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Original Article

Effect of Traditional Nixtamalization and Extrusion Processes on Bioaccessibility and Antioxidant Activity of Phenolic Compounds in Blue Maize Tortillas During *In Vitro* Fermentation by Human Colonic Microbiota

Efecto del Proceso de Nixtamalización Tradicional y Extrusión sobre la Bioaccesibilidad y Actividad Antioxidante de Compuestos Fenólicos de Tortillas de Maíz Azul Durante la Fermentación *In Vitro* por la Microbiota Colónica Humana

Alma Haydee Astorga-Gaxiola[1], Cuauhtémoc Reyes-Moreno[2,3], Maribel Jiménez-Edeza[2], Edith Oliva Cuevas-Rodríguez[2,3] and Saraid Mora-Rochín [2,3]*

[1] Facultad de Ciencias de la Nutrición y Gastronomía, Universidad Autónoma de Sinaloa, Av. Cedros S/N y Calle Sauces, Los Fresnos Fracc, C.P. 80019 Culiacán Rosales, Sinaloa, México.
[2] Programa de Posgrado Integral en Biotecnología, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Ciudad Universitaria, Culiacán, Sinaloa, C.P. 80030, México.
[3] Maestría en Ciencia y Tecnología de Alimentos, Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Ciudad Universitaria, Culiacán, Sinaloa, C.P. 80030, México.

ABSTRACT

In recent years, tortillas made with pigmented maize have garnered interest due to their contribution of polyphenols, considered natural antioxidant compounds with antihypertensive, antidiabetic, and anti-carcinogenic properties. In maize, the greatest contribution of polyphenols is in insoluble form. These secondary metabolites are released by the colonic microbiota making them more bioaccessible to the organism. In the present work, chemical composition, bioaccessibility, release of phenolic compounds, and antioxidant activity by colonic microbiota in tortillas, made with nixtamalized (NMT) and extruded (EMT) blue maize, were evaluated and compared. EMT had higher protein, lipid, and ash content than NMT. However, NMT had higher anthocyanin content than its EMT counterpart (4.01 and 2.28 mg CGE/100 g, dw). The bound phenolic fraction in both tortillas represents > 80 %. At 5 h of *in vitro* fermentation by colonic microbiota, NMT showed the highest average in phenolic release, bioaccessibility, and antioxidant activity [11.78 mg GAE/ g, dw, 88.23 %, and 569.82 (ORAC) and 26.76 (ABTS) µmol TE/g] than EMT. The use of traditional nixtamalization to produce maize tortillas will continue to be the main process that brings health benefits to consumers.

Keywords: Tortillas, blue maize, phenolics, bioaccessibility, antioxidants.

RESUMEN

En los últimos años las tortillas elaboradas con maíces pigmentados han despertado interés debido al aporte de polifenoles, los cuales son considerados antioxidantes naturales y poseen propiedades antihipertensivas, antidiabéticas, y anticarcinogénicas. En maíz, el mayor aporte de polifenoles se encuentra en forma insoluble. Estos metabolitos secundarios son liberados por la microbiota del colón haciéndolos más bioaccesibles para el organismo. En el presente trabajo se evaluó y comparó composición química, bioaccesibilidad, liberación de compuestos fenólicos y actividad antioxidante por la microbiota colónica en tortillas elaboradas con maíz azul nixtamalizado (NMT) y extrudido (EMT). Las EMT presentaron mayor contenido de proteína, lípidos y cenizas, con respecto NMT. Sin embargo, NMT presentó mayor contenido de antocianinas que su contraparte EMT (4.01 y 2.28 mg ECG/100 g, bs). La fracción de fenólicos ligados en ambas tortillas representa > 80 %. A las 5 h de fermentación *in vitro* por la microbiota del colon, NMT mostró el mayor promedio de liberación y bioaccesibilidad de fenólicos, y actividad antioxidante [11.78 mg EAG/g, bs, 88.23 %, y 569.82 (ORAC) y 26.76 (ABTS) µmol ET/g] que EMT. El proceso de nixtamalización tradicional para elaborar tortillas seguirá siendo clave importante para el aporte de beneficios en la salud de los consumidores.

Palabras clave: Tortillas, maíz azul, fenólicos, bioaccesibilidad, antioxidantes.

INTRODUCTION

Nowadays, products elaborated with pigmented maize, such as blue maize tortillas, have received considerable attention from a health benefit perspective due to their high content of secondary metabolites, such as phenolic compounds, anthocyanins, and carotenoids (Mora-Rochin *et al*., 2016; Colín-Chávez *et al*., 2020). These compounds have been shown to possess potential for the prevention of chronic noncommunicable diseases, such as cancer, arterial hypertension, and diabetes, through their antioxidant capacity (Mora-Rochin *et al*., 2019; Domínguez-Hernández *et al*., 2022). However, this property, provided by these secondary metabolites or phytochemicals present in maize kernels, depends not only on their concentration but also on their bioaccessibility after

*Autor para correspondencia: Sara Monzerrat Ramírez Olvera
Correo-e: **smora@uas.edu.mx**
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ingestion (Astorga-Gaxiola *et al*., 2023; Menchaca-Armenta *et al*., 2023).

Maize in Mexico is processed mainly by the ancestral method inherited from the Aztecs, nixtamalization, where the maize kernel is put in water with lime, cooked at high temperatures, and left to stand for long periods of time. These characteristics of the process significantly change the nutritional content, concentration of phytochemicals, antioxidant properties, as well as the bioaccessibility of different biomolecules (Enriquez-Castro *et al*., 2020). On the other hand, grain cooking using extrusion technology has been used as an effective processing method to improve the nutritional quality of cereals and legumes, where it has been reported that extrusion modifies physicochemical properties of insoluble fiber, resulting in an increase in soluble fiber due to the shearing forces. Additionally, protein inhibitors and other anti-nutritional factors (trypsin inhibitors, tannins, oxalates, and lectins) are inactivated or denatured, and it has been reported that extrusion significantly modifies the content of phytochemicals with antioxidant properties (Choton *et al*., 2020; Kamau *et al*., 2020; Bonilla-Vega *et al*., 2022).

