for strawberries coated with cassava starch and coconut oil, stored for 12 days at controlled temperature (5±1ºC).

Figure 1. Psychrotrophic (A) and mold and yeast (B) found in strawberries coated with cassava starch and different proportions of coconut oil at 5±1ºC, for 12 days



Averages of three repetitions, expressed in colony forming unit per gram of sample: (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; and (T4) 3.0% cassava starch and 2% coconut oil.

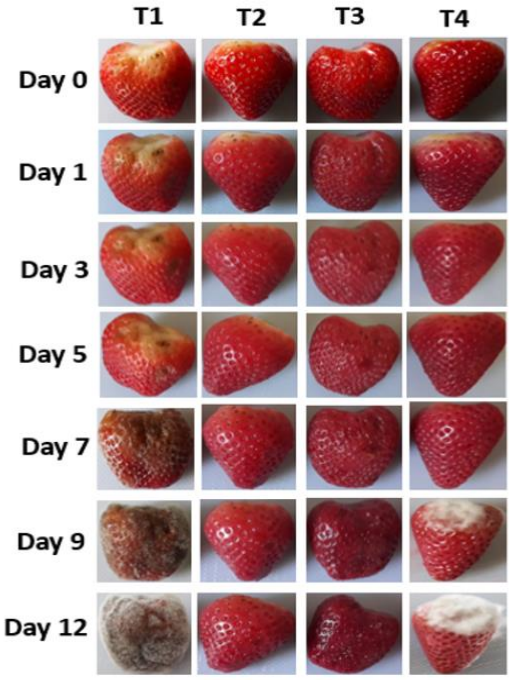


Figure 2. Appearance of strawberries without and with cassava starch-based coatings containing different concentrations of coconut oil over the days of storage

Figure 1 (A) shows the growth of psychrotrophic organisms during the 12 days storage period. As observed, there was an accentuated growth of these microorganisms with the passing of the storage days, and the control treatment (T1) presented a higher growth (5.5 log/CFU.g^{-1}), differing statistically from the others at the end of the experiment. The T2, T3 and T4 treatments did not differ from each other during the evaluated period.

As much as there are no standards in psychrotrophic bacteria and for mold and yeast in Brazil, it has been suggested that foods with populations above 10^5 and 10^6 CFU.g^{-1} (Verzeletti *et al*., 2010) may be unsuitable for humans due to loss of nutritional value, organoleptic changes, and risk of deterioration and infection (Lima *et al*., 2011). In this study, only the control treatment was above these limits from the 9th day of storage, demonstrating that the use of coatings was efficient to reduce the growth of psychrotrophic microorganisms. Similar results were observed by Chevalier *et al*. (2018), when they worked with coatings based on protein isolate of tilapia applied on minimally processed melons, and the control treatment also showed values above 10^5 from the 9th day of storage, and the treatments with protein isolate only exceeded this limit on the 12th day of storage.

Figure 1 (B) shows the growth of molds and yeasts in strawberries minimally processed during 12 days of storage. There was an increase in mold and yeast growth for all the evaluated treatments, and the T1 and T4 treatments showed the highest growth over the days of storage and at the end of 12 days these treatments did not differ statistically. For the treatments T2 and T3 an increase was also observed, but less than the control treatment and the treatment with cassava starch and 2% coconut oil.

The high growth for molds and yeasts presented by the T4 treatment can be explained by the fact that this treatment has the highest concentration of coconut oil, which may have caused a higher humidity of the sample and thus contributed to a significant increase of these microorganisms. On the other hand, the T2 and T3 treatments that presented lower

concentration of coconut oil (1.0% and 1.5%, respectively) left the strawberries with lower humidity, contributing to a lower growth of molds and yeasts.

When Perdones *et al*. (2012) worked with chitosan along with lemon essential oil; they obtained a positive effect in reducing the fungal activity in strawberries stored at 5ºC. Moreover, Vu *et al*. (2011) reported a higher antifungal effect of limonene with chitosan in strawberries in 12 days of storage compared to the control sample. These studies present similar results to the present study, where low concentrations of coconut oil were effective for lower microbial growth throughout the days of analysis.

Figure 2 shows the appearance of strawberries over the days of storage. As we can see, the strawberries developed a darker color (redder) with the passing of the days of storage; besides, the T1 and T4 treatments showed apparent mold growth (days 9 and 12). These observations can be confirmed when we evaluate Figure 1 (B), in which there was greater growth of mold and yeast for these treatments and also when we analyze the data obtained during the sensory analysis (Table 7), in which the judges provided grades between 3 (which was considered as the limit grade for fruit acceptability) or below this value from the ninth day for T1 and T4.

3.8. Sensory evaluation

Table 7 presents the data obtained for the sensory evaluation of strawberries coated with different proportions of coconut oil together with cassava starch, stored for 12 days at controlled temperature.

There was a significant difference in all samples and attributes evaluated after 12 days of storage. The treatments that received coverage with cassava starch and coconut oil were equal to or greater than 3 at the end of 12 days, and are therefore considered acceptable.

It can be observed that for all the evaluated attributes there was loss of visual acceptance with the passing of the days of storage. On the days 0 and 1 of storage there was no preference for coated fruits, since the average scores of acceptances did not differ significantly between treatments. Hernández-Muñoz *et al*. (2008), when evaluating chitosan and calcium-coated strawberries, obtained a higher acceptance of coated strawberries. These authors explain that the brilliance conferred by the use of this coating was attributed by the tasters for its greater initial acceptance. This observation was not a differential for the present study, since the marks attributed by the judges did not differ between the treatments at the beginning of the evaluation days.

T1 treatment presented the biggest reduction, and from the ninth day it had already surpassed the stipulated minimum value of quality (except for the aroma attribute). Hernández-Muñoz *et al*. (2008) observed that from the sixth day of storage, the control strawberries (without coatings) had already exceeded the stipulated limit, but it is worth noting that these authors were working with a storage temperature of 10ºC. The T2 and T3 treatments presented the highest acceptability at the end of 12 days of storage, and at the end of this period; they were still with values above 3.

Restrepo and Aristizábal (2010) found that the strawberry control sample had a higher odor in 5 days of storage when compared to the samples containing edible carnauba wax-based coating and also mucilage. They attributed this to the fact that the coatings used acted as a barrier that reduced the passage of aromatic compounds by reducing the perception of odor by the judges. This was not observed in this study, since when the aroma attribute was evaluated, it was verified that the samples that received the cassava starch coating with different concentrations of coconut oil presented higher values for this attribute at the end of 12 days of storage.

Table 7. Sensory evaluation of strawberries coated with cassava starch and different proportions of coconut oil at 5±1ºC, for 12 days

Analyzed Parameters	Days	Treatments			
		T1	T2	T3	T4
Texture	0	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}
	1	4.72±0.00^{bA}	4.81±0.18^{abA}	4.83±0.14^{aA}	4.84±0.00^{aA}
	3	4.41±0.15^{cA}	4.43±0.23^{bcA}	4.71±0.12^{abA}	4.43±0.13^{bA}
	5	4.00±0.00^{dC}	4.34±0.14^{cAB}	4.43±0.09^{bA}	4.20±0.00^{cBC}
	7	3.29±0.05^{eB}	4.11±0.18^{cdA}	4.02±0.13^{cA}	3.34±0.07^{dB}
	9	2.85±0.12^{fB}	3.89±0.01^{dA}	3.79±0.20^{cA}	3.14±0.15^{deB}
	12	2.60±0.00^{gC}	3.29±0.05^{eA}	3.14±0.13^{dAB}	3.00±0.00^{eB}
Color	0	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}
	1	4.88±0.00^{aA}	4.87±0.09^{aA}	4.95±0.22^{aA}	4.71±0.15^{bA}
	3	4.51±0.02^{bB}	4.80±0.05^{abA}	4.81±0.12^{abA}	4.60±0.02^{bB}
	5	4.14±0.15^{cC}	4.50±0.00^{bAB}	4.55±0.10^{bA}	4.30±0.05^{cBC}
	7	3.00±0.00^{dC}	4.08±0.25^{cAB}	4.12±0.10^{cA}	3.75±0.04^{dB}
	9	2.87±0.15^{dB}	3.98±0.19^{cA}	4.0±0.05^{cA}	3.00±0.00^{eB}
	12	2.62±0.08^{eC}	3.45±0.00^{dA}	3.43±0.10^{dA}	2.80±0.00^{fB}
Aroma	0	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}
	1	4.70±0.00^{bA}	4.71±0.05^{bA}	4.73±0.13^{abA}	4.70±0.00^{bA}
	3	4.21±0.08^{cB}	4.59±0.12^{bA}	4.48±0.06^{bcA}	4.43±0.13^{cAB}
	5	4.00±0.00^{dB}	4.45±0.09^{bA}	4.43±0.09^{cdA}	4.23±0.03^{dAB}
	7	3.29±0.15^{eB}	4.12±0.03^{cA}	4.14±0.07^{deA}	4.00±0.00^{eA}
	9	3.05±0.00^{fB}	3.81±0.15^{dA}	3.75±0.13^{efA}	2.98±0.00^{fB}
	12	2.67±0.00^{gC}	3.54±0.12^{eA}	3.43±0.10^{fA}	2.90±0.05^{gB}
Overall evaluation	0	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}	5.00±0.00^{aA}
	1	4.85±0.02^{aA}	4.87±0.02^{abA}	4.87±0.00^{aA}	4.86±0.03^{aA}
	3	4.57±0.10^{bA}	4.63±0.08^{bA}	4.71±0.10^{abA}	4.54±0.04^{aA}
	5	3.86±0.15^{cB}	4.29±0.10^{cA}	4.43±0.07^{bcA}	4.00±0.08^{bB}
	7	3.00±0.00^{dC}	4.00±0.12^{cdA}	4.16±0.23^{cdA}	3.40±0.00^{cB}
	9	2.85±0.15^{dB}	3.73±0.19^{dA}	3.82±0.05^{dA}	3.00±0.0^{dB}
	12	2.15±0.04^{eD}	3.05±0.01^{eB}	3.14±0.04^{eA}	2.85±0.03^{eC}

Averages of 3 repetitions ± standard deviation, followed by the same lower-case letter in the column and upper-case letter in the line do not differ by Tukey's Test ($p<0.05$): (T1) control (strawberry uncoated); (T2) 3.0% cassava starch and 1.0% coconut oil; (T3) 3.0% cassava starch and 1.5% coconut oil; (T4) 3.0% cassava starch and 2% coconut oil.

Mendonça *et al*. (2020) also obtained a decrease in the sensory attributes evaluated when they studied the use of microemulsions, microemulsions with citronella essential oil, microemulsions with avocado oil, and emulsion of avocado oil with water in minimally processed strawberries. These authors observed that in 11 days of storage at 4 ºC, the appearance, color and odor attributes presented by the control sample and the sample that received the emulsion prepared with avocado oil with water were classified as good by the judges. In turn, samples that had microemulsions along with citronella essential oil and microemulsions with

avocado oil were classified as regular and the sample containing only microemulsions was considered bad at the end of 11 days of storage. This work demonstrated that the use of oils together with the microemulsions were efficient to maintain the sensory characteristics of strawberries for longer. In the present study, it was also verified that the use of coconut oil together with cassava starch was promising for a longer sensorial life of minimally processed strawberries.

4. Conclusions

Of the evaluated treatments, T2 (1% coconut oil) and T3 (1.5% coconut oil) were the most efficient treatments in the conservation of minimally processed strawberries, when compared to the control sample and also the sample that presented the highest concentration of coconut oil in the formulation (T4).

The T2 and T3 treatments proved to have great potential to be applied as coatings as they reduced mass loss by approximately 85%, maintained physical and chemical characteristics, and reduced microbiological changes, especially in mold and yeast growth.

However, the use of coconut oil in low concentrations (1% and 1.5%), kept the minimally processed strawberries safe for consumption for a longer time when stored at 5 ± 1 ºC.

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ANTIBACTERIAL AND PHYTOCHEMICAL SCREENING OF VARIOUS FRUITS EXTRACTS OF *ABELMOSCHUS MANIHOT* TRADITIONALLY USED FOR THE TREATMENT OF CHRONIC BRONCHITIS

Mohammad M. Al Said[1], Jamal N. Al-Sabahi[2], Yahya A. Al Rashdi[1], Afaf M. Weli[1]✉

[1]College of Pharmacy and Nursing, University of Nizwa, Sultanate of Oman, P.O. Box 33, Postal code 616, Nizwa, Sultanate of Oman

[2] Central Instrument Laboratory, college of Agricultural and Marine Sciences, Sultan Qaboos University, Sultanate of Oman

✉afaf@unizwa.edu.om

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ABSTRACT

Abelmoschus manihot (*A. manihot*) is a flowering plant belong to the Malvaceae family that has been used traditionally in Oman to cure bronchitis, wound and toothache. Therefore, the goal of this present study is to prepare the various crude extracts from the fruits of the plant to identify the phytochemical constituents by using Gas Chromatography-Mass Spectrometry (GC-MS) and antibacterial activity by disc diffusion method. The powder of the fruits was extracted with methanol by using maceration method for 24 to 48 hours and then the extract was filtered by using Buchner apparatus. The methanol was removed under reduced pressure and the methanol crude residue was dissolved in a mixture of alcohol-water solution and successively fractionated with hexane, chloroform, ethyl acetate, and butanol to give the corresponding fractions. All solvents were removed from under reduced pressure and the crude various extracts was used to determined their antibacterial activity against *Escherichia coli (E. coli), Klebsiella pneumoniae (K. pneumoniae), Proteus vulgaris (P. vulgaris), Streptococcus pneumoniae (S. pneumoniae), and Staphylococcus aureus (S. aureus)* using the disc diffusion method. In addition, the phytochemicals of each extract were identified by using GC-MS. The results showed that among the extracts hexane and water extracts had the highest antibacterial activity with the range of inhibition zone 0-10.6 mm against the applied bacterial strains. The primary phytochemicals found in the extracts were unsaturated fatty acids, terpenoids derivatives, normal and aromatic hydrocarbons, alkaloid, and flavonoids derivatives. In conclusion, the best antibacterial activity extract of the selected plant could be used for the development of antibacterial agents.

1.Introduction

Plants are undeniably important in our daily lives. Plants are used by many creatures to carry out their functions. Humans, as well as some animals, ingest plant components, which are then eaten by humans. Plants were also employed to construct homes and treat a variety of illnesses. Plants were once thought to be the primary and only therapy for a variety of ailments, long before synthetic drugs were developed. Originally, drugs were made from extracts of various plant parts such as seeds, leaves, fruits, and roots (Todarwal et al, 2011). In the past, all medical therapies involved

manipulating plants in a precise way, such as boiling, grinding, or drying a specific component of a medicinal plant to cure various illnesses. Medicinal plants have been used to prevent and treat ailments since ancient times because they are biological sources of various minerals and bioactive elements that are both useful and safe. As a result, traditional medicine is preferred by 80% of the world's population. Only in Kenya, nearly 70% of the population relies on traditional medicine and home remedies as a first line of treatment for their illnesses (Abdullahi, 2011). More than 50,000 plant species are used for medical purposes all around the world (Chen et al, 2016). *Abelmoschus manihot* (*A. manihot*) is one of the well-known medicinal plant, which have so many medicinal values. This plant can be used for many conditions as it contains many active ingredients that are useful as anti-inflammatory, antibiotic, antidiabetic, antioxidant and many other uses (Onakpa,2011 & Dwivedi,et al 2013).

A. manihot which is commonly known as okra, edible hibiscus or sunset muskmallow belongs to the Malvaceae (Wen, et al 2015, Dorr & Wiersema 2010) family and it is considered to be a flowering plant. In addition, it is considered herbaceous perennial plant. It has palmate leaves that are profoundly analyzed and dissected (Wen, et al 2015). It has also profound projections found deeply called lobes and they are five to nine in number. The leaves vary in their size at the base of the plant they are wide. Also, they have different shapes, pigmentation, and colors. The leaves are alternate in the direction, and they are one of the simplest types of leaves (Onakpa, 2013). The length of the petiole can be from 3 to 25 cm. The leaf blade of this plant is linear and contains deep lobed segments which can be 3 to 7 in number. The stem is branching, woody and erect. The root is 30 to 40 cm long and it is known to be shallow and adventitious. The flowers are bell shaped and they consist of overlapping five petals. The petals are yellow in color and the flower has red center. The fruit is ovoid capsule covered by hair and has a narrow tip (Onakpa, 2013, & Dwivedi, et al 2013).

A. manihot is considered to be native to southwestern Asia. It is cultivated as vegetables especially in tropical areas. The plant has also wide distribution in Eastern European countries and Asia like India, Indonesia and Southern China (Chen et al, 2016 & Onakpa 2013).

A. manihot is one of the most important medicinal plants as it contains many active ingredients that have valuable therapeutic benefits. Many studies revealed that the plant has been used in traditional medicine in the past and also in the modern medicine as it contains a lots of chemical constituents which made the plant so special. This plant is known to have anti-inflammatory (Huang et al 2008) antifungal (Grosvenor et al 1995), antibacterial (Zamrul et al, 2019) antidiabetic (Dubey& Mishra 2017, Chen,et al 2015, Alam et al 2019, Zhao et al 2020) antioxidant and other activities (Wang,et al 2020, Gul et al 2011, Tahseen et al 2010). For example, in traditional medicine this plant is still used for cuts specially the bark of the plant which is mixed with water to produce a paste and this paste that can be applied to wounds. The applied paste will keep the wound clean and accelerate wound healing process. Recently, the juice of flowers is used to treat chronic bronchitis in addition to its benefits in the treatment of toothache. Furthermore, the roots of *A. manihot* are used in Nipal for the treatment of sprains (Luan et al, 2020). The literature available nowadays reveals that there are many chemical components obtained from this plant, they are estimated by 128 including polysaccharides, steroids, amino acids, flavonoids, nucleosides, volatile oils and many additional chemicals. It is also proven that the plant contains alkaloids (Liu et al 2020). The *A. manihot* is known to have many active ingredients that can be involved in the production of many drugs like antibiotics, antiviral, antidiabetic, antioxidant, diuretics, anti-inflammatory and many other activities (Xue et al 2011). Also, many studies revealed that this plant can be used for wound healing process, as well as protection against osteoporosis. Another study has shown that the Okra has hepatoprotective properties (Wang et al 2020).

Antibacterial effects are the ability to kill or inhibit the growth of bacteria, and they can be detected by allowing bacteria (G+ and G-) to colonize and providing suitable incubation conditions for the bacteria to grow, and extracts of various polarities will be inserted into the plate, and then observations and calculations will be made according to the zones of inhibitions, which are the areas in which bacterial growth is inhibited (Luan et al 2020). There is no previous research on the antibacterial activity of the fruit of *A. manihot* in Oman but species from other countries have shown to have antibacterial activity. Therefore, the goal of this present study is to prepare the various crude extracts from the fruits of the plant and determine the phytochemicals and antibacterial activity by using GC-MS and disc diffusion method.

2. Materials and methods

2.1. Materials

All chemicals and solvents were of analytical quality and were utilized without additional purification. Chem Solute in Germany provided the ethanol absolute (99.5 percent). Methanol, hexane, chloroform, butanol, and DMSO were purchased from Fisher Scientific in the United Kingdom, while ethyl acetate was procured from Carbon Group in Ireland. The University of Nizwa's DARIS Center distilled the water. Scharlau, Chemie Company Whatman Grade 1 Qualitative filter papers, plastic petri plates, and nutritional agar

Three gram-negative bacterial strains such as *Escherichia coli (E. coli), Proteus vulgaris (P. vulgaris), and Klebsiella pneumoniae (K. pneumonia)* and two gram-positive bacterial strains *Staphylococcus aureus (S. aureus) and Streptococcus pneumoniae (S. pneumonia)* were obtained from Nizwa Hospital and those were cultured at the DARIS Center, University of Nizwa. The cultured bacterial strains were used for the determination of antibacterial activity.

2.1.1. Collection of the Samples

The fruits of the *A. manihot* shrub were gathered in the Dhofar mountains. It's an untamed variety. Dhofar is located in southern Oman and is ideal for medicinal plants. The plant was sent to the lab for further processing. Dr. Syed Abdullah Gilani, Department of Biological Sciences and Chemistry, College of Arts and Sciences, University of Nizwa, recognized the samples, and a voucher specimen was deposited in the university's herbarium. The fruits samples were first cleaned and dried on newspapers in the shade at room temperature. A ball mill was used to grind the dried materials into a coarse powder.

2.1.2. Preparation of extracts

The coarse powder (130 gm) was placed into a beaker and extracted with methanol solvent (500 ml) for 48 hours to allow the extraction process to take place. The extract was then filtered by using a Buchner funnel, and the methanol solvent was removed under reduced pressure. The obtained semi-solid residue (8.2 gm; yield 8.2%) from the fruits coarse powder was suspended in an alcohol-water mixture (90:10) before fractionated. The solution was then placed into a separatory funnel. The relevant fractions, which comprise the remaining water fraction, were then obtained by adding hexane, chloroform, ethyl acetate, and butanol in order of increasing polarity. By evaporating all solvents with a rotary evaporator, hexane, chloroform, ethyl acetate, butanol, and water extracts were obtained. All of the extracts were subjected to antibacterial and phytochemical testing (Weli et al, 2020).

2.1.3. Antibacterial activity

The antibacterial activity of various plant fruits extracts of *A. manihot* was determined against a number of gram-positive and gram-negative bacterial strains. In this study, the disc diffusion method was employed to investigate activity. The stock solution of each extract was prepared by adding 10 mg of extract with 10 ml of DMSO solvent. From the stock solution of each extract, four different concentrations such as 1000, 500, 250 and 125 µg/ml were prepared using dilution method by adding DMSO. Petri dishes were made by adding agar to the dish, which formed some solid surface rich in nutrition for bacteria. A sterile cotton swab was then used to inoculate the germs onto the plates.

Discs (6 mm) were prepared from the filter paper and insert the discs in all prepared concentration of each extract. Then, extract-impregnated disc papers were carefully put onto the agar plate's surface. The paper discs were inserted in all prepared concentrations and kept for 30 minutes. Then, the disc was applied on the agar gel plate against the selected bacterial strains. As a positive and negative controls, the antibiotic levofloxacin and DMSO were used (Weli et al, 2018). All the plates were incubated inside the incubator for 24 hours at constant temperature 37 degrees Celsius. After 24 hours, all the plates were taken out and measured their inhibition by using the scale.

2.1.4. Phytochemicals of A. manihot

GC-MS analysis for phytochemicals was performed in Sultan Qaboos University, College of Agricultural and Marine Sciences, Central laboratories, on a Perkin Elmer Clarus, fitted with a SP-2560 Supelco capillary column (100 m × 0.250 mm i.d. × 0.2μm film thickness) coupled to Clarus 600C MS. As a carrier gas, helium (purity 99.9999%) was used at a constant flow of 1.0 ml/min. The temperatures for injection, transfer line and ion source were adjusted at 250, 240 and 240ºC, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 35-500 amu. The sample (1 μl) was injected into the injector with a split ratio of 50:1. The oven temperature program was 60ºC (holds for 1 minutes) and accelerated at a rate of 8ºC/min until 280ºC hold for 25 minutes. The unknown compounds in the fruits crude extracts were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition) and further confirmed with C7-C30 saturated alkane's standard (cat. # 49451-U).

3.Results and discussion

As a sources of bioactive compounds, the medicinal plants are considered one of the main resources for the discovery of medicine either natural or synthetic drugs. In addition, medicinal plants play a significant role for the progress of human cultures all over the world. Due to the importance of medicinal plant the present study was undertaken to conduct one of the traditionally used medicinal plant *A. manihot* in Oman. Therefore, the selected plant was collected from the southern part of Oman. After necessary process the coarse powder samples was extracted with methanol by using a maceration method and afterwards fractionated by various polarities of solvents. The percentage of yield of each extract from the methanol extract was presented in Table 1.

Table 1. % of yields of each extract from the methanol extract of *A. Manihot*

Name of extract	Amount(g)	%Yield
Hexane	10.70±0.15	8.20±0.11
Chloroform	5.63±0.22	4.33±0.72
Ethyl acetate	2.08±0.39	1.60±0.19
Butanol	3.90±0.09	3.00±0.10
Water	7.33±0.13	5.64±0.26

3.1. Antibacterial activity

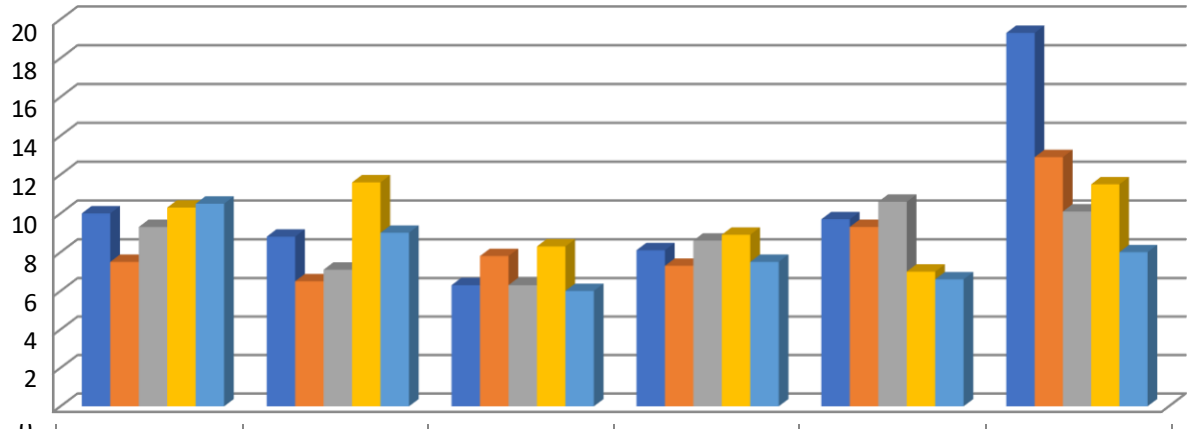
The antibacterial activity was evaluated by disc diffusion method describe by several authors (Weli et al 2018). All the various polarities fruits extracts of *A. manihot* at different concentrations were evaluated against the two-Gram positive bacterial strains *S. aureus* and *S. pneumonia* and three-Gram negative bacterial strains *E. coli, P. vulgaris,* and *K. pneumonia.* The results of antibacterial activity of the prepared various fruits extracts at different concentration was presented in Table 2. Based on the experimental results showed that in general all the extracts from the fruits of *A. manihot* gave the significant zones of inhibition

against the employed bacterial strains. The highest activity was observed in hexane extract against all tested bacteria with the range of 0-15 mm except *S. aureus* it gave moderate activity about 6.5 mm at the highest concentration of 1000µg/ml. The chloroform residue was most active against *K. pneumonia* with the zone of inhibition 12 mm at the highest concentration 1000µg/ml. The ethyl acetate residue showed comparatively best activity with the zone of inhibition 8.5 mm against *S. aureus* at the concentration 1000 µg/ml and weaker activity against other employed bacteria at all prepared concentrations. However, the butanol extract gave good inhibition against all applied Gram (– and +) bacteria strains with the range of 0-9 mm. In addition, the water extract residue also showed very good activity against Gram-negative tested bacteria strains and moderate to weak activity against Gram-positive tested bacteria strains Table 2.

Table 2. Antibacterial activity of different polarities extracts of *A. manihot* against *S. pneumonia, S. aureus, P. vulgaris, K. pneumonia and E. coli*

Extracts	**Concentration µg\ml**	***S. pneumonia***	***S. aureus***	***P. Vulgaris***	***K. pneumonia***	***E. Coli***
Hexane	1000	10	6	15	13.5	9.5
	500	9	7	7.5	9	13
	250	11	6.5	9	9	10
	125	10	10.5	6	10	9.5
Levofloxacin	300	14	12	9.5	10	8
Chloroform	1000	7.5	7	6	12	9
	500	7	6	6	12	9
	250	11	7	10.5	10.5	6
	125	10	6	6	12	12
Levofloxacin	300	18.5	6.5	6	10	6
Ethyl acetate	1000	6	8.5	6	7	6
	500	6.5	8	6	9	6
	250	6.5	8	6	10	6
	125	6.5	7	7	7	6
Levofloxacin	300	15	10	8	10	6
Butanol	1000	8.5	8	8.5	8	9
	500	7.5	8	9	8.5	7.5
	250	8.5	7	7.5	9	7.5
	125	8	6	9.5	10	6
Levofloxacin	300	12.5	10	7	9	6
Water	1000	9.5	10	8.5	7	6
	500	9.5	9	16	7	6
	250	8.5	8.5	6	7	6.5
	125	10	8.25	12	7	8
Levofloxacin	300	17	13	10	7	6

From the results the order of inhibition zone against *S. pneumonia* hexane>water>chloroform>butanol>ethyl acetate, Zone of inhibition against *S. aureus*: water >ethyl acetate>hexane>butanol>chloroform, Zone of inhibition against *P. vulgaris*: water>hexane>butanol>chloroform>ethyl acetate, Zone of inhibition against *K. pneumonia*: chloroform>hexane>butanol>ethyl acetate>water, and inhibition zone against *E. coli*: hexane >chloroform>butanol >water>ethyl acetate Figure 1.



	Hexane	Chloroform	Ethyl acetate	Butanol	water	Levofloxacin
S. pneumonia	10	8.8	6.3	8.1	9.7	19.3
S.aureus	7.5	6.5	7.8	7.3	9.3	12.9
P. Vulgaris	9.3	7.1	6.3	8.6	10.6	10.1
K. pneumonia	10.3	11.6	8.3	8.9	7	11.5
E.Coli	10.5	9	6	7.5	6.6	8

Figure 1. Data analysis represented by average inhibition zones by mm of antibacterial potentials of different polarities extracts of *Abelmoschus manihot*

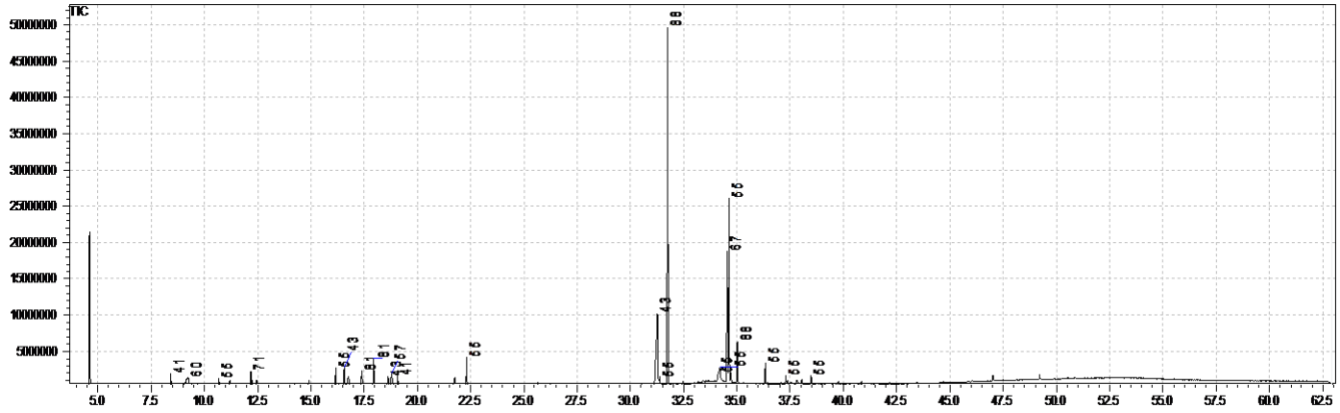
3.2. Phytochemicals

3.2.1. Hexane extract

The highest percentage phytochemicals in the hexane extract are octadecanoic acid, methyl ester, erucic acid, 9-octadecadienoic acid ethylester, n-hexadecanoic acid, 9,12-octadecadienoic acid and lower percent are 2-heptenal, hexanoic acid, 3-octen-2-one, 2,3'-bifuran, octahydro-, cyclopropane, octyl-, 2-decenal, 2,4-decadienal, 2,4-decadienal, dec-3-en-2-one, octane, 2,4,6- trimethyl-, 2-undecenal, nonanoic acid, 9-oxo-, ethyl ester, ethyl 9-hexadecenoate, ethyl ester, erucic acid, cis-10 nonadecenoic acid, Z-8-methyl-9-tetradecenoic acid. The percentage of each phytochemical are presented in Table 3 and Figure 2.

Table 3. Compounds present in the hexane extract

#	Compound name	R.Time (min)	Area	Area %	Cal KI	NIST KI
1	2-Heptenal, (Z)-	8.421	3838514	1.01	963.1365	932
2	Hexanoic acid	9.246	4605197	1.22	993.7184	973
3	3-Octen-2-one	10.679	2526867	0.67	1045.451	1015
4	2,3'-Bifuran, octahydro-	12.186	5089419	1.34	1099.856	1079
5	Cyclopropane, octyl-	16.163	5617027	1.48	1249.533	1115
6	2-Decenal, (Z)-	16.579	5941056	1.57	1265.72	1227
7	2,4-Decadienal, (E,E)-	17.395	5588290	1.48	1297.471	1288
8	2,4-Decadienal	17.95	8848750	2.34	1320.082	1270
9	Dec-3-en-2-one	18.624	3143083	0.83	1347.705	1233
10	Octane, 2,4,6-trimethyl-	18.793	4685995	1.24	1354.631	1277
11	2-Undecenal	19.07	2987578	0.79	1365.984	1350
12	Nonanoic acid, 9-oxo-, ethyl ester	22.311	8893830	2.35	1505.068	1507
13	n-Hexadecanoic acid	31.265	51134796	13.51	1953.911	1942
14	Ethyl 9-hexadecenoate	31.38	2398319	0.63	1960.335	1955
15	Octadecanoic acid, ethyl ester	31.754	121128966	31.99	1981.229	2181
16	Erucic acid	34.195	7019802	1.85	2124.085	2546
17	9,12-Octadecadienoic acid (Z,Z)-	34.543	43987128	11.62	2145.305	2095
18	(E)-9-Octadecenoic acid ethyl ester	34.632	60218239	15.9	2150.732	2174
19	Octadecanoic acid, ethyl ester	35.019	13250251	3.5	2174.329	2167
20	cis-10-Nonadecenoic acid	36.332	6649612	1.76	2256.815	2256
21	Z-8-Methyl-9-tetradecenoic acid	38.476	3712100	0.98	2397.086	2104



F**igure 2.** GC-MS chromatogram of Hexane fruit extract of *Abelmoschus manihot*

3.2.2. Chloroform extract

Nonanoic acid was the major constituent in the chloroform extract about (20.5%) followed by dimethyl sulfoxide, hexanoic acid, 8-nonynoic acid, hexadecanoic acid, ethyl ester and linoleic acid ester up to 3.63% and to lesser amounts from other components that are presented in Table 4 and Figure 3.

Table 4. Compounds present in the chloroform extract

#	Compound name	R. Time (min)	Area	Area%	Cal KI	NIST KI
1	Hexanal	4.623	1877661	3.96	808.2288	806
2	Dimethyl Sulfoxide	5.265	8670103	18.29	831.9188	820
3	Dimethyl sulfone	7.434	2649863	5.59	926.7159	914
4	2-Heptenal, (Z)-	8.426	1243096	2.62	963.321	932
5	Hexanoic acid	9.113	3883513	8.19	988.6716	973
6	Heptanoic acid	11.603	390294	0.82	1078.809	1073
7	Nonanal	12.459	460385	0.97	1109.963	1081
8	Octanoic acid	14.208	762521	1.61	1174.741	1173
9	Pantolactone	14.876	313822	0.66	1199.481	1148
10	2-Decenal, (Z)-	16.578	1124355	2.37	1265.681	1229
11	Nonanoic acid	16.8	9708212	20.5	1274.319	1268
12	8-Nonynoic acid	17.361	2542464	5.36	1296.148	1270
13	2,4-Decadienal, (E,E)-	17.954	370391	0.78	1320.246	1288
14	1-Undecene, 8-methyl-	18.627	895560	1.89	1347.828	1124
15	gamma.-Nonalactone	19.105	1230906	2.6	1367.418	1325
16	Undec-10-ynoic acid	19.658	763741	1.61	1390.082	1469
17	9-Oxononanoic acid	21.714	1832321	3.87	1478.87	1483
18	Nonanoic acid, 9-oxo-, ethyl ester	22.312	291758	0.62	1505.114	1507
19	Ethyl hydrogen suberate	23.391	910306	1.92	1554.384	1553
20	Z-10-Tetradecen-1-ol acetate	28.084	330310	0.7	1782.437	1787
21	Hexadecanoic acid, ethyl ester	31.725	2303600	4.86	1979.609	1985
22	9,12-Octadecadienoic acid (Z,Z)-	34.532	705093	1.49	2144.634	2095
23	(E)-9-Octadecenoic acid ethyl ester	34.615	537516	1.13	2149.695	2185
24	Linolenic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (Z,Z,Z)-	42.068	1720786	3.63	2652.09	2721
25	U.I	42.748	1875650	3.96	2702.923	

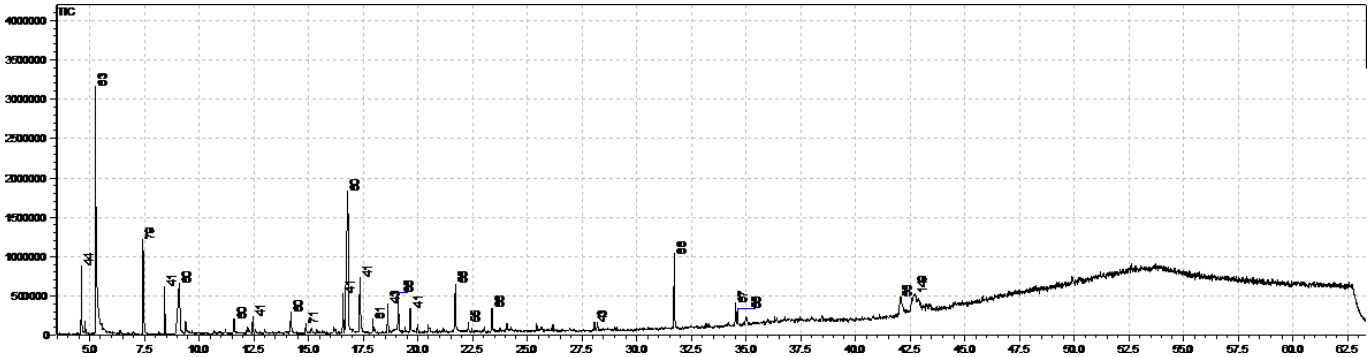


Figure 3. GC-MS chromatogram of Chloroform fruit extract of *Abelmoschus manihot*

3.2.3. Ethyl acetate extract

In the chromatogram **Figure 4**, it is clearly showed that no peaks appeared in the ethylaceate extract. It could be due to the compounds are not sensitive or not separated in the SP-2560 Supelco capillary column. In addition, inside the ethyl acetate extract might be all compounds are high molecular weight.

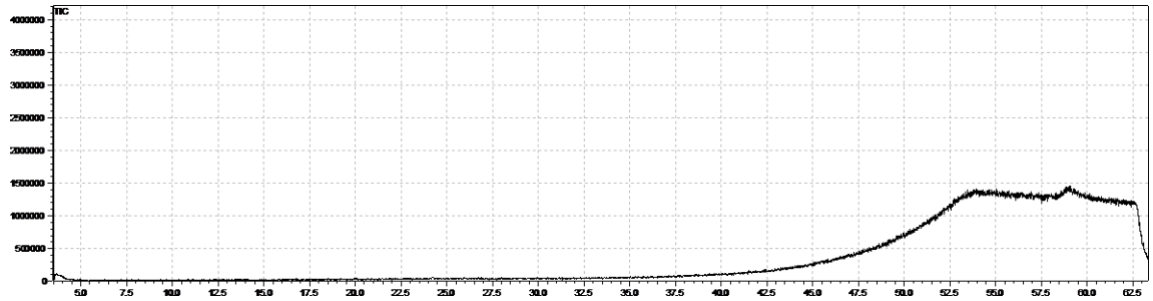


Figure 4. GC-MS chromatogram of ethyl acetate fruit extract of *Abelmoschus manihot*

3.2.4. Butanol extract

Butanoic acid, butyl ester, 2-heptanol, 2-methyl and docosanoic acid, docosyl ester were present in high percent in the butanol extract 28.5, 28.2 and 16.64 percent respectively. In addition to palmitic acid, ethyl ester about 8.86% that are presented in Table 5, Figure 5.

Table 5. Compounds present in the butanol extract

#	Compound name	R.Time (min)	Area	Area %	Cal KI	NIST KI
1	Propanoic acid, 2-methyl-, butyl ester	8.345	692258	3.84	960.3321	952
2	Propanoic acid, 2 methyl-, 2-methylpropyl ester	8.405	1383514	7.67	962.5461	925
3	Butanoic acid, butyl ester	9.494	5139690	28.5	1002.671	969
4	2-Heptanol, 2-methyl-	12.975	5086154	28.2	1129.074	919

5	Butane, 1,1-dibutoxy-	16.409	753347	4.18	1259.105	1229
6	Palmitic acid, methyl ester	30.543	381329	2.11	1913.575	1908
7	Palmitic acid, ethyl ester	31.723	1598305	8.86	1979.497	1968
8	Docosanoic acid, docosyl ester	49.955	3000779	16.64	3304.206	4547

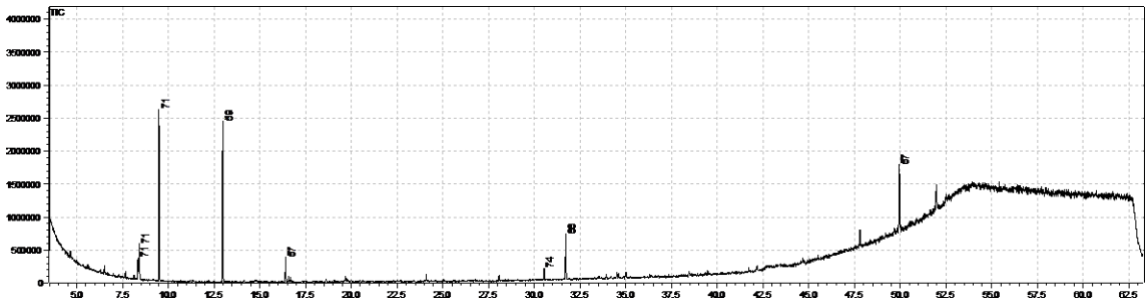


Figure 5. GC-MS chromatogram of Butanol fruit extract of *Abelmoschus manihot*

4. Conclusions

The antimicrobial activity and phytochemicals of each fruits extract were determined by disc diffusion method and GC-MS. In this study, the highest antibacterial activity was observed in hexane and water extracts with the range of inhibition zone 0-10.6 mm against the applied bacterial strains. Unsaturated fatty acids, terpenoids derivatives, normal and aromatic hydrocarbons, alkaloid, and flavonoids derivatives were the main phytochemicals detected in the different polarity extracts. Finally, the selected plant's extract with the highest antibacterial activity might be employed to develop antibacterial agents.

Major compounds found in butanol extract are butanoic acid, butyl ester, 2-heptanol, 2-methyl. In the chloroform extract nonanoic acid was the major constituent (20.5%). The hexane extract showed the presences of octadecanoic acid, methyl ester (31.99%), erucic acid, E- 9-octadecadienoic acid ethyl ester (15.9%), n-hexadecanoic acid (13.51%), and 9,12-Octadecadienoic acid (Z,Z) (11.62%). However, the ethyl acetate extract did not show the presences of any compounds.

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OPTIMIZATION AND KINETICS OF THE SUPERCRITICAL FLUID EXTRACTION OF TRITERPENOIDS FROM *GANODERMA LUCIDUM* WITH CO_2 AND ETHANOL AS COSOLVENT

Van Man Phan[1]✉, Minh Suong Ngo Thi[2], Duc Duy Tran[3]

[1]Faculty of Food Technology, Ba Ria–Vung Tau College of Technology, Vung Tau city, Vietnam.
[2]School of Animal Sciences, Can Tho University, Can Tho City, Vietnam.
[3]Faculty of Food Science and Technology, HCMC University of Food Industry, Ho Chi Minh City, Vietnam.
✉pvman.dbv@moet.edu.vn

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ABSTRACT

This research aims to optimize ethanol-modified supercritical carbon dioxide extraction (SC-CO_2) conditions for extracting triterpenoids from *G. lucidum* using a response surface methodology (RSM). A central composite face-centered design (CCF) was employed to investigate the influences of three independent variables, including ethanol concentration in SC-CO_2 (X_1), extraction pressure (X_2), and temperature (X_3) on the response, triterpenoid content (Y). The results showed that the optimal RSM-based SC-CO_2 conditions were 380 bar, 7%v/v, and 60°C, achieving the maximum value of 1.49g/100g. Under these conditions, the predicted values for triterpenoids agreed well with the experimental results, confirming the validity of the generated model. The SC-CO_2 extraction technique showed clear advantages over conventional maceration extraction and soxhlet extraction in terms of high triterpenoid recovery and antioxidant activity. The kinetics of the solvent-based triterpenoid extraction processes were subsequently assessed via the first-order and second-order kinetic models. The second-order kinetic model was more sufficient to describe the extraction mechanism of triterpenoids from *G. lucidum* in comparison to the first-order kinetic extraction model. According to these findings, SC-CO_2 extraction is a promising and efficient method for triterpenoid extraction from *G. lucidum*.

1. Introduction

Ganoderma lucidum (*G. lucidum*) is a traditional medicinal mushroom in China, Japan, and other Asian countries, which has been utilized for more than 2000 years (Li et al., 2020; Zhu et al., 2020). The *G. lucidum* spore contains many bioactive compositions such as triterpenoids, steroids, phenolics, and nucleotides (Mau et al., 2001; Zhu et al., 2020; Li et al., 2016). Among these compounds, triterpenoids have been known as strong antioxidant compounds in *G. lucidum* (Taofiq et al., 2017; Zhang et al., 2008). Triterpenoids have many effective therapeutic actions including antitumor, anti-inflammatory, anti-hepatitis, antimetastatic, and antihyperlipidemic (Cai et al., 2016; Zhu et al., 2020; Pan et al., 2013).

In the last few decades, the conventional extraction methods (i.e., maceration, soxhlet, and heat reflux extraction) are commonly used for industrial extraction of triterpenoids from *G. lucidum* (Taofiq et al., 2017; Plazas et al., 2020). The main disadvantages of conventional soxhlet extraction are very time-consuming and require large volumes of solvents (Rodrigues et al., 2021; Xu et al., 2017; Ibáñez and Cifuentes,

2015; Blicharski and Oniszczuk, 2017). In comparison, supercritical carbon dioxide (CO_2) fluid extraction has obvious advantages such as low operation temperature, low cost, and no solvent residue. However, CO_2 is not a suitable solvent for the extraction of triterpenoid compounds because triterpenoids are usually non-polar compounds. Therefore, the use of ethanol as a co-solvent extraction has been considered for enhancing the solubility of the triterpenoid compounds in the solvent (Herrero et al., 2010; Pieczykolan et al., 2019).

