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Photoacoustic characterization of wheat bread mixed with *Moringa oleifera*.

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ABSTRACT

Photoacoustic spectroscopy is applied to evaluate the impact of *Moringa* at different concentrations (0, 1.25, 2.5, 5 and 10%) on the elaboration, sanity, texture, and color of wheat bread. It was found that: i) Photoacoustic signal amplitude values of bread significantly increase from 37 to 90% when moringa powder concentration raises from 1.25% to 10%, at 300 nm wavelength. ii) Comparing the photoacoustic signal values at 300, 330, and 370 nm wavelengths, produced by the different bread types, there were statistically significant differences. iii) The sanitary quality of bread mixed with a 2.5% of moringa is relatively higher than the ones obtained for other concentrations, such that the number of fungal colonies were reduced by 99% in comparison with the control bread without moringa, after six days of storage. Moringa at 2.5% of concentration could thus improve the sanitary quality of wheat bread. iv) The addition of moringa for making bread slows down its textural changes (hardness, elasticity, cohesiveness, resilience, and chewiness) during storage. v) Finally, the highest correlation between the photoacoustic amplitude and the moringa concentration occurs at the wavelengths of 300 and 330 nm, which could be related to significant changes in the content of flavonoids and phenolic acids.

1. Introduction

The need to consume foods fortified with natural substances has been intensified due to their nutritional potential and therapeutic effects (Shahidi, 2009;El Sohaimy, 2015). Stress, pollution, infections, and other factors cause that the body's nutrient reserves decrease, leaving people susceptible to contract several diseases, such as Covid-19 (Palacios et al., 2019). An optimal immune response depends on a proper diet and nutrition, a weak nutritional state affects the immune system due to insufficient consumption of multiple micronutrients and bioactive compounds such as omega 3 fatty acids, water, probiotics, carotenoids, and phenolic compounds among others (Iddir et al., 2020; Palacios et al., 2019). Polyphenols have been attributed anti-inflammatory and antioxidant properties. Antioxidants inhibit and scavenge free radicals, providing protection to humans against Infections and degenerative diseases (Mahmood et al., 2010). Flavonoids such as quercetin, naringin, hesperetin and catechins have been reported with antibacterial, antifungal, and antiviral properties (Gyawali et al., 2020).

One of the plants with many bioactive compounds and that strengthens the immune system is the Moringa oleífera, universally referred to as the miracle plant or the tree of life (Oyeyinka and Oyeyinka, 2018), which is recommended its consumption in disease prevention (Rastogi et al., 2020). Among many other compounds contains carbohydrates, fatty acids, proteins and functional peptides and phenolic compounds. This has led that the World Health Organization (WHO) to promote it as alternative to treat malnutrition, since at least one person in five lacks the necessary micronutrients such as iodine, zinc, iron, folic acid, calcium, and vitamins A and B. This lack of micronutrients is known as "hidden hunger" and is a problem that prevails in several world regions (Mahmood et al., 2010;Berenji et al., 2008; Habeych et al., 2016). Then, it is necessary to consume nutritious food, a strategy of its consumption is through fortified foods. Among the applications of interest of the moringa is as additive to elaborate fortified food products, which could increase the consumption of antioxidants in the population and prevention diseases. Bioactive compounds added to staple foods as bread, it could be a way for the population to

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consume them (Aghamirzaei et al., 2017;Martínez-Monzó et al., 2013).

In the Literature there are only some authors that have reported the addition of Moringa oleifera in bakery products such as cookies (Ogunsina et al., 2011;Nwakalor et al., 2014;Igbabul et al., 2018), Ogi and amala (Abioye and Aka, 2015;Karim, 2015), rice crackers (Manaois et al., 2013), snack (Devisetti et al., 2016), gluten-free bread (Hayat et al., 2018) and wheat bread (Sengev et al., 2013; Bolarinwa et al., 2019). Differences due to phenolic compounds have not been reported in the literature in box breads added with moringa; nor have the antifungal effects of moringa been evaluated, due to the increase in flavonoids (Sengev et al., 2013; Bolarinwa et al., 2019). Likewise, the use of photoacoustic spectroscopy for the characterization of moringa bread has not been reported.

The used techniques for bread characterization have been among others, UV-VIS spectroscopy and chemical methods. Sengev et al. (2013) used the Absorption Spectrophotometer to obtain an increase in beta-carotene content of moringa breads, compared with control breads. From absorbance at 452 nm, Bolarinwa et al. (2019) obtained beta-carotenes and from these determined the vitamin A of added and not added breads, showing that vitamin A is increased in the Moringa breads. In conventional methods chemical and reagents are required to carry out these measurements (Sellami *et al.*, 2012). Methods that could avoid the use of these chemical reagents have been reported in the literature, such as is the case of Photoacoustic spectroscopy technique (Hernández-Aguilar et al., 2019a).

Photoacoustic spectroscopy (PAS) does not use chemicals to prepare the material for its measurement (Hernández-Aguilar et al., 2019a, 2019b) and could be used for characterization of bread. In this research it was characterized, by photoacoustic spectroscopy, wheat bread added with different Moringa concentrations, relating the photoacoustic signal amplitude values at 300 nm, 330 nm, 370 nm, 515 nm, and 650 nm wavelengths with the used moringa powder concentrations and the number of fungi colonies developed at 4 and 6 days. Complementing the study, other properties of moringa bread were evaluated, as the bread texture.

In general, it should be noted that, several spectroscopic techniques have been applied for food analysis as Raman, Nuclear magnetic resonance, Fluorescence, atomic absorption spectroscopy, Near infrared, visible/infrared, and UV/VIS (Pojić and Mastilović, 2013; Karoui and Blecker, 2011; Pankaj et al., 2018). These techniques have been applied to characterize pesticides, fumonisins, differentiation of grains, coffee, oils, minerals, adulterations of wines and spices, fermentations and bioactive elements of food and plants (Sowoidnich et al., 2010; Lee and Herrman, 2016; Pojić and Mastilović, 2013; Foca et al., 2011; Lu et al., 2011; Aleixandre-Tudo et al., 2017; Hernández *et al.*, 2019a; 2020a; Bolarinwa et al., 2019).

UV-VIS spectroscopy has been applied to determine concentration of total phenols and ferulic acids by obtaining their absorbance spectra from 240 nm to 360 nm, where the ferulic acid absorption band ranged from 310 nm to 340 nm, with the maximum absorption peak at 320 nm (Tian et al., 2021). However, phenolic compounds are characterized by a peak absorption at 280 nm, where there is a lower absorption band (Pan et al., 2002). It is worth mentioning, the authors used extraction methods to obtain these bioactive and chemical elements. According to the literature, among the bioactive compounds found in nutraceutical and/or fortified food and/or bread are phenolic acids and flavonoids, which are characterized by their optical absorption spectra (Hernandez-Aguilar et al., 2020; 2021). In this way quercetin, gallic acid, resveratrol, polyphenols have been characterized by absorption spectroscopy in the ultraviolet and visible region (200–800 nm) (Kroon et al., 2016; Song et al., 2015; Ren et al., 2019; Masek et al., 2019; Qi e*t al.* Al., 2015).

