

**University of São Paulo
“Luiz de Queiroz” College of Agriculture”**

**Ozone technology as an alternative for reducing mycotoxin
contamination in wheat products**

Allana Patrícia Santos Alexandre

Thesis presented to obtain the degree of Doctor in
Science. Area: Food Science and Technology

**Piracicaba
2018**

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RESUMO

Tecnologia do ozônio como alternativa para reduzir a contaminação de micotoxinas em produtos de trigo

O objetivo deste estudo foi avaliar a redução nos níveis de micotoxinas em produtos e subprodutos de trigo: desoxinivalenol (DON) em farinha de trigo integral, farelo de trigo e efluente de moagem da farinha de trigo e zearalenona (ZEA) em farelo de trigo. No primeiro momento, a redução da contaminação por DON foi estudada em farinha de trigo integral, naturalmente contaminada e considerando diferentes níveis de umidade, bem como no efluente de moagem úmida da farinha. Além disso, o impacto do processo de ozonização nas propriedades reológicas da farinha processada foi avaliado. Em segundo lugar, estudou-se o farelo de trigo naturalmente co-contaminado com DON e ZEA, considerando a degradação de ambas as micotoxinas e o impacto do processo de ozonização no conteúdo do composto fenólico do farelo e na capacidade antioxidante. A degradação de DON na farinha de trigo integral aumentou tanto com o tempo de processamento quanto com o teor de umidade. Ao alterar esses parâmetros de processo, foi possível obter produtos de acordo com os limites legais do Brasil e da União Européia, mesmo com a concentração 2-4 vezes superior aos limites legais. Contudo, as propriedades reológicas da farinha de trigo integral foram afetadas pelo processo, provavelmente devido a modificações de proteínas. A concentração de DON no efluente de moagem úmida foi linearmente reduzida pela ozonização. Em farelo de trigo naturalmente contaminado e em sua umidade de equilíbrio, a ozonização reduziu a contaminação DON e ZEA. A degradação do ZEA foi maior e mais rápida que a degradação do DON, o que poderia ser explicado pelas suas estruturas moleculares. Observou-se também que o processo de ozonização não afetou negativamente os compostos fenólicos e a capacidade antioxidante, o que é altamente desejável do ponto de vista nutricional. Consequentemente, este trabalho conclui que o processo de ozonização foi efetivo na redução de DON e ZEA em diferentes produtos de trigo e efluentes. Vale ressaltar que os resultados obtidos são promissores para futuros estudos e elucidar o mecanismo de ação do ozônio sobre micotoxinas e constituintes dos alimentos.

Palavras-chave: Micotoxinas; Ozonização; Grãos; Efluente; Qualidade; Propriedades reológicas

ABSTRACT

Ozone technology as an alternative for reducing mycotoxin contamination in wheat products

The objective of this study was to evaluate the reduction on the levels of mycotoxins in wheat products and by-products: deoxynivalenol (DON) in whole wheat flour, wheat bran and the effluent from wet milling of wheat flour, and zearalenone (ZEN) in wheat bran. Firstly, the reduction of DON contamination was studied on whole wheat flour, naturally contaminated, and considering different moisture levels, as well as in wet milling effluent of wheat flour. Further, the impact of the ozonation process on the rheological properties of the processed flour was evaluated. Secondly, the wheat bran naturally co-contaminated with DON and ZEN was studied, considering the degradation of both mycotoxins and the impact of the ozonation process on the bran phenolic compound content and on the antioxidant capacity. The DON degradation in the whole wheat flour increased with both processing time and moisture content. By changing these process parameters, it was possible to obtain products in accordance with the legal limits of Brazil and the European Union, even starting with concentration 2-4 times higher than the legal limits. However, the rheological properties of the whole wheat flour were affected by the process, probably due to protein modifications. The DON concentration on the wet milling effluent was linearly reduced by the ozonation. In wheat bran naturally contaminated and in its equilibrium moisture, the ozonation reduced both DON and ZEN contamination. The degradation of ZEN was higher and faster than the degradation of DON, which could be explained by their molecular structures. It was also observed that the ozonation process did not negatively affect the phenolic compounds and the antioxidant capacity, which is high desirable from a nutritional point of view. Consequently, this work concludes that the ozonation process was effective in reducing DON and ZEN in different wheat products and effluent. It is noteworthy that the results obtained are promising for future studies and to elucidate the mechanism of action of ozone on mycotoxins and constituents of food.

Keywords: Mycotoxins; Ozonation; Grains; Effluent; Quality; Rheological properties

RESUMEN

La tecnología de ozono como alternativa para reducir la contaminación de micotoxinas en productos derivados de trigo

El objetivo de este estudio fue evaluar la reducción en los niveles de micotoxinas de productos y subproductos del trigo: desoxinivalenol (DON) en harinas de trigo integral, salvado de trigo y efluentes de la molienda húmeda de la harina de trigo; y zearalenona (ZEA) en salvado de trigo. En primer lugar, se estudió la reducción del contenido de DON en harina de trigo integral naturalmente contaminada, considerando diferentes niveles de humedad, así como en el efluente de la molienda húmeda de la harina de trigo. Además, se evaluó el efecto del proceso de ozonización en las propiedades reológicas de la harina. En segundo lugar, se estudió la reducción de DON y ZEA usando ozono en el salvado de trigo naturalmente contaminado, así como su impacto en los compuestos fenólicos y la capacidad antioxidante. La degradación de DON en la harina de trigo integral aumento cuando mayor fue el tiempo de procesamiento y la humedad de las muestras. Al variar dichos parámetros del proceso, fue posible obtener productos con contenidos de micotoxinas de acuerdo con los límites legales de Brasil y la Unión Europea, procesando incluso muestras con concentraciones de 2 a 4 veces superiores a los límites legales. Por otro lado, las propiedades reológicas de la harina de trigo integral fueron afectadas por el proceso, debido probablemente a cambios en las proteínas. La concentración de DON en el efluente de la hidratación fue linealmente reducida por la ozonización. Con respecto al salvado de trigo naturalmente contaminado y en su humedad de equilibrio, la ozonización redujo la contaminación tanto de DON como de ZEA. La degradación de ZEA fue mayor y más rápida que la degradación de DON, lo que pudo ser explicado a sus estructuras moleculares. Se observó también que el proceso de ozonización no afectó negativamente los compuestos fenólicos ni la capacidad antioxidante, lo que es altamente deseable desde el punto de vista nutricional. Consecuentemente, este trabajo concluye que el proceso de ozonización fue efectivo en la reducción de DON y ZEA en diferentes productos del trigo y efluentes. Cabe resaltar que los resultados obtenidos son prometedores para futuros estudios específicamente donde se aclaren los mecanismos de acción del ozono sobre las micotoxinas y otros componentes de los alimentos.

Palabras clave: Micotoxinas; Ozonización; Grano; Efluente; Calidad; Propiedades reológicas

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1. INTRODUCTION

Grains provide significant amounts of nutrients (ZIELIŃSKI; KOZŁOWSKA, 2000) and wheat, one of the world's most important cereals, is high in carbohydrates, proteins, minerals, lipids, vitamins and fibers (KOEHLER; WIESER, 2013). The interest in health benefits provided by grain consumption has led to a higher focus on their phytochemical content, including derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds (ADOM; LIU, 2002; ADOM; MARK; SORRELLS, 2003). The phenolic compounds present in the grains have antioxidant properties associated with the health benefits; the flavonoids possess potent antioxidant and anticancer activities (ADOM; LIU, 2002). The consumption of these compounds reduces the risk of chronic diseases, such as cardiovascular diseases and cancer (ADOM; MARK; SORRELLS, 2003).

However, the cereal crop can be affected due to the presence of contaminants, such as mycotoxins. Mycotoxins are secondary metabolites produced under specific conditions by some fungi and may be present at various stages of agricultural production, causing damage to human and animal health, economic losses to agribusiness. In addition, mycotoxins are stable for most food processing and can concentrate on specific fractions of the products obtained. In commercial milling there is a redistribution of the mycotoxins in obtained grain fractions (SCUDAMORE et al., 2008), which could be larger than that found in whole grains and above tolerable limits in national or international legislation. Even with the adoption of good agricultural and manufacturing practices, identifying the main critical points of control in the pre- and post-harvest, encompassing every production chain of a given product, there are situations where the presence of some mycotoxin is unavoidable. During the development of the crops in the field exceptional climatic conditions can occur that favor fungal infections (CALORI-DOMINGUES et al., 2016). Also, the impact of climate change on fungal growth and mycotoxin production has been studied, as well as the interaction of different factors such as water activity, temperature and CO₂ concentration (MEDINA et al., 2015). Fungal growth and mycotoxin contamination are consequences of the interaction between fungi, substrate (host) and the environment. An appropriate combination of these factors

determines the infection and colonization of the substrate, as well as the type and amount of mycotoxin produced.

The most studied mycotoxins, due to their occurrence and toxic effects, are aflatoxins (B₁, B₂, G₁ and G₂), fumonisins B₁ and B₂ (FB₁ and FB₂), trichothecenes (deoxynivalenol (DON), nivalenol, T-2 toxin, diacetoxiscirpenol), ochratoxin A (OTA) and zearalenone (ZEN). These substances have already been detected in corn, peanuts, cottonseed, rice, wheat, beans, dried fruit, soy, milk, cheese, beer, animal feed, wine, among others (CAST, 2003). The occurrence of DON and/or ZEN in grains has been reported over the years in different grains and derivatives. In Brazil, the occurrence was evaluated in the following grains and/or their derivatives: wheat (CALORI-DOMINGUES et al., 2007, 2016; SANTOS et al., 2013; ALMEIDA-FERREIRA et al., 2013), corn (MILANEZ et al., 2006; SILVA; VARGAS, 2001), rice (DORS; BIERHALS; BADIALE-FURLONG, 2011), soybean (CALORI-DOMINGUES et al., 2014; BARROS et al., 2012) and barley and wheat (MALLMANN et al., 2017).

Although there are geographic and climatic variations in the production and occurrence of mycotoxins, exposure to these substances occurs worldwide and it is estimated that a significant part of the world's food supply is positive for some mycotoxins. To minimize the risks associated with mycotoxin contamination it is important the application of a contamination management system that involves prevention, control and monitoring. For this reason, there is a demand for alternatives to reduce mycotoxin contamination in agricultural products, their derivatives and by-products. Consequently, one important objective of the food and feed industries worldwide is the research to obtain a decontamination process that is commercially applicable. For this reason, there is a demand for alternatives to reduce mycotoxin contamination in agricultural products, their derivatives and co-products.

Among the possible methods used to reduce the concentration of mycotoxins (physical, chemical and biological), the use of ozone (O₃) presents important advantages, being considered an emerging technology. Ozone is a powerful oxidant, which can be simply generated at the moment of use (from O₂) and leaves no residues further than oxygen, being an interesting alternative in the management of mycotoxin contamination. Based on that, ozone is considered a Green Chemical Process, and a safe substance - *Generally Recognized As Safe* (GRAS). Its use, under gas or aqueous phase, is regulated by the Food and Drug Administration (FDA) in agricultural products *in natura* (FDA, 2001; RICE; GRAHAM, 2001).

The ozonation has been evaluated in grains and derivate to control insects (KELLS et al., 2001) and fungi (RAILA et al., 2006; WU; DOAN; CUENCA, 2006), as well as in the decontamination of aflatoxins (DE ALENCAR et al., 2012; LUO et al., 2014a, 2014b; PRUDENTE JR.; KING, 2002), their naturally contaminated derivatives and co-products. For DON, the first studies evaluating the ozone efficiency on DON degradation showed promising results for ground corn (YOUNG et al., 1986), but small reduction in wheat grain samples (YOUNG, 1986). Recently, studies of naturally and artificially contaminated wheat grains (spiked in the product) with DON (SAVI et al., 2014; WANG et al., 2016a, 2016b), showed effective reductions in mycotoxin concentration.

Artificial contamination mainly evaluates the toxins present in the outer layers of the grains (DE ALENCAR et al., 2012). Due to the characteristics of the fungal infection, the highest concentrations of hyphae are generally found in the outer layers of the grains, however, the fungus is distributed along the grain (LEE et al., 1987; SEITZ; BECHTEL, 1985). In fact, studying naturally contaminated samples (rather than artificially contaminated grains) is of great importance, which is in line with the actual field conditions and the natural distribution of mycotoxins in the contaminated grains (localization in tissues and cells, as well as interaction with other molecules).

Although the application of ozone in the decontamination of mycotoxins is promising and there are some studies with this objective, there are few studies related to the degradation of DON and ZEN using ozonation in naturally contaminated wheat products, as well as there is a lack of information regarding the impact on the product quality.

Consequently, the present Thesis was conducted in order to expand the knowledge in these subjects.

1.1 Objectives

1.1.1. General Objectives

The objective of this work was to apply the ozone technology to reduce the contamination of deoxynivalenol (DON) and zearalenone (ZEN) in wheat products and co-products, as well as to evaluate the impact of this process on the products quality.

1.1.2. Specific Objectives

- Study the effect of ozonation on the reduction of DON contamination in whole wheat flour naturally contaminated and wet milling effluent wheat flour;
- Evaluate the effect of ozonation on the degradation of DON and ZEN on wheat bran naturally contaminated;
- Analyze the impact of ozonation on rheological properties of whole wheat flour;
- Evaluate the nutritional quality of wheat bran after the ozonation process.

To achieve these objectives, this Thesis is presented in chapters. The first chapter involves the Introduction, Objectives and Literature Review, being designed to contextualize the reader. The second chapter evaluated the ozone processing of whole wheat flour and wet milling effluent, being published at the Journal of Environmental Science and Health Part B - Pesticides, Food Contaminants and Agricultural Wastes (<http://dx.doi.org/10.1080/03601234.2017.1303325>). The third chapter evaluated the ozone processing of wheat bran, being currently under review. The third chapter also presents the Conclusions and Suggestions for Future Studies. Finally, Appendix A presents the work carried out at the Catholic University of Portugal, as a sandwich period of this Ph.D.

1.2. 1.2 Literature review

1.2.1. General considerations

Among the *Fusarium* mycotoxins, zearalenone (ZEN) and deoxynivalenol (DON) have been found with relative frequency in agricultural products, both alone and together. ZEN is a toxin mainly produced by *Fusarium graminearum* and also by *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. crookwellense* and *F. semitectum* (GIL-SERNA, et al., 2014). DON is produced by *F. graminearum*, and related species to *F. culmorum* (BEATTIE, et al., 1998; GREENHALGH; NEISH; MILLER, 1983) it is also known as vomitoxin belonging to group B of trichothecenes being prevalent within the

group. The fungal species that produce these mycotoxins are plant pathogens found in soils and cereal crops in the field such as wheat, corn, barley, rye, but may also occur in storage (EFSA, 2014).

Diseases caused by mycotoxins are called mycotoxicosis that can affect men and animals; the results of most animal poisoning are loss of production. Most mycotoxins have specific effects on a given system, and they can affect several systems simultaneously (CAST, 2003).

Toxic effects of DON on animals cause damage mainly to the immune system and gastrointestinal tract; acute doses are characterized by effects such as diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock and ultimately death; chronic doses are characterized by refusal of food, reduction in weight gain and nutrient absorption and neuroendocrine and immunological changes (LARSEN et al., 2004; PESTKA; SMOLINSKI, 2005; PITT; MOTARJEMI, 2014). The toxic potential of DON in humans implies acute gastroenteritis with vomiting beyond the chronic effect on growth immune and reproductive functions (PESTKA; SMOLINSKI, 2005).

ZEN causes several toxic effects in animals, especially those related to the reproductive system (CAST, 2003). Further, according to the International Agency for Research on Cancer (IARC), ZEN can binds to estrogen receptors in human breast cancer cells (IARC, 1993). Pigs are the most commonly affected animal, with estrogenic effect, but there are few epidemiological studies related to this effect in humans (PITT; MOTARJEMI, 2014). Studies have suggested ZEN as the cause in the early development of puberty in children in Porto Rico (SAENZ DE RODRIGUEZ; BONGIOVANNI; CONDE DE BORREGO, 1985; SAENZ DE RODRIGUEZ, 1984) and in girls in Italy (MASSART et al., 2008).

Among the foods consumed in both human and animal feed, corn, wheat, rice and soybeans are prominent, as well as derived products and co-products such as flours, meal, bran, pre-cleaning waste, etc. in which the mycotoxins provided for in the legislation have already been detected.

