FEDERAL UNIVERSITY OF RIO GRANDE
SCHOOL OF CHEMISTRY AND FOOD
POSTGRADUATE PROGRAM IN FOOD SCIENCE AND ENGINEERING

THE FATE OF AFLATOXINS AND FUMONISIN B1 DURING THE PROCESSING OF CORN: A BIOACCESSIBILITY APPROACH

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Dedico este trabalho aos meus pais Cleocir e Claudete, irmãs Carol e Luana e esposo Thiago.

Não deve haver limites para o esforço humano. Somos todos diferentes.
Por pior do que a vida possa parecer, sempre há algo que podemos fazer em que podemos obter sucesso. Enquanto houver vida, haverá esperança.

Steven Hawking

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GENERAL ABSTRACT

Corn grains are subject to contamination by mycotoxin-producing fungi, which cause deleterious health effects. This is an important fact for food safety, as this cereal is present in the human diet in various forms. Physical, chemical, or biological methods have been tested to mitigate contamination. Heat treatments can modify the structure of mycotoxins due to interactions with food constituents, affecting the bioaccessibility of the contaminant. In this work, the objective was to study the distribution of aflatoxins and fumonisin in milling processes and their bioaccessibility after heat treatments. A method for simultaneous extraction of these mycotoxins was validated and during the heat treatments of hydrothermal cooking and extrusion, the parameters that determined the formation of resistant starch in corn products were studied. In corn grains naturally contaminated with aflatoxins B1 and B2 and fumonisin B1, their distribution during dry and wet-milling was evaluated. The effect of heat treatments and the correlation of product composition with mycotoxin levels and their subsequent *in vitro* bioaccessibility were determined by multivariate analysis. The validated method of extraction and detection of mycotoxins was shown to be indicative of analytical efficiency in determining their concentration in the different forms of corn processing, according to international recommendations. The highest resistant starch content was obtained by cooking the fine cornmeal and water at 120 °C for 40 min with a proportion of 1:5 (w/v) and 3% soybean oil, in the extrusion process of coarse cornmeal with a screw speed of 210 rpm, 22% initial moisture, and addition of 20% high amylose corn starch. Wet and dry milling processes reduced the concentration of aflatoxins B1 and B2 in the endosperm (98–100%) and dry milling reduced the contamination of fumonisin B1 in the endosperm (< 0.4 μg g^{-1}). The distribution factor of fumonisin B1 to the germ and pericarp showed an increase by more than three times the contamination levels in these fractions. Hydrothermal cooking resulted in a 0–52% reduction in aflatoxin levels and 39–59% in fumonisin B1 levels, however the extrusion process promoted further reductions in aflatoxin (74.7–89.9%) and fumonisin B1 levels (66.0–74.9 %). In both heat treatments, the reduction of aflatoxins and fumonisin showed a positive correlation with the presence of starch and resistant starch. However, high aflatoxin (63–91%) and low fumonisin B1 (27–35%) bioaccessibility occurred in cooked products, and similar bioaccessibility behavior for aflatoxins (43–95%) and fumonisin B1 (35–54%) was identified in the extruded products. Possibly, during these processes, aflatoxins form bonds that are broken during *in vitro* digestion, and the modified fumonisin is stable under digestive conditions. Milling processes are effective in reducing aflatoxins and fumonisin in the endosperm fraction, extrusion heat treatment is the most efficient for simultaneously reducing aflatoxins and fumonisin levels, and the presence of resistant starch reduces fumonisin B1 bioaccessibility.

Keywords: Extrusion. Milling process. Mycotoxins. Resistant starch. Thermal treatment.

RESUMO DO TRABALHO EM PORTUGUÊS

Grãos de milho estão sujeitos à contaminação por fungos produtores de micotoxinas, as quais causam efeitos deletérios a saúde. Fato importante para a segurança alimentar, pois este cereal está presente na dieta humana sob várias formas. Métodos físicos, químicos ou biológicos vem sendo testados para mitigar a contaminação. Tratamentos térmicos podem modificar a estrutura da micotoxina em decorrência de interações com constituintes do alimento, afetando a bioacessibilidade do contaminante. Neste trabalho, o objetivo foi estudar a distribuição de aflatoxinas e fumonisina em processos de moagem e a bioacessibilidade delas após tratamentos térmicos. Foi validado um método para extração simultânea destas micotoxinas e durante tratamentos térmicos de cozimento hidrotérmico e extrusão foram estudados os parâmetros que determinavam a formação de amido resistente em produtos à base de milho. Em grãos de milho naturalmente contaminados com aflatoxinas B1 e B2, e fumonisina B1 foram avaliadas a distribuição delas durante as moagens seca e úmida. O efeito dos tratamentos térmicos e a relação da composição dos produtos nos níveis de micotoxinas e a posterior bioacessibilidade *in vitro* delas foram determinados por análise multivariada. O método de extração e detecção de micotoxinas validado apresentou indicativos de eficiência analítica para determinar a concentração delas nas diferentes formas de processamento do milho, conforme recomendações internacionais. O maior teor de amido resistente foi obtido com cozimento da farinha de milho fina e água a 120 °C por 40 min na proporção de 1:5 (w/v) e 3% de óleo de soja, enquanto no processo de extrusão da farinha de milho foi com velocidade de parafuso de 210 rpm, 22% de umidade e adição de 20% de amido com alto teor de amilose. Os processos de moagem úmida e seca reduziram a concentração de aflatoxinas B1 e B2 (98-100%) e a moagem seca reduziu a contaminação de fumonisina B1 no endosperma (< 0.4 μg g^{-1}). O fator de distribuição de fumonisina B1 para gérmen e pericarpo mostrou um aumentou em mais de três vezes nos níveis de contaminação nessas frações. O cozimento hidrotérmico resultou em redução de 0–52% nos níveis de aflatoxinas e de 39–59% nos níveis de fumonisina B1, no entanto o processo de extrusão promoveu maiores reduções nos níves de aflatoxinas (74.7–89.9%) e fumonisina B1 (66.0–74.9%). Em ambos os tratamentos térmicos a redução das aflatoxinas e fumonisina mostraram correlação positiva com a presença de amido e amido resistente. No entanto, a bioacessibilidade de aflatoxinas (63–91%) foi elevada e da fumonisina B1 (27–35%) foi diminuida nos produtos cozidos, comportamento similar de bioacessibilidade para aflatoxinas (43–95%) e fumonisina B1 (35–54%) foi identificado nos produtos extrusados. Possivelmente, durante esses processos as aflatoxinas formam ligações que são desfeitas durante a digestão *in vitro*, e a fumonisina modificada é estável em condições digestivas. Processos de moagem são efetivos para reduzir aflatoxinas e fumonisina na fração do endosperma, o tratamento térmico extrusão é o mais eficiente para redução simultânea dos níveis de aflatoxinas e fumonisina e a presença do amido resistente reduz a bioacessibilidade de fumonisina B1.

Palavras-chave: Amido resistente. Extrusão. Micotoxinas. Moagem. Tratamento térmico.

1 INTRODUCTION

Corn is the most produced grain in the world, reaching 1.12 billion tons in the 2018/19 harvests (USDA, 2019). In Brazil, it is the second crop, which makes the country the third largest world producer with a forecast of 98.4 million tons for the 2019/20 crop (CONAB, 2019). The demand for cultivation results from the diversity of application of this cereal for animal consumption, as well as a raw material for plastics, syrups, biofuels, and human food (ABIMILHO, 2019). Human consumption of corn has increased due to its high starch content, low gliadin protein content, lack of gluten, low allergen content, dietary fiber, and delicate taste. With these characteristics, it is the most flexible raw material for the manufacture of food products such as popcorn, polenta, tortillas, porridge, breakfast cereals, snacks, bakery products, cornmeal, and pasta (SHI et al., 2016; BLANDINO et al., 2017).

Due to its physicochemical composition and morphological characteristics, corn and its derivatives are susceptible to fungal contamination. Some species of filamentous fungi, under unfavorable conditions, can produce secondary metabolites known as mycotoxins. The main fungal toxins found in corn are aflatoxins, ochratoxin A, trichothecenes, fumonisins, and zearalenone (BULLERMAN; BIANCHINI, 2014). These are mainly produced by fungal species belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*, which may be present in the field, during grain drying, and storage (PITT, 2006; NEME; MOHAMMED, 2017).

Mycotoxins are considered in worldwide food quality control programs due to their toxicity and deleterious effects on human and animal health. Among them, aflatoxins and fumonisins stand out due their frequent contamination of corn and its derivates (OLIVEIRA et al., 2017) and are classified as carcinogenic in humans (IARC, 2002). The pre-harvest and post-harvest measures that are being adopted do not guarantee the absence of mycotoxins in food (JACOBSEN, 2014). It is necessary to use physical, chemical, or biological methods during food processing to reduce the levels of these contaminants. Physical methods include cleaning, grinding, separation, and heat treatment procedures (KARLOVSKY et al., 2016).

Corn products are consumed after some heat treatment, so the use of this strategy to reduce mycotoxins is applicable, as studies have shown a reduction in these contaminants by both hydrothermal cooking and extrusion (DE GIROLAMO et al., 2016; SOHI SUKHMAN et al., 2015). The degree of mycotoxin reduction achieved is variable and depends on the heat treatment conditions and the composition of the food matrix. The mechanisms that promote these reductions are not well known, but may involve thermal decomposition, modification of mycotoxin structure due to interactions with other food constituents (HUMPF; VOSS, 2004;

FALAVIGNA et al., 2012; DALL'ASTA; BATTILANI, 2016). This interaction may be beneficial as it reduces contaminant bioaccessibility as once ingested, modified mycotoxin may be stable (FALAVIGNA et al., 2012) or unwanted because it increases bioaccessibility by being converted back to its free form and becoming available for absorption (DALL'ASTA et al., 2010).

This concern raised the need for *in vitro* studies to evaluate the fate of modified mycotoxins, simulating natural conditions during digestion. The correlation of mycotoxins to undigested macro components of food, such as resistant starch, has not been studied in corn products. Available studies have analytically evaluated the reduction in mycotoxin levels during processing and may not reflect actual exposure to them. The resistant starch formed could retain part of the contaminant, reducing its bioaccessibility by promoting the formation of complexes that hinder the action of digestive enzymes (KARUNARATNE; ZHU, 2016). Knowing this reality allows us to adopt strategies to mitigate the risk of exposure.

2 INTRODUÇÃO

O milho é o grão mais produzido no mundo, alcançando 1,12 bilhões de toneladas nas safras de 2018/19 (USDA, 2019). No Brasil é a segunda maior cultura, o que torna o país o terceiro maior produtor mundial com previsão de 98,4 milhões de toneladas na safra 2019/20 (CONAB, 2019). A demanda de cultivo decorre da diversidade de aplicação deste cereal para consumo animal, além de matéria-prima para plásticos, xaropes, biocombustíveis e alimentação humana (ABIMILHO, 2019). O consumo de milho na alimentação humana tem aumentado devido ao alto teor de amido, proteínas com baixo teor de fração gliadina, ausência de glúten, baixos teores de alergênicos, alto conteúdo de fibras dietéticas e sabor delicado. Com essas características é a matéria prima mais flexível para fabricação de produtos para alimentação humana: pipoca, polenta, tortilhas, mingau, cereais matinais, *snacks*, produtos de panificação, farinha de milho e massas (SHI et al., 2016; BLANDINO et al., 2017).

Devido a sua composição físico-química e características morfológicas, o milho e seus derivados são susceptíveis a contaminação fúngica. Algumas espécies de fungos filamentosos, em condições desfavoráveis, podem produzir metabólitos secundários conhecidos como micotoxinas. As principais toxinas fúngicas encontradas em milho são aflatoxinas, ocratoxina A, tricotecenos, fumonisinas e zearalenona (BULLERMAN; BIANCHINI, 2014). Essas são produzidas principalmente por espécies de fungos pertencentes ao gênero *Aspergillus*, *Penicillium* e *Fusarium*, os quais podem estar presentes no campo, durante a secagem e armazenamento dos cereais (PITT, 2006; NEME; MOHAMMED, 2017).

As micotoxinas estão consideradas em programas mundiais de controle de qualidade de alimentos devido à sua toxicidade e efeitos deletérios a saúde humana e animal. Dentre elas destacam-se as aflatoxinas e fumonisinas que frequentemente contaminam o milho e derivados (OLIVEIRA et al., 2017) e são classificadas como cancerígenas em seres humanos (IARC, 2002). As medidas de pré-colheita e pós-colheita que vem sendo adotadas não garantem a ausência de micotoxinas em alimentos (JACOBSEN, 2014), sendo necessária a utilização de métodos físicos, químicos ou biológicos durante o processamento de alimentos para reduzir os níveis destes contaminantes. Dentre os métodos físicos encontram-se procedimentos de limpeza, moagem, separação e tratamento térmico (KARLOVSKY et al., 2016).

Produtos derivados de milho são consumidos após algum tratamento térmico, sendo assim, o uso dessa estratégia para reduzir micotoxinas é aplicável, visto que estudos tem mostrado redução destes contaminantes, tanto por cozimento hidrotérmico como por extrusão (DE GIROLAMO et al., 2016; SOHI SUKHMAN et al., 2015). O grau de redução de

micotoxinas alcançado é variável e depende das condições do tratamento térmico e da composição da matriz alimentar. Os mecanismos que promovem essas reduções não são bem conhecidos, mas podem envolver decomposição térmica e/ou modificação da estrutura da micotoxina em decorrência de interações com outros constituintes do alimento (HUMPF; VOSS, 2004; FALAVIGNA et al., 2012; DALL'ASTA; BATTILANI, 2016). Essa interação pode ser benéfica por reduzir a bioacessibilidade do contaminante, pois uma vez ingerida, a micotoxina modificada pode ser estável (FALAVIGNA et al., 2012) ou indesejada devido ao fato de aumentar a bioacessibilidade por ser convertida novamente em sua forma livre e tornar-se disponível para absorção intestinal (DALL'ASTA et al., 2010).

Essa preocupação norteia a necessidade de estudos *in vitro* para avaliar o destino da micotoxina modificada, simulando as condições naturais durante a digestão. A relação das micotoxinas com macro componentes não digeríveis do alimento, como o amido resistente, ainda não foi estudada em produtos à base de milho. Os estudos disponíveis avaliaram analiticamente a redução dos níveis das micotoxinas durante os processamentos e podem não refletir a real exposição a elas. O amido resistente formado poderia reter parte do contaminante reduzindo a sua bioacessibilidade por promover a formação de complexos que dificultam a ação de enzimas digestivas (KARUNARATNE; ZHU, 2016). Conhecer esta realidade permite adotar estratégias para mitigar o risco de exposição.

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

To evaluate the distribution of aflatoxins G2, G1, B2, and B1, and fumonisin B1 in corn milling processes and the bioaccessibility of these mycotoxins after heat treatments.

3.2 SPECIFIC OBJECTIVES

To standardize and validate a method for simultaneous extraction of aflatoxins and fumonisin in corn and its derivatives;

To determine the distribution of aflatoxins and fumonisin during dry and wet corn milling and to identify the milling parameters that contribute to the decrease of these contaminant levels in the endosperm fraction;

To study the formation of resistant starch in corn-based products obtained by the heat treatments, hydrothermal cooking and extrusion;

To identify the effect of composition and resistant starch formation on aflatoxin G2, G1, B2, and B1, and fumonisin B1 levels, and bioaccessibility in corn products obtained by hydrothermal cooking and extrusion;

To recommend treatment that provides greater reductions in mycotoxin levels and lower bioaccessibility in heat-treated corn products.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar a distribuição de aflatoxinas G2, G1, B2 e B1 e fumonisinas B1 em processos de moagem de milho e a bioacessibilidade destas micotoxinas após tratamentos térmicos.

4.2 OBJETIVOS ESPECÍFICOS

Padronizar e validar método de extração simultânea de aflatoxinas e fumonisina em milho e seus derivados;

Determinar a distribuição de aflatoxinas e fumonisina durante as moagens seca e úmida de milho e identificar os parâmetros que contribuem para a diminuição dos níveis destes contaminantes na fração do endosperma;

Estudar a formação de amido resistente em produtos à base de milho obtidos por tratamentos térmicos cozimento hidrotérmico e extrusão;

Identificar o efeito da composição e da formação de amido resistente nos níveis e bioacessibilidade de aflatoxinas G2, G1, B2 e B1 e fumonisinas B1 em produtos de milho obtidos por cozimento hidrotérmico e extrusão;

Recomendar tratamento que propicie maiores reduções nos níveis de micotoxinas e menor bioacessibilidade delas em produtos de milho tratados termicamente.

CHAPTER II

LITERATURE REVIEW

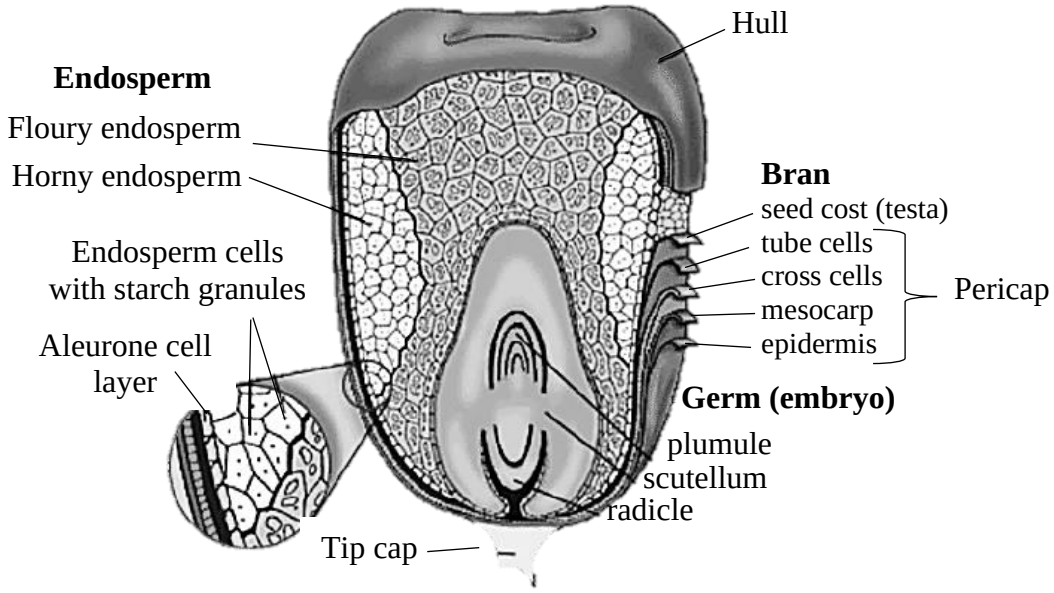
5 LITERATURE REVIEW

5.1 CORN

Corn (*Zea mays* L.), also known as corn, is a grass belonging to the family *Gramineae*. This grain is the third cereal most cultivated cereal in the world. Brazil is the world's third largest corn producer and accounted for 98.4 million tons in 2019 (CONAB, 2019), after the United States of America and China. The main producer states of the country are Paraná, Mato Grosso, Minas Gerais, and Rio Grande do Sul. In 2019, the industrial utilization was 6 million tons to dry and wet-milling, 1.6 million tons to human consumption, and 1.5 million tons to *in nature* use (ABIMILHO, 2019).

Corn kernel, is botanically a caryopsis, formed by four main physical structures: endosperm, germ, pericarp, and tip cap (**Figure 1**), which differ in chemical composition and also in the organization within the grain.

Figure 1 – Structure anatomy of corn kernel



Source: PAES (2006)

The largest constituent of corn grain is the endosperm (Table 1), which consists mainly of starch (86.6%) and is formed by granules with an average diameter of 20 μm, and ranging from polyhedral to spherical (DELCOUR; HOSENEY, 2010).

Endosperm, based on the distribution of starch granules and protein matrix, is classified into two types: floury and horny. In the floury type, the starch granules are rounded and scattered, with no protein matrix surrounding these structures. On the other hand, in the horny endosperm, the protein matrix is dense, with structured protein bodies surrounding the polygonal starch granules, preventing spaces between these structures. In the endosperm, there are also prolamins which are reserve proteins called zein. These proteins form the protein bodies that make up the matrix that surrounds the starch granules within the cells in the endosperm (DELCOUR; HOSENEY, 2010). Five groups of corn are known: popcorn, flint corn, dent corn, flour corn, and sweet corn, the main difference between the types being the shape and size of the grains, defined by the structure of the endosperm and the size of the germ (PAES, 2006).

Table 1 - Weight and composition of component parts of dent corn kernels

Percent dry weight of whole kernel		**Composition of kernel parts (% dry basis)**				
		Starch	**Fat**	**Protein**	**Ash**	**Sugar**
Germ	11.5	8.3	34.4	18.5	10.3	11.0
Endosperm	82.3	86.6	0.86	8.6	0.31	0.61
Tip cap	0.8	5.3	3.8	9.7	1.7	1.5
Pericarp	5.3	7.3	0.98	3.5	0.67	0.34
Whole kernel	100	72.4	4.7	9.6	1.43	1.94

Source: ECKHOFF; WATSON (2009)

Corn needs some transformation to be consumed, except for consumption when the grains are in a milk stage. Dried grains are processed by wet and dry grinding. This cereal is part of the basic diet in Latin America, Asia, and Africa, where it is used for the preparation of traditional foods such as tortillas, *arepas*, couscous, and porridge. In addition, consumption has increased in developed countries as it is used as an ingredient for breakfast cereals, snacks, dietetic products, and in particular for gluten free foods whose consumption is increasing (BLANDINO et al., 2017). In Brazil, corn on the cob is consumed cooked and in the preparation of *pamonha*; while, cornmeal is used in typical preparations such as couscous, cornbread, *angu*, soup, breads, cakes, *farofa*, cream, and polenta. Polenta, consumed mainly in southern region of Brazil, consists of a process of cooking cornmeal with water, salt and oil, which can also be subsequently fried.

Corn is used for human nutrition due to its specific physicochemical properties, such as high levels of starch, proteins with low levels of gliadin fraction, gluten free, dietary

lipids, hypoallergenic, phytochemicals, dietary fiber, and a delicate flavor (WÓJTOWICZ; KOLASA; MOŚCICKI, 2013). Among the phytochemicals, there are the phenolic compounds, which are found in both free (mostly flavonoid) and bound (mainly phenolic acids) forms. Ferulic acid is the predominant phenolic acid bound to cell wall polysaccharides (MONTILLA et al., 2011). In the aleurone layer and horny endosperm, carotenoids are present, which give color to grains and are important in preventing macular degeneration and as precursors of vitamin A (PAES, 2006).

5.2 STARCH AND RESISTANT STARCH

The main corn component is starch (SOMAVAT et al., 2016). Among polysaccharides, starch is the main energy reserve substance in higher plants, and it is formed by two macromolecules, amylose and amylopectin. The proportions in which these macromolecules occur differ among the sources, varieties, and degree of maturity of the plant. Corn starch contains 25–28% amylose; while some waxy corn varieties do not have amylose, being entirely composed of amylopectin. Other starches have an amylose content above 50% and are called high amylose (WEBER; COLLARES-QUEIROZ; CHANG, 2009).

Starch can be classified as glycemic and resistant. Glycemic is degraded to glucose by enzymes in the digestive tract and can be classified as either rapidly (hydrolyzed to glucose within 20 min) or slowly digestible starch (converted to glucose between 20 and 110 min). The resistant starch (RS) is the sum of starch and starch degradation products not absorbed in the small intestine of healthy individuals (ENGLYST; HUDSON, 1996), which are fermented by colonic bacteria to produce short-chain fatty acids and gases in the large intestine (TOPPING; CLIFTON, 2001). The RS properties are similar to those of dietary fiber, presenting a prebiotic effect and acting on lipid metabolism, reducing cholesterol, risk of ulcerative colitis, colon cancer (HU et al., 2016), hypoglycemic effects, inhibiting fat accumulation (HASJIM et al., 2010), and increasing absorption of minerals (MORAIS et al., 1996).

RS has been classified into five types, RS1: physically inaccessible starch entrapped in a non-digestible matrix, such as whole or partly milled grains or seeds; RS2: some types of raw starch granules and high amylose starches; RS3: retrograded starch, either processed from unmodified starch or resulting from food processing applications; RS4: chemically modified starch (ENGLYST; HUDSON, 1996); and RS5: lipid-amylose complexes (HASJIM et al., 2010). In addition to these five types of RS, studies have shown that interactions between starch and phenolic compounds affect starch digestibility by forming an amylose phenol complex (RS

similar to type 5), which affects the enzymatic hydrolysis of starch granules by α-amylase (KARUNARATNE; ZHU, 2016; ZHU, 2015).

The amount of RS in foods depends on the botanical source, amylose content, moisture, storage time (KIM; TANHEHCO; NG, 2006), amylose/amylopectin ratio (LI et al., 2015), and processing (EGGUM et al., 1993). It is extremely important to know the types of RS formed during processing, as it may influence its physical properties (OKUMUS et al., 2018) and health benefits (HASJIM et al., 2010). Most of the starch is consumed in a gelatinized form. Processes in food technology reduce or eliminate RS1 and RS2, but potentially form RS3, such process include thermal treatment, particularly if high amylose content starches are used (MUTLU; KAHRAMAN; ÖZTÜRK, 2017) or optimizing extrusion conditions are employed (FARAJ; VASANTHAN; HOOVER, 2004; KIM; TANHEHCO; NG, 2006). RS5 is formed when processing is carried out with the addition of lipid and high moisture content (CHEN et al., 2017). Based on the health benefits and functional properties attributed to RS, product development with higher levels of RS are trending (CHEN et al., 2017; MUTLU; KAHRAMAN; ÖZTÜRK, 2017).

The *in vitro* method used to quantify RS first remove the digestible fraction by enzymatic treatment, simulating hydrolysis that occurs in the upper digestive tract (mouth, stomach, and small intestine). After this step, the remaining (resistant) starch is solubilized with potassium hydroxide or dimethyl sulfoxide, and further hydrolyzed by amylolytic enzymes (CHAMP, 1992). In the last step of hydrolysis, the glucose released is quantified and converted to starch by the use of a conversion factor of 0.9.

5.3 MYCOTOXINS

Cereal starch is susceptible to mycotoxins contamination, which are toxic compounds. They are a group of molecules with diverse structures and generally low molecular weight produced by the secondary metabolism of different genera of toxigenic fungi, which under appropriate temperature and humidity conditions can grow in various foods. Mycotoxins, due to their range of distribution, damage to the economy, and risks to human and animal health, have been increasingly investigated (NEME; MOHAMMED, 2017).

The main fungal toxins important for agribusiness are aflatoxins (AFLAS), ochratoxin A (OTA), trichothecenes (TRs), fumonisins (FBs), and zearalenone (ZEA) (NEME; MOHAMMED, 2017). These toxins are mainly produced by fungal species belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. *Fusarium* species are plant pathogens

producing mycotoxins before or immediately after harvest. *Penicillium* species are most common as contaminants in commodities and food during drying and storage. *Aspergillus* species are organisms that can produce mycotoxins in the field or during drying and storage (PITT, 2006).

Aflatoxins and fumonisin are the mycotoxins frequently found in corn (HENDEL et al., 2017; MURASHIKI et al., 2017; WANG et al., 2016) and due to its toxicity and deleterious effects on human and animal health, aflatoxin B1 is classified by the International Cancer Research Agency (IARC) as carcinogenic (group 1) and fumonisin B1 as probably carcinogenic to humans (group 2B) (IARC, 2002). To protect consumers, many countries have set stringent regulations concerning mycotoxins levels permitted in food commodities (Table 2).

Table 2 - Maximum tolerable limits of aflatoxins and fumonisins in corn foods in Brazil, United States of America and the European Union

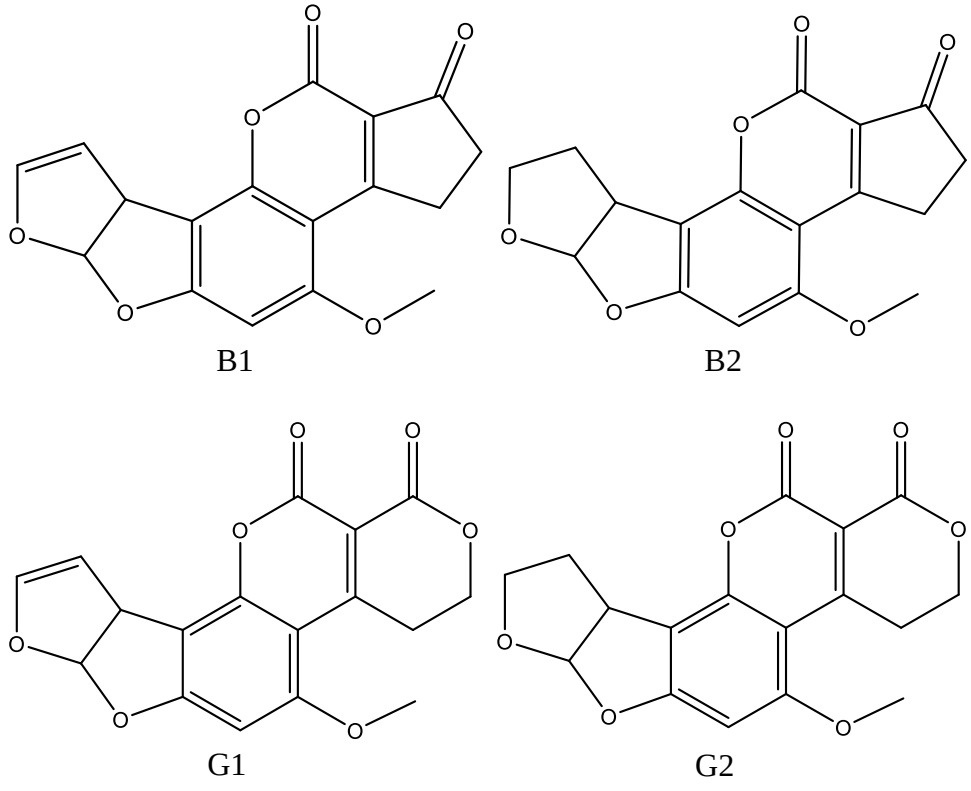
Human Foods	**ANVISA**	**FDA**	**EU**
	Aflatoxins (G2, G1, B2, and B1)		
	Maximum tolerable limits (μg kg^{-1})		
Corn and corn products	20	20	10
	Fumonisin (B1 + B2)		
	Maximum tolerable limits (mg kg^{-1})		
Unprocessed corn	5,000	-	4,000
Cornmeal, corn cream, flakes, hominy	1,500	2,000	1,000
Corn starch and other corn products	1,000	-	-
Breakfast cereals and snacks	-	-	800
Corn destined to baby food	-	-	200
Cleaned corn intended for popcorn	-	3,000	-
Cleaned corn intended for masa production, dry milled corn bran and whole or partially degermed dry milled corn products	-	4,000	-

Source: ANVISA - Agência Nacional de Vigilância Sanitária (2011, 2017); EU - European Commission (2006, 2007); USFDA - Food and Drug Administration (2001).
"-" = Fumonisin levels are not stablished for this food.

5.3.1 Aflatoxins

Aflatoxins, the most investigated and dangerous mycotoxins since first being discovered in the 1960s, are compounds synthesized mainly by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (SWEENEY; DOBSON, 1998) under optimum conditions of temperature, humidity, and plant–host interactions (ABBAS et al., 2013). These compounds are a class of secondary metabolites also known as bis-furano-coumarins (CAMPBELL; HAYES, 1976) and the main known AFLAS are called B1, B2, G1, and G2, based on their fluorescence under ultraviolet light (B = Blue, G = Green) and their mobility during thin layer chromatography. They are soluble in polar solvents, such as chloroform and methanol, have molecular weights of 312.3 g mol^{-1} for B1, 314.3 g mol^{-1} for B2, 328.3 g mol^{-1} for G1, and 330.3 g mol^{-1} for G2 (SWEENEY; DOBSON, 1998). The AFLAS chemically have similar structures (Figure 2), forming a single group of heterocyclic compounds (furocoumarin complex). The “G” and “B” groups differ in the presence of a 3-lactone ring in the place of the cyclopentanone ring and in the presence of a double bond 9,9 as a vinyl ether on the furan terminal ring in AFLAS B1 and G1, but not in AFLAS B2 and G2 (JAIMEZ et al., 2000).

Figure 2 - Aflatoxins B1, B2, G1 and G2 chemical structure



Source: JAIMEZ et al. (2000)

Aflatoxins B1 and G1 molecules can be metabolized by biotransformation of the terminal furan ring double bond by cytochrome P-450 enzymes to toxic AFB exo-8,9-epoxides (BEDARD; MASSEY, 2006) with AFB1 having the highest hepatocarcinogen activity in several species of animal (FIRMIN et al., 2010). Toxicity is attributed to the ability of the highly reactive exo-8,9-epoxide to induce the formation of DNA adducts and subsequently hepatocellular carcinoma (WILD; TURNER, 2002) and epidemiological studies have demonstrated that the aflatoxin B1 is responsible for acute liver intoxications (IARC, 2002).

AFLAS have been found in cornmeal in Italy (ALBORCH et al., 2012) and Turkey (ALGÜL; KARA, 2014), in corn in China (WANG et al., 2016), the United States (HENDEL et al., 2017), and Zimbabwe (MURASHIKI et al., 2017), and in corn products in Spain (CANO-SANCHO et al., 2012). In cornmeal analyzed in Brazil, contamination above the legislated limit was found in samples collected in Recife (KAWASHIMA; SOARES, 2006). In samples from São Paulo, AFLAs were found in 42% of corn based products, with higher incidence in cornmeal, but at levels acceptable by Brazilian legislation (0.051–2.8 μg kg^{-1}) (JAGER et al., 2013). Of the 148 corn samples analyzed from southern states, AFLA B1 and G1 were detected in 38 and 11 samples, respectively (OLIVEIRA et al., 2017).

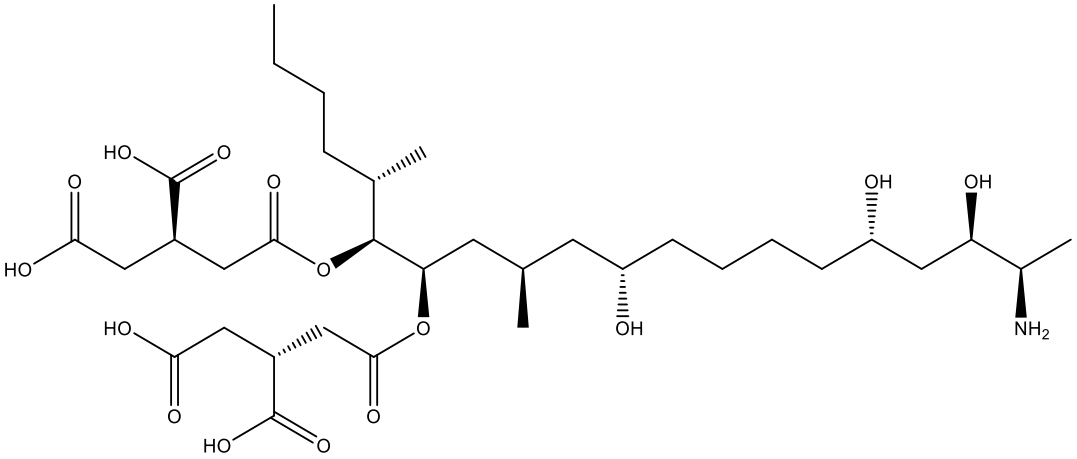
5.3.2 Fumonisins

FBs are mycotoxins synthesized mainly at the pre-harvest stage due to high moisture conditions (MUNKVOLD, 2003) by the fungi *Fusarium verticilloides* and *Fusarium proliferatum*, which are common organisms associated with the microflora present on corn throughout the growth cycle of the crop (NESIC; IVANOVIC; NESIC, 2014). These compounds are characterized as a propane-1,2,3-tricarboxylic acid diester and a pentahydroxyheicosane containing a primary amino group and have structural similarity to sphingosine and sphinganine. The structural similarity by FBs to these sphingolipids is responsible for the disruption of sphingolipid metabolism due to the inhibition of sphingosine N-acyltransferase and the subsequent accumulation of sphingoid bases resulting in unscheduled DNA synthesis. Cytotoxicity of FB1 has also been linked to the disruption of protein metabolism and the urea cycle (NESIC; IVANOVIC; NESIC, 2014).

They are strongly polar compounds, soluble in water, methanol and acetonitrile/water, with a melting point of approximately 105 °C and a molecular weight of 721 g mol^{-1}. Since first being discovered in 1988, eighteen different types of fumonisins have already been isolated, differing in hydroxyl groups identified. Fumonisin B1 (FB1) and

fumonisin B2 (FB2) are the most commonly found in corn (OLIVEIRA et al., 2017; SCUSSEL et al., 2014). However, the most toxic molecular form produced by *Fusarium moniliforme* is FB1 (Figure 3) (SEO; LEE, 1999).

Figure 3 - Fumonisin B1 chemical structure



Source: NESIC; IVANOVIC; NESIC (2014)

The incidence of FBs in corn was verified in China (WANG et al., 2016), the United States (HENDEL et al., 2017), and Zimbabwe (MURASHIKI et al., 2017), and in corn products in Spain (CANO-SANCHO et al., 2012). Occurrence studies of FBs conducted in Brazil showed that all corn samples analyzed from the southern region (n = 148) were contaminated with FB1 and FB2, with levels ranging from 64 to 66,274 μg kg^{-1} (OLIVEIRA et al., 2017). In another study, FBs were detected in 46.6% of the samples (n = 108), with values from 66 to 7,832 μg kg^{-1} for FB1 and 110 to 1,201 μg kg^{-1} for FB2 (SCUSSEL et al., 2014). Of the 50 samples from the state of Minas Gerais, 44% presented contamination with FB1 (PASCHOAL et al., 2016); while 76% of samples from Sao Paulo were contaminated with FB1. Corn based products samples collected in Sao Paulo had FB1 levels ranging from 33 to 1,208, which were all below the limits of Brazilian legislation (1,500 mg kg^{-1}) (BORDIN et al., 2014).

Most of the time, more than two mycotoxins can occur together in grains. In Nigeria, 65% of corn grains had co-occurrence of aflatoxins and fumonisins (ADETUNJI et al., 2014) and in Brazil, a study also showed this co-occurrence (OLIVEIRA et al., 2017). This is a matter of concern, as both fumonisin and aflatoxin are recognized as carcinogenic to humans, which can lead to a mechanism of action of complementary toxicity (BRYDEN, 2007).

5.3.3 Mycotoxin determination

The trend in mycotoxin analysis is the development of multi mycotoxin methods capable of extracting several mycotoxins in a single analysis. An example is the extraction proposed by Anastassiades et al. (2003), known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), successfully used to determine mycotoxins in different matrices such as dried fruits (AZAIEZ et al., 2014; HARTWIG; KUPSKI; BADIALE-FURLONG, 2019), eggs (FRENICH et al., 2011), and cereals (FERNANDES et al., 2015; KUPSKI; BADIALE-FURLONG, 2015). In these approaches, the mycotoxins are extracted with acetonitrile or a mixture of water and ethyl acetate, followed by a partition step after the addition of salts and a cleanup step with dispersive solid phase extraction.

Another technique is matrix solid phase dispersion (MSPD), where a small amount of sample is homogenized with a solid support, then packaged in a cartridge and solvent elute (BARKER; LONG; SHORT, 1989). The elution stage of the sample is commonly conducted in cartridges; however, seeking to reduce the time during packaging and the variations resulting from this process, modifications have been performed, such as vortex assisted MSPD (VA-MPSD). In this modification, after dispersion of the sample with the solid support, the mixture is placed into polypropylene tubes and the elution solvent is added and vortexed (CALDAS et al., 2013). In this technique, sample preparation and cleanup are performed in the same step using small amounts of solid support and solvent, thereby reducing the cost and time of analysis. In addition, it can be used for solid, semi-solid, and highly viscous samples (BARKER, 2007). Despite its advantages, MSPD is not yet a common sample preparation technique in the field of mycotoxins, being limited to a few studies (DE OLIVEIRA et al., 2017; RUBERT et al., 2012; RUBERT; SOLER; MAÑES, 2011; SERRANO et al., 2012).

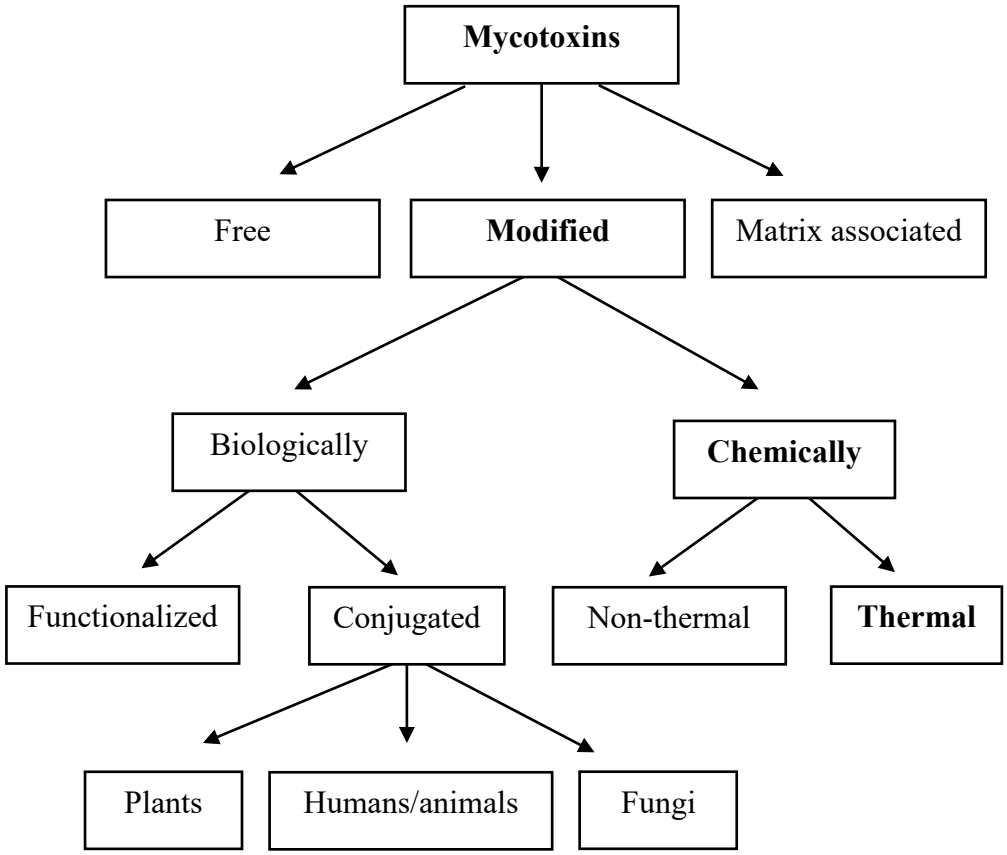
For the detection and quantification of mycotoxins, immunoassay (ELISA) (BHATTACHARYA; BHATTACHARYA; DHAR, 1999), high performance liquid chromatography (HPLC) (JAIMEZ et al., 2000), gas chromatography (GC), and GC or HPLC combined with mass spectrometry techniques are the common approaches (CUNHA; FERNANDES, 2010).

5.3.4 Modified mycotoxin

Mycotoxins, can occur as “free” compounds in food and feed, but can also be converted in various ways into products with altered chemical structures with different

physicochemical, chemical, and biological properties (HUMPF; RYCHLIK; CRAMER, 2019). Rychlik et al. (2014) proposed a definition for modified mycotoxins, which can be classified at a higher level as "free", “matrix-associated”, and “modified” mycotoxins (Figure 4).

Figure 4 – Definition of modified mycotoxins



Source: RYCHLIK et al. (2014)

“Free” mycotoxins include all “unmodified” or parent mycotoxins, which are produced as secondary metabolites by various fungi. “Matrix-associated” mycotoxins include mycotoxins which are covalently or non-covalently bound to matrix compounds such as proteins or starch. Non-covalent interactions, also described as “physical entrapment”, are mediated by hydrogen, ionic, or any other kind of non-covalent binding. Such physical entrapment is especially described for fumonisins and is regarded as analytical issue as it leads to low recovery rates and an underestimation of fumonisin levels (DALL’ASTA; BATTILANI, 2016).

“Modified mycotoxins” normally remain undetected during the testing for free mycotoxin. These modified forms of mycotoxins are called “biologically modified” when produced by fungi or generated as part of the defense mechanism of the infected plant. However, when modified forms occur during food processing they are called “chemically

modified" (HUMPF; RYCHLIK; CRAMER, 2019). The type of process and the composition of the food affect the modified mycotoxin levels present in the final product (FREIRE; SANT'ANA, 2018).

Chemically modified mycotoxins can be forms generated by "thermal" and "non-thermal" reactions. Thermal reactions include baking, roasting, frying, and extruding food processing technologies. During food processing, the tricarballylic acid side chains of fumonisins can react with polysaccharides and proteins leading to covalently bound forms of fumonisins (SEEFELDER; KNECHT; HUMPF, 2003). Covalently bound forms of mycotoxins have been confirmed by Seefelder, Knecht and Humpf (2003) in model experiments. Thermally modified fumonisin occurs in a Maillard-type reaction between fumonisins and reducing sugars forming the N-(1-deoxy-Dfructos-1-yl)-fumonisin B1 (NDF-FB1) and N-(carboxymethyl)-fumonisin B1 (NCM-FB1) (HUMPF; VOSS, 2004). However, some modified mycotoxins, like hydrolyzed fumonisin B1 (HFB1) can be classified into different categories. HFB1 is "chemically modified" as it is formed during nixtamalization, which is a traditional alkaline cooking process of corn to produce masa and tortilla chips (HUMPF; VOSS, 2004). HFB1 can also be classified as "biologically modified" as it was identified together with partially hydrolyzed FB1 as an intestinal metabolite of FB1 in piglets (FODOR et al., 2008).

The reconversions of modified mycotoxins to free forms depend on the nature of the modified compound. Covalently bound fumonisins such as *N*-(deoxy-D-fructos-1-yl)-FB1 (NDF) or *N*-carboxymethyl-FB1 have been found to resist *in vitro* digestive conditions (FALAVIGNA et al., 2012); however, there is evidence that some modified mycotoxins can be converted back to their free forms during digestion. The concentration of modified mycotoxins may exceed the level of free mycotoxin in processed foods (KOVAČ et al., 2018). Even though toxicological data are scarce, the possibility of modified mycotoxin conversion to its free form may result in a potential risk to human and animal health (FREIRE; SANT'ANA, 2018).

5.4 THE FATE OF MYCOTOXINS DURING THE FOOD PROCESSING

Food processing covers all physical, chemical, or biological processes undergone by raw grains in the manufacturing of food products. Processing techniques are commonly used to provide a means to address food safety concerns in foods, including mycotoxins. Several studies have been conducted on the fate of mycotoxins during food processing all over the world, which includes sorting, cleaning, grinding, fermenting, baking, frying, preserving, peeling, alkaline cooking, nixtamalization, and extrusion (BULLERMAN; BIANCHINI, 2007;

KAUSHIK, 2015). Knowing the mycotoxin behavior during processes allows for the adjustment of processing conditions to obtain safe products, which in the case of mycotoxins, considering the values of the legislated maximum permitted levels. It is important to mention that the majority of the guideline levels are for raw material.

5.4.1 Corn milling processes

Milling is one of the oldest forms of food processing, mainly used for corn grains, because they are complex, consisting of many distinctive parts. It is the process of crushing grains to obtain flours, in which the outer structures, the husk, the pericarp, germ, and tip cap are removed to expose the endosperm, which is then used to produce various grinding fractions (RAUSCH et al., 2009). It can be achieved by a dry or wet process.

Dry milling is the removal of the external parts of the grain with minimal disruption of the endosperm by the physical process. The germ and pericarp are removed, which are intended for oil extraction and animal feed, respectively. The internal fraction, endosperm, is intended for the production of grits, flour, breakfast cereal, snacks, hominy, and others (BORDINI et al., 2017; RAUSCH et al., 2009). After the dry milling process, mycotoxins persist and may concentrate or reduce in some fractions. Studies have shown that FBs and AFLAS tend to be concentrated in the germ and pericarp, and a lower concentration was found in the endosperm and its derivatives (BORDINI et al., 2017; BURGER et al., 2013; CASTELLS et al., 2008; GENEROTTI et al., 2015). The bran layer has the potential to act as a physical barrier preventing the mycelia from penetrating further into the kernel structure and the transferring of mycotoxins to the inner part of the kernel (CASTELLS et al., 2008). Furthermore, mycotoxins are produced at the site of fungal growth (YOUNG et al., 1984).

Aflatoxin B1 and B2 were higher in the bran than the germ when the industrial dry milling of corn was evaluated using both conventional and organic corn (BRERA et al., 2006). Contrary to that, Pietri, Zanetti and Bertuzzi (2009) verified higher aflatoxin levels in germ than bran. The variability between these studies could be caused by heterogeneity in the aflatoxin-contamination of corn kernels, besides the location of the fungus in the tip cap and germ areas just beneath the pericarp (KATTA et al., 1997). As a matter of fact, the germ is externally located in the kernel and rich in lipids, which favors the attack of molds and the subsequent mycotoxin production (BRERA et al., 2006). Lillehoj et al. (1976) demonstrated that growth of *Aspergillus flavus* occurs from the external part of the grain to the endosperm.

