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Colloidal properties and stability of olive oil-in water emulsions stabilized by starch particles

Umer Farooq, Carla Di Mattia*, Marco Faieta, Federica Flamminii, Paola Pittia

Faculty of Bioscience and Technology for Agriculture, Food and Environment, University of Teramo, Teramo, Italy

***Corresponding Author:** Carla Daniela Di Mattia, Faculty of Bioscience and Technology for Agriculture, Food and Environment, University of Teramo, Via Renato Balzarini 1, 64100 Teramo, Italy. Email: cdimattia@unite.it

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PAPER

Abstract

In this study, olive oil-in-water emulsions (30% oil, v/v) were prepared by using high-pressure homogenization and different concentrations of modified corn starch particles (6–10% w/v). After a preliminary physical characterization, the modified starch particles were used to produce olive oil-in water (o/w) emulsions whose droplet size and distribution, flow behavior, microstructure, and physical stability were evaluated. The stabilization by Pickering phenomena was observed, as well as the formation of a starch network able to entrap oil particles. Increasing the starch concentration enhanced the emulsion physical stability by improving the oil particles' stabilization within the starch network.

Keywords: corn starch; high pressure homogenization; olive oil; o/w emulsions; Pickering emulsions

Introduction

Oil-in-water (o/w) emulsions are amongst the most widespread multiphasic systems and, because of their role in providing a proper structure, desirable appearance, and mouthfeel properties to lipid-containing matrices, they are of crucial importance in a wide range of emulsified products from the food, pharmaceutical, and cosmetic industry (Yang *et al*., 2017).

In recent years, studies on Pickering stabilization of the oil/water interface by solid particles which get absorbed on the interfacial layer and act as a physical barrier have been progressively increasing (Tavernier *et al*., 2016; Xiao *et al*., 2016). Indeed, due to the high adhesion energy at the liquid–liquid interface, particle adsorption is considered to be much stronger in comparison to low molecular weight surfactants (McClements and Gumus, 2016; Ravera *et al*., 2020; Wu and Ma, 2016). Solid particles can thus reside at the interface of droplets, thereby providing Pickering emulsions with high resistance against coalescence and instability phenomena like Ostwald ripening.

In preliminary works, inorganic particles such as clay, silica, and latex were used as stabilizers in Pickering emulsions (Abend *et al*., 1998; Aveyard *et al*., 2003; Binks and Lumsdon, 2000); however, due to rapid biodegradability, poor bioavailability, and above all, the increasing attention of consumers toward more natural ingredients, their use in the food industry was largely limited, highlighting the need of using natural biopolymer particles as stabilizers in Pickering emulsions (Berton-Carabin and Schroën, 2015; Dickinson, 2012).

Recently, starch has obtained an increased interest as a potential stabilizer biopolymer in Pickering emulsions for both food and pharmaceutical applications thanks to its low cost, high natural availability, nontoxicity, ease in production, and high bioavailability (Ge *et al.*, 2017; Lee and Chang, 2019; McClements and Gumus, 2016; Tavernier *et al.*, 2016). However, native starch particles are generally

not hydrophobic and present a low capacity to absorb at oil/water interfaces. Among the methods available to increase starch hydrophobicity, esterification is one of the most commonly used (Khan and Ahmad, 2013). Indeed, esterification improves the performance of starches by making them more amphiphilic and hence more suitable for the stabilization of emulsions. Esterification is a commonly used process in which an acid or its derivative is used to react with the hydroxyl groups of starch, replacing them with ester groups which are more hydrophobic (Fang *et al*., 2002). As a result, a more hydrophobic starch with reduced retrogradation and improved emulsifying properties is produced (Ghanbarzadeh *et al*., 2011; Xie and Liu, 2004; Zhou *et al*., 2016). For esterification purposes, among the various organic acids used, citric acid, a trifunctional carboxylic acid, is most widely used due to its low cost, high effectiveness, and environmental sustainability (Jiangping *et al.*, 2019).

High pressure homogenization (HPH) is a technology applied for obtaining finely dispersed o/w emulsions characterized by higher physical stability and resistance. In general, a pre-homogenized, coarse, pre-emulsified mix of the oil and water phases is passed through a small valve under dynamic pressure to produce the final fine dispersion (Friberg *et al*., 2003). It is well known that HPH induces starch gelatinization; however, this high-pressure-induced gelatinization is significantly different form the heat-induced gelatinization as starch granules retain their structure and only little amount of amylose oozes out (Rubens and Heremans, 2000; Stute *et al*., 1996). Moreover, high-pressure-treated starch granules comprise two different zones: the inner zone, which is completely destroyed due to high pressure and forms a gel-like network, and an outer zone, which largely remains undisturbed (Błaszczak *et al*., 2005). So far the use of HPH is largely focused on stabilizing the traditional emulsions using the chemical surfactants and biomolecules while the use of HPH in stabilizing the Pickering emulsions is rarely investigated. To the best of authors knowledge so far no study is published which investigate the use of HPH in stabilizing the Pickering emulsions with starch particles, using olive oil as dispersed phase. While HPH is largely used to produce emulsions with conventional emulsifiers, its use in the formation of Pickering emulsions has been scarcely investigated and, to the best of authors' knowledge, no studies are available in the literature on its application to olive oil o/w emulsions stabilized with starch particles. Currently, very few works are available on starch-stabilized olive oil emulsions as well (Farajpour *et al*., 2020; Qian *et al*., 2020); in the work by Quian and coworkers, the use of corn starch nanocrystals with increasing levels of acetylation was explored to produce, by means of a rotor-stator device, Pickering olive oil emulsions characterized by different levels of viscoelasticity. In another work, Pickering olive oil emulsions stabilized by starch-zein nanoparticle complexes were studied as starting material to produce biocomposite edible films (Farajpour *et al.*, 2020; Qian *et al.*, 2020).

The aim of this work was, thus, to study the colloidal properties, flow behavior, and physical stability of olive oil-in-water emulsions obtained by means of HPH and stabilized with corn starch granules modified by esterification with citric acid. In a preliminary step, the physical properties, microstructure, and size of corn starch granules after esterification were initially evaluated. Moreover, to evaluate any likely effect induced by high dynamic pressure, the properties of the modified starch subjected to HPH at conditions similar to those applied during the emulsion preparation were also studied. Finally, the o/w emulsions were characterized for droplet size and distribution, flow properties, and physical stability over a storage time of 30 days.

Material and Methods

Experimental plan

The investigation was carried out according to the following steps:

Step I: Starch particles' modification by esterification with citric acid.
Step II: Emulsifying activity of modified starch toward olive oil.
Step III: Characterization of emulsions' colloidal properties and physical stability.

Materials

Native corn starch was provided by Roquette Italia S.P.A. (Cassano Spinola, Italy). Commercial extra virgin olive oil was purchased from a local market. Anhydrous citric acid (99.5% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

Methods

Starch particles' modification by esterification

Native corn starch (NS) was esterified with citric acid according to the method of Kim *et al.* (2017) with some modifications. Briefly, citric acid (30% on starch dry basis) was dissolved in 50 mL distilled water and subsequently the pH of the solution was adjusted to 3.5 using 10M NaOH. An aliquot of 50 g of NS was added in the citric acid solution with continuous mixing and the mixture was then left at room temperature for 16 h. The mixture

was than dried in a hot air oven at 60°C for 3–4 h till the 5–10% (w/w) moisture content was achieved followed by grinding and drying at 140°C for 4 h. The dried starch was then ground using a pestle and mortar and washed with distilled water thrice to remove the leftover citric acid. Finally, the starch was dried at 45°C for 24 h and finely powdered by a ball-miller (Fritsch, Idar-Oberstein, Germany) and passed through a 90-micron sieve.

Preparation of o/w Pickering emulsions

The 30% (v/v) o/w emulsions were prepared by mixing the modified starch (MS) suspension at different concentrations (6.0, 8.0, 8.5, 9.0, 9.5, and 10.0%) (w/v) and olive oil with a rotor-stator device (DI 25 basic, IKA, Stufen, Germany) at 20,000 rpm for 2 min; the pre-emulsions were then submitted to HPH using a Panda Plus 2000 homogenizer (GEA, Parma, Italy) at 55 bar with a circulation time of 5 min.

Degree of substitution of esterified starch

The method of Jeon *et al*. (1999) with some modifications was used to determine the degree of substitution (DS) of the modified starch. Both the native and modified starches (1 g) were slowly dissolved in the 10 mL solution of DMSO at 70°C for 10 min and left to cool down at ambient temperature. Then, after the addition of a few drops of phenolphthalein indicator, the solution was titrated against a standardized 0.05M NaOH solution till a light pink color was attained as the end point. The DS values were calculated using the following equation (Zhang *et al*., 2017):

$$DS = \frac{0.162(A \times M / W)}{1 - [0.210\ (A \times M / W)]} \quad (1)$$

where, A = volume of NaOH used, M = molarity of NaOH, and W = weight of the starch sample used.

Particle size measurement of starch granules and o/w emulsions

The particle size distribution of starch granules and oil droplets in the emulsions was measured by laser diffraction (Mastersizer 3000, Malvern Instruments Ltd. Malvern, UK). For starch, a refractive index of 1.52 was used (Loisel *et al.*, 1998). For emulsions, refractive indices of 1.33 and 1.59 were used for water and olive oil, respectively. Droplet size measurements are reported as particle size distribution curves and surface mean diameter ($D_{3,2}$).

Flow behavior of o/w emulsions

The flow curves of Pickering emulsions were determined by using a controlled stress–strain rheometer (MCR 302, Anton Paar, Graz Austria) equipped with a concentric cylinder configuration. Flow curves were measured at 20°C at increasing shear rates from 3 to 300 s^{-1}. The experimental data were modeled using the Power Law equation (Eq. 2)

$$\sigma = K\gamma^n \quad (2)$$

where σ = shear stress (Pa), K = consistency index, γ = shear rate (1/s), and n = flow behavior (dimensionless).

Physical stability of o/w emulsions

The physical stability of emulsions was measured using the creaming index (CI) during a storage period of 30 days at room temperature. Emulsions were photographed at 1-week intervals and the images were processed using ImageJ software. The cream volume was measured and the creaming index was obtained as a ratio between the total volume (V_t) and cream volume (V_c) using the following equation

$$\text{Creaming Index} = \frac{V_c}{V_t} \times 100 \quad (3)$$

Optical microscopy

The microscopic observation of the Pickering emulsions was carried out using an Olympus-BX53 light microscope at 10× and 40× magnification. The photographs were taken with a 12-bit digital camera (QIQAM Fast 1394, Surrey, Canada) connected with the microscope. The starch was stained with Lugol's solution; samples were placed on the microscope slide and covered with a glass cover before observation.

Scanning Electron Microscopy (SEM)

A Scanning Electron Microscope (model Leica Cambridge, UK) at 5kV was used to evaluate the microstructural properties of esterified starch. About 100 mg of sample with double-sided adhesive tape was mounted on the SEM stub, and to avoid the charging effect due to electron beam it was sputter coated with gold.

Statistical analysis

All experiments were performed at least in triplicates. The analysis of variance (ANOVA) was performed using SPSS version 24 (SPSS, Chicago, USA). Ducan's test was used for reporting the significance difference results at 95% confidence level ($P < 0.05$).

Results and Discussion

Effect of citric acid treatment and HPH on corn starch particles

The emulsifying ability of starch granules as Pickering particles toward o/w emulsions is highly dependent on

their composition, structure, and granule size (Timgren *et al*., 2013). In order to evaluate any likely effect of HPH on starch particles' morphology and functionality, corn starch was preliminarily modified by using citric acid and then subjected to the homogenization process conditions used for emulsion preparation. It is known that HPH can significantly affect starch properties and induce gelatinization (Wang *et al.*, 2008) ; however, in this work, pressure of homogenization was kept low (55 bar) to limit state transition of the starch particles dispersed in the aqueous phase.

In Figure 1, the microstructural properties of native (NS) and citric-acid-modified corn starch (MS) subjected to HPH (HPH-NS and HPH-MS) are shown, while in Figure 2 their particle size is reported as $D_{3,2}$. Regardless of the treatments applied, all the starch samples were characterized by a poly-dispersed population. However, HPH significantly ($P < 0.05$) reduced the particle size of both the native starch (Figure 1B) and modified corn starch (Figure 1D), as also confirmed by the results of the volume/surface ($D_{3,2}$) and particle size of the samples (Figure 2). The size reduction was more evident on the citric-acid-modified corn starch whose median value was significantly lower ($P < 0.05$) compared to native corn starch (12.54 ± 0.04 μm and 20.84 ± 0.12 μm, respectively), likely due to the structural modification of corn starch induced by the citric acid pretreatment.

The hydrophobic properties of starch particles as a consequence of both esterification and esterification in combination with the HPH process were evaluated by measuring the degree of substitution (DS). During the esterification process, hydrophobic groups are introduced to the amylose residues, causing an increase of the DS, resulting in enhanced surface active properties (Zhou *et al*., 2016). The DS of MS was 0.22 ± 0.01, significantly higher than that of NS whose DS value was equal to 0.01 ± 0.00, confirming the efficacy of the esterification process applied. This result is in agreement with literature data where significantly higher DS values were reported after esterification of maca starch with citric acid (Lee and Chang, 2019). HPH did not affect the hydrophobic properties of the starch particles of both native and modified starches as the DS of both HPH-NS and HPH-MS was not significantly different than the not homogenized sample (data not shown).

The changes induced in the morphology and microstructure of the NS and MS are highlighted in the SEM images

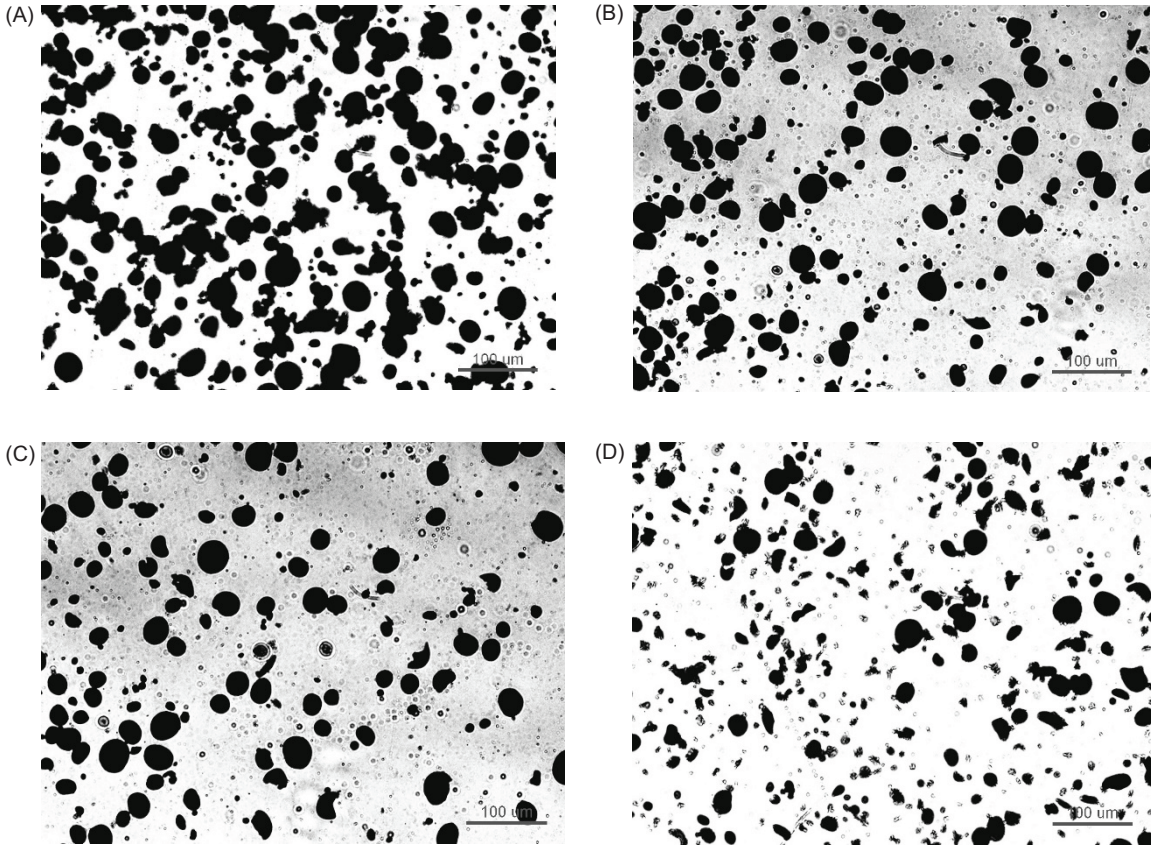

Figure 1. Micrographs of starch particles taken by light microscope: NS (A), HPH-NS (B), MS (C), HPH-MS (D). HPH: high pressure homogenization; NS: native starch; MS: modified starch.

reported in Figure 3. NS granules (Figure 3A) present a smooth outer surface while after the citric-acid-modification slight groves and corrosion appeared on the surface of the MS granules (Figure 3B). However, overall, the esterification did not significantly alter the main structure of the starch granules or their size. These results are similar to those reported by other authors reporting that chemical modification of native starch only affects the outer surface of the granules while no changes in the morphology and structure of the starch granules occur (Mbougueng *et al*., 2012; Zhang *et al*., 2017). The surface modification of the starch granules induced by the esterification has been related to the weathering effect of acid hydrolysis (Alimi and Workneh, 2018).

Emulsifying capacity of modified starch toward olive oil

In order to study the emulsifying capacity of starch particles toward olive oil, suspensions of both native and modified starches at increasing concentrations were used to prepare o/w emulsions made of olive oil as dispersed phase (fixed concentration 30%, v/v), homogenized by means of HPH. Regardless of the concentration tested, no emulsifying capacity was observed when NS was used as the emulsions readily destabilized; hence, NS was not further taken into consideration. On the contrary, fine olive oil o/w emulsions were obtained when MS was used as emulsifier: Figure 4 thus shows the size class and particle size distribution of the olive oil o/w emulsions prepared with MS in the concentration range of 6.0–10.0% (w/v). Such a result can be related to the improvement of the emulsifying capacity due to the introduction of ester groups during modification (Lee and Chang, 2019). Indeed, some authors evidenced that esterification increases the amphiphilic properties of starch granules and this leads to a decrease of the surface tension between the oil–water interface with respect to that observed in the corresponding native, unmodified ones (Królikowska *et al*., 2017), favoring the formation and stabilization of the fine oil droplets formed during the homogenization process.

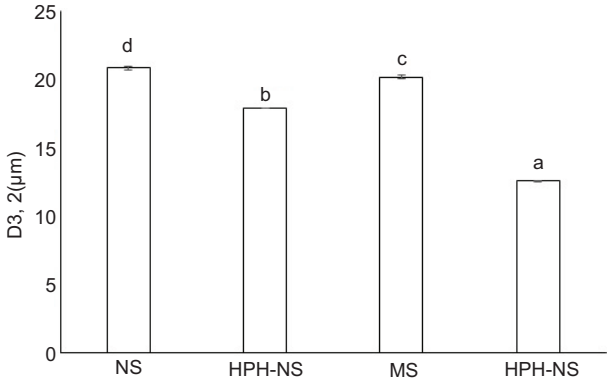

Figure 2. Particle size ($D_{3,2}$) of the differently treated corn starch particles. Different letters on each bar indicate significant differences among the mean values (P < 0.05). NS: native starch; MS: modified starch; HPH: high pressure homogenization.

With regard to the dispersion degree, of all the MS concentrations tested, the particle size distribution was characterized by a high degree of polidispersity (Figure 4A) with at least three oil droplet populations with a relative maximum average at particle sizes of around 2, 5, and 50 mm, respectively. At increasing starch concentrations, a progressive higher particle size was observed along with a relative higher amount of larger particles, with no variation in the size range distribution. This behavior was also reflected in the results of the surface mean droplet size $D_{3,2}$ of the emulsified samples (Figure 4B) with the smaller droplet diameter observed in the emulsions made with lower starch concentration (5.94 ± 0.73 μm). Then, by increasing the starch concentration, the droplet particle size increased up to a plateau value of about

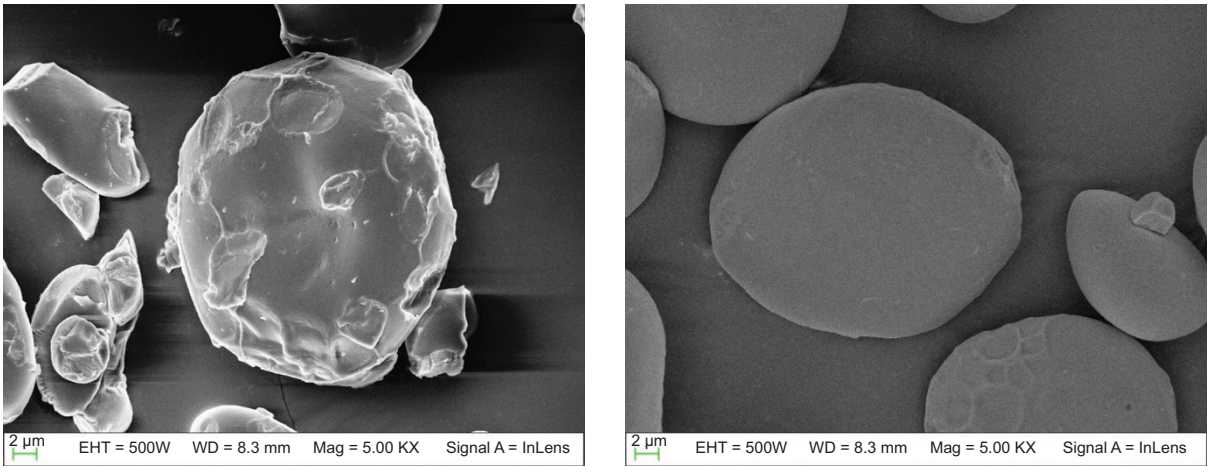

Figure 3. SEM micrographs of NS (A) and MS (B) particles (NS: native starch; MS: modified starch).

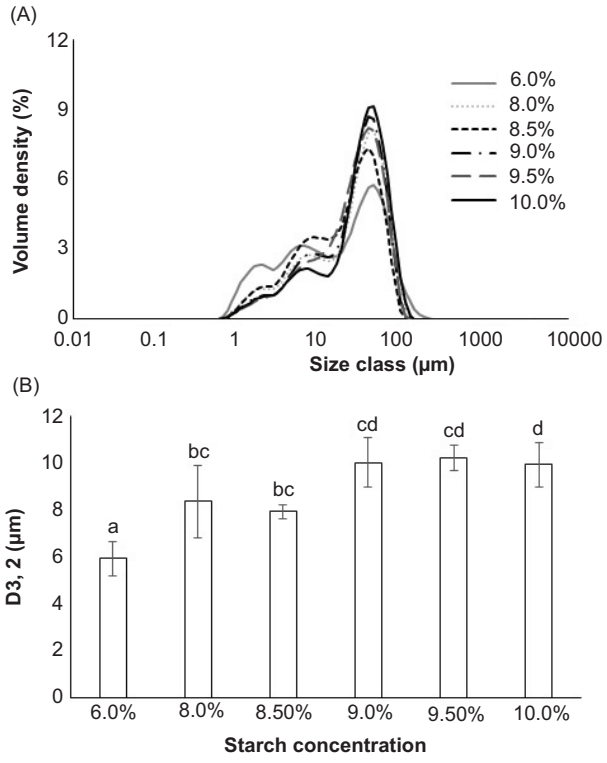

Figure 4. Droplet size distribution (A), and surface mean droplet size (B) of o/w Pickering emulsions prepared with different concentrations of modified corn starch. Different letters in the same columns indicate significant differences (P < 0.05) (n = 3).

10 µm. This result is in disagreement with data reported in the literature, as higher starch concentration allows more particles to be able to absorb at the oil/water interface and stabilize the interfacial layer and thus decrease the particle size (Li *et al.*, 2013; Marefati *et al.*, 2017). This disagreement could be due to several causes, such as a difference in the starch particles' properties and size; the emulsified systems, like the oil to water volume ratio; and the homogenization conditions used with respect to the current study.

However, it is worth observing that emulsions presented a surface mean droplet size lower or equal to 10 µm, which, according to some authors, indicates good emulsification capacity and emulsion stability toward physical destabilization phenomena such as creaming and flocculation, when compared to emulsion with larger droplets (Saari *et al.*, 2016). So, it can be generally affirmed that in the range of concentrations tested and under the process conditions applied, the modified starch particles exerted good emulsifying properties, determining the formation of fine o/w olive oil dispersed systems.

Images of emulsions made with 9.5% MS (w/v) evaluated by light microscopy captured at two different magnifications are reported in Figure 5, as an example of systems' microstructure. The micrographs highlight that the emulsions present a complex structure where small oil droplets are dispersed in an aqueous phase that contains a large amount of starch (black spots in the images) that are in some cases interacting (Figure 5A), likely due to the effect of the high dynamic pressure during emulsification. Moreover, it could be clearly evidenced that oil droplets are stabilized by adsorbed starch particles (Figure 5B). As previously observed in the literature, the overall emulsified dispersed state of the emulsions can be due to both the Pickering mechanism by starch particles adsorbed on the o/w interface as well as the stabilization and immobilization of oil droplets within the starch, gel-like granules network formed during the HPH process (Dickinson, 2020). Indeed, it could be affirmed that during the high-pressure homogenization, the Pickering emulsions were stabilized by several mechanisms occurring simultaneously, which are the adsorption of starch particles on the oil/water interface, the release of amylose from the starch granules which formed complexes with the oil droplets, and the formation of the gel-like network of starch granules which immobilized the oil droplets. Indeed, these stabilization phenomena have been previously reported in Pickering emulsions prepared with corn starch under HPH (Meng *et al.*, 2014; Villamonte *et al.*, 2016).

Flow behavior of Pickering olive oil emulsions

Flow behavior of Pickering emulsions at different MS concentrations is reported in Figure 6. All dispersed systems presented a non-Newtonian shear-thinning behavior as, at increasing MS concentration, the shear stress increased. The shear-thinning behavior can be explained by a weak droplet association where the network underwent breakage as the shear force was increased, the rate of starch network deformation was higher than the reformation rate, resulting in lower viscosity because of reduced intermolecular resistance, behavior which has been previously observed for starch-stabilized o/w emulsions (Ye *et al.*, 2017).

The experimental data were fitted by using a power law model ($\sigma = K\gamma^n$) where σ is the shear stress (Pa), K the consistency index, γ the shear rate (1/s), and n = flow behavior (dimensionless). The extent of shear-thinning behavior in a non-Newtonian fluid is calculated using the flow behavior (n). If the value of n is <1, it is shear-thinning fluid and >1 indicates a shear-thickening fluid (Rezaei *et al.*, 2017). In Table 1, the flow behavior (n) and consistency index (k) values of the differently made emulsions are summarized. All samples presented an n value <1 confirming the shear-thinning behavior; it significantly (P > 0.05) increased from 6.0 to 8.0% starch

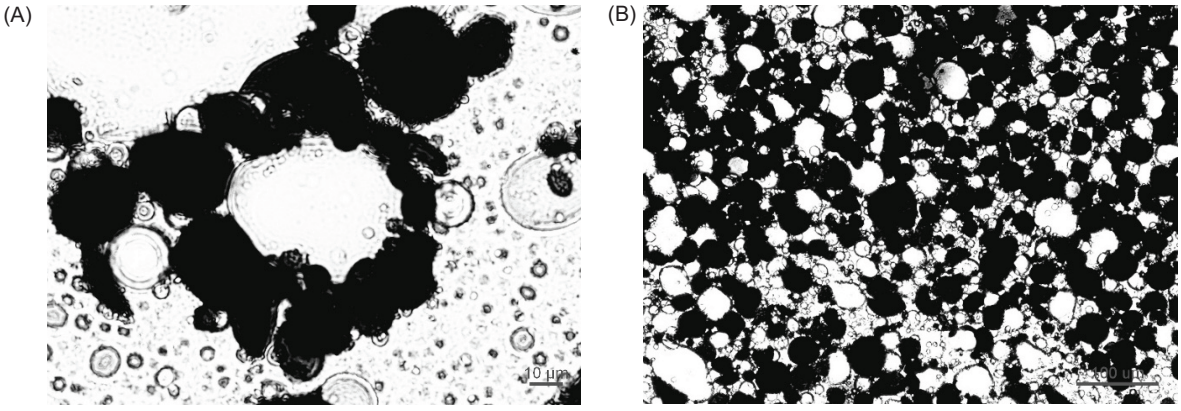

Figure 5. Optical microscope images of Pickering emulsions prepared with 9.5% MS at different magnifications (10× in A, 40× in B). MS granules appear to be black in color (MS: modified starch).

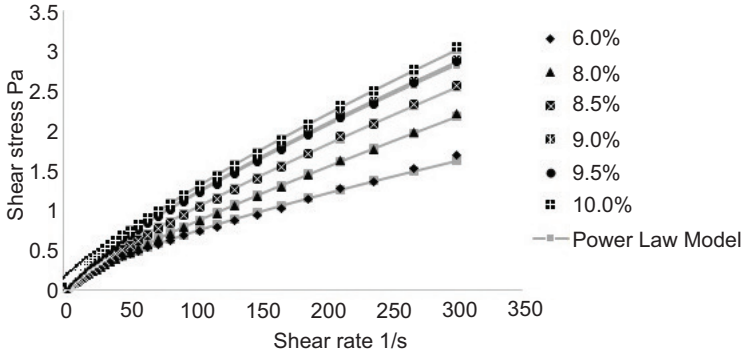

Figure 6. Flow behavior of the o/w Pickering emulsions prepared with different concentrations of modified corn starch (6.0–10.0% w/w).

Table 1. Consistency index and flow behavior of the 30% o/w Pickering emulsions stabilized by high pressure homogenization and MS particles.

	MS 6.0%	MS 8.0%	MS 8.5%	MS 9.0%	MS 9.5%	MS 10.0%
K	$0.027^{a} \pm 0.01$	$0.017^{a} \pm 0.01$	$0.022^{a} \pm 0.00$	$0.034^{a} \pm 0.01$	$0.033^{a} \pm 0.01$	$0.036^{a} \pm 0.01$
n	$0.740^{a} \pm 0.03$	$0.856^{b} \pm 0.06$	$0.83^{ab} \pm 0.02$	$0.783^{ab} \pm 0.04$	$0.784^{ab} \pm 0.02$	$0.783^{ab} \pm 0.03$

K = consistency index, n = flow behavior, *R^2 value for all the measurements was greater than 0.977. Different letters in the same rows indicate significant difference ($P < 0.05$) (n = 3). MS: modified starch.

concentration while no significant increase was observed afterward. The lowest consistency index (K) was found in emulsions stabilized with 6.0% of MS while an increase was observed at all the remaining concentrations, even though with no significant differences among them ($P < 0.05$). From these data, it could be concluded that consistency index largely remained unaffected by increasing starch concentration while the flow behavior was only significantly affected up to a certain level (6–8% of MS) and afterward it also remained unaffected by increasing starch concentrations.

Creaming stability of o/w Pickering emulsions

Stability of the Pickering emulsions was investigated by the evaluation of the creaming index (CI) as a parameter that could indicate the extent of droplet aggregation in an emulsion, which in turn affects its physical stability. The higher the creaming index, the more the droplets clump together (Sun-Waterhouse *et al.*, 2013). Figure 7 shows the CI of o/w Pickering emulsions over a storage period of 1 month at room temperature. It is possible to note that the higher the starch concentration, the lower

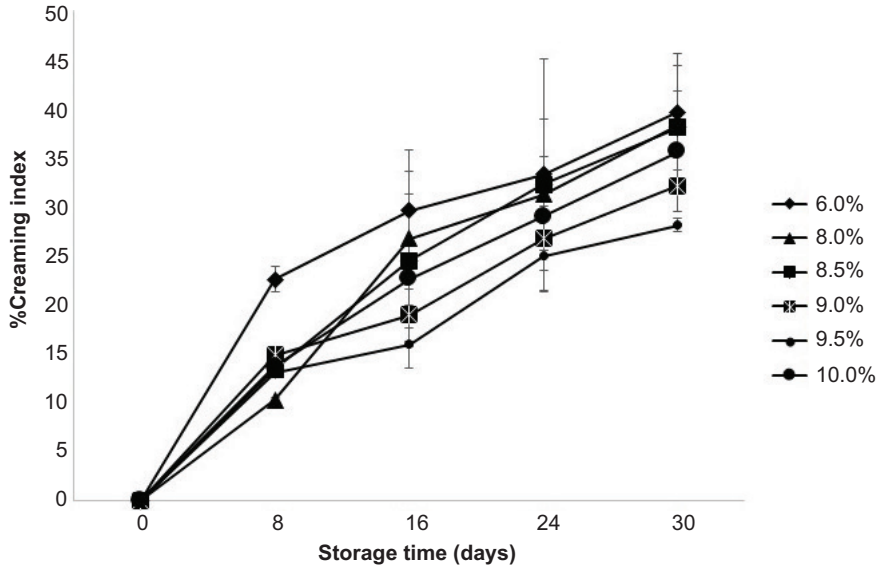

Figure 7. Effect of storage time on creaming index stability of o/w Pickering emulsions prepared with high dynamic pressure and modified corn starch at different concentrations (6.0–10.0%).

the CI observed. On the 30th day of storage, the 6.0% MS emulsion showed the highest CI of 40% ± 1 while the 9.5% MS systems showed the lowest CI (28% ± 1) and such differences were statistically significant compared to the other MS concentrations ($P < 0.05$). These results are in agreement with the findings of other authors who evaluated the stability of Pickering emulsions stabilized with native and modified lauroylated starch and concluded that increasing the modified starch concentration increases the stability of the Pickering emulsions (Leal-Castañeda *et al.*, 2018).

The enhanced creaming stability as a consequence of increased starch concentration may be related to a higher emulsion viscosity and to the formation of a starch particles network in which oil droplets are embedded and immobilized, as previously observed in Figure 5 and also demonstrated by other authors (Villamonte *et al.*, 2016). A further mechanism of stabilization may be ascribed to the occurrence of starch-fatty acid complexes promoted by the homogenization process, which provided effective stabilization against coalescence and enhanced the Pickering emulsion stability (Meng *et al.*, 2014).

Conclusions

In this preliminary study, the emulsification properties of corn starch particles modified by esterification with citric acid toward olive oil in model systems prepared by using HPH were studied for the first time. The emulsification of olive oil by using starch particles was successfully performed. The esterification of corn starch increased the emulsification properties toward olive oil and finely dispersed systems were obtained. The microscopic images demonstrated the emulsions' stabilization by the occurrence of a mixed mechanism based on Pickering phenomena by solid starch particles absorbed on the o/w interface and the formation of a starch network able to entrap oil particles. The flow properties of the Pickering emulsions showed a shear-thinning non-Newtonian behavior; increasing the starch concentration enhanced the emulsion physical stability as evaluated by the creaming index by improving the oil particles entrapment within the starch network. Further work is needed to explore the emulsifying properties and exploitability of starch particles in more complex food systems formulated with olive oil as the lipid phase.

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Paulownia tomentosa flower polysaccharide as an effective immunopotentiator to enhance immune responses for Newcastle disease vaccine in mice

Xiaolan Chen[1]*, Junjie Jin[2], Fuxing Hao[1], Haifeng Yang[1], Hongxiang Sun[3], Chunmao Jiang[1]

[1]Jiangsu Agri-animal Husbandry Vocational College, Taizhou, China; [2]Wenzhou Vocational College of Science and Technology, Wenzhou, China; [3]Zhejiang University, Hangzhou, China

***Corresponding author:** Xiaolan Chen, Jiangsu Agri-animal Husbandry Vocational College, Taizhou, Jiangsu Province, China. Email: cxl7972563@163.com

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PAPER

Abstract

To investigate the immunomodulatory activity and explore the mechanism of *Paulownia tomentosa* flower polysaccharides (PTFP). PTFP was orally administrated to mice for seven successive days before and after Newcastle disease vaccination. The results demonstrated that compared with the vaccine control (VC) group, PTFP enhanced the inhibition of hemagglutination assay antibody titers, promoted the antigen-specific immunoglobulin (Ig)G, IgG1, IgG2a, and IgG2b antibodies responses, enhanced proliferation of spleen T and B lymphocytes, increased the secretions of interferon-γ and interleukin-10 cytokines of spleen lymphocytes, and promoted the activation of natural killer cells. Therefore, PTFP, as an effective immunopotentiator, could induce a mixed T-helper (Th)1 and Th2 immune responses and an innate immune response.

Keywords: immune responses, immunopotentiator, Newcastle disease vaccine, *Paulownia tomentosa* flower polysaccharides

Introduction

In recent years, polysaccharides from Chinese medicinal herbs have drawn more attention because of their effective immune enhancement, favorable safety, and excellent biocompatibility (Sun *et al.,* 2018; Tang *et al.,* 2019; Chen *et al.,* 2020). Various polysaccharides, such as *Astragalus* polysaccharide, Lentinan, *Angelica sinensis* polysaccharide, and *Lycium barbarum* polysaccharide, have been proved to possess potent immunomodulatory activity (Su *et al.,* 2014; Wang *et al.,* 2016; Sun *et al.,* 2018; Chen *et al.,* 2020; Ren *et al.,* 2021). The *Paulownia tomentosa* (*P. tomentosa*), as Chinese herbal medicine, has been widely used to treat stomach disorders, diarrhea, gonorrhea, erysipelas, hypertension, enteritis, tonsillitis, bronchitis, and dysentery (Dai *et al.,* 2015; Liu *et al.,* 2017; Lee *et al.,* 2018; Wang *et al.,* 2019). Recent researches have proved that *P. tomentosa* possesses various pharmacological activities, such as antibacterial, anti-inflammatory, antiphlogistic, antitussive, antiasthmatic, immunomodulatory, antioxidant, antiviral, and anticholinesterase activities (Dai *et al.,* 2015; Liu *et al.,* 2017; Wang *et al.,* 2019). *P. tomentosa* flower polysaccharides (PTFP), which are extracted from the *P. tomentosa* flower, are the main water-soluble component of this flower. It has been reported that PTFP could serve as a new immunopotentiator to enhance humoral and cellular immune responses (Wang *et al.,* 2019).

Newcastle disease (ND), one of the most contagious and devastating diseases in the poultry industry around the world, is caused by an avian paramyxovirus type 1 serotype of the genus *Avulavirus* in the Paramyxoviridae family (Zhai *et al.,* 2011(a); Yuan *et al.,* 2020; Chen *et al.,* 2021). This disease reduced the production of eggs, led to respiratory and central nervous infections, death

of poultry, and caused an immense economic loss in the poultry industry (Ma *et al.,* 2019; Yuan *et al.*, 2020). Vaccination is the most effective and productive approach to prevent and control the spread of ND (Yang *et al.,* 2020). The commercial vaccines available were live or inactivated virus-based vaccines. So to enhance immune responses for the ND vaccine, immunopotentiators or adjuvants were commonly required (Zhai *et al.,* 2011(b); Ma *et al.*, 2019; Yuan *et al.,* 2020).

The immunomodulatory activity of PTFP was determined in our previous study by orally administrating it into the ND-vaccinated chickens. . The *in vivo* experiment results demonstrated that PTFP could improve lymphocyte proliferation, increase antibody response, and enhance the secretion of interferon (IFN)-γ, indicating that PTFP has the potential to improve immune responses in ND-vaccinated chickens(Yang *et al.,* 2019). However, the mechanism of PTFP enhancing immune responses for the ND vaccine is still unknown. Hence, to further investigate the immunomodulatory activity and mechanism of PTFP for improving the ND vaccine response. Mice used as the model animal were orally administrated with the immunopotentiator PTFP. Later they were immunized with ND-vaccine twice at an interval of 14 days. After the second dose of vaccination, inhibition of hemagglutination assay (IHA) titers against ND and antigen-specific immunoglobulin (Ig)G and isotypes (IgG1, IgG2a, and IgG2b) antibodies were determined. Meanwhile, for further immune responses evaluation, spleen lymphocytes proliferation, together with cytokines, and natural killer (NK) cells activity were measured.

Materials and Methods

Materials

Dried-cultured *P. tomentosa* flower were obtained from Bozhou Guoxin Pharmaceutical Co., Ltd (Anhui, China). Attenuated ND- vaccine (LaSota strain, No. 1170121) was purchased from Guangxi Liyuan Biotechnology Co., Ltd (China). Fetal bovine serum was obtained from Gibco (Carlsbad, CA). Roswell Park Memorial Institute (*RPMI*)-1640 medium was purchased from HyClone, Logan, UT. Concanavalin A (Con A) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO). The antigen and positive control sera used for the ND virus (NDV)-specific IHA was purchased from Qingdao YEBIO Biological Technology Co., Ltd (China). Horse radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody was purchased from Sigma-Aldrich. HRP-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b antibodies were obtained from Southern Biotechnology Associates (Birmingham, AL). IFN-γ and interleukin (IL)-10 enzyme-linked immunosorbent assay (ELISA) kits were obtained from BOSTER Biological Technology Co., Ltd (China). All other regents and chemicals were of analytical grade.

Preparation of PTFP

PTFP was prepared by water decoction and ethanol precipitation as previously described (Yang *et al.,* 2019). In brief, dried cultured *P. tomentosa* flowers were extracted twice with boiling water for 2 hours and 1 hour, respectively. After filtration, the merged decoction was then condensed, and the suspension was precipitated with 95% ethanol four times for a total of 12 hours. The solution was then centrifuged and concentrated to a specific volume, dried under reduced pressure at 60°. The carbohydrate concentration (%) of the total PTFP was 48 compared with D-glucose, and the luteolin and apigenin concentration (%) of the total PTFP was 3.12 and 4.35, respectively.

Cells and animals

Human leukemia cell line K562 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences.

Institute of Cancer Research mice (5–6 weeks, 18–22 g, male and female) were purchased from Shanghai Slake Laboratory Animal Co., Ltd and housed in Zhejiang University. The mice were maintained under pathogen-free conditions and acclimatized for 7 days before experiments. The animal experiments were conducted at the Zhejiang University. All animal experiments were conducted in compliance with the guide for the care and use of laboratory animals and approved by the Animal welfare and ethics committee, Zhejiang University (January 16, 2019, authorization number is No.18227). All the animals were anesthetized with ether, the blood samples were drawn from the eyes, and the mice were killed by the cervical dislocation method.

Method

Experiment design

The mice were randomly divided into five groups: blank control (BC), vaccine control (VC), PTFP-low dose (PTFP-L), PTFP-medium dose (PTFP-M), and PTFP-high dose (PTFP-H) groups with 12 animals per group. The experimental procedure is represented as a schematic illustration in Figure 1. The mice (except the BC group) were subcutaneously immunized with 0.1mL ND vaccine ($10^{6.0}$ EID_{50}/0.1mL) on day 4 and boosted with the same dose on day 18. The mice in PTFP groups were continuously orally administrated with different doses of PTFP (30, 60, and 120 mg/kg) for 7 days (from day 1 to day 7), once a day. In addition, the oral administration

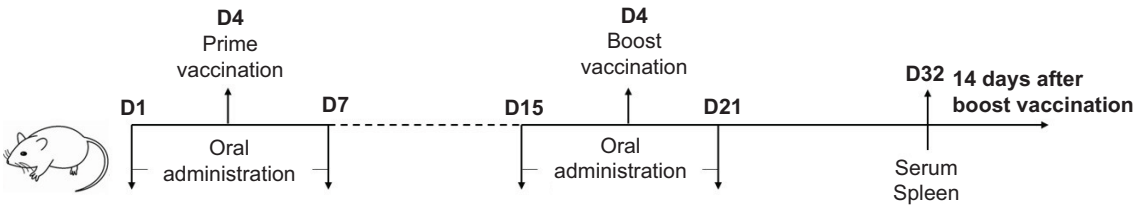

Figure 1. The schematic illustration of the vaccination and treatment schedule of the experiment.

procedure was performed again for 7 days from day 15 to day 21. The mice in BC and VC groups were administrated with the same volume of physiological saline orally. About 14 days after the boost vaccination (at day 32), the mice were sacrificed, serum samples were collected, and spleens were harvested for subsequent immunological tests.

Inhibition of hemagglutination assay (IHA)

IHA titers against the NDV in the serum samples were carried out according to the IHA procedure as previously described (Wu *et al.*, 2012; Wang *et al.,* 2013; Liu *et al.,* 2014; Zhang et al., 2014). Briefly, serum samples of different groups were collected at 14 days after boosting immunization and were heat-inactivated at 56° for 30 minutes. The serum samples (25 μL) were serially two-fold diluted using phosphate buffer saline (PBS) in a V-shaped microtiter plate. Later 25 μL NDV antigen (4 hemagglutinating units (HAU) was added and incubated for 30 minutes at room temperature. Here, PBS was used as the negative control. Finally, 25 μL of 1% chicken erythrocyte suspension was added, and the samples were reincubated for 30 minutes at room temperature. The IHA titer was expressed as the reciprocal value of the highest serum dilution, which completely inhibited the hemagglutination of chicken erythrocytes.

Determination of antigen-specific antibodies in serum

NDV-specific IgG and its isotypes (IgG1, IgG2a, and IgG2b) antibodies in the serum were measured on day 14 after boosting vaccination by ELISA (Wu *et al.,* 2012; Sun *et al.,* 2020). Briefly, 96 well ELISA plates were coated with 100 μL NDV antigen (0.5 HAU/mL, carbonate solution, pH = 9.6) per well at 4° overnight. After washing thrice with PBS containing 0.5% Tween 20, ELISA plates were blocked with PBS containing 1% bovine serum albumin and incubated for 2 hours at 37°. Then, the plates were washed thrice, and 100 μL of serially diluted serum samples were added and reincubated for 2 hours at 37°. After rewashing thrice, the HRP-conjugated anti-mouse IgG (1:8000 diluted), IgG1 (1:6000 diluted), IgG2a (1:4000 diluted), or IgG2b (1:4000 diluted) antibodies were added into plates and incubated for 2 hours at 37°. Then ELISA plates were washed thrice, and 100 μL of tetramethylbenzidine was added to the plates. After incubation for 15 minutes, 50 μL of 2 M sulphuric acid was added to stop the reaction. The optical density (OD) was measured at 492 nm using a Bio-Rad 680 ELISA reader (Bio-Rad, Hercules, CA).

Determination of spleen lymphocytes proliferation

At 14 days after boosting immunization, splenocytes were harvested from the mice (Wang *et al.,* 2016; Kumar *et al.,* 2017; Huang *et al.,*2020; Lu *et al.,*2020; Yu *et al.,* 2020; Gan *et al.,* 2021). Briefly, the mice were sacrificed by cervical dislocation, and the spleens from different groups were aseptically separated. Later, they were crushed and passed through a 200-mesh sterile cell strainer, and the red blood cells were separated using cells lysis solution. The collected spleen lymphocytes (5×10^6 cells/mL) were seeded into a 96-well plate with stimulated NDV antigen (0.0625 HAU/mL), Con A (final concentration 5 μg/mL), or LPS (final concentration 10 μg/mL), respectively. The cells incubated with RPMI-1640 medium were used as the negative control. After cultivation for 44 hours at 37° in 5% carbon-di-oxide atmosphere, 50 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 2 mg/mL) was added and further cultured for 4 hours at 37°. Subsequently, the supernatant was removed, and 150 μL dimethyl sulfoxide (DMSO) was added. The absorbance at 570 nm (A_{570}) was measured by using a microplate reader. The stimulation index (SI) was calculated as the ratio of absorbance values of ND antigen, Con A, or LPS stimulated cells to untreated cells (negative control group) as shown in Equation (1).

$$SI = \frac{A_{570}(\text{stimulated experimental group})}{A_{570}(\text{negative control group})} \quad (1)$$

Determination of spleen lymphocytes cytokines by ELISA

The spleen lymphocytes (5×10^6 cells/mL) from different groups were incubated in 24-well plates and restimulated with NDV antigen (0.0625 HAU/mL). After incubation at 37° for 72 hours, the productions of cytokines INF-γ and IL-10 were detected in the supernatants collected after performing ELISA according to the manufacturer's instructions.

Determination of NK cells activity

The NK cells activity was determined as previously described (Xu *et al.,* 2019; Zhang *et al.,* 2020). In brief, the spleen lymphocytes (1×10^7 cells/mL)) of mice from

different groups were collected as the effector cells, and the human leukemia K562 cells (2×10^5 cells/mL) were used as the target cells. About 100 μL of spleen lymphocytes were added into a 96-well plate with K562 cells (100 μL) in the ratio of 50:1 (effector cells: target cells) and incubated for 20 hours at 37°. The MTT method was used to measure the cell viability of NK cells wherein 50 μL of MTT (2 mg/mL) was added to the plates and incubated for 4 hours. Then the supernatant was removed, DMSO was added, and the absorbance was determined at 570 nm by a microplate reader. The cell viability of NK cells was calculated according to Equation (2).

$$\text{NK cells viability}=\frac{A570_T-(A570_S-A570_E)}{A570_T}\times100\% \quad (2)$$

where $A570_T$: absorbance value of target cells control; $A570_S$: absorbance value of samples; and $A570_E$: absorbance value of effector cells control.