Regarding to nutraceutical foods, turmeric as a nutritional supplement, has been studied by UV-VIS spectroscopy. Optical absorption spectra, from 250 nm to 550 nm wavelength of pure and adulterated (with metanil yellow) turmeric, were obtained. The authors reported that by increasing the adulterant percentage in turmeric, from 5% to 50%, the absorbance increases, since the spectrum of metanil yellow has an absorption band from 420 nm to 450 nm wavelength. Also, the optical absorption spectrum of pure turmeric and metanil yellow showed maximum absorption peaks at 420 nm and 445 nm, respectively (Das et al., 2019). UV-VIS spectroscopy has also been used to obtain absorbance spectra in other nutritional supplements as moringa, the bread additive used in this research. Carbajal et al. (2020) obtained optical absorption spectra of moringa extracts at different concentrations (1.56, 3.12, 6.25 and 12.5 mg mL − 1). In the reported optical absorption spectra, from 200 nm to 400 nm wavelength, phenolic acids are found. To obtain the spectra, moringa powder was diluted in water to obtain semi-transparent liquids, necessary condition to be possible the measurement. In case of characterizing solid samples, UV-VIS spectroscopy with integrating sphere should be used. Hernandez-Aguilar et al. (2011) reported the reflectance spectra, from 620 nm to 700 nm wavelength of different layers of corn, obtained by UV-VIS spectroscopy with integrating sphere. In the present study photoacoustic spectroscopy is proposed to qualitatively observe the changes in the absorption bands, corresponding to secondary metabolites contained in moringa bread.

2. Materials and methods

2.1. Formulations of moringa powder bread

Five types of bread formulations were elaborated (Fig. 1) with common ingredients as egg (average weight 48 g), salt (1.25 g), sugar (4 g), olive oil (70 g), and yeast (11 g). Moringa powder (MP) was incorporated 0, 7.5, 15, 30 and 60 g, in 600, 592.5, 585, 570 and 540 g wheat flour, respectively. At different concentrations in relation to wheat flour weight (0, 1.25, 2.5, 5 and 10%). The final five formulations expressed by percentage are showed in Table (1). The ingredients were mixed during 5 min, in a blender with a spiral hook. After its fermentation in 300 g warm water (30 °C), the yeast was incorporated into the mixture, which was then kneaded for 10 min and placed in a mold covered with a moistened cotton fabric during 30 min. Next, the dough was again kneaded during 10 min and put in a greased mold for bread, where it increased its volume during 25 min. Afterwards, each type of the doughs was placed in different molds and simultaneously baked in a pre-heated electric oven (Black + Decker) at 180 °C, for 50 min. After baking, the bread was allowed to cool down at room temperature during 24 h and subsequently cut it into slices (1.5 cm thick) through an electric knife (Hamilton beach type EK08, 121 V, 11 Hz).

2.2. Photoacoustic spectroscopy experimental setup

Fig. 2 shows the PAS experimental setup which consists of a series of interconnected and instrumented elements to produce the photoacoustic effect within a photoacoustic cell, detect it and record it. This instrumentation a Xenon lamp used as the light excitation source, a monochromator, to obtain a monochromatic light beam, and a mechanical chopper, employed to modulate the light beam at fixed $f = 17$ Hz frequency, at which the electret microphone has the best response. The modulated beam is focused onto an optical fiber to guide it to the photoacoustic (PA) cell, whose signal is then detected by an electret microphone (photoacoustic sensor) connected to the cell through a fine channel (1 mm diameter). The PA signal is amplified by means of a lock-in amplifier (EG&G, Mod. 5210) and then recorded in a personal computer.

2.3. Antifungal effects

Sanitary quality test adapted according to Neergaard (1979) and Perez et al. (2015), was established under a randomized complete block design with 10 repetitions for each bread sample. We randomly selected 10 slices of each bread type (0, 1.25, 2.5, 5 and 10% of MP

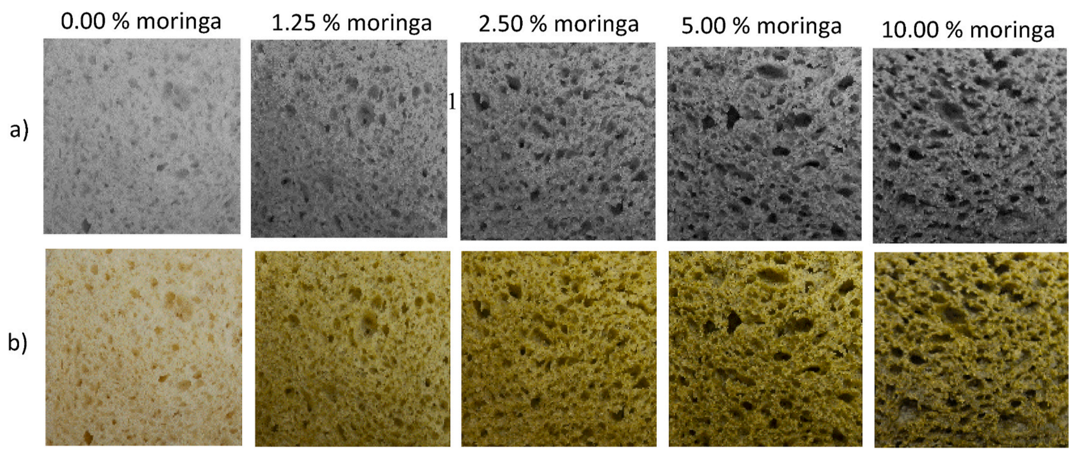


Fig. 1. Optical image of wheat bread added with moringa (0, 1.25, 2.5, 5 and 10%). a) in gray scales, b) color images.

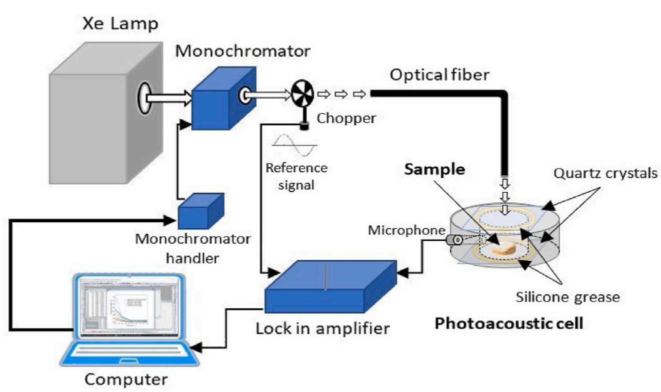


Fig. 2. Scheme of PAS experimental setup used in our experiments.

concentration) and subsequently place them in a hermetically sealed container of polystyrene properly sterilized. The incubated samples were then placed in a closed room at 25 °C average temperature. The observation and quantification of the existing fungal colonies were performed daily during the 6 days of incubation. Incubation elapsed; observations were performed by a stereoscopic microscope to record the development of fungal colonies in the breads.