Ethanol, a green solvent, is suggested for the extraction of triterpenoids from *G. lucidum* due to its availability, low cost, and environmentally friendly solvent (Rodrigues et al., 2021; Li et al., 2017). Some previous studies showed that various extraction factors such as co-solvent extraction, extraction temperature, and extraction pressure significantly influence the extraction efficacy of triterpenoids (Zhu et al., 2020; Yim et al., 2019). However, to the best of our knowledge, there are limited or no studies, available in the literature on optimization and kinetic extraction of triterpenoid from *G. lucidum*. In this study, CO_2 solvent with the addition of ethanol was used for triterpenoid extraction from the Vietnamese *G. lucidum*. The optimization of different SC-CO_2 extraction conditions (extraction pressure, ethanol content in SC-CO_2, and extraction temperature) on the recovery of triterpenoids was investigated. A response surface methodology (RSM) was used to find the optimal extraction conditions and interpret the interactions among these independent variables by establishing a model from experimental data. Furthermore, the first and second-order kinetic models were utilized to explain and describe the kinetic behavior of the SC-CO_2 process extracting triterpenoids with a mixture of SC-CO_2 and ethanol. Then the radical scavenging activity of the extracts was compared with the soxhlet extraction (SE) and maceration extraction (ME). It is expected that the developed SC-CO_2 processes will contribute to improving the efficiency of the overall extraction process and be applied in the *Ganoderma* industry.

2. Materials and methods

2.1. Materials

The dried *G. lucidum* was obtained from a L'ang farm store (Da Lat city, Lam Dong province, Vietnam) as dry material. All samples were ground in a Waring blender and passed through a 20-mesh sieve before extraction.

Ursolic acid, perchloric acid 70 %, glacial acetic acid, and ethanol (99.8%) were obtained from Merck (Dam-stadt, Germany). Carbon dioxide (99.9%) was purchased from the Daxing Gas Co., Beijing, China. All other chemical reagents used were of analytical grade.

2.2. Extraction procedure

The SC-CO_2 method was experimented using a supercritical fluid system (SFE-500F2-C50, Waters, USA). In this study, SC-CO_2 with ethanol addition was used to investigate the effect of extraction parameters on the recovery of triterpenoid from *G. lucidum*. Firstly, the effect of the addition of ethanol as a polar cosolvent extraction on the triterpenoid content was analyzed. In each run, approximately 10 g *G. lucidum* powder was introduced into the extraction vessel, and the extraction was performed at 50°C, with the different ethanol concentrations in SC-CO_2 (0-12%v/v). The extraction pressure was set at the desired value of 400 bar. Secondly, the influence of extraction pressure on the recovery of triterpenoids was experimented on between the range of 200 and 500 bar at 50°C using 6%v/v of ethanol in SC-CO_2. Finally, the influence of temperature extraction on the recovery of triterpenoids was performed. The temperature extraction was in a range of 30 to 80°C, and the extraction process was performed with 6%v/v of ethanol at 400 bar. All the extractions were performed with a constant CO_2 flow rate of 0.45 mL/min for 2 hours. After extraction, the extracts were collected and the solvent was then evaporated in a rotatory evaporator (Buchi R210, Flawil Switzerland). The extracts were kept refrigerated at -20°C for further analysis.

2.3. Experimental design

Central composite face-centered design (CCF) was used to determine the optimum conditions for cosolvent extraction of triterpenoid content. Three independent variables were ethanol concentration in SC-CO_2 (X_1, %v/v), extraction pressure (X_2, bar), and extraction temperature (X_3, °C).

Table 1. Coded and actual levels of three variables

Variables	**Coded levels of variables**		
	Low (-1)	Medium (0)	High (+1)
Ethanol content (X_1, %v/v)	2	7	12
Pressure extraction (X_2, Bar)	200	350	500
Extraction temperature (X_3, °C)	30	55	80

Table 2. Experimental and predicted gamma oryzanol recovery under variable ethanol content (X_1, %v/v), pressure extraction (X_2, Bar), and extraction temperatures (X_3, °C)

Run	Coded variable						Experimental value	Predicted value
	X_1	X_2	X_3	X_1	X_2	X_3	g/100g	g/100g
1	-1	-1	-1	2	200	30	0.513±0.01	0.506±0.02
2	-1	-1	+1	2	500	30	0.772±0.02	0.735±0.01
3	-1	+1	-1	2	200	80	1.050±0.01	1.033±0.01
4	-1	+1	+1	2	500	80	0.938±0.01	0.928±0.03
5	+1	-1	0	12	200	30	0.791±0.01	0.794±0.01
6	+1	-1	+1	12	500	30	1.098±0.02	0.986±0.02
7	+1	+1	0	12	200	80	0.596±0.01	0.578±0.01
8	+1	+1	+1	12	500	80	0.810±0.01	0.811±0.02
9	0	0	0	7	200	55	1.231±0.02	1.128±0.01
10	0	0	+1	7	500	55	1.510±0.02	1.507±0.01
11	0	-1	0	7	350	30	1.281±0.01	1.260±0.02
12	0	+1	0	7	350	80	1.539±0.01	1.460±0.03
13	-1	0	0	2	350	55	1.196±0.01	1.068±0.01
14	+1	0	0	12	350	55	1.356±0.02	1.308±0.01
15	0	0	0	7	350	55	1.511±0.01	1.450±0.02
16	0	0	0	7	350	55	1.512±0.01	1.516±0.01
17	-1	-1	-1	2	200	30	0.513±0.01	0.510±0.02

The triterpenoid content was selected as the response (Y) for the combination of the independent variables. Statistic MODDE software (version 13; Umetri, Umeå, Sweden) was used for the regression analysis of the experimental data. The variation of these factors was varied at three levels (low, moderate, and high), and coded as -1, 0, and +1, respectively (Table 1), and the experimental design consisted of 17 experimental runs (Table 2). A second-order polynomial equation was established to predict for optimization of the triterpenoid content.

$$Y = \beta_0 + \sum_{i-1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_i X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X^2 \qquad (1)$$

where Y is the predicted response; β_0 is a constant; X_1, X_2 and X_3 are independent variables and βi, βij and βii are the linear

coefficients, interaction coefficients, and quadratic coefficients, respectively. The model adequacy was evaluated based on an F-test and the coefficient of determination (R^2) using analysis of variance (ANOVA).

2.4. Kinetic extraction

In this study, the first- and second-order kinetic models were employed to explain the extraction behaviors. The kinetic analysis was conducted based on two scenarios: (i) variable extraction temperature (30°C, 55°C, 80°C), given 350 bar SC-CO_2 pressure; and (ii) variable SC-CO_2 pressure (200 bar, 350 bar, 500 bar), given 55°C extraction temperature. Ethanol was used as cosolvent extraction (the RSM-based optimal ethanol concentration was 7%v/v), while the extraction time was varied between 0 min, 20 min, 40 min, 60 min, 80 min, 100 min, and 120 min.

2.4.1. First-order kinetic extraction

The first-order kinetic extraction was described well by Hobbi et al. (2021) as follows:.

$$\frac{dC_t}{dt} = k.(C_e - C_t) \quad (2)$$

where C_t is the extraction capacity at different extraction time t, and C_s is the triterpenoid content of G. lucidum at saturation. k (min^{-1}) is the extraction rate constant. Eq. 2 was then integrated at the boundary conditions Ct = 0 at t = 0 and Ct = Ct at t = t to obtain Eq. 3.

$$\ln\frac{C_e}{C_e - C_t} = k.(C_e - C_t) \quad (3)$$

2.4.2. Second order kinetics

In this study, the second-order model was employed to describe the kinetics behavior of solid-liquid extraction, and can be written as follows:

$$\frac{dC_t}{dt} = k_s.(C_e - C_t)^2 \quad (4)$$

where k_s is the second-order rate constant (100g/g min), C_t is the weight of the triterpenoids in the SC-CO_2 extraction at a given time t (g/100g), and Cs is the equilibrium concentration of the triterpenoids extracted by the SC-CO_2 (g/100g). Eq. 4 can be integrated at the initial and boundary condition (t = 0 to t, and C = 0 to C), and then Eq. 5 can be re-arranged as:

$$\frac{t}{C_t} = \frac{1}{k.C_e^2} + \frac{t}{C_e} \quad (5)$$

When *t* approaches 0, the initial extraction rate, *h*, is written as in Eq. 6.

$$h = k.C_e^2 \quad (6)$$

The initial extraction rate (h), the extraction capacity (C_S), and the second-order extraction rate constant (k) can be determined from the slope and intercept by plotting t/C_t versus t.

2.5. Conventional extraction

The efficiency of extraction of soxhlet extraction (SE) and ethanol maceration extraction (ME) was carried out to compare with the SC-CO_2 extraction method. For soxhlet extraction, 10 g *G. lucidum* powder was performed with 500 mL of ethanol 99.8% for 6 hours (Rodrigues and Silva 2021). For ethanol maceration, 100g G. lucidum powder was extracted with 1 L ethanol 99.8% for 24 hours in a shaking water bath set at 160 rpm and 32±0.5°C, based on the preliminary experiment, showing a suitable extraction condition. Then, the solution was filtered through a Whatman No. 1 filter paper to collect the extracts. The solvents were removed using a rotary evaporator (Buchi R210, Flawil Switzerland), and the extracts were then stored at −20 °C for further analysis. The experiments were performed in triplicate.

2.6. Determination of triterpenoid content

The determination of total triterpenoids was performed following the method of Wei et al., 2015 with minor modifications. Specifically, a 0.16ml extract was mixed with 0.4 mL of 5% vanillin/glacial acetic acid (w/v) in the screw cap test tube. After that, 1.0 mL of perchloric acid solution was added and incubated at 60°C for 30 min using a water bath (Memmert WNB, GmbH & Co. KG, Germany). The mixture was rapidly cooled and added 5.0 mL of glacial acetic acid, and then measured at 573 nm using a UV spectrophotometer/NIR (Shimazu, UV-2600,

Japan). For triterpenoid analysis, ursolic acid was used as the standard solution. To construct the calibration curves, the standard solutions of triterpenoid (0.1–1.0 g/100mL in methanol) were used. The results were calculated in mg of ursolic acid equivalents per g of dw.

2.7. Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) (JEOL Model JSM-6490LV, Peabody, MA, USA) was used to depict the effect of different extraction methods on the morphologic structure of *G. lucidum*. The dried samples were placed on an adhesive carbon tab and coated with a thin layer of gold (Cressington 108 auto, Ted Pella, Redding, CA, USA) by sputtering. The most representative SEM images were obtained at 1000x magnifications with an accelerating voltage of 15kV.

2.8. Statistical analysis

The statistical analyses were conducted using Stagraphic Centrution XV (Statsoft Inc., Umeå, Sweden), and the results were expressed as mean ± SD, and. The data from the response surface methodology (RSM) were analysed using Modde (version 10; Umetri, Umeå, Sweden) by F-test and ANOVA.

3.Results and discussions

3.1. The effect of ethanol as a polar cosolvent extraction on the triterpenoid content

Triterpenoids are less soluble in pure SC-CO_2 (Yang and Wei, 2015; Domingues et al., 2013). The addition of a polar cosolvent is thus necessary to increase the solubility of triterpenoids in the SC–CO_2 solvent. Fig 1. illustrates the effect of ethanol content in SC-CO_2 on the recovery of triterpenoids extracted from G. lucidum using the SC-CO_2 method, where the ethanol contents in SC-CO_2 varied between 0%v/v, 2%v/v, 4%v/v, 6%v/v, 8%v/v, 10%v/v, and 12%v/v.

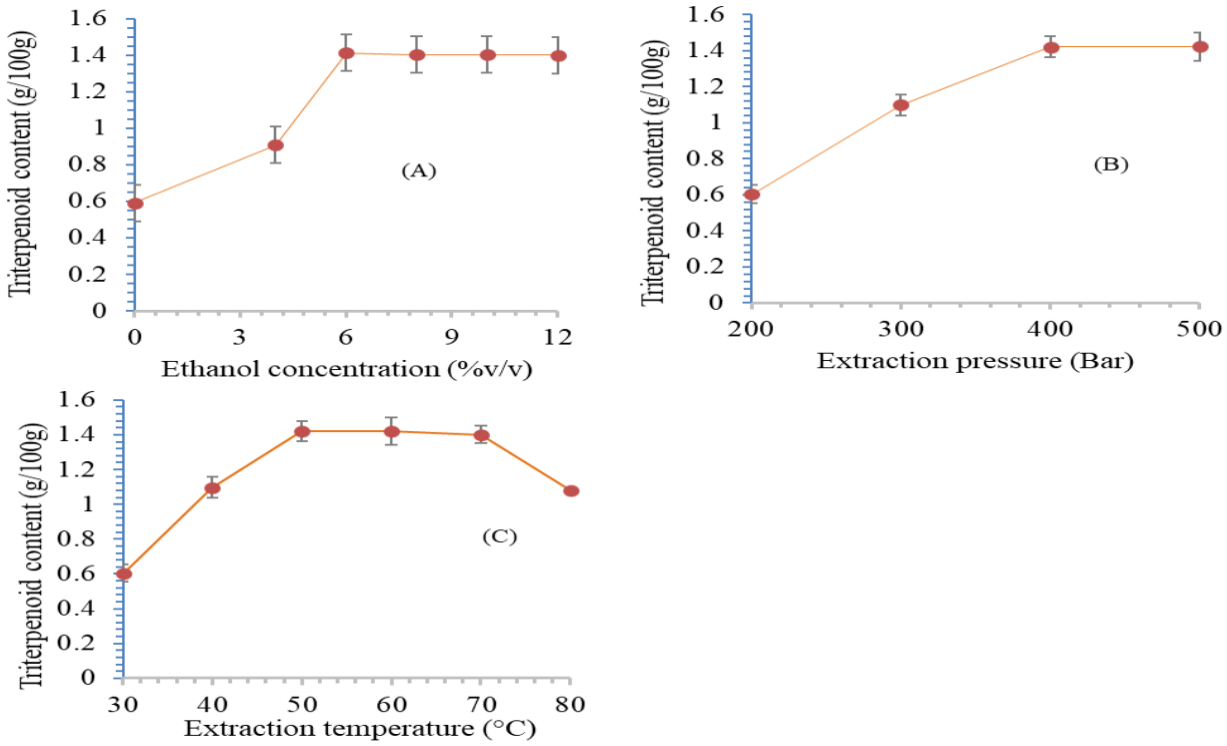


Figure 1. The effects of extraction variables on triterpenoid content: (A) effect of ethanol concentration in SC-CO_2 on the triterpenoid content; (B) effect of extraction pressure on the triterpenoid content; (C) effect of extraction temperature on the triterpenoid content.

As shown in Fig 1A, it can be seen that the triterpenoid contents obtained from the extracts significantly increased when the ethanol content varied from 2%v/v to 8 %v/v. At 6%v/v of ethanol, the triterpenoid content achieved the maximum values of 1.411g/100g, approximately 1.3 times higher than that of the extracted sample without ethanol (0%v/v) and 2%v/v. The results could be attributed to the interaction of ethanol with the matrix of the cell plant, which enhanced the solubilization of triterpenoids into the extraction solvent. Its finding is similar to Pieczykolan et al., 2019, who documented that the addition of ethanol enhanced the SC-CO_2 solvent power, and caused the swelling of the matrix, thus increasing the extraction yield. However, the addition of ethanol in SC-CO_2 was higher than 6%v/v, and the dissolution of triterpenoids was slightly decreased, as shown in Fig 1A. Taking all of the results into consideration, within the ranges of the parameters studied, the best ethanol concentration in SC-CO_2 for extraction was 6%v/v.

3.2. The effect of extraction pressure on triterpenoid content

The SC-CO_2 pressure can help triterpenoids dissolve out of cells. As presented in Fig 1B, it can be seen that the triterpenoid contents obtained from the SC-CO_2 extracts significantly increased when the extraction pressure ranged from 200 to 500 bar. The highest recovery of triterpenoids was 1.422 g/100g as the extraction pressure was in a range of 400-500 bar. This could be explained that an increase in pressure extraction at a constant temperature leads to a higher density of solvent extraction, resulting in the enhancement of the release of triterpenoids into the solvent, thus producing a higher yield of the triterpenoids. A similar result was reported by Yim et al., 2019, who showed that the higher the pressure used for extraction, the more solvent entered inside the cells and more bioactive compounds dissolved into the solvent. In 2015, Yang and Wei reported that an increase in pressure extraction caused changes in the mass liquid transfer. Consequently, the bioactive compounds are easily released into the solvent. Nevertheless, high pressure was unsafe and expensive, and the extract purification and analysis were more complicated. Thus, the best extraction pressure in this study was 400bar.

3.3. The effect of extraction temperature on triterpenoid content

Experiments were conducted to evaluate the effect of extraction temperature on the recovery of triterpenoids. The extraction was performed with 400bar extraction pressure and 6%v/v ethanol at different extraction temperatures (30, 40, 50, 60, and 70°C, respectively). In Fig 1C, the content of triterpenoids markedly increased as the temperature increased from 30 to 70°C, and achieved the maximum content at 50°C (1.421 g/100g). However, the content of triterpenoids slightly decreased when the extraction temperature continued to increase (exceeded 70 °C). It could be attributed to an increase in temperature (over 60°C) that destroyed the triterpenoid molecular structure of the five rings (Cai et al., 2019). Additionally, an increase in extraction temperature elevated the volatility of the solvent, reducing the diffusivity of the solutes to be extracted. Therefore, the extraction temperature of 50 °C was selected for supercritical fluid extraction of triterpenoid from *G. lucidum*.

3.4. Model fitting

In this study, RSM design (based on CCF) was used to investigate the effects of three independent factors on the recovery of triterpenoids. These parameters included ethanol content in SC-CO_2 (X_1), extraction pressure (X_2), and extraction temperature (X_3). Y is the response for the triterpenoid content. The 17 experiments of the design matrix and the measured average of triterpenoid are shown in Table 2. The experimental and predicted triterpenoid values were in the range of 0.513–1.512g/100g. The ANOVA results were used to check the adequacy of the suggested model and shown in Table 3.

Table 3. Results of the ANOVA for the response surface quadratic model

Tritepenoid content	Degree of free dom	Sum of squares	Mean square	F-value	p-value
Total Corrected	17	20.3167	1.19510		
Regression	9	1.80772	0.200857	38.6237	0.000
Residual	7	0.03640	0.005200		
Lack of Fit	5	0.03602	0.007203	37.5195	0.056
Pure Error	2	0.00039	0.000192		

R^2 = coefficient of determination = 0.983; adjusted R^2 = 0.957; Model predictive ability Q^2 = 0.819; $p < 0.05$ indicates statistical significance.

The model's F-value of 38.46 and the p-value of 0.0001 implied that the model is significant. The lack of fit with the p-value of 0.07 was not significant, which indicated the suitability of the model to predict the variations.

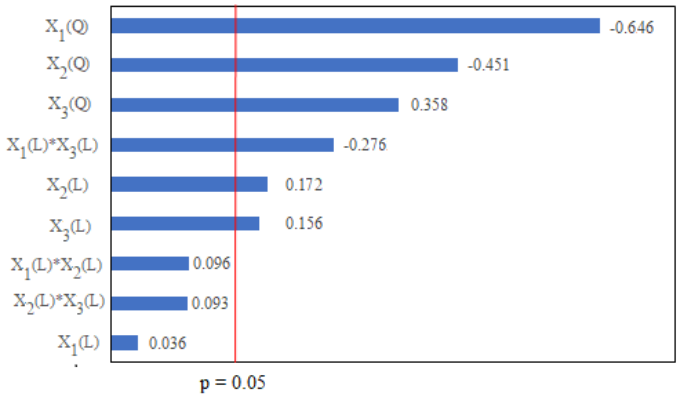


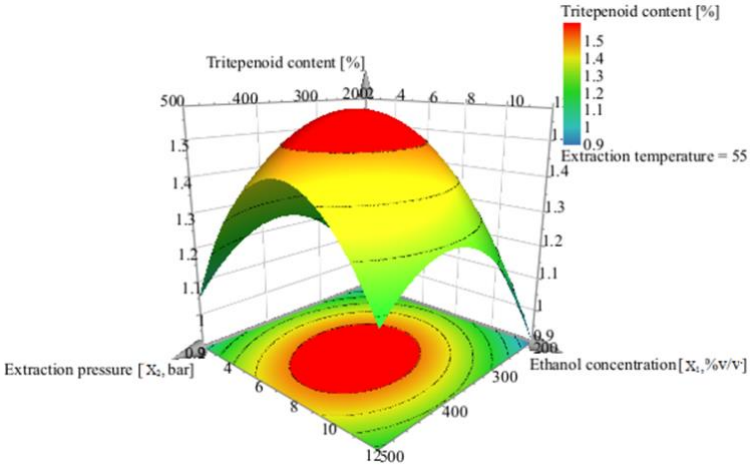
Figure 2. Pareto chart of the data analysis ($p<0.05$)

The fitness and predictive ability of the model are evaluated by the coefficient of determination (R^2), the adjusted R^2, and model predictive ability (Q^2) (Phan et al., 2020). According to our results, the R^2 and adjusted R^2 were 0.983 and 0.957, respectively, showing that 98.3% and 95.7% of the variability in the response was explained by the model. The Q^2 was 0.819, and the difference between R^2 (0.957) and Q^2 (0.819) was less than 0.3, which confirmed the validity of the predicted model (Phan et al., 2020). Furthermore, to evaluate the interactions of each variable on the recovery of triterpenoids, the Pareto analysis was used. As shown in Fig 2, the linear terms (X_2, X_3), the interaction terms (X_1*X_3), and the quadratic terms (X_2^2, X_3^2) had a considerable influence on the recovery of triterpenoid. Meanwhile, the linear term (X_1), the interaction terms (X_1*X_2, X_2*X_3) did not affect the triterpenoid recovery and were eliminated. A polynomial equation was then generated as follows.

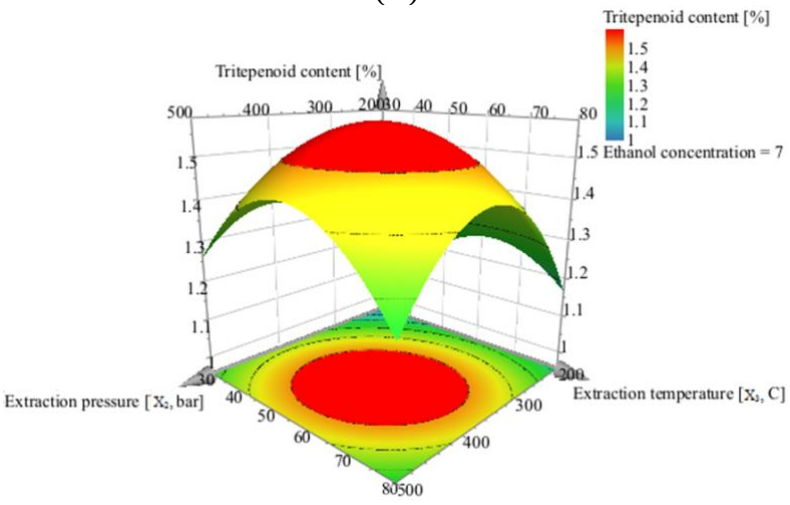
$$Y = 1.476 - 0.323X_1^2 + 0.229X_2^2 - 0.179X_3^2 + 0.086X_2 + 0.078X_3 - 0.138X_1 * X_3 \quad (6)$$

where the negative sign in front of the terms indicates the antagonistic effects and the positive sign indicates the synergistic effects of the factors. The relation between different variables and responses is elucidated by the 3D response surface plots as a function of two variables by maintaining the other variable at a central level. Fig 3A presents the interaction between the ethanol content in SC-CO_2 (X_1) and extraction pressure (X_2) on the recovery of

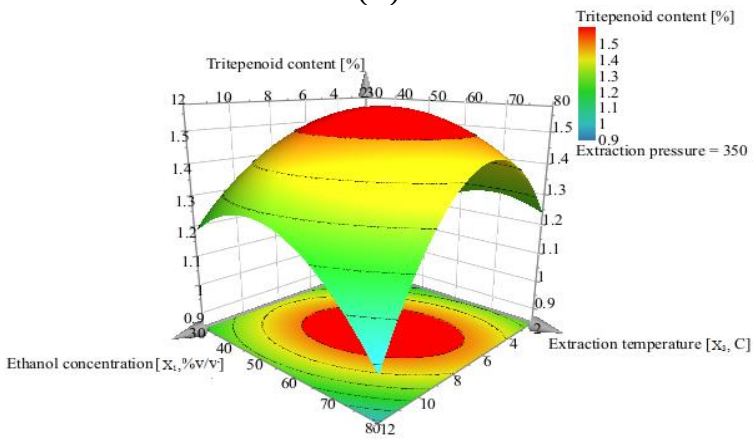
triterpenoids, given a 55 °C temperature. The results showed that an increase in X_1 (2-7%v/v) and X_2 (200-400bar) enhanced the recovery of triterpenoids.



(A)



(B)



(C)

Figure 3. Response surface plots of relationships between triterpenoid recovery and ethanol contentration in SC-CO_2 (X_1, %v/v), extraction pressure (X_2, Bar), and extraction temperature (X_3, °C), given: (A) Extraction temperature of 55 °C; (B) ethanol concentration of 7%v/v; (C) extraction pressure of 350 bar

The maximum triterpenoid content (1.48g/100g) was achieved with X_1 at 6.9%v/v and X_2 at 375 bar. The triterpenoid content was not significantly increased as the extraction pressure and ethanol content increased from 375 to 500bar and 7%v/v to 12%v/v, respectively. Similar results were also reported for the extraction of triterpenoids from *Eucalyptus globulus* using the SC-CO_2 with the addition of ethanol as co-solvent extraction (Domingues et al., 2013). These authors suggested that an increase in ethanol content and extraction pressure lead to the change of the solvent polarity, which enhanced the interactions between ethanol and the triterpenoids within the cell, thus improving the recovery of triterpenoids. Nevertheless, in Fig 3A, a circular shape shows a low significant interaction between X_1 and X_2.

Fig. 3B indicates the simultaneous effects of X_2 and X_3 on the recovery of triterpenoid. With a fixed level of X_1 (7%v/v), the triterpenoid content increased and achieved the maximum value (1.59g/100g) at X_2 (374bar) and X_3 (60°C). The increase in extraction temperature was attributed to differences in the values of solvent density, which promoted a considerable increase in extraction yield (Yang and Wei, 2015; Marinho et al., 2019). However, when X_2 and X_3 were higher than 374bar and greater than 60 °C the triterpenoid recovery decreased. This could be attributed to the degradation of triterpenoids by the high extraction temperature and pressure. In addition, an increase in pressure and temperature causes a decrease in the effective diffusivity, thus reducing the triterpenoid recovery (Yang and Wei, 2015; Uwineza and Waśkiewicz, 2020) suggested that the SC-CO_2 extraction temperature of bioactive compounds should be fixed between 35 and 60°C to avoid degradation, and the pressure should be around 400 bar.

Figure 3C depicts the relation between X_1 and X_3. It can be seen that the increase in both X_1 and X_3 resulted in greater solubility of triterpenoids in the extract. The triterpenoid content reached its highest value with X_1 at 7%v/v and X_3 at 60 °C. The triterpenoid content was slightly reduced when X_1 rose from 7%v/v to 12%v/v and X_3 increased from 60 to 80°C. The high extraction temperature facilitates solvent volatilization, thus reducing the triterpenoid recovery. In addition, higher temperatures might cause thermal degradation of triterpenoid compounds. The result was according to other studies (Tran et al., 2021; Yang and Wei, 2015).

3.5. Optimization of reaction and model validation

Optimal extraction conditions for maximum recovery of triterpenoids from *G. lucidum* were further predicted using RSM mathematical models. The optimal extraction conditions (i.e, cosolvent content, extraction pressure, and extraction temperature) for triterpenoid extraction from the *G. lucidum* were 380 bar, 60°C, and 7%v/v, achieving 1.49g/100g. Under the optimal conditions, the experimental triterpenoid content was 1.51/100g which was very near to the predicted value, illustrating that the models fitted very well.

Table 4. Extraction conditions and triterpenoid recovery of the SC-CO_2, SE, ME methods

Methods	Extraction conditions				Triterpenoid content (g/100g)
	Pressure (bar)	Extraction time (hours)	Ethanol content (%v/v)	Temperature (°C)	
SC-CO_2	379	2	7	57.3±0.5	1.21^{b}±0.01
SE	-	6	-	70±1.0	1.51^{a}±0.02
ME	-	24	-	32±0.5	0.45^{c}±0.01

Different letters in each column denote statistically significant differences between treatments ($p < .05$). The values are the mean of three replications SD.

In this study, the SE and ME methods were compared for their efficiency in extraction recoveries of triterpenoids. As shown in Table 4, it can be seen that there was a significant difference in the recovery of triterpenoids among the SC-CO_2 (1.51g/100g), SE (1.21g/100g), and ME methods (0.45g/100g). It could be attributed to the morphological changes of the *G. lucidum* cell wall during the extraction processes.

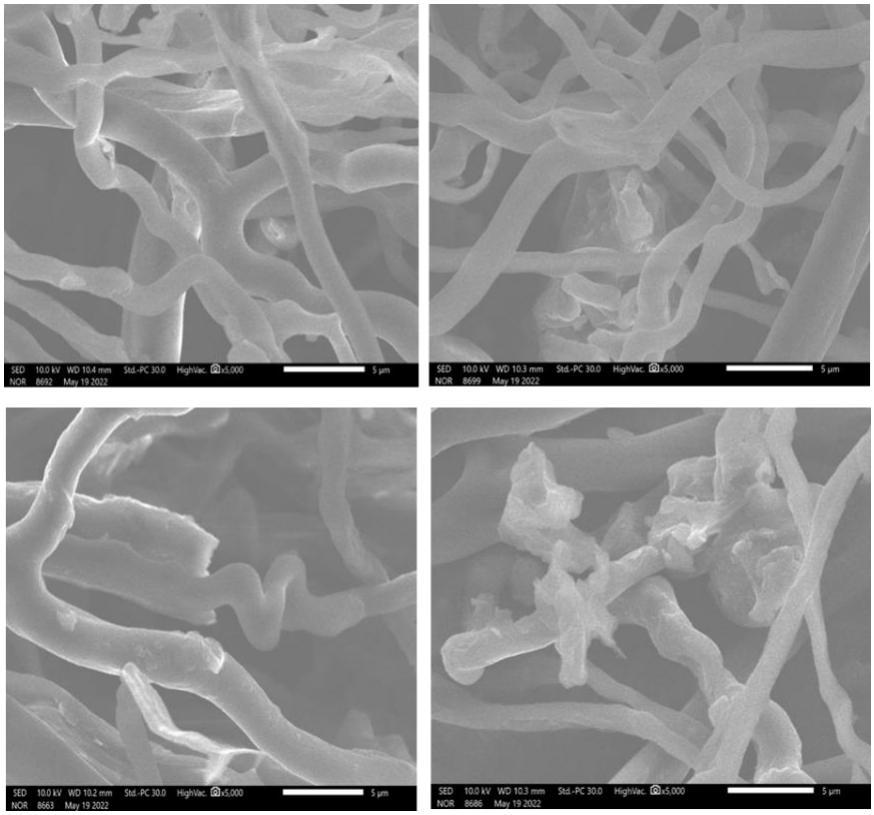


Figure 4. SEM images of untreated *G. lucidum* sample (a), SE treated sample (b), ME-treated samples (c), SC-CO_2 treated sample (magnification 5000) (d).

In Fig 4A, the surface of the native sample had a smooth surface. In Figs 4B-C, some cracks on the SE and ME-treated *G. lucidum* cell walls resulted in the enhancement of the solution of triterpenoids in the extracts. Meanwhile, the surface of the samples treated with SC-CO_2 modified with ethanol exhibited numerous cells that were completely broken (Fig 4C). Therefore, significant benefits in terms of extraction conditions indicated that the SC-CO_2 method is a useful extraction method for triterpenoids from *G. lucidum.*

3.6. Kinetic of SC-CO_2 extraction process

In this study, both the first- and second-order kinetic models were used to describe the effects of the SC-CO_2 parameters on the extraction of triterpenoids from *G. lucidum*, given an extraction time of 0-120min. For the first-order kinetic model, the plot of log (C_s/(C_s-C_t)) versus t gave a slope and intercept that was used to determine k (min^{-1}) and C_s. Based on Table 5, it can be seen that the k values increased with increasing SC-CO_2 extraction parameters and obtained values are 0.799 to 2.470 (100g/g.min). However, the first-order model presented low coefficients of determination (R^2) values, which can not represent well the experimental results of triterpenoid extraction. Compared with the first-order extraction models, the second-order model presented very high R^2 values, thus the second-order kinetic is a more suitable model for describing the kinetic extraction process of triterpenoids. As shown in Table 6, the

parameters hs and C_s values appreciably increased with increasing extraction temperature (30-55°C) or pressure extraction (200-350 bar), and then C_s and h slightly decreased.

Table 5. The first-order kinetic parameters of tritepenoid extraction from *G. lucidum* at various temperatures and extraction pressures

Extraction conditions		k (100g/g.min)	C_s (g/100g)	R^2
Temperature (°C)	30	1.548724	1.21	0.8115
	55	2.388239	1.58	0.8621
	80	1.998606	1.09	0.8634
Extraction pressure (bar)	200	0.798944	1.21	0.8435
	350	2.470431	1.55	0.8348
	500	2.339356	1.49	0.8648

These values were obtained from Microsoft Excel1. Cs= triterpenoid concentration at saturation; *k* = extraction rate constant; R^2 = coefficient of determination

Table 6. Second order kinetic parameters of triterpenoid recovery under different SC-CO_2 extraction pressures and extraction temperatures, given 120 min extraction time

Extraction conditions		*h* (g/100g)	*k* (100g/g.min)	C_e (g/100g.min)	R^2	RSME
Temperature (°C)	30	0.408	0.377	1.04	0.984	0.040
	55	1.829	0.802	1.51	0.989	0.079
	80	0.987	0.697	1.19	0.991	0.081
Extraction pressure (bar)	200	0.554	0.466	1.09	0.945	0.031
	350	1.758	0.792	1.49	0.966	0.045
	500	1.083	0.752	1.20	0.985	0.063

These values were obtained from Microsoft Excel1. Cs= triterpenoid concentration at saturation; *k* = extraction rate constant; *h* = initial extraction rate; R^2 = coefficient of determination; RMSE = root mean square error

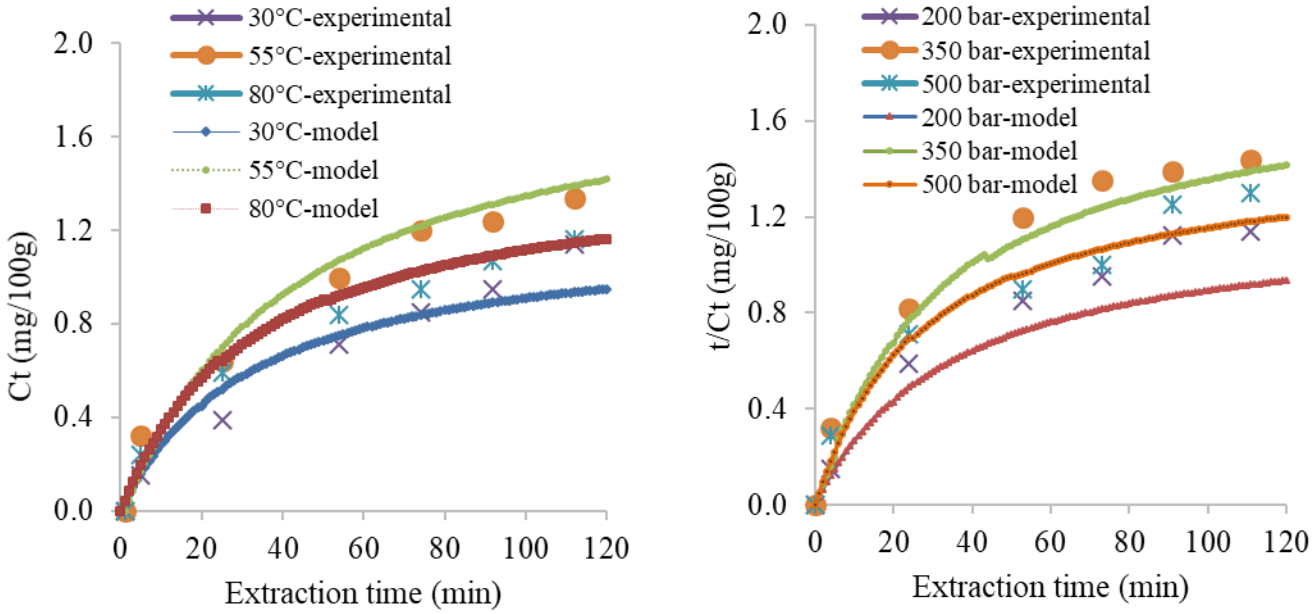


Figure 5. Second-order kinetic models (A–B) of extraction of triterpenoids from *G. lucidum* at different extraction temperatures and pressures

In Figs 5A-C, it also can be seen that the two- thirds of the triterpenoid content was recovered in the early SC-CO_2 extraction stage (0 to 100 min), and reached equilibrium after 100 min extraction. Specifically, the triterpenoid content increased from 0.036 to 1.43 mg/100g as the temperature raised from 30-55°C, or pressure extraction from 200-350 bar, respectively. The results could be explained that rising temperature and pressure enhanced the density of ethanol concentration in SC-CO_2, which thereby improves the solubility of triterpenoid in the solvent. Nevertheless, the recovery of triterpenoids declined with the increasing temperature to 55°C and pressure to 500 bar, consistent with the RSM results (Fig. 3A-C). The high temperature and pressure extraction lead to heat exposure, and other compounds were extracted more, reducing the triterpenoid content (Hobbi et al., 2021). According to our results, the second-order extraction models are correlated to the RSM results. Therefore, it can be concluded that the second-order kinetic extraction model was suitable for characterizing the effect of SC-CO_2 parameters on triterpenoid recovery.

3.7. Comparison of antioxidant activity of the triterpenoid-rich extracts among optimized SC-CO_2, SE, and ME processes

The DPPH and ABTS methods have been widely used to determine the free radical-scavenging activity of natural products. In this study, the $DPPH^{\bullet+}$and $ABTS^{\bullet+}$ scavenging activities of triterpenoids obtained by the optimized SC-CO_2 were compared with the ascorbic acid (AA) and conventional processes (ME and SE). The $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical scavenging activities of the extracts and ascorbic acid are shown in Figs 5A-B. As shown in Figs 6A-B, it can be seen that the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radical scavenging activities of the extracts correlated positively with their concentration in the medium. In addition, the antioxidant activity of the extracts at the optimal SC-CO_2 conditions was higher than ME and SE in the same range of concentrations. When the concentrations of triterpenoids were 0.15 and 0.25 g/ml, the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ values of SC-CO_2 increased from 15.15% to 92.50% and 13.71% to 73.56%, respectively.

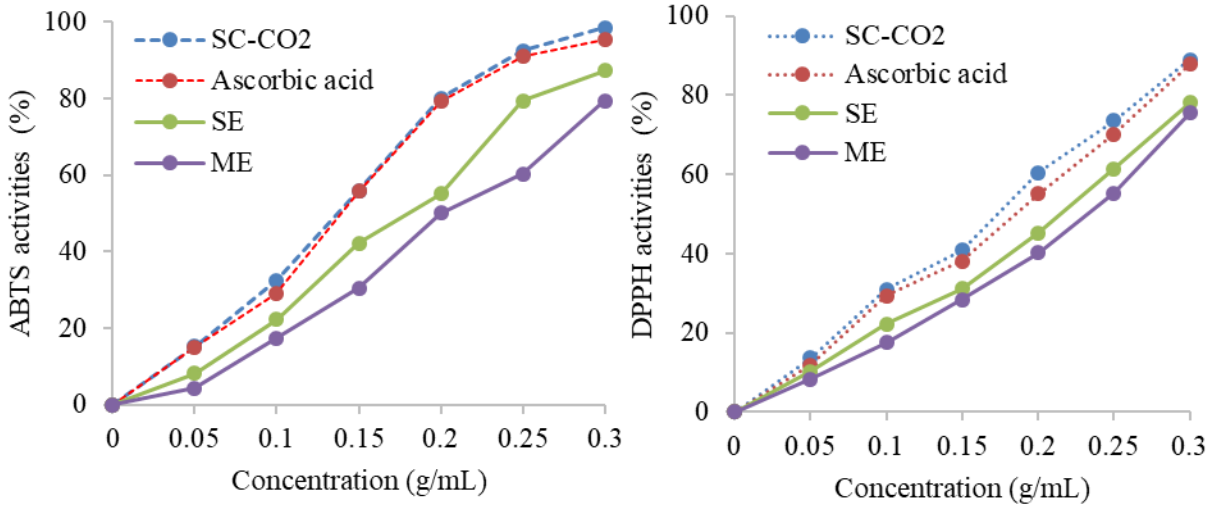


Figure 6. (A) ABTS and (B) DPPH radical scavenging activities of the extract and ascorbic acid

Meanwhile, the $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ values of ME and SE extracts were in a range of 4.35 to 79.45% and 8.35% to 61.45%. Based on the relationship curve between the triterpenoid concentration, ascorbic acid, and the percentage of antioxidant activity, the value of IC50 was

determined. The results showed that the measured IC50 values of the optimized SC-CO_2 (0.141-0.178 g/ml) resembled the antioxidant capacity of ascorbic acid (0.14-0.17g/ml). Meanwhile, the SE and ME extracts were in a range of 0.20-0.22g/ml. A reasonable explanation is that the SC-CO_2 allows the extraction of different triterpenoid fractions responsible for the high antioxidant activity of the extracts. Our findings were similar to Porto et al for extracting proanthocyanidins from grap marc. However, this study only indicated the total content of triterpenoids which was responsible for the antioxidant activity. Thus, further studies should be conducted to investigate the bioactive compounds from triterpenoid fractions to establish the biochemical mechanisms.

4. Conclusions

In this work, the influences of the SC-CO_2 process parameters (ethanol concentration, extraction temperature, and extraction pressure) on the recovery of triterpenoids were investigated. The RSM was applied to determine the optimal extraction conditions (i.e., ethanol concentration in SC-CO_2, extraction temperature, and extraction pressure) for the maximum recovery of triterpenoids from *G. lucidum*. The first- and second-order kinetic extraction models were used to interpret the kinetic extraction behavior. According to our results, the optimal condition for triterpenoid extraction was 380 bar, 7%v/v, and 60°C, achieving the highest triterpenoid content (1.49 mg/100g). These results agreed with the experimented value of 1.51 mg/100g, indicating the success of RSM in the optimization of extraction parameters in the prediction of triterpenoid from *G. lucidum*. The second-order gave better fitting to the experimental data than the first-order kinetic model. The fit of the second order model is satisfactory and it was able to predict in reasonably way the recovery of the extraction process. Compared with the other two conventional methods, the SC-CO_2 method is faster, cleaner, and more efficient. The antioxidant activity of *G. lucidum* triterpenoids extracted by the SC-CO_2 method had a stronger ability to remove DPPH free radicals and ABTS free radicals than the two conventional extraction methods. Thus, the SC-CO_2 method is recommended for the extraction of triterpenoids from *G. lucidum*. However, in this study, the triterpenoids responsible for the antioxidative activity are not explained. Thus, further investigation is needed to clarify the antioxidant compounds present in the *G. lucidum* extract.

5. References

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Conflicts of interest

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QUALITY FEATURES OF FAT TISSUE AS A PLATFORM FOR "IDEAL" BACKFAT VIRTUAL MODEL: A REVIEW

Irina Chernukha[1]✉, Marina Nikitina[1], Nadezhda Kupaeva[1], Liliya Fedulova[1]

[1]*V. M. Gorbatov Federal Research Center for Food Systems, Talalikhina st., 26, Moscow 109316, Russia*
✉*imcher@inbox.ru*

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ABSTRACT

The intensification of livestock farming and increased selection for "lean meat" breeds has led to the predominance of pigs with altered characteristics of adipose tissue. The meat industry faces the difficult task of providing consumers with high-quality meat and a variety of meat products. While scientists have to develop an optimal strategy to obtain high-quality new meat products with improved nutritional profiles and methods for measuring quality indicators of fat in order to meet both the requirements for a healthy diet of consumers and the technological requirements of the manufacturer.

This review describes the main methods of studying fat and the parameters of the quality of backfat are considered: the thickness of the dorsal fat, the color of the fat, Solid fat content, the determination of the fatty acid composition, and the ratio of fatty acids, iodine number, oxidative degradation. As a result of the systematization and literature analysis on the fatty acid composition of backfat, as well as the recommendations of the WHO, scientists, and nutritionists, a structural-parametric model of the "ideal" fat was formulated. Model summarizes and presents the characteristics of pork adipose tissue, that are optimal for obtaining meat products health-promoting.

1.Introduction

The attitude to fats has changed dramatically over the past 100 years from positive (high nutritional value, energy reservoir) to sharply negative. Since the 1950s, nutritionists have recommended reducing total fat intake in order to decrease the potentially adverse effects of diet on diseases development such as obesity and coronary heart disease resulting from the consumption of excess fat, especially animal origin. Traditionally, animal fat, especially pork fat, is a source of such concerns. However, in recent decades, fat has been considered not only as a source of negative health effects. Taking into account the main potentials of fats, the recommendations of fat intake have moved from quantity of fat toward its quality (Kucha *et al.*, 2018).

2. The current state of pig livestock in Russia

Pork is the most consumed meat. Pig farming is intensively developing and occupying more than 30% of the total meat production. From 2016 to 2018 about 3.36-3.74 million tons of pork in carcass weight were produced in Russia, while in 2019 pork production overlapped 4 million tons and accounted for more than 37% of the total meat production. In 2019 about 2505.7 thousand tons of pork in live weight were produced on the territory of the Central Federal District. Enterprises of the Central Black Earth region occupy the largest share of pork production, locate in the Belgorod (17.8%), Kursk (7.9%) and Voronezh (5.9%) regions. Pig processing companies of the Volga region produce 813.7

thousand tons of pork, locate in the Republic of Tatarstan (2.0%), Bashkortostan (1.8%) and Mordovia (1.6%), respectively, in the gross volume of pig production in the country. Siberian producers rank third in the rating of federal districts with a share of 10.0% and a gross volume of 493.7 thousand tons, and enterprises of the Krasnoyarsk Territory, Omsk Region and Altai Territory achieve significant success (Tikhomirov, 2021).

The intensification of pig livestock in Russia and the imposed restrictive measures on the products from a number of foreign countries led to a sharp reduction in pork imports from 619.8 to 79.0 thousand tons and an increase in the cost of a unit of imported products by 80.2%. In 2019, pork occupied the largest share in the structure of the volume of imported supplies (87.2% of the total supply), followed by backfat (8.6%), and offal (4.3%). Brazil (51.8% of all supplies), Chile (23.8%) and Argentina (15.4%) were the main pork suppliers to the Russian market, followed by Paraguay (4.6%), Serbia (2.3%), Belarus (1.6%), Kazakhstan (0.5%); Chile (68.8% of total imports) was the main supplier of backfat (Plugov, 2021).