Data analysis

All data were expressed as the mean ± standard deviation (SD). The statistical significance of differences was assessed by analysis of variance and Tukey's multiple comparisons. A probability value of $P < 0.05$ was considered statistically significant.

Results

Antibody responses

IHA antibody titers

Figure 2 shows the significant increase in the IHA antibody titers in PTFP groups compared with the VC group ($P < 0.05$). IHA antibody titers in PTFP-M group were higher than that of PTFP-L and PTFP-H groups and were not detected in the BC group (with no vaccination), whose data is not shown. The results demonstrated that PTFP, as an immunopotentiator, could promote IHA antibody responses in mice against ND-vaccine.

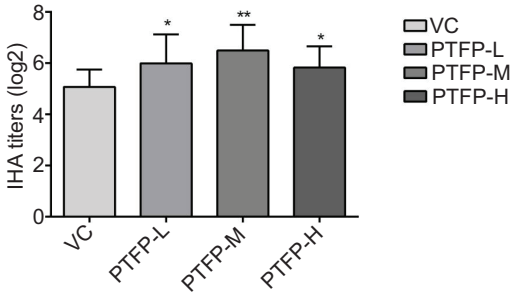

Figure 2. Effect of PTFP on IHA antibody response in the mice immunized with ND vaccine. The IHA antibody titers (log2) in serum from mice of different groups at 14 days after the boost immunization were determined by IHA assay. The values were presented as mean ± SD (n =12). Significant differences with the VC group were designated as *$P < 0.05$ and **$P < 0.01$. IHA, inhibition of hemagglutination assay; VC, vaccine control; PTFP, *Paulownia tomentosa* flower polysaccharides; PTFP-L, PTFP-low dose; PTFP-M, PTFP-medium dose; PTFP-H, PTFP- high dose.

Antigen-specific IgG and their isotypes (IgG1, IgG2a, and IgG2b) responses

The antigen-specific IgG and isotypes (IgG1, IgG2a, and IgG2b) antibodies were determined by ELISA to further evaluate the humoral responses induced by PTFP in mice immunized with ND-vaccine. As shown in Figure 3A, PTFP in all groups significantly induced stronger antigen-specific IgG titers than the VC group ($P < 0.05$). IgG titers in the PTFP-M group were higher than the PTFP-L and PTFP-H groups. The results of IgG titers were consistent with IHA titers results (Figure 2).

The levels of antigen-specific IgG isotypes (IgG1, IgG2a, and IgG2b) in different groups were determined to investigate the effects of PTFP on T-helper (Th)1 or Th2 immune responses. The IgG1 antibody was associated with a Th2-biased immune response. As shown in Figure 3B, IgG1 antibody titers in all the PTFP groups were significantly promoted compared with the BC group ($P < 0.05$). The levels of IgG1 antibody induced by the PTFP-M group were higher than the PTFP-L and PTFP-H groups. The results of IgG2a and IgG2b (Th1-associated) antibodies titers are shown in Figures 3C and 3D, respectively. IgG2a and IgG2b titers in all PTFP groups markedly increased (especially IgG2b) than the BC group. Moreover, IgG2a and IgG2b titers were induced by the PTFP-M group were higher than that in PTFP-L and PTFP-H groups. The results of IgG isotypes (IgG1, IgG2a, and IgG2b) in different groups had a similar trend to IgG titers (Figure 3A). In addition, ND-specific IgG and its isotypic (IgG1, IgG2a, and IgG2b) antibodies were not detected in the BC group (with no vaccination), and the data were not shown.

Spleen lymphocytes proliferation

To further investigate the effects of PTFP on cellular immune responses in mice against the ND-vaccine, spleen lymphocytes from mice were collected at 14 days after the boost vaccination, and the lymphocytes proliferation was stimulated with NDV- antigen, Con A, or LPS and determined. As shown in Figure 4A, after incubation with NDV antigen for 44 hours, lymphocytes proliferation in the PTFP groups (PTFP-L, PTFP-M, and PTFP-H groups) was significantly promoted compared with the VC groups ($P < 0.05$). The lymphocyte proliferation in the PTFP-M group was higher than the PTFP-L and PTFP-H groups. The result of lymphocytes proliferation with Con A-stimulation is shown in Figure 4B. The lymphocytes proliferation in the PTFP-L, PTFP-M, and PTFP-H

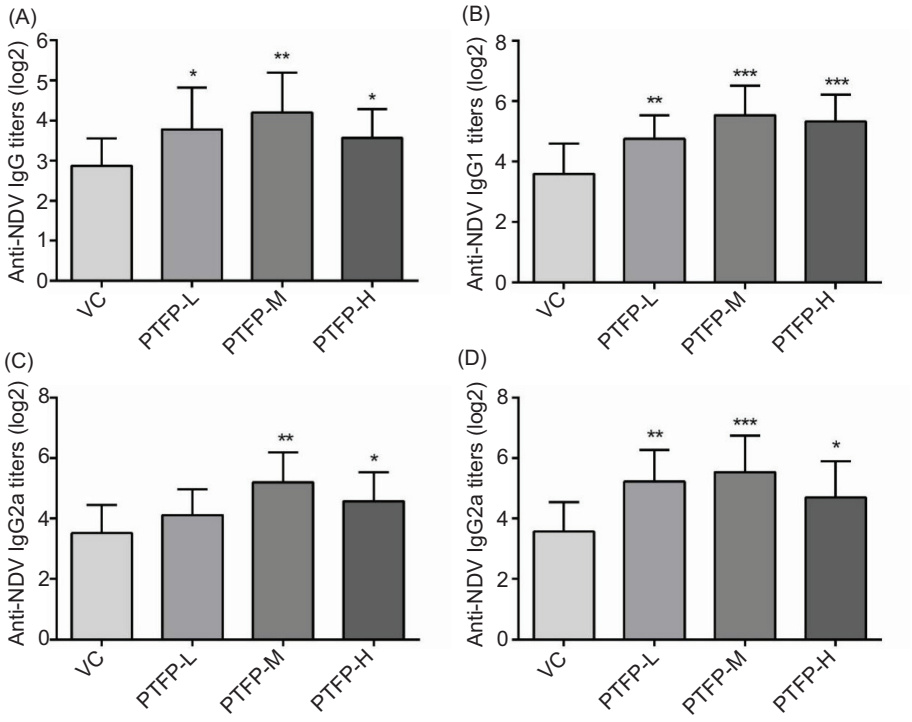

Figure 3. Effects of PTFP on antigen-specific antibodies responses in the mice immunized with ND vaccine. The antigen-specific IgG (A) and isotypes IgG1 (B), IgG2a (C), and IgG2b (D) titers in the serum were measured by an indirect ELISA at 14 days after the secondary immunization. The values are presented as mean ± SD (n = 12). Significant differences with VC group were designated as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. ND, Newcastle disease; NDV, ND-vaccine; VC, vaccine control; PTFP, *Paulownia tomentosa* flower polysaccharides; PTFP-L, PTFP-low dose; PTFP-M, PTFP-medium dose; PTFP-H, PTFP- high dose.

groups, especially the PTFP-M group, were improved compared with the VC group ($P < 0.05$). The trend of lymphocytes proliferation with LPS stimulation was similar to Con A stimulation (Figure 4C). All PTFP groups significantly promoted higher lymphocytes proliferation than the VC group ($P < 0.05$). The effect of lymphocytes proliferation with LPS stimulation in the PTFP-M group was better than those in the PTFP-L and PTFP-H groups.

Spleen lymphocytes cytokines

Spleen lymphocytes from immunized mice were collected and incubated with the NDV antigen for 72 hours 14 days after the second vaccination. The secretion of cytokines IFN-γ and IL-10 were measured, and the results are shown in Figures 5 A and 5B. The levels of IFN-γ in PTFP-L, PTFP-M, and PTFP-H groups were significantly increased compared with the VC group ($P < 0.05$). The IFN-γ in PTFP-M groups was higher than that in the PTFP-L and PTFP-H groups. The IL-10 expressions in all PTFP groups were significantly higher than that in the VC group ($P < 0.05$), and IL-10 levels in PTFP-L and PTFP-M groups were higher vs. PTFP-H group.

NK cells activity

To further investigate the effect of PTEP on immunological enhancement, the cytotoxic activity of NK cells against human leukemia K562 cells was determined. The NK cells activity of splenocytes significantly increased in mice orally administrated with PTFP compared with the VC group (Figure 6) and was more strongly induced in PTFP-L and PTFP-M groups vs. the PTFP-H group.

Discussion

Recently, natural polysaccharides have been considered as novel immunopotentiators or immunomodulators because of their potent immune enhancement and low toxicity (Sun *et al.,* 2018; Zeng *et al.,* 2019; Zhao *et al.,* 2020;). Numerous studies have demonstrated that polysaccharides extracted from Chinese medicinal herbs could enhance immune responses and promote the efficiency of vaccines by oral administration (Xie *et al.,* 2012; Feng *et al.,* 2013; Tang *et al.,* 2019; Zhang *et al.,* 2020). In our previous study, PTFP, extracted from *P. tomentosa* flower, had been proved to possess the immunomodulatory activity and enhance immune responses for orally administered ND-vaccine in chickens (Yang *et al.,* 2019). Wang *et al.* (2019) have reported that PTFP as a new immunopotentiator or adjuvant could enhance humoral and cellular responses in chickens by injection administration. However, the mechanism of PTFP promoting

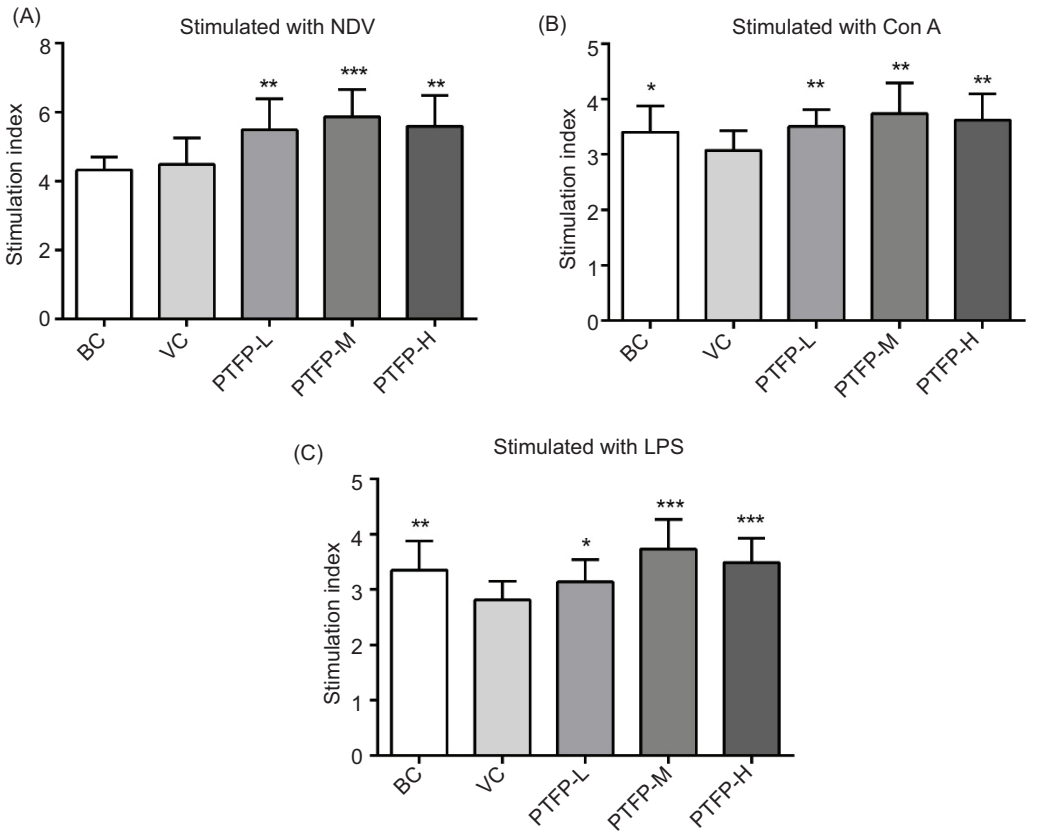

Figure 4. Effects of PTFP on spleen lymphocytes proliferation in the mice immunized with ND-vaccine. Splenocyte proliferation was detected by the MTT method after stimulation with ND antigen (A), Con A (B), or LPS (C) for 44 hours. The values are presented as mean ± SD (n = 12). Significant differences with the VC group were designated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. ND, Newcastle disease; NDV, ND-vaccine; LPS, lipopolysaccharide; Con A, concanavalin A; BC, blank control; VC, vaccine control; PTFP, *Paulownia tomentosa* flower polysaccharides; PTFP-L, PTFP-low dose; PTFP-M, PTFP-medium dose; PTFP-H, PTFP-high dose.

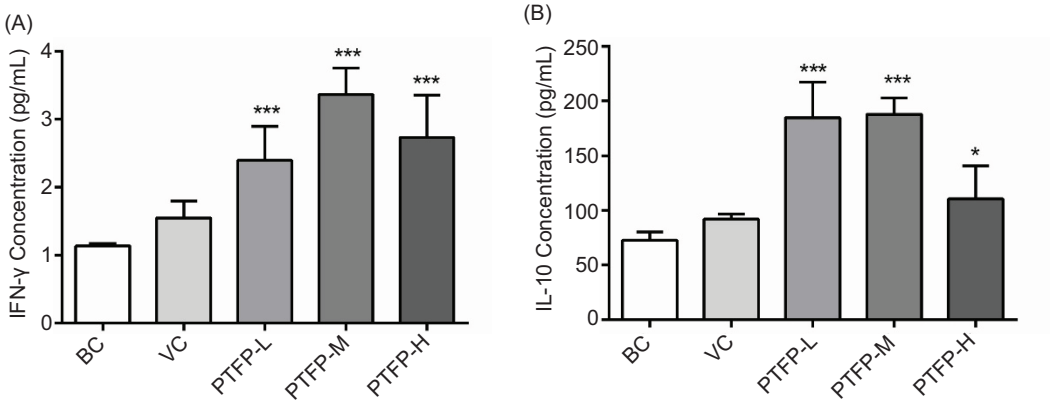

Figure 5. Effects of PTFP on spleen lymphocytes cytokines in the mice immunized with ND vaccine. The spleen lymphocytes from immunized mice were incubated with ND antigen for 72 hours. The IFN-γ (A) and IL-10 (B) cytokines in supernatants of spleen lymphocytes were determined by ELISA. The values are presented as mean ± SD (n = 12). Significant differences with VC group were designated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. IFN, interferon; IL, interleukin; BC, blank control; VC, vaccine control; PTFP, *Paulownia tomentosa* flower polysaccharides; PTFP-L, PTFP-low dose; PTFP-M, PTFP-medium dose; PTFP-H, PTFP- high dose.

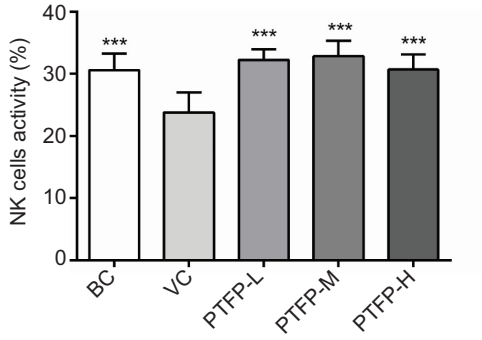

Figure 6. Effect of PTFP on NK cell activity in the splenocytes from the mice immunized with ND vaccine. Splenocytes from different groups were collected 14 days after the second immunization, and the NK cells activity was measured by MTT assay. The values are presented as mean ± SD (n = 12). Significant differences with the VC group were designated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. NK, natural killer; BC, blank control; VC, vaccine control; PTFP, *Paulownia tomentosa* flower polysaccharides; PTFP-L, PTFP-low dose; PTFP-M, PTFP-medium dose; PTFP-H, PTFP-high dose.

and regulating immune responses for ND vaccine by oral administration was still unexplored. Therefore, to further evaluate the immunomodulatory activity and investigate the mechanism of PTFP for ND-vaccine, mice were used as the model animal. Different doses of PTFP were orally administrated to it, and they were later immunized with ND vaccine.

NDV-specific antibody immune responses play an important and indispensable role in protecting the body from ND virus infection. The IHA was performed to determine the levels of specific antibodies against the hemagglutinin-neuraminidase protein (Yuan *et al.,* 2020). Compared with the VC group, the mice orally administered with PTFP, especially with the PTFP-M dose, significantly increased the ND virus-specific IHA titers (Figure 2), indicating that PTFP with oral administration enhanced the antibody responses against the ND vaccine. In addition, the PTFP significantly promoted higher anti-NDV IgG antibody titers than the VC group (Figure 3A), which was consistent with the results of IHA in Figure 2. To further investigate the Th1 and Th2 immune responses induced by PTFP, antigen-specific IgG1, IgG2a, and IgG2b antibodies were determined. IgG1 antibody is associated with Th2-type immune, whereas IgG2a and IgG2b antibodies are associated with Th1-biased immune response (Feng *et al.,* 2013). The PTFP promoted the productions of Th2-biased IgG1 antibody and IgG2a and IgG2b antibodies (Th1-biased) compared with the VC group (Figures 3B–D). These results suggested that PTFP induced a strong humoral immune response and elicited a mixed Th1 and Th2 response.

The stimulation of lymphocytes proliferation indicates the capacity of effective T and B lymphocytes immunity and hence are commonly used as an indicator to reflect the state of cellular immunity (Yang *et al.,* 2008; Feng *et al.,* 2013; Huang *et al.,* 2013). Con A and LPS were cooperated to stimulate T and B lymphocytes proliferation, respectively (Yang *et al.,* 2008; Wang *et al.,* 2016). In Figure 4, the PTFP markedly increased the lymphocytes proliferation with the stimulation of NDV antigen, Con A, or LPS vs. the VC group. The result indicated that PTFP could effectively promote the NDV antigen-stimulated lymphocytes proliferation response, Con A-stimulated T lymphocytes proliferation, and LPS-stimulated B lymphocytes proliferation.

Th1 lymphocytes mainly secreted IFN-γ, IL-2, and IL-12 cytokines, whereas the Th2 cells predominantly produced IL-4, IL-6, and IL-10 cytokines *(*Liu *et al.,* 2009). To further investigate the cellular immune response induced by PTFP, the spleen lymphocytes cytokines IFN-γ and IL-10 were measured. IFN-γ, one of the main cytokines representing cellular immunity, could promote the antibody isotype switching to IgG2a and improve the differentiation and proliferation of Th1 cells (Zhang *et al.,* 2020; Coutant *et al.,* 2017). IL-10, a Th2-type cytokine, is considered an anti-inflammatory cytokine that regulates immune response (Courant *et al.,* 2017). The PTFP both significantly improved the secretion of IFN-γ and IL-10 cytokines when compared with the BC and VC groups (Figure 5A,B), indicating that PTFP both enhanced the Th1 and Th2 immune responses, which was consistent with the result of IgG1, IgG2a, and IgG2b antibodies (Figure 3B–D).

NK cells (the cytolytic effector lymphocytes of innate immunity) could recognize and eliminate virus-infected cells and tumor cells and are crucial in the innate immune system (Park *et al.,* 2017; Xie *et al.,* 2019). They act as a defense line against viral infections, and the activation of these cells plays a crucial role in regulating immune responses (Park *et al.,* 2017; Xu *et al.,* 2019). In Figure 6, compared with the VC group, PTFP significantly enhanced the NK cells activity by increasing the lysing of human leukemia K562 cells, indicating that PTFP with oral administration could markedly promote the activation of NK cells and improve immune responses.

Based on the results presented above, we investigated the effects of PTFP by oral administration on the mice immunized with ND vaccine and explained the mechanism of PTFP influences on the immune responses. First, the oral administration of PTFP enhanced the ND virus-specific IHA titers (Figure 2), increased the levels of anti-NDV IgG antibody titers (Figure 2A), and thereby improved the humoral immune response for the ND vaccine. Meanwhile, the PTFP promoted the productions of anti-NDV IgG1(Th2-type), IgG2a, and IgG2b (Th1-type)

antibodies (Figures 3B–D), together with the secretions of IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine; Figure 5), indicating that PTFP induced both the Th1 and Th2 immune responses. Moreover, it promoted the proliferation of T and B lymphocytes (Figure 4) and enhanced the cellular immune response. At the same time, the PTFP also activated the NK cells (Figure 6), thereby improving innate immunity. Hence, the oral administration of PTFP could enhance the humoral and cellular immune responses and initiate the innate immunity for ND vaccine in mice, which further demonstrated the possibility that PTFP could be the candidate of a new-type immunopotentiator in chickens. However, the values in the PTFP-H group were consistently lower than those in the PTFP-M group. No serious adverse effects of PTFP were observed after administration. It is necessary to design more groups to narrow the concentration gap between groups to find the best dose and study the side effects of PTFP in mice shortly. The cost of *P. tomentosa* flower is cheap, and the production process of PTFP is also simple. PTFP, as a new-type immunopotentiator, could play a significant role and have great developing space in poultry breeding.

Conclusion

In this study, to further investigate the immunomodulatory activity and explore the mechanism of PTFP for the ND vaccine, the mice were used as the model animals. Different doses of PTFP were orally administered to mice, and the ND vaccine was subcutaneously injected into them. Our finding demonstrated that orally administered PTFP enhanced the antigen-specific antibody immune responses and induced mixed Th1 and Th2 immune responses. Meanwhile, the PTFP also increased the proliferation of T and B lymphocytes, promoted the productions of IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine), and improved the cellular immune response. In addition, it promoted the activation of NK cells thereby, improving innate immunity. Therefore, oral administration of PTFP could possess the immunomodulatory activity and improve immune responses by enhancing humoral immune response and cellular immune responses by inducing mixed Th1 and Th2 immune responses and promoting the innate immune response.

Disclosure Statement

The authors declare no competing interests.

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Local food consumption during the covid-19 pandemic

Zohra Ghali-Zinoubi[1,2]

[1]*College of Administrative and Financial Sciences, Saudi Electronic University, Riyadh, Saudi Arabia;* [2]*Higher Institute of Management of Tunis, University of Tunis, Le Bardo, Tunisia*

***Corresponding author:** Zohra Ghali-Zinoubi, College of Administrative and Financial Sciences, Saudi Electronic University, Riyadh, Saudi Arabia & Higher Institute of Management of Tunis., University of Tunis., Le Bardo, Tunisia. Email: zohragh@yahoo.fr

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PAPER

Abstract

The influence of intrinsic quality, health consciousness, environmental awareness, local support, and proximity of process on consumers' intention to consume local food during the COVID-19 pandemic was tested, with food availability as a moderator. Online survey results were analyzed using a two-step structural equation modelling (SEM). Health consciousness was the major reason for consuming local food. Intrinsic quality and proximity of process were also significant drivers. Local support and environmental awareness have little impact on the intention to purchase local food. This study contributes to knowledge regarding the main factors driving local food consumption during a health crisis, providing directions.

Keywords: consumer behavior; COVID-19 pandemic; local food; purchase intention; Tunisia

Introduction

The Coronavirus Disease of 2019 (COVID-19) pandemic has dramatically upset people's everyday lives, brought about a global recession that the world had not witnessed since the Second World War, and created a health crisis around the world (Smith and Wesselbaum, 2020). The food sector and its stakeholders are particularly in the spotlight (Ben Hassen *et al*., 2021a; Galanakis, 2020) because food is essential for human survival, and therefore it cannot be placed under lockdown. Indeed, the emergence of COVID-19 has significantly affected the global food systems at several levels, i.e., for producers, distributors, and consumers (Cranfield, 2020; Qi *et al*., 2020; Xie *et al*., 2020). Notably, both consumption patterns and consumers' behavior changed as the pandemic progressed (Celimli and Adanacioglu, 2021; Eger *et al*., 2021; Yuen *et al*., 2020). In this context, what should be eaten has become one of the main concerns for customers who seek to improve their immunity and increase their food security. In April 2020, worldwide Google searches for "food delivery" and "local food" reached an all-time high (Shveda, 2020). Thus, the crisis forced people to reexamine the sources of their groceries and the mode through which they procured food.

In a globalized era, the distribution of food from the producer to geographically dispersed consumers relies on a large and complex supply chain. The coronavirus crisis has disrupted this supply chain and caused gaps in food delivery. Moreover, the lockdowns instituted in response to the COVID-19 pandemic have led to shortages of labor, interruptions of logistics, and inconsistency in the demand for and supply of food (Pantano *et al*., 2020; Rizou *et al*., 2020). This has resulted in empty supermarket shelves, food wastage, and hindered food procurement due to interruptions in exports and imports (Aday and Aday, 2020). Local food systems can alleviate this growing threat of the global food system crisis (Memery *et al*., 2015; Peterson, 2013; Pressman *et al*., 2020). The existing literature names several reasons for the increased demand for local food providers during the COVID19

pandemic. First, during a health crisis, the most crucial issue for any consumer is to consume healthy, nutritious, and safe food, such as local food (Arsil *et al*., 2018; Skallerud and Wien, 2019). Second, during the pandemic, consumers have become more interested in what they consume and from where they source them (Severo *et al*., 2020; Sheth, 2020). The intrinsic quality of food matters more than ever (Ben Hassen *et al*., 2020). Local food is consumed quickly (Aprile *et al*., 2016; Loiseau *et al*., 2020). This ensures that it is fresher and more nutritious, with stronger immunity-boosting properties (Ozturk and Akoglu, 2020). Third, the distribution chain of local food is transparent, short, safe, and fair, because it is produced in the same geographic zone (Aprile *et al*., 2016; Roy *et al*., 2019; Toukabri and Ghali-Zinoubi, 2020). This reassures consumers and leads them to perceive the reduced risk of product contamination during its journey from the producer to the final consumer. Fourth, shorter distances between producers of local food and consumers are associated with reduced carbon impacts and waste production. Moreover, local production stimulates accountability among farmers, meaning that they will be more likely to engage in environmentally friendly practices (Mesić *et al*., 2020). Therefore, local foods are perceived as both sustainable and environmentally friendly products (Ozturk and Akoglu, 2020; Rousseau and Deschacht, 2020). Fifth, consuming locally available foods during times of crisis is a positive behavior that supports local producers and distributors, while consequently making consumers aware of their responsibility (Jribi *et al*., 2020; Mesić *et al*., 2020). However, the consumption of local food still depends on availability (Sowers *et al*., 2019). This is because local food increases customer trust, in addition to being accessible and easy to find (Kumar and Kashyap, 2018, Verma, 2020), thereby benefiting the retailer (Steinhart *et al*., 2013).

Tunisia is a Mediterranean country located in North Africa. Agriculture plays a vital role in its economy, and 10.1% of its Gross Domestic Product (GDP) in 2019 came from agricultural products. According to the Moderator Intelligence Report (MIR) of 2020, agricultural produce constitutes almost 6% of the total Tunisian exports. In 2019, the vegetable production of this country exceeded 3.0 million metric tons, constituted by 545 metric tons of citrus, 288.7 metric tons of dates, and 130 metric tons of tomatoes. This country is the second-biggest exporter of organic foods (olive, date, vegetables, vines, orange, apple, etc.) in Africa with almost 80% of its production (MIR, 2020). However, Tunisia does not have food sovereignty, despite the important and varied local food production. It has had a trade deficit in agriculture production for several years (Almayed, 2019). For example, in 2020, Tunisia imported food for over 2.56 billion US dollars while its exports amounted only to 1.95 billion US dollars (Salah, 2021). Its leading agriculture imports are wheat (over 431 million US dollars), sugar ($204 million), soybeans ($162 million), vegetable oils ($160 million), corn ($145 million), and barley ($134 million).

With the emergence of the COVID-19 pandemic and the breakdown, social distinction, and other restrictive measures taken in Tunisia and across the globe, the food supply faced several issues, while the food availability on the local market became a vital concern (Ben Hassen *et al*., 2021a; Kirkm and Kirkin, 2020; Pantano *et al*., 2020; Sheth, 2021). In this context of global breakdown and interruption of international transport, local food became the single viable alternative. Indeed, several studies conducted during the COVID-19 pandemic confirmed the growth of local food consumption amidst the pandemic (Ben Hassen *et al*., 2021b; Celimli and Adanacioglu, 2021; Duda-Chodak *et al*., 2020; Loiseau *et al*., 2020; Mesić *et al*., 2020). However, to the best of our knowledge, no studies have examined the factors driving this growth. Furthermore, from a marketing perspective, there are no studies focusing on the behavior of Tunisian consumers toward local food during the COVID-19 pandemic.

The current study was conducted in the aforementioned context to fill this gap. It examines motivations for local food consumption during the COVID-19 pandemic. The main objective of this paper was to examine the main motivations for local food consumption during the COVID-19 pandemic and the moderating role of food availability. This paper begins with a literature review, from which five hypotheses are derived. Next, the research methodology was developed. The discussion of the results and their implications are presented in the last section, which also includes the study's limitations and directions for future research.

Literature Review

What is local food?

The concept of "local food" has been the focus of several studies in the literature (Bazzani and Canavari, 2017; Bentsen and Pedersen, 2020; Meyerding *et al*., 2019; Picha *et al*., 2017). However, there is still no agreed-upon definition of this concept (Memery *et al*., 2015; Skallerud and Wien, 2019). For instance, several studies have used an objective approach, defining local food in relation to the geographic distance that the food travels from the producer to the final customer. In other words, the term "local food" has been used to describe food systems or short supply chains wherein the food is produced in areas close to where the consumers live (Meyerding *et al*., 2019; Skallerud and Wien, 2019). However, this approach has been criticized because

consumers may struggle to accurately assess the distance between producers and markets (the places of purchase), especially at higher levels of congruence. A perceptual approach has been developed as an alternative. It considers food as "local" based on the consumer perception, in other words it is local if the consumer subjectively believes it to be so. This approach is more versatile but has issues regarding reliability, especially when neighbors living on the same street estimate the same product differently. In this context, Bazzani and Canavari (2017, p. 514) suggested that the meaning of "local" should be expressed in terms of connection to a geographical area rather than in terms of food miles. Considering the advantages and disadvantages of both approaches, we will use the definition developed by the Industry of Global Distribution (IGD) (2005, p. 3), where "local food must be grown or produced within 30 miles of where the buyer lives."

Motivation for local food consumption

Intrinsic quality

The existent literature in marketing shows that intrinsic quality is an ambiguous and multidimensional concept. (Fandos and Flavian, 2006). It is among the most important criteria that consumers use to evaluate food products (Memery *et al*., 2015). The literature distinguishes between extrinsic and intrinsic quality. Intrinsic attributes consist of an objective quality assessment, are intangible, specific to every product, and cannot be altered without modifying the nature of the product (Fandos and Flavian, 2006). Several studies have found that consumers perceive local food as having superior intrinsic quality in terms of freshness, taste, naturalness, nutrition, health, and safety (Mesić *et al*., 2020; Meyerding *et al*., 2019; Roy *et al*., 2019). However, local food only presents these attributes if purchased from local producers, especially in their production season (Ozturk and Akoglu, 2020).

During the COVID-19 pandemic, strengthening immunity has become a major concern for consumers. Local foods meet these objectives for consumers because they consider them free from preservatives and chemicals, perceiving them as natural and wholesome. Such attributes are believed to provide health benefits (Memery *et al*., 2015). Therefore, intrinsic quality is important motivation for consumers to purchase local food during the pandemic (Mesić *et al*., 2020; Shveda, 2020). Given the above information, the first hypothesis of the study is as follows:

> ***H1.*** During the COVID-19 pandemic, intrinsic quality positively influences consumers' intention to purchase local food.

Health consciousness

Health consciousness is the degree to which issues related to personal health are of concern to an individual (Pu *et al*., 2020). It also refers to the degree to which health-related actions are integrated into a person's everyday activities (Janetius and Krithik, 2020). Customers with high health consciousness are more likely to engage in healthy habits and take proactive measures to protect their health (Mesić *et al*., 2020; Pu *et al*., 2020). In addition, people have improved their health consciousness during the pandemic.

Local food is characterized by having a short distribution chain, freshness, and nutritional value, and by being harvested at the optimal stage (Roy *et al*., 2019). The consumption of local food during the coronavirus pandemic has grown because it is considered safer and healthier (Xie *et al*., 2020). Based on the arguments above, we hypothesize that:

> ***H2.*** During the COVID-19 pandemic, health consciousness positively influences consumers' intention to purchase local food.

Proximity to the production process

Proximity to production refers to sharing knowledge of the internal functioning of trade (quality, origin, the production process, and the distribution chain) with customers (Bergadaà and Del Bucchia, 2009). In other words, proximity to the production is based on formal and informal exchanges of relevant and timely information to meet wishes and expectations of the actors involved in a transaction (Anderson and Narus, 1990). This proximity dimension shows customers the transparency of the manufacturing process and the useful, sanitary, and nutritious qualities of the product (Merle and Piotrowski, 2012). In geographically dispersed food systems, production and distribution chains are complex, large, and include several intermediaries (Loiseau *et al*., 2020; Roy *et al*., 2019; Todorovic *et al*., 2018). This means that the processes of production and distribution are not transparent to customers. Thus, easy access to the source of production leads to greater food security (Mazieres and Gauthier, 2015; Toukabri and Ghali-Zinoubi, 2020). Consequently, proximity to the production, which focuses on the relationship between the producer and consumer, becomes important to reassure customers, especially as safety and nutritious attributes of products are regarded (Loiseau *et al*., 2020; Skallerud and Wien, 2019). Local food chains have been found to meet these requirements (Todorovic *et al*., 2018).

During COVID-19, customers have become more concerned with health and the environment. Short and transparent production, distribution processes, and smaller supply chain have become essential criteria to

reassure customers, minimize contamination risks, and increase food security (Hobbs, 2020; Mesić *et al*., 2020). Therefore, local food has fostered increasing interest among customers, and its consumption has grown prominently (Shveda, 2020). Thus, we hypothesize that:

> ***H3.*** During the COVID-19 pandemic, proximity to the production process positively influences consumers' intention to purchase local food.

Local support

Consumption of local food has empathic, social, and local loyalty motivations (Skallerud and Wien, 2019). In fact, individuals consume local food to help local producers compete with national producers and imports (Mesić *et al*., 2020; Meyerding *et al*., 2019). It is a moral obligation (Peterson, 2013) and behavior in favor of the local community and retailers (Memery *et al*., 2015). An increase in local food consumption revitalizes the economy and plays a role in reducing the environmental damage that occurs while shipping food (e.g., carbon emissions) (Ozturk and Akoglu, 2020).

During the coronavirus pandemic, local restaurants and stores were temporary or extendedly closed. However, the "shop local" movement also increased during such times. Several studies have stated that consumers are more aware of the impact of the COVID-19 crisis on local suppliers, retailers, and the wider community (Severo *et al*., 2020). To support them and reduce the impact of the crisis on their activities, consumers are more motivated to purchase local food. This has been translated into favorable purchasing intention (Mesić *et al*., 2020). Based on this argument, we hypothesize that:

> ***H4.*** During the COVID-19 pandemic, local support positively influences consumers' intention to purchase local food.

Environmental awareness

According to the World Health Organization (2020) report, the COVID-19 pandemic has highlighted that maintaining sanitation and hygienic conditions, providing safe water, and reducing air pollution are all crucial measures for protecting human health during any infectious disease outbreak. Rousseau and Deschacht (2020) analyzed the online search behavior of 20 European countries and found that public awareness of nature and the environment increased during the COVID-19 pandemic. This is because protecting the environment is an important factor in reducing the risk of infection (Severo *et al*., 2020). Consuming local food is a way to reduce environmental pollution and improve food safety, as it reduces the risk of contamination associated with a long supply chain, in addition to being more sustainable (Ozturk and Akoglu, 2020). Overall, a short distribution chain is associated with fewer negative environmental impacts (e.g., carbon emissions and other pollution forms), once there is less packaging and no need for shipping facilities, packing facilities, or refrigeration. Similarly, Jribi *et al*. (2020) identified a positive impact of COVID-19 on Tunisian consumer awareness in terms of attitudes and behaviors toward food waste. Therefore, we posit the following:

> ***H5.*** During the COVID-19 pandemic, environmental awareness positively influences consumers' intention to purchase local food.

Moderating role of food availability

Food availability is a proxy of the service level provided to end customers (Lovel *et al*., 2005; Verma, 2020). Consumers have a positive perception of local food if it is accessible and available (Steinhart *et al*., 2013). The higher the availability of local food in the market, the higher is the familiarity of the consumer with its characteristics and, consequently, the perception of its values (Toukabri and Ghali-Zinoubi, 2020). Therefore, product availability increases the intention to purchase it. During the COVID-19 pandemic period, which was characterized by a general lockdown and restrictions on international transport, the demand for local food grew prominently (Duda-Chodak *et al*., 2020; Loiseau *et al*., 2020; Mesić *et al*., 2020). Food availability becomes crucial for local retailers to satisfy the increasing demand of their customers. This is because local food became the single viable alternative available for customers in several countries during the COVID-19 pandemic. Consumers consume local food to protect their health, environment, and the local community (Memery *et al*., 2015). Consequently, the following hypotheses are proposed:

> ***H6.*** During the COVID-19 pandemic, local food availability strengthens the relationship between the purchase intention of the consumer and its predictors.
>
> ***H6.a.*** During the COVID-19 pandemic, local food availability strengthens the relationship between intrinsic quality and purchase intention.
>
> ***H6b.*** During the COVID-19 pandemic, local food availability strengthens the relationship between health consciousness and purchase intention.
>
> ***H6c.*** During the COVID-19 pandemic, local food availability strengthens the relationship between proximity to the production process and purchase intention.

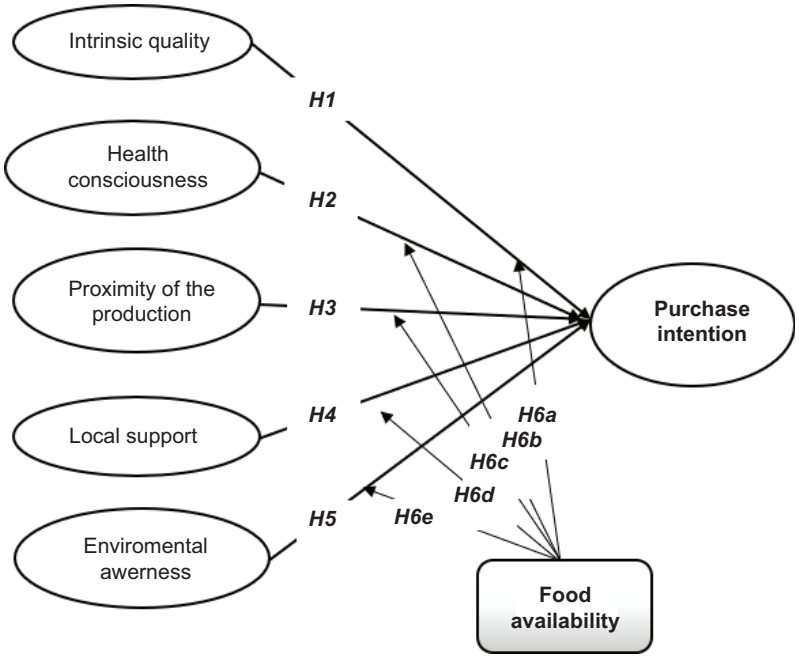

Figure 1. Proposed conceptual model.

H6d. During the COVID-19 pandemic, local food availability strengthens the relationship between local support and purchase intention.

H6e. During the COVID-19 pandemic, local food availability strengthens the relationship between the environmental awareness and purchase intention.

Materials and Methods

Survey design

The survey was designed using references from local food literature. The variable health consciousness was measured using Dutta-Bergman's (2004) five-item scale adapted to the coronavirus context (Pu *et al.*, 2020). Environmental awareness was measured using Severo *et al.*'s (2020) six-item scale. Local support was measured using Megicks *et al.*'s (2012) scale, which included three items. Proximity to the production was measured by adapting the Mazieres and Gautier (2015) scale. Intrinsic quality was measured using six items adapted from Megicks *et al.* (2012). Purchase intention was measured using the two-item scale of Lee *et al.* (2010), and food availability was measured using the four-item scale of Arsil *et al.* (2018). To adapt these measurement scales to the context of the coronavirus pandemic, the prefix "during the COVID-19 pandemic" was added to the different items as following Pu *et al.* (2020). All items were rated on a five-point Likert scale ranging from *strongly agree* to *strongly disagree*.

Data collection

The research context we chose was the country of Tunisia. The survey forms were made available online, using Google Forms. They were distributed mainly using email and social media networks (Facebook, LinkedIn, WhatsApp, and Instagram). The survey questionnaire was distributed using the convenience sampling approach. This speedy, easy, and low-cost technique does not allow the generalization of results (Yadav, 2016). However, it has provided pertinent and reliable findings in the context of food consumption studies (Ghali-Zinoubi, 2020, 2021).

A pre-study, including 23 surveys that were distributed to our family members and colleagues, was conducted to check the clarity and understandability of the survey. No revisions were made to the structure and content of the survey because respondents did not report any ambiguity or difficulty in understanding the questions.

The data were collected from May 03, 2020, to September 29, 2020. At the beginning of the survey, we provided examples of local food such as vegetables (potatoes, onions, spinach, parsley…), fruits (orange, apple, figs, peaches, apricots, etc.), eggs, meals, milk, and olive oil. They are the main local food produced in the different regions of Tunisia. In the end, 287 responses were returned out of 430 distributed questionnaires. However, we considered only 272 responses after eliminating incomplete or inappropriate (all answers were similar) responses. Table 1 summarizes the demographic profile of the sample.

Table 1. Demographic description of the sample.

Variables/criteria	N	%	Variables/criteria	N	%
Gender			***Education***		
Female	158	58.08	Elementary and middle school	23	8.45
Male	114	41.92	High school	54	19.85
Age			Graduate	122	44.85
18–35	68	25	Postgraduate	60	22.05
36–59	156	57.35	PhD	13	4.78
Older than 60					
Main household			***Household monthly income (TD)****		
Purchaser	48	17.65	Less than 1000	61	22.42
Myself	113	41.54	From 1001 to 1500	139	51.10
Other members	102	37.5	More than 1500	72	26.47
Both	57	20.96			

*TD: Tunisian Dinar. 1 TD = 0.36 US $ in June 2021.

Results and Discussion

Data were analyzed using the SEM procedure. This method is considered appropriate as it examines simultaneous dependency among relationships (Severo *et al*., 2020, p. 6). To test the hypotheses of dependent relationships, both measurement and structural models will be assessed.

Measurement model

The reliability, and convergent and discriminant validities were tested using a Confirmatory Factor Analysis (CFA). The internal consistency of the survey items was measured using Cronbach's alpha (α). The values ranged from 0.789 to 0.887, which exceeded the threshold of 0.7 established by Hair *et al*. (2013). The Composite Reliability (CR) scores ranged from 0.743 to 0.888, exceeding the threshold established by Bagozzi *et al.* (1998). Therefore, the reliability of the scales of all constructs of the conceptual model was confirmed.

The convergent validity was tested through the factor loading and the Average Variance Extracted (AVE). The factor loading of all items was above 0.6 (ranging from 0.623 to 0.889), meeting Chin *et al*.'s (1997) criterion. In addition, the AVE value ranged from 0.611 to 0.785, which was within the acceptable limit of 0.5 (Hair *et al*., 2013). Hence, convergent validity was confirmed for every construct. These results are summarized in Table 2.

The discriminant validity was also assessed by measuring the square root of AVE of each construct (Bagozzi and Yi, 1988). The results showed that they were higher than its correlation value (as shown in Table 3). This criterion was met for every construct. Thus, the discriminant validity is confirmed.

Structural model

Goodness of fit indices

The goodness of fit indices of the conceptual model was assessed using a structural model. The SEM results showed that the absolute, incremental, and parsimonious index values (X^2 = 206.429, X^2/df = 2.788, FI = 0.912; TLI = 0.892; CFI = 0.911; IFI = 0.912; RMSEA = 0.04) were well above the recommended thresholds (Bagozzi and Yi, 1988). Therefore, the conceptual model presents a good fit.

Hypothesis testing and discussion

Test of direct impact

To test the hypotheses, we examined β-values (the association between the independent and dependent constructs), t-values, and P-values. The path coefficients indicated that "intrinsic quality" and "health consciousness" had a positive significant influence on purchase intention (β = 0.428, t-value = 17.334; P < 0.01 and β = 0.449, t-value = 22.267; P < 0.01, respectively). Therefore, ***H1*** and ***H2*** were supported. These findings were in line with previous findings which showed that the health and the personal well-being of consumers are their main motivations for purchasing local food (Birch *et al*., 2020; Memery *et al*., 2015). This interest in maintaining health becomes more important in the era of COVID-19 (Duda-Chodak *et al*., 2020; Janetius and Krithika, 2020). Furthermore, the "proximity to the production" also had a positive and significant influence on purchase intention (β = 0.388, t-value = 24.584; P < 0.05).

Table 2. Measurement model: reliability and convergent validities.

Measurement items	Factor loadings	t-values	Cronbach's α
Intrinsic quality (IQ): CR = 0.888; AVE = 0.733			
IQ1. During the COVID-19 pandemic, I buy local food because it is free from preservatives.	0.834	24.734**	0.827
IQ2. During the COVID-19 pandemic, I buy local food because it is free from chemicals.	0.823	33.754**	0.823
IQ3. During the COVID-19 pandemic, I buy local food because it is natural.	0.788	34.842**	0.823
IQ4. During the COVID-19 pandemic, I buy local food because it is wholesome.	0.887	31.783**	0.851
IQ5. During the COVID-19 pandemic, I buy local food because it has a good appearance.	0.754	26.647*	0.827
IQ6. During the COVID-19 pandemic, I buy local food produce because it lasts longer.	0.742	24.752**	0.816
Health consciousness (HC): CR = 0.821; AVE = 0.785			
HC1. During the COVID-19 pandemic, I feel that living life in the best possible health is very important to me.	0.823	36.754**	0.887
HC2. During the COVID-19 pandemic, eating right, exercising, and taking preventive measures will keep me healthy for life.	0.814	36.184**	0.827
HC3. During the COVID-19 pandemic, my health depends on how well I take care of myself.	0.889	13.762**	0.876
HC4. During the COVID-19 pandemic, I actively try to prevent disease and illness.	0.837	24.818**	0.828
HC5. During the COVID19-19 pandemic, I do everything I can to stay healthy.	0.847	19.528*	0.856
Environmental awareness (EA): CR = 0.789; AVE = 0.611			
EA1. The COVID-19 Pandemic has made me increase the separation of organic and recyclable waste.	0.778	23.766*	0.803
EA2. The COVID-19 pandemic has caused me to reduce water consumption further, as this is a finite environmental resource.	0.792	27.337*	0.789
EA3. The COVID-19 Pandemic made me worry even more about the natural resources for future generations.	0.763	36.547**	0.823
EA4. The COVID-19 Pandemic made you realize the reduction in air pollution.	0.811	17.831*	0.811
EA5. The COVID-19 Pandemic made me realize, even more, the environmental impact caused on the planet.	0.801	22.781**	0.793
EA6. The COVID-19 Pandemic has increased my environmental awareness.	0.788	24.972*	0.801
Local support (LS): CR = 0.816; AVE = 0.743			
LS1. During the COVID-19 pandemic, I buy local food to support local producers.	0.623	28.627**	0.844
LS2. During the COVID-19 pandemic, I buy local food to support local retailers.	0.688	26.738**	0.822
LS3. During the COVID-19 pandemic, I buy local food to support the local community.	0.724	24.343*	0.773
Proximity of process (PP): CR = 0.833; AVE = 0.713			
PP1. During the COVID19 pandemic, I am interested to know very well the rules of production and distribution of the local foods.	0.854	28.972**	0.851
PP2. During the COVID-19 pandemic, I am interested in being very familiar with the production methods used by the producers who produce this local food.	0.836	27.384**	0.802
PP3. During the COVID-19 pandemic, I am interested to know as to who the producers of this local food are.	9.812	31.827**	0.826
Purchase intention (PI): CR = 0.743; AVE = 0.776			
PI1. During the COVID-19 pandemic, I am willing to buy local food while shopping.	0.845	18.993**	0.881
PI2. During the COVID-19 pandemic, I will make an effort to buy local food in the near future.	0.844	21.366**	0.823
Availability: CR = 0.811; AVE = 0.783			
AV1. During the COVID-19 pandemic, local food is available.	0.833	17.529**	0.842
AV2. During the COVID-19 pandemic, local food is easier to find.	0.833	12.333**	0.827
AV3. During the COVID-19 pandemic, local food has cheaper price.	0.853	21.023**	0.852
AV.4. During the COVID-19 pandemic, local food provides an assurance of product origin.	0.845	21.333**	0.783

Note: **P < 0.01. *P < 0.05.

Table 3. Discriminant validity test.