2.4. Texture profile analysis (TPA)

The texture profile analysis for the 5 breads added with moringa powder were evaluated using a Texture Analyzer Brookfield Model CT3 25 K USA with a 25 kg load cell. The samples were cut into 3 × 3 × 2.5 cm cubes, 10 min before their measurement and stored in hermetic polyethylene bags. The analysis was performed for two 20% compression cycles by means of a TA General Probe Kit with a TA25/1000 with a cylinder, 50.8 mm and 20 mm diameter and length respectively: operating at 1.7 mm/s speed. The TPA analysis was performed on the day after its preparation, also 3 and 6 days later. Four replicates were carried out at 24 ± 1°.

2.5. Statistical analysis and principal component analysis (PCA)

2.5.1. Variance analysis

Moringa bread texture, spectroscopy photoacoustic and sanitary quality were carried out under an experimental design with four and ten repetitions of each bread type (0%, 1.25%, 2.5%, 5%, and 10% MP concentrations). The mean difference between evaluated variables of each bread treatment was determined by using analysis of variance (ANOVA), followed by a Tukey test at 95% confidence level, using Minitab® version 17 statistical software (Minitab Inc., State College PA, USA) and SAS GLM procedures (SAS, V9). The least significant difference test (LSD) at the 5% probability level was used for comparing treatments (Steel and Torrie, 1980), see Table 1.

2.5.2. Principal component analysis (PCA)

The PCA was applied to the variables evaluated in this study in the wheat bread elaborated with 0%, 1.25%, 2.5%, 5% and 10% MP concentration (CB, MBa, MBb, MBc, MBd). The analysis was performed using the R Project software version 0.10–47, with R Commander and factoMiner and Fitopac. The used data matrix come from the measurements of photoacoustic signals at 300, 330, 370, 450, 515 and 650 nm wavelengths, fungal colonies evaluated on days 1, and texture variables (hardness, elasticity, resilience, cohesiveness, and chewiness). Finally, the linear correlation equation and the linear correlation intensity (r) between the variables: normalized photoacoustic signal, at 300 nm, 330 nm and 370 nm wavelengths, and MP concentrations, was determined (Table 2). The r parameter was quantified through Pearson's linear correlation coefficient. It was calculated from the quotient of the covariance divided by the standard deviations of the obtained data from the variables (Vila et al., 2004).

2.5.3. Statistical analysis of photoacoustic spectra using kernel estimator

Statistical analysis of the data was performed by using the probability density function (PDF), which specifies how the probabilities are distributed over the values adopted by the random variable (Soong, 2004). The PDF is calculated by using a non-parametric method: kernel estimator, which to estimate a continuous and uniform distribution from a finite set of observed points (Rojas-Lima et al., 2016). The data processing was carried out using MatLab R2020a software.

Table 1
Moringa bread formulations.

Ingredients	CB	MB-1.25	MB-2.5	MB-5.0	MB-10
	(%)	(%)	(%)	(%)	(%)
Wheat flour	81.72	80.7	79.68	77.63	73.55
Olive oil	9.53	9.53	9.53	9.53	9.53
Egg	6.54	6.54	6.54	6.54	6.54
Salt	0.17	0.17	0.17	0.17	0.17
Sugar	0.54	0.54	0.54	0.54	0.54
Yeast	1.5	1.5	1.5	1.5	1.5
Moringa powder	0	1.02	2.04	4.09	8.17

CB: Control bread (without addition of turmeric), MB: Moringa **bread.**

Table 2
Equations used to calculate estimated density, linear correlation, and correlation coefficient.

Description	Equation
Linear equation of correlation	$y = ax + b$ $a = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2}$ $b = \frac{\sum y - a\sum x}{n}$
Pearson's linear correlation coefficient (r)	$-1 \le r = \frac{Cov(X,Y)}{S_x S_y} =$ $\frac{\sum_{t=1}^{n}(X_t - X)^*(Y_t - Y)}{\sqrt{\sum_{t=1}^{n}(X_t - X)^2} * \sqrt{\sum_{t=1}^{n}(Y_t - Y)^2}} \le +1$

3. Results and DISCUSSION

3.1. Photoacoustic spectroscopy

Fig. 3 shows (a) the photoacoustic (PA) spectra, (b) the second derivative of PA signals and probability distribution densities for the wheat bread elaborated with 0%, 1.25%, 2.5%, 5% and 10% MP concentrations. Fig. 2a shows that the PA spectra of control bread (without addition of MP) has the lowest PA signal amplitude in the evaluated spectral region (300 nm–650 nm). The PA signal greatest changes of control bread (CB) and MP added breads were obtained in the range from 300 nm to 450 nm wavelength. This region, according to the literature, is characterized by the optical absorption of phenolic acids and flavonoids (Vongsak et al., 2013). Natural wheat bread (0% of MP) has qualities for consumption by the population, but with low nutritional value and antioxidant activity (Zhou et al., 2015). In this way the content of phenolic compounds in bread is modified with the addition of moringa. The bread added with 10% of MP (MBd) concentration showed the highest PA signal, in this absorption band range. It is observed that the PA signal amplitude, as a function of wavelength, is also a function of the MP concentration added to the bread.

Table 3 shows the PA signal amplitude (mV) at 300 nm, 330 nm, 360 nm, 370 nm, 450 nm, 515 nm, and 650 nm wavelengths, of breads added with 0%, 1.25%, 2.5%, 5% and 10% MP concentrations. It was observed that at 300 nm, 330 nm and 370 nm, there were statistically significant differences ($\rho \le 0.05$) between the control bread and MP added breads. For the case of 300 nm wavelength, the PA signal increased by 37%, 68%, 79% and 90%, for 1.25%, 2.5%, 5% and 10% MP concentrations, respectively. The PA signal amplitude increases as a function of the MP concentration added to the bread.

Similarly, in the wavelengths of 330 and 370 nm, there were increases in the level of the photoacoustic signal obtained in relation to that found in the control bread in 25, 62, 73, 87% and 44, 66, 77 and 83%. However, at values equal to or greater than 450 nm, no statistically significant differences were found between the values of the photoacoustic signal obtained. Compounds of bread have characteristic optical absorption bands centered at different wavelengths, as carotenoids (450 nm), anthocyanins (525 nm) and chlorophylls (650 nm) [Hernández-Aguilar et al. (2005) and Siddhuraju and Becker (2003)], and these bands do not present statistically significant differences ($\rho \le$