In maize, phenolic compounds are bound to cell wall structures, mainly in the pericarp (> 80 %), which inhibits their digestion in the upper part of the gastrointestinal tract (Méndez-Lagunas *et al*., 2020; Astorga-Gaxiola *et al*., 2023). Thus, upon reaching the colon, these compounds are metabolized by the colonic microbiota, allowing their release, and their conversion into smaller secondary metabolites and subsequent absorption, achieving local and systemic beneficial effects (Tomás-Barberán and Espín, 2019; Astorga-Gaxiola *et al*., 2023). Phenolic compounds regulate the composition and functionality of gut microbiota, which can have significant implications for human health. Gut bacteria perform various essential roles, including food fermentation, pathogen defense, immune system stimulation, and vitamin synthesis. Foods rich in phenolic compounds may exhibit effects comparable to those of classical prebiotics. Additionally, the metabolites derived from phenolic transformation contribute to regulating redox balance and metabolism, while demonstrating antioxidant, antimicrobial, anti-inflammatory, and anticancer properties (Hou *et al*., 2022; Kwon *et al*., 2024).

It is important to mention that, to date, there is little scientific evidence in regard to the effect of the different processes for the elaboration of blue maize tortillas on the structure of the food matrix that facilitates the metabolization of phenolic compounds by the colonic microbiota. Therefore, the objective of the present work is to evaluate and compare the bioaccessibility, the release of phenolic compounds by the colonic microbiota, and antioxidant activity in blue maize tortillas made by the ancestral process of nixtamalization and by the extrusion process.

MATERIAL AND METHODS

Materials

Whole blue maize was sourced from a local market in Culiacán, Sinaloa, Mexico. Seeds were cleaned and stored in airtight containers at 4 °C until use.

Production of blue maize flour by traditional nixtamalization

The flour was obtained with the procedure described by Mora-Rochín *et al*. (2010). Batches of 100 g of blue maize were cooked for 30 min in a lime solution [5.4 g Ca $(OH)_2$/L distilled water] at 85 °C, using a grain to water ratio of 1:3 (w/v) and set to stand for 8 h. After the setting period, the cooking liquid (nejayote) was removed, and the nixtamalized grain was washed under running water to remove excess lime and pericarp. The nixtamal was dried at 55 °C for 12 h in a forced air oven. After cooling for 30 min at room temperature, it was ground using a cyclone mill with an 80 mesh (0.180 mm) sieve. The resulting flour was stored at 4 °C until use.

Production of blue maize flour by extrusion

The methodology proposed by Milán-Carrillo *et al*. (2006) was followed. Briefly, 500 g of blue maize seeds were ground and passed through a 40 US mesh (0.074 mm) sieve. The resulting flour was mixed with lime (0.21 g Ca $(OH)_2$/100 g flour). H_2O was added until 28 % moisture was achieved. The extrusion cooking process was conducted using a 20 DN extruder (CW Brabender Instruments, Inc, NJ, USA) with a single strand of 19 mm in diameter. The operating conditions were: a temperature of 85 °C and a screw speed of 240 rpm. The extrudates were cooled to room temperature and ground to 80-US mesh (0.180 mm). Finally, everything was stored in polyethylene bags at 4 °C until further use.

Preparation of blue maize tortillas

The protocol described by Mora-Rochín *et al*. (2016) was followed. Four hundred g of flour and 400 mL of water were mixed until a dough of suitable consistency was obtained. Small portions of dough (30 g) were pressed and shaped into flat discs (15 cm) using a manual press (Casa Herrera, Mexico DF, Mexico). The disks were baked on a hot griddle (270 ± 10 °C) for 15 s on one side and 30 s on the other side. Finally, the first side was cooked again until a puffed tortilla was observed. The fresh tortillas were dried and ground (UD Cyclone Sample Mill, UD Corp. Boulder, CO, USA) to pass through an 80-US mesh sieve (0.180 mm). The resulting tortilla flours were stored in plastic bags at - 20 °C until use.

Proximate chemical composition

The proximate chemical composition was determined according to AOAC (2005) standards. Moisture was obtained by drying the samples at 105 °C for 24 h. Protein content was determined using the micro-Kjeldahl method (Nx6.25). Lipid content was determined using a Soxhlet apparatus, with petroleum ether as solvent, and ash was determined incinerating the samples at 550 °C.

Determination of free and bound phenolic compounds

Extractions were performed following the protocol by Mora-Rochín *et al*. (2010). In summary, the free fraction was obtained from 1 g of flour. The free fraction was added to 10 mL of a cold ethanol-water mixture (80:20, v/v), stirred for 10

min and subsequently centrifuged at 2500 *x g* for 10 min. The supernatant was concentrated using a rotary evaporator at 45 °C and low pressures. The bound phenolic fraction was obtained from the residue of the initial extraction, where 10 mL of NaOH (2 mol/L) were added in a water bath at 95 °C for 30 min, followed by stirring at room temperature for 1 h. The mixture was acidified with concentrated HCl, and stirred for 30 min. Hexane was added for lipid removal. The residue was subjected to 5 extractions with ethyl acetate, evaporated at 45 °C, and finally reconstituted with 50 % methanol. The free and bound phenolic extracts were stored at -20 °C. The phenolic compound content of the different fractions was quantified by the Folin-Ciocalteu method described by Singleton *et al*. (1999). The results were expressed in milligram gallic acid equivalents (GAE) per 100 g dry weight (dw).