The volume of exports of pork, offal, backfat and commercial pigs from the Russia also increased from 2017 to 2020. For example, the total volume of exports in 2019 amounted to 108.3 thousand tons, exceeding the volumes for 5 years by 27.5%, for 10 years - by 238.6 times. The structure of total exports was previously dominated by offal, but in 2019 pork accounted for 54.8% in the total volume of export, pork offal - 40.0%, backfat - 4.8%, commercial pigs - 0.4%. Export volumes increased by 947.2% over 5 years, and by 19.9 times over 10 years, while the value of exports amounted to $ 7.6 million. The total volume of pork backfat exports from Russia amounted to 5.2 thousand tons in 2019, which is 115.7% more than in 2018 (Grigoriev, 2021).

At the same time, there is a demand from domestic and foreign producers for backfat, which is necessary for the production of many types of meat products: the mass fraction of backfat in the recipes of boiled sausages is up to 30%, in semi-smoked, boiled-smoked, raw smoked sausages - up to 60% (Semenova *et al.*, 2015). The annual demand of the Russian meat industry for backfat is about 450 thousand tons. Currently, backfat is produced in Russia in extremely small volumes, because of the predominance of pigs with altered characteristics of adipose tissue in the agro-industrial livestock. Thus, there is a growing market demand for healthier sources of fat. To improve the fat content and its composition, several strategies are used, including feed varying and selection (Martins *et al.*, 2018). The meat industry faces a difficult task to provide consumers with high-quality meat and a variety of meat products, while scientists have to develop an optimal approach to obtaining high-quality products and methods for measuring quality indicators of fat in order to meet both the requirements for a healthy diet of consumers and the technological requirements of the manufacturer. The aim of the article was to review the most recent works on methods of studying adipose tissue and develop the concept of a "ideal" backfat virtual model.

3.Qualitative characteristics of the backfat

The quality of the backfat is evaluated after slaughter. The thickness of dorsal fat and color characteristics is measured, chemical and spectral analysis is carried out (Hoa *et al.*, 2019), as well as the fatty acid (FA) composition is determined and quality coefficients are calculated. The main traits of pig backfat and methods for their studying are presented in Table 1.

Table 1. The main traits for assessing the quality of pig backfat

Trait	The principle of determination or equation	Reference
The thickness of dorsal backfat	Measured with a flexible ruler with an accuracy of 0.5 mm: - at the 6-7 thoracic vertebrae; - at the 10-11 thoracic vertebrae;	Garskaya, Peretyatko, 2021
	- midline, between the 4th and 5th lumbar vertebra level;	Wang *et al.*, 2021
	- at the 14th rib level.	Ayuso *et al.*, 2020
Color of backfat	- CIELAB or CIEXYZ: In CIELAB color space L * (the lightness/darkness); a * (the redness/greenness); b * (the yellowness/blueness): b * value is considered the ideal objective measurement of the yellowness of a fat surface; L * value correlates well with the FAs composition.	Brewer *et al*., 2001
	- colorimetric, spectrophotometric.	Hoa *et al.*, 2019
Solid Fat Content	SFC is the percentage of solids in fat at specified temperatures and determined by dilatometry; NMR and differential scanning calorimetry.	Kucha *et al.*, 2018
SFA :UFA	Ratio of saturated fatty acids to unsaturated fatty acids	Nistor *et al.*, 2012
Iodine value	- iodometric Wijs/ Hanuš method - calculated according to the equations: IV *(for triglicerides)* = [C16:1] × 0.95 + [C18:1] × 0.86 + [C18:2] × 1.732 + [C18:3] × 2.616 + [C20:1] × 0.785 + [C22:1] × 0.723; brackets indicate the concentration of FA in %; IV *(for free FA)* = [C16:1] × 0.9976 + [C18:1] × 0.8986 + [C18:2] × 1.810 + [C18:3] × 2.735 + [C20:1] × 0.8175 +[C22:1] × 0,7497	Kyriakidis and Katsiloulis, 2000; ISO 3961:2018.
	- coefficient for free FA is calculated according to the equation: $K_{FAi} = \frac{Mj2 \times k}{MFAi}$, where M_{j2} – molar mass of molecular iodine, g, $M_{FA}i$ – molar mass of the i-th FA, k – the number of double bonds in the molecule of the i-th FA - coefficient for triglycerides is calculated according to the equation: $K_{FA}i = \frac{Mj2 \times k}{(MFAi + \frac{1}{3}Mgl - M_{H2O})}$, where Mgl – molecular weight of glycerin, g, M_{H2O}– molecular weight of water, g; IV *(for free FA)* = $\sum K_{FA}i \times m_{Fai}$, where $K_{FA}i$ – coefficient for i-th FA determined for free FA or for triglycerides, m_{Fai} – mass content of i-th FA in the sample, %.	Spiridonov et al., 2016
	- regression equations.	Paulk et al., 2015
Fatty acid composition	- Fatty acid methyl esters analysis using GC/MS; - Near infrared reflectance (appropriate for C16:0, C18:0, C18:1 and C18:2); - Raman spectroscopy	Ayuso *et al.*, 2020; De Marchi *et al.*, 2012; Ros-Freixedes *et al.*, 2014
Oxidative Degradation	- peroxide value (PV); - malondialdehyde (MDA)	Barriuso *et al.*, 2013; Qiu *et al.*, 2013.

3.1.The thickness of subcutaneous backfat is traditionally determined after slaughter, but modern methods of ultrasound examination in real time allow predicting the thickness of subcutaneous and intramuscular fat *in vivo* (Jung *et al.,* 2015). The thickness of dorsal backfat is traditionally measured in Russia at the 6-7 and 10-11 thoracic vertebrae (Garskaya and Peretyatko, 2021), in China – midline, between the 4th and 5th lumbar vertebra level (Wang *et al.,* 2021), while in Italy – at the 14th rib level (Ayuso *et al.*, 2020).

3.2. Backfat Color of mainly depends on the freshness and fatty acids composition. Fresh fat has a white or slightly pinkish color. A high content of unsaturated fatty acids causes a grayish tinge of fat and occurs in young animals. Unusual color, an orange for example, as a rule, is a result of the reaction of unsaturated fatty acids with molecular oxygen by a free radical mechanism (Domínguez *et al.*, 2019; Pereira and Abreu, 2018). Various equipment is used for the studying the color of fat, which evaluate the following CIE L* a* b parameters: for lightness from black (0) to white (100) - L *; from green (-) to red (+) - a * and from blue (-) to yellow (+)b * (CIE, 1986; Hoa *et al.,* 2019). The b * value is considered the ideal objective measurement of the yellowness of a fat surface (Brewer *et al.*, 2001). In subcutaneous fat CIEL*a*b* variables significantly affect color with L* and chroma being the most affected. L* value correlates well with the FAs composition (Kucha *et al.*, 2018). In (Maw *et al.*, 2003) a correlation was found between increased yellowness and an elevated percentage of linoleic and α-linolenic acid, which accompanied with a decrease in the levels of palmitic, palmitoleic and oleic acids. Elevated transparency and softness of fat correlated with a decrease in the percentage of palmitic, stearic and oleic acid while increasing the ratio of linoleic and α-linolenic acid. At the same time, an increased percentage of myristic acid was associated with a decrease in red color.

3.3.Solid fat content (SFC) could be defined regarding firmness and the percentage of solids in fat at specified temperatures.

Firmness is usually measured by several instrumental techniques for instance by a penetrometer, a texture analyzer and Instron materials testing machine (Dransfield and Jones, 1984; Warnants *et al.*, 1998; Glaser *et al.*, 2000). SFC is an important feature of fat that affects the appearance, flavor, melting rate, shelf life and stability of fat-based foods. It is known that the melting point of FA with an even and odd number of carbon atoms differs. The C′, C″ and C forms are the only ones which melt. Namely, A′, B′, C′, C″ and D′ forms can be observed for the odds, and A, B, C and E forms for the evens (Gbabode *et al.*, 2010). The length of FA positively correlated with the melting point. Thus, melting point for C14 average 54.0°C, while for C26 – 88.2°C (Francis *et al.*, 1930). According to the melting point, it is possible to empirically proposing the FA composition of adipose tissue and vary the melting point by lifetime or technological manipulation.

SFC is particularly of primary importance when considering fat quality because soft fats could be difficult to dimension and process thus causing the decrease of high-quality cuts that could lead to monetary loss of value. The softness or oiliness of fat depends on the FA composition which also affects the SFC of pork fat at any given temperature. It was found that the melting point of lipids, as well as the hardness of fat, are closely related to the concentration of stearic acid (18: 0) and palmitic acid (C16:0), and has a linear correlation with iodine value at 20°C (Davenel *et al.*, 1999).

Products with an ideal SFC is desirable during processing, which provide the product to remain solid at room temperature, but at the same time give consumers the desired texture in the mouth. In other words, the melting point of backfat is recommended not lower than 35°C for the technological processing in the production of meat products using heat treatment (cooking, smoking, etc.), while the melting point of fat in the finished product may

be lower (32 - 36°C). Thus, the SFC is an important parameter of fat quality control to achieve high quality of the final product.

Traditionally, dilatometry is used to study SFC in routine practice. New methods, such as Nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC), are also developed. NMR results are calculated based on the relative number of protons present in the triglycerides in the solid and liquid phases, while DSC data are obtained by the melting enthalpies of these triglycerides (Márquez *et al.*, 2013). NMR methods are fast, non-destructive and rarely required recalibration over a long period of measurement. However, sample preparation (tempering) is required before measuring at each required temperature. DSC provides the possibility of tempering fat at various temperatures before measurement, providing a thermogram that includes the entire temperature range, as a result of a single measurement, from which an SFC can be obtained by partially integrating the thermogram. Thus, DSC provides information about the thermal transitions that the fat may undergo during processing since adding fat to a product without the desired melting profile could cause encapsulation of other ingredients. DSC measurement of melting characteristics is an internationally accepted conventional method by the American Oil Chemists Society.

Currently, NMR and DSC methods still require sample preparation and cannot be used for rapid assessment of fat SFC. Therefore, attempts are still being made to find more suitable methods for determining the SFC (Kucha *et al.*, 2018).

3.4.The ratio of saturated to unsaturated fatty acids is one of the ways to describe the relative composition of the fatty acid profile (Azain, 2001; Nistor *et al.*, 2012). The total amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid and the ratio between FA, especially SFA:PUFA, are often used to determine the quality of fat with higher levels of saturation indicating a more desirable quality, while increased unsaturation indicate an undesirable quality of fat. The ratio of all PUFA to SFA, the target value for which is from 0.4 to 0.58, for pork fat may exceed these values and this may be a favorable factor for pigs and other monogastric animals compared to ruminants (Nieto and Ros, 2012).

It is considered that a good quality fat from the standpoint of technological suitability should have a composition of 12% or more of C18: 0, the proportion of C18: 2 should be 12-15% of the total amount of FA. Thus, the ratio of these two key FA should be 1:[1-1.25] (FAO Food and Nutrition Paper, 2010). The total ratio of unsaturated ω6/ω3 FA is preferable to be more than 3.5-4.5 (Debrecéni *et al.*, 2017). In addition, according to (Pascual *et al.*, 2007) C18: 2w6 can be considered as a marker of the feed, and C16:0 as a marker of de novo synthesis. It is advisable to pay attention to their content in pork.

3.5.The iodine value (IV) characterizes the degree of unsaturation of carbon bonds in the structure of fatty acids. This parameter is used for standardization of the characteristics and composition of lipids in fat and is the most commonly used industry standard for quantifying the degree of unsaturation of pork. The advantage of the iodine value is that it corresponds to the amount of iodine that can interact with fatty acids by double bonds (White and Latour, 2008). IV also characterizes the resistance of fat to oxidation (Kyriakidis and Katsiloulis, 2000). A standardized method for calculating the iodine value is used by titration, and the calculated value for free fatty acids is also used ISO 3961:2018. In (Spiridonov *et al.*, 2016) a new method for calculating the IV was proposed, taking into account the content of all unsaturated fatty acids identified in the fat. Acceptable IVs range from less than 70 g/100 g to 75 g/100 g (Corominas *et al.*, 2014). In (Paulk *et al.*, 2015) based on the literature data, equations were proposed to predict the IV in the pork carcass back, belly, and jowl fat, when fattening with various sources of FA.

A high IV and a decrease in the ratio of saturated to unsaturated FA indicate a decrease in the hardness of fat. Many producers use IV for evaluation of carcass quality and IV> 65 for some processes may be unacceptably high, while IV> 75 is the threshold value for many technological processes. Currently, most US enterprises set the IV standard at a level below 74 g/100 g. (Seman *et al*., 2013). In Russia, fat suitable for long-term storage and for the production of delicatessen meat products should have a IV of no more than 70 g / 100 g (Spiridonov *et al.*, 2016).

3.6.FA profile of pork fat. Fatty acid methyl esters (FAMEs) analysis using GC/MS is the most common method for determining the FA profile of pork fat. The FA composition is usually expressed as a set of percentages corresponding to the relative content of each FA in the total number of determined FA (Ros-Freixedes and Estany, 2014). FA are classified into saturated and unsaturated (UFA) according to the chemical structure. UFAs are divided into monounsaturated and polyunsaturated. The FA proportions determine the physical properties and distinguish one fat from others: SFA are solid at room temperature, have a higher melting point than MUFA, PUFA, which contain at least one double bond in their structure. Moreover, as the number of double bonds increases, the fat becomes more unsaturated with a lower melting point and a softer consistency at room temperature. Various factors such as feed, gender, breed and age influence on FA composition.

Among the modern methods for assessing the FA profile, NIRS is used and let to develop quantitative models for FAs and the FA classes including SFA, MUFA, and PUFA. This method allows analyzing fat samples both in homogenized or intact forms. Better predictions were obtained for palmitic acid (C16: 0), stearic acid (C18: 0), oleic acid (C18: 1) and linoleic acid (C18: 2), while poor prognosis obtained for the remaining FA was associated with low concentrations of FA in the sample (De Marchi *et al.*, 2012).

Raman Spectroscopy (RS) is a vibrational spectroscopy method based on the shifts in the wavelength or frequency of an exciting incident beam of radiation that result from inelastic scattering on the interaction between the photons and the sample molecules. RS is also used to evaluate fatty acid classes using partial least square regression (PLSR) in combination with various data preprocess. Olsen, Ruke, Egelandsdal and Isaksson (2008) used RS to develop prediction models for the quantification of omega-3 and omega-6 in adipose tissue by analyzing Raman spectra of pork subcutaneous fat. An accuracy of R 20.97 and 0.91 and a prediction error of 0.99 and 0.23 were achieved for omega-3 and omega-6, respectively, based on preprocessed PLSR and first derivative spectra and selected wavelengths. Surprisingly, the study showed poor results in predicting the ratio of omega-6 and omega-3 in non-extracted fat with a determination coefficient of 0.31.

In another study on the determination of FA groups using RS (Berhe *et al.*, 2016) the method of multidimensional PLSR modeling was used on the fingerprint region of the spectra of subcutaneous pork fat samples and obtained reliable results for predicting SFA, MUFA, PUFA and IV, achieving reliable R 2 with a reasonable root mean square error of prediction. In addition, individual fatty acids including C14: 0; C16: 0; C16: 1 cisΔ9; C17: 0; C18: 0; C18: 1 cisΔ9; C18: 1 cisΔ11; C18: 2 cisΔ9, 12; C18: 1 cisΔ9, 12, 15; C120: 0; C20: 1 trans Δ11; C20: 2 cisΔ11, 14; C20: 3 cisΔ8, 11, 14; C20: 4 cisΔ11; C20: 1 cisΔ5, 11, 14 were predicted with R2 of 0.67, 0.89, 0.56, 0.07, 0.72, 0.82, 0.43, 0.90, 0.87, 0.18, 0.46, 0.78, 0.35, 0.60, 0.87 and prediction errors of 0.06, 0.20, 0.20, 0.09, 0.87, 1.3, 0.18, 1.84, 0.22, 0.05, 0.09, 0.10, 0.05, 0.05, and 0.22, respectively. This study showed RS's ability to determine the quality of pork fat. Moreover, Berhe *et al.* (2016) revealed that the poor prediction of the ratio of omega-6 to omega-3 obtained by Olsen, Rukke, Egelandsdal and Isaksson could be linked to the modelling of FAs based on the same Raman spectra information arising as a consequence of

strong correlation of a less abundant FA to a more abundant FA or their groups (IV, SFA, MUFA, PUFA). In fact, this means that the high coefficient of determination obtained for both less common and more common FAs can be changed or for the entire group of FAs. The study considers that a good prediction model for FA can be obtained by RS when using the PLSR algorithm. It is important to investigate the correlation structure of individual FAs and the degree of unsaturation using other non-destructive spectroscopic methods that demonstrate high collinearity of their spectra (Kucha *et al.*, 2018).

3.7.Oxidative degradation of lipids is an important indicator of the quality of fats, meat and meat products, which degree effects on sensory properties (color, aroma, taste, texture) and nutritional value of foods, can negatively affect human health. Lipid oxidation products are harmful to the body due to carcinogenic and atherosclerotic effects, changes in the composition of cell membranes and a decrease in high-density lipoproteins (Reitznerová *et al.*, 2017).

PUFA are more prone to oxidation than MUFA and SFA, and form hydroperoxides with conjugated double bonds (conjugated diene hydroperoxides and conjugated triene hydroperoxides). UFA with two double bonds form conjugated dienes hydroperoxide, while fatty acids with three double bonds form conjugated trienes hydroperoxide (Papuc et al., 2018).

Further, the primary decomposition products (hydroperoxides) decompose to form various low molecular weight secondary compounds, including aldehydes, ketones and alcohols – MDA, pentanal, propanal and hexanal. These compounds are responsible for the development of rancidity, undesirable odor and color changes (Barriuso *et al.*, 2013). 4-hydroxy-2-nonenal is the most permanent aldehyde substance formed during the peroxidation of PUFA ω6, such as C18:2ω6 and C20, and accumulates in membranes at concentrations of 5-10 μM. Lipid oxidation of PUFA ω3 leads to the formation of 4-hydroxy-2-hexanal, the concentration of which in meat can reach 120 μM (Kanner, 2007). Dialdehyde, including malonic dialdehyde (MDA), glyoxal and acrolein are other important reactive aldehydes formed as a result of the peroxidation of linolenate lipids and arachidonic acid. In many cases, MDA is the most common individual aldehyde, which is formed in foods undergoing lipid peroxidation. Its concentration in meat products can reach more than 300 μM (Reitznerová *et al.*, 2017).

Modern methods for lipid oxidation assessment can measure changes in primary products (changes in FAs and formation of lipid hydroperoxides and conjugated dienes or trienes) and changes in secondary products (formation of carbonyls, aldehydes, volatiles, MDA). The most common approach to assessing the degree of oxidation is to measure both primary and secondary oxidation compounds (Domínguez *et al.*, 2019). A significant decrease in the content of UFAs is expected during lipid oxidation, since they are the main substrate for oxidative reactions. However, the analysis of FAs is an additional indicator of the degree of oxidation, since the effective loss of UFA can be quantified only at the last stage of the oxidation process (Guyon *et al.*, 2016). The measurement of the hydroperoxides generation, also called the peroxide value (PV), has long been used as the main indicator of the formation of primary oxidation compounds in meat and meat products. A low PV may indicate both early and late oxidation (Domínguez *et al.*, 2015). In this regard, the determination of the PV is effective only at the initial stages of oxidative processes. It is recommended to monitor the change in the PV over time in order to get complete information about the state of lipid oxidation and to know whether the lipid is at the site of growth or decay of the hydroperoxide concentration curve (Yang and Boyle, 2016). Methods for determining peroxides are based on their reducing ability (Barriuso *et al.*, 2013). This requires extraction of lipids, which contain hydroperoxides that easily oxidize inorganic ions such as iodine or

iron. Iodometric titration and Ferric-xylenol Orange (FOX) are two main methods used for the determination of peroxides. FOX is a simpler method, does not depend on the availability of oxygen (Gómez and Lorenzo, 2013). The results obtained by the FOX method correlate better with other oxidation parameters than iodometric titration (Nuchi *et al.*, 2009).

Another marker of primary oxidation is the formation of conjugated compounds (dienes and trienes), the measurement of which is used to monitor oxidation in meat and meat products. The method of its determination includes extraction of conjugated compounds with a small amount of organic solvent (hexane/ isopropanol or chloroform / methanol mixture), and then the concentration of conjugated dienes and trienes is measured in the organic phase at 234 and 268 nm, respectively, on a spectrophotometer (Domínguez *et al.*, 2019). This method is simple and does not require expensive reagents; small amount of sample is used. However, this method has a significant number of disadvantages, including low sensitivity, so it is recommended to use other methods together with the measurement of conjugated compounds.

7-ketocholesterol, 20α-hydroxy-cholesterol, 25-hydroxycholesterol, α, β-epoxycholesterol, and 7α, 7β-hydroxycholesterol are the most commonly presented in meat and meat products. Concentrations between 57 to 71 µg/100 g can be found in meat and meat products (Domínguez *et al.*, 2019). GC-MS is the most accurate and frequently used method for quantifying cholesterol oxidation products (Chiu *et al.*, 2018). However, the method requires expensive equipment and a number of complex processes, including extraction, saponification, purification and derivatization of lipids in order to increase their volatility and thermal stability, as well as reduce contamination (traces of cholesterol and /or partial glycerides). Measurement of primary oxidation products provides reliable information only in the early stages, since they are unstable and decompose rapidly, reducing their content as oxidation increases. Therefore, the measurement of secondary compounds is more suitable for determining the degree of oxidation of meat or meat products. Secondary compounds are stable, and they also cause the appearance of rancid taste and smell, which affects the quality of meat.

MDA and hexanal are the most important and widespread aldehydes used as lipid peroxidation indices (Qiu *et al.*, 2013). In fact, different studies established values of 2–2.5 mg MDA/kg as the accepted limit in which there is no rancidity in fat and meat (Campo *et al.*, 2006; Zhang *et al.*, 2019). The main method of quantitative determination of MDA is the method based on MDA, as specific product of lipid peroxidation, reaction with thiobarbituric acid (TBA) lead to formation of a colored complex with maximum absorption at 532 nm (Mousavi *et al.*, 2018).

However, the reaction with TBA is not specific to MDA. There are several aldehydes and other oxidation products that also react with TBA, so the method is called thiobarbituric acid reactive substances (TBARS) (Yang and Boyle, 2016) and is used to evaluate total lipid oxidation, rather than quantifying MDA (Banerjee *et al.*, 2017). There are several variants of the TBARS method with different conditions for extracting MDA from food samples.

HPLC and GC methods provide better specificity and sensitivity when detecting MDA. However, the TBARS spectrophotometric methods are the most widely used to assess the oxidative state of meat and fat in routine analysis due to their simplicity and low cost (Pereira and Abreu, 2018). This method has a good correlation with sensory deterioration of the products quality (Domínguez *et al.*, 2019).

In addition to MDA, several aldehydes, including alkanols, 2-alkenals and 2, 4-alkadienals, are formed during lipid hydroperoxides decomposition. Determination of p-Anisidine value is a general spectroscopic method for measuring the amount of secondary lipid oxidation products formed during the

decomposition of peroxides and hydroperoxides (Majchrzak *et al.*, 2018). This method requires extraction of lipids, however, the p-Anisidine value is considered a good indicator, since it correlate with other indicators of both primary (PV) and secondary oxidation (TBARS), as well as with deterioration of organoleptic qualities (Barriuso *et al.*, 2013). The measurement of carbonyls (aldehydes and ketones) also makes it possible to track secondary lipid oxidation processes. Methods for determining carbonyls are based on interaction with 2,4-dinitrophenylhydrazine in an aqueous solution, as a result of which carbonyls turn into orange-colored hydrazones extracted by hexane. This is a simple and fast method that correlates well with the deterioration of the taste resulting in rancid taste (Domínguez *et al.*, 2019).

More recently, other analytical methods have been developed for the determination of lipid oxidation products in meat and meat products (chemiluminescence, fluorescence emission, RS, infrared spectroscopy or NMR). It is worth noting that the oxidative susceptibility is more influenced by the unsaturation of fat than the amount of fat.

4.Fatty acid composition of adipose tissue vs consumer health: "ideal" backfat virtual model

Consumer concern about the composition of the food product, the desire for healthy food has become synonymous with low-fat, or even free-fat food. Moreover, this approach primarily concerns animal fats. Anxiety about the harmful effects of cholesterol and saturated fatty acids on the cardiovascular system has led to changes in pig livestock technologies everywhere. Pig breeds with the thickness of the backfat lower 1.0 cm have been bred. Combined feeds contribute to a change in the FA composition of fat towards a decrease in the proportion of saturated fats. In this regard, fat was also evaluated by its atherogenicity (AI) and thrombogenicity (TI) indices.

$$AI = \frac{C12:0 + C14:0 + C16:0}{n\text{-}3PUFA + n\text{-}6PUFA + MUFA}, \quad (1)$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{0.5 \times MUFA + 0.5 \times n\text{-}6PUFA + 3 \times n\text{-}3PUFA + n\text{-}3PUFA/n\text{-}6PUFA} \quad (2)$$

Currently, these indicators are calculated according to the equations proposed by (Ulbricht and Southgate, 1991) in various modifications, for example, according to the equation described by Campo et al. (2013).

The values of both indicators are linked to certain FA composition. The positive role of omega 3 FA in reducing the risk of atherosclerosis is known, as well as C 12:0, C 14:0 и C 16:0 FA contributes to an increase of low-density cholesterol level in the blood, whereas C 18:0 does not demonstrate such an effect.

Systematization and analysis of literary (Fallon S., 1999; Pascual *et al.*, 2007; Domínguez *et al.*, 2015; Semenova *et al.*, 2019; Huang *et al.*, 2020) and own data on the FA composition assessment of pork fat, as well as WHO, scientists and nutritionists recommendations (FAO Food and Nutrition Paper, 2010) resulted in Table 2 in the form of a structural-parametric model, which are summarized and presented characteristics of pork adipose tissue optimal for obtaining products with health-promoting properties.

The structural-parametric model is a square matrix and is formed in the blocks (Ivashkin, 2004). They are placed along the main diagonal and combine operators of functional relationships within the selected groups of parameters reflecting the variety of existing known and theoretically defined relationships

between the content of individual FAs and the characteristics of fat. If the parameters belonging to the same group are independent, the corresponding block on the main diagonal is a unit matrix. If there are relationships between parameters, the extra-diagonal elements of the selected blocks describe interaction operators both within groups and between parameters of other groups. Then the extra-diagonal square of the original block matrix correspond to the operators of direct and indirect intergroup influence of individual parameters belonging to different functional groups.

Table 2. "Ideal" backfat structural-parametric model

k	Traits of "ideal" backfat								SFA														MUFA						PUFA								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	x																			*		*	!!	⁂	⁂	!!	⁂	⁂	!!	!!	⁂	⁂	*			*
2		x							*	*	*	*		*		*	*	*	*	*	*	*	*	*	*	!!	⁂	!!	⁂	⁂		⁂	*			*
3			x											!!		!!		!!				⁂	!!	*	!!	!!	⁂	⁂	⁂	⁂	⁂	!!	!!			!!
4				x										⁂		⁂		⁂		!!			!!	⁂	⁂	⁂	⁂	⁂	!!	⁂	⁂	!!	⁂			⁂
5					x																		⁂	*	⁂	!!	⁂	*	!!	*	*	*	*			*
6						x			*	*	*	⁂		⁂		!!		!!		!!			⁂	⁂	⁂	⁂	⁂	*	⁂	⁂	*	*	*			*
7							x									!!	!!	!!	!!	!!													!!			!!
8								x																					!!	!!	⁂	⁂	!!			!!
9		*				*			x																											
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29	!!	⁂	⁂	!!	!!	⁂		!!																					x								
30	!!	⁂	⁂	⁂	*	⁂		!!																						x							
31	⁂		⁂	⁂	*	*		⁂																							x						
32	⁂	⁂	!!	!!	*	*		⁂																								x					
33	*	*	!!	⁂	*	*	!!	!!																									x				
34																																		x		
35																																			x	
36	*	*	!!	⁂	*	*	!!	!!																													x

Note:

1 – IV; 2 – Trans fat content; 3 – AI; 4 – IA/IT ratio; 5 – Saturated index (SI); 6 – Melting point; 7 – Index ω 3; 8 – ω 6: ω 3; 9 – C4:0; 10 – C6:0; 11– C8:0; 12 – C10:0; 13 – C11:0; 14 – C12:0; 15 – C13:0; 16 – C14:0; 17 – C15:0; 18 – C16:0; 19 – C17:0; 20 – C18:0; 21 – C19:0; 22 – C20:0; 23 – C14:1; 24 – C 15:1; 25 – C16:1; 26 – C18:1; 27 – C20:1; 28 – C 22:1; 29 – C18:2 ω 6; 30 – C18:3 ω 3; 31 – C 20:2 ω 6; 32 – C20:4 ω 6; 33 – C 20:5 ω 3; 34 – C 22:2 ω 6; 35 – C 22:5 ω 3; 36 – C 22:6 ω 3

* – low correlation; ⁂ – medium correlation; !! – significant correlation.

$$\Phi(x) = 1 - \sqrt{\frac{1}{n}\sum_{i=1}^{n} b_i \left(\frac{x_i - x_i^0}{\Delta x_i^l}\right)} \rightarrow max \qquad (3)$$

where n – the number of estimated indicators, according to the structural–parametric model, their number is equal to 8; x_i, x_i^0 – actual and desired value; Δx_i^l – the maximum deviation from the desired value for the *l*–th quality level; b_i – the weighting coefficient of the *i*–th parameter.

Parameters of FA composition are presented in three blocks: SFA (k=9 to 22), MUFA (k=23 to 28), PUFA (k=29 to 34). Knowledge and analysis of patterns,

relationships, including structural ones, allow creating a model of "ideal" backfat. Block "Traits of "ideal" backfat" includes indicators forming the quality functionality (Nikitina *et al.*, 2019). Qualitative indicators can make an equivalent contribution to the formation of the quality of an "ideal" backfat, or they can have their own weight value (contribution) different from other indicators. The determination of weight coefficients can be carried out by methods of expert assessments, factor experiment, etc.

The value of the quality functional changes from 1 when the obtained values completely coincide with the optimal ones (the best quality) to 0 when the quality level limit is reached (the limit value).

The FA composition of adipose tissue has a significant effect on the quality traits of the "ideal" backfat: blocks of SRA, MUFA, PUFA in the structural–parametric model. The presence of a correlation in the matrix table is marked with signs (*, ⁂, ⁑, !!), which corresponds to a low, medium and significant correlation between fatty acids and indicators of the quality of "ideal" backfat. Numerical values can be obtained using correlation and regression analysis.

The equations, ratios, criteria used in the assessment of FA composition are presented in Table 3, and the optimal values are also given, which must be achieved in order to have the most satisfying (sufficient, significant) effect on the qualitative traits the "ideal" backfat.

5.Discussions

Various factors should be taken into account in order to meet industry requirements and consumers demands for a quality of product during the developing an optimal pig livestock system. On the one hand, saturated animal fats contribute to the cardiovascular diseases development, on the other hand, fat are an important component of the human diet, being the main source of energy, and contains a range of essential fatty acids that have a positive effect on consumer health. At the same time, adipose tissue is important and determines the quality of pork. However, less attention has been paid to the qualitative characteristics of fat in recent decades compared to other characteristics of meat quality, including pH, moisture loss, moisture content, tenderness and color. Traditionally, approaches for assessing the quality of fat, such as gas chromatography, iodometric titration and spectrophotometric methods, are widely popular for assessing fatty acids, iodine value (IV) and fat resistance to oxidation. The IV not exceeding 0.74 g/100g can be considered as the optimal value (Seman et al., 2013). The creation of an effective method for assessing the traits of fat quality and the development of criteria and indicators of the "ideal" backfat are very relevant today.

Table 3. Optimal backfat traits for a healthy diet

Trait / Reference	Value	Equations/ criteria of optimality*
MUFA:PUFA (ω3, ω6):SFA ratio	1:1:1	$\sum_{k=9}^{22} q_k lx = \sum_{k=23}^{28} q_k lx$
FAO Food and Nutrition Paper, 2010		$\sum_{k=9}^{22} q_k lx + \sum_{k=23}^{28} q_k lx = 2\sum_{k=29}^{36} q_k lx$
MUFA:PUFA:SFA ratio	0.48:0,12:0.4	$0.3\sum_{k=9}^{22} q_k lx = 1.2\sum_{k=23}^{28} q_k lx$
Fallon S., 1999		$\sum_{k=9}^{22} q_k lx + \sum_{k=23}^{28} q_k lx = 1.5\sum_{k=29}^{36} q_k lx$
PUFA:SFA ratio	0.4–0.6	$0.4 \le \frac{\sum_{k=29}^{34} q_k lx}{\sum_{k=9}^{22} q_k lx} \le 0.6$
Nieto&Ros, 2012; Ulbricht & Southgate, 1991		
MUFA:PUFA ratio	2,5–3,5	$2.5 \le \frac{\sum_{k=23}^{28} q_k lx}{\sum_{k=29}^{34} q_k lx} \le 3.5$
Lisitsyn *et al*., 2013		

SI Martins et al., 2015	≤1	$SI = \frac{q_{16} + q_{18} + q_{20}}{\sum_{k=23}^{28} q_k + \sum_{k=29}^{36} q_k}$
Σ C14 and C 16	25%	$(q_{16} + q_{18}) = 0.25 \sum_{k=9}^{36} q_k$
C18:0 content	≥12 %	$q_{20} \geq 0.12 \sum_{k=9}^{36} q_k$
C18:2 content	12–15 %	$q_{20} \geq 0.12 \sum_{k=9}^{36} q_k$
18:3n–3:18:2n–6 ratio Lisitsyn *et al*., 2013	1–(3–5)	$1 \leq \frac{q_{30}}{q_{29}} \leq 5$
C18:0:C18:2 ratio FAO Food and Nutrition Paper, 2010	1.1–1.25	$1.1 \leq \frac{q_{20}}{q_{29}} \leq 1.25$
Σ C 11, C 13, C 15, and C 17 (Mainly due to the pentadecanoic FA)	0.4–0.6%	$0.04 \sum_{k=9}^{36} q_k \leq (q_{13} + q_{15} + q_{17} + q_{19}) \leq 0.06 \sum_{k=9}^{36} q_k$
Σ C22:5 and C22:6n3	≥0.25%	$(q_{35} + q_{36}) = 0.025 \sum_{k=9}^{36} q_k$
Arachidonic cis–5, 8, 11, 14– C20:4ω6 content	0.8–1.1	$0.8 \leq q_{32} \leq 1.1$
FA medium chain (C11 – C16): FA long chain (>C17) ratio	1.6–2.0	$1.6 \leq \frac{\sum_{k=13}^{18} q_k}{\sum_{k=19}^{22} q_k + \sum_{k=26}^{36} q_k} \leq 2$
ω6:ω3 ratio Debrecéni *et al.*, 2017	3,5–4,5	$3.5 \leq \frac{q_{29} + q_{31} + q_{32} + q_{34}}{q_{30} + q_{33} + q_{35} + q_{36}} \leq 4.5$

Note: * according to the structural–parametric model (in corresponding units): q – FA content of fatty acid in the "ideal" backfat; k – FA identifier, for example q_{32} – corresponds to C20:4, a q_{22} – C20:0, etc.; l – the amount of fat in the "ideal" backfat; x – the amount of the "ideal" backfat.

This article describes the criteria for the optimality of pig fat tissue – the first stage on the way to creating "ideal" backfat virtual model.

A comprehensive assessment of the fatty acid (FA) composition and the application of accumulated knowledge to the identification of quality–determining FA have great potential. So, for example, it is known that myristic and myristooleic FA have an effect on the melting of fat. Some monounsaturated FA may protect against the risk of cardiovascular diseases development (Briggs *et al.*, 2017). Odd-numbered FAs are rarely detected in pork fat, or are found in trace amounts, for example, C:19 and C:21 FA (Lisitsyn et al., 2013). The total increase in odd-numbered FAs content begins with C:15. The recommended PUFA:SFA ratio is higher than 0.4. Such values can be achieved by the in vivo influence on pig adipose tissue through the use of certain ingredients in feed. J.V. Pascal et al obtained pork with a PUFA:SFA ratio of 0.6–0.74, using linolenic acid–rich feed when raising pigs of the Landrace and Duroc breed (Pascual *et al.*, 2007). The ω–6:ω–3 ratio is an important trait. Experts point out that the ω–6 – ω–3 FA should be at the level of 4–5% of the fat component of the diet. At the same time, scientists differ in determining the optimal ω–6:ω–3 ratio, which ranges from 3:1 to 10:1, while nutritionists claim: the ω–6:ω–3 ratio of a healthy adult diet should be in the range 4:1–6:1 (FAO Food and Nutrition Paper, 2010).

The negative attitude towards trans fats is well known. WHO recommends the use of technologies that do not cause the formation of trans fats. This is means, that the product should be free of trans–fatty acids of non–natural origin. However, trans isomers of FA

are found in nature. For example, vaccenic FA, trans–11–octadecenoic acid (C18:1 ω7) is synthesized in ruminants in significant quantities. At the same time, some authors note its presence in pork (Domínguez *et al.*, 2019; Aboagye *et al.*, 2020).

Vaccenic acid is metabolized to form rumenic, or conjugated linoleic acid – polyunsaturated FA (ω –6), in organism. The "omega 3 index" indicator proposed by Harris and von Schacky is included in the number of fat characteristics and calculated as the sum of eicosapentaenoic and docosahexaenoic fatty acids (Harris and von Schacky, 2004). These FA are essential and contribute to the elimination of low–density lipoproteins, reduction of inflammatory processes, decrease the risk of cardiovascular diseases. Docosahexaenoic acid (DHA) is found in large quantities in brain tissues and the retina of the eye, being their structural component (Arterburn *et al.*, 2006).

Eicosopentaenoic acid is a precursor of prostaglandins, promotes the formation of anti–inflammatory protective function of the organism. In adipose tissue, these FA are contained in very small amounts, which indicates the need to replenish these FA. Although these acids can be synthesized from alpha–linolenic FA, this process is very complex and inefficient with coefficient of efficiency only of 0.1. At the same time, it has been shown that the content of these two FA in human blood in an amount of 8% dramatically reduces the risk of mortality from cardiovascular diseases (Huang *et al.*, 2021), and in concentrations above 4% of the total fatty acid content they could contribute to the normal metabolism of brain cells, retina, heart tissue. This indicator should tend to 1 in the adipose tissue of pigs. It is advisable to pay attention to such a long–chain FA as eicosotriene (C20:3 ω–3), which is usually either not detected during analysis, or is detected in trace amounts. The presence of this FA in detectable amounts indicates the priority activity of synthesis of ω – 3 PUFA due to ω – 6 PUFA (Pascual *et al.*, 2007).

As mentioned above, many doctors consider fat as a potential source of cholesterol and a promoter of cardiovascular diseases. It is worth noting that currently not all cholesterol is considered harmful to health, but its individual fractions, especially cholesterols of low density lipoproteins. The equation proposed by Ulbricht TLV and Southgate DAT in 1991 for calculating the atherogenicity of a food product by the ratio of individual fatty acids divided fatty acids into those that have a significant effect on the risk of cardiovascular diseases development and others that, on the contrary, contribute to the removal of excess fats from the body. Previously, we determined the atherogenicity and thrombogenicity indices of various pork samples obtained from animals of different breeds and location on carcasses. There was no correlation between the total fat content and the atherogenicity of the studied samples. However, the correlation between the indices and the location on carcasses has been established. The minimum value of AI = 0.53 was found in the outer part of the hip of carcass, and the maximum value was 0.84 in the spinal–lumbar part of the middle carcass. At the same time, the atherogenicity of pork is lower than that of beef. Own and literary data allowed determining the optimal ratio of AI:TI ranges from 0.4 to 0.6. The focus on the indicators of thrombogenicity and atherogenicity of pork made it possible to formulate more precisely the requirements for pigs and the obtained raw materials.

6. Conclusions

Summarizing, pork backfat meeting the arisen in the review traits as much as possible, will contribute, in addition to its main purpose – energy nutrition of the body, also to maintaining its normal functioning, reduction the risk of cardiovascular diseases development and other alimentary–dependent diseases.

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QUALITY, ACCEPTABILITY AND SHELF LIFE OF CHICKEN NUGGETS PREPARED FROM DIFFERENT CHICKEN MEAT TYPES

Opeyemi Abiala✉, Babatunde Omojola and Moses Abiala

[1]*Department of Animal Science, Faculty of Agriculture, University of Ibadan, Oyo State, Nigeria.*
[2]*Department of Biological Sciences, College of Basic and Applied Sciences, Mountain Top University, Prayer City, Ogun State, Nigeria.*
✉*leadroleva@gmail.com*

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ABSTRACT

Quality, acceptability, and shelf-life of chicken nuggets prepared from different chicken meat types (broiler, spent layer and cockerel) were investigated. Raw chicken nugget pieces (n = 60, per chicken meat type) were deep-fried and representative samples were analysed for proximate composition, total cholesterol, lipid oxidation and microbial load. Product yield was calculated, and samples were assessed for sensory properties. Broiler chicken nugget had outstanding ($P<0.05$) crude protein content and product yield in comparison to spent layer and cockerel chicken nuggets. Interestingly, spent layer chicken nugget had remarkable ($P<0.05$) low ether extract and cholesterol content in comparison to other chicken meat types. Apart from the variation in the shelf-life based on microbial load especially from day 10 to 15, the chicken nuggets from different chicken meat types were equally accepted. Thus, spent layer and cockerel chicken meat types could also be useful raw materials for production of chicken nuggets. Most importantly, spent layer could be a ready choice for the production of products with reduced fat and cholesterol content which could be a more acceptable choice for the ever-increasing health conscious consumers.

1. Introduction

Globally, the production and consumption of poultry meat has increasingly progressed and in many nations of the world, per capita consumption (Yogesh et al., 2012) of poultry meat continues to grow. On similar note, poultry meat relevance have been applauded by United Nations Food and Agricultural Organization that speak of it as a readily available, inexpensive food especially in developing countries where it help in meeting up short falls in essential food nutrients. Its consumption also improves the quality of diets consumed in certain ages and conditions such as during pregnancy, lactation, and geriatric ages and during growth and development in young children (Cricelli et al., 2015).

Poultry products are universally popular, due to the fact that they are not subject to cultural or religious constraints and the meat itself is perceived as wholesome, healthy and nutritious, being relatively low in fat and with more desirable unsaturated fatty acid content (Khaksar et al., 2010; Issara et al., 2014). In addition, the bland flavour and soft texture characteristics of poultry meat makes it readily acceptable by meat processors for the development of further processed products (Petracci and Bianchi, 2012). This is as a result of its high soluble collagen, light colour as well as low-fat and high-protein content. Their ability to retain water (the one naturally present or added during processing) and the ability to achieve the desired final texture which in return increases meat water holding capacity (WHC) has made poultry meat highly sought after in the development of meat products (Cricelli et al., 2015).

Among the poultry meat products is chicken nugget. It is a ready – to – eat emulsion based food item that is gaining popularity with consumers. It is made from breast meat of broiler chickens and often battered or breaded before being deep-fried or baked. (Shai, 2015). However, due to the very tender nature of broiler chicken meat, many consumers are interested in the use of other chicken meat types which could improve the firmness and overall acceptability of chicken nuggets made from it (Mir et al., 2017). Thus, the use of other chicken meat types such as spent layers and cockerels in chicken nugget formulations need to be considered.

Recently, due to food insecurity, many countries have developed interest in processing spent layer meat into a more sustainable and profitable meat products (Petracci and Bianchi, 2012). In Brazil, a study at University of Sâo Paulo revealed that the meat of spent layer add healthier value to the mortadella-type sausage due to the fact that, the end product contains high polyunsaturated fatty acids and with a good polysaturated-to-saturated fatty acid ratio (Souza et al., 2011; Haris, 2019). Regarding cockerel, apart from the fact that it is hardy, tasty and well accepted, the meat is low in fat and cholesterol in comparison to broilers meat (Nonye, 2021). On this note, considering the health benefit in terms of low fat content and consumer preference for taste, spent layers and cockerels were considered as raw materials for chicken nugget in this study. We evaluated the quality, acceptability and shelf life of the chicken nuggets prepared from broiler, spent layers and cockerels.

2. Materials and methods

2.1. Meat source and preparation

Ten each of live broiler, spent layer and cockerel chickens weighing between 1.5-2kg were purchased from Zartech Farms in Ibadan, Nigeria. The birds were slaughtered, dressed and cut into primal parts. The breast meat were trimmed of skin, external fats and visible connective tissues. The meat samples were kept in the refrigerator (before chicken nugget preparation) at 4°C to keep the microbial load relatively low.

2.2. Preparation of non-meat ingredients

The dry spices (curry, thyme and red pepper) were sorted of extraneous matters, ground individually into powdery form and sieved through a 2 mm diameter sieve and kept in well covered containers until use. The fresh spices which include garlic and onion bulbs were cleaned, ground separately in a blender (model PNA 00582NW) and used on wet basis. Others which include powdered milk, soya oil, curing salt (NaCl), sugar, monosodium glutamate, and dry white corn seeds used for corn flour preparation were obtained. The dry corn seeds were sorted carefully to remove any extraneous matter such as stones, glass beads and dirt before grinding using grinder (Model BLSTMG, PN, 133093-002). The coarse particles were removed using a sieve of 2 mm mesh diameter. The fine powder was kept in an air- tight container until use. The chicken nugget recipe (g/100g) were as follows; chicken meat (70%), vegetable oil (7%), corn flour (10%), powdered milk (4%), curing salt

(0.8%), sugar (0.1%), ice flakes (6.0%), seasoning (2.1%).

2.3. Chicken nugget preparation

The recipe was used for chicken nugget production following standard procedures as described by Suradkar et al. (2013*)*. Chilled breast meat from each chicken type were ground using a Super Wolf (MADO MEW 513, Maschinferfabrik Domhan, Gmbh, Germany) grinder through 3mm sieve plate. Dry ingredients such as corn flour and powdered milk were added to each portion of the ground meat types. Slurry of curing salt, soya oil, ice water and seasoning as shown above was prepared and also added to each portion of the ground meat types. The ingredients were thoroughly mixed with the ground meat manually until a desired consistency was obtained. Batter from each chicken type was moulded into a round shape of 20 g per sample. A total of sixty chicken nugget pieces were prepared per chicken type. Chicken nugget samples were coated with rusk (ground oven dried bread) and then frozen at 10°C for 15 minutes. Raw chicken nuggets were deep fried using soya oil to a core temperature of 72°C using a probe type meat thermometer Model No 3504-66. The cooked chicken nuggets were kept at 4°C and sampled every five days throughout a period of 15 days for evaluation. The experiment was a complete randomized design (CRD). Each chicken meat type represent a treatment, making a total of three treatment. Each treatment was replicated six times.