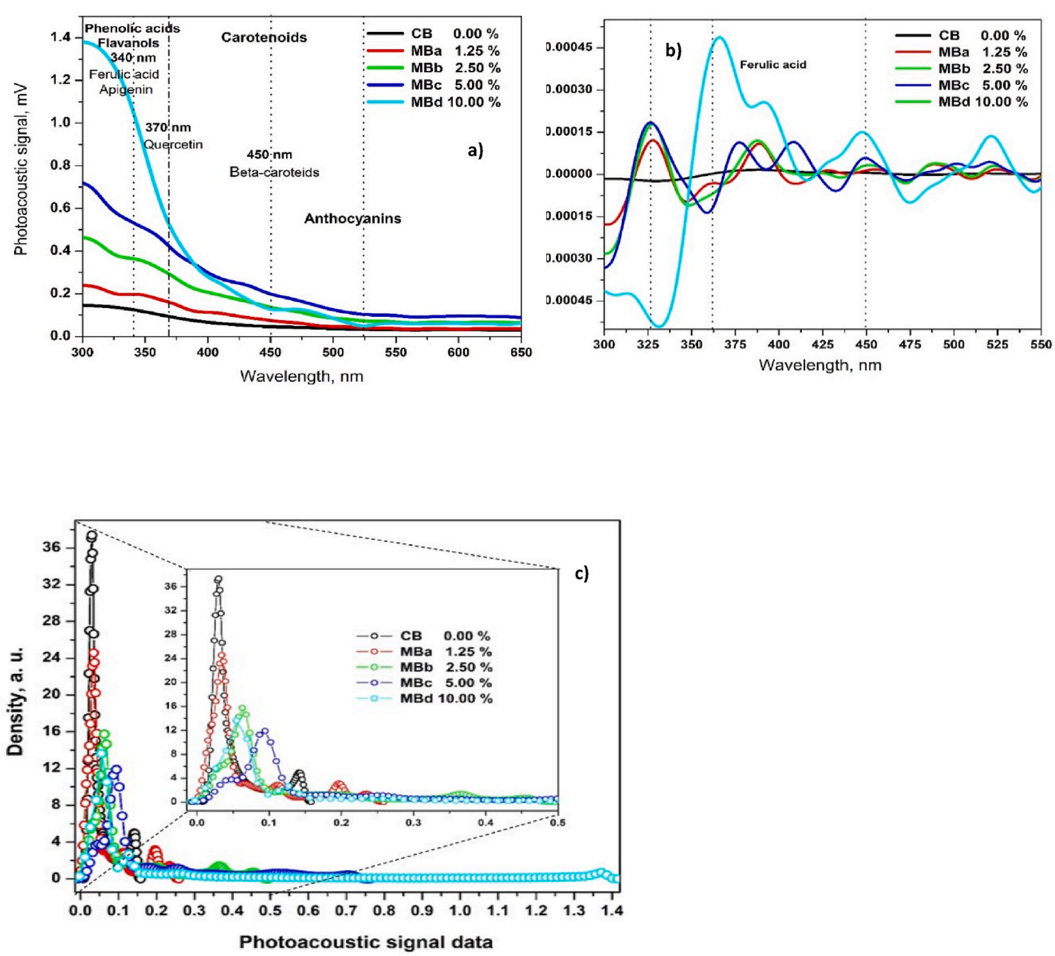
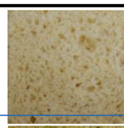
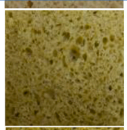
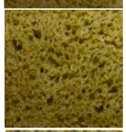
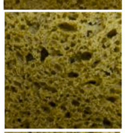
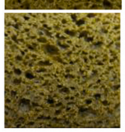


Fig. 3. Characterization of *Moringa oleifera* bread with different TP concentrations (0, 1.25, 2.5, 5 and 10%): a) Spectra of the photoacoustic signals and their b) second derivative c) density functions.

Table 3
Photoacoustic signal levels at 300, 330, 370, 450, 515, 650 nm and effects on the sanitary quality of bread..
PA-s: Photoacoustic signal; d: day; The different letters in the columns indicates that the values are statistically different ($\rho \leq 0.05$).

% Concentration of turmeric at wheat bread	Digital image of the bread	PA -s 300 nm mV	PA -s 330 nm mV	PA -s 370 nm mV	PA-s 450 nm mV	PA -s 515 nm mV	PA- s 650 nm mV	No. of Colonies 4 d	No. of Colonie s 5 d	Image Entropy
(a) 0.00% (White)		0.15c	0.15c	0.1c	0.08a	0.04a	0.02a	0.6b	21.6b	6.03
(b) 1.25%		0.24cb	0.2b	0.18b	0.1ba	0.05a	0.02a	0.9b	3.6c	6.61
(c) 2.50%		0.47cb	0.4b	0.3b	0.18a	0.06a	0.03a	0b	0.2c	6.7
(d) 5.00%		0.71ba	0.56a	0.45ba	0.23a	0.11a	0.1a	2b	4.5c	7.07
(e) 10.00 %		1.38a	1.2a	0.6a	0.18a	0.04a	0.08a	13.4a	40.7a	7.08

0.05); however, it is possible to observe a tendency to increase the photoacoustic signal amplitude at these wavelengths for MBa, MBb, MBc and MBd breads, when compared with white bread (control). Some of them are Gallic acid, Chlorogenic acid, Luteolin, Rutin, Quercetin, Kaempferol, Apigenin and Ferulic acid (Singh et al., 2013); in addition, it contains beta-carotenes and antioxidants (Leone et al., 2015).

Moringa oleifera has been reported to consist of various phenolic compounds such as: phenolic acids and isoflavonoids and flavonoids (Valdez-Solana et al., 2015; Sulastri et al., 2018). Some of them are Gallic acid, Chlorogenic acid, Luteolin, Rutin, Quercetin, Kaempferol, Apigenin and Ferulic acid (Singh et al., 2013); in addition, it contains beta-carotenes and antioxidants (Leone et al., 2015); in addition, it contains beta-carotenes, antioxidants, etc. (Leone et al., 2015). The concentration of compounds can vary depending on where the moringa is grown. But they also vary when they are added to food to generate nutraceutical foods, depending on its concentration during the production process (Hernandez-Aguilar et al., 2019b). Some flavonoids and phenolic acids have been identified at specific wavelengths such as: 340 nm for ferulic acid and apigenin; and 370 nm for myricetin, quercetin, butein, kaempferol, isorhamnetin, and rhamnetin (Siddhuraju and Becker, 2003) and phenolic acids caffeic, p-Coumaric and ferulic have been identified with the maximum peaks at 322, 309 and 322 nm (Pari et al., 2007). On the other hand, beta carotenes have been identified at 450 and 470 nm and at 650 nm the chlorophyll *b*. As can be seen in Fig. 2a, at the wavelength of 450 nm or close to it, the amplitude level of the photoacoustic signal is lower for the control bread when compared with the bread added with moringa. Vitamin A is a function of beta carotenes (Bolarinwa et al., 2019). Therefore, it could be said that vitamin A in moringa added bread also increases, as has been reported by other studies, where an increase from 2.2 to 4.3–8.5 mg/100 g has been indicated, depending on the concentration of moringa (Bolarinwa et al., 2019). In general, moringa is high in vitamins and minerals, high in protein and low in carbohydrates (Olushola, 2006). It has been used to feed babies in countries such as the Philippines (Kolawole et al., 2013), in addition to this it is associated with medicinal properties (Matic et al., 2018; Daba, 2016). It is classified within medicinal plants that could offer protection against coronavirus-induced inflammation, in addition, due to its high content of carotenoids, vitamin C or flavonoids can act as an immune-stimulant (Oladele et al., 2020).