Latent variables	IQ	HC	EA	LS	PP	PI	AV
IQ	0.856						
HC	0.534**	0.886					
EA	0.675**	0.373**	0.781				
LS	0.531**	0.462**	0.422**	0.861			
PP	0.674*	0.561**	0.372*	0.467**	0.844		
PI	0.554**	0.333**	0.323*	0.556**	0.324*	0.880	
AV	0.621**	0.234**	0.341**	0.433	0.233	0.33**	0.793
MEAN (S.D)	*4.266 (0.4316)*	*4.333 (4.363)*	*4.233 (0.466)*	*4.666 (0.522)*	*4.666 (0.492)*	*4.301 (0.449)*	*4.001 (0.692)*

Notes: **P < 0.01; *P < 0.05. S.D: Standard Deviation. The square roots of AVEs are the numbers written in bold on the diagonal. Numbers below the diagonal represent constructs' correlations.

Hence, ***H3*** was supported. This finding was in line with Toukabri and Ghali-Zinoubi (2020). The common point among the three predictors was that they expressed self-interest ("what is good for me") by focusing on concerns over health, safety, and well-being, which translated into self-centered motivations (Birch *et al*., 2018). During the COVID-19 pandemic, this interest in health and safety gained additional attention (Pu *et al*., 2020). The strong association between self-centered motivations and behavioral intentions is justified by the fact that local food meets health and safety requirements (Birch *et al*., 2018; Mesić *et al*., 2020; Meyerding *et al*., 2019).

The influence of "local support" on purchase intention was positive and significant (β = 0.111, t-value = 10.233; P < 0.05). Therefore, ***H4*** was supported. This finding was in line with Memery *et al.* (2015) and Arsil *et al.* (2018). The construct "environmental awareness" had a positive influence on purchase intention, but the relation was weak (β = 0.043, t-value = 1.633; P > 0.05). Therefore, ***H5*** was not supported. These two variables expressed a "do good" for the environment and wider community, which translated into altruistic motivations (what is good for "us"). The findings of this study showed a weak impact of the altruistic motivations compared with self-centered ones, although local support was a significant driver of intention of purchasing local food. Likewise, Rousseau and Deschacht (2020) analyzed online search behavior in the European Union and found that the COVID-19 crisis did not affect public awareness of environmental issues. Birch *et al.* (2018) also reported a stronger impact of self-centeredness than of altruism in the context of local food consumption. However, Severo *et al.* (2020) conducted a study in Portugal and Brazil and found that the pandemic was instrumental in people's behavioral change, increasing environmental awareness and sense of social responsibility. These results are summarized in Table 4.

Test of the moderating role of local food availability

The moderating role of food availability (Table 5) was significant for the relationships between intrinsic quality and purchase intention, health consciousness and purchase intention, and proximity to the production and purchase intention. However, it was weak in the relationship between local support and purchase intention, and environmental awareness and purchase intention. These results show that regardless of food availability, the

Table 4. Hypothesis testing.

	Path	β-values	t-statistics	Relationship
H1	IQ → PI	0.428	17.334**	Supported
H2	HC → PI	0.449	22.267**	Supported
H3	PP → PI	0.388	24.584*	Supported
H4	LS → PI	0.111	10.233*	Supported
H5	EA → PI	0.043	1.633	Not supported

Note: **P < 0.01. *P < 0.05, IQ: Intrinsic quality; HC: Health consciousness, PP: Proximity of process, LS: Local support, EA: Environmental awareness, PI: Purchase intention.

Table 5. Hypothesis testing.

	Path	β-values	t-statistics	Relationship
H6a	IQ * AV → PI	0.223	6.333*	Supported
H6b	HC * AV → PI	0.154	3.667**	Supported
H6c	PP * AV → PI	0.111	11.628*	Supported
H6d	LS * AV → PI	0.033	1.333	Not supported
H6e	EA * AV → PI	0.004	1.066	Not supported

Note: **P < 0.01. *P < 0.05; IQ: Intrinsic quality; HC: Health consciousness, PP: Proximity of process, LS: Local support, EA: Environmental awareness, PI: Purchase intention, AV: Availability.

consumer was more self-centered than altruistic during the COVID-19 pandemic. In other words, the consumer intent to purchase local food is mainly due to its good quality and health benefits, irrespective of its availability. These results are in line with Toukabri and Ghali-Zinoubi (2020).

Conclusion, Implications, and Limitations

The findings indicate that, during the COVID-19 pandemic, health consciousness is the highest motive for consuming locally. Intrinsic quality and proximity to the production chain are also significant drivers of local food consumption. However, local support and environmental awareness have little impact on the intention to purchase local food. Food availability plays an important moderating role in three relationships (health consciousness/purchase intention, intrinsic quality/purchase intention, and proximity to the production/purchase intention). Many people have become more concerned with what they eat and from where they purchase their food. These findings also have several theoretical and managerial implications.

Theoretical implications

This study enhanced the theoretical background of local and healthy food. First, several studies have argued that local food consumption is more important than ever, during the COVID-19 pandemic (Ben Hassen *et al*., 2021b; Cranfield, 2020; Duda-Chodak *et al*., 2020; Naja and Hamadeh, 2020; Rizou *et al*., 2020). Other studies have argued that this growth is because local food is safe, healthy, and accessible (Aprile *et al*., 2016; Arsil *et al*., 2018; Meyerding *et al*., 2019; Ozturk and Akoglu, 2020). However, this study tested more predictors of local food consumption, such as proximity to the production and local support. Then, it may be considered among the first to test these predictors in the context of the COVID-19 pandemic. Second, this paper examined the impact of both self-centered and altruistic motivators on the intention to purchase local food. The findings showed that self-centered motivations are important drivers for local food consumption while the effect of altruistic motivations is still too weak. The study was developed in the context of a developing country (Tunisia), where environmental consciousness is still in its early stage (Ghali-Zinoubi, 2021). These findings are in contrast to those of several other studies which were conducted in developed countries and argued that both motivators are important (Rousseau and Deschacht, 2020; Skallerud and Wien, 2019). Third, this study is among the few studies that examine the moderating role of food availability on consumers' purchase intention during the COVID-19 pandemic. The findings indicated that the pandemic strengthened the relationships between self-centered motivations and purchase intention. However, it was not significant for altruistic relationships. This means that during the COVID-19 pandemic, local food, if available, is mainly purchased for health protection, accessibility, and its high quality.

Managerial implications

The findings of this study showed that during the coronavirus pandemic, consumers purchased local food because of its superior intrinsic quality, health benefits, and accessibility. Practitioners would be wise to focus their marketing campaigns considering these features to boost the consumption of their products during, as well as after, the pandemic. Appropriate packaging, effective labeling strategies, and coherent branding promoting these benefits of local foods should be implemented to attract customers and increase their local food consumption. Moreover, advertising should highlight known features, such as superior intrinsic quality, supporting local food, and healthy ingredients. Marketers of local food should communicate slogans such as "proudly eating local food" or "tastier, healthier, more nutritious and safer food." Local retailers may also extend their support to local producers by providing details pertaining to the local source of produce and enhancing the transparency of their supply chain. Moreover, local food producers and the wider community can act as a unique platform to improve sales of existing food products and invent new products both during and after the current health crisis.

Governments should also play an active role to make local food more available by supporting local farmers with the required infrastructure, providing seeds and other financial aids. Furthermore, a suitable program should be added to local curricula to educate the next generation of consumers about the importance of local products. Local support has been found to have a significant impact on consumer intention to purchase local food. Several scholars have argued that COVID-19 has disrupted and changed consumer habits, lifestyles, and long-term behaviors (Naja and Hamadeh, 2020; Picha *et al*., 2017; Pressman *et al*., 2020; Qi *et al*., 2020). The pandemic brought the community closer together and highlighted the need to address social inequalities. Marketers should emphasize the importance of supporting local businesses and the wider community while building branding messages and communication strategies that focus on the attributes we presented. Such messages should inform and educate the public about the economic, environmental, and social benefits to be

gained by purchasing local food. Because local food is considered a facet of local tradition, its consumption can be encouraged through traditional, cultural, and religious events. Environmental consciousness has been found to have a weak association with the intention to purchase local food. Marketers should build aggressive awareness campaigns to enhance the interest of eco-friendly consumers in local foods. These steps would increase local food consumption during and after the pandemic.

Limitations and future research directions

This paper fills the gap in the literature regarding the motivation for local food consumption during the COVID-19 pandemic, particularly in a developing market. However, some limitations should be underlined to guide future studies. First, data were collected through convenience sampling. This method is speedy, easy, and low cost; however, it hinders generalizability and replicability of results. Second, this study was developed in a single country. Cross-cultural studies would increase the inclusiveness of the research. Third, in this study, the motivations for purchasing local food were limited to five. However, several other motivations can be studied, such as short supply chains, local identities, ethical identities, past uses, and interest in traceability. Fourth, in this study, the focus was on the main drivers of local food purchase intention while the barriers were not studied. Future research should examine barriers that could inhibit consumers from purchasing local food, including high price, availability, and diversity.

Finally, the COVID-19 issue has taught us that individual action yields benefits that are multiplied when implemented on a global scale. We can master the crisis and flatten the curve of its progression if we work together. The influence of human behavior on personal and public well-being, including health protection, environmental preservation, and local support, has been highlighted by the COVID-19 pandemic. Humans must focus on being altruistic rather than self-centered to have a beneficial impact on those areas.

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Conflict of Interest

There are no conflicts of interest. The author strictly followed all ethical guidelines.

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Analysis of aroma compounds of nine autochthonous and non-autochthonous loquat cultivars grown in Sicily

Diego Planeta, Vittorio Farina, Paola Bambina, IleanaTinebra, Roberta Passafiume, Luciano Cinquanta, Onofrio Corona*

Department of Agricultural, Food, and Forest Sciences (SAAF), Università degli Studi di Palermo, Viale delle Scienze, Palermo, Italy

***Corresponding author:** Onofrio Corona, Department of Agricultural, Food and Forest Sciences (SAAF), Università degli Studi di Palermo, Viale delle Scienze, Palermo, 90128 Italy. Email: onofrio.corona@unipa.it

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PAPER

Abstract

Loquat cultivation in Sicily is mainly based on nonnative cultivars and local ecotypes characterized by high nutraceutical value and appreciable physicochemical characteristics. Increased interest in commercial loquat production has increased the intention to provide premium quality loquat cultivars that include volatile substances capable of conditioning the sensorial properties and, therefore, the acceptability of fruits by consumers. This study determined the content of volatile compounds in nonnative and local loquat fruits grown in Sicily. Analyses were performed on five international cultivars and four local cultivars.

Keywords: international and local cultivars, loquat, volatile compounds

Introduction

In recent years, there has been a growing demand for fruit products from tropical and subtropical countries. Among the attractive characteristics that fuel this demand from consumers is the increased interest in products with a high nutraceutical value, importantly their characteristic taste and flavour (Gentile *et al.*, 2019). It is well known that the taste and quality of food are determined by aromatic compounds, which in turn, influence consumer preferences and attract the attention of farmers who require more information and analytical tools to enable them to select the most suitable cultivars to grow (Baldwin, 2004; Schwab *et al.*, 2008). The aroma is a critical quality parameter that differentiates one fruit from the other and it is associated with many volatile compounds (Lo Bianco *et al.*, 2010; Ye *et al.*, 2017; Yuan *et al.*, 2018) belonging to different chemical groups, such as esters, alcohols, terpenes, ethers, aldehydes, etc.

One of the fruits belonging to the subtropical species, which is well adapted to the temperate zones of the Mediterranean and whose production is concentrated in Spain and Italy, is the Loquat (*Eriobotrya japonica* Lindl). It is an evergreen subtropical species (Family Rosaceae - Subfamily Mathat loideae) that originates from Southern China. Today, 90% of the cultivation is concentrated in the region of Sicily, particularly in the province of Palermo (Farina *et al.*, 2016), with an extended harvest period (from April to June) based on several cultivars and local ecotypes (Farina *et al.*, 2011; Gentile *et al.*, 2016). Loquat cultivation in Sicily is based especially on nonnative cultivars and local ecotypes characterized by a high nutraceutical value (Gentile *et al.*, 2019) and appreciable physico-chemical characteristics. Today, the interest in loquat commercial production has risen, and it is geared towards loquat cultivars of premium quality (Badenes *et al.*, 2013). The most important characteristic for the market is fruit size. The value of the crop (Goulas *et al.*, 2014) is in line with the

commercial classification (Testa *et al.*, 2020). As a result, fruits are divided into four classes based on their diameter: GGG for fruits over 53 mm; GG for fruits between 46 and 52 mm; G for fruits between 32 and 45 mm; and M for fruits between 31 and 28 mm. Quality is a complex of chemical and physical parameters and aromatic composition. Volatile flavor compounds are likely to play a key role in determining the perception and acceptability of products by consumers (Pott *et al.*, 2020). Fruits produce a range of volatile compounds that make up their characteristic aromas and contribute to their flavor. The differences in volatile compounds may be because of their ripening phase during the harvest time (Agozzino *et al.*, 2007) and differences in the studied cultivars (Farina *et al.*, 2020). Many studies on the volatile component of many fruits can be found in literature, while only a few studies have been conducted to date for the Mediterranean loquat. In this regard, Shaw and Wilson (1982) identified the following volatile compounds in loquat fruits; 2-phenylethanol, 3-hydroxy-2-butanone, phenylacetaldehyde, isomeric hexen-1-ols, ethyl acetate, methyl cinnamate, and β-ionone. Hexanal and (E)-2-hexenal and benzaldehyde have also been identified by Fröhlich and Schreier (1990). Chen *et al.* (2011) reported that β-ionone, decanoic acid, propanoic acid, bicycle nonane, and heptadecane are the major volatile compounds in the Zaozhong 6 cultivar. These studies highlighted that volatile compounds such as 2E-hexenol, 3Z-hexenol, and hexanol contribute to green notes; methyl cinnamate and eugenol contribute to the spicy note; ethyl and methyl 2-methylbutanoates are responsible for fresh fruity notes; a sweet watery aroma was also detected by traces amount of phenylacetaldehyde, vanillin, and β-ionone (Hideki *et al.* 1998). Finally, Takahashi *et al.* (2000) reported that the Tanaka cultivar presents phenylacetaldehyde as the most aromatic compound, while small traces of hexanal, (E)-2-hexanal, hexanoic acid, and β-ionone have also been found. Nevertheless, many other volatile compounds are present in traces which can be detected not only by analytical instruments but also by human olfaction (Goff *et al.*, 2006).

The aim of this study was to determine for the first time the content of volatile compounds in non-native and local loquat fruits grown in Sicily.

Material and Methods

Plant material

Two hundred and seventy loquat fruits (*Eriobotrya japonica* Lindl.) were harvested in an experimental orchard located in Santa Maria di Gesù (Palermo, Italy, 38°04'N, 13°22'E, 150 m a.s.l.) between April and June at commercial ripening, using fruit peel color as a ripening index (807-809 degree of Biologische Bundesantalt, Bundessortenamt and Chemische Industrie (BBCH) Scale). The analyses were carried out on four local cultivars (BRT20, Claudia, Sanfilippara, and Nespolone di Trabia) and five international cultivars (Algerie, Bueno, El Buenet, Golden Nugget, and Peluche). A sample of 30 fruits per cv was submitted for laboratory analyses.

Commercial classification

Primarily the loquat fruits were divided into international and local cultivars and later were classified based on the flesh color (yellow and white) and diameter (GGG, GG, G, and M), according to Testa *et al.* (2020).

Volatile compounds analysis

Three replicates of the pulp (about 200 g) of 10 fruits were separated from the peel and seeds with the addition of 100 mg of sodium metabisulfite. The pulps were crushed with a laboratory blender by a high-speed Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany) and centrifuged twice at 4500gand 4°C for 15 minutes and later the solid residue was washed with 70 mL of ethanol:water solvent (12:88). The final extract (250 mL) was then clarified with 0.1 g of the pectolytic enzyme without secondary glycosidase activity (Rapidase X-Press, DSM, The Netherlands) at room temperature for 2 hours. 1-Heptanol was added as internal standard (0.2 mL of 40 mg/L in 10% ethanol) to the samples and was loaded onto a 5-g C18 reversed-phase solid-phase extraction (SPE) cartridge (Isolute, SPE Columns, Uppsala, Sweden), previously activated with 20 mL of methanol,50 mL of deionized water using a flow-rate of ca. 3 mL/min, and then rinsed with 100 mL of deionized water to eliminate sugars, acids, and other low molecular weight polar compounds. The free aromatic fraction was then eluted with 25 mL of dichloromethane. The eluate was dried over anhydrous sodium sulfate (Na_2SO_4) and was concentrated to about 0.2 mL under a stream of nitrogen. This extract, containing free volatile compounds, was immediately analyzed by gas chromatography/mass spectrometry (GC/MS). Then, the glycoconjugates aromas were finally eluted from the cartridge with 20 mL of methanol, and the eluate was concentrated to dryness using a vacuum rotary evaporator set at 30 °C (Buchi R-210, Switzerland). These dried glycosides extract were dissolved in 5 mL of citrate-phosphate buffer (0.2 M, pH 5) and subjected to enzymatic hydrolysis with 50 mg of an AR-2000 commercial preparation with glycosidase side activities (DSM Oenology, The Netherlands) at 40 °C for 24 hours. Later, 0.2 mL of 1-heptanol was added as internal standard, and the volatiles generated by the enzymatic hydrolysis of glycosylated precursors were then extracted following the SPE method previously described. The dichloromethane

extract obtained was dried using anhydrous Na_2SO_4, concentrated to 0.2 mL, and kept at −20 °C until further analysis. GC/MS analysis was performed with an Agilent 6890 Series GC system and Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Palo Alto, CA) equipped with a DB-WAX column (30 m, 0.250 mm i.d., film thickness 0.25 µm; Agilent Technologies).

The GC-MS conditions used were as reported by Corona *et al.* (2019). The detection was carried out by electron impact mass spectrometry in total ion current (TIC) mode using ionization energy of 70 eV. The mass acquisition range was m/z 30–330. Volatile organic compounds were identified by comparing their mass spectra and GC retention times with those of the pure commercial standard compounds and those within the NIST/EPA/NIH Mass Spectral Library database (Version 2.0d, build 2005). The concentration (µg/kg pulp) of volatile compounds was determined as 1-heptanol equivalents.

All solvents and reagents were purchased from WWR International (Milan, Italy).

Statistical analysis

The data were presented as mean ± standard deviation and analysed using the Tukey test at $P \leq 0.05$. All statistical analysis was conducted using XLSTAT software version 9.0 (Addinsoft, Paris, France).

Results And Discussion

Commercial classification

According to commercial classification, our data showed that local ecotypes sizes were larger than international ecotypes (Amorós *et al.*, 2003; Testa *et al.,* 2020). However, only Peluche had the highest value as a well-known large fruit cultivar (Barone *et al.*, 2010).

Varieties with a larger size are more appreciated by consumers because of their small portion size and high sugar/acid ratio. (Agusti *et al.* 2000; Testa*et al.* 2020). More sugar in local fruits versus international cultivars shows an increased degree of acidity (Gentile *et al.,* 2016).

In this study, all the international cultivars had yellow flesh, whereas, among the local cultivars, only Nespolone di Trabia showed more similar behavior with that of the international cultivars (yellow flesh). On the other hand, BRT 20, Claudia, and Sanfilippara, showed white flesh with highest commercial classification (Table 1).

Table 1. Commercial classification based on origin, size, and color of analyzed loquat fruits: GGG> 53mm, GG 46-52mm, G 32-45 mm, and M 31-28 mm.

Origin	Cultivars	Commercial classification	Color classification
Local	BRT 20	GGG	White flesh
	Claudia	GGG	White flesh
	Sanfilippara	GG	White flesh
	Nespolone di trabia	GG	Yellow flesh
International	Algerie	GG	Yellow flesh
	Bueno	G	Yellow flesh
	El buenet	G	Yellow flesh
	Golden nugget	GG	Yellow flesh
	Peluche	GGG	Yellow flesh

Volatile compounds analysis

GC-MS analysis of the concentrated flesh extract of SPE was performed to evaluate the aromatic compounds of loquat fruit flesh, and 35 free volatile compounds (Table 2) and 17 glycosylated compounds (Table 3) were detected. Of which, 14 were acids, 10 alcohols, two aldehydes, one benzenoid, and eight esters. Among which four were terpenes, four C13-norisoprenoids, and nine benzenoids. The latter were released after enzymatic hydrolysis from aromatic precursors linked to sugars.

During maturation, the volatile compounds of the two cultivars, Algerie and Golden nugget, have been studied, and a strong similarity was identified in terms of aroma, flavor, and parameters related to physiological-qualitative traits (Pino *et al.,* 2002; Besada *et al.*, 2013, 2017).

The heritability of loquat aromas was assessed by Jiang *et al.* (2014) by examining the composition of the volatile compounds of Xiantgtian and Jiefangzhong cultivars and two-hybrid progenies (Xiangzhong No.11 and Zhongxiang No. 25). They concluded that the level of volatile compounds in the fruit of the progeny was like the values known in their parents. Previous studies showed that the maturity stage determines the qualitative and quantitative volatile substances of many fruit species (Mattheis *et al.* 1992; Perez *et al.* 1992), for loquat, very little is known about their association with other ripening or quality characteristics that vary between cultivars (Jiang *et al.*, 2014).

Free volatile compounds

Among the free volatile compounds, minimal presence of volatile compounds that can characterize the aroma of loquat fruit was observed. It is a predominance of compounds belonging to the class of acids and alcohols, followed by esters, aldehydes, and finally a benzenoid

Table 2. Free volatile compounds released by enzymatic hydrolysis of glycosylated precursors (µg/kg pulp).

Compounds	Local cultivars*				International cultivars*				
	BRT 20	Claudia	Sanfilippara	Nespolone di trabia	Algerie	Bueno	El buenet	Golden Nugget	Peluche
Acids									
Butyric acid	4.0 ± 0.0	6.2 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	3.6 ± 0.2	6.4 ± 0.4	n.d.	0.5 ± 0.1
Pentanoic acid	10.3 ± 1.6	7.2 ± 0.1	3.5 ± 0.1	9.4 ± 0.3	8.1 ± 0.7	5.5 ± 0.5	15.7 ± 1.8	8.9 ± 0.7	8.5 ± 0.7
Hexanoic acid	1060.9 ± 44.7^{b}	1190.9 ± 31.0^{b}	554.4 ± 34.3^{a}	511.1 ± 29.4^{a}	475.2 ± 57.6^{ab}	645.8 ± 49.3^{ab}	358.4 ± 7.3^{a}	598.5 ± 50.4^{ab}	506.1 ± 34.9^{ab}
Heptanoic acid	2.2 ± 0.6^{b}	2.2 ± 0.2^{b}	2.2 ± 0.1^{b}	0.8 ± 0.0^{a}	0.1 ± 0.0^{a}	3.4 ± 0.2^{b}	3.8 ± 0.2^{b}	0.8 ± 0.0^{a}	0.4 ± 0.0 ^{a}
Octanoid acid	13.6 ± 1.3^{b}	11.9 ± 1.1^{b}	9.9 ± 0.1^{a}	11.9 ± 0.2^{b}	13.8 ± 2.0^{b}	10.5 ± 1.6^{ab}	12.1 ± 0.7^{ab}	4.2 ± 0.2^{a}	10.1 ± 0.7 ^{ab}
Nonanoic acid	18.4 ± 1.7^{b}	17.4 ± 1.9	17.4 ± 0.7	16.4 ± 1.2	13.2 ± 1.5	20.4 ± 1.5	18.9 ± 1.8	20.8 ± 1.3	19.0 ± 1.5
Decanoic acid	16.8 ± 1.0^{b}	8.0 ± 0.1^{a}	8.2 ± 0.5^{a}	17.4 ± 0.2^{b}	33.5 ± 1.3^{b}	12.0 ± 0.7^{a}	8.0 ± 0.3^{a}	20.1 ± 0.8^{ab}	11.4 ± 1.1^{a}
Dodecanoic acid	3.9 ± 0.1^{a}	2.4 ± 0.3^{a}	4.2 ± 0.1^{a}	9.9 ± 0.1^{b}	13.3 ± 0.8^{ab}	8.4 ± 0.6^{ab}	39.1 ± 3.6^{b}	12.8 ± 0.2^{ab}	4.4 ± 0.1^{a}
Tetradecanoic acid	7.5 ± 0.1	1.9 ± 0.2	3.7 ± 0.2	3.2 ± 0.0	7.4 ± 0.4	14.1 ± 1.4	15.6 ± 1.2	6.7 ± 0.7	1.8 ± 0.2
Pentadecanoic acid	7.6 ± 0.3^{a}	19.8 ± 1.2^{b}	15.9 ± 0.2^{b}	45.3 ± 1.0^{c}	4.0 ± 0.0^{a}	10.8 ± 0.8^{abc}	5.3 ± 0.2^{ab}	25.9 ± 0.1^{d}	1.3 ± 0.1^{a}
Hexadecanoic acid	240.6 ± 17.4^{a}	219.4 ± 21.1^{a}	444.9 ± 95.5^{b}	341.7 ± 21.3^{b}	636.4 ± 16.0^{b}	667.6 ± 26.8^{b}	617.8 ± 13.7^{b}	525.0 ± 10.5^{ab}	105.0 ± 4.2^{a}
Heptadecanoic acid	4.3 ± 0.2	n.d.	0.3 ± 0.0	2.1 ± 0.1	5.4 ± 0.1	3.4 ± 0.1	3.8 ± 0.3	1.5 ± 0.1	n.d.
Octadecanoid acid	124.5 ± 11.6	120.3 ± 10.2	133.4 ± 21.7	116.1 ± 4.8	143.4 ± 4.2^{b}	171.7 ± 12.0^{b}	137.0 ± 10.3^{b}	118.8 ± 13.7^{b}	73.9 ± 2.6^{a}
Benzoic acid	1053.3 ± 53.0^{b}	1185.2 ± 130.4^{b}	547.8 ± 33.5^{a}	504.5 ± 25.7^{a}	464.2 ± 55.2^{ab}	640.5 ± 61.0^{ab}	353.6 ± 7.9^{a}	594.4 ± 40.2^{ab}	502.8 ± 35.2^{ab}
Total	2567.8 ± 133.6^{b}	2792.8 ± 197.9^{b}	1746.3 ± 187.0^{a}	1590.6 ± 84.4^{a}	1818.4 ± 138.9^{ab}	2217.4 ± 157.7^{ab}	1595.3 ± 49.7^{ab}	1938.4 ± 118.9^{ab}	1245.2 ± 81.4^{a}
Alcohols									
1-Pentanolo	2.4 ± 0.0^{b}	2.9 ± 0.1^{b}	1.6 ± 0.1^{a}	2.7 ± 0.0^{b}	1.7 ± 0.1^{ab}	1.7 ± 0.1^{ab}	5.6 ± 0.2^{b}	2.7 ± 0.0^{ab}	4.3 ± 0.2^{ab}
3-Heptanol	0.6 ± 0.0	n.d.	0.4 ± 0.0	n.d.	0.3 ± 0.0^{a}	0.7 ± 0.0^{a}	2.8 ± 0.1^{b}	n.d.^{a}	0.2 ± 0.0^{a}
2-Hexanol	1.3 ± 0.0	n.d.	0.4 ± 0.0	1.6 ± 0.0	1.3 ± 0.1^{a}	54.7 ± 4.3^{b}	6.3 ± 0.2^{ab}	1.3 ± 0.1^{a}	2.0 ± 0.1^{a}
1-Hexanol	1.5 ± 0.0^{a}	1.4 ± 0.0^{a}	4.7 ± 0.1^{b}	5.5 ± 0.0^{b}	12.4 ± 0.3^{b}	2.0 ± 0.0^{a}	3.9 ± 0.1^{a}	3.5 ± 0.1^{a}	3.3 ± 0.2^{a}
3-Ethyl-3-heptanol	0.3 ± 0.0	n.d.	n.d.	n.d.	n.d.	0.2 ± 0.0	0.2 ± 0.0	n.d.	0.3 ± 0.1
Cis-3-hexen-1-ol	2.0 ± 0.0^{a}	12.5 ± 0.1^{bc}	6.7 ± 0.1^{b}	22.2 ± 1.2^{c}	20.0 ± 1.4^{ab}	5.9 ± 0.3^{ab}	2.8 ± 0.1^{a}	5.7 ± 0.2^{ab}	4.8 ± 0.0^{ab}
2-Butoxyethanol	5.4 ± 0.0^{b}	3.4 ± 0.1^{a}	3.5 ± 0.0^{a}	2.4 ± 0.0^{a}	5.0 ± 0.1^{ab}	3.9 ± 0.1^{ab}	2.0 ± 0.0^{a}	2.3 ± 0.1^{ab}	3.3 ± 0.3^{ab}
Trans-3-hexen-1-ol	2.7 ± 0.0^{a}	6.5 ± 0.1^{b}	4.0 ± 0.0^{a}	9.4 ± 2.6^{b}	26.5 ± 0.3^{c}	11.2 ± 0.2^{ab}	8.1 ± 0.1^{ab}	7.5 ± 0.2^{ab}	14.6 ± 0.2^{b}
2-Ethyl hexanol	5.3 ± 0.0	2.4 ± 0.0	3.9 ± 0.1	2.3 ± 0.0	5.6 ± 0.2	4.7 ± 0.2	2.5 ± 0.1	3.3 ± 0.1	2.8 ± 0.1

Total	21.6 ± 0.0^{a}	29.1 ± 0.4^{a}	25.4 ± 0.4^{a}	46.2 ± 3.9^{b}	72.5 ± 2.5^{c}	85.1 ± 5.1^{bc}	34.2 ± 0.9^{abc}	26.5 ± 0.8^{abc}	35.6 ± 1.2^{abc}
Aldehydes									
Cis-2-hexenal	6.2 ± 0.1^{a}	8.7 ± 0.1^{a}	9.2 ± 0.2 ^{a}	20.5 ± 0.2^{b}	92.1 ± 8.9^{b}	7.9 ± 0.6^{a}	n.d.^{a}	5.0 ± 0.1^{a}	30.3 ± 1.7^{ab}
Nonanal	1.6 ± 0.0 ^{ab}	3.5 ± 0.1^{b}	2.4 ± 0.1^{ab}	1.0 ± 0.0^{ab}	0.6 ± 0.0^{a}	1.8 ± 0.1^{ab}	0.4 ± 0.0^{a}	1.4 ± 0.2^{ab}	1.3 ± 0.2^{ab}
Total	7.8 ± 0.1^{a}	12.3 ± 0.2^{ab}	11.6 ± 0.3^{ab}	21.5 ± 0.2^{b}	92.7 ± 8.9^{b}	9.7 ± 0.7^{ab}	0.4 ± 0.0^{a}	6.4 ± 0.3^{a}	31.6 ± 1.9^{ab}
Benzenoids									
Vanillin	11.7 ± 0.2^{b}	8.7 ± 0.1^{a}	8.0 ± 0.1^{a}	8.2 ± 0.0^{a}	11.1 ± 1.1^{ab}	27.9 ± 2.4^{b}	n.d.^{a}	11.8 ± 0.2^{ab}	10.1 ± 0.1^{ab}
Esters									
Ethyl hexanoate	1.9 ± 0.1^{a}	n.d.^{a}	5.0 ± 0.1^{ab}	0.6 ± 0.0^{a}	3.3 ± 0.5^{a}	3.8 ± 0.4^{a}	9.6 ± 0.3^{b}	1.0 ± 0.0 ^{a}	3.2 ± 0.2^{a}
Ethyl octanoate	3.4 ± 0.0^{a}	1.4 ± 0.0^{a}	9.4 ± 0.3^{a}	3.7 ± 0.0^{a}	11.3 ± 1.8^{a}	6.7 ± 0.3^{a}	25.2 ± 0.3^{b}	3.1 ± 0.2^{a}	7.4 ± 0.2^{a}
Ethyl-3-hydroxy butyrate	230.8 ± 8.7^{d}	10.0 ± 0.1^{a}	181.1 ± 9.6^{cd}	7.1 ± 0.1^{a}	83.3 ± 4.5^{b}	203.9 ± 23.1^{d}	120.8 ± 10.8^{c}	5.2 ± 0.3^{a}	4.6 ± 0.0^{a}
Ethyl decanoate	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	1.3 ± 0.0^{ab}	1.2 ± 0.0^{ab}	2.8 ± 0.2^{b}	0.5 ± 0.0^{a}	2.4 ± 0.1^{b}	0.6 ± 0.0^{a}	0.5 ± 0.1^{a}
Diethyl succinate	0.8 ± 0.0^{a}	n.d.^{a}	1.3 ± 0.1^{ab}	0.1 ± 0.0^{a}	3.3 ± 0.1^{b}	0.7 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	2.5 ± 0.1^{b}
Butanoic Acid-2-methyl	137.4 ± 10.9^{a}	118.7 ± 9.1^{a}	8.7 ± 0.1^{a}	65.7 ± 1.8^{a}	623.9 ± 7.1^{c}	415.1 ± 36.2^{b}	308.0 ± 28.6^{b}	7.3 ± 0.2^{a}	301.3 ± 3.8^{b}
Hexadecanoic acid methyl ester	4.4 ± 0.4^{a}	25.3 ± 0.2^{b}	2.7 ± 0.2^{a}	7.4 ± 0.2^{a}	4.3 ± 0.3^{a}	4.6 ± 0.3^{a}	2.9 ± 0.1^{a}	1.6 ± 0.1^{a}	6.4 ± 0.2^{b}
Hexadecanoic acid ethyl ester	1.6 ± 0.0	2.6 ± 0.0	1.4 ± 0.1	2.4 ± 0.2	7.0 ± 0.3	1.5 ± 0.1	5.4 ± 0.2	2.4 ± 0.2	2.8 ± 0.1
Total	380.5 ± 20.1^{cd}	158.2	210.9 ± 10.5^{abc}	88.1 ± 2.3^{a}	739.3 ± 14.8^{c}	636.7 ± 60.4^{c}	474.7 ± 40.4^{b}	21.5 ± 1.0^{a}	328.8 ± 4.7^{b}

*Mean ± standard deviation (n = 3). Different superscripted letters indicate significant differences for P ≤ 0.05 (analysis of variance or Tukey test).
n.d., not determinable.

Table 3. Glycosylated volatile compounds released by enzymatic hydrolysis of glycosylated precursors (µg/kg pulp).

	Local cultivars*				International cultivars*				
Compounds	**BRT 20**	**Claudia**	**Sanfilippara**	**Nespolone d' trabia**	**Algerie**	**Bueno**	**El buenet**	**Golden nugget**	**Peluche**
Terpenes									
Trans-8-diidrolinalool	6.1 ± 0.0^{b}	8.0 ± 0.1^{b}	4.0 ± 0.0^{a}	3.3 ± 0.0^{a}	10.7 ± 0.2^{c}	6.9 ± 0.0^{b}	3.4 ± 0.1^{ab}	6.1 ± 0.2^{b}	1.4 ± 0.0^{a}
Trans-8-hydroxylinalool	5.5 ± 0.1^{a}	15.1 ± 0.3 ^{b}	7.0 ± 0.2^{a}	15.5 ± 0.3^{b}	12.8 ± 0.1^{bc}	9.5 ± 0.2^{b}	7.1 ± 0.2^{b}	16.0 ± 1.4^{c}	1.0 ± 0.0^{a}
Cis-8-hydroxylinalool	1.5 ± 0.0^{a}	4.1 ± 0.1 ^{ab}	8.5 ± 0.2^{b}	7.6 ± 0.3^{b}	3.2 ± 0.0^{b}	4.3 ± 0.2^{bc}	5.7 ± 0.1^{c}	3.7 ± 0.2^{b}	1.1 ± 0.0^{a}
Geranial	3.4 ± 0.0^{ab}	2.6 ± 0.2^{a}	6.9 ± 0.2^{b}	4.2 ± 0.2^{ab}	6.0 ± 0.0^{bc}	3.8 ± 0.1^{a}	4.8 ± 0.1^{b}	7.6 ± 0.2^{c}	5.6 ± 0.2^{b}
Total	16.5 ± 0.1^{a}	29.8 ± 0.7^{b}	26.4 ± 0.6^{b}	30.6 ± 0.8^{b}	32.7 ± 0.3^{c}	24.5 ± 0.5^{b}	21.1 ± 0.5^{b}	33.4 ± 2.0^{c}	9.2 ± 0.2^{a}
C13-norisoprenoids									
3-oxo-a-ionol	59.2 ± 4.8^{a}	62.4 ± 1.2^{a}	94.4 ± 4.9^{ab}	207.5 ± 23.0^{b}	461.0 ± 41.5^{d}	286.6 ± 19.8^{c}	217.9 ± 19.3^{b}	373.4 ± 34.0^{c}	112.3 ± 9.8^{a}
3-4-dihydro-3-oxo-a-ionol	19.8 ± 1.1^{a}	41.9 ± 1.1^{ab}	32.7 ± 2.9^{ab}	74.1 ± 14.0^{b}	92.1 ± 7.2^{c}	55.6 ± 4.2^{b}	27.7 ± 2.0^{a}	72.9 ± 17.1^{bc}	24.8 ± 1.1^{a}
3-OH-b-ionone	2.0 ± 0.0^{a}	28.5 ± 1.3^{ab}	34.1 ± 1.6^{b}	64.7 ± 3.7^{c}	23.4 ± 2.9^{c}	13.0 ± 1.8^{bc}	7.8 ± 1.0^{ab}	22.2 ± 1.6^{c}	2.6 ± 0.1^{a}
Vomifoliol	135.0 ± 10.4^{a}	149.2 ± 0.8^{a}	178.0 ± 12.2^{a}	283.4 ± 23.8^{b}	581.6 ± 26.7^{c}	79.9 ± 2.2^{a}	101.2 ± 9.1^{a}	283.8 ± 10.9^{b}	120.0 ± 11.5^{a}
Total	215.9 ± 16.3^{a}	281.9 ± 4.4^{ab}	339.2 ± 21.6^{b}	629.6 ± 64.5^{c}	1158.1 ± 78.3^{c}	435.1 ± 28.0^{ab}	354.5 ± 31.4^{a}	752.3 ± 63.6^{bc}	259.7 ± 22.5^{a}
Benzenoids									
Eugenol	32.3 ± 1.6 ^{a}	21.7 ± 0.1^{b}	15.9 ± 0.2^{a}	27.9 ± 0.3^{b}	55.0 ± 3.6^{b}	59.2 ± 4.1^{b}	24.7 ± 1.9^{a}	121.3 ± 9.5^{c}	16.1 ± 0.2^{a}
4-vinylguaiacol	54.4 ± 4.1^{c}	35.2 ± 0.3^{b}	30.1 ± 0.2^{a}	32.5 ± 0.4^{a}	29.0 ± 2.4^{a}	25.1 ± 2.1^{a}	21.4 ± 0.7^{a}	78.0 ± 8.4^{b}	16.9 ± 0.9^{a}
Isoeugenol	2.6 ± 0.1^{a}	7.3 ± 0.1^{b}	7.9 ± 0.1^{b}	5.2 ± 0.1^{a}	23.0 ± 0.1^{c}	22.0 ± 1.0^{c}	24.5 ± 1.5^{d}	10.8 ± 0.7^{ab}	7.0 ± 0.1^{a}
Methylvanillate	30.5 ± 0.2^{a}	192.5 ± 11.9^{b}	24.7 ± 0.2^{a}	45.1 ± 0.4^{a}	194.9 ± 10.7^{c}	32.0 ± 2.9^{a}	19.8 ± 1.7^{a}	134.8 ± 0.4^{b}	11.8 ± 0.2^{a}
Benzylalcohol	5.9 ± 0.2^{a}	7.9 ± 0.2^{a}	6.8 ± 0.1^{a}	12.2 ± 0.2^{b}	11.3 ± 1.1^{c}	3.4 ± 0.2^{ab}	4.8 ± 0.2^{b}	12.2 ± 0.1^{c}	2.5 ± 0.1^{a}
2-phenylethanol	225.4 ± 13.6^{b}	443.8 ± 24.3^{c}	177.4 ± 13.7^{a}	234.1 ± 21.1^{b}	285.1 ± 18.7^{c}	92.4 ± 2.1^{b}	84.7 ± 2.0^{b}	241.7 ± 13.0^{c}	77.2 ± 6.2^{a}
Vanillin	18.8 ± 0.2^{c}	13.8 ± 0.7^{b}	3.9 ± 0.2^{a}	13.4 ± 1.8^{b}	12.4 ± 0.8^{ab}	10.2 ± 1.0^{ab}	9.1 ± 0.7^{ab}	25.4 ± 2.3^{c}	2.8 ± 0.1^{a}
Methoxyeugenol	5.7 ± 0.2^{b}	5.3 ± 0.1^{b}	3.9 ± 0.1^{a}	2.2 ± 0.2^{a}	2.3 ± 0.0^{b}	3.4 ± 0.1^{c}	5.6 ± 0.3^{d}	0.4 ± 0.0^{a}	3.8 ± 0.1^{c}
Syringaldehyde	56.3 ± 0.4^{ab}	4.6 ± 0.1^{a}	45.9 ± 2.2^{ab}	36.8 ± 3.2^{ab}	68.7 ± 3.4^{b}	16.7 ± 0.9^{ab}	26.3 ± 1.9^{ab}	10.2 ± 0.7 ^{ab}	7.0 ± 1.0^{a}
Total	431.9 ±20.6^{b}	732.3 ± 37.8^{c}	316.5 ± 17.0^{a}	409.4 ± 27.7^{b}	681.7 ± 40.8^{c}	264.3 ± 14.4^{ab}	221.0 ± 10.9^{ab}	634.8 ± 35.1^{c}	145.1 ± 8.9^{a}

*Mean ± standard deviation (n=3). Different superscripted letters indicate significant differences for P ≤ 0.05 (analysis of variance or Tukey test). n.d., not determinable.

(vanillin only, according to Hideki *et al.* 1998). The identified acids range from C4 to C18, the esters from C4 to C10 and C16; they contribute to the taste sensations perceptible in the mouth during tasting and eating of the flesh, giving taste, fat sensation, and different aromatic sensations, ranging from fruity to floral (Table 4). The greater acids concentration are: hexanoic (1060.9±44.7 in BRT20, local cultivar), benzoic (1185.2 mg/kg flesh fruit in Claudia, local cultivar), hexadecanoic (667.6 mg/kg flesh fruit in Bueno, international cultivar), and octadecanoic (171.7 mg/kg flesh fruit also in Bueno cultivar).

In terms of bad tastes, the highest values of butyric and decanoic acid, commonly detectable in cheese and fat

Table 4. Odor descriptor and odor threshold volatile compounds.

Compuonds	Odor descriptor	Odour threshold (ppb)	Reference
Acids			
Butyric acid	Rancid, cheese	173	Fariña *et al.* (2015)
Pentanoic acid	Sweet	70	Pino and Mesa (2006)
Hexanoic acid	Fatty, cheese	420	Fariña *et al.* (2015)
Heptanoic acid	Waxy, cheese, fruity	–	www.thegoodscentscompany.com/
Octanoic acid	Fatty, cheese	500	Fariña *et al.* (2015)
Nonanoic acid	Green, fatty	3000	Pino and Mesa (2006)
Decanoic acid	Rancid, fatty	1000	Fariña *et al.* (2015)
Dodecanoic acid	Fatty, waxy	–	www.thegoodscentscompany.com/
Tetradecanoic acid	Waxy, oily, fatty	–	www.thegoodscentscompany.com/
Pentadecanoic acid	Waxy	–	www.pherobase.com
Hexadecanoic acid	Oily	–	www.pherobase.com
Heptadecanoic acid	Oily	–	www.pherobase.com
Octadecanoid acid	Oily	–	www.pherobase.com
Benzoic acid	Balsamic	–	
Alcohols			
1-Pentanol	Green, grassy, powerful	4,000	Pino and Mesa (2006)
3-Heptanol	Herbal	–	www.thegoodscentscompany.com/
2-Hexanol	Chemical, winey	500	Pino and Mesa (2006)
1-Hexanol	Fatty, green, resin, flower, sweet	500	Bonneau *et al.* (2016)
3-Ethyl-3-heptanol		–	
cis-3-hexen-1-ol	Green, moss, fresh	110	Bonneau *et al.* (2016)
2-butoxyethanol		–	
trans-3-hexen-1-ol	Green, grass, fruity	70	Bonneau *et al.* (2016)
2-Ethyl-hexanol	Oily, rose, sweet	–	Li *et al.* (2011)
Aldehydes			
Trans-2-hexenal	Green, banana-like	17	Pino and Mesa (2006)
Nonanal	Fatty, citrus, green, floral, sweet, soapy	1	Bonneau *et al.* (2016)
Benzenoid			
Vanillin	Vanilla-like, sweet	25	Bonneau *et al.* (2001)
Esters			
Ethyl hexanoate	Apple peel, fruity	1	Pino and Mesa (2006)
Ethyl octanoate	Fruity, fat	194	Pino and Mesa (2006)
Ethyl-3-hydroxy butyrate	Fruity, grape	1000	Moyano *et al.* (2002)
Ethyl decanoate	Sweet, oily, nutlike, grape	6300	Pino and Mesa (2006)
Diethyl succinate	Overripe melon, lavender	100,000	Fariña *et al.* (2015)
2-methylbutanoic acid	Cheese	250	Fariña *et al.* (2015)
Methyl hexadecanoic	Waxy	–	www.thegoodscentscompany.com/
Ethyl hexadecanoic	Waxy	–	www.thegoodscentscompany.com/

notes was observed only in Bueno and El buenet (both international cultivars).

Among the esters, higher amounts of ethyl-3-OH-butyrate and 2-methyl-butanoic acid were observed.

The very limited presence of C6 alcohols, deriving from the enzymatic activities of lipoxygenase, highlights how loquat is poorly endowed with these enzymes, which lead to the formation of herbaceous aromas that are not always pleasant, or that the sample preparation was done correctly. The different cultivars examined show significant differences in ester and alcohol content: international Algerie and Bueno cultivars tend to have the highest values.

The total contents of acids, aldehydes and benzenoids show a similar profile in most cultivars. Significant differences were recorded for total acid content in Claudia (local cultivar) which represent the highest values (2792 mg/kg flesh fruit) and in Peluche (international cultivar), which represent the lowest values (1245 mg/kg flesh fruit); in total aldehydes, Algerie has significantly higher values and BRT 20, El buenet and Golden nugget are lower; finally, benzenoids are present in higher amounts in Bueno.

Glycosylated volatile compounds

A limited number of glycosylates, released by enzymatic hydrolysis, have been recorded in the studied cultivars, demonstrating that loquat does not have a high supply of sugar-related flavors, which can be released and perceived after swallowing the flesh (flavor).

Among the identified glycosylates, there is a clear predominance of compounds belonging to the class of benzenoids and C13-norisoprenoids followed by terpenes, the latter is present at low concentrations and with significant differences between cultivars.

Among the benzenoids, the presence of eugenol, 4-vinylguaiacol, vanillin, syringaldehyde, methylvanillate, and 2-phenylethanol was shown according to the study outcome of Chen *et al.* (2011) and Shaw and Wilson (1982). Among the C13-norisoprenoids and terpenes presence of vomifoliol and 3-oxo-α-ionol and their dehydroxylated forms is recorded. Important compounds that contribute to aromatic sensations range from fruity to floral (Table 5). The international cultivars tend to have a higher concentration of these compounds, especially Algerie and Golden nugget. Among the local cultivars, Claudia has the highest values. Higher values for total benzenoids are observed in Claudia (local cultivar), Golden nugget, and Algerie (international cultivars); Higher C13-norisoprenoids in Algerie and Golden nugget; the latter also had higher total terpenes. As in all cultivars, the synthesis of terpene compounds is shifted towards dehydroxylated forms (trans-8-Hydroxylinalool and cis-8-Hydroxylinalool), and geraniol is present only among monohydroxylates.

Table 5. Odor descriptor and odor threshold volatile compounds.

Compounds	Odor descriptor	Odor threshold (ppb)	Reference
Terpenes			
Linalool	Floral, lavender	6	Pino and Mesa (2006)
Trans-8-hydroxy-linalool	Sweet, floral, creamy	–	www.thegoodscentscompany.com/
Cis-8-hydroxy-linalool	Sweet, floral, creamy	–	www.thegoodscentscompany.com/
Geranial	Citrus-like, flowery, fruity	32	Pino and Mesa (2006)
C13-norisoprenoids			
3-oxo-a-ionol	Spicy, woody, violet	–	www.pherobase.com
3,4-dihydro-3-oxo-actinidol 1		–	www.pherobase.com
3-OH-b-ionone	Flower, violet	–	www.thegoodscentscompany.com/
Vomifoliol	Fruity	–	www.pherobase.com
Benzenoids			
Eugenol	Clove, spicy, balsamic	6	Pino and Mesa (2006)
4-Vinylguaiacol	Clove, curry	3	Pino and Mesa (2006)
Isoeugenol	Flower	6	Escudero *et al.* (2007)
Methyl vanillate	Caramel, butterscotch, vanilla	990	Escudero *et al.* (2007)
Benzylalcohol	Sweet, flower	–	www.pherobase.com
2-Phenylethanol	Hawthorne, honey, sweet	1100	Pino and Mesa (2006)
Methoxyeugenol	Sweet, flower	–	www.pherobase.com
Syringaldehyde	Sweet, cocoa, chocolate	50,000	Escudero *et al.* (2007)

Conclusion

Among local cultivars, only cv Claudia had higher values of glycosylated compounds highlighting the floral and acidic notes that are appreciated in the market. Regarding the international cultivars, all yellow fleshed cultivars had a higher number of free aromatic compounds that showed cheese and fat notes (butyric and decanoic acid) and other odors and flavors less appreciated by consumers. They have fewer acids and a greater number of glycosylated compounds that showed characteristic floral and woody notes. This article tends to highlight the importance of local and international cultivars in Mediterranean environments that are grown with the best market characteristics.