Antioxidant capacity

ORAC and ABTS assays were performed as described by Re *et al*. (1999) and Mora-Rochín *et al*. (2010). For the measurement of this property by the ORAC method, peroxyl radicals were generated using the AAPH reagent, and the loss of fluorescence caused by the free radicals was recorded in a microplate reader (Synergy HT microplate multi-detection reader; BioTek Instruments, Inc., Winooski, VT, USA). Excitation and emission absorbance measurements were recorded at 485 and 538 nm, respectively.

The ABTS radical ($ABTS^{+}$) method was performed by the oxidation of 2 mM ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution for 12 h. Absorbance of all samples with the $ABTS^{+}$ radical was performed at 734 nm on a multi-detection microplate reader Synergy HT (BioTek Instruments, Inc., Winooski, VT, USA) 6 min after initial mixing. A Trolox standard curve was used as a control in both assays. The antioxidant capacity was reported as µmol Trolox equivalents (TE) per g dry weight (dw).

***In vitro* fermentation of blue maize tortilla flours by colonic microbiota**

The *in vitro* fermentation experiments for the nixtamalized and extruded blue maize tortilla flours were carried out as described by Campos-Vega *et al*. (2009), with minor modifications. Most of the soluble phenolics in the tortilla samples used for fermentation were removed by a wash treatment with cold ethanol-water (80:20, v/v).

Fresh fecal samples were donated by four healthy individuals (marked with letters A-D) ranging in age from 18 to 28 years, with no previous reports of intestinal diseases or antibiotic treatment for at least 3 months. Fecal samples were preserved in sterile containers within 2 h of collection. Two g of each sample were homogenized with 18 mL of 0.1 mol/L sodium phosphate buffer (pH 7.0). This fecal inoculum was used as a fermentation starter. Sterile tubes (15 mL) were filled with 9 mL of sterile basal culture medium. The tubes were sealed and kept under H_2-CO_2-N_2 conditions (10:10:80, by volume), free of O_2 for 24 h. The tubes were inoculated with 1 mL of fecal matter and 0.1 g of tortilla flour, except for the blanks. The samples were shaken for 30 s and placed in a 37 °C water bath. Meanwhile, two different controls were performed under the following conditions: (i) the flour was incubated in buffer solution without feces to determine the possible release of phenols, and (ii) the fecal suspension was incubated without flour as a negative control. The samples and controls were collected at 0, 1, and 5 h. Fermentation was terminated by placing the tubes in a -70 °C freezer. The phenolics released by fermentation and the antioxidant activity were analyzed by the aforementioned assays.

Percentage of bioaccessibility

The percentage of phenolic compounds (PC) that could be available for uptake following the *in vitro* fermentation was obtained using the following formula (Blancas-Benítez *et al*., 2018):

$$\%\ B = \frac{(PC\ RCF) - (SPC)}{(PC\ RCF) + (IPC)} \mathbf{X\ 100} \qquad \text{Eq (1)}$$

Where the PC released in colonic fermentation is PC RFC; the PC associated with the soluble fraction is SPC, and the PC of the insoluble fraction is IPC. The quantification of each phenolic fraction was calculated following said methodology.

Statistical analysis

Experiments were performed in triplicate, and data were presented as mean ± standard deviation (SD). Comparisons between means were analyzed using Graphpad Prism v8 statistical software. An analysis of variance (ANOVA), followed by a Tukey test, were carried out to evaluate the differences between the means of the measured parameters. A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Proximate chemical analysis of NMT and EMT

The results of the proximate chemical analysis of tortillas made with nixtamalized (NMT) and extruded blue maize flour (EMT) are shown in Table 1. The results show similar moisture values ($p > 0.05$) among tortillas. However, EMT presented the highest values ($p < 0.05$) for protein (9.31 ± 0.06), lipids (2.03 ± 0.20), and ash (1.41 ± 0.09), while NMT showed the highest value ($p < 0.05$) for carbohydrate content (83.34 ± 0.08).

The higher protein content in EMT compared to NMT, is due to the protein denaturation and the improvement of protein digestibility caused by the high temperatures and

Table 1. Proximate chemical analysis of nixtamalized and extruded blue maize tortillas.
Tabla 1. Análisis químico proximal de tortillas de maíz azul nixtamalizado y extrudido.

Tortillas	Humidity	Proteins	Lipids	Ash	Carbohydrates
NMT	48.0 ± 0.0^{a}	9.0 ± 0.20^{b}	1.38 ± 0.10^{b}	1.36 ± 0.05^{b}	83.34 ± 0.08^{a}
EMT	48.0 ± 0.0^{a}	9.31 ± 0.06^{a}	2.03 ± 0.20^{a}	1.41 ± 0.09^{a}	82.63 ± 0.10^{b}

Means ± SD. Consecutive means not sharing the same letter are significantly different ($p < 0.05$). The results are expressed as percentage (%). NMT= nixtamalized maize tortilla, and EMT= extruded maize tortilla.

shearing forces during the extrusion cooking process, which lead to anti-nutritional factors inactivation (such as phytates, tannins, hemagglutinins, and trypsin inhibitors) and thermal unfolding of proteins, changes that enhance bioavailability of sulfur amino acids, as they increase the surface area accessible to enzymatic action (Kamau *et al*., 2020). On the other hand, during the traditional nixtamalization process to produce tortillas, several physical and chemical transformations occur in the kernels during alkaline cooking and subsequent soaking. This process improves the protein quality, niacin availability, and calcium content of the grain while softening the pericarp of the seed. The increased pH and high cooking temperatures help grains humidify, soften their endosperm, which leads to pericarp release. Therefore, proteins and other components of the maize kernel, such as soluble carbohydrates, dietary fiber, and vitamins, are leached into the cooking liquid, causing their loss by removing the nejayote, leaving the nixtamalized maize products nutritionally deficient (Cervantes-Ramirez *et al*., 2020; Kamau *et al*., 2020; Hassan *et al*., 2023).

Gámez-Valdez *et al*. (2021) evaluated the proximate composition of extruded and nixtamalized blue maize tortillas fortified with amaranth. These authors observed that tortillas made with extruded flours showed higher protein (10.08 and 7.83 %) and lipid (6.11 and 2.40 %) contents compared to tortillas made with nixtamalized flours. In addition, protein content in maize kernels could be influenced by such factors as endosperm type, kernel hardness, genetics, climatic conditions, soil type, and agricultural practices employed (Sánchez-Nuño *et al*., 2024). Furthermore, plant fertilization with nitrogen, or the abundant presence of this compound in the soil, increases the total protein content in plant and grain (Salinas-Moreno *et al*., 2017).

On the other hand, it can be observed that EMT has the highest lipid content with respect to the nixtamalized tortilla (2.03 and 1.38 %). The lower lipid content in nixtamalized products is determined in part by different times and temperatures used in cooking the maize kernels, as well as losses of fractions such as pericarp and germ (the fraction with the highest lipid content in maize) during soaking and washing of the kernels (Hassan *et al*., 2023). In contrast, the saponification of the oils contained in the maize kernel, due to the effect of the alkali used during the nixtamalization process, causes a decrease in lipids (Salinas-Moreno *et al*., 2017; Escalante-Aburto *et al*., 2020).

With regard to ash content, a significant part of the ash present in maize tortilla comes from calcium incorporated during the nixtamalization process, which is accumulated mainly in the pericarp and germ fraction of the nixtamalized grain (Salinas-Moreno *et al*., 2017). Part of these components, however, are partially lost during the soaking and washing stages of the nixtamal while the maize kernel is processed integrally during extrusion. Lime is added to obtain the same flavor as flour obtained by the traditional nixtamalization (Aguayo-Rojas *et al*., 2012). In this study, it can be observed that the tortilla made with extruded blue maize flour has the highest ($p < 0.05$) ash content (1.41 %) compared to the tortilla made with the traditional nixtamalization process (1.36 %).

The carbohydrate values present in this study coincide with those reported by Escalante-Aburto *et al*. (2020), who mention that the total carbohydrate content of traditionally processed tortillas ranges from 44.08 to 87.56 %. However, these values depend mainly on the type of maize used and the nixtamalization conditions. Total carbohydrate content shows a complex behavior during the transformation of maize kernels to tortilla; it can increase, remain constant, or decrease due to excessive loss of starch, lipids, proteins, and pericarp components during cooking, soaking, and washing (Escalante-Aburto *et al*., 2020). Several studies have indicated that nixtamalized samples contain higher levels of amylose compared to raw corn starch, which is attributed to the depolymerization of amylopectin during the nixtamalization process. Some authors proposed that the rise in amylose content in pigmented samples from raw corn to nixtamal results from the separation of amylose and anthocyanins. This separation occurs due to the breaking of ester bonds, which are unstable in alkaline conditions (Serna-Saldívar, 2021; Enríquez-Castro *et al*., 2022).

Bioactive compounds

The blue color of the different varieties of maize is the result of the presence of water-soluble phytochemicals known as anthocyanins, which have a high capacity to eliminate and/or neutralize free radicals and reactive oxygen species, chelate metals, control signaling pathways, and reduce proinflammatory markers. As a result, they reduce the risk of cardiovascular pathologies, cancer, neurodegeneration, among others (Sánchez-Nuño *et al*., 2024).

Table 2 shows the results of anthocyanins and phenolic compounds content (free, bound and total) of blue maize tortillas, obtained by traditional nixtamalization and an alternative technology such as extrusion cooking. The tortilla made with nixtamalized blue maize (NMT) (4.01 ± 0.09 mg CGE/100 g, dw) presented the highest value ($p < 0.05$) of anthocyanins compared to the tortilla made with extruded blue maize (EMT) (2.28 ± 0.15 mg CGE/100 g, dw). The lower

Table 2. Bioactive compounds present in nixtamalized and extruded blue maize tortillas.
Tabla 2. Compuestos bioactivos presentes en tortilla de maiz azul nixtamalizado y extrudido.

		Phenolic Compounds[2]		
Tortillas	**Anthocyanins[1]**	**Free**	**Bound**	**Total**
NMT	4.01 ± 0.09[a]	32.30 ± 3.45[a] (20 %)	125.8 ± 16.01[b] (80 %)	158.1 ± 5.2[c]
EMT	2.28 ± 0.1[5]b	24.42 ± 2.89[b] (9 %)	247.0 ± 23.48[a] (91 %)	271.42 ± 3.2[a]

Means ± SD. Consecutive means not sharing the same letter are significantly different ($p < 0.05$). [1] mg CGE/100 g dw; [2] mg GAE/100 g, dw. The values in parentheses represent the contribution of this fraction to the total content. NMT= nixtamalized maize tortilla, and EMT= extruded maize tortilla.

anthocyanin content in EMT can be attributed to the conditions used (temperature and screw speed) during extrusion, as well as to the moisture content of the raw material. High temperatures during extrusion lead to glycosylating sugar loss with consequent opening of the anthocyanin ring and production of colorless chalcones (Ruíz-Gutiérrez *et al*., 2018; Menchaca-Armenta *et al*., 2023).

It is important to mention that the blue maize was fragmented before being fed to the extruder, which caused an increase in the contact area of the anthocyanins with the high temperatures used during extrusion, causing a greater loss of this secondary metabolite (Aguayo-Rojas *et al*., 2012; Menchaca-Armenta *et al*., 2023). In contrast, our results are lower than those reported by Bonilla-Vega *et al*. (2022) who evaluated the effect of different extrusion conditions on blue maize anthocyanins. The authors observed values ranging from 6.37 (T: 170 °C and SS: 145 rpm) to 26.04 (T: 68 °C and SS: 78 rpm) mg CGE/100 g dw, depending on the temperature and screw speed used. Similarly, our values are lower than those reported by Colín-Chávez *et al*. (2020), who show an anthocyanin content of 21.80 mg CGE/100 g dw, in nixtamalized blue maize tortillas.

A diet rich in phenolic compounds has been linked to the prevention of different diseases (different types of cancer, neurodegenerative, and cardiovascular diseases). Of the cereals consumed worldwide, maize has the highest content of phenolic compounds, mainly in their insoluble form or bound to cell wall materials. However, maize must be processed prior to human intake, so it is of vital importance to understand the effects of processing on its nutritional value and bioactive compound content (Butts-Wilmsmeyer *et al*., 2018).

The content of NMT and EMT bound phenolic compounds accounted for 80 and 91 % of the total phenolics present in the tortillas, respectively. These values are in agreement with those reported by Roccheti *et al*. (2022) who mention that most of the phenolic compounds present in cereals are bound, constituting approximately 85 % of the total phenolics present in maize. In contrast, there are reports that mention that different processes applied to foods can enhance the release of bound phenols, among them are fermentation and malting, as well as thermomechanical processes such as extrusion cooking (Roccheti *et al*., 2022). EMT presented the highest content of phenolic compounds in the bound (91 %) and total fraction (247.0 ± 23.48 and 271.42 ± 3.2 mg GAE/100 g, respectively) when compared to NMT (125.8 ± 16.01 and 158.1 ± 5.2 mg GAE/100 g, respectively).

The results found in this study agree with those reported by Gámez-Valdéz *et al*. (2022), who in a study with commercial blue maize tortillas found values of free and bound phenolic compounds of 61.85 ± 0.58 and 134.59 ± 0.83 mg GAE/100 g, dw, and in extruded blue maize tortillas of 64.18 ± 0.37 and 218.43 ± 2.83 mg GAE/100 g, dw, respectively. Similarly, our results are consistent with those reported by Bonilla-Vega *et al*. (2022) who evaluated 13 extrusion conditions in a blue maize variety, observing values of total phenolic compounds from 268.06 to 330.53 mg GAE/100 g, dw. The extrusion conditions of this study, nonetheless, differ from those reported by these authors who observed the highest phenolic compound content (330.53 mg GAE/100 g, dw) at a temperature of 50 °C and a screw speed (SS) of 145 rpm. In contrast, the lowest content (268.06 mg GAE/100 g, dw) was obtained at 152 °C and 78 rpm, leading to the conclusion that lower temperatures and higher SS yield the highest total phenolic content.

During extrusion cooking, the cell wall is disrupted, exposing high molecular weight complex phenolic compounds to lower molecular weight compounds, resulting in higher extractability of phenolic compounds. However, during this process, the phenolic compound content is also influenced by the origin of the raw material and numerous variables, such as raw material moisture, screw speed, and extruder configuration, i.e., die and screw size, temperature, and exposure time of the sample inside the extruder (Šárka *et al*., 2021). Likewise, it is important to mention that during extrusion cooking, the maize kernel is processed integrally, and no loss of anatomical parts and leaching of compounds are generated, leaving the extruded products with a higher content of phenolic compounds than those obtained by traditional nixtamalization, in which partial loss of the pericarp (fraction with more than 80 % of the phenolics present in the maize kernel) occurs, and phenolics sensitive to thermal (≥ 85 °C) and alkaline (pH ≥ 12) conditions are reduced, respectively (Dewanto, 2002; Kamau *et al*., 2020; Astorga-Gaxiola *et al*., 2023).

Release of phenolic compounds and bioaccessibility during colonic fermentation

Table 3 and 4 show the values of phenolic compounds released by the colonic microbiota from the different donors and the percentage of bioaccessibility, respectively. Tables 3 and 4 show a similar behavior, in which the longer the interaction time between bacteria (contained in feces) from the colon and tortilla flours made with nixtamalized and extruded blue maize, the higher the content of phenolic compounds and their bioaccessibility. At the initial time (0 h) of the evaluation of phenolic compounds released, values were observed between donors (EMT from 0.01 to 0.07 mg GAE/g, and NMT from 0.01 to 0.08 mg GAE/g), while in the bioaccessibility of phenolics no activity could be detected. Interestingly, donors C and D show the highest release of phenolics. In contrast, low release and bioaccessibility values were observed, which may be due to the fact that the interaction time was not sufficient for the microbiota contained in the fecal samples to carry out the process of metabolizing the phenolic compounds of the bound or insoluble fraction contained in the different tortillas under study. This behavior is similar to that reported by Blancas-Benítez *et al*. (2018) who were able to observe a phenolic release of 4 to 6 mg GAE/g after 20 min of colonic microbiota interaction in guava flours.

During the 1 h fermentation period of this study, a greater release activity of phenolic compounds by bacteria

Table 3. Phenolic compounds released during *in vitro* colonic fermentation of nixtamalized and extruded blue maize tortillas.
Tabla 3. Compuestos fenólicos liberados durante la fermentación colónica *in vitro* de tortillas de maíz azul nixtamalizado y extrudido.

	NMT			EMT		
	Fermentation time (h)					
DONORS	0	1	5	0	1	5
A	0.01 ± 0.01^{cxB}	2.56 ± 0.06^{byB}	10.78 ± 0.27^{ayC}	0.01 ± 0.01^{cxB}	3.44 ± 0.12^{bxA}	13.61 ± 0.25^{axB}
B	0.03 ± 0.02^{cxB}	2.70 ± 0.04^{bxA}	13.81 ± 0.08^{axB}	0.02 ± 0.01^{cxB}	0.67 ± 0.03^{byD}	4.09 ± 0.06^{ayD}
C	0.05 ± 0.04^{cxA}	1.23 ± 0.05^{byD}	7.71 ± 0.05^{axD}	0.07 ± 0.04^{cxA}	1.61 ± 0.02^{bxC}	7.57 ± 0.02^{axC}
D	0.08 ± 0.03^{cxA}	2.32 ± 0.02^{byC}	14.83 ± 0.15^{axA}	0.05 ± 0.03^{cxA}	2.62 ± 0.18^{bxB}	14.38 ± 0.13^{ayA}
Average	0.042	2.20	11.78	0.037	2.08	9.91
SD	0.03	0.66	3.21	0.02	1.20	4.93

Means ± SD at 0, 1 and 5 hours (h) of fermentation. Results are the average of at least 3 independent experiments, expressed as mg GAE/g. Consecutive means not sharing the same letter are significantly different ($p < 0.05$). $^{a-c}$ Comparison of means between times of the same flour. $^{x-z}$ Comparison of means between the same time of the different tortillas, $^{A-C}$ Comparison of means between donors of each time and tortilla. NMT= nixtamalized maize tortilla, EMT: extruded maize tortilla.

Table 4. Bioaccessibility (%) of phenolic compounds during the *in vitro* colonic fermentation in nixtamalized and extruded blue maize tortillas.
Tabla 4. Bioaccesibilidad (%) de los compuestos fenólicos durante la fermentación colónica *in vitro* en tortillas de maíz azul nixtamalizado y extrudido.

	NMT			EMT		
	Fermentation time (h)					
DONORS	0	1	5	0	1	5
A	ND	59.97 ± 0.01^{bxB}	87.97 ± 0.02^{axC}	ND	40.3 ± 0.02^{byB}	74.96 ± 0.23^{ayC}
B	ND	60.45 ± 0.02^{bxA}	88.83 ± 0.21^{axB}	ND	20.53 ± 0.01^{byD}	63.53 ± 0.22^{ayD}
C	ND	43.73 ± 0.01^{bxD}	85.07 ± 0.15^{axD}	ND	38.4 ± 0.01^{byC}	76.53 ± 0.12^{ayB}
D	ND	59.49 ± 0.03^{bxC}	91.05 ± 0.05^{axA}	ND	48.38 ± 0.01^{byA}	83.87 ± 0.15^{ayA}
Average	ND	55.91	88.23	ND	36.90	74.72
SD	ND	8.12	2.47	ND	11.74	8.41

Means ± SD at 0, 1 and 5 hours (h) of fermentation. Results are the average (%) of at least 3 independent experiments. Consecutive means not sharing the same letter are significantly different ($p < 0.05$). $^{a-b}$ Comparison of means between times of the same flour. $^{x-z}$ Comparison of means between the same time of the different tortillas, $^{A-C}$ Comparison of means between donors of each time and tortilla. ND= not detected, NMT= nixtamalized maize tortilla, EMT: extruded maize tortilla.

from the colon microbiota was observed, where EMT showed values from 0.67 to 3.44 mg GAE/g, and NMT from 1.23 to 2.70 mg GAE/g, being donor A in EMT, and donor B in NMT the ones that presented the highest phenolic release. During this fermentation time, bioaccessibility of the phenolics released in the different tortillas under study was observed. Bioaccessibility percentages were higher in NMT with values ranging from 43.73 to 60.45 %, while EMT presented values ranging from 20.53 to 48.38 %.

Finally, at 5 h both tortillas presented the highest phenolic release and bioaccessibility (EMT = 7.57 to 14.38 mg GAE/1 g, 63.53 to 83.87 % and NMT = 7.71 to 14.83 mg GAE/1 g = 85.07 to 91.05 %). Based on these results, it was be observed that at 5 h, the average phenolic release (11.79 mg GAE/g) and bioaccessibility (88.23 %) among all donors were higher in NMT. Different authors mention that several factors affect the bioaccessibility of phenolic compounds, such as the type of process and food matrix interactions (Tomás-Barberán and Espín, 2019; Martini *et al*., 2021). The highest bioaccessibility values in the tortilla obtained from the nixtamalized grain could be attributed to the characteristics of the process, where the maize grain is subjected to high temperature cooking, alkaline pH (> 12), and long resting times. The alkaline conditions that take place during this process can modify or weaken the cell wall structure (formed by cellulose and lignin polymers) favoring enzymatic hydrolysis by the colonic microbiota, thus facilitating the release of phenolic-bound compounds in the different polymers (cellulose, lignin and hemicellulose) that constitute the cell wall (Ying *et al*., 2018; Baky *et al*., 2022)

In cereals, phenolic compounds are covalently bound to cell wall polysaccharides, which results in a very low and limited bioavailability for their bioaccessibility in the organism. These complex polysaccharides cannot be hydrolyzed by enzymes in the gastrointestinal tract, therefore, complexed phenolics are not released and absorbed at the

intestinal level (Tomás-Barberán and Espín, 2019). However, the colonic microbiota will release and bioconvert phenolic compounds attached to the cell wall, producing compounds of smaller molecular size and microbial metabolites, some of which have been shown to be more bioactive and more easily absorbed than their precursors (Tomás-Barberán and Espín, 2020; Astorga-Gaxiola *et al*., 2023). Likewise, the transformation of phenolic compounds by the colonic microbiota modulates their bioactivity, exerting an anti-inflammatory, antioxidant and anticarcinogenic action (Domínguez-Ávila *et al*., 2020). On the other hand, several authors have mentioned that technological and biotechnological processes for food production, can induce chemical or physical modifications in foods, or directly influence polyphenols to improve their bioaccessibility and bioavailability (Ribas-Agustí *et al*., 2018; Calvo-Lerma *et al*., 2020). These modifications include changes in the food matrix structure leading to the release of phenolic compounds from the matrix and conversion to postbiotic metabolites by intestinal microbial strains (Polia *et al*., 2022). However, a more comprehensive research approach is still needed to reach valid conclusions on the effects of food processing and polyphenols on human health, mediated by the colonic microbiota (Tomás-Barberán and Espín, 2019).

Incidentally, the results found in this study show similarity to those observed by Juániz *et al*. (2017), who evaluated colonic fermentation at different times (15 min, 5 and 24 h) in raw, fried (olive oil and sunflower oil) and roasted cardon. These authors were able to observe how the type of processing influenced the release of phenolic compounds during *in vitro* fermentation, detecting considerable microbial metabolic activity as fermentation time increased, observing maximum phenolic release values at 24 h in cardon fried with sunflower oil (8.814 µmol of phenolic compounds/g, dw) compared to unprocessed cardon (0.714 µmol of phenolic compounds/g, dw). Similarly, our results agree with Inada *et al*. (2020) who evaluated the bioaccessibility of phenolic compounds from jaboticaba peel and seeds after gastrointestinal digestion and *in vitro* fermentation with colonic microbiota. These authors observed an increase in the release of total phenolic compounds in the first 4 h of fermentation (49 %), followed by a decrease after 24 h (17 %). These authors observed that after gastric and intestinal digestion, the overall bioaccessibility of phenolic compounds reached 49 %. Unlike other studies that can be found in the literature, our work did not pool the fecal samples, and we evaluated the fermentation of phenolic compounds and bioaccessibility in 5 donors (A-D) individually, being donor D the one who presented the highest values for both evaluations. Several authors have shown that interventions on the microbiota do not have uniform effects in different subjects, but that their outcome depends on the individual's baseline microbiota, both *in vivo* and *in vitro* assays. Thus, by pooling fecal microbiota, interindividual differences are completely eliminated and an "artificial" community with unpredictable competition and balance between taxa is created. In contrast to the recent suggestion that microbiota should be pooled for pooled fermentations, the use of individual, well-protected, fresh fecal microbiota as inoculum for *in vitro* human gut microbiota experiments, is recommended to avoid unpredictable tendencies (Hou *et al*., 2020; Perez-Burillo *et al*., 2021; Isenring *et al*., 2023).

Antioxidant activity

In our study, antioxidant activity was evaluated by ORAC and ABTS chemical methods. In the ORAC assay (Figure 1) it was observed that, at the initial time (0 h), the antioxidant activity of phenolics released by the microbiota was similar between the NMT and EMT ($p > 0.05$). However, after 1 h, significant differences were observed, with EMT presenting the highest antioxidant activity values (A = 137.16 ± 13.39, B = 150.07 ± 3.80, C = 71.02 ± 1.36, D = 100.09 ± 6.38 µmol TE/g). In contrast, at 5 h post-fermentation the highest activity was observed mainly in NMT (A = 676.12 ± 76.45, B = 804.57 ± 23.62, C = 282.81 ± 16.75, D = 515.77 ± 11.22 µmol TE/g).

In the ABTS assay, the antioxidant activity showed a similar behavior. During the initial time (0 h), no significant differences ($p > 0.05$) were observed between tortillas and individuals. However, this changed after 1 h, where the values were very variable depending on the individual, having a similar behavior among tortillas (Figure 2). Finally, at 5 h the NMT reported, for the most part, the highest ($p < 0.05$) values (A = 24.73 ± 0.14, B = 24.26 ± 0.195, C = 29.32 ± 0.468, and D = 28.73 ± 0.49 µmol TE/g) compared to its EMT counterpart (A = 24.99 ± 0.14, B = 20.19 ± 0.28, C = 18.31 ± 0.14, D = 27.01 ± 0.30). As in the phenolic released during colonic fermentation assay, in the evaluation of antioxidant capacity, different individuals had the highest values along the assays (ORAC and ABTS) for both NMT and EMT. Pérez-Burillo *et al*. (2021) mention that it is important to consider interindividual variability during the metabolism of phenolic compounds, as it may lead to different results (different metabolites and/or physiological effects). Therefore, what is beneficial for one person may be less positive or even unnecessary for another.

In both antioxidant activity assays, it was observed that NMT presented the highest average of all donors results at 5 h (569.82 µmol TE/g, by ORAC, and 26.76 µmol TE/g, by ABTS). Kamau *et al*. (2020) mention that ferulic acid, the most abundant phenolic acid in maize with a high antioxidant potential, is mainly found in the *trans* esterified form to arabinoxylans and hemicelluloses, so more than 90 % is bound in insoluble form. In traditional nixtamalization, the lime in the soaking water can cleave the ester bound, thus releasing this phenolic acid. This phenomenon explains the increase of ferulic acid during nixtamalization as reported by Mora-Rochín *et al*. (2010), who observed a higher percentage of free ferulic acid in tortillas nixtamalized from white maize when compared to tortillas obtained from extruded maize.

Natural antioxidants are often varied, so it is sometimes a problem to use a one-dimensional method to evaluate this property. The use of more than one methodology to assess the antioxidant activity of foods is essential as they are complementary, and their sensitivity depends on the different

macromolecules and bioactive compounds present in the food (Bello-Pérez *et al*., 2015; Chen *et al*., 2022). Our results agree with Aguayo-Rojas *et al*. (2012) who reported that total phenolic compounds are the most important contributors to antioxidant capacity, and that hydrophilic antioxidant activity accounts for approximately 98 % of total antioxidant activity in maize. Additionally, our results contrast with Gaxiola-Cuevas *et al*. (2017) who evaluated total phenolics and antioxidant activity of different creole blue maize processed by extrusion and traditional nixtamalization. These authors reported that, in both cooking procedures, antioxidant activity decreased compared to raw kernels. However, in their study tortillas made with extruded blue maize retained a higher proportion of total phenolics (83.5 ± 2.1 %) and cellular antioxidant activity (77.5 ± 2.5 %), compared to traditional tortillas (49.8 ± 1.6 and 48.7 ± 1.5 %). In addition, phenolic compounds interact with macromolecules such as proteins, carbohydrates, and lipids, significantly influencing the nutritional and nutraceutical properties of food (Sęczyk *et al*., 2021b). These interactions are governed by various factors, including external conditions like temperature, pH, and ionic strength, as well as intrinsic properties such as the type, structure, and concentration of both the phenolic compounds and the food matrix components. Despite their well-recognized antioxidant activity, the binding of phenolics to food matrices or their interactions with low molecular weight components, such as other phenolics, vitamins, and minerals, often diminishes this activity (Shahidi *et al*., 2018; Sęczyk *et al*., 2021a; Sęczyk *et al*., 2021b).

The composition and physicochemical properties of food matrices, along with processing methods, play a crucial role in modulating the bioaccessibility and antioxidant activity of phenolic compounds. Innovative processing techniques that enhance the stability of phenolics and optimize food matrix properties, can significantly improve their release and availability during digestion (Ribas-Agustí *et al*., 2018). Moreover, the gut microbiota exerts a substantial influence on polyphenol bioaccessibility, through metabolic conversions and enzymatic breakdown of food matrix components. However, since the majority of microbial fermentation occurs in the colon, while absorption predominantly takes place in the stomach and small intestine, the overall bioavailability of polyphenols may be limited (Dufour *et al*., 2018; Ribas-Agustí *et al*., 2018; Cao *et al*., 2021). Although *in vitro* models offer valuable preliminary insights into the bioaccessibility of phenolic compounds, human studies remain the gold standard for generating accurate and specific conclusions about their behavior and potential health benefits (Sęczyk *et al*., 2021).

Correlation analysis of processing, phenolic compounds, bioaccessibility and antioxidant activity during colonic fermentation

A correlation analysis was performed between the type of processing (extrusion and nixtamalization), antioxidant activity (ORAC and ABTS), bioaccessibility and phenolic compounds released at different fermentation times (Table 5). A strong correlation (R^2 = 0.72 - 0.903) ($p < 0.05$) was observed between fermentation time and bioaccessibility (% B), antioxidant activity and phenolic compounds released. In contrast, when evaluating the correlation of treatment (or maize processing) and bioaccessibility, antioxidant activity and phenolic compounds released, the results were negative (R^2= -0.029 to -0.156). However, the results obtained show a higher correlation of antioxidant activity and phenolic compounds released by the ORAC (R^2 = 0.855) than the ABTS (R^2 = 0.709) assays. This positive correlation with the ORAC assay may be due to the principle of the assay, which measures the radical chain-breaking capacity of antioxidants by monitoring the inhibition of peroxyl radical-induced oxidation, whereas the ABTS assay measures the ability of antioxidants to scavenge free radicals (Cheng *et al*., 2020; Astorga-Gaxiola *et al*., 2023).

The positive correlation between time and antioxidant activity in our study agrees with Cheng *et al*. (2020), who report that the release of phytochemical compounds with antioxidant activity is closely associated with fermentation time. Likewise, these authors observed a positive correlation between total phenolic compounds and antioxidant activity (DPPH and ABTS) after fermentation with *Lactobacillus casei*, showing that fermentation can increase the antioxidant phenolic compounds involved in the DPPH reduction reaction (R^2 = 0.998) through proton donation, while the positive correlation with the ABTS assay (R^2 = 0.959) may be due to the hydroxyl and phenolic groups that converted ABTS-+ to ABTS. Bello-Pérez *et al*. (2015) evaluated the correlation between the content of phenolic compounds and tannins

Table 5. Correlation matrix of the variables involved in colonic fermentation in nixtamalized and extruded blue maize tortillas.

Tabla 5. Matriz de correlación de las variables involucradas en la fermentación colónica en tortillas de maíz azul nixtamalizado y extrudido.

	Time	*Phenolics*	*Bioaccesibility*	*ORAC*	*ABTS*	*Treatment*
Time	1					
Phenolics	0.9032175	1				
Bioaccesibility	0.884294	0.84653016	1			
ORAC	0.90266572	0.85507923	0.78798375	1		
ABTS	0.7174433	0.70915823	0.90049208	0.63874146	1	
Treatment	0	-0.05583935	-0.15683367	-0.02977879	-0.09059565	1

with two different antioxidant activity methods (DPPH and FRAP). Pearson's coefficients indicated positive correlations between extractable phenolic compounds and DPPH inhibition ($R^2 = 0.93$), and between condensed tannins with FRAP ($R^2 = 0.62$), suggesting a direct relationship between phenolic compound content and antioxidant activity. The differences in Pearson's coefficients between the antioxidant methods used in this study, are due to the polarity of the compounds and the sensitivity characteristics of each technique.

CONCLUSIONS

The results of the present research highlight that, while both traditional nixtamalization and extrusion processes provide substantial benefits for releasing bioactive compounds, extrusion proves more effective in releasing bound phenolic compounds and enhancing total antioxidant activity. This makes extrusion a promising technology for maximizing the nutraceutical potential of maize-based foods. Notably, traditional nixtamalization remains valued today for its ability to preserve various bioactive compounds with antioxidant benefits that contribute to health protection. This ancestral process, commonly used in tortilla production, preserves the cultural and sensory properties of maize. Additionally, it produces highly bioavailable phenolic compounds after colonic fermentation, which could play a crucial role in the digestive health and prevention of chronic diseases. For this reason, traditional nixtamalization remains the gold standard in tortilla production, preserving not only the nutritional quality and nutraceuticals, but also the culinary heritage.

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CONFLICTS OF INTEREST

The author declare no conflicts of interest.

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