In the present study, it was found how the photoacoustic signal of the breads added with moringa are modified with respect to the control breads. This is observed both in, the photoacoustic spectrum and the analysis of the signals using the second derivative and the kernel estimator (Fig. 3). In the second derivative, an almost constant behavior is observed in the curve corresponding to the control bread for all wavelengths, indicative of the low content of bioactive compounds (Fig. 3b). However, in the curves corresponding to the moringa added bread, depending on the concentration, there is a behavior that goes through various maximum peaks in the curves, which are associated with the existence, in general, of phenolic compounds: phenolic acids and flavonoids (Fig. 3b). The spectra of the photoacoustic signal and its second derivative show changes in flavonoid content. The increase in flavonoid content has indicated an increase in oxidant activity. Some authors have pointed out that vitamin C, quercetin, rutin, and ferulic acid are related to antioxidant activity (Siddhuraju and Becker, 2003).

Fig. 3c shows the probability distribution function estimated by the Gaussian kernel of each of the photoacustic spectra of the breads. Note that wheat bread CB (0% MP) exhibits a maximum value around 0.025 mV of the amplitude of the photoacoustic signal (photoacoustic signal data), within a defined region between 0 and 0.05 mV, the which indicates that in this region the values occur with a frequency high. It corresponds to the range of values located in the wavelength range of

500–650 nm (Fig. 3a). In general, as the concentration of moringa powder increases in the bread, the maximum frequency of data is decreasing and at the same time occupying a level of higher values of amplitude of the photoacoustic signal (Fig. 3c). The photoacoustic signal amplitude values with the lowest data occurrence occur within the amplitude range of 0.2–1.4 mV, which corresponds to the wavelength range of 300–450 nm (see Fig. 3a and c).

For the bread MBb (2.5%), the range of highest occurrence of values occurs from 0 to 0.1 mV, having the maximum peak value of occurrence at 0.05 mV, where these values are in the wavelength range of 490–650 nm. The values with the lowest occurrence correspond to the photoacoustic signal amplitude values of 0.1–0.5 mV. These are in the spectral region of 300–490 nm, corresponds to the range mainly of phenolic compounds (including quercetin at 370 nm) according to the literature and beta-carotenes are located at 450 nm (Siddhuraju and Becker, 2003). It is possible to mention that kernel curves of bread with and without moringa showed different pattern of behavior, in such a way that they could serve as characteristic patterns to distinguish the types of bread according to their concentration.

On the other hand, it is interesting to point out that photoacoustic spectroscopy, in difference of UV–Vis or NIR and MIR, give us an amplitude and phase of the photoacoustic signal, for each wavelength. If a sample with two components A and B are considered, then due to the different non-radiative relaxation times of each component, or even if the components are in different layers, there should have a time lag between the signals arising from A and B components (Conde-Gallardo et al., 2004). Therefore, the difference in the time to reach the gas layer, in the photoacoustic cell, produces a phase shift ϕ between the two signals. By using the Phase Resolved Method it is possible to separate different absorption bands in a sample with several components (Alvarado-Noguez et al., 2018). Here it is also worth mentioning that photoacoustic spectroscopy makes it possible to obtain the non-radiative relaxation time of molecules, which are related to some optical absorption band, by means of the photoacoustic signal phase (Domínguez-Pacheco et al., 2017). These are some of the advantages of using the photoacoustic spectroscopy technique.

The data analysis, from signals and images, can be performed by several algorithms and filtering techniques. In the case of PA spectra, the obtained data are linear and analyzes can be performed as a probability of density of a function (pdf), density estimator of a function (kdf), histogram, adjustment of distribution values, and several types of filters are used for images, including histograms, brightness, contrast, among many others that can show differences in porosity, roughness, and differences in areas of sample surface.

Regarding the accuracy, precision, selectivity, and traceability are directly related to how the technique obtains data from the analyzed samples. In PAS technique a wavelength scan is performed to obtain the optical absorption spectrum of the sample. In the case of the PA signal data acquisition, the precision and selectivity depend on selection of the wavelength range and the data acquisition program. In the PA instrumentation, used in this study, allows to average ten acquired data for each wavelength.

Although in the present investigation only the PA signal amplitude was used, in future studies it could be considered the use of PA signal phase to generate different information. In the present investigation, it was used the PA signal amplitude, which is proportional to the optical absorber concentration, in this case with the moringa concentration. Also, the optical absorption spectra obtained by PAS change, mainly in bread added with moringa, in the wavelength ranges where flavonoids and phenolic acids, as well as beta carotenes, have optical absorption bands. In future works, a wide range of concentrations of moringa added to bread and portable systems that can be validated could be established.

PAS is an experimental method that since its inception in 1973, Rosencwaig demonstrated its possible utility in the characterization of homogeneous and non-homogeneous materials in different areas of knowledge. It is a non-invasive and non-destructive method; the analyzed samples do not require prior preparation to be characterized. By this technique it is possible to obtain optical and thermal properties as optical absorption coefficient, thermal diffusivity and thermal effusivity (Satour and Zegadi, 2020; Mandelis, 1983; Lamastra et al., 2018; Britto Dhas et al., 2007), including also complex materials that contain different structures and layers (Hernandez-Aguilar et al., 2015).

Bicanic (2011) mentioned that PAS is a sort of spectroscopy, nondestructive based on photothermal phenomena, which allows spectroscopic studies. The conventional configuration uses Xe lamps, mainly in the UV-VIS range, and this configuration has been applied to the foodstuff analysis (obtaining PA spectra, as a function of wavelength) including plants and agricultural seeds.

Validation of the photoacoustic spectroscopy technique to characterize biological samples, such as corn seeds, and compared with spectra obtained by UV–Vis spectroscopy with an integrating sphere, demonstrated the usefulness of the photoacoustic spectroscopy technique to characterize foodstuffs such as corn kernels (Hernandez-Aguilar et al., 2011). In this way, it is effectively a reliable method, reported in the scientific literature, although this contribution is intended to give an idea of the possibility of using it for other applications in the foodstuff area. In this way to be able to transit, to its use especially in developing countries where it is necessary to develop technology, since most of the instrumentation comes from abroad. Generating knowledge related to these applications and many others of instrumentation based on laser technology will be relevant for the following years. Scientific and technological advances allow to done portable instrumentations using elements of low-cost, small size and stable (Rabasović *et al.*, 2009; Hariri *et al.*, 2018; Attia et al., 2019; Medina *et al.*, 2019).

In the review by Hernandez-Aguilar et al. (2019b), several applications of PAS for foodstuff characterization are mentioned, even though they are complex materials, due to their structure and components (Power *et al.*, 2019). Then PAS has been used in combination with mathematical and statistical analyzes, with favorable results that allow differentiating characteristic patterns of these complex samples. Foodstuffs that have been investigated by using PAS are cereals, legumes, vegetables, algae, fruits, and other liquid, semi-liquid, or solid foods, for example, milk, water, juices, mustard, tomato sauce and “tortillas” (De Oliveira et al., 2018; Scotter *et al.*, 1997; Bicanic, 2011; Rico *et al.*, 2013; Paniagua *et al.*, 2018; Hernández *et al.*, 2019b).