Contamination by mycotoxins in cereals represents a public health problem as well as an economic obstacle in several countries since the trade balance is based on the export of commodities (ROCHA et al., 2014).

1.2.2. Mycotoxins in grains and derivatives

According to the Food and Agriculture Organization of the United Nations (FAO) world cereal production in 2017/18 is expected to reach 2,591 million tonnes (FAO, 2017). In Brazil the production of grains reached 238.78 million tons, and wheat production shall be 5,221.4 thousand tons (CONAB, 2017). According to the Brazilian Agriculture Yearbook, the national wheat production up to September 2017 was 5,185,500 tons (AGRIANUAL, 2017).

The occurrence of DON and ZEN in cereals was reported by Tanaka et al. (1988), who showed their occurrence in cereal samples collected from 19 countries. Since then, several works have been performed showing the presence of DON and ZEN in different cereals and derivatives (EFSA-CONTAM, 2013). In Brazil the occurrence of DON was evaluated by Calori-Domingues et al. (2007), which analyzed 50 samples of national wheat (São Paulo, Paraná and Rio Grande do Sul) and 50 imported ones (Argentina and Paraguay). In the evaluated samples, 60% of the Brazilian wheat had contamination of 90 to 1000 $\mu\text{g kg}^{-1}$, while the Argentinean and Paraguayan wheat, 42% of the contamination was 90 to 349 $\mu\text{g kg}^{-1}$. Del Ponte et al. (2012) observed that 12 of the 64 analyzed wheat samples from Rio Grande do Sul were above 1000 $\mu\text{g kg}^{-1}$. Santos et al. (2013) evaluated 113 wheat samples produced in the northeast, southwest and central regions of Paraná and observed the presence of DON in 66.4% of the samples. In the years from 2006 to 2008, Santos et al. (2011) evaluated 36 wheat samples from the States of Paraná and Rio Grande do Sul, of which 72.2% were positive for DON, with a maximum concentration of 1592.21 $\mu\text{g kg}^{-1}$. Lamardo; Navas; Sabino (2006) analyzed samples of wheat and wheat flour in the state of São Paulo. Fifty per cent of the wheat flour samples and 36% wheat were contaminated with DON with maximum concentrations of 600 and 1500 $\mu\text{g kg}^{-1}$, respectively.

The presence of other types of mycotoxins have also been studied in cereals, such as Oliveira et al. (2002) who studied the incidence of aflatoxins in peanuts and derivatives, ZEN in corn and rice and DON in bakery products, flour and wheat bran, in the State of Minas Gerais. Of the 120 peanut samples, 56 were above the limit established by the Ministry of Agriculture Legislation with total aflatoxin. DON was present in 32 samples from the 47 analyzed, with a maximum concentration of 1205 $\mu\text{g kg}^{-1}$ and ZEN was detected in only one sample.

Silva and Vargas (2001) analyzed 380 maize samples, where they observed the presence of ZEN in 30 samples (7.8%) with a mean concentration of $232 \mu\text{g kg}^{-1}$. Dors et al. (2011) analyzed parboiled rice for the presence of DON and ZEN. Of the 32 samples analyzed, 22% and 19%, respectively, were above maximum limit (MT). Heidtmann-Bemvenuti et al. (2012) evaluated two methods (QuEChERS and partition) for determination of DON and ZEN in natural and parboiled rice. The QuEChERS method presented a better recovery for the two mycotoxins studied (91% for DON, 105% for ZEN).

Calori-Domingues et al. (2016) analyzed 745 samples of wheat grains produced in Brazil (2009 and 2010 crop years), for the presence of ZEN, DON and nivalenol (NIV). They observed that the incidence of ZEN, DON and NIV in 2009 was higher than 2010 (85%, 90% and 77%, respectively). However for DON the incidence in 2010 was 83% but the mean concentration ($1690 \mu\text{g kg}^{-1}$) was much lower than in 2009 ($407 \mu\text{g kg}^{-1}$). This is justified because of different climatic conditions between the years that may influence crop infection and result in a higher incidence of *Fusarium* spp and mycotoxin production. When evaluating the co-occurrence of mycotoxins, the researchers observed that in 2009 ZEN, DON and NIV was 74%, ZEN and DON 11% on the samples, in 2010 the presence of ZEN, DON and NIV was 12%, ZEN and DON 14% on the samples. In summary, it can be observed that the concentrations of mycotoxins can vary each year and the monitoring of contamination needs to be permanent.

Iqbal et al. (2014) analyzed aflatoxins, ochratoxin A and zearalenone in samples of breakfast cereals in Pakistan. About 8% were contaminated with ZEN, 30% for ochratoxin A and 41% with aflatoxins were above the maximum limit allowed by the European Union. Alkadri et al. (2014) investigated the co-occurrence of mycotoxins in wheat in Syria and Italy. In general, the researchers observed that the incidence of trichothecenes was highest in the wheat produced in Italy, while aflatoxin and ochratoxin A in wheat from Syria. Ennouari et al. (2013) evaluated 81 samples of durum wheat in Morocco, 11.1% were contaminated with deoxynivalenol and the maximum observed concentration was $1310 \mu\text{g kg}^{-1}$.

In fact, although there are geographic and climatic variations in the production and occurrence of mycotoxins, exposure to these substances occurs

worldwide, once it is estimated that about 25% of the world food supply are contaminated with mycotoxins (PARK; NJAPAU; BOUSTRIF, 1999).

The studies showed a wide range of contamination, varying according to the year of the survey, fact that shows the seasonal occurrence of the contamination of the agricultural products, since depend on the environmental conditions.

1.2.3. Methods of mycotoxin decontamination

Preventing mycotoxin contamination in cereals prior to harvesting, post-harvesting and storage by adopting the Good Agricultural Practices is the best choice. However, even following Good Agricultural Practices it is not always possible, due to external factors such as environmental conditions during the production stages. As an alternative, decontamination methods are used to reduce mycotoxin contamination to maximum limits for use in food or feed (KABAK; DOBSON; VAR, 2006). Among the methods used in the decontamination of mycotoxins in agricultural and processed products are the physical, chemical and biological methods (LUO et al., 2014b). Some examples are described as follows.

Moreau et al. (2013) used pulsed light to decrease the concentration of ZEN and DON in solution with efficiency of 84.5% and 72.5%, respectively. Atalla et al. (2004) employed UV and fluorescent light was on artificially contaminated wheat grains, where they observed reductions of 87 to 100% for aflatoxins, ochratoxin A, ZEN, nivalenol, deoxynivalenol, T-2 and DON toxin. The reduction of DON and ZEN was effective after 30 minutes of treatment, with 87% reduction of ZEN. Ibarz et al. (2015) studied the photo-degradation of ocratoxina A by UV irradiation. The pH of the aqueous solutions influenced the photo-degradation of mycotoxin, being higher at pH 7 than at pH 4. The photo-degradation of patulin was also studied (IBARZ; GARVÍN; IBARZ, 2017). In this case, the more acidic the solution, higher the kinetic constant, increasing the rate of reaction.

However, the UV radiation obtained has only a superficial effect and presents several drawbacks, especially the difficult application due to the shadow areas, with limited practical application. In fact, the photo-processing is a promising technology for fluids; however, it shows many difficulties of application on solid and particulate products.

Zhang et al. (2012) studying the decontamination of aflatoxin B₁ in peanuts, with acidic electrolyzed oxidizing water, observed a reduction of 85%. Fan et al. (2013) also studied the decontamination of aflatoxin B₁ in vegetable oils with water alkaline electrolysis with different pHs, after 5 minutes almost 100% of the mycotoxin was removed. Martins et al. (2017) studied the degradation of aflatoxin during peanut roasting. After 20 minutes at 180°C, the maximum reduction was 81%. By increasing the temperature to 200°C, the maximum aflatoxin reduction was 89.7% after 20 minutes. Yumbe-Guevara; Imoto; Yoshizawa (2003) studied the effect of heating of DON, ZEN and NIV on barley and wheat. In barley powder, the reduction of NIV was 70% (200°C, 58 min); ZEN 80% (220°C, 18 min) and 50% DON (220°C, 8 min).

Sangare et al. (2014) studied the efficiency of *Pseudomonas aeruginosa* culture in the reduction of AFB₁. The reduction of AFB₁ with the supernatant culture after 12 hours was 43.3%. After 72 hours, AFB₁ was reduced in 72.5% compared to viable cells (40%) and cell extracts (24.4%). After 7 days, 94.3% of AFB₁ was degraded; the reduction was observed to vary at different temperatures, and the ideal temperature was 55°C (90.2%).

Decontamination using ozone gas (O₃), which is a potent oxidant, is an interesting alternative due to the advantages of its application. It can be easily generated at the moment of use, from oxygen (pure or from the air) and it does not leave residues. Being considered a green chemical process (Green Chemical Process), the ozonation is a technology of great interest for the processing of food products.

1.2.4. Ozone

Ozone is a substance naturally found in our atmosphere, but it can also be produced synthetically from oxygen. The word "ozone" is derived from the Greek word "ozein" which means "to smell" (MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005). Ozone (O₃) is the triatomic oxygen formed by addition of a free radical from oxygen to molecular oxygen (Figure 1.1) (GREENE; GÜZEL-SEYDIM; SEYDIM, 2012), commercially the ozone is formed from the electric discharge, the most known method is the corona discharge (Equation 1.1) (GUZEL-SEYDIM;

GREENE; SEYDIM, 2004). In the laboratory, for higher ozone yields, oxygen must be used as the feed instead of air (MILLER; SILVA; BRANDÃO, 2013).

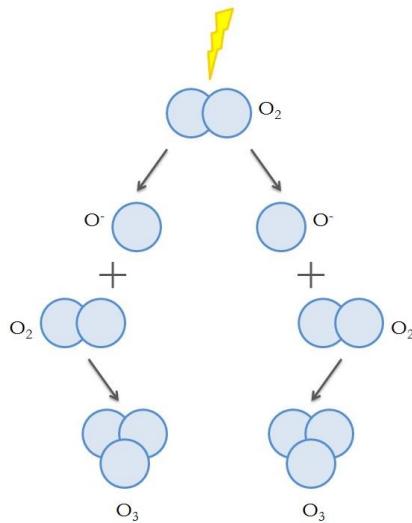


Figure 1.1 Formation of the ozone molecule

The corona effect occurs by the electric discharge method, due to the passage of air or high concentration of pure oxygen between two electrodes subjected to a high potential difference of approximately 10 kV (ALMEIDA et al., 2004).



Ozone was discovered in 1839 by Schönbein, noting that the electrolysis of water produced a gas that exuded a characteristic odor. However, it was not until 1906 in France that ozone was first commercially used as a drinking water disinfectant (TIWARI; RICE, 2012). In 1982 the Food and Drug Administration (FDA) recognized ozone as a safe (*Generally Recognised As Safe - GRAS*) for disinfection of bottled water and as a disinfectant in bottled water process and bottling facilities (TIWARI; RICE, 2012). In 1997, ozone was confirmed with GRAS status for direct food contact (GÜZEL-SEYDIM; BEVER JR.; GREENE, 2004), and in 2001 the FDA approved its use as a food additive and antimicrobial agent; three years later the FDA issues guidelines and recommendations to the juice and apple cider processing industries (TIWARI; RICE, 2012).

1.2.4.1. Properties of ozone

The strong reactivity of ozone is due to its molecular structure, composed of three oxygen atoms. In the valence layer of each oxygen atom there are two unpaired electrons, each occupying the 2p orbital (GREENE; GÜZEL-SEYDIM; SEYDIM, 2012) (Figure 1.2). The molecule is unstable and highly reactive.

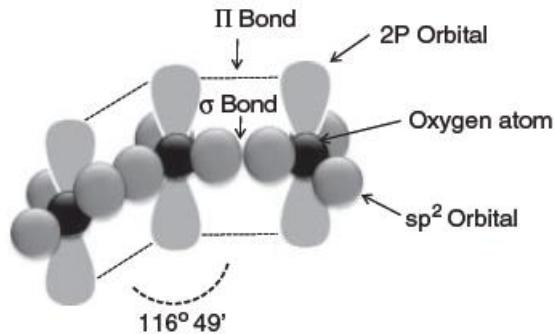


Figure 1.2 Molecular structure of ozone (GREENE; GÜZEL-SEYDIM; SEYDIM, 2012)

Ozone is liquid at -111.9°C and solid at -192.7°C having an opaque blue-black color (ÇATAL; İBANOĞLU, 2012). It has a high electrochemical potential ($E^0 = 2.07V$), indicating that ozone is an excellent oxidizing agent (MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005). Table 1.1 summarizes the physical properties of ozone.

Table 1.1 Physical properties of ozone (MAHAPATRA; MUTHUKUMARAPPAN; JULSON, 2005)

Physical properties	Value
Boiling point	-111.9°C
Density	2.14 kg/m ³
Heat of formation	144.7 kJ/mole
Melting point	-192.7°C
Molecular weight	47.9982 g/mole
Oxidation strength	2.075 V
Solubility in water	3 ppm (at 20°C)

Ozonation is an alternative method as a sanitizer in some products, so this gas can be an alternative to conventional methods (ÇATAL; İBANOĞLU, 2014). In conditions of neutral or alkaline pH, the ozone decomposes generating hydroxyl radical (*OH), as described on Equation 1.2 (DEZOTTI, 2008; ALMEIDA et al., 2004).



In fact, many studies were conducted focusing on the microbial inactivation using ozone. For example, Güzel-Seydim, Bever Jr. and Greene (2004) studied the bactericidal potential of ozone, demonstrating that spores are more resistant than vegetative cells. Alwi and Ali (2014) studied the efficacy of ozone to reduce foodborne pathogens such as *Escherichia coli* O157, *Listeria monocytogenes* and *Salmonella enterica* sv. *Typhimurium* in minimally processed peppers. The exposure to 9 mg L⁻¹ of ozone for 6 hours reduced pepper pathogens, demonstrating the high potential of ozone to be an alternative in sanitizing treatments. Amorim et al., (2013) studied the inactivation of *Bacillus subtilis* spores and *Escherichia coli* cells in cassava starch, with different moisture contents, ozone concentration and exposure

time. There was an inactivation of >7 log cycles of *Escherichia coli* with 40 mg L⁻¹ of ozone for 60 minutes and *Bacillus subtilis* of >5 log cycles with 118 mg L⁻¹ of ozone for 120 minutes.

Another contaminant common to cereals are insects, the ozone fumigant effect has also been evaluated in reducing it. Kells et al. (2001) evaluated the ozone at disinfest maize, when applying 50 mg L⁻¹ for 3 days the researchers obtained a reduction of 92 to 100% in the population of the studied insects (*Plodia interpunctella*, *Tribolium castaneum* and *Sitophilus zeamais*).

Ozone efficacy was also studied associated with ultraviolet light as barriers against *Listeria monocytogenes* in brines (KUMAR et al., 2016). A process of 10 min of ozonation and 10 min of UV radiation resulted in reduction >9 log CFU mL⁻¹ in fresh brine; 60 minutes of ozonation and 10 minutes of UV exposure resulted in 5 log CFU mL⁻¹ of reduction in *Listeria monocytogenes* cells in the past brine. The authors concluded that the efficiency of the process depends on the quality of brine used, duration of exposure and dose. Alexandre et al. (2011) studied the efficacy of ozone in the treatment of aqueous solution evaluating the inactivation of three microorganism/food combinations: *Listeria innocua* in red bell peppers (artificially inoculated), total mesophiles in strawberries and total coliforms in watercress. The highest microbial reductions were obtained for the highest ozone concentration with the highest treatment time (2 mgL⁻¹ for 3 min), which resulted on 2.8, 2.3 and 1.7 log cycles in peppers, strawberries and watercress, respectively. However, a portion of the microorganisms were reduced only by washing water without ozone. Consequently, the authors demonstrated that the presence of ozone added an additional reduction of 0.5-1.0 log cycles.

Furthermore, ozone was used to promote desirable modifications in different products. For example, ozone was studied as an alternative for oxidation of starch in substitution of hypochlorite, in order to reduce the residue production (CHAN; BHAT; KARIM, 2009). Gozé et al. (2016) oxidized wheat starch with ozone, while Castanha; Matta Jr; Augusto (2017) studied the modification of potato starch using ozonation. The researchers observed that the process modified the structural and functional properties of the starch, proving that ozone is an excellent alternative to the methods currently used.