2.4. Sensory evaluation of chicken nuggets produced from different chicken types

This was conducted using a 20 member semi-trained taste panels at each stage according to the method described by AMSA (1995). The taste panelists were made up of male and female students and workers in the Department of Animal Science, University of Ibadan in the age range of 25-45 years. Unsalted cracker biscuit and water were provided for mouth cleansing in between treatments. The room was well ventilated and devoid of all forms of distractions that could affect panelist. Chicken nuggets were blind coded and the orders of serving were randomized. Chicken nuggets were assessed using a 9- point hedonic scale for colour, juiciness, flavour, aroma, hotness, tenderness and overall acceptability.

2.5. Cholesterol content of chicken nuggets prepared from different chicken types

Cholesterol of the chicken nugget was carried out by adding 5 mL of chloroform into a conical flask containing 5 g of the sample and then ground. Additional 5 mL of chloroform and 10 mL of distilled water were added and mixed thoroughly. The mixture was poured into a separating flask and the lower layer was released into a test tube. 1mL of acetic anhydride and 1ml of concentrated sulphuric acid (H_2SO_4) were poured into the separated solution. Green colour was observed at the interface. Absorbance wavelength of the solution was measured in spectrophotometer at 640 nm (Nawar et al., 1991).

2.6. Proximate composition and product yield of chicken nuggets

Proximate analysis of all samples were determined according to AOAC (2000). Product yield was determined by measuring the difference in the sample weight before and after cooking. Product yield (%) = [Weight of cooked nugget / Weight of uncooked nugget] X 100.

2.7.Thiobubaturic acid reactive substances (Tbars) content in prepared chicken nuggets

The degree of lipid oxidation was determined for each meat and meat product sample at days 0, 5, 10 and 15. Thiobubatunic Acid Reactive Substances (TBARS) assay was done using the method of Zeb and Ullah (1990). 5g of each sample were weighed into the conical flask, 10 mL of distilled water was added and homogenized for 2 minutes. 2 mL of 10% Trichloroacetic Acid (TCA) was added

and each was filtered through Whatman No 1 filter paper. Freshly prepared Thiobubatunic Acid (TBA) was added to each sample filtrate on ratio 1:1. A blank of 10 mL distil water, 2 mL of 10 % TCA and freshly prepared TBA were prepared in another conical flask. The solutions of each sample and the blank were stirred for 4 -5 seconds and stored in the dark for 1 hour to develop the colour (slightly reddish). Absorbance wavelength was measured using an (UV-Vis spectrophotometric CE1020 model, cecic-UK) at 530 nm. The results were expressed as mg malonaldehyde (MDA) per kg products using the formulae: TBA = K + OD5 nm, where K = 9.242.

2.8. Microbial load of of chicken nuggets prepared from different chicken types

Three different culture media were used to carry out the microbial analysis of chicken nugget samples. These were the nutrient agar, MacConkey agar and potato dextrose agar. Microbial assay was carried out using pour plating method and the plate was incubated at 37^{o}C for 48 hours. Bacterial and fungi counts were determined from plates bearing the colonies. All analysis were carried out in triplicates for days 0, 5, 10 and 15.

2.9. Statistical analyses

Experimental treatments were compared using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). For each of the experiment, replicated data sets were subjected to the analysis of variance (ANOVA) technique according to the experimental design to find out the significance of the treatments. ANOVA was also used to determine the effect of treatments and error associated with each experiment. Mean comparison of traits was used and carried out by protected LSD (p = 0.05; Students-Newman-Keuls Test) where the error mean square served as the standard error of differences between mean.

3. Results and discussions

3.1. Proximate composition of prepared chicken nggets

There were significant ($P<0.05$) variation in the proximate composition and total cholesterol content across the chicken nuggets prepared from broiler, spent layer and cockerel (Table 1). Specifically, the broiler meat had the highest moisture content (43.10%) and spent layer chicken nugget had the lowest (42.31%). The moisture content obtained for the chicken nugget from the different chicken meat types were higher than that of Ismed et al. (2009) from commercial chicken nuggets and also lower in comparison to that of Darwish et al. (2011) that had 52.4% in cooked beef burger. The differences in the moisture content obtained could be as a result of the differences in the WHC (Abd-El-Aziz et al., 2021) of the different chicken meat types used in this study.

The broiler chicken nugget had high crude protein content in comparison to the spent layer and cockerel chicken nuggets, this could be linked to both physical and chemical properties of the raw broiler chicken meat. At least to a certain extent, the spent layer (Souza et al., 2011) and cockerel chicken nuggets were comparatively similar to that of broiler chicken nugget. This suggest that when occasion arises, preference for protein from spent layer and cockerel chicken nugget could be considered. Interestingly, the chicken nuggets from spent layer had the lowest cholesterol content in comparison to broiler and cockerel chicken nuggets. This observation in the diet of birds have the potential to influence the amount of fat deposition especially in the raw meat (Souza et al., 2011; Verma et al., 2012; Kim et al., 2015). This implies that the low total cholesterol content of raw meat from spent layer could have resulted in a lower cholesterol content of cooked chicken nugget samples prepared from it.

The ether extract of chicken nuggets from broiler chicken meat had the highest value. This is however expected as broiler chicken meat are known for more accumulation of fat which could have resulted in higher value

recorded. Spent layer chicken nugget, followed by cockerel chicken nugget had well pronounced nitrogen free extract in comparison to broiler chicken nugget with unremarkable nitrogen free extract. This agreed with the work of Reddy et al. (2016) that documented similar observation for spent layer meat against broiler meat types.

3.2. Yield, pH and acceptability of prepared chicken nuggets

Product yield is one of the very crucial factors in meat industry as it predicts the behaviour of a product when cooked. (Pietrasik and Li-chan, 2002; Souza et al., 2011). In this study, yield (%) of chicken nuggets from broiler meat were significantly (P<0.05) higher (80.14 %) than spent layer (77.89 %) and cockerel (78.50 %) which were comparably similar (Figure 1). This implies higher product yield (%) obtained in chicken nuggets prepared from broiler chicken meat could be linked to the ability of the chicken nugget to retain more moisture and fat during cooking thereby making it of higher economic value as the amount of marketable product produced is more than that of chicken nuggets prepared from cockerel and spent layers.

Next, we determined the pH. There were no significant (P< 0.05) differences in the values recorded for pH (emulsion and cooked nuggets) across the treatments (Figure 1). Most importantly, the pH values of the cooked chicken nuggets were higher than that of the emulsion (Verma et al., 2015). Though, not as high as 6.90 which could result in a number of negative changes, the most visible is seen in colour and microbiological stability of such product. (Aidani et al., 2014).

In order to assess acceptability of chicken nuggets from the different chicken meat types, sensory evaluation was carried out using scientific approach which include measuring, analysing and interpreting food characteristics as perceived by sense of smell, touch, sight and others (Grammatina et al., 2012). When meat goes into the mouth, certain characteristics which include juiciness, aroma, texture and flavour are factors that affects product organoleptic quality. All chicken nuggets produced from different chicken meat types were significantly (P< 0.05) similar and accepted. Specifically and without exception, aroma, colour, flavour, juiciness, tenderness and hotness enhanced sensory acceptability of nugget samples (Table 2). More often, consumers score chicken product acceptability based on colour (Rosli et al., 2011). Meat colour also depends on a number of factors which include chemical characteristics of meat pigment, its concentration, physical characteristics and presence of nonmeat ingredients, air, humidity, storage temperature, packaging method and type of package used (Sayago-Ayerdi et al., 2009). The aroma, flavour, juiciness and tenderness of chicken nuggets are known to increase with more fat in meat product. In all the eating qualities assessed for in this study, the panelists did not record significant (P<0.05) difference in the eating qualities of chicken nuggets (Raeisi et al., 2021) from different chicken meat types which probably is an indication that any of the chicken type could be used for nugget preparation without any adverse effect on eating quality.

3.3. Quality and shelf life of chicken nuggets as affected by chicken types and storage days

Lipid oxidation leads to lipid degeneration and development of oxidative rancidity in meat and its products (Jimenez et al., 2016). In this study, Lipid oxidation in terms of TBARS values were estimated over a period of 15 days. TBARS values increased as storage days increased. Chicken nuggets from broiler chicken meat were observed to have lower values from day 0 to 10. At day fifteen, the lowest TBARS values were obtained in chicken nugget prepared from spent layers with value 0.0378 mg/MDA/kg. However cockerel chicken nuggets had the highest TBARS which ranged from day 0 – 15 (Table 3). Worthy to note that all values recorded were lower than the threshold value of TBARS of 2.0

mg/MA/kg recommended by Witte et al. (1970). This could be attributed to the antioxidative property of soyabean oil which was used in frying the chicken nugget samples as it is known for its vitamin E content. Hence, all chicken nuggets estimated for TBARS are fit for consumption till day 15th of storage.

Thereafter, microbial quality of the cooked chicken nuggets were assessed (Egan et al., 2007). Microbiological results revealed that different chicken types influenced microbiological state of the chicken nuggets. Specifically, there were differences ($P<0.05$) in the values obtained for coliform, total bacteria and moulds across the treatments and over the period of storage which lasted for 15 days. It was observed that the microbial load increased with days of storage. On a more specific note, from day 5 – 15, cockerel chicken nuggets had highest coliform load in comparison to broiler and spent layer chicken nuggets. The broiler chicken nuggets attracts less bacterial load from day 0 – 15 compared to spent layer and cockerel chicken nuggets with more bacterial load over the period of 15 days. Surprisingly, chicken nugget from spent layers did not attract mould at all from days 0 - 15 (Table 4) while cockerel chicken nugget had more mould counts, followed by that of broiler chicken nugget (Sugiharto, 2019). This however could be attributed to the pH of the cooked nuggets as lower pH values have been reported to contribute to reduction in microbial activity in meat and meat products and vice versa (Aidani et al., 2014). However, the microbial load obtained for all chicken types are within the acceptable limits of 6.0 $\log_{10}$ cfu/g as reported by Shapton and Shapton (1991).

Table 1. Proximate of nutrient composition and cholesterol content of chicken nugget prepared from different chicken types

Parameters	Broiler	Spent layer	Cockerel	SEM
Moisture (%)	43.10^{c}	42.31^{a}	42.96^{b}	0.007
Crude protein (%)	32.92^{a}	32.20^{b}	32.20^{b}	0.006
Ether extract (%)	9.80^{a}	9.42^{b}	9.70^{a}	0.015
Ash (%)	4.01^{a}	3.60^{b}	3.61^{b}	0.002
Crude fibre (%)	0.89^{b}	0.92^{a}	0.89^{b}	0.002
Nitrogen Free Extract (%)	9.28^{a}	11.55^{c}	10.64^{b}	0.017
Cholesterol (mg/100g)	1.76^{a}	1.34^{b}	1.48^{a}	0.010

^{abc}: Means in the same row with varying superscripts are significantly ($P<0.05$) different according to Student-Newman- Keuls Test (n=3). SEM: Standard Error of Mean

Table 2. Sensory evaluation of chicken nugget prepared from different chicken types

Parameters	Broiler	Spent layer	Cockerel	SEM
Aroma	4.75	4.63	4.75	0.172
Colour	4.70	4.63	4.28	0.288
Flavour	4.80	4.75	5.73	0.151
Juiciness	3.65	4.48	4.33	0.136
Tenderness	5.05	4.90	4.45	0.153
Ropiness	6.05	5.45	6.45	0.118
Overall Acceptability	6.48	6.45	5.98	0.104

^{abc}: Means in the same row with varying superscripts are significantly ($P<0.05$) different according to Student-Newman- Keuls Test (n=3). SEM: Standard Error of Mean

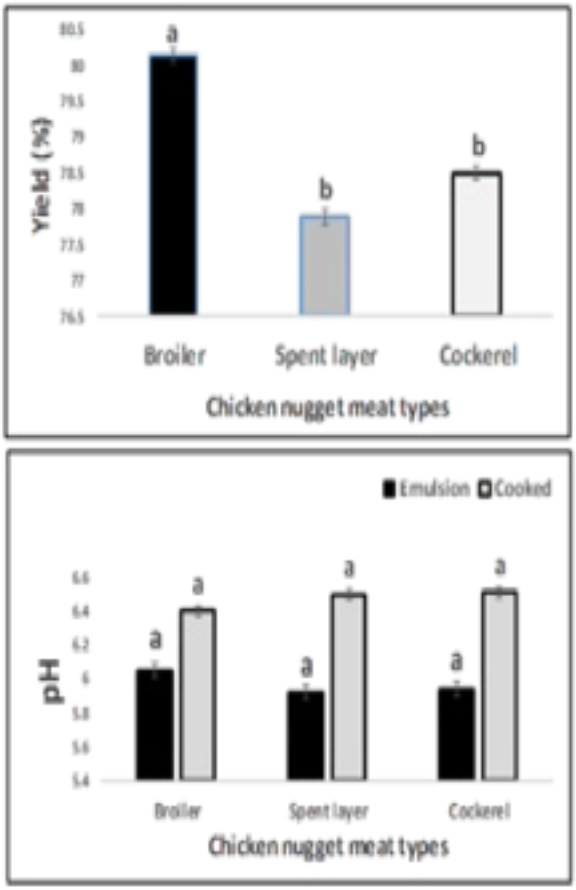

Figure 1. Yield (%) and pH (emulsion and cooked) of chicken nuggets from different chicken meat types. Means values with different letters among the treatments are significantly (P<0.05) different.

Table 3. Lipid oxidation of chicken nugget prepared from different chicken types

Treatment	TBARS (mgMA/1000g)
Broiler	0.0226[b]
Spent layer	0.0233[a]
Cockerel	0.0236[a]
Standard Error of Means	0.00004
Time (Days)	
0	0.0093[d]
5	0.0171[c]
10	0.0276[b]
15	0.0387[a]
Standard Error of Means	0.00006
Interaction between chicken types and time (Days)	
Broiler 0	0.0095[f]

	5	0.0168^{e}
	10	0.0255^{d}
	15	0.0385^{ab}
Spent layer	0	0.0090^{f}
	5	0.0175^{e}
	10	0.0288^{c}
	15	0.0378^{b}
Cockerel	0	0.0093^{f}
	5	0.0170^{e}
	10	0.0285^{c}
	15	0.0398^{a}

$^{abc…f}$ Means in the same column with varying superscripts are significantly (P<0.05) different according to Student-Newman-Keuls Test (n=3).

Table 4. Microbial counts (cfu/g x 10^3) of chicken nugget as affected by chicken types and storage days

Treatment		Coliform	Total Bacteria	Mould
Broiler		0.500 ^{b}	4.458^{b}	0.625^{b}
Spent layer		0.625^{ab}	5.500^{a}	0.000 ^{c}
Cockerel		0.875^{a}	5.833^{a}	1.000^{a}
SEM		0.0625	0.1749	0.0442
Time (Days)				
0		0.000 ^{c}	2.167^{c}	0.000 ^{c}
5		0.333^{b}	2.833^{c}	0.500^{b}
10		1.000^{a}	4.500^{b}	0.500^{b}
10		1.333^{a}	11.556^{a}	1.167^{a}
SEM		0.0833	0.2332	0.0589
Interaction between chicken types and time (Days)				
Broiler	0	0.000 ^{c}	2.000^{e}	0.000 ^{d}
	5	0.000 ^{c}	3.000^{de}	0.500^{cd}
	10	1.000^{ab}	3.500^{cde}	0.500^{cd}
	15	1.000^{ab}	9.300^{b}	1.500 ^{ab}
Spent layer	0	0.000 ^{c}	2.500^{de}	0.000 ^{d}
	5	0.500^{bc}	3.000^{de}	0.000 ^{d}

	10	1.000^{ab}	4.500^{cd}	0.000 ^{d}
	15	1.000^{ab}	12.000^{a}	0.000 ^{d}
Cockerel	0	0.000 ^{c}	2.000^{e}	0.000 ^{d}
	5	0.500^{bc}	2.500^{de}	1.000^{bc}
	10	1.667^{a}	5.500^{c}	1.000^{bc}
	15	1.500^{a}	13.333^{a}	2.000^{a}

^{abce}: Means in the same column with varying superscripts are significantly (P<0.05) different according to Student-Newman- Keuls Test. The results shown are means ± standard error (n=3).

4. Conclusions

Apart from the fact that the chicken nuggets prepared using spent layers, cockerels and broiler chicken meat had equal over all acceptability ratings, each chicken meat type showed unique attributes; cockerel chicken nugget had improved product yield, while that of spent layer chicken nugget had reduced fat and cholesterol content. The three chicken meat types can be used in the preparation of quality and acceptable chicken nuggets. The spent layer meat could be a ready choice for production of products with reduced fat and cholesterol contents. Thus, using different chicken meat types for chicken nugget would increase availability of more raw materials for food/meat processors in the production of chicken nuggets and as well help reduce seasonal overproduction of these birds by converting them into storable ready to eat products.

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Ethical Statements

The authors declare that they have no conflicts of interest

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NEW TECHNOLOGICAL METHODS TO CONTROL HMF FORMATION IN DATE SYRUP DURING PROCESSING

Shahinaz A. Helmy[1]✉, Aya Y. Mostafa[2], Adel Z. M. Badee[1], Serag A. Farag, [2] and Mohamed E. Abdel-Aziz [1]

[1]Food Science Department, Faculty of Agriculture Cairo University, Giza, Egypt.
[2]Food Irradiation Research Department, Industrial Irradiation Division, National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (AEAE), Cairo, Egypt
✉shahinaz29@cu.edu.eg

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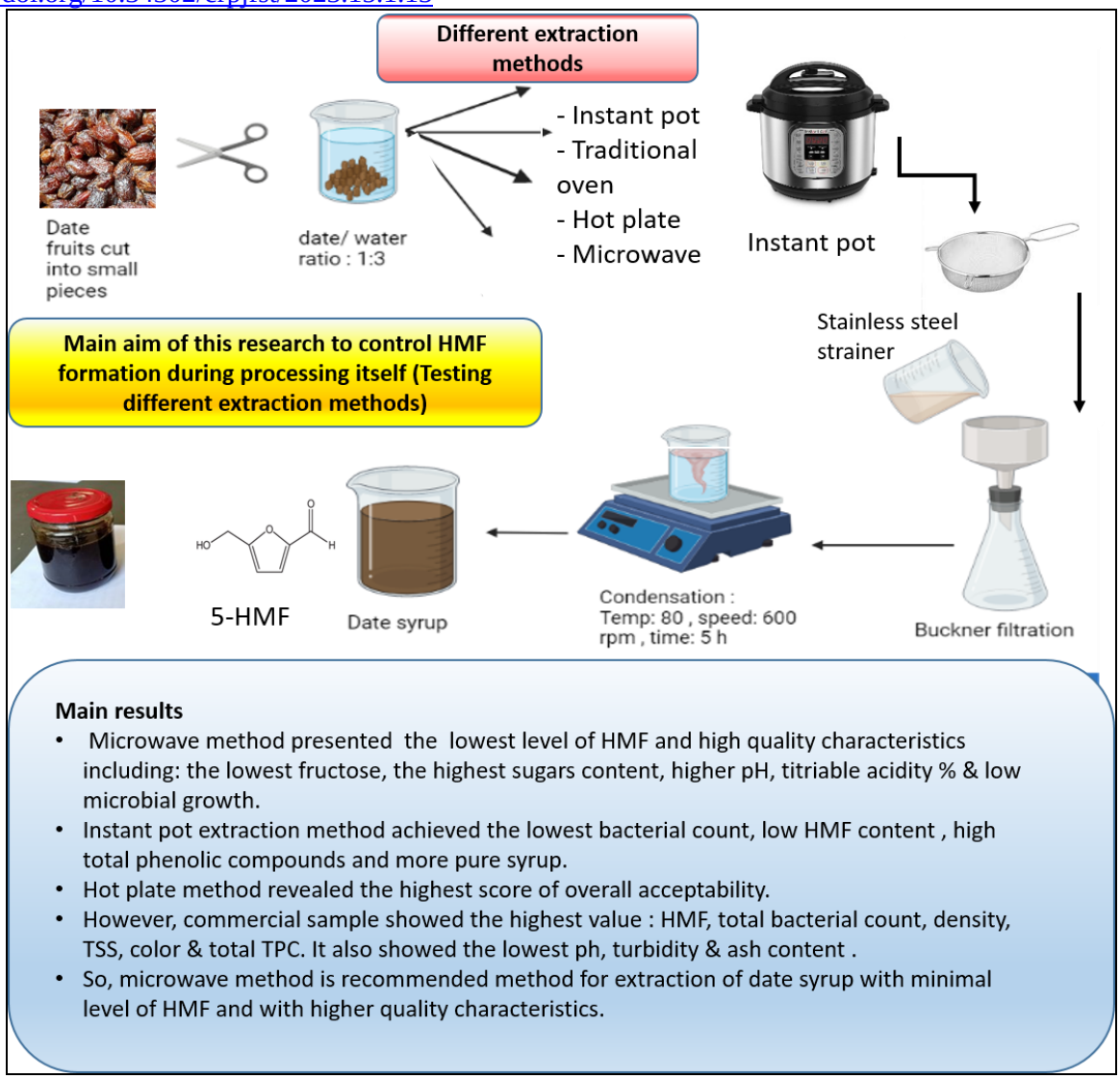

Figure 1. Graphical abstract clarify date syrup processing steps and the new proposed techniques (New extraction methods) that may reduce formation of HMF during manufacture processing itself.

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ABSTRACT

Date occupies a position between many crops with its nutritional and medicinal values. The higher fruit loss induces the processing of these fruits to convert to useful products, such as date syrup and date paste. Date syrup is characterized by its high nutritional value. However, it contains a toxic-hazard compound (5-hydroxyl methyl furfural). This compound is usually formed as a result of the dehydration of sugars under higher temperatures during processing (Millard reaction). So, the present paper aimed to reduce HMF levels in date syrup during processing using different extraction methods. The obtained results emphasized that the extraction method has a great influence on HMF levels of the lab-produced date syrup compared with the commercial sample. Where instant pot, traditional oven, hot plate, microwave methods, and commercial sample recorded HMF levels ranged (from 503.63, 285.38, 1010.00, 240.13 & 1844.30 mg/ kg, respectively). In this regard, the microwave extraction method presented the best results concerning with safety of the product: the lowest level of toxic hazard (5- HMF) and high-quality characteristics including Fructose, pH, titriable acidity, and a high score of overall acceptability of Panel test. Meanwhile, the instant pot extraction method was characterized by a low level of HMF and the lowest microbial count. While the commercial sample featured elevated levels of (5-HMF), the highest bacterial count, and the darkest color. The commercial sample also showed the highest score in the following: TSS, density, specific weight, color, and total phenolic compounds. However, it showed the lowest score in turbidity, EC, total, and sulfated ash %. Also, sensory evaluation by Hedonic scale showed consumers' appreciation of date syrup especially extracted using microwave methods. So, the microwave method is recommended method for the extraction of date syrup with a minimal level of HMF and higher quality characteristics.

1. Introduction

Dates (*Phoenix dactylifera* L.) represent one of the most important fruit trees in many states worldwide, particularly in the middle east and Arabian countries as Hashem, *et al.* (2017) clarified. Nowadays, this fruit has also gained great importance in global trade. Within the recent period, world date production has doubled; this trend is expected to continue as per FAO projections according to Ghnimi *et al.* (2018). Egypt occupies the first position among the top five date-producing countries. Where Egypt's annual production of date surpasses 1.7 million tons (FAO, 2020). Dates (Semi-dry) resemble about 19.2% and the Siwi date is considered the most important semi-dry cultivar in Egypt as declared El-Samahy, *et al.* (2005). The rejected ratio of Siwi date reached about 15% (3937 tons) of the total date production of Baharia oasis, mainly used in animal feeding. So, it is very important to utilize these quantities in date dibs production The majority of date fruits are used up directly at different stages (Khalal, Rutab, and Tamr), with minimal or no processing. Nowadays, great attention is paid to the improvement of date processing. Various date products such as date syrup, liquid sugar, alcohol, and vinegar are successfully marketed (Yousif, *et al.*, 1987). One of the important popular products of dates is date syrup, which is principally produced from second-grade and non-marketable dates as Ramadan (1998) mentioned. Date syrup processing seems to be a suitable way for preserving the fruits and minimizing transportation and storage costs and could open new prospects for date fruits. A promising usage of date syrup recently emerges in food industries. It is used as a natural sweetener in many food products instead of harmful sugar such as beverages, jam, jelly, ice cream, yogurt, bakery products, date bars, date sheets, and confectionary (Abd El-Hady *et al.*, 2014; El-Samahy & Youssef, 2009; Hou et al.,

2022). Where debis is a very rich source of natural sugar, polyphenol, antioxidant activity, vitamins (Niacin), and minerals (Fe, Ca, Na, Mg, Zn). So, date syrup is a great source of essential nutrients and various health benefits as declared by Shahein et al. (2022). However, dates processing face a health hazard concern. Where date fruits exposes to elevated temperature during the processing of date syrup which can influence the quality, nutritional value, and product safety. Higher heat stimulates the formation Millard reaction products like furan and 5-hyroxy methyl furfural A higher concentration of HMF causes cytotoxic, oxidative stress and genotoxic effects on human health (Hou et al., 2022). 5-HMF is one of the most important heat-induced food toxicant (food contaminants) formed in various foods including honey, jams, fruit juices, milk, spirits, coffee, cereals, bakery products, and vinegar (Farag et al., 2020). HMF is a heterocyclic product (Choudhary et al., 2021). It is yellow, low-melting solid and highly water-soluble. The molecule composed of furan is a ring, having aldehyde and alcohol functional groups. HMF formed by the caramelization of sugars as well. HMF is a quality parameter to monitor heating (mode & rate) or storage (period & conditions) in many foods (Rahimzadeh, et al., 2014).The toxicity of 5-HMF has been experimented by many researchers. They reported that 5-HMF is a potential toxin, mutagen, and carcinogen for humans (Eshete & Eshete, 2019; Farag et al., 2020; Michail et al., 2007). Where HMF is metabolized by human body (catalyzed by sulfotransferase) to genotoxic and carcinogenic compound called 5-sulfoxymethylfurfural (SMF) (Capuano & Fogliano, 2011; Severin et al., 2010; Shapla et al., 2018). SMF is also considered nephrotoxic (Bakhiya, Monien, Frank, Seidel, & Glatt, 2009). Unfortunately, SMF is considered a non-excretable compound and may accumulate in human body. Therefore, HMF contaminant take over a great attention from many researchers (Shapla et al., 2018).Different carcinogenic effects of HMF were reported on various living things and organs (Yang, *et al.*, 2019): highly toxic to honey bees (Le Blanc et al., 2009). 5-HMF raise the occurrence of deviant crypt foci in rat colon, skin papillomas in mice, lipomatous tumors in rat kidney (Capuano & Fogliano, 2011), small intestine adenomas in mice (Svendsen, *et al.*, 2009), hepatocellular adenomas in female mice, and has mutagenic effects on *S. typhimurium* (Lee, *et al.*, 1995). Where 5-SMF also induces small intestine adenomas potential in mice (Svendsen et al., 2009) and regeneration and atypical hyperplasia of tubules and hepatotoxic effects and sororities of peritoneal tissues (Bauer-Marinovic, *et al.*, 2012). Humans revealed higher sensitivity to 5-HMF because the existence of sulfotransferase, which can convert 5-HMF into SMF, is appeared in extra hepatic tissues at higher levels than rodents (Teubner, *et al.*, 2007).

Safety margin of HMF is also called (preclinical level), in which no toxic effects have been perceived. The estimated daily intake of 5-HMF for humans is approximately 2.5 mg/kg body (Capuano & Fogliano, 2011). HMF consumption beyond the safety limit is also cytotoxic to humans and causes irritation to the mucous membranes of the upper respiratory tract, skin and eyes etc. as declared by Choudhary *et al.* (2021); (Pastoriza de la Cueva et al., 2017; Shapla et al., 2018). Doses differ 2–30 mg / person/day according to human consumption (Abraham et al., 2011; Pastoriza de la Cueva et al., 2017).This range was found to be safe and does not cause health risks to humans (Janzowski, *et al.*, 2000).CODEX (Codex Alimentarius Commission) established a maximum limit for HMF in the honey of 40 - 80 mg/kg in tropical honey (Abraham et al., 2011). HMF levels should not surpass 80 mg/kg in tropical zones as mentioned by Commission. (Amended in 2019); (Eshete & Eshete, 2019)

In another study, Shapla et al. (2018) stated tolerable daily intake (TDI); at daily doses ranging from 80 to 100 mg/kg body weight. In fact, it is important to get ways to reduce 5-HMF levels in processed food so that

the total intake of 5-HMF can be reduced for humans.

Higher concentrations of HMF were found in date syrup and its fresh products ranged between 1000 to 2675 mg⁄kg (Jafarnia, *et al.*, 2016). This higher amount of HMF was produced in date syrup when the high temperatures (110℃) processing method was followed (Naknean, *et al.*, 2009). The same findings were reported for other foods such as bakery products (Ramírez-Jiménez, *et al.*, 2000) and the formation of HMF is dependent on the time of process in boiling juice. A highly-variable amount of HMF, changing from 12.8 to 3500 µg/kg, was found in boiled juices (Jafarnia et al., 2016). The challenge that concerns the present study is the formation of HMF with higher levels in date syrup and how to interfere and control HMF formation during the processing steps. So, the present study aimed to investigate the possibility to reduce such toxic compounds (5-HMF) during processing (using different extraction methods) and the influence of different extraction methods on the physicochemical and microbial characteristics of date syrup.

2. Materials and methods

2.1. Materials

Raw Materials & Chemicals: Mature and fresh date fruits (semi- dry, Siwi cv.) were purchased from local markets, Giza, without any impact and physical damage. The fruits were immediately packed in poly ethylene bags after sorting, washing with tap water and storing until processing. Commercial date syrup samples were purchased from the local markets, Giza. Standard of 5-HMF (97%) was purchased from Sigma Aldrich Company, (St. Louis, Missouri, USA). All chemicals used in this investigation were of analytical grade.

2.2. Methods

2.2.1. Preparation of date juice and syrup

First, date juice samples were prepared with four different extraction methods. The treatments were carried out in comparison with a commercial date syrup sample from local market (Giza governorate). In all previous extraction methods, date juice (date extract) was prepared according to constant ratio (1:3 for date: water, respectively) according to Hashem et al. (2017). Date syrup preparation including steps of clarification were illustrated in Fig.1.

2.2.2. Extraction methods

a. Instant pot: In this novel method, Electrical pressure cooker (PALSON instant pot: made in china, model: CR-34J, Capacity: 8 liters, power: 1200- 1400 W, frequency: 220- 240 V, 50/ 60 Hz) was used for extraction of date juice. TSS $_{initial}$ of date extract or date juice = 20 %. Work conditions: Pressure, 15 psi (pounds/ square inch), temperature: 130 ^{o}C, time: 20 min (Fig.1).

b. Traditional oven: date juice was extracted using traditional oven (100 ± 10 ^{o}C for 2 hours). TSS initial of date extract or date juice = 15 %.

c. Hotplate: date juice was extracted using hot plate (JOANLAB hotplate Stirrer) with the same pervious ratio at 100±5° C. This mix was heated on a high temperature (100 ± 5 ^{o}C) for 30 min. to produce date extract (date juice). TSS $_{initial}$ of date extract = 17 %.

d. Microwave: about 250 g date fruits /750 ml tap water. This mixture was put into the microwave oven (JAC microwave, Mode: NGM-2002, Capacity 20 liters, Power: 1200 W, frequency: 230V- 50 Hz) using medium temperatures level (ranging: 70 -80 ^{o}C) for 20 min. TSS $_{initial}$ of date extract = 15 %.

***2.2.3. Date juice filtration*:** different extracted samples are filtered and concentrated to produce date syrup (TSS= 68 %) as follows:

Coarse filtration: This procedure was done using Muslin cloth (4 layers) and coarse stainless steel strainer to get rid of date fruit parts.

Fine filtration: This procedure was applied using Muslin cloth, followed by filtration with

filter paper (pore size: 102 um) using Buckner funnel (Fig.1).

2.2.4. Date extract concentration: Date syrup was prepared by concentration of date juice to (TSS= 68 %) using hot plate: Concentration conditions: Temp: 80 ± 5 °C, Speed: 600 rpm and time: 5 hours as mentioned in Fig.1.

***2.2.5.* Physicochemical characteristics of date syrup**

2.2.5.1. Determination of HMF:

5-HMF content of lab-produced date syrup was determined after processing comparing with the commercial date syrup sample. HMF stock solution and serial dilutions were prepared (7 points of standard solution) and standard curve was plotted (Calculating R2 predicted: it should be ≥ 85 %).

Extraction of HMF: 5-HMF completely dissolves in water, so it is extracted easily in distilled water. The extraction ratio is 2 ml of date syrup / 25 ml of distilled water.

Purification procedures were applied using Carrez I solution (15 g of potassium hexacyano ferrate dissolved in 100 ml of distilled water) and Carrez II (30 g of zinc acetate dissolved in 100 ml of distilled water). About 0.5 ml of each carrez I and CarrezII were added to the HMF extract. The mixture was vortexed for 2 min. allowing the samples to precipitate. Remark the filtrate (containing HMF), then it was filtered using filter paper (qualitative filter paper 102 moderate (110 mm)) excluding the precipitate. After that, extracted samples were filtered using syringe membrane filter (0.45 um). Finally, filtrated samples were kept (in dark small vials) frozen until to be injected in HPLC- UV instrument. Suitable injection volume in HPLC instrument: 20 ul. Sample preparation with carried out according to Baltaci and Akşit (2016) with some modification.

Device Specification: Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector. About10 µl was taken of standard vial & unknown sample and injected to HPLC. Also, about 10 µl of un-known sample was injected. HPLC analysis conditions: Column C18 Inertsil: 4.6x250 mm, 5µm, Mobile phase: Water: Methanol. (85%: 15%), Mode of elution: Isocratic, Flow rate: 1ml/min, and ambient temperature at wavelength 285 nm.

2.2.5.2. Total soluble solids (TSS %):

Total soluble solids (TSS%) expressed as °Brix or percentage, were measured at 20±0.5°C by a Hand Refractometer (model (ATAGO, Japan)as described by Ranganna (1977).

2.2.5.3. pH values:

pH of date extract and syrup were measured using a digital pH-meter (Hanna, HI 902 meter, Germany).

2.2.5.4. Density & specific weight (SP):

Density (g/ cm^3) of syrup was measured by using a pycnometer. The results were calculated according to method mentioned in (Lullah-Deh, Khan, & Eneji, 2018). Determination of specific weight (Ratio): The specific weight of date syrup was calculated as the following: Specific weight = Weight of sample in pycnometer / weight of the same volume of water (SP = W of 25ml of date syrup / W of 25 ml of water). This determination was done according to Lullah-Deh et al. (2018).

2.2.5.5. Titratable acidity (TA):

It was titrated with 0.1N sodium hydroxide to the endpoint in the presence of phenolphthalein indicator and the results were expressed as gram malic acid per 100 gram sample (A.O.A.C, 2000).

2.2.5.6. Turbidity:

Turbidity was measured using pen turbidity meter (Model: Milwaukee MI 415-Romania). The results were expressed in NTU (Nephelometeric Turbidity Units).

2.2.5.7. Electrical conductivity (EC):

Date syrup samples is very viscose, so it is better to be diluted, so, 1 ml of date syrup was diluted with 25 ml of d.w., the mixture was

vortexed for 5 min. and was measured using Electrical conductivity meter (digital electrical Cond. & TDS: mode: AZ8361., China), according to AOAC (1999) with some modification. The results were recorded in mS/ cm.

2.2.5.8. Total ash & sulphated ash:

Total ash (%) was calculated based on EC value as described by Sancho, Muniategui, Sánchez, Huidobro, and Simal (1991) using EC equation: Total Ash % = 0.083 EC - 0.092. However, sulphated ash % also calculated based on EC value according the following equation: Sulphated Ash (%) = 0.121 EC - 0.097.

2.2.5.9. Non-Enzymatic browning (420 nm):

Non-enzymatic browning was determined as a color indication at wave length (420 nm) using spectrophotometer (Model Labomed, Inc, New York, USA) according to Fathi, *et al*.(2013) with some modifications.

2.2.5.10. Non-reducing and reducing sugars:

Non- reducing and reducing sugars were determined using HPLC according to Al-Farsi (2003) with slight modification based on the injection of external standards: Sucrose, glucose and fructose as external standard solutions, which were prepared as described (Sesta, 2006; Zhang, Aldosari, Vidyasagar, Shukla, & Nair, 2015). The results were expressed as g / 100 ml date syrup. Measurements were done using Waters 2690 Alliance HPLC system equipped with a Waters RI detector (Refractive index). 20 µl of each standard and unknown sample were injected. HPLC analysis conditions: Column BP 100 Ca: 300 x7.8 mm, mobile phase was acetonitrile /Water (75 / 25 v/v). Mode of elution: Isocratic, flow rate: 0.4ml/min. temperature was 65 ^{o}C.

2.2.5.11. Total phenolic compounds (T.P.C):

Preparation of phenolic extract :phenolic extract preparation was carried out according to Rasheed, Cobham, Zeighmami, and Ong (2012) with some modification.

Preparation of Gallic acid standard curve: (Serial dilution of gallic acid solutions): Dissolve about 30 mg of Gallic acid in 50 ml solvent (methanol 80 %) according to Mistrello et al.(2014).

Determination of total phenolic content (T.C.P):determination of T.C.P were done according to the Folin-Ciocalteu method (Farhadi et al., 2016). Add 750 ul Folin solution (diluted 10 %) to 200 ul of phenolic extract and vortex. Let the mixture for 10 minutes. Then about 750 ul of aqueous sodium carbonate (2 %) was added. The samples were vortexed and kept at room temperature for 60 mint in the dark. Spectrophotometer measurements: The absorbance was recorded at wave length 765 nm (model: UVKON 860). Using the gallic acid calibration curve, calculate the total phenol concentration (using graph and linear equation. The results were presented as mg of gallic acid equivalent (mg GAE / 100 g sample) according to Farhadi et al. (2016).

2.2.6. Determination of antioxidant activity: (DPPH methods):

Radical scavenge activity was determined using DPPH (1.1-diphenyl-2- picrylhydrazyl). DPPH scavenging analysis was carried out following the (Brand-Williams et al., 1995). Antioxidant activity (AO %) was expressed as a percentage of DPPH scavenging relative to control (DPPH only). DPPH scavenging activity = [(Absorbance of control – Absorbance of sample)/ Absorbance of control] x 100 (Shahdadi, Mirzaei, & Daraei Garmakhany, 2015).IC $_{50}$ of DPPH: The IC_{50} is the concentration of phenolic extract that achieve inhibition for 50 % of DPPH radicals. This value was calculated from standard curve (Scavenging activity versus phenolic concentration of sample) as Farahnaky, Mardani, Mesbahi, Majzoobi, and Golmakani (2016)clarified.

2.3. Microbial examination:

The total plate counts as well as total yeasts and molds of different-extracted date syrup were determined using pour plate technique on

Nutrient agar media and Potato Dextrose Agar, respectively (Sulieman & Elkashif, 2009). Date syrup samples were serially diluted using sterile saline (0.85% NaCl) and the appropriate dilution was then plated. The plates for total count were incubated for 48 h at 35°C, however, the plates for yeasts and molds were incubated for 5 days at 28 °C. Colonies were then counted. Each test was performed in duplicate and the results were expressed as colony forming units (CFU) per milliliter.

2.4. Sensory evaluation:

Date syrup samples were given three-digit codes and organoleptically examined by ten panelists from Food Radiation Lab., Food irradiation research dept., National Center for Radiation Research and Technology (NCRRT), Industrial Irradiation Division, Egyptian Atomic Energy Authority (EAEA). Panelists were asked to evaluate each attribute (1-5).All samples were evaluated for color, purity, flowing rate (viscosity), sweetness, odor, taste, consistency and sugar crystal. The panelists were also asked to tell the final decision about the overall acceptability of date syrup using 9.point hedonic scale (Hyvönen & TÖRMÄ, 1983). Where samples Scores ranging from like extremely (9) to dislike extremely (1).Water and neutral wafers were also served for cleaning palate between samples.

2.5. Statistical analysis:

A complete randomized design was followed with one factor (4 different extraction methods were tested comparing with commercial date syrup sample). Each treatment was performed in 3 replications the number of treatment (n=15).

The obtained results (mean values of three separate experiments, unless otherwise stated, were statically analyzed using Minitab (18) software (Minitab 18, 2021). One way Analysis of Variance (ANOVA) and standard deviation (SD) followed by comparison test (Tukey test) were calculated. Pearson method (R^2) test were done to test correlation between some variables. All tests were performed at ($p < 0.05$).

3. Results & discussion

The present research aimed to reduce HMF formation in palm syrup during processing *via* different extraction methods and determine the effects of these methods on physico-chemical, microbial properties and organoleptic characteristics as well.

3.1. Effect of different extraction methods on HMF level in date syrup

The obtained results (Table 1 & Figure 2) emphasized that the extraction methods of date juice and heating levels play an important role in formation of HMF compound in processed date syrup. Where the commercial sample recorded the highest score followed by heat extraction sample (1844.30 & 1010.00 mg/ kg, respectively). Meanwhile, date syrup sample extracted by microwave methods showed the lowest HMF content followed by syrup extracted by traditional oven (240.13 & 285.38 mg/ kg). These findings were in a similar trend with pervious study (El-Nagga & Abd El–Tawab, 2012). Where they found that Microwave method showed the lowest concentration of HMF when compared with rotary extraction and water bath method. So, microwave extraction method is considered the safest method for date juice extraction (Containing low levels of such toxic compound, HMF).

Table 1. Effect of using different extraction methods on 5-HMF, Non-reducing & reducing sugars concentration of processed date syrup

Extraction methods	HMF content (ug/ mg) or (mg/ kg)	Non-reducing sugars(g/ 100 ml)	Reducing sugars (g/ 100 ml)		Total reducing sugars
		Sucrose	Glucose	Fructose	
Instant pot	503.63	35.69	30.38	13.14	43.52
Traditional oven	285.38	38.50	32.56	11.87	44.43
Hot plate	1010.00	41.92	34.41	12.31	46.72
Microwave	240.13	48.92	39.71	11.68	51.39
Commercial sample	1844.30	37.91	32.61	12.77	45.38
Pearson correlation between HMF & different sugars	—	**-0.34**	**-0.29**	**0.502**	

Instant pot: Electrical pressure cooker, Data were statically analyzed using Minitab (18) program.

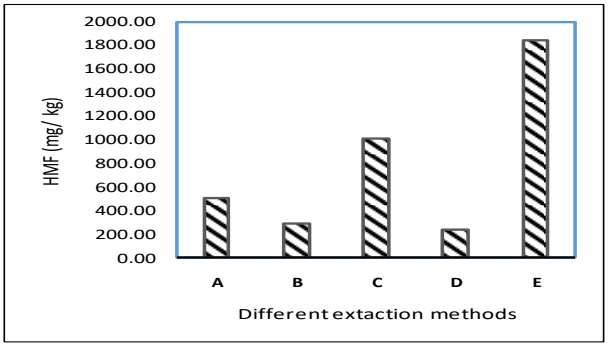


Figure 2. Effect of different extraction methods on HMF content (mg/ kg) of date syrup
Where, A: instant pot (Electrical pressure cooker), B: Traditional oven, C: hotplate method, D: Microwave method, and E: commercial date syrup sample.

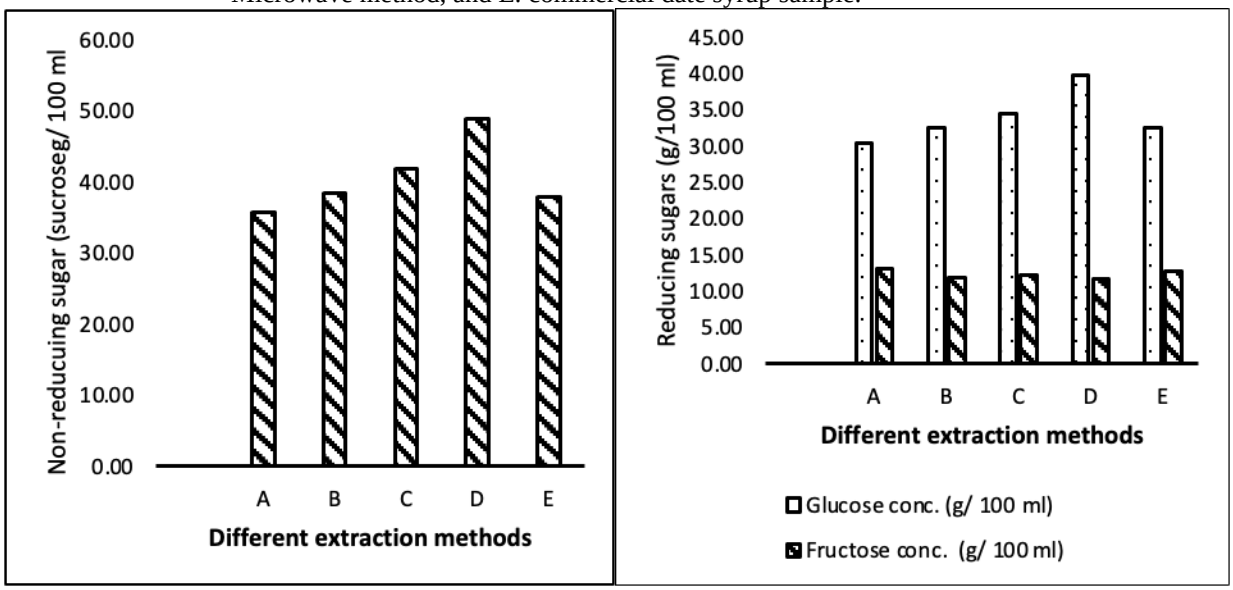


Figure 3. Effect of different extraction methods on reducing and non-reducing sugars of date syrup
Where, A: instant pot (Electrical pressure cooker), B: Traditional oven, C: hotplate method, D: Microwave method, and E: commercial date syrup sample.

3.2. Effect of different extraction methods on physico-chemical characteristics

3.2.1.Reducing and non-reducing sugars **:**it is noteworthy that reducing sugars, being the main components in date fruits varied 52.1–62.8 g/100 g fruit weight with smaller variation between cultivars (6.2%) as reported by Ghnimi et al. (2018).The obtained results in Table 1& Figure 3. demonstrate that extraction methods that followed higher & direct temperatures led to increase in fructose content thus increase in HMF content. So, sample extracted using microwave method (lower temperature) recorded the lowest fructose content followed by traditional oven methods (11.68 & 11.87 g/ 100 ml respectively).The explanation for that the enolization incident of glucose molecules to fructose under higher temperature causes increase formation rate of HMF subsequently. In this context, ***there were two ways of formation HMF*****: direct way:** Dehydration of fructose under higher temperature and converted to HMF

Indirect way: Conversion of glucose molecule to fructose (Enolization) under elevated heat conditions as Ståhlberg, Sørensen, and Riisager (2010) clarified.