PAS has the advantage that with the same instrumentation, liquids and solids can be optically characterized in different configurations. In the case of liquids, it does not need to be diluted, to turn semi-transparent, as the case of optical characterization by UV-VIS spectroscopy. In PAS it is possible to measure the optical transmission spectrum of liquid samples, without diluted, which allows to obtain the complementary absorption photoacoustic spectrum (Hernández *et al.*, 2009). Recently PAS has been applied for the baking industry, in this sense, bread added with germinated lentil (0%, 5% and 10%) was evaluated by PAS. Absorption spectra was obtained at the wavelength range from 300 nm to 750 nm, which presented an increase in the amplitude level of the photoacoustic signal in the absorption band from 300 nm to 425 nm, depending on the increase in the concentration of lentil sprouts added in bread preparation. The authors reported additional tests to identify the presence of secondary metabolites and it was indeed demonstrated that in lentil sprouts, phenols and flavonoids are increased and saponins decreased. It was observed in the absorption spectra of lentil sprouts and un-germinated seed, at the band of saponins (430 nm–544 nm), that photoacoustic signal amplitude tends to decrease (Hernández et al., 2019a). Also, these observations were corroborated by HPLC tests, where it was shown that the addition of lentil sprouts increases the amount of quercetin in bread, mainly when 10% of lentil sprouts was added. Other studies in fortified bread have shown the relationship of photoacoustic absorption spectra with bread antioxidant activity, when added with turmeric. The found findings showed that the bread antioxidant activity is directly proportional to the concentration of turmeric powder added to bread, and to the photoacoustic signal amplitude at

515 nm (Hernandez-Aguilar et al., 2020).

Other advantages of photoacoustic spectroscopy have been reported in plant science (Pinchasov-Grinblat and Dubinsky, 2012). Photoacoustic methods have been used for *in vivo* measurements, for example, the inhibition of photosynthetic activity under water stress (Havaux *et al.*, 1987). In this way PAS is added as a powerful technique with multiple tools to characterize and analyses foodstuffs in its different aspects and needs. In the present research it is important to highlight that in box breads, added with moringa, PAS allows us to visualize the nutritional improvements that this fortified bread has. In system interventions in the real world, it could be useful to demonstrate the improvement of bread added with moringa. In a society with a pandemic of metabolic syndrome, in addition to COVID-19, it is important to add nutritional elements and antioxidants to people's diet, since in countries as Mexico one of the main problems is the low consumption of vegetables. Adding these nutrients to staples as bread could be an occasion for their consumption.

3.2. Antifungal effects

According to the analysis of variance carried out, it is possible to say that significant statistical differences were found ($\rho \leq 0.05$) between the number of colonies of fungi existing among the 5 types of breads made, on days 4, 5 and 6. From the day 4, the appearance of fungi began (Fig. 3). Moringa bread at a concentration of 2.5% (MBb) is the one with the least number of fungi, followed by a concentration of 1.25 (MBa) and 5% (MBc). It seems that there is a fungicidal effect due to the Moringa added in the bread, finding that according to the applied concentration, there is an inhibition or delay in the growth of fungi.

The behavior pattern of the curve that represents the number of fungal colonies in the control bread (CB) per day, increases with the passage of time. In the first three days without any fungus on the bread, and from the fourth day the number of colonies on the bread begins to increase, where it should be noted that the higher number of fungal colonies was concentrated on the crust of the bread. The most contaminated breads were the control and the corresponding to 10% of moringa powder concentration. Due to the concentration of moringa and the temperature where the experiment was established, the humidity increased, favoring a higher development of fungi in this type of bread, appearing both in the crumb and in the crust. Bread at 2.5% concentration had, on average, a lower number of fungal colonies (0.2), compared to those found in control bread (21.6) on the fifth and sixth day. Bread with a concentration of 10%, the difference in the number of fungal colonies was higher, since it reached an average total number of fungal colonies in moringa bread of 40.7 (Fig. 4). ***Moringa oleifera*** has been reported with antifungal effects (Phiri and Mbewe, 2010), due they have a combination of secondary metabolites such as: zeatin, quercetin, b-sitsterol, caffeoylquinic acid and kaempferol (El-Mohamedy and Abdalla, 2014). In this investigation it was found, the region where the greatest changes of the photoacoustic signal (Fig. 3a) existed was precisely in the region of phenolic compounds, where phenolic acids are found (Gallic, ellagic, chlorogenic and ferulic acid) and flavonoids (kaempferol, quercetin, isoquercetin, astragalin and rutin) among others (Muhammad et al., 2016). According to the literature, changes in the sanitary quality of bread can be associated with the content of flavonoids. Flavonoids are secondary metabolites, identified as broad classes of polyphenols, which have been reported with anti-bacterial, antiviral, and antifungal effects, among others (Aboody and Mickymaray, 2020; Mickymaray, 2019). Numerous phytochemical studies in moringa have shown the existence of these secondary metabolites (Muhammad et al., 2016). Then, by adding moringa to a product such as bread, it was found in this research that it could improve the quality of the food product, as well as extend its useful life. So, adding moringa to bread not only has a benefit in the nutritional dimension, but also in the health quality dimension, both dimensions having a beneficial impact on the health of the population that can consume it. Consumer preferences are linked to

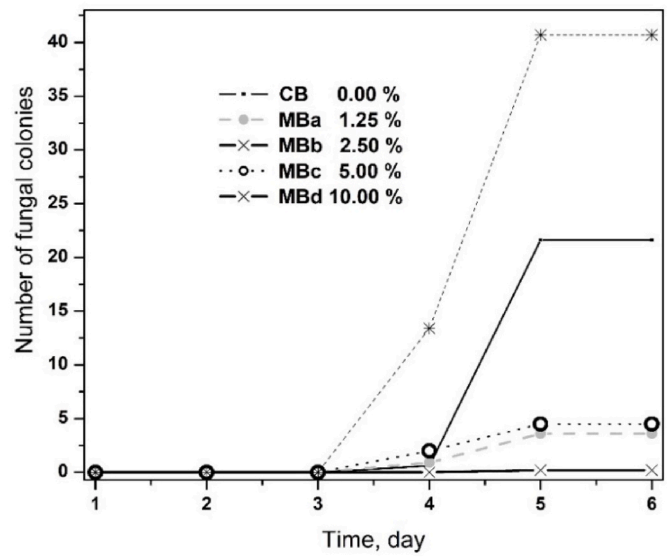


Fig. 4. Antifungal effects of moringa in bread (at different concentrations) on different days of storage.

low levels of moringa concentration (due to the presence of tannins that gives an undesirable flavor) (Murdiana et al., 2018). In this study, the best results of moringa concentration with antifungal effects was demonstrated with 2.5% moringa powder addition. Other authors using moringa seed to fortify sliced bread, reported the best preferences in sensory test in bread with 5% added moringa (Bolarinwa et al., 2019). As well as it is a way to improve nutrition in poor countries where there is no access to meat, since it is rich in protein and helps vitamin A deficiency, since it increases the content of betacarotenoids. In this study, an increase in the amplitude value of the photoacoustic signal is observed in the wavelength of carotenoids (450 nm) with the increase of the moringa powder added to the bread.