During wet-milling, clean and dry corn is steeped (hydrated), and processed to obtain pure starch and other byproducts such as germ, protein, and fiber. In the steeping, the grains are immersed in a water solution with 0.1% to 0.2% sulfur dioxide (with sodium metabisulfite added) and lactic acid at temperatures between 45 and 55 °C. These conditions allow the growth of *Lactobacillus sp*., which converts sugars and leach soluble starch from corn kernels. Lactic acid helps to soften the grain and facilitates the entry of sulfur dioxide, which solubilizes and disperses the protein matrix that is bound to starch grains, aiding the separation of these components. The corn kernels are immersed in this solution for 24 to 60 hours to aid starch and protein separation (RAUSCH et al., 2019).

In wet-milling, washing or steeping can reduce mycotoxin levels in contaminated corn, because the mycotoxin could be transferred from the corn to the water during the wet-milling process (PARK et al., 2018). Starch fractions (endosperm) tend to be lower in mycotoxins than the germ and pericarp; this distribution is due to the mostly superficial colonization of intact corn kernels by toxigenic fungi. In damaged kernels, however, the endosperm is much more likely to be colonized, resulting in higher mycotoxin levels in the starch fractions. Dry and wet-milling processing results in a concentration of both fumonisins and aflatoxins in the pericarp and germen, which are widely used in the production of animal feed (SAVI et al., 2016). At the same time, these cereal milling fractions (pericarp and germ) represent a novel category of promising ingredients for human nutrition and health, due to other interesting functional properties (SCHAFFER-LEQUART et al., 2017). The use of whole grains for food products is considered healthier than refined grains, the outer layers of the cereal grains are more likely to be exposed to mycotoxins contaminants.

5.4.2 Thermal processes

The application of heat to cook and preserve products is the basis of all thermal processes. Thermal treatments currently employed by the food industry include ordinary cooking, boiling, baking, frying, roasting, canning, and extrusion. Corn products are consumed after some heat treatment, so the use of this processing is the most applicable strategy to reduce the mycotoxins levels.

5.4.2.1 Cooking

Cooking is the act of preparing food for eating by the application of heat. It encompasses a vast range of methods depending on customs and traditions, the availability and the affordability of the resources (CARMODY; WRANGHAM, 2009). Several studies report on the effect of cooking on mycotoxin levels. Mycotoxin reduction in the cooking process depends on the type and the concentration of the mycotoxin, the heating temperature along with the time employed, the moisture content as well as the degree of heat penetration, pH, and type of food (BECKER-ALGERI et al., 2013; DE GIROLAMO et al., 2016).

Cooking rice contaminated by AFLA B1 resulted in a 34% loss of toxin (JE; LEE; KIM, 2005) and even greater reductions (78–88%) when the grain was cooked under pressure (JE; KIM, 2006). Hydrothermal treatment reduced FB1 contamination in rice by 80% (BECKER-ALGERI et al., 2013); while 23% reduction in fumonisin levels was found in the preparation of salted cornmeal and boiled for 20 min in water (SHEPHARD et al., 2002). In rice hydrothermally treated by electric plate with the addition of salt and oil a 87% reduction was observed for aflatoxins (NOGUEIRA et al., 2019).

Most mycotoxins are chemically and thermally stable. While conventional food preparation at temperatures around 100 °C has little effect on most mycotoxins, higher temperatures used in frying, roasting, baking, and extruding can reduce contamination (KARLOVSKY et al., 2016) or lead to modified mycotoxins (HUMPF; RYCHLIK; CRAMER, 2019).

5.4.2.2 Extrusion

Extrusion cooking, a continuous high-temperature, pressure, and short-time process, is the most versatile processing technology used in the food industry to develop products with better nutritional, functional, and sensory characteristics (DALBHAGAT; MAHATO; MISHRA, 2019). This cooking process applies high pressure, torque, and heat to uncooked cereal to produce snacks, breakfast cereals, and textured foodstuffs (JOZINOVIĆ et al., 2016). The process gelatinizes the starch, degrades the protein, and forms complexes among lipids, starch, and proteins (ARRIBAS et al., 2017).

Extrusion cooking is one of the fastest growing food-processing operations in recent years due to several advantages over traditional methods. Apart from its main goal of improving the quality of intermediate and final processed products, it may incidentally also

improve safety because of the potential to reduce mycotoxin levels in cereals (MOLLA; ZEGEYE, 2014; SOHI SUKHMAN et al., 2015). This reduction depends on different factors such as extruder type, screw type, configuration, initial mycotoxin concentration, barrel temperature, screw speed, moisture content, and use of additives (CASTELLS et al., 2006, 2009). Corn is considered as one of the most suitable cereals for commercial production of extruded products because of the low cost and the good expansion properties due its high starch content (ARRIBAS et al., 2017).

Different levels of aflatoxins reductions are achieved by the extrusion process. Examples include: 75% (B1) and 72.5% (B2) in corn (SOHI SUKHMAN et al., 2015), 93.8% (B1) and 83.5% (B2) in corn-peanut extrudates and flakes (MOLLA; ZEGEYE, 2014), 77.6% (B1) in peanut meal (ZHENG et al., 2015), and only 25% of aflatoxin B1 in corn flour (CAZZANIGA; BASÕ, 2001). The type of food and its composition has a significant effect on the reduction of aflatoxin as a result of the food processing. Additionally, reduction also varies depending on the extrusion processing parameters, such as the moisture, temperature, screw speed, and the initial aflatoxin concentration. The presence of moisture in foods may enhance aflatoxin B1 degradation by hydrolyzing the lactone ring. At a critical moisture concentration and temperature this hydrolytic opening of the lactone ring forms a terminal carboxylic acid, which then undergoes heat-driven decarboxylation to the lesser toxic aflatoxin D1 (SAMARAJEEWA et al., 1990). Castells et al. (2006), when extruding artificially contaminated rice meal, observed that the moisture content had a significant influence ($p < 0.05$) on reducing aflatoxins B2, G1, and G2 whereas it was not a significant factor for aflatoxin B1 reduction. Zheng et al. (2015), when extruding contaminated peanut meal with aflatoxin B1, showed that the barrel temperature and moisture have significant influences ($p < 0.01$) on the degradation rate of aflatoxin, while the screw speed has no significant influence.

Extrusion was effective in reducing fumonisins in corn grains by 34–95%, and the highest reduction rates resulted from temperature increase, screw speed decrease, and glucose addition (BULLERMAN; BIANCHINI, 2007). Some authors showed that the extrusion processing efficacy can be further improved by the addition of reducing sugars (JACKSON et al., 2011) and sodium chloride (CASTELLS et al., 2009). However, results have shown that this decrease could be ascribed to possible modifications of the mycotoxin structure by interaction with other food components leading to the formation of conjugates (FALAVIGNA et al., 2012). Kovač et al. (2018) showed some evidence concerning mycotoxins masked/modified by technological processes, especially in cereal-based products.

Some authors have suggested that fumonisins may react with some components of the food matrix during technological processes (HUMPF; VOSS, 2004). FB1 reacts with glucose and other reducing sugars to form N-(deoxy-D-fructos-1-yl) FB1 and N-carboxymethyl FB1 compounds (HOWARD et al., 1998). Park et al. (2004) detected protein-bound FB1 in corn flakes and corn-based breakfast cereals, and another study demonstrated that FB1 is also able to bind to proteins and starch compounds via their two tricarballylic acid side chains (SEEFELDER; KNECHT; HUMPF, 2003).

FB1 is a reactive compound, its structure is a diester of propane-1,2,3-tricarboxylic acid (TCA) and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxy-icosane, in which the C-14 and C-15 hydroxyl groups form esters with the terminal carboxy group of TCA. The FB1 amino groups and TCA groups facilitate interactions with other compounds, resulting in various modified forms of fumonisin (FALAVIGNA et al., 2012). Additionally, the TCA side chains are commonly cleaved forming the hydrolyzed FB (DE GIROLAMO et al., 2016).

Physical treatment such as extrusion or cooking applied to food can seldom completely eliminate mycotoxins from a food product and their perceived disappearance due to the lack of detection does not necessarily mean detoxification. Upon processing, structural modification or conversion into a "modified" forms may occur (KARLOVSKY et al., 2016). As a consequence of the masking phenomenon, the total concentration of mycotoxin in processed corn-based food could be underestimated and certain “modified” mycotoxins forms may be hydrolyzed into their free forms upon ingestion. Therefore, further research is needed to fully clarify their fate upon ingestion (KOVAČ et al., 2018).

5.5 BIOACCESSIBILITY

Bioaccessibility is defined as the fraction of a compound that is released from the food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption. This is the first process to determine the oral bioavailability of a compound, the second stage is the intestinal transport (transport through the intestinal epithelium to other organs), and the last stage is the metabolism (degradation of the compound in the liver and intestine) (VERSANTVOORT et al., 2005). Therefore, the release of the contaminant from the ingested product in the gastrointestinal tract is a prerequisite for absorption and bioavailability in the body, so the oral bioavailability of compounds present in the food may be significantly different depending on the food product, food processing, or preparation (VAN HET HOF et al., 2000).

The process of digestion of a compound within the digestive system begins in the mouth, where food is ground and, due to the action of salivary amylase, starch is converted into maltose. Afterwards, the bolus goes toward the stomach, which due to the production of hydrochloric acid has high acidity, facilitating the conversion of pepsinogen to pepsin, which hydrolyzes proteins to amino acids, oligopeptides, and polypeptides. After a portion of the acidified food is digested in the stomach, it is directed to the small intestine, where enzymes and fluids are added to the chyme via the pancreatic canal and the liver. The action of bicarbonate, secreted by the pancreas, neutralizes the acidified chyme, preventing damage to the intestinal mucosa. The intestinal epithelium, together with the pancreas, secretes fluids containing enzymes (amylase, lipase, protease, peptidase) that act on the digestion of starch, proteins, carbohydrates, and fats. The liver adds bile, which is a complex mixture of bile salts, and agents that aid in the digestion of fat. At the end of the process, there is the absorption in the small intestine of the products generated by this sequence of enzymatic breaks, as well as the absorption of contaminants such as mycotoxins that were free to be absorbed. Water is absorbed in the large intestine and soon after, there is defecation. After this complex digestion process, the contaminant may be absorbed, metabolized (SILVERTHORN, 2017), and may cause a toxic effect on the body.

The food matrix primarily affects bioaccessibility, while absorption and metabolism depend more on the specific properties of the compound and on the physiology of the animal (BRANDON et al., 2006).

The second bioavailability process, intestinal transport, can be evaluated using dialysis membranes under physiological conditions that simulate the intestinal environment (pH). The human small intestine has three segments: the duodenum (25 to 30 cm), the jejunum (2 m), and the ileum (3 to 4 m), an absorptive area of approximately 100,000 cm^2, without considering the area of the microvilli (EKMEKCIOGLU, 2002). Absorption in the small intestine preferably occurs in the duodenum and proximal portion of the jejunum. The high absorption rate centralized in this region occurs because this intestinal surface has certain peculiarities such as: kerckring folds, villi, and microvilli that facilitate the nutrient absorption process (BRANDON et al., 2006).

About 90% of all nutrient absorption occurs in the small intestine, the other 10% in the stomach and large intestine. Any undigested or unabsorbed material left in the small intestine passes into the large intestine (EKMEKCIOGLU, 2002).

In vitro digestion models simulate, in a simplified way, the process of human digestion at oral, gastric, and intestinal levels, allowing the determination of bioaccessibility of

food components from their matrix during gastrointestinal transit. Versantvoort, Van de Kamp and Rompelberg (2004) developed an *in vitro* digestion model to measure the bioaccessibility of contaminants. Since that, this digestion model has been applied by many scientists to measure the bioaccessibility of mycotoxins ingested from peanut slurry (VERSANTVOORT et al., 2005), corn flakes (MOTTA; SCOTT, 2007, 2009), peanut, pistachio, hazelnut, dried figs, paprika, wheat, corn (KABAK; OZBEY, 2012), cooked contaminated pasta (RAIOLA et al., 2012), processed cereals for children (ASSUNÇÃO et al., 2016), bread (SALADINO et al., 2018), chicken breast muscles (SOBRAL et al., 2019), and corn after cooking (LIN et al., 2019). Furthermore, the *in vitro* digestion model has been used to evaluate the stability of modified mycotoxins in the human digestive system (FALAVIGNA et al., 2012).

CHAPTER III

WORK DEVELOPMENT

WORK OVERVIEW

The study “The fate of aflatoxins and fumonisin B1 during the processing of corn: a bioaccessibility approach” was performed in the Laboratory of Science and Mycotoxins in partnership with other laboratories of the School of Chemistry and Food, and the Analytical Center at the Federal University of Rio Grande. It was also developed in part in the Department of Food Science and Technology, at the University of Nebraska – Lincoln. Here, it was presented in eight main chapters, where experimental parts of chapters 1–3 and 5–6 where conducted at FURG and those from chapters 4, 7, and 8 at the University of Nebraska.

1. Optimization of matrix solid-phase dispersion method for extraction of aflatoxins from cornmeal.
2. Simultaneous distribution of aflatoxins B1 and B2 and fumonisin B1 in corn fractions during dry and wet-milling.
3. Particle size and physical-chemical characteristics of hydrothermally treated cornmeal on resistant starch content.
4. Resistant starch in cooked cornmeal-based food.
5. Increasing the resistant starch content in corn-based products through extrusion.
6. Resistant starch and hydrothermal treatment of cornmeal: factors in aflatoxins and fumonisin B1 reduction and bioaccessibility.
7. Fate of aflatoxins in cornmeal during single-screw extrusion: a bioaccessibility approach.
8. Stability of fumonisin B1 and its bioaccessibility in extruded corn-based products.

ARTICLE I

OPTIMIZATION OF MATRIX SOLID-PHASE DISPERSION METHOD FOR EXTRACTION OF AFLATOXINS FROM CORNMEAL

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Optimization of matrix solid-phase dispersion method for extraction of aflatoxins from cornmeal

ABSTRACT

An extraction method for simultaneous determination of aflatoxins (AFLAs) G2, G1, B2, and B1 in cornmeal, based on vortex-assisted matrix solid-phase dispersion (MSPD) and high-performance liquid chromatography (HPLC) with fluorescence detection was optimized by a central composite design, validated and applied. Multivariate analysis was performed to evaluate the effect of cornmeal composition on AFLA extraction. The amount and proportion of solid support (celite and C18) and volume of elution solvent (methanol and acetonitrile) were the variables tested. The mobile phase of methanol:acetonitrile:water (24:14:62, *v/v/v*) in isocratic elution mode provided satisfactory AFLA separation. The best recoveries (85.7% to 114.8%) were obtained when the sample preparation contained 25 mg C18 as solid support and 10 mL of elution solvent. The limits of detection ranged from 0.01 to 0.04 ng g^{-1}, and the limits of quantification varied from 0.02 to 0.1 ng g^{-1}. The optimized method was suitable for coarse and medium grind cornmeal. Multivariate correlation analysis showed that the main interferers for AFLA recovery were proteins and sugars.

Keywords: Interferers. Multivariate correlation. Recovery. Solid support.

1 INTRODUCTION

Corn (*Zea mays* L.) is the third most cultivated cereal on the planet. One of the main problems of the corn production chain is the susceptibility to contamination by toxigenic fungi, which can produce mycotoxins under stress conditions (WANG et al. 2016). Among the mycotoxins that can contaminate corn (MUTIGA et al., 2015; OLIVEIRA et al., 2017) and corn flours (ALBORCH et al., 2012; ALGÜL; KARA, 2014) are the aflatoxins (AFLAs).

AFLAs are the most toxic compounds produced by fungi and display strong immunosuppressive, mutagenic, teratogenic and carcinogenic effects (EFSA, 2007). AFLA B1 has been reported to be the most toxic of the AFLAs and has been classified as a group 1 (human) carcinogen by the International Agency for Research on Cancer (IARC, 2012). To protect consumers, many countries have set stringent regulations about the level of AFLAs permitted in food commodities. European Union legislation has mandated maximum limits of 5 μg kg^{-1} for AFLA B1 and 10 μg kg^{-1} for the sum of AFLAs G2, G1, B2 and B1, in corn and corn products (EUROPEAN COMMISSION, 2006). In Brazil, a level of 20 μg kg^{-1} has been established as the limit for corn contamination with AFLAs (G2, G2, B1 and B2) (ANVISA 2011).

In order to evaluate exposure to AFLAs, reliable data on their occurrence in various corn products are required. The development of multi-mycotoxin methods able to detect, in a single analysis, several mycotoxins, thereby promoting the lowest impact on the environment, is a trend in mycotoxin analysis (KARAMI-OSBOO; MIRABOLFATHI, 2017; ZACHARIASOVA et al., 2010). A promising alternative is the matrix solid-phase dispersion (MSPD) technique, which is little explored for mycotoxins (HU et al., 2006; MANOOCHEHRI; ASGHARINEZHAD; SAFAEI, 2015), especially for corn and its products. Capriotti et al. (2013) highlighted that the key factors for the success of MSPD are its feasibility, flexibility, versatility, low costs and rapidity.

In MSPD, a small amount of sample and solid support are mixed homogenously; the powder obtained is then eluted with a solvent (CALDAS et al., 2013; KRISTENSON; RAMOS; BRINKMAN, 2006). In this technique, sample preparation and cleaning are performed in the same step, using small amounts of solid support and solvent and thus reducing the cost and time of analysis. In addition, the use of a vortex instead of a column for the elution step protects the analyst from solvent exposure and sample handling, contributing to green chemistry strategies. The efficiency and selectivity of the extraction process are conditioned by the solid support characteristics, sample/solid support ratio, solvents and sample/solvent ratio,

as well as the concentration and chemicals properties of the analytes (CAPRIOTTI et al., 2010). The choice of the solid support depends on the analyte polarity and the potential co-extracted components of the matrix. Several solid supports are available (DE OLIVEIRA et al., 2017; RUBERT; SOLER; MAÑES, 2011), however C18 is the most used for mycotoxin analysis in cereals because its lipophilic characteristic allows good disruption, dispersion and retention of lipophilic species (BARKER, 2007; RUBERT; SOLER; MAÑES, 2012; SERRANO et al., 2012). There is no information in the literature about the effect of sample particle size in the MSPD method performance, unlike that for the dispersant (CAPRIOTTI et al., 2013).

This study contributes to the adaptation of a fast and eco-friendly analytical extraction method for simultaneous determination of AFLAs G2, G1, B2 and B1 in cornmeals, consuming few reagents with no effect on sensitivity and analytical performance. The method has been validated according to SANTE/11945/2015 guidelines (SANTE 2016). Besides, the applicability of the validated method in further matrices, to determine the impact of particle size on the sample recoveries, as well as an investigation identifying the interferences in AFLAs extraction, using multivariate correlation analysis, were studied.

2 MATERIAL AND METHODS

2.1 REAGENTS AND SAMPLES

The AFLAs (G2, G1, B2 and B1) standards (purity >98%) were supplied by Sigma–Aldrich and the working standard solutions were prepared by diluting the standards with toluene:acetonitrile (MeCN) (98:2, *v/v*). After preparation, the mycotoxin standards were dried under nitrogen and stored at -18 °C, to ensure their stability. The working solutions were prepared from stock solutions, which were quantified before use in a spectrophotometer, according to the AOAC (2000). The solvents (purity >99.9%) used as mobile phase in the chromatographic system (MeCN and methanol [MeOH]) were supplied by JT Baker and were passed through a 0.45 m cellulose filter. Ultrapure H_2O (>18.2 MΩ cm^{-1} resistivity) was purified using a Milli-Q® SP Reagent Plus water system (Millipore Corp., Bedford, USA). The mobile phase solvents were previously degassed in an ultrasonic bath.

Cornmeals (fine, medium and coarse) were purchased commercially from supermarkets located in the city of Rio Grande (Brazil). The corn used for the wet- and dry-milling processes was purchased directly from farmers of Parana State (Brazil).

2.2 CHROMATOGRAPHIC CONDITIONS

The AFLA standards were dissolved in 1 mL of ultrapure H_2O:MeCN (90:10, *v/v*) and 20 µL was injected into a high-performance liquid chromatography system coupled with fluorescence detection (HPLC-FD) and on-line post-column photochemical derivatization (Romer Derivatization Unit RDU^{TM}), which pre-excites the analytes with UV light at 254 nm. The chromatographic elution from a Kromasil C18 column (5 µm, 150 mm × 4.6 mm) was performed at 40 ºC, at a flow rate of 1.0 mL min^{-1} and the excitation and emission wavelengths were set at 365 and 440 nm, respectively. The separation of the AFLAs was evaluated using different proportions of the mobile phase (ultrapure H_2O, MeCN and MeOH) and the best condition was defined based on retention (k) and separation (α) factors.

The method limit of detection (LOD) and limit of quantification (LOQ) for each AFLA were obtained considering 3 and 10 times the signal-to-baseline (noise) ratio, respectively. The linearity was evaluated through standard calibration curves in the concentration range of the LOQ of each AFLA to a concentration equivalent to 100-fold the LOQ value.

2.3 PRELIMINARY TESTS

Initially, three AFLA extraction methods were tested (Table 1) and after, a control sample (without solid support) was tested for method 1 (RUBERT; SOLER; MAÑES, 2011). The method that provided the greatest recovery of the four AFLAs was studied, using the central composite design (CCD).

Table 1 - Evaluation of extraction methods for AFLAs.

Method	Sample amount (g)	Solvent	Salts	Sorbent
1	1	20 mL MeCN:MeOH (50:50, v/v)	-	1 g C18
2	5	20 mL MeCN:H_2O (84:16, v/v, with 1% acetic acid)	$MgSO_4$:NaCl (2:0.5 w/w)	0.03 g C18
3	10	25 mL MeOH:MeCN:H_2O (60:20:20, v/v/v)	$MgSO_4$:NaCl (2:1.5 w/w)	0.03 g C18

1: Rubert, Soler and Mañes (2011); 2: Wang et al. (2016); 3: Paschoal et al. (2016)

2.4 OPTIMIZATION OF AFLAS EXTRACTION

The experiments were performed according to Rubert, Soler and Mañes (2011). Aliquots of 1 g of the sample (cornmeal) were transferred to a mortar (30 mL capacity), spiked with the standard solution and, after solvent evaporation, gently homogenized with a pestle, using different amounts and proportions of the solid support (Table 2) for 5 min to homogeneity. The mixture was poured into a polypropylene centrifuge tube; different volumes of MeCN:MeOH (50:50, *v/v*) were added, and the content was thoroughly vortexed for 3 min. The tubes were centrifuged at 3220 *g* for 10 min, the extract was collected, dried at 60 °C, dissolved in 1000 µL of ultrapure H_2O:MeCN (90:10, *v/v*), and injected into the HPLC-FD. The variables, namely the amount and solid support proportion (celite and C18), and volumes of extractor solvent (MeOH and MeCN) were defined using a 2^3 CCD, where AFLA recovery was the response variable. Medium cornmeal spiked with 4 ng g^{-1} of AFLAs mixture (1.0 ng g^{-1} G1 and G2, 0.6 ng g^{-1} B2 and 1.4 ng g^{-1} B1) was used in the optimization.

2.5 METHOD VALIDATION

The accuracy of the method was evaluated regarding the recovery assays, in compliance with SANTE/11945/2015 (SANTE 2016) and ANVISA (2003). Aliquots of 1 g of the sample (cornmeal) were spiked with standard AFLAs at three levels for each AFLA. The levels of fortification were a concentration equivalent to the LOQ, 5-fold LOQ and 10-fold LOQ for AFLA G2, and 2-, 10- and 20-fold LOQ for AFLA G1, B2 and B1, respectively. Each fortification level was extracted in triplicate and injected three times (n=9).

The precision of the method was evaluated regarding the repeatability, with nine determinations; extraction of the sample by MSPD was carried out at three different fortification levels, in triplicate.

The study of the matrix effect (ME) was performed according to equation 1, by comparing the slopes in matrix-matched calibration solutions prepared in blank cornmeal extract and calibration solutions prepared in solvent. The extent of the effects due to the matrix components was rated according to the percentage of signal enhancement (+) or suppression (-).

$$\text{ME (\%)} = 100 \times \left(1 - \frac{Sm}{Ss}\right) \quad (1)$$

where *Ss* is the slope in solvent, and *Sm* is the slope in matrix. No ME is observed when the ME (%) is equal to 100%. Values above 100% indicate enhancement, and values below 100% mean suppression.

2.6 APPLICABILITY OF THE METHOD

For the application of the validated method in other matrices (fine and coarse commercial cornmeals, and cornmeals obtained by dry- and wet-milling,), the validation was carried out at a concentration of 10-fold LOQ for G2 and 20-fold LOQ for the other AFLAs.

Dry-milling of the corn was performed according to Somavat et al. (2016), and wet-milling was achieved based on Malumba et al. (2015). In the dry-milling, cornmeals of fine, medium and coarse granulometry were obtained, and in the wet-milling, only fine cornmeal was prepared. Table 3 provides the granulometric profile of the different cornmeals.

Table 3 - Granulometric profile of cornmeals.

Particle sizes	**Flours**		
	Coarse	**Medium**	**Fine**
Between 0.71 and 0.50 mm (%)	8.5 (0.1)	0	0
Between 0.50 and 0.355 mm (%)	70.1 (0.0)	26.0 (0.1)	11.2 (0.1)
Between 0.355 and 0.25 mm (%)	20.4 (0.0)	32.7 (0.0)	54.1 (0.0)
Between 0.25 and 0.147 mm (%)	0.6 (0.4)	21.1 (0.0)	18.8 (0.1)
Smaller than 0.147 mm (%)	0.4 (0.4)	20.2 (0.1)	15.9 (0.1)

Results expressed as mean (RSD) n=3. RSD= relative standard deviation.

All flours were characterized for protein, ash and lipid contents (AOAC 2000); sugar was determined by the phenol–sulfuric acid method (DUBOIS et al., 1951), and the total starch was measured by iodometry, using a starch calibration curve.

2.7 STATISTICAL ANALYSES

Statistical analysis was performed using Statistica 7.0 software at 95% significance level, considering the recovery of the four AFLAs as a response to the variables studied. The experimental plans were obtained from the 2^3 CCD, from which, a set of combinations were experimentally evaluated. The experimental results were used to find a statistical mathematical

model, as a function of all the influenced factors. Multivariate analysis was also performed to establish correlations between the different components of the matrices and the recovery of AFLAs by the MSPD method using HPLC-FD.

3 RESULTS AND DISCUSSION

3.1 CONDITIONS FOR SIMULTANEOUS AFLA G2, G1, B2 AND B1 DETERMINATION

Different ratios of mobile phase were tested in the chromatographic system, with the aim of achieving excellent separation of the AFLAs and an efficient elution time. The three proportions of H_2O, MeCN and MeOH allowed obtaining a short elution time of around 15 min. Both, the retention (*k*) and separation (α) factors were estimated, to decide the best proportion of the elution components (Table 4).

Table 4 - Chromatographic parameters from AFLAs separation in different proportions of mobile phase

H_2O: MeCN: MeOH	**Retention time (t_r) (min)**				**Retention factor (k)**				**Separation factor (α)**		
	G2	**G1**	**B2**	**B1**	**G2**	**G1**	**B2**	**B1**	**G2-G1**	**G1-B2**	**B2-B1**
62:20:18	7.0	8.3	9.2	11.2	1.51	2.00	2.31	3.03	1.32	1.15	1.31
62:13:25	5.9	6.9	7.2	8.6	1.68	2.14	2.28	2.93	1.27	1.07	1.29
62:14:24	7.5	8.9	10.2	12.5	2.44	3.13	3.69	4.75	1.28	1.18	1.29

The separation factor (α) shows the selectivity of the chromatographic system in relation to the four AFLAs that elute at adjacent peaks, and values higher than 1 are recommended. Both parameters (*k* and α) indicated that the column and the H_2O:MeCN:MeOH (62:14:24, *v/v/v*) mobile phase allowed good separation of the four AFLAs (Fig. 2a) in 14 min, which is suitable for the routine application of the four AFLAs determinations concomitantly.

After the AFLA separation, the LOD and LOQ of the instrument, and the analytical curves of the standards were determined (Table 5). Results showed the chromatographic procedure was adequate for quantification of the AFLAs, with correlation coefficients for all calibration curves higher than 0.999. The instrument LOQ for AFLA G2 was the same as that found by Telles, Kupski and Badiale-Furlong (2017) (0.05 ng mL^{-1}). However, the LOQs of AFLA B1 and B2 were 0.04 and 0.01 ng mL^{-1}, respectively, which are lower than those verified

by the mentioned authors (2.00 and 0.03 ng mL^{-1}). Our study applied a detector equipped with post-column photochemical derivatization, which, as stated above, pre-excites the analytes with UV light at 254 nm. Therefore, the instruments LOQs are satisfactory to determine the four AFLAs, and the linearity shows a wide application range.

Table 5 - Analytical parameters of AFLAs G2, G1, B2 and B1 in HPLC-FD

Analytical parameters	**G2**	**G1**	**B2**	**B1**
Analytical curve	y=43181x +520.73	y=119472x +1040	y=211335x +1453.1	y=49838x -658.76
Linearity (ng mL^{-1})	0.05 – 5	0.02 - 4	0.01 - 2	0.04 - 6
Correlation coefficient (R)	0.9999	0.9999	0.9999	0.9999
Determination coefficient (R^2)	0.9999	0.9999	0.9999	0.9999
LODi (ng mL^{-1})	0.02	0.01	0.005	0.02
LOQi (ng mL^{-1})	0.05	0.02	0.01	0.04

LODi: instrument limit of detection; LOQi: instrument limit of quantification

3.2 PRELIMINARY TESTS

The highest recoveries values for AFLAs G2, G1, B2 and B1 (83.4%–102.5%) were achieved by method 1 (Tables 1 and 6).

Table 6 - AFLAs extraction conditions and their recoveries.

AFLAs	**Recoveries (%)**		
	Method 1*	**Method 2***	**Method 3***
G2	99.5 (18.5)	90.3 (5.0)	48.9 (1.4)
G1	83.7 (4.7)	41.8 (2.9)	36.3 (7.7)
B2	102.5 (17.5)	88.8 (1.4)	81.4 (0.2)
B1	83.4 (2.5)	71.5 (7.8)	2.3 (27.0)

Results expressed as mean (RSD). n=3 RSD= relative standard deviation
*1: Rubert, Soler and Mañes (2011); 2: Wang et al. (2016); 3: Paschoal et al. (2016)

Acceptable recoveries (94.4%–112.5%) were obtained by the method that did not use the solid support. In that procedure, the structure of the physical sample acted as an abrasive

and promoted the disruption of the matrix sample, providing extraction of AFLAs, but the cleanliness of the extracts was not satisfied, so it was necessary to add a solid support to eliminate some interfering components during the extraction. Method 1 (RUBERT; SOLER; MAÑES, 2011) was subject to a CCD, to improve the recoveries and reduce the amount of solvent and solid support used. In addition, a different solid support (celite) was evaluated to substitute the traditional and costly, C18.

3.3 OPTIMIZATION OF AFLAS EXTRACTION CONDITIONS

Table 2 presents the AFLA recoveries (%) in the CCD for the three variables under study (solvent volume, solid support amount, C18:celite ratio). In this design, the AFLA recoveries were in the range 43.0–113.3% (G2), 36.4–107.6% (G1), 51.7–122.8% (B2) and 38.3–100.0% (B1). For the four AFLAs, trials 1, 2, 5 and 6 demonstrated the best results, all of which showed less solid support amount, at level -1 (0.1 g), solvent volume at level -1 (10 mL) or +1 (30 mL), and C18:celite proportion at level -1 (0:1) or +1 (1:0), than the other trials.

Table 2 - CCD 23 (coded and real values) and the responses AFLAs recoveries (%).

Trial	X_1	X_2	X_3	G2	G1	B2	B1	AFLAs
1	-1 (10)	-1 (0.1)	-1 (0:1)	107.8	100.2	114.7	95.7	104.6
2	+1 (30)	-1 (0.1)	-1 (0:1)	102.9	99.6	113.4	94.7	102.7
3	-1 (10)	+1 (1.0)	-1 (0:1)	43.0	36.4	51.7	38.3	42.4
4	+1 (30)	+1 (1.0)	-1 (0:1)	46.2	37.6	69.5	45.4	49.7
5	-1 (10)	-1 (0.1)	+1 (1:0)	113.3	107.1	115.7	93.8	107.5
6	+1 (30)	-1 (0.1)	+1 (1:0)	110.5	107.6	122.8	100.0	110.2
7	-1 (10)	+1 (1.0)	+1 (1:0)	112.7	107.3	108.7	84.6	103.3
8	+1 (30)	+1 (1.0)	+1 (1:0)	96.4	92.6	103.0	81.1	93.3
9	0 (20)	0 (0.45)	0 (0.5:0.5)	59.7	46.2	89.9	66.4	65.6
10	0 (20)	0 (0.45)	0 (0.5:0.5)	74.0	58.1	93.8	72.2	74.5
11	0 (20)	0 (0.45)	0 (0.5:0.5)	66.8	52.2	91.8	69.3	70.3

X_1: Solvent volume (mL); X_2: Solid support amount (g); X_3: Proportion of C18:celite; AFLAs: mean recoveries the four AFLAs

The main effect can be estimated by evaluating the difference in process performance caused by a change from low (-1) to high (+1) levels (HAALAND, 1989), which provides a model that fulfills the recovery of the four AFLAs. Therefore, the main effect was

assessed using the average of the recoveries of the four AFLAs, and the *p*-value was used to verify the significance of the factors under study. The solid support amount was identified as the most relevant variable for the AFLA recoveries. Increasing the solid support amount from 0.1 to 1 g, decreased the AFLAs recoveries by an average of 34.1%. An alteration in the ratio of C18:celite, from 0:1 to 1:0, led to an average increase of 28.8% in the AFLA recoveries. The combined effect of solid support amount and their proportion (C18:celite) resulted in an average increase of 23.5% in the AFLA recoveries. The solvent volume, as well as their combinations with other variables, did not present any significant effect. Variance analysis (ANOVA) was performed, using the significant effects only (Table 7).

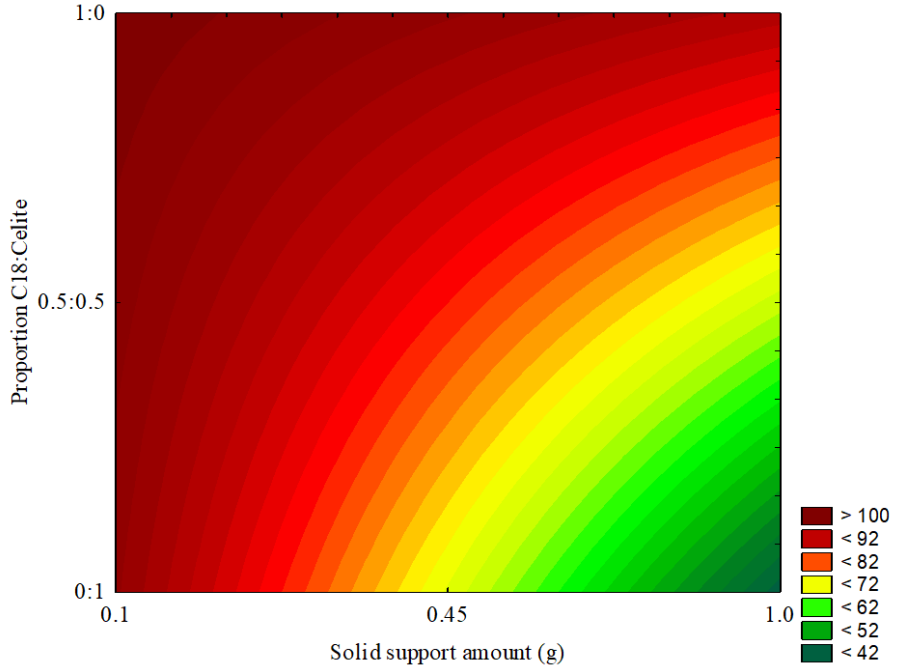
Table 7 - ANOVA for AFLAs recoveries for CCD

Variation	Sum of squares	Degrees of freedom	Means squares	*F*-ratio
Regression	5086.45	3	1695.48	12.84[a]
Residual	924.57	7	132.08	
Total	6011.02	10		

Regression coefficients= 0.85; F 0.95; 7; 3 = 4.34; [a]*F*-ratio (regression/residual).

Based on the F-test, the model proved predictive, since its $F_{calculated}$ (12.84) was greater than $F_{tabulated}$ (4.34), and significant, given the regression coefficient (0.85) was close to unity. The coded model was used to generate the contour diagram (Figure 1).

Figure 1 - Contour diagram of AFLAs recoveries as a function of amount and ratio of C18 and celite.



AFLA recovery (%) = 83.9 - 34.0.X_2 + 28.7.X_3 + 23.5.X_2.X_3 (2)

To determine the optimal values for the significant variables (X_2, X_3 and their interaction), the first derivative of Equation 2 was obtained, and the optimal values of 25 mg of the solid support (X_2) and 1:0 the ratio of C18:celite (X_3) were obtained. Under these conditions and setting a solvent volume of 10 mL, the model validation was performed, resulting in a 103.3% experimental result and a predictive of 125.5%, with a deviation of 21.5% from the experimental to the predictive value. These validated conditions were used for analytical method validation and applicability of the method in different cornmeals.

3.4 METHOD VALIDATION

The method LOD and LOQ were 0.01–0.04 and 0.02–0.1 ng g^{-1}, respectively (Table 8). These values were lower than those verified by Quinto et al. (2009), of LOD 0.035–0.2 ng g^{-1} and LOQ 0.1–0.63 ng g^{-1} for solid-phase microextraction and quantification of AFLAs in cereal flours by HPLC-FD with post-column photochemical derivatization.

Table 8 - Indicative of merit for AFLAs G2, G1, B2 and B1 extraction method in medium cornmeal

AFLAs	LODm* (ng g^{-1})	LOQm* (ng g^{-1})	Concentration (ng g^{-1})	Recovery (%)
G2	0.04	0.10	0.10	114.8 (4.1)
			0.50	107.8 (9.9)
			1.00	98.4 (1.3)
G1	0.02	0.05	0.08	106.2 (12.6)
			0.40	85.7 (9.6)
			0.80	89.3 (2.8)
B2	0.01	0.02	0.04	94.1 (7.3)
			0.20	91.3 (1.0)
			0.40	88.5 (2.8)
B1	0.04	0.07	0.12	86.9 (12.2)
			0.60	109.5 (19.9)
			1.20	86.7 (6.6)

Results expressed as mean (RSD). RSD=relative standard deviation. *LODm: method limit of detection. *LOQm: method limit of quantification.

Similarly, they were also lower than those documented by Rubert, Soler and Mañes (2011), of LOD 0.1–1 ng g^{-1} and LOQ 0.25–1.5 ng g^{-1} for MSPD extraction and quantification

of AFLAs in cereal flours by HPLC-mass spectrometry (MS). In another study, Rubert, Soler and Mañes (2010) optimized the extraction conditions of cereals, resulting in 0.3–0.4 ng g^{-1} for the LOD and the LOQ in the range of 1 ng g^{-1}.

Paschoal et al. (2017) optimized and applied a solid–liquid extraction of AFLAs from cornmeal using MeOH:H_2O:MeCN (60:20:20, *v/v/v*) prior to HPLC-MS quantification, and the LOD ranged from 0.24 to 0.32 ng g^{-1} and the LOQ from 0.80 to 1.05 ng g^{-1}. According to Pereira, Fernandes and Cunha (2014), it is possible to obtain a LOD for HPLC-FD comparable to or better than those achieved by HPLC-tandem MS. Therefore, as the proposed method showed low values of LOD and LOQ, this is an advantage when it is desired to determine trace compounds, such as mycotoxins.

The recoveries at the three levels for the four AFLAs ranged from 85.7% to 114.8% (Table 8), which are within the criteria approved by the European Regulatory Committee. For the acceptance of analytical methods for the AFLAs, it was established that for concentrations of less than 1 μg kg^{-1}, the recoveries should range between 50% and 120%, and for concentrations between 1 and 10 μg kg^{-1}, the recoveries should vary between 70% and 110% (EUROPEAN COMMISSION, 2006).

Rubert, Soler and Mañes (2010) reported recoveries ranging from 64% to 91% with the relative standard deviation (RSD) <19% for MSPD extraction and HPLC-MS quantification of AFLAs in various cereals. In 2011, the same authors extracted 19 mycotoxins from cereal flours by MSPD and acquired AFLA recoveries between 68.8% and 80.3% (RSD <12%). The elution step used in our work was by vortex, whereas the other authors packed the mixture (sample and solid support after maceration) in cartridges and then eluted with solvent. In the vortex-assisted MSPD method proposed in this work, after dispersing the sample with the solid support, the mixture is placed in tubes, and the elution solvent is added, followed by vortexing. This elution form has the advantage of reducing the packing time and the variations resulting from this process.

Repeatability represents the agreement between the results of successive measurements of the same method, conducted under the same measurement conditions, called repeatability conditions: same procedure; same analyst; the same instrument used under the same conditions; same location; repetitions in a short interval of time. The RSDs were <20% and thus conformed to the accepted limits for the concentrations used in the repeatability test (EUROPEAN COMMISSION, 2006).

The ME was studied to verify if the matrix interferes with the signal of the contaminant at interest. For AFLAs G2 and G1, the ME was close to 20% (18.2% and 20.1%,

respectively) and for B2 and B1, this value was about 11% (11.1% and 11.8%, respectively). According to SANTE/11945/2015 (SANTE, 2016), results that show a 20% ME are considered acceptable for analyzes of contaminants in foods at trace level. Therefore, due to the low ME values for the four AFLAs, the quantification of the levels of the AFLAs in the samples can be undertaken, using the curve in the solvent. Moreover, this acceptable effect also avoided the false positive results.

The chromatograms in Figure 2 represent the instrument LOD (Figure 2a), the blank sample (medium cornmeal; Figure 2b), and the cornmeal matrix fortified with G2, G1, B2 and B1, at 3.75, 3.0, 1.5 and 4.5 ng mL^{-1}, respectively (Figure 2d). It can be observed that the interferers are present at the start of the chromatogram and, after 5 min, only the AFLAs at the same retention times as the standards are seen (Figure 2c), which shows that the extraction and detection method is reliable to predict the contamination levels of these mycotoxins in cornmeal.

Figure 2 – Chromatogram of AFLAs G2, G1, B2 and B1 limit of detection (a), blank sample (b), standards (c) and chromatogram of standard in the extract of medium cornmeal, G2 3.75 $ng.mL^{-1}$, G1 3.0 $ng.mL^{-1}$, B2 1.50 $ng.mL^{-1}$ and B1 4.5 $ng.mL^{-1}$ (d)

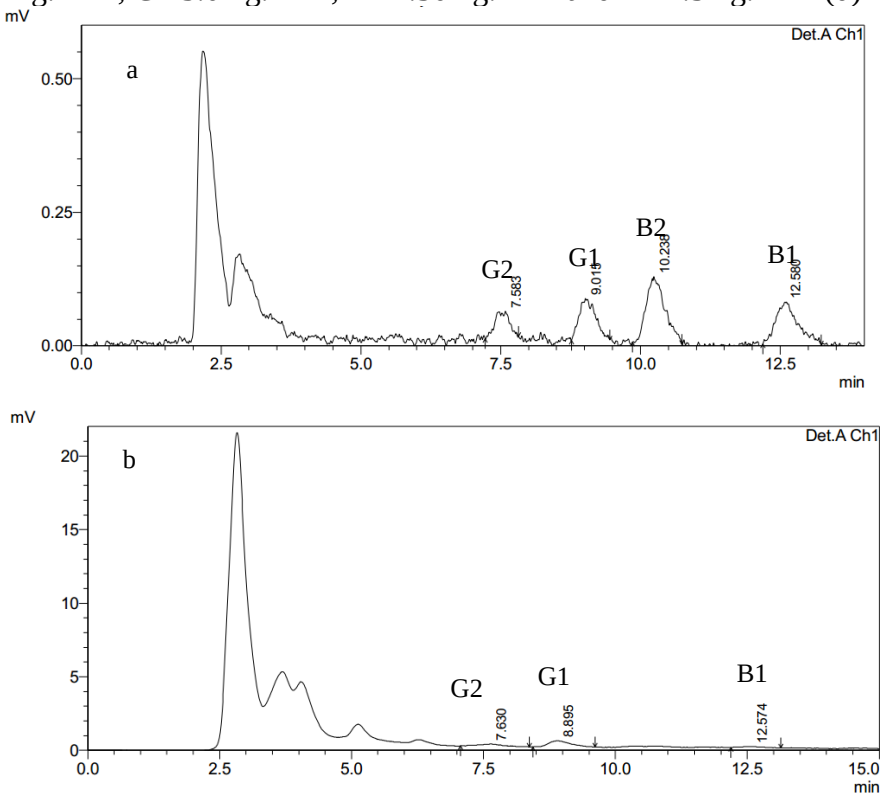
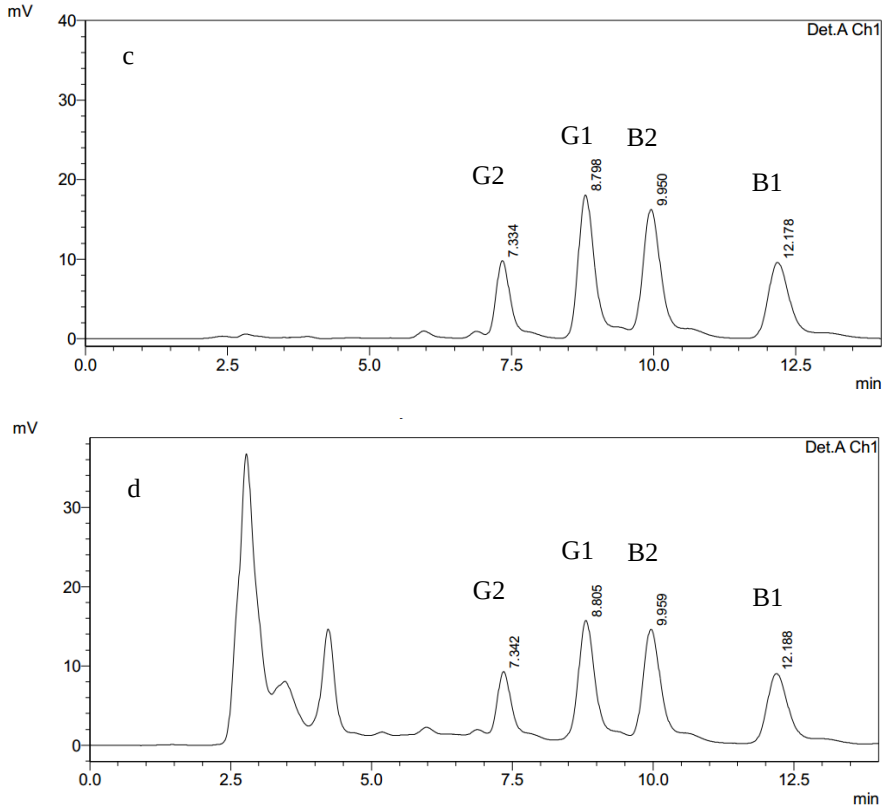


Figure 2 – Chromatogram of AFLAs G2, G1, B2 and B1 limit of detection (a), blank sample (b), standards (c) and chromatogram of standard in the extract of medium cornmeal, G2 3.75 ng.mL^{-1}, G1 3.0 ng.mL^{-1}, B2 1.50 ng.mL^{-1} and B1 4.5 ng.mL^{-1} (d) Continuação



3.5 APPLICABILITY OF THE METHOD

Various cornmeals were used to evaluate the applicability of the proposed method. The extraction method was adequate for coarse cornmeals, both commercial and dry-milling, with recoveries from 60.6% to 93.7% (Table 9).

Table 9 - Chemical composition of cornmeals and AFLAs recoveries by HPLC-FD

Cornmeal	Ashes (%)	Lipids (%)	Protein (%)	SS* (%)	TS* (%)	Recoveries (%)			
						G2	G1	B2	B1
Fine commercial	0.9	2.8	7.4	2.9	81.4	88.5	81.0	59.5	52.3
Medium commercial	0.4	0.3	6.9	2.1	88.8	98.4	89.3	88.5	86.7
Coarse commercial	0.2	0.1	8.0	3.5	89.7	93.7	68.0	81.6	73.9
Fine DM*	1.4	5.0	9.8	4.1	68.4	41.5	51.2	51.7	54.5
Medium DM*	1.0	4.0	10.5	4.2	72.1	87.9	96.6	94.3	95.3
Coarse DM*	1.4	3.6	10.0	32.0	77.5	64.5	88.8	72.3	60.6
Fine WM*	0.4	1.2	8.6	4.5	90.6	64.0	40.0	84.2	62.2

*DM= dry-milling, WM= wet-milling, SS= soluble sugar, TS= total starch; Results expressed as mean. n=3.