Sartori et al. (2017) studied the clarification of sugarcane juice from ozonation as an alternative to the usual sulphation method. The researchers used the process to decrease gallic acid, a pigment widely found in the broth. They observed that ozone was efficient in reducing this pigment.

The destruction of pesticides can also be carried out with ozonation. Bourgin; Albet; Violeau (2013) studied the degradation of two types of pesticides in seeds, observing that the preliminary humidification of the seeds increased the effectiveness of the process. Chen; Lin; Kuo (2013) studied the removal of residues of pesticides present in plants, observing a removal of 70% of the initial content. Savi; Piacentini; Scussel (2015) studied pesticide reduction (deltamethrin and fenitrothion) on stored wheat grains. After 180 minutes of treatment (60 µmol/mol), a reduction of 84.8% of deltamethrin and 66.7% of fenitrothion was achieved. Ozone is also used in wastewater treatment, for example in the works as Nöthe; Fahlenkamp; Von Sonntag (2009), Qi et al. (2018) and Snyder et al. (2006).

In addition to its capacity for destruction of microorganisms, insects, pesticides and modification of foods, the ozone oxidizing property have been studied for the decontamination of mycotoxins in different products.

1.2.5. Reduction of mycotoxin concentration by ozonation

There are physical, chemical and biological methods (BANU, 2010; MOREAU et al., 2013; SANGARE et al., 2014) employed to decontaminate mycotoxins on agricultural or processed products. Decontamination using ozone (O_3) is an interesting alternative, being successfully used in the control of toxigenic fungi in wheat (EL-DESOUKY et al., 2012 SAVI et al., 2014; WU; DOAN; CUENCA, 2006), peanut (DE ALENCAR et al., 2012) and barley (ALLEN; WU; DOAN, 2003), among others.

The reduction of aflatoxins with ozone has been studied extensively in recent years in several products. Inan; Pala; Doymaz (2007) studied the reduction of aflatoxin B_1 in flaked and chopped red pepper contaminated with 20 $\mu\text{g kg}^{-1}$ and 32 $\mu\text{g kg}^{-1}$, respectively. In the flaked pepper the concentration was reduced to 4 $\mu\text{g kg}^{-1}$ (33 mg $L^{-1} O_3$ after 60 min) and in chopped pepper it was reduced to 2 $\mu\text{g kg}^{-1}$ (66 mg

L^{-1} after 60 min) – ensuring final products with contamination below the limit of the Turkish Food Codex.

Zorlügenç et al. (2008) evaluated the ozonation process in microbial flora in water and the degradation of aflatoxin B₁ in dried figs. In ozonized water, the microorganisms studied (aerobic mesophilic bacteria, *Escherichia coli*, coliform, yeast and mold counts) were completely inactivated after 15 minutes of processing. In the dried fig, the reduction of aflatoxin B₁ was 95.21% after 180 minutes of process and 13.8 mg L^{-1} ozone gas concentration.

Kamber et al. (2016) studied the reduction of aflatoxin B₁ contamination in red pepper, considering artificially contaminated samples. The maximum aflatoxin reduction obtained after the treatment was 74% (80 mg L^{-1} for 40 min).

Considering the cereals it is observed that the ozonation has been studied more in the reduction of aflatoxins in corn (PRUDENTE JR.; KING, 2002) and (LUO et al., 2014a) with a reduction of 92% and 72-88%, respectively, and peanut (DE ALENCAR et al., 2012) with a reduction of 30%.

LUO et al. (2014a) observed that the moisture, ozone concentration and time of exposure had a significant impact on the rate of degradation of aflatoxins in corn flour. In maize grains naturally contaminated with aflatoxins, ozonation decreased their concentration, showing small changes in the most important nutrients (McKENZIE et al., 1998).

Ozone gas demonstrated efficiency in the reduction of ZEN in aqueous solution (DUDZIAK, 2012; LEMKE et al., 1999; McKENZIE et al., 1997). The reduction of toxic effects was also studied. McKENZIE et al. (1997) observed the ozone efficiency to degrade aflatoxin B₁ (AFB₁), patulin, cyclopiazonic acid, secalonic acid D, ochratoxin A, and ZEN. The detoxification was also reduced, which was observed after performing a bioassay with *Hydra attenuata* (McKENZIE et al., 1997). Lemke et al. (1999) verified the toxicological effect of ZEN in the mouse uterine bioassay. The animals were dosed orally daily with ozonized corn oil artificially contaminated with ZEN. It was observed that ozonized corn oil did not increase uterine weights in mice, indicating that ZEN was rapidly degraded in non-estrogenic like products.

In the first studies evaluating the effect of ozonation on the concentration of DON, Young (1986) obtained reductions of 70 to 90% in DON levels of sample corn

that were inoculated with fungus *Fusarium graminearum*, and treated with moist ozone and dry ozone, respectively. It was observed that the percentage of reduction depends on the conditions of the sample. Young et al. (1986) studied wheat naturally contaminated with DON, observing a small reduction in contamination, which were attributed to low grain moisture (12%). Moisture improves the efficiency of the process: highest moisture values facilitate the absorption of ozone by the surface of the sample, in addition, the process is catalyzed by free radicals, such as the hydroxyl ions present in the water, the higher the moisture content, more reactive ions are present (DE ALENCAR et al., 2012; LI; GUAN; BIAN, 2014).

Savi et al. (2014) evaluated the effectiveness of ozone in reducing the contamination with DON in artificially contaminated wheat samples in the pericarp and endosperm. The authors observed a highest percentage of DON reduction as the exposure time and dose increased, without causing physical and biochemical changes in the grain. They pointed out that the higher impact of DON reduction was on the external of the grain (pericarp).

Trombete et al. (2016) evaluating the efficacy of ozone in the decontamination of mycotoxins and fungus in soft wheat grains artificially contaminated, observed a reduction of 64.3 and 48% for DON and total aflatoxins, respectively. In the same process, the total fungal count decreased 3 log cycles.

Wang et al. (2016a) studied the effect of ozone on DON in naturally contaminated wheat grains (15-16% of moisture) and time of exposure up to 90 minutes, obtaining degradation ranging from 26.4 to 53.5%. The authors also observed that there were no significant differences in protein content, starch content, amino acids and fatty acids after ozone processing. In another study, Wang et al. (2016b) evaluated the effect of ozone on the degradation of DON in naturally contaminated whole wheat flour obtaining maximum degradation of 78.7%.

The reduction of the zearalenone contamination has been little studied. The observed studies were carried out mainly by the use of ozone in aqueous solutions (LEMKE et al., 1999; MCKENZIE et al., 1997). Qi et al. (2016) studied the decontamination of ZEN and ochratoxin A in corn naturally co-contaminated using ozone. When the corn (19.6% moisture) was processed with 100 mg L⁻¹ of ozone for 180 min, ZEN concentration decreased from 2903.9 to 405.7 µg kg⁻¹ (86%) and ochratoxin A from 67.6 to 24.1 µg kg⁻¹ (64.2%). In order to compare ozonation

processes, the oxygen flow used, ozone concentration, process time and sample weight should be also considered.

Despite the similarities of the presented works, it is observed that there are still few studies that discuss the reduction in the contamination of mycotoxins in co-products of grains, as well as two mycotoxins in the same products. Further, it is highlighted the need to evaluate the technological and nutritional quality of the product after ozone processing.

Therefore, further studies are needed regarding the decontamination of DON and ZEN in naturally contaminated cereals and their effects on the quality of the ozonized product, as discussed in this Thesis.

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2. OZONATION OF WHOLE WHEAT FLOUR AND WET MILLING EFFLUENT: DEGRADATION OF DEOXYNIVALENOL (DON) AND RHEOLOGICAL PROPERTIES¹

Abstract

The combination of environmental factors can result in fungal plant infection and possible mycotoxin production, even after adopting good practices in the various stages of production, processing, and storage. Deoxynivalenol (DON) is a mycotoxin commonly found in grains and derivatives, compromising the safety and commercialization of the products. Wet milling process wastewater may contain this mycotoxin, with possible negative impacts to the effluent treatment system, besides contaminating aquatic environments, soil, and plants. Thus the reduction of DON contamination in these products is desirable, and ozone (O_3) stands out as a promising alternative. Ozonation is an emerging technology easy to use, safe, with low cost and low environmental impact. The objective of this study was to evaluate the reduction of DON levels in whole wheat flour with different moisture levels and wet milling effluent through ozone processing, as well as the impact of ozonation on the rheological properties of flour. The results have shown that the reduction of DON was improved with increasing moisture and exposure time of whole wheat flour and wet milling effluent to ozone. The maximum reduction was about 80%, proving that ozonation is an effective and promising technology in reducing mycotoxins in different products. However, the process altered the rheological profile of whole wheat flour, thus further studies are needed to better understand the process.

Keywords: Mycotoxins; Ozone; Grains; Effluent

2.1. INTRODUCTION

Mycotoxins are secondary metabolites produced by some fungi, leading to harmful effects to humans and animals, as well as economic losses to agribusiness. These metabolites are produced under specific conditions and can be present at

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various stages of agricultural production, being stable to most processes (RUBERT et al., 2012). Although there are geographic and climatic variations in the production and occurrence of mycotoxins, exposure to these substances occurs worldwide, once it is estimated that about 25% of the world food supply are contaminated with mycotoxins (PARK; NJAPAU; BOUSTRIF, 1999). Therefore, mycotoxins in food represent both a public health problem and economic and industrial losses.

Trichothecenes (deoxynivalenol (DON), nivalenol, T-2 toxin, diacetoxyscirpenol) are produced by fungi of the genus *Fusarium*. They often contaminate foods such as corn, rice, wheat, soy, barley, rye and animal feed (CAST, 2003). Trichothecenes can inhibit protein synthesis, including DNA and RNA, affect cell division, besides interfering with phospholipid metabolism in the cell membrane and alter serotonin activity, related to food intake regulation (YAZAR; OMURTAG, 2008). The toxic potential of DON in humans implies acute gastroenteritis with vomiting, in addition to the chronic effect on growth and immune and reproductive functions (PESTKA, 2007).

One way to minimize some of the risks associated with mycotoxin contamination is to apply a quality management system. However, there are situations where the presence of mycotoxins is unavoidable, even adopting all good production practices. For example, during crop development, climatic conditions can favor fungal infections, resulting in contamination of the grains (CALORI-DOMINGUES et al., 2016). Therefore, it is extremely important to study alternatives that reduce mycotoxin contamination in agricultural products, their derivatives, and co-products.

A promising method of decontamination is the use of ozone (O_3), a gas with high oxidizing potential, generated electrically at the time of use and decomposing into O_2 . It is considered a green chemical process and is recognized as GRAS - *Generally Recognized As Safe*, with use regulated by the FDA for agricultural products *in natura* (FDA, 2001; RICE; GRAHAM, 2001).

Although ozonation may be a promising alternative in the reduction of DON, with many studies about this issue, there are few studies describing such degradation in cereals and their naturally contaminated derivatives. Studying naturally contaminated samples (rather than artificially contaminated grains through contact with mycotoxin solutions) is of great importance, which is in line with the

actual field conditions and the natural distribution of mycotoxins in the contaminated grains (localization in tissues and cells, as well as interaction with other molecules).

Starch and cereal protein concentrates are produced by wet milling processes (i.e., in the presence of water, under suspension). Wheat starch has significant production, corresponding to 36% of the starch production in the European Community in 2005 (MANINGAT et al., 2009). Therefore, due to the affinity of the DON with water (SOBROVA et al., 2010), the wet milling effluent may contain this mycotoxin, with negative impacts to the effluent treatment system and the environment.

The objective of this study was to evaluate the use of ozone to reduce deoxynivalenol (DON) in whole wheat flour and wet milling effluent, as well as the effect of the process on the rheological properties of the processed flour.

2.2. MATERIAL AND METHODS

2.2.1. Preparation of whole wheat flour

Whole wheat flours (WWF) from naturally contaminated grains from commercial production fields in Southern Brazil (Rio Grande do Sul, Santa Catarina and Paraná States) were evaluated. The flours contained (in g 100g⁻¹ dry basis) 1.62 ± 0.03% ash, 16.62 ± 0.54% protein and 64.22 ± 1.64% starch.

As the ozone reactivity is higher in the presence of water (WU; DOAN; CUENCA, 2006), two moisture levels were studied: 10.6 ± 0.2%, 24.5 ± 0.6 % (samples coded as 10% and 25% moisture). For that, the initial moisture of the whole wheat flour was increased by spraying water and packing it in a sealed container under refrigeration (5°C) for 5 days, thus ensuring homogeneity in the water distribution. The moisture content was determined using the Moisture Analyzer (A & D company, AND MX-50, Japan).

The initial DON concentration in naturally contaminated whole wheat flour ranged from 2,711 - 3,046 µg kg⁻¹, exceeding the maximum limit (ML) by Brazilian Legislation (BRASIL, 2017) and European Commission (EC) (EUROPEAN COMMISSION, 2006). EC has established a limit of 750 µg kg⁻¹ and 1,250 µg kg⁻¹ in Brazil. Thus, the samples under study can be an effective technological problem.

2.2.2. Preparation of wet milling effluent

As DON is water-soluble (SOBROVA et al., 2010), the effluent with contaminated grains should also contain this mycotoxin, thus justifying the degradation study.

The effluent was obtained from previously processed whole wheat flour, following conditions based on the industrial processes (SAYASLAN, 2004). Water was added to the whole wheat flour in the ratio of 1:2 (w:w), followed by stirring for 1 hour on a shaker table (Marconi, model MA 139/CFT, Piracicaba, Brazil), and centrifugation at 2,000 g (Eppendorf 5810 R, Hamburg, Germany). The decanted fraction (wheat flour insoluble fraction) was discarded, and the supernatant containing DON and the other soluble fractions were considered as the effluent to be studied.

2.2.3. Ozone processing

The ozonation system used in this study consisted of an ozone generating unit (Ozone & Life, O & L 3.0 RM, São José dos Campos, Brazil), which converts part of the O₂ into O₃ through electric discharge, coupled to a glass reactor (one specific for solids and other for liquids). In all experiments, industrial gas oxygen (95% purity, Air Liquide, Campinas, Brazil) was used at a flow rate of 0.5 L min⁻¹ and the ozone concentration in the gas stream was 65 mg L⁻¹. After passing through the samples, the gas stream was led out of the reactor and converted to oxygen in a thermal ozone destroyer (Ozone & Life, São José dos Campos, Brazil). The procedures were performed at 25 ± 1°C (room temperature) and the process conditions were defined after pre-testing.

2.2.4. Ozonation of whole wheat flour samples

About 13 g of sample (corrected for moisture of 10%, keeping the ratio of dry matter in the reactor fixed) was poured into a stainless steel screen base, in a cylindrical glass reactor (29 cm x 5.5 cm) coupled to an ozone generator (Figure 2.1).

The ozone-containing gas stream was inserted into the upper end of the reactor, percolating the sample and then discarded into the ozone destructor.

Ozonation was performed for 60, 120, and 180 minutes for each moisture level (resulting in one process for each condition) in four replicates. After processing, the samples were packed in polyethylene bags which were sealed and stored at -18°C until the time of analysis.

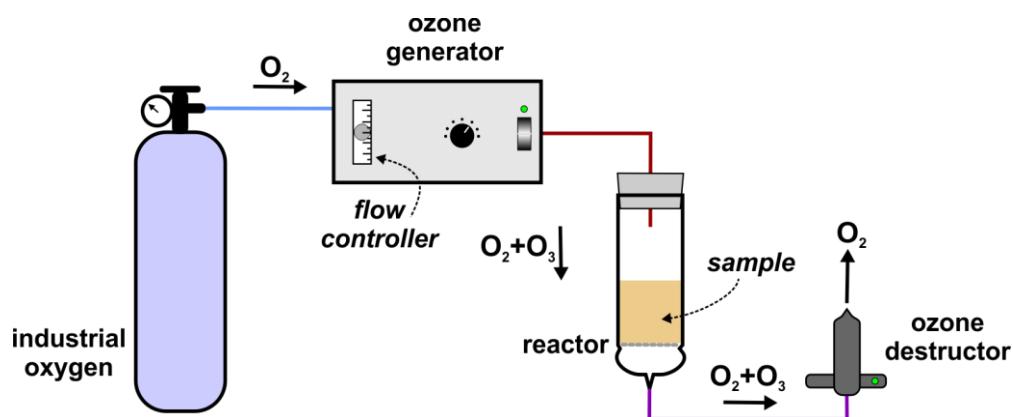


Figure 2.1 Ozonation system of whole wheat flour

2.2.5. Ozonation of wet milling effluents

About 300 mL of the effluent were mixed with 400 mL of distilled water in a vessel, and 0.2% of antifoam agent (Surfata®, PTI 77069, Ata Tensoativos, São Paulo, Brazil) was added. The suspension was poured into a glass reactor (69 cm x 16 cm) coupled to the ozone generator (Figure 2.2), and stirred continuously with a magnetic stirrer. The ozone-containing gas stream was bubbled into the sample, and the headspace of the reactor was discarded into the ozone destructor.