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Factors affecting the cooking quality of stored carioca beans (*Phaseolus vulgaris*)

Juliana Aparecida Correia Bento[1], Karen Carvalho Ferreira[1], Priscila Zaczuk Bassinello[2]*, B. Dave Oomah[3†]

[1]School of Agronomy, Federal University of Goiás—UFG, Goiânia, Brazil; [2]Embrapa Rice and Beans, Santo Antônio de Goiás, Brazil; [3]Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, BC, Canada

[†]Retired.

***Corresponding Author**: Priscila Zaczuk Bassinello, Embrapa Rice and Beans, PO Box 179, Santo Antônio de Goiás, Goiás, 75375-000, Brazil. Email: priscila.bassinello@embrapa.br

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REVIEW

Abstract

The culinary quality of carioca beans is related to their market value and consumer acceptability. The depreciation of the cooking/technological quality of the product occurs mainly because of the integument browning and the longer cooking time of the grains, which are influenced by the storage time and conditions. The loss of culinary quality reduces the market value of carioca beans because consumers reject darkened grains that are attributed to a longer cooking time. As a result, cooking time (resistance to cooking), the color of the integument, and the texture of the cooked beans are determinant factors in the acceptance of carioca bean cultivars. The browning of the grain integument and the cooking time mainly depends on the environmental conditions, storage time, the tegument of each genotype, and the chemical and physical properties of the cotyledons. Therefore, this review aims to survey the scientific literature on the extrinsic and intrinsic factors that affect the culinary quality of carioca beans.

Keywords: browning; cooking time; polyphenolic compounds; quality loss; storage

Introduction

Beans are cultivated during the whole agricultural year, in distinct ecosystems, principally in developing countries, which are responsible for 86.7% of world consumption. In 2019, five countries (Myanmar, India, Brazil, China, and the United Republic of Tanzania) accounted for 59% of the world's dry bean production (~30.2 million tons; Food and Agriculture Organization of the United Nations, 2019). Beans are rich in essential nutrients like (i) proteins with high lysine content (essential amino acid); (ii) high complex carbohydrate content as oligosaccharides, and dietary fibers with their recognized hypocholesterolemic and hypoglycemic effects; and (iii) complex vitamins and minerals (calcium [Ca], iron [Fe], copper [Cu], zinc [Zn], phosphorus [P], potassium [K], and magnesium [Mg]); bioactive antioxidant compounds including saponins, polyphenols, and anthocyanins (Ganesan and Xu, 2017; Lovato *et al.*, 2017; Oliveira *et al.*, 2017; Celmeli *et al.*, 2018; Yang *et al.*, 2018; Jeepipalli *et al.*, 2020; Liu *et al.*, 2020;). Therefore, regular consumption of this pulse benefits human health. The US Department of Health and Human Services (US-DHHS, 2015) recommends eating about three cups of beans like pinto, kidney, or black beans/per week because of their health benefits.

Brazil is the largest global producer of common beans, mainly for domestic consumption, with an average per capita bean consumption of 16 kg in 2013 (Rawal and

Navarro, 2019). The national market in Brazil predominantly consists of two classes of beans: carioca (70%) and black bean (20%; Souza *et al.*, 2020). Carioca beans are seasonally produced beans in Brazil and many other countries. So, to maintain bean supply throughout the year and prevent scarcity between harvests, the storage of this variety is crucial. However, improper storage can cause undesirable changes in the carioca beans leading to consumer rejections (Carbonell *et al.*, 2010; Scariot *et al.*, 2017; Alvares *et al.*, 2020). These changes occur in the integument of some carioca bean cultivars because some genotypes darken very quickly, and the bean also hardens itself rapidly. These processes reduce the culinary quality of the carioca beans economically, depreciating the product (Bento *et al.*, 2020a, 2020b; de Farias *et al.*, 2020; Bento *et al.*, 2021a).

The loss of culinary quality reduces the market value of carioca beans because consumers reject darkened grains that are considered resistant to cooking. Consequently, determinant factors in the acceptance of carioca bean cultivars are cooking time (resistance to cooking), the color of the integument, and the texture of the cooked beans. Therefore, this review aims to survey the scientific literature on factors that affect the culinary quality of carioca beans during storage.

Culinary Quality

Culinary quality of beans refers to their sensory attributes, technological properties, such as water absorption before and after cooking, cooking time or resistance to cooking, percentage of soluble solids in the broth, integument color, and the broth. Crop production, postharvest drying, and storage conditions are responsible for bean quality when it reaches its destination, either at the consumer's table or back in the field to be used as seed. Generally, beans subjected to improper handling or even storage conditions will influence the culinary quality, resulting in consumer rejection of the product.

Breeding new carioca bean cultivars emphasize high technological and cooking quality prioritizing preferential selection for lighter grains, 250–300 g 1000 seed weight, elliptical shape, semi-full degree of grain flatness, and cooking time less than 25 minutes (Kaur *et al.*, 2009; Wani *et al.*, 2013, 2017; Yadav *et al.*, 2018; Ribeiro *et al.*, 2019;). Bean coats are rich in water-insoluble fibers and polyphenols. Their cotyledons have higher soluble fibers, oligosaccharides, and resistant starch (Singh, 2017). Hardening of the husk during bean storage occurs mainly when the air humidity is high, hindering hydration during preparation for consumption. The increased air humidity accompanied by high temperatures increases the incidence of a hardshell and hard-to-cook (HTC) grains, thereby reducing consumer's acceptance, digestibility, and protein absorption (Oliveira *et al.*, 2011).

Briefly, 52% of surveyed consumers preferred commercial brands of carioca beans for their technological (lighter color grains [53 and 6.2; L^* and a^* values, respectively], medium-sized beans [28 g], elliptical shape, and semi-full flatness), cooking (fast cooking time ~15 min), and nutritional (high protein and minerals [K, P, Ca, Zn and Cu] contents) quality traits (Ribeiro *et al.*, 2019).

Resistance to cooking/cooking time

The time for beans to reach the desired degree of softness, determined as cooking time, is a critical attribute for consumers. Cooking time can also be evaluated by the resistance to cooking generally determined using the Mattson cooker (Figure 1; Proctor and Watts, 1987; Bento *et al.*, 2020a). Freshly harvested grains have less resistance to cooking. However, when stored under ambient conditions, carioca beans increase their resistance to cooking or take longer to cook (Alvares *et al.*, 2020; Bento *et al.*, 2020a, 2020b; de Farias *et al.*, 2020; Alvares *et al.*, 2020; Bento *et al.*, 2021a).

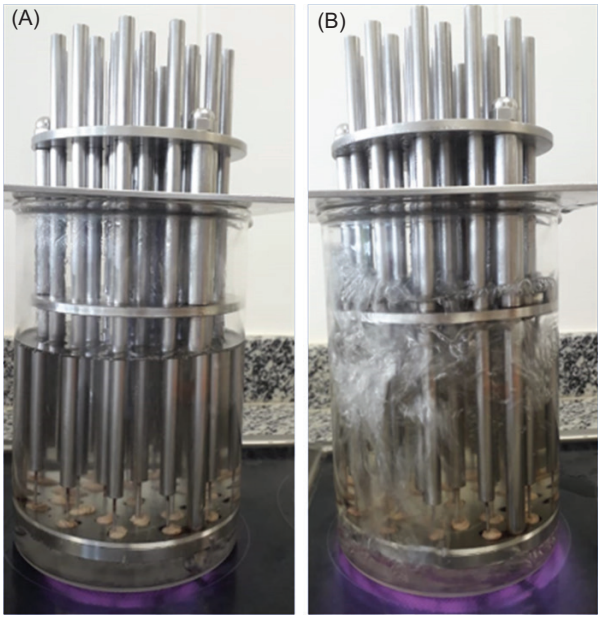

Figure 1. Mattson apparatus for bean resistance classification to cooking based on the scale defined by Proctor and Watts (1987). The time (t_{13}) is recorded until the drop of the 13th rod and is converted into a rank (RMC) of resistance to cook, as described by Bento *et al.* (2020a). Beans in the Mattson apparatus (A). The cooking progress of beans in Mattson apparatus (B).

Beans are soaked in water (1–12 h [overnight]) before cooking to reduce the cooking time. The beans are hydrated, swollen, and smooth during immersion, which reduces cooking time (Yadav *et al.*, 2018). Immersion also promotes the uniform expansion of the seed coat and cotyledon. The extent of hydration and swelling of the grains during immersion depends on the hydration capacity of the grains and varies among different bean cultivars and is related to cooking quality (Singh *et al.*, 2004; Kaur *et al.*, 2009). Therefore, the bean water absorption capacity is a pivotal factor for the technical quality of the grains and is causally related to their resistance to cooking, as the cooking time decreases because of its occurrence before cooking (Kaur *et al.*, 2009). Hence factors like the type of grain (i.e., size or shape), moisture level, genetics, storage time, and storage conditions influence the bean water absorption rate (Delfino and Canniatti-Brazaca, 2010).

The hydration kinetics of carioca beans is complex and mostly related to the seed coat. They are associated with bean composition (fat and K contents, protein to lipid ratio that correlates with the lag phase time) and structure (specific surface and seed coat impermeability to water; Miano *et al.*, 2018). Alkaline conditions (pH 6–12) increase hydration kinetics (rate and water absorption) of the carioca bean (cv. IAC Eté) and reduce the lag phase (83%), affecting the mass transfer behavior in both the seed coat and cotyledons, indicating variations in proteins and polysaccharides (Oladele *et al.*, 2018). Hydration with ferrous sulfate ($FeSO_4$; 0.271% w/v) solution accelerates carioca bean softening and cooking, particularly with ultrasound (91 W/L; 25 kHz) at 25 °C (Miano and Augusto, 2018). This process also fortifies the beans with iron (60 vs. 34 mg Fe/100 g wet basis [510 min, with and without ultrasound]). Moreover, the iron content of the seed coat, cotyledons, and whole carioca beans (cv. IAC Imperador) increased 68, 5, and 16 folds, respectively when soaked in $FeSO_4$solution for 510 minutes compared to conventional hydrate (distilled water) for the same time.

One of the defects (hardshell) that is observed in grains stored under conditions of inadequate temperature and humidity is alleviated bean water absorption, even after long periods of maceration. The hardshell phenomenon can be a consequence of grain storage in high humidity and high temperatures, which also causes the HTC phenomenon (Yadav *et al.*, 2018). That refers to grains requiring a longer cooking time to soften or fail to soften even when subjected to boiling water for extended periods. Storage under ambient conditions of temperature and humidity for 6 months induces HTC phenomenon in common beans and consequently increases the cooking time. For most carioca bean cultivars, only 3 months of storage is sufficient to observe enhanced hardness (Alvares *et al.*, 2020; Bento *et al.*, 2020a; Alvares *et al.*, 2021). The HTC defect manifests itself differently depending on the cultivar, planting, and storage conditions. These defects reduce the culinary quality of beans, causing depreciation and often a rejection of the product (Njoroge *et al.*, 2015, 2016).

The HTC phenomenon has not yet been completely elucidated, despite several studies trying to understand it. Some theories have been postulated to explain the HTC phenomenon: (i) polymerization of phenolic compounds or lignification, (ii) production of insoluble pectates or phytase-phytate-pectin, (iii) changes in protein and starch, and (iv) a multiple mechanism theory (Nasar-Abbas *et al.*, 2008; Siqueira *et al.*, 2016a; Jombo *et al.*, 2018; Siqueira *et al.*, 2018). In the lignification theory of cotyledon tissues, the development of grain hardening is related to the polymerization of phenolic compounds, mainly from phenolic-rich seed coats. The polymerization reaction is mediated by oxido-reducing enzymes and by cross-links formation between the phenolic compounds and the cotyledon cell wall proteins (Nasar-Abbas *et al.*, 2008). However, other studies by Siqueira *et al.* (2014, 2016a, 2018) showed the inadequacy of the lignification mechanism. Demonstrating that the HTC in carioca beans during storage cannot be attributed to changes in the total phenol content or the oxidoreductase activities. According to the most widely accepted "phytase-phytate-pectin" theory (Jombo *et al.*, 2018; Yang *et al.*, 2018), water-soluble pectin allows water absorption by legume seeds. Both phytates and carboxyl groups of soluble pectin can complex with Ca or Mg ions, but phytates preferably bind to divalent cations. If phytates complex with Ca or Mg ions, legume seeds are easy to cook. However, phytates can be hydrolyzed by phytase during storage, reducing their chelating potential. Then the Ca or Mg ions complexes with the carboxyl groups of the soluble pectin to form insoluble Ca and Mg pectates which are not readily dissolved when heated, thus restricting cell separation and inhibiting water absorption resulting in HTC defect (Jombo *et al.*, 2018; Yang *et al.*, 2018; Bento *et al.*, 2020a). Finally, the theory that associates changes in starch during storage (Rupollo *et al.*, 2011) was discredited. Since the noninvolvement of starch or protein in the hardening of carioca bean genotypes was known. In addition, the authors obtained results confirming that the hardening of carioca beans is related to structural changes in the cell wall and middle lamella. Genotypes susceptible to the HTC phenomenon display intense structural changes (Shiga, 2004; Siqueira *et al.*, 2018).

Integument color

Consumers have different requirements. But generally attribute the dark color in carioca beans to prolonged

cooking, and therefore low culinary quality (Ribeiro *et al.*, 2008; Coelho *et al.*, 2009; Cichy *et al.*, 2019; Rodrigues *et al.*, 2019; Alvares *et al.*, 2020; Bento *et al.*, 2020a; de Farias *et al.*, 2020;). According to Bolsinha de SP (Brazilian platform with bean market price information), the market negotiates the price of carioca beans, considering the color of the grain. The color is evaluated visually and subjectively based on an unofficial ascending color grade scale that ranges between 5 and 10, and in practice, the product with a grade below 8.5 is devalued (Bento *et al.*, 2020b; Bolsinha, 2020). In short, carioca beans with dark coloring are depreciated, as they are considered of low culinary quality.

Studies generally show that the luminosity (L^*) of the grains and the Hue angle (H°, color angle) decrease during storage and are influenced by storage time. On the other hand, the value of Chroma (C^*), a variable that measures the opacity of the grains and the total color difference (ΔE), increase during storage (Siqueira *et al.*, 2014, 2016b; Bento *et al.*, 2020a; Coelho *et al.*, 2020; de Farias *et al.*, 2020;). These changes in the instrumental color parameters indicate the darkening of the carioca bean; the grains leave the cream color with brown streaks and become dark brown (Figure 2).

Changes in color parameters during storage are often associated with grain genotype, indicating that cultivars have a genetic predisposition to faster or slower browning (darkening) when stored. da Silva *et al.* (2008) verified that storage time accentuated the differences between quick and moderate browning carioca beans. Moreover, genotype influenced the browning/darkening rate of five carioca bean cultivars stored for 5 months under environmental conditions (Siqueira *et al.*, 2014). In short, genotypes are sensitive to the browning process, exhibiting difference in intensity among genotypes even when stored under high relative humidity, a factor known to promote browning (Rani *et al.*, 2013).

The rate of grain darkening is linked to the genetics of the strains, storage conditions, and storage time (Coelho *et al.*, 2009, 2013; Rani *et al.*, 2013; Spitti *et al.*, 2019; de Farias *et al.*, 2020). Considering that small farmers do not always have the technology or resources to invest in controlled storage of beans (under low temperature and humidity), commercial damage is inevitable, leading to the accumulation of low-value grains in the producers' warehouses, filling stations, and supermarkets. Thereby limiting the production and commercialization of good-quality beans. However, some bean genotypes that darken regardless of storage conditions do not necessarily harden or lose nutritional and functional value, and vice versa (Alvares *et al.*, 2020; Bento *et al.*, 2020b; de Farias *et al.*, 2020).

The browning of stored beans has been extensively studied, although its association with culinary quality is limited in routine breeding programs (Cichy *et al.*, 2019; Rodrigues *et al.*, 2019; Miklas *et al.*, 2020), which can lead to the development of apparently suitable materials in terms of appearance (color). These results in genotypes that are resistant or tolerant to factors that accelerate grain browning or darkening, but not necessarily resistance to the hardening process, masking its culinary quality (Alvares *et al.*, 2020). Therefore, bean breeding programs must consider nutritional properties, culinary properties, and color when selecting genotypes.

Texture

Textural parameters are also used to assess the culinary quality of beans, as grain hardness or resistance

Figure 2. Freshly harvested carioca beans from slow darkening cultivar BRSMG Madrepérola (A), TAA Dama (B), darkening cultivar BRS Notable (C), and IAC Imperador (D). The corresponding grains after 6 months storage (27.5 ± 1.6 °C / 56.9 ± 10.0% relative humidity-RH) (E–H).

to compression strength is one of the primary sensory properties of foods. It refers entirely to the feeling of hardness that the food presents during chewing and can be objectively measured by mechanical means in fundamental units of mass or strength.

The texture parameters for beans refer to the evaluation of the hardness of raw or cooked grains, generally using the texturometer with a return-to-start analysis method that determines the strength required to compress/puncture the bean (Revilla and Vivar-Quintana, 2008; Siqueira *et al.*, 2013, 2014, 2016a; Bento *et al.*, 2020a). Other studies evaluate grain texture through texture profile analysis (TPA) that provides information on hardness, cohesiveness, elasticity, gumminess, and resilience of bean samples (Koriyama and Kasai, 2019; Wani *et al.*, 2013, 2017; Yadav *et al.*, 2018). The best evaluation of carioca bean texture is the method that measures the force necessary to drill the grain with a 2 mm probe (P2) according to Revilla and Vivar-Quintana (2008) and Siqueira *et al.* (2013). The small area of this probe penetrates the integument. They can differentiate similar samples, even when these grains have soft cotyledons but hard integument.

The hardness of raw and cooked common beans varies (10–30 and 0.2–0.8 kgf, respectively). The texture of the cooked grains depends on the degree of cooking, and consequently if the grain is difficult to cook or exhibits HTC defect that will present greater hardness. Physical and chemical changes such as protein denaturation, carbohydrate solubilization, and starch gelatinization occur when the beans are cooked. Starch can exhibit different gelatinization patterns. Furthermore, depending on the cultivar, these changes can occur with greater or lesser intensity, favoring the reduction of grain hardness (Wani *et al.*, 2017; Yadav *et al.*, 2018). Moreover, Carbonell *et al.* (2010) associated the increase in the hardness of some cultivars with genetic characteristics of the grain or the susceptibility in the interaction of genetic and environmental factors, which can accelerate with inadequate storage.

The method of bean preparation to evaluate the texture of the cooked grain interferes with the results obtained. The difference in hardness of fresh and aged carioca beans after distilled water (1:2 w/v) soaking (18 hours, 25 °C) has been evaluated by five cooking methods: Mattson Bean Cooker, hot air oven, hotplate, boiling water bath, and autoclave (Siqueira *et al.*, 2013). Generally, cooking time and temperature affect bean hardness. Mattson Bean Cooker and hot air oven undercook the beans with hardness above 4 Newtons (N). Increasing cooking time from 30 to 60 minutes on a hotplate reduces bean hardness whereas, mild autoclave condition (105 °C/10 minutes) differentiates fresh and aged bean hardness (3 N vs. 3.4 N) and severe environment (115 °C/20 minutes) produce softer beans (0.8 N vs. 1N for fresh and aged beans, respectively). The hotplate (45 or 60 minutes) and autoclave (110 °C/15 minutes) cooking promote grain softening and discriminate fresh and aged beans. Hence are, therefore, suitable procedures to prepare carioca beans for instrumental texture analyses (Marles *et al.*, 2008). Hardness has also been used to evaluate the effects of irradiation on widely consumer accepted commercial carioca beans. Irradiation (5 kGy) almost doubled the rupture force or hardness of cooked (autoclave 121 °C/15 minutes) carioca beans compared with nonirradiated grains. This increase in grain hardness is presumably associated with starch retrogradation after cooking (Mendes *et al.*, 2011). Industrial canning (rotating autoclave 120 °C, 2 hours/35 minutes cooking) showed no significant difference in the texture or maximum compression (90% of initial height using 50 kg texture meter analyzer) force (0.60–0.75 N/grain) of carioca beans from three cultivars (Schoeninger *et al.*, 2020).

Bean hardness increases in raw and cooked grains when stored (Siqueira *et al.*, 2014, 2016a). This increase in hardness of aged bean is attributed to the loss of cell membrane integrity, which increases soluble solids loss in HTC grains during the immersion and cooking processes and may explain the changes in water permeability (Siqueira *et al.*, 2013, 2014, 2016a, 2018). These results demonstrate that carioca beans hardening is related to structural changes in the cell wall and middle lamella during storage.

Field Management/Environmental Influence

The management of bean cultivation and grain harvest is the first control point for good culinary quality beans acquisition. Adverse environmental conditions, inadequate harvest time, incomplete drying, and severe threshing showed grain breakage (or the appearance of cracks in the seed coat), which favors rotting, the development of fungi, and bruchid attacks (Mutungi *et al.*, 2020). Grains grown in tropical conditions (for example, high temperature and high humidity) may have long cooking times, and cotyledons have higher resistance to softening during cooking (Cichy *et al.*, 2019; Kigel, 1999). Cichy *et al.* (2019) recommended future research to differentiate the effect of the growth and storage environment on the culinary quality of beans. In short, the growth conditions affect pests and microflora development cycles and regulate progression rates of the biochemical reactions of bean grains, thereby influencing grain quality (Mutungi *et al.*, 2020). However, some genotypes exhibit stable cooking times in different production environments (Cichy *et al.*, 2019).

The best harvest period for most grains is close to their physiological maturity, wherein the grains

present maximum dry matter accumulation and quality. At this stage the grains have high (30–45%) water contents depending on the type and cultivar (Faroni *et al.*, 2006; Scariot *et al.*, 2017). Thus, the grains are left in the field until they absorb water appropriate for threshing or mechanized harvesting. However, field drying can compromise grain quality since the grains are susceptible to the environmental climate, such as temperature and relative humidity variations that accelerate the respiratory rate of grains, increasing the consumption of reserves and attacks by insects and fungi. According to Scariot *et al.* (2017), beans harvested with 16.6% water content had lower physiological quality and 1000 seed weight than those with higher water content (25.2% and 35.2%). Similarly, Faroni *et al.* (2006) confirmed that beans harvested with 11.7% water content had lower technological classification over those with higher water content (18.7%), demonstrating the negative effects of delayed harvest.

Postharvest Management

Drying

The reduction of water content limits the availability of water for physical-chemical and biological processes of the grains, and the development of fungi, bacteria, and insects is mainly responsible for degradation and loss during storage (Mutungi *et al.*, 2020). Therefore, drying is extremely important before enhanced safe storage. The first stage of bean drying occurs initially at the plant itself in the field, before harvesting, and is called predrying. This is not sufficient to maintain the grain for prolong storage. Drying in the sun or at high temperatures (10 °C above ambient air) under tarpaulins or land, mainly by small farmers, is carried out. Here the grain layer must not exceed 5 cm, and periodical mixing of grains is necessary to standardize drying. This method may not reduce the water content of the grains to ideal levels or delay this process, causing losses of culinary quality in the product and are contaminated by fungi (Rani *et al.*, 2013; Scariot *et al.*, 2017).

The removal of water performed inappropriately by drying can negatively influence the physical, physiological, chemical, and cooking characteristics of beans. The main factors that affect the quality of the grains are the drying air temperature and the initial water content of the grains. The use of high temperatures for drying the grains, mainly combined with high water contents, can cause damage such as cracks and fissures, which can become a gateway for fungal attack during storage, in addition to reducing quality after drying (Faroni *et al.*, 2006; Rani *et al.*, 2013; Scariot *et al.*, 2017). Drying beans at temperatures above 40 °C reduces their physical and physiological quality, such as 1000 seed weight, germination, and vigor (Scariot *et al.*, 2017).

There are no current reports on drying methods that aim to reduce the loss of the culinary quality of beans. Additionally, few studies report the effects of the drying process on the organoleptic, nutritional, and culinary characteristics of the grains. However, increasing the grain drying temperature to 60 °C increased the cooking time after 225 days of storage than the lower drying temperatures (Elias *et al.*, 2016).

Storage

The last step before the commercialization of dry grains is storage. During this period, monitoring of some factors is pivotal to maintain bean quality and quality. While stored, grains undergo chemical and physical changes that alter their characteristics(loss of cooking quality and weight reduction). These are linked to the consumption of dry matter because of breathing and attack by pests. The absence of effective grain handling and storage techniques significantly decreases the quantity and quality of grains during the postharvest phases (Affognon *et al.*, 2015; Mutungi *et al.*, 2020). Hardening and reduction of the permeability of the tegument occur when grains are stored in inadequate conditions of temperature and relative humidity, increasing their resistance to cooking. These quality losses can be considered a hardshell when the tegument loses its water absorption ability and HTC when beans have increased resistance to cooking (Oliveira *et al.*, 2011).

Temperature and relative humidity also influence grain quality during storage. The high temperature, combined with high relative humidity (RH) of the air during storage, favors an increase in respiratory rate, culminating in accelerated deterioration rate, as well as increased fungi and insect contamination. Therefore, low temperature and relative humidity are necessary to maintain grain quality during storage (Rani *et al.*, 2013; Coelho *et al.*, 2020; de Farias *et al.*, 2020). Grains stored in cold rooms (8 °C and 45% RH, 360 days) show no increase in cooking time. On the other hand, grains stored under normal environmental conditions increased their cooking time with longer storage time (Morais *et al.*, 2010). Moreover, de Almeida *et al.* (2017) reported shorter cooking times in beans stored for 108 days at 15 °C and 45% RH than those stored at 27 °C and 75% RH.

Some countries, such as Australia, Brazil, Africa, and Argentina, have adopted a system of hermetic storage for pulses using polyethylene (PET) silo bags. The silo bag is constructed with high-density polyethylene in three layers: two black internal layers and one white external layer

made of titanium dioxide. The level of oxygen within the bag drastically falls, whereas carbon dioxide increases once the product is wrapped and the bag is sealed (Hell *et al.*, 2014; Freitas *et al.*, 2016). Thus, this technology is based on creating storage environments harmful to pests by one of the following methods: bio-generated modified atmosphere and hermetic vacuum or gas hermetic fumigation (Freitas *et al.*, 2011; Hell *et al.*, 2014; Mutungi *et al.*, 2015; Freitas *et al.*, 2016). An advantage of the hermetic silo bags is the nonchemical alternative to postharvest quality control in terms of moisture content, specific mass, germination, and electrical conductivity, for up to 120 days (Mutungi *et al.*, 2015; Magalhaes and de Sousa, 2020). Common beans (red beans) storage in silo bags with 17.8% RH presented higher cooking time than those stored in PET bottles or a glass recipient (closed with organza fabric; Freitas *et al.*, 2011). Information is limited on the hermetic storage using PET silo bags, silo bags with temperature control, or even PET bottles influence in cooking culinary quality of carioca beans.

Type of Cultivar/Genotype

The darkening of common beans is an undesirable characteristic for consumers, generally associated with old beans and consequently extended cooking time. However, the factors that influence darkening include environmental and genetic factors. The latter do not show any darkening pattern among different grain types and do not strongly affect the environment (Alvares *et al.*, 2019; Spitti *et al.*, 2019). Seed coat postharvest darkening depends on beans genotype (Islam *et al.*, 2020).

Postharvest darkening (PHD) of seed coat gradually changes the seed coat color of some dry bean market classes during storage. Genotypic and environmental factors influence the rate and extent of PHD, and darkening occurs rapidly in environments subjected to high temperatures, humidity, and light exposure. There are at least three PHD phenotypes: (i) non-darkening (ND), (ii) slow darkening (SD), and (iii) regular darkening (RD). SD and ND genotypes have already been identified in common beans (Elsadr *et al.*, 2011).

According to Spitti *et al.* (2019), darkening is closely linked to the growing environment regardless of the cultivated genotype, or the genotype and environment interaction affect the bean seed coat darkening, requiring the characteristic evaluation in various climates of genetic control and strains selection (Silva *et al.*, 2014). Seed coat darkening and cooking resistance increased in different carioca bean genotypes over 6 months of storage under varied environmental conditions (Siqueira *et al.*, 2014).

Darkening gene

Genetic variation occurs in seed coat darkening of many bean classes including carioca beans (Rodrigues *et al.*, 2019). The variation can be monogenic or oligogenic, which means their inheritance is controlled by one or a few genes (Silva *et al.*, 2018). In carioca beans, the darkening phenotype is controlled by a single recessive gene "Sd," the slow darkening is represented by the "sd" recessive allele (Silva *et al.,* 2008). However, oligogenic control has also been proposed (Elsadr *et al.*, 2011; Silva *et al.*, 2014). A model with two genes interacting under epistasis was suggested by Elsadr *et al.* (2011), where the "J" gene modulates darkening or interacts with a second Sd gene, responsible for regulating the darkening rate. Similarly, Spitti *et al.* (2019) described that this characteristic is oligogenic or even polygenic from significant results obtained by the genotype and environment interaction.

Islam *et al.* (2020) demonstrated yet another allele in the P gene (Psd) responsible for the SD trait in common beans, "P" is a transcription factor that restores the seed coat color. The sequence comparison of this gene in several beans differing in the seed coat postharvest darkening provided insights into the molecular mechanism that governs this characteristic and the development of new specific gene markers for potential use in bean breeding programs. Selecting lines with slow seed coat darkening is possible despite conflicting results regarding the control of beans darkening (Silva *et al.*, 2014). However, selection based on the evaluation of only one environment can generate sufficient gains for this characteristic, even in the presence of genotype and environment interaction, which would explain the inconsistencies between the patterns of genetic control in each location (Alvares *et al.*, 2016).

The elucidation of the genetic control of grain darkening is of fundamental importance to establish breeding programs for developing cultivars with slow seed coat darkening during storage (Silva *et al.*, 2014). Microsatellites are notable in genetic diversity studies. Polymerase chain reaction (PCR)-based markers are developed for a many plant species, including commercial crops. A panel of 24 microsatellites has been built for the common bean specifically for studying the genetic diversity available to the scientific community and has been routinely used for this type of analysis in Brazil (Métais *et al.*, 2002; Blair *et al.*, 2003; Morais *et al.*, 2016). Thousands of single nucleotide polymorphism markers are currently available for the common bean. This number increased immensely because of the species genome sequence publication (*Phaseolus vulgaris* v1.0; http://www.phytozome.net; Schmutz *et al.*, 2014; Morais *et al.*, 2016). Even if the choice of breeding lines and the development of cultivars

with the slow darkening trait are relatively simple, given its high heritability and simple genetics, the genetic complexity of many interesting characteristics makes this selection difficult in breeding programs. However, the slow darkening trait is expressed maternally and inherited recessively (Silva *et al.*, 2018; Alvares *et al.*, 2019).

Oxidative Enzymes Related to Beans Aging

During storage, cell damage occurs with advanced grain aging, and the physical barrier that separates enzymes and substrates is lost, enabling the oxidation of phenolic compounds by oxidoreductases. Complex processes and enzymatic reactions are activated in postharvest beans and intensify during storage, initiating the aging and darkening phenomenon (Siqueira *et al.*, 2016b). Postharvest darkening of the bean seed coat, both enzymatic and nonenzymatic, has already been attributed to the presence of phenolic compounds (Spitti *et al.*, 2019) because of their involvement in oxidative steps and subsequent changes in the flavonoid skeleton, as well as by the formation of quinones or similar enzyme-mediated reactions (Marles *et al.*, 2008; Siqueira *et al.*, 2016b; Bento *et al.*, 2021a).

Polyphenol oxidase is associated with the enzymatic activity of peroxidase. It produces a dark compound that causes grain integument darkening called melanin (Siqueira *et al.*, 2016b). RD is strongly associated with increased polyphenol oxidase activity in some strains of beans (Marles *et al.*, 2008). Likewise, Alves *et al.* (2021) evaluating, a fast darkening and hardening cultivar that demonstrated high peroxidase and polyphenol oxidase activity in the process. Higher polyphenol oxidase activity is noted in bean cultivars with lighter tegument than in dark tegument cultivars during the complete aging process. This activity confirms that oxidative processes of phenolic compounds linked to polyphenol oxidase activity contribute to color changes in the bean grain coat (Siqueira *et al.*, 2016b). Polyphenol oxidase and peroxidase activities are attenuated during controlled bean storage at cooling temperature, thereby inhibiting/suppressing grain darkening (Demito *et al.*, 2019). Therefore, the highest color change occurs at higher storage temperatures because of high enzymatic activity, mainly polyphenol oxidase, which degrades polyphenols and reduces the bioactive value of these grains.

Oxidation by environmental oxygen can trigger the darkening and hardening process of carioca beans. Previous research by Bento *et al.* (2020a) found the predominant superoxide dismutase (SOD) activity in the grain integument. But its byproduct, hydrogen peroxide, was only noted in the integument. The high SOD activity suggests that the seed tissue (like cotyledons) maintains strict airway control. Another study by Siqueira *et al. (*2016b) also stressed the importance of analyzing the separate cotyledon integument because of these enzyme variations activities. Nevertheless, according to Bento *et al.* (2020a), the presence and activity of SOD indicate that oxidative stress occurs during storage.

The hardening of beans during storage is also related to low humidity and hydration defects. High temperatures and low relative humidity in the presence of light and oxygen are the main factors that hinder water absorption, in which shows grain hardening consequently, contributing to the HTC phenomenon because of changes in phenolic content related to lignification and loss of phytates (Junk-Knievel *et al.*, 2008). The lignification process relates the hardening with the polymerization of the phenolic compounds from the integument, mediated by polyphenol oxidases, and the formation of cross-links between phenolic compounds and cell wall proteins of the cotyledon. Peroxidase is involved in the polymerization reaction of phenolic compounds. An increase in its activity may be associated with the cell wall lignification process (Alves *et al.*, 2021). The hardening process can also be explained by the SOD activity in oxidative stress (Bento *et al.*, 2020a).

Polyphenolic Compounds

Polyphenolic compounds are associated with the plant defense system, ensuring resistance to pest attacks at considerable levels but, they can accelerate the grain darkening process (Spitti *et al.*, 2019). According to Chen *et al.* (2015), bean darkening is rapid and more common in cultivars with high phenolic content in the tegument. Other studies have also associated bean darkening to the content of phenolic compounds, where darkening is more prevalent in cultivars with high phenolic content in the tegument, and the degree of darkening is proportional to the loss of phenolics (Martín-Cabrejas *et al.*, 1997; Beninger *et al.*, 2005; Luthria and Pastor-Corrales, 2006; Nasar-Abbas *et al.*, 2009). In a recent study, the content of phenolic compounds decreased for fast darkening grains but did not change or increased in slow darkening carioca beans during storage (Bento *et al.*, 2021a). Furthermore, kaempferol was suggested as the marker to differentiate fast and slow darkening cultivars since it decreased in quick darkening cultivars during storage.

The bean darkening has also been associated with the presence of proanthocyanidins (condensed tannins) in the seed coat (Junk-Knievel *et al.*, 2007), which accumulate at higher levels in regular darkening than in the slow darkening genotypes, just like most flavonoids (Duwadi *et al.*, 2018). Proanthocyanidins are oligomeric flavonoids composed mainly of catechin and epicatechin

units. The synthesis of proanthocyanidin shares the flavonoids pathway with anthocyanins to leukocyanidin/cyanidin (Duwadi *et al.*, 2018). The presence of catechin and kaempferol was identified by Beninger *et al.* (2005), and the significant increase in aging suggesting that the formation of these compounds occurred during the oxidative process. Therefore, these compounds can interfere with the culinary quality of beans once the dimer procyanidin B-type contributes to the seed coat darkening process because of the oxidation of proanthocyanidin in reactive quinones (brown colored compound; Ranilla *et al.*, 2007; Bento *et al.*, 2021a).

Alternative Uses of Beans with Low Culinary Quality

The aged beans with low culinary quality and consequently low commercial value can be used as ingredients in food formulation. In the same way, bean byproducts (i.e., broken beans) may be used for food development since they present similar nutritional value compared with whole grains. Both can be transformed into flours that constitute a new way of using materials with low added value that can contribute to the sustainability of the bean production chain. The bean flour usage is aligned with the current trends and consumption habits, based on sensory quality, practicality, diversity, and healthiness. Bean flours can also be used in the gluten-free and vegan products development, one of the most successful markets in the food industry. Thus, various studies report an effective way to apply aged dry beans flour as a base ingredient in many foods, such as tempeh (Bento *et al.*, 2020c), baked snacks (tortillas), and instant pasta (Bento *et al.*, 2021b), vegan tempeh burger (Bento *et al.*, 2021c), mix for cakes (Gomes *et al.*, 2015; Bassinello *et al.*, 2020). In these studies, the aged beans were heat treated (e.g., extrusion, cooking in an autoclave, or the traditional pressure-cooking method) to mitigate some unpleasant flavors in the bean flours (Pasqualone *et al.*, 2020).

Other studies have also used bean as ingredient for the development of spaghetti and ravioli (Gallegos-Infante *et al.*, 2010; Ringuette *et al.*, 2018), extruded snacks (Bassinello *et al.*, 2015), light red kidney bean porridge (Nyombaire *et al.*, 2011), snack bars (Ramírez-Jiménez *et al.*, 2018), cookies (Pérez-Ramírez *et al.*, 2018), bread and chips (Hooper *et al.*, 2019), and extruded snacks made with maize and bean (7:3; Félix-Medina *et al.*, 2021). In addition, there is significant interest presently in dry and wet fractionation of pulses into starch, protein, and fiber concentrates for use in both food and nonfood products (Tyler *et al.*, 2017). Additionally, the consumption of bean products is associated with health benefits such as the reduced risk for cardiovascular disease and cancer, the management of type 2 diabetes, metabolic syndrome, and obesity, and contributes to overall health and wellness (Tyler *et al.*, 2017; de Lima *et al.*, 2019; Mullins and Arjmandi, 2021).

Conclusion

The culinary quality of beans is influenced by intrinsic and extrinsic factors and involves sensory attributes and technological properties that directly reflect consumer choice. The cooking time, color, and texture are the determining properties in the acceptance of the grain, which can be affected during storage mainly by temperature and humidity. The type of cultivar directly influences the darkening and hardening of the grain. The literature presents this control as oligogenic or monogenic. Moreover, different cultivars have a varied genetic makeup for darkening. Specific phenolic compounds present in the tegument play an important role as a substrate for oxidation reactions, and the type of pigments determine the final color of the grain, that is, the post-harvest darkening rate. The future perspectives to enhance bean quality may be related to the use of different techniques for enhanced preservation of the grains appearance and composition, such as the system of hermetic storage using polyethylene silo bags. Additionally, the breeding programs can recommend new bean cultivars in the market, which are resistant to long-term storage without losing their culinary quality. A study of factors influencing bean properties acceptable by the final consumer is critical to encourage breeding programs and increase the demand for this high nutritional food. The use of bean flour as a food ingredient is an alternative for the use of aged beans with low culinary quality and improves food industry diversity. In addition, they are the excellent raw material for protein extraction in plant-based products applications, a market that has been growing in recent years. Additionally, aged beans or broken grains have potential for use in gastronomy (as flour) because of their technological, nutritional, and functional properties, as well as their versatile application. It is also an option to enrich school meals and the diet of low-income populations, in addition to specific demands (gluten-free, low glycemic index, vegan, etc.).

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Effects of the drying method for flowers of *Cynara cardunculus* var. *Altilis* on milk coagulating properties

Carmela Tripaldi[1], Giuliano Palocci[1]*, Sabrina Di Giovanni[1], Miriam Iacurto[1], Roberto Steri[1], Maria Concetta Campagna[2], Cristina Di Russo[2], Tiziana Zottola[2]

[1]Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Centro di ricerca Zootecnia e Acquacoltura, Monterotondo, Italy; [2]Istituto Zooprofilattico Sperimentale del Lazio e Toscana, Roma, Italy

***Corresponding Author**: Giuliano Palocci, Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Centro di ricerca Zootecnia e Acquacoltura, Monterotondo, Italy. Email: giuliano.palocci@crea.gov.it

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PAPER

Abstract

In the production of some traditional cheeses from vegetable rennet, raw extracts of *Cynara cardunculus* flowers are used as the coagulant. During the preparation of this rennet, there are many factors that can influence its coagulation activity. We studied the flowers of *Cynara cardunculus* var. *altilis* to evaluate the effects of some of these factors: ripening stage of the flower at harvest, type of drying, part of the flower subjected to drying, toasting of the pistils, and maceration time of the pistils. The results show that it is possible to improve the coagulation activity of the traditional preparation of *Cynara cardunculus* flowers through some practices such as the rapid drying of the flowers/pistils at a controlled temperature, the toasting treatment of the pistils carried out after the slow drying of the flowers, and the extension of the extraction time to 24 h.

Keywords: clotting activity; *Cynara cardunculus*; drying process; maceration time

Introduction

The use of vegetable rennet in cheese production is limited to a few cheeses. The excessive proteolytic nature of plant coagulants can negatively affect the cheese-making process and favor a reduction in the cheese yield and the presence of some defects in flavor and texture. This may have limited the diffusion of vegetable rennet (Lo Piero *et al*., 2002).

An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers, which is the most common vegetable rennet in the Mediterranean region (Barros *et al*., 2001).

The milk-clotting activity of this plant extract has been known for centuries and has been successfully used to produce cheeses from ovine and caprine milk (Silva *et al*., 2003), which are highly appreciated.

These cheeses are widespread mainly in Spain and Portugal, where in many cases only the raw extract of dried *Cynara cardunculus* flowers is used as a coagulant (Almeida and Simões, 2018). Furthermore, some of them (Ibores, Flor de Guia, La Serena, Torta del Casar, Azeitao, Castelo Branco, Evora, Nisa, Serpa, Serra da Estrela) have been recognized as Protected Designation of Origin (PDO), as evidence of their close link with origin area and traditional practices of cheese-making. In Italy, only a few traditional ovine cheeses are made with vegetable rennet (Caciofiore, Casoperuto).

Cheeses produced with *Cynara cardunculus* differ from those produced with animal rennet due to their softer texture, more intense odor, and flavor, including the bitter taste (Alavi and Momen, 2020; Barbosa *et al*., 1981). It has been observed that the formation of bitter taste peptides is caused by a strong proteolytic activity of thistle enzymes (Agboola *et al*., 2004; Alavi and Momen,

2020). In sheep and goat cheeses produced with Cynara cardunculus extracts, a slightly bitter taste was detected (Conceição *et al*., 2018; Roseiro *et al*., 2003). Cheeses made with cow's milk tend to develop a bitter taste (Alavi and Momen, 2020; Barbosa *et al*., 1981).

It was found that ovine caseins are less likely than bovine caseins to form hydrophobic bitter peptides following proteolytic action (Pelissier and Manchon, 1976). According to Macedo *et al*. (1996), the bitter taste of the ovine cheese could be due to the formation of several peptides identifiable in the digests of isolated bovine alfa-s and beta-casein from ovine milk. The concentration of bitter peptides (those with a molecular size of 165–6500 g·mol^{-1}) was the lowest in ovine cheese made from calf rennet; however, cheese made from cardoon coagulant was perceived to be less bitter by a sensory panel (Agboola *et al*., 2004).

Cynara cardunculus flowers produce cardosins and cyprosins, aspartic proteases that accumulate in mature flowers; in fact, the concentration of the aspartic proteases in the fresh flower is lower (Cordeiro *et al*., 1994).

To date, nine different aspartic proteases have been found at the protein level: six cardosins and three cyprosins (Folgado and Abranches, 2020).

Cardosins A and B were extracted from the stigmae and stylets of dried flowers of *Cynara cardunculus* (Silva *et al*., 2003). Cardosins A and B are among the aspartic proteases that are found in many varieties of plant species. Aspartic proteases are involved in protein degradation during the plant development process, protein storage mechanisms, responses to stress and pathogens, reproduction, and plant senescence (Cordeiro *et al*., 1994; González-Rábadea *et al*., 2011; Pissarra *et al*., 2007). Cardosin B is more proteolytic than cardosin A. In terms of activity and specificity, cardosin A is similar to chymosin as it cleaves the same peptide bond (Phe105-Met106) of κ-casein. Cardosin B is similar to pepsin; in ovine caseins, as1-casein is cleaved by cardosin B at bonds Leu156-Asp157 and Trp164-Tyr165, whereas β-casein is cleaved at peptide bonds Leu127-Thr128, Leu165-Ser166, and Leu190-Tyr191 (Macedo *et al*., 1993; Silva and Malcata, 1999; Silva *et al*., 2006; Veríssimo *et al*., 1995).

Cardosin A is often highlighted as being more suitable for promoting milk clotting due to its higher specificity and lower proteolytic activity than cardosin B. However, the amount of cardosin A needed for milk clotting is 10-fold higher than that of cardosin B, i.e., the specific activity of cardosin A is lower than that of cardosin B (Silva *et al*., 2003). According to some authors (Silva and Malcata, 1998, 1999; Silva *et al*., 2003), although thistle aspartic proteases cut the same peptide bond as chymosin, their proteolytic activity is more extensive. According to Conceição *et al*. (2018), cardosins reveal a more intense secondary proteolytic action on cheese αs- and β-casein than other coagulants, with impact on the cheeses' biochemical and sensory properties. In addition, three cyprosins from dried flowers of *Cynara cardunculus*, which were isolated, purified, and characterized by Heimgartner *et al*. (1990), have milk-clotting activity.

Generally, vegetable rennet enzymes have been obtained by aqueous extraction of various plant organs, such as flowers, seeds, roots, and leaves. There are several different ways to prepare aqueous extracts of plant material (Sousa and Malcata, 1996).

In one method, dried whole or crushed cardoon flowers are soaked in water at room temperature for a variable time. Then, the filtrate is collected, and this crude extract is used to coagulate milk (Roseiro *et al*., 2003). An alternative method of extraction is grinding the dried flowers with crude kitchen salt, laying the paste on a cotton cloth, and solubilizing the enzymes by percolation with warm milk (Sousa and Malcata, 2002). The crude extract can also be further purified to obtain partially purified enzyme or pure enzyme depending upon the degree of purification (Shah *et al*., 2014). Milk-clotting proteases have also been produced by in vitro techniques (Shah *et al*., 2014).

The activity of the plant extract (Almeida and Simões, 2018) depends on numerous factors, such as the thistle flower ecotype, the part of the flower used, its stage of maturity, the drying time, the final moisture content, the pH of the buffer, the salt concentration of the buffer, and the homogenization time (Correia *et al*., 2016; Folgado and Abranches, 2020; Guiné *et al*., 2016; Sousa and Malcata, 1996). In particular, the different profiles of aspartic proteases found among flowers of different genotypes further increase the variability of the flower extracts and their clotting time (Folgado and Abranches, 2020). This results in cardoon extract preparations that may vary in terms of clotting and proteolytic activities, and may influence the yield and final characteristics of cheese (Sousa and Malcata, 2002).

For all these reasons, the objective of this paper was to evaluate the effects of some factors influencing the milk clotting activity of cardoon rennet to reduce its variability. Two kinds of drying methods for cardoon flowers were compared: the slow drying method adopted by a farm and an experimental fast drying method. Second, the effects of the stage of the flower on harvesting and the part of the flower subjected to drying were estimated. Finally, the effect of the extraction time of the crude extract was assessed in all samples.

Materials and Methods

Plant materials

The flowers of *Cynara cardunculus* var. *altilis* were harvested from plants grown on a farm near Rome (Agricoltura Nuova Cooperative; Rome, Italy) in 2019. On this farm, for some years, vegetable rennet has been produced from *Cynara cardunculus*. This rennet, used to produce certain sheep farm cheeses, is prepared as illustrated in Figure 1. The flowers of *Cynara cardunculus* var. *altilis*, collected in the maturity stage, were dried in a cool and dry room for approximately 3 weeks (slow drying method), and then the pistils were separated from the flowers and subjected to heat treatment at 120°C for 15 min (toasting treatment).

Samples of flowers of *Cynara cardunculus* var. *altilis* intended for the experimental trial (Figure 2) were collected from the same farm both in the pre-ripening phase (not fully violet) and in the maturity stage (fully violet). All samples were subjected to fast drying of both the whole flowers and the pistils. In the first case, the pistils were separated after drying; in the second case, they were separated from the flower before drying and immediately after being collected. Fast drying was carried out at a temperature of 35°C with a dryer prototype, which was created by "Evoluzione Natura®" company for withering, drying, dehydration, and sanitizing of plant material. The system was based on controlled forced ventilation, dehumidification, and emission of ultraviolet radiation. Water activity (a_w) was measured before, during, and at the end of drying by Aqualab 4te Meter Group, Inc., USA. The experimental drying lasted about 60 h for the whole flowers and about 12 h for the pistils. During the preliminary tests, it was found that these were the times required to reach an average a_w of less than 0.500. In the end, all pistils were vacuum-packed and kept at room temperature.

