CHARACTERIZATION OF INDIGESTIBLE FIBER IN DIETS BASED ON CORN AND SOYBEAN MEAL OR CORN, SOYBEAN MEAL, AND DISTILLER DRIED GRAINS WITH SOLUBLES AND IN FECES FROM PIGS FED THESE DIETS

BY

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THESIS

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ABSTRACT

Three experiments were conducted to determine concentrations of non-starch polysaccharides, total dietary fiber and phenolic acids in a diet based on corn and soybean meal (**SBM**), and in a diet based on corn, SBM, and distiller dried grains with solubles (**DDGS**), as well as in feces from pigs fed these diets. The in-vitro disappearance of DM without and with inclusion of exogenous enzymes in both diets was also determined. In Exp. 1, total NSP and dietary fiber in diets based on corn and SBM or corn, SBM, and DDGS and in fecal samples from pigs fed these diets were quantified. The hypothesis that the concentration of NSP in the diet based on corn, SBM, and DDGS, and in feces from pigs fed this diet is greater than in the corn and SBM diet and in feces from pigs fed this diet, was tested. Concentration of total NSP in the diet containing corn, SBM, and DDGS was greater than in the diet based on corn and SBM. In contrast, the concentration of total NSP in the feces from pigs fed these diets was not different. The concentration of xylose, arabinose, and cellulose was predominant in both diets and feces from the pigs fed both diets. However, the digestibility of total NSP in the diet based on corn and SBM was greater than the digestibility from the diet based on corn, SBM, and DDGS. In Exp. 2, the ferulic and coumaric acid concentration in a diet based on corn and SBM and in a diet based on corn, SBM, and DDGS as well as in feces from pigs fed these diets was quantified. The hypothesis that the concentration of coumaric acid and ferulic acid is greater in the corn, SBM, and DDGS diet than in the corn and SBM diet, as well as in feces from pigs fed the corn, SBM, and DDGS diet compared with feces from pigs fed the corn and SBM diet was tested. The diet based on corn, SBM, and DDGS contained approximately 3 times as much coumaric acid and ferulic acid than the diet based on corn and SBM. Analysis of coumaric acid and ferulic acid in feces from pigs fed both diets indicated that only around one third of the

bound phenolic acids are fermented in the intestinal tract of pigs indicating that coumaric and ferulic acids are major barriers to fermentation of arabinoxylans in corn fiber. In Exp. 3, the disappearance of DM in the indigestible fraction of a diet based on corn and SBM and in a diet based on corn, SBM, and DDGS was determined. Both diets were digested without inclusion of enzymes, with inclusion of four exogenous enzymes: arabinofuranosidase, feruloyl esterase, endo-xylanase, and cellobiohydrolase; and with 10 different combinations of these enzymes. The hypothesis that inclusion of xylanase by itself or in combination with cellobiohydrolase, arabinofuranosidase, or feruloyl esterase increases the digestibility of DM in the two diets was tested. Endo-xylanase may increase hydrolysis of arabinoxylan in corn and SBM diets and in corn, SBM, and DDGS diets when the enzyme is included in the diets, contributing to improved ATTD of DM. Inclusion of feruloyl esterase, cellobiohydrolase, and arabinofuranosidases individually or in combinations did not improve the ATTD of DM.

Key words: Arabinoxylan, enzymes, fiber, non-star polysaccharides, pig

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CHAPTER 1: INTRODUCTION

Corn is the most extensively cultivated and consumed cereal grain in the world due to the nutritional composition, high yield capacity and its many potential applications. Corn production in the USA in 2019 was 347,765,649 metric tons (USDA, 2020a). Iowa and Illinois were the states with the largest production ($65,631,060 and 46,893,480 metric tons respectively; USDA, 2020a).

Corn is mainly used as a feed ingredient in diets for animals and in bioethanol production. In 2019, 45 million metric tons of corn were exported, 140 million metric tons were used as animal feed, and 106 million metric tons were used as fuel ethanol (USDA, 2020b). In the ethanol process, nonfermented coproducts are produced; those are known as distiller dried grains with solubles (**DDGS**) or cereal coproducts of the distillation process, which may be used as an ingredient in diets fed to pigs (Stein and Shurson, 2009).

Corn is used as a feed ingredient in diets for pigs and poultry due to its palatability, relatively low cost, and because it is one of the best sources of metabolizable energy (ME) among cereal grains (Loy and Lundy, 2019). Starch and fat are the main energy components in corn because 72% of the corn kernel is starch (DM-basis; Hamaker et al., 2019) and total dietary fiber is approximately 13.73% (NRC, 2012). Oil in corn is approximately 3.4% (DM basis), is highly unsaturated, and it is a rich source of linoleic acid, which is an essential fatty acid. Oil in corn may contribute 10 to 12% of the ME (Loy and Lundy, 2019). Protein is also an important component in corn, and protein in corn is high in sulfur-containing amino acids, but is low in Lys and Trp, which are two of the indispensable amino acids (Loy and Lundy, 2019). The protein

content is 8.24% as fed basis (NRC, 2012), but it may vary because of climatic conditions, genetic modifications, and soil fertility (Asghari and Hanson, 1984).

Distiller dried grains with solubles have been used as an ingredient in diets for pigs and poultry because it contributes energy, amino acids, and phosphorus (Stein and Shurson, 2009). Nutrient composition of DDGS may vary depending on year of production, among production plants, and also among batches within the same plant, which is why chemical analysis should be performed on a regular basis (Liu, 2012).

Fiber is one of the main components in DDGS and may positively impact gut health. However, fiber in diets for pigs reduces energy and protein digestibility (Bach Knudsen, 1997; NRC, 2012). Fiber can be soluble or insoluble, but the majority of fiber in corn and corn DDGS is insoluble fiber (Jaworski et al., 2015). Insoluble fiber in cereal grains is primarily composed of lignin, cellulose, and arabinoxylans, which is formed by arabinose and xylose (Jaworski et al., 2015). Pigs lack digestive enzymes capable of digesting dietary fiber, but dietary fiber may be fermented in the hindgut by the microbiota to obtain energy (Navarro et al., 2018).

Due to the lack of digestive enzymes secreted by pigs, supplementation of fiber digesting enzymes in diets for pigs, may facilitate hydrolysis of the bonds between monosaccharides in the arabinoxylan molecule, which may improve fiber digestibility and ME (Casas and Stein, 2016; Abelilla and Stein, 2019). The enzymes that are potentially needed to hydrolyze arabinoxylan are: β-(1-4) endoxylanases, β-xylosidases, β-galactosidases, α-arabinofuranosidases, arabinoxylan α-arabinofuranohydrolases, α-glucuronidases, acetyl xylan esterases, ferulic and coumaric acid esterases (Abelilla and Stein, 2019). At this point, enzymes or enzyme mixtures that can hydrolyze corn fiber have not been identified and the majority of fiber in corn and corn co-products is, therefore, not fermented in the pig (Abelilla and Stein, 2019). However, knowing

the exact composition of insoluble fiber in corn and the structure of arabinoxylans in corn and the fiber components that cannot be digested by pigs, may contribute to determine which enzymes are needed to improve digestibility of insoluble dietary fiber in corn.

Therefore, the overall aim of the present work is to determine the complete composition of dietary fiber in diets based on corn and corn DDGS, and the fiber composition of fecal samples from pigs fed diets based on corn and DDGS. Based on the fiber profile of the unfermented fiber in feces, enzymes needed to hydrolyze these fibers will be identified. In vitro work will then be conducted to determine if the identified enzymes are effective in hydrolyzing the unfermentable fiber.

LITERATURE CITED

Abelilla, J. J., and H. H. Stein. 2019. Degradation of dietary fiber in the stomach, small intestine, and large intestine of growing pigs fed corn- or wheat-based diets without or with microbial xylanase. J. Anim. Sci. 97:338-352. doi:10.1093/jas/sky403

Asghari, M., and R. G. Hanson. 1984. Climate, management, and N effect on corn leaf N, yield, and grain N. Agron. J. 76:911-916. doi:10.2134/agronj1984.00021962007600060012x

Bach Knudsen, K. E. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67:319-338. doi:10.1016/S0377-8401(97)00009-6

Casas, G. A., and H. H. Stein. 2016. Effects of microbial xylanase on digestibility of dry matter, organic matter, neutral detergent fiber, and energy and the concentrations of digestible and metabolizable energy in rice coproducts fed to weanling pigs. J. Anim. Sci. 94:1933-1939. doi:10.2527/jas.2015-0064

Hamaker, B. R., Y. E. Tuncil, and X. Shen. 2019. Carbohydrates of the kernel. In: S. O., and Serna-Saldivar, editors, Corn: chemistry and technology. Elsevier Inc, Cambridge, MA, United States. p. 305-318.

Jaworski, N. W., H. N. Lærke, K. E. Bach Knudsen, and H. H. Stein. 2015. Carbohydrate composition and in vitro digestibility of dry matter and nonstarch polysaccharides in corn, sorghum, and wheat and coproducts from these grains. J. Anim. Sci. 93:1103-1113. doi:10.2527/jas.2014-8147

Liu, K. 2012. Chemical composition of DDGS. In: K. Liu, and K. A. Rosentrater, editors, Distillers grains production, properties, and utilization. CRC Press Taylor & Francis group, United States of America. p. 143-178.

Loy, D. D., and E. L. Lundy. 2019. Nutritional properties and feeding value of corn and its coproducts. In: S. O., and Serna-Saldivar, editors, Corn: chemistry and technology. Elsevier Inc, Cambridge, MA, United States. p. 633-654.

Navarro, D. M. D. L., E. M. A. M. Bruininx, L. de Jong, and H. H. Stein. 2018. Effects of physicochemical characteristics of feed ingredients on the apparent total tract digestibility of energy, DM, and nutrients by growing pigs. J. Anim. Sci. 96:2265-2277. doi:10.1093/jas/sky149

NRC. 2012. Nutrient requierements of swine. 11th rev. Natl. Acad. Press ed, Washington, D.C.

Stein, H. H., and G. C. Shurson. 2009. Board-invited review: the use and application of distillers dried grains with solubles in swine diets. J. Anim. Sci. 87:1292-1303. doi:10.2527/jas.2008-1290

USDA. 2020a. Crop production 2019 summary. https://www.nass.usda.gov/Publications/Todays_Reports/reports/cropan20.pdf Accessed November 6, 2020.

USDA. 2020b. Feed Outlook. https://www.ers.usda.gov/webdocs/outlooks/95689/fds-20a.pdf?v=2997.5 Accessed November 6, 2020.

CHAPTER 2. CARBOHYDRATES FROM UNFERMENTABLE CORN FIBER INCLUDED IN FEED INGREDIENTS COMMONLY USED IN COMMERCIAL DIETS FOR PIGS: A REVIEW

INTRODUCTION

Corn is one of the main feed ingredients used in diets for pigs, and many coproducts from the corn processing industry are used as well (Jaworski et al., 2015). Carbohydrates from corn supply the majority of the energy in most diets, although pigs do not have a specific requirement for carbohydrates (NRC, 2012).

Carbohydrates in corn are classified into two main groups: endosperm starch and nonstarch polysaccharides **(NSP)**, which are primarily present in the cell walls (Lundy, 2019), but also in the endosperm. Together with lignin, NSP is defined as total dietary fiber (Slavin, 2013), which may be classified as soluble dietary fiber **(SDF)** and insoluble dietary fiber (**IDF;** Zhang et al., 2013).

Insoluble dietary fiber is not as fermentable as SDF (Urriola et al., 2010) and exogenous enzymes may be needed to improve fermentability of IDF in diets containing corn and distillers dried grains with solubles **(DDGS).** The fiber composition can be analyzed with techniques that quantify neutral detergent fiber **(NDF)**, acid detergent fiber **(ADF),** total dietary fiber **(TDF),** SDF, IDF, and lignin (Wolfrum et al., 2009). However, more information about the chemical composition of the specific structures in dietary fiber in corn and DDGS is needed to gain a better understanding of the fermentability and energy provided. Analysis of non-starch polysaccharides, low molecular weight sugars, and phenolic acids in corn and DDGS as well as in feces from pigs fed diets containing corn and DDGS may contribute to that objective.

PHYSICAL COMPOSITION OF CORN GRAIN AND DISTILLERS DRIED GRAINS WITH SOLUBLES

Corn is the most important cereal grain in the feeding of livestock and poultry in USA (Loy and Lundy, 2019) due to its favorable palatability and relatively low cost. The composition of corn may vary because of the place of cultivation, hours of sun light, climatic conditions, genetic modifications, and soil fertility (Asghari and Hanson, 1984).

The corn grain has three main parts: germ, endosperm, and pericarp or bran (Fig 2.1, Lara et al., 2019). The pericarp contains several layers composed of empty cells with lignified secondary walls (Izydorczyk, 2017) and protects the germ and the endosperm. The greatest concentration of non-starch polysaccharides in the kernel is located in the outer pericarp layer (Hamaker et al., 2019). The bran also contains arabinoxylans, which are connected with diferulate cross-links. The germ is composed of two parts: the embryo and the scutellum. The germ contains albumin and globulin proteins, and around 85% of the total fat of the kernel is located in the germ. The endosperm is divided into the aleurone layer and the starchy endosperm, which is composed of horny and floury endosperm (Lara et al., 2019).

Distillers dried grains with solubles is a byproduct of the etahnol industry. Variability in the chemical composition of DDGS is often observed because DDGS is produced by different plants with different conditions of operation. Concentration of nutrients like protein, fat, vitamins, minerals and crude fiber in corn DDGS are increased after fermentation except for starch. In DDGS, the concentration of nutrients is aproximatelly: crude protein (314 g/Kg DM), fat (91 g/Kg DM), crude fiber (77 g/Kg DM), and starch (60 g/Kg DM). Non starch polysacharides in

DDGS are 25 to 30% and the two major components of NSP in DDGS are arabinoxylan and cellulose (Pedersen et al., 2014).

CELL WALLS

Plant cell wall polysaccharides consist of pentoses (arabinose and xylose), hexoses (glucose, galactose and mannose), 6-deoxyhexoses (rhamnose and fucose), and uronic acids (glucuronic and galacturonic acids), which are chemically linked to each other through glycosidic bonds to form NSP (Pluske et al., 2001). Noncovalent interactions and covalent cross-links reinforce the cell wall (Izydorczyk, 2017).

Besides polysaccharides, the cell walls also contains lignin and proteins, but the composition may vary depending on the plant, cell phase of development, and tissue location (Izydorczyk, 2017). The unlignified walls or primary walls of the cells in the endosperm and aleurone layer are rich in arabinoxylans and (1-3)(1-4) β-D- glucans. In contrast, the secondary or lignified walls from the bran and pericarp are rich in cellulose and arabinoxylans, and a small amount of (1-3)(1-4) β-D- glucans (Izydorczyk, 2017). Because of the lack of lignification of the arabinoxylans in the primary cell walls, these components are partly soluble whereas the secondary cell walls are insoluble.

NON-STARCH POLYSACCHARIDES

Non-starch polysaccharides are composed of up to several hundred thousand monosaccharide units (Lundy, 2019). In cereal grains, non-starch polysaccharides provide rigidity to the outer layer and are key constituents needed for physiological process as the grain develops (Hamaker et al., 2019). Non-starch polysaccharides do not contain α-(1-4)-linked

glycosyl units that are characteristic for starch (Englyst et al., 2007), but are often associated with phenolic lignified polymers, protein and starch (Pluske et al., 2001). Non-starch polysaccharides in corn and DDGS consist of cellulose and hemicelluloses with hemicelluloses primarily consisting of arabinoxylans and β-glucans (Fig 2.2).

Cellulose

Cellulose is the most abundant organic compound in the biosphere and it is the major polysaccharide of glucose in plants (Tymoczko et al., 2015; Held et al., 2015). Cellulose is composed of unbranched chains of β-(1-4)-linked D-glucose that bind between 7,000 and 15,000 glucose units in a flat ribbon form (Fig 2.3). This particular conformation allows cellulose chains to maintain the conformation and form intramolecular hydrogen bonds between the hydroxyl groups at the C3 positions of neighboring glucose residues (Hamaker et al., 2019). This structure and the associated H bonds also allow the cellulose chains to stack together to form larger microfibrils, which facilitate the association with other carbohydrates and lignin moieties that allow the formation of a rigid strong cell wall structure (Tymoczko et al., 2015; Lundy, 2019).

Cellulose contains both crystalline and amorphous domains (Fig 2.4), and the structure of the cellulose molecules influences the physical properties and chemical behavior. Reactants and enzymes may penetrate the amorphous region, and it is, therefore, primarily the amorphous regions of the cellulose that may be fermented. In contrast, the crystalline regions are largely resistant to fermentation (Ciolacu et al., 2011).

Hydrolysis of cellulose requires endoglucanases, which randomly hydrolyze β-(1-4) glycosidic bonds from the internal part of the amorphous region, forming new reducing or non-reducing ends that may be hydrolyzed by exoglucanases (Duan and Feng, 2010; Zhang, 2013). Exoglucanases cleave from the reducing or non-reducing ends of cellulose (Zhang, 2013) to

release oligosaccharides or disaccharides. For complete hydrolysis, two additional enzymes are needed: cellodextrinases and β-glucosidases. Cellodextrinases hydrolyze soluble cello-oligosaccharides, which generates cellobiose (Duan and Feng, 2010), and β-glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Zhang, 2013).

In corn, cellulose is mainly present in the bran. Conventional yellow dent corn contains 1.7% cellulose, but the cellulose proportion in the corn bran is 22.5 to 23% of total weight (Jaworski et al., 2015). Crystallinity of crude cellulose in corn is approximately 43% (Xu et al., 2009; Lundy, 2019), which indicate that more than 50% of the cellulose is in the amorphous form and therefore, potentially can be fermented.

In DDGS, the cellulose concentration is approximately 7%, but this may be cross-linked with hemicellulose and/or lignin, which forms a stable three-dimensional structure. Its crystallinity is approximately 49% being similar to the corn kernel (Xu et al., 2009).

Hemicelluloses

Hemicelluloses are polysaccharides composed of a backbone of heterogeneous groups of pentose and hexose sugars such as xylose, arabinose, mannose and galactose, and branches composed of arabinose, galactose, glucose, and glucuronic acid (Hamaker et al., 2019). In corn, the main hemicelluloses are arabinoxylans and β-glucans.

Arabinoxylans are composed of 51.2% D-xylose, 27.4% L-arabinose, 7.5% galactose, 1.6 % glucose, and 4.5% uronic acids (Rumpagaporn et al., 2015). Arabinoxylan has a D-Xylose linear backbone, and β-xylopyranosyl units of the backbone are substituted with α-arabinofuranose units at their O-2 and /or O-3 positions (Fig 2.5; Hamaker et al., 2019).

Other substitutes such as D-glucuronic acid and acetyl groups may be attached to the xylan backbone. Xylose units may also be attached to arabinose monomers in the sidechains, and

these xylose units may be further substituted with galactose. Strong heterogeneous intermolecular complexes are formed due to the arabinoxylan cross-linking. These complexes affect enzymatic degradation and potential encapsulation of nutrients within the cell wall (Lapierre et al., 2001; Pedersen et al., 2014).

In corn bran, ferulic acid, p-coumaric, dehydrodiferulic, and dehydrotriferulic acids are covalently bound to cell wall arabinoxylans through ester linkages to the arabinose side chains. Most of the arabinoxylans can be extracted with alkali and alkaline H_2O_2 treatment (Yadav et al., 2007b) via the hydrolyzation of the ester linkages.

In corn, arabinoxylans are present in the cell wall material from the endosperm and in the bran. The chemical structure of arabinoxylan varies depending on where it is located. Arabinoxylan from the bran has a lower arabinose/xylose ratio compared with arabinoxylan from intact corn fiber, which includes both bran and endosperm (Yadav et al., 2007a). Therefore, the arabinoxylan that is located in the endosperm is more branched or have a less heterogeneous structure with more arabinose sidechains than the arabinoxylan from the bran portion (Hamaker et al., 2019). The concentration of ferulic acid in the endosperm is also less than in the bran portion (Yadav et al., 2007a). The phenolic acid content in corn is high and in corn bran, it can be up to 5%, primarily ferulic acid (Saulnier and Thibault, 1999).

Ferulic acid residues allow arabinoxylan chains to cross-link with each other through di and tri ferulic acid bridges, which together with linkages to lignin, and association with proteins limit the solubility of arabinoxylan. These linkages and associations with lignin also precludes fermentation of the arabinoxylans. Nevertheless, after extraction of arabinoxylan with alkaline solution, the majority of corn arabinoxylans become soluble in water. Addition of hydrogen peroxide contribute to delignification and facilitate the alkali extraction of arabinoxylan.

However, after alkali extraction, some ferulic acids remain esterified to the solubilized corn arabinoxylan (Hamaker et al., 2019).

Due to the complex structure of arabinoxylans several enzymes are required for complete hydrolysis. β-(1-4) Endoxylanases cleave the xylan backbone via hydrolysis of the β-(1-4) glycosidic bonds between xylose units (Dodd and Cann, 2009), which results in shorter chains of xylose that may be cleaved by β-D-xylosidase, which hydrolyses short xylooligosaccharides from the non-reducing end to release xylose (Bedford and Schulze, 1998; Belkacemi and Hamoudi, 2003). α-Glucuronosidases hydrolyze the side chains of the arabinoxylans, cleaving the α-(1-2) bond between the xylan backbone and the glucuronic acid in the side chain, and β-galactosidases cleave the glycosidic bond between galactose and xylose residues in the side chains. The α –arabinofuranosidases hydrolyze the α-(1,5) glycosidic bond between 2 L-arabinose units in the side chain, and α –arabinofuranohydrolases hydrolyze the α-(1-2) glycosidic bond between the xylose backbone and arabinose side chain units (Abelilla, 2018). Acetyl xylan esterases cleave the glycosidic bond between O-acetyl and xylose, whereas ferulic/coumaric acid esterases hydrolize the linkage between L-arabinose and ferulic acid or coumaric acid, respectively (de Vries, 2003).

Beta-glucans are linear homopolysaccharides of β-D- glucopyranosyl residues linked via a mixture of (1-3) and (1-4) glycosidic linkages, with blocks of (1-4) linked units (oligomeric cellulose-like segments) separated by (1-3) linkages (Fig 2.6; Izydorczyk, 2017; Hamaker et al., 2019). The beta-glucan quantity in corn ranges from 0.8% to 1.7% (dry basis; Hamaker et al., 2019). Blocks of (1-4) linkages may exhibit a tendency for inter-chain aggregation via strong hydrogen bonds within these cellulose-like segments. Nevertheless, the structure confer

solubility to the molecule due to the kinks that are formed because of the (1-3) linkages (Izydorczyk, 2017).

In corn, β-glucan is composed of approximately 67.5% cellotrimer and 26.7 cellotetramer segments. The remaining part of the molecule consists of 0.3% cellobiose segments and 5.4% cellodextrin-like oligosaccharides containing more than four consecutive 4-O-linked glucose residues (Yoshida et al., 2014; Hamaker et al., 2019). Hydrolysis of mixed linked β-glucan requires endo-1,4-β-D-glucanase, which hydrolyses the (1→4)-β-D-glycosidic linkages, and endo-1,3(4)-β-glucanase, which acts on of (1→3)- or (1→4)-linkages when the glucose is substitude at C-3 (Paloheimo et al., 2010).

In the beta-glucan structure, the β-(1-3) linkages form coils that create an asymmetric conformation that prevents intermolecular alignment of chains into cellulose microfibrils. The asymmetric conformation allows the molecule to form water-soluble gel-like matrices that reinforce the cellulosic microfibrils in the wall (Burton and Fincher, 2009).

Beta-glucans may increase viscosity in aqueous solutions, and increased viscosity in the gut may reduce fat, cholesterol, and bile acid absorption because of reduced nutrient movement in the small intestine (Izydorczyk, 2017). However, this effect is primarily a problem in poultry (Ayres et al., 2019), whereas increased viscosity does not seem to affect nutrient absorption in pigs (Navarro et al., 2018).

Lignin is not considered a polysaccharide, but is present in the cell wall and the sum of lignin and NSP equals the amount of TDF in a feed ingredient. Based on its structure and presence in the cell wall, lignin has two main functions: 1) it binds and attach the cellulose microfibrils and other matrix polysaccharides; and 2) it prevents biochemical degradation and physical damage of cell walls (Bach Knudsen, 1997).