Statistic for Table (1) emphasized that there were a proportional relationship between HMF & fructose content. (Pearson correlation = 0.502 at p-value = 0.139). Thus, increasing of fructose content raise the formation of HMF. Concerning with sucrose & glucose content, it was noticed a weak reversible relationship between HMF and sucrose & glucose content (Table1).

3.2.2. TSS %: there were no significant difference between different extraction methods, but the three significant difference between extraction methods and commercial that recorded the highest TSS % (73 .00 %) as shown in Table 2.

3.2.3. pH: hot plate method showed the highest pH value followed by microwave method (4.60 & 4.58 respectively). Meanwhile, commercial sample recorded the lowest pH value (4.40) as present in Table 2 and Figure 4. Correlation analysis (Pearson method) was performed between TSS% &pH. It was illustrated that there were a reversible relationship between TSS% &pH. (Pearson correlation = -0.837 at p-value = 0.077).

This relation was emphasized by Farahnaky et al. (2016). There were a reversible relationship between Brix &pH. Higher concentrations of date syrup caused greater pH reduction, which is due to date syrup when become diluted (the more moisture content %) this able to reduce organic acids in sample.

Table 2. Effect of using different extraction methods on TSS%, pH &TA % of date syrup

Extraction methods	TSS % (Brix)	pH	Titratable acidity %
Instant pot	67.00 ^{b} ± 1.00	4.49 ^{ab} ± 0.07	0.367 ^{ab} ± 0.058
Traditional Oven	67.33 ^{b} ± 0.58	4.54 ^{ab} ± 0.00	0.500 ^{ab} ± 0.183
Hot plate	67.00 ^{b} ± 0.82	4.60 ^{a} ± 0.07	0.225 ^{b} ± 0.050
Microwave	67.50 ^{b} ± 0.50	4.58 ^{a} ± 0.07	0.675 ^{a} ± 0.275
Commercial sample	73 .00 ^{a} ± 1.00	4.40 ^{b} ± 0.00	0.500 ^{ab} ± 0.100

*Means (±SD) followed by different superscripts within each column are significantly different (p≤0.05). Data were statistically analyzed using Minitab (18) program

Instant pot: Electrical pressure cooker

3.2.4. Titeriable acidity %: data in Table 2 and Figure 4. demonstrate that date syrup extracted using microwave methods recorded the highest acidity (0.675 %), while sample extracted using hot plate recorded the lowest (0.225 %) as shown in Table.2). The proposed explanation for that direct & higher heat may contribute to degradation of mallic acid (the dominant acid in date syrup).

3.2.5. Density (g/ cm^3): The importance of measuring density summarized in its relation to moisture content. When density of date syrup is less than particular value, the syrup is could be cheated. As shown in Table 3 and Figure 4 , the commercial sample recorded the highest density value followed by syrup extracted using instant pot (1.64 & 1.62 g/ cm3 respectively), while sample extracted using traditional oven recorded the lowest (1.59 g/ cm3).

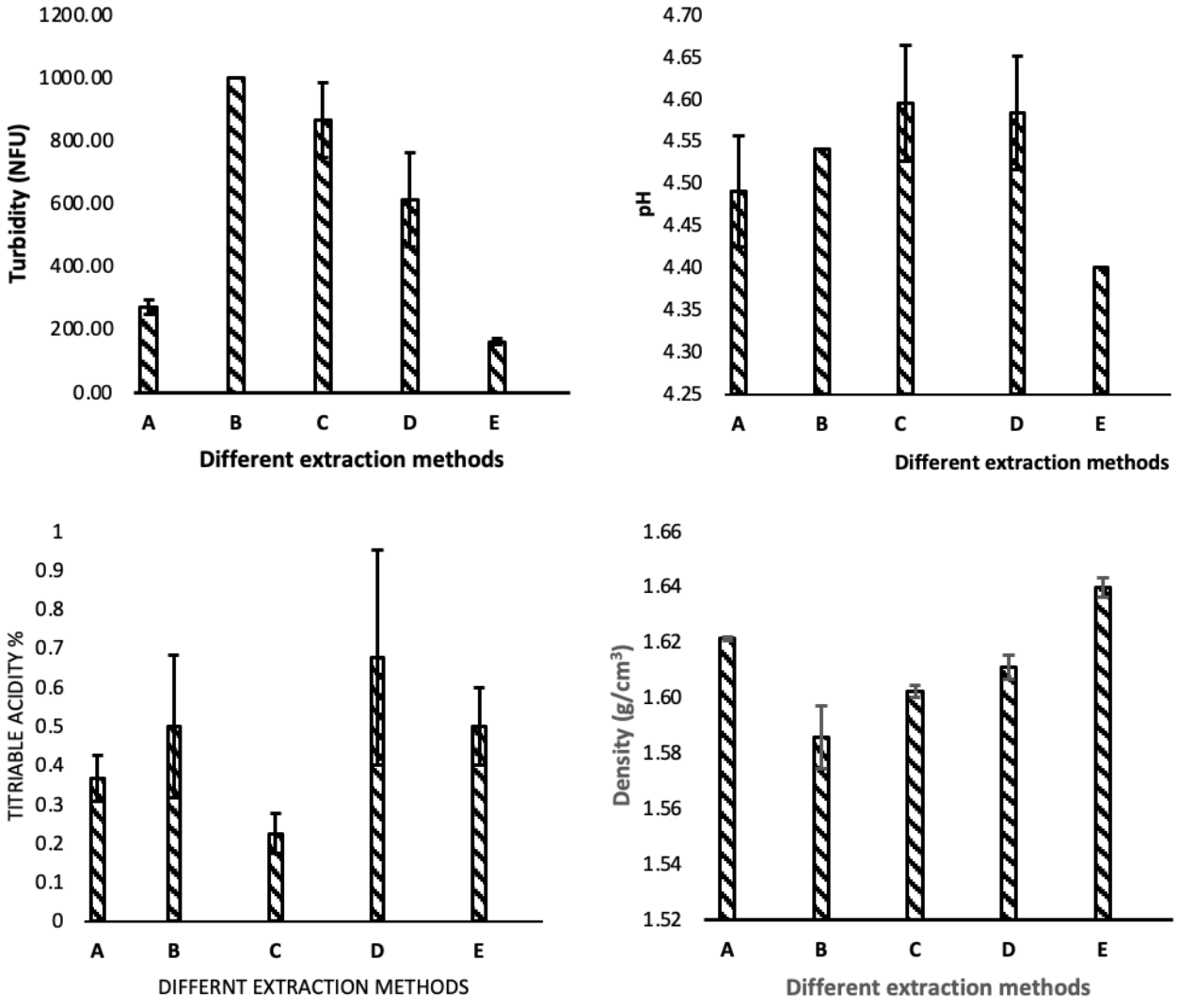


Figure 4. Effect of different extraction methods on pH, turbidity, TA % & density of date syrup
Where, A: instant pot (Electrical pressure cooker), B: Traditional oven, C: hotplate method, D: Microwave method, and E: commercial date syrup sample.

3.2.6. Specific weight: this parameter related to density. Commercial sample recorded the highest specific weight followed by syrup extracted using instant pot (1.36 & 1.35 respectively), while sample extracted using traditional oven recorded the lowest (1.32) as presented in Table 3.

3.2.7. Turbidity (NFU): Turbidity is important parameter that indicates the purity of date syrup, statistical analysis for Table (3) proved that there were significant differences between different extraction methods. Where the oven-extracted sample revealed the highest score followed by heat-extracted sample (1000.00 & 863.33 NFU respectively). While the commercial showed the lowest followed by instant pot (161.00 & 270.33 NFU, respectively.) as shown in Figure 4. Regardless the effect of extraction methods on turbidity and purity of date extract, elevated heat induced coagulation for proteins, causing easier separation, leaving a more clear extract, the higher absorbance and lower turbid (El-Nagga & Abd El–Tawab, 2012). That explains why commercial sample and instant pot-extracted sample recoded lower turbidity values.

Table 3. Effect of using different extraction methods on density, specific weight (Sp) & turbidity of date syrup

Extraction methods	Density (g/cm^3)	Specific weight	Turbidity (NFU)
Instant pot	1.62 ^{ab} ± 0.00	1.35 ^{ab} ± 0.00	270.33 ^{c} ± 24.09
Traditional Oven	1.59 ^{c} ± 0.01	1.32 ^{c} ± 0.01	1000.00 ^{a} ± 00.00
Hot plate	1.60 ^{bc} ± 0.00	1.33 ^{bc} ± 0.00	863.33 ^{a} ± 118.51
Microwave	1.61 ^{b} ± 0.00	1.34 ^{b} ± 0.00	611.67 ^{b} ± 149.25
Commercial sample	1.64 ^{a} ± 0.00	1.36 ^{a} ± 0.00	161.00 ^{c} ± 8.72

*Means (±SD) followed by different superscripts within each column are significantly different ($p \leq 0.05$). Data were statistically analysis using Minitab (18) program. Instant pot: Electrical pressure cooker

3.2.8. Electrical conductivity: it gains an especial importance it indicate the minerals content in liquid food sample. Data tabulated in Table 4 and drawn in Figure 6 proved that different extraction method influenced the EC of different samples. The highest value was for oven-extracted followed by heat-extracted (35.44 & 35.25 mS/cm respectively). Meanwhile, commercial sample showed the lowest followed by microwave extracted sample (26.73 & 30.47 mS/cm respectively). It was supposed that higher heat in commercial sample and microwave radiation in microwave method may causes reduction in minerals content.

Moreover, Pearson correlation was carried out for the relation between two variables (EC & turbidity). It was observed that there were a positive relationship between EC & turbidity (Pearson correlation = 0.692 at p-value = 0.195).

3.2.9. Total ash %: As a result for difference incident in EC between different methods of extraction. Subsequently, there was a significant difference between date syrup samples extracted by different methods. Oven-extracted sample recorded the highest minerals content (ash) followed by heat extracted sample (2.85 & 2.83 % respectively). While commercial sample showed the lowest followed by microwave- extracted sample (2.13

& 2.44 % respectively) as shown in Table 4 and Figure 6. Calculated total ash % in this study was in agreement with Farahnaky et al. (2016). Where they determined Total Ash % in date syrup in similar value (2.18 ± 0.01). For explanation for the different ash content of different extracted samples. It could be notice that minerals content greatly affected by tannin content. Where tannin compounds reduces the bioavailability of minerals (Marin, Siqueira, & Arruda, 2009) lead to decreasing ash. It was appeared that elevated heat (in commercial sample) and microwave may increase tannin content within possible chemical polymerization, thus mineral and ash content decreased. On the other side, tannin content may didn't change in oven-extracted sample. So, it recorded the highest minerals content (ash). In this context, Hassan, Osman, Rushdi, Eltayeb, and Diab (2009) found out duel reversed responses to gamma irradiation. Where irradiation increased tannin content in sorghum (That is in agreement with our findings). While gamma irradiation significantly reduced tannin content of maize cultivar. It worth to mention that the change in tannin content affected by heating temperature, irradiation and food material. In previous study (Duodu, *et al.,* 1999), it is reported that cooking and gamma irradiation caused significant reduction in phytic acid level of sorghum. Similarly, treatment of soybean seeds with gamma irradiation, alone or in combination with soaking reduced the level of phytate compared to untreated seeds (Sattar & Akhtar, 1990).

3.2.10. Sulphated ash %: It tends to have done so total ash% (Table 4 & Figure 6) the relationship between EC and sulphated ash summarized in following equation:

Sulphated Ash % = 0.121 EC - 0.097 (1)
(Sancho et al., 1991).

Table 4. Effect of using different extraction methods on EC, total ash & sulphated ash of date syrup

Extraction methods	Electrical conductivity (EC)	Total Ash (%)	Sulphated Ash (%)
Instant pot	34.33 ^{ab} ± 0.37	2.76 ^{ab} ± 0.03	4.06 ^{ab} ± 0.04
Traditional Oven	35.44 ^{a} ± 1.06	2.85 ^{a} ± 0.09	4.19 ^{a}± 0.13
Hot plate	35.25 ^{ab}± 3.17	2.83 ^{ab} ± 0.26	4.17 ^{ab}± 0.38
Microwave	30.47 ^{bc} ± 2.25	2.44 ^{bc} ± 0.19	3.59^{bc}± 0.27
Commercial sample	26.73 ^{c} ± 0.05	2.13 ^{c} ± 0.00	3.14 ^{c}± 0.01

EC should be expressed in mS / cm or mS / ml. EC should be measured at 20 ^{o} C. If temp > 20, we should use correction factor. Total Ash % = 0.083 EC - 0.092, Sulphated Ash % = 0.121 EC - 0.097.
Data were statistically analyzed using Minitab (18) program.
*Means (±SD) followed by different superscripts within each column are significantly different ($p \leq 0.05$).

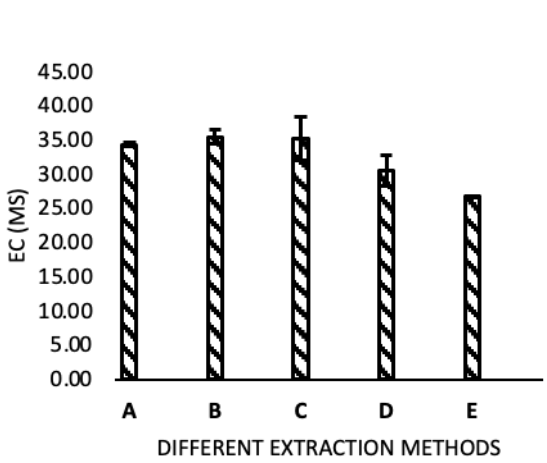
Table 5. Effect of using different extraction methods on color, total phenol & antioxidant activity of processed date syrup

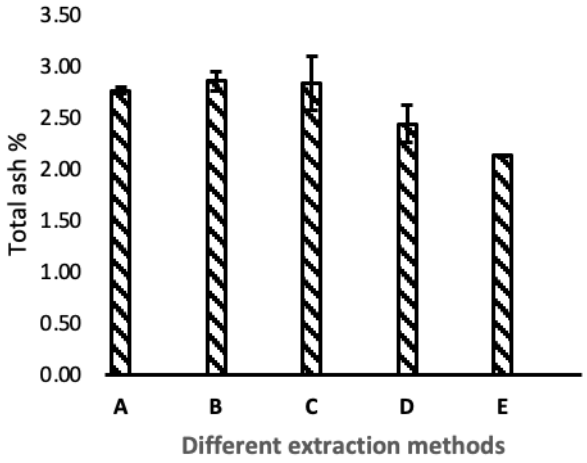
Extraction methods	Color (non-enzymatic browning)	Total phenol (TPC) (mg GAE/ 100 g)	Antioxidant Activity %(AO)
Instant pot	0.614 ^{a} ± 0.002	395.43 ^{b} ± 12.12	43.78 ^{a} ± 8.51
Traditional Oven	0.561 ^{b} ± 0.004	249.60 ^{c} ± 13.52	38.10 ^{a} ± 6.67
Hot plate	0.511 ^{c} ± 0.022	291.14 ^{c} ± 1.09	44.53 ^{a} ± 6.48
Microwave	0.551 ^{bc} ± 0.011	288.62 ^{c} ± 28.28	40.56 ^{a} ± 4.74
Commercial sample	0.656 ^{a} ± 0.006	493.34 ^{a} ± 13.52	35.64 ^{a} ± 1.84

TCP= c * V / m, c : Gallic acid concentration obtained from std. curve (mg/ L), V : Volume of extract in ml (or DF : dilution factor = 20 ml), m: mass of extraction in g. For conversion of (mg / L) to (mg / 100 g): TPC=(c * V)* 100 / 1000.

*Means (±SD) followed by different superscripts within each column are significantly different ($p \leq 0.05$). Data were statistically analyzed using Minitab (18) program.

Figure 5. The appearance (color) of different – extracted date syrup (1: Instant pot, 2: Traditional oven, 3: Hot plate (100± 5 C), 4: microwave)





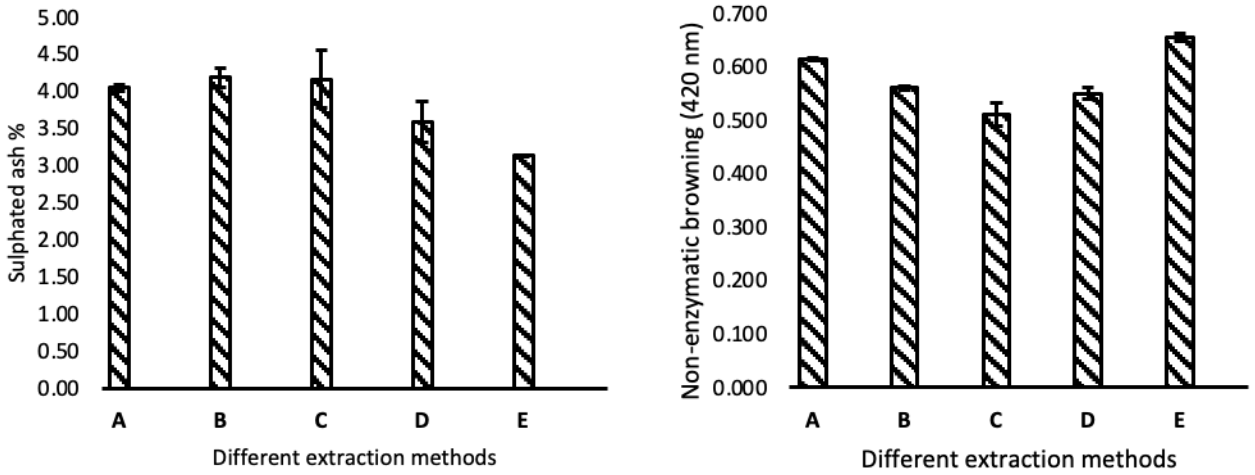


Figure 6. Effect of different extraction methods on EC, total ash %, sulphated ash %, and browning of date syrup. Where, A: instant pot (Electrical pressure cooker), B: Traditional oven, C: hotplate method, D: Microwave method, and E: commercial date syrup sample.

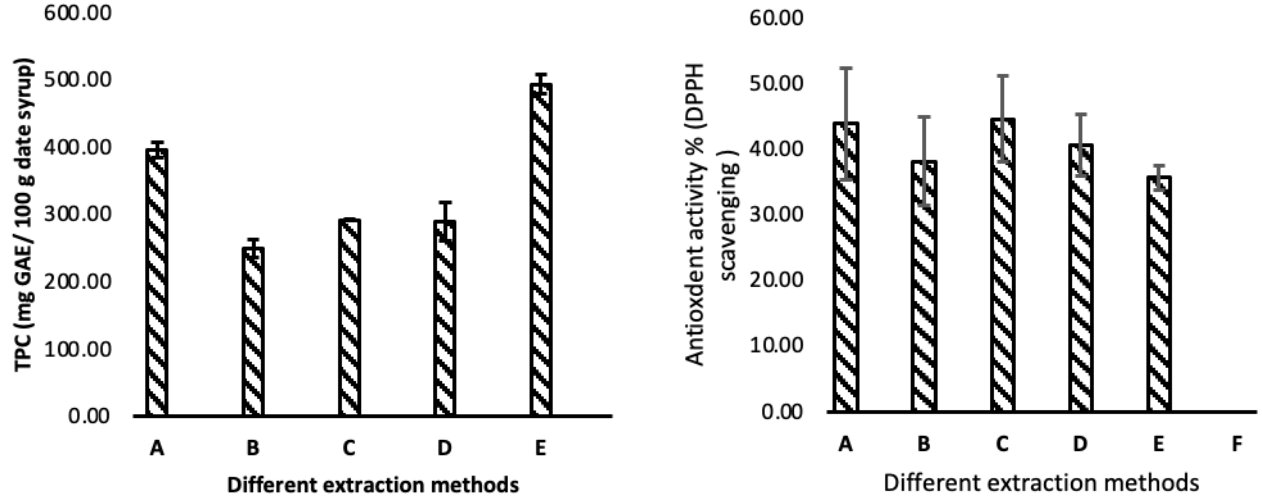


Figure 7. Effect of different extraction methods on total phenolic compounds (T.P.C) & Antioxidant activity % (AO %) of date syrup. Where, A: instant pot (Electrical pressure cooker), B: Traditional oven, C: hotplate method, D: Microwave method, and E: commercial date syrup sample.

3.2.11. Color (non-enzymatic browning): Data shown in Table 5 and Figure 5 & 6demonstrated that different extraction methods showed significantly different degrees of color (non- enzymatic browning). The highest value for commercial followed by instant pot; 0.656 and 0.614, respectively. In agreement with what Hashem et al. (2017) have reported, commercial sample recorded the highest value of color (browning). Where the lowest value were for sample extracted using heater (100± 5 °C) followed by microwave and traditional oven; 0.511, 0.551 & 0.561 respectively. That could be explained that extraction methods that following higher temperature may increase the extraction of pigments and polyphenols of processed fruits unlike the treatments that do not use higher temperature like microwave and traditional oven. In this context, Millard reaction products

increases by increase of extraction temperature thus may increase non-enzymatic browning.

3.2.12.Total phenol content (TPC): data presented in Table 5 and Figure 7 revealed that the commercial date syrup sample has the highest T.P.C. (mg GAE/100 g) followed by sample extracted with instant pot and extracted by heat (100 ± 5 °C): 493.34, 395.43 & 291.14 respectively. While date syrup extracted using traditional oven recorded the lowest T.P.C content followed by sample extracted by microwave: 249.60 & 288.62 respectively. In a previous study carried out by Farahnaky et al. (2016), T.P.C content of date syrup determined in similar values (T.P.C. of date syrup: 453.04 (mg GAE/100g sample). Statistical analysis revealed that there were a strong relationship between Color (non- enzymatic browning and T.P.C. (Pearson correlation = 0.89 at p-value = 0.043).

3.2.13. Antioxidant activity % (AO %): as presented in Table 5, the statistical analysis proved that there were no significant difference ($p \geq 0.05$) between different extraction methods and commercial sample in antioxidant activity (AO %). Thus the extraction methods did not significantly affect the antioxidant activity (Fig.6). IC_{50} of phenolic extract of date syrup was generally calculated (IC_{50}= 540 ug/ml). A lower IC_{50}indicates better radical scavenging as of date syrup as Farahnaky et al. (2016) clarified.

3.3. Effect of different extraction methods on microbial properties of date syrup

Microbial analysis of date syrup was examined and tabulated in Table 6. Regardless total bacterial count, the commercial date syrup sample showed the highest total bacterial count, followed by sample extracted using traditional oven (2.08 x 10^3 &1.62 x 10^3cfu/ ml respectively). While date syrup extracted using instant pot contained the lowest bacterial count (1.16 x 10^3cfu / ml) as shown in Figure.8. That refers to the pressure and higher temperature conditions of instant pot method that reduces the microbial growth in date syrup sample to minimum level. It can be concluded that instant pot methods produced the most sanitized date syrup sample with minimum level of bacterial count.

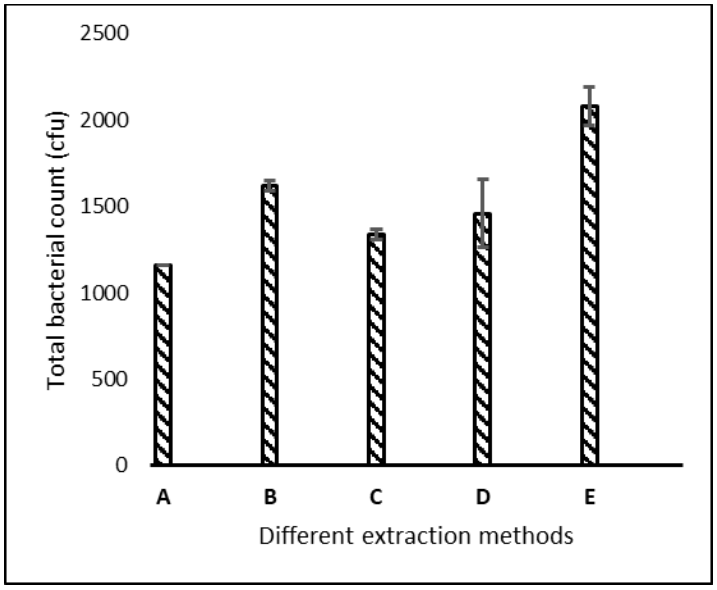


Figure 8. Effect of different extraction methods on total bacterial count (CFU) of date syrup
Where, A: instant pot (Electrical pressure cooker), B: Traditional oven, C: hotplate method, D: Microwave method, and E: commercial date syrup sample.

Table 6. Effect of different extraction methods on total microbial count of processed date syrup compared with commercial sample

Extraction methods	Total bacterial count in date syrup (CFU x10^3/ ml)	Total Fungal count (CFU/ ml)
Instant pot	1.16	Nil
Traditional Oven	1.62	5x10
Hot plate	1.34	Nil
Microwave	1.46	Nil
Commercial sample	2.08	Nil

CFU: Colony forming unit (cell/ml) of Siwi dibs.
Data were statistically analyzed using Minitab (18) program.
Instant pot: Electrical pressure cooker.

Total mold & yeast counts: Generally the elevated of sugar concentration in date syrup (68 - 75 %) is considered a preservative agent and inhibit the yeast & mold growth.

So the different extracted date syrup sample did not contain fungal growth.

Except for the samples extracted using traditional oven (Table 6). This may be due to the sample contained air bubbles (where aeration conditions increase fungal growth).

3.4. Effect of different extraction methods on sensory evaluation of date syrup

Sensory evaluation of different samples of date dibs were evaluated and the obtained results were tabulated in Table 7.

The data ascertained that the highest color scores were recorded for commercial sample followed by instant (5.00 & 4.00 respectively), while the lowest score for oven extracted (2.33) as shown in Figure 9.

For purity, the most pure sample was instant pot sample followed by commercial (4.67 & 4.33 respectively).

While the lowest purity was recorded by oven- extracted sample (Table7 & Fig.9).

Regarding with viscosity, commercial sample showed the highest viscosity followed by microwave method, however instant pot showed the lowest (Figure 9).

Concerning with sweetness, the commercial sample pertained the highest sweet taste, but the other samples were not significantly different (Table 7). As for consistency: Hot plat sample showed the highest consistency taste (Figure 9).

Although extraction method affected the odor intensity of date syrup slightly, these difference were non-significant ($p \geq 0.05$), commercial sample characterized with burned sugar odor (Table 7).

Finally, the panelists were asked to assess the overall acceptability of date syrup using (9.point hedonic scale), where hot plate sample achieved the highest score (7± 1) followed by oven (7 ± 0.58).

While both of microwave and instant pot methods recorded the same score (6.00± 0.58). However, commercial sample showed the lowest (5± 1.15) as clarified in Table7.

Table 7. Effect of different extraction methods on sensory properties of date syrup

Extraction methods	Appearance			Taste			Odor			Overall acceptability
	Color (1-5)	Purity (1-5)	Flowing rate (1-5)	Sweetness (1-5)	Consistency (Softness; 1-3)	Sugar crystal	Good smell	Burned sugar smell	Stran -ge smell	
Instant pot	4.00 ^{a} ± 0.00	4.67 ^{a} ± 0.58	1.00 ^{c} ± 0.00	3.00 ^{b} ± 0.00	2.00 ^{b} ± 0.00	No	3.67 ^{a} ± 1.15	No	No	6.00 ^{ab} ± 0.58
Oven	2.33 ^{b} ± 0.58	2.33 ^{b} ± 0.58	2.33 ^{bc} ± 1.15	3.00 ^{b} ± 0.00	2.00 ^{b} ± 0.00	No	3.33 ^{a} ± 0.58	No	No	7.00 ^{ab} ± 0.58
Hot plate	2.67 ^{b} ± 0.58	3.33 ^{ab} ± 1.15	2.33 ^{bc} ± 1.15	3.00 ^{b} ± 0.00	3.00 ^{a} ± 0.00	No	3.00 ^{a} ± 1.00	No	No	7.00 ^{a} ± 1.00
Microwave	2.67 ^{b} ± 0.58	3.67 ^{ab} ± 0.58	3.67 ^{ab} ± 1.15	3.00 ^{b} ± 0.00	2.00 ^{b} ± 0.00	No	2.00 ^{a} ± 1.00	No	No	6.00^{ab} ± 0.58
Commercial	5.00 ^{a} ± 0.00	4.33 ^{a} ± 0.58	5.00 ^{a} ± 0.00	5.00 ^{a} ± 0.00	1.00 ^{c} ± 0.00	No	3.67 ^{a} ± 1.53	Yes	No	5.00 ^{b} ± 1.15

Means (±SD) followed by different superscripts within each column are significantly different (p≤0.05). Data were statistically analyzed using Minitab (18) program

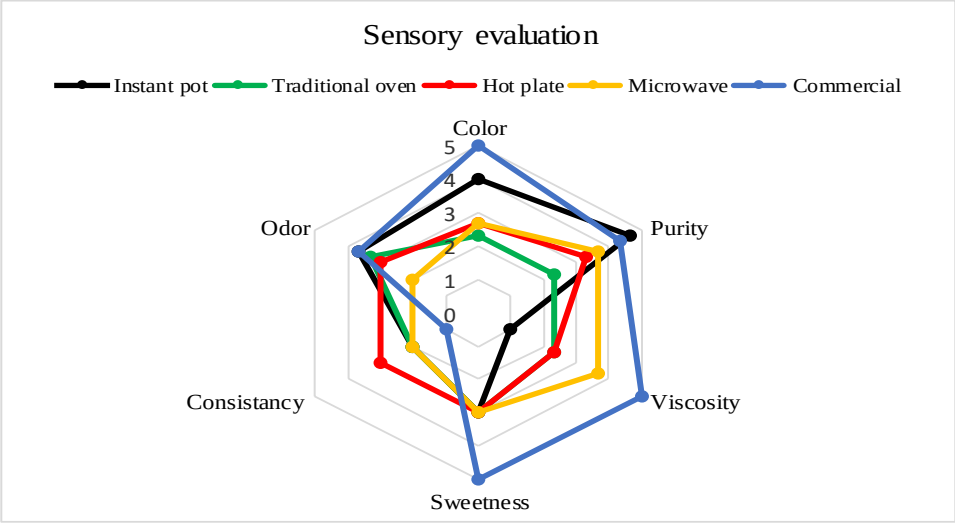


Figure 9. Spider web clarifying sensory attributes of date syrup extracted by different methods

4. Conclusions

From the obtained results, it can be concluded that among lab-processed date syrup, microwave extraction method presented the best results concerning with safety of the product: the lowest level of toxic hazard (5-HMF) and high quality characteristics including: the lowest level of fructose, the highest reducing sugar content, pH and titriable acidity %. Hot plate method has shown the highest score of overall acceptability of Panel test. Meanwhile, instant pot extraction methods revealed moderate level of HMF, density, specific weight, turbidity, color and total phenolic compounds.

Furthermore, instant pot extraction method achieved the lowest bacterial count due to temperature & pressure conditions (Most sanitized date syrup sample).

On the other hand, traditional oven extraction method showed good results regarding with low HMF level, the highest EC, Total ash %, sulphated ash %. However, this method showed the highest fungal count. Regarding with other quality characteristics, it revealed the highest score of TSS %, density, specific weight, color and total phenolic compounds. It also recorded the lowest score in turbidity, EC, total ash %, sulphated ash %. So, microwave extraction method is recommended method for extraction of date syrup with minimal level of HMF and with higher quality characteristics.

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PREBIOTICS AND PROBIOTICS: A FOCUSED REVIEW OF APPLICATIONS IN RESPIRATORY DISORDERS

Md Sadique Hussain[1], Arun Sharma[2], Rajesh Kumar[2]✉

[1]School of Pharmaceutical Sciences, Jaipur National University, Jagatpura, Jaipur, Rajasthan, India.
[2]School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India.
✉rajksach09@gmail.com

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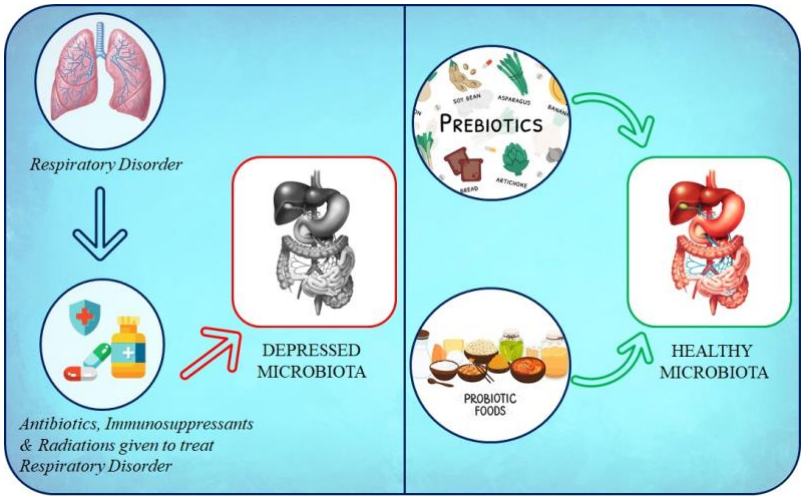
ABSTRACT

The principal function of food is to provide sufficient nutrients to achieve healthy diets and give a sense of fulfillment and health to people. The prevalence, seriousness, predicted patterns and economic effects of chronic respiratory conditions such as asthma, COPD, COVID-19, and other such diseases pose a serious public health challenge. The use of, among many other therapies, antibiotics, immunosuppressants, and radiation can induce alterations and influence the gastrointestinal biome. Therefore, it would be a very enticing choice to re-establish microbial balance and avoid disease if favorable microorganisms are introduced in the GIT. Probiotic and prebiotic ingredients have been the focus of substantial studies in recent decades in human nutrition with therapeutic potentials. The number of studies on possible health advantages that come via the use of probiotics and prebiotics has improved dramatically in the last few years. The concept of probiotic products has emerged from a live active culture that enhances the balance of the intestinal microbiota composition and the immunomodulatory capacity of clearly specified strains, to specific results. Prebiotics are short-chain carbohydrates that beneficially alter the composition or metabolism of intestinal microbiota. Therefore, prebiotics is supposed to improve wellness like probiotics but at the same time are economic, less toxic, and easier to introduce into the diet than probiotics. These are used to prevent and cure different medical problems and to encourage general well-being.

GRAPHICAL ABSTRACT



1.Introduction

Health statements about living microbes particularly lactic acid bacteria in food have a rich history. In an old-tape Persian version, it says that "Abraham owed the consumption of sour milk to his longevity." In 76 BC, the Roman historian Plinius suggested that gastroenteritis be treated with fermented dairy products (Schrezenmeir & De Vrese, 2001). Epidemiological studies suggest that food has an important effect on human wellness: diets with low fat and high fruit and vegetables have been associated with a reduced prevalence of certain ailments, including cardiovascular disease (CVD) and colorectal carcinoma. Such a diet involves not only components readily ingested in the small intestine but also digestion-free ingredients by the pancreas and small intestine enzymes (Blaut, 2002). It is only in the last 40 years that diets have played an important role in emerging illnesses such as CVDs and tumors. In this sense, among the most metabolically active parts of the human body is the colon which has a rather diverse microbial environment, which is not only a barrier to infection but also effectively contributes to the energy conservation from diets which cannot be influenced by enzymes of the human body (Kolida & Gibson, 2011).

The major cause of mortality and morbidity is airway diseases which affect the lives of more than a billion individuals worldwide. Chronic respiratory diseases (CRDs) are a broad type of major disease that is associated with the anatomy of the respiratory system. CRDs are regarded as a primary cause of accidental mortality in the world's population (KUMARI et al., 2020). Over time, the morbidity and mortality of CRDs are growing for infants and young children who are highly sensitive (Burney et al., 2015). Millions are dying owing to the inadequacy of healthcare without accessibility to immunizations (Ferkol & Schraufnagel, 2014). The responsibility of CRD and diagnosis messes up the frameworks of patients' daily lives and reduces activities (Dobler, 2019). CRDs include chronic obstructive pulmonary disorder (COPD), asthma, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), carcinoma of the lung, etc. COPD and asthma are the most prevalent of all these disorders with a very high degree of incidence and death. COPD is one of the leading international and Indian non-communicable causes of death (Salvi et al., 2018). More than 4 million deaths per year and 4% of the global prevalence of infectious diseases are CRDs (Wang et al., 2016). CRD risk factors are frequent and widespread: at least 2 billion people are vulnerable to the harmful effects of the use of biomass fuels, 1 billion to outdoor air emissions, and 1 billion smokers are exposed to the detrimental effects of second-hand smoking in a quasi-equivalent proportion in the population. Although CRDs are not curable, different types of treatment can help manage symptoms, improve the quality of life of patients and prevent adverse effects that are associated with severe morbidity, impairment, and risk of death (Soriano et al., 2020).

The use of, among other forms of treatment, antibiotics, immunosuppressive therapy, and radiation will induce alterations in the flora and composition. Thus, it may be very tempting to preserve microbial balance and avoid disease by adding beneficial bacterial organisms into the GI tract (Gupta & Garg, 2009). Probiotics have been identified in different ways according to our understanding of their pathways for effect on human well-being. Lilly and Stillwell coined the term probiotic to describe the substances developed by one microorganism which stimulate another's development (Salminen et al., 1999). These are live microbes that support the wellbeing of the individual if given in sufficient quantity. Many bacteria are used for a variety of applications in clinical practice. The most widespread and extensively studied species are *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* (Benjamin Kligler & Andreas Cohrssen, 2008).

First, a prebiotic was described as a "non-digestible food component that has a positive effect on the host by selectively stimulating bacterial growth and/or operation in the colon and thereby improving the health of the

host (Ringø et al., 2010; Roberfroid, 2007). Some recognized prebiotics are low-digestible carbohydrates and have impaired gastrointestinal (GI) resistance, particularly in large amounts when consumed while other prebiotic fibres (e.g., wheat dextrin, polydextrose) show high GI resilience (Slavin, 2013). Though there is many prebiotics available in the world market, not all of these have been extensively studied, and thus, the scope for discovering new prebiotics is very much intrinsic. These can possess desirable attributes that are not present in the current generation (Rastall & Maitin, 2002).

Synbiotics have both probiotic and prebiotic features and have been developed to address the potential survival problems in the GI tract. A supreme impact can therefore, in contrast to the behavior of probiotic or prebiotic alone, be assured by the optimum combination of both components in one product (Markowiak & Ślizewska, 2017). Different health benefits of both pro- and prebiotics are shown in figure 1.

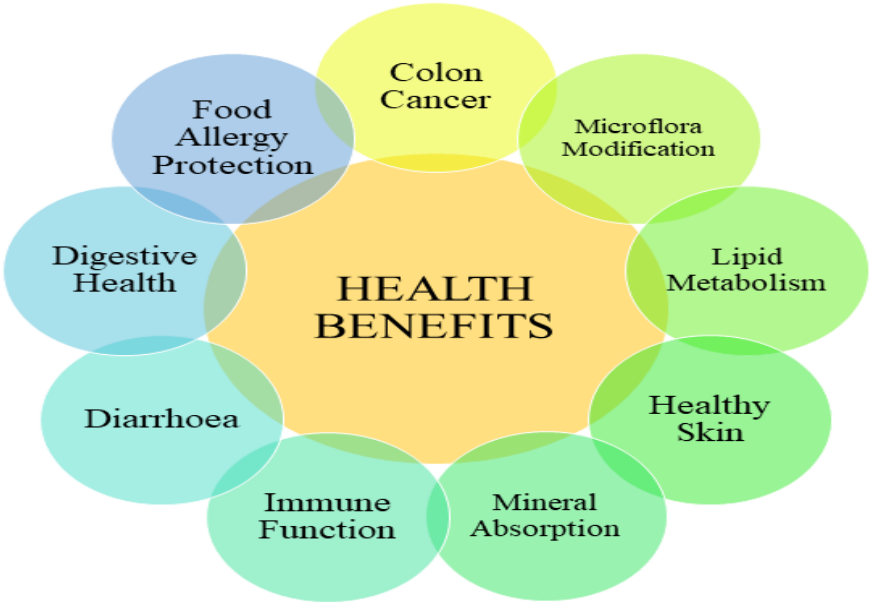


Figure 1. The health benefits of probiotics and prebiotics

2. Probiotics

In 1989, R Fuller popularised the term probiotics, which was defined as 'living micro-organisms that exert health benefits beyond general nutrition when ingested in certain numbers (Arthur C Ouwehand et al., 2002). These bacteria, normally non-motile and of different shapes, are fermentative anaerobic microbes. They generate lactic acid typically. Their biological characteristics allow them to predominate and prevail in the human digestive tract over potentially pathogenic microorganisms. It is currently speculated that these organisms produce low molecular metabolic by-products, including short-chain fatty acids such as butyrate, which have a favorable regulatory influence on host metabolic processes. Sometimes, these metabolic by-products are called "postbiotics" and can biologically function as immune modulators (Dan W Thomas & Frank R Greer, 2010). The terminology is drawn from early experiments that examine the influence on the overall structure of the human intestinal microbiota of some yogurt bacteria. Probiotics were first used for intestinal microbiota modification to affect both human and animal health. The basic ingredients of live microbial foods and their health impacts are currently researched in food matrices and as individual or mixed-cropped preparations (Isolauri et al., 2004).

Analysis and market interest in probiotics have risen dramatically in recent years. Increasing scientific data confirms certain of its medical benefits, notably when treating certain diarrheal disorders, associated with the use of probiotics. Yeast or bacteria consist of probiotics regulated as nutritional supplements and foods. They are sold as pills, tablets, packages, or powders, most commonly in yogurt or milk beverages. A single microorganism or a combination of several species can be included in probiotic products (Williams, 2010).

The rising numbers of modern illnesses, such as malignancies, atherosclerosis, heart attacks, high blood pressure, and HIV infection, have stimulated attention to probiotics. A multitude of beneficial effects have been documented in the probiotic intake, including improved immune reactions, controlled colonic microbiota effects, vaccine adjuvant effects, decreased fecal enzymes involved in initiating cancer, travel-related diarrhoea treatment and anti-biotic medication, rotavirus regulation, and the Clostridium difficile colitis and ulcer prevention associated with Helicobacter pylori (Kaur et al., 2002). The processes underlying the use of probiotics to exercise biological effects remain unclear, but the unidentified factors such as resistance to colonization and competition exclusion sometimes explain their method of action (Soccol et al., 2010). Table 1 enlists the known microbial species which are used as probiotics.

Table 1. List of micro-organisms used as Probiotics (Chow, 2002)

***Bifidobacterium* species**	***Lactobacillus* species**	***Saccharomyces* species**	***Streptococcus* species**	**Other species**
B. adolescentis *B. bifidum* *B. breve* *B. infantis* *B. lactis* *B. longum*	*L. acidophilus* *L. bulgaricus* *L. casei* *L. fermentum* *L. gasseri* *L. johnsonii*	*S. boulardii*	*S. thermophilus* *S. salivarius subsp. thermophilus*	*Bacillus cereus* *Bacillus subtilis* *Escherichia coli* *Enterococcus* *Propionibacterium freudenreichii*

3. Prebiotics

In 1995, Gibson and Roberfoid presented the idea of prebiotics as an effective solution to gut microbiota modulation (Charalampopoulos & Rastall, 2012). The FAO/WHO describes prebiotics as an unsustainable food element, which provides the host with health care benefits connected with microbiota modulation. Prebiotics are a community of complex, unidentified carbohydrate ingredients based on their source, fermentation characteristics, and dose about health benefits. Prebiotic sources include breast milk, soy, inulin, raw oats, unrefined wheat, unrefined barley, non-digestible carbs especially non-digestible oligosaccharides (Pandey et al., 2015). Currently, all the prebiotics are short-chain carbohydrates with a polymerization of between 2 and 60, which are known to be non-digestible with human or animal digestive enzymes (Cummings J.H.* & Macfarlane, 2002). The importance of prebiotics is due to:

a. the growing belief that a stable or healthy intestinal microbiota exists
b. the indication that the microbiota makeup may be altered by prebiotics toward a healthier profile
c. An alternate to probiotics difficult to control in certain foodstuffs, but whose health benefits are increasingly well known as regards the prevention of diarrhoea and immunomodulation

d. Since currently used prebiotics, particularly inulin and galactooligosaccharides (GOS) are fairly inexpensive to produce and collect from crops and have beneficial effects on the gut microbiota and host, these are also useful functional components in foods with the potential to enhance organoleptic propagation on fat and dairy products (Macfarlane et al., 2006).

When taken in comparatively small quantities (5-20 g/day) of the inulin, fructooligosaccharides (FOS), trans-GOSs, and lactulose, the development of organisms (responsible for health promotion) belonging to the genera Bifidobacterium and lactobacillus in human beings was clearly shown in the studies (Gibson et al., 2004).

Prebiotics travel into the small intestine and become available without the need for other intestinal bacteria for probiotic bacteria. The frequently used prebiotics in human diets is lactulose, GOS, FOS, inulin and hydrolysates, malto-oligosaccharides, and resistant starch. A prebiotic for one or a small quantity of probiotics is the selective substratum. Probiotics are allowed to develop and produce short prebiotic chain fatty acids. The Prebiotic will then shift the host's colonic microbiota to a healthy condition (Al-Sheraji et al., 2013). Table 2 details the various types and sources of prebiotics.

Table 2. Types and sources of prebiotics (Al-Sheraji et al., 2013).