Samples (10 mL) were withdrawn over the course of the procedure (60 min, 120 min, and 180 min) for further analysis. The procedure was performed in quadruplicate.

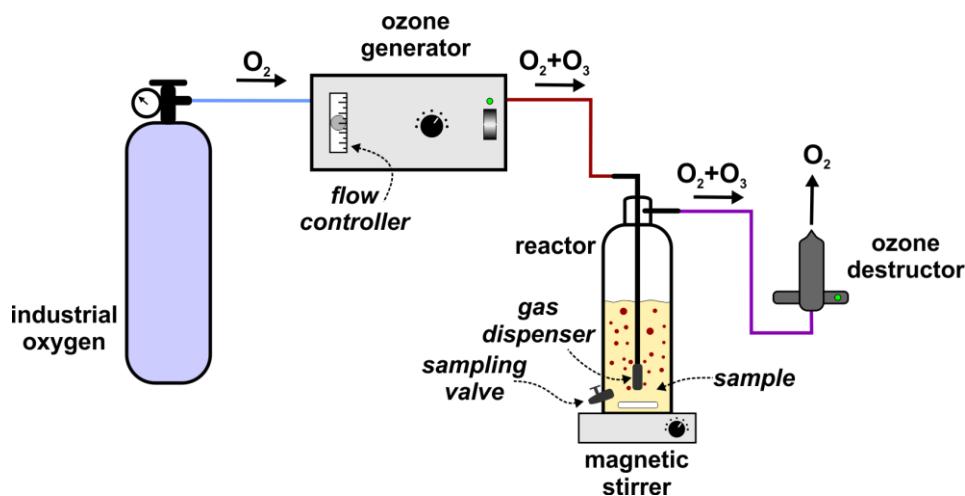


Figure 2.2 Ozonation system of wet milling effluent

2.2.6. Extraction, purification and determination of don in both whole wheat flour and wet milling effluent

The DON determination was based on the methodology described by (PASCALE et al., 2014) with modifications as recommended by the immunoaffinity column supplier (NEOCOLUMN DON, [s.d.]). Samples (5 g) were extracted with 40 mL of distilled water for 1 hour on a shaker table (Marconi, MA 139/CFT, Piracicaba, Brazil), and the extract solution was centrifuged at 2,000 g (Eppendorf, 5810 R, Hamburg, Germany). For analysis of the effluent, the aqueous extract was added directly to the immunoaffinity column.

Purification of the extracts was performed on an immunoaffinity column (NeoColumn™ 8340 to DON, NeogenCo.Ayr, UK) and eluted with methanol. The purified extract was collected in a 10 mL flask and evaporated at 50 °C (under air flow) using a heating block (MA 4006, Marconi, Sao Paulo, Brazil). The dried residue was reconstituted in 500 µL of water: acetonitrile (92: 8 v/v), followed by filtration using a Millex-GV filter (0.22 x 13 mm) (Millipore, Bedford, MA, USA) before HPLC injection.

Separation and quantification were performed by liquid chromatography (HPLC) and diode array detector (DAD). The chromatograph (Shimadzu, Kyoto, Japan) consisted of a pump (LC-20AT) with solvent mixing chamber (FCV-10AL vp), degasser (DGU-20A5), automatic injector (SIL-20A) maintained at 40°C. Chromatographic separation of DON was performed using Hypersil ODS column

(size: 250 x 4.6 mm, particle size: 3 µm - Thermo, Bellefonte, PA, USA). The mobile phase consisted of water: acetonitrile (92: 8 v/v) at a flow rate of 0.8 mL min⁻¹ and an injection volume of 50 µL. The DON identity was confirmed by overlapping the absorption spectrum at the representative peak of the samples and reference solutions ($\lambda = 219$ nm), and by checking the similarity.

The concentrations before and after each process were expressed on dry basis to evaluate the effective decrease in contamination. Finally, the results were analyzed by relative concentration (C/C_0), where C is the DON concentration in the sample and C_0 is the initial concentration, as a function of the ozone processing time.

2.2.7. Analytical control

The method used for DON separation, and the validation parameters (selectivity, linearity, precision, and accuracy) were adequate. Linearity was evaluated by the correlation coefficient (r) by the calibration curve for each DON concentration, with a mean correlation coefficient of 0.999. The Limit Of Detection (LOD - 3 times the noise) and the Limit Of Quantification (LOQ - 2 times the LOD) was 50 µg kg⁻¹ and 100 µg kg⁻¹, respectively. The mean recovery was 92.87%. The calibration curve solutions for DON quantification with different concentrations varied from 0.1 ng µL⁻¹ to 2.1 ng µL⁻¹ for DON. The average of 3 injections of each solution was used to construct the calibration curve used in the quantification.

2.2.8. Rheological properties of whole wheat flour

The rheological properties of the whole wheat flour (WWF) were evaluated through the Rapid Viscometer Analyser (RVA, Newport Scientific Pvt. Ltd., Australia) using the software Thermocline for Windows (version 3.0, Newport Scientific, Warriewood, Australia). For that, 3 g sample (corrected to 14% moisture) were mixed to 25 g distilled water. The suspension was maintained at 50°C for 1 minute, heated for 4 minutes to reach 95°C and maintained at this temperature for about 2 minutes. It was then cooled for 4 minutes until reaching 50 °C, remaining at this temperature for 2 minutes. The apparent viscosity of the suspension was determined over the

cycle. Considering the reduction of DON in whole wheat flour with 25% moisture, the rheological parameters were evaluated for only this moisture condition.

2.2.9. Statistical analysis

The average values were calculated and the Tukey's multiple comparison test was used considering a significance level of 5%, using Statistica 13.0 (StatSoft, USA) software and R Core Team (R DEVELOPMENT CORE TEAM, 2016).

2.3. Results and Discussion

2.3.1. Effect of ozonation on the DON concentration in whole wheat flour

The effect of ozonation on whole wheat flours with 10%, 25% moisture subjected to different processing times is shown in Figure 2.3. In general, it can be observed that the moisture levels and process time have affected the reduction of DON.

DON concentration was reduced from $2,748 \pm 769 \mu\text{g kg}^{-1}$ to $2,053 \pm 504 \mu\text{g kg}^{-1}$ during processing of whole wheat flour with 10% moisture at the maximum exposure time (180 min), corresponding to a reduction of only ~ 20% of the initial concentration (Figure 2.3). In addition, the reduction of mycotoxin was more relevant in the first hour of the process, with no significant differences ($p \leq 0.05$) among the further evaluated times.

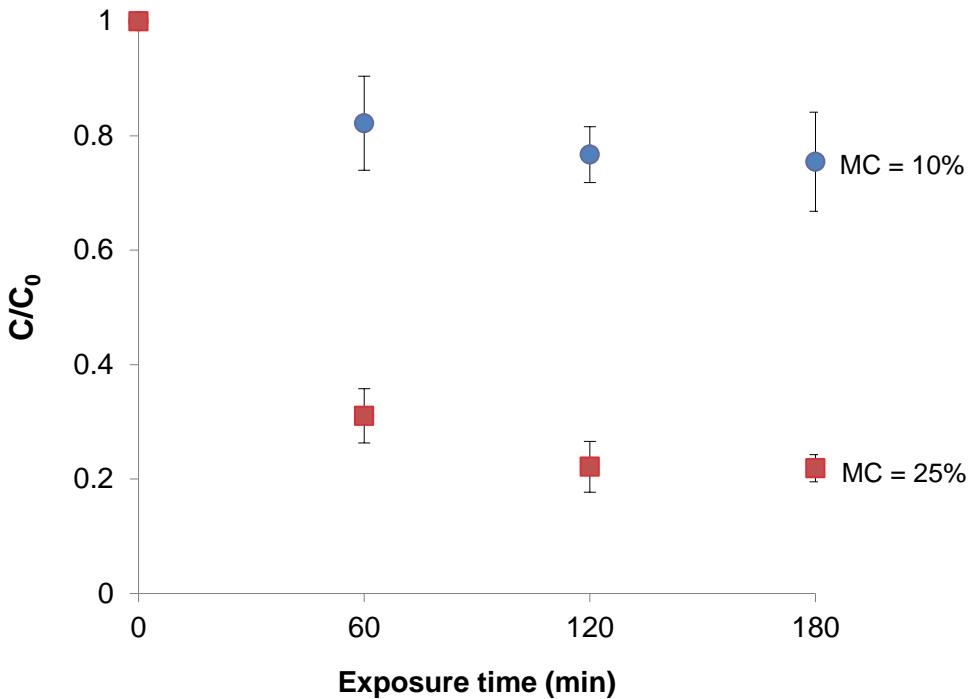


Figure 2.3 Reduction of DON (concentration relative to the initial value - C/C_0) in whole wheat flour with different moisture contents (MC) subjected to ozone processing. Dots represent the mean experimental values and bars represent the standard deviations.

On the other hand, when the moisture of whole wheat flour was increased to 25%, the ozonation process led to a marked degradation in DON, with a reduction of ~ 70% at 60 minutes of the process, maintaining the levels after 120 and 180 minutes. The maximum reduction was $78 \pm 2\%$ at 180 minutes of the process. In fact, even when exposed to 60 minutes of ozonation, the sample remained within the legal limits ($1,250 \text{ } \mu\text{g kg}^{-1}$ (BRASIL, 2017); $750 \text{ } \mu\text{g kg}^{-1}$ (EUROPEAN COMMISSION, 2006)). Therefore, the DON reduction was significantly ($p \leq 0.05$) improved with increasing moisture and ozone exposure.

It is observed that the increase in moisture implies enhances the process efficiency, due to different mechanisms. For example, higher moisture values facilitate the uptake of ozone by the sample surface (CHEN et al., 2010; ALENCAR et al., 2012), since ozone is water-soluble, thus increasing the contact between the gas and the components of the sample. Further, ozonation is catalyzed by free radicals, such as the hydroxyl ions present in water. Consequently, samples with

higher moisture content tend to generate more reactive ions (LI; GUAN; BIAN, 2014).

However, higher moisture contents associated with room temperature may favor the development of toxin-producing fungi during storage of whole wheat flour, compromising the product's quality and adversely affecting the ozonation process. Thus, to minimize the risks of microbial contamination, the whole wheat flour ozonation should be applied just before using, which is viable from the technological point of view, once a moist dough is needed in the manufacture of bread and biscuits.

In addition, ozonation has been used for different purposes and products during grains processing, especially for insect (KELLS et al., 2001) and microorganisms (KOTTAPALLI et al., 2008; RAILA et al., 2006; WU et al., 2006) inactivation. In the case of mycotoxins, ozonation has been studied mainly in the reduction of aflatoxins in different products, such as wheat (TROMBETE et al., 2016), corn (PRUDENTE JR.; KING, 2002), corn flour (LUO et al., 2014b), red pepper (INAN; PALA; DOYMAZ, 2007), dry fig (ZORLUGENÇ et al., 2008), peanut (CHEN et al., 2014), and pistachio (AKBAS; OZDEMIR, 2006).

The decrease in DON concentration has been successfully evaluated in artificially (SAVI et al., 2014; TROMBETE et al., 2016) and naturally contaminated (LI; GUAN; BIAN, 2014; LUO et al., 2014; WANG et al., 2016a, 2016b) wheat grain products.

The importance of studying naturally contaminated samples is consistent with the actual distribution conditions of mycotoxins in the field and grain. Although higher hyphae and mycotoxins concentrations are found in the outer layers of the grains due to the characteristics of the fungal infection, both the fungus and the mycotoxins are distributed throughout the grain (LEE et al., 1987; SEITZ; BECHTEL, 1985). On the other hand, artificial contamination is performed by placing the grain in contact with mycotoxin solution or by inoculating toxin-producing fungi; in both cases, toxins remain mainly in the outer layers (ALENCAR et al., 2012).

Wang et al. (2016a) studied ozonation on wheat grains naturally contaminated with DON and evaluated the mycotoxin distribution in the different fractions after milling. Although the authors have observed a reduction of ~ 50% in DON concentration in both grain and the outer layers, this behavior was not observed for the refined flour (that is, containing only the inner layers of the grain), with no

significant differences between the initial and final DON concentration. This may be due to the ozone preferably reaches the outer layers of the grain during the process.

On the other hand, the ozonation process applied directly to ground or crushed products is more effective because of the larger contact area. Luo et al. (2014) observed a reduction of 70-80% of aflatoxins (total and B₁, G₁, and B₂) in ozonized corn flour, as well as Wang et al. (2016b) obtained a reduction of 78.6% in whole wheat flour naturally contaminated under similar conditions to our study. Thus, ozonation of ready-to-eat products or in industrial applications may be an alternative for less exposure of the population to DON. Despite that, studies on the extent of ozonation in the technological properties of flour are needed, as well as the decontamination of the effluents generated in the process of obtaining wheat products.

Finally, it can be observed that ozonation is a promising and effective technology for mycotoxins degradation, and consequently, in the reduction of contamination. However, monitoring along the production chain should not be neglected for the purpose of subsequent decontamination, once ozonation should be used only as an additional tool in the management of mycotoxins.

2.3.2. Effect of ozonation on the DON concentration in wheat wet milling effluent

The effect of the ozonation on wet milling effluent subjected to ozone for 60, 120, and 180 minutes is shown in Figure 2.4. Ozone exposure significantly affected ($p \leq 0.05$) the DON concentration, which was $0.72 \pm 0.04 \mu\text{g mL}^{-1}$ at the beginning of the process and was reduced by 32% after 60 minutes. It is also observed that at the end of the process (180 minutes), the reduction was even more pronounced, with 82% reduction in DON concentration, with an apparent linear decrease.

Literature studies have demonstrated the degradation of different mycotoxins by ozonation applied in aqueous solutions, obtained by solubilizing pure toxin standards. This is the case of DON (LI; GUAN; BIAN, 2014; YOUNG; ZHU; ZHOU, 2006), aflatoxins, cyclopiazonic acid, ochratoxin A, and zearalenone (LEMKE, 1999; MCKENZIE, 1997). Ozonation was effective not only in reducing mycotoxin concentration but also by reducing the toxic effects associated with zearalenone

(DUDZIAK, 2012; LEMKE, 1999; MCKENZIE, 1997), once these compounds were not detected after ozonation.

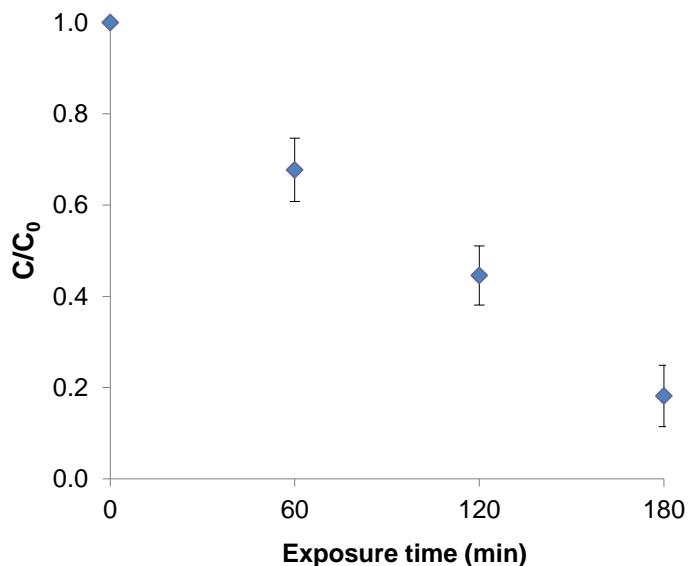


Figure 2.4 Reduction of DON in the wet milling effluent as a function of ozonation time. Concentration relative to the initial value (C/C_0). Dots represent the mean experimental values and the bars represent the standard deviations.