Lignin is an organic polymer formed by phenyl propane units organized in a tri-dimensional structure. Coniferyl, sinapyl and p-coumaryl alcohols, which can be transformed into guaiacyl (G unit), syringyl (S unit) and p-hydroxyphenyl (H unit), are the precursors needed to synthesize lignin (He et al., 2018), and these units are connected to each other by aryl-ether and carbon−carbon linkages (Van Erven et al., 2019). The concentration of lignin in the corn grain is around 1.1% (weigh basis), which is lower than in other cereal grains (Bach Knudsen, 1997; Hamaker et al., 2019).

Lignin is bound to coumaric acid, but small amounts of lignin may be bound to other polysaccharides. Ferulic acid is also bound to lignin, but it is mostly bound to cell wall polysaccharides such as arabinoxylans or pectins (Cuevas et al., 2011). Digestive or microbial enzymes cannot degrade lignin, and lignified parts of fiber are, therefore, unfermentable. As a consequence, fermentability of arabinoxylan from the bran of the corn grain is low due to the linkages between lignin and the phenolic acids in arabinoxylan in the cell wall.

DIETARY FIBER

Dietary fiber is defined as the sum of "non-digestible soluble and insoluble carbohydrates with 3 or more monomeric units and lignin that are intrinsic and intact in plants, and isolated or synthetic non-digestible carbohydrates with 3 or more monomeric units determined to have physiological effects that are beneficial to human health" (FDA, 2016). The positive effects of fiber in human health include lowering blood glucose and cholesterol, reduced calorie intake, and increasing the frequency of bowel movements (FDA, 2016). Total dietary fiber include all non-digestible carbohydrates such as oligosaccharides, cellulose hemicelluloses, and resistant

starch (Bach Knudsen et al., 2013). Based on functional, chemical, and physical properties, total dietary fiber may be divided into SDF and IDF.

Soluble dietary fiber may form a viscous gel due to the solubility in water and is composed of pectins, gums, inulin-type fructans and other hemicelluloses like β-glucans (Lattimer and Haub, 2010). Soluble dietary fiber is partially or completely fermented in the cecum producing short chain fatty acids **(SCFA;** Slavin, 2013; Jaworski and Stein, 2017).

Insoluble dietary fiber does not form gels due to its water insolubility and is composed of cellulose, lignin, and insoluble hemicelluloses (Lattimer and Haub, 2010; Dreher, 2001). Insoluble dietary fiber may be fermented in the large intestine, where SCFA are formed, and those can be absorbed and contribute to the body's energy needs (Bach Knudsen, 2011; Stipanuk and Caudill, 2013). However, IDF is much less fermentable than the soluble fraction (Urriola et al., 2010).

ANALYSIS OF DIETARY FIBER

Analysis of dietary fiber in feed ingredients may be divided into three main methods: enzymatic gravimetric methods, non-enzymatic–gravimetric methods, and enzymatic –chemical methods (Fig 2.7). Non-enzymatic–gravimetric methods include two procedures: The Weende method, which uses acid hydrolysis to extract sugars and starch, and alkaline hydrolysis to remove protein, some hemicelluloses, and lignin. The other procedure is the Van-Soest method, which quantifies NDF and ADF (Champ et al., 2003), also known as the detergent fiber analysis.

Enzymatic gravimetric methods include the use of enzymes to remove protein and starch followed by precipitation and isolation of soluble dietary fiber and a correction for protein and

ash in the residue (McCleary, 2003). The most used enzymatic gravimetric methods to analyze feed ingredients are analyses that quantify IDF, SDF, TDF, and lignin.

In enzymatic chemical methods, amylase is added to the sample to remove starch, followed by separation of soluble and insoluble fractions and determination of polysaccharides by chromatography (McCleary, 2003). The enzymatic-chemical methods allows for quantification of the individual sugars included in NSP. Phenolic acids and lignin may also be analyzed (Fig 2.8).

Crude fiber (Weende method)

The crude fiber method is a chemical-gravimetric method, which separates carbohydrates into crude fiber and nitrogen free extract. A sample is digested with 1.25% sulfuric acid and 1.25% sodium hydroxide and the residue after digestion is quantified (Urriola et al., 2013). The crude fiber method is an official method for analyzing fiber (AOAC 962.09), and it is robust and repeatable. However, this procedure may not be accurate because only approximately 80% of the hemicelluloses, 90% of the lignin, and 30 to 50% of the cellulose may be removed during the digestion and therefore, the recovery of the fiber is not be complete (Alyassin and Campbell, 2019, Urriola et al., 2013).

ADF

This method was first published in 1963 by Van Soest, and the main objective was to analyze cellulose and lignin, but the analysis does not include insoluble hemicelluloses (Jung, 1997; Udén et al., 2005). In this method protein is removed from the fibrous residue using cetrimonium bromide **(CTAB)**, and samples are also treated with 1 *N* H_2SO_4 to remove non-fibrous compounds (Mertens, 2003). The remaining residue is dried and the ADF fraction is calculated as the final weight of the sample divided by the initial sample weight.

NDF

Because the ADF method does not quantify total fiber due to the exclusion of hemicelluloses, the NDF method was developed by Van Soest and Wine in 1967 (Udén et al., 2005). Neutral detergent fiber represents the insoluble fraction, including insoluble hemicellulose, cellulose, and lignin, but exclude soluble β-glucans and other soluble hemicelluloses.

The neutral detergent fiber analysis is performed using a chemical solubility-gravimetric method. Samples are treated with an anionic detergent, which is mainly composed of sodium dodecyl sulfate (USP), Ethylenediaminetetraacetic disodium salt dehydrate, and sodium lauryl sulfate. These reagents extract the proteins and fats are removed by hot water and acetone (Mertens, 2003). During extraction, thermostable α amylase is also added to remove starch and this is followed by filtration and washing (Mongeau and Brooks, 2003). Samples are then dried and the NDF fraction is calculated as the final weight of the sample divided by the initial sample weight.

TDF

Fiber may be characterized using the Weende analysis procedure, which is repeatable, but this procedure is not complete because the recovery of cellulose, hemicellulose, and lignin is partial (Urriola et al., 2013). Analysis for detergent fiber does not recover soluble dietary fiber components such as pectins, mucilages, gums, soluble arabinoxylans, and β-glucans. To overcome these limitations the TDF procedure (AOAC Int., method 985.29) was developed by Prosky. This is a gravimetric method, which measures soluble and insoluble polysaccharides and lignin, however, it only quantifies a part of the resistant starch, and inulin and polydextrose are not quantified. Later, AOAC method 985.29 was modified and replaced by method 991.43,

which is an enzymatic-gravimetric method to quantify total soluble fiber, and total insoluble dietary fiber in foods and feed ingredients. (Champ et al., 2003).

Fiber research continued as well as research in characterization of fiber, having the need to quantify and understand other fiber components including resistant starch, fructans, polydextrose, and resistant maltodextrins. Therefore the TDF method was updated (AOAC Int., method 2009.01; McCleary et al., 2019) to include total high molecular weight dietary fiber (**HMWDF**), which is composed of IDF + soluble dietary fiber that remains soluble in the presence of 76% aqueous ethanol (**SDFP**) and is quantified by a gravimetric method and soluble dietary fiber that remains soluble in the presence of 76% aqueous ethanol (**SDFS**), which is quantify by chromatography (McCleary et al., 2019). The TDF method (AOAC 2011.25) was proposed in 2011, and this method is an enzymatic-gravimetric method which uses chromatography to measure separately insoluble, soluble, and total dietary fiber, including resistant starch and the water: alcohol soluble non-digestible oligosaccharides and polysaccharides of DP ≥3 (McCleary et al., 2019; Phillips et al., 2019). The latest official TDF method is the (AOAC 2017.16), which measures TDF by a gravimetric and HPLC procedures and includes both resistant starch and isomaltoologosaccharide in the soluble fraction. This method differs from the AOAC 2009.01 because the incubation time with pancreatin α-amylase and amyloglucosidase is reduced from 16 to 4 hours and a greater concentration of enzymes is used.

Lignin

Lignin can be analyzed or calculated as the difference between TDF and NSP. One of the most common analytical methods is the Klason lignin procedure. In this analysis, samples are treated with amylase and amyloglucosidase to remove starch followed by a precipitation using 80%

ethanol. Afterwards, samples are treated with 12*M* sulfuric acid, and the non-hydrolyzed residue is collected and ashed. Klason lignin is determined as the difference in weight between the residue before ashing and after ashing (Hatfield et al., 1994).

An alternative method is the acid detergent lignin procedure (Ankom Technology method 9), which involves analysis of ADF prior to lignin analysis. The residue from the ADF analysis is treated with 12*M* sulfuric acid for 3 hours to remove non-lignin components. At the end of digestion, the residue is dried and lignin is calculated as the weight of the sample after the treatment divided by the weight of the sample before the treatment (Fukushima et al., 2015 ; Ankom Daisy^{II} Incubator, Ankom Technology, Macedon, NY).

Due to the complexity of lignin, analysis that may be more specific and accurate have been proposed including the pyrolysis-GC-MS (py−GC−MS) procedure. This analysis quantifies the lignin and characterizes the structure of the lignin in grasses, hardwoods and softwoods (Van Erven et al., 2019). However, the procedure has not been adapted to analysis for lignin in cereals grains, oilseeds, or grain coproducts.

Monosaccharide analysis and quantification of non-starch polysaccharides

Chromatography is used to separate an individual component from a mixture of chemical compounds using a mobile phase and a stationary phase (Poole and Poole, 1991; Feng et al., 2019). The method and the type of chromatography that are used is determined based on the compounds to be isolated. Therefore, information about the component to be isolated needs to be known to choose the correct standards, technique, parameters, and method. Some samples contain a complex mix of compounds; therefore, more than one method will be required to isolate these compounds (de Coning and Swinley, 2019c). Results from chromatography are

based on identification of the peaks obtained, and the comparison of these peaks with suitable standards (de Coning and Swinley, 2019b).

One of the basic principles in chromatography is chemical separation of the components in a mixture, which requires a stationary phase and a mobile phase (Braithwaite and Smith, 1985). Chromatography classification (Table 1) is based on the type of phases used.

Gas liquid chromatography is a technique for separation of volatile compounds that are thermally stable at the temperatures required for their vaporization (Poole, 2020), and a gas chromatographer is used to complete this separation. A chromatographer consists of different modules (Fig 2.9). Supply is considered the first module of the GLC unit, and the second module is the sample introduction unit. Samples are contained inside a vial and inserted into the chromatographer using an injector. The injector is an isothermal vaporizing chamber, in which the evaporated sample is mixed with a carrier gas, and it is then divided into a stream that enters the column and a second stream that is vented to the outside (de Coning and Swinley, 2019a; Poole, 2020). After sample injection, separation is achieved using a column. Different columns are used for the separation of mono- and disaccharides by GLC. Columns are usually produced from materials such as glass, stainless steel, aluminum, or copper (Izydorczyk, 2017). The column is located inside the oven and must have a stable temperature control (de Coning and Swinley, 2019a).

After separation, detection of the components is achieved using a detector. The most common detector is the flame ionization detector (FID) because of the better selectivity and higher sensitivity compared with other detectors (Izydorczyk, 2017; Poole, 2020). The detector has an analogue output, which is electronically converted to a digital signal that can be processed by a software (de Coning and Swinley, 2019a).

High performance liquid chromatography (HPLC) is a physical separation technique of the liquid phase (Fig 2.10). The separation by components is performed using two phases: a mobile phase and a stationary phase (Fallon et al., 1987; Dong, 2006). This technique uses high pressure that generates a flow through the column (Izydorczyk, 2017). Once the sample is separated between phases based on their affinity, an in-line detector monitors the concentration of each separated component in the effluent and generates a chromatogram (Dong, 2006).

The HPLC method is reliable, simple, nondestructive, and it is the dominating method for analysis performed in biochemistry and biology. It may also be used for analysis of monosachharides due to the efficiency in separation, identification, and quantification at very low concentrations of a sugar that has the ability to be dissolved in a solvent (Izydorczyk, 2017).

Non-starch polysaccharides may be analyzed with a chemical gravimetric method performed in three parallel extraction 1) total non-starch polysaccharides, 2) non-cellulosic polysaccharides, and 3) insoluble non-starch polysaccharides (Bach Knudsen, 1997). This procedure starts with the removal of starch using an enzymatic procedure that includes: 1) α-amylase to cleave α-(1-4) linkages to maltose, maltriose and maltodextrins, and 2) amyloglucosidase to cleave dextrins (Bach Knudsen, 1997). Afterwards, solutes are removed using a sodium phosphate buffer (pH = 7), and NSP is precipitated using ethanol. Sulfuric acid is then used to swell the cellulose and hydrolyze the NSP (Bach Knudsen, 1997; Jaworski et al., 2015).

To analyze sugars in NSP by gas chromatography it is necessary to perform a derivatization (Chen et al., 2009), which is a chemical modification that allows for analysis of carbohydrates because of the instability of polysaccharides at high temperatures. Derivatization confers volatility to the carbohydrates without structural changes, and improves chromatographic condition for detection (Izydorczyk, 2017). There are different techniques for derivatization, but

specifically for the analysis of monosaccharides in fiber, an acetylation of alcohols is used in the presence of 1-methyl imidazole as a catalyzer (Bach Knudsen, 1997).

Uronic acids are analyzed separately using a colorimetric method (Scott, 1979). Five standards with different concentrations of uronic acid are used to build a calibration curve to know the concentration of uronic acids in the samples. The standards and the samples react with 96% sulfuric acid in the presence of a solution composed of sodium chloride and boric acid as a catalyzer. Then, 3.5 dimethyl phenol is added to form a chromogen because this reagent is selective for 5-formyl-2-furancarboxylic acid, which is formed from uronic acids (Scott, 1979). After this reaction, the absorbance of the sample as well as the absorbance of five standards is read on a spectrophotometer (Bach Knudsen, 1997).

Following quantification of monosaccharides and uronic acids, total NSP is calculated as the sum of the analyzed monosaccharides plus the uronic acids.

Phenolic acid analyses are needed to estimate fermentation and digestibility of arabinoxylan. Phenolic acids analysis is divided in bound and free ferulic and coumaric acids, and starts with treatment of the samples with 0.5*M* KOH to extract the total (free and bound) ferulic and coumaric acids, then samples are incubated at 37°C in the dark during 20 h. Bound phenolic acids are then extracted by suspending the sample in sodium acetate buffer (pH 5.7). Quantification of the phenolic acids is performed using ultra high performance liquid chromatography and mass spectrometric (Appeldoorn et al., 2010; Van Dongen et al., 2011).

CONCLUSION

Corn and DDGS fiber is composed primarily of insoluble fiber, which reduces energy and protein digestibility in pigs. Insoluble fiber is composed mainly of NSP, which contain

cellulose and hemicelluloses, including arabinoxylan and β-glucans. Analysis of non-starch polysaccharides in diets containing corn or DDGS, and feces from pigs fed those diets, will contribute to generating information about the chemical composition of the carbohydrates in the insoluble dietary fiber and the components in feces that are not fermented by pigs. Non-starch polysaccharide analysis can be performed using chromatography techniques such as HPLC and GLC, and use of the procedures is needed to quantify monosaccharides in feed ingredients and diets. Uronic acids in fiber may be quantified using a colorimetric method and by adding monosaccharides and uronic acids, the concentration of NSP can be calculated. This information will contribute to the identification of enzymes required to hydrolyze the insoluble fiber in diets containing corn and DDGS. This information will also contribute to generating data for the fermentability of fiber and the potential energy that may be provided by fiber will be generated.

TABLE

Table 2. 1. Classification of chromatography

Stationary phase	Mobile phase	Type of Chromatography
Liquid	Liquid	High performance liquid chromatography (HPLC)
		Ultra high performance liquid chromatography (UHPLC)
		Thin-layer chromatography (TLC)
Solid	Liquid	Gel-filtration chromatography
		With molecular mechanism added, but same principle:
		Reversed phase chromatography
		Ion-exchange chromatography
Solid	Gas	Gas solid chromatography (GSC)
Solid	Liquid	Gas liquid chromatography (GLC)