However, for the fine cornmeals, commercial, and dry- and wet-milling, the recoveries (40.0% to 88.5%) were below the criteria approved by the European Regulatory Committee (EUROPEAN COMMISSION, 2006). Thus, although all matrices were cornmeals, the AFLA recoveries varied from 40.0% to 98.4% (Table 9). Given the fine cornmeals presented the lowest recoveries, the composition and recoveries data of these cornmeals were used for multivariate correlation, to verify which components of the matrix interfere in the recovery so that the extraction method can be modified accordingly.

3.6 CORRELATION BETWEEN MATRIX COMPONENTS AND AFLA RECOVERY IN FINE CORNMEAL

According to Kupski and Badiale-Furlong (2015), it is fundamental to study the major components of matrices that may positively or negatively affect the mycotoxins extraction, so changes in the method may be made according to the presence or absence of the target component. To evaluate these interferences, the multivariate statistical technique was used to determine the correlation between the components of the fine cornmeal matrix and the G2, G1, B2 and B1 AFLA recoveries (Table 10). For this, the results presented in Table 9 were used.

Table 10 - Correlation between fine flours components and AFLAs recoveries.

AFLAs	Ashes	Lipids	Starch	Proteins	Sugars
G2	-0.47	-0.56	0.56	**-0.99**[a]	-0.76
G1	0.26	0.18	-0.17	-0.70	**-0.99**[a]
B2	-0.96	-0.93	0.93	-0.23	0.48
B1	-0.74	-0.68	0.67	0.21	0.81

[a] significant factor $p < 0.05$

Proteins demonstrated a significant interference in the recovery of AFLA G2, with a correlation of -0.99 while sugars were the significant interferers (r=-0.99) in G1 recovery (Table 10). For the recovery of AFLAs B1 and B2, these compounds did not significantly affect the determination, an important fact considering the frequency of these two AFLAs in food matrices.

In performing the extraction for the multivariate analysis of the AFLA recovery from the fine cornmeal, anhydrous sodium sulfate (0.25 g) was added as the solid support, and the C18 amount was increased to 0.1 g, to minimize the effect of significant interferences, like

sugars and protein. With these modifications, recoveries of 75.9%, 97.7%, 59.4% and 59.5% were obtained for G2, G1, B2 and B1, respectively. AFLA G2 recovery increased from 41.5% to 75.9%. As observed in Table 9, the proteins obtained a correlation of -0.99 with the G2 recovery. Among the proteins present in corn, zein is highlighted, which has a hydrophobic molecular structure (SHUKLA; CHERYAN, 2001). Thus, this increase of 34.4% in the recovery of G2 can be attributed to the increase in C18 (from 25 to 100 mg), since this limits the effect of apolar compounds (ANASTASSIADES et al., 2003). AFLA G1 recovery increased from 51.2% to 97.7%. According to Prestes et al. (2009), anhydrous sodium sulfate improves extraction of polar compounds, which may have contributed to alleviating the effect of interfering sugars (correlation of -0.99 with G1). This behavior was also evidenced by Kupski and Badiale-Furlong (2015) in the extraction of ochratoxin A from cereal by the QuEChERS method. For AFLAs B1 and B2, method modifications did not lead to significant increases in the recoveries, since none of the major components of the cornmeals had a significant impact on the recovery of these mycotoxins (Table 9).

4 CONCLUSION

A precise and accurate method for the extraction of AFLAs G2, G1, B2 and B1 was established using a low solvent volume and solid support amount. Acceptable recoveries (85.7% to 114.8%) were obtained under the following extraction conditions: 1 g sample, 25 mg C18 and 10 mL MeCN:MeOH (50:50, *v/v*). The optimized method was suitable for coarse and medium grind cornmeals. The multivariate correlation analysis identified were proteins (r=-0.99) and sugars (r=-0.99) as the main interferers in the determination of AFLA G2 and G1 in fine cornmeal.

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ARTICLE II

SIMULTANEOUS DISTRIBUTION OF AFLATOXINS B1 AND B2, AND FUMONISIN B1 IN CORN FRACTIONS DURING DRY AND WET-MILLING

Simultaneous distribution of aflatoxins B1 and B2, and fumonisin B1 in corn fractions during dry and wet-milling

ABSTRACT

One of the limitations for the use of corn in the food chain is its contamination with mycotoxins. Reduction in the levels of these contaminants can be achieved by processing the grain, which in the case of corn can be achieved by wet or dry milling. The aim of this study was to compare the distribution of aflatoxins B1 and B2, and fumonisin B1 in corn fractions (germ, pericarp and endosperm) obtained by dry and wet milling, aiming to identify conditions to mitigate the risk of exposure to these contaminants. Naturally contaminated corn kernels were subjected to laboratory milling. For wet-milling, an experimental design was used to define the process conditions that would affect the reduction of these mycotoxins in the endosperm, the fraction of greatest interest for human consumption. The wet-milling conditions containing 1% lactic acid in the steeping solution and 18 h of steeping were the most efficient for mycotoxin reduction in the evaluated fraction. After the wet-milling process, aflatoxins B1 and B2 were detected below the limit of quantification (LOQ B2 0.02 ng g^{-1} and B1 0.07 ng g^{-1}) in the endosperm fraction. Dry-milling reduced the concentration of these mycotoxins in the endosperm (98-99%). Fumonisin B1 contamination increased in the germ and pericarp fraction by more than three times in both dry and wet milling. Dry-milling reduced fumonisin B1 contamination in the endosperm to levels below the limit of quantitation. Wet and dry milling processes can be an efficient control method to reduce aflatoxins and fumonisin in the corn endosperm fraction.

Keywords: Milling fractions. Mycotoxins. Wet milling.

1 INTRODUCTION

Corn (*Zea mays* L. ssp. m*ays*) is part of the basic diet in Latin America, Asia and Africa. Besides, corn consumption has increased in developed countries as it is used as an ingredient for breakfast cereals, snacks, dietetic products and in particular for gluten-free foods whose consumption is increasing. Given the role of ground corn products as a staple food, their quality characterization becomes extremely important. From a nutritional point of view, corn and its products are good sources of starch, proteins, lipids and different bioactive compounds (BLANDINO et al., 2017).

The corn production chain includes the dry and wet milling processes that separate the grain into three main components: the germ (outer fraction), pericarp (outer fraction) and endosperm (inner fraction). These fractions are used mainly for oil extraction, feed production and human consumption, respectively. Wet milling differs fundamentally from dry milling as it contains a steeping step in which physical and chemical changes occur in the basic constituents, leading to the production of pure starch for industrial and food uses. Additionally, protein, fiber and germ are obtained as by-products of the wet milling process (MALUMBA et al., 2015).

However, corn quality and safety may be reduced by the presence of mycotoxins, as kernels may be affected by fungi, that are capable of producing various metabolites in the field and during storage (GRENIER; OSWALD, 2011). The association of corn with different fungi may lead to the co-occurrence of mycotoxins in this crop. In Nigeria 65% of corn grains showed co-occurrence of aflatoxins and fumonisins (ADETUNJI et al., 2014). Similarly, this co-occurrence was observed in Korea (PARK et al., 2018), EUA (CURRY et al., 2019) and Brazil (FRANCO et al., 2019; OLIVEIRA et al., 2017). This is a matter of concern, since both fumonisin and aflatoxin are known human carcinogens and their co-occurrence may lead to a mechanism of action of complementary toxicity (BRYDEN, 2007).

During the industrial milling process, mycotoxins are not destroyed and are redistributed between fractions (CASTELLS et al., 2008; SAVI et al., 2016). In general, fumonisins and aflatoxins tend to be concentrated in the germ and pericarp and to a smaller proportion in the endosperm and its derivatives (BORDINI et al., 2019a; GENEROTTI et al., 2015; PARK et al., 2018). However, these mycotoxin distributions are also dependent on the grinding process used, mycotoxin class and grain contamination level. Therefore, the milling process can be studied through multivariate techniques such as experimental design, where

mathematical models are used to achieve the best reduction of these contaminants in the different fractions.

Considering the risk to human and animal health associated with the occurrence of mycotoxins, maximum limits are legislated by several countries. In Brazil and United States the maximum tolerable limits for the sum of aflatoxins B1, B2, G1 and G2 is 20 μg kg^{-1} and 10 μg kg^{-1} in European Union for corn or cornmeal. The limits for fumonisins (B1 and B2) for corn destined for further processing is 5000 μg kg^{-1} in Brazil, 4000 μg kg^{-1} in United States and European Union. For cornmeal, corn cream, flakes, hominy the limit is 1500 μg kg^{-1} in Brazil, 2000 μg kg^{-1} in United States and 1000 μg kg^{-1} in European Union. For cornstarch and others corn products the limit is 1000 μg kg^{-1} in Brazil and for breakfast and snacks the limit is 800 μg kg^{-1} in European Union (ANVISA, 2011, 2017; USFDA, 2001; EUROPEAN UNION, 2006, 2007).

Although the distribution of aflatoxins and fumonisins in corn milling processes has been evaluated in other studies, the simultaneous determination of these mycotoxins for the redistribution of these contaminants has been poorly performed. The aim of this study was to investigate and compare the simultaneous distribution of mycotoxins produced by field (fumonisin B1- FB1) and storage fungi (aflatoxins B2 (AFLA B2) and B1 (AFLA B1)) in fractions obtained by two milling processes performed under laboratory conditions and using experimental design as a tool to find the best conditions for mycotoxin reduction in wet-milling process.

2 MATERIAL AND METHODS

2.1 STANDARDS, REAGENTS AND SAMPLES

Aflatoxins standards (B2 and B1) and fumonisin B1 were supplied by Sigma-Aldrich (Saint Louis, MO, USA) with purity >98%. Aflatoxin stock solutions were prepared by dissolving the standards with toluene/acetonitrile (98:2, v/v); while fumonisin was dissolved in methanol:acetonitrile (50:50, v/v). After preparation, the mycotoxin standards were dried under nitrogen and stored at −18°C, to ensure their stability. The working solutions were prepared from stock solutions, and before use aflatoxins were quantified in a spectrophotometer, according to the AOAC (2000).

The solvents used as mobile phase in the chromatographic system (acetonitrile and methanol) with purity > 99.9% were supplied by JT Baker (Goiânia, GO, Brazil). Ultrapure H_2O (>18.2 MΩ cm^{-1} resistivity) was purified using a Milli-Q® SP Reagent Plus water system

(Millipore Corp., Bedford, USA). The 0.1 M phosphate buffer pH 3.15 used as mobile phase in the chomatographic system for fumonisin B1 analysis was prepared diluting 13.8 g of sodium phosphate monobasic in 1 L of ultrapure water, and adjusting the pH to 3.15 with 2 M hydrochloride acid.

The OPA-MCE derivatization reagent used during quantification of fumonisin B1 was prepared daily according to Kong et al. (2012) by mixing 100 mg ortho-phthaldialdehyde (OPA), 20 mL methanol, 500 µL 2-mercaptoethanol (MCE), and 950 mL of sodium tetraborate solution 0.05 M. This mixture was then brought to 1L by addition of ultrapure water. The OPA-MCE reagent was stored in a brown glass bottle and, when not in use, kept at 4 °C for up to 2 days. The mobile phase and derivatization reagent were always filtered through a 0.45 µm cellulose filter and then degassed in an ultrasonic bath before use.

Corn (*Zea mays* L.) samples were obtained in 2016 from a local farm in Matelândia (Paraná, Brazil). Ears of corn (100 ears) with the husks intact were stored after harvesting in a ventilated storage place for 10-12 months. Kernels were shelled and stored frozen in plastic containers until use.

2.2 WET MILLING CONDITIONS

The wet milling until steeping was performed according to Malumba et al. (2015). The corn kernels were cleaned to remove broken grain, husk and other impurities. The clean grains proceeded to steeping step, which was evaluated by a CCRD 2^2 experimental design where the steeping time and lactic acid concentration were the variables of interest (Table 1). Steeping was carried out at a constant temperature of 50±2 ºC. Corn kernels (15 g) were added in the proportion (1:2.4 w/v) to the steeping solution containing lactic acid and sodium metabisulfite (0.6%), to release 0.2% SO_2. After the steeping step, the corn grain was separated into 3 fractions: germ and pericarp (external fractions) and endosperm (internal fraction) (Figure 1).

Figure 1 - Wet milling fractions.

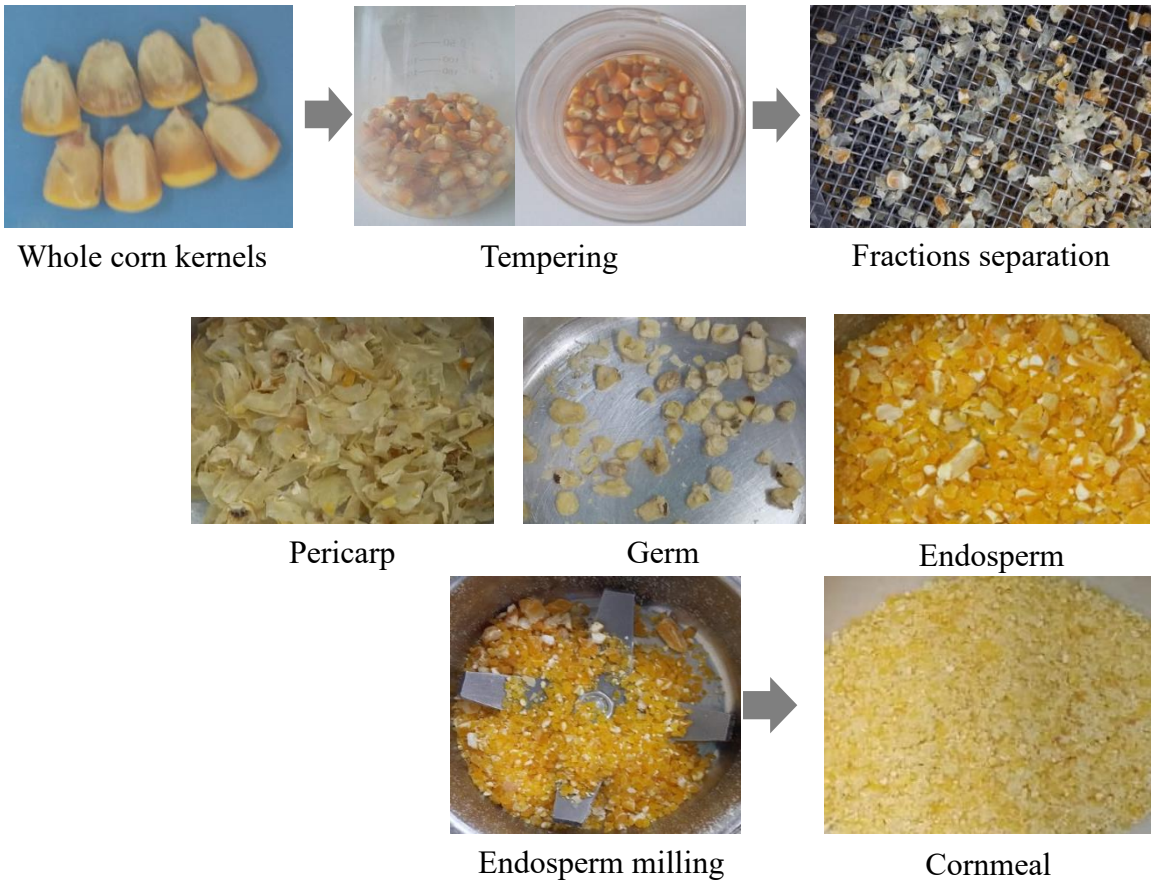
Corn kernels after steeping | Endosperm | Germ | Pericarp

Endosperm samples were ground to 0.50 mm and homogenized for mycotoxin determination. The dependent variable was mycotoxin concentration in the endosperm, since this fraction is destined for the production of breakfast cereals or ground for cornmeal production.

2.3 MYCOTOXIN DISTRIBUTION IN WET AND DRY MILLING

Laboratory milling of naturally contaminated corn with aflatoxins and fumonisin was performed by the dry milling technique according to Somavat et al. (2016), with the following steps: cleaning the corn grains; tempering (moisture adjustment to 23.5% with addition of water and orbital agitation at 200 rpm for 20 min); first grind; drying at 49 °C for 2 h; separation of fractions; endosperm milling and sieving to obtain the cornmeal (Figure 2).

Figure 2 - Dry milling.



The wet milling was performed according to the conditions defined in item 2.2, where the corn kernels (30 g) were added to the steeping solution in the proportion (1:2.4 w/v), containing 1% lactic acid and 0.6% sodium metabisulphite for 0.2% SO_2 release. Steeping was performed for 18 hours. After that, the steeping water was separated from the kernels, which were then separated into 3 fractions: germ, pericarp and endosperm.

2.3.1 Aflatoxins and fumonisin B1 determination

Aflatoxins B2 and B1 and fumonisin B1 were extracted from the milling fractions according to Massarolo et al. (2018). In short, 1 g of sample was macerated with 0.5 g of adsorbent C18, transferred to Falcon tube and added 10 mL of acetonitrile:methanol (50:50, v/v). The mixture was vortexed for 3 min and centrifuged at 3220 xg for 10 min. Two aliquots of the supernatant (one for aflatoxins and another for fumonisin B1) were removed and dried at 60 °C. For the determination of aflatoxins, the extract was resuspended in a mixture of ultrapure water and acetonitrile (90:10, v/v) and quantification in HPLC-FL with post column derivatization was performed. The chromatographic conditions for identification and quantification of aflatoxins B2 and B1 were performed as Massarolo et al. (2018).

Fumonisin B1 was first identified and quantified in a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with post column derivatization (unpublished data). After quantification, confirmation was performed using an Alliance Sampler Liquid Chromatograph equipped with an auto-sampler and Sequential Mass Detector Electrospray ionization mode following the chromatographic conditions of Scaglioni et al. (2018).

2.3.2 Distribution factor and distribution in the grain

Due to the variability associated with testing corn for mycotoxins, the estimation of mycotoxin concentration in the whole corn was carried out using mass balance according to previous studies (BELLUCO et al., 2017). The distribution factor was adopted to express the describe the redistribution of mycotoxin content in each fraction (endosperm, germ, pericarp) compared to their respective concentrations estimated in whole kernels. The distribution factor is defined as the ratio between the concentration of mycotoxins in each fraction and the concentration of mycotoxins in whole corn (BORDINI et al., 2017).

The distribution in the grain, which is defined as the multiplication of the mycotoxin content in the processed fraction and the percentage of the fraction in the whole grain, divided by the sum of the mycotoxin in all fractions (calculated using the percentage of the fraction).

2.4 STATISTICAL ANALYSIS

Data normality was verified by the Kolmogorov-Smirnov test. In order to evaluate the influence and interaction of the independent variables of interest (steeping time and lactic

acid concentration) in relation to the response (mycotoxin concentration in the endosperm), the analysis of the effects on to the dependent variable measured (mycotoxin concentration in the endosperm), a statistical analysis was performed. Once the significance of the independent variables was verified, then an analysis of variance was performed to determine the mathematical model associated with the process, the F value and the significance of the regression equation obtained. Response surfaces were generated to the process variables that led to the lowest levels of mycotoxins in the endosperm. Analyzes were performed using *Statistica* 6.0 software.

3 RESULTS AND DISCUSSION

3.1 CO-OCCURRENCE OF MYCOTOXINS IN CORN

The corn used for this research was naturally contaminated with fumonisin B1 (Figure 3) and aflatoxins B1 and B2 (Figure 4), as can be observed in the chromatograms by the retention times of the compounds in the sample and their respective standards. As corn did not have the natural presence of aflatoxins G1 and G2, these were not evaluated in this work.

Figure 3 - Chromatogram of standard fumonisin B1 1µg mL^{-1} (a) and fumonisin B1 in the whole corn 0.81 µg g^{-1} (b).

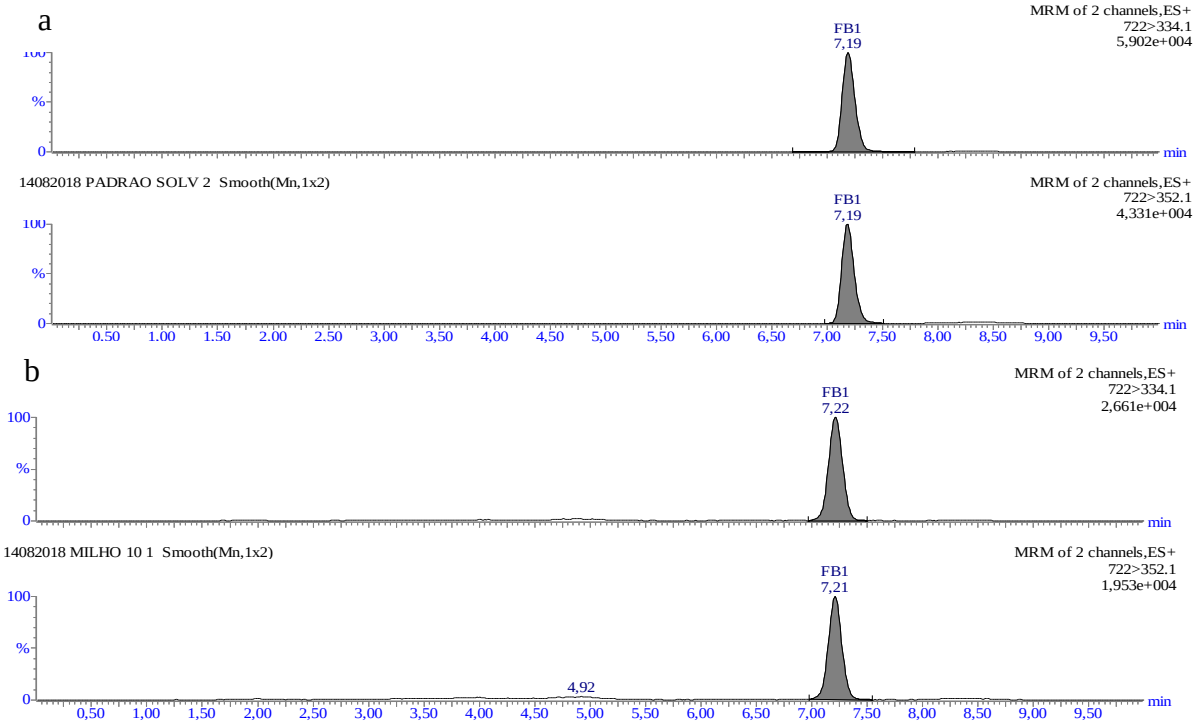
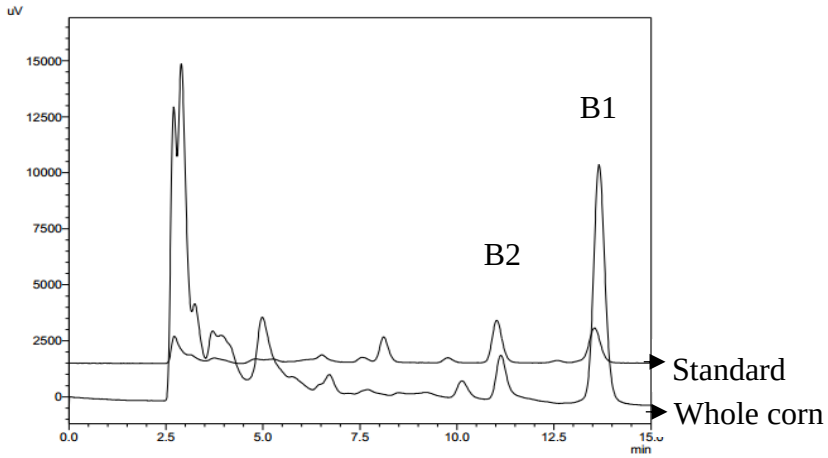


Figure 4 - Chromatogram of standard aflatoxins B1 (0.35 ng mL^{-1}) and B2 (0.10 ng mL^{-1}) and in the whole corn B1 (7.08 ng g^{-1}) and B2 (3.48 ng g^{-1}) .



The use of naturally contaminated sample present advantages over the use of spiked samples, as the study of processing in naturally contaminated corn is more applicable and representative of an industrial process. In addition, a naturally contaminated sample illustrates the actual behavior of the contaminant in corn, including possible interactions with corn macromolecules.

3.2 WET MILLING CONDITIONS

The results of the reductions of aflatoxins B1 and B2 and fumonisin B1 in endosperm obtained by the wet milling evaluated by CCRD for the 2 variables under study (steeping time and lactic acid concentration) are shown in Table 1.

Table 1 – CCRD matrix (coded and actual values) with the response of aflatoxins B1 and B2 and fumonisin B1 concentration.

Assay	X_1	X_2	AFLAB2 (ng g^{-1})	AFLAB1 (ng g^{-1})	FB1 (µg g^{-1})
1	-1 (6)	-1 (0.6)	0.40	0.01	1.53
2	+1 (18)	-1 (0.6)	0.42	0.35	0.68
3	-1 (6)	+1 (1.0)	0.38	<LOD	0.43
4	+1 (18)	+1 (1.0)	0.01	<LOD	0.82
5	-1.41 (3.54)	0 (0.8)	0.01	0.01	0.50
6	+1.41 (26.46)	0 (0.8)	0.19	<LOD	1.75
7	0 (12)	-1.41 (0.52)	0.08	<LOD	0.53
8	0 (12)	+1.41 (1.28)	0.01	0.01	0.21
9	0 (12)	0 (0.8)	3.78	10.28	0.71
10	0 (12)	0 (0.8)	3.98	12.87	0.88
11	0 (12)	0 (0.8)	3.80	10.01	0.79

X_1: Steeping time (h); X_2: Lactic acid concentration (%). LOD AFLA B1 0.04 ng g^{-1}.

An estimate of the main effects was obtained by assessing the differences in process performance caused by a change from a lower level (-1.41) to a higher level (+1.41). The p value was used to verify the significance of the factors under study (Table 2). FB1 did not present an empirical model.

Table 2 – Main effects and interaction analysis for aflatoxins B1 and B2 and fumonisin B1 levels.

Factor	Effect (%)			Standard error			*t*-Value			*p*-Value		
	B2	**B1**	**FB1**	**B2**	**B1**	**FB1**	**B2**	**B1**	**FB1**	**B2**	**B1**	**FB1**
Mean	**3.85**	**10.27**	**0.79**	**0.07**	**1.43**	**0.21**	**58.39**	**7.20**	**3.68**	**<0.01[a]**	**0.02[a]**	**0.01[a]**
X_1 (L)	-0.02	0.08	0.33	0.08	1.75	0.26	-0.27	0.05	1.23	0.81	0.97	0.27
X_1 (Q)	**-3.65**	**-10.26**	0.39	**0.10**	**2.09**	0.32	**-37.77**	**-4.91**	1.24	**<0.01[a]**	**0.04[a]**	0.27
X2 (L)	-0.13	-0.09	-0.35	0.08	1.75	0.26	-1.65	-0.05	-1.34	0.24	0.97	0.24
X2 (Q)	**-3.70**	**-10.25**	-0.37	**0.10**	**2.09**	0.32	**-38.34**	**-4.91**	-1.16	**<0.01[a]**	**0.04[a]**	0.30
X_1 x X_2	-0.20	-0.17	0.62	0.11	2.47	0.37	-1.72	-0.07	1.66	0.22	0.95	0.16

[a] significant factor $p<0.05$. X_1: Steeping time (h); X_2: Lactic acid concentration (%).

Steeping time and lactic acid concentration quadratic were the most significant variables for the reduction of aflatoxins B2 and B1, where aflatoxin levels decreased by 3.7% for B2 and 10.3% for B1 with increasing steeping time and amount of lactic acid. For fumonisin B1 none of the variables under evaluation were significant at the 5% level. Therefore, process conditions that resulted in the greatest reduction in aflatoxins was a steeping time of 18 hours and acid lactic at 1.0%.

Since the values obtained for the correlation between the dependent and independent variables were 0.99 for AFLA B2 and 0.95 for AFLA B1, mathematical models (Equations 1 and 2) were obtained from the coefficients presented in **Table 2**.

$$Y_{AFLAB2} = 3.85 - 3.65X_1^2 - 3.7X_2^2 \quad (1)$$

$$Y_{AFLAB1} = 10.27 - 5.13X_1^2 - 5.13X_2^2 \quad (2)$$

Where: Y= aflatoxin concentration, X_1= steeping time and X_2= lactic acid concentration.

The calculated F-value was higher than the tabulated F and the determination coefficient obtained was close to 1 for aflatoxins B2 and B1. Thus, it is possible to state that the model is predictive and significant (Table 3). From the equations obtained a contour curve was generated to illustrate the effect of the processing variables on the concentration of aflatoxin B1 and B2 (Figure 5).

Table 3- ANOVA for aflatoxin B1 and B2 concentration.

Aflatoxin	Variation	Sum quadratic	Degrees of freedom	Quadratic mean	*F*-value
B2	Regression	29.30092	2	14.65	406.94[a]
	Residue	0.28919	8	0.036	
	Total	29.59011	10		
B1	Regression	228.1379	2	114.07	74.07[a]
	Residue	12.3336	8	1.54	
	Total	240.4715	10		

Regression coefficient= 0.99 (B2) and 0.95 (B1); $F_{0.95;\,2;\,8}$ =4.46; [a] *F*-value (regression/residue).

Figure 5 – Contour diagram of aflatoxin B1 (A) and aflatoxin B2 (B) concentration as function of lactic acid concentration and steeping time.

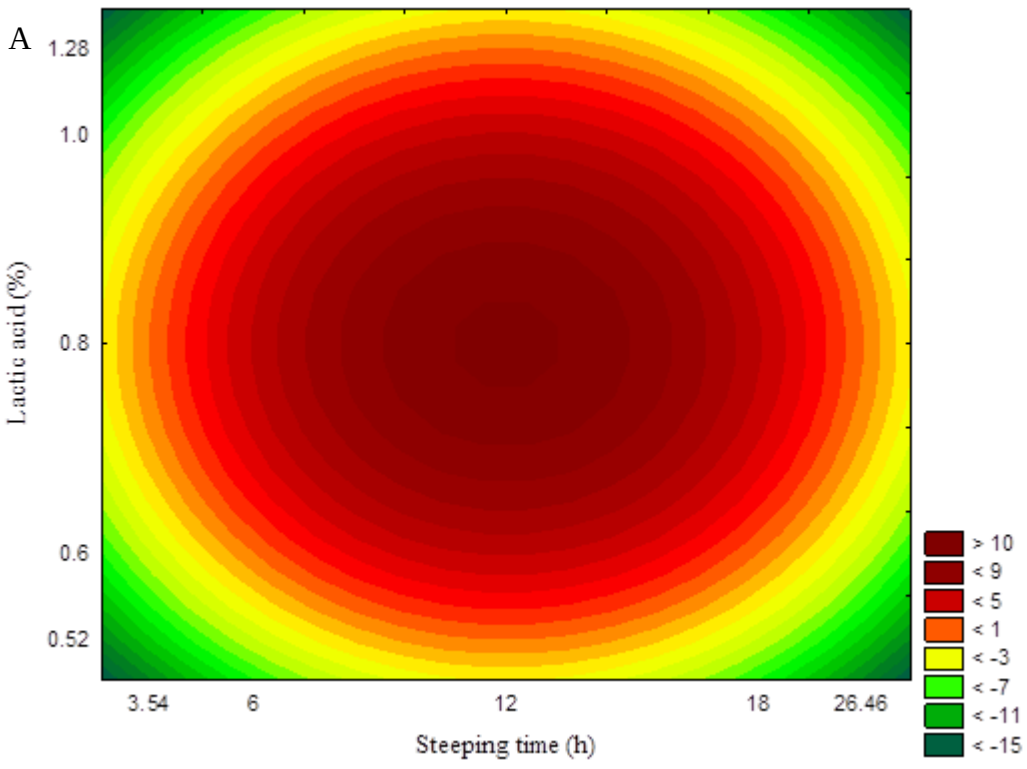
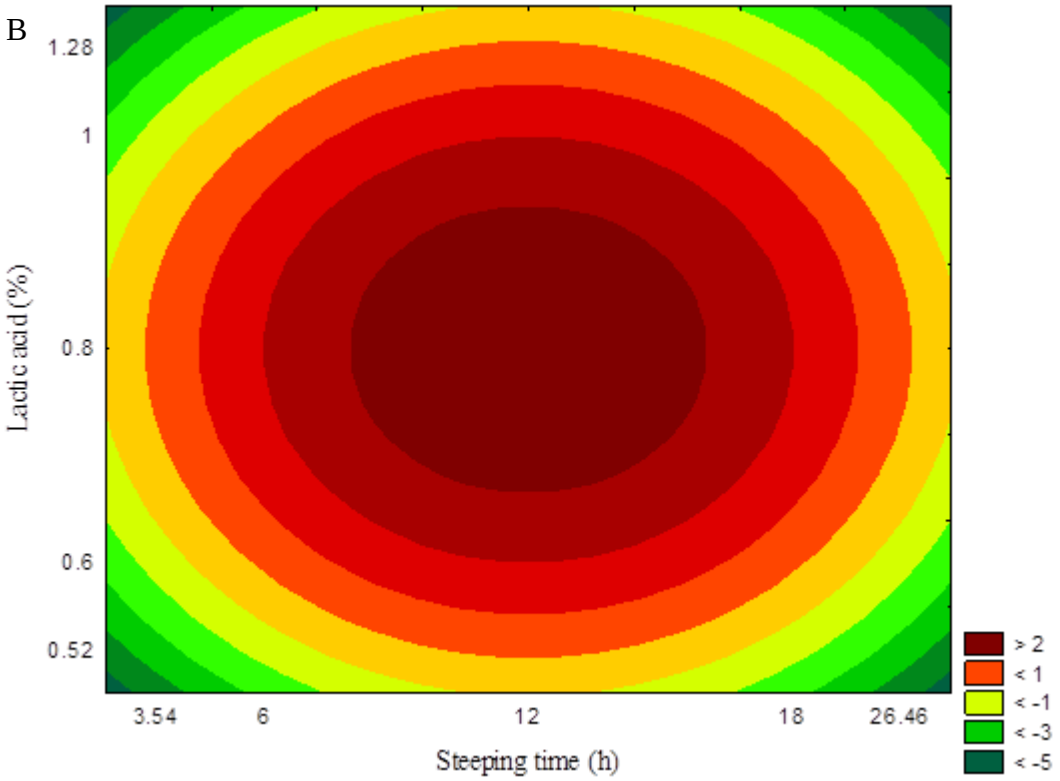


Figure 5 – Contour diagram of aflatoxin B1 (A) and aflatoxin B2 (B) concentration as function of lactic acid concentration and steeping time



The lowest levels of aflatoxin in the endosperm was obtained with the steeping time of 18 hours and a lactic acid concentration of 1%. These conditions led to a reduction of aflatoxin concentration in the endosperm by 99.8% for B2 and 100% for B1. Thus, these steeping conditions were applied to evaluate the distribution of mycotoxins during corn wet milling, as it results in the lowest aflatoxin concentration in the endosperm fraction, which is of greater interest for human consumption.

3.3 MYCOTOXIN DISTRIBUTION IN DRY AND WET MILLING

3.3.1 Fumonisin distribution

The fumonisin B1 content was determined in each fraction of the milled corn. In the wet milling the greatest fumonisin content was observed in the germ fraction, followed by the pericarp and endosperm fractions. After the dry milling the content of FB1 in the endosperm was below the limit of quantification, and the greatest FB1 content was detected in the pericarp followed by the germ fraction (Table 4).

Table 4 – Distribution of fumonisin B1 in wet and dry milling.

Fraction and % in the grain	Wet milling			Dry milling		
	FB1 (µg $g^{-1}_{fraction}$)	**D.F (%)**	**D.G (%)**	**FB1 (µg $g^{-1}_{fraction}$)**	**D.F (%)**	**D.G (%)**
Germ (11%)	6.10 (6.4)	369	40.6	2.53 (21.1)	331	47.4
Pericarp (7%)	4.40 (12.2)	266	18.7	4.41 (29.4)	751	52.6
Endosperm (82%)	0.82 (15.2)	41	40.7	<LOQ	-	-

Mean (CV) CV= coefficient of variation, n=3. LOD= 0.2 µg g^{-1}, LOQ= 0.4 µg g^{-1}. D.F= distribution factor, D.G= distribution in the grain.

The results are shown as distribution factor, defined as the ratio between the mycotoxin content in the processed fraction and the mycotoxin content in whole corn, and as distribution in the grain, which is defined as the multiplication of the mycotoxin content in the processed fraction and the percentage of the fraction in the whole grain, divided by the sum of the mycotoxin in all fractions (calculated using the percentage of the fraction).

After the wet milling, 40% of the fumonisin B1 was quantified in the germ and in the endosperm and only 18% in the pericarp; however, after the dry milling 52% was quantified in the pericarp and 47% in the germ.

The distribution factor for germ and pericarp ranged from 266 to 751%, which means that contamination in these fractions was increased in more than three-fold. The higher FB1 concentrations in germ and pericarp might be due to the location of the fungus in the tip cap and germ areas just beneath the pericarp (KATTA et al., 1997). As a matter of fact, the germ is externally located in the kernel and rich in lipids, which favors the attack of molds and the subsequent mycotoxin productions (BRERA et al., 2006).

The results of distribution factor in the germ (331%) in this study is similar to the results reported for industrial dry milling that was evaluated by Bordini et al. (2017). In the industrial evaluation the fate of fumonisins (B1 and B2) were evaluated in a dry milling process for two lots of non-transgenic corn. The fractions evaluated included germ, pericarp, endosperm, cornmeal and grits. Samples were collected from one of the major Brazilian milling industries in 2014 (n=120) and 2015 (n=120). The authors verified that fumonisins were concentrated in the germ and pericarp at a rate of 322% and 188% (lot 1) and 311% and 263% (lot 2), respectively.

Contamination trends for the different corn fractions in this study were similar to those found in other studies (BORDINI et al., 2017; CASTELLS et al., 2008; GENEROTTI et al., 2015), in which an increase of fumonisin concentration in pericarp and germ fractions and

a decrease in endosperm, a product intended for human consumption, was reported. Although fumonisin levels were higher in the corn germ, the alkaline treatment usually performed in the oil refining industry led to a degradation of these mycotoxins and negligible amounts of fumonisins were detected in refined oil and margarine for human consumption (ESCOBAR et al., 2013).

The content of FB1 in the endosperm during the wet milling was higher than in the dry milling, probably due the fact that mycotoxins may have been carried by the steeping water into the endosperm. In the dry milling the surface parts of the grain is removed with minimum breakage of the endosperm by a physical process, and lower levels of mycotoxin is observed in the endosperm. The pericarp layer has the potential to act as a physical barrier preventing the mycelia from penetrating further in the kernel structure and transferring of fumonisins to the inner part of the kernel (CASTELLS et al., 2008).

3.3.2 Aflatoxin distribution

The wet milling decreased the aflatoxins concentration in all fractions (Table 5), both aflatoxin B1 and B2 could not be measured in the endosperm.

Table 5 – Distribution of Aflatoxins B1 and B2 in wet and dry milling.

Fraction and % in the grain	**Wet milling**					
	B1 (ng $g^{-1}_{fraction}$)	**D.F (%)**	**D.G (%)**	**B2 (ng $g^{-1}_{fraction}$)**	**D.F (%)**	**D.G (%)**
Germ (11%)	0.15 (8.4)	620	68.2	0.07 (15.1)	556	58.7
Pericarp (7%)	0.11 (12.2)	455	31.8	0.07 (10.6)	556	41.3
Endosperm (82%)	<LOD	-	-	<LOQ	-	-
Fraction and % in the grain	**Dry milling**					
	B1 (ng $g^{-1}_{fraction}$)	**D.F (%)**	**D.G (%)**	**B2 (ng $g^{-1}_{fraction}$)**	**D.F (%)**	**D.G (%)**
Germ (11%)	0.09 (21.0)	61	6.7	0.21(20.9)	228	9.9
Pericarp (7%)	1.15 (11.9)	778	54.5	0.52 (20.7)	563	16.1
Endosperm (82%)	0.07 (19.7)	47	38.8	0.04 (14.5)	43	74.0

Mean (CV) CV= coefficient of variation. LOD= B2 0.01 ng g^{-1}, B1 0.04 ng g^{-1}, LOQ= B2 0.02 ng g^{-1}, B1 0.07 ng g^{-1}. D.F= distribution factor, D.G= distribution in the grain.

After the wet milling 68% and 58% of the aflatoxin B1 and B2, respectively, were quantified in the germ and 31% and 41% of these aflatoxins in the pericarp. The relatively low contamination levels of aflatoxin in corn fractions possible can be explained by the low contamination in the whole corn and by the aflatoxin migration from kernels into steeping solution. Research conducted by Park et al. (2018) showed that aflatoxins were transferred from corn to steep water during the wet milling process.

A different aflatoxin distribution was observed in dry milling, and after this process aflatoxin B1 and B2 were detected in all fractions of the kernel. Regarding aflatoxin B1, 54.5% was quantified in the pericarp, 38% in the endosperm and only 6.7% in the germ; however, 74% of aflatoxin B2 was found in the endosperm, 16% in the pericarp and only 9.9% in the germ.

Higher aflatoxin levels were observed in pericarp and germ, and it could be the result of mold growth progress in the kernels. Lillehoj et al. (1976), showed that growth of *Aspergillus flavus* occurs from the external part of grain into the endosperm. The aflatoxin B1 and B2 levels were also higher in the pericarp than the germ in an industrial dry milling process using both conventional and organic corn (BRERA et al., 2006). Contrary to that, Pietri, Zanetti and Bertuzzi (2009) verified higher aflatoxin levels in germ than pericarp, and the conflicting results among these studies could be caused by the heterogeneity in the aflatoxin-contamination of corn kernels.

Dry and wet milling processing resulted in a concentration of both fumonisins and aflatoxins in pericarp and germen, which is widely used in the production of animal feeds. At the same time, these cereal milling fractions (pericarp and germ) represent a novel category of promising ingredients for human nutrition and health, due to other interesting functional properties (SCHAFFER-LEQUART et al., 2017). Therefore, even though consumption of whole grain is recommended due to its functional properties, the outer layers of the cereal grain are more likely to be exposed to mycotoxins contaminants. The average industrial yields obtained are approximately 70%, 28% and 2% for endosperm, germ and pericarp, respectively (BORDINI et al., 2017).

The observed variation in the levels of aflatoxins and fumonisin in the corn fractions can be associated with both the yield of the milling process and the distribution of mycotoxins in the various parts of the grain as a consequence of the fungal attack.

4 CONCLUSION

The wet-milling process with 1% lactic acid concentration and 18 h of steeping under laboratory conditions resulted in the largest redistribution of aflatoxins in the endosperm fraction, however for fumonisin B1 dry-milling is the most indicated. Considering that the mycotoxin levels in unprocessed corn do not reflect the contamination in their fractions obtained after milling, it is essential to evaluate the effect of milling on mycotoxin redistribution.

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ARTICLE III

PARTICLE SIZE AND PHYSICAL-CHEMICAL CHARACTERISTICS OF HYDROTHERMALLY TREATED CORNMEAL ON RESISTANT STARCH CONTENT

MASSAROLO, K. C.; FERREIRA, C. F. J.; BORBA, V. S.; KUPSKI, L.; BADIALE-FURLONG, E. Particle size and physical-chemical characteristics of hydrothermally treated cornmeal on resistant starch content. **Food chemistry**, v. 283, p. 39-45, 2019.

Particle size and physical-chemical characteristics of hydrothermally treated cornmeal on resistant starch content

ABSTRACT

The influence of particle size and chemical composition of three different cornmeals on the resistant starch (RS) content formed during the hydrothermal treatment was studied. The changes in reducing sugars and phenolic acids in different fractions caused by the hydrothermal treatment were correlated with RS content. The cornmeal with smaller particle size had higher RS content (202 mg g^{-1}). The hydrothermal treatment promoted a reduction in the total reducing sugars (46.7–84.5%), availability of free phenolic acids (256.7%) and reduction of the bound phenolic acids (71.1%). The correlation between RS contents and phenolic acids was determined by multivariate statistics and principal component analysis (PCA) showed that hydroxybenzoic and ferulic acids influence the formation of RS similar to type 5, amylose-phenol complex. The texture profile parameters (TPA) of cornmeal were negatively correlated with RS and differential scanning calorimetry (DSC) analysis showed consistency with this behavior.

Keywords: Amylose-phenol complex. Ferulic acid. Principal component analysis.

1 INTRODUCTION

The resistant starch (RS) is the sum of starch and starch degradation products non-absorbed in the small intestine of healthy individuals (ENGLYST; HUDSON, 1996), which are fermented by colonic bacteria, to produce short-chain fatty acids and gases in the large intestine (TOPPING; CLIFTON, 2001).

The RS properties are similar to those of dietary fiber, presenting prebiotic effect and acting on lipid metabolism, reducing cholesterol, risk of ulcerative colitis, colon cancer (LE LEU; HU; YOUNG, 2002), hypoglycemic effects, inhibiting fat accumulation (HASJIM et al., 2010) and increasing absorption of minerals (MORAIS et al., 1996).

The RS is constituted by heterogeneous compounds, and its classification depends on the physical structure and the susceptibility to the enzymatic attack. In this way, it is divided into five types, being the RS1: physically inaccessible starch entrapped in a non-digestible matrix, such as whole or partly milled grains or seeds; RS2: raw starch granules, consisted in non-gelatinized granules; RS3: retrograded starch produced when the starch is cooled after gelatinization and again forms a partially crystalline structure, insoluble and resistant to enzymatic digestion; RS4: starches that are chemically modified to obtain resistance to enzymatic digestion; RS5: complexes formed between amylose and lipids, which slows the swelling of the granules during heating in excess of water, reducing the accessibility of the enzyme to hydrolyze the starch granules (DUPUIS; LIU; YADA, 2014; ENGLYST; HUDSON, 1996). In addition to these five types of RS, studies have shown that interactions between starch and phenolic compounds affect starch digestibility by forming an amylose phenol complex (RS similar to type 5), which affects the enzymatic hydrolysis of starch granules by α-amylase (KARUNARATNE; ZHU, 2016; ZHU, 2015).

The amount of RS in foods depends on the botanical source, amylose content, moisture, storage time (KIM; TANHEHCO; NG, 2006),amylose/amylopectin ratio (LI et al., 2015) and processing (grinding, cooking and cooling) (EGGUM et al., 1993). It is extremely important to know the types of RS formed during processing, once it may influence the physical properties (OKUMUS et al., 2018) and the health benefits (HASJIM et al., 2010). Many processes in food technology reduce or eliminate RS1 and RS2, but potentially form RS3, such as hydrothermal treatment, particularly if high amylose content starches are used (MUTLU; KAHRAMAN; ÖZTÜRK, 2017) and RS5 when it is carried out with addition of lipid and high moisture content (CHEN et al., 2017).

RS has attracted interest for its health benefits and functional properties reflecting in researchers seeking to identify mechanisms to increase the RS content in foods (CHEN et al., 2017; MUTLU; KAHRAMAN; ÖZTÜRK, 2017). In this study the hypothesis is that the formation of RS is affected by the hydrothermal treatment and physical-chemical characteristics of cornmeals. Therefore, the objective was first to investigate the conditions of hydrothermal treatment (with and without previous hydration of the cornmeals) in the RS content and right after apply multivariate analysis to correlate the particle size and chemical composition of the cornmeals and the contents of RS, in order to infer the type of RS formed.

2 MATERIAL AND METHODS

2.1 STANDARDS AND SAMPLES

Cornmeals with different particle sizes and sodium chloride were purchased from a local supermarket. The fungal α-amylase and protease enzyme produced by *Aspergillus oryzae*, amyloglucosidase enzyme produced by *Aspergillus niger* and the amylose type III from potato standard were obtained from Sigma-Aldrich. The starch P.A – ACS standard was obtained from Dinamica Quimica Contemporânea Ltda. The phenolic acids standards namely: caffeic, chlorogenic, *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, protocatechuic, syringic acids and vanillin were obtained from Sigma-Aldrich. The methanol (purity >99.9%) used as mobile phase in the chromatographic system was supplied by JT Baker and were passed through a 0.45 µm nylon filter. Ultrapure H_2O (>18.2 MΩ cm^{-1} resistivity) was purified using a Milli-Q® SP Reagent Plus water system (Millipore Corp., Bedford, USA). The mobile phase solvents were previously degassed in an ultrasonic bath.

2.2 CHARACTERIZATION OF CORNMEALS

The granulometric profile of different cornmeals (fine, medium and coarse) was verified in sieves with openings of 0.71; 0.50; 0.35; 0.25 and 0.14 mm. The centesimal composition was defined by the percentage of moisture, crude protein, ash, and lipid content according to AOAC (AOAC, 2000) methods. Total soluble sugars were determined by phenol-sulfuric method (DUBOIS et al., 1951).