LI; GUAN; BIAN (2014) obtained a reduction of ~ 80% in the DON concentration in aqueous solution after 16 minutes of process under ozonation conditions similar to those used in the present study. However, a direct comparison cannot be made due to several factors, including: (i) only solubilized ozone reacts with the molecules; (ii) solubilization of the gas in the solution is highly dependent on the characteristics of the reactor, process, and solution components; (iii) the solubility of the gases is hampered by the presence of soluble solids in the solution; (iv) the authors studies a solution of only DON and water. Even so, it is observed that a lower exposure time is required when using an aqueous solution when compared with the effluent under study (a ~ 10-fold longer time was required to achieve the same degradation level). This is due to the fact that the effluent contains several other soluble organic molecules from the wheat composition (about 3.5% soluble solids), leading to oxidation of both DON and other molecules.

The treatment of water contaminated with toxic compounds has gained prominence worldwide. The reduction of the pollutant load from corn wet milling effluent was studied by Yasri; Yaghmour; Gunasekaran, (2015), observing a

reduction of 99.2% on the chemical oxygen demand using oxidation and electrochemical adsorption methods. However, those authors did not study the presence of mycotoxins. Oh et al. (2014) and Liu et al. (2014) used ozonation to reduce the concentration of antibiotics in effluents, observing reductions of up to 99%.

Mycotoxins can be present in grains and derivatives and migrate to various environments. The European Union has included the analysis of mycotoxins (GROMADZKA et al., 2015) in the monitoring of surface waters (i.e. - waters that flow or accumulate on the soil surface, rivers, streams, or lakes). The presence of DON in soil and plants (SCHENZEL et al., 2012), zearalenone in agricultural wastewater (HARTMANN et al., 2007; WAŚKIEWICZ et al., 2012) and aflatoxin B₁ and DON in wastewater from rice production (LEITE et al., 2012) were also observed by several authors. Such studies indicate that effluents can carry toxins naturally present in the grains, thus compromising the environment.

Due to the extension of the problem, the ozonation process can be an alternative in the management of the effluents from agricultural crops, being a promising and effective technology for the reduction of DON contamination. Consequently, studies on other mycotoxins are required.

2.3.3. Effects of the ozonation on the quality of the whole wheat flour

The ozone high oxidative capacity can result in both positive and negative effects on food processing. As shown in the present study, it can be used in the product decontamination, and also to achieve the desirable changes in food properties (for example improving starch properties, as discussed by CASTANHA; MATTA Jr; AUGUSTO, 2017). However, a negative aspect is the oxidation of some food constituents, with undesirable effects such as changes in color, loss of compounds, and undesirable odor (TIWARI et al., 2010). The viscoamylographic profile is considered one of the most important wheat flour properties since it provides the viscous properties of the cooked flour and allows to correlate its functionality and structural properties. In this study, the viscous properties of the WWF samples were measured through an RVA equipment, to evaluate possible changes caused by the ozone processing.

The main viscous parameters of the WWF samples with 25% moisture and their corresponding curves are shown in Table 2.1 and Figure 2.5, respectively. The RVA profile was similar to that reported for wheat flour (CHEN et al., 2010).

Table 2.1 RVA results for the whole wheat flour (WWF) samples at 25% moisture before and after ozone processing for different times

Processing Time	Peak Apparent Viscosity (mPa.s)	Final Apparent Viscosity (mPa.s)	Relative Breakdown (%)	Relative Setback (%)	Pasting Temperature (°C)
0 min	441 ± 6 a	997 ± 0 a	20.9 ± 0.7 b	65.0 ± 0.1 a	89.0 ± 0.1 b
60 min	315 ± 2 b	282 ± 1 b	55.3 ± 0.5 a	50.2 ± 0.0 c	90.1 ± 0.6 ab
120 min	282 ± 2 b	256 ± 1 b	55.1 ± 0.1 a	50.2 ± 0.4 c	91.3 ± 0.1 a
180 min	281 ± 28 b	246 ± 20 b	58.3 ± 2.9 a	52.5 ± 0.6 b	91.0 ± 0.4 a

Averages in the same column followed by the same letter do not differ significantly at the level of 5% ($p < 0.05$).

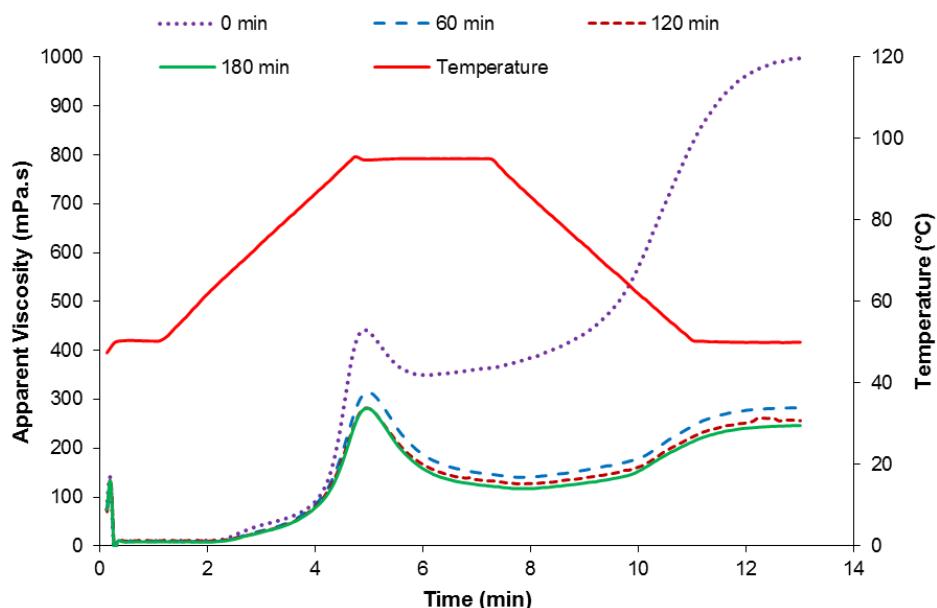


Figure 2.5 RVA curves of the whole wheat flour (WWF) samples with 25% moisture before and after ozone processing for 60, 120, and 180 minutes.

Significant ($p<0.05$) changes were observed in the pasting properties of the WWF subjected to ozone processing when compared with the control sample (unprocessed flour). Ozonation resulted in a decrease in the peak and final apparent viscosities and relative setback parameters, and an increase in the relative breakdown and pasting temperature. In general, no significant differences ($p<0.05$) were observed for all parameters of the processed samples at different times. The changes in the RVA profile can be due to modifications on the WWF main components.

Wheat grain is mainly composed of 63-72% starch (major component) and about 12% proteins (divided into two groups: gluten (80-85%) and non-gluten (15-20%) proteins, being the gluten proteins composed by gliadin and glutenin). Lipids, non-starch polysaccharides, among other small compounds, are considered negligible components (BELITZ; GROSCH, 1999; LINEBACK; RASPER, 1988; VAN DER BORGHT et al., 2005). In fact, the proximate composition showed similar results to those described in the literature (in dry basis: $16.62 \pm 0.54\%$ protein and $64.22 \pm 1.64\%$ starch). Therefore, the present study considered that the main changes in the WWF RVA parameters after ozone processing have occurred mainly in the two major components, starch and gluten proteins.

Chen et al., (2010) studied the effect of gluten on the pasting properties of wheat starch. The authors observed that the starch fraction was the main responsible for the RVA curve profile and that the increasing on gluten content led to a reduction in the values of the main parameters of the RVA curve (peak apparent viscosity, trough apparent viscosity, final apparent viscosity, setback, and peak time). Thus, probably the ozonation in the present study degraded the starch fraction rather than proteins. As reported by Chen et al., (2010), the RVA parameters have increased with protein degradation. However, the degree of protein oxidation was not investigated in this study.

Considering only the degradation of the starch fraction, and based on previously published studies (CASTANHA; MATTA Jr; AUGUSTO, 2017; SANDHU; MANTHEY; SIMSEK, 2012), it can be considered that most of the modifications observed in the RVA were due to a molecular depolymerization and formation of carboxylic groups. The RVA parameters discussed below, therefore, have considered only the effects of ozonation on the starch fraction.

The peak apparent viscosity represents the maximum apparent viscosity reached by the starch granules while swelling. After that point, there is a “rupture” of the granule, releasing polymers, which leads to a consequent decrease in the apparent viscosity until reaching the trough apparent viscosity, another RVA parameter (LIU, 2005). As can be observed in Table 2.1, the peak apparent viscosity decreased with the ozone processing, indicating that, under stirring and heating, the oxidized starch fraction from the WWF presented a reduced capacity to swell and to maintain its integrity when compared to the starch from the unprocessed flour. This reduction can be due to changes occurring in the starch molecules, leading to molecules with lower resistance to shear forces and, consequently, decreased the swelling capacity.

The breakdown parameter is calculated as the difference between two RVA parameters: the peak apparent viscosity and the trough apparent viscosity. Considering that both parameters change at the same time, the relative breakdown was defined by Castanha; Matta Jr; Augusto (2017) to better understand the breakdown and avoiding a misinterpretation: it is defined as the ratio between the breakdown and the peak apparent viscosity. After the ozone processing, an increase was observed in the relative breakdown of the WWF samples (Table 2.1), indicating that the starch granules become less resistant and easier to rupture with the sample oxidation, as also reported by Mei et al. (2016), probably due to the molecular cleavage and formation of carboxyl groups.

The setback parameter, as the breakdown, is also calculated as a difference between two RVA parameters: final apparent viscosity and trough apparent viscosity (LIU, 2005). Similarly, it represents the retrogradation tendency of the starch molecules. Again, this is not the best form to compare the variation in apparent viscosity among the samples, since both parameters change simultaneously. Thus, a relative setback was settled, being the ratio between the setback value and the final apparent viscosity (Table 2.1). Considering that at this point the samples were cooled to 50°C, the relative setback represents the molecular association tendency (retrogradation) during cooling, especially the amylose molecules, due to the higher mobility and consequent capacity of interaction. As can be observed in Table 1, the relative setback of the ozonated samples was lower than those observed for the unprocessed sample, indicating a lower tendency to retrogradation. This result

indicates a lower molecular association after cooling, probably due to the electrostatic repulsion caused by the formation of electronegative carboxyl groups.

Finally, the pasting temperature indicates when the sample starts to present a higher apparent viscosity. The pasting temperatures of the ozone-processed samples slightly increased when compared to the control (Table 2.1). This increase may be due to the molecular depolymerisation of starch since smaller size molecules require more energy to gelatinize than larger-sized molecules (ÇATAL; İBANOĞLU, 2012).

Starch depolymerization can have positive technological effects, depending on the amount and process conditions. Amylases have been used in several applications, including bread-making to generate dextrin of different sizes (depending on the amylase specificity), which may be associated with an anti-staling effect (by interfering with the retrogradation properties of starch). They can also be used to increase the levels of fermentable sugars, improving yeast activity, as well as Maillard reaction products (GOESAERT et al., 2005). If the ozone has been effective under the conditions studied, the depolymerization of the starch molecules may have led to the formation of fermentable sugars, favoring the enzymatic activity.

Regarding the gluten fraction, changes are also expected due to the oxidation reactions, which can affect some of the technological applications of whole wheat flour. However, these changes were not assessed in the present study, and literature information concerning this subject is scarce.

Furthermore, the present results can have positive technological effects, depending on the changes and possible applications.

Sandhu et al., (2011) studied ozonation to replace potassium bromate as flour oxidant. It is well known that the rheological properties of the wheat flour are remarkably affected by oxidation, since it can interfere in the disulfide bonds between the glutenin molecules, which are the main responsible to form networks, providing resistance and deformation to the dough (improving gas retention and increasing the loaf volume). The most common oxidants are chemical agents, including ascorbic acid, iodate, azodicarbonamide (ADA), and bromated (BLOKSMA, 1972; GOESAERT et al., 2005; YAMADA; PRESTON, 1992). However, these agents may form undesirable residues in food, as the potassium bromate, a genotoxic and potential carcinogenic compound, which can leave a residual amount in food due to an incomplete decomposition to its stable form (potassium bromide) (KAYA;

TOPAKTAŞ, 2007; KUROKAWA et al., 1990). In this scenario, ozone can play an important role due to its high oxidative power, which can change parts of the glutenin molecules without the need of high levels of chemical compounds. Mei et al. (2016) studied the effects of processing wheat flour with ozone regarding the flour quality and its performance on steamed bread-making. The authors concluded that the ozone can be satisfactorily used to improve dough properties, presenting positive effects on quality scores, volume and structure of steamed bread, also affecting flour whiteness. However, the process conditions should be carefully selected, since, at high levels, the oxidation by ozonation can lead to undesirable consequences, once a high-degraded flour can show less initial water absorption and consequent poorest gluten formation during mixing. Further, the dough consistency and its gas-retentive capacity may decrease (MEI et al., 2016).

Considering the information above, the whole wheat flour ozonation can be considered an advantage regarding the gluten fraction. However, the severity of the oxidation should be taken into account, since the ozonation used for the potassium bromate substitution can be milder when compared to that used in the DON decontamination.

An alternative to reduce the changes in the properties of the whole wheat flour is to apply the ozone on the whole wheat grain, before milling. However, it can lead to a lower efficiency in DON decontamination. Gozé et al., (2016) studied the effects of ozone oxidation on the starch characteristics of wheat grains. They observed that the properties of the wheat starch were not significantly affected by the ozonation in the experimental conditions studied (starch extracted from ozonated whole wheat grain). The authors concluded that, in the whole grain ozonation, other potentially more reactive molecules may have been attacked earlier, thus protecting the starch granules. Further, the starch granules were not directly exposed to ozone. Mendez et al. (2003) reported that, after exposing hard wheat grains to an ozone-rich atmosphere for 30 days, the bread-making properties of the flour (tolerance of the dough to over mixing, water absorption, dough weight, and proof height) were not significantly affected. Ibanoglu (2002) also reported that washing soft and hard wheat samples with ozonated water did not significantly alter the chemical, physical, and rheological properties of their respective flours.

Thus, ozonation may be an alternative for the DON decontamination in the whole wheat flour. However, the oxidation may lead to changes in the flour

properties. Consequently, further studies are needed to evaluate whether these modifications in the flour paste properties significantly interfere with their technological properties, especially regarding baking.

2.4. Conclusions

The ozonation process has proven to be an effective alternative in reducing DON contamination in both whole wheat flour and wheat wet milling effluent. In the flour, the increase in moisture had a positive correlation with the efficacy of the process. It was possible to obtain products in accordance with the legal limits of Brazil and the European Union, even starting with concentration 3-4 times higher than the limits established by law. The DON concentration in the wet milling effluent significantly reduced after ozonation. However, it is important to emphasize that, under the conditions studied, significant changes were observed in the rheological properties of whole wheat flour, thus other studies are necessary to evaluate the effects of these changes on industrial applications.

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3 OZONE TREATMENT TO REDUCE DEOXYNIVALENOL (DON) AND ZEARALENONE (ZEN) CONTAMINATION IN WHEAT BRAN AND ITS IMPACT ON NUTRITIONAL QUALITY²

Abstract

Wheat bran is an important source for human and animal feed. Its nutritional aspects include a high content of fiber and minerals, as well as phenolic compounds that help prevent chronic diseases. However, wheat can be susceptible to contamination by fungus, which can produce mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEN), causing adverse health effects. Therefore, methods should be developed to reduce possible contamination. Ozone can be used for this purpose as it is considered safe and environmentally friendly. The aim of this study was to evaluate the reduction of DON and ZEN concentrations in wheat bran using the ozonation process, as well as to evaluate the effect of ozonation on the nutritional quality of bran. Considering this, wheat bran naturally contaminated with both DON and ZEN was processed using ozone at different conditions. The nutritional quality of the bran was evaluated after processing considering the following aspects: the total phenolic content and the bran antioxidant capacity (by using both radicals DPPH and ABTS). The results showed that the degradation of ZEN was higher and faster than the degradation of DON, which could be explained by their molecular structures. The total phenolic content and antioxidant capacity of the bran were not affected by the ozonation process, which is preferable from a nutritional point of view. Therefore, the ozone showed to be an alternative to reduce mycotoxins in wheat bran – although more studies are needed in order to better understand and optimize processing and the product quality.