FIGURES

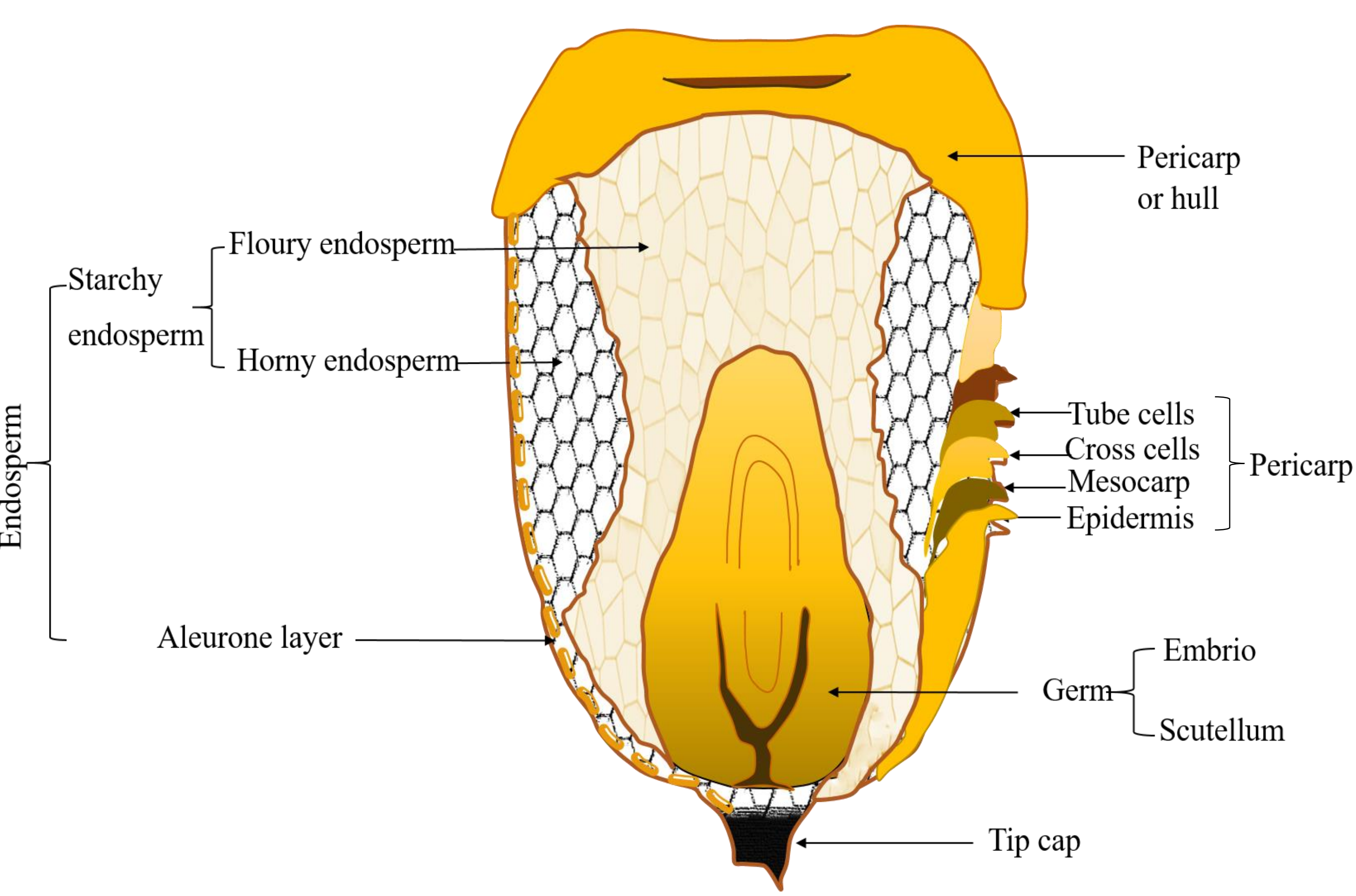

Figure 2. 1. Grain Structure

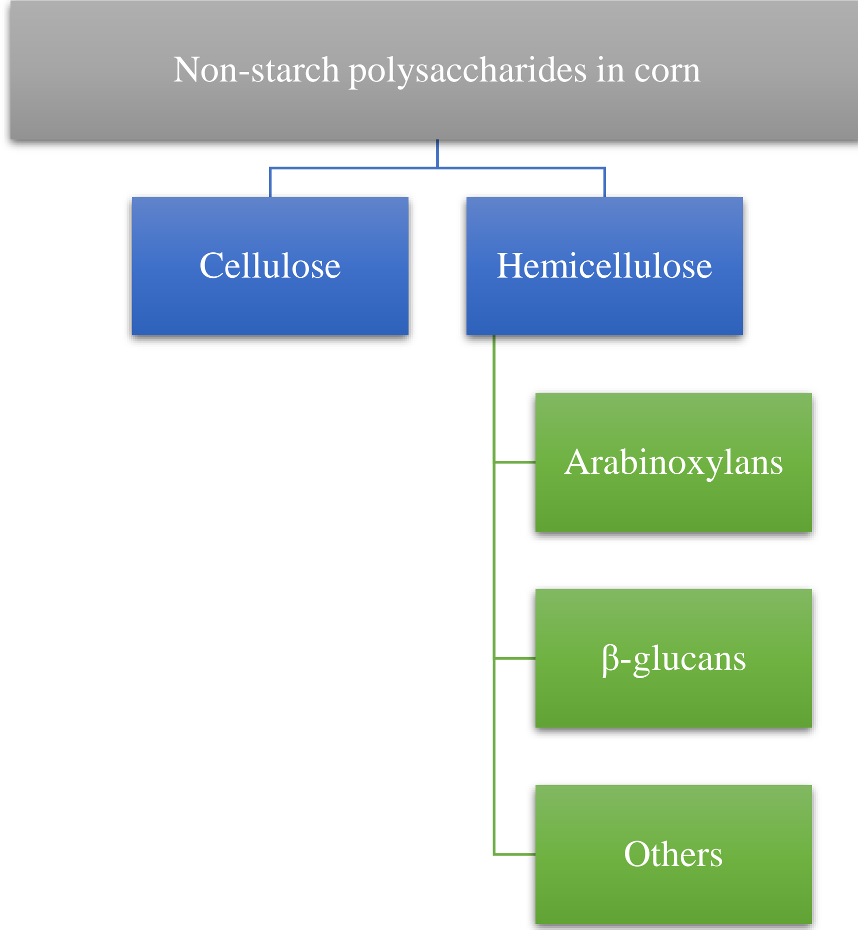

Figure 2. 2. Non-starch polysaccharides in corn

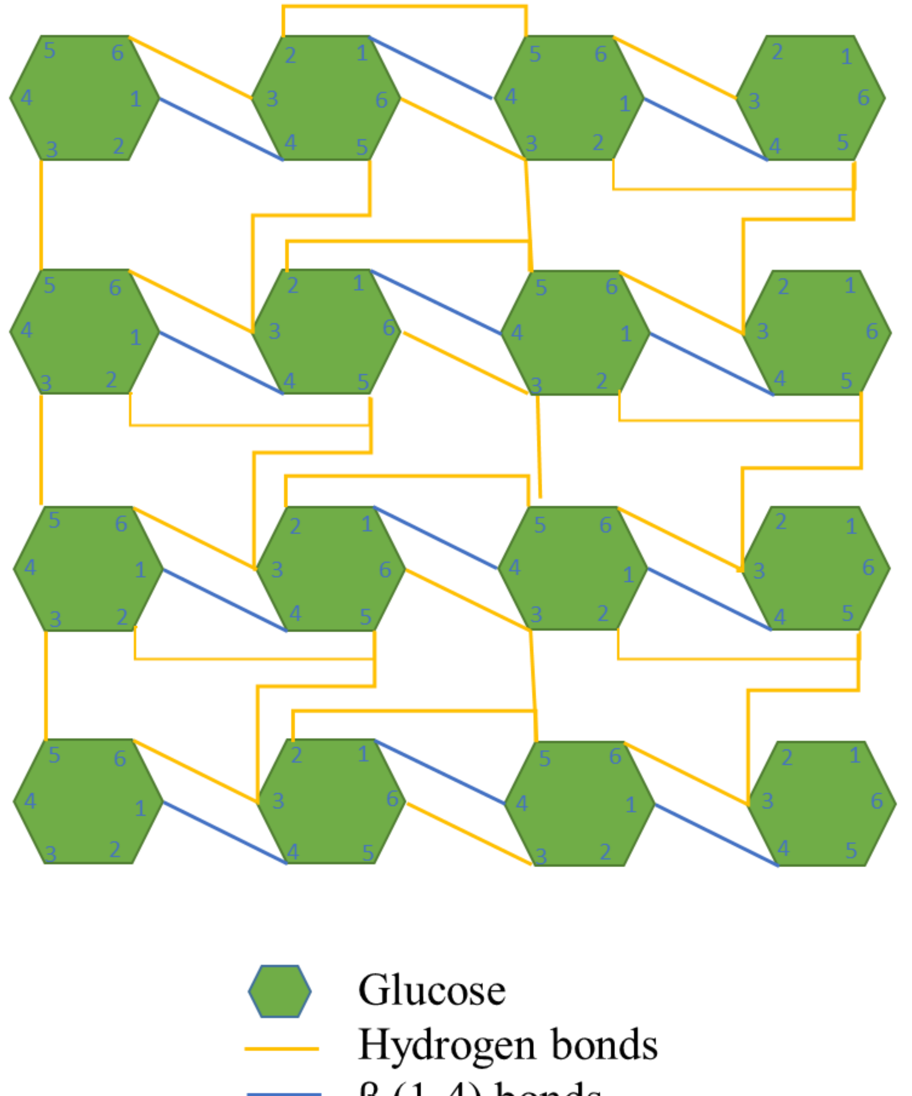

Figure 2. 3. Cellulose structure

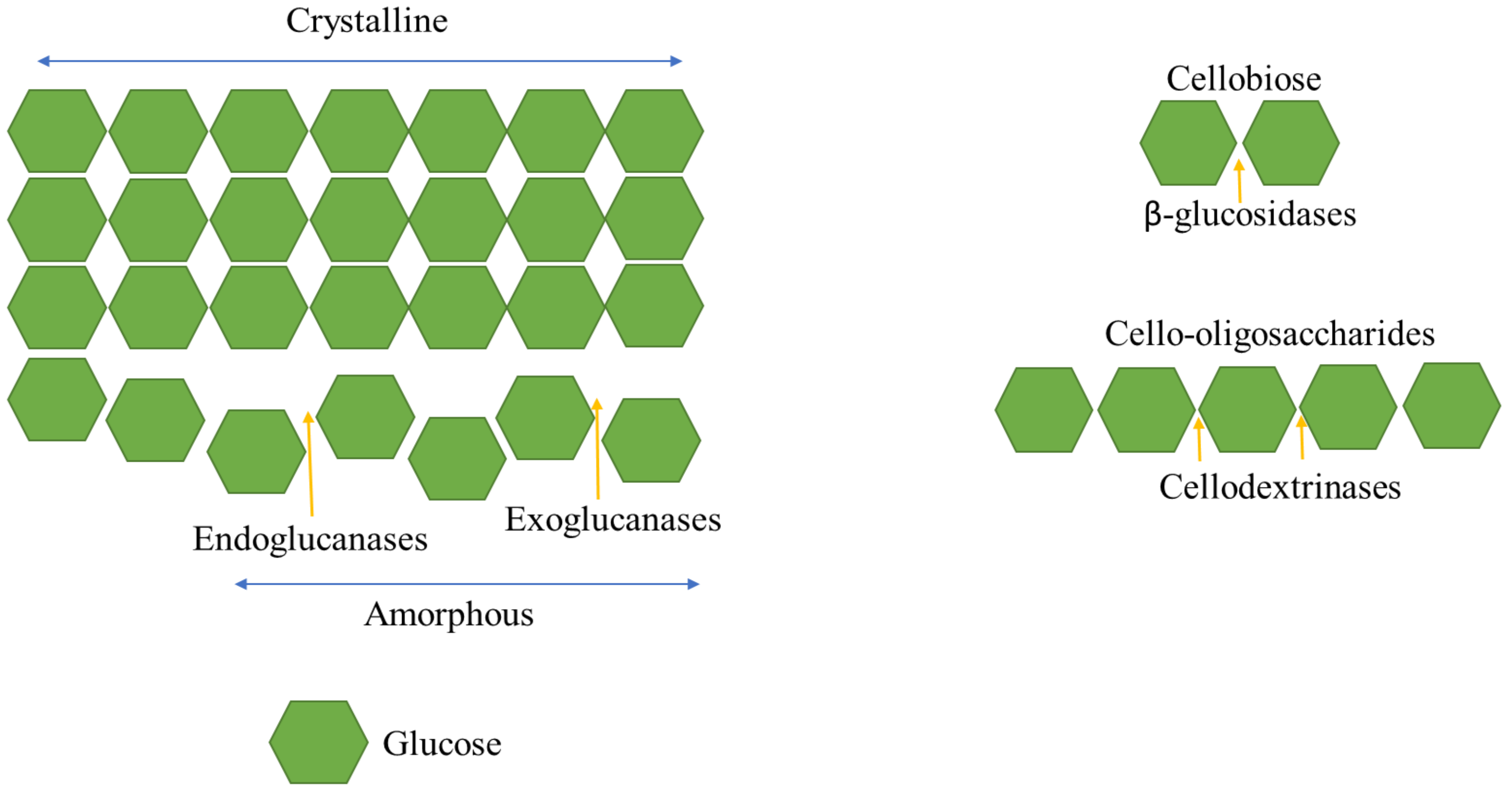

Figure 2. 4. Enzymes to hydrolyze the cellulose structure

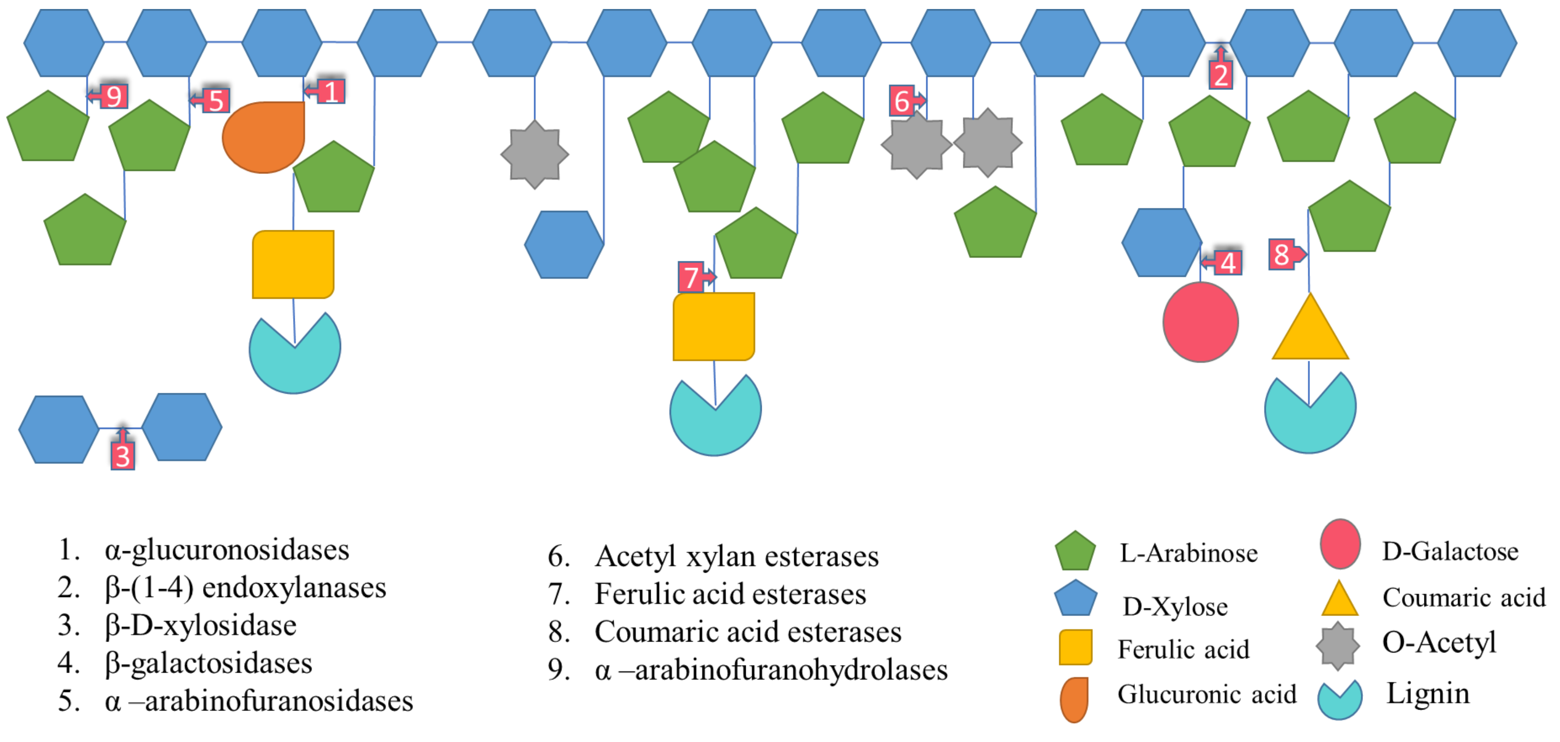

Figure 2. 5. Enzymes required for hydrolysis of arabinoxylan

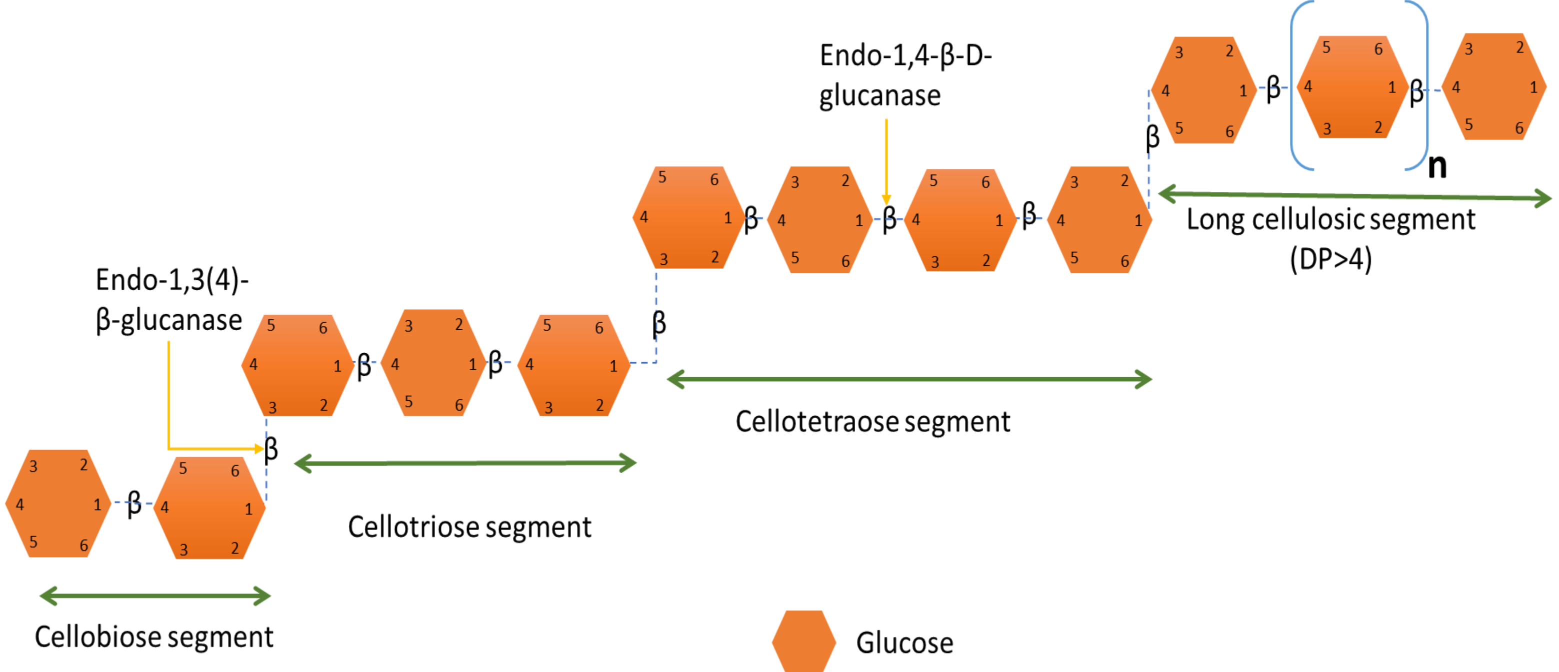

Figure 2. 6. Structure of β glucans.

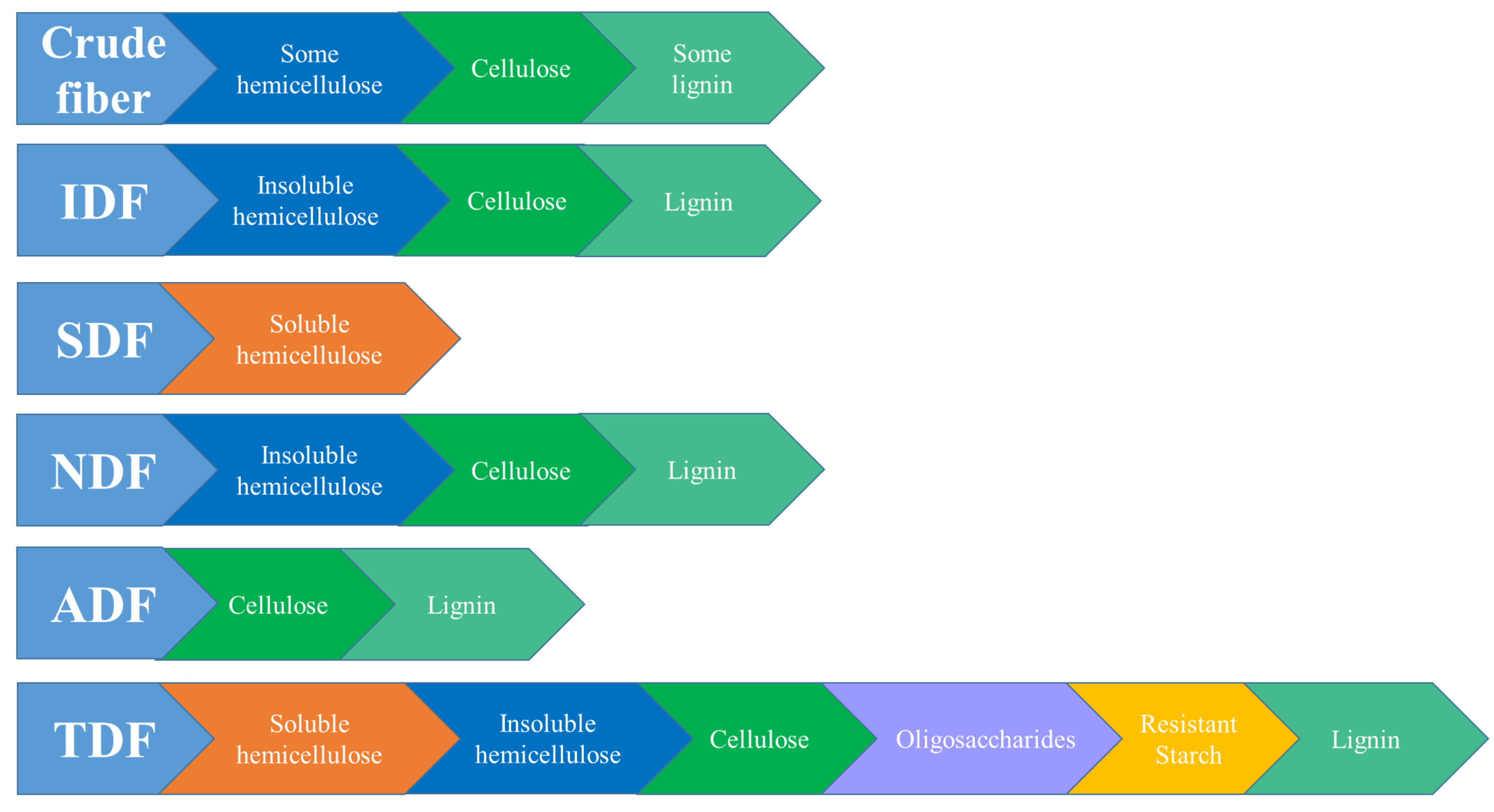

Figure 2. 7. Methods to analyze fiber diet

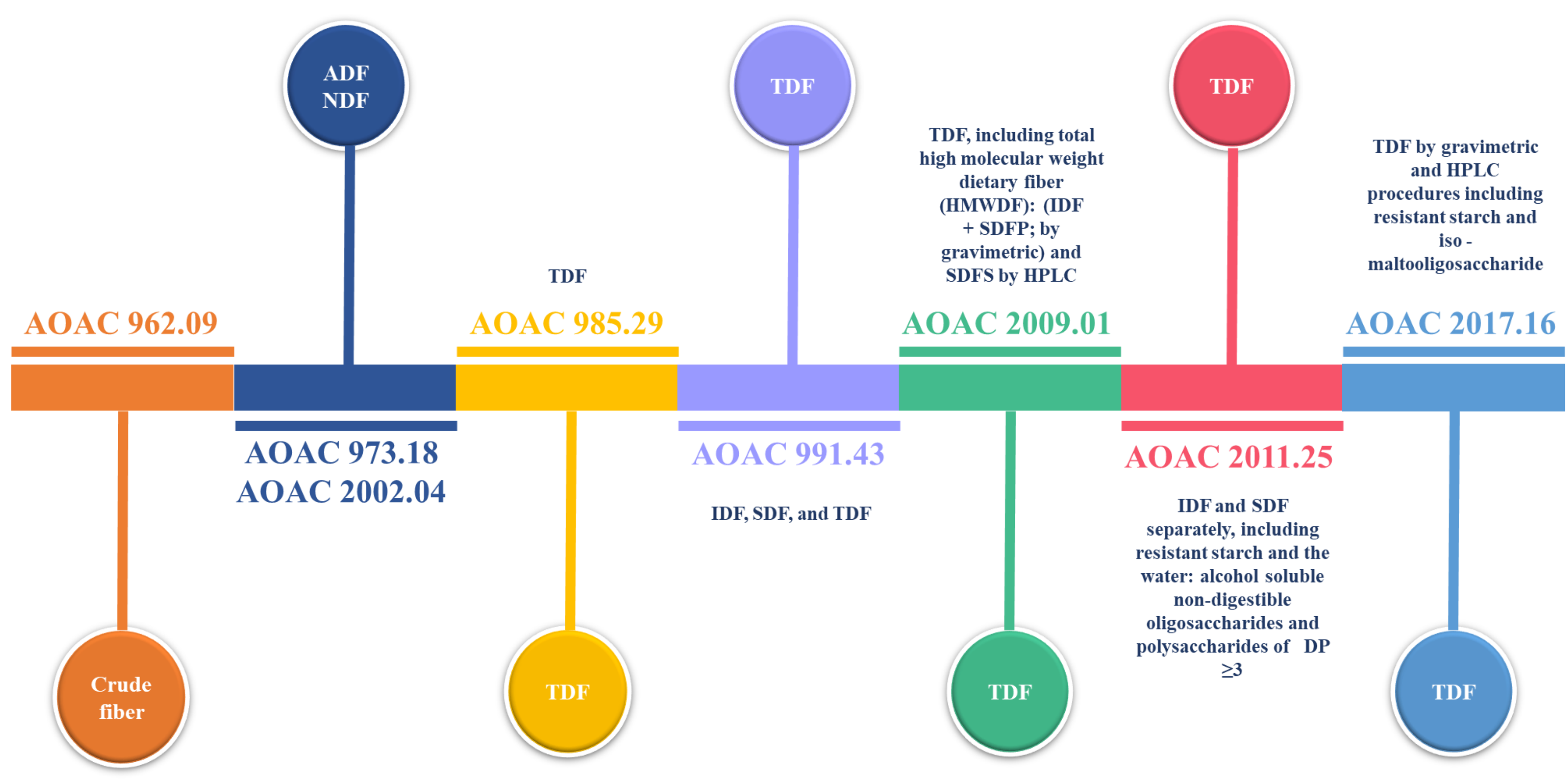

Figure 2. 8. Fiber analysis AOAC methods

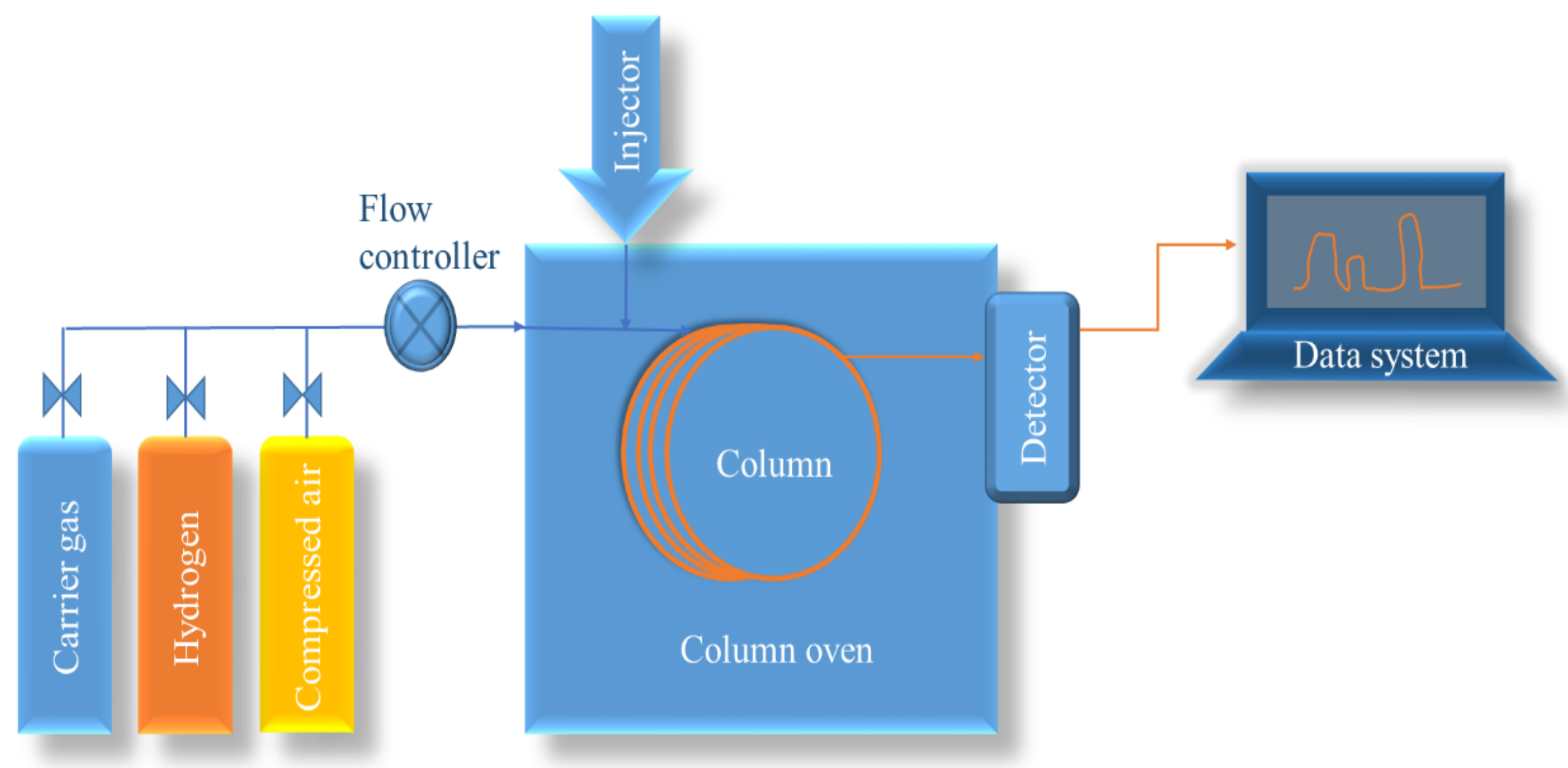

Figure 2. 9. Gas chromatographer

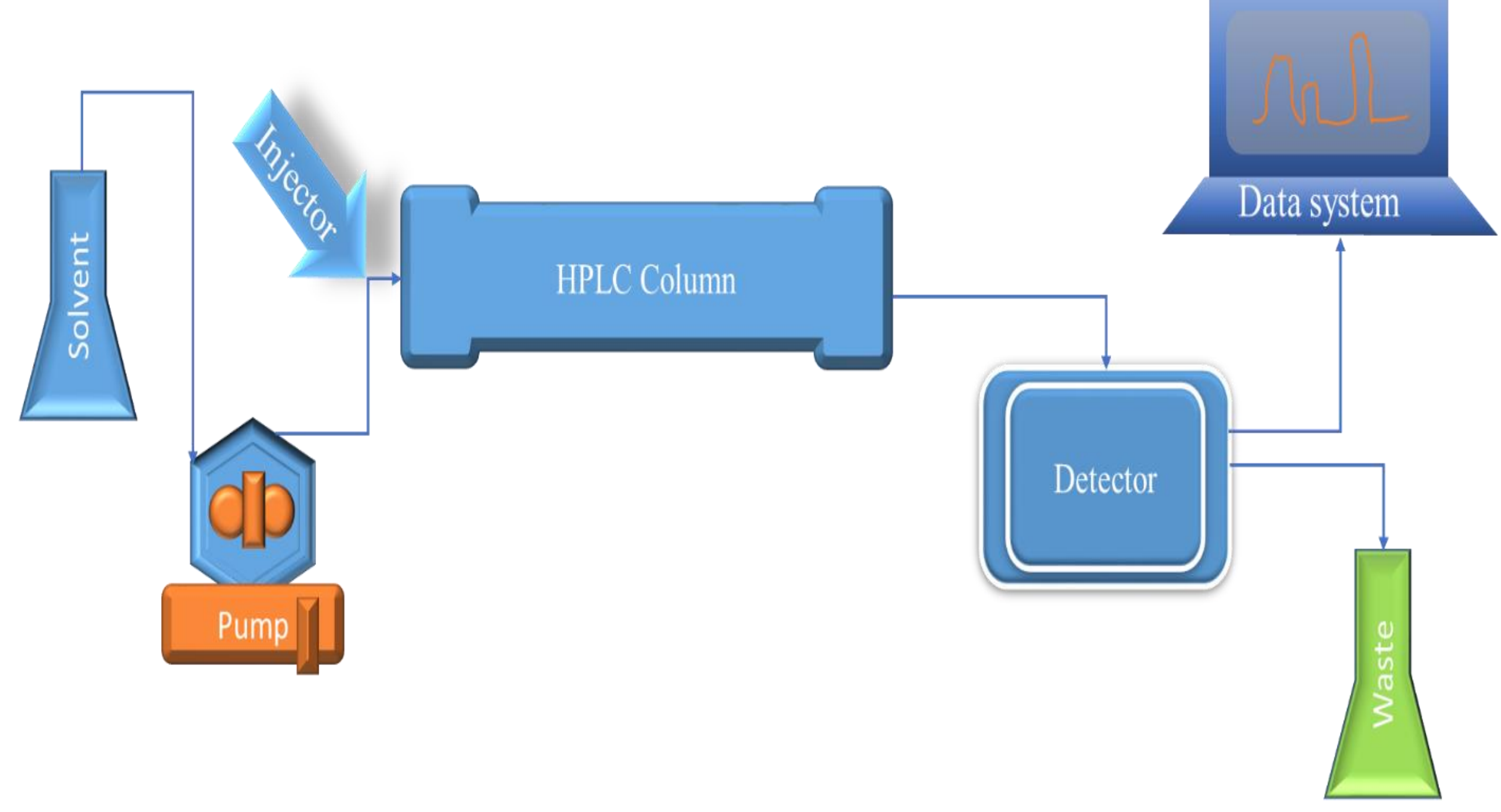

Figure 2. 10. Gas chromatographer

LITERATURE CITED

Abelilla, J. J. 2018. Fermentation and energetic value of fiber in feed ingredients and diets fed to pigs. PhD Diss, Illinois Univ., Urbana-Champaign.