The amylose content was determined according to the colorimetric method proposed by Martinéz and Cuevas (1989) with adaptations. The cornmeals amylose extraction was performed with 95% ethyl alcohol (v/v) and NaOH 1 mol L^{-1}. The colorimetric reaction

was performed with 2% (w/v) iodine solution and the amylose content was determined with standard amylose curve (4-24 μg mL^{-1}) in a spectrophotometer at wavelength 620 nm.

The total starch content of each cornmeal was determined by iodometry and the starch-iodine complex was quantified in a spectrophotometer at 620 nm using a standard starch curve (0.05-0.21 mg mL^{-1}). Amylopectin was estimated by the difference of total starch and amylose.

2.3 HYDROTHERMAL TREATMENT

The hydrothermal treatment for cornmeals with different particle sizes (fine, medium and coarse) was performed by an electric cooking plate at 120 °C for 40 min. Two preparation methods were used for each cornmeal. On the first preparation, the cornmeal was added in boiling water (1:5, w/v) with sodium chloride (1%) and homogenized until the end of the cooking process. The second method of preparation was carried out with a previous hydration of the cornmeal, according to the commercial package instructions. On this preparation, the cornmeal was previously dissolved in cold water (30%), added to the rest of the boiling water and sodium chloride (1%), being homogenized during the cooking process.

2.4 STARCH DIGESTIBILITY

Resistant and non-resistant starch contents were measured according to AOAC method 996.11 modified by Walter, Silva and Perdomo (2005). The determination of the starch fractions (resistant and non-resistant) after hydrolysis was performed by quantifying the glucose released in each step by the 3,5 dinitrosalicylic acid reduction method (DNS) (MILLER, 1959) converting to starch by the conversion factor of 0.9.

2.5 REDUCING SUGAR

The reducing and total reducing sugars of raw cornmeals and after the hydrothermal treatment were determined by 3,5 dinitrosalicylic acid reduction method (DNS) (MILLER, 1959).

2.6 PHENOLIC COMPOUNDS

Phenolic compounds soluble in methanol, in ethanol 80% (free form) and bound form were extracted from raw cornmeals and after hydrothermal treatment according to Scaglioni, de Souza, Schmidt and Badiale-Furlong (2014). The identification of the phenolic acids in the different extracts was performed using Sigma-Aldrich reference standards, named: gallic, protocatechuic, chlorogenic, *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric, ferulic acids and vanillin. The extracts were diluted in methanol: water (1:1, v/v) and analyzed by High-Performance Liquid Chromatography, according to Scaglioni, de Souza, Schmidt and Badiale-Furlong (2014) with adaptations for Photodiode Array Detector (PDA). The detection was monitored with photodiode system with wavelength band between 230-340 nm for 25 min.

2.7 TEXTURAL MEASUREMENTS

The pastes formed during hydrothermal treatment were transferred to cylindrical plexiglass holders (30 mm inner diameter and 13 mm depth), covered to avoid weight loss and held at 25 ºC for 1 h. The textural measurements were performed with TA-XT2 texturometer (Stable Micro Systems, UK) fitted with a cylindrical probe (20 mm diameter). Gel was compressed to a distance of 10.0 mm, a cylindrical probe with a diameter of 20 mm and a test speed of 5.0 mm s^{-1} were used. The textural parameters recorded were hardness, adhesiveness, cohesiveness and springiness.

2.8 DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Fine and coarse cornmeal (5 mg $\pm$ 0.01) were weighed into aluminum pan and added 25 mL of distilled water using a micropipette. The sample pan was hermetically sealed and equilibrated at room temperature for 1 h before analysis. A sealed empty pan was used as a reference. The samples were analyzed by a DSC (Shimadzu TGA-60, Japan) by equilibrating at 20 ºC for 2 min before ramping to 180 ºC at a rate of 10 ºC min^{-1}.

2.9 DATA ANALYSIS

The analysis of variance (ANOVA) of the data, followed by Tukey mean difference test, was performed using Software Statistica 6.0. Differences with a probability value of p

<0.05 were considered significant. Principal Component Analysis (PCA) was performed in Past software (folk.uio.no/ohammer/past) to establish correlations between changes in different matrix components and the RS content after hydrothermal treatment. Also, correlation studies, between texture profile parameters (TPA) and RS, were conducted by Past software and p value of 0.10 is considered as the level of statistical significance unless otherwise specified.

3 RESULTS AND DISCUSSION

3.1 CHARACTERIZATION OF CORNMEALS

The distribution of the particle sizes for the cornmeals is as described on the packaging by the supplier (Table 1), which classifies the cornmeals as fine, medium and coarse without defining the particle size for each class of cornmeal. The coarse cornmeal presented 78.6% of particles larger than 0.35 mm, in the medium cornmeal 58.7% of particles presented sizes between 0.25 and 0.50 mm and the fine cornmeal presented 88.8% of particles smaller than 0.35 mm. In addition to present differences in particle sizes, the cornmeals present a difference in their composition (Table 2).

Table 1 - Granulometric profile of cornmeals.

Particles sizes	**Cornmeal**		
	Coarse	**Medium**	**Fine**
Between 0.71 and 0.50 mm (%)	8.5 (0.1)	0	0
Between 0.50 and 0.355 mm (%)	70.1 (0.0)	26.0 (0.1)	11.2 (0.1)
Between 0.355 and 0.25 mm (%)	20.4 (0.0)	32.7 (0.0)	54.1 (0.0)
Between 0.25 and 0.147 mm (%)	0.6 (0.4)	21.1 (0.0)	18.8 (0.1)
Smaller than 0.147 mm (%)	0.4 (0.4)	20.2 (0.1)	15.9 (0.1)

Data presented as mean (RSD) n=3. RSD= relative standard deviation

Table 2 – Composition of cornmeals with different particle sizes.

Cornmeal	**Ashes (%)**	**Lipids (%)**	**Proteins (%)**	**TRS (%)**	**ALS (%)**	**AMP (%)**	**ALS/ AMP**	**TS (%)**
Fine	0.9^{a}	2.8^{a}	7.4^{a}	2.9^{ab}	27.2^{a}	54.2^{b}	0.50	81.4^{b}
Medium	0.4^{b}	0.3^{b}	6.9^{a}	2.1^{b}	31.4^{a}	57.3^{ab}	0.55	88.8^{a}
Coarse	0.2^{c}	0.1^{b}	8.0^{a}	3.5^{a}	23.7^{a}	66.0^{a}	0.36	89.7^{a}

The mean values of triplicate analyses are given. Values with different letters in the same column differ significantly ($p<0.05$). TRS=total reducing sugar, ALS=amylose, AMP=amylopectin, ALS/AMP=amylose/amylopectin ratio, TS= total starch.

The fine cornmeal had the higher content of ashes (0.9%) and lipids (2.8%) when compared to the other cornmeals. About the protein content, there was no significant difference ($p>0.05$) between cornmeals. Coarse cornmeal has the highest total soluble sugars (3.5%) and amylopectin content (66.0%). It also had the lowest amylose/amylopectin ratio (0.36), whilst medium cornmeal had the highest value (0.55), followed by the fine cornmeal (0.50). These differences in cornmeals composition may contribute to changes in RS content mainly due to amylose/amylopectin ratio and lipid content (LI et al., 2015).

3.2 HYDROTHERMAL TREATMENT AND RESISTANT STARCH

Factorial analysis of hydrothermal treatment and cornmeals types showed that different hydrothermal treatments (with and without hydration) for the 3 types of cornmeals (fine, medium and coarse) did not have significant difference ($p>0.05$) in the RS content (Table 3), whereas among the cornmeals types there was a significant difference ($p<0.05$). The fine cornmeal had a higher RS content (202.0 mg g^{-1}) and the coarse cornmeal had a lower RS content (142.7 mg g^{-1}). The lower digestibility of starch with smaller particles (greater RS) was also verified in the study that evaluated the *in vitro* digestibility of durum wheat starch in different particle sizes, in which the digestibility was higher for the cooked flour larger (0.25-0.50 mm) than for flours with smaller particle sizes (0.15-0.25 mm and less than 0.15 mm). This result can be explained by the possible interactions between the flour components during heating, with greater interaction occurring in flours with a smaller particle size (GUO et al., 2018).

Table 3 – Influence of cornmeals hydration on the resistant starch in dry basis.

Cornmeals	Treatment	Resistant starch (mg g^{-1})
Fine	Without hydration	201.6^{a}
	With hydration	202.0^{a}
Medium	Without hydration	178.3^{ab}
	With hydration	195.8^{ab}
Coarse	Without hydration	142.7^{b}
	With hydration	181.8^{ab}

The mean values of triplicate analyses are given. Values with different letters differ significantly ($p<0.05$) between cornmeals and treatment.

In this study, the fine cornmeal presented higher lipid content (Table 2) what could interact with starch during hydrothermal treatment and form an amylose-lipid complex, thus forming RS5. Chen et al. (2017) studied the effect of adding corn oil and soy protein to starch digestibility and found out that the addition of corn oil and soy protein decreased the rapidly digestible starch content and increased the sum of the slowly digestible starch and RS content. The physical barrier of corn oil, amylose-lipid complex and protein-starch matrix can promote resistance to starch digestion (CHEN et al., 2017).

Based on the results obtained, the characterization of the cornmeals was carried out regarding the contents of reducing sugars and phenolic compounds, in order to infer about the type of RS that was being formed during the hydrothermal treatment.

3.3 HYDROTHERMAL TREATMENT AND THE EFFECT ON CONTENTS OF REDUCING SUGARS AND PHENOLIC COMPOUNDS IN CORNMEALS

The experiments to evaluate the effect of the hydrothermal treatment on the contents of reducing sugars and phenolic compounds were carried out with the hydrothermal treatment without previous hydration because it did not present significant difference in the RS content and it is the most usual hydrothermal treatment.

Factor analysis showed that for both reducing sugar and total reducing sugar contents the variable type of cornmeal and hydrothermal treatment presented significant difference ($p<0.05$). In the fine cornmeal, the hydrothermal treatment promoted a 4.5-fold increase in the reducing sugars, while in the medium and coarse cornmeals a reduction of 13.2 and 9.1% was observed, respectively (Table 4).

Table 4 – Effect of hydrothermal treatment on reducing sugar contents.

Cornmeals	**Reducing sugar (mg g^{-1})**		**Total reducing sugar (mg g^{-1})**	
	R	**HT**	**R**	**HT**
Fine	2.68^{b}	12.28^{a}	29.57^{A}	15.75^{B}
Medium	0.91^{c}	0.79^{c}	13.94^{C}	3.39^{D}
Coarse	0.77^{c}	0.70^{c}	7.98^{E}	1.24^{F}

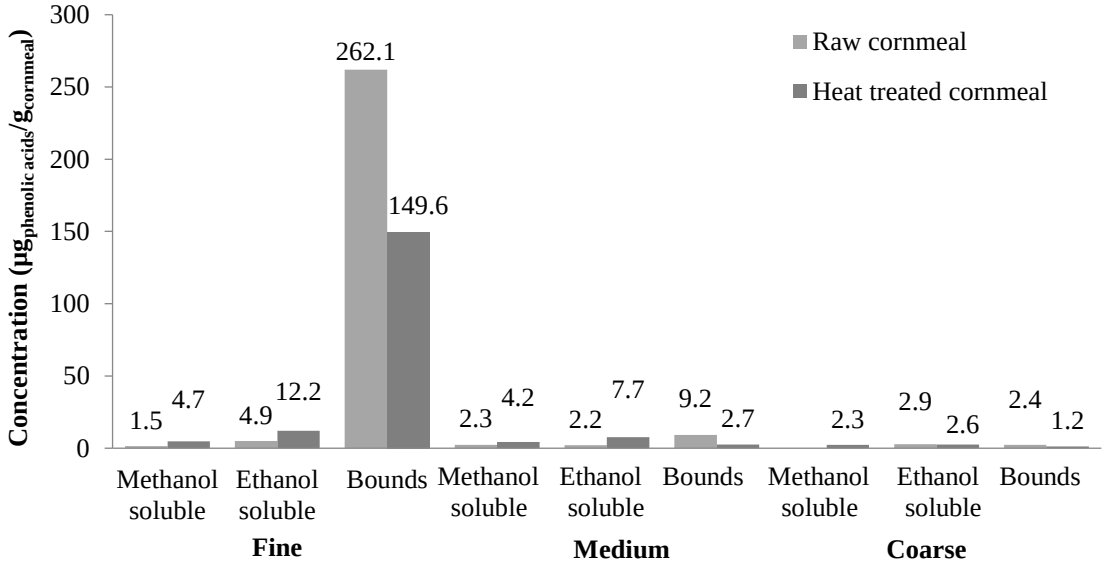
R=raw HT= heat treated. The mean values of triplicate analyses are given. Values with different small letters differ significantly ($p<0.05$) to reducing sugar and different capital letters differ significantly to total reducing sugar ($p<0.05$).

Kataria and Chauhan (1988) observed an increase in reducing sugars after cooking legume seeds, and this increase may have been caused by starch hydrolysis to oligosaccharides and monosaccharides due to wet heating. In addition to the heating, in this study the particle size of the fine cornmeal may have provided greater hydrolysis of the starch by offering a larger surface for the hydrolytic process.

The total reducing sugars were reduced by 46.7%, 75.7% and 84.5% in the fine, medium and coarse cornmeals, respectively, after the hydrothermal treatment. This fact may be related to the formation of Maillard reaction products because during thermal processing the reducing sugar interacts easily with the amino acids. This reaction is initiated when the carbonyl group of a sugar reacts with an amine group, the initial products of the reaction are N-glycosylamines or N-fructosylamines, which give rise to intermediate products and final heterocycliation and polymerization (STANIMIROVA; BOUCON; WALCZAK, 2011).

Phenolic acids soluble in methanol and ethanol (free) and bound were determined in the cornmeals of different particle sizes (fine, medium and coarse) in the raw form and after hydrothermal treatment. The phenolic acids, both in the free (soluble in methanol and ethanol 80%) and bound form were present in greater quantity in the fine cornmeal followed by the medium and smaller amount in the coarse cornmeal (Figure 1). The grinding processes increased the solvent-extractable phenolic content because this process increases the specific surface area of the particle and thus increases the accessibility of the phenolic compounds to the extraction solvents (GUTIÉRREZ-GRIJALVA et al., 2016).

Figure 1 - Effect of hydrothermal treatment on free and bound phenolic acids.



The hydrothermal treatment promoted an increase in the phenolic acids soluble in methanol (205% fine, 81.5% medium and 225% coarse) and in ethanol (146.2% fine and 256.7% medium) and reduction in bound phenolic acids (42.9% fine, 71.1% medium and 49.6% coarse). De La Parra, Serna Saldivar and Liu (2007) reported that cooking with lemon, roasting and tortilla frying increased the concentrations of free phenolic compounds and decreased the bounds in corn. The modifications in the types of phenolic acids present in each fraction were demonstrated by the phenolic acid profile (Table 5).

Table 5 – Effect of hydrothermal treatment in the free and bound phenolic acids profile.

Phenolic acids	Soluble in methanol (µg g^{-1})						Soluble in ethanol (µg g^{-1})						Bound (µg g^{-1})					
	Fine		Medium		Coarse		Fine		Medium		Coarse		Fine		Medium		Coarse	
	R	HT	R	HT	R	HT	R	HT	R	HT	R	HT	R	HT	R	HT	R	HT
Gallic	<LOQ	2.07	1.54	2.07	n.d	n.d	1.64	n.d	n.d	n.d	0.68	n.d	1.02	0.87	0.94	0.87	1.02	0.80
Chlorogenic	<LOQ	<LOQ	n.d	n.d	n.d	n.d	n.d	1.03	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
p-hydroxybenzoic	n.d	0.68	n.d	1.05	n.d	1,00	n.d	3.21	<LOQ	2.71	n.d	1.73	0.82	0.55	n.d	n.d	n.d	n.d
Caffeic	<LOQ	n.d	n.d	n.d	n.d	n.d	0.82	0.36	0.62	0.59	<LOQ	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Syringic	n.d	n.d	n.d	n.d	n.d	n.d	<LOQ	n.d	<LOQ	3.24	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Vanillin	<LOQ	0.67	n.d	n.d	n.d	0.57	<LOQ	2.06	n.d	n.d	n.d	n.d	4.19	1.63	1.21	0.64	n.d	n.d
p-coumaric	0.73	0.54	0.38	0.57	<LOQ	0.28	1.08	2.13	0.80	0.52	0.76	0.25	15.96	9.52	0.94	0.31	<LOQ	0.43
Ferulic	0.80	0.71	0.41	0.54	n.d	0.4	1.40	3.37	0.75	1.27	0.84	0.63	240.08	137.02	6.08	0.83	1.40	0.42

Protocatechuic was not detected. R= raw HT= heat treated n.d= not detected LOQ=limit of quantification.

The treatment provided the soluble phenolic acids identification as gallic, *p*-hydroxybenzoic acids and vanillin. Vanillin can be produced by the thermal decomposition of ferulic acid (PELEG et al., 1992) and the increase of other acids may be due to the incorporation of water during the hydrothermal treatment, which facilitated the extraction of these compounds. Some phenolic compounds are accumulated in the

cell vacuoles (CHISM; HAARD, 1996) and the hydrothermal processing can release the unavailable ones, which may explain the increase of free phenolic acids. In the study by Gutiérrez-Grijalva et al., (2016) thermal processing demonstrated the potential to increase the extraction of phenolic compounds due to the increase of free phenolic acids.

The reduction of the bound phenolic acids after the hydrothermal treatment, verified in our study, also occurred in the study of Žilić et al. (2013) ($p<0.05$), in which infrared radiation heating significantly reduced ($p<0.05$) the levels of total phenolic, ferulic and *p*-coumaric acids in corn. These reductions may be due to the polymerization of some compounds, since high temperatures during cooking may lead to the polymerization of some phenolic compounds, reducing the phenolic acids extractable content (WANG; HE; CHEN, 2014).

Processing of food containing ferulic acid may release it from cell compartments and bound forms and in this way can interact with other components, such as protein and starch, affecting food digestibility (KARUNARATNE; ZHU, 2016). Among the molecules bound by phenolic compounds, the starch shows this incorporation characteristic. Phenols are capable to form clathrate compounds with amylose molecules since the amylose molecule has six glucose units per helical turn, the helix is internally hydrophobic and the enclosed ferulic acid also has lipophilic properties (BETA; CORKE, 2004). Another author suggests that the interactions between the starch and the phenolic compounds, in addition to the formation of the type V amylose inclusion complex, may also be through the non-inclusive complex with the formation of weaker bonds (hydrogen bonding) (ZHU, 2015).

As cornmeals have high starch content (Table 2) and ferulic acid (Table 5) it is possible that this enclosed of ferulic acid by amylose occurs. The complex formed resembles the complex of lipid and amylose, which may promote resistance to starch digestion (CHEN et al., 2017) and is related to the formation of RS5.

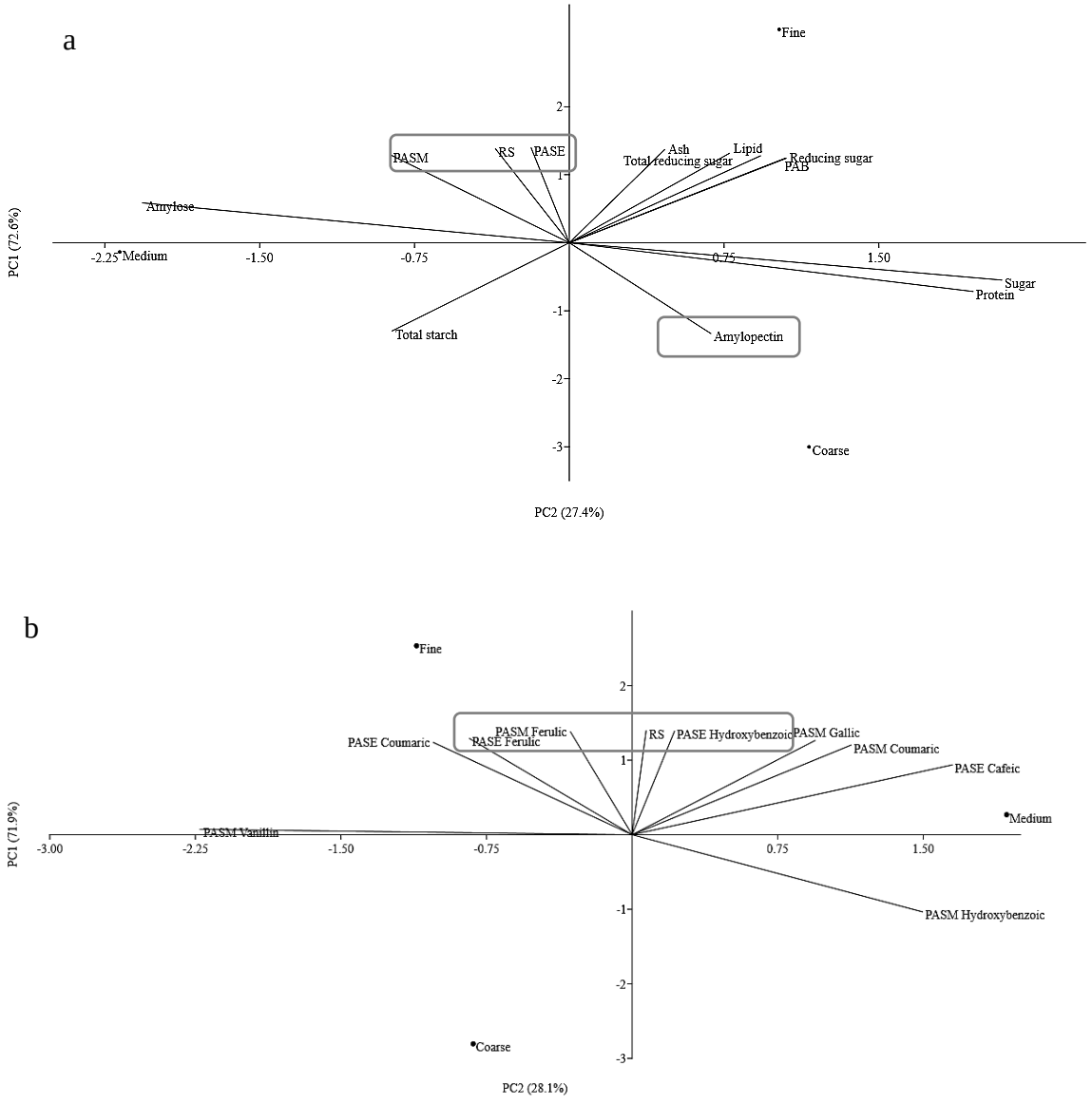
3.4 EFFECT OF CORNMEALS COMPOSITION IN THE RESISTANT STARCH FORMATION

In order to evaluate the association between RS formation and cornmeal components, multivariate analysis was employed (Figure 2a). The PC1 (principal component) (72.6%) and PC2 (27.4%) together explained 100.0% of the results variation, with the variables ashes, RS, phenolic acids soluble in ethanol (PASE) and amylopectin showed higher correlation with PC1, with R values of 0.98, 0.98, 0.99 and -0.95, respectively, and the sugar (R= 0.92) and amylose (R= -0.90) higher correlation with PC2.

The phenolic acids soluble in methanol (PASM) and ethanol (PASE) variables influenced the formation of resistant starch, because angles smaller than 90° represent a good correlation, however, the amylopectin showed a negative correlation of 0.98 with the formation of RS, according to regions shown in the graph (Figure 2a). This fact indicates that phenolic acids are mainly responsible for the formation of RS.

With PCA (Figure 2a) it was possible to verify the correlation of PASM and PASE with the formation of RS, which motivated the performance of another PCA to identify which phenolic acids affect the RS formation (Figure 2b).

Figure 2 - Effect of cornmeals constituents in the resistant starch contents (a) and effect of phenolic acids in the resistant starch formation (b).



PASM= phenolic acid soluble in methanol, PASE= phenolic acid soluble in ethanol, FAB= phenolic acid bound, RS= resistant starch.

The variables RS, PASE *p*-hydroxybenzoic, PASM and PASE ferulic contributed positively to the variability associated with PC 1. PASM vanillin (R= 0.99) showed a higher correlation with PC2. It can be observed, as pointed out by the highlighted regions in the graph (Figure 2b), that the ferulic acid content of the soluble fraction in methanol (R= 0.98) and the *p*-hydroxybenzoic acid (R= 0.99) and ferulic acid contents (R= 0.91) of the soluble fraction in ethanol showed a high positive correlation with RS formation. That is, higher the content of these phenolic acids, the greater RS content formed. It can be inferred that the hydrothermal treatment process causes a modification in the RS content, with the possible formation of RS similar to type 5, amylose-phenol complex. Zhu (2015) has shown that interactions between starch and phenolic compounds can affect the physicochemical and nutritional properties of foods, Karunaratne and Zhu (2016) verified that among phenolic acids the ferulic affects the enzymatic hydrolysis of starch granules by α-amylase.

3.5 GEL TEXTURE ANALYSIS

Cornmeals gels were subjected to texture profile analysis (Table 6). The fine cornmeal showed the lower hardness (987 g) and this cornmeal has more phenolic compounds, mainly ferulic acid (Table 5), indicating this textural property can be influenced by the cornmeal composition. Karunaratne and Zhu (2016) showed that the hardness value decreased with increasing ferulic acid content, indicating that the textural properties of gels depend on the starch constituents and amylose, the volume and deformation of the granules and the interaction between the continuous and dispersed phases (CHOI; KERR, 2003).

Table 6 - Gel textural properties of different cormeals.

Cornmeal	Hardness (g)	Adhesiveness (g.s)	Springiness	Cohesiveness
Fine	987^{c} (3.7)	-196^{a} (37.5)	0.88^{b} (0.1)	0.14^{a} (23.3)
Medium	1395^{b} (1.9)	-244^{a} (33.9)	0.85^{b} (3.1)	0.15^{a}(14.6)
Coarse	1512^{a} (0.8)	-203^{a} (27.1)	0.94^{a} (1.1)	0.16^{a} (13.9)

The mean values of triplicate analyses are given. Values with different letters differ significantly ($p<0.05$).

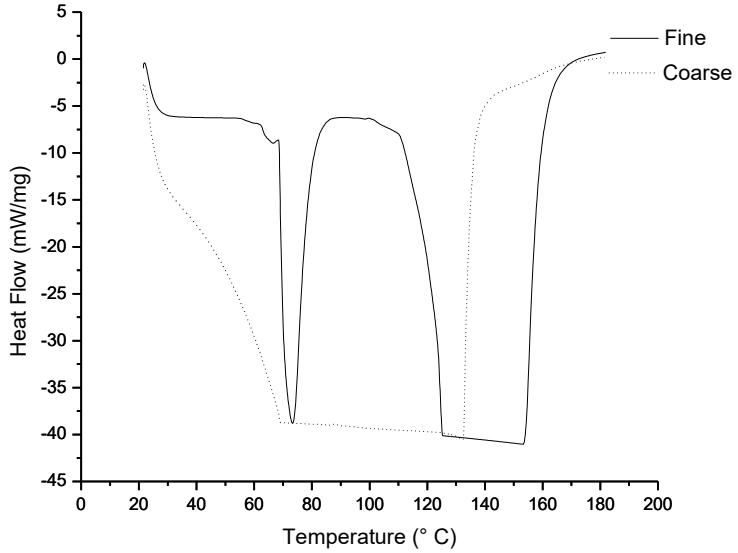
All of the TPA texture profile parameters of cornmeal were negatively correlated with RS. RS was found to be significantly associated with cohesiveness of cornmeal with R= -0.99 and p=0.07, while RS also showed a modest correlation to hardness (R= -0.92,

p=0.25) and springiness (R= -0.74, p=0.47). All of the correlations were negative, so increasing RS in the cornmeal the hardness, cohesiveness and springiness values are reduced.

3.6 GELATINIZATION CHARACTERISTICS

The thermal transitions of fine and coarse cornmeal were determined by DSC. The DSC thermograms of fine cornmeal showed two endothermic peaks (Figure 3), the first peak was attributed to the melting of amylose double helices (CHUNG; JEONG; LIM, 2003) and the second peak can be the amylose-lipid complex, which was reported to form at around 101.3 °C for corn starch (AI; HASJIM; JANE, 2013) or can be an amylose-ferulic complex. Zhu (2015) mention the melting point of ferulic acid in the presence of water (solid to water ratio 1:3) is 114 ºC as measured by DSC. Chen et al. (2017) studied starch corn and its blends with corn oil and/or soy protein and their DSC thermograms displayed two separated peaks, which were attributed to the dissociation of double helix of amylopectin and the dissociation of amylose-lipid complex, respectively.

Figure 3 – DSC thermograms of fine and coarse cornmeal.



The composition of cornmeal can greatly influence the outcome of gelatinization. Zhu et al. (2009) showed that the type and structure of starch play an important role in the interactions and also it depends on the concentration and type of the polyphenols.

4 CONCLUSION

The previous hydration of the cornmeals did not affect the RS content and the fine cornmeal had a higher RS content (202.0 mg g^{-1}) after the hydrothermal treatment. It was demonstrated that the cornmeal with smaller particle size had a higher resistant starch content and the phenolic acids influenced the formation of resistant starch. Besides, the correlation between resistant starch contents and phenolic acids was determined by principal component analysis and it was verified that the ferulic (R= 0.91) and *p*-hydroxybenzoic (R= 0.99) acids showed a positive correlation with resistant starch formation, indicating that the hydrothermal treatment promotes the formation of resistant starch similar to type 5, amylose-phenol complex. The texture profile parameters (TPA) of cornmeal were negatively correlated with RS and differential scanning calorimetry (DSC) analysis showed consistency with this behavior.

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ARTICLE IV

RESISTANT STARCH CONTENT IN COOKED CORNMEAL-BASED FOOD

MASSAROLO, K. C.; FERREIRA, C. F. J; KUPSKI, L.; BADIALE-FURLONG, E. Effects of particle size, soybean oil and water content during cooking on the *in vitro* resistant starch of cornmeal. **Nutrition & Food Science International Journal**, v. 7, p. 1-5, 2018.

Resistant starch content in cooked cornmeal-based food

ABSTRACT

Cornmeal is consumed after being submitted to some processing and preparation, which can affect the resistant starch content in the final product. Resistant starch has attracted interest due to conferring functional properties to foods. The aim of this work was to find the interference of cornmeal´s particle size on resistant starch content and to establish the conditions of cooking that promote the highest resistant starch content when preparing cornmeal based foods, such as cornmeal:water proportion and the amount of soybean oil used. To achieve the aim, the *in vitro* starch digestibility of hydrothermal treated cornmeals was evaluated by experimental design CCD 2^2, carried in order to optimize the cornmeal:water proportion and the amount of soybean oil conditions that promote the major resistant starch formation. The best condition of hydrothermal treatment was applied in cornmeals with different particle sizes. The fine-ground cornmeal showed the highest resistant starch content (201.6 mg g^{-1}). Applying hydrothermal treatment in the cornmeal:water proportion of 1:5 w/v and 3% of soybean oil, the content of resistant starch has increased significantly (21.9%). Under these conditions, the resistant starch content was increased 39.8% and 45.4% for medium-ground and coarse-ground cornmeals, respectively. These findings can inspire other studies with different cooking conditions and starch and other ingredients interactions during processing, to obtain starch-based foods with lower glycemic index.

Keywords: Amylose-lipid complex. Digestibility. Hydrothermal treatment.

1 INTRODUCTION

Corn (*Zea mays*) is used for human nutrition due to its specific physicochemical properties, such as high levels of starch, proteins with low levels of gliadin fraction, gluten free, dietary lipids, hypoallergenics, phytochemicals, dietary fiber and a delicate flavor (WÓJTOWICZ; KOLASA; MOŚCICKI, 2013).

The use of cornmeals has been increased by the demand to produce gluten free products, a protein found in the endosperm of some cereals such as wheat, barley and rye (DE LA HERA et al., 2013), which can cause health problems in some individuals in population. Cornmeals are obtained by corn milling processes (RAUSCH et al., 2009) and have different particle sizes, that can affect physicochemical characteristics and the product quality (BOLADE; ADEYEMI; OGUNSUA, 2009; SHI et al., 2016).

The main cornmeal component is starch (SOMAVAT et al., 2016), which digestibility is modified by hydrothermal treatment (CHUNG; LIU; HOOVER, 2009). Starch digestion and absorption are crucial for the control of blood sugar levels in metabolic disorders (WOLEVER; MEHLING, 2002). Therefore, resistant starches to digestive enzymes have been recommended for diets that prevent the damage of the syndrome.

Resistant starch is not digested in the small intestine, it goes to the large intestine where it is fermented by bacterial microflora (SAJILATA; SINGHAL; KULKARNI, 2006). Its content in food is related to properties such as amylose content (HSU et al., 2015; SAJILATA; SINGHAL; KULKARNI, 2006), amylopectin branch chain length (JANE et al., 2003), amylose/amylopectin ratio (LI et al., 2015), processing conditions such as milling, fermentation, quantity of water, time and temperature storage and its interactions with other compounds in food (SAJILATA; SINGHAL; KULKARNI, 2006; STUTE, 1992).

Studies shown that extrusion cooking process increases the resistant starch content in foods, with positive correlation with humidity, storage time (KIM; TANHEHCO; NG, 2006) and amylose/amylopectin ratio (LI et al., 2015); hydrothermal treatment of corn starch also promotes formation of resistant starch (CHUNG; LIU; HOOVER, 2009), particularly when preformed with lipid addition associated to high moisture contents (CHEN et al., 2017).

Interactions between starch and other compounds such as lipids have been in focus of researches due to their impacts on food properties and nutrition (CHEN et al., 2017; ZHU, 2015), as starch-lipid complexes and other similar compounds shown resistance to hydrolysis by amylases (KARUNARATNE; ZHU, 2016; SENEVIRATNE; BILIADERIS, 1991).

There is an interest on developing products with low glycemic index carbohydrates. In this context, resistant starch emerges as an alternative to reduce energy availability, therefore, the increase on its content in food promotes benefits on health and on food functional properties (ZHANG; JIN, 2011). Therefore, preparation of foods with higher resistant starch contents from cornmeals is important, so the aim of this work was to verify the cornmeal particle size (fine, medium and coarse), moisture and amount of soybean oil that promotes a higher content of resistant starch in cooked cornmeal-based food.

2 MATERIALS AND METHODS

2.1 REAGENTS AND SAMPLES

Cornmeals with different particle sizes (fine, medium and coarse) and soybean oil where obtained on Rio Grande - RS, Brazil's local market. Fungal alpha-amylase enzyme (30 U mg^{-1}) produced by *Aspergillus oryzae* was obtained from Novozymes Corp., Sigma-Aldrich Co., Switzerland, fungal protease enzyme (500 U g^{-1}) produced by *Aspergillus oryzae* was obtained from Novozymes Corp., Sigma-Aldrich Co., Denmark and fungal amyloglucosidase enzyme (6 U mL^{-1}) produced by *Aspergillus niger* was obtained from Sigma-Aldrich Co., Germany.

2.2 CORNMEALS CHARACTERIZATION

Particle-size distribution of fine, medium and coarse grounded cornmeals was determined using sieve openings of 0.71; 0.50; 0.35; 0.25 and 0.14 mm. Composition was determined based on their moisture (AOAC 934.01), crude protein (AOAC 955.04C; conversion factor 5.75), ash (AOAC 900.02) and lipid (AOAC 920.85) content (AOAC, 2000).

Amylose content was determined according to colorimetric method by Martinez e Cuevas (1989), adapted. Extraction of cornmeal's amylose was performed with ethyl alcohol 95% (v/v) and NaOH 1M solution. Colorimetric reaction was performed with iodine 2% solution (w/v) and the amylose content was determined using amylose standard curve (4-24 µg mL^{-1}) in a spectrophotometer with a wavelength of 620 nm.

The total starch content of the solution of each gelatinized sample was determined by iodometry and the starch-iodine complex was quantified in a spectrophotometer at 620 nm using a starch standard curve (0.05-0.21 mg mL^{-1}) (GARDA-BUFFON; BARAJ; BADIALE-

FURLONG, 2010). Amylopectin was estimated by the difference between total starch and amylose.

2.3 EXPERIMENTAL DESIGN TO STUDY THE EFFECT OF THE ADDITION OF SOYBEAN OIL AND WATER ON THE RS FORMATION DURING HYDROTHERMAL TREATMENT

The hydrothermal treatment was performed by electric plate cooking, according to Giacomelli et al. (2013). The fine cornmeal was added in boiling water with sodium chloride (1%) and homogenized until the end of the treatment. The electric plate temperature was maintained at 120 °C for 40 min. The variables cornmeal:water proportion and amount of soybean oil were defined by CCD 2^2 (Table 1), with the RS content as the response variable.

2.4 RESISTANT AND AVAILABLE STARCH

The determination of available and RS was performed according to AOAC method 996.11 modified by Walter, Silva and Perdomo (2005). The *in vitro* method quantifies the RS after the removal of starch digestible fraction by enzymatic treatment, simulating the hydrolysis that occurs in the digestive tract (mouth, stomach and small intestine), with α-amylase, protease and amyloglucosidase enzymes. The remaining starch (resistant) was solubilized with dimethylsulfoxide and again hydrolyzed by amylolytic enzymes.

The determination of the starch fractions (available and resistant) after hydrolysis was performed by quantifying the glucose released in each step using the 3,5 dinitrosalicylic acid (DNS) reduction (MILLER, 1959) and using a conversion factor of 0.9, to convert free glucose into starch.

2.5 STATISTICAL ANALYSIS

Data analysis of variance (ANOVA), followed by Tukey Mean-Difference test, were performed in *Statistica* 6.0 program. Differences with probability value of $p<0.05$ were considered significantly.

3 RESULTS AND DISCUSSION

3.1 CORNMEAL CHARACTERIZATION

The particle-size distribution for the 3 types of cornmeals is as described on the packaging by the supplier. The coarse grounded presented 78.6% of the particles larger than 0.35 mm, the medium had 58.7% of the particles with sizes between 0.25 and 0.50 mm and the fine 88.8% of the particles smaller than 0.35 mm (Table 2).

Table 2 – Particle-size distribution of cornmeals.

Particle-size	**Flours**		
	Coarse	**Medium**	**Fine**
Between 0.71 and 0.50 mm (%)	8.5 (0.1)	0	0
Between 0.50 and 0.355 mm (%)	70.1 (0.0)	26.0 (0.1)	11.2 (0.1)
Between 0.355 and 0.25 mm (%)	20.4 (0.0)	32.7 (0.0)	54.1 (0.0)
Between 0.25 and 0.147 mm (%)	0.6 (0.4)	21.1 (0.0)	18.8 (0.1)
Smaller than 0.147 mm (%)	0.4 (0.4)	20.2 (0.1)	15.9 (0.1)

Results expressed as mean (RSD) n=3. RSD= relative standard deviation.

In addition to the difference in particle size, cornmeals present a difference in its composition. The fine cornmeal had a higher content of ash (0.9%) and lipids (2.8%) when compared to other cornmeals (Table 3).

Table 3 – Cornmeal composition.

Cornmeal	**Ash (%)**	**Lipid (%)**	**Protein (%)**	**AS (%)**	**AP (%)**	**Relation AS/AP**	**AT (%)**
Fine	0.9^{a}	2.8^{a}	7.4^{a}	27.2^{a}	54.2^{b}	0.50^{b}	81.4^{b}
Medium	0.4^{b}	0.3^{b}	6.9^{a}	31.4^{a}	57.3^{ab}	0.55^{b}	88.8^{a}
Coarse	0.2^{c}	0.1^{b}	8.0^{a}	23.7^{a}	66.0^{a}	0.36^{a}	89.7^{a}

Results expressed as mean (n=3). Different superscript letters in the same column indicate significant differences ($p<0.05$). AS=amylose, AP=amylopectin, AT=total starch

There is no significant difference ($p> 0.05$) in the protein content of the cornmeals, in the study by Shi et al. (2016) similar results were verified since the milling process does not affect the primary structure and the protein content in cornmeal.

The coarse cornmeal has the highest content of amylopectin (66.0%). The same cornmeal had the lowest amylose/amylopectin ratio (0.36), the medium cornmeal had the highest value (0.55), followed by the fine cornmeal (0.50). The amylose/amylopectin ratio in the cornmeal composition may contribute to changes in RS content (LI et al., 2015). In this way, the fine and medium cornmeal, because of the higher amylose/amylopectin ratio, have a higher potential to form a higher RS content.

3.2 OPTIMIZATION OF THE HYDROTHERMAL TREATMENT FOR RS FORMATION

The results of RS in the CCD for the 2 variables under study (soybean oil amount, and proportion of cornmeal:water) are shown in Table 1.

Table 1 – Matrix of the central composite design (coded and real values) with the response in terms of resistant starch.

Trial	**X_1**	**X_2**	**RS (mg g^{-1})**
1	-1 (0.00)	-1 (1:5)	201.0
2	+1 (0.30)	-1 (1:5)	240.1
3	-1 (0.00)	+1 (1:15)	119.1
4	+1 (0.30)	+1 (1:15)	83.2
5	0 (0.15)	-1 (1:10)	122.5
6	0 (0.15)	-1 (1:10)	126.2
7	0 (0.15)	+1 (1:10)	129.9

X_1:Soybean oil amount (g); X_2: Proportion of cornmeal:water; RS= resistant starch

In this design, the RS content ranged from 83.2 to 240.1 mg g^{-1}, however the best result for the RS was found in trial 2, which showed less proportional cormeal:water, at lower level (1:5) and more soybean oil amount at higher level (0.3g).

The proportional cornmeal:water was found to be the most relevant variable for RS content (Table 4). The RS content decreased on an average of 119.4% by increasing the proportional cornmeal:water from 1:5 to 1:15. The combined effect of soybean oil amount and proportional cornmeal:water resulted in an average decrease of 37.5% in the RS. The soybean oil amount did not present any significant effect ($p>0.05$).

Table 4 – Main effects and interaction analysis for RS.

Factor	Effect (%)	Standard error	*t*-Value	*p*-Value
Media	**145.98**	**1.39**	**104.96**	**<0.01[a]**
X_1 (L)	1.6	3.68	0.43	0.70
X_2 (L)	**-119.40**	**3.68**	**-32.45**	**<0.01[a]**
X_1 x X_2	**-37.49**	**3.68**	**-10.19**	**0.01[a]**

[a] significant factor $p<0.05$. X_1: Soybean oil amount (g); X_2: Proportion cornmeal:water.

A variance analysis (ANOVA) was performed using the significant effects only (Table 5).

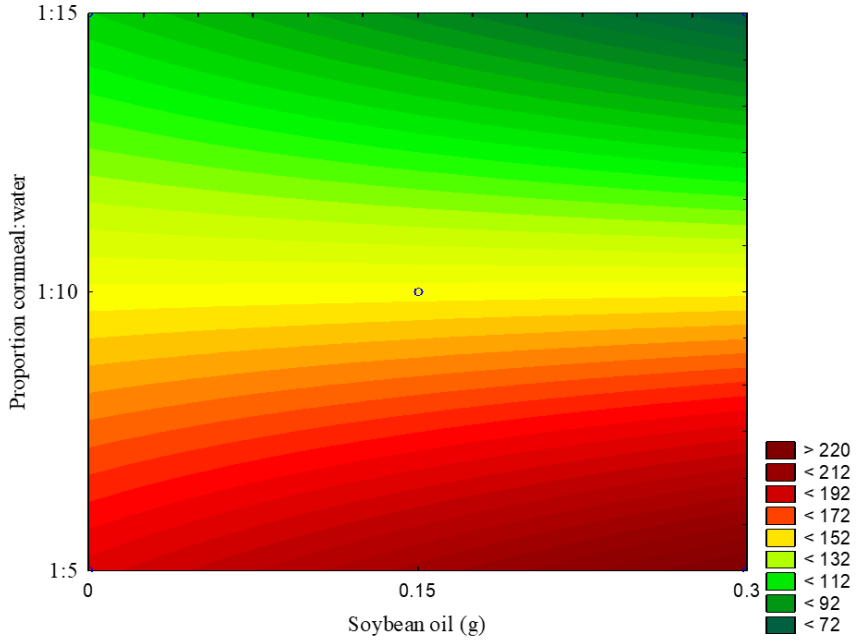
Table 5- ANOVA to the RS formation to CCD.

Variation	Sum of squares	Degrees of freedom	Means squares	*F*-value
Regression	17725.6	4	4431.4	327.28[a]
Residual	27.08	2	13.54	
Total	17752.69	6		

Regression coefficients= 0.88; $F_{0.95;\,4;\,2}$ =19.25; [a] *F*-value (regression/residual).

Based on the *F*-test, the model is predictive, since its $F_{calculated}$ (327.28) was greater than F_{tabled} (19.25); and significant, once the regression coefficient (0.88) is close to unity. The coded model was used to generate the contour diagram (Figure 1).

Figure 1 - Contour diagram of RS content as a function of soybean oil amount and proportion cornmeal and water.



$$RS\ (mg\ g^{-1}) = 145.9-119.4X_2-37.5X_1.X_2 \quad (1)$$

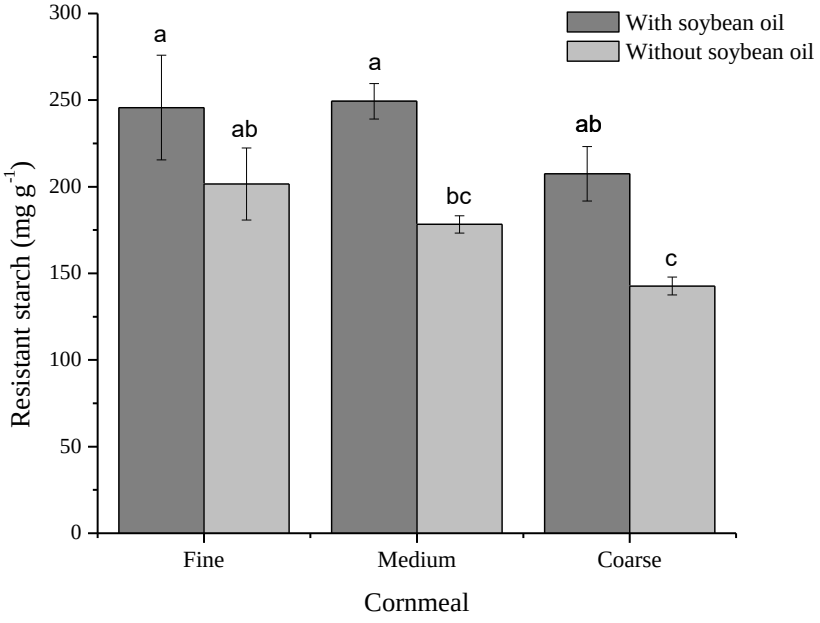
The greater values of the significant variables were not determined (X_2 and interaction X_1 and X_2) because there is no possibility to reduce water in the hydrothermal treatment, which simulates the cornmeal domestic cooking process. Therefore, the proportion of cornmeal:water was defined as 1:5 and soybean oil amount 3%.

These hydrothermal treatment conditions were applied for the 3 cornmeals (fine, medium and coarse) and also treatment without oil were realized to evaluate the soybean oil addition effect on the RS content. Factor analysis showed that both the oil addition and cornmeal particle size had a significant difference ($p<0.05$). When the treatment without oil was carried out, the fine cornmeal showed higher RS content (201.6 mg g^{-1}) and the coarse cornmeal lower content (142.7 mg g^{-1}) (Figure 2).

Lower digestibility of starch with smaller particles (greater RS) was also verified in the study by (GUO et al., 2018), in which the *in vitro* starch digestibility from milled durum wheat grains with different particle sizes was evaluated. In it, the digestibility was greater for the cooked flour with larger granules (0.25-0.50 mm) than for the flours with smaller particle sizes (0.15-0.25 mm and less than 0.15 mm). Therefore, higher interactions between the components of the cornmeal during heating occur in flours with the smaller particle size (GUO et al., 2018).

Besides the particle size, the fine cornmeal composition may contribute to the higher RS content, since this cornmeal had higher lipid content (Table 2) that can interact with the starch during the hydrothermal treatment and form an amylose-lipid complex, forming RS type 5.

Figure 2 – Influence of soybean oil in the RS content.



For the 3 cornmeals, the highest RS content was verified in the treatment with oil addition (Figure 2), with higher content of RS to fine cornmeal (245.7 mg g^{-1}) and medium (249.3 mg g^{-1}) and lowest to coarse (207.5 mg g^{-1}), increases were 21.9%, 39.8% and 45.4%, respectively.

A study with different food lipids (triglycerides, phospholipids and free fatty acids of different chain-lengths and numbers of double bonds) showed that all lipids, after cooking with starches, promoted a significant decrease in starch hydrolysis, except for corn starches waxy low amylose (AI; HASJIM; JANE, 2013). In the study by Chen et al. (2017) the addition of corn oil and soy protein decreased the rapidly digestible starch content and increased the sum of the slowly digestible starch and RS content. It has been demonstrated that the physical barrier of corn oil, amylose-lipid complex and protein-starch matrix can promote resistance to starch digestion (CHEN et al., 2017; SENEVIRATNE; BILIADERIS, 1991). This fact can be interesting for the elaboration of food products with reduced glycemic content.