Keywords: Mycotoxin; Deoxynivalenol; Zearalenone; Ozone; Bran; Quality

3.1 INTRODUCTION

Wheat is one of the most important cereals in the world. According to the Food and Agriculture Organization (FAO), the estimated world cereal production in 2017 was 2,597 million tonnes, in which ~30% was represented by wheat (FAO, 2017). Wheat grains comprise three parts: bran, germ and endosperm. Bran constitutes

² This chapter is currently under review:

ALEXANDRE, A. P. S.; VELA-PAREDES, R. S.; SANTOS, A. S.; COSTA, N. S. CANNIATTI-BRAZACA, S. G.; CALORI-DOMINGUES, M. A.; AUGUSTO, P. E. D. Ozone treatment for reduction of deoxynivalenol (DON) and zearalenone (ZEN) contamination in wheat bran and its impact on nutritional quality. **Food Additives and Contaminants, Part A**, 2017.

about 14% of the grain weight and it contains a high amount of fiber and ash; the germ, the embryonic part of wheat where most of the lipids and many of the essential nutrients of the grain are concentrated, constitutes only about 3%; the inner portion of the grain, called endosperm, represents 83% of its weight and is characterized by its high starch and protein content (i.e., gluten) (FINNIE; ATWELL, 2016).

Due to its high nutritional value, wheat bran can be used for animal feed and it is also an interesting ingredient for human nutrition (EDWARDS et al., 2011). In fact, a diet rich in cereals has been suggested to help prevent chronic diseases, probably due to the fiber, vitamins, minerals and phytochemical content of phenolic acids and sterols.(ADOM; LIU, 2002; KIM et al., 2006). In grains, the phenolic compound content include derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones and flavanones (ADOM; LIU, 2002). Wheat bran contains several phenolic acids, including vanillic, p-coumaric, and mainly ferulic acid (KIM et al., 2006). These compounds are not evenly distributed in the grains. For example, ferulic acid is predominantly found in the external layer of the grain, in the bran (KIM et al., 2006; VERMA et al., 2009). Zhou and Yu (2004) reported that ferulic acid is a major contributor to the antioxidant activity in wheat, serving as a marker for the quality of the wheat.

The grains can be contaminated by fungi in the field or during storage, and some of these fungi can produce toxic metabolites called mycotoxins (Scudamore et al., 2007). The co-occurrence of different mycotoxins can be explained because different toxigenic genera/species of fungi are capable of infecting the plant and the same species producing different mycotoxins such as *Fusarium graminearum* that produces deoxynivalenol (DON) and zearalenone (ZEN) (SMITH et al., 2016). In fact, even adopting all good production practices, there are situations where the presence of mycotoxins is unavoidable. For example, plant infection depends on the weather conditions (precipitation and temperature) (CALORI-DOMINGUES et al., 2016). Therefore, it is important to study alternatives to reduce mycotoxin contamination in agricultural products.

Mycotoxins have a range of toxic effects for humans and animals, and they are concentrated at the bran fraction of wheat grains (EDWARDS et al., 2011; BELLUCO et al., 2017). Toxic effects of DON on animals and humans imply acute gastroenteritis and affect the immune system, as well as the chronic effect on growth and immune and reproductive functions (PESTKA; SMOLINSKI, 2005). ZEN causes

various toxic effects in animals, especially those related to the reproductive system (CAST, 2003). Furthermore, according to the International Agency for Research on Cancer (IARC), ZEN may bind to estrogen receptors in human breast cancer cells (IARC, 1993).

Among the alternatives to reduce the mycotoxin contamination in agricultural products, ozone gas (O_3) has been used successfully to control different mycotoxins in products such as peanuts, wheat, corn and flour (ALENCAR et al., 2012; LUO et al., 2014a, 2014b; WANG et al., 2016a; ALEXANDRE et al., 2017). Ozone is a gas with high oxidizing potential, generated electrically at the time of use and decomposing into O_2 . It is considered a green chemical process and is GRAS - Generally Recognized As Safe, with use regulated by the FDA for agricultural products in natura (FDA, 2001; RICE; GRAHAM 2001). Consequently, ozone can be used to help produce safe food.

However, due to its oxidizing properties, ozone can promote changes in the constituents of wheat (GOZÉ et al., 2017). Ozone can partially demolish the cell structure (ZOU et al., 2015) and oxidize carotenes, tannins, ascorbates, flavoproteins or polyphenols and other constituents (OBADI et al., 2017). Ozonation can induce the oxidation of some constituents of wheat bran by forming free radicals (OBADI et al., 2017), highlighting the importance to evaluate its nutritional quality. Therefore, it is important to study not only the effect of the ozonation process on the mycotoxin degradation, but also the effect on the product quality.

Even considering the studies of using ozone to reduce the mycotoxin decontamination in grains, there are few studies considering naturally contaminated products. Even more rare are studies which evaluate the simultaneous ozone decontamination of different mycotoxins – to the best of our knowledge, there is only one study (QI et al., 2016) in the literature. This is important because the same product can be contaminated with different mycotoxins, and their behavior during the ozonation process can be distinct.

Consequently, the aims of this research were to study the reduction of DON and ZEN contamination in naturally contaminated wheat bran, as well as to evaluate the effect of ozone on its nutritional quality considering the total phenolic content and antioxidant capacity of bran.

3.1. MATERIAL AND METHODS

3.1.1. Wheat bran

This study was conducted using wheat bran samples from naturally contaminated grains, cultivated in commercial production fields in Southern Brazil (Paraná State). It is important to highlight that this approach is in line with the actual field conditions and natural distribution of mycotoxins in contaminated grains (localization in tissues and cells, as well as interaction with other molecules), rather than artificially contaminated grains, whose mycotoxin distribution is a function of the solution absorption (ALEXANDRE et al., 2017).

The samples were previously homogenized in a type Y homogenizer (TE 200, Tecnal, Brazil) and then separated to compose subsamples to be processed. The wheat bran was processed with the initial moisture of 13.8%, which is in the range of commercialization moisture according to Brazilian Legislation (BRASIL, 2005). The moisture content was determined using the Moisture Analyzer (A & D company, AND MX-50, Japan). The wheat bran composition (in g 100g⁻¹ dry basis) was: 3.64 ± 0.24% of ash (AOAC, 2006), 12.20 ± 2.02% of proteins (AOAC, 2006), 37.70 ± 2.23% of starch (Kit Magazyme K-TSTA for total starch, based on AOAC 996.11 (2006) and AACC 76-13.01 (1976) methods), 3.84% of soluble fiber and 32.53% of insoluble fiber (method 991.43, AOAC, 2012).

The initial DON concentration ranged from 2,090 – 3,009 µg kg⁻¹, while the ZEN concentration ranged from 1,117 – 1,429 µg kg⁻¹. Both mycotoxins exceeded the maximum limit (ML) by Brazilian Legislation for wheat bran (1,250 µg kg⁻¹ for DON ; 200 µg kg⁻¹ for ZEN) (BRASIL, 2017) and European Commission (EC) (750 µg kg⁻¹ for DON ; 75 µg kg⁻¹ for ZEN) (EUROPEAN COMMISSION, 2006a).

3.1.2. Ozone processing

The ozonation system used in this study is shown in Figure 3.1 (schematic diagram, without scale). It consisted of an ozone generating unit (Ozone & Life, O & L 3.0 RM, São José dos Campos, Brazil), which converts part of the O₂ into O₃ through electric discharge, coupled to a glass reactor. In all experiments, industrial gas oxygen (95% purity, Air Liquide, Campinas, Brazil) was used at a flow rate of 0.5

L min^{-1} and the ozone concentration in the gas stream was 62 mg L^{-1} in the reactor inlet. The amount of ozone that reacted with the sample was calculated by the difference between the amount of ozone present in the gas stream at the inlet and the outlet of the reactor using an ozone monitor (2B Technologies Model 106-H, USA). After passing through the samples, the gas stream was led out of the reactor and converted to oxygen in a thermal ozone destroyer (Ozone & Life, São José dos Campos, Brazil). The procedures were performed at $25 \pm 1^\circ\text{C}$ and the process conditions were defined after pre-testing.

Around 30 g of sample was transferred to a stainless steel net base with filter paper in a cylindrical glass reactor (29 cm x 5.5 cm) coupled to the ozone generator (Figure 3.1). The ozone-containing gas stream was inserted into the upper end of the reactor, percolating the sample and was then discarded into the ozone destructor.

Ozonation of wheat bran was performed up to 240 minutes. After processing, the wheat bran was finely ground (20 mesh; model TE 020, Tecnal, Brazil) to be analyzed and then packed in polyethylene bags, which were sealed and stored at -18°C until analysis.

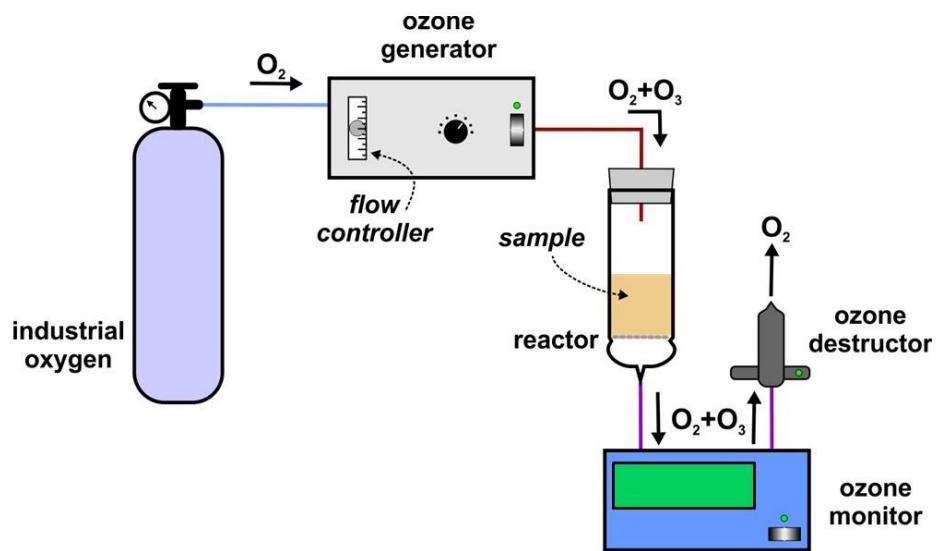


Figure 3.1 Ozonation system for the wheat bran (schematic representation, without scale).

3.1.3. Extraction, purification and determination of DON in wheat bran

DON determination was based on the methodology described by Pascale et al. (2014), with modifications, as recommended by the immunoaffinity column supplier (Neocolumn DON, [s.d.]). Samples (5 g) were extracted with 40 mL of distilled water during 1 hour of agitation in an orbital shaker (240 rpm) (Marconi, MA 139/CFT, Piracicaba, Brazil). The dispersion was centrifuged at 2,000 g (Eppendorf, 5810 R, Hamburg, Germany) for 10 minutes at 25°C and the supernatant was separated for purification.

Purification of the extracts was performed on an immunoaffinity column (NeoColumm™ 8340 to DON, NeogenCo.Ayr, UK), eluted with methanol. The purified extract was collected in a 10 mL flask and evaporated at 50°C (under air flow) using a heating block (MA 4006, Marconi, São Paulo, Brazil). The dried residue was reconstituted in 500 µL of water:acetonitrile (87.5:12.5 v/v), followed by centrifugation at 7,100 g for 10 minutes (Eppendorf, MiniSpin® plus, Germany) before high performance liquid chromatography (HPLC) injection.

Separation and quantification were performed by HPLC and diode array detector (DAD). The chromatograph (Shimadzu, Kyoto, Japan) consisted of a pump (LC-20AT) with solvent mixing chamber (FCV-10AL vp), degasser (DGU-20A5), automatic injector (SIL- 20A) maintained at 40°C. Chromatographic separation of DON was performed using Shim-pack GIST (4.6 x 250 mm, particle size: 3 µm, Shimadzu, Kyoto, Japan). The mobile phase consisted of water:acetonitrile (87.5:12.5 v/v) at a flow rate of 0.8 mL minutes⁻¹, 17 minutes of race time and an injection volume of 50 µL. The DON identity was confirmed by overlapping the absorption spectrum at the representative peak of the samples and reference solutions ($\lambda = 219$ nm) and by checking the similarity.

3.1.4. Extraction, purification and determination of ZEN in wheat bran

The ZEN determination was based on the methodology described and recommended by the immunoaffinity column supplier (Neocolumn ZEN, [s.d.]). Samples (5 g) were extracted with 25 mL of methanol:water (80:20 v/v) during 1 hour of agitation in an orbital shaker (240 rpm) (Marconi, MA 139/CFT, Piracicaba, Brazil).

The dispersion was centrifuged at 2,000 g (Eppendorf, 5810 R, Hamburg, Germany) for 10 minutes at 25°C and the supernatant was separated for purification.

Purification of the extracts was performed on an immunoaffinity column (NeoColumn™ 8140 to ZEN, NeogenCo.Ayr, UK) and eluted with methanol. The purified extract was collected in a 10 mL flask and evaporated at 50°C (under air flow) using a heating block (MA 4006, Marconi, São Paulo, Brazil). The dried residue was reconstituted in 500 µL of methanol:water (70:30 v/v), followed by centrifugation at 7,100 g for 10 minutes (Eppendorf, MiniSpin® plus, Germany) before HPLC injection.

Separation and quantification were performed by (HPLC), photodiode array detector (SPD-M20A) and fluorescence detector (RF-10AXL). The chromatograph (Shimadzu, Kyoto, Japan) consisted of a pump (LC-20AT) with solvent mixing chamber (FCV-10AL vp), degasser (DGU-20A5), automatic injector (SIL- 20A) maintained at 40°C. Chromatographic separation of ZEN was performed using Shim-pack GIST (4.0 x 250 mm, particle size: 5 µm, Shimadzu, Kyoto, Japan) and pre-column (10 x 4.6 mm). The mobile phase consisted of methanol:water (70:30 v/v) at a flow rate of 1 mL minutes⁻¹, 15 minutes of race time and an injection volume of 50 µL. The ZEN identity was confirmed by overlapping the absorption spectrum at the representative peak of the samples and reference solutions ($\lambda = 236$ nm) and by checking the similarity.

3.1.5. Analytical control (*in house validation*)

To validate the mycotoxin methodology, the parameters evaluated were: selectivity, linearity, accuracy, precision and limits of detection and quantification. The selectivity was evaluated by the retention time for each mycotoxin associated with their UV spectral data analyses. The linearity was evaluated by the correlation coefficient (r). The coefficient of determination (r^2) was 0.999 for DON and ZEN. The limit of detection (LOD) was considered as the lowest concentration value that would result in a peak height greater than 3 times the amplitude of the analyzed noise interval (2 minutes before and 2 minutes after TR) in 7 replicates.

The limit of quantification (LOQ) was calculated as $2 \times \text{LOD}$ for each mycotoxin in 7 replicates. Accuracy and precision were evaluated by the recovery

tests for DON and ZEN (Table 3.1) carried out with three different concentrations for each mycotoxin, using non contaminated wheat grain ground samples in 3 to 4 replicates on different days. The recovery of the methodology using two wheat grain reference materials was also evaluated: for DON (QCM2W2 Biopure, Romer Labs, Tulln, Austria) containing $1,431 \pm 256 \mu\text{g kg}^{-1}$ and for ZEN (TRZ100, TRILOGY®, Trilogy analytical laboratory, Washington) with $98.3 \pm 14.3 \mu\text{g kg}^{-1}$. The precision of the method was evaluated by the relative standard deviation of repeatability (RSDr) calculated with the performed repetitions. The results obtained are presented in Table 1 where it can be observed that they are within the acceptable ranges at the concentrations evaluated using the performance criteria established by the Commission Regulation (EC) N° 401 (EUROPEAN COMMISSION , 2006b).

Table 3.1 Performance characteristics for DON and ZEN

	Concentration ($\mu\text{g kg}^{-1}$)	Recovery (%)	RSDr (%)
DON			
LOD	50	-	-
LOQ	100	90	14
Spiked wheat samples	500	90	16
	1000	74	16
	2000	92	7
Reference material - wheat grain (QCM2W2)	1431	95	17
ZEN			
LOD	5	-	-
LOQ	10	88	16
Spiked wheat samples	200	86	8.1
	400	78	7.8
	800	79.4	12.6
Reference material - wheat grain (TRZ100, TRILOGY®)	98	91.75	4.06

* Equation for the linear regression (where $x = \text{area obtained in the chromatogram}; y = \text{injected mass in ng}\text{:}$ DON $y = 7.4453e-005 X + 1.2530$; ZEN $y = 1.9307e-005 X - 0.24239$.