Alyassin, M., and G. M. Campbell. 2019. Challenges and constraints in analysis of oligosaccharides and other fibre components. In: G. Gonzalez-Ortiz, M. R. Bedford, K. E. B. Knudsen, C. M. Courtin, and H. L. Classen, editors, The value of fibre engaging the second brain for animal nutrition. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 257-272.

AOAC Int. 2019. Official methods of analysis of AOAC international. 21st ed. AOAC international, Rockville, MD, United States.

Appeldoorn, M. M., M. A. Kabel, D. Van Eylen, H. Gruppen, and H. A. Schols. 2010. Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation. J. Agric. Food. Chem. 58:11294-11301. doi:10.1021/jf102849x

Asghari, M., and R. G. Hanson. 1984. Climate, management, and N effect on corn leaf N, yield, and grain N. Agron. J. 76:911-916. doi:10.2134/agronj1984.00021962007600060012x

Ayres, V. E., J. N. Broomhead, X. Li, R. M. Raab, and J. S. Moritz. 2019. Viscosity and growth response of broilers fed high fiber diets supplemented with a corn-produced recombinant carbohydrase. J. Appl. Poult. Res. 28:826-836. doi:10.3382/japr/pfz039

Bach Knudsen, K. E. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67:319-338. doi:10.1016/S0377-8401(97)00009-6

Bach Knudsen, K. E. 2011. Triennial growth symposium: effects of polymeric carbohydrates on growth and development in pigs. J. Anim. Sci. 89:1965-1980. doi:10.2527/jas.2010-3602

Bach Knudsen, K. E., H. N. Lærke, and H. Jørgensen. 2013. Carbohydrates and carbohydrate utilization in Swine. In: L. I. Chiba, editor, Sustainable Swine Nutrition. John Wiley & Sons, Inc., Ames, IA, United States. p. 109-137.

Bedford, M. R., and H. Schulze. 1998. Exogenous enzymes for pigs and poultry. Nutr. Res. Rev. 11:91-114. doi:10.1079/NRR19980007

Belkacemi, K., and S. Hamoudi. 2003. Enzymatic hydrolysis of dissolved corn stalk hemicelluloses: reaction kinetics and modeling. J. Chem. Technol. Biotechnol. 78:802-808. doi:10.1002/jctb.865

Braithwaite, A., and F. J. Smith. 1985. Theoretical considerations. In: A. Braithwaite, F. J. Smith, and R. Stock, editors, Chromatographic Methods. Chapman and Hall, New York, NY, United States. p. 17-43.

Burton, R. A., and G. B. Fincher. 2009. (1,3;1,4)-β-D-Glucans in cell walls of the poaceae, lower plants, and fungi: a tale of two linkages. Mol. Plant. 2:873-882. doi:10.1093/mp/ssp063

Champ, M., A.-M. Langkilde, F. Brouns, B. Kettlitz, and Y. L. B. Collet. 2003. Advances in dietary fibre characterisation. Definition of dietary fibre, physiological relevance, health benefits and analytical aspects. Nutr. Res. Rev. 16:71-82. doi:10.1079/NRR200254

Chen, Y., M.-Y. Xie, Y.-X. Wang, S.-P. Nie, and C. Li. 2009. Analysis of the monosaccharide composition of purified polysaccharides in Ganoderma atrum by capillary gas chromatography. Phytochem. Anal. 20:503-510. doi:10.1002/pca.1153

Ciolacu, D., F. Ciolacu, and V. Popa. 2011. Amorphous cellulose - structure and characterization. Cell. Chem. Tech. 45:13-21.

Cuevas, E., S. Hillebrand, A. Antezana, and P. Winterhalter. 2011. Soluble and bound phenolic compounds in different bolivian purple corn. J. Agric. Food. Chem. 59:7068-7074. doi:10.1021/jf201061x

de Coning, P., and J. Swinley. 2019a. The gas chromatograph. In: P. de Coning, and J. Swinley, editors, A practical guide to gas analysis by gas chromatography. Elsevier Inc., Cambridge, MA, United States. p. 77-98.

de Coning, P., and J. Swinley. 2019b. Overview and theory. In: P. de Coning, and J. Swinley, editors, A practical guide to gas analysis by gas chromatography. Elsevier Inc., Cambridge, MA, United States. p. 1-43.

de Coning, P., and J. Swinley. 2019c. A practical gas analysis by gas chromatography. Elsevier Inc., Cambridge, MA, United States.

de Vries, R. 2003. Regulation of Aspergillus genes encoding plant cell wall polysaccharide-degrading enzymes; relevance for industrial production. Appl. Microbiol. Biotechnol. 61:10-20. doi:10.1007/s00253-002-1171-9

Dodd, D., and I. K. O. Cann. 2009. Enzymatic deconstruction of xylan for biofuel production. Glob. Change. Biol. Bioenergy. 1:2-17. doi:10.1111/j.1757-1707.2009.01004.x

Dong, M. W. 2006. Introduction. In: M. W. Dong, editor, Modern HPLC for practicing scientists. John Wiley & Sons, Inc, Hoboken, New Jersey. p. 1-14.

Dreher, M. L. 2001. Dietary fiber overview. In: S. S. Cho, and M. L. Dreher, editors, Handbook of dietary fiber. Marcel Dekker Inc. , New York. p. 1-16.

Duan, C.-J., and J.-X. Feng. 2010. Mining metagenomes for novel cellulase genes. Biotechnol. Lett. 32:1765-1775 journal article. doi:10.1007/s10529-010-0356-z

Englyst, K. N., S. Liu, and H. N. Englyst. 2007. Nutritional characterization and measurement of dietary carbohydrates. Eur. J. Clin. Nutr. 61:S19 Review. doi:10.1038/sj.ejcn.1602937

Fallon, A., R. F. G. Booth, and L. D. Bell. 1987. The theory of HPLC. In: A. Fallon, R. F. G. Booth, and L. D. Bell, editors, Laboratory techniques in biochemistry and molecular biology No. 17. Elsevier, New York, NY, United States. p. 8-22.

FDA. 2016. CFR - Food labeling: Revision of the nutrition and supplement facts labels Code of Federal Regulations Title 21. p. 111. FDA, Silver Spring, MD, United States.

Feng, T., M. Sun, S. Song, H. Zhuang, and L. Yao. 2019. Gas chromatography for food quality evaluation. In: J. Zhong, and X. Wang, editors, Evaluation technologies for food quality. Woodhead Publishing, Cambridge, MA, United States. p. 219-265.

Fukushima, R. S., M. S. Kerley, M. H. Ramos, J. H. Porter, and R. L. Kallenbach. 2015. Comparison of acetyl bromide lignin with acid detergent lignin and Klason lignin and correlation with in vitro forage degradability. Anim. Feed Sci. Technol. 201:25-37. doi:10.1016/j.anifeedsci.2014.12.007

Hamaker, B. R., Y. E. Tuncil, and X. Shen. 2019. Carbohydrates of the kernel. In: S. O., and Serna-Saldivar, editors, Corn: chemistry and technology. Elsevier Inc., Cambridge, MA, United States. p. 305-318.

Hatfield, R. D., H.-J. G. Jung, J. Ralph, D. R. Buxton, and P. J. Weimer. 1994. A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. J. Sci. Food Agric. 65:51-58. doi:10.1002/jsfa.2740650109

He, Y., T. M. Mouthier, M. A. Kabel, J. Dijkstra, W. H. Hendriks, P. C. Struik, and J. W. Cone. 2018. Lignin composition is more important than content for maize stem cell wall degradation. J. Sci. Food Agric. 98:384-390. doi:10.1002/jsfa.8630

Held, M. A., N. Jiang, D. Basu, A. M. Showalter, and A. Faik. 2015. Plant cell wall polysaccharides: structure and biosynthesis. In: K. G. Ramawat, and J.-M. Mérillon, editors, Polysaccharides bioactivity and biotechnology. Springer International Publishing AG Switzerland, Switzerland. p. 3-54.

Izydorczyk, M. S. 2017. Functional properties of cereal cell wall polysaccharides. In: A.-C. Eliasson, editor, Carbohydrates in food. Taylor & Francis Group, Boca Raton, FL, United States. . p. 193-246.

Jaworski, N. W., H. N. Lærke, K. E. Bach Knudsen, and H. H. Stein. 2015. Carbohydrate composition and in vitro digestibility of dry matter and nonstarch polysaccharides in corn, sorghum, and wheat and coproducts from these grains. J. Anim. Sci. 93:1103-1113. doi:10.2527/jas.2014-8147

Jaworski, N. W., and H. H. Stein. 2017. Disappearance of nutrients and energy in the stomach and small intestine, cecum, and colon of pigs fed corn-soybean meal diets containing distillers dried grains with solubles, wheat middlings, or soybean hulls1. J. Anim. Sci. 95:727-739. doi:10.2527/jas.2016.0752

Jung, H.-J. G. 1997. Analysis of forage fiber and cell walls in ruminant nutrition. J. Nutr. 127:810S-813S. doi:10.1093/jn/127.5.810S

Lapierre, C., B. Pollet, M.-C. Ralet, and L. Saulnier. 2001. The phenolic fraction of maize bran: evidence for lignin-heteroxylan association. Phytochemistry. 57:765-772. doi:10.1016/S0031-9422(01)00104-2

Lara, S. G., C. C. Hernandez, and S. O. S. Saldivar. 2019. Development and structure of the corn kernel, Corn: chemistry and technology. Elsevier, Cambridge, MA, United States. p. 147-162.

Lattimer, J. M., and M. D. Haub. 2010. Effects of dietary fiber and its components on metabolic health. Nutrients. 2:1266-1289. doi:10.3390/nu2121266

Loy, D. D., and E. L. Lundy. 2019. Nutritional properties and feeding value of corn and its coproducts. In: S. O., and Serna-Saldivar, editors, Corn: chemistry and technology. Elsevier Inc., Cambridge, MA, United States. p. 633-654.

Lundy, D. D. L. a. E. L. 2019. Corn : chemistry and technology. Third ed. Elsevier Inc., Cambridge, MA, United States.

McCleary, B. V. 2003. Dietary fibre analysis. Proc. Nutr. Soc. 62:3-9. doi:10.1079/PNS2002204

McCleary, B. V., J. W. DeVries, J. I. Rader, G. Cohen, L. Prosky, D. C. Mugford, M. Champ, and K. Okuma. 2019. Determination of insoluble, soluble, and total dietary fiber (CODEX definition) by enzymatic-gravimetric method and liquid chromatography: collaborative study. J. AOAC Int. 95:824-844. doi:10.5740/jaoacint.CS2011_25

Mertens, D. R. 2003. Challenges in measuring insoluble dietary fiber. J. Anim. Sci. 81:3233-3249. doi:10.2527/2003.81123233x

Mongeau, R., and S. P. J. Brooks. 2003. Dietary fiber determination. In: B. Caballero, editor, Encyclopedia of food sciences and nutrition Academic Press, Oxford, UK. p. 1823-1833.

Navarro, D. M. D. L., E. M. A. M. Bruininx, L. de Jong, and H. H. Stein. 2018. Analysis for low-molecular-weight carbohydrates is needed to account for all energy-contributing nutrients in some feed ingredients, but physical characteristics do not predict in vitro digestibility of dry matter. J. Anim. Sci. 96:532-544. doi:10.1093/jas/sky010

NRC. 2012. Nutrient requierements of swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Paloheimo, M., J. Piironen, and J. Vehmaanperä. 2010. Xylanases and cellulases as feed additives. In: M. R. Bedford, and G. G. Partridge, editors, Enzymes in farm animal nutrition. CAB Internationa, London, UK. p. 12-53.

Pedersen, M. B., S. Dalsgaard, K. E. B. Knudsen, S. Yu, and H. N. Lærke. 2014. Compositional profile and variation of distillers dried grains with solubles from various origins with focus on non-starch polysaccharides. Anim. Feed Sci. Technol. 197:130-141. doi:10.1016/j.anifeedsci.2014.07.011

Phillips, K. M., D. B. Haytowitz, and P. R. Pehrsson. 2019. Implications of two different methods for analyzing total dietary fiber in foods for food composition databases. Journal of Food Composition and Analysis. 84:103253. doi:10.1016/j.jfca.2019.103253

Pluske, J., J. C. Kim, D. E. McDonald, D. Pethick, and D. Hampson. 2001. Non-starch polysaccharides in the diet of young weaned piglets. In: M. A. Varley, and J. Wiseman, editors, The weaner pig: nutrition and management. CABI Publishing, New Tork, NY, United States. p. 81-112.

Poole, C. F. 2020. Conventional gas chromatography: basic principles and instrumental aspects. In: P. Q. Tranchida, editor, Advanced gas chromatography in food analysis. The royal society of chemistry, Croydon, UK. p. 83-130.

Poole, C. F., and S. K. Poole. 1991. Fundamental relationships of chromatography. In: C. F. Poole, and S. K. Poole, editors, Chromatography today. Elsevier Inc., Amsterdam, The Netherlands. p. 1-95.

Rumpagaporn, P., B. L. Reuhs, A. Kaur, J. A. Patterson, A. Keshavarzian, and B. R. Hamaker. 2015. Structural features of soluble cereal arabinoxylan fibers associated with a slow rate

of in vitro fermentation by human fecal microbiota. Carbohydr. Polym. 130:191-197. doi:10.1016/j.carbpol.2015.04.041

Saulnier, L., and J.-F. Thibault. 1999. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. J. Sci. Food Agric. 79:396-402. doi:10.1002/(SICI)1097-0010(19990301)79:3<396::AID-JSFA262>3.0.CO;2-B

Scott, R. W. 1979. Colorimetric determination of hexuronic acids in plant materials. Anal. Chem. 51:936-941. doi:10.1021/ac50043a036

Slavin, J. 2013. Fiber and prebiotics: mechanisms and health benefits. Nutrients. 5:1417-1435. doi:10.3390/nu5041417

Stipanuk, M., and M. Caudill. 2013. Biochemical physiological, and molecular aspects of human nutrition. 3rd ed. Elsevier Inc., St. Louis, MO, United States.

Tymoczko, J. L., J. M. Berg, and L. Stryer. 2015. Carbohydrates. In: E. Geller, editor, Biochemestry a short course. W. H. Freeman & Company, New York, NY, United States. p. 163-183.

Udén, P., P. H. Robinson, and J. Wiseman. 2005. Use of detergent system terminology and criteria for submission of manuscripts on new, or revised, analytical methods as well as descriptive information on feed analysis and/or variability. Anim. Feed Sci. Technol. 118:181-186. doi:10.1016/j.anifeedsci.2004.11.011

Urriola, P. E., S. K. Cervantes-Pahm, and H. H. Stein. 2013. Fiber in swine nutrition. In: L. I. Chiba, editor, Sustainable Swine Nutrition. John Wiley & Sons, Inc., Ames, IO, United States. p. 255-276.

Urriola, P. E., G. C. Shurson, and H. H. Stein. 2010. Digestibility of dietary fiber in distillers coproducts fed to growing pigs. J. Anim. Sci. 88:2373-2381. doi:10.2527/jas.2009-2227

Van Dongen, F. E. M., D. Van Eylen, and M. A. Kabel. 2011. Characterization of substituents in xylans from corn cobs and stover. Carbohydr. Polym. 86:722-731. doi:10.1016/j.carbpol.2011.05.007

Van Erven, G., R. de Visser, P. de Waard, W. J. H. Van Berkel, and M. A. Kabel. 2019. Uniformly 13C labeled lignin internal standards for quantitative pyrolysis−GC−MS analysis of grass and wood. ACS Sustain. Chem. Eng. 7:20070-20076. doi:10.1021/acssuschemeng.9b05926

Wolfrum, E. J., A. J. Lorenz, and N. deLeon. 2009. Correlating detergent fiber analysis and dietary fiber analysis data for corn stover collected by NIRS. Cellulose. 16:577-585. doi:10.1007/s10570-009-9318-9

Xu, W., N. Reddy, and Y. Yang. 2009. Extraction, characterization and potential applications of cellulose in corn kernels and distillers dried grains with solubles (DDGS). Carbohydr. Polym. 76:521-527. doi:10.1016/j.carbpol.2008.11.017

Yadav, M. P., D. B. Johnston, A. T. Hotchkiss, and K. B. Hicks. 2007a. Corn fiber gum: a potential gum arabic replacer for beverage flavor emulsification. Food. Hydrocoll. 21:1022-1030. doi:10.1016/j.foodhyd.2006.07.009

Yadav, M. P., R. A. Moreau, and K. B. Hicks. 2007b. Phenolic acids, lipids, and proteins associated with purified corn fiber arabinoxylans. J. Agric. Food. Chem. 55:943-947. doi:10.1021/jf0624493

Yoshida, T., Y. Honda, T. Tsujimoto, H. Uyama, and J.-i. Azuma. 2014. Selective isolation of β-glucan from corn pericarp hemicelluloses by affinity chromatography on cellulose column. Carbohydr. Polym. 111:538-542. doi:doi.org/10.1016/j.carbpol.2014.04.050

Zhang, W., D. Li, L. Liu, J. Zang, Q. Duan, W. Yang, and L. Zhang. 2013. The effects of dietary fiber level on nutrient digestibility in growing pigs. J. Anim. Sci. Biotechnol. 4:17 journal article. doi:10.1186/2049-1891-4-17

Zhang, X.-Z. Z. a. Y.-H. P. 2013. Cellulases: characteristics, sources, production, and applications. In: S.-T. Yang, H. A. El-Enshasy, and N. Thongchul, editors, Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. John Wiley & Sons, Inc., Hoboken, NJ, United States. p. 131-146.

CHAPTER 3. CHARACTERIZATION OF NON-STARCH POLYSACCHARIDES IN DIETS BASED ON CORN AND SOYBEAN MEAL OR CORN, SOYBEAN MEAL, AND DISTILLERS DRIED GRAINS WITH SOLUBLES AND IN FECES FROM PIGS FED THESE DIETS

ABSTRACT

An experiment was conducted to quantify the non-starch polysaccharides in a diet based on corn and soybean meal (**SBM**), and in a diet based on corn, SBM, and distiller dried grains with solubles (**DDGS**), as well as in feces from pigs fed these diets. A total of 24 pigs (initial body weight: 61.71 ± 5.39 kg) were allotted to the diets in a randomized complete block design with 4 blocks, and 3 replicate pigs per diet in each block. Therefore, there were 12 replicate pigs per diet. Diets were fed to pigs for 22 d. During the initial 12 d diets were provided on an *ad libitum* basis, and collection of feces started on d 17 and ended on d 22. The concentration of total non-starch polysaccharides (**NSP**) in diets and collected fecal samples was calculated based on the concentration of individual sugars, which were measured using gas-liquid chromatography. Lignin was measured as acid detergent lignin and total dietary fiber was calculated as the sum of insoluble dietary fiber (**IDF**) and soluble dietary fiber. Results indicated that the concentration of NSP in the diet containing corn and SBM was 11.41%, whereas in the diet containing corn, SBM, and DDGS the concentration of NSP was 12.94%. Greater values for insoluble xylose, arabinose, and cellulose than for other individual sugars were analyzed in both diets and in feces from pigs fed the diets. There were no differences in NSP concentration in feces regardless of the diet being fed, but the digestibility of total NSP in the diet based on corn and SBM was greater than the digestibility from the diet based on corn, SBM, and DDGS

Keywords: enzymes, insoluble dietary fiber, lignin, non-starch polysaccharides, pigs.

INTRODUCTION

Corn and corn distillers dried grains with solubles (**DDGS**) are extensively used in diets fed to pigs (Jaworski et al., 2015). Corn distillers dried grains with solubles are used as an alternative ingredient due to the increased demand and increased cost of conventional raw materials (Pedersen et al., 2015b). Corn and DDGS contain dietary fiber, but in diets fed to pigs, fiber reduces energy and protein digestibility (Bach Knudsen, 1997; NRC, 2012).

Insoluble fiber in corn and corn-coproducts contain lignin and non-starch polysaccharides, which are composed mainly of cellulose, arabinoxylans, and β-glucans with arabinoxylan being the main fiber component in corn grain and corn co-products (Jaworski et al., 2015). Arabinoxylan has a xylose backbone that contains side chains of arabinose, galactose, and glucuronic acid, and ferulic acid and coumaric acid may be linked to some of the arabinose units via ester bonds. Ferulic acid and coumaric acid may link arabinoxylan to lignin (Bach Knudsen et al., 2013; Mnich et al., 2020).

There are several methods to analyze fiber including analysis to quantify crude fiber, detergent fiber, and total dietary fiber. The objective of these methods is to estimate the total amount of fiber, and each analysis is based on measuring the weight of the undissolved residue after chemical or enzymatic treatment. However, more details are needed to study structure, composition, and physiological effects of fiber (Alyassin and Campbell, 2019), and other techniques have been developed to provide details on the composition of the fiber fraction. One of these techniques is based on determination of the concentrations of individual sugars in fiber using gas-liquid chromatography (**GLC**). This method requires removal of starch, hydrolysis of

the polysaccharides, and quantification of their individual sugar constituents as alditol acetates using GLC (Bach Knudsen, 1997).