4 CONCLUSION

The cornmeal particle size interferes in the RS content, however the proportion cornmeal:water was the variable more relevant, followed by combined effect of oil amount and proportion cornmeal:water. All cornmeals, after hydrothermal treatment with soybean oil, showed an increase in the RS content in the final product, it can be associated the production of RS type 5. The interaction between starch and oil may contribute to obtaining carbohydrate-based food with low glycemic index.

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ARTICLE V

INCREASING THE RESISTANT STARCH CONTENT IN CORN-BASED PRODUCTS THROUGH EXTRUSION

Increasing the resistant starch content in corn-based products through extrusion

ABSTRACT

Resistant starch (RS) has gained attention for its potential health benefits and functional properties. The purpose of this study was to find extrusion conditions that can increase the RS content in cornmeal, leading to improved functionality of corn products. The highest RS content was achieved at 210 rpm screw speed and 22% initial moisture. The amount of RS in the extrudate was reduced by the process, when compared to the initial cornmeal. However, the RS content in the extruded cornmeal added with 20% high-amylose corn starch (HACS) was significantly increased (by 21%) when compared to the product extruded in the absence of HACS. The physical (diameter and expansion ratio) and functional (water absorption) properties of the extrudates were not significantly affected by the addition of HACS. Thermogravimetric analysis (TGA) indicated that the RS in extruded cornmeal is a retrograded starch. The proposed extrusion conditions, along with addition of 20% HACS, showed to be a promising processing alternative to increase the RS content in extruded products. This study contributes to a better understanding of the effect of extrusion conditions and addition of HACS on the production of resistant starch in corn extruded products.

Keywords: Cornmeal. Extrusion. High-amylose. Thermogravimetric analysis.

1 INTRODUCTION

Resistant starch (RS) is a fraction of starch that cannot be digested by α-amylase enzymes and absorbed in the small intestine. This carbohydrate has been further classified into five types: physically inaccessible starch (RS1); some types of raw starch granules and high-amylose starches (RS2); retrograded starch, either processed from unmodified starch or resulting from food processing applications (RS3); chemically modified starch (RS4) (ENGLYST; HUDSON, 1996); and lipid-amylose complexes (RS5) (HASJIM et al., 2010). Of these, RS3 has been of special interest due to its thermal stability, which allows it to maintain its stability during the food preparation process (FARAJ; VASANTHAN; HOOVER, 2004).

Due to its structural characteristics, resistant starch is not absorbed in the small intestine and passes almost entirely intact through this part of the digestive tract, reaching the large intestine where it is fermented by colonic bacteria (RAATZ et al., 2016). Further, RS has been shown to reduce diabetes, occurrences of obesity, and cardiovascular disease and (2) to prevent the development of colon cancer (HASJIM et al., 2010; HU et al., 2016). Based on these health benefits and functional properties, mechanisms to increase the content of RS in food are of general interest (CHEN et al., 2017; MUTLU; KAHRAMAN; ÖZTÜRK, 2017). Different processing technologies, such as extrusion, can reduce or eliminate RS1 and RS2, but potentially form RS3, particularly if high amylose content starches are used (MUTLU; KAHRAMAN; ÖZTÜRK, 2017) or optimized extrusion conditions are employed (FARAJ; VASANTHAN; HOOVER, 2004; KIM; TANHEHCO; NG, 2006).

Extrusion cooking is a continuous high-temperature and short-time process, which physically modifies starch through the combination of high temperature, pressure and shear forces. This process is used to obtain expansible starchy products, snacks and corn flakes with a wide array of both organoleptic (SINGH; GAMLATH; WAKELING, 2007) and functional properties (VON BORRIES-MEDRANO et al., 2018). Corn is considered one of the most suitable cereals for commercial production of extruded products because of its low cost and good expansion properties due its high starch content.

Different chemical changes can take place during extrusion, including gelatinization of starch molecules, loss of crystallinity, molecular degradation of amylose and amylopectin, and retrogradation (YAN et al., 2019). The degree of retrogradation of different types of starches depends on the quantity of amylose released during the gelatinization process, the interactions between chains of amylose-amylose and amylose-amylopectin, as well as the segmented movement of chains of amylose and amylopectin (AMBIGAIPALAN et al., 2013).

The retrogradation of gelatinized starch induces the formation of resistant starch type 3 (YAN et al., 2019).

In this study the hypothesis is that the formation of RS can be improved by extrusion conditions and addition of amylose into the cornmeal used for extrusion. Therefore, the objectives of this research were as follows: 1) to optimize the conditions of extrusion (screw speed and moisture) for the production of RS in corn-based extrudates; 2) to evaluate the effect of adding high-amylose corn starch into cornmeal before extrusion on the RS values of extrudates; 3) to evaluate physical and functional properties of corn-based extrudates; and 4) to evaluate the levels of starch retrogradation in extruded products using the thermogravimetric analysis (TGA).

2 MATERIAL AND METHODS

2.1 SAMPLE PREPARATION

Commercial coarse cornmeal (Lincoln, NE, USA) was used. The moisture content of the cornmeal was adjusted to 21%, 22%, 25%, 28% and 29%, in wet basis. The samples were kept in sealed plastic bags and stored at 4 °C for 24 h to reach a homogeneous moisture distribution. Before extrusion, cornmeal samples were allowed to reach ambient temperature (25 °C), homogenized and tested to confirm the desired adjusted moisture content using a moisture analyzer (Model MB27, Ohaus) set at 105 °C.

2.2 EXTRUSION OF CORNMEAL

A single-screw extruder laboratory-scale GR-8 (C.W. Brabender Instruments, South Hackensack, NJ, U.S.A.) with a nozzle diameter of 3 mm and compression 3:1, was used for sample processing. The temperature of the first zone (feed) was set to 50 °C, whereas the second (transition) and third (metering) zones were set to 140 °C and 160 °C, respectively (JACKSON et al., 2011). Five different screw speeds were used during the experiment (Table 1).

For each trial (sample), 800 ± 1 g of cornmeal was extruded, and parameters including torque and product temperature during extrusion were recorded by the data acquisition system of the extruder. After stable extruder conditions were established, the extruded product was collected and stabilized at room temperature (25 °C) for 24 h, then ground and sealed in polyethylene bags until subsequent testing.

2.3 EXPERIMENTAL DESIGN

A Central Composite Rotatable Design (CCRD) was applied for the experiment with two independent variables, feed moisture content (X_1) and screw speed (X_2), using STATISTICA® software (Statsoft, USA). The levels of each variable were established according to preliminary trials for suitable extrusion cooking. The 2^2 CCRD design was used, consisting of 4 assays (−1 and +1), 3 center points and 4 axial points (−1.414 and +1.414), and resulted in an orthogonal distribution, for a total of 11 experiments.

The experimental plans were obtained from the Central Composite Rotatable Design (CCRD 2^2), from which a set of variable combinations (Table 1) were performed. The results were analyzed using the response surface method and the experimental results were used to determine a statistical mathematical model as a function of all the influenced factors.

Table 1 - Central Composite Rotatable Design (CCRD 2^2)

Variables	**Coded levels**				
	-1.414	-1	0	1	1.414
Screw speed (rpm)	90	110	160	210	231
Moisture (%)	21	22	25	28	29

2.4 EFFECT OF AMYLOSE ON *in vitro* DIGESTIBILITY OF EXTRUDED CORN-BASED PRODUCTS

Cornmeal was extruded in the presence (20%, w/w) and absence of high-amylose corn starch (HYLON VII), using the optimal extrusion conditions as defined by the CCRD 2^2. These experiments had the objective to measure the effect of amylose on the amount of resistant starch (RS) produced by extrusion, as well as on the characteristics of the extrudates. Hylon VII is an unmodified corn starch derived from high amylose corn and it contains approximately 70% amylose. The inclusion level used (high-amylose starch at 20%) was established in preliminary trials. The experiments in the presence and absence of high-amylose starch were performed in duplicate.

2.5 PHYSICAL-CHEMICAL CHARACTERISTICS OF EXTRUDED PRODUCTS

2.5.1 Moisture

Moisture of ground extrudates (5 ± 0.05 g) was measured using a moisture analyzer (Model MB27, Ohaus) set at 105 °C, in triplicate.

2.5.2 Resistant and digestible starch

Resistant and digestible starch contents of cornmeal and the extrudates were measured according to AOAC method 996.11 modified by Walter, Silva and Emanuelli (2005). The *in vitro* method quantifies the RS after the removal of the digestible starch fraction by enzymatic treatment, and simulating the hydrolysis that occurs in the digestive tract (mouth, stomach and small intestine) with α-amylase, protease and amyloglucosidase enzymes. The remaining starch (resistant) was solubilized with dimethyl sulfoxide and further hydrolyzed by amylolytic enzymes.

The determination of the starch fractions (resistant and non-resistant) after hydrolysis was performed by quantifying the glucose released in each step via glucose oxidase assay (GAGO-20 Sigma). Glucose values were then converted to starch using the conversion factor of 0.9. Testing was performed in triplicate.

2.5.3 Expansion ratio (ER)

The expansion ratio of the extruded product in the presence and absence of high-amylose corn starch was determined as the diameter of dried extrudate divided by the die diameter (PAN; ZHANG; JANE, 1998). The diameter of the extruded product was measured with a caliper and the ER calculated based on the mean diameter of 15 random extruded samples.

2.5.4 Water absorption index (WAI)

The WAI of samples extruded in the presence and absence of high-amylose corn starch was determined according to Anderson (1982). Briefly, 2.5 g of ground sample were suspended in 30 mL of water at 30±1 °C in a 50 mL tared centrifuge tube. Samples were stirred intermittently for 30 min and centrifuged at 3000 *x* g for 10 min. The supernatant liquid was

removed and the remaining gel was weighed. The WAI (g g^{-1} dry solid) was calculated as the ratio of the weight of the gel after removal of supernatant to the dry weight of the sample. Analyses were done in triplicate.

2.5.5 Thermogravimetric analysis (TGA)

The thermogravimetric analysis of cornmeal and extruded cornmeal samples was performed by using the TGA-50 (Shimadzu, Japan). The analysis was performed under nitrogen atmosphere from 30 °C to 500 °C at a heating rate of 10 °C min^{-1} (TIAN et al., 2011).

2.6 STATISTICAL ANALYSIS

Statistical analysis was performed at 95% significance level, considering the resistant starch content of extruded products as the response to the studied variables. The experimental results were used to determine a mathematical model that explains RS levels in extruded product, as a function of all the variables evaluated. Statistical comparisons between average values were performed using the Student t-test (two-sided) to evaluate the effect of high-amylose corn starch on the RS content and physical-chemical characteristics of extruded products. The results associated with the TGA analysis were evaluated using ORIGIN 8.0 (OriginLab Inc., USA).

3 RESULTS AND DISCUSSION

3.1 EXTRUSION OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY

The results for resistant starch in samples extruded according to the Central Composite Rotatable Design (CCRD) for the 2 variables under study (screw speed and moisture) are shown in Table 2.

Based on the results for this experimental design, the resistant starch content of the samples ranged from 6.9 to 8.2%. The main effect can be estimated by evaluating the difference in process performance caused by a change from low (−1.414) to high (+1.414) levels which provides a model that fulfills the resistant starch content (Table 3).

Table 2 - Matrix of the central composite rotatable design (coded and real values) with the response in terms of resistant starch.

Trial	Variable		Response
	Screw speed (rpm)	**Moisture content (%)**	**Resistant starch (%)**
1	110 (-1)	22 (-1)	7.9
2	210 (1)	22 (-1)	8.2
3	110 (-1)	28 (1)	7.6
4	210 (1)	28 (1)	7.6
5	90 (-1.41)	25 (0)	7.8
6	231 (1.41)	25 (0)	8.0
7	160 (0)	21 (-1.41)	7.8
8	160 (0)	29 (1.41)	7.2
9	160 (0)	25 (0)	7.1
10	160 (0)	25 (0)	6.9
11	160 (0)	25(0)	7.1

The *p* value was used to verify the significance of the factors under study and the analysis of variance (ANOVA) was performed using the significant effects only. ANOVA results are included in Table 3. Based on the *F*-test, the model is predictive ($F_{calculated}$ (45.65) greater than F_{table} (4.35)) and significant (regression coefficient = 0.95).

Table 3 - Coefficients of variables in the predictive model for response variables.

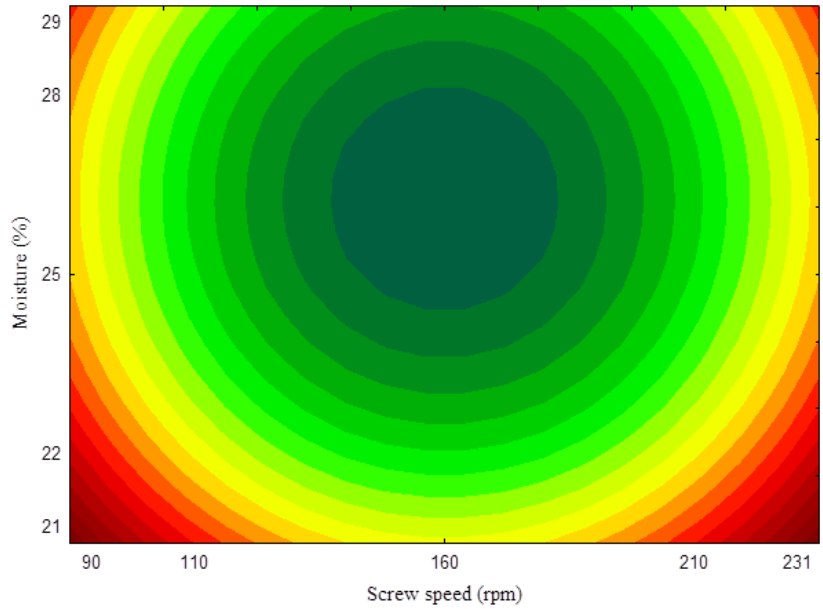
Variables	Resistant starch
Mean	**7.04[a]**
Screw speed (L)	0.12
Screw speed (Q)	**0.94[a]**
Moisture (L)	**-0.42[a]**
Moisture (Q)	**0.50[a]**
Screw speed x Moisture	-0.11
Model (F-value)	45.65
F-tabled	4.35
R^2	0.95

[a] significant factor (p<0.05). L = linear Q = quadratic

The surface response plot (Figure 1**)** based on the results obtained shows two different regions that contribute to an increased level of RS in the extrude product. One of these regions is associated with lower screw speed, while the other is related to higher screw speeds. In both cases those screw speeds were running at the lowest moisture levels tested.

The content of RS in food products is highly influenced by food processing (RAIGOND; EZEKIEL; RAIGOND, 2015). The increase in resistant starch with increasing screw speed was also observed by Kim, Tanhehco and Ng (2006) when extruding pastry wheat flour. The decrease in the amount of resistant starch associated with thermally processed foods, such as extruded products, is the result of the interactions of heat, starch and water. Under these conditions, the crystalline region of the starch molecule breaks allowing water to enter, leading to granule disruption and gelatinization of the native granular structure (BOTHAM et al., 1995). The result is a starch molecule that becomes more easily accessible to the action of digestive enzymes.

Figure 1 - Surface plot of resistant starch (RS) as a function of moisture and screw speed.

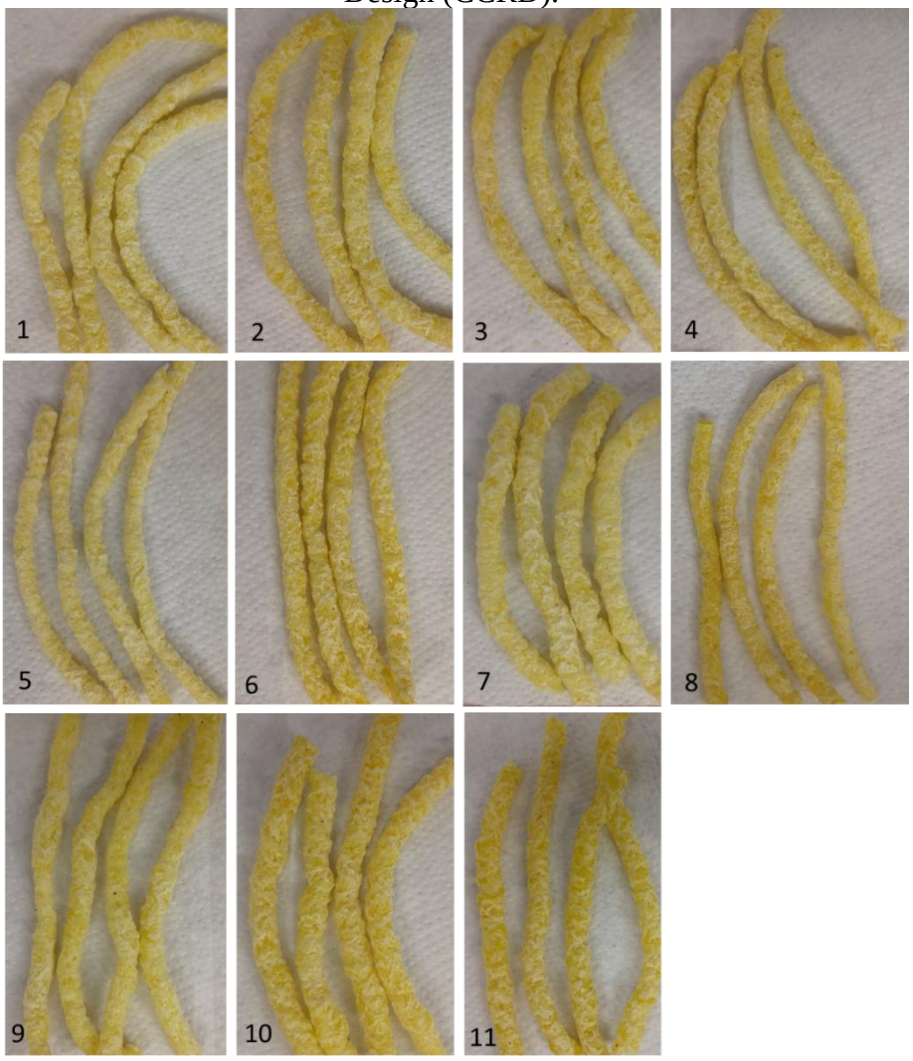


$$RS = 7.04 + 0.94X_1^2 - 0.42X_2 + 0.50X_2^2 \quad (1)$$

Based on the results and preliminary trials, the best extrusion conditions for the production of RS were 22% of moisture and 210 rpm for screw speed. Preliminary trials, trying to operate at lower levels of moisture and screw speed caused processing issues, such as clogging of the extruder, burning of material and poor product expansion. Within the ranges tested here for moisture and screw speed, the extruded products (Figure 2) showed an acceptable appearance.

Even though some of the extrusion conditions tested led to significant differences on the resistant starch content, the extruded products still showed a lower than desired RS content. Therefore, in order to further increase the amount of resistant starch in the extruded products, additional research was done. In subsequent extrusions, addition of 20% high-amylose corn starch to the cornmeal used for extrusion was considered in association with the extrusion conditions defined by the CCRD 2^2. This approach was chosen as previous studies have shown that high-amylose corn starch granules are robust and exhibit little damage and gelatinization at most extrusion conditions, resulting in low digestibility of the extrudate (SHRESTHA et al., 2010; ZHANG et al., 2015).

Figure 2 - Appearance of extruded products obtained from the Central Composite Rotatable Design (CCRD).

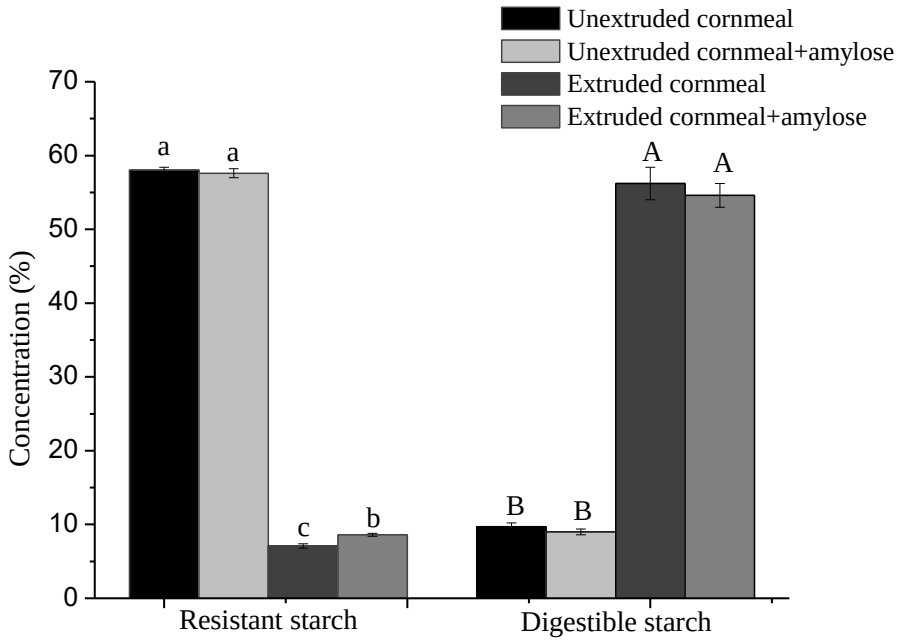


(1) 110 rpm screw speed and 22% moisture, (2) 210 rpm screw speed and 22% moisture, (3) 110 rpm screw speed and 28% moisture, (4) 210 rpm screw speed and 28% moisture, (5) 90 rpm screw speed and 25% moisture, (6) 231 rpm screw speed and 25% moisture (7) 160 rpm screw speed and 21% moisture, (8) 160 rpm and 29%, (9-11) 160 rpm screw speed and 25% moisture.

3.2 EFFECT OF AMYLOSE ON *in vitro* DIGESTIBILITY OF EXTRUDED CORN-BASED PRODUCTS

Digestibility results for non-extruded cornmeal and cornmeal with high-amylose corn starch indicated that these samples had the highest RS (57.6% and 58.0%) and the lowest digestible starch (DS) (9.7% and 9.0%) values among all samples (Figure 3). Different levels of RS have been reported on corn starch, such as 4.6% by Chung, Liu and Hoover (2009) and 17.8% by Chung, Jeong and Lim (2003); while in high-amylose corn starch those values were 60% according to Htoon et al. (2009) or 42.7% by Mutlu, Kahraman and Öztürk (2017). Part of the inconsistencies in these reported values could be attributed to the differences in the *in vitro* method used: boiling step used to gelatinize the starch, the use of heat-stable α-amylase, or the use of gravimetric assays. All of these differences contribute to make any direct comparison difficult.

Figure 3 - Digestibility of unextruded and extruded cornmeal, in the presence or absence of high amylose corn starch.



Bar values with different lower letters differ significantly ($p<0.05$) resistant starch and different capital letter differ significantly ($p<0.05$) digestible starch.

The extrusion process increased the DS in the cornmeal from 9.7% to 56.2% and in the cornmeal with high-amylose corn starch from 9.0% to 54.6%. Extrusion cooking generally increases starch digestibility because it disrupts the physical structure of the starch granule and cause gelatinization. Starch granules are broken by the action of thermal and mechanical energy

in the extrusion process, increasing the accessibility of amylases during digestion (HAGENIMANA; DING; FANG, 2006).

Even though, as discussed, digestibility of starch improves during cooking, not all of the starch present in a food product is digestible (RAIGOND; EZEKIEL; RAIGOND, 2015). Any starch present in the diet, that escapes digestion and absorption in the small intestine, is called resistant starch. RS can be found in both raw and processed food materials, the difference is the type of RS. The type RS1 is physically inaccessible to digestion due to its entrapment in a non-digestible matrix; while RS2 is an ungelatinized starch, usually present in raw ingredients. Another type of RS, RS3 is a retrograded starch (mainly amylose) formed during cooling of gelatinized starch, and it can be found in processed foods. When considering those fractions of starch that can withstand digestion, consumption of products rich in RS3 is advantageous to diabetics, since this type of starch does not contribute to an increase in blood glucose (HASJIM et al., 2010). Also, Ranhotra, Gelroth and Glaser (2002) showed a significant lowering of blood cholesterol and triglyceride levels in hamsters fed with resistant starch.

In foods, most of the starch is consumed in the gelatinized form. In this study, the amount of RS in the extruded product was lower than the levels found in the raw ingredient (cornmeal), which was somewhat expected since extrusion leads to starch gelatinization and increased digestibility (Figure 3). Food processing that involves heat and moisture, in most cases, destroys RS1 and RS2, but may form RS3. Cornflakes are good examples of products where RS3 is formed during extrusion (RAIGOND; EZEKIEL; RAIGOND, 2015). In cornflakes, after cooling the gelatinized starch, the amylose and amylopectin chains undergo a molecular reassociation process and, as a result, may become resistant to enzymatic digestion (HAGENIMANA; DING; FANG, 2006).

The RS content in the extruded cornmeal with 20% high-amylose corn starch was significantly increased (by 21%) when compared to the RS content of extruded cornmeal without high-amylose. The results support previous findings where the RS levels were increased with higher amylose contents (ROBIN et al., 2016). High-amylose starches are resistant to hydrolysis by digestive enzymes due to their low water absorption, which leads to low gelatinization when they are used to produce cooked low-moisture foods (BRUMOVSKY; THOMPSON, 2001). Furthermore, a higher content of RS in products made with cornmeal and high-amylose starch could be attributed to the inherent high resistance to thermal treatment and mechanical shear of the crystalline structures of the high-amylose starch granules (ROBIN et al., 2016).

3.3 PHYSICAL-CHEMICAL CHARACTERISTICS OF EXTRUDED PRODUCTS

During the extrusion, some processing parameters were measured and they are included in Table 4**.** Also, after the extrusion, the physical-chemical characteristics of the extruded products were measured and the results are shown in Table 5. The torque applied to the dough by the extruder was higher in trials 2, 5 and 6 (Table 4), which used the highest (210 and 231 rpm) and lowest screw speed (90 rpm), respectively. All of these trials were run at a moisture level below 25%. The higher torque applied by the extruder, under these conditions, indicates a more viscous dough with higher shear forces.

In general, the product temperature was above 184 °C and the final moisture ranged from 5.6% to 6.9%. The reduction in product moisture is caused by flash evaporation of water as the product leaves the extruder. Due to changes in pressure experienced by the product as it leaves the equipment, not only moisture is reduced by also product is expanded (MOŚCICKI; VAN ZUILICHEM, 2011). In the extrusion process, an increase in the screw speed may result in an increase of shear rate, leading to higher product expansion. And this was observed within the conditions tested in this research. However, when the screw speed was increased over a critical value (210 rpm), the product tends to have both lower residence time in the equipment and degree of gelatinization, which results on a decreased ER (Table 4).

Table 4 - Parameters measured during and after extrusion when following the Central Composite Rotatable Design (CCRD).

	Variable		**Extruder**		**Corn-based Extruded Product**		
Trial	**Screw speed (rpm)**	**Moisture (%)**	**Torque (Nm)**	**Temperature (°C)**	**Moisture (%)**	**Diameter (mm)**	**Expansion ratio**
1	110	22	32.6	197	5.6	5.40	1.80
2	210	22	52.0	196	5.8	5.87	1.96
3	110	28	20.7	187	6.0	5.00	1.67
4	210	28	41.6	195	6.3	5.43	1.81
5	90	25	46.9	194	6.1	4.97	1.66
6	231	25	50.0	188	6.2	5.67	1.89
7	160	21	22.9	196	6.1	6.27	2.09
8	160	29	18.1	187	6.1	4.97	1.66
9	160	25	19.0	186	5.8	6.43	2.14
10	160	25	40.0	194	5.8	6.30	2.10
11	160	25	21.0	184	5.7	6.57	2.19

Water is an essential reaction component in gelatinization and plays a key role in controlling extrudate expansion. Increased water content in the ingredients would soften the amylopectin structure, reducing its elastic characteristics, and resulting in a lower degree of starch gelatinization and lower expansion (CHIU et al., 2013; LAUNAY; LISCH, 1983). Indeed, some authors using different formulations, indicated that higher feed moisture content led to decreased radial expansion ratio (OKE; AWONORIN; WORKNEH, 2013; ONWULATA; KONSTANCE, 2006). Similar results were observed in this research, as an increase in the moisture content of the cornmeal used for extrusion was associated with decreased ER (Table 4).

The physical (diameter and expansion ratio) properties of the extrudates were not affected by the addition of high-amylose corn starch to the cornmeal used for extrusion. However, the functional property (water absorption) was reduced when amylose was added (Table 5).

Table 5 - Parameters measured in the presence and absence of 20% high-amylose starch.

Treatment	Moisture (%)	Diameter (mm)	ER	WAI (g g^{-1} dry solid)
Cornmeal - Extruded	6.82 (0.18)^{b}	5.9 (0.74)^{a}	1.98 (0.25)^{a}	5.18 (0.24)^{a}
Cornmeal and 20% hylon VII - Extruded	6.83 (0.20)^{b}	5.9 (0.71)^{a}	1.97 (0.24)^{a}	4.92 (0.04)^{b}
Cornmeal - Raw	22 (0.19)^{a}	-	-	2.60 (0.03)^{c}
Cornmeal and 20% hylon VII - Raw	22 (0.23)^{a}	-	-	2.52 (0.03)^{c}

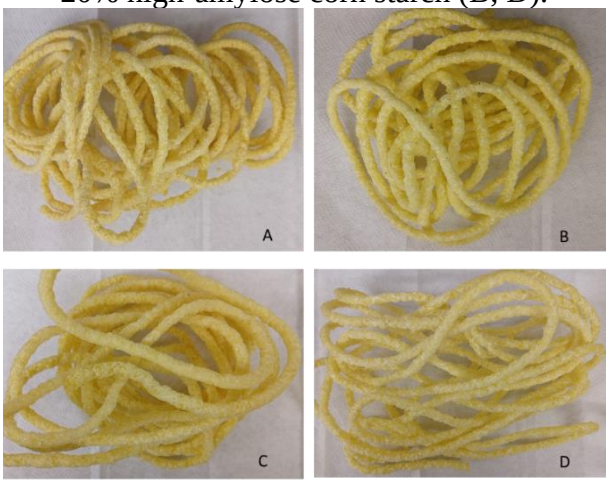
Results expressed as mean (SD) SD= standard deviation (n=6). Values with different letters in the same column show significant ($p<0.05$) differences between treatments.

In general, the WAI was higher in the extruded products, when compared to non-extruded products. This characteristic has been generally attributed to the higher dispersion of starch, in excess water, observed with thermally processed starch-based products. Researchers have shown that starch dispersion increases with the degree of starch damage due to gelatinization during extrusion (JOZINOVIĆ et al., 2016). Figure 4 shows some images of the extruded corn-based products, in the presence and absence of high-amylose starch.

Extrusion of cornmeal modifies starch digestion kinetics by creating different secondary structures in the extrudates. The amount of RS generated in the extruded corn-based product depends on the extrusion parameters, as well as the amylose content in the ingredients.

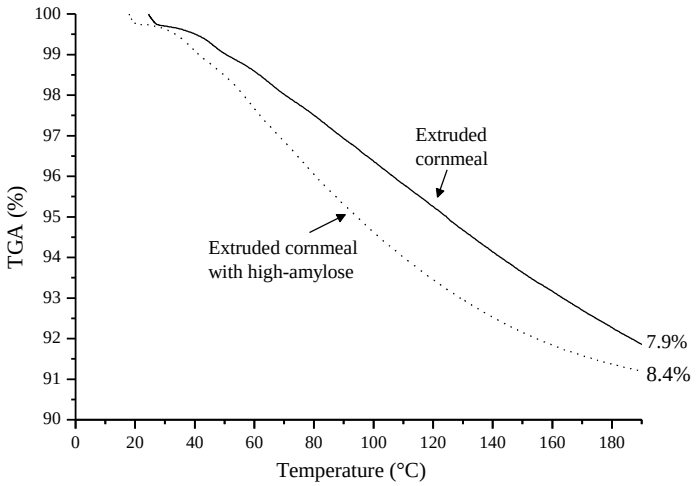
This is attributed to one or more factors, such as the amount of preserved native crystalline structures and retrograded starches in the final product.

Figure 4 - Extruded corn-based products containing 0% high-amylose corn starch (A, C) or 20% high-amylose corn starch (B, D).



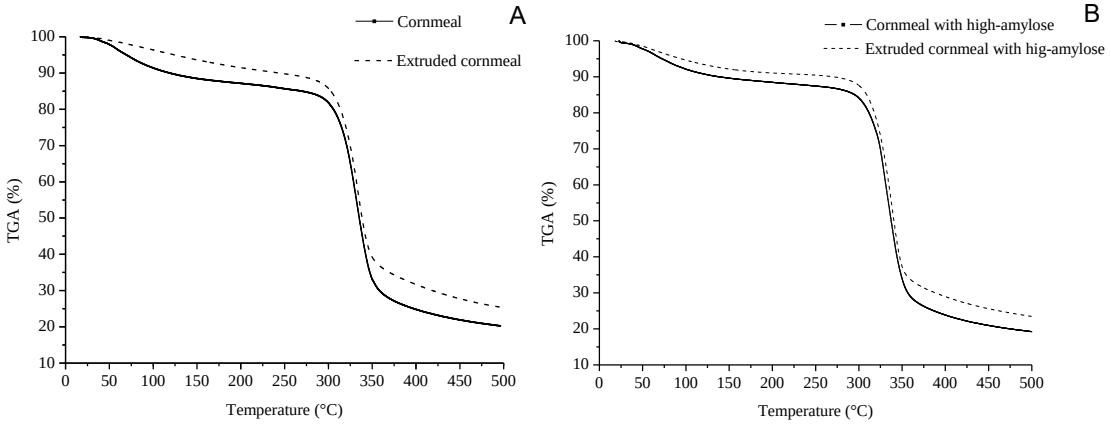
The retrogradation in the extruded product was characterized by thermogravimetric analysis (TGA), by measuring the mass loss of bound water at the first decomposition stage (30-190 °C) (TIAN et al., 2011). The mass loss that occurs in this step may be attributed to the evaporation/dehydration of bound water (KUMAR VARMA; PANPALIA; KUMAR, 2014). Additionally, researchers have reported that bound water and starch retrogradation are positively correlated, therefore the measurement of bound water is considered a method to monitor starch retrogradation (TIAN et al., 2011). Based in this association between bound water and starch retrogradation, the TGA results (Figure 5) indicate that the sample with high-amylose corn starch had more retrograded starch (mass loss of 8.4%) than the extruded cornmeal without high-amylose (mass loss of 7.9%). Additionally, the content of retrograded starch is an indication of RS3 formation in the extruded product.

Figure 5 - Curves of sample mass loss plotted against heating temperature for extruded cornmeal, in the presence or absence of 20% high-amylose corn starch.



The thermal stability of un-extruded and extruded cornmeal (in the presence and absence of high-amylose starch) was analyzed by TGA (Figure 6 – A and B), the results showed that the profile of each curve was similar, with three main mass losses.

Figure 6 - Curves of sample mass loss plotted against heating temperature for raw and extruded cornmeal (A) and cornmeal with 20% high-amylose corn starch (B).



The first region of losses begins at room temperature and continues until around 190 °C. This represents the evaporation of the water and volatile compounds. Once dehydrated, the second and third main regions in the TGA curves are due to degradation of the organic matter with the formation of a final residue (ash). The second mass loss started at a temperature of 250 °C and ended around 370 °C, with the third region continuing until 500 °C. Similar thermal stability curves of native and retrograded starch was observed by Kumar Varma, Panpalia and Kumar (2014).

4 CONCLUSION

The designed experiment using a Central Composite Rotatable Design was successful in elucidating the effect of process variables (moisture content and screw speed) on the formation of resistant starch in corn-based extruded products. The addition of 20% high-amylose corn starch into the cornmeal used for extrusion promoted an increase (by 21%) in resistant starch (RS), while producing corn-based extrudates with acceptable physical and functional properties. The TGA showed differences in the retrogradation profiles of cornmeal extruded in the presence and absence of high-amylose starch. Based on the results shown by this research, using optimum extrusion conditions for RS formation (22% moisture and 210 rpm), along with the addition of 20% high-amylose corn starch to the formulation, an increased RS value can be achieved in the corn-based extruded products.

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ARTICLE VI

RESISTANT STARCH AND HYDROTHERMAL TREATMENT OF CORNMEAL: FACTORS IN AFLATOXINS AND FUMONISIN B1 REDUCTION AND BIOACCESSIBILITY

MASSAROLO, K. C.; FERREIRA, C. F. J; COLLAZZO, C. C.; BIANCHINI, A.; KUPSKI, L.; BADIALE-FURLONG, E. Resistant starch and hydrothermal treatment of cornmeal: factors in aflatoxins and fumonisin B1 reduction and bioaccessibility. **Food Control**, p. 107274, 2020.

Resistant starch and hydrothermal treatment of cornmeal: factors in aflatoxins and fumonisin B1 reduction and bioaccessibility

ABSTRACT

The objective of the study was to evaluate the effect of hydrothermal treatment on the levels of aflatoxins (G2, G1, B2, B1) and fumonisin B1 in cornmeal, and to investigate the chemical constituents of cornmeal that could affect the mycotoxins bioaccessibility. For this, cornmeal (fine and coarse) were spiked with mycotoxins, and then subjected to hydrothermal treatment. Mycotoxin quantification was determined before and after the treatments; while *in vitro* mycotoxins bioaccessibility was evaluated after the hydrothermal treatments. Treated samples were also characterized based on their components and resistant starch. The hydrothermal treatment of mycotoxin-spiked coarse cornmeal resulted in 36-52% reduction in aflatoxin and 39% in fumonisin B1 levels; while fine cornmeal showed reductions of 0-41% in aflatoxin and 59% in fumonisin B1 levels. The reduction of the aflatoxins G2, B1 and B2 was significant correlated ($p<0.1$) with the presence of the starch. Reductions in fumonisin B1 levels were significant correlated ($p<0.05$) with resistant starch and reducing sugars. In the fine cornmeal, 63% of the aflatoxins (G2, B2 and B1) and 27% of the fumonisin B1 were free for absorption in the small intestine; while the remaining fractions were degraded or bounded to some component of the matrix. In coarse cornmeal the percentage of bioaccessibility was higher (91% aflatoxins and 35% fumonisin B1). The non-bioaccessible fumonisin B1 fraction that remained bound to the matrix, possibly was associated to the non-digestible starch, since the reduction of this mycotoxin showed a high correlation with the resistant starch.

Keyword: Hydrothermal process. Mycotoxin reduction. Non-digestible starch.

1 INTRODUCTION

Corn (*Zea mays*) is susceptible to fungal contamination by some species of fungi belonging to the genre *Aspergillus*, *Penicillium* and *Fusarium* that under certain conditions can produce mycotoxins (PITT, 2006). These toxic compounds are part of the worldwide concern regarding food safety due to their toxicity and deleterious effects on human and animal health. Among these toxins, aflatoxins and fumonisins frequently contaminate corn (HENDEL et al., 2017; MURASHIKI et al., 2017; WANG et al., 2016) and corn based products (CANO-SANCHO et al., 2012; KOWALSKA; HAJOK; PIEKUT, 2017).

Pre-harvest and post-harvest management do not guarantee the absence of mycotoxins in food (JACOBSEN, 2014), requiring the use of physical, chemical or biological methods during food processing to further reduce mycotoxin levels. Among the physical methods are cleaning, grinding, separation and heat processes (KARLOVSKY et al., 2016).

The reduction of mycotoxins by heat treatments depends on the level of initial contamination, the heating temperature, the time of exposure to heat, the type of food and mycotoxins, as well as product moisture and pH (BECKER-ALGERI et al., 2013; DE GIROLAMO et al., 2016). However, a better understanding of the true effects of thermal treatments on mycotoxin degradation or binding to proteins, sugars or other compounds present in the food matrix is still required. Further, the bioaccessibility of mycotoxins in raw agricultural products, processed foods and prepared foods ready for consumption also needs to be evaluated. A study by Falavigna et al. (2012) suggests that reductions induced by thermal processes in fumonisin B1 content in foods may result from changes in its chemical structure, as a result of interactions with other food constituents to form conjugates.

The chemical/physical basis of these reductions is not yet fully elucidated, but because of the presence of a primary amino group and four carboxyl groups, fumonisins may undergo various chemical changes, such as those resulted from Maillard or ester/amide type reactions. Maillard reactions may occur between the fumonisin molecule and carbohydrates; while the ester/amide reactions would involve the toxin and proteins, lipids or carbohydrates (DALL'ASTA; BATTILANI, 2016).

The potential for binding mycotoxins to the matrix components may be beneficial depending on their effect on bioavailability, the toxicity of the bound products or the extent to which the attached mycotoxins will be released into the gastrointestinal tract (DALL'ASTA; BATTILANI, 2016; HUMPF; VOSS, 2004). In view of these possibilities, studies on the bioaccessibility of mycotoxins in ready-to-eat starchy products are necessary, since the

relationship between mycotoxins and macro non-digestible food components, such as resistant starch, has not yet been fully studied. Much less is understood about these interactions in corn-based products.

Strategies to mitigate the risk of exposure to mycotoxins, considering that resistant starch could bind part of the contaminant, could be feasible, caused by the formation of complexes between mycotoxins and food components that hinder the action of digestive enzymes, reducing their bioaccessibility. The objective of the present study was to evaluate the effect of hydrothermal treatment on the residual levels of aflatoxins (G2, G1, B2, B1) and fumonisin B1 in cornmeal, and to investigate the chemical constituents of cornmeal that could affect potential reductions during *in vitro* digestion.

2 MATERIAL AND METHODS

2.1 STANDARDS, REAGENTS AND SAMPLES

Aflatoxins standards (G2, G1, B2 and B1) and fumonisin B1 were supplied by Sigma-Aldrich (Saint Louis, MO, USA) with purity >98%. Aflatoxin stock solutions were prepared by dissolving the standards with toluene/acetonitrile (98:2, v/v); while fumonisin was dissolved in methanol:acetonitrile (50:50, v/v). After preparation, the mycotoxin standards were dried under nitrogen and stored at −18°C, to ensure their stability. The working solutions were prepared from stock solutions, and before use aflatoxins were quantified in a spectrophotometer, according to the AOAC (2000).

The solvents used as mobile phase in the chromatographic system (Acetonitrile and methanol) with purity > 99.9% were supplied by JT Baker (Goiânia, GO, Brazil). Ultrapure H_2O (>18.2 MΩ cm^{-1} resistivity) was purified using a Milli-Q® SP Reagent Plus water system (Millipore Corp., Bedford, USA). The 0.1 M phosphate buffer pH 3.15 used as mobile phase in the chomatographic system for fumonisin B1 analysis was prepared diluting 13.8 g of sodium phosphate monobasic in 1 L of ultrapure water, and adjusting the pH to 3.15 with 2 M hydrochloride acid.

The OPA-MCE derivatization reagent used during quantification of fumonisin B1 was prepared daily according to Kong et al. (2012) by mixing 100 mg ortho-phthaldialdehyde (OPA), 20 mL methanol, 500 µL 2-mercaptoethanol (MCE), and 950 mL of sodium tetraborate solution 0.05 M. This mixture was then brought to 1L by addition of ultrapure water. The OPA mixture was stored in a brown glass bottle and, when not in use, kept at 4 °C for up to 2 days.

The mobile phase and derivatization reagent were always filtered through a 0.45 µm cellulose filter and then degassed in an ultrasonic bath before use.

The enzymes and chemicals for the digestive fluids, pepsin from porcine pancreas, α-amylase from *Aspergillus oryzae*, pancreatin and lipase from porcine pancreas, mucin from porcine stomach, bile salts, phosphoric acid, citric acid, uric acid, D-glucosamine hydrochloride and D-glucuronic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) (Inlab Confiança, São Paulo, SP, Brasil), Hydrochloric acid (HCl) and Sodium hydroxide (Labsynth, Diadema, SP, Brasil) were also sourced for this research. Dialysis tubing cellulose membrane was obtained from Sigma-Aldrich (St. Louis, MO, USA) with a molecular mass cut-off of 12 kDa and 25 mm diameter.

Cornmeal (coarse and fine), corn starch and soy oil were purchased at local markets in the South region of Brazil. The coarse cornmeal showed a particle size distribution of 78.6% bigger than 0.35 mm; while the fine cornmeal had 88.8% of particles smaller than 0.35 mm (MASSAROLO et al., 2018b).

2.2 METHODS

2.2.1 Aflatoxins and fumonisin B1 determination in raw and hydrothermally treated cornmeal

Aflatoxins and fumonisin B1 (FB1) in raw and hydrothermally treated cornmeal were extracted by MSPD (Matrix Solid Phase Dispersion) according to Massarolo et al. (2018b) with modifications for hydrothermal treated samples. For those, the amount of sample used was 5 g. For aflatoxin determination, the extract was reconstituted in ultrapure water and acetonitrile (90:10, v/v) and injected into a HPLC-FD (High Performed Liquid Chromatographic with Fluorescence detector) for separation and detection of the contaminants. The chromatographic conditions for identification and quantification of aflatoxinas G2, G1, B2 and B1 were those suggested by Massarolo et al. (2018b) and the analytical standard curve was built using aflatoxin standards of different concentrations (G2 from 0.05 to 5 ng mL^{-1}; G1 from 0.02 to 5 ng mL^{-1}; B2 from 0.01 to 2 ng mL^{-1} and B1 from 0.04 to 6 ng mL^{-1}). All standard injections into the HPLC-FD for standard curve preparation were done in triplicate.

Fumonisin B1 chromatographic separation was performed on a HPLC system with fluorescence detector and post-column chemical derivatization (40 °C and reagent flow rate of 0.4 mL min^{-1}). The column used was Ascentis C18 (150 mm x 4,6 mm i.d., particle size 3 µm) purchased from Supelco (E.U.A) and the column temperature was maintained at 40 °C. The

mobile phase consisted of methanol (60%) and 0.1 M phosphate buffer at pH 3.15 (40%). Flow rate was maintained at 0.6 mL min^{-1} and the injection volume was 20 µL. Fluorescence detection was performed at excitation and emission wavelengths of 343 and 445 nm, respectively.

Fumonisin B1 standards solutions, diluted in methanol and 0.1 M phosphate buffer pH 3.15 (60:40, v/v), were used to build a standard calibration curve in the concentration range from 0.2 to 2.0 µg mL^{-1}. Each standard was injected into the HPLC in triplicate. The extracts obtained from cornmeal samples were re-suspended, prior to injection into the HPLC, in methanol and 0.1 M phosphate buffer pH 3.15 (60:40, v/v).

2.2.1.1 Method validation

The validation of the mycotoxin analysis method was performed according to the European Commission (SANTE, 2016) and ANVISA (2003). The linearity, correlation coefficient, limit of detection (LOD) and quantification (LOQ) were all determined. The instrument LOD and LOQ were determined based on the signal-to-baseline (noise) ratio of the equipment. Once the noise was determined, then the LOD and LOQ were calculated as three and ten times that value, respectively. The method LOD and LOQ were estimated considering the extraction method.

The accuracy (recovery) was evaluated by spiking samples with aflatoxin standards (G2, G1, B2 and B1) at three levels (3, 5 and 10-fold LOQ) and FB1 (1 µg mL^{-1}), in triplicate. The precision (repeatability) was evaluated by carrying out nine independent determinations. For this, samples spiked at three different levels were extracted by MSPD and quantified in triplicate. The matrix effect (ME) on the quantification of aflatoxins was evaluated by comparing the slopes obtained when the standard curve was prepared either using blank cornmeal extract for preparation of calibration standards or solvents. The matrix effect on fumonisin B1 was evaluated according to Malachová et al. (2014).

2.2.2 Hydrothermal treatment and its effect on mycotoxins

The hydrothermal treatment of cornmeal (fine and coarse) containing mycotoxins was performed using a heating plate set to 120 °C for 40 min, according to Massarolo et al. (2018a), the temperature of the product ranged from 70 °C to 85 °C during the cooking process. Cornmeal samples (fine and coarse) were spiked with 5 mycotoxins to achieve the maximum

level allowed by Brazilian regulatory authorities: aflatoxins at 20 ng g^{-1}, by the combination of 8 ng g^{-1} AFLA B1, 4 ng g^{-1} of each AFLA B2, G2 and G1; and fumonisin B1 at 3.0 µg g^{-1}. The spiked cornmeal sample was added to boiling water (1:5, w/v), along with sodium chloride (1%) and soybean oil (3%). The mixture was stirred during the process. In the hydrothermal treatment of the coarse cornmeal soybean oil was not added, in an attempt to avoid the formation of resistant starch (MASSAROLO et al., 2018a).

Mycotoxins were determined before and after the hydrothermal treatment. Mycotoxin reduction was calculated based on the amount of toxin in the uncooked product and the prepared food, always considering the mycotoxin concentration in dry basis.