Type	**Sources**
Arabinoxylo oligosaccharides	Wheat bran
Cyclodextrins	Water-soluble glucans
Fructo oligosaccharides	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, and rye
Galacto oligosaccharides	Human’s milk and cow’s milk
Isomaltulose	Sucrose
Isomalto oligosaccharides	Starch
Soybean oligosaccharide	Soybean

4. Common Diseases

a. Asthma

Asthma is one of the most prominent non-communicable disorders which has a serious influence on the living quality of many people. About 300 million people worldwide have asthma, and another 100 million are expected to be affected by 2025 (Dharmage et al., 2019). Asthma is a chronic inflammatory disorder of the respiratory tract in which many of the cells in the adaptive and innate immune systems work alongside the cells of the epithelium to create bronchial hyperreactivity (BHR), excess production of mucus, remodeling of the respiratory wall, and narrowing of the respiratory tract. This contributes to repeated breathing problems, wheezing, and tightness of the chest in vulnerable patients (Lambrecht & Hammad, 2015). These symptoms temporarily fluctuate and may intensify during times of exacerbation resulting in respiratory failure (Lambrecht et al., 2019). The most critical symptom in the detection of asthma is wheezing (Ferrante & La Grutta, 2018), however, there may be separate cases for the relative severity, type of inflammatory cell, and location of

inflammatory infiltrate. A significant number of cells participate in the immune and inflammatory reactions to asthma allergens including T-cells, eosinophils, mast, and neutrophils (Hamid & Tulic, 2009). Effective management and care of asthmatic patients can eliminate the mortality of the disease, while one in 250 attributes to the global mortality of asthma (P. Kumar & Ram, 2017). Amid the progress made in asthma care throughout the past few decades, changes in patient preparation, the use of innovative medical methods, and personalized support services have yet to be completed.

b. Chronic Pulmonary obstructive disease (COPD)

Because of its higher incidence and resulting impairment and death, COPD is a major public health problem. Worldwide, the third leading cause of death is COPD; 3.2 million deaths were reported in 2017, and a total of 4.4 million per year is expected by 2040 (Bartolomé R. Celli & Wedzicha, 2019). The disease has been generally recognized as an illness caused by cigarette smoke. The classic idea was to develop an irregular inflammatory reaction in vulnerable people to destroy the airways and alveoli (emphysema), speed up the physiologic drop in lung capacity with age, and reduce breathing limitation and CR symptoms (Agustí & Hogg, 2019). Patients with COPD have a distinct inflammatory pattern- the more usually type 1 immunity and type 3 immunity-predominantly macrophages and neutrophils with elevated CD8+ cytotoxic T cell levels, CD4+ TH1 cell, TH17 cell, ILC3, and B cells, and are grouped in peripheral airways with T cells in local lymphatic follicles (Barnes, 2018).

c. Covid-19

A current coronavirus (CoV), known as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has developed an on-going global threat, reported by international health agencies (Malik et al., 2020; Mohit & Hussain, 2021). The sudden onset and rapid spread of the infection have led to an outbreak. The root of the virus is suspected in numerous animals eaten as food in China, but not been proven. The initial infection research revealed that there is a correlation between both the local and wild animal markets in China with most of the early infections. Subsequently, new infections are often transmitted through transmission from human to human (Chhikara et al., 2020). However, research suggests that asymptomatic patients also can spread the virus through droplets in the air produced while sneezing or coughing (Agarwal et al., 2020). About 26-32 kilobases in size, the most commonly recognized RNA virus, are enveloped by CoV of the Coronaviridae family (CoVs) with a single strand, RNA-positive sense genome. The term 'coronavirus' refers to the presence of the CoV virus when viewed under an electron microscope, which gives the appearance of a crown or a corona in Latin to spike projections from the virus membrane (Su et al., 2016). COVID-19 covers a wide variety of symptoms, from high respiratory problems to dangerous pneumonia associated with acute respiratory distress syndrome (ARDS). Fever, weakness, dry cough, myalgia, and dyspnea are the most common symptoms displayed. Patients with headache, hemoptysis, diarrhea, or pleuritic of the chest are less often seen. At present, in the case of upper and lower respiratory track specimens, a reference procedure for diagnosing COVID-19 is a real-time reverse transcriptase polymerase chain reaction (RT-PCR) (Jajodia et al., 2020). Globally, the CoV incubation period is 3-7 days. About 80% of infectious cases persist as mild or asymptomatic, 15% are severe, and 5% are critical, with a requirement for ventilation (Hussain et al., 2021).

d. Lung Cancer

Lung carcinoma has evolved in the last century, from a rare, dim disease to the world's most advanced carcinoma and the most widespread cause of tumor mortality. The identified lung cancer risk factors include behavioral, environmental, and genetic factors, all of which have a role in the growth of the disease and also influence the response

capability of particular patients (de Groot et al., 2018). The second-most prominent gender-based carcinoma diagnosis behind prostate cancer in men and women's breast cancer is lung carcinoma. In 2018, lung carcinoma accounted for 14 percent of new male and 13 percent of new female cancers in the United States (Siegel et al., 2018). Per year, lung cancer affects 1.8 million people and the illness causes 1.6 million deaths. Five-year survival of lung cancer populations ranges from 4% to 17%, based on stage and geographic variations (Hirsch et al., 2017). The risk of developing lung carcinoma in smokers is 20-30 times significantly greater than in those who do not smoke. Lung carcinoma rates are rising parallel to tobacco use in developing nations. Future preventive measures and research can focus on and clarify state-of-the-art exposures including non-cigarette products to modifiable non-tobacco factors. The 2004-2008 data for Surveillance, Epidemiology, and End Result (SEER) indicated that lung carcinoma has been detected at a median age of 71 years (S & Hussain MS, 2021).

5. Mechanisms of Action of Probiotics in Various Diseases

a. Immunomodulation

Various studies conducted on animals and humans reported findings that variant strains of lactic acid bacteria (LAB) can induce and manage natural and adaptive immune responses. Bifidobacteria and lactobacilli strains were found to have distinctive capabilities to adjust and regulate immune responses (H. Gill & Prasad, 2008). The mechanism of action of probiotics is not completely known. The immune reaction towards the probiotics depends upon the variant and distinction because of the availability of disparate presence of protein/carbohydrate in the cellular walls (Mortaz et al., 2013). The advantageous effectiveness of probiotics is partially a consequence of the capability of probiotics, to modulate the formation of anti- and proinflammatory cytokines as well as the equilibrium between kinds of T-cell responses like T-helper 1 (Th1), Th2, and, Th17 (Ghadimi et al., 2008; Helwig et al., 2006; Hosono et al., 1986). Cytokines are one of the greatest mediators showing their role in inflammation and immunity responses. Cytokines mediate the beginning, sustenance as well as resolution of the natural and adaptive immune response. Various studies have shown that probiotics increase levels of interferon-γ (IFN-γ), IFN-α and interleukin-2 (IL-2) in individuals administered with probiotics (Halpern et al., 1991; Kishi et al., 1996; Solis Pereyra & Lemonnier, 1993; Wheeler et al., 1997). Regular intake of yogurt also leads to the elevated formation of IL-1β, IL-6, IL-10, tumor necrosis factor (TNF-α), and IFN-γ (Aattouri & Lemonnier, 1997; Halpern et al., 1991; Miettinen et al., 1996; Solis Pereyra & Lemonnier, 1993). This stimulation of cytokines and interferons in epithelial and dendritic cells (DCs) acts as a key way to tackle viral infections by removing viruses through the mediation via cell-to-cell and also the adaptive immunity (Lehtoranta et al., 2014). As per a randomized control trial, when the various probiotic strains were given to serious sepsis-suffering children, it was reported that proinflammatory cytokines such as IL6, and TNF-α were diminished and anti-inflammatory cytokines such as IL-10 levels were elevated in comparison to those who received placebo (Suresh K. Angurana et al., 2018). LAB when given by oral route not only adjusts and regulates the cytokines in the intestinal region but also at the systemic level (Noverr & Huffnagle, 2005). Immunity responses are elevated by various LAB variant strains to constitute the proliferation of T-lymphocyte and antitumor capability of natural killer cells (NK) as well as the phagocytic activity of mononuclear cells (Harata et al., 2009). Phagocytic cells are efficacious in removing pathogenic microbes and NK cells are important for safe guarding against cancer cells as well as different viruses. Several studies have revealed the capability of probiotics to enhance the phagocytic actions of leucocytes (Harsharnjit S. Gill, 2003). As per a recent hypothesis, probiotic bacteria such as LAB can

interact with Gut-associated lymphoid tissue which is present in payer's patches in the gut and can increase respiratory immunity and probiotics need not be directly given in the airway for airway-related diseases (Izumo et al., 2010). For natural immune receptors for instance toll-like receptors (TLRs) which are generally executed on epithelial cells as well as immune cells of mucosa, probiotics generally act as ligands to affect different signal paths consisting of nuclear transcription factor nuclear factor-kappa B (NF-kB) as well as peroxisome proliferator-activated receptor-γ (PPAR-γ) (Bermudez-Brito et al., 2012; Thomas & Versalovic, 2010). According to a study conducted on new born rats, it was shown that L. reuteri DSM 17938 considerably elevated the survival and lessen the occurrence and seriousness of experimental Necrotizing enterocolitis (NEC) in rat intestine by restriction of TLR4 and NF-kB signal path. These actions of probiotics lead to declined formation of TNF-α and IL-1β (Y. Liu et al., 2012). Increment in the NK activity and escalation in overall percentage of NK cells by routine taking of probiotic food like yogurt and curd was showed in various studies (Chiang et al., 2000; H. S. Gill et al., 2001; Olivares et al., 2006; Sheih et al., 2001). Cell interceded and antibody intervened responses are involved in adaptive immune responses and it is quite particular in action and has memory. Intake of some particular probiotics reported to increase antibody reactions and also local and systemic immunizations (Fukushima et al., 1998; Isolauri et al., 1995; Kaila et al., 1992; Link-Amster et al., 1994; Majamaa et al., 1995). According to a randomized study conducted by Kaila *et al*., greater levels of particular serum and mucosa antibody reactions in children infected with rotavirus administered with L. rhamnosus GG fermented milk were observed when compared to children administered with only placebo (Kaila et al., 1992). In those cases, with salmonella vaccination, where probiotics such as B. bifidum were administered, considerably greater levels of particular serum immunoglobulin A (IgA) and IgA secreting cell response were reported (Fang et al., 2000; Link-Amster et al., 1994).

b. Antiviral/Antimicrobial Effects of Probiotics

Primarily viruses bind to the host cell and then the disease progresses; thus, if this step is interrupted, it may lead to a decline in disease progression and can be advantageous to the host. Probiotics may precisely bind to viruses and restrict them from binding with host cells. For example, particular variants of LAB have been found to inhibit the attachment of flu-like stomatitis virus in in-vitro conditions (Botić et al., 2007). Anti-microbial activities are reported to be shown by probiotics by forming various compounds such as bacteriocins, hydrogen peroxide, and various organic acids. In a study, metabolic intermediates of bacteria in the yogurt exhibited antiviral action against duplication of the influenza virus (Choi et al., 2009). In the case of the influenza virus, probiotics were found to modulate the immune responses and help in viral elimination as well as advantageous effects on inflammation caused by lung damage (Zelaya et al., 2016). In an in-vitro study conducted by Ang *et al*., comprising colon cells and skeletal muscle of humans, it was reported that L. reuteri exhibited considerable dose-dependable anti-viral action against enterovirus seventy-one strain and coxsackie virus type A (CA) six and sixteen strain (Ang et al., 2016). Commonly viruses may lead to upper respiratory tract infections. A Cochrane review of 12 randomized controlled trials found that in comparison to a placebo, probiotics were better at reducing acute upper respiratory tract infection (Hao et al., 2015). The flowchart (fig. 2) given below represents the mechanism of action of probiotics.

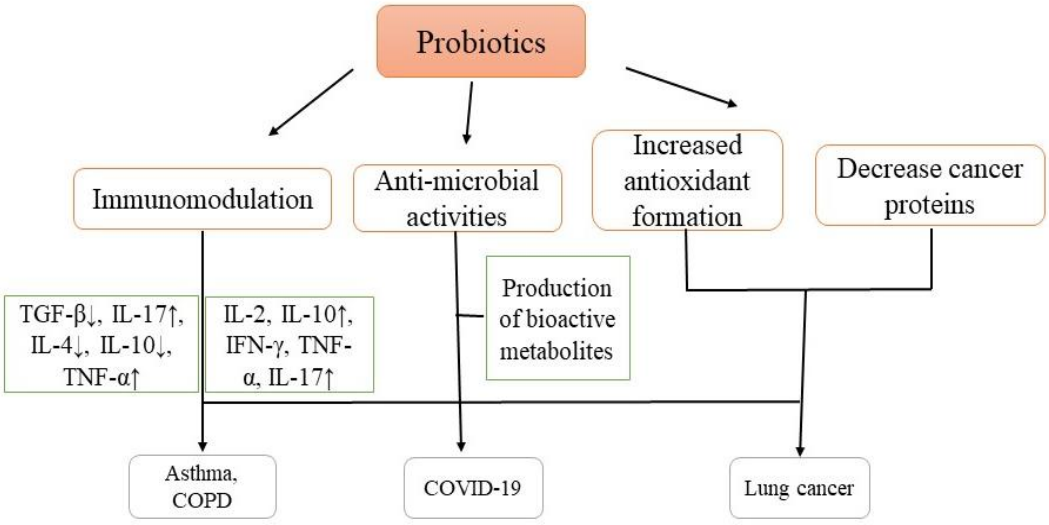


Figure 2. Mechanism of action of probiotics in respiratory disorders

c. Production of Bioactive Metabolites

Several bacteria present in the gut or particular probiotics have been shown to form various bioactive metabolites such as histamine, rheuterin, and butyrate with anti-inflammatory effects. L. reuteri variants obtained from humans form reuterin which has wide-spectrum antimicrobial activity against various intestinal microbes (Casas & Dobrogosz, 2000). Glycerol is broken down into smaller components by L. reuteri to form vitamin B12-reliant reuterin (Y. Liu et al., 2018). L. reuteri ATCC PTA6475 variant which is derived from humans, produces histamine which restrains inflammatory response, through activating type-2 receptor of histamine in intestinal parts of mammals, which leads to the restraining of inflammation of intestinal parts as well as colorectal tumorigenesis (Ganesh et al., 2018; Gao et al., 2017). Probiotics also can break down particular kinds of fibres to form short-chain fatty acids (SCFAs) like butyrate. SCFAs act as protection of host and have an important part in immune responses in addition to antioxidant action and activities against malignancy (Peng & Biswas, 2017). Butyrate intervenes inflammatory signal path to manage the formation of cytokines and also restricts the histamine deacetylase for adjusting the exhibition of several proinflammatory genes (Y. Liu et al., 2018).

6. Effects of Probiotics on Various Diseases

a. Asthma

The effectiveness of probiotics in the cure or prevention of asthma has been focused on by a few studies. A clinical trial was conducted on 1187 children by Giovannini *et al*. to study the efficacy of fermented milk consisting of L. casei on the occurrence of asthma and allergic rhinitis. It was observed that there was no distinction between the controlled and experiment groups in the case of asthma in 12 months of the trial (Giovannini et al., 2007). Another randomized controlled study of adults and juveniles reported that there was no betterment in signs in those who were administered with yogurt consisting of S. thermophilus and L. bulgaricus in the absence or presence of L. acidophilus and also there was no distinction in the inflammatory markings (Wheeler et al., 1997). In a double randomized, placebo-regulated study including toddlers with the possibility of allergy, it was reported that repetition of wheezing episodes was not reduced because of probiotic intake, and also it could not have any effect on the pervasiveness of asthma till two years of age

(Abrahamsson et al., 2007; Taylor et al., 2007). In another case, asthma-related signs were averted in toddlers with atopic dermatitis, and with the administration of probiotics, there was a considerable reduction in the expiration flow (Van Der Aa et al., 2011). In a mouse model, intake of probiotics via the oral route weakened the signs of allergic asthma, stimulated by the adjustment of immune responses by the regulatory T (Treg) mechanism, and reduced air passage hypersensitiveness (Jang et al., 2012). Several infections related to respiratory parts specifically viral infections precisely can cause comorbidities as well as can lead to fatality and asthma-like conditions also. It has been contemplated that if probiotics are recognized can prevent or manage virus infections, then in the initial years of life, asthma can be prevented (Holtzman et al., 2009; Yoo et al., 2007).

b. Chronic Pulmonary obstructive disease (COPD)

The most significant role is played by smoking as a lifestyle-associated factor in the development of COPD (Barnes, 2010). The seriousness of COPD in patients is related to the degree of inflammation in an air passage which is crucially associated with the pathogenesis of COPD in experimental conditions [100]-[102]. Characterizations such as escalated shortness of breath, increased phlegm, elevated inflammation, and a decrease in lung activity are seen in COPD (Bhowmik et al., 2000; Sapey & Stockley, 2006). The worsening of COPD symptoms is primarily because of viral infections in forty to sixty percent of cases (B. R. Celli & Barnes, 2007; Sapey & Stockley, 2006). In animal models, increased respiratory and enhanced airway apoptosis is caused by virus infection post-exposure to cigarette smoke (B. R. Celli & Barnes, 2007). Commonly it is acknowledged that, after virus infection, primary immunity response depends upon the detection of pathogen-associated molecular pattern molecules by TLRs such as TLR7 (Kang et al., 2008; Newman & Riley, 2007). These receptors are present in DCs and inflammation cells and induction of these causes stimulation of NK cells by forming IL-12, IL-15, etc. (Kawai & Akira, 2006; Lucas et al., 2007; Newman & Riley, 2007). In managing virus infections in primary phases, NK cell stimulation is crucial (Strowig et al., 2008). NK cells were considered just killer cells because of their capability to damage virus-affected cells (Ortaldo et al., 1991). Now consideration is also being given to the noncytotoxic effects of NK cells (Strowig et al., 2008). Stimulated NK cells form greater concentrations of IFN-γ (Schroder et al., 2004). IFN-γ stimulated by NK cells is crucial for the inflammation process that keeps virus infections in check (Orange et al., 1995; Scharton & Scott, 1993). NK cells and the mediators are thought to be crucial in COPD condition worsening symptoms. The use of cigarettes leads to hindered cytotoxic actions of NK cells and the generation of cytokines (Mian et al., 2008). Those individuals who do smoke have lesser activity of NK cells than those who do not smoke (Morimoto et al., 2005). Everyday routine administration of L. casei probiotics leads to enhanced NK cells (Naruszewicz et al., 2002). Thus, it is advised that probiotics can be beneficial in COPD-suffering individuals, especially those who have repetitive virus infections (Morimoto et al., 2005).

c. COVID-19

Lactobacillus and Bifidobacterium are probiotics that may act at various phases in the case of COVID-19 unlike antiviral drugs as well as drugs used to cope with inflammation which act in a few phases. Probiotics may play a role in the reclamation of the microbiome of the gut region, managing cytokine storm, averting other virus and fungus invasions as well as having antiviral activities (Suresh Kumar Angurana & Bansal, 2020). Such activities of probiotics can help in averting and/or improving the signs associated with COVID-19 and provides passive proof to use probiotics in the management of the novel coronavirus disease. Also, the probiotics are inexpensive, and effortlessly accessible and administration is not difficult in comparison to

drugs utilized in COVID-19 against viruses (Infusino et al., 2020). When food ferments, bioactive peptides are formed by probiotics, and those compounds can restrict angiotensin-converting enzyme (ACE) by hindering the active sites. The litter of dead probiotic cells plays antagonizing role for the ACE enzyme (Olaimat et al., 2020). Probiotics can impede the ACE receptor which plays a role in access of severe acute respiratory syndrome corona virus-2 (SARS-CoV-2) to host gastrointestinal cells. For this virus, no standard management regimes are available yet. The utilization of probiotics in managing COVID-19 has not been confirmed by any trials, but managing COVID-19 clinically can be an appropriate plan. Various trials are being conducted to access the efficacy of probiotics in curing COVID-19-suffering individuals (Infusino et al., 2020). Few individuals suffering from COVID-19 showed dysbacteriosis in the intestine represented by lesser probiotics such as lactobacillus etc., which suggests that those individuals may have feeble immunity thus, administration of probiotics can be beneficial to stabilize the imbalanced microflora and also in reducing the possibility of infection (Xu et al., 2020). Intake of probiotic foods such as fermented products can improve symptoms of COVID-19. According to research, the intake of fermented milk with probiotics may considerably decrease the occurrence of upper respiratory tract infections (Makino et al., 2010; Merenstein et al., 2010; Shida et al., 2017; Taipale et al., 2011). Because of the role of probiotics in several virus infections, it can be considered in COVID-19 management without concrete proof. It has been established with an escalation of age that there is a decline in gastrointestinal microflora and its variation also reduces. This reduction is a cause of various ailments in elderly people such as diseases linked to inflammation, obesity, etc. Individuals with imbalanced microbial flora and old aged people are more prone to be infected with COVID-19. Thus, in such cases, probiotics can act beneficial and help strengthen immunity by helping the intestinal microflora to modulate the immune responses (Olaimat et al., 2020).

d. Lung cancer

Around the world, the occurrence of cancer and death rates have elevated over the previous 10 years, and probiotics act in safeguarding against several cancers and it spellbound the science society. Various findings have shown the utilization of probiotics in preventing and managing distinct kinds of cancers (M. Kumar et al., 2010). The attainable multiple health-related effects of probiotics can be antimicrobial, antitumor, retarding the development of a tumor and enhancing natural and acquired immunity, precise restriction of food-originated microbes by competing as well as help in alleviating adverse effects of chemotherapy (P. C. Liu et al., 2017; R. M. Patel & Denning, 2013; Raman et al., 2013).

7. Direct Effect of Probiotics on Lung Cancer

According to a study, if the flora of the intestine is in equilibrium then it acts as a safeguard in managing malignancy (Iida et al., 2013). The use of probiotics in lung cancer is being considered nowadays. In research including 30 lung cancer-suffering individuals, it was examined whether gut microbiome got enhanced or not upon treating with chemotherapy along with probiotic supplementation. The group in which chemotherapy and probiotics were given in a combined way was reported to have enhanced gut microbiome and declined intestine indigestion whereas, in the case of another group with only chemotherapy, the individuals reported to have constipation and decline in lactobacillus and Bacteroides and disease-causing bacterial strains were elevated (Serkova et al., 2013). Another study was conducted in-vivo on the lewis lung cancer (LLC) bearing mice to elaborate utilization of probiotics. Lung malignant cells were in 3 different groups, and it was found that groups with cisplatin as well as cisplatin/antibiotic combinations such as vancomycin, ampicillin, and neomycin, found to have lesser continuity rate than the group with

cisplatin/probiotics (L. acidophilus). In this case (cisplatin/probiotics), the continuity rate of life was lengthier. Furthermore, the activity of probiotics on cancer-suppressing as well as oncogenes was also examined and it was found that the exhibition of oncogenes declined and the exhibition of cancer-suppressing genes was diminished (Gui et al., 2015). In another study on tumor cell lines such as lung carcinoma cell lines (SK-MES-1), breast adenocarcinoma (AGS), and colon carcinoma (HT-29), the actions of strain Lactococcus lactis KC24 were observed. Rapid multiplication of SK-MES-1, AGS, and HT-29 was restricted by 86.53 %, 90.12 %, and 68.30 % sequentially (Lee et al., 2015). A study on probiotic L. lactis NK34 showed that the strain showed anti-cancer activity as well as anti-inflammation properties against different carcinoma cell lines such as SK-MES-1, AGS, etc. As a consequence of managing with L. lactis, it led to firm inhibition of cell rapid multiplication; in the case of SK-MES-1, it was 96.71 % and for AGS it was 82.07 %. Because of its anti-inflammatory activity, L. lactis NK34 was found to have decreased proinflammation cytokines (Han et al., 2015). In a study conducted to evaluate the effectiveness of a vaccine with probiotics against cancer solid sarcoma 37 (S37) and lewis lung carcinoma, it was found that consolidation of vaccine and probiotic strains mixture of *Enterococcus faecium* and *Saccharomyces cerevisiae* 14 K led to a combined enhanced effect of vaccine and probiotics in treating the S37 in mice and lewis lung carcinoma. The consolidated effect was turned up by 2-2.5 in comparison to the vaccine alone (Tanasienko et al., 2005). In another study, in which fermented milk with L. casei CRL 431 was given to BALB/c mice and it was found that there was a restriction of cancer development, and reduced lung metastasis (Aragón et al., 2015). According to a study conducted to evaluate the effect of probiotic-containing fermented milk products on lung metastasis, fermented milk products showed, toxicity toward 4T1 breast tumor cells (Zamberi et al., 2016).

8. Mechanism of Action of Prebiotics

The assumed mode of action of prebiotics can be in distinct ways, such as direct and indirect approaches. In the case of an indirect approach, sustenance is provided to gut microflora by prebiotics which leads to natural development, thus, leading to health advantages. In direct mode, there can be precise restriction of various disease-causing bacteria, prohibition of malignancy, etc. (Al-Sheraji et al., 2013; S. Patel & Goyal, 2012).

9. Therapeutic Effects of Prebiotics

a. Effects against pathogens

Probable utilization of prebiotics in different animal studies has been shown, concerning gastric infections. Against several diseases causing bacteria such as *Escherichia coli* by the usage of different modes including the formation of restrictive factors such as bacteriocin, SCFA and elimination by competing, etc. (Emanuel Vamanu & Adrian Vamanu, 2010; Licht et al., 2012). Actions of prebiotics such as inulin, dahlia, raffinose, and lactulose on the formation of bacteriocins have been studied from L. paracasei CMGB16 variant. It was reported that on supplementing the medium with inulin, raffinose, and lactulose, there was a considerable elevation in the actions of bacteriocin (Emanuel Vamanu & Adrian Vamanu, 2010). Bacteriocin formation by Pediococcus acidilactici LAB 5 is effected positively by prebiotic sorbitol (Mandal et al., 2009). In prohibiting pathogens, SCFA has a key role in decreasing the pH of the gastrointestinal tract. Lowered pH leads to a decrease in the decomposition of peptides (Mohanty et al., 2018). Colonic crypt cells are induced by SCFA, which reduces the possibility of intestinal mutation and helps in elevating the biomass by escalating protein formation (Cavaglieri et al., 2003; Coles et al., 2005; Fooks & Gibson, 2006).

b. Activities against Cancer

Prebiotics play the guarding role against cancer-causing substances in case of colon cancer. Propionate is SCFA which has

properties against inflammation in colon cancer cells. Galactooligosaccharide (GOS) fermentation forms butyrate which manages apoptosis and decreases the metastasis in colon cancer cells. It improves the exhibition of enzymes that causes detoxification leading to safeguarding from cancer-causing compounds (Nurmi et al., 2005; Pool-Zobel, 2005; Pool-Zobel & Sauer, 2007). Lists of patents in prebiotics and probiotics are shown in Table 3 (Dixit et al., 2016). Table 4 lists the Commercially available probiotics and prebiotics and their information regarding the manufacturer, source, and origin (Douglas & Sanders, 2008; Mishra et al., 2018).

Table 3. Patents involved with different probiotics/prebiotics

Probiotics/Prebiotics	**Patent Involved**	**Inventors**
Bifidobacterium longum	EP2318513A1	Jens Kildsgaard, Thomas Dyrmann Leser, Thomas Gunnarsson, Mette Weise, Ditte Marie Folkenberg, Thomas Janzen, Benedicte Flambard
Lactobacillus plantarum	US20160151434A1	Young Kwack, Se Jin You, Tae-Hun Park, Bum Jin Lee, Kye Ho Shin, Jin Oh Chung, Jun Cheol Cho
Streptococcus sanguis	US20140023620A1	Natalya Ioudina
Bacillus coagulans	US8697055B2	Sean Farmer
Enterococcus faecium	US20070098744A1	Ruth Knorr, Christoph Cavadini, Jalil Benyacoub, Ebenezer Satyaraj
Oligosaccharide	US20120294980A1	Albertus Alard Van Dijk, Yulia M. Efimova, Margot Elisabeth Francoise Schooneveld-Bergmans, Natalja Alekseevna Cyplenkova

Table 4. Commercially available probiotics and prebiotics

Sources/ Strain	**Brand/ Trade name**	**Type**	**Manufacturer**	**Origin**
Lactobacillus casei Immunitas	Actimel	Probiotics	Danone	France
Short-chain fructooligosaccharides	Ensure Fiber	Prebiotics	Abbott Nutrition	United States
Lactobacillus reuteri	Rela	Probiotics	Ingman Foods	Finland
Oligofructose, inulin, or combination	Cereal bars, meal replacement	Prebiotics	South Beach Diet	United States

	bars, and snacks			
Bacillus sp. strain IP5832	Bactisubtil	Probiotics	Synthelabo	Belgium
Lactobacillus strain	Jovita Probiotisch	Probiotics	H & J Bruggen	Germany
isomaltooligosaccharides	VitaFiber	Prebiotics	BioNeutra	United States
Lactobacillus casei Shirota	Yakult	Probiotics	Yakult	Japan
Lactobacillus strain	Vifit	Probiotics	Campina	The Netherlands

10. Conclusions

Approximately 10^{14} bacterial cells can hold the human intestine which can impact individual wellbeing. The regulation of the microorganisms in the intestines by diets (e.g., pro- and prebiotics) can be seen as a wonderful opportunity to affect people's health favorably. However, with certain health arguments made for pre- and probiotics, there are no definitive proofs and there is no appropriate explanation for their mechanism of action to describe these results. To conclude, a mixture of fundamental and applied science is desperately required to test intensively the health arguments made for pro and prebiotics and to learn about the actual mechanistic approach. Well before final declarations on the importance of pro- and prebiotics can be made- several unanswered issues need to be addressed.

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EFFECT OF GERMINATION ON CHEMICAL COMPOSITION, ANTI-NUTRITIONAL FACTORS, FUNCTIONAL PROPERTIES AND NUTRITIONAL VALUE OF KIDNEY BEAN (*PHASEOLUS VULGARIS*)

Mandeep Singh Sibian[1,2*], Charanjit Singh Riar[2]

[1]University Institute of Applied Health Sciences, Chandigarh University, Gharuan, (Mohali), India
[2]Department of Food Engineering and Technology, Sant Longowal Institute of Engineering and Technology, Longowal (Sangrur), India
mdeepsibian@yahoo.com

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ABSTRACT

The aim of this study was to analyze the impact of germination on the proximate composition, trace elements, anti-nutritional factors and amino acid profile of kidney bean. Results revealed positive effect of germination on the composition and nutritional attributes. Anti-nutritional factors (trypsin inhibitor, phytic acid, tannins, polyphenols and oxalates) decreased during germination which ensure the high bioavailability of minerals and other nutritional components. Protein content of sprouted beans was higher and leads to more available amino acids and its nutritional value. Essential amino acid content of beans increased after germination and inter-conversion of amino acids lead to lower non-essential amino acids. Amino acid profile revealed higher essential amino acid index (EAAI), protein efficiency ratio and nutritional index after germination. The nutritional value of amino acid was further analyzed by observing the amino acid score w.r.t. the pattern described by FAO, which showed improved nutritional value of essential and limiting amino acids.

1.Introduction

Legumes are the edible grains utilized by both humans and animals as food stuff throughout the history. Legumes are also known as poor man's meat due to its high protein content (20-40%) and low cost as compared to meat (Manay and Shadaksharaswamy, 2008). Like other beans, the kidney beans possess high nutritional value with good amount of starch, protein, dietary fiber and minerals. Fiber content also provides support to digestive system by flourishing the beneficial bacteria in colon (Tang, 2008). However, contribution of nutrition through edible legumes to the consumer is limited due to the presence of some toxic factors, enzyme inhibitors and anti-nutritional factors which limits their digestibility (El-Adawy, 2002). The presence of these anti-nutritional factors or secondary metabolites causes interference to digestibility and availability of nutrients (Zhang et al., 2015). In developing countries, foods are rarely modified at the household level to increase nutrient density to meet the needs of infants. The nutritional value of edible grains and its products (like porridges) depend primarily on their nutrient availability or presence/absence of anti-nutritional factors and thus have impact on physical and cognitive development of consumer (Neumann *et al.*, 2002). Various techniques have been currently applied by plant breeding experiments to lower the effect of these

anti-nutritional factors and secondary metabolites. Recent trend has also shown the utilization of processing of edible grains to enhance the nutritional quality of a product. Processing techniques may include germination of grains (Nkhata et al., 2018), fortification (Stabnikova et al., 2019), and fermentation (Bourré et al., 2019) of edible grains. Germination is one of best and inexpensive method to enhance the nutritional qualities and functional characteristic of grains. Germination allows the digestion of some components of grain like carbohydrates and also enhance free amino acids (Hallén, Ibanoglu, & Ainsworth, 2004).

2. Materials and methods

2.1. Materials

2.2.1. Raw material preparation

Kidney bean (light speckled kidney beans) were procured from certified seed agency. Grains were germinated as per the method described by Sibian et al. (2016-a). The sprouts were cleaned and rinsed in water after germination. Drying of sprouts was initially carried out at 80°C for 15 min to halt the enzyme activity, and then final drying was done at 60±5°C to attain moisture content of 8.00 ± 2% (db). Dried sprouts and un-germinated kidney bean grains were grinded separately in lab grinder to form flour and passed through 60 mesh sieves (US size 60 mesh = 250 μm). Both samples were kept at ambient condition for further analysis.

2.2.2. Composition analysis

The proximate analysis for the components like moisture content, protein (Kjeldahl method), crude fat (solvent extraction), crude fiber, ash and dietary fiber of raw and germinated kidney bean was carried out in triplicates using standard AOAC (2005) methods. Starch content was estimated by the modified anthrone method using variable range of glucose as standard solution.

Folic acid content of sample was estimated using IS 7234 (BIS, 1974) colorimeter at 660nm. Folic acid content was estimated by interpolation of graph readings on standard curve. Following relationship was used to calculate the folic acid per gram of sample.

$$\mu g \text{ of folic acid per g of sample} = \frac{\text{Average}(\mu g/ml) \times \text{Dilutionfactor}}{\text{Mass of sample}} \quad (1)$$

2.2.3. Trace element analysis

Sodium, phosphorus, calcium, magnesium and iron were estimated by the standard method described by AOAC (2005). The samples were ashed at 550 °C. The ash was boiled with 10 ml of 20% hydrochloric acid in a beaker and then filtered into a 100 ml standard flask. This was made up to the mark with deionized water. The minerals were determined from the resulting solution. Sodium [Na] was determined using the standard flame emission photometer using NaCl as the standards. Phosphorus was determined calorimetrically using KH_2PO_4 as the standard. Calcium [Ca], Magnesium [Mg] and Iron [Fe] were determined using Atomic Absorption Spectrophotometer.

2.2.4. Microstructure analysis

Microstructure of kidney bean flour from raw and un-germinated grains were observed using scanning electron micrographs obtained from scanning electron microscopy (SEM, JEOL, Tokyo, Japan, Model No. JSM 6610-LV) at varying range of magnification. Dried flour sample were directly positioned on S.E.M stub using two faced cellophane adhesive tape and then smeared with gold pladinum (60:40 g/g) by means of auto fine coater (JEOL-JFC-1600).

2.2.5. Functional properties

Effect of germination on functional properties of kidney bean flour was observed by analyzing various physical attributes of flour. Water absorption capacity was calculated by the method described by Yamazaki (1953) and expressed as water absorbed by 1.0 g of sample. Oil absorption capacity was estimated by the method of Lin et al. (1974) as oil absorbed by per gram of flour. Bulk density was measured as volume of 100 g of sample in 250 ml volumetric cylinder and values were expressed as g/ml of sample. Sedimentation value was estimated by calculating the swelling power of flour suspended in lactic acid as described in ICC

116/1 standard method (Zeleny's method). Percentage foaming capacity was calculated according to the method described by Mizubuti et al. (2000). Emulsification capacity was calculated by the method of Naczk, Diosady, and Rubin (1985) and expressed as ml of oil emulsified by 1.0 g of the sample. The emulsion stability was determined by heating the emulsified sample for 15 min at 85°C followed by cooling and centrifugation (5000×g) for 5 min. The emulsion stability was expressed in percentage activity of emulsion remained after heating.

2.2.6. Antinutritional factor analysis

The trypsin inhibition activity (TIA) was estimated as inhibition of bovine trypsin using the substrate benzoyl-DL-arginine-pnitrianilide (BAPNA) hydrochloric (Kakade et al., 1969). Tannin content was evaluated by vanillin-HCl methods (Price et al., 1978). For analysis sample was defatted and extracted for tannin in methanol (acidic). Vanillin-HCl reagent was added to develop color in the solution. Catechin was used as standard and run along with the sample. Spectrophotometer reading was measured at 500 nm. Results of tannins were expressed as mg/100g dry weight. Oxalate content was determined by AOAC (2005) method. The concentration of oxalate in each sample was obtained from the equation as 1 ml of 0.1 N permanganate = 0.006303 g oxalate. Phytic acid was estimated by the method of Davies and Reid (1979). Extraction was carried out with nitric acid and reacted with ferric ammonium sulphate in a boiling water bath. After cooling of solution, isoamyl alcohol and ammonia solution were added. Solution was centrifuged at 3000 rpm for 10 min and the alcoholic layer was separated. Spectrophotometer reading was measured at 465 nm with amyl alcohol taken as blank. The results were expressed as mg phytic acid/100g dry weight.

Polyphenols were estimated using method described by Singleton et al. (1999), with slight modification. Defatted sample was extracted using 1% HCl in methanol and content was refluxed for 2 h. Volume was made up to 100ml with water and 0.2ml extract was taken, to this 0.5ml Folin-denis reagent was added and mixed with saturated sodium carbonate again volume was made to 10 ml with water. The OD was taken at 765 nm after 30 min. The results were calculated as mg gallic acid equivalent/g sample and expressed as mg/100g on dry weight basis.

2.2.7. Amino acid analysis

Raw and un-germinated samples were analyzed for amino acid profile, using the physiological kits for gas chromatography flame ionization detection (Phenomenex, USA). The grain samples were milled to flour (60 mesh) and then hydrolyzed with concentrated HCl. Analysis was performed as directed in the kit's manual. The GC column used was the ZB-AAA GC column, which was provided in the kits and standard analysis conditions were used, as described in the kit's manual. Amino acids were further analyzed for estimation of essential amino acid index (EAAI), protein efficiency ratio (PER-1 and 2), nutritional index (NI) and biological value (BV). EAAI was calculated by using the ratio of relative essential amino acids in the test protein as compared to the respective values in whole egg protein used as reference value. Essential amino acids (g/16g N) were converted into g/g nitrogen basis and then the ratio of test to reference was calculated. The whole calculation was done according to the equation of Oser, (1959) as:

$$\mathrm{EAAI} = \sqrt{\frac{\mathrm{Lys_a} \times \mathrm{Tyr_a} \times \ldots\ldots \times \mathrm{His_a}}{\mathrm{Lys_b} \times \mathrm{Tyr_b} \times \ldots\ldots \times \mathrm{His_b}}} \quad (2)$$

Where “a” is the amino acid in test sample and “b” is the amino acid in reference protein sample.

Biological value, Protein efficiency ratio and nutritional index was calculated according to the method described by Alsmeyer et al. (1974) and Ijarotimi (2012).

Amino acid score for infants (pre-school) and adult were calculated as the ratio of observed value of amino acid to the appropriate reference patterns as provided by FAO (2013). Indispensable amino acid values (g/16 gN) were taken as test amino acids and individually each amino acid was observed for scoring.

$$\text{Amino acid score} = \frac{\text{Value of observed amino acid in test sample}}{\text{Value of amino acid in reference sample}} \quad (3)$$

3.Results and discussions

3.1. Proximate composition

Proximate composition of kidney bean was observed and as shown in table 1. Composition of flour varied significantly as a result of germination. After germination the protein content increased significantly and varied from 20.77±0.04 to 23.36±0.06 g/100g flour. Increment in the protein content could be attributed to the biosynthesis of amino acids (Zhang et al., 2015). Insignificant increment in the ash content of was observed. Chiemela et al. (2009), observed similar results after germination of tiger-nut seed flour. Carbohydrate content of kidney bean varied significantly after germination. Decrease in the complex carbohydrate molecules like starch, amylose and amylopectin was observed. The change might be attributed to the increase activity of amylase and other hydrolyzing enzymes (Zhang et al., 2015).

Non-reducing sugars and reducing sugar, enhanced as a result of increase in total sugar. Non-reducing sugar in raw kidney bean was observed as 2.86±0.03 g/100g which increased to 4.07±0.03 after germination. Reducing sugar was also high in germinating kidney bean and varied from 0.29±0.01 to 0.43±0.09 g/100g. Activity of α-amylase promotes the breakdown of carbohydrates and converted the complex molecules into more easily digestible polysaccharides sugar. Various researchers observed the same pattern and conclusions for increment of sugars after germination (Zhang et al., 2015; Nkhata et al., 2018).

Table 1. Proximate composition of raw and germinated kidney bean flour

Constituents (%)	Kidney bean (Raw)	Kidney bean (Germinated)
Protein	20.77±0.04^{b}	23.36±0.06^{a}
Ash	1.28±0.03^{a}	1.36±0.04^{a}
Carbohydrates	72.84±0.03^{a}	67.76±0.03^{b}
Starch	67.32±0.02^{a}	63.54±0.03^{b}
Amylose	17.83±0.02^{a}	16.24±0.03^{b}
Amylopectin	49.49±0.02^{a}	47.30±0.03^{b}
Sugar	3.15±0.02^{b}	4.51±0.02^{a}
Non-reducing sugar	2.86±0.03^{b}	4.07±0.03^{a}
Reducing sugar	0.29±0.01^{b}	0.43±0.09^{a}
Fats	1.20±0.01^{a}	1.18±0.03^{a}
Crude fiber	5.28±0.03^{b}	6.18±0.04^{a}
Dietary Fibers	10.28±0.02^{b}	15.13±0.03^{a}
Folic Acid (mcg/100g)	153.17±0.32^{b}	182.12±0.12^{a}

*n=3, Results are expressed as mean values ± standard deviations. Means in a row with different superscripts are significantly different ($p<0.05$)

Fat content of kidney bean decreased during germination. Fat content in raw kidney bean flour was higher and ranged from 1.20±0.01 to 1.18±0.03 g/100 in germinated kidney bean. Some researchers attributed this decrease to total solid loss during soaking process (Sibian et al., 2016-a). Crude fiber and dietary fiber increased as a result of germination. Increase in the fiber content might be due to synthesis of cell wall components and other polysaccharide based substances required for synthesis of cell structures, which enhanced the fiber content. Crude and dietary fibers in raw kidney bean were reported as 5.28±0.03 and 10.28±0.02 g/100g and after germination the values varied to 6.18±0.04 and 15.13±0.03 g/100g respectively. Increase in the total fiber content was observed by Jan, Saxena, & Singh (2017) in *Chenopodium album* after germination. Folic acid content was also high in germinated kidney bean. Folic acid content varied from 153.17±0.32 to 182.12±0.12 mcg/100g after germination. Folic acid plays an important role in seed germination by promoting the biochemical and functional development in plant cells, therefore during germination growth of seedling took place and could be correlated to enhanced folic acid content. Kariluoto et al. (2006), observed the prominent increment in folates of germinating rye and observed that thermal treatments could cause folate loss.

3.2. Trace element analysis

Significant increase in all trace elements was observed as shown in table 2. Calcium, iron, magnesium, phosphorus, sodium and zinc were observed for the deviation after germination. Minerals of kidney bean flour varied as calcium 78.32±0.05 to 82.09±0.04; iron-5.67±0.03 to 5.84±0.04, magnesium-135.51±0.09 to 146.39±0.05, phosphorus- 64.61±0.05 to 69.28±0.05, sodium- 14.74±0.05 to 16.21±0.04 and zinc- 3.53±0.04 to 3.96±0.07 mg/100g after germination. Increment in mineral content with germination of some cereals and legumes have been reported previously by Desai et al. (2010) and Laxmi et al. (2015).

3.3. Microstructure analysis

Effect of germination on the morphology of starch was observed as shown in figure 1 (a-b). Germination enhanced the protein content and degraded starch therefore, as a result morphological changes occurred. Undamaged starch molecules were observed before germination with smooth surface and in less association with protein matrix. Germination brought changes in the starch structure molecules and also increment in protein which was observed as matrix and in association with starch molecules. As a result of germination starch molecules become distorted and smooth surfaces become rough. Scanning electron micrographs of wheat showed similar observations after germination due to the action of alpha amylase (Sibian et al., 2016-b).

Table 2. Changes in the trace elements of kidney bean (cranberry bean) after germination

Trace elements (mg/100g flour sample)	**Kidney bean (Raw)**	**Kidney bean (Germinated)**
Calcium	78.32±0.05^{b}	82.09±0.04^{a}
Iron	5.67±0.03^{b}	5.84±0.04^{a}
Magnesium	135.51±0.09^{b}	146.39±0.05^{a}
Phosphorus	64.61±0.05^{b}	69.28±0.05^{a}
Sodium	14.74±0.05^{b}	16.21±0.04^{a}
Zinc	3.53±0.04^{b}	3.96±0.07^{a}

*n=3, Results are expressed as mean values ± standard deviations. Means in a row with different superscripts are significantly different ($p<0.05$)

3.4. Functional properties

Effect of germination on the functional properties of kidney bean is as shown in table 3. Germination enhanced the functional capabilities of kidney bean flour. Functional properties are determinant factor for evaluating the flour components and its behavior during processing or food formulation (Siddiq *et al.*, 2009). Water absorption capacity of kidney bean (g/g water absorbed) was higher in germinated flour sample. The value of water absorption capacity ranged from 1.13±0.01 to 1.27±0.02.

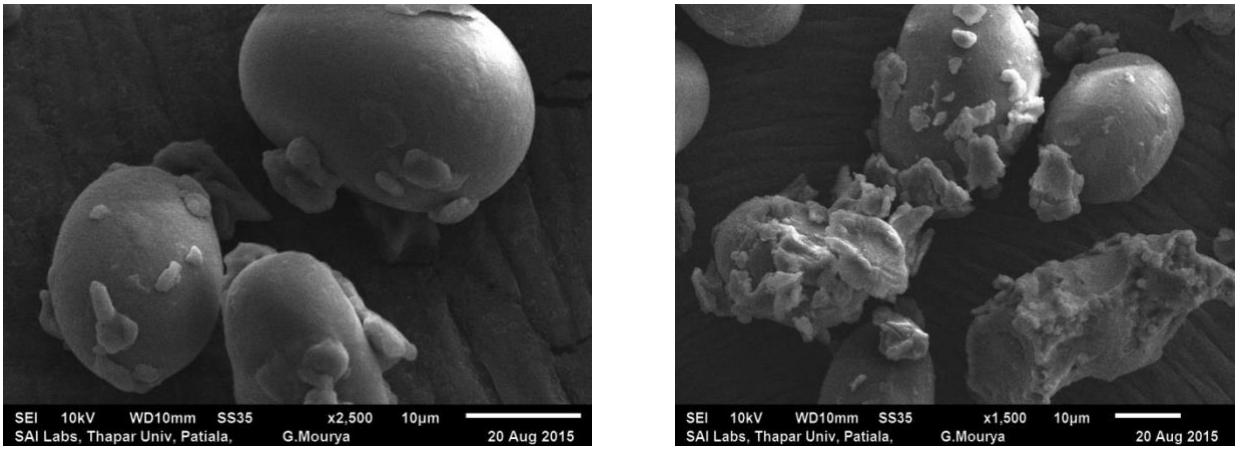

Figure1 (a-b): Effect of germination on the microstructure of kidney bean

Table 3. Effect of germination on functional properties of kidney bean

Functional parameters	**Kidney bean (Raw)**	**Kidney bean (Germinated)**
Water absorption capacity (g/g water absorbed)	1.13 ± 0.01^{b}	1.27 ± 0.02^{a}
Oil absorption capacity (g/g oil absorbed)	1.31 ± 0.02^{b}	1.39 ± 0.03^{a}
Bulk density (g/cm^3)	0.81 ± 0.02^{a}	0.77 ± 0.02^{b}
Foaming capacity (%)	26.01 ± 0.01^{b}	38.67 ± 0.04^{a}
Sedimentation value (ml)	9.03 ± 0.02^{ab}	11.02 ± 0.01^{a}
Emulsification activity (%)	55.67 ± 0.06^{b}	61.67 ± 0.05^{a}
Emulsification capacity (ml oil/g sample)	128.67 ± 0.05^{b}	151.12 ± 0.06^{a}
Emulsification stability (%)	46.34 ± 0.05^{b}	58.08 ± 0.06^{a}

*n=3, Results are expressed as mean values ± standard deviations. Means in a row with different superscripts are significantly different (P<0.05)

Improvement in the protein content during germination and breakdown of complex carbohydrates led to increment in the water absorption capacity (Sibian et al., 2020). Raw kidney bean flour showed lower oil absorption capacity (g/g oil absorbed) which was reported as 1.31±0.02 and enhanced to 1.39±0.03 after germination. Oil absorption capacity is function of hydrophobic protein molecules; increase in the protein content enhanced the oil absorption capacity of kidney bean flour (Chiemela et al., 2009). Due to the change in the conformation of physical structure of particle bulk density decreased. Bulk density depends on the number of factor like structure of particle, moisture content of flour, dispensability and particle size. Milling conditions and techniques also affected bulk density. Foaming capacity of kidney flour showed increment after germination. Germination enhanced the protein properties and therefore lowered the surface tension at the interface of air and water to effectively form foam (Kaur *et al*., 2010). Foaming capacity of raw kidney bean flour was reported as 26.01±0.01 and 38.67±0.04% in germinated kidney bean flour sample. Sedimentation value of raw kidney bean flour was 9.00 ml which increased slightly to 11.00 ml. Sedimentation values might be lower due to lack of gluten like protein in legume flour.