Studies have been conducted to quantify the total non-starch polysaccharides (**NSP**) fraction from ingredients and diets fed to pigs (Jaworski et al., 2015) and to determine the effect of inclusion of xylanases and glucanases on the digestibility of energy (Abelilla and Stein, 2019). However, there is no information about the NSP in the feces from pigs fed diets based on corn or DDGS. Due to this lack of information, it is difficult to determine the components that are not fermented by pigs. Knowing the fraction of the NSP in diets fed to pigs as well as in the feces may provide a better understanding of the individual carbohydrates from fiber that have not been fermented by pigs, which will facilitate the selection of enzymes needed to improve fiber fermentation. Therefore, the objective of the current experiment was to quantify the total NSP in diets based on corn and soybean meal (**SBM**) or corn, SBM, and DDGS and in fecal samples from pigs fed these diets. The second objective was to test the hypothesis that the concentration of NSP in the diet based on corn, SBM, and DDGS, and in feces from pigs fed this diet was greater than in the corn and SBM diet and in feces from pigs fed this diet.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

The institutional Animal Care and Use Committee at the University of Illinois reviewed and approved the protocol for the animal part of the experiment. Pigs used in the experiment were the offspring of L 359 boars mated to Camborough females (PIC, Hendersonville, TN).

A diet based on corn and SBM, and a diet based on corn, SBM, and DDGS (Archer Daniels Midland, Decatur, IL) were used (Table 3.1). Twenty-four pigs (initial body weight:

61.71 ± 5.39 kg), were allotted to a randomized complete block design with 2 diets, 4 blocks of 6 pigs, and 3 pigs per diet in each block. Therefore, there were 12 replicate pigs per diet and a total of 24 fecal samples were collected.

The 24 pigs were housed in individual pens equipped with slatted floors, a self-feeder, and a nipple waterer. During the initial 12 d, diets were provided on an *ad libitum* basis. On d 13, pigs were moved to individual metabolism crates and were adapted to the crates for 4 d (d 13 to 16). Pigs were restrictedly fed during the time they were housed in the metabolism crates and each day, pigs received an amount of feed that was equal to 3.2 times the maintenance energy requirement (NRC, 2012), which were provided in 2 equal meals at 0800 and 1700 h. On d 17 in the morning, a color marker was included in the meal fed to each pig and fecal collections started as soon as the marker appeared in the feces (Kong and Adeola, 2014). On d 22 in the morning, a second color marker was fed and fecal collections ceased when the second marker appeared in the feces. Throughout the study, pigs had *ad libitum* access to water. Fecal samples were thawed and mixed within pig and diet, and then dried in a 65°C forced air drying oven (Model 8, Metalab, Equipment Corp., Hicksville, NY) prior to analysis.

Chemical Analyses

All chemical analyses were performed in duplicate. Fecal and diet samples were analyzed for dry matter (**DM**; method 930.1; AOAC Int., 2007). Crude protein in the diets and fecal samples was calculated as N × 6.25 and N was analyzed by combustion (AOAC Int., 2007; method 990.03) using a LECO FP628 Nitrogen Analyzer (LECO Corp., Saint Joseph, MI). Acid hydrolyzed ether extract **(AEE)** in the diets and fecal samples was analyzed by acid hydrolysis using 3*N* HCl (Ankom HCl Hydrolysis System, Ankom Technology, Macedon, NY) followed by fat extraction (Ankom XT-15 Extractor, Ankom Technology, Macedon, NY). Acid detergent lignin was

determined using Ankom Technology method 9 (Ankom Daisy II Incubator, Ankom Technology, Macedon, NY). Insoluble dietary fiber (**IDF**) and soluble dietary fiber (**SDF**) were analyzed in diets and fecal samples according to method 991.43 (AOAC Int., 2007) using the Ankom^{TDF} Dietary Fiber Analyzer (Ankom Technology, Macedon, NY). Total dietary fiber (**TDF**) was calculated as the sum of IDF and SDF.

Non Starch Polysaccharide Analysis

Non-starch polysaccharides were analyzed based on their individual sugar constituents as alditol acetates using GLC, and uronic acids were analyzed using a colorimetric technique (Bach Knudsen, 1997). The NSP analysis was conducted in a three-parallel extraction procedure: 1) total non-starch polysaccharides (**T-NSP**), 2) non-cellulosic polysaccharides (**NCP**), and 3) insoluble non-cellulosic polysaccharides (**I-NCP**) as outlined by Bach Knudsen (1997) and Bach Knudsen, 1997; Jaworski et al. (2015). Dietary fiber was calculated as the sum of total NSP and lignin.

Extraction 1: Total NSP: Diets and feces were ground through a 0.5 mm screen, and 250 mg were added to 50 mL tubes. Samples were treated with thermostable α amylase (EC 3.2.1.1 Megazyme Ltd. Cat. No. E-BLAAM 3,000 Units/mL) during 1 h at 100 ºC. Amyloglucosidase (EC 3.2.1.3 Megazyme Int., Cat. No. E-AMGDF, 3,260 units/ mL) was added afterwards and samples were incubated for 2 h. After the removal of starch, the soluble non-starch polysaccharides (**S-NSP**) were precipitated by adding 99% ethanol while the samples were kept in an ice bath during 1 h. Afterwards, samples were centrifuged and the supernatant was removed. The residues were washed twice with 85% ethanol and once with acetone, and left to dry overnight. Then, 5.0 mL of 12 *M* H_2SO_4 was added and samples were incubated for 1 h at 35 °C to swell the cellulose, and the NSP were hydrolyzed to monosaccharides incubating the

samples using 30.0 mL of 2 *M* H_2SO_4 for 1 h at 100°C. The acid hydrolysates were filtered, and the hydrolysate was collected.

Extraction 2: NCP: The procedure described for T-NSP was followed with the exception that the starch-free residue was directly hydrolyzed to monosaccharides with 2 *M* H_2SO_4 without prior swelling of the cellulose with 12 *M* H_2SO_4.

Extraction 3: I-NCP: The procedure described for T-NSP was followed with the exception that S-NSP were extracted from the starch-free residue using 40 mL of a 0.2 *M* phosphate buffer.

Once the hydrolysates from the 3 parallel extractions were obtained, an internal standard (allose, 2 mg L^{-1}) was added to each tube. Sugars were reduced to alcohols with potassium borohydride and acetylated to alditol acetate derivatives using 1-methylimidazole to catalyze the reaction (Connors and Pandit, 1978). Sugars were quantified using a gas chromatographer (Agilent 8860, Agilent technology, Santa Clara, CA) with a flame ionization detector and an HP Cyanopropyaryl-polysiloxane column (250ºC 30m × 250µm × 0.2µm 225 mm, 88% Cyanopropyaryl-polysiloxane).

Uronic Acid Analysis

Uronic acids were analyzed in the T-NSP and I-NCP hydrolysates via a colorimetric method (Scott, 1979). D-glucuronic acid standards with 5 different concentrations (0, 6.25, 12.5, 25.0, 37.5, and 50 g/mL) were used to generate a calibration curve. Samples and standards were treated with a NaCl/H_3BO_3 solution as a catalyzer, and 96% H_2SO_4 before being digested at 70 ºC for 40 min. Afterwards, 3.5 dimethylphenol was added to the samples and the standards, and the absorbance of the solution was read on a spectrophotometer (GENESYS™ 40 Vis/UV-Vis,

Thermofisher technology, Waltham, MA) both at 400 and 450 nm. Sulfuric acid 96% was used for baseline determination.

Calculations of Carbohydrate Composition

Total non-starch polysaccharides were calculated using equation 1 (Bach Knudsen, 1997):

T-NSP, % = rhamnose + fucose + arabinose + xylose + mannose + galactose + glucose + uronic acids [1]

Cellulose was calculated using equation 2 (Bach Knudsen, 1997):

Cellulose, % = (glucose from total NSP extraction) – (glucose from NCP extraction) [2]

Insoluble NCP were calculated using equation 3 (Bach Knudsen, 1997):

I-NCP, % = [rhamnose + fucose + arabinose + xylose + mannose + galactose + glucose + (uronic acids from I-NCP)] – cellulose [3]

Soluble NSP were calculated using equation 4 (Bach Knudsen, 1997):

S-NSP, % = total NSP – cellulose – I-NCP [4]

Total dietary fiber (TDF) was calculated using equation 5 (Bach Knudsen, 1997):

TDF, % = total NSP + lignin [5]

Insoluble dietary fiber (IDF) was calculated using equation 6 (Bach Knudsen, 1997):

IDF, % = cellulose + I-NCP + lignin [6]

Soluble dietary fiber (SDF) was calculated using equation 7 (Bach Knudsen, 1997):

SDF, % = TDF – IDF [7]

Statistical Analysis

Normality of residuals and assumptions of the model were tested using the UNIVARIATE procedure of SAS (SAS Inst. Inc., Cary, NC). Data were analyzed using the PROC MIXED of SAS with the pig as the experimental unit. The model included diet as the fixed effect and block and pig within block were considered random effects. Treatment means were calculated using the LSMEANS statement and means were separated using the PDIFF option of SAS. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

RESULTS AND DISCUSION

Concentration of S-NCP was greater in the diet based on corn, SBM, and DDGS, than in the diet based on corn and SBM (Table 3.2), which is in agreement with reported data (Jaworski et al., 2015). The concentration of glucose in the S-NCP fraction in the diet based on corn, SBM, and DDGS was greater than the concentration of other sugars. Concentrations of soluble non cellulosic arabinose, xylose, mannose and glucose were too low to be detected in the corn and SBM diet. Soluble non cellulosic polysaccharides in both diets were lower than I-NCP due to the high concentrations of cellulose, lignin, and insoluble hemicelluloses in the insoluble fraction. Soluble non cellulosic polysaccharides in pigs are important because S-NCP provide energy to the pigs more rapidly than I-NCP (Bach Knudsen, 2011), and most soluble fiber is easily fermented by pigs (Urriola et al., 2010; Jaworski and Stein, 2017).

The concentration of I-NCP was slightly greater in the corn and SBM diet than in the diet containing corn, SBM, and DDGS. Arabinose, xylose, and cellulose were the predominant sugars in both diets, and mannose was present in a lower concentration in both diets, which is in

agreement with reported values (Bach Knudsen, 1997; Jaworski et al., 2015). Arabinose and xylose concentrations were high in both diets because cereals like corn, rye, and wheat are rich in arabinoxylan (Knudsen and Vangsøe, 2019). Concentration of arabinose was greater than xylose in the diet based on corn and SBM due to the contribution of arabinose from the SBM because the concentration of arabinose may be greater than xylose in SBM (Karr-Lilienthal et al., 2005; Choct, 2015; Maharjan et al., 2019). Concentration of the individual sugars in the diet based on corn, SBM, and DDGS was lower than values reported in the literature (Pedersen et al., 2014; Céspedes, 2015). This difference may be a result of the fact that DDGS is a byproduct from the production of ethanol, and during the fermentation process different enzymes are added, which may alter the concentration of insoluble sugars because some of the neutral detergent fiber may be degraded, and therefore, the concentration of insoluble fiber is reduced (Espinosa et al., 2019).

The concentration of cellulose in the diet based on corn, SBM, and DDGS was greater than in the diet based on corn and SBM, which is in agreement with reported data (Patience and Petry, 2019), and the values for cellulose in the diet containing corn, SBM, and DDGS are in agreement with the values reported by Jaworski et al. (2015) for corn DDGS, but lower than the values reported by Pedersen et al. (2014). Differences in the concentration of cellulose may be due to differences in nutrient concentration among different sources of DDGS, because DDGS is produced by different plants with different conditions of operation (Luthria et al., 2012; Pedersen et al., 2015a).

Concentration of lignin was greater in the diet containing corn, SBM, and DDGS than in the corn and SBM diet. The concentration of lignin in the diet based on corn, SBM, and DDGS is in agreement with values reported for DDGS (Pedersen et al., 2014; Jaworski et al., 2015;

Navarro et al., 2018). Variation in the analysis of lignin is also possible because of the complexity of the molecule, and possibly because the presence of other non-lignin components like protein and hemicelluloses can contaminate the determination of lignin during analysis (Fahey et al., 2019). Lignin analysis are challenging and there is a lack of consensus about which procedure results in the most accurate results (Hatfield and Fukushima, 2005; Lupoi et al., 2015).

Total non-starch polysaccharides, IDF, and dietary fiber were greater in the corn, SBM, and DDGS diet than in the corn and SBM diet, which has also been reported (Céspedes, 2015; Jaworski et al., 2015). Similarly, values for IDF, SDF, and TDF analyzed using the Ankom^{TDF} Dietary Fiber Analyzer were greater in the diet based on corn, SBM, and DDGS than in the diet based on corn and SBM. This is likely because NSP in the distilled grain are concentrated during processing because starch and dextrose are converted to ethanol (Liu, 2012).

No differences in crude protein or ash were observed between the feces from the pigs fed either the corn and SBM diet or the corn, SBM, and DDGS diet (Table 3.3). Acid hydrolyzed ether extract was greater in the feces from pigs fed the diet based on corn and SBM than in the feces from pigs fed the diet based on corn, SBM, and DDGS. The reason for this observation likely is that the volume of feces voided by pigs was greater when the corn, SBM, and DDGS diet was feed compared with the corn and SBM diet. The indigested fat was, therefore, diluted by the greater volume.

No differences in the concentration of NCP in feces from pigs fed the 2 diets were observed, and the concentration of I-NCP and NSP was also not different. However, the concentration of mannose in S-NCP and in I-NCP was greater ($P < 0.05$) in feces from pigs fed the corn and SBM diet than in feces from pigs fed the corn, SBM, and DDGS diet. Mannose concentration was higher in the feces from pigs fed the corn and SBM diet because the inclusion

of SBM in that diet is greater than in the corn, SBM, and DDGS diet. Soybean meal contains β-mannan, which is a polymer made from mannose and most of the mannan in SBM is located in the NSP fraction (Hsiao et al., 2006). There was also a tendency (P = 0.056) for the concentration of cellulose to be greater in feces from pigs fed the corn and SBM diet. However, no difference in lignin concentration was observed in feces from pigs fed the two diets.

Values for IDF and SDF calculated from the sum of the individual sugars differed from the values obtained from the Ankom^{TDF} Dietary Fiber Analyzer, which has also been reported for DDGS by Céspedes (2015). These differences may be a result of the two procedures not capturing all fiber components in the samples (Céspedes, 2015; Fahey et al., 2019). The observation that the sum of analyzed CP, ash, fat and TDF was less than 100% for both procedures demonstrates that there are components in the samples that were not accounted for in the analysis. However, the fact that if fiber was quantified using the Ankom procedure rather than the NSP and lignin analysis, the sum of all analyzed components was closer to 100 than if the NSP and lignin analysis were used indicates that the Ankom procedure captures a greater part of the total fiber compounds.

Digestibility of I-NCP, arabinose, xylose, galactose, and cellulose was not different between pigs fed the corn and SBM diet or the corn, SBM, and DDGS diet (Table 3.4). In contrast, the digestibility of mannose, glucose, and uronic acids was greater ($P < 0.05$) from pigs fed the corn, SBM, and DDGS diet. Digestibility of these sugars may be greater than other sugars like arabinose or xylose because they may be free sugars that are not linked to a big molecule such as arabinoxylan. Digestibility of total NSP and dietary fiber in the corn and SBM diet was greater than in the corn, SBM, and DDGS diet, which demonstrates that greater content of NSP and dietary fiber in the diet reduces digestibility. This observation also concurs with the

greater values for NSP and dietary fiber analyzed in the corn, SBM, and DDGS diet than in the corn and SBM diet. Digestibility of SDF, IDF, and TDF using the Ankom procedure was not different between the corn and SBM diet or the corn, SBM, and DDGS diet.

Analysis of NSP in the diets and in the feces from pigs provided indications about the individual sugars that pigs cannot digest or ferment. This knowledge may aid in the understanding of which enzymes are needed to hydrolyze fiber in pig diets because the greater concentration of fiber in feces appears to be primarily a result of greater amounts of arabinoxylan and cellulose. Development of enzymes needed to improve fermentability of fiber in diets fed to pigs should, therefore focus on enzymes that can hydrolyze cellulose and arabinoxylan.

CONCLUSION

Concentration of total NSP in the diet containing corn, SBM, and DDGS was greater than in the diet based on corn and SBM, but concentration of total NSP in the feces from pigs fed these diets was not different. The concentration of xylose, arabinose, and cellulose was predominant in both diets and feces from the pigs fed the two diets. However, the digestibility of total NSP in the diet based on corn and SBM was greater than the digestibility from the diet based on corn, SBM, and DDGS indicating that pigs may better digest the NSP from a diet based on corn and SBM than a diet based on corn, SBM, and DDGS. Therefore, inclusion of exogenous cellobiohydrolases, endo-xylanases, α arabinofuranosidases, and feruloyl esterases, which may hydrolyze the arabinose and xylose from arabinoxylan as well as cellulose from corn and DDGS may improve digestibility of nutrients in pigs.

TABLES

Table 3.1 Compositions of experimental diets

Ingredients, %	Diets	
	Corn-soybean meal	Corn-soybean meal-distiller dried grains with solubles
Ground corn	63.82	46.17
Distiller dried grains with solubles	-	40.00
Soybean meal	33.00	10.00
Soybean oil	1.00	1.00
Ground limestone	1.30	1.50
Dicalcium phosphate	0.30	0.15
Salt	0.40	0.40
Vitamin mineral premix[1]	0.15	0.15
L-Lysine, HCl, 78 % Lys	-	0.52
L-Threonine	-	0.05
L-Tryptophan	-	0.03
Phytase premix[2]	0.03	0.03
Total	100.00	100.00

[1]The vitamin-micromineral premix provided the following quantities of vitamins and microminerals per kg of complete diet: vitamin A as retinyl acetate, 11,150 IU; vitamin D_3 as cholecalciferol, 2,210 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate,

Table 3.1. (Cont.)

1.10 mg; riboflavin,6.59 mg; pyridoxine as pyridoxine hydrochloride, 1.00 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.6 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate; Fe, 125 mg as ironsulfate; I, 1.26mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganoussulfate; Se, 0.30mg as sodium selenite and selenium yeast; and Zn, 125.1mg as zinc sulfate.

[2]The phytase premix (Optiphos 2,000, Huvepharma, Sofia, Bulgaria) contained 2,000 phytase units per g. At 0.03% inclusion, the premix provided 600 units of phytase per kg in the complete diet.

Table 3. 2 Dry matter and analyzed nutrient composition of experimental diets, dry matter basis (DM)[1]

Item, %	Corn-soybean meal	Corn-soybean meal-distiller dried grains with solubles
DM	87.84	87.69
Crude protein	20.74	19.46
Acid hydrolyzed ether extract	3.24	5.11
Ash	3.54	4.29
S-NCP[2]	0.30	1.99
Arabinose	N.D	0.36
Xylose	N.D.	0.39
Mannose	0.21	0.23
Galactose	0.09	0.19
Glucose	N.D.	0.57
Uronic Acids	N.D.	0.25
I-NCP[3]	9.70	8.37
Arabinose	2.05	1.90

Table 3.2. (Cont.)

Xylose	1.85	2.78
Mannose	0.26	0.51
Galactose	2.13	0.92
Glucose	1.37	0.75
Uronic acids	2.05	1.50
Cellulose	1.41	2.58
Total NSP[4]	11.41	12.94
Lignin	1.55	2.29
SDF [5]	0.30	1.99
IDF[6]	12.66	15.82
Dietary fiber[7]	13.24	17.81
SDF Ankom[8]	0.46	1.71
IDF Ankom[9]	12.64	25.09
TDF Ankom[10]	13.09	26.80

[1]Total rhamnose and fucose in all ingredients ranged from non-detected (N.D.) to 0.1% and thus were excluded from the table.

[2]S-NCP = soluble non-cellulosic polysaccharides.

Table 3.2. (Cont.)

[3]I-NCP = insoluble non-cellulosic polysaccharides.

[4]Total NSP = total non-starch polysaccharides

[5]SDF = soluble dietary fiber

[6]IDF = insoluble dietary fiber

[7]Dietary fiber = soluble dietary fiber + insoluble dietary fiber.

[8]Soluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[9] Insoluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[10] Total dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

Table 3. 3 Dry matter and analyzed nutrient composition of feces from pigs fed a diet based on corn and soybean meal or corn, soybean meal, and distillers dried grains with solubles (DDGS), dry matter basis [1]

Item, %	Corn-soybean meal diet	Corn-soybean meal-distiller dried grains with solubles diet	SEM	*P*-Value
Dry matter	94.84	94.90	0.72	0.020
Crude protein	24.45	21.74	0.70	0.275
Acid hydrolyzed ether extract	18.19	10.11	0.63	0.022
Ash	13.66	11.53	0.34	0.801
S-NCP[2]	1.62	1.34	0.75	0.716
Arabinose	0.17	0.08	0.14	0.424
Xylose	0.17	0.03	0.28	0.159
Mannose	0.14	0.15	0.03	0.057
Galactose	0.26	0.20	0.06	0.850
Glucose	N.D.	0.08	0.28	0.867
Uronic Acids	0.92	0.81	0.32	0.630

Table 3.3. (Cont.)

I-NCP[3]	17.54	17.92	0.86	0.387
Arabinose	4.57	4.42	0.22	0.462
Xylose	9.22	9.61	0.46	0.446
Mannose	0.16	0.06	0.02	0.037
Galactose	1.85	1.58	0.10	0.101
Glucose	1.42	0.76	0.40	0.987
Uronic Acids	1.42	1.71	0.18	0.401
Cellulose	8.33	7.07	0.54	0.056
Total NSP[4]	26.02	25.97	1.40	0.459
Lignin	3.61	3.45	0.27	0.530
SDF [5]	1.62	1.34	0.75	0.716
IDF[6]	28.01	28.07	1.31	0.700
Dietary fiber[7]	29.63	29.42	1.38	0.484
SDF Ankom[8]	3.46	2.87	0.49	0.808
IDF Ankom[9]	35.46	47.43	0.93	0.124

Table 3.3. (Cont.)

TDF Ankom[10]	38.92	49.93	1.12	0.293
Sum NSP[11]	82.16	69.03	1.32	0.579
Sum TDF[12]	95.06	92.99	1.22	0.583

[1] Data are least squares means of 12 observations per diet.

[2]S-NCP = soluble non-cellulosic polysaccharides.

[3]I-NCP = insoluble non-cellulosic polysaccharides.

[4]Total NSP = total non-starch polysaccharides.

[5]SDF = soluble dietary fiber.

[6]IDF = insoluble dietary fiber.

[7]Dietary fiber = soluble dietary fiber + insoluble dietary fiber.

[8]Soluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[9] Insoluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[10] Total dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[11] Sum1 = Sum of ash, CP, AEE, and NSP.

[12] Sum2 = Sum of ash, CP, AEE, and TDF.

Table 3.4 Digestibility of nutrients in feces from pigs fed a diet based on corn and soybean meal or corn, soybean meal, and distiller's dried grains with solubles (DDGS), dry matter basis (DM)[1]

Item, %	Feces			
	Corn-soybean meal diet	Corn-soybean meal-distiller dried grains with solubles diet	SEM	*P*-Value
I-NCP[3]	82.56	66.99	2.06	0.340
Arabinose	80.13	64.20	2.00	0.185
Xylose	55.65	46.73	3.49	0.119
Mannose	92.35	98.45	1.34	<0.001
Galactose	92.30	73.91	1.19	0.868
Glucose	88.52	91.54	7.49	0.007
Uronic Acids	93.83	88.91	1.27	0.031
Cellulose	65.57	58.12	3.30	0.310
Total NSP[4]	77.33	67.58	1.86	0.037

Table 3.4. (Cont.)

IDF[6]	78.54	67.02	1.87	0.374
Dietary fiber[7]	77.58	69.02	1.71	0.010
SDF Ankom[8]	35.64	74.34	5.70	0.563
IDF Ankom[9]	74.32	71.38	1.72	0.279
TDF Ankom[10]	72.94	71.78	0.985	0.635

[1] Data are least squares means of 12 observations per diet.

[2] S-NCP = soluble non-cellulosic polysaccharides.

[3] I-NCP = insoluble non-cellulosic polysaccharides.

[4] Total NSP = total non-starch polysaccharides.

[5] SDF = soluble dietary fiber.

[6] IDF = insoluble dietary fiber.

[7] Dietary fiber = soluble dietary fiber + insoluble dietary fiber.