2.2.3 Mycotoxin and starch correlation

Cornmeal is a complex product with several types of carbohydrates, varying from single monosaccharides to complex chains of monosaccharides. Therefore, if the effect of starch was to be considered individually on mycotoxin reductions, then ideally this molecule should be tested by itself in the presence of mycotoxins. Therefore, another hydrothermal treatment (120 °C/40 min) was conducted using a corn starch solution (58 mg mL^{-1}) spiked with aflatoxin (G1, G2 and B2 1 ng mL^{-1}, B1 2 ng mL^{-1}) and fumonisin B1 (5 µg mL^{-1}) standards. Once again, mycotoxins quantification was carried out before and after the hydrothermal treatment and reductions were calculated in dry basis.

2.2.4 Characterization of fine and coarse cornmeal

Cornmeal samples were characterized based on their moisture (AOAC 934.01), crude protein (AOAC 955.04C; conversion factor 5.75), ash (AOAC 900.02) and lipid (AOAC 920.85) content (AOAC, 2000). Total starch was determined by iodometry using a starch standard curve.

The amylose content was determined according to the colorimetric method proposed by Martinéz and Cuevas (1989) with adaptations. The cornmeal amylose extraction was performed with 95% ethyl alcohol (v/v) and 1 M NaOH, the colorimetric reaction was performed with 2% (w/v) iodine solution, and the amylose content was determined using a standard amylose curve (0.004–0.024 mg mL^{-1}) in a spectrophotometer at wavelength 620 nm.

Resistant starch content in cornmeal hydrothermally treated was measured according to AOAC method 996.11 modified by Walter, Silva and Perdomo (2005). The

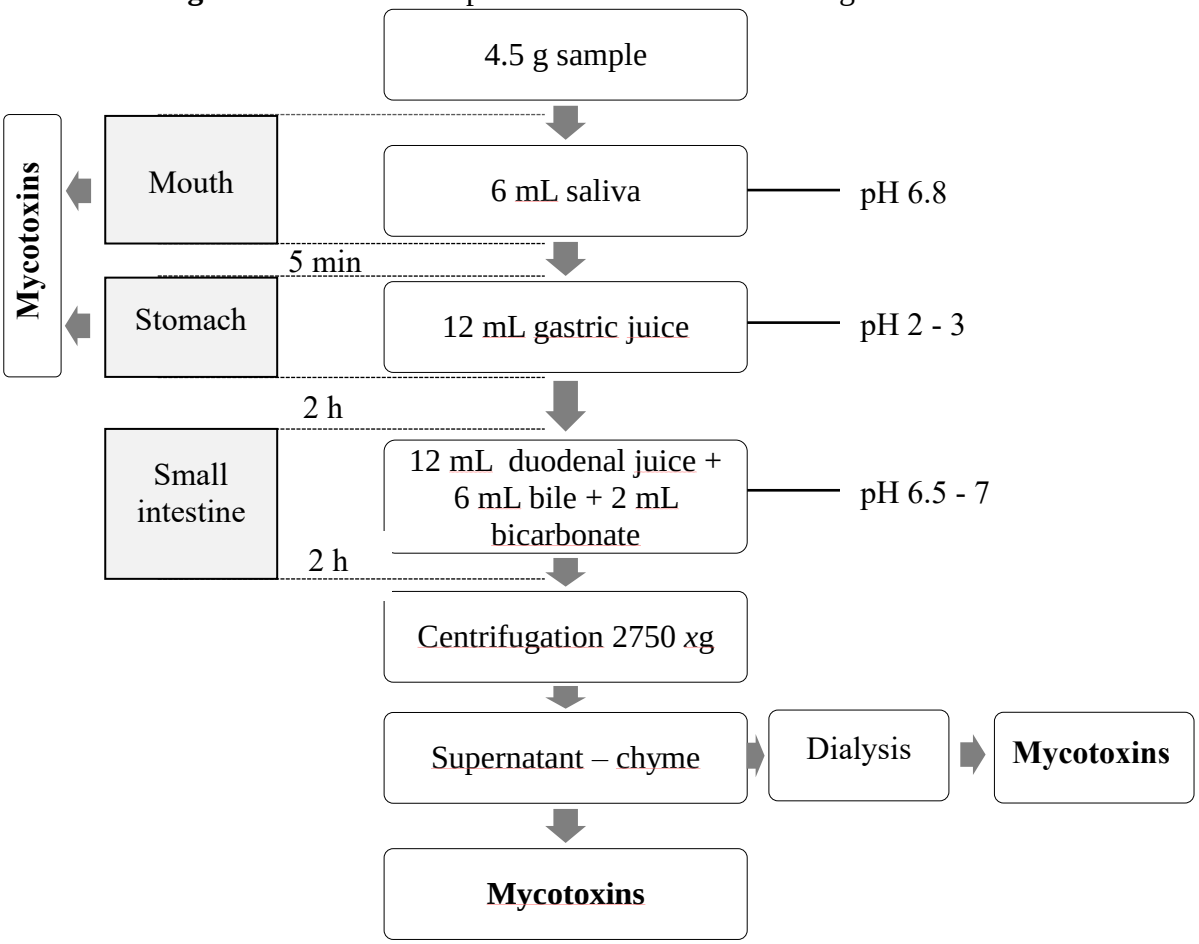
determination of the resistant starch after hydrolysis was performed by quantifying the glucose released by the 3,5 dinitrosalicylic acid reduction method (DNS) (MILLER, 1959) and converting to starch using the conversion factor of 0.9.

In these cornmeal samples the total soluble sugars were also determined by the phenol-sulfuric method (DUBOIS et al., 1951), as well as reducing and total reducing sugars by the 3,5 dinitrosalicylic acid reduction method (DNS) (MILLER, 1959).

2.2.5 Bioaccessibility and intestinal transport of mycotoxins in cornmeal products hydrothermally treated

The mycotoxins bioaccessibility after hydrothermal treatment was performed according to *in vitro* digestion model (Figure 1) developed by Versantvoort, Van de Kamp and Rompelberg (2004).

Figure 1 - Schematic representation of the *in vitro* digestion model.



The model was developed to simulate the biochemistry conditions in the gastrointestinal tract of adult individuals and describes a three-step procedure simulating the digestive processes in mouth, stomach and small intestine. The digestion model is carried out using synthetic solutions containing the same components of the different portions of the digestive tract of monogastric animals (Table 1).

Table 1 – Composition of synthetic juices used for each step in the simulated digestive model applied to hydrothermally processed cornmeal.

	Saliva	**Gastric juice**	**Duodenal juice**	**Bile juice**
Composition (per liter)	0.9 g KCl	2.75 g NaCl	7.01 g NaCl	5.26 g NaCl
	0.2 g KSCN	0.27 g NaH_2PO_4	3.39 g $NaHCO_3$	5.79 $NaHCO_3$
	0.9 g NaH_2PO_4	0.82 g KCl	0.08 g KH_2PO_4	0.38 g KCl
	0.57 g Na_2SO_4	0.4 g $CaCl_2.2H_2O$	0.56 g KCl	0.15 mL HCl (37%)
	0.3 g NaCl	0.31 g NH_4Cl	0.05 g $MgCl_2$	0.25 g urea
	1.7 g $NaHCO_3$	6.5 mL HCl (37%)	0.18 mL HCl (37%)	0.22 g $CaCl_2.2H_2O$
	0.2 g urea	0.65 g glucose	0.1 g urea	1.8 g BSA
	290 mg α-amylase	0.02 g glucuronic acid	0.2 g $CaCl_2$ $2H_2O$	30 g bile
	15 mg uric acid	0.085 g urea	1 g BSA	
	25 mg mucin	0.33 g glucoseamine hydrochloride	9 g pancreatin	
		1 g BSA	1.5 g lipase	
		2.5 g pepsin		
		3 g mucin		
pH	6.57	2.00	7.51	7.49
Time	5 min	2 h	2 h	

The small intestine step was simulated with the use of dialysis tubing (membrane) of known porosity (12.4 kDa), to simulate the absorption of the compound of interest in the intestinal epithelium (GONZÁLEZ-ARIAS et al., 2015). Therefore, after the digestion period, an aliquot of the digested sample (15 mL) was placed into a dialysis bag of 12 kDa, that had been previously activated (water bath for 10 min). The bags were closed at the ends and placed in 100 mL graduated cylinders, containing ultrapure water and sodium bicarbonate (60 mL) at pH 7.4 (physiological pH). Samples were then placed in a 37 °C shaking water bath for 2 h. After this period, the dialysis bags were removed from the graduated cylinder, and both the

dialysate (external volume of the membrane) and the non-dialysate fraction (internal volume of the membrane) were collected in separate vials for the determination of mycotoxins.

Hydrothermally treated cornmeal samples were incubated at 37 °C, then the digestion process was started by adding synthetic solutions that mimic the action of saliva. Samples were maintained incubated for 5 min, then the gastric digestion was simulated by adding gastric juice and homogenized in an orbital shaker for 2 h. In the third phase the duodenal digestion was carried out, where duodenal juice, bile and bicarbonate solution (1 M) were added simultaneously and the mixture was agitated (55 rpm at 37 °C ± 2 °C) for another 2 h (Figure 1).

All digestive juices were heated to 37 ± 2 °C and had their pH determined prior to utilization (Table 1). After each digestion process step (mouth, stomach and small intestine), the digestion tubes were centrifuged and two fractions were obtained: chyme fraction (the supernatant - digestible material) and the digested matrix fraction (pellet – non digestible material). Mycotoxin quantification was carried out in each of the chyme fractions.

2.2.5.1 Determination of mycotoxin in the digestible fractions

Aflatoxins from digestible fractions from mouth, stomach and small intestine digestion steps and the dialysate fraction were extracted by liquid-liquid partition with ethyl acetate, centrifuged 3220 x*g* for 5 min, and then the solvent fraction was transferred to amber flask. This process was repeated 3 times to maximize mycotoxin extraction. The solvent was then evaporated at 60 °C. The dried aflatoxin extract was solubilized in water and acetonitrile (90:10) and quantified in HPLC-FD according to Massarolo et al. (2018b).

FB1 extraction from digestible fractions from stomach and small intestine digestion steps and the dialysate fraction was carried out according to Sobral et al. (2019) with modifications. Briefly, an aliquot from the digested sample was transferred into a Falcon tube, added with ethyl acetate acidified with formic acid 1% (v/v), 1.75 g of $MgSO_4$ anhydrous and 0.5 g of NaCl. The tube was immediately vortexed for 30 s to prevent agglomeration of the salts, and then centrifuged at 3220 x*g* for 5 min. The organic phase was transferred to an amber flask and the solvent evaporated at 60 °C. The extract was resuspended in phosphate buffer pH 3.15 and methanol (40:60, v/v) and then analyzed by HPLC-FD with post-column derivatization. The chromatographic conditions used for fumonisin B1 identification and quantification were those described in item 2.2.1, with a modification in the mobile phase: methanol (63%) and 0.1 M phosphate buffer at pH 3.15 (37%).

The methods used for mycotoxin quantification were evaluated based on their ability to recover mycotoxin from spiked samples. Therefore, the method recovery was determined by spiking the mix of the juice solutions with aflatoxins B1 and G1 (2.25 ng mL^{-1}), B2 (0.56 ng mL^{-1}), G2 (1.12 ng mL^{-1}) and FB1 (2 µg mL^{-1}). The recovery tests were done in triplicate. The matrix effect was evaluated by comparing the area under the chromatographic curves obtained for the sample extracts spiked with mycotoxins (standard matrix area) and the area obtained for solvents spiked with mycotoxins (standard solvent area), according to Malachová et al. (2014).

2.2.6 Statistical analysis

Statistical analysis was performed using *Statistica* 6.0 software, where distributed variables one-way ANOVA and t-Student post hoc test were performed. A significance level of 95% was applied to all tests. Additionally, Principal Component Analysis (PCA) was performed in Past software (folk.uio.no/ohammer/past) to establish correlations between cornmeal components, mycotoxins reductions and toxin bioaccessibility after the hydrothermal treatment.

3 RESULTS AND DISCUSSION

3.1 METHOD VALIDATION

3.1.1 Aflatoxins

The method validation for aflatoxins in raw cornmeal was performed by Massarolo et al. (2018b). Method recoveries, at the three levels tested for the four aflatoxins, in both fine and coarse cornmeal hydrothermally treated ranged from 66.3% to 168.5% (Table 2).

Most of the recovery values obtained are within the criteria approved by the European Regulatory Committee (ERC). The criteria establishes that for mycotoxin concentrations of less than 1 µg kg^{-1}, recoveries should range between 50 and 120%, and for concentrations between 1 and 10 µg kg^{-1}, recoveries should vary between 70 and 110% (EUROPEAN COMMISSION, 2006). Recovery values for aflatoxins G2 and G1 when near the limit of quantification of the method were higher than the maximum recommended by ERC. This type of behavior may have been observed either because the toxin levels under evaluation were very low or because of a significant matrix effect for these two aflatoxins.

Table 2 – Parameters considered during the evaluation of the method used for quantification of aflatoxins G2, G1, B2 and B1 in cornmeal hydrothermally treated.

Aflatoxins	Concentration ($ng\ g^{-1}$)	Recovery (%)		Matrix effect (%)	
		Fine	Coarse	Fine	Coarse
G2	0.30	120.4 (2.2)	132.9 (3.7)	15.0	-24.3
	0.50	109.7 (2.2)	122.8 (1.6)		
	1.00	94.4 (6.8)	107.3 (1.0)		
G1	0.14	120.0 (6.4)	168.5 (4.6)	33.5	-44.6
	0.24	120.9 (17.3)	81.3 (14.4)		
	0.48	94.3 (16.7)	129.1 (0.6)		
B2	0.07	119.0 (0.6)	97.2 (1.9)	11.6	2.7
	0.12	87.8 (5.4)	86.8 (0.9)		
	0.24	72.6 (2.5)	82.7 (0.6)		
B1	0.22	80.5 (14.6)	86.8 (4.9)	4.0	0.0
	0.36	72.6 (3.1)	78.0 (6.3)		
	0.72	66.3 (2.8)	83.9 (0.4)		

Results shown as mean (RSD). RSD= relative standard deviation.

Repeatability represents the agreement between the results obtained for consecutive measurements of different preparations of the same sample carried out under the same conditions. Results indicated that the Relative Standard Deviations (RSDs) of the methods under evaluation were <20% and thus conformed to the accepted limits for the concentrations used in the repeatability test (EUROPEAN COMMISSION, 2006). The matrix effect on the quantification of aflatoxins G2, B2 and B1 in the fine cornmeal hydrothermally treated was lower than 20% (15.0%, 11.6% and 4.0%, respectively). According to the European Commission (SANTE, 2016), a matrix effect of 20% or less is considered acceptable for analyses of contaminants in food at trace level. However, the matrix effect for aflatoxin G1 was -33.5%, which then required the quantification of this mycotoxin to be done using a standard curve prepared in the presence of the matrix components.

For the quantification of aflatoxins B2 and B1 in coarse cornmeal hydrothermally treated no matrix effect was observed. However, when quantifying aflatoxins G2 and G1 an effect of -24.3% and -44,6%, respectively, was observed. Therefore, for the quantification of these two aflatoxin species in coarse cornmeal that had been hydrothermally treated, standard curves prepared in the presence of matrix components were used.

3.1.2 Fumonisin B1

The analytical parameters obtained showed that the identification and quantification of fumonisin B1 was adequate when using HPLC-FD with post-column derivatization. The standard curve showed a linearity of 0.2 to 2.0 μg mL^{-1} and a correlation coefficient greater than 0.99. The limit of quantification of the instrument and method (0.2 μg mL^{-1} and 0.4 μg g^{-1}, respectively) were satisfactory for the determination of fumonisin B1, since the maximum legislated limit in Brazil is 1.5 μg g^{-1}.

The recovery values for the method used for fumonisin B1 quantification in raw and hydrothermally treated fine cornmeal were 93.3% and 85.6%, respectively (Table 3); while in raw and hydrothermally treated coarse cornmeal, they were 75.5% and 119%, respectively. These percentages, for the most part, are within the criteria approved by the European Regulatory Committee, which recommends that for concentrations greater than 0.5 μg g^{-1} recoveries can vary between 70-110% (EUROPEAN COMMISSION, 2006).

Table 3 - Parameters considered during the evaluation of the method used for quantification of fumonisin B1 in raw and hydrothermally treated cornmeal.

Cornmeal		Recovery (%)	Matrix effect (%)
Raw	Fine	93.3 (4.9)	3.2
	Coarse	75.5 (13.0)	2.1
Hydrothermally treated	Fine	85.6 (10)	-1.0
	Coarse	119 (7)	12.0

Results shown as mean (n=3) and RSD. RSD= relative standard deviation.

The repeatability of the method for quantification of fumonisin B1 was evaluated based on the RSDs and results indicated that those values were lower than 20%, which is in agreement with the accepted limits for the concentrations used in the repeatability test (EUROPEAN COMMISSION, 2006). The matrix effect results for the fine and coarse cornmeal, both raw and hydrothermally treated, were acceptable (<20%) (Table 3).

3.1.3 Digestible fraction

The recoveries values for the mix of the digestible juices were 70% (AFG1), 108% (AFG2), 110% (AFB2 e AFB1) and 82% (FB1) and the matrix effect values were lower than 20%, indicating no matrix effect on the quantification of mycotoxins in the digestible fraction.

3.2 MYCOTOXIN REDUCTION BY HYDROTHERMAL TREATMENT

The hydrothermal treatment evaluated led to mycotoxin reductions in both fine and coarse cornmeal (Table 4). The reductions ranged from 35.9% to 51.7% for the coarse cornmeal on a dry basis (db) and in the fine cornmeal, the reductions in dry basis were between 0 and 59.4%. The highest reduction values in the coarse cornmeal was observed for aflatoxin G1 (51.7%, db); while for the fine cornmeal, fumonisin B1 showed the largest values (59.4%, db). However, when statistical analysis was carried out, none of the different toxins showed reduction values statistically higher ($p > 0.05$) than the others in the same type of cornmeal.

Table 4 – Mycotoxin level reduction with hydrothermal treatment

Mycotoxins	**Reduction in dry basis (%)**	
	Coarse cornmeal	**Fine cornmeal**
AFLA G2	36.0 (15.7)^{aA}	29.1 (18.2)^{aA}
AFLA G1	51.7 (16.6)^{aA}	0^{bB}
AFLA B2	35.9 (14.3)^{aA}	34.6 (7.8)^{aA}
AFLA B1	44.9 (9.0)^{aA}	41.1 (9.9)^{aA}
Fumonisin B1	38.6 (7.4)^{bA}	59.4 (8.2)^{aA}

Results expressed as mean and RSD. RSD= relative standard deviation. Values with different small letters in the same line differ significantly ($p<0.05$). Values with different capital letters in the same column differ significantly ($p<0.05$).

Other studies also showed different levels of reduction for different mycotoxins species upon hydrothermal treatment (DE GIROLAMO et al., 2016; SHEPHARD et al., 2002). Some authors have suggested that mycotoxin reductions promoted by heat treatments may be a result of changes in mycotoxin structure as a consequence of interactions with other food constituents to form conjugates (DALL'ASTA; BATTILANI, 2016; FALAVIGNA et al., 2012).

The differences observed on mycotoxins reductions in this study may be due to differences in the composition of the different cornmeals evaluated. Therefore, the macro components of these products were determined and then a multivariate analysis was carried out to verify which components may be related to the reduction of mycotoxins by heat treatment.

3.3 CORRELATION BETWEEN MYCOTOXIN REDUCTION AND CORNMEAL COMPONENTS

The fine cornmeal had higher contents of ash (0.9%), lipids (2.8%), resistant starch (245.71 mg g^{-1}), reducing sugar (12.28 mg g^{-1}) and total reducing sugar (15.75 mg g^{-1}) when compared to coarse cornmeal. No significant difference ($p>0.05$) for the levels of protein was observed in both types of samples (Table 5).

Table 5 – Cornmeal composition

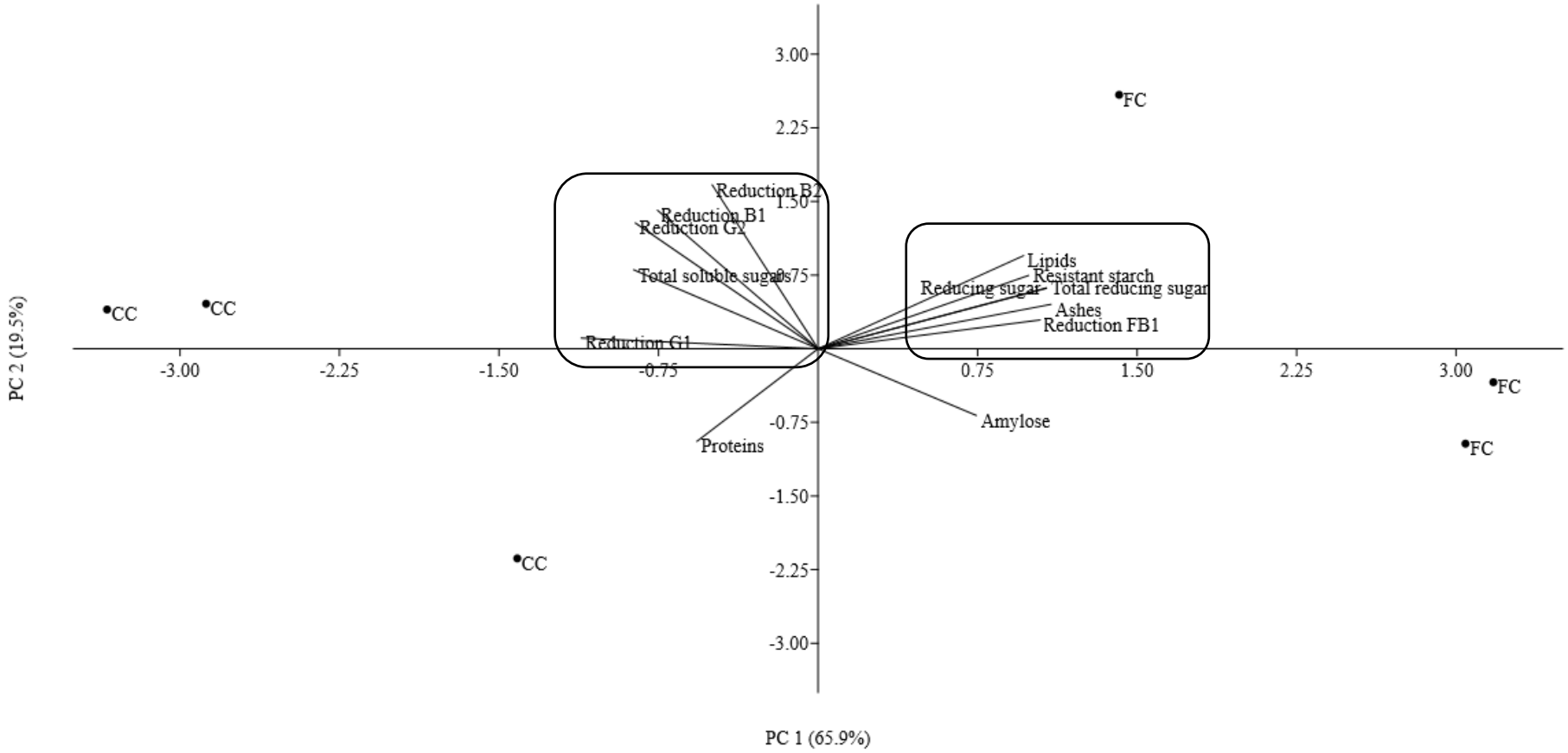
Cornmeal	RS (mg g^{-1})	RR (mg g^{-1})	TRR (mg g^{-1})	Amylose (%)	Ashes (%)	Lipids (%)	Proteins (%)	TSS (%)
Fine	245.71[a]	12.28[a]	15.75[a]	27.2[a]	0.9[a]	2.8[a]	7.4[a]	2.9[a]
Coarse	142.70[b]	0.71[b]	1.24[b]	23.7[a]	0.2[b]	0.1[b]	8.0[a]	3.5[a]

RS= resistant starch, RR= reducing sugar, TRR= total reducing sugar, TSS= total soluble sugar. The mean values of triplicate analyses are given. Values with different letters in the same column differ significantly ($p < 0.05$).

The reduction of mycotoxins and their association with certain components of the hydrothermally treated cornmeal was evaluated using Principal Component Analysis (PCA), as shown in Figure 2, in which the major components 1 (65.9%) and 2 (19.5%) explained 85.4% of the results variability. Based on the PCA for aflatoxins, total soluble sugars had a significant positive correlation ($p <0.1$) with the reduction of aflatoxins G2, G1 and B1, with R values of 0.80, 0.82 and 0.75, respectively.

Resistant starch (R= -0.84), reducing sugar (R= -0.93), total reducing sugar (R= -0.93), amylose (R= -0.74), ashes (R= -0.96) and lipids (R= -0.80) showed a negative correlation with aflatoxin G1 reduction. When the PCA was carried out for fumonisin, resistant starch (R= 0.75), reducing sugars (R= 0.94), total reducing sugars (R= 0.94), ashes (R= 0.93) and lipids (R= 0.94) showed a positive effect on the reduction of fumonisin B1 levels, as highlighted in the graph (Figure 2). The correlation between each of these components and fumonisin B1 showed angles smaller than 90°, which represent a good correlation.

Figure 2 – Principal components analysis for reduction of mycotoxins and components of hydrothermally treated cornmeal samples.



FC= fine cornmeal and CC= coarse cornmeal

As observed in the PCA, carbohydrates in the form of total and reducing soluble sugars, as well as resistant starch contribute to the reduction of mycotoxins. However, the cornmeal products used in this research had a complex mixture of carbohydrates. Therefore, to better understand the nature of the starch-mycotoxin interaction, a heat treatment (120 °C/40 min) was done in the presence of a corn starch solution (58 mg mL^{-1}) and mycotoxins (aflatoxins and fumonisin). Once again, the mycotoxins were determined before and after the hydrothermal treatment.

The hydrothermal treatment with the corn starch solution led to the reduction of aflatoxins G2 (39.6% RSD 7.8), B2 (15.1% RSD 6.4) and B1 (48.5% RSD 7.9). Only aflatoxin G1 did not show any reduction. Although aflatoxin reduction was observed, it is not possible to infer whether it was due to thermal degradation or starch binding. There are no studies that demonstrate the possible interactions between starch and aflatoxins.

Fumonisin B1 was reduced by 66.0% (RSD 0.7) in the presence of the starch solution, indicating some association between fumonisin and this macromolecule. This behavior is consistent with the work done by Seefelder, Knecht and Humpf (2003), where fumonisin B1 was subjected to heating (150 ºC/40 min) in a model solution with methyl-α-D-glucopyranoside (as starch model). After the treatment, nuclear magnetic resonance spectrometry of the formed product helped elucidate its structure. Results showed that binding of the starch and fumonisin was achieved by the two side chains of the fumonisin tricarballylic acid.

Table 6 shows the main results of all the experiments described so far. Mycotoxin reduction results, in cornmeal and starch solution, are presented along with the components evaluated in both types of cornmeal products. Comparisons among these variables were made and those that showed significant correlations ($p < 0.1$) are described in Table 6.

The reductions in aflatoxin G2 and B1 levels in all treatments tested, did not show significant differences and soluble sugars showed a relationship with these aflatoxin reductions. Aflatoxin G1 was not reduced in the fine cornmeal or corn starch solution, indicating that this mycotoxin does not interaction well with starch. In the case of aflatoxin B2, the levels of reduction were higher in the cornmeal samples than those obtained in the presence of the starch solution, therefore the reduction of this mycotoxin cannot be fully explained by the interactions with starch. Other components of the cornmeal may have contributed to further reduce AFLA B2 in cornmeal samples. Nogueira et al. (2019) showed that the hydrothermal treatment leads to the association of aflatoxins and other macromolecules of the matrix such as resistant starch in rice samples.

Table 6 – Comparison of reductions of mycotoxins in cornmeals and in corn starch with hydrothermal treatment and their respective correlations with the constituents of cornmeals.

Mycotoxin	Reduction (%)			Constituents	
	Coarse cornmeal	Fine cornmeal	Corn starch	Positive correlation	Negative correlation
AFLA G2	36.0 (15.7)[a]	29.1 (18.2)[a]	39.6 (7.8)[a]	Soluble sugars	-
AFLA G1	51.7 (16.6)[a]	0[b]	0[b]	Soluble sugars	Resistant starch, amylose, ashes, lipids and reducing sugars
AFLA B2	35.9 (14.3)[a]	34.6 (7.8)[a]	15.1 (6.4)[b]	-	-
AFLA B1	44.9 (9.0)[a]	41.1 (9.9)[a]	48.5 (7.9)[a]	Soluble sugars	-
Fumonisin B1	38.6 (7.4)[b]	59.4 (8.2)[a]	66.0 (0.7)[a]	Resistant starch, reducing sugar, ashes and lipids	-

Results shown as "mean (RSD)", where. RSD = relative standard deviation. Values with different letters in the same line differ significantly ($p<0.05$).

The reduction of fumonisin B1 levels was lower in the coarse cornmeal than in the fine cornmeal and starch solution. Additionally, fumonisin B1 reductions in cornmeal were correlated with levels of resistant starch and reducing sugars. Previous studies have also shown a positive correlation between fumonisin B1 reductions and the level of reducing sugars during baking and extrusion (BULLERMAN; BIANCHINI, 2007; CASTELO et al., 2001; JACKSON et al., 2011), but there is no information about cooking. Castelo et al. (2001) when extruding cornmeal in the presence of glucose found higher reduction levels of fumonisin B1 (45.3 to 71%) than when fructose (29.5 to 53%) or sucrose (19.2 to 39%) was used. Also, baking cornmeal muffins containing glucose led to significantly lower levels of fumonisin B1 in the product than muffins made with sucrose, fructose or no added sugar (CASTELO et al., 2001).

Bullerman and Bianchini (2007) found reductions in fumonisin levels (75-85%) when extruding corn grits with addition of glucose. In another study Jackson et al. (2011) analyzing the mass balance for mycotoxin reduction during extrusion, showed that 38% to 46% of the fumonisin B1 species detected in corn extruded with glucose were converted to N-(deoxy-D-fructos-1-yl)-FB1, a compound less toxic than fumonisin B1 (HAHN et al., 2015).

The interaction between the mycotoxins and the matrix components may be beneficial, depending upon the stability of these associations when they go through the gastrointestinal tract (DALL'ASTA; BATTILANI, 2016; HUMPF; VOSS, 2004). In view of

this, the bioaccessibility of these mycotoxins was evaluated, emphasizing their association with macro non-digestible components of the food. Better understanding these associations and their stability during digestion can contribute to the development of mitigation strategies that would more reliably reduce the risk of exposure to these contaminants.

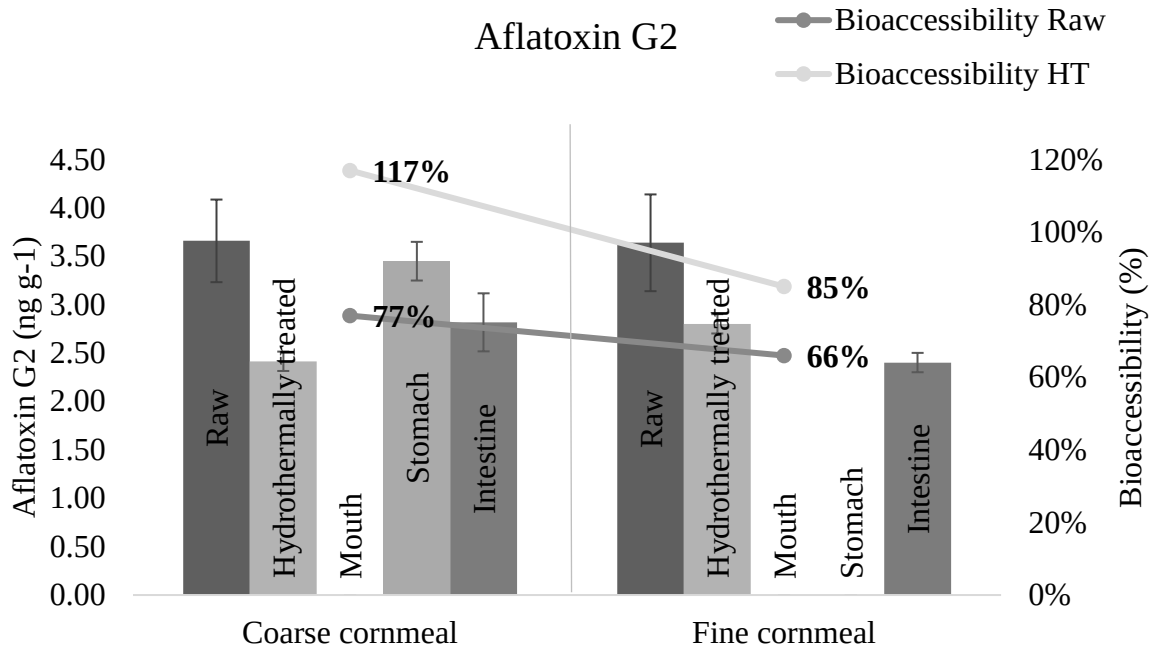
3.4 MYCOTOXINS BIOACCESSIBILITY

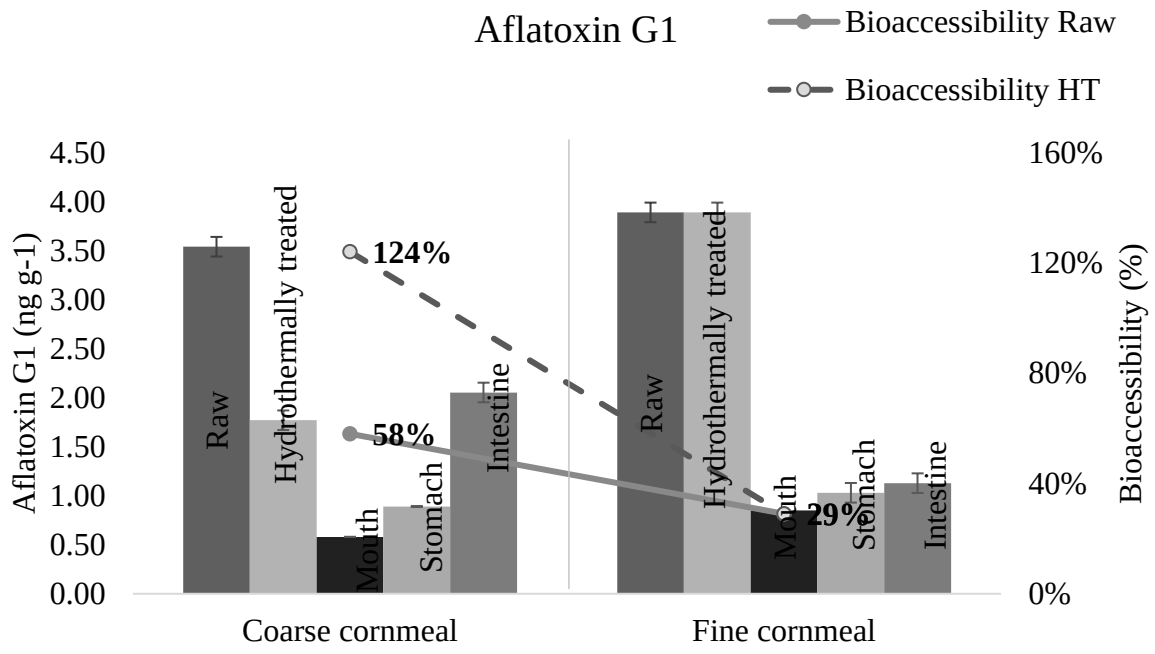
Bioaccessibility is defined as the fraction of a compound that is released from the food matrix in the gastrointestinal tract, thus becoming available for intestinal absorption. The release of a contaminant from the product ingested into the gastrointestinal tract is a prerequisite for absorption and its bioavailability in the body (VERSANTVOORT; VAN DE KAMP; ROMPELBERG, 2004).

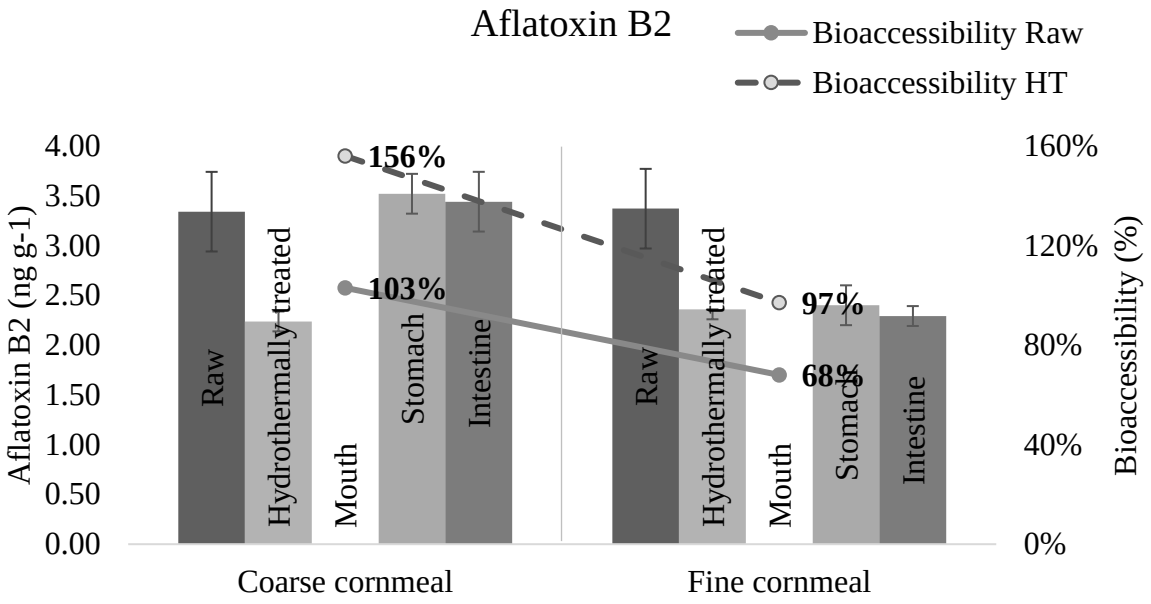
The process of digestion of a compound within the digestive system begins in the mouth, where the grinding of the food occurs. Also in the mouth due to the action of the salivary amylase, the first degradation of the starch is obtained, with its conversion into maltose (SILVERTHORN, 2017). In this work only aflatoxin G1, present in both flours, was released in the mouth digestion step (Figure 3).

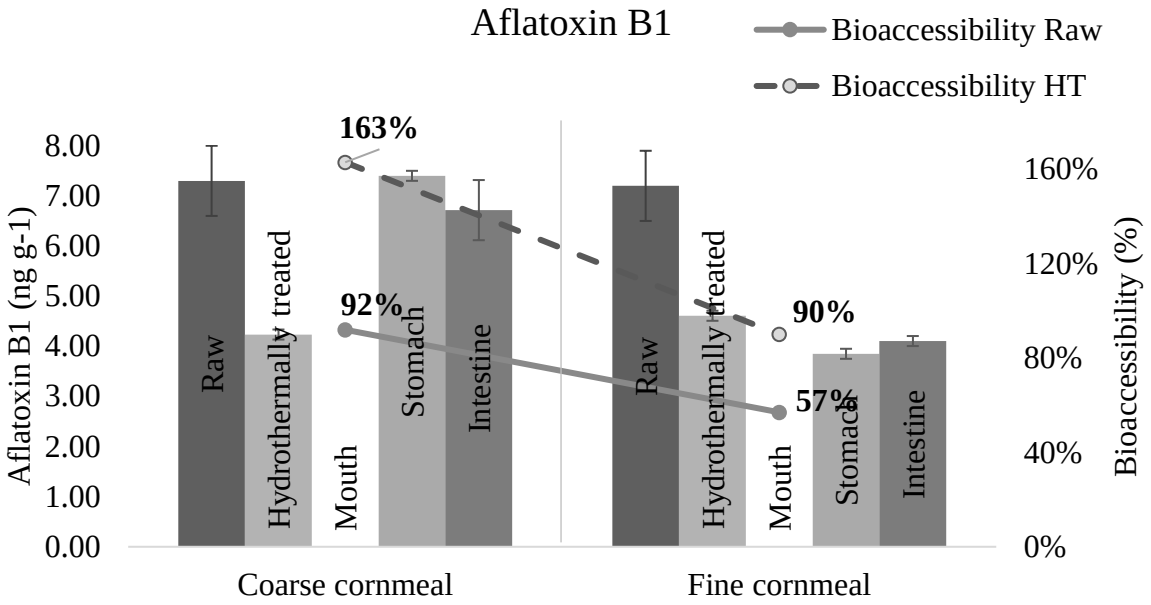
The next digestive step is the transfer of the bolus of food to the stomach, which has a high acidity due to the production of hydrochloric acid. This low pH environment facilitates the conversion of pepsinogen into pepsin, which then hydrolyzes proteins to amino acids, oligopeptides and polypeptides (SILVERTHORN, 2017). In this experiment aflatoxins (G2, B2 and B1) were released in the stomach (Figure 3), as previously shown by another study the bound aflatoxins can be released from their binders and converted to the free forms as a result of acid or enzymatic treatments (VIDAL et al., 2018). In the stomach where the pH is acid (pH 2.0) and proteolytic enzymes are present, aflatoxins that are bound to proteins can be released from the matrix to their free forms. The identification of this behavior of aflatoxins is important, since future work could evaluate the interaction of aflatoxins with other macromolecules, such as proteins.

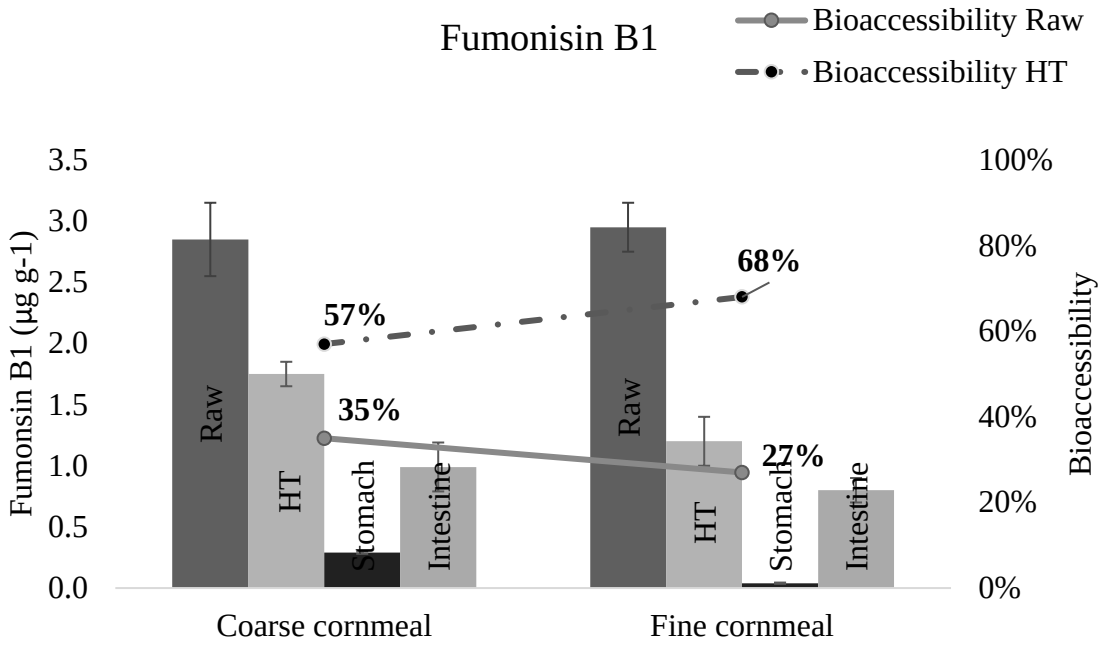
Figure 3 – Mycotoxins concentration in raw, hydrothermally treated (HT), mouth, stomach and small intestine and duodenal bioaccessibility.






Aflatoxin B2
Bioaccessibility Raw
Bioaccessibility HT
Aflatoxin B2 (ng g-1)
Bioaccessibility (%)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
160%
120%
80%
40%
0%
156%
103%
97%
68%
Raw
Hydrothermally treated
Mouth
Stomach
Intestine
Raw
Hydrothermally treated
Mouth
Stomach
Intestine
Coarse cornmeal
Fine cornmeal


Aflatoxin B1
Bioaccessibility Raw
Bioaccessibility HT
Aflatoxin B1 (ng g-1)
Bioaccessibility (%)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
160%
120%
80%
40%
0%
163%
92%
90%
57%
Raw
Hydrothermally treated
Mouth
Stomach
Intestine
Raw
Hydrothermally treated
Mouth
Stomach
Intestine
Coarse cornmeal
Fine cornmeal



Fumonisin B1 was poorly released in the stomach, which is in line with the characteristic of the fumonisin extraction methods that suggest an alkaline hydrolysis to best release this toxin from food matrices (BRYŁA et al., 2016).

After digestion by acidification is completed, the food is directed to the small intestine, where enzymes and fluids are added to chyme through the pancreatic canal and liver. The action of bicarbonate, secreted by the pancreas, neutralizes the acidified chyme, preventing damage to the intestinal mucosa. The intestinal epithelium, along with the pancreas, secretes fluids containing enzymes (amylase, lipase, protease, and peptidase) that act on the digestion of starch, proteins, carbohydrates and fats. The liver adds bile, which is a complex mixture of bile salts, and agents that aid in the digestion of fats. By the end of this process, absorption of the products generated by this sequence of enzymatic conversions occurs in the small intestine. At this point in the gastrointestinal tract, absorption of contaminants, like mycotoxins, would occur if they are free to be absorbed. Water is then absorbed in the large intestine, and soon after, non-digestible and non-absorbable components are excreted through defecation (SILVERTHORN, 2017).

The bioaccessibility is usually calculated in relation to the concentration of mycotoxin in the product before the digestion process, however in this study it was also calculated in relation to concentration of mycotoxin in the raw material (cornmeal before the hydrothermal treatment). This was added because the values of bioaccessibility calculated in relation to the aflatoxin concentration in hydrothermal treated product were higher than 100%,

this behavior can be explained because after the digestion process the aflatoxin levels detected in samples was increased, partially reverting the hydrothermal treatment effect.

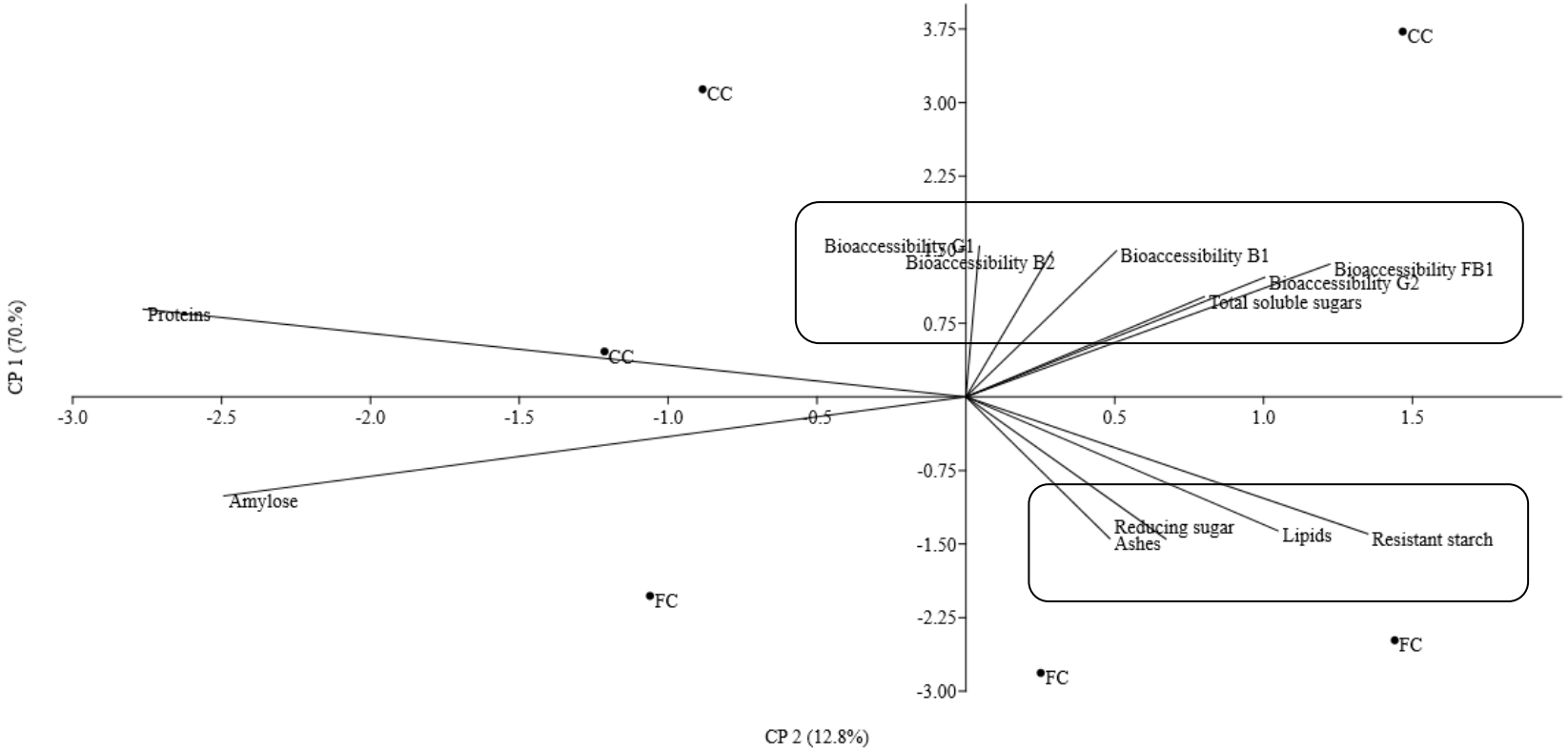
Aflatoxins bioaccessibility values ranging from 29 to 163% (Figure 3), in all instances the results showed a higher bioaccessibility of aflatoxins in the coarse cornmeal. The bioaccessibility values that were higher than 100%, when considered mycotoxin concentration in the product after the heat treatment, are may reflect potential interactions between aflatoxins and the matrix promoted by the heat treatment. Because at the end of the digestive process more toxin was free for detection, it indicates that bonds created by the thermal treatment were undone by the acids, salts and enzymes used.