Significant increase in the emulsification properties was observed as an effect of germination. Emulsion forming and stability behavior of flour depends on the amphiphilic nature of protein. Emulsion stability is function of lipid-protein interaction, more the protein-lipid interaction more would be the stability of emulsion formed (Sibian et al., 2017). Emulsion activity in raw flour was 55.67±0.06% and enhanced to 61.67±0.05% after germination. Emulsion capacity (ml oil/g sample) was also increased from 128.67±0.05 to 151.12±0.06. Emulsion stability was lower in raw kidney bean flour and was reported as 46.34±0.05% which increased to 58.08±0.06% after germination. Similar observations in improvement of the emulsion properties were already observed in germinated sorghum and brown rice flour (Elkhalifa *et al*., 2010).

Table 4. Variability of anti-nutritional factors in kidney bean

	Trypsin Inhibitor activity (TIU)	**Phytic Acid (g/100g)**	**Tannins (mg/100g)**	**Polyphenol (mg/100g)**	**Oxalate (mg/100g)**
Kidney bean (Raw)	158.05±0.03^{a}	0.780±0.06^{a}	0.330±0.04^{a}	0.564±0.03^{a}	1.54±0.05^{a}
Kidney bean (Germinated)	86.54±0.04^{b}	0.495±0.07^{b}	0.219±0.03^{b}	0.317±0.05^{b}	0.76±0.03^{b}
Variability (%)	45.24	36.53	33.63	43.79	50.64

*n=3, Results are expressed as mean values ± standard deviations. Means in a column with different superscripts are significantly different (P<0.05)

3.5. Effect of germination on anti-nutritional factors of kidney bean

Effect of germination and variability of anti-nutritional factors in kidney bean was observed as shown in table 4. Significant reduction in trypsin inhibitor, phytic acid, tannin, polyphenol and oxalate was observed as result of germination (Nkhata et al., 2018). Trypsin inhibitor activity in raw kidney bean was observed as 158.05±0.03 TIU and after germination it was reduced to 86.54±0.04 TIU with overall variability of 45.24%. Similar results were also observed by Zhang et al. (2015), after germination of buckwheat. Phytic acid was reduced by 36.53% and the value of raw and germinated kidney bean varied from 0.780±0.06 to 0.495±0.07 g/100g sample. Germination of legumes activates phyates enzyme which digests phytate (Luo et al., 2014). Tannin content was reduced by 33.63% with

reduction from 0.330±0.04 to 0.219±0.03 mg/100g. Polyphenol content of raw legume was observed 0.564±0.03 mg/100g which reduced to 0.317±0.05 mg/100g after germination. The percentage variability in reduction of polyphenol was observed as 43.79%. Oxalate content of kidney bean was also reduced as a result of germination. Raw kidney bean contains 1.54±0.05 mg/100g which reduced significantly to 0.76±0.03 with variability of 50.64%. The reason for reduction of water soluble secondary metabolites could be solid leaching during soaking due to their hydrophobic interactions (Dongyan *et al*., 2014).

3.6. Effect of germination on total amino acid content of kidney bean

Legumes are considered as good source of proteins and essential amino acids. Total amino acid profile was observed for both raw and germinated kidney bean as shown in figure 2. Total non-essential amino acid proportion decreased as a result of germination and varied from 47.17±0.03 to 39.74±0.04 g/100g protein. On the other hand total essential amino increased from 52.83±0.07 to 60.26±0.06 g/100g protein. Increment in the amino acid content was also observed by Bhathal & Kaur (2015). Aromatic amino acid and acidic amino acid content in germinated kidney bean was observed with lower values, whereas basic amino acid content increased. Leucine to isoleucine ratio was maintained during germination due to proportionate increment in both branched chain amino acids.

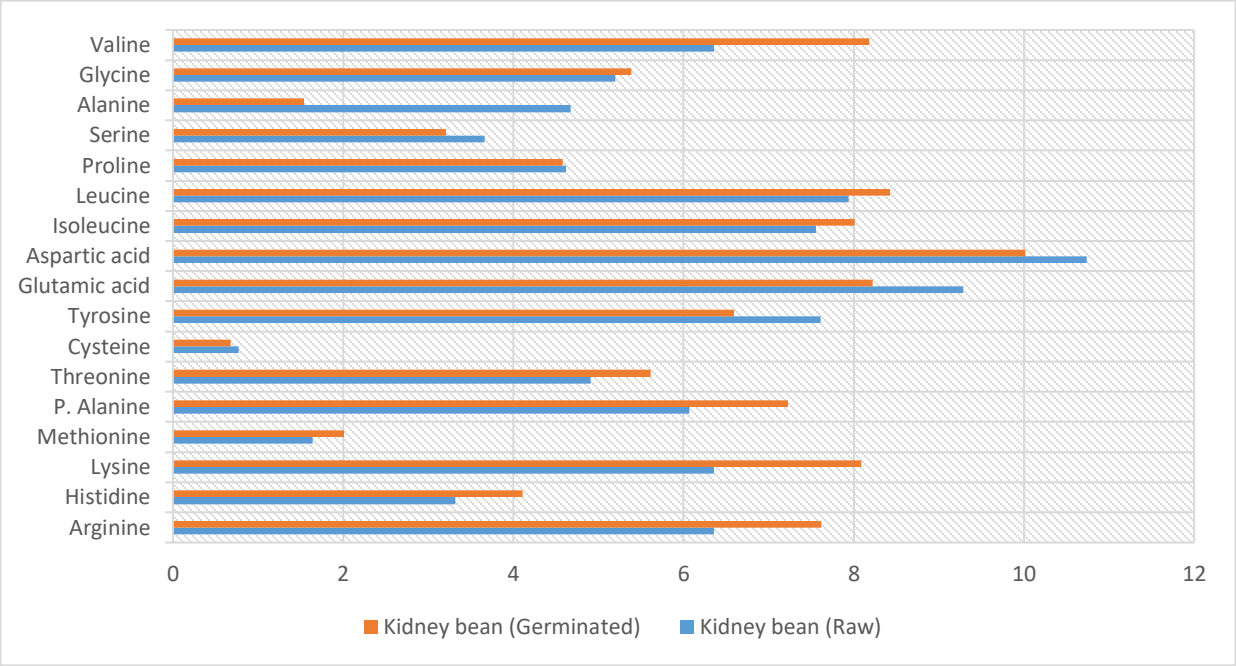

Figure 2. Amino acid contents of raw and germinated kidney bean

Essential amino acid index, biological value, protein efficiency ratios, nutritional index are the parameters to evaluate quality of protein on the basis of amino acids. Food arbitrated on the basis of protein profile should have biological value between 70 to 100% and essential amino acid index above 90% (Larson & Beevers, 1965). Essential amino acid index of kidney bean was reported 80.26±0.09 in raw kidney bean which was further improved by germination to 91.05±0.06 (Table 5). Biological value of kidney bean protein was also reported higher after germination. Biological value of kidney bean protein varied from 75.78±0.06 to 87.54±0.07. Protein efficiency ratios were also improved during germination and varied from 2.72±0.04 to 2.94±0.01 and 2.34±0.04 to 2.66±0.03. With the increase in essential amino

acid and improvement in protein content, nutritional index of kidney bean protein was reported higher in germinated kidney bean (Sibian et al., 2016-a). Nutritional index increased from 16.67±0.02 to 21.27±0.05. Similar observations in the increment of amino acid profile of wheat, brown rice and triticale was observed by Sibian et al. (2017).

Table 5. Effect of germination on the total amino acid profile and nutritional profile of kidney bean (cranberry bean)

Amino acid profile (%)	Kidney bean (Raw)	Kidney bean (Germinated)
Total non-essential amino acid	47.17±0.03^{a}	39.74±0.04^{b}
Total essential amino acid	52.83±0.07^{b}	60.26±0.06^{a}
Total Aromatic Amino acid	14.09±0.06^{a}	13.89±0.06^{b}
Total Acidic amino acid	20.63±0.02^{a}	18.32±0.03^{b}
Total Basic Amino Acid	16.52±0.08^{b}	19.91±0.06^{a}
Leucine/Isoleucine ratio (BCAA)	1.05±0.03^{ab}	1.05±0.06^{ab}
Essential amino acid index	80.26±0.09^{b}	91.05±0.06^{a}
Biological Value	75.78±0.06^{b}	87.54±0.07^{a}
PER-1	2.72±0.04^{b}	2.94±0.01^{a}
PER-2	2.34±0.04^{b}	2.66±0.03^{a}
Nutritional index	16.67±0.02^{b}	21.27±0.05^{a}

*n=3, Results are expressed as mean values ± standard deviations. Means in a row with different superscripts are significantly different (P<0.05)

3.7. Effect of germination on amino acid score

Amino acid score of raw and germinated kidney bean was observed as per the reference values of FAO (2013) as shown in Table 6. The detrimental factor for food protein quality greatly depends on the content and availability of amino acids (Graciela Caire-Juvera et al., 2013).

Table 6. Amino acid score for infants/preschool and adults (FAO, 2013) in raw and germinated kidney bean

Amino Acid Score (for infants/pre-school(1-2 yrs)	**FAO (2013)**	**Kidney bean**	
		Raw	**Germinated**
Isoleucine	3.2	236.22	250.16
Leucine	6.6	120.36	127.78
Lysine	5.7	111.50	141.94
Methionine + Cystiene	2.7	89.16	99.89
Phenylalanine + Tyrosine	5.2	262.95	265.90
Threonine	3.1	158.42	180.90
Valine	4.3	147.80	190.15
Amino Acid Score (for adults)	**FAO (2013)**	**Kidney bean**	
		Raw	**Germinated**
Isoleucine	3.0	251.97	266.84
Leucine	5.9	134.65	142.94
Lysine	4.5	141.23	179.79
Methionine + Cysteine	2.2	109.42	122.59
Phenylalanine + Tyrosine	3.8	359.83	363.87
Threonine	2.3	213.52	243.82
Valine	3.9	162.96	209.65
Histidine	1.5	221.47	273.97

All the amino acids has shown higher amino acid score and were reported above 100 except sulfur containing amino acids. Sulfur containing amino acids were found limited in both raw and germinated kidney bean in amino acid scoring pattern of infants/pre-school, which could be attributed to lower value of methionine in legume. Sulfur containing amino acid content improved with germination. The values for methionine plus cystine varied from 89.16 to 99.89 in infant amino acid scoring pattern whereas the amino acid score for sulfur containing amino acid was varied from 109.42 to 122.59 in adult amino acid scoring pattern. The difference in the values were attributed to the reference values in both cases. Isoleucine and aromatic amino acid content of kidney bean was found quite higher in both amino acid scoring patterns of infants/pre-school and adults. Overall amino acid score showed increment as a result of germination (Sibian et al., 2017).

4. Conclusions

Germination proved as an effective processing method in the improvement of nutritional attributes of kidney bean. Overall nutritional profile including proteins, amino acids, sugars, and fiber content improved significantly during germination. Functional properties showed the increased water and oil absorption capacities, which could be beneficial in formulation of number of products. Decrease in anti-nutritional factors would contribute to improved bio-availability and digestibility. Improved protein quality and amino acid profile further enhances the nutritional value of legume.

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MICROWAVE ASSISTED EXTRACTION OF CUSTARD APPLE (*ANNONA SQUAMOSAL* L.) PEEL

Trang Nguyen Thi[1,2] and Huan Phan Tai[1*]

[1]Nong Lam University - Ho Chi Minh City, Faculty of Chemical Engineering and Food Technology, Thu Duc, Ho Chi Minh city 700000, Vietnam.

[2]Industrial University of Ho Chi Minh City, Institute of Biotechnology and Food Technology, Go Vap district, Ho Chi Minh city 700000, Vietnam.

pthuan@hcmuaf.edu.vn

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ABSTRACT

In recent years, custard apple fruit has been applied in food processing with various products. The purpose of this study aimed to valorize the peel as organic food waste produced by fruit processing. Microwave assisted extraction (MAE) of bioactive polyphenols from custard apple peel was performed at different aqueous ethanol composition, extraction irradiation time, solvent to solid ratio, and microwave power. Total polyphenols content (TPC) and antioxidant activities of the extracts were investigated. Response surface methodology was applied to find the optimal condition according to the central composite design with ethanol concentration ranged from 50 to 70%, extraction time from 3 to 7 min, solvent to solid ratio from 20 to 30 mL/g, and microwave power from 154 to 274 W. A quadratic model was respectively developed to correlate the investigated variables to the TPC and radical scavenging activity by DPPH and ABTS of the extracts. Optimum condition was successfully selected at an ethanol concentration of 60%, extraction time of 5 min, solvent-solid ratio of 25 mL/g, and microwave power of 214 W. With a good correlation between predicted values and actual experimental results, the developed response surface model can be used to optimize the extraction of polyphenols from custard apple peel by MAE.

1.Introduction

Annona squamosal L., commonly known as custard apple, is cultivated mainly for its edible fruit. The plant is also attributed with antifertility, antitumor and abortifacient properties, and can be traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, haemorrhage, antibacterial infection, dysuria, fever, and ulcer (Amudha & Varadharaj, 2017; Kaleem, Medha, Ahmed, Asif, & Bano, 2008). Custard apple is climacteric fruit, characterized by high respiration and rapid softerned after harvest, and are chilling sensitive (Pareek, Yahia, Pareek, & Kaushik, 2011).

Todays, fruit by-products are one of the main sources of organic waste that cause environmental pollution (Carciochi et al., 2017). Previous study showed that the custard apple peel contains polyphenols (Prajapti, Purohit, Sharma, & Kumar, 2006). Polyphenols have considerable significance as bioactive compounds with substantial health benefits because of their antioxidant potential to scavenge free radicals (Nag & Sit, 2018). It was reported that polyphenols from custard apple peel can be extracted by conventional solvent extraction methods and may have several medicinal properties (Sharma, Sharma, Chand, Khardiya, & Agarwal, 2013). However,

conventional solvent extraction method is associated with high solvent consumption and negative environmental impact, longer extraction times and generating relatively low yields (Jovanović et al., 2017). Recently, there have been many advanced techniques developed for extracting phenolic compounds from plant materials such as ultrasound assisted extraction, microwave assisted extraction (MAE), pressurized liquid extraction, enzymatic extraction and supercritical fluid extraction (Marchese et al., 2016). Among the techniques, MAE method has attracted significant interest because of its advantages in shorter extraction time, less energy and organic solvent consumption, reasonable cost and high efficiency (Kaderides, Papaoikonomou, Serafim, & Goula, 2019; Mellinas, Jiménez, & Garrigós, 2020; Sarfarazi, Jafari, Rajabzadeh, & Galanakis, 2020). There are several variables can influence the MAE. This work included a study of MAE of custard apple peel at different solvent composition, extraction irradiation time, solvent to solid ratio, and microwave power. Total polyphenols content, antioxidant activity according to DPPH and ABTS assays were investigated and the extraction process was optimized using response surface methodology. Up to now, there is no literature on optimization of the process for MAE of polyphenols from custard apple peel.

2. Materials and methods

2.1. Materials

2.1.1. Samples

The custard apples were from Tay Ninh Province, Vietnam. The peel was separated and rinsed with water to remove fleshy residues, then dried by hot-air at 60°C until the moisture content obtained ≤ 12%. The dried peel was powder and sieved through a 0.5 mm sieve before stored in PE bags with an average mass of 5 ± 0.03 g. PE samples were sealed and stored in the freezer at -20°C for subsequent experiments.

2.1.2. Chemicals and reagents

Folin-Ciocalteu reagent (≥ 99.8%) and standard gallic acid (GA) (≥ 99.9%) were supplied by Merck (Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (≥ 97%) reagent were purchased from Sigma-Aldrich (USA). All other chemicals were analytical grade.

2.2. Methods

2.2.1. Extraction procedure

The polyphenols extraction was carried out by using a domestic microwave oven (Sanyo, Japan). For a typical experimental extraction procedure, 1.0 gram of custard apple peel powder was infused in aqueous ethanol and introduced to the oven equipped with reflux condenser in order to condense the vapors generated during MAE. The extraction variables evaluated were ethanol proportion (40-70%), solvent to solid material ratio (20:1- 35:1 v/w), extraction time (1-7 min) and microwave power (95 - 284 W). After MAE treatment, the extracts obtained were filtered through Whatman No. 4 filter paper then their total polyphenol content and antioxidant activity were determined.

2.2.2. Analysis of total phenolic content

Total polyphenol content (TPC) was determined according to the Folin Denis method as described in study of (Sripakdee, Sriwicha, Jansam, Mahachai, & Chanthai, 2015) with modification. Sample extract (0.1 mL) was reacted with 1.8 mL Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and incubated at room temperature for 5 min followed by the addition of 1.2 mL of sodium carbonate (15%, w/v). After 90 min absorbance was measured at 765 nm at room temperature. The results were expressed as mg gallic acid equivalent per g dry sample (mg GAE/g DW).

2.2.3. Determination of radical scavenging activity by DPPH

The free radical scavenging activity of custard apple peel extract on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was determined according to the method described by (Sripakdee et al., 2015) with modifications. Sample extract (0.1 ml) was added to 4 ml of

DPPH reagent and kept in darkness conditions at room temperature for 30 min. The absorbance was measured at 517 nm using a UV–visible spectrophotometer (Thermo, Genesys 10 UV). Trolox was used as standard and the results were expressed as μmol Trolox equivalents per gram dry sample (μmol TE/g DW).

2.2.4. Determination of radical scavenging activity by ABTS

The antioxidant capacity against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was measured according to the method described by (Sripakdee et al., 2015) with modifications. 0.1 mL of extract was then added to 3 mL of ABTS solution and mixed at room temperature for 15 min in darkness conditions. The absorbance was determined at 734 nm using a UV–visible spectrophotometer (Thermo, Genesys 10 UV). Trolox was used as standard and the results were expressed as μmol Trolox equivalents per gram dry sample (μmol TE/g DW).

2.2.5. Statistical analysis

All results were subjected to statistical analyses. For screening individual process variables, experiments were carried out in triplicate and average values with standard deviations were computed by Statgraphics (Centurion XV). Significant difference was defined at $p<0.05$.

2.2.6. Experimental design and optimization

Response surface methodology (RSM) was used to determine optimal process parameters (variables) to extract polyphenols from custard apple peel. Equation (1) is the general form of a response surface of response variable Y as a function of n independent process variables from X_1 to X_n.

$$Y = B_o + \sum_{i=1}^{n} B_i X_i + \sum_{i=1}^{n} B_{ii} X_i^2 + \sum_{\substack{i,j=1 \\ i \neq j}}^{n} B_{ij} X_i X_j \quad (1)$$

where B_o is the constant; B_i is the linear coefficient; B_{ii} is the quadratic coefficient; and B_{ij} is the cross-product coefficient.

Table 1. Independent variables and selected levels used in the CCD for polyphenols extraction from custard apple peel.

Parameters/Variables	-1	0	+1
Ethanol concentration, X_1 (%)	50	60	70
Extraction time, X_2 (min)	3	5	7
Solvent-solid ratio, X_3 (mL/g)	20:1	25:1	30:1
Microwave power, X_4 (W)	154	214	274

A central composite design (CCD) was employed to the experimental data with different independent variables and selected levels (Table 1).

In this study, four independent process variables were ethanol concentration (% v/v; X_1), extraction time (min; X_2), solvent-solid ratio (mL/g; X_3), and microwave power (W; X_4). The selected response variables were TPC (mg GAE/g DW; Y_1), radical scavenging activity (RSA) by DPPH (μmol TE/g DW; Y_2), and RSA by ABTS (μmol TE/g DW; Y_3).

Different experimental combinations of levels were carried out in duplicate, and six experiments were performed at the center points of the design to allow the estimation of pure error. JMP version 10 was used to fit the quadratic response surface model (Equation 1 with n=4) to the experimental data.

3. Results and discussions

Influences of processing parameters including aqueous ethanol composition, extraction irradiation time, solvent to solid ratio, and microwave power on the TPC, antioxidant activity according to DPPH and ABTS assays of the extracts by MAE of custard apple peel are presented in Table 2.

Table 2. Effect of processing parameters on polyphenol content and antioxidant activity.

Parameter	Level	TPC (mg GAE/g DW)	RSA by DPPH (μmol TE/g DW)	RSA by DPPH (μmol TE/g DW)
Ethanol concentration	40 (%)	$82.06^{a} \pm 2.07$	$489.90^{d} \pm 6.65$	$1119.52^{g} \pm 7.05$
	50 (%)	$91.39^{b} \pm 1.17$	$536.04^{e} \pm 20.25$	$1159.12^{g} \pm 24.84$
	60 (%)	$96.27^{c} \pm 0.74$	$589.46^{f} \pm 17.39$	$1242.98^{h} \pm 21.79$
	70 (%)	$89.06^{b} \pm 0.57$	$582.21^{f} \pm 15.65$	$1122.48^{g} \pm 42.14$
Solvent-solid ratio	20:1 (mL/g)	$92.09^{b} \pm 1.92$	$550.22^{d} \pm 25.57$	$1267.39^{f} \pm 21.69$
	25:1 (mL/g)	$96.12^{c} \pm 0.21$	$617.35^{e} \pm 15.54$	$1361.38^{g} \pm 19.91$
	30:1 (mL/g)	$91.16^{ab} \pm 2.13$	$587.55^{e} \pm 13.57$	$1298.38^{f} \pm 9.07$
	35:1 (mL/g)	$88.94^{a} \pm 0.61$	$546.99^{d} \pm 4.37$	$1261.69^{f} \pm 30.99$
Extraction time	1 (min)	$92.13^{a} \pm 0.56$	$549.03^{c} \pm 4.38$	$1159.24^{e} \pm 13.72$
	3 (min)	$95.02^{b} \pm 0.74$	$561.38^{c} \pm 9.00$	$1236.51^{f} \pm 8.29$
	5 (min)	$98.63^{c} \pm 1.05$	$603.02^{d} \pm 18.73$	$1283.58^{g} \pm 11.52$
	7 (min)	$95.37^{b} \pm 0.84$	$587.59^{d} \pm 6.91$	$1180.64^{e} \pm 15.47$
Microwave power	95 (W)	$90.07^{a} \pm 1.06$	$518.29^{d} \pm 6.94$	$1009.40^{g} \pm 5.44$
	166 (W)	$91.40^{a} \pm 1.67$	$573.94^{e} \pm 11.41$	$1198.78^{i} \pm 11.32$
	214 (W)	$97.62^{b} \pm 1.72$	$613.15^{f} \pm 13.89$	$1279.37^{j} \pm 60.61$
	284 (W)	$91.76^{a} \pm 1.89$	$463.51^{c} \pm 2.21$	$1121.40^{h} \pm 11.75$

Within one parameter group, different superscripts within the same column indicate significant differences between values ($p < 0.05$).

3.1. Effect of ethanol concentration

Ethanol and water are among the most commonly used solvents to extract polyphenols from plant because of their effectiveness and environmental friendliness (Chan et al., 2009; Mustafa & Turner, 2011). It was reported that by creating a more polar medium and breaking hydrogen bonding, addition of a quantity of water to ethanol facilitated the extraction of polyphenols from both high and low polarity ends (Jovanović et al., 2017). Therefore, aqueous ethanol composition is an importance factor to be optimized in order to obtain a good extraction yield with economic advantage.

As shown in Table 2, the effect of ethanol concentration with different polarities on TPC is presented. The result shows that increasing the ethanol concentration from 40% to 60%, the total polyphenol content (TPC) increases gradually from 82.06 mg GAE/g DW to 96.27 mg GAE/g DW. Meantime, radical scavenging activity by DPPH increased from 489.90 to 589.46 μmol TE/g DW and activity by ABTS was from 1119.52 to 1242.98 μmol TE/g DW. This can be explained by the difference in dielectric properties of the solvents towards microwave heating. The dielectric constant of water is higher than ethanol; therefore, increase in ethanol concentration led to a slower microwave energy absorption and reduced heating of the sample with a limited thermal degradation of the extracted phenolic compounds (Dahmoune et al., 2014). A similar effect was reported for the extraction of polyphenols from other plant sources (Dahmoune, Nayak, Moussi, Remini, & Madani, 2015; Li et al., 2012).

However, continuing increase concentration of ethanol to 70%, the TPC decreased to 89.06 mg GAE/g DW, DPPH and ABTS radical scavenging activities decreased to 582.21 (μmol TE/g DW) and 1122.48 (μmol TE/g DW), respectively. A high ethanol concentration will reduce the polarity of the solvent and molecular movement resulting in light dissolution of polyphenol compounds and decrease of

solubility (Jovanović et al., 2017). Therefore, to achieve the optimal polyphenol extraction efficiency and antioxidant activity, the 60% ethanol concentration was finally selected as center point for the next RSM trials.

3.2. Effect of solvent-solid ratio

In the MAE extraction method, the ratio of solid material to solvent is an important factor affecting the extraction efficiency. Solvent can diffuse through the porous matrix of dried peel material and extract the interested compounds. The solvent-solid ratio influences the mass transfer. Using a high ratio also provides an increase in the gradient concentration of the polyphenols between surface and interior part of the dried custard apple peel. The influence of ratio of solvent to solid on the polyphenol content and antioxidant activities of the extract is shown in Table 2.

TPC content increased from 92.09 mg GAE/g DW at solvent-solid ratio of 20:1 to a maximum of 96.12 mg GAE/g DW at the solvent-solid ratio of 25:1. With the same trend, DPPH scavenging activity increased from 550.22 μmol TE/g DW to 617.35 μmol TE/g DW and ABTS scavenging activity increased from 1267.39 μmol TE/g DW to 1361.38 μmol TE/g DW. However, when the solvent-solid ratio continued to rise, the TPC and antioxidant activity gradually decreased. The total polyphenol content decreased from 91.16 mg GAE/g DW at solvent-solid ratio of 30:1 and continued to decrease to 88.94 mg GAE/g DW at the ratio of 35:1. The radical scavenging activity by DPPH decreased from 587.55 μmol TE/g DW to 546.99 μmol TE/g DW and the activity by ABTS was also reduced from 1298.38 μmol TE/g DW to 1261.69 μmol TE /g DW. At a certain amount of solvent, the bioactive substances will not continue to increase when the extraction reaches equilibrium. The more solvent used, the greater the amount of dissolved oxygen in it. As a result, the presence of oxygen not only reduces the TPC but also weakens the antioxidant activity of polyphenols (Chan et al., 2009; Thoo, Ho, Liang, Ho, & Tan, 2010). Therefore, when increasing the solvent-solid ratio to 30:1 and 35:1, the total polyphenol content and antioxidant activity decrease. The solvent-solid ratio of 25:1 was statistically different from the remaining ratios and this is the highest for TPC and antioxidant activity. Therefore, to achieve the best extraction efficiency, this will be selected for the next optimization experiments.

3.3. Effect of extraction irradiation time

Effect of extraction irradiation time on polyphenols content and antioxidant activity is also shown in Table 2. Increased extraction time from 1 minute to 5 minutes, the total polyphenol content (TPC) increased from 92.13 mg GAE/g DW to a maximum of 98.63 mg GAE/g DW. With the same trend, the antioxidant activity according to DPPH and ABTS assays increases from 549.03 μmol TE/g DW to 603.02 μmol TE/g DW and from 1159.24 μmol TE/g DW to 1283.58 μmol TE/g DW, respectively. In contrast, when extraction time continued to increase to 7 minutes, TPC decreased to 95.37 mg GAE/g DW and antioxidant activity also reduced to 587.59 μmol TE/g DW (DPPH assay) and to 1180.64 μmol TE/g DW (ABTS assay). Thus, extraction time is an important factor and affects the microwave extraction of custard apple peels. The TPC content and antioxidant activities increase as the extraction time increases, but too long extraction time may lead to degradation of the polyphenols resulting in a decrease of these important values. This is consistent with the study of (Zhao, Zhang, Li, Meng, & Li, 2018) showing that in microwave-assisted extraction of phenolic compounds from *Melastoma sanguineum* fruit the TPC value increased with duration increasing from 15 to 45 min; while the extraction efficacy decreased with extended duration to 60 min (Zhao et al., 2018). Therefore, to achieve the best extraction efficiency during polyphenol extraction, the irradiation duration of 5 minutes was selected for the optimization process.

3.4. Effect of microwave power

Microwave power is also an important factor in the extraction of polyphenols by MAE

method. Results (Table 2) showed that microwave power significantly influenced the TPC and antioxidant activities in the tested condition. There were correlations among TPC, antioxidant activities and microwave power. As microwave power increased from 95 W to 214 W, the total phenol and antioxidant capacity also increased. A maximum extraction of 97.62 (mg GAE/g DW) total poly phenol content, together with scavenging against DPPH and ABTS (613.15 μmol TE/g and 1279.37 μmol TE/g DW) was obtained at 214 W. It was reported that the main effect of microwaves in many cases of MAE is the heating effect (Dahmoune et al., 2014). Therefore, this can be explained that as temperature increases at high microwave power, the solubility and diffusion of the compounds from the material matrix into the solvent will be enhanced. In addition, increasing temperature will decreases viscosity of the solvent, facilitate solvent to penetrate deeply into the material matrix and increase the contacting surface area.

However, when continue to increase the microwave power to 284 W, the total polyphenol content and antioxidant activity according to DPPH and ABTS assays decreased to 91.76 mg GAE/g DW, 463.51 μmol TE /g DW and 1121.4 μmol TE /g DW, respectively. The results obtained were according with (Alara, Abdurahman, Ukaegbu, & Azhari, 2018), who studied microwave-assisted extraction of *Vernonia cinerea* leaves and observed a reduction in the TPC and antioxidant activity at a very high microwave power because of thermal degradation of phenolic compounds in the plant sample. Therefore, with the maximum TPC and antioxidant activity obtained at 214 W, this microwave power was considered proper for further experiments.

3.5. Optimization of polyphenol extraction

A total of 54 runs were used to optimize the four individual parameters in the CCD applied to total extracted polyphenols and antioxidant activities. The response values at different experimental combination were listed in Table 3.

Table 3. Experimental matrix design for response surface and results obtained of different response variables.

No.	X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	50	3	20	154	90.26	404.53	1228.76
2	70	3	20	154	92.87	570.90	1208.06
3	50	7	20	154	93.04	419.36	1255.71
4	70	7	20	154	91.43	453.05	1240.78
5	50	3	30	154	91.70	400.21	1223.41
6	70	3	30	154	92.59	557.90	1206.49
7	50	7	30	154	94.98	452.00	1236.03
8	70	7	30	154	91.52	426.06	1241.90
9	50	3	20	274	91.40	453.41	1275.61
10	70	3	20	274	92.63	487.62	1231.49
11	50	7	20	274	92.37	439.58	1279.87
12	70	7	20	274	91.65	504.33	1280.31
13	50	3	30	274	90.69	417.16	1280.38
14	70	3	30	274	91.04	436.57	1245.83
15	50	7	30	274	92.21	444.32	1313.37
16	70	7	30	274	90.56	427.88	1231.12
17	50	5	25	214	96.37	593.92	1341.40
18	70	5	25	214	97.92	595.01	1316.25
19	60	3	25	214	96.75	646.84	1344.58

20	60	7	25	214	96.76	586.46	1317.84
21	60	5	20	214	96.98	559.53	1348.46
22	60	5	30	214	97.47	547.12	1336.50
23	60	5	25	154	98.81	589.88	1310.44
24	60	5	25	274	97.28	593.98	1351.33
25	60	5	25	214	100.5	680.26	1415.12
26	60	5	25	214	100.24	697.03	1410.65
27	60	5	25	214	100.42	692.75	1409.95
28	50	3	20	154	90.68	409.21	1241.65
29	70	3	20	154	92.27	575.45	1225.38
30	50	7	20	154	92.73	419.15	1231.11
31	70	7	20	154	91.61	457.47	1240.78
32	50	3	30	154	90.45	404.89	1236.31
33	70	3	30	154	91.55	553.34	1207.21
34	50	7	30	154	95.40	451.50	1229.89
35	70	7	30	154	91.10	430.63	1262.75
36	50	3	20	274	91.40	476.23	1281.34
37	70	3	20	274	92.75	462.18	1231.49
38	50	7	20	274	92.76	444.46	1265.79
39	70	7	20	274	91.44	538.06	1285.26
40	50	3	30	274	90.69	421.68	1281.15
41	70	3	30	274	90.01	436.44	1255.34
42	50	7	30	274	92.52	457.89	1308.67
43	70	7	30	274	91.70	419.41	1225.44
44	50	5	25	214	96.98	581.61	1341.40
45	70	5	25	214	98.23	594.95	1322.79
46	60	3	25	214	96.13	638.54	1349.27
47	60	7	25	214	96.76	591.86	1319.41
48	60	5	20	214	96.87	563.67	1339.89
49	60	5	30	214	97.57	547.61	1336.50
50	60	5	25	154	98.91	585.03	1304.88
51	60	5	25	274	97.18	580.41	1356.03
52	60	5	25	214	99.35	650.43	1396.44
53	60	5	25	214	99.35	649.98	1419.88
54	60	5	25	214	101.05	667.55	1405.48

[X_1: ethanol concentration (% v/v); X_2: extraction time (min); X_3: solvent-solid ratio (mL/g); X_4: microwave power (W) and Y_1: TPC (mg GAE/g DW); Y_2: radical scavenging activity by DPPH (μmol TE/g DW); Y_3: radical scavenging activity by ABTS (μmol TE/g DW)].

Effects of linear, quadratic and cross-product coefficients of second-order models on TPC, antioxidant activity according to DPPH and ABTS assays are shown in Table 4. The R^2 values of the TPC, antioxidant activity according to DPPH and ABTS assays were found to be 0.968, 0.928 and 0.916 respectively, whereas the adjusted R^2_{Adj} values were 0.957, 0.902 and 0.885 respectively. The adjusted determination coefficients R^2_{Adj} were comparable to determination coefficients R^2 indicating that the models were highly significant (Myers & Montgomery, 2002).

Table 4. Estimated regression coefficients of second-order models of different response variables.

Term	TPC, Y_1 (mg GAE/g DW)		RSA by DPPH, Y_2 (μmol TE/g DW)		RSA by ABTS, Y_3 (μmol TE/g DW)	
	Regression coefficient	***P*-value**	**Regression coefficient**	***P*-value**	**Regression coefficient**	***P*-value**
Constant B_o	99.54179	<.0001*	647.75611	<.0001*	1381.1099	<.0001*
Linear						
B_1	-0.104444	0.3735	23.226111	<.0001*	-10.92167	0.0029*
B_2	0.4077778	0.0011*	-10.82306	0.0263*	5.8966667	0.0947
B_3	-0.038611	0.7411	-11.26611	0.0211*	-0.929167	0.7887
B_4	-0.322778	0.0083*	-3.304167	0.4850	18.007778	<.0001*
Quadratic						
B_{11}	-1.861852	<.0001*	-43.76167	0.0011*	-36.41148	0.0003*
B_{22}	-2.636852	<.0001*	-19.20917	0.1295	-34.09648	0.0006*
B_{33}	-2.014352	<.0001*	-80.65167	<.0001*	-26.53398	0.0059*
B_{44}	-1.191852	0.0004*	-47.80917	0.0004*	-36.20148	0.0003*
Cross-product						
B_{12}	-0.7325	<.0001*	-17.63906	0.0010*	3.913125	0.2905
B_{13}	-0.330625	0.0105*	-10.76719	0.0365*	-3.65125	0.3235
B_{23}	0.265625	0.0371*	1.4240625	0.7761	-1.336875	0.7163
B_{14}	0.06375	0.6074	-15.81844	0.0029*	-7.824375	0.0384*
B_{24}	-0.15125	0.2264	14.120312	0.0071*	-1.7025	0.6436
B_{34}	-0.355625	0.0063*	-9.747812	0.0571	1.199375	0.7443
R^2	0.968259		0.92794		0.915536	
R^2_{Adj}	0.956865		0.902073		0.885215	

* *Terms with P-value < 0.05 are significant at α = 0.05.*

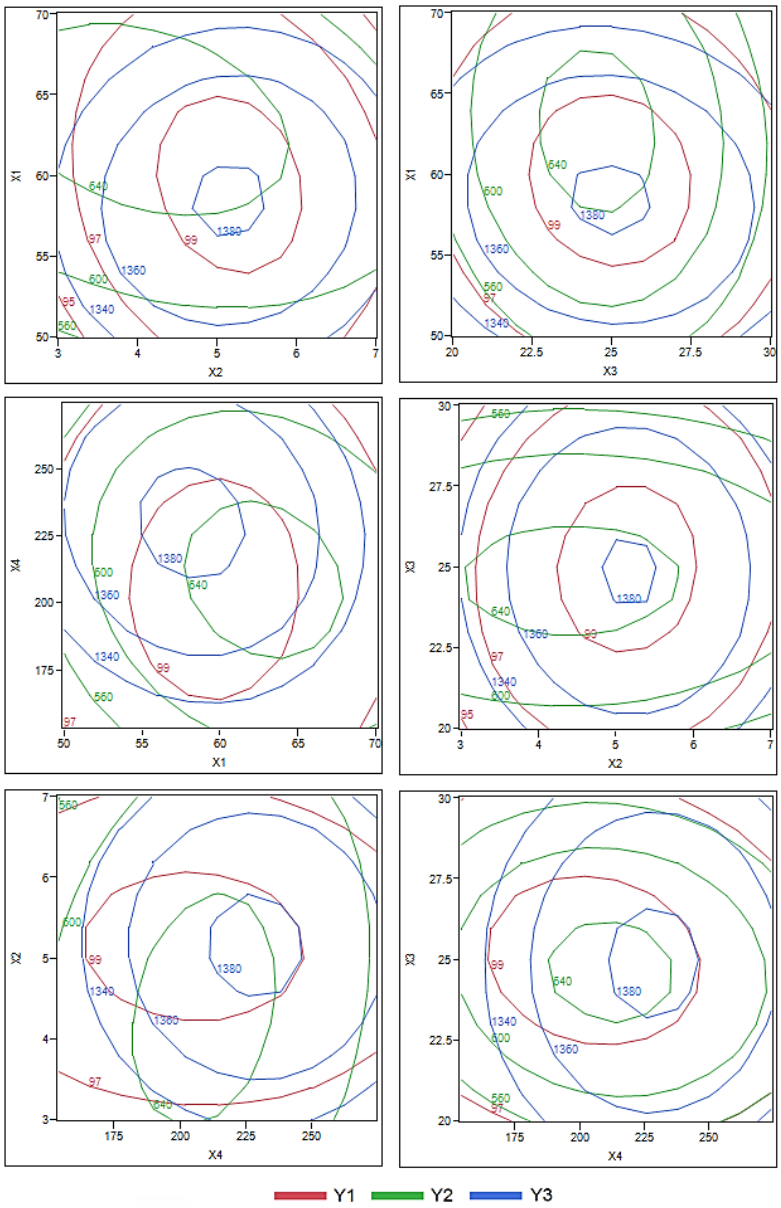

Figure 1. Effects of four investigated factors on three chosen response values. [X_1: ethanol concentration (% v/v); X_2: extraction time (min); X_3: solvent-solid ratio (mL/g); X_4: microwave power (W) and Y_1: TPC (mg GAE/g DW); Y_2: radical scavenging activity by DPPH (μmol TE/g DW); Y_3: radical scavenging activity by ABTS (μmol TE/g DW)].

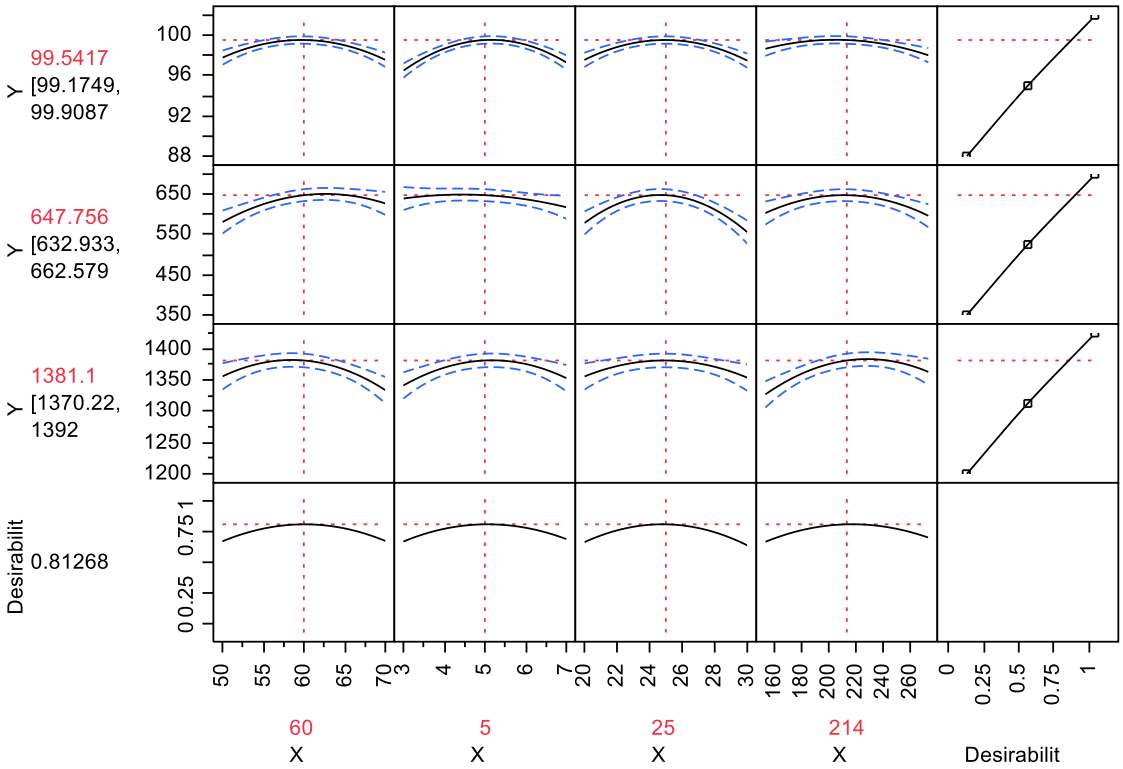

Figure 2. Prediction profiler of the optimization of polyphenol extraction. [X_1: ethanol concentration (% v/v); X_2: extraction time (min); X_3: solvent-solid ratio (mL/g); X_4: microwave power (W) and Y_1: TPC (mg GAE/g DW); Y_2: radical scavenging activity by DPPH (µmol TE/g DW); Y_3: radical scavenging activity by ABTS (µmol TE/g DW)].

Omitted terms which are insignificant at $p \geq 0.05$, the linear regression equations in terms of coded factors for the three models developed are as follows:

$Y_1 = 99.54 + 0.41X_2 - 0.32X_4 - 1.86X_1^2 - 2.64X_2^2 - 2.01X_3^2 - 1.19X_4^2 - 0.73X_1X_2 - 0.33X_1X_3 + 0.27X_2X_3 - 0.36X_3X_4$

$Y_2 = 647.76 + 23.23X_1 - 10.82X_2 - 11.27X_3 - 43.76X_1^2 - 80.65X_3^2 - 47.81X_4^2 - 17.64X_1X_2 - 10.77X_1X_3 - 15.82X_1X_4 + 14.12X_2X_4$

$Y_3 = 1381.11 - 10.92X_1 + 18.01X_4 - 36.41X_1^2 - 34.1X_2^2 - 26.53X_3^2 - 36.2X_4^2 - 7.82X_1X_4$

The model equations allowed the prediction of the effects of the four investigated factors on the TPC, antioxidative activity according to DPPH and ABTS assays. Six independent contour plots showing the effects of four investigated factors on three chosen response values are presented in Figure 1. These contour plots of response surfaces can be used to explore the dependence of chosen response values on the changes of process parameters around the center values developed in the CCD design.

As shown in Figure 1, an increase in ethanol concentration (X_1), extraction time (X_2), solvent-solid ratio (X_3), microwave power (X_4) up to a threshold level led to increased total polyphenol content (Y_1), radical scavenging activity by DPPH and ABTS assays (Y_2 and Y_3). Beyond this level, all the response values slightly decreased, which indicated that a greater TPC and antioxidant activity could be obtained when the moderate variables were selected.

Optimization of polyphenol extraction from custard apple peel was done to obtain maximum chosen response values. As observed, the developed contours allow the optimum combination of process variables to be ascertained. The desirability function was used as a tool for optimization in order to find the combinations of process parameters, which would achieve maximum TPC, antioxidative activity according to DPPH and ABTS assays.

The solution having highest desirability of 0.81 was finally selected (Figure 2).

An ethanol concentration of 60%, extraction time of 5 minutes, solvent-solid ratio of 25:1 (mL/g), and microwave power 214 W are predicted and selected as conditions for the polyphenol extraction from custard apple peel. Under the selected optimal conditions, experimental results showed an optimum TPC of 100.15 ± 0.68 mg GAE/g DW, 673.0 ± 20.44 μmol TE/g DW (DPPH assay) and 1409.6 ± 8.11 μmol TE/g DW (ABTS assay), compared to the predicted values of TPC of 99.54 mg GAE/g DW, 647.76 μmol TE/g DW (DPPH assay) and 1381.11 μmol TE/g DW (ABTS assay). The very good correlation between these results confirmed that the developed quadratic response surface model was adequate for reflecting the predicted optimization.

4. Conclusions

MAE can be successfully used to extract polyphenols from the custard apple peel. Ethanol composition, extraction irradiation time, solvent to solid ratio and microwave power show a strong effect on the TPC and antioxidant activities of the extracts. The response surface method can be used as a good tool to describe the dependency of investigated factors on the response values. Optimum conditions are selected at an aqueous ethanol concentration of 60%, extraction time of 5 minutes, solvent-solid ratio of 25:1 mL/g, and microwave power of 214 W. The model was successfully validated as the results of actual and predicted values of TPC, RSA by DPPH and ABTS showed a very good correlation.