[8] Soluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[9] Insoluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[10] Total dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

LITERATURE CITED

Abelilla, J. J., and H. H. Stein. 2019. Degradation of dietary fiber in the stomach, small intestine, and large intestine of growing pigs fed corn- or wheat-based diets without or with microbial xylanase. J. Anim. Sci. 97:338-352. doi:10.1093/jas/sky403

Alyassin, M., and G. M. Campbell. 2019. Challenges and constraints in analysis of oligosaccharides and other fibre components. In: G. Gonzalez-Ortiz, M. R. Bedford, K. E. B. Knudsen, C. M. Courtin, and H. L. Classen, editors, The value of fibre engaging the second brain for animal nutrition. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 257-272.

AOAC Int. 2007. Official methods of analysis of AOAC international. 18th ed. AOAC international, Gaithersburg, MD.

Bach Knudsen, K. E. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67:319-338. doi:10.1016/S0377-8401(97)00009-6

Bach Knudsen, K. E. 2011. Triennial growth symposium: effects of polymeric carbohydrates on growth and development in pigs. J. Anim. Sci. 89:1965-1980. doi:10.2527/jas.2010-3602

Bach Knudsen, K. E., H. N. Lærke, and H. Jørgensen. 2013. Carbohydrates and carbohydrate utilization in Swine. In: L. I. Chiba, editor, Sustainable Swine Nutrition. John Wiley & Sons, Inc., Ames, IA, United States. p. 109-137.

Céspedes, N. A. G. 2015. Quantification of digestive utilization of dietary fiber from corn co-products in growing pigs. Doctoral disertation, Iowa, United States.

Choct, M. 2015. Feed non-starch polysaccharides for monogastric animals: classification and function. Anim. Prod. Sci. 55:1360-1366. doi:10.1071/AN15276

Connors, K. A., and N. K. Pandit. 1978. N-Methylimidazole as a catalyst for analytical acetylations of hydroxy compounds. Anal. Chem. 50:1542-1545. doi:10.1021/ac50033a038

Espinosa, C. D., S. A. Lee, and H. H. Stein. 2019. Digestibility of amino acids, energy, acid hydrolyzed ether extract, and neutral detergent fiber, and concentration of digestible and metabolizable energy in low-oil distillers dried grains with solubles fed to growing pigs. Transl. Anim. Sci. 3:662-675. doi:10.1093/tas/txz025

Fahey, G. C., L. Novotny, B. Layton, and D. R. Mertens. 2019. Critical factors in determining fiber content of feeds and foods and their ingredients. J. AOAC Int. 102:52-62. doi:10.5740/jaoacint.18-0067

Hatfield, R., and R. S. Fukushima. 2005. Can lignin be accurately measured? Crop. Sci. 45:832-839. doi:10.2135/cropsci2004.0238

Hsiao, H. Y., D. M. Anderson, and N. M. Dale. 2006. Levels of β-Mannan in soybean meal. Poult. Sci. 85:1430-1432. doi:10.1093/ps/85.8.1430

Jaworski, N. W., H. N. Lærke, K. E. Bach Knudsen, and H. H. Stein. 2015. Carbohydrate composition and in vitro digestibility of dry matter and nonstarch polysaccharides in corn, sorghum, and wheat and coproducts from these grains. J. Anim. Sci. 93:1103-1113. doi:10.2527/jas.2014-8147

Jaworski, N. W., and H. H. Stein. 2017. Disappearance of nutrients and energy in the stomach and small intestine, cecum, and colon of pigs fed corn-soybean meal diets containing distillers dried grains with solubles, wheat middlings, or soybean hulls. J. Anim. Sci. 95:727-739. doi:10.2527/jas.2016.0752

Karr-Lilienthal, L. K., C. T. Kadzere, C. M. Grieshop, and G. C. Fahey. 2005. Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: A review. Livest. Prod. Sci. 97:1-12. doi:10.1016/j.livprodsci.2005.01.015

Knudsen, K. E. B., and C. Vangsøe. 2019. Fibre- how and which structures can be modified by enzymes. In: C. G. Ortiz, M. R. Bedford, K. E. B. Knudsen, C. M. Courtin, and H. L. Classen, editors, The value of fibre engaging the second brain for animal nutrition. Wageningen Academic Publishers, The Netherlands. p. 85-95.

Kong, C., and O. Adeola. 2014. Evaluation of amino acid and energy utilization in feedstuff for swine and poultry diets. Asian Australas. J. Anim. Sci. 27:917-925. doi:10.5713/ajas.2014.r.02

Liu, K. 2012. Chemical composition of DDGS. In: K. Liu, and K. A. Rosentrater, editors, Distillers grains production, properties, and utilization. CRC Press Taylor & Francis group, Boca Raton, FL, United States of America. p. 143-178.

Lupoi, J. S., S. Singh, R. Parthasarathi, B. A. Simmons, and R. J. Henry. 2015. Recent innovations in analytical methods for the qualitative and quantitative assessment of lignin. Renew. Sust. Energ. Rev. 49:871-906. doi:10.1016/j.rser.2015.04.091

Luthria, D. L., K. Liu, and A. A. Memon. 2012. Phenolic acids and antioxidant capacity of distillers dried grains with solubles (DDGS) as compared with corn. J. Am. Oil. Chem. Soc. 89:1297-1304. doi:10.1007/s11746-012-2025-y

Maharjan, P., M. Mayorga, K. Hilton, J. Weil, A. Beitia, J. Caldas, J. England, and C. Coon. 2019. Non-cellulosic polysaccharide content in feed ingredients and ileal and total tract non-cellulosic polysaccharide digestibility in 21 and 42 day old broilers fed diets with and without added composite enzymes. Poult. Sci. 98:4048-4057. doi:10.3382/ps/pez079

Mnich, E., N. Bjarnholt, A. Eudes, J. Harholt, C. Holland, B. Jørgensen, F. H. Larsen, M. Liu, R. Manat, A. S. Meyer, J. D. Mikkelsen, M. S. Motawia, J. Muschiol, B. L. Møller, S. R. Møller, A. Perzon, B. L. Petersen, J. L. Ravn, and P. Ulvskov. 2020. Phenolic cross-links: building and de-constructing the plant cell wall. Nat. Prod. Rep. 37:919-961 10.1039/C9NP00028C. doi:10.1039/C9NP00028C

Navarro, D. M. D. L., E. M. A. M. Bruininx, L. de Jong, and H. H. Stein. 2018. Analysis for low-molecular-weight carbohydrates is needed to account for all energy-contributing nutrients in some feed ingredients, but physical characteristics do not predict in vitro digestibility of dry matter. J. Anim. Sci. 96:532-544. doi:10.1093/jas/sky010

NRC. 2012. Nutrient requierements of swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Patience, J. F., and A. L. Petry. 2019. Fibre, carbohydrases and performance in swine. In: C. G. Ortiz, M. R. Bedford, K. E. B. Knudsen, C. M. Courtin, and H. L. Classen, editors, The value of fibre engaging the second brain for animal nutrition. Wageningen Academic Publishers, The Netherlands. p. 99-112.

Pedersen, M. B., M. Bunzel, J. Schäfer, K. E. B. Knudsen, J. F. Sørensen, S. Yu, and H. N. Lærke. 2015a. Ferulic acid dehydrodimer and dehydrotrimer profiles of distillers dried grains with solubles from different cereal species. J. Agric. Food. Chem. 63:2006-2012. doi:10.1021/jf505150g

Pedersen, M. B., S. Dalsgaard, S. Arent, R. Lorentsen, K. E. B. Knudsen, S. Yu, and H. N. Lærke. 2015b. Xylanase and protease increase solubilization of non-starch polysaccharides and nutrient release of corn- and wheat distillers dried grains with solubles. Biochem. Eng. J. 98:99-106. doi:10.1016/j.bej.2015.02.036

Pedersen, M. B., S. Dalsgaard, K. E. B. Knudsen, S. Yu, and H. N. Lærke. 2014. Compositional profile and variation of distillers dried grains with solubles from various origins with focus on non-starch polysaccharides. Anim. Feed Sci. Technol. 197:130-141. doi:10.1016/j.anifeedsci.2014.07.011

Scott, R. W. 1979. Colorimetric determination of hexuronic acids in plant materials. Anal. Chem. 51:936-941. doi:10.1021/ac50043a036

Urriola, P. E., G. C. Shurson, and H. H. Stein. 2010. Digestibility of dietary fiber in distillers coproducts fed to growing pigs. J. Anim. Sci. 88:2373-2381. doi:10.2527/jas.2009-2227

Vangsøe, C. T., E. Bonnin, M. Joseph-Aime, L. Saulnier, V. Neugnot-Roux, and K. E. Bach Knudsen. 2020. Improving the digestibility of cereal fractions of wheat, maize, and rice by a carbohydrase complex rich in xylanases and arabinofuranosidases: an in vitro digestion study. J. Sci. Food Agric. doi:10.1002/jsfa.10806

CHAPTER 4. FERULIC AND COUMARIC ACID IN DIETS BASED ON CORN AND SOYBEAN MEAL OR CORN, SOYBEAN MEAL, AND DISTILLER DRIED GRAINS WITH SOLUBLES AND IN FECES FROM PIGS FED THESE DIETS

ABSTRACT

An experiment was conducted to quantify the ferulic and coumaric acid in a diet based on corn and soybean meal (**SBM**) and in a diet based on corn, SBM, and distillers dried grains with solubles (**DDGS**), as well as in feces from pigs fed these diets. A total of 24 pigs (initial body weight: 61.71 ± 5.39 kg) were allotted to the diets in a randomized complete block design with 4 blocks, and 3 replicate pigs per diet in each block. Therefore, there were 12 replicate pigs per diet. Diets were fed to pigs for 22 d. During the initial 12 d diets were provided on an *ad libitum* basis, but feed was restricted to 3.2 times the maintenance requirement for metabolizable energy from d 13 to 22; collection of feces started on d 17 and ended on d 22. The concentration of ferulic acid and coumaric acid was analyzed using reversed phase ultrahigh-performance liquid chromatography photodiode array with in-line electrospray ionization mass spectrometry. Diferulic acid (**DFA**) and triferulic acid (**TriFA**) were detected using the same method. Results indicated that the concentration of bound ferulic and coumaric acid in diets was greater in the corn, SBM, and DDGS diet and in feces from pigs fed this diet than in the diet containing corn and SBM, and feces from pigs fed this diet. Concentration of diferulic acid was greater than triferulic acid in both diets and feces. Disappearance of free coumaric acid and bound ferulic acid in the intestinal tract of pigs was not different between the 2 diets. In contrast, disappearance of bound coumaric acid was greater ($P < 0.05$) in the corn and SBM diet than in the corn, SBM, and DDGS diet. In conclusion, a diet based on corn and SBM and feces from pigs fed this diet,

contain less phenolic acids than a diet based on corn, SBM, and DDGS, and feces from pigs fed this diet. However, bound ferulic acid appears to be poorly fermented in the intestinal tract of pigs, whereas some coumaric acid is fermented.

Key words: arabinoxylan, corn, coumaric acid, distiller dried grains with solubles, ferulic acid, pigs.

INTRODUCTION

Arabinoxylan is the main fiber component in corn and corn co-products (Jaworski et al., 2015). Arabinoxylan has a β-(1-4) xylose backbone that contains side chains of arabinose, galactose, and glucuronic acid, and some of the arabinose units in the side chain maybe linked to ferulic acid or coumaric acid via ester bonds (Stein, 2019). Ferulic acid in corn is esterified to the C (O) 5- hydroxy group of arabinose residues (Appeldoorn et al., 2010; Hamaker et al., 2019; Mnich et al., 2020), and coumaric acid is esterified through covalent bonds (Hamaker et al., 2019).

Coumaric acid and ferulic acid are phenolic acids, which are compounds with one or more aromatic rings and one or more hydroxyl groups in the structure (Liu, 2013). In corn, arabinoxylan is located in the endosperm and bran cell walls. Arabinoxylan from the endosperm has more branches than arabinoxylan from the bran, but contains less ferulic acid compared with corn bran (Yadav et al., 2007; Hamaker et al., 2019). Lignin and phenolic acids are related because during synthesis of lignin, phenolic radicals in lignin can merged with other phenolic acids, including ferulate dehydrodimers, and lignin may also be acylated with p-coumarate (Mnich et al., 2020).

Phenolic acids in corn bran are composed of ferulic acid (4-hydroxy-3-methoxycinnamic acid), smaller amounts of p-coumaric acid (4-hydroxy-cinnamic), diferulic acids (**DFA**), and triferulic acids (**TriFA**) that are covalently bound to cell wall arabinoxylan (Yadav et al., 2007; Appeldoorn et al., 2010; Gálvez et al., 2017). Esterified ferulic acid can form ferulate dimers, trimers, and tetramers due to the ability to form crosslinks by oxidative coupling, which obstructs enzymatic degradation of the cell wall (Mnich et al., 2020).

Because of the resistance to enzymatic degradation of arabinoxylan, which is linked to ferulic acid or coumaric acid, it is likely that the majority of ferulic acid and coumaric acid is excreted in feces of pigs fed diets containing phenolic acids linked to arabinoxylan. However data to confirm this have not been reported. The objective of this experiment, therefore, was to quantify the ferulic and coumaric acid in a diet based on corn and soybean meal (**SBM**), and in a diet based on corn, SBM, and DDGS, as well as in feces from pigs fed these diets. The hypothesis that the concentration of coumaric acid and ferulic acid is greater in the corn, SBM, and DDGS diet than in the corn-SBM diet, as well as in feces from pigs fed the corn, SBM, and DDGS diet compared with feces from pigs fed the corn-SBM diet was tested.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

The institutional Animal Care and Use Committee at the University of Illinois reviewed and approved the protocol for the animal part of the experiment. Pigs used in the experiment were the offspring of L 359 boars mated to Camborough females (PIC, Hendersonville, TN).

A diet based on corn and SBM, and a diet based on corn, SBM, and DDGS (Archer Daniels Midland Company, Decatur, IL) were used (Table 4.1). Twenty-four pigs (initial body

weight: 61.71 ± 5.39 kg), were allotted to a randomized complete block design with 2 diets, 4 blocks of 6 pigs, and 3 pigs per diet in each block. Therefore, there were 12 replicate pigs per diet, and a total of 24 fecal samples were collected.

The 24 pigs were housed in individual pens equipped with slatted floors, a self-feeder, and a nipple waterer. During the initial 12 d diets were provided on an *ad libitum* basis. On d 13, pigs were moved to individual metabolism crates and were adapted to the crates for 4 d (d 13 to 16). Pigs were restrictedly fed during the time they were housed in the metabolism crates and each day, pigs received an amount of feed that was equal to 3.2 times the maintenance energy requirement (i.e., 197 kcal ME per kg $BW^{0.60}$; NRC, 2012), which was provided in 2 equal meals at 0800 and 1700 h. On d 17 in the morning, a color marker was included in the meal fed to each pig and fecal collections started as soon as the marker appeared in the feces (Kong and Adeola, 2014). On d 22 in the morning, a second color marker was fed and fecal collections ceased when the second marker appeared in the feces. Throughout the study, pigs had ad libitum access to water. Fecal samples were thawed and mixed within pig and diet, and then dried in a 65°C forced air drying oven (Model 8, Metalab, Equipment Corp., Hicksville, NY) prior to analysis.

Chemical Analysis

Fecal and diet samples were analyzed in duplicate for dry matter (**DM**); method 930.1; (AOAC Int., 2007), and for nitrogen using a combustion procedure according to method 990.03 (AOAC Int., 2007) on a LECO FP628 (LECO Corp., Saint Joseph, MI). Crude protein was calculated as Nitrogen × 6.25. Samples were also analyzed for soluble dietary fiber and insoluble dietary fiber according to method 991.43 (AOAC Int., 2007) using the AnkomTDF Dietary Fiber Analyzer (Ankom Technology, Macedon, NY).

Extraction of free and bound ferulic acid and coumaric acid. Extraction and quantification of the phenolic acids were performed in triplicate. Approximately 100 mg of sample was weighed and suspended in 10 mL of 0.5*M* KOH solution to extract the total (free and bound) ferulic and coumaric acid. Approximately 100 mg of sample was weighed and suspended in 10 mL of 50mM sodium acetate buffer (pH 5.7) to extract the bound ferulic and coumaric acids. Both suspensions were incubated for 20 h at 37°C, and then centrifuged (5,000G) for 20 min at 4°C. Afterwards, supernatants containing KOH were diluted 5 times in purified and deionized water. Supernatants containing sodium acetate buffer were not diluted.

Reversed phase ultrahigh-performance liquid chromatography photodiode array with in-line electrospray ionization mass spectrometry (RP-UHPLC-PDA-ESI-MS) analysis. After extraction, ferulic and coumaric acids were analyzed using a Vanquish UHPLC system (Thermo Scientific, Waltham, MA), equipped with a pump, degasser, auto-sampler, and photodiode array detector. An Acquity UPLC BEH C18 column (150 × 2.1 mm, particle size 1.7 µm) with a VanGuard column of the same material (Waters, Milford, MA) was used. Water (A) and acetonitrile (B) were used as eluents, both acidified with 0.1% formic acid.

Mass spectrometry was performed on a LTQ Velos Pro mass spectrometer (Thermo Scientific, Waltham, MA) coupled to the UHPLC and equipped with an electrospray ionization (ESI) probe. Standard curves (1-50 µg/mL) for quantification of coumaric and ferulic acids were constructed by using pure ferulic acid and *p*-coumaric acid standards. Free and bound coumaric and ferulic acid fractions were quantified. The free ferulic and coumaric acid fractions were calculated by subtracting the bound fraction from the concentration of total ferulic and coumaric acids.

Diferulic acid and TriFA were identified (Table 4.2), but not quantified due to the lack of available standards. No molar response factors are known for these compounds, but their abundance is expressed as peak area per milligram of sample.

Calculations and Statistical Analysis

Disappearance of ferulic and coumaric acid were calculated using equation 8 (Jaworski et al., 2015):

$$Disapearance\ (\%) = \frac{Phenolic\ acid\ intake - Phenolic\ acid\ in\ feces}{Phenolic\ acid\ intake} * 100 \quad [8]$$

The normality of residuals and assumptions of the model were tested using the UNIVARIATE procedure of SAS (SAS Inst. Inc., Cary, NC). Data were analyzed using the PROC MIXED of SAS with the pig as the experimental unit. The model included diet as the fixed effect, whereas block and pig within block were considered random effects. Treatment means were calculated using the LSMEANS statement and means were separated using the PDIFF option of SAS. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

RESULTS AND DISCUSSION

The main phenolic compounds in cereal grains like corn are present in bound and free forms. The diet based on corn and SBM contained 0.03 µg/mg free coumaric acid, whereas the diet based on corn, SBM, and DDGS contained 0.04 µg/mg (Table 4.3). Free phenolic acids are rapidly absorbed in the small intestine (Das and Singh, 2016), but free ferulic acid was not detected in any of the diets. Concentration of bound coumaric acid in the diet based on corn and SBM was 0.17 µg/mg, whereas the diet based on Corn, SBM, and DDGS contained 0.41 µg/mg.

Greater values for bound phenolic acids than free phenolic acids were expected for both corn and DDGS (Kandil et al., 2012; Luthria et al., 2014; Siyuan et al., 2018). Bound ferulic acid were analyzed in greater concentrations than free and bound coumaric acid in both diets, which is in agreement with previous data (Siyuan et al., 2018; Hamaker et al., 2019).

The bound coumaric acid concentration in the diet containing corn and SBM was in agreement with values reported for sweet corn, but lower than values reported in popcorn (Das and Singh, 2016). Values for bound ferulic acid in the diet containing corn and SBM was also in agreement with values reported by Das and Singh (2016) for sweet corn, but greater than in popcorn, baby corn, and quality protein maize (Das and Singh, 2016). Bound phenolic acids, in contrast with the free phenolic acids are not absorbed in the small intestine, and proceed to the cecum and colon, where they may be released by action of microorganisms, which may result in certain health benefits (Das and Singh, 2016; Gálvez et al., 2017). As an example, ferulic acid, has antioxidant and bioactive properties, which may be associated with health benefits (Butts-Wilmsmeyer and Bohn, 2016). However, absorbed phenolic acids, which are not utilized, are excreted in the urine.

Values for bound ferulic acid in the corn, SBM, and DDGS diet were 3.4 times greater than in the diet containing corn and SBM, which concurs with published data (Luthria et al., 2014; Pedersen et al., 2015). Greater values for ferulic and coumaric acid in the diet containing corn, SBM, and DDGS compared with the diet containing corn and SBM is a result of the increased concentration of fiber in DDGS compared with corn. During the ethanol production process, corn undergoes grinding, cooking, liquefaction, saccharification, fermentation, and distillation. The concentration of phenolic acid increases during fermentation because of the conversion of starch into ethanol, and phenolic acids are further concentrated in the whole

stillage because of removal of ethanol (Luthria et al., 2014). Phenolic acids in DDGS may vary among sources and processing plants due to differences in the starting material and processing conditions (Luthria et al., 2012; Pedersen et al., 2015), but concentrations of total coumaric and ferulic acid in the DDGS diet used in this experiment are in agreement with values reported for DDGS by Luthria et al. (2012).

Values of bound DFA and TriFA in diets were summarized as peak area per milligram of sample, and the presence of DFA was greater than TriFA in both diets. Greater values for DFA than TriFA in corn and DDGS have been reported (Ayala-Soto et al., 2014; Pedersen et al., 2015). A tentative characterization of the DFA and TriFA indicated the presence of 8-8'-(furan)-diferulic acid, 8-8'-(aryl)-diferulic acid, 8-5'-diferulic acid, 5-5'-diferulic acid, and 8-O-4'-diferulic acid. The DFA and TriFA tentatively identified in diets used in this study are in agreement with the profile reported by Pedersen et al. (2015) for DDGS samples.

To our knowledge, no data for the concentration of phenolic acid in feces have been published. However, total (free and bound) coumaric acid concentrations in feces from pigs fed the diet based on corn, SBM, and DDGS was greater ($P < 0.05$) than in feces from pigs fed the diet based on corn and SBM (Table 4.4). The concentration of bound coumaric acid in feces from pigs fed the diet based on corn, SBM, and DDGS was also greater ($P < 0.05$) than in feces from pigs fed the diet based on corn and SBM. There were no differences in the concentration of free ferulic acid in feces from pigs fed the corn and SBM diet compared with the feces from pigs fed the diet based on corn, DDGS, and SBM. However, the concentration of bound ferulic acid in feces from pigs fed the corn, SBM, and DDGS diet was greater ($P < 0.05$) than in feces from pigs fed the diet containing corn and SBM. The concentration of DFA and TriFA in feces from

pigs fed the diet based on corn, SBM, and DDGS was not different from that in feces from pigs fed the corn and SBM diet.

Ferulic acid and coumaric acid are bound via ester bonds to arabinose in the side chains of the arabinoxylan molecule (Appeldoorn et al., 2010; Hamaker et al., 2019; Mnich et al., 2020). The concentration of arabinose is greater in DDGS than in corn, and therefore, it was expected that the concentration of bound ferulic and coumaric acid in the diet based on corn, SBM, and DDGS was greater than in the corn and SBM diet. The observation that the concentration of bound ferulic and coumaric acid in feces from pigs fed the corn, SBM, and DDGS diet were greater than in feces from pigs fed the corn and SBM diet demonstrates that bound ferulic or coumaric acid is poorly fermented by pigs. Moreover, arabinose units in the side chains may also be cross-linked through DFA and TriFA, which together with binding proteins and lignin, make the molecule insoluble, which prevents fermentation (Siyuan et al., 2018; Hamaker et al., 2019).

The greater concentration of ferulic and coumaric acids in feces than in diets is a result of the fact that other sugars, protein, and starch are absorbed in the small intestine, whereas bound phenolic acids that are linked to arabinoxylan are enzymatically hydrolyzed in the small intestine only to small degree and partly resist microbial fermentation in the hindgut (Das and Singh, 2016). Phenolic acids in the diet may have negative effects on the digestibility of fiber and energy because phenolic compounds have an inhibitory effect on enzymatic starch hydrolysis by α-amylase and amyloglucosidases, due to the ability of polyphenols to decrease enzyme activity by binding enzymes proteins (Kandil et al., 2012).

Diets based on corn, SBM, and DDGS have a lower digestibility of energy than diets based on corn and SBM (Urriola et al., 2010) due to the insoluble fiber in DDGS, which is

mainly composed of cellulose, hemicelluloses, and lignin. The presence of phenolic acids in the feces is an indication of the low solubility of the fiber in those diets, which may be explained by the crosslinking between arabinoxylan and lignin, which is primarily facilitated by diferulate (Bunzel et al., 2001; Mnich et al., 2020; Vangsøe et al., 2020).