When considering the calculated bioaccessibility results for aflatoxins based on the concentration of mycotoxin in the raw material, it was observed that in the fine cornmeal, on average, 63% of these aflatoxins (G2, B2 and B1) were free for absorption in the small intestine; while the remaining balance (approximately 37%) were either degraded or remained attached to some matrix component. In the case of coarse cornmeal, the bioaccessibility was higher (82%), with only 18% of the aflatoxins remaining bound or degraded by the heat treatment.

Part of the fumonisin B1 was released in the step that simulates the reactions of the stomach, however it was most released in the reactions of the intestine (Figure 3). In the fine cornmeal, 27% of the fumonisin B1 was bioaccessible; while in the coarse cornmeal the value was 35%. Most likely in the fine cornmeal the mycotoxin remained attached to some component of the matrix, possibly to the non-digestible starch, which was present in greater percentage in this type of cornmeal (Table 5).

Correlation analysis among the bioaccessibility of the mycotoxins and the components of the cornmeal was performed in order to elucidate which constituents could contribute to reduce their bioaccessibility. According to the results, resistant starch, reducing sugars, ashes and lipids seem to have influenced mycotoxin bioaccessibility negatively, according to the regions shown in the graph (Figure 4) and correlation values presented in Table 7. Based on this, the higher the content of these components in the thermally treated product, the less mycotoxin is bioaccessible.

Figure 4 – Principal components analysis for bioaccessibility of mycotoxins and constituents of hydrothermally treated cornmeals.



FC= fine cornmeal and CC= coarse cornmeal

Table 7 – Correlations among variables and their respective significance

Correlation		Resistant starch	Reducing sugars	Amylose	Ashes	Lipids	Proteins	Total soluble sugars
Bioaccessibility	G2	-0.42 (0.39)	-0.54 (0.26)	-0.52 (0.28)	-0.51 (0.29)	-0.59 (0.21)	0.30 (0.55)	0.22 (0.66)
	G1	**-0.82 (0.04)**	**-0.85 (0.03)**	-0.60 (0.20)	**-0.82 (0.04)**	**-0.84 (0.03)**	0.55 (0.25)	0.48 (0.32)
	B2	**-0.73 (0.09)**	**-0.75 (0.08)**	-0.55 (0.25)	-0.73 (0.10)	**-0.74 (0.09)**	0.55 (0.25)	0.47 (0.34)
	B1	**-0.74 (0.09)**	**-0.75 (0.08)**	-0.62 (0.19)	**-0.74 (0.09)**	**-0.73 (0.09)**	0.49 (0.32)	0.50 (0.31)
	FB1	-0.63 (0.17)	-0.63 (0.18)	-0.66 (0.15)	-0.66 (0.15)	-0.50 (0.31)	0.37 (0.47)	**0.76 (0.07)**

Data presented as correlation values (R) and significance value (p). Values $p<0.10$ significant.

According to Versantvoort, Van de Kamp and Rompelberg (2004) three processes are required to determine the oral bioavailability of a compound: 1) Release of the compound from its matrix into digestive juice in the gastrointestinal tract (bioaccessibility); 2) Transport across the intestinal epithelium into the vena Portae (intestinal transport); and 3) Degradation of the compound in the liver (and intestine)(metabolism).

In this work, in addition to bioaccessibility, the intestinal transport of bioaccessible mycotoxins was also evaluated. For this, dialysis membranes were used, under physiological conditions that simulate the intestinal environment (pH).

About 90% of all nutrient absorption occurs in the small intestine, the other 10% in the stomach and large intestine. Any undigested or non-absorbed material, left in the small intestine, passes into the large intestine (EKMEKCIOGLU, 2002). Since *in vivo* food digestion and absorption of compounds occurs mainly in the small intestine (VERSANTVOORT et al., 2005), the absorption in the tract of the large intestine was not taken into account in this study.

The degree of aflatoxin adsorption through the simulated intestinal transport varied depending upon the mycotoxin species. Among aflatoxins, G2 was absorbed the most, followed by B2 and G1; while aflatoxin B1 was the one absorbed the least (Table 8). However, when all

mycotoxins were considered, fumonisin B1 was associated with the lowest dialyzable fraction inferring that this mycotoxin, among those studied here, would be the one absorbed the least by the small intestine. Therefore, another important factor to consider when aiming to enhance the safety associated with food products and/or processes.

Table 8 – Dialysate fraction of mycotoxins

Mycotoxins	**Dialysate fraction (%)**	
	Fine cornmeal	**Coarse cornmeal**
AFLA G2	100.0	100.0
AFLA G1	76.9	83.8
AFLA B2	97.8	61.9
AFLA B1	78.9	95.4
FB1	51.2	62.5

The bioavailability of a compound also depends upon its physicochemical forms that are present at the site of absorption to overcome the intestinal barrier. Since the food matrix mainly affects bioaccessibility, while absorption and metabolism depend more on the specific properties of the toxin and the physiology of the animal of interest, the food matrix is expected to have less influence on these processes (BRANDON et al., 2005).

The hydrothermal treatment used in the study was shown to be a good way to mitigate fumonisin B1. For aflatoxins the search continues for other potential processes that could be used to mitigate mycotoxins and the risk of exposure to these contaminants through food.

4 CONCLUSION

The hydrothermal treatment resulted in mycotoxin reduction in the cooked product and these reductions were affected by the constituents of the matrix, such as soluble and reducing sugars and resistant starch. After the digestion process in the coarse cornmeal the concentration of aflatoxins in the small intestine were higher than in the cooked product, on contrary most of the bound fumonisin B1 resisted the *in vitro* digestion. In the fine cornmeal 64% of the aflatoxins (G2, B2 and B1) and 27% of the fumonisin B1 were free for absorption in the small intestine; while in the coarse cornmeal 91% of the aflatoxins and 35% of fumonisin B1 would be available. Thus, the hydrothermal treatment promotes the binding of fumonisin

B1 with macro non-digestible components of the food that are able to resist digestion. These mycotoxin-matrix interactions, when properly applied as part of mycotoxin mitigation strategies, could help in reducing the risk of exposure to these contaminants. From all the results shown here, it is clear that in order to truly estimate levels of mycotoxin exposures through foods, the study of their interaction with the components of the matrix is indispensable.

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ARTICLE VII

FATE OF AFLATOXINS IN CORNMEAL DURING SINGLE-SCREW EXTRUSION: A BIOACCESSIBILITY APPROACH

Fate of aflatoxins in cornmeal during single-screw extrusion: a bioaccessibility approach

ABSTRACT

The potential reduction of aflatoxins B1, B2, G1 and G2 levels through extrusion process as well as their bioaccessibility in corn-based products were investigated. Cornmeal samples were spiked with aflatoxins (50 ng g^{-1}) and extruded in the absence and presence of high-amylose corn starch (20%, w/w). Aflatoxins were quantified before and after the extrusion process, and the extruded products were subjected to *in vitro* digestion process where the toxin bioaccessibility was later determined. Aflatoxin quantification in all samples was done via HPLC-fluorescence detector. Extrusion of cornmeal samples led to a reduction of aflatoxins levels in the extruded product (B1 - 83.7%, B2 - 80.5%, G1 - 74.7% and G2 - 87.1%), and when high-amylose corn starch was added to the formulation, resistant starch was increased and higher aflatoxins reductions were observed (B1 - 89.9%, B2 - 88.6%, G1 - 75.0% and G2 - 89.9%). Extrusion cooking seems to be a promising food processing technology to mitigate the risk of exposure to aflatoxins in food products. The *in vitro* study of bioaccessibility indicates that part of the aflatoxin reduction observed after the extrusion process may be caused by interactions between aflatoxins and the food matrix macromolecules, and once the digestion is completed, part of these toxins become available for absorption in the small intestine. Therefore, it is recommended to include bioaccessibility testing of cereals after thermal treatment processes used to prepare food because they can form the modified mycotoxins, that are not possible to be detected using a conventional method.

Keywords: Extrusion. *In vitro* digestion. Modified mycotoxins.

1 INTRODUCTION

Corn crops are susceptible to fungal contamination in the field, after harvest and during storage. Due to specific environmental and storage conditions of grains, fungi may produce toxic compounds known as mycotoxins, which can induce toxic responses in human and animals after ingestion of contaminated grains (HUSSEIN; BRASEL, 2001). A group of mycotoxins called aflatoxins are compounds produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* (SWEENEY; DOBSON, 1998), and are the most widely investigated due to their dangerous potential. These compounds have the highest hepatocarcinogen activity in several animal species (FIRMIN et al., 2010; WOGAN; EDWARDS; SHANK, 1967) and epidemiological studies have demonstrated that aflatoxin B1 is associated with acute liver intoxications (IARC, 2002). Although there are different prevention methods for contaminated grains, different post-harvest treatments are required to reduce this contamination to acceptable levels. Since most foods are consumed after a heat treatment, the evaluation of this type of processing is worthwhile.

Extrusion cooking, a continuous high-temperature, pressure and short-time process, is the most versatile processing technology used in the food industry to develop products having better nutritional, functional and sensory characteristics (DALBHAGAT; MAHATO; MISHRA, 2019). This process gelatinizes the starch, degrades the protein and forms complexes among lipids, starch and proteins (ARRIBAS et al., 2017). Besides, it reduces aflatoxins in corn (SOHI SUKHMAN et al., 2015), corn-peanut meals and flakes (MOLLA; ZEGEYE, 2014). Most mycotoxins are chemically and thermally stable at temperature close to 100 ºC, and only higher temperatures used in frying, roasting, baking and extrusion can reduce contamination (KARLOVSKY et al., 2016).

However, mycotoxin reduction through extrusion does not necessarily mean detoxification as there is a possibility for toxins to be converted into a “modified” form, preventing them to be detected by conventional analytical methods. Once the contaminated product is consumed, certain “modified” mycotoxins can be hydrolyzed into their free forms after the ingestion. Consequently, further research is needed to fully understand the fate of mycotoxins upon ingestion (KOVAČ et al., 2018).

In light of this, the study of the bioaccessibility of mycotoxins, defined as the fraction of a compound that is released from the food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (BENITO; MILLER, 1998) can aid to better understand the effect of processing on mycotoxin. Research has shown the use of *in vitro*

digestion models to measure the bioaccessibility of aflatoxins ingested from peanut slurry (VERSANTVOORT et al., 2005), peanut, pistachio, hazelnut, dried figs, paprika, wheat, corn (KABAK; OZBEY, 2012), bread (SALADINO et al., 2018) and chicken breast muscles (SOBRAL et al., 2019), as an indicator of gastrointestinal bioavailability. However, most studies performed the bioaccessibility in the raw cereal, or spiked the sample before the digestion model, so it was not possible to evaluate the behavior of the potencially "modified" mycotoxin formed due to thermal processes.

The present study aimed to evaluate the impact of extrusion cooking process in the presence and absence of high-amylose corn starch, on the levels of the aflatoxins G1, B1, G2 and B2, and their subsequent *in vitro* bioaccessibility, in order to infer the potential of this process to mitigate the exposure to aflatoxins.

2 MATERIAL AND METHODS

2.1 CORNMEAL SAMPLE PREPARATION

Commercial coarse cornmeal (Lincoln, NE, USA) and corn starch with high amylose (HYLON VII) was used in the study. Hylon VII is an unmodified high amylose corn starch which contains approximately 70% amylose. The moisture content of the samples was adjusted to 22% and 29% (w/w) with the addition of distilled water. The samples were kept in sealed plastic bags and stored at 4 °C for 24 h to allow moisture equilibration and mycotoxin homogeneous distribution. Prior to the extrusion process, samples were allowed to reach ambient temperature (25 °C) and were thoroughly mixed. The moisture content was determined with an halogen moisture analyzer (MB27, Ohaus Corporation, Pine Brook NJ) set at 105 °C.

2.2 EXTRUSION PROCESS

Cornmeal samples were extruded in a laboratory-scale GR-8 single-screw extruder (C.W. Brabender Instruments, South Hackensack, NJ, U.S.A.). The extruder screw had a compression ratio of 3:1 with length to diameter ratio of 20:1. Feed rate of extruder was kept at a pace such that the feeding compartment was full at all times of operation. The temperature of the first feeding zone was set to 50 °C, whereas the transition and metering zones were set to 140 °C and 160 °C, respectively. Different conditions of screw speed (160 and 210 rpm), moisture (22 and 29%) and hylon VII (0 and 20%) were used during the experiment.

For each run, 800 g of cornmeal or cornmeal with hylon VII were passed through the extruder. Work was done in duplicate. After stable extruder conditions were reached, extrudates were collected and stabilized at room temperature (25 °C) for 24 h, ground and sealed in polyethylene bags until further testing.

2.3 AFLATOXIN IN UNEXTRUDED AND EXTRUDED CORNMEAL

Standards of aflatoxins B1, B2, G1 and G2 (Sigma-Aldrich, Saint Louis, MO, USA) were used for sample spiking. Aflatoxin stock standard solutions were prepared with acetonitrile (Fisher Scientific, Fair Lawn, NJ). Further, working solutions were prepared from stock solutions and quantified before use in a Genesys 150 UV-visible spectrophotometer (Thermo Scientific USA), according to AOAC (2000). Upon confirmation of stock solution concentration, cornmeal samples were spiked with different aflatoxin levels to achieve 50 ng g^{-1} (20 ng g^{-1} of B1 and 10 ng g^{-1} for B2, G1 and G2) using a spray nozzle bottle.

Aflatoxins in unextruded and extruded cornmeal were extracted according to USDA-GIPSA (2016) method. Briefly, 50 g of ground sample and 5 g NaCl (VICAM. Waters, USA) were blended with 100 mL methanol:water (80:20, v/v) at high speed for 1 min, and the resulting extract was poured into a fluted filter paper (#31240. VICAM. Waters, USA). Filtrate was collected in a clean vessel. Ten milliliters of the filtered extract were pipetted into a clean vessel containing 20 mL purified water and mixed. The diluted extract was filtered through a 1.5 µm glass microfiber filter (#31955. VICAM. Waters, USA) into a clean vessel.

The filtered extract was passed through an AFLAtest immunoaffinity column (G1010 Vicam, Watertown, MA) previously prepared according to manufacturer's instructions. The column was washed twice with 1 mL purified distilled water and sample was eluted into an amber flask by adding 1 mL HPLC grade methanol (A452-4, Fisher scientific, Pittsburgh, PA, USA). Extract was dried using a Reacti-Vap evaporating unit (18780, Pierce Chemical Company, Rockford, IL). Dried flasks were kept under refrigeration (4-8 °C) until analysis.

For aflatoxin quantification, extracts were derivatized prior to HPLC determination. Derivatization solution comprised of 1 mL of TFA (Sigma –Aldrich Inc., St. Louis, MO) and 4 mL HPLC grade acetonitrile (Fisher Scientific, Fair Lawn, NJ) in ultrapure water (10:90, v/v). Dry extracts were exposed to 250 µL of the derivatization solution, mixed for 30 s using a vortex and allowed to react for 30 min. The samples were filtered through a 0.2 µm membrane (PALL, Life Sciences) before injected into the HPLC.

Aflatoxin quantification was performed by HPLC UltiMate 3000 Series (Dionex, Sunnyvale, CA), coupled with a fluorescence detector (RF 2000). The HPLC system was equipped with a Nova-Pak C18 column (4 µm, 3.9 x 150 mm, WAT086344, Waters Corporation, Milford, MA), and a Nova-Pak C18 guard column (4 µm 3.9x20 mm, WAT044380, Waters Corporation, Milford, MA). Solvents used as mobile phase in the chromatographic system (acetonitrile and methanol) with purity > 99.9% were supplied by Fisher Chemical (HPLC grade, Fisher Scientific, Fair Lawn, NJ). Ultrapure water (>18.2 MΩ cm^{-1} resistivity) was obtained using a Milli-Q® SP Reagent Plus water system. The chromatographic conditions were followed according to Bianchini (2010), with column temperature 35 °C, mobile phase consisting of acetonitrile, methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ) and ultrapure water (17:17:66, v/v/v), flow rate 0.5 mL min^{-1}, with 20 µL of sample per injection. Excitation was set at 360 nm and emission at 440 nm.

Samples for aflatoxin quantification were taken before (unextruded) and after (extruded) the extrusion process. Aflatoxin reduction was determined according to equation 1, based on the amount of toxin in the unextruded product and the extruded corn-product, always considering the aflatoxin concentration in dry basis.

$$\text{Aflatoxin reduction (\%)} = 100 * \left(\frac{[\text{ aflatoxin SC}] - [\text{aflatoxin EC}]}{[\text{aflatoxin SC}]}\right) \quad (1)$$

Where: [aflatoxin SC] = aflatoxin concentration on spiked unextruded cornmeal,
[aflatoxin EC] = aflatoxin concentration on extruded cornmeal.

2.3.1 Method validation

The validation was performed according to European Commission (SANTE, 2016) and ANVISA (2003). The linearity, determination and correlation coefficient, as well as the limit of detection (LOD) and quantification (LOQ) were determined. The LOD and LOQ of the instrument were obtained considering three and ten times the signal-to-baseline (noise) ratio, respectively. Both the LOD and LOQ of the method were estimated considering the described extraction method.

An analytical curve was constructed using the aflatoxin standard solutions (G2 from 0.4 to 40 ng mL^{-1}; G1 from 1 to 100 ng mL^{-1}; B2 from 0.2 to 20 ng mL^{-1} and B1 from 0.4 to 40 ng mL^{-1}) in triplicate. The accuracy (recovery) was evaluated by spiking samples with

aflatoxins standards (B1, B2 and G2) at levels equivalent to 10-fold LOQ and 6-fold LOQ (G1), in triplicate. The precision (repeatability) of the method was expressed as the relative standard deviation (RSD) calculated from two triplicates of the spiked samples. The matrix effect (ME) of unextruded and extruded cornmeal was evaluated according to Malachová et al. (2014).

2.4 RESISTANT AND DIGESTIBLE STARCH

Resistant (RS) and digestible starch (DS) contents of extruded cornmeal were quantified according to AOAC 996.11 modified by Walter, Silva and Perdomo (2005) in triplicate. Briefly, the *in vitro* method quantifies the RS after the removal of DS by enzymatic treatment, simulating the hydrolysis that occurs in the upper digestive tract (mouth, stomach and small intestine), with α-amylase, protease and amyloglucosidase enzymes. The enzymes α-amylase and protease were obtained from MP Biomedicals (Solon, OH, USA) and amyloglucosidase from Sigma-Aldrich (St. Louis, MO, USA).

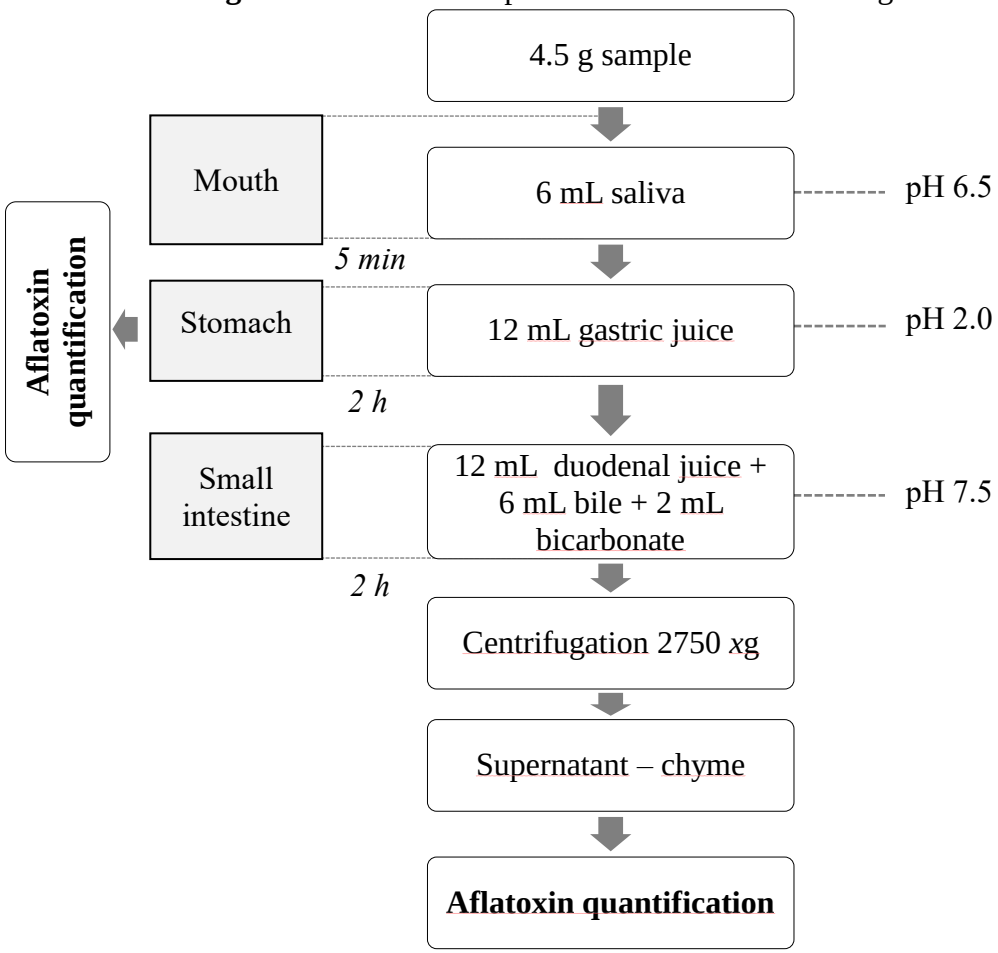
The remaining starch (resistant) is solubilized with dimethylsulfoxide and further hydrolyzed by amylolytic enzymes. The determination of the starch fractions (resistant and digestible) after hydrolysis was performed by quantifying the glucose released in each step by a glucose oxidase assay (GAGO-20 Sigma), converting readings to starch by the used of factor of 0.9.

2.5 BIOACCESSIBILITY OF AFLATOXINS IN EXTRUDED PRODUCTS

The bioaccessibility assay was performed according to the *in vitro* digestion model developed by Versantvoort, Van de Kamp and Rompelberg (2004), described in Figure 1.

This model simulates the biochemical conditions of the gastrointestinal tract in adult humans, and describes a three-step procedure simulating digestive processes in the mouth, stomach and small intestine. To accomplish this, synthetic solutions of the different portions of the digestive tract of monogastric animals were used (Table 1).

Figure 1 - Schematic representation of the *in vitro* digestion model



The enzymes pepsin from porcine gastric mucosa, α-amylase from *Bacillus subtilis*, pancreatin and lipase from porcine pancreas for the digestive fluids were obtained from MP Biomedicals (Solon, OH, USA). The mucin from porcine stomach type III was sourced from HiMedia (Mumbai, Maharashtra, India) and bile salts from Sigma–Aldrich (St. Louis, MO, USA). Uric acid, D-glucosamine hydrochloride and magnesium dichloride were obtained from Alfa Aesar; while D-glucuronic acid was purchased from Acros organics and bovine serum albumin (BSA) from Fisher Scientific, part of Thermo Fisher Scientific (Tewksbury, MA, USA).

All digestive juices were kept at 37 ± 2°C and pH was adjusted as indicated in Table 1. In the stomach and small intestine digestion process step, the digestion tubes with the samples were centrifuged and two fractions were obtained: chyme (supernatant - digestible material) and the digested matrix fraction (pellet – non digestible material). The pH of the chyme (supernatant) was in the range of 6.8 and 7. The mycotoxin extraction and quantification was done for the chyme fraction.

Table 1 – Synthetic juices constituents and concentrations

	Saliva	**Gastric juice**	**Duodenal juice**	**Bile juice**
Composition (per liter)	0.9 g KCl	2.75 g NaCl	7.01 g NaCl	5.26 g NaCl
	0.2 g KSCN	0.27 g NaH_2PO_4	3.39 g $NaHCO_3$	5.79 $NaHCO_3$
	0.9 g NaH_2PO_4	0.82 g KCl	0.08 g KH_2PO_4	0.38 g KCl
	0.57 g Na_2SO_4	0.4 g $CaCl_2.2H_2O$	0.56 g KCl	0.15 mL HCl (37%)
	0.3 g NaCl	0.31 g NH_4Cl	0.05 g $MgCl_2$	0.25 g urea
	1.7 g $NaHCO_3$	6.5 mL HCl (37%)	0.18 mL HCl (37%)	0.22 g $CaCl_2.2H_2O$
	0.2 g urea	0.65 g glucose	0.1 g urea	1.8 g BSA
	290 mg α-amylase	0.02 g glucuronic acid	0.2 g $CaCl_2$ $2H_2O$	30 g bile
	15 mg uric acid	0.085 g urea	1 g BSA	
	25 mg mucin	0.33 g glucoseamine hydrochloride	9 g pancreatin	
		1 g BSA	1.5 g lipase	
		2.5 g pepsin		
		3 g mucin		
pH	6.51	2.10	7.50	7.60
Time	5 min	2 h	2 h	

2.5.1 Aflatoxin quantification of digestible fraction

Aflatoxin content in the gastric and duodenal fluids was determined, and the bioaccessibility was then calculated. Samples collected from the *in vitro* digestion model (2.5 mL of stomach liquid fraction, and 3 mL of small intestine supernatant fraction) were cleaned-up prior to HPLC quantification. For each liquid fraction, each volume was passed through an AFLAtest immunoaffinity column (G1010 Vicam, Watertown, MA) previously prepared according to manufacturer's instructions. The column was washed twice with an equal volume of purified distilled water and sample was eluted into an amber flask by adding 2 mL of HPLC grade methanol (A452-4, Fisher scientific, Pittsburgh, PA, USA). Resulting extracts were dried and kept in refrigeration until quantification as described in Section 2.3.

In order to evaluate the aflatoxin recovery during the bioaccessibility assay, non-spiked cornmeal (control blank) was extruded and the biological fluids obtained from digestion (chyme) of uncontaminated extruded product were spiked with standard aflatoxins B1, B2, G1 and G2, 10-fold LOQ in triplicate. The matrix effect (ME) of the chyme fraction was evaluated according to Malachová et al. (2014).

2.6 STATISTICAL ANALYSIS

The extrusion experiment for aflatoxin reduction and resistant starch formation was repeated twice. Of these, the aflatoxin and resistant starch testing were done in triplicate. An analysis of variance (ANOVA) was carried out using *Statistica* 6.0, with the difference of least square means as post-comparison test. Significance of treatments was considered only when the statistical significance level of the test was at least 5% ($p<0.05$). Correlation analysis was performed in Past software (folk.uio.no/ohammer/past) to establish correlations between aflatoxins reduction, bioaccessibility and extrusion variables, where a p value of 0.10 is considered as the level of statistical significance.

3 RESULTS AND DISCUSSION

3.1 ANALYTICAL METHOD PERFORMANCE

The range of linearity, linear regression equation and correlation coefficient for each aflatoxin are given in Table 2.

Table 2 – Indicative of merit for aflatoxins G1, B1, G2 and B2 extraction method in unextruded and extruded cornmeal.

Figure of merit		**Aflatoxins**			
		G1	**B1**	**G2**	**B2**
Linearity (ng mL^{-1})		1 – 100	0.4 – 40	0.4 – 40	0.2 – 20
R^2		0.9996	1.0000	0.9992	0.9998
R		0.9998	1.0000	0.9996	0.9999
Linear regression equation		y = 3595.6x – 1616.7	y = 9027.2x – 1221.7	y = 5355.4x + 229.35	y = 10673x + 339.68
LODi (ng mL^{-1})		0.30	0.12	0.12	0.06
LOQi (ng mL^{-1})		1.00	0.40	0.40	0.20
LODm (ng g^{-1})		0.45	0.18	0.18	0.09
LOQm (ng g^{-1})		1.50	0.60	0.60	0.30
Recovery (%)	Unextruded	76.8 (7.1)	70.0 (8.1)	80.9 (3.1)	79.2 (1.0)
	Extruded	75.2 (1.0)	75.9 (0.1)	83.2 (1.8)	82.2 (1.9)
Matrix effect (%)	Unextruded	-6.6	-17.5	21.9	7.3
	Extruded	11.8	0.5	10.6	0.0

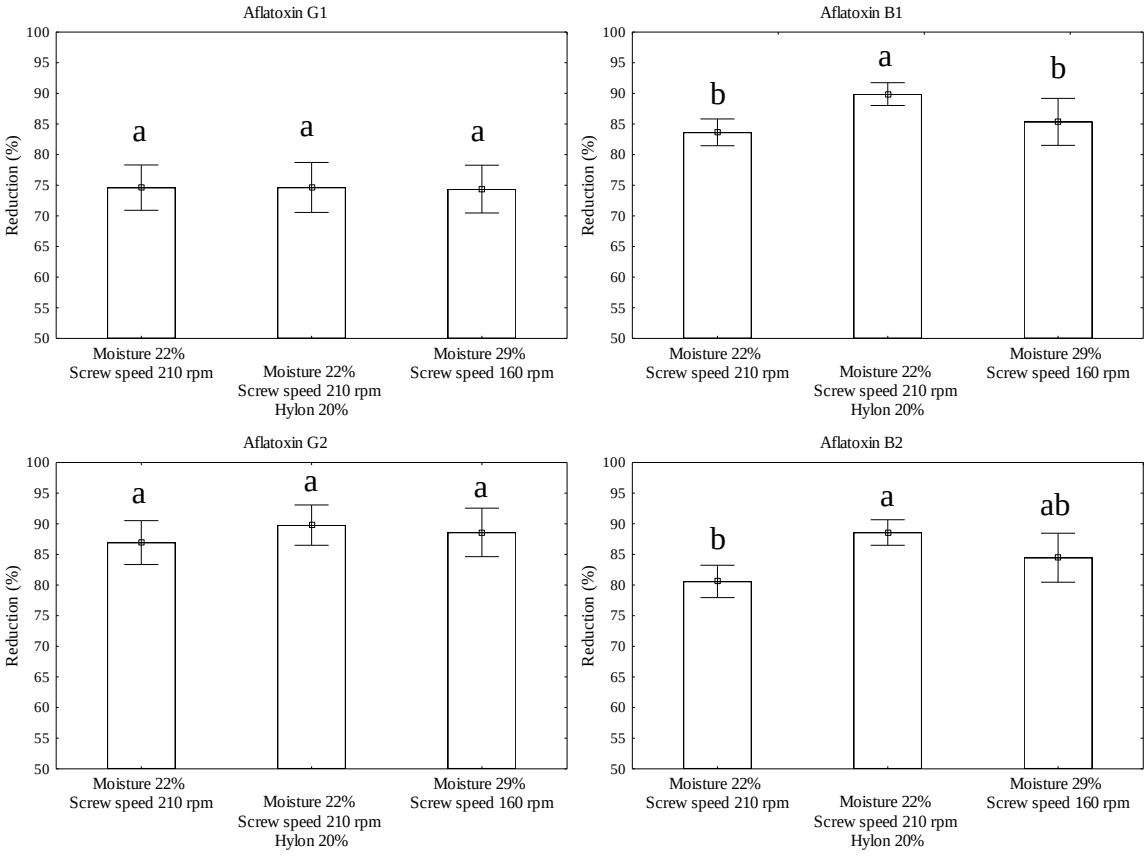
Results expressed as mean (RSD) (n=3). RSD= relative standard deviations in %. LODs (S/N= 3) and LOQs (S/N= 10). LODi= instrument limit of detection, LOQi= instrument limit of quantification, LODm= method limit of detection, LOQm= method limit of quantification.

It was shown adequate in all cases as the correlation coefficients were higher than 0.999. The limit of detection and quantification for all aflatoxins evaluated are also shown in Table 2. The level of spiking was from 3 to 9 μg kg^{-1} and recoveries for the four aflatoxins, both unextruded and extruded cornmeal, ranged from 70% to 83.2% (Table 2). All values are within the criteria approved by the European Regulatory Committee, which established that for concentrations between 1 and 10 μg kg^{-1}, the recoveries should vary between 70 and 110% (EUROPEAN COMMISSION, 2006). Further, relative standard deviations (RSDs) associated with the recovery tests were <20% and thus conformed to the accepted limits for the concentrations used in a repeatability test (EUROPEAN COMMISSION, 2006). No matrix effect was observed for either unextruded or extruded products based on the criteria suggested by SANTE (2016).

3.2 AFLATOXIN CONTENT AFTER EXTRUSION

The extrusion process decreased aflatoxins levels (Figure 2). The largest reductions were in aflatoxin G2 (84.1%-92.5%) and B1 (82.1%-91.1%), followed by B2 (79.9%-88.8%) and G1 (70.8%-78.3%).

Figure 2 - Apparent reduction of aflatoxins in extruded corn-based product.



Mean ± standard deviation (n= 6). Different letters indicate significant differences among treatments ($p<0.05$).

Previous studies also showed similar reduction trends involving extrusion. Corn extrusion resulted in aflatoxin reductions of 75% (B1) and 72.5% (B2) (SOHI SUKHMAN et al., 2015); while in corn-peanut extrudates and flakes the reduction observed was 93.8% (B1) and 83.5% (B2) (MOLLA; ZEGEYE, 2014). In peanut meal a reduction of 77.6% was observed for B1 (ZHENG et al., 2015); while in corn flour only 25% of aflatoxin B1 was reduced (CAZZANIGA; BASÕ, 2001).

In this study, the term “apparent reduction” will be used to refer to reduction promoted by the extrusion treatment, because it is not known if the reduction in aflatoxin concentrations is caused by degradation of the toxin or if it involves modification or binding mechanisms. Factorial analysis of extrusion conditions (moisture and screw speed) and amylose addition showed that different extrusion conditions did not have significant difference ($p>0.05$) in the aflatoxin apparent reduction (Figure 2), whereas changes on amylose content showed a significant difference ($p<0.05$) for B1 and B2.

Cornmeal with 20% hylon showed higher reduction of aflatoxins B1 and B2 after extrusion process, showing that aflatoxins are affected by physical treatments, that promote the interactions of the toxins with food components (SAMARAJEEWA et al., 1990).

Castells et al. (2006) reported that moisture content had a significant influence on reducing aflatoxins B2, G1, and G2 in extruded artificially contaminated rice meal, whereas it was not a significant factor for aflatoxin B1. Moreover, Zheng et al. (2015) showed that the barrel temperature and moisture have significant influences ($p<0.01$) on the degradation rate of aflatoxin B1 in extruded contaminated peanut meal, while the screw speed has no significant influence. The type of food and their composition have a significant effect on aflatoxin reduction and their reduction also vary depending on the extrusion processing parameters, such as the moisture, temperature, screw speed, and the initial aflatoxin concentration. Food moisture may enhance aflatoxin B1 degradation by hydrolyzing the lactone ring. At a critical moisture concentration and temperature this hydrolytic opening of the lactone ring forms a terminal carboxylic acid, which then undergoes heat-driven decarboxylation to the less toxic aflatoxin D1 (SAMARAJEEWA et al., 1990).

Physical treatment, such as extrusion, applied to foods may transform any mycotoxin present into a different chemical structure, or by means of binding to food matrix components, may render not detectable by conventional analytical methods (KARLOVSKY et al., 2016). Tabata et al. (1992) suggested that in the presence of reducing saccharides (such as glucose), proteins and amino acids, aflatoxins may bind to the macromolecules during processing.

The decrease in aflatoxins levels in the presence of hylon (i.e. high-amylose content) can be due to the production of certain compounds during or after the extrusion process. It has been previously reported that extrusion may lead to the formation of resistant starch (RS) type 3, particularly if high amylose content starches are incorporated (MUTLU; KAHRAMAN; ÖZTÜRK, 2017). Therefore, the levels of resistant starch in all extrudates were quantified, and a correlation analysis was carried out to verify if RS could be involved in the reduction of aflatoxins after extrusion.

3.2.1 Resistant starch and aflatoxins level

A correlation analysis (Table 3) was performed in order to establish strength of association between each variable (screw speed, moisture, amylose addition, resistant starch, and digestible starch) and the reduction of aflatoxins by the extrusion treatment. The variables, moisture and screw speed, did not show a significant ($p>0.1$) correlation with the reduction of aflatoxins.

Table 3 – Correlations coefficients among extrusion variables and their respective significance ($p<0.1$).

Correlation parameter	**Aflatoxin reduction**			
	G1	**B1**	**G2**	**B2**
Moisture	-0.03 (0.90)	-0.19 (0.46)	0.03 (0.89)	-0.01 (0.96)
Screw speed	0.03 (0.90)	0.19 (0.46)	-0.03 (0.89)	0.01 (0.96)
Hylon	0.02 (0.94)	**0.71 (0.00)**	0.28 (0.25)	**0.68 (0.00)**
Resistant starch	0.12 (0.67)	**0.66 (0.00)**	0.28 (0.26)	**0.54 (0.02)**
Digestible starch	0.40 (0.09)	0.04 (0.87)	0.27 (0.27)	0.03 (0.91)

The addition of 20% hylon and levels of resistant starch showed a significant ($p<0.1$) positive correlation with the reduction of aflatoxins B1 and B2, with R values higher than 0.54. The interaction among aflatoxins B1 and B2, and amylose or resistant starch promoted by the extrusion process may have led to the formation of modified aflatoxin. Kovac et al. (2018) showed some evidence that mycotoxins could be masked or modified by using food-processing equipment, especially in cereal-based products.

Mechanical or thermal processing was shown to promote mycotoxin interactions with macromolecules such as polysaccharides and proteins. Modified forms of aflatoxin may

be considered a contribution to reduce the risk of exposure to these contaminants in the gastrointestinal tract depending on the degree to which these toxins are bound to other matrix molecules. Because once ingested, modified mycotoxins can revert to their free forms (DALL'ASTA; BATTILANI, 2016; HUMPF; VOSS, 2004), the bioaccessibility of aflatoxins in these extruded products must be evaluated.

3.3 AFLATOXIN BIOACCESSIBILITY

The validation of a method to determine aflatoxins in the chyme (digested fraction) is necessary to demostrate that the method can produce accurate and reliable results. The mean recoveries of the chyme fraction for aflatoxin G1, B1, G2 and B2 were considered adequate, being above 80% (RSD<6%) (Table 4), meeting the requirements of recovery values for aflatoxins in foodstuffs set by European Commission (2006). The matrix effect was also evaluated, being below 9.4%, thus demonstrating that the clean-up of the chyme was efficient.

Table 4 – Analytical performance for aflatoxin G1, B1, G2 and B2 in blank chyme fraction.

Aflatoxin	**Recovery (%)**	**RSD (%)**	**Matrix effect (%)**
G1	91.2	3.4	-7.1
B1	86.8	4.6	-9.4
G2	100.5	5.4	-4.6
B2	96.1	2.8	-8.1

Results expressed as mean (n=3). RSD= relative standard deviation.

According to Versantvoort, Van de Kamp and Rompelberg (2004) to determine the oral bioavailability of a compound, three processes are required. The first one is the bioaccessibility, the second is the intestinal transport and the last one is the metabolism.

The food matrix mainly affects the bioaccessibility, whereas absorption and metabolism depend more on the toxin-specific properties and on the animal physiology and, therefore, the food matrix is expected to have less influence on these (BRANDON et al., 2006). Furthermore, this study did not take into account the large intestine, as absorption of mycotoxins mainly takes place in the small intestine (GONZÁLEZ-ARIAS et al., 2013). Based on this fact and the higher reduction on aflatoxins levels (>70%) promoted by the extrusion process, an *in vitro* digestion model was used to determine aflatoxin bioaccessibility. This type of analysis

can help elucidate if the aflatoxin reduction during the extrusion is partly due to its association with food components.

The digestion of a compound begins in mouth, where the grinding of the food occurs and, due to the action of the salivary amylase, the first degradation of the starch is obtained. Then, the bolus travels toward the stomach, in which due to the production of hydrochloric acid the conversion of pepsinogen into pepsin is facilitated, and proteins are hydrolyzed to amino acids, oligopeptides and polypeptides (SILVERTHORN, 2017).

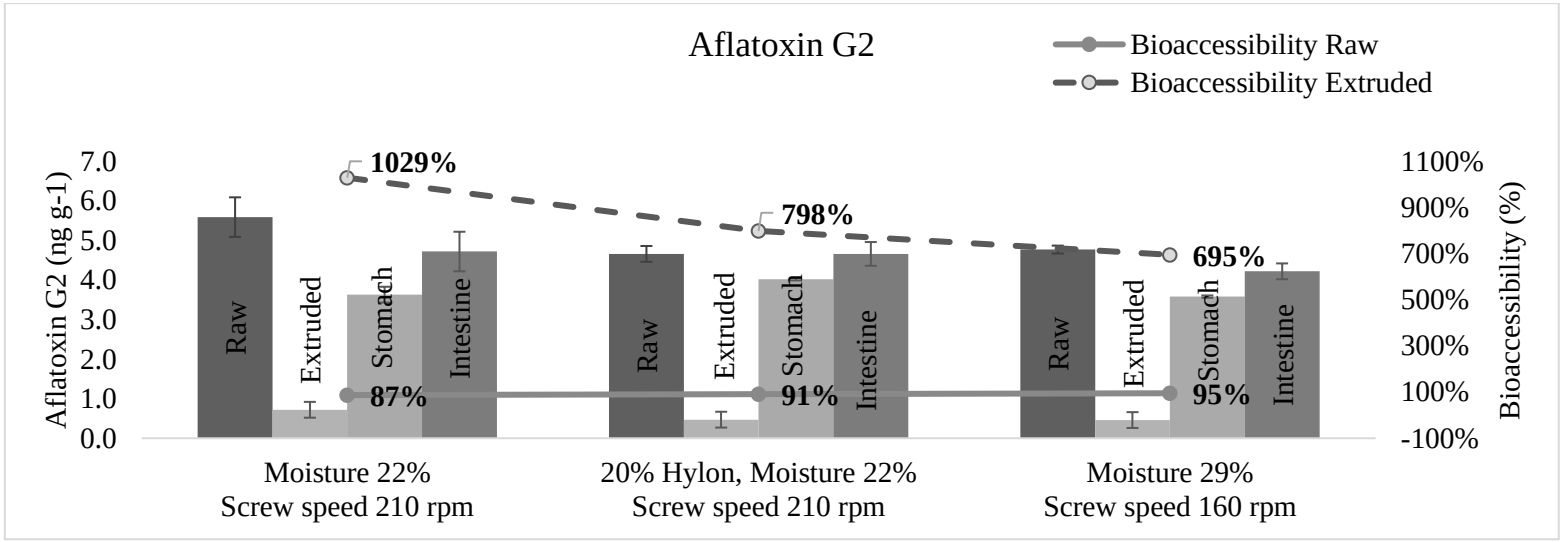
After a portion of the acidified food is digested in the stomach, the next step is the small intestine, where enzymes and fluids are added to chyme through the pancreatic canal and liver. The action of bicarbonate, secreted by the pancreas, neutralizes acidified chyme, preventing damage to the intestinal mucosa. The intestinal epithelium, along with the pancreas, secretes fluids containing enzymes (amylase, lipase, protease, peptidase) that act on the digestion of proteins, carbohydrates and fats. The liver adds bile, a complex mixture of bile salts, that aids in the digestion of fats. At the end of the process, the absorption of the products, including free mycotoxins, generated by the sequence of enzymatic break occurs in the intestinal epithelium (BENITO; MILLER, 1998). After the absorption, any toxins will be metabolized and may exert their toxic effect in the body.

The bioaccessibility is usually calculated considering the concentration of mycotoxin in the product before the digestion process, however in this study it was also calculated based on the aflatoxin concentration in the unextruded cornmeal. This was added as the bioaccessibility calculated in association to the aflatoxin concentration in extruded product, both gastric and duodenal, previous values higher than 100%. This behavior can be explained by the possible interaction of the aflatoxins with the matrix components due to the heat treatment of cornmeal.

The gastric bioaccessibility of aflatoxin G2 calculated based on the extruded (158-215%) and unextruded (70-83%) product was higher than other aflatoxins (Figure 3). It also was observed that the aflatoxin G2 was mostly released in the stomach, where the pH is acidic (pH 2.0). By the action of proteolytic enzymes, aflatoxins that were bound to the proteins were released to their free forms. These results support the theory that the extrusion process provides an apparent reduction in aflatoxin levels, since aflatoxin G2 showed the largest reductions after extrusion.

The duodenal bioaccessibility (small intestine) of all aflatoxins was higher than 100% when calculated based on the initial aflatoxin concentration in extruded products (Figure 3). This indicates that after the digestion process the aflatoxins levels detected in sample

Figure 3 - Aflatoxin concentration in raw, extruded, stomach, small intestine and duodenal bioaccessibility



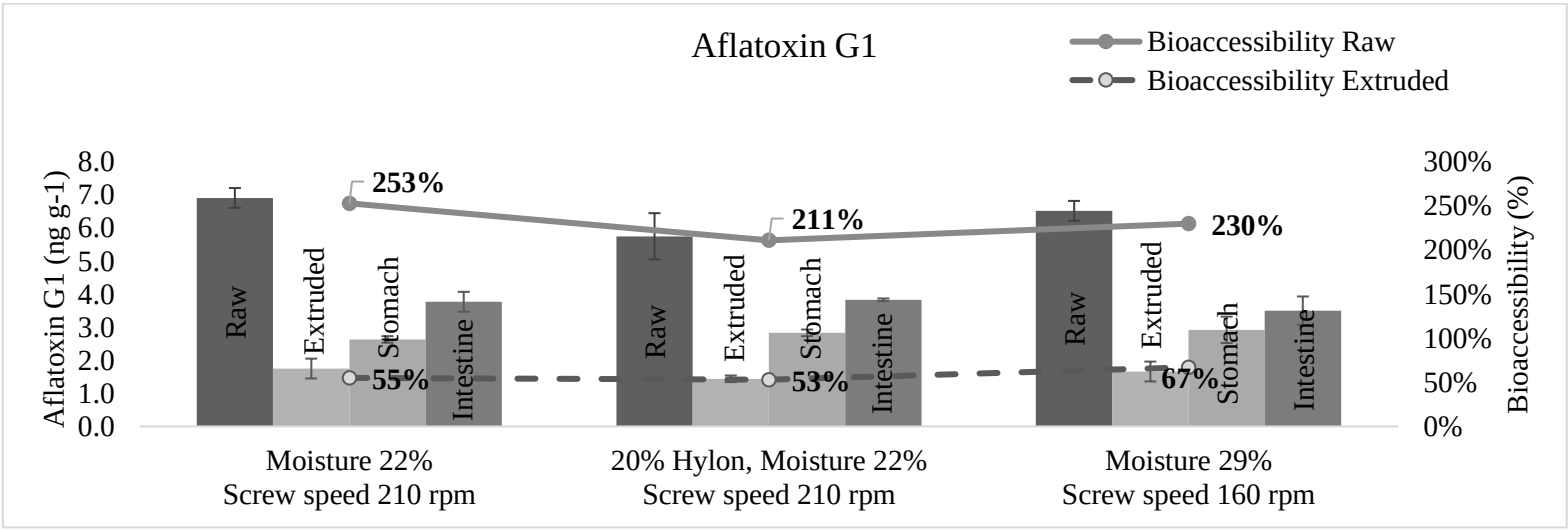
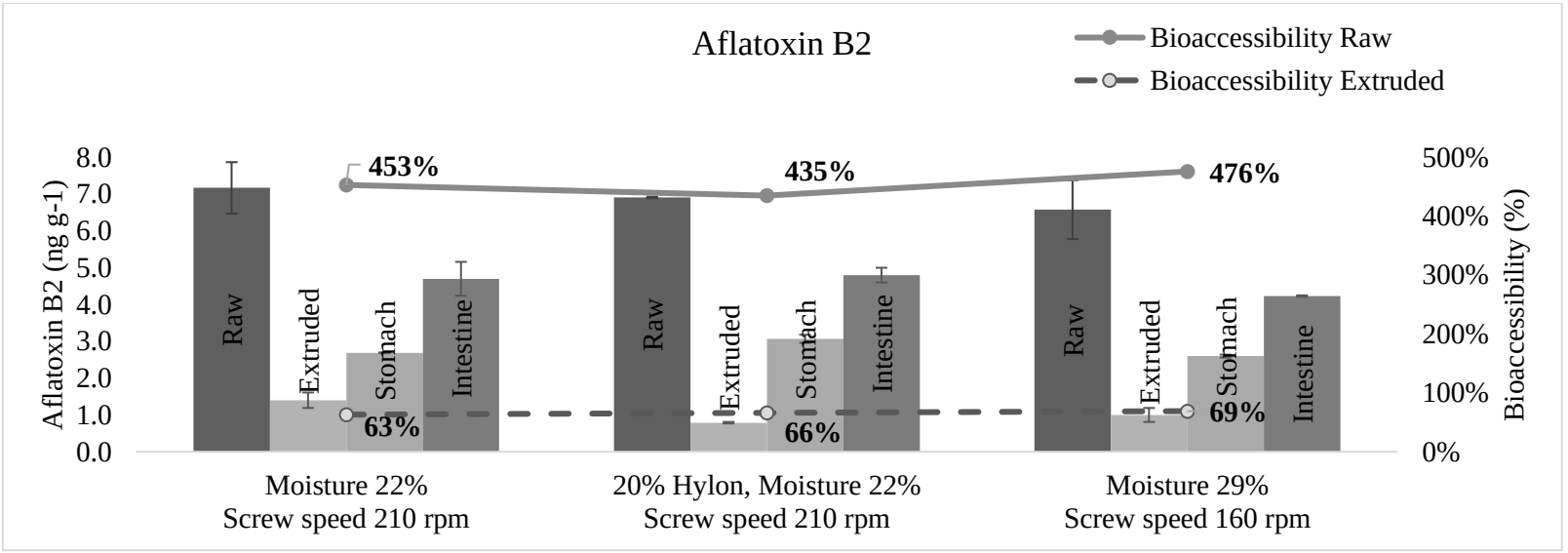
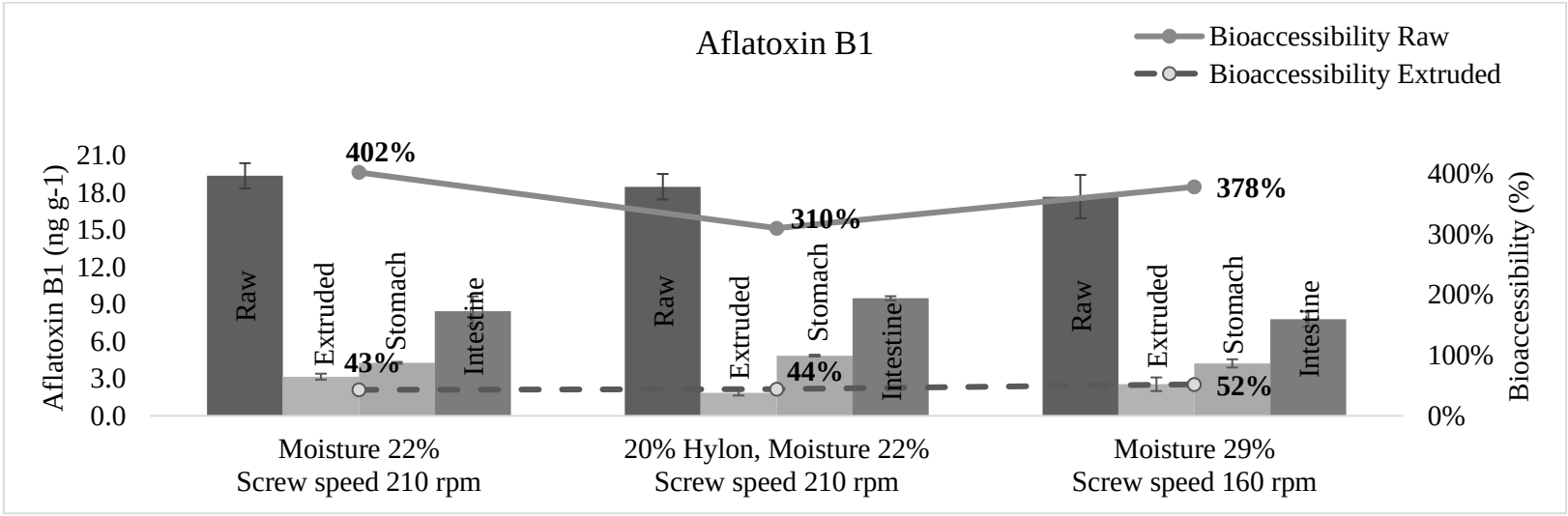


Figure 3 - Aflatoxin concentration in raw, extruded, stomach, small intestine and duodenal bioaccessibility Cont.





was increased, partially due to a reversal of the extrusion effect (Figure 3). This *in vitro* study indicates that part of the aflatoxin reduction promoted by thermal extrusion process may be caused by interactions between aflatoxins and the food matrix macromolecules, and once the digestion is completed, part of these toxins become available for absorption in the small intestine.

However, when the duodenal bioaccessibility is calculated based on the initial aflatoxin concentration in the unextruded cornmeal, aflatoxin B1 (43-52%), G1 (53-67%) and B2 (63-69%) showed lower bioaccessibility than G2 (87-95%), interestingly exhibiting the opposite trend based on their toxicity (IARC, 2002). The higher the toxicity, the less bioavailable the toxin appeared to be in the product. Remaining aflatoxins that were not bioaccessible indicate that upon digestion some mycotoxins still remain bound to some matrix component.

3.3.1 Processing parameters influencing aflatoxin bioaccessibility

A correlation analysis (Table 5) was carried out to identify which extrusion component could impact the bioaccessibility of aflatoxins. The variable moisture negatively (R= -0.54) and screw speed positively (R= 0.54) influenced the aflatoxin bioaccessibility, but only for G1 in a significant ($p<0.05$) manner.

Table 5 – Correlations coefficients among extrusion variables and their respective significance ($p<0.05$).

Correlation	**Aflatoxins bioaccessibility**			
	G1	**B1**	**G2**	**B2**
Moisture	**-0.54 (0.02)**	-0.33 (0.17)	-0.10 (0.71)	-0.14 (0.56)
Screw speed	**0.54 (0.02)**	0.33 (0.17)	0.10 (0.71)	0.14 (0.56)
Hylon	**0.93 (0.00)**	**0.95 (0.00)**	**0.59 (0.01)**	**0.86 (0.00)**
Resistant starch	**0.91 (0.00)**	**0.83 (0.00)**	**0.48 (0.04)**	**0.68 (0.00)**
Digestible starch	-0.16 (0.52)	-0.14 (0.57)	0.19 (0.45)	-0.06 (0.79)
Aflatoxin reduction	0.34 (0.16)	**0.81 (0.00)**	**0.82 (0.00)**	**0.86 (0.00)**

Resistant starch and hylon in the product showed positive and significant correlation with the bioaccessibility. Previously, it was shown that the addition of 20% hylon and the levels of resistant starch showed a positive correlation with the reduction of aflatoxins

B1 and B2. Thus, in order to evaluate if the greater reduction of aflatoxins levels after the extrusion process also promotes a lower bioaccessibility, a correlation of the data was performed. The reduction of aflatoxin levels showed a positive correlation with their bioaccessibility, G1 (R= 0.34 p= 0.16), B1 (R= 0.81 p= 0.00), G2 (R= 0.82 p= 0.00) and B2 (R= 0.86 p= 0.00). Therefore, the higher the reduction during extrusion the greater the bioaccessibility, confirming that not all aflatoxin binding to the food matrix is stable during digestion in the gastrointestinal tract.

Therefore, the reduction of free mycotoxin levels after a thermal treatment does not indicate a decrease in total levels of mycotoxins (HUMPF; VOSS, 2004), since a realistic fraction of these toxins may still be absorbed in the small intestine.

4 CONCLUSION

The extrusion process promoted an apparent reduction of aflatoxin levels, particularly in the presence of high-amylose corn starch and resistant starch. The *in vitro* digestion assay showed that part of the aflatoxin reduction promoted by the thermal extrusion process might be caused by interactions between aflatoxins and food matrix macromolecules, forming modified aflatoxins. Once the digestion is completed, part of these toxins become available once again for absorption in the small intestine.

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ARTICLE VIII

STABILITY OF FUMONISIN B1 AND ITS BIOACCESSIBILITY IN EXTRUDED CORN-BASED PRODUCTS

Stability of fumonisin B1 and its bioaccessibility in extruded corn-based products

ABSTRACT

Fumonisins are a group of mycotoxins commonly associated with corn-based products. While there are some approaches to control mycotoxins during processing, innovative alternatives are still of interest for food processors. The objective of this research was to determine the effect of amylose and resistant starch on fumonisin B1 (FB1) levels in extruded corn-based products as well as the toxin bioaccessibility upon digestion. Cornmeal alone or combined with high-amylose corn starch (hylon) (20%, w/w) were extruded in the presence of FB1 (1.5 μg g^{-1}). FB1 was quantified both in the unextruded and extruded products by HPLC-fluorescence detector with pre-column derivatization. Samples were then subjected to an *in vitro* digestion model to evaluate the stability of FB1. The addition of hylon further reduced FB1 (74.9%), when compared to the effect of the extrusion alone (66.0%), indicating the possible binding of fumonisin with this macromolecule or resistant starch. The bound fumonisin became stable upon gastric digestion, and the duodenal bioaccessibility of free FB1 was lower than 35% when hylon ingredient was used in the product. Principal component analysis (PCA) showed that hylon and resistant starch content influenced the reduction of FB1 and its duodenal bioaccessibility. This study showed for the first time the increase in FB1 reduction with addition of hylon in extruded corn-based product, and that the stability of modified fumonisin under digestive conditions is likely to occur. Extrusion cooking may contribute to mitigate FB1 contamination in foods, with the addition of amylose, possibly, further minimizing the risk of exposure.

Keywords: Modified fumonisin, extrusion, *in vitro* digestion

1 INTRODUCTION

Fumonisins are a group of mycotoxins distributed worldwide, and particularly associated with grain commodities, including corn. These fungal toxins are produced mainly by *Fusarium verticilloides* and *Fusarium proliferatum* species. Although different fumonisin types have been identified, FB1 is the most prevalent in food (BORDINI et al., 2019b). Exposure to fumonisin-contaminated foods may contribute to various adverse health outcomes such as cancer and birth defects (WORLD HEALTH ORGANIZATION, 2018). For this reason, the International Agency for Research on Cancer (IARC) has classified FB1 as probable carcinogenic for humans (group 2B) (IARC, 2002). Therefore, strategies for mitigating fumonisin in corn products are of utmost importance.

Corn products are commonly prepared by some heat treatment, so the use of a thermal approach is an applicable strategy to reduce the mycotoxins levels. Previous findings have showed a reduction of FB1 when corn is cooked by extrusion (BULLERMAN; BIANCHINI, 2007). Extrusion applies high pressure, torque and heat to raw ingredients and this cooking process is the most versatile processing technology used by the food industry to produce snacks, breakfast cereals and textured foodstuffs (JOZINOVIĆ et al., 2016). The degree of FB1 reduction achieved is variable and depends on extrusion conditions and food matrix composition. The mechanisms that promote these reductions are not well understood, but it involves thermal decomposition or binding to food matrix components (HUMPF; VOSS, 2004), forming “modified mycotoxins”. These mycotoxins normally remain undetected during the testing for free mycotoxin (HUMPF; RYCHLIK; CRAMER, 2019). The type of process and the composition of the food affect the modified mycotoxin levels present in the final product (FREIRE; SANT’ANA, 2018).

FB1 is a reactive compound, containing a diester of propane-1,2,3-tricarboxylic acid (TCA) as well as a 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxy-icosane, in which the C-14 and C-15 hydroxyl groups form esters with the terminal carboxyl group of TCA. The FB1 amino groups and TCA groups promote interactions with other compounds, resulting in various modified forms of fumonisin (FALAVIGNA et al., 2012; FREIRE; SANT’ANA, 2018). Additionally, the TCA side chains are commonly cleaved forming the hydrolyzed FB (DE GIROLAMO et al., 2016).

The transformation mechanisms of FB1 in extrusion process still have not been completely clarified. Some authors have suggested that fumonisins may react with some components of the food matrix during processing (HUMPF; VOSS, 2004), where the

interactions can be further improved by the addition of reducing sugars (JACKSON et al., 2011). In these interactions FB1 reacts with glucose and other reducing sugars to form N-(deoxy-D-fructos-1-yl) FB1 and N-carboxymethyl FB1 compounds (HOWARD et al., 1998). This toxin has also been reported to form adducts via its tricarballylic acid moieties when heated with a model protein and starch compounds (SEEFELDER; KNECHT; HUMPF, 2003).

However, once ingested, the modified fumonisin can be stable (FALAVIGNA et al., 2012) or converted back to their free forms and became available for intestinal absorption (DALL'ASTA et al., 2010). The total amount of an ingested contaminant does not always reflect the amount that is available to the body (VERSANTVOORT et al., 2005). This concern has raised the need for *in vitro* studies to examine the fate of modified fumonisin by mimicking natural conditions during digestion.

Based on these aspects, it is hypothesized that fumonisins might bind to corn starch or resistant starch during extrusion. And if so, that this binding could influence the FB1 bioaccessibility. The purpose of this study was to determine if the addition of high-amylose corn starch and formation of resistant starch during the extrusion of cornmeal reduces the amount of FB1 in extrudates and how it influences the mycotoxin *in vitro* bioaccessibility.

2 MATERIAL AND METHODS

2.1 CORNMEAL SAMPLE PREPARATION

Commercial coarse cornmeal (Lincoln, NE, USA) and high-amylose corn starch (Hylon VII) were used in the study. Hylon VII ((Ingredion Incorporated, Westchester, IL, EUA) is an unmodified corn starch derived from high amylose corn. It contains approximately 70% amylose, and is used in a variety of food applications.

The moisture content of the samples was adjusted to 22% and 29% (w/w) with the addition of distilled water and samples were spiked with 1.5 µg g^{-1} of FB1 using a spray nozzle bottle. The samples were kept in sealed plastic bags and stored at 4 °C for 24 h to allow moisture equilibration and mycotoxin homogeneous distribution. Prior to the extrusion process, the products were allowed to reach ambient temperature (25 °C) and were mixed to ensure homogeneity. The moisture content was determined with a halogen moisture analyzer (MB27, Ohaus Corporation, Pine Brook NJ) set at 105 °C.

2.2 EXTRUSION PROCESS

Cornmeal samples were extruded in a laboratory-scale GR-8 single-screw extruder (C.W. Brabender Instruments, South Hackensack, NJ, U.S.A.) according to Jackson et al., (2011) with some modifications. The extruder screw had a compression ratio of 3:1 with length to diameter ratio of 20:1. Feed rate of extruder was kept at a pace such that the feeding compartment was full at all times of operation. The temperature of the first feeding zone was set to 50 °C, whereas the transition and metering zones were set to 140 °C and 160 °C, respectively. Different screw speed (160 and 210 rpm), moisture (22 and 29%) and hylon concentration (0 and 20%) were used during the experiment.

For each sample, 800 g of cornmeal and cornmeal combined with hylon were passed through the extruder. Work was done in duplicate. After stable extruder conditions were established, extrudates were collected and stabilized at room temperature (25 °C) for 24 h, ground and sealed in polyethylene bags until further testing. Samples for fumonisin quantification were taken before (unextruded) and after the extrusion process (extruded).

2.3 BIOACCESSIBILITY OF FB1 IN EXTRUDED PRODUCTS

The bioaccessibility assay was performed according to the *in vitro* digestion model developed by Versantvoort, Van de Kamp and Rompelberg (2004). The model simulates the biochemical conditions of the gastrointestinal tract in adult humans, and describes a three-step procedure simulating digestive processes in the mouth, stomach and small intestine. To accomplish this, synthetic solutions containing the same components of the different portions of the digestive tract of monogastric animals were used and all digestive juices were kept at 37 ± 2°C and pH determined before use. A detailed juice composition is shown in Table 1.

The enzymes pepsin from porcine gastric mucosa, α-amylase from *Bacillus subtilis*, pancreatin and lipase from porcine pancreas for the digestive fluids were obtained from MP Biomedicals (Solon, OH, USA). The mucin from porcine stomach type III was sourced from HiMedia (Mumbai, Maharashtra, India) and bile salts from Sigma–Aldrich (St. Louis, MO, USA). Uric acid, D-glucosamine hydrochloride and magnesium dichloride were obtained from Alfa Aesar; while D-glucuronic acid was purchased from Acros organics and bovine serum albumin (BSA) from Fisher Scientific, part of Thermo Fisher Scientific (Tewksbury, MA, USA).

Table 1 – Synthetic juices constituents and concentrations

	Saliva	**Gastric juice**	**Duodenal juice**	**Bile juice**
Composition (per liter)	0.9 g KCl	2.75 g NaCl	7.01 g NaCl	5.26 g NaCl
	0.2 g KSCN	0.27 g NaH_2PO_4	3.39 g $NaHCO_3$	5.79 $NaHCO_3$
	0.9 g NaH_2PO_4	0.82 g KCl	0.08 g KH_2PO_4	0.38 g KCl
	0.57 g Na_2SO_4	0.4 g $CaCl_2.2H_2O$	0.56 g KCl	0.15 mL HCl (37%)
	0.3 g NaCl	0.31 g NH_4Cl	0.05 g $MgCl_2$	0.25 g urea
	1.7 g $NaHCO_3$	6.5 mL HCl (37%)	0.18 mL HCl (37%)	0.22 g $CaCl_2.2H_2O$
	0.2 g urea	0.65 g glucose	0.1 g urea	1.8 g BSA
	290 mg α-amylase	0.02 g glucuronic acid	0.2 g $CaCl_2$ $2H_2O$	30 g bile
	15 mg uric acid	0.085 g urea	1 g BSA	
	25 mg mucin	0.33 g glucoseamine hydrochloride	9 g pancreatin	
		1 g BSA	1.5 g lipase	
		2.5 g pepsin		
		3 g mucin		
pH	6.51	2.10	7.50	7.60
Time	5 min	2 h	2 h	

The digestive process started by adding the simulated saliva formulation (6 mL) to the sample (4.5 g) and allowing a 5-min incubation at 37 ± 2°C. The gastric digestion was simulated with the addition of gastric juice (12 mL), followed by a 2 h mixing at 55 rpm at 37 ± 2°C using a shaking incubator (MaxQ 4000, Thermo Scientific, USA). The duodenal digestion was accomplished with the simultaneous addition of the duodenal juice (12 mL), bile (6 mL) and 1 M bicarbonate solution (2 mL), followed by a 2 h mixing with the same conditions as described above. The pH determined in the chyme (supernatant) was in the range of 6.8 and 7.

In the stomach and small intestine digestion process steps, the digestion tubes with the samples were centrifuged and two fractions were obtained: chyme (supernatant - digestible material) and the digested matrix fraction (pellet – non digestible material). FB1 was quantified in the chyme which allowed for bioaccessibility determination, according to equation 1.

$$\text{Bioaccessibility (\%)} = 100 * \left(\frac{[\text{FB1 chyme}]}{[\text{FB1 extruded cornmeal}]}\right) \quad (1)$$

Where: [FB1 chyme] = fumonisin B1 concentration of chyme in the stomach or small intestine. [FB1 extruded cornmeal] = fumonisin B1 concentration of extruded cornmeal.

2.4 EXTRACTION AND CLEAN-UP OF FB1

2.4.1 Unextruded and extruded cornmeals

A fumonisin B1 standard (Cayman Chemical, Ann Arbor, MI, USA) was used for sample spiking and standard solution was prepared with acetonitrile:methanol (1:1, v/v) according to product information.

FB1 in unextruded and extruded cornmeal were extracted according to FumoniTest™ HPLC method (VICAM, [s.d.]) for corn. Briefly, 50 g of ground sample and 5 g NaCl (VICAM. Waters, USA) were weighed inside a blender jar, and 100 mL methanol:water (80:20, v/v) were added. Sample was then blended at high speed for 5 min, and the resulting extract was poured into a fluted filter paper (#31240. VICAM. Waters, USA). Ten mL of the filtered extract were pipetted into a clean vessel with 40 mL PBS (Phosphate-buffered saline) (#G1113, VICAM, Waters, USA) and mixed well. The diluted extract was filtered through a 1.5 µm glass microfiber filter (#31955. VICAM. Waters, USA).

For the clean-up, 10 mL of the filtered extract were passed through a FumoniTest™ immunoaffinity column (Vicam, Watertown, MA) previously prepared according to manufacturer's instructions. The column was washed with 10 mL PBS and sample was eluted into an amber flask by adding 1 mL HPLC grade methanol (A452-4, Fisher scientific, Pittsburgh, PA, USA) and then 1 mL purified water. Extract was dried using a Reacti-Vap evaporating unit (18780, Pierce Chemical Company, Rockford, IL). Dried flasks were kept under refrigeration (4-8 °C) until analyzed.

2.4.2 Digestible fraction

The chyme collected from the *in vitro* digestion model was filtered and then 5 mL of each step (stomach or small intestine) were diluted in 5 mL 0.1% Tween-20/2.5% PEG (polyethylene glycol) PBS mixed well and then 5 mL passed through a FumoniTest™ immunoaffinity column (Vicam, Watertown, MA). The column was washed with 5 mL of PBS (#G1113, VICAM, Waters, USA) and sample was eluted into an amber flask by adding 2 mL of HPLC grade methanol (A452-4, Fisher scientific, Pittsburgh, PA, USA) and then the extract was dried and kept under refrigeration (4-8 °C) until analysis.

2.4.3 Method validation

The validation was performed according to European Commission (SANTE, 2016) and guidelines from the Brazilian Health Regulatory Agency (ANVISA, 2003). The linearity, determination and correlation coefficient, as well as the limit of detection (LOD) and quantification (LOQ) were determined. The instrument limit of detection (LOD) and quantification (LOQ) were obtained considering three and ten times the signal-to-baseline (noise) ratio, respectively. The method LOD and LOQ were estimated considering the dilution in the extraction method.

The analytical curve was constructed using FB1 standard solutions with increasing concentrations, from 0.025 to 1 µg mL^{-1} in triplicate. The accuracy (recovery) was evaluated spiking a known blank sample with a FB1 standard (0.25 µg g^{-1}) in triplicate. The precision (repeatability) was evaluated with recovery determination. The matrix effect (ME) of both unextruded and extruded cornmeal was evaluated according to Malachová et al. (2014).

In order to evaluate the FB1 recovery during the bioaccessibility assay, un-spiked unextruded cornmeal (control blank) was extruded and digested. The supernatant (chyme) was spiked with FB1 at levels equivalent to 10-fold the LOQ in triplicate to evaluate the analytical method in terms of accuracy and precision. The matrix effect (ME) of the chyme fraction was evaluated according to Malachová et al. (2014).

2.5 FB1 QUANTIFICATION

For the quantification of FB1, the samples were derivatized prior to HPLC injection according to Becker-Algeri et al. (2013) with some modifications. FB1 was quantified using an HPLC UltiMate 3000 Series (Dionex, Sunnyvale, CA), with a fluorescence detector (RF 2000) at an excitation of 335 nm and emission of 440 nm. Separation was done on a Nova-Pak C18 column (4 µm, 3.9 x 150 mm, WAT086344, Waters Corporation, Milford, MA) at 25 °C, with a Nova-Pak C18 guard column (4 µm, 3.9 x 20 mm, WAT044380, Waters Corporation, Milford, MA). The mobile phase was prepared according to Becker-Algeri et al. (2013), with acetonitrile and water (50:50, v/v) and the pH adjusted at 2.45 with acetic acid (HPLC grade, Fisher Scientific, Fair Lawn, NJ). Ultrapure water (>18.2 MΩ cm^{-1} resistivity) was obtained using a Milli-Q® SP Reagent Plus water system and acetonitrile was sourced from Fisher Scientific, Fair Lawn, NJ. The flow rate was set to 0.5 mL min^{-1}, with 20 µL of reconstituted sample per injection.

The OPA-MCE reagent was prepared dissolving 40 mg ortho-phthalaldehyde (OPA) (Sigma-Aldrich, St. Louis, MO, USA) in 1 mL methanol. OPA was later diluted with 5 mL of 0.1 M sodium borate decahydrate (Sigma-Aldrich, St. Louis, MO, USA) adjusted to pH 9.5. Fifty microliters of 2-mercaptoethanol (MCE) (Sigma-Aldrich, St. Louis, MO, USA) were incorporated to complete the derivatization solution. The solution was well mixed with a vortex and stored in dark conditions for up to one week at 8 °C in an amber vial (MOTTA; SCOTT, 2007). The dry extract was dissolved in 0.5 mL water:acetonitrile (70:30, v/v) and the extract was filtered. One hundred microliters of the filtered extract were transferred to a clean vial and 100 µL of OPA-MCE reagent was added, followed by mixing using a vortex and allowing for a 2-min reaction prior to quantification.

2.6 RESISTANT AND DIGESTIBLE STARCH

Resistant and digestible starch contents of unextruded and extruded cornmeal were quantified according to AOAC method 996.11 modified by Walter, Silva and Perdomo (2005) in triplicate. Briefly, the *in vitro* method quantifies the RS after the removal of digestible starch by enzymatic treatment, simulating the hydrolysis that occurs in the upper digestive tract (mouth, stomach and small intestine), with α-amylase, protease and amyloglucosidase enzymes. The remaining starch (resistant) is solubilized with dimethylsulfoxide and further hydrolyzed by amylolytic enzymes.

The determination of the resistant starch after hydrolysis was performed by quantifying the glucose released by a glucose oxidase assay (GAGO-20 Sigma), converting readings to starch with a factor of 0.9.

2.7 STATISTICAL ANALYSIS

Extrusion was carried out in duplicate, while quantifications (FB1 reduction, resistant starch formation and bioaccessibility) were performed in triplicate. Analysis of variance were carried out using Software Statistica 6.0, followed by Tukey mean difference test. Differences with a probability value of $p < 0.05$ were considered significant. A principal component analysis (PCA) was performed in Past software (folk.uio.no/ohammer/past) to establish correlations among FB1 reduction and bioaccessibility, and variables association with extrusion, resistant starch, digestible starch and hylon content.

3 RESULTS AND DISCUSSION

3.1 ANALYTICAL METHOD PERFORMANCE

The analytical parameters obtained showed that both the identification and quantification of FB1 were suitable with the HPLC-FD method, which included pre-column derivatization. The analytical curve exhibited linearity of 0.025 to 1.0 μg mL^{-1} and correlation coefficient greater than 0.99. The LOQ of the instrument and method (0.025 μg mL^{-1} and 0.025 μg g^{-1}) were satisfactory for the determination of FB1, being well below the recommended maximum levels for fumonisins in degermed dry milled corn products in Brazil that is 1.5 μg g^{-1} (ANVISA, 2017) and in U.S which is 2.0 μg g^{-1} (USFDA, 2001).

The recovery values of FB1 for unextruded, extruded cornmeal and duodenal chyme were 90.9%, 99.7% and 91.5%, respectively. These percentages are within the criteria approved by the European Regulatory Committee, which recommends that for lower or equal concentrations than 0.5 μg g^{-1} recoveries can vary between 60-120% (EUROPEAN COMMISSION, 2006).

The repeatability was evaluated by the relative standard deviation (RSD) and the values were 8.2%, 12.3% and 15.4% for unextruded, extruded cornmeal and chyme, respectively. All RSD values were lower than 20%, which is accepted limits for the concentrations used in a repeatability test (EUROPEAN COMMISSION, 2006), and are therefore also in agreement with the results of the recovery. The matrix effects for unextruded (13.9%), extruded (-19.9%) and chyme (-16.6%) indicate that the solvent curve can be used to determine FB1 levels with reliability (ME <+ - 20%) (SANTE, 2016).

3.2 FUMONISIN REDUCTION WITH EXTRUSION PROCESS

The addition of hylon was expected to be a factor influencing the levels of resistant starch in extruded products (unpublished results). Additionally, based on the health benefits and functional properties associated with resistant starch, development of extruded products with higher levels of resistant starch are of current interest (CHEN et al., 2017; MUTLU; KAHRAMAN; ÖZTÜRK, 2017). Being an undesirable contaminant in corn-based products, this study aimed to understand the stability of FB1 in extruded cornmeal when hylon was added to the mixture. Hylon is a common ingredient used in extruded products.

Extrusion cooking resulted in reductions of FB1 (Table 2). The addition of hylon further reduced FB1 (75%), when compared to the effect of the extrusion alone (66%),

indicating the possible association of fumonisin with this macromolecule. The extrusion parameters (moisture content and screw speed) did not seem to affect the reduction of FB1.

It has been long recognized that extrusion cooking can reduce FB1 concentrations in foods. The reduction in fumonisin content can be due to structural modifications and/or interactions with food matrices, resulting in modified forms of the toxin that are not detectable by conventional methods of analysis (DALL'ASTA et al., 2009).

Table 2 - Fumonisin B1 (FB1) content in unextruded and extruded samples, as well as reduction of FB1 by extrusion

Treatment	FB1 content (μg g^{-1})		Reduction (%)*
	Unextruded	Extruded	
Moisture 22%, Screw speed 210 rpm	2.22 (7.9)	0.75 (9.6)	66.0[b]
20% Hylon, Moisture 22%, Screw speed 210 rpm	2.24 (10.6)	0.56 (10.8)	75.0[a]
Moisture 29%, Screw speed 160 rpm	1.76 (4.9)	0.58 (5.7)	66.9[b]

Mean (RSD %) RSD= standard relative deviation (n=6).
*Different letters indicate significant differences ($p<0.05$).

Some authors showed that the extrusion processing efficacy can be further improved by the addition of reducing sugars (JACKSON et al., 2011) and sodium chloride (CASTELLS et al., 2009). The reduction in fumonisin in some of these cases was probably due to bound forms promoted by chemical interactions or reactions between the amino group of FB1 and glucose or other reducing sugar. The result is the formation of N-(deoxy-D-fructos-1-yl)-FB1 and N-carboxymethyl-FB1 (HOWARD et al., 1998).

Another modified fumonisin found in processed food is the hydrolyzed form. This is the result of a cleavage of the tricarballylic side chains of FB1, and it is commonly obtained upon alkali treatment (DE GIROLAMO et al., 2016). Although Jackson et al. (2011) detected little amounts of hydrolyzed fumonisin and N-carboxymethyl-FB1 after extrusion, the formation of N-(deoxy-D-fructos-1-yl)-FB1 via maillard type reactions was a major modification pathway upon sugar addition. Nonetheless, it was verified that unknown (i.e. other modified) forms were predominant (>50%) when comparing the FB1 originally present in corn, and the amount of fumonisin-related compounds measured after extrusion. Park et al. (2004) detected protein-bound FB1 in corn flakes and corn-based breakfast cereals. Previously

research had shown that this toxin is able to bind to proteins via their two tricarballylic acid side chains (SEEFELDER; KNECHT; HUMPF, 2003).

These results reinforce the hypothesis that thermal processes applied to food can form modified fumonisins, including fumonisins covalently bound to starch, proteins, and reducing sugars (FALAVIGNA et al., 2012). To date, there is no information about the reduction of FB1 in corn-based products with addition of high-amylose corn starch in extrusion process. The higher reduction verified in this study with this ingredient can be explained by the association of fumonisin with this particular macromolecule. A study performed in model systems indicate the formation of covalent bounds between carboxyl groups of fumonisins and a hydroxyl group present in the starch molecule (SEEFELDER; KNECHT; HUMPF, 2003). Moreover, the FB1 may also be physically entrapped into the macromolecular structure of starch (BRYLA et al., 2014). Even though the entrapping mechanism is still not clear from a physicochemical point of view, the existing data indicates that biopolymers such as amylose and amylopectin can form inclusion complexes with fumonisin (KOVAČ et al., 2018).

The apparent reduction shown through extrusion can be partly reversible under some conditions (KOVAČ et al., 2018). Therefore, there is a need for bioassays when evaluating the efficacy of the extrusion process on mycotoxin reduction. In response to this knowledge gap, bioaccessibility assays can be used to investigate if modified fumonisins are released during digestion in the gastrointestinal tract.

3.3 FB1 CONTENT IN THE GASTRIC AND DUODENAL FLUIDS FRACTIONS

For a more accurate risk assessment related to hazard exposure, it is important to evaluate if mycotoxins are bioaccessible to be absorbed and to act on the different organs or tissues of the human body. Bioaccessibility is defined as the fraction of a compound that is released from the food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (VERSANTVOORT et al., 2005). Therefore, the release of the contaminant from the ingested product in the gastrointestinal tract is a prerequisite for absorption and bioavailability in the body. Therefore the bioavailability of compounds present in the food may be significantly different depending on the food product, food processing or preparation (VAN HET HOF et al., 2000). Further, knowledge about the toxicological relevance of modified mycotoxins is still modest. By mimicking natural conditions during digestion, *in vitro* studies are able to better evaluate the fate of modified mycotoxins as a result of their interaction with stomach and duodenal juices. In order to evaluate a more realistic

exposure to FB1 through the consumption of extruded corn-based products, a bioaccessibility component was included in the present study.

In humans, digestion is a sequential process that begins in the mouth, where food is chewed and mixed with saliva, rich in amylases, that helps breaking down polysaccharides. Then, the stomach continues to break food constituents in the presence of hydrochloric acid, facilitating protein degradation to lower weight peptides. The third part of the process is in the small intestine, where enzymes and fluids are added to the chyme through the pancreatic canal and liver. The action of bicarbonate, secreted by the pancreas, neutralizes the acidified chyme, preventing damage to the intestinal mucosa. The intestinal epithelium, along with the pancreas, secretes fluids containing enzymes (amylase, lipase, protease, peptidase) that act on the digestion of starch, proteins, and other food components. The liver adds bile, which is a complex mixture of bile salts, and agents that aid in the digestion of fats. At the end of the process, absorption of nutrients (SILVERTHORN, 2017), including free mycotoxins, by the cells of the intestinal epithelium take place (BENITO; MILLER, 1998).

After the absorption, the contaminant will be metabolized and then may cause the toxic effect in the body. The food matrix mainly affects the bioaccessibility, whereas absorption and metabolism depend more on the toxin-specific properties and on the animal physiology. Therefore, the food matrix is expected to have less influence on absorption and metabolism (BRANDON et al., 2006). This study does not take into account the large intestine, as absorption of mycotoxins takes mainly place in the small intestine (GONZÁLEZ-ARIAS et al., 2013). Table 3 shows the results regarding the gastric and duodenal bioaccessibility expressed as FB1 concentration and as percentage related to the initial FB1 concentration in extruded samples.

Table 3 - Bioaccessibility of fumonisin B1 for extruded cornmeal samples after gastric and duodenal digestion.

Treatment	**FB1 (μg g^{-1})**			**Bioaccessibility (%)***	
	Extruded	**Stomach**	**Intestine**	**Gastric**	**Duodenal**
Moisture 22% Screw speed 210 rpm	0.75 (9.6)	0.42 (21.9)	0.33 (20.0)	55^{c}	43^{b}
20% Hylon, Moisture 22% Screw speed 210 rpm	0.56 (10.8)	0.47 (24.5)	0.20 (10.8)	83^{b}	35^{c}
Moisture 29% Screw speed 160 rpm	0.58 (5.7)	0.59 (23.1)	0.32 (15.3)	100^{a}	54^{a}

Mean (RSD, %) (n= 6) RSD= relative standard deviation. Statistically significant differences ($p<0.05$) shown by different letters in the same column.

Bioaccessility values for both gastric and duodenal fractions were lower than 100% indicating that any potential modified fumonisin formed during the extrusion process was stable under the *in vitro* digestion. This suggested that digestive enzymes were not able to cleave the covalent bonds in these toxin-matrix conjugates. Falavigna et al. (2012) also showed that FB1 conjugates formed with reducing sugars, proteins and starch are stable under *in vitro* digestion. From a chemical stand point, FB1 bound to glucose by an amino group is a secondary amine, which is largely stable upon heating as well as under acidic and alkaline conditions (FALAVIGNA et al., 2012).

The results shown here suggest that fumonisins covalently bound to matrix components are difficult to be released in the digestive tract indicating that extrusion of contaminated products will likely render the resulting extrudates safer than their raw counterparts. Besides, it was shown that a maximum of 54% of the FB1 present in extruded samples arrived unmodified to the intestine, available for the absorption by the cells of the intestinal epithelium. Other studies support these findings, showing a low bioaccessibility of FB1 in chyme on corn flakes (37–64%) where hydrolyzed FB1 was not detected (MOTTA; SCOTT, 2007, 2009).

The low duodenal bioaccessibility of FB1 in the present study may also be a consequence of the ability of FB1 to bind to bile salts, a behavior consistent with an *in vivo* study that demonstrated the excretion of FB1 in bile (PRELUSKY; TRENHOIM; SAVARD, 1994). Furthermore, molecular mechanics from previous work showed that the FB1 interaction with bile components, such as cholesterol and bile salts, (MAHFOUD et al., 2002) is likely due to the structural similarity of fumonisin with sphingoid bases, known to interact with cholesterol and sphingolipids (MAHFOUD et al., 2002).

A study about fumonisin absorption and transformation in humans showed that FB1 is actually excreted mainly in faeces and, at lower amount, in urine (RILEY et al., 2012). This indicates a toxicokinetic similar to that reported for animals, where the majority of ingested FB1 is excreted via faeces unchanged or after partial hydrolysis of the tricarballylic acid side chains. Shier (2000) reported toxicokinetics of FB1 indicating a low oral bioavailability in rats, pigs, chickens, cows and monkeys. In these studies, the toxin was rapidly cleared by the biliary route and excreted largely unchanged in the feces. Even after feeding periods as long as 12 days, only traces of FB1 were found in liver and kidney, and less was found in other organs.

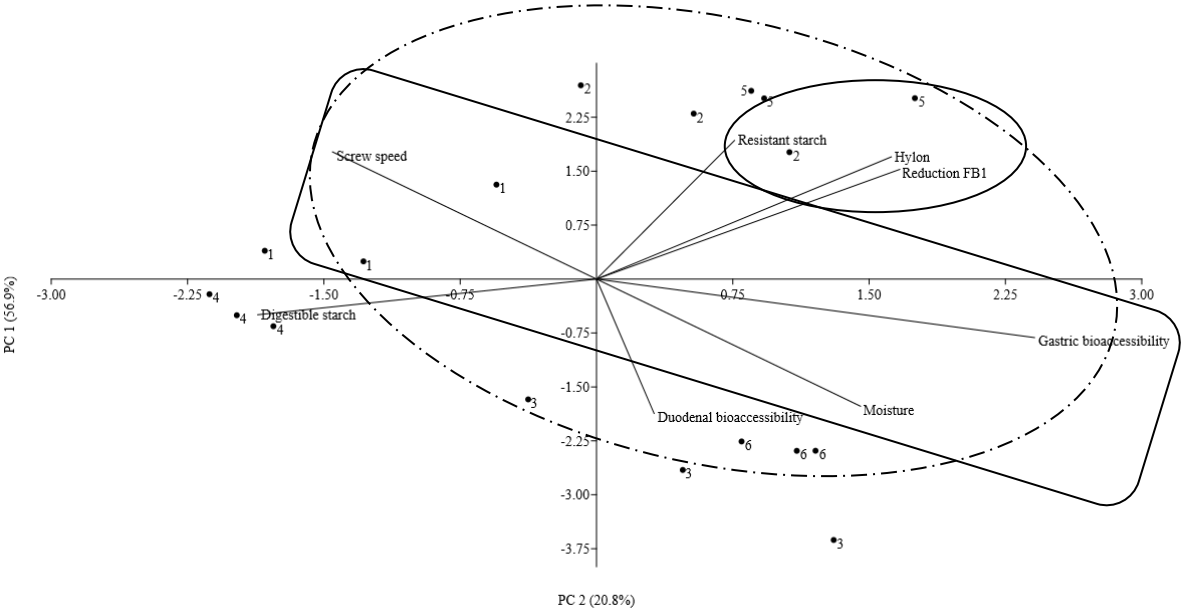
The food matrix and preparation process can affect the FB1 reduction and bioaccessibility. Therefore, a correlation analysis with the different variables within the

extrusion process become of importance to identify which parameter could contribute to the reduction of FB1, as well as its bioaccessibility.

3.4 INFLUENCE OF EXTRUSION PARAMETERS ON FB1 CONTENT AND BIOACCESSIBILITY

In order to evaluate the association among extrusion conditions, resistant starch and FB1 reduction and bioaccessibility, a multivariate analysis was employed (Figure 1).

Figure 1 - Effect of extrusion condition and resistant starch on fumonisin reduction and bioaccessibility



The principal component (PC) 1 (56.9%) and PC2 (20.8%) together explained 77.7% of the results variation. The resistant starch and hylon variables influenced the reduction of FB1 (circle with solid lines) after the extrusion process, because angles smaller than 90° indicate an adequate correlation. Additionally, these variables showed a positive and significant correlation (Table 4) with the reduction of FB1. It can be observed, as pointed out by the highlighted regions in the graph (Figure 1), that the gastric bioaccessibility of FB1 showed a significant ($p<0.05$) positive correlation with the moisture ($R= 0.49$) and a negative correlation with screw speed ($R= -0.49$), showed in the rectangle with solid lines.

The moisture content (0.72) positively influenced the duodenal bioaccessibility, however the screw speed (-0.72), hylon content (-0.65), resistant starch (-0.73) and FB1 reduction (-0.65) showed a significant negative correlation (circle with dotted lines) with the

bioaccessibility. Therefore, the higher the content of hylon, resistant starch and the reduction of FB1 after the extrusion cooking, the lower its bioaccessibility in the small intestine.

Table 4 – Correlations among extrusion cooking variables and fumonisin B1 reduction and bioaccessibility

Parameter	Fumonisin B1		
	Reduction	Gastric Bioaccessibility	Duodenal Bioaccessibility
Moisture	-0.33 (0.18)	**0.49 (0.04)**	**0.72 (0.00)**
Screw speed	0.33 (0.18)	**-0.49 (0.04)**	**-0.72 (0.00)**
Hylon	**0.78 (0.00)**	0.08 (0.75)	**-0.65 (0.00)**
Resistant starch	**0.75 (0.00)**	-0.07 (0.78)	**-0.73 (0.00)**
Digestible starch	-0.35 (0.16)	-0.04 (0.88)	0.29 (0.24)
Reduction	-	-0.08 (0.74)	**-0.65 (0.00)**

Data presented as correlation values (R) with significance when $p<0.05$.

This study showed for the first time the positive effect on food safety associated with extrusion of FB1 contaminated corn in the presence of hylon, as well as the stability of modified fumonisins under digestive conditions. This research highlights that the food matrix in which mycotoxins may be present can have an effect on their bioavailability, as complex and diverse reactions occur during thermal processes during food production. These interactions between mycotoxin and food matrix components will likely result in different levels of free toxin available for absorption in the intestinal tract.

4 CONCLUSION

The addition of high-amylose corn starch further reduced FB1 (75%), when compared to the effect of the extrusion alone (66%), indicating the possible binding of fumonisin with this polysaccharide chain. Upon *in vitro* digestion, modified fumonisin became stable and the duodenal bioaccessibility of free FB1 was as low as 35% when hylon ingredient was used as part of the formulation of the extruded corn-based product. This indicates that adding amylose to thermally processed foods may contribute to both a greater reduction in fumonisin levels and to a lower toxin bioaccessibility. Therefore, more specific studies should

be undertaken in order to clarify potential toxic effects associated with these modified mycotoxins.

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CHAPTER IV

FINAL CONSIDERATION AND CONSIDERAÇÕES FINAIS

FINAL CONSIDERATIONS

A method of simultaneous extraction of aflatoxins G2, G1, B2, and B1 and fumonisin B1 precisely, accurately, with minimum waste generation, and reliably was validated to evaluate mycotoxin levels during milling, thermal, and bioaccessibility processes.

The dry milling process under laboratory conditions showed a redistribution in mycotoxin levels in the external fractions of the corn grain (germ and pericarp). The wet-milling process with a 1% lactic acid concentration and 18 h of maceration promoted a reduction in aflatoxin B1 and B2 concentrations in the endosperm (99.8–100%).

The hydrothermal treatment (cooking) of fine cornmeal and water using the proportion of 1:5 (w/v) and 3% soybean oil was the best condition for higher resistant starch content in the final product. In the extrusion process, the highest resistant starch content was achieved with a 210 rpm screw speed, 22% initial moisture, and the addition of 20% high amylose corn starch (70%).

The hydrothermal treatment cooking promoted a 0–52% reduction in aflatoxin levels and 39–59% in fumonisin B1 levels. The reduction of aflatoxins was significantly correlated ($p < 0.1$) with starch and reduction of fumonisin B1 levels were correlated ($p < 0.05$) with resistant starch and reducing sugars. The greatest effects on mycotoxin reduction in the samples were observed in the extrusion treatment, with reductions of 74.7 – 89.9% in aflatoxin levels and 66 – 75% in fumonisin B1 levels. Further reductions during extrusion were observed in extruded products with the addition of high amylose corn starch and consequently higher resistant starch content.

Aflatoxin bioaccessibility was high in cooked corn products and extruded products and for fumonisin was low in both cooked (27–68%) and extruded products (35–54%), indicating possible binding of these mycotoxins with macromolecules such as starch and resistant starch.

The extrusion heat treatment was the most efficient for simultaneously reducing aflatoxins and fumonisin B1 levels in the products, while the presence of high amylose starch and resistant starch contributed to the greater reduction of them and lower bioaccessibility of fumonisin B1.

CONSIDERAÇÕES FINAIS

Um método de extração simultânea de aflatoxinas G2, G1, B2 e B1 e fumonisina B1 preciso, exato, com mínima geração de resíduos e confiável foi validado para avaliar os níveis de micotoxinas durante os processos de moagem, térmico e bioacessibilidade.

O processo de moagem do milho a seco em condições de laboratório mostrou uma redistribuição nos níveis de micotoxinas nas frações externas do grão de milho (gérmen e pericarpo). O processo de moagem úmida com concentração de 1% de ácido lático e 18 h de maceração promoveu redução na concentração de aflatoxinas B1 e B2 no endosperma (99,8-100%).

O tratamento hidrotérmico (cozimento) da farinha de milho fina e água na proporção 1:5 (w/v) e 3% de óleo de soja foi a melhor condição para formação de amido resistente no produto final. No processo de extrusão o maior conteúdo de amido resistente foi alcançado com 210 rpm de velocidade de parafuso, 22% de umidade inicial e adição de 20% de amido de milho com alto teor de amilose (70%).

O tratamento hidrotérmico cozimento promoveu redução de 0-52% nos níveis de aflatoxinas e 39-59% nos níveis de fumonisina B1. A redução das aflatoxinas apresentou correlação significativa ($p<0,1$) com a presença de amido e redução dos níveis de fumonisina B1 foram correlacionados ($p <0,05$) com amido resistente e açúcares redutores. O maiores efeitos na redução de micotoxinas nas amostras foram observadas quando foi empregada extrusão, promovendo reduções de 74,7 a 89,9% nos níveis de aflatoxinas e de 66 a 75% nos níveis de fumonisina B1. Maiores reduções foram verificadas em produtos extrusados com adição de amido de milho com alto teor de amilose e consequentemente maior teor de amido resistente.

A bioacessibilidade das aflatoxinas foi elevada nos produtos a base de milho cozido e nos produtos extrusados e para fumonisina foi baixa tanto nos produtos cozidos (27-68%) como nos extrusados (35-54%), indicando possíveis ligações destas micotoxinas com macromoléculas como o amido e amido resistente.

O tratamento térmico extrusão foi o mais eficiente para redução simultânea dos níveis de aflatoxinas e fumonisina B1 nos produtos, enquanto que a presença de amido com alto teor de amilose e amido resistente contribuíram para maior redução delas e menor bioacessibilidade de fumonisina B1.

CHAPTER V

FUTURE SUGGESTIONS

FUTURE SUGGESTIONS

To evaluate the behavior of non-bioaccessible mycotoxins in the small intestine in the intestinal microbiota;

To identify mycotoxins modified by heat treatment and the digestive process;

To expand the milling scale to verify the distribution of mycotoxins and the characteristics of cornmeal in industrial processes;

To use other ingredients such as lipids, proteins, sugars, and phenolic compounds during heat treatments and evaluate the reduction and bioaccessibility of mycotoxins;

To evaluate the bioaccessibility of mycotoxins in corn products obtained by other heat treatments such as baking and frying.

SUGESTÕES FUTURAS

Avaliar o comportamento das micotoxinas não bioacessíveis no intestino delgado na microbiota intestinal;

Identificar as micotoxinas modificadas pelo tratamento térmico e processo digestivo;

Ampliar a escala de moagem para verificar a distribuição de micotoxinas e as características das farinhas em processo industrial;

Utilizar outros ingredientes como lipídios, proteínas, açúcares e compostos fenólicos durante os tratamentos térmicos e avaliar a redução e bioacessibilidade das micotoxinas;

Avaliar a bioacessibilidade de micotoxinas em produtos à base de milho obtidos por outros tratamentos térmicos como assamento e fritura.

CHAPTER VI

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