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DETERMINATION OF PRESERVATIVES AND PHYSICOCHEMICAL PROPERTIES OF FRUIT JUICE-BASED BEVERAGES

Vern Mein Wong[1], Lejaniya Abdul Kalam Saleena[1], Pui Liew Phing[1]✉

[1]Department of Food Science and Nutrition, Faculty of Applied Sciences, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia

✉puilp@ucsiuniversity.edu.my; zoepui123@gmaill.com

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ABSTRACT

Fruit juices and juice type beverages may have benzoates, sorbates and sulphur dioxide as preservatives. Five different categories of fruit juice-based beverages, including fruit juices, fruit nectars, fruit juice drinks, fruit drinks, and fruit cordials, were analyzed for benzoic acid, sorbic acid, and physicochemical properties such as pH, titratable acidity, degree Brix, and sugar-to-acid ratio. 15 samples were detected to contain benzoic acid while 12 samples were found to contain sorbic acid. A combination of benzoic and sorbic acids were detected in 12 samples and the remaining 36 samples did not contain any benzoic acid or sorbic acid. All the fruit juice-based beverages complied with Food Regulations 1985 for benzoic acid or sorbic acid. Brand K tropical fruit juice drink base is the only product that did not comply with the specification of CODEX standard. No violation of labelling requirement was observed in all samples. All samples tested were considered as acid food as their pH readings were below 4.6. The titratable acidity of fruit juice-based beverages ranged from 0.14 to 2.71 % (w/v). The range of Brix values measured was from 10.2 to 60.9 °Brix. Sugar-to-acid ratios calculated were ranged from 16.9 to 275.7.

1.Introduction

Fruits are perishable entities. They cannot be kept intact over long periods and tend to deteriorate. As an alternative, juices are extracted from their respective fruits to reduce these losses and add value to agricultural export (Bates *et al.,* 2001). One of the major aims of food preservation was to limit or prevent the establishment of unwanted microbial flora in food items. A variety of preservation technologies have been developed to increase the shelf-life of food goods, not only by suppressing microbial growth, but also by preserving the antioxidant capacity to meet the demands of customers (Sreerupa *et al.,* 2014). Today, the demand for these beverages increases, where the world trade has accelerated over the last decade with developing countries achieving over 60% of fruit juice exports. There are different types of fruit juice-based beverages in the market. Fruit juice is the fluid expressed from fruits by comminute, crushing, and pressing or the reconstituted product of concentrated juice and potable water (Bates *et al.,* 2001; Legal Research Board, 2010). Fruit nectar is generally made by blending a proscribed minimum percentage of fruit juices, ranging from 25 to 50 % by weight, with water and permitted sweetening substance (Bates *et al.,* 2001). On the other hand, according to Food Regulations (1985), fruit juice drink contains at least 35 % (w/v) of fruit juice, while fruit drink contains not less than 5 % (w/v) of fruit juice and fruit cordial is composed of syrup and the juice of one or more types of fruit. All fruit cordials

require dilution before drinking (Legal Research Board, 2010).

Beverage manufacturing companies should ensure that every batch of their product meets consumer demand and is safe for consumption. In order to meet these quality targets, test parameters such as preservatives, pH, titratable acidity and soluble solids are used as one of the major indicators to evaluate the manufactured fruit juice products (Taylor, 2007). Sorbic acid and its derivatives are widely used to inhibit the growth of yeasts, molds, and some aerobic Gram-positive bacteria (Tucker and Featherstone, 2011). Benzoic acid and its derivatives have a similar mode of action to sorbates, but they are generally used to inhibit the growth of yeasts and molds (Taylor, 2006). Since their inhibitory effects are more effective at low pH values, they are suitable to be incorporated into fruit juice-based beverages. Chemical preservatives potassium sorbate and sodium benzoate are widely found in fruit juice and soft beverages (Magomya *et al.*, 2020). Although preservatives such as benzoic acid and sorbic acid are permitted in fruit juice beverages, the levels should not exceed the safety limits as they can be harmful to human health at high concentrations (Dong and Wang, 2006). The higher the concentration of Benzoic acid the lower the rate of growth of the microbial isolates (Oladipo *et al.*, 2010). Besides meeting quality targets, manufacturers should also ensure their product is in compliance with its respective label claim. Some unethical manufacturers tend to actively hide the actual content of fruit juices and adulterate juices with sugar, water or inferior juices (Bates *et al.*, 2001). In some cases, products labelled with "no preservatives added" may actually contain detectable number of preservatives. However, the advancements in analytical chemistry and instrumentation today make adulteration easier to detect (Nagy and Wade, 1995).

Commercially accessible fruit juices are drunk by people of all ages all over the world, and if not properly handled, this healthful drink can be harmful to human health (Ahmed *et al.*, 2018). This research aims to study the amount of benzoic and sorbic acids present in fruit juice-based beverages and to compare their amount with Food Regulations 1985 and CODEX general standard. Besides determining the amount of these preservatives in fruit juice beverages, parameters such as pH, soluble solids content and titratable acidity are evaluated in this research.

2. Materials and methods

2.1. Materials

A total of 75 samples were purchased from local supermarkets and hypermarkets in Klang Valley. In this research, fruit juices and juice beverages were classified into five categories: fruit juice, fruit nectar, fruit cordial, fruit juice drink and fruit drink. 15 samples of five varieties were chosen from each category for analysis.

2.1.1. Preparation of Samples

Ready-to-drink fruit juice beverages were centrifuged at 4500 rpm, at the temperature of 20°C for 15 minutes (Eppendort, Germany) and the supernatant diluted with 1: 10 ratio. On the other hand, fruit beverage concentrates, were reconstituted with ultrapure water (1: 100 ratio) before centrifugation.

2.2. HPLC Analysis

The HPLC analysis was performed using Agilent 1200 series HPLC system (Agilent Technologies, United States of America) equipped with vacuum degasser, G1311A Quaternary Pump, G1329A Auto-sampler, G1316A Column Thermostat, column compartment and G1315D Diode Array Detector. The chromatographic separation was done on ZORBAX Eclipse XDB-C18 analytical column (5 µm, 150 mm x 4.6 mm) at the wavelength of 235 nm. Methanol-acetate buffer (pH 4.4) were used as mobile phase (Saad *et al.*, 2005), while benzoic and sorbic acid were used a standard, with equation of 30.289x+5.1221 and 59.931x+41.833, respectively with R^2= 0.99. Each sample was analyzed for 15 minutes, with a flow rate of 1 mL per minute, and the injection volume was 10 µL.

2.3. pH and Titratable Acidity

The pH of the samples was measured using a digital pH meter (Mettler Toledo, USA) (Chang *et al.,* 2020). Titratable acidity was estimated according to Pui *et al.* (2018), where fruit juice was titrated with 0.1 M NaOH solution until the color turned from clear to pink.

2.4. Brix value and Brix-to-Acid ratio

Three drops of the juice were placed measured with refractometer (0-32 °Brix) in accordance to Pui *et al.* (2018). The Brix-to-acid ratio of fruit juice-based beverages was determined following the equation below:

Equation (1):

$$\text{Brix-to-acid Ratio} = \frac{\text{Degree Brix value } (^{\circ}Bx)}{\text{Titratable acidity (g per 100 mL)}}$$

2.5. Statistical analysis

All the data were analyzed using Minitab 17 statistical software (Minitab Inc., USA). One-way analysis of variance (ANOVA) and Tukey's HSD test was used to determine the significant differences ($p \leq 0.05$) among the fruit juice beverage samples tested. All the parameters tested in this study were determined in triplicate.

3.Results and discussions

3.1. Analysis of Benzoic Acid and Sorbic Acid in Fruit juice-based Beverages with HPLC

Table 1, figure 1 showed levels of benzoic and sorbic acids in five different categories of fruit juice-based beverages, which include fruit juice, fruit nectar, fruit juice drink, fruit drink, and fruit cordial. Among these 75 samples, 15 samples were detected to contain benzoic acid, while 12 samples were found to contain sorbic acid. A combination of benzoic and sorbic acids was detected in 12 samples, and the remaining 36 samples did not contain any benzoic acid or sorbic acid. As shown in Table 1, Brand K's tropical fruit juice drink base has the highest level of benzoic acid with the concentration of 690.0 ppm while Brand D orange drink has the highest level of sorbic acid with the concentration of 256.9 ppm. In general, fruit juice drinks have the highest average amount of benzoic acid, followed by fruit cordials, fruit drinks, and fruit juices. On the other hand, fruit drinks contained the highest average concentration of sorbic acid, followed by fruit juice drinks, fruit cordials, and then fruit juices. From the label claims Brand C pineapple juice and Brand D orange juice were purely extracted from fruits. Others were made by reconstituting concentrated juices. Brand E apple juice contained both benzoic and sorbic acids at the concentrations of 149.4 ppm and 104.7 ppm, respectively. All the tested fruit nectars complied with their label claims and did not violate Food Regulations 1985 and CODEX general standards for benzoic acid or sorbic acid. All the tested fruit juice drinks also complied with Food Regulations 1985. Brand K's tropical fruit juice drink base did not comply with the specification of the CODEX standard as its benzoic acid level has exceeded 600 ppm.

Drinks made of different fruits will give different intrinsic properties such as acidity and chemical composition. The differences in these properties will affect the type, and the number of chemical preservatives added. Here, some category of fruit juice drink has only declared the presences of permitted preservatives without indicating the type of preservative incorporated. However, both Brand K tropical fruit juice drink base and blackcurrant fruit juice drink claimed the usage of sulphur dioxide in their respective products. Since both of these beverages were found to contain benzoic acid, it could be predicted that these two products were added with more than one type of preservatives. Hence, further inspection of the products must be performed to measure other preservatives that may present. None of the fruit drinks tested in this study has exceeded the legal limits for benzoic acid or sorbic acid imposed by Food Regulations 1985. Among all the five types of fruit cordials, Table 1 presented, the highest amount of benzoic acid was detected in Brand R mixed fruit cordial at the concentration of 494.3 ppm. Brand S lychee cordial has the highest amount of sorbic acid at the concentration of 106.9 ppm. All the products in this group were

by the requirement of Food Regulations for both preservatives and have complied with CODEX general standard for benzoic acid. According to fruit cordial label, another preservative known as sodium metabisulphite (E 223) was added. Thus, for preservatives besides benzoic and sorbic acids, other methods must be developed to measure and quantify other kinds of preservatives and also to determine the compliance of products with label claims and regulations.

The study indicated a preference for incorporating benzoates over sorbates into fruit juice-based beverages even though sorbates are less toxic and less obstructive in terms of taste and allergic reactions than benzoates (Taylor, 2006; World Health Organization, 2000; Tfouni and Toledo, 2002). This may be due to the lower price of benzoates and the higher solubility of benzoates (World Health Organization, 2000; Mahindru, 2008). Preservatives such as benzoic acid, sorbic acid, methyl paraben, and propyl paraben were identified and quantified concurrently in 50 different fruit juice products using a unique RP-HPLC technique that was designed, verified, and used (Islam *et al.*, 2019). Mahmoud *et al.* (2017) analysis sorbic acid and benzoic acid in different food commodities using reversed- phase high performance liquid chromatography (RP-HPLC). High-pressure liquid chromatography was used to detect sorbic acid and benzoic acid in yoghurt, tomato and pepper paste, fruit juices, chocolates, soups, and chips in Turkey (HPLC) (Cakir and Cagri-Mehmetoglu, 2014).

Brand E apple juice, Brand O tropical fruit drink base, Brand S lychee cordial, and Brand T mango cordial contained both benzoic and sorbic acids. This is because benzoic acid can act synergistically with other preservatives. The combinations of benzoic acid and sorbic acid have been reported to inhibit many bacterial strains better than either of these alone (Tucker and Featherstone, 2011; Taylor, 2006; Fellows, 2000). Ekanem and Ekanem (2018) suggest that a combination of chemical and natural preservation, as well as cooling, was ideal for the long-term preservation of apple juice. A total of 36 fruit juice beverage samples did not contain any benzoic and sorbic acids. Manufacturers today apply hurdle principles to preserve the quality of fruit juice beverages (Tucker and Featherstone, 2011; Taylor, 2006; Fellows, 2000). All the samples analyzed are low acid food. By applying pasteurization, spoilage micro-organisms can be destroyed (Bates *et al.*, 2001; Fellows, 2000). Also, for some products, for instance, the cranberry and mixed fruit juices in this study were produced by using aseptic technology. This processing, together with other barriers that combat spoilage are sufficient to destroy harmful micro-organisms, and therefore, benzoates and sorbates, can be omitted to reduce the cost. Among all the 36 samples that could not detect the presence of benzoic acid or sorbic acid, only 6 samples have claimed to contain permitted preservatives. In this case, further studies should be implemented to quantify other possible preservatives.

Even though some category of fruit juice has declared the absence of preservative on their respective label claims. However, a traceable amount of benzoic acid was detected in cranberry and mixed fruit juice because cranberries contain a natural amount of benzoic acid at approximately 150 ppm when calculated as sodium benzoate (Coppola and Starr, 1988; Pylypiw and Grether, 2000). Due to the natural occurrence of benzoic acid in cranberry juice, it was then concluded that all the fruit juice products analysed in this study did not violate the regulation of labelling. The differences in level of preservatives for the same type of product could be caused by the variation in the combined or synergistic activity of several additives, intrinsic product parameters such as composition and acidity, and extrinsic factors such as processing temperature, storage atmosphere, and temperature (Dauthy, 1995). No benzoic acid or sorbic acid was detected in all beverages categorized under the group of fruit nectar. This was because the intrinsic characteristics of these sample products, together with effective processing methods, were adequate to combat spoilage. Therefore,

the usage of benzoic acid or sorbic acid as chemical preservatives could be omitted (Dauthy, 1995). Non-thermal treatment seems to be a promising and practical method for preserving fruit juice and beverages. The goods made using these processes have a number of advantages over typical thermal processing, including the preservation of sensory attributes and nutritional contents (Rupasinghe and Yu, 2012).

Food Regulations 1985 has classified fruit juice drinks, fruit drinks, and fruit cordials as soft drinks. The maximum level of benzoic acid or sorbic acid permitted by this regulation is 350 ppm for ready-to-drink soft drinks and 800 ppm for soft drinks requiring dilution. CODEX general standard has defined fruit juice drinks, fruit drinks, and fruit cordials as non-carbonated water-based flavoured drinks. The maximum level of benzoic acid allowed by CODEX commodity committees is 600 ppm. However, this standard did not specify the maximum allowable concentration of sorbates in these non-carbonated water-based flavoured drinks. This is because sorbates are less toxic than benzoates (Taylor, 2006). Sorbates are generally considered to be among the safest food preservatives in use, and therefore, only Acceptable Daily Intake (ADI) of sorbic acid was estimated at 25 mg/kg body weight (Taylor, 2006; Wood *et al.,* 2004).

As per the FAO/WHO Expert Panel on Food Additives, daily intakes of benzoic acid and sorbic acid should be 5 mg/kg/d and 25 mg/kg/d, respectively. Excessive amounts, on the other hand, might result in metabolic acidosis, seizures, asthma, and allergic responses, among other things (Chaojian *et al.,* 2019). Different countries have there on regulations, the addition of any preservatives to fruit juices is likewise prohibited by the Turkish Food Codex. The permissible daily intakes in Turkey for both preservatives were 0–5 mg benzoic acid intake/kg bodyweight and 0–25 mg sorbic acid intake/kg bodyweight. Similarly, the typical Portuguese population's ADIs for benzoic acid and sorbic acid are 0.25 mg intake/kg bodyweight and 0.17 mg intake/kg bodyweight, respectively, representing 4.9 percent and 0.68 percent of the ADI. Furthermore, the typical consumer's estimated benzoate and sorbate intakes in Brazil were found to be substantially below the ADIs, ranging from 0.3 to 0.9 mg/kg body weight and 0.2 mg/kg body weight to 0.3 mg/kg body weight, respectively (Cakir and Cagri-Mehmetoglu, 2014).

3.2. pH Measurement of Fruit juice-based Beverages

Table 2 exhibits the pH readings of fruit juice-based beverages tested, where it ranged from 3.09 to 4.26. Brand K tropical fruit juice drink base has the lowest pH value, and Brand Q lychee cordial has the highest pH value. On the other hand, Figure 2 illustrated the differences in pH values for five different categories of fruit juice-based beverages.

According to Table 2, Brand A cranberry and mixed fruit juice have the lowest pH value among all the fruit juices. The manufacturer of Brand A cranberry and mixed fruit juice has claimed that the particular product was made up of 55% grape juice, 30% apple juice, and lastly, 15% of cranberry juice. The presence of cranberry juice has lowered the overall pH of the beverage blend. It also indicated the differences in pH values between apple juices of two different brands. According to the beverages' respective labels, Brand B apple juice was not added with sugar while Brand E apple juice was added with cane sugar. In the absence of additional sugar, the pH value of Brand B apple juice was lower than that of Brand E apple juice. The pH reading for Brand C pineapple juice was 3.59, and it was similar to the pH value of the pineapple fruit fleshes studied by Bartolome *et al.* (1994), where the pH values for both Red Spanish and Smooth Cayenne cultivars of pineapple fruits were 3.49 and 3.54, respectively. The reason was that the manufacturer of Brand C pineapple juice produced the juices from fresh pineapple fruits, not from concentrate.

Brand K tropical fruit juice drink base has the lowest pH reading not only in the category of fruit juice drink but also among all the

beverages experimented. Under the same category as Brand K products, Brand B orange juice drink without sugar has the highest pH value of 4.03. For fruit drink, there were two types of orange drinks with different brands. Even though both drinks were made from the same fruit juice type, the pH values were different. As presented in Table 2, Brand Q lychee cordial has the highest pH value of 4.26 among all the tested fruit cordials, while Brand R lemon cordial has the lowest pH value of 3.45.

The natural pH of fruits may differ depending on the fruit cultivar, cultivation practices, harvest season, maturity, and the handling of harvest and post-harvest (Bates *et al.,* 2001). Table 2 exhibits the pH readings and Figure 2 illustrated the differences in pH values for five different categories of fruit juice-based beverages. Fruit cordials were shown to have the highest pH value, followed by fruit juices, fruit nectars, fruit juice drinks, and lastly fruit drinks. Fruit cordials contained the highest level of sugar and can only be consumed upon dilution. Hence, their pH values would be higher as compared to ready-to-drink products. Wilbur and Ronald, (2001) stated that cranberry juice alone had the lowest pH value among all the other fruit juices. Due to its low pH and high tartness level, it was often blended with other types of fruit juice to produce beverage blends. Grape juice was found to be more acidic than orange juice and pineapple juice when the three liquids were compared. The pH dropped the most after drinking grape juice, followed by orange and pineapple juice, in that order (Mehta *et al.,* 2019).

Bates *et al.* (2001) also had pointed out that sour cherry juice was amenable to blends with less acidic juices or as nectar with added sugar due to its tartness level. Since sour cherry fruit was more acidic than other fruits used to make fruit nectar in this study, its pH reading was the lowest. A study by Grenby *et al.* (1989) has shown that the pH of orange drink and low-sugar orange drink were 2.7 and 3.5, correspondingly. Both orange drinks analysed in this study have higher pH values than the product samples. Lemon cordial was made from lemon juice. Since the natural pH of lemon fruit is lower than other fruits used to produce the tested fruit cordials, the pH of lemon cordial would be lower than other fruit cordials (Bates *et al.,* 2001; Wilbur and Ronald, 2001).

3.3.Titratable Acidity of Fruit juice-based Beverages

The titratable acidity for all the samples tested were shown in Table 2. The titratable acidity of fruit juice-based beverages ranged from 0.14 to 2.71% (w/v), with Brand O tropical fruit drink base having the highest percentage of predominant acid of 2.7% (w/v) while Brand Q lychee cordial having the lowest percentage of predominant acid of 0.1% (w/v). The percentages of predominant acid contained in five different categories of fruit juice-based beverages were shown in Figure 2. On average, the highest percentage of predominant acid was found in fruit juice drinks, followed by fruit drinks, fruit cordials, fruit juices, and lastly fruit nectars. For pineapple juice and cranberry and mixed fruit juice, the titratable acidity, calculated as anhydrous citric acid, shall not exceed 3.5% (w/v). Both of them did not violate the legal limit, Food Regulations 1985. Brand D pure orange juice did not violate the legal limit as it contained an average of 0.71 g of anhydrous citric acid in 100 mL. Values of Brand C pineapple juice, which contained only 0.64 g of acid per 100 mL.

In the group of fruit nectar product, sour cherry nectar tested contained the highest percentage value of acid with 0.79 % (w/v). The lowest acid content was found in guava nectar containing an average of 0.18 % (w/v) of anhydrous citric acid. As indicated in Table 2, Brand K tropical fruit juice drink base has the highest percentage value of predominant acid in the category of fruit juice drink. The percentage of acid presented in Brand B pink guava juice drink was the lowest as it contained only 0.28 % (w/v) of anhydrous citric acid. Brand O tropical fruit drink base containing 2.71 % (w/v) of anhydrous citric acid has the highest percentage of acid not only in the category of fruit drink product but also among all the beverages in the

study. The lowest acid content was found in Brand M apple drink with 0.16 g of malic acid in 100 mL. On the other hand, Brand R lemon cordial contained the highest amount of anhydrous citric acid content with 1.94 % (w/v) due to the natural tartness level of lemon fruits (Bates *et al.*, 2001). Brand Q lychee cordial has the lowest percentage value of predominant acid, with only 0.14 % (w/v). As indicated in Table 2, the total acid contents for lychee cordials with two different brands were similar to each other.

Juices are liquids that many people take on a regular basis, with children being among the most avid users. Fruit and vegetable juices are made by extracting the natural liquid from the fruits or vegetables. The endogenous pH, titratable acidity, and ascorbic acid content of juices widely ingested by children are evaluated by Ogbeide *et al.* (2020). The Malaysian government has set the titratable acidity standard for fruit juices (Legal Research Board, 2010). No acidity specification was set for other categories of beverages studied. For apple juice, titratable acidity was calculated as malic acid. The value shall not be less than 0.3 g and not more than 0.8 g of acid in 100 mL measured at 20°C. Malaysian Food Regulations also stated that the titratable acidity of orange juice, calculated as anhydrous citric acid, shall contain not less than 0.65 g and not more than 1.5 g of acid in 100 mL. Bartolome *et al.* (1994) had researched the titratable acidity of fresh pineapple. The titratable acidity of Red Spanish pineapple and Smooth Cayenne pineapple were 1.2 and 0.9 grams of acid per 100 mL, respectively. The differences in processing, fruit cultivar, and fruit maturity level are the factors that cause these variations in pH (Bates *et al.*, 2001). Sour cherries are generally more acidic than peaches, guavas, and apricots (Bates *et al.*, 2001). For this category, the titratable acidity of concentrated fruit juice drink was higher than that of ready-to-drink products. According to Cairns *et al.* (2002), the titratable acidity of drinks was reduced as the drink became more dilute.

Table 1. Amount of benzoic acid and sorbic acid present in five different categories of fruit juice-based beverages

Category	Product name	Label Claim	Benzoic acid (ppm)*	Sorbic acid (ppm)*
Fruit juice	Cranberry and mixed fruit juice (Brand A)	No preservatives	6.1 ± 0.3^{bG}	ND^{bH}
	Apple juice (Brand B)	No preservatives	ND^{cG**}	ND^{bH}
	Pineapple juice (Brand C)	No preservatives	ND^{cG}	ND^{bH}
	Orange juice (Brand D)	No preservatives	ND^{cG}	ND^{bH}
	Apple juice (Brand E)	Permitted preservative	149.4 ± 0.6^{aF}	104.7 ± 0.7^{aEF}
Fruit nectar	Peach nectar (Brand F)	No preservatives	ND^{aG}	ND^{aH}
	Guava nectar (Brand G)	No preservatives	ND^{aG}	ND^{aH}
	Sour cherry nectar (Brand H)	No preservatives	ND^{aG}	ND^{aH}
	Apricot nectar (Brand I)	No preservatives	ND^{aG}	ND^{aH}
	Multivitamin 12 fruit nectar (Brand J)	No preservatives	ND^{aG}	ND^{aH}
Fruit juice drink	Orange juice drink without sugar (Brand B)	Permitted preservative	ND^{dG}	238.4 ± 2.3^{aB}
	Pink guava juice drink (Brand B)	Permitted preservative	ND^{dG}	182.3 ± 1.0^{bC}

	Tropical fruit juice drink base (Brand K)	Permitted preservatives (contains sulfur dioxide)	690.0 ± 2.8^{aA}	ND^{cH}
	Blackcurrant fruit juice drink base (Brand K)	Permitted preservatives (contains sulfur dioxide)	484.5 ± 3.8^{bB}	ND^{cH}
	Pomegranate and apple juice drink (Brand L)	Sodium benzoate (E211)	232.2 ± 1.4^{cE}	ND^{cH}
Fruit drink	Apple drink (Brand M)	No preservatives	ND^{bG**}	ND^{dH}
	Orange drink (Brand D)	Permitted preservative	ND^{bG}	256.9 ± 1.1^{aA}
	Orange drink (Brand N)	Permitted preservative	ND^{bG}	79.8 ± 1.0^{cG}
	Tropical fruit drink base (Brand O)	Permitted preservatives	312.4 ± 3.4^{aD}	148.7 ± 3.2^{bD}
	Tropical mixed fruit drink (Brand P)	No preservatives	ND^{bG}	ND^{dH}
Fruit cordial	Lychee cordial (Brand Q)	Permitted preservative	ND^{dG}	ND^{cH}
	Lemon cordial (Brand R)	Sodium metabisulphite (E223)	ND^{dG}	ND^{cH}
	Mixed fruit cordial (Brand R)	Sodium benzoate (E211) and sodium metabisulphite (E223)	494.3 ± 4.3^{aB}	ND^{cH}
	Lychee cordial (Brand S)	Permitted preservatives	382.0 ± 4.2^{cC}	106.9 ± 2.7^{aE}
	Mango cordial (Brand T)	Permitted preservative	469.6 ± 4.2^{bB}	95.9 ± 2.9^{bF}

***** Average ± S.E.M. of three determinations.
****** ND, no detection
[a-e] Means with different letters within the same category were significantly different at $p \leq 0.05$.
[A-H] Means with different letters between categories were significantly different at $p \leq 0.05$.

Table 2. Physicochemical properties of fruit juice-based beverages

Category	Product name	pH*	Predominant acid	Percentage of acid (%) *	Brix value (°Brix) *	Brix-to-acid ratio*
Fruit juice	Cranberry and mixed fruit juice (Brand A)	3.31 ± 0.01^{eK}	Citric acid	0.41 ± 0.00^{cH}	14.5 ± 0.0^{aI}	35.5 ± 0.3^{aG}
	Apple juice (Brand B)	3.63 ± 0.01^{cF}	Malic acid	0.39 ± 0.01^{dH}	11.8 ± 0.1^{dK}	32.1 ± 0.3^{cG}
	Pineapple juice	3.59 ±	Citric acid	0.64 ±	14.2 ±	22.3 ±

	(Brand C)	0.01^{dG}		0.00^{bF}	0.0^{bJ}	0.2^{dH}
	Orange juice (Brand D)	4.01 ± 0.02^{aB}	Citric acid	0.71 ± 0.00^{aE}	12.0 ± 0.0^{cK}	16.9 ± 0.1^{eI}
	Apple juice (Brand E)	3.72 ± 0.01^{bD}	Malic acid	0.33 ± 0.00^{eHI}	11.0 ± 0.0^{eL}	34.5 ± 0.3^{bG}
Fruit nectar	Peach nectar (Brand F)	3.73 ± 0.01^{BD}	Citric acid	0.31 ± 0.00^{cI}	13.8 ± 0.0^{cJ}	44.4 ± 0.3^{bF}
	Guava nectar (Brand G)	3.96 ± 0.02^{aC}	Citric acid	0.18 ± 0.00^{dJ}	14.7 ± 0.1^{aI}	80.5 ± 2.3^{aCD}
	Sour cherry nectar (Brand H)	3.20 ± 0.03^{dM}	Malic acid	0.79 ± 0.00^{aE}	13.0 ± 0.0^{dJ}	17.3 ± 0.1^{dI}
	Apricot nectar (Brand I)	3.71 ± 0.01^{bD}	Malic acid	0.32 ± 0.00^{cI}	14.1 ± 0.0^{bJ}	45.5 ± 0.3^{bF}
	Multivitamin 12 fruit nectar (Brand J)	3.55 ± 0.02^{cH}	Citric acid	0.49 ± 0.00^{bG}	11.8 ± 0.0^{eK}	24.0 ± 0.2^{cH}
Fruit juice drink	Orange juice drink without sugar (Brand B)	4.03 ± 0.01^{aB}	Citric acid	0.53 ± 0.00^{cG}	10.2 ± 0.0^{eL}	19.2 ± 0.1^{eHI}
	Pink guava juice drink (Brand B)	3.72 ± 0.02^{bD}	Citric acid	0.28 ± 0.00^{eI}	11.4 ± 0.0^{dK}	40.7 ± 0.1^{aFG}
	Tropical fruit juice drink base (Brand K)	3.09 ± 0.01^{eN}	Citric acid	2.40 ± 0.03^{aB}	60.9 ± 0.1^{aA}	25.4 ± 0.3^{dGH}
	Blackcurrant fruit juice drink base (Brand K)	3.37 ± 0.01^{dJ}	Citric acid	1.31 ± 0.02^{bD}	52.0 ± 0.1^{bC}	39.7 ± 0.7^{bFG}
	Pomegranate and apple juice drink (Brand L)	3.63 ± 0.01^{cF}	Malic acid	0.39 ± 0.01^{dH}	13.2 ± 0.0^{cJ}	35.6 ± 0.9^{cG}
Fruit drink	Apple drink (Brand M)	3.36 ± 0.01^{dJ}	Malic acid	0.16 ± 0.00^{cJ}	12.2 ± 0.0^{bK}	81.3 ± 0.0^{aC}
	Orange drink (Brand D)	3.61 ± 0.01^{aFG}	Citric acid	0.28 ± 0.01^{bcI}	12.6 ± 0.1^{bK}	44.8 ± 0.6^{cF}
	Orange drink (Brand N)	3.55 ± 0.01^{cH}	Citric acid	0.32 ± 0.00^{bHI}	11.8 ± 0.1^{bK}	37.5 ± 0.5^{dG}
	Tropical fruit drink base (Brand O)	3.24 ± 0.02^{eL}	Citric acid	2.71 ± 0.10^{aA}	60.1 ± 0.1^{aB}	22.2 ± 0.8^{eH}
	Tropical mixed fruit drink (Brand P)	3.57 ± 0.01^{bG}	Citric acid	0.19 ± 0.00^{cJ}	11.6 ± 0.1^{bK}	59.5 ± 1.0^{bE}
Fruit cordial	Lychee cordial (Brand Q)	4.26 ± 0.01^{aA}	Malic acid	0.14 ± 0.01^{cJ}	37.4 ± 0.0^{dG}	275.7 ± 13.7^{aA}
	Lemon cordial (Brand R)	3.45 ± 0.01^{dI}	Citric acid	1.94 ± 0.04^{aC}	41.0 ± 0.1^{cF}	21.2 ± 0.5^{dHI}

	Mixed fruit cordial (Brand R)	3.67 ± 0.01^{bE}	Citric acid	0.60 ± 0.02^{bE}	41.6 ± 0.1^{bE}	69.2 ± 2.2^{cD}
	Lychee cordial (Brand S)	4.23 ± 0.01^{aA}	Malic acid	0.17 ± 0.01^{cJ}	32.4 ± 0.1^{eH}	200.6 ± 13.2^{bB}
	Mango cordial (Brand T)	3.60 ± 0.01^{cGF}	Citric acid	0.63 ± 0.02^{bF}	50.6 ± 0.0^{aB}	80.5 ± 2.7^{cC}

$^{a-e}$ Means with different letters within the same category were significantly different at $p \leq 0.05$.
$^{A-N}$ Means with different letters between categories were significantly different at $p \leq 0.05$.

Table 3. Label instructions of dilution for fruit cordials found in respective sample labels

Sample name	Dilution instruction on the label
Lychee cordial (Brand Q)	1 part of cordial to 5 parts of water
Lemon cordial (Brand R)	1 part of cordial to 4 parts of water
Mixed fruit cordial (Brand R)	1 part of cordial to 4 parts of water
Lychee cordial (Brand S)	1 part of cordial to 4 parts of water
Mango cordial (Brand T)	1 part of cordial to 7 parts of water

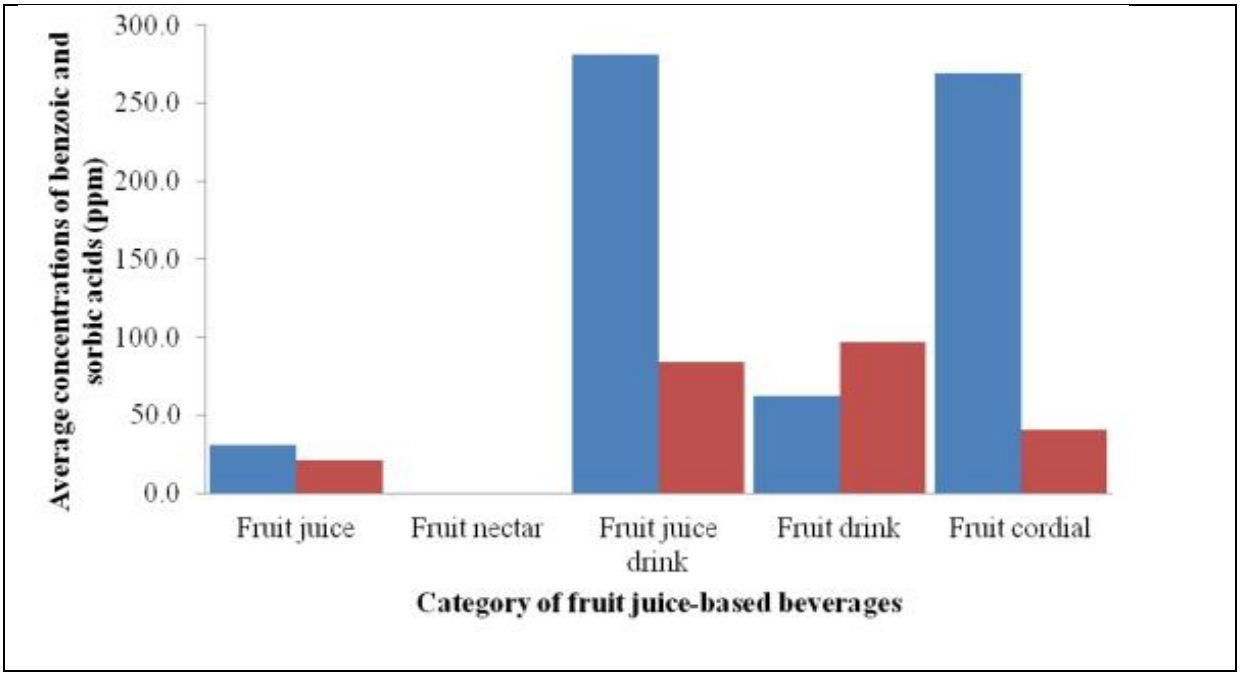

Figure 1. The concentrations of benzoic (blue) and sorbic acids (red) (ppm) found in five different categories of fruit juice-based beverage

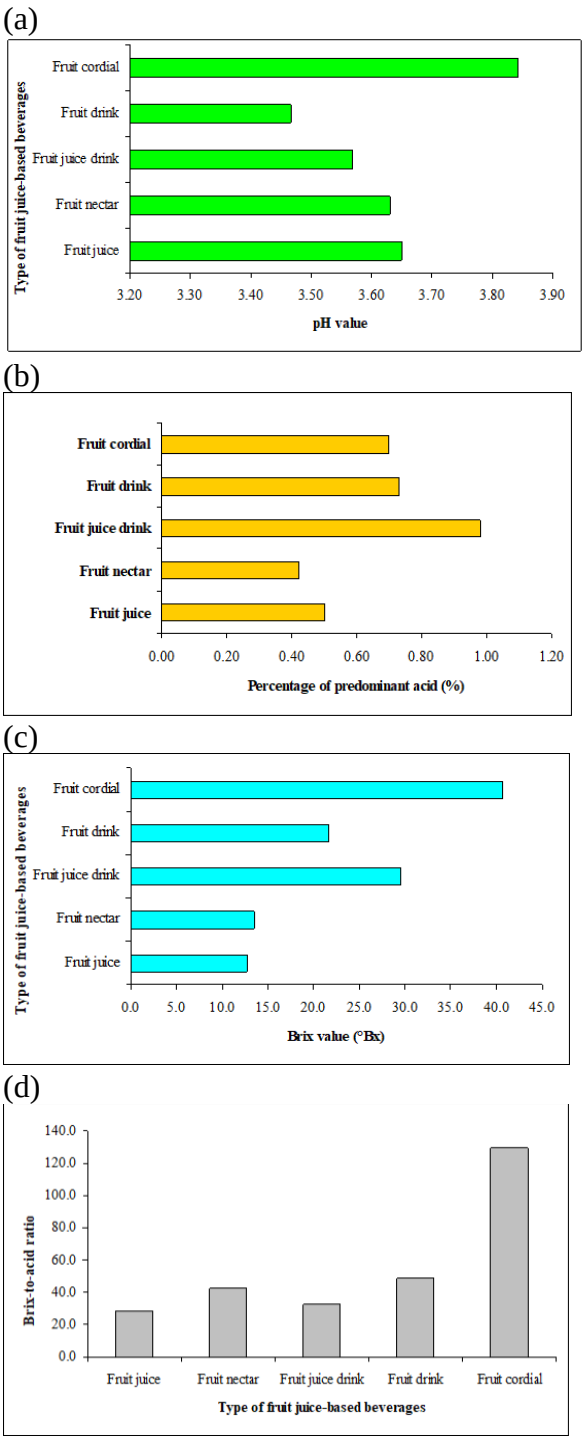

Figure 2. pH values (a), percentages of predominant acid (b), Brix (c) and Brix-to-acid ratios (d) of five different kinds of fruit juice-based beverages

3.4.The Brix value of Fruit juice-based Beverages

Table 2 presented the Brix values for five different categories of fruit juice-based beverages measured by using handheld refractometers. On average, Brand B orange juice drink without sugar has the lowest Brix value, which was 10.2 °Brix. Brand K tropical fruit juice drink base product was in the same group as Brand B orange juice drink but was marketed in the form of concentrate. It has the highest Brix value of 60.9 °Brix among all the samples tested. The Brix values for all the fruit juices tested ranged from 11.0 to 14.5 °Brix with the highest value found in Brand A cranberry and mixed fruit juice, and the lowest value found in Brand E apple juice. Brand E apple juice did not comply with the requirement as its value was below the minimum level imposed by Food Regulations 1985. The soluble solids content of orange juice was established not to be less than 10.5 g in 100 mL. Since Brand D orange juice, Brand A cranberry and mixed fruit juice and Brand C pineapple juice also did not violate the legal limits of Food Regulations 1985 as their Brix levels have exceeded the minimum percentage required. The Brix value must be more than 11.2°Brix for fruit juices made from concentrates. Brand B apple juice was following the specification, while the Brix level of Brand E apple juice was lower than the requirement. In the category of fruit nectar, the Brix levels of samples ranged from 11.8 to 14.7 °Brix. Mostly products label claim has complied with the labelling regulation of Food Regulations 1985.

As shown in Table 2, the highest Brix value of 50.6 °Brix was detected in Brand T mango cordial, and the lowest Brix value of 32.4 °Brix was detected in Brand S lychee cordial. Table 3 showed the label instructions of dilution for five different types of fruit cordials. According to the label instructions, a higher amount of water is required to dilute Brand T mango cordials to prepare their ready-to-drink soft drinks. This means that the dilution factor was the highest in Brand T mango cordials. Brand Q and Brand S fruit cordials were made from the same type of fruit juices. However, their Brix values were different from each other. The Brix level of Brand Q lychee cordial was higher than that of Brand S lychee cordial because a higher amount of water was needed to dilute the cordial.

Average Brix readings for five different kinds of fruit juice-based products were given in Table 2. Among all the 75 samples tested, fruit cordials have the highest average Brix value, followed by fruit juice drinks, fruit drinks, fruit nectars, and lastly fruit juices. Fruit cordials are concentrated products. When water is removed from beverages, the juice solid will gradually increase up to 10-fold (Bates *et al.,* 2001), and therefore increases the total soluble solid contents. According to Food Regulations 1985, apple juice shall contain not less than 11.5 g of soluble solids in 100 mL measured at 20 °C. CODEX general standard for soluble solids is classified into two: Brix level for reconstituted juice from concentrate and Brix level for single strength juice not from concentrate. The minimum Brix level requirement set by CODEX commodity committees was applicable only for single strength juice. Therefore, Brand A product containing different types of fruit juices was not compared with CODEX general standard. Under the current Food Regulations 1985, the total soluble solids content of fruit nectar shall not be less than 12 %. All of the fruit nectars tested were conformed to their specification limit. The minimum Brix level established by CODEX commodity committees for orange juice and pineapple juice is 10.0 °Brix and 11.2 °Brix, correspondingly. Both Brand C pineapple juice and Brand D orange juice have met the quality requirement.

The Brix levels for all the tested fruit juice drinks ranged from 10.2 to 60.9. Tropical fruit juice drink base has the highest Brix value, and orange juice drink without sugar has the lowest Brix value. Taylor (2007) has pointed out that Brix value was related directly to both the sugars and fruit acids. Since the percentage of fruit juice is not high in fruit juice drinks and the effect of fruit acids is not significant, the amount of added sugar becomes the main contributor to the Brix level. Fruit juice drinks are soft drinks. In Malaysia, the minimum Brix level required for soft drinks is not specified. Hence, no comparison was made between all the soft drinks and Food Regulations 1985. While for fruit drinks, the highest Brix value was found in tropical fruit drink base product as it was the only fruit drink which marketed in concentrated form. Other fruit drinks were sold in prepared forms. All the fruit cordials were manufactured in different concentration levels by different beverage companies. The differences in concentration of fruit cordials were compared by reading their respective label instructions for dilution.

3.5. The Brix-to-Acid ratio of fruit Juice-Based Beverages

The Brix-to-acid ratios of fruit juice-based products were presented in Table 2. According to the table, Brand D orange juice has the lowest ratio value of 16.9. Hence, its taste would be sourer as compared to others. Brand Q lychee cordial has the highest Brix-to-acid ratio of 275.7. Its sweetness taste was the highest among all the products tested. Figure 2 also shows the differences in the Brix-to-acid ratios between five different kinds of fruit juice-based beverages.

The Brix-to-acid ratios of fruit juices ranged from 16.9 to 35.5, with the highest value found in Brand A cranberry and mixed fruit juice and the lowest value found in Brand D orange juice. Food Regulations 1985 has specified the standard for the Brix-to-acid ratio of orange juice. The Brix-to-acid ratios for other types of fruit juice were not included in the standard. Cranberry juice provides tartness mouth feel to consumers who drink it. The strong sour sensation of pure cranberry juice may reduce consumer preferences towards the product. Therefore, it is more commonly blended with other types of fruit juice. For instance, the cranberry juice in Brand A product was blended with apple and grape juices.

Among all the fruit nectars, guava nectar has the highest sugar-to-acid ratio value. Its value was much higher than its counterparts of the same group. Sour cherry nectar has the lowest Brix-to-acid ratio. Fructose syrup was one of the ingredients added in Brand H sour cherry nectar. It played an important role in increasing the Brix-to-acid ratio of the product to an acceptable

level. On the other hand, in the group of fruit juice drink, Brand B pink guava juice drink has the highest Brix-to-acid ratio, and Brand B orange juice drink has the lowest Brix-to-acid ratio. Sugar was not added to the Brand B orange juice drink. Thus, its Brix value was the lowest in this group.

The sugar-to-acid ratios of fruit drinks ranged from 22.2 to 81.3, with the highest value found in Brand M apple drink and lowest value found in Brand O tropical fruit drink base. Table 2 has also shown that the Brix-to-acid ratios differed for orange drinks with different brands. Brand Q lychee cordial has the highest Brix-to-acid ratio of 275.7. Both Brand Q and Brand S lychee cordials have very high sugar-to-acid ratios as compared to other fruit cordial samples. The lowest sugar-to-acid ratio was found in Brand R lemon cordial with only 21.2. Lemon juice was more acidic as compared to other fruit juices contained in fruit cordials tested. As a result, its high percentage of acid has reduced the sugar-to-acid ratio.

Generally, the acidity of fruit juices would decrease with increasing maturity of fruits, or with increasing levels of sugars in resulting juice (Taylor, 2007). In the beverage industry, the Brix-to-acid ratio could be used to establish standard sensory, maintain the qualities of products, and also to minimize the effect of seasonal variation. The higher the Brix value as compared to the acid content of the juice, the higher the ratio value and the sweetness taste would increase as well. Fruit cordials have the highest average Brix-to-acid ratio value, followed by fruit drinks, fruit nectars, fruit juice drinks, and lastly fruit juices. This is because fruit cordials are concentrated products. Since their degree Brix values will be much higher than other ready-to-drink fruit juice-based products, the ratio of Brix to acid will be much higher. By using the blending method, the sugar-to-acid ratio can be adjusted to a value that meets consumer demand (Bates *et al.*, 2001). According to Bates *et al.* (2001), cherry cultivars range from extremely sour to low sweet acid with sugar-to-acid ratios from 7 to 35. The Brix value would then lower the average sugar-to-acid ratio.

According to the United States Code of Federal Regulations, juices extracted straight from a fruit or vegetable are considered 100% juice and must be stated as such. When reconstituted from juice concentrate, however, the US FDA defines 100% juice as Brix concentrations that are typical of those extracted from the fruit. Physical qualities including Brix concentration, acidity, Brix:acid ratio, colour, and flavour all have an impact on overall 100% juice quality (Roger *et al.*, 2015).

4. Conclusions

It was concluded that among all the 75-fruit juice-based beverage samples, 15 samples were found to contain benzoic acid with Brand K tropical fruit juice having the highest content, while 12 samples were found to contain sorbic acid. Brand D orange juice that contains highest content of sorbic acid. A preference for incorporating benzoates (cheaper price) over sorbates into fruit juice-based beverages were noted. A combination of benzoic and sorbic acids was detected in 12 samples for synergistic effect of bacterial inhibition, and the remaining 36 samples did not contain any benzoic acid or sorbic acid. All the fruit juice-based beverages studied did not violate the legal limit for benzoic acid or sorbic acid imposed by Food Regulations 1985 and no violation of the labelling requirement was observed. In general, physicochemical properties of fruit juice-based beverages such as pH, titratable acidity, total soluble solids content, and sugar-to-acid ratio were affected by factors such as fruit, specifications and company. Therefore, physicochemical properties may differ for products made of the same fruit juice type. Also, the natural pH of lemon and cranberry fruits was lower than other fruits. Thus, their physicochemical properties' values were most likely to be lower than other fruit juice-based beverages.

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