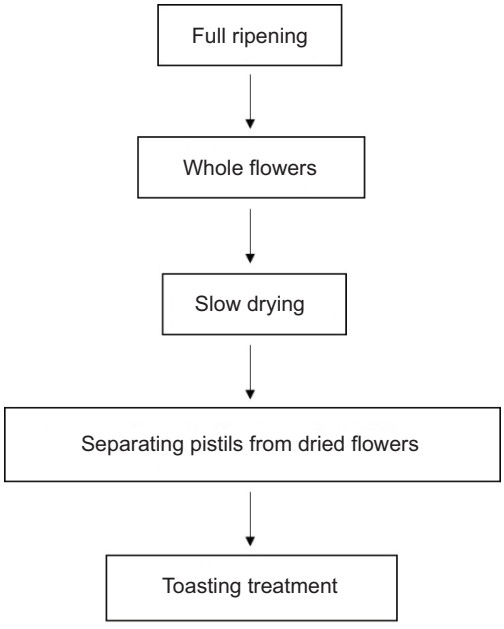

Figure 1. Method used by the farm for the preparation of rennet from *Cynara cardunculus* flowers.

Rennet extraction

To prepare the raw extracts, 2.5 g of pistils was soaked in 100 mL of tap water. The amount of pistils was the same as that used on the farm to prepare a rennet solution for traditional vegetable rennet cheese.

The pistils were kept in tap water for three maceration times: 2, 15, and 24 h at a temperature of 15°C. In total, 12 raw extracts were prepared for each sample and were analyzed in duplicate for a total of 24 raw extracts.

At the end of the extraction, the extracts were filtered, and the pH was determined. The samples were then frozen at –20 °C and maintained until analysis.

Milk coagulation properties

The milk clotting properties were determined by the Zannoni and Annibaldi (1981) method using the

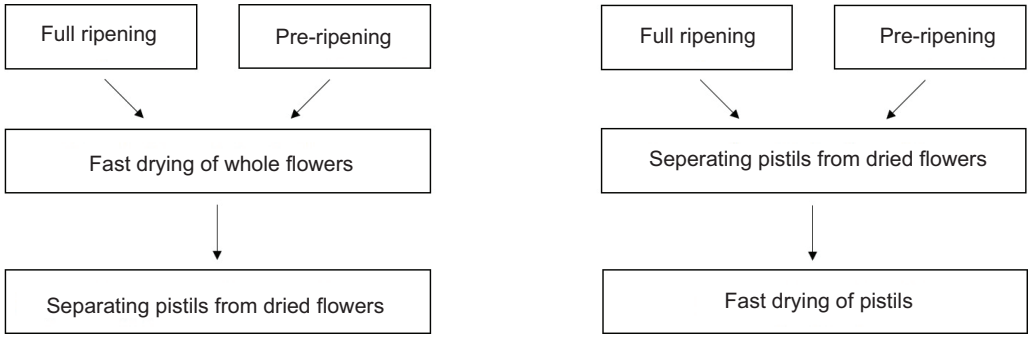

Figure 2. Experimental design for the preparation of rennet from *Cynara cardunculus* flowers.

FORMAGRAPH instrument (Maspres, Firenze, Italy). Measurement is based on the movement of small pendulums immersed in linearly oscillating samples of milk. Minute forces are applied to the pendulums because of the formation of a gel in the moving milk sample. The result is the registration of the coagulation properties of the milk (McMahon and Brown, 1982). The Formagraph parameters are milk clotting time (r, min), curd firming time (k20, min), and curd firmness (a, mm). The parameter r measures the time from the addition of the rennet to the milk up to the point where the baseline starts to increase in width (Bittante, 2011). Repeatability values within laboratories of milk clotting time were 96% (Duranti *et al*., 2003). The parameter k20 is the interval from the start of gel development until an oscillation width of 20 mm is attained. The curd firmness is the width of the graph at a definite time from rennet addition (Bittante, 2011). This last parameter is measured 30 min after rennet addition or at twice the clotting time (a2r) (Delacroix-Buchet *et al*., 1994). To better estimate the curd firmness of our samples, which coagulate more slowly than those using animal rennet (Liburdi *et al*., 2019), we measured this parameter at 60, 75, and 90 min (a60, a75, a90) after adding the rennet.

The amount of pistils (50 g/100 L of milk) used to evaluate the milk clotting activity was the same as used on the farm dairy to prepare rennet solution to produce traditional vegetable rennet cheese. For each batch of samples analysis, two reference commercial coagulants were used. First: animal rennet (Hansen standard 160 IMCU/mL, 80% chymosin and 20% pepsin) used according to Formagraph method (200 µl of 1.6% rennet solution for 10 mL of milk). Second: commercial vegetable rennet (Galium, Prodor) used according to the dose indicated on the label (100 g of rennet/100 L of milk). All samples were analyzed in duplicate.

Standardized milk powder used as substrate was prepared according to ISO 23058 IDF 199: 2006. Then, 110 g of low-heat, low-fat, and spray-dried milk powder was added to 1 l of $CaCl_2$ solution (0.5 g/L). The final pH of substrate was approximately 6.5. The temperature during pre-heating and analysis of the milk samples was 35 °C.

Statistical analysis

The effects of the drying method: slow method applied in farm and experimental fast method, toasting treatment applied in farm, ripening phase, part of the flower, and maceration time were analyzed using the following linear model:

$$Y = \mu + T + e$$

where Y is the dependent variable; µ is the overall mean; T represents the treatments; and e is the residual error.

Statistical analysis was performed using the General Linear Model (GLM) procedure in SAS software (SAS Institute, 2011) version 9.3. The level of significance was set at $P < 0.05$.

The effect of a_w on clotting time was analyzed. The relationship between these factors was shown by performing the Boxplot procedure (SAS software) on the mean data of groups. In fact, the a_w in the experimental method was used as a threshold value (below 0.500) to fix the end of drying. In the farm, the end of drying carried out in a cool and dry room is determined by time (3 weeks) and the average final a_w values were higher than 0.500.

Results

Table 1 compares the effects of the two drying methods on the coagulation properties of the crude extracts. For these two samples, we started from fully ripened whole flowers being submitted to the farm method (slow drying) and then to the experimental method (fast drying). After both kind of drying, the pistils were separated from the other parts of the flower and only in the farm method the pistils were toasted. Then, to prepare the raw extracts, the toasted pistils and pistils from the fast dried flowers were used.

All the crude extracts were subjected to the three maceration times and the data shown in the tables are the results of all the maceration times.

The raw extracts prepared with farm and experimental methods reach the lowest value of curd firmness after 60 min from the addition of the rennet and the highest at 90 min. The samples produced by the experimental method showed a lower curd firming time (11.89 vs 13.88 min) and higher curd firmness at 90 min after rennet addition (44.18 vs 41.27 mm).

In general, better rennet capability is characterized by a brief clotting and curd firming time and an elevated curd firmness. The fast-drying rennet, named experimental method, had a better clotting ability than the rennet produced by using the slow drying method.

In Table 2, we evaluated the clotting activity of samples subjected to slow drying. Both samples were from fully mature, whole flowers. After the flowers were dried, the pistils were separated. The pistils of the sample prepared according to the farm method were toasted, while the pistils of the second sample were not toasted, in order to evaluate the specific effect of the toasting treatment.

Table 1. Effect of drying method on milk coagulation properties.

	Method of drying	
	Farm method	Experimental method
No. of samples	24	24
r (min)	40.19	42.83
K20 (min)	13.88[a]	11.89[b]
A60 (mm)	25.37	26.09
A75 (mm)	35.79	37.41
A90 (mm)	41.27[b]	44.18[a]

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

Table 2. Effect of toasting treatment included in the farm method on milk coagulation properties.

	Toasting treatment	
	Yes	No
No. of samples	24	24
r (min)	40.19[b]	59.53[a]
K20 (min)	13.88	14.62
A60 (mm)	25.37[a]	13.26[b]
A75 (mm)	35.79[a]	21.02[b]
A90 (mm)	41.27[a]	32.96[b]

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

Table 3. Effect of ripening phase and part of the flower submitted to experimental fast drying on milk coagulation properties.

	Experimental fast-drying method			
	Ripening phase		Part of the flower	
	Pre	Full	Whole	Pistils
No. of samples	48	48	48	48
r (min)	46.85[a]	39.96[b]	47.03[a]	39.77[b]
K20 (min)	12.24[a]	11.11[b]	12.73[a]	10.62[b]
A60 (mm)	22.50[b]	30.47[a]	22.42[b]	30.55[a]
A75 (mm)	33.16[b]	38.79[a]	32.28[b]	39.66[a]
A90 (mm)	41.31[b]	45.15[a]	40.33[b]	46.14[a]

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

Table 4. Effect of maceration time of the crude extracts on milk coagulation properties.

	Hours		
	2 h	15 h	24 h
No. of samples	64	64	64
pH	5.97[a]	5.76[ab]	5.66[b]
r (min)	48.71[a]	44.82[b]	40.67[c]
K20	13.62[a]	12.11[b]	11.03[c]
A60 (mm)	22.10[b]	25.05[ab]	28.17[a]
A75 (mm)	29.27[c]	34.13[b]	39.11[a]
A90 (mm)	37.20[c]	41.80[b]	45.80[a]

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

The characteristics evaluated were significantly different for the two types of treatments, except for the curd firming time. The coagulation time was reduced by 32% (40.19 vs 59.53 min) in the toasted samples. The curd firmness measured after 60, 75, and 90 min was significantly higher in flowers subjected to the toasting treatment (25.37 vs 13.26 mm; 35.79 vs 21.02 mm; 41.27 vs 32.96 mm). Therefore, according to our results, toasting of the pistils after traditional slow drying of the flowers improves the coagulation characteristics of the thistle rennet.

Table 3 shows the effects on the crude extract characteristics of both the ripening phase and the part of flower in the samples subjected to experimental fast-drying. Significantly, lower milk coagulation times were achieved with flowers in the complete ripening phase compared to pre-ripened flowers and using directly dried pistils compared to whole dried flowers (39.96 vs 46.85 min and 39.77 vs 47.03 min, respectively). The firming time values had the same tendency as the clotting time values and were lower in the complete phase and in the pistils (11.11 vs 12.24 min and 10.62 vs 12.73 min). In the pistils and in complete phase samples, the higher curd firmness values were found: 60 (30.47 vs 22.50 mm and 30.55 vs 22.42 mm), 75 (38.79 vs 33.16 mm and 39.66 vs 32.28 mm), and 90 min after rennet addition (45.15 vs 41.31 mm and 46.14 vs 40.33 mm).

The effects of the maceration times of the raw extracts at 2, 15, and 24 h are shown in Table 4. The evaluation of the maceration time was carried out on all the samples and included all the treatments (pre-ripening and ripening phase, whole flowers and pistils, farm and experimental drying, toasting or not). For all parameters, the results obtained at the three maceration times were significantly different. The best coagulation aptitude was obtained after 24 h of maceration: clotting and curd firming times of crude extracts were lower, and curd firmness at 60, 75, and 90 min was higher. pH values decreased with increasing extraction time.

The correlation coefficients among the Formagraph parameters were analyzed for all samples. We found that all correlation coefficients were significant ($P < 0.0001$): +0.70 between r and k20; −0.73, −0.86, −0.90 between

k20 and a60, a75, a90, respectively; –0.94, –0.95, –0.88 between r and a60, a75, a90, respectively. Formagraph parameters from raw extracts were highly correlated, i.e., clotting time and curd firming time were positively correlated, and both were negatively correlated with curd firmness. In raw extracts and standard rennet, when the coagulation time is longer, the time necessary to firm the curd is extended. The greater the curd firmness, the more the milk coagulates in a short time. The coagulation activity is good when the coagulation time is shorter, and the curd consistency is greater.

Similarly, the correlation coefficients ($P < 0.0001$) found for cow milk coagulated with animal rennet (Mariani *et al.*, 1997) were +0.58 between r and k20; –0.80 between k20 and a30; and –0.89 between r and a30.

All samples during Formagraph analysis were compared with the reference coagulants, i.e., animal and commercial vegetable rennet whose average data were, respectively: r 14.52 and 11.38 min; k20 2.79 and 2.23 min; A30 44.70 and 50.30 mm; A2r 44.52 and 44.20.

Figure 3 shows the box plot of the distribution of clotting time according to a_w classes. We observed that the two samples with higher a_w had higher coagulation time. These samples came from whole flowers collected at a pre-ripening stage and quickly dried (PR-WF-FD) (a_w = 0.623) and from whole flowers slowly dried and not toasted (FR-WF-SD-NT) (0.557). The other samples had lower a_w and faster clotting times. These included FR-WF-SD-T slowly dried and toasted samples (0.457); PR-P-FD pistils quickly dried from premature (0.401) and FR-P-FD mature flowers (0.324); and FR-WF-FD whole flowers dried quickly and collected when fully ripe (0.323).

Discussion

According to the classification of the milk coagulation properties with animal rennet by the method of Zannoni and Annibaldi (1981), good-quality cow milk has a clotting time between 11:30 and 18:00 min.

The results obtained from the raw extracts showed very long milk coagulation times: from 40.19 min with the farm method to 42.83 min with the experimental method, compared to 14.52 min with reference animal rennet. Regarding curd firmness, 90 min after adding the rennet (A90), it ranged from 41.27 mm (farm method) to 44.18 mm (experimental method). These values are comparable

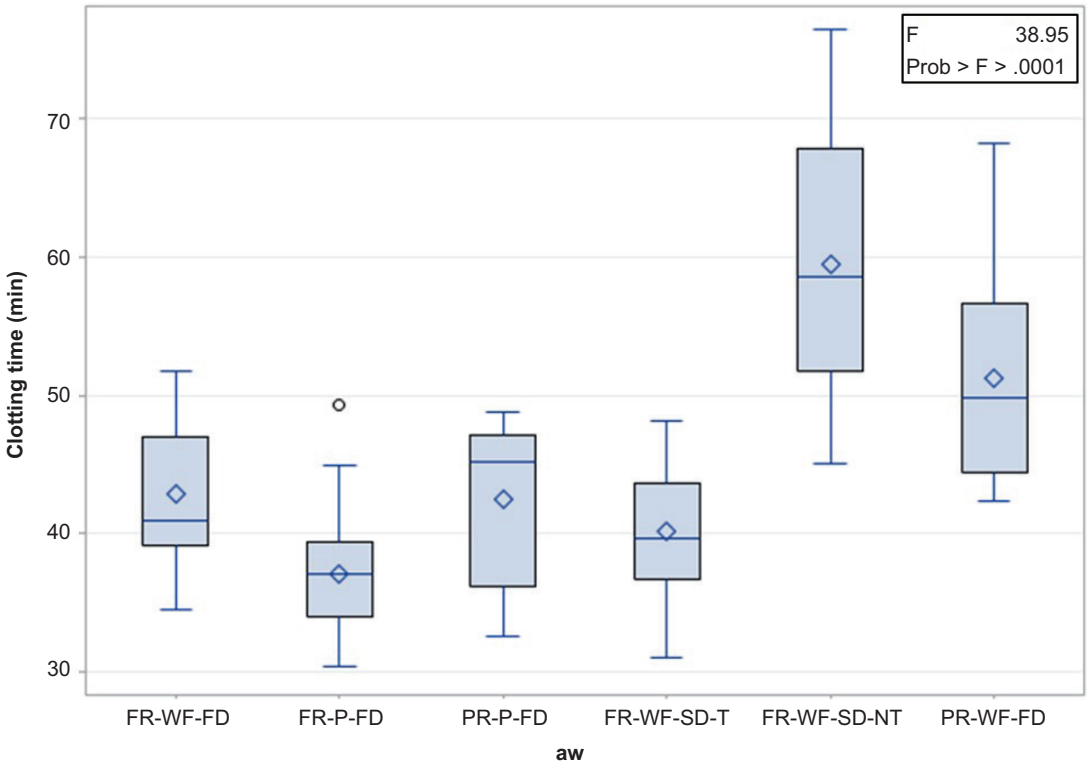

Figure 3. Box Plot of distribution of clotting time according to a_w classes. FR: full ripening; WF: whole flower; FD: fast drying (experimental method); P: pistils; PR: pre-ripened; SD: slow dried; T: toasted; NT: not toasted.

with those obtained 30 min after the addition of the animal rennet (44.70 mm). These results suggest that, by coagulating cow's milk with raw extracts prepared both with the farm and experimental methods, it is possible to obtain curd firmness similar to that obtained with animal rennet by extending the total coagulation time by 1 h.

Similar results were found by Liburdi *et al*. (2019) for samples of cow's milk coagulated with *Cynara cardunculus* and analyzed by Formagraph: coagulation time of 42.30 min and firmness of the curd after 60 min equal to 29.45 mm.

In cheese from animal rennet, the parameters of milk clotting activity are related to cheese yield; it was demonstrated that a faster coagulation time and firmer curd were positively correlated with cheese yield (Aleandri *et al*., 1989; Johnson *et al*., 2001; Ng-Kwai-Hang *et al*., 1989; Okigbo *et al*., 1985; Pretto *et al*., 2012). Moreover, the reduction in coagulation time is an undoubted advantage, as it contributes to decreasing the duration of the process.

In a recent paper, Ben Amira *et al*. (2017) studied the technological properties of milk gels produced by chymosin and wild cardoon rennet (*C. cardunculus var. sylvestris*). Higher curd firmness, similar to chymosin values, was obtained following the optimization of extraction conditions of wild cardoon rennet. It would be interesting to evaluate the effects of different coagulating activities of the raw extracts of *Cynara cardunculus* on the yield and characteristics of the cheese. Regardless, the effects of any changes in the preparation of vegetable rennet on the characteristics of traditional cheese should not be overlooked.

The results of the correlation between the a_w of the flowers/pistils and the clotting time suggest that the a_w should be less than 0.45 so that the clotting time is less than 45 min. In traditional practice, which always starts from fully ripe flowers, the addition of toasting of the pistils, which follows the slow drying of the flowers, is very effective in reducing the a_w and the clotting time. If fast drying is performed, the factors that favor the reduction of a_w are the collection of flowers at the fully ripe stage and the direct drying of the pistils. The best result, in terms of clotting time, was obtained from the pistils obtained from the ripe flowers and dried directly. However, separation of the pistils from the flowers before drying is time-consuming, which means higher costs to produce vegetable rennet.

In the flowers, the quantity of enzyme increased during development and was mainly present in the purple parts of styles and corollas. The maximum activity of enzymes observed in mature flowers may indicate involvement in the senescence process (Cordeiro *et al*., 1994). Clotting times decreased as flower development progressed due to increased enzymatic activities in the extracts (Cordeiro *et al*., 1994).

According to Martins *et al*. (1996), the drying time of thistle flowers subjected to the traditional method gave the following results. The a_w decreased from fresh (a_w 0.866) to medially dried (1 day): 0.674 and dried (30 days): 0.592. Better clotting activity was observed in medially dried flowers on a wet and dry matter basis. In contrast, Ordiales *et al*. (2012) investigated the milk-clotting activity of *Cynara cardunculus* at three ripening stages: opening of flower, flower fully open, and flower beginning to dry out. Milk clotting activities of aqueous extracts after maceration for 1 and 24 h did not vary significantly according to the ripening stages.

On the other hand, *Cynara cardunculus*, including its variety *altilis* (Ramos *et al*., 2014), is a source of phenolic compounds. The content of phenolic compounds was highest in the early stages of maturation and decreased as the maturity stage progressed (Mandim *et al*., 2020). Phenolic compounds could have a role in the clotting activity of flowers. Protein–polyphenol interactions were reported to modify the functional properties of foods (Yildirim-Elikoglu and Erdem, 2018). Phenolic compounds are easily oxidized to form pigments. These pigments attach to proteins, including native enzymes, leading to inactivation of these enzymes (Barros *et al*., 2001). The interactions between casein micelles and polyphenols decrease the enzymatic gelation properties at both the first and second stages of the renneting process (Haratifar and Corredig, 2014).

Considering that the crude extract is often contaminated by other floral compounds, such as phenolic compounds (Conceição *et al*., 2018), it can be assumed that the best performance of mature flowers could be due to both the greater content of proteases and to the lower content of polyphenols.

Among the different treatments used to prepare vegetable rennet from *Cynara cardunculus* flowers, the toasting treatment was very effective at improving clotting activity. Some hypotheses can be made to explain this positive effect. In a study (Wang *et al*., 2008) on bitter gourd, it was shown that aspartic protease was activated by heating treatment. Only few APs of the plant have been functionally characterized. For most APs of the plant, a definitive role was not assigned (Wang *et al*., 2008).

The results obtained with the different maceration times indicate that the longer the extraction time, the greater the coagulating activity of the raw extract. The same trend was observed in *Cynara cardunculus* flowers by

Ordiales *et al*. (2012), who reported better results after 24 h than after 1 h of maceration. Ben Amira *et al*. (2017), studying a model based on four variables to optimize the extraction conditions of *Cynara cardunculus*, found that the best extraction time was 50 min compared to 145 and 240 min.

Conclusions

The results indicate that it is possible to improve the coagulating activity of the crude extract of *Cynara cardunculus* by modifying the rennet preparation process. The most effective innovations compared to the traditional process were the toasting treatment following the slow drying of the flowers and the fast drying of the flowers/pistils at a controlled temperature. Extending the extraction time can further improve clotting activity. During the production of typical cheeses, it is necessary to evaluate whether innovations in the preparation of vegetal rennet can lead to changes in the characteristics of the cheese.

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Med-index: a food product labeling system to promote adherence to the mediterranean diet encouraging producers to make healthier and more sustainable food products

Maria Lisa Clodoveo[1]*, Elvira Tarsitano[2], Carlo Sabbà[1], Loreto Gesualdo[3], Filomena Corbo[4]

[1]Interdisciplinary Department of Medicine, Piazza Giulio Cesare, University of Bari, Bari, Italy; [2]Department of Veterinary Medicine, University of Bari A. Moro, Bari, Italy; [3]Department of Emergency and Organ Transplantation—Piazza Giulio Cesare, University of Bari, Bari, Italy; [4]Department of Pharmacy-Drug Sciences, University of Bari, Bari, Italy

***Corresponding Author:** Maria Lisa Clodoveo, Interdisciplinary Department of Medicine, Piazza Giulio Cesare, University of Bari, Bari, Italy. Email: marialisa.clodoveo@uniba.it

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OPINION PAPER

Abstract

Consumers are increasingly demanding transparency in food labeling as they want more and better information about what they are eating and where their food comes from. Several food indexes have been developed in the last decades to promote healthy eating with the aim of reducing certain diseases such as obesity, cancer, and diabetes. The Mediterranean diet is known to be one of the healthiest dietary patterns, and it is associated with a lower incidence of mortality from all-causes, and it is also related to a lower incidence of cardiovascular diseases, type 2 diabetes, certain types of cancer, and neurodegenerative diseases; however, a comprehensive index that quantifies the Mediteraneaness of foods is still missing. The real European challenge is to identify a uniform labeling system for the whole of Europe which promotes a healthy lifestyle. This article describes the development of the Mediterranean Index (MI), which aims to accurately measure the degree of food Mediterraneaness. The MI simultaneously integrates nutritional and sustainability characteristics of foods. The MI may provide an objective basis for the use of the "Mediterraneaness" label on food products, which can ultimately promote adherence to the Mediterranean diet encouraging producers to make healthier and more sustainable food products. Growing consumer concern toward health foods for better health can be a factor useful to promote the applicability of the precision nutrition principles by means of conscious choice.

Keywords: disease prevention; front-of-pack nutrition label; healthy eating choices; Mediterranean diet; sustainable production.

Introduction

According to EU statistics, in 2017, over 950,000 deaths were attributable to unhealthy diets. Nutrient-based warning labels can help in finding healthy diets. Nutritional labeling, in fact, is a very important tool because it immediately provides consumers with the information necessary to compare one product with another and to assess whether or not it meets their dietary needs, allowing them to make an informed choice (Mhurchu *et al*., 2017). The goal is to develop a method to combat diseases related to incorrect eating habits (Joint WHO/FAO Expert Consultation, 2003). The nutritional profiles related to foods, in turn, serve to distinguish those unbalanced in the supply of energy, nutrients, and sodium. The nutritional profiles related to foods were introduced by the regulation on Nutrition & Health Claims (Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006) with the express purpose of preventing that HFSS

foods (with high fat, sugar and sodium content) could boast alleged health benefits, protecting consumers from misleading communications that jeopardize a fair comparison between products.

As part of the "Farm to Fork Strategy," the path designed to lead toward a healthier and more sustainable food system, the European Commission intended to propose a compulsory nutritional labeling system harmonized at the EU level, which should be adopted by the end of 2022. The European challenge is to identify a uniform labelling system for the whole of Europe, which is not penalizing but promotes a healthy lifestyle. The objectives of the Farm to Fork Strategy clearly recognize that our health begins with the quality of the food we eat. Promoting healthy eating also means practicing prevention to defeat certain diseases such as obesity, especially childhood obesity, cancer, and diabetes (no less important is to add "sustainability" as an additional quality to be certified through labels (Narciso and Fonte, 2021)).

Currently, in the European Union, there are four systems, three of which are based on colors but significantly different from each other. The first simplified nutrition label is the one adopted in June 2013 by Great Britain, which uses the three traffic light colors, taking as a reference the amount of calories, sugar, salt, fat, and saturated fat in 100 g of product. Color is applied to each of these ingredients, except for the calories. The usefulness of traffic lights is evident on HFSS foods.

The British traffic light labeling system (Figure 1) explains whether a food has high, medium, or low amounts of fat, saturated fat, sugars, and salt (Machín *et al*., 2018).

Then, there is the Nutri-Score label (Figure 2) adopted by France (Chantal *et al*., 2017), which expresses the overall nutritional quality of foods through the use of five colors, from green to red, which correspond to five letters of the alphabet, from "A" to the "E." The color is attributed to the food as a whole, considering the presence of ingredients and nutrients to be limited, such as simple sugars and salt, but also those positive for health, such as fibers, fruits, and vegetables.

Between these two systems adopted by the British and French governments was the initiative of six big names in the food industry—Nestlé, Coca-Cola, PepsiCo, Unilever, Mondelez, and Mars—who launched their own traffic light label (Julia and Hercberg, 2018), inspired by the British one, with an apparently insignificant difference but with important consequences. While the label adopted by Great Britain refers to the number of calories, sugar, salt, fat, and saturated fat referring to 100 g of product, that of the industries, called Evolved Nutrition Labeling, refers to a single portion, which however is established by the company.

Italy argues that the "traffic light" indications penalize the Mediterranean diet and more generally, therefore, the "Made in Italy" products. The proposed Italian alternative is called Nutrinform Battery (Lorenzoni *et al*., 2021) and evaluates not individual foods, but rather their incidence within the diet. The label is designed as a battery and indicates all the values relating to a single portion consumed. The symbol, therefore, indicates the percentages of energy, fats, saturated fats, sugars, and salt provided by the individual portions compared to the recommended daily amount. In practice, the percentage of energy or nutrients contained in the single portion is represented by the charged part of the battery, so as to visually quantify them.

Nutrinform battery (Figure 3) is considered a difficult system because it proposes a generic reference to the "portion" and uses a misleading image, as in the common logic if the battery is more charged it is better, but not in the case of fats, sugars and salt , for which the best solution is represented by a flat battery.

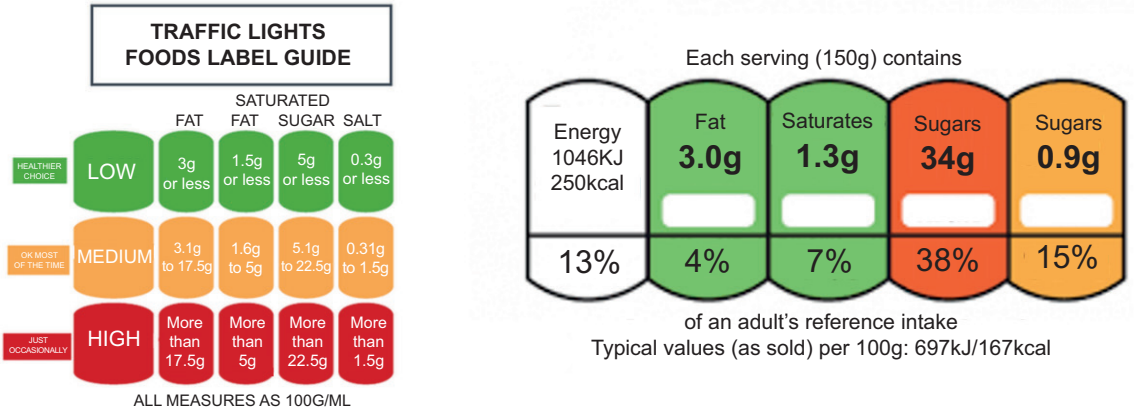
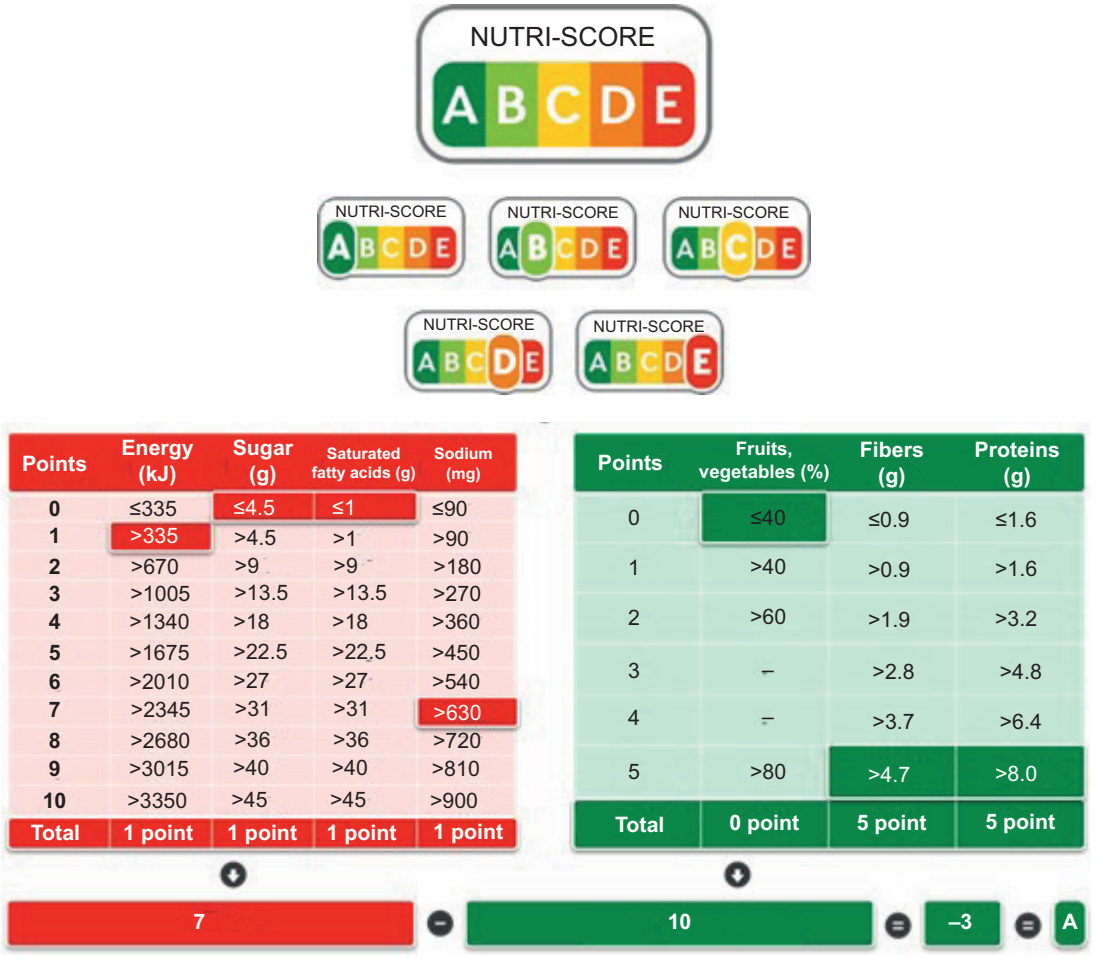


Figure 1. The British traffic light labeling system will tell you whether a food has high, medium, or low amounts of fat, saturated fat, sugars, and salt.



Points	Energy (kJ)	Sugar (g)	Saturated fatty acids (g)	Sodium (mg)
0	≤335	≤4.5	≤1	≤90
1	>335	>4.5	>1	>90
2	>670	>9	>9	>180
3	>1005	>13.5	>13.5	>270
4	>1340	>18	>18	>360
5	>1675	>22.5	>22.5	>450
6	>2010	>27	>27	>540
7	>2345	>31	>31	>630
8	>2680	>36	>36	>720
9	>3015	>40	>40	>810
10	>3350	>45	>45	>900
Total	1 point	1 point	1 point	1 point

Points	Fruits, vegetables (%)	Fibers (g)	Proteins (g)
0	≤40	≤0.9	≤1.6
1	>40	>0.9	>1.6
2	>60	>1.9	>3.2
3	–	>2.8	>4.8
4	–	>3.7	>6.4
5	>80	>4.7	>8.0
Total	0 point	5 point	5 point

7 − 10 = −3 = A

Figure 2. The Nutri-Score is a nutrition label that converts the nutritional value of products into a simple code consisting of five letters, each with its own color. Each product is awarded a score based on a scientific algorithm.

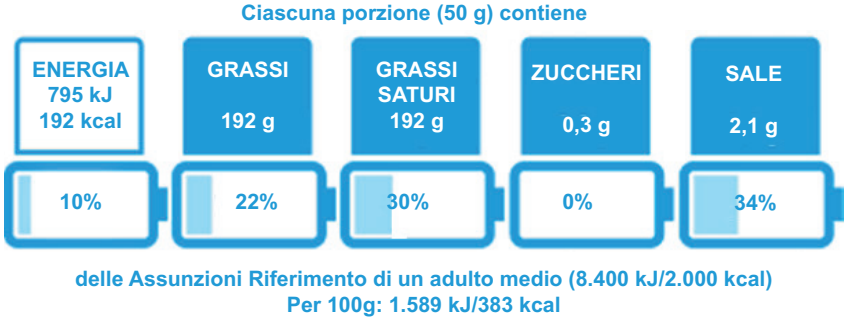


Figure 3. In the Nutrinform Battery nutrition label, each of the five batteries offers the consumer both a percentage and quantitative indication of the variable it represents (calories, sugars, etc.) in relation to a portion of the product to be purchased.

Both Nutri-Score (referred to 100 g of food) and Nutrinform Battery (referred to an arbitrary portion of food) essentially repeat what is already reported in the mandatory "nutritional declaration" reported in the rear part of the package in accordance with Regulation 1169/2011 starting from 13 December 2016, and do not constitute an effective tool for adapting the choices in relation to the quantitative and qualitative need of each individual.

National authorities of France, Belgium, Germany, Luxembourg, Netherlands, Spain, and Switzerland announced the establishment of a transnational coordination mechanism to facilitate the use of the front-of-pack nutrition label Nutri-Score.

Meanwhile, some member states of the EU (Italy, Czech Republic, Cyprus, Greece, Hungary, Latvia, and Romania,

which could soon be joined by Poland and Slovakia), have set up a united front against Traffic Light Label/ Nutri-Score labels, considered a distorting element of the market that can cause economic and image damage to Mediterranean products and provide scarce benefits for consumers, as it does not represent an effective and complete tool to promote a healthy diet and a balanced eating style.

In the European single market, there are nutrition labeling systems that do not give "red cards" but are based on a positive classification, such as the "keyhole" label in force in the Scandinavian countries—Sweden, Norway, and Denmark (van der Bend and Lissner, 2019). The keyhole has only green, indicating healthy products, recognized on the basis of specific requirements.

Not All "Calories" Are Created Equal

It is well known that food molecules not only serve as nutrients but can also modulate the body's physiological functions (Chakrabarti *et al*., 2018; Rescigno *et al*., 2017; Vamanu and Gatea, 2020). One of the final goals of the promising field of precision nutrition is the design of nutritional recommendations tailored to treat or prevent metabolic disorders (Toro-Martín *et al*., 2017). More specifically, precision nutrition aims to develop more comprehensive and dynamic nutritional recommendations based on variable and interacting parameters in a person's internal and external environment throughout life (Toro-Martín *et al*., 2017).

Weight problems and obesity are increasing at a rapid rate in most of the EU Member States (Garrido-Miguel *et al*., 2019). Obesity is a serious public health problem, as it significantly increases the risk of chronic diseases such as cardiovascular disease, type-2 diabetes, hypertension, coronary heart diseases, and certain cancers (Frasca *et al*., 2017). For specific individuals, obesity may further be linked to a wide range of psychological problems. For society as a whole, it has substantial direct and indirect costs that put a considerable strain on healthcare and social resources.

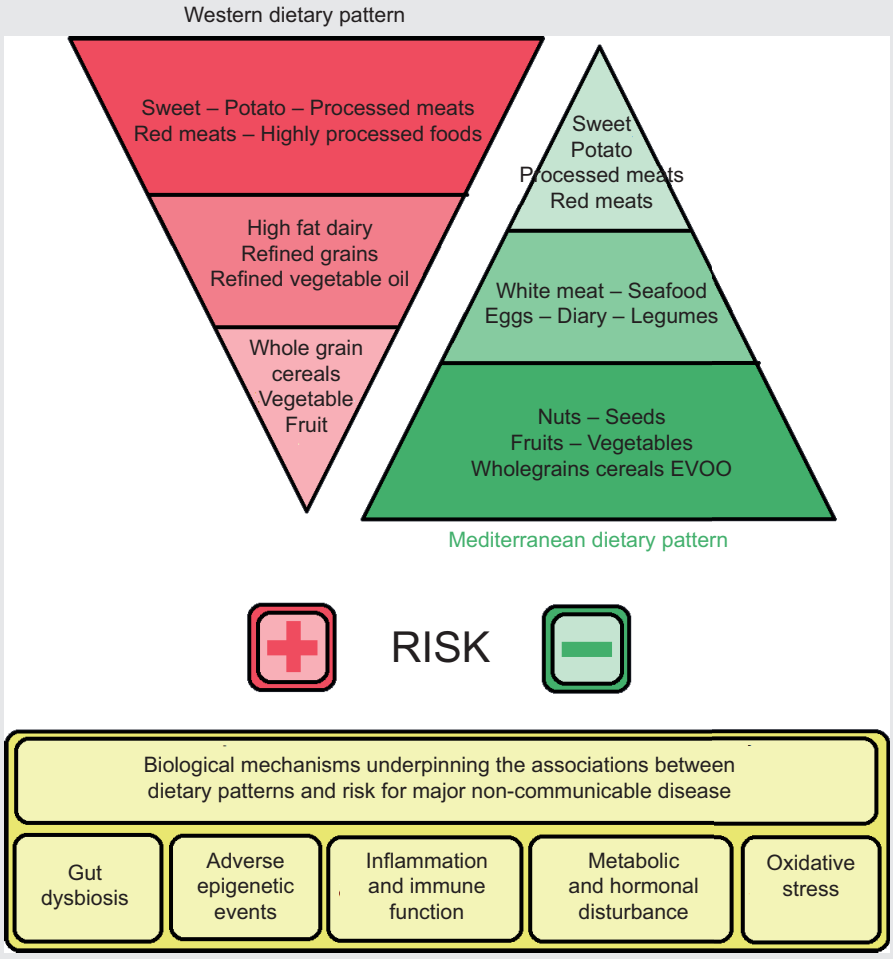
Excess weight and obesity are in 95% of cases the result of an energy imbalance between calories input and calories consumed by the body (Romieu *et al*., 2017). The principles of thermodynamics applied to the energy of the human body show that the susceptibility to weight gain varies among individuals due to the inter-individual differences in energy expenditure and energy intake, two factors that balance and determine the daily energy balance and, ultimately, the change in body weight (Piaggi, 2019). Excess energy is stored by the body in the form of fat; therefore, the British traffic light label, the French Nutri-Score label, and the Italian Nutrinform Battery are strongly focused on calorie content in foods. The simplified representation of a food in absolute terms of calories is an oversimplification of the functioning of the human body which is not simply a machine that burns fuel. The human body decodes the introduced molecules and considers food, more than a source of plastic and energy molecules, above all, as a source of information.

As defined, a calorie is the amount of energy it takes to raise 1 kg of water by 1 °C. However, the value of a food goes beyond calories, including macronutrients like fats, carbohydrates, and proteins, and micronutrients like vitamins, minerals, and antioxidants. So, consuming 100 calories of candy is not the same as consuming 100 calories of fruit, vegetables, and legumes that contain dozens of essential nutrients and tens of thousands of bioactive substances. So, food quality is more important than quantity. The nutrigenomics studies demonstrated that food molecules modify the extent to which different genes are expressed and thereby modulates the incidence of numerous chronic diseases linked to eating patterns. In fact, "food matrices" contain different compounds that interact in a coordinated way in the human body, determining the positive or negative effect of food. The researchers of European Nutrigenomics Organization support the hypothesis that all diseases can be reduced to imbalances in four overarching processes: inflammatory, metabolic, oxidative, and psychological stress. Diseases arise because of genetic predispositions to one or more of these stressors.

Food quality affects the first three of these four areas and explains the effects that two dietary patterns such as the Mediterranean Dietary Pattern (MDP) and Western Dietary Pattern (WDP) have on human health (Table 1). Moreover, food molecules are the main significant determinants of the microbial multiplicity of the gut and its metabolic activities.

Caloric restriction, understood as the pillar of the prevention of chronic noncommunicable diseases, is by no means the only discriminating factor as many studies have shown, in particular the European project PREDIMED (Martínez-González *et al*., 2015), which highlighted that in their primary prevention trial, an energy-unrestricted Mediterranean diet, supplemented with extra-virgin olive oil or nuts, resulted in a substantial reduction in the risk of major cardiovascular events among high-risk persons. These results can support the principles that the benefits of the Mediterranean diet for the primary prevention of cardiovascular disease are not exclusively dependent on the quantity of assumed foods but on the quality of the product that composed the basket of food choices.

Table 1. Comparison among Mediterranean Dietary Pattern (MDP) and Western Dietary Pattern (WDP).



Western dietary pattern: frequent intakes of whole dairy products, meats (i.e., red meat, viscera, low fat processed meat, and high fat processed meat), seafood, potatoes, refined grain bread, rice, pasta, non-extra virgin olive oils, non-olive oils, bakery (i.e., homemade and also commercial bakery), chocolate, sugar, processed meals, sauces, spices, salt, and commercial juices.

Western style diet or low adherence to Mediterranean diet increases risk of chronic human disease and is associated with aberrant microbiota profiles and harmful microbial activities, e.g., Trimethylamine, trimethylamine N-oxide, leaky gut, inflammation, branched chain fatty acids, genotoxins, carcinogens, ammonia.

Mediterranean dietary pattern: high intakes of fruits, vegetables, olive oils, wholegrains, legumes, nuts, unsaturated fatty acids and fish, with restrictions in red meat and dairy products, moderate but regular consumption of red wine, and low consumption of red meat and discretionary foods including sugar-sweetened food and drink.

Adherance to Meditteranean diet (or other similar healthy diets) reduces risk of chronic disease and increases healthy associated microbiota activities, e.g., short-chain fatty acids, polyphenol catabolites, bile acid deconjugation, immune homeostasis, gut barrier, nutrient absorption, K and B vitamins, lipid biohydrogenation.

The Diet for a Healthy Life in a Healthy Planet

Sustainable diets have low environmental impact and contribute to food and nutrition security and a healthy life for present and future generations of human resources (Nelson *et al.*, 2016). Europe enters the 2030 agenda for sustainable development with an ambitious policy document: the Green Deal (Haines and Scheelbeek, 2020). The objectives contained therein aim to promote the efficient use of resources by moving to a clean and circular economy, to restore biodiversity and reduce environmental pollution, promoting what is called "green transition," transforming the problems of climate and environmental challenges into opportunities across all sectors and making the transition just and inclusive for all (Sikora, 2021). Educating about the Mediterranean diet also means spreading and enhancing a sustainable consumption model capable of ensuring food security, promoting healthy lifestyles, sharing good food practices, and contributing to the achievement of the Sustainable Development Goals (SDGs) set by the UN 2030 Agenda and those established by the European Commission in the Green Deal (Dernini and Berry, 2015; Medina, 2021; Serra-Majem *et al.*, 2020).

The real and progressive increase of the environment, climate, food, and health crisis of our century requires

reconstructing the connections among agriculture, nutrition, and ecology. To be able to conjugate the "tradition" with the "technology" is a challenge of our millennium for the benefit of quality and sustainability of good life and well-being.

In 2015, the global community adopted the 17 Global SDGs to improve people's lives by 2030. Worth mentioning is the Goal 2—Zero Hunger—a commitment to end hunger, to achieve food security and improved nutrition, and promote sustainable agriculture. Actually, the world has made great progress in reducing hunger, but if we want to see a world free from hunger by 2030, governments, citizens, civil society organizations, and the private sector must work together to invest, innovate, and create lasting solutions through resilient communities. Reducing food waste is one of the major challenges faced by the Agenda 2030 through the Goal 12, "Ensuring sustainable production and consumption patterns." Worth mentioning too are ambitious targets that have been set out by the UN including the implementation of a 10-year framework of activities linked to sustainable production and consumption, aimed at reducing food loss by 2030, as well as along the entire food chain, and above all halving per capita food waste in terms of retail sales and domestic consumption (SDG 12.3).

The Med-Index

The Mediterranean Index (MI) has been developed as a tool to accurately measure the degree of food Mediterraneaness. The MI simultaneously integrates nutritional and sustainability characteristics of foods. The MI may provide an objective basis for the use of the "Mediterraneaness" label on food products, which can ultimately promote adherence to the Mediterranean diet among citizens, encouraging producers to make healthier and more sustainable food products.

"The Mediterranean way" through a food product labeling system (Med-Index) is, in fact, also a tool for achieving these goals of the 2030 Agenda for Sustainable Development. In fact, the Mediterranean diet and the Med-Index stimulate, in a simple and intuitive way, the interest of the community in a correct diet; it brings citizens closer to the productive world; it stimulates interest and curiosity from adults to younger groups so that they acquire greater awareness of the importance of sustainability as a primary tool for sustainable development, well-being, and good life for every society (de Vries, 2020; Tarsitano *et al*., 2020) by launching the foundations for the creation of a network of sustainable and resilient communities.

Combining tradition and innovation through the multifunctionality and diversification of agriculture such as the quality of products, rather than only production, reproducibility of resources, protection and valorization of the landscape, and cultural and ethical values in a perspective of sustainability is the real challenge Agriculture must return to open itself toward other knowledge, like new connections between air and water, earth and living organisms (vegetal and animal), because they represent the real foundation of ecological knowledge. It is about knowing, recognizing, and appreciating the ancient flavors of our agri-food tradition and trying to safeguard our different ecosystems throughout the Mediterranean area. The local product is linked to the land where it is produced, to its environmental resources, to its historical processes, to its community networks, and to people who live there.

Healthy eating is the most direct and complete way to relate to the surrounding world, to express our culture through the choice of food and the methods of consumption. It must be taken into account that the state of health of people, their equilibrium, and psycho-physical well-being are closely linked to the relationship with the environment in which they live and to interactions with others, including animals and plants, waters, smells and flavors: it would be like distancing people from their natural history, turning them away from ancestral experiences that linked them to the mother earth and shaped their tastes and choices, including food.

The distinctive character must be that of a sustainable, holistic, and systemic approach to healthy eating: we believe that lifestyle modifications and individual behavior can lead us on the path of awareness for the improvement of quality and sustainability of life. The Mediterranean diet/way adapts easily to changes by improving its current perception not only as a healthy diet but also as a sustainable lifestyle (Tarsitano *et al*., 2018).

"The Mediterranean way" and the correct adoption of the Med-Index becomes a model of resilient and sustainable communities (Table 2). Inspired by this sustainable, holistic, and systemic notion of the Mediterranean diet, the authors identify the importance of sustainable food culture, scientific research, training and creation of wide and comprehensive partnership networks, with the aim of developing instruments for the defense of human rights, such as the right to adequate nutrition for everyone, a healthy well-being and good life, an intact and healthy environment, as well as the respect for and enjoyment of common goods and specific cultural identities and biodiversity of the territories, including the food production according to principles of sustainability, dignity, and equity for all people involved.

Nutritional labeling should not only be a tool that provides the consumer with the information about

Table 2. Elements of sustainability of the Mediterranean diet.