The percent disappearance of bound ferulic acid was low and not different between the two diets, which confirms the low capacity of the pig to digest and ferment bound ferulic acid from complex arabinoxylan either from corn or DDGS. The reason for this observation likely is that ferulic acid is bound to lignin through covalent bonds, and to arabinose in the arabinoxylan sidechain. Because of the binding of ferulic acid to the arabinose, microbial enzymes do not have easy access to the individual sugars in corn arabinoxylan, which is the reason for the low digestibility of arabinoxylan in corn fiber. The presence of ferulic acid in arabinoxylan therefore, is a major hindrance to fermentation.

Disappearance of bound coumaric acid was greater ($P < 0.05$) in the corn and SBM diet than in the corn, SBM, and DDGS diet. However, disappearance of bound coumaric acid in both diets was less than 50% indicating that coumaric acid also is resistant to enzymatic hydrolysis, and thus, contributes to the low digestibility of arabinoxylans in corn fiber.

The very low disappearance of phenolic acids in the digestive tract of pigs clearly indicate that fractions in fiber that are linked to phenolic acids are mostly unfermentable. As a consequence if exogenous enzymes that can hydrolyze the ester bonds in corn between arabinose units and phenolic acids can be developed, increased fermentation of corn fiber may be achieved. Overall, successful use of enzymes that can hydrolyze the ester bond between arabinose and phenolic acids may result in improving digestibility of energy in diets for pigs.

CONCLUSIONS

The diet based on corn, SBM, and DDGS contained approximately 3 times as much coumaric acid and ferulic acid than the diet based on corn and SBM, but the concentration of the two phenolic acids as a percentage of total fiber was not different. Analysis of coumaric acid and ferulic acid in feces from pigs fed both diets indicated that only around one third of the bound phenolic acids are fermented in the intestinal tract of pigs indicating that coumaric and ferulic acids are major barriers to fermentation of arabinoxylans in corn fiber. As a consequence, if esterases that hydrolyze the ester bond between coumaric and ferulic acid and arabinose can be identified and included in pig diets, pigs may be able to ferment a greater portion of fiber in corn and corn co-products.

TABLES

Table 4. 1. Compositions of experimental diets

Ingredients, %	Diets	
	Corn-soybean meal	Corn-soybean meal-distiller dried grains with solubles
Ground corn	63.82	46.17
Distiller dried grains with solubles	-	40.00
Soybean meal	33.00	10.00
Soybean oil	1.00	1.00
Ground limestone	1.30	1.50
Dicalcium phosphate	0.30	0.15
Salt	0.40	0.40
Vitamin mineral premix[1]	0.15	0.15
L-Lysine, HCl, 78 % Lys	-	0.52
L-Threonine	-	0.05
L-Tryptophan	-	0.03
Phytase premix[2]	0.03	0.03
Total	100.00	100.00

[1]The vitamin-micromineral premix provided the following quantities of vitamins and microminerals per kg of complete diet: vitamin A as retinyl acetate, 11,150 IU; vitamin D_3 as cholecalciferol, 2,210 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine

Table 4.1. (Cont.)

mononitrate, 1.10 mg; riboflavin,6.59 mg; pyridoxine as pyridoxine hydrochloride, 1.00 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.6 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate; Fe, 125 mg as ironsulfate; I, 1.26mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganoussulfate; Se, 0.30mg as sodium selenite and selenium yeast; and Zn, 125.1mg as zinc sulfate.

[2]The phytase premix (Optiphos 2,000, Huvepharma, Sofia, Bulgaria) contained 2,000 phytase units per g. At 0.03% inclusion, the premix provided 600 units of phytase per kg in the complete diet.

Table 4. 2. Tentative identification of phenolic acid compounds by using reversed phase ultrahigh-performance liquid chromatography photodiode array with in-line electrospray ionization mass spectrometry

(Tentative) identification	Retention time (min)	MS^2 fragments	Reference[1,2]
p-coumaric acid	7.50	119 (100)	-
Ferulic acid	8.45	134 (100), 149 (88)	-
8-8'-(furan)-diferulic acid	9.13	193 (100), 341 (38)	(Vismeh et al., 2013)
8-8'-(aryl)-diferulic acid	9.22	341 (100)	(Vismeh et al., 2013)
8-8'-(furan)-diferulic acid	9.50	233 (100), 297 (89), 151 (44), 359 (33), 207 (28), 218 (25), 136 (24), 282 (16), 315 (16), 148 (14), 163 (14)	(Xiang et al., 2019)

Table 4.2. (Cont.)

8-8'-(furan)-diferulic acid	9.84	233 (100), 359 (67), 151 (54), 207 (33), 136 (28)	(Xiang et al., 2019)
8-5'-diferulic acid	9.66	341 (100), 297 (68)	(Vismeh et al., 2013)
5-5'-diferulic acid	11.14	341 (100), 326 (41), 282 (27)	(Vismeh et al., 2013)
8-*O*-4'-diferulic acid	12.23	193 (100), 313 (86), 341 (48), 326 (8)	(Vismeh et al., 2013)
Triferulic acid	12.67	533 (100), 355 (35), 311 (18), 489 (12)	(Xiang et al., 2019)

[1] Vismeh, R., F. Lu, S. P. Chundawat, J. F. Humpula, A. Azarpira, V. Balan, B. E. Dale, J. Ralph, and A. D. Jones. 2013. Profiling of diferulates (plant cell wall cross-linkers) using ultrahigh-performance liquid chromatography-tandem mass spectrometry. Analyst. 138:6683-6692. doi:10.1039/c3an36709f

[2] Xiang, J., M. Zhang, F. B. Apea-Bah, and T. Beta. 2019. Hydroxycinnamic acid amide (HCAA) derivatives, flavonoid C-glycosides, phenolic acids and antioxidant properties of foxtail millet. Food. Chem. 295:214-223. doi:10.1016/j.foodchem.2019.05.058

Table 4. 3. Analyzed phenolic acids in experimental diets[1]

Item	Corn-soybean meal	Corn-soybean meal-distiller dried grains with solubles
Dry matter	87.84	87.69
CP	20.74	19.46
SDF Ankom[2]	0.11	1.71
IDF Ankom[3]	15.48	25.09
TDF Ankom[4]	15.60	26.80
Free ferulic acid, µg/mg	N.D[5]	N.D
Bound ferulic acid, µg/mg	1.22	4.12
Free p-coumaric acid, µg/mg	0.03	0.04
Bound p-coumaric acid, µg/mg	0.17	0.41
Diferulic acid bound, MS Area/mg [,6,7,8]	1,170	4,287
Triferulic acid bound, MS Area/mg [6,7]	113	332
Peak area ratio (DFA+ triFA)/(FA)	2.0	2.2

Table 4.3. (Cont.)

[1]Diferulic acid (DFA) and triferulic acid (TriFA) were identified (Table 4.2), but not quantified due to the lack of available standards. No molar response factors are known for these compounds, but their abundance is expressed as peak area per milligram sample.

[2]Soluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[3]Insoluble dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[4] Total dietary fiber analyzed with Ankom procedure (Ankom Technology, Macedon, NY).

[5]N.D. = Not detected

[6]Diferulates and triferulates not detected in unsaponified samples.

[7]Expressed as extracted-ion peak areas per mg of sample ($[M\text{-}H]^-$ = 385 and 403 for diferulic acid; $[M\text{-}H]^-$ = 577 for triferulic acid)

[8]Summed area of all diferulic acids shown in Table 4.2.

Table 4. 4. Analyzed phenolic acids in feces from pigs fed the experimental diets and disappearance of phenolic acids during digestion[1]

Item	Corn-soybean meal diet	Corn-soybean meal-distiller dried grains with solubles diet	SEM	*P*-value
Dry matter, %	94.85	94.90	0.72	0.020
Free ferulic acid, µg/mg	0.05	0.13	0.01	0.439
Bound ferulic acid, µg/mg	10.08	17.86	0.81	0.033
Free p-coumaric acid, µg/mg	0.02	0.03	0.002	0.001
Bound p-coumaric acid, µg/mg	1.04	2.04	0.09	0.002
Diferulic acids, MS Area/mg [2,3,4] bound	12,005	24,546	1,403	0.563
Triferulic acid, MS Area/mg[2,3] bound	1,239	2,540	136	0.465
Peak area ratio (DFA+triFA)/(FA)	2.50	2.92	0.08	0.700
Disappearance of bound ferulic acid, %	36.30	35.35	4.12	0.695
Disappearance of free coumaric acid, %	93.95	88.04	0.91	0.125
Disappearance of bound coumaric acid, %	48.12	31.69	3.74	0.012

Table 4.4. (Cont.)

[1]Diferulic acid (DFA) and triferulic acid (TriFA) were identify (Table 4.2), but not quantify due to the lack of available standard. No molar response factors are known for these compounds, their abundance is expressed as peak area per mg sample.

[2]Diferulates and triferulates not detected in unsaponified samples.

[3]Expressed as extracted-ion peak areas per mg of sample ($[M\text{-}H]^-$ = 385 and 403 for diferulic acid; $[M\text{-}H]^-$ = 577 for triferulic acid)

[4]Summed area of all diferulic acids shown in Table 4.2.

LITERATURE CITED

AOAC Int. 2007. Official methods of analysis of AOAC International. 18th ed., 2005. ed. AOAC Int., Gaithersburg, MD.

Appeldoorn, M. M., M. A. Kabel, D. Van Eylen, H. Gruppen, and H. A. Schols. 2010. Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation. J. Agric. Food. Chem. 58:11294-11301. doi:10.1021/jf102849x

Ayala-Soto, F. E., S. O. Serna-Saldívar, S. García-Lara, and E. Pérez-Carrillo. 2014. Hydroxycinnamic acids, sugar composition and antioxidant capacity of arabinoxylans extracted from different maize fiber sources. Food. Hydrocoll. 35:471-475. doi:10.1016/j.foodhyd.2013.07.004

Bunzel, M., J. Ralph, J. M. Marita, R. D. Hatfield, and H. Steinhart. 2001. Diferulates as structural components in soluble and insoluble cereal dietary fibre. J. Sci. Food Agric. 81:653-660. doi:10.1002/jsfa.861

Butts-Wilmsmeyer, C. J., and M. O. Bohn. 2016. High-throughput extraction of insoluble-bound ferulic acid in maize. Crop. Sci. 56:3152-3159. doi:10.2135/cropsci2015.12.0763

Das, A. K., and V. Singh. 2016. Antioxidative free and bound phenolic constituents in botanical fractions of Indian specialty maize (Zea mays L.) genotypes. Food. Chem. 201:298-306. doi:10.1016/j.foodchem.2016.01.099

Gálvez, L., A. Christopher, D. Sarkar, K. Shetty, R. Chirinos, and D. Campos. 2017. Phenolic composition and evaluation of the antimicrobial activity of free and bound phenolic

fractions from a peruvian purple corn. J. Food. Sci. 82:2968-2976. doi:10.1111/1750-3841.13973

Hamaker, B. R., Y. E. Tuncil, and X. Shen. 2019. Carbohydrates of the kernel. In: S. O., and Serna-Saldivar, editors, Corn: chemistry and technology. Elsevier Inc., Cambridge, MA, United States. p. 305-318.

Jaworski, N. W., H. N. Lærke, K. E. Bach Knudsen, and H. H. Stein. 2015. Carbohydrate composition and in vitro digestibility of dry matter and nonstarch polysaccharides in corn, sorghum, and wheat and coproducts from these grains. J. Anim. Sci. 93:1103-1113. doi:10.2527/jas.2014-8147

Kandil, A., J. Li, T. Vasanthan, and D. C. Bressler. 2012. Phenolic acids in some cereal grains and their inhibitory effect on starch liquefaction and saccharification. J. Agric. Food. Chem. 60:8444-8449. doi:10.1021/jf3000482

Kong, C., and O. Adeola. 2014. Evaluation of amino acid and energy utilization in feedstuff for swine and poultry diets. Asian Australas. J. Anim. Sci. 27:917-925. doi:10.5713/ajas.2014.r.02

Liu, R. H. 2013. Dietary bioactive compounds and their health implications. J. Food. Sci. 78:A18-A25. doi:10.1111/1750-3841.12101

Luthria, D. L., K. Liu, and A. A. Memon. 2012. Phenolic acids and antioxidant capacity of distillers dried grains with solubles (DDGS) as compared with corn. J. Am. Oil. Chem. Soc. 89:1297-1304. doi:10.1007/s11746-012-2025-y

Luthria, D. L., A. A. Memon, and K. Liu. 2014. Changes in phenolic acid content during dry-grind processing of corn into ethanol and DDGS. J. Sci. Food Agric. 94:1723-1728. doi:10.1002/jsfa.6481

Mnich, E., N. Bjarnholt, A. Eudes, J. Harholt, C. Holland, B. Jørgensen, F. H. Larsen, M. Liu, R. Manat, A. S. Meyer, J. D. Mikkelsen, M. S. Motawia, J. Muschiol, B. L. Møller, S. R. Møller, A. Perzon, B. L. Petersen, J. L. Ravn, and P. Ulvskov. 2020. Phenolic cross-links: building and de-constructing the plant cell wall. Nat. Prod. Rep. 37:919-961 10.1039/C9NP00028C. doi:10.1039/C9NP00028C

NRC. 2012. Nutrient requierements of swine. 11th rev ed. Natl. Acad. Press, Washington, D.C.

Pedersen, M. B., M. Bunzel, J. Schäfer, K. E. B. Knudsen, J. F. Sørensen, S. Yu, and H. N. Lærke. 2015. Ferulic acid dehydrodimer and dehydrotrimer profiles of distillers dried grains with solubles from different cereal species. J. Agric. Food. Chem. 63:2006-2012. doi:10.1021/jf505150g

Siyuan, S., L. Tong, and R. Liu. 2018. Corn phytochemicals and their health benefits. Food. Sci. Hum. Wellness. 7:185-195. doi:10.1016/j.fshw.2018.09.003

Stein, H. H. 2019. Multi vs. single application of enzymes to degrade fibre in diets for pigs. In: G. Gonzalez-Ortiz, M. R. Bedford, K. E. B. Knudsen, C. M. Courtin, and H. L. Classen, editors, The value of fibre engaging the second brain for animal nutrition. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 117-122.

Urriola, P. E., G. C. Shurson, and H. H. Stein. 2010. Digestibility of dietary fiber in distillers coproducts fed to growing pigs. J. Anim. Sci. 88:2373-2381. doi:10.2527/jas.2009-2227

Vangsøe, C. T., N. P. Nørskov, M.-F. Devaux, E. Bonnin, and K. E. Bach Knudsen. 2020. A carbohydrase complex rich in xylanases and arabinofuranosidases affects the autofluorescence signal and liberates phenolic acids from the cell wall matrix in wheat, maize, and rice bran: An in vitro digestion study. J. Agric. Food. Chem. doi:10.1021/acs.jafc.0c00703

Yadav, M. P., R. A. Moreau, and K. B. Hicks. 2007. Phenolic acids, lipids, and proteins associated with purified corn fiber arabinoxylans. J. Agric. Food. Chem. 55:943-947. doi:10.1021/jf0624493

CHAPTER 5. IN-VITRO DISAPPEARANCE OF DRY MATTER IN DIETS WITH CORN AND SOYBEAN MEAL AND CORN, SOYBEAN MEAL, AND DISTILLERS DRIED GRAINS WITH SOLUBLES WITHOUT AND WITH INCLUSION OF ENZYMES

ABSTRACT

An in-vitro experiment was conducted to determine the disappearance of DM in the indigestible fraction of a diet based on corn and soybean meal (**SBM**) and in a diet based on corn, SBM, and distillers dried grains with solubles (**DDGS**). Both diets were digested without the inclusion of enzymes; with the inclusion of four exogenous enzymes: arabinofuranosidase, feruloyl esterase, endo-xylanase, and cellobiohydrolase; and with 10 different combinations of these enzymes. Effects of exogenous enzymes on the disappearance of DM was determined. Results indicated that inclusion of endo-xylanase in both diets tended ($P < 0.10$) to increase the disappearance of DM, whereas the disappearance of DM was not affected by the inclusion of feruloyl esterase in the diets. Reduced ($P < 0.05$) disappearance of DM was observed when arabinofuranosidase was included in the corn and SBM diet, and when cellobiohydrolase was included in the diet based on corn, SBM, and DDGS. Disappearance of DM was either not changed or was reduced ($P < 0.05$) when enzymes were used in combinations. In conclusion, endo-xylanase tended to increase the disappearance of DM, but the combinations of enzymes did not increase the disappearance of DM. Inhibitory effects between enzymes, microbial origin of the enzymes, physical characteristics of the substrate, and conditions of the medium of the essay may have contributed to the inconsistent responses to the enzymes.

Key words: arabinoxylan, corn, distiller dried grains with solubles, exogenous enzymes, in-vitro, pigs.

INTRODUCTION

Diets containing corn and distillers dried grains with solubles (**DDGS**) are high in insoluble fiber, which is mainly composed of cellulose and insoluble hemicelluloses such as arabinoxylan (Urriola et al., 2010; Jaworski et al., 2015). Pigs lack enzymes to digest these molecules, and therefore, cannot take advantage of the energy from the sugars contained in these fibers. Therefore, the use of exogenous enzymes may be an alternative to increase the digestibility of fiber (Bach Knudsen et al., 2013; Petry et al., 2020).

Cellulose is a complex structure, which requires four enzymes for complete degradation (Stein, 2019): 1) Endo-1,4-β-glucanases, which cleave internal bonds in the amorphous cellulose chain of glucose units; 2) exo-1,4-β-glucanases, also known as cellobiohydrolases, which hydrolyze the reducing or non-reducing end of the cellulose to obtain cellobiose; 3) cellodextrinases, which hydrolyze soluble cello-oligosaccharides and generate cellobiose (Duan and Feng, 2010); and 4) β-glucosidases, which hydrolyze soluble cellodextrins and cellobiose to glucose (Zhang, 2013).

The arabinoxylan molecule requires several enzymes for complete hydrolysis. β-(1-4) endoxylanases cleave the xylan backbone via hydrolysis of the β-(1-4) glycosidic bonds between xylose units (Dodd and Cann, 2009). This results in shorter chains of xylose oligosaccharides that may be cleaved by β-D-xylosidase, which hydrolyses short xylooligosaccharides from the non-reducing end to release xylose (Bedford and Schulze, 1998; Belkacemi and Hamoudi, 2003).

The enzyme α-glucuronosidase, hydrolyzes the α-(1-2) bond between glucuronic acid and xylose in the sidechain of arabinoxylan, and β-galactosidases cleave the glycosidic bond between galactose and xylose residues in the side chains. The enzyme α –arabinofuranosidase hydrolyzes the α-(1,5) glycosidic bond between 2 L-arabinose units in the side chain, and α-arabinofuranohydrolase hydrolyzes the α-(1-2) glycosidic bond between the xylose backbone and arabinose side chain units (Abelilla, 2018). Acetyl xylan esterases cleave the glycosidic bond between O-acetyl and xylose, whereas ferulic/coumaric acid esterases hydrolyze the linkage between L-arabinose and ferulic acid or coumaric acid, respectively (de Vries, 2003).

In-vitro digestibility procedures simulate the digestive process of the pig, and results from this procedure are often in agreement with values for digestibility obtained in pigs (Boisen and Fernández, 1997). An in-vitro procedure to determine the disappearance of DM was developed by Boisen and Fernández (1997) and modified by Jaworski et al. (2015). It is possible that this procedure may be used to determine the efficacy of exogenous enzymes to increase DM disappearance. The objective of this experiment, therefore, was to conduct an in-vitro experiment that included 15 treatments to estimate the disappearance of DM from a diet based on corn and soybean meal (**SBM**) and a diet based on corn, SBM, and DDGS. The hypothesis that inclusion of xylanase by itself or in combination with cellobiohydrolase, arabinofuranosidase, or feruloyl esterase increases the disappearance of DM in the two diets was tested.

MATERIALS AND METHODS

In-vitro procedure

A diet based on corn and SBM, and a diet based on corn, SBM, and DDGS (Archer Daniels Midland company, Decatur, IL) were used (Table 5.1).

The in-vitro disappearance of DM in the two diets was determined using the procedure of Jaworski et al. (2015). Samples were ground through a 0.5 mm screen, and 0.5 g of each sample was added to a conical flask. Four conical flasks were used for each treatment. Twenty five mL of phosphate buffer (0.1 *M*; pH 6.0) and 10 mL of 0.2 *M* HCl were added to each flask, and pH was adjusted to 2 using 1 *M* HCl or 1 *M* NaOH. One mL of freshly prepared pepsin solution (25 mg of pepsin/mL; P7000, Sigma Aldrich, St. Louis, MO) and 0.5 mL of chloramphenicol solution (0.5 g chloramphenicol, Sigma No. C-0378, per 100 mL ethanol) was then added to each flask to prevent bacterial growth. Samples were incubated in a water bath at 40°C for 75 min with constant stirring.

After 75 min of incubation, 10 mL of phosphate buffer (0.2 M; pH 6.8) and 5 mL of 0.6 *M* NaOH were added to each flask, and pH was adjusted to 6.8 ± 0.01 by adding 1 *M* HCl or 1 *M* NaOH. One mL of pancreatin solution (100 mg of pancreatin/mL; P1750, Sigma Aldrich, St. Louis, MO) was added to each flask and samples were incubated in a water bath with constant stirring at 40°C for 3.5 h.

After 3.5 h, 10 mL of a 0.2 *M* EDTA solution was added to each flask and the pH was adjusted to 4.8 ± 0.01 using 30% acetic acid. One mL of Viscozyme (Viscozyme, Sigma-Aldrich, St. Louis, MO) was added to each flask and flasks were incubated in a water bath with constant stirring for 18 h at 40°C. The same in-vitro procedure was repeated with the inclusion of

4 enzymes: Feruloyl esterase from *Clostridium Thermocellum*, 40 U per Kg, family CE1 (Megazyme, Ireland); endo xylanase from *Trichoderma Viride*, 20,000 U per Kg, family GH11 (Megazyme, Ireland); arabinofuranosidase from *Bifidobacterium Adolescentis*, 800 U per Kg, family GH43 (Megazyme, Ireland); and cellobiohydrolase I from *Trichoderma Longibrachiatum*, 3,750 U per Kg, family: GH7 (Megazyme, Ireland). Ten combinations of these enzymes (Table 5.2) were also included in the experiment.

At the end of incubation, the undigested residues from the samples were filtered through Gooch crucibles, which were previously weighed. Celite (0.400 g ± 5 mg) was added to the crucibles prior to filtration. The undigested material collected in the crucibles was washed with 10 mL of 96% ethanol and then with 10 mL of acetone. Crucibles were dried overnight at 102°C in an isothermal oven, cooled in a desiccator, and weighed to measure DM residues (Boisen and Fernández, 1997). Two blanks were included in the procedure. The DM of the blanks were used to correct the final DM of the residues.

Calculations and Statistical Analysis

In-vitro disappearance of DM was calculated using equation 9 (Jaworski et al., 2015):

$$\text{In vitro ATTD of DM, \%} = \frac{[\text{Sample DM} - (\text{Residue DM} - \text{Blank DM})]}{\text{sample DM}} \times 100 \quad [9]$$

The normality of residuals and assumptions of the model were tested using the UNIVARIATE procedure of SAS (SAS Inst. Inc., Cary, NC). Data were analyzed using the PROC MIXED of SAS with the conical flask as the experimental unit. The model included enzyme or enzyme combination as the fixed effect, whereas replicate was considered the random effect. Treatment means were calculated using the LSMEANS statement and means were

separated using the PDIFF option of SAS. Contrast statements were used to compare the effect of the inclusion of enzymes. Statistical significance and tendency were considered at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

RESULTS

Disappearance of DM in the diet based on corn and SBM was 92.77 (Table 5.3). Disappearance of DM in the diet based on corn and SBM was greater ($P < 0.05$) without the inclusion of arabinofuranosidase, than with the inclusion of the enzyme individually or in combination with cellobiohydrolase or endo-xylanase. In contrast, inclusion of arabinofuranosidase in combination with feruloyl esterase did not change the disappearance of DM.

Inclusion of cellobiohydrolase by itself or in combination with endo-xylanase did not affect the disappearance of DM of the diet based on corn and SBM, but the disappearance of DM was greater ($P < 0.05$) without inclusion of enzymes than if a combination of cellobiohydrolase and feruloyl esterase was added to this diet. Inclusion of individual feruloyl esterase did not affect the disappearance of DM in the diet based on corn and SBM, but the disappearance of DM was reduced ($P < 0.05$) if a combination of feruloyl esterase and endo-xylanase or a combination of feruloyl esterase, arabinofuranosidase, and cellobiohydrolase was added to the control diet.