The calibration curve solutions for ZEN and DON quantification were prepared from the standards Z2125 (Sigma Aldrich, USA) and DON (001101, Biopure, Tulln, Austria) with different concentrations varying from 0.015 to 0.880 ng μL^{-1} of ZEN and 0.1 ng μL^{-1} to 2.0 ng μL^{-1} for DON. The average of 3 injections of each solution was used to construct the calibration curve used in the quantification.

3.1.6. Nutritional aspects

The nutritional aspects were evaluated by determining the total phenolic content and the bran antioxidant capacity which exert an inhibitory effect against oxidation processes (SHAHIDI; ZHONG, 2015). This choice is justified by the interest in the health benefits provided by the bran and the possibility of alteration with treatment.

3.1.7. Extract preparation

The extracts were obtained according to the method by Adom et al. (2003) and Verma (2009). Samples (1 g) were extracted with 20 mL of ethanol:water (80:20 v/v) solution during 10 minutes of agitation in an orbital shaker (240 rpm) (Marconi, MA 139/CFT, Piracicaba, Brazil). The dispersion was centrifuged at 2,000 g (Eppendorf, 5810 R, Hamburg, Germany) for 20 minutes at 25°C and the supernatant was separated.

3.1.8. Determination of total phenolic content

The total phenolic content of each extract was determined using the method described by Singleton et al. (1999), with modifications. Briefly, bran extracts were oxidized with the Folin–Ciocalteu reagent by phenolic compounds under an alkaline condition. Folin-Ciocalteu reagent contains phosphomolybdic/phosphotungstic acid complexes, which are based on the transfer of electrons and are reduced to yield a blue chromophore with maximum absorption at 750-765 nm (MAGALHÃES et al., 2008; SHAHIDI; ZHONG, 2015). Therefore, the absorbance of the solution was measured at 765 nm and phenolic concentrations were determined against external

standards of gallic acid (GAE). The phenolic content was expressed as $\mu\text{g GAE g}^{-1}$ dry matter.

3.1.9. Determination of antioxidant capacity using radical DPPH

The antioxidant activity was determined using the 1,1-diphenyl-2-pycrylhydrazyl (DPPH) method, as described by Brand-Williams et al. (1995). Briefly, DPPH is a radical chromogen stable with a deep purple color, it is based on electron donation of antioxidants to neutralize the radical. The reaction is accompanied by a color change and discoloration acts as an indicator of the antioxidant efficacy (SHAHIDI; ZHONG, 2015). The results were expressed as μmol of antioxidant capacity Trolox equivalent (TEAC) g^{-1} dry matter.

3.1.10. Determination of antioxidant capacity using radical ABTS

Antioxidant activity using the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was performed as described by De Camargo et al. (2012) and Re et al. (1999). Briefly, the ABTS reagent is a blue-green chromophore which decreases in its intensity in the presence of antioxidants. Antioxidants can neutralize the radical cation ABTS by either direct reduction via electron donation or by radical quenching via hydrogen atom donation (SHAHIDI; ZHONG, 2015), Moreover, it can determine the antioxidant capacity of both hydrophilic and lipophilic compounds/samples (MAGALHÃES et al., 2008). The results were expressed as μmol of Trolox equivalent (TEAC) g^{-1} dry matter.

3.1.11. Experimental design and statistical analysis

This work was conducted using two wheat bran samples which were previously homogenized in a type Y homogenizer (TE 200, Tecnal, Brazil) and then separated to compose subsamples to be processed. Each process condition was conducted between three and five replicates (individually processed) and the analyses were carried out in five replicates. The average values were calculated and

the Tukey's multiple comparison test was used considering a significance level of 5%, software and R Core Team (R DEVELOPMENT CORE TEAM, 2016).

The concentrations of DON and ZEN before and after each process were expressed on a dry basis to evaluate the effective decrease in contamination. The results were analyzed by relative concentration (C/C_0), where C is the DON concentration in the sample and C_0 is the initial concentration, as a function of the ozone processing time.

3.2. RESULTS AND DISCUSSION

3.2.1. Effects of ozonation on DON and ZEN concentration in wheat bran

Figure 3.2 shows the results of DON and ZEN concentration in wheat bran naturally contaminated when processed with ozone. It can be observed that the degradation of both mycotoxins occurs until a specific concentration, from which it tends to stabilize. In the evaluated conditions, the degradation of ZEN was higher than DON. Furthermore, the reduction rate was different, and the degradation of ZEN was faster than DON.

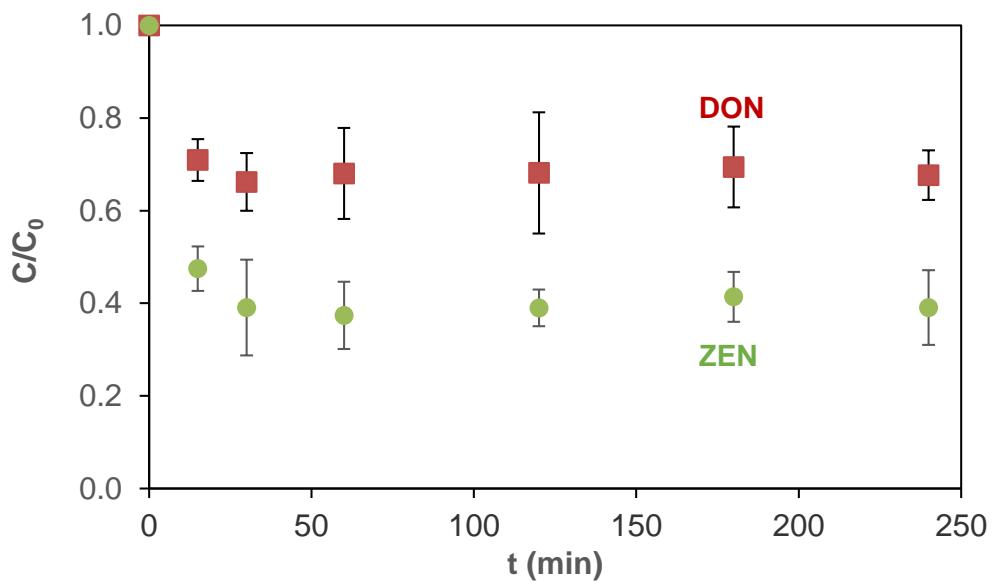


Figure 3.2 Reduction of DON and ZEN (concentration relative to the initial value - C/C_0) in wheat bran with different ozone processing time. Dots represent the mean experimental values and bars represent the standard deviations.

After 15 minutes of ozonation, the decrease in ZEN concentration (52%) was approximately twice that of DON (~29%).

In the first hour of ozonation, the DON concentration decreased from $2416 \pm 389.7 \text{ } \mu\text{g kg}^{-1}$ to $1667 \pm 269.5 \text{ } \mu\text{g kg}^{-1}$. It is observed that after the maximum ozonation time (240 minutes), the concentration of DON decreased to $1662 \pm 204.2 \text{ } \mu\text{g kg}^{-1}$, corresponding to a reduction of only ~32% of the initial concentration (Figure 3.2).

On the other hand, in the first 15 minutes of processing, the ZEN concentration decreased from $1290 \pm 146.86 \text{ } \mu\text{g kg}^{-1}$ to $555 \pm 64.8 \text{ } \mu\text{g kg}^{-1}$. After the maximum ozonation time (240 minutes), the concentration reached $520 \pm 70.6 \text{ } \mu\text{g kg}^{-1}$, with a maximum reduction of 61%.

Consequently, the ozone processing can reduce both ZEN and DON contamination in wheat bran, which can be useful for food safety. The Panel on Contaminants in the Food Chain (CONTAM) has established the value of $0.25 \text{ } \mu\text{g kg}^{-1}$ body weight per day as the Tolerable Daily Intake (TDI) for ZEN (EFSA-CONTAM 2011); whereas this value is $1.0 \text{ } \mu\text{g kg}^{-1}$ body weight (b.w.) per day for DON (EFSA-CONTAM 2017). Some studies suggest that part of the population can be exposed to mycotoxin contamination and that especially the risk groups (children) deserve even more attention (Efsa-Contam, 2011; Mally et al., 2016; Efsa-Contam 2017). Therefore, the ozonation process can contribute positively to reduce the mycotoxin of exposure. However, further studies are necessary to evaluate the safety of the products after ozonation due to the intermediate and final products formed during processing. Even so, previous studies of mycotoxin degradation by ozone showed no toxic effects for *Hydra attenuata* (in aqueous solution, McKENZIE et al., 1997) or mouse (corn oil, LEMKE et al., 1999).

It is observed that the ozonation process was efficient in its initial proposal, i.e., reduction in mycotoxin contamination. However, other parameters also affect the reduction of sample contamination, such as the physical characteristics of the sample (grit, bran, milling, grain; particle size distribution, exposed area, etc.), initial contamination, exposure time, gas flow rate and moisture content. Consequently, different products, systems and process conditions may lead to different mycotoxin degradation, as well as alterations in product quality – highlighting the importance of studies such as the present one.

Ozone has been studied for the reduction of mycotoxins in different products such as wheat, corn, corn flour, peanuts and pistachio (PRUDENTE JR.; KING, 2002; AKBAS; OZDEMIR, 2006; ALENCAR et al., 2012; CHEN et al., 2014; LUO et al., 2014a, 2014b; TROMBETE et al. 2016). However, most of the studies were carried out for aflatoxins. Using ozone on DON degradation has been studied only in wheat and whole wheat flour (LI et al., 2014; SAVI et al., 2014; WANG et al., 2016a, 2016b; ALEXANDRE et al., 2017).

Qi et al. (2016) studied the ozonation on corn grains naturally contaminated with ZEN and ochratoxin A. They observed a reduction of 86% in ZEN and 68% in ochratoxin A after 180 minutes of processing. This high value of reduction can be related with the higher concentration of ozone in their work, which was ~60% higher than in the present study, and the differences among products and reactors.

According to our results (Figure 3.2), the lower reduction on contamination of DON compared to ZEN can be attributed to the different molecular structures of these mycotoxins.(Figure 3.3). During the process, the Ciegee Mechanism occurs, where the ozone reacts with the double bond. This leads to the formation of ozonides, which are reorganized to molozonide, an unstable compound, producing a variety of carbonyl compounds (aldehydes and ketones) or organic acids (McKENZIE et al., 1998; OLIVEIRA; WOSCH, 2012; YOUNG et al., 2006). The DON molecule has one double bond on the benzene ring, while ZEN has three double bonds in the ring, giving the molecule more reactive sites to ozone, which may justify further reduction of ZEN (YOUNG et al., 2006).

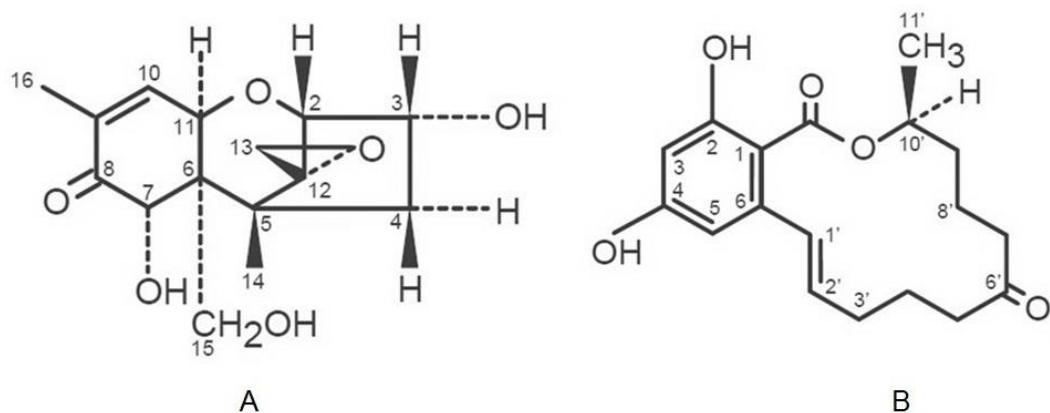


Figure 3.3 Chemical structures of deoxynivalenol (A) and zearalenone (B) Reference: (CAST, 2003).

On the other hand, the reduction of mycotoxin contamination cannot be explained by only one factor. The adsorption and penetration of ozone in the sample rely on intrinsic and extrinsic factors such as the characteristics of the sample, initial contamination, exposure time, gas flow rate, column height and moisture content (RAILA et al., 2006; STEPONAVIČIENĖ et al., 2012; TIWARI et al., 2010).

Moisture plays an important role in ozone reactivity, because water solubilizes ozone and increases the contact between gas and grain (TIWARI et al., 2010), generating more reactive ions due to interaction with water (GLAZE, 1986). In fact, many studies showed evidence of the positive correlation of moisture with the reduction in mycotoxin concentration during ozonation, such as those of Li et al. (2014) and Wang et al. (2016a) in wheat grains and Alexandre et al. (2017) in whole wheat flour.

However, although the increase in the product moisture can be positive for mycotoxin degradation, it may favor the proliferation of other microorganisms by affecting the product quality. Consequently, although it is expected that the increase in the moisture of grains and derivatives enhances the mycotoxin degradation through ozone, the product humidification is not favorable from an industrial point of view. Due to this, the wheat bran used in the present study was in agreement with the commercialization moisture required by Brazilian legislation, i.e., <15%. However, this could be a determining factor limiting the reduction of DON and ZEN.

The concentration of ozone that leaves the reactor (i.e., in the gas flow after reacting with the sample) was monitored throughout the process, as well as the sample moisture (Figure 3.4). As the inlet gas flow consisted of pure O₂ and O₃ (see the Material and Methods section), its moisture was negligible. Consequently, the gas flow promoted a partial drying of the sample during processing, which may have also contributed to the observed reduction in the rate of mycotoxin degradation (Figure 3.2).

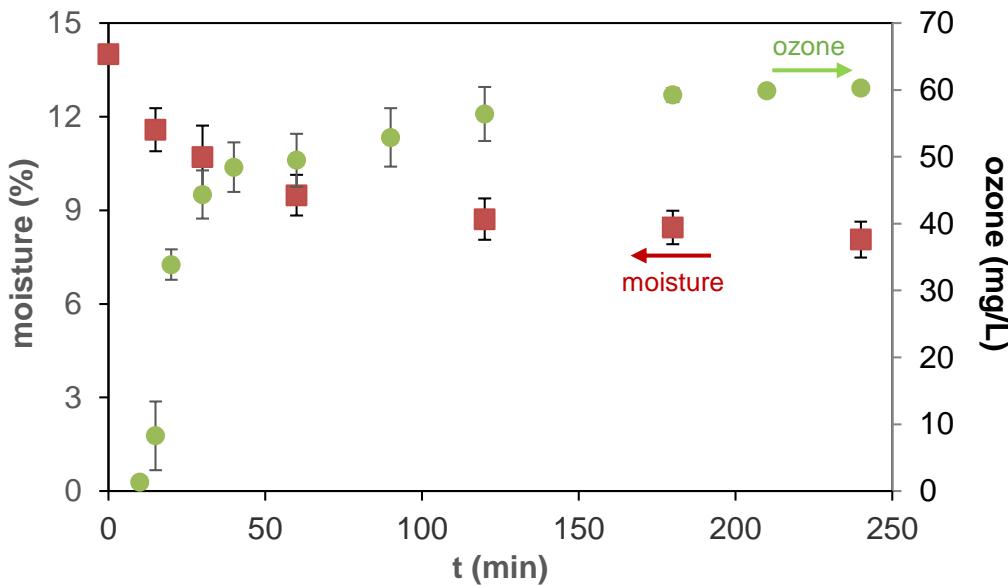


Figure 3.4 Relationship between wheat bran moisture content and ozone concentration in the gas flow during the ozone process.

It can be observed that at the beginning of the process, the bran moisture was 13.8%. As the process was carried out and the sample reacts with the gas, the concentration of ozone in the stream leaving the reactor increased, while the sample moisture decreased (~8% after 240 minutes of process, Figure 3.4). The decrease in sample moisture can be due to two mechanisms. The simplest one is the sample drying due to the absence of moisture in the gas flow. Additionally, ozone can bind to free water when reacting with the sample (LUO et al., 2014; QI et al., 2016).