Sustainability of the Mediterranean diet	
The Mediterranean food model is healthy for both people and the environment. It is estimated on average that to obtain 100 calories, the Mediterranean diet causes an environmental impact of about 60% less than a Western-style diet, based to a greater extent on meat and animal fats, rather than on vegetables and cereals. *The Mediterranean food model, as already underlined by UNESCO, goes beyond the concept of food. The term diet itself derives from the ancient Greek diaita (lifestyle) to indicate the social and cultural value of the Mediterranean diet. Considering the positive effects on the social, economic and environmental spheres, the Mediterranean diet can be considered a sustainable food model.*	**ENVIRONMENTAL BENEFITS** **Use of natural resources.** The Mediterranean diet involves a high consumption of cereals, fruit, vegetables and legumes, the production of which requires a less intensive use of natural resources (soil, water) and greenhouse gas emissions compared to a diet based mostly on the consumption of meat and animal fats. **Seasonality.** The Mediterranean diet provides for the consumption of food respecting the seasonality of the same. This translates into a reduction in greenhouse crops and related environmental impacts, as well as supply and transport costs from distant countries (food miles). **Biodiversity.** The Mediterranean diet respects the territory and biodiversity, through different sowing in each area and crop rotation, in order to also guarantee food security. **Frugality.** The Mediterranean diet includes moderate portions and consumption of whole and fresh, lightly processed foods. Both the quantities consumed and the minor transformations undergone by food contribute to reducing the environmental impacts of eating behaviors. **SOCIAL BENEFITS** **Health**. The Mediterranean diet, together with physical activity, helps prevent cardiovascular disease, diabetes and some types of cancer (colorectal, breast, prostate, pancreas, endometrium). In addition, the intake of fresh and whole foods allows greater availability and use of micronutrients and antioxidants. **Awareness**. The Mediterranean diet promotes greater food awareness and link with the territory, knowledge of seasonality, biodiversity and naturalness of foods. **Conviviality**. The Mediterranean diet promotes social interaction, common meals are the cornerstone of the holidays and of our social traditions. **Identity.** The Mediterranean diet is an expression of the entire historical and cultural system of the Mediterranean. It is a millenary food tradition that has been handed down from generation to generation, promoting not only the quality of foods and their territorial characterization, but also the dialogue between peoples. **ECONOMIC BENEFITS** **Health expenditure.** A greater adherence of eating habits to the Mediterranean model would improve the general state of health of the population, which would translate into a decrease in national health expenditure. **Household spending**. Adherence to the Mediterranean food model, favoring seasonal foods, mainly cereals and vegetables, would allow a decrease in household food spending. **Business enhancement.** The spread of the Mediterranean food model would result in an increase in the commercial demand for natural products (fruit, vegetables, cereals, legumes ...) and their derivatives (olive oil, wine, pasta, bread ...), creating income and employment for companies in the Mediterranean regions. **Enhancement of territories.** The spread of the Mediterranean food model would enhance the agro-eno-gastronomic offer of our territories, contributing to the seasonal adjustment of the tourist offer.

nutritional needs but also an instrument to compare one product with another with the aim of assessing the element of sustainability because human health is inextricably linked to the health of the planet. In order to interrupt the information asymmetry that often characterizes food purchases and to offer the possibility of a better understanding of the information on the products useful for following a healthy and sustainable diet based on the principles of the Mediterranean diet, a new model of nutritional labeling, called Med Index, has been developed.

Med-Index: The Shape

Nutrition labeling must be able to speak to consumers by making immediately clear the purpose for which it was designed. The first phase of the design involved the choice of the geometric shape to be assigned to the label in such a way that it immediately and uniquely brings to mind the topic for which it is designed: information relating to nutritional aspects. The triangle is a symbol of action; it recalls the indications of the road signs and induces you to think about the direction to take. Geometric shapes quickly bring to mind past experiences. The triangle is immediately associated with the stable image of a mountain or a pyramid. Here, the food pyramid comes to mind and evokes the concepts of balanced food choices aimed at well-being. The dissemination of messages can also take place through forms and images; it is not necessary to add anything else, no words and no numbers.

The triangle, like the iconography of the food pyramid (Figure 4), has been divided into three parallel bands to identify two fields that return quantitative information to the user and one field for qualitative information.

The apex of the triangle (Figure 5) was intended to inform about the doses that make up the sales unit. The individual dose is defined by means of icons that identify

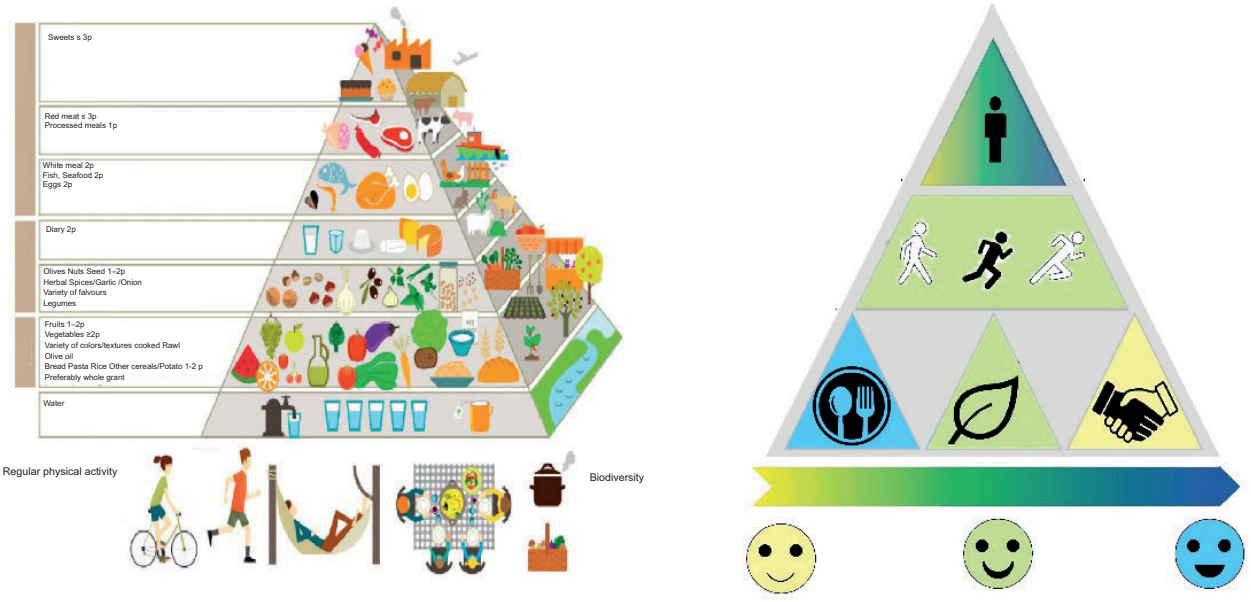


Figure 4. Med-index: the shape.

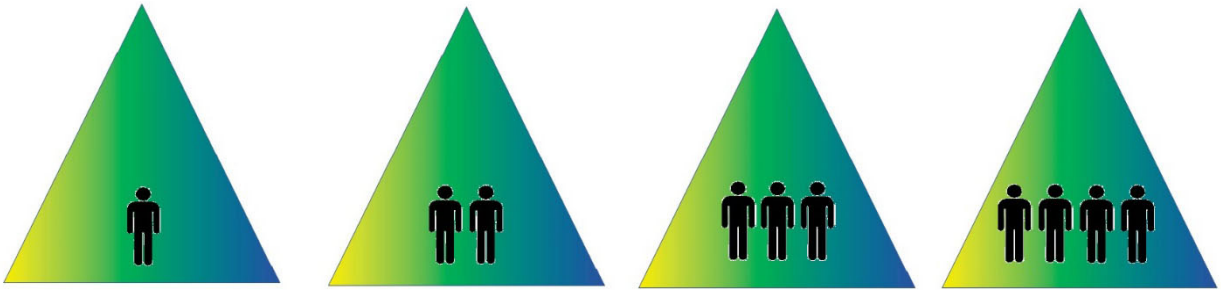


Figure 5. Med-index: apex of the triangle was intended to inform about the doses that make up the sales unit or the number of food portions.

the number of diners. The prescription of the portion in grams often represents an operational and psychological binding for diet compliance.

Obesity mostly affects individuals belonging to less affluent social classes associated with a lower level of education: culture, awareness, nutrition, and education are needed to fully understand the parameters chosen in most nutritional labels, often related to metabolic effects, and expressed in absolute or percentage numerical terms, which acquire meaning in a context of knowledge of the reference values. In the absence of skills, they do not contribute to positively changing food choices and behaviors.

While listing calories could have a detrimental effect on people with eating disorders, the caloric intake, instead of being expressed quantitatively through the number of kcal, is expressed in the form of intensity of

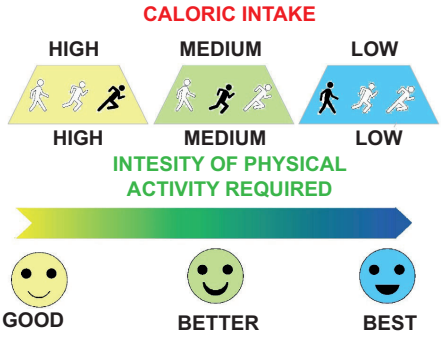


Figure 6. The central band of the triangle provides information on the calorie intake of the portion referring to the intensity of physical activity necessary to balance the intake of calories.

recommended physical activity (Figure 6), namely, low, medium, and high, so that a balance between calories consumed and calories ingested can be actuated, because it is necessary to strike a balance between informing and

educating people to make healthier choices, while not negatively impacting people with eating disorders, or those in recovery. In fact, there is a broad consensus in the literature that a sedentary lifestyle is one of the main factors contributing to the epidemic of cardiometabolic diseases (Hill *et al*., 2012).

To make the communication clearer, three icons represent walking, light running, and intense running. The caloric intake is deduced from the icon highlighted in black and from the background color, which is in line with the color scale used to indicate the lowest caloric intake value with walking and the blue color which symbolizes the best choice, while the high caloric intake value with intense running is represented by the yellow color.

In addition to unhealthy diets, inadequate physical activity is an important factor in the increasing trend of obesity and noncommunicable diseases. The implementation of physical activity recommendations in nutrition labeling may influence the consumers' food choices. The Med-Index in full spirit of the principles of the Mediterranean diet encourages the practice of physical activity. Physical inactivity is in fact identified globally as the fourth most important risk factor for mortality. In many countries, levels of physical inactivity are increasing, with important repercussions on the prevalence of non-communicable diseases and on the state of health of the general population around the world.

The base of the triangle (Figure 7) provides information on the nutritional and sustainability characteristics of the product through the use of three small triangles that express the degree of nutritional, environmental, and social sustainability by means of three different colors.

This iconographic information is in line with the opinion of the European Economic and Social Committee (EESC, 2017), a global EU food policy, adopted in December 2017 and then integrated in February 2019, with the released opinion entitled Promoting healthy and sustainable diets in the EU (EESC, 2019), which suggest combining healthy diets with environmental, social, and economic sustainability through the coordination of policy measures.

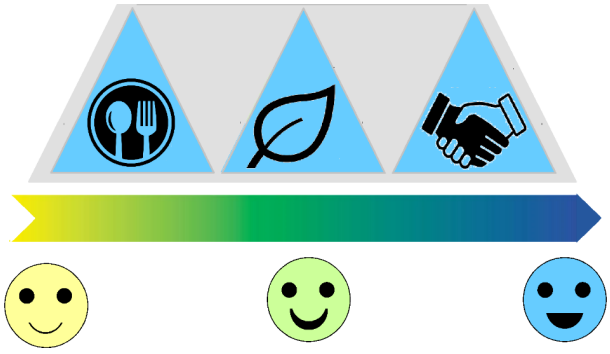


Figure 7. The base of the triangle provides information on the nutritional and sustainability characteristics of the product (NS: nutritional sustainability; ES: environmental sustainability; SS: social sustainability).

Med-Index: The Colors

The meaning of colors varies culturally. Traffic lights on the roads are different in Japan and Europe. The Japanese replace green with blue. Care must be taken when the message is intended for audiences from different nations as is the EU's intention to harmonize nutrition labeling; therefore, no proposal should be approved without a transnational pilot trial. Color is a real code, which changes from culture to culture.

In the Western society, these peculiarities are recognized in the main colors: in marketing, yellow is used to attract attention and convey optimism; red increases the heart rate, creates urgency and/or concern, and is an energetic color; blue creates the feeling of confidence and security; green is often a color that is easy to process for the eye, it provides relaxation because it does not weigh down the eyes; orange prompts action and is an aggressive color.

In the MI the colors are used to define a positive scale of values (Table 3) that excludes negative alarm signals as happens in the traffic light labeling when the red and orange colors appear that refer to the danger, generating an alert state. The message is always in a positive key because it starts from the assumption that the Med-Index

Table 3. The meaning of the colors used in the Med-index.

GOOD	Yellow is the color of optimism and clarity; it attracts attention and intrigues and can arouse positive and reassuring emotions (unlike red).
BETTER	Green is the color of freshness, well-being, and relaxation. It expresses authenticity and naturalness and inspires confidence.
BEST	Blue is the color that most of all conveys security, tranquility, and trust. It is a very positive color, and it is also the color most loved by people.

can only be applied to products that fall within the Mediterranean basket of products.

To support food security for current and future generations, there is a need to understand the relation between sustainable diets and the health of a population (Nelson *et al*., 2016). A set of criteria (Table 4) can be used to attribute color to the three triangles that will provide information on nutritional, environmental, and social sustainability.

If all the criteria of a category (e.g., good or better) are satisfied, even just one criterion of the next category (e.g., better or best) is sufficient for the triangle to take on the color of the best category. If for a category of sustainability, different from nutritional sustainability, no parameter is satisfied, the triangle remains white.

By way of example, Figure 8, summarizing the iconographic interpretation, shows the extremes of the value that the Med-Index can assume.

An Index Consistent with the LARNs, the Pyramid of the Mediterranean Diet, and the Guidelines for Healthy Eating

A balanced diet provides the right amounts of different nutrients to preserve the health and well-being of the body. Proteins, carbohydrates, fats, vitamins, minerals, and water are all "nutrients." Each nutrient has a specific function in the human body. The amount of each nutrient that is needed to keep an individual healthy is called the "nutrient requirement." Nutrient requirements vary according to age and gender. The level of physical activity, the physiological state in which one is (e.g., pregnancy), dietary habits, and genetic heritage are also important factors.

Since eating in a healthy, balanced, and appropriate way, suitable to the individual well-being and energy expenditure, not only imply the choice of foods but also their quantity and frequency of consumption, it must be remembered that to follow a correct diet, it is essential to know how to quantify and evaluate the quality of what an individual eats.

The Med Index is a tool that used alone informs the consumer about the "standard portions," the specific quantity of a food taken as a reference unit of measurement and defined by experts for the main types of food groups, considering their content in nutritional principles, the average food consumption of the population, consistent with the food tradition and reported in the recent revision of the LARN (Nutrient and Energy Reference Intake Levels) (SINU, 2014). The LARN already in 1996, as well as in the last revision (2014), proposed a list of "measures for common use" for the quantification of standard portions, which the Med Index incorporates and typifies in iconographic form in the apical portion of the triangle.

To ensure proper nutrition, it is also necessary to establish how often (daily or weekly) a portion of a given food can or should be consumed according to different energy needs. Quantity and frequency of consumption must be adapted to age, sex, height, and level of physical activity.

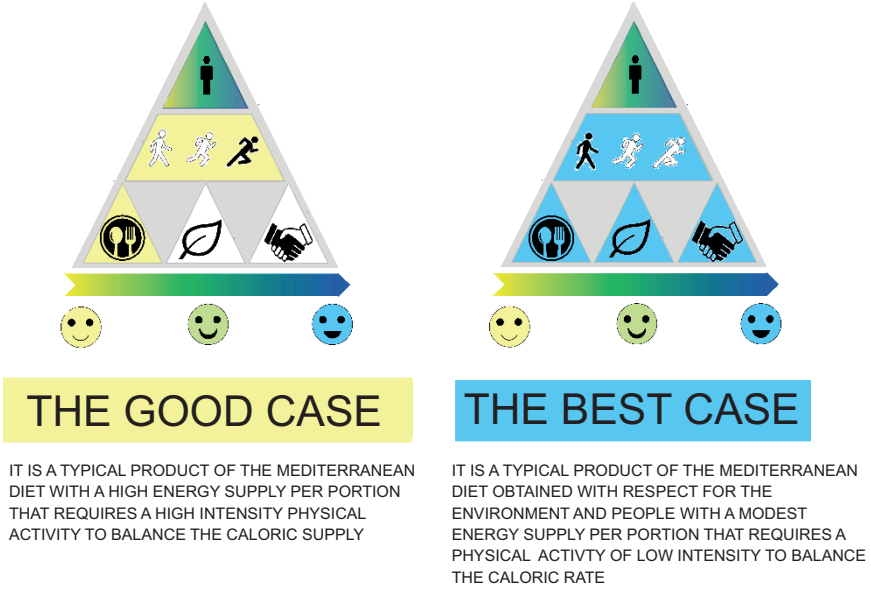


Figure 8. The extremes of the value that the Med-Index can assume.

Table 4. Criteria for attributing, in each category of sustainability, nutritional, environmental, and social, the color that indicates the three degrees of positive evaluation, namely, good, better, and best.

THE COLOR IS ATTRIBUTED ACCORDING TO THE PRESENCE OF AT LEAST ONE CHARACTERISTIC IN THE BEST CATEGORY		
 **NS Nutritional Sustainability**		1. The product is in the traditional basket of products of the Mediterranean diet 2. The Mediterranean product respects the biodiversity of food 3. The Mediterranean product respects the seasonal availability
		4. The Mediterranean product has a balanced ratio of macronutrients 5. The Mediterranean product has a nutritional claim 6. The Mediterranean product is recognized by a certification of origin
		7. The Mediterranean product contains prebiotics 8. The Mediterranean product contains probiotics 9. The Mediterranean product has a health claim
THE COLOR IS ATTRIBUTED ACCORDING TO THE PRESENCE OF AT LEAST ONE CHARACTERISTIC IN THE BEST CATEGORY		
 **ES Environmental Sustainability**		1. It is a local food product certified by a traceability system 2. The production is conducted process in full respect of the environmental regulations 3. It is a zero-residue product from conventional farming systems
		4. It is a zero-residue product from organic farming systems 5. It comes from an eco-friendly management of all waste throughout the entire production cycle 6. It is designed to limit the waste associated with its consumption
		7. The production process uses renewable energies 8. The product has an environmental sustainability certification relating to the carbon footprint 9. The product has an environmental sustainability certification relating to the water footprint
THE COLOR IS ATTRIBUTED ACCORDING TO THE PRESENCE OF AT LEAST ONE CHARACTERISTIC IN THE BEST CATEGORY		
 **SS Social Sustainability**		1. The production process is conducted in full respect of the labour regulations 2. The income deriving from the product is equally divided among the players in the supply chain 3. The production systems protect the Mediterranean landscape and its identity role
		4. The producer implements food education actions 5. The producer invests in R&D aimed to support people's well-being and quality of life 6. The producer measures the impacts of the production process on society (social report, SLCA, etc.)
		7. The producer takes positive action to promote gender equity 8. The producer takes positive action to reduce inequality between generations 9. The producer implements positive actions to create new jobs and and reduce forced migration of human capital that deplete the territory

The Med Index combines with other tools such as the "Pyramid of the Mediterranean Diet" and the "Guidelines for healthy eating" to understand the "frequency of consumption of standard portions," which defines the number of standard portions recommended for each group (or subgroup/type) of foods to be consumed daily or weekly, to ensure the adequacy of the diet. The "Pyramid of the Mediterranean Diet" is a graphic symbol that shows with great immediacy which foods to consume, in what quantity, and how often to make them immediately understandable for the consumer.

Since the frequency of consumption cannot be the same for all individuals, but varies according to age, sex, physical activity, and physiological state, the "Guidelines for a healthy diet" address the needs:

(i) of woman in different stages of life, namely, fertile age, conception, pregnancy, and menopause;
(ii) of individuals in the age of growth, infants, children, and adolescents;
(iii) of the elderly;
(iv) of sportsmen and athletes;

and in combination with the Med Index, it makes food purchasing choices more aware and immediate consistent with the "fast" lifestyle habits that reduce the time dedicated to food shopping and preparing meals.

From Farm to Fork

In the United Kingdom, large catering companies will need to display food calorie information on restaurant menus and on labels of prepackaged ready-to-eat food and ready meals sold in commercial establishments starting from April 2022, in order to make it easy for people to make healthier food choices for themselves and their families, both at the restaurant and at home. The obligation will apply to large companies with 250 or more employees in England, including bars, restaurants, and takeaways, which will have to indicate the calories of

nonprepackaged food and nonalcoholic drinks prepared for customers. This information will need to be displayed on menus, including online menus, on food delivery platforms, and on food labels (Littlewood *et al.*, 2016).

The Med-Index can be applied in all stages of the supply chain and to the different product ranges (Figure 9):

(i) Primary products: products of primary production including products from the land, livestock, hunting, and fishing.
(ii) Processed products: food products obtained from the processing of unprocessed products. These products may contain ingredients necessary for their processing or to give them specific characteristics.
(iii) Finished product: product that is not subjected to further processing or transformation by the company.
(iv) Gastronomic preparation: a complex of ingredients that has undergone a series of gastronomic preparation processes that are consumed at home, in collective catering, and in restaurants (Clodoveo *et al.*, 2020).

This multi-sectoral and multilevel approach links food to education, agricultural practices, and different sources of sustainability and accountability.

Extra Virgin Olive Oil: A Case Study

Observational cohort studies and a secondary prevention trial have shown an inverse association between adherence to the Mediterranean diet and cardiovascular risk. PREDIMED researchers conducted a randomized trial of this diet pattern for the primary prevention of cardiovascular events. In this trial, an energy-unrestricted Mediterranean diet supplemented with either extra-virgin olive oil or nuts resulted in an absolute risk reduction of approximately three major cardiovascular events per 1000 person-years, for a relative risk reduction of approximately 30%, among high-risk persons who were initially free of cardiovascular disease. These results support the benefits of the Mediterranean diet for cardiovascular risk reduction. They are particularly relevant given the challenges of achieving and maintaining weight loss.

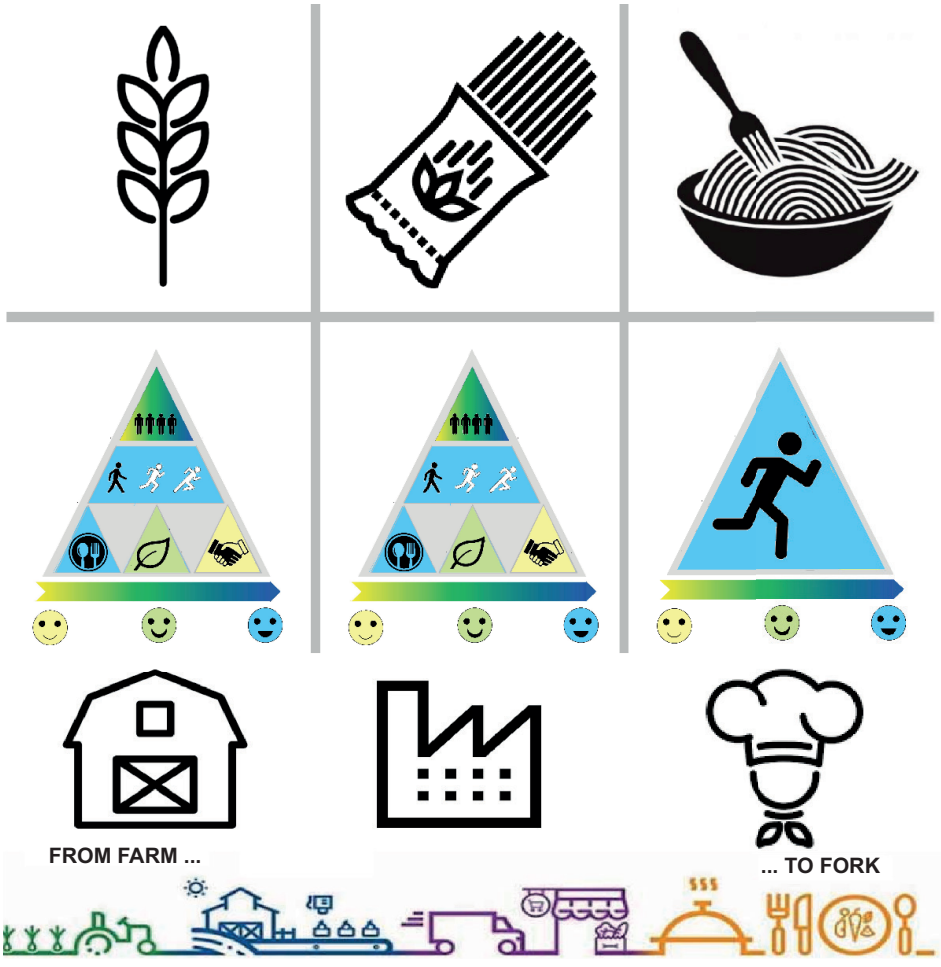


Figure 9. Applicability of Med-Index from farm to fork.

The beneficial effects of EVOO (Lammi *et al.*, 2020; Mallamaci *et al.*, 2021) are not clearly communicated through the nutrition labels currently evaluated in the European harmonization process, in particular the Nutri-Score. In fact, Nutri-Score is calculated via an algorithm developed by a team of researchers, which combines both the nutrients to limit because considered unhealthy (i.e., calories, saturated fat, sugars and salt) and those elements to favor because considered healthy (such as fibers, proteins, nuts, fruit and vegetables) and thus should allow consumers to choose the healthy option comparing foods of the same category.

Under the system, extra virgin olive oil (EVOO) receives a yellow C rating. This, according to members of the olive oil industry, does not take the health benefits of EVOOs into due account and ultimately penalizes the product. Under this system, EVOO receives a yellow C rating. This, according to stakeholders of the olive oil industry, fails to account the health benefits of EVOOs (respect to refined olive oil and pomace olive oil, characterized by the same score) penalizing the image and the value of the product. Italian extra virgin olive oil producers believe that the Nutri score label on the front of the pack (FOPL) can't help European consumers understand the benefits of adhering to the Mediterranean diet because condemns EVOOs. Conversely, its simplistic classification could even turn citizens away from a food, the EVOO, that has so many scientifically proven health benefits. Conversely, its simplistic classification could even turn customers away from a food that has so many scientifically proven health benefits. Assitol, Italian Association of the Oil Industry, and many Italian olive oil producers believe that the possible introduction of Nutri-Score on domestic and foreign markets could hinder the extra virgin olive oil trade, especially in those countries where there is no native culture of the usage of olive oil that can compete with what they see as misleading information.

The Spanish National Designation of Origin Olive Oil Sector has requested that the Nutri-Score grant virgin and extra virgin olive oil the highest nutritional classification. This request received the support of the International Olive Oil Council (COI).

This request is not appropriate as it brackets extra virgin olive oil in the category of a mere commodity (Commodity is an economical term to identify a fungible product. This means that each unit of a commodity is considered exactly like every other unit, without element

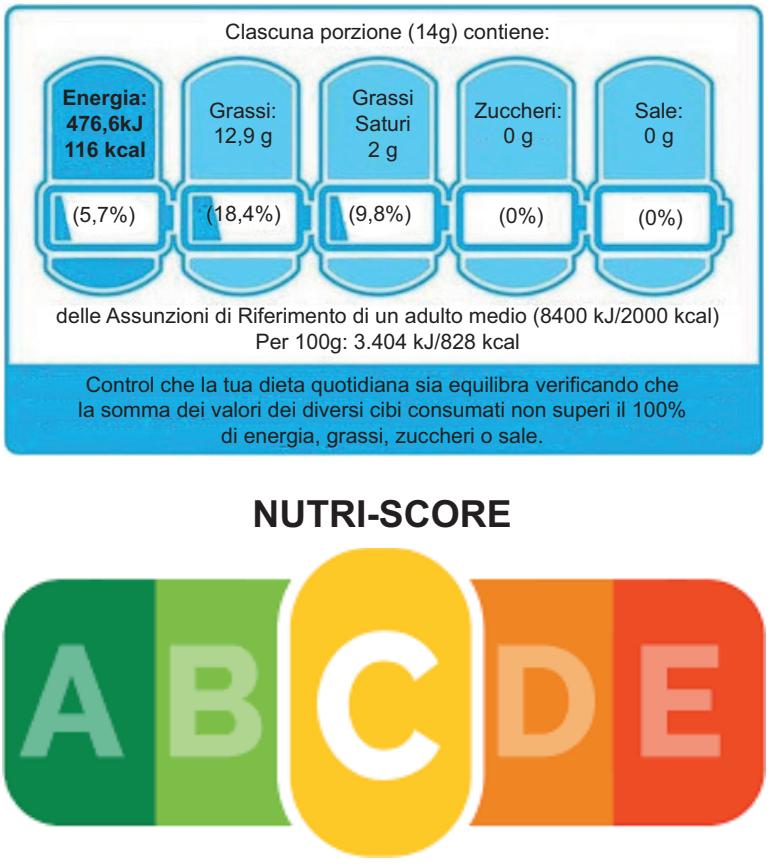
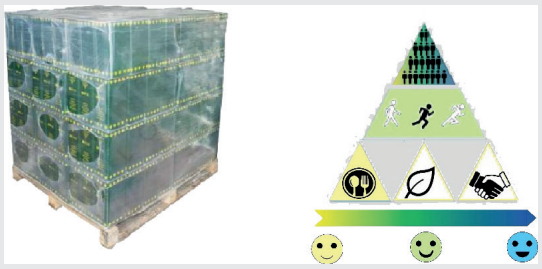


Figure 10. Nutrinform battery and Nutri-Score labels applied to extra virgin olive oil.

Table 5. The Med index applied to two different types of extra virgin olive oil, an EVOO in promotion (A) and a high quality EVOO (B).

(A)	(B)
	
The Med Index of the EVOO in promotion (A) thanks to the iconographic code instantly delivers the following information to consumers: extra virgin olive oil is a condiment and as such the package contains numerous doses. The recommended daily dose, also by EFSA, 20 g/day, being made up of triglycerides, will require to be balanced by moderate intensity physical activity in order to achieve the balance between ingested calories and energy expenditure. While it does not have distinctive elements relating to certifications of origin, environmental or health claims, it is a good choice because extra virgin olive oil is the main lipid source in the basket of products suitable for the Mediterranean diet.	The high quality EVOO Med Index (B) thanks to the iconographic code instantly delivers the following information to consumers: extra virgin olive oil is a condiment and as such the package contains numerous doses. The recommended daily dose, also by EFSA, 20 g/day, being made up of triglycerides, will require to be balanced by moderate intensity physical activity in order to achieve the balance between ingested calories and energy expenditure. The product in question, extra virgin olive oil, in addition to being the main lipid source in the basket of products suitable for the Mediterranean diet, is equipped with numerous distinctive elements relating to certifications of origin (DOP), environmental certifications (it is an organic oil with sustainability certification) and reports three health claims (polyphenols, vitamin E and oleic acid), elements that overall make it the best choice, and in terms of value guarantee a premium price.

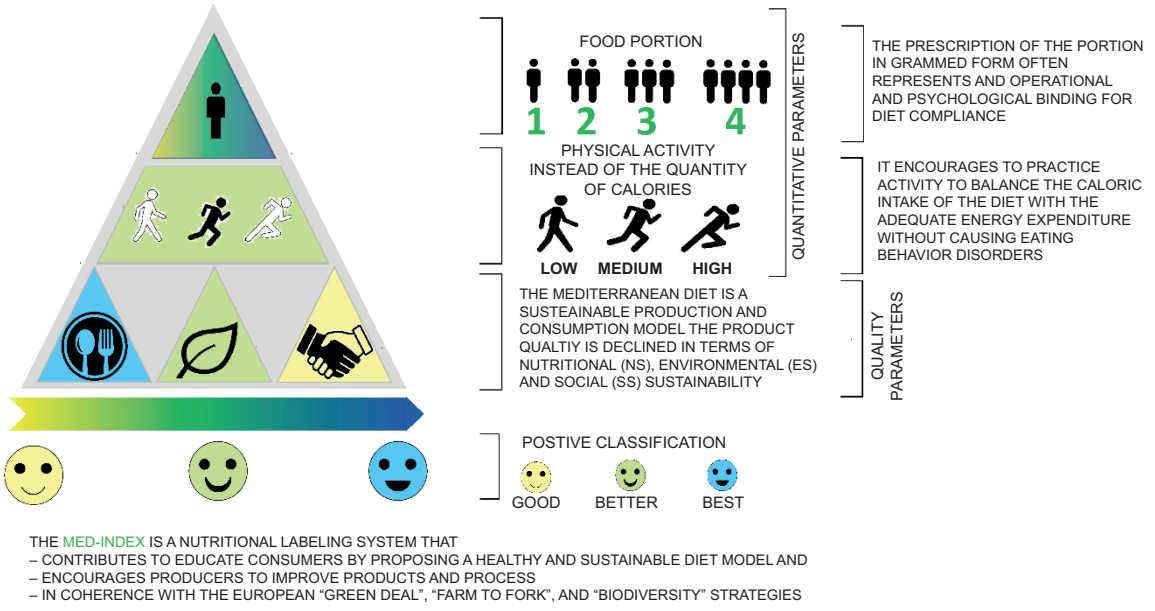


Figure 11. Med index graphic summary.

of differentiation that can determine a range of different values and prices, useful to award the best producer with a premium price). In fact, it perpetuates the misunderstanding that all extra virgin olive oils are the same from a health point of view. According to EFSA, the extra virgin olive oil, which is promoted for consumption as part of the Mediterranean diet for its beneficial effects against diseases of the cardiovascular system, is a product rich in oleic acid, polyphenols, and tocopherols.

This condition depends on the latitude of the production area, the variety of olives, and the agronomic and technological practices (Amirante *et al.*, 2008, 2010a, 2010b); therefore, a nutritional label should allow the consumer to recognize with certainty an extra virgin oil that performs a mere function of condiment from the functional product, effective in reducing the risk of pathologies.

The Med-Index responds to this need in a complete and effective way and is a real food education tool that increases consumer awareness and encourages producers to improve the product and the process because it breaks the information asymmetry (Table 3) and facilitates in recognizing a premium price for the best product (Roselli *et al.*, 2017).

Conclusions

Citizens would benefit from extension to food nutritional labeling by including environmental and social information in order to drive consumers' choices toward healthier and more sustainable options (European Economic and Social Committee, 2019). The World Health Organization recommends member states to implement nutrition labels on packaging to guide citizens toward healthier food choices, as part of a comprehensive strategy to prevent noncommunicable food-related diseases. Overcoming these goals and integrating the objectives of Green Deal, in particular Farm to Fork and Biodiversity strategies, we have developed a food product labeling system, the Med-Index, which informs consumers not only by providing guidance on whether a food is good or bad for health but also as to whether the dietary choice can be both nutritious and sustainable. The use of the Med-Index, from Farm to Fork, can effectively create the conditions for Mediterranean food chains (farmers, processors, retailers, foodservice, and businesses) to produce, work, distribute, and sell healthier and more sustainable foods. The Med-Index is a positive label that guides consumers to the best choices.

The implementation of the Med-Index can create a constructive dialog with the industry because it is a simple and positive tool for consumers that can inform both on the quality aspects of food products and on the impact of the product production system.

The Med-Index supplements do not replace the Regulation (EU) No 1169/2011 that requires the vast majority of prepacked foods to bear a nutrition declaration providing the energy value and the amounts of fat, saturates, carbohydrate, sugars, protein, and salt of the food, with information presented in a legible tabular format on the packaging. In fact, the Med-Index wants to be inclusive, because it doesn't present monochrome numerical information that many consumers cannot understand and use like information to improve awareness in the choice, particularly those with lower nutrition literacy, education, or members of minority ethnic groups.

Med-Index can be useful to meet the present and future food needs of both citizens and the planet and can help citizens to shift food choices and patterns, stimulate companies to implement existing strategies, and help develop new agricultural production practices that reduce ecological effects and conserve resources while continuing to meet food and nutritional needs, thereby reducing food waste (Medina, 2019).

Author Contributions

Maria Lisa Clodoveo conceptualized the study, did the formal analysis, prepared the original draft, and created the graphic illustrations; Maria Lisa, Clodoveo, Elvira Tarsitano, and Filomena Corbo decided on the methodology of the study; Maria Lisa Clodoveo along with Elvira Tarsitano, and Filomena Corbo were involved in reviewing and editing; Supervision was done by Carlo Sabbà and Loreto Gesualdo; Maria Lisa Clodoveo and Filomena Corbo were in charge of project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Effect of addition of Tunisian *Zizyphus lotus* L. Fruits on nutritional and sensory qualities of cookies

Youkabed Zarroug[1]*, Jazia Sriti[2], Dorra Sfayhi[3], Bechir Slimi[3], Wafa Allouch[a], Kamel Zayani[4], Khaoula Hammami[2], Maryam Sowalhia[1] and Mohamed Kharrat[1]

[1]Université de Carthage, Laboratoire des Grandes Cultures (LR16INRAT02), Institut National de Recherche Agronomique de Tunisie (INRAT), Ariana, Tunisie; [2]Laboratoire des Substances Bioactives, Centre de Biotechnologie, Technopole Borj-Cedria, Hammam-Lif, Tunisie; [3]Laboratoire des Nanomatériaux et Systèmes pour les Energies Renouvelables (LANSER), Centre de Recherches et des Technologies de l'Energie Technopole Borj Cedria, Hammam-Lif, Tunisie; [4]Centre National des Recherches en Sciences des Matériaux (CNRSM), Technopôle de Borj-Cédria, Soliman, Tunisie

***Corresponding Author:** Youkabed Zarroug,, Université de Carthage, Laboratoire des Grandes Cultures (LR16INRAT02), Institut National de Recherche Agronomique de Tunisie (INRAT), Ariana, Tunisie. Email: zarrougyoukabed@yahoo.fr

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PAPER

Abstract

Zizyphus lotus, which belongs to Rhamnaceae family, has been widely used to formulate many healthy food products. The aim of this work was to formulate new functional cookies enriched with different amounts of *Zizyphus lotus* powder (ZLP; 15%, 30%, 45% and 100%). The chemical properties of ZLP were also determined. The formulated cookies were evaluated for their physicochemical, textural and sensory characteristics. Results revealed that ZLP contained various bioactive components, fatty acids, and antioxidants. ZLP-added cookies demonstrated higher phytochemical and antioxidant activities than control cookies prepared without ZLP. The activity of ZLP cookies was enhanced with increase of ZLP level. Hardness and fracturability (**brittleness**) of cookies increased with increasing amount of ZLP. Results of Fourier-transform infrared spectroscopy and thermogravimetric analysis also revealed the presence of many bioactive compounds in formulated cookies. All cookie samples were generally accepted, but the panelists indicated a higher preference for cookies containing 15% ZLP.

Keywords: antioxidants; cookies; phytochemical compounds; sensory characteristics; texture; *Zizyphus lotus*.

Introduction

Bakery products such as cookies are widely consumed around the world. This is mainly due to their higher energy, low cost, ready-to-eat nature, availability in different tastes and extended shelf life (Ajila *et al*., 2008). In addition, cookies may represent an excellent model product for enhancement of nutritional value and fortification. The main ingredients used for production of cookies are wheat flour, fat and sugar. To obtain homogeneous dough, water is also added. Nowadays, the enrichment of these cookies with vitamins, minerals, polyphenols and fibers is considered as a good alternative to produce high nutritional value foods to improve human health. To achieve this goal, many fruits and vegetable powders are used for the formulation of cookies such as papaya pulp flour, choke-berry extract, grape marc extract, sour cherry pomace extract and Japanese quince (Antoniewska *et al*., 2019; Molnar *et al*., 2015; Pasqualone *et al*., 2014; Šaponjac *et al*., 2016; Varastegani *et al*., 2015). In addition, many fruits being used are gaining attention because of attractive flavor as well as diverse antioxidant, anticarcinogenic and antimutagenic substances included in them (Antoniewska *et al*., 2019). Besides, the consumption of these products has health benefits against several chronic diseases, including cardiovascular disease and certain types of cancers (Ludwig *et al*., 2018). Genus Zizyphus belonging to Rhamnaceae family is widespread in tropical and subtropical regions: Asia, Africa, North America, South America, Oceania and Europe, with the center of diversity in Asia

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(Richardson *et al*., 2004). Besides, previous studies have described the secondary metabolites and pharmacology of genus Zizyphus. Traditionally, Zizyphus plant has been used in medicines to treat different diseases such as diarrhea, cholera, bronchitis, diabetes, hypertension, inflammation and intestinal spasms (El Maaiden, 2020; Le-Floc'h, 1983). In Tunisia, the commonly known species of Zizyphus shrub is *Zizyphus lotus*, which is named as 'Sedra', and its edible fruit is called 'Nbeg' (Mkadmini *et al*., 2015). Various studies have described the nutritional and beneficial effects of *Zizyphus lotus* fruits (Yamada *et al*., 1985). This small and round fruits are a rich source of sugar (Ghedira, 2013), minerals (calcium, magnesium, iron, sodium, potassium and phosphorus), carbohydrates, fatty acids and proteins (Abdeddaim *et al*., 2014). Authors have also highlighted the presence of large amounts of vitamins A, C and D (Chouaibi *et al*., 2011; Ghedira, 2013). Therefore, Owing to the presence of high healthy functional components, such as polyphenols, fibers, bioactive compounds, including micronutrients and phytochemicals (Da Silva *et al*., 2007), *Zizyphus lotus* fruits are widely consumed in many countries and used to formulate many food products such as pastes, purees, syrups and confections. Moreover, *Zizyphus* fruits are usually used as food additive in many bakery products to improve their technological quality and nutritional value. In this context, the aim of the present work was to characterize *Zizyphus lotus* fruit powder and to evaluate its effect on physicochemical properties and sensory evaluation of cookies.

Materials and methods

Preparation of *Zizyphus lotus* powder

Zizyphus lotus fruits were collected in July 2020 from Goubollat region of Beja in the northwest of Tunisia. The edible part of the fruits were manually isolated from seeds, sun-dried for 120 h, and milled. The obtained samples were then sieved through a 75-µm sieve to get fine powder of *Zizyphus lotus* fruits (ZLP) and finally stored in plastic containers at 4°C until further use.

Chemical composition

Moisture, protein, fat and ash contents of ZLP and cookie samples were determined according to the method described by Association of Official Analytical Chemists (AOAC, 2005). Total carbohydrate content was calculated by difference (100 – sum of protein, fat, ash and moisture). The results were expressed as g per 100 g of dry weight. Energy was calculated as follows:

$$\text{Energy (kcal/100 g)} = 4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g fat}).$$

The water activity (Aw) was determined using Aqua Lab (Meter, AquaLab Series 3TE, USA) at 25°C. Each assay was carried out in triplicate.

Techno-functional properties of ZLP

Water absorption capacity (WAC) was determined according to the method described by Anderson *et al*. (1970). In a pre-weighed centrifuge tube, 1-g sample was mixed with 10-mL distilled water. The tube was stirred for 2 min and then centrifuged at 3,000 rpm for 15 min. The supernatant was decanted into a tared evaporating dish for determining its solid content and the sediment was weighed. The WAC was calculated as weight of sediment (g) per weight of sample (g) on a dry basis. Oil absorption capacity (OAC) was determined according to the method described by Kaur and Singh. (2005). An amount of 0.5 g of sample was mixed with 6 mL of corn oil in preweighted centrifuge tubes, stirred for 1 min, left for 30 min and centrifuged at 3,000 g for 25 min. The separated oil volume was recorded and the tubes were inverted for 25 min to drain out the oil prior to reweighting. The OAC was expressed as gram of oil bound per gram of sample on a dry basis. Swelling index (SI) was determined by the procedure described by Rosell *et al*. (2009). An amount of 1 g of sample was transferred into a graduated cylinder, it was gently leveled and the volume was noted. Then 10 mL of distilled water was added to the sample; the cylinder was swirled and left to stand for 60 min while the swelling (change in volume) was recorded every 15 min. The swelling index of samples was determined as a multiple of original volume.

Fatty acid extraction and analysis

Zizyphus lotus powder (20 g) was extracted in a Soxhlet apparatus with 250 mL of ether of petroleum at 40°C. Between each step, the extract was filtered with a Whatman filter paper (N°4), concentrated under rotary vacuum evaporator (Rotovapor-El, Labortechnik AG, Büchi, Switzerland) at 40 C and conserved at 4°C for analysis

Fatty acid composition of ZLP lipid fraction was determined as fatty acid methyl esters using gas chromatography (Agilent 19091S-433) equipped with a flame ionization detector and a polar phenylmethyl-siloxane capillary column (60 m × 25 mm × 0.25 µm film thickness) as described by Zarroug *et al*. (2015).

Formulation of cookies enriched with ZLP

Cookies were formulated according to the method described of Tyagi *et al*. (2020) with minor modification.

The ingredients used for preparing cookies are as follows: wheat flour, ZLP, 25-g powdered sugar, 26-g margarine, 0.6-g baking powder, 12-mL water, 0.7-g salt and 18-g whole eggs. Control cookie (CC) samples were prepared with wheat flour (100%) and other ingredients whereas the enriched cookie samples were formulated using combinations of wheat flour and ZLP. The ZLP was incorporated into cookies at the following five levels: 0% (CC), 15% (C15), 30% (C30), 45% (C45) and 100% (C100) by replacing the equivalent amount of wheat flour from the formulation. For preparation of cookies, the process consisted of mixing wheat flour (or ZLP), margarine and sugar using a mixer (Kenwood, Poland) for 7 min. Then whole eggs and required amount of water were added to this mixture with the rest of ingredients to obtain homogeneous dough. Prepared dough was rolled out to 2-mm thick circular shapes and biscuit dough was cut with a circular cutter to obtain pieces of 5-cm diameter. Finally, cookies were baked in oven (Convection, Type De Dietrich) at 175°C for 15 min. The baked cookies were cooled for 30 min at ambient temperature and stored in air-tight bags for further analysis.

Physical, color and textural analysis of cookies

Physical parameters, including weight (g), thickness (T), diameter (D) and spread ratio (D/T) were determined for cookie samples according to the procedure described by Hussai *et al*. (2006). Color measurement of the surface of the studied cookie samples was determined by a Chromameter (Konika Minolta, Sensing INC, Japan) using the hunter L^*, a^* and b^*. The L^* values measure black (0) to white (100), a^* values measure redness (+100) and greenness (-100), and b^* values measure yellowness (+100) and blueness (-100). The hardness of formulated baked cookies was studied by three-point bending tests at room temperature. A texture analyzer TAXT2i (Perten, TVT 6700, UK) was used with 1,000-N load cell and a sharp blade cutting probe (HDP/BSK blade set with knife). The distance between support bars was 4 cm and the probe travelling speed was set at 1 mm/s. Texture analysis were carried out in triplicate for each cookie formulation.

Thermogravimetric analysis (TGA)

Thermogravimetric analysis of cookie samples was carried out in TGA-4000 Perkin Elmer (CURIE GRANT) and analyzed by the Pyris Manager software. The analysis was carried out by standard protocol. Weight loss (weight%) was observed with respect to temperature, and graphs were obtained for each sample with the Pyris software.

Fourier-transform infrared spectroscopy (FTIR)

Functional groups present in cookie samples were characterized by the Perkin Elmer FTIR instrument equipped with the software Perkin Elmer Spectrum Version 10.4.3. The samples were grinded with potassium bromide pellets and measured at a wavelength range of 400–4,000 cm^{-1}.

Scanning Electron Microscopy (SEM)

Microstructure of baked cookies was evaluated using SEM (JEOL/JSM-5400) as described by Adebiyi *et al*. (2016). Cookies were milled, sieved and then observed using two magnification levels of ×400 and ×1,500.

Phytochemical Analysis and Antioxidant Activities of ZLP and Formulated Cookies

Extract preparation

Zizyphus lotus powder was extracted by maceration using the following solvents: acetone (60%), ethanol (60%), methanol (60%) and water. This extraction was done according to the method described by Mau *et al*. (2001). Triplicate samples of 2.5 g of dry matter were extracted by mixing with 25 mL of solvent. The mixture was stirred for 30 min and kept in darkness for 24 h at 4°C. Finally, this mixture was filtered with a Whatman filter paper (N°4) and concentrated under rotary vacuum evaporator at 40°C. The dry residues were stored at 4°C for further analysis. Each extraction was done in triplicate. For cookie samples, extracts were prepared using the method described by Blanco Canalis *et al*. (2020). Cookies were milled and defatted in 30-mL hexane at 70°C for 20 min. Cookie sample, 100 mg, was extracted with 1 mL of acetone:water (70:30 v/v) mixture, and agitation in vortex was applied for 5 min at room temperature. Then the extracts were centrifuged for 10 min at 800× g and supernatants were collected. The supernatants were filtered and stored for further analysis.

Determination of total polyphenols (TPC), total flavonoids (TFC) and condensed tannins content (CTC)

Content of TPC was determined using the Folin–Ciocalteu spectrophotometric method (UV–VIS) as described by Dewanto *et al*. (2002). TPC was expressed as milligram of gallic acid equivalent per gram of dry matter (mg EAG/g DW) through the calibration curve with gallic acid. For determining TFC, 250 µL of methanolic extract was combined with 75-µL $NaNO_2$ (5%) (Dewanto *et al*., 2002). TFC levels were expressed in

milligram of quercetin equivalent per gram of dry matter (mg EC/g DW). The protocol followed in the extraction of CTC was that recommended by Sun *et al*. (1998). CTC was expressed in milligram of catechin equivalent per gram of extract (mg CE/g DW).