Moringa has been found to control *Aspergillius* fungi, depending on the concentration of the moringa (Arowora and Adetunji, 2014). Other authors showed a fungicidal effect of moringa in vitro experiments, where it has been reported reduction of pathogens as a function of the increase in moringa extract (El-Mohamedy and Abdalla, 2014). In the present study, the reduction of fungal colonies was a function of concentration of moringa powder, except for bread added to the concentration of 10%, because its humidity was increased. The found fungi in the bread slices were: *Penicillium* sp. (growing colonies in green color with dense conidia*), Rhizopus* sp. (white cottony mycelia, with black dots) and *Aspergillus* sp. (yellow or yellowish green colonies*),* with *Penicillium* fungus was the most abundant. In this research it is observed that adding moringa to bread could improve its sanitary quality and therefore, be of better quality for human consumption.

3.3. Texture profile analysis

Fig. 5 shows the texture profile analysis (TPA) for the 5 breads, where it is clearly observed that for the parameters (hardness, cohesiveness, elasticity, and chewiness) there is a significant difference ($\rho \leq 0.05$) between the different types of breads. In the hardness attribute for day 0 there was no significant difference ($\rho > 0.05$) between the control bread and the 1.25% moringa bread; however, between the control bread and the breads with moringa at 2.5%, 5% and 10%, the hardness increased by 86%, 23% and 290%, respectively. In this regard, Ogunsina et al. (2011). mentioned that the firmness of bread is greater when the percentage of moringa powder in bread increases. Likewise, the 10% moringa bread on day 3 showed the highest value (34 N). It is also observed that the 5% moringa bread did not show significant difference in hardness between the different days in which it was evaluated (days 3

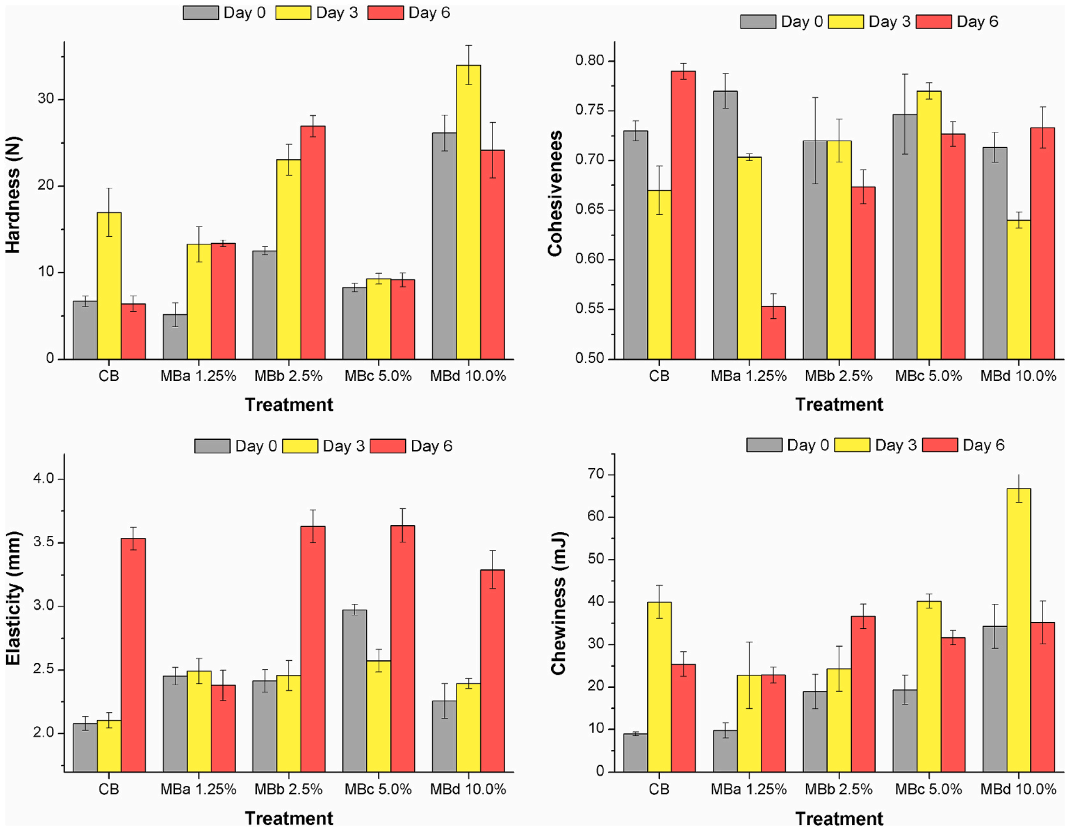


Fig. 5. Bread texture variables a). Hardness, b) Cohesiveness, c) Elasticity, d) Chewiness.

and 6), (8.25–9.3 N). Hardness is a parameter that is measured by the force required to deform the bread without disintegrating it (Gaytán--Martínez et al., 2011).

Therefore, it could be assumed that the 5% moringa bread remained firm for the 6 days. For the control and 10% moringa breads, the hardness behavior was higher on day 3 and decreased on day 6. These results presented a behavior like reported by Chia-Ling et al. (2012), who evaluated the hardness of bread made with wheat flour, rice, and rice porridge at different storage days. In cohesiveness, which represents the limit up to which a material can deform, indicating the force between the internal bonds of the material during compression, there was no significant difference ($\rho > 0.05$) on day 0, between the different types of bread. However, for 1.25% moringa bread, cohesiveness decreases significantly with increasing days of storage. It is also observed (Fig. 4) that in control bread and 10% moringa bread, cohesiveness decreases from day 0 to day 3 by 8 and 10%, respectively, while from day 3–6, the cohesiveness increases by 18 and 15%, respectively. Analyzing the cohesiveness results of these two breads with those obtained in hardness, it is shown that an opposite behavior occurs, that is, the hardness in the breads (control and with 10% moringa) increases from day 0 to day 3 and decreases in the day 6. These results indicate that for the day 3 the breads tend to lose moisture and therefore the hardening of the bread during storage may be caused by the migration of moisture from the bread crumb to the crust (Baik and Chinachoti, 2000). Other authors indicated that when the humidity decreases, the formation of cross links between starch and protein is accelerated, therefore the firmness of the bread is greater (He and Hoseney, 1990). But as the cohesiveness decreases (Fig. 4.), the internal force between these bonds is weaker. Now then, on day 6 the hardness decreases which is mainly attributed to the possible presence of microorganisms. Fungi are the main microorganisms that deteriorate bread, mainly due to its composition (humidity, around 40%, water activity 0.94 to 0.98 and intermediate acidity pH 5.5 to 6.0, as well as storage conditions, which influences life useful of bread that is between 3 and 7 days, if the bread does not contain preservatives (Saranraj and Geetha, 2012; García et al., 2019). This fact is attributed to those breads made for this research do not contain preservatives or additives that could capture water, then during the hardening of the bread the migration of water towards the crust occurred. In this regard, Smith et al. (2004), mention that the condensation of water on the bread creates conditions for the growth of fungi, such as *Neurosporacrassa* spp and *Penicillum* spp. From the results shown, it is observed that the 5% moringa bread was the one that did not show changes in hardness during the days of storage. In his research, Bolarinwa et al. (2019), showed that the sensory evaluation carried out on bread with wheat flour and bread fortified with 5% moringa seed powder were very similar in all the evaluated attributes, such as firmness and texture. These results suggest that the moringa plant at certain concentrations of addition to bread is a viable alternative to fortify and prolong the shelf life of wheat bread. Regarding the elasticity that represents the height recovery of the food between the first and second compression cycles and evaluates how much structure of the food has been broken by the initial compression (Rosenthal, 1999), there was no significant difference for the 1.25% moringa bread between days of storage. Likewise, elasticity increased significantly in control bread and moringa bread at 2.5, 5 and 10% on day 6. This gives an indication that on day 6 there was a higher recovery of the material when it was subjected to the first compression cycle, which indicates changes in the structure of the bread due to possible chemical and microbiological reactions. Regarding chewiness, the work required for the disintegration of the bread, and it can be swallowed (Civille and Szczesniak 1976; Szcsesniak, 2002) was the 10% moringa bread (65 mJ) on day 3, which had the highest value (Fig. 4d). Sengev et al. (2013) reported that by increasing the percentage of moringa powder in bread, the texture of the crumb is less acceptable by a group of panelists. Therefore, it is not recommended to add high percentages of the moringa plant in the bread formulation. In this research, there was a coincidence with respect to what was reported by other authors. It was not recommended to add 10% of moringa, since the quality of the structure is lost, likewise, a greater quantity of fungi developed, since when the moringa interacted with other ingredients of the bread, at the temperature (25–26 °C) at which the fungi test experiment was established.