Although the decrease in sample moisture may be an advantage for the stored grains by reducing the propagation of microorganisms (TIWARI et al., 2010) when the ozonation process is carried out industrially, it would probably use air (atmospheric oxygen) instead of pure oxygen. Consequently, due to the humidity present in the air, the sample drying would probably not be a concern.

Therefore, before beginning ozonation, one must consider the factors that influence the process. Even so, the ozonation process has shown to be effective for the reduction of DON and ZEN. The remaining question is if this process affects the product quality.

3.2.2. Effects of ozonation on wheat bran nutritional quality

The nutritional quality of wheat bran before and after 60 minutes of ozone processing is shown in Table 3.2.

Table 3.2 Total phenolic compounds and antioxidant activity (DPPH and ABTS) in wheat bran before and after ozonation

Processing Time (minutes)	Total phenolic content ($\mu\text{g GAE g dry matter}^{-1}$)	DPPH ($\mu\text{mol TE g dry matter}^{-1}$)	ABTS ($\mu\text{mol TE g dry matter}^{-1}$)
0	2470.91 ± 182.79 a	29.98 ± 8.74 a	18.80 ± 9.95 a
60	2611.97 ± 331.13 a	21.45 ± 3.0 a	14.76 ± 8.7 a

*Averages in the same column followed by the same letter do not differ significantly at the level of 5% ($p \leq 0.05$).

**DPPH and ABTS: antioxidant activity expressed as trolox equivalent (TE)

Wheat bran contains most of the phenolic compounds present in grain. In our research, they were expressed as micrograms of gallic acid equivalent per gram of dry sample. The unprocessed wheat bran was $2470.91 \pm 182.79 \mu\text{g GAE. g}^{-1}$ dry matter, which is in agreement with the literature: the reported content of phenolic compounds in wheat bran varies from 1258 to 3157 $\mu\text{g GAE. g}^{-1}$ (WANG et al., 2008; VAHER et al., 2010; ABOZED et al., 2014).

It is observed that there was no significant difference between the wheat bran processed and not processed with ozone. This confirms that the ozonation process, at the evaluated level, did not interfere with the phenolic contents of the samples, preserving their quality.

The antioxidant capacity by the ABTS method evaluates the radical elimination activity of hydrophilic and lipophilic compounds (DE CAMARGO et al., 2015), while the DPPH method evaluates only the hydrophilic compounds (BOUTENNOUN et al., 2017).

When evaluating the antioxidant capacity of wheat bran by the DPPH method, it can be observed that before and after the ozonation process, the concentration was 29.98 ± 8.74 and $21.45 \pm 3.0 \mu\text{mol TE g}^{-1}$ dry matter, respectively (Table 3.2). Once again, no significant differences were observed ($p \leq 0.05$). The antioxidant

capacity of wheat bran is reported in the literature from 14.4 to 71.2 $\mu\text{mol TE g}^{-1}$ (BAUER et al., 2013; POVILAITIS et al., 2015).

Similarly, the antioxidant capacity by the ABTS method (Table 3.2) shows that there were no significant differences ($p \leq 0.05$) between the samples before ($18.80 \pm 9.95 \mu\text{mol TE g}^{-1}$ dry matter) and after ($14.76 \pm 8.7 \mu\text{mol TE g}^{-1}$ dry matter) ozone processing. According to the literature, the antioxidant capacity observed in wheat bran ranged from 4.04 to 17.44 $\mu\text{mol g}^{-1}$ dry matter (NARWAL et al., 2014; VERMA; HUCL; CHIBBAR, 2009).

Therefore, it can be observed that the ozonation process did not negatively affect the antioxidant capacity of the wheat bran. Wheat grain corresponds to an important part of the human diet in different products (ADOM et al., 2003). Consequently, the preservation of phenolic compounds is highly favorable, as long as the phenolic compounds found in grains are not present in fruit and vegetables (ADOM et al., 2003).

As the phytochemicals from wheat can act as antioxidants, providing health benefits when consumed (ADOM et al., 2003), our results indicated that processing with ozone is an interesting alternative to enhance the wheat bran safety.

3.3. Conclusion

It can be concluded that the ozonation process was effective in the decontamination of two mycotoxins in the same product, which were naturally contaminated in the field. Zearalenone (ZEN) was faster degraded than deoxynivalenol (DON), which could be explained by their molecular structures. The ozonation did not alter the total phenolic compound content and antioxidant activity of wheat bran, which shows the possibility of using the ozonation process to reduce the contaminants. However, further studies are needed to enhance the mycotoxin degradation without affecting the nutritional quality to understand the effect of this technology on food quality and further components, as well as to clarify which products are generated during ozonation.

Acknowledgements

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4. CONCLUSIONS

The ozonation process is a promising technology and has been effective in reducing the contamination of DON and ZEN in different wheat products. Firstly, the effect of the ozonation process on the reduction of DON in whole wheat flour with different moisture contents was evaluated, where it was observed that the reduction of DON was higher in the flour with high moisture content (25%).

We observed that in the wet milling of wheat flour, DON can migrate to the solvent (water) due to its solubility, contaminating the water resources when discarded. Therefore, we observed that the ozonation process was also effective in decontaminating the effluent.

When the wheat bran containing DON and ZEN was ozonized, we observed that the rate of reduction was different, probably because of the molecular structure of mycotoxins.

Although there are many studies on the reduction of different mycotoxins and products, the impact of the process on quality is limited. Thus, when evaluating the rheological quality of whole wheat flour, it was observed a modification with the process. The main alterations occur in the two main components, starch and gluten proteins. However, the nutritional quality (phenolic compounds and antioxidant capacity) of wheat bran was not affected by the ozonation process.

The results obtained indicated that the ozonation process was effective in the reduction of contaminants, however the monitoring of the production chain should not be neglected, ozonation should only be used to manage the production of grains and derivatives.

5. SUGGESTIONS FOR FUTURE STUDIES

In future works, the effect of the ozonation process can be studied in other co-products (bran, grits) of different grains, and with other mycotoxins, combined or not. The effect of process variables (ozone concentration, flow, diffusion, temperature, etc) must be better understood, as well as the reactor design and scale up procedure.

In addition, one can study how the diffusion of ozone in the grain occurs and analyze the effect of the process on the starch content and the rheological properties, as well as study the effect of ozone on other parameters of nutritional quality. Moreover, the effect of ozone on other components such as proteins, lipids, carbohydrates in solution containing mycotoxin and using food models.

Finally, it is necessary to study the products generated during the ozonation process and the possible toxic effects.

APPENDIX

Appendix A – abstract presented at the Latin American Symposium on Food Science (SLACA, 2017)

IMPACT OF OZONIZATION ON QUALITY PARAMETERS, NUTRITIONAL ASPECTS AND INACTIVATION OF *LISTERIA INNOCUA* IN BEANS

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Beans are one of the most consumed legumes, due to their high content of proteins, complex carbohydrates, fibers, vitamins and also antioxidant substances, such as polyphenols. Antioxidants are substances that can slow down or inhibit oxidative damage by playing a role in preventing chronic diseases. However, the bean quality can be affected by microbial contamination or the methods used to avoid it. Ozone (O_3) is a gas with high oxidizing potential, considered a “green chemical process” and recognized as GRAS (Generally Recognized As Safe). It can be used for microbial inactivation in different agricultural products, as well as for mycotoxins degradation in grains and derivate. However, it can also negatively impact the product quality due to oxidative processes. Consequently, the objective of this study was to evaluate the effect of processing beans with ozone on their phenolic compounds and antioxidant capacity, as well as their impact on the physical properties (water activity, color) and inactivation of *Listeria innocua*, as a target microorganism. Three different beans were evaluated: black, red and *catarino* beans with 10 and 40% moisture content. The samples were processed, in a reactor using a gas flow of 5 g h^{-1} with ozone concentration of 37.06 g L^{-1} , for up to 240 min. The total phenolic content was evaluated through the method of Folin–Ciocalteu, while the antioxidant capacity was evaluated through the method of ABTS. The results demonstrates that it was possible to reduce 2.0 Log cycles of *Listeria innocua* after 240 min of processing. The *catarino* bean was affected in the phenolic content when moistened. On the other hand, the ozone processing did not affected ($p \leq 0.05$) the total phenolic content, antioxidant capacity, and color of the beans. Therefore, the present study demonstrated that the ozonation can reduce the microbiology content in beans, without impact its nutritional quality.

Keywords: Beans, Ozone, Quality

Appendix B - Simple abstract

English

Mycotoxins are substances that can occur naturally in the field. However, they can cause problems to human and animal health. An alternative to reduce contamination of mycotoxins is the use of ozone. This technology is already used for different purposes and does not generate toxic waste. In this work, it was possible to study the reduction of two different mycotoxins in two important wheat products (whole wheat flour and wheat bran) for human and animal feed, as well as the effluent from wheat milling. Thus, it was observed that, as the moisture of the product increased, the mycotoxin reduction was higher. We also observed that, when processing a product with two mycotoxins, the reduction behaviors were different. When evaluating the quality of the ozonized products, we conclude that, even when the process affects the technological quality, the product can be used for different purposes. The nutritional quality of wheat bran was also not affected by the ozonation process. Therefore, ozonation technology is effective and promising in reducing mycotoxins without compromising product quality.

Keywords: Mycotoxin, Quality, Ozone, Whole Wheat Flour, Bran

Português

As micotoxinas são substâncias que ocorrem naturalmente no campo, no entanto, elas podem causar danos à saúde humana e animal. Uma alternativa para reduzir a contaminação de micotoxinas é a utilização do ozônio. Essa tecnologia já é utilizada para diferentes fins e não gera resíduos tóxicos. Neste trabalho, foi possível estudar a redução de micotoxinas em dois importantes produtos de trigo (farinha de trigo integral e farelo de trigo) que estão presentes na alimentação humana e animal. Assim, observou-se que, à medida que a umidade do produto aumentava o teor de redução foi proporcional. Observamos também que, ao processar um único produto com duas micotoxinas, os comportamentos na redução foram distintos. Ao avaliar a qualidade dos produtos ozonizados, concluímos que, apesar de afetar a qualidade tecnológica, o produto pode ser usado para diferentes fins. A qualidade nutricional do farelo de trigo também não foi afetada pelo processo de ozonização. Portanto, a tecnologia de ozonização é eficaz e promissora na redução de micotoxinas sem comprometer a qualidade do produto.

Palavras-chave: Micotoxinas, Qualidade, Ozônio, Farina de trigo integral, Farelo de trigo

Español

Las micotoxinas son sustancias que se encuentran naturalmente en el campo, las cuales pueden causar daños a la salud humana y animal. Una alternativa para reducir la contaminación de micotoxinas es la utilización de ozono. Esta tecnología ya es utilizada para diferentes fines e no genera residuos tóxicos. En este trabajo fue posible estudiar la reducción de micotoxinas en dos importantes productos de trigo (harina de trigo integral y salvado de trigo) que están presentes en la alimentación humana y animal. Además, se observó que a medida que la humedad del producto aumenta, la reducción de micotoxinas es mayor. Observamos también que, al procesar un único producto con dos diferentes micotoxinas, los comportamientos de reducción fueron distintos. Al evaluar la calidad de los productos ozonizados, concluimos que, a pesar de afectar la calidad tecnológica, el producto puede ser usado para diferentes fines. La calidad nutricional del salvado de trigo también no fue afectada por el proceso de ozonización. Consecuentemente, la tecnología de ozonización es eficaz y prometedora en la reducción de micotoxinas sin comprometer la calidad del producto final.

Palabras clave: micotoxinas, calidad, ozono, harina de trigo integral, salvado de trigo.

Appendix C – First page of the published Chapter 2

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Ozonation of whole wheat flour and wet milling effluent: Degradation of deoxynivalenol (DON) and rheological properties

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ABSTRACT

The objective of this study was to evaluate the reduction on the levels of the mycotoxin deoxynivalenol (DON) in whole wheat flour (WWF) with different moisture levels, on the wet milling effluent through ozone (O_3) processing, as well as the impact of ozonation on the rheological properties of flour. The results have shown that the reduction of DON was improved with increasing moisture and exposure time of WWF and wet milling effluent to ozone. The maximum reduction was about 80%, proving that ozonation is an effective and promising technology in reducing mycotoxins in different products. However, the process altered the rheological profile of WWF. Therefore, further studies are needed to better understand the process.

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KEYWORDS

Mycotoxins; ozone; grains; effluent

Introduction

Mycotoxins are secondary metabolites produced by some fungi, leading to harmful effects to humans and animals, as well as economic losses to agribusiness. These metabolites are produced under specific conditions and can be present at various stages of agricultural production, being stable to most processes.^[1] Although there are geographic and climatic variations in the production and occurrence of mycotoxins, exposure to these substances occurs worldwide, once it is estimated that about 25% of the world food supply are contaminated with mycotoxins.^[2] Therefore, mycotoxins in food represent both a public health problem and economic and industrial losses.

Trichothecenes (deoxynivalenol (DON), nivalenol, T-2 toxin, diacetoxyscirpenol) are produced by fungi of the genus *Fusarium*. They often contaminate foods such as corn, rice, wheat, soy, barley, rye and animal feed.^[3] Trichothecenes can inhibit protein synthesis, including DNA and RNA, affect cell division, besides interfering with phospholipid metabolism in the cell membrane and alter serotonin activity, related to food intake regulation.^[4] The toxic potential of DON in humans implies acute gastroenteritis with vomiting, in addition to the chronic effect on growth and immune and reproductive functions.^[5]

One way to minimize some of the risks associated with mycotoxin contamination is to apply a quality management system. However, there are situations where the presence of mycotoxins is unavoidable, even adopting all good production practices. For example, during crop development, climatic conditions can favor fungal infections, resulting in contamination of the grains.^[6] Therefore, it is extremely important to study

alternatives that reduce mycotoxin contamination in agricultural products, their derivatives, and co-products.

A promising method of decontamination is the use of ozone (O_3), a gas with high oxidizing potential, generated electrically at the time of use and decomposing into O_2 . It is considered a green chemical process and is recognized as GRAS—*Generally Recognized As Safe*, with use regulated by the FDA for agricultural products *in natura*.^[7,8]

Although ozonation may be a promising alternative in the reduction of DON, with many studies about this issue, there are few studies describing such degradation in cereals and their naturally contaminated derivatives. Studying naturally contaminated samples (rather than artificially contaminated grains through contact with mycotoxin solutions) is of great importance, which is in line with the actual field conditions and the natural distribution of mycotoxins in the contaminated grains (localization in tissues and cells, as well as interaction with other molecules).

Starch and cereal protein concentrates are produced by wet milling processes (i.e., in the presence of water, under suspension). Wheat starch has significant production, corresponding to 36% of the starch production in the European Community in 2005.^[9] Therefore, due to the affinity of the DON with water,^[10] the wet milling effluent may contain this mycotoxin, with negative impacts to the effluent treatment system and the environment.

The objective of this study was to evaluate the use of ozone to reduce deoxynivalenol (DON) in whole wheat flour (WWF) and wet milling effluent, as well as the effect of the process on the rheological properties of the processed flour.

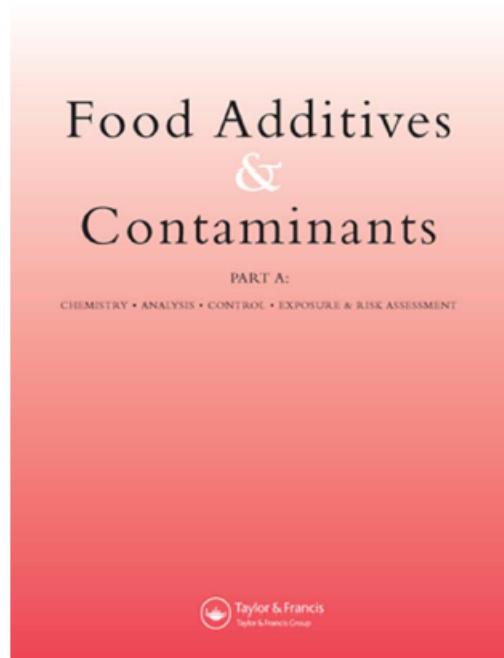
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Appendix D - First page of the submitted (under review) chapter 3

Food Additives and Contaminants



Ozone treatment to reduce deoxynivalenol (DON) and zearalenone (ZEN) contamination in wheat bran and its impact on nutritional quality

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