Antiradical activity

Methanol extract, 1,000 µL, was added to 500 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.2 mM; Hatano *et al*., 1988). After vigorous stirring, the mixture was kept at room temperature for 30 min in the dark and the absorbance was measured at 517 nm. The antiradical activity was calculated as percentage of inhibition (PI) of DPPH using the following formula:

$$\text{Percentage of inhibition}(\text{PI}) = \frac{\text{Do}_{\text{control}} - \text{Do}_{\text{extract}}}{\text{Do}_{\text{control}}},$$

where $DO_{control}$ is the absorbance of control at 30 min and $DO_{extract}$ is the absorbance of extract. The antiradical activity was finally expressed as IC_{50} (µg mL^{-1}). A lower IC_{50} value corresponds to a higher antioxidant activity of the extracted sample (Patro *et al*., 2005). All samples were analyzed in three replications.

Reducing power

This method consists of mixing 1,000 µL of fraction at different concentrations with 1,250 µL of phosphate buffer (0.2 mol/L, pH 6.6) and 1,250 µL of $K_3Fe(CN)_6$ (1%) (Ferreira *et al*., 2007). The solution was then kept in water bath for 20 min at 50°C. To stop reaction, 1,250 µL of trichloroacetic acid (TCA) (10%) was added to the solution followed by centrifugation at 650 g for 10 min at 25°C. Finally, at the intake of 1,250 µL of supernatant, 1,250 µL of H_2O and 250 µL of $FeCl_3$ (0.1%) were added to the solution and the absorbance was measured at 700 nm.

Sensory analysis of cookies

Sensory analysis of cookies was conducted by 15 semi-trained panelists (research students and laboratory staff) from the Field Crops Laboratory, INRAT, Tunisia. They were asked to evaluate cookie samples for flavor, color, texture, after taste and overall acceptability. After cooling, the cookies were coded and served to panelists in plastic containers with mineral water to cleanse the palate between each tasted sample. The scores were based on the following criteria: 9 = liked extremely; 8 = liked very much; 7 = liked moderately; 6 = liked slightly; 5 = neither liked nor disliked; 4 = disliked slightly; 3 = disliked moderately; 2 = disliked very much; 1 = disliked extremely. Average of the scores was calculated and rounded off to the nearest whole number. Sensory analysis of cookie samples was carried out in triplicate for each sample.

Statistical analysis

Each analytical determination was performed at least in triplicate. Values of different parameters were expressed as mean ± standard deviation ($\bar{X} \pm SD$). Statistical analyses were performed with the STATISTICA software. The Duncan's test was used to evaluate the significance of differences between mean values at $p < 0.05$.

Results and Discussion

ZLP characterization

Chemical composition and techno-functional properties of ZLP

Results of chemical composition and techno-functional properties of ZLP are presented in Table 1.

The ZLP had a moisture content of 9.55%, which was lower than those reported for *Z*L fruits from Algeria (Saadoudi *et al*., 2017), but was within the recommended moisture contents (<14%) for safe storage, minimal microbial growth and chemical deterioration, leading to longer shelf life. Protein, fat and ash contents of ZLP were 2.64%, 5.37% and 3.01%, respectively. Fat and ash contents in Tunisian ZLP were higher than those found by Choi *et al*. (2016) for Korean jujubes. However, the protein content in ZLP was higher than those found by Ghalem *et al*. (2014) (2.10%) and Saadoudi *et al*. (2017) (1.43%) in *Zizyphus lotus* from Algeria. The carbohydrates content in Tunisian ZLP, about 79.43%, was lower than those found by Li *et al*. (2007) in five Chinese *Zizyphus lotus* cultivars (ranged from 80.86% to 85.63%). The observed difference in chemical composition of ZLP depended on the location and the used species. In comparison to other fruit powders, the protein and ash contents in ZLP were lower than those found in the *Tinospora cordifolia* stem powder (proteins: 5.21% and ash: 6.26%) by Tyagi *et al*. (2020).

Referring to the techno-functional properties of ZLP, the values of WAC, SI and OAC in ZLP were about 2.81 g/g, 5.5 mg/g and 2.96 g/g, respectively. The obtained WAC (about 1.32 g/g) and OAC (about 1.20 g/g) values were higher than those detected in pearl millet flour (Adebiyi *et al*., 2016). The WAC of ZLP was lower than those found in some cereals, such as in rice bran (around 5.21 g/g) (Sangnark and Noomhorm, 2004) and fruit coproducts such as passion fruit (13.5 g/g) and pineapple

Table 1. Chemical composition and techno-functional properties of ZLP.

Parameters	ZLP
Moisture (%)	9.55 ± 0.07
Fat (%)	5.37 ± 0.42
Protein (%)	2.64 ± 0.07
Ash (%)	3.01 ± 0.02
Carbohydrates (%)	79.43 ± 1.6
Energy (kcal/100 g)	388.65 ± 2.92
SI (mg/g)	5.5 ± 0.02
WAC (g/g)	2.81 ± 0.07
OAC (g/g)	2.96 ± 0.22
Fatty acids (% total fatty acid)	
Palmitic acid (C16:0)	13.12 ± 0.41
Stearic acid (C18:0)	4.72 ± 0.22
Arachidic acid (C20:0)	1.00 ± 0.01
Behenic acid (C22:0)	0.92 ± 0.06
Tetracosanoic acid (C24:0)	0.60 ± 0.02
Oleic acid (C18:1)	55.73 ± 0.85
10-Octadecenoic acid methyl ester (C19:1)	0.96 ± 0.05
Gadoleic acid (C20:1)	2.64 ± 0.01
Linoleic acid (C18:2)	20.31 ± 0.24
∑ Saturated fatty acids (%)	20.36
∑ Monousaturated fatty acids (%)	59.33
∑ Polyunsaturated fatty acids (%)	20.31
Ratio: Unsaturated:saturated fatty acids	3.91

Values are expressed as mean ± SD of three determinations.
SI: swelling index; WAC: water absorption capacity; OAC: oil absorption capacity.

(14.6 g/g) (Martínez *et al*., 2012). As noted, the hydration properties of ZLP were related to the chemical structure of polysaccharides, proteins and the fruit source. However, OAC is the capability of vegetable apolar chain protein to physically bind lipids by capillary attraction. This interaction is essential in food applications, because oil is known as retainer of flavor and enhances mouthfeel of food-formulated products. In addition, a better SI improved functionality of flour, which would ultimately yield a good product (Adebiyi *et al*., 2016).

Composition of fatty acids

In ZLP, saturated fatty acids represented 20.36% of total fatty acids, while monounsaturated fatty acids accounted for 79.64%. Nine fatty acids were identified, where oleic acid was the major one accounting for 55.73%, followed by linoleic acid with 20.31% of total fatty acids (Table 1). Since ZL oil was rich in both oleic and linoleic acids, it might be considered healthier for human consumption. It has long been recognized that plant oils containing relatively low concentrations of omega-6 and higher levels of monounsaturated fatty acids (mainly oleic acid) may contribute to the lower rate of coronary heart disease (CHD) and a nutritional perspective (Ryan *et al*., 2007). Other representative fatty acids were palmitic (13.12%), stearic (4.72%) and gadoleic (2.64%) acids. In addition, arachidic, behenic, tetracosanoic and 10-octadecenoic acid methyl ester acids were minor fatty acids with contents varying from 0.60% to 1%. These results are similar to those reported by Chouaibi *et al*. (2011) for ZL seed oil.

Phytochemical composition and antioxidant activities of ZLP

Total polyphenols, TFC and CTC of different extracts are summarized in Figure 1. The highest contents of TPC, TFC and CTC were obtained in the acetone (60%) extract with values of 3.12 mg GAE/g DW, 0.49 mg CE/g DW and 1.09 mg CE/g DW, respectively. The lowest contents were recorded in the methanol fraction. According to Wojdyło *et al*. (2016), ZL fruit is considered as a rich source of bioactive components, including polyphenols, triterpenic acids, polysaccharides, nucleosides and nucleobases. In general, phenolic compounds in ZL fruit contribute to the antioxidant potential of its powder extracts. Authors have reported correlation between phenolic compounds and antioxidant activity (Elfalleh *et al*., 2011). Antioxidant activity of different extracts of ZLP was examined by its radical scavenging activity using the stable radical DPPH and by reducing powers (Table 2). Methanol (60%) and acetone (60%) fractions exhibited the uppermost antiradical capacities with IC_{50} reaching 70 µg/mL and 75 µg/mL, respectively. The obtained results are lower than those found in Moroccan ZL (IC_{50} = 477.6 µg/mL) using methanol solvent (70%) (Bakhtaoui *et al*., 2014). Previous study conducted on ZL fruit from another region of Tunisia revealed IC_{50} = 289 µg/mL (Mkadmini *et al*., 2015) and 310 µg/mL (Ghazghazi *et al*., 2014). The *Zizyphus mauritiana* fruits from Nigeria exhibited IC_{50} = 338.45 µg/mL (Okala *et al*., 2014), which was higher than the value obtained in the present study. The consequences of solvent on the antioxidant ability were also analyzed by determining reducing powers (Figure 1). The highest antioxidant potential was observed in acetone fractions (330 µg/mL). The obtained result was similar to that found in southern Tunisian ZL fruits (289 µg/mL) (Mkadmini *et al*., 2015). The observed variability of phytochemical contents and antioxidant activities of *Zizyphus* genus could be explained by various factors, such as the geographical provenance, grown conditions, types of species and the nature of solvent used for extraction (Ksouri *et al*., 2008).

Cookies characterization

Nutritional composition of formulated cookies

Nutritional composition of enriched cookies with ZLP is presented in Table 2. Results established that the addition

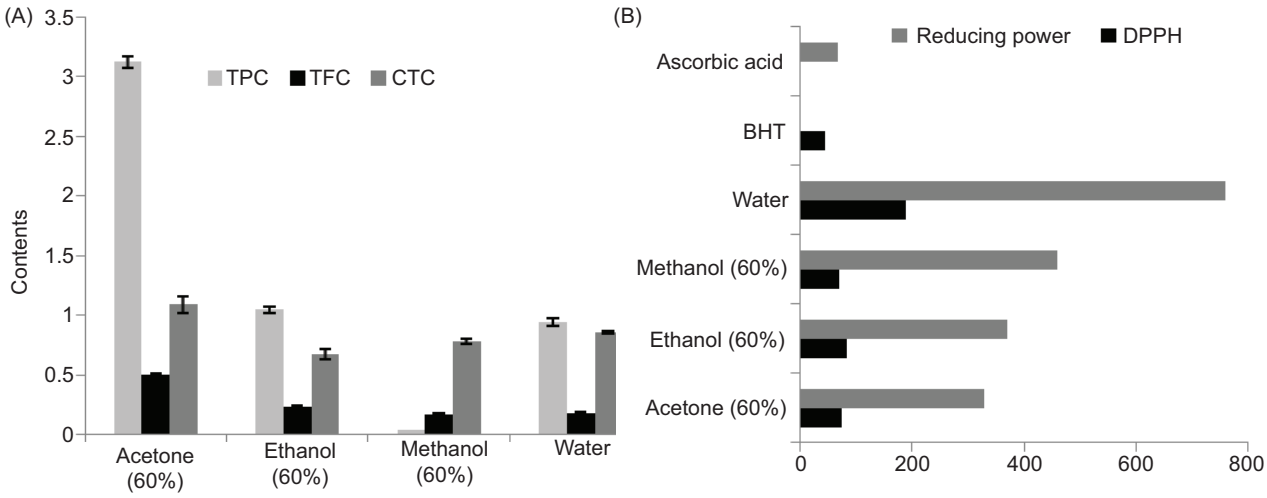

Figure 1. (A) TPC, TFC and CTC of different solvent extracts, and (B) antioxidant activities of ZLP. TPC: total polyphenols content (mg GAE/g MS); TFC: total flavonoids content (mg CE/g MS); CTC: condensed tannins content (mg CE/g MS); DPPH (IC_{50}, µg/mL); reducing power (EC_{50}, µg/mL); GAE: gallic acid equivalents; CE: catechin equivalents.

Table 2. Nutritional analysis of formulated cookies.

Cookies	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Carbohydrates (CHO %)	Water activity (Aw)	Energy (kcal/100 g)
CC	5.14 ± 1.20^{e}	17.10 ± 0.01^{a}	7.10 ± 0.03^{e}	0.84 ± 0.02^{a}	69.82 ± 1.22^{a}	0.50 ± 0.01	461.58 ± 4.57^{a}
C15	6.55 ± 0.32^{d}	18.90 ± 0.01^{b}	6.60 ± 0.02^{d}	0.92 ± 0.03^{b}	67.03 ± 0.34^{b}	0.53 ± 0.02	464.62 ± 2.13^{a}
C30	7.29 ± 0.37^{c}	19.50 ± 0.02^{c}	6.35 ± 0.02^{c}	1.10 ± 0.03^{c}	65.76 ± 0.41^{c}	0.55 ± 0.13	463.94 ± 1.33^{a}
C45	7.62 ± 2.48^{b}	19.70 ± 0.01^{d}	6.06 ± 0.02^{b}	1.03 ± 0.01^{d}	65.59 ± 2.48^{c}	0.57 ± 0.01	463.90 ± 9.29^{a}
C100	7.64 ± 0.53^{a}	19.90 ± 0.05^{e}	4.94 ± 0.01^{a}	1.50 ± 0.01^{e}	66.02 ± 0.51^{d}	0.59 ± 0.01	462.94 ± 1.21^{a}

Mean values with different superscript alphabets in the same column differ significantly. CC: control cookies; C15, C30, C45, C100 are cookie samples containing wheat flour:ZLP in the ratio of 85:15, 70:30, 55:45 and 0:100, respectively.

of ZLP significantly increased ($p < 0.05$) the moisture, ash, fat and carbohydrates contents in cookies when compared to control cookies whereas protein values were decreased significantly.

In all formulated cookies, carbohydrates were the major component, followed by fat, protein, water and ash. Results demonstrated statistically significant differences ($p < 0.05$) between formulated cookies, especially for moisture, fat, protein and ash contents. Only for carbohydrates content, a nonsignificant difference was observed. Similar results revealed increase in ash and moisture contents in cookies enriched with spinach (5–15%) (Galla *et al*., 2017) and *Tinospora cordifolia* stem (2–12%) powders (Tyagi *et al*., 2020). In the same context, Bhat *et al*. (2020) reported consistent results in cookies containing tomato powder and crude lycopene. The energy values of enriched cookies ranged from 461.58 kcal in CC sample to 464.62 kcal in C15 sample. These results were in consistency with previous studies carried by Kaur *et al*. (2017) and Saadoudi *et al*. (2017) on cookies enriched with ZLP from Algeria and raw flaxseed flour. About water activity, similar to that observed for moisture content, addition of ZLP in cookies provoked a significant increase in this parameter. The highest water activity value was observed in C100 sample (0.59), but it was still below than that allowed for growth of microorganisms (Aw > 0.6) (Chieh, 2006). These findings were higher than those reported by Milićević *et al*. (2020), who revealed water activity values ranging from 0.297 to 0.411 in cookies prepared with wheat bran gels.

Physical, textural and color characteristics of formulated cookies

According to Pareyt and Delcour (2008), quality of good cookies is related to large piece diameter, tender but snapping final product, and uniform surface-cracking pattern. The physical, textural and color characteristics of formulated cookies are depicted in Table 3. The weight of cookies enriched with ZLP varied from 12.5 g in C15 sample to 13.27 g in C100 sample. After addition of ZLP, the weight of cookies increased significantly ($p < 0.05$) in

Table 3. Physical, textural and color analysis of formulated cookies

Cookies	Diameter (D) (mm)	Thickness (T) (mm)	Weight (g)	Spread ratio (D/T)	L*	a*	b*	Hardness (N)	Fracturability (mm)
CC	57.60 ± 0.24^{a}	5.03 ± 0.02^{a}	13.13 ± 0.21^{b}	2 .60 ± 0.24^{a}	66.72 ± 0.56^{c}	4.17 ± 0.12^{a}	47.83 ± 0.17^{e}	22.33 ± 1.53^{a}	0.58 ± 0.03^{a}
CI5	56.70 ± 0.92^{a}	5.09 ± 0.09^{a}	12.50 ± 0.95^{ab}	1.70 ± 0.92^{a}	46.62 ± 0.02^{d}	10.06 ± 0.07^{b}	36.97 ± 0.014^{d}	40.00 ± 4.58^{b}	0.99 ± 0.06^{ab}
C30	57.10 ± 0.43^{a}	5.50 ± 0.32^{b}	12.70 ± 0.36^{ab}	2.10 ± 0.43^{a}	39.72 ± 0.25^{c}	11.58 ± 0.08^{c}	34.56 ± 0.17^{c}	51.33 ± 1.53^{c}	1.41 ± 0.05^{b}
C45	57.23 ± 0.66^{a}	5.55 ± 0.33^{b}	12.80 ± 0.21^{b}	2.23 ± 0.66^{a}	36.88 ± 0.82^{b}	11.43 ± 0.29^{c}	32.86 ± 0.14^{b}	65.00 ± 2.65^{d}	2.45 ± 0.06^{c}
C100	57.32 ± 0 46^{a}	6.15 ± 0.10^{c}	13.27 ± 0.38^{b}	2.32 ± 0.46^{a}	32.53 ± 0.31^{a}	12.33 ± 0.18^{b}	29.64 ± 0.05^{a}	74.33 ± 2.52^{c}	6.80 ± 0.66^{d}

Mean values with different superscripts on the same column differ significantly (Duncan's LSD test, $p < 0.05$). Where CC: control cookies, C15, C30, C45, C100 are cookies samples containing wheat flour: ZLP (85:15, 70:30, 55:45, 0:100).

comparison to CC sample. This increase in the weight of cookies was related to the presence of dietary fibers in ZLP, which enables to bind with water molecules and prevents loss of moisture during baking process. These results are in the trend of those observed by Saadoudi *et al*. (2017) on biscuits made with Algerian ZLP. In all formulated cookies, the diameter ranged from 56.70 mm to 57.60 mm. Maximum diameter was observed in CC sample, while minimum was in C15 cookie sample. These variations could be due to the dilution of gluten present in wheat flour. Results also demonstrated increase in the thickness of cookies from 5.03 mm (CC sample) to 6.15 mm (C100 sample). The obtained results of cookies enriched with ZLP are in accordance with the studies reported by Kaur *et al*. (2017) and Saadoudi *et al*. (2017) on some fortified cookies. A decrease in the spread ratio of cookies was observed with the inclusion of ZLP; this reduction could be due to the water absorption capacity of fibers present in ZLP. Similar results were obtained by Das Chagas *et al*. (2020), Ismail *et al*. (2014) and Toledo *et al*. (2017) when evaluating the effect of addition of by-products of pomegranate peels, pineapple, apple, melon, and camu-camu in cookies.

In order to evaluate the acceptability of prepared cookies by consumers, it is very important to determine their color parameters. The photographic images (Figure 2) of formulated cookies revealed that ZLP was the main ingredient that developed dark color, and C100 sample was the much darker cookies, looking like biscuits made with chocolate. The statistical analysis revealed a significant difference ($p < 0.05$) between all cookie samples regarding L^* and b^* values. In addition, as observed in Table 4, CC sample had the highest L^* and b^* values as compared to the cookies enriched with ZLP. Moreover, addition of ZLP decreased L^* and b^* values but increased a^* values of formulated cookies. These effects could be explained by the dark color of cookies because of added ZLP, which is related to its richness in natural pigments such as polyphenols (Ajila *et al*., 2008; Takeungwongtrakul and Benjakul. 2017). According to Masmoudi *et al*. (2021), the dark color of cookies could be also due to the more pronounced Maillard and caramelization reactions during the baking process, when wheat flour was substituted by ZLP containing higher content of sugar. These findings are in agreement with those of biscuits supplemented with Doum dietary fiber (Aboshora *et al*., 2019) and bamboo shoot powder (Ajila *et al*., 2008).

Regarding texture analysis, results revealed that the hardness and fracturability (**brittleness**) of cookies were increased significantly ($p < 0.05$) with the addition of ZLP. In all formulated cookies, C100 sample had the highest hardness and fracturability values (74.33 N and 6.8 mm) compared to CC sample (22.33 N and 0.58 mm). Similar results were also observed in previous findings in

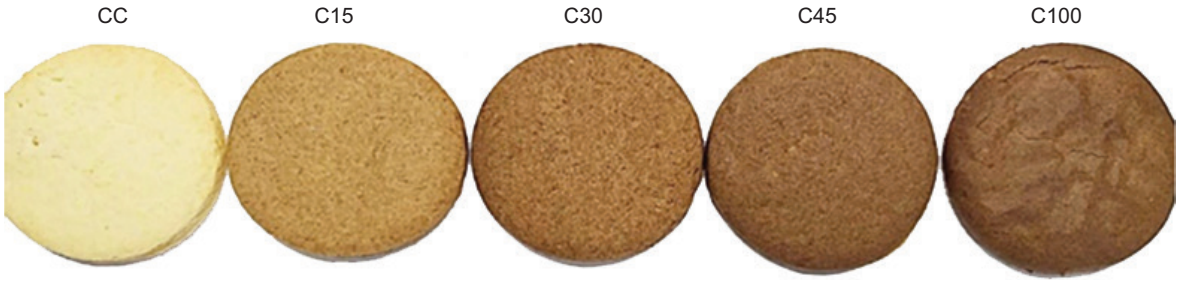

Figure 2. Photographic images of cookie samples. CC: control cookies, C15, C30, C45, C100 are cookie samples containing wheat flour:ZLP in the ratio of 85:15, 70:30, 55:45 and 0:100, respectively.

which cookies were enriched with bamboo shoot, spinach, *Tinospora cordifolia* stem and tomato powder (Ajila *et al*., 2008; Bhat *et al*., 2020; Galla *et al*., 2017; Tyagi *et al*., 2020). This increase in texture parameters are explained by the presence of ZLP, with high content of polysaccharides, which may lead to the dilution of gluten and extensive gluten structure. In addition, substituting ZLP for wheat flour in cookies formulation decreased its gluten content responsible for the viscoelastic character of dough. Das Chagas *et al*. (2020) affirmed in their study on cookies enriched with camu-camu coproduct powder that reduction of glutenin and gliadin proteins could have a remarkable effect on the formation of viscoelastic dough, which may possibly lead to increased hardness.

TPC, TFC and CTC of cookies

Table 4 reveals the TPC, TFC and CTC values of defatted methanolic extract of formulated cookie samples. Results revealed that the addition of ZLP increased significantly ($p < 0.05$) TPC, TFC and CTC in enriched cookie samples when compared to CC sample, and this increase was effectively due to the high content of phenolic compounds such as protocatechuic acid, gallic acid, chlorogenic acid, caffeic acid and ascorbic acid present in ZLP (Koley *et al*., 2016). In all formulated cookies, C100 sample contained the highest contents of TPC (0.11 mg GAE/g), TFC (0.05 mg QE/g) and CTC (1.07 mg QE/g). Our results were generally in agreement with the findings of other studies regarding the substitution of wheat flour in cookies by some fruit and vegetable powders such as apple, pineapple and melon coproducts (Toledo *et al*., 2017), watermelon rind powder (Naknaen *et al*., 2016) and camu-camu coproduct (Das Chagas *et al*., 2020).

Antioxidant potential of cookies

Table 4 indicates that the addition of ZLP in cookies significantly ($p < 0.05$) increased the free radical scavenging activity and reducing power as compared to CC sample. This implies that the addition of higher level of ZLP improved the antioxidant activity of cookie products. In all formulated cookies, the highest free radical scavenging activity and reducing power were observed in C100 sample. This observation was in accordance with previous studies carried out with incorporation of

Table 4. Phytochemical analysis and antioxidant activities of formulated cookies.

Cookies	Phytochemical parameters			Antioxidant activities	
	TPC[a]	TFC[b]	CTC[b]	DPPH[c]	Reducing power[d]
CC	<0.01	0.01 ± 0.01[a]	0.57 ± 0.14[e]	454 ± 0.54[e]	950 ± 3.42[e]
C15	0.01 ± 0.02[a]	0.02 ± 0.01[b]	0.80 ± 0.15[d]	355 ± 0.21[a]	820 ± 2.45[d]
C30	0.02 ± 0.01[b]	0.03 ± 0.02[c]	0.93 ± 0.1[b]	280 ± 0.15[b]	410 ± 1.34[c]
C45	0.05 ± 0.01[c]	0.04 ± 0.01[d]	1 ± 0.05[a]	165 ± 0.1[c]	380 ± 3.5[b]
C100	0.11 ± 0.04[d]	0.05 ± 0.01[e]	1.07 ± 0.01[c]	95 ± 0.24[d]	320 ± 2.45[a]
Synthetic standards					
BHT (IC_{50}, mg/mL)	–	–	–	46.6 ± 0.08	-
Ascorbic acid (EC_{50}, mg/mL)	–	–	–	–	68 ± 0.06

[a](mg GAE/g), [b](mg QE/g), [c](IC_{50}, µg/mL), [d](EC_{50}, µg/mL).
BHT: Butylated hydroxytoluene; TPC: total polyphenols content ; TFC: total flavonoids content ; CTC: Condensed tannins content; CC: control cookies; C15, C30, C45, and C100: cookie samples containing the wheat flour:ZLP in the ratio of 85:15, 70:30, 55:45, 0:100, respectively.BHT: butylated hydroxytoluene. an values with different superscripts in the same column differ significantly (Duncan's LSD test, $p < 0.05$).

freeze-dried Japanese quince fruits (Antoniewska *et al.*, 2019) and tomato powder in cookies (Bhat *et al.*, 2020). Several authors also demonstrated that the DPPH radical scavenging activity and reducing power increased in cookies with baking temperature, which could be due to the formation of melanoidins produced in the Maillard reaction (Bhat *et al.*, 2020). In addition, high concentration of bioactive compounds in ZLP lead to an increase in the antioxidant potential of formulated cookies. This could be of interest for human health and could provide an extended shelf life of cookies for food industry.

FTIR analysis

The FTIR spectra of cookie samples in the range of 400–4,000 cm^{-1} are revealed in Figure 3. Results revealed that cookie samples demonstrated common peaks with small variations in intensity. In all cookie samples, major peaks were observed at 3,500 cm^{-1} and 2,800 cm^{-1}, which indicated the stretch vibrations of O–H groups. In addition, the observed peak at 2,943 cm^{-1} resulted from the stretching vibration of C–H bond. However, the peaks found between 1,645 cm^{-1} and 1,742 cm^{-1} were attributed to the C–O stretch vibration of α,β-unsaturated compound. Similar results were reported by Tyagi *et al.* (2020) on cookies enriched with *Tinospora cordifolia* stem powder. The observed peaks at 1,485.71 cm^{-1} were essentially assigned to water molecules absorbed in the amorphous region. The established peaks at a range of 1,142–1,000 cm^{-1} could be attributed to the C–O bond and aliphatic C–N stretching; these peaks were with higher intensities in cookie samples enriched with ZLP compared to CC sample. These peaks are related to the presence of phenolic compounds, β-glycoside and glucoside (Tyagi *et al.*, 2020), which were essentially present in ZLP. Besides, decrease in the intensity of peaks in cookie samples reflects their change in crystallinity.

Thermogravimetric analysis

In general, when materials are heated, they lose weight because of simple processes, such as drying, or because of chemical reactions that release gasses (Blanco Canalis *et al.*, 2018). The observed weight loss, which was related to reduction of water by evaporation can provide valuable information about the volatile and heat labile compounds present in cookie samples and their thermal stability. The TGA and differential TGA (Dr-TGA) curves of cookie samples are revealed in Figure 4(a) and Figure 4(b). The TGA of all studied cookie samples demonstrated four typical stages of weight loss. Figure 4(b) indicates that no significant differences in shape and intensity were observed between C45 and C100 samples. Comparison between the cookies enriched with ZLP and control samples indicated that the maximum rate of weight loss was in C45 sample (92.69%) followed by C100 (91.72%). The first stage of weight loss ranged from 190°C to 211°C, and was attributed to the loss of free and bound water with increasing temperature. It is suggested that inclusion of ZLP in cookies reduced the amount of water available for protein hydration, allowing faster water evaporation. In addition, the observed increase in weight loss suggested the formation of a weak gluten network in cookies dough. According to Blanco Canalis *et al.* (2018), water in cookies dough is shared between different components (such as starch, gluten and sucrose) responsible for trapping water until it is released because of heating. The second, third and fourth stages ranged from 229.54°C to 254.38°C, 311.85°C to 373.63°C and 426.29°C to 470.3°C, respectively. The second stage represented

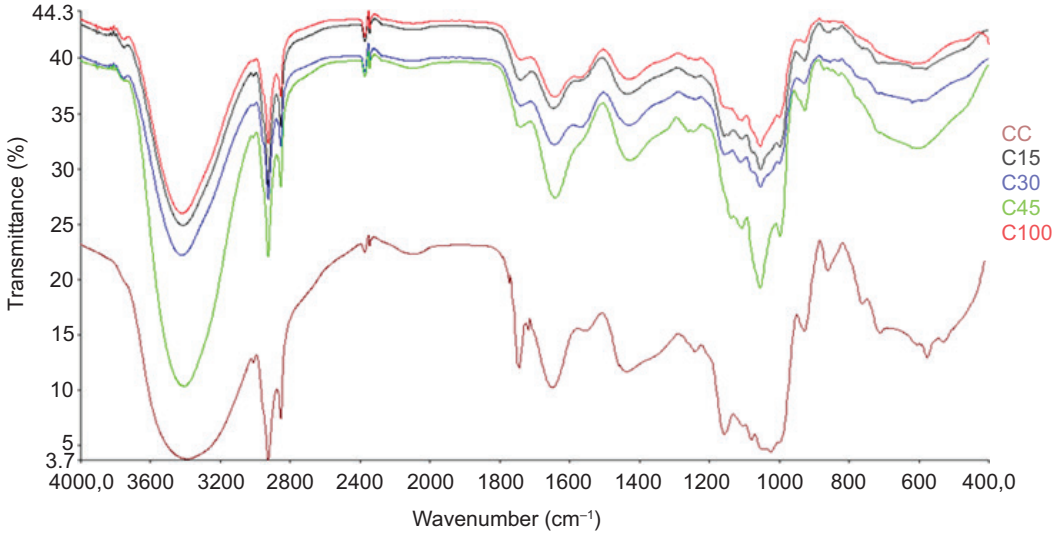

Figure 3. FTIR spectra of cookie samples; CC: control cookies; C15, C30, C45 and C100 are cookie samples containing wheat flour:ZLP in the ratio of 85:15, 70:30, 55:45 and 0:100, respectively.

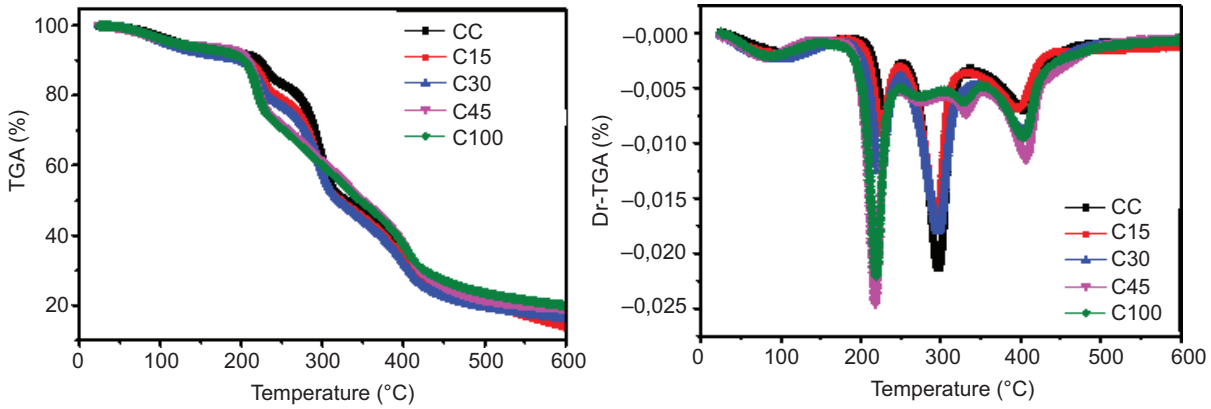

Figure 4. (A) Thermogravimetric, and (B) differential thermogravimetric curves of cookie samples; CC: control cookies; C15, C30, C45 and C100 are cookie samples containing wheat flour:ZLP in the ratio of 85:15, 70:30, 55:45, 0:100, respectively.

the depolymerization and degradation of organic matter (starch, gluten and polysaccharides). However, the third stage of weight loss represented the oxidation of organic matter that lead to the formation of ash residue. In the last range of temperature (close to 500°C), the total weight loss measured for cookie samples was not noticeably different.

Microstructure of cookie samples

Scanning electron microscopy was used to study the microstructure of baked cookie samples. The microstructure observation indicated that the protein network matrix was well developed and the starch granules were more intact in the case of control cookies (Figure 5). However, a change in the structure of cookies was observed with increase in ZLP content. This change was strongly observed in the case of C100 sample. Alteration in the microstructure of cookies could be due to the richness of ZLP in polysaccharides, which have good water retention capacity; this could also be due to the gelatinization of starch and denaturation of protein matrix. These results are in agreement with those found by Adebiyi *et al.* (2016), who reported a similar microstructure of cookies enriched with millet flours.

Sensory evaluation of cookies

The flavor, color, texture, after-taste and overall acceptability scores recorded for the cookies enriched with ZL powder are depicted in Table 4. Results of sensory evaluation revealed that color, flavor and after-taste parameters increased with increase of ZLP in cookies, reaching the values of 7.87, 8.89 and 3.20, respectively, noted essentially in C100 sample. The sensory evaluation of cookies' color surface paralleled the colorimetric measurement (Table 3), with ZLP-enriched cookies being darker than the control. In addition, increase in ZLP concentration demonstrated a decrease in texture parameter in comparison to control cookies; these results were in agreement with the instrumental data of hardness and fracturability

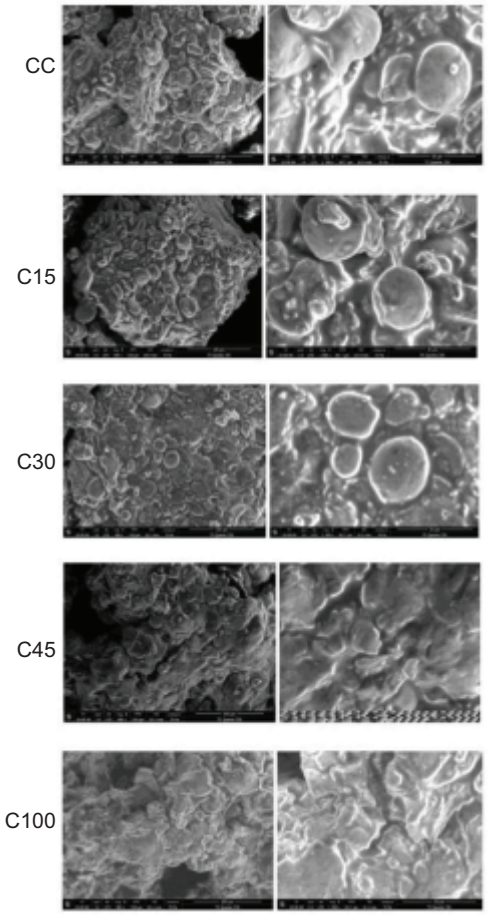

Figure 5. SEM of cookies samples. Magnification ×400 and ×500; CC: control cookies. C15, C30, C45, C100 are cookies samples containing wheat flour: ZLP (85:15, 70:30, 44:45, 0:100).

(Table 3). Similar effect was reported by Krystyjan *et al.* (2015), Saadoudi *et al.* (2017) and Šaponjac *et al.* (2016) with cookies enriched with bee pollen, jujube, and sour cherry pomace extract, respectively. In all enriched cookies, the C15 sample had a sensory score near to the CC

Table 5. Sensory acceptability of both control and formulated cookies.

Cookies	Color	Flavor	After-taste	Texture	Overall acceptability
CC	4.27 ± 1.12[a]	6.54 ± 0.71[c]	8.47 ± 1.30[a]	6.60 ± 1.60[b]	7.20 ± 2.04[a]
C15	5.20 ± 1.00[b]	7.33 ± 0.63[c]	7.93 ± 0.96[a]	5.66 ± 1.13[a,b]	6.47 ± 1.30[b]
C30	5.60 ± 0.64[b]	7.41 ± 1.03[c]	7.33 ± 1.05[a]	5.06 ± 1.03[c]	5.00 ± 1.31[a,b]
C45	6.67 ± 0.63[c]	8.27 ± 0.52[d]	5.36 ± 1.39[b]	4.87 ± 1.44[a]	4.68 ± 1.25[a]
C100	7.87 ± 0.63[c]	8.89 ± 0.41[d]	3.20 ± 2.01[b]	4.07 ± 1.74[a]	4.20 ± 2.61[a]

Values are expressed as mean ± standard deviation of three determinations. Mean values with different superscripts in the same column differ significantly ($p < 0.05$). CC: control cookies; C15, C30, C45 and C100 are cookie samples containing wheat flour:ZLP in the ratio of 85:15, 70:30, 55:45, 0:100, respectively.

sample. For the overall acceptability of results, CC and C15 samples were most acceptable, while C100 sample was not preferred by panelists. Masmoudi *et al*. (2021) established that biscuits supplemented with jujube flour had as acceptable quality as the control for all its doses (5%, 10% and 15%). Reduction in acceptability of cookies at higher level of substitution with ZLP was due to more plant undertones contributed by ZLP. Similar effect was reported for cookies supplemented with flaxseed flour (Kaur *et al*., 2017) and Japanese quince fruit (Antoniewska *et al*., 2019).

Conclusion

The results of the present study reveal that ZLP contains several bioactive components, fatty acids, phenolics and antioxidants that are beneficial for human health. Results demonstrate that this powder has a great nutritional potential to be used in food industry and to substitute the conventional wheat flour in the formulation of new functional cookies. Addition of ZLP improves the phytochemical composition and the antioxidant potential of formulated cookies as revealed by the increase in DPPH and reducing power values in cookies. The FTIR and TGA results revealed the presence of many bioactive compounds in ZLP and formulated cookies. Besides improving the nutritional value, addition of ZLP affects the appearance, physicochemical, textural and sensorial properties of formulated cookies. Based on the sensory analysis, it is concluded that the acceptability of formulated cookies is directly dependent on the amount of added ZLP, and cookies enriched with 15% ZLP (C15) seems to have gathered the overall preference of panelists.

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Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the research reported in this paper.

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Effect of different plants' aromatic essential oils on frozen Awassi lamb meat's chemical and physical characteristics

Ahmed S.A. Al-Obaidi[1], Ayad B. Mahmood[2], Zaid K. Khidhir[2,*], Hemn G. Zahir[2], Ziyad T. Al-doori[3], Hiewa Othman Dyary[4,*]

[1]Department of Animal Production, College of Agriculture, University of Diyala, Iraq; [2]Department of Animal Sciences, College of Agricultural Engineering Sciences, University of Sulaimani, Iraq; [3]Department of Public Health, College of Veterinary Medicine, University of Tikrit, Iraq; [4]Department of Basic Sciences, College of Veterinary Medicine, University of Sulaimani, Sulaymaniyah, Iraq

***Corresponding Authors**: Zaid K. Khidhir, Department of Animal Sciences, College of Agricultural Engineering Sciences, University of Sulaimani, Iraq. Email: Zaid.khzir@univsul.edu.iq; Hiewa Othman Dyary, Department of Basic Sciences, College of Veterinary Medicine, University of Sulaimani, Sulaymaniyah, Iraq. Email: dyary.othman@univsul.edu.iq

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PAPER

Abstract

The effect of drenching Awassi lambs with three aromatic essential oils from sage (*Salvia officinalis L.*), clove (*Syzygium aromaticum L.*), and laurel (*Laurus nobilis L.*) was investigated on meat chemical and physical characteristics, and oxidative and deterioration measurements. Twenty-four Awassi lambs, five to six months old, were divided into four groups. A concentrated diet was provided to the lambs at a rate of 3% of the body weight. The treatments were as follows: T1 was served as the untreated control, while T2, T3, and T4 were drenched with oils of sage, clove, and laurel, respectively. Drenching was carried out using water-soluble capsules containing 500 mg oil/capsule/day. Treatments lasted 90 days. At the end of the treatment period, the animals were fasted overnight and slaughtered. The carcasses were cleaned and kept at 4°C for 24 h. The longissimus dorsi (LD) muscle was then separated and preserved in a plastic bag for three preservation periods: no freezing and 30 days and 60 days freezing at –18°C. Several physical, fat, and protein stability analyses of meat were done after the preservation periods. The results indicated no significant effect of drenching Awassi lambs with different aromatic essential oils on the meat's physical and chemical characteristics. However, these oils, especially clove oil, affected fat and protein stability with increasing preservation period by freezing.

Keywords: Awassi; essential oil; *Laurus nobilis L.*; meat; *Salvia officinalis L.*; *Syzygium aromaticum L.*

Introduction

Meat is classified as a perishable commodity due to its high moisture content and nutrient availability, making it suitable for microbial growth (Kumar *et al.*, 2015, 2017). Meat contamination might occur at different stages, starting from the field and ending with the preparation for consumption, including slaughter, transportation, handling, storage, processing, marketing, and consumer's handling (Niyonzima *et al.*, 2015). Treatments used to keep meat from contamination and spoilage vary with each stage, including heating, refrigeration, hydrostatic pressure, packaging, ionizing radiation, chemical preservatives, salts, and bioactive compounds. Selecting appropriate treatments to maintain the meat and meat products' hygiene depends on several factors (Chen *et al.*, 2012).

Fats' oxidation and destruction by free radicals in cell membranes are natural processes affecting membrane

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transport and functions. Cell membrane phospholipids are rapidly affected by the oxidation process closely related to the fatty acids' saturation level. Free radicals react with these fatty acids and produce hydroperoxides that decompose to volatile aromatic compounds such as alkanes and aldehydes. These toxic substances affect animal products' nutritional value (Aminzare *et al.*, 2019), safety for consumption, quality of meat, organoleptic characteristics, and storage period (Fernandes *et al.*, 2018). Controlling free radicals' biological damage has recently become a topic of interest to researchers. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used commercially to prevent or reduce lipid peroxidation's unwanted effects. However, the demand to find or use plant-based natural compounds has recently increased, with consumers trying to protect meat and meat products from oxidation, spoilage, and pathogens (Aminzare *et al.*, 2019; Veneziani *et al.*, 2017). Natural antioxidants play an essential role in this field, protecting food from spoilage and maintaining public health. Essential oils are natural sources rich in antioxidants that inhibit free radicals such as phenolic compounds. These oils preserve animal fatty tissue and protect animal products from off-flavor and reactive oxygen resulting from oxidation of polyunsaturated fatty acids (Nitiema *et al.*, 2012). Bioactive compounds such as essential oils improve food quality and protect consumers from the adverse effects of oxidative stress and microbial spoilage (Simitzis and Deligeorgis, 2011). Essential oils are complex mixtures of many ingredients and are mainly composed of terpenoids with low molecular weight aliphatic hydrocarbons such as aromatic aldehydes and phenols (Dorman and Deans, 2000). They have antimicrobial activities, as many studies have confirmed the antimicrobial effects of these oils when used with different foods, which extends the shelf life by reducing spoilage (Calo *et al.*, 2015). For example, adding 0.25% cassia oil to refrigerated fresh chicken sausages increases the shelf life by 5–6 days and lowers the microbial count (Sharma *et al.*, 2017). In another study, it was found that wrapping chicken meat burgers with edible film incorporated with 0.10% oregano and 0.15% thyme essential oils increased shelf life up to 30 days (Soni *et al.*, 2018).

Feed processing with antioxidants such as essential oils is one of the easiest ways to deliver these antioxidants to membrane phospholipids, reducing the oxidation from free radicals and protecting animal products such as meat from fat oxidation (Fasolato *et al.*, 2015). Therefore, this method is considered the easiest and the most effective compared to the treatment of postmortem meat. There is a lack of sufficient research on using essential oils with Awassi sheep, the most common breed raised in Iraq. Fresh meat or meat refrigerated for 2–3 days is used for cooking. Finding a method to naturally increase the shelf-life without affecting the meat's quality and taste is important. Therefore, this research aimed to investigate the effects of three aromatic essential oils from sage (*Salvia officinalis L.*), clove (*Syzygium aromaticum L.*), and laurel (*Laurus nobilis L.*) on meat chemical and physical characteristics, and oxidative and deterioration measurements after different freezing periods.

Materials and Methods

Extraction of volatile oil

Dried seeds of clove (*Syzygium aromaticum L.*) and green leaves of sage (*Salvia officinalis L.*) and laurel (*Laurus nobilis L.*) were collected from local markets in Sulaymaniyah. The plants' identities were confirmed at the Department of Horticulture, Faculty of Agricultural Sciences, University of Sulaimani, where voucher specimens were deposited. The plants were dried in a freeze dryer and ground in a laboratory grinder. A hot oil extraction technique was used to extract oils. Then, water was added at a ratio of 5:1, and the mixture was subjected to hydro-distillation for 3 h using a Clevenger-type apparatus (Clevenger, 1928). The volatile oil content was calculated as a relative percentage (v/w). Later, the essential oil was extracted from the milled sample by the hydro-distillation method using the Clevenger set in 1000 mL distilled water and refrigerated until use (Ranjitha and Vijiyalakshmi, 2014).

Animals and treatments

Twenty-four Awassi lambs, 5–6 months old, with an average weight of 28.4 kg, were divided according to weight into four treatment groups. The lambs were raised in individual cages and acclimatized for 2 weeks, followed by a treatment period lasting 90 days. During the experimental period, the lambs were fed a concentrated diet consisting of 35% wheat flour, 40% barley, 12% wheat bran, 10% soybean meal, 2.9% salt and limestone, and 0.1% premix. The energy content was 2791 cal/kg, and the protein content was 13.75/kg. Feed was provided to the lambs at 3% of their body weight. The lambs were subjected to weekly weight measurements, and the amount of feed was adjusted according to weight change.

The first group of lambs (T1) served as a control without drenching. Groups T2, T3, and T4 were drenched with sage oil, clove oil, and laurel oil, respectively. The drenching process was carried out using plastic syringes attached to a rubber tube. Each animal was given 0.5 mL of the extracted oil daily.

After the treatment period, the animals were fasted overnight and slaughtered. The carcasses were cleaned and

kept for 24 h at 4°C. Afterward, the longissimus dorsi (LD) muscle was separated, divided into several parts as appropriate, and each part was preserved in a plastic bag. Three preservation periods were used: zero freezing (P1), and 30-day freezing (P2) and 60-day freezing (P3) at –18°C.

Physical measurements

Several physical measurements were recorded on meat samples for each storage period, including pH, water holding capacity (WHC) (Dolatowski and Stasiak, 1998), thaw loss, and cooking loss (Purchas and Barton, 2012).

Chemical measurements

Several chemical measurements were also made on the stored meat samples at the end of each storage period, including meat chemical composition (moisture, protein, ether extract, and ash) (Horwitz and Latimer, 2005), thiobarbituric acid (TBA) (Gheisari *et al.*, 2010), total volatile nitrogen (TVN) (Pearson and Muslemuddin, 1971), and free fatty acids (FFA) (Pearson and Dustson, 1985).

Statistical analysis

SAS statistical analysis program was used to determine the effect of oil drenching and storage period on the studied measurements. Duncan's test was used to analyze the data to determine the effect of oil drenching and storage period on the studied measurements. Probability values of ≤0.05 were considered statistically significant.

Results

There was no significant effect of oil treatments on the LD muscle pH in all preservation periods. In contrast, a significant increase in pH values ($P \leq 0.05$) occurred for most treatments with increasing freezing periods (Table 1). Also, different oils did not affect the LD muscle WHC according to the control for all preservation periods, while a significant decrease in WHC was observed for most treatments with increasing freezing periods.

Values are presented as mean ± SEM. Different lowercase letters indicate significant differences between means within columns, while different uppercase letters indicate significant differences between means within rows ($P \leq 0.05$). WHC, water holding capacity.

No significant effects on thaw loss were observed for different oils and preservation periods, while a significant linear increase ($P \leq 0.05$) in thaw loss was observed for all oils with increasing preservation periods of up to 60 days (Table 1). No significant cooking loss was observed in the treated groups for all preservation periods, while a significant decrease was diagnosed with increasing preservation periods.

Table 2 illustrates the effect of different oils and freeze-preservation periods on TBA, FFA, and TVN values of the LD muscle. The results indicated a decreasing effect of oil treatments on TBA, FFA, and TVN values in all treatment groups. Group T3 drenched with clove oil recorded the lowest values than the rest of the oil drenching treatments and the control for all preservation periods. Data also recorded a significant linear increase in TBA, FFA, and TVN values for all treatments, increasing the preservation period to 60 days.

Values are presented as mean ± SEM. Different lowercase letters indicate significant differences between means within columns, while different uppercase letters indicate significant differences between means within rows ($P \leq 0.05$). TBA, thiobarbituric acid; TVN, total volatile nitrogen; FFA, free fatty acids.