3.4. Principal component analysis

Fig. 6a shows the plot of the PCA, it is possible to observe that 4 groups (clusters) of data were formed, where only one group showed two types of bread with similarities, which corresponds to the types of wheat bread elaborated with 0 and 1.25% of moringa concentration (CB and MBa). Note that the similarities of these two types of bread are due to a similar behavior of the variables: PA300, PA330, PA370, PA450, PA650, chewiness and concentration. The other three clusters formed (2-bread (2.5% of MP), 3-bread (5%) and 4-bread (10%)) had no similarities in behavior of variables. Note, according to the PCA (quadrant 3), moringa bread (10%) is characterized by the existence of fungal colonies for the days evaluated (4 and 6). Conversely, for the bread of cluster 1 (2.5% of MP), showed opposite behavior (fewer fungal colonies). Regards the correlations of the variables, it is possible to observe that the variables concentration MP and amplitude of the photoacoustic signal (at 300 and 330 nm) and chewiness are highly correlated, since they form an angle of less than 45° between them. On the other hand, the variables of the number of fungal colonies on the fourth and sixth day are more correlated with the variables related to the values of photoacoustic signals at 300, 330 and 515 nm and the concentration of moringa of the breads.

Finally, the PA signal (normalized) at 330, 330 and 370 nm was also related with the concentration of bread by linear equation of the correlation and the intensity of linear correlation (r). Obtaining the equation: $y = ax + b$; where a = 11.05, 11.12, 11.42; b = −0.97, −0.90, −2.45; and R^2 = 0.99, 0.98, 0.94 (see Fig. 6b). Fig. 5b shows that when the concentration of MP increases, the photoacoustic signal value at 300, 330 and 370 nm is increased. In this way, there is a relationship between these values. It can be observed that the increase in the concentration of bread mainly improves its content of phenolic acids and flavonoids, according to the photoacoustic spectra obtained. In this sense, Moringa bread can be consumed by the population as a type of nutraceutical bread.

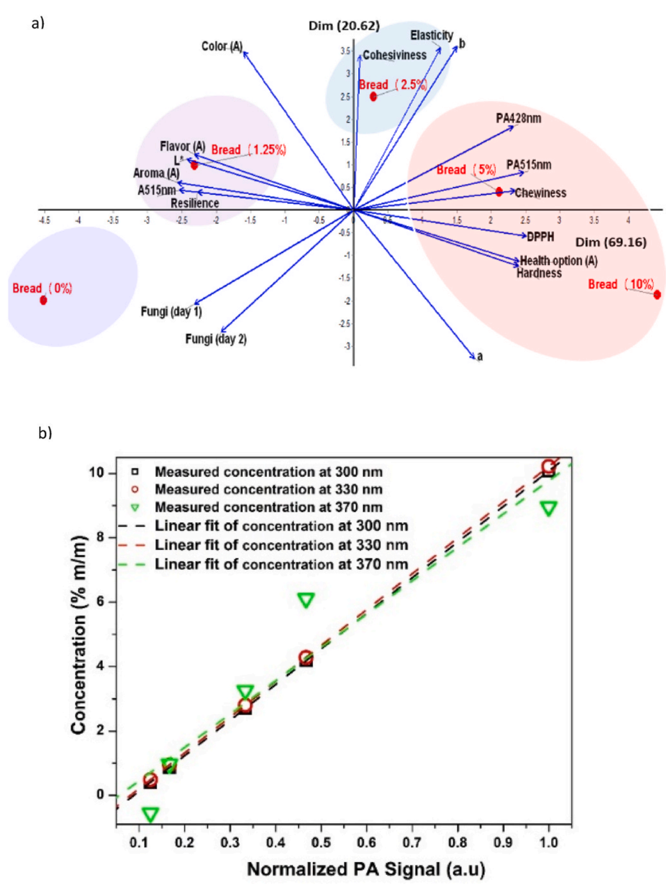


Fig. 6. Principal component analysis and correlation: a) Principal component analysis (Dim 1 (69.16%) x Dim 2 (20.62%)) of the variables evaluated in the bread samples at 0, 1.25, 2.5, 5 and 10%. b) Correlation between the normalized PA signal at 300, 330 and 370 nm of the sample with its concentration.

4. Conclusions

The photoacoustic spectra of wheat bread mixed with *Moringa oleifera* at different concentrations (0, 1.25, 2.5, 5 and 10%) have been recorded and analyzed for wavelengths from 300 to 650 nm. Significant statistical differences between the control bread and the moringa-added ones have been observed in the region of 300–450 nm mainly. it has been shown that: i) The photoacoustic signal amplitude of bread increases with the moringa concentration, such that its values increase from 37% to 90% when moringa powder concentration raises from 1.25% to 10%, at 300 nm wavelength. ii) The strongest positive correlation between the photoacoustic signal and the number of fungal colonies in bread appears at the wavelengths of 300, 330, 370 and 515 nm iii) The sanitary quality of bread mixed with a 2.5% of moringa is relatively higher than the ones obtained for other concentrations, such that the number of fungal colonies were reduced by 99% in comparison with the control bread without moringa, after six days of storage. Moringa at 2.5% of concentration could hence improve the sanitary quality of wheat bread. iv) The texture of bread changes over time, such that slower variations show up for higher concentrations of moringa. Bread with 10% of moringa is thus not recommended, as it does not maintain an adequate texture over time.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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