Inclusion of endo-xylanase by itself tended ($P = 0.080$) to increase the disappearance of DM in the diet based on corn and SBM, but the inclusion of endo-xylanase combined with feruloyl esterase and cellobiohydrolase or the combination of endo-xylanase and feruloyl esterase and arabinofuranosidase did not affect the disappearance of DM. Inclusion of a

combination of feruloyl esterase, endo-xylanase, arabinofuranosidase, and cellobiohydrolase also did not affect the disappearance of DM in the corn and SBM diet.

Disappearance of DM in the diet based on corn, SBM, and DDGS was 83.91 if no enzymes were used (Table 5.4). Inclusion of arabinofuranosidase to this diet did not affect the disappearance of DM and inclusion of arabinofuranosidase in combination with feruloyl esterase, cellobiohydrolase, or endo-xylanase also did not result in differences in the disappearance of DM. Disappearance of DM was reduced ($P < 0.05$) if cellobiohydrolase was included in the diet and the combination of cellobiohydrolase and feruloyl esterase tended ($P = 0.093$) to decrease the ATTD of DM in the corn, SBM, and DDGS diet. However, inclusion of cellobiohydrolase in combination with endo-xylanase did not affect the disappearance of DM.

Inclusion of feruloyl esterase by itself or in combination with endo-xylanase did not affect the disappearance of DM in the corn, SBM, and DDGS diet, and the same was true for all combinations of feruloyl esterase and other enzymes. Inclusion of endo-xylanase by itself tended ($P = 0.080$) to increase the disappearance of DM in the corn, SBM, and DDGS diet, but the disappearance of DM was reduced ($P < 0.05$) if the combination of feruloyl esterase, endo-xylanase, arabinofuranosidase, and cellobiohydrolase were added to that diet.

DISCUSSION

The disappearance of DM in the diet based on corn and SBM concurs with published data for corn (Jaworski et al., 2015), but the ATTD of DM in the diet based on corn, SBM, and DDGS was greater than reported for DDGS by Jha et al. (2011) and Jaworski et al. (2015), but lower than reported by Huang et al. (2017). It is expected that corn and SBM have greater disappearance of DM than DDGS, and it was, therefore, not surprising that the diet based on

corn and SBM had a greater disappearance of DM than the diet based on corn, SBM, and DDGS. Additionally, the in-vitro procedure used by Jha et al. (2011) was different than the in-vitro procedure used in this experiment, which may also have affected results.

Arabinofuranosidase isolated from *Bifidobacterium adolescentis,* family GH43, catalyzes the release of (1→3)-α-L-linked arabinofuranose units from doubly substituted xylose residues (Sørensen et al., 2006). However, no improvement in the ATTD was observed when arabinofuranosidase was used, which may be a result of the activity of arabinofuranosidases being influenced by the degree of substitution of the arabinoxylan with arabinose units, the positions of the arabinose units, and the presence of other substitutions (Fehér et al., 2015). The arabinoxylan molecule from the analyzed diets may have more xylose substituted by individual arabinose units, than double substituted arabinose units, therefore, the enzyme did not release many arabinose units from the backbone. Furthermore, arabinofuranosidase activity may be negatively affected by reagents like EDTA, metals like Cu^{2+}, and the presence of glucose (Kim and Yoon, 2010; Broek et al., 2005), and EDTA was used in the simulation of conditions of the large intestine.

Cellobiohydrolase I from *Trichoderma longibrachiatum,* from family 7, hydrolyzes (1,4)-β-D-glycosidic linkages in cellulose, releasing cellobiose from the reducing or non-reducing end of the chains (Liu et al., 2011; Fox et al., 2012; Horn et al., 2012). The observation that inclusion of cellobiohydrolase in the diet did not increase the disappearance of DM may be the result of inhibition of the enzyme by the presence of glucose and cellobiose, which acts as a competitive inhibitor of cellobiohydrolase (Zhang and Lynd, 2004; Levine et al., 2010; Fox et al., 2012). Furthermore, cellulose is a complex molecule to hydrolyze because the presence of hemicelluloses in the samples may contribute to recalcitrance of the cellulose (Horn et al., 2012).

Inclusion of feruloyl esterase has positive effects on the removal of ferulic acid arabinoxylan in DDGS (Márquez Escalante and Carvajal Millan, 2019). The effectiveness of the enzyme was evaluated by the amount of ferulic acid hydrolyzed by the enzyme, followed by a chromatographic analysis to quantify the remaining ferulic acid. Therefore, the effect of other enzymes like pancreatin and pepsin was not taken into account. In-vitro trials have also been conducted to evaluate the effects of the feruloyl esterase on nutrient digestibility (Jayaraman et al., 2015), however, the feruloyl esterase used by Jayaraman et al. (2015) was from a different culture, and enzymes from different cultures may be more active under different conditions. Additionally, studies of feruloyl esterases in pigs are limited, however, in poultry it was demonstrated that feruloyl esterase improve nutrient digestibility (Liu et al., 2015).

Xylanase from *Trichoderma Viride*, from family GH11, hydrolyzes the (1,4)-β-D bonds in arabinoxylan, and an increase in the digestibility was expected when this enzyme was added. The observation that the disappearance of DM tended to increase when endo-xylanase was added to both diets was, therefore, expected. In a similar experiment, no differences in the disappearance of DM in a diet based on corn and SBM were observed when xylanase was used (Saleh et al., 2004). Inclusion of xylanase to diets based on corn co-products such as DDGS have not resulted in increased disappearance of DM in in-vivo experiments (Ndou et al., 2015; Jang et al., 2017). In contrast, in an in-vitro study using corn-based diets, an increase in the disappearance of DM was observed when xylanase was included (Tapingkae et al., 2008). Thus, results of experiments in which xylanase was added to corn-based diets have been inconsistent (Jang et al., 2017), which may be attributed to the microbial origin of the enzymes and the substrate (Ndou et al., 2015). In DDGS, the effectiveness of xylanases may be related to the source, the formation of aggregates of resistant and non-fermentable starchy and non-starchy

complexes formed during DDGS production, the heat damage during the processing, and physical characteristics of the substrate like porosity (Jha et al., 2015).

In- vitro procedures include proteases and proteases influence the performance of exogenous enzymes (Saleh et al., 2004; Vangsøe et al., 2020). Furthermore, pH also influences the enzymatic activity because if pH < 3 or pH > 5 the relative activity of xylanase is reduced (Lafond et al., 2014; Vangsøe et al., 2020) and in the procedure used in this experiment, the pH ranged between 2.0 and 6.8. Therefore the enzyme activity may be reduced under the conditions of the current experiment. Experiments to evaluate the disappearance of DM are usually performed using commercial mixes of enzymes or engineered individual enzymes, which are resistant to a wider range of temperature and pH. It is, therefore, possible that the pH and other conditions in the traditional in-vitro procedure need to be change if enzymes are need to be evaluated.

The observation that the disappearance of DM was not affected by inclusion of enzymes individually or in combinations with other enzymes is in agreement with results of other in-vitro studies, which used in diets with corn, SBM, and DDGS, or corn and SBM (Zhong et al., 2020). However, a positive impact on digestibility of nutrients when enzymes are included on DDGS have also been reported (Kiarie et al., 2012).

For in-vitro studies yield more consistent results, more studies are needed to make the enzymes more resistant to different essay conditions. Additionally, more research is needed to evaluate the synergistic effects when enzymes are used in combinations and to elucidate times of action. Moreover, genetic modifications including cloning and expression on the microorganism, which produces the enzymes, may contain a complex of enzymes, which hydrolyze the cell wall, or enzymes that due to the genetic and protein configuration may act synergistically with others.

CONCLUSION

Endo-xylanase may hydrolyze arabinoxylan in corn and SBM diets and corn, SBM and DDGS diets, which may contribute to improving the disappearance of DM. Inclusion of feruloyl esterase, cellobiohydrolase and arabinofuranosidases individually or in combinations did not improve the disappearance of DM. However, results may have been affected by factors related to the incubation conditions used. Therefore, conditions of incubation may need to be optimized and additional steps for the removal of end products at the end of the second step may be needed.

TABLES

Table 5. 1. Compositions of experimental diets

Ingredients, %	Diets	
	Corn-soybean meal	Corn-soybean meal-distiller dried grains with solubles
Ground corn	63.82	46.17
Distiller dried grains with solubles	-	40.00
Soybean meal	33.00	10.00
Soybean oil	1.00	1.00
Ground limestone	1.30	1.50
Dicalcium phosphate	0.30	0.15
Salt	0.40	0.40
Vitamin mineral premix[1]	0.15	0.15
L-Lysine, HCl, 78 % Lys	-	0.52
L-Threonine	-	0.05
L-Tryptophan	-	0.03
Phytase premix[2]	0.03	0.03
Total	100.00	100.00

[1]The vitamin-micromineral premix provided the following quantities of vitamins and microminerals per kg of complete diet: vitamin A as retinyl acetate, 11,150 IU; vitamin D_3 as cholecalciferol, 2,210 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin

Table 5.1. (Cont.)

K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 1.10 mg; riboflavin,6.59 mg; pyridoxine as pyridoxine hydrochloride, 1.00 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.6 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate; Fe, 125 mg as ironsulfate; I, 1.26mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganoussulfate; Se, 0.30mg as sodium selenite and selenium yeast; and Zn, 125.1mg as zinc sulfate.

[2]The phytase premix (Optiphos 2,000, Huvepharma, Sofia, Bulgaria) contained 2,000 phytase units per g. At 0.03% inclusion, the premix provided 600 units of phytase per kg in the complete diet.

Table 5. 2. Enzymes used in the in-vitro procedure and mixes of enzymes

	ENZYMES			
Batch	Feruloyl esterase[1]	Endo Xylanase[2]	Arabinofuranosidase[3]	Cellobiohydrolase[4]
1	Not included	Not included	Not included	Not included
2	Not included	Not included	Not included	Included
3	Not included	Not included	Included	Not included
4	Not included	Included	Not included	Not included
5	Included	Not included	Not included	Not included
6	Included	Included	Not included	Not included
7	Included	Not included	Included	Not included
8	Included	Not included	Not included	Included
9	Not included	Included	Included	Not included
10	Not included	Included	Not included	Included
11	Not included	Not included	Included	Included
12	Included	Included	Included	Not included
13	Included	Not included	Included	Included
14	Included	Included	Not included	Included
15	Included	Included	Included	Included

1. From *Clostridium Thermocellum*, 40U per Kg. Megazyme, Ireland.
2. From *Trichoderma Viride*, 20,000U per Kg. Megazyme, Ireland.
3. From *Bifidobacterium adolescentis*, 800U per Kg. Megazyme, Ireland.
4. From *Trichoderma longibrachiatum*, 3,750U per Kg. Megazyme, Ireland.

Table 5. 3 Disappearance of DM of a diet based on corn and SBM without or with inclusion of exogenous enzymes.

ATTD of DM, %	Corn-soybean meal	Contrast *P*-Value[1]
No enzyme	92.77	-
Arabinofuranosidase	89.91	0.020
Cellobiohydrolase	93.97	0.313
Feruloyl esterase	92.37	0.741
Endo xylanase	94.88	0.080
Arabinofuranosidase - feruloyl esterase	91.00	0.143
Arabinofuranosidase - cellobiohydrolase	89.29	0.005
Arabinofuranosidase - endo xylanase	90.03	0.026
Feruloyl esterase - endo xylanase	87.33	<0.001
Feruloyl esterase - cellobiohydrolase	88.04	<0.001
Endo xylanase - cellobiohydrolase	93.35	0.623
Feruloyl esterase - arabinofuranosidase - cellobiohydrolase	88.21	<0.001
Feruloyl esterase - endo xylanase - cellobiohydrolase	91.90	0.469
Feruloyl esterase - endo xylanase - arabinofuranosidase	91.86	0.446

Table 5.3. (Cont.)

Feruloyl esterase - endo xylanase – arabinofuranosidase - cellobiohydrolase	90.81	0.106
SEM	0.84	-

[1] Orthogonal contrasts, no enzyme inclusion vs. inclusion of enzyme or mixes of enzymes

Table 5.4 Disappearance of DM of a diet based on corn, SBM, and DDGS without or with inclusion of exogenous enzymes.

ATTD of DM, %	Corn-soybean meal-DDGS	Contrast *P*-Value[1]
No enzyme	83.91	-
Arabinofuranosidase	81.42	0.229
Cellobiohydrolase	79.08	0.023
Feruloyl esterase	83.63	0.893
Endo xylanase	87.58	0.080
Arabinofuranosidase- feruloyl esterase	84.42	0.840
Arabinofuranosidase- cellobiohydrolase	84.28	0.867
Arabinofuranosidase- endo xylanase	82.80	0.618
Feruloyl esterase - endo xylanase	82.49	0.491
Feruloyl esterase - cellobiohydrolase	80.10	0.093
Endo xylanase - cellobiohydrolase	81.16	0.185
Feruloyl esterase - arabinofuranosidase - cellobiohydrolase	83.01	0.663
Feruloyl esterase - endo xylanase - cellobiohydrolase	79.65	0.043

Table 5.4 (Cont.)

Feruloyl esterase - endo xylanase - arabinofuranosidase	82.69	0.553
Feruloyl esterase - endo xylanase – arabinofuranosidase - cellobiohydrolase	78.30	0.009
SEM	1.01	-

[1] Orthogonal contrasts, no enzyme inclusion vs. inclusion of enzyme or mixes of enzymes

LITERATURE CITED

Bach Knudsen, K. E., H. N. Lærke, and H. Jørgensen. 2013. Carbohydrates and carbohydrate utilization in Swine. In: L. I. Chiba, editor, Sustainable Swine Nutrition. John Wiley & Sons, Inc., Ames, IA, United States. p. 109-137.

Boisen, S., and J. A. Fernández. 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. Anim. Feed Sci. Technol. 68:277-286. doi:10.1016/S0377-8401(97)00058-8

Broek, L., R. Lloyd, G. Beldman, J. Verdoes, B. McCleary, and A. Voragen. 2005. Cloning and characterization of arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from Bifidobacterium adolescentis DSM20083. Appl. Microbiol. Biotechnol. 67:641-647. doi:10.1007/s00253-004-1850-9

Fehér, C., B. Gál, A. Fehér, Z. Barta, and K. Réczey. 2015. Investigation of commercial enzyme preparations for selective release of arabinose from corn fibre. J. Chem. Technol. Biotechnol. 90:1329-1337. doi:10.1002/jctb.4440

Fox, J. M., S. E. Levine, D. S. Clark, and H. W. Blanch. 2012. Initial and processive cut products reveal cellobiohydrolase rate limitations and the role of companion enzymes. Biochemistry. 51:442-452. doi:10.1021/bi2011543

Horn, S. J., G. Vaaje-Kolstad, B. Westereng, and V. Eijsink. 2012. Novel enzymes for the degradation of cellulose. Biotechnol. Biofuels. 5:45. doi:10.1186/1754-6834-5-45

Huang, Z., P. E. Urriola, and G. C. Shurson. 2017. Use of in vitro dry matter digestibility and gas production to predict apparent total tract digestibility of total dietary fiber for growing pigs. J. Anim. Sci. 95:5474-5484. doi:10.2527/jas2017.1964

Jang, Y. D., P. Wilcock, R. D. Boyd, and M. D. Lindemann. 2017. Effect of combined xylanase and phytase on growth performance, apparent total tract digestibility, and carcass characteristics in growing pigs fed corn based diets containing high fiber coproducts. J. Anim. Sci. 95:4005-4017. doi:10.2527/jas.2017.1781

Jaworski, N. W., H. N. Lærke, K. E. Bach Knudsen, and H. H. Stein. 2015. Carbohydrate composition and in vitro digestibility of dry matter and nonstarch polysaccharides in corn, sorghum, and wheat and coproducts from these grains. J. Anim. Sci. 93:1103-1113. doi:10.2527/jas.2014-8147

Jayaraman, S., R. Mukkalil, and H. Chirakkal. 2015. Use of ferulic acid esterase to improve performance in monogastric animals. US patent No. 20150118361A1

Jha, R., J. Bindelle, A. Van Kessel, and P. Leterme. 2011. In vitro fibre fermentation of feed ingredients with varying fermentable carbohydrate and protein levels and protein synthesis by colonic bacteria isolated from pigs. Anim. Feed Sci. Technol. 165:191-200. doi:10.1016/j.anifeedsci.2010.10.002

Jha, R., T. A. Woyengo, J. Li, M. R. Bedford, T. Vasanthan, and R. T. Zijlstra. 2015. Enzymes enhance degradation of the fiber–starch–protein matrix of distillers dried grains with solubles as revealed by a porcine in vitro fermentation model and microscopy. J. Anim. Sci. 93:1039-1051. doi:10.2527/jas.2014-7910

Kiarie, E., A. Owusu-Asiedu, A. Péron, P. H. Simmins, and C. M. Nyachoti. 2012. Efficacy of xylanase and β-glucanase blend in mixed grains and grain co-products-based diets for fattening pigs. Livest. Sci. 148:129-133. doi:10.1016/j.livsci.2012.05.020

Kim, Y. A., and K. H. Yoon. 2010. Characterization of a Paenibacillus woosongensis beta-xylosidase/alpha-arabinofuranosidase produced by recombinant escherichia coli. J. Microbiol. Biotechnol. 20:1711-1716. doi:10.4014/jmb.1010.10040

Lafond, M., O. Guais, M. Maestracci, E. Bonnin, and T. Giardina. 2014. Four GH11 xylanases from the xylanolytic fungus Talaromyces versatilis act differently on arabinoxylans. Appl. Microbiol. Biotechnol. 98:6339-6352. doi:10.1007/s00253-014-5606-x

Levine, S. E., J. M. Fox, H. W. Blanch, and D. S. Clark. 2010. A mechanistic model of the enzymatic hydrolysis of cellulose. Biotechnol. Bioeng. 107:37-51. doi:10.1002/bit.22789

Liu, Q., D. Y. Zhou, L. Chen, R. Q. Dong, and S. Zhuang. 2015. Effects of feruloyl esterase, non-starch polysaccharide degrading enzymes, phytase, and their combinations on in vitro degradation of rice bran and nutrient digestibility of rice bran based diets in adult cockerels. Livest. Sci. 178:255-262. doi:10.1016/j.livsci.2015.06.007

Liu, Y.-S., J. O. Baker, Y. Zeng, M. E. Himmel, T. Haas, and S.-Y. Ding. 2011. Cellobiohydrolase hydrolyzes crystalline cellulose on hydrophobic faces. J. Biol. Chem. 286:11195-11201. doi:10.1074/jbc.m110.216556

Márquez Escalante, J., and E. Carvajal Millan. 2019. Feruloylated arabinoxylans from maize distillers dried grains with solubles: effect of feruloyl esterase on their macromolecular characteristics, gelling, and antioxidant properties. Sustainability. 11:6449. doi:10.3390/su11226449

Ndou, S. P., E. Kiarie, A. K. Agyekum, J. M. Heo, L. F. Romero, S. Arent, R. Lorentsen, and C. M. Nyachoti. 2015. Comparative efficacy of xylanases on growth performance and digestibility in growing pigs fed wheat and wheat bran or corn and corn DDGS based

diets supplemented with phytase. Anim. Feed Sci. Technol. 209:230-239. doi:10.1016/j.anifeedsci.2015.08.011

Petry, A. L., N. F. Huntley, M. R. Bedford, and J. F. Patience. 2020. Xylanase increased the energetic contribution of fiber and improved the oxidative status, gut barrier integrity, and growth performance of growing pigs fed insoluble corn-based fiber. J. Anim. Sci. 98. doi:10.1093/jas/skaa233

Saleh, F., A. Ohtsuka, T. Tanaka, and K. Hayashi. 2004. Carbohydrases are digested by proteases present in enzyme preparations during in-vitro digestion. J. Poult. Sci. 41:229-235. doi:10.2141/jpsa.41.229

Sørensen, H. R., C. T. Jørgensen, C. H. Hansen, C. I. Jørgensen, S. Pedersen, and A. S. Meyer. 2006. A novel GH43 alpha-L-arabinofuranosidase from Humicola insolens: mode of action and synergy with GH51 alpha-L-arabinofuranosidases on wheat arabinoxylan. Appl. Microbiol. Biotechnol. 73:850-861. doi:10.1007/s00253-006-0543-y

Stein, H. H. 2019. Multi vs. single application of enzymes to degrade fibre in diets for pigs. In: G. Gonzalez-Ortiz, M. R. Bedford, K. E. B. Knudsen, C. M. Courtin, and H. L. Classen, editors, The value of fibre engaging the second brain for animal nutrition. Wageningen Academic Publishers, Wageningen, The Netherlands. p. 117-122.

Tapingkae, W., M. Yachai, W. Visessanguan, P. Pongtanya, and P. Pongpiachan. 2008. Influence of crude xylanase from Aspergillus niger FAS128 on the in vitro digestibility and production performance of piglets. Anim. Feed Sci. Technol. 140:125-138. doi:10.1016/j.anifeedsci.2007.02.001

Urriola, P. E., G. C. Shurson, and H. H. Stein. 2010. Digestibility of dietary fiber in distillers coproducts fed to growing pigs. J. Anim. Sci. 88:2373-2381. doi:10.2527/jas.2009-2227

Vangsøe, C. T., E. Bonnin, M. Joseph-Aime, L. Saulnier, V. Neugnot-Roux, and K. E. Bach Knudsen. 2020. Improving the digestibility of cereal fractions of wheat, maize, and rice by a carbohydrase complex rich in xylanases and arabinofuranosidases: an in vitro digestion study. J. Sci. Food Agric. n/a. doi:10.1002/jsfa.10806

Zhang, Y.-H. P., and L. R. Lynd. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol. Bioeng. 88:797-824. doi:10.1002/bit.20282

Zhong, R. Q., L. X. Gao, L. L. Zhang, Q. H. Huang, L. Chen, and H. F. Zhang. 2020. Effects of optimal carbohydrases cocktails screened using an in vitro method on nutrient and energy digestibility of different fiber source diets fed to growing pigs. Anim. Feed Sci. Technol.:114728. doi:10.1016/j.anifeedsci.2020.114728

CHAPTER 6: CONCLUSION

A diet based on corn, soybean meal (**SBM**), and distillers dried grains with solubles (**DDGS**) had a greater concentration of total non-starch polysaccharides (**NSP**) than a diet based on corn and SBM. Concentration of xylose, arabinose, and cellulose was predominant in both diets and in feces from pigs fed both diets. However, the digestibility of total NSP in the diet based on corn and SBM was greater than the digestibility of total NSP in the diet based on corn, SBM, and DDGS indicating that pigs may digest NSP from a diet based on corn and SBM to a greater degree than from a diet based on corn, SBM, and DDGS. More studies to elucidate effects of concentration of NSP in diets containing corn and corn coproducts on digestibility of NSP are needed to determine the most effective inclusion levels.

A diet based on corn, SBM, and DDGS contains approximately 3 times as much coumaric acid and ferulic acid than a diet based on corn and SBM. Analysis of coumaric acid and ferulic acid in feces from pigs fed both diets indicated that only around one third of the bound phenolic acids are fermented in the intestinal tract of pigs indicating that coumaric and ferulic acids are major barriers to fermentation of arabinoxylan in the fiber from corn and corn coproducts. For a better understanding of factors influencing the digestibility of ferulic and coumaric acid more studies about ileal digestibility and total tract digestibility of phenolic acids are needed. Furthermore, standards for DFA and TFA are needed to improve the characterization of ferulic acid in feed ingredients and more studies to elucidate the influence of DFA and TFA on the digestibility of arabinoxylan should be conducted.

Endo-xylanase may release xylose from arabinoxylan in corn and SBM diets and in corn, SBM, and DDGS diets, which may contribute to improved apparent total tract digestibility (**ATTD**) of DM. However, under conditions of the present experiments, ATTD of DM was not

further improved by combining xylanase with other carbohydrases. Inclusion of feruloyl esterase, cellobiohydrolase, and arabinofuranosidase individually or in combinations also did not improve the ATTD of DM. However, there is a lack of information about the synergistic effects and time of action when enzymes are used in combinations. Moreover, genetic modifications including cloning and expression on the microorganism, which produces the enzymes maybe considered to obtained a complex of enzymes that could hydrolyze the cell wall of the cereal. In addition, studies about the microbiota and the mechanisms of fermentation of the phenolic acids in the hindgut are needed as well as studies about the physical characteristics of the diet such as porosity.