Figure 1 shows the effect of different oil treatments and freeze-preservation periods on the LD muscles' chemical composition. There was no significant effect of oil treatments and preservation periods on the muscles' chemical components, despite decreasing moisture content and increased protein and fat content for all treatments with increasing preservation period.

Discussion

Maintaining meat's ultimate pH is very important because of its relationship to meat quality, color, and shelf life. Parvar *et al.* (2018) stated that feed additives, such as essential oils, had no significant effect on meat's final pH 24 h postmortem. Smeti *et al.* (2018) noted no significant effect of rosemary essential oils on lamb meat's final pH. Moreover, de Oliveira Monteschio *et al.* (2017) reported no significant effect of feeding clove and rosemary essential oils on final meat pH. These results agree with the results achieved in this research (Table 1). The results also agreed with Rivaroli *et al.* (2020) and Ornaghi *et al.* (2020), who indicated that the average pH of LD is between 5.5 and 5.8. This value is influenced by chilling and the stress to which the animal is subjected before slaughter. The research data indicated that the meat pH of the animals was within the normal limits, indicating the animals were calm when slaughtered and the carcasses were cooled well. Microorganisms and meat enzymes cause proteolysis, producing organic sulfides, ammonia, and amines, which raise the pH (Muela

Table 1. Effect of different oils and freeze-preservation periods on longissimus dorsi muscles' pH, WHC, thaw lass, and cooking loss.

Parameter	pH			WHC (%)			Thaw loss (%)			Cooking loss (%)	
Periods Groups	No freezing (P1)	30-day freezing (P2)	60-day freezing (P3)	No freezing (P1)	30-day freezing (P2)	60-day freezing (P3)	30-day freezing (P2)	60-day freezing (P3)	No freezing (P1)	30-day freezing (P2)	60-day freezing (P3)
Control (T1)	aB 5.72 ± 0.04	aAB 5.80 ± 0.05	aA 5.91 ± 0.02	aA 34.52 ± 0.74	aAB 34.32 ± 0.35	aB 33.85 ± 0.96	aB 3.89 ± 0.04	aA 4.49 ± 0.08	aA 34.75 ± 0.24	aA 34.16 ± 0.30	aB 33.41 ± 0.08
Sage oil (T2)	aB 5.58 ± 0.06	aAB 5.77 ± 0.03	aA 5.95 ± 0.02	aA 34.33 ± 0.96	aAB 34.10 ± 0.96	aB 33.56 ± 0.74	aB 3.75 ± 0.04	aA 4.10 ± 0.03	a A 34.53 ± 0.14	aAB 33.95 ± 0.15	aB 33.10 ± 0.08
Clove oil (T3)	aB 5.40 ± 0.06	aA 5.71 ± 0.12	aA 5.78 ± 0.05	aA 34.63 ± 1.00	aA 34.53 ± 0.96	aA 33.75 ± 0.96	aB 3.48 ± 0.03	aA 4.28 ± 0.07	aA 34.49 ± 0.18	aA 34.10 ± 0.08	aB 33.38 ± 0.08
Laurel oil (T4)	aA 5.63 ± 0.04	aA 5.81 ± 0.31	aA 5.79 ± 0.35	aA 34.55 ± 0.74	aB 34.22 ± 0.74	aC 33.85 ± 0.96	aB 3.62 ± 0.10	aA 4.37 ± 0.01	aA 34.69 ± 0.07	aAB 34.22 ± 0.10	aB 33.65 ± 0.05

Table 2. Effect of different oils and freezing periods on longissimus dorsi muscles' TBA, FFA, and TVN.

Parameter	TBA mg malonaldehyde/kg			FFA (%)			TVN mg N/100 g		
Periods Groups	No freezing (P1)	30-day freezing (P2)	60-day freezing (P3)	No freezing (P1)	30-day freezing (P2)	60-day freezing (P3)	No freezing (P1)	30-day freezing (P2)	60-day freezing (P3)
Control (T1)	aC 0.96 ± 0.01	aB 1.35 ± 0.01	aA 1.58 ± 0.02	aC 0.90 ± 0.31	aB 1.19 ± 0.71	aA 1.42 ± 0.28	aC 8.92 ± 0.05	aB 10.87 ± 0.18	aA 12.15 ± 0.39
Sage oil (T2)	bC 0.66 ± 0.01	bB 1.06 ± 0.08	bA 1.34 ± 0.03	bcC 0.66 ± 0.25	cB 0.84 ± 0.36	cA 0.98 ± 0.21	abC 7.89 ± 0.04	bB 9.45 ± 0.16	bA 10.94 ± 0.58
Clove oil (T3)	dB 0.47 ± 0.02	dA 0.77 ± 0.02	dA 0.76 ± 0.08	cC 0.51 ± 0.17	dB 0.68 ± 0.85	dA 0.82 ± 0.34	cB 5.24 ± 0.59	dA 6.75 ± 0.19	dA 7.22 ± 0.43
Laurel oil (T4)	cC 0.56 ± 0.01	cB 0.92 ± 0.01	cA 1.08 ± 0.01	abC 0.82 ± 0.20	bB 0.96 ± 0.43	bA 1.21 ± 0.22	bC 7.04 ± 0.19	cB 8.17 ± 0.23	cA 9.32 ± 0.30

et al., 2010). This research agreed with the results that indicated increasing pH values corresponding to an increased storage period. Increase in pH value with increasing storage period of oil treatments was not at the same level in the control group. The increase in control treatment pH value may have resulted from proteolysis, reduced by the effect of oils and their active substances. The antimicrobial activity of essential oils is due to the presence of hydroxyl groups. This antimicrobial activity is mediated by several mechanisms, including influence on the cytoplasmic membrane and active transport (Sharma *et al.*, 2020).

WHC is one of the most important measurements that determine meat quality and other characteristics. It is affected by many postmortem factors, including the extent of pH decline, proteolysis, and others (Huff-Lonergan and Lonergan, 2005). The results obtained in this research (Table 1) agree with Rivaroli *et al.* (2020), who indicated no significant effect of different essential oils on meat WHC. Ripoll *et al.* (2012) confirmed that WHC is greatly affected by meat pH. The average pH values of the treated lambs' meat may explain the absence of differences in WHC values between these treatments. The results also agree with Muela *et al.* (2015), who observed a decrease in WHC with an increased freezing period. The degradation of meat protein and its decreased ability to hold water may explain this outcome. There is a positive relationship between WHC and pH, as lactic acid production leads to a decrease in pH. As the pH reaches the isoelectric point of meat proteins (especially myosin), the excess charges that bind the protein with water are minimal, resulting in decreased WHC. With an increase in the pH and its rise above the isoelectric point of proteins, the negative or positive charges that are not associated with the protein increase, enabling the proteins to bind more water molecules, resulting in increased WHC. In the case of freezing (as in this research), although the pH increased as a result of proteolysis, a decrease in WHC occurred.

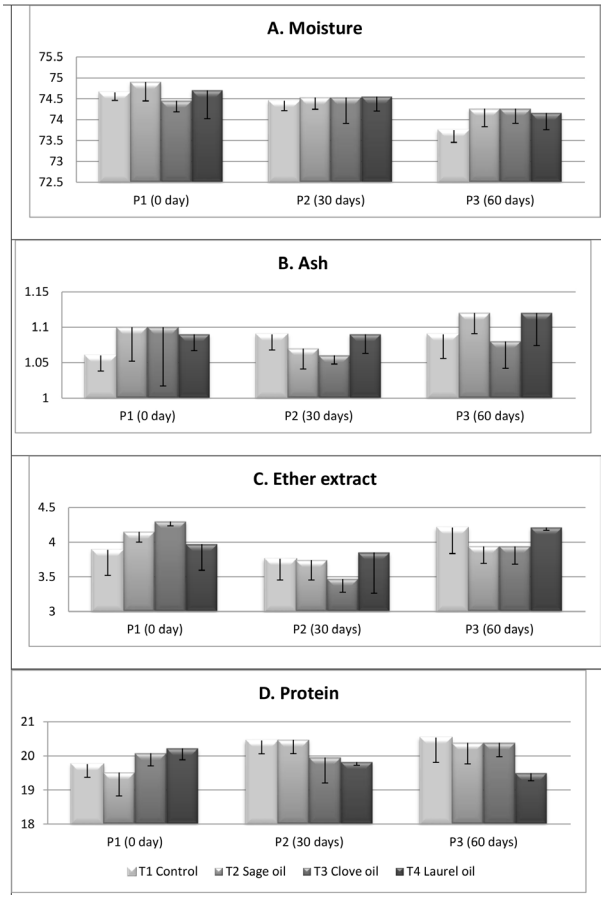

Figure 1. Effect of different oil treatments on the percentages of A. moisture, B. Ash, C. Ether extract, and D. Protein content of the treated lambs' longissimus dorsi muscles after different preservation periods.

This decrease may be due to the myofibril proteins shrinkage resulting from the presence of ice crystals that cause damage to muscle cells and denatured proteins, which increases in size with increasing storage period (Ripoll *et al.*, 2012).

This study indicated no significant effect of treatment with essential oils in cooking loss, which agrees with previous studies (de Oliveira Monteschio *et al.*, 2017; Smeti *et al.*, 2018). The results confirmed thawing losses with increasing preservation period and indicated a rise in total thaw loss with increased freezing time. These results coincide with previous studies (Muela *et al.*, 2015; Ornaghi *et al.*, 2020). Freezing increases thaw loss due to muscle membranes' mechanical damage by ice crystals, which leads to increased loss. Membrane damage may lead to increased protein denaturation and decreased WHC. Also, prolonged freezing causes increased water loss in meat by ice crystals damaging the cell membranes (Lu *et al.*, 2019).

Fat and protein oxidations affect meat quality and are related to meat deterioration. TBA level is an indicator of the extent of lipid oxidation through malondialdehyde concentration and color intensity measurement. A TBA content of 0.6–2.0 mg malondialdehyde/kg is considered to be within normal limits (Falowo *et al.*, 2014). The results revealed that the different essential oils decreased TBA levels. These results agreed with Parvar *et al.* (2018) and Ranucci *et al.* (2019). The oxidative indicator's decrease is because the essential oils are absorbed into the circulatory system ingestion, then distributed to muscles and the rest of the tissues (Velasco and Williams, 2011). Phenolic compounds are considered antioxidants due to their ability to inhibit free radicals by incorporating them into the aromatic ring (Maqsood *et al.*, 2014). Nieto *et al.* (2010) stated that feed additives allow antioxidants such as essential oils to get into tissues and cellular membranes, protecting them from oxidative stress by reactive oxygen. This process has proven to be more effective than treating meat with antioxidants postmortem (Kumar *et al.*, 2018).

The even distribution of antioxidants within the tissues and cells ensures the effectiveness of these compounds against fat and protein oxidation. The results in Table 2 also agreed with that reported by Politeo *et al.* (2006), who ranked 12 essential oils in the descending order according to their capacity as antioxidants, including the oils used in this research. They showed that the antioxidant efficacy of clove oil was the highest. Other studies recorded an increase in TBA relative to the increased storage time (Ranucci *et al.*, 2019; Rivaroli *et al.*, 2020). O'sullivan *et al.* (2004) stated that increased storage time leads to increased lipid oxidation, breakdown of peroxides, and increased secondary compounds resulting from oxidation, represented by malondialdehyde.

FFA is formed from fat and oil hydrolysis and is considered a measure of degradation by microorganisms and lipolytic enzymes (Rahman *et al.*, 2015). The FFA results in Table 2 agreed with Ozogul *et al.* (2017), who observed a significant decrease in FFA for the different nano oil treatments and all preservation periods, compared with the control treatment. The results also indicated that a decrease in FFA is due to additives' ability to inhibit lipolytic bacteria growth (Maqsood *et al.*, 2015; Rahman *et al.*, 2020). Several hypotheses explain the essential oils and phenolic compounds' antimicrobial activity, but they are not yet proven (Kalogianni *et al.*, 2020). Olatunde and Benjakul (2018) reported that essential oils could destroy bacteria by interacting with bacterial cell wall proteins. This interaction increases membranes' permeability and leaking of cytoplasmic structures and potassium ions, causing cell death (Bajpai *et al.*, 2008). At the same time, other researchers reported that the decrease in pH and increase of phenols increased essential oils' hydrophobicity, favoring their attachment to pathogen's lipid cell membranes and increasing antimicrobial activity (Gutierrez *et al.*, 2009). Due to their hydrophobic properties, phenolic compounds are bound with microbial membrane lipids causing their disruption (Devi *et al.*, 2010; Trombetta *et al.*, 2005). This disruption leads to intracellular compounds efflux, protein functional dysregulation, and cell death (Devi *et al.*, 2010).

TVN is a product of meat and nonprotein nitrogenous substance degradation and a measure of meat deterioration. Degradation of nitrogenous substances is caused by microbial and endogenous proteolytic enzyme activity. This study's results agreed with Saleh *et al*. (2021), who reported decreased TVN values by feed additives, and Ozogul *et al.* (2017), who indicated a decrease in TVN following different nano oil treatments and storage times. The maximum permissible meat TVN content is 150 mg/kg. Research reports the antimicrobial effectiveness of phenolic compounds, which possibly destabilizes the bacterial cell membrane (Pisoschi *et al.*, 2018), leading to permanent damage of the cell membrane and intracellular organelles and bacterial internal enzyme inhibition causing cell death. Besides, phenolic compounds' antioxidant efficacy may increase proteins' stability and reduce TVN by reducing radicals (Moroney *et al.*, 2013). Our results confirmed increases in TVN values with increasing storage period. Custódio *et al.* (2018) indicated that TVN increases due to protein degradation by the internal meat enzymes and microorganisms' action.

This study's results revealed no significant effect of essential oils as feed additives on LD chemical composition. Other studies also reported similar results (Ranucci *et al.*, 2019; Rivaroli *et al.*, 2020). A decrease in moisture content with increasing storage time might occur due to protein denaturation, lack of WHC, and decomposition by microorganisms. This decrease in moisture leads to increased dry matter (protein ratios, ether extract, and ash) content (Al-Rubeii *et al.*, 2009; Sharma *et al.*, 2015).

Conclusion

The results of this research indicated no significant effect of drenching Awassi lambs with different aromatic essential oils of sage, clove, and laurel at a concentration of 500 mg/head/day on the physical and chemical characteristics of meat. However, these oils' effect was positive on fat oxidation and protein stability, increasing the preservation period, especially when using clove oil.

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Goldenberry (*Physalis peruviana* L.) seed oil: press extraction, optimization, characterization, and oxidative stability

Pedro P. Ugarte-Espinoza[1], Victor Delgado-Soriano[1], Lorenzo Estivi[2], Alyssa Hidalgo[2]*, Gloria Pascual-Chagman[1]

[1]Departamento de Tecnología de Alimentos y Productos Agropecuarios, Facultad de Industrias Alimentarias, Av. La Universidad s/n. La Molina, Universidad Nacional Agraria La Molina (UNALM), Lima, Perú; [2]Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Milan, Italy

***Corresponding Author:** Alyssa Hidalgo, Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Italy. Email: alyssa.hidalgovidal@unimi.it

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PAPER

Abstract

In order to optimize the screw-press extraction conditions of oil from goldenberry (*Physalis peruviana* L.) seeds obtained from nectar processing waste, a face centered design was applied. The oil was extracted at different temperatures (60, 80, and 100°C) and seed moisture contents (8, 10, and 12%). Oil recovery (OR) increased and residual oil in the cake decreased significantly as moisture content and temperature were reduced; oil moisture and volatile matter as well as acid value, K_{232}, K_{268}, and *p*-anisidine, respectively, decreased proportionally with the moisture extraction. Thus, the highest OR (86.4%) and the best quality were obtained at 8% moisture content and 60°C pressing temperature. Under these conditions, the extracted oil presented high linoleic acid (76.0%), iodine value (140.0 mg I_2/g), and refractive index (1.4769). The oil stability index, measured by Rancimat, varied from 3.65 h (120°C) to 14.87 h (100°C); the predicted shelf life at 25°C was 120.4 days and the activation energy was 85.6 kJ/mol. The results highlighted that screw-pressing of goldenberry seeds provides quality oil without employing polluting and hazardous solvents.

Keywords: cape gooseberry; expeller; oil recovery; oxidation kinetics; Rancimat; shelf life

Introduction

Physalis peruviana L., commonly known as goldenberry or Cape gooseberry, is a perennial herb native of the Andean highlands belonging to the *Solanaceae* family. *P. peruviana* has become one of the most promising tropical fruits and has received growing interest from all over the world due to its potential as an intensive crop with a high content of bioactive compounds (Etzbach *et al.*, 2018; Ramadan, 2020).

The fruit when fresh is used as decoration in meals, salads, and desserts; when processed is used in sauces, jam, syrup, and yogurt (Chasquibol Silva and Yácono Llanos, 2015; Ramadan and Mörsel, 2003); and when dehydrated is used in baked foods, cocktails, snacks, and cereal breakfast (Vásquez-Parra *et al.*, 2013). The food industry produces juices and nectars from goldenberry pulp, discarding seeds and peels (ca. 27% fruit fresh weight, Ramadan, 2020) as by-products. The amount of seeds compared to the fresh fruit weight is rather variable: Ramadan and Mörsel (2003) reported that seeds constituted 17% of the fruit's weight, whereas Popova *et al.* (2020) found this to be 7.3 and 11.5% in two different genotypes. The seeds are a potential raw material for oil production because of their high oil content (Aslanov *et al.*, 1995; Chasquibol Silva and Yácono Llanos, 2015; Popova *et al.*, 2020) and high nutritional value. In addition,

they are an important source of linoleic acid (omega 6) and vitamins A, E, and K (Chasquibol Silva and Yácono Llanos, 2015; Ramadan and Mörsel, 2003), as well as of phenolic compounds. Furthermore, no adverse effects or toxicity are reported (Nocetti *et al*., 2020).

In previous studies, oil from goldenberry pomace (seeds, peels, and pulp remnants) was obtained by aqueous enzymatic extraction and solvent extraction (Mokhtar *et al*., 2018; Ramadan *et al*., 2008; Ramadan and Moersel, 2009). However, these methods have certain disadvantages related to performance, economy, and safety. For instance, the aqueous enzymatic extraction has low yields and high costs of enzymes (Mwaurah *et al*., 2020). On the other hand, solvent extraction requires expensive facilities and equipment, and has the risk of fire and explosion associated with the flammable nature of solvents (Deli *et al*., 2011). They are also harmful to both human health and environment as pollutants (Mwaurah *et al*., 2020).

Hence, mechanical extraction using a screw press is a cheaper, safer, and simpler alternative. Although goldenberry seed oil (GSO) has already been extracted using this method (Chasquibol Silva and Yácono Llanos, 2015), the effect of extraction conditions on oil yield and quality was not investigated. Likewise, no characterization of GSO oxidation kinetic or shelf-life prediction by Rancimat is available in literature.

Therefore, the objective of this research was to optimize the screw-press extraction process of oil from goldenberry seeds, as well as to characterize and evaluate the oxidative stability by Rancimat of the oil obtained at the best extraction conditions.

Materials and Methods

Raw material

The goldenberry pomace (peel, seed, and pulp remains) obtained during the nectar production process at the Agroindustrial Development Institute-INDDA (Lima-Peru) was used. After removing the peels and pulp residual by washing with water, the seeds were dried at 50°C for 17 h, sieved, and stored at 4°C in hermetic low-density polyethylene bags until the extraction trials.

Optimization trials for oil screw-press extraction

Response surface methodology was used to evaluate the effect of different extraction conditions on the response variables, namely, oil recovery (OR), residual oil (RO), oil moisture and volatile matter, acid value (AV), peroxide value (PV), specific extinction at 232 and 268 nm (K_{232} and K_{268}), and *p*-anisidine value (*p*-AV). The experiments were carried out following a face centered design (FCD), considering temperature (–1 = 60°C, +1 = 100°C) and moisture (–1 = 8%, +1 = 12%) as independent variables. The FCD was performed considering three central points, with a total of 11 runs according to Table 1. To avoid systematic errors, the experiments were performed randomly.

Oil extraction

The seeds were hydrated with distilled water until moisture levels of 8, 10, and 12%, according to the methodology indicated by Singh and Bargale (2000). The hydrated seeds were packed in hermetic containers and stored at room temperature for approximately 48 h to reach equilibrium. The containers were shaken at regular intervals to distribute the moisture evenly throughout the seeds. The amount of water necessary for hydration was determined by applying the following formula (Mridula *et al*., 2019): $M_W = M_S \frac{(H_1 - H_0)}{100 - H_1}$, where M_W is the mass of water to be added (g), M_S is the mass of seeds to be hydrated (g), H_0 and H_1 are, respectively, the initial and the final moisture content (wb) of the seeds.

The seeds were pressed at 60, 80, and 100°C, using a KOMET screw press (CA 59 G, IBG Monforts, Germany), at a screw speed of 15 RPM and a nozzle diameter of 4 mm. Before introducing the seeds into the feed hopper, the press was operated for 15 min with heating through the electric resistance ring fixed around the press head to raise the temperature of the cylinder to the selected temperature. After each run, all press devices were cleaned and dried. The oils obtained were centrifuged (ROTOFIX 32A, Hettich, Germany) at 2,701 *g* for 30 min and subsequently stored in amber bottles at 4°C until analysis. The cakes obtained were stored at 4°C in hermetic low-density polyethylene bags until analysis.

Analyses

All the following determinations were performed in triplicate.

Seed characterization

The moisture, crude fat, ash, crude fiber, and crude protein (N × 6.25) of the seeds were determined following the AOAC methods, 935.29, 945.16, 950.49, 962.09, and 950.48 (AOAC International, 2016), respectively. The total carbohydrate content was determined by difference, i.e. by subtracting all the above mentioned compounds from the total.

Table 1. Experimental design and average results for oil recovery (%), residual oil (% dm), moisture and volatile matter (%), acid value (mg KOH/g), *p*-anisidine value, extinction coefficients K_{232}, K_{268} of goldenberry seed oil obtained at different press extraction conditions (temperature, °C; moisture, g/100 g).

Standard order	Independent variables		Response variables						
	Temperature	Moisture	Oil recovery	Residual oil	Moisture and volatile matter	Acid value	*p*-anisidine	K_{232}	K_{268}
1	60 (−1)	8 (−1)	86.43^{a}	6.62^{h}	0.072^{d}	0.237^{c}	0.72^{d}	1.33^{e}	0.17^{e}
2	100 (1)	8 (−1)	65.46^{d}	15.66^{e}	0.075^{d}	0.234^{c}	0.73^{d}	1.36^{de}	0.19^{de}
3	60 (−1)	12 (1)	56.15^{f}	18.57^{d}	0.097^{a}	0.360^{ab}	1.00^{b}	1.49^{a}	0.25^{a}
4	100 (1)	12 (1)	34.98^{i}	25.26^{a}	0.097^{a}	0.382^{a}	1.04^{a}	1.50^{a}	0.26^{a}
5	60 (−1)	10 (0)	78.27^{b}	10.01^{g}	0.087^{b}	0.350^{ab}	0.83^{c}	1.40^{bc}	0.21^{c}
6	100 (1)	10 (0)	48.95^{g}	21.01^{c}	0.087^{b}	0.373^{a}	0.84^{c}	1.42^{b}	0.22^{b}
7	80 (0)	8 (−1)	73.03^{c}	13.13^{f}	0.075^{d}	0.236^{c}	0.73^{d}	1.34^{e}	0.19^{de}
8	80 (0)	12 (1)	43.51^{h}	22.45^{b}	0.097^{a}	0.361^{ab}	0.99^{b}	1.49^{a}	0.26^{a}
9	80 (0)	10 (0)	57.35^{e}	18.46^{d}	0.086^{bc}	0.338^{b}	0.84^{c}	1.41^{b}	0.21^{bc}
10	80 (0)	10 (0)	56.42^{f}	18.82^{d}	0.088^{b}	0.369^{ab}	0.84^{c}	1.38^{cd}	0.20^{cd}
11	80 (0)	10 (0)	56.83^{ef}	18.79^{d}	0.084^{c}	0.371^{ab}	0.84^{c}	1.40^{bc}	0.20^{cd}

Different letters in the same column indicate significant differences (P ≤ 0.05) among trials following the LSD test.

Oil recovery (OR) and residual oil (RO)

The oil recovery (OR) of the oil extracted was calculated using the formula indicated by Mridula *et al.* (2019):

$$\mathrm{OR}(\%) = \left[1 - \frac{\text{Oil content on cake (g)}}{\text{Oil content in seeds (g)}}\right] \mathrm{x}\,100$$

The oil content in the seeds before pressing extraction and in the cake was assessed following method 945.16 (AOAC International, 2016). The residual oil (RO) was determined from the oil content in the cake after pressing.

Physicochemical analyses of the oils

Moisture and volatile matter, acid value (AV), peroxide value (PV), refractive index at 20°C, *p*-anisidine value (*p*-AV), iodine value (IV), saponification value (SV), and unsaponificable matter were determined following the methods Ca 2d-25, Ca 5a-40, Cd 8-53, Cc 7-25, Cd 18–90, Cd 1d-92, Cd 3-25, and Ca-40 (AOCS, 1998), respectively. Specific extinction at 232 and 268 nm (K_{232} and K_{268}) was determined following the method, ISO 3656 (ISO, 2011).

The fatty acids' (FA) composition was determined as fatty acid methyl esters (FAME) by gas chromatography after transesterification of the oils with 2 N KOH in methanol, according to IUPAC Standard Method 2.302 (IUPAC, 1987). The fatty acid profile was determined by gas chromatography as described by Rodríguez *et al.* (2021).

Evaluation of oil oxidative stability (OSI)

Rancimat test

The OSI of each oil and of the blends were evaluated by the method AOCS Cd 12b-92 (AOCS, 1998) using a 743 Rancimat equipment (Metrohm Schweiz AG, Zofigen, Switzerland). The assays were carried out using 3.0 ± 0.1 g of oil sample with an air flow of 20 L/h at 100, 110, and 120°C.

Oil shelf life

The prediction of shelf life was determined by extrapolation of the linear correlation of the logarithm of OSI vs temperature (T) for a temperature of 25°C, as described by Heidarpour and Farhoosh (2018): log OSI = aT + b, where a and b represent the slope and intercept, respectively.

Oxidation kinetics

The reaction rate constant (k) was calculated as the reciprocal of OSI (k = 1/OSI), as indicated by Aktar and Adal (2019). The temperature coefficient (Q_{10}), which indicates the increase in reaction rate due to a 10°C rise in temperature, was calculated according to Symoniuk *et al.* (2017): $Q_{10} = (k_2/k_1)^{10/(T_2-T_1)}$. The relationship between k and temperature was defined by the Arrhenius equation:

$\ln k = \ln A - Ea/RT$, where A is the frequency factor (h^{-1}), Ea is the activation energy (kJ/mol), R is the universal gas

constant (8.314 J/mol K), and T is the absolute temperature (K).

Statistical analysis

The analyses of variance (ANOVA) of data and construction of response surface plots were performed using Design Expert software v.12 (Stat-Ease Inc., Minneapolis, USA). Before the ANOVA, normal data distribution was verified. The data were also processed by one-way ANOVA; when significant differences at $P \leq 0.05$ were found, Fisher's Least Significant Difference (LSD) at $P \leq 0.05$ was determined. These statistical analyses were performed with the Statgraphics® Centurion XVI program (Statpoint Technologies, USA). The data are presented as mean ± standard deviation (SD) of three replicates, computed using the software Excel (Microsoft® Office Excel 2016).

Results and Discussion

Seed composition

The proximate chemical composition of the goldenberry seeds was 33.63 ± 0.03 g/100 g dry matter for crude fat, similar to the level (32.7 g/100 g, i.e., 18.09 g/100 g extracted oil + 14.63 g/100 g in the cake) observed by Chasquibol Silva and Yácono Llanos (2015), but much higher than the value (18 g/100 g) reported by Aslanov *et al*. (1995) in seeds from Azerbaijan. The seeds also contained 14.46 ± 0.05 g/100 g dm crude protein, 2.45 ± 0.01 g/100 g dm ash, 32.48 ± 0.40 g/100 g dm crude fiber, and 16.98 ± 0.04 g/100 g dm total carbohydrates. Similar protein, ash, and fiber levels were reported in the seed cake by Chasquibol Silva and Yácono Llanos (2015).

Optimization trials for oil screw-press extraction

Table 1 reports the results of the oil extraction trials, while Figure 1 shows the response surface plots. The ANOVA (Table 2) highlighted significant effects of temperature and moisture on oil recovery and residual oil in the cake; the moisture presented the highest influence. While oil recovery showed a linear behavior, the residual oil showed a quadratic effect of temperature. Even if the lack-of-fit for both variables was significant, the adjusted-R^2 and predicted-R^2 were very high. During the extraction, the oil quality parameters were mainly influenced by the seed moisture and by its quadratic term for acid value and *p*-anisidine. All these models showed non-significant lack of fit and high R^2.

The OR increased and the residual oil decreased significantly as the moisture content of the seeds and the pressing temperature decreased from 12 to 8% and from 100 to 60°C, respectively (Figure 1). Similar trends were reported by Mridula *et al*. (2015) and Singh *et al*. (2002) during the screw-pressing oil extraction from, respectively, linseed (moisture 6–10% wb; 50–90°C) and crambe seeds (moisture 3.6–9.2% dm; 120°C). Similarly, Silvia *et al*. (2012) observed an increase of OR from nigella seeds with a pressing temperature decrease from 100 to 50°C. According to Martínez *et al*. (2017) and

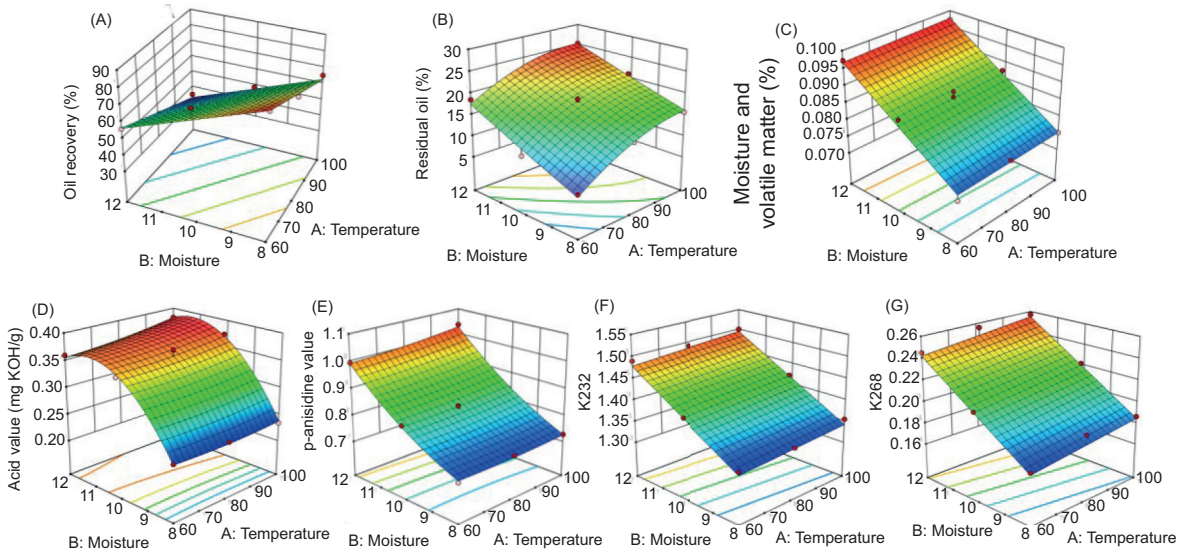

Figure 1. Response surface plots for oil recovery (A), residual oil in the cake (B), and physicochemical quality (moisture and volatile matter, C; acid value, D; *p*-anisidine value, E; K_{232}, F; K_{268}, G) of goldenberry seed oil as a function of moisture and temperature of press extraction.

Table 2. Analysis of variance (mean square and significance) for oil recovery, residual oil in the cake, and physicochemical quality of goldenberry seed oil.

Source	df	Oil recovery	Residual oil	Moisture and volatile matter	K_{232}	K_{268}	df	Acid value	*p*-anisidine
A-Temperature	1	850.9***	119.2***	1.4×10^{-6}	0.0004	0.00023	1	0.00029	0.00059*
B-Moisture	1	1358.1***	158.9***	0.00076***	0.035***	0.00737***	1	0.026***	0.12***
AB			1.37				1	0.00015	0.00023
A^2			11.95*				1	0.00004	0.00006
B^2			0.03				1	0.009***	0.00205**
Residual	8	10.8	1.27	2.3×10^{-6}	0.00018	0.00005	5	0.00016	0.00009
Lack of Fit	6	14.4*	2.10*	1.6×10^{-6}	0.00018	0.00007	3	0.00003	0.00015
Pure Error	2	0.22	0.04	4.1×10^{-6}	0.00019	0.00001	2	0.00035	2.7×10^{-6}
R^2		0.96	0.98	0.98	0.96	0.95		0.98	1.00
Adj-R^2		0.95	0.96	0.97	0.95	0.93		0.96	1.00
Pred-R^2		0.93	0.81	0.96	0.93	0.92		0.94	0.96
C.V. %		5.50	5.58	1.75	0.96	3.46		3.85	1.11

df, degrees of freedom; Adj-R^2, R^2 adjusted by df; Pred-R^2, R^2 in prediction; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Savoire *et al*. (2012), both high moisture content and high pressing temperature may result in poor OR due to excessive softening of the tissues, which makes seeds to stick and reduces friction.

The moisture and volatile matter were, respectively, in the range of 0.072 and 0.097%, below the maximum limit (0.2%) established by the Codex Alimentarius (1999a) for vegetable oils. The AVs were in the range of 0.234 and 0.382 mg KOH/g, below the maximum limit established by the Codex Alimentarius (1999a) for cold-pressed oils (4 mg KOH/g) and refined oils (0.6 mg KOH/g). We obtained quite low AV when compared to Mokhtar *et al*. (2018; 2.36 mg KOH/g), indicating that the applied treatments did not greatly increase the hydrolysis of fatty acids. In fact, unlike Mokhtar *et al*. (2018), in the present study, the seeds were separated from the pomace before grinding, probably reducing the contact between the oil and the endogenous hydrolytic enzymes.

The hydroperoxides were below the detection limit (0.5 mEq O_2/kg; Frankel, 2012), suggesting that the treatments did not accelerate the oxidative process, likely due to the presence of natural antioxidants that may have delayed it. In fact, GSO is extremely rich in total tocopherols (2400–5100 mg/kg), with β>γ-δ>α (Popova *et al*., 2020, 2021; Ramadan *et al*., 2008).

Specific extinctions in UV (K_{232} and K_{268}) are highly sensitive indices that measure the extent of oil oxidation. Conjugated dienes are detected at 232 (or 234) nm and derive from primary oxidation of linoleic acid, following the same trend of peroxides. Conjugated trienes are detected at 268 (or 270) nm and derive from primary oxidation of linolenic acid as well as from dehydration of hydroxy-linoleate or -linolenate (Frankel, 2012). The K_{232} and K_{268} ranged from 1.33 to 1.50 and 0.17 to 0.26, respectively (Table 1). These values are in contrast with those of Ramadan *et al*. (2008), who reported K_{232} = 0.57 and K_{268} = 1.02–1.12 in goldenberry pomace oil processed by enzyme-aided aqueous and solvent extractions. However, the oil obtained by Ramadan *et al*. (2008) appeared to contain secondary oxidation products. In fact, as reported by Spatari *et al*. (2017) for several edible oils (soybean, olive, corn, linseed, sunflower, and peanut), UV absorbance spectra are always higher around 230 nm than around 270 nm, and the primary oxidation mainly affects the absorbance in the former region.

The *p*-AVs were in the range of 0.72 and 1.04, which is below the maximum limit (10.0) indicated by Matthäus (2010) for refined oils. The *p*-anisidine test detects high molecular weight carbonyl compounds (Frankel, 2012), thus a low value indicates that the oil is not in an advanced stage of oxidation, thereby supporting the fact that the applied treatments did not induce oil oxidation.

The values of all the physicochemical parameters decreased with the reduction of seeds' moisture from 12 to 8% except for AV, whose values remained almost constant between 12 and 10% and decreased from 10 to 8% following a quadratic relation. In general, this parameter was not influenced by the pressing temperature (Table 2). Lipase, lipoxygenase, and phospholipase are activated at high moisture (>10%; Gupta, 2002): this may explain why a predominant moisture effect on oil degradation indicators was observed. Besides, lipase exhibits hydrolytic activity only if sufficient water is available both as

reactant and to form a water–oil interface (Brockman, 2013).

To find out the experimental conditions that maximize oil recovery and minimize other responses, a multi-response optimization by RSM was performed by using the desirability function. The solution we found had a desirability equal to 0.98 and corresponded to the treatment performed with 8% moisture at 60°C. The maximum oil recovery (86.43%) was far higher than the yield obtained by Ramadan and Moersel (2009; 42.1%) with an enzymatic-aided aqueous extraction; this might be due to nonfatty matter retaining oil in the pomace. The OR observed in the present research was similar to those reported for screw-pressed flaxseed (82.9%) and sesame (74.2%) seeds (Martínez *et al*., 2017; Mridula *et al*., 2015). Therefore, screw-pressing of goldenberry seeds separated from pomace guarantee higher yields and better quality of oil.

Characterization of the oil extracted at the optimized conditions

Physicochemical characteristics

Table 3 shows the physicochemical characteristics of the GSO extracted at 60°C with 8% of final moisture. The iodine value, 140.5 g I_2/100 g, is consistent with the theoretical value calculated as the average, weighted on the fatty acid composition, of the values provided by Gunstone (2004; 85.6, 173.2, and 260.3 for methyl oleate, linoleate, and linolenate, respectively). This places the goldenberry oil in a straddling position between semi-siccative and siccative oils. The degree of unsaturation was also reflected by RI (1.4769 at 20°C). The IV and RI values were higher than those (116.3 g I_2/100 g and 1.4481, respectively) reported by Aslanov *et al*. (1995), likely due to a lower degree of unsaturation of the oil they analyzed. In fact, both IV and RI increase as the number of double bonds increases (Raziq *et al*., 2012). Mokhtar *et al*. (2018) reported an IV of 109.5 g I_2/100 g, despite their fatty acid composition being very similar to ours.

Table 3. Physicochemical characteristics of goldenberry seed oil corresponding to the treatment with the highest oil recovery and the best quality (8% of final moisture, 60°C).

Parameter	
Iodine value (g I_2/100 g)	140.50 ± 0.24
Refractive index (20°C)	1.4769 ± 0.0001
Saponification value (mg KOH/g)	188.10 ± 0.20
Unsaponifiable matter (g/100 g)	1.67 ± 0.02
Color coordinates:	
*L**	30.21 ± 0.08
*a**	−2.13 ± 0.01
*b**	10.03 ± 0.11

We determined a saponification value of 188.1 mg KOH/g, similar to those observed by Mokhtar *et al*. (2018) in goldenberry pomace oil and by Anwar *et al*. (2002) in safflower oil, i.e., 186.2 and 189.0 mg KOH/g, respectively. The GSO's SV was lower than the SV of coconut, babassu, and palm kernel oils, where medium chain fatty acids (mainly lauric and myristic; Codex Alimentarius, 1999b) predominate. This indicates that long-chain fatty acids (C18) were more abundant in GSO, as SV decreases with fatty acid chain length.

The unsaponificable matter is the sum of minor but valuable components such as tocopherols, carotenoids, squalene, and phytosterols, which not only impart oxidative stability to the oils but also enhance their nutritional value (Raziq *et al*., 2012). The value we found, 1.67 g/100 g, is the average among edible oils, being very close to soybean, sunflower (both regular than high oleic), safflower, coconut, and cottonseed oil; conversely, corn oil has superior values, near to 3 g/100 g (Codex Alimentarius, 1999b; Gunstone, 2004). Higher values were reported by Ramadan *et al*. (2008) and Popova *et al*. (2021): 2.13–2.25 and 3.02 g/100 g, respectively. According to Popova *et al*. (2021), the peel is the part richest in unsaponificable matter (61.33 g/100 g of peel oil), despite containing twofold less tocopherols and far less sterols than seeds. In our opinion, this is explained by the wax covering the fruit, which distorts the result. Instead, our experiments were conducted separating the seeds from the rest of the pomace.

The *L**, *a**, and *b** coordinates were 30.21, −2.13, and 10.03, respectively. This indicated that the oil was slightly dark with the presence of yellow and, in lower degree, green compounds that likely correspond to pigments such as carotenoids and chlorophylls, respectively. The GSO coordinates in the CIELab system were lower than those reported for other oils, such as chia (Ixtaina *et al*., 2011), pistachio (Ling *et al*., 2016), and linseed (Varas Condori *et al*., 2020).

Fatty acid composition

Linoleic acid (C18:2 ω-6) was the most abundant fatty acid, followed by oleic (C18:1), palmitic (C16:0), stearic (C18:0), and vaccenic (C18:1 ω7) acids (Table 4). The remaining unsaturated fatty acids (palmitoleic and α-linolenic), as well as the long-chain saturated fatty acids (arachidic, behenic, and lignoceric) were found in low concentrations (<0.5%). Similar results were reported by Mokhtar *et al*. (2018), Ramadan and Moersel (2009), Ramadan and Mörsel (2003), and Chasquibol Silva and Yácono Llanos

Table 4. Fatty acid profile (% of total FAME) of goldenberry seed oil corresponding to the treatment with the highest oil recovery and best quality (8% of final moisture, 60°C).

Fatty acid	
Palmitic acid (C16:0)	6.43 ± 0.01
Palmitoleic acid (C16:1 ω-7)	0.40 ± 0.02
Stearic acid (C18:0)	3.23 ± 0.01
Oleic acid (C18:1 ω-9)	10.97 ± 0.06
Vaccenic acid (C18:1 ω-7)	1.67 ± 0.01
Linoleic acid (C18:2 ω-6)	75.99 ± 0.02
α-linolenic acid (C18:3 ω-3)	0.23 ± 0.00
Arachidic acid (C20:0)	0.40 ± 0.01
Behenic acid (C22:0)	0.16 ± 0.01
Lignoceric acid (C24:0)	0.16 ± 0.00
Saturated fatty acids	10.37 ± 0.01
Unsaturated fatty acids	89.25 ± 0.11
Monounsaturated	13.03 ± 0.04
Polyunsaturated	76.22 ± 0.02

(2015), although they found lower contents of α-linolenic and vaccenic acids. Aslanov *et al*. (1995) reported higher values of palmitic, oleic, and α-linolenic acids, lower values of linoleic acid, but similar percentage of stearic acid. Our results were also quite similar to Embaby *et al*. (2022), who found slightly higher amounts of stearic, behenic, lignoceric, and α-linolenic acids offset by lower contents of linoleate and palmitate. Differences in oil composition may be explained by different origin, environment, growing conditions, and oil extraction process (Varas Condori *et al*., 2020). In fact, for two genotypes of goldenberry, Popova *et al*. (2020) reported extremely discordant values for fatty acids: palmitic 20.6–20.9%, stearic 13.0–17.5%, oleic 5.4–29.4%, linoleic 5.3–11.3, and α-linolenic 5.4–9.2%. Comparing other oil species, GSO appears very similar to regular safflower oil (Anwar *et al*., 2002; Codex Alimentarius, 1999b; Gunstone, 2004).

Linoleic acid derivatives serve as structural components of the plasma membrane and as precursors of some metabolic regulatory compounds. In addition, studies suggest that linoleic acid consumption is inversely correlated with the risk of cardiovascular diseases (Marangoni *et al*., 2020); thus, introducing GSO in diet could help maintain an adequate health.

Shelf-life prediction

The OSI at 100°C was 14.87 h (Table 5), higher than the values reported for other crude oils such as camelina (5.21 h; Ratusz *et al*., 2016), linseed (4.07 h; Varas Condori *et al*., 2020), chia (3.03 h; Villanueva *et al*., 2017), and sacha inchi (1.59 h; Rodríguez *et al*., 2015) but lower than crude pumpkin oil (18.2 h; Vujasinovic *et al*., 2010). These differences could be due to the different fatty acids' profile, since OSI decreases as the degree of unsaturation increases (Shadyro *et al*., 2017).

The relationship between OSI and temperature was linearized through logarithm transformation. The line of regression showed a high coefficient of determination (R^2 = 0.9994), thus 99.94% of OSI variation was explained by the model. The predicted shelf life at 25°C was 120 days (approximately 4 months), very close to the 118.9–123.2 days of two chia:sesame oil blends studied by Rodríguez *et al*. (2020), higher than the 91 days reported for crude linseed oil by Varas Condori *et al*. (2020), but lower than the 386 and 211 days of, respectively, pistachio (Dini *et al*., 2016) and avocado (Aktar and Adal, 2019) crude oils. In comparison with commercial oils, whose shelf life generally is between 12 and 15 months under normal storage conditions, GSO predicted shelf life was low (Kochhar and Henry, 2009). Taking into account that the GSO started with very low levels of deterioration (Table 1), the short time obtained can be attributed to the high concentration of linoleic acid, which oxidizes 10– 40 times faster than the oleic acid (Symoniuk *et al*., 2016).

Oxidation kinetics

The *k* constant increased as a function of the temperature (Table 5) since the oxidation rate was accelerated by the temperature increase. The magnitude of the effect of temperature on *k* was demonstrated by Q_{10}, which had a value of 2.02. Similar values were reported in refined soybean oils (1.99–2.08; Farhoosh, 2007) and crude linseed oils (1.99–2.05; Symoniuk *et al*., 2017), while lower values were reported in crude canola oils (1.84–1.86; Symoniuk *et al*., 2016). A high Q_{10} implies that small changes in temperature induce a greater increase in the reaction rate, so that high Q_{10} values indicate lower oxidative stability (Farhoosh *et al*., 2008; Symoniuk *et al*., 2016).

The GSO oxidation kinetics obeyed the Arrhenius equation (Table 5) in the temperature range from 100 to 120°C (R^2 = 0.9988). The activation energy gives an indication of the minimum amount of energy needed to initiate the oxidation reaction. The *Ea* of GSO was 85.56 kJ/mol, slightly higher than the levels reported for camelina (70.39–79.08 kJ/mol; Ratusz *et al*., 2016), linseed (74.03–77.76 kJ/mol; Symoniuk *et al*., 2016), canola (75.73–77.64 kJ/mol; Symoniuk *et al*., 2017), and chia (82.0 kJ/mol; Rodríguez *et al*., 2020) crude oils but below the *Ea* of sesame (96.2 kJ/mol; Rodríguez *et al*., 2020), hazelnut (94.75 kJ/mol; Gülmez and Şahin, 2019), and avocado (99.6 kJ/mol; Aktar and Adal, 2019) crude oils. These differences may be due to several factors, such as the degree of unsaturation and the presence of different

Table 5. Oxidative stability index (OSI) at different temperatures and oxidation kinetic parameters of goldenberry seed oil corresponding to the treatment with higher oil recovery and better quality (8% of final moisture, 60°C). Shelf life at 25°C (OSI_{25}) was extrapolated.

	Temperature (°C)			Line of regression	Slope	Intercept	R^2	Shelf life OSI_{25} (d)	Q_{10}	*Ea* (kJ/mol)
	100	110	120							
OSI (h)	14.87 ± 0.08	7.52 ± 0.11	3.65 ± 0.06	log OSI = aT + b	−0.0305 ± 0.0003	4.223 ± 0.032	0.9994	120.44		
$k \times 10^3$ (h^{-1})	67.27 ± 0.35	132.9 ± 1.9	273.8 ± 4.8	$\ln k = \ln A - Ea/RT$	−10291 ± 145	24.87 ± 0.38	0.9986		2.02	85.56

endogenous antioxidants or prooxidants (Symoniuk *et al*., 2017).

Conclusions

The pressing temperature and the moisture content of the seeds exerted a significant, but negative, effect on the OR. On the other hand, the seeds' moisture content affected the physicochemical quality of the oil to a greater extent than the pressing temperature. All the extraction conditions allowed to obtain oils with good physicochemical quality, but the best quality and the highest OR were achieved at 60°C with 8% of moisture content. Under these extraction conditions, the oil exhibited a yellow tone with low luminosity, presented high iodine value and refractive index, and low saponification value. In addition, GSO consisted mainly of unsaturated fatty acids, with a high percentage of linoleic acid (ω-6), which makes it an important source of this essential fatty acid. The oil presented a low oxidative stability that resulted in a shelf life of 120 days at 25°C; the *Ea* was 85.56 kJ/mol. Finally, the results highlighted that screw-pressing of goldenberry seeds provides quality oil without employing polluting and hazardous solvents.

Authors' Contribution

Pedro P. Ugarte-Espinoza designed the study, collected and analyzed the data, and drafted the manuscript; Victor Delgado-Soriano supervised the work, collected and analyzed the data, and revised the manuscript; Lorenzo Estivi and Alyssa Hidalgo statistically elaborated the data, and drafted and revised the manuscript; Gloria Pascual-Chagman planned and designed the study, supervised the work, provided resources, and revised the manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest concerning this article. There was no financial support, except those mentioned in the acknowledgments.

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