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**Ozone processing of peanut “milk”: degradation of aflatoxins, impact on quality attributes and the potential effect on peanut allergens**

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**Abstract:**

Demand for plant-based products has been expanding over the last few years leading to the development of vegetable products analogous to conventional animal-products, such as plant-based “milks”. Although peanuts can be used to produce these plant-based “milks”, it is important to develop alternatives to reduce their allergens and the risk of mycotoxin contamination. This work studied the peanut “milk” ozonation process as an alternative to achieve these goals. We evaluated the kinetics of degradation of aflatoxins AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>, and the effect on lipid oxidation and peanut allergens. Ozonation was efficient to degrade >98% of AFB<sub>1</sub> and AFG<sub>1</sub> within 30 min, which are the most toxic evaluated aflatoxins, while AFB<sub>2</sub> and AFG<sub>2</sub> were more resistant even after 210 min. In addition, the ozonation of peanut “milk” showed the potential to reduce the assessed peanut allergens by roughly 80%. However, ozonation also degraded fatty acids and produced off-flavours. Therefore, further studies using ozonation combined with other strategies are encouraged to obtain safer vegetable “milk” with desirable sensory attributes.

**Keywords:** plant-based, peanut protein, allergenic proteins, mycotoxins, oxidation, green technologies.

## 1. Introduction

The market for plant-based products has been expanding in recent years due to the consumers tendency to vegetarianism, healthier and/or a more sustainable lifestyles. Plant-based “milks” are products analogous to cow’s milk produced from water suspensions and emulsions based on different sources such as soy, coconut, almond, hemp, rice, oats, and peanut (Shori and Zahrani, 2021; Rojas et al., 2022).

The aqueous extract of peanut kernels (*Arachis hypogea* L.) is a plant-based beverage commonly known as peanut “milk”. This product has a high content of proteins, non-soluble fibers, minerals, and fatty acids such as linoleic and oleic acids (Shori and Zahrani, 2021). Peanut “milk” is basically obtained by soaking and grinding raw (in general), roasted or partially defatted peanuts with water (Diarra et al., 2005). In addition to sensorial and nutritional studies to support the acceptance of peanut “milk” by consumers, there is a need to develop enhanced processes to achieve safe and high-quality products.

Two main concerns about peanut “milk” are the possible presence of mycotoxins and the allergenicity of its proteins. Peanuts can be contaminated by fungi in the field or during storage, possibly producing mycotoxins. Aflatoxins are carcinogenic, genotoxic and immunosuppressive mycotoxins whose presence has already been reported in the literature (Miranda et al., 2007; Magrini et al., 2011; Hoeltz et al., 2012). If present in the peanut kernel, aflatoxins can be carried into the extract during processing, contaminating the peanut “milk” and compromising product safety. Once peanut “milk” is basically a water extract of dissolved and disintegrated plant material, the mycotoxins eventually found in peanut kernel particles can be carried to peanut extract. Mycotoxins are resistant to various procedures for detoxification, but some emerging approaches, such as ozone processing, can be used to decontaminate solid or liquid foods (Pandieselvam et al., 2019, Alexandre et al., 2017). However, this technique has yet to be evaluated in plant-based “milks”.

Ozonation is an emerging technology in food processing, whose efficiency, low complexity and relatively low cost make it a good alternative to obtain products with enhanced properties. Ozone can decompose many organic compounds without producing secondary

pollutants during its degradation to oxygen (Pandieselvam et al., 2020), it does not remain in foods submitted to ozonation process due to its short half-life (Pandieselvam et al., 2017). However, during ozonation, the ozone can react with other compounds in the food, showing possible advantages and drawbacks. For instance, some consumers are allergic to peanut proteins. In these consumers, these proteins are recognized by the immune system as a threat and lead to an allergic reaction. Therefore, if ozone oxidizes specific epitopes in these proteins, ozonation has the potential to alter the allergenicity of peanut-based products. Although promising, this evaluation has not been conducted so far.

This work studied the application of the ozonation process on peanut “milk”, evaluating the kinetics of degradation of the aflatoxins AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>, as well as their effect on lipid oxidation and peanut allergens.

## **2. Experimental Section**

### **2.1. Sample and sample preparation**

Commercial peanut “milk” samples (same brand and batch) were purchased from local stores in the region of Piracicaba, São Paulo, Brazil. The peanut “milk” composition includes 2.1% of carbohydrates, 1.5% of protein, 2.7% of total fat, and 0.3% of fibre and did not present detectable levels of aflatoxins. This approach allowed standardization and the study of a real commercial product.

For each replicate (3) of ozone processing, a 700 mL of peanut “milk” was artificially contaminated with a mix of aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> (Sigma Aldrich) standards and processed in the ozonation system. The average concentrations of aflatoxins obtained in the “milk” after standard fortification were: AFB<sub>1</sub> = 43 µg L<sup>-1</sup>; AFB<sub>2</sub> = 38 µg L<sup>-1</sup>; AFG<sub>1</sub> = 59 µg L<sup>-1</sup>; AFG<sub>2</sub> = 30 µg L<sup>-1</sup>.

### **2.2. Ozonation of peanut “milk”**

The system used for the ozonation of peanut “milk” is presented in Figure 1. It consisted of an ozone generating unit (Ozone & Life, O&G Model RM 3.0, São José dos Campos, Brazil) and a monitor equipped with software to measure ozone concentration in the gas flow (2B

Technologies Inc., 106 -H). The ozone was produced by the coronal-discharge method from industrial oxygen (95% purity). The gas flow was  $1.0 \text{ L min}^{-1}$  with an ozone concentration of  $47 \text{ mg L}^{-1}$ . The gas stream containing ozone and oxygen was bubbled within the peanut “milk”, which was constantly stirred with a magnetic stirrer inside a glass reactor. The gas headspace was continuously removed from the reactor and transferred to an ozone thermal destruction. The process was conducted at room temperature ( $21 \pm 1 \text{ }^\circ\text{C}$ ).

Ozonation was carried out in triplicate at treatments times 0, 5, 10, 20, 30, 60, 90, 120, 150, 180, and 210 min. At each time point, aliquots of approximately 7 mL of peanut “milk” were withdrawn to analyse the concentration of aflatoxins. Sampling was performed by opening the collect valve of the reactor, which quickly released a volume of peanut “milk” from the base of the reactor. After obtaining the 7 mL, the collect valve was closed and there was no observed ozone loss during sampling (Figure 1).

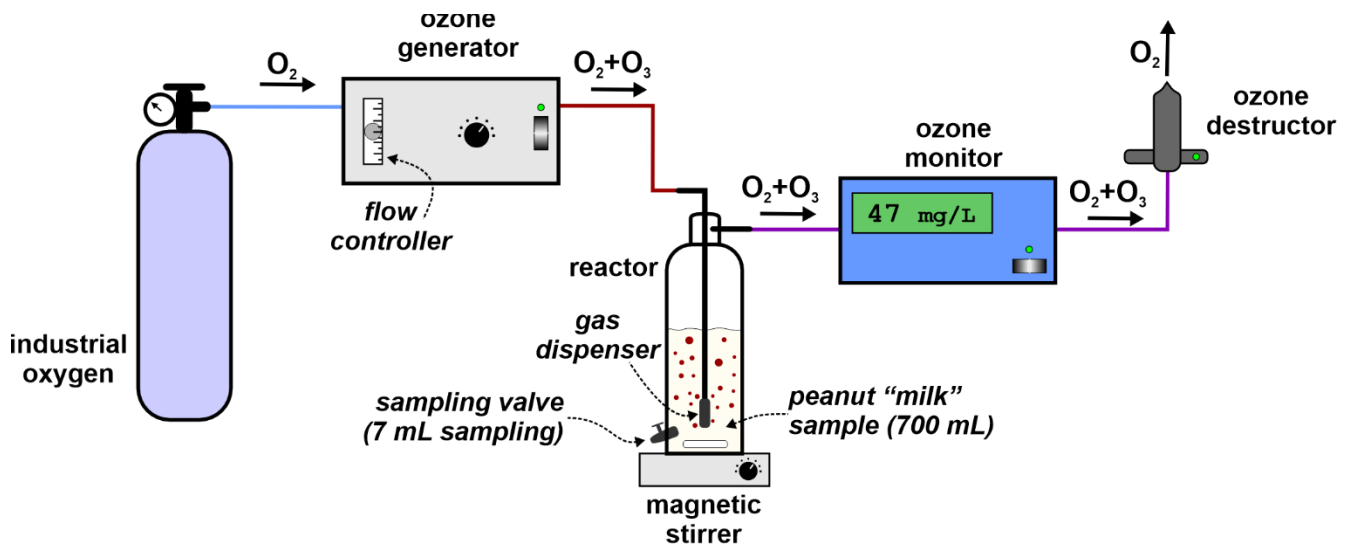


Figure 1. Ozonation system (representative, without scale)

The reduction in the initial concentration of aflatoxins AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> in the different ozonation times was evaluated by the  $C/C_0$  ratio, obtained by the concentration of aflatoxin after ozonation in each treatment ( $C$ ) and the initial concentration of aflatoxin, before processing ( $C_0$ ).

The ozone consumed by the reaction was calculated by measuring the ozone concentration at the inlet of the reactor (ozone produced) minus the ozone measured at the

outlet of the reactor (excess of ozone, not reacted, leaving the reactor), considering the gas stream flow.

### **2.3. Aflatoxin analysis**

Aflatoxin extraction was performed with methanol (2:3 ratio, “milk”: methanol, v/v). The extract was placed in a shaker for 30 min (Marconi, MA 139/CFT, Piracicaba, Brazil) and filtered throughout filter paper. Before adding the filtrate to the immunoaffinity column, the extract was diluted in phosphate buffer saline (PBS, 2:3 ratio, filtered: buffer, v/v), and then centrifuged at 10,000 g for 10 min (Eppendorf, MiniSpin® plus, Germany). Purification was carried out using NeoColumn™ for Aflatoxins (Neogen®) and the elution was carried out with methanol:acetonitrile (1:1, v/v). Chromatographic separation was performed using a Shimadzu HPLC equipped with a fluorescence detector (RF-20AXL), configured for 440 nm emission and 362 nm excitation, and X-Terra RP18 column (4.6 x 150 mm, 5 µm, Waters, Milford, MA, USA). The mobile phase comprised methanol, acetonitrile, and water (1:1:2, v/v). The mobile phase flow was 1.0 mL min<sup>-1</sup> and the injection volume was 50 µL. The limit of detection (LOD) of the method was 0.11 µg L<sup>-1</sup> for AFB<sub>1</sub> and AFG<sub>1</sub> and 0.07 µg L<sup>-1</sup> for AFB<sub>2</sub> and AFG<sub>2</sub>. The limit of quantification was set as 1.1 µg L<sup>-1</sup> for AFB<sub>1</sub> and AFG<sub>1</sub> and 0.7 µg L<sup>-1</sup>, for AFB<sub>2</sub> and AFG<sub>2</sub>.

### **2.4. Quality attributes: pH, colour, and lipid oxidation**

The pH value of the ozonated samples was measured using a digital pH meter (PA 200 model, Marconi) in three replicates of the ozonation process.

The effect of ozonation on the “milk” colour was evaluated using a colorimeter (CR-400 model, Konica Minolta). The colour parameters L\* (lightness), a\* (redness-greenness), and b\* (yellowness-blueness) were measured in duplicate of each ozonated sample.

The fatty acid composition was assessed, as well as the formation of nonanal and hexanal, which were selected as off-flavour markers of lipid oxidation (see Sections 2.6 and 2.7 for more details).

### **2.5. Oil extraction for fatty acid composition determination**

Peanut oil extraction was carried out based on Bligh and Dyer (1959), with modifications. Each process was conducted in two independent replicates and the assays



were conducted in duplicate. Peanut “milk” (100 mL), chloroform (100 mL), and methanol (200 mL) were mixed and shaken manually for 2 min. Chloroform (100 mL) was added and the mixture was shaken manually for 30 s. Then, the mixture was filtered in a Buchner funnel coupled to a vacuum bomb with filter paper. The solid residue on the filter paper was cleansed with 25 mL chloroform. The filtered mixture was transferred to a separation funnel, and the lower phase was collected. The chloroform phase was dried in anhydrous sodium sulfate and rotaevaporated at 473 mbar/40°C in the Rotavapor R-215 (Buchi, Flawil, Switzerland). The dry residue was resuspended in 20 mL hexane, shaken, and transferred to a separation funnel. After the formation of two phases, the upper phase was collected and rotaevaporated at 360 mbar/40°C.

## **2.6. Fatty acid composition by gas chromatography-flame ionization detection (GC-FID)**

Fatty acid methyl esters (FAME) were prepared based on the method described by Hartman and Lago (1973). A GC-2010 Plus gas chromatography system (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector was used. Separation was achieved on a Supelco column (100 m × 0.25 mm, 0.25 µm) (Sigma-Aldrich, St. Louis, USA). Nitrogen was the carrier gas at a flow rate of 1.2 mL min<sup>-1</sup>. The injected sample volume was 1 µL (split 1:10). The initial column oven temperature (140°C) was held for 5 min, then raised to 240°C at 4 °C min<sup>-1</sup> and held for 15 min. Injector and FID temperatures were set at 230 °C and 240 °C, respectively. FAME were identified using the peak retention times of a standard FAME mix C8-C22 (Sigma-Aldrich, St. Louis, USA), as a reference. Quantification was carried out according to Joseph and Ackman (1992), using the methyl tridecanoate (Sigma-Aldrich, St. Louis, USA) as an internal standard, and calculating theoretical correction factors for the peak intensities, as well as factors to convert the methyl esters into fatty acids.

## **2.7. Formation of low molecular-weight volatile compounds by solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS)**

Aliquots of 5 mL of peanut “milk” samples were placed in 20 mL glass vials and capped with gas-tight magnetic screw caps with a polytetrafluoroethylene/silicone septum for needle

pierce. The septum of the screw caps was pierced with the needle of an SPME manual holder assembly (Supelco, Bellefonte, PA, USA) after 40 min of equilibrium at 60°C in a water bath and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm) fiber (Sigma-Aldrich, St. Louis, USA) was exposed (2 cm) in the headspace for 10 min for volatile adsorption. Samples were manually injected into a GC-2010 coupled with a QP 2010 Plus mass spectrometer with 3 min of volatile desorption.

Volatiles were separated in an Rtx-5MS column (30 m x 0.25 mm x 0.25 µm, Restek, Bellefonte, PA, USA). The injection temperature and ion source temperature were set at 250°C and 200°C, respectively, at the split mode of 10.0. The oven temperature started at 32°C and was held for 3 min, increased to 60°C at 5°C min<sup>-1</sup>, increased to 200°C at 8°C min<sup>-1</sup>, and finally increased at 290°C at 20°C min<sup>-1</sup>, remaining for 5 min at that temperature. The carrier gas was helium at a flow rate of 1.0 mL min<sup>-1</sup>. The mass spectrometer ionization energy was 70 eV, and the spectrum was recorded from 40 m/z to 300 m/z. Volatiles were tentatively identified by their similarity with spectra from libraries (WILEY8.LIB and FFNSC1.3lib), and by calculating their Linear Retention Indexes (LRIs), which were compared with the available literature. Nonanal and hexanal identities were also confirmed by comparison with analytical standards (Sigma-Aldrich, St. Louis, USA), which were used to build calibration curves for their quantification.

## **2.8. Effect of ozone on peanut allergens**

The effect of ozonation on peanut allergens was evaluated by a sandwich enzyme-linked immunosorbent assay (ELISA) (AgraQuant Peanut ELISA test kit, Romer Labs Tulln, Austria), following the manufacturer's instructions and applying a conversion factor of 0.2587. The kit detects mainly the most abundant peanut allergen Ara h 3 (Jayasena et al., 2015); thus, it must be highlighted that not all peanut allergens are detected by this assay. The peanut allergen levels were assessed after 0, 10, 30, 60, and 120 min of the ozonation process, using a standard curve ranging from 1 to 40 µg mL<sup>-1</sup>. Briefly, for each processing time, 1 mL of peanut "milk" was added to the extraction buffer placed for 15 min in a shaker, and centrifuged at 2000 g for 10 min. From the supernatant, 100 µL were placed in antibody-coated microwells and

incubated according to the protocol for analysis. The absorbance was read on a microplate reader SpectraMax1 M3 (Molecular Devices, LLC, Sunnyvale, CA, USA) at 450 nm.

## 2.9. Experimental design and statistical analysis

Each process was conducted in three independent replicates and the analyses were conducted in triplicate or duplicate. The results were statistically evaluated (SAS University Edition, 2021) by ANOVA followed by Tukey's post hoc test (0.05% of significance) to verify if the different times of ozonation produced significant differences in the evaluated parameters.

After observing the behaviour of aflatoxin degradation during ozone processing, a first order kinetic considering a fractional conversion of mycotoxin concentration was used to describe the phenomena - Equation 1, where  $C_t$  represents the concentration of each aflatoxin at any processing time  $t$ ,  $C_0$  is its initial concentration,  $C_\infty$  is its residual concentration in long processes,  $t$  is the ozonation time and  $k$  the rate constant. The model parameters were iteratively adjusted to the experimental data by minimizing the sum of squared residues (SSR) between the experimental (three replicates together) and predicted values using the generalized reduced gradient algorithm (GRG, nonlinear solving method) of the 'Solver' tool of Excel 365 (v2204, Microsoft, USA). The parameters of each model were considered valid when Solver reported that the GRG method found a locally optimal solution (to a set convergence of 0.00001).

$$C_t/C_0 = (C_\infty/C_0) + (1 - C_\infty/C_0) \cdot e^{-kt} \quad \text{Equation 1}$$

## 3. Results and Discussion

### 3.1. Effect of ozone processing on aflatoxin degradation in peanut "milk"

Figure 1 shows the reduction of the initial concentration of aflatoxins ( $C/C_0$ ; AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>) according to the ozonation time. Ozonation quickly reduced the concentration of AFB<sub>1</sub> and AFG<sub>1</sub> at the beginning of processing. Within the first 5 min, 53% of the initial concentration of aflatoxin G<sub>1</sub> and 43% of B<sub>1</sub> were degraded by ozone. Furthermore, the ozonation almost totally eliminated AFG<sub>1</sub> (99%) and AFB<sub>1</sub> (98%) concentrations after 30 min

of processing ( $\text{LOD} = 0.11 \mu\text{g L}^{-1}$ ). On the other hand,  $\text{AFG}_2$  and  $\text{AFB}_2$  were more resistant to be degraded by ozone as they only started to be reduced between 60 ( $\text{AFG}_2$ ) and 90 min ( $\text{AFB}_2$ ) of processing. The greatest reduction of  $\text{AFG}_2$  was 41% at 210 min, while  $\text{AFB}_2$  had a maximum reduction of 32% at 180 min.

This observed behaviour can be described by a first order kinetic considering a fractional conversion of aflatoxins concentration during ozonation processing, similar to that observed for previous studies with other systems (Alexandre et al., 2019, Wang et al., 2016a and 2016b). The experimental data shows a good fit to this model (Equation 1;  $\text{SSR} < 0.08$ ) (Figure 2). The ratio  $C^\infty/C_0$  represents the aflatoxin concentration after sufficient long processes, describing the maximum degradation reached by this approach. This ratio for each aflatoxin was  $4 \cdot 10^{-5}$  ( $\text{AFB}_1$ ), 0.72 ( $\text{AFB}_2$ ),  $4 \cdot 10^{-3}$  ( $\text{AFG}_1$ ) and 0.66 ( $\text{AFG}_2$ ), which agrees with the observed behaviour of quick degradation of  $\text{AFB}_1$  and  $\text{AFG}_1$ , with a residual concentration of  $\text{AFB}_2$  and  $\text{AFG}_2$ . In fact, the kinetic parameter ( $k$ ) of  $\text{AFB}_1$  and  $\text{AFG}_1$  presented one order of magnitude higher than the values for  $\text{AFB}_2$  and  $\text{AFG}_2$ :  $k \text{ (min}^{-1}\text{)} = 0.1196$  ( $\text{AFB}_1$ ), 0.0161 ( $\text{AFB}_2$ ), 0.1569 ( $\text{AFG}_1$ ) and 0.0173 ( $\text{AFG}_2$ ).

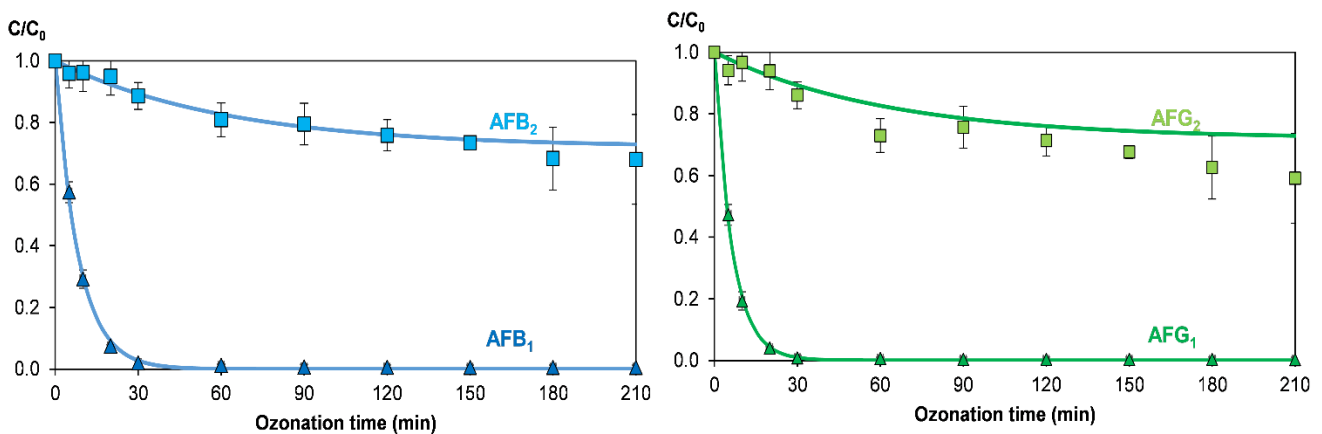


Figure 2. Kinetics of aflatoxin ( $\text{AFB}_1$ ,  $\text{AFB}_2$ ,  $\text{AFG}_1$ ,  $\text{AFG}_2$ ) degradation in peanut “milk” as a function of ozonation processing time (dots and bars are average  $\pm$  standard deviation; curves are the kinetic model – Equation 1)

The structure of each aflatoxin can explain the different behaviours during processing.  $\text{AFB}_1$  and  $\text{AFG}_1$  are more toxic than  $\text{AFB}_2$  and  $\text{AFG}_2$  due to the high electrophilic reactivity promoted by the double bonds at positions 8 and 9 in the furan ring (Maeba et al., 1988). Since

this region has a higher electron density, it is also more susceptible to electrophilic attacks by ozone (Maeba et al., 1988). Conversely, AFB<sub>2</sub> and AFG<sub>2</sub> have an olefin double bond in the terminal furan ring, which is the reactive site for the ozone reaction (Heathcote and Hibbert, 1974). Then, the oxidation reaction is slow as the lactone ring must be opened during the ozone exposure, modifying the molecule to reduce its toxicity (Samarajeewa et al., 1990).

In fact, Agripolou et al. (2016) demonstrated the total degradation of AFB<sub>1</sub> and AFG<sub>1</sub> in an ozonated aqueous medium (distilled water), but obtained only partial degradation of AFB<sub>2</sub> and AFG<sub>2</sub>. Even after an ozonation time 4-5 longer than for the degradation of AFG<sub>1</sub> and AFB<sub>1</sub>, the authors reported the greatest reduction was 53.9% for AFG<sub>2</sub> and 29.6% for AFB<sub>2</sub>. It is noteworthy that the peanut “milk” has many components to compete with AFs for the reaction with ozone, compared with distilled water. This explains the observed exponential decay with fractional conversion for aflatoxin degradation. Moreover, even so, the degradation trend of AFB<sub>2</sub> and AFG<sub>2</sub> in the present work is similar to that reported in distilled water (Agripolou et al., 2016), regardless of the differences in process conditions.

Similarly, Maeba et al. (1988) had already demonstrated the same dynamics for the ozone degradation of AFB<sub>1</sub> and AFG<sub>1</sub> diluted in 4% dimethyl sulfoxide which concentrations, after the process, were no longer detected, while AFB<sub>2</sub> and AFG<sub>2</sub> required longer processing time and higher ozone concentrations.

In addition to being less susceptible to ozone, AFB<sub>2</sub> and AFG<sub>2</sub> presented greater variability between the replicates than AFB<sub>1</sub> and AFG<sub>1</sub>. This variability might contribute to the lack of significant differences of the treatments when compared to the control.

Therefore, although ozonation proved to be efficient to degrade the aflatoxins with greater toxicity significance (AFB<sub>1</sub> and AFG<sub>1</sub>) in peanut “milk”, AFB<sub>2</sub> and AFG<sub>2</sub> were resistant to ozone degradation. Despite this, the expected concentrations of AFB<sub>2</sub> and AFG<sub>2</sub> in naturally contaminated samples are much lower than those found for AFB<sub>1</sub> and AFG<sub>1</sub> (and in concentrations much lower than here applied; it is worth mention our samples had a much higher concentration of aflatoxins than typically found in food products, because we needed to quantify them even after long process conditions). In general, the concentration of AFB<sub>1</sub>

naturally found in food and feed is more than twice the sum of AFB<sub>2</sub> and AFG<sub>2</sub> concentrations (Kensler et al., 2011; Matumba et al., 2015). For example, Kensler et al. (2011) reported the proportion of AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>, as, respectively, 1.0:0.1:0.3:0.03.

The regulatory action guidance for total aflatoxins in peanut and peanut products established in United States is 20 µg kg<sup>-1</sup> (FDA, 2016). The Codex Alimentarius established 15 µg kg<sup>-1</sup> as the maximum limit (ML) of aflatoxins for peanuts intended for further processing (Codex, 2019), while the European Community (EU, 2010) set reduced ML for peanut and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs (2 µg kg<sup>-1</sup> for AFB<sub>1</sub> and 4 µg kg<sup>-1</sup> for total aflatoxins). Gil-Serna et al. (2020) observed that the mycotoxins can be presented in the raw products that are commonly used to produce plant-based "milks". Despite this, there are still few studies evaluating the occurrence of mycotoxin contamination in plant-based "milks". Hamed et al. (2017) evaluated eight oat "milk" commercial samples, three of them containing deoxynivalenol, demonstrating that mycotoxin contamination from the raw oat can be transferred to the beverages. Miró-Abella et al. (2017) evaluated nine commercial samples of plant-based beverages (oat, soy and rice) and observed that seven were positive for mycotoxins. Therefore, considering consumer health, it is of scientific interest to conduct studies on possible methods to reduce, or even eliminate, the concentration the mycotoxins in plant-based "milks".

Previous studies concerning the formation of degradation products of AFB<sub>1</sub> during ozonation showed that conjugate reaction on the double bond of the terminal furan ring is responsible for reducing the toxicity of this mycotoxin (Luo et al., 2013). Since oxidation by ozone promotes the breakdown of these structures responsible for aflatoxins toxicity, this metabolic route is not expected to occur. Luo et al. (2013) showed that degradation products of AFB<sub>1</sub> formed after the ozonation lacked the terminal furan ring characteristic of the parental compound; thereby, the expected toxicity for these products was significantly lowered. Similarly, an *in vivo* toxicity study of ozonated kernels of soybean and corn showed that ozonation reduced the deleterious effect of aflatoxins on physiological and biochemical liver

and kidney parameters in mice and rats (Luo et al., 2018; Shahat et al., 2017). Nevertheless, it is worth to mention that ozonation can affect the nutritional quality of foods (Erdman, 1997; Sharma and Graham, 2010), causing side effects related to nutrient loss (Shahat et al., 2017). Some adverse metabolic effects were reported in rats fed with ozonated casein due to the amino acid profile imbalance from the diet promoted by ozonation (Kasai et al., 1993; Kasai et al., 1994; Newel, 1997). In conclusion, the effect of ozonation on the other properties of peanut “milk”, such as nutritional and toxicological, needs further evaluation before industrial application with this claim. Moreover, the elucidation of the chemical structures of degradation products by metabolomic approaches using spectrometric and spectroscopic techniques, concomitantly to toxicity assays, may be helpful to confirm the safety of the ozonation of peanut “milk”.

Once the main aflatoxin degradation occurred in the first 120 min of ozone processing, this time range was selected to evaluate the effect on peanut allergens.

### **3.2. Effect of ozone on peanut allergens of peanut “milk”**

Whole peanuts have a high crude protein content (22-30% w.b.) and despite their recognized nutritional value, peanuts belong to the group of eight foods with the highest allergenic potential, according to the US Food and Drug Administration (FDA, 2021). This allergenic potential is due to the presence of proteins such as 2S albumins, conglutinins (Ara h 2, Ara h 6, Ara h 7), vicilin (Ara h 1), and glycinins (Ara h 3) (Zhuang and Dreskin, 2013), among which the major allergens (Ara h 2 and Ara h 6) are heat resistant (Baumgartner et al., 2007). Consequently, even the peanut “milk” adequately processed by the conventional approach (thermal processing) can still be allergenic.

The ozonation process for 30 min was able to reduce the detection of peanut allergens by 29%, while a more significant reduction (up to 80%) was achieved after 120 min of processing, indicating changes in the epitopes of mainly Ara h 3. The Ara h 3 is the most abundant allergen found in peanut extract and is the most detected by the ELISA test used in this experiment (Jayasena et al., 2015), although Ara h 2 and h 6 are also responsible for most of the allergic effects found in a crude peanut extract (Zhuang and Dreskin, 2013).

Food allergies are IgE-mediated responses to food proteins recognized as a foreign substance. The portion of food protein associated with IgE binding is named epitope, which is a contiguous or non-contiguous amino acid sequence in a three-dimensional/structural motif (Sathe and Sharma, 2009). Despite the immunochemical association suggesting that the IgE binding epitopes of the peanut allergens play an important role on the allergic reaction, allergic symptom development after peanut “milk” intake depends on the effector function of peanut proteins, i.e., the ability of allergens to effectively lead to activation of the mast cells and basophils (Zhuang and Dreskin, 2013). In the present study, the allergenicity of peanut proteins was not measured, but the detection of peanut allergens upon ozonation process indicates the potential of this technology for this purpose.

The peanut “milk” ozonation can modify the protein structure, and thus reduces the peanut allergen detection. In aqueous medium, the ozonation may promote direct oxidation by molecular ozone, as well as indirect oxidation by free radicals formed during ozone decomposition (Young et al., 2006). Amino acids may be attacked directly either on the primary amine nitrogen atom as in the R group (Greene et al., 2012). In addition, ozonation can alter the protein structure by attacking the disulphide bonds between sulfhydryl groups, which are responsible for maintaining tertiary structures in proteins (Gong et al., 2016, Cataldo, 2003). Thus, although not measured by this assay, Ara h 2, the major peanut allergen that presents high number of disulphide bonds (Sen et al., 2002), might have been affected by the ozonation process. Future studies using antibodies specific for this allergen can address this impact.

Although ozonation can change protein structure, it does not necessarily decrease the protein’s allergenicity. Ozonation, as other food processing operations, may only reduce the detectability of the protein by an immunoreactivity test. In a previous study on almond milk allergens using an ELISA method, the loss of protein solubility rather than epitope degradation was suggested to be responsible for the decrease of its immunoreactivity, meaning that the allergenicity may have remained (Dhakal et al., 2014). In fact, treatments such as heating and pressure could lower protein solubility by aggregation, making it more difficult to be extracted, and consequently, reducing its immunoreactivity (Dhakal et al., 2014; Fu and Maks, 2013)



while not necessarily reducing allergenicity. Ara h 3 is particularly known to be highly susceptible to aggregation during heating. In general, temperature conditions applied in industrial food processing do not decrease the IgE-binding of Ara h 6 or its isoforms, and the heat-induced conformational changes are limited (Jong et al., 2020). Otherwise, thermal treatments could enhance the allergenicity of peanut proteins by increasing the exposition of allergenic binding sites that were previously unexposed (Maleki et al., 2000). Therefore, it is important to make clear that our results, although indicating a reduction in peanut protein detectability, do not rule out a possible effect of the ozonation on peanut proteins other than reducing their allergenicity. Further studies to confirm it are encouraged.

During the ozonation process, the peanut “milk” sample showed gradual acidification. The pH changed from 6.5 ( $t_0$ ) to 5.0 ( $t_{120}$ ). In the present work, ozonation reduced the peanut allergen levels regardless of the pH, showing ozone has the capability to react with peanut proteins even when the pH was higher than 5.0. Previous studies showed that ozone acts attacking towards the aromatic amino acid units of proteins, converting the thiol group of cysteine into disulphides. The S-S crosslinks formed denature the proteins, modifying secondary and tertiary structures (Cataldo, 2003). Therefore, the pH reduction throughout the processing can contribute to improving the ozone reaction, by opening the protein structure.

Individual sensitivity to allergens varies among the population, and the threshold to activate the immune response to peanut proteins also varies according to the literature. The FAO/WHO recommends the use of an index based on research evaluating the dose of peanuts able to elicit a reaction in the 5% most sensitive segment of the population. The ED05 for a single dose of peanut is estimated at 2.1 mg (Remington et al., 2020). In this study, we show the reduction of the peanut allergens to  $138 \mu\text{g mL}^{-1}$  after 120 min of ozonation, 20% of the initial level (without ozonation), highlighting the potential of ozonation to reduce peanut allergens. Nevertheless, despite the significant reduction in the initial allergen level, we acknowledge that the ELISA test used in this study most detected the Ara h 3 (Jayasena et al., 2015), while Ara h 2 and h 6, the major elicitors of anaphylaxis among the peanut proteins (Zhuang and Dreskin, 2015), are underdetermined. In addition, the effective allergenic

potential (i.e. immune response) of these proteins after ozonation must be assessed to confirm the use of ozonation as a viable alternative to reduce the allergenicity of peanut proteins.

In fact, future studies can evaluate the immune response to the ozone-treated peanut “milk”. The reduction of allergenicity on ozonated peanut “milk” should be evaluated by other methods such as an RBL cell-based immunological assay, which was used to identify Ara h 2 as the most potent peanut allergen (Dibbern Jr. et al., 2003; Sun et al., 2014). Hence, the ozonation should be further investigated in future studies to confirm its feasibility as a technique to reduce peanut proteins' allergenicity before industrial application with this claim.

### 3.3. Effect of ozone processing on quality parameters of peanut “milk”

The colour value ( $L^*$ ,  $a^*$ ,  $b^*$ ) change is a quality parameter that could impair the peanut “milk” consumer acceptance. The ozonated peanut “milk” samples showed different degrees of colour impairment. However, all of the treatments produced visual changes ( $\Delta E$ ) in colour compared to the original sample. The  $\Delta E^*$  was calculated according to the formula:  $\Delta E^* = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$  (Table 1).

Table 1. Effect of ozonation on the peanut “milk” colour through ozonation time

Ozonation (min)	pH	Colour (CIELAB)			$\Delta E^*$ value
		$L^*$	$a^*$	$b^*$	
0	6.5	53.35 <sup>a</sup>	-0.49	2.10 <sup>a</sup>	
10	6.1	46.69 <sup>b</sup>	-0.35	3.57 <sup>ab</sup>	4.30
20	5.8	46.74 <sup>b</sup>	-0.59	3.34 <sup>ab</sup>	3.26
30	5.7	44.67 <sup>b</sup>	-0.56	2.38 <sup>ab</sup>	5.48
60	5.4	45.48 <sup>b</sup>	-0.40	1.95 <sup>ab</sup>	8.47
90	5.2	45.36 <sup>b</sup>	-0.64	0.98 <sup>b</sup>	7.93
120	5.0	46.92 <sup>b</sup>	-0.81	0.91 <sup>b</sup>	9.26

Different letters on the same column shows a significant difference ( $P < 0.05$ )

Ozonated peanut “milk” showed a reduction in lightness ( $L^*$  parameter) of approximately 12% compared to the control during the process. While the parameter  $a^*$  was

not affected by ozonation, the reduction on the  $b^*$  parameter indicated a yellowness reduction of 55% compared with the control.

The reaction between ozone and fatty acids is expected to promote the formation of off-flavour (Hung et al., 2005); thereby, this is the main limiting factor to extend the exposure to ozone. In our study, the fatty acids and the off-flavour volatile compounds of the peanut “milk” were primarily quantified between the interval of 0 and 30 min of ozonation, which was the minimum ozonation time required to eliminate AFB<sub>1</sub> and AFG<sub>1</sub> from the peanut “milk”.

The total identified fatty acids corresponded to almost 100% of the total ion chromatogram – TIC in the untreated sample, but continuously decreased up to 91% after 30 min ozonation, as shown in Table 2. On the other hand, their concentrations, corresponding to 93 mg/100 mg of oil in the untreated sample, decreased up to 43% after 30 min of ozonation (Table 2). Therefore, the ozonation led to fatty acid degradation, and formation of other compounds not identified by the GC-FID analysis, as shown in the obtained chromatograms, including two low molecular-weight compounds eluted between 12.5 and 15 min (Figure S1).

Oleic acid was by far the major fatty acid found in peanut “milk”, clearly corresponding to the majority of the monounsaturated fatty acids’ concentration (Table 2). Hence, the peanut genotype used for peanut “milk” production was possibly high oleic, because although the ratio of oleic acid/linoleic acid varies according to the peanut genotype, normal-oleic acid peanut genotypes contain relevant and similar amounts of both oleic and linoleic acids (Lozano et al., 2019). The oleic acid concentration continuously decreased over ozonation time. The significant decrease on oleic acid concentration with increasing ozonation time was previously reported in a study with pure oleic acid droplets, where the concomitant formation of low molecular-weight compounds was observed (Hung et al. 2005).

Despite the increase in the relative areas in the TIC, the concentrations of  $\alpha$ -linolenic acid and saturated fatty acids also decreased (Table 2), suggesting the deleterious effect of ozone on those fatty acids as well. Saturated fatty acids are known to have their C-C bonds cleaved by heating, such as during deep-frying, and no studies about the effect of ozonation on these fatty acids was found.

Table 2. Concentration of fatty acids in grams of fatty acid per 100 mg of oil in samples treated with different ozonation times. The % of the total ion chromatogram (TIC) area is shown between parentheses.

Fatty acid	Ozonation time (min)			
	0	10	20	30
Palmitic - C16:0	5.44 ±0.00 (5.71 ±0.00)	4.37 ±0.06 (5.97 ±0.01)	4.37 ±0.13 (6.34 ±0.09)	4.14 ±0.67 (6.96 ±0.05)
Margaric - C17:0	0.09 ±0.00 (0.09 ±0.00)	0.07 ±0.00 (0.10 ±0.00)	0.07±0.00 (0.10 ±0.00)	0.07 ±0.01 (0.11 ±0.00)
Stearic - C18:0	2.38 ±0.00 (2.53 ±0.00)	1.90 ±0.01 (2.63 ±0.01)	1.90 ±0.06 (2.77 ±0.06)	1.78 ±0.27 (3.04 ±0.05)
Arachidic - C20:0	1.15 ±0.01 (1.24 ±0.01)	0.91 ±0.00 (1.28 ±0.02)	0.92 ±0.03 (1.35 ±0.03)	0.83 ±0.11 (1.43 ±0.06)
Behenic - C22:0	2.39 ±0.01 (2.60 ±0.01)	1.84 ±0.12 (2.66 ±0.01)	1.89 ±0.08 (2.82 ±0.09)	1.71 ±0.22 (2.99 ±0.13)
Lignoceric acid - 24:0	1.54 ±0.01 (1.69 ±0.01)	1.22 ±0.01 (1.73 ±0.01)	1.19 ±0.04 (1.81 ±0.06)	1.11 ±0.14 (1.95 ±0.08)
∑ Saturated fatty acids (% TIC area)	13.00 (13.86)	10.30 (14.36)	10.34 (15.19)	9.63 (16.48)
Palmitoleic - C16:1(9)	0.08 ±0.00 (0.09 ±0.00)	0.06 ±0.00 (0.09 ±0.00)	0.05 ±0.00 (0.08 ±0.00)	0.06 ±0.02 (0.10 ±0.03)
<i>cis</i> -10-Heptadecenoic acid – C17:1 (10)	0.09 ±0.00 (0.10 ±0.00)	0.07 ±0.00 (0.10 ±0.01)	0.06 ±0.01 (0.09 ±0.01)	0.06 ±0.02 (0.10 ±0.01)
Elaidic - C18:1 (trans-9)	0.04 ±0.00 (0.05 ±0.00)	0.02 ±0.02 (0.03 ±0.03)	0.04 ±0.00 (0.06 ±0.00)	0.04 ±0.01 (0.06 ±0.00)
Oleic - C18:1 (9)	75.84 ±0.03 (81.25 ±0.09)	56.57 ±0.57 (78.88 ±0.21)	50.43 ±0.55 (74.17 ±0.61)	40.54 ±7.18 (69.43 ±0.69)
Gadoleic - C20:1 (9)	1.66 ±0.01 (1.80 ±0.01)	1.24 ±0.01 (1.74 ±0.02)	1.10 ±0.01 (1.63 ±0.03)	0.85 ±0.15 (1.48 ±0.01)

Erucic - C22:1 (9)	0.15 ±0.01 (0.16 ±0.01)	0.11 ±0.00 (0.15 ±0.00)	0.10 ±0.00 (0.14 ±0.00)	0.07 ±0.01 (0.13 ±0.00)
∑ Monounsaturated fatty acids (% TIC area)	77.87 (83.45)	58.07 (80.99)	51.78 (76.17)	41.62 (71.30)
Linoleic - C18:2 (9,12)	2.09 ±0.01 (2.25 ±0.01)	1.59 ±0.08 (2.23 ±0.08)	1.34 ±0.03 (1.99 ±0.01)	1.55 ±0.52 (2.63 ±0.46)
α-Linolenic - C18:3 (9,12,15)	0.05 ±0.00 (0.06 ±0.01)	0.05 ±0.00 (0.08 ±0.01)	0.06 ±0.00 (0.10 ±0.01)	0.10 ±0.03 (0.19 ±0.03)
∑ Polyunsaturated fatty acids (% TIC area)	2.13 (2.31)	1.64 (2.31)	1.40 (2.09)	1.65 (2.82)
∑ Identified (% TIC area)	93.00 (99.62)	70.02 (97.66)	63.52 (93.45)	52.91 (90.60)

The low molecular-weight compounds formed by the ozonation of peanut “milk” were tentatively identified by untargeted SPME-GC-MS analysis and are shown in Table S1. One pyrrole, one pyridine, one pyrazine, and gamma-terpinene, which are flavour volatiles commonly found in peanut oil (Hu et al., 2021; De Camargo et al., 2016), were detected only in the untreated peanut “milk”. Limonene, which is another flavour compound found in peanut, was detected only up to 10 min of ozonation. Conversely, saturated aldehydes such as heptanal and nonanal, as well as 1-octanol and nonanoic acid, which are all related to off-flavour, were detected only in the ozonated samples, indicating that ozonation negatively affected the sensory attributes of the peanut “milk”. Moreover, nonanal and nonanoic acid have been reported to be degradation products of ozonated oleic acid droplets (Hung et al. 2005).

Since off-flavour volatiles normally show very low thresholds, the concentrations of nonanal and hexanal, which were major low molecular-weight compounds in all ozonated samples, were determined (Figure 3). In our study, the untreated sample showed no nonanal and a very low concentration of hexanal (0.01 ppm). However, nonanal and hexanal concentrations increased approximately 10, 16 and 20 times in the treatments of 10 min, 20 min and 30 min of ozonation, respectively. The concentrations of nonanal and hexanal found in the ozone treated samples (in ppm) are far higher than their thresholds, which are estimated to be 1 and 5 ppb, respectively (Choi and Lee, 2021), meaning that consumers would certainly perceive sensory alterations in the ozone treated peanut “milk”. Given the deleterious effect of ozonation on the lipid fraction of peanut “milk” up to 30 min, it was not assessed in treatments involving higher periods of ozone exposition in this study.

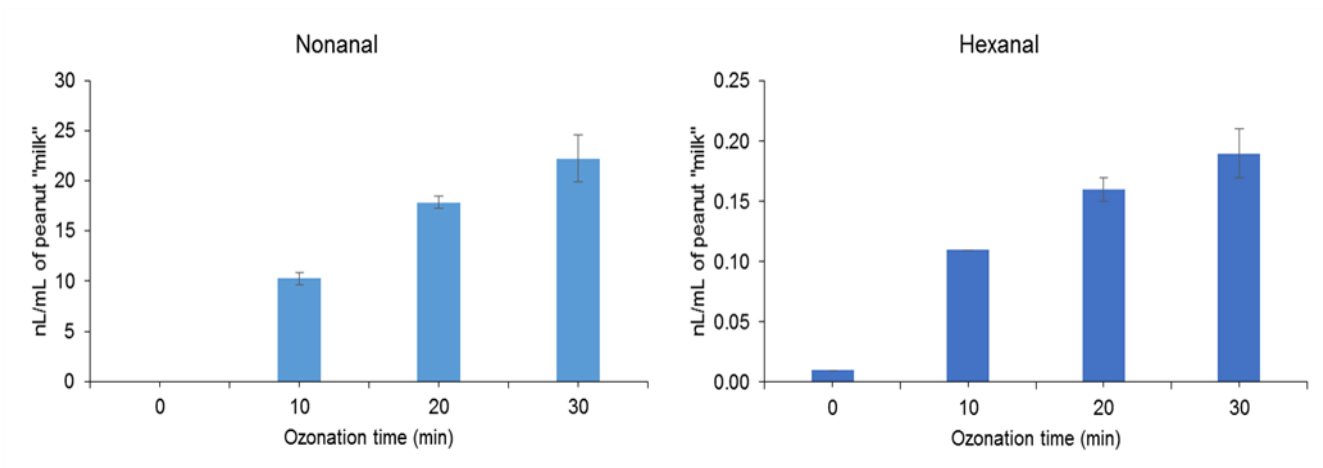


Figure 3. Effect of ozonation on the concentration of nonanal and hexanal in peanut “milk” (\*n.d.: not detectable according to the limit of detection of the method, and considered as zero for the statistical calculation. Error bars represent the standard deviation).

Therefore, studies focusing on strategies to protect the fatty acids of the peanut “milk” against the oxidation promoted by ozonation must be encouraged. These strategies could include adding antioxidants resistant to the ozonation treatment, process engineering approaches (such as separation of currents, performing different treatments in different compositions), among others. Moreover, further studies regarding the effects of combined treatments, such as mild heating and different ozone concentrations at lower exposition times on sensory attributes in addition to aflatoxin levels and allergenicity should be encouraged as well.

### 3.4. Ozone consumption during processing

Inside the reactor, ozone is primarily consumed in reactions with organic compounds that are more susceptible to oxidation. At the same time, the interaction with less susceptible compounds occurs over the processing time. The ozone consumed by the reaction is the difference between the ozone at the reactor inlet (ozone produced) and the gas at the reactor outlet (surplus ozone). By evaluating the ozone consumption over processing time, the reaction intensity can be discussed. Figure 4 shows the ozone consumed during the ozonation of peanut “milk”, highlighting the moments where most of the evaluated effects were observed.

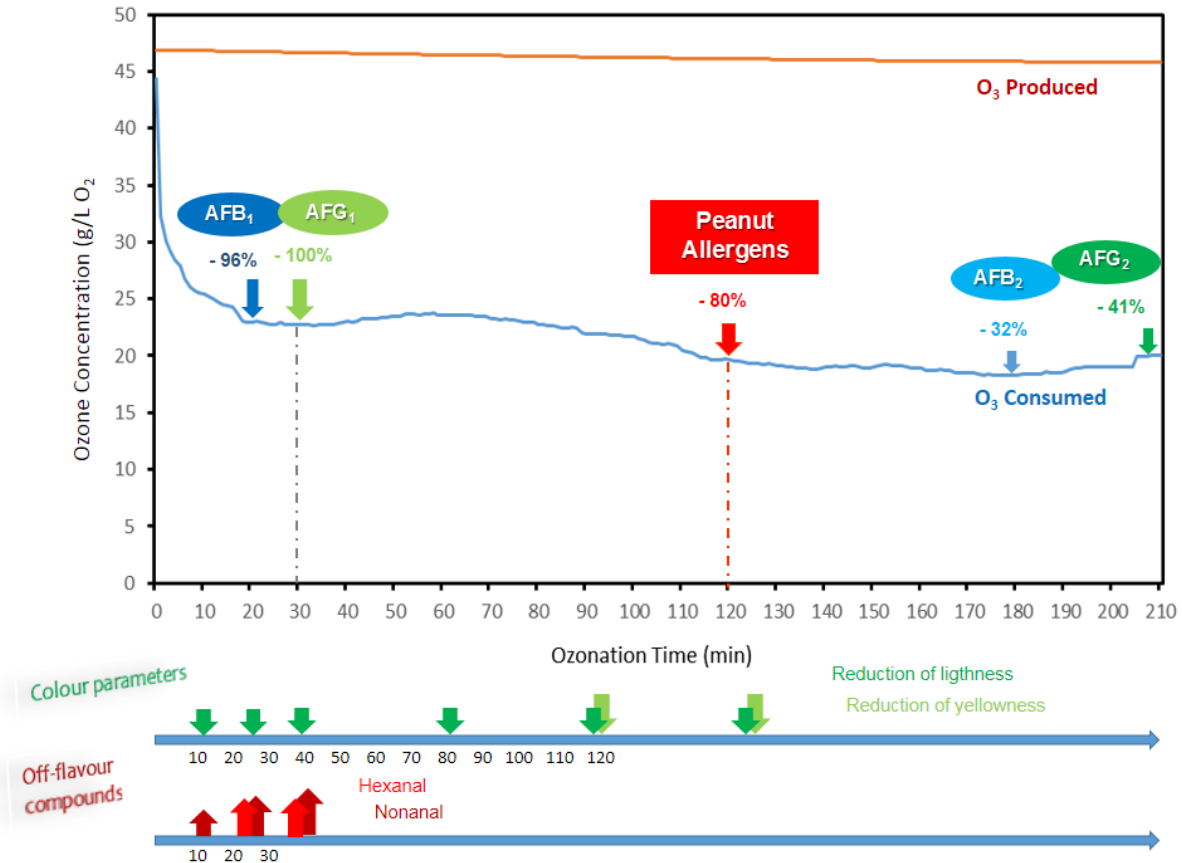


Figure 4. Ozone consumed during the ozonation process of peanut “milk” (blue line) and effects on the parameters evaluated over time: aflatoxins, peanut allergens, colour, and fatty acids, off-flavour volatile compounds.

The reduction followed by the stabilization of the ozone, consumed during peanut “milk” processing, seems to occur after 60 min. This increase in the surplus ozone leaving the reactor suggests the more susceptible compounds were degraded. In fact, the most sensitive aflatoxins were degraded in the first 30 min of processing. Despite the fact that 30 min is enough to degrade AFB<sub>1</sub> and AFG<sub>1</sub>, peanut allergens were more resistant to ozone oxidation.

However, since the ozone is a non-selective oxidizer, the reaction with other compounds occurs concurrently. Ozone can act as an oxidizing agent promoting an electrophilic attack or as a radical reagent (Bailey, 1958).

The sensitivity of fatty acids found in peanut “milk” was shown by the formation of undesirable off-flavour volatile compounds up to the first 10 min of ozonation. Nonanal and hexanal formation found in this study suggest a drawback of using ozonation as a technique



to degrade AFB<sub>1</sub> and AFG<sub>1</sub> in peanut “milk”, highlighting the need for future studies applying collaborative approaches to avoid fatty acid oxidation. On the other hand, ozonation seems to be a possibility to change protein conformation, altering conformational epitopes, and thus leading to a reduction in the recognition of allergens in peanut “milk”, which clearly represents a potential to decrease the allergenicity of peanut proteins.

In the present study, we aimed at investigating the effect of ozonation on peanut allergens as a potential technology to be applied for hypoallergenic products. However, more studies are needed to thoroughly assess ozonation potential to obtain such products. The combination of complementary strategies to ozonation might improve the exposure of the epitopes to readily ozone access. This approach can not only reduce the time needed to achieve the reduction of allergen levels with lower off-flavours development, but also might lead to even lower allergenicity. Future studies can determine an ideal combination of technologies to achieve an ideal; nevertheless, the results presented useful information to the scientific community to base future approaches.

#### **4. Conclusion**

This work evaluated the ozonation process of peanut “milk” contaminated with four aflatoxins, also evaluating the effect of this technology in peanut allergens and quality parameters (lipid oxidation). Ozonation was efficient to degrade AFB<sub>1</sub> and AFG<sub>1</sub> within 30 min, but AFB<sub>2</sub> and AFG<sub>2</sub> were more resistant even after 210 min. A discussion was provided concerning their molecular differences and reactivity, as well as their relative importance in actual products. The peanut allergen detection was reduced up to 77.4% after 120 min of ozonation. However, the lipid oxidation occurred at undesirable levels even in lower times of ozonation, highlighting the need to combine other technologies with ozonation to process peanut “milk”.

Therefore, there is a need for future studies and industrial developments to find the best compromise among the potential for aflatoxin degradation (considering actual products and their contamination) and lipid oxidation (also considering the use of antioxidants) in each context. In addition, the potential of ozonation to reduce the peanut allergens (when desirable)

must be better evaluated by comprehensive studies focusing on chemistry, toxicology and process, including the major allergen Ara h 2.

However, we highlight the obtained results as a contribution to future developments in improving the safety and quality of plant-based “milks”, including further studies regarding ozone effects on food allergens.

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### **Author